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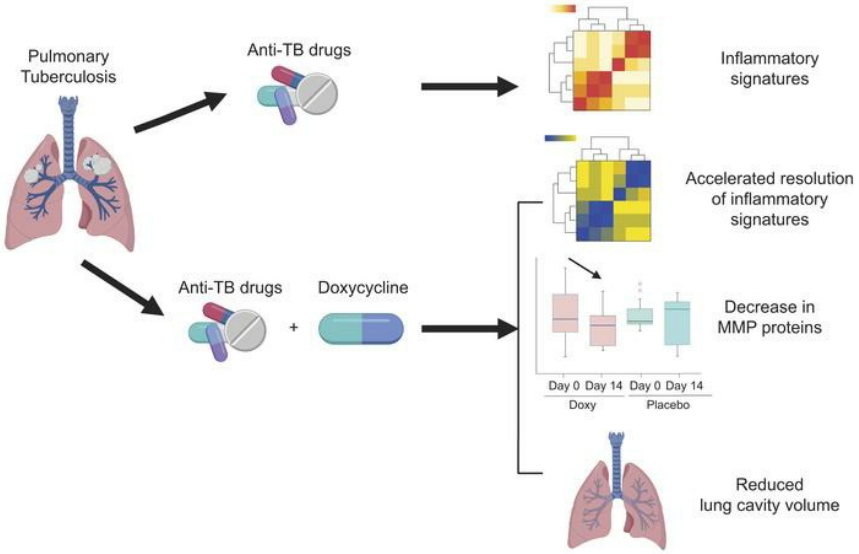
Graphical abstract



Title:

Doxycycline host-directed therapy in human pulmonary tuberculosis

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Abstract (247 / 250 word limit)

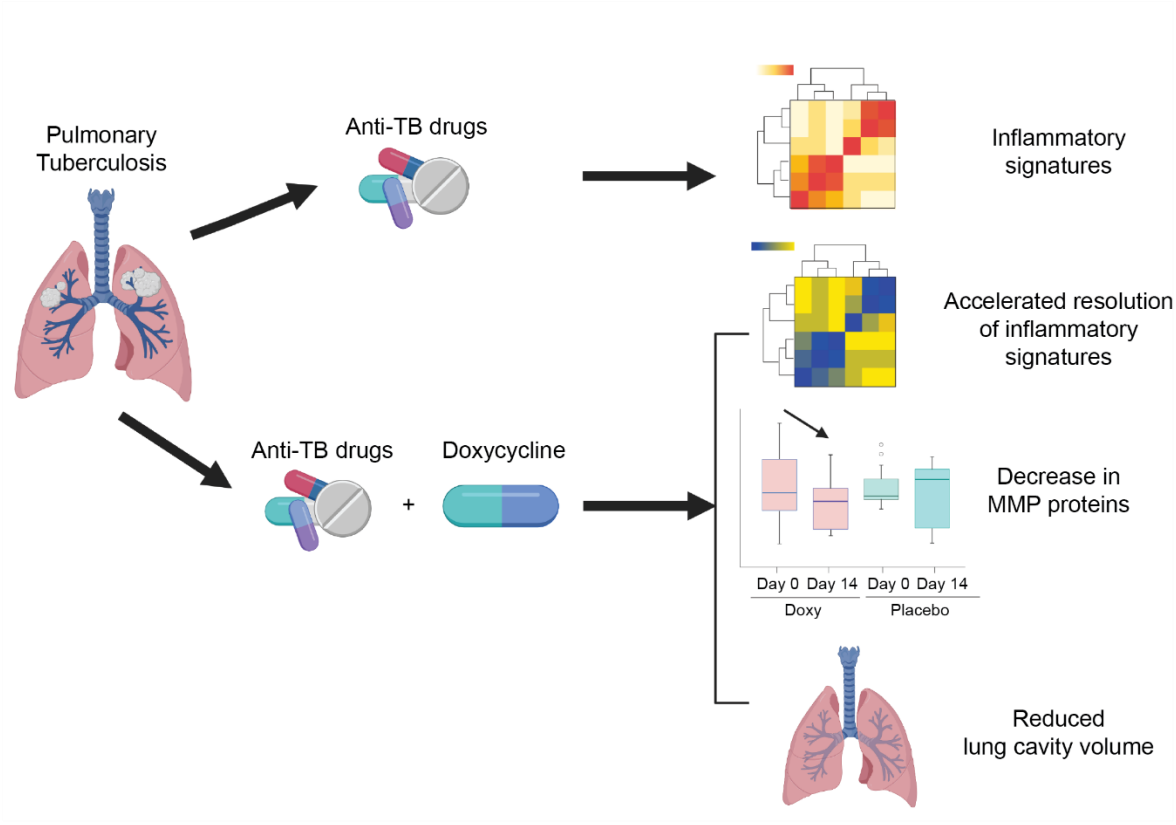
BACKGROUND. Matrix metalloproteinases (MMPs) are implicated as key regulators of tissue destruction in tuberculosis (TB) and may be a target for host-directed therapy. Here, we conducted a Phase 2 randomized, double-blind, placebo-controlled trial investigating doxycycline, a licensed broad spectrum MMP inhibitor, in pulmonary TB patients.

METHODS. Thirty pulmonary TB patients were enrolled within 7 days of initiating anti-TB treatment and randomly assigned to receive either doxycycline 100 mg or placebo twice a day for 14 days in addition to standard care.

RESULTS. There were significant changes in the host transcriptome, and suppression of systemic and respiratory markers of tissue destruction with the doxycycline intervention. Whole blood RNA-sequencing demonstrated that doxycycline accelerated restoration of dysregulated gene expression patterns in TB towards normality, with more rapid down-regulation of type I and II interferon and innate immune response genes and concurrent up-regulation of B-cell modules relative to placebo. The effects persisted for 6 weeks after doxycycline was discontinued, concurrent with suppression of plasma MMP-1. In respiratory samples, doxycycline reduced MMP-1, -8, -9, -12 and -13 concentrations, suppressed type I collagen and elastin destruction, and reduced pulmonary cavity volume despite unchanged sputum *Mycobacterium tuberculosis* loads between the study arms. Two weeks of adjunctive doxycycline with standard anti-TB treatment was well-tolerated, with no serious adverse events related to doxycycline.

CONCLUSION. These data demonstrate that adjunctive doxycycline with standard anti-TB treatment suppresses pathological MMPs in pulmonary tuberculosis patients, and suggest that larger studies on adjunctive doxycycline to limit immunopathology in TB are merited.

GRAPHICAL ABSTRACT.



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Introduction

Globally, an estimated 10 million people develop tuberculosis (TB) each year and TB remains the leading cause of death from a single infectious agent (1). Standard short-course anti-TB treatment still requires antimicrobial drugs of at least 6 months, and drug-resistant TB is an increasing public health threat. Even after microbiological cure of TB, patients often suffer from significant sequelae, such as residual or secondary lung diseases (2). A recent meta-analysis revealed that TB survivors have approximately 3 to 4 times greater mortality than their local population (3). Consequently, there is interest in adjunctive host-directed therapies that may modulate host immune responses to *Mycobacterium tuberculosis* (*Mtb*) to improve the efficacy of anti-TB drugs, shorten treatment duration, and limit associated tissue damage (4-12). These aims are unlikely to be met by antimicrobial treatment alone.

Mtb causes apical pulmonary disease in the immunocompetent host and drives destructive pathology resulting in pulmonary cavity formation (13-15). Cavities are sites of high mycobacterial burden, and are poorly penetrated by anti-TB drugs, leading to the persistence of drug-tolerant bacilli and contribute to the transmission of infectious bacilli (16-18). After completion of anti-TB treatment, the sequelae of tissue damage include permanent respiratory dysfunction in the form of pulmonary fibrosis or post-TB bronchiectasis, which can lead to decreased effort tolerance, infectious exacerbations resulting in repeated hospitalizations and reduced quality of life (6, 19-21). Furthermore, several population-based studies have demonstrated that a history of treated TB increases the risk of obstructive airway disease, independent of smoking and other clinical factors (22-24).

Pathological destruction of the highly stable network of collagen fibrils in the lung parenchyma is mainly mediated by proteases, in particular, MMPs (10, 15, 17, 18, 25, 26). MMPs

are a family of zinc-dependent proteases that may collectively degrade all fibrillar components of the extracellular matrix at neutral pH and are involved in diverse physiological processes, including tissue modeling, organ development and regulation of immune responses (27, 28). MMP concentrations [MMP-1, -2, -3, -7, -8, -9, and -10] and their matrix degradation products, procollagen III N-terminal propeptide (PIIINP) and desmosine, are consistently found to be elevated in respiratory samples from pulmonary TB patients compared to patients with other respiratory diseases and healthy volunteers (29-32). Increased MMP concentrations associate with markers of pulmonary TB disease severity, such as sputum smear status, radiographic disease extent and presence of cavitation (29, 33). In addition, *Mtb* infection and/or stimulation by conditioned media from *Mtb*-infected monocytes induced secretion of MMPs with proteolytic activity in cellular models of human bronchial epithelial cells, monocyte-derived macrophages and neutrophils (14, 34-38), as well as in animal models (26, 39). These observations implicate MMPs as dominant effectors of lung matrix destruction in pulmonary TB and consequently MMPs are attractive host targets for adjunctive host-directed therapies.

Currently, the only licensed MMP inhibitor is doxycycline, a tetracycline antibiotic with broad spectrum MMP inhibitory activity (40). Doxycycline suppresses *Mtb*-induced MMP secretion in cellular models (29) and limits collagen destruction by *Mtb*-induced MMPs (14, 31). Doxycycline treatment inhibits MMP activity in periodontal disease at 20 mg twice daily (41) and improves lung function in inflammatory lung disorders, such as chronic obstructive pulmonary disease (COPD) (42) and asthma (43). In a guinea pig model of TB, doxycycline monotherapy reduced the lung mycobacterial burden in a dose-dependent manner (29), and in the mouse model of TB, MMP inhibition improved drug efficacy (39). However, adjunctive doxycycline treatment in TB patients has not been evaluated.

Here, we performed a first-in-human pilot Phase 2 randomized, double-blind, placebo-controlled trial to investigate the effects of doxycycline on the host transcriptome, mycobacterial burden and biological markers of tissue destruction, including pulmonary cavitation, as well as concentrations of MMPs, tissue inhibitor of metalloproteinases (TIMPs) and matrix degradation products in patients with drug-susceptible pulmonary TB. Concurrently, we assessed the safety of adjunctive doxycycline treatment with standard anti-TB therapy.

Results

Study profile and safety analyses.

A total of 143 pulmonary TB patients who were HIV negative were pre-screened, of which 33 were assessed for eligibility. 30 pulmonary TB patients were enrolled within 7 days of initiating anti-TB treatment and randomly assigned to either doxycycline or placebo (Figure 1). The baseline clinical, laboratory and radiologic characteristics at enrolment were similar between the study arms (Table 1). Six (20%) of the 30 TB patients were female and nine (30%) had diabetes mellitus with a mean HbA1c of 11.3%. The median chest X-ray (CXR) score was 2.8 (IQR 1.9 – 4.6) and 19 (63%) patients had pulmonary cavities. Two patients had isoniazid mono-resistant *Mtb* while the remaining 28 had fully drug-sensitive *Mtb*.

In the doxycycline arm, two patients withdrew due to adverse events (both Grade 1 nausea and vomiting), while in the placebo arm, one patient developed neutropenia and was withdrawn by the managing physician, and one by the investigator's decision as the patient was found to have cognitive impairment after randomization. Thus, there were 13 patients in each arm, who completed the allocated intervention (took at least 24 out of the 28 doses of the study drug), and were followed up for 2 months (Figure 1). Subsequently, one patient in the placebo arm declined phlebotomy at Day 14, and another patient's neutrophil and monocyte samples in the doxycycline arm was not processed due to electrical failure at the laboratory, which led to analysis of 12 blood samples in each arm.

Among all the 30 enrolled patients, there was no difference between the study arms due to any adverse events (relative risk [RR] 1.1, 95% CI 0.69 – 1.76, Supplemental Table 1), or presumed toxic effects (any grade 3 or 4 events, or serious adverse events). Patients in the doxycycline arm tended to experience more nausea and vomiting (RR 3.0, 95% CI 0.72 – 12.56)

and rash (RR 3.0, 95% CI 0.35 – 25.69), but there was no statistical significance between the arms (*p*-value = 0.21 and *p*-value = 0.60 by Fisher's exact test, respectively, Supplemental Table 1). One patient in the doxycycline arm developed Grade 4 neutropenia attributed to anti-TB treatment which persisted after completion of the intervention. Two patients in the placebo arm developed serious adverse events: one had prolonged hospitalization due to paradoxical reaction and another developed dyspnea requiring a visit to the Emergency Department that resolved.

Ten healthy volunteers (5 males and 5 females) were also enrolled and received doxycycline 100 mg twice a day for 14 days. All 10 volunteers completed at least 24 out of the 28 doses of doxycycline and were followed for 2 months. No subject discontinued participation in the study due to a doxycycline-related adverse event and no serious adverse events were observed (Supplemental Table 2). Two out of ten volunteers developed Grade 1 nausea and vomiting which resolved on completion of the drug course (Supplemental Table 2).

Clinical, radiological and microbiological outcomes were similar between study arms.

There were no difference in median BMI, and chest X-ray (CXR) score between doxycycline and placebo-treated patients at any time point (Table 1). Although doxycycline is bacteriostatic to *Mtb in vitro* (29, 44, 45), sputum *Mtb* burden was unchanged between the study arms. Two patients in the placebo arm and one in the doxycycline arm had positive sputum *Mtb* cultures at 2 months into anti-TB treatment (Table 1, *p*-value = 1.00). Pulmonary cavities resolved in 4 out of seven patients (57%) in the doxycycline arm compared to 2 out of nine (22%) in the placebo arm by the end of 2 months, but this finding was not statistically significant.

Gene expression of MMPs and immuno-regulatory genes are dysregulated in patients with TB.

To analyze the effects of *Mtb* infection and doxycycline in an unbiased manner, we first studied baseline differences between blood transcriptomes of TB patients and healthy volunteers. After normalization and filtering, principal component analysis (PCA) showed differentiation between healthy volunteers and TB patients, although with significant inter-individual variation, as expected from human disease with diverse severity (Supplemental Figure 1A). Differential gene expression (DEG) analysis using generalized linear models (EdgeR) (46) identified 1657 DEGs, including 853 genes up-regulated in TB patients. As evaluated by gene ontology analyses, these genes mostly encoded for immune response and detection of bacterial molecules (Supplemental Figure 1B), while pathway enrichment identified immune, infectious and inflammatory pathways (Supplemental Figure 1C). To visualize the extent of changes induced by *Mtb* infection in the blood cell transcriptome, significantly regulated genes were plotted onto the diagrams of individual KEGG pathways (47-49), including the TB pathway (Supplemental Figure 2) and the TNF signalling pathway (Supplemental Figure 3), which demonstrated extensive changes in gene expression in each pathway.

Co-expression module analysis using CEMITool (50) identified 3 modules (module 1, 2 and 4) to be differentially enriched in TB patients compared with healthy volunteers (Supplemental Figure 4A). Modules 2 and 4 were specifically overexpressed in TB, with normalized enrichment scores of 1.96 and 1.45, respectively. Module 2 was predominantly enriched for immune activation, neutrophils, extracellular matrix and dendritic cells, all of which have been described to be important pathways in TB (Supplemental Figure 4B). Module 4 was dominated by B cell signatures, which are being recognized as of increasing importance in the host immune response to TB (Supplemental Figure 4C). Of note, expression of matrix metalloproteinases was increased

in module 2, including *MMP8* and *MMP9*, as well as *PLAUR*, encoding a receptor for urokinase plasminogen activator involved in extracellular matrix degradation (Supplemental Figure 4D). In module 4, genes were involved in immuno-regulation, including *IFIT3*, *MX2* and *TNFAIP6* (Supplemental Figure 4E). Quantitative PCR (qPCR) validated up-regulation of these genes in TB (Supplemental Figures 4F and G).

Doxycycline modulates diverse host immune pathways, with effects persisting 6 weeks after doxycycline discontinuation.

Next, we investigated the effect of doxycycline therapy over time on the blood transcriptome. There was significant donor-to-donor variability, consistent with studying human disease. In PCA analysis, the inter-individual differences were reduced between Day 0 and Day 14 as analyzed in the doxycycline arm (Figure 2A), but in the placebo arm, greater dispersal between patients occurred between Day 0 and Day 14 (Figure 2B).

To investigate this phenomenon further, we performed paired analysis in each study arm to identify genes that were differentially expressed over time. Doxycycline led to a more rapid normalization of immune response genes towards expression levels in healthy volunteers, such as *SLC26A8*, *IGSF6*, *PYGL*, *GBP6* and *CLEC12B* (Supplemental Figures 5A-E). Similarly, in a parallel unbiased co-expression network analysis using GraphiaPro (51), 27 co-expressed clusters were identified (Figure 2C; Supplemental Table 3). These clusters comprised genes highly expressed in TB that were down-regulated during the course of treatment with doxycycline but were up-regulated or unaltered in the placebo arm (Figures 2D-I). Of note, one cluster encoded type I interferon signalling pathway (adjusted *p*-value = 5.20×10^{-19}), while another cluster encoded cellular response to interferon-gamma (adjusted *p*-value = 5.05×10^{-14}) and innate immune

responses (adjusted *p*-value = 2.17×10^{-12}). In addition, the latter cluster comprised *IRF1*, *APOL1*, *FCGR1A*, *FCGR1B*, *GBP5*, and *GBP6*, genes all related to the innate immune response, indicating doxycycline selectively modulates innate immunity (Figures 2D-I). Furthermore, although doxycycline was only administered for the first 14 days of treatment, the effects on gene expression were still observed at Day 56. Similar observations were found in the qPCR validation of these genes (Supplemental Figures 5F-K).

To further assess the effect of doxycycline on specific signalling pathways in comparison with placebo, we performed Ensemble of Gene Set Enrichment Analyses (EGSEA) (52), combining results from eleven algorithms to calculate collective gene set scores for biological relevance of the highest ranked gene sets. Mapping of the divergently regulated genes onto the KEGG TB pathway (EGSEA adjusted *p*-value = 0.0047 for doxycycline treatment) visualized that doxycycline modulated numerous different stages of the pathway, with expression changes often in the opposite direction to placebo (Figure 3). A parallel analysis of biological processes and pathway enrichment in genes differentially regulated between Day 14 and Day 0 in doxycycline and placebo arms across all available Gene Ontology and Hallmark signatures demonstrated multiple pathways divergently regulated by doxycycline (Supplemental Figures 6A and B).

To further dissect which pathways were significantly changed in the doxycycline arm but not placebo, a comparison within linear model was used for identification of doxycycline-specific DEGs (EdgeR, contrasts: (Doxy_Day14-Doxy_Day0)-(Placebo_Day14-Placebo_Day0)). Gene Set Enrichment Analysis using Camera (53) with blood transcriptional modules (54) showed that several pathways were more significantly regulated in the doxycycline arm than the placebo arm (Figure 4A; Supplemental Figure 6C), including interferon responses (Figure 4B), T cell responses and innate responses. In addition, B cell responses increased with doxycycline treatment (Figure

4A). Finally, we studied gene expression of extracellular matrix related genes and showed a trend towards reduction with doxycycline (Figure 4C). Within this pathway, doxycycline led to a significant reduction of *MMP9* gene expression compared to placebo (Figure 4D; adjusted *p*-values = 1.00 and 0.003 for placebo and doxycycline arms, respectively).

Systemic MMPs are inhibited by doxycycline

Since the unbiased blood transcriptomic analysis identified up-regulated extracellular matrix-related genes in TB, and doxycycline suppressed *MMP9* expression, we next investigated the effect of adjunctive doxycycline on plasma MMPs. Plasma MMP-1 was significantly suppressed at Day 56 by doxycycline (Figure 5A, adjusted *p*-value < 0.05). A similar trend was observed for plasma MMP-8 (Figure 5B, adjusted *p*-value = 0.06 at Day 56). These data show that systemic MMP-1 continues to be suppressed at a protein level at a late time point, even after doxycycline treatment was stopped after 14 days, consistent with the observations in the blood transcriptomic analysis (Figures 2D-I). Fold change of the tissue inhibitor of MMPs, TIMP-1 and -2, in the plasma were not different between both arms (Figures 5C and D). Other plasma MMPs were unchanged between arms (Supplemental Figures 7A-F).

Next, we analyzed the fold change of circulating matrix degradation products of Type III collagen, PIIINP (32) and desmosine from elastin fibers (55). Both were unchanged in TB patients' plasma (Supplemental Figures 7G and H). In addition, *Mtb*-induced MMP secretion from *ex vivo* culture of neutrophils (Supplemental Figures 8A and B) and monocytes (Supplemental Figures 9A-G) were not different between doxycycline and placebo-treated TB patients.

Doxycycline suppresses MMPs in respiratory secretions and decreases extracellular matrix degradation with a concurrent decrease in pulmonary cavity volume

Since MMP activity is tightly regulated and may be compartmentalized (56), we next investigated MMPs in respiratory samples to determine the effect of doxycycline in the lung. Sputum MMP-1, -8, -9, -12 and -13 fold changes were significantly decreased by doxycycline relative to placebo (Figures 6A-C; Supplemental Figures 10A and B), while other MMPs were unchanged (Supplemental Figures 10C-G). From Day 0 to Day 14, collagenases MMP-1, -8 and gelatinase MMP-9 were decreased in the doxycycline arm (mean fold change of Day 14 vs. Day 0 ± s.e.m.; MMP-1: 0.45 ± 0.16, MMP-8: 0.38 ± 0.10, MMP-9: 0.45 ± 0.10), while in the placebo arm, they remained unchanged or increased (mean fold change of Day 14 vs. Day 0 ± s.e.m.; MMP-1: 1.46 ± 0.50, MMP-8: 1.62 ± 0.62, MMP-9: 2.19 ± 0.90). Inhibitors TIMP-1 and -2 were not significantly different between the two arms (Figures 6D and E), indicating an overall suppression of sputum MMP activity by doxycycline.

Next, we examined the effects of doxycycline on sputum enzymatic activity degrading Type I collagen and elastin, which are key lung extracellular matrix proteins (57, 58). Type I collagen is the substrate of MMP-1 and -8, while elastin is a substrate of MMP-9 (59). In doxycycline-treated TB patients, sputum Type I collagenase and elastase activities were significantly decreased compared to placebo-treated patients (Figures 6F and G), consistent with the observed decrease in sputum MMP-1, -8 and -9 concentrations (Figures 6A-C). Furthermore, a decrease in pulmonary cavity volume was observed in the doxycycline arm at Day 56, while no difference was observed in the placebo arm (Figure 6H; adjusted *p*-value = 0.045). Total sputum PIIINP fold change was unchanged between the two arms (Supplemental Figure 10H), while

sputum desmosine showed a non-significant trend towards suppression in the doxycycline arm (Supplemental Figure 10I; adjusted *p*-value = 0.06).

Discussion

Our phase II randomized controlled trial is an exploratory study to investigate the effects of adjunctive doxycycline to standard anti-TB treatment in 30 pulmonary TB patients. Doxycycline significantly regulated the host transcriptome and biological markers of tissue destruction. We demonstrate for the first time in TB patients that adjunctive treatment with doxycycline regulated expression of immune and inflammatory response genes, and these effects persisted for six weeks after completion of two weeks doxycycline intervention. Pathway analyses revealed that doxycycline down-regulated type I/II interferon and innate immune response genes and up-regulated genes involved in B-cell biology. Gene expression of the extracellular matrix-related gene set showed a trend towards reduction in the doxycycline arm, with significant MMP-9 gene suppression. At the protein level, doxycycline suppressed systemic MMP-1, while in the respiratory compartment, collagenases MMP-1 and -8 and gelatinase MMP-9 were reduced. Doxycycline reduced total functional Type 1 collagenase and elastase activities in TB patients' sputum with a concurrent significant decrease in pulmonary cavity volume. There was no change in BMI which is reassuring as a low BMI is associated with mortality (60). Our findings in TB patients extend previous *in vitro* observations that doxycycline inhibits MMP activity in cellular TB models (14, 29) and suggest that MMP inhibition may be associated with decreased tissue damage in TB. Finally, we show that adjunctive doxycycline with standard anti-TB treatment was safe.

Whole blood RNA-sequencing dissected the molecular pathways affected by adjunctive doxycycline in pulmonary TB patients. Using co-expression network analysis, we found immune response genes, such as *IRF1*, *APOL1*, *FCGR1A*, *FCGR1B*, *GBP5* and *GBP6* to be specifically down-regulated by doxycycline treatment towards the expression levels in healthy volunteers. Of

note, these genes are often elevated in TB patients and have been repeatedly identified in blood gene signatures proposed to diagnose TB with high sensitivity and specificity (61-64).

Co-expression network analysis revealed that doxycycline led to greater down-regulation of type I/II interferon and innate immune response genes. A parallel blood transcriptional module analysis of the linear model showed type I interferon and innate response genes were more down-regulated, while conversely genes involved in B-cell biology were more up-regulated after doxycycline treatment. In several other blood transcriptional studies, interferon-inducible genes (both type I and II interferon signaling) and innate immune-related genes are highly expressed in TB patients (65-67) and their expression diminishes to that of healthy individuals after successful standard anti-TB treatment (67-70). In addition, B-cell markers increase in expression only at the later phases of treatment during disease resolution (69). Thus, the greater change of these pathways after 14 days of additional doxycycline treatment suggests that doxycycline hasten the normalization of gene expression relative to standard anti-TB treatment.

The doxycycline course lasted for two weeks, but the change in expression levels of TB-associated immune response genes persisted for six weeks after doxycycline discontinuation. Similar long-term suppressive effect was also observed on plasma MMP-1, which is often elevated in active TB patients (31). These findings suggest that doxycycline host-directed therapy sustains modulations to the host immune responses.

Adjunctive doxycycline specifically suppresses MMPs and their functional activity in pulmonary TB patients. In particular, MMP-1, -8, -9, -12, and -13, and cleavage of Type I collagen and elastin in the respiratory compartment were down-regulated by doxycycline, while the tissue inhibitor of MMPs, TIMP-1 and -2, remained unchanged. This MMP suppression is likely due to the direct effect of doxycycline (71), since sputum *Mtb* loads were unchanged. Multiple studies

have demonstrated collagenases MMP-1 and -8 and gelatinase MMP-9 to be elevated in respiratory samples of TB patients, and are closely associated with parameters of immunopathology, such as cavitation and chest radiograph infiltration (14, 29, 30, 33). Although humans express more than 20 MMPs (72), the luminex bead array can only analyze for 10 MMPs, including EMMPRIN, and so our analysis does not cover all MMPs. Overall, our findings of MMP suppression by doxycycline suggest it may be useful in limiting TB immunopathology, and may also reduce the risk of pulmonary impairment after TB (20), as has been proposed for other inflammatory diseases (73, 74). Although PIIINP was listed as primary outcome measure at trial conception, no difference was observed between groups. These may be due to the interval before analysis, as PIIINP has been reported to be unstable in serum or EDTA plasma after 3 months of storage (75).

In serial 18-F fluorodeoxyglucose positron emission tomography-computed tomography (18-F FDG PET-CT) scans of pulmonary TB patients, high cavity volume is strongly associated with poor treatment outcome (76). In murine studies, treatment with another broad spectrum MMP inhibitor marimastat, increases the potency of frontline TB drugs isoniazid and rifampicin, and enhances the delivery and/or retention of TB drugs in the infected tissue due to improved vascular integrity (39). Conversely, monotherapy of cipemastat, a selective inhibitor of MMP-1, -7, -8 and -13, paradoxically increased cavitation, immunopathology, and mortality in a murine model of cavitary TB (77). Similarly, in the rabbit model of cavitary TB, cipemastat monotherapy also did not prevent cavitation (78). In the current study, adjunctive doxycycline decreased the average volume of pulmonary cavities from 23,694 mm^3 at Day 0 to 1,136 mm^3 at Day 56, suggesting an inhibitory effect on cavitation by MMPs suppression when combined with standard anti-TB treatment. Further investigation of MMP inhibition by doxycycline on TB immunopathology and chronic lung tissue damage are merited.

Doxycycline at 100 mg twice daily is the maximum clinical dose and approved by the U.S. Food and Drug Administration (79), and so was selected in our placebo-controlled trial. The dose required for MMP inhibition in periodontal disease is 20 mg twice daily (80). Murine studies have shown that doxycycline has high organ penetration and reached higher concentrations in both cellular and necrotic TB lesions compared to plasma (81). Combined treatment with rifampin can reduce the levels of doxycycline by approximately 67% in plasma, while clearance of doxycycline was increased approximately 2-fold in patients receiving concurrent rifampin (82). However, the interaction of doxycycline with standard anti-TB treatment was not evaluated in this study, and the pharmacokinetics of doxycycline should be considered in future trials. Regardless, adjunctive doxycycline with standard anti-TB treatment in pulmonary TB patients is safe from our initial analyses and the safety profile is consistent with recent clinical trials examining doxycycline therapy in other diseases (83, 84).

Our study has limitations, including the sample size of 30 pulmonary TB patients and the short doxycycline treatment regimen of 2 weeks. Sputum *Mtb* colony forming units (cfu) and chest X-ray (CXR) score remained the same in both arms, although a higher proportion of patients in the doxycycline arm had resolved pulmonary cavities, this did not reach statistical significance, whereas analysis of cavity volume did differ. PET-CT would be more sensitive in detecting changes in cavity size and lung inflammation than CXR analysis used in this study. However, due to concerns of substantial radiation exposure by repeated scans and its high operating costs, PET-CT was not used in our study. A larger sample size to sufficiently power future Phase 3 clinical trials evaluating the effect of doxycycline on TB immunopathology is needed, which could incorporate weekly collection of sputum for TB cultures to document time to culture conversion.

Lung function tests, pulmonary cavitation, time to conversion of sputum culture, pharmacokinetics studies, and adverse drug effects should be considered as outcome measures.

The duration of intervention was based on a prospective study showing the rapid decrease of collagenases MMP-1, -3, and -8 levels after 2 weeks of anti-TB treatment (33). Given the current data that doxycycline is safe and suppressed biological markers of tissue destruction, a longer treatment duration in a larger cohort could be considered, similar to other clinical trials, which administered doxycycline for at least 4 weeks (41-43). Doxycycline, the only U.S. FDA licensed drug for MMP inhibition, is routinely used as a broad-spectrum antibiotic, and may have off-target effects other than MMPs. Indeed, doxycycline was shown to have comprehensive immunomodulation effects on cytokines and chemokines levels and production (85, 86), and may also influence the composition of the gastrointestinal microbiota (87).

In summary, adjunctive doxycycline with standard anti-TB treatment is associated with accelerated normalization of the increased type I/II interferon response and innate immune response genes in TB, and up-regulation of suppressed B-cell markers. Doxycycline specifically suppresses systemic MMP-1 and respiratory MMP-1, -8 and -9, inhibits matrix destruction in respiratory samples of pulmonary TB patients and reduces pulmonary cavity volume. Adjunctive doxycycline is well-tolerated in TB patients on standard anti-TB treatment. Overall, this study is a stepping stone for larger studies to further evaluate and validate the benefits of adjunctive doxycycline treatment for TB patients. Doxycycline may prove to be a cheap and widely available host-directed therapy targeting TB-associated tissue destruction and the associated dysregulated inflammatory immune response.

Methods

Study design and participants

This was a randomized, double-blind, placebo-controlled trial of doxycycline as an adjunct to standard anti-TB treatment in pulmonary TB patients at the National University Hospital (NUH) and the Tuberculosis Control Unit (TBCU), Singapore. Patients were eligible if they were aged 21–70 years old, have confirmed pulmonary TB with positive acid-fast bacilli smear and/or positive GeneXpert and/or culture results, have a chest radiograph demonstrating pulmonary involvement, and were within 7 days of initiating anti-TB treatment. Patients with HIV co-infection, previous pulmonary TB, and severe pre-existing lung diseases were excluded. Other exclusion criteria included pregnancy or breast feeding; allergies to tetracyclines; on retinoic acid, neuromuscular blocking agents and pimozide treatment; autoimmune disease and/or on systemic immunosuppressants; hemoglobin lower than 8 g/dl; creatinine 2 times upper limit of normal (ULN); ALT above 3 times ULN; severe depression, schizophrenia or mania. Healthy volunteers aged 21–70 years and in general good health were enrolled at Investigational Medicine Unit, NUH. The complete list of eligibility criteria can be found in the study protocol (available at https://scholarbank.nus.edu.sg/handle/10635/171211). In total, 30 pulmonary TB patients and 10 healthy volunteers were recruited from 2015 to 2017. Due to unintentional administrative oversight, the trial was registered in ClinicalTrials.gov (NCT02774993) after the full ethical approval, Singapore Health Science Authority clinical trial certificate (CTC1400221) and study start date. Monitoring of the clinical trial was performed by the Singapore Clinical Research Institute (SCRI).

Study procedures

TB patients were randomly assigned (1:1) using www.randomisation.com to receive daily self-administered doxycycline 100 mg bd or matching placebo (Beacons Pharmaceuticals) for 14 days (Figure 1). Participants, clinicians, and the study team were masked to treatment allocations during the conduct of the trial. TB patients were prescribed standard anti-TB treatment, in conjunction with the study drug. Healthy volunteers were given 14 days of doxycycline and were not randomized.

Participants were followed up at Day 14 and 56 ± 2 days for safety monitoring and sample collection. Criteria for cessation of study intervention include serious adverse events related to the study drug, participant withdrawal of consent, and other reasons specified in the study protocol. A pill count was performed at the end of study intervention to determine adherence. Participants were excluded from downstream assessment if they missed 4 doses of the study drug. Induced sputum was collected from TB patients at Day 0 and 14, while chest radiograph and whole blood were taken at Day 0, 14, and 56. For healthy volunteers, blood collection was performed at Day 0, 14, and 56. All samples were sent for immediate processing in the BSL-3 laboratory at National University of Singapore (NUS).

Samples collection and processing

Sputum induction was performed in designated collection rooms, where TB patients were nebulized with 5% saline in 5-minute cycles, up to 20 minutes as tolerated. Sputum samples were liquefied as previously described (29) and sterile filtered through 0.22 μm Durapore PVDF membrane (Merck Millipore) to remove *Mtb* (88).

Whole blood was collected separately into Tempus™ Blood RNA tubes (Applied Biosystems) for RNA-sequencing, and 50 mL tube containing EDTA (1st BASE) for plasma processing, as well as neutrophils and monocytes isolation. Plasma obtained after blood centrifugation was sterile filtered through 0.22 μm Durapore PVDF membrane. Neutrophils and monocytes were respectively isolated from blood samples using CD15- and CD14-antibody conjugated pluriBeads (pluriSelect), following manufacturer's instructions. Cell viability was > 99% by trypan blue assay, and neutrophil and monocyte purities were > 95% and > 80% by flow cytometry respectively.

Outcome measures

The primary outcome measure was change of PIIINP in TB patients. Other outcome measures include change of MMPs and TIMPs in plasma and sputum samples, change of collagenase and elastase activities in sputum samples, change of *Mtb*-induced MMP secretion from isolated neutrophils and monocytes, change of whole blood transcriptome, change of *Mtb* cfu in sputum samples

Chest radiograph analysis and *Mtb* cfu measurement

Chest radiographs of TB patients were scored for extent of pulmonary consolidation as described previously (29) by 2 specialists blinded to the intervention (CWMO and PTE) and the absence or presence of cavitation by 2 out of 3 specialists (CWMO, PTE and FSWT). Chest-X-ray infiltration (CXR) was scored on a scale of 0 – 10 depending on the number of segments involved, on a modified scoring system developed by Lawson *et al* (29, 89). Pulmonary cavity volume was

estimated by measuring its radius in ImageJ (90), and calculated as $V = \frac{4}{3}\pi r^3$. Liquefied sputum was serially diluted and plated for cfu enumeration on Middlebrook 7H11 agar (BD Biosciences) supplemented with OADC growth enrichment, polymyxin B, carbenicillin, amphotericin B and trimethoprim (Sigma-Aldrich).

Whole blood RNA-sequencing

Blood samples collected in Tempus™ Blood RNA tubes were processed following the manufacturer's protocol to obtain the RNA pellet in Step 8 (Thermo Fisher Scientific). The pellet was re-suspended in 1 mL TRIzol (Thermo Fisher Scientific), and total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction followed by Qiagen RNeasy Micro clean-up procedure. All RNAs were analyzed on Agilent Bioanalyzer for quality assessment. Only 15 TB patients (8 placebo and 7 doxycycline) and 6 healthy volunteers had a complete set of samples (Day 0, 14 and 56) that qualified for RNA-sequencing, with RNA Integrity Number (RIN) ranging from 6.4 to 9.2 and median RIN of 8. cDNA libraries were prepared as described previously (91) using SMARTSeq v2 protocol (54). The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip GX system. All samples were subjected to an indexed paired-end sequencing run of 2x151 cycles on an Illumina HiSeq 4000 (69 samples in 4 lanes).

Paired-end sequence reads were quantified to transcript abundance using Kallisto (92) with bias correction, and 50 bootstrap samples. Reads were mapped to ENSEMBL release 95. On average, the percentage of aligned reads is 89.3%. The transcript abundance was then summarized to gene level using Sleuth (93).

Differential Gene Expression (DEG) analysis

Raw counts from RNA-sequencing were processed in Bioconductor package EdgeR (46), variance was estimated, and size factor normalized using Trimmed Mean of *M*-values (TMM). Genes with minimum 2 reads at minimum 50% samples were included in the downstream analyses. All fit models included a term to model individual variation. For the identification of DEGs over time (Day 14 vs. Day 0), a paired model was used, whereas for the comparison of treatment effect between placebo and doxycycline, a nested design was applied ((Doxy_Day14-Doxy_Day0)-(Placebo_Day14-Placebo_Day0)). Genes with a false discovery rate (FDR)-corrected *p*-value < 0.05 were identified as differentially expressed, resulting from a likelihood ratio test using a negative binomial generalized linear model fit.

Transcript-to-transcript co-expression analysis

Transcript-to-transcript co-expression analysis were done on filtered TMM normalised genes using GraphiaPro (51) (Pearson r = 0.8, Markov Clustering Algorithm MCL = 1.7) and CEMITool (50) (min_ngen = 30, diss_thresh = 0.8, r^2 = 0.8247, beta = 3).

Gene ontology and pathway enrichment analysis

Gene ontology and pathway enrichment analysis were done using Camera (53) or Ensemble of Gene Set Enrichment Analyses (EGSEA) (52). Additionally, blood transcriptional modules (BTMs) (54) were used as gene sets. BTM activity was calculated using the BTM package (Version 1.015) in Python using the normalized counts as input.

ex vivo culture and stimulation

Mtb H37Rv laboratory strain was first cultured in supplemented Middlebrook 7H9 medium (BD Biosciences) and used at mid-logarithmic growth (optical density of 0.60) for infection of the isolated neutrophils and monocytes as described (14). Neutrophils were infected at a multiplicity of infection (MOI) of 10 for 4 hours, while monocytes were infected at MOI of 1 for 24 hours. Culture supernatants were sterile filtered through 0.22 μm Durapore PVDF membrane to remove *Mtb*.

ELISAs for TIMP-1/2, PIIINP, and desmosine

TIMP-1 and -2 concentrations were measured using Duoset ELISA Development System (R&D Systems), with minimum detection limit of 31.2 pg/mL for both; PIIINP using human PIIINP ELISA kit (Cloud-Clone Corp.), with 25.9 pg/mL detection limit; desmosine using human desmosine ELISA kit (Cusabio), with 39.0 pg/mL detection limit. All ELISAs were performed according to the manufacturer's instructions. Plasma samples were diluted to 1:200 for TIMP-1 and -2, 1:100 for PIIINP, and 1:300 for desmosine. Sputum samples were diluted to 1:500 for TIMP-1, 1:5 for TIMP-2, 1:150 for PIIINP, and 1:100 for desmosine. Results from sputum samples were normalized by their total protein concentrations that were qualified by Bradford assay (Bio-Rad).

Luminex array for MMPs

EMMPRIN, MMP-1, -2, -3, -7, -8, -9, -10, -12, and -13 concentrations were analyzed by the Magnetic Luminex Performance Assay (R&D Systems) on the Bio-Plex analyzer (Bio-Rad),

according to the manufacturer's protocol. The minimum detection limit for the 10 analytes were 5.6, 1.1, 12.6, 2.9, 6.6, 7.8, 5.7, 3.2, 0.7 and 63.5 pg/mL, respectively. Culture supernatants from *ex vivo* neutrophils cultures were diluted to 1:50 for both MMP-8 and -9, while supernatants from monocytes cultures were assayed at neat for all analytes. Plasma and sputum samples were diluted to 1:5 for all analytes, except for plasma MMP-2 (1:50), and sputum MMP-8 and -9 (1:200 for both). Results from sputum samples were normalized by their total protein concentrations.

DQ collagen and elastin degradation assays

Type I collagen and elastin degradation by sputum samples were respectively assessed at 1:8 dilution using EnzChek® Gelatinase/Collagenase and EnzChek® Elastase assay kits (Molecular Probes), as described previously (14). Samples were activated with 2 mM of p-aminophenylmercuric acetate (APMA) for 1 hour at 37°C, before mixing with the DQ collagen (10 μg) or elastin (5 μg). Matrix degradation activity was measured after 24 hours with a fluorescence plate reader (BioTek Instruments). Results were normalized by the total protein concentration.

Statistics

Results of the trial were reported in accordance with the CONSORT guidelines. Data were analyzed using GraphPad Prism (version 7, GraphPad Software). Multiple intervention experiments were compared using two-way ANOVA with Sidak's multiple comparisons, while continuous variables between 2 sets of data were assessed using two-tailed Mann-Whitney *U* test.

Categorical data were analyzed using Fisher's exact test. Data are expressed as mean ± s.e.m. unless stated otherwise. Adjusted p-value < 0.05 was considered statistically significant.

Data and materials availability

All the notebooks used for the RNA-sequencing analysis are available at https://github.com/afvallejo/Doxycycline-as-host-directed-therapy-in-pulmonary-tuberculosis. The raw data was deposited at the European Nucleotide Archive (ENA) under the accession number PRJEB38126.

Study approval

The Domain Specific Review Board from National Healthcare Group Singapore approved this study (Reference: 2014/0222) and written informed consent or a thumb-print was obtained from all participants prior to inclusion in the study. Two people on a data safety and monitoring board provided oversight during the study.

Author contributions

C.W.M.O., J.S.F. and P.T.E. conceived the clinical trial. Y.D., F.S.W.T., A.D.Y.W., S.H.G., H.W.S., C.B.E.C., Y.T.W. and C.W.M.O. carried out the trial. J.S.F., N.I.P., P.A.T., P.T.E. and C.W.M.O. analyzed the clinical trial. J.L., A.T. and A.S. performed the RNA-sequencing. Q.H.M., Y.W., J.M.H., C.B. and H.R.L. performed the biological assays. Q.H.M., P.T.E., T.Z.T., A.F.V., M.E.P., A.S., J.S.F. and C.W.M.O. analyzed the RNA-sequencing data and

biological data. Q.H.M., P.T.E. and C.W.M.O. wrote the first draft of the paper which was reviewed and revised by all authors.

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Table 1. Baseline characteristics and outcomes of pulmonary TB patients in trial

Baseline characteristics	**Placebo (n = 15)**	**Doxycycline (n = 15)**	**Total (n = 30)**	***p*-value ***
Median age, years (IQR)	49 (42 – 59)	42 (26 – 58)	47.5 (34.8 – 58.3)	0.29
Male : Female^	13 : 2	11: 4	24: 6	0.65 #
Diabetes (%)	6 (40%)	3 (20%)	9 (30%)	0.43 #
Mean HbA1c, % ǂ	11.1	11.7	11.3	
Median weight, kg (IQR)	55.4 (41.2 – 73.4)	51.5 (46.7 – 63.2)	54.4 (46.6 – 67.6)	0.42
Median BMI, kg/m^2 (IQR)	21.0 (18.5 – 24.6)	20.7 (18.7 – 22.1)	20.9 (18.7 – 23.2)	0.53
Median WBC (IQR)	8.5 (6.3 – 10.4)	7.7 (5.9 – 9.0)	8.3 (6.1 – 9.5)	0.22
Median Hb, g/dL (IQR)	13.9 (12.6 – 14.5)	13.7 (11.8 – 14.7)	13.8 (12.3 – 14.6)	0.47
Median ALT (IQR)	20 (16 – 24)	19 (12 – 24)	19 (13– 24)	0.38
Median AST (IQR)	23 (21 – 28)	22 (16 – 25)	23 (19 – 26)	0.50
Median Creatinine (IQR)	66 (57 – 71)	78 (62 – 85)	69 (59 – 82)	0.11
Median AFB smear (IQR)	2 (0 – 3)	2 (1 – 3)	2 (1 – 3)	0.85
Isoniazid mono-resistant *Mtb* (%)	1 (7%)	1 (7%)	2 (7%)	1.00 #
Median *Mtb* CFU/ml (IQR)	8,500 (25 – 152,500)	6,125 (0 – 75,000)	8,500 (25 – 106,250)	0.46
Median CXR score (IQR)	3.5 (1.5 – 5.0)	2.5 (2.0 – 4.5)	2.8 (1.9 – 4.6)	0.91
Cavities present (%)	11 (73%)	8 (53%)	19 (63%)	0.45 #
Outcomes	**Placebo (n = 13)**	**Doxycycline (n = 13)**	**Total (n = 26)**	
Clinical outcomes				
Median BMI, Day 14, kg/m^2 (IQR)	22.2 (19.4 – 24.5)	20.6 (18.5 – 22.8)	20.8 (19.1 – 23.2)	0.31
Median BMI, Day 28, kg/m^2 (IQR)	22.0 (20.1 – 24.3)	21.1 (19.1 – 22.8)	21.3 (20.0 – 23.5)	0.34
Median BMI, Day 56, kg/m^2 (IQR)	22.3 (20.2 – 24.2)	21.5 (19.3 – 23.2)	21.9 (20.1 – 23.8)	0.36
Radiological outcomes				
Median CXR score, Day 14 (IQR)	2.5 (1.0 – 4.5)	2.5 (1.3 – 4.5)	2.5 (1.0 – 4.5)	0.87
Median CXR score, Day 56 (IQR)	2.0 (1.0 – 3.0)	1.5 (1.0 – 3.0)	2.0 (1.0 – 3.0)	0.73
Cavities resolved, Day 56 (%)	2 (22%) §	4 (57%) ¶	6 (38%)	0.30 #
Microbiological outcomes				
Median *Mtb* CFU/ml at Day 14 (IQR)	100 (0 – 1,063)	25 (0 – 125)	38 (0 – 513)	0.32
TB culture positive at 2nd month (%)	2 (15%)	1 (8%)	3 (12%)	1.00 #

*Clinical variables between placebo and doxycycline arms were compared by Mann-Whitney *U* test unless stated otherwise.

#Analysis was performed using Fisher's exact test.

^The classification was made by the investigators.

ǂMean HbA1c was calculated only among diabetes patients.

§2 patient with pulmonary cavities in the placebo arm withdrew; ¶1 patients with pulmonary cavities in the doxycycline arm withdrew.

AFB, acid-fast bacilli; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CFU, colony forming unit; CXR, chest X-ray; Hb, haemoglobin; HbA1c, glycated haemoglobin; IQR, interquartile range; *Mtb*, *Mycobacterium tuberculosis*; WBC, white blood cell count.

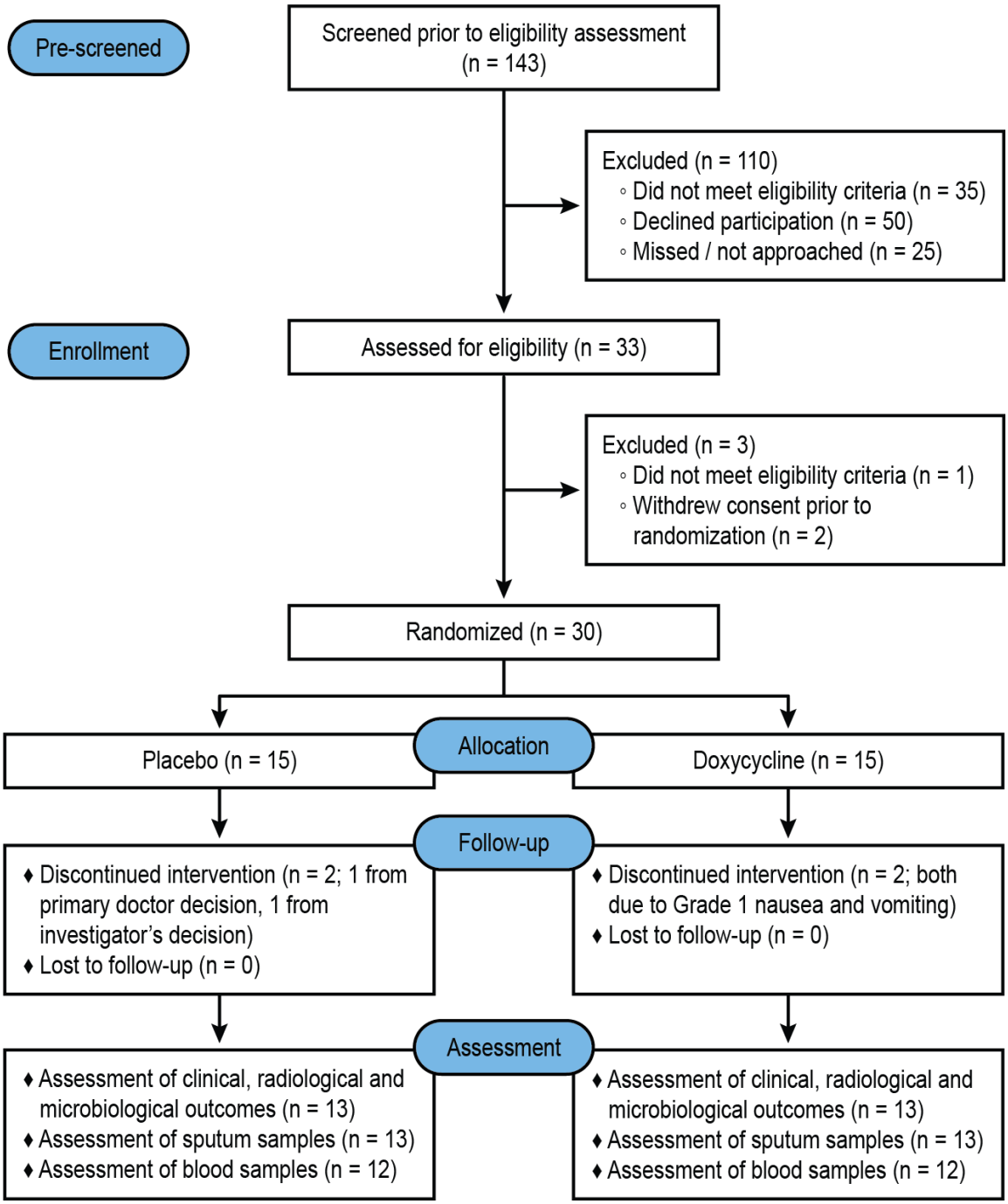

Figure 1. Trial profile (94). The safety analysis included all participants who underwent randomization and received an initial dose of placebo or doxycycline. The downstream assessment included all participants who underwent randomization, took at least 24 out of the 28 doses of placebo or doxycycline, and returned for follow-up at Day 14 and Day 56.

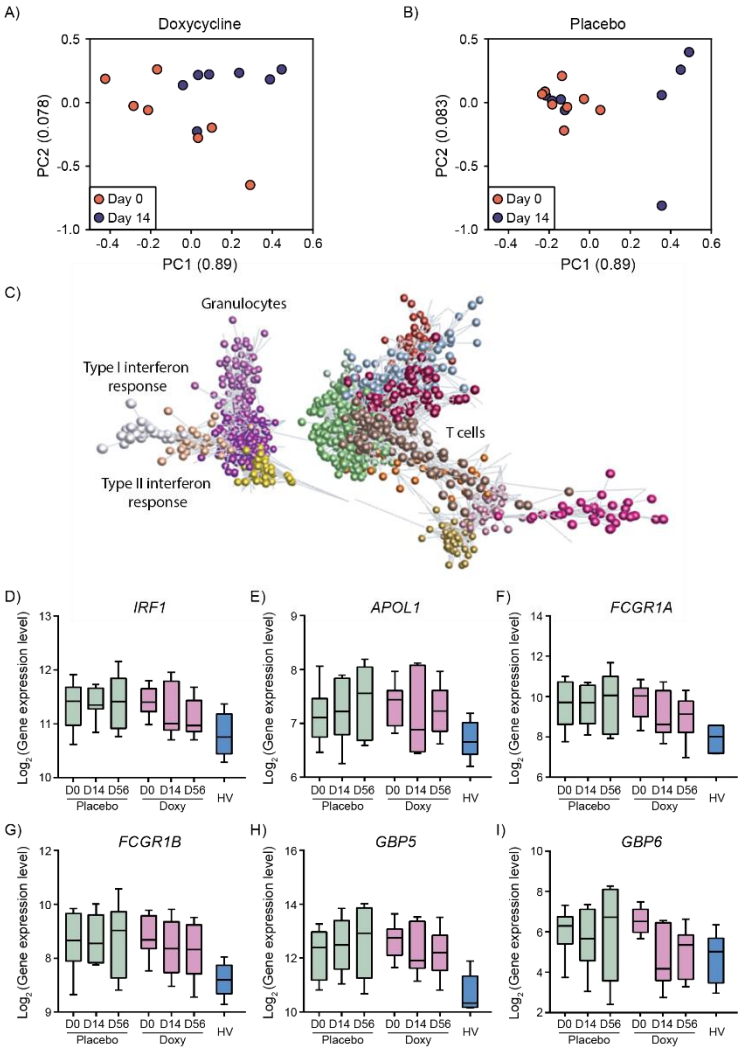

Figure 2. Doxycycline results in faster normalization of type II interferon and innate immune response genes relative to placebo. (A-B) PCA analysis of doxycycline **(A)** and placebo **(B)** treated patients at Day 0 (orange) and Day 14 (blue). First two components of PCA are shown and their variances are shown in parenthesis. Doxycycline reduces variation between individuals over the first two weeks of treatment. Only patients with Day 0 and 14 samples are plotted. n = 8 placebo, n = 7 doxycycline. **(C)** Transcript to transcript clustering of 12,977 genes filtered and normalized using Trimmed Mean of *M*-values (TMM) identified 27 co-expressed clusters (Pearson r = 0.8; Markov Clustering Algorithm, MCL = 1.7; n gene/cluster ≥ 10) over the course of doxycycline treatment. The 15 largest clusters (Supplemental Table 3) are shown in different colors. Lines represent the similarity between transcripts, circles represent individual genes. Two major groupings identify genes preferentially expressed in innate immune response (Granulocytes, Interferon response) and adaptive immune response (T cells). **(D-I)** Longitudinal analysis of selected genes, *IRF1* **(D)**, *APOL1* **(E)**, *FCGR1A* **(F)**, *FCGR1B* **(G)**, *GBP5* **(H),** and *GBP6* **(I)** from a cluster encoding for type II interferon and innate immune responses. TMM normalized gene expression at Day 0, 14, and 56 of TB patients in placebo (n = 8, green) and doxycycline (Doxy, n = 7, purple) arms, and baseline expression of healthy volunteers (HV, n = 6, blue) are plotted. Box represents 25th and 75th percentile, line is median, with whiskers denoting extremes.

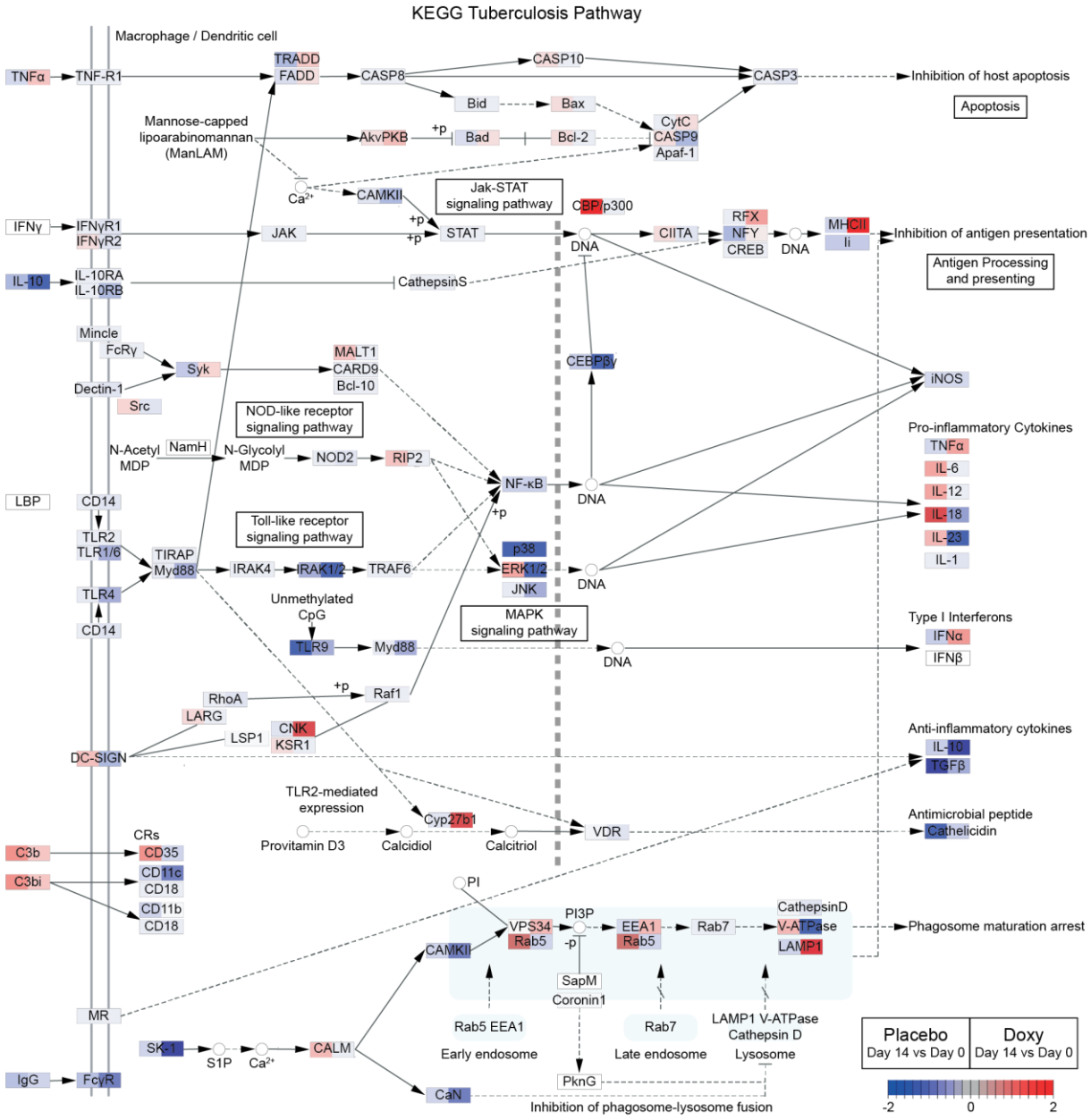

Figure 3. Doxycycline differentially regulates numerous genes in TB pathway relative to placebo. Comparison of gene expression changes (Day 14 vs. Day 0) between placebo and doxycycline (Doxy) arms for genes in the KEGG Tuberculosis pathway (map05152) (47-49). The pathway was identified by Ensemble of Gene Set Enrichment Analyses (EGSEA) (52) to be significantly regulated by doxycycline treatment (adjusted *p*-value = 0.0047). Gene expression changes (Day 14 vs. Day 0) in the placebo and doxycycline arms are respectively shown on the left and right side of each gene box. Red represents up-regulation and blue represents down-regulation of gene expression.

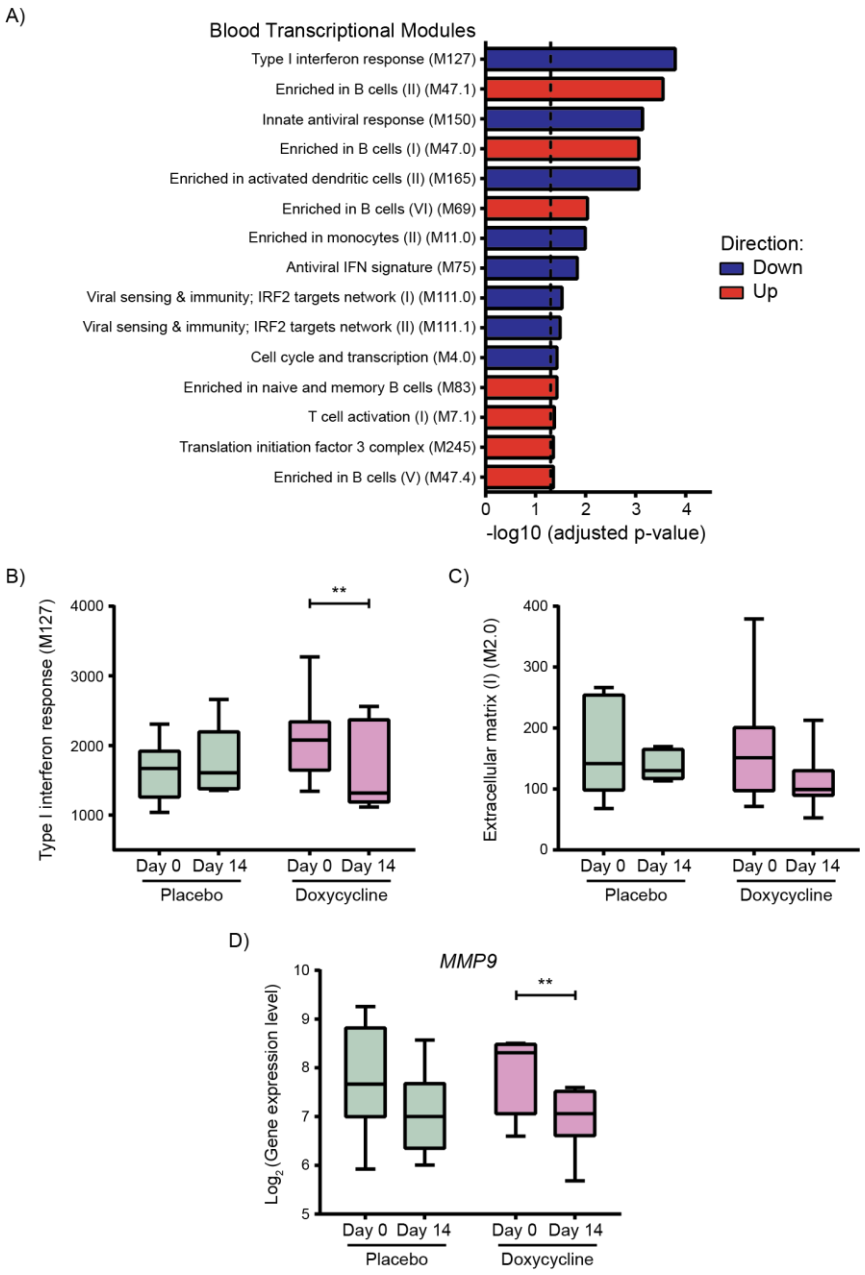

Figure 4. Doxycycline leads to greater down-regulation of type I interferon responses and extracellular matrix genes, and up-regulation of B cells markers relative to placebo. (A) Gene set enrichment analysis of differentially regulated genes specific to doxycycline treatment ((Doxy_Day14-Doxy_Day0)-(Placebo_Day14-Placebo_Day0)). Blood transcriptional modules (54) were used as gene sets. The top 15 enriched gene sets are shown. Dotted line marks adjusted *p*-value = 0.05. Red represents up-regulation and blue represents down-regulation of gene sets. **(B-D)** Longitudinal analysis of type I interferon response gene set (M127) **(B)**, extracellular matrix gene set (M2.0) **(C)**, and *MMP9* **(D)** at Day 0 and 14 of placebo (n = 8, green) and doxycycline (n = 7, purple) arms. Median expression levels of genes in the gene sets are plotted, while TMM normalized gene expressions are shown for *MMP9*. Box represents 25th and 75th percentile, line is median, with whiskers denoting extremes. **Adjusted *p*-value < 0.01.

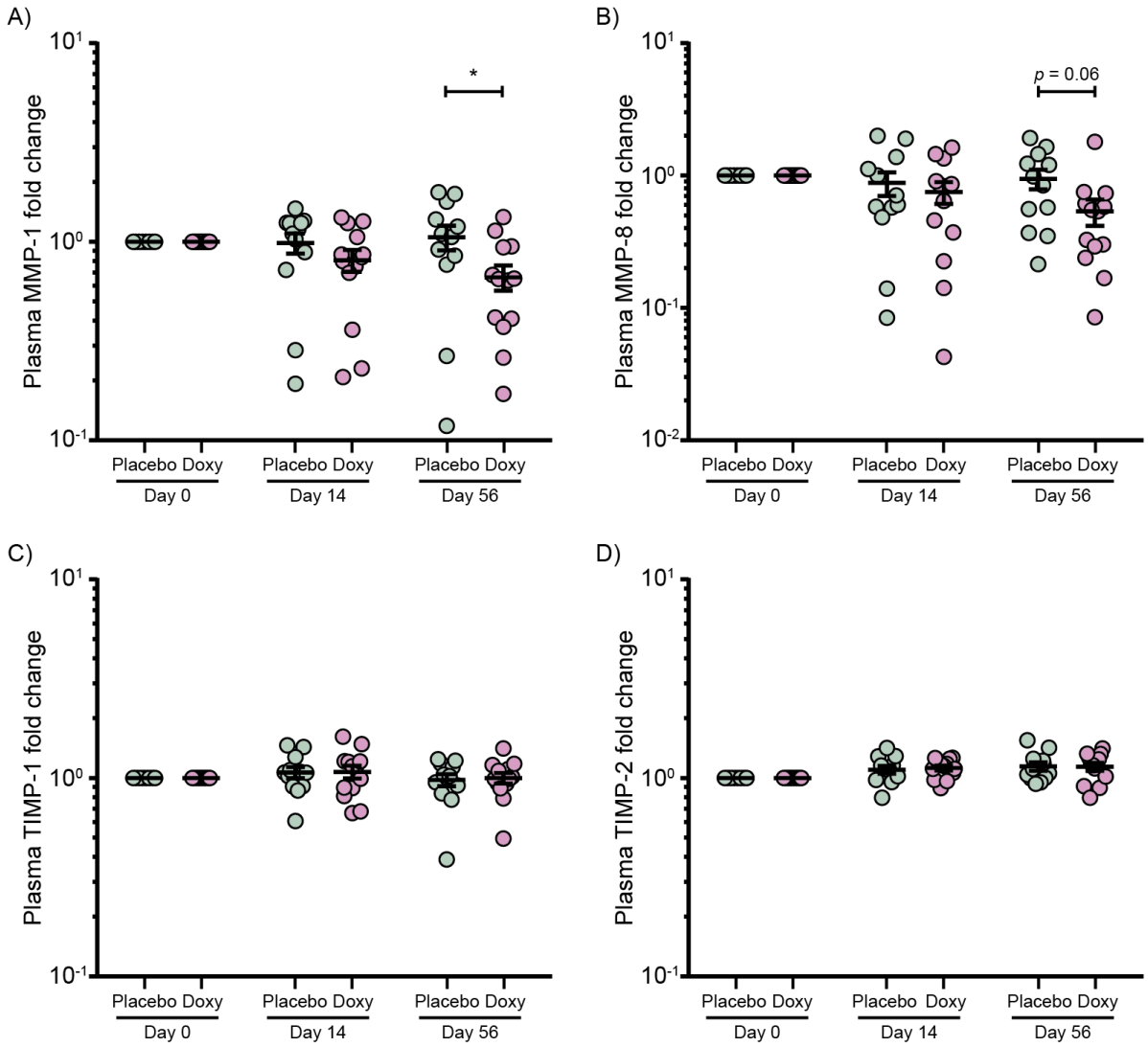

Figure 5. Plasma MMP-1 is suppressed by doxycycline but TIMP-1 and -2 are not affected. (A-D) Longitudinal analysis of plasma MMP-1 **(A)**, MMP-8 **(B)**, TIMP-1 **(C)**, and TIMP-2 **(D)** concentrations at Day 0, 14, and 56 of placebo (n = 12, green) and doxycycline (Doxy, n = 13, purple) arms. Protein concentrations of each subject were normalized to their Day 0 values. Analysis by 2-way ANOVA with Sidak's multiple comparisons. *Adjusted *p*-value < 0.05. Bars represent mean ± s.e.m.

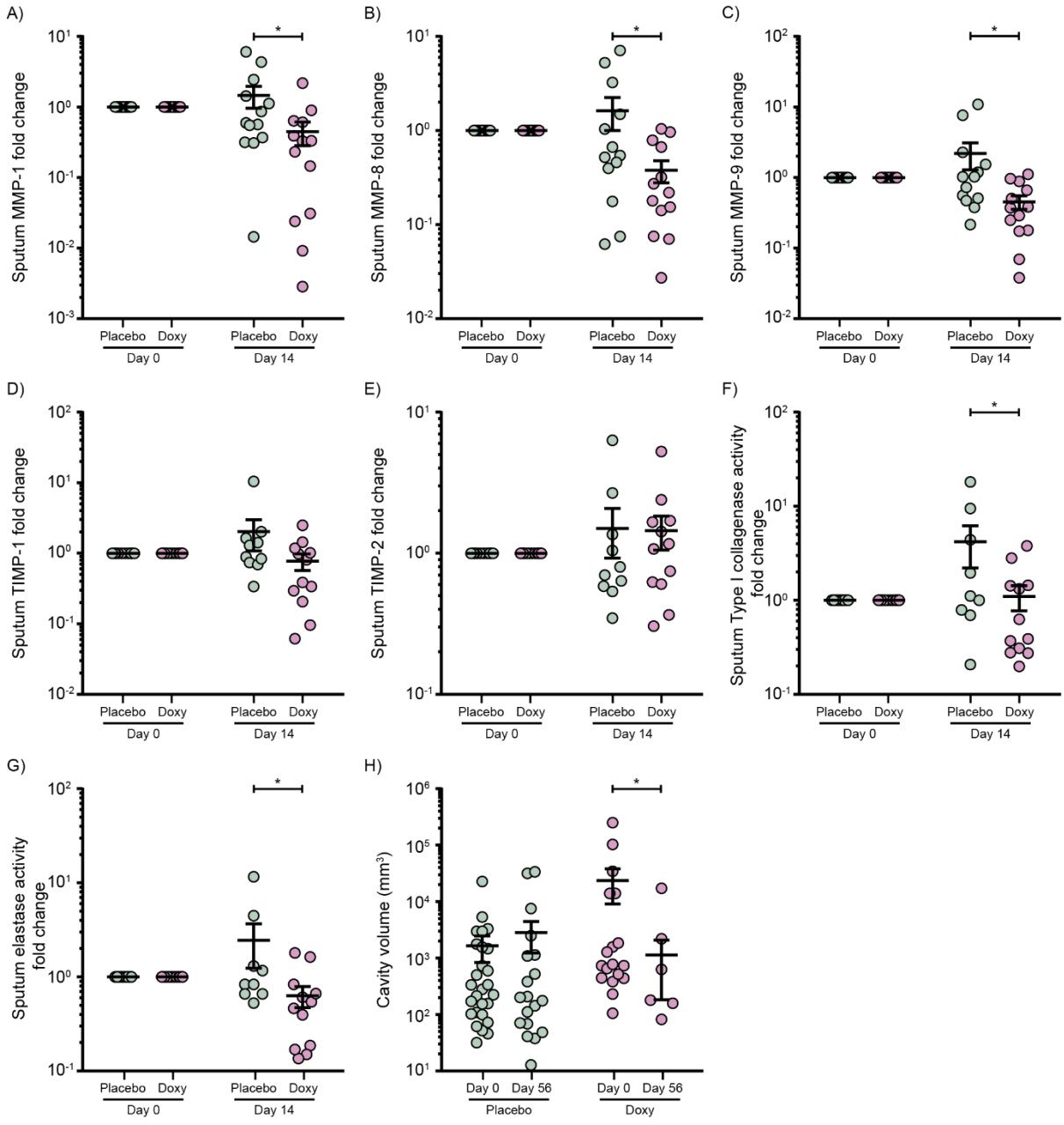

Figure 6. Sputum MMPs and extracellular matrix degradation activity in TB patients treated with doxycycline are suppressed with a concurrent decrease in cavity volume. (A-G) Longitudinal analysis of sputum MMP-1 (**A**), MMP-8 (**B**), MMP-9 (**C**), TIMP-1 (**D**), TIMP-2 (**E**), Type I collagenase activity (**F**), and elastase activity (**G**) at Day 0 and 14 of placebo (green) and doxycycline (Doxy, purple) arms. Protein concentrations, as well as functional activity, for each subject were normalized to their Day 0 values. n = 13 placebo and n = 13 doxycycline for MMPs; n = 10 placebo and n = 12 doxycycline for TIMPs; n = 12 placebo and n = 9 doxycycline for functional activity. **(H)** Longitudinal analysis of cavity volume in patients with pulmonary cavities at Day 0 and 56. n = 9 placebo (green) and n = 7 doxycycline (Doxy, purple). Analysis by 2-way ANOVA with Sidak's multiple comparisons. *Adjusted *p*-value < 0.05. Bars represent mean ± s.e.m.