Cathepsin K Contributes to Cavitation and Collagen Turnover in Pulmonary Tuberculosis

André Kubler,1,3,4̊ Christoffer Larsson,7,4̊ Brian Luna,3,4̊ Bruno B. Andrade,6 Eduardo P. Amaral,5 Michael Urbanowski,3 Marlene Orandle,6 Kevin Bock,6 Nicole C. Ammerman,2 Laurene S. Cheung,2 Kathryn Winglee,3 Marc Halushka,4 Jin Kyun Park,4 Alan Sher,5 Jon S. Friedland,1,b Paul T. Elkington,1,2,b and William R. Bishai,1,a

1Infectious Diseases and Immunity, Imperial College London, and 2Faculty of Medicine, University of Southampton, United Kingdom; 3Center for Tuberculosis Research and Department of Pathology, Johns Hopkins University School of Medicine, Baltimore; 4Immunobiology Section, Laboratory of Parasitic Diseases, and 5Infectious Diseases Pathogenesis Section, Comparative Medicine Branch, National Institutes of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland; 6Department of Molecular Biology, Umeå University, Sweden; and 7Division of Rheumatology, Department of Internal Medicine, Seoul National University Hospital, South Korea

Cavitation in tuberculosis enables highly efficient person-to-person aerosol transmission. We performed transcriptomics in the rabbit cavity tuberculosis model. Among 17 318 transcripts, we identified 22 upregulated proteases. Five type I collagenases were overrepresented: cathepsin K (CTSK), mast cell chymase-1 (CMA1), matrix metalloproteinase 1 (MMP-1), MMP-13, and MMP-14. Studies of collagen turnover markers, specifically, collagen type I C-terminal propeptide (CICP), urinary deoxypyridinoline (DPD), and urinary helical peptide, revealed that cavitation in tuberculosis leads to both type I collagen destruction and synthesis and that proteases other than MMP-1, MMP-13, and MMP-14 are involved, suggesting a key role for CTSK. We confirmed the importance of CTSK upregulation in human lung specimens, using immunohistochemical analysis, which revealed perigranulomatous staining for CTSK, and we showed that CTSK levels were increased in the serum of patients with tuberculosis, compared with those in controls (3.3 vs 0.3 ng/mL; P = .005).

Keywords. tuberculosis; cathepsin K; rabbit; collagen; collagenolysis; RNAseq.

Tuberculosis is a common, debilitating infectious disease that, if left untreated, has a fatality rate of >50% [1]. Extensively drug-resistant tuberculosis accounts for 9% of the 300 000 multidrug-resistant tuberculosis cases and costs on average $554 000 per patient to treat [2]. Tuberculosis transmission is increased by cavitary disease, which contributes to antibiotic failure and the emergence of antibiotic resistance [3–5]. Bilateral cavity formation is the most significant predictor of treatment failure for extensively drug-resistant tuberculosis [6].

Cavity formation is rarely studied, partly because it is so difficult to recreate under controlled conditions. Our current understanding is therefore derived from observational studies of human specimens, in which the histopathological hallmarks are caseous, liquefactive, and coagulative necrosis [7, 8]. The mechanisms of these processes remain elusive.

Several theories attempt to explain cavity formation. The most widely taught is that maturing granulomas invade the airway, spilling their necrotic debris and leaving a cavity at the granuloma site [9, 10]. This ignores the observation that cavities occur in a number of nongranulomatous conditions and are encountered less frequently in nontuberculous granulomatous conditions than during tuberculosis [11]. Additionally, it is known that pneumonic change is the most common pathology associated with postprimary tuberculosis in humans [12]. Other, less commonly accepted theories of cavitation include cystic dilation, infarction, postobstructive pneumonia, and postobstructive increases in intra-alveolar pressure [11].

In this study, we use our ability to manipulate cavity formation to explore effectors of cavity formation in a rabbit model of postprimary tuberculosis [13]. Next-generation transcriptional profiling with RNAseq in a number of pathologies revealed that collagen-degrading proteases were among the most highly transcribed genes in cavitary disease. This is significant, as the key structural components of lung extracellular matrix (ECM) are the fibrillar collagens (type I and type III) [14], and only 9 enzymes are known to cleave the two alpha components of type I collagen, and only matrix metalloproteinase 1 (MMP-1), MMP-8, MMP-13, MMP-14, and cathepsin K (CTSK) can cleave the mature triple helical form [14–17].

We discovered that CTSK, a serine protease with the unique ability to cleave type I collagen both inside and outside its helical region [17], was strongly associated with tuberculosis pathology and that changes in collagen-breakdown products suggestive of CTSK activity were unique to rabbits in which active cavitation was taking place. Finally, we demonstrated that CTSK expression is associated with human immunopathology.
and that an increased level of plasma CTSK is a feature of active pulmonary tuberculosis. Together, these findings suggest that collagen degradation mediated by CTSK plays an important role in cavity formation, adding to a growing body of evidence that collagen destruction is the key mechanism underlying cavity formation.

MATERIALS AND METHODS

Animal Procedures
Female New Zealand white rabbits, weighing 3–3.5 kg (Covance Research Products, Gaithersburg, Maryland), were sensitized with five 0.2-mL subcutaneous injections of $1 \times 10^7$ (lower dose) or $1 \times 10^8$ (higher dose) heat-killed *Mycobacterium bovis*, emulsified 1:1 in Freund’s adjuvant. Twenty-one days after sensitization, skin test reactivity was determined by injection of 5 IU of purified protein derivative (Tubersol; Sanofi-Aventis, Bridgewater, New Jersey) and measured at 48 hours. Animals were maintained in biosafety level 3 conditions in accordance with protocols (RB11M466) approved by the Institutional Animal Care and Use Committee at Johns Hopkins University (Baltimore, Maryland). Serum/plasma specimens were collected from the central ear artery, and urine samples were obtained by bladder pressure; all specimens were collected after animals were anesthetized.

Rabbit Infection
Rabbits were anesthetized by intramuscular administration of ketamine (10 mg/kg) and xylazine (20 mg/kg) prior to endotracheal tube intubation. A 3.0-mm flexible Pentax FB-8V pediatric bronchoscope (Pentax, Montvale, New Jersey) was guided into 1 lower lobe, and 400 µL of 7.5 × 10³ (lower dose) or 1 × 10⁸ (higher dose) log-phase *Mycobacterium tuberculosis* strain H37Rv was inoculated via catheter.

Rabbit Imaging
Computed tomography and positron emission tomography (CT/PET) were performed at times specified in Figure 1. Anesthetized animals were administered 0.2mCu 2-deoxy-2-(18F)fluoro-D-glucose (18F-FDG) by intravenous injection. The animal was placed in an air-tight, HEPA-filtered cylinder, and anesthesia was switched to isoflurane and oxygen. PET/CT was performed using a clinical 8-slice CT scanner (CereTom) and a Philips Mosaic HP Small Animal PET Imager. CT and PET images were coregistered using Amira (Visualization Sciences Group, Burlington, Massachusetts). Amira was also used to calculate the mean and maximum standard uptake values in rabbit lungs.

Rabbit Immune Suppression and Reconstitution
At 140 days after infection, rabbits were administered 10 mg/kg dexamethasone and then continuation dose of 0.1 mg/kg/day for 27 days as described in a previous protocol [18].

Necropsy and Tissue Samples
Rabbits were euthanized following anesthesia by intravenous injection of Euthasol (Virbac, Fort Worth, Texas). Tissues for RNAseq analysis were transferred to RNAlater (Ambion, Life technologies, Grand Island, New York) within 10 minutes. Tissue for protein analysis was snap frozen in liquid nitrogen. Samples for histological analysis were fixed in 10% formalin or in phosphate-buffered saline (PBS) for enumeration of colony-forming units.

RNA Preparation
After 24 hours in RNAlater, samples were frozen at −80°C. RNA was extracted by 3-mm bead beating in Trizol and column purified (Qiagen, Valencia, California). RNA was treated with Ambion Turbo DNA-free DNase (Life Technologies, Grand Island, New York). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California), and messenger RNA was enriched using the RIBO-Minus kit (Life Technologies). Successful removal of ribosomal RNA (rRNA) was verified by a second Bioanalyzer assay.

RNA Deep Sequencing
Starting with 200–500 ng of rRNA-depleted total RNA, fragmentation of the whole transcriptome RNA was by chemical induction, as described in Applied Biosystems SOLiD Total RNA-Seq Kit protocol (Applied Biosystems, Carlsbad, California). Fragmented RNA was purified (RiboMinus Concentration Module; Life Technologies), and size distribution and yield were assessed by Bioanalyzer, using the RNA 6000 pico Chip Kit (Agilent Technologies) and Qubit Fluorometer (Life Technologies), respectively. Construction of the amplified whole-transcriptome library was performed as directed in the Applied Biosystems SOLiD Total RNA-Seq Kit protocol. After reverse transcription, each sample was barcoded with a unique 3′ primer during library amplification. Libraries were run on a DNA1000 chip, using the Agilent Bioanalyzer, to assess library size distribution and quality. Quantification of each library was performed by quantitative polymerase chain reaction (qPCR) analysis, and equimolar concentrations of each library were pooled together to proceed with emulsion PCR and sequencing on the AB 5500xl SOLiD sequencer (Applied Biosystems). CTSK expression was verified by real-time PCR. Briefly, RNA was reverse transcribed by a complementary DNA synthesis kit (Agilent, California) with random primers, and real-time PCR was carried out using SYBR green and primers specific for CTSK and β-actin (iQ5 cycler; BioRad, California). Results have been submitted to the gene expression omnibus at the National Center for Biotechnology Innovation (GEO: GSE68206).

Data Analysis
Reads were aligned to the *Oryctolagus cuniculus* genome, using the Bioscope 1.3 Whole Transcriptome Analysis Pipeline (Applied Biosystems, Carlsbad, California). The number of reads mapped to each transcript was calculated using
stained serial tissue sections in the absence of primary antibody. Secondary antibody binding or chromogen development, we considered significant if $P < .05$.

The complex was coupled to alkaline phosphatase by biotin and avidin (Vector Laboratories, Burlingame, California; reference PK-6100). Tissues were incubated for 30 minutes in serum-free protein blocking agent (Dako, Carpinteria, California; reference H-3300). Tissues were washed in xylene, rehydrated and heated by microwave for 20 minutes in unmasking solution (Vector Laboratories, Rockford, Illinois). For CTSK immunohistochemical analysis, 5-µm serial sections were washed in xylene, rehydrated and heated by microwave for 20 minutes in unmasking solution (Vector Laboratories, Burlingame, California; reference H-3300). Tissues were incubated for 30 minutes in serum-free protein blocking agent (Dako, Carpinteria, California; reference X0909) and incubated with monoclonal mouse anti-human CTSK primary antibody (AbD Serotec, Oxford, United Kingdom; reference MCA5232Z; 60 minutes) and then with biotinylated goat anti-mouse Fc secondary antibody (30 minutes). The complex was coupled to alkaline phosphatase by biotin and avidin (Vector Laboratories, Burlingame, California; reference AK-5000). The development step used the Vulcan Fast Red chromagen (Biocare, Concord, California; reference FR805) and hematoxylin counterstain. To control for nonspecific signal from secondary antibody binding or chromogen development, we stained serial tissue sections in the absence of primary antibody.

RESULTS

Proteases That Cleave Type I Collagen Are Abundantly Expressed in Rabbit Cavity Tissue

A lower-dose sensitizing regimen model was used to generate a range of clinical disease phenotypes in rabbits (Supplementary Figure 1). Three rabbits that developed extensive cavitation as defined by CT were necropsied, and tissue specimens were collected for RNA sequencing. Whole-transcriptome analysis was performed on RNA extracted from 9 samples: 3 from the cavity wall, 3 from grossly normal tissue in the same animals, and 3 from uninfected control animals (Figure 1 and Supplementary Figure 2). Of 17 318 transcripts mapped, expression of 1410 was found to change by $>2$-fold in the cavity wall, as compared to normal infected tissue (Figure 1A), and expression of only 86 protein-coding transcripts changed by $>2$-fold between normal infected tissue and uninfected tissue (Supplementary Figure 2). A total of 77 protease genes (GO:008 233) were identified among the cavity transcripts (Figure 1D and Supplementary Figure 3). Of the 22 proteases that showed significantly different transcription (Figure 1B and 1D) in cavity and noncavity tissue, 5 are known to cleave fibrillar collagen (Figure 1C).

Measurement of Plasma Levels of CTSK in Humans

The CTSK concentration in plasma samples stored with ethylenediaminetetraacetic acid were assessed by ELISA (USCN Life Science). Samples were from 12 patients (6 males; median age, 33 years [range, 25–52 years]) with a diagnosis of active pulmonary tuberculosis confirmed by sputum culture and from 10 healthy blood donors (5 males; median age, 30 years [range, 10–58 years]) who were matched for age and sex and recruited between 2012 and 2014. Median M KK values were used as measure of central tendency, and values were compared between the groups using the Mann–Whitney U test.
MMP-12, which can cleave type III collagen, was the most abundantly expressed protease in cavity tissue (Figure 1A and 1D) [15]. As CTSK has the unique ability to cleave type I collagen both inside and outside the helical domain and has not been previously investigated in tuberculosis, we focused our subsequent studies on this host protease.

CTSK Expression Is Associated With Immunopathology in Rabbits

To confirm and expand upon our sequencing findings, we evaluated a range of lesions from higher-dose sensitization and infected animals for CTSK expression levels by quantitative real-time PCR (Figure 2 and Supplementary Figure 4). Compared with nonpathological tissue, CTSK transcription was

![Figure 1.](https://academic.oup.com/jid/article-abstract/213/4/618/2459385) Collagen and collagenase activity are deregulated at the cavity wall, compared with noncavity tissue. A, By using RNAseq, 17,318 transcripts (gray dots) were identified in either or both cavity wall (y-axis) and infected noncavity tissue (x-axis). Seventy-seven proteases (GO:0008322) were identified (larger dots). Levels of transcripts to the left of the green line are 2-fold greater in cavity tissue, compared with noncavity tissue, and levels of transcripts to the right of the red line are 2-fold greater in noncavity tissue, compared with cavity tissue. Five type I collagen–degrading enzymes (as identified by substrate analysis in MEROPS) were identified (red dots). All of these collagenases were significantly upregulated in cavity tissue. Matrix metalloproteinase 12 (MMP12; green dot) was the most abundant protease in cavity tissue. B, Cathepsin K (CTSK) was the most abundant protease capable of cleaving type I collagen. MMP-1, MMP-13, and MMP-14 were significantly upregulated, as was chymase I, which cleaves procollagen during fibril synthesis. C, MMP-1, MMP-13, and MMP-14 cleave type I collagen alpha subunits at very specific sites, whereas CTSK can cleave type I collagen at multiple sites. The remaining upregulated collagenases consisted of other MMP and cathepsin family proteins, but none of these are implicated in physiological degradation of structural collagens. Statistical analysis was performed using 2-way analysis of variance with the Bonferroni post hoc test for comparison within identified proteases. Abbreviation: FPKM, fragments per kilobase of exon per million kilobases mapped.
substantially greater, by 9.8-fold, in pathological tissue, both cavitary and granulomatous (P < .001; Figure 2). We confirmed the localization of CTSK by performing carefully developed immunohistochemical staining in rabbit pulmonary tissue (Figure 3 and Supplementary Figures 5 and 6). We observed that CTSK protein expression is localized to the periphery of necrotizing, cavitary lesions (Figure 3), within the lesion margins, but not at the necrotic centers (Figure 3B–D). We also noted that, in small granuloma-like lesions, CTSK was abundantly expressed (Supplementary Figure 5).

Collagen Degradation and Synthesis Are Elevated During Cavitary Tuberculosis

To identify the contribution of collagenases to collagen breakdown in vivo we examined markers of type I collagen breakdown in plasma and urine from animals with a range of clinical manifestations of tuberculosis (Figure 4). Three principal pathologies were defined according to the extent of the cavity (Supplementary Figure 7): (1) cavities that represented immediate risk to the animal’s health (terminal cavities; >100 mm³; these animals became moribund), (2) small cavities were not considered life threatening (nonterminal cavities; all <10 mm³; these animals were nonmoribund), and (3) noncavitary consolidation (noncavities). We kept animals with nonterminal cavities for prolonged follow-up (Supplementary Figures 8–10). Serum CICP, a marker released during both degradation and synthesis of collagen, was increased by 3-fold in animals with terminal cavities when compared to the other animals (Figure 4B; P = .0148). The amount of free urinary DPD, also a marker of degradation, was increased by 4-fold in cavitary animals as compared to noncavitary animals (Figure 4C; P = .068). Weight change and 18F-FDG uptake did not differ in these animals (Supplementary Figure 8). These data suggested that increased collagen breakdown and resynthesis occurs during cavitation. This was confirmed by examining fibrillar collagen expression (COL1A1, COL1A2, and COL3A1) in cavitary tissue in the sequencing data (Figure 4D).

CTSK Is Specifically Expressed in a Circumferential Pattern in Human Tuberculosis Lesions

We next studied CTSK expression in patients with pulmonary tuberculosis. As in the rabbit model, CTSK expression localized to the periphery of lesions (Figure 5 and Supplementary Figure 11) and mononuclear cells at the surface of cavities (Figure 5A and 5B). Additionally, we observed CTSK expression at the margin of central caseating material and the fibrous cuff (Figure 5B). We also confirmed previous observations of abundant CTSK expression in multinucleated giant cells (Figure 5D). Finally, we measured levels of CTSK in the plasma of patients with active tuberculosis and compared levels to those in healthy controls. Mean CTSK levels were 13-fold greater (P = .0013) in the peripheral circulation of patients with active tuberculosis, compared with healthy controls (Figure 6).

Figure 2. Cathepsin K (CTSK) is expressed in granulomatous and cavitary pathology in a rabbit model that consistently produces human-like pathology, including cavity formation. A, Quantitative polymerase chain reaction (qPCR) analysis revealed an increase of >9-fold in ctsk expression in granulomatous and cavity tissue, compared with control tissue (n = 8). Data were analyzed by 1-way analysis of variance with the Tukey post hoc comparison test. B, Primers used for qPCR and the sequenced product of these primers after qPCR. Abbreviation: NCBI, National Center for Biotechnology Information.

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DISCUSSION

Tuberculosis continues to plague humankind, and the increasing burden of antibiotic-resistant disease is forcing a return to low-potency, toxic therapeutic regimens [20]. These regimens are extremely costly and have limited efficacy [20]. By dissecting the destructive and protective components of the host immune response, appropriately targeted host-directed therapies may restore immune function and facilitate antibiotic efficacy or offer novel therapeutic advantages in resistant disease [21].

Type I collagen is the key structural component of lung tissue and is distributed throughout the distensible lung regions and provides the mechanical scaffold, while type III collagen is the principal component of alveolar walls [22]. The role of collagen extends beyond the mechanical, facilitating cellular polarity, immune activation, and cellular migration [22]. Collagen degradation in the lung is currently irreversible, and it is typically followed by fibrotic scarring that impairs functionality [22, 23]. Because of this, ECM degradation is tightly regulated [22]. In tuberculosis, extensive fibrosis develops around cavities, which usually persists even after cure of infection, resulting in significantly impaired lung function [24]. In our study, we demonstrated upregulation of multiple collagens and the formation of thick-walled cavities in rabbits, further demonstrating that, in tuberculosis, the matrix remodeling that occurs is dysregulated and obliterates the normal microarchitecture of the lung. Further studies will be required to determine whether earlier prevention of lung destruction by protease inhibition will reduce subsequent fibrosis and impairment of lung function.

We demonstrated that type I collagen cleavage is a feature of cavitation, in agreement with a previous hypothesis regarding cavity formation in pulmonary disease [25]. We identified that CTSK was the most abundantly expressed protease capable of type I collagen cleavage, with levels greater than MMP-1, a collagenase associated with active tuberculosis in multiple cohorts [26–28]. In humans with tuberculosis, CTSK was

Figure 3. Cathepsin K (CTSK) is expressed in nonnecrotic regions of cavitary lesions. CTSK expression in rabbit pulmonary lesions as assessed by immunohistochemical analysis (B and D, compared with hematoxylin-eosin (H-E)-stained sections (A and C). A, H-E-stained section demonstrating a large caseous lesion (green dotted line) with regions of early cavitation (yellow arrows), surrounded by lipoid pneumonia (black dotted line). B, Red anti-CTSK staining localized to the region of lipoid pneumonia. C, Enlarged region showing both necrotic tissue and the adjacent mononuclear infiltrate and lipoid pneumonia. D, CTSK was expressed primarily in the mononuclear infiltrate and occasionally in cells infiltrating the necrotic debris. Thick bars, 5 mm; thin bars, 200 μM.
**Figure 4.** Collagen metabolites vary with disease phenotype in the rabbit model. 

A. The level of C-propeptide of type I procollagen (CICP) in serum was measured at the time of cavitation (day 21) and compared to day 0 serum CICP levels. An increase in CICP level was seen in all infected animals, but this increase was significantly greater in animals with terminal cavities, compared with those without. 

B. A decrease in the level of urinary helical peptide, a fragment of COL1A1, was observed in all infected animals when urine specimens obtained on day 21 were compared to those obtained on day 0 but was lower in animals with terminal cavities. 

C. The level of urinary deoxypyridinoline (DPD) did not change significantly with infection, and there was an insignificant reduction in the level of DPD in animals that did not cavitate. 

D. Fibrillar collagen expression was significantly increased in cavity wall tissue. Expression of all components of type I collagen (COL1A1 and COL1A2) and type III collagen (COL3A1) was increased, as analyzed by RNAseq.
The observation that PIIINP levels (a marker of type III collagen) increased in cavitary animals. Our results are consistent with peptide released during type I collagenases were identified. Levels of CICP, a collagen peptide released during fibril formation and destruction, were increased in cavitary animals. Our results are consistent with the observation that PIIINP levels (a marker of type III collagen turnover) are increased in active tuberculosis [33]. MMP-1, MMP-13, and MMP-14 are known to be upregulated in rabbit cavitary tissue [13], an observation confirmed in our sequencing data (Figure 1B) and correlative with findings from human studies [26–28]. We found macrophage elastase MMP-12 to be highly expressed and upregulated in cavity tissue, although the significance of this finding is unclear as MMP-12 is not induced in human tuberculosis [26, 34]. MMP-1, MMP-13, and MMP-14 all cleave collagen at a unique site located at amino acid 953 (Figure 1C) [15, 35]. The reduction in helical peptide levels that we observed suggest that this fragment is being degraded and indicates that collagen is cleaved outside of the helical region during active tuberculosis. These findings suggested a previously unidentified role for non-MMP collagenolysis in active tuberculosis, implicating CTSK.

Regulators of ECM remodeling, including pirfenidone, nintedanib, odanacatib, and mongersen, are entering the clinical arena [36–39]. Our understanding of these drugs’ effects on ECM-related immune function remains limited. Our data suggest that ECM degradation is a key component of active tuberculosis and, therefore, that these drugs, as well as more-specific MMP and CTSK inhibitors, such as odanacatib, cipemastat, and doxycycline [17, 26, 27, 40], may affect immune balances in pulmonary pathologies [41]. Further study of the role of matrix degradation as a key contributor and potential therapeutic target is warranted, given the potential to repurpose this growing arsenal of drugs to treat destructive pulmonary pathologies. Clearly, caution must be taken if we are to manipulate ECM turnover in the context of infection, as it is known to play a role in immune cell function and cellular migration [42, 43].

Tuberculin skin test (TST) positivity predicted cavity formation in our model (Figure 4B) and is associated with excessive collagenase activity in our model. We did not demonstrate an association between TST reactivity and cavity size, although this may reflect a lack of power due to the sample size or alternatively the inherent variability of outbred rabbits. Prolonged follow-up of our animals revealed that animals that developed cavities were more likely to have progressive active disease (as defined by 18F-FDG PET scanning) than those without (Supplementary Figures 9 and 10). However, 18F-FDG uptake did not predict bacterial burden (Supplementary Figure 12). This suggests a significant role for immune activity, rather than bacterial burden, in driving active disease.

In summary, we investigated cavitary disease, revealing that collagenolytic enzyme expression and collagen turnover are key components of cavitation in a rabbit model of tuberculosis. We identified a role for CTSK in the immunopathogenesis of cavitary tuberculosis in animals and showed correlative findings in humans. Collagenolysis is not known to be a functional component of any immune response, and so there is a possibility that inhibition of this process may benefit the host without...
adversely affecting immune control of bacterial replication. Further experiments to see whether targeting CTSK can prevent cavitation without inhibiting tissue repair are required. The postprimary rabbit model provides an optimal system for such investigations, and the investigation of other therapeutics that modulate extracellular matrix degradation [13, 44]. Such studies will have the dual benefit of assisting our understanding of the importance of matrix regulation in immune responses, as well as providing an avenue, if justified, to bring these therapeutic agents to the clinic.

Supplementary Data
Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes
Acknowledgments. We thank Dr Irini Sereti (NIAID, NIH) for providing the serum samples from patients with tuberculosis. A. K., B. L., C. L., N. C. A., L. S. C., and K. W. designed and performed the animal experiments. A. K., B. L., C. L., M. U., and K. W. designed and conducted RNA analysis. B. B. A., M. O., K. B., M. H., and J. K. P. developed, designed, performed, and interpreted the histopathological assessment of rabbit and human tissues. E. P. A. and B. B. A. designed and performed human serum collection and analysis. A. S., J. S. F., P. T. E., and W. R. B. contributed to study design, manuscript preparation, and grant acquisition. All authors reviewed and contributed to manuscript preparation.

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Financial support. This work was supported by the Howard Hughes Medical Institute (to W. R. B.), the National Institutes of Health (NIH; R01 AI 079590, R01 AI037856, and R01 AI036973 to W. R. B. and R33 A1102239 to P. E.), the NIH Intramural Research Program (to A. S., B. L., C. L., M. U., and K. W.), and the NIH (to C. L.).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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