



Platelets regulate pulmonary inflammation and tissue destruction in tuberculosis

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Platelets regulate pulmonary inflammation and tissue destruction in tuberculosis

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Contributions

KAF, DEK and JSF conceived the study; KAF designed and carried all the cellular experiments and analyzed sputum samples; DEK and RHG obtained clinical data concerning patient plasma samples; JCP carried out immunohistochemistry and associated imaging; NK and BDR carried out the murine experiments from which samples were processed for immunohistochemistry and advised on the data. KAF, DEK, AMW, RHG and JSF drafted the manuscript. All authors reviewed and agreed the final version of the manuscript.

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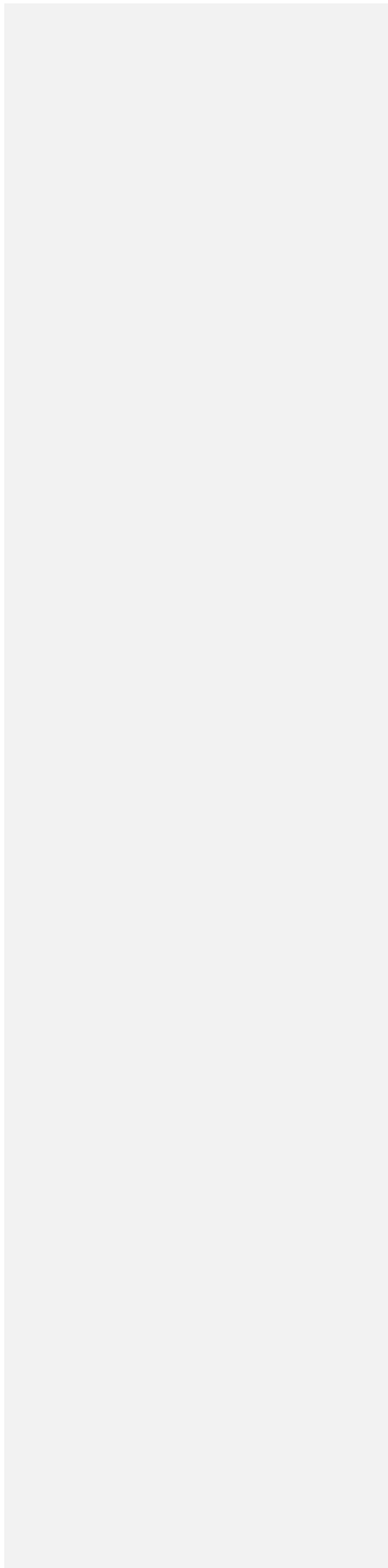
At a Glance Commentary

Scientific Knowledge on the Subject: In addition to their role in coagulation, platelets influence immune responses. However, their role in cellular networks of inflammatory tissue destruction in tuberculosis is unknown.

What This Study Adds to the Field: Platelets drive significant human monocyte-dependent inflammation and collagenase activity in TB, resulting in tissue destruction in both a cellular model of infection and in TB patients.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

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Abstract

Rationale: Platelets may interact with the immune system in tuberculosis (TB) to regulate human inflammatory responses that lead to morbidity and spread of infection.

Objectives: To identify a functional role of platelets in the innate inflammatory and matrix degrading response in TB.

Methods: Markers of platelet activation were examined in plasma from 50 TB patients pre-treatment, and 50 controls. 25 patients were followed longitudinally. Platelet-monocyte interactions were studied in a co-culture model infected with live, virulent *Mycobacterium tuberculosis* (*M.tb*) and dissected using qPCR, Luminex multiplex arrays, matrix degradation assays and colony counts.

Immunohistochemistry detected CD41 expression in a pulmonary TB murine model and secreted platelet factors were measured in bronchoalveolar lavage fluid (BALF) from 15 TB patients and matched controls.

Measurements and Main Results: Five of six platelet-associated mediators were upregulated in plasma of TB patients compared to controls, with concentrations returning to baseline by day 60 of treatment. Gene expression of the monocyte collagenase MMP-1 was upregulated by platelets in *M.tb* infection. Platelets also enhanced *M.tb*-induced MMP-1 and -10 secretion which drove Type I collagen degradation. Platelets increased monocyte IL-1 and IL-10 and decreased IL-12 and monocyte-derived chemokine (MDC, also known as CCL-22) secretion, as consistent with an M2 monocyte phenotype. Monocyte killing of intracellular *M.tb* was decreased. In the lung, platelets were detected in a TB mouse model and

secreted platelet mediators were upregulated in human BALF, and correlated with MMP and IL-1 β concentrations.

Conclusions: Platelets drive a pro-inflammatory, tissue-degrading phenotype in TB.

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Introduction

Approximately 2 billion people are infected with *Mycobacterium tuberculosis* (*M.tb*), which killed around 1.8 million people in 2015 alone (1). In pulmonary tuberculosis (TB), inflammatory tissue damage leads to cavity formation and ultimately morbidity, mortality, and onward transmission (2). This journal has recognized the importance of the host response to *M.tb* throughout its 100 year history (3) and, with drug resistance increasing worldwide and limited new therapies, there is an increasing interest in host-directed therapy for TB. Further understanding of TB immunopathology is needed to support progress in this area.

In addition to their classic role in haemostasis, platelets modulate innate and adaptive immune responses (4). Platelet interaction with monocytes regulates cell recruitment, maturation, and secretion of cytokines and MMPs (5-7). High platelet numbers are found in pulmonary vessels, and the lung (the main target organ for *M.tb*) is a major site of platelet production (8). Both platelet count and activity are increased in TB (9, 10). Platelets express surface markers such as CD41 (11), and upon activation secrete mediators from their granules. These include platelet factor-4 (PF4), which is primarily platelet-secreted, and platelet-derived growth factor (PDGF)-BB, RANTES, P-selectin and MMP-9 which are also secreted by other cell types (12-14). Pentraxin-3 (PTX3) interacts with platelet-derived P-selectin to regulate platelet activity (15).

Central to TB pathology is development of a tissue degrading phenotype due to innate immune cell activation (2, 16). *M.tb* infection upregulates intercellular networks and secretion of cytokines such as IL-1 β , IL-10 and IL-12, which influence replication, spread, and persistence of *M.tb* (17). Innate immune responses also

drive secretion of matrix metalloproteinases (MMPs) such as the collagenase MMP-1, and these degrade the pulmonary extracellular matrix (ECM) to cause tissue destruction (18). As well as transcriptional regulation, MMP activity is regulated post-translationally by tissue inhibitors of metalloproteinases (TIMPs) and other MMPs such as MMP-10, which regulates MMP-1 (19, 20).

Monocytes migrate early from the circulation into *M.tb*-infected tissue, where they differentiate to specialized cell types that control bacterial replication, granuloma formation and inflammatory tissue destruction (21-23). Platelets may influence monocyte maturation and cytokine responses to *M.tb* antigens and Bacillus Calmette-Guérin (BCG) stimulation, and they drive development of multinucleated giant cells (24). However, platelet regulation of monocyte functions is not understood in the context of TB immunopathology.

In this study, we investigate the hypothesis that platelets have a key role in inflammatory tissue destruction in pulmonary TB. We demonstrate platelet activation in the circulation of pulmonary TB patients. We establish platelets to be key regulators of *M.tb*-driven monocyte collagen degradation, and demonstrate a role of platelets in the control of intracellular monocytic bacterial replication. Finally, we show platelets at the site of infection in pulmonary TB.

Methods

Further information on methodology is in the supplementary methods section.

Clinical Studies

Plasma samples were collected in prospective studies involving adult TB patients in Lima, Peru with a new diagnosis of culture-positive, drug-sensitive pulmonary TB (25). 50 age- and sex-matched healthy controls were asymptomatic and purified protein derivative (PPD)-negative. All patients provided written informed consent, and the study had ethical approval from Universidad Peruana Cayetano Heredia, the Asociación Benéfica PRISMA, and each participating centre's ethics committee.

Appetite was assessed using the Short Nutritional Assessment Questionnaire (SNAQ). Plasma and sputum samples were obtained at Day 0 and plasma samples were collected from 25 patients longitudinally at days 14, 28, and 60 of treatment.

Bronchoalveolar lavage fluid (BALF) was collected from patients in a previously described study (26).

Cell Isolation and Culture

Human platelets were isolated and activated with 0.1 U/ml thrombin (27). Monocytes were isolated using either adhesion purification or, for confocal microscopy, the Pan Monocyte Isolation (Human) Kit (Miltenyi Biotec, Bisley, UK). *M. tuberculosis* H37Rv Pasteur (*M.tb*) was cultured in Middlebrook 7H9 medium (28).

Autologous freshly isolated platelets and monocytes from healthy volunteers were co-cultured at a ratio of 100:1 unless otherwise stated, and infected with *M.tb* at a multiplicity of infection (MOI) of 1.

Secreted cytokine and MMP analysis

Clinical samples were analyzed by Luminex magnetic bead array (R&D Systems, Abingdon, UK). ELISA or Luminex array were used to measure cell culture concentrations of MMPs, TIMPs (both R&D Systems, Abingdon, UK) and cytokines (Millipore, Watford, UK).

Cytokine and MMP gene analysis

RNA was extracted and qRT-PCR performed using β -actin as a reference gene. The 1-Step RT-PCR master mix (Qiagen, Crawley, UK). PCR plasmid standards were used to calculate copy number of MMP-1 and β -actin mRNA, and fold change compared to controls.

Colony forming unit (CFU) analysis

Lysed adherent cells or culture supernatants were plated onto Middlebrook 7H11 agar and incubated at 37 °C for 2-4 weeks prior to CFU counting.

Functional assay of tissue destruction by confocal microscopy

Cells were cultured on slides coated with DQ collagen type 1 (ThermoFisher Scientific, Hemel Hempstead, UK) and imaged with a Leica confocal microscope. Images were processed using Leica LAS AF Lite 2.6.0 and Image J 1.43U software (NIH, USA).

Immunohistochemistry

Immunohistochemistry using the Discovery XT instrument and the DAB Map Kit (Ventana medical systems, Tucson, AZ, USA) was performed on paraffin embedded day 28 post-infection lung samples from Balb/C mice collected as part of a previously published study (29).

Statistics

Analyses were performed using PRISM Version 6 (GraphPad, La Jolla, CA, USA) and STATA 12 (Stata Corp., College Station, TX, USA). Clinical data are medians and interquartile ranges (IQR) and analyzed using Wilcoxon matched pairs signed rank test or the Skillings-Mack statistic. Pearson D'Agostino testing demonstrated data were not normally distributed. Cell culture data are mean \pm standard deviation

(SD) and analyzed by One Way Anova and Tukey's correction for multiple comparisons. Graphs are representative of at least three independent experiments. Correlations were calculated with Spearman correlation. $p < 0.05$ was considered significant.

Results

Active platelet-associated mediators circulating in TB patients

Clinical characteristics of the 50 newly diagnosed TB patients and 50 healthy controls matched for age and sex are presented in Table 1. TB cases weighed less and had a significantly lower body mass index (BMI) than controls at recruitment (BMI 20.4 vs 27.2 respectively; $p < 0.0001$), and appetite measured by SNAQ, was poor compared to controls ($p < 0.001$).

PDGF-BB, RANTES and PF4 concentrations were significantly upregulated in patients compared to controls (all $p < 0.0001$; Fig 1A-1C). MMP-9 concentrations were higher in untreated TB patients versus healthy controls (89,124 pg/ml and 68,057 pg/ml respectively) ($p < 0.05$; Fig 1D) as were PTX3 concentrations (433 pg/ml and 94.0 pg/ml respectively) ($p < 0.001$; Fig 1E). There was no difference in P-selectin concentration ($p > 0.05$; Fig 1F).

In TB patients, there was strong correlation between concentrations of PDGF-BB and PF4 ($R = 0.89$), PDGF-BB and RANTES ($R = 0.65$), and PF4 and RANTES ($R = 0.70$). There was a moderate correlation between PF4 and MMP-9 concentrations ($R = 0.54$) and a weaker correlation between concentrations of MMP-9 and PTX3 ($R = 0.47$), MMP-9 and PDGF-BB ($R = 0.45$), PTX3 and PDGF-BB ($R = 0.48$),

PDGF-BB and P-selectin ($R=0.45$), and PF4 and PTX3 ($R=0.44$). Among control subjects, there was strong correlation between levels of RANTES and PDGF-BB ($R=0.93$), RANTES and PF4 ($R=0.87$), and between PDGF-BB and PF4 ($R=0.88$). There was moderate correlation between concentrations of PTX3 and PDGF-BB ($R=0.56$) and between PTX3 and PF4 ($R=0.50$), and weaker correlation between PTX3 and RANTES ($R=0.49$, all $p<0.05$).

Longitudinal changes in platelet-derived mediators during treatment of TB patients

Next, we performed a longitudinal assessment of platelet activity over the first 60 days of TB treatment in 25 patients. Biometric and microbiological data are shown in Table E1 in the online data supplement. 31/50 patients (62%) remained sputum culture-positive at Day 14, 20 patients (40%) at Day 28, and 5 patients (10%) at Day 60. Mean BMI of TB cases steadily increased from 20.4 at baseline to 22.0 by Day 60, and mean SNAQ score from 13.3 to 15.9. There was no difference in age, sex, mean BMI, or mean SNAQ score between TB cases who were followed up compared to those who were not (data not shown).

Over 60 days of treatment, plasma concentrations of PF4 and PDGF-BB changed significantly in TB patients ($p<0.05$; Fig 2A-B). There was a trend towards an increase at Day 14 but concentrations subsequently decreased to near control values by Day 60 (Fig 2A-B). P-selectin concentrations, which were not upregulated at presentation with TB, did not alter during treatment (Fig 2C).

Platelet-monocyte interactions drive MMP-1 & -10 gene expression and secretion in TB

Since there was good evidence of platelet activation in TB patients, we developed a co-culture model to investigate the functional role of platelets in regulating the human

inflammatory immune response. A major outcome of this response is MMP-driven ECM destruction in which the collagenase MMP-1 is key (16). Monocytes co-stimulated with platelets and *M.tb* had a 4.3-fold increase in *Mmp-1* expression compared to *M.tb*-stimulated monocytes alone ($p < 0.0001$; Fig 3A). Platelets did not significantly upregulate *Mmp-1* expression in uninfected monocytes. MMP-1 secretion was increased 6.6-fold from *M.tb*-stimulated monocytes co-cultured with platelets compared to *M.tb*-infected monocytes alone ($p < 0.0001$; Fig 3B), although platelets did not alter MMP-1 secretion in control cells. This effect was dependent on the ratio of platelet:monocytes (P:M) (Fig 3C), increasing up to 100:1.

The MMP-1 activator MMP-10 was similarly upregulated by platelets. MMP-10 concentrations increased from $318 \pm 106.9.1$ pg/ml to 1972 ± 405.4 pg/ml upon exposure to *M.tb*. This increased further to 3423 ± 363.2 pg/ml with exposure to *M.tb* in the presence of platelets (both $p < 0.001$; Fig 3D). Significant MMP-10 upregulation was seen with P:M ratios of 50:1 ($p < 0.01$) and this increased further at a ratio of 100:1 (Fig 3E). However, MMP-10 secretion decreased with P:M ratio of 500:1 to below that seen with monocytes stimulated by *M.tb* alone ($p < 0.0001$; Fig 3E). In addition, platelet co-culture increased monocyte secretion of MMP-3, -7 and -9, while MMP-8 was not changed. Similar to MMP-1 and -10, platelet upregulation of MMP-3 and -7 required *M.tb* exposure (see Figure E1 in the online data supplement). TIMP-1 and -2 secretion from monocytes in the presence or absence of *M.tb* were not altered by platelet co-culture (Fig 3F-G).

Platelets regulate monocyte-dependent collagen destruction in TB

To investigate the impact of platelets on monocyte's functional MMP-1 activity, monocytes were cultured on type I collagen that fluoresces on degradation. Confocal

microscopy revealed monocytes alone had minimal collagenase activity when uninfected, regardless of platelet co-culture. *M.tb* stimulation of monocytes induced some functional collagenase response but this was significantly increased in monocytes stimulated by *M.tb* in the presence of platelets (Fig 4A). Quantitative analysis revealed a 2.5-fold increase in collagenase activity of *M.tb*-exposed co-cultured cells compared with *M.tb*-exposed monocytes in the absence of platelets ($p<0.01$; Fig 4B). Platelet-only controls produced no measurable collagen degradation (not shown).

Platelet regulation of monocyte inflammatory responses

Next, we investigated the effect of platelet-monocyte interactions on the secretion of inflammatory cytokines. There was a marked increase in IL-1 β secretion from *M.tb*-stimulated monocytes in the presence of platelets compared with *M.tb*-stimulated monocytes alone ($p<0.0001$; Fig 5A). This trend was not seen in the inflammatory cytokines interferon- γ (Fig 5B) or TNF- α (Fig 5C). IL-10 was upregulated 7.5-fold in *M.tb*-infected platelet co-culture compared to single culture monocytes ($p<0.0001$; Fig 5D). In contrast, *M.tb*-infected monocytes in platelet co-culture secreted 6.4-fold less IL-12 and 6.1-fold less monocyte-derived chemokine (MDC) (both $p<0.001$; Fig 5E-F). There was no platelet regulation of the immunomodulatory cytokine IL-6 or the chemokine IL-8 (see Figure E2 in the online data).

Platelet co-culture increases monocyte intracellular M.tb replication

In addition to regulation of monocyte-derived inflammatory mediators, monocyte co-culture with platelets altered the intracellular killing of *M.tb*. Although extracellular *M.tb* did not change in platelet co-culture, 2.5-fold more viable intracellular *M.tb* was detected in co-cultured cell lysates ($p<0.001$; Fig 6).

Active platelets are present at the site of infection in pulmonary TB.

Next, we investigated whether platelets were present at the site of TB infection itself. Like most murine TB models, Balb/C mice do not form organised granulomas with central necrosis but *M.tb* infection and dissemination through tissue does result in large areas of highly inflamed pulmonary tissue. Therefore, we stained lung tissue from a murine model of infection for the platelet marker CD41 (Fig 7A-B). Platelets are anucleate cells and this is predominately reflected by the staining. Interestingly, there were large numbers of platelets detected in the alveoli. Limited CD41 association with nucleated cells can be seen which is largely restricted to areas of granuloma-like structure and giant multinucleate cell formation. In peripheral lung areas with limited inflammation there was minimal detection of platelets (Fig 7C).

To confirm that platelets were active at the site of infection, human BALF samples obtained during a previous clinical study (26) were analyzed for platelet-derived mediators and compared to respiratory symptomatic control patients. There was a significant upregulation of PDGF-BB concentration in BALF from TB cases compared to controls ($p < 0.01$; Fig 7D). Both P-selectin and RANTES concentrations were also increased in TB patients ($p < 0.01$; Fig 7E-F) but PF4 concentrations were not (Fig 7G).

In human BALF, P-selectin concentrations correlated significantly with increasing concentrations of IL-1 β and MMP-1 (both $p < 0.001$), and MMP-7, -8 and -9 (all $p < 0.01$) ($R = 0.5$; Fig 8A-E). P-selectin correlation with MMP-3 was also statistically significant ($R = 0.4$; $p < 0.05$; Fig 8F). In addition, BALF PDGF-BB concentrations correlated with MMP-1, -3, -8 and -9 (see Figure E3 in the online data supplement). BALF RANTES concentrations correlated with MMP-1, -8 and -9 as well as IL-1 β (see Figure E4 in the online data supplement).

Discussion

This study used a longitudinal patient study, a human cellular model, a murine model, and a more invasive bronchoscopy-based clinical study to investigate the activation and function of platelets in the innate inflammatory response to TB. Plasma concentrations of platelet-associated factors were significantly upregulated in a cohort of patients with pulmonary TB. This is consistent with reports that concentrations of PTX3 (30) and PF4 (10, 31, 32) increase in pulmonary TB along with various platelet indices such as platelet count and mean platelet volume (9, 33). In our study, there was a transient increase in platelet-associated mediators when patients received anti-TB treatment followed by a decrease towards plasma concentrations of healthy controls. These mediators are not necessarily exclusively produced by platelets. However, the concentrations of different platelet-derived mediators were significantly correlated and this is consistent with co-storage and co-secretion. Interestingly, plasma PF4 levels have been previously correlated with TB disease activity (31).

As the secretion of the collagenase MMP-1 is a key driver of tissue destruction of the lung observed in TB (18), we investigated the role of platelets on monocyte MMP-1 secretion in detail using platelet monocyte ratios similar to those observed in man.

Platelets were added to monocytes in a thrombin-activated form, reflecting the activated state we observed in our clinical studies and in keeping with techniques standard across the literature (34-36). In macrophages produced *in vitro* from platelet-matured monocytes, *Mmp-1* is among the top five upregulated genes (24). Platelets alone do not regulate monocyte MMP-1 gene expression or secretion,

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which may reflect control mechanisms limiting platelet proinflammatory activity. However, platelets significantly upregulated monocyte MMP-1 mRNA accumulation and protein secretion in the presence of the additional stimulus of *M.tb* infection. There was a concurrent increase in the MMP-1 activating enzyme MMP-10, and upregulation was not associated with any concurrent increase in TIMP secretion. Consequently, functional collagen degradation was increased markedly in platelet-monocyte co-culture.

The platelet-dependent upregulation of monocyte IL-1 β further demonstrates that platelets drive monocyte inflammatory responses since IL-1 β is essential for nitric oxide production, phagosomal acidification and maturation, and adhesion molecule expression (37). Knockouts of IL-1 activity in pulmonary TB murine models have been used to demonstrate a protective role of this cytokine, which can be associated with polymorphonuclear leukocytes. IL-1 β regulates the number and development of granulomas, limits *M.tb* growth, and is associated with proper induction of other cytokine responses (38, 39). The upregulation of IL-1 β was specific and not observed in either interferon- γ or TNF- α secretion. Upregulation of IL-1 β has previously been shown to be dependent on caspase-1 processing in *M.tb* infection (40) and the data may therefore suggest a role of platelets in the upregulation of inflammasome complexes, such as the caspase-1-NLRP3 inflammasome.

In a novel observation, we found that *M.tb*-stimulated monocyte MDC secretion is decreased by over 50% when co-cultured with platelets. MDC acts through the C-C chemokine receptor type 4 (CCR4) and is associated with pulmonary inflammation (41). Such changes in IL-10, IL-12, and MDC are typically attributed to monocytes maturing with an M2 macrophage phenotype. This phenotype is associated with significant tissue remodelling properties, including secretion of MMPs involved in

both tissue damage and tissue repair (42-45). Prolonged *M.tb* infection tilts monocyte-derived cells towards an M2 phenotype, which is the major phenotype found in TB granulomas (46). Our data indicate that platelets may be involved in driving such differentiation. M2 type monocyte-derived macrophages are also associated with increased bacterial replication and progression of disease in TB patients (47, 48). We observed increased IL-10, which enhances mycobacterial intracellular survival and growth by suppressing innate and adaptive immune responses (49, 50) but decreased IL-12, which is strongly associated with *M.tb* killing (51). CFU analysis of cell lysates reflected this, as we observed a 2.5-fold increase in intracellular survival of *M.tb* in monocytes with platelet co-culture compared to without co-culture. [The lack of effect by platelets on extracellular CFU counts suggests that platelets do not affect internalization of *M.tb* by monocytes.](#)

In the murine model, we demonstrated platelets concentrated at the site of TB pathology in the lung. There were platelets in some alveolar air pockets and this is consistent with the breakdown of the epithelial barrier resulting in the common symptom of haemoptysis. The images also showed platelets associated with nucleate cells. This is consistent with both tight platelet adherence to recruited cells as they extravasate from the blood vessels, and platelet phagocytosis during monocyte maturation (52). Further magnification revealed that most of the CD41 staining was associated with nuclei of developing multinucleate cells. This supports previous immunohistochemistry of human TB lung detecting the platelet integrin CD42b, which demonstrated that monocyte phagocytosis of platelets drives development of multinucleated giant cells (24).

Finally, the analysis of human TB patient BALF samples demonstrated upregulation of PDGF-BB, P-selectin, RANTES and CD40L consistent with platelet activation in

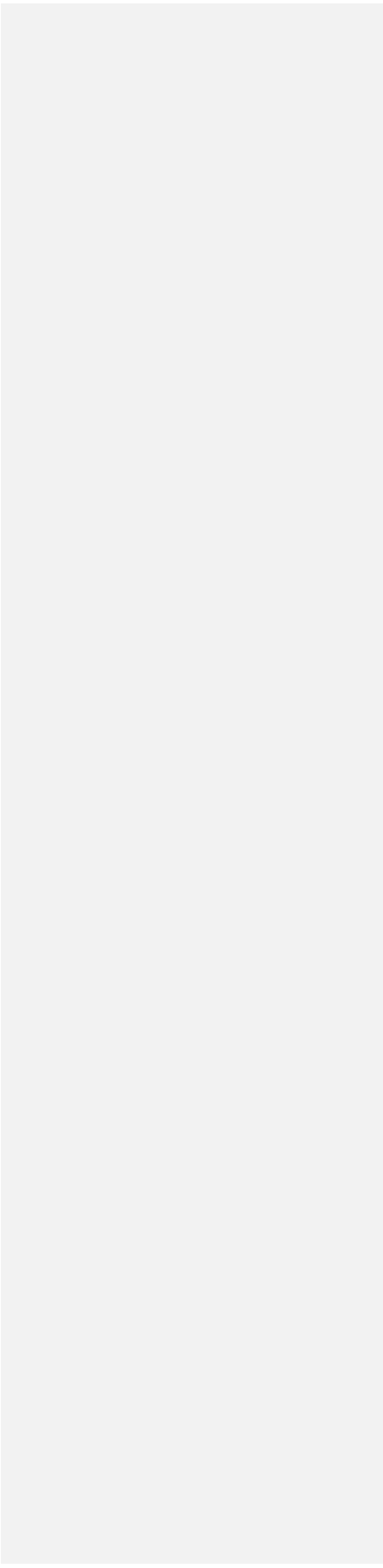
pulmonary TB. These mediators correlated with activity of specific MMPs, including the important collagenase MMP-1, and is consistent with our cellular data. Previous work found that PDGF-BB concentrations in the epithelial lining fluid after six months of treatment correlated with worsening pulmonary fibrosis scores (53). The role of platelets in long-term immunopathology and fibrosis following TB infection merits further study.

In summary, we demonstrated that markers of platelet activity are increased in plasma of patients with pulmonary TB compared to healthy controls, and these are then normalized with anti-mycobacterial treatment. We demonstrate that platelets alter the proinflammatory response resulting in increased tissue destruction and development of an IL-1 β , IL-10 high, IL-12 low milieu that is associated with increased pathogen growth. Platelets are present within the infected lung tissue itself and active in human disease. In TB, large-scale tissue damage and bacterial replication is associated with onward transmission, morbidity, and mortality. Our data indicate a previously unappreciated role for platelets in driving this pathology, which may have implications for host-directed therapies in the era of rising drug resistance.

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Figure Legends

Figure 1. Plasma concentrations of platelet-derived mediators in pre-treatment TB patients and control subjects.

(A) PDGF-BB, (B) RANTES, (C) Platelet Factor 4 (PF4), (D) MMP-9, and (E) Pentraxin (PTX)-3 were higher in patients with pulmonary TB prior to treatment (grey bars shaded boxes) compared to age- and sex-matched healthy controls (black bars open boxes). (F) Plasma concentrations of P-selectin were unchanged in TB patients compared to controls. Data presented as

median and IQR. Whiskers represent 10th and 90th percentiles and outline values are shown. P-values calculated using the Wilcoxon matched-pairs signed rank test.

N=50 for both groups. *p<0.05, ***p<0.001, ****p<0.0001.

Figure 2: Plasma concentrations (pg/ml) of (A) Platelet Factor 4 (PF4) and (B) PDGF-BB but not (C) P-selectin decrease to baseline after the first 60 days of

treatment in TB patients (black bars shaded boxes). Age- and sex-matched healthy controls are shown by grey bars open boxes. Data presented as median and IQR. Whiskers represent 10th and 90th percentiles and outline values are shown.

Significance of change over time assessed using Skillings-Mack statistic. N=25 for both groups. *P<0.05.

Figure 3. Monocyte MMP-1 and MMP-10 is regulated by platelets in *M.tb*

infection. Monocytes were cultured with autologous platelets (Plt) and virulent *Mycobacterium tuberculosis* (*M.tb*) before analysis. (A) Monocytes were cultured at platelet:monocyte ratios (P:M) of 100:1 for 18 hours. Y axis shows MMP-1 mRNA copy number normalized to β -actin and displayed as a fold change of the unstimulated control monocytes. Bars represent mean \pm SD for N=3. (B-G)

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Monocytes were cultured with Plt and virulent *M.tb* for 24 hours. Y axis shows secreted MMPs or TIMPs in pg/ml. (B) Monocytes were cultured at P:M ratio of 100:1 with *M.tb* stimulus. Platelets significantly increased MMP-1 secretion with *M.tb* stimulus but not controls. (C) Monocytes were cultured with increasing P:M to show a dose-dependent response of *M.tb*-stimulated monocytes up to P:M 500:1. (D) Monocytes cultured at P:M of 100:1 with *M.tb* stimulus had significantly increased MMP-10 secretion compared with monocytes alone. (E) Monocytes cultured with increasing P:M had increasing MMP-10 secretion until 100:1, after which secretion returned to baseline. (F-G) Monocytes cultured at P:M of 100:1 did not have altered TIMP-1 or TIMP-2 secretion compared with controls. Data are mean \pm SD and are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Monocyte collagenase activity in *M.tb* infection is upregulated by platelet co-culture. Monocytes were cultured with autologous platelets (Plt) and virulent *M.tb* for 24 hours on DQ collagen type I, which fluoresces upon degradation. A) Monocyte-dependent collagenase activity at a high level is only seen in *M.tb*-infected cells cultured in the presence of platelets. Although some collagenase activity is detected in *M.tb*-stimulated monocytes alone, none is found in the absence of *M.tb*. B) Collagen fluorescence of each image was quantified in triplicate and normalised to cell number. Data are mean \pm SD and are representative of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

Figure 5. Platelet interaction alters cytokine and chemokine secretion from human monocytes stimulated by *M.tb*. Monocytes were cultured with autologous

platelets (Plt) and live virulent *M.tb* for 24 hours and proinflammatory mediators measured in cell culture medium. Y axis shows secreted analytes in pg/ml. (A) IL-1 β was significantly increased in *M.tb* stimulation, and increased further by platelet co-culture. (B) Interferon (IFN)- γ was not significantly regulated by platelet stimulation. (C) TNF- α was not significantly regulated by platelet stimulation. (D) IL-10 was significantly upregulated by platelets in *M.tb* stimulated monocytes but not controls. (E) *M.tb* upregulation of IL-12p70 was reduced in the presence of platelets. (F) *M.tb* upregulation of macrophage derived chemokine (MDC) was reduced in the presence of platelets. Data are mean \pm SD and are representative of at least three independent experiments. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

Figure 6. Platelet co-culture increases intracellular bacterial viability in

monocytes. Human primary monocytes were cultured with autologous platelets (Plt) and infected live virulent *M.tb* for 24 hours. Bacterial killing assessed by colony forming unit (CFU) counts of *M.tb* obtained from both the intracellular (Intracell) and extracellular (Extracell) environment. Whilst extracellular CFU remained the same, significantly more bacteria was rescued from platelet-cultured monocytes than controls. Data are mean \pm SD and are representative of three independent experiments. *** p <0.001.

Figure 7. Active platelets are upregulated at the site of infection in pulmonary

TB. (A-C) Platelets are localized to the lung in murine TB. CD41 staining was performed on regions of (A-B) significant *M.tb*-inflammation and tissue remodelling and (C) peripheral lung with minimal inflammation in *Mtb*-infected Balb/C mice. CD41 is highly expressed in detectable in lung areas with significant TB pathology of

inflammation and tissue remodelling. Whilst most CD41 staining was associated with anucleate cells consistent to platelet morphology, some staining of nucleated cells may represent expression of the marker or phagocytosis of platelets or adherence of platelets. Images from a single mouse and representative of N=5 mice. (D-G)

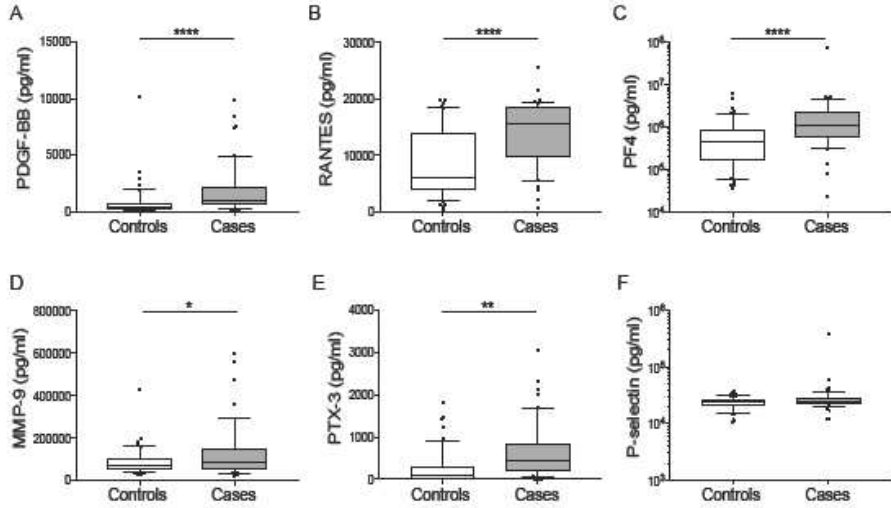
Platelet-derived mediators are upregulated in bronchoalveolar lavage fluid (BALF) from TB patients compared to people with other respiratory illnesses. Bronchial lavage fluid from 15 TB patients (cases) and 15 respiratory symptomatic controls (control) were analysed by multiplex analysis for: (D) PDGF-BB, (E) P-selectin, (F) RANTES and (G) Platelet Factor 4 (PF4). Data are median and IQR. Whiskers represent 10th and 90th percentiles and outline values are shown. **p<0.01

Figure 8. P-selectin expression correlates with MMP and IL-1 β concentrations in BALF. P-selection concentrations measured in BALF of 15 TB patients and 15 respiratory symptomatics by multiplex assay correlated with secretion of (A) IL-1 β , (B) MMP-1, (C) MMP-7, (D) MMP-8, (E) MMP-9 and (F) MMP-3 was assessed using Spearman rank correlation (R). Scatter plots are presented using a log scale. P-values and R-values are shown inset.

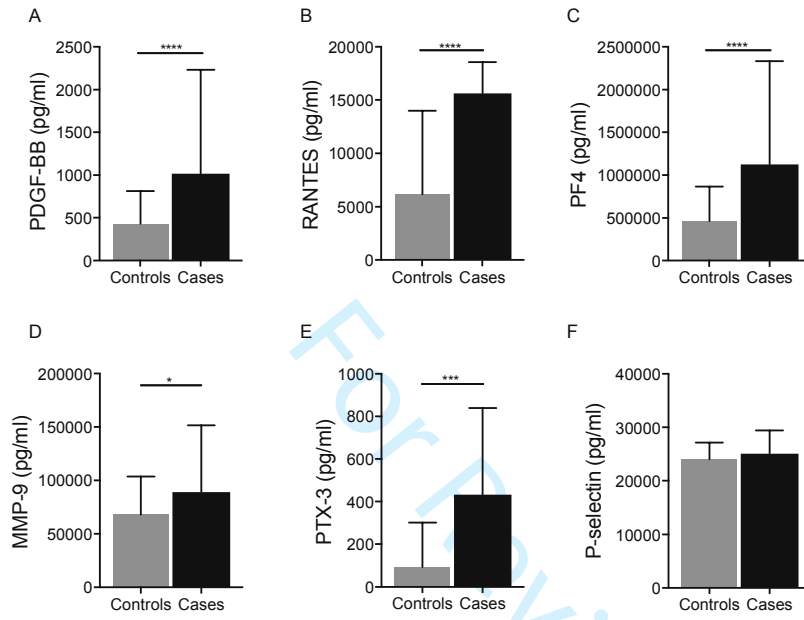
Table 1. Demographics and clinical characteristics of tuberculosis cases pre-treatment and healthy controls.

	Cases, pre-treatment (n=50)	TB-negative controls (n=50)	P-value
Age, mean (SE) ^a	35 (1.90)	35 (1.93)	0.72
Males, n (%)^b	34 (68)	34 (68)	1.0
Height, cm. Mean (SE) ^{a*}	163.6 (1.53)	159.1 (1.29)	0.022
Weight at diagnosis, kg. Mean (SE) ^{a*}	54.6 (2.28)	68.6 (1.94)	<0.0001
Body Mass Index (BMI). Mean (SE) ^{a*}	20.4 (0.82)	27.2 (0.86)	<0.0001
SNAQ score. Mean (SE) ^a	13.3 (0.33)	15.0 (0.27)	0.0008
^a P-value calculated using paired two sample t-test; ^b P-value calculated using Fisher's exact test *Weight, Height, and BMI data missing for 2 cases. BMI data excluded for one case (lower limb amputation)			

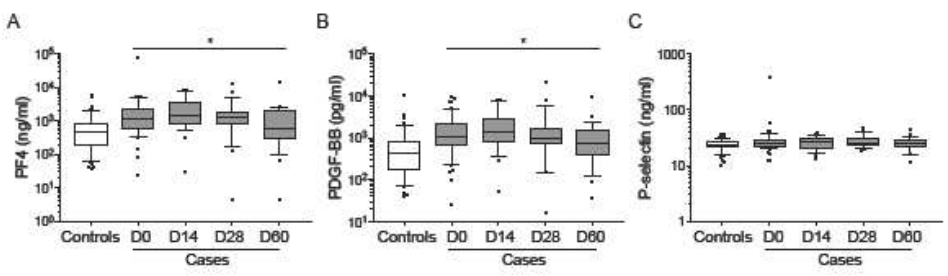
Figure 1



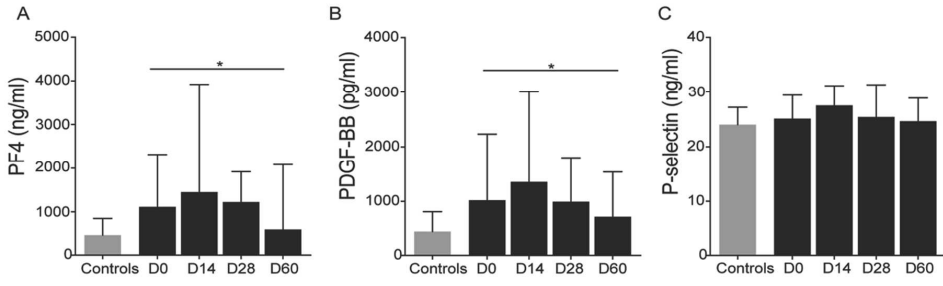
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Figure 3

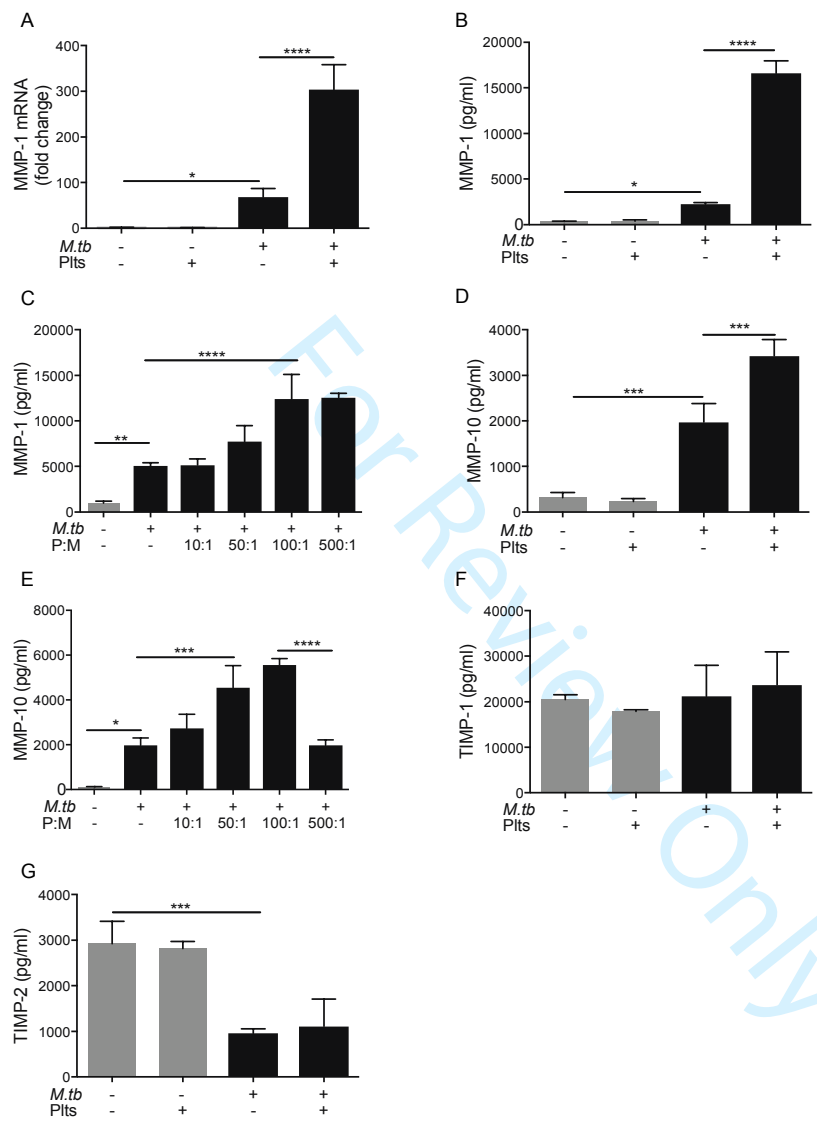


Figure 5

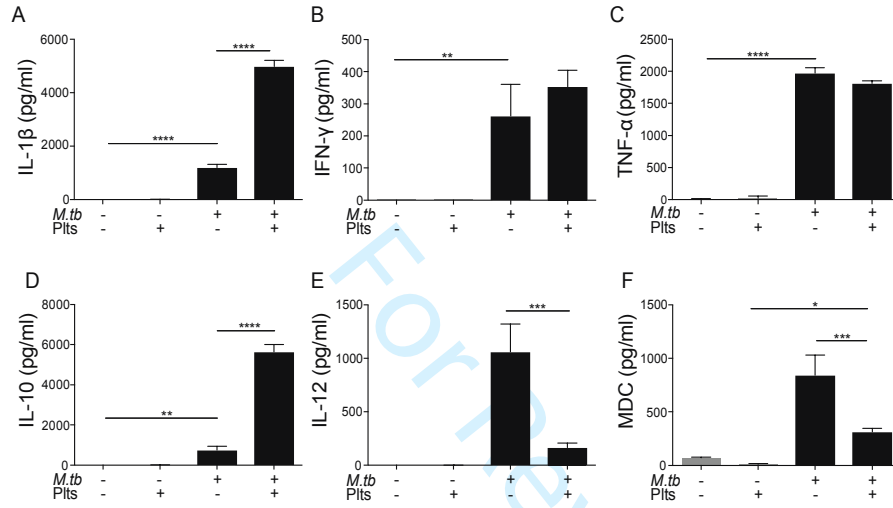
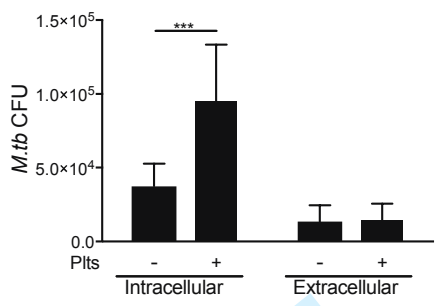
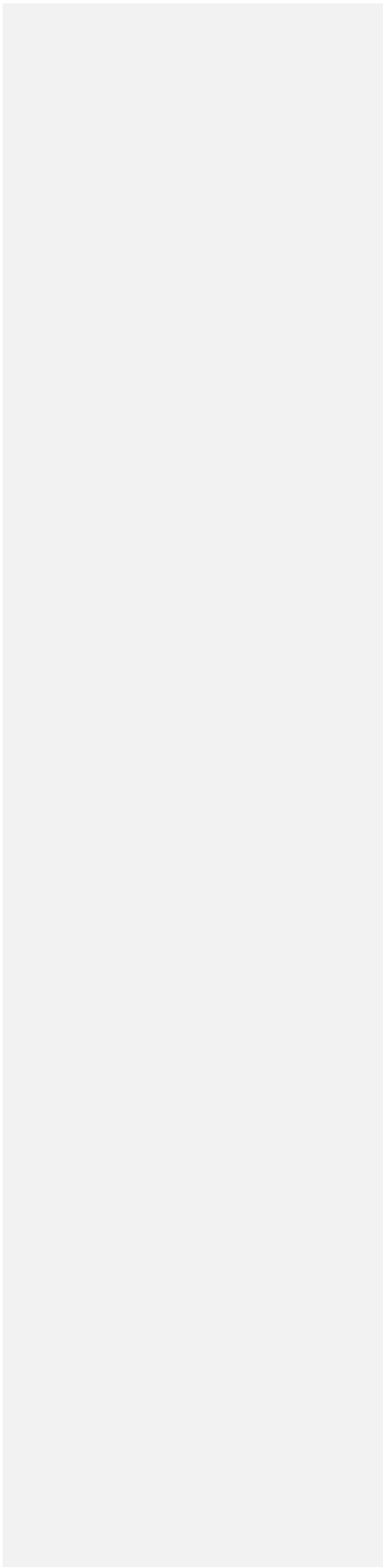


Figure 6



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| Figure 87

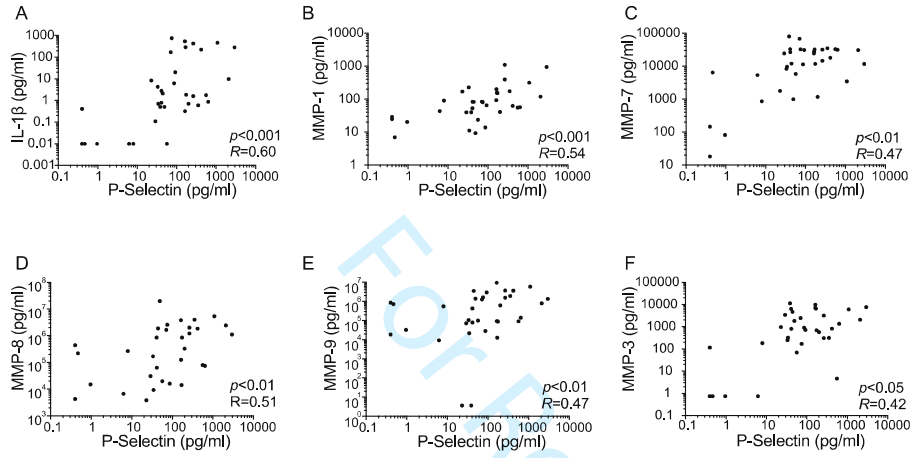
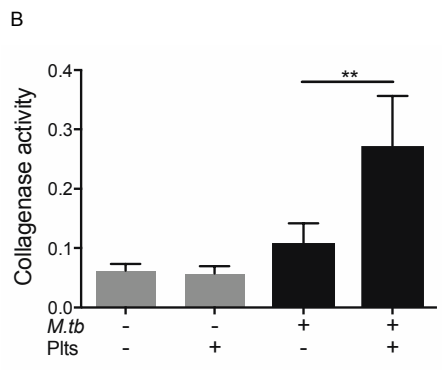
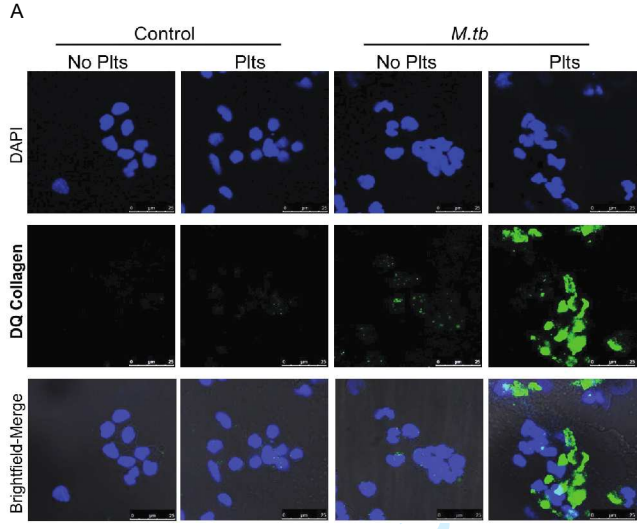


Figure 4



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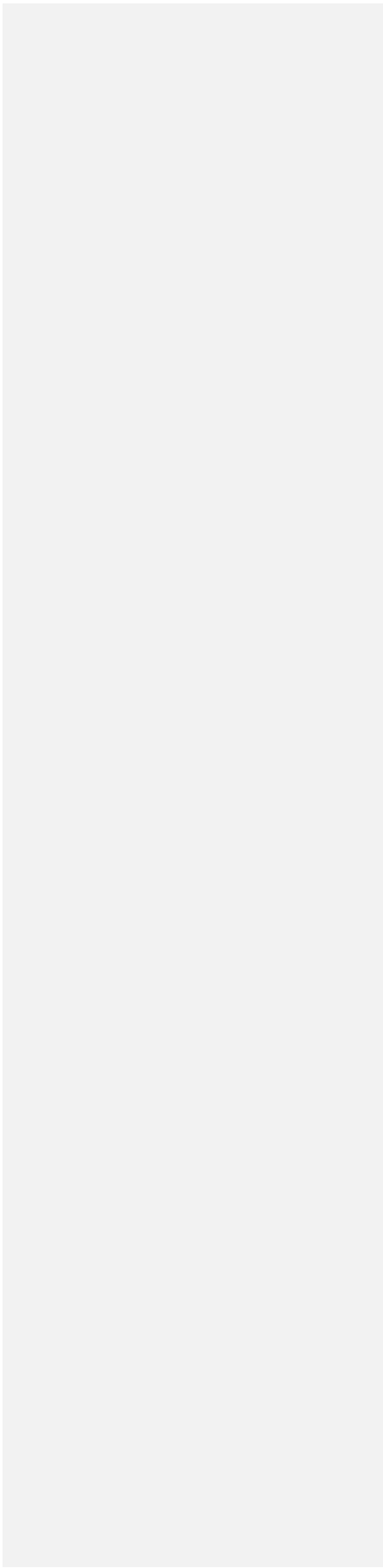
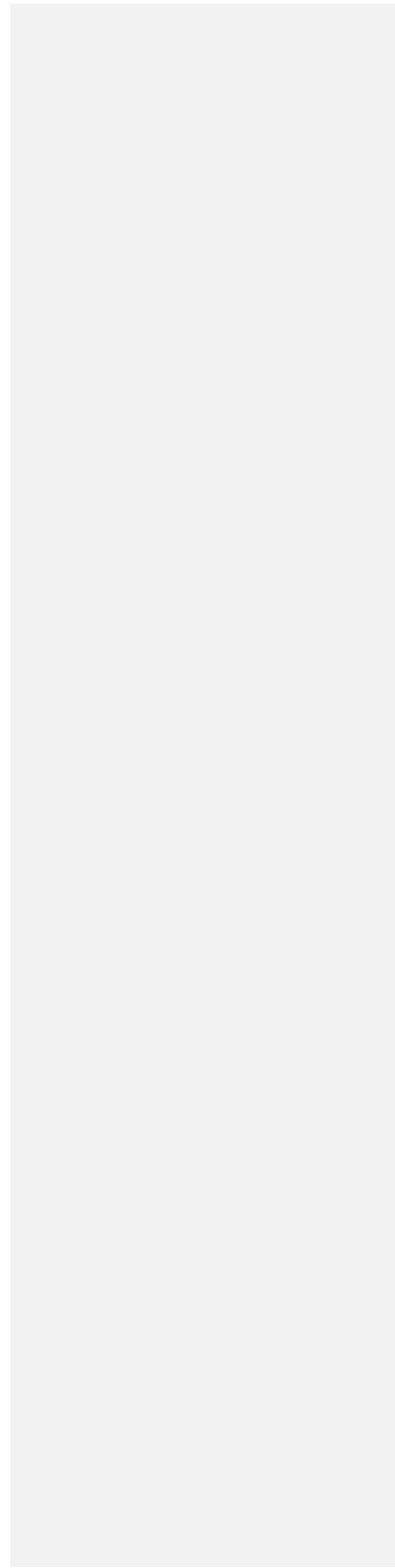
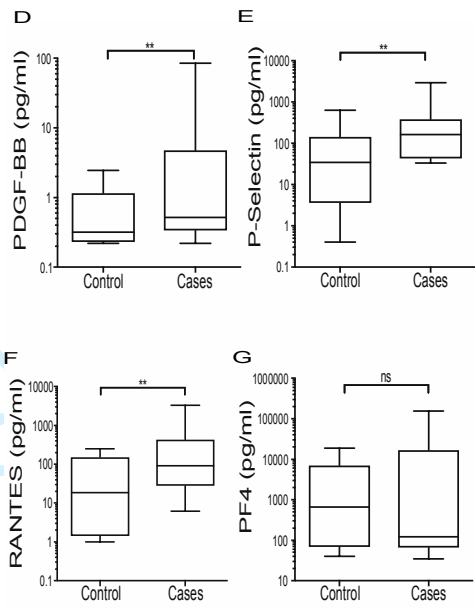
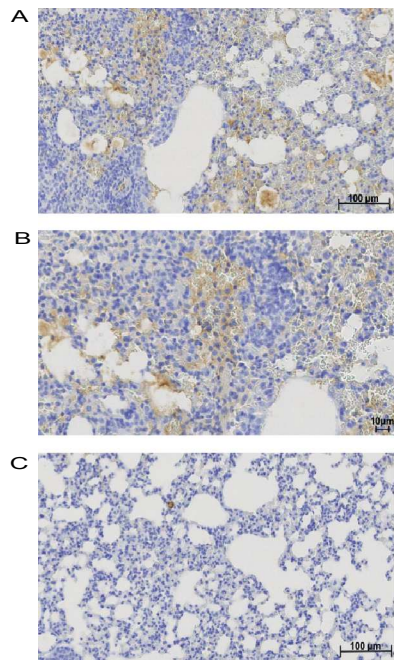


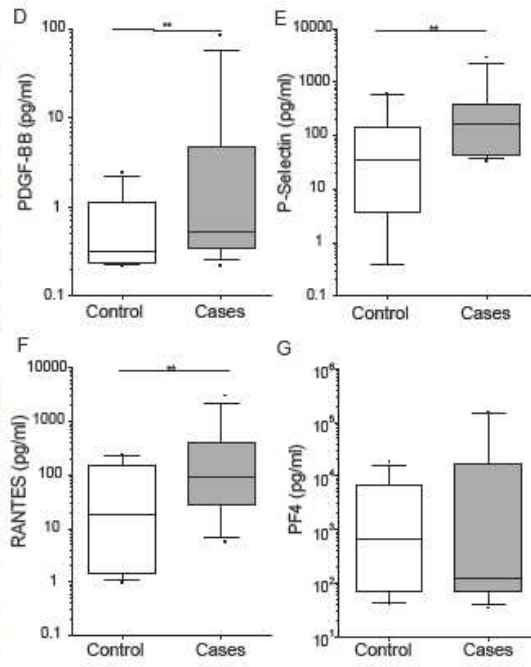
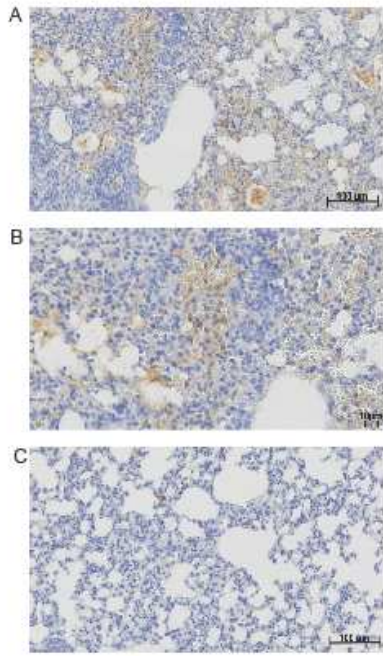
Figure 7

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Methods for supplementary submission

Clinical Studies

Plasma samples were collected as part of a prospective cohort study of cough and biomarkers in the treatment response to TB performed in Lima, Peru. The study population comprised adult patients with tuberculosis and healthy controls were asymptomatic and PPD-negative individuals recruited from the same population.

Inclusion and exclusion criteria have been published elsewhere (1).

At each visit, height and weight were documented and appetite was assessed using the Short Nutritional Assessment Questionnaire (SNAQ) (2, 3). 10 mls of blood was collected into tubes containing EDTA, centrifuged at 1,000 g, and aliquots of plasma were placed into dry ice within 30 minutes of blood extraction then transported to a specimen biobank maintained at -70°C. Sputum samples were also collected from TB patients for smear microscopy and culture with simultaneous Isoniazid- and Rifampicin-resistance testing using the Microscopic-Observation Drug-Susceptibility (MODS) assay. TB patients underwent evaluation at baseline (pre-treatment), and at regular intervals thereafter, whereas controls had a single evaluation only.

For analysis, 50 patients with culture-positive pulmonary tuberculosis (TB), whose *Mycobacteria tuberculosis* isolate was found to be sensitive to isoniazid and rifampicin according to the MODS assay, and who had completed follow-up to Day 60, were selected. In addition, 50 controls were age- and sex-matched from the database of healthy controls. Plasma concentrations of platelet-derived growth factor (PDGF)-BB, RANTES, P-selectin, platelet factor-4 (PF4), MMP-9, and Pentraxin-3 (PTX3) were measured from plasma samples obtained at Day 0 from all cases and all controls. In addition, longitudinal samples obtained at days 14, 28, and 60 were

also analysed for 25 (50%) randomly selected cases age- and sex-matched to the controls in a 1:2 ratio. ~~All patients had provided written informed consent, and the study had ethical approval from Universidad Peruana Cayetano Heredia (UPCH), the Asociación Benéfica PRISMA, and each participating centre's ethics committee.~~

Bronchoalveolar lavage fluid (BALF) was collected from patients being routinely investigated for respiratory symptoms at Nalanda University Hospitals, Patna, India. Demographic, clinical, hematologic, and radiologic data were collected using a standardized proforma. Blood-stained samples were excluded from the study. Details of the study have been previously published (4).

Samples of both studies were analysed using Luminex multiplex magnetic bead array (R&D Systems) in accordance with the manufacturer's instructions.

Mycobacterium tuberculosis culture

Ampicillin resistant *Mycobacteria tuberculosis* H37Rv Pasteur (*M.tb*) was grown from stock stored at -80 °C in 30-50 % glycerol. It was cultured in Middlebrook 7H9 medium supplemented with 10% enrichment medium, 0.2% glycerol, 0.02% Tween 80, and 2.5 µg/ml Amphotericin with agitation at 10rpm. Culture growth was monitored with a Biowave cell density meter (WPA, Cambridge) and the *M.tb* sub-cultured when the optical density reached 1.00. For infection experiments, culture at mid log growth at an optical density of 0.60 was used, which corresponded to 1×10^8 – 2×10^8 colony forming units (CFU)/ ml. Optical density was correlated with CFU by performing colony counts in triplicate on Middlebrook 7H11 agar supplemented with OADC enrichment medium and 0.5 % glycerol.

Cell isolation

Procedures for the isolation of platelets were adapted from previously published methods (5). Blood was drawn from healthy volunteers into tubes containing preservative-free sodium citrate. The blood was gently inverted and used within 2 hours. The blood was diluted in wash buffer (10 mM HEPES, 5 mM glucose, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄ and 145 mM NaCl, pH 7.4) and centrifuged at 200 g for 15 min. After centrifugation the top layer of blood was removed and centrifuged with 1 µM prostaglandin E1 (Calbiochem, San Diego, CA, USA) to avoid activation. The cells were resuspended, washed, and activated in wash buffer with 0.1 U/ml thrombin (Sigma Aldrich, Poole, UK). They were washed before immediate use. Cell viability was assessed with trypan blue staining. Cell purity was approximately 95% according to flow cytometry and cell activation verified with CXCL4 secretion compared to resting platelets.

Monocytes were isolated using Histopaque density centrifugation followed by either adhesion purification or, for the confocal analysis, the Pan Monocyte Isolation (Human) Kit following the manufacturer's instructions (Miltenyi Biotec, Bisley, UK).

Monocyte-platelet co-culture

Autologous freshly isolated monocytes and platelets were combined at platelet: monocyte of 100:1 unless otherwise stated, in DMEM (Gibco®, Life Technologies, Paisley, UK) supplemented with ampicillin. Cells were then infected with *M.tb* at a monocyte multiplicity of infection (MOI) of 1 and incubated at 5% CO₂ at 37°C.

MMP and cytokine analysis

Supernatant was harvested after 24 hours by centrifugation and sterile filtration. This process does not interfere with detection of MMPs and cytokines (6). Concentrations of MMPs and TIMPs were measured by ELISA (R&D systems Abingdon, UK).

Luminex multiplex magnetic bead array was used for multiplex analysis of MMPs (R&D Systems) and cytokines (Millipore, Watford, UK). Platelet contributions were assessed using a platelet-only control for all measurements and found to be minimal. Unless otherwise stated, values have been presented in graphs following subtraction of platelet-only concentrations for normalisation.

CFU analysis

Cellular killing of *M.tb* was assessed through colony counts performed on Middlebrook 7H11 agar. Culture supernatants were used to assess extracellular killing, and adherent cells lysed with water to assess intracellular killing. The plates were incubated at 37°C for 2-4 weeks before counts were obtained.

MMP-1 Quantitative reverse transcription PCR (qRT-PCR)

After 18 hours of incubation, Trizol cell lysis was performed and RNA was collected. qRT-PCR was performed using the 1-Step RT-PCR master mix (Qiagen, Crawley, UK) according to the manufacturer's instructions on a Stratagene Mx3000P platform. The RT-PCR cycling conditions comprised 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. For MMP-1, the forward primer was 5'- AAGATGAAAGGTGGACCAACAATT -3'; the reverse primer was 5' -CAAGAGAATGGCCGAGTTC -3'; and the probe was 5'- FAM-CAGAGAGTACAACCTTACATCGTGTTGCGGCTC-TAMRA -3'. VIC-labelled β -actin control reagent (Applied Biosystems, Manchester, UK) was used as a reference gene. PCR plasmid standards were used to calculate MMP-1 and B-actin copy numbers.

Confocal Microscopy

Monocytes were cultured with platelets on slides coated with 150 µg/ml of DQ collagen type 1 (ThermoFisher Scientific, Hemel Hempstead, UK) for 24 hours before adherent cells were fixed with 4% paraformaldehyde overnight. Collagen degradation was visualised using a Leica confocal microscope (Leica TCS SP5) and processed using Leica LAS AF Lite 2.6.0 (Leica Microsystems, Germany) and Image J 1.43U (NIH, USA) software. Numerical analysis was performed by assigning arbitrary units to the DQ collagen fluorescence using Image J 1.43U (NIH, USA) and normalising this to the cell number present. A platelet-only control was also included at equivalent concentrations but produced no measurable fluorescent signal.

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded lung samples from five Balb/C *M.tb*-infected mice, harvested after 28 days post infection. All animal procedures were performed under the licence issued by the UK Home Office (PPL 70/7160) and in accordance with the Animal Scientific Procedures Act of 1986. Eight to ten week old female BALB/c mice (Charles River Ltd, UK) were maintained in biosafety containment level 3 facilities according to institutional protocols. Mice were infected with 10⁴CFU of the *M.tuberculosis* clinical isolate, East-Asian/Beijing strain 333 via the intranasal route (n=5 mice per group). At 28 days post-infection, mice were humanely culled and the upper left lobe of the lung was fixed in 10% neutral buffered formalin (Sigma-Aldrich, United Kingdom) for histopathological analysis. (7)

Immunohistochemistry was done in collaboration with Professor Joanna Porter at the University College of London using the Discovery XT instrument and DAB Map Kit (both Ventana). Heat induced epitope retrieval was performed in a pressure cooker using citrate buffer pH6. Anti-CD41 (Abcam, Cambridge, UK) primary antibody incubation was for 12 hrs using a 1 in 40 dilution. Rabbit anti-Rat (Dako, Glostrup,

Denmark) secondary antibody incubation was for 60 mins, using a 1 in 200 dilution. Heat induced epitope retrieval was performed in a pressure cooker using citrate buffer pH6. Slides were haematoxylin counterstained, dehydrated and mounted.

Statistics

Statistical analyses were performed using PRISM Version 6 (GraphPad, La Jolla, CA, US) and STATA 12 (Stata Corp., College Station, TX, USA). Clinical data are represented as median and interquartile range (IQR). Analysis between groups was performed using Wilcoxon matched pairs signed rank test or, for longitudinal data, evaluated over time using the Skillings-Mack statistic. For all *in vitro* experiments, multiple interventions were analyzed by the One Way Anova followed by Tukey's multiple comparison. Graphs are representative of experiments performed from at least three separate donors, with experiments performed in triplicate for each donor, at least three independent experiments performed in triplicate unless otherwise stated, and data are presented as mean \pm standard error. Correlations were calculated with Spearman correlation and scatter plots generated with a log scale. A two tailed P-value was used to assess significance; $p < 0.05$ was considered significant for all statistics.

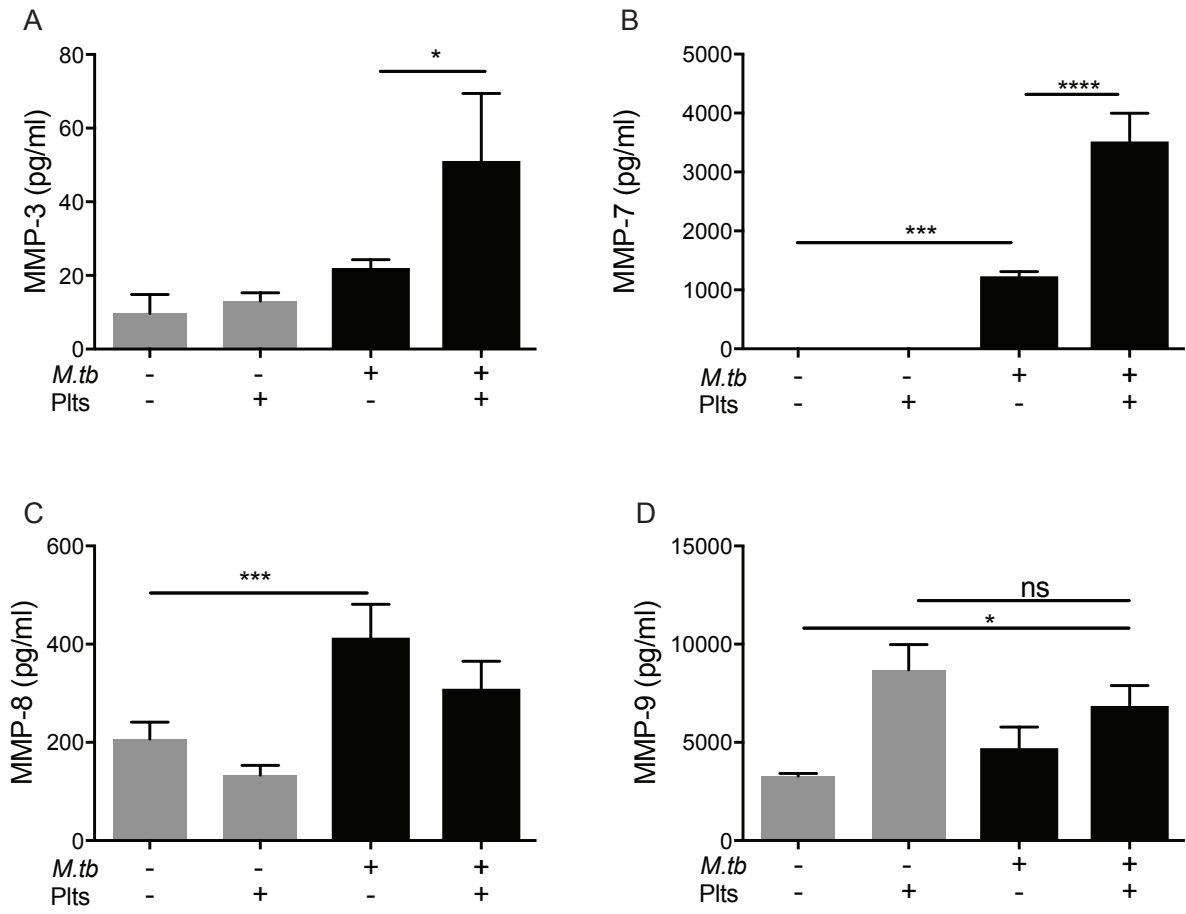
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Figure E1



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Figure E2

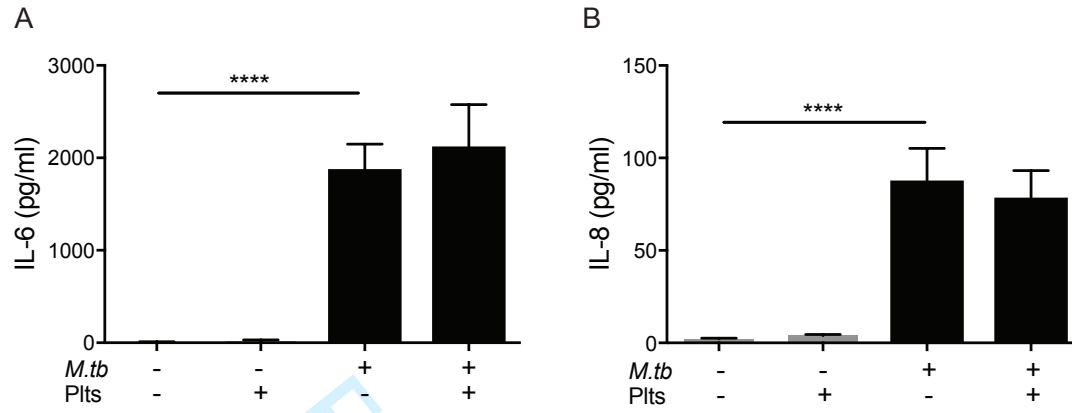
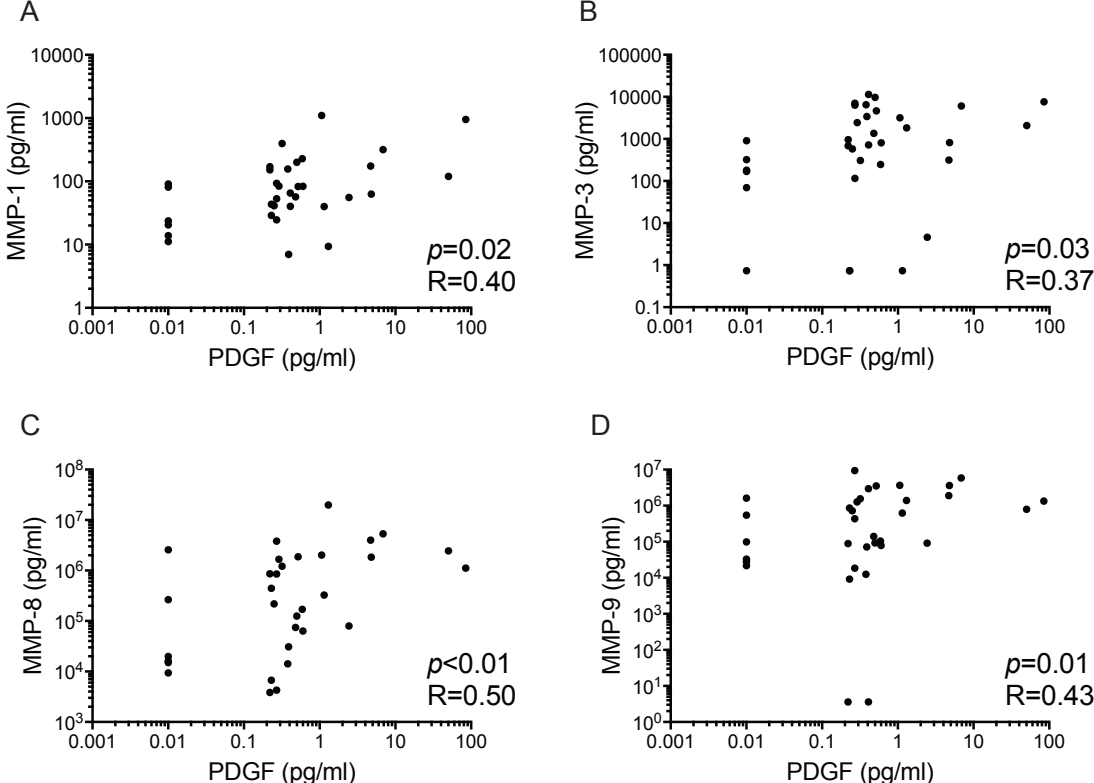
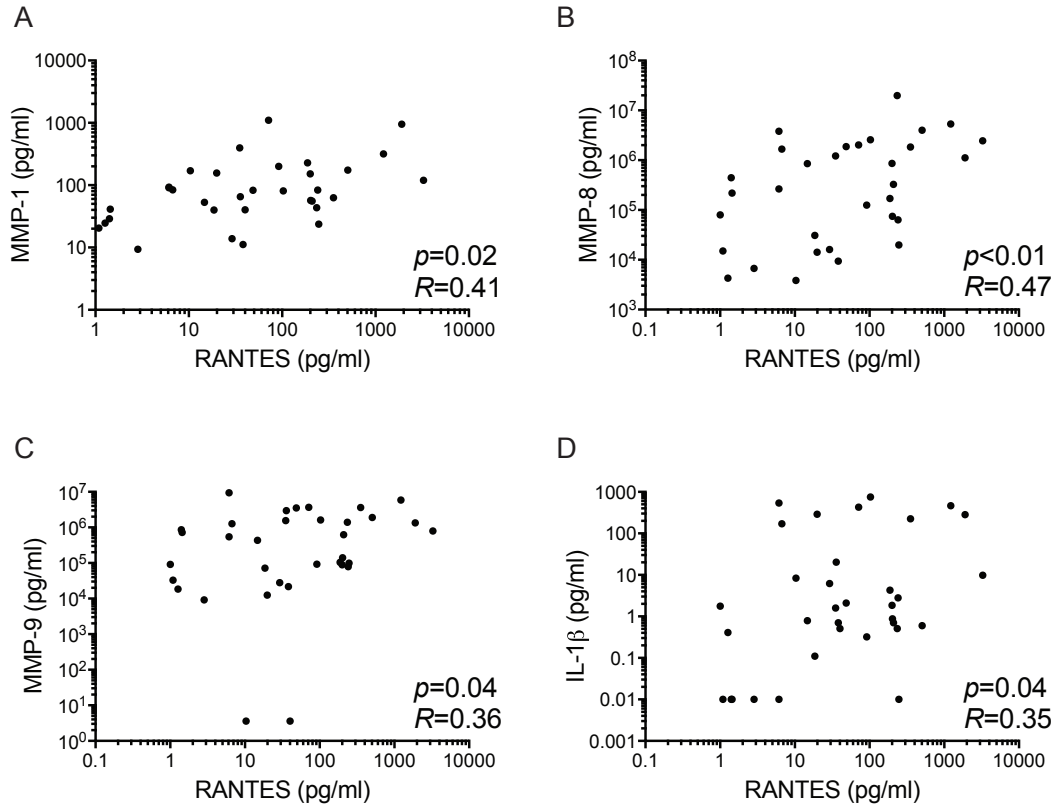


Figure E3



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Figure E4



Platelets regulate pulmonary inflammation and tissue destruction in tuberculosis

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Contributions

KAF, DEK and JSF conceived the study; KAF designed and carried all the cellular experiments and analyzed sputum samples; DEK and RHG obtained clinical data concerning patient plasma samples; JCP carried out immunohistochemistry and associated imaging; NK and BDR carried out the murine experiments from which samples were processed for immunohistochemistry and advised on the data. KAF, DEK, AMW, RHG and JSF drafted the manuscript. All authors reviewed and agreed the final version of the manuscript.

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Running head: Platelets regulate inflammation in tuberculosis

Subject category

7.08 Inflammation: cell-cell interactions

Word Count: 3186

At a Glance Commentary

Scientific Knowledge on the Subject: In addition to their role in coagulation, platelets influence immune responses. However, their role in cellular networks of inflammatory tissue destruction in tuberculosis is unknown.

What This Study Adds to the Field: Platelets drive significant human monocyte-dependent inflammation and collagenase activity in TB, resulting in tissue destruction in both a cellular model of infection and in TB patients.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

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Abstract

Rationale: Platelets may interact with the immune system in tuberculosis (TB) to regulate human inflammatory responses that lead to morbidity and spread of infection.

Objectives: To identify a functional role of platelets in the innate inflammatory and matrix degrading response in TB.

Methods: Markers of platelet activation were examined in plasma from 50 TB patients pre-treatment, and 50 controls. 25 patients were followed longitudinally. Platelet-monocyte interactions were studied in a co-culture model infected with live, virulent *Mycobacterium tuberculosis* (*M.tb*) and dissected using qPCR, Luminex multiplex arrays, matrix degradation assays and colony counts.

Immunohistochemistry detected CD41 expression in a pulmonary TB murine model and secreted platelet factors were measured in bronchoalveolar lavage fluid (BALF) from 15 TB patients and matched controls.

Measurements and Main Results: Five of six platelet-associated mediators were upregulated in plasma of TB patients compared to controls, with concentrations returning to baseline by day 60 of treatment. Gene expression of the monocyte collagenase MMP-1 was upregulated by platelets in *M.tb* infection. Platelets also enhanced *M.tb*-induced MMP-1 and -10 secretion which drove Type I collagen degradation. Platelets increased monocyte IL-1 and IL-10 and decreased IL-12 and monocyte-derived chemokine (MDC, also known as CCL-22) secretion, as consistent with an M2 monocyte phenotype. Monocyte killing of intracellular *M.tb* was decreased. In the lung, platelets were detected in a TB mouse model and

secreted platelet mediators were upregulated in human BALF, and correlated with MMP and IL-1 β concentrations.

Conclusions: Platelets drive a pro-inflammatory, tissue-degrading phenotype in TB.

Word Count: 245 words

Key Words:

Tuberculosis

Matrix metalloproteinases

Platelets

Human

Innate immunity.

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Introduction

Approximately 2 billion people are infected with *Mycobacterium tuberculosis* (*M.tb*), which killed around 1.8 million people in 2015 alone (1). In pulmonary tuberculosis (TB), inflammatory tissue damage leads to cavity formation and ultimately morbidity, mortality, and onward transmission (2). This journal has recognized the importance of the host response to *M.tb* throughout its 100 year history (3) and, with drug resistance increasing worldwide and limited new therapies, there is an increasing interest in host-directed therapy for TB. Further understanding of TB immunopathology is needed to support progress in this area.

In addition to their classic role in haemostasis, platelets modulate innate and adaptive immune responses (4). Platelet interaction with monocytes regulates cell recruitment, maturation, and secretion of cytokines and MMPs (5-7). High platelet numbers are found in pulmonary vessels, and the lung (the main target organ for *M.tb*) is a major site of platelet production (8). Both platelet count and activity are increased in TB (9, 10). Platelets express surface markers such as CD41 (11), and upon activation secrete mediators from their granules. These include platelet factor-4 (PF4), which is primarily platelet-secreted, and platelet-derived growth factor (PDGF)-BB, RANTES, P-selectin and MMP-9 which are also secreted by other cell types (12-14). Pentraxin-3 (PTX3) interacts with platelet-derived P-selectin to regulate platelet activity (15).

Central to TB pathology is development of a tissue degrading phenotype due to innate immune cell activation (2, 16). *M.tb* infection upregulates intercellular networks and secretion of cytokines such as IL-1 β , IL-10 and IL-12, which influence replication, spread, and persistence of *M.tb* (17). Innate immune responses also

drive secretion of matrix metalloproteinases (MMPs) such as the collagenase MMP-1, and these degrade the pulmonary extracellular matrix (ECM) to cause tissue destruction (18). As well as transcriptional regulation, MMP activity is regulated post-translationally by tissue inhibitors of metalloproteinases (TIMPs) and other MMPs such as MMP-10, which regulates MMP-1 (19, 20).

Monocytes migrate early from the circulation into *M.tb*-infected tissue, where they differentiate to specialized cell types that control bacterial replication, granuloma formation and inflammatory tissue destruction (21-23). Platelets may influence monocyte maturation and cytokine responses to *M.tb* antigens and Bacillus Calmette-Guérin (BCG) stimulation, and they drive development of multinucleated giant cells (24). However, platelet regulation of monocyte functions is not understood in the context of TB immunopathology.

In this study, we investigate the hypothesis that platelets have a key role in inflammatory tissue destruction in pulmonary TB. We demonstrate platelet activation in the circulation of pulmonary TB patients. We establish platelets to be key regulators of *M.tb*-driven monocyte collagen degradation, and demonstrate a role of platelets in the control of intracellular monocytic bacterial replication. Finally, we show platelets at the site of infection in pulmonary TB.

Methods

Further information on methodology is in the supplementary methods section.

Clinical Studies

Plasma samples were collected in prospective studies involving adult TB patients in Lima, Peru with a new diagnosis of culture-positive, drug-sensitive pulmonary TB (25). 50 age- and sex-matched healthy controls were asymptomatic and purified protein derivative (PPD)-negative. All patients provided written informed consent, and the study had ethical approval from Universidad Peruana Cayetano Heredia, the Asociación Benéfica PRISMA, and each participating centre's ethics committee. Appetite was assessed using the Short Nutritional Assessment Questionnaire (SNAQ). Plasma and sputum samples were obtained at Day 0 and plasma samples were collected from 25 patients longitudinally at days 14, 28, and 60 of treatment. Bronchoalveolar lavage fluid (BALF) was collected from patients in a previously described study (26).

Cell Isolation and Culture

Human platelets were isolated and activated with 0.1 U/ml thrombin (27). Monocytes were isolated using either adhesion purification or, for confocal microscopy, the Pan Monocyte Isolation (Human) Kit (Miltenyi Biotec, Bisley, UK). *M. tuberculosis* H37Rv Pasteur (*M.tb*) was cultured in Middlebrook 7H9 medium (28).

Autologous freshly isolated platelets and monocytes from healthy volunteers were co-cultured at a ratio of 100:1 unless otherwise stated, and infected with *M.tb* at a multiplicity of infection (MOI) of 1.

Secreted cytokine and MMP analysis

Clinical samples were analyzed by Luminex magnetic bead array (R&D Systems, Abingdon, UK). ELISA or Luminex array were used to measure cell culture concentrations of MMPs, TIMPs (both R&D Systems, Abingdon, UK) and cytokines (Millipore, Watford, UK).

Cytokine and MMP gene analysis

RNA was extracted and qRT-PCR performed using β -actin as a reference gene. The 1-Step RT-PCR master mix (Qiagen, Crawley, UK). PCR plasmid standards were used to calculate copy number of MMP-1 and β -actin mRNA, and fold change compared to controls.

Colony forming unit (CFU) analysis

Lysed adherent cells or culture supernatants were plated onto Middlebrook 7H11 agar and incubated at 37 °C for 2-4 weeks prior to CFU counting.

Functional assay of tissue destruction by confocal microscopy

Cells were cultured on slides coated with DQ collagen type 1 (ThermoFisher Scientific, Hemel Hempstead, UK) and imaged with a Leica confocal microscope. Images were processed using Leica LAS AF Lite 2.6.0 and Image J 1.43U software (NIH, USA).

Immunohistochemistry

Immunohistochemistry using the Discovery XT instrument and the DAB Map Kit (Ventana medical systems, Tucson, AZ, USA) was performed on paraffin embedded day 28 post-infection lung samples from Balb/C mice collected as part of a previously published study (29).

Statistics

Analyses were performed using PRISM Version 6 (GraphPad, La Jolla, CA, USA) and STATA 12 (Stata Corp., College Station, TX, USA). Clinical data are medians and interquartile ranges (IQR) and analyzed using Wilcoxon matched pairs signed rank test or the Skillings-Mack statistic. Pearson D'Agostino testing demonstrated data were not normally distributed. Cell culture data are mean \pm standard deviation

(SD) and analyzed by One Way Anova and Tukey's correction for multiple comparisons. Graphs are representative of at least three independent experiments. Correlations were calculated with Spearman correlation. $p < 0.05$ was considered significant.

Results

Active platelet-associated mediators circulating in TB patients

Clinical characteristics of the 50 newly diagnosed TB patients and 50 healthy controls matched for age and sex are presented in Table 1. TB cases weighed less and had a significantly lower body mass index (BMI) than controls at recruitment (BMI 20.4 vs 27.2 respectively; $p < 0.0001$), and appetite measured by SNAQ, was poor compared to controls ($p < 0.001$).

PDGF-BB, RANTES and PF4 concentrations were significantly upregulated in patients compared to controls (all $p < 0.0001$; Fig 1A-1C). MMP-9 concentrations were higher in untreated TB patients versus healthy controls (89,124 pg/ml and 68,057 pg/ml respectively) ($p < 0.05$; Fig 1D) as were PTX3 concentrations (433 pg/ml and 94.0 pg/ml respectively) ($p < 0.001$; Fig 1E). There was no difference in P-selectin concentration ($p > 0.05$; Fig 1F).

In TB patients, there was strong correlation between concentrations of PDGF-BB and PF4 ($R = 0.89$), PDGF-BB and RANTES ($R = 0.65$), and PF4 and RANTES ($R = 0.70$). There was a moderate correlation between PF4 and MMP-9 concentrations ($R = 0.54$) and a weaker correlation between concentrations of MMP-9 and PTX3 ($R = 0.47$), MMP-9 and PDGF-BB ($R = 0.45$), PTX3 and PDGF-BB ($R = 0.48$),

PDGF-BB and P-selectin ($R=0.45$), and PF4 and PTX3 ($R=0.44$). Among control subjects, there was strong correlation between levels of RANTES and PDGF-BB ($R=0.93$), RANTES and PF4 ($R=0.87$), and between PDGF-BB and PF4 ($R=0.88$). There was moderate correlation between concentrations of PTX3 and PDGF-BB ($R=0.56$) and between PTX3 and PF4 ($R=0.50$), and weaker correlation between PTX3 and RANTES ($R=0.49$, all $p<0.05$).

Longitudinal changes in platelet-derived mediators during treatment of TB patients

Next, we performed a longitudinal assessment of platelet activity over the first 60 days of TB treatment in 25 patients. Biometric and microbiological data are shown in Table E1 in the online data supplement. 31/50 patients (62%) remained sputum culture-positive at Day 14, 20 patients (40%) at Day 28, and 5 patients (10%) at Day 60. Mean BMI of TB cases steadily increased from 20.4 at baseline to 22.0 by Day 60, and mean SNAQ score from 13.3 to 15.9. There was no difference in age, sex, mean BMI, or mean SNAQ score between TB cases who were followed up compared to those who were not (data not shown).

Over 60 days of treatment, plasma concentrations of PF4 and PDGF-BB changed significantly in TB patients ($p<0.05$; Fig 2A-B). There was a trend towards an increase at Day 14 but concentrations subsequently decreased to near control values by Day 60 (Fig 2A-B). P-selectin concentrations, which were not upregulated at presentation with TB, did not alter during treatment (Fig 2C).

Platelet-monocyte interactions drive MMP-1 & -10 gene expression and secretion in TB

Since there was good evidence of platelet activation in TB patients, we developed a co-culture model to investigate the functional role of platelets in regulating the human

inflammatory immune response. A major outcome of this response is MMP-driven ECM destruction in which the collagenase MMP-1 is key (16). Monocytes co-stimulated with platelets and *M.tb* had a 4.3-fold increase in *Mmp-1* expression compared to *M.tb*-stimulated monocytes alone ($p < 0.0001$; Fig 3A). Platelets did not significantly upregulate *Mmp-1* expression in uninfected monocytes. MMP-1 secretion was increased 6.6-fold from *M.tb*-stimulated monocytes co-cultured with platelets compared to *M.tb*-infected monocytes alone ($p < 0.0001$; Fig 3B), although platelets did not alter MMP-1 secretion in control cells. This effect was dependent on the ratio of platelet:monocytes (P:M) (Fig 3C), increasing up to 100:1.

The MMP-1 activator MMP-10 was similarly upregulated by platelets. MMP-10 concentrations increased from $318 \pm 106.9.1$ pg/ml to 1972 ± 405.4 pg/ml upon exposure to *M.tb*. This increased further to 3423 ± 363.2 pg/ml with exposure to *M.tb* in the presence of platelets (both $p < 0.001$; Fig 3D). Significant MMP-10 upregulation was seen with P:M ratios of 50:1 ($p < 0.01$) and this increased further at a ratio of 100:1 (Fig 3E). However, MMP-10 secretion decreased with P:M ratio of 500:1 to below that seen with monocytes stimulated by *M.tb* alone ($p < 0.0001$; Fig 3E). In addition, platelet co-culture increased monocyte secretion of MMP-3, -7 and -9, while MMP-8 was not changed. Similar to MMP-1 and -10, platelet upregulation of MMP-3 and -7 required *M.tb* exposure (see Figure E1 in the online data supplement). TIMP-1 and -2 secretion from monocytes in the presence or absence of *M.tb* were not altered by platelet co-culture (Fig 3F-G).

Platelets regulate monocyte-dependent collagen destruction in TB

To investigate the impact of platelets on monocyte's functional MMP-1 activity, monocytes were cultured on type I collagen that fluoresces on degradation. Confocal

microscopy revealed monocytes alone had minimal collagenase activity when uninfected, regardless of platelet co-culture. *M.tb* stimulation of monocytes induced some functional collagenase response but this was significantly increased in monocytes stimulated by *M.tb* in the presence of platelets (Fig 4A). Quantitative analysis revealed a 2.5-fold increase in collagenase activity of *M.tb*-exposed co-cultured cells compared with *M.tb*-exposed monocytes in the absence of platelets ($p < 0.01$; Fig 4B). Platelet-only controls produced no measurable collagen degradation (not shown).

Platelet regulation of monocyte inflammatory responses

Next, we investigated the effect of platelet-monocyte interactions on the secretion of inflammatory cytokines. There was a marked increase in IL-1 β secretion from *M.tb*-stimulated monocytes in the presence of platelets compared with *M.tb*-stimulated monocytes alone ($p < 0.0001$; Fig 5A). This trend was not seen in the inflammatory cytokines interferon- γ (Fig 5B) or TNF- α (Fig 5C). IL-10 was upregulated 7.5-fold in *M.tb*-infected platelet co-culture compared to single culture monocytes ($p < 0.0001$; Fig 5D). In contrast, *M.tb*-infected monocytes in platelet co-culture secreted 6.4-fold less IL-12 and 6.1-fold less monocyte-derived chemokine (MDC) (both $p < 0.001$; Fig 5E-F). There was no platelet regulation of the immunomodulatory cytokine IL-6 or the chemokine IL-8 (see Figure E2 in the online data).

Platelet co-culture increases monocyte intracellular M.tb replication

In addition to regulation of monocyte-derived inflammatory mediators, monocyte co-culture with platelets altered the intracellular killing of *M.tb*. Although extracellular *M.tb* did not change in platelet co-culture, 2.5-fold more viable intracellular *M.tb* was detected in co-cultured cell lysates ($p < 0.001$; Fig 6).

Active platelets are present at the site of infection in pulmonary TB.

Next, we investigated whether platelets were present at the site of TB infection itself.

Like most murine TB models, Balb/C mice do not form organised granulomas with central necrosis but *M.tb* infection and dissemination through tissue does result in large areas of highly inflamed pulmonary tissue. Therefore, we stained lung tissue from a murine model of infection for the platelet marker CD41 (Fig 7A-B). Platelets are anucleate cells and this is predominately reflected by the staining. Interestingly, there were large numbers of platelets detected in the alveoli. Limited CD41 association with nucleated cells can be seen which is largely restricted to areas of granuloma-like structure and giant multinucleate cell formation. In peripheral lung areas with limited inflammation there was minimal detection of platelets (Fig 7C).

To confirm that platelets were active at the site of infection, human BALF samples obtained during a previous clinical study (26) were analyzed for platelet-derived mediators and compared to respiratory symptomatic control patients. There was a significant upregulation of PDGF-BB concentration in BALF from TB cases compared to controls ($p < 0.01$; Fig 7D). Both P-selectin and RANTES concentrations were also increased in TB patients ($p < 0.01$; Fig 7E-F) but PF4 concentrations were not (Fig 7G).

In human BALF, P-selectin concentrations correlated significantly with increasing concentrations of IL-1 β and MMP-1 (both $p < 0.001$), and MMP-7, -8 and -9 (all $p < 0.01$) ($R = 0.5$; Fig 8A-E). P-selectin correlation with MMP-3 was also statistically significant ($R = 0.4$; $p < 0.05$; Fig 8F). In addition, BALF PDGF-BB concentrations correlated with MMP-1, -3, -8 and -9 (see Figure E3 in the online data supplement). BALF RANTES concentrations correlated with MMP-1, -8 and -9 as well as IL-1 β (see Figure E4 in the online data supplement).

Discussion

This study used a longitudinal patient study, a human cellular model, a murine model, and a more invasive bronchoscopy-based clinical study to investigate the activation and function of platelets in the innate inflammatory response to TB.

Plasma concentrations of platelet-associated factors were significantly upregulated in a cohort of patients with pulmonary TB. This is consistent with reports that concentrations of PTX3 (30) and PF4 (10, 31, 32) increase in pulmonary TB along with various platelet indices such as platelet count and mean platelet volume (9, 33). In our study, there was a transient increase in platelet-associated mediators when patients received anti-TB treatment followed by a decrease towards plasma concentrations of healthy controls. These mediators are not necessarily exclusively produced by platelets. However, the concentrations of different platelet-derived mediators were significantly correlated and this is consistent with co-storage and co-secretion. Interestingly, plasma PF4 levels have been previously correlated with TB disease activity (31).

As the secretion of the collagenase MMP-1 is a key driver of tissue destruction of the lung observed in TB (18), we investigated the role of platelets on monocyte MMP-1 secretion in detail using platelet monocyte ratios similar to those observed in man. Platelets were added to monocytes in a thrombin-activated form, reflecting the activated state we observed in our clinical studies and in keeping with techniques standard across the literature (34-36). In macrophages produced *in vitro* from platelet-matured monocytes, *Mmp-1* is among the top five upregulated genes (24). Platelets alone do not regulate monocyte MMP-1 gene expression or secretion,

which may reflect control mechanisms limiting platelet proinflammatory activity. However, platelets significantly upregulated monocyte MMP-1 mRNA accumulation and protein secretion in the presence of the additional stimulus of *M.tb* infection. There was a concurrent increase in the MMP-1 activating enzyme MMP-10, and upregulation was not associated with any concurrent increase in TIMP secretion. Consequently, functional collagen degradation was increased markedly in platelet-monocyte co-culture.

The platelet-dependent upregulation of monocyte IL-1 β further demonstrates that platelets drive monocyte inflammatory responses since IL-1 β is essential for nitric oxide production, phagosomal acidification and maturation, and adhesion molecule expression (37). Knockouts of IL-1 activity in pulmonary TB murine models have been used to demonstrate a protective role of this cytokine, which can be associated with polymorphonuclear leukocytes. IL-1 β regulates the number and development of granulomas, limits *M.tb* growth, and is associated with proper induction of other cytokine responses (38, 39). The upregulation of IL-1 β was specific and not observed in either interferon- γ or TNF- α secretion. Upregulation of IL-1 β has previously been shown to be dependent on caspase-1 processing in *M.tb* infection (40) and the data may therefore suggest a role of platelets in the upregulation of inflammasome complexes, such as the caspase-1-NLRP3 inflammasome.

In a novel observation, we found that *M.tb*-stimulated monocyte MDC secretion is decreased by over 50% when co-cultured with platelets. MDC acts through the C-C chemokine receptor type 4 (CCR4) and is associated with pulmonary inflammation (41). Such changes in IL-10, IL-12, and MDC are typically attributed to monocytes maturing with an M2 macrophage phenotype. This phenotype is associated with significant tissue remodelling properties, including secretion of MMPs involved in

both tissue damage and tissue repair (42-45). Prolonged *M.tb* infection tilts monocyte-derived cells towards an M2 phenotype, which is the major phenotype found in TB granulomas (46). Our data indicate that platelets may be involved in driving such differentiation. M2 type monocyte-derived macrophages are also associated with increased bacterial replication and progression of disease in TB patients (47, 48). We observed increased IL-10, which enhances mycobacterial intracellular survival and growth by suppressing innate and adaptive immune responses (49, 50) but decreased IL-12, which is strongly associated with *M.tb* killing (51). CFU analysis of cell lysates reflected this, as we observed a 2.5-fold increase in intracellular survival of *M.tb* in monocytes with platelet co-culture compared to without co-culture. The lack of effect by platelets on extracellular CFU counts suggests that platelets do not affect internalization of *M.tb* by monocytes.

In the murine model, we demonstrated platelets concentrated at the site of TB pathology in the lung. There were platelets in some alveolar air pockets and this is consistent with the breakdown of the epithelial barrier resulting in the common symptom of haemoptysis. The images also showed platelets associated with nucleate cells. This is consistent with both tight platelet adherence to recruited cells as they extravasate from the blood vessels, and platelet phagocytosis during monocyte maturation (52). Further magnification revealed that most of the CD41 staining was associated with nuclei of developing multinucleate cells. This supports previous immunohistochemistry of human TB lung detecting the platelet integrin CD42b, which demonstrated that monocyte phagocytosis of platelets drives development of multinucleated giant cells (24).

Finally, the analysis of human TB patient BALF samples demonstrated upregulation of PDGF-BB, P-selectin, RANTES and CD40L consistent with platelet activation in

pulmonary TB. These mediators correlated with activity of specific MMPs, including the important collagenase MMP-1, and is consistent with our cellular data. Previous work found that PDGF-BB concentrations in the epithelial lining fluid after six months of treatment correlated with worsening pulmonary fibrosis scores (53). The role of platelets in long-term immunopathology and fibrosis following TB infection merits further study.

In summary, we demonstrated that markers of platelet activity are increased in plasma of patients with pulmonary TB compared to healthy controls, and these are then normalized with anti-mycobacterial treatment. We demonstrate that platelets alter the proinflammatory response resulting in increased tissue destruction and development of an IL-1 β , IL-10 high, IL-12 low milieu that is associated with increased pathogen growth. Platelets are present within the infected lung tissue itself and active in human disease. In TB, large-scale tissue damage and bacterial replication is associated with onward transmission, morbidity, and mortality. Our data indicate a previously unappreciated role for platelets in driving this pathology, which may have implications for host-directed therapies in the era of rising drug resistance.

Acknowledgments

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Figure Legends

Figure 1. Plasma concentrations of platelet-derived mediators in pre-treatment TB patients and control subjects. (A) PDGF-BB, (B) RANTES, (C) Platelet Factor 4 (PF4), (D) MMP-9, and (E) Pentraxin (PTX)-3 were higher in patients with pulmonary TB prior to treatment (shaded boxes) compared to age- and sex-matched healthy controls (open boxes). (F) Plasma concentrations of P-selectin were unchanged in TB patients compared to controls. Data presented as median and IQR. Whiskers represent 10th and 90th percentiles and outline values are shown. P-values calculated using the Wilcoxon matched-pairs signed rank test. N=50 for both groups. *p<0.05, ***p<0.001, ****p<0.0001.

Figure 2: Plasma concentrations (pg/ml) of (A) Platelet Factor 4 (PF4) and (B) PDGF-BB but not (C) P-selectin decrease to baseline after the first 60 days of treatment in TB patients (shaded boxes). Age- and sex-matched healthy controls are shown by open boxes. Data presented as median and IQR. Whiskers represent 10th and 90th percentiles and outline values are shown. Significance of change over time assessed using Skillings-Mack statistic. N=25 for both groups. *P<0.05.

Figure 3. Monocyte MMP-1 and MMP-10 is regulated by platelets in *M.tb* infection. Monocytes were cultured with autologous platelets (Plt) and virulent *Mycobacterium tuberculosis* (*M.tb*) before analysis. (A) Monocytes were cultured at platelet:monocyte ratios (P:M) of 100:1 for 18 hours. Y axis shows MMP-1 mRNA copy number normalized to β -actin and displayed as a fold change of the unstimulated control monocytes. Bars represent mean \pm SD for N=3. (B-G) Monocytes were cultured with Plt and virulent *M.tb* for 24 hours. Y axis shows

secreted MMPs or TIMPs in pg/ml. (B) Monocytes were cultured at P:M ratio of 100:1 with *M.tb* stimulus. Platelets significantly increased MMP-1 secretion with *M.tb* stimulus but not controls. (C) Monocytes were cultured with increasing P:M to show a dose-dependent response of *M.tb*-stimulated monocytes up to P:M 500:1. (D) Monocytes cultured at P:M of 100:1 with *M.tb* stimulus had significantly increased MMP-10 secretion compared with monocytes alone. (E) Monocytes cultured with increasing P:M had increasing MMP-10 secretion until 100:1, after which secretion returned to baseline. (F-G) Monocytes cultured at P:M of 100:1 did not have altered TIMP-1 or TIMP-2 secretion compared with controls. Data are mean \pm SD and are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Monocyte collagenase activity in *M.tb* infection is upregulated by platelet co-culture. Monocytes were cultured with autologous platelets (Plt) and virulent *M.tb* for 24 hours on DQ collagen type I, which fluoresces upon degradation. A) Monocyte-dependent collagenase activity at a high level is only seen in *M.tb*-infected cells cultured in the presence of platelets. Although some collagenase activity is detected in *M.tb*-stimulated monocytes alone, none is found in the absence of *M.tb*. B) Collagen fluorescence of each image was quantified in triplicate and normalised to cell number. Data are mean \pm SD and are representative of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

Figure 5. Platelet interaction alters cytokine and chemokine secretion from human monocytes stimulated by *M.tb*. Monocytes were cultured with autologous platelets (Plt) and live virulent *M.tb* for 24 hours and proinflammatory mediators

measured in cell culture medium. Y axis shows secreted analytes in pg/ml. (A) IL-1 β was significantly increased in *M.tb* stimulation, and increased further by platelet co-culture. (B) Interferon (IFN)- γ was not significantly regulated by platelet stimulation. (C) TNF- α was not significantly regulated by platelet stimulation. (D) IL-10 was significantly upregulated by platelets in *M.tb* stimulated monocytes but not controls. (E) *M.tb* upregulation of IL-12p70 was reduced in the presence of platelets. (F) *M.tb* upregulation of macrophage derived chemokine (MDC) was reduced in the presence of platelets. Data are mean \pm SD and are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. Platelet co-culture increases intracellular bacterial viability in monocytes. Human primary monocytes were cultured with autologous platelets (Plt) and infected live virulent *M.tb* for 24 hours. Bacterial killing assessed by colony forming unit (CFU) counts of *M.tb* obtained from both the intracellular (Intracell) and extracellular (Extracell) environment. Whilst extracellular CFU remained the same, significantly more bacteria was rescued from platelet-cultured monocytes than controls. Data are mean \pm SD and are representative of three independent experiments. *** $p < 0.001$.

Figure 7. Active platelets are upregulated at the site of infection in pulmonary TB. (A-C) Platelets are localized to the lung in murine TB. CD41 staining was performed on regions of (A-B) significant *M.tb*-inflammation and tissue remodelling and (C) peripheral lung with minimal inflammation in *Mtb*-infected Balb/C mice. CD41 is highly expressed in detectable in lung areas with significant TB pathology of inflammation and tissue remodelling. Whilst most CD41 staining was associated with

anucleate cells consistent to platelet morphology, some staining of nucleated cells may represent expression of the marker or phagocytosis of platelets or adherence of platelets. Images from a single mouse and representative of N=5 mice. (D-G) Platelet-derived mediators are upregulated in bronchoalveolar lavage fluid (BALF) from TB patients compared to people with other respiratory illnesses. Bronchial lavage fluid from 15 TB patients (cases) and 15 respiratory symptomatic controls (control) were analysed by multiplex analysis for: (D) PDGF-BB, (E) P-selectin, (F) RANTES and (G) Platelet Factor 4 (PF4). Data are median and IQR, Whiskers represent 10th and 90th percentiles and outline values are shown. **p<0.01

Figure 8. P-selectin expression correlates with MMP and IL-1 β concentrations in BALF. P-selection concentrations measured in BALF of 15 TB patients and 15 respiratory symptomatics by multiplex assay correlated with secretion of (A) IL-1 β , (B) MMP-1, (C) MMP-7, (D) MMP-8, (E) MMP-9 and (F) MMP-3 was assessed using Spearman rank correlation (R). Scatter plots are presented using a log scale. P-values and R-values are shown inset.

Table 1. Demographics and clinical characteristics of tuberculosis cases pre-treatment and healthy controls.

	Cases, pre-treatment (n=50)	TB-negative controls (n=50)	P-value
Age, mean (SE) ^a	35 (1.90)	35 (1.93)	0.72
Males, n (%)^b	34 (68)	34 (68)	1.0
Height, cm. Mean (SE) ^{a*}	163.6 (1.53)	159.1 (1.29)	0.022
Weight at diagnosis, kg. Mean (SE) ^{a*}	54.6 (2.28)	68.6 (1.94)	<0.0001
Body Mass Index (BMI). Mean (SE) ^{a*}	20.4 (0.82)	27.2 (0.86)	<0.0001
SNAQ score. Mean (SE) ^a	13.3 (0.33)	15.0 (0.27)	0.0008
^a P-value calculated using paired two sample t-test; ^b P-value calculated using Fisher's exact test *Weight, Height, and BMI data missing for 2 cases. BMI data excluded for one case (lower limb amputation)			

Figure 1

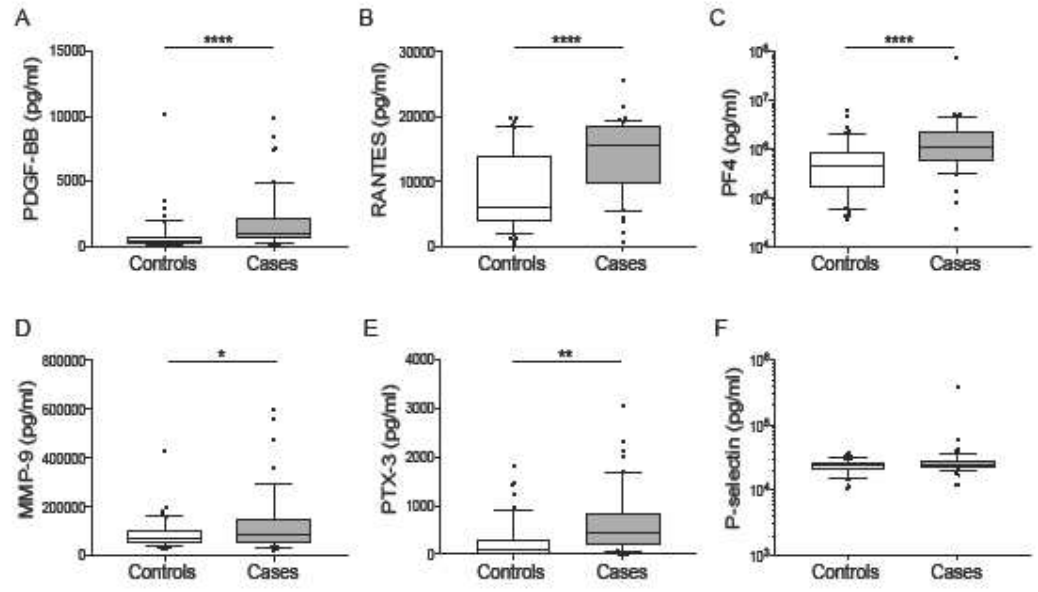


Figure 2

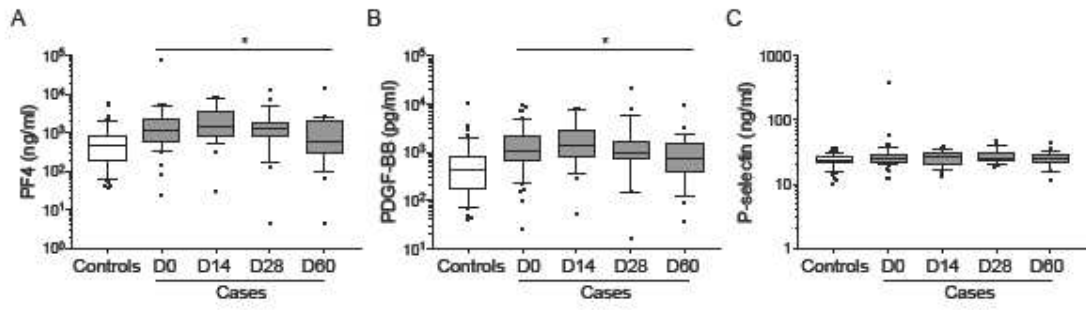


Figure 3

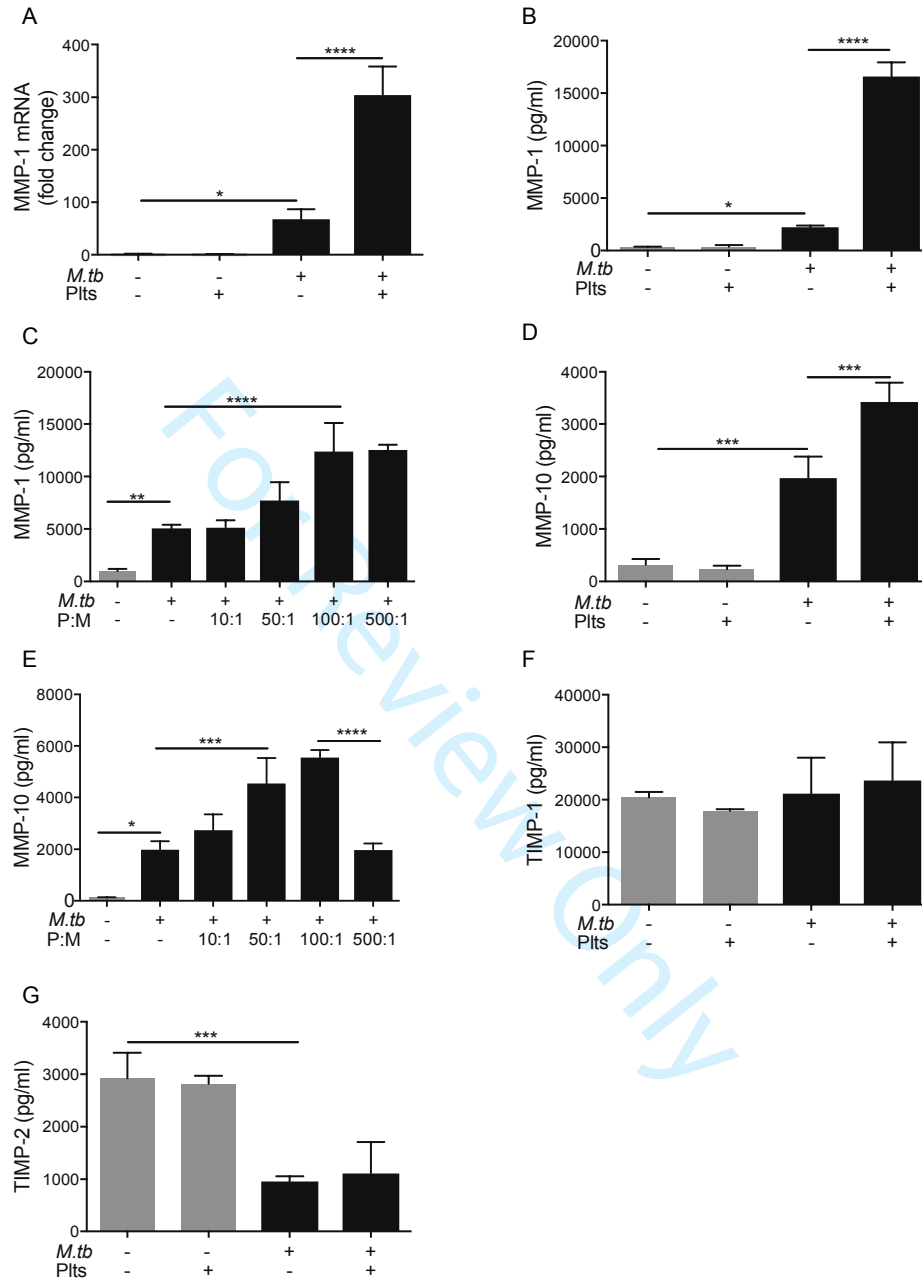


Figure 5

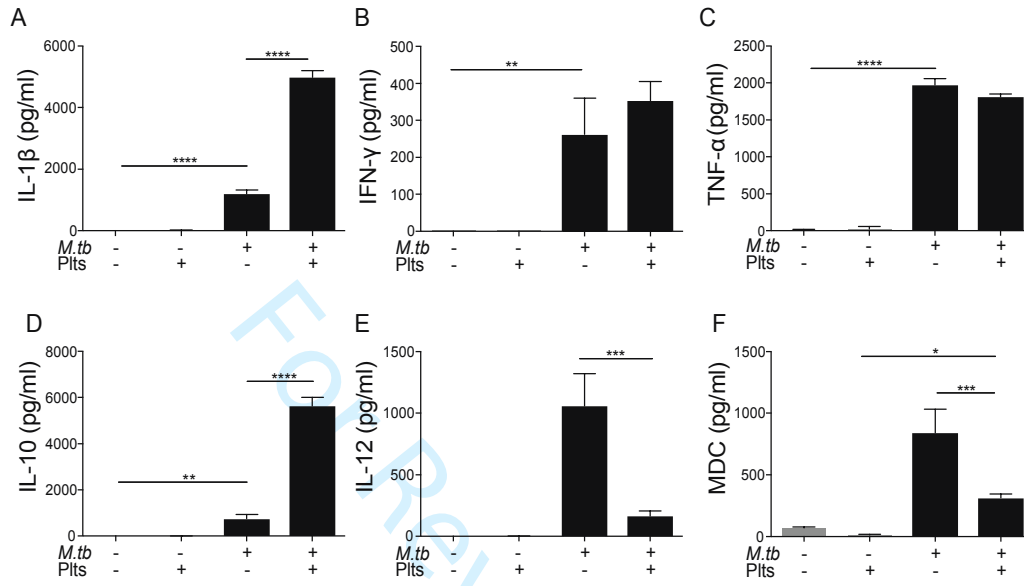
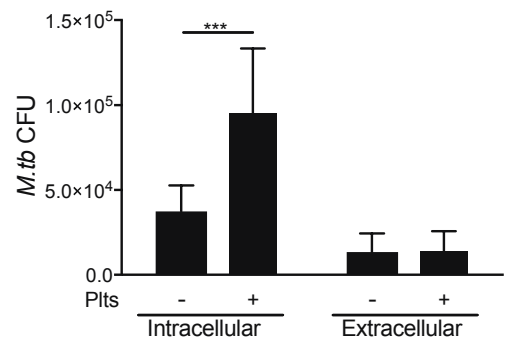


Figure 6



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Figure 8

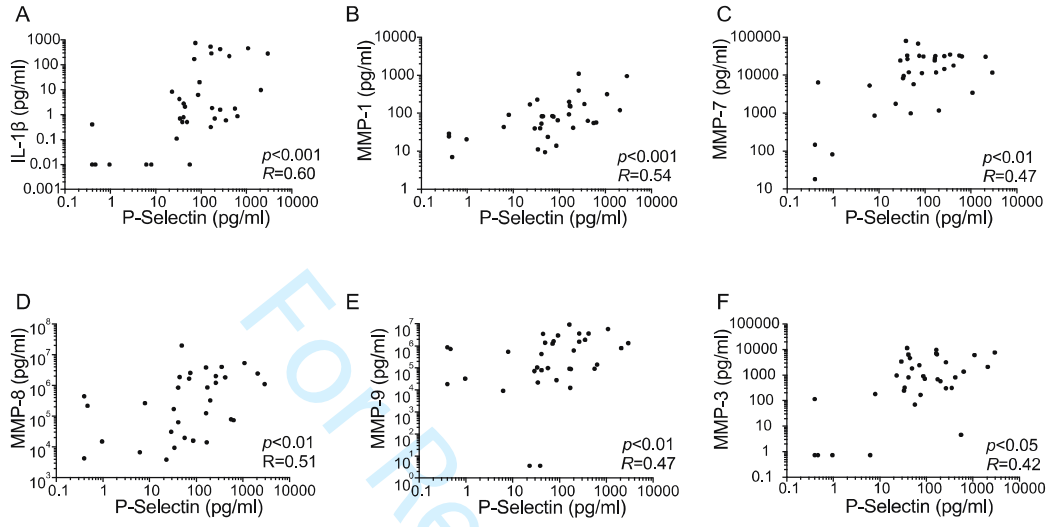


Figure 4

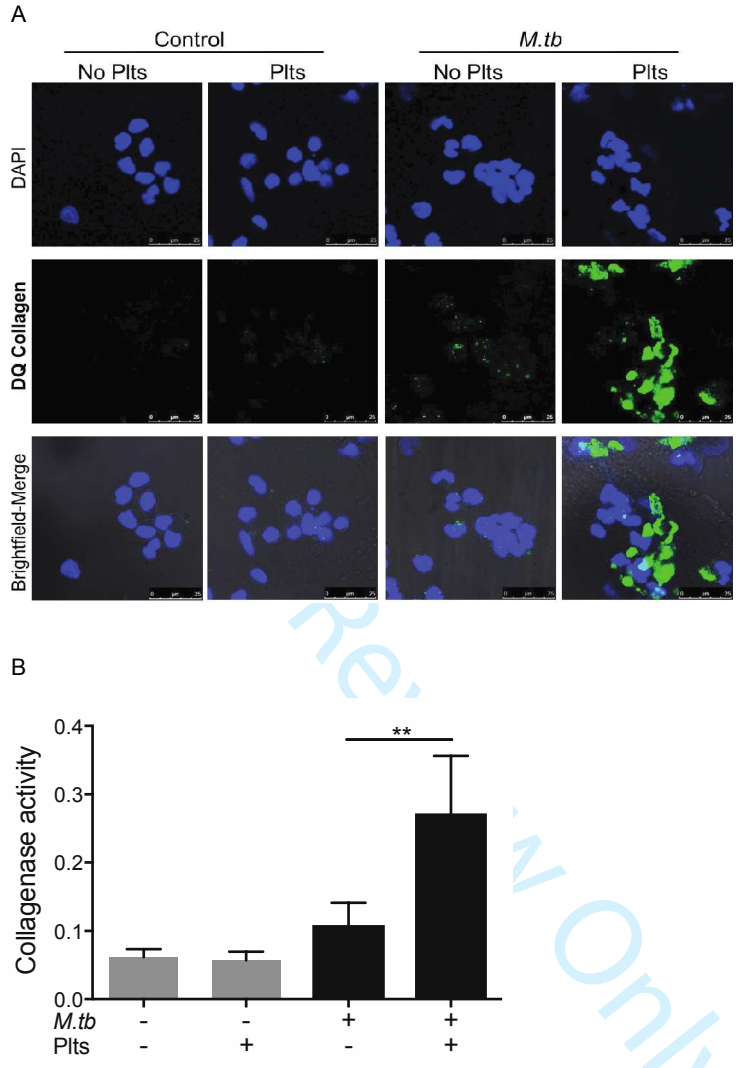
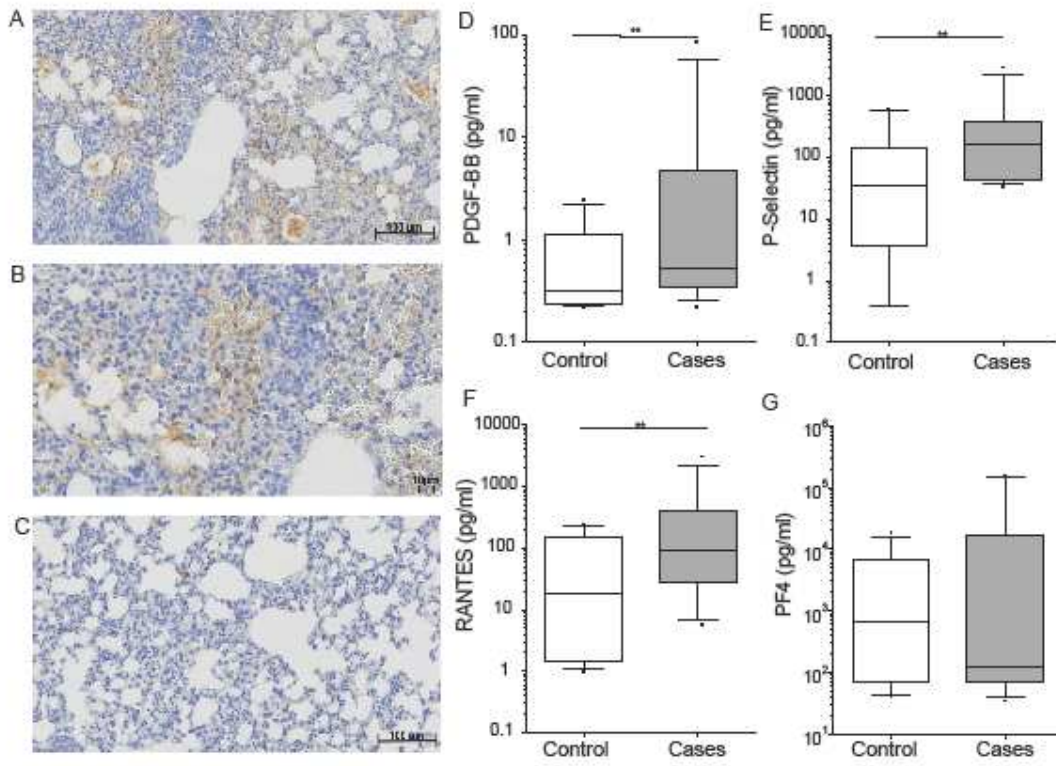


Figure 7



Methods for supplementary submission

Clinical Studies

Plasma samples were collected as part of a prospective cohort study of cough and biomarkers in the treatment response to TB performed in Lima, Peru. The study population comprised adult patients with tuberculosis and healthy controls were asymptomatic and PPD-negative individuals recruited from the same population. Inclusion and exclusion criteria have been published elsewhere (1).

At each visit, height and weight were documented and appetite was assessed using the Short Nutritional Assessment Questionnaire (SNAQ) (2, 3). 10 mls of blood was collected into tubes containing EDTA, centrifuged at 1,000 g, and aliquots of plasma were placed into dry ice within 30 minutes of blood extraction then transported to a specimen biobank maintained at -70°C. Sputum samples were also collected from TB patients for smear microscopy and culture with simultaneous Isoniazid- and Rifampicin-resistance testing using the Microscopic-Observation Drug-Susceptibility (MODS) assay. TB patients underwent evaluation at baseline (pre-treatment), and at regular intervals thereafter, whereas controls had a single evaluation only.

For analysis, 50 patients with culture-positive pulmonary tuberculosis (TB), whose *Mycobacteria tuberculosis* isolate was found to be sensitive to isoniazid and rifampicin according to the MODS assay, and who had completed follow-up to Day 60, were selected. In addition, 50 controls were age- and sex-matched from the database of healthy controls. Plasma concentrations of platelet-derived growth factor (PDGF)-BB, RANTES, P-selectin, platelet factor-4 (PF4), MMP-9, and Pentraxin-3 (PTX3) were measured from plasma samples obtained at Day 0 from all cases and all controls. In addition, longitudinal samples obtained at days 14, 28, and 60 were

also analysed for 25 (50%) randomly selected cases age- and sex-matched to the controls in a 1:2 ratio.

Bronchoalveolar lavage fluid (BALF) was collected from patients being routinely investigated for respiratory symptoms at Nalanda University Hospitals, Patna, India.

Demographic, clinical, hematologic, and radiologic data were collected using a standardized proforma. Blood-stained samples were excluded from the study.

Details of the study have been previously published (4).

Samples of both studies were analysed using Luminex multiplex magnetic bead array (R&D Systems) in accordance with the manufacturer's instructions.

Mycobacterium tuberculosis culture

Ampicillin resistant *Mycobacteria tuberculosis* H37Rv Pasteur (*M.tb*) was grown from stock stored at -80 °C in 30-50 % glycerol. It was cultured in Middlebrook 7H9 medium supplemented with 10% enrichment medium, 0.2% glycerol, 0.02% Tween 80, and 2.5 µg/ml Amphotericin with agitation at 10rpm. Culture growth was monitored with a Biowave cell density meter (WPA, Cambridge) and the *M.tb* sub-cultured when the optical density reached 1.00. For infection experiments, culture at mid log growth at an optical density of 0.60 was used, which corresponded to 1×10^8 – 2×10^8 colony forming units (CFU)/ ml. Optical density was correlated with CFU by performing colony counts in triplicate on Middlebrook 7H11 agar supplemented with OADC enrichment medium and 0.5 % glycerol.

Cell isolation

Procedures for the isolation of platelets were adapted from previously published methods (5). Blood was drawn from healthy volunteers into tubes containing preservative-free sodium citrate. The blood was gently inverted and used within 2

hours. The blood was diluted in wash buffer (10 mM HEPES, 5 mM glucose, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄ and 145 mM NaCl, pH 7.4) and centrifuged at 200 g for 15 min. After centrifugation the top layer of blood was removed and centrifuged with 1 µM prostaglandin E1 (Calbiochem, San Diego, CA, USA) to avoid activation. The cells were resuspended, washed, and activated in wash buffer with 0.1 U/ml thrombin (Sigma Aldrich, Poole, UK). They were washed before immediate use. Cell viability was assessed with trypan blue staining. Cell purity was approximately 95% according to flow cytometry and cell activation verified with CXCL4 secretion compared to resting platelets.

Monocytes were isolated using Histopaque density centrifugation followed by either adhesion purification or, for the confocal analysis, the Pan Monocyte Isolation (Human) Kit following the manufacturer's instructions (Miltenyi Biotec, Bisley, UK).

Monocyte-platelet co-culture

Autologous freshly isolated monocytes and platelets were combined at platelet: monocyte of 100:1 unless otherwise stated, in DMEM (Gibco®, Life Technologies, Paisley, UK) supplemented with ampicillin. Cells were then infected with *M.tb* at a monocyte multiplicity of infection (MOI) of 1 and incubated at 5% CO₂ at 37°C.

MMP and cytokine analysis

Supernatant was harvested after 24 hours by centrifugation and sterile filtration. This process does not interfere with detection of MMPs and cytokines (6). Concentrations of MMPs and TIMPs were measured by ELISA (R&D systems Abingdon, UK). Luminex multiplex magnetic bead array was used for multiplex analysis of MMPs (R&D Systems) and cytokines (Millipore, Watford, UK). Platelet contributions were assessed using a platelet-only control for all measurements and found to be minimal.

Unless otherwise stated, values have been presented in graphs following subtraction of platelet-only concentrations for normalisation.

CFU analysis

Cellular killing of *M.tb* was assessed through colony counts performed on Middlebrook 7H11 agar. Culture supernatants were used to assess extracellular killing, and adherent cells lysed with water to assess intracellular killing. The plates were incubated at 37°C for 2-4 weeks before counts were obtained.

MMP-1 Quantitative reverse transcription PCR (qRT-PCR)

After 18 hours of incubation, Trizol cell lysis was performed and RNA was collected. qRT-PCR was performed using the 1-Step RT-PCR master mix (Qiagen, Crawley, UK) according to the manufacturer's instructions on a Stratagene Mx3000P platform. The RT-PCR cycling conditions comprised 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. For MMP-1, the forward primer was 5'- AAGATGAAAGGTGGACCAACAATT -3'; the reverse primer was 5' -CAAGAGAATGGCCGAGTTC -3'; and the probe was 5'- FAM-CAGAGAGTACA ACTTACATCGTGTGCGGCTC-TAMRA -3'. VIC-labelled β -actin control reagent (Applied Biosystems, Manchester, UK) was used as a reference gene. PCR plasmid standards were used to calculate MMP-1 and B-actin copy numbers.

Confocal Microscopy

Monocytes were cultured with platelets on slides coated with 150 μ g/ml of DQ collagen type 1 (ThermoFisher Scientific, Hemel Hempstead, UK) for 24 hours before adherent cells were fixed with 4% paraformaldehyde overnight. Collagen degradation was visualised using a Leica confocal microscope (Leica TCS SP5) and

processed using Leica LAS AF Lite 2.6.0 (Leica Microsystems, Germany) and Image J 1.43U (NIH, USA) software. Numerical analysis was performed by assigning arbitrary units to the DQ collagen fluorescence using Image J 1.43U (NIH, USA) and normalising this to the cell number present. A platelet-only control was also included at equivalent concentrations but produced no measurable fluorescent signal.

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded lung samples from five Balb/C *M.tb*-infected mice, harvested after 28 days post infection. All animal procedures were performed under the licence issued by the UK Home Office (PPL 70/7160) and in accordance with the Animal Scientific Procedures Act of 1986. Eight to ten week old female BALB/c mice (Charles River Ltd, UK) were maintained in biosafety containment level 3 facilities according to institutional protocols. Mice were infected with 10⁴CFU of the *M.tuberculosis* clinical isolate, East-Asian/Beijing strain 333 via the intranasal route (n=5 mice per group). At 28 days post-infection, mice were humanely culled and the upper left lobe of the lung was fixed in 10% neutral buffered formalin (Sigma-Aldrich, United Kingdom) for histopathological analysis. (7)

Immunohistochemistry was done in collaboration with Professor Joanna Porter at the University College of London using the Discovery XT instrument and DAB Map Kit (both Ventana). Heat induced epitope retrieval was performed in a pressure cooker using citrate buffer pH6. Anti-CD41 (Abcam, Cambridge, UK) primary antibody incubation was for 12 hrs using a 1 in 40 dilution. Rabbit anti-Rat (Dako, Glostrup, Denmark) secondary antibody incubation was for 60 mins, using a 1 in 200 dilution. Heat induced epitope retrieval was performed in a pressure cooker using citrate buffer pH6. Slides were haematoxylin counterstained, dehydrated and mounted.

Statistics

Statistical analyses were performed using PRISM Version 6 (GraphPad, La Jolla, CA, US) and STATA 12 (Stata Corp., College Station, TX, USA). Clinical data are represented as median and interquartile range (IQR). Analysis between groups was performed using Wilcoxon matched pairs signed rank test or, for longitudinal data, evaluated over time using the Skillings-Mack statistic. For all *in vitro* experiments, multiple interventions were analyzed by the One Way Anova followed by Tukey's multiple comparison. Graphs are representative of experiments performed from at least three separate donors, with experiments performed in triplicate for each donor, unless otherwise stated, and data are presented as mean \pm standard error. Correlations were calculated with Spearman correlation and scatter plots generated with a log scale. A two tailed P-value was used to assess significance; $p < 0.05$ was considered significant for all statistics.

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