STAT3, p38 MAPK, and NF- κ B Drive Unopposed Monocyte-Dependent Fibroblast MMP-1 Secretion in Tuberculosis

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Tissue destruction characterizes infection with *Mycobacterium tuberculosis* (*Mtb*). Type I collagen provides the lung's tensile strength, is extremely resistant to degradation, but is cleaved by matrix metalloproteinase (MMP)-1. Fibroblasts potentially secrete quantitatively more MMP-1 than other lung cells. We investigated mechanisms regulating *Mtb*-induced collagenolytic activity in fibroblasts *in vitro* and in patients. Lung fibroblasts were stimulated with conditioned media from *Mtb*-infected monocytes (CoMTb). CoMTb induced sustained increased MMP-1 (74 versus 16 ng/ml) and decreased tissue inhibitor of metalloproteinase (TIMP)-1 (8.6 versus 22.3 ng/ml) protein secretion. CoMTb induced a 2.7-fold increase in MMP-1 promoter activation and a 2.5-fold reduction in TIMP-1 promoter activation at 24 hours ($P = 0.01$). Consistent with this, TIMP-1 did not co-localize with fibroblasts in patient granulomas. MMP-1 up-regulation and TIMP-1 down-regulation were p38 (but not extracellular signal-regulated kinase or c-Jun N-terminal kinase) mitogen-activated protein kinase-dependent. STAT3 phosphorylation was detected in fibroblasts *in vitro* and in tuberculous granulomas. STAT3 inhibition reduced fibroblast MMP-1 secretion by 60% ($P = 0.046$). Deletion of the MMP-1 promoter NF- κ B-binding site abrogated promoter induction in response to CoMTb. TNF- α , IL-1 β , or Oncostatin M inhibition in CoMTb decreased MMP-1 secretion by 65, 63, and 25%, respectively. This cytokine cocktail activated the same signaling pathways in fibroblasts and induced MMP-1 secretion similar to that induced by CoMTb. This study demonstrates in a cellular model and in patients with tuberculosis that in addition to p38 and NF- κ B, STAT3 has a key role in driving fibroblast-dependent unopposed MMP-1 production that may be key in tissue destruction in patients.

Keywords: stromal cells; monocytes/macrophages; human; signal transduction; inflammation

Extensive tissue damage is the hallmark of pulmonary tuberculosis (TB). Cavitation and fibrosis contribute both to bacillary spread and survival during the acute illness and to chronic morbidity (1). The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that degrade extracellular matrix (ECM) proteins and cleave cytokines. Although MMPs

CLINICAL RELEVANCE

This article identifies sustained up-regulation of the collagenolytic enzyme matrix metalloproteinase-1 by fibroblasts in tuberculosis, with accompanying down-regulation of its major inhibitor, tissue inhibitor of metalloproteinase-1. This is mediated by monocyte-dependent activation of the p38 and STAT3 intracellular signaling pathways in fibroblasts, and by NF- κ B. These experiments provide novel insight into the regulation of matrix destruction in tuberculosis, and suggest that inhibiting these proinflammatory pathways may have a therapeutic role in limiting the tissue damage that characterizes this disease.

are required for leukocyte recruitment to sites of injury and infection, high level MMP activity is implicated in matrix breakdown and tissue remodeling in lung diseases such as emphysema, bronchiectasis, pulmonary fibrosis (2–4), and in TB (5, 6). Our group demonstrated that human macrophages infected with *Mycobacterium tuberculosis* (*Mtb*) but not vaccine strain BCG secrete MMP-1 (7). At neutral pH, MMP-1 (interstitial collagenase) is the most potent enzyme capable of degrading type I collagen, which provides the lung's tensile strength (8). An MMP-1 promoter polymorphism associated with increased transcriptional activity has been linked with more extensive radiologic change and cavity formation in patients with TB (9, 10). These data suggest MMP-1 may be a key mediator of tissue destruction in TB.

In addition to inflammatory leukocytes, parenchymal cells are increasingly recognized as important sources of MMP activity in disease (11, 12). In the lung, fibroblasts may secrete the highest MMP-1 concentrations (13). Little is known about the role of fibroblasts in TB. Medium or lysates from virulent and avirulent strains of *Mtb* may induce changes in fibroblast MMP-1/13 and tissue inhibitor of metalloproteinase (TIMP)-1 expression (14), but the net effect of medium or lysates on fibroblast-mediated matrix degradation is unclear, and this study did not investigate the effects of whole live bacilli (14). *In vivo* fibroblasts can become infected with *Mtb* without causing local tissue breakdown or remodeling (15).

The granuloma that forms in *Mtb* infection is surrounded by a peripheral mantle of fibroblasts. The main function of fibroblasts has been assumed to be to wall off and isolate bacilli by secreting collagen at the granuloma periphery, though we demonstrated sustained fibroblast CXCL8 secretion in TB both *in vitro* and *in vivo* (16). Most granuloma fibroblasts are not directly infected with *Mtb* but are exposed to proinflammatory mediators secreted by *Mtb*-infected mononuclear cells. We have previously shown that oncostatin M (OSM) secreted by monocytes and macrophages in response to *Mtb* synergizes with

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inflammatory cytokines such as TNF- α to drive fibroblast MMP-1 secretion, and in a model of the monocyte-fibroblast interaction in the granuloma in TB have shown that conditioned media from *Mtb*-infected monocytes (CoMTb) caused a dose-dependent increase in MMP-1 secretion by fibroblasts at 72 hours (17).

In this study, we now investigate regulation of fibroblast MMP-1 secretion in this model of tuberculosis. We demonstrate TB-induced monocyte/macrophage-dependent sustained MMP-1 gene expression and secretion by both normal adult and fetal lung fibroblasts. There is an accompanying down-regulation in TIMP-1 gene expression and secretion that results in an invasive phenotype. Conditioned medium from *Mtb*-infected monocytes induce rapid and sustained phosphorylation of p38 and c-Jun N-terminal kinase (JNK) but not extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK), and consistent with our data that OSM may be a key stimulus to MMP-1 induction in the fibroblasts in TB (17), CoMTb drives phosphorylation of STAT3 at its Tyr705 (and Ser 727) position. We show for the first time that STAT3 phosphorylation occurs in human TB granuloma fibroblasts. Inhibition of STAT3 or p38 phosphorylation with chemical inhibitors reduces fibroblast MMP-1 secretion. NF- κ B is key in regulating CoMTb-stimulated fibroblast MMP-1 gene expression as demonstrated by promoter-reporter studies using site-specific deletion constructs. TNF- α , IL-1 β , and OSM are the intercellular regulators of the monocyte-fibroblast network which may have a key role in MMP-1-mediated tissue damage in pulmonary TB.

MATERIALS AND METHODS

Fibroblast Cell Culture

Human fetal (MRC5) cells and normal adult human (HL) lung fibroblasts were cultured in Modified Eagle's Medium Essential (MEME; Invitrogen, Paisley, UK) or Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) as described (17). For experiments 1×10^5 cells were suspended in a 0.5-ml type I collagen gel, and allowed to polymerize before bathing in MEME/DMEM 1% fetal calf serum (FCS). For inhibition experiments, fibroblasts were pre-treated with signaling pathway inhibitors for 2 hours before stimulation with CoMTb. Specific inhibitors for p38, ERK, and JNK MAP kinase (SD203580, PD98059, SP600125, respectively) and piceatannol, STAT3 inhibitor, were obtained from Calbiochem. All materials used were LPS-free and were from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

Generation of Conditioned Media from *Mtb*-Infected Monocytes

Monocytes were isolated from single donor buffy coat (UK National Blood Transfusion Service, Collindale, UK) or whole blood from healthy volunteers by density centrifugation across Ficoll-Paque (Amersham, Amersham, UK) and adherence as described (7, 16). *Mtb* H37Rv was cultured in 7H9 broth. Monocytes were infected with *Mtb* at multiplicity of infection (MOI) of 10 or stimulated with the equivalent volumes of uninfected 7H9 broth. At 24 hours, culture supernatants were harvested and filtered through a 0.2- μ m tissue culture filter (18) to produce conditioned media from *Mtb*-infected monocytes (CoMTb) or conditioned media from uninfected monocytes (CoMcon). MOI was confirmed by colony counting in triplicate on Middlebrook 7H11 agar. Monocyte-derived macrophages (MDMs) were generated by maturing monocytes in RPMI containing 10% FCS and 20 ng/ml M-CSF for 5 days, before washing with HBSS and adding serum-free medium. Conditioned media from *Mtb*-infected macrophages (CoMDMTb) was then generated as for monocytes.

Zymography for Detection of MMP-1

Casein zymography was performed as described by our group (7) to detect MMP-1. Gels were digitally imaged and the density of bands

obtained at zymography quantified by Scion image analysis. We have previously determined that densitometric measurements of MMP-1 on casein gels by this method are linear over the range of 10 to 100 ng/ml. All gels were run with a standard aliquot (12.5 ng/ml) of MMP-1. Comparison of the density of the standard band compared with test bands allowed quantification.

Western Blotting for MMPs and Signaling Molecules

MRC5 or HL cells were stimulated with CoMcon or CoMTb or cytokines. Supernatants were harvested at given time points or cells lysed in 200 μ l lysis buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.015 Bromophenol blue), scraped, and frozen at -80°C , before analysis by Western blot as described (19). MMP-1 and MMP-13 antibodies were from the Binding Site (Birmingham, UK); antibodies for total and phosphorylated forms of p38, ERK, JNK, STAT1, and STAT3 were from Cell Signaling, New England Biolabs (Hitchin, UK).

RNAse Protection Assay

MRC5 or HL cells stimulated with CoMTb or CoMcon were lysed using Tri-Reagent (Sigma-Aldrich) and total RNA was extracted by a modified chloroform-phenol-ethanol method. The RPA was performed using a non-radioactive technique and the BD Riboquant human MMP1 or MMP2 kit according to manufacturer's instructions, and the chemiluminescent signal developed using the Pierce Nucleic acid detection module.

TIMP-1 ELISA

TIMP-1 ELISA (Duoset; R&D Systems Europe, Abingdon, UK) was performed according to manufacturer's instructions. The range of detection was 15.6 to 2,000 pg/ml. Tissue culture samples were diluted 1/10 and 1/100 for analysis.

Fibroblast Migration Assay

In brief, according to the methods of Yang and Liu (20), 8- μ m Transwell inserts pre-coated with type I collagen were seeded with MRC5 cells in a monolayer. Cells were stimulated with CoMTb or CoMcon for 24 hours before removing the Transwells, fixing in paraformaldehyde, and staining with crystal violet. The number of cells that migrated through to the undersurface of the insert was counted per 20 high-power fields (hpf).

Promoter-Reporter Studies

Wild type TIMP-1 promoter and six of the seven truncation constructs of the MMP-1 promoter linked to firefly luciferase in pGL3 basic vector were a kind gift from Ian Clark (University of East Anglia, UK). The $-2,942$ truncation MMP-1 construct was a gift from Matthew Vincenti (Dartmouth, NH). MRC5 cells were transfected with MMP-1 plasmid and pRL-TK vector (which is the control for transfection efficiency) at 10:1 ratio (Promega, Southampton, UK) using Eugene 6 transfection reagent (Roche Applied Science, Burgess Hill, UK) as described (16, 21). Cells were stimulated and lysates harvested at stated time intervals. Promoter activity was detected using the dual luciferase assay kit (Promega) (16, 21).

Generation of Mutation Promoter Constructs

MMP-1 promoter constructs with deleted NF- κ B or AP-1 sites were generated from the WT promoter in pGL3 Basic using the Quik-Change II XL Site-Directed Mutagenesis Kit (Stratagene, Leicester, UK) according to the manufacturer's instructions. The MMP-1 promoter sequence has been previously published (22). The NF- κ B-binding site between $-2,878$ and $-2,886$ bp was mutated from ATGGAAAAA to ATGGCCCAA, confirmed by sequencing. Plasmid DNA was then grown in DH5 α *Escherichia coli*, extracted and purified using the Qiagen Maxi Kit (Crawley, UK) according to manufacturer's instructions.

NF κ B Transcription Factor Assay

Nuclear extracts of MRC5 cells were prepared as described. NF- κ B subunit translocation to the nucleus were determined by the TransAm NF- κ B p65 transcription factor assay kit (Active Motif, Carlsbad, CA), according to manufacturer's instructions (16).

Immunohistochemistry

Paraffin-embedded pleural biopsy samples from 6 patients with known culture positive pleural tuberculosis were stained for TIMP-1 (Serotec), vimentin (CLONE v9; Sigma-Aldrich), CD68 (Dako, Ely, UK) and STAT1/3 (Cell-Signaling, New England Biolabs) using methods previously described (17). Ethical approval for the use of archived biopsy material was from A. B. Prisma and the Hammersmith Hospitals Research Ethics Committee.

Statistical Analysis

Graphs were generated using Microsoft Excel; statistical comparisons were made using SPSS v12.0. The Student's *t* test was used for paired comparisons. ANOVA with *post hoc* Tukey's comparison was used for multiple group comparisons. Unless stated, data are mean $\pm$ SEM representative of at least two experiments performed in triplicate.

RESULTS

Mtb-Infected Monocytic Cells Drive Sustained Unopposed Fibroblast MMP-1 Secretion

Stimulation of MRC5 or HL fibroblasts directly with *Mtb* did not induce gene expression or secretion of MMP-1 (data not

shown), indicating that cell-cell networks are required to drive fibroblast MMP-1 secretion. CoMTb was previously shown to drive MMP-1 production by fibroblasts in a dose-dependent manner (17). MRC5 cells were stimulated with 1/10 dilution of CoMTb and kinetics of MMP-1 secretion investigated. There was a greater than 4-fold increase in MMP-1 secretion within 24 hours as identified on zymography and confirmed by Western analysis. MMP-1 secretion increased over 72 hours, after which it plateaued ($*P < 0.05$; Figures 1A and 1B). Since granulomas contain both mature macrophages and monocytes, we next demonstrated that CoMDMTb also stimulated sustained fibroblast MMP-1 secretion (Figure 1C). Neither CoMTb nor CoMDMTb used at 1/10 dilution contained detectable MMP-1 concentrations (data not shown). Fibroblast MMP-1 mRNA accumulation increased up to 48 hours (Figure 1D), consistent with prolonged MMP-1 promoter activation that increased over a 24-hour period by 2.7 fold (both $P < 0.01$; Figure 1E), falling thereafter. Similar MMP-1 secretion kinetics were obtained for adult HL fibroblasts (Figure 1F): a similar dose-response for MRC5 and HL cells in response to CoMTb was previously noted

Figure 1. Kinetics of matrix metalloproteinase (MMP)-1 induction in fibroblasts in tuberculosis (TB). (A) Representative casein zymogram and densitometric analysis of MRC5 cells stimulated with conditioned media from *Mycobacterium tuberculosis* (*Mtb*)-infected monocytes (CoMTb). CoMTb (black line) or conditioned media from control uninfected monocytes (CoMcon; gray line) were used to stimulate MRC5 cells at 1/10 dilution. Culture supernatants were analyzed by casein zymography. The arrows on the representative zymogram gel show the glycosylated (upper arrow) and nonglycosylated (lower arrow) forms of MMP-1. $*P < 0.05$ for CoMTb versus CoMcon-stimulated fibroblasts at each given time point. (B) Western blot confirming MMP-1, and showing persistent high MMP-1 levels at 120 hours. (C) Conditioned media from *Mtb*-infected macrophages (CoMDMTb) induces similar sustained MMP-1 secretion from fibroblasts. $*P < 0.05$ for CoMTb versus CoMcon-stimulated fibroblasts at each given time point. (D) MMP-1 gene expression. RNA was taken at 24 and 48 hours from MRC5 cells stimulated with CoMcon/CoMTb and analyzed by RPA. (E) CoMTb activates the MMP-1 promoter. MRC5 cells were transiently transfected with plasmid DNA containing the MMP-1 promoter linked to Firefly luciferase, and stimulated with CoMTb or CoMcon. Lysates were analyzed for luminescence (expressed as relative luminescence units [RLU]). $*P < 0.05$ for CoMTb versus CoMcon (F) Kinetics for adult human (HL) fibroblasts stimulated with CoMTb. Representative zymogram shown indicating similar pattern of glycosylated and nonglycosylated MMP-1 as for MRC5 cells. HL cells were stimulated with 1/10 CoMTb (dark line) or CoMcon (gray line). Supernatants were collected at 24-hour intervals and analyzed by casein zymography for MMP-1. $*P < 0.05$ CoMTb versus CoMcon at given time point.

(17). MMP-13, the other significant fibroblast-derived collagenase, was not induced by CoMTb (data not shown).

TIMP-1 is the major secreted inhibitor of MMP-1. MRC5 cells and HL cells (not shown) constitutively secreted TIMP-1, with accumulating protein in the supernatant of untreated cells over a 120-hour period (data not shown). This was unaltered in the presence of CoMcon (where the protein continued to accumulate). In contrast, CoMTb reduced TIMP-1 secretion compared with CoMcon-treated fibroblasts (or unstimulated fibroblasts [data not shown]) from 48 hours onward (Figure 2A). There was constitutive TIMP-1 promoter activity in unstimulated fibroblasts (not shown), which was unaltered in the presence of CoMcon. As with protein secretion, there was concurrent down-regulation of TIMP-1 promoter activity and mRNA accumulation at 24 hours (Figures 2B and 2C). Further, in biopsies from patients with TB, TIMP-1 mainly localized to inflammatory cells in the center of the granuloma (Figure 2D), and dual staining with the mesenchymal marker vimentin and TIMP-1 showed little co-localization (Figures 2Dii and 2Diii).

The net effect on fibroblast invasive potential was investigated. Fibroblast migration through a type I collagen gel was measured in response to CoMTb or CoMcon at 72 hours. CoMTb at 1/5 dilution caused 3-fold greater fibroblast migration compared with controls ($P = 0.04$; Figure 2E).

M. tuberculosis-Infected Monocytic Cells Activate STAT- and MAPK-Dependent Signaling Paths in Fibroblasts

Previously, we have shown sustained activation of NF- κ B in fibroblasts stimulated with CoMTb, with nuclear translocation of p65 for up to 24 hours (16). Furthermore, we showed that MAPK p38 is phosphorylated in human tuberculous granuloma (7); phosphorylation was detectable *in vivo* at the periphery of granuloma, suggesting possible activation of this signaling pathway in fibroblasts. CoMTb stimulation of MRC5 fibroblasts resulted in rapid p38 phosphorylation (Figure 3). There was also rapid phosphorylation of the p46 and p54 isoforms of JNK. Both p38 and JNK phosphorylation peaked at 30 minutes (Figure 3). Subsequently, absolute

Figure 2. CoMTb reduces fibroblast tissue inhibitor of metalloproteinase (TIMP)-1 secretion. (A) Kinetics. MRC5 cells were stimulated with CoMTb (black line) or CoMcon (gray line) at 1/10 dilution. TIMP-1 was measured by ELISA. * $P < 0.05$ for CoMTb versus CoMcon at given time point. (B) TIMP-1 promoter activation. The TIMP-1 promoter is constitutively activated in fibroblasts. The reduced promoter activity in CoMcon-treated cells after 48 hours reflects transient transfection. * $P < 0.05$ for CoMTb (black line) versus CoMcon (gray line) at given time point. (C) CoMTb and TNF- α reduce TIMP-1 gene expression. Northern blot showing reduced MMP-1 expression. Loading control 18 s and 28 s RNA. (Di) Immunohistochemistry showing TIMP-1 localization in the tuberculous granuloma. TIMP-1 is brown against a blue background. Maximal staining occurs in the center of the granuloma. (Dii and Diii) Co-staining for the mesenchymal marker vimentin (blue) and TIMP-1 (red). (Diii) Higher magnification of the outlined area in Dii, showing that most vimentin-positive cells (blue; solid arrows) do not stain for TIMP-1 (red; hollow arrow). This is consistent with *in vitro* findings that TIMP-1 is down-regulated in fibroblasts that

interact with *Mtb*-infected monocytes. (E) CoMTb induces a net invasive collagenolytic phenotype. Fibroblasts were seeded on a collagen-gel on a Transwell and stimulated with CoMTb or CoMcon. Fibroblast invasion through the gel to the undersurface of the Transwell insert was assessed at 96 hours. Data are mean number cells per high-power-field (hpf) for 20 hpf. * $P < 0.05$ versus CoMcon (first lane).

Figure 3. CoMTb-dependent mitogen-activated protein kinase (MAPK) signaling in pulmonary fibroblasts. (A) p38 and c-Jun N-terminal kinase (JNK) MAPK are phosphorylated in response to CoMTb. Lysates from MRC5 cells stimulated with CoMcon or CoMTb were probed by Western blotting for total and phosphorylated p38, JNK, and extracellular signal-regulated kinase.

phosphorylation decreased but remained elevated in CoMTb-stimulated cells compared with controls for up to 4 hours. A sustained increase in p38 phosphorylation remained detectable at 24 hours (data not shown). ERK p42 and p44 isoforms were constitutively phosphorylated in these cells (Figure 3). Similar data were obtained in adult normal human lung fibroblasts (data not shown).

Since we found that OSM drives fibroblast MMP-1 secretion *in vitro*, and is expressed in human tuberculous granulomas (17), we next focused on STAT1/3, which are known to be activated by OSM receptor binding. Granulomas were identified by hematoxylin and eosin staining of patient biopsies (Figure 4Ai) and then immunostained for phosphorylated STAT1 and STAT3. Widespread STAT1 phosphorylation was identified, predominantly in CD68-positive cells but not in fibroblasts, suggesting that this signaling pathway may be relatively unimportant in granuloma fibroblasts *in vivo* (Figure 4Aii). In contrast, there was strong immunoreactivity for STAT3 phosphorylated both on Tyr705 (Figure 4B) and on Ser727 (Figure 4C) in spindle-shaped CD68-negative cells (fibroblasts) surrounding granulomas.

We next investigated STAT1/3 phosphorylation in fibroblasts *in vitro* in response to CoMTb. The STAT1 α splice variant became phosphorylated within 15 minutes, with sustained phosphorylation at 4 hours (Figure 5A). STAT3 was constitutively phosphorylated at the Tyr705 position, and this increased markedly within 15 to 30 minutes, followed by depletion (Figure 5B). Similarly, increased phosphorylation at the Ser 727 position was detected within the first 15 to 30 minutes.

Next, we pre-incubated MRC5 cells with SB203580, SP600125, and PD980590, specific inhibitors for p38, JNK, and ERK pathways, respectively (23). Inhibition of p38 MAPK with SB203580 10 μ M reduced CoMTb-induced MMP-1 secretion by 40% ($P = 0.0001$; Figure 5C) and reversed CoMTb-induced inhibition of TIMP-1 secretion (Figure 5D). Inhibition of the JNK pathway was cytotoxic to these cells at concentrations of 1 μ M SP600125

Figure 4. STAT phosphorylation in the human tuberculous granuloma. (Ai) Hematoxylin and eosin section demonstrating fibrotic granuloma. (Aii) STAT1 phosphorylation. Samples were used as in Figure 2D. STAT1 (nickel; black) phosphorylation (arrowed) was readily demonstrated throughout the granuloma, and co-localized mainly with CD68-positive cells (alkaline phosphatase; red). (Bi and Bii) Phosphorylated STAT3 Tyr 705 (black stain). Phosphorylated STAT Tyr 705 was identified in some of the CD68-positive cells in the granuloma (hollow arrow) in a nuclear staining pattern, but mainly was localized to the peripheral spindle-shaped CD68-negative cells (solid arrow). (Ci and Cii) Phosphorylated STAT3 Ser727 was also found in both CD68-positive cells (hollow arrow) and in the peripheral spindle-shaped CD68-negative cells (solid arrow). Scale bar indicates 50 μ m.

Figure 5. (A) STAT1 signaling *in vitro* in fibroblasts. MRC5 cells were stimulated with 1/10 CoMTb or CoMcon and lysates collected at the given time (' = minutes or hr = hours). The STAT antibody binds both the α (upper band) and β (lower band) isoform of STAT1. (B) STAT3 phosphorylation *in vitro* in fibroblasts stimulated with CoMTb or CoMcon. (C) Effect of inhibiting p38 MAPK with SB203580 (μ M) on MMP-1 secretion. MRC5 cells were pretreated with SB203580 for 2 hours before stimulation with CoMTb or CoMcon (-). Supernatants were collected at 72 hours and analyzed by zymography for MMP-1. Control and CoMTb-stimulated wells were also treated with the same volume of vehicle (DMSO) as used to dissolve the inhibitor. DMSO had no effect on MMP-1 secretion. * $P < 0.05$ for SB203580 10 μ M versus CoMTb. (D) Effect of inhibiting p38 MAPK with SB203580 (μ M) on TIMP-1 secretion. Supernatants from C were analyzed by ELISA for TIMP-1. * $P < 0.01$ for SB203580 10 μ M versus CoMTb. (E) The effect of STAT3 tyrosine phosphorylation inhibition by piceatannol on MMP-1 secretion. Fibroblasts were preincubated with piceatannol (μ M) for 2 hours before stimulation with CoMTb or CoMcon (-). Supernatants were collected at 72 hours and MMP-1 measured by casein zymography. Data are mean of CoMTb-induced MMP-1, * $P < 0.05$ versus CoMTb.

and above, and there was no effect on MMP-1 or TIMP-1 secretion at lower concentrations. Inhibition of the ERK MAP kinase pathway with PD980590 or U0126 had no effect on MMP-1 or TIMP-1 secretion (data not shown).

Inhibiting the tyrosine phosphorylation of STAT3 with piceatannol (24) reduced MMP-1 secretion by 60% ($P < 0.05$; Figure 5E and discussed previously [17]). There was no further inhibition with higher doses of piceatannol.

NF- κ B Promoter Binding Is Necessary for CoMTb-Induced Fibroblast MMP-1 Expression

To further investigate transcriptional regulation of MMP-1 in response to CoMTb, we performed promoter-reporter assays, using a series of deletion luciferase-reporter constructs of the MMP-1 promoter. The wild-type (WT) promoter is 4,382 bp long upstream from the transcriptional start site, and a schematic diagram of the promoter is shown in Figure 6A. Truncation of the promoter at -3,830 bp increased CoMTb-induced MMP-1 promoter activity, suggesting that this area of the promoter contains inhibitory binding sites. Truncation at -2,942 bp further increased promoter activation, indicating further transcriptional-repressive elements are present in the region between -2,942 and -3,830 bp. Truncation at -2,001 bp significantly reduced promoter activation to less than basal activation in control cells (Figure 6B), showing that the region between -2,001 and -2,942 bp is key in basal and CoMTb-induced MMP-1 promoter activation. The single NF- κ B-binding site at -2,878 to -2,886 bp in the MMP-1 promoter (22) is within this critical -2,942 and -2,001 region. Specific site-directed mutagenesis of this binding site blocked up-regulation of promoter activity in response to CoMTb, demonstrating that NF- κ B binding is essential for CoMTb-induced fibroblast MMP-1 gene expression (Figure 6C).

Monocyte-Derived TNF- α , IL-1 β , and OSM Regulate MMP-1 and TIMP-1 Secretion by Fibroblasts

Mtb-infected monocytes secrete many cytokines, including TNF- α , IL-1 β , IL-6, CXCL8, and CCL2 (19, 25-27), and as we first reported, OSM (17), and together these cytokines have the ability to activate the signaling pathways up-regulated by CoMTb. In a series of inhibition experiments, 5 μ g/ml anti-TNF- α antibody reduced CoMTb-induced fibroblast MMP-1 by >65%, ($P = 0.02$; Figure 7A), and restored TIMP-1 secretion (data not shown). Inhibition of IL-1 β by 10 ng/ml IL-1Ra resulted in 48% decrease in MMP-1 secretion ($P = 0.04$), which increased to 63% inhibition with 100 ng/ml IL-1Ra ($P < 0.001$) (Figure 7A), but did not affect TIMP-1 secretion. Inhibition of both IL-1 and TNF- α had no additive effect. Pre-incubation of MRC5 cells with pertussis toxin (which inhibits G_s-protein-coupled receptors [GPCRs], including chemokine receptors, and some prostaglandin receptors, prior to stimulation with CoMTb) had no effect on MMP-1 or TIMP-1 secretion (data not shown).

Preincubation of CoMTb with blocking anti-OSM antibody resulted in dose-dependent reduction in MMP-1 secretion with maximal blockade, causing a significant 25% reduction in CoMTb-induced MMP-1 ($P = 0.04$; Figure 7B). Inhibition of IL-6, another member of the same cytokine family as OSM, had no effect on MMP-1 secretion, and we have previously shown that direct stimulation of fibroblasts with IL-6 alone or in combination with TNF- α did not drive MMP-1 secretion (17). IFN- γ is also well known to activate STATs, but is not present in CoMTb and does not drive fibroblast MMP-1 secretion alone or in combination with other cytokines (data not shown).

Next, we investigated the effect of TNF- α , OSM, and IL-1 β on phosphorylation of the signaling intermediates that drove

Figure 6. MMP-1 promoter regulation. (A) Schematic diagram of the MMP-1 promoter. Wild-type (WT) promoter is 4,372 bp upstream from the transcriptional start site. In the experiments described, a luciferase gene begins at position 0 replacing the MMP-1 gene. Truncated constructs of the promoter linked to luciferase were available at each of the sites arrowed. The diagram is not to scale and shows only key transcription factor-binding sites. (B) CoMTb stimulation using a series of deletion constructs of the MMP-1 promoter. MRC5 cells were transiently transfected with plasmids containing the luciferase-linked MMP-1 promoter as described in Figure 1. Lysates were collected at 24 hours. Data are expressed as a factor of luminescence induced in CoMcon-stimulated cells containing the WT promoter. (C) The effect of point mutation of the NF-κB-binding site in the MMP-1 promoter. MRC5 cells were transiently transfected with mutant constructs and stimulated with CoMTb. Luciferase activity at 24 hours is expressed as a multiple of that measured in WT CoMcon-stimulated cells. * $P < 0.05$ for the deleted NF-κB-containing MMP-1 promoter compared with WT stimulated with CoMTb.

unopposed fibroblast MMP-1 secretion, namely p38 and STAT-3. All three cytokines alone phosphorylated p38 in lung fibroblasts within 30 minutes (Figure 7C). Antagonism between OSM and IL-1β has been reported in synovial fibroblasts (28), but was not observed. Low basal Tyr705-phosphorylated STAT3 was up-regulated in response to OSM at 30 minutes, but was not affected by TNF-α or IL-1β (Figure 7D). STAT3 phosphorylation at the 727 Serine site was also detected basally, and this increased in response to both TNF-α and OSM with no antagonism by IL-1β.

Figure 7. CoMTb induces MMP-1 secretion from fibroblasts via cytokine-dependent networks. (A) MMP-1 induction is TNF-α- and IL-1-dependent. CoMTb was pre-incubated for 2 hours with anti-TNF-α antibody (μg/ml) before stimulating MRC5 cells. For IL-1 inhibition, cells were pretreated with IL-1Ra (ng/ml) for 2 hours before stimulation with CoMTb for 72 hours. MMP-1 was measured by casein zymography. (B) OSM inhibition reduces MMP-1 secretion. CoMTb was pre-incubated with anti-OSM antibody (μg/ml) for 2 hours before stimulation of MRC5 cells. MMP-1 was measured by casein zymography. Data are expressed as fraction of that induced by CoMTb at 72 hours (CoMTb = 1), * $P < 0.05$ compared with CoMTb. (C) TNF-α, IL-1β, and OSM induce p38 phosphorylation. MRC5 cells were stimulated with TNF-α, IL-1β, OSM (all 10 ng/ml) for 30 minutes, and lysates probed for phosphorylated and total p38. (D) OSM phosphorylates STAT1 and 3. Western blots of lysates harvested at 30 minutes are shown.

DISCUSSION

We have identified up-regulation of unopposed, functionally active fibroblast-derived MMP-1 secretion in an *Mtb*- and monocyte-dependent network. This indicates a novel role for the granuloma fibroblast, confirming that these cells, which we

have previously found to drive chemokine secretion (16), play a role in regulating matrix turnover and resultant tissue destruction. This process is regulated in part by p38 MAPK as in lung epithelial cells (19), but we also show a key role for the STAT3 intracellular signaling pathway in regulating fibroblast-dependent MMP-1 secretion.

Direct infection of fibroblasts *in vitro* with *Mtb* had no effect on MMP gene expression and secretion, which is consistent with *post mortem* data showing that mycobacterial DNA can be isolated from parenchymal cells in the absence of macroscopic evidence of inflammation or tissue damage (15). Mycobacteria up-regulate transcription-dependent MMP secretion via monocyte/macrophage-dependent networks. This is consistent with the spatial relationship in the TB granuloma where mycobacteria are central, often within monocytic cells, and fibroblasts are located at the periphery. The magnitude of MMP-1 secretion by fibroblasts stimulated by CoMTb was significantly greater than that secreted by monocytes or macrophages in response to *Mtb* infection, since MMP-1 was not detected in neat samples of CoMTb or CoMDMTb. The monocyte-fibroblast interaction therefore amplifies the amount of MMP-1 secreted for a given number of infecting bacilli. Similar MMP-1 secretion was demonstrated in adult normal lung fibroblasts as in MRC5 cells, indicating that MRC5 cells are a good model in which to study mechanisms regulating fibroblast MMP activity. MRC5 cells have the advantage of allowing more frequent passages and facilitating transfection studies, which we were unable to achieve reproducibly in HL cells.

Low levels of MMP-1 were detectable in the tissue culture medium of CoMcon-stimulated or unstimulated fibroblasts. This probably reflects the culture of these cells in a three-dimensional collagen matrix. This induces cytoskeletal changes resulting in increased MMP secretion compared with tissue culture on plastic (29), but this system is more representative of the *in vivo* microenvironment of fibroblasts in chronically infected tissue. Fibroblasts constitutively secrete TIMP-1 *in vitro*. CoMTb-induced increased MMP-1 secretion was associated with decreased activity of the TIMP-1 promoter with reduced gene expression and secretion, suggesting a net matrix degrading phenotype. The invasion assay through a type I collagen gels suggests that as a result of CoMTb but not CoMcon stimulation, fibroblasts are able to break down collagen, but it is possible that other factors such as altered cytoskeletal activity may contribute to their motile invasive phenotype in this assay as well. The *in vivo* patient studies confirmed that fibroblasts in the tuberculous granuloma stained poorly for TIMP-1, further demonstrating that in TB fibroblasts are shifted toward matrix destruction.

Rapid and sustained phosphorylation of p38 MAPK was induced in fibroblasts and was associated with prolonged up-regulation of MMP-1 promoter activity and mRNA accumulation. MMP-1 secretion and TIMP-1 down-regulation were both p38 dependent. Inhibitor experiments are potentially limited due to nonspecific off-target effects. However, the p38 inhibitor both reduced CoMTb-induced MMP-1 and increased TIMP-1 secretion, indicating that this is not a nonspecific down-regulatory effect. Furthermore, stimulation of fibroblasts with this inhibitor alone had no effect on TIMP-1 secretion.

In contrast to p38, we showed only transient phosphorylation of JNK. ERK was constitutively phosphorylated both in fetal fibroblasts and in primary adult human lung fibroblasts. Fibroblasts from other organs such as skin do not usually show constitutive ERK phosphorylation, and it is possible that constitutive ERK phosphorylation reflects the presence of low concentrations of serum in these experiments. We performed all cultures in the presence of 1% FCS, since complete serum deprivation may induce AP-1 activation in many cells, and AP-1

is implicated in MMP gene expression. Inhibition of ERK had no effect on MMP-1 or TIMP-1 secretion, which is divergent from published data showing ERK-dependent MMP-1 secretion by fibroblasts and other cells (30, 31), indicating cell- and stimulus-specific effects.

We demonstrate STAT phosphorylation at both Tyr 705 and Ser727 in TB granulomas from patients for the first time. Tyr705 phosphorylation is known to occur first in response to gp130 and OSM receptor binding, and the earlier role of this signaling pathway is probably reflected by the more transient phosphorylation of STAT3 Tyr705 *in vitro*. We have previously identified OSM, but not IL-6, as a potential stimulus driving MMP-1 secretion by fibroblasts in tuberculosis (17), and these *in vivo* data showing STAT3 Tyr705 in addition to the *in vitro* STAT3 Tyr705 phosphorylation in response to CoMTb further suggest that OSM is functionally active in the monocyte/macrophage-fibroblast interaction in the granuloma in TB. STAT3 ser727 phosphorylation occurs later in response to MAPK phosphorylation. Sustained p38 phosphorylation was found *in vitro* as was sustained Ser727 STAT3 phosphorylation. This represents a point at which MAPK and STAT pathways may interact, explaining why inhibition of either pathway alone does not completely abolish MMP-1 induction. Reduction of MMP-1 secretion by both the STAT3 tyrosine phosphorylation inhibitor piceatannol and p38 inhibitor SB203580 supports this hypothesis. Ser727 phosphorylation was also noted in CD68-positive cells, which is to be expected, since we have shown that MAPK activation occurs in monocytes in TB (17), and Ser727 phosphorylation occurs downstream of MAPK activation.

STAT1 mediates IFN- γ -induced responses, but these will not be important in monocyte/macrophage-fibroblast interactions, since CoMTb contains no detectable IFN- γ (32). STAT1 was phosphorylated in the granuloma CD68-positive cells, consistent with the known role for IFN- γ -dependent lymphocyte-macrophage/monocyte interactions in protective immunity to *Mtb* (33). Fibroblasts did not stain for STAT1 in the patient samples.

Other studies have suggested that the proximal region of the MMP-1 promoter (–517 construct) is sufficient to drive MMP-1 promoter activity in response to inflammatory cytokines such as IL-1 and OSM (34). However, our data show that more distal activation of the MMP-1 promoter sequence is required for CoMTb-induced fibroblast MMP-1 secretion, with the critical region lying between –2,001 and –2,942 bp upstream of the transcriptional start site. An absolute requirement for NF- κ B binding for MMP-1 transcription in this model was demonstrated by the site-specific mutagenesis study, and may reflect the role of inflammatory cytokines such as TNF- α in CoMTb in driving this response. Truncation of the promoter at –3,830 bp upstream of transcriptional origin removes an Oct A3 site, two PSi1 sites, and a PEA-3 site, and resulted in increased promoter activation in response to CoMTb, suggesting that some of these transcription factors have an inhibitory role. Truncation at –2,942 bp removes a further five PSi1 sites, a PEA3 site and an AP-1 site, and resulted in a further increase in CoMTb-induced promoter activation, suggesting that this promoter contains multiple repressive sequences that may limit MMP-1 gene transcription in the presence of complex cytokine stimulation such as CoMTb.

We used the signaling data to assist identification of the mediators in CoMTb which drive functionally unopposed MMP-1 secretion by fibroblasts. Direct infection of fibroblasts with *Mtb* did not induce MMP secretion, so residual mycobacterial antigens in CoMTb are not responsible for driving MMP secretion.

Both TNF- α and IL-1 β are key in driving MMP-1 secretion in this monocyte-fibroblast network. This is distinct from

monocyte–epithelial networks, where MMP-1 secretion is TNF- α – but not IL-1 β –dependent (19). Confirming the cell-specific nature of MMP-1 induction, we demonstrated that OSM inhibition reduced fibroblast but not epithelial MMP-1 up-regulation in TB (19). OSM inhibition studies are limited in that the inhibitory capacities of available anti-OSM antibodies are poor, as we have described (17), and the maximal inhibition of CoMTb-induced MMP-1 we could achieve in this study was 25%. However, by using piceatannol to inhibit the Tyr-705 STAT3 phosphorylation, which we now and others have shown to be phosphorylated in response to OSM, MMP-1 secretion was reduced by over 60% (discussed also in Ref. 17). Although piceatannol can also inhibit the nonreceptor tyrosine kinase Syk, Syk is found in hematopoietic cells, but not lung fibroblasts.

Human cells and tissues were used exclusively in this study of fibroblast-dependent collagenolytic activity rather than a mouse model. Murine knockout models have been used to study MMP function in the lung, but are of limited value in TB since mice do not form cavities during infection with TB but develop fibrotic reactions (35), which may be a reflection of the murine MMP-1 ortholog having limited type I collagenolytic activity (36).

In summary, we now show that fibroblasts are a major source of sustained and functionally unopposed MMP-1 in an *in vitro* model of TB, suggesting that they may propagate tissue destruction in this disease. Furthermore, while we have shown that MMP-1 induction is critically dependent on NF- κ B and p38-dependent signaling, we demonstrate that STAT3 Tyr 705 phosphorylation occurs in fibroblasts in human tuberculous granulomas, and is necessary for MMP-1 induction *in vitro*. These data provide further evidence for the role of OSM interacting with other inflammatory cytokines in driving MMP-1 activity in TB. Targeting STAT3 signaling pathways may have the potential to decrease collagen destruction in tuberculosis, and requires further evaluation in *in vivo* models of TB.

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