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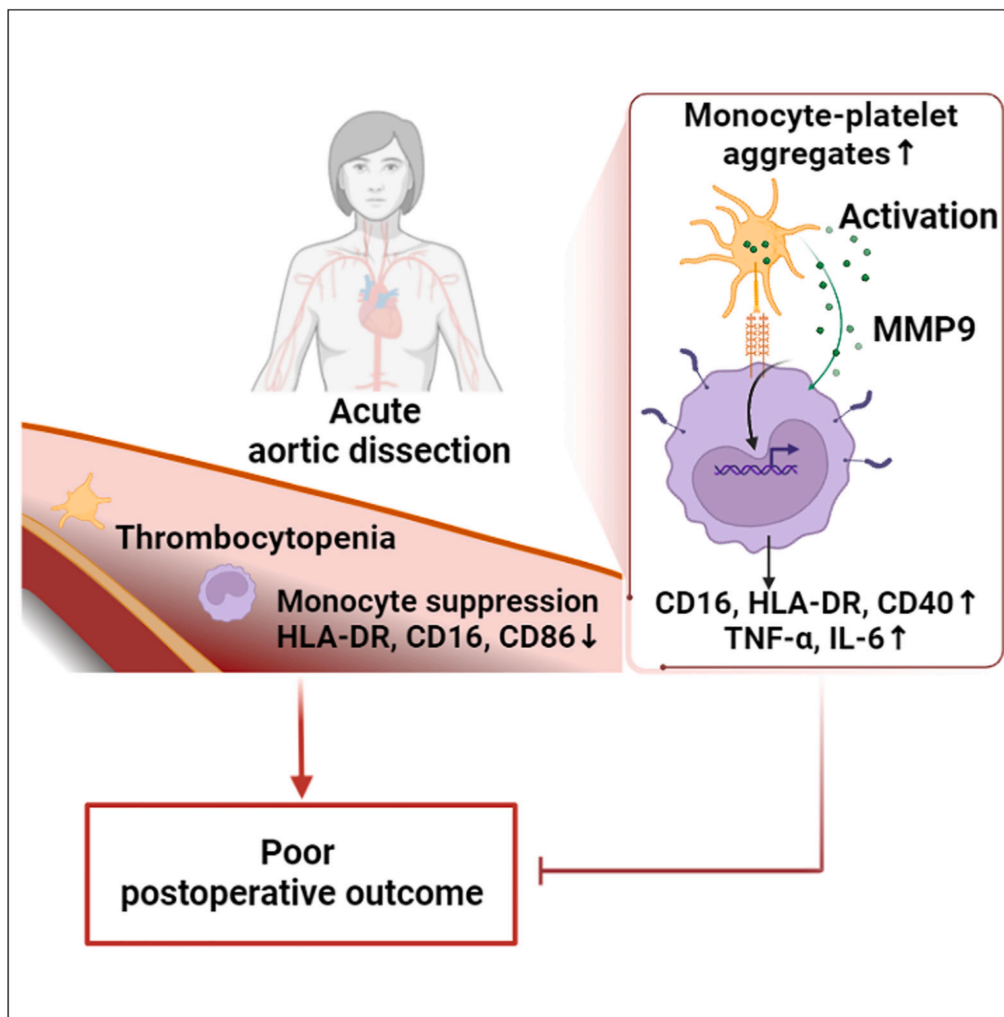

Wei-Yun Shen, Hui Li, An-Hui Zha, Ru-Yi Luo, Yan-Ling Zhang, Cong Luo, Ru-Ping Dai

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Highlights

Platelets were activated, and thrombocytopenia predicts surgical prognosis in AAD

AAD patients displayed reduced pro-inflammatory phenotypes in monocytes

Platelets-monocyte aggregates benefit postoperative recovery in AAD

Platelets restore monocyte function partly in an MMP-9-dependent way in AAD

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Platelets reprogram monocyte functions by secreting MMP-9 to benefit postoperative outcomes following acute aortic dissection

Wei-Yun Shen,[1,2] Hui Li,[1,2] An-Hui Zha,[1,2] Ru-Yi Luo,[1,2] Yan-Ling Zhang,[1,2] Cong Luo,[1,2,3,*] and Ru-Ping Dai[1,2,3,4,*]

SUMMARY

Platelets have a great ability to modulate immune responses. Monocyte-platelet aggregates (MPAs) are associated with the pathogenesis of cardiac disease. Notably, a low preoperative platelet count often indicates poor postoperative recovery following acute aortic dissection (AAD). The functions of platelets and MPAs in AAD, however, remain poorly understood. We found that, despite decreased platelet counts, platelets were also activated in AAD patients, with significant alterations in immune-modulating mediators. Of interest, monocytes in AAD patients had a suppressed immune status, which was correlated with poor outcomes following surgery. Interestingly, platelets preferentially aggregated with monocytes, and the levels of MPAs were related to recovery after surgical repair in AAD patients. Platelets restored suppressed monocyte functions in AAD patients by forming aggregates and partly by secreting matrix metalloproteinase-9 (MMP-9). Thus, the results point to a previously unknown mechanism for platelets involving monocyte reprogramming, which may improve postoperative outcomes following complex cardiovascular surgery.

INTRODUCTION

Platelets circulate to maintain hemostasis and to modulate various immune responses.[1,2] For example, by forming monocyte-platelet aggregates (MPAs), platelets skewed pro-inflammatory phenotypical and functional features of monocytes to M1 polarization and increased survival in septic mice.[3] In autoimmune diseases, such as diabetes and rheumatoid arthritis, MPAs promote disease progression by over-activating monocytes.[4,5] Moreover, elevated MPAs also predict poor outcomes and increase cardiovascular events following myocardial infarction, ischemic stroke, and chronic kidney disease.[6–9]

Recently, there has been a surge in interest in the role of platelets and MPAs in patient outcomes after cardiac surgery. One month after coronary artery bypass grafting, studies found a sustained increase in MPAs.[10] In addition, platelet activation and MPAs were shown to be associated with poor recovery after valvular surgery.[11] However, in more complex cardiovascular diseases such as acute aortic dissection (AAD), the effect is different. In AAD, a low platelet count at admission predicted postoperative pneumonia and increased 30-day mortality.[12–14] Our study found an association between cluster of differentiation (CD) 4^{+} T lymphopenia and major adverse events after type A AAD surgery, indicating a possible suppressed immune status.[15] Nevertheless, the effects of platelets and MPAs on postoperative outcomes in complex cardiovascular surgeries remain equivocal.

The present study revealed that platelets could improve postoperative outcomes after type A AAD surgery by enhancing monocyte functions by forming aggregates and secreting matrix metalloproteinases-9 (MMP-9).

Methods

The Institutional Medical Ethics Review Board of the Second Xiangya Hospital and enrollment in the Chinese Clinical Trial Registry both approved the study (No. ChiCTR2100047573). All participants provided written, informed consent to donate their blood samples. Additional methods and materials are displayed in the STAR Methods section.

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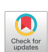

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Table 1. Clinical characteristics of study patients with AAD and healthy donors

Characteristics	HD (n = 88)	AAD (n = 85)	p value
Age (years)	51.81 ± 11.55	51.60 ± 10.14	0.239
Male	68 (77.27)	65 (76.47)	0.653
Body mass index (kg/m^2)	20.55 ± 1.32	25.16 ± 3.22	<0.0001
Complication			
Hypertension	/	82 (96.5)	/
Diabetes mellitus	/	7 (8.24)	/
Marfan syndrome	/	3 (3.53)	/
Laboratory manifestation			
PLC (10^9/L)	202 (172, 236)	185 (153, 220)	< 0.001
MPV (fL)	10.79 ± 0.86	10.75 ± 0.95	0.279
PTC (%)	0.23 (0.20, 0.26)	0.21(0.15, 0.24)	0.000
PDW (fL)	13.56 ± 3.41	12.79 ± 2.37	0.592
MPV/PLC ratio	4.91 (4.22, 5.96)	5.74 (4.80,7.06)	0.001
WBC (10^9/L)	5.94 ± 1.29	12.24 ± 3.68	< 0.001
Neutrophils (10^9/L)	3.44 ± 1.01	10.03 ± 3.60	< 0.001
Lymphocytes (10^9/L)	0.31 (0.25, 0.40)	1.15 (0.81, 1.48)	< 0.001
Monocytes (10^9/L)	0.33 ± 0.10	0.70 ± 0.31	< 0.001
PCT (ng/mL)	/	0.13 (0.06, 0.32)	/
CRP (mg/mL)	/	29.6 (9.09, 86.2)	/
D-dimer (μg/mL)	/	2.72 (1.46, 5.00)	/
Hospital mortality, n (%)	/	8 (4.94)	/

Results are expressed as mean ± SD for normally distributed continuous variables, median (interquartile range) for non-normal continuous variables, and n (%) for categorical variables. PLC: platelet count; PTC: plateletcrit; MPV: mean platelet volume; PDW: platelet distribution width; PCT: procalcitonin; CRP: C-reactive protein; D-dimer.

RESULTS

Preoperative platelets are aberrant and able to modulate immunoinflammatory responses in AAD patients

In total, 88 AAD patients and 85 matched healthy donors (HDs) were enrolled in this study. Consistent with previous studies, AAD patients showed significantly altered immune cell constitutions, manifested by an increased number of neutrophils and monocytes but a decreased count of lymphocytes. AAD patients displayed decreased platelet counts (PLCs) and plateletcrit (PTC) but an increased ratio of mean platelet volume (MPV) to PLC (MPV/PLC) (Table 1), indicating a platelet abnormality in AAD patients.

Platelets from AAD patients showed stronger Annexin V staining than HDs, suggesting activation and degranulation (Figures 1A–1D and S1A). Moreover, preoperative PLC was negatively correlated with intensive care unit (ICU) stays in AAD patients (Figure 1E). PLC at admission was also negatively correlated with 24-h postoperative C-reactive protein (CRP) and procalcitonin (PCT) (Figures 1F and 1G), but no significant association with the postoperative D-dimer was found (Figure S1B). Together, these findings suggest a close association between preoperative platelets and postoperative outcomes following complex cardiovascular surgery.

To determine how platelets are involved in postoperative outcomes, whole transcriptome levels of purified platelets in AAD patients were measured using RNA sequencing (RNA-seq) (Table S1 and Data S1). Differential expression analysis identified 232 upregulated and 112 downregulated genes from platelets in AAD patients compared to HDs (Figure 1H, $P < 0.05$ and fold-change >2). Interestingly, gene ontology (GO) enrichment analysis revealed that the upregulated genes were involved in neutrophil degranulation, responses to lipopolysaccharides (LPSs), positive regulation of inflammatory responses, the chronic inflammatory response, and neutrophil chemotaxis (Figure 1I). Next, the AAD patients were stratified into groups with high or low levels of platelets.[12] Functional enrichment analysis revealed that the differentially expressed genes (DEGs) were significantly enriched in immune response regulation (Figure 1J). Overall, platelet transcriptional

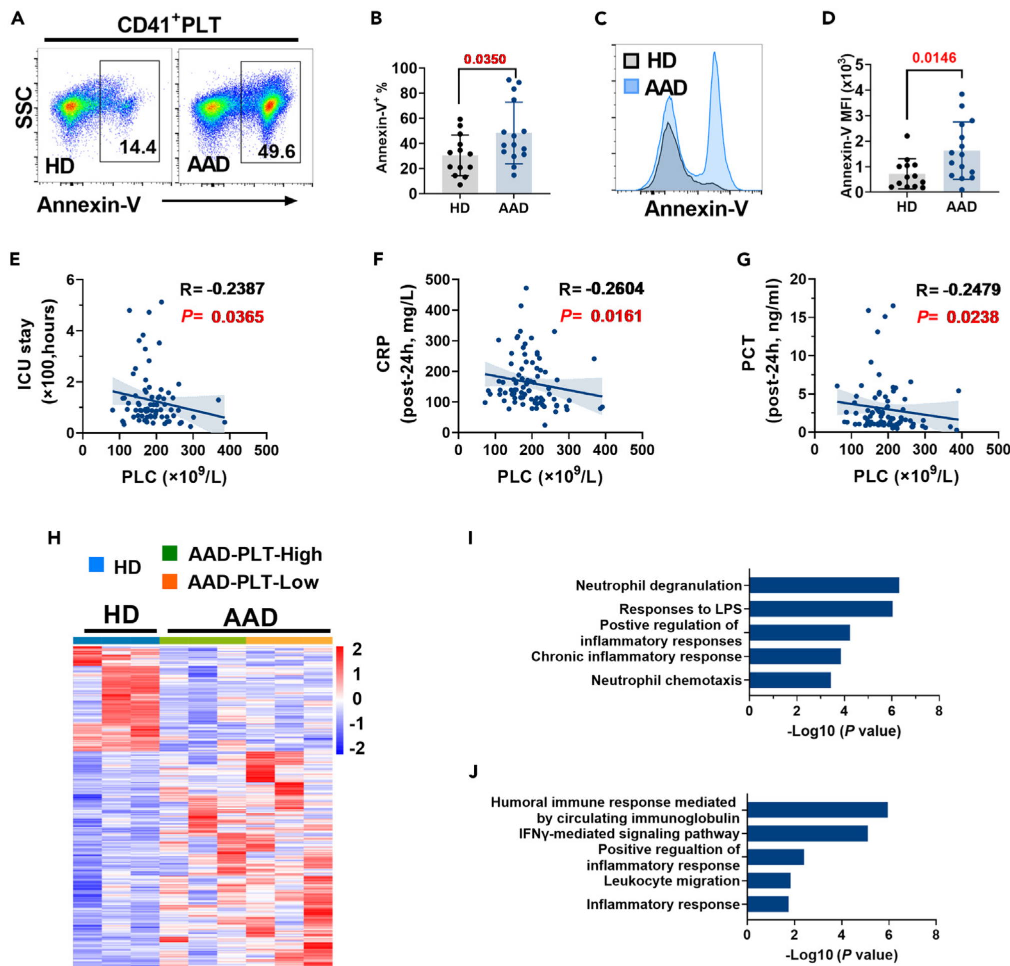

Figure 1. Preoperative platelets are aberrant and able to modulate immunoinflammatory responses in AAD patients

Platelet-rich plasma from AAD patients and matched HDs were stained with Annexin V and CD41 and measured using flow cytometry.

(A and B) Representative flow cytometric images (A) and statistical analysis (B) demonstrate the frequency of Annexin V^+ cells in $CD41^+$ platelets.

(C and D) Annexin V MFI in $CD41^+$ platelets. Data are represented using an image (C) and summary graph (D).

(E–G) Clinical data in AAD patients were obtained, and correlation analyses were performed between the preoperative platelet numbers and postoperative parameters, including the length of ICU stay (E), CRP (F), and PCT (G) 24 h after surgery.

(H and L) Platelet samples from 6 AAD patients and 3 HDs were subjected to RNA-Seq, and the results were subjected to gene set enrichment analysis. (H) Heatmap of significantly differentially expressed platelet transcripts from AAD patients and HDs. Red indicates increased relative expression, and blue indicates decreased relative expression.

(I) Five immune-related GO terms were upregulated in AAD platelets compared to HDs platelets.

(J) Five immune-related GO terms were upregulated in the AAD-PLT-Low group compared to the AAD-PLT-High group.

Data are expressed as the mean ± SD. Two-tailed Student's *t* tests (B, D) and Spearman correlation analysis (E-G) were conducted. AAD: acute aortic dissection; AAD-PLT-High: aortic dissection patients with high platelet; AAD-PLT-Low: aortic dissection patients with low platelet; CRP: C-reactive protein; HDs: healthy donors; PCT: procalcitonin; PLC: platelet count; post-24h: 24 h after surgery.

mapping suggested that platelets could modulate AAD patients' immunoinflammatory responses and influence postoperative outcomes.

PMAs shortened the ICU and hospital stays of AAD patients by restoring monocyte immune responses

Platelets modulate the phenotype and functions of monocytes in various inflammatory diseases. In AAD patients, the proportion of human leukocyte antigen (HLA)-DR^+, $CD86^+$, $CD40^+$, $CD16^+$, and $CX3CR1^+$

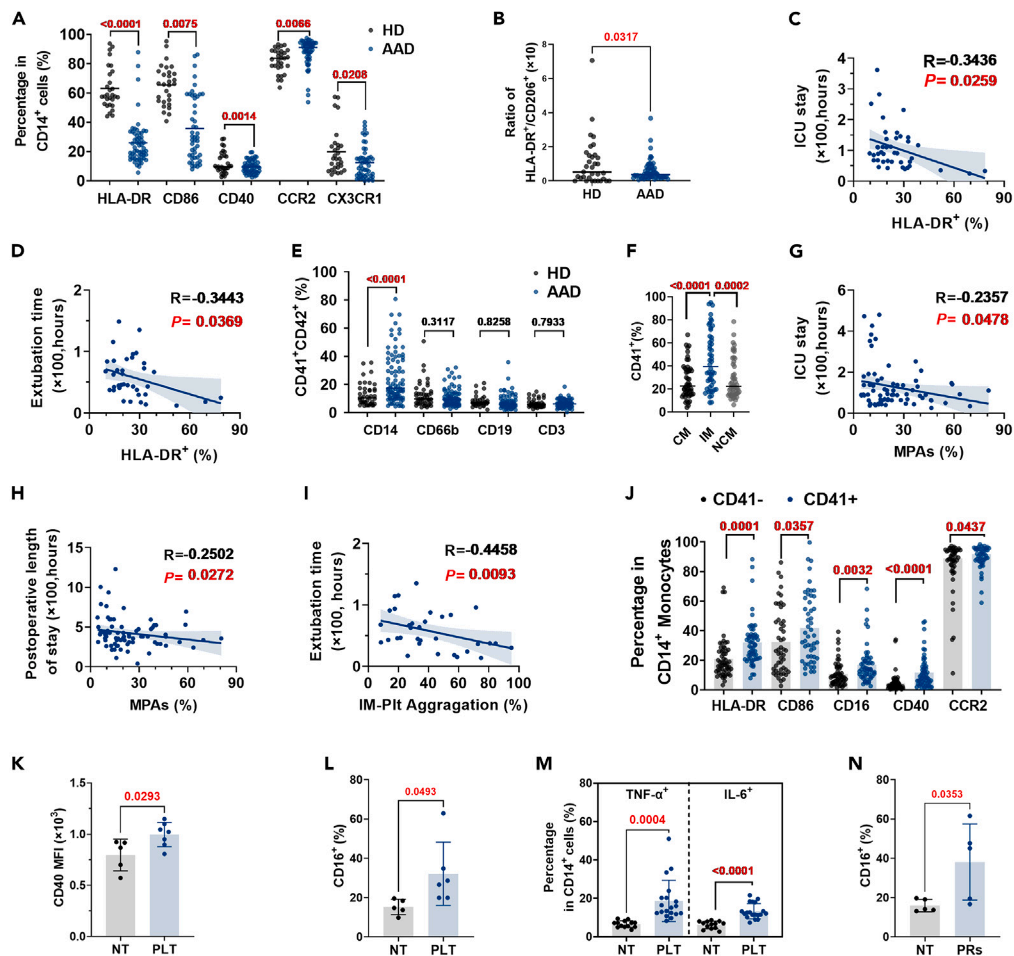

Figure 2. Platelet-monocyte aggregates improved postoperative outcomes of AAD patients by restoring the monocyte immune response

Flow cytometry was performed on whole blood from AAD patients and matched HDs by staining for CD41 and immune cell markers.

(A) Flow cytometry analysis showed the percentage of HLA-DR$^+$, CD86$^+$, CD40$^+$, CCR2$^+$, and CX3CR1$^+$ monocytes in AAD patients compared to HDs.

(B) The ratio of HLA-DR$^+$ to CD206$^+$ monocytes in AAD patients compared to HDs.

(C and D) Correlation analysis of the percentage of HLA-DR$^+$ monocytes and postoperative indicators of the length of ICU stay (C) and the time to tracheal extubation (D).

(E) Flow cytometric analysis showing the percentage of MPAs (CD14$^+$CD41$^+$CD42$^+$), platelet-neutrophile aggregates (CD66b$^+$CD41$^+$CD42$^+$), platelet-T cell aggregates (CD3$^+$CD41$^+$CD42$^+$), and platelet-B cell aggregates (CD19$^+$CD41$^+$CD42$^+$) in the circulation.

(F) Flow cytometric analysis showing the percentage of MPAs in classical monocytes (CD14^{bright}CD16$^-$, CM), intermediate monocytes (CD14$^+$CD16$^+$, IM), and nonclassical monocytes (CD14$^+$CD16^{++}, NCM).

(G and H) The correlation analysis of the percentage of MPAs and postoperative indicators, including length of ICU (G) and hospital (H) stays.

(I) Correlation analysis of the percentage of platelet-intermediate monocyte aggregates and the time to tracheal extubation.

(J) Flow cytometry analysis comparing the expression of HLA-DR, CD86, CD16, CD40, and CCR2 in monocytes with or without platelet aggregation.

(K–M) PBMCs from HDs were cultured with platelets from AAD patients at a 1:100 ratio for 24 h. CD40 expression (K), percentage of CD16$^+$ monocytes (L) and cytokines TNF-α and interleukin-6 (IL-6) expression (M) were measured using flow cytometry.

(N) Monocytes from HDs were isolated using magnetic beads and stimulated with PRs from AAD patients for 24 h. Flow cytometric analysis showing the percentage of CD16$^+$ monocytes changes. Data are expressed as the mean $\pm$ SD.

Figure 2. *Continued*

Two-tailed Student's *t* tests (A-B, E, J-N), one-way ANOVA (F), and Spearman correlation analysis (C-D, G-I) were performed. AAD: acute aortic dissection; HD: healthy donors; CM: classical monocytes; IM: intermediate monocytes; NCM: nonclassical monocytes; MPAs: platelet-monocyte aggregates; NT: no treatment; PLT: platelet from AAD patients; PRs: platelet releasates.

monocytes was reduced, while the C-C chemokine receptor (CCR)2^{+} subsets were increased (Figure 2A). In addition, the ratio of HLA-DR^{+} to CD206^{+} monocytes was lower in AAD patients compared to HDs (Figure 2B). In addition, there were increased subpopulations of CD14^{bright}CD16^{-} classical monocytes but decreased CD14^{+}CD16^{++} nonclassical monocytes in AAD patients (Figures S2A and S2B), a finding in good agreement with a previous report.[16] Notably, correlation analysis showed that the decreased percentage of HLA-DR^{+} monocytes at admission predicted a prolonged length of (ICU stay and a delayed extubation time (Figures 2C and 2D). These results strongly indicate that monocytes have reduced pro-inflammatory functions in AAD patients.

Platelets can aggregate with monocytes and thus enhance their pro-inflammatory immune responses.[1,2] Therefore, MPAs levels were investigated in AAD patients. Interestingly, MPAs levels were higher in AAD patients compared to HDs (Figure 2E). Further subpopulation analysis revealed that platelets preferentially aggregated with pro-inflammatory intermediate monocytes (CD14^{+}CD16^{+}) compared to classical (CD14^{bright}CD16^{-}) and nonclassical monocytes (CD14^{+}CD16^{++}) in AAD patients (Figures 2F and S2A). Moreover, MPAs levels were negatively associated with the postoperative length of ICU (Figure 2G) and hospital (Figure 2H) stays. In addition, pro-inflammatory intermediate MPAs were also inversely correlated with the extubation time for AAD patients (Figure 2I). Neutrophils have irreplaceable roles in AAD. In our study, an increase in the percentage of neutrophil-platelet aggregates (NPAs) in the neutrophils in AAD patients compared to healthy controls was not detected (Figure 2E). There was only a tendency for a negative association between the level of NPAs and the postoperative hospital and ICU stays (p=0.1487 and p=0.1202, respectively) (Figures S2C and S2D). These findings suggested that platelets may bind to monocytes and benefit AAD patients. Indeed, compared to CD41^{-}CD14^{+} monocytes, CD41^{+}CD14^{+} monocytes had a higher expression of antigen-presenting and inflammatory molecules, including HLA-DR, CD40, CD86, CD16, and CCR2 (Figure 2J). The percentage of MPAs was also positively correlated with the percentage of CD86, HLA-DR, CD16, and CD40 monocytes at admission (Figures S2E–S2H). Next, AAD patients' platelets were cocultured with monocytes from HDs to investigate whether platelets could modulate the functions of monocytes. As expected, platelets from AAD patients increased the expression of CD40 (Figure 2K) and CD16 (Figure 2L) in monocytes, as well as upregulating the expression of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Figure 2M). When platelet releasates (PRs)[17] from AAD patients were added to cultured monocytes, the percentage of CD16^{+} monocytes also increased (Figure 2N).

Platelets maintain the immune functions of monocytes in AAD patients in an MMP-9-dependent manner

Analysis of DEGs in platelets showed that MMP-9 was significantly upregulated in AAD patients, especially those with lower PLCs (Figures 3A–3C). Generally, MMP-9 is known to degrade the extracellular matrix and regulate inflammation.[18] Additionally, increased serum MMP-9 has been shown to affect AAD patient outcomes independently.[19,20] A previous study reported that platelet-derived MMP-9 could influence coronary disease progression.[21] In the present study, enhanced MMP-9 mRNA concentrations and elevated MMP-9 protein levels were identified in platelets of AAD patients (Figures 3D–3H), suggesting a possible role of platelet-derived MMP-9 as immunomodulators in AAD patients.

Our findings indicated that platelets could alter monocyte functions and that MMP-9 might be a critical mediator. To that end, healthy monocytes were treated with PRs, and a direct MMP-9 influence was observed on the functional ability of monocytes. Indeed, the MMP-9 inhibitor (MMP-9i) blocked the upregulation of CD16 induced by PRs in AAD patients (Figure 3I). To the best of our knowledge, no previous studies have reported on the role of MMP-9 in monocyte functioning. Therefore, monocytes were collected, and RNA-seq analysis was conducted. In total, there were 102 DEGs in PRs-treated monocytes in the presence of MMP-9i or vehicle (Figure 3J, Table S1, and Data S2), among which CD16, CD163, CCR2, CCR5, and macrophage receptor with collagenous structure (MARCO) transcripts were downregulated (Figure 3K). GO analysis revealed significant enrichment in the cellular response to LPS, the chemokine-mediated signaling pathway, inflammatory responses, chemotaxis, positive regulation of IL-6, and others (Figure 3L). Taken together, these findings

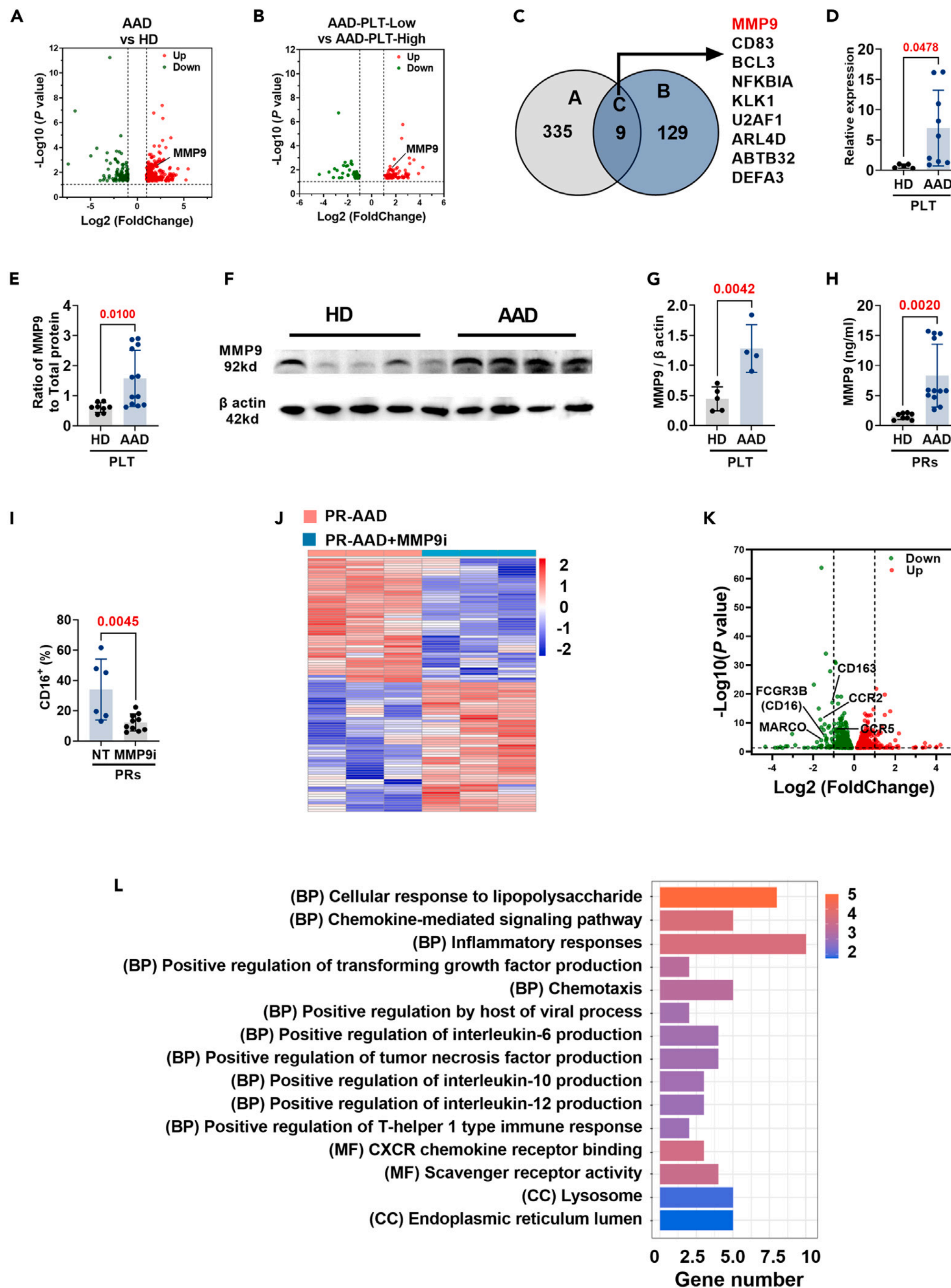

Figure 3. Platelets maintain the immune functions in AAD patients in an MMP-9-dependent manner

(A and B) Platelet samples from 6 AAD patients and 3 HDs were subjected to RNA-Seq. Volcano plots for differential genes between HDs and AAD patients (A) and comparing AAD patients with high (AAD-PLT-High) and low (AAD-PLT-Low) platelet (B). The red and green dots indicate upregulated and downregulated DEGs, respectively, with $p < 0.05$.

(C) Venn diagram generated to show an overlap of mapped DEGs from different comparison groups, including between HD and AAD patients (Set A) and between high-platelet and low-platelet patients (Set B). Sets A and B share an intersecting set (Set C) of 9 genes, including MMP-9.

(D) MMP-9 mRNA in platelets from AAD and HDs subjects was measured by qPCR.

(E) The ratio of MMP-9 contents to total protein in platelets. MMP-9 and total protein levels were measured using ELISA and BCA, respectively.

(F and G) Representative western blot images (F) and statistical analysis of the gray value (G) showing MMP-9 protein levels in platelets.

Figure 3. *Continued*
(H) ELISA of MMP-9 in PRs from AAD patients and HDs.
(I) Pure HD monocytes were cultured for 24 h with PRs from AAD patients, followed by MMP-9 inhibition. The percentage of $CD16^{+}$ monocytes was measured using flow cytometry.
(J–L) RNA sequencing was performed on pure HD monocytes cultured with or without an MMP-9i following stimulation of PR from AAD patients.
(J) Heatmap depicting differentially expressed transcripts. Red indicates increased relative expression, and blue indicates decreased relative expression.
(K) The volcano plot shows differentially expressed genes. The red and green dots indicate upregulated and downregulated DEGs, respectively, with p-value <0.05.
(L) The top 15 GO terms of differential gene expression data from biological processes (BPs), molecular function (MF), and cellular component (CC) are listed. Data are expressed as the mean $\pm$ SD. Two-tailed Student's *t* tests (D, E, G-I) were performed. AAD: acute aortic dissection; HD: healthy donors; IM: intermediate monocytes; MMP-9i: inhibitor of matrix metalloproteinase-9; NT: no treatment; PLT: platelet from AAD patients; PRs: platelet releasates.

suggest that platelet-derived MMP-9 might maintain or enhance monocyte functions during the formation of MPAs.

DISCUSSION

In the present study, decreased preoperative PLC and suppressed pro-inflammatory status of monocytes were shown to be associated with poor postoperative outcomes in AAD patients. More importantly, it was further found that platelets could restore monocyte functions in AAD patients by forming aggregates and secreting MMP-9. Therefore, we surmised a mechanism through which platelets could reprogram monocyte function by aggregation and secreting MMP-9, exerting a protective effect in AAD patients after they had undergone complex cardiovascular surgery.

AAD is a life-threatening condition caused by a tear or bleeding of the aortic wall and leads to high morbidity and mortality if not timely managed through surgery.[22,23] Despite recent surgical advances, the perioperative period is still associated with a high mortality rate.[24,25] Numerous factors contribute to the postoperative prognosis, among which platelets are believed to play an essential role through their coagulation function. AAD patients with decreased PLC at admission have been associated with postoperative pneumonia and increased 30-day mortality in retrospective studies.[12–14] Moreover, the MPV/PLC ratio, a marker for estimating the function of platelets, was shown to be an independent predictor of prognosis in AAD patients.[26] In the present study, we further demonstrated that platelets are highly activated, resulting in the dysfunctional immune-regulatory transcriptome in AAD patients. In addition, low PLC was closely related to postoperative CRP and PCT. These pieces of evidence suggest that platelet activation and consumption have a significant impact on postoperative systemic inflammatory status.

During the development of cardiovascular disease, monocytes are often overactivated, causing vascular inflammation and exacerbating the particular underlying condition.[27,28] Nevertheless, the characteristics of preoperative monocytes in AAD patients and their impact on postoperative prognosis remain poorly understood. Our previous study found that AAD patients had altered monocyte subsets dominated by M2-like monocytes but a decrease in M1-like monocytes.[29] Here, we further demonstrated that monocytes from AAD patients displayed suppressed pro-inflammatory phenotypes, with downregulation of HLA-DR, CD86, and CD40. HLA-DR is a robust marker of immune dysfunction in critically ill patients, and a low HLA-DR level is often associated with increased postoperative complications after non-infectious insults. In the present study, downregulated HLA-DR expression in monocytes was closely correlated with poor postoperative recovery of AAD patients. Despite the unclear mechanism, several possibilities may explain how monocyte functions affect the outcome of AAD patients. First, the proportions of subsets of monocytes in AAD patients may be changed. For example, there is decreased CD16 expression, in particular the $CD14^{bright}CD16^{+}$ intermediate subset, in AAD patients compared to patients with coronary heart disease.[16] The reduced intermediate monocytes would weaken the monocytes' immune response since CD16 is well known as an inflammatory mediator. In contrast, the increased aggregation of platelets with intermediate monocytes will recover the immune functions of monocytes and improve the postoperative outcome as indicated by a shortened length of intubation postoperatively. Second, Cifani et al. suggested that $CD14^{bright}CD16^{+}$ intermediate monocytes contributed to stimulating the proliferation of T cells.[16] Of note, lymphopenia has been reported in AAD patients. Our recent study also proved that $CD4^{+}$ T lymphopenia was a strong predictor of postoperative major adverse events in AAD patients.[15]

Thus, it is likely that reduced monocyte functions may also contribute to lymphopenia and reduced immune defense ability of AAD patients. Therefore, it is possible that maintaining the defensive immune function of monocytes in the perioperative period in AAD patients would be critical for preserving immunologic homeostasis.

Importantly, we found that MPAs could restore the suppressed pro-inflammatory phenotypes of monocytes in AAD patients, as indicated by the higher HLA-DR, CD40, and CD16 expression in monocytes with aggregates. The cross talk between platelets and monocytes stimulates monocytes to secrete pro-inflammatory cytokines, which is proatherogenic in chronic vascular disease.[30] In contrast, platelets play a protective role by reprogramming monocytes to a pro-inflammatory phenotype to promote bacterial clearance during sepsis.[4] In the present study, the level of MPAs was negatively correlated with the length of ICU and postoperative stays. Thus, the interaction between platelets and monocytes may benefit postoperative outcomes in AAD patients with immunocompromised monocytes. Besides monocytes, there are pivotal roles of neutrophils in AAD. Several reports have indicated that the neutrophil to lymphocyte ratio at admission is an important risk factor and independently associated with in-hospital mortality of type A AAD patients.[31,32] Of interest, an increase in the neutrophil count has been reported to be able to discern patients with a high probability of developing aortic dissection.[32] Neutrophils have also been reported to initiate the tearing of the aorta by infiltration into the aortic intima and release of MMP-9.[33] We also found a tendency for a negative association between the level of NPAs and the postoperative hospital and ICU stays for AAD patients. However, the recognition of the role of platelets aggregated with neutrophils is limited and well worth identifying in future studies.

We further found that platelet-derived MMP-9 could reprogram the pro-inflammatory status of monocytes in AAD patients. The expression of MMP-9 in human platelets has been studied by Sheu et al.[34] In their article, MMP-9 was observed on the plasma membrane, a-granules, and open canalicular system and within the cytoplasm both in resting and activated platelets. The expression of MMP-9 was able to act on platelet (PLT) itself and play an important role as a negative feedback regulator during platelet activation.[34] In addition to degrading the extracellular matrix, MMP-9 is involved in regulating inflammation and is implicated in the pathogenesis of the vascular disease, including aortic dissection and coronary illness.[18–21] Several proteases play a central role in platelet activation and inflammation, such as thrombin, cathepsin G, plasmin, and matrix metalloproteinase.[35,36] These proteases can activate platelet secretion and promote platelet-leukocyte interaction through human platelets' protease-activated receptors (PARs). For example, thrombin-activated platelets through PARs are central to their hemostatic and prothrombotic functions.[37,38] In addition, MMP-9 is also a protease and could regulate the activation of platelets and the formation of thrombus. For instance, blood MMP-9 and angiotensin II concentrations are elevated significantly in AAD patients. In response to angiotensin II, AAD is initiated by neutrophils that have infiltrated the aortic intima and released MMP-9.[33] In vascular disease, MMP-9 may also accelerate the aggregation of platelets and the leukocyte response to inflammation.[39] However, the effect of MMP-9 in platelets on monocyte functions in AAD remains unclear. In the present study, platelets or PRs from AAD patients were shown to upregulate the expression of CD40, CD16, cytokines, and chemotaxis signals in healthy monocytes. Additionally, the PRs-activated pro-inflammatory cytokines and chemotaxis signals were inhibited by an MMP-9i. Hence, we hypothesize that platelet-derived MMP-9 can reprogram monocytes toward a pro-inflammatory phenotype, which then exerts a protective effect on the postoperative outcome in AAD patients. Further in-depth studies are warranted to decipher the upstream signaling pathways that trigger the secretion of MMP-9 and the mechanisms of MMP-9 involvement in the aggregation of leukocytes, including monocytes and neutrophils, and how it promotes leukocyte immune activity.

Collectively, the present study found that in addition to its coagulation function, platelets could aggregate with monocytes and maintain their functions by secreting MMP-9. This phenomenon may be critical for complex cardiovascular diseases such as AAD, where monocyte functions are suppressed. Taken together, our results support the hypothesis that maintaining platelet immune functions is essential for improving postoperative outcomes after complex cardiovascular surgery.

Limitations of the study

The current study revealed that platelets form aggregates with monocytes, enhancing their defensive capabilities. Although the critical role of monocytes was emphasized, neutrophils also played a significant

part in acute aortic dissection pathology. The understanding of platelet aggregation with neutrophils is limited, and further investigation is warranted. Additionally, we discovered that MMP-9 may be an essential molecule secreted by platelets, influencing monocyte functions. However, more in-depth studies are required to elucidate the mechanisms of how it enhances leukocyte immune activity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106805.

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AUTHOR CONTRIBUTIONS

W.Y.S. conducted most of the experiments. R.P.D. and C.L. designed the study, analyzed the data, and wrote and revised the manuscript together with W.Y.S. A.H.Z. and R.Y.L. helped collect information about patients and carried out some experiments on flow cytometry. H.L. and Y.L.Z. provided critical suggestions on the experiment design and manuscript writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC/Cy7 anti-human CD45	Biolegend	304014
PE/Cy7 anti-human CD3e	Biolegend	344816
FITC anti-human CD14	Biolegend	301804
BV421 anti-human CD14	Biolegend	563742
Percp5.5 anti-human CD16	BD Pharmngen	302028
PE anti-human CD206	Biolegend	321106
PE anti-human CD40	Biolegend	334307
APC-anti-human CD41	Biolegend	303710
Percp5.5 anti-human CD42b	Biolegend	303918
Alexa Fluor 647 anti-human CD66b	BD Pharmngen	561645
BV421 anti-human CD86	BD Pharmngen	562432
BV421 anti-human CX3CR1	BD Pharmngen	565800
PE/Cy7 anti-human CCR2	Biolegend	150611
PE/Cy7 anti-human HLA-DR	Biolegend	307616
BV421 anti-human TNF-α	Biolegend	502932
FITC anti-human IL-6	Biolegend	501104
Anti-MMP-9 mouse mAb	Servicebio	Gb12132-1
HRP-Conjugated Beta Actin Monoclonal Antibody	Proteintech	HRP-60008
HRP-conjugated Goat Anti-Mouse IgG(H+L)	Proteintech	SA00001-1
Chemicals, peptides, and recombinant proteins		
CD14 MicroBeads, human	Miltenyi Biotec	130-050-201
Fixation buffer	Invitrogen	00-8222-49
Compensation Beads	Invitrogen	01-3333-42
Red cells lysis buffer	Biosharp	21282768
1X PBS (without Ca^{2+})	Biosharp	BL302A
Advanced Tyrode's solution	Leagene	CZ0063
Advanced Tyrode's solution (without Ca^{2+})	Leagene	CZ0064
Prostaglandin E1	Cayman	13010
Fibrinogen	Sigma	F3860
Thrombin	Sigma	T7009
Protein transport inhibitor cocktail (500X)	Invitrogen	00-4980
MMP-9 inhibitor	Selleck	S0769
Lymphoprep	Stemcell	07851/07861
DMDM basic (1X)	Gibco	C11995500BT
Fetal bovine serum	Gibco	10091-148
Streptomycin/penicillin	Gibco	15140-12
L-Glutamine	Gibco	A2916801
TRIzol	Ambion	15596076
SDS-PAGE	Epizyme	PG112
RIPA lysis buffer	Bioss	C5029

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
MMP-9 ELISA kit	Boster	EK0465
RevertAid First-strand cDNA Synthesis kit	Thermo Scientific	K1622
FITC Annexin V Apoptosis Detection Kit	BD Pharmngen	556547
Enhance CBA protein assay kit	Beyotime	P0010
2X NovoStart SYBR qPCR superMix plus	Novoprotein	E096
Deposited data		
Human/patient platelet RNA-sequencing data	This study	SRA: PRJNA956436
Human monocytes RNA-sequencing data	This study	SRA: PRJNA956496
Experimental models: Cell lines		
Human: Primary PBMCs	N/A	N/A
Human:Primary monocytes	N/A	N/A
Oligonucleotides		
MMP9 Reverse primer- 5′ACGCAGACATCGTCATCC 3′	This paper	N/A
MMP9 Reverse primer- 5′CCAGGGACCACAACTCG 3′	This paper	N/A
Software and algorithms		
PRISM	GraphPad	Version 8
FlowJo	FlowJo	Version 10
Image J	Rawak Software	https://imagej.nih.gov/ij/
Other		
BD trucount tubes	BD	340334
ACD Vacutainer tubes	BD	364606
PVDF transfer membranes	Immobilon	IPVH00010

RESOURCE AVAILABILITY

Lead contact

All requests for reagents and resources should be directed to the lead contact, Ru-Ping Dai (xyeyyrupingdai@csu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- RNA-sequencing data of this work have been deposited at Sequence Read Archive (SRA) data repository and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

All participants provided informed written consent to donate their blood samples for research. The Institutional Medical Ethics Review Board of the Second Xiangya Hospital approved the study (No.

LYF2021081), which was enrolled in the Chinese Clinical Trial Registry (No. ChiCTR2100047573). Subject demographic data and other relevant clinical variables are reported in Tables 1 and S1.

Between December 2019 and December 2021, 85 patients with type A acute aortic dissection were admitted to the Second XiangYa Hospital and enrolled in our study. AAD diagnosis was confirmed using computed tomographic angiography (CTA) within 14 days of symptom onset. Exclusion criteria were as follows: (1) infectious diseases; (2) autoimmune diseases; (3) malignant tumors; (3) previous aortic surgery; (4) treatment with non-steroidal anti-inflammatory drugs or steroids. Control blood samples were obtained from age- and sex-matched healthy donors (HDs).

Primary cell cultures

The PBMCs described in this study were collected from a healthy donor (Male, 55 years old). And monocytes from PBMCs were purified by magnetic separation using human CD14 microbeads. Cells were resuspended in IMDM complete medium (10% fetal bovine serum, 1% streptomycin/penicillin) in 37°C and 5% CO_2.

METHOD DETAILS

Clinical data collect

Clinical data were recorded from electronic hospital records. Laboratory results of age-and sex-matched HDs were extracted from the physical examination center of the Second Xiangya Hospital. Blood routine tests, C-reactive protein (CRP) and procalcitonin (PCT) were drawn at admission and 1 day after surgery. Hospital length of stay, length of intensive care unit (ICU) stays, and time to tracheal extubation was recorded. For correlation analysis, patients with a hospital length of stay greater than 30 days or who died in the hospital were excluded.

Preparation of platelet-rich plasma (PRP), platelet, and platelet releasate

Human platelets were obtained as previously described.[17] A total of 20 ml of whole blood were collected in acid citrate dextrose (ACD) vacutainer tubes and then centrifuged at 120 g for 20 min to collect platelet-rich plasma (PRP). Then, PRP was centrifuged at 360 g for 20 min to obtain platelets. To obtain platelet releasates, the platelets were suspended and washed in Tyrode's buffer containing PGE1 and adjusted to $4 \times 10^5/\mu l$ in Tyrode's buffer containing calcium chloride for 30 minutes. To stimulate platelets, resting platelets were stimulated with fibrinogen (0.25 mg/ml) for 5 minutes, followed by thrombin (1 U/ml). The supernatants of platelet releasates were then collected after platelet aggregation.

RNA isolation and RNA sequencing of human platelets

For RNA-seq analysis of platelets, platelets were isolated from AAD patients and matched HDs (Table S1) within 30 min for blood sampling using a standardized differential centrifugation protocol, as reported previously.[40] Platelet purity was >95%, as evaluated by flow cytometry (Figure S1C). The RNA-seq analysis of an index leukocyte transcript (PTPRC, CD45) confirmed that the samples were highly purified platelets. We measured equivalent PTPRC levels from all samples that were >1000 times lower in fragments per kilobase of transcript per million mapped reads (FPKM) than the index platelet transcript PF4. RNA purity and quantification were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was measured using an Agilent bioanalyzer (Agilent Technologies, USA). RNA integrity number scores were similar among all samples. According to the manufacturer's instructions, the libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA). OE Biotech Co., Ltd. (Shanghai, China) conducted the transcriptome sequencing and analysis. The clean reads were mapped to the human genome (GRCh38) using HISAT2. FPKM of each gene was calculated using Cufflinks, and the read counts of each gene were obtained using HTSeq count. Differential expression analysis was performed using the DESeq (2012) R package. P-value < 0.05 and fold-change > 2 or fold-change < 0.5 was set as the threshold for significantly differential expression. Hierarchical cluster analysis of differentially expressed genes (DEGs) was performed to demonstrate the expression pattern of genes in different groups and samples. GO enrichment analysis of DEGs was performed using R based on a hypergeometric distribution.

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Flow cytometry

To test platelet-leukocyte aggregates, 100μl of whole blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes and stained with the following antibodies for 20 min at room temperature: percp5.5 anti-human CD41a (platelet glycoprotein alpha-IIb), FITC anti-human CD41, APC/Cy7 anti-human CD45, CD14 anti-human BV421, PE anti-human CD19, PE/Cy7 anti-human CD3, and Alexa Fluor 647 anti-human CD66b. To test the phenotype of monocytes, cells were stained with the following antibodies for 20 min: APC/Cy7 anti-human CD45, APC anti-human CD41, FITC anti-human CD14, PE/Cy7 anti-human HLA-DR, PE/Cy7 anti-human CCR2, PE anti-human CD40, PE anti-human CD206, percp5.5 anti-human CD16, BV421 anti-human CD86, and BV421 anti-human CX3CR1. To detect the expression of cytokines, cells were fixed with fixation buffer and then permeabilized with permeabilization buffer. Then cells were incubated with the following antibodies for 30 min: FITC anti-human IL-6 and BV421 anti-human TNF-α. After rinsing, the fluorescence was read on a flow cytometer (Cystek, USA), and the data were analyzed using FlowJo vX0.7 software.

Labeling of phosphatidylserine (PS) with Annexin V in platelets

Isolated PRP (50μl) was added with 50 μl binding buffer and incubated with CD41-APC and Annexin V-FITC antibody for 20 min at room temperature in the dark. The reaction was stopped with 400 μl binding buffer, and the sample was immediately subjected to flow cytometry analysis within 30 min. The gating scheme is illustrated in Figure S1.

RT-PCR

Platelet RNA was isolated using TRIzol, then reverse-transcribed and pre-amplified. The MMP-9 primer sequence was as follows: Fwd-5′ACGCAGACATCGTCATCC-3′, Rev-5′-CCAGGGACCACAACTCG-3′. The change in mRNA expression was calculated using the comparative change-in-cycle method (BioRad, USA).

Cell culture

Whole blood was collected from HDs, and PBMCs were isolated using density gradient centrifugation. According to the manufacturer's instructions, monocytes from PBMCs were purified by magnetic separation using human CD14 microbeads. The purity of $CD14^+$ cells was >95%, as assessed by flow cytometry. Cells were resuspended in IMDM complete medium (10% fetal bovine serum, 1% streptomycin/penicillin) in 96-well plates, with each well containing 4×10^5 cells in 100 μl medium and cultured in 37 °C and 5% CO_2; 100 μl platelet cell suspensions (4×10^5/ul) or 100 μl platelet releasates were added to the cultured cells and stimulated for 24 hours. The protein transport inhibitor was added for the final 6 hours of culture for intracellular staining. To measure the effect of MMP-9 on monocytes, the cultured cells were treated with MMP-9 inhibitor (200μm) or vehicle for 24 hours, and cells were collected to stain and read using flow cytometry, or we extracted RNA for RNA-sequencing analysis as described above. Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol.

Western blot

Platelets were homogenized in ice-cold RIPA buffer containing proteinase inhibitor for protein extraction. According to the manufacturer's instructions, the total protein was quantified using a Coomassie protein assay kit. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) was performed with specific amounts of protein, electrophoresed, and electrotransferred to PVDF membranes. Membranes were then incubated with anti-MMP-9 antibody (1:500) followed by peroxidase-conjugated secondary antibody or incubated with HRP-conjugated beta-actin monoclonal antibody. Blots were detected using an imaging system (CLINX, China).

Enzyme-linked immunosorbent assay (ELISA)

MMP-9 was detected in releasate and lysate of platelet using an enzyme-linked immunosorbent assay according to the manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviations (SD) for normally distributed continuous variables, median (interquartile range) for non-normal continuous variables, and n (%) for categorical variables. We

performed the analysis of significance using Prism (GraphPad, USA). All numerical variables were tested for normality of distribution using the Shapiro-Wilk test. For comparisons between two groups, statistical significance was determined using Student's t-test (normally distributed data) or the Mann–Whitney test for the non-normally distributed data. Differences between the groups were assessed using one-way analysis of variance (ANOVA) with Tukey post hoc test (normally distributed data) or Kruskal-Wallis test with Dunn's post hoc test (nonnormal distribution). Correlation coefficients were calculated using the Pearson correlation test for parametric distributions and the Spearman correlation test for nonparametric distributions. $P < 0.05$ indicated statistically significant differences.

ADDITIONAL RESOURCES

The study was approved by the Institutional Medical Ethics Review Board of the Second Xiangya Hospital (No. LYF2021081) and registered in the Chinese Clinical Trial Registry (No. ChiCTR2100047573) (https://www.chictr.org.cn/).