Rapamycin Modulates Pulmonary Pathology in a Murine Model of Mycobacterium tuberculosis Infection

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ABSTRACT

In this study we employed C3HeB/FeJ mice as an experimental model to investigate the potential role of rapamycin, an mTOR inhibitor, as an adjunctive therapy candidate during the treatment of Mycobacterium tuberculosis infection with moxifloxacin. We report that administration of rapamycin with or without moxifloxacin reduced infection-induced lung inflammation, and the number and size of caseating necrotic granulomas. Results from this study strengthen the potential use of rapamycin and its analogs as adjunct TB therapy and importantly underscore the utility of the C3HeB/FeJ mouse model as a pre-clinical tool to evaluate HDT candidates in TB treatment.

Key words: tuberculosis, host directed therapy, rapamycin, inflammation
INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a significant global burden. Worldwide an estimated 10.0 million people developed TB disease in 2019, and there were an estimated 1.4 million TB deaths (Chakaya et al., 2021). Several drug regimens are available for the treatment of TB; however, the duration of the regimen is lengthy (6 months for drug-susceptible TB and more than 18 months for drug-resistant TB). Moreover, these treatment modalities are often associated with severe side effects (Hosford et al., 2015, Sayada, 2010). Patients that complete treatment regimens successfully, nonetheless, frequently experience permanent lung damage due to aberrant inflammation in response to Mtb (Pasipanodya et al., 2010, Wallis and Hafner, 2015, Zumla et al., 2015). For example, in patients who completed treatment for multidrug resistant disease (mean 21 months), FEV1 was only 63% of predicted value, indicating significant loss of lung function (de Vallière and Barker, 2004).

To circumvent the complications associated with microbial drug resistance and to mitigate Mtb-induced inflammatory damage to the host, investigational research has turned towards host directed therapies (HDTs), some of which are already being tested for clinical use (Kaufmann et al., 2014, Hawn et al., 2013). Four focus areas that drive the development of novel HDTs against TB include: 1) improving the efficacy of anti-microbial mechanisms and decreasing the length of TB treatment; 2) reducing inflammation during TB treatment and permanent lung damage at end of treatment; 3) improving memory immune responses against the pathogen; and 4) designing drugs that readily penetrate the tuberculous granuloma for better access to the bacilli (Young et al., 2020, Tsenova and Singhal, 2020). That said, developing a standard HDT regimen for TB is challenging since the disease presents itself as a broad spectrum, ranging from latent to incipient to sub-clinical disease prior to progression to clinical symptomatic TB disease (Pai et al., 2016). Yet, drugs that target core immuno-metabolic pathways in the host, can potentially have a significant impact on the treatment outcome (Hnizdo et al., 2000, Ravimohan et al., 2018, Pasipanodya et al., 2007).

Autophagy has been widely studied for its role in protection during Mtb infection (Deretic, 2014, Gutierrez et al., 2004, Watson et al., 2012). Autophagy competent mice have lower lung bacterial load and reduced lung pathology compared to autophagy deficient mice, both during Mtb Erdman and Mtb H37Rv infections (Castillo et al., 2012, Bonilla et al., 2013). These findings suggest that targeting autophagy to ameliorate chronic lung inflammation during TB treatment can potentially lead to the development of novel adjunct therapies. One of the core regulatory pathways that mediates autophagy is the mammalian target of rapamycin (mTOR). Rapamycin,
a known immunosuppressive drug, binds to mammalian target of Rapamycin complex 1 (MTORC1), blocking its downstream signaling and leading to induction of autophagy (Jung et al., 2010, Noda and Ohsumi, 1998, Dumont and Su, 1996, Thomson et al., 2009). Rapamycin treatment induced autophagy decreases intracellular mycobacterial survival as seen in murine bone marrow-derived macrophages and primary human monocytes (Gutierrez et al., 2004). A similar reduction in intracellular bacterial load was also reported in THP1 macrophages treated with rapamycin (Gupta et al., 2014). We (Verma et al., 2019) and others (Harper et al., 2011, Kramnik et al., 2000, Pan et al., 2005, Ordonez et al., 2016, Irwin et al., 2015, Ong et al., 2014) have shown that the C3HeB/FeJ mice develop caseating, necrotic granulomas in the lung during chronic Mtb infection, closely resembling pulmonary pathology seen in TB patients. Hence, we employed this mouse strain as our experimental model to study the effect of rapamycin treatment on lung inflammation and immunopathological disease, and the potential use of rapamycin as an adjunct HDT candidate during TB treatment.

In this study, we observed that treatment with rapamycin alone or as adjunct therapy led to a significant reduction in lung inflammation and immunopathology in C3HeB/FeJ mice during Mtb infection. This treatment did not have any substantial impact on lung and spleen bacterial burden. Overall, these findings highlight the use of C3HeB/FeJ mice as a valuable model system to evaluate HDT candidates targeted against infection-induced pathology and further emphasize the opportunity that adjunct therapeutic agents, such as rapamycin and its analogs, present in modulating disease outcomes in a real-world setting.

MATERIALS AND METHODS

Ethics statement
All animal experiments described in this study conform to the Rutgers University Biomedical Health Sciences-Newark (RBHS) and Institutional Animal Care and Use Committee (IACUC) Guidelines as well as NIH and USDA policies on the care and use of animals in research and teaching. Efforts were taken to ensure minimal animal pain and suffering and when applicable, approved anesthesia methods were employed for the same.

Mice and aerosol infection
6-8 weeks old female C3HeB/FeJ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and infected with a low dose (~50 to 100 CFU) of Mtb Erdman strain (Trudeau Institute, Saranac, NY) using Glass-Col Full Body Inhalation Exposure. For all infections, the
actual infection dose was determined by plating total lung homogenates from a minimum of 3 mice on Middlebrook 7H11 plates at 24 hours after aerosol exposure, and colony forming units (CFU) were determined after 3-4 weeks incubation at 37°C. Mtb-infected mice were housed in the animal biosafety level 3 (BSL3) facility, and guidelines from Rutgers-NJMS Institutional Animal Care and Use Committee (IACUC) were followed in handling the mice.

**Estimation of the bacterial burden from lung and spleen**
Whole lungs and spleens were excised from the infected mice at the indicated time points and homogenized in PBS with 0.05% Tween 80. Lung and spleen homogenates were plated on 7H11 agar in serial dilutions. Colony forming units from lung and spleen were determined after incubation at 37°C for 3-4 weeks.

**Rapamycin diet and moxifloxacin administration**
We obtained microencapsulated rapamycin (eRAPA) from Rapamycin Holding Inc, Texas. The control diet contained an equivalent amount of Eudragit S100 enteric polymer (eudragit), the encapsulating polymer. Diet containing this eudragit microencapsulated rapamycin was custom manufactured by TestDiet (St. Louis, MO). The eRAPA diets contained active rapamycin at either 14ppm (for experiments presented in Figures 3 to 8; Supplementary Figure 2) or 42 ppm (for experiments presented in Figures 1 and 2; Supplementary Figures 1 and 5) (Harrison et al., 2009). Mice were fed these eRAPA or eudragit diets daily for the indicated time period. Solution of moxifloxacin (Sigma) in distilled water was prepared weekly and stored at 4°C. Moxifloxacin at a concentration of 100mg/kg in distilled water was administered to mice by oral gavage for five days per week, at indicated time intervals.

**Cytokine and chemokine estimation in lung homogenates**
Whole lungs from infected animals were homogenized in 0.05% PBS tween. After homogenization, a fraction of the lung homogenate was treated with 2X protease inhibitor (ThermoFisher Scientific) at the time of collection and frozen at -80°C. Mouse cytokine ultrasensitive immunoassay (mouse pro-inflammatory V panel catalog# K15048D-2; Meso-Scale Discovery, Gaithersburg, MD) was used to quantify cytokine and chemokine levels. The plates were read on the MSD detector (Sector Imager 2400, MSD, Gaithersburg, MD). The assays are based on the principle of electrochemiluminescence (ECL) sandwich ELISA. The calculations to establish calibration curves and determine analyte concentrations were carried out using the MSD DISCOVERY WORKBENCH analysis software.
Histopathological assessment
Post-mortem, lungs of Mtb-infected mice were perfused with sterile PBS and subsequently fixed in 4% paraformaldehyde for seven days, followed by paraffin embedding. For histopathological analysis, 5- to 7-μm sections were cut and stained using a standard H&E protocol. Leica SCN400 F whole-slide scanner (Experimental Pathology Research Lab, NYU Langone Health) was used for scanning histological sections and images were analyzed using Aperio ImageScope. For quantitation of granulomatous inflammation in the lung section, Image-Pro Discovery Software was used to create a grid overlay onto each photomicrographs of H&E stained lung section and numbers of points hitting areas of granulomatous infiltration were counted. Mosaic images were created using Surveyor software with Turboscan by objective imaging at 20X. Histopathological evaluations were performed with blinding to the type of diet that the animals were on.

Rapamycin measurement
Uninfected 6-8 weeks old female C3HeB/FeJ mice were fed either eudragit control or eRAPA diets, for 17 days. Mice were then euthanized, and blood was collected via cardiac puncture in microtubes coated with heparin (Sarstedt # 41.1393.105). Samples were stored at -80°C until shipment. Tubes were later shipped to University of Texas HSC Biological Psychiatry Analytical Lab for the estimation of rapamycin using liquid chromatography mass spectrometric analysis. This analysis was repeated twice in two independent tests.

Flow cytometry
At indicated time points, lungs were perfused with 10 ml of PBS and then middle and inferior lobes of the right lung were harvested. Lung tissue was digested in 2 mg/ml collagenase D (Roche) at 37°C for 30 minutes and 10 mM EDTA was added to halt the reaction. The digested tissue was then mashed through 40 um, nylon filter using plunger of the syringe. The single-cell suspension was then centrifuged at 1200 rpm for 10 minutes and the cell pellet was subjected to RBC lysis using ammonium chloride potassium (ACK) lysis buffer (Quality Biologicals Inc.). Live cell count was then determined using the trypan blue exclusion method. 1 X10^6 live single cells from each sample were then surface stained with directly conjugated fluorochrome-labeled anti-mouse CD4-V450 (clone RM4-5; BD Horizon), anti-mouse CD8-AF488 (clone 53-6.7; BD Pharmingen), anti-mouse B220-PECF594 (clone RA3-6B2; BD Pharmingen), anti-mouse CD11b-APC Cy7 (clone M1/70; BD Pharmingen), anti-mouse Ly6G-BV605 (clone 1A8; BD Pharmingen), anti-mouse CD11c-AF700 (clone HL3; BD Pharmingen), and anti-mouse
Ly6C-APC (clone HK1.4; Biolegend). Following surface staining, cells were fixed in 4% paraformaldehyde and then acquired on BD Fortessa flow cytometer. Analysis was performed using FlowJo software (Tree star). Gating for the myeloid population was based on fluorescence minus one control.

**Immunohistochemistry**

Tissue sections were deparaffinized in xylene and hydrated with ethanol gradations and water. Ly6G, CD20, and CD3 epitopes were retrieved by the heat-induced antigen retrieval method using 10 mM citrate buffer pH6. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide and then subsequently blocked with 1X Power block (Bio Genex). Sections were then incubated with the primary goat anti-mouse CD20 antibody (clone M-20; Santa Cruz), rat anti-mouse Ly6G antibody (clone 1A8; Bio legend), rat anti-mouse CD3 antibody (clone 17A2, Biolegend) at 1:100 dilution overnight at 4ºC in Power Block. Sections were then washed with PBS-0.05% Tween 20 and then incubated with biotinylated secondary antibodies (Goat anti-Rat IgG, BD 559286; Rabbit anti-goat IgG BA-5000 Vector Laboratories) at 1:100 dilution for 45 minutes. Streptavidin horseradish peroxidase (Biogenex, HK3305K) was used to label the secondary antibody for immunodetection by DAB chromogen (Biogenex). After counterstaining with Mayer’s hematoxylin (Biogenex), the samples were dehydrated with ethanol gradations, dipped in xylene, mounted using Cytoseal (Thermo scientific).

**Statistics**

Graph Pad Prism software was used to perform statistical analyses. Unpaired t-test was used to compare two groups. One-way ANOVA was performed to determine the statistical significance for more than two groups. In all experiments, a p-value <0.05 or lower was considered statistically significant.

**RESULTS**

**Rapamycin treatment alone decreases immunopathology in Mtb infected mice**

Addition of rapamycin to *in vitro* macrophage cultures induces autophagy and results in significant control of intracellular bacterial replication (Gutierrez et al., 2004). We therefore asked whether rapamycin treatment similarly would reduce bacterial burden *in vivo* in Mtb infected mice. The phosphorylation of ribosomal protein S6 is widely used as an indicator of
mTOR activity. Therefore, to confirm that rapamycin was active in the lungs of infected mice, lung tissue sections from Mtb-infected mice fed either eudragit or eRAPA diets for two weeks were stained with antibodies reactive against phosphorylated ribosomal protein S6. eRAPA treatment led to significant reduction in phospho-S6 expression in bronchial epithelial lining compared to control animals treated with eudragit (Supplementary Fig. 1). This indicates that the mTORC1 pathway is inhibited in eRAPA-treated animals and confirms that rapamycin is active in Mtb-infected lungs.

We first evaluated whether rapamycin alone would reduce bacterial burden and in addition alleviate disease pathology. Mice were started on eudragit only diet or rapamycin in eudragit diet (eRAPA) at different time points following Mtb infection. To rule out the possibility of discrepancies arising from differential rapamycin uptake, we also assessed the serum concentration of rapamycin in uninfected animals and found very little variance in steady state serum rapamycin levels (Supplementary table 1).

We found that when mice were administered eRAPA starting at week 2 post Mtb infection, all 5 mice in the eRAPA-treated group had to be euthanized around 2 weeks since they showed signs of becoming moribund. Gross observation of the lungs indicated severe immunopathology. This suggests that initiating rapamycin treatment early on likely affects the development of T cell immunity and so this treatment regimen was not used later for testing adjunct activity of rapamycin with moxifloxacin. In the succeeding experiment, eRAPA was administered for four weeks starting at week 4 post Mtb infection and referred to as regimen 1 (Supplementary Fig. 2). Evaluation of bacterial numbers in the lung at end of treatment (8 week following Mtb infection) showed that contrary to what was observed in vitro, eRAPA treated mice actually exhibited a small increase in bacterial burden in lung and spleen, albeit not significant (Supplementary Fig. 3).

Initiation of rapamycin treatment in the chronic phase most closely resembles the clinical situation where HDT would be initiated during active disease. In the next regimen (regimen 2; Supplementary Fig. 2), eRAPA treatment was therefore initiated in the chronic phase at week 7 post-Mtb infection and administered for only two weeks. In this second treatment regimen, we found a trend towards decreased CFU in eRAPA treated mice but compared to control mice the difference between the two groups was not statistically significant (Fig. 1A). Flow cytometric analysis showed that the percentage of live cells in lung single cell suspensions was significantly higher in eRAPA treated group, but the percentage of neutrophils (CD11^b^Ly6G^hi^) was significantly lower in this group compared to the eudragit group (Fig. 1B). Expression of
Interferon (IFN)γ, interleukin (IL)-1β, IL-6, CXCL1 (KC) and Tumor Necrosis Factor (TNF) was similar in the two groups (Fig. 1C). Histopathological examination of lung tissue (left lung lobe) revealed significant dampening of immunopathology in eRAPA treated mice. As shown in Figure 2, lungs from 4 of 7 mice (Fig. 2-M2, M4, M5, M7) in the eudragit treated group showed focal-to-multifocal caseating necrotic granulomas that were encapsulated with central cores of acellular necrotic cell debris. In the eRAPA treated mice 3 of the 8 mice (M2, M6, M7) showed caseating necrotic granulomas but they were smaller and not coalescing to occupy major areas of the lung (Fig. 2). Next, we determined if rapamycin given by oral gavage would be more effective than that given in the diet. Following regimen 2, Mtb infected mice at 7 weeks of infection were administered via gavage with either orange juice alone or rapamycin (0.04 mg/mouse) in orange juice (oRAPA) for 2 weeks. Comparison of lung histology revealed marked differences in the number of necrotic lesions and overall parenchymal inflammation between the oRAPA treated and control groups (Supplementary Fig. 4). Of the nine mice in the oRAPA treated group, three (M3, M4, M9) did not develop any necrotic lesions, three (M1, M5 and M7) had one to two lesions and three (M2, M6, M8) had greater than three lesions. In contrast, all the eight control mice developed necrotic lesions with 4 (M4, M2, M7, M8) exhibiting between one and two lesions and the remaining 4 (M1, M2, M3, M6) showing greater than three lesions. Despite the recognized unevenness in necrotic lesion development in C3HeB/FeJ mouse strain, the data presented so far provide firm evidence that in mice receiving rapamycin there is a conspicuous reduction in lung inflammation and immunopathology.

**Prolonged four weeks of rapamycin treatment with moxifloxacin significantly reduces immunopathology**

The data presented so far indicate that rapamycin treatment did not reduce bacterial numbers in Mtb infected mice, but there was appreciable decrease in immunopathology. The length of TB treatment is extremely long i.e., 6 months for drug susceptible TB and more than 18 months for drug resistant TB. We argued that the ability of rapamycin to modulate immunopathology may be beneficial during TB treatment by enhancing drug efficacy. So, we next evaluated if adjunct treatment with rapamycin would affect treatment outcome as measured by bacterial burden and immunopathology. Rapamycin’s adjuvant activity with the TB drug moxifloxacin was first tested using regimen 1 (Supplementary Fig. 2) which is four weeks of eRAPA treatment initiated at week four post Mtb infection in C3HeB/FeJ mice. Moxifloxacin was administered five times a week via oral gavage and given for the same length of time as eRAPA. One cohort of infected
and treated mice was used for determining bacterial burden and another for histopathological evaluation. As expected, mice receiving eudragit diet and moxifloxacin treatment significantly reduced the bacterial burden in both lungs and spleen, compared to control animals that received only eudragit diet (Fig. 3A). In the presence of moxifloxacin also, mice receiving eRAPA showed marginally higher bacterial CFU, albeit not significant, when compared to control mice receiving eudragit diet and moxifloxacin (Fig. 3A). Despite the marginal increase in CFU in the moxifloxacin + eRAPA treated group compared to moxifloxacin + eudragit group, IFN$\gamma$ expression was however similar in both the groups (Fig. 3B). Histopathological evaluation showed a significant decrease in disseminated lung inflammation and associated immunopathology in animals that received combination treatment of eRAPA and moxifloxacin as compared to those that received moxifloxacin with eudragit control diet (Fig. 3C and 3D). Of the six mice in the group receiving moxifloxacin treatment in control eudragit diet, four developed necrotic lesions whereas only two of six mice in the eRAPA+moxifloxacin group exhibited necrotic lesions. Of note, both the mice in the eRAPA treated group had only one lesion each.

Two-week adjunct rapamycin treatment with moxifloxacin during chronic phase does not substantively augment Mtb control

Next, we administered eRAPA diet to C3HeB/FeJ mice for 2 weeks, starting at a relatively chronic stage of infection (7 weeks post Mtb-infection). At this time, moxifloxacin was also administered five times a week via oral gavage, while the animals were either on eRAPA or eudragit control diet (Supplementary Fig. 2). Here too we had two cohorts, and whole lungs and spleen were harvested from euthanized animals at week 9 from one cohort to determine bacterial burden and from the other cohort all lung lobes were harvested for histopathological evaluation. Not surprisingly, moxifloxacin administration led to a significant decrease in both lung and spleen bacterial burden in animals on eRAPA and eudragit diet (Fig. 4A). Consistent with decreased cfu, mice in both the moxifloxacin treated groups had significant reduction in pulmonary expression of IFN$\gamma$, IL-1$\beta$, IL-6 and TNF compared to the group without moxifloxacin (Fig. 4B). Rapamycin is an immunosuppressive drug and inhibits immune responses including CD4$^+$ T cell activation (Thomson et al., 2009) and thus could significantly impact the host’s ability to control Mtb infection. Although not statistically significant, lower bacterial burden in the eRAPA+moxifloxacin compared to the eudragit + moxifloxacin group (Fig. 4A), nonetheless,
suggests that the immunosuppressive property of eRAPA is voided if administered later during infection.

**Two-week adjunct rapamycin treatment with moxifloxacin during chronic infection decreases infiltrative pulmonary pathology and formation of necrotic lesions**

Since mice treated with eRAPA in the absence of TB drugs had reduced necrotic lesions in chronic infection, we next determined if rapamycin given as adjunct therapy would enhance treatment outcome with moxifloxacin. Evaluation of H&E stained, lung sections from Mtb infected animals treated with eRAPA and moxifloxacin at week 7 post-infection through week 9, showed notably fewer numbers of necrotic lesions, compared to those on eudragit and moxifloxacin diet (Fig. 5A). Of the five mice that were treated with moxifloxacin and on eudragit diet, three (M1, M2, M4) had greater than two lesions, one (M3) had 1 lesion and one (M5) had no lesion. In contrast, of the five mice receiving moxifloxacin and eRAPA, three (M1, M2, M5) developed necrotic lesions but of note none of the three had greater than 2 lesions. Quantification of lung inflammation showed a significant decrease in inflammation in the eRAPA group (Fig. 5B). Representative lung images show that the animals on moxifloxacin monotherapy and control eudragit diet had highly infiltrative pulmonary pathology (Fig. 5D i), as compared to the animals on the eRAPA and moxifloxacin regimen (Fig. 5D ii). Animals that received moxifloxacin with eudragit diet had significant thickening of the lung interstitium, likely due to cellular infiltration (Fig. 5D iii). In mice that received adjunct treatment with eRAPA, there were significantly more open alveolar spaces with less inflamed alveolar septae (Fig. 5D iv).

Representative necrotic lesions (Supplementary Fig. 5) from both groups were closely evaluated to determine if eRAPA adjunct treatment with moxifloxacin not only reduced the number of necrotic lesions but also modified them qualitatively. H&E staining of paraffin-embedded lung sections revealed that the necrotic lesions from both groups had a central acellular caseum that contained large numbers of neutrophil karyorrhectic debris and were well-defined, circumscribed lesions (Supplementary Fig. 5). The parenchymal area surrounding the lesion in eRAPA treated appeared more normal compared to that from eudragit treated group (Supplementary Fig. 5). Higher magnification revealed that the necrotic lesions from both groups were surrounded by a ring of foamy macrophages (FM) and an outer layer of fibroblasts (F) (Fig. 6). Immunohistochemical staining was performed to further characterize the immune infiltrates surrounding the necrotic lesions. Examination of the large necrotic lesions from both
groups showed that the necrotic lesion in eudragit and moxifloxacin treated mice was surrounded by a rim of intact and necrotic neutrophils in the foamy macrophage and fibroblast layers (Fig. 6). In contrast, in the eRAPA + moxifloxacin treated group, intact neutrophils were seen around the lesion, albeit significantly fewer than that observed in the rim from eudragit+ moxifloxacin treated mice. Lesions from both groups showed presence CD20⁺ B cells, but no CD3⁺ T cells were discernible (Fig. 6).

Moxifloxacin and eRAPA treated mice also exhibited smaller necrotic lesions (Fig. 7) and therefore we next analyzed the staining pattern in this lesion type and found that it contained a central neutrophilic core with surrounding fibrotic area and an outer rim of B cell aggregates (Fig. 7). Small indistinct CD3⁺ clusters were present within the B cell aggregates (right CD3 panel). Similar small necrotic lesions were not apparent in eudragit-treated mice. Examination of inflammatory regions outside of the necrotic lesions revealed distinct composition of cells within the cellular aggregates. As seen in Fig. 8, the cellular aggregate in eudragit+moxifloxacin treated mice included small numbers of CD20⁺ B and CD3⁺ T cells but contained numerous neutrophils whereas in eRAPA+moxifloxacin treated mice they predominantly comprised of CD20⁺ B cells and contained no neutrophils. Overall, the mice that received eRAPA adjunct therapy with moxifloxacin developed granulomas that appeared as tight, well-contained cellular aggregates and showed minimal lung inflammation even during chronic stages of Mtb infection.

**DISCUSSION**

Autophagy-targeted HDTs are attractive since data from several studies indicate that activation of this pathway not only enhances bacterial killing (Watson et al., 2012, Gutierrez et al., 2004, Parihar et al., 2014, Stanley et al., 2014, Schiebler et al., 2015) but also dampens harmful inflammation (Castillo et al., 2012, Bonilla et al., 2013). The mTOR signaling pathway inhibits autophagy (Reviewed in (Kubota et al., 1968)) and consequently mTOR inhibitors provide a host-directed therapeutic strategy to promote autophagy induction in Mtb infection. In this regard, the AMP-activated protein kinase (AMPK) stimulates autophagy by inhibition of mTOR phosphorylation through tumor suppressor complex (TSC) (Gwinn et al., 2008, Inoki et al., 2003). Thus, metformin that stimulates AMPK to mediate inhibition of mTOR signaling has been shown in the mouse model to restrict intracellular growth of Mtb and reduce immunopathology (Singhal et al., 2014).
In this study we evaluated the performance of rapamycin, an mTOR inhibitor, on modulating bacterial burden and lung pathological disease during Mtb infection. The data presented here demonstrate that mice that received rapamycin alone or as adjunct treatment with moxifloxacin had significantly less lung inflammation compared to those on eudragit diet or moxifloxacin and eudragit diet. Lung architecture significantly improved in mice on eRAPA diet as seen from decreased cellular infiltration around necrotic lesions, presence of discrete solid lesions and more open alveolar spaces. Albeit not statistically significant, a greater number of mice on eRAPA diet had no or fewer necrotic granulomas both at weeks 8 and 9 post-infection thus showing an overall reduced occurrence of these lesions when compared to animals on eudragit control diet. Interestingly, eRAPA treated mice exhibited B cell aggregates with concomitant decrease in neutrophilic inflammation. Given that B cells regulate neutrophilia during Mtb infection (Kozakiewicz et al., 2013, Maglione et al., 2007), future studies should examine the mechanisms of how suppression of inflammation by rapamycin can promote B cell aggregation and how that contributes to tempering immunopathology. Initiation of rapamycin treatment in the chronic phase for 2 weeks had overall better outcome in the mice suggesting that the timing of rapamycin initiation and the duration of treatment is critical for its performance as adjunct therapy. The C3HeB/FeJ model system will allow further interrogation of these variables along with rapamycin analogs. The model system will also provide a system to study whether modulating necrotic lesion development with rapamycin analogs will impact on the evolution of bacterial drug resistance. Overall, the findings provide a strong basis for future studies to dissect mechanisms of necrotic lesion evolution and the impact of rapamycin and its analogs to this process.

Pulmonary TB can lead to a plethora of post-disease complications including alteration of lung parenchyma, bronchiectasis, and scarring of the lung (Ravimohan et al., 2018). Even after successful completion of treatment, severe lung impairment can potentially impact the patient’s quality of life (Meghji et al., 2020). Of note, is the complex interplay between co-morbid diseases such as chronic obstructive pulmonary disease (COPD) and bronchiectasis with TB, which can further exacerbate the dysregulation of host immune responses and often lead to deleterious outcomes for the host (Byrne et al., 2015). A population-based cohort study indicated that the relative risk of developing active TB and subsequent risk of mortality in COPD patients was higher versus the general population (Inghammar et al., 2010). Prior history of TB can also play an important role in the natural course of COPD, as it has been reported that patients with past TB who were diagnosed with COPD, died 5 years earlier as compared to the patients without TB (Yakar et al., 2017). Also, treatment with inhaled corticosteroids in patients with COPD
Huang et al., 2020), has been associated with a significantly higher risk of TB, as shown in meta-analysis of clinical trials (Dong et al., 2014) and non-randomized studies (Castellana et al., 2019). Thus, the contribution and impact of pulmonary TB to the etiology of other chronic respiratory diseases, and vice-versa, needs to be critically considered when designing treatment modalities in TB-endemic areas.

Dampening excessive, systemic inflammation and downstream tissue damage is by far the most important metric to improving outcomes for patients with active TB. Biological agents or repurposed drugs that can interfere with biologically relevant cellular checkpoints, can have high potential to act as ‘target-organ saving’ strategy (Zumla et al., 2015). Anti-inflammatory agents such as vitamin D, phenylbutyrate, prednisolone and others have been shown to play a role in improving sputum smear conversion rate and chest radiological appearance, making them noteworthy and viable HDT candidates (Hayford et al., 2020). Also of interest is the potentially beneficial role of non-steroidal anti-inflammatory drugs (NSAIDs), in mitigating disease severity and reducing lung inflammation when used as adjunct treatment along with standard anti-TB drugs (Kroesen et al., 2017).

Published work has shown that treatment with rapamycin inhibits inflammation and airway hyperreactivity in the disease models of COPD and asthma, which is consistent with our findings (Zhang et al., 2019, Mushaben et al., 2011, Mitani et al., 2016). Here, in this study we demonstrate in a mouse model that treatment with rapamycin during chronic infection noticeably ameliorates lung inflammation without deleterious effects on bacterial burden. However, there are several important issues that still need to be addressed for the application of mTOR inhibitors as HDT against TB. Treatment with everolimus induced reactivation of latent tuberculosis in organ transplant patients (Guirao-Arrabal et al., 2016). It would be important to understand how mTOR inhibitors (including everolimus) function as effective adjunct therapy in diverse clinical settings since rapamycin analogs including everolimus are currently being investigated as HDTs against TB in phase 2 clinical trials (Kim et al., 2004). CC-11050 and everolimus were safe and reasonably well tolerated as adjunctive therapies for tuberculosis, and analysis of preliminary efficacy suggests they might also enhance the recovery of FEV₁, a key measure of lung function and predictor of all-cause mortality (Wallis et al., 2021).

Pulmonary cavitation has been observed in Mtb aerosol infected C3HeB/FeJ mice through serial computed tomography (CT) imaging (Ordonez et al., 2016), further indicating that with advanced imaging tools these mice could serve as a very helpful tool for studying TB progression, pathogenesis and cavitary disease. Thus, the key similarities with human disease,
make C3HeB/FeJ mice a valuable small animal model for studying efficacy of anti-tuberculosis interventions in bacterial control and mitigation of disease pathology.

CONFLICT OF INTEREST
The authors declare no conflict of interest

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Figure 1. Modulation of bacterial burden and pulmonary cell infiltration in mice treated with eRAPA alone during chronic Mtb infection.

C3HeB/FeJ mice were infected with ~50-100 CFU of Mtb Erdman. Seven weeks post Mtb infection, mice were divided into two groups and fed either eudragit (microencapsulation polymer) control diet or eRAPA (eudragit encapsulated rapamycin) diet for two weeks. Thereafter, the animals were euthanized and whole spleen as well as superior lung lobe were homogenized for estimating the bacterial burden (A). Single cell lung preparations were obtained, and trypan blue exclusion method was used to calculate the total live cells in the lungs of the mice at 9 weeks post-infection (B). Lung cells from both groups of mice were also surface stained with directly conjugated antibodies to quantitate immune cell subsets by flow cytometry.
(B). Lung lysates were obtained from both groups of mice on either eudragit or eRAPA diets, after homogenizing lung tissue in 1ml PBS and 2X protease inhibitor (ThermoFisher). Levels of different immune mediators were evaluated in filtered cell-free lysates using multiplex MesoScale Discovery (MSD) platform (C). Eudragit group had n=7 mice, and eRAPA group had n=8. Data are presented as mean +/- SEM. Statistical significance was calculated using one-way ANOVA with Kruskal Wallis test and significance is shown as ** P < 0.005; *** P < 0.0001.
Figure 2. Differential granulomatous response and necrotic lesion development in mice treated with eRAPA alone during chronic Mtb infection.

Mice were infected with low dose (~50-100 CFU) of Mtb Erdman and at 7 weeks post Mtb infection, they were either fed eudragit diet or eRAPA diet for two weeks. Corresponding mosaic H&E images of lung tissue from each animal in the two groups are shown here. These images were created using Surveyor software with Turboscan by objective imaging at 20X.
Figure 3. Treatment with eRAPA and moxifloxacin during acute Mtb infection modulates lung immunopathology.

Six- to eight-week-old C3HeB/FeJ mice were infected with ~50-100 CFU of Mtb Erdman and then categorized into three groups (eudragit control diet with no treatment, moxifloxacin + eudragit and moxifloxacin + eRAPA). These mice were treated for four weeks starting week 4 post infection till week 8, and then euthanized. Bacterial burden in the lungs and spleen was determined (A). Levels of IFNγ were evaluated in filtered cell-free lysates using multiplex MSD (B). Multi-lobe lung histopathological analysis was conducted, and necrotic granulomas are marked with blue asterisk (C). Total lung area under infection-induced inflammation (D) was also determined. Each group included 5-6 mice. Data are presented as means ± standard errors of the means. Statistical significance was calculated using either unpaired t test (two groups), one-way ANOVA (three groups) with Bonferroni’s correction or one-way ANOVA with Kruskal Wallis test, and significance is represented as * P < 0.5; *** P < 0.005; **** P < 0.0005.
At week 7 post infection, Mtb Erdman infected C3HeB/FeJ mice were divided into three groups (eudragit control diet with no treatment, moxifloxacin + eudragit and moxifloxacin + eRAPA) and were then treated for two weeks up to week 9, when they were euthanized. Bacterial burden (A) and cytokine analysis in homogenized lung lysates (B) were carried out at this timepoint. Five mice were included in each group. Data are presented as means ± standard errors of the means. Statistical significance was calculated using one-way ANOVA with Kruskal Wallis test and significance is shown as * P < 0.05; ** P < 0.005.
Figure 5. eRAPA adjunct treatment with moxifloxacin reduces number of necrotic lesion development and infiltrative pulmonary pathology

H&E images of lung tissues from Mtb infected C3HeB/FeJ received either moxifloxacin with eudragit or moxifloxacin and eRAPA from week 7 to week 9 post-infection were scanned using Leica SCN-400 F whole slide scanner up to 40X magnification. Images were captured at various magnifications using Aperio ImageScope to show histopathological differences between the two groups. Presence of necrotic granulomas is indicated with blue asterisk (A). Inflamed lung area was calculated using Image J (B). Differences in pulmonary pathology (C i and ii) and thickening of lung interstitium (C iii and iv) between the two groups are shown using representative images at 200µm (10X) and 70µm (30X) magnification. Data are presented as means ± standard errors of the means. Statistical significance was calculated using unpaired t test, and significance is represented as ** P < 0.005. Five mice were included in each group.
Figure 6. Distinct cellular infiltrates surround necrotic lesions in eRAPA and eudragit treated mice.

Mtbd infected C3HeB/FeJ mice were fed either eudragit or eRAPA diets, along with oral gavage of moxifloxacin from week 7 to week 9 post-infection. Formalin-fixed paraffin-embedded lung sections from these groups were then H&E stained or immunostained for Ly6G+ (neutrophils), B220+ (B cells) and CD3+ (T cells). These sections were scanned using Leica SCN-400 F whole slide scanner up to 40X magnification. Five mice were included in each group. Representative images shown here at the 200 µm scale and 20X magnification.
Figure 7. Small necrotic lesions and B cell aggregates observed in eRAPA and moxifloxacin treated mice.

Standard H&E staining and immunostaining with anti-Ly6G, anti-CD20, or anti-CD3 was performed on formalin-fixed paraffin-embedded lung section from Mtb Erdman infected C3HeB/FeJ mice that were fed eRAPA diet during moxifloxacin monotherapy. Representative scanned images were captured to show a small necrotic lesion with B cell aggregates surrounding it, as indicated with the red square. These sections were scanned up to 40X using Aperio ImageScope. Representative images shown here are at 500 µm scale at 4.5X zoom (H&E, left column) and 70 µm scale at 30X (right column). Five mice were included in this group.
Figure 8. Reduced neutrophil infiltration in lungs of eRAPA and moxifloxacin treated mice.

Formalin-fixed paraffin-embedded lung sections were obtained from Mtb Erdman infected C3HeB/FeJ mice that were given either control diet or eRAPA (starting at week 7 post-infection) along with moxifloxacin monotherapy. Standard H&E staining and immunostaining with anti-Ly6G, anti-CD20, or anti-CD3 was performed on these lung sections. Representative images were captured to show inflamed granulomatous areas in the lung. These sections were scanned up to 40X using the Leica SCN-400 F whole slide scanner. All images shown here are at 70 µm scale and 30X magnification. Five mice were included in each group.
Fig. S1. Treatment with eRAPA leads to reduced expression of phospho-S6 ribosomal protein in alveolar bronchiole epithelial cell lining.
C3HeB/FeJ mice infected with low dose of Mtb Erdman were either fed eudragit or eRAPA diets, starting at week 2 post-infection. The animals were then euthanized 2 weeks after the initiation of treatment regimen. PhosphoS6 protein (Santa Cruz, sc-293144) was detected using immunofluorescence in formalin fixed paraffin embedded lung sections of these eudragit (control) or eRAPA treated mice. Nuclear counterstaining was done using DAPI. Images were captured on Nikon A1R confocal laser scanning microscope and analyzed using NIS Elements. This image is representative of 5-6 animals in each group.
Fig. S2. Experimental scheme.
Schematic overview of the timeline for Mtb infection, eRAPA treatment and tissue harvest for moxifloxacin monotherapy.

Fig. S3. Bacterial burden in Mtb-infected animals receiving eudragit or eRAPA at 4 weeks following Mtb infection.
C3HeB/FeJ mice were infected with low dose of Mtb Erdman. Four weeks post Mtb infection, mice were either fed eudragit or eRAPA diets. The animals were sacrificed 4 weeks after the initiation of treatment regimen. Whole lungs and spleens were homogenized for bacterial burden estimation. Six to eight animals were included in each group. Data are presented as mean ± standard errors of the means. Statistical significance was calculated using one-way ANOVA with Kruskal Wallis test.
Fig. S4. eRAPA delivered by oral gavage leads to significant changes in histopathology. At week 7 post Mtb infection, C3HeB/FeJ mice were given eRAPA in orange juice at a daily dose of 0.04mg for two weeks, via oral gavage. All lung lobes processed for histology. H&E stained sections were scanned up to 40X using the Leica SCN-400 F whole slide scanner. Necrotic lesions are marked with blue asterisk and each group had 8 to 9 animals.
Fig. S5. H&E stained sections comparing necrotic lesions from eudragit and moxifloxacin versus eRAPA and moxifloxacin groups. Representative scanned H&E images of areas in and around necrotic lesions of lung tissue sections of mice from eudragit and eRAPA treated groups at week 9 postinfection. The scale is 1 mm, and five mice were included in each group.
Table S1. Estimation of rapamycin in murine serum samples

<table>
<thead>
<tr>
<th>Test</th>
<th>Volume</th>
<th>Rapamycin (ng/mL)</th>
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</thead>
<tbody>
<tr>
<td><strong>Test I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eudragit</td>
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<td>&lt; 1.56</td>
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<tr>
<td></td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Rapamycin mean (+/-SD)</td>
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</tr>
<tr>
<td><strong>Test II</strong></td>
<td></td>
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</tr>
<tr>
<td>Eudragit</td>
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<td>&lt; 1.56</td>
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<tr>
<td></td>
<td>50 µl</td>
<td>237</td>
</tr>
<tr>
<td>Rapamycin mean (+/-SD)</td>
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<td></td>
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</tbody>
</table>

Note: values <1.56 are below detectable limit.