MAJOR ARTICLE

Platelets Direct Monocyte Differentiation Into Epithelioid-Like Multinucleated Giant Foam Cells With Suppressive Capacity Upon Mycobacterial Stimulation

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(See the editorial commentary by Lugo-Villarino and Neyrolles on pages 1687–90.)

Background. Epithelioid, foam, and multinucleated giant cells (MNGCs) are characteristics of tuberculosis granulomas, yet the precise genesis and functions of these transformed macrophages are unclear. We evaluated the role of platelets as drivers of macrophage transformation in mycobacterial infection.

Methods. We employed flow cytometry and microscopy to assess cellular phenotype and phagocytosis. Immune assays allowed quantification of cytokines and chemokines, whereas gene microarray technology was applied to estimate global transcriptome alterations. Immunohistochemical investigations of tuberculosis granulomas substantiated our findings at the site of infection.

Results. Monocytes differentiated in presence of platelets (MP-Macs) acquired a foamy, epithelioid appearance and gave rise to MNGCs (MP-MNGCs). MP-Macs up-regulated activation markers, phagocytosed mycobacteria, and released abundant interleukin 10. Upon extended culture, MP-Macs shared transcriptional features with epithelioid cells and M2 macrophages and up-regulated CXCL5 transcripts. In line with this, CXCL5 concentrations were significantly increased in airways of active tuberculosis patients. The platelet-specific CD42b antigen was detected in MP-Macs, likewise in macrophages, MNGCs, and epithelioid cells within tuberculosis granulomas, along with the platelet aggregation-inducing factor PDPN.

Conclusions. Platelets drive macrophage differentiation into MNGCs with characteristics of epithelioid, foam, and giant cells observed in tuberculosis granulomas. Our data define platelets as novel participants in tuberculosis pathogenesis.

Keywords. platelet; macrophage; multinucleated giant cell; tuberculosis; CXCL5.

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In addition to their well-known role in hemostasis and thrombosis, platelets play a critical role in chronic inflammatory diseases. Platelet activation promotes atherosclerosis [1], tumor metastasis [2] and arthritis [3]. Recently, immune modulatory activities of platelets have been revealed [4]. Platelets can directly trap pathogens [5], activate neutrophils to release neutrophil extracellular traps [6], recruit and activate phagocytes by contact-dependent and -independent mechanisms [7] and modulate T and B lymphocyte functions through their broad range of granule-derived cytokines and chemokines [8].

Platelets and their granule contents influence cell differentiation. Phagocytosis of lipid-loaded platelets by human $CD34^+$ progenitor cells [9], monocytes [10], or monocytic THP-1 cells [11] results in the formation of foam cells. This process represents a novel mechanism of pathogenesis, which contributes to stability of atherosclerotic plaques in vascular disease [11]. In this article we interrogated the immunoregulatory roles of platelets in the context of bacterial infection with a focus on tuberculosis.

Histopathologically, tuberculosis is characterized by granulomas, which contain a broad spectrum of transformed macrophages such as multinucleated giant cells (MNGCs), epithelioid cells, and foam cells. We hypothesized that platelets contribute to progression of mononuclear phagocytes to the above-mentioned cell phenotypes, thereby affecting the functions of the mononuclear phagocyte to the benefit of the pathogen. We demonstrate that coculture with platelets induces differentiation of human monocytes into epithelioid-like MNGCs (MP-MNGCs) with suppressive sequellae. Immunophenotyping and whole-genome expression profiling allowed further characterization of these cells, and revealed similarities with MNGCs from tuberculosis granulomas. We propose that platelets contribute to tuberculosis pathogenesis by inducing transformation of mononuclear phagocytes into foam cells, epithelioid cells or MNGCs, thereby modifying the cellular composition at the site of infection and inflammation.

METHODS

Isolation of Monocytes and Platelets

Human mononuclear cells were obtained from buffy coats of healthy donors by density gradient centrifugation and subsequent immunoaffinity selection (Monocyte Isolation Kit II; Miltenyi). The purity of monocytes was above 98% as verified with anti-CD14 antibody by flow cytometry. Human platelets were isolated from buffy coats as described [9] and resuspended in Tyrodes-HEPES buffer (pH 7.4). This isolation procedure resulted in more than 93% purity of platelets with partial activation (Supplementary Figure 1*A*).

Monocyte–Platelet Coculture

Monocytes were cultured in high-glucose DMEM medium supplemented with 10% human serum AB (Lonza), at 37°C and 5% CO_2 for 2 hours. Freshly isolated platelets (monocyte to platelet 1:100) were added to monocytes, and half of the culture medium was exchanged with fresh complete medium every 2 days. After culture for 5 days, floating cells or platelets were washed away.

Oil Red O Staining for Foamy Cells

Cells were fixed with 10% formalin for 1 hour, stained with 0.35% Oil Red O in isopropanol for 10 minutes at room temperature, counterstained with hematoxylin, mounted on glass slides, and imaged.

Quantification of MNGCs

MNGCs were enumerated based on microscopic images of 5-day culture M-Macs (7 fields) and MP-Macs (11 fields) from 4 donors. Frequencies of MNGCs were calculated using Adobe Photoshop CS6 software.

Phenotyping of MP-Macs

Adherent cells after 5-day culture were collected after treatment with 5 mM EDTA-phosphate-buffered saline (PBS) containing 20% FCS. Cells were stained with anti-HLA-DR-PE, anti-CD80-PE, anti-CD40-PE, anti-CD36-APC or anti-E-cadherin-PE antibodies (BD Pharmingen) and acquired on a FACS Canto flow cytometer (Becton Dickinson).

Stimulation With Lipopolysaccharide, Mycobacteria or Latex Beads

Mycobacterium bovis Bacille Calmette Guérin (BCG)-expressing green fluorescent protein (GFP; BCG-GFP) and *Mycobacterium tuberculosis* $H_{37}Rv$ were cultured in Middlebrook 7H9 broth (Becton Dickinson) supplemented with 10% albumin dextrose catalase (ADC). Five-day differentiated cells were stimulated with lipopolysaccharide (LPS, 100 ng/mL, Sigma), *M. tuberculosis* (MOI 5, 10) for 48 hours, BCG-GFP (MOI 10) for different time periods, or fluorescent red latex beads (2-μm diameter, Sigma-Aldrich) for 1.5 hours. After stimulation, cells were collected and fixed with 1% PFA overnight at +4°C. Phagocytosis ratios (BCG-GFP^+ or latex bead-PE^+ cells) were determined using an Accuri C6 (Becton Dickinson) cytometer.Tumor necrosis factor α (TNF-α), interleukin 10 (IL-10), and CCL2 levels in supernatants were detected with enzyme-linked immunosorbent assay (ELISA) kits (eBiosciences).

Quantitative RT-PCR

Total RNAs were isolated and purified from 21-day culture of M-Macs and MP-Macs with Trizol (Invitrogen) and converted to complementary DNA (cDNA) using the Revertra Ace® qPCR RT kit (Toyobo). Gene expression assays of CXCL5, CXCL7/PPBP, and GAPDH were carried out using SYBR Green Realtime PCR Master Mix-Plus kit (Toyobo) in an ABI 7500 machine. All messenger RNA (mRNA) quantifications were normalized to the GAPDH signal and calculated as fold expression to M-Macs. The primers used in this research are listed in Supplementary Table 1.

Microarray Analysis

Twenty-one-day differentiated cells were washed and lysed with TRIzol reagent (Invitrogen). Microarray experiments were performed as dual-color hybridizations (Agilent Technologies). To compensate for dye-specific effects, an independent dye-reversal color-swap with 4 samples from 2 independent biological experiments was applied [12]. Raw microarray image data were processed with the Image Analysis/Feature Extraction software G2567AA (Version A.10.5.1, Agilent Technologies) and

analyzed with Rosetta Resolver Biosoftware, Build 7.2.2 SP1.31. Ratio profiles comprising single hybridizations were combined in an error-weighted fashion to create ratio experiments. A 1.5-fold change expression cutoff for ratio experiments was applied together with anticorrelation of ratio profiles rendering the microarray analysis highly significant (P value > .01), robust, and reproducible. Microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE45960). Genes differentially expressed between M-Macs and MP-Macs were ranked according to absolute fold change of gene expression and were analysed by Gorilla [13]; DAVID bioinformatics resources 6.7 was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis [14].

Immunohistochemistry and Immunofluorescent Detection of Platelet Markers

Formalin-fixed, paraffin-embedded blocks of lung tissue from 5 pulmonary tuberculosis patients with pulmonary lobectomy were investigated. Samples were obtained from the Sample Bank of Shanghai Pulmonary Hospital. Five-µm lung biopsy sections were processed for staining against CD42b (clone EPR6995, Epitomics) and PDPN (D2-40, M2A, Proteintech). Sections were then incubated with Supersensitive 1-step polymer-HRP detection system (Biogenex). Immunostaining was visualized with 3,3 diaminobenzidine (DAB, Biogenex) substrate; after counterstaining in hematoxyline, sections were mounted and examined.

Twenty-one-day cultured MP-Macs were fixed with 4% PFA, permeabilized with 0.2% Triton X-100, blocked with 10% goat serum cells, incubated with anti-CD42b antibody or the isotype control (ab125938), followed by staining with Alexa fluor 488-conjugated affinipure goat anti-rabbit immunoglobulin G (IgG; Proteintech) and nuclei counterstained with DAPI. Expression of CD42b was detected with Cellomics ArrayScan Vti (Thermo Scientific).

BAL Fluid Collection and Chemokine Detection

Individuals studied included tuberculosis patients (n = 12, mean age 39.4 ± 14.9 years, 6 females, 6 males, 8 confirmed with secondary pulmonary tuberculosis, 4 newly diagnosed with tuberculosis); nontuberculosis nongranulomatous disease controls (n = 12, mean age 45.2 ± 12.7 years, 5 females, 7 males) included interstitial lung disease (n = 7), tracheal obstruction (n = 1), bronchiectasis with infection (n = 3), and lung adenocarcinoma (n = 1). Healthy controls were individuals with high risk of pneumoconiosis (n = 7, mean age 47 ± 10.9 years, all males). All individuals were human immunodeficiency virus (HIV) negative. None were under antimycobacterial and/or corticosteroids therapy. Tuberculosis was diagnosed by smear observation and/or bacterial culture and corroborated with clinical symptoms.

Bronchoscopy was performed for diagnostic purposes. Lavage was performed using 25-mL aliquots of prewarmed standard saline solution. Cell-free supernatants (bronchoalevolar lavage fluid [BALF]) were stored at −80°C for subsequent chemokine (CXCL5, CXCL7) determination using DuoSet ELISA kits from R&D Systems.

Ethical Approval

Studies on clinical samples were conducted in accordance with ethical guidelines of the institutional review board of Tongji University, and with the Declaration of Helsinki. Participants with bronchoscopy were provided with written information about the study and gave written consent. All materials were by-products of regular bronchoscopy or pulmonary lobectomy and were obtained after completion of all medical treatment, according to institutional guidelines of Tongji University. Specimens were anonymized for the study.

Statistics

Results shown are mean ± SD and are representatives of at least 3 repeats. Significant differences were analyzed using an independent Student t-test.

RESULTS

Monocytes Cocultured With Platelets Are Activated and Differentiate Into Multinucleated Giant Foam Cells

After 1 day of coculture, phagocytosis of platelets by monocytes became apparent, because platelet-free areas formed around some monocytes, as previously reported [9] and larger cells appeared solely in cocultures (Supplementary Figure 1*B*). After 4 or 5 days, macrophages differentiated in the presence of platelets (MP-Mac) became larger and expressed a more complex cellular pattern (Figure 1*A* and 1*B*) with higher FSC and SSC values as detected by flow cytometry (Figure 1*C*), compared with platelet-free differentiated macrophages (M-Mac). The presence of platelets prompted macrophage progression to the heterokaryon stage, because the giant cell phenotype prevailed in cocultures (Figure 1*D*). Oil red O staining indicated formation of platelet/monocyte-derived multinucleated giant cells (MP-MNGCs) with multinucleated centers, surrounded by lipid-rich droplets (Figure 1*A*, *inserted photo*). Intriguingly, lipoarabinomannans from *M. tuberculosis*, which have been reported to induce MNGCs [15], further increased the capacity of MP-Macs to progress to MNGCs (Supplementary Figure 1*C*). MP-Macs were activated as indicated by higher surface density of molecules associated with antigen presentation (MHC-II, CD80, and CD40), slightly up-regulated fusion-related markers [16], such as CD36, E-cadherin, and increased secretion of the monocyte/macrophage chemoattractant CCL2 (Figure 1*E*). MP-MNGCs share characteristics of transformed macrophages observed in tuberculosis, such as foamy and epithelioid cells

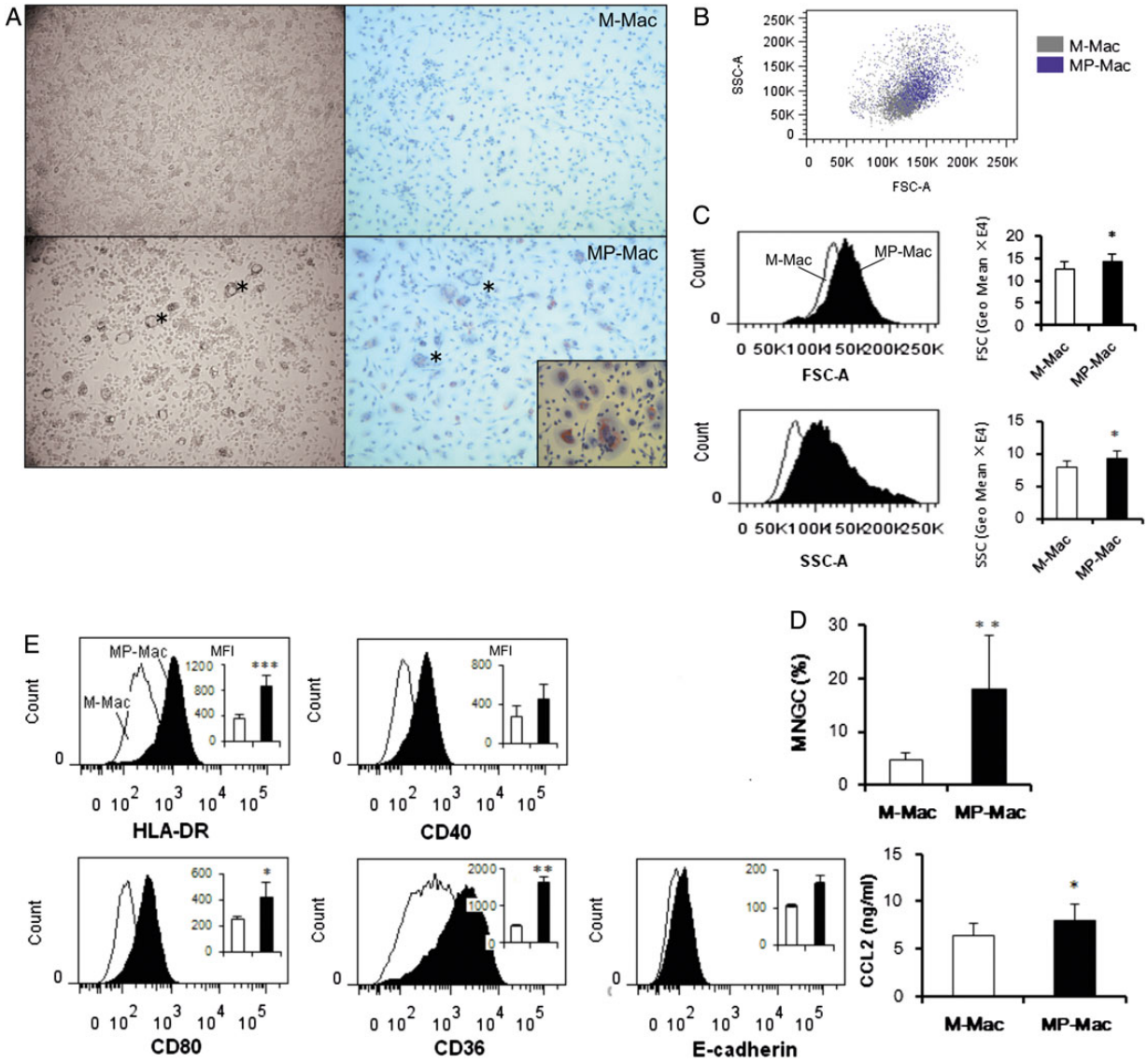

Figure 1. Platelet-directed monocyte differentiation into multinucleated giant foam cells. *A*, Monocytes were cultured in presence (*lower panels*) or absence (*upper panels*) of platelets for 5 days. Morphological observation without (*left*) or with (*right*) oil red O staining. Magnification 100×. Inserted photo: morphology of lipid-rich MP-MNGCs. Magnification 200×. Asterisk indicates lipid-rich MNGCs. *B*, Light scatter properties of MP-Macs (*blue dots*) and M-Macs (*gray dots*) visualized as dot-plot and (*C*) representative histograms of FSC and SSC with bar graphs (mean + SD, n = 7). *D*, Percentages of MNGCs in M-Macs and MP-Macs cultures (mean ± SD, n = 4). *E*, Expression of cell surface markers on M-Macs and MP-Macs. Inserted bars are MFI mean + SD of 3 independent experiments (n = 3). CCL2 levels in supernatants were detected using ELISA; mean + SD of 3 independent experiments. **P* < .05, ***P* < .01, ****P* < .001. Abbreviations: ELISA, enzyme-linked immunosorbent assay; MFI, mean fluorescent intensity; M-Mac, monocyte-differentiated macrophage; MNGC, multinucleated giant cell; MP-Mac, platelet/monocyte-differentiated macrophage; SD, standard deviation.

surrounding the necrotic core of granulomas and giant cells. We conclude that platelets induced transformation propensity in monocytes.

MP-Macs Vigorously Phagocytose Mycobacteria and Release Abundant Anti-Inflammatory IL-10 Following LPS or Mycobacteria Stimulation

Compared with platelet-free-differentiated M-Macs, MP-Macs presented augmented phagocytosis of both mycobacteria (BCG-GFP) and foreign bodies (latex beads) as shown in Figure 2*A* and 2*B* and Supplementary Figure 2*B*. LPS and BCG-GFP induced similar levels of TNF-α in cells of the two groups, but stimulated significantly higher interleukin 10 (IL-10) production in MP-Macs. MP-Macs scantly changed their cellular morphology following LPS stimulation. In contrast, most M-Macs became fusiform and showed long cellular protrusions, typical morphological changes of classically activated macrophages (Supplementary Figure 2*A*). Neither mycobacteria nor

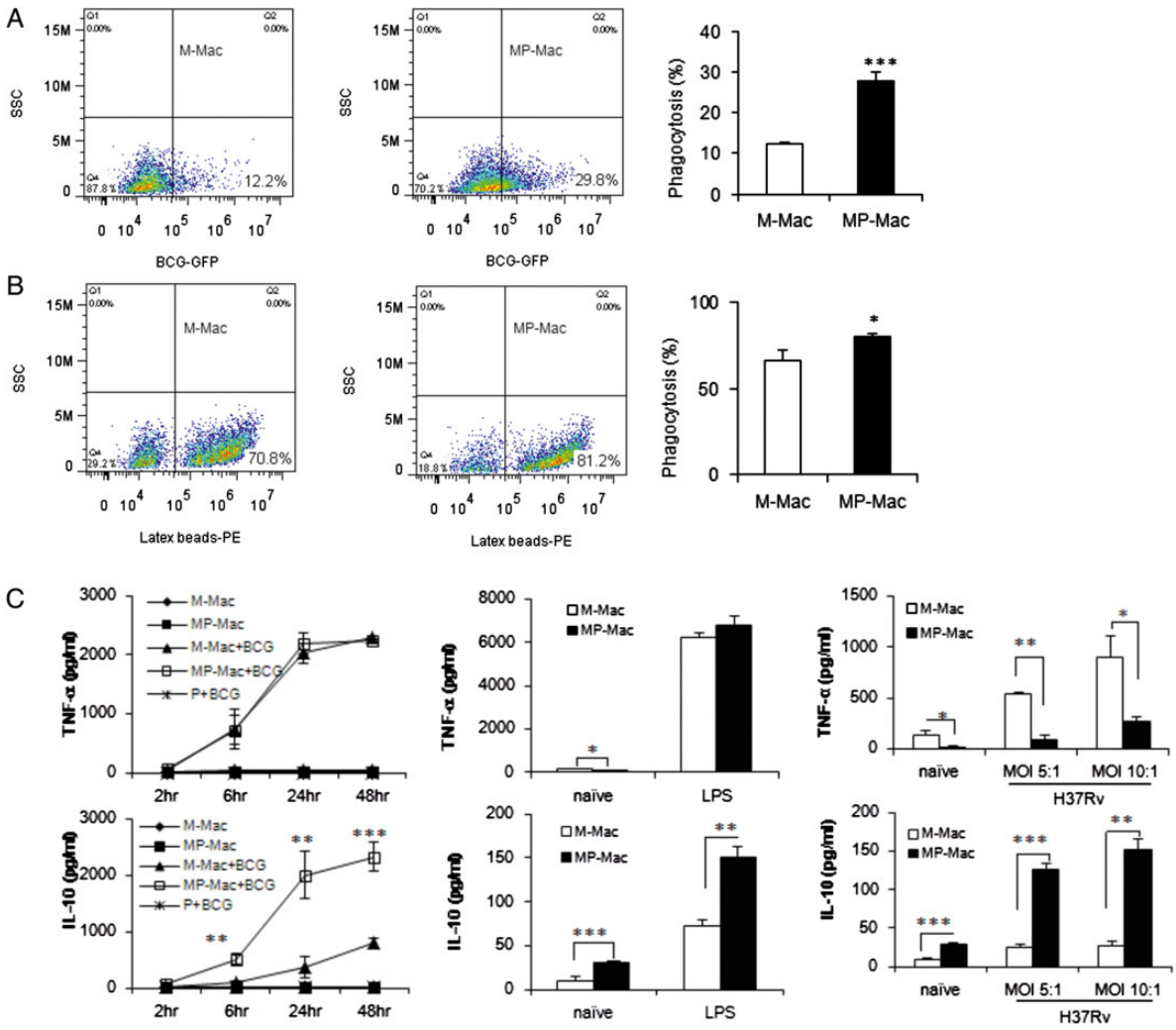

Figure 2. Platelet-transformed macrophages readily phagocytose mycobacteria and release IL-10. M-Macs and MP-Macs were cultured with (*A*) BCG-GFP for 4 hours or (*B*) latex beads-PE for 1.5 hours, and phagocytosis was estimated by flow cytometry. Data are representative of 3 independent experiments with 3 replicates each, mean + SD. *C*, Abundance of TNF-α and IL-10 in cell free supernatants collected from M-Macs and MP-Macs infected with BCG at MOI 10, or stimulated with LPS (100 ng/mL) or *Mycobacterium tuberculosis H37Rv* at various MOI for 48 hours. Data are mean + SD and are representative of at least 3 experiments $*P < .05$, $**P < .01$, $***P < .001$. Abbreviations: BCG-GFP, Bacille Calmette Guérin-expressing green fluorescent protein; IL, interleukin; M-Mac, monocyte-differentiated macrophage; MOI, multiplicity of infection; MP-Mac, platelet/monocyte-differentiated macrophage; SD, standard deviation; TNF, tumor necrosis factor.

LPS stimulated detectable cytokine production in platelets. The immunosuppressive property was more apparent upon infection with *M. tuberculosis*: MP-Macs released a higher level of IL-10 accompanied by significantly reduced TNF-α production (Figure 2*C*). These data demonstrate that platelet-transformed macrophages sense pathogens resulting in an anti-inflammatory phenotype of potential relevance for outcome of infection.

Microarray Analysis Indicates Epithelioid and M2 Features of MP-Macs

Because of the profound morphological differences between M-Macs and MP-Macs, we prolonged coculture time to 21 days to allow extensive cell differentiation. During this timeframe cells maintained viability as indicated by scant release of lactate dehydrogenase in the culture media (Supplementary Figure 1*D*). A remarkable difference in gene expression patterns between M-Macs and MP-Macs was observed. Compared with M-Macs, 2121 genes were up-regulated and 1819 genes down-regulated in MP-Macs. Figure 3 depicts the top 10 of the most up- and down-regulated genes in MP-Macs. The most up-regulated genes CXCL5, and PPBP/CXCL7, were verified by qPCR at day 21 of differentiation of MP-Mac (Figure 3*B*). Consistent with their suppressive response to bacterial stimulation, MP-Macs up-regulated M2 gene signatures [17], as shown in Table 1. Interestingly, some platelet-specific genes [18] like PPBP/CXCL7, PF4 (platelet factor 4)/CXCL4, and F13A1

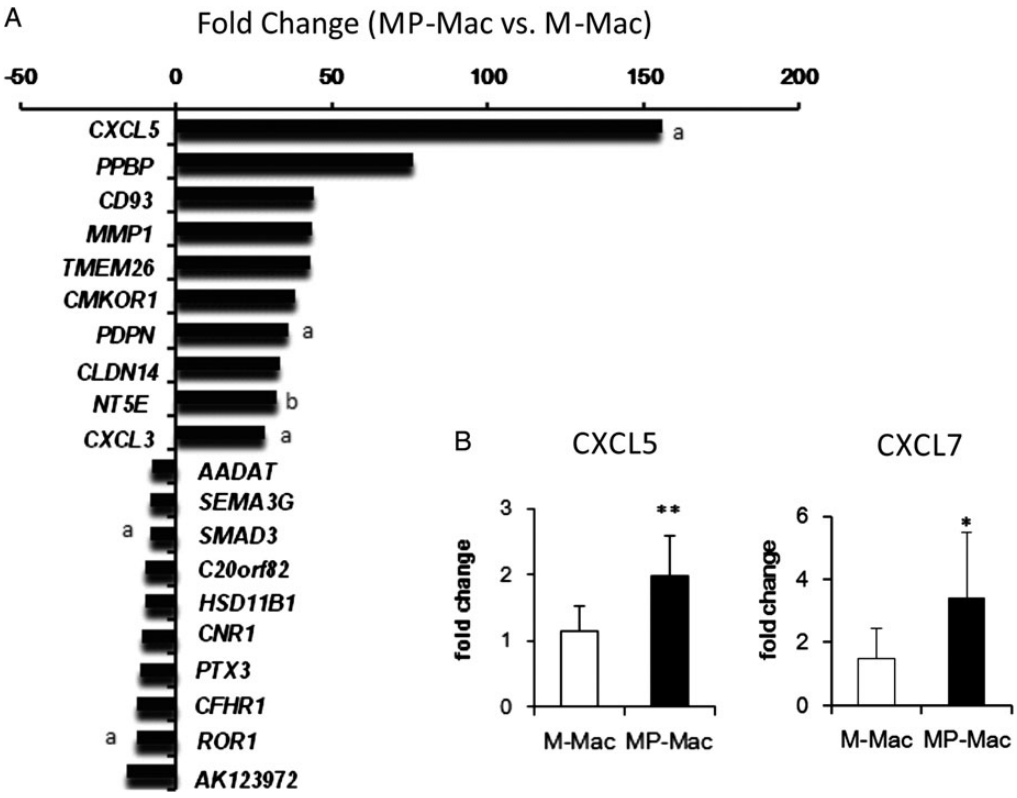

Figure 3. Top 10 up-regulated and down-regulated genes in MP-Macs vs M-Macs identified by microarray analysis. *A*, Graph depicts the fold change of genes expressed in MP-Macs vs M-Macs. Data were obtained from 2 donors: *a.* represents mean result of the depicted gene detected by 2 different probes; *b.* represents mean result of the depicted gene detected by 3 different probes. *B*, Quantitative RT-PCR analysis of CXCL5 and PPBP/CXCL7 in 21-day differentiated M-Macs and MP-Macs. Target gene expression was normalized with human GAPDH expression to calculate the relative expression ratio (mean + SD, n = 9). **P* < .05, ***P* < .01. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M-Mac, monocyte-differentiated macrophage; MP-Mac, platelet/monocyte-differentiated macrophage; RT-PCR, real-time polymerase chain reaction; SD, standard deviation.

could be detected in MP-Macs (Table 2). Further gene ontology (GO) analysis of differentially expressed genes identified enrichment for genes involved in leukocyte chemotaxis, in response to leptin stimulation and to oxygen levels, as well as genes associated with epithelial cell proliferation (Figure 4*A*). KEGG pathway analysis revealed that up-regulated genes were correlated with extracellular matrix (ECM)-receptor interaction ($P = 2.39E-05$), hematopoietic cell lineage ($P = 2.90E-04$), arginine and proline metabolism ($P = .00607$), endocytosis ($P = .018439$; Figure 4*B*), and partially with the TGF-β signaling pathway ($P = .066$).

Epithelioid Cells and MNGCs in Tuberculosis Granuloma Express Platelet-Specific and MP-Mac-Related Markers

Epithelioid cells can merge into MNGCs, which represent a hallmark of granulomas in tuberculosis patients. We observed morphologic similarities between MP-MNGCs and MNGCs in tuberculosis granulomas, and between foam cells found in both instances. To further compare in vitro MP-Mac-derived MP-MNGCs and MNGCs in tuberculosis granulomas, we investigated expression patterns of major markers of MP-Macs and platelet-specific antigens in lung biopsies from tuberculosis patients. Our immunohistochemical examination indicated that epithelioid cells and MNGCs in tuberculosis granulomas were strongly positive for CD42b, a platelet-specific marker, and PDPN, a cell surface antigen and a marker of MP-Macs as revealed by our study (Figure 5*A* and 5*B*). Other markers of MP-Macs, NT5E and ARG1, were detected on foam cells and MNGCs of tuberculosis granulomas (Supplementary Figure 2*C*). In addition, concentration of CXCL5, which prevailed in MP-Macs, was statistically higher in BALF from active tuberculosis patients compared to healthy controls ($P < .05$, Figure 5*C*). Notably, expression of CD42b was detected in MP-Macs and MP-MNGCs (Figure 5*D*). These findings suggest a hitherto unappreciated role of platelets in the genesis of transformed macrophages, such as MNGCs and epithelioid cells, with potential impact on disease processes in pulmonary tuberculosis.

DISCUSSION

Our study demonstrates that platelets drive macrophage activation and transition to epithelioid-like MNGCs. The MP-Macs

Table 1. Up-Regulated M2 Gene Signatures in MP-Macs vs M-Macs

M2 Gene Signature [17]	Accession No.	Fold Change (MP-Macs vs M-Macs)	*P* Value
CXCL3	NM_002090	48.43(8.21)[a]	<1E – 45
ARG1	NM_000045	19.04	<1E – 45
CXCL1	NM_001511	13.35	<1E – 45
CD163	NM_004244	8.42	<1E – 45
CXCL2	NM_002089	7.76(14)[a]	<1E – 45
F13A1	NM_000129	7.37	<1E – 45
FN1	NM_212482	5.15	<1E – 45
CCL20	NM_004591	2.95	.00014
TGFβ2	NM_003238	11.27	<1E – 45
TGFβ3	NM_003239	2.72	2.44E – 15
CLECSF6	NM_016184	2.09	<1E – 45

Data generated from 2 donors.

Abbreviations: M-Mac, monocyte-differentiated macrophage; MP-Mac, platelet/monocyte-differentiated macrophage.

[a] Results of 2 different probes on the array.

maintained phagocytic capacity yet exhibited suppressive features upon mycobacterial stimulation. Moreover, the platelet-conditioned transformed macrophages released chemokines and displayed gene profiles suggesting involvement in leukocyte chemotaxis, cellular transformation and tissue remodelling. Identification of platelet-specific markers in MNGCs and epithelioid cells within tuberculosis granulomas, along with presence of MP-Mac markers substantiate our concept that platelets contribute to tuberculosis pathogenesis by fine-tuning macrophage functions.

Activation of thrombocytopoiesis and platelets in active tuberculosis has been shown by elevated peripheral platelet counts [19], up-regulation of CD62P (unpublished observations), and serum levels of PF4/CXCL4 [20]. As lung endothelial cells constitutively express ICAM-1 and αvβ3 integrins and are prone to tether activated platelets [21], platelets could intimately interact with lung immune cells during *M. tuberculosis* infection. Using an in vitro coculture system we confirmed the propensity of platelets to confer monocytes with a foam phenotype and further demonstrated that longer incubation switched on a differentiation program common to epithelioid and giant cells. These MP-Macs were endowed with enhanced capability to engulf mycobacteria at an early stage of differentiation, and to produce IL-10 abundantly in response to LPS stimulation or mycobacterial infection. Both LPS and BCG induced TNF-α in M-Macs and MP-Macs to a similar degree. In contrast, *M. tuberculosis* induced lower TNF-α release. Differential influence of nonvirulent and virulent strains of mycobacteria on TNF-α secretion by infected macrophages resembles previous findings with *M. ulcerans* [22]. Infection with *M. tuberculosis* suppressed TNF-α, and stimulated IL-10 production in MP-Macs, indicating immune suppressive capacities of MP-Macs. Although we observed up-regulation of CD80 and CD86 on MP-Macs, similar to some previous investigations [23], our data contrast with studies reporting proinflammatory properties of platelet-conditioned macrophages [24–26]. This could be due to the extended culture period applied in our study (more than 5 days compared to 2 days in previous studies). This assumption is supported by the observation that cellular transformation is a time-dependent process. However, recent studies reported that activated platelets enhance IL-10 and reduce TNF-α in monocytes stimulated with extracellular bacteria through CD40–CD40L interactions [27]. The isolation procedure resulted in partial activation of platelets, which might be another reason for the anti-inflammatory phenotype of MP-Macs observed in our system.

MNGCs are detected in various tissues facing persistent insults, including inert particles [28]. The heightened phagocytic properties of MP-Macs, including MP-MNGCs, were observed in response to both mycobacteria and latex beads suggesting common traits of these cells at early stage of differentiation. Differences in particle internalization were yet more pronounced for BCG, suggesting involvement of phagocytic receptors specialized for mycobacterial uptake, presumably CD36

Table 2. The 7 Most Up-Regulated Platelet-Specific Genes in MP-Macs

Gene Symbol [18]	Accession No.	Description	Fold Change (MP-Macs vs M-Macs)	*P* Value
PPBP	NM_002704	PBP/CXC chemokine 7	75.98	<1E – 45
PF4	NM_002619	PF4/CXC chemokine 4	13.75	<1E – 45
F13A1	NM_000129	Coagulation factor XIII, A1	7.37	<1E – 45
GNG11	NM_004126	Guanine nucleotide binding protein 11	4.59	<1E – 45
STX1A	NM_004603	Syntaxin 1A (brain)	3.82	<1E – 45
HES2	NM_019089	Hairy and enhancer of split homolog 2	2.66	<1E – 45
NRGN	NM_006178	Neurogranin (PKC substrate, RC3)	1.91	4.15E – 21

Data shown are means of 2 repeats.

Abbreviations: M-Mac, monocyte-differentiated macrophage; MP-Mac, platelet/monocyte-differentiated macrophage.

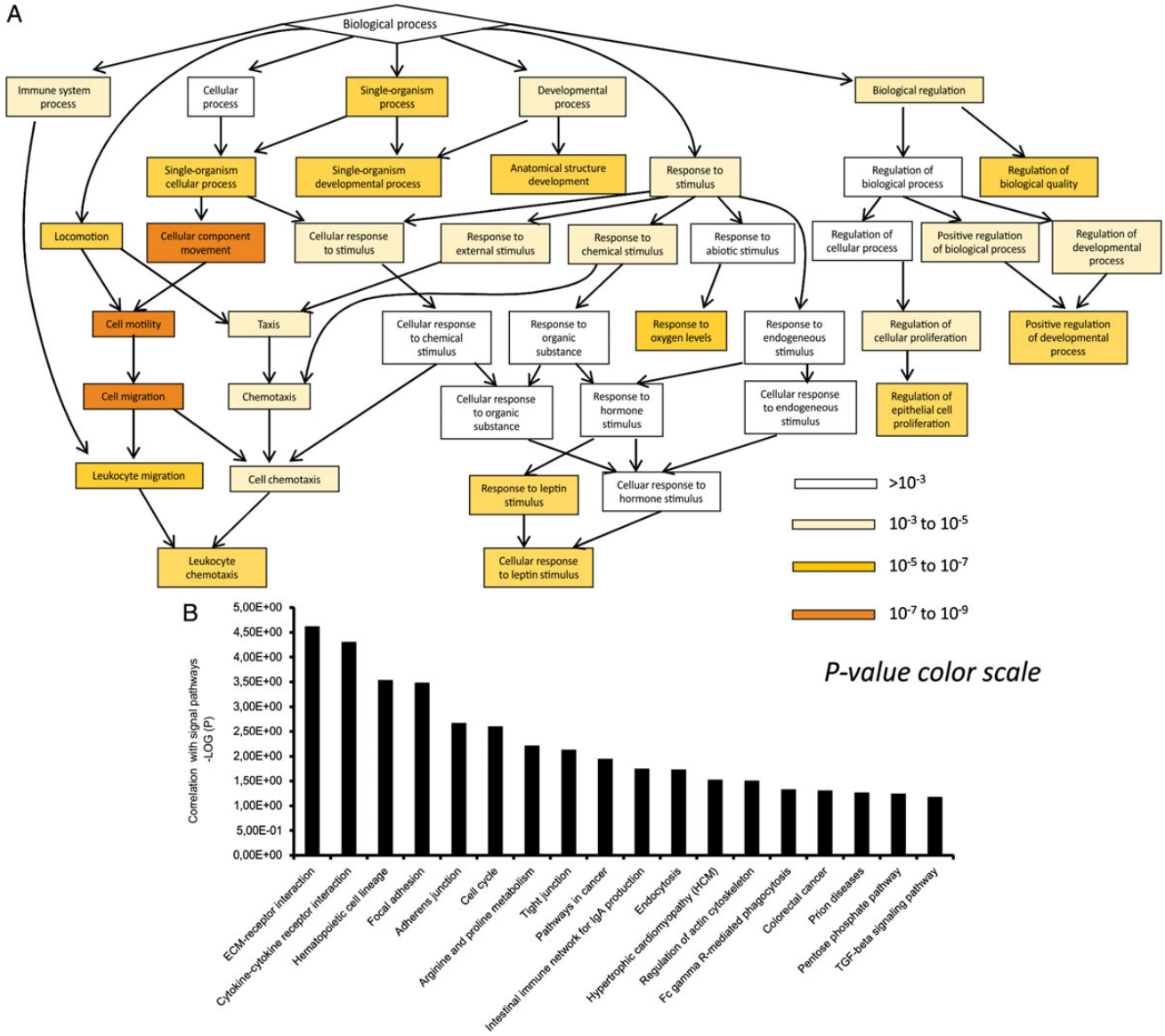

Figure 4. Enrichment of genes in MP-Macs and their correlation with signaling pathways. *A*, Graph of biological process built by GO analysis on groups of genes significantly enriched, *P* value of 10^{-5} was set as the threshold. *B*, KEGG analysis was carried out to identify the correlated signalling pathway in up-regulated gene clusters in MP-Macs. Data shown were –log *P* values of signalling pathways with most significant correlation. Abbreviations: ECM, extracellular matrix; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MP-Mac, platelet/monocyte-differentiated macrophage; TGF, transforming growth factor.

[29], which were highly expressed by MP-Macs. CD36 is also involved in cell fusion [16]. Platelets fostered MP-Macs fusion into MNGCs, and this process was further promoted by mycobacterial lipoarabinomanan. At later stages of differentiation, MNGCs may lose their phagocytic abilities, as reported by Lay et al [30]. PDPN, the platelet aggregation-inducing factor, was significantly up-regulated in fully differentiated MP-Macs and observed on macrophages, epitheliod-like cells and MNGCs in tuberculosis granulomas. Interestingly, $PDPN^{+}$ macrophages induced by systemic zymosan delivery poses heightened phagocytic potential [31], thus further supporting the involvement of platelets in regulation of mycobacterial uptake by transformed macrophages. Future investigations will identify the key molecules involved in platelet-induced augmentation of phagocytosis by macrophages and progression to heterokaryon.

Granuloma formation in tuberculosis is tightly dependent on the accumulation of cells permissive for bacteria. In our studies, the presence of platelets significantly augmented CCL2 release by monocytes, a chemokine involved in macrophage tissue recruitment. Platelets constitutively store CCL5, another chemoattractant for monocytes/macrophages. Further, global gene

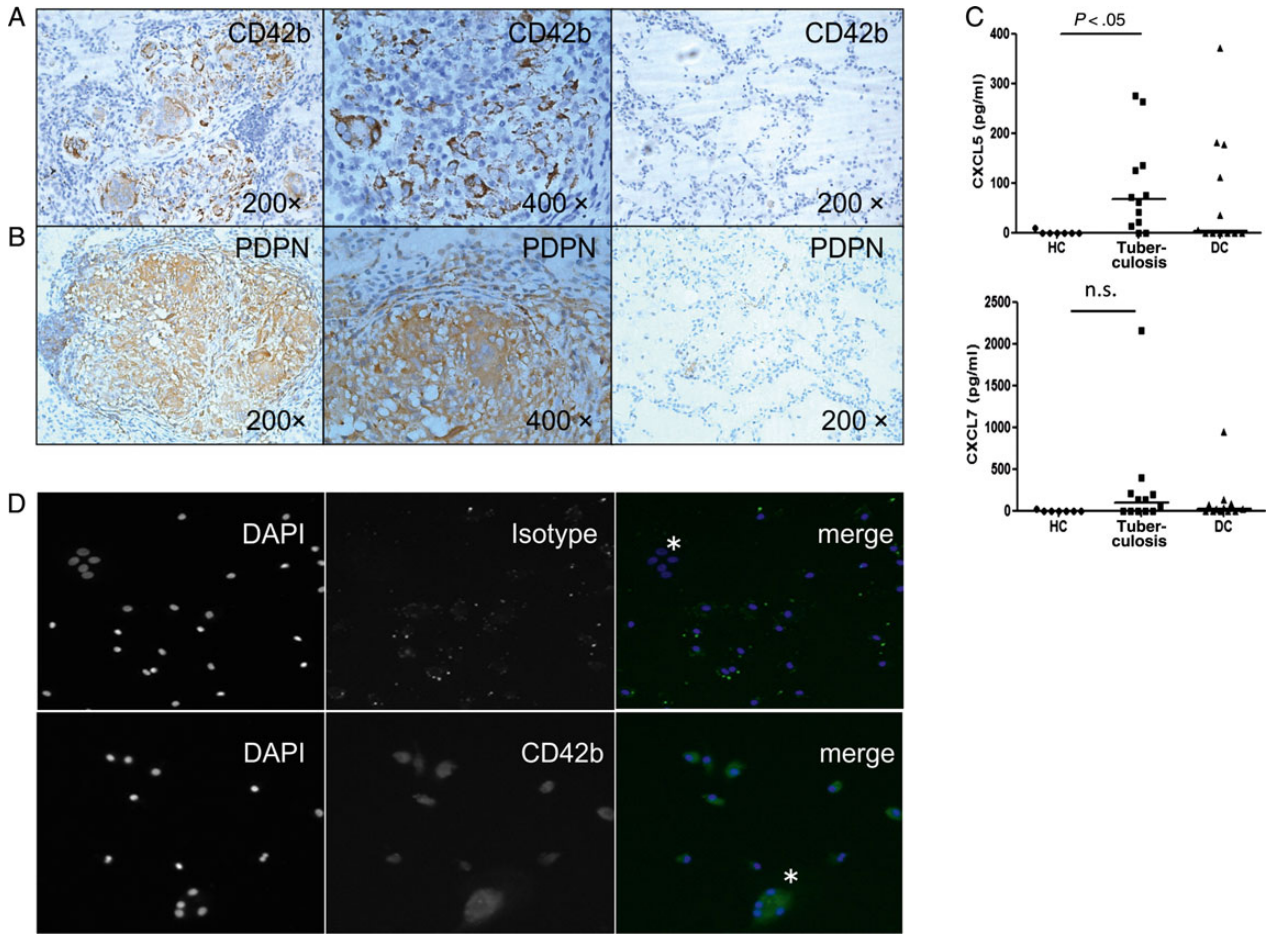

Figure 5. Expression of platelet and MP-Mac markers in MP-MNGCs and granuloma biopsies from tuberculosis patients. Immunohistochemical detection of (*A*) CD42b and (*B*), PDPN in lung biopsies from tuberculosis patients. Pictures are representative of 5 certified tuberculosis patients. *C*, Levels of CXCL5 and CXCL7 in BALF from healthy controls (HC, n = 7), active tuberculosis (n = 12) and non-tuberculosis respiratory disease controls (DC, n = 12). Horizontal line represents median value of each group. *P* < *.05*, NS, not significant. *D*, Immunofluorescent detection of CD42b in MP-Macs cultures (CD42b, *green*; nuclei, *blue*). Magnification 200×. Asterisk indicates MNGCs. Pictures are representative of four experiments. Abbreviations: BALF, bronchoalevolar lavage fluid; MNGC, multinucleated giant cell; MP-Macs, platelet/monocyte-differentiated macrophages; MP-MNGC, platelet/monocyte-differentiated multinucleated giant cell.

expression profiling revealed gene clusters associated with leukocyte chemotaxis in fully differentiated MP-Macs. The most profoundly regulated gene in MP-Macs encodes CXCL5, a chemokine that stimulates chemotaxis of neutrophils during pulmonary inflammation [32]. CXCL5 levels correlate with angiogenesis and influence various inflammatory diseases, such as acute respiratory distress syndrome [33], atherosclerosis [34], and cancer [35]. Pre-platelet basic protein (PPBP), also known as CXCL7, regulates neutrophil adhesion and transmigration [36]. Both CXCL5 and CXCL7 are expressed by platelets [36, 37], and their expression was heightened in MP-Macs. Moreover, we detected abundant CXCL5 in BAL from active tuberculosis patients, suggesting that secretion of these cytokines by platelet-transformed macrophages, perhaps along with other lung-resident cell types [38], contributes to pulmonary recruitment of neutrophils. In tuberculosis, lung recruitment of neutrophils parallels disease progression, and identification of novel molecular pathways controlling their chemotaxis is of particular interest [39].

Cell transformation and progression to pathologic phenotypes has been observed for macrophages within persistent inflammatory foci. Epithelioid cells and MNGCs are characteristic for tuberculosis lesions [40]. We observed cumulative features of transformed macrophages in monocytes cocultured with platelets. MP-Macs and MP-MNGCs presented characteristics of giant foam cells, as shown by lipid staining. Platelets can promote foam cell formation by modified (ie, acetylated or oxidized) low-density lipoprotein [41]. Concurrent with the foam phenotype we detected up-regulated epithelioid genes in MP-Macs, such as CD93 and CMKOR1. CD93 is an epidermal growth factor (EGF)-like domain-containing transmembrane protein, which is predominantly expressed in endothelium

and is involved in cell adhesion, migration, phagocytosis, and angiogenesis [42]; CMKOR1 encodes for CXCR7, which is highly expressed in endothelial progenitor cells, and serves as a receptor for the proangiogenic chemokine CXCL12 [43]. In addition, fully differentiated MP-Macs up-regulated numerous M2 (ARG1, CXCL2, CXCL3, CD163, FN1 and TGF-β) [17] or M2-related (MMP1 and COL1A2) [44, 45] genes. The platelet-induced M2 switch along with anti-inflammatory IL-10 release could contribute to susceptibility to infection.

Genesis of foamy, epithelioid, and MNGCs in MP-Mac cultures was independent of mycobacteria, yet progression to MNGCs was increased by mycobacterial stimulation. This observation is consistent with studies describing that mycobacterial lipomannans induce macrophage fusion through TKR2, ADAM9, and β1 integrins [15]. Our data point to platelets as additional critical modulators of macrophage differentiation. Intriguingly, heightened expression of CD42b and PDPN in epithelioid cells and MGNCs of tuberculosis granulomas indicate involvement of platelets in macrophage transformation toward pathologic cell phenotypes in pulmonary tuberculosis. We propose that activated platelets impose on monocytes a continuum of pathologic phenotypes, all detectable in tuberculosis, most likely mirroring the transformation kinetics upon infection.

Multiple lines of evidence suggest that platelet-triggered macrophage transformation facilitates *M. tuberculosis* persistence. The release of IL-10 along with macrophage polarization toward M2 cells could contribute to susceptibility to tuberculosis. The foamy nature of macrophages in tuberculosis is caused by intracellular lipid accumulation within lipid bodies, which constitutes a nutrient-rich reservoir for *M. tuberculosis* persistence [46]. The source of these lipids is still debated. Engulfed platelets may induce intracellular lipid accumulation [47] and perhaps contribute to maintenance of the bacterial persister populations. Expression of platelet-specific antigens in MP-Macs, especially within the lipid-rich area surrounding the nuclei of MP-MNGCs, substantiates this hypothesis.

Our study evokes involvement of platelets in macrophage transformation and subsequently modulation of inflammation and possibly tuberculosis granuloma remodelling. These biological processes require diversified cellular functionality per se or transfer of novel functions to cells endowed with appreciable plasticity, such as macrophages. Although platelets are anucleate, they contain mRNA, mRNA splicing machinery, and fully functional miRNA machinery, which regulates the translation from mRNA into proteins relevant to hemostasis and inflammation [48, 49]. Similar to CXCL5 and CXCL7, transcripts for MMP1 are present in platelets, likely explaining the high abundance of these mRNA in MP-MNGCs. Platelet-transferred MMP1 could modulate lung immunopathology in tuberculosis and contribute to local tissue remodelling [50]. Platelets could transfer not only mRNA but also granular content upon interaction with monocytes/macrophages. Platelets are a rich source of latent TGF-β, and this could explain their effects on macrophage phenotype skewing (M2). Consistent with this notion, KEGG analysis indicated involvement of the TGF-β signalling pathway in MP-Macs at a later stage of differentiation. Further studies will elucidate the precise molecular interactions between platelets and mononuclear phagocytes, which promote cell transformation concurrent with gain of novel immune functions.

Based on our findings we envisage the following scenario: phagocytosis of platelets by monocytes recruited to the site of infection induces activation of these phagocytes. Subsequently, the platelet granular content stimulates their differentiation into epithelioid cells. Continuous activation of the epithelioid cells drives fusion into foamy MNGCs expressing M2 phenotype, which contain mycobacteria and restrain inflammation. Our data indicate novel functions for platelets in tuberculosis pathogenesis. In more general terms, they also suggest specific modulation of platelet interactions as host-directed strategy in granulomatous diseases.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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