Antiretroviral therapy timing impacts latent tuberculosis infection reactivation in a tuberculosis/simian immunodeficiency virus coinfection model

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Conlicts of Interest Statement
The authors have declared that no conflict of interest exists.
Abstract

Studies using the nonhuman primate model of *M. tuberculosis* /Simian Immunodeficiency Virus co-infection have revealed protective CD4+ T cell-independent immune responses that suppress LTBI reactivation. In particular, chronic immune activation rather than the mere depletion of CD4+ T cells correlates with reactivation due to SIV co-infection. Here, we administered cART at 2 weeks post-SIV co-infection to study if restoration of CD4+ T cell immunity occurred more broadly, and if this prevented reactivation of LTBI compared to cART initiated at 4 weeks post-SIV. Earlier initiation of cART enhanced survival, led to better control of viral replication and reduced immune activation in the periphery and lung vasculature thereby reducing the rate of SIV-induced reactivation. We observed robust CD8+ T effector memory responses and significantly reduced macrophage turnover in the lung tissue. However, skewed CD4+ T effector memory responses persisted and new TB lesions formed post SIV co-infection. Thus, reactivation of LTBI is governed by very early events of SIV infection. Timing of cART is critical in mitigating chronic immune activation. The novelty of these findings mainly relates to the development of a robust animal model of human *Mtb*/HIV co-infection that allows the testing of underlying mechanisms.
Introduction

The tuberculosis (TB) and Human Immunodeficiency Virus (HIV) co-pandemic continues to pose a major healthcare burden in resource-limited countries (1). HIV co-infection predisposes the host to reactivation of latent tuberculosis infection (LTBI) resulting in worsening of disease conditions, and in mortality. Epidemiological studies in the United States estimate that HIV-infected persons have a nine-times higher chance of progressing to active TB from LTBI compared to HIV-uninfected individuals (2). About 0.08-2% of people with LTBI experience reactivation following HIV co-infection and cART treatment (3, 4). The reason that some reactivate while others do not is not completely understood with the sequence of TB and HIV infections playing a pivotal role in humans. The best characterized impact of HIV on immune function is the depletion of CD4+ T cells in lymphoid tissues and peripheral blood (5, 6). Additionally, the frequency and depletion of CD4+ T cells in lungs and lymph nodes is a strong predictor of progression to TB. Studies using the nonhuman primate (NHP) model of *M. tuberculosis* (*Mtb*)/Simian Immunodeficiency Virus (SIV) co-infection have revealed that while CD4+ T cell dependent mechanisms are important in LTBI reactivation in a SIV co-infection setting (8), there are CD4+ T cell-independent immune responses that suppress and therefore protect against LTBI reactivation (9, 10). However, antibody-mediated depletion of CD4+ T cells in TB/SIV co-infection studies have revealed that the virus-mediated TB-specific disruption of immune response goes beyond CD4+ T cell depletion (11). These studies suggest a role of granuloma specific responses as more critical in LTBI reactivation than just peripheral CD4 counts (12). We have previously shown that there are direct cytopathic effects of SIV resulting in chronic immune activation, altered effector T cell phenotypes and dysregulated T cell homeostasis that causes LTBI reactivation (10, 13). Further, highly effective combinatorial antiretroviral therapy (14), while effective in reducing viral loads in the periphery and lungs of *Mtb*/SIV co-infected macaques, failed to reduce the rate of reactivation of LTBI (15). Thus, it is important to understand the driving forces behind chronic immune activation in a relevant co-infected preclinical model. This will lead to the discovery of key biomarkers predicting LTBI reactivation and therefore point the way to intervention strategies (13, 16).

We aim to leverage our NHP model of *Mtb*/SIV co-infection to study which concurrent cART/anti-TB regimens are most effective in controlling the chronic immune activation and subsequent LTBI reactivation. Previously, a greater effect of combined cART and isoniazid...
preventive therapy (IPT) on reducing the risk of TB has been shown compared to cART alone (17-19). The TEMPRANO ANRS clinical trial demonstrated that a six month IPT and early cART each independently reduced mortality in HIV-infected people in Cote d’Ivoire with high CD4 counts (20). Additionally, IPT with early cART provided marked protection from a serious HIV-related event or death. Despite IPT being the cornerstone of protection against TB in HIV+ patients during cART, it’s plausible that protection is lost after ending therapy (21). We hypothesized that it is imperative to optimize the timing of anti-viral therapy to effectively control immune activation before initiating anti-bacterial therapy. The optimal time to initiate cART in adults who are infected with HIV is as early as possible based on data from several human randomized clinical trials (22-26). The general practice to defer initiation of cART in HIV+ asymptomatic individuals has changed over time (27). The Strategic Timing of Antiretroviral Therapy (START) study concluded that there was a substantial benefit in immediate initiation of cART in HIV+ patients irrespective of CD4 count (22). In addition, the Starting Antiretroviral Therapy at Three Points in Tuberculosis (SAPIT) trial provided compelling evidence of the benefit of initiating cART during anti-TB therapy in HIV co-infected patients (24). However, it is to noted that the rapidity of the restoration of *Mtb* responses upon cART is fairly rapid (28, 29). This is evidenced by the fact that TB patients who develop immune reconstitution disease typically do so during the first few weeks after the initiation of cART (30). However, long-term recovery of TB-specific immune function is incomplete(31). To study the impact of the timing of cART on the immune activation, we utilized our established cART treated *Mtb*/SIV co-infected rhesus macaque model.

The aim of this study was to determine if administering cART at peak viremia (2 weeks post-SIV co-infection) compared to cART at chronic phase of the virus (4 weeks post-SIV co-infection) is able to rescue from the virus-induced immune activation and prevent LTBI reactivation. Though our *Mtb* infection mimics the human route and is likely only a log different than the human dose (we infect with ~10 CFU via aerosol), we do not infect using either the natural route or the physiologically relevant dose of HIV (we infect with 300 TCID50 SIVmac239 via IV route) in our model. This approach is necessary to reduce both the time and number of animals in these experiments while appropriately powering them. In the present study, we identified an immune correlate LTBI reactivation, namely, macrophage turnover, in macaque lungs.

While initiating cART earlier leads to better survival in humans, we show for the first time that initiating cART earlier results in reduced macrophage turnover in the lungs of co-infected
rhesus macaques. The updated guidelines for use of cART in TB/HIV co-infected individuals recommends the use of once daily dolutegravir (DTG) or twice daily raltegravir 400 mg daily in combination with tenofovir disoproxil fumarate/emtricitabine with once-weekly isoniazid plus rifapentine for 3 months (32). Most highly active antiretroviral therapy (HAART) treatment regimens for humans include drugs from at least two of the three classes cART (nucleoside analog reverse transcriptase (RT) inhibitors, non-nucleoside analog RT inhibitors, and protease inhibitors) (33). In accordance with the recommendations, we utilized an established cART regimen comprising of a formulation of a three-drug cocktail containing RT inhibitors tenofovir (20 mg/kg), emtricitabine (30 mg/kg), and 2.5 mg/kg of the integrase inhibitor, DTG (34-39). To determine if the early timing of cART enhanced survival and/or had an impact on the bacterial control, we compared the data from the current study (cART administered 2 weeks post-SIV co-infection) with the published data from co-infected macaques that were administered cART 4 weeks post-SIV co-infection (15). We aimed to identify the components of TB immunity in the blood and lung compartment that remain impaired after cART, versus those that are restored by cART administered at 2 weeks post-SIV co-infection (cART/2 week) and at 4 weeks post-SIV co-infection (cART/4 week) in our model.

We were able to demonstrate that cART/2 week enhanced the general well-being of the study macaques as evidenced by an increased survival during the protocol. cART/2 week controlled the viral replication and significantly reduced immune activation in BAL and blood thereby reducing the rate of SIV-driven LTBI reactivation. This was consistent with improved lung pathology in this group compared to the cART-naïve or the group cART/4 week group. Computed Tomography (CT) imaging of the lungs demonstrated a severe bronchial lymphadenopathy upon cART administration at 4 weeks post-SIV. Despite lowering inflammation and pathology due to an earlier and effective control of SIV infection, cART/2 week failed to reconstitute the skewed effector memory responses in the lung compartment. Depleted CD4+ T cells were only partially restored and marked increase in Th1 responses was still observed in the cART/2 week group. A higher percentage of CXCR3+ and CCR6+CD4+ T cells was observed in cART/2 week group in both BAL and whole blood at the end of the study. Further studies aiming at i) concurrent therapies to contain bacterial burden and ii) to study the impact of early initiation of cART to maintain the gut integrity and reduce microbial translocation are needed to have an optimum translational intervention.
Results

cART at peak viremia (cART/2 week) increases survival. To assess the impact of differential timing of cART on LTBI reactivation, we utilized 5 new macaques (cART/2 week, n = 4; cART/4 week, n = 1) infected with a low dose of ~10 CFU Mtb CDC1551 and reused published data from LTBI (n = 4), cART naïve co-infected macaques (n = 8), cART/4 week (n = 4) (15, 40) (Supplemental Table 1). The study design is outlined in Fig. 1A. All the macaques were infected with low dose of Mtb (~10 CFU deposited in the lungs) and SIV (300 TCID$_{50}$ SIVmac$_{239}$). Infection was confirmed by a positive Tuberculin Skin Test (41) at weeks 3 and 5 post TB infection. All macaques on the study developed asymptomatic LTBI infection characterized by less than 1-2 Log$_{10}$CFU of Mtb in the BAL at weeks 3, 5 and 7 post-TB infection, serum C-reactive protein (CRP) $\leq 5$ µg/mL (Fig. 1B) and no significant difference in percentage body temperature (Supplementary Fig. 1A) and body weight (Supplementary Fig. 1B) up to 9 weeks post-TB infection. Upon establishment of latency, macaques were co-infected with 300 TCID$_{50}$ SIVmac$_{239}$ (50% tissue culture infective dose) via the intravenous route 9 weeks post-TB infection (9, 10, 15). Once confirmation of SIV infection was evidenced by plasma viral loads measured via reverse transcription quantitative PCR (RT-qPCR), the macaques were treated with cART. The clinical, pathological and immunological responses were studied in the 4 experimental groups; LTBI, cART naïve, cART/2 week and cART/4 week.

Survival was a critical correlate impacted by the timing of cART in this study. There was a significant difference in the survival curves of the 4 experimental groups ($P = 0.0006$; Mantel-Cox test). Macaques in group cART/2 week survived in good body condition with adequate body muscling and fat until the predetermined study endpoint (Fig. 1C), significantly longer than macaques in group cART/4 week ($P = 0.02$). Conversely, macaques in group cART/4 week were humanely euthanized based on pre-specified endpoints starting as early as 1 week post cART initiation (Fig. 1C). The macaques in group cART/2 week survived longer than cART naïve and macaques in group cART/4 week survived for a reduced period compared to cART naïve. These differences were not significant as determined by Mantel-Cox and Gehan-Breslow-Wilcoxon tests. The clinical signs of active TB in humans and NHPs is often associated with elevated serum CRP levels, declining body weight and increased body temperature (42, 43). CRP is an inflammatory
marker of disease severity that correlates to bacterial burden in nonhuman primates (9, 43). CRP levels were significantly lower in the macaques in group cART/2 week at study endpoint compared to the macaques from cART/4 week group ($P < 0.001$) and cART naïve controls ($P < 0.0001$) (Fig. 1B). It is to be noted that the low CRP levels in the LTBI group are expected due to the low bacterial burden (43). The macaques in group cART/2 week maintained low CRP values with not more than 5-7% body weight loss or fever (Fig. 1B).

**cART effectively controls viral replication.** To evaluate the efficacy of cART regimen, viral loads were measured in the plasma and BAL supernatant of all the co-infected and treated macaques. No significant difference in plasma and BAL supernatant viral loads was observed in the cART naïve co-infected macaques between the peak viremia (wk 11 post-TB or 2 weeks post-SIV) and study endpoint (Fig. 1D, E). A significant and rapid decrease in the viral loads of plasma and BAL supernatant was observed at necropsy compared to peak of viremia in both; cART/2 week and cART/4 week groups (~4 log, $P < 0.0001$) (Fig. 1D, E).

**Reduced bacterial burden with no extrapulmonary spread of TB upon earlier cART initiation.** To determine the impact of cART timing on bacterial burden, BAL fluid, lung, spleen, bronchial lymph node and lung granuloma was plated on agar plates as described previously (9, 44). The macaques in group cART/2 week had significantly lower bacterial burden ($P = 0.0003, <10^2$ CFU/g in 3 out of 4 macaques sampled) compared to cART naïve and cART/4 week group at necropsy (Fig. 2A). cART naïve macaques ($P = 0.0182, \sim 10^4$ CFU/ g) and macaques in group cART/4 week displayed a significantly higher burden ($P = 0.0002, \sim 10^3$ CFU/ g) in the lung tissue when compared to LTBI controls and cART/2 week group (Fig. 2B). The bacterial burden was significantly higher in the lung granulomas of macaques in the cART naïve ($P = <0.0001$), cART/2 week ($P = 0.001$) and cART/4 week ($P = 0.0009$) groups compared to LTBI controls (Fig. 2C). However, the burden in the lung granulomas of macaques in group cART/2 week was significantly ($P = 0.0087$) lower than in group cART/4 week (Fig. 2C). Similar to lungs and lung granulomas, a significantly higher bacterial burden was observed in the bronchial lymph node of macaques in the cART naïve ($P = 0.0053$), cART/2 week ($P <0.0001$) and cART/4 week ($P = 0.02$) compared to the LTBI controls (Fig. 2D). The bacterial burden was significantly reduced ($P < 0.0001$) in bronchial lymph nodes of macaques in group cART/2 week compared to the macaques in groups
cART naïve and cART/4 week (Fig. 2D). No extrapulmonary spread of bacteria was observed in the spleen of all the 4 macaques in group cART/2 week (Fig. 2E). On the contrary, 3 out of 5 macaques in group cART/4 week displayed ~10^2 CFU/ g in the spleen at necropsy (Fig. 2E).

Reduced granuloma formation and improved lung pathology upon earlier cART initiation. To determine the impact of timing of cART on the lung pathology, lung tissue was collected at necropsy and subjected to H&E staining to study the cellular and granulomatous pathology (Fig. 3). The pathological findings correlated well with the clinical and microbiological findings. The LTBI group expectedly had few to no granulomas, with an average of 4-5% lung involvement (Fig. 3A, E). This group also displayed reduced TB disease-related pathology including edema, pneumonia and generalized foci of inflammation (Fig. 3A). In contrast, the co-infected cART naïve group demonstrated a significantly higher (P < 0.05) lung involvement than LTBI control group (Fig. 3B, E). This group demonstrated the lesions consistent with SIV-induced pathology including interstitial pneumonia and septal thickening, increased accumulation of foamy alveolar macrophages and lymphangitis (Fig. 3B). The macaques in group cART/2 week demonstrated rare small granulomas (Fig. 3C, E) and minimal enlargement of hilar and bronchial lymph nodes (data not shown). Overall, the macaques in this group had good body condition, with fewer granulomas and disease pathology (Supplementary Fig. 1C). Gross pathology demonstrated that the macaques in group cART/4 week harbored numerous large granulomas with significantly higher (P = 0.004) percent lung involvement compared to LTBI controls (Fig. 3D, E). H&E staining demonstrated confluent granulomas with necrotic cores in both cART naïve and cART/4 week groups (Fig. 3B, D). Overall, earlier initiation of cART resulted in significantly (P = 0.03) reduced lung involvement in group cART/2 week compared to group cART/4 week (Fig. 3E).

Earlier cART initiation does not prevent the formation of new TB lesions post-SIV infection. Computed tomography (CT) imaging was performed on the macaques in group cART/2 week at different time points throughout the study to examine TB lesions pre-, post-SIV and post-cART (Fig. 3F). The findings were compared to the CT images of the macaque in group cART/4 week (Fig. 3G). CT helped identify the lesions, TB reactivation and the granulomatous regions of the lungs. CT scans demonstrated that the worsening of the pathology was significantly mitigated in group cART/2 week, earlier cART initiation was unable to rescue from new TB lesions (Fig. 3F;
wk 12 and wk 17 post-TB; Supplementary Fig. 1D). The lung lesions in TB infection were characterized by solitary focal soft tissue attenuating nodules usually sub-centimeter in size (Fig. 3G; wk 4 post-TB). In the longitudinal scans studied, the TB lung lesions did not show progression in numbers or size. During 2-3 weeks post intravenous SIV challenge (Fig. 3F; wk 12, Supplementary Fig. 1D; wk 8 and wk12 post-TB), an increase in the size of the pre-existing nodules along with additional pulmonary nodules across several lung lobes was observed. As the disease progressed post-SIV challenge, numerous, large and irregular nodules extending into the lung periphery and the pleural margins were observed in the macaque in group cART/4 week (Fig. 3G; wk 8 and necropsy). Macaque in group cART/4 week demonstrated clinical signs of TB reactivation, developed severe alveolar pulmonary patterns with adjacent nodules, while some large nodular mass ranging from 1 to 1.5 cm in size were also recorded (Fig. 3G; wk 8 and necropsy). Additionally, the macaque in group cART/4 week (Fig. 3G) demonstrated a severe disease progression in the lymph nodes at necropsy (1 week of cART). It demonstrated a severe lymphadenopathy highlighting the possibility of a paradoxical reaction to TB post cART named Immune Reconstitution Inflammatory Syndrome (TB-IRIS) (Fig. 3G). However, future studies with additional markers are needed to verify this occurrence of IRIS like phenotype in co-infected and cART treated macaques.

Earlier initiation of cART fails to restore CD4 effector memory responses. cART treatment resulted in partial restoration of CD4+ T cells in the lung tissue (Fig. 4A). Despite treatment, the percentage of CD4+ T cells remained significantly lower than LTBI control group (P <0.0001). However, earlier initiation of cART demonstrated a significantly higher percentage of CD4+ T cells in the lung tissue (Fig. 4A) and BAL (Fig. 4B) compared to both cART naïve (P = 0.003) and cART/4 week groups (P = 0.01). Additionally, there was a significant difference in both, lung (P = 0.02) and BAL CD8+ T cells (P = 0.02) between cART/2 week and cART/4 week groups (Fig. 4C, D). No significant impact of the timing of cART was observed on the CD4+ and CD8+ T cell responses in whole blood (Supplementary Fig. 2A, B), bronchial lymph nodes (Supplementary Fig. 2C, D) and spleen (Supplementary Fig. 2E, F). Further longitudinal phenotyping of the replenished BAL CD4+ T cells in cART/2 week group demonstrated higher percentage of central memory phenotype (Fig. 4E). However, earlier initiation of cART could not rescue from the skewed CD4+ T effector memory response as evidenced by the significantly lower percentage of
this subset at study end point ($P = 0.017$) compared to the pre-SIV levels (Fig. 4F). There was no significant impact of early timing of cART initiation on CD8$^+$ T central memory response with the levels being maintained at similar percentage throughout the study period (Fig. 4G). In contrast to CD4$^+$ T cells, initiating cART at 2 weeks post-SIV significantly increased the CD8$^+$ T effector memory response (Fig. 4H) compared to the LTBI phase. There was no significant impact of initiating cART/2 week on peripheral CD4$^+$ T central and effector memory response (Supplementary Fig. 3A, B). Though early initiation of cART resulted in a significant increase in CD8$^+$ T central memory response in periphery (Supplementary Fig. 3C), there were no significant changes in CD8$^+$ T effector memory (Supplementary Fig. 3D). In conclusion, earlier initiation of cART is unable to restore the CD4$^+$ T effector memory response in the BAL to levels maintained during LTBI phase possibly leading to reactivation despite adequate CD8$^+$ T cell responses.

Further analysis of the restoration of CD4$^+$ and CD8$^+$ T cells in granulomas of the two treatment groups demonstrated a significantly higher ($P < 0.0001$) restoration of CD4$^+$ T cells (Supplementary Fig. 3E) and a lower percentage of CD8$^+$ T cells (Supplementary Fig. 3F) in cART/2 week compared to cART/4 week group. Since we observed a failure of restoration of adequate effector memory phenotype in the BAL with earlier cART initiation, we analyzed the levels of memory phenotype in different compartments of macaques in cART/2 week group. A significantly lower percentage ($P < 0.05$) of both, CD4$^+$ T central (Fig. 4I) and effector (Fig. 4J) memory response was observed in the bronchial lymph nodes compared to the lung compartment. No significant difference was observed in the CD8$^+$ T central memory response in different compartments but there were significant differences in the CD8$^+$ T effector memory responses (Supplementary Fig. G, H). Further studies aimed at studying the restoration of these responses in an anti-TB+cART treatment model would better define the critical role of efficient effector memory response in controlling LTBI reactivation.

Next, *Mtb*-specific CD4$^+$ T central and effector memory responses in the lung, BAL, bronchial lymph node and PBMCs of macaques initiated on cART/2 week were analyzed. ~60% *Mtb*-specific CD4$^+$ T central (Fig. 4K) and ~15% CD4$^+$ T effector (Fig. 4L) memory cells was observed in the lung and BAL of macaques in cART/2 week group. While no significant differences were observed in the *Mtb*-specific CD8$^+$ T central memory responses (Supplementary Fig. I), there were significantly lower percentage of CD8$^+$ T effector memory cells in BAL, bronchial lymph node and periphery compared to the lung tissue (Supplementary Fig. J). No
significant difference was observed in the gamma producing \( Mtb \) specific cells at necropsy in the different compartments (data not shown). We aim to perform a comparison of the \( Mtb \)-specific responses between LTBI, cART naïve, cART/2 week and cART/4 week in our future studies to correlate these findings with the bacterial load and LTBI reactivation.

**cART initiated at peak viremia better controls immune activation.** In our previous study, cART was initiated 4 weeks post-SIV (13 weeks post-\( Mtb \)) (15). This intervention fails to control chronic immune activation. To study the impact of cART timing on immune activation, we examined HLA-DR\(^+\), CD69\(^+\) and PD-1\(^+\) CD4\(^+\) T cells in BAL and whole blood at peak viremia (wk 11 post-\( Mtb \)) and at necropsy in the 4 experimental groups (Fig. 5). Early initiation of cART at peak viremia significantly reduced the activation markers, HLA-DR\(^+\) \((P = 0.0019)\) and CD69\(^+\)CD4\(^+\) T cells \((P = 0.01)\) in both BAL (Fig. 5A, B) and periphery (Fig. 5C, D). A comparable decrease was not observed in the macaques that initiated cART 4 weeks post-SIV co-infection (Fig. 5A, B, C, D). No significant difference was observed in the immune exhaustion marker, PD-1 in BAL and periphery between peak of viral replication and necropsy in any of the 4 experimental groups (Supplementary Fig. 4A, B). Activation markers were also examined in the lung, BAL, periphery, granuloma of the two treatment groups (Fig. 5). A significantly higher \((P = 0.001)\) percentage of CCR5\(^+\)CD4\(^+\) T cells was observed in the BAL and periphery of macaques in cART/2 week compared to cART/4 week group (Fig. 5E) at study end-point. Further, markers associated with specific cytokine function of CD4\(^+\) T cells; CXCR3(Th1) and CCR6 (Th17) were examined in the tissues and periphery of the treatment groups. As expected, cART treatment resulted in a reversal of the SIV-induced decrease of CXCR3\(^+\) CD4\(^+\) T cells in the periphery indicative of viral control (Fig. 5F) with no significant differences between the 2 groups. Additionally, a significantly higher \((P < 0.0001)\) percentage of Th17 responses \((CCR6\(^+\)CD4\(^+\) T cells) were observed in the granuloma of cART/2 week group (Supplementary Fig. 4C). Concordant with our findings in BAL and periphery, there was a significantly reduced immune activation and immune exhaustion in lungs and granulomas of cART/2 week group compared to cART/4 week group (Supplementary Fig. 4D, E, F). In conclusion, initiation of cART at peak viremia better controls immune activation and thus LTBI reactivation, though the long-lasting impact will need to be studied post cART termination in longer tenure NHP studies.
**Early cART initiation fails to reduce inflammation.** To investigate the impact of initiating cART on inflammation, we examined the percentage of CXCR3^+CCR6^+CD4^+ T cells in BAL (Fig. 5G) and periphery (Fig. 5H) of macaques in all 4 experimental groups at peak viremia (week 11 post-TB) and at necropsy. A significantly higher ($P = 0.0006$) percentage of CXCR3^+CCR6^+CD4^+ T cells was observed in the BAL and periphery of both the treatment groups compared to LTBI and cART-naïve at study end-point. Despite a decrease in activation markers HLA-DR and CD69 in the cART/2 week group (Fig. 5A, B), the early timing of cART initiation failed to diminish inflammation both, locally and in periphery (Fig. 5G, H). These findings are in concordance with our earlier studies (15) and with human findings (45).

**Early cART reduces macrophage turnover in TB/SIV co-infection:** Immunohistochemistry was performed to study the impact of the timing of cART on macrophage proliferation by staining BrDU^+CD163^+CD68^+ macrophages in the lungs of cART naïve (Fig. 6A), cART/2 week group (Fig. 6B) and cART/4 week group (Fig. 6C). A significantly lower ($P < 0.05$) percentage of macrophage turnover was observed in the lung of macaques in cART/2 week group (Fig. 6B, D) compared to the macaques in cART/4 week group (Fig. 6C, D) and cART naïve group (Fig. 6A, D). Presence of BrDU^+ nuclei (green) within macrophages (red) as indicated by the white arrow was observed in the lungs of macaque that received cART 4 weeks post-SIV (Fig. 6C). This phenomenon was considerably reduced in the macrophages in the lungs of cART/2 week group (Fig. 6B).

**Early cART initiation reduced IDO production in the granulomatous region:** We have previously shown that macrophage expressing indoleamine 2,3 – dioxygenase (IDO-1) in the macaque model of *M. tuberculosis* infection abrogates CD4^+ T cell and *Mtb* antigen presenting cell interaction (15, 46). In addition, increased bacterial burden and poor formation of inducible bronchus-associated lymphoid tissue (iBALT) correlates with higher expression of IDO-1 in lung tissue (46). Initiating cART at 4 weeks post-SIV resulted in an increased IDO-1 expression in macrophages surrounding the granuloma with poor iBALT formation (Fig. 7A, B). Earlier initiation of cART drastically reduced the IDO-1 production in the lung tissue (Fig. 7C, D) that corresponds to the lower bacterial burden (Fig. 2B), improved lung pathology (Supplementary Fig. 1C), reduced IFN-γ and TNF-α
production (Supplementary Fig. 5A, B) and increased protective IL-17 levels in BAL supernatant (Supplementary Fig. 5C).

**Discussion**

This is the first study to examine the impact of timing of cART on LTBI reactivation in a biologically and physiologically relevant nonhuman primate model. Initiation of cART in HIV infected individuals has not been standardized with limited clinical trial data to determine the initiation of cART in adults (47-49). Compared with humans who likely develop LTBI with a substantially lower infectious dose of *Mtb* (1-2 CFU), we infected the macaques with ~10-15 CFU *Mtb* CDC1551. While rhesus macaques infected with this dose/strain combination exhibit control of infection akin to human LTBI, the presented dose is clearly higher than the physiologically relevant human infectious dose. That this results in ~70% reactivation in absence of treatment is therefore expected. Hence, our results are indicative of the worst outcomes in co-infected humans. cART remains the cornerstone of HIV care, though our previous study demonstrated that while cART substantially reduced viral loads, it did not reduce the relative risk of SIV-induced TB reactivation (15). The long-term impact of cART is dependent on the degree of immunodeficiency at which it is initiated (50, 51). In this study, we sought to determine if initiating cART 2 weeks earlier than the previous study (15), at the time of peak viremia, would better control immune activation and prevent LTBI reactivation. Earlier initiation of cART substantially increased the survival rate of the study macaques with reduced disease severity. In addition, it significantly reduced bacterial burden in lungs and granulomas with no extrapulmonary spread of the bacteria following in spleen or liver. In concordance with these findings, we also observed an improved lung pathology with smaller, rare granulomas and reduced percentage lung involvement in the macaques in cART/2 week group compared to cART naïve controls and cART/4 week group. Hence, in our model, an earlier initiation of cART resulted in decreased mortality, less disease severity and improved survival.

Poor CD4+ T cell recovery is often associated with a persistent immune activation and inflammation (52, 53). We hypothesized that the improved survival could be attributed to an enhanced CD4 recovery in the macaques initiated on cART earlier. In the present study, initiating cART as early as 2 weeks post-SIV co-infection restored the lung CD4+ T cells to substantially
higher levels compared to cART/4 week group. A better CD4+ T cell recovery could be attributed to for the improved well-being and longevity of the macaques initiated on cART/2 week co-infection. These results reflect human data wherein, earlier initiation of cART in individuals with reduced depletion of CD4+ T cells results in limiting chronic immune activation (54). We hypothesize that while earlier initiation of cART is able to restore the CD4 responses better in BAL, the residual chronic immune activation after cART treatment in Mtb/SIV co-infected macaques interferes with the complete restoration of CD4+ T cell responses in the lung compartment as well as extrapulmonary organs. Overall, while the timing of cART positively impacts CD4 response restoration, HIV suppression results in better control of CD8 responses in the primary infection site as well as in extrapulmonary organs.

We next determined the phenotype of the replenished CD4+ T cells in the lungs of macaques initiated earlier on cART. For this, we characterized the CD4+ T cells in the BAL (surrogate for lung) into effector (CD28-CD95+) and central (CD28+CD95+) memory phenotype longitudinally throughout the study. cART initiated as early as 2 weeks post-SIV failed to restore the effector memory responses in the BAL to the levels maintained in LTBI. On the other hand, the central memory responses were restored to levels higher or similar to those in LTBI phase. Further studies should distinguish this affected effector phenotype into Th1, Th2 or a mix of Th1 and Th2 to identify the biomarkers and design therapeutics in conjunction with cART. Similar to BAL, we observed a marked increase in the central memory responses in both CD4+ and CD8+ T cells in the periphery at the end of the study period compared to the effector responses. Thus, cART is unable to restore the CD4+ T effector response in the BAL which results in the failure to restore the bacterial control leading to reactivation despite adequate CD8 responses. We next examined the Mtb-specific central and effector memory responses in the primary site of infection, lungs and BAL and in the extrapulmonary compartments such as bronchial lymph nodes and blood of the macaques initiated on cART 2 weeks post-SIV co-infection. Not surprisingly, the percentage of Mtb-specific CD4+ T effector memory cells were at ~10% while the Mtb-specific CD4+ T central memory cells were at more than 60% in the lungs and BAL. There was no significant difference in the Mtb-specific CD4+ T cells producing IFN-γ in the cART/2 week group at necropsy indicating that while cART has an impact on the Mtb-specific effector memory phenotype, there is no marked impact on the production of IFN-γ. It appears that there are factors other than Th1 induced IFN-γ responses that play a role in the control of Mtb upon SIV infection and cART administration.
Paradoxically, studies using the macaque model of TB/SIV co-infection have revealed protective CD4+ T cell-independent immune responses that suppress LTBI reactivation (9, 10). Recent work shows that the mere depletion of CD4+ T cells is insufficient to cause LTBI reactivation in SIV co-infected macaques. Instead, chronic immune activation appears to be critical for reactivation (10). Hence, we studied the impact of the timing of cART on the immune activation in the study macaques. An important marker of immune activation following Mtb/HIV co-infection in humans is elevated frequencies of HLA-DR+CD4+ T cells, reflecting chronic T cell activation (55, 56). In the macaques initiated on cART/2 week, there was a significant decrease in HLADR+ and CD69+CD4+ T cells in BAL and in the lungs, from week 11 to necropsy. This was not observed in macaques initiated on cART 4 weeks post-SIV co-infection. Early initiation of cART controlled immune activation in BAL to a better extent than periphery concordant with reduced disease pathology in this group. However, long-term prospective studies are still needed to determine whether early cART translates to a marked reduction in serious non-AIDS events and mortality. CCR5+CD4+ T cells, a subset of memory/activated CD4+ T cells is both a preferential target of virus replication and a marker of immune activation (57). We observed a substantial number of CCR5+CD4+ T cells at the end of the study that correlates to the viral replication control by cART and subsequent restoration of CCR5+CD4+ T cells in all the tissues except in bronchial lymph node. We also assessed the markers that are associated with specific cytokine function of CD4+ T cells: CXCR3(Th1) and CCR6(Th17). In peripheral blood, SIV infection lowers the frequencies of total CXCR3+ (Th1) and CCR6+ (Th17). In co-infected cohort of macaques that are treated with cART at peak viremia, we observed a reversal of the decrease in CXCR3+CD4+ T cells with higher levels in periphery than in the tissue at necropsy indicative of viral control. In untreated HIV infection, CCR6+CD4+ T cells are targets of HIV and SIV replication (58). A higher percentage of this subset in cART/2 week group is indicative of a better viral control in the lungs and granulomas. This highlights the need to study the impact of timing of cART on viral reservoirs.

CXCR3+CCR6+ CD4+ subset referred to as Th1* appears to play a critical role in mycobacterial infections in humans (29, 59). A significantly higher percentage of this subset was observed in both the treated groups compared to LTBI and cART-naïve groups. However, in people co-infected with pulmonary TB and HIV, immune reconstitution inflammatory syndrome (IRIS) occurs upon initiation of cART. This TB-IRIS state is characterized by a preferential expansion of CXCR3+ and CCR6+ populations as observed in this study. Earlier studies have
shown that SIV-driven blood monocyte turnover concurrent with macrophage death is a better correlate of LTBI reactivation than CD4+ T cell depletion [8]. Similar to humans, SIV differentially impacts alveolar macrophages (in BAL) and interstitial macrophages in lungs [15]. We observed that an earlier initiation of cART at 2 weeks post SIV-co-infection in LTBI macaques resulted in a reduced macrophage turnover concurrent to reduced disease pathology. Dissecting the impact of timing, duration of cART on macrophages in lung vasculature is critical to identifying key subsets involved in immune activation and immunosenescence in Mtb/SIV co-infection. One of the limitations was that the cART/4 week study was performed prior to cART/2 week study. Future studies need to be performed to correlate the rate of macrophage turnover to ensuing skewed CD4+ T-effector functions in the lung and BAL of cART treated Mtb/SIV co-infected macaques.

In conclusion, initiating cART as early as 2 weeks post-SIV co-infection substantially reduced SIV-driven LTBI reactivation by improving survival and health of the macaques. It effectively controlled SIV replication, improved lung pathology, reduced T cell activation in the primary site of infection and periphery while maintaining CD8+ T effector memory responses. However, it only partially restored CD4+ T cells in the lungs and did not rescue from the skewed CD4+ T effector memory responses. Earlier initiation of cART could arrest the increase in size of older TB lesions but could not prevent new TB lesions. It failed to contain the virus-induced inflammatory responses in lungs and BrLN highlighting the role of SIV reservoirs. We hypothesize that future studies on co-administration of anti-TB therapy, such as the WHO recommended 3HP treatment of LTBI with cART that could result in enhancing Mtb-specific immunity and prevention of LTBI reactivation compared to cART alone.

Methods

Animal infection. This study included a total of 21 Indian-origin rhesus (Macaca mulatta) macaques from two different studies. Data was included from completed studies wherein, 17 animals had been enrolled from a specific-pathogen free colony maintained at TNPRC (10, 15) and a total of 4 specific pathogen free Indian-origin rhesus macaques were enrolled from SNPRC colony. All macaques were infected with a low dose of approximately 10 CFU M. tuberculosis CDC1551 (BEI Resources, catalog NR13649) via aerosol as described before
TST was performed at weeks 3 and 5 post TB infection to confirm infection. All the macaques were monitored for CRP, percent body weight and body temperature weekly through the study period. 17 of the LTBI macaques were then co-infected with 300 TCID$_{50}$ SIVmac$_{239}$ via the intravenous route 9 weeks post-TB infection (9, 10, 15) (provided by the Preston Marx Laboratory, TNPRC, Covington, Louisiana, USA). All the procedures were conducted a board-certified veterinary clinician. The remaining 4 macaques served as LTBI controls for the study. The viral infection was confirmed through plasma viral loads via reverse transcription quantitative PCR (RT-qPCR). Upon confirmation of SIV infection, the 17 macaques were then divided into 3 groups: the first group of 8 macaques served as co-infected controls with no cART administration; the second group of 4 macaques were started on cART at 2 weeks post-SIV co-infection or 11 weeks post TB infection (cART at peak viremia) and the third group of 5 macaques started cART at 4 weeks post-SIV co-infection or 13 weeks post TB infection (cART at chronic phase of the virus). All the macaques in the ART at chronic phase group had to be euthanized within 9-11 weeks of cART treatment (by week 24) due to clinical signs of TB reactivation. The macaques in the cART at peak viremia group were euthanized after 9 weeks of cART treatment to match the treatment tenure with the cART at chronic phase group. The study demographics and the statistical comparison between the two studies are presented in Supplementary Tables 1 and 2.

**cART regimen.** Co-infected NHPs received a drug regimen consisting of 20 mg/kg of (R)-9-(2-phosphonylmethoxypropyl) adenine (PMPA, tenofovir, Gilead Sciences), 30 mg/kg of 2’, 3’-dideoxy-5-fluoro-3’-thiacytidine (FTC, emtricitabine, Gilead Sciences) and 2.5 mg/mL of the integrase inhibitor, DTG, Dolutegravir (ViiV Healthcare). The drugs were administered daily via subcutaneous injection of a cocktail of these three drugs in the vehicle kleptose at previously published doses (15).

**Viral load and bacterial burden measurement.** Bacterial burden in BAL was measured throughout the study period as previously described (9). Viable Mtb burden was also measured at necropsy in BAL, lung, spleen, bronchial lymph node and individual granulomas collected at necropsy (Fig. 2A-E) (9, 10). Viral loads in acellular BAL supernatant and plasma were determined by RT-qPCR at peak viremia (2 weeks post-SIV or 11 weeks post TB-infection) and at necropsy (Fig. 2 F, G). The measurements were performed by NIAID, DAIDS, Nonhuman Primate Core Virology
Laboratory for AIDS Vaccine Research and Development). A lower limit of 100 copies/sample was set for quantification of SIV copies in this assay.

**Computed Tomography imaging.** The macaques were anaesthetized (Telazol 2-6 mg/kg) and intubated to perform end-respiratory breath-hold using a remote breath-hold switch. The anesthesia was maintained during imaging by inhalation of isoflurane delivered through the Hallowell 2002 ventilator anesthesia system. Lung field CT images were acquired using Multiscan LFER150 PET/CT (MEDISO) scanner. 3D ROI Tools available in Vivoquant (Invicro) was used for image analysis. The ventral lung lobes were described as caudal and the upper lung lobes were described as cranial. The CT resolution was fair with moderate beam hardening/streak artifacts due to cone beam technology. Axial/transverse reconstruction series were provided in soft tissue windows. The studies were reviewed using Sectra IDS7 viewing software in a lung window with centerline -230.0 and window of 2250.0. The CT disease was graded subjectively but utilized the following scheme for image interpretation and grading of mild, moderate or severe disease: 0; normal, 1; mild interstitial, ground glass opacity or nodules <5mm, 2; moderate interstitial, dense ground glass opacity with crazy waving pattern or nodules 5-10mm, 3; alveolar (uniform soft tissue; unable to see vessels) or nodules >1cm. There was some subjectivity between mild and moderate interstitial, and utilizing HU may have been beneficial however was not measured in these studies. If the attenuation in the lung was not uniform and pulmonary vascular margins could readily be delineated, it was graded as mild interstitial/ground glass. If the attenuation in the lung was increased and vascular margins were indistinct or not defined but the attenuation was not uniform soft tissue attenuating, it was classified as moderate. If the pulmonary attenuation was uniform soft tissue with adjacent soft tissue structures it was classified as alveolar. Pulmonary nodules were graded based on their size: mild <5mm, Moderate= 5-10mm and severe= >1cm diameter.

**High parameter flow cytometry.** High parameter flow cytometry was performed on BAL cells and whole blood at pre-infection, pre-SIV (wk 3, 9), post-SIV, pre-cART (wk 11) and post-cART (wk 20 or necropsy). Lung, bronchial lymph nodes and granulomas were harvested at necropsy and processed as described earlier (9, 10). The single cells prepared were then stained with surface and intracellular markers to study various cell phenotypes (Supplementary Table 3). The freshly
collected BAL cells and peripheral blood mononuclear cells (PBMCs) were stimulated \textit{ex vivo} with \textit{Mtb}-specific antigens, ESAT-6/CFP-10 and \textit{Mtb} Cell Wall Fraction (BEI Resources, 10 μg/mL) for a total of 16 h. Brefeldin A (0.5 μg/mL, SIGMA) was added 2 h after the onset of stimulation. After stimulation, the cells were stained with LIVE/DEAD fixable Near-IR stain (ThermoFisher) and stained subsequently with the surface antibodies: CD4-PerCP-Cy5.5 (BD Biosciences; Clone L200; Cat. No. 552838), CD8-APC (BD Biosciences; Clone RPA-T8; Cat. No. 555369), CD3-AlexaFlour 700 (BD Biosciences; Clone SP34-2; Cat. No. 557917), CD95-BV421 (BD Biosciences; Clone DX2; Cat. No. 562616), CD28-PECy7 (BD Biosciences; Clone CD28.2; Cat. No. 560684) and CD45- BV395 (BD Biosciences; Clone D058-1283; Cat. No. 564099). Cells were then fixed, permeabilized and stained with intracellular antibodies: IFN$\gamma$ - APC-Cy7 (Biolegend; Clone B27; Cat. No. 506524), IL-17-BV605 (Biolegend; Clone BL168; Cat. No. 512326) and TNF$\alpha$ - BV650 (Biolegend; Clone Mab11; Cat. No. 502938). Cells were washed, suspended in BD stabilizing fixative buffer and acquired on BD FACS Symphony flow cytometer. Analysis was performed using FlowJo (v10.6.1) using previously published gating strategy (Supplementary Fig. 6, 7) (9, 15, 44).

\textit{Gross pathology}. The macaques were anesthetized for necropsy and lung lobes, spleen, liver, bronchial lymph nodes, BAL and blood were collected. All the tissues were weighed at the time of collection. Tissues were fixed in 10% neutral-buffered formalin, paraffin embedded, sectioned at 5 μm thickness and stained with haematoxylin and eosin using standard methods. Steriology scores were prepared on percentage lung affected by a board-certified veterinary pathologist.

\textit{Immunohistochemistry staining}. Fluorescent immunohistochemistry was performed on formalin-fixed, paraffin-embedded lung and bronchial lymph node tissues as previously described (10, 15, 43, 62). The stained slides were scanned in the Axio Scan Z1 and the images were analyzed using HALO software.

\textit{Statistics}. Statistical analysis was performed using an unpaired Student’s t test, 1-way ANOVA with Sidak’s or Tukey’s correction as applicable in GraphPad Prism (version 8.4.1). A $P$ value of <0.05 was considered as statistically significant. $^*P < 0.05; ^{**}P < 0.01; ^{***}P < 0.001; ^{****}P < 0.0001$. Data are represented as Mean \textpm SEM.
Study Approval: All infected macaques were housed under Animal Biosafety Level 3 facilities at the Southwest National Primate Research Center, where they were treated according the standards recommended by AAALAC International and the NIH guide for the Care and Use of Laboratory Animals. The study procedures were approved by the Animal Care and Use Committee of the Texas Biomedical Research Institute.

Author Contributions

RS, DK, SAK, SM, JR designed the study. RS conducted the sample processing and analyzed the flow data with assistance from SRG, DKS, ANB. RS, XA, VS performed and analyzed confocal imaging. MG, CA, JF, AB, RT, BS, RE assisted with necropsy sampling. VV contributed to data interpretation. RS, DK wrote the manuscript. JC supervised the veterinary work. SRG performed the CT scans and aerosol Mtb infections. ED and VS performed the necropsies and histopathology analysis. VS performed the scanning of the confocal slides and the cell counts on the scanned slides. SH was the attending veterinarian on the study.

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32. Services DoHaH. Panel on antiretroviral guidelines for adults and adolescents.


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Figure 1. Comparison of clinical parameters in LTBI, cART naïve, cART/2 week and cART/4 week. (A) Study outline. (B) Serum CRP levels, (C) Survival curve representing the 4 study groups; LTBI (n = 4), cART naïve (n = 8), cART/2 week (n = 4) and cART/4 week (n = 5). The survival curves were compared using the log-rank, Mantel-Cox or Gehan-Breslow-Wilcoxon tests. Viral loads in (D) plasma and (E) BAL supernatants of the treated macaques were measured longitudinally throughout the study. (B), (D), (E) were analyzed using 1 – way ANOVA with Tukey’s multiple-comparisons test in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SEM.
Figure 2. Impact of cART treatment on 
*Mtb* burden in tissues at necropsy. Bacterial burden (Log_{10} CFU/mL or Log_{10} CFU/g) was determined in the (A) BAL, (B) Lung, (C) lung granuloma, (D) bronchial lymph node and (E) spleen at necropsy by homogenizing the tissues and plating on agar plates. Significance was determined in LTBI (*n* = 4), cART naïve (*n* = 8), cART/2 week (*n* = 4) and cART/4 week (*n* = 5) using 1-way ANOVA with Tukey’s multiple-comparisons test in GraphPad Prism v8.4.1. A *P* value of <0.05 was considered as statistically significant. *P* < 0.05; **P* < 0.01; ***P* < 0.001; ****P* < 0.0001. Data are represented as Mean ± SEM.
Figure 3. Impact of timing of cART on lung pathology and TB lesions in Mtb/SIV co-infected macaques. To determine the impact of cART timing on the lung pathology, lung tissue was collected at necropsy and subjected to H&E staining to study the cellular and granulomatous pathology in (A) LTBI \((n = 4)\), (B) cART naïve \((n = 8)\), (C) cART/2 week \((n = 4)\) and (D) cART/4 week \((n = 5)\). (E) Percentage lung involvement was calculated by board certified pathologist by quantification of the number of lesions per lobe of the lungs. (F) Computed tomography imaging was performed on the macaques that received cART 2 weeks post-SIV co-infection at different time points throughout the study to examine the TB lesions pre-, post-SIV and post-cART. (G) Computed tomography imaging was performed on the macaque that received cART 4 weeks post-SIV co-infection at different time points throughout the study to examine the TB lesions pre-, post-SIV and post-cART. Significance was determined using 1 – way ANOVA with Tukey’s multiple-comparisons test in GraphPad Prism v8.4.1. A \(P\) value of \(<0.05\) was considered as statistically significant. \(*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001\). Data are represented as Mean ± SEM.
Figure 4. Earlier initiation of cART fails to restore CD4 effector memory responses. To assess the impact of cART treatment on CD4+ T cell restoration, cells were stained with flow cytometry surface antibodies and acquired on BD Symphony. Percentage of CD4+ T cells in (A) lungs, (B) BAL and percentage of CD8+ T cells in (C) lungs, (D) BAL was determined in LTBI (n = 4), cART naïve (n = 8), cART/2 week (n = 4) and cART/4 week (n = 5). Phenotyping of (E) BAL CD4+ Tcm cells and (F) CD4+ Tem was performed by staining for CD28+CD95+ (Tcm) and CD28-CD95+ (Tem) in cART/2 week (n = 4). Percentage of BAL (G) CD8+ Tcm and (H) CD8+ Tem cART/2 week (n = 4). (I) Percentage of CD4+ Tcm and (J) CD4+ Tem in lung, granuloma and BrLN of cART/2 week (n = 4). (K) Percentage of Mtb-specific CD4+ Tcm and (L) CD4+ Tem in lung, BAL, BrLN and PBMCs of macaques in cART/2 week group (n = 4) at necropsy. Significance was determined using 1-way ANOVA with Sidak’s or Tukey’s correction as applicable in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SEM.
Figure 5. cART initiated at peak viremia better controls immune activation. To study the impact of cART timing on immune activation, we examined the percentages of (A) BAL HLA-DR⁺, (B) BAL CD69⁺ (C) whole blood HLA-DR⁺ (D) whole blood CD69⁺CD4⁺ T cells at peak viremia (wk 11 post-TB) and at necropsy. (E) CCR5⁺CD4⁺ and (F) CXCR3⁺CD4⁺ T cells were examined in the lung, BAL, whole blood, granuloma of cART/2 week (n = 4) and cART/4 week (n = 5) at necropsy. Percentage of CXCR3⁺CCR6⁺CD4⁺ T cells were examined in the (G) BAL and (H) whole blood at peak viremia (week 11 post-TB) and at necropsy in LTBI (n = 4), cART naive (n = 8), cART/2 week (n = 4) and cART/4 week (n = 5). Significance was determined using 1-way ANOVA with Tukey’s correction in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SEM.
Figure 6. Early cART reduces macrophage turnover in Mtb/SIV co-infection. Immunohistochemistry was performed to study the impact of the timing of cART on macrophage turnover by staining for BrDU+ nuclei (green) of macrophages (CD163+CD68+ red) per µm² of lung sections of (A) cART naïve (n = 3), (B) cART/2 week (n = 3) and (C) cART/4 week (n = 3) at necropsy. The images were captured on Axio Scan Z1 and analyzed using HALO software. Significance was determined using 2-tailed Student’s t test in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SEM.
Figure 7. Early cART initiation reduced IDO production in the granulomatous region. Immunohistochemistry was performed to study the impact of the timing of cART on IDO-1 production in the lung tissue by staining for IDO-1$^+$ cells (red), macrophages (CD68$^+$ green) and B cells (CD20$^+$ blue). Panels A and B represent study animals from cART/4 week ($n = 5$), while panels C and D represent study animals from cART/2 week ($n = 4$) at necropsy. The arrow marks point to the IDO-1 production in the granulomatous region and in the lung tissue. The images were captured on Axio Scan Z1 and analyzed using HALO software.
**Supplementary Figure 1.** (A) Percentage temperature change in °F and (B) percentage change in body weight (kg) at pre-infection, post-TB infection, post-SIV, pre-cART and post-cART in *Mtb/SIV* co-infected macaques. (C) H&E staining of lung sections collected at necropsy from cART treated macaques representing the percent lung involvement. (D) Representative CT scan images from cART/2 week (*n*=4) at pre-infection, weeks 4, 8 and 12 post TB infection demonstrating the increase in the size of the older lesions. (A) and (B) were analyzed using 1-way ANOVA with Tukey’s comparison in GraphPad Prism v8.4.1. A *P* value of <0.05 was considered as statistically significant. *P* < 0.05; **P* < 0.01; ***P* < 0.001; ****P* < 0.0001. Data are represented as Mean ± SEM.
Supplementary Figure 2. CD4+ T and CD8+ T cells enumerated in the (A, B) whole blood, (C, D) BrLN and (E, F) spleen at necropsy in LTBI (n = 4), cART naïve (n = 8), cART/2 week (n = 4) and cART/4 week (n = 5). Significance was determined using 1-way ANOVA with Tukey’s comparison in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *P < 0.05; **P <0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SEM.
Supplementary Figure 3. Phenotyping of whole blood (A) CD4+ Tcm cells, (B) CD4+ Tem cells, (C) CD8+ Tcm and (D) CD8+ Tem was performed by staining for CD28+CD95+ (Tcm) and CD28-CD95+ (Tem) in cART/2 week (n = 4). Percentage of (E) CD4+ T and (F) CD8+ T cells in the granulomas of cART/2 week (n = 4) and cART/4 week (n = 5) at necropsy. (G) Percentage CD8+ Tcm and (H) CD8+ Tem in lung, granulomas and BrLN of cART/2 week (n = 4). Percentage of (I) Mtb-specific CD8+ Tcm and (J) CD8+ Tem responses in lung, BAL, BrLN and PBMCs in cART/2 week (n = 4) at necropsy. Significance was determined using 1-way ANOVA with Sidak’s or Tukey’s correction as applicable in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SEM.
**Supplementary Figure 4.** Percentage of (A) BAL PD-1⁺ and (B) whole blood PD-1⁺CD4⁺ T cells in LTBI (n = 4), cART naïve (n = 8), cART 2 weeks post-SIV (n = 4) and cART 4 weeks post-SIV (n = 5) at week 11 post-TB and necropsy time point. Percentage of (C) CCR6⁺CD4⁺ T cells, (D) CD69⁺CD4⁺ T cells, (E) HLA-DR⁺CD4⁺ T cells, (F) PD-1⁺CD4⁺ T cells in lungs and granuloma of cART/2 week (n = 4) and cART/4 week (n = 5). Significance was determined using 1-way ANOVA with Tukey’s comparison in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SEM.
Supplementary Figure 5. Levels of (A) IFN-\( \gamma \), (B) TNF-\( \alpha \) and (C) IL-17 were measured in BAL supernatant at pre-infection, weeks 7, 11 and at necropsy using NHP XL Cytokine Luminex Performance Premixed Kit (R&D systems). The data was analyzed using Belysa software. Significance was determined using 1-way ANOVA with Tukey’s comparison in GraphPad Prism v8.4.1. A P value of <0.05 was considered as statistically significant. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \); ****\( P < 0.0001 \). Data are represented as Mean ± SEM.
Supplementary Figure 6. Representative gating strategy for CD4 and CD8 T cell memory responses. Lymphocytes were first gated on Live/Dead and CD45 to select for live cells and perform RBC discrimination. This was followed by singlet gating on SSC and FSC -area, width and height. CD66a (CEACAM1), CD66b (CEACAM8), CD66c (CEACAM6), and CD66e (CEACAM5) are differentially expressed on some epithelial cells as well as on T cells, NK cell subsets, and granulocytes. Since our antibody consists of all (CD66a, b, c and e), we gated on total CD66 positive followed by gating out the NK cells and selecting only the total CD3+ population. Next, we gated the CD4 and CD8 on total CD3. Lastly, we gated on CD28 and CD95 for naïve (CD28+CD95-), central memory (CD28+CD95+), effector memory (CD28-CD95+) CD4 and CD8 T cells.
Supplementary Figure 7. Representative gating strategy for CD4 and CD8 T cell transient markers in BAL at week 7 post-Mtb infection.
**Supplementary Table 1.** Age, sex, timing of cART and Mamu type of the study macaques.

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Supplementary Table 2: Comparison of study demographics

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<th>Variable</th>
<th>Study 1*</th>
<th>Study 2**</th>
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<tr>
<td>N</td>
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<td>5</td>
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</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>6.35 ± 2.00</td>
<td>6.02 ± 1.15</td>
<td>0.732</td>
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<tr>
<td>cART Duration (weeks)</td>
<td>6.25 ± 2.5</td>
<td>8.00 ± 2.23</td>
<td>0.302</td>
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<tr>
<td>Sex (N, %)</td>
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<tr>
<td>Female</td>
<td>0 (0%)</td>
<td>3 (60%)</td>
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<tr>
<td>Male</td>
<td>17 (100%)</td>
<td>2 (40%)</td>
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<tr>
<td>Plasma SIV burden at week 11 (peak viremia)</td>
<td>7.07 ± 0.63</td>
<td>7.6 ± 0.30</td>
<td>0.421</td>
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<tr>
<td>BAL Supernatant SIV burden at week 11</td>
<td>4.54 ± 1.52</td>
<td>5.02 ± 0.33</td>
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<td>Mtb burden at week 3 post infection (Log10 CFU/mL)</td>
<td>0.50 ± 0.82</td>
<td>0.34 ± 0.68</td>
<td>0.731</td>
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</table>

*KV48, LA04, LA46, LE24, KR44, LC88, JH07, JF23, KG40, IP88, JI68, JE48, GP50, JF46, HV02, JD72 (14)
**33343, 33994, 34741, 35974, 32026 (current study)
P values for numerical variables are based on Kruskal-Wallis test across all groups.
For pairwise comparisons, P-values are based on Wilcoxon test, adjusted for multiple comparisons (Holm)
A P value of <0.05 was considered as statistically significant.
*P < 0.05; **P <0.01; ***P < 0.001; ****P < 0.0001. Data are represented as Mean ± SD

Supplementary Table 2: Comparison of study demographics
<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorochrome</th>
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<td>CD28</td>
<td>BUV395</td>
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<td>CD4</td>
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<td>CD196(CCR6)</td>
<td>BUV661</td>
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<td>BUV805</td>
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<td>CD183(CXCR3)</td>
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<td>CD185(CXCR5)</td>
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<td>CD69</td>
<td>BV750</td>
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**Supplementary Table 3.** High parameter flow cytometry panel.
Graphical Abstract