Platelets Regulate Pulmonary Inflammation and Tissue Destruction in Tuberculosis

Katharine A. Fox¹, Daniela E. Kirwan¹, Ashley M. Whittington¹, Nitya Krishnan², Brian D. Robertson², Robert H. Gilman^{3,4}, José W. López^{5,6}, Shivani Singh¹, Joanna C. Porter⁷, and Jon S. Friedland¹

¹Infectious Diseases and Immunity, Wellcome Trust Centre for Global Health Research, and ²Medical Research Council Centre for Molecular Bacteriology and Infection, Department of Medicine, Imperial College London, United Kingdom; ³Department of International Health, Johns Hopkins University, Baltimore Maryland; ⁴Laboratorio de Investigación en Enfermedades Infecciosas, Universidad Peruana Cayetano Heredia, Lima, Peru; ⁵Laboratorio de Bioinformática y Biología Molecular, Universidad Peruana Cayetano Heredia, Lima, Peru; ⁶Instituto Nacional de Salud del Niño, Lima, Peru; and ⁷Centre for Inflammation and Tissue Repair, Respiratory Medicine, University College London, United Kingdom

ORCID IDs: 0000-0003-3989-0795 (D.E.K.); 0000-0001-7789-9649 (J.S.F.).

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 patients with TB before treatment and 50 control subjects. Twenty-five patients were followed longitudinally. Platelet-monocyte interactions were studied in a coculture model infected with live, virulent *Mycobacterium tuberculosis* (*M.tb*) and dissected using qRT-PCR, Luminex multiplex arrays, matrix degradation assays, and colony counts. Immunohistochemistry detected CD41 (cluster of differentiation 41) expression in a pulmonary TB murine model, and secreted platelet factors were measured in BAL fluid from 15 patients with TB and matched control subjects.

Measurements and Main Results: Five of six platelet-associated mediators were upregulated in plasma of patients with TB compared with control subjects, with concentrations returning to baseline by Day 60 of treatment. Gene expression of the monocyte collagenase MMP-1 (matrix metalloproteinase-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 MDC (monocyte-derived chemokine; 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 BAL fluid and correlated with MMP and IL-1 β concentrations.

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

Keywords: tuberculosis; matrix metalloproteinases; platelets; human; innate immunity

(Received in original form October 24, 2017; accepted in final form February 8, 2018)

K.A.F. had a Wellcome Trust Ph.D. studentship with J.S.F. J.S.F. was supported by the Imperial College National Institute for Health Research (NIHR) Biomedical Research Centre. D.E.K. was supported by an NIHR Academic Clinical Fellowship. D.E.K., A.M.W., and S.S. were supported by MRC Clinical Research Training Fellowships. J.C.P. is funded by a Medical Research Council New Investigator Research Grant and supported by the University College London Hospitals NIHR Biomedical Research Centre. B.D.R. was supported by the Imperial College Trust. The project was also supported by the charity Breathing Matters.

Author Contributions: K.A.F., D.E.K., and J.S.F. conceived the study; K.A.F. designed and carried all the cellular experiments and analyzed sputum samples; D.E.K., R.H.G., and J.W.L. obtained clinical data concerning patient plasma samples; S.S. obtained BAL fluid samples; J.C.P. performed immunohistochemistry and associated imaging; N.K. and B.D.R. performed the murine experiments from which samples were processed for immunohistochemistry and advised on the data; K.A.F., D.E.K., A.M.W., R.H.G., and J.S.F. drafted the manuscript. All authors reviewed and agreed the final version of the manuscript.

Correspondence and requests for reprints should be addressed to Jon S. Friedland, Ph.D., F.R.C.P., Infectious Diseases and Immunity, Commonwealth Building, Hammersmith Campus, Imperial College London, Du Cane Road, London W12 0NN, UK. E-mail: j.friedland@imperial.ac.uk.

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

Am J Respir Crit Care Med Vol 198, Iss 2, pp 245–255, Jul 15, 2018

Copyright © 2018 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201710-2102OC on February 8, 2018

Internet address: www.atsjournals.org

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 (TB) 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 patients with TB.

Approximately 2 billion people are infected with *Mycobacterium tuberculosis* (*M.tb*), which killed approximately 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 hemostasis, platelets modulate innate and adaptive immune responses (4). Platelet interaction with monocytes regulates cell recruitment, maturation, and secretion of cytokines and MMPs (matrix metalloproteinases) (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 (cluster of differentiation 41) (11), and on activation secrete mediators from their granules. These include PF4 (platelet factor-4), which is primarily platelet secreted, and PDGF-BB (platelet-derived growth factor-BB), RANTES (regulated upon activation, normal T cell expressed and secreted), P-selectin, and MMP-9, which are also secreted by other cell types (12–14). PTX3 (pentraxin-3) 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 MMPs, such as the collagenase MMP-1, and these degrade the pulmonary extracellular matrix to cause tissue destruction (18). As well as transcriptional regulation, MMP activity is regulated post-translationally by TIMPs (tissue inhibitors of metalloproteinases) 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 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 patients with pulmonary TB. 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 online supplement.

Clinical Studies

Plasma samples were collected in prospective studies from 50 adult patients with TB in Lima, Peru with a new diagnosis of culture-positive, drug-sensitive pulmonary TB (25). Fifty age- and sex-matched healthy control subjects were asymptomatic and 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 center'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.

BAL 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). *M. tuberculosis* H37Rv Pasteur (*M.tb*) was cultured in Middlebrook 7H9 medium (28).

Autologous freshly isolated platelets and monocytes from healthy volunteers were cocultured at a ratio of 100:1 unless otherwise stated and infected with *M.tb* at a multiplicity of infection of 1.

Secreted Cytokine and MMP Analysis

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

Cytokine and MMP Gene Analysis

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

Colony-Forming Unit Analysis

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

Functional Assay of Tissue Destruction by Confocal Microscopy

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

Immunohistochemistry

Immunohistochemistry using the Discovery XT instrument and the DAB Map Kit (Ventana Medical Systems) was performed on paraffin-embedded Day 28 postinfection lung samples from Balb/C mice collected as part of a previously published study (29).

Statistics

Analyses were performed using PRISM Version 6 (GraphPad) and STATA 12 (Stata Corp.). Clinical data are medians and interquartile ranges 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 $\pm$ SD and analyzed by one-way ANOVA and Tukey 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 Patients with TB

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

PDGF-BB, RANTES, and PF4 concentrations were significantly upregulated

in patients compared with control subjects (all $P < 0.0001$; Figures 1A–1C). MMP-9 concentrations were higher in untreated patients with TB versus healthy control subjects (89,124 pg/ml and 68,057 pg/ml, respectively) ($P < 0.05$; Figure 1D), as were PTX3 concentrations (433 pg/ml and 94.0 pg/ml, respectively) ($P < 0.001$; Figure 1E). There was no difference in P-selectin concentration ($P < 0.05$; Figure 1F).

In patients with TB, 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 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 Patients with TB

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 supplement. A total of 31 of 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 patients with TB steadily increased from 20.4 at baseline to 22.0 by Day 60, and mean SNAQ score increased from 13.3 to 15.9. There was no difference in age, sex, mean BMI, or mean SNAQ score between patients with TB who were followed up and those who were not (data not shown).

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

Platelet-Monocyte Interactions Drive MMP-1 and -10 Gene Expression and Secretion in TB

Because there was good evidence of platelet activation in patients with TB, we developed a coculture model to investigate the functional role of platelets in regulating the human inflammatory immune response. A major outcome of this response is MMP-driven extracellular matrix destruction, in which the collagenase MMP-1 is key (16). Monocytes costimulated with platelets and *M.tb* had a 4.3-fold increase in *Mmp-1* expression compared with *M.tb*-stimulated monocytes alone ($P < 0.0001$; Figure 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 cocultured with platelets compared with *M.tb*-infected monocytes alone ($P < 0.0001$; Figure 3B),

Table 1. Demographics and Clinical Characteristics of Tuberculosis Cases before Treatment and Healthy Control Subjects

	Cases, before Treatment (n = 50)	TB-Negative Control Subjects (n = 50)	P Value
Age, yr*	35 (1.90)	35 (1.93)	0.72
Men, n (%)†	34 (68)	34 (68)	1.0
Height, cm**	163.6 (1.53)	159.1 (1.29)	0.022
Weight at diagnosis, kg**	54.6 (2.28)	68.6 (1.94)	<0.0001
BMI**	20.4 (0.82)	27.2 (0.86)	<0.0001
SNAQ score*	13.3 (0.33)	15.0 (0.27)	0.0008

Definition of abbreviations: BMI = body mass index; SNAQ = Short Nutritional Assessment Questionnaire; TB = tuberculosis.

Data are presented as mean (SE) unless otherwise noted.

*P value calculated using paired two-sample t test.

†P value calculated using Fisher exact test.

**Weight, height, and BMI data missing for two cases. BMI data were excluded for one case (lower limb amputation).

Figure 1. Plasma concentrations of platelet-derived mediators in patients with tuberculosis (TB) and control subjects before treatment. (A) PDGF-BB (platelet-derived growth factor-BB), (B) RANTES (regulated upon activation, normal T cell expressed and secreted), (C) PF4 (platelet factor 4), (D) MMP-9 (matrix metalloproteinase-9), and (E) PTX-3 (pentraxin-3) were higher in patients with pulmonary TB before treatment (gray boxes) than age- and sex-matched healthy control subjects (white boxes). (F) Plasma concentrations of P-selectin were unchanged in patients with TB compared with control subjects. Data are presented as median and interquartile range. Whiskers represent 10th and 90th percentiles, and outlier 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.

although platelets did not alter MMP-1 secretion in control cells. This effect was dependent on the ratio of platelets to monocytes (P:M) (Figure 3C), increasing up to 100:1.

The MMP-1 activator MMP-10 was similarly upregulated by platelets. MMP-10 concentrations increased from 318 ± 106.9 pg/ml to $1,972 \pm 405.4$ pg/ml on exposure to *M.tb*. This increased further to $3,423 \pm 363.2$ pg/ml with exposure to *M.tb* in the presence of platelets (both *P* < 0.001;

Figure 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 (Figure 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; Figure 3E). In addition, platelet coculture increased monocyte secretion of MMP-3, -7 and -9, whereas MMP-8 was not changed. Similar to MMP-1 and -10, platelet upregulation of MMP-3 and -7

required *M.tb* exposure (Figure E1). TIMP-1 and -2 secretion from monocytes in the presence or absence of *M.tb* was not altered by platelet coculture (Figures 3F and 3G).

Platelets Regulate Monocyte-Dependent Collagen Destruction in TB

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

Figure 2. Plasma concentrations (pg/ml) of (A) PF4 (platelet factor 4), and (B) PDGF-BB (platelet-derived growth factor BB), but not (C) P-selectin decrease to baseline after the first 60 days of treatment in patients with tuberculosis (gray boxes). Age- and sex-matched healthy control subjects are shown by white boxes. Data are presented as median and interquartile range. Whiskers represent 10th and 90th percentiles, and outlier values are shown. Significance of change over time was assessed using Skillings-Mack statistic. *n* = 25 for both groups. **P* < 0.05. D = Day.

Figure 3. Monocyte MMP-1 (matrix metalloproteinase 1) and MMP-10 are regulated by platelets in *Mycobacterium tuberculosis* (*M.tb*) infection. Monocytes were cultured with autologous platelets (Plts) and virulent *M.tb* before analysis. (A) Monocytes were cultured at a platelet:monocyte ratio (P:M) of 100:1 for 18 hours. The 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 ($n = 3$). (B–G) Monocytes were cultured with Plts and virulent *M.tb* for 24 hours. The y-axis shows secreted MMPs or TIMPs (tissue inhibitors of metalloproteinases) in pg/ml. (B) Monocytes were cultured at a 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 and 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 *Mycobacterium tuberculosis* (*M.tb*) infection is upregulated by platelet coculture. Monocytes were cultured with autologous platelets (Plts) and virulent *M.tb* for 24 hours on DQ collagen type I, which fluoresces on 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 normalized to cell number. Data are mean $\pm$ SD and are representative of three independent experiments. ** $P < 0.01$. Scale bars, 25 μ m.

microscopy revealed monocytes alone had minimal collagenase activity when uninfected, regardless of platelet coculture. *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 (Figure 4A). Quantitative analysis revealed a 2.5-fold increase in collagenase activity of *M.tb*-exposed cocultured cells compared with *M.tb*-exposed monocytes in the absence of platelets ($P < 0.01$; Figure 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 ($P < 0.0001$; Figure 5A). This trend was not seen in the inflammatory cytokines IFN- γ (Figure 5B) or tumor necrosis factor (TNF)- α (Figure 5C). IL-10 was upregulated 7.5-fold in *M.tb*-infected platelet

coculture compared with single-culture monocytes ($P < 0.0001$; Figure 5D). In contrast, *M.tb*-infected monocytes in platelet coculture secreted 6.4-fold less IL-12 and 6.1-fold less monocyte-derived chemokine (MDC) (both $P < 0.001$; Figures 5E and 5F). There was no platelet regulation of the immunomodulatory cytokine IL-6 or the chemokine IL-8 (Figure E2).

Platelet Coculture Increases Monocyte Intracellular *M.tb* Replication

In addition to regulation of monocyte-derived inflammatory mediators, monocyte coculture with platelets altered the intracellular killing of *M.tb*. Although extracellular *M.tb* did not change in platelet coculture, 2.5-fold more viable intracellular *M.tb* was detected in cocultured cell lysates ($P < 0.001$; Figure 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 organized 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 (Figures 7A and 7B). 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 (Figure 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 with respiratory symptomatic control patients. There was a significant upregulation of PDGF-BB concentration in BALF from patients with TB compared with control subjects ($P < 0.01$; Figure 7D). Both P-selectin and RANTES concentrations were also increased in patients with TB ($P < 0.01$;

Figure 5. Platelet interaction alters cytokine and chemokine secretion from human monocytes stimulated by *Mycobacterium tuberculosis* (*M.tb*). Monocytes were cultured with autologous platelets (Plts) and live virulent *M.tb* for 24 hours and proinflammatory mediators measured in cell culture medium. The y-axis shows secreted analytes in pg/ml. (A) IL-1 β was significantly increased in *M.tb* stimulation and increased further by platelet coculture. (B) IFN- γ was not significantly regulated by platelet stimulation. (C) TNF- α (tumor necrosis factor- 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 MDC (macrophage-derived chemokine) 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.

Figures 7E and 7F), but PF4 concentrations were not (Figure 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; Figures 8A–8E). P-selectin correlation with MMP-3 was also statistically significant (R = 0.4; P < 0.05; Figure 8F). In addition, BALF PDGF-BB concentrations correlated with MMP-1, -3, -8, and -9 (Figure E3). BALF RANTES

concentrations correlated with MMP-1, -8, and -9 as well as IL-1 β (Figure E4).

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 toward plasma concentrations of healthy control subjects. These mediators are not necessarily exclusively produced by platelets.

Figure 6. Platelet coculture increases intracellular bacterial viability in monocytes. Human primary monocytes were cultured with autologous platelets (Plts) and infected live virulent *Mycobacterium tuberculosis* (*M.tb*) for 24 hours. Bacterial killing assessed by colony-forming unit counts of *M.tb* obtained from both the intracellular and extracellular environment. Although extracellular colony-forming unit counts 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 tuberculosis (TB). (A–C) Platelets are localized to the lung in murine TB. Cluster of differentiation (CD) 41 staining was performed on regions of (A and B) significant *Mycobacterium tuberculosis* (*M.tb*) inflammation and tissue remodeling, and (C) peripheral lung with minimal inflammation in *M.tb*-infected Balb/C mice. CD41 is highly expressed in lung areas with significant TB pathology of inflammation and tissue remodeling. Although most CD41 staining was associated with anucleate cells consistent with platelet morphology, some staining of nucleated cells may represent expression of the marker or phagocytosis of platelets or adherence of platelets. Images are from a single mouse and are representative of five mice. (D–G) Platelet-derived mediators are upregulated in BAL fluid (BALF) from patients with TB compared with people with other respiratory illnesses. BALF from 15 patients with TB (cases) and 15 respiratory symptomatic control subjects (control) were analyzed by multiplex analysis for: (D) PDGF-BB (platelet-derived growth factor-BB), (E) P-selectin, (F) RANTES (regulated upon activation, normal T cell expressed and secreted), and (G) PF4 (platelet factor 4). Data are median and interquartile range. Whiskers represent 10th and 90th percentiles, and outlier values are shown. ** $P < 0.01$

However, the concentrations of different platelet-derived mediators were significantly correlated, and this is consistent with costorage and cosecretion. 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 humans. 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 coculture.

The platelet-dependent upregulation of monocyte IL-1 β further demonstrates that platelets drive monocyte inflammatory

Figure 8. P-selectin expression correlates with MMP (matrix metalloproteinase) and IL-1 β concentrations in BAL fluid (BALF). P-selection concentrations measured in BALF of 15 patients with tuberculosis and 15 respiratory symptomatic subjects 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.

responses, because 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 IFN- 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 more than 50% when cocultured with platelets. MDC acts through the CCR4 (C-C chemokine receptor type 4) 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 remodeling 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 patients with TB (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). Colony-forming unit analysis of cell lysates reflected this, as we observed a 2.5-fold increase in intracellular survival of *M.tb* in monocytes with platelet coculture compared with those without coculture. The lack of effect by platelets on extracellular colony-forming unit 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 hemoptysis. The images also showed platelets associated with nucleated 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 multinucleated 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 BALF samples from human patients with TB 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 are consistent with our cellular data. Previous work found that PDGF-BB

concentrations in the epithelial lining fluid after 6 months of treatment correlated with worsening pulmonary fibrosis scores (53). The role of platelets in long-term immunopathology and fibrosis after TB infection merits further study.

In summary, we demonstrated that markers of platelet activity are increased in plasma of patients with pulmonary TB compared with healthy control subjects, and these are then normalized

with antimycobacterial 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 are 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 increasing drug resistance. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the patients and staff who contributed to the clinical studies.

References

- World Health Organization. Global tuberculosis report 2016. France: World Health Organization; 2016.
- Elkington PT, D'Armiento JM, Friedland JS. Tuberculosis immunopathology: the neglected role of extracellular matrix destruction. *Sci Transl Med* 2011;3:71ps6.
- Schluger NW. AJRCCM: 100-year anniversary: focus on tuberculosis. *Am J Respir Crit Care Med* 2017;195:1112–1114.
- Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol* 2011;11:264–274.
- Sreeramkumar V, Adrover JM, Ballesteros I, Cuartero MI, Rossaint J, Bilbao I, et al. Neutrophils scan for activated platelets to initiate inflammation. *Science* 2014;346:1234–1238.
- Rong MY, Wang CH, Wu ZB, Zeng W, Zheng ZH, Han Q, et al. Platelets induce a proinflammatory phenotype in monocytes via the CD147 pathway in rheumatoid arthritis. *Arthritis Res Ther* 2014;16:478.
- Stephen J, Emerson B, Fox KA, Dransfield I. The uncoupling of monocyte-platelet interactions from the induction of proinflammatory signaling in monocytes. *J Immunol* 2013;191:5677–5683.
- Lefrançois E, Ortiz-Muñoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature* 2017;544:105–109.
- Lee MY, Kim YJ, Lee HJ, Cho SY, Park TS. Mean platelet volume in *Mycobacterium tuberculosis* infection. *Biomed Res Int* 2016;2016:7508763.
- Büyükaşık Y, Soyulu B, Soyulu AR, Özcebe OI, Canbakan S, Haznedaroğlu IC, et al. In vivo platelet and T-lymphocyte activities during pulmonary tuberculosis. *Eur Respir J* 1998;12:1375–1379.
- Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science* 2010;327:580–583.
- von Hundelshausen P, Petersen F, Brandt E. Platelet-derived chemokines in vascular biology. *Thromb Haemost* 2007;97:704–713.
- Demoulin JB, Montano-Almendras CP. Platelet-derived growth factors and their receptors in normal and malignant hematopoiesis. *Am J Blood Res* 2012;2:44–56.
- Morrell CN, Aggrey AA, Chapman LM, Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. *Blood* 2014;123:2759–2767.
- Deban L, Russo RC, Sironi M, Moalli F, Scanziani M, Zambelli V, et al. Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nat Immunol* 2010;11:328–334.
- Ong CW, Elkington PT, Friedland JS. Tuberculosis, pulmonary cavitation, and matrix metalloproteinases. *Am J Respir Crit Care Med* 2014;190:9–18.
- Hossain MM, Norazmi MN. Pattern recognition receptors and cytokines in *Mycobacterium tuberculosis* infection—the double-edged sword? *Biomed Res Int* 2013;2013:179174.
- Elkington P, Shiomi T, Breen R, Nuttall RK, Ugarte-Gil CA, Walker NF, et al. MMP-1 drives immunopathology in human tuberculosis and transgenic mice. *J Clin Invest* 2011;121:1827–1833.
- Wilkins-Port CE, Ye Q, Mazurkiewicz JE, Higgins PJ. TGF- β 1 + EGF-initiated invasive potential in transformed human keratinocytes is coupled to a plasmin/MMP-10/MMP-1-dependent collagen remodeling axis: role for PAI-1. *Cancer Res* 2009;69:4081–4091.
- Saunders WB, Bayless KJ, Davis GE. MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. *J Cell Sci* 2005;118:2325–2340.
- Volkman HE, Pozos TC, Zheng J, Davis JM, Rawls JF, Ramakrishnan L. Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. *Science* 2010;327:466–469.
- O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. *Annu Rev Immunol* 2013;31:475–527.
- Lugo-Villarino G, Neyrolles O. Manipulation of the mononuclear phagocyte system by *Mycobacterium tuberculosis*. *Cold Spring Harb Perspect Med* 2014;4:a018549.
- Feng Y, Dorhoi A, Mollenkopf HJ, Yin H, Dong Z, Mao L, et al. Platelets direct monocyte differentiation into epithelioid-like multinucleated giant foam cells with suppressive capacity upon mycobacterial stimulation. *J Infect Dis* 2014;210:1700–1710.
- Proano A, Bravard MA, Lopez JW, Lee GO, Bui D, Datta S, et al. Tuberculosis Working Group in Peru. Dynamics of cough frequency in adults undergoing treatment for pulmonary tuberculosis. *Clin Infect Dis* 2017;64:1174–1181.
- Singh S, Saraiva L, Elkington PT, Friedland JS. Regulation of matrix metalloproteinase-1, -3, and -9 in *Mycobacterium tuberculosis*-dependent respiratory networks by the rapamycin-sensitive PI3K/p70(S6K) cascade. *FASEB J* 2014;28:85–93.
- Araki J, Jona M, Eto H, Aoi N, Kato H, Suga H, et al. Optimized preparation method of platelet-concentrated plasma and noncoagulating platelet-derived factor concentrates: maximization of platelet concentration and removal of fibrinogen. *Tissue Eng Part C Methods* 2012;18:176–185.
- Elkington PT, Nuttall RK, Boyle JJ, O'Kane CM, Horncastle DE, Edwards DR, et al. *Mycobacterium tuberculosis*, but not vaccine BCG, specifically upregulates matrix metalloproteinase-1. *Am J Respir Crit Care Med* 2005;172:1596–1604.
- Krishnan N, Malaga W, Constant P, Caws M, Tran TH, Salmons J, et al. *Mycobacterium tuberculosis* lineage influences innate immune response and virulence and is associated with distinct cell envelope lipid profiles. *PLoS One* 2011;6:e23870.
- Basu S, Andrews JR, Poolman EM, Gandhi NR, Shah NS, Moll A, et al. Prevention of nosocomial transmission of extensively drug-resistant tuberculosis in rural South African district hospitals: an epidemiological modelling study. *Lancet* 2007;370:1500–1507.
- van Asten L, Langendam M, Zangerle R, Hernández Aguado I, Boufassa F, Schiffer V, et al. Tuberculosis risk varies with the duration of HIV infection: a prospective study of European drug users with known date of HIV seroconversion. *AIDS* 2003;17:1201–1208.
- Antonucci G, Girardi E, Raviglione MC, Ippolito G. Risk factors for tuberculosis in HIV-infected persons: a prospective cohort study. The Gruppo Italiano di Studio Tubercolosi e AIDS (GiSTA). *JAMA* 1995;274:143–148.

33. Assinger A. Platelets and infection - an emerging role of platelets in viral infection. *Front Immunol* 2014;5:649.
34. Poullis M, Manning R, Laffan M, Haskard DO, Taylor KM, Landis RC. The antithrombotic effect of aprotinin: actions mediated via the protease-activated receptor 1. *J Thorac Cardiovasc Surg* 2000;120:370–378.
35. Day JR, Malik IS, Weerasinghe A, Poullis M, Nadra I, Haskard DO, *et al.* Distinct yet complementary mechanisms of heparin and glycoprotein IIb/IIIa inhibitors on platelet activation and aggregation: implications for restenosis during percutaneous coronary intervention. *Heart* 2004;90:794–799.
36. Hamzeh-Cognasse H, Cognasse F, Palle S, Chavarin P, Olivier T, Deléazay O, *et al.* Direct contact of platelets and their released products exert different effects on human dendritic cell maturation. *BMC Immunol* 2008;9:54.
37. Sasindran SJ, Torrelles JB. *Mycobacterium tuberculosis* infection and inflammation: what is beneficial for the host and for the bacterium? *Front Microbiol* 2011;2:2.
38. Juffermans NP, Florquin S, Camoglio L, Verbon A, Kolk AH, Speelman P, *et al.* Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. *J Infect Dis* 2000;182:902–908.
39. Yamada H, Mizumo S, Horai R, Iwakura Y, Sugawara I. Protective role of interleukin-1 in mycobacterial infection in IL-1 alpha/beta double-knockout mice. *Lab Invest* 2000;80:759–767.
40. Kleinnijenhuis J, Joosten LA, van de Veerdonk FL, Savage N, van Crevel R, Kullberg BJ, *et al.* Transcriptional and inflammasome-mediated pathways for the induction of IL-1beta production by *Mycobacterium tuberculosis*. *Eur J Immunol* 2009;39:1914–1922.
41. Richter JR, Sutton JM, Belizaire RM, Friend LA, Schuster RM, Johannigman TA, *et al.* Macrophage-derived chemokine (CCL22) is a novel mediator of lung inflammation following hemorrhage and resuscitation. *Shock* 2014;42:525–531.
42. Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, *et al.* An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest* 2011;121:985–997.
43. Zdrengea MT, Makrinioti H, Muresan A, Johnston SL, Stanciu LA. The role of macrophage IL-10/innate IFN interplay during virus-induced asthma. *Rev Med Virol* 2015;25:33–49.
44. Quatromoni JG, Eruslanov E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *Am J Transl Res* 2012;4:376–389.
45. Sathymoorthy T, Tezera LB, Walker NF, Brilha S, Saraiva L, Mauri FA, *et al.* Membrane type 1 matrix metalloproteinase regulates monocyte migration and collagen destruction in tuberculosis. *J Immunol* 2015;195:882–891.
46. Huang Z, Luo Q, Guo Y, Chen J, Xiong G, Peng Y, *et al.* *Mycobacterium tuberculosis*-induced polarization of human macrophage orchestrates the formation and development of tuberculous granulomas in vitro. *PLoS One* 2015;10:e0129744.
47. Balboa L, Romero MM, Laborde E, Sabio Y, García CA, Basile JJ, Schierloh P, *et al.* Impaired dendritic cell differentiation of CD16-positive monocytes in tuberculosis: role of p38 MAPK. *Eur J Immunol* 2013;43:335–347.
48. Lastrucci C, Bénard A, Balboa L, Pingris K, Souriant S, Poincloux R, *et al.* Tuberculosis is associated with expansion of a motile, permissive and immunomodulatory CD16(+) monocyte population via the IL-10/STAT3 axis. *Cell Res* 2015;25:1333–1351.
49. Turner J, Cyktor J, Carruthers B, Kominsky R, Stromberg P, Beamer G. Deficiency in interleukin 10 reverses the susceptibility phenotype of CBA/J mice during infection with *Mycobacterium tuberculosis*. *J Immunol* 2012;188(1 Suppl):43.23.
50. Abdalla AE, Lambert N, Duan X, Xie J. Interleukin-10 family and tuberculosis: An old story renewed. *Int J Biol Sci* 2016;12:710–717.
51. Cooper AM, Solache A, Khader SA. Interleukin-12 and tuberculosis: an old story revisited. *Curr Opin Immunol* 2007;19:441–447.
52. Pawelski H, Lang D, Reuter S. Interactions of monocytes and platelets: implication for life. *Front Biosci (Schol Ed)* 2014;6:75–91.
53. Ameglio F, Casarini M, Capoluongo E, Mattia P, Puglisi G, Giosuè S. Post-treatment changes of six cytokines in active pulmonary tuberculosis: differences between patients with stable or increased fibrosis. *Int J Tuberc Lung Dis* 2005;9:98–104.