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Immunity to infection

Research Article

Platelets accumulate in lung lesions of tuberculosis patients and inhibit T-cell responses and *Mycobacterium tuberculosis* replication in macrophages

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Platelets regulate human inflammatory responses that lead to disease. However, the role of platelets in tuberculosis (TB) pathogenesis is still unclear. Here, we show that patients with active TB have a high number of platelets in peripheral blood and a low number of lymphocytes leading to a high platelets to lymphocytes ratio (PL ratio). Moreover, the serum concentration of different mediators promoting platelet differentiation or associated with platelet activation is increased in active TB. Immunohistochemistry analysis shows that platelets localise around the lung granuloma lesions in close contact with T lymphocytes and macrophages. Transcriptomic analysis of caseous tissue of human pulmonary TB granulomas, followed by Gene Ontology analysis, shows that 53 platelet activation-associated genes are highly expressed compared to the normal lung tissue. In vitro activated platelets (or their supernatants) inhibit BCG-induced T- lymphocyte proliferation and IFN-γ production. Likewise, platelets inhibit the growth of intracellular macrophages of *Mycobacterium (M.) tuberculosis*. Soluble factors released by activated platelets mediate both immunological and *M. tuberculosis* replication activities. Furthermore, proteomic and neutralisation studies (by mAbs) identify TGF-β and PF4 as the factors responsible for inhibiting T-cell response and enhancing the mycobactericidal activity of macrophages, respectively. Altogether these results highlight the importance of platelets in TB pathogenesis.

Keywords: Cytokines · Lymphocytes · Macrophages · *Mycobacterium tuberculosis* · Platelets

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

The World Health Organization (WHO) in 2020 estimated 10 million new tuberculosis (TB) cases worldwide. TB mortality with 1.5 million estimated deaths increased compared to 2019, thus, ranking TB as the main cause of mortality from a single pathogen excluding COVID-19 [1]. After exposure to *Myobacterium tuberculosis*, among those infected 5–10% can rapidly develop active disease, while 90% control the replication of the pathogen [2]. Reactivation of *M. tuberculosis* replication may occur in around 10% in the course of the whole life of the infected individuals, due to different factors impairing the immune system [3–5].

More efficient disease control requires more effective prevention, diagnosis, and therapy measures. WHO is trying to reach this goal through the program END TB Strategy that, by 2035, aims to reduce mortality and incidence of active disease by 95 and 90%, respectively, compared with 2015 [6–8].

TB is a chronic inflammatory disease mainly affecting the lungs, characterized by a typical local inflammatory infiltrate, the granuloma. This tissue reaction is accompanied by the change of specific hematological parameters in the bloodstream. Therefore, the dynamic changes of the blood cells found in active TB may represent a potential biomarker to support the diagnosis in addition to clinical, microbiological, and radiological tools [9–12]. Among the several proposed biomarkers, the monocytes to lymphocytes ratio (ML ratio) [13] and neutrophils to lymphocytes ratio (NL ratio) [14–16] have been promising approaches. However, the usefulness of other indexes as the absolute count of platelets or the platelets to lymphocytes ratio (PL ratio) is still unclear. Platelets are known for their role in hemostasis, but they also play an important role in chronic inflammation since their granules contain different types of mediators as chemokines, cytokines, and growth factors that may be involved in the lung damage associated with active disease, as shown in the murine model [17, 18].

Therefore, we aimed to evaluate the role of platelets in TB pathogenesis, starting with the analysis of platelets in peripheral blood of subjects at different TB stages and characterizing their phenotype and functional properties also at the site of TB disease. We also evaluated in vitro the impact of the platelets in modulating *M. tuberculosis* replication and antigen-specific T-cell activation.

Results

High platelet count and PL ratio are associated with active TB disease

We evaluated the PL ratio in patients at different TB clinical stages and in healthy donors (HD) (Fig. 1A). A significantly higher PL ratio was found in active TB patients (active TB) compared to HD ($p < 0.0001$), subjects with latent TB infection (LTBI) ($p < 0.0001$) and cured TB patients ($p < 0.0001$) (Fig. 1A and Supporting information Table S1). Conversely, we did not observe significant differences among the other groups.

The increased PL ratio found in active TB was due to a significantly elevated absolute platelet counts (Fig. 1B) and significantly decreased absolute lymphocyte counts (Fig. 1C), as compared with LTBI ($p < 0.0001$ and 0.0001, respectively), HD subjects ($p < 0.0001$ and $p = 0.0001$, respectively), and cured TB patients ($p < 0.0001$ and $p = 0.0045$, respectively). Furthermore, the Spearman-Rank test showed that the increased PL ratio in active TB directly correlated to the absolute platelet counts (r: 0.701, $p < 0.0001$) and inversely correlated to the absolute lymphocyte counts (r: 0.651, $p < 0.0001$), demonstrating that variations of both platelet and lymphocyte absolute counts contribute to the increased PL ratio (Fig. 1D and E).

To evaluate the accuracy of the PL ratio to discriminate patients with active TB from other tested groups, we used Receiver Operating Characteristic (ROC) curves analysis. PL ratio significantly discriminated active TB from HD, LTBI, and cured TB ($p < 0.0001$) in all comparisons (Supporting information Fig. S1A–C and Table S2). Similar results were obtained using the absolute platelet counts (Supporting information Fig. S1D–F and Table S3).

Finally, we evaluated the expression of lineage and activation molecules on the surface of platelets. Expressions of CD61, CD42a, and CD41b as lineage markers and PAC-1 and CD62p as activation markers were evaluated. MFI was used to quantify surface molecule expression. Expression of either CD61 or CD42a was significantly higher in circulating platelets in active TB compared to HD ($p = 0.0047$ and 0.0012, respectively). Figure 2A and C shows cumulative data from nine patients with active TB and nine HD, while Fig. 2B and D shows a representative MFI analysis of one TB patient and one HD.

Levels of platelet-associated mediators increase in the peripheral blood of active TB patients

To better analyze the status of platelets in TB patients, we evaluated the level of platelet-derived cytokines, chemokines, and growth factors in plasma samples of active TB compared to HD (Fig. 2). Levels of IL-1β (Fig. 2E) and MIP-1α (Fig. 2F) significantly increased in active TB ($p = 0.004$ and $p < 0.0001$, respectively) when compared to HD, while VEGF levels although increased in active TB did not reach statistical significance (Fig. 2G). The increased plasma levels of IL-1-β and MIP1-α observed in active TB likely reflect the release of these cytokines from monocytes in addition to platelets.

Plasma concentrations of IL-6 (Fig. 2H) and GM-CSF (Fig. 2I), two cytokines known to be involved in platelet production, were significantly upregulated in active TB compared to HD ($p < 0.0001$), suggesting that they may contribute to the increased platelet production in the BM and/or in the lungs during active TB. These results confirm our previous findings that PDGF-BB levels were significantly higher in TB patients than in non-TB patients [19].

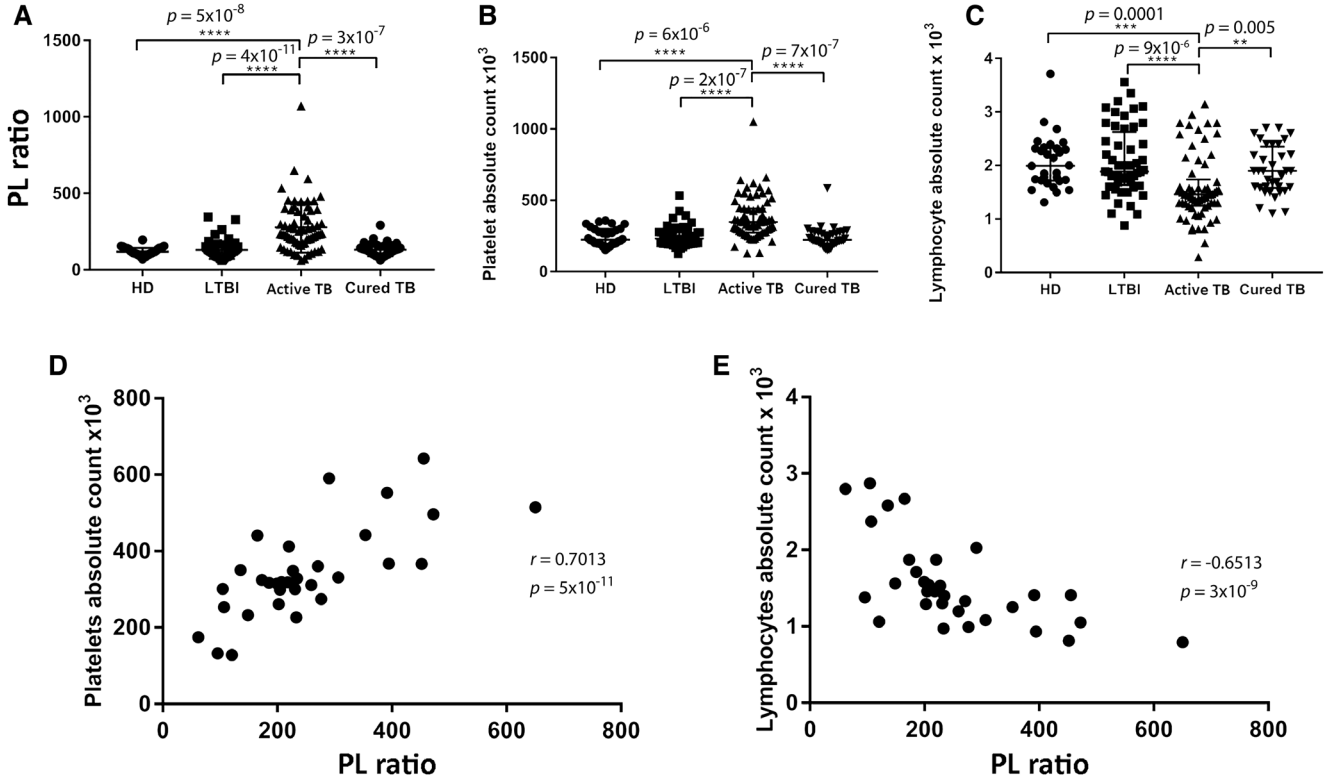

Figure 1. PL ratio, platelet, and lymphocyte absolute count of the different cohort groups. Absolute platelet and lymphocyte numbers were evaluated in whole blood by a hematology analyzer for diagnostic use. PL ratio **(A)**, platelet absolute count **(B)**, and lymphocyte absolute count **(C)** of HD, LTBI subjects, patients with active TB disease and cured TB patients. Correlation between the PL ratio and absolute platelet **(D)** and lymphocyte count **(E)**. Each dot represents one individual subject out of 30 HD, 52 LTBI, 66 active TB, and 36 cured TB patients. Each horizontal bar represents the median of each group. Correlation between the PL ratio and absolute lymphocyte count was analyzed by Spearman rank correlation test. Significance of differences between groups was compared using Kruskal–Wallis test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

In active TB, platelets colocalize with macrophages and lymphocytes in LN and lung lesions

To investigate whether platelets were localized at the disease site, we performed immunohistochemistry (IHC) on LN and lung specimens from patients with active TB. As shown in Fig. 3, platelet clusters surrounded the area of granuloma lesions in both lymph nodes (A, B) from lymph nodal TB and in lungs (C, D) from in pulmonary TB. Moreover, platelets were localized close to macrophages in LNs (E, F) in lymph nodal TB and lungs (G, H) in pulmonary TB. Finally, platelet clusters were also observed at the periphery of the granuloma, intermingling with lymphocytes in both LNs (I, J) and lung tissues (K, L) of active TB patients (Supporting information Fig. S2). Interestingly, platelet infiltration was not found in LNs of patients with nonspecific reactive follicular hyperplasia (M, N, O, P).

Similar results were obtained in a different cohort of patients with pulmonary TB evaluated at Istituto Nazionale di Malattie Infettive (INMI), showing colocalization of platelets and macrophages in lung lesions of TB patients. This finding was TB-specific because it was not observed in lungs from patients with non-TB pneumonia (Supporting information Fig. S3A). Moreover, the internalization of platelets by macrophages in lung lesions from active TB was also observed (Supporting information Fig. S3).

Transcriptomic analysis of caseous tissue shows signs of platelet activation

After demonstrating the colocalization of platelets with monocytes and lymphocytes in the granulomatous lesion, we aimed to characterize platelets present at the site of the granulomatous lesion. For this purpose, we analyzed the gene expression profiles of caseous human pulmonary TB granulomas. A total of 11 494 differentially expressed genes were identified from gene chip GSE20050 at a significance level cut-off of 0.05, and a Volcano plot was drawn for those differentially expressed genes. Interestingly, 2815 genes were significantly upregulated, and 8681 genes were significantly downregulated in TB granuloma as compared to normal lung tissue ($p < 0.05$, fold-change > 2) (Fig. 4A). At the next level of resolution, we used Gene Ontology (GO) terms in the biological process of platelet activation for this study (GO:0030168). This GO term contains 161 annotations [20] used to screen the differentially expressed genes associated with platelets in humans (Fig. 4B and Supporting information Table

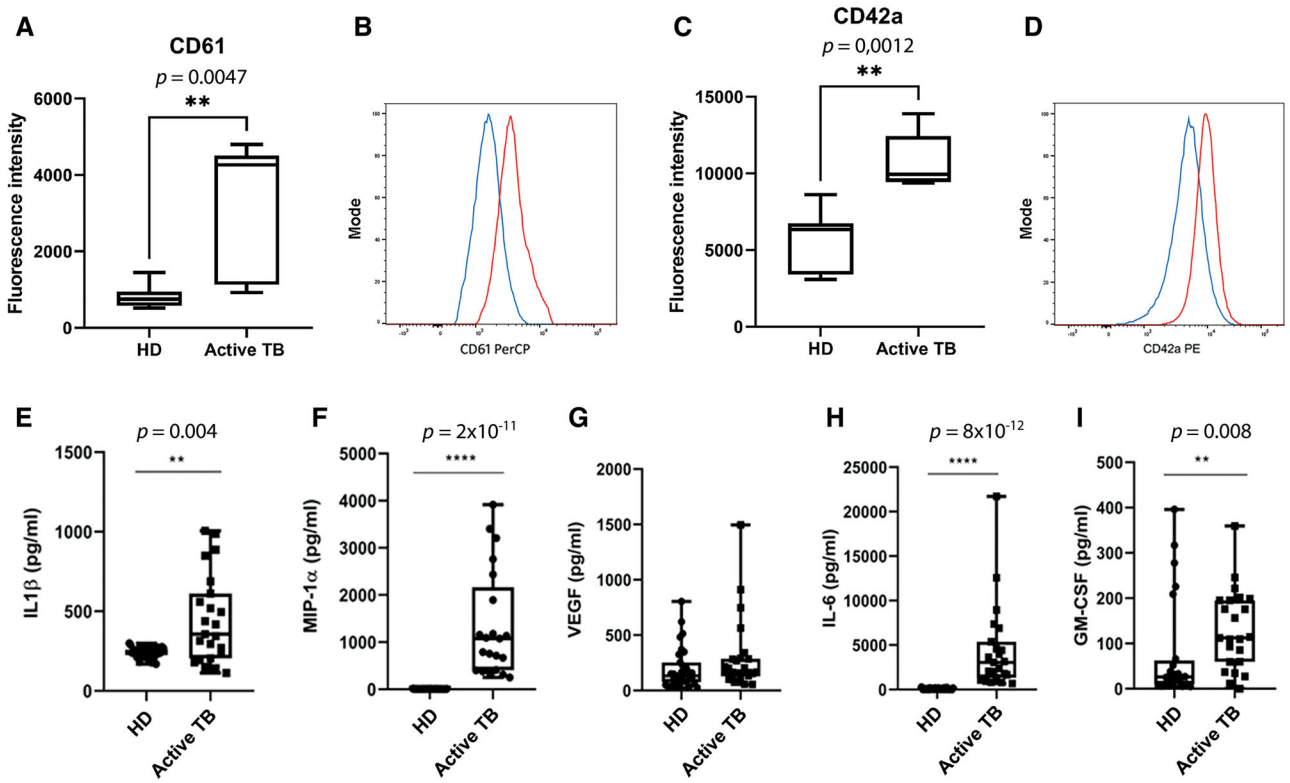

Figure 2. Phenotype of platelets and overlays between HD and active TB of CD61 and CD42a marker expression and cytokine levels in sera of HD and active TB. Box and whiskers plot **(A)** and the representative overlay **(B)** of comparison of CD61 MFI between HD and active TB platelets. Box and whiskers plot **(C)** and the representative overlay **(D)** of comparison of CD42a MFI between HD and active TB platelets. The box and whiskers plots represent the pooled data (median, interquartile ranges, and 10-90 percentiles) from nine experiments with a total of nine subjects for the two groups. The significant differences between groups were analyzed using Mann–Whitney test, $^{**}p < 0.01$, $^{***}p < 0.001$. Box and whiskers plot of comparison of IL-1β **(E)**, MIP-1α **(F)**, VEGF **(G)**, IL-6 **(H)**, and GM-CSF **(I)** between HD and active TB. The box and whiskers plots represent the pooled data (median, interquartile ranges, and 10-90 percentiles) from one experiment with 25 active TB and 40 HD subjects for the two groups. The significant differences between groups were analyzed using Mann–Whitney test, $^{**}p < 0.01$, $^{****}p < 0.0001$.

S4). GO analysis showed 53 platelet-associated genes (Supporting information Table S4A) highly expressed in caseous human pulmonary TB granulomas, compared to normal lung tissue (Supporting information Table S4B), and of note, among these were *ITGB3*, *TGFB1*, and *PF4* coding for CD61, TGF-β, and platelet factor-4 (PF4, CXCL4), respectively.

Platelets inhibit T-lymphocytes functions but enhance *M. tuberculosis* clearance in macrophages

The finding that platelets localize in close contact with macrophages and lymphocytes in TB lesions, both in LNs and lungs, prompted us to investigate the impact of platelets on the effector functions of these cells.

To study the effect of platelets on T cells, we stimulated PBMCs of patients with active TB with BCG in the presence or absence of either resting or thrombin-activated autologous platelets and measured T-cell proliferation and IFN-γ production. Resting platelets did not affect BCG-induced $CD3^+$ T-cell proliferation, but thrombin-activated platelets consistently inhibited $CD3^+$ T-lymphocyte proliferation ($p = 0.0013$) in response to BCG stimulation (Fig. 5A).

In addition to their effect, T-cell proliferation, thrombin-activated platelets inhibited BCG-induced IFN-γ production by $CD3^+$ T lymphocytes ($p = 0.0099$, Fig. 5B). Conversely, and in agreement with the T-cell proliferation results, resting platelets did not affect BCG-induced IFN-γ production. Moreover, intracellular cytokine staining analysis showed that activated platelets inhibited IFN-γ production by both $CD4^+$ and $CD8^+$ T lymphocytes (Supporting information Fig. S4). Activated, but not resting platelets, similarly inhibited $CD3^+$ T-cell proliferation ($p = 0.03$, Supporting information Fig. S5A) and IFN-γ production ($p = 0.03$, Supporting information Fig. S5B) induced by SEB.

To address whether soluble factors released after activation-induced degranulation mediated the inhibitory activity of platelets, we generated supernatants from platelets activated with thrombin (sn. Act. PLT) and tested their suppressive activity on T-cell immunity. Results showed that the supernatant of activated platelets significantly reduced T-cell proliferation ($p = 0.03$, Fig. 5C) and IFN-γ production ($p = 0.02$, Fig. 5D) of BCG-stimulated $CD3^+$ T cells and virtually abrogated both SEB-induced proliferation ($p = 0.04$) and IFN-γ production

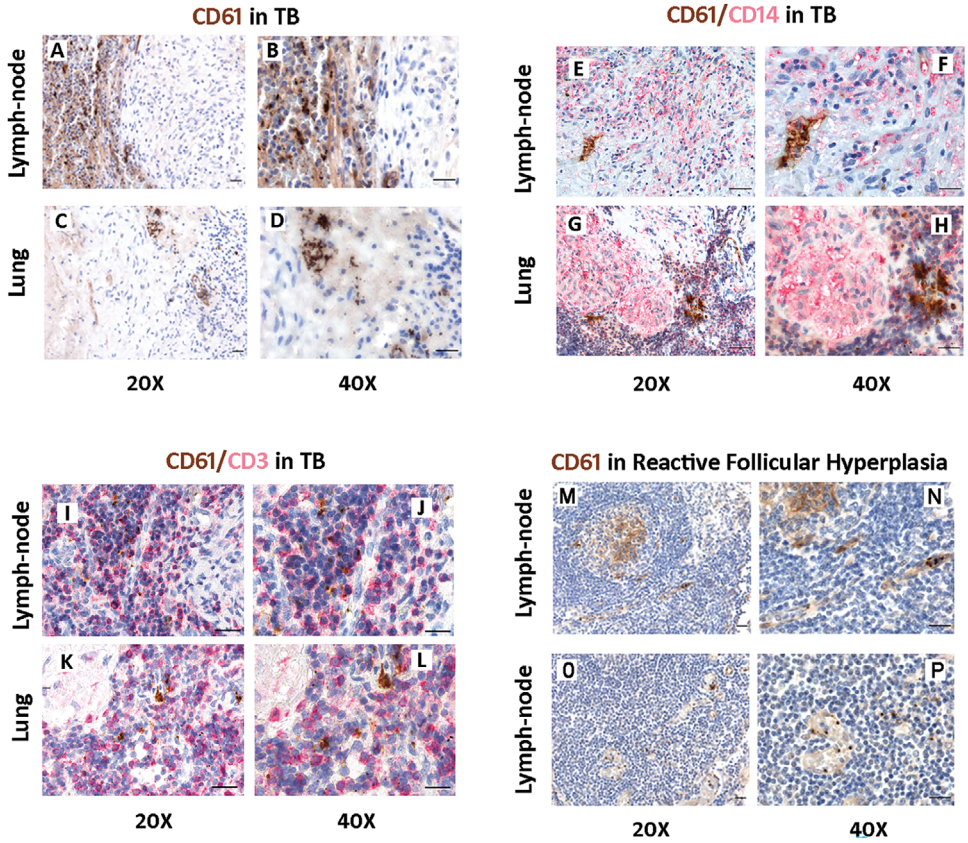

Figure 3. IHC of LN and lung tissue autopsy samples of one patient with lymph-nodal TB and one patient with pulmonary TB. Representative IHC immunostainings on LN and lung parenchyma of two TB patients, performed in two different experiments, highlighting the presence of $CD61^+$ (brown signal) platelet clusters intermingling with $CD3^+$ (purple signal) T-cell infiltrates within peripheral areas of granulomatous foci. Scale bars, 100 μm. Platelets clusters surrounding the area of granulomatous lesion in lymph nodal TB (A, B) and pulmonary TB (C, D); platelets were localized close to macrophages in LNs (E, F) in lymph nodal TB and in lungs (G,H) in pulmonary TB. Platelet at the periphery of the granulomatous lesions, intermingling with lymphocytes in both LN (I, J) and pulmonary tissues (K, L) of TB-infected individuals. No platelet infiltration was observed in control LNs of patients with nonspecific reactive follicular hyperplasia (M,N,O,P).

($p = 0.05$) (Supporting information Fig. S5C and D). Together, these results indicate that platelets modulate cell proliferation and immune response by soluble mediators.

Given the capacity of activated platelets and their supernatants to inhibit *M. tuberculosis*-specific $CD3^+$ T-cell proliferation and IFN-γ release, and because a previous study has reported that platelets increase *M. tuberculosis* intracellular survival in monocytes [17], we investigated the effects of platelets on the ability of macrophages to modulate *M. tuberculosis* growth. Therefore, we infected THP1-derived macrophages with *M. tuberculosis* strain H37Rv in the presence or absence of resting or thrombin-activated platelets and evaluated the mycobacterial burden as CFU at 24, 48, and 72 h after in vitro infection. Resting platelets did not modulate *M. tuberculosis* replication, but activated platelets reduced the growth of intracellular *M. tuberculosis* any time after infection (Fig. 6A), although the antimycobacterial effect was significant only at 72 h postinfection ($p = 0.0002$ and 0.005 in Fig. 6A and B, respectively). Notably, neither resting nor activated platelets affected the viability of macrophages (data not shown).

Since the inhibitory activity of platelets on T-cell proliferation and IFN-γ production was mediated by soluble molecules, we wondered whether the effects of platelets on *M. tuberculosis*-infected macrophages were similarly due to soluble mediators.

Therefore, we cultured *M. tuberculosis*-infected THP1-derived macrophages in the presence of supernatants from thrombin-activated platelets for 3 days and then measured *M. tuberculosis* growth by CFU. The results show that the addition of supernatant from activated platelets significantly reduced *M. tuberculosis* replication ($p = 0.02$) indicating that the microbicidal activity of platelets relies on soluble factors released upon activation (Fig. 6C). As with intact platelets, supernatants from activated platelets did not reduce macrophage viability (data not shown).

These results were confirmed using monocyte-derived macrophage (MDM) as targets of *M. tuberculosis* infection. As observed with THP1-derived macrophages, we found that activated platelets significantly reduced the bacterial burden of *M. tuberculosis*-infected MDM, as compared to *M. tuberculosis*-infected MDM cultured either in the absence of platelets or in the presence of resting platelets ($p = 0.03$ and 0.02, respectively) (Supporting information Fig. S6).

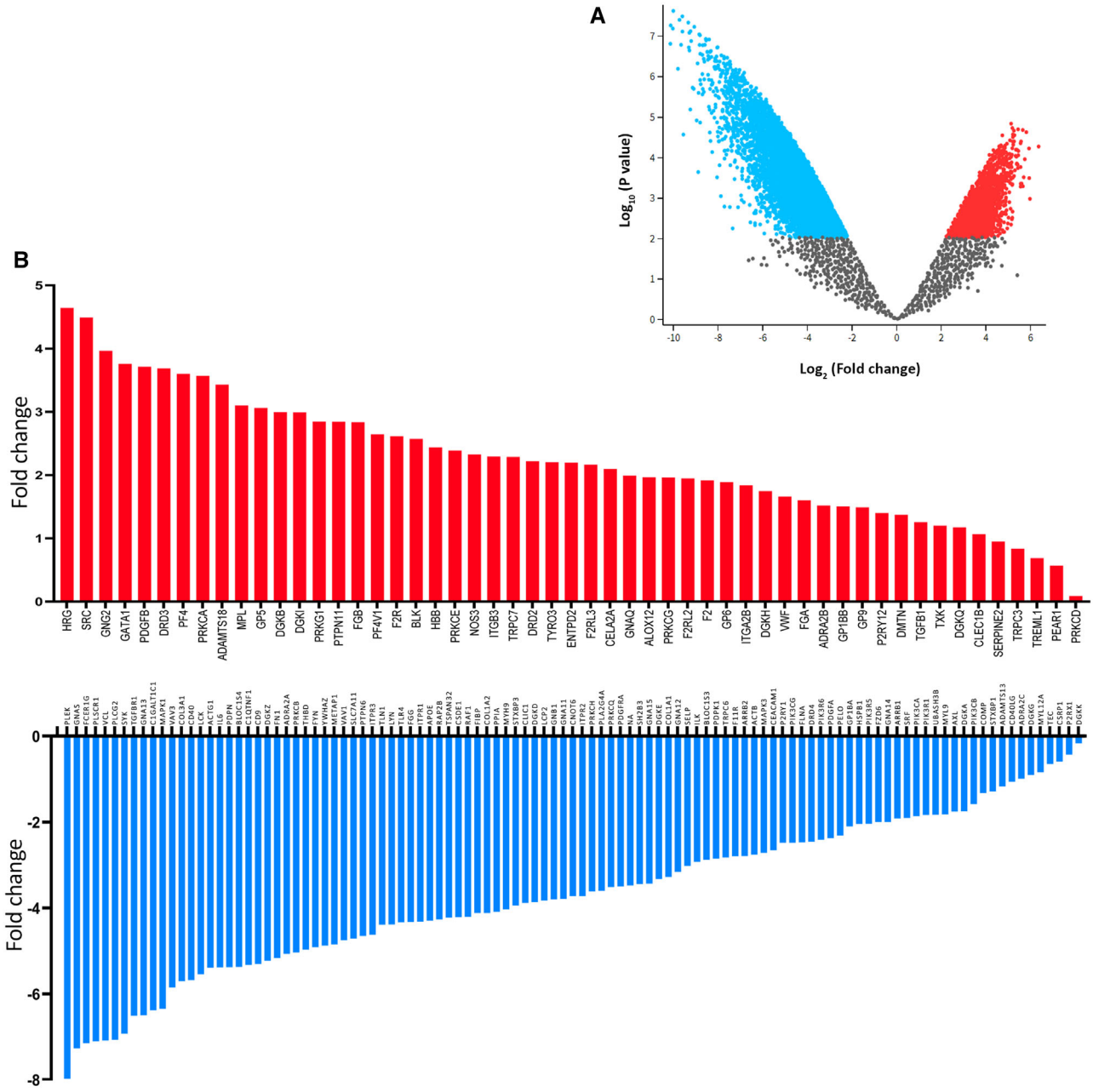

Figure 4. Gene expression analysis of caseous human pulmonary TB granulomas from TB patients. **(A)** Volcano plot of gene expression profiles of five caseous TB lung tissues and two normal lung tissues from one experiment, where blue represents significantly downregulated and red represents upregulated genes. **(B)** Expression profiles of platelet activation-associated genes, where the positive values indicate that genes are highly expressed in caseous respect to normal lung tissue and negative values indicate that genes are highly expressed in normal respect to caseous lung tissue.

TGF-β and PF4 partially mediate the immunomodulatory functions of activated platelets

Having identified, in the transcriptomic analysis, TGF-β and PF4 as markers of platelets activation, and having demonstrated the colocalization of platelets with monocytes and lymphocytes in the granuloma lesion, we further studied the soluble factors released by activated platelets that may account for the inhibition of T-cell functions and mycobactericidal activity. We data mined an independent cohort of 19 proteomics ["platelet releasate," (PR)] acquired on mass spectrometry and downloaded from the ProteomeX change dataset repository, with the identification code PXD009310. Of the 277 core proteins quantified across all donors in the PR microarray dataset, we selected the 72 most abundant proteins in the thrombin-activated PR. GO analyses of those proteins showed significant enrichment for terms related

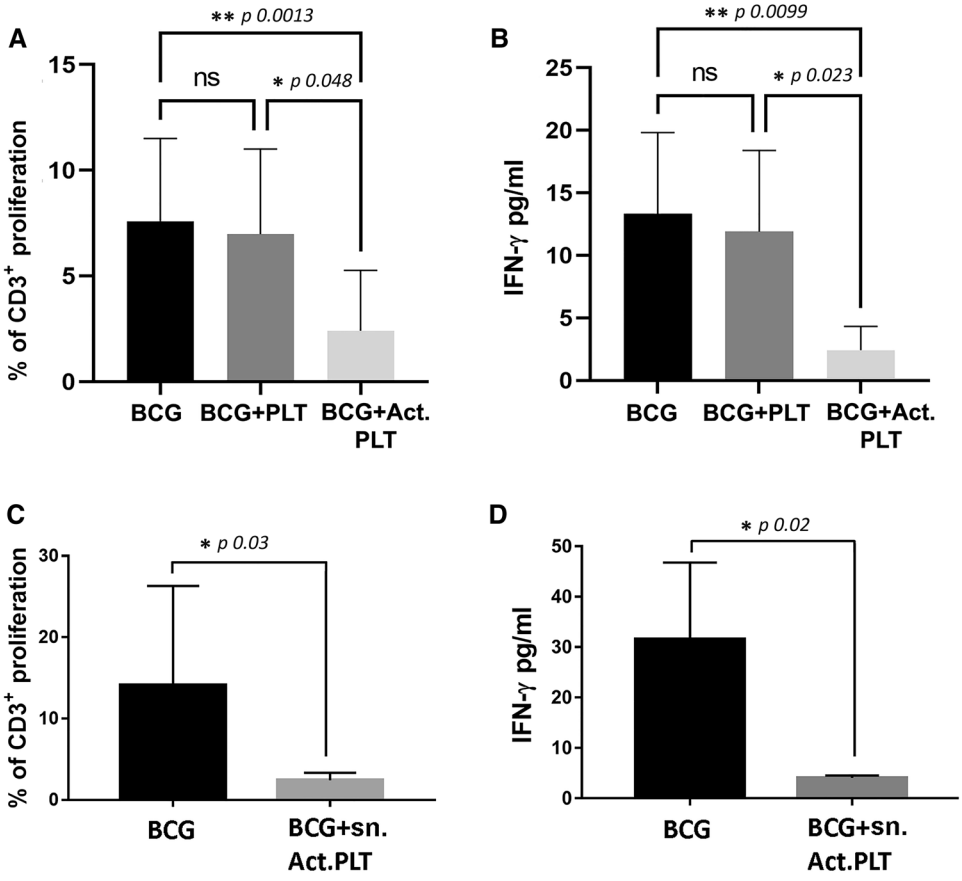

Figure 5. Thrombin-activated platelets and supernatant of activated platelet downregulates IFN-γ production from PBMC of active TB. **(A)** $CD3^+$ T cells proliferation from PBMC stimulated with BCG or with BCG plus platelets or thrombin-activated platelets or BCG. **(B)** IFN-γ production from PBMC stimulated overnight with BCG or with BCG plus platelets or thrombin-activated platelets. The graphs A and B represent the pooled data (median and IQR) from seven active TB. **(C)** $CD3^+$ T cells proliferation from PBMC stimulated with BCG in RPMI medium or with BCG in RPMI medium conditioned with supernatant of thrombin-activated platelets. **(D)** IFN-γ production from PBMC stimulated overnight with BCG in RPMI medium or with BCG in RPMI medium conditioned with supernatant of thrombin-activated platelets. The graphs C and D represent pooled data (median and IQR) from three active TB patients. The significant differences between groups were analyzed using Friedman's test and Mann– Whitney test (one tail), $^*p \leq 0.05$, $^{**}p \leq 0.01$.

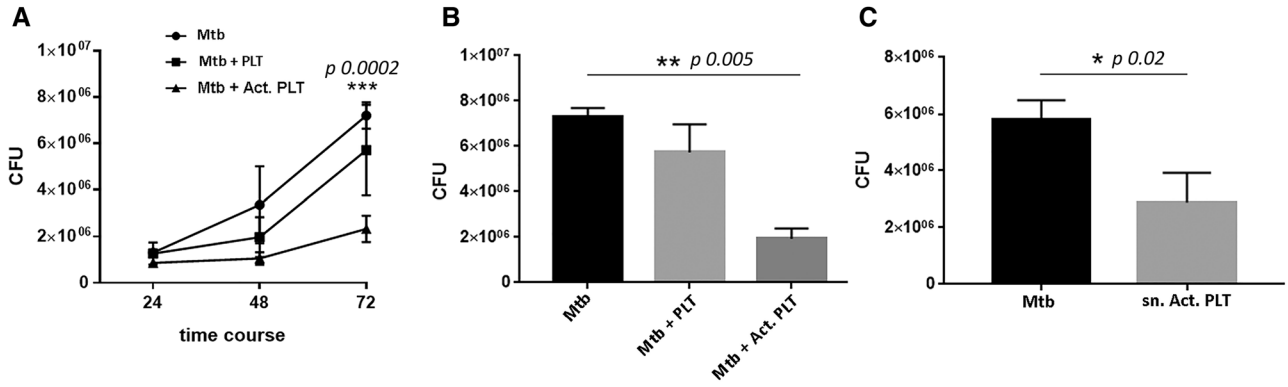

Figure 6. Thrombin-activated platelets enhance the clearance of intracellular *M. tuberculosis* in THP1-derived macrophages. **(A)** Time course of bacterial burden measurement at 24, 48, and 72 h in THP1-derived macrophages cultured in RPMI medium without antibiotics and with platelets or activated platelets from one experiment with platelets of four active TB. The significant differences between groups were analyzed using Kruskal-Wallis test $^{***}p < 0.001$. **(B)** Bacterial burden counted at 72 h in THP1-derived macrophages cultured in RPMI medium without antibiotics and with platelets or activated platelets of one experiment with six active TB; the significant differences between groups were analyzed using Kruskal–Wallis test $^{**}p < 0.01$. **(C)** Bacterial burden counted at 72 h in THP1-derived macrophages cultured in RPMI medium without antibiotics added with 20% of supernatant of activated platelets of one experiment with six active TB. The significant differences between groups were analyzed using Mann–Whitney test, $^*p < 0.05$. Data in **(A)**, **(B)**, and **(C)** are shown as mean + SEM.

to immunological functions of T lymphocytes and macrophages (Fig. 7A and Supporting information Table S5).

To predict possible interactions between the relevant proteins in platelet release, we used STRING to evaluate direct (physical) and indirect (functional) associations of molecules from primary databases elaborated by computational prediction. As shown in Fig. 7B, inputting PF4 and setting to visualize only the "evidence" interactions with a maximum of 10 interactors for the first shell and 20 for the second shell, we found 41 nodes based on the following setting: “meaning of network edges: confidence”; “active interaction source: experiments (pink line), databases (light blue line), coexpression (black line), text mining (green line)”; “protein homology (violet line).” Among the cluster generated, the direct interaction between platelets and macrophages was the most statistically significant ($p < 0.001$) (Fig. 7B).

We found that TGF-β and PF4 are representative protein signatures, confirming the result of the transcriptomic analysis and allowing us to hypothesize for these two molecules a potential role as mediators of the modulatory effects by activated platelets in vitro [21].

Since the GO analysis showed an abundance of TGF-β and PF4 in the supernatants of activated platelets, we assumed that the TGF-β was responsible for inhibiting proliferation [22] and IFN-γ production [23], while PF4 was responsible for inhibiting *M. tuberculosis* replication into macrophages.

To test these possibilities, we used anti-TGF-β and anti-PF4 monoclonal antibodies to neutralize the respective factors eventually present in the supernatants of activated platelets. We found that TGF-β neutralization partially but significantly restored T-cell proliferation (BCG vs. BCG + sn. Act. PLT $p = 0.002$, and BCG + sn. Act. PLT vs. BCG + sn. Act. PLT + anti TGF-β $p = 0.04$) (Fig. 8A) and IFN-γ release (BCG vs. BCG + sn. Act. PLT $p = 0.0005$, and BCG + sn. Act. PLT vs. BCG + sn. Act. PLT + anti TGF-β $p = 0.04$) (Fig. 8B) upon BCG stimulation. Moreover, neutralizing PF4 led to a partial but significant increase of *M. tuberculosis* load (MTB vs. MTB + sn. Act. PLT $p = 0.003$; MTB + sn. Act. PLT vs. MTB + sn. Act. PLT + anti-PF4 $p = 0.007$) (Fig. 8C). In both settings, the isotype control mAbs did not affect the capacity of supernatants from activated platelets to either inhibit T-cell proliferation or IFN-γ production or modulate intracellular *M. tuberculosis* growth (Fig. 8C).

Discussion

In this study, we evaluated the role of platelets in TB pathogenesis. We show that patients with active TB have an elevated PL ratio that, combined with the low lymphocyte number, can be used as a helpful tool to discriminate among the different TB stages. Furthermore, platelets express an activated phenotype. Moreover, mediators promoting platelet differentiation or associated with platelet activation are increased in active TB. At the site of TB, platelets are localized around the granuloma lesions in close contact with T lymphocytes and macrophages. Functionally, platelets inhibit BCG-induced T-lymphocyte proliferation and IFN-γ production, as well as intracellular *M. tuberculosis* replication, by soluble factors including TGF-β and PF4. Altogether these results highlight the importance of platelets in TB pathogenesis.

Pulmonary TB is an infectious disease whose diagnosis is complex, needs clinical and radiological evaluations confirmed by the microbiological analysis carried out on sputum by molecular and culture tests. LTBI diagnosis is based on immunological tests (TST or IGRA) that have the limitations to not discriminate between LTBI and active TB. Identifying plasma or cellular biomarkers may help in supporting a rapid diagnosis and appropriate therapeutic interventions.

Our interest in platelets is based on the evidence we generated evaluating blood cellular components in patients at different stages of TB infection/diseases. We found that patients with active TB have elevated numbers of circulating platelets. While this finding had been already reported [24, 25], we identified only few studies evaluating the role of platelets in subjects at different TB stages. Here, we found that the absolute count of platelets was significantly increased whereas the lymphocytes count was decreased in active TB, as compared to HD and LTBI subjects. Consequently, active TB had a very high PL ratio, in analogy with the ML and the NL ratios [13, 26, 27]. Hence, the PL ratio can be an additional laboratory tool to help discriminating active TB from LTBI.

Depending on the specific pathogen, a microbial infection may impact platelet production by megakaryocytes in the BM [28–31]. Indeed, one of the most common abnormalities of the blood count is a high platelet number, and the most common cause of thrombocytosis is a reaction to an inflammatory insult [32]. It is known that cytokines, such as IL-6 and TNF-α, produced in response to inflammatory conditions enhance the platelet production by triggering megakaryocytopoiesis [33, 34]. In vitro IL-6 appears to act as a megakaryocyte maturation factor [35]. Intravenous infusion of IL-6 in mice and during clinical trials in humans leads to modest thrombocytosis [36, 37]. Initially, it was suggested that inflammatory thrombocytosis was due to IL-6; however, differently, several investigators have shown that IL-6 induces hepatic thrombopoietin production and thrombocytosis associated with IL-6 is eliminated by thrombopoietin blockade. Elevated platelet counts are typically found in infections and inflammation and generally return to normal after resolution. In both these conditions, elevated platelets count occurs in response to cytokines produced by leukocytes. Accordingly, our study found that plasma levels of IL-6 and GM-CSF were significantly increased in samples from active TB compared to HD. IL-6 is an inflammatory cytokine but, together with GM-CSF, can also promote megakaryocytopoiesis, thus, accounting for the increased absolute platelet number found in active TB.

In active TB patients, platelets had an increased surface expression of CD61 and CD42a, two molecules involved in platelet adhesion and activation, indicating that circulating platelets display a phenotype associated with adhesiveness and aggregation. Accordingly, levels of other proinflammatory cytokines, such as

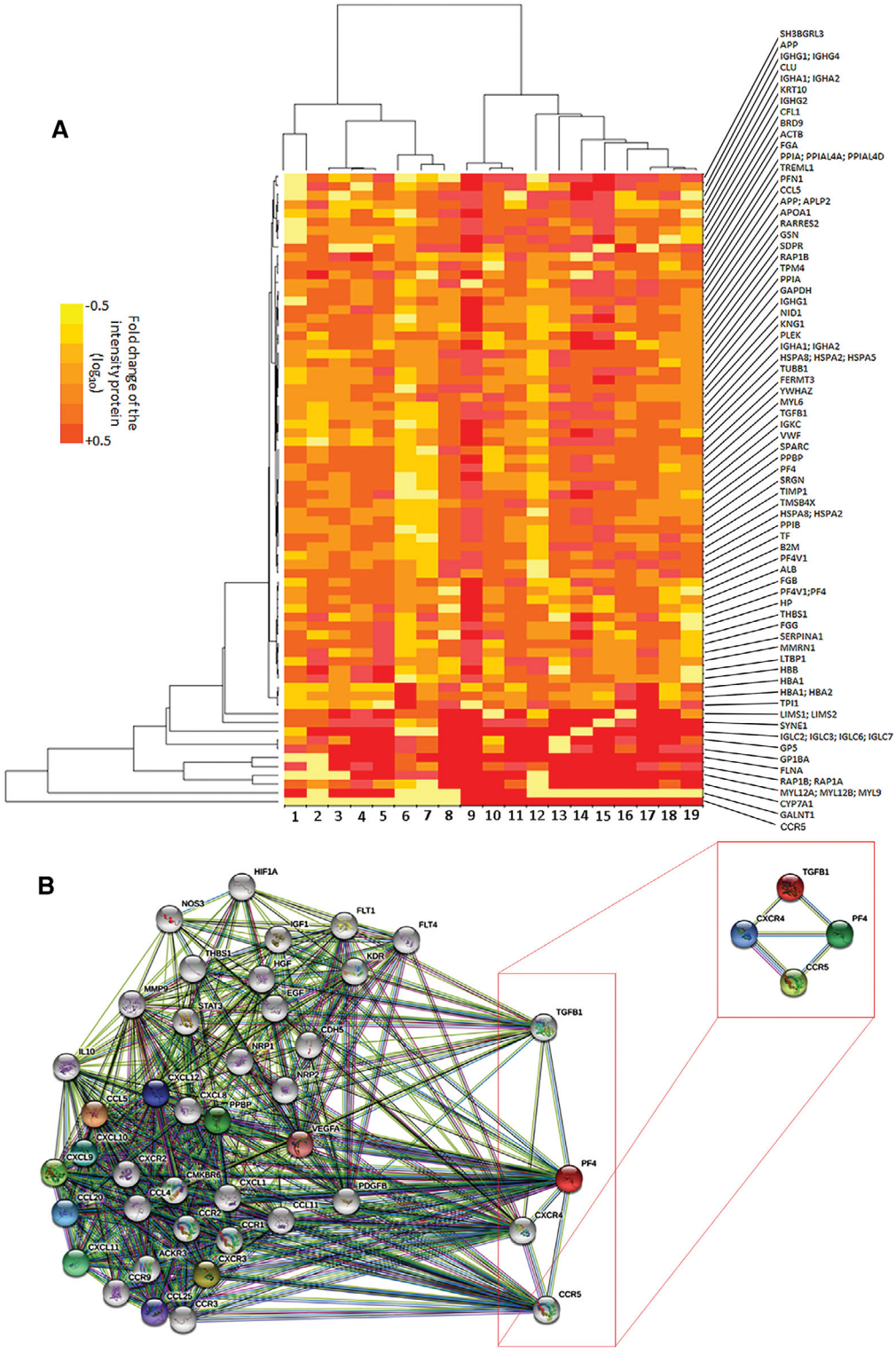

Figure 7. Direct (physical) and indirect (functional) associations of molecules released by platelets from primary databases elaborated by computational prediction. **(A)** PF4 network proteins/interacting partners. The protein network was built using String 11.0., inputting PF4 and setting to visualize only the "evidence" interactions with a maximum 10 interactors for the first shell and 20 for the second shell, we found 41 nodes based of the following setting: "meaning of network edges: confidence"; "active interaction source: experiments (pink line), databases (light blue line), coexpression (black line), text mining (green line)"; "protein homology (violet line)." Among the cluster generated, the direct interaction between platelets and macrophages was the most statistically significant ($p < 0.001$) (Figure 7B, right panel). **(B)** Heat maps of the most abundant proteins in platelets released (intensity value in log_{10}); yellow indicates the lowest intensity, while red indicates the highest intensity (72 proteins analyzed).

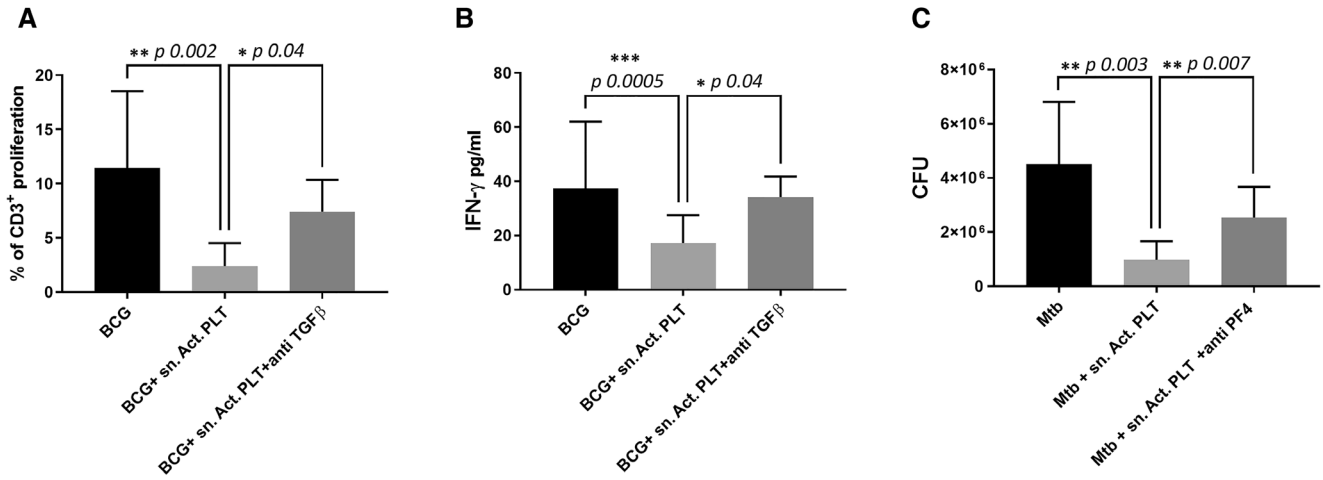

Figure 8. Thrombin-activated platelets and supernatant of activated platelet proliferation and IFN-γ production from PBMC of active TB patients. **(A)** $CD3^+$ T-cells proliferation from PBMC stimulated with BCG, with BCG in RPMI medium conditioned with supernatant of thrombin-activated platelets or with BCG in RPMI medium conditioned with supernatant of thrombin-activated platelets depleted of TGF-β. The graph represents the pooled data (median and IQR) from seven different experiments performed with samples of seven active TB. **(B)** IFN-γ production from PBMC stimulated with BCG in RPMI medium conditioned with supernatant of thrombin-activated platelets depleted of TGF-β. The graph represents the pooled data (median and IQR) from nine different experiments performed with samples of nine active TB. **(C)** Bacterial burden counted at 72 h in THP1-derived macrophages infected with chemiluminescent. *M. tuberculosis* and cultured in RPMI medium without antibiotics, RPMI medium added with 10% of supernatant of activated platelets from active patients or RPMI medium added with 10% of the same supernatant of activated platelets depleted of PF4. The graph represents the pooled data (median and IQR) from one experiment with eight active TB. The significant differences between groups were analyzed using Kruskal–Wallis test, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

IL-1-β, MIP-1α, and VEGF, were significantly higher in the circulation of active TB patients than HD. Monocytes likely released these molecules but activated platelets could contribute to their increased plasma levels, thus, amplifying inflammation and tissue damage.

Most notably, we found that large numbers of platelets were present at the site of *M. tuberculosis* infection itself. Platelet clusters were detected at the periphery of the granulomatous lesions, intermingling with lymphocytes in both pulmonary and LN tissues from TB patients. Only a few scattered platelets aggregates were detected in a control LN with reactive follicular hyperplasia.

Altogether these findings highlight that platelets are important actors of the human TB pathology.

Previous in vitro studies have demonstrated that platelets contribute to the differentiation of monocytes to the foam phenotype and switch to epithelioid and giant cells [38], and that platelets are concentrated at the site of TB lungs in a murine model [17, 39].

In view of this evidence, and to better understand the role of platelets in mycobacterial setting, we used an in vitro coculture system to investigate the influence of activated platelets in regulating proliferation and IFN-γ production by BCG-stimulated T lymphocytes. We demonstrated that activated, but not resting platelets, reduced both proliferation and the production of IFN-γ by T lymphocytes, indicating that in vitro activated platelets display immunosuppressive activity. In addition, activated platelets significantly enhanced the killing of intracellular *M. tuberculosis*, which was particularly evident at 72 h after infection. This was found either using THP1-differentiated or monocyte-derived macrophages infected in vitro with *M. tuberculosis*. This microbicidal activity was not associated with a significant macrophage killing.

While the inhibitory activity of platelets on T-lymphocyte proliferation and IFN-γ production has been shown in other systems, the finding that they enhance macrophage bactericidal response to *M. tuberculosis* was unexpected. A previous study demonstrated that monocytes cocultured with platelets reduced the intracellular killing of *M. tuberculosis* by 2.5-fold [17]. Several reasons are possible for the discrepancy with our findings, as different MOI were used (which was 1) [17], the infection performed in primary monocytes infected with nonactivated platelets [17], and *M. tuberculosis* growth measured on 1 day postinfection [17]. Conversely, in the present study, we used the THP1 cell line and confirmed the results in monocyte-derived macrophages, employed a higher MOI (which was 10), performed a daily measurement of CFU up to day 3 postinfection, and very importantly, and added activated (not resting) platelets.

It is notorious that platelets contain and release several proinflammatory and anti-inflammatory molecules that interact with many different immune cells (e.g., DCs, neutrophils, and lymphocytes) and can, thus, modulate or regulate innate and adaptive immune responses [40–44].

In our study, we found that activated platelets differently modulate T lymphocytes and macrophages responses to *M. tuberculosis* and these distinct effects were associated with the release of soluble factors. We first interrogated 277 core human proteins identified in the releseate of thrombin-activated platelets reported by Parsons et al. [45] and downloaded from ProteomeXchange, and performed enrichment analyses of GO annotations and cell-signal pathways for biological processes. Analysis indicated

that two of the most abundant proteins in the activated-platelet releseate, TGF-β and PF4, had biological activities compatible with the supernatant of activated platelets. In fact, it has been demonstrated that PF4 released from platelets mediates microbial growth reduction, that is, inhibition of *Plasmodium falciparum* into erythrocytes by neutrophil activation [46, 47]. Moreover, the network between PF4 and macrophages has been reported in atherosclerosis as PF4 induces the polarization of a unique subset of macrophages [4, 48] equipped with properties distinct from classical M1 and alternative M2 macrophages and, for these reasons, defined as M4-polarized macrophages [3, 49].

Platelet-derived TGF-β is known to be involved in the regulation of the immune response improving the function of regulatory cells and inhibiting T-cell responses to control the excessive inflammatory response [50], and PF4 and CCL5 were potentiating the phagocytic cells to clear the pathogens by phagocytosis or neutrophil extracellular traps formation [51, 52]. These two different roles on the adaptive and innate immune systems could coexist and be driven by platelets.

Transcriptomic analysis of caseous human pulmonary TB granulomas revealed the upregulation of 53 platelet- associated genes. Identification of biological pathways by transcriptomics enhances the understanding of the potential role of platelets in the pulmonary granulomatous TB diseases with respect to normal lung tissue. Moreover, upregulation of following three genes correlated with platelets was observed: *ITGB3*, *TGFB*1, and *PF4*.

The link between TGF-β, PF4, and macrophages has been well explained previously [5, 7, 53, 54]. In our study, the interaction between platelets and macrophages was first documented by IHC experiments in the TB granuloma where we showed that the platelets aggregated in close contact with macrophages and lymphocytes. Indeed, activated platelets are responsible for the induction of CD16 on monocytes because TGF-β is released at tissue damage sites and enhances the functions of these effector cells. Finally, the relationship between CCR5, CXCR4, and PF4 is related to the multifaceted platelet functions. PF4 not only acts as an inflammatory mediator but also downregulates chemotactic receptors, such as CCR1, CCR2, and CCR5, on isolated monocytes, thereby interfering with their migration [6, 55]. In this way, platelets are dynamic actors in the balance between negative and positive feedback mechanisms during bacterial infection (as shown in the "GO process" in Supporting information Table S4) and by their presence at the site of the lung disease (transcriptomic analysis Fig. 4).

In light of the results obtained from the proteomic and transcriptomic analysis, and the functional results, we show that TGF-β and PF4 are involved in inhibiting TB immune response and *M. tuberculosis* replication. Obviously, other molecules present in the supernatants from activated platelets [18, 56, 57] may contribute to further modulate T-lymphocytes and macrophages activities during TB.

In conclusion, these results highlight the importance of platelets in TB pathogenesis. Additional studies are needed to confirm these findings.

Materials and methods

Characteristics of participants enrolled

We recruited 184 subjects in four different groups: LTBI, active TB, cured TB, and HD from the Department of Infectious Disease, University Hospital of Palermo (Supporting information Table S6). Tissue samples from active TB, from patients with pneumonia and from cases that died from other causes than pneumonia, were collected at INMI-IRCCS"L. Spallanzani in Rome, Italy (Supporting information Table S7). None of the patients or HD was affected by coagulation or platelet pathologies at the study time. Exclusion criteria were hematologic disorders, coagulopathies, peripheral vascular disease, thrombocytopenia autoimmune, anticoagulant, or anti-inflammatory therapies.

Microbiological-confirmed TB cases were based on positivity to at least one of these tests: *M. tuberculosis* culture (sputum, broncholavage, pleural fluid, abscesses); *M. tuberculosis*-specific RNA amplification (TRC Ready M.TB, Tosoh, Japan), and/or *M. tuberculosis*-specific NAT (Home-made PCR (IS6110) GeneXpert, Cepheid; Genotype MTBDRPlus Hain Lifescience); histopathological findings and presence of acid-fast bacilli in tissues. TB patients were enrolled no later than the first week of TB treatment. In the absence of clinical, microbiological, and radiological signs of active-TB, LTBI definition was based on a positive QFT-Plus (Qiagen, Hilden, Germany) score. LTBI individuals were enrolled before starting preventive therapy. Cured TB patients were those with a previous microbiological diagnosis who were successfully treated after at least 6 months of therapy. The Ethical Committee approved the study of the University Hospital in Palermo (approval number 13/2013) and at INMI (approval number 72/2015). All participants signed a written informed consent.

QFT-Plus assay

QFT-Plus kits were donated by Qiagen and used according to the manufacturer's instructions. Levels of IFN-γ were quantified by ELISA and analyzed by a QFT-Plus Analysis Software {3708 QuantiFERON®-TB Gold Plus, ELISA Package Insert, QUIAGEN}.

Complete differential counts of peripheral blood

Whole blood specimens were collected in EDTA vacutainer tubes. Peripheral blood samples were analyzed using an automatic hematology analyzer for diagnostic use (Coulter 4.500, Germany) to carry out a complete differential blood count. The analysis was performed by a clinical diagnostic laboratory accredited by the Italian National Accreditation System following international standards ISO17025/2005 and ISO 15189/2007. Complete blood cell counts were measured within 6 h after collection, following the guidelines established by the quality controls.

Luminex multiplex immunoassay

By Luminex assay, we measured the concentrations of platelet production mediators IL-6, GM-CSF, and released factors by activated platelets, such as IL1-β, MIP-1β, and VEGF, in plasma samples from active TB and HD. Experiments were performed according to the instructions of the manufacturer. Briefly, following prewetting of plates, 50 μL of precombined beads of all individual cytokines or chemokines were added and washed twice with wash buffer. Plasma samples (25 μL) were diluted 1:2 with the kit serum matrix and added to the plate. The plate was shaken for 30 s at 1000 rpm and then incubated for 1 h on a plate shaker at 300 rpm at room temperature. The plates were washed twice, and 25 μL of detection antibody was added per well and incubated for 1 h on a plate shaker. After 1 h of incubation, the streptavidin-PE conjugate (50 μL per well) was added and incubated for 30 min at room temperature. Finally, the plate was washed three times, and 150 μL of sheath fluid was added to each well, then the plate was read on the Bio-Plex platform (Bio-Rad), with Bio-Plex Software version 6.1 used for bead acquisition and analysis.

Platelets staining and FACS analysis

The evaluation of platelets phenotype was performed on HD and active TB using anti-human mAbs to CD61 PerCP (Clone RUU-PL7F12), CD62p PE (clone AC1.2), CD42b APC (clone HIP1), CD41a FITC (clone HIP8), CD42a PE (clone ALMA.16), PAC-1 FITC (clone PAC-1), and CD45 APC-H7 (clone 2D1), all from BD Bioscience (San Diego, USA) and the relative isotype controls [57, 58]. For the surface staining, 20 μL of platelets was used for a tube containing 20 μL of each mAb used in different combinations. After 20 min of incubation at room temperature, the samples were resuspended in PBS acquired using a FACSCANTO II (BD Biosciences). FACS plots were analyzed using FlowJo software (version 6.1.1; Tree Star, Ashland, OR, USA). The sequential gating strategy is shown in Supporting information Fig. S7.

Immunohistochemistry and immunofluorescence analysis

Two independent analyses were carried out at the University of Palermo, and at INMI.

At the University of Palermo, fixed paraffin embedded (FFPE) human tissue samples of pulmonary and lymph nodal tuberculosis were collected according to the Helsinki Declaration, and the study was approved by the University of Palermo Ethical Review Board (approval number 09/2018). Briefly, sections 2.5/3 micron-thick were cut from paraffin blocks, dried, dewaxed and rehydrated. Then, the antigen unmasking technique was performed using target retrieval solutions, pH 9 EDTA-based buffer in a thermostatic bath at 98°C for 30 min. After the sections were brought to room temperature, the neutralization of the endogenous peroxidase with 3% H_2O_2 and protein blocking by a specific protein block was performed.

Tissue samples were incubated with the following primary antibodies: mouse monoclonal anti-human CD61 (dilution 1:50, pH 9, Clone 2f2 Leica Novocastra), rabbit monoclonal CD14 (dilution 1:50, pH 9, Clone EPR3653 Cell Marque). The single-marker IHC was revealed by either a polymer detection method (Novolink Polymer Detection Systems Novocastra Leica Biosystems Newcastle Ltd Product No: RE7280-K), and 3,3'diaminobenzidine (DAB) substrate was used as the chromogen. Double-marker IHC was carried out by incubation overnight at 4°C with a cocktail of the two above-mentioned primary antibodies. Staining was revealed using MACH2 Double Stain detection kit (Biocare Medical), DAB, and Vulcan Fast Red as substrate chromogens. The slides were counterstained with Harris hematoxylin (Novocastra).

At INMI, formalin FFPE lung tissue samples were collected and entered into this study. These comprised histologically and PCR proven cases of pulmonary TB (n.10) and a comparison group of samples from subjects with pneumonia of different etiologies (n.10) and samples from cases died from other causes than pneumonia (n.6) such as hepatitis, endocarditis, etc. (controls) (Supporting information Table S7). Autopsy lung tissue samples were fixed in the neutral buffered-formalin immediately for histological and immunohistochemical analysis. For molecular studies, DNA was selectively extracted (QIAamp) Tissue Kit, Qiagen GmbH) from paraffin sections and PCR detection of mycobacterium DNA was performed.

CD61 IHC was performed on tissue sections using ready to use Leica mouse monoclonal anti-CD61 (clone 2f2), revealed with a Ventana VU Universal DAB detection kit in a Bench Mark ULTRA IHC/ISH processor (Ventana Medical Systems, Tucson, AZ) according to the manufacturer protocol. Antigen retrieval was a standard automated process on the Ventana Bench Mark ULTRA. Sections were counterstained with hematoxylin, dehydrated, and mounted.

For immunofluorescence, deparaffinised and rehydrated lung sections were immersed in 10 mM sodium citrate, pH 6.0, and microwaved for antigen retrieval. Samples were incubated with anti-CD61 Leica (2f2) and anti-CD68 Ventana (KP-1), primary antibodies overnight at 4°C.

Sections were then incubated with Alexa Fluor 488 or Alexa Fluor 594 conjugated secondary antibodies. Samples were counterstained with DAPI (Invitrogen, Thermo Fisher Scientific). Confocal fluorescence microscopy images were acquired on a Zeiss 900 LSM confocal equipped with an Airyscan2 detector and processed using Zen software (Zeiss, Germany).

Platelet separation

Blood was collected in EDTA tubes centrifuged at 120 × *g* for 10 min at room temperature to prepare platelet-rich plasma (PRP). The erythrocyte- and leukocyte-free PRP was collected

and transferred in another tube without anticoagulant for second centrifugation at 800 × *g* for a further 5 min [21]. Finally, the serum component was removed, and the platelets were resuspended in 2 mL of PBS 1×, pH 7.4 and used for further experiments.

Effect of platelets on T-lymphocyte proliferation and IFN-γ production

We have stimulated PBMC from TB patients with BCG and with SEB. We then analyzed the stimulated cells in the same manner for both stimuli. A total of 1×10^5 PBMCs were stimulated with BCG (BCG MEDAC, MOI 1 μg/mL) and put in coculture in round-bottom 96-well plate with platelets (ratio 1:100) activated and nonactivated ex vivo with 0.5 U/mL of thrombin (Hemos IL Product, Bedford, USA) at a final volume of 200 μL of RPMI supplemented with 10% FCS, L-glutamine (2 mM), HEPES buffer (10 mM), and penicillin/streptomycin 1×. We also tested supernatants of activated platelets obtained after 5 min of stimulation with 0.5 U/mL of thrombin in RPMI complete medium at 37°C and collected after centrifugation at 1200*g*. Supernatants of activated platelets were used on BCG-activated T lymphocytes at a dilution of 1:4 and on polyclonal-activated T lymphocytes at a dilution of 1:2 with complete medium (RPMI 1640 medium, Euroclone, Pero, MI, Italy) supplemented with 20 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% heat-inactivated FCS, 2 mM L-glutamine. After overnight incubation at 37°C with 5% CO_2, supernatants derived from cultures performed with activated platelets or supernatants of activated platelets were harvested and analyzed for the IFN-γ concentration by an ELISA test according to manufacturer indications (www.quantiferon.com). The data obtained as IU per milliliter has been converted to pg per milliliter. The IFN-γ production at the different conditions was compared to the controls' production without any stimulation. The same experiments were carried out to evaluate T-lymphocytes proliferation. For this purpose, PBMCs have been prelabelled with CFSE (Biolegend, San Diego, CA) at a final concentration of 1 μM and subsequently stimulated with SEB as described above. After 6 days of incubation, PBMCs were harvested and stained with mABs anti-CD3 PE-Cy7 (clone SK7) from BD (Becton Dickinson, USA) for 20 min at room temperature (RT). After the incubation, cells were washed and resuspended in 500 μL of FACSFLOW, acquired to FACSCANTO II and analyzed by FlowJo software (version 6.1.1; Tree Star). Cells were gated on morphological parameters FSC versus SSC, followed by gating on $CD3^+$T lymphocytes to analyze proliferation.

Effect of platelets on *M. tuberculosis* replication into macrophages

A total of 2.5×10^5 cells per well of a monocyte line (THP1, from ATCC) or monocytes from peripheral blood were differentiated toward macrophages in RPMI complete medium plus PMA at a concentration of 50 ng/mL in 24-well plate, for 3 days. After differentiation, THP1-derived macrophages or MDM were infected with chemiluminescent *M. tuberculosis* transfected with bacterial luciferase at MOI of 10:1 bacteria/cell. After 3 h of infection, cells were washed several times with PBS to eliminate extracellular *M. tuberculosis*; then, 1 mL of complete RPMI medium or 800 μL of complete RPMI medium, and 200 μL of supernatant of activated platelets were added to the cultures, according to the experimental layout. Luminometric evaluation of bacterial burden was performed at 24, 48, and 72 h using VeritasMicroplate Luminometer Promega (Turner Biosystems; Sunnyvale, CA, USA). After the culture time and before the bacterial burden evaluation, we assessed the viability of THP1-derived macrophages or MDM by dye exclusion test, using Trypan Blue staining (Thermo Fisher Scientific Gibco).

The medium was removed, and cells were washed three times with PBS to detect the intracellular mycobacteria. Then, 500 μl per well of 1× permeabilization solution (Invitrogen by Thermo Fisher Scientific, eBioscience) was added. After 15 min of incubation at RT, 200 μL of supernatant from the cell cultures of each well was harvested and put into 96-well microplate for luminometric assay. Next, 20 μL of 1% decyl aldehyde (decanal) solution was injected in each well containing the supernatant obtained from *M. tuberculosis*-infected macrophages, and after 1 s of delay, photon emission was detected for 2 s; a ladder made with different concentrations of *M. tuberculosis* was used to determine the concentration of *M. tuberculosis* in supernatants from different experimental points.

In selected experiments, we added anti-human TGF-β or anti-human PF4 mABs in the activated platelet supernatants to deplete these endogenous cytokines. We coated 96-well flat-bottom plates with the mABs at above-mentioned concentration of 10 μg/mL and overnight incubated 200 μL of activated platelet supernatants. TGF-β-depleted activated platelet supernatant was used to evaluate the cell proliferation of $CD3^+$T lymphocytes obtained from PBMC previously stimulated with BCG or SEB. PF4-depleted supernatant from the activated platelet was used to evaluate the ability to modulate *M. tuberculosis* growth in the THP1-derived macrophages.

Bioinformatic analysis of platelets releasate

We data mined an independent cohort of 19 proteomics "platelet releasate" (PR) [24], from healthy adult volunteers, acquired on mass spectrometry and downloaded from the ProteomeXchange dataset repository, with the identification code PXD009310. We calculated the weighted mean of the 277 core proteins quantified across all donors in the PR microarray data set and considered those protein values that were over the weighted mean to be overexpressed. Then, we selected the 72 most abundant proteins in the thrombin-activated PR that were plotted with heat maps (range −0.5 +0.5 log10 intensity value).

These 72 most abundant proteins were subjected to GO analysis, and all RefSeq gene accession numbers and their GO terms

were collected with R Bioconductor (https://bioconductor.org), using R Bioconductor package bioma Rt. This set was used for the gene enrichment analysis for GO with R Bioconductor package top GO.

To assess the functional relationships between the 72 most abundant proteins, we used STRING [33, 34] with TGF-β1 or PF4 data input and the following setting parameters: "meaning of network edges: confidence"; "active interaction source: experiments, databases, coexpression, text mining"; "minimum required interaction score: highest confidence (0.900)"; "max number of interactors to show:1stshell–no more than 10 interactors, 2ndshell–20 interactors."

Gene expression profiles of caseous human pulmonary TB granulomas

Data of gene chip GSE20050 were obtained from GEO database. The data were from the Microbiology and Immunology Department of Cornell University with five cases of caseous granulomas from TB patients as an experimental group and two cases of normal samples as a control group. From these data, we have performed Affymetrix Expression Console and RMA algorithm for quality control, normalization, and log2 conversion for the raw data of gene chips (Supporting information Fig. S8). Microarray data analysis package (GEO query, Limma, umap) in "R" software was used to screen the differentially expressed genes from raw data of gene chip. *p*-value and adjusted p-value were calculated with Benjamini & Yekutieli Adjustment test. "R" software was used to screen the differentially expressed genes from raw data of gene chip. *p*-value and adjusted *p*-value were calculated with Benjamini & Yekutieli Adjustment test [59].

Statistical analysis

The PL ratio was calculated by dividing the absolute platelet count permicroliter by the absolute lymphocyte count per microliter. The median or geometric mean was used for descriptive statistics for each parameter.

The nonparametric Kruskal-Wallis test was performed, comparing the medians of absolute platelet count, lymphocyte absolute count, and PL ratio among different groups. The relationship between platelets and lymphocytes was analyzed by the Spearman rank correlation test. For each test, $p < 0.05$ was considered statistically significant. The ROC curve analysis was performed to evaluate the potential of platelets or PL ratio as diagnostic TB biomarkers and to set cut-off to discriminate among different TB stages. The nonparametric Mann-Whitney Friedman and Kruskal–Wallis tests were used to compare the levels of cytokines in plasma of active TB versus HD and to analyze the downregulation of IFN-γ in T lymphocytes and the bacterial burden among the groups in macrophages.

Data were analyzed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA).

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Author contributions: FD and NC conceived and designed the experiments and wrote the article. MPL, VO, GB, BT, BB, LP, and LF performed the experiments. FD, NC, MPL, VO, DG, MSA, EL, and PD made intellectual contributions to the work. MPL, VO, GB, BB, LP, MSA, EL, and LF analyzed the data. PD, ML, BT, and VO enrolled the patients and collected the clinical information. PD, BT, DG, and ML supervised the laboratory collection of the clinical samples. FD, NC, MPL, and DG supervised the writing of the article.

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Abbreviations: **DAB**: 3,3'diaminobenzidine · **FFPE**: fixed paraffin embedded · **GO**: gene ontology · **HD**: healthy donors · **IHC**: immunohistochemistry · **INMI**: Istituto Nazionale di Malattie Infettive · **LTBI**: latent TB infection · **MDM**: monocyte-derived macrophage · **ML ratio**: monocytes to lymphocytes ratio · **NL ratio**: neutrophils to lymphocytes ratio · **PL ratio**: platelets to lymphocytes ratio · **PR**: platelet releasate · **PRP**: platelet-rich plasma. · **ROC**: Receiver Operating Characteristic · **WHO**: World Health Organization · **TB**: tuberculosis

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