**Mycobacterium tuberculosis** dysregulates MMP/TIMP balance to drive rapid cavitation and unrestrained bacterial proliferation

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**Abstract**

Active tuberculosis (TB) often presents with advanced pulmonary disease, including irreversible lung damage and cavities. Cavitary pathology contributes to antibiotic failure, transmission, morbidity and mortality. Matrix metalloproteinases (MMPs), in particular MMP-1, are implicated in TB pathogenesis. We explored the mechanisms relating MMP/TIMP imbalance to cavity formation in a modified rabbit model of cavitary TB. Our model resulted in consistent progression of consolidation to human-like cavities (100% by day 28), with resultant bacillary burdens (>107 CFU/g) far greater than those found in matched granulomatous tissue (105 CFU/g). Using a novel, breath-hold computed tomography (CT) scanning and image analysis protocol, we showed that cavities developed rapidly from areas of densely consolidated tissue. Radiological change correlated with a decrease in functional lung tissue, as estimated by changes in lung density during controlled pulmonary expansion ($R^2 = 0.6356, p < 0.0001$). We demonstrated that the expression of interstitial collagenase (MMP-1) was specifically greater in cavitary compared to granulomatous lesions ($p < 0.01$), and that TIMP-3 significantly decreased at the cavity surface. Our findings demonstrated that an MMP-1/TIMP imbalance is associated with the progression of consolidated regions to cavities containing very high bacterial burdens. Our model provided mechanistic insight, correlating with human disease at the pathological, microbiological and molecular levels. It also provided a strategy to investigate therapeutics in the context of complex TB pathology. We used these findings to predict a MMP/TIMP balance in active TB and confirmed this in human plasma, revealing the potential of MMP/TIMP levels as key components of a diagnostic matrix aimed at distinguishing active from latent TB (PPV = 92.9%, 95% CI 66.1–99.8%, NPV = 85.6%; 95% CI 77.0–91.9%).

**Keywords:** tuberculosis; matrix metalloproteinase; computed tomography; cavity

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Introduction

Tuberculosis (TB) is a leading cause of death worldwide, costing the global economy 100–300 billion dollars and orphaning 10 million children annually [1–3]. The majority of disease results from Mycobacterium tuberculosis infection of immunocompetent adults, characterized by complex destructive immunopathology, including cavity formation [4–6]. Cavities contain the majority of the bacillary burden in human disease and play a pivotal role in disease transmission [7,8]. Within the cavity, immune responses are impaired and antibi-otic efficacy altered; treatment failure occurs in up to 15.8% of patients with cavities compared to just 2.6% of those without [7,9–11]. Despite this critical role in TB pathogenesis, cavity formation is poorly under-stood.

Cavitary TB is associated with delayed-type hypersensitivity (DTH) reactions, which are functionally assessed with tuberculin skin tests (TSTs) [12,13]. DTH contributes to effective bacterial control and tissue destruction [14]. Differentiating destructive from antibacterial mechanisms is essential for the development of safe vaccines and immunotherapies. Matrix metalloproteinases (MMPs) are emerging as central mediators of the tissue destructive response in TB [15,16]. MMPs can cleave all extracellular matrix (ECM) components [6]. In humans, MMP-1, which can degrade the most resilient fibrillar components of the ECM (types I and III collagen), and its activator MMP-3, are more abundant in respiratory secretions of TB patients than controls [15,17,18]. Conversely, their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), are not substantially increased [15,18]. In M. tuberculosis-infected MMP-1 transgenic mice, collagen degradation is greater than in controls, although these mice do not develop cavities, possibly because mice have limited functional DTH responses, as determined by responsiveness to PPD after infection (a maximal 0.3 mm swelling occurred after injection of 10 000 tuberculin units (TU) [13–15,19]. These data suggest a dual importance of DTH and pulmonary MMP-1 expression. Investigation of the underlying mechanisms of this relationship are challenging, because cavity formation does not occur in in vitro mouse, guinea-pig or zebrafish models of TB and is sporadic in other models [20,21]. Rabbits and macaques infected with M. tuberculosis develop DTH responses and also occasionally develop cavities [20,22]. Like humans, these animals can also contain infection [23]. The most consistent cavity TB model is a post-primary rabbit model, in which DTH responses are induced via presensitization [24]. This model required prolonged infection times and disease progression was inconsistent, leading to difficulties in quantifying outcomes [24,25].

We developed a reliable model of cavitary disease in rabbits and confirmed pathological and molecular correlates of human disease. We designed an in vivo imaging strategy to observe and quantify the events leading to cavitation. We show that cavities develop rapidly within areas of dense consolidation. This was associated with an MMP-1/TIMP imbalance and high intracavitary bacterial burdens. We provide evidence that M. tuberculosis specifically induces MMP-1/3 and that MMP/TIMP imbalance is a feature of active TB.

Materials and methods

Extended methods are included in Supplementary materials and methods (see supplementary material).

Mycobacterial culture

M. tuberculosis H37Rv and M. bovis Ravenel were grown as in [24].

Animals

Female New Zealand rabbits, weight 3–3.5 kg (Covance Research Products, Princeton, NJ, USA) were housed in accordance with protocols of the Institutional Animal Care and Use Committee at Johns Hopkins University, Baltimore, MD, USA.

Sensitization and infection

As in [24], except: (a) 10^8 instead of 10^7 bacilli were used for each presensitization injection; (b) a target inoculum of 10^3 CFU was utilized; (c) intradermal injection of 0.1 ml purified protein derivative (5 Tuberculin Units) (Tubersol, Sanofi-Aventis, Bridge-wat, NJ, USA) was used to assess DTH. Briefly, sensitization was achieved using five injections of M. bovis, equally spaced over 14 days, and infection with M. tuberculosis occurred 21 days later.

Lung sampling

Within 5 minutes of euthanasia (described in [24]), tissue biopsies were taken. Macroscopically matched samples were dissected by gross appearance and: (a) snap-frozen in liquid nitrogen; (b) transferred to RNAlater (Invitrogen/Life Technologies, Carlsbad, CA, USA); (c) fixed in 10% formalin; (d) 3 mm punch biopsies (six/tissue/animal) were weighed and then homogenized in phosphate-buffered saline (PBS) prior to CFU enumeration on 7H11 selective medium. The remaining lung lobe (>90% by mass) was weighed and then homogenized, using a Polytron homogenizer (Kinematica, CH), in 40 ml PBS prior to CFU enumeration by serial dilution on selective 7H11 medium.

Rabbit imaging

Breath-hold image acquisition took place as in Figure S3 and image analysis is described in Figures S4, S6 and S7 (see supplementary material). Tissue type rules are described in [26]. Calculations of lung mass was
performed as described in Figure S5 (see supplementary material) and [40].

Quantitative polymerase chain reaction (qRT-PCR)
qPCR was performed on 0.5 μg/μl RNA samples converted to cDNA using superscript cDNA synthesis kit (Agilent Technologies), according to the manufacturer’s protocols with an additional DNAase incubation. Expression was measured utilizing SYBR II (Bio-Rad, Hercules, CA, USA) and an iQ5 platform (Bio-Rad; for primers, see supplementary material, Table S2) and and −ΔΔCt and fold changes calculated.

Zymography
Homogenized, sterile-filtered samples were assessed using casein zymography [27].

Histology
Tissues was processed by Histoserv, imaged on an Olympus BX51 microscope and photomicrographs were taken using an Olympus DP70 camera.

Ethics
Written informed consent was obtained from all participants, and the study was performed in accordance with the principles expressed in the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of the National Institute for Research in Tuberculosis (NIRT; Protocol Nos NCT01154959 and NCT00342017).

Study population
Cryopreserved heparinized plasma samples were collected from 97 subjects with active pulmonary TB (PTB), 14 with latent TB infection (LTBI) and 20 matched, uninfected, healthy controls (HC), described in detail in [28].

Immunoassays
Human MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in plasma samples using a Luminex Kit from R&D systems (Minneapolis, MN, USA). Rabbit MMP-1 was measured using a commercialized ELISA kit (SEA097Rb, USCN Life Sciences, Hubei, China).

In vitro experiments
Performed as in [27], UV-killed M. tuberculosis generated by 90 min of UV transillumination (wavelength 365 nm; UVP, Upland, CA, USA).

Data analysis
Data analysis was performed as described in the figures using JMP 11 (SAS, Cary, NC, USA), STATA 10 (StataCrop LP, College Station, TX, USA) and Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Results
High-dose presensitization causes rabbit skin test positivity and is required for pulmonary cavity formation
First, we developed a high-dose sensitization regime with heat-killed M. bovis, since we hypothesized that this would generate cavities more reproducibly than in previous studies [24,29]. This regimen resulted in universal skin-test positivity (Figure 1A; see also supplementary material, Table S1), whereas lower-dose sensitization did not (data not shown). Cavity formation, detected by serial CT scanning, occurred in all rabbits (Figure 1B, C) after infection with 10^3 CFU M. tuberculosis H37Rv. All cavities appeared between days 14 and 28 (Figure 1D) and were large in volume (Figure 1E). Histologically, the cavities were similar to human cavities, comprising a fibrotic wall surrounding a necrotic core (Figure 1F, G). Numerous acid-fast bacilli were present within the necrotic debris (Figure 1H). Several other histopathological features typical of human TB also developed, including: (a) small paucibacillary cellular granulomas, which were observed in the tissue surrounding the cavity, at sites distal to the cavity and occasionally in lobes that were not directly infected (Figure 1I; see also supplementary material, Figures S1, S2); (b) multinucleated giant cells at the periphery of cavitary lesions (Figure 1J); and (c) direct connection of the cavity to the bronchi (Figure 1B; see also supplementary material, Figure S1).

Cavities contain high bacillary burdens
Next, we investigated the association between radiographic findings, pathology and bacterial burden. CT appearances were strongly representative of gross pathology (Figure 2A). The majority of the bacterial burden was within the lobe containing the cavity (Figure 2B). We investigated the precise location of the maximal bacterial burden and obtained multiple biopsies from the cavity wall, the surrounding granulomatous material (in the same lobe) and normal-appearing tissue in the lobe which was adjacent to the initially infected lobe but in the contralateral lung of each animal (see supplementary material, Figure S2). Quantitative culture demonstrated an approximately 100-fold increase of bacterial burden at the cavity surface when compared to the granulomatous regions (p < 0.001) (Figure 2C). Acid-fast staining of these regions confirmed that bacteria were located within the necrotic debris at the cavity surface, whereas the remaining lung pathology was paucibacillary, with very few acid-fast bacilli visualized within granulomas (see supplementary material, Figure S2).

Tissue destructive MMPs are abundant at the cavity surface
Having demonstrated histological similarity to human disease, we sought to investigate molecular correlates
Figure 1. A reproducible model of TB that demonstrates human-like pathology and cavity formation within 28 days. (A) Sensitization and infection protocol: rabbits were sensitized by administering five injections over 14 days, each containing $10^8$ heat-killed CFU M. bovis strain Ravenel. After 21 days, PPD testing was performed to confirm the development of delayed-type hypersensitivity to M. tuberculosis complex antigens. Infection with $10^3$ CFU M. tuberculosis was then performed 3 days after PPD testing under bronchoscopic guidance. The animals were monitored by serial CT imaging and then humanely sacrificed at predetermined time points (for detailed description, see Supplementary materials and methods). (B) Representative CT scan of a rabbit with a cavity; red arrows, cavity; yellow arrows, gastric air. (C) A 3D reconstruction of a representative animal scan, using a customized scale in which low-density regions (cavities and airways) are represented in red and high-density regions (blood vessels and consolidation) in a green-to-yellow scale, where yellow regions are more dense. (D) Proportion of animals with cavities at each time point, as assessed by CT scan (n = 12). (E) Cavity size was assessed using an automated cavity segmentation algorithm [45]; maximum cavity sizes for each animal are shown. (F–J) Slides of rabbit lung tissue, demonstrating all the pathognomonic features of human TB (stained with haematoxylin and eosin unless stated): (F) low-power image of cavity, with air-filled centre, necrotic rim and a peripheral fibrotic cuff; (G) necrotic debris at cavity surface; (H) Ziehl–Neelsen staining demonstrates numerous bacteria within necrotic debris; (I) small cellular granuloma without central caseation; (J) multinucleate giant cell within granuloma. HU, Hounsfield units; scale bars = (F) 1 mm; (G–J) 200 μm
Protease dysregulation in cavitary tuberculosis

Figure 2. Bacterial burden is 100-fold higher at the cavity surface than in granulomatous lung tissue. (A) Matched findings from six animals (A–E and K), showing correlations between pre-mortem CT scans, post-mortem gross pathology and *M. tuberculosis* burden. CT scans reliably demonstrate the lobe containing the cavity. The bacterial burden is represented on a five-directional axis, with each axis representing a lobe of the rabbit lung. Bacterial burden is highest in the lobe with the most extensive pathology. (B) The majority of the bacteria are found within the cavitary lobe (*n* = 12): red, lobe containing cavity; blue, ipsilateral adjacent (relative to cavity) lobe; light grey, non-adjacent (to cavity) lobes; no triangle, no bacilli cultured. (C) Bacterial burden within the cavitary lobe was assessed by taking 3 mm punch biopsies from the surface of the cavity, non-cavity infected tissue from the same lobe, and tissue from the contralateral lower lobe (six samples/region/animal, *n* = 11). The bacterial burden was almost exclusively at the cavity surface, representing 99.9% of bacteria. Error bars represent standard deviations (SDs); *p* values were calculated using one-way ANOVA with Tukey post-comparison test.


doing tissue destruction. We investigated MMPs implicated in tissue destruction during human infection (see supplementary material, Table S2). Multiple MMPs showed increased transcription in regions of pathological change in the pulmonary architecture, including MMP-1, −3, −7, −12 and −13, (Figure 3A, B). MMP-1 (interstitial collagenase) showed the greatest fold increase in transcription and was the only MMP investigated that was more highly expressed in cavity walls than in granulomatous tissue (*p* < 0.01).
The specific inhibitor TIMP-3 had lower expression in cavity than in non-cavity tissue \( (p < 0.001) \) (Figure 3C). Because MMP-1 is highly implicated as a marker of both disease progression and cavitation in man [15], we sought to confirm that its increased transcription correlated with protein abundance. We demonstrated a nearly 10-fold increase in MMP-1 protein concentration around the cavity (Figure 3D). Furthermore, MMP-1 proteolytic activity was detected in infected lung tissue (Figure 3E), which was inhibited by incubation with the collagenase inhibitor Ro32-3555 (Cipemastat) (Figure 3F).

**In vivo imaging demonstrates that cavitation originates from dense consolidation**

To investigate the sequence of events that lead to cavitation formation, we performed longitudinal image analysis of *M. tuberculosis*-infected rabbits (Figure 4A–H). Initial bronchoscopic infection caused localized consolidation (Figure 4B), which became less diffuse over the following 7 days (Figure 4C). Subsequently, dense consolidation formed, and then cavitation occurred rapidly within the regions of dense consolidation. In the majority of cases, cavities appeared and reached their maximum size within a 7 day period (Figure 4D, E). The first cavities appeared by day 21 and all animals were cavitated by day 28. After formation, the cavities remained relatively stable in size (Figure 4E, F), although some resolution occasionally occurred by day 49 (Figure 4G).

**Breath-hold CT scanning permits quantification of dynamic changes in pulmonary architecture**

To perform detailed quantitative measurement of lung infiltration, we constructed a bio-safety level-3 (BSL-3) respiratory support device and developed a breath-hold scanning methodology (see supplementary material, Figure S3). The system permitted accurate measurement of overall volumes of tissue within given density ranges. The lower pressure represents an approximately normal inhalation volume, while the higher pressure causes expansion of the lung to beyond physiological norms, allowing the identification of poorly inflated regions that remained consolidated even with this increased expansion (higher pressures allow for even greater expansion, but were not used to reduce the effect of barotrauma). Two scans of each animal were taken before infection and at days 21, 28 and 35, at inflation pressures of 10 cmH\(_2\)O (lower pressure) and 20 cmH\(_2\)O (higher pressure).

Higher-pressure expansion \( (n = 54) \) led to highly consistent distribution of tissue densities (Figure 4H). We observed an increase in poorly aerated \( (−100 to 100 \text{ HU}) \) and hyperexpanded \( (−1000 to −900 \text{ HU}) \) tissue volumes, corresponding to consolidation and cavitation change, respectively, during the course of infection (Figure 4I).

Assessment of lung consolidation facilitates the estimation of functional lung tissue

We then assessed whether lung consolidation after infection could be measured non-invasively, and whether the pressure changes permitted measurement of functional tissue. First, we assessed lung recruitment by comparing lung consolidation between the lower and higher inflation pressure measurements. Functional lung tissue decreased significantly after infection and continued to fall until day 35 post-infection (Figure 5B).

Low-density pathological change includes both cavitary and hyperexpansive pathology

Next, we analysed the CT images to differentiate specific pathological changes driven by *M. tuberculosis* infection in our model. This was achieved by comparing the proportion of voxels at all densities, before and after infection, in the more consistent high-pressure scans. A proportion of voxels containing very low-density tissue \( (−1022 and −865 \text{ HU}, \text{peak} −889 \text{ HU}) \), representing cavity formation, and a proportion of voxels containing high-density tissue \( (−719 to 212 \text{ HU}, \text{peak} 35.19 \text{ HU}) \), representing dense consolidation, showed significant increases (Figure 5C). Conversely, the proportion of tissues \( (−866 to −712 \text{ HU}, \text{peak} −715 \text{ HU}) \) within the normal aerated range was reduced. Although the high-density peak was across a broad range of HU, 47% of this peak was in the non-aerated range \( −100 to 100 \text{ HU} \), with the remainder of the peak being evenly distributed from \( −700 to −100 \text{ HU} \).

We first focused on the low-density peak. Unexpectedly, the distribution of voxels was different between the two inflation pressures, with a bifid peak in this region \( (−1022 to −865 \text{ HU}; \text{Figure} 5C) \) occurring at high-pressure expansion (see supplementary material, Figure S6A). To investigate the cause of this phenomenon, we identified a cavity-specific region by comparing scans of animals from day 21 and found a significant change in very low-density regions \( (−1024 to −924 \text{ HU}; \text{see supplementary material, Figure} S6B) \) that was diagnostic of cavity formation in higher-pressure scans (Figure S6C). By further evaluating the distribution of the remaining portion of the bifid peak in 3D reconstructions \( (−924 to −865 \text{ HU}; \text{see supplementary material, Figure} S6E, F) \), we established that, after infection, a diffuse increase in low-density regions was occurring (Figure S6E, F). This indicates that additional lung hyperexpansion was occurring after infection when the lungs were highly inflated.

TB-driven lung inflammation can be quantified accurately

The change in the volume of dense tissue was small when expressed as a proportion of total lung voxels, but represented a significant change from baseline (Figures 4I, 5A). Since cavities appear to originate in these dense tissues, and these same areas are unlikely to
Figure 3. Tissue-destructive MMPs are up-regulated in the infected lung, particularly within the cavity wall. (A) Multiple MMPs implicated in human TB immunopathology are up-regulated in infected lung tissue when evaluated by qRT–PCR. (B) Heat chart showing approximate MMP expression levels, as evaluated by average difference in cycle threshold value relative to β-actin (BACT). (C) TIMP-1 and TIMP-3 expression in cavity wall, granulomatous and normal tissue. (D) MMP-1 protein concentration, analysed by ELISA, demonstrated a significant increase in the cavity wall as compared to normal tissue. (E) MMP-1 activity was demonstrated by casein zymography, with a band of proteolytic activity at 35 kDa in infected lung tissue, similar to active recombinant MMP-1. (F) Ro32-3556 completely inhibits caseinolytic activity of both M. tuberculosis-infected rabbit lung tissue and human macrophages (Mφ) infected with M. tuberculosis. Error bars represent SDs; ct, cycle threshold; p values were calculated using one-way ANOVA with Tukey post-comparison test.
facilitate gas exchange, we investigated the progression of these regions over time. The region of unique change in proportional volume between pre- and post-infection was identified (−84 to 56 HU; see supplementary material, Figure S7A). The changes in volume of these high-density regions were small, but these relatively low volumes represent substantial tissue mass. We analysed the mass of high-density tissue pre- and post-infection and identified a larger zone in the range −184 to +156 HU, which demonstrated consistently increasing volume after infection (see supplementary material, Figure S7B). By highlighting this zone in 3D reconstructions, we demonstrated that these loci represented areas of new tissue consolidation (Figure 5D).

Both cavitation (p < 0.01; Figure 5E) and consolidation (p < 0.05; Figure 5F) signatures within the TB lesions increased progressively during the early stages after infection (days 21–35). Interestingly, neither the mass nor the volume of the regions of consolidation decreased during the appearance of cavitation, suggesting that while cavitation originates in these areas, cavities do not lead to a substantial reduction in consolidated tissue. We used the combined volumes of both cavitory and consolidated tissues to generate a total disease score. This score changed substantially between days 21 and 35 after infection (p < 0.001; Figure 5G) and correlated strongly with the functional decline in tissue (p < 0.0001; see supplementary material, Figure S8).

MMP/TIMP imbalance is a bacterial-driven signature of active TB

UV-irradiated M. tuberculosis induced substantial levels of MMP-1, −3 and −7 expression (Figure 6A) from human blood-derived monocyte macrophages. The addition of M. tuberculosis culture supernatant
Figure 5. Disease progression in individual animals can be quantitatively analysed in vivo. (A) Distribution of tissue mass in lungs, inclusive of tissue recruited during expansion from 10 to 20 cmH₂O scans: black, not aerated; darkest grey, poorly aerated; middle grey, potentially recruited; light grey, normally aerated; white, hyperexpanded. (B) Proportional loss of functional lung tissue by mass during the course of infection; functional tissue is sum of normally aerated tissue and potentially aerated tissue. (C) Change in proportional voxel density distribution after infection (n = 42). (D) Visualization of pathological changes: yellow, high density, consolidation, −184 to +156 HU; red, low-density, cavity, −1024 to −924 HU. (E–G) In vivo quantification of pulmonary pathological change. (E) Proportion of lung containing a cavity signature to day 35 post-infection. (F) Proportion of lung containing a consolidation signature. (G) Overall diseased lung signature, incorporating both consolidated and cavitary tissue. p values were calculated using repeated-measures ANOVA with Tukey post-comparison test.
Figure 6. MMP/TIMP imbalance in humans: (A) MMP-1, −3 and −7 levels were measured in supernatants from human monocyte-derived macrophages infected with UV-killed M. tuberculosis in the presence or absence of released bacterial products. (B) Plasma concentrations of several MMPs and TIMPs were assessed by ELISA in samples from ATT-naïve active pulmonary TB patients (PTB; n = 97), individuals with latent TB infection (LTBI; n = 14) and age- and gender-matched healthy controls (HC; n = 20). The ratios between levels of MMP-1 and each one of the TIMPs are shown. (C) An unsupervised two-way clustering analysis (Ward’s method) was employed, using the plasma concentrations of each MMP and TIMP. Individuals from the PTB, LTBI or HC groups were listed in rows and each biomarker was placed in a different column. The squares in the heat map represent values below or above the geometric mean values (log10-transformed) of a given biomarker in the entire study population (n = 131), with dark red indicating an increase in expression and dark blue a decrease. (D) A representative profile of geometric mean values (log10-transformed) for MMPs and TIMPs in plasma is shown for each clinical group. (E) The performance of the combined assessment of plasma concentrations of several MMPs and TIMPs in distinguishing the different clinical groups was tested using a canonical correlation analysis (CCA) model. The statistical significance of the CCA model was tested using standardized tests, and p values for each of them are shown; small circles, 95% confidence region to contain true mean of each group; large shaded ellipses, region estimated to contain 50% of the population of each group. (F) The performance of the CCA model in distinguishing the different clinical groups, as well as details of sensitivity, specificity and predictive values of the test distinguishing PTB from LTBI are shown. In (B), data were analysed using Kruskal–Wallis test with Dunn’s multiple comparisons post hoc test. In (F), data were analysed using χ² (left panel) and Fisher’s exact (right panel) tests; ** p < 0.0001; CI, confidence interval.
significantly enhanced MMP-1 and −3 secretion, but not that of MMP-7 (Figure 6A). Finally, we took a hypothesis-driven approach to evaluating MMP/TIMP imbalances in peripheral blood from a cohort in India, including pulmonary TB (PTB), latent TB (LTBI) and healthy controls (HC) previously used to investigate biomarkers for of active PTB diagnosis ([28] and Andrade et al. unpublished data). We showed that MMP-1/TIMP-1-4 ratios were greater in pulmonary TB (PTB) patients than LTBI and healthy controls (Figure 6B). We then utilized hierarchical clustering of tissue-degrading MMPs and TIMPs to assess the associations between MMP/TIMP levels and patients with active TB (Figure 6C). Overall increases in MMP-1, −8 and −9 were observed (Figure 6D) and a canonical correlation analysis (CCA) model confirmed that peripheral MMP/TIMP imbalance is a prominent feature of active disease (Figure 6E, F).

**Discussion**

The majority of the global TB burden occurs in immunocompetent adults, where tissue destruction and consequent cavity formation are associated with treatment failure, the emergence of drug resistance, transmission of infection, morbidity, mortality and long-term respiratory impairment [1,8,10,11,30,31]. Despite its clinical significance, pulmonary cavitation is poorly understood and the effect of therapeutics in cavitory TB is not established in preclinical trials. To address this gap, we have developed a novel rabbit model of pulmonary cavitation and an imaging strategy that provides objective, quantitative, real-time measure of disease. We demonstrate that cavities develop rapidly from areas of dense consolidation, facilitating massive bacterial proliferation in the context of a protease-antiprotease imbalance.

Our model replicates the classical pathology observed in human TB, including cavity and granuloma formation, in a rapid and reliable manner (Figures 1, 2). These pathologies develop in the presence of the characteristic DTH responses of post-primary TB [13,32,33]. TST acts as a functional measure of DTH, as its development is dependent on not only CD4+ T cell responses but also the subsequent recruitment and activation of monocytes, NK cells and CD8+ T cells, as well as IFNγ- and IL-12-mediated inflammatory cascades [12]. Pre-existing DTH to *M. tuberculosis* is common in endemic regions where 90% of healthy adults are TST-positive [33]. In low-prevalence settings, where the population prevalence of TST positivity is low, TST positivity is present in 90% of individuals with active TB [32]. Experimental data from rabbits indicate that DTH, as measured by any induration of TST, either from pre-sensitization or through prolonged infection, predicts cavity formation [24,25,29,34]. By recapitulating post-primary DTH, our model rapidly generates cavities that are highly representative of human pathology: there is a fibrotic layer surrounded by monocytes, within which lies a layer of epithelioid macrophages that are progressively more necrotic towards the centre of the cavity, which contains high numbers of culturable, acid-fast bacilli [5,12,13]. This model complements existing mouse, guinea-pig, rabbit and non-human primate models, adding to their utility in the study of multiple drug regimens, vaccine efficacy, granuloma progression and human-like immune responses, respectively, the possibility of studying cavitary disease preclinically [29,35–37].

Clinical studies can only provide a snapshot of disease progression and consequently do not inform us of the precise sequence of events during cavity formation. To address this, we developed novel methods to accurately quantify tissue destruction *in vivo* (Figures 4, 5).

Previously, positron emission tomography (PET) using the glucose analogue 2-deoxy-2-[18 F]fluoro-D-glucose ([18 F-FDG] has been used in clinical and preclinical trials [38–40]. In comparison to PET, CT scanning has several key technical benefits: CT gives detailed structural information; does not require intravenous injections; does not use radioisotopes; can be acquired in < 30 seconds, allows for high resolution (in this case, 0.5 × 0.5 × 0.625 mm) quantitation, and is more readily available [41,42]. Previous CT analyses have primarily focused on pattern recognition and, to date, no technique to quantitatively assess TB disease severity by CT has been developed [43–46]. Hunter’s exhaustive review of human specimens and post-mortem studies indicated that cavities emerge from regions of confluent consolidation, and not from individual granulomas [5]. Our model recapitulates this process, revealing that cavitation is a very rapid process. Further studies are required to determine the exact nature of the precavitary lesion.

Pulmonary extracellular matrix (ECM) is highly resilient and its rapid destruction requires proteolytic enzymes [47,48]. Previous studies have identified correlates between active TB and increased MMP expression in respiratory secretions, but not a direct link between cavity formation and MMP activity [15,16,18]. In this study, we established a unique and specific relationship between MMP-1 expression and cavitary pathology (Figure 3). Our finding that, in rabbits, MMP-1, −3, −7, −12 and −13 transcriptional levels are increased in granulomatous and cavitary pathologies correlate with the finding that several of these MMPs are increased in human respiratory secretions and *in vivo* models [16,49]. TIMP-1 did increase in our model, although not substantially, a finding also consistent with human data [18]. The reduction in TIMP-3 is predicted by *in vitro* human monocyte infection models, but has not been observed in tissue [27]. TIMP-3 deficiency in mice is, in isolation, sufficient to lead to lung ECM degradation [50]. This suggests that, in rabbits, MMP-1/TIMP-3 imbalance is specifically associated with cavity formation.

Previously, we have demonstrated that MMP-1 and −3 expression is greater in macrophages infected with pathogenic *M. tuberculosis* than in attenuated
vaccine strains [27]. We provide evidence that secreted and cellular components of *M. tuberculosis* drive the collagen-degrading MMP-1 and its activator MMP-3, but not non-collagenolytic MMPs (Figure 6A). This evidence from the rabbit and macrophage infection models, as well as previous studies, indicates that MMP/TIMP imbalance is a key feature of cavity disease [18].

Our final evaluation was of the diagnostic potential of MMP/TIMP imbalances in human plasma. Prior studies used induced sputum or bronchoalveolar lavage samples, which are not easily obtainable diagnostic materials. In this study we utilized plasma, which is easier to obtain and process. We first confirmed that MMP-1/TIMP imbalance was significantly altered in active disease (Figure 6B) and demonstrated that, although MMP/TIMP imbalances alone are not diagnostic of active disease, they have potential as peripheral markers of active pulmonary TB (Figure 6E, F).

In summary, our model, in combination with previous studies of both human and rabbit disease, suggests that DTH reactions as well as MMP/TIMP imbalances are key components of cavity development [15,24]. Through direct observation of cavitation, we reveal that dense consolidation erodes to leave a cavity in which bacterial multiplication is unchecked and the airways can be accessed (see supplementary material, Figure S1). We provide evidence that a bacterial-driven host-protease imbalance drives cavity formation. The protease–antiprotease imbalance distinguishes active from latent disease in man. Although not of diagnostic power, this may be relevant to screening or diagnostic pathways. We predict that restoration of this imbalance may be of benefit in TB treatment. Our model provides a strategy for investigating protease-directed (and other) therapies in the context of human-like pathology. Such studies will provide: (a) a mechanistic insight into TB pathogenesis; (b) the function of the targeted pathways in immunity; and (c) the therapeutic potential of these drugs as adjunctive TB therapy.

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**Author contributions**

AK and BL, and senior authors JSF, PTGE and WRB, contributed equally to the work; AK, BL, CL, NCA, JSF, PTGE and WRB designed and performed the experiments; BBA, KWB and MO performed histopathological analysis; ZX, UB, DJM, MK, SKJ, BL and AK contributed to image acquisition and analysis; JB and AK designed and manufactured the breath-hold chamber; JM, KW, BAA, LC, BL, AK, CL and NCA performed rabbit experiments; BBA, NPK, SB and AS acquired human samples and performed analyses; and AK prepared the manuscript.

**References**

Figure S6. Justification of low-density regions of interest in CT scans
Figure S5. Formula for calculating tissue mass from CT density
Figure S4. Segmentation of the lung region from CT images using AMIRA
Figure S3. Equipment configuration for performing breath-hold scans of rabbits
Figure S2. Representative images demonstrating tissue selected for bacillary, qRT–PCR and protein analyses
Figure S1. Rupture of necrotic debris into a bronchus

The following supplementary material may be found in the online version of this article:

Supplementary materials and methods
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Figure S7. High-density regions in lungs of rabbits upon M. tuberculosis infection
Figure S8. Proportional changes in diseased tissue and functional lung volume correlate
Table S1. Skin test responses post-sensitization with heat-killed M. bovis
Table S2. Primers for qRT–PCR quantification of MMPs and TIMPs

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