Adjunct antibody administration with standard treatment reduces relapse rates in a murine tuberculosis model of necrotic granulomas

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Abstract

Matrix metalloproteinase (MMP)-9 is a zinc-dependent protease associated with early immune responses to \textit{Mycobacterium tuberculosis} infection, macrophage recruitment and granuloma formation. We evaluated whether adjunctive inhibition of MMP-9 could improve the response to standard TB treatment in a mouse model that develops necrotic lesions. Six weeks after an aerosol infection with \textit{M. tuberculosis}, C3HeB/FeJ mice received standard TB treatment (12 weeks) comprising rifampin, isoniazid and pyrazinamide alone or in combination with either anti-MMP-9 antibody, etanercept (positive control) or isotype antibody (negative control) for 6 weeks. Anti-MMP-9 and the isotype control had comparable high serum exposures and expected terminal half-life. The relapse rate in mice receiving standard TB treatment was 46.6%. Compared to the standard TB treatment, relapse rates in animals that received adjunctive treatments with anti-MMP-9 antibody or etanercept (positive control) or isotype antibody (negative control) for 6 weeks. Anti-MMP-9 and the isotype control had comparable high serum exposures and expected terminal half-life. The relapse rate in mice receiving standard TB treatment was 46.6%. Compared to the standard TB treatment, relapse rates in animals that received adjunctive treatments with anti-MMP-9 antibody or etanercept were significantly decreased to 25.9\% ($P = 0.006$) and 29.8\% ($P = 0.019$) respectively, but were not different from the arm that received the isotype control antibody (25.9\%). Immunostaining demonstrated localization of MMP-9 primarily in macrophages in both murine and human lung tissues infected with \textit{M. tuberculosis}, suggesting the importance of MMP-9 in TB pathogenesis. These data suggest that the relapse rates in \textit{M. tuberculosis}-infected mice may be non-specifically improved by administration of antibodies in conjunction with standard TB treatments. Future studies are needed to evaluate the mechanism(s) leading to improved outcomes with adjunctive antibody treatments.
Introduction

*Mycobacterium tuberculosis* is a global threat and a major cause of human morbidity and mortality [1]. The development of multi-drug resistant strains and the limited arsenal of effective tuberculosis (TB) treatment regimens, which need to be administered for prolonged periods (6 months for fully drug susceptible TB), are major challenge to controlling TB leading to an urgent need for shorter regimens. Most of the current components of TB treatments target actively replicating bacteria and thus require protracted treatments to eliminate the slow-replicating “persistent” bacteria and achieve stable cure. Moreover, it is possible that the early host-responses to *M. tuberculosis*, i.e., necrosis, hypoxia, granuloma formation and cavitation, could be detrimental to bacterial clearance [2].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases known to degrade collagen and remodel the extracellular matrix and basement membranes [3]. Multiple MMPs have been associated with TB pathogenesis, including MMP-9, an extracellular gelatinase that specifically degrades type IV and V collagen [4–7]. In humans with pulmonary, pleural or CNS TB, increased MMP-9 activity in serum and CSF correlates with increased disease severity and worse outcomes [8–10]. Antisense knock down of MMP-9 in zebrafish infected with *Mycobacterium marinum* attenuated granuloma formation and bacterial growth [11]. In *M. tuberculosis*-infected mice, disruption of MMP-9 expression or activity reduces macrophage infiltration into the lungs, leading to the dysregulation of granuloma formation and reduced disease burden [12]. Therefore, inhibiting MMP-9 expression or activity could disrupt TB-associated granulomas thereby enhancing the activity of antimicrobials against persistent bacteria. Here, we evaluated an inhibitory antibody against MMP-9 [13] as adjunctive treatment in combination with standard TB treatments in *M. tuberculosis*-infected C3HeB/FeJ mice, which develop well-organized, hypoxic TB granulomas as well as cavitary lesions after aerosol infection [7, 14, 15].

Materials and methods

All procedures were approved by the ethics and Animal Care and Use committees of Johns Hopkins University. All experiments with *M. tuberculosis* were performed according to biosafety procedures in the animal biological safety level-3 (ABSL-3) facility at Johns Hopkins University.

Animal experiments

In vivo aerosol infection. The experimental scheme is outlined in S1 Fig. Four to six-week-old female C3HeB/FeJ (Jackson Laboratory) mice were aerosol infected with frozen titrated bacterial stocks of *M. tuberculosis* H37Rv [5.66 ± 0.13 log_{10} colony-forming units (CFU)/ml], using the Middlebrook Inhalation Exposure System (Glas-Col). The animals were housed inside the ABSL-3 facility with water and food available *ad libitum* and monitored daily for signs of distress after infection. Mice were sacrificed using isoflurane (Henry Schein) overdose one day after infection (to assess implantation) and at the start of treatment at six weeks after infection. Mice were also sacrificed 2, 6, 8, 10 and 12 weeks after starting TB treatments. A minimum of seven mice were used per group and for each time-point. Whole lungs were removed aseptically, homogenized in PBS and plated by serial dilution in duplicate, onto Middlebrook 7H11 agar plates (Becton Dickinson). Plates were incubated at 37˚C for 4 weeks before CFU were counted.

Multi-drug TB treatments. Standard TB treatment entailed the administration of rifampin (R, 10mg/kg/day), isoniazid (H, 10mg/kg/day) and pyrazinamide (Z, 150mg/kg/day) by gavage, 5 days per week for a total of 12 weeks as described previously [16]. Pyrazinamide was
administered for the first 8 weeks only, as is standard for TB treatment in humans [17]. The mice were split among five treatment groups: no treatment, standard TB treatment (RHZ), standard TB treatment with 15mg/kg etanercept (Amgen) injected intraperitoneally twice weekly (positive control) [16], standard TB treatment with isotype control antibody (AB5123, Gilead Sciences, Inc.) (negative control), and standard TB treatment with antibody against MMP-9 (AB0046, Gilead Sciences, Inc.) for 6 weeks. Both AB0046 and AB5123 were administered at 20 mg/kg intraperitoneally, twice weekly after a single injection of 50 mg/kg at the start of treatment as a loading dose.

**Relapse.** To assess for stable, relapse free cure, additional cohorts of mice were held for 16 weeks after cessation of treatment. At this time, lungs and spleens were removed aseptically, homogenized and plated on Middlebrook 7H11 agar plates. The complete homogenate for each organ was plated across several plates.

**Serum antibody concentrations.** An indirect ELISA was utilized for the quantitation of anti-MMP-9 (AB0046) and isotype control (AB5123) in mouse serum. Briefly, standard 96-well microtiter plates were coated with mouse MMP-9 (R&D Systems) and CHOoAg (ProSpec) to capture AB0046 and AB5123, respectively. AB0046 and AB5123 in mouse serum were detected using goat anti-mouse IgG horseradish peroxidase conjugate (HRP) followed by incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) substrate solution to produce a color reaction stopped by the addition of 1 M HCl. The absorbance was measured at 450 nm using a standard plate reader. The intensity of the color produced is proportional to the amount of AB0046 and AB5123 present in the serum sample extrapolated against a standard curve established with AB0046 and AB5123, respectively, using a 4-parameter logistic curve fit.

**Histopathology.** Lungs were harvested after systemic perfusion with phosphate-buffered saline under deep anesthesia, fixed in 4% paraformaldehyde and sectioned to 5μm thickness. Hematoxylin-eosin, acid-fast and Masson’s trichrome staining was performed as described previously [7, 18]. Images shown are representative of sections obtained from a minimum of four animals per group for each time-point.

**Immunohistochemistry.** Paraffin embedded sections were rehydrated in graded alcohols, steamed in citrate buffer at pH 6 and probed at room temperature for 2 hours using the MMP-9 (rabbit polyclonal; 1:250; Abcam [AB38898]) and processed with a polymer-HRP kit (Biogenex) with diaminobenzidine development and Mayer hematoxylin counterstaining. Lungs from uninfected, and infected but untreated animals without primary antibody served as negative controls. Slides were scanned using the Apeiro digital scanner (Leica). For human tissue immunohistochemistry, de-identified, post mortem samples from patients with pulmonary TB were obtained from the Johns Hopkins Hospital Department of Pathology collection and processed similarly for immunohistochemistry with the human anti-MMP-9 antibody (AB76003, rabbit monoclonal; 1:250; Abcam [EP1254]).

**Computed tomography imaging.** A subset of live *M. tuberculosis*-infected animals from the relapse cohort were imaged within a sealed bio-containment bed (Minerve) modified in-house to be compliant with biosafety level 3 (BSL3) containment, as described previously [7, 19]. A standard small animal anesthesia machine was used to deliver a mixture of isoflurane and oxygen to anesthetize during imaging. Computed tomography (CT) scans were then immediately performed using the NanoSPECT/CT (Mediso) *in vivo* animal imager. The images were analyzed using VivoQuant 2.5 (Invicro). The whole lung field was segmented and the frequency of voxels with a given density (measured as Hounsfield Units, HU) was determined. A cavity was defined as a macroscopic region of air (density < -900 HU) within diseased lung parenchyma.

**Statistical analysis.** Statistical comparison between groups was performed using a two-tailed Fisher’s Exact or a two-tailed Mann-Whitney test in Prism 6 version 6.07 (GraphPad).
Data are presented on a logarithmic scale as mean ± standard deviation (SD) for CFU counts, except where stated.

Results
After a low-dose aerosol infection with *M. tuberculosis*, the pulmonary bacterial implantation in mice was $1.60 \pm 0.39 \log_{10}$ CFU. Six weeks after the infection when TB treatments were initiated the pulmonary bacterial burden was $6.87 \pm 0.11 \log_{10}$ CFU.

MMP-9 expression in *M. tuberculosis*-infected tissues
To evaluate the expression of MMP-9 after *M. tuberculosis* infection we qualitatively assessed MMP-9 using immunohistochemistry in mouse and human lung tissues. Immunostaining demonstrated localization of MMP-9 mostly with macrophages in both human and mouse tissues (Fig 1). However, in mouse lungs there was also an increased MMP-9 signal associated with neutrophils.

Serum antibody concentrations
Anti-MMP-9 (AB0046) and isotype control (AB5123) had comparable high serum exposures, ranging from approximately 300–600 μg/mL by 6 weeks of dosing. After dosing, the antibodies displayed the expected terminal half-lives based on historical data, with AB0046 showing faster elimination due to target mediated disposition (Fig 2). No significant apparent immunogenicity was noted in the animals based on aberrant declines in antibody titers during dosing and antibody elimination.

Adjunctive treatments improve bacterial clearance
The addition of adjunctive treatments did not modify the bacterial burden during the initial phase of TB treatment, when most of the bacteria are actively replicating (Fig 3A). However, at later time-points (6 and 8 weeks of treatment), when a significant proportion of bacteria are dividing slowly (persisters), the addition of adjunctive treatments resulted in a significantly lower bacterial burden compared to the standard treatment alone ($P = 0.012$ and $<0.001$ at 6 and 8 weeks, respectively) with a trend towards better outcome in the arm receiving anti-MMP-9 treatment (Fig 3B and 3C). Post-mortem gross pathological lungs samples at 6 (panel A) and 8 (panel B) weeks of treatment are shown in S2 Fig.

Additional cohorts of mice were held for 16 weeks after cessation of treatment to assess for stable cure (Fig 4). The relapse rate in mice that were administered standard TB treatment for 12 weeks was 46.6% (27 of 58). Consistent with our prior data [17], adjunctive use of etanercept significantly reduced the relapse rates to 29.8% (17 of 57; $P = 0.019$). Animals that received adjunctive treatments with anti-MMP-9 also had a significantly lower relapse rate 25.9% (15 of 58; $P = 0.006$) but no different from the arm that received the isotype control antibody (25.9%; 15 of 58; $P = 1.000$). There were no significant differences between the groups in the number of cavities as evaluated by CT (S3 Fig).

In summary, we evaluated an inhibitory antibody against MMP-9 as adjunctive treatment in combination with standard TB treatments in *M. tuberculosis*-infected C3HeB/FeJ mice, which develop well-organized, hypoxic TB granulomas as well as cavitary lesions after aerosol infection. After 6 weeks of treatment, the addition of adjunctive treatments significantly reduced the bacterial burden compared to the standard treatment alone. During the relapse phase, animals that received adjunctive treatments with antibody against MMP-9 as well as the control isotype antibody had a significantly lower relapse rate.
Discussion

While current TB treatments are highly effective in targeting actively replicating bacteria, killing the slow-replicating, persistent microbes requires extended treatment with multiple drugs. Early host-responses (inflammation, necrosis and subsequent hypoxia) may promote survival of persistent bacteria and limit the effectiveness of TB treatments [20]. Therefore, adjuvant host-directed therapies that modulate the immune response may be beneficial during early

Fig 1. MMP-9 expression in mouse and human *M. tuberculosis*-infected tissues. (A) MMP-9 immunohistochemistry in the lung tissue of a C3HeB/FeJ mouse, 10 weeks after *M. tuberculosis* aerosol infection. MMP-9 was visualized in macrophages (black solid arrows) and neutrophils (black dotted arrows). (B) Expression of MMP-9 in the human lung tissue with cavitary TB. MMP-9 was expressed in macrophages (black arrow) and multinucleated giant cells (red arrows).

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phases of TB treatments [21]. There is strong evidence based on randomized controlled trials that adjunctive use of corticosteroid for the initial 6–8 weeks improves survival in HIV-negative patients with TB meningitis [22]. However, the value of host-directed treatments is not well established for other forms of TB, though some studies suggest there are some benefits [23–25]. For example, tumor necrosis factor alpha (TNF-α) has a key role in the host response against TB[26], and adjunctive use of TNF-α inhibitors during TB treatments may paradoxically be beneficial [16, 27–30]. TNF-α levels increase shortly after initiation of TB treatment [31], causing tissue destruction, which could favor bacterial survival, and thus inhibition of TNF-α or other such pro-inflammatory pathways may be useful during the early phase of TB treatment.

We have previously shown MMP-9 expression in necrotic and cavitary lesions of *M. tuberculosis*-infected mice, with a corresponding decrease during TB treatment [7]. Therefore, in the current study we evaluated MMP-9 expression (utilizing immunohistochemistry) in *M. tuberculosis*-infected murine tissues as well as post-mortem specimens from TB patients. While MMP-9 was predominantly associated with macrophage lineage in human lung tissues, MMP-9 expression was also noted in neutrophils in murine lung tissues. It should be noted that we utilized species-specific antibodies for immunohistochemistry—AB3889 directed against murine MMP-9 and AB76003 directed against human MMP-9. However, since these antibodies target different epitopes in murine and human MMP-9, differential staining patterns could be observed in murine and human tissues. Moreover, given that MMP expression and distribution is likely to be species specific [32, 33], these patterns could also be attributed to biological differences between the two mammalian species.

MMP-9 has been implicated in the pathogenesis of several diseases including TB [8–10], and selective inhibition of MMP-9 (using AB0046) was efficacious in preclinical models of ulcerative colitis and colorectal cancer [13]. We therefore evaluated whether inhibition of MMP-9 during the early phase of TB treatments in mice with established granulomatous pulmonary TB, a situation analogous to human patients presenting with active TB, could hasten bacterial clearance and provide stable cure. The anti-MMP9 and the isotype control arms had relatively high serum exposures in mice and no significant immunogenicity was noted. Importantly and as expected, the addition of adjunctive treatments did not alter bactericidal activity during the initial phase of TB treatment. However, at later time-points (6 and 8 weeks of
treatment), the addition of adjunctive treatments resulted in a significantly lower bacterial burden as compared to the standard treatment alone with a trend towards a better outcome in the arm receiving anti-MMP-9.

Additional cohorts of mice were held for 16 weeks after cessation of treatment to assess for stable (relapse-free) cure. Consistent with our prior data, adjunctive use of etanercept (positive control) significantly reduced the relapse rates compared to the standard TB treatment arm [16]. Similarly, animals that received adjunctive treatments with anti-MMP-9 had a
significantly lower relapse rate of 25.9% (versus 46.6% for the standard treatment arm), but it was no different from the animals that received the isotype control antibody. This was an unexpected finding. Non-specific polyclonal antibodies, such as intravenous immunoglobulins (IVIG) have shown some protective effects in mouse models of *M. tuberculosis* [34–37]. There is also evidence for the potential of antibody-based treatments in improving TB treatments [38, 39], with a faster reduction of the pulmonary bacillary burden when IVIG was administered in combination with standard TB treatment [40]. While the exact mechanism(s) of action are not entirely known, it is possible that both the neutralization of pathogens as well as the immune-modulatory activities may be contributing to this effect [40–42]. However, given that none of the adjunctive antibody treatments utilized in the current study were specific for mycobacterial antigens, this effect is most likely mediated by a non-specific ability of the antibodies to modulate inflammation. The protective effect of IVIG in TB has been suggested to be dependent on antibody binding to the fragment crystallizable region receptor (FcR), which significantly increased phagocytosis and intracellular killing of *M. tuberculosis* by macrophages when exposed to IVIG in vitro [37, 40]. Furthermore, the presence of sialylated glycans in the Fc-portion of antibodies increases binding affinity to the FcR, leading to increased antibody-dependent cellular cytotoxicity [43], which could have an effect on the survival of the mycobacteria.

In summary, we evaluated the potential of adjunctive MMP-9 inhibition in combination with standard TB treatments in a murine model that develop well-organized hypoxic TB granulomas and cavitary lesions. Animals that received adjunctive treatments with anti-MMP-9 antibody had a significantly lower relapse rate but it was no different from the arm that received the isotype control antibody. Given that none of the adjunctive treatments utilized in

Fig 4. Relapse rates associated with each treatment arm. Additional cohorts of mice were held for 16 weeks after cessation of treatment to assess for stable, relapse free cure. Results are presented as proportion of mice with any viable bacteria in the lungs. RHZ = standard TB treatment comprising rifampin (R), isoniazid (H) and pyrazinamide (Z) administered by gavage.

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the current study were specific for mycobacterial antigens, these improved treatments are likely a host-directed effect. Future studies will evaluate the mechanism(s) for this outcome.

Supporting information

**S1 Fig. Experimental scheme.** Four to six-week-old female C3HeB/FeJ mice were aerosol infected with *Mycobacterium tuberculosis*. Mice were sacrificed one day after infection (to assess implantation) and at the start of treatment at six weeks after infection. Mice received multi-drug TB treatments with and without adjunctive treatments for 12 weeks and were split among six treatment groups: no treatment, standard TB treatment (administered rifampin, isoniazid and pyrazinamide by gavage), or standard TB treatment with either adjunctive etanercept (positive control), isotype antibody (negative control) or anti-MMP-9 antibody for 6 weeks. Pyrazinamide was administered for the first 8 weeks only, as is standard for TB treatment in humans. Additional cohorts of mice were held for 16 weeks after cessation of treatment to assess for stable, relapse free cure.

(DOCX)

**S2 Fig. Post-mortem gross pathological lungs samples.** Mice were sacrificed at 6 (A) and 8 (B) weeks of treatment and the lungs were harvested, fixed in 4% paraformaldehyde and gross images were acquired. RHZ = standard TB treatment comprising rifampin (R), isoniazid (H) and pyrazinamide (Z) administered by gavage.

(DOCX)

**S3 Fig. Presence of cavities in each treatment arm during relapse.** Additional cohorts of mice were held for 16 weeks after cessation of treatment to assess for stable, relapse free cure. 20 animals per group were CT scanned to evaluate for the presence of cavitary lesions. The transverse, coronal and sagittal views of a CT from a representative mouse scanned 16 weeks post-treatment are shown in panel A. The crosshairs indicate the cavitary lesion. A cavity was defined as a macroscopic region of air (density < -900 HU) within diseased lung parenchyma. (B) The number of cavitary lesions was quantified by CT in each of the scanned mice within the relapse groups. RHZ = standard TB treatment comprising rifampin (R), isoniazid (H) and pyrazinamide (Z) administered by gavage.

(DOCX)

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