Title: ESAT-6 drives MMP-10 gene expression and secretion in tuberculosis

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Running title: MMP-10 expression in tuberculosis.

Abstract Word count: 250 words.

Total word count: 3135 words.

Keywords: Matrix Metalloproteinases; ESAT-6; Tuberculosis

Author Contributions:

PTE and JSF conceived the study. TS, NFW, SS and RJW organized the clinical studies, recruited patients and collected data and samples. SB, TS, LHS and RCM performed all in vitro experiments. SB, TS, PTE and JSF analysed all data. All authors participated in drafting and revising the manuscript and have approved the final version of the manuscript.

Abstract

Tuberculosis (TB) causes disease worldwide and multi-drug resistance is an increasing problem. Matrix metalloproteinases (MMPs), particularly the collagenase MMP-1, cause lung extracellular matrix destruction which drives disease transmission and morbidity. The role in such tissue damage of the stromelysin MMP-10, a key activator of the collagenase MMP-1, was investigated in direct *Mycobacterium tuberculosis* (*Mtb*) infected macrophages and in conditioned medium from Mtb infected monocytes (CoMtb)-stimulated cells. *Mtb* infection increased MMP-10 secretion from primary human macrophages 29-fold, while CoMtb increased secretion by 4.5-fold from pulmonary epithelial cells and 10.5-fold from fibroblasts. Inhibition of MMP-10 activity decreased collagen breakdown. In two independent cohorts of TB patients from different continents, MMP-10 was increased in both induced sputum and bronchoalveolar lavage fluid compared to controls and patients with other respiratory diseases (both $p < 0.05$). *Mtb* drove 3.5-fold greater MMP-10 secretion from human macrophages than the vaccine strain BCG ($p < 0.001$), whereas both mycobacteria upregulated TNFα secretion equally. Using overlapping short linear peptides covering the sequence of ESAT-6, a virulence factor secreted by *Mtb* but not BCG, we found that stimulation of human macrophages with a single specific 15 amino acid peptide sequence drove 3-fold greater MMP-10 secretion than any other peptide ($p < 0.001$). *Mtb*-driven MMP-10 secretion was inhibited in a dose-dependent manner by p38 and ERK MAPK blockade ($p < 0.001$ and $p < 0.01$ respectively), but was not affected by inhibition of NF-κB.

In summary, *Mtb* activates inflammatory and stromal cells to secrete MMP-10 and this is partly driven by the virulence factor ESAT-6, implicating it in TB-associated tissue destruction.

Introduction

There were 9.6 million cases and 1.5 million deaths attributed worldwide to tuberculosis (TB) in 2014, and rising drug resistance is of great concern (1). To develop new therapeutic approaches, it is critical to understand underlying mechanisms of disease. In pulmonary TB, extracellular matrix (ECM) breakdown causes cavitation and facilitates *Mtb* transmission (2), drug resistance (3) and also increases morbidity and mortality (4). Matrix metalloproteinases (MMPs) are zinc dependent proteolytic enzymes, which are involved in pulmonary ECM turnover and have diverse immunological roles such as chemokine processing (5). These enzymes may be secreted by leukocytes and stromal cells during *Mtb*-infection (6, 7). Triple helical collagens (types I – III) are the primary structural fibrils of the lung, and MMP-1 and MMP-8 are the predominant secreted collagenases in TB (8, 9). Accumulating evidence from murine and human studies implicates the interstitial collagenase or MMP-1 as a key effector in tissue destruction and cavity formation in pulmonary TB (10, 11).

MMP-10, also known as Stromelysin-2, may promote breakdown of collagen within the ECM, by regulating MMP-1 collagenase activity at both the gene expression level and via zymogen activation (12-14). MMP-10 has also been shown to cleave fibronectin, laminin and type IV collagen in *in vitro* studies (15). A pathological role for MMP-10 in other pulmonary diseases involving ECM degradation has been reported, including lung malignancy (16) and emphysema (17). MMP-10 activation of MMP-1 is of importance in disease models of angiogenesis (14), and MMP-10 gene expression has been shown to be induced by some bacterial infections (18). Besides ECM breakdown, MMPs may have additional functional roles in the immune

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response to infections by modulating cytokine processing, defensin activation (5), or leukocyte influx (19). Recently, a novel role for MMP-10 on macrophage polarization was identified in *Pseudomonas aeruginosa* infection (20). However, MMP-10 has not been systematically investigated in TB.

The region of difference 1 (RD1) is a key determinant of mycobacterial pathogenicity and is absent from all avirulent strains of *M. bovis* BCG and *M. microti.* Deletion of RD1 from *Mtb* results in significant strain attenuation (21). The early secretory antigenic target-6 (ESAT-6) and the culture filtrate protein-10 (CFP-10) are encoded by RD1 genes Rv3874 and Rv3875, are secreted by *Mtb* and are highly immunogenic. They have pleiotropic effects on both innate and adaptive immune responses. For example, ESAT-6 induces macrophage apoptosis and interacts with TLR2 to decrease IL-12 secretion, which can favour a Th2 phenotype (22). ESAT-6 and ESAT-6:CFP-10 complexes may cause pneumocyte cell lysis facilitating *Mtb* dissemination in the lung (23). ESAT-6 also regulates migratory gradients via MMP-9 (24), but the effect of ESAT-6 on MMP-derived tissue destruction has not been investigated.

In this study, we investigated MMP-10 secretion from human macrophages and stromal cells in TB. We measured MMP-10 concentrations in patients, studying sputum and bronchoalveolar lavage fluid (BALF) from two geographically distinct groups, and analysed the mechanisms regulating MMP-10 gene expression and secretion, identifying a novel role for ESAT-6 in driving MMP secretion.

Materials and Methods

Clinical TB studies

Studies were approved by the University of Cape Town (HREC Refs 509/2009 and 516/2011) and Nalanda University Hospitals Research Ethics Committees (REC SS/0810/TB). Informed consent was obtained in all cases. In Ubuntu clinic, Cape Town, TB and control patient recruitment, recording of data, sputum induction and processing was performed as described (25). In the Nalanda University Hospital, Patna, TB and respiratory symptomatic patient recruitment and BALF collection and processing were performed as described (26).

Reagents

SB203580, PD98059 and SC-514 (Millipore, Beeston, UK) were used to block activity of p38 mitogen activated protein kinase (MAPK), extracellular signal related kinase (ERK) and nuclear factor-κB (NF-κB) respectively.

Anti-human MMP-10 (Abcam, Cambridge, UK) and HRP-conjugated anti-rabbit IgG (Cell Signaling, Boston, USA) antibodies were used for western blot. MMP10 neutralizing antibody and IgG2B isotype control antibody (R&D Systems, Abingdon, UK) were used for functional assays.

Lipoarabinomannan (LAM, Colorado State University, USA) and peptides covering the entire ESAT-6 sequence (Pepceuticals, Enderby, UK) were used to stimulate macrophages. Peptides sequences are shown in Table 1. Peptide purity was>90% by mass spectrometry.

Cell culture

Human monocytes were matured over 5 days in RPMI with 100ng/ml M-CSF (R&D Systems), 2mM glutamine, 10µg/ml ampicillin and 10% heat inactivated FBS. Macrophages in serum-free media were infected with *Mtb* H37Rv or BCG at multiplicity of infection (MOI) 1, which were cultured in Middlebrook 7H9 medium (BD Biosciences, Oxford, UK) and used at mid-log growth.

Normal human bronchial epithelial (NHBE) cells (Lonza, Slough, UK) were cultured according to suppliers' instructions. MRC-5 lung fibroblasts were cultured in MEM with 2mM glutamine, 10µg/ml ampicillin, 1% sodium pyruvate, non-essential amino acids and 10% FBS. Experiments commenced at 80% confluence.

Conditioned Medium from *Mtb* infected Monocytes (CoMtb)

CoMtb was prepared as previously described (27). Briefly, monocytes were infected with *Mtb* H37Rv at a MOI 1, incubated for 24h and supernatants sterile filtered . Medium from uninfected monocytes was termed CoMCont.

MMP-10, TNFα ELISA and Luminex assays

ELISA Duoset kits (R&D Systems) were used to measure MMP-10 and TNFα concentrations in cell supernatants. Lower detection limits were 31.2pg/ml and 15.6pg/ml respectively. MMP-10 Fluorokine beads (R&D Systems) were used to measure concentrations of MMP-10 in induced sputum and BALF samples on the Luminex 200 platform (Bio-Rad, Hertfordshire, UK). Lower detection limit was 3.2pg/ml. Assays were performed as per manufacturer's instructions.

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using the Purelink RNA Mini Kit (Life Technologies) and reverse transcribed with the Quantitec Reverse Transcription Kit (Qiagen, Manchester, UK). RT-PCR was performed on a Stratagene Mx3000Pro using MMP-10 and β-actin primers and probes (Applied Biosystems, Warrington, UK). Analysis was performed using ΔΔCt method.

Collagenase activity

MMP-1 collagenolytic activity was measured using EnzChek DQ type I collagen assay (Life Technologies) performed as per manufacturer's instructions. DQ collagen coated slides were used for confocal microscopy.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Mann-Whitney U, Student's unpaired t-test or one-way analysis of variance with Tukey's post hoc analysis were used as appropriate. $p < 0.05$ was considered significant.

Results

Mtb Infection drives MMP-10 secretion from human macrophages, respiratory epithelial cells and fibroblasts

First, MMP-10 secretion from *Mtb*-infected macrophages was investigated. *Mtb* caused a dose dependent upregulation in MMP-10 secretion, causing a 20.2-fold increase (370±331.6pg/ml to 7461.7±2073.7pg/ml) increase at a MOI of 0.5 and 29-fold (10719.3±1713.2pg/ml) at a MOI of 1 compared to uninfected macrophages (both $p < 0.001$). Low dose infection at MOI of 0.1 did not upregulate MMP-10 (Fig. 1A). Kinetic studies showed a 5.1-fold upregulation of MMP-10 secretion from *Mtb*-infected macrophages at 48 hours ($p < 0.01$) increasing to 31.5-fold at 72 hours ($p < 0.001$; Fig. 1B). Western blotting confirmed that *Mtb* stimulation drives MMP-10 secretion from macrophages at 72 hours (Fig. 1C). Increased secretion was secondary to increased gene expression, as *Mtb* increased MMP-10 mRNA accumulation by 182-fold compared to uninfected macrophages at 24 hours ($p < 0.0001$; Fig.1D).

Next, we investigated MMP-10 secretion from CoMtb-simulated pulmonary epithelial cells and fibroblasts to investigate the effects of monocyte-dependent intercellular networks. CoMtb-stimulation increased MMP-10 secretion by 4.5-fold (from 2525±208.5pg/ml to 11250±886.1pg/ml; $p < 0.0001$) from epithelial cells (Fig. 1E) and 10.5-fold from fibroblasts (from 641.7±152.9pg/ml to 6715.7±391.9pg/ml; $p < 0.0001$; Fig. 1F) compared to unstimulated cells.

To confirm that MMP-10 was functionally active and affected MMP-1 collagenolytic activity, 15µg/ml MMP-10 neutralizing antibody was used to block MMP-10 activity. Collagenolytic activity was analysed using DQ type I collagen, which fluoresces in areas of collagen degradation. A significant increase in collagen breakdown was observed in macrophages infected with Mtb by confocal microscopy, compared with uninfected controls. Inhibition of MMP-10 activity led to inhibition of DQ collagen degradation by Mtb infected macrophages (Fig. 2A). Similarly, in CoMtb-stimulated NHBE cells, addition of 15µg/ml MMP-10 neutralizing antibody significantly decreased collagen breakdown ($p < 0.0001$; Fig. 2B).

MMP-10 is increased at the site of infection in patients with pulmonary TB

To investigate the relevance of MMP-10 in clinical disease, we measured MMP-10 levels in the respiratory secretions of two separate patient cohorts. We studied induced sputum of patients with TB (mixed HIV serostatus) and non-TB controls from South Africa, and BALF of HIV-uninfected TB patients compared to patients with non-tuberculous respiratory disease from India. MMP-10 concentrations were increased in patients with TB compared to controls groups for both these cohorts (Fig. 3; $p < 0.05$). In the induced sputum study of South African patients, MMP-10 concentrations were 5067pg/ml (1440–19077pg/ml) in TB patients compared to 2648pg/ml (797–5914pg/ml) in controls. In BALF, MMP-10 concentrations were 6531pg/ml (637-11929pg/ml) in TB patients compared to 783pg/ml (76-5473pg/ml) in respiratory symptomatic controls. MMP-10 mRNA accumulation was also increased by 60% in induced sputum of TB patients compared with controls, normalised to β-actin (Fig. 3B; $p < 0.05$). Therefore, these findings show that MMP-10 is present at the

site of disease in pulmonary TB and that concentrations are higher in TB compared to other respiratory diseases.

MMP-10 Expression in Human macrophages is driven by virulent *Mtb* but not by lipoarabinomannan or the Vaccine Strain BCG

Next, we investigated whether MMP-10 secretion was driven specifically by virulent *Mtb* or was a non-specific response to infection. LAM is a mycobacterial cell wall component and principally a TLR2 agonist known to be important in inflammatory responses to *Mtb* (28). Stimulation of macrophages with 10µg/ml LAM resulted in MMP-10 secretion which was 5% of that observed after stimulation with live *Mtb* (Fig. 4A). In contrast, TNFα secretion from macrophages in response to LAM and direct *Mtb* infection were similar (Fig. 4B). LAM stimulated MMP-1 secretion which was 30% of that driven by direct *Mtb* infection (data not shown).

Tissue destruction is not usually a feature of pulmonary infection with the vaccine strain *M. bovis* BCG (29). MMP-10 concentrations following macrophage infection with BCG were 586.3±96.7pg/ml compared to 2047.7±347pg/ml from *Mtb*-infected macrophages at 72 hours (Fig. 4C, $p<0.001$). *Mtb* infection of macrophages upregulated MMP-10 gene expression 4–fold more than BCG infection at 24 hours (Fig. 4D, $p< 0.001$), demonstrating that the divergent effects of BCG and *Mtb* on MMP-10 secretion are transcriptionally regulated. In contrast, *Mtb* and BCG stimulated similar TNFα secretion from macrophages at 72 hours (Fig. 4E). Colony counting confirmed equal infectious doses of *Mtb* and BCG were used in these experiments (data not shown).

MMP-10 secretion is dependent on a specific 15 amino acid peptide sequence in ESAT-6 of *Mtb*

Since BCG did not drive MMP-10 secretion and lacks the RD1 region in its genome (30), we investigated ESAT- 6, a protein encoded by the RD1 region which is implicated in *Mtb* virulence (31) and modulation of the host immune response (32, 33). Macrophages were stimulated with a series of short linear peptide sequences from ESAT-6 (Table 1). Stimulation of macrophages with the total pool of all ESAT-6 peptides at a concentration of 10µg/ml caused a 17.6-fold increase in MMP-10 secretion (from 7.8±7.9pg/ml to 137.2±56.5pg/ml), while 1µg/ml ESAT-6 peptides increased MMP-10 by 4.7-fold (36.3±25.9pg/ml) compared with unstimulated macrophages at 72 hours (Fig. 5A, p<0.001). Next, overlapping pools containing 3-4 peptides at a total concentration of 10µg/ml, defined by the matrix shown in Fig. 5B, were used to stimulate macrophages. Pools 1 and 8 were the only pools that caused significantly higher MMP-10 secretion at 72 hours than unstimulated macrophages, with a 5.2-fold and 3.9-fold upregulation respectively (Fig. 5C p<0.001 and p<0.01). The single peptide common to both these pools is ESAT-6 peptide 4, which has the sequence SAIQGNVTSIHSLLD (Table 1). Stimulation of macrophages with 10µg/ml ESAT-6 peptide 4 alone drove a 3-fold increase in MMP-10 secretion of 66.7±11.7pg/ml compared to compared to the randomly selected ESAT-6 peptide 13 (present in non-stimulatory peptide matrix pools 3 and 7) and an 4-fold increase in MMP-10 when compared to control, unstimulated macrophages at 72 hours (Fig 5D p<0.001), confirming peptide 4 was the dominant regulator of MMP-10.

MMP-10 Secretion from *Mtb*-stimulated macrophages is MAPK-dependent but NF-κB independent

Next, we investigated the role of key signalling pathways in control of *Mtb*-driven MMP-10 secretion. p38 MAPK inhibition caused a significant dose-dependent decrease in *Mtb*-driven MMP-10 secretion from macrophages at 72 hours (Fig. 6A). ERK MAPK inhibition caused a less marked but significant decrease in MMP-10 secretion with 10μM PD98059 (Fig. 6B, p<0.01). Similarly, blockade of both the p38 and ERK MAP kinases inhibited MMP-10 gene expression (Fig. 6C, p<0.01). No significant cell death was observed with the concentrations of inhibitors used.

NF-κB regulates gene expression of diverse MMPs including collagenases in TB (34, 35). MMP-10 does not have a consensus NF-κB binding site in its promoter although non-canonical binding was shown to the promoter of the related stromelysin MMP-3 (36). NF-κB inhibition by the IKK-2 inhibitor SC-514 did not alter *Mtb*-driven MDM MMP-10 secretion (Fig. 6D). As a control, we confirmed that 10μM SC-514 reduced *Mtb* driven MMP-1 secretion by 51% (Fig. 6E), demonstrating that MMP-10 regulation is NF-κB independent.

Discussion

The stromelysin MMP-10 regulates activity of the collagenase MMP-1, which plays a central role in TB pathogenesis (8), but has not previously been investigated in TB. In the present study, we showed that *Mtb* infection drives macrophage MMP-10 gene expression and secretion and investigated its regulation. MMP-10 secretion was also increased by CoMtb stimulation of pulmonary epithelial cells and fibroblasts, which emphasizes a key role for monocyte-dependent networks in stimulating stromal cells, thereby amplifying the matrix-degrading response. Furthermore, MMP-10 may be an important driver of type I collagen breakdown via its regulation of MMP-1 activity, since we demonstrated that inhibition of MMP-10 decreases collagen breakdown with Mtb-stimulation. Our data is consistent with other studies on the role of MMP-10 on pro-collagenase activation (13, 14), although *in vivo* studies that address these findings are still limited, possibly due to experimental challenges in providing *in vivo* correlates.

Networking effects are similarly important in driving MMP-1 expression in TB (27, 35, 37). ESAT-6, a secreted virulence factor not expressed by the attenuated vaccine strain *M. bovis* BCG, upregulated MMP-10 secretion, implicating this pathway in mycobacterial pathology. Furthermore, increased MMP-10 expression has been shown to drive macrophage polarization towards an M2-like phenotype (20), an alternative-activated form of macrophage that is be present within TB granulomas and associated with Mtb persistence (38).

In clinical studies, increased MMP-10 concentrations were detected in the respiratory secretions of patients with pulmonary TB relative to non-TB controls, and compared to patients with non-tuberculous pulmonary diseases. These data were consistent

between two populations from different continents. To understand tissue destruction in human TB, it is important to study TB patients since most animal models do not accurately mimic the cavitatory disease seen in man (39). Our data are consistent with the observation that MMP-10 gene expression was 14-fold higher in human TB granulomas compared to normal lung tissue on microarray analysis (40). Our finding of increased levels of the MMP-10 by Luminex bead array in induced sputum and BALF of TB patients shows that such increased gene expression translates to secreted protein which may augment pathology in clinical disease. Furthermore, the finding in the Indian cohort of patients that MMP-10 is increased in the BALF of TB patients compared to patients with other respiratory diseases indicates that there is some specificity in the increased MMP-10 concentrations in TB. MMP-10 activity has been implicated in other destructive pulmonary pathology, such as COPD (41), and was associated with disease severity and prognosis in patients with idiopathic pulmonary fibrosis (42). Although our study implicated MMP-10 in the protease network upregulated in TB, as MMP-10 was increased in respiratory secretions of TB patients and at gene expression level, we did not precisely define the cellular source. However, it is most likely due to an MMP-amplification network involving multiple cell types within the granuloma including macrophages, respiratory epithelial cells and fibroblasts.

Tissue destruction and cavitation are a feature of pulmonary TB, whereas administration of vaccine BCG does not cause lung pathology in immunocompetent individuals (29). *Mtb* and BCG caused similar upregulation in TNFα secretion, showing that BCG causes a pro-inflammatory reaction, but did not upregulate MMP-10. This finding suggested that specific factors present in *Mtb* but not BCG were

responsible for driving MMP-10 expression. RD1 is a region absent in the genome of all BCG strains and avirulent mycobacteria, but present in virulent *M. bovis* and *Mtb* (30). Mice infected with a RD-augmented BCG in showed an increased bacterial growth rate and inflammatory cell infiltration with granuloma formation, confirming its role in pathogenesis, but an emerging paradigm is that RD1 has multiple effects to cause pathology (24, 31). We employed an overlapping pool of peptides spanning the entire ESAT-6 sequence to identify a single linear 15aa sequence which alone was sufficient to drive MMP-10 secretion from macrophages. Thus, ESAT-6-dependent MMP-10 secretion is an additional pathological mechanism through which this protein promotes *Mtb* virulence. This peptide, along with other ESAT-6 fragments, has been shown previously to induce specific IFN-γ -secreting T cells (43). Therefore, it is possible that in macrophages this peptide may act as a pathogen associated molecular pattern (PAMP) to activate pattern recognition receptors (44, 45).

There are multiple signalling pathways involved in the innate immune response to *Mtb*. TLR activation by *Mtb* infection drives activation of signalling molecules MyD88, p38, ERK, PI3K and NF-κB pathways (46, 47). These pathways have been shown to regulate diverse MMPs in *Mtb* infection and other diseases, but their importance in controlling MMP-10 expression has not been assessed. MMP-10 secretion from *Mtb* infected macrophages was regulated by both p38 and ERK MAP kinase signalling. ESAT-6, which we showed activated MMP-10 secretion, is also known to increase p38 and ERK MAPK phosphorylation in macrophages. MAP kinases often activate inflammatory mediators in a NF-kB dependent manner (37, 48). Both Mtb and LAM stimulation activated TLR2 leading to similar TNF-α induction via MAP/ NF-kB

signalling. However, our data shows that MMP-10 upregulation appears independent of NF-kB. It is possible that the independence of MMP-10 expression from the NF-κB /TLR2 signalling pathway may be part of a regulatory network to limit inflammation, as less MMP-10 at the site of disease may prevent excessive tissue destruction. Activator Protein 1 (AP-1) and Signal Transducer and Activator of Transcription (STAT), both of which have consensus binding sites in the MMP-10 promoter (49), may be more important factors and are known to regulate ERK-dependent MMP-10 expression in cardiomyocytes (50).

In summary, MMP-10 is upregulated by macrophage *Mtb* infection and by TB-dependent networks acting on respiratory epithelial cells and fibroblasts. MMP-10 is elevated in induced sputum and BALF of patients with TB. MMP-10 expression during *Mtb* infection is specifically driven by a central peptide sequence within ESAT-6, which is absent in BCG, causing ERK and p38 MAPK-dependent expression of MMP-10. In pulmonary TB, MMP-10 may have a key role in promoting tissue destruction and cavitation, acting at the apex of a proteolytic cascade resulting in matrix degradation.

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Tables

Table 1- ESAT-6 overlapping peptide sequences.

Peptide number	Sequence
1	MTEQQWNFAGIEAAA
2	WNFAGIEAAASAIQG
3	IEAAASAIQGNVTSI
4	SAIQGNVTSIHSLLD
5	NVTSIHSLLDEGKQS
6	HSLLDEGKQSLTKLA
7	EGKQSLTKLAAAWGG
8	LTKLAAAWGGSGSEA
9	AAWGGSGSEAYQGVQ
10	SGSEAYQGVQQKWDA
11	YQGVQQKWDATATEL
12	QKWDATATELNNALQ
13	TATELNNALQNLART
14	NNALQNLARTISEAG
15	NLARTISEAGQAMAS
16	ISEAGQAMASTEGNV
17	QAMASTEGNVTGMFA

Figure Legends

Figure 1- *Mtb* **Infection drives MMP-10 secretion from macrophages, respiratory epithelial cells and fibroblasts.**

A MMP-10 secretion from macrophages infected with increasing MOI of *Mtb*. B Kinetics of MMP-10 secretion from *Mtb* infected macrophages (MOI 1). C Western blot on cell supernatants were performed to analyse secreted MMP-10 by macrophages after 72h of *Mtb*-infection. MMP-10 corresponds to a band of 54kDa. D MMP-10 gene expression in *Mtb* infected macrophages. MMP-10 transcript abundance was measured by RT-PCR and was normalized to β-actin. Copy numbers were calculated from standard curves with known concentrations of MMP-10 and β-actin. E MMP-10 secretion from CoMtb (1:5 dilution) stimulated pulmonary epithelial cells at 72h. F MMP-10 secretion from CoMtb (1:10 dilution) stimulated pulmonary fibroblasts at 72h. Data corresponds to mean±SD and are representative of at least 2 independent experiments performed in triplicate.**p<0.01, ***p<0.001, ****p<0.0001.

Figure 2- Mtb-driven MMP-10 is required for MMP-1 collagenolytic activity in macrophages and respiratory epithelial cells.

A Confocal microscopy figures of collagen breakdown in control macrophages, infected with Mtb (MOI 1) and where MMP-10 activity was inhibited by 15µg/ml neutralizing antibody. 15µg/ml of an IgG2B isotype was used as control. Nucleic acids were stained with DAPI (blue), while DQ type I collagen breakdown is shown by release of FITC fluorescence (green). Overlay of DAPI, FITC and bright field is

also shown in the last row of panels. Scale bar: 50µm. B Supernatants of NHBE cells stimulated for 72h with CoMCont, CoMtb and where MMP-10 activity was inhibited by 15µg/ml neutralizing antibody where used on a DQ type I collagen assay. 15µg/ml of an IgG2B isotype was used as control. Increase in fluorescence corresponds to increase in DQ collagen breakdown. Data corresponds to mean±SD and are representative of at least 2 independent experiments performed in triplicate. ****$p < 0.0001$; ns- not significant.

Figure 3- MMP-10 is elevated in pulmonary TB in two independent cohorts of distinct ethnicity.

A Induced sputum samples were collected prospectively from patients with active pulmonary TB (n=21) and non-TB controls (n=21) in Cape Town, South Africa. B BALF samples were collected prospectively from patients with active pulmonary TB (n=14) and patients with other non-tuberculous pulmonary diseases (n=17) in Patna, India. MMP-10 concentrations were measured by Luminex, and in each cohort MMP-10 was elevated in patients with TB. C Induced sputum samples were collected prospectively from HIV-negative patients with active pulmonary TB (n=11) and healthy controls (n=17) in Cape Town, for RNA extraction. MMP-10 mRNA accumulation was analysed by reverse transcription RT-PCR using β-actin mRNA as control. Statistical analysis was performed using a Mann-Whitney U test (*$p < 0.05$).

Figure 4- MMP-10 secretion in human macrophages is driven by *Mtb* but not by mycobacterial LAM or vaccine BCG strain.

MMP-10 and TNFα secretion from macrophages in response to *Mtb* infection (MOI 1) or stimulation with lipoarabinomannan (LAM; 10µg/ml). A MMP-10 secretion from macrophages at 72h post-stimulation with LAM or *Mtb*. B TNFα secretion from macrophages at 72h post-stimulation with LAM or Mtb. C MMP-10 secretion at 72h from *Mtb* (MOI 1) infected macrophages compared to BCG-infected macrophages (MOI 1). D MMP-10 gene expression at 24h from *Mtb* infected macrophages compared to BCG infected macrophages (normalised to β-actin). E. TNFα secretion from *Mtb* infected macrophages and BCG infected macrophages at 72h. Data corresponds to mean±SD and are representative of 3 independent experiments performed in triplicate. **$p<0.01$, ***$p <0.001$.

Figure 5- A 15 amino acid peptide sequence of ESAT-6 drives MMP-10 secretion from human macrophages.

A MMP-10 secretion from macrophages stimulated with complete pool of 17 overlapping ESAT-6 peptides spanning the entire protein sequence (see Table 1). B Matrix indicating peptides present in pools 1 to 9 pools 1-9 containing all peptides (P1-P17). C MMP-10 secretion from macrophages stimulated with the ESAT-6 peptide pools from the matrix. D MMP-10 secretion from macrophages stimulated with ESAT-6 peptide 4 or ESAT-6 peptide 13 individually. Data corresponds to mean±SD and are representative of 3 independent experiments performed in triplicate.**$p<0.01$, ***$p<0.001$.

Figure 6- MMP-10 expression in *Mtb*-infected human macrophages is p38 and ERK MAPK dependent but NF-κB independent.

A, B MMP-10 secretion at 72h post- *Mtb* infection of human macrophages treated with chemical inhibitors of p38 (A) and ERK MAPK (B). macrophages were pre-incubated with the p38 MAPK inhibitor SB203580 (SB) or the ERK MAPK inhibitor PD98059 (PD) at 1μM or 10μM for 2h prior to *Mtb* infection (MOI 1). C MMP-10 gene expression normalized to β-actin in *Mtb* infected macrophages SB (p38) or PD (ERK) chemical inhibitors. D, E MMP-10 (D) and MMP-1 (E) secretion from *Mtb* infected human macrophages with chemical inhibition of NF-κB signalling. macrophages were pre-incubated with SC-514 at 1μM or 10μM for 2h prior to *Mtb* infection. Data corresponds to mean±SD and are representative of 3 independent experiments performed in triplicate. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.
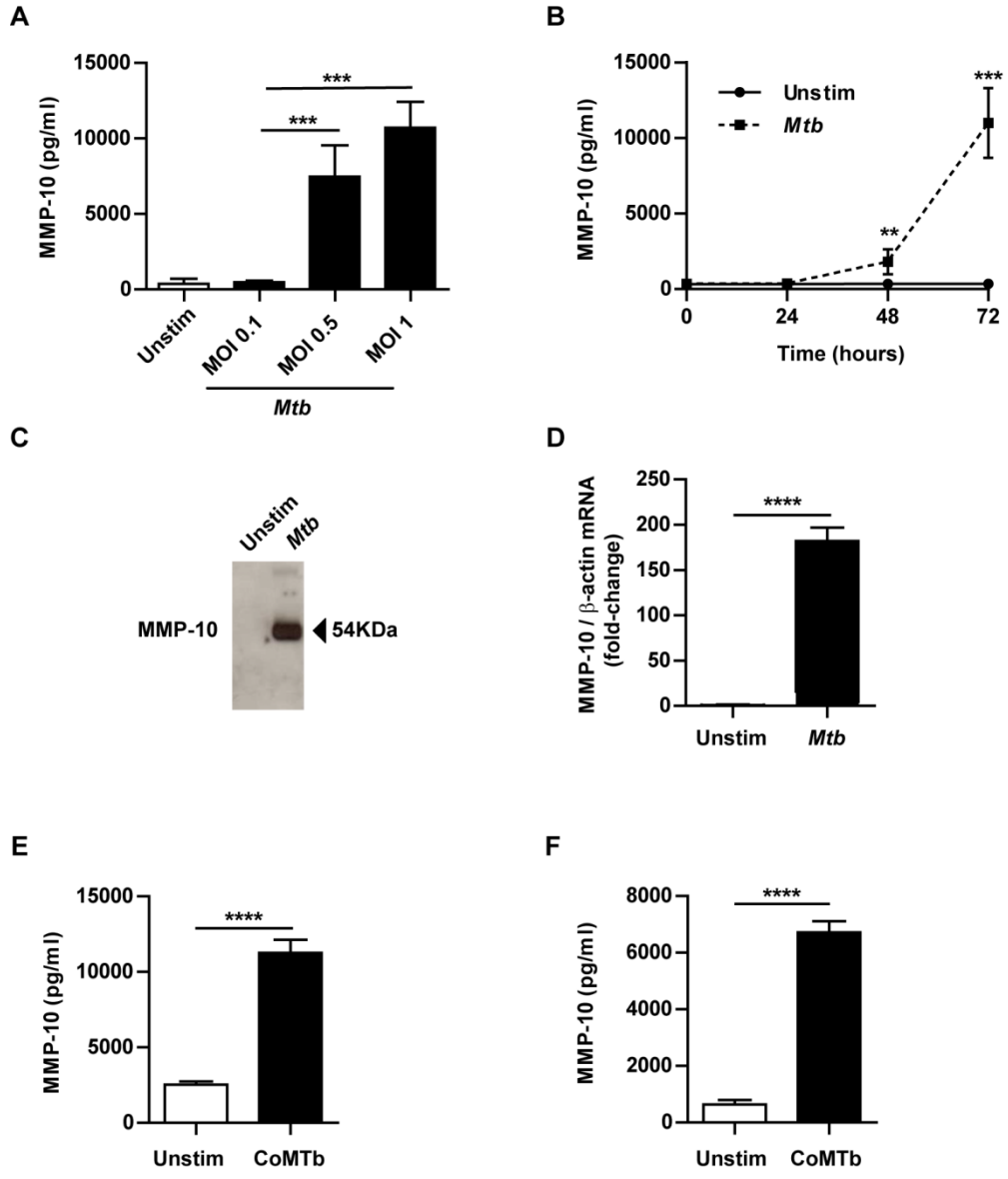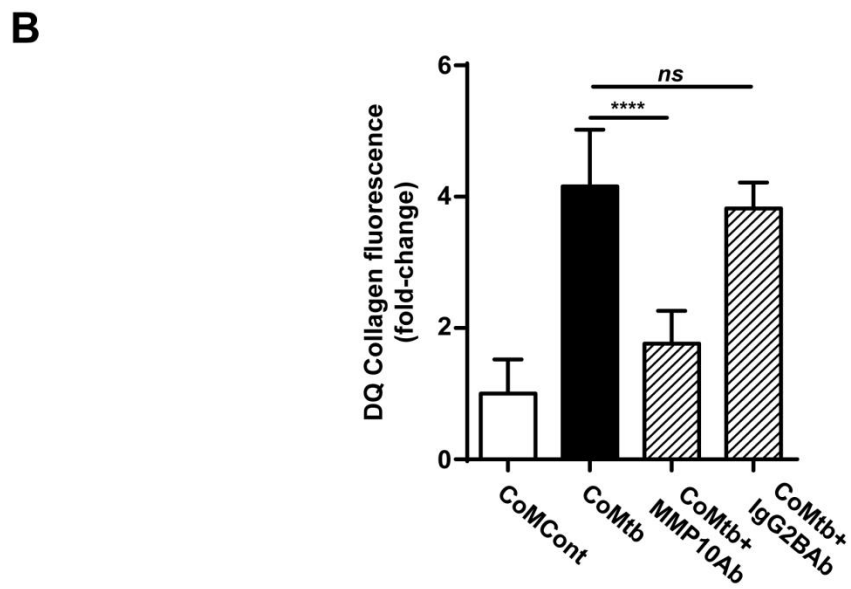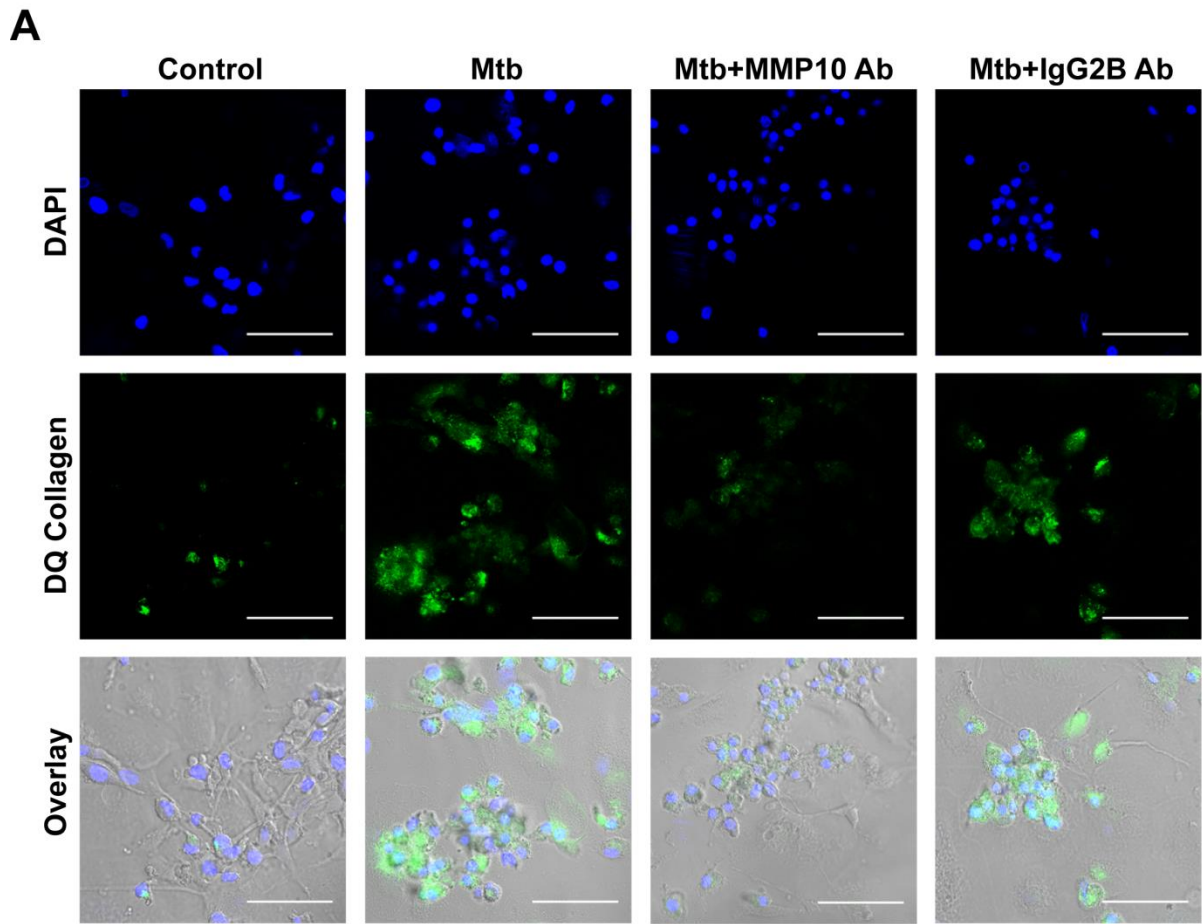
FIG. 1

Fig. 2

A

B

FIG. 3

A — Induced sputum (South Africa) — MMP-10 (pg/ml) — Control vs TB — *

B — Bronchoalveolar lavage fluid (India) — MMP-10 (pg/ml) — Respiratory symptomatic vs TB — *

C — RNA from induced sputum (South Africa) — MMP-10/β-actin mRNA — Control vs TB — *

Fig. 4

A

B

C

D

E

FIG. 5

A

ESAT-6 all peptides pool

B

Peptide pool					
	5	**6**	**7**	**8**	**9**
1	P1	P2	P3	P4	P5
2	P6	P7	P8	P9	P10
3	P11	P12	P13	P14	P15
4	P16	P17			

C

ESAT-6 matrix peptide pools
10 µg/ml
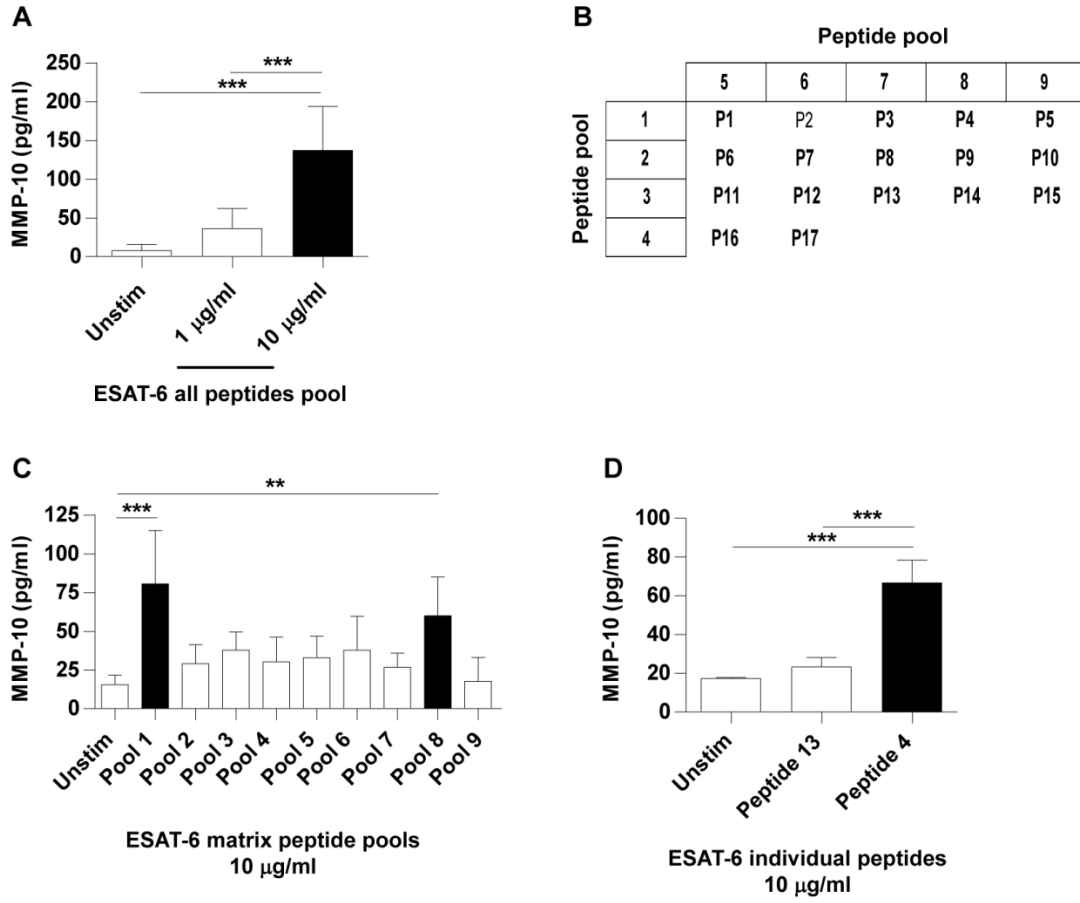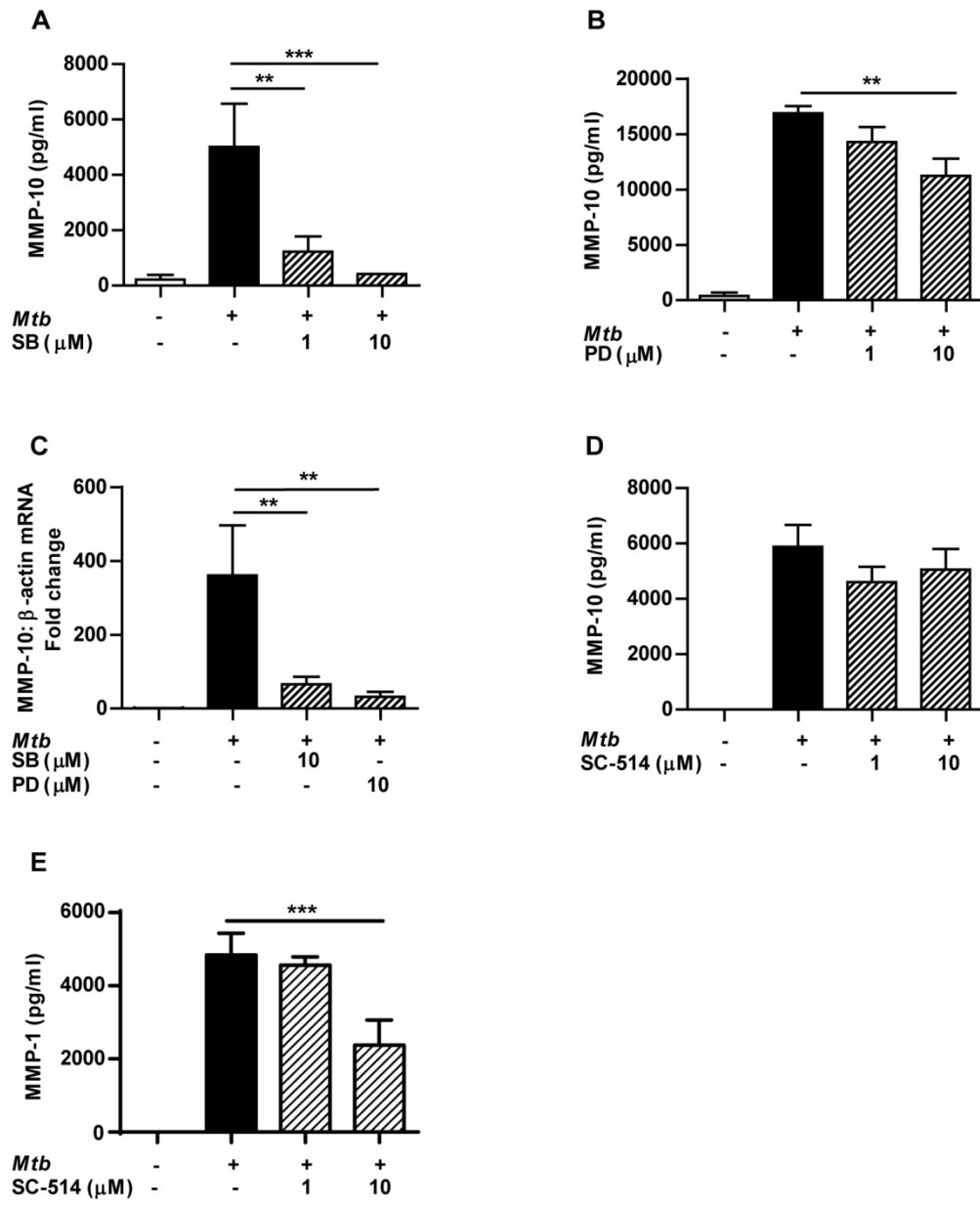
D

ESAT-6 individual peptides
10 µg/ml

FIG 6.

Supplementary Material and Methods

Western blotting

Cell supernatants in loading buffer were run on NuPAGE 4-12% Bis-Tris gels (Life Technologies, Paisley, UK). Proteins were electro-transferred to nitro-cellulose membranes, which were blocked and incubated with rabbit anti-human MMP-10 (Abcam, Cambridge, UK) and HRP-conjugated goat anti-rabbit IgG (Cell Signalling, Boston, USA) antibodies, placed in chemiluminescent substrate and exposed to chemiluminescence film (ECL, GE Healthcare, Little Chalfont, UK).

Confocal microscopy

4-well glass slides were pre-coated with DQ type I collagen (Life Technologies). Macrophages were seeded and infected with Mtb (MOI= 1). MMP-10 activity was blocked using mouse anti-human MMP-10 neutralizing antibody (R&D systems). Mouse IgG2B antibody (R&D systems) was used as a control.

Slides were fixed with 4% paraformaldehyde and DAPI was used as nuclear counterstain. Slides were scanned using a 40x oil immersion objective and to avoid bleed-through effects, each dye was scanned independently in a Leica TCS SP5 confocal microscope equipped with 405nm diode laser, 488nm argon laser, 543nm and 633nm HeNe lasers and using the Leica Application Suite 2.6.2 software (Milton Keynes, UK). Images were edited using ImageJ software v1.46r (NIH, Maryland, USA).

RNA extraction and Real-Time Polymerase Chain Reaction (RT-PCR) from induced sputum

Sputum induction using nebulised 5% saline has previously described. 1ml of RNA later (Qiagen) was added on site to the samples which would be processed for RNA extraction. Mucolysis was performed by adding an equal volume of 0.1% Dithiothreitol (Sigma-Aldrich)

and agitating gently at room temperature for 20 min. The mucoid layer was filtered through 100μm pore size strainer and centrifuged at 500g for 10 min. The cell pellet was aspirated and 1.5ml of cold TRI reagent added before vortexing. Total RNA was extracted using the Purelink RNA Mini Kit (Life Technologies) and reverse transcription RT-PCR was performed using 15ng RNA and the OneStep RT-PCR Kit (Qiagen), on a Stratagene Mx3000Pro using MMP-10 and β-actin primers and probes (Applied Biosystems, Warrington, UK). Analysis was performed using the Pfaffl comparative Ct method, applying the equation: Ratio (MMP-10/ β-actin mRNA)= $E_{MMP10}^{\Delta Ct(calibrator-sample)}/E_{\beta\text{-actin}}^{\Delta Ct\ (calibrator-sample)}$; E is the real-time PCR efficiency of one cycle in the exponential phase, calculated according to the equation: $E= 10^{[-1/slope]}$. Samples without Ct for MMP-10 after 43 cycles but with Ct for the reference gene were considered negative.