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Heme Oxygenase-1 Regulation of Matrix Metalloproteinase-1 Expression Underlies Distinct Disease Profiles in Tuberculosis

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Pulmonary tuberculosis (TB) is characterized by oxidative stress and lung tissue destruction by matrix metalloproteinases (MMPs). The interplay between these distinct pathological processes and the implications for TB diagnosis and disease staging are poorly understood. Heme oxygenase-1 (HO-1) levels were previously shown to distinguish active from latent TB, as well as successfully treated *Mycobacterium tuberculosis* infection. MMP-1 expression is also associated with active TB. In this study, we measured plasma levels of these two important biomarkers in distinct TB cohorts from India and Brazil. Patients with active TB expressed either very high levels of HO-1 and low levels of MMP-1 or the converse. Moreover, TB patients with either high HO-1 or MMP-1 levels displayed distinct clinical presentations, as well as plasma inflammatory marker profiles. In contrast, in an exploratory North American study, inversely correlated expression of HO-1 and MMP-1 was not observed in patients with other nontuberculous lung diseases. To assess possible regulatory interactions in the biosynthesis of these two enzymes at the cellular level, we studied the expression of HO-1 and MMP-1 in *M. tuberculosis*-infected human and murine macrophages. We found that infection of macrophages with live virulent *M. tuberculosis* is required for robust induction of high levels of HO-1 but not MMP-1. In addition, we observed that CO, a product of *M. tuberculosis*-induced HO-1 activity, inhibits MMP-1 expression by suppressing c-Jun/AP-1 activation. These findings reveal a mechanistic link between oxidative stress and tissue remodeling that may find applicability in the clinical staging of TB patients. *The Journal of Immunology*, 2015, 195: 2763–2773.

Upon respiratory exposure to *Mycobacterium tuberculosis*, individuals can develop a broad range of disease manifestations, varying from asymptomatic latent tuberculosis (TB) infection (LTBI) to aggressive pulmonary forms with extensive

lung damage (1). Major challenges in TB diagnosis include the ability to distinguish active from latent infection (2, 3), as well as the discrimination between TB and other lung diseases with similar clinical presentation, such as sarcoidosis and nontuberculous my-

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Abbreviations used in this article: AFB, acid-fast bacilli; CoPPIX, cobalt (III) protoporphyrin IX dichloride; CORM-II, CO-releasing molecule Ru₂Cl₄(CO)₆, also known as tricarbonyldichlororuthenium (II) dimer; CRP, C-reactive protein; HbO₂, oxyhemoglobin; HO-1, heme oxygenase-1; LTBI, latent tuberculosis infection; MMP, metalloproteinase; NHLBI, National Heart, Lung, and Blood Institute; NIAID, National Institute of Allergy and Infectious Diseases; NIH, National Institutes of Health; NIRT, National Institute for Research in Tuberculosis; NTM, nontuberculous mycobacterial; OR, odds ratio; PCA, principal component analysis; PTB, pulmonary tuberculosis; RD1, region of deletion 1; ROC, receiver operator characteristic; SA, succinylacetone; SAA, serum amyloid protein-A; SnPPPIX, tin (IV) protoporphyrin IX dichloride; TB, tuberculosis; TST, tuberculin skin test; WT, wild-type.

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cobacterial (NTM) infection (4–6). Clinical and experimental animal studies demonstrated that active TB is accompanied by systemic oxidative stress and augmented lipid peroxidation (7, 8). We showed previously that plasma levels of heme oxygenase-1 (HO-1), a major antioxidant that is highly expressed in the lungs, can accurately distinguish active from latent TB cases or uninfected controls in both adult (9) and pediatric (10) populations in South India. These studies indicated that HO-1 can serve as an important biomarker of TB disease.

The pathology of pulmonary TB (PTB) involves enzymatic degradation of lung tissue by matrix metalloproteinases (MMPs) (11–13). This process is reflected in the detection of increased MMP levels in sputum (14) and plasma (15) samples from TB patients, which was shown to correlate with clinical disease severity. Among the different MMPs, MMP-1 (interstitial collagenase) is thought to play a critical role in driving immunopathology in PTB (11) and appears to be selectively induced by *M. tuberculosis* infection (16). MMP-1 gene expression involves activation of the transcription factor AP-1, as well as JNKs, ERKs, and p38 kinases (17), factors that were described to be induced in response to oxidative stress (18). Interestingly, biliverdin and CO, products of the reaction catalyzed by HO-1, were shown to suppress the expression of ERKs and p38 kinases in experimental models (19, 20). These observations led us to hypothesize that the clinical presentation of *M. tuberculosis* infection may be influenced by stress-induced HO-1 acting on MMP-driven lung damage/remodeling.

In the current study, we demonstrate that circulating levels of HO-1 and MMP-1 are elevated in active PTB patients compared with individuals with LTBI from two distinct South Indian and Brazilian cohorts and that expression of these two biomarkers is inversely correlated in active TB disease but not in North American subjects with pulmonary NTM infection or sarcoidosis. More importantly, our data reveal that the pattern of expression of HO-1 and MMP-1 in plasma identifies two subpopulations of active TB patients who exhibited different inflammatory profiles and clinical presentations. The inverse relationship between HO-1 and MMP-1 levels in TB patients was reflected in the dichotomous expression of the two enzymes in *M. tuberculosis*-infected macrophages, which we were able to link with the suppression of MMP-1 production by HO-1-induced CO. Together, these findings reveal a pathway by which oxidative stress can negatively regulate tissue remodeling and demonstrate combined measurement of HO-1 and MMP-1 as a potential strategy for clinical staging of TB.

Materials and Methods

Ethics statement

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participants or their legally responsible guardians before enrolling into the substudies. The South Indian study was approved by the Institutional Review Board of the National Institute for Research in Tuberculosis (NIRT; protocol numbers NCT01154959 and NCT00342017). The Brazilian study was approved by the Ethical Committee of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (protocol number: 003.0.225.000-11). TB and NTM infection samples from the North American study were collected according to protocols approved by the Institutional Review Board of the National Institutes of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) (protocol numbers: NCT00001355, NCT01212003, and NCT01611402). Sarcoidosis subjects selected for this study were screened for a pulmonary sarcoidosis treatment study, National Heart, Lung, and Blood Institute (NHLBI) protocol 06-H-0072 (NCT00279708). After written informed consent was obtained, sarcoidosis subjects were clinically screened under NHLBI protocol 82-H-0032 (NCT00001183), and research blood samples were collected under NHLBI protocol 96-H-0100 (NCT00001532).

Indian study

Cryopreserved heparinized plasma samples were collected from 97 subjects with active PTB, 39 individuals with LTBI, and 40 uninfected healthy controls

recruited as part of a TB cohort study at the Government Stanley Medical Hospital and at TB clinics supported by NIRT, as described previously (9) (Fig. 1). TB diagnosis was based on culture positivity of sputum samples. Three sputum samples/subject were examined by fluorescence microscopy, processed by the modified Petroff's method, and cultured on Lowenstein-Jensen medium. Presence of acid-fast bacilli (AFB) in sputum smears was also documented. A posteroanterior chest x-ray was performed to determine the extent of lung disease (unilateral versus bilateral lesions), which was scored by three independent expert physicians from NIRT. LTBI diagnosis was based on Quantiferon TB-gold ELISA and tuberculin skin test (TST) positivity (≥ 10 mm in diameter), absence of chest radiography abnormalities or pulmonary symptoms, and negative sputum smears and cultures. Healthy controls were health care professionals recruited at Government Stanley Medical Hospital and at TB clinics who agreed to participate in the study. To be designated as a healthy control, an individual needed to be asymptomatic with normal chest radiograph and negative sputum smears and cultures, Quantiferon results (< 0.35), and TST induration (< 5 mm in diameter). All individuals were negative for type 2 diabetes based on American Diabetes Association criteria (HbA1c levels $> 6.5\%$ and random plasma glucose > 200 mg/dl). At the time of enrollment, all individuals were HIV⁻ (all patients were actively screened), had received no treatment for TB, had received a BCG vaccine, and had no record of prior TB disease.

Brazilian study

Cryopreserved heparinized plasma samples were collected from a cohort of 63 subjects with active PTB, 15 individuals with LTBI, and 10 healthy controls recruited between May and November 2012 at the Hospital Especializado Octávio Mangabeira, Salvador, Brazil (Fig. 1). PTB diagnosis included positive AFB in sputum smears and positive *M. tuberculosis* sputum cultures. Three sputum samples/subject were examined by fluorescence microscopy, processed by the modified Petroff's method, and cultured on Lowenstein-Jensen medium. LTBI was diagnosed in contacts of active TB cases who agreed to participate in the study and was based on TST positivity (≥ 10 mm in diameter), absence of chest radiography abnormalities or pulmonary symptoms, and negative sputum cultures. Healthy control individuals (health care professionals and medical students from the Hospital Especializado Octávio Mangabeira who agreed to participate) were asymptomatic with normal chest radiograph and negative sputum cultures and TST induration (< 5 mm in diameter). At the time of enrollment, all individuals were HIV⁻ (all patients were actively screened), had received a BCG vaccine, and had no record of prior TB disease or anti-TB treatment.

North American study

We assessed cryopreserved EDTA plasma samples from 18 individuals with culture-confirmed TB and 11 individuals with NTM infection who were recruited at the NIH Clinical Center under protocols from the NIAID, NIH. Samples from 48 individuals with confirmed diagnosis of pulmonary sarcoidosis were recruited under a protocol from NHLBI, NIH. Plasma samples from healthy controls were obtained from blood donors at the NIH Clinical Center. Recruited TB patients exhibited positive AFB, as determined by smear, culture, or biopsy. Diagnostic criteria for pulmonary NTM infection followed American Thoracic Society guidelines (21), with microbiologic and radiographic evidence of active pulmonary NTM infection. All patients tested negative for Abs to HIV, had normal numbers of CD4⁺ T cells, and no HIV risk factors. Sarcoidosis patients were recruited from a clinical protocol that accrues treatment-requiring patients with parenchymal lung disease and excludes those with manifest cardiac and neurologic involvement and other serious disorders, such as HIV disease, TB, and cancer. Forty-eight adult subjects who had a history compatible with sarcoidosis and a tissue biopsy demonstrating noncaseating granulomas were included in this analysis. Healthy controls were healthy blood donors from the NIH blood bank (Fig. 1).

Immunoassays

Levels of HO-1 (Assay Designs, Ann Arbor, MI), Ferritin-H chain (Abnova, Taipei City, Taiwan), IL-17, IFN- γ , and TNF- α (R&D Systems, Minneapolis, MN) were measured using ELISA kits. Levels of C-reactive protein (CRP), serum amyloid protein-A (SAA), haptoglobin, and $\alpha 2$ macroglobulin were determined using a multiplex ELISA system (Bio-Rad, Hercules, CA). Levels of MMP-1, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 were measured using a Luminex kit from R&D Systems. Total heme concentrations were measured using a colorimetric assay from BioAssay Systems (Hayward, CA). Assessment of the expression of 34 human proteases in culture supernatants was performed using a standardized array (Proteome Profiler; R&D Systems), following the manufacturer's instructions. Murine MMP-1a levels were determined using an ELISA kit (USCN Life Science, Houston, TX).

MMP1 gene expression assay

Total RNA was isolated from human macrophages using the RNeasy Mini Kit, and residual DNA was digested using RNase-free DNase (both from QIAGEN, Valencia, CA). The RNA samples were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Gene expression was measured using SYBR Green–based real-time quantitative PCR, and 18S mRNA was used as the housekeeping gene. The following oligonucleotide primers were used: 18S, forward, 5'-CACGGCCGGTACAGTGAAAC-3' and reverse, 5'-CCCCTCGGCATGTATTAGCT-3'; and MMP1, forward, 5'-GCTAACCTTTGATGCTATAACTACGA-3' and reverse, 5'-TTTGTGC-GCATGTAGAATCTG-3'. Fold induction of *MMP1* gene expression was calculated using the $\Delta\Delta$ threshold cycle method, normalizing mRNA levels for each sample to levels of 18S and comparing with mRNA levels in unstimulated cells.

In vitro assays

CD14⁺ column-purified human elutriated monocytes were obtained from peripheral blood of healthy donors from the NIH blood bank. Macrophages were generated by culturing monocytes in the presence of RPMI 1640 media containing 10% human AB serum and M-CSF 50 ng/ml (PeproTech, Rocky Hill, NJ) for 7 d; fresh media with growth factor were added every 48 h, as previously described (22). This method of macrophage differentiation was chosen based on a recently published guideline (23). Bone marrow cells from wild-type (WT) or *Hmx1*^{-/-} mice on the C57BL/6 genetic background (a gift from Dr. Miguel Soares, Instituto Gulbenkian de Ciências, Oeiras, Portugal) were cultured for 7 d in 30% L929 supernatant media to differentiate bone marrow–derived macrophages. Cells were plated at a concentration of 10⁶ cells/well in 24-well plates. Cells were exposed to WT H37Rv, ESAT6 knockout (a gift from Dr. Volker Briken, University of Maryland, College Park, MD), or region of deletion 1 (RD1) knockout (a gift from Dr. Steven Derrick, U.S. Food and Drug Administration, College Park, MD) *M. tuberculosis* strains at different multiplicities of infection for 3 h, washed to remove extracellular bacteria, and cultured in serum-free media for 24 h in the presence or absence of the indicated concentrations of the HO-1 inducer cobalt (III) protoporphyrin IX dichloride (CoPPiX; Frontiers Scientific, Logan UT), the inhibitor of HO-1 activity tin (IV) protoporphyrin IX dichloride (SnPPiX; Frontiers Scientific), the MAPK inhibitor SB 203580 (Tocris Bioscience, Bristol, U.K.) used to inhibit MMP-1 production, FeSO₄ (Sigma, St. Louis, MO) as a source of iron (Fe²⁺), the inhibitor of heme biosynthesis succinylacetone (SA; Sigma), the CO-resealing molecule Ru₂Cl₂(CO)₆, also known as tricarbonyldichlororuthenium (II) dimer (CORM-II; Sigma), a molecule with similar structure to CORM-II but with no CO releasing capability RuCl₃ (Sigma), the CO scavenger oxymyoglobin (HbO₂; Sigma), and the inhibitor of AP-1 activity SR 11302 (Tocris Bioscience). In some experiments, recombinant ESAT6 was delivered into the cytosol of infected macrophages using a fusion protein system with the N-terminal fragment of the lethal factor of *Bacillus anthracis*. LFn-ESAT6-His6 consists of the N-terminal region of *B. anthracis* lethal factor fused at the C terminus to ESAT6, which contains six histidine residues at its C terminus. A DNA fragment encoding ESAT6, followed by a six-residue linker and then a C-terminal hexa-histidine tag was synthesized by GeneArt (Life Technologies). The fragment was subcloned into the expression plasmid FP59AGGpYS (24) using MluI and XmaI restriction sites in frame with the first 255 N-terminal amino acids of anthrax lethal factor. A *B. anthracis* strain deficient in extracellular proteases (25) was transformed with the resulting plasmid, and LFn-ESAT6-His6 fusion protein was purified from culture supernatants by nickel-affinity chromatography. The molecular mass of the protein was confirmed by electrospray ionization mass spectrometry. Anthrax-protective Ag was prepared as described previously (26). Indicated doses of the proteins were used in cell cultures 3 h after *M. tuberculosis* infection and left for 24 h. Culture supernatants were collected, sterile filtered, and stored at -80°C until use. Cell extracts were prepared following instructions for the HO-1 ELISA kit. Nuclear extracts were obtained using a kit from Active Motif (Carlsbad, CA). Activation of the transcription factors NFE2L2 and c-JUN/AP-1 was determined in nuclear extracts using the TransAM DNA-binding ELISA kits (Active Motif), following the manufacturer's instructions. Cell viability was estimated using the XTT assay kit (Cayman Chemical Ann Arbor, MI), following the manufacturer's instructions.

Data analysis

The median values with interquartile ranges were used as measures of central tendency. For the in vitro experiments, bars represent mean and SD. The Mann-Whitney *U* test (for two groups) or the Kruskal-Wallis test with the Dunn multiple-comparison or linear trend post hoc test (for more than two groups) were used to compare continuous variables. The Fisher or χ^2 test was

used to compare variables displayed as percentages. Spearman rank tests were used to assess correlations. Receiver operator characteristic (ROC) curves were used to test the power of individual or combined markers to distinguish active from latent TB. Three models of principal component analysis (PCA) indicated in the text were designed to assess how different combinations of plasma mediators contributed to the differentiation between patients with distinct expression profiles of HO-1 and MMP-1. Unsupervised two-way hierarchical cluster analyses (Ward's method) with bootstrap, where dendrogram branch spaces are proportional to distance, were used to test whether PTB patients with different expression profiles of HO-1 and MMP-1 could be grouped separately. Multinomial logistic regression analyses adjusted for age and gender were performed to assess the odds ratios (ORs) of the associations between hematological and clinical parameters and the different expression profiles of HO-1 and MMP-1. A *p* value < 0.05 was considered statistically significant. The statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA), STATA 9.0 (StataCorp, College Station, TX), JMP 10.0 (SAS, Cary, NC) and R 3.1.0 (R Foundation, Vienna, Austria) programs.

Results

Patients with active TB display higher plasma levels of both HO-1 and MMP-1 than do those with LTBI

We showed previously that plasma HO-1 levels are increased in active PTB patients compared with noninfected individuals and those with LTBI in a cohort from South India (9) (Fig. 1). To validate these results, we measured HO-1 levels in a geographically distinct cohort from the northeast region of Brazil. Again, HO-1 concentrations were higher in patients with active PTB disease than in noninfected individuals (*p* < 0.001) or those with LTBI (*p* < 0.001; Fig. 2). Although median HO-1 levels were higher in active PTB than in LTBI cases, we observed that some patients from these different clinical groups exhibited similar values (9) (Fig. 2).

Using the larger cohort of patients from India, we next tested whether the expression of MMP-1 in plasma also identifies individuals with active TB. Parallel to our findings with HO-1, we observed significantly higher levels of MMP-1 in active PTB patients than in healthy controls (*p* < 0.001) or LTBI cases (*p* < 0.001) (Fig. 3A). Again, similar findings were obtained in the Brazilian validation cohort (Fig. 2B). Interestingly, as observed for HO-1 values, some patients with active TB exhibited MMP-1 levels that were nondistinguishable from those observed in LTBI cases. These results indicate that although HO-1 or MMP-1 alone can distinguish active from latent TB, some disease cases that have values that overlap with LTBI can still be misclassified.

Expression of HO-1 and MMP-1 in plasma delineates two subpopulations of patients with active TB

Having demonstrated that plasma levels of both HO-1 and MMP-1 are elevated in active PTB compared with LTBI, we addressed whether combined measurement of HO-1 and MMP-1 would increase the power to discriminate these two patient groups. We found that although each marker individually displayed a high degree of accuracy, the combined assessment resulted in close to maximum (100%) performance in distinguishing active TB from LTBI (Figs. 2D, 3C). In Brazil, because the isolated markers were already shown to be accurate in distinguishing active TB from LTBI, the gain in accuracy with the combined approach was less pronounced than in the Indian cohort (Figs. 2D, 3C). Based on this observation, we hypothesized that the levels of these two biomarkers might be positively correlated. Surprisingly, we observed a striking negative correlation between HO-1 and MMP-1 expression in patients with active PTB but not in individuals with LTBI (Figs. 2C, 3B). Thus, patients with PTB in both the Indian and Brazilian cohorts expressed either very high levels of HO-1 (HO-1^{hi}MMP-1^{lo}) or MMP-1 (HO-1^{lo}MMP-1^{hi}), revealing a dichotomy within this clinical group (Figs. 2C, 3B). Of note, the median values of HO-1 and MMP-1 used to define the expression profiles were different between the two patient cohorts.

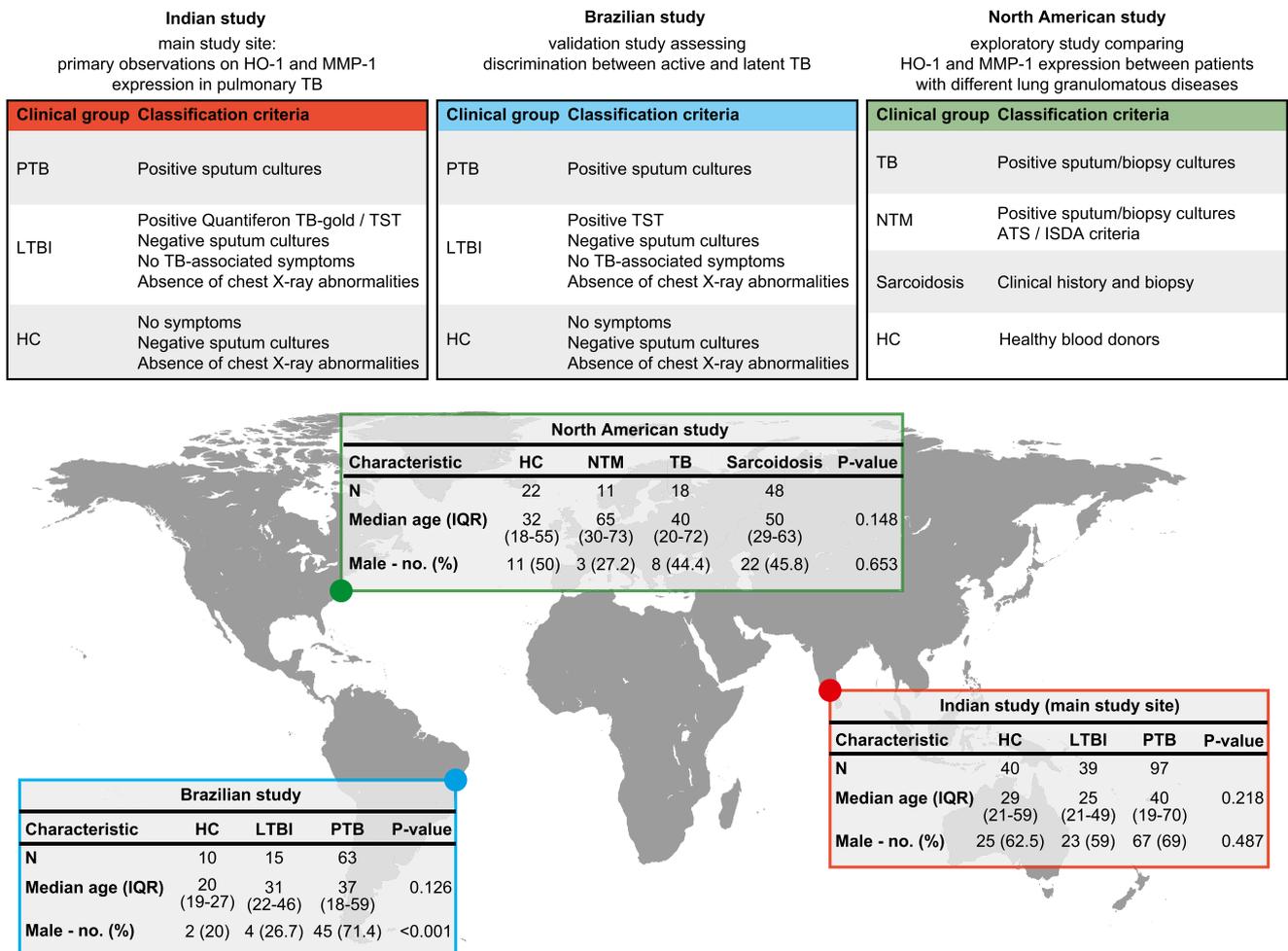


FIGURE 1. Classification criteria and characteristics of the study participants. This study used data obtained from clinical investigation of patient cohorts from two geographically distinct TB-endemic areas (India and Brazil), as well as from North America (see *Materials and Methods* for details). Age was compared between the groups using the Kruskal–Wallis test. Frequency of male gender was compared using the χ^2 test. All individuals enrolled in the different studies were negative for HIV infection.

These assays were performed separately in the different countries and not simultaneously in a single facility. The distinct median values likely represent interassay variability and/or genetic and environmental differences. Importantly, despite the distinction in median values, the expression of the biomarkers relative to each other was very similar in the two geographically distinct patient groups.

Patients with active TB, atypical mycobacterial infection, or sarcoidosis show distinct HO-1 and MMP-1 expression profiles

In an exploratory study of a limited number of patients from North America, we next examined whether the expression profile of HO-1 and MMP-1 in plasma differs between TB and other lung granulomatous diseases. HO-1 levels were increased in patients with active TB, NTM infection, or sarcoidosis compared with healthy controls (Fig. 4A). Although active TB patients exhibited markedly higher HO-1 expression than did NTM infection patients ($p = 0.008$), they were not significantly different from sarcoid patients (Fig. 4A). In contrast, systemic concentrations of MMP-1 were dramatically elevated in TB patients compared with healthy controls ($p < 0.001$) and sarcoid patients ($p < 0.001$), but they were not significantly different from NTM infection patients ($p = 0.051$, Fig. 4B). On average, plasma MMP-1 values in sarcoid patients were indistinguishable from healthy controls (Fig. 4B). A hierarchical clustering analysis of the plasma concentrations of these two enzymes revealed

the existence of two major subgroups of TB patients with very distinct HO-1 and MMP-1 expression profiles, whereas NTM infection and sarcoid patients exhibited more heterogeneous profiles (Fig. 4C). Importantly, an inverse correlation between HO-1 and MMP-1 levels was observed in TB but not in the other lung diseases examined (Fig. 4D). As described in the India and Brazil cohorts, patients with active TB preferentially displayed HO-1^{hi}MMP-1^{lo} or HO-1^{lo}MMP-1^{hi} expression profiles, whereas individuals in the other clinical groups exhibited a more heterogeneous profile ($p < 0.0001$, χ^2 test, Fig. 4E). Thus, these preliminary findings indicated that the pattern of the relationship between plasma levels of HO-1 and MMP-1 is different between TB and other granulomatous lung diseases.

Active TB patients with distinct HO-1 versus MMP-1 expression patterns display markedly different inflammatory profiles

The data shown above demonstrated an inverse correlation between HO-1 and MMP-1 expression among patients with active TB in Indian, Brazilian, and North American cohorts but not in healthy controls, individuals with latent TB, or those with other lung diseases. We next examined the associations between HO-1 and MMP-1 and other biomarkers of inflammation or tissue damage/remodeling in PTB patients from our major study site in southern India ($n = 97$). Interestingly, the TB patients could be separated into two major

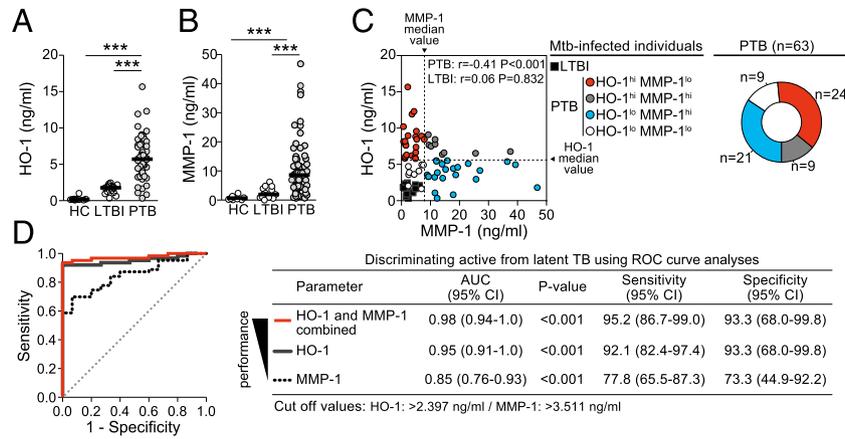


FIGURE 2. Plasma levels of HO-1 and MMP-1 in a Brazilian TB patient cohort. Plasma levels of HO-1 (A) and MMP-1 (B) were measured in a cohort of individuals with PTB ($n = 63$) or LTBI ($n = 15$), as well as in healthy controls (HC; $n = 10$) from northeast Brazil. Horizontal lines represent median values. (C) Correlation graph of plasma MMP-1 and HO-1 in individuals with PTB. Data were analyzed using the Spearman rank correlation test. Dotted line on the x-axis represents the median value of MMP-1 within the group of patients with pulmonary disease, whereas the dotted line on the y-axis indicates the median value for HO-1 (left panel). Using this approach, it was possible to stratify the PTB patients according to the patterns of expression of MMP-1 and HO-1 in plasma (right panel). (D) ROC curves were used to assess the performance of HO-1, MMP-1, or both in discriminating active from latent TB disease. *** $p < 0.001$, Kruskal–Wallis test with the Dunn multiple-comparison post hoc test. CI, confidence interval; hi, values higher than median in PTB patients; lo, values lower than median in PTB patients.

clusters based on plasma protein expression of these markers (Fig. 5A). Within these populations, HO-1 levels displayed significant negative correlations with other MMPs, such as MMP-8 and MMP-9, as well as TNF- α and serum amyloid protein-A. In contrast, HO-1 concentrations were positively correlated with TIMP-1, TIMP-4, CRP, haptoglobin, IFN- γ , and IL-10 (Fig. 5B). In addition, the correlations involving HO-1 resulted in the identification of several unique expression profiles for a number of these markers (Supplemental Fig. 1). Visualization of the data using density plots clearly showed that the subpopulation of high HO-1-expressing individuals displayed relatively decreased levels of MMP-1, MMP-8, and MMP-9 compared with the group of individuals exhibiting low plasma values of HO-1, but they also accounted for all of the patients with greatly elevated CRP and haptoglobin levels (Supplemental Fig. 1B). Strikingly, the correlations involving plasma MMP-1 concentrations had a strongly inverted profile compared with those found for HO-1 levels (Fig. 5B, 5C).

To better assess which of the two processes, inflammation or tissue remodeling, is more relevant in describing differences between patients with HO-1^{hi}MMP-1^{lo} and HO-1^{lo}MMP-1^{hi} expression profiles, we used PCA. In the first model, we inputted data on all of the biomarkers (Supplemental Fig. 2A, Supplemental Table I). Using this approach, we found that the groups of patients with either HO-1^{hi}MMP-1^{lo} or HO-1^{lo}MMP-1^{hi} clustered separately, although there was a notable dispersion of the data points within each group (Supplemental Fig. 2B). In the second model, which incorporated only the biomarkers of inflammation, we observed that the groups remained separately clustered with considerably less dispersion (Supplemental Fig. 2B). The third model used data from markers of tissue remodeling alone. In this analysis, both the intersection between the groups and the dispersion of the data points within each group were significantly greater than those observed with the other two models (Supplemental Fig. 2B). The above PCA analyses indicated that patients with HO-1^{hi}MMP-1^{lo} or HO-1^{lo}MMP-1^{hi} can

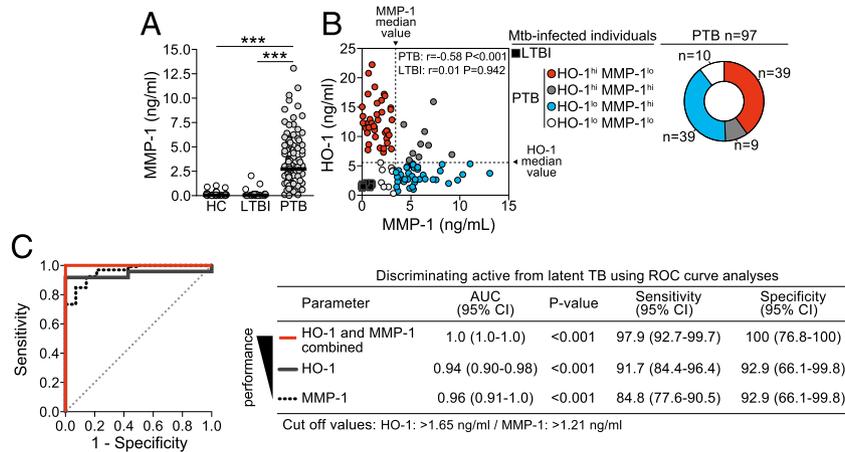


FIGURE 3. Combined assessment of HO-1 and MMP-1 plasma levels distinguishes active TB infection from LTBI. (A) Plasma levels of MMP-1 were compared in 97 individuals with active PTB, 39 individuals with LTBI, and 40 healthy controls (HC) matched for age and gender using the Kruskal–Wallis test with the Dunn multiple-comparison post hoc test (see Fig. 1 for details about the study groups). (B) Correlation between MMP-1 and HO-1 plasma concentrations in individuals with LTBI or PTB was assessed using the Spearman rank test. The dotted line on the x-axis represents the median value of MMP-1 within the group of patients with pulmonary disease, whereas the dotted line on the y-axis indicates the median value for HO-1. Using this approach, it was possible to stratify the PTB patients according to the patterns of expression of MMP-1 and HO-1 in plasma. (C) ROC curve analyses were used to assess the performance of HO-1, MMP-, or both in discriminating active from latent TB cases. *** $p < 0.001$. CI, confidence interval; hi, values higher than median in PTB patients; lo, values lower than median in PTB patients.

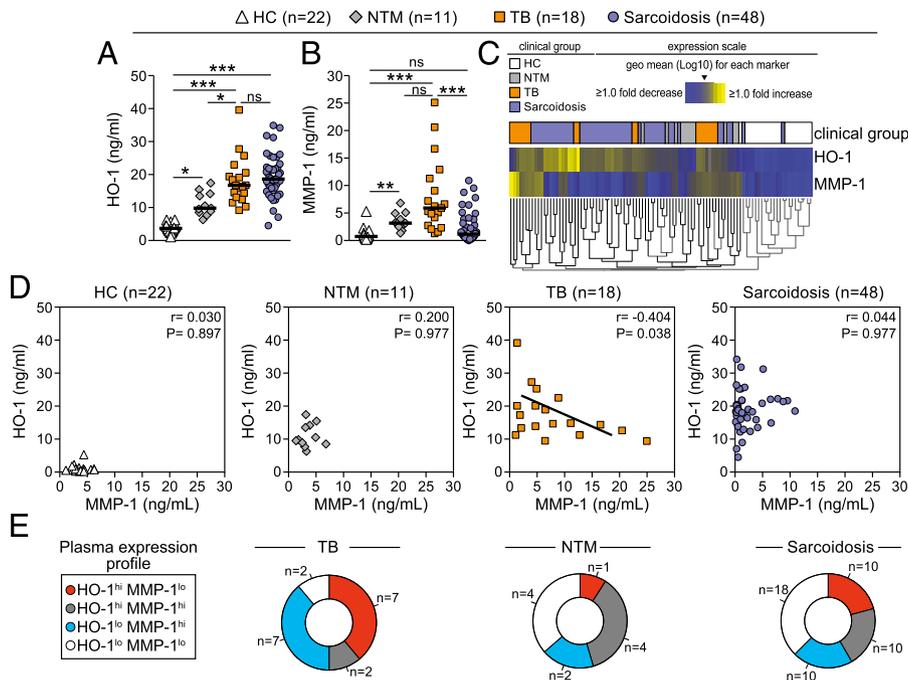


FIGURE 4. HO-1 and MMP-1 expression profiles in plasma from patients with active TB and individuals with other granulomatous lung diseases. Plasma levels of HO-1 (A) and MMP-1 (B) were quantified in patients with confirmed active TB, NTM infection, sarcoidosis, and age- and gender-matched healthy controls (HC) from North America. Horizontal lines represent median values. (C) An unsupervised cluster analysis (Ward's method) was used to identify overall differences in the expression profiles of HO-1 and MMP-1 in this study population. In the heat map, individual patients are listed in columns, and each biomarker was placed in a different row. The squares represent values below or above the geometric mean levels (log10) of a given biomarker in the study population. (D) Correlation between MMP-1 and HO-1 plasma concentrations was assessed using the Spearman rank test in the different study groups. (E) Distribution of the patients from the different lung disease groups with regard to expression profiles of HO-1 and MMP-1 in plasma. Data were compared using the χ^2 test ($p < 0.0001$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal–Wallis test with the Dunn multiple-comparison post hoc test. hi, values higher than median in the indicated clinical group; lo, values lower than median in the indicated clinical group; ns, nonsignificant.

be better distinguished on the basis of their inflammatory marker profiles than by the differential expression of tissue-remodeling markers in plasma. This conclusion was supported by hierarchical clustering, as well as ROC curve analyses of the different combinations of biomarkers used in the PCA models (Supplemental Fig. 2C, 2D).

HO-1^{hi}MMP-1^{lo} and HO-1^{lo}MMP-1^{hi} active TB patients display distinct disease-presentation profiles

We next compared the HO-1^{hi}MMP-1^{lo} and HO-1^{lo}MMP-1^{hi} patient subpopulations with regard to clinical, microbiological, hematological, and radiological parameters to test whether these populations diverge in terms of TB disease extension/severity. HO-1^{hi}MMP-1^{lo} and HO-1^{lo}MMP-1^{hi} patients did not differ significantly with regard to age, gender, and most hematological parameters (Fig. 6A). HO-1^{hi}MMP-1^{lo} patients displayed lower body mass index and more frequently exhibited positive AFB sputum smears and bilateral lung lesions compared with HO-1^{lo}MMP-1^{hi} patients (Fig. 6A). Among the entire population of active PTB patients, individuals presenting with positive sputum smears and bilateral lung lesions simultaneously exhibited the highest levels of plasma HO-1 and the lowest MMP-1 values (Fig. 6B). Total leukocyte and absolute neutrophil counts in the blood were higher in the subpopulation of TB patients with HO-1^{lo}MMP-1^{hi} than in those with HO-1^{hi}MMP-1^{lo} expression profiles (Fig. 6A). Nevertheless, there were no significant differences in neutrophil or total leukocyte counts between the subgroups of patients with positive or negative sputum smears (Fig. 6C, 6D), as well as between individuals with unilateral or bilateral lung lesions (Fig. 6C, 6D). Together, these analyses revealed that the patient subpopulations identified by combined measurement of plasma HO-1 and MMP-1 exhibit distinct disease presentations.

Regulation of HO-1 and MMP-1 expression in M. tuberculosis-infected macrophages

The above clinical observations on HO-1 and MMP-1 levels led us to hypothesize that the expression of these two biomarkers might be cross-regulated. We directly tested this hypothesis in *in vitro* studies using human and murine macrophages. Macrophages were described as an important source of HO-1 in several disease models, including murine mycobacterial infection (27), and MMPs and other tissue proteases are known to be induced by *M. tuberculosis* infection in the same myeloid cell type (11). In the current study, we observed that MMP-1 is induced in a more selective manner than other MMPs or proteases in human macrophages infected with *M. tuberculosis* (Supplemental Fig. 3). For this reason, we assessed the expression of intracellular protein HO-1 in cell lysates and the secreted protein MMP-1 in supernatants from macrophages infected with increasing multiplicities of virulent *M. tuberculosis*. We observed a significant dose-dependent induction of both proteins in the cell cultures (Fig. 7A, 7B). In the case of HO-1, the response obtained was dependent on replicating bacilli, because irradiated *M. tuberculosis* triggered only minor levels of the enzyme (Fig. 7A). In contrast, *M. tuberculosis* irradiation failed to diminish the MMP-1 response in the same cultures (Fig. 7B), suggesting that the two biomarkers have distinct bacterial triggers.

A recent study indicated that the *M. tuberculosis* virulence-associated secreted protein ESAT6 plays a role in the induction of HO-1 in a murine macrophage cell line (28). In the current study, we observed that infection of human macrophages with mutant *M. tuberculosis* strains lacking the RD1 or the ESAT6 protein (29) induced significantly lower HO-1 production (Fig. 7C, 7D) but higher MMP-1 secretion (Fig. 7E) than did the WT H37Rv

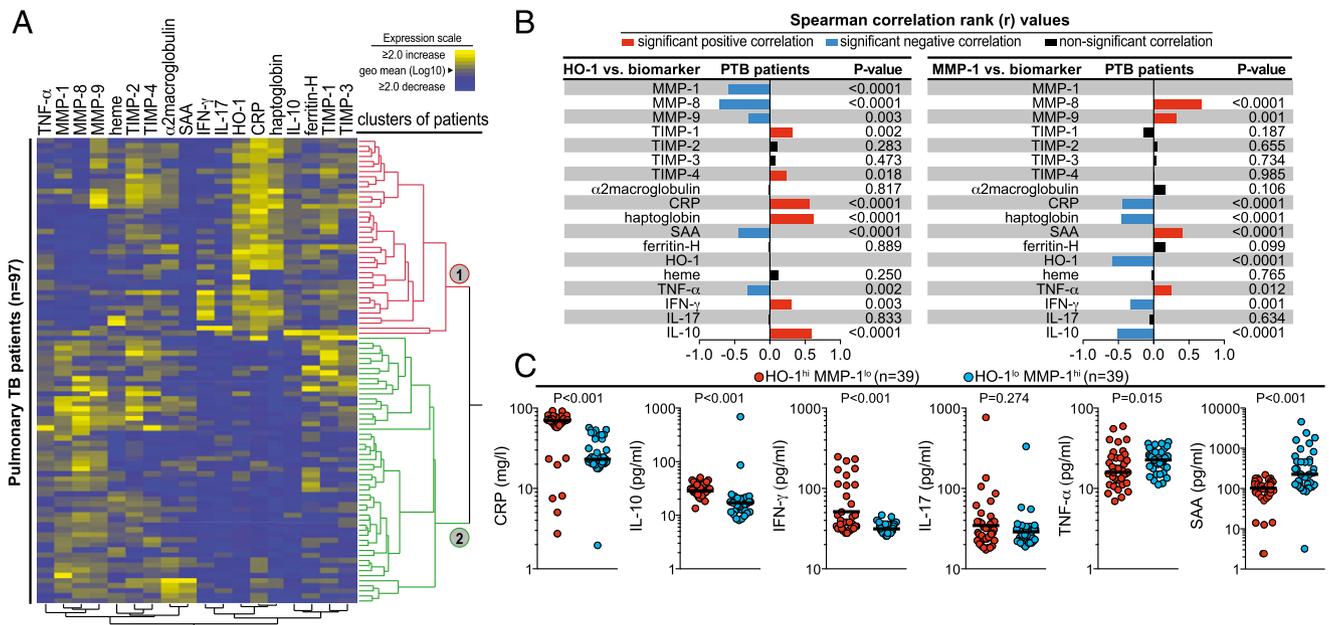


FIGURE 5. PTB patients with different HO-1 and MMP-1 ratios display distinct plasma inflammatory biomarker profiles. **(A)** Protein levels of several plasma biomarkers of inflammation and tissue damage/remodeling were determined by ELISA in active TB patients from India ($n = 97$). An unsupervised hierarchical cluster with bootstrap analysis was used to identify overall differences in the expression profiles of the markers in this population. To visualize the clusters, the dendrogram branch spacing was set to be proportional to the hierarchical distance. The two larger clusters are highlighted with colors. In the heat map, individual patients are listed in rows and each biomarker was placed in a different column. The squares represent values below or above the geometric mean levels (log10) of a given biomarker in the study population. **(B)** Plasma levels of HO-1 (*left panel*) and MMP-1 (*right panel*) were tested for correlations with each one of the biomarkers measured. Bars represent the strength of associations (Spearman rank values). **(C)** Circulating levels of the indicated plasma mediators were compared between the groups of patients exhibiting opposite patterns of expression of HO-1 and MMP-1 in plasma using the Mann–Whitney U test (see Supplemental Table I for details).

M. tuberculosis strain. Delivery of ESAT6 recombinant protein into the cytosol of macrophages infected with ESAT6-deficient *M. tuberculosis* using a fusion protein with the N-terminal fragment of the lethal factor of *B. anthracis* restored HO-1 induction and led to a decrease in MMP-1 secretion to levels similar to those induced by infection with the WT H37Rv *M. tuberculosis* strain (Fig. 7F–H).

Given that HO-1 is described as a potent antioxidant and immunomodulator and that induction of MMP-1 by *M. tuberculosis* in macrophages involves activation of inflammatory transcription factors (17), we hypothesized that, in conditions characterized by high HO-1 expression, MMP-1 production would be downregulated. Indeed, drug-induced overexpression of HO-1 triggered by CoPPIX caused a major (>2 -log) reduction in MMP-1 concentrations in supernatants of *M. tuberculosis*-infected macrophages (Fig. 8A, 8B). Conversely, treatment of macrophages with SnPPIX, a potent inhibitor of HO-1 activity, resulted in a pronounced increase in MMP-1 production upon *M. tuberculosis* infection (Fig. 8B). Interestingly, treatment of macrophages with a MAPK inhibitor (SB 203580) caused a significant increase in HO-1 expression while strongly inhibiting MMP-1 production by infected macrophages (Fig. 8C). This data demonstrated that HO-1 and MMP-1 are differentially regulated in *M. tuberculosis*-exposed macrophages and suggested that HO-1 induction is strongly associated with inhibition of MMP-1 expression in these cells.

CO derived from HO-1 activity downregulates MMP-1 expression by decreasing activation of c-JUN/AP-1

We next asked whether downstream products of HO-1 activity, such as free iron (Fe^{+2}) and CO, or heme catabolism by HO-1 might explain the downmodulation of MMP-1 expression induced by the former enzyme. To do so, we cultured *M. tuberculosis*-infected macrophages with FeSO_4 to mimic the increased availability of Fe^{+2} , but this treatment failed to alter MMP-1 production (Fig. 8D).

In contrast, *M. tuberculosis*-infected cells treated with CORM-II, a CO-releasing molecule, led to a dramatic reduction in MMP-1 production that was not reproduced by the control drug RuCl_3 , which lacks CO-releasing activity ($p < 0.001$, Fig. 8D). The CORM-II concentration used was not cytotoxic (cell viability in treated cultures: $92.5 \pm 3.8\%$ versus $95.6 \pm 5.2\%$ in untreated cells, $p = 0.857$). We also treated the macrophage cultures with SA, an inhibitor of heme biosynthesis, to mimic the reduction in heme availability triggered by HO-1 overexpression. We observed a substantial decrease in MMP-1 production by *M. tuberculosis*-infected macrophages in the SA-treated cultures ($p = 0.022$, Fig. 8D), but the levels were still much higher than those seen in cultures treated with CO-releasing molecule ($p < 0.001$, Fig. 8D). We next infected bone marrow-derived macrophages from WT or *Hmox1*^{-/-} mice and assessed secretion of MMP-1a, the murine ortholog of human MMP-1. *M. tuberculosis*-infected macrophages from *Hmox1*^{-/-} mice secreted significantly higher amounts of MMP-1a than did WT cells (Fig. 8E). The secretion of MMP-1a by *Hmox1*^{-/-} macrophages was dramatically reduced by treatment with the CO-releasing molecule but only partially so following addition of the inhibitor of heme biosynthesis, SA (Fig. 8E), reinforcing the role of CO in the modulation of MMP-1 secretion.

In further experiments, we verified that CO affects MMP-1 expression at the transcriptional level, because mRNA levels of *MMP1* were dramatically reduced in *M. tuberculosis*-infected macrophages treated with CORM-II and were restored by the addition of HbO_2 , a CO scavenger (Fig. 8F). Given the central importance of the transcription factor AP-1 in MMP-1 expression in the context of *M. tuberculosis* infection (30), we hypothesized that CO derived from HO-1 activity would impair MMP-1 expression by interfering with AP-1 activation. We confirmed in our *in vitro* system that human macrophages infected with *M. tuberculosis* display increased activation of c-JUN/AP-1 in nuclear extracts 12 h postinfection

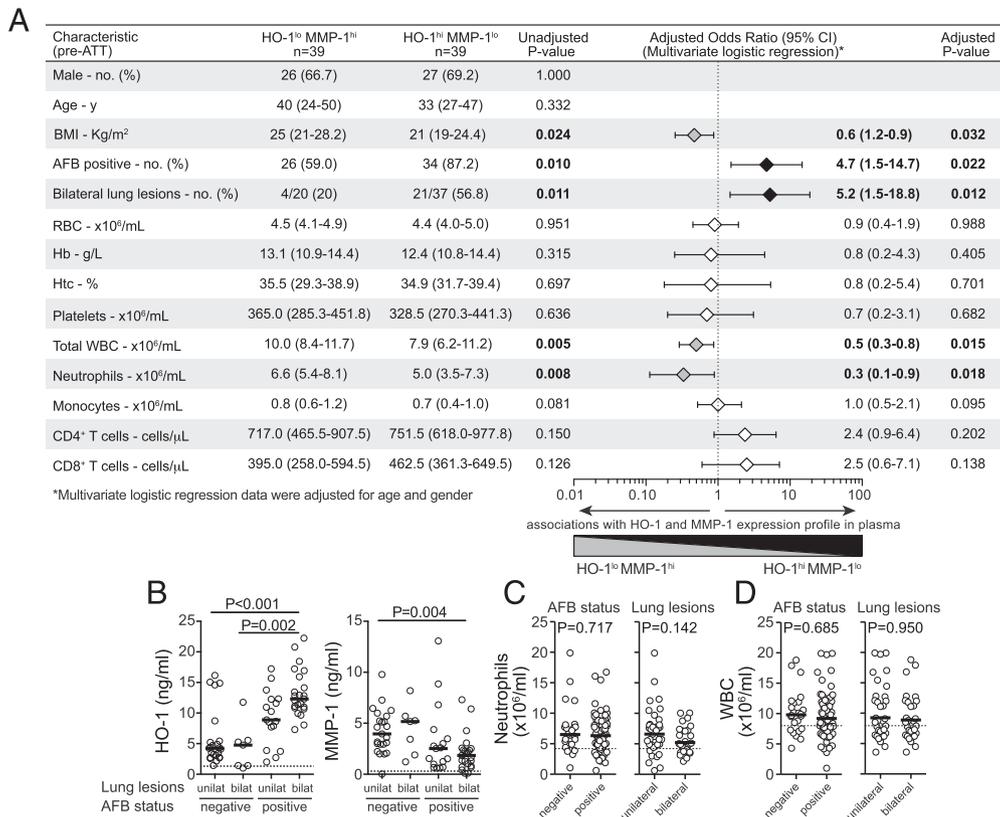


FIGURE 6. HO-1^{hi}MMP-1^{lo} and HO-1^{lo}MMP-1^{hi} active TB patients display distinct disease-presentation profiles. **(A)** Patients with active PTB exhibiting different patterns of expression of HO-1 and MMP-1 in plasma were compared with regard to demographical, clinical, and laboratory characteristics, using the Mann–Whitney *U* test (for continuous variables) or the Fisher exact test (for categorical variables). The associations between the variables and the pattern of HO-1/MMP-1 expression were also assessed using multivariate logistic regression adjusted for age and gender. ORs are per SD increase after log transformation. ORs for the percentage of AFB⁺ and bilateral lung lesions are for comparisons between AFB⁺ versus AFB[−] and for unilateral versus bilateral lung lesions, respectively. **(B)** PTB patients were stratified according to sputum positivity and lung disease extension, and plasma levels of HO-1 (*left panel*) and MMP-1 (*right panel*) were compared using the Kruskal–Wallis test, with the Dunn multiple-comparison post hoc test. **(C)** Absolute neutrophil counts in the blood were assessed in TB patients according to the AFB positivity in sputum (*left panel*) and the location of lung lesions (*right panel*). **(D)** Absolute WBC counts in the blood were assessed in PTB patients according to the AFB positivity in sputum (*left panel*) and the location of lung lesions (*right panel*). In (B)–(D), horizontal lines represent median values, and dotted lines denote median values observed in the group of healthy controls. In (C) and (D), data were analyzed using the Mann–Whitney test. bilat, bilateral; CI, confidence interval; Hb, hemoglobin; hi, higher than median values; Htc, hematocrit; lo, lower than median values; unilat, unilateral.

(Fig. 8G). Interestingly, we detected a significant reduction in c-JUN/AP-1 activation in infected macrophages treated with the HO-1 inducer (Fig. 8G), accompanied by increases in the expression of the *Hmox1* transcription factor NFE2L2 (Fig. 8H). The inhibitory effects on c-JUN/AP-1 activation observed with treatment with the HO-1 inducer were reproduced when cells were treated with the CO-releasing molecule (Fig. 8G). Importantly, inhibition of HO-1 activity or removal of CO by HbO₂ restored the activation of c-JUN/AP-1 in infected macrophages (Fig. 8G). These findings argue that HO-1 activity in *M. tuberculosis*-infected macrophages downmodulates MMP-1 expression via CO-mediated suppression of c-JUN/AP-1 activation.

Discussion

The development of reliable biomarkers for active TB is important for identifying patients in need of antibiotic therapy and for a better understanding of the pathological mechanisms involved in the progression of *M. tuberculosis* infection to active disease that could serve as targets for immunotherapies. In this study, we demonstrate that combined measurement of HO-1 and MMP-1 in plasma reveals a dichotomy in PTB patients that reflects their different disease-presentation profiles. This dichotomy was absent in LTBI individuals, as well as in the patients with other lung diseases, highlighting potential differences in immunopathology among these clinical conditions.

HO-1 is a potent antioxidant enzyme associated with cytoprotection in a number of disease settings. In contrast, MMP-1 is a major collagenase involved in tissue remodeling. Because both enzymes have been used as biomarkers for TB (9, 12, 14, 15), it was of interest to determine whether their expression is linked. We found in human macrophages infected with *M. tuberculosis* that, although HO-1 and MMP-1 are induced, MMP-1 expression is regulated by HO-1. Previous data showed that HO-1 can regulate MMP-1 production in a human chondrocyte cell line stimulated with IL-1 β by CO-driven inhibition of the NOX4 pathway (31). This finding suggested that HO-1-mediated inhibition of MMP-1 expression in *M. tuberculosis*-infected macrophages might involve a similar CO-dependent mechanism. In support of this hypothesis, we showed that HO-1-driven CO downregulates MMP-1 expression at the transcriptional level by inhibiting activation of c-JUN/AP-1. In addition, the observed suppression of MMP-1 by CO in macrophages was associated with increased activation of the HO-1 transcriptional regulator NFE2L2, suggesting an upstream interaction between the pathways controlling these two transcription factors. Additional experiments are necessary to confirm this relationship between HO-1 and MMP-1 at the single-cell level and to further delineate its molecular basis.

The finding that CO is a major regulator of MMP-1 expression in *M. tuberculosis* infection has important implications for pathogenesis.

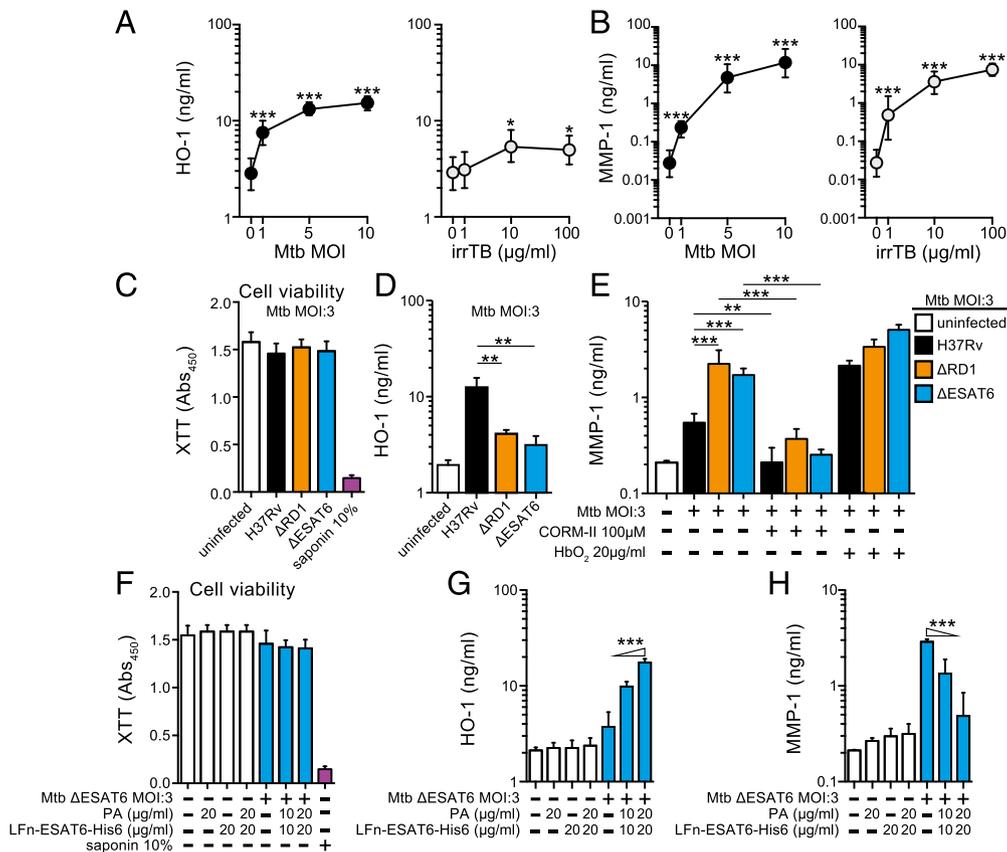


FIGURE 7. Infection of human macrophages with live virulent *M. tuberculosis* is required for robust induction of high levels of HO-1 but not MMP-1. (A) Levels of HO-1 were determined in cell lysates of human monocyte-differentiated macrophages after 24 h of infection with *M. tuberculosis* H37Rv strain (Mtb) at different multiplicities of infection (MOI) (left panel) or stimulation with irradiated *M. tuberculosis* at distinct concentrations (right panel) using ELISA. (B) MMP-1 levels were determined in culture supernatants of the same conditions as in (A). Macrophages were infected with H37Rv, Δ RD1, or Δ ESAT6 *M. tuberculosis* strains for 24 h in the presence or absence of the CO-releasing molecule CORM-II or the CO scavenger HbO₂, and cell viability (XTT assay) (C), levels of HO-1 in cell lysates (D), and levels of MMP-1 in culture supernatants (E) were assessed. Cells infected with the Δ ESAT6 *M. tuberculosis* strain were treated with different doses of the fusion Lfn-ESAT-6 with the anthrax-protective Ag cytosolic delivery system, and cell viability (XTT assay) (F), levels of HO-1 (G), and levels of MMP-1 (H) were measured after 24 h. In cell viability assays, saponin 10% was used as a positive control to induce cell lysis and death. Data are from at least three experiments using cells from a total of up to six healthy donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal-Wallis test with the Dunn multiple-comparison and/or linear trend post hoc analyses (triangles indicate the direction of the trend variation).

In vitro exposure of several bacterial species, such as *Pseudomonas aeruginosa* and *Escherichia coli*, to increasing doses of CO results in microbial death by inhibition of critical enzymes involved in respiratory electron transport chains (32, 33). The importance of CO in controlling mycobacterial growth in vitro was investigated previously (34). HO-1-derived CO was shown to alter *M. tuberculosis* gene transcription and activate the mycobacterial dormancy regulon in experimental studies with mouse macrophages (27, 35). The recent identification of a gene mutation in *M. tuberculosis* strains that confers resistance to CO and leads to increased pathogenicity capacity in a murine TB model (36) suggests that CO resistance may be critical for *M. tuberculosis* survival and persistence in vivo. Our results demonstrating that CO strongly inhibits the expression of MMP-1 argue that, in addition to its antimicrobial effects, this metabolic product could have regulatory effects on collagen degradation in the lungs of TB patients. Inhibition of MMP-1 was proposed as an adjunct treatment strategy for TB and other lung diseases (37). The findings presented in this article suggest that pharmacological induction of HO-1 expression leading to CO production could be used as a strategy for MMP-1 inhibition in TB and perhaps other fibrotic diseases.

We observed that the majority of patients with active TB express either high HO-1 and low MMP-1 or vice versa. However, up to 19.5% of the TB patient population in India and 28.6% in Brazil did not fall

into these HO-1^{hi}MMP-1^{lo} or HO-1^{lo}MMP-1^{hi} categories. In addition, in our in vitro experiments, significant induction of HO-1 was seen only when macrophages were exposed to live virulent ESAT6 expressing *M. tuberculosis*, whereas MMP-1 production was potently induced by both live and irradiated mycobacteria, as well as by *M. tuberculosis* lacking ESAT6 expression. Thus, the dichotomous expression of HO-1 and MMP-1 may reflect some aspect of the cellular response triggered by actively replicating virulent *M. tuberculosis*, such as the ability to escape from the macrophage phagosome. In addition, we observed that pharmacologic inhibition of MMP-1 with SB 203580 results in increased HO-1 expression in *M. tuberculosis*-infected macrophages, suggesting that MMP-1-related pathways could also inhibit HO-1 production and that the expression of these two critical enzymes is indeed cross-regulated. Such a mechanism triggered by *M. tuberculosis* infection might explain why a distinct HO-1 and MMP-1 expression profile with a strong inverse correlation in plasma values occurs in the majority of active TB cases and is not clearly observed in patients with other granulomatous lung diseases, such as sarcoidosis and NTM infection.

The two subpopulations of PTB patients identified by dual measurement of HO-1 and MMP-1 are of special interest because they could reflect distinct stages of TB disease progression (early versus advanced disease) or differences in disease severity or anatomical

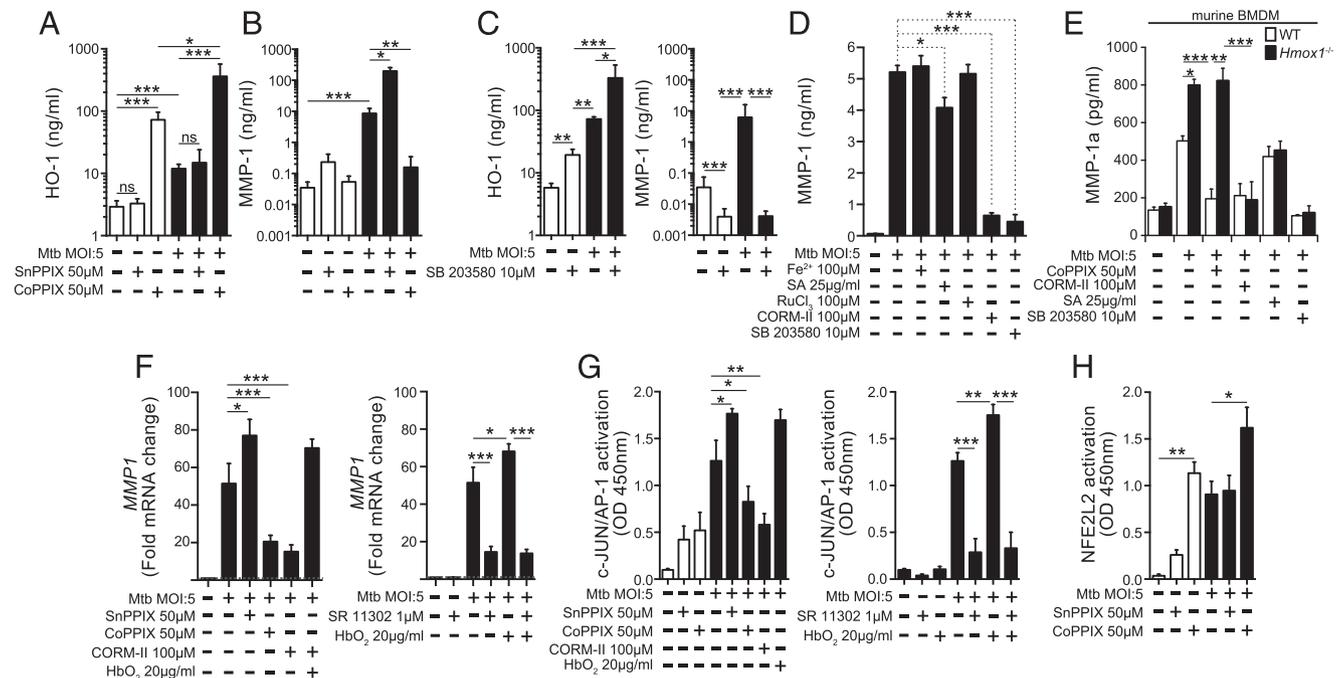


FIGURE 8. HO-1 and MMP-1 expression are differentially regulated in *M. tuberculosis*-infected human macrophages. Human monocyte-differentiated macrophages were infected with H37Rv *M. tuberculosis* for 24 h in the absence or presence of an inhibitor of HO-1 activity (SnPPIX) or a potent HO-1 inducer (CoPPIX), and levels of HO-1 in cell lysates (**A**) and levels of MMP-1 in supernatants (**B**) were quantified. (**C**) Macrophages were also treated with a MAPK inhibitor (SB 203580) in the presence of *M. tuberculosis* for 24 h, and the levels of HO-1 and MMP-1 were determined in cell lysates and supernatants, respectively. (**D**) MMP-1 protein in supernatants was quantified in cells treated with free iron (Fe²⁺; FeSO₄), an inhibitor of heme biosynthesis (SA), a CO-releasing agent (CORM-II), a molecule similar to CORM-II but with no CO-releasing capability (RuCl₃), SB 203580, SnPPIX, CoPPIX, and the CO scavenger (HbO₂) for 24 h post-*M. tuberculosis* infection. (**E**) These in vitro experiments were repeated using bone marrow derived macrophages (BMDMs) from C56BL/6 WT mice or *Hmox1*^{-/-} animals, and MMP-1a (an ortholog of the human MMP-1) was measured in culture supernatants by ELISA. (**F**) *MMP1* mRNA levels were assessed in cultures of *M. tuberculosis*-infected human macrophages after 24 h of the indicated treatments. For mRNA analysis, fold induction over mRNA levels in untreated cells is shown. Activation of the transcription factors c-JUN/AP-1 (**G**) and NFE2L2 (**H**) was determined 12 h postinfection and/or stimulation using a colorimetric DNA-binding ELISA kit. Data are mean and SD and were compared using the Kruskal–Wallis test, with the Dunn multiple-comparison posttest. Data are from at least three experiments using cells from a total of up to six healthy donors. In (**E**), four experiments were performed, with samples run in triplicates. Data from different biological groups were analyzed using the Kruskal–Wallis test, with the Dunn multiple-comparison test, whereas matched analyses were performed using the Wilcoxon matched-pairs test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. ns, nonsignificant.

location. In the current study, patients were recruited at the time of disease presentation (none were exposed to antituberculous treatment prior to or at study enrollment), and for this reason we did not have reliable data on the history of TB disease prior to plasma collection. However, we were able to compare HO-1^{hi}MMP-1^{lo} and HO-1^{lo}MMP-1^{hi} patients with regard to clinical, microbiological, hematological, and radiological parameters to test whether these populations diverge in terms of TB disease severity. Multivariate regression analysis confirmed that lower body mass index, AFB positivity in sputum smears, and presentation with bilateral lung lesions in chest x-rays were strongly associated with the HO-1^{hi}MMP-1^{lo} expression profile, whereas total leukocyte and neutrophil counts were linked to the HO-1^{lo}MMP-1^{hi} profile. This observation is consistent with a scenario in which HO-1^{hi}MMP-1^{lo} patients are those with increased TB disease severity. A longitudinal study would be needed to determine whether these individuals derive from HO-1^{lo}MMP-1^{hi} patients at an earlier stage of disease progression. Interestingly, single nucleotide polymorphisms that impact both HO-1 and MMP-1 expression were described previously in a variety of conditions (38–45); thus, it is possible that the two patient populations described in this article reflect this genetic heterogeneity. Finally, as alluded to above, the distinct HO-1^{hi}MMP-1^{lo} and HO-1^{lo}MMP-1^{hi} patient sets may reflect differences in the virulence of the *M. tuberculosis* strains infecting individuals in each subgroup within the cohort. Genetic analyses of the

infected patients and their corresponding *M. tuberculosis* isolates could be used to address the latter two hypotheses.

Together, our results reveal two distinct subgroups of active TB, each marked by increased HO-1 or MMP-1 expression and deriving from a potential cross-talk between these molecules. Both the nature of these clinical subpopulations and the possible role of HO-1 and MMP-1 in their pathogenesis await further definition.

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Disclosures

The authors have no financial conflict of interest.

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