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Thromboinflammation: Crosstalk between thrombosis and inflammation in cardiovascular disease

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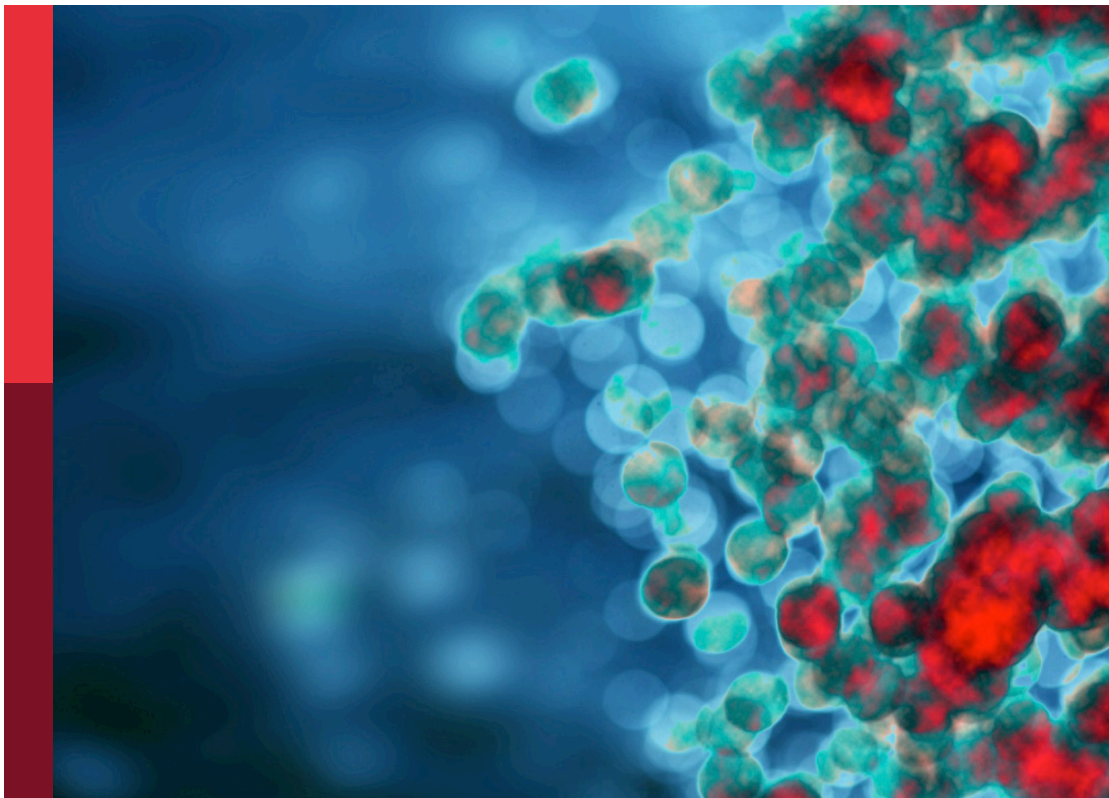

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Thromboinflammation: Crosstalk between thrombosis and inflammation in cardiovascular disease

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Antigen-Presenting Cell-Like Neutrophils Foster T Cell Response in Hyperlipidemic Patients and Atherosclerotic Mice

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Neutrophils constitute abundant cellular components in atherosclerotic plaques. Most of the current studies are focused on the roles of granular proteins released by neutrophils in atherosclerosis. Here, we revealed a unique subset of neutrophils which exhibit the characteristics of antigen-presenting cell (APC) (which were called APC-like neutrophils afterwards) in atherosclerosis. The roles of APC-like neutrophils and relevant mechanisms were investigated in hyperlipidemic patients and atherosclerotic mice. Higher percentages of neutrophils and APC-like neutrophils were found in peripheral blood of hyperlipidemic patients than that of healthy donors. Meanwhile, we also identified higher infiltration of neutrophils and APC-like neutrophils in atherosclerotic mice. Ox-LDL induced Phorbol-12-myristate-13-acetate (PMA)-activated neutrophils to acquire the APC-like phenotype. Importantly, upon over-expression of APC-like markers, neutrophils acquired APC functions to promote the proliferation and interferon-γ production of $CD3^+$ T cells *via* HLA-DR/CD80/CD86. In accordance with what found *in vitro*, positive correlation between neutrophils and $CD3^+$ T cells was observed in hyperlipidemic patients. In conclusion, our work identifies a proinflammatory neutrophil subset in both hyperlipidemic patients and atherosclerotic mice. This unique phenotype of neutrophils could activate the adaptive immune response to promote atherosclerosis progression. Thus, this neutrophil subset may be a new target for immunotherapy of atherosclerosis.

Keywords: neutrophils, APC-like phenotype, $CD3^+$ T cells, interferon-γ, atherosclerosis

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INTRODUCTION

Atherosclerosis is a chronic inflammatory disease that occurs in the walls of blood vessels, and is the pathological basis for cardiovascular diseases (1). Its morbidity and mortality rates are higher than those of any other diseases in the world (2). Hyperlipidemia, especially hypercholesterolemia, leads to accumulation of plasma low-density lipoprotein (LDL) in the artery wall; LDL and its components elicit vascular inflammation that drives the build-up of lipid-laden atherosclerotic plaques (3). Hyperlipidemia is one of the risk factors for atherosclerosis (4) and represents the initiation stage of atherosclerosis (5). The accumulation of lipids in the arterial wall, as well as the infiltration of a large number of inflammatory cells such as macrophages, neutrophils and T cells, are the main characteristics of atherosclerosis (6, 7). The atherosclerotic lesion contains lipid oxides such as oxidized LDL (ox-LDL) (8) and a variety of inflammatory cytokines. The innate immune response and adaptive immune response both play important roles in the genesis of atherosclerosis (9). The innate immune system is triggered by the activation of vascular endothelial cells (10, 11) and monocytes/macrophages (12),while the adaptive immune system is initiated by antigen-presenting cells presenting multiple antigens to effector T cells (13).

Neutrophils are the most abundant leukocytes in peripheral blood and one of the first innate immune cells to arrive at the site of inflammation (14). Neutrophils contribute to the occurrence and progression of atherosclerosis by releasing granular proteins such as matrix metalloproteinase (15, 16), myeloperoxidase (17, 18), elastase (19) and forming neutrophil extracellular traps (NETs) (20, 21). There are a growing body of evidences suggesting that neutrophils have a highly variable transcriptome profile depending on their tissue location and microenvironment (22). As a result, under the condition of different stimulating factors, neutrophils may exhibit different phenotypes and exert different functions. For example, in the tumor microenvironment, tumor-associated neutrophils are proposed to be polarized into an anti-tumor or pro-tumor phenotype (23, 24). In allergic diseases, IL-33 can stimulate neutrophils to produce Th1 cytokines such as IL-5, IL-9, IL-13, and so on, thus promotes disease progression (25). Neutrophils could possess different functions and phenotypes depending on the disease model (26–29).

Antigen presenting cells (APCs) are required for the priming of adaptive immune system (30). APCs uptake and present antigens to T cells, triggering the adaptive immune response (31). The major histocompatibility complex (MHC), and the costimulatory molecules CD80 and CD86, are the key molecules responsible for T cell activation (31, 32). Strikingly, the presence of APC-like neutrophils has been discovered in infectious diseases (33), allergic diseases (28), and tumors (34). For instance, in patients with rheumatoid arthritis, neutrophils in the synovial fluid express large amounts of class II MHC molecules and then stimulate T cell proliferation (35). APC-like neutrophils gain the ability to activate T cells, resulting in the production of inflammatory cytokines, and ultimately, promote or dampen disease progression. So, whether APC-like neutrophils exist in atherosclerotic plaques and the potential roles of this unique neutrophil subset in atherosclerosis are of great importance for further understanding of this inflammatory disease.

Herein, we show that APC-like neutrophil subset exists in peripheral blood of hyperlipidemic patients and atherosclerotic plaques of $LDLr^{-/-}$ mice. Upon exposing to ox-LDL, PMA-activated neutrophils upregulate the expression of HLA-DR, CD80 and CD86, exhibiting an APC-like phenotype. In turn, APC-like neutrophils enhance the proliferation and interferon-γ (IFN-γ) production of $CD3^+$ T cells *via* HLA-DR/CD80/CD86. Clinical data show a positive correlation between APC-like neutrophils and $CD3^+$T cells, which implies APC-like neutrophils may promote atherosclerosis progression through activating adaptive immune system.

MATERIALS AND METHODS

Human Samples

Peripheral blood of 90 hyperlipidemic patients were collected from University-Town Hospital of Chongqing Medical University. Hyperlipidemic patients with high blood pressure or diabetes mellitus were excluded. Peripheral blood of 90 healthy donors were used as control. Informed consent was signed by each subject. The study was approved by the Ethics Committee of Chongqing Medical University.

Mice

Six-to-eight-week-old male $LDLr^{-/-}$ mice and C57BL/6 mice (wild-type mice, WT mice) were purchased from Beijing huafukang Biotechnology Co. All mice were bred in specific pathogen-free conditions. All animal experiments were undertaken with review and approval from the Animal Ethical and Experimental Committee of Chongqing Medical University.

Atherosclerosis Mouse Model

$LDLr^{-/-}$ mice were fed with high-fat-diet (HFD) containing 0.15% cholesterol (medicine Ltd, China) for 12 weeks to generate lipid-induced atherosclerosis ($LDLr^{-/-}$ HFD). WT and $LDLr^{-/-}$ mice fed with normal diet were used as control (WT ND and $LDLr^{-/-}$ ND). Each group contained eight mice. After feeding for 12 weeks, $LDLr^{-/-}$ mice and WT mice were anesthetized and sacrificed. Blood in heart and aorta were flushed with sterile phosphate-buffered saline (PBS)-sodium heparin solution *via* cardiac puncture. Hearts were excised and fixed with 4% paraformaldehyde. Aortas were digested for 1 h at 37°C using an enzyme mixture containing 450 U/ml collagenase Is (Sigma-Aldrich, USA), 125 U/ml collagenase XI (Sigma-Aldrich, USA), 60 U/ml DNase I (Sigma-Aldrich, USA), and 60 U/ml hyaluronidase (Sigma-Aldrich, USA) as previously reported (36). Mouse peripheral blood was collected in the Eppendorf tubes containing Heparin sodium. Digested aorta, bone marrow from femur and tibia and spleen were prepared into a single cell suspension by grinding for further use.

Mouse Blood Lipids Analysis

Plasma samples were collected from LDLr$^{-/-}$ mice and WT mice for lipids measurement. Total cholesterol (TC), total triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) in mouse plasma were assayed with corresponding assay kit (Mindray, China) by using biochemical analyzer (Mindray, China).

Assessment of Atherosclerotic Lesion

Frozen sections of aortic sinuses were stained with Oil-red O (Solarbio, China) to determine the lipids deposition. Total lesion areas defined as intimal atherosclerotic areas and lesion lipid deposition areas were evaluated by Image J. Paraffin-embedded hearts were cut into 6-8-μm thick slides for Hematoxylin-Eosin staining and Masson's staining to visualize the necrotic core areas and the collagen contents. Necrotic core areas and collagen contents of atherosclerotic lesions were measured by ImageJ.

Isolation of Neutrophils and CD3$^+$ T Cells

Peripheral blood was obtained from healthy adult volunteers. Human neutrophils were isolated by density gradient centrifugation using Ficoll (Solarbio, China) according to manufacturer's instructions. The purity of neutrophils was up to 98% (**Supplementary Figure S1A**). Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation using Ficoll (Solarbio, China). CD3$^+$ T cell from PBMC were sorted by Easysep^{TM} human T cell isolated kit (Stemcell, Canada). The viability of sorted CD3$^+$ T cells was higher than 90% and their purity was up to 97% (**Supplementary Figure S1B**).

Neutrophil Stimulation

Freshly isolated neutrophils were treated with ox-LDL, PMA, ox-LDL plus PMA, respectively. As for PMA and ox-LDL plus PMA groups, neutrophils were pretreated with 1 nmol/L PMA (Solarbio, China) for 30 min and the cells were washed with RPMI1640 medium. Then the cells were incubated with 40 μg/ml ox-LDL (Yiyuanbiotech, China) for 12h, 24h, 36h, 48h, separately, and harvested for flow cytometric analysis.

In Vitro Neutrophil-T Cell Co-Culture System

Purified CD3$^+$ T cells (3×10^6 cells/ml) were labelled with carboxyfluorescein succinimidyl ester (CFSE) and co-culture with neutrophils pre-stimulated with ox-LDL, PMA, or ox-LDL plus PMA at a 1:1 ratio in 200 μL RPMI-1640 medium containing rhIL-2 (20IU/mL) (Peprotech), anti-CD3 (2 μg/mL) (Biolegend), and anti-CD28 (1 μg/mL) (Biolegend) antibodies, with or without human CD80 neutralizing antibody (20 μg/mL) (Biolegend), human CD86 neutralizing antibody (20 μg/mL) (Biolegend) and human HLA-DR neutralizing antibody (20 μg/mL) (Biolegend). After 5-day incubation, the cells were collected for intracellular cytokine staining.

Flow Cytometry

Flow cytometric analysis was performed according to standard protocols. Cell surface markers were stained with fluorescence labeled antibodies for 30 min at 4°C. Anti-human CD45, CD66b, CD80, CD86, HLA-DR, CD11c antibodies were applied to analyze the phenotype of neutrophils in hyperlipidemic patients and healthy donors. Anti-mouse CD45, Ly6G, CD80, CD86, MHC-II antibodies were applied for detecting neutrophil phenotype in atherosclerotic mice. For intracellular staining, the cells were stimulated for 6 hours with Cell Activation Cocktail with Brefeldin A (Biolegend, USA) (5). Intracellular cytokine staining was performed after the cells were fixed and permeabilized with fixation/permeabilization buffer (eBioscience, USA) for 20 min (37). Anti-human CD45, CD3, IFN-γ antibodies and anti-mouse CD45, CD3, IFN-γ antibodies were used for intracellular cytokine staining. All flowcytometric antibodies except anti-IFN-γ antibody (Invitrogen, USA)were purchased from Biolegend (USA).

Statistical Analysis

Each experiment was performed at least three times. Data were expressed as mean ± SEM. Differences between hyperlipidemic patients and healthy donors were analyzed by the unpaired, Student's test. Correlations between parameters were assessed using the Pearson correlation analysis and linear regression analysis as appropriate. The animal data and *in vitro* data were analyzed by non-parametric analysis. GraphPad Prism 7.0 was used for all statistical analysis. All data were analyzed using two-tailed tests, and $p<0.05$ was considered statistically significant.

RESULTS

Neutrophils Increase in Peripheral Blood of Hyperlipidemic Patients and Express Characteristic Markers of APCs

To identify neutrophil subsets in hyperlipidemic patients, we first used flow cytometry to analyze the percentage of CD45$^+$CD66b$^+$ neutrophils within the total CD45$^+$ leukocytes in different samples from 90 hyperlipidemic patients. 90 healthy donors were used as control. It showed that patients with hyperlipidemia had a greater proportion of neutrophils in peripheral blood than healthy donors (**Figure 1A**). Since neutrophils in patients with cancer or infectious diseases have the ability to heterogeneously express some co-stimulatory molecules, we postulated that there might be a subset of neutrophils with characteristics of APCs in atherosclerosis. So, the expression of APC-like markers on neutrophils were examined by flow cytometry. As expected, increased percentages of peripheral neutrophils in hyperlipidemic patients expressed APC signature markers including HLA-DR (**Figure 1B**), CD80 (**Figure 1C**) and CD86 (**Figure 1D**), but few neutrophils expressed dendritic cell (DC) marker CD11c (**Supplementary Figure S2**). Further analysis of the co-expression of HLA-DR, CD80 and CD86 on neutrophils showed that the percentage of HLA-DR$^+$ CD80$^+$ CD86$^+$ neutrophils in peripheral blood of hyperlipidemic patients is significantly higher than that of healthy donors (**Supplementary Figure S3**). These results imply that a subset of APC-like

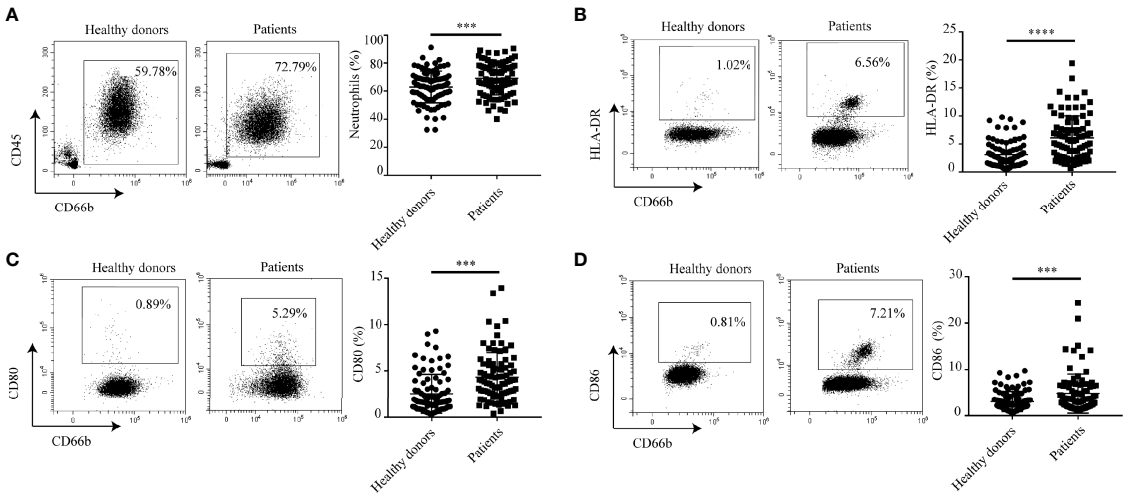

FIGURE 1 | Neutrophils express characteristic surface molecules of APCs in hyperlipidemic patients. **(A)** The percentage of neutrophils in peripheral blood of patients with hyperlipidemia and healthy donors. **(B–D)** Flow cytometry analysis of the percentages of HLA-DR$^+$, CD80$^+$, CD86$^+$ neutrophils between patients with hyperlipidemia and healthy donors. ***p < 0.001, ****p < 0.0001.

neutrophils exists in hyperlipidemic patients. As most of the atherosclerosis cases developed from hyperlipidemia, we speculate that APC-like neutrophils may play pivotal roles in atherosclerosis.

APC-Like Neutrophils Are Enriched in Murine Atherosclerosis

To assess the distribution of APC-like neutrophils in atherosclerosis, we established a murine model of atherosclerosis by feeding LDLr$^{-/-}$ mice on a high-fat diet (HFD) for 12 weeks (LDLr$^{-/-}$ HFD cohort). LDLr$^{-/-}$ mice and WT mice fed with normal diet (ND) were set as control groups (LDLr$^{-/-}$ ND cohort; WT ND cohort). Oil Red O staining of the whole aorta from aortic arch to abdominal aorta showed that LDLr$^{-/-}$ mice with HFD had increased atherosclerotic areas than control groups (**Supplementary Figure S4A**). Oil Red O staining of aortic root also showed the same trend (**Supplementary Figure S4B**). Masson trichrome staining and HE staining showed increased collagen contents and enlarged necrotic core areas appeared in aortic root of LDLr$^{-/-}$ mice with HFD (**Supplementary Figures S4C, D**). Blood lipid levels including TC, TG, LDL-C and HDL-C of LDLr$^{-/-}$ mice with HFD were significantly higher than control groups (**Supplementary Figure S4E**). Besides, the weight of LDLr$^{-/-}$ mice fed with HFD was much higher than control (**Supplementary Figure S4F**). These results indicate that the mouse model of atherosclerosis is established successfully.

Then, the distribution of neutrophils in aorta, blood, spleen and bone marrow of atherosclerotic mice were analyzed by flow cytometry. As shown in **Figure 2A**, neutrophils were enriched in aorta of LDLr$^{-/-}$ mice feeding with HFD. Similarly, atherosclerotic mice showed a higher neutrophil percentage in peripheral blood (**Figure 2B**) and spleen (**Figure 2C**) than control groups. However, no such differences were observed in the bone marrow (**Figure 2D**). Next, the subsets of neutrophils in different tissues were detected. In accordance with what found in hyperlipidemic patients, neutrophils in aorta of LDLr$^{-/-}$ mice with HFD showed higher expression of APC-like markers including MHC-II molecules (**Figure 3A**), CD80 (**Figure 3B**) and CD86 (**Figure 3C**). The same trends were also observed in neutrophils from peripheral blood (**Figures 3D–F**) and spleen (**Figures 3G–I**) of atherosclerotic mice, while neutrophils in the bone marrow expressed none such markers (**Figures 3J–L**). Taken together, these results clarify the existence of APC-like neutrophils in atherosclerotic mice. The different distribution of APC-like neutrophils between peripheral tissues and bone marrow suggests that the APC-like neutrophils may be regulated under hyperlipidemic conditions other than generated in the bone marrow.

Ox-LDL Induces PMA-Activated Neutrophils Differentiating Into APC-Like Phenotype

Since ox-LDL is one of the most important lipids in atherosclerosis, we wonder whether it could modulate the differentiation of APC-like neutrophils. To verify this hypothesis, neutrophils were isolated from peripheral blood of healthy donors and stimulated with ox-LDL, PMA, ox-LDL plus PMA for 12 hours, respectively. Surprisingly, neutrophils treated with ox-LDL alone showed little expression of HLA-DR, CD80, and CD86 (**Figures 4A–C**). However, these markers were dramatically upregulated on neutrophils treated with ox-LDL plus PMA (**Figures 4A–C**). The same results were observed when neutrophils were stimulated for 24h, 36h, 48h, respectively (**Supplementary Figure S5**). These findings demonstrate that when neutrophils are activated by PMA, they can differentiate into APC-like phenotype by ox-LDL, which implies other factors

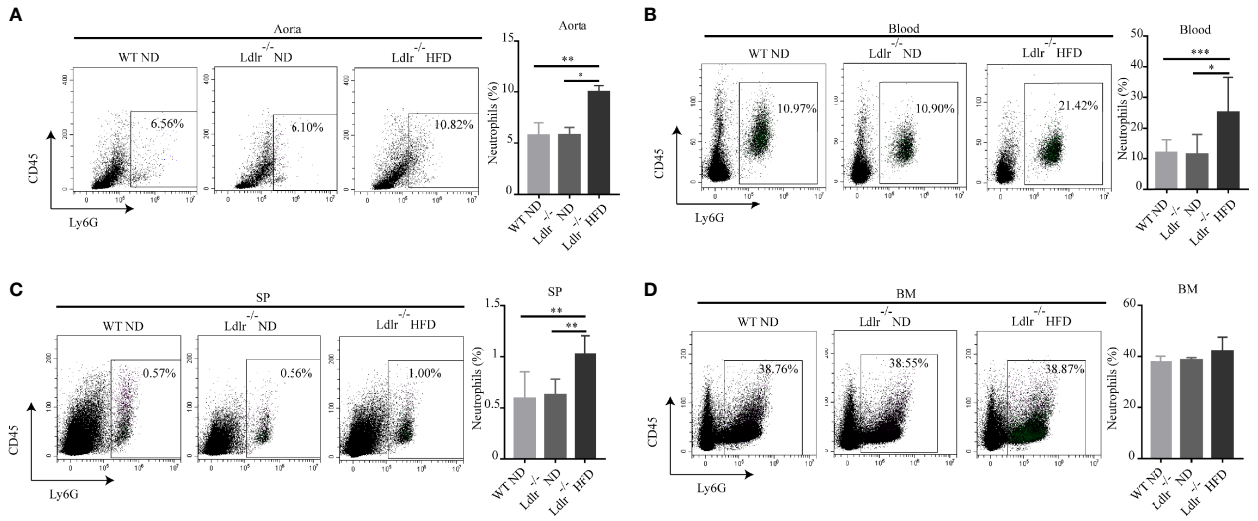

FIGURE 2 | Percentage of neutrophils was higher in LDLr$^{-/-}$HFD mice than control groups. Distribution of neutrophils in **(A)** aorta, **(B)** blood, **(C)** spleen (SP), **(D)** bone marrow (BM) of atherosclerotic mice and control groups were detected by flow cytometry. n=8. *p < 0.05, **p < 0.01, ***p < 0.001.

in atherosclerosis may work together with ox-LDL to regulate the differentiation of APC-like neutrophils.

APC-Like Neutrophils Possess the Ability to Activate T Cell Response

APCs are the key players in the immune response since they are capable of presenting antigens to T cells thereby initiating T cell responses (38). To determine the role of APC-like neutrophils in T cell function, CFSE-labeled CD3$^+$ T cells were co-cultured with conditioned neutrophils which were pretreated with ox-LDL plus PMA. Then, T cell proliferation and IFN-γ production were measured by flow cytometry. Neutrophil/T-cell co-cultures showed that APC-like neutrophils (induced by ox-LDL and PMA) significantly promoted the proliferation and IFN-γ production of T cells, which could be significantly attenuated by blockade of HLA-DR, CD80 and CD86 on neutrophils (**Figure 5**).

To verify these findings *in vivo*, we analyzed the distribution of T cells in atherosclerotic mice. Gate strategies of T cells and IFN-γ$^+$CD3$^+$ T cells were shown in **Supplementary Figure S6**. Consistently, we found that more CD3$^+$ T cells were infiltrated in aorta of LDLr$^{-/-}$ mice with HFD than control groups (**Figure 6A**). The percentage of IFN-γ$^+$CD3$^+$ T cells in the aorta of LDLr$^{-/-}$ mice with HFD was also much higher than that of control groups (**Figure 6B**). Increased CD3$^+$ T cells and IFN-γ$^+$CD3$^+$ T cells were found in blood, spleen and bone marrow of atherosclerotic mice as well (**Figures 6C–H**). The *in vitro* and *in vivo* results together suggest that APC-like neutrophils may play an essential role in T cell activation, but further research is needed to verify it.

APC-Like Neutrophils Positively Correlate With T Cells in Hyperlipidemic Patients

The demographic and clinical characteristics of the patients with hyperlipidemia were outlined in **Supplementary Table S1**. The distribution of T cells in human peripheral blood was analyzed. The results showed that the percentage of CD3$^+$ T cells in peripheral blood of hyperlipidemic patients was much higher than that in healthy donors (**Figure 7A**). Furthermore, a greater proportion of IFN-γ$^+$ CD3$^+$ T cells was found in blood of hyperlipidemic patients comparing to that of healthy donors (**Figure 7B**). Within the patient cohort, APC-like neutrophils were positively correlated with CD3$^+$ T cells and IFN-γ$^+$ CD3$^+$ T cells, respectively (**Figures 7C, D**). The results were in accordance with that observed in neutrophil/T-cell co-cultures, which implies a stimulatory role of APC-like neutrophils in the early stage of human atherogenesis.

DISCUSSION

In this study, we have identified a unique neutrophil subset which expresses APC associated markers including HLA-DR (human)/ MHC-II(mice), CD80 and CD86. We show that the percentage of APC-like neutrophils significantly increases both in hyperlipidemic patients and atherosclerotic mice. We uncover that ox-LDL play pivotal roles in the differentiation of PMA-activated neutrophils into APC-like phenotype. What's more, APC-like neutrophils foster T cell response *via* HLA-DR, CD80 and CD86 *in vitro*, implying an immunostimulatory effect of APC-like neutrophils in atherosclerosis. Our clinical data also support the concept because there is a positive correlation between neutrophils and CD3$^+$T cells and IFN-γ$^+$ CD3$^+$ T cells, separately, in hyperlipidemic patients. Despite the fact that neutrophils have previously been described in atherosclerosis, to our knowledge this is the first demonstration for the existence of APC-like neutrophils in atherosclerosis.

The involvement of neutrophils in the pathogenesis of atherosclerosis has recently received a lot of attention.

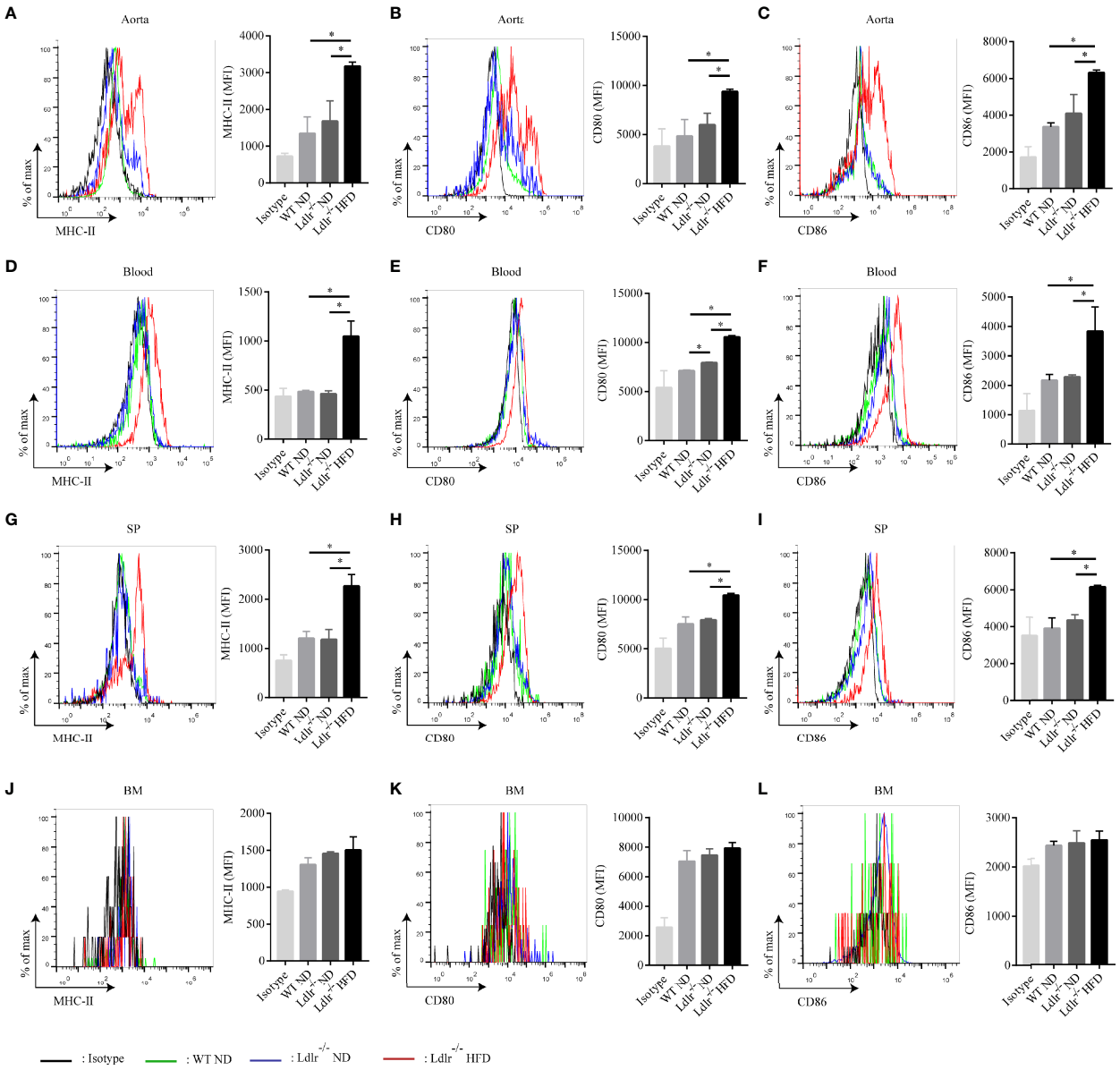

FIGURE 3 | Neutrophils with characteristics of APC are present in atherosclerotic mice. Expression of MHC-II, CD80, CD86 on neutrophils in the **(A–C)** aorta, **(D–F)** blood, **(G–I)** spleen (SP), and **(J–L)** bone marrow (BM) of WT ND (green line), LDLr$^{-/-}$ ND (blue line) and LDLr$^{-/-}$ HFD mice (red line) (n=8). Black line: isotype control. MFI, mean fluorescence intensity. *p < 0.05.

However, most of the current studies are focused on the granule proteins and cytokines released by neutrophils in atherosclerosis (39). Here, we show that neutrophils in the blood of hyperlipidemic patients exhibited an APC-like phenotype characterized by expression of HLA-DR, CD80 and CD86. The same subset of neutrophils were also found in aorta, blood and spleen of atherosclerosis mice. This discovery is accordance with what found in inflammatory lesions (40) and allergic conditions (41), implying APC-like neutrophils may exist widely in inflammatory diseases including atherosclerosis.

In the progression of atherosclerosis, ox-LDL regulates the phenotype and function of various cells such as macrophages and T lymphocytes (42–45). However, there have been few studies focusing on the effect of ox-LDL on neutrophil phenotypes. Our work reveal that PMA-activated neutrophils can differentiate into APC-like neutrophils *via* ox-LDL.There are two possible reasons to explain this phenomenon. Firstly, because it is reported that ox-LDL induces NET formation in human neutrophils via toll like receptor (TLR)-PKC-IRAK-MAPK and NADPH-oxidase activation (46), we speculate that ox-LDL is likely to mediate the differentiation of neutrophils

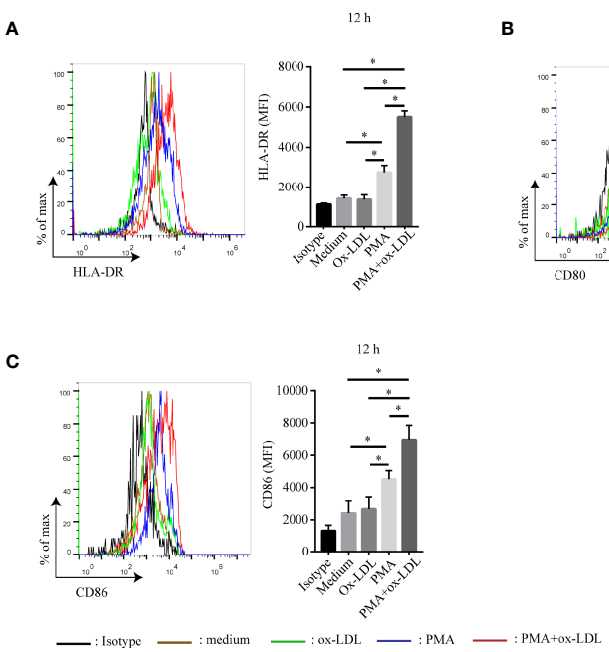

FIGURE 4 | Ox-LDL induces PMA-activated neutrophil to express HLA-DR, CD80 and CD86. Isolated neutrophils were treated under different conditions: no treatment (brown line), 40 μg/ml ox-LDL alone (green line), 1 nmol/L PMA (blue line), 40 μg/ml ox-LDL plus 1 nmol/L PMA (red line). After 12h, expression of **(A)** HLA-DR, **(B)** CD80, **(C)** CD86 on neutrophils were analyzed by flow cytometry. Black line: isotype control. *p < 0.05.

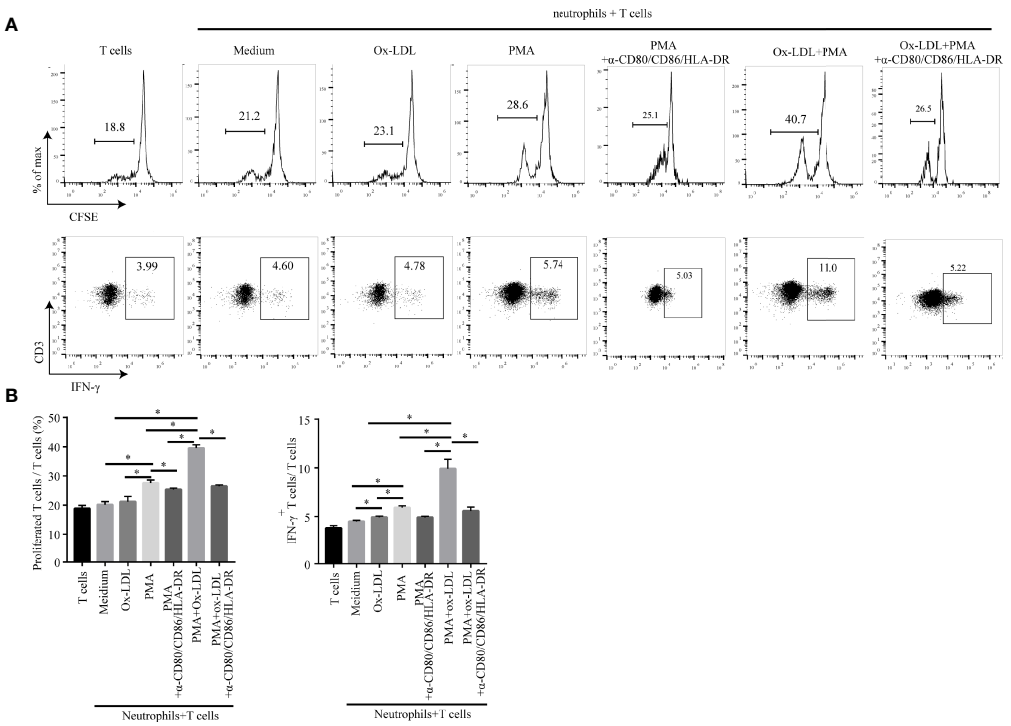

FIGURE 5 | APC-like neutrophils increase T cell proliferation and IFN-γ production *in vitro*. Isolated CFSE-labelled peripheral $CD3^+$ T cells of healthy donors were co-cultured for 5 days with neutrophils treated under different conditions: medium, ox-LDL, PMA, ox-LDL +PMA, with or without blocking antibodies (human HLA-DR/CD80/CD86 neutralization antibodies). Representative graphs **(A)** and statistical analysis **(B)** of T cell proliferation and IFN-γ production were shown (n=3). *p < 0.05.

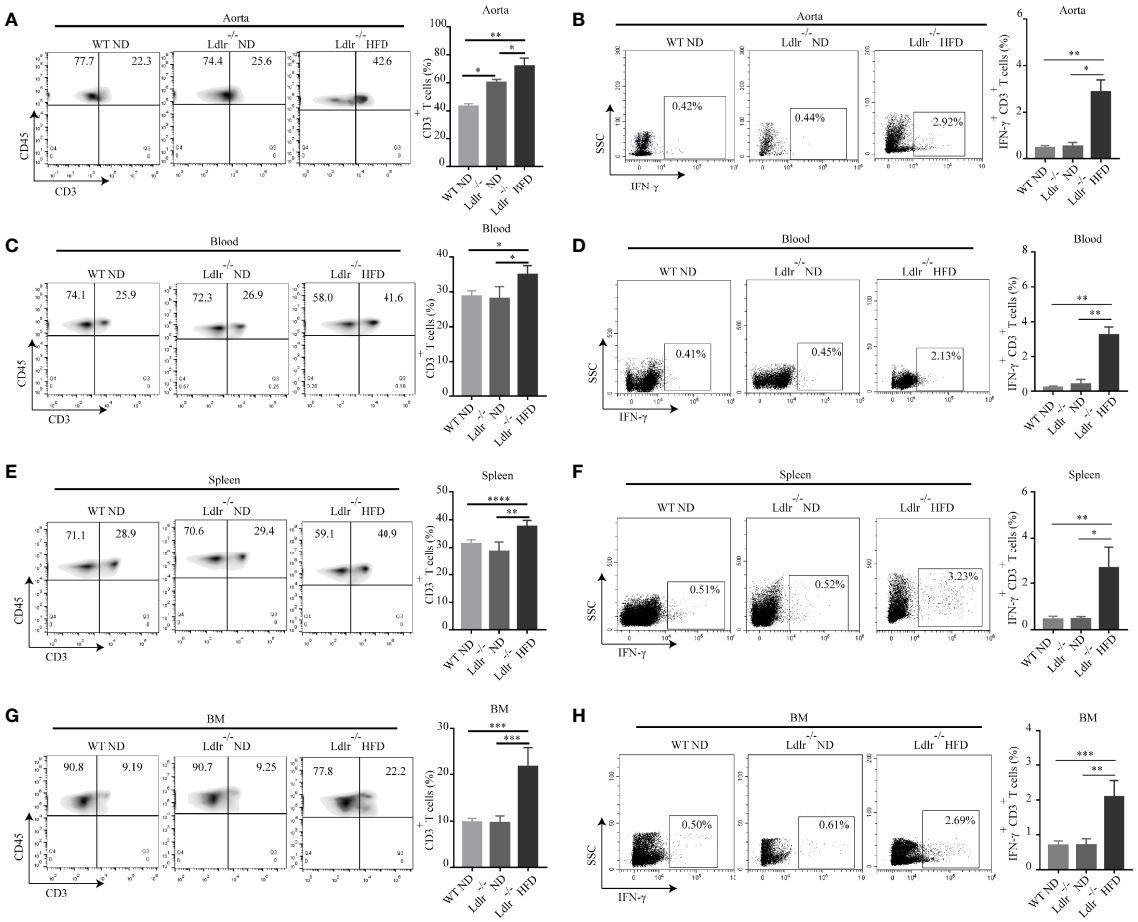

FIGURE 6 | Percentages of $CD3^+$ T cells and IFN-γ^+ T cells are higher in $LDLr^{-/-}$HFD mice compared to control groups. The distribution of $CD3^+$ T and IFN-γ^+ T cell in the **(A, B)** aorta, **(C, D)** blood, **(E, F)** spleen (SP), and **(G, H)** bone marrow (BM) of WT ND, $LDLr^{-/-}$ND and $LDLr^{-/-}$HFD mice were measured by flow cytometry. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

through TLRs; however, without PMA activation, the expression of TLRs on the surface of fresh neutrophils is extremely low, so ox-LDL alone cannot differentiate neutrophils into APC-like subset. The second explanation is that some inflammatory factors in atherosclerosis may work together with ox-LDL, playing a similar role to PMA *in vitro*, to regulate the differentiation of APC-like neutrophils. Further research is needed to validate these hypotheses.

The antigen presentation function of APCs has long been recognized as a pivotal component in T cell activation (40). HLA-DR/MHC-II-antigen crosstalk provides the first signal while costimulatory pathway provides the second signal of T cell activation (47). In this study, we show that APC-like neutrophils promote T cell proliferation and IFN-γ production through HLA-DR, CD80 and CD86 *in vitro*. In consistence with this, increased percentages of $CD3^+$ T cells and IFN-γ^+ $CD3^+$ T cells are found in atherosclerotic mice and hyperlipidemic patients. Correlation analysis reveals a positive correlation between APC-like neutrophils and $CD3^+$ T cells/ IFN-γ^+ $CD3^+$ T cells. IFN-γ is the only member of type II IFN secreted mainly by T cells and macrophages (48). It is involved in the initiation and modulation of a variety of immune responses, many of which are pro-atherogenic (49). Atherogenic effects of IFN-γ have been shown in murine models where exogenous administration enhances atherosclerotic lesion formation while knockout of IFN-γ or its receptor reduces lesion size (50). Thus, APC-like neutrophils described here in atherosclerotic mice and hyperlipidemic patients may play a pro-atherogenic role through induction of IFN-γ by $CD3^+$ T cells.

Atherosclerosis involves an ongoing inflammatory response. The atherosclerotic plaque consists of large amounts of inflammatory cells and the interaction of these immune cells plays a vital part in atherosclerosis progression. Our study identifies a novel pro-atherogenic APC-like neutrophil subset in atherosclerosis, and reveal that APC-like neutrophil subset

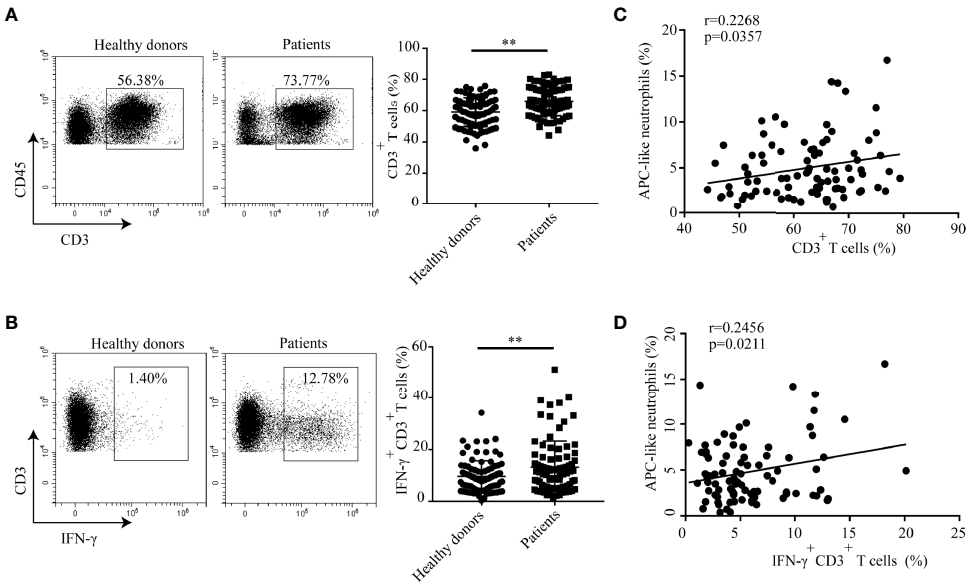

FIGURE 7 | Percentages of $CD3^+$ T cells and IFN-γ^+CD3^+ T cells are higher in hyperlipidemic patients than that of healthy donors. Percentage of **(A)**T cells and **(B)** IFN-γ^+ T cell in blood of patients with hyperlipidemia and healthy donors were detected by flow cytometry. **(C)** Correlation analysis between APC-like neutrophils and $CD3^+$ T cells in hyperlipidemic patients. **(D)** Correlation analysis between APC-like neutrophils and IFN-γ^+ T cells in hyperlipidemic patients. In this set, HLA-DR^+ neutrophils were defined as APC-like neutrophils. $^{**}p < 0.01$.

contributes to T cell activation, which is consistent with our clinical correlation analysis. Thus, our study provides a new perspective for the immune theory of atherosclerosis and provides new targets for anti-atherosclerosis therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Chongqing Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Ethical and Experimental Committee of Chongqing Medical University.

AUTHOR CONTRIBUTIONS

TW, CY: conception and design, data analysis, manuscript revision. TZ: experiment conduction, data analysis, drafting the manuscript. QJ: data analysis, manuscript revision. WL: blood samples and clinical data collection. YW, YZ, XC: experiment conduction. TD, ZY, LM, and RY: technical support and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.851713/full#supplementary-material

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Succinate/IL-1β Signaling Axis Promotes the Inflammatory Progression of Endothelial and Exacerbates Atherosclerosis

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Inflammation is an important driver of atherosclerosis. Succinate is a new extracellular inflammatory alarm released by activated macrophages. Succinate is sensed by succinate receptor 1 (Sucnr1) and then transferred to effector cells. It is worth exploring whether succinate is capable of facilitating the inflammatory response in atherosclerosis. In this study, we firstly found that arterial serum of Coronary Heart Disease (CHD) patients contained significantly higher succinate and interleukin (IL)-1β than Health control (HC) subjects, and succinate was positively correlated with IL-1β. As demonstrated by the *in vitro* study, succinate/hypoxia-inducible factor 1α (Hif)-1α/IL-1β signal axis existed and significantly facilitated the inflammatory program in human umbilical vein endothelial cells (HUVECs). Under the coculture, activated macrophages released succinate, which would be transferred to HUVECs *via* Sucnr1 and then activate Hif-1α to produce a greater amount of IL-1β. Likewise, the aortic sinus's inflammatory phenotype was found to be more significant within Apoe$^{-/-}$ mice that were injected with succinate. Furthermore, Sucnr1 inhibitor (NF-56-EJ40) could significantly interrupt succinate/IL-1β signal in HUVECs and macrophages. As revealed by this study, glycolytic metabolism following the release of succinate could be found in atherosclerotic pathology, and succinate would drive succinate/IL-1β signal dependent on Sucnr1 and then exacerbate inflammatory responses. Sucnr1 might be a novel target for cutting off the transduction of succinate signal to prevent the inflammation of atherosclerosis.

Keywords: atherosclerosis, succinate, Sucnr1, Hif-1α, IL-1β, inflammation

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INTRODUCTION

Atherosclerosis has been reported as the underlying pathology of cardiovascular diseases, which can be significantly driven by inflammation (1). Macrophage deposition and endothelial activation are recognized as the preconditions for arterial inflammation (2). When blood vessels are invaded or injured, macrophages deposit and convert their metabolism from oxidative phosphorylation to

aerobic glycolysis (e.g., Warburg effect) after the activation using surface receptors (TLRs, toll-like receptors), which underpins the rapid activation of macrophages and the synthesis of immune mediators required for perpetuating inflammation (3–5). The mentioned variations of energy metabolism can be performed in normoxic and hypoxic conditions (6, 7). Succinate has been found as a major metabolite accumulating in this procedure, as well as a novel extracellular inflammatory driver, which can be sensed extracellularly by succinate receptor 1 (Sucnr1) (8). Intracellular succinate in macrophages has been confirmed to stabilize hypoxia-inducible factor 1α (Hif-1α) for facilitating enhanced pro-inflammatory IL-1β production in the normoxic condition, since it can replace the function of inhibiting prolyl hydroxylase domain (PHD) enzyme activity under hypoxia (9). For this reason, extracellular succinate locally activates Sucnr1-expressing macrophages to initiate or intensify the immune response.

As confirmed by existing studies, succinate is abundantly detected in two immune diseases, i.e., synovial fluid (SF) of rheumatoid arthritis (RA) (3, 10) and serum of Crohn's disease (11, 12). To be specific, the former has been proven as the upstream of IL-1β in SF. Endothelial cells, the inherent barrier of the vascular wall, are capable of releasing numerous inflammatory mediators and interacting with macrophages for amplifying inflammatory responses (13–15). However, the initial interaction between endothelial and macrophage remains unclear. On that basis, this study hypothesized that macrophages can locally deposit and release succinate after vascular endothelial injury, and then the accumulating succinate promotes vascular endothelial cells to impact the inflammatory response *via* succinate/Hif-1α/IL-1β signaling axis and facilitate the pathological characteristics of atherosclerosis.

MATERIALS AND METHODS

Human Samples

All volunteers originated from cardiovascular clinics and wards of Shandong Provincial Qianfoshan Hospital. All of the volunteers signed informed consent forms. This study, complying with all the requirements of the Declaration of Helsinki, was reviewed and approved by the Ethics and Research Committee of the First Affiliated Hospital of Shandong First Medical University. Radial arterial blood was collected before coronary angiography. Subsequently, the volunteers fell into the positive (CHD group, >50%, n = 34) and negative groups (HC group, n = 38) in accordance with the tested degrees of coronary arteriostenosis. The blood was centrifugated at 3,000 rpm at 4°C for 10 min, and the serum was collected and kept at -80°C. **Supplementary Table 1** presents the statistics of clinical parameters.

Mice

All mice were applied by complying with Regulations on the Administration of Experimental Animals under the promulgation and implementation of the State Science and Technology Commission. Beijing Vital River Laboratory Animal Technology Co., Ltd., offered $Apoe^{-/-}$ and C57BL/6J mice, male, overall aged 8 weeks. C57BL/6J mice took the ordinary diet as the control (CON, n = 12), while $Apoe^{-/-}$ mice (n = 26) had the western high-fat diet. After 12 weeks, 2 mice were randomly chosen to prove the formation of atherosclerosis plaque. After the model was made, $Apoe^{-/-}$ mice were randomly divided into $Apoe^{-/-}$ group (n = 12) and $Apoe^{-/-}$+Suc group (n = 12). Succinate (0.039 mg/kg) in 0.9% normal saline (NS) was injected into $Apoe^{-/-}$+Suc group mice once every other day (16), while 0.9% NS was injected into CON and $Apoe^{-/-}$ group for comparison. The total of 7 injections lasted for 14 days. After another 7 feeding days, the mice were overall anesthetized with 10% hydrated chlorine aldehyde and perfused through the left ventricle with 0.9% NS. The blood sample was collected from cardiac apex and then centrifuged at 2,500 rpm for 5 min. Next, the serum was collected for ELISA. For the respective mice, the artery from heart to iliac artery was dissected and kept. Several fresh specimens were stored at -80°C to perform Western blots, and the others were fixed with 4% paraformaldehyde at the ambient temperature for 24 h. For the fixed specimens, the artery from aorta root to the iliac artery was stained with Oil-Red. The heart was embedded in optimal cutting temperature (OCT), and the aortic sinus was sectioned into 10-μm slices to perform Oil-Red, Masson, and H&E stain. Besides, the heart was embedded in paraffin, and the aortic sinus was sectioned into 6-μm slices to perform immunohistochemistry (IHC).

Cell Culture and Treatments

Macrophage cell was differentiated from human monocyte THP-1 cell. THP-1 cell line was purchased from National Collection of Authenticated Cell Culture (ATCC) and then cultured within RPMI-1640 medium supplemented with 10% fatal bovine serun (FBS) using the incubator at 37°C with 5% CO_2. Passages 10–15 were applied to perform the subsequent experiment. THP-1 cells were differentiated by employing 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich p8139#) for 24 h to obtain M0, and M0 is induced to M1 through Lipopolysaccharide (LPS) (100 ng/ml 24 h) stimulation. Since M1 expressed IL-1β, IL-6, tumor necrosis factor (TNF)-α, C-X-C motif ligand 8 (CXCL8), and IL-12B, rt-PCR was performed to verify the successful induction of M1 (17) (**Supplementary Figure 3**). Non-adherent macrophages were cleaned using phosphate buffered solution (PBS), and the adherent cells were cultured using fresh RPMI-1640 medium.

Human umbilical vein endothelial cells (HUVECs) were purchased from Sciencell and then maintained in Endothelial Cell Medium (ECM) media (Sciencell, USA) at 37°C using 5% CO_2. All HUVECs used here were between passages 3 and 7.

Macrophages and HUVECs were counted by employing the hemocytometer and subsequently seeded in 6-, 12-, or 96-well plates to conduct the next study. Here, 0.25% trypsin-EDTA (Gibco, Canada) digested cells, and Serum-Free Cell Freezing Medium (NCM, China) cryopreserved cells. LPS (Sigma-Aldrich), PMA, and succinate were dissolved in PBS solution, and NF-56-EJ40 (MCE) was dissolved in Dimethyl Sulfoxide (DMSO). In the analysis of the pro-inflammatory function, cells were seeded in well plates added with LPS (100 ng/ml), succinate

(800 μM) (3), or NF-56-EJ40 (4 μM) in accordance with the demand.

Cell Counting Kit 8 Assay

The Cell Counting Kit 8 (CCK8) assay was performed for measuring the cell viability by complying with the manufacturer's protocols (Beyotime, China). Macrophage cells were seeded in 96-well plate under a density of 10^4 per well and adhering for 24 h. Next, the cell medium was changed to fresh medium supplemented with succinate or PBS, NF-56-EJ40, or DMSO. Following the experiment design, the cell activity was examined at 24 and 48 h of the incubation. The OD 450 absorbance was measured for the assessment of the cell activity. HUVECs' seeded density reached 5,000 per well, and the remaining steps complied with those of macrophages.

Hypoxia-Inducible Factor-1α Silencing

Macrophages and HUVECs were transiently transfected with NC siRNA or targeted Hif-1α silencer siRNA (20 nM for macrophages and 10 nM for HUVECs) in the presence of ribo FECT transfection reagent (Ribobio, China) for 36 h. The sequence of siRNA used is as follows: Sense GGAACCUGAUGCUUUAACUt and anti-sense AGUUAAAGCAUCAGGUUCCtt.

ELISA

Succinate Detection

The levels of succinate in the human serum were measured by performing ELISA (Y-S Biotechnology, China) in accordance with the manufacturer's instructions. The assay sensitivity reached 2.5 ng/L.

Interleukin-1β Detection

The levels of IL-1β in human serum and cell condition medium were examined by performing ELISA (BOSTER, China). The assay sensitivity was 1.95 pg/ml, and the OD 450 absorbance was examined to assess the indicators.

Quantitative RT-PCR

Total RNA was extracted from cells with TRIzol (ambion by Life technologies, USA) method. Total RNA quantity was examined at 260 nm. cDNA was prepared with the use of the Reverse Transcription System (Bioer Technology, China) by performing SuperReal PreMix Plus assay (TIANGEN BIOTECH, China). ChemiDoc Imaging System (Bio-Rad) was employed to perform quantitative PCR. The results were determined based on the comparative Ct method. Primers of Sucnr1, Hif-1α, IL-1β, IL-6, TNF-α, CXCL8, IL-12B, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were presented below: Sucnr1-S, TATGGGATTGAGTTCGTTGTGG; Sucnr1-A, GAGAATGC TGGTATAGAGGTT GGC; Hif-1α-S, GCTCATCAGTT GCCACTTCCAC; Hif-1α-A, CCAAATCACCAGCAT CCAGAAG; IL-1β-S, GGCTTATTACAGTGGCAATGAGG; IL-1β-A,GTAGT GGTGGTCGGAGATTCGT; GAPDH-S, GAAGGTGAAGGTCG GAGTC; GAPDH-A, GAAGATGGTGA TGGGATTTC; TNF-α-S, CTGCCTFCAC TTTFFAG; TNF-α-A, ACATGGGCTACAGGCTTGTCACT; CXCL8-S, CACTGTG TGTAAACATGACTTCCAA; CXCL8-A, TGTGGTCCACTCTC AATCACTCTC; IL-12B-S, CTTGGAGCGAATGGGCATC; IL-12B-A, TGGGTCTATTCCGTTGT GTCTTTA. All the primers originated from Sangon Biotech (China).

Western Blot

Total protein was extracted from cells or tissues with the use of RIPA Vs protease inhibitor cocktail (100:1), while cytoplasmic proteins and nucleoproteins were extracted by employing extraction kits (BOSTER, China). Protein concentration was quantified based on BCA kit (Beyotime, China). Equal proteins (15–25 μg) were separated on 10%–12% Sodium dodecyl sulfate (SDS) gels and then transferred onto Polyvinylidene fluoride (PVDF) (0.22/0.45 μm) membranes. After being blocked with 5% skim milk, the membranes were incubated with primary antibodies (1:1,000) overnight at 4°C. Primary antibodies consisted of Sucnr1 (Abcam, ab272856), Hif-1α (CST, #36169), IL-1β (CST, #12242), NLRP3 (Proteintech, #19771-1-ap), NF-κB (Proteintech, 66535-1-lg), Lamin B1 (Abcam, ab16048), Caspase-1 (Proteintech, 22915-1-ap), and β-Actin (Affinity, AF7018). Subsequently, the membranes were incubated with secondary antibody [goat anti-rabbit IgG HRP/ goat anti-mouse IgG Horseradish Peroxidase (HRP) (1:10,000, ZSGP-BIO, China)] at Room temperature (RT) for 1 h. Bands were visualized with Enhanced chemiluminescence (ECL) reagent (MilliporeSigma, USA) and then captured on FluoChem E or ChemiDoc Imaging System. The optical density of bands was analyzed with the use of ImageJ software.

Immunohistochemistry

Paraffin sections of the aortic sinus from mice were made for IHC after dewaxing and antigen repair. Subsequently, the sections were incubated with primary antibody (1:100) at 4°C overnight. The primary antibody included F4/80 (CST, #70076), CD31 (Affinity, AF6191), and antibodies in Western blot section. Next day, the respective section was incubated in secondary antibody at the ambient temperature for 30 min and then cleaned thoroughly with PBS. The tissues were stained with Diaminobenzidine (DAB) reagent and then observed under Leica DM1000 microscope. The images were investigated with ImageJ software.

Statistical Analyses

For the clinical information, the values had the expression of mean ± SD. Continuous variables were determined by performing unpaired t-test, categorical variable was analyzed by performing χ^2 test, and the correlation was explored by applying Pearson's correlation coefficient. For *in vitro* and *in vivo* data, the values were expressed as mean ± SEM and then determined based on one-way ANOVA. P values <0.05 had statistical significance. Data were analyzed with GraphPad Prism 8.0 software.

RESULTS

Expression of Succinate and Interleukin-1β in Atherosclerosis

The clinical parameters were analyzed as **Supplementary Table S1**. CHD patients and the controls showed no difference between

age, weight, Body Mass Index (BMI), gender, and smoking rate, whereas the two groups were noticeably different in alcohol drinking (P = 0.049). As reported by ELISA results of serum, succinate and IL-1β significantly increased in CHD patients in comparison with those in the HC group. Furthermore, Pearson's correlation analysis was conducted on the two data, and results revealed a linear correlation between succinate and IL-1β in the two groups (R = 0.5443, R^2 = 0.2962, 95% confidence interval is 0.2414–0.7505, P = 0.0013) (**Figures 1A–C**).

Succinate Stimulates Human Umbilical Vein Endothelial Cells Producing Interleukin-1β

According to the verification result, succinate/IL-1β signaling axis existed in HUVECs. HUVECs were stimulated with LPS (100 ng/ml) and succinate (800 μM) as published literature. All drug doses of HUVECs here were the same as macrophages, and CCK8 was adopted to detect the toxicity of succinate to HUVEC, as shown in **Supplementary Figure 1**. Supernatant and holoprotein were extracted for Hif-1α and IL-1β detection among the dual stimulation group (LPS and succinate), the LPS group, and the blank control group. According to the result of ELISA, IL-1β of supernatant notably increased in the dual stimulation group (**Figure 2A**), and Western blots showed a similar result that Hif-1α and IL-1β were elevated under LPS and succinate stimulation (**Figures 2B–E**). Moreover, nuclear protein was extracted, and the results indicated that the expression level of Hif-1α in HUVEC nucleus was more significant under dual stimulation, which confirmed that succinate stimulation upregulated the expression of Hif-1α in HUVEC nucleus and facilitated its function as a transcription factor (**Figures 2F, G**).

Sucnr1 Antagonist Inhibits Succinate/Interleukin-1β Signal Axis in Human Umbilical Vein Endothelial Cells and Macrophages

The succinate pathway was cut off to observe the expression of succinate downstream substances. As Sucnr1 is the membrane receptor of succinate, succinate should be combined with Sucnr1 to exert a pro-inflammatory activity. In this study, a high-affinity and highly selective antagonist (NF-56-EJ40, NF) was first adopted to interrupt Sucnr1 to cut off the signal axis. HUVECs served as the test cells to establish the concentration gradient and time gradient of NF intervention, and Sucnr1 levels were tested by Western blots (**Figures 3A, B**). The optimal concentration and time of NF-56-EJ40 intervention were 4 μM and 24 h. CCK8 results indicated that macrophage and HUVEC activities were not affected under the optimal concentration and time (**Supplementary Figure 1**). HUVECs were interfered with using NF for 24 h and LPS compared with succinate for the next 24 h. Sucnr1, Hif-1α, and IL-1β expression levels were tested using ELISA, rt-PCR, and Western blot (**Figures 3C–J**). Nuclear protein was also extracted to assess Hif-1α activation (**Figures 3K, L**). According to the results, NF-56-EJ40 could significantly inhibit Sucnr1 and lead to Hif-1α and IL-1β decreasing. In addition, the function of NF-56-EJ40 in macrophages was verified (**Figure 4**). As macrophages could produce succinate under LPS intervention, we only interfered it with NF-56-EJ40 4 μM for 24 h and then stimulated with LPS for another 24 h. All the results were similar as in HUVECs, which confirmed that NF-56-EJ40 could significantly reduce Hif-1α activation and IL-1β production through Sucnr1 inhibition in HUVECs and macrophages.

The Significance of Hypoxia-Inducible Factor-1α in Succinate/Interleukin-1β Signal Axis

As succinate can stimulate Hif-1α and IL-1β in macrophages and DCs in prior literature, we verified the significance and location of Hif-1α in HUVECs in this signal directly. Hif-1α siRNA was transiently transfected into HUVECs to downregulate Hif-1α, and the results indicated that the levels of Hif-1α and IL-1β decreased significantly in comparison with those in the NC siRNA group on the premise of succinate and LPS stimulation (**Figure 5**). Transfection efficiency of Hif-1α siRNA was verified by rt-PCR,

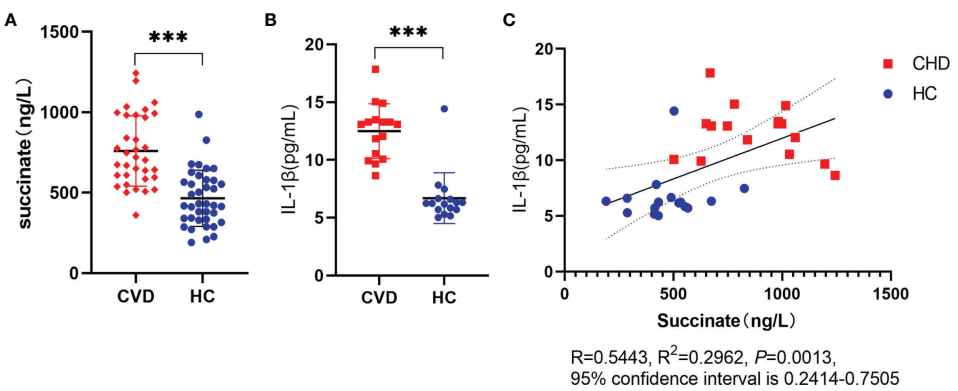

FIGURE 1 | Expression of succinate and IL-1β in CHD. Radial arterial blood is collected before coronary angiography, then volunteers are divided into CHD group and HC group based on the results of coronary arteriostenosis. **(A, B)** Succinate and IL-1β were assessed *via* ELISA. Succinate detection (CHD group, n = 34; HC group, n = 38), IL-1β detection (CHD group, n = 16; HC group, n = 16), and all samples of this section had succinate detection. **(C)** Clinical correlation was analyzed in human samples using Pearson's correlation coefficient (R = 0.5443, R^2 = 0.2962, P = 0.0013, 95% confidence interval is 0.2414–0.7505). ***P < 0.001.

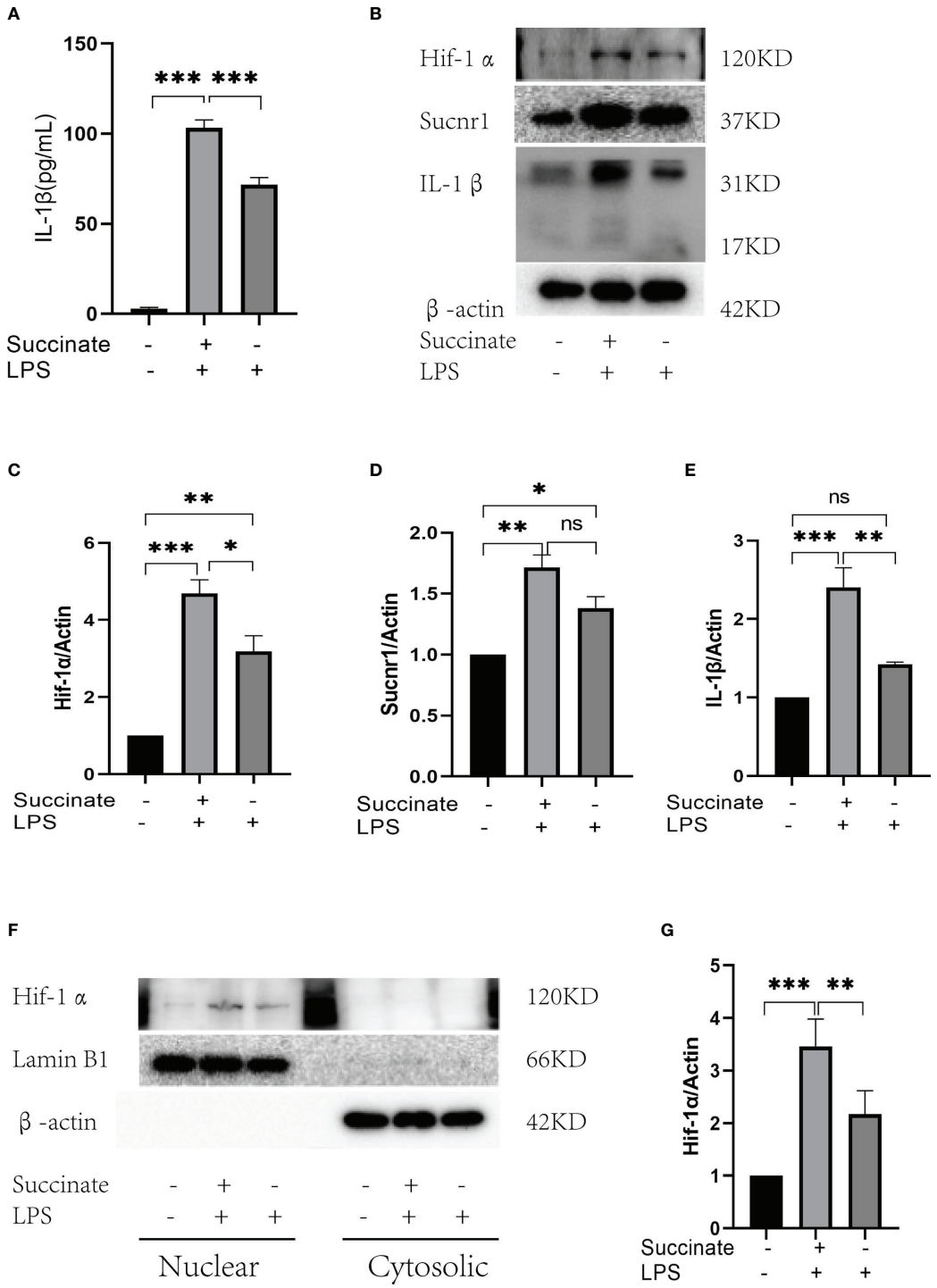

FIGURE 2 | Sucnr1/Hif-1α/IL-1β signal axis in HUVECs. HUVECs were divided into three groups and given intervention according to design: Blank control was given equal amount of PBS, LPS+succinate group was given LPS (100 ng/ml) and succinate (800 μM), and LPS group was given LPS (100 ng/ml) for 24-h intervention, then supernatant medium and proteins were collected for experiment. **(A)** IL-1β of supernatant medium was detected *via* ELISA. **(B–E)** Expression levels of Sucnr1, Hif-1α, and IL-1β were displayed *via* Western blots and analyzed graphs. **(F, G)** Expression levels of Hif-1α in nucleus were detected using Western blots, in which Lamin B1 was the reference protein. Western blot data present mean ± SEM from three independent experiments at least. ELISA results represent mean ± SD from three different experiments at least. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns means no statistical difference.

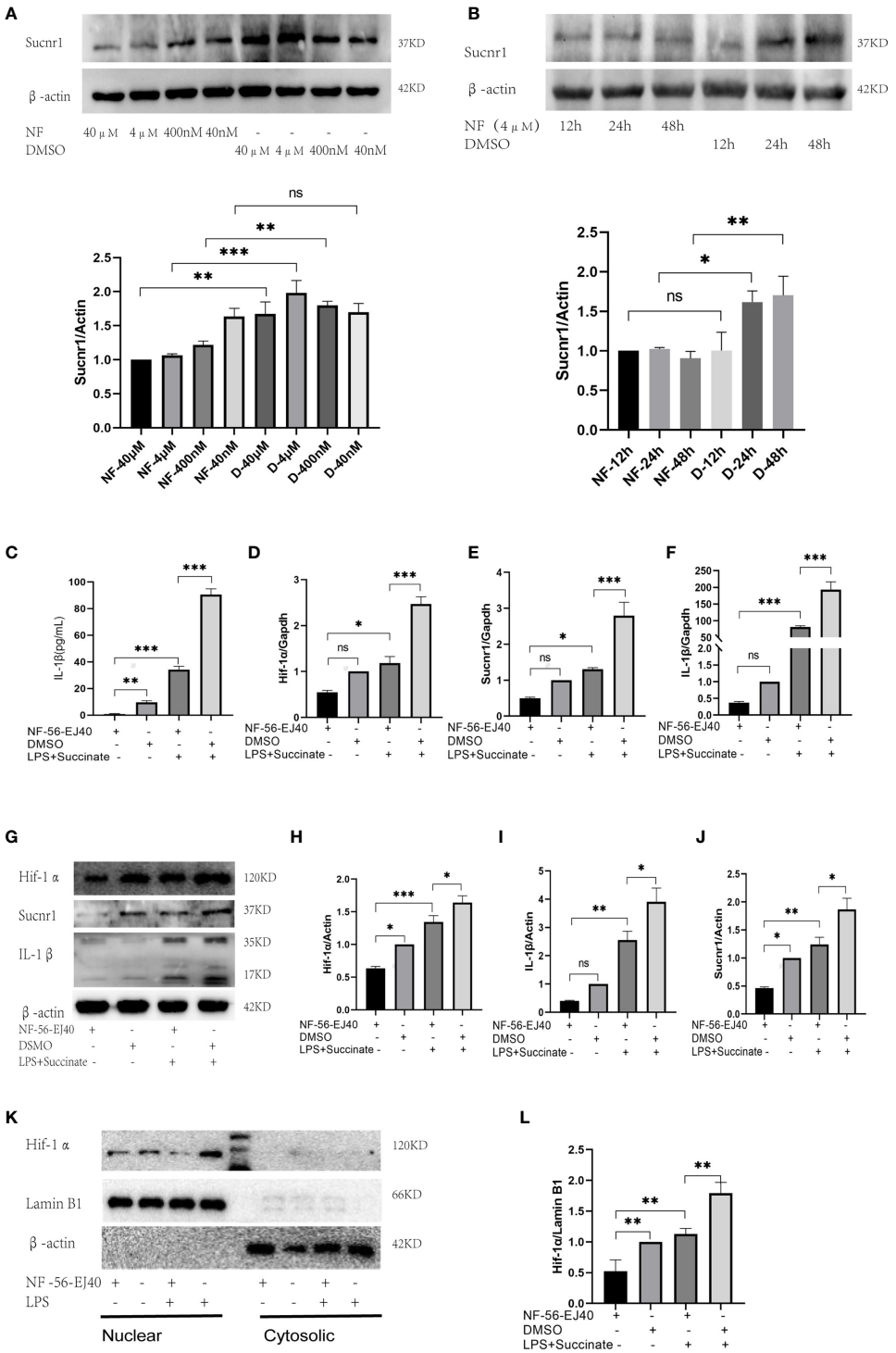

FIGURE 3 | Sucnr1 antagonist inhibits succinate/IL-1β signal axis in HUVECs. **(A, B)** HUVECs were used as test cells to determine the optimum time and concentration of NF-56-EJ40 by Western blot. HUVECs were divided into four groups and given intervention according to design: NF-56-EJ40 group, DMSO group, NF-56-EJ40 and LPS+succinate group, DMSO and LPS+succinate group. **(C)** IL-1β of supernatant medium was detected *via* ELISA. **(D–F)** mRNAs of Sucnr1, Hif-1α, and IL-1β were tested using rt-PCR, in which Gapdh was the reference mRNA. **(G–J)** Expression levels of Sucnr1, Hif-1α, and IL-1β were displayed *via* Western blots. **(K, L)** Expression levels of Hif-1α in nucleus were detected using Western blots. Western blot data present mean ± SEM from three independent experiments at least. ELISA results represent mean ± SD from three different experiments at least. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns means no statistical difference.

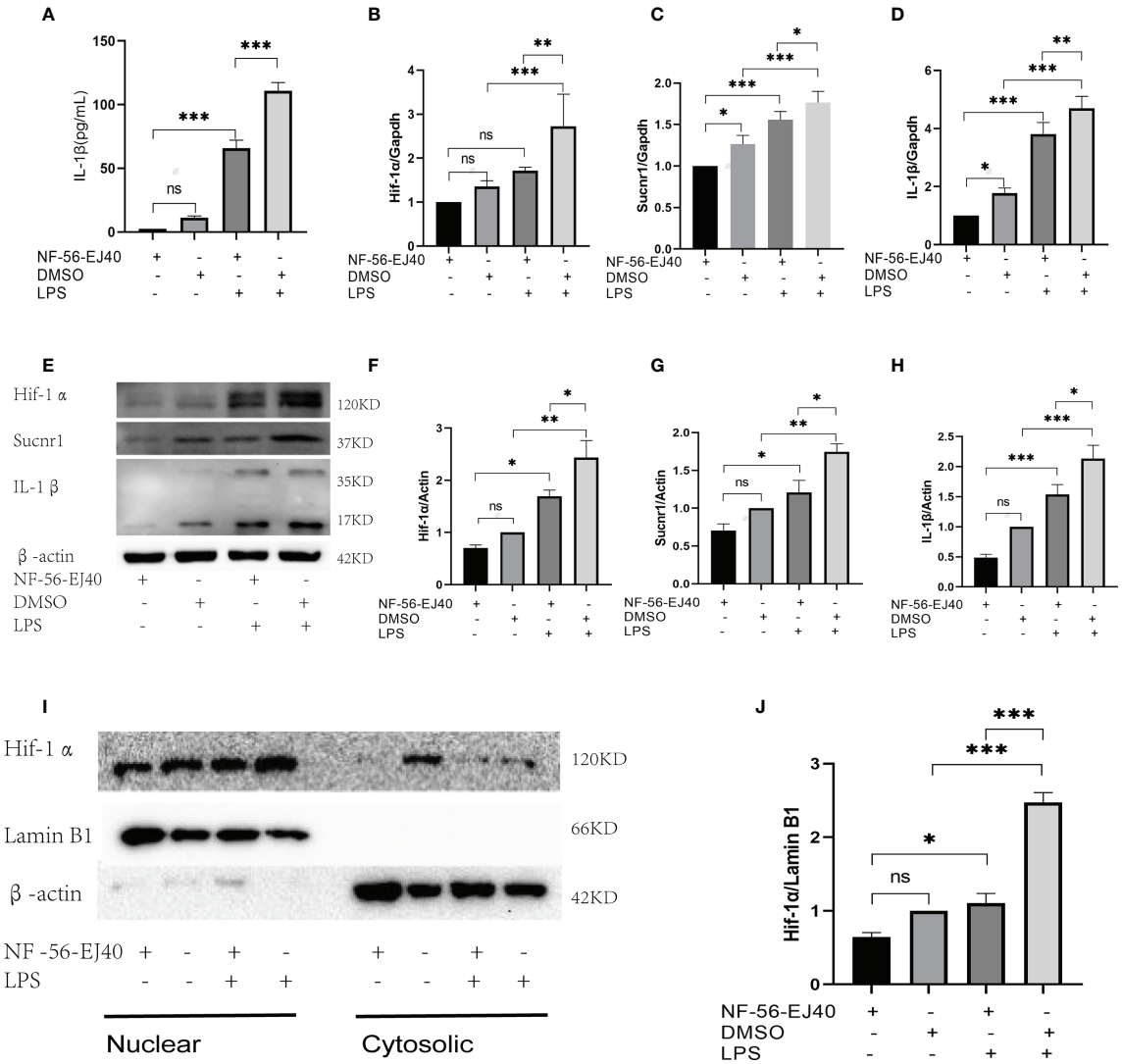

FIGURE 4 | Sucnr1 antagonist inhibits succinate/IL-1β signal axis in macrophages. Macrophages were divided into four groups and given intervention according to design: NF-56-EJ40 group, DMSO group, NF-56-EJ40 and LPS group, DMSO and LPS group. **(A)** IL-1β of supernatant medium was detected *via* ELISA. **(B–D)** mRNAs of Sucnr1, Hif-1α, and IL-1β were tested using rt-PCR. **(E–H)** Expression levels of Sucnr1, Hif-1α, and IL-1β were displayed *via* Western blots. **(I, J)** Expression levels of Hif-1α in nucleus were detected using Western blots. Western blot data present mean ± SEM from three independent experiments at least. ELISA results represent mean ± SD from three different experiments at least. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns means no statistical difference.

as presented in **Supplementary Figure 2D**. All the results confirmed that in HUVECs, succinate upregulated the expression of inflammatory factor IL-1β by activating transcription factor Hif-1α.

Nucleotide-Binding Oligomerization Domain(Nod)-Like Receptor 3 and Caspase-1 Are Involved in the Activation of Pro-Interleukin-1β Regulated by Hypoxia-Inducible Factor-1α

Referring to IL-1β, the NF-κB pathway cannot be bypassed. Thus, we explored whether there is a mutual influence between succinate/IL-1β signal axis and NF-κB pathway.

Firstly, we tested whether succinate intervention affected protein expression in the NF-κB pathway. HUVECs were divided into two groups: one was LPS combined with succinate group; the other was LPS group. The stimulation lasted for 24 h, and NF-κB, NLRP3, and Caspase-1 were tested through Western blots. According to the results, NLRP3 and Caspase-1 notably increased after succinate stimulation, whereas the transcription factor NF-κB remained unchanged (**Figures 6A–E**).

Subsequently, we observed the effect of Sucnr1 inhibition on NF-κB pathway. Inhibitor (NF-56-EJ40) was used to interrupt Sucnr1 for 24 h, and then LPS and succinate were added for another 24 h. Western blots displayed that NLRP3 and Caspase-1 markedly decreased compared with those in the non-inhibitor

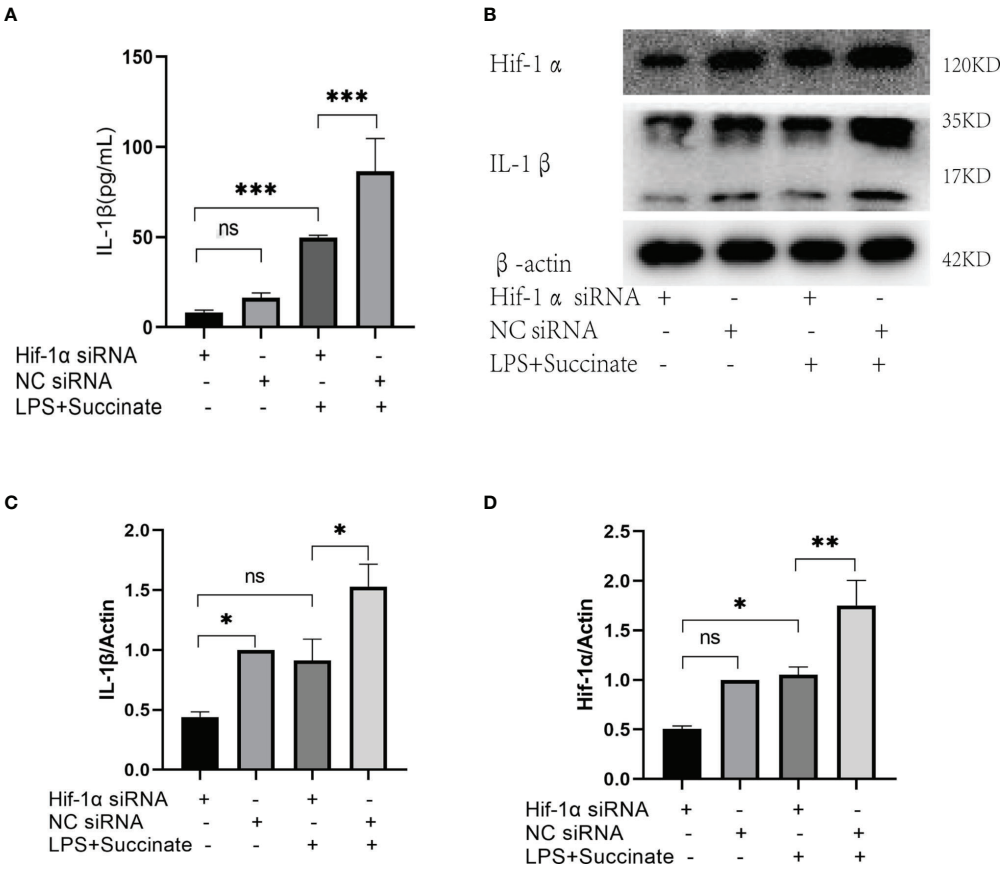

FIGURE 5 | The expression of IL-1β after Hif-1α knockout. Hif-1α siRNA was transfected into HUVECs to downregulate Hif-1α as follows: Hif-1α siRNA group, NC siRNA group, Hif-1α siRNA and LPS+succinate group, NC siRNA and LPS+succinate group. **(A)** IL-1β of supernatant medium was detected *via* ELISA. **(B–D)** Expression levels of Hif-1α and IL-1β were assessed *via* Western blots. Western blot data present mean ± SEM from three independent experiments at least. ELISA results represent mean ± SD from three different experiments at least. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns means no statistical difference.

group, whereas NF-κB had no obvious change (**Figures 6F–J**), which demonstrated that the two proteins were the downstream of succinate, and their location should be investigated during this axis.

Finally, we further examined whether this change occurred in Hif-1α/IL-1β section. Again, we silenced Hif-1α in HUVECs and intervened with LPS and succinate for 24 h. The expression levels of NLRP3 and Caspase-1 decreased compared with those in the NC siRNA group, and Hif-1α silencing did not affect NF-κB (**Figures 6K–O**), which demonstrated NLRP3 and Caspase-1 as the downstream of transcription factor Hif-1α and the upstream of activating IL-1β.

Expression of Succinate/Interleukin-1β Signal in Human Umbilical Vein Endothelial Cells Cocultured With Macrophages

In abnormal vessels, macrophages generally act as inflammatory trigger cells to affect endothelium, and together they exacerbate the immune inflammatory response. Thus, in this study, HUVECs were cocultured with macrophages given LPS stimulation to evaluate HUVEC inflammatory response in succinate/IL-1β signal axis. The coculture environment is presented in **Figure 7A**.

First, HUVECs were stimulated with LPS in the presence or absence of macrophages for 24 h, and then total protein of HUVEC was extracted. All the proteins of succinate/IL-1β signal axis were assessed by Western blots, and IL-1β of coculture supernatant was detected by ELISA. The results (**Figures 7B–J**) displayed that M1 could stimulate succinate/IL-1β signal axis of HUVECs to produce more IL-1β, as LPS could activate M0 differentiating to M1, which led to the release of succinate. The trends of NLRP3 and Caspase-1 were found to be consistent with those of Hif-1α and IL-1β, and NF-κB had no change in either coculture or pro-inflammatory stimulation.

Subsequently, we used Sucnr1 inhibitors to rescue the inflammatory process. HUVECs were divided into four groups (I: LPS stimulated; II: cocultured with macrophages and LPS stimulated; III: NF-56-EJ40 inhibited and LPS stimulated; IV: NF-56-EJ40 inhibited, cocultured with macrophages, and LPS stimulated). After intervention, total protein of HUVECs was extracted and supernatant was collected. As indicated from the result of Western blots, NF-56-EJ40 could significantly

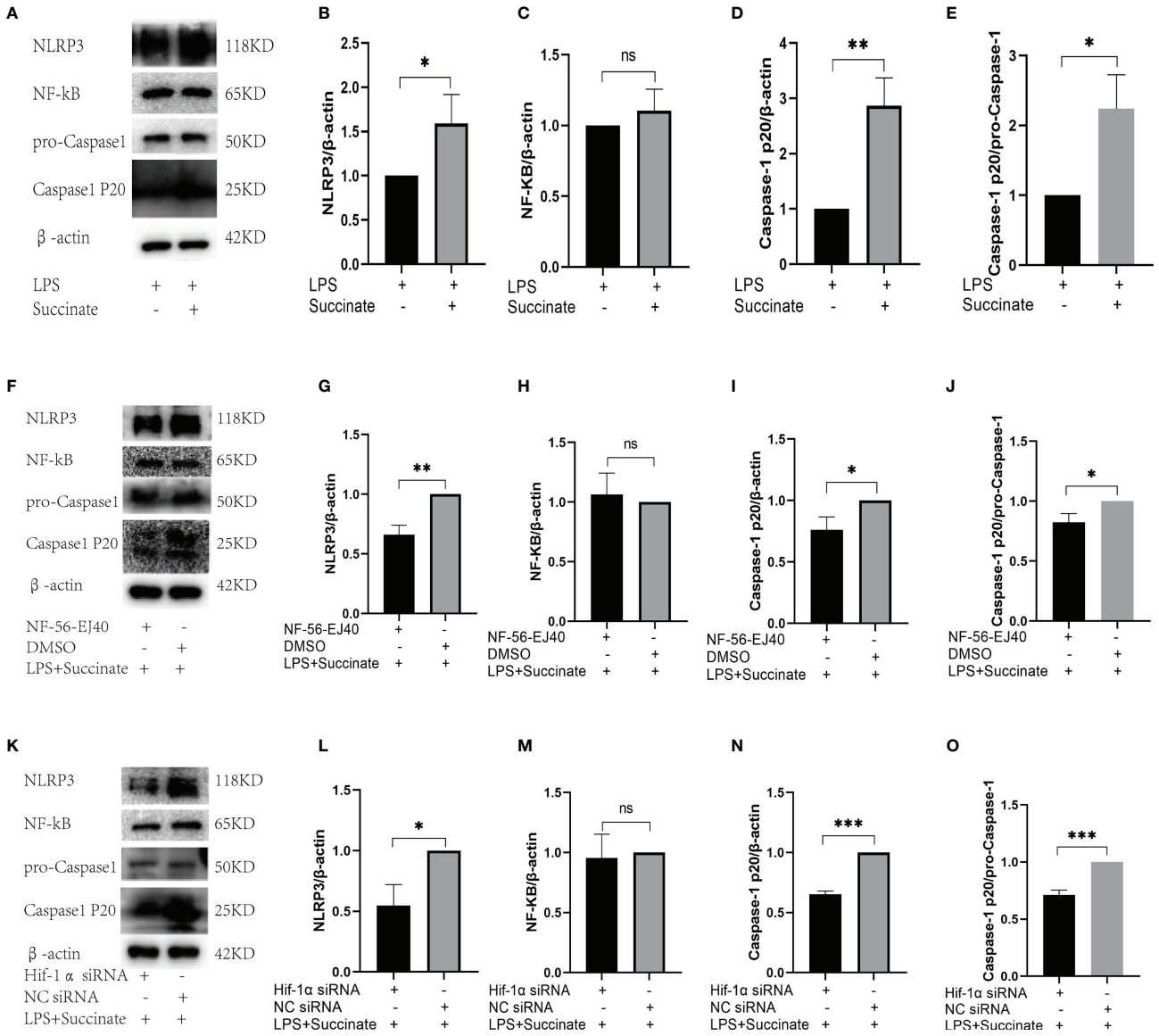

FIGURE 6 | The relation between NF-κB pathway and succinate/IL-1β signal axis. **(A–E)** The expression levels of NF-κB, NLRP3, and Caspase-1 in HUVECs after succinate stimulation. **(F–J)** The expression levels of NF-κB, NLRP3, and Caspase-1 in HUVECs after NF-56-EJ40 inhibition. **(K–O)** The expression levels of NF-κB, NLRP3, and Caspase-1 in HUVECs after Hif-1α knockout. Western blot data present mean ± SEM from three independent experiments at least. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns means no statistical difference.

downregulate the expressions of Hif-1α, NLRP3, Caspase-1, and IL-1β compared with those in the non-inhibitor intervention group, and ELISA proved that NF-56-EJ40 effectively reduced IL-1β production (**Figures 7K-Q**).

Succinate/Interleukin-1β Signal Axis *In Vivo*

Next, the investigation was conducted on how succinate activates inflammatory responses to atherosclerosis *in vivo*. C57BL/6J mice had the normal diet, while Apoe$^{-/-}$ had the Western diet for 12 weeks and were divided into 2 groups. Subsequently, intravenous succinate was injected into the Apoe$^{-/-}$+Suc group, and 0.9% NS was injected into the Apoe$^{-/-}$ group.

As indicated by serum ELISA, succinate and IL-1β significantly increased in the Apoe$^{-/-}$+Suc group compared with those in the Apoe$^{-/-}$ group and CON group. Furthermore, Pearson's correlation analysis was conducted on the two data, and the results showed that there was also a positive linear correlation between succinate and IL-1β ($R = 0.7365$, $R^2 = 0.5425$, 95% confidence interval was 0.004189–0.01084, $P <$

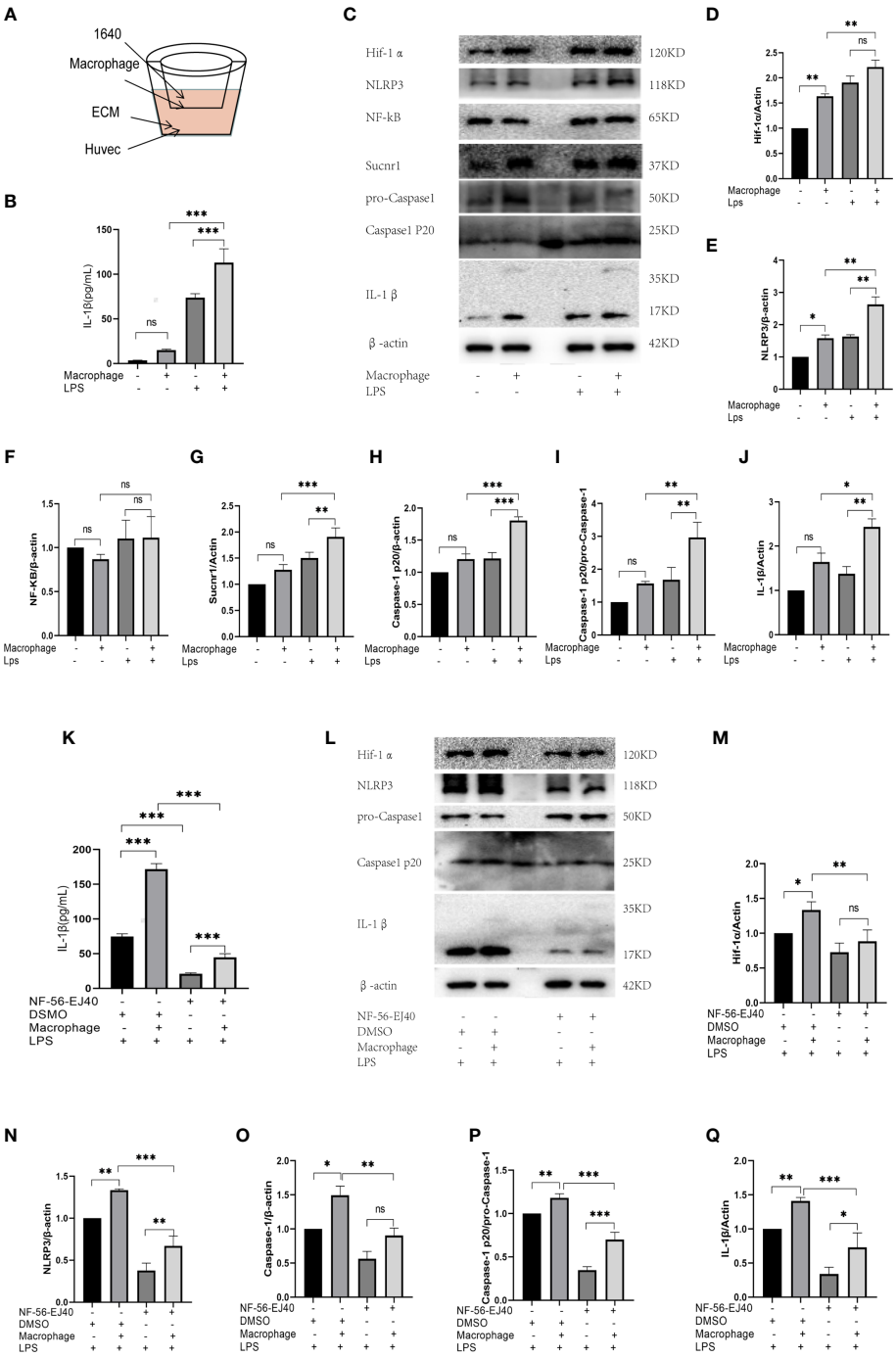

FIGURE 7 | The expression of succinate/IL-1β signal axis during coculture system. **(A)** HUVECs were cocultured with macrophages in Transwell system, as shown in the figure. HUVECs were seeded in the down plate with 2.5 ml ECM complete medium, and macrophages were seeded in the upper fibrous membranes with 1.6 ml RPMI-1640 complete medium. The groupings of the coculture system were designed as follows: HUVECs with coculture medium (ECM and RPMI-1640), HUVECs cocultured with macrophages, HUVECs with coculture medium and LPS, HUVECs cocultured with macrophages and LPS, then the supernatant medium and holoprotein of HUVECs were extracted. **(B)** IL-1β of supernatant medium was detected *via* ELISA. **(C–J)** Expression levels of succinate/IL-1β signal axis and NF-κB were tested *via* Western blots. NF-56-EJ40 tried to rescue the inflammatory response of coculture system, and HUVECs were grouped as follows: I: LPS stimulated; II: cocultured with macrophages and LPS stimulated; III: NF-56-EJ40 inhibited and LPS stimulated; IV: NF-56-EJ40 inhibited, cocultured with macrophages, and LPS stimulated. Similarly, the supernatant medium and holoprotein of HUVECs were extracted. **(K)** IL-1β of supernatant medium was detected *via* ELISA. **(L–Q)** Expression levels of succinate/IL-1β signal axis and NF-κB were tested *via* Western blots. Western blot data present mean ± SEM from three independent experiments at least. ELISA results represent mean ± SD from three different experiments at least. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns means no statistical difference.

0.0001) (**Figure 8A**). According to **Figures 8B, C**, the expressions of Hif-1α and IL-1β in the Apoe$^{-/-}$+Suc group were largely upregulated in comparison with those in the Apoe$^{-/-}$ group and CON group. Consistent with the above observation, after succinate injection, the expression levels of succinate/IL-1β signal axis including NLRP3 and Caspase-1 were mostly higher than those in the Apoe$^{-/-}$ group and the Control (**Figures 8A, B**).

As revealed from the Oil Red, Masson, and H&E staining, succinate stimulation could accelerate the formation of atherosclerotic plaque and cause more severe fibrosis of wall and lumen narrowing (**Figures 9A–C, G**). Aortic root IHC was used to evaluate the inflammatory injured ability of succinate *in vivo*, and results indicated that M1 content (F4/80), Hif-1α, and IL-1β increased significantly in comparison with those of the two control groups (**Figures 9D–G**).

DISCUSSION

This study reported that the upregulation of serum IL-1β showed positive correlations with succinate in CHD patients. As indicated from in-depth study, succinate released by activated macrophages could drive the succinate/IL-1β signaling axis of endothelial cells and its own to amplify the intravascular inflammatory response and eventually could aggravate atherosclerotic inflammation.

Inflammation refers to the typical response of the host immune system to microbial infection or tissue injury (18, 19). However, excessive and unresolved inflammation generally causes detrimental chronic inflammatory diseases (3). Atherosclerosis refers to a cholesterol-induced inflammatory disease with macrophages and endothelial cells as the major

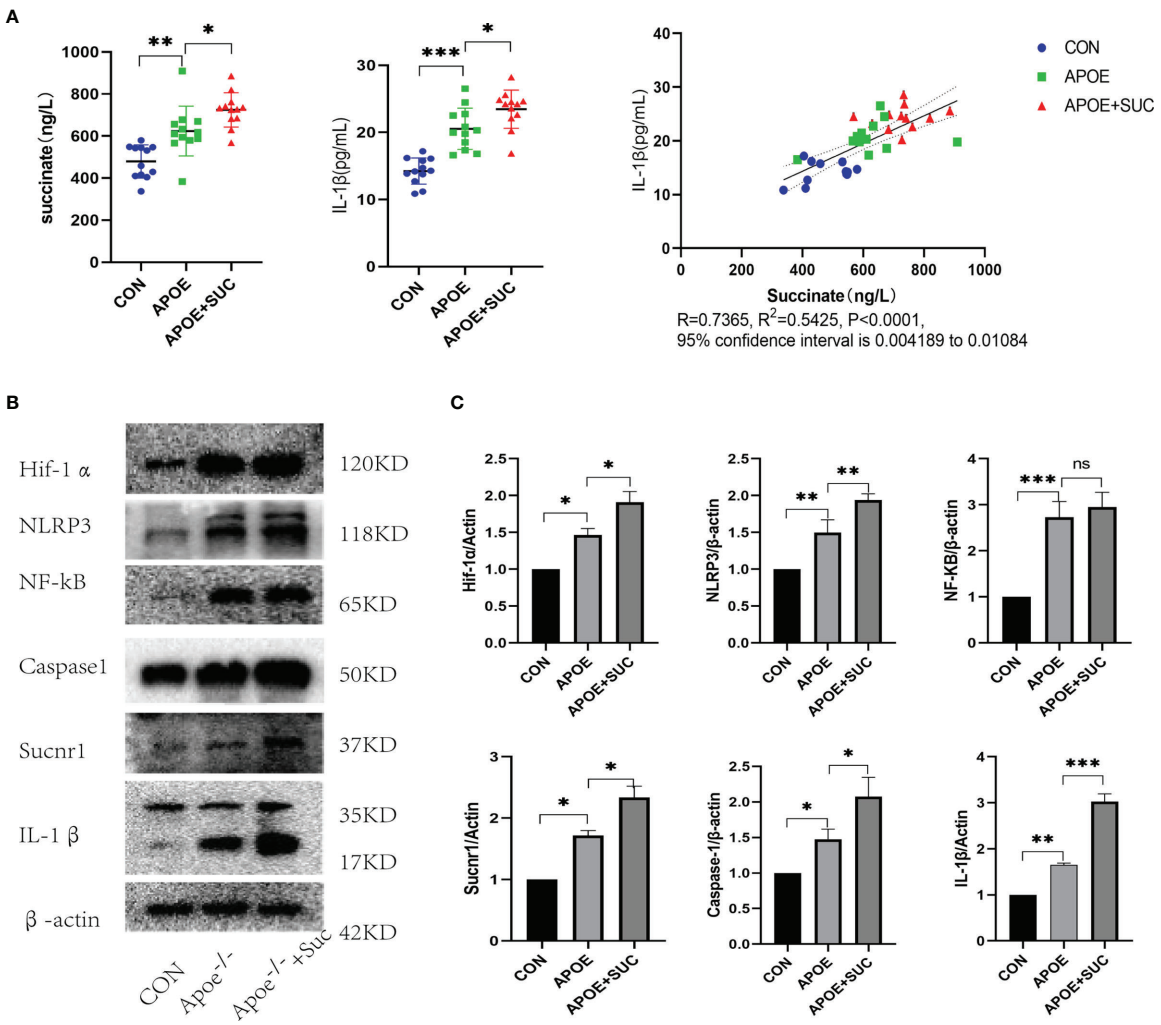

FIGURE 8 | Succinate/IL-1β signal axis in atherosclerosis model (protein level). C57BL/6J mice were blank control (CON), while Apoe$^{-/-}$ mice were randomly divided into two groups (Apoe$^{-/-}$ group and Apoe$^{-/-}$+Suc group). For each mouse, the artery from heart to iliac artery was dissected and kept. **(A)** Succinate and IL-1β of serum were detected by ELISA. ELISA results were mean ± SD from at least three different experiments. *P < 0.05; **P < 0.01; ***P < 0.001. **(B, C)** Expression levels of succinate/IL-1β signal axis and NF-κB were tested by Western blots. Western blot data present mean ± SEM from three independent experiments at least. *P < 0.05; **P < 0.01; ***P < 0.001; ns means no statistical difference.

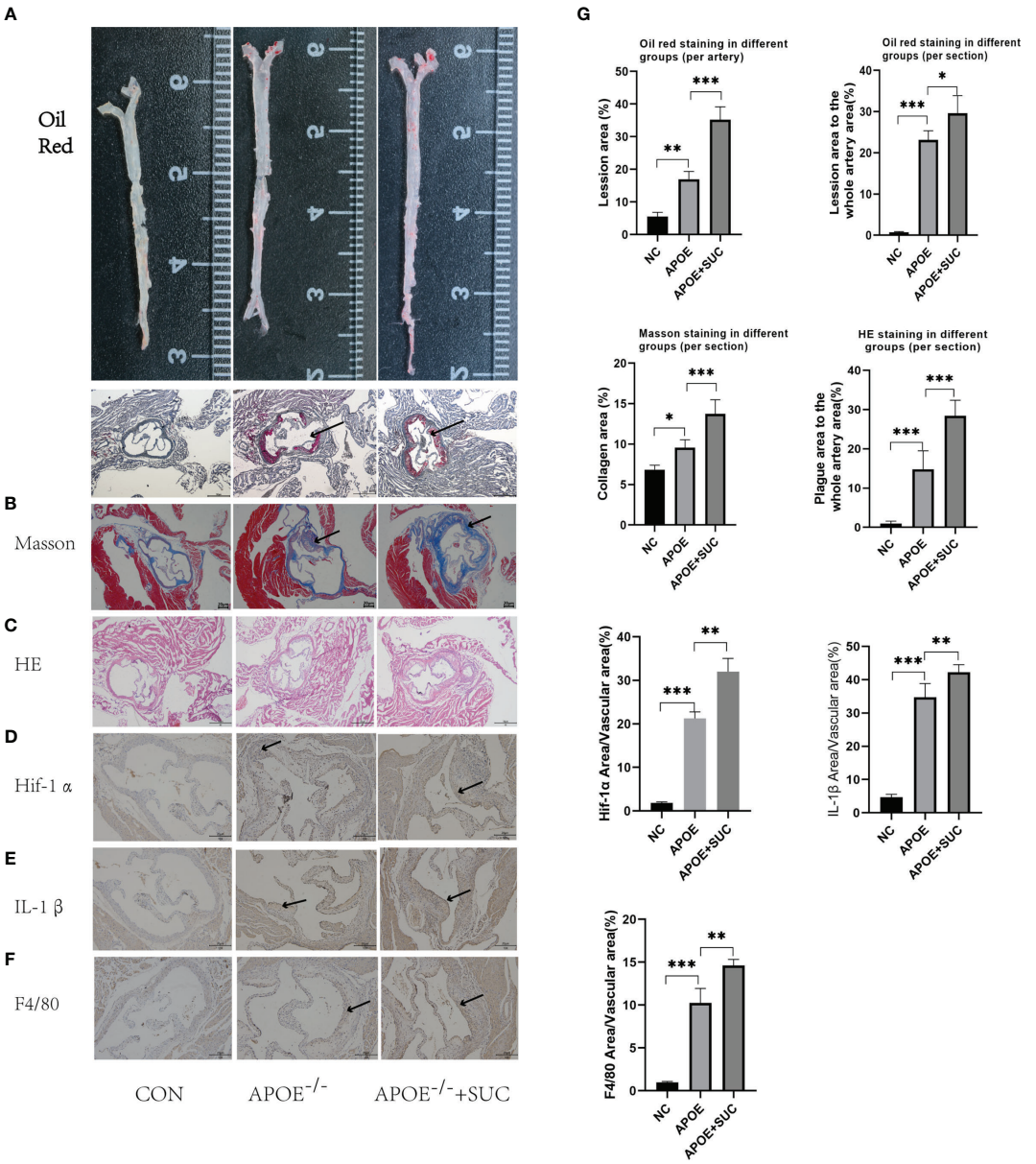

FIGURE 9 | Succinate/IL-1β signal axis in atherosclerosis model (stainings). **(A, B)** Representative images of Oil Red O staining of the whole aorta (n = 3, per group) and aortic roots (n = 6–9, per group). **(C)** Representative images of H&E staining aortic roots (n = 6–9, per group). **(D)** Representative images of Masson staining aortic roots (n = 3–5). **(E–G)** Representative images of Hif-1α, IL-1β, and F4/80 staining aortic roots (n = 5–9). The areas of lesion or target proteins were marked with black arrows. **(A–C)** Magnification ×40; **(D–F)** magnification ×100. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

protagonists (20, 21). During early atherogenesis, monocytes are recruited from the blood to the injured arterial vascular wall and locally differentiate into inflammatory macrophages (M1). Macrophages, inflammatory triggering cells, are capable of secreting considerable pro-inflammatory factors and stimulating the endothelium to impact the inflammatory response to amplify the inflammatory vascular injury (20). Since both types of cells exhibit pro-inflammatory functions, the sources of pro-inflammatory factors in existing studies could not be distinguished. Accordingly, how macrophages that activate endothelium causes a sustained inflammatory state in atherosclerosis remains unclear.

When macrophages are being activated, its energy metabolism will vary from oxidative phosphorylation to glycolysis concomitantly (22, 23). Succinate has been reported as a vital intermediate in glycolysis metabolism, which was demonstrated as a novel inflammatory signal in chronic inflammatory diseases [e.g., rheumatoid arthritis (3) as well as

Crohn's disease (12)]. Indeed, extracellular succinate increases because of local tissue ischemia or hypoxia (24), so both at the early stage of macrophage attachment to the endothelium and in the middle and late stages of plaque and thrombosis, excess succinate can be reasonably present in the pathological process of atherosclerosis.

This study first demonstrated that succinate significantly elevated in arterial serum in CHD patients compared with HC based on targeted detection, and the inflammatory marker IL-1β (2, 25) presented a consistent trend. Subsequently, as revealed by the Pearson correlation analysis, a positive relationship was found between the mentioned two materials. According to existing studies, succinate could induce IL-1β through Hif-1α in activated macrophages (19), whereas whether endothelial cell IL-1β is directly correlated with succinate increasing and whether it can act as a bridge between macrophages and endothelial cells to explain the internal mechanism of the enlarged inflammatory response should be explored in depth.

Succinate, an inflammatory signal, only has a conducting effect in the presence of Sucnr1. Sucnr1/GPR91 refers to a G protein-coupled cell surface sensor in terms of extracellular succinate (26–29), which is stably expressed on immature Dendritic cells (DCs) and macrophages in immune systems (28). As reported by the cell experiments, Sucnr1 could be stably expressed in HUVECs, thereby creating a pathway for uptaking external succinate. Subsequently, the data of this study indicated that exogenous succinate successfully activated Hif-1α and stabilized its nuclear expression in HUVECs, whereas the IL-1β level rose notably. However, when Hif-1α was disrupted, IL-1β production was significantly reduced in HUVECs. The mentioned results revealed that extracellular succinate alone led to a moderate induction of Hif-1α in HUVECs and significantly enhanced its transcription function, thereby ultimately potentiating IL-1β production.

Next, we demonstrated the necessity of Sucnr1 in this inflammatory signaling axis using an antagonist of Sucnr1 NF-56-EJ40. NF-56-EJ40 blocked succinate signal transduction and led to a reduction of Hif-1α production and transcription activity, thereby directly causing IL-1β production to decrease in HUVECs and macrophages. This result confirmed Sucnr1 as the only pathway that succinate mediated Hif-1α/IL-1β signal, and this study found the possibility of reducing the production of the inflammatory factor IL-1β by blocking Sucnr1.

However, the production of IL-1β is complicated, and NF-κB pathway is the most famous and classical one. Our previous results can only explain Hif-1α as a transcription factor that regulates pro-IL-1β production, whereas activated IL-1β should be generated in the presence of Caspase1, which serves as the major material of NF-κB pathway (1, 30, 31). We found that the downstream nodes of NF-κB pathway NLRP3 and Caspase-1 increased significantly in HUVECs after succinate stimulation, whereas the level of transcription factor NF-κB remained almost unchanged. Next, the NF-56-EJ40 intervention experiment displayed that NLRP3 and Caspase-1 notably decreased after the interruption of succinate transduction, while NF-κB was stable. The mentioned results suggested that succinate combined with Sucnr1 could activate NLRP3/Caspase-1 cascades, whereas it showed no correlation to their upstream NF-κB. Next, we further determined the position and origin of NLRP3/Caspase-1 in succinate signal axis. In the following study, the downregulation of Hif-1α *via* siRNA led to a decrease in NLRP3 production at baseline, thereby proving that Hif-1α is a transcription factor that could positively regulate NLRP3 gene synthesis. NLRP3 could activate and recruit adapters to form inflammasome complexes in response to inflammation or other imprints, then inflammasome joints to pro-caspase-1 (1, 30–32). Caspase-1 is activated by autoproteolysis and formation of the enzymatically active heterotetramer, and then it catalyzes pro-IL-1β cleavage to IL-1β (33–35).

All of the above data were based on the increased succinate in vascular microenvironment, so we cocultured HUVECs with activated macrophages to verify the existence of succinate/IL-1β signal axis in HUVECs. The results confirmed that in injured vessel microenvironment, macrophages were converted into M1 and attached to the vascular endothelium, thereby releasing considerable succinate with the transformation of energy metabolism. Succinate could bind with Sucnr1 entering HUVECs and then activate the transcription factor Hif-1α to localize to the nucleus, which could induce the production of pro-IL-1β and NLRP3. NLRP3 is the major of NLRP3 inflammasome, which could link to pro-caspase-1 and release active caspase-1. Lastly, active caspase-1 catalyzed IL-1β processing by exacerbating the inflammatory cycle (**Figure 10**).

Based on the mentioned results, we also confirmed the pro-inflammatory effect of succinate *in vivo*. In atherosclerosis, external succinate can trigger the succinate/IL-1β signaling axis and intensify the inflammatory response, which leads to the thickening of atherosclerotic plaques, the fibrosis of artery wall, and the narrowing of the lumen. In this pathological mechanism, succinate stimulation was also reported to enhance NLRP3 and caspase-1 expression, whereas NF-κB was not visibly affected. The mentioned results confirmed that glycolysis only stabilized the activity of transcription factor Hif-1α, whereas the expression of NF-κB was not affected. Second, glycolysis can affect the assembly of NLRP3 inflammasome through Hif-1α and ultimately regulate IL-1β production.

In brief, glycolytic metabolism already exists when macrophages recruit and attach to vascular endothelium at the early stage of atherosclerosis, accompanied by succinate production. On the one hand, succinate enters the circulation. On the other hand, it enters the endothelium and macrophages and starts succinate/IL-1β signal axis to produce considerable inflammatory factors, thereby exacerbating the inflammatory process of atherosclerosis. According to cell studies, NF-56-EJ40 has been reported as an effective, high-affinity, and significantly selective human Sucnr1 antagonist, capable of interrupting succinate/IL-1β signal axis in endothelium and macrophages for the suppression of inflammation responses. Our study provides a point that energy metabolism switch can initiate an immune response to affect the surrounding tissue to exacerbate an inflammatory response. Sucnr1 may act as a novel

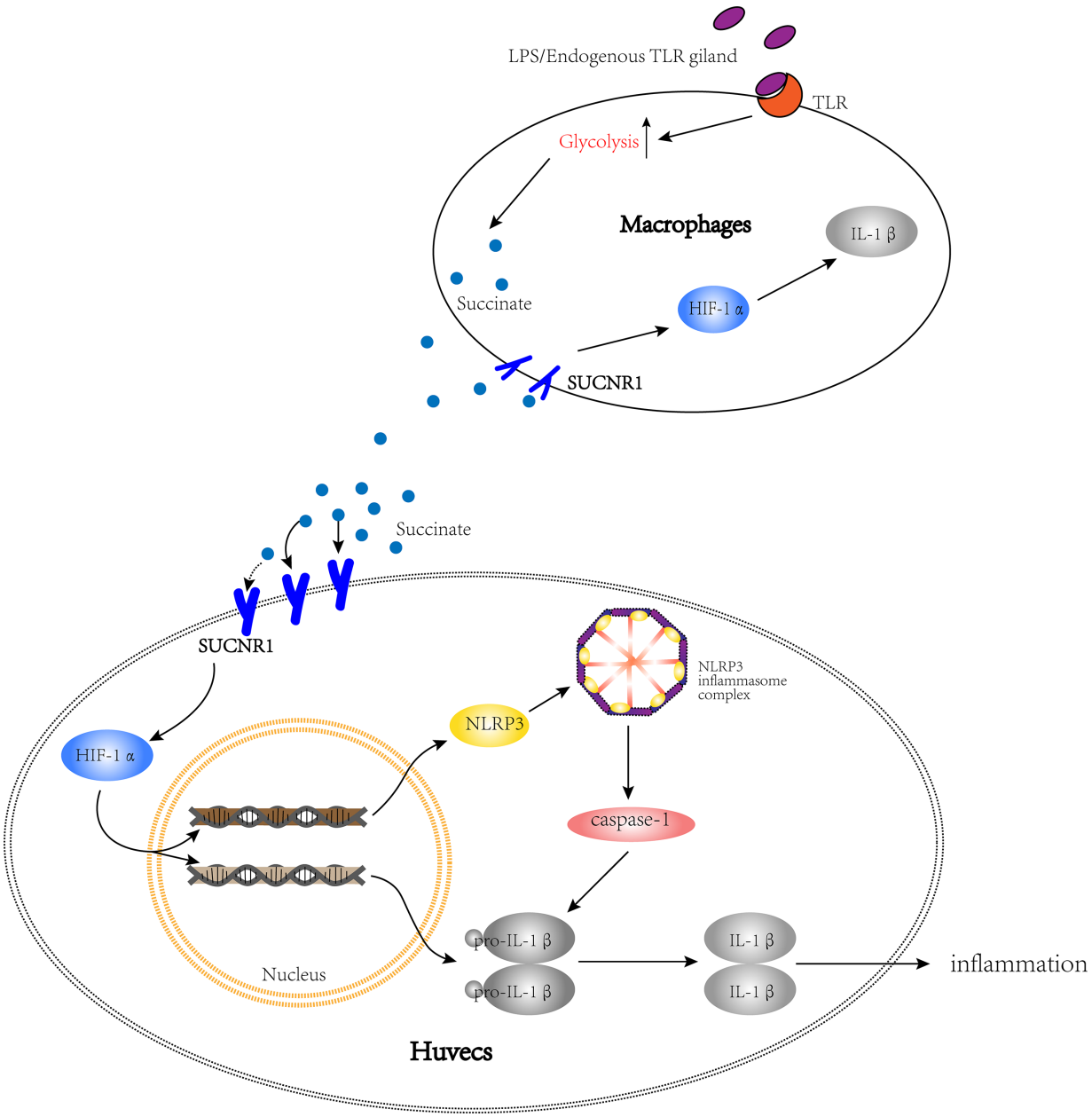

FIGURE 10 | The path diagram of succinate/IL-1β signal axis between HUVECs and macrophages. The bigger cell is HUVEC, while the smaller one is macrophage. This figure shows that after being activated, macrophages release succinate and stimulate the succinate/IL-1β signal axis in HUVECs to promote an inflammatory response.

target for cutting off succinate signal transduction to prevent the inflammatory process of atherosclerosis.

Limitations

Since frozen slices are rare, immunofluorescence and co-location analysis of Hif-1α and IL-1β were not performed. Otherwise, Sucnr1 antagonist (NF-56-EJ40) is currently only available in human cells, and Sucnr1$^{-/-}$/Apoe$^{-/-}$ mice were not created to prove the significance of Sucnr1 *in vivo*. Subsequently, the significance of this signal axis for atherosclerosis will be further explored in animal experiments.

DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paper are present in the paper and/or the **Supplementary Material**.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact YH.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics and Research Committee of the First Affiliated Hospital of Shandong First Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics and Research Committee of the First Affiliated Hospital of Shandong First Medical University.

AUTHOR CONTRIBUTIONS

JX and YH designed the research. JX performed most of the experiments and analyzed the data. YBZ, YQZ, YJZ, XW, HL, AZ, WW, and JW gave according guidance or support to the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.817572/full#supplementary-material

Supplementary Table 1 | Clinical parameter statistics.

Supplementary Figure 1 | Cell viability assay for drug intervention *via* CCK8.

Supplementary Figure 2 | Transfection efficiency of Hif-1α siRNA *via* rt-PCR.

Supplementary Figure 3 | Expression of representative M1 marker genes of THP-1 macrophage after 24 h of LPS polarization.

Supplementary Figure 4 | Raw data review incoming and outgoing emails.

Supplementary Figure 5 | Repeated experiment of caspase-1 in a blot.

Supplementary Figure 6 | Sucnr1 blots between DMSO and blank control.

Supplementary Figure 7 | En face staining of Aortic endothelium.

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MINI REVIEW
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Implication of Platelets in Immuno-Thrombosis and Thrombo-Inflammation

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In addition to their well-described hemostatic function, platelets are active participants in innate and adaptive immunity. Inflammation and immunity are closely related to changes in platelet reactions and enhanced platelet function in thrombo-inflammation, as well as in microbial and virus infections. A platelet's immune function is incompletely understood, but an important balance exists between its protective and pathogenic responses and its thrombotic and inflammatory functions. As the mediator of vascular homeostasis, platelets interact with neutrophils, bacteria and virus by expressing specific receptors and releasing granules, transferring RNA, and secreting mitochondria, which controls hemostasis and thrombosis, infection, and innate and adaptive immunity. This review focuses on the involvement of platelets during immuno-thrombosis and thrombo-inflammation.

Keywords: platelets, immuno-thrombosis, thrombo-inflammation, NETosis, extracellular vesicles (EVs)

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INTRODUCTION

Platelets are small (2–4 μm in diameter) anucleated cells derived from their megakaryocyte's precursors with 7–10 days lifespan. Nearly one trillion platelets sentinel the blood vessels to monitor and preserve the integrity of the vasculature. There is no nucleus in platelets, but they are prepacked with proteins and various forms of RNA from their precursor cells. When damage to blood vessels occurs, it triggers the formation of a thrombus to stop bleeding (1). The discoid shape changes to a spherical one, resulting in long filopodia that facilitate adhesion. In order for platelets to function, they must involve an array of adhesive and activation receptors, secreted granule reservoirs, and dynamic cytoskeletal proteins (2). Extracellular vesicles (microparticles) can also play a role in the formation of thrombus mediated by platelets, as they provide anionic phospholipids that aid in the coagulation process (3). The role of platelets is not restricted to the hemostatic/thrombotic response, but platelets play a crucial role in inflammatory and immune responses (4–8). Indeed, not only do platelets express an array of molecules serving wound repair, but they also bear immune and inflammatory molecules such as interleukin IL1 (9), and an array of receptors including toll-like-receptors (TLRs), CD154, or CD40L (4), Fc receptor for IgG (FcγRIIA) (10), IgA (FcαRI) and IgE (FcεRI) (11, 12).

In response to harmful pathogens, platelets contribute to the immune system either directly, by producing cytokines and antimicrobial peptides, or indirectly, through interactions with neutrophils, monocytes, lymphocytes, and other cells (13, 14). Immuno-thrombosis may negatively

affect hemostatic and immunological processes during a bacterial infection, resulting in adverse clinical outcomes (15). In this review, we will focus on the role of platelets in immuno-thrombosis and thrombo-inflammation.

PLATELET-ASSOCIATED IMMUNOPATHOLOGY: IMMUNO-THROMBOSIS AND THROMBO-INFLAMMATION

Mechanisms of Neutrophil Extracellular Trap-Induced Thrombosis

It is unsurprising that platelet-neutrophil interactions are greatly increased during inflammatory responses (16–19). For the most part, soluble mediators initiate these interactions, which directly activate these cells. Platelets and neutrophils co-incubated with septic patient plasma induced platelet adhesion to neutrophils mediated by the TLR4 receptor (20, 21).

Neutrophils are the most abundant subset of leukocytes in arterial thrombi from patients with myocardial infarction (22). Activated neutrophils express adhesion molecules belonging to the selectin and integrin families promoting platelets and the endothelium binding (23). As well, activated platelets express adhesion molecules on their surface membrane, such as P-selectin that mediates binding of platelets to its main receptor on neutrophils, P-selectin-glycoprotein-ligand-1 or PSGL-1 (24). Indeed, neutrophils and activated platelets can recruit each other to inflamed or injured tissues, thereby causing thrombo-inflammation (25). In this regard, we found that platelets can modulate neutrophil adhesion to the injured arterial wall and that both elements influence the degree of post-injury vasoconstriction in *in vivo* porcine model involving arterial injury by angioplasty (26). More recently, we have revealed that platelet activation and binding to neutrophils enhance the secretion of platelet MMP-2 via an adhesive interaction between P-selectin and PSGL-1, which contributes to increase platelet-neutrophil aggregation (27). Inhibition of platelet-leukocyte binding, using a recombinant PSGL-1 reduced restenosis (28) and prevented in in-stent restenosis via reduction of thrombo-inflammatory reactions (29).

Neutrophil extracellular traps (NETs) are composed of DNA, histones, and antimicrobial peptides, and they are produced as part of an antimicrobial mechanism, which is affected by immune/immune-related cells during NETosis (30). Indeed, NETosis appears to be associated with many inflammatory disorders, including infections, cancers, endothelial dysfunction, atherosclerosis, thrombosis, and ischemia (31, 32).

Neutrophil extracellular traps contribute to thrombosis through direct and indirect mechanisms. Although the vast majority of studies use NET components rather than intact NETs, the role of intact versus NET component in activating coagulation is controversial (33). In addition to their ability to promote thrombin formation, NETs were also known for providing a scaffold for pro-coagulant molecules such as VWF, fibrinogen, FXII, and tissue factor, as well as pro-coagulant extracellular vesicles TF-bearing EVs for instance (34–38).

Platelets can induce dysregulation of NET, resulting in tissue damage, hypercoagulability, and thrombosis (34). In addition, NETosis is well documented in its role in the pathogenesis of sepsis and ARDS, causing vascular tissue damage and spreading microthrombi that eventually cause multiorgan failure and death (39, 40).

The studies on NETosis provide growing evidence that NETosis is connected to inflammation, atherosclerosis, and atherothrombosis, as well as poor prognoses of ischemic/reperfusion injuries (41).

Mouse studies have examined the effects of NETosis and platelet aggregation on outcome after ischemia/reperfusion (42). Then, Cf-DNAs trigger DNA-platelet and DNA-platelet-granulocyte colonization. This form of NET and platelet aggravation leads to NETosis.

There are no doubts that NETs play a significant role in thrombosis and hemostasis (**Figure 1**). Both arterial and venous thrombosis are affected by NETs, and NETs are implicated in stenosis that regulate thrombosis in different ways (43, 44). Additionally, preventing the formation of NETs can reduce thrombogenicity, which could be useful in preventing thrombosis (44). A thorough understanding of how new therapeutic options targeting NETs affect thrombosis will require both preclinical and clinical trials.

Platelet Extracellular Vesicles

In view of the critical role for platelets in thrombosis, studying platelet function may provide novel biomarkers for arterial thrombosis (45). There are numerous platelet function tests available in clinical practice, but most commonly, aggregometry-based tests are carried out (46). The aggregometry method, however, provides information on platelet functionality in the presence of exogenous and soluble agonists, which does not represent *in vivo* platelet activation. Thus, so far results from platelet function tests *in vitro* have limited value as biomarkers of arterial thrombosis.

A biomolecule or metabolite associated with activated platelets may be able to provide information about arterial thrombosis (47). Platelet-derived extracellular vesicles (PEV) are among these biomarkers, covering platelet microparticles/microvesicles and exosomes (48).

A number of benefits attributed to platelets are likely mediated by platelet-derived extracellular vesicles (PEVs), which are small vesicles released from activated platelets. Indeed, the release of platelet α-granules have been considered as small vesicles, as revealed by electron microscopic analyses (49). Also known as microparticles, this release of vesicles from the platelet plasma membrane occurs as a result of the extrusion of the platelet cytomembrane structures (50). Later studies using electron microscopy confirmed the presence of the two types of PEVs: small vesicles with diameters of 80–200 nm, and larger vesicles with diameters of 400–600 nm, which retained procoagulant activity mediated by factor V-like activity and tissue factor (51).

Platelet-derived extracellular vesicle can serve as biomarkers for autoimmune diseases, cancer, cardiovascular diseases, and

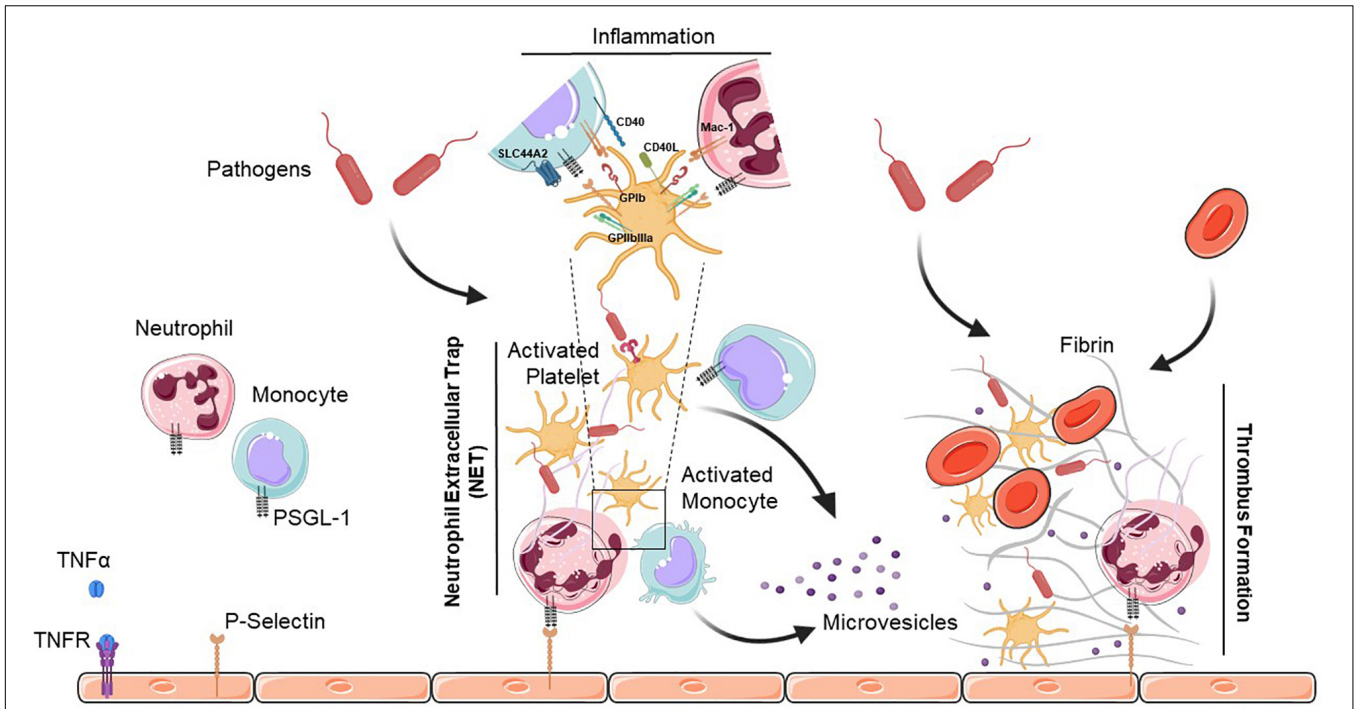

FIGURE 1 | Neutrophil extracellular trap (NET)-mediated platelet thrombosis. In the presence of pathogens, TNFα binds to its receptor (TNFR) to initiate diverse cellular responses, and platelets, neutrophils and monocytes collaborate to form NETs that are highly thrombotic and resistant to tissue plasminogen activator-mediated fibrinolysis. NETs also help to recruit platelets which support the immuno-thrombotic process by promoting fibrin generation along with endothelial cells. Activated platelets release large amounts of pro-inflammatory cytokines in platelet extracellular vesicles. Interactions between platelets and neutrophils or monocytes occur via direct contact between cell surface receptors, or by binding of secreted ligands. PSGL-1, P-selectin glycoprotein ligand-1; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; GPIb, glycoprotein Ib; SLC44A2, solute carrier family 44 member 2; CD40L, cluster of differentiation 40 lignad; CD40, cluster of differentiation 40; Mac-1, macrophage-1 antigen; GPIIbIIIa, glycoprotein IIb/IIIa.

infectious diseases (52–55). Rheumatoid arthritis patients have PEV detected in their synovial fluid (56), and increased levels of circulating PEV correlate with disease activity (57). Additionally, in murine models of atherosclerosis and autoimmune arthritis, PEV concentrations have been found to be increased in lymph (52, 58). As a result of systemic lupus erythematosus (SLE), PEV levels in blood can be increased. Higher levels of PEV have been associated with declining kidney function (59). Recently, an increase in PEV circulating in blood of COVID-19 patients has been observed (55, 60, 61).

Aside from transporting and producing inflammation-promoting mediators such as prostaglandins and leukotrienes, the PEV is also the transporter and producer of many lipid mediators (62).

RECEPTORS IN PLATELET–IMMUNE CELL INTERACTIONS

Platelets enhance leukocyte recruitment by expressing an arsenal of complement system receptors including cC1qR, gC1qR, C3aR, and C5aR, and storing complement proteins and regulators such as C3 and factor H. Platelets stimulate the classical and alternative pathways of complement, causing the accumulation of opsonin C3b as well as the release of anaphylatoxins C3a and C5a, which chemoattract innate immune cells (63, 64).

Among innate immune system receptors, TLRs are pattern recognition receptors that link various pathogen-shared molecules, including lipopolysaccharide (LPS), lipoproteins, and other bacterial wall constituents (65). As a consequence of expressing TLRs involved in the innate immunity response, platelets may contribute to the immune response and to infections by secreting a number of inflammatory mediators and pro-inflammatory factors (5, 66, 67).

Recent studies found significantly elevated levels of CXCL1, CXCL8, CXCL12, CCL2, CCL3, CCL5, EGF, VEGF, and PDGF-AB/BB in plasma and BAL fluid of patients with severe COVID-19, all of which can be found in platelet granules, indicating that platelets may play a role in hyperinflammation observed in patients with ARDS (68, 69). These factors mediate the inflammatory response by acting synergistically, through both autocrine and paracrine pathways.

There is a wealth of evidence suggesting that platelets modulate both the innate and adaptive immune systems. Platelets and platelet-derived microparticles are active in killing foreign pathogens in addition to directing a cascade of proteases and inhibitors to sites of vascular injury and inflammation (70). As well, secreted chemokines and cytokines (RANTES, IL-1β, and MCP-1) and platelet-expressed P-selectin promote leukocyte

recruitment, adhesion, and transmigration at sites of vascular injury (**Figure 1**).

A CD40L-expressing platelet is capable of not only increasing inflammatory responses in the endothelium, but also triggering antigen-presenting cells (dendritic cells and macrophages), resulting in enhanced antigen delivery to T lymphocytes. As demonstrated in CD40L deficient mice infected with viruses, CD40L on activated platelets also facilitates B cell differentiation and Ig class switching (71, 72). Also, patients with immune thrombocytopenia have reduced levels of regulatory T (T_{Reg}) cells, and therapeutic increases in platelet counts restore T_{Reg} cell numbers and functions in such individuals (73–75). The effect of TGFβ secreted by platelets in T_{Reg} formation is unclear, but since differentiation of T_{Reg} cells requires TGFβ, platelets may contribute to T_{Reg} formation.

PLATELETS IN BACTERIAL AND VIRAL INFECTIONS

In Bacterial Infections

A wide variety of bacteria can interact with platelets, including the *Staphylococci family*, *Neisseria gonorrhea*, *Porphyromonas gingivalis*, and *Helicobacter pylori* (76–78). GPIIb/IIIa, FcγRIIa, and IgG receptors on platelets help them adhere and aggregate around bacteria, as well as fibrinogen and fibronectin (79, 80). TLR1, 2, 4, 6, and 9 within platelets enable them to bind bacteria, which will either cause platelets to secrete thrombocidins (antibacterial proteins within platelet α-granule, including thrombocidin 1 and 2) or aggregate around bacteria to trap them for elimination by phagocytes (20, 81–84). Upon interacting with bacteria, platelets release antimicrobial compounds that are contained within their granules. Alpha toxin released by *S. Aureus* mediates the release of β-defensin from platelets, which is responsible for NET formation (85). Platelets binding to Gram-negative bacteria releases a PF4 with exposed heparin-like epitopes, increasing antibody binding to the surface of bacteria and possibly leading to neutrophil opsonization and phagocytosis (86, 87). Some Gram-negative bacteria, such as *Yersinia pestis*, are insensitive to mammalian TLR4s, making this mechanism crucial (88, 89). Based on *in vivo* experiments with another Gram-negative bacteria, *Porphyromonas gingivalis*, it has been shown that during infection, platelets interact with neutrophils forming heterotypic aggregates in a TLR2-dependant fashion and that TLR2 promotes the aggregation of platelets (90). These study findings indicate that platelets can trigger platelet thrombotic pathway and/or inflammatory pathway activation upon recognizing bacterial components (90).

In Viral Infections

On the other hand, platelets interact with various types of viruses and their phenotype may vary depending on the type of viral infection (91). Thrombocytopenia and even thrombosis can accompany viral infections (92). Viruses can be divided into those that have either DNA or RNA genomes. Furthermore, RNA viruses can be divided into double-stranded and single-stranded viruses. Several DNA viruses, such as the herpes simplex virus type 1 (HSV1), cytomegalovirus (CMV) and vaccinia, have been identified associated with platelets. However, it is unknown if these viruses can be internalized by platelets (93–95). In contrast, RNA viruses such as HIV, hepatitis C virus (HCV), dengue, influenza, CVB, and EMCV have smaller sizes and are easily internalized by platelets (96–101).

There was recently evidence that SARS-CoV-2, a single-stranded RNA virus, can increase platelet activity and formation of platelet-monocyte aggregates facilitated by TF expression on monocytes (55, 102–105). Koupenova et al. (106) reported that SARS-CoV-2 promotes programmed cell death in platelets in another aspect of platelet-SARS-CoV-2 interaction. According to this study, RNA sequence analysis for SARS-CoV-2 shown by ARTIC v3 sequencing, transmission electron microscopy and immunofluorescence showed that SARS-CoV-2 virions internalized when attached to microparticles. As a consequence of such internalization, apoptosis, necroptosis, and EV release occur, which contribute to impaired immunity and thrombosis (106).

Several studies suggest that platelets may associate with SARS-CoV-2 RNA molecules, and that this event may be more likely to occur in older patients (55, 105, 107). However, Bury et al. did not detect viral RNA in platelets from COVID-19 patients (108). The disparity could be due to the size of the cohort which is significantly larger in the studies that detected traces of viral RNA in platelets of some COVID-19 patients.

CONCLUSION

Anucleated megakaryocyte-derived platelets play an important role in hemostasis and thrombosis. Platelets serve as additional mediators of inflammation beyond hemostasis and contribute to several aspects of immune response, including priming of other immune cells and integration of extrinsic immunological stimuli. Indeed, platelets initiate innate immunity as well as adaptive immunity, which is beneficial for host defenses at certain stages of infection. The functions of platelets are thus diversified and rooted firmly in their interactions with vascular and circulating immune cells. Ultimately, uncontrolled endothelial damage and inflammation caused by viral infection progression can result in enhanced platelet reactions which amplify thrombosis and inflammation leading to higher cardiovascular, cerebral and lung pathologic events.

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YZ and YM wrote and edited the manuscript, read, and agreed to the published version of the manuscript.

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REVIEW
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Thrombosis and Inflammation—A Dynamic Interplay and the Role of Glycosaminoglycans and Activated Protein C

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Hemostasis, thrombosis, and inflammation are tightly interconnected processes which may give rise to thrombo-inflammation, involved in infectious and non-infectious acute and chronic diseases, including cardiovascular diseases (CVD). Traditionally, due to its hemostatic role, blood coagulation is isolated from the inflammation, and its critical contribution in the progressing CVD is underrated, until the full occlusion of a critical vessel occurs. Underlying vascular injury exposes extracellular matrix to deposit platelets and inflammatory cells. Platelets being key effector cells, bridge all the three key processes (hemostasis, thrombosis, and inflammation) associated with thrombo-inflammation. Under physiological conditions, platelets remain in an inert state despite the proximity to the endothelium and other cells which are decorated with glycosaminoglycan (GAG)-rich glycocalyx (GAGs). A pathological insult to the endothelium results in an imbalanced blood coagulation system hallmarked by increased thrombin generation due to losses of anticoagulant and cytoprotective mechanisms, i.e., the endothelial GAGs enhancing antithrombin, tissue factor pathway-inhibitor (TFPI) and thrombomodulin-protein C system. Moreover, the loss of GAGs promotes the release of mediators, such as von Willebrand factor (VWF), platelet factor 4 (PF4), and P-selectin, both locally on vascular surfaces and to circulation, further enhancing the adhesion of platelets to the affected sites. Platelet-neutrophil interaction and formation of neutrophil extracellular traps foster thrombo-inflammatory mechanisms exacerbating the cardiovascular disease course. Therefore, therapies which not only target the clotting mechanisms but simultaneously or independently convey potent cytoprotective effects hemming the inflammatory mechanisms are expected to provide clinical benefits. In this regard, we review the cytoprotective protease activated protein C (aPC) and its strong anti-inflammatory effects thereby preventing the ensuing thrombotic complications in CVD. Furthermore, restoring GAG-like vasculo-protection, such as providing heparin-proteoglycan mimetics to improve

regulation of platelet and coagulation activity and to suppress of endothelial perturbance and leukocyte-derived pro-inflammatory cytokines, may provide a path to alleviate thrombo-inflammatory disorders in the future. The vascular tissue-modeled heparin proteoglycan mimic, antiplatelet and anticoagulant compound (APAC), dual antiplatelet and anticoagulant, is an injury-targeting and locally acting arterial antithrombotic which downplays collagen- and thrombin-induced and complement-induced activation and protects from organ injury.

Keywords: thrombo-inflammation, neutrophil extracellular traps (NETs), activated protein C (aPC), glycosamine glycans, platelet-neutrophil complexes, platelet activation

INTRODUCTION

Cardiovascular disease (CVD) is the foremost cause of death worldwide, accounting for estimated 17.9 million deaths each year (1). CVDs are a group of disorders that mainly affect heart and blood vessels. CVDs include acute atherothrombotic complications, i.e., myocardial infarction (MI) and ischemic stroke, as well as venous thromboembolic (VTE) disease. Atherosclerosis is the primary underlying disease process driven by lipid accumulation in the arterial wall, persistent inflammation, and vascular endothelial dysfunction (2–4). As it progresses, plaque rupture can occur, to expose blood with the subendothelial matrix. The deliberated plaque content creates an imbalance between the pro- and anticoagulant homeostasis which causes formation of occlusive thrombi (2). Alongside, these thrombotic events not only cause an increased risk for myocardial ischemia and stroke, but they also trigger an interplay between platelets and innate immune cells thereby promoting mechanisms of sterile inflammation. Inflammation and thrombosis are therefore central pathological processes involved in atherosclerosis and associated vascular complications.

Both inflammation and thrombosis in CVD have commonly been investigated independently but been recently integrated within the new concept of vascular thrombo-inflammation (5). In the following sections, we will first outline the thrombotic mechanisms of endothelial damage followed by events leading to activation of inflammatory cardiovascular complications. Finally, we will introduce cytoprotective functions of both conventional and novel therapies, which are known to inhibit to coagulation system, but convey an anti-inflammatory effect independently or aside of blood clotting. One such novel strategy is the provision of heparin proteoglycan-like mimetics, antiplatelet and anticoagilant compound (APAC) (6).

THE INTERPLAY BETWEEN PLATELETS, COAGULATION REGULATORS AND INFLAMMATION

The Interaction Between Platelets and Endothelium

Under physiological conditions, inert platelets circulate unnoticed by other blood cells and vascular endothelium (7). The negatively charged proteoglycans, glycoproteins and glycolipids, together glycocalyx, decorate surfaces of both blood cells and luminal vessel wall and are critical to maintain a quiescent state (8). The protective glycocalyx engages dynamic interaction with its environment, resulting in spatial and organ-specific differences. Majority of the endothelial glycocalyx constituents are protein-bound glycosaminoglycans (GAG), composed of mainly heparan sulfate as well as chondroitin -and dermatan sulfate and non-protein bound hyaluronic acids (9). Endothelial cells carry several mechanisms to regulate and localize the injury, including platelet-inhibitory ectoADPases (CD39), nitric oxide and prostacyclin (10, 11). These mediators not only inhibit platelets, but also tune the vasoactivity and protect from vasoconstriction, local reactions modulating shear rates.

Platelets are the first blood cells to respond to vascular injury, regardless of the cause of damage; intervention, or disease state (12, 13). Injury causes the loss, shedding or alteration of the balanced glycocalyx structure and, thus, exposure of underlying adhesive proteins to interact with blood (**Figure 1**) (14–16). Platelets adhere to injury-exposed subendothelial matrix components, where selected binding depends on the shear rate of the flowing blood (7). Under arterial and microvascular shear rates platelets are first arrested from the blood flow by interaction of glycoprotein receptor (GP) Ib-V-IX and von Willebrand factor (VWF) and tightened by GPIIb/IIIa, engaging, also, fibrinogen and fibrin while the blood flow is altered by the growing thrombi (**Figures 1**, **2**). Also, inflammatory cells roll and tether on the inflamed endothelium and bind to fibrin via their αMβ2 (CD11b/18) integrin receptors (**Figure 3**) (17).

Permanent platelet adhesion is secured by the early binding to matrix proteins, mainly collagen, fibronectin, and laminin via GPIa/IIa, GPVI and integrin α6β1 receptors, respectively (18, 19). In turn, under venous shear rates, platelets may bind directly to the forming fibrin, once the natural anticoagulation, provided by antithrombin, thrombomodulin – protein C and protein S as well as tissue factor pathway inhibitor (TFPI), starts to fail. The subsequent activation of platelets is enhanced by thrombin, and platelet-and red cell-released adenosine diphosphate (ADP), and enzymatic generation of thromboxane A_2 from arachidonic acid (18, 20–24). Recruitment of coagulation factors to foster thrombin formation, platelet aggregation and contraction, as well as fibrin mesh are all stabilizing the platelet plug at the vascular injury site. In addition to their role in coagulation, versatile

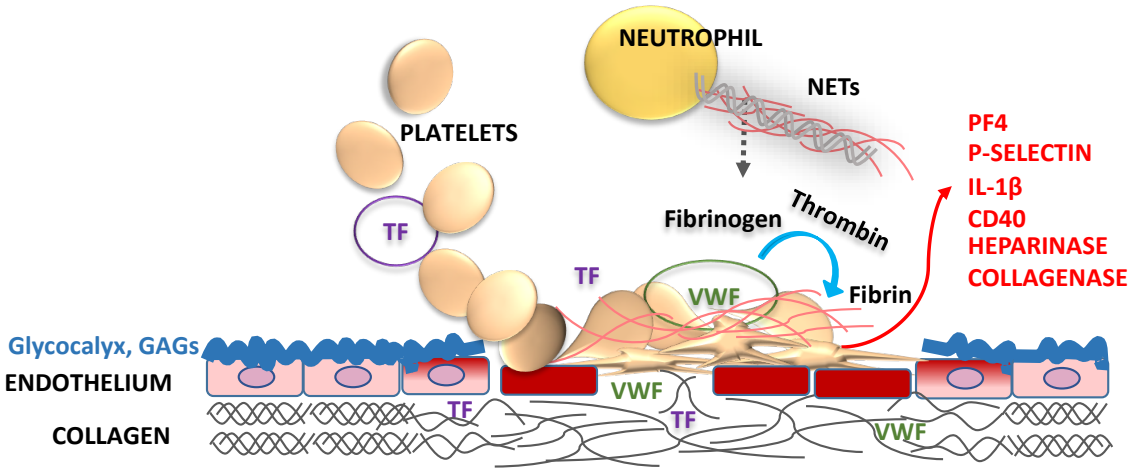

FIGURE 1 | Role of endothelial glycocalyx and platelets in vascular injury. Glycocalyx lines the vascular endothelial cell surface facing the vessel lumen forming a barrier between blood and endothelium maintaining the steady state, but also protects vasculature from pathogens. Glycocalyx supports anticoagulation by enhancing the natural anticoagulants antithrombin (AT), tissue factor pathway inhibitor (TFPI), protein C and S. Disruption of the glycocalyx exposes endothelial cell adhesion molecules [endothelial cell intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin] and extracellular matrix components, i.e., von Willebrand factor (VWF), collagen and tissue factor (TF) in a deeper injury (smooth muscle cells, adventitia). These structures capture blood cells, activating the crosstalk between the inflammatory cells and the coagulation system. Activated platelets release heparinase, metalloproteinases and collagenase to progress the local injury. Activation of platelets feeds back further expression of procoagulant P-selectin, platelet factor 4 (PF4), interleukin-1β (IL-1β), and CD40 ligand to foster the thrombo-inflammatory interactions. Enhanced activation of platelets and leukocytes induces neutrophil extracellular traps (NETs) formation, which in turn activates both inflammatory and hemostatic arms. Vascular endothelial injury and related procoagulant and inflammatory activities may be downplayed by replacing the defective glycocalyx structure with glycosaminoglycan (GAG) moieties, such as APAC.

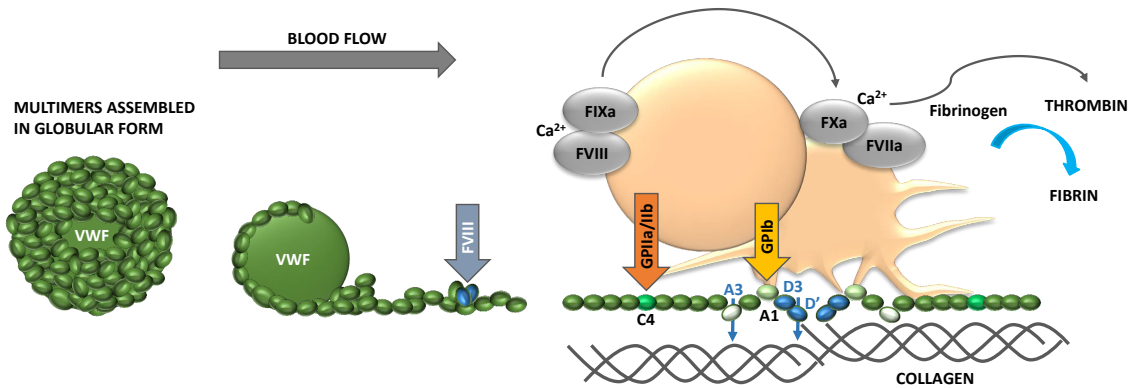

FIGURE 2 | Properties of von Willebrand factor (VWF). VWF obtains globular conformation under static conditions, while it unfolds and elongates under blood flow. The higher the hematocrit, the faster the flow conditions and the smaller the vessel lumen (vasoconstriction), the higher the shear forces and contribution of adhesive platelets. VWF multimer size is controlled by ADAMTS13. While unfolding VWF exposes domains with binding sites for several extracellular proteins, including collagen, and glycosaminoglycans (GAG), and platelet glycoprotein (GP) Ib and GPIIb/IIIa to foster platelet adhesion and activation. VWF carries coagulation factor VIII (FVIII) and liberates it to the tenase complex on platelet surface to support generation of thrombin and formation of fibrin. VWF binds to fibrin as well. The multiple coordinated actions of VWF in platelet recruitment and coagulation pathway are critical in microvascular thrombosis, including thrombo-inflammatory settings.

platelets contribute widely to inflammation and immunity (12, 25, 26). Inflammation-associated endothelial damage underlies several cardiovascular, hematological, and kidney complications: ischemia reperfusion injuries (IRI), sepsis, thrombo-inflammation, and microangiopathy causing organ hypoxia. In response to hypoxia, endothelial cells will release further adhesive components, i.e., VWF/FVIII to the vascular surface and circulation (27) maintaining pro-inflammatory and thrombotic conditions (**Figures 1**, **2**). Damage to endothelium is associated with shedding of heparan sulfate from glycocalyx due to heparinases, which are released by activated platelets and provided by several bacteria.

Thus, functional endothelium and its glycocalyx and other regulatory capacities keep the platelet, coagulation and inflammatory response localized and finely balanced to prevent thrombosis from spreading.

Damage to the endothelium results in release of mediators both locally on vascular surfaces and to circulation, further enhancing platelet adhesion to the affected sites, especially in microcirculation (**Figures 1**, **2**). These key mediators being both

platelet- and endothelium-derived, include VWF, Platelet Factor 4 (PF4, CXCL4) and P-selectin (28). These mediators interact with neutrophils at the injury site to arrest them from blood flow to clear up the inflammatory plaque (**Figure 3**). In addition to its role in platelet adhesion and aggregation, and fibrin binding, VWF also mediates extravasation of leukocytes, an important element of inflammation with tissue injury (29). In this section, we will outline the contributions of VWF, PF4 and P-selectin, for thrombo-inflammatory pathways.

Von Willebrand Factor

Von Willebrand factor obtains globular conformation under static conditions to unfold under active blood flow (**Figure 2**) (30). This is relevant to the bench studies which overlook blood flow. The largest multimers are released from activated platelets, and in case the size regulating ADAMTS13 enzyme is deficient, such as occurs in thrombotic thrombocytopenic purpura (TTP) (31). Sticky VWF binds to several extracellular proteins, including collagen, and GAG structures and to platelet GP Ib and IIb/IIIa to foster platelet adhesion and activation and to resist blood flow forces. This VWF-platelet interaction is highly relevant under high blood flow conditions, including microcirculation and stenosed larger arteries. The larger the multimers, the higher the hematocrit, the faster the flow conditions and the smaller the vessel diameter, the higher are the physical shear rates and the more platelets deposit on VWF (19).

Von Willebrand factor also carries coagulation factor FVIII to platelet surfaces to support generation of thrombin and formation of fibrin, where VWF also binds to. The multiple coordinated actions of VWF in platelet recruitment and coagulation pathway are critical in microvascular thrombosis, including thrombo-inflammatory settings (32). Due to neutrophil-induced oxidation VWF cleavage by ADAMTS13 is impaired, creating large multimers which deposit platelets on micro-vasculature and cause organ damage and thrombocytopenia (**Figure 1**). VWF is also involved in mediating extravasation of leukocytes (33). One example of infection is adenovirus-induced endothelial activation which leads to the above mediator release and platelet activation, and subsequent binding of virus to platelets causes their clearance and thrombocytopenia (34).

Platelet Factor 4

Platelet factor 4 is the early platelet-released chemoattractant for neutrophils, but also for monocytes and fibroblasts (35). Upon activation, platelets release procoagulant PF4, which neutralizes negatively charged heparin-like GAGs and their anticoagulant potential, including the glycocalyx, and the therapeutic unfractionated heparin (UFH), but less so the lower molecular weight species (LMWH, low-molecular weight heparin).

As a chemokine, PF4 has a well-established role in inflammation, but it also poses an immunological epitope together with polyanionic structures, including DNA or GAGs (36). PF4-polyanion interactions trigger formation of antibodies of IgG4 class to induce prothrombotic heparin-induced thrombocytopenia (HIT), and recently discovered adenoviral vectored vaccine-induced thrombocytopenic and severe thrombotic syndrome (VITT), without exogenous heparin exposure (37).

Platelet factor 4 together with P-selectin provides a signal for leukocyte rolling on endothelial surfaces and serves as the early ligand for platelet-neutrophil interactions (**Figure 3**). Moreover, PF4 triggers vascular smooth muscle cell proliferation, which upon regulation is involved in vascular healing, however, when escaping from its regulation by, e.g., GAGs, PF4 will turn into a harmful player inducing proliferation of arterial wall, introduction of pulmonary hypertension, as one devastating example (38).

In this regard, therapeutic heparin has several anti-inflammatory properties, and when administered early to patients with infection, i.e., SARS-CoV-2 lately at treatment doses, even without visible venous thromboembolism, heparin is lifesaving. Heparin been shown to reduce not only early PF4-related activities, but also P-selectin-mediated leukocyte recruitment under blood flow, and further even cancer metastasis has been shown to be attenuated by a background of P-selectin deficiency or by treatment with heparin (39).

P-Selectin

CD62P, P-Selectin is a thrombo-inflammatory molecule which is exposed on the platelet and endothelial surface upon activation (**Figures 3**, **4**). It is therefore a key molecule that further promotes activation and aggregation of platelets. It is not solely an adhesive molecule, its binding to the P-selectin glycoprotein ligand-1 (PSGL-1) induces platelet activation and enhances aggregation (40). P-selectin blocking antibodies are therefore helpful in preventing both venous thrombosis and vessel wall inflammation (41). In addition, also heparan sulfate, heparin proteoglycans and UFH inhibit P- and L-selectin binding with ability to interfere with PSGL-1 (42). Importantly, the receptor PSGL-1 is widely expressed by leukocytes. Upon thrombo-inflammation, platelets can therefore influence leukocyte migration and activation. Interestingly, neutrophils abundantly express PSGL-1 and search for P-selectin expressing platelets and to coordinate thrombo-inflammation. Such platelet-neutrophil complexes promote the expression of endothelial intercellular adhesion molecule 1 (ICAM-1), which is necessary for neutrophil extravasation into the organ (43). These processes have been explained in several organ and disease systems, i.e., pulmonary infection, renal IRI or bacterial infection in the liver (44–46). An interaction, which was initially thought to promote phagocytic clearance of bacteria, is increasingly known as formation of neutrophil extracellular traps (NETs), which will be discussed next.

NEUTROPHIL EXTRACELLULAR TRAPS AT THE CROSS-ROADS OF THROMBO-INFLAMMATION IN CARDIOVASCULAR DISEASES

An interaction of platelets with immune cells promotes the onset of inflammatory processes (**Figures 3**, **4**). In infectious diseases, neutrophils upon activation result in formation of neutrophil

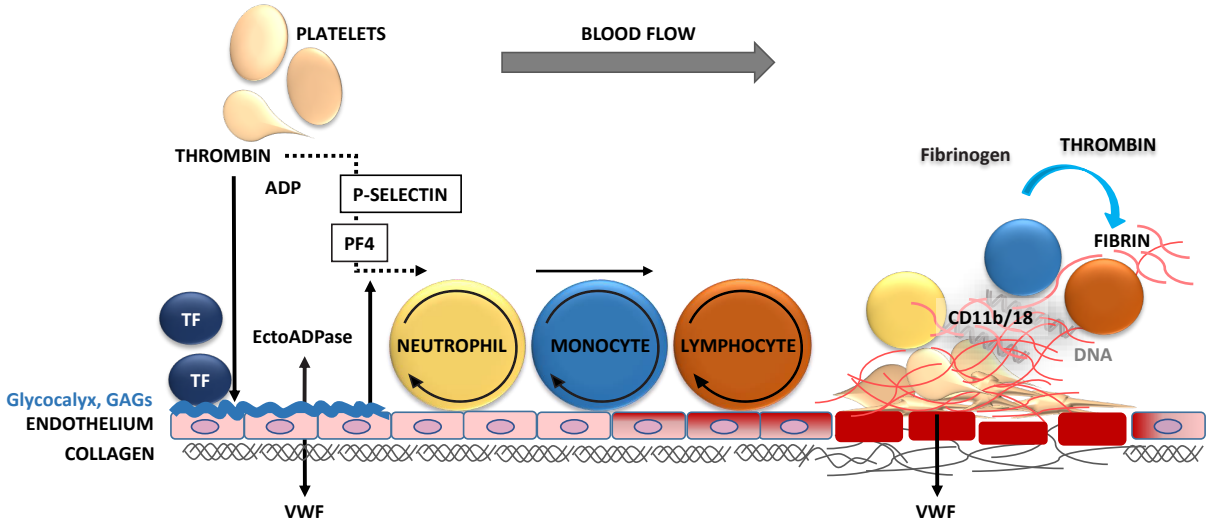

FIGURE 3 | Inflammation-triggered coagulation and thrombosis in vasculature. Upon mechanical injury or systemic inflammation of the vessel wall, activated endothelial cells and monocytes release tissue factor (TF). TF induces the generation of activated forms of coagulation factors FVII, FX, and thrombin, leading to fibrin formation. Thrombin induces the release of P-selectin, which binds to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils, monocytes and lymphocytes mediating their tethering and rolling on to the exposed endothelium. The release of procoagulant platelet factor 4 (PF4) and von Willebrand factor (VWF) promote the recruitment, adhesion, and activation of platelets and leukocytes at the injury site. Thrombin cleaves fibrinogen to form fibrin, which stabilizes thrombosis. The firm adhesion of leukocytes to fibrin occurs via CD11b/18 αMβ2, macrophage-1 antigen (MAC-1) or complement receptor 3 (CR3).

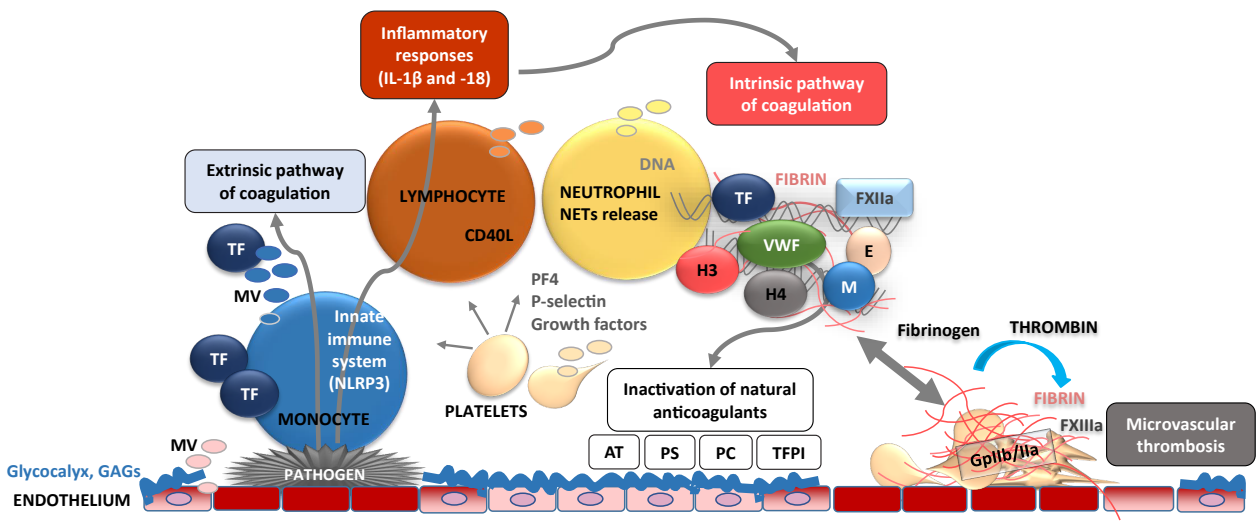

FIGURE 4 | Dynamic cellular and molecular interplay underlying thrombo-inflammation. Pathogen- or danger-signal mediated activation of endothelial cells at the site of disrupted glycocalyx results in adhesion of immune cells (e.g., monocytes) on the cell surface. Activated endothelial and immune cells release microvesicles (MV) bearing, e.g., tissue factor (TF) which induces activation of extrinsic coagulation pathway. TF is also capable to activate NLRP3 inflammasome [subsequent activation of interleukin (IL)-1β, and IL-18] and inflammatory response. Furthermore, endothelial cell activation results in adhesion of platelets which act as chemo-attractants (e.g., via P-selectin) to neutrophils. As consequence, neutrophil activation results in formation and release of neutrophil extracellular traps (NETs) composed of several pro-inflammatory molecules, neutrophile elastase (E), myeloperoxidase (M), histones (H3 and 4), which perpetuate endothelial dysfunction. NETs activate coagulation FXII and the intrinsic pathway of coagulation. Subsequent enhanced fibrin formation leads to the capture and kill of pathogens in fibrin-strengthened NETs. Procoagulant platelets also release platelet factor 4 (PF4) which inactivates glycosaminoglycans (GAGs) such as heparin. On the other hand, these thrombo-inflammatory mechanisms are accompanied by inactivation of cytoprotective anticoagulants such as activated protein C, antithrombin (AT), tissue factor pathway inhibitor (TFPI), and protein S.

extracellular traps (NETs) to sequester or kill the pathogens (13). As outlined in the previous section, platelet activation and surface expression of P-selectin and secretion of PF4 enable platelets to bind to neutrophils, which result in their activation and further recruitment to sites of tissue damage. This will initiate the formation of neutrophil extracellular traps (NETs).

However, NET formation is not limited to infectious diseases but plays a role in both acute as well as chronic sterile inflammatory diseases such as atherosclerosis, diabetes and kidney diseases (47). An exacerbated release of NETs has both pro-thrombotic and pro-inflammatory effects and induces endothelial dysfunction. Platelets are a part of NETs and are major contributors to acute innate inflammation. Platelets express pattern recognition receptors, resulting in their activation upon contact with danger-associated molecule patterns. Interactions between neutrophils and platelets trigger and accelerate NET formation, as well as thrombosis due to aggregation of platelets. In addition, abnormal activation of neutrophils may lead to endothelial damage during autoimmune or exaggerated inflammatory responses by releasing neutrophil serine proteases into the circulation, which activate specific cell surface receptors (48, 49).

Neutrophil extracellular traps are present within human and mouse atherosclerotic lesions (50). Their presence next to the apoptotic endothelial and smooth muscle cells within the plaques imply that they contribute to plaque disruption (51). Another study showed that neutrophils and NETs localized in all types of complicated lesions, without differences between ruptures, erosions, and intraplaque hemorrhages (52). On the other hand, NETs were not present within intact plaques, but they were numerous within adjacent perivascular tissue of complicated plaques. Despite an association and experimental evidence for the involvement of NETs in atherothrombosis, the mechanisms whereby they are either a cause or a consequence of plaque instability remain to be shown (52).

In further studies, mitochondrial oxidative stress has been associated with NETs and lesion size. Thus, a causal link was identified between endogenous neutrophil mitochondrial oxidative stress level with NETosis and atherosclerotic lesions in aged mice (53). However, these studies do not identify an association with thrombo-inflammatory mechanisms and NET formation. On the other hand, several studies show that NETs are capable of aggravating thrombotic complications of atherosclerotic plaques, including plaque disruption (54). Furthermore, plasma levels of myeloperoxidase (MPO) and MPO-DNA complex correlate with a risk of coronary artery disease and other major adverse cardiac events suggesting that NETs, and associated biomarkers can be used to predict a risk for atherosclerotic disease burden and events (54, 55).

Neutrophil extracellular traps not only promote thrombin generation, but also possess prothrombotic molecules which include tissue factor (TF), FXII, histones H3 and H4, and fibrin(ogen) (56). Therefore, they play a major role in arterial as well as venous thrombosis. An interaction of neutrophils and platelets at the site of plaque rupture promotes NET formation, thereby increasing TF abundance and prothrombotic events. Neutrophil-mediated platelet aggregation via integrins (α9β1) promotes arterial thrombosis. Vice versa, platelets promote NETosis. As outlined above, P-selectin is an important mediator which along with other adhesion molecules mutually mediates the interaction between platelets and neutrophils. Accordingly, beyond the beneficial actions of routine heparin use, P-selectin blocking antibody inclacumab has been proven to be beneficial in non-STEMI patients in preventing myocardial damage. Similarly, inhibition of NETs by PAD4 deletion has been shown to abrogate NET associated atherosclerosis burden and inflammatory response (57). Furthermore, PAD4 dependent NETosis is associated with plaque rupture and erosion (51). These observations suggest that NETs participate in thrombotic complications of atherosclerosis. Furthermore, studies in mouse models show that NETs impair endothelial cell survival under such conditions (58). Taken together, an interplay between neutrophils, platelets, and hemostatic factors are important mediator of the pathophysiology of cardiovascular diseases (**Figures 3**, **4**).

Simultaneously with promoting activation of the hemostatic system, NETs also contribute to activation of inflammatory pathways enhancing the atherothrombotic processes. NET-mediated activation of the inflammasome can amplify the inflammatory response through a feed-forward loop. The inflammasome stimulation triggers synthesis and release of interleukin (IL)-18 and IL-1β, which in turn enhance NET formation (59). NLRP3 inflammasome-associated activation of IL-1β and IL-18 is identified as an essential pathogenic mechanism in CVD and its inhibition reduces IL-6 synthesis (60, 61), in addition to that of fibrinogen. Also, the association with inflammasome, neutrophils possess armaments which include reactive oxygen species (ROS), lipid mediators (e.g., eicosanoids) as well as granular proteins such as alarmins (e.g., cathelicidins, defensins), MPO and serine proteases. These enzymes bind to NETs and promote/support local inflammatory functions at the site of NET release. Mechanistic studies on how these granular proteins, when conjugated with NETs, modify organ function are not yet reported.

Neutrophil Extracellular Traps and Glycosaminoglycans

Glycosaminoglycans and sialylated glycans play an important role in cellular signaling and immunological events. Regarding neutrophils and NETs, it has been observed that glycophorin A, a glycoprotein on erythrocyte, acts as a natural inhibitor of neutrophil activation in circulation (62). Thus, neutrophils are more susceptible to undergo NETosis under isolated cell culture conditions. Besides sialoglycoproteins, and neutrophil activation, NET formation is largely dependent on heparin sulfate. In this regard, heparin, a potent anticoagulant, is known to inhibit neutrophil elastase activity (63). In this study, sulfation was required for inhibition of neutrophil aggregation and elastase activity, since a non-sulfated GAG, hyaluronic acid and neutral dextran, were unable to support the elastase -induced inhibition of neutrophils (63).

Besides inhibiting elastase activity, heparin is able to destabilize histones and destroy the NET scaffold, thereby preventing thrombus formation (64, 65). Endothelial GAGs support the chemotaxis of neutrophils (66). Furthermore, GAGs on the surface of neutrophils are known to synergistically act with GAGs on the inflamed tissues mediating their migration. This indicates that GAGs or GAG-mimetics are a promising

therapeutic approach to prevent neutrophil-mediated thrombo-inflammatory effects. However, a direct evidence of GAG-dependent neutrophil activation and NETs in CVD remains to be evaluated.

ACTIVATED PROTEIN C AND THROMBO-INFLAMMATION IN CARDIOVASCULAR DISEASES

In addition to procoagulant function, thrombin is also an integral component of thrombomodulin, protein C (TM-PC) system. Protein C is serine protease zymogen that is synthesized in the liver, circulates in the plasma and has a high affinity to endothelial protein C receptor (EPCR). Activation of protein C (aPC) is mediated by the thrombin and TM (thrombomodulin) complex on the endothelial surface and is, therefore, thrombin-dependent at its moderate concentrations (67). aPC is well known for its anticoagulant and cytoprotective functions (68–70). The anti-coagulant activity of aPC is dependent on its ability to inactivate FVa and FVIIIa, thereby inhibiting thrombin generation (71–73). Furthermore, aPC confers cytoprotective signaling largely via protease activated receptor 1 (PAR1) on endothelial cells (**Figure 5**). Interestingly, other PAR receptors in combination of cellular receptors (e.g., integrins) have also been shown to contribute to cytoprotective mechanisms of aPC.

Plasma aPC levels decline and are inversely linked with clinical severity of coronary artery atherosclerosis and with cardiac ischemic injury in patients (74). Likewise, TM and the endothelial cell protein C receptor are downregulated on endothelial cells overlying the atherosclerotic plaque in coronary arteries (75). Several preclinical studies have shown cardio-protective effects of aPC (76–79). aPC restricts cardiomyocyte cell death in myocardial IRI, a hallmark of thrombo-inflammation (77, 78).

These protective effects were dependent on PAR-1 and were paralleled by an anti-inflammatory effect based on lowered IL-6 levels and leukocyte infiltration (78) (**Figure 5**). Furthermore, to mediate these anti-inflammatory, cardioprotective effects in cardiac IRI, independent of its anticoagulant activities aPC stimulates AMPK signaling but inhibits NF-κB and JNK signaling (79). The generation of signaling competent aPC variants (e.g., 3K3A, PC-2Cys) which do not interfere with the hemostatic activities has enabled to strengthen these cytoprotective effects of aPC in CVD (79–81). Moreover, the non-anticoagulant variants 3K3A-aPC and PC-2Cys, but not the non-signaling aPC-E170A mutant, restricted induction of pro-inflammatory TNF-α and IL-6 following myocardial IRI (79, 81). Interestingly, aPC conveys autophagic activities through AMPK driven metabolic changes thereby mediating cardio-protection in IRI (82, 83). Congruent to these observations, aPC administration limited myocardial IRI-triggered NLRP3 inflammasome activation via an AMPK -driven mechanism involving restricting of rapamycin kinase complex 1 (TORC1) signaling and hexokinase 1 (**Figure 5**). The protective effect of aPC was mimicked by 3K3A-aPC or parmodulin-2, a biased PAR-1 modulator (81).

Compatibly, aPC was shown to limit hypoxia reoxygenation (H/R)-induced NLRP3 inflammasome activation in neonatal murine cardiomyocytes and cardio-fibroblasts, the vital cells of the heart (81). These studies established that aPC is an endogenous negative regulator of NLRP3 inflammasome activation following IRI, uncovering a new anti-inflammatory mechanism of aPC. In our recent follow-up studies, we have investigated the transcriptomic profile of aPC treatment in murine myocardial IRI in comparison to direct oral anticoagulants FXa inhibitor (FXai, rivaroxaban) and FIIa inhibitor (FIIai, dabigatran). In this regard, the dosing regimens for both anticoagulants were experimentally determined to provide comparable anticoagulant effects and the infarct sizes (84). The results from our study show that the gene expression profile of aPC-treated mice resembled that of mice treated with FXa inhibitor (FXai, rivaroxaban) (84). On the contrary, mice treated with FIIa inhibitor (FIIai, dabigatran) had a markedly different gene expression profile compared to FXai or aPC treated mice. Alike aPC, FXai prevented the NLRP3 inflammasome activation following IRI. These protective anti-inflammatory effects of FXai, depended on aPC generation and were lost following endogenous aPC blockade. While both FXai and FIIai are potent anticoagulants and used to prevent cardiovascular complications, the observed gene expression changes in our study were independent of their anticoagulation efficacy.

Activated protein C-mediated cardio-protection is not limited to myocardial IRI model. Since AMPK is known to

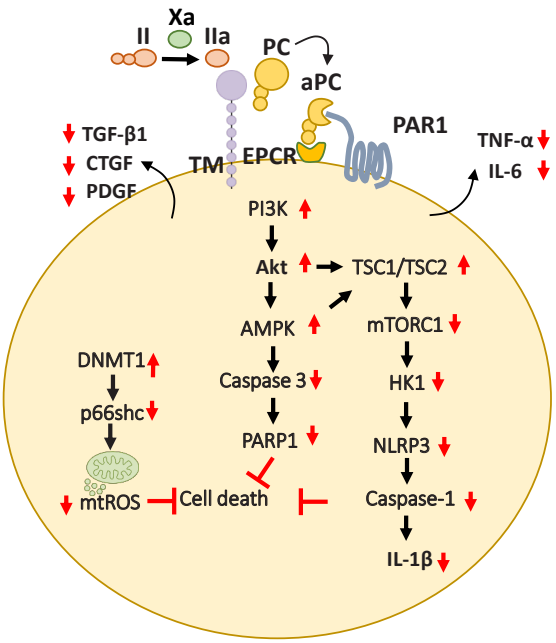

FIGURE 5 | Overview of cytoprotective signaling mechanisms in the myocardium. aPC triggers a complex signaling network of PI3K thereby inhibiting cell death and promote cardioprotection by preventing the mitochondrial ROS and pro-apoptotic as well as inflammasome associated caspases described in multiple cell types of the myocardium. Additionally, aPC has been shown to prevent the release of pro-inflammatory cytokines (TNF-α, IL-6) as well as pro-fibrotic factors (TGF-β, CTGF, PDGF) from the myocardium. Bottom pointing red arrows, downregulation; Upward pointing red arrows, upregulation; Black arrows, pathway connectors.

play a protective role in pressure-overload induced cardiac hypertrophy, aPC was found to confer cardio-protection in mouse models (85). Interestingly, in this model, aPC was able to prevent macrophage infiltration and the activity of redox enzyme $p66^{shc}$, thereby inhibiting ROS accumulation. Moreover, we have recently demonstrated that aPC reduces epigenetically sustained redox regulator $p66^{Shc}$ to avert diabetes-induced accelerated atherosclerosis (86). Mechanistically, in this study aPC mediated reversal of glucose-induced CpG hypo-methylation within the $p66^{Shc}$ promoter by induction of the DNA methyltransferase-1 (DNMT1) was demonstrated as the critical signaling axis in the athero-protection (86) (**Figure 5**). aPC also ameliorated angiotensin II- triggered myocardial remodeling by limiting expression of the pro-fibrotic cytokines transforming growth factor beta 1 (TGF-β1), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF) (87) (**Figure 5**). In this context, aPC was found to confer cardio-protection by acting on the infiltrating immune cells. Overall, these studies suggest a general anti-inflammatory, cytoprotective role of aPC in mediating cardio-protection independent of its role in blood clotting.

Taken together, these studies highlight a coagulation-independent role of aPC in preventing inflammatory mechanisms in CVD. The current studies have focused on the role of aPC in cellular signaling in specific cell types. As a result of these cytoprotective mechanisms, the overall cardiovascular health is restored thereby avoiding further thrombotic complications.

Activated Protein C and Glycosaminoglycans

Proteoglycans have been long known to regulate thrombin. Chondroitin moieties are important for the binding of thrombin and thrombomodulin, which is necessary to produce aPC. Heparin and chondroitin sulfate interact with arginine residues on thrombin and regulate its activity, thereby also controlling protein C activation (88, 89). Furthermore, serpins which are predominant protease inhibitors, capable of effectively inhibiting aPC, are in turn regulated by interactions with GAGs, such as heparin or heparan-sulfate (90). Interestingly, an interaction of FXa with anionic phospholipids, influences its binding to GAGs, thereby allosterically modulating the active site of FXa, and enhances its capacity to activate protein C (91). These findings suggest that modulating the GAG function can indirectly modulate protein C activation, to regulate thrombosis as well as anti-inflammatory cellular mechanisms. Therefore, therapies which can mediate these effects, could act as a double-edge sword against cardiovascular complications.

HEPARIN PROTEOGLYCAN-MIMETIC, ANTIPLATELET AND ANTICOAGILANT COMPOUND

Antiplatelet and anticoagulant compound APAC is a heparin proteoglycan mimetic, in which UFH chains are covalently conjugated to globular protein core of albumin. The conjugation reaction can be modified to provide highly negatively charged concentrated GAG moieties with tailored number of heparin chains with functional impacts (6, 92). APAC has the dual properties of inhibiting both collagen- and thrombin-induced platelet activation and aggregation and act as an anticoagulant, by virtue of the heparin moieties (6). Intravenously injected APAC dose-dependently prolonged activated partial thromboplastin time (APTT) in plasma without any accumulation effect during 2-week daily repeated dosing in rodents and non-human primates (93). APAC is more potent anticoagulant than UFH when measured with thrombin time in human and animal plasma. APAC followed similar clearance route to UFH, mainly via liver and kidneys (94).

In a severe model of local thrombosis on TF and collagen surface, human blood collected to APAC alone (without any other anticoagulation) and subjected to high shear blood flow, lead to reduced deposition of both platelets and fibrin (92). In a baboon Folts model of arterial thrombosis upon severe stenosis (30–90%) APAC maintained patency of the artery when administered locally (6). As such, APAC acts as a dual platelet and thrombin inhibitor, to control VWF-mediated thrombus growth under high shear force-blood flow conditions.

Direct binding of purified VWF to APAC was shown by immunoprecipitation analysis (92). In addition, based on atomic force microscopy studies, recombinant VWF supplemented to APAC solution reduced APAC binding on muscovite mica surface, supporting competitive interaction of VWF to APAC (95). APAC can also interact with platelet- and megakaryocyte-specific receptor G6b-B, the immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor, which is critical for platelet production and activation (96). The effect of APAC and UFH, on wild type and G6b-B deficient mouse platelets was studied using flow cytometry and by detecting GPIIb/IIIa activation-induced fibrinogen binding and by platelet degranulation (TLT-1 expression) with or without stimulation of C-type lectin like receptor 2, CLEC-2 antibody (96–98). CLEC-2 triggers the downstream semi-immunoreceptor tyrosine-based activation motif (ITAM) pathway, and dimerization of CLEC-2 leads to binding of Spleen Tyrosine Kinase (Syk) and subsequent further progression of downstream tyrosine phosphorylation events and eventually platelet aggregation (97). The multiple functions of CLEC-2 have been discussed in several reviews including addressing its role to control collagen-induced platelet activation and thrombo-inflammation (99–102). APAC, unlike UFH, inhibited CLEC-2 receptor stimulation-induced platelet activation and degranulation in wild-type platelets, but not with G6b-B deficient platelets. Thus, APAC may suppress CLEC-2 mediated platelet activation by inducing an inhibitory signal via G6b-B (96). Using platelets from humans and genetically modified mice, interaction of G6b-B to heparins, and more so to APAC, inhibited platelet and megakaryocyte functions (96). Toxicology studies did not show thrombocytopenia (93).

Upon APAC exposure, the injury-induced inflammatory complications can be avoided or decreased in the ruptured vascular endothelium model. An intriguing suggestion is to limit

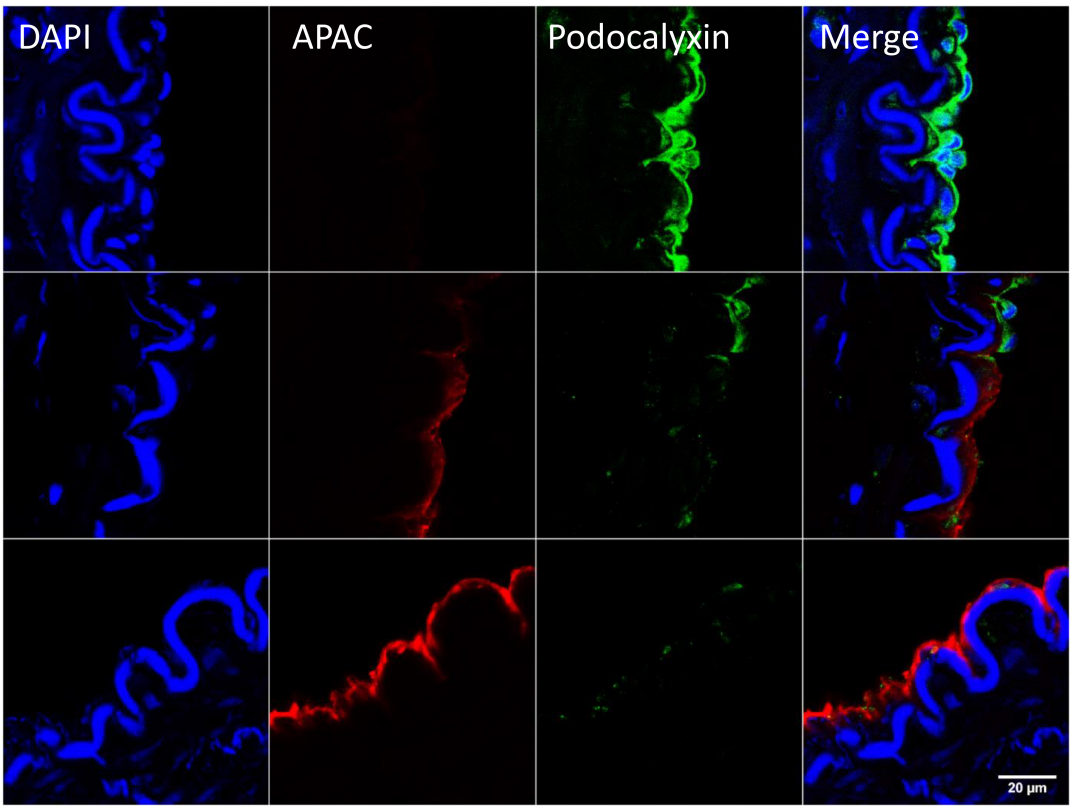

FIGURE 6 | Example of targeting and binding of the heparin proteoglycan mimetic, APAC at the injured arterial wall *in vivo*. APAC and podocalyxin double staining on the arterial injury of vascular anastomosis in pig (103). The anastomotic area was treated with biotinylated APAC (0.3 mg) before exposing to circulation. After 30 min of restored blood flow the artery was resected and processed for histology. Histological samples were stained for nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (blue), APAC (red), and podocalyxin (green). Podocalyxin is a glycosylated cell surface sialomucin expressed, e.g., by vascular endothelial cells and hematopoietic progenitors (107). Disrupted endothelium is depicted by the binding of APAC and the intact endothelium by the binding of podocalyxin. APAC signal was absent at the sites of podocalyxin (merge). Scale bar corresponds to 20 μm.

subsequent damages by regeneration of the glycocalyx with an injury site- targeting glycoprotein structures, such as APAC, to speed up the healing process (103). In animal models, both locally and systemically administered APAC was, indeed, shown to target to the surgically crafted injury under normal and high flow conditions in an acute setting (6, 103). Notably, APAC but not UFH, rescued kidney function, and inflammation alleviated in an IRI rat model of acute kidney injury (94). Also, inhibition of complement (anti-Complement compound 5 antibody, BB5.1) reduces glycocalyx shedding and IRI damage in mice model of acute kidney injury (104).

After exposure of internal elastic lamina during severe balloon injury, immunohistochemical analysis of the biotin-labeled APAC confirmed the co-localization of highly negatively charged APAC with positively charged VWF (103). In contrast, at the site of preserved endothelium, i.e., when platelet endothelial cell adhesion molecule (PECAM-1, CD31) (105) or podocalyxin (106) was present, APAC was absent (103) (**Figure 6**). The similar colocalization of APAC with VWF and laminin was depicted at anastomosis sites of arteriovenous fistula (AVF) surgery (103). In AVF, very high shear rates prevail, and maintenance of the conduit is jeopardized by a high risk of closure already prior its uptake for clinical use (maturation failure), and when used for hemodialysis access after repeated (thrice a week) exposure of blood to collagen with dialysis needle punctures. These indications provide a clinical unmet need, which APAC treatment may address.

Moreover, data are evolving that APAC also targets the injury site from blood circulation in two mouse models of vascular surgery and laser injury, both preventing and delaying thrombus formation. In the reversible kidney IRI model, APAC reduced expression of innate immunity ligand, hyaluronan, tubule-interstitial injury marker, Kim-1, and alleviated structural damage of the renal cortex (94). Specifically, neutrophil gelatinase-associated lipocalin, a marker of renal epithelial injury was reduced, while serum creatinine and urea nitrogen showed a timely fall. Furthermore, in this irreversible IRI model, APAC reversed the kidney failure and reduced serum levels of vascular destabilizing and pro-inflammatory angiopoietin-2 and syndecan-1. UFH was unable to provide this array of protection (94). In conclusion, reno-protection effect of APAC was evident following both a reversible IRI and even after a severe, irreversible IRI by attenuating vascular injury and innate immune activation.

Given its association to GAGs, APAC not only mediates antiplatelet and anticoagulant properties but can confer additional cytoprotection by promoting the activation of protein C. However, it remains to be shown if APAC treatment will enhance of aPC at the site of inflammation or in circulating blood.

CONCLUSION

The hemostatic and the inflammatory systems are interconnected. Coagulation regulators, classically known to solely contribute to the hemostasis machinery and mediate blood clotting have been increasingly recognized to regulate cellular processes including inflammation. Activated platelets are the key players during thrombosis, and in turn trigger inflammation and immune responses, either directly or via activation of immune cells. In this regard, neutrophil activation, and formation of NETs importantly contribute to the pathophysiology of cardiovascular disease by linking thrombosis and inflammation, popularly termed as thrombo-inflammation. Likewise, activation of inflammatory response is paralleled by altered regulation of coagulation proteases and platelet activation. Therefore, antithrombotic therapies which have a dual mode of action, and not only prevent clotting but can confer cytoprotective effects are beneficial in resolving thrombo-inflammation. One such strategy could be heparin proteoglycan mimetic, which locally targets several above key protective functions at sites of vascular damage.

AUTHOR CONTRIBUTIONS

SK, KS, AJ, HH, BI, and RL contributed to the interpretation of the available data and writing of the manuscript. All authors approved the manuscript.

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REVIEW
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Persistent Lung Injury and Prothrombotic State in Long COVID

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Lung injury may persist during the recovery period of COVID-19 as shown through imaging, six-minute walk, and lung function tests. The pathophysiological mechanisms leading to long COVID have not been adequately explained. Our aim is to investigate the basis of pulmonary susceptibility during sequelae and the possibility that prothrombotic states may influence long-term pulmonary symptoms of COVID-19. The patient's lungs remain vulnerable during the recovery stage due to persistent shedding of the virus, the inflammatory environment, the prothrombotic state, and injury and subsequent repair of the blood-air barrier. The transformation of inflammation to proliferation and fibrosis, hypoxia-involved vascular remodeling, vascular endothelial cell damage, phosphatidylserine-involved hypercoagulability, and continuous changes in serological markers all contribute to post-discharge lung injury. Considering the important role of microthrombus and arteriovenous thrombus in the process of pulmonary functional lesions to organic lesions, we further study the possibility that prothrombotic states, including pulmonary vascular endothelial cell activation and hypercoagulability, may affect long-term pulmonary symptoms in long COVID. Early use of combined anticoagulant and antiplatelet therapy is a promising approach to reduce the incidence of pulmonary sequelae. Essentially, early treatment can block the occurrence of thrombotic events. Because impeded pulmonary circulation causes large pressure imbalances over the alveolar membrane leading to the infiltration of plasma into the alveolar cavity, inhibition of thrombotic events can prevent pulmonary hypertension, formation of lung hyaline membranes, and lung consolidation.

Keywords: COVID-19, long COVID, thrombosis, phosphatidylserine, therapy, anticoagulation

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INTRODUCTION

While the majority of patients with coronavirus disease 2019 (COVID-19) will develop only mild, self-limited illness, up to 20% will progress to a more serious form, including severe pneumonia, acute respiratory distress syndrome (ARDS), and pulmonary fibrosis (1–8). The potential risk of pulmonary impairment and parenchymal fibrosis in long COVID is of particular concern (9–13), and studies of multiple treatment options for COVID-19 do not consider their effects on subsequent risk and progression of long-term COVID-19 symptoms (14). Multiple mechanisms of lung injury in COVID-19 patients have been tentatively described, but the long-term pathogenicity of SARS-

CoV-2 in discharged patients remains unclear. It has been reported that the consequences of severe COVID-19 are similar to those of severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) in terms of clinical sequelae, respiratory function, mental illness, and health-related quality of life (15–17). After infection, virus-induced immunopathological events are believed to be responsible for the pulmonary manifestations of SARS and MERS. Specifically, the virus replicates rapidly, infects type II alveolar epithelial cells and vascular endothelial cells, and increases the production of proinflammatory cytokines and chemokines. These, in turn, recruit fibroblasts and induce their differentiation into myofibroblasts, resulting in impaired O_2 and CO_2 exchange (18). In addition, viral antagonism and delayed interferon responses further aggravate inflammation (19, 20).

When SARS-CoV-2 replicates in large numbers, immune cells and inflammatory mediators respond strongly, forming cytokine storms and damaging alveolar structures. The virus invades vascular endotheliocytes from the blood-air barrier. As the disease progresses, endothelial dysfunction leads to more rigid and therefore vulnerable pulmonary vessels. Vascular endothelium expresses more protease activated receptor 1, tissue factor (TF), P-selectin and phosphatidylserine (PS) on the membrane surfaces, releasing microparticles, von Willebrand Factor (vWF), and factor VIII (21). These alterations, together with soluble thrombomodulin (sTM) and increased surface chemokines, causes platelet overactivation and thrombosis (22). With the enhanced permeability of the alveolar membrane, the pulmonary edema causes further hypoxemia and deterioration (23, 24). Pulmonary (micro)thrombus is key to severe hypoxemia, multiple organ dysfunction, and prolonged COVID-19 syndrome (25–33). Microthrombi can block microvessels in the alveolar capillaries, making it difficult for red blood cells to pass through. Slow blood flow and local congestion lead to elevated pulmonary capillary pressure and then to pulmonary hypertension (4). The pressure difference between the two sides of the blood-air barrier increases, while severe inflammation causes diffuse alveolar damage, resulting in the inability of the alveolar membrane to maintain normal permeability (34). Various components in the blood, including macromolecules (mainly albumin and globulin), enter the alveolar cavity. This fluid in the alveolar cavity then induces aggravated dyspnea (35–37). The alveolar liquid evaporates under airflow action, leaving behind plasma proteins and necrotic alveolar epithelial debris to form a transparent membrane, leading to lung consolidation (3–6, 38, 39). Although hypoxemia results from a combination of many mechanisms, the amplifying effects of hypoxia promote the exacerbation of cytokine storms, endothelial injury and thrombosis (23, 24). In long COVID, patients often show substandard six-minute walk test (6mWT), abnormal chest imaging findings (such as bilateral interstitial infiltration, ground-glass opacity (GGO), and fibrosis), and lung diffusing capacity for carbon monoxide (DLCO) < 80%, all indicating persistent lung damage (11–13, 40). In a follow-up study of 113 COVID-19 patients with ARDS, 55% reported dyspnea eight months after diagnosis. Adjusted for age, more than 50% of patients who undertook a 6mWT reached less than 80% of the theoretical distance. Abnormal chest radiographs were reported in 49% of cases, with bilateral interstitial infiltration predominating (87.5%). Chest computerized tomography (CT) scans showing GGO (55%) and fibrosis (19%) were common. Additionally, DLCO was less than 80% in 77.8% of patients (41).

The frequently reported pulmonary arterial, venous, and capillary thrombotic events at autopsy suggest that the transformation of stable vascular endothelial cells to the prothrombotic state is not negligible (8, 25, 27–29, 35, 38–47). We therefore hypothesize that the effects of thrombosis may persist long after the patient has met the criteria for discharge (27). In the recovery stage, it is worth considering whether the prothrombotic status is neglected in patients without thrombotic complications and whether procoagulant factors (such as PS exposure) return to normal in patients with thrombosis. We will investigate pulmonary susceptibility in long COVID and the possibility that prothrombotic states may influence long-term pulmonary symptoms.

CONTINUOUS SHEDDING OF THE VIRUS

Tarhini et al. reported cases of severely immunocompromised COVID-19 patients shedding infectious virus up to four months following symptom onset. In one instance, they reported a single persistent infection with high load culture-positive virus and positive reverse transcription polymerase chain reaction (RT-PCR) on day 103 (27 days after readmission). This study also included a discharged patient who developed post-COVID pneumonia with active virus replication in the lower respiratory tract and finally developed a double infection after second admission (48). The immune system can be suppressed or even depleted by fighting off the increasing viral load (23, 49). Therefore, even if patients have no compromised immune system before SARS-CoV-2 infection, they may show similar symptoms during the disease. In these cases, the virus may retain the ability to transmit over time, as evidenced by positive viral cultures. A variety of scenarios (such as asymptomatic carrier, symptom resolution, or secondary infection) allow for prolonged infectious virus emission (12, 50). In a cross-sectional study, 10 of 60 discharged COVID-19 patients tested positive for SARS-CoV-2 by RT-PCR 4-24 days after discharge. Since all discharged patients were instructed to stay at home and local cases were rare, the researchers assumed that the positive result was persistent virus shedding rather than reinfection (51). Viral persistence is associated with more extensive tissue invasion and worse recovery outcomes. Another study found that the average shedding time of the virus was 19 days in asymptomatic patients, ranging from 6-45 days. While circulating antibodies to other coronaviruses (such as SARS-CoV or MERS-CoV) have been shown to last for at least a year, antibodies to SARS-CoV-2 wane relatively quickly. In both the asymptomatic and symptomatic groups, IgG levels fell by more than 70% in more than 90% of cases during the early recovery period (8 weeks after

discharge) (52). In a cohort study, the positive serum rate and median titer of neutralizing antibodies were significantly lower in the convalescent follow-up than during acute infection (12). A report assessed 30,082 patients with mild-to-moderate COVID-19 indicated that antibody titers remained stable at three months but declined slightly at the 5-month time point (49). There is evidence that the antigenicity of SARS-CoV-2 spike protein changes. Spike of amino acid substitutions and deletions impact neutralizing antibodies and the variants are resistant to antibody-mediated immunity elicited by vaccines (53). Therefore, the risk of reinfection should be monitored, especially in patients with prolonged viral shedding (54). SARS-CoV-2 is likely to persist in certain tissues to drive chronic symptoms. In a follow-up trial, approximately 4-6 months after diagnosis, positive SARS-CoV-2 ribonucleic acid (RNA) was detected in olfactory mucosa samples from four patients with negative nasopharyngeal swabs for SARS-CoV-2 RNA (55).

PULMONARY VULNERABILITY

Early Stage

SARS-CoV-2 enters local alveolar type II cells along the airway, replicates, and damages the targeted cells. Inflammatory mediators are produced when sentinel cells (pulmonary macrophages in the lung interstitium) surrounding the injured tissue recognize the damaged target cells. These mediators trigger neutrophils and monocytes in the blood circulation to migrate to the injured site under the action of chemokines. Various immune cells are mobilized and activated wherever the virus goes, inducing the release of inflammatory factors such as monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interferon-γ (IFN-γ), etc (56). Leukocytes, while necessary to phagocytose inflammatory substances, can also release lysosomal enzymes, reactive oxygen species, and free radicals into the

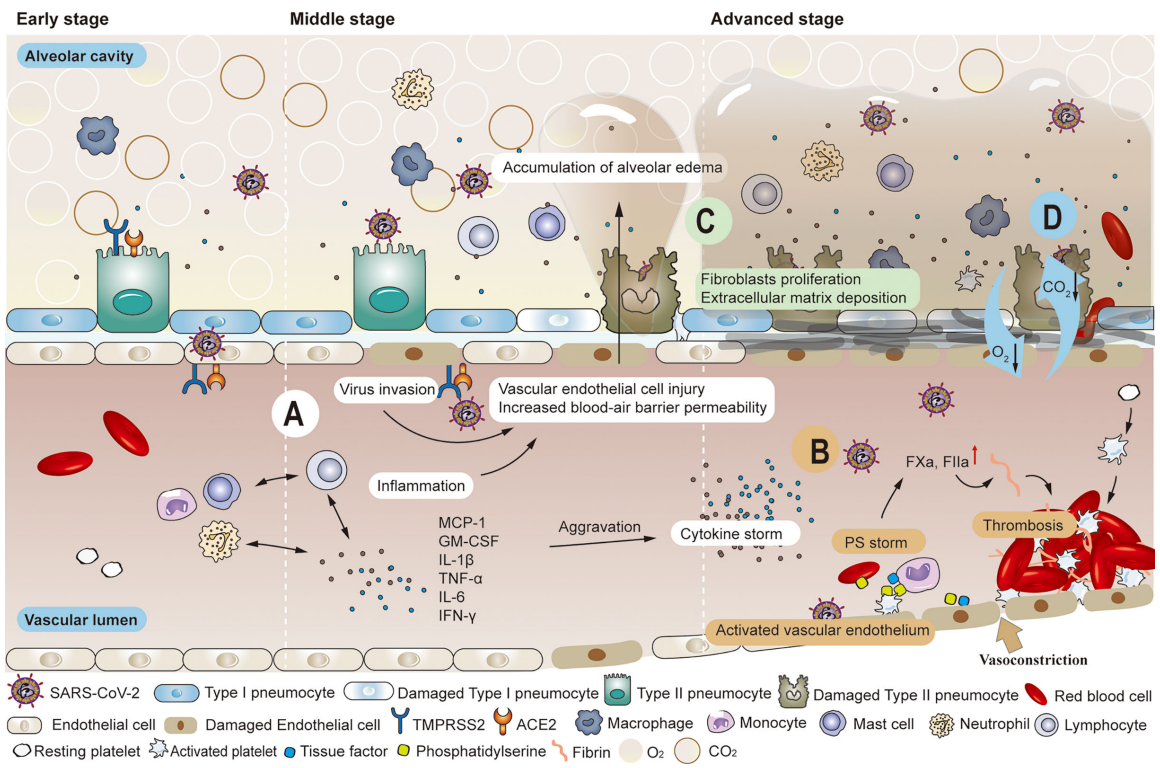

FIGURE 1 | Common pathological changes in the lungs of patients with COVID-19. **(A)** SARS-COV-2 enters local alveolar type II cells along the airway, replicates, and damages the targeted cells. Various immune cells are mobilized and activated wherever the virus goes, inducing the release of inflammatory factors such as MCP-1, GM-CSF, IL-1β, TNF-α, IL-6, IFN-γ, etc. The inflammatory response produced in killing the virus also leads to the injury of alveolar type I and type II cells. When high viral load accompanies the inflammatory response, the air-blood barrier is destroyed on the alveolar side. The virus has the opportunity to invade vascular endothelial cells by crossing the local air-blood barrier, and strong cytokine responses can also spread to vascular endothelial cells. As the permeability of the blood-air barrier increases, blood components enter the alveolar cavity, forming pulmonary edema. **(B)** Significantly enhanced thrombin and elevated levels of endothelial cell biomarkers (vWF: Ag, vWFpp, FVIII, and sTM) were observed in the convalescence period. Impaired pulmonary vascular endothelium can cause uncontrolled activation of coagulation cascades, further leading to vascular thrombosis or fatal pulmonary fibrosis. **(C)** The initial response to the destruction of the alveolar epithelial-endothelial barrier is edematous infiltration in the alveoli and interstitial portion, followed by proliferation as the alveolar barrier is rebuilt by removing exudate. Extracellular matrix deposition occurs. Fibroblasts migrate and transform into muscle cells. **(D)** The damaged blood-air barrier, impaired pulmonary blood perfusion, reduced effective volume of alveolar cavities and the appearance of fibrosis all lead to the obstruction of the exchange of oxygen and carbon dioxide.

extracellular stroma, damaging normal alveolar type I and II cells. Another function of leukocytes is to recognize membrane expression of PS, an 'eat me' signal for macrophages lest more extensive pro-coagulant surfaces appear (57, 58). Ideally, SARS-CoV-2 is gradually cleared by three defensive walls consisting of airway secretions, ciliary oscillations, and innate and acquired immune cells. Even if local ciliated goblet cells, mucous goblet cells, and alveolar epithelial cells are damaged, mild conditions will not evolve into persistent symptoms, and virus numbers may quickly start to decrease. The damaged target cells in the airway can be repaired by the proliferation and differentiation of basal cells. Still, alveolar cells are difficult to regenerate, which is often the precursor to long-term symptoms (59). The virus has the opportunity to invade vascular endothelial cells by crossing the local air-blood barrier (60, 61) (**Figure 1A**). However, the extent of the damage to the local vascular endothelial cells is difficult to predict in early stage. Nevertheless, these impaired vascular endothelial cells often serve as the initial site of thrombosis. At this time, activated vascular endothelial cells are often overlooked, but they are the vulnerable basis of long COVID after discharge (31, 33, 39, 43). Although vascular endothelial damage is difficult to distinguish clinically, it can be used as a differentiating point in pathology. This is important, because the chain reaction caused by damaged endothelial cells will affect the vascular and blood system (45).

Middle Stage

Particular attention should be paid to patients with intermediate disease who have not developed to a severe stage. The increasing viral loads infect alveolar capillary endothelial cells at the blood-air barrier through damaged alveolar epithelial cells and alveolar interstitium (21). The enhanced defense system will inevitably cause tissue damage while killing the virus. However, the overall impact of the defense system is more positive than negative. The injured vascular endothelial cells initiate the coagulation cascade system, activating coagulation factor X and promoting thrombin production. Then, this catalyzes the conversion of fibrinogen to fibrin and form pulmonary microthrombi (21, 31, 33, 39, 43–47, 61–65). As weak parts of the alveolar membrane are destroyed, blood components such as water molecules, plasma proteins, and platelets enter the alveolar cavity, forming pulmonary edema (6) (**Figure 1A**). Many histopathological findings showed that the most frequently reported morphological feature of COVID-19 disease is diffuse alveolar damage, characterized by a variable degree of edema in the exudate phase (42, 66, 67). As long as reduction of alveolar volume is compensated and thrombosis can be prevented or dissolved, the trend towards severe complications can be blocked. However, even if the criteria for discharge are met after effective treatment, the influence of impaired alveolar ventilation and pulmonary microcirculation in the course of COVID continues, and the pulmonary tissues are vulnerable to further damage (11–13, 40, 41). In another study, no significant differences in forced expiratory volume in 1s (FEV1), forced vital capacity (FVC), or their rates were observed nearly one month after discharge, regardless of the severity of COVID-19. DLCO values decreased significantly with increasing severity of clinical symptoms (total 47.2%, mild 30.4%, moderate 42.4%, severe pneumonia 84.2%) (68). Huang et al. observed that 30 days after discharge, DLCO values of patients were notably different (< 80%), 42.5% in non-critical patients versus 75.6% in severe patients (69).

Advanced Stage

When the disease progresses to the severe/critical stage, the primary clinical task is to prevent and treat multiple organ failures and prolong life regardless of the risk of subsequent sequelae. With the exponential increase in SARS-CoV-2 particles, a large number of immune cells activate and release cytokines while gathering around and infiltrating into the lung tissue, thus initiating relevant transduction pathways and a cascade of inflammatory reactions. This creates a vicious cycle that eventually leads to a cytokine storm (70–77). Severe cases can also be accompanied by lymphocytic depletion, leading to suppression or even failure of the immune system (2, 78). Direct and rapid cytotoxic effects of plasma from critically ill patients on umbilical cord blood tubule cells were found *in vitro* (79). Researchers extracted plasma from healthy donors, non-intensive care unit (non-ICU) patients with COVID-19, intensive care unit (ICU) patients with COVID-19, and convalescent patients with COVID-19. Results showed that plasma from both COVID-19 patients and convalesced patients significantly reduced human pulmonary microvascular endothelial cells activity compared to healthy plasma, but plasma from ICU patients induced the greatest cytotoxicity. Blood vessel involvement through endotheliitis is also one of the distinguishing features of COVID-19. Microthrombi within alveolar capillaries, precapillary arteries, and postcapillary venules were frequently reported (66). Studies have shown that alveolar capillary microthrombi were 9 times more common in patients with COVID-19 than in patients with influenza, and the amount of new pulmonary vessel growth were 2.7 times higher than in patients with influenza (80). Damaged vascular endothelium contributes to a pre-thrombotic state, further activating the clotting pathway, accelerating (micro) thrombogenesis, and reducing alveolar blood flow. Impaired pulmonary vascular endothelium can cause uncontrolled activation of coagulation cascades, further leading to vascular thrombosis or fatal pulmonary symptoms of fibrosis (21) (**Figure 1B**). Many studies have suggested that severe and critical COVID-19 is associated with an increased incidence of diffuse thrombosis or pulmonary blood vessel thrombosis (2, 81–83). In severe cases, hypoxic capillary constriction and pulmonary microthrombus, thrombosis, and/or embolism cause slow blood flow, local blocked microvasculature, elevated pulmonary capillary pressure, and overall pulmonary hypertension (84). Changes in the vasculature, coupled with extensive inflammation of lung tissue, enhance the permeability of the air-blood barrier, resulting in vascular leakage with plasma and blood cells entering the alveolar cavity. Pulmonary hyaline membrane formation, acute respiratory distress, and pulmonary fibrosis exacerbate dyspnea (1–8) Impaired pulmonary vascular endothelium can cause uncontrolled activation of coagulation cascades, further leading to vascular thrombosis or fatal pulmonary symptoms of fibrosis (21) (**Figure 1C**). Therefore,

besides damaged alveolar structure, reduced effective volume of the alveolar cavity, and difficulty in gas dispersion (85), (**Figure 1D**) insufficient alveolar blood flow caused by thrombus also needs timely improvement. However, severe or critical illness can present challenges. The incidence of bleeding events and sequelae is relatively high and monitoring is essential to maintain the patient's health status (86–92). The importance of thrombus and embolic events in severe and critically ill patients is widely recognized (93–95). But the occurrence of hypoxemia even with good lung compliance in the early stage also indicates that early abnormal pulmonary blood perfusion may also exist (26, 96, 97). Autopsy results showed microthrombi in the lung but no destruction of surrounding alveolar structures (98), further suggesting that pulmonary blood perfusion is of great importance in the formation of microthrombi. Since this circumvention of traditional ARDS formation has been found, attention should also be paid to lung damage caused by microthrombi in patients recovering from COVID-19.

LUNG INJURY IN LONG COVID

The most commonly reported lingering symptoms of COVID-19 at discharge are fatigue, muscle weakness, sleep disturbances, abnormal lung dispersion, anxiety, and depression (12). Although fatigue and weakness are the most common effects in long COVID, some survivors also report persistent severe symptoms and organ dysfunction (88). A meta-analysis of 16 cohort studies showed that discharged patients could develop residual symptoms in multiple organs, including cardiopulmonary (chest pain, dyspnea, cough, sore throat, and palpitations), nerve (dysmnesia, cognitive disorder, headache, dysgeusia, and dysosmia), gastrointestinal tract (diarrhea, vomiting, abdominal pain, and anorexia), eyes (conjunctivitis), skin (urticaria), musculoskeletal system (myalgia, and arthralgia), etc. (86) In addition to the psychological impact, there is overwhelming evidence that the lung is the most severely affected organ in COVID-19 patients, both in the progressive and convalescent stages (99, 100). In a 6-month follow-up study involving 1733 discharged patients, those requiring high flow nasal catheter (HFNC), non-invasive ventilation (NIV), or intermittent mandatory ventilation (IMV) had an odds ratio (OR) of 4.60 (after multivariable adjustment) for diffusion disorders compared with those requiring no supplemental oxygen. 36% of patients in the severest group had dyspnea with a modified Medical Research Council (mMRC) score > 1 (severe dyspnea) at six months. 50% of patients who completed high-resolution computed tomography chest scans across different severity scales had at least one CT anomaly, with GGO being the most common, followed by irregular lines (12). Revisiting the survivors after 12 months showed a slight increase in the rate of dyspnea. There was no improvement in pulmonary diffusion impairment. And the incidence of pulmonary diffusion impairment was 23% in the no oxygen group, 31% in the oxygen-required group, and 54% in the group with HFNC, NIV, or IMV. The proportion of CT abnormalities decreased significantly over time. But 76% of patients in the severe group still had GGO, and the proportion of patients with thickened interlobular septa increased significantly (11). Wu et al. tested lung function in 83 survivors of severe COVID-19 pneumonia. Although the 6mWT and dyspnea score showed significant improvement at 12 months, 33% had DLCO < 80%, and 24% had GGO radiological abnormalities (13). While most studies have focused on the long-term effects of COVID-19 on hospitalized patients, little is known about the statistics of long COVID in patients with mild or asymptomatic disease. SARS-CoV-2 infection can have subtle effects on the body, even if the patient does not require hospitalization. A study of 8,983 non-hospitalized patients two weeks after a positive test showed that these individuals had a slightly increased risk of initial diagnosis of dyspnea (1.2% vs. 0.7%) and venous thromboembolism (0.2% vs. 0.1%) compared with matched SARS-CoV-2-negative individuals. However, similar results were not found for increased risk of serious complications (such as ischemic stroke, encephalitis, psychosis, or multisystem inflammatory syndrome in children), as previously seen in severe COVID-19 hospitalizations. Positive patients were more likely to initiate bronchodilator therapy, particularly short-acting beta2 agonists (17% vs. 13%), which may be associated with dyspnea (40).

In one meta-analysis of 894 subjects from seven studies, lung function tests showed that low diffusion ability was the most common abnormality, followed by reduced lung volume, while airflow obstruction was relatively uncommon (86). Damage and repair of the blood-air barrier play an essential role in long COVID. Alveolar epithelium is a single layer of epithelial cells in which a subpopulation of alveolar type II cells undergoes self-repair after injury and act as precursors of type I cells. Alveolar type II epithelia are the dominant target cells for SARS-CoV-2. Therefore, impaired type II cells can significantly impede epithelial repair mechanisms, resulting in incomplete repair, scarring, and fibrosis (59). The initial response to the destruction of the alveolar epithelial-endothelial barrier is edematous infiltration in the alveoli and interstitial portion. This is followed by proliferation as the alveolar barrier is rebuilt by removing exudate. However, in some patients, it progresses to excessive fibrosis rather than dissipating inflammation. During the recovery of influenza and SARS, evidence of parenchymal fibrous bands and tractive bronchiectasis has been observed (101, 102). Studies have also found that elevated growth factor receptor B1 mediates extracellular interstitial protein deposition, chemotactic fibroblast migration, and the transformation of fibroblasts into myocytes (4). It has also been suggested that respiratory virus infection may induce significant fibroblast activation during convalescence (12). It is unclear whether COVID-19-associated ARDS causes irreversible pulmonary fibrosis. Studies have observed dramatic increases in the number of lung fibroblasts and collagen deposits in cases of fatal COVID-19 disease (1–8). However, whether long COVID fibrosis will stabilize and subside in subsequent years remains uncertain (5).

When injured host cells release damage-related molecular patterns, the pro-inflammatory molecules and activated immune cells lead to endothelial cell damage, hypoxia, and dysfunction in pulmonary vessels (21). Hypoxia is a driver of vascular

remodeling, inducing the activation of endothelial, mesenchymal, and immune cells and promotes thrombotic fibrosis and epithelial-mesenchymal transformation in COVID-19 patients. Similar vascular remodeling can occur in pulmonary hypertension and chronic obstructive pulmonary disease, but the degree of remodeling is greater in patients with COVID-19. In COVID-19, the endothelial cells transform into smooth muscle cells. Proliferation, migration, and hypertrophy of vascular smooth muscle cells were observed at the cellular level (7). In addition, overexpression and high levels of pro-angiogenic factors (such as vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1α (HIF-1α), IL-6, tumor necrosis factor receptor superfamilies 1a and 12, and angiotensin-converting enzyme 2(ACE2)) have been found in both living and dead COVID-19 patients (103). In one study, compared with healthy volunteers, patients with COVID-19 had more pulmonary vessels with a 5-30mm^2 cross-sectional area and fewer small vessels (0-< 5mm^2). However, there was no difference in overall lung blood volume, suggesting blood redistribution between blood vessels of different sizes (43). Histological evaluation of early COVID-19 showed an abnormal increase in the number of pulmonary blood vessels, accompanied by hyperemia, dilation, and distortion. CD4$^+$ T lymphocytes infiltrated the edema wall and thickened post-capillary venules (44). Compared with the normal vascular endothelium, these new blood vessels, together with the damaged vascular endothelium, are still activated in the convalescence period. They are therefore unable to fully fulfill their role in maintaining the normal blood-air barrier in an anti-coagulant state. As a result, patients can still suffer from respiratory insufficiency and other pulmonary symptoms following COVID-19.

NEW POINT: PS AND THROMBOSIS

Pulmonary vascular endothelial cells prevent thrombosis by binding to TF pathway inhibitors (TFPIs) and blocking the action of the FVIIa-TF complex (104). The presence of various endothelial injury biomarkers, including extracellular vesicles, confirms the persistence of vascular damage in convalescent COVID-19. Significantly elevated thrombin and endothelial cell biomarkers (vWF antigen (vWF: Ag), vWF propeptide (vWFpp), FVIII, and sTM) were observed in the convalescence period. At this point, most patients have normalized acute phase markers, including C-reactive protein, neutrophil counts, white blood cell counts, IL-6, and sCD25 levels (105). Persistent endothelial lesions were also observed during recovery in non-hospitalized patients (31). Impaired pulmonary vascular endothelium can cause uncontrolled activation of coagulation cascades, further leading to vascular thrombosis or fatal pulmonary symptoms of fibrosis (21). Evasio et al. monitored serological markers in 75 patients who had been discharged from the hospital for two months after COVID-19. They found high concentrations of D-dimer, and this persistent change raised the long-term risk of thromboembolic disease in convalescence patients (106).

Ongoing monitoring of COVID-19 patients after discharge from the hospital is necessary to understand the breadth and severity of long-term effects. However, COVID-19 has not existed long enough to complete large-scale cohort studies to examine its long-term impacts on infected patients in detail. Although a critical factor in the development of disease, thrombus-related indicators have rarely been comprehensively studied (106). Thrombosis is a pathological outcome of the local microenvironment. The research on thrombosis should not be limited to its subsequent influence on tissues or organs but should also include the mechanisms involved in thrombosis formation, such as the close connection with vascular endothelium, damage to blood cells, formation of the procoagulant state, and the existence of microthrombi (83, 107–110). Fibrin clumps formed in hypercoagulable conditions are difficult to detect, in contrast to thrombus formation (111). But during the transition period between disease progression and recovery period, it is difficult to judge the extent of the risk of locally developing arteriovenous thrombosis. It is also difficult to confirm whether there has been improvement of the local endothelial injury and return to an anti-coagulant state (21). However, these processes are undeniably common in COVID-19. Therefore, in long COVID, the influence of thrombosis should be assessed from the onset of the prothrombotic state. The degree of early injury and the progression from functional to organic lesions are associated with dyspnea that affects long-term quality of life.

Elevated endothelial stress products are present in the circulating blood of COVID-19 patients. Although endothelial alterations are not specific, thrombogenesis caused by endothelial alterations in COVID-19 results in fibrin deposition in small blood vessels in the lungs and other organs. An early step in the thrombogenesis process is the expression of the pro-coagulant phospholipid PS. In normal conditions, PS is confined to the inner layer of cell membranes by the actions of floppase and flippase. When intracellular Ca^{2+} increases, the ATP-dependent translocation enzyme is blocked, and scramblase is activated, resulting in a random distribution of PS to both sides of the membrane (112). Once exposed on the outer membrane, PS mediates TF decryption and activation, initiating the coagulation cascade (113). PS also provides an active catalytic surface for the formation of the TF-FVIIa, factor X-enzyme (FIXa-FVIIIa-Ca^{2+}-PL), and prothrombinase (FXa-FVa-Ca^{2+}-PL) complexes, leading to the conversion of fibrinogen to fibrin. Pulmonary microthrombi can further develop into pulmonary arteriovenous thrombosis and decrease alveolar blood flow (**Figure 2B**). In addition to providing a negatively charged surface to initiate and maintain clotting functions, PS also acts as a signal to be engulfed by macrophages, avoiding the activation of inflammation and autoimmunity (58). There are two modes of recognition between macrophages and PS-expressing cells. One is direct recognition by the phagocytic receptors: brain-specific angiogenesis inhibitor 1 (BAI1), T cell immunoglobulin mucin 4 (TIM-4), and Stabilin 2. The other is indirect recognition. The bridging molecules milk fat globule epidermal growth factor 8 (MFG-E8, also known as lactadherin) and growth arrest-specific 6 (GAS6) bind to PS (114, 115), which

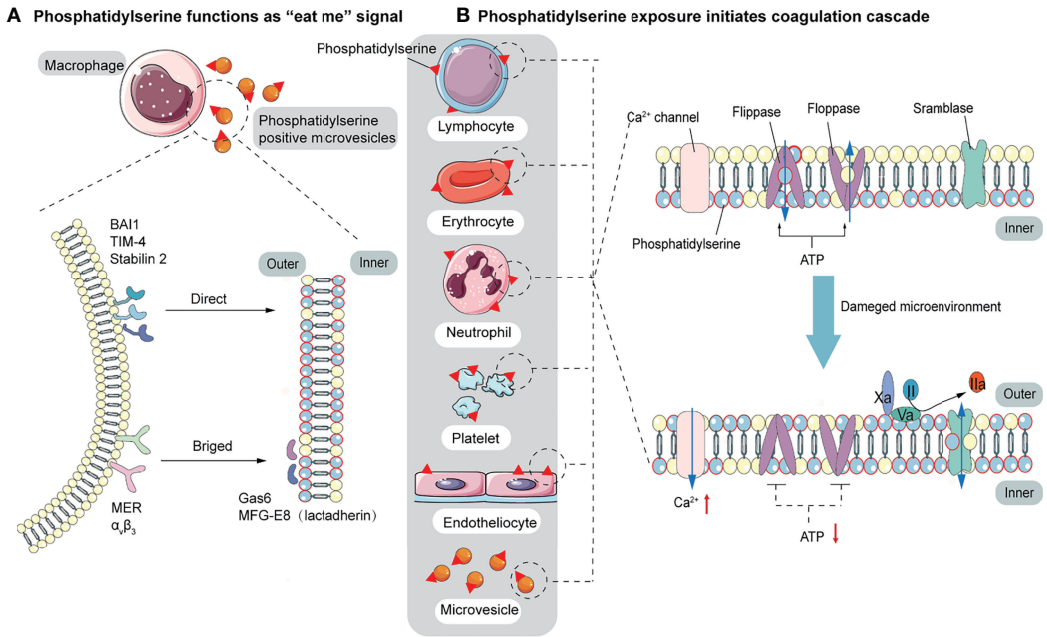

FIGURE 2 | The function of PS. **(A)** PS acts as a signal to be engulfed by macrophages with two recognition modes. One is directly recognized by phagocytic receptors BAI1, TIM-4, and Stabilin 2. The other is indirect recognition. The bridging molecules MFG-E8 (also known as lactadherin) and GAS6 bind to PS, which is recognized by membrane proteins MER and $\alpha_V\beta_3$. These two recognition patterns are not mutually exclusive and may co-occur. **(B)** PS provides a negatively charged surface to initiate and maintain coagulation. In normal conditions, PS is sequestered in the inner layer of cell membranes by the action of floppase and flippase. When intracellular Ca^{2+} increases, the ATP-dependent translocation enzyme is blocked, and the scramblase is activated, resulting in a random distribution of PS to both sides of the membrane. PS, initially located in the cell's inner membrane, is exposed to the outer side. On the outer membrane, PS provides active clotting catalytic surfaces for forming TF-FVIIa complex, factor X-enzyme complex (FIXa-FVIIIa-Ca^{2+}-PL), and prothrombinase complex (FXa-FVa-Ca^{2+}-PL).

is recognized by membrane proteins MER and $\alpha_V\beta_3$. These two recognition patterns are not mutually exclusive and may co-occur (57) (**Figure 2A**). Biomarkers of platelet activation are associated with thrombosis and mortality risk in COVID-19 (116, 117). Several studies have pointed to microvesicles (EVs) and platelet-derived microvesicles (pEVs) as potential biomarkers in COVID-19. Elevated levels of circulating pEVs have been observed in patients with SARS-CoV-2 infection and significantly elevated levels of pEVs in patients with severe disease (118–122). One study using flow cytometry of patient samples found that the frequency of PS^+ cells in the blood of all COVID-19 patients within a week of diagnosis was considerably higher than that of peripheral blood mononuclear cells (PBMC) from healthy or recovered donors. The number of PS^+ PBMC is strongly correlated with the severity of disease and can better predict the need for respiratory support (123). Corresponding autoantibodies to the PS/prothrombin complex have also been found in COVID-19 patients (124). EVs, approximately 100 to 1000 nm in diameter, are produced by budding and shedding of the plasma membrane of various blood cells. Since EVs are unable to maintain membrane asymmetry, they are characterized by PS externalization and can affect the regulation of coagulation and inflammation (125–127). In both sepsis and COVID-19, upregulation of PS exposure can occur on cell surfaces (including endothelial cells, platelets, red blood cells, neutrophils, and lymphocytes) and extracellular particles (128–130). Karina et al. reported significantly increased depolarization of mitochondrial inner transmembrane potential and cytosolic Ca^{2+} and PS externalization in ICU patients compared with healthy controls and non-ICU patients with COVID-19 (131). Since the localization of PS on subcellular organelles was first reported in 2008, we have been focusing on PS-induced hypercoagulability and thrombotic events. The presence of PS^+ blood cells, endothelial cells, and particles has been found in experimental studies of acute promyelocytic leukemia, nephrotic syndrome, sepsis, inflammatory bowel disease, acute stroke, and triple-negative breast cancer, suggesting that PS-induced procoagulant activity may be common in various diseases (132–136).

INHIBITING THE PROTHROMBOTIC STATE

In terms of thrombosis, thrombogenesis should be blocked from the beginning, and the procoagulant state should be alleviated to reduce the incidence of sequelae. D-dimer is commonly used as a marker for thrombosis, and a higher D-dimer level is independently associated with a higher risk of death. In some studies, changes in D-dimer level are used to distinguish the

severity of COVID-19 in the middle and late stages (137–140). Because existing anti-thrombotic interventions appear to have limited effects in the severe and critical stage, it is crucial to take measures at moderate or even mild stage to improve patient outcomes and reduce the occurrence of sequelae. In late stage COVID-19 patients, the maximum solubility assessed by rotary thromboelastometer analysis was significantly less in patients with thrombus compared with patients without thrombotic events (141–143). Meanwhile, the increased levels of tissue plasminogen activator and plasminogen activator inhibitor-1 in patients' blood circulation further suggest impaired fibrinolysis (144). It is important to relieve thrombus formation tendency or remove (micro) thrombi early. Therefore, the focus should be on early antithrombotic therapy, including anticoagulation, anti-platelet activation, and thrombolytic therapy as appropriate.

As a means to prevent thrombosis and relieve hypercoagulability, anticoagulant therapy has been studied primarily in the acute stage of COVID-19. Currently, all guidelines agree that low-molecular-weight heparin thromboprophylaxis should be used in all hospitalized patients with COVID-19, recognizing that hypercoagulability can contribute to more severe disease progression (145–149). The commonly used anticoagulant drugs are low molecular weight heparin (LMWH) (such as enoxaparin) and direct oral anticoagulants (such as Rivarxaban and dabigatran). Heparin can also play a part in controlling leukocyte migration and complement activation (150). Treatment with high-dose prophylactic anticoagulation was associated with a significantly reduced risk of pulmonary embolism (hazard ratio, 0.72, 95%CI, 0.53-0.98) in a study of patients admitted to ICU 14 days after COVID-19 diagnosis (151). A New York study, using the Cox proportional risk model to assess the effect of therapeutic-dose anticoagulation on in-hospital mortality, found that patients treated with anticoagulant had a 22.5% in-hospital mortality and a median survival of 21 days, compared with 22.8% who did not receive anticoagulant and a median survival of 14 days. Systemic therapeutic dose anticoagulation may be associated with improved outcomes in hospitalized patients with COVID-19 (152). One study found that for non-critical patients with COVID-19, therapeutic anticoagulation improved hospital discharge survival without organ support (153). Theoretically, inhibition of hypercoagulability can block the occurrence of microthrombotic events, especially with early treatment. As the site of initial infection, lungs are the most susceptible organ where the effects of inflammation and thrombosis appear early. With early anticoagulation, the pulmonary circulation remains unblocked so that inflammatory substances formed in the lungs that enter systemic circulation can be removed by the immune system. Limiting the level of inflammation in the lungs can reduce the damage to the alveoli and prevent the generation of cytokine storms and PS storms. Effective control of inflammation and improvement of hypoxia reduces damage to endothelial cells and prevents a large number of endothelial cells from transitioning to a defensive state.

Some studies have failed to show prolonged survival time or improved survival rate as a result of anticoagulant therapy. In these studies, most samples are patients with severe or critical disease. A large number of clots have formed, resulting in the depletion of clotting factors resulting in a low fibrinolytic state. Under these conditions, anticoagulants do not protect the patient (138, 154). After the necessary thrombolysis or thrombectomy to narrow and remove the clot, the alveolar perfusion blood flow is improved, and function is gradually restored. However, the inflammatory storm and PS storm, which were originally confined to the lung, can also quickly enter the systemic circulation, accelerate injury to the extrapulmonary organs, and even lead to death. Another possible explanation is the dosage of anticoagulant therapy. Some studies have used therapeutic doses of LMWH for thromboprophylaxis in critically ill patients with thrombotic risk factors (146). The incidence of bleeding events with therapeutic dose anticoagulants was 3.0%, and 1.7% with the prophylactic dose. Among all bleeding events, the gastrointestinal tract was the most common (50.7%), followed by mucosa (19.4%), bronchia (14.9%), and intracalvarium (6%), but fatal bleeding events were rare (155–157). This suggests that therapeutic dose anticoagulants improve overall survival. Meanwhile, some studies have shown that therapeutic doses do not increase the risk of bleeding (158). However, some studies have avoided an increased dose of thromboprophylaxis due to a slight increase in bleeding events (159, 160). The difficulty of using anti-thrombotic therapy during the period of severe and critical illness highlights the importance of timely comprehensive antithrombotic therapy before the late stage. From this perspective, treatment to prevent mild or moderate disease from progressing to a severe or critical condition will significantly improve the overall prognosis. Temporary appropriate comprehensive treatment can effectively block the trend of severe disease development and reduce the occurrence of sequelae in the long run (161). In a prospective study in France, analysis using propensity score matching confirmed that pre-hospital anticoagulant treatment was associated with a better outcome, with a risk of 0.43(95% CI, 0.29-0.63) for admission to intensive care (162). The difference in therapeutic anticoagulant efficacy between moderate and critically ill patients may be attributed to severe inflammatory responses, when thrombotic complications in critically ill patients are too pronounced to recover. In non-ICU patients, therapeutic anticoagulant therapy may still help maintain an appropriate balance (163). As for the risk of bleeding events, because the vascular endothelial cells are relatively undamaged and the coagulation factors are not yet depleted, the risk of antithrombotic bleeding is lower than the risks associated with respiratory distress syndrome, multiple organ failure, and/or sequelae. Christopher T Rentsch and colleagues found that patients who received prophylactic anticoagulation within 24 hours of admission had a 27% reduction in 30-day mortality (hazard ratio, 0.73; 95% confidence interval, 0.66 to 0.81) compared with those who did not. Receiving prophylactic anticoagulant therapy was not associated with an increased risk of bleeding requiring transfusion (hazard ratio, 0.87,0.71 to 1.05) (164). Unobstructed blood flow ensures adequate blood perfusion to

the alveoli, reducing hypoxemia incidence. Unimpeded pulmonary circulation slows or prevents the leakage of plasma into the alveolar cavity, which is aggravated when there is a large pressure difference between the two sides of the alveolar membrane. Thus improved circulation prevents pulmonary hypertension, lung hyaline membrane formation, and lung consolidation, thereby reducing the risk of death and sequelae. Current guidelines have no routine precautions for discharged patients. Some recommend anticoagulant prophylaxis (LMWH or direct oral anticoagulants) in high-risk patients with a low risk of bleeding (165).

The application of antiplatelet drugs mainly includes aspirin (cyclooxyganese inhibitor), clopidogrel (P2Y12 inhibitor), or dipyridamole (adenosine deaminase and phosphodiesterase) (166, 167). It has been reported that clopidogrel may interact with antiviral drugs and should be used with caution. Dipyridamole may be considered for antiplatelet therapy in the presence of renal insufficiency to reduce bleeding due to drug build-up. Aspirin is the most commonly used antiplatelet drug with anti-inflammatory, antipyretic, analgesic and antiplatelet functions. In infectious diseases, aspirin is associated with a reduction in thrombotic inflammation, clinical complications, and in-hospital mortality (168). In a retrospective cohort study of COVID-19, aspirin had some benefits in reducing the risk of mechanical ventilation, ICU admission, and in-hospital mortality (169). Similar results were found in a small observational cohort study of adults with COVID-19 when aspirin was taken at least seven days before or within 24 hours of hospitalization compared with no aspirin (170). In another recent observational study, 730 patients who received antiplatelet therapy had lower mortality and shorter mechanical ventilation duration during hospitalization than 6986 patients who did not receive antiplatelet treatment (171). However there are clinical trials showing that aspirin is not associated with a reduced risk of ARDS (172). Aspirin, as an irreversible platelet inhibitor, is cheap and available, and trials are investigating its effect on the risk of thrombosis.

PS: NOVEL THERAPEUTIC TARGETS

PS, an initial factor of the coagulation cascade, could potentially be used as a new therapeutic target. Lactadherin combined with PS is a more targeted "eat me" signal that could prevent or reduce hypercoagulability (173). PS^+ EVs and cells provide a platform for the anchoring of coagulation factors. Annexin V and lactadherin interrupt coagulation cascades by selectively binding PS (174). Lactadherin is structurally homologous to FVIII and FV and effectively blocks the availability of PS for coagulation reactions (175). The levels of PS^+ cells and pEVs in COVID-19 patients were higher than those in healthy controls and are positively correlated with the severity of the disease. Due to the high likelihood of diffuse microthrombi and arteriovenous thrombus in critically ill patients and the procoagulant role of PS, we think that Annexin V or lactadherin could reduce the incidence of thrombosis in COVID-19 patients (118, 123). In the presence of a large amount of PS, the inhibition of upstream FXIa, FXIIa, and FXa generation can block IIa generation and avoid the formation of thrombus. While the anticoagulant effect of lactadherin has been confirmed *in vitro*, it's anticoagulant effect *in vivo* requires additional study (176). As an under-recognized hemostatic regulator, lactadherin is a potential therapeutic agent in preventing COVID-19 thrombosis (177).

DISCUSSION

Due to the high prevalence of respiratory failure and the need for mechanical ventilation in COVID-19, a significant number of patients will be at risk of long-term complications following severe lung disease. Currently, the world has limited knowledge of long-term lung disease in survivors, and long COVID is still a public health concern. There may be variation in the phenotypes of long COVID, and different post-infection states exist in survivors of COVID-19 ARDS. Many people continue to experience respiratory symptoms for months after an acute infection, especially those with underlying asthma. Other subgroups of patients appear to worsen within three to four weeks of initial infection and brief recovery. Some patients start with a very mild course of illness (do not require medical care or hospitalization) but go on to develop infectious symptoms and ARDS after a few weeks. Thrombus is a crucial factor in the progression of COVID-19 to severe disease, and its importance in long COVID has not been fully assessed. Early treatment of microthrombi can reduce not only mortality but also reduce the incidence of sequelae. PS expression is common when cells are damaged or undergoing apoptosis. And epidemic diseases often have a large number of cell damage and death, forming PS storms and promoting thrombosis. While vaccines are essential in preventing severe disease, effective treatment of COVID-19 remains essential given the rise of virus variants and the waning effectiveness of vaccines.

AUTHOR CONTRIBUTIONS

MX prepared figures and wrote the manuscript. HJ and CW provided helpful comments and acquired data. JS came up with the project, designed the study, contributed to successive drafts, and reviewed this manuscript. VN gave the revision advice and polished this review. All authors read and approved the final manuscript.

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REVIEW
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Endothelial Cell Phenotype, a Major Determinant of Venous Thrombo-Inflammation

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Reduced blood flow velocity in the vein triggers inflammation and is associated with the release into the extracellular space of alarmins or damage-associated molecular patterns (DAMPs). These molecules include extracellular nucleic acids, extracellular purinergic nucleotides (ATP, ADP), cytokines and extracellular HMGB1. They are recognized as a danger signal by immune cells, platelets and endothelial cells. Hence, endothelial cells are capable of sensing environmental cues through a wide variety of receptors expressed at the plasma membrane. The endothelium is then responding by expressing pro-coagulant proteins, including tissue factor, and inflammatory molecules such as cytokines and chemokines involved in the recruitment and activation of platelets and leukocytes. This ultimately leads to thrombosis, which is an active pro-inflammatory process, tightly regulated, that needs to be properly resolved to avoid further vascular damages. These mechanisms are often dysregulated, which promote fibrinolysis defects, activation of the immune system and irreversible vascular damages further contributing to thrombotic and inflammatory processes. The concept of thrombo-inflammation is now widely used to describe the complex interactions between the coagulation and inflammation in various cardiovascular diseases. In endothelial cells, activating signals converge to multiple intracellular pathways leading to phenotypical changes turning them into inflammatory-like cells. Accumulating evidence suggest that endothelial to mesenchymal transition (EndMT) may be a major mechanism of endothelial dysfunction induced during inflammation and thrombosis. EndMT is a biological process where endothelial cells lose their endothelial characteristics and acquire mesenchymal markers and functions. Endothelial dysfunction might play a central role in orchestrating and amplifying thrombo-inflammation thought induction of EndMT processes. Mechanisms regulating endothelial dysfunction have been only partially uncovered in the context of thrombotic diseases. In the present review, we focus on the importance of the endothelial phenotype and discuss how endothelial plasticity may regulate the interplay between thrombosis and inflammation. We discuss how the endothelial cells are sensing and responding to environmental cues and contribute to thrombo-inflammation with a particular focus on venous thromboembolism (VTE). A better understanding of the precise mechanisms involved and the specific role of endothelial cells is needed to characterize VTE incidence and address the risk of recurrent VTE and its sequelae.

Keywords: venous thromboembolism, endothelial cell, inflammation, endothelial plasticity, fibrosis

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INTRODUCTION

A growing body of evidence reveals the functional interdependence of inflammation and thrombosis in cardiovascular diseases. The intricate relationship between these two processes, where inflammation begets thrombosis, and in turn, thrombosis amplifies inflammation, is mediated by the endothelium, leukocytes and platelets in a process termed immuno-thrombosis (1). In physiological conditions, activation of the coagulation cascade by inflammation is part of a natural defense mechanism against pathogens. However, it now well established that similar mechanisms are involved in aberrant activation of inflammatory-dependent thrombosis. This process can occur in sterile conditions and is characterized by a cascade of signals leading to the recruitment and activation of neutrophils, monocytes and platelets that is partially orchestrated by the endothelium (2). The integrity of the endothelium is an important protective mechanism that ensures circulatory homeostasis and prevents thrombus formation. Endothelial dysfunction is characterized by an imbalance between pro- and anti-coagulant factors and between pro- and anti-inflammatory mediators (3). This is directly responsible for various cardiovascular diseases including hypertension, atherosclerosis, stroke, heart disease, diabetes, pulmonary arterial hypertension and venous thrombosis. Recent studies have better characterized one specific mechanism, namely the endothelial-to-mesenchymal transition (EndMT), contributing to endothelial dysfunction during inflammation (4). EndMT is a complex mechanism leading to phenotypic switching of the endothelium associated with acquisition of mesenchymal makers and properties leading to pathological states including tissues fibrosis. EndMT occurs consequently to activation of endothelial cells by pro-inflammatory factors including cytokines but also to changes in the environment such as hypoxia and released in the extracellular space of damage-associated molecular patterns (DAMPs). Endothelial cells are equipped with specific receptors and sensors allowing them to respond to these signals and consequently adapt their phenotype. Here, we discuss how the endothelial phenotype is central to the interactions between inflammation and thrombosis in cardiovascular diseases with a general focus on venous thromboembolism (VTE). Better characterization of these processes will be crucial to prevent recurrent events and long-term complications associated with VTE.

VTE AND STERILE INFLAMMATION

VTE, which encompasses deep venous thrombosis (DVT) and pulmonary embolism (PE), is a frequent and life-threatening disease. VTE is the third leading cause of cardiovascular death after myocardial infarction and stroke with a mortality rate of 10% at 3 months after PE. VTE affects 1 to 2 per 1,000 persons per year and is associated with long-term complications affecting the life expectancy and the quality of life of patients. Main complications of VTE include recurrent (non-fatal and fatal) VTE and long-term sequelae due to incomplete clot resolution in the leg veins [the post-thrombotic syndrome (PTS)] and/or in pulmonary arteries [chronic thromboembolic disease [CTED] and chronic thromboembolic pulmonary hypertension (CTEPH)]. Hence, VTE is a major health issue representing 240 million euros per year in France (5, 6).

VTE is a multifactorial and complex disease involving individual predispositions, environmental parameters and genetic determinants. It is defined as an exaggerated hemostatic response, leading to the formation of an occlusive blood clot obliterating blood flow in the venous system (7). Virchow's triad was traditionally invoked to describe pathophysiological mechanisms fostering thrombosis, alleging concerted roles for hypercoagulability, endothelial dysfunction, and hemodynamic changes (stasis and turbulence) in the development of venous thrombosis (8). It is now well established that sterile inflammation drives VTE formation and resolution (**Figure 1**).

Sterile inflammation occurs in acute conditions including ischemia reperfusion injury and trauma and depends on a well-orchestrated migration sequence of leukocytes to and from the site of injury (9). Sterile inflammation refers to inflammation triggered by tissue injury in the absence of infection. It causes reactive oxygen species (ROS), cytokines, chemokines and DAMP production following endothelial, platelet and leukocyte interactions. Consequently, necrotic events are initiated leading to the release of self-DAMPs including nucleic acids, high-mobility group box 1 (HMGB1), heat shock proteins and purine metabolites such as ATP (10). The recruitment of neutrophils and monocytes at early stages and probably lymphocytes at latter stages is in part orchestrated by the endothelium through cytokine secretion and adhesion molecule expression. Endothelial dysfunction plays a crucial role in VTE, both in thrombus formation and resolution. The endothelium is a major regulator of vascular homeostasis. Its main function is to form a barrier controlling the transport of molecules and cells between the bloodstream and the vessel wall. In addition, under physiological conditions, the endothelium responds to a series of chemical and biomechanical cues by secreting factors regulating vascular tone, smooth muscle cells proliferation and migration, thus preventing immune cell adhesion, thrombosis and inflammation. Endothelial dysfunction is an early predictor of subsequent cardiovascular events or mortality including in VTE (11). Hence, it has been associated with spontaneous VTE in patients further highlighting the importance of the endothelial phenotype in modulating thrombo-inflammatory events (12).

ENDOTHELIAL DYSFUNCTION AND MECHANISMS OF THROMBO-INFLAMMATION

Homeostasis

Endothelial cells form a monolayer called the endothelium covering the inner surface of blood vessels. When discovered in 1865, the endothelium was described as an inert barrier between blood and tissues. However, it is now well recognized that endothelial cells are capable of plasticity and phenotypical changes according to the microenvironment. At the whole-organism level, endothelial cells share essential functions

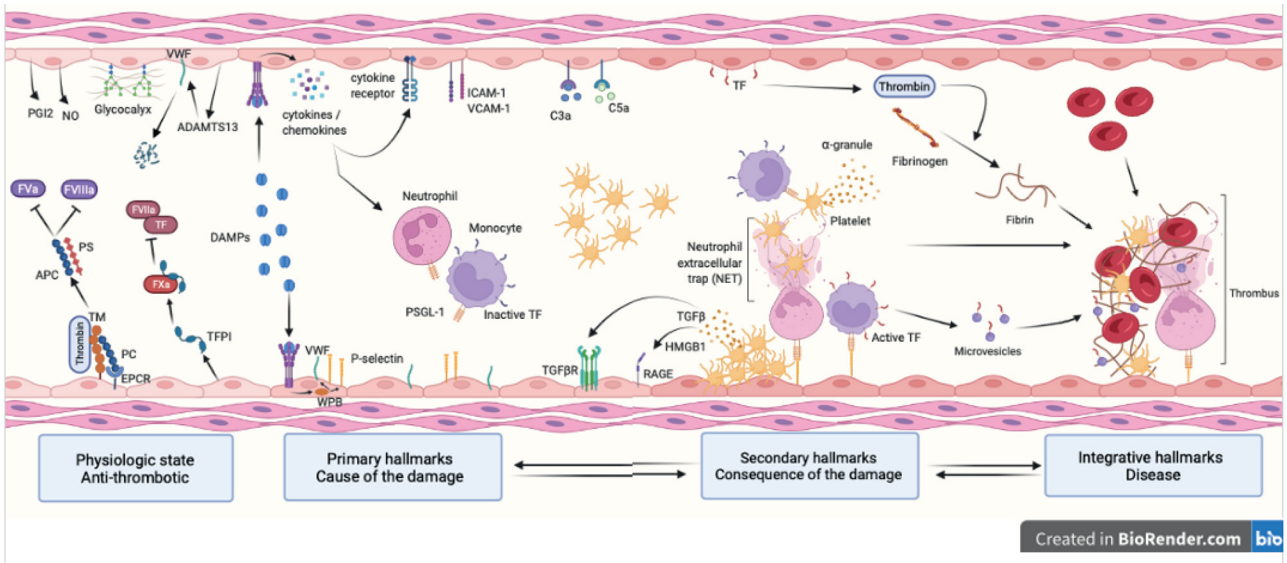

FIGURE 1 | Hallmarks of venous thromboembolism. Healthy endothelial cells provide an anti-coagulant surface by expressing anti-coagulant factors (TM, TFPI, EPCR and PC) limiting thrombin generation. Intact glycocalyx and production of NO and PGI_2 also protect against venous thrombosis. Thrombo-inflammation is triggered by the release in the bloodstream of DAMPs following cell injury. DAMPs interact with the endothelium and promote the release of cytokines, chemokines and WPB content and expression of adhesion molecules (primary hallmarks). This leads to endothelial dysfunction characterized by platelet and leukocyte recruitment that will in turns become activated and secrete pro-inflammatory and pro-coagulant molecules further contributing to thrombosis (secondary hallmarks). Together with the complement system, platelets and endothelial cells induce NET formation and TF expression in monocytes through interactions between P-selectin and PSGL-1. This initiates the coagulation cascade through both the intrinsic and extrinsic pathways and ultimately leads to thrombin-induced fibrin generation and formation of an obstructive clot (integrative hallmarks). APC, activated protein C; TM, thrombomodulin; TFPI, tissue factor pathway inhibitor; EPCR, endothelial protein C receptor; PC, protein C; NO, nitric oxide; PGI_2, prostaglandin I 2; DAMP, damage-associated molecular pattern; WPB, Weibel Palade body; NET, neutrophil extracellular trap; TF, tissue factor; PSGL-1, P-selectin glycoprotein ligand-1; RAGE, receptor for advanced glycation endproducts; HMGB-1, high mobility group box-1.

in a multitude of physiological processes. They regulate vasomotor tone, vascular permeability, angiogenesis, immune cell trafficking, inflammation, coagulation and transport of nutrients, hormones, growth factors and particles. Importantly, the endothelium also exerts a number of organ-specific functions which conditioned the heterogeneity of endothelial cells (13). However, when dysfunctional, they strongly contribute to the pathogenesis of cardiovascular disease, including VTE (14).

In physiological conditions, endothelial cells guarantee an anti-coagulant and anti-inflammatory state through the integration of complex mechanisms to avoid contact between proteins from the coagulation cascade and blood cells. An intact endothelium actively regulates the coagulation response through potent inhibitory processes. Endothelial cells express various anticoagulant molecules, such as tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), endothelial protein C receptor (EPCR), and heparin-like proteoglycans constituting the glycocalyx. The endothelium also expresses ectonucleoside triphosphate diphosphohydrolase-1 (ENTPDase1/CD39) which metabolizes ATP into adenosine preventing platelet activation and aggregation (15). Other mechanisms include endothelial synthesis of nitric oxide (NO) and prostacyclin I2 (PGI_2). These molecules inhibit platelet adhesion and aggregation and vasodilation of the vessel (16).

TFPI limits the action of tissue factor (TF), responsible for the activation of the extrinsic pathway. TFPI acts via factor Xa (FXa) to inhibit the formation of the TF-FVIIa complex (17). In addition, the TM/thrombin complex binds and activates protein C, which in turns binds to protein S and inactivates FVa and FVIIIa (18). Endothelial cells also regulate coagulation by producing the serine proteases, tPA (tissue-type plasminogen activator) and uPA (urokinase-type plasminogen activator) that cleave plasminogen into plasmin (19, 20). Finally, the glycocalyx, which is negatively charged, repels negative molecules thus preventing activation of the coagulation cascade (21). The glycocalyx also fosters the formation of complexes between antithrombin III and thrombin (factor II) or with other serine proteases (factor IXa, Xa, XIa and XIIa) (22).

Additionally, the glycocalyx provides the endothelium with a negatively charged surface contributing to the anti-adhesive properties of the endothelial cell surface. Initial platelet adhesion to VWF is also prevented by a disintegrin-like and metalloprotease with thrombospondin type I repeats−13 (ADAMTS13) cleaving VWF multimers released from the Weibel-Palade bodies (WPBs) (23, 24). *In vivo* studies using experimental models of inflammation showed that ADAMTS13 deficiency is associated with enhanced WPB secretion and increased leukocyte adhesion and extravasation (25). This was associated with increased endothelium-platelet interactions and spontaneous thrombus formation in veins (26). Importantly, platelet depletion in ADAMTS13 deficient mice reduced leukocyte rolling in unstimulated veins suggesting that platelet

and endothelial VWF promote leukocyte arrest thus facilitating thrombus formation (25).

Activation

Sustained activation of endothelial cells by inflammatory stimuli, including circulating pathogen-associated molecular pattern (PAMPs), DAMPs, cytokines, chemokines, complement proteins and ROS cause alteration of the endothelial function (27, 28). The end result is an impairment of endothelial anti-coagulant, anti-inflammatory and immune-dependent properties, which are hallmarks of endothelial dysfunction (**Figure 1**). Experimental models of VTE showed that interferon-γ (IFN γ), transforming growth factor-β (TGF β), tumor necrosis factor-α (TNF α), interleukin-6 (IL-6), IL-17, IL-9, IL-1β contribute to thrombus formation and resolution through endothelial activation. Chemokines such as CXCL8/CXCL1 and CCL2 are also involved (29). It is important to note that endothelial cells interact with the complement system via the expression of specific receptors (C1q, C3a or C5a receptors) and complement proteins (C1, C3, C5, factor B) further contributing to inflammation (30). Lastly, oxidative stress mediated by ROS induce several signaling pathways leading to endothelial dysfunction (31).

Due to its localization, the endothelium plays a crucial role in supporting thrombus formation and resolution in collaboration with platelets and leukocytes. During the initiation phase of thrombus formation, endothelial cells, platelets and leukocytes (especially neutrophils and monocytes) activate each other leading to a highly pro-coagulant environment and the recruitment of additional blood cells. Interactions and mutual activation during thrombo-inflammation between endothelial cells, neutrophils, monocytes and platelets has been extensively reviewed elsewhere (32–35). Briefly, leukocyte recruitment to regions of thrombo-inflammation is primarily initiated by direct interaction between leukocytes and the inflamed endothelium. The activated endothelium expresses P-selectin allowing the rolling and the adhesion of neutrophil via the P-selectin glycoprotein ligand-1 (PSGL1). The upregulated expression of VCAM-1 (vascular cell adhesion protein) and ICAM-1 (intercellular adhesion molecule) allows a stronger adhesion of neutrophils on the endothelial surface. A key process linking inflammation to thrombosis is the formation of neutrophil extracellular traps (NETs) by diverse stimuli including cytokines, activated platelets via P-selectin, DAMPs and ROS. NET formation is conditioned to chromatin decondensation *via* H3 and H4 histone citrunillation by peptidylarginine deiminase 4 (PAD4). NETs trigger coagulation *via* the release of enzyme including neutrophil elastase (NE), cathepsin-G or myeloperoxidase (MPO). This induces FXII activation and inactivation and degradation of TFPI and TM. NETs also contain TF and favor platelet recruitment and activation via VWF, histones, fibrinogen and fibronectin (1, 36–38).

The inflamed endothelium releases WPBs containing VWF, P-selectin and other pro-coagulant and pro-inflammatory components (cytokines and chemokines) in the extra-cellular environment (23). Platelet adhesion and activation are related to integrin binding on endothelial cell surface. Interaction of GPIbα with VWF is crucial for platelet accumulation along the inflamed endothelium mostly in arterial thrombosis. Hence, high shear stress exposes A1 domain of VWF multimers, important for GPIbα binding. This mechanism was also found to drive the onset of thrombus initiation in venous thrombosis in the stenosis model of VTE. To explain these interactions, it was suggested that GPIbα interacts with VWF multimers with pre-exposed A1 domain (39). Platelet GPIIb/IIIa binds to fibrinogen, thereby crosslinking platelets with platelets and platelets with endothelial cells. Once activated, platelets release their granule content further fueling platelet and endothelial activation. Among these factors, selectins are important for platelet-endothelial and platelet-leukocyte interactions. In addition, platelet secrete several soluble factors affecting leukocyte function. These molecules including IL-8, platelet factor 4 (PF4), CCL5, CCL7, CCL3, CXCL4 and CXCL5 are important for the recruitment and tethering of neutrophils to the nascent thrombus in association with selectins. Importantly, enzymes secreted by the activated neutrophils mediate fibrin formation by platelets. The integrin CD11b/CD18, activated following neutrophil adhesion to platelet, further develops the pro-coagulant activity of activated platelets (9, 34, 40). Platelets also participate to neutrophil activation and NET formation. The reciprocal interactions between platelets and neutrophils have been demonstrated both in *in vitro* and *in vivo* experimental models including in VTE (2).

In parallel, monocytes are recruited to the inflamed endothelium through a CCL2/CCR2 mechanism and adhere to the endothelium through P- and E-selectin/PSGL-1 interactions. They initiate coagulation through TF expression, release of TF-positive microvesicles and interactions with platelets (41, 42). Monocytes/macrophages are also important for thrombus resolution by clearance of apoptotic and necrotic cells and matrix debris, profibrinolytic activity and thrombus neovascularization. Monocytes/macrophages are an heterogenous population of leukocytes. Importantly, it was recently suggested that monocyte conversion from pro-inflammatory Ly6Chi to patrolling Ly6Clo monocytes is important for thrombus resolution (43, 44). Finally, endothelial cell, platelet and leukocyte activation culminate to fibrin formation stabilizing the thrombus (**Figure 1**) (2, 45–48).

ENDOTHELIAL SENSING OF ENVIRONMENTAL CUES

Endothelial cells play critical roles in regulating immune functions including cytokine secretion, phagocytic function, antigen presentation, PAMPs and DAMPs sensing, immune cell trafficking and cell extravasation. Increased permeability of the endothelium during inflammation is an important underlying cause of almost all endothelial-related destructive sequelae to the vascular wall. Breakdown of the complex balance between pro- and anti-coagulant systems as a result of acquired disturbances often result in thrombosis.

Danger molecules or alarmins such as DAMPs released during tissue damage in the extravascular environment are recognized by patterns recognition receptors (PRRs). Several type of PPRs can be activated including toll-like receptors (TLRs), NOD-like

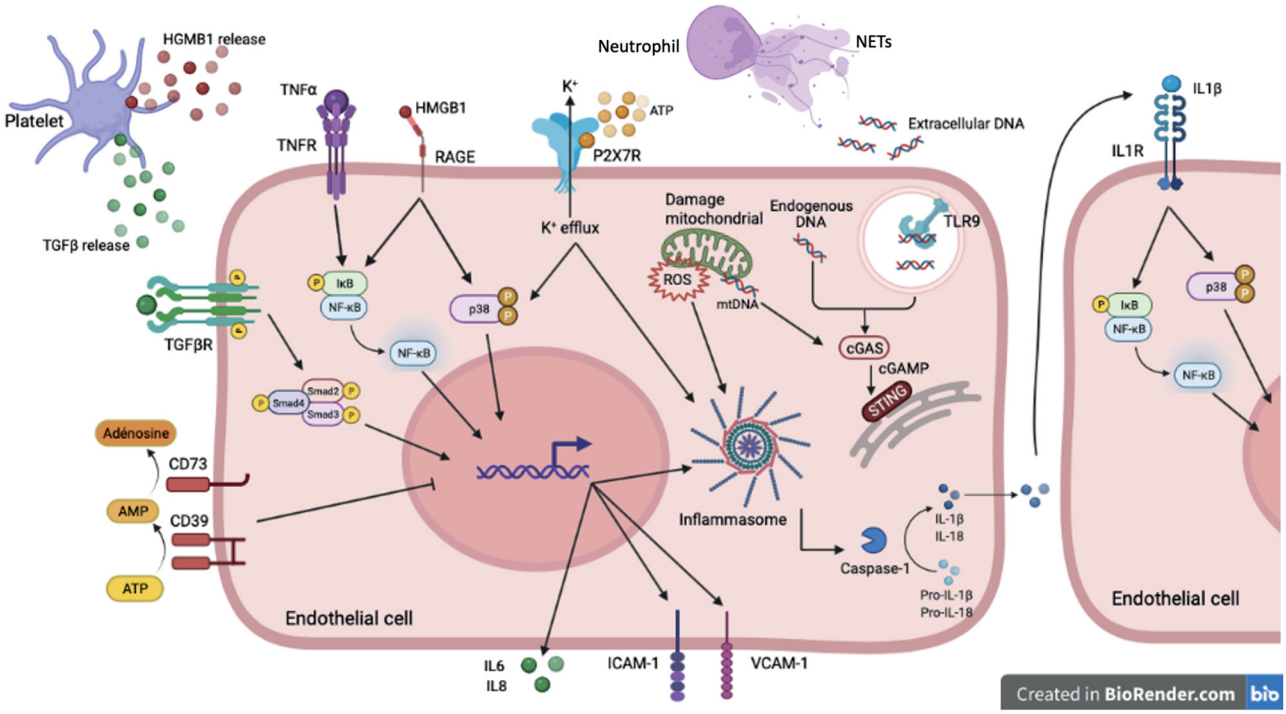

FIGURE 2 | DAMP sensing by endothelial cells and signaling pathways induced downstream PRRs in sterile inflammation. In sterile inflammation, damage-associated molecular patterns (DAMPs) can activate pattern recognition receptors (PRRs). These extra- or intracellular DAMPs are recognized by many cell types including endothelial cells. Among PPRs, some are membrane receptor such as TLRs, cytokines receptors, purinergic receptor and some are intracellular receptor including cytoplasmic NLRP3 or reticuloplasmic STING. Cytokines (TGFβ, TNFα, IL-1β) activate via their receptors (TGFβR, TNFα R, IL1βR) many signaling pathways such as NFκB, p38 MAPK and SMAD. These pathways promote pro-inflammatory gene activation including IL-6 and IL-8 but also ICAM-1 and VCAM-1 involved in recruitment and adhesion of immune cells on the endothelium. The high-mobility group box 1 protein (HMGB1) mediates its pro-inflammatory effects through binding to RAGE and activation of MAP kinases and NFκB pathways. The assembly of the NLRP3 inflammasome, which allows the cleavage of the cytokines IL-1β and IL-18 into their active form, is activated by several stimuli. Binding of extracellular ATP to P2X7R results in Ca^{2+} influx and K^+ efflux. K^+ efflux can induce NLRP3 inflammasome activation. To counteract pro-inflammatory effects of ATP, the ecto-nucleotidase CD39/CD73 hydrolyzes ATP to adenosine thus preventing its binding to P2XR7. The reactive oxygen species (ROS) production by the mitochondria also contribute to NLRP3 inflammasome assembly. Extracellular DNA derived from cell damage can be recognized by TLR9. Intracellular and mitochondrial DNA activate cGAS signaling in the cytoplasm. Activation of cGAS induces the production of cyclic second messenger GMP—AMP (cGAMP), which binds and activates STING at the endoplasmic reticulum.

receptors (NLRs) but also non-PRR DAMP receptors including receptor for advanced glycation endproducts (RAGE) and ion channels (P2X receptors). Downstream signaling pathways activated by sterile inflammation lead to phenotypical changes in endothelial cells contributing to endothelial dysfunction (**Figure 2**; **Table 1**) (27, 28).

Purine Nucleotides and Inflammasome Activation

During tissue injury, purine nucleotides (ATP, ADP) are released in the extravascular environment. These molecules are recognized as danger signals by a variety of cells including endothelial cells, platelets, neutrophils and monocytes and activate inflammatory and thrombotic pathways (55, 56). The purine nucleotide metabolism is regulated by two systems that have opposite actions: CD73/CD39 that hydrolyzes ADP into adenosine has an anti-inflammatory and anti-thrombotic role; and P2X7 receptor, that binds ATP, activates the leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome pathway and promotes pro-inflammatory and pro-thrombotic effects (15).

The inflammasome is extensively involved in non-sterile inflammation-dependent thrombosis. Recently, Wu et al. described a mechanism by which bacterial infection induces blood coagulation through activation of inflammatory responses by the host. They identified that inflammasome activation enhances the release of TF-positive microvesicles from macrophages in a mechanism dependent on pyroptosis. This ultimately leads to systemic activation of the coagulation (57). The importance of the inflammasome in different thrombotic-associated diseases along with infection or not has been reviewed elsewhere (53).

Activation of the inflammasome has also been demonstrated in sterile inflammation. Hence, microparticles from mice deficient for CD39 induce a pro-inflammatory and pro-thrombotic response from endothelial cells illustrated by

TABLE 1 | Endothelial responses to molecules from the microenvironment and thrombo-inflammatory consequences.

Molecular pattern	Receptor	Endothelial activation	Thrombo-inflammatory manifestations	References
Purine nucleotides				
ATP	P2X7	↑ p38 phosphorylation ↑ IL-8, E-selectin	Production of cytokines and adhesion molecules Atherosclerosis	(40)
ATP	CD39	↑ NLRP3 ↑ IL-1β, TF ↑ Thrombus	Production of cytokines and activation of coagulation pathway Venous thrombosis	(39)
ATP	CD39	↑ IL-1β, IL-6, P-selectin ↑ Leukosequestration Neutrophil accumulation	Production of cytokines and adhesion molecules → leukocyte and neutrophil recruitment Venous thrombosis	(15)
DAMPs				
HMGB1	RAGE	↑ NFκB nuclear translocation ↑ phosphorylation ERK1 ↑ IL-8 ↑ ICAM-1, VCAM-1, E-selectin ↑ neutrophil adhesion	Production of cytokines and adhesion molecules → neutrophil adhesion on endothelium	(48)
HMGB1	RAGE	↑ phosphorylation ERK1, JNK, p38 Activation NFκB, Sp1 ↑ ICAM-1, VCAM-1 ↑ IL-8, TNFα, CCL2	Production cytokines, chemokines, and adhesion molecules	(44)
DNA mtDNA	STING	↑ Type I interferon pathway ↑ ICAM-1, VCAM-1 ↑ TF	Production of adhesion molecules and activation of coagulation pathway	(49–51)
RNA	TLRs	↑ TF ↑ permeability ↑ adhesion ↑ leukocyte recruitment ↑ angiogenesis	Activation of coagulation pathway, modification of endothelium metabolism → leukocyte recruitment	(52)
Cytokines				
IL-1β	IL1R	↑ oxidative stress Production of pro-coagulant mediator Production of IL-6	Production of cytokines, modification of endothelium metabolism Atherosclerosis	(53)
TNFα	TNFR	↑ P-selectin Production of ROS	Production of adhesion molecules, modification of endothelium metabolism	(54)
TNFα	TNFR	↑ TF, PAI-1 ↓Thrombomodulin	Activation of coagulation pathway, suppression of anti-coagulation effector → Acceleration of clotting time	(54)

ICAM-1, inter cellular adhesion molecule-1; IL, interleukin; PAI-1, plasminogen activator inhibitor; RAGE, receptor for advanced glycation endproducts; ROS, reactive oxygen species; STING, stimulator of interferon genes; TF, tissue factor; TLR, toll like receptor; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion protein-1. ↑, Increase; ↓, decrease; →, leading to.

increased expression of the adhesion molecules, ICAM-1 and VCAM-1, and the release of VWF and TNFα (54). More importantly, CD39 has an important protective role in venous thrombogenesis. Hence, haplo-insufficient mice develop much larger thrombus characterized by increased leukocyte recruitment, NET formation and increased fibrin and TF expression. Mechanistically, CD39 deficiency was associated with accumulation of extracellular ATP, activation of the NLRP3 inflammasome and subsequent IL-1β secretion (15, 55). Hence, when purine nucleotides are not metabolized by CD39, they might accumulate in the circulation and activate the P2X7 receptor expressed by endothelial or circulating cells leading to activation of NLRP3. Thus, CD39 has an important role in inhibiting NLRP3 and IL-1β signaling during VTE.

Additionally, activation of endothelial P2X7 induces p38 phosphorylation leading to IL-8, P-selectin and E-selectin expression in atherosclerotic plaques (56). The lack of P2X7 in mice abolished inflammasome activation and monocyte recruitment to atherosclerotic lesions (49). P2X7 receptors were also found to aggravate cardiac dysfunction in myocardial infarction, hypertension and diabetic retinopathy. Interestingly, P2X7 exerts pro-coagulant functions by inducing platelet activation and TF expression in macrophages, by enhancing the formation of the prothrombinase complex and by inducing the generation of phosphatidyl serine rich microvesicles. In an experimental model of arterial thrombosis, time to occlusion was delayed in P2X7 deficient mice compared to control animals (50). Since P2X7 is crucial for IL-1β production, this study suggests an important link between TF expression and activation of the inflammasome. Interestingly, it has recently been shown that in hypoxic conditions, such as exposure to high altitude, occurrence of thromboembolic events was increased. Thrombosis was the result of inflammation and enhanced expression of NLRP3, IL-1β, IL-18 and caspase-1 through a mechanism dependent of HIF1α (51). IL-1β release plays an important role in the production of additional pro-inflammatory mediators and

upregulation of adhesion molecules on endothelial cells through autocrine and paracrine effects that would contribute to thrombus formation (53).

High-Mobility Group Box 1

HMGB1 is a non-histone DNA-binding nuclear protein implicated in the regulation of DNA structure to facilitate transcription, replication, and repair (52). HMGB1, which is secreted by stimulated macrophages and activated platelets, is also one of the prototypical DAMPs. HMGB1 binds to RAGE, expressed on the endothelium, smooth muscle cells and monocytes/macrophages (52). Once release in the extracellular compartment, HMGB1 regulates leukocyte recruitment through RAGE-dependent production of cytokines, including TNFα, IL-6, CCL3, CCL4 and CXCL12, and expression of ICAM-1 and VCAM-1 (58). In a mouse model of VTE, HMGB1 accumulates at the endothelial surface as early as 1 h after induction of thrombosis. Increased HGMB1 deposition overtime was correlated with platelet-leukocyte aggregates. Pharmacological inhibition of HMGB1 resulted in significant decreased in thrombus size. Bone marrow or platelet transplantation experiments showed that platelets are the main source of HMBG1 thereby promoting monocyte recruitment and NET formation (59). In addition, Dyer et al. (60) confirmed that platelet specific deletion of HMGB1 reduced NET formation and corresponding thrombus size. Two studies further characterized the mechanism of action of HMGB1 by demonstrating that it is involved in increased cytokine secretion, especially IL-8. This was correlated with higher ICAM-1 and VCAM-1 expression, allowing neutrophil recruitment and adhesion to endothelial cells (52, 61).

Reactive Oxygen Species

Oxidative stress is a major contributor to endothelial dysfunction in cardiovascular diseases. ROS have deleterious effects when produced in excess and participate in the production and secretion of cytokines linking them to inflammation and endothelial dysfunction. Major sources of ROS are NADPH oxidase, the mitochondria and eNOS (endothelial nitric oxide synthase) uncoupling. Hence, mitochondria are important contributors to intracellular ROS production and play a central role in endothelial function (62). As mentioned below, mitochondrial dysfunction and associated liberation of mitochondrial DNA (mtDNA) was shown to promote inflammation (63). In endothelial cells, mitochondrial ROS production is also amplified by activation of NLRP3 and in turns NLRP3 promotes ROS generation. NLRP3 activation increases the secretion of pro-inflammatory molecules, such as IL-1β, IL-18 and HMGB1, which might eventually lead to endothelial cell death through pyroptosis. In addition, eNOS uncoupling is an important characteristic of endothelial dysfunction. Hence, eNOS normally prevents platelet aggregation and adhesion. NO has also anti-inflammatory actions by inhibiting leukocyte interactions with the vessel wall, thereby reducing pathological inflammation and thrombosis. Importantly, in an experimental model of VTE, thrombus burden was reduced by inhibition of TF expression consequent to eNOS activation (64). Ischemic conditions created by the formation of thrombi in veins is also associated with the production of ROS from endothelial cells and recruited leukocytes. In turns, ROS promote the formation of NETs by neutrophils, induce expression of cytokines and adhesion molecules by the endothelium and TF expression by monocytes (9). Thus, ROS are involved in almost all deleterious effects promoting thrombosis and endothelial dysfunction including EndMT. ROS mediate their effect either by directly influencing TGFβ signaling or indirectly by activating NFκB and cytokine production. TGFβ is also inducing oxidative stress in endothelial cells through mitochondrial dysfunction (65).

Cytokines and Chemokines

The role of cytokines and chemokines in VTE has been reviewed elsewhere (29). We will here only discuss IL-1β and TNFα that are important regulators of thrombo-inflammation and endothelial phenotype. IL-1β is involved in many pathologies related to inflammation and results from NLRP3 activation. IL-1β is produced by endothelial cells and platelets and directly affects endothelial functions. For example, IL-1β increases production of ROS, pro-coagulants mediators and impairs vasodilatation. IL-1β expression is initiated following activation of the endothelium by a priming signal such as TNFα leading to NFκB activation; and a second activation signal such as extracellular ATP or DAMPs. The first signal is important for expression of NLRP3, pro-caspase-1 and IL-1β and IL-18 in their inactive forms. The second signal allows the assembly of the inflammasome which leads to the cleavage of pro-caspase-1 into caspase-1. When pro-IL-1β is cleaved by caspase-1 to its active form, it is secreted in the extravascular environment and binds to its receptor. It has been shown in atherosclerosis that IL-1β directly activates endothelial cells and increases the production of thrombogenic mediators such as IL-6 (66).

TNFα is a central cytokine in the regulation of inflammation. TNFα has two receptors: TNF receptor type 1 (TNFR1) or TNF receptor type 2 (TNFR2). TNFR1 is ubiquitously expressed whereas TNFR2 is mostly expressed by immune and endothelial cells. TNFα receptor activation is associated with NFκB translocation to the nucleus and expression of pro-inflammatory genes (29). In presence of TNFα, the expression of P-selectin is upregulated on HUVEC surface allowing platelet rolling suggesting a pro-thrombotic role. Moreover, TNFα downregulates TM expression and increased TF and plasminogen activator inhibitor-1 (PAI-1) expression. All these endothelial changes might result in an acceleration of blood clotting induce by TNFα (67).

Importantly, IL-1β and TNFα are also potent inducers of EndMT. This will be discussed in the paragraph below together with TGFβ.

Endogenous Nucleic Acids

As for purine nucleotides, intracellular DNA, RNA, rRNA and miRNA become disseminated into the circulation or retained at the site of cell activation and/or injury in the extracellular space. These extracellular nucleic acids elicit profound pro-inflammatory and pro-thrombotic effects. Analysis of extracellular DNA provided insight into their alarmin

properties including highly pro-coagulant NETs and extracellular mitochondrial DNA, derived from cell injury and recognized as a DAMP by TLR9 to foster sterile inflammatory responses. Endogenous nuclear and mitochondrial DNA are also recognized by intracellular receptors including the stimulator of interferon genes (STING). STING is ubiquitously expressed in a variety of cells including endothelial cells. Recent studies demonstrated that STING is activated by mtDNA, free fatty acids and TNFα in endothelial cells resulting in control of immune cell transmigration (68–70). STING-induced endothelial dysfunction has been associated with elevated expression of endothelial inflammatory markers including TF and adhesion molecules. Importantly, thrombotic coagulopathy in COVID-19 patients have been attributed to STING over-activation and subsequent endothelial dysfunction in these patients (69). Despite a potential involvement in coagulopathy related to endothelial cell dysfunction, this mechanism has yet not been involved in VTE and would require future research.

As extensively studied by Klaus Preissner's group, different forms of extracellular RNA (eRNA), derived from activated or injured cells, are detectable in the extracellular compartment and contribute to the pathogenesis of cardiovascular diseases [reviewed in (71)]. Although, the exact nature of these eRNA remain to be defined, they contribute to a variety of mechanisms also involved in VTE. eRNA were first found to have pro-coagulant functions in arterial thrombosis by activating the contact phase pathway. It was later describe that they contribute to endothelial activation, leukocyte recruitment, vascular permeability, angiogenesis, macrophage polarization and cell death (72). A recent study showed that acute hypoxia induces TF expression in the vasculature in a TLR3-dependent manner. However, no functional characterization of the direct involvement of this pathway in VTE was investigated in this study (73). **Table 1** summarizes endothelial responses to molecules from the microenvironment and which thrombo-inflammatory events are induced following endothelial activation.

ENDOTHELIAL PHENOTYPICAL CHANGES: ENDOTHELIAL-TO-MESENCHYMAL TRANSITION

Endothelial dysfunction may be orchestrated by a process called EndMT. In this process, endothelial cells lose their endothelial markers to express instead mesenchymal markers. Endothelial cells also acquire mesenchymal characteristics including morphological changes, increased motility and cytoskeletal re-arrangements (74). They become more proliferative, thrombogenic and produce high amounts of extracellular matrix proteins such as fibronectin and collagen and express several adhesion proteins involved in leukocyte recruitment (75, 76). In addition, EndMT leads to altered endothelial cell junction organization, loss of cell polarity, increased cell proliferation and high invasive and proliferative potential (77). Specifically, when endothelial cells undergo EndMT, VE-cadherin, CD31, PECAM-1, EGF (TIE1 and TIE2), VWF and eNOS expression decreases and smooth muscle actin (SMA), smooth muscle protein 22-alpha (SM22α), vimentin, fibroblast specific protein-1 (FSP1), N-cadherin, type I collagen, fibronectin, nestin, CD73, matrix metalloproteinase- 2 and 9 (MMP-2 and MMP-9) and fibronectin expression increases (77–83).

Although, this is a physiological process during embryogenesis, in adulthood, EndMT is associated with various cardiovascular pathologies (78, 84). Hence, it was reported that EndMT is involved in the process of cardiac development and might be also important for the vascular system. However, when EndMT is induced in adult organisms, it is associated with atherosclerosis, pulmonary arterial hypertension, transplant arteriopathy, valvular disease and vein graft remodeling (84, 85). EndMT is induced by a number of factors and stimuli such as pro-inflammatory cytokines, hypoxia, abnormal mechanical forces but also as a secondary event following activation of pathways described in previous paragraphs by DAMPs. Multiple extracellular ligands are involved in the initiation and progression of EndMT programs (86). Also, epigenetic modifications might participate in EndMT and play an important role in cardiovascular diseases. DNA methylation, histone modifications and RNA interference are recognized as the most involved (82). However, EndMT is a complex process and due to the lack of standardization, relative expression of endothelial versus mesenchymal markers should be graduated and reversible or transient characteristics should be evaluated when one wants to show EndMT (87).

MOLECULAR MECHANISMS AND REGULATION OF EndMT

EndMT Inducers and Signaling Pathways

TGFβ is the most described inducer of EndMT but IL-1β, TNFα, IFNγ, hypoxia, high glucose and abnormal shear stress are also potent activators of this extreme phenomenon in the spectrum of endothelial activation (88). TGFβ binds with high affinity to type II serine/threonine kinase receptor (TGFβRII) and to activin-like kinases (ALK) 1 and ALK5 that are the predominant type I receptors (TGFβRI) in endothelial cells (76, 89). TGFβ signals through receptor complexes combining 2 type I and 2 type II components. Type I activation results in the activation of the canonical SMAD signaling pathway. Activated SMAD proteins form heterodimeric complexes with SMAD4 and translocate into the nucleus (82). There, they interact with various transcription factors such as Snail, Twist, Zeb and Slug and regulate the transcription of genes involved in EndMT (75). TGFβ might also induce non-canonical SMAD-independent signaling pathways including mitogen-activated protein kinase (MAPK; p38, JNK, ERK), phosphatidylinositol-3-kinase/Akt (PI3K/Akt), mammalian target of rapamycin (mTOR), Hippo/YAP, β-catenin/Wnt, protein kinase C and Rho-like GTPase (76, 78, 84). There are multiple signaling pathways induced by TGFβ alone or in combination with other inflammatory cytokines, including IL-1β and TNFα, hypoxia, abnormal shear stress or high glucose leading to EndMT (88, 90, 91). For example,

during hypoxia the degradation of the hypoxia-inducible factor (HIF)1α is inhibited which then accumulates into the nucleus. Interestingly, HIF1α regulates the expression of Snail, Twist and ALK5 which might explain why hypoxic endothelial cells are sensitive to TGFβ signaling (76). Other cytokines can act as stimuli for EndMT, such as IFNγ, which acts via the Janus kinase (JAK) pathway and activation of STAT. Elevated HDL levels show anti-fibrotic effects by blocking the TGFβ/SMAD/Slug/ZEB signaling pathway. On the contrary, high glucose conditions have been shown to induce EndMT through the Akt/PI3K/NFκB pathway (82). Several studies showed that cytokine combination is more powerful in inducing EndMT. It has been particularly demonstrated for TGFβ, IL-1β and TNFα that converge to Sp1-dependent expression of EndMT-related genes (4). Since TGFβ, IL-1β, TNFα are released upon platelet activation along with other pro-inflammatory cytokines, it reinforces the important role of platelets in both thrombosis and inflammation and suggests that platelets participate in EndMT.

Regulation of EndMT and Link With VTE

EndMT is a tightly regulated process in normal physiology. However, endogenous factors that inhibit EndMT have been less investigated. Several studies have highlighted the important role played by the fibroblast growth factor (FGF) in the regulation of TGFβ signaling (92). Hence, decreased FGF signaling leads to increased expression of TGFβ ligands and receptors and enhances TGFβ signaling resulting in EndMT (93). FGF controls EndMT and TGFβ signaling by maintaining high expression of Let-7 family of miRNAs, which downregulates ALK5 expression. However, in inflammatory conditions where FGF signaling is often reduced, the dramatic decrease of Let-7 miRNAs leads to rapid increase of ALK5 and amplification of TGFβ signaling. In addition, FGF directly inhibits ALK5-dependent expression of EndMT genes through activation of the Ras/MEK1 pathway (94). Accordingly, disruption of FGF signaling has been associated with aggravation of EndMT and atherosclerosis progression (95). BMP7 signaling is a second pathway that negatively regulates EndMT. It can antagonize TGFβ signaling through activation of SMAD1 leading to SMAD2/3 inhibition or by induction of ID proteins which can heterodimerize with SMAD2/3, thus inactivating their transcriptional activities (96).

Different types of non-coding RNA such as circular RNAs (circRNAs), long-non coding RNAs (lncRNAs) and miRNAs have been implicated in the regulation of EndMT. CircRNA are widely described as playing a role in epithelial-to-mesenchymal transition (EMT), a cellular transition process similar to EndMT and were found upregulated by 20-fold after TGFβ1 treatment (97). The circRNA DLGAP4 (DLG-associated protein 4) expressed in mice brain endothelial cells inhibits EndMT by interacting with miR-143 and thereby regulating expression of mesenchymal markers (98). Moreover, the CircHECTD1 was found to regulate the migratory capacity of endothelial cells by decreasing the expression of HECTD1 protein (99). The lncRNAs MALAT1 and GATA6-AS are also regulating EndMT. MALAT1 expression is increased in endothelial progenitor cells upon TGFβ1 treatment leading to downregulation of miR-145 and expression of SMAD3 and TGFβR2, thereby facilitating EndMT. GATA6-AS decreases TGFβ2-induced EndMT in human umbilical vein endothelial cells (HUVECs) by reducing SMA and calponin expression thus preventing VE-cadherin loss. GATA6-AS also interacts with LOXL2, which regulates endothelial gene expression via changes in histone methylation (H3K4me3) (97). However, little is currently known about how circRNAs and lncRNAs are regulated and functionally relevant in EndMT-related cardiovascular diseases.

Similarly, TGFβ signaling is directly controlled by miRNA that either downregulate growth factor receptor-bound 2 (Grb2), involved in fibrosis, (i.e. miR-200a) or the transcription factors Snail1 (i.e. miR-200b and miR-532) and Snail2 (i.e. miRNA-630) (84, 97, 100). TGFβ can also directly modulate expression of miRNAs that suppress inhibitors of mesenchymal gene expression or that affect endothelial gene expression. Normally, mesenchymal gene expression is kept inactive by transcriptional repressors SKI [SKI proto-oncogene (c-Ski)] and the ternary complex factor ELK1. SKI that inhibits the formation of the SMAD complex is the target of miR-155 and ELK1 is repressed by miRNA-27b thus inducing mesenchymal gene transcription. Finally, TGFβ increases the expression of miRNAs that suppress endothelial protein expression, including miR-21. PTEN is a target of miR-21 and an inhibitor of Akt which facilitates EndMT. Several other miRNAs were found to be modulated in EndMT but their precise role has not been defined yet (82, 100).

Interestingly, as for other cardiovascular diseases, miRNAs have been implicated in the pathogenesis of VTE. To date, only 4 studies have quantified miRNA in patients with venous thrombosis in an attempt to correlate miRNA expression to thrombotic events (101–104). Other studies have investigated miRNA expression profile in pulmonary embolism, recurrent venous thrombosis or experimental model of VTE. The most recent study in VTE patients showed that 9 miRNAs were significantly associated with venous thrombosis. Four of these miRNAs were found to regulate proteins of the coagulation cascade and might represent predictors of thrombotic events (104). Although this required further investigation, it has important clinical perspectives since miRNAs can be easily modulated. In addition, some miRNAs associated with VTE in these different studies were also found to be associated with EndMT in separate works (85, 105). For instance, miR-27b that promotes EndMT was found upregulated in pulmonary embolism. In chronic obstruction pulmonary disease, miR-145 was found to negatively regulate TGFβ signaling by decreasing SMAD3 phosphorylation, thus potentially interfering with EndMT (106). Interestingly, miR-145 was also associated with inhibition of TF expression thus preventing venous thrombosis in experimental models (107). These works suggest that miRNAs might link EndMT to VTE. Although, validation of causal implication of these miRNAs in EndMT-related VTE is required, it opens interesting research avenues.

EPIGENETIC REGULATION OF EndMT

Histone Modifications

Histone methylation, which is associated with both transcriptional activation and repression, is an important regulator of EndMT. EZH2 (enhancer of homologous zest 2) is the major histone methyltransferase responsible for

the deposition of trimethylation marks on lysine 27 of H3 (histone 3; H3K27me3) leading to transcriptional repression. Co-stimulation of endothelial cells with TGFβ2 and IL-1β results in decreased expression of EZH2 and number of H3K27me3 marks at the SMA promoter. This induces SMA expression enabling EndMT. In addition, during cardiac development, histone deacetylase 3 (HDAC3) recruits EZH2 to prevent transcription of TGFβ1, and block physiological EndMT, which is an essential step to complete cardiac development. The association between HDAC3 and EZH2 might therefore be a mechanism involved in EndMT in cardiovascular diseases (97). Another histone demethylase that has been involved in the epigenetic control of EndMT is Jumonji domain-containing protein 2B (JMJD2B). This protein transcriptionally activates gene expression by demethylation of the repressive histone mark H3K9me3 and contributes to the methylation of the activating histone mark H3K4me3. JMJD2B is activated by EndMT-promoting stimuli including TGFβ1, TGFβ2, IL-1β and hypoxia. Using ChIP-sequencing, changes in H3K9me3 marks were found at various promoters upon induction of EndMT. Normally, expression of mesenchymal gene such as calponin is repressed by H3K9me3 marks. However, this repression can be reversed by JMJD2B-mediated demethylation following induction of EndMT (108).

Because aberrant HDAC expression and activity can promote EMT in cancer and that HDAC inhibitors prevent EMT, these proteins have been studied in the context of EndMT. Acetylation of H4 (histone 4) positively regulates SMAD3 upon combined TGFβ and Notch signaling stimulation. However, it remains to be defined which histone acetyltransferase (HAT) may be involved in this mechanism. Interestingly, treatment with TGFβ2 of cardiac endothelial cells induces histone acetyltransferase p300 expression, which is well known to be upregulated in fibrotic tissues. Therefore, p300 might be responsible for H4 acetylation and upregulation of specific SMAD3 target genes upon TGFβ1 stimulation (97). HDACs have also been studied in the regulation of gene expression during EndMT. As mentioned above, HDAC3 appears involved in repression of mesenchymal gene expression. In coronary endothelial cell lines, increased HDAC9 expression correlated with a generalized reduction of histone acetylation during EndMT. Importantly, class IIa HDAC inhibition prevented EndMT, whereas HDAC9 over-expression promoted EndMT. Endothelial-specific Hdac9 deficiency in mice was associated with reduced EndMT and a more stable atherosclerotic plaque phenotype (109). Others HDACs have been involved in EMT but their role in EndMT has not been studied yet.

DNA Methylation

DNA methylation refers to the presence of methyl 1 groups on cytosine bases in the CpG islands of DNA. These CpG islands are mainly located in gene promoter regions, which are responsible for transcriptional activation. Addition of methyl groups via DNA methyltransferases (DNMTs) to these CpG islands is the most potent epigenetic regulatory mechanism to stably silence gene expression. Treatment with TGFβ1 of human coronary endothelial cells resulted in aberrant methylation of the promoter RASAL1 (RAS protein activator like 1) (a Ras signaling inhibitor), which contributes to EndMT (97).

Regulation of EndMT involving epigenetic mechanisms to push toward expression of mesenchymal genes still requires investigation (**Figure 3**). A better understanding of the different regulatory networks involved might be useful to determine if maladaptive EndMT can be prevented or reversed to reduce or abrogate vascular remodeling. Aberrant regulation of epigenetic mechanisms is also strongly associated with inflammation and thrombosis. Some studies have investigated how DNA methylation and histone modifications were involved in VTE. Expression of several proteins from the coagulation cascade including FVII, FVIII and tPA was regulated by DNA methylation mechanisms leading to alteration of their plasmatic levels (110). Whole-blood DNA methylation analysis identified a potential association between methylation marks and quantitative biomarkers of thrombotic disorders (111). Regarding histone modifications, one particular histone modification, the citrullination, occurring in the formation of NETs have been extensively implicated in VTE. More importantly, studies have shown that the tPA gene expression was sensitive to histone modifications in endothelial cells and modified by HDAC inhibition (112, 113). *In vivo*, HDAC inhibition with valproic acid was associated with reduced thrombus burden supporting the idea that it can act as an anti-thrombotic agent (114). Elucidation of the epigenetic pathways involved in EndMT-related VTE will help in the discovery of new therapeutic targets.

OTHER FACTORS INFLUENCING EndMT

As discussed, TGFβ induces only partial EndMT and more potent effects are observed in association with other cytokines or stimuli including high glucose, oxidative stress, hypoxia or hypoxia caused by extreme environmental conditions. This implies that pathways independent of TGFβ might be activated during thrombo-inflammation that contribute to endothelial dysfunction and phenotypical changes. For example, thromboembolic complications were found to occur more frequently at high-altitude. The high altitude environment was associated with higher plasmatic levels of platelet and endothelial activation markers contributing to an hypercoagulative state (115). Hypoxia is also an important factor contributing to ischemic stroke and ischemia/reperfusion injury. In these pathological conditions, EndMT was found to contribute to the development of vascular fibrosis. In an experimental model of ischemic stroke, endothelial cells in infarct lesion undergo EndMT characterized by higher expression of TGFβR1, α-SMA, fibronectin, FSP-1, and Snail. Vascular brain damages due to EndMT were reversed by overexpression of the miRNA Let-7i (116). Additional miRNAs were found to regulate EndMT in the context of ischemic brain injuries, including the CircHECTD1 miRNA discussed above (117). In addition, it has been suggested that the endothelial dysfunction induced by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) might initiate pulmonary fibrosis and vascular remodeling through EndMT

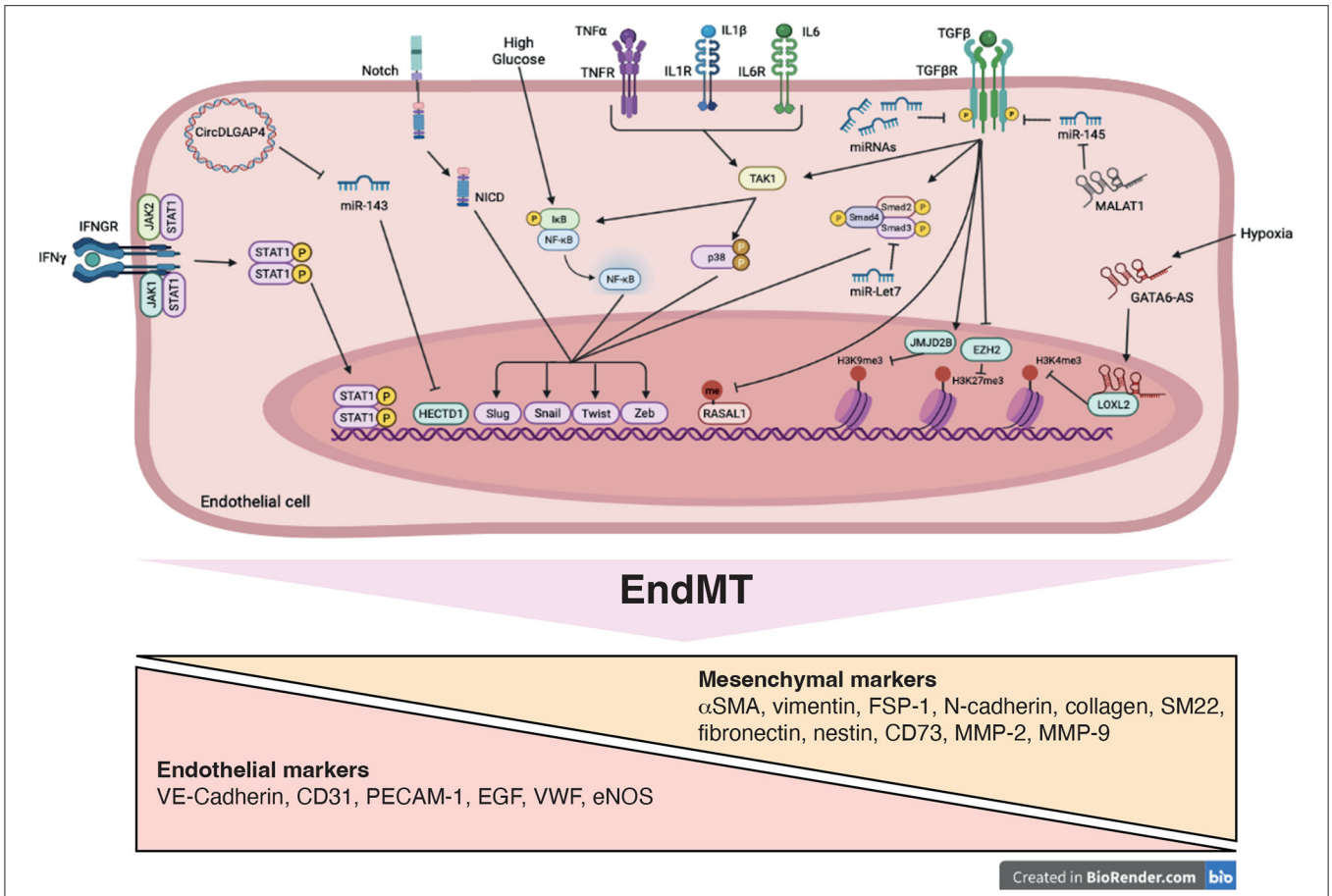

FIGURE 3 | Signaling pathways and mechanistic drivers of endothelial-to-mesenchymal transition (EndMT). Stimulation with transforming growth factor-β (TGFβ), cytokines, Notch ligands, high glucose and hypoxia induce expression of transcription factors, such as Twist, Slug, Zeb and Snail resulting in EndMT. Epigenetic mechanisms including modifications of histone methylation marks and methylation of CpG island are induced. These processes are mediated by variety of actors such as microRNAs (miRNAs), long non-coding RNA (GATA6-AS and MALAT1), circularRNA (DLGAP4) and signaling pathways including SMAD, STAT, NFκB, and p38, promoting EndMT.

(118, 119). Although mechanisms by which EndMT is activated are still undefined, the interplay between hypercoagulopathy in COVID-19 patients and inflammasome activation might need to be further addressed (120).

EndMT IN CARDIOVASCULAR DISEASES

Underlying pathological mechanisms in cardiovascular diseases involve multiple cell types, including endothelial cells, fibroblasts, smooth muscle cells, macrophages and other immune cells. Understanding not only how these various cell types interact during pathologic progression but also how the endothelial phenotype and more specifically EndMT are contributing is key to the molecular regulation of these diseases and has gain immense interest in the past years. The importance of EndMT in cardiovascular diseases has been recently reviewed elsewhere (96). Briefly, lineage tracing experiments have been used determine the contribution of EndMT to cardiovascular pathologies including atherosclerosis and pulmonary arterial hypertension (PAH) that will be discussed below.

Atherosclerosis

Atherosclerosis is characterized by the accumulation of extracellular matrix proteins, mainly collagen and fibronectin, contributing to structural vascular remodeling, thickening of the arterial wall, and plaque formation. EndMT is thought to be one key process that leads to the differentiation of endothelial cells into pro-atherogenic cells leading to plaque formation (88). Recently, single-cell transcriptomic analyses supported evidence that EndMT features were observed in atherosclerotic plaque (121). Since, EndMT has been mostly associated and characterized in area of the vasculature exposed to disturbed flow where atherosclerotic plaque tends to form. Many sensors and pathways involved in transducing mechanical forces in endothelial cells contribute to endothelial dysfunction. Demos et al. recently identified Piezo, a mechanosensor that differentially modulates responses to laminar and disturbed

flow. Piezo is a nonselective cation channel that activates PI3K-eNOS pathway which preserved the endothelial phenotype. On the contrary, disturbed flow activates the NFκB pathway contributing to the endothelial pro-atherogenic phenotype (122). The TGFβ-Alk5 is also important for shear-dependent activation of EndMT. Hence, endothelial-specific deletion of both TGFβR1 (Alk5) and TGFβR2 reduced plaque growth and induced plaque regression revealing an important link between EndMT and atherosclerosis (123). A direct connection between mechanical forces, mechanosensing and EndMT in the regulation of endothelial plasticity has recently been investigated. Results from this study showed that inhibition of Alk5 using siRNA in endothelial cells was associated with reduced flow-induced phosphorylation of SMAD2 and downstream expression of mesenchymal genes. Mechanistically, Alk5 association with Shc (Src homology and collagen) modulated EndMT and plaque formation in atheroprone areas (124). Other studies have confirmed that EndMT features were associated with plaque formation induced by disturbed flow, including expression and activation of Twist and Snail (125). It remains that other mechanosensors are probably involved in EndMT in atherosclerosis and would require further work.

Pulmonary Arterial Hypertension

PAH is a complex pathophysiological adaptation characterized by vascular remodeling and neointimal thickening of pulmonary vessels with increased migratory, inflammatory and metabolically impaired states of smooth muscle cells following endothelial dysfunction and infiltration of immune cells. Direct link between PAH and EndMT has been evidenced in human and experimental models of pulmonary hypertension (126). Mechanistically, studies have demonstrated that EndMT is induced by several factors in the context of PAH including hypoxia, inflammation, TGFβ signaling and ROS production. As mentioned, hypoxia induces HIF1α that can directly bind to Twist and promotes the expression of mesenchymal genes. However, it was found that silencing HIF2α, which is highly expressed in endothelial cells from PAH patients, reduced Snail and Slug expression in association with increased expression of mesenchymal makers and decreased endothelial markers. Interestingly, endothelial-specific deletion of HIF2α prevented the development of hypoxia-dependent pulmonary hypertension in mice (127). However, it remains to determine if HIF2α deletion *in vivo* would rescue or prevent the development of EndMT. Pulmonary arterial endothelial cells appear to respond differently to IL-1β, TGFβ1, TGFβ2 than other vascular beds. Inflammation contributes to dysregulation of TGFβ-BMP signaling, which has been associated with predisposition to PAH. BMPR2 signaling in particular is responsible for the activation of SMAD1/5/8 that has antagonistic effects on TGFβ signaling. Loss of function mutations in BMPR2 gene are associated with increased EndMT-related expression of Twist1 and decreased expression of VE-cadherin. Reduced expression and function of BMPR2 is also associated with BMP9-induced EndMT through higher IL-6 expression. Interestingly, neutralizing IL-6 blocked BMP9 and consequent EndMT (96, 128).

EndMT AND VTE

Studies have also suggested that EndMT may be an important mechanism involved in long term complications of VTE. It is well established that thrombus resolution is associated with vascular fibrosis. However, mechanisms of vein wall fibrosis have only been partially characterized and the role of EndMT in these processes still require intense investigation. When studying the role of CCR7 signaling on vein wall fibrotic remodeling overtime in VTE models, Laser et al. found markers of EndMT in the vein wall. VTE was associated with increased expression of TGFβ, SMA, SM22, FSP-1, collagen I and III and MMP-2. Surprisingly, CCR7 from leukocytes confers protection against EndMT in VTE without affecting thrombus resolution. Although mechanisms of action of CCR7 were not elucidated, this study was the first to implicate EndMT as a potential explanation for fibrotic vein wall repair following VTE (129). Later, Bochenek et al. analyzed and characterized tissue sample from patients with chronic thromboembolic pulmonary hypertension (CTEPH) for structural and cellular composition. Along with the analysis of thrombosis resolution in mice, the authors proposed that fibrosis and scar formation in CETPH result from a sequence of events ranging from fresh to organized thrombus, myofibroblast and endothelial cell activation. These data suggest that fibrotic pulmonary lesions in CTEPH might result from unresolved thrombotic material. However, confirmation in experimental model using lineage tracing experiments would have definitely proved EndMT contribution to vascular fibrosis (130). Further mechanistic studies from this group demonstrated that endothelial TGFβ signaling and EndMT are important drivers of CTEPH. VTE was induced in mice with platelet-specific TGFβ1 deficiency and in mice with an inducible endothelial-specific deletion of TGFβ TGFβRII. The absence of TGFβ1 from platelets was associated with faster thrombus resolution. Surprisingly, endothelial-specific deletion of TGFβ RII significantly delayed thrombus resolution. Thrombi produced in mice with an endothelial-specific deletion of TGFβRII expressed characteristics of EndMT with increased fibrosis, collagen expression and CD31 positive cells co-expressing FSP-1 or SMA. In human CETPH specimen, immunohistochemistry analysis showed overactivation of TGFβ signaling characterized by higher Alk5/SMAD pathway activation. This was explained by higher circulating levels of TGFβ and overexpression of TGFβ. Interestingly, endothelin-1 was also overexpressed and blocking endothelin-1 receptor reversed EndMT and improved thrombus resolution. This study suggest that EndMT may be clinically relevant in CETPH patients contributing to obstruction of pulmonary artery branches with unresolved thrombo-fibrotic material (131). It was also suggested that EndMT might contribute to VTE. In a stenosis model of the iliac vein, thrombosis was associated with higher expression of mesenchymal markers and reduced expression of endothelial markers confirming previous observations. Interestingly, this study demonstrated that the vein wall of mice treated with rivaroxaban showed less markers of EndMT suggesting that anticoagulation therapy might reduce EndMT occurrence (132). These observations might have important

TABLE 2 | Major anticoagulant therapy efficacy, mode of action and potential effects on endothelial function.

Anticoagulant		Action	Efficiency	Potential effects on endothelium
Heparin	Unfractionated heparin (UFH)	Binding to antithrombin III (ATIII) → inhibition of thrombin, FXa and other clotting serine proteases.	Does not reduce mortality, organ damage, or hospital stay with increased risk of bleeding Beneficial effects on mortality in patients with sepsis-induced DIC	Anti-inflammatory effects: • Prevents binding of cytokines and chemokines to endothelial cells and consequent leukocyte recruitment. • Inhibits complement activation. (133)
	Low-molecular-weight heparin (LMWH)		Reduced sepsis severity, decreased 28-day mortality but increased bleeding	
ATIII	Kybernin P	Inhibition α-thrombin, FXa, FIXa, FVIIa, FXIa and FXIIa Binding endothelial GAGs → enhance PGI2 production	No significant reduction in mortality. Coadministration of heparin and ATIII exacerbates bleeding risk	Anti-inflammatory effects: • Increases PGI_2: prevents platelet adhesion and activation. • Inhibition of cytokines and TF expression from endothelial cells and monocytes. (134)
NOACs	Rivaroxaban	Anti-FXa		Anti-inflammatory effects: • Reduces inflammatory genes induced by thrombin in endothelial cells (135). • Attenuation of EndMT in experimental model of VTE (130).
	Dabigatran	Anti-thrombin		Anti-inflammatory effects: • Reduces inflammatory genes induced by thrombin in endothelial cells (135).
APC	rhAPC: Xigris TM	Protein C binding α-thrombin → generate APC Soluble APC → Cleavage and inactivation FVa and FVIIIa	Preclinical model: reduction tissue damage and death Clinical trials: no reduction of death and increased risk for serious bleeding	Anti-inflammatory and cytoprotective effects: • Reduces thromboinflammation. • endothelial barrier stabilizing activities. • Cleavage of PAR-1 on endothelial cells induces protective genes.
	rAPC variants: 3K3A-APC	APC bound to endothelial protein C receptor → cleavage and activation of PAR1	Safer treatment	
Soluble rhTM	ART-123	Activation of PC Suppression of complement, endotoxin, and HMGB-1 protein	Less bleeding improved efficacy and safety in the treatment of DIC compared to heparin Phase 2b trials of sepsis patients: Lower D-dimer, prothrombin fragment and TAT concentration	Anti-inflammatory effects: • Complement and HMGB1 inhibition.
rTFPI	Tifacogin 1	Inhibition FXa and FVIIa/FT complex	Phase 2 trial: Reduction in TAT and IL6 level trend toward reduction in mortality phase 3 trial: not effect on mortality. Attenuated prothrombin fragment and TAT levels, leading to serious bleeding complications	Anti-inflammatory effects: • Reduces cytokine production.

ATIII, antithrombin III; NOAC, novel oral anticoagulant; APC, activated protein C; TM, thrombomodulin; TFPI, tissue factor pathway inhibitor.

repercussions when considering that fibrosis of the vein wall is potentially involved in enhanced risk of VTE recurrence. Additionally, when establishing a model of VTE recurrence in mice, Andraska et al. (133) observed that recurrent venous thrombosis was associated with important vascular remodeling with increased thickness of the vein wall and upregulation of TGFβ, IL-6, elastin and metalloproteinases expression. However, further investigations are still required to decipher precise mechanisms and chronological events occurring during VTE that promote the formation of vascular fibrosis following EndMT activation.

CONCLUDING REMARKS

Reducing the deleterious impact of inflammation and endothelial dysfunction during VTE remains a major therapeutic challenge. This is not surprising considering that both mechanisms are intimately intricated and reflect

complex interactions between immune and hemostatic responses. However, the possibility of reducing long term effect of thrombo-inflammation on the vascular wall would greatly prevent occurrence of PTS, CTED, CETPH and recurrent VTE. Endothelial dysfunction has been extensively studied in different cardiovascular diseases. The relatively recent discovery of the EndMT process highlighted the plasticity of the endothelial phenotype and have open new therapeutic perspectives. Understanding what is triggering EndMT might be as important as investigating mechanisms activated within the endothelium and how this translates into endothelial dysfunction and fibrosis. With accumulating evidence of the impact of EndMT in cardiovascular diseases, therapeutic strategies to prevent or reverse EndMT need to be considered.

A wide range of potential EndMT inducers are release during thrombo-inflammation. IL-1β, for example, represent an interesting target since it has been demonstrated in the CANTOS trial that its inhibition with canakinumab, might prevent recurrent cardiovascular events (134). Thus, future studies should consider similar strategies in the context of VTE. In addition, selective small P2X7 inhibitors were developed and may be used to block excessive IL-1β secretion during VTE as it has been suggested for atherosclerosis (49). IFNγ is also an inducer of EndMT. We and others have demonstrated that IFNγ was important for NK and T cell pro-coagulant activities in VTE. However, no studies have yet investigated if IFNγ was directly contributing to EndMT during VTE.

As recently reviewed by Choi et al., several drugs tested in clinical trials have been reported to inhibit EndMT in various animal disease models [see Table 3 of (65)]. These drugs block EndMT by targeting signaling molecules of the TGFβ pathway. An interesting strategy might be to prevent interactions between the endothelium, platelets and leukocytes that contribute to thrombosis, inflammation and EndMT. Severe coronavirus disease 2019 (COVID-19) infection is associated with endothelial pro-inflammatory and pro-thrombotic state that may be caused by release of VWF and P-selectin. Thus, a clinical trial is currently testing a monoclonal antibody targeting P-selectin, Crizanlizumab. This treatment might decrease thrombo-inflammation by blocking platelet and leukocyte interaction with endothelial cells thus reducing thrombo-inflammation (ClinicalTrials.gov Identifier: NCT04435184). Interestingly, given the interactions between coagulation and endothelial inflammation highlighted in this review, it is reasonable to assume that anti-coagulant therapies may have protective effects on endothelial phenotype. Heparin, a widely used anti-coagulant, have anti-inflammatory effects. Heparin and low molecular weight heparin (LMWH) can bind most of chemokines and cytokines. By competing with endothelial cell surface for cytokines, heparin and LMWH disrupts interactions between endothelial glycocalyx and cytokines thus preventing activation and trafficking of leukocytes (135). Antithrombin III might also exerts anti-inflammatory effects through upregulation of PGI_2 by interacting with heparan sulfate on endothelial surface and inhibition of cytokines and TF production in endothelial cells and monocytes (136). New oral anticoagulants (NOACs), in particular rivaroxaban and dabigatran, show anti-inflammatory and endothelial protective effects. Rivaroxaban or dabigatran significantly reduced pro-inflammatory genes induced by thrombin in endothelial cells (137). Importantly, as mentioned above, rivaroxaban attenuates EndMT in a model of iliac stenosis (132). Alternative therapeutic strategies using the natural anti-coagulant properties of the endothelium have been developed. Thus, recombinant APC, TM and TFPI molecules were tested in experimental models and clinical trials (138). These molecules appear to modulate the endothelial inflammatory response that might potentially prevent interactions with platelets and leukocytes (**Table 2**).

It is unlikely that one drug may be used to treat all forms of EndMT in cardiovascular diseases. Because there is different kind of endothelium, it is highly probable that therapy would have to be adapted to specific features of each pathology. A better understanding of the mechanisms and regulatory networks controlling thrombo-inflammation-dependent development of VTE is still required.

AUTHOR CONTRIBUTIONS

MP, EO, VG-L, FC, and CL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Identification of Key Genes as Early Warning Signals of Acute Myocardial Infarction Based on Weighted Gene Correlation Network Analysis and Dynamic Network Biomarker Algorithm

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Purpose: The specific mechanisms and biomarkersunderlying the progression of stable coronary artery disease (CAD) to acute myocardial infarction (AMI) remain unclear. The current study aims to explore novel gene biomarkers associated with CAD progression by analyzing the transcriptomic sequencing data of peripheral blood monocytes in different stages of CAD.

Material and Methods: A total of 24 age- and sex- matched patients at different CAD stages who received coronary angiography were enrolled, which included 8 patients with normal coronary angiography, 8 patients with angiographic intermediate lesion, and 8 patients with AMI. The RNA from peripheral blood monocytes was extracted and transcriptome sequenced to analyze the gene expression and the differentially expressed genes (DEG). A Gene Oncology (GO) enrichment analysis was performed to analyze the biological function of genes. Weighted gene correlation network analysis (WGCNA) was performed to classify genes into several gene modules with similar expression profiles, and correlation analysis was carried out to explore the association of each gene module with a clinical trait. The dynamic network biomarker (DNB) algorithm was used to calculate the key genes that promote disease progression. Finally, the overlapping genes between different analytic methods were explored.

Results: WGCNA analysis identified a total of nine gene modules, of which two modules have the highest positive association with CAD stages. GO enrichment analysis indicated that the biological function of genes in these two gene modules was closely related to inflammatory response, which included T-cell activation, cell response to inflammatory stimuli, lymphocyte activation, cytokine production, and the apoptotic signaling pathway.

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DNB analysis identified a total of 103 genes that may play key roles in the progression of atherosclerosis plaque. The overlapping genes between DEG/WGCAN and DNB analysis identified the following 13 genes that may play key roles in the progression of atherosclerosis disease: SGPP2, DAZAP2, INSIG1, CD82, OLR1, ARL6IP1, LIMS1, CCL5, CDK7, HBP1, PLAU, SELENOS, and DNAJB6.

Conclusions: The current study identified a total of 13 genes that may play key roles in the progression of atherosclerotic plaque and provides new insights for early warning biomarkers and underlying mechanisms underlying the progression of CAD.

Keywords: CAD, monocyte, transcriptomics, WGCNA, DNB

INTRODUCTION

Coronary artery disease (CAD) remains the leading cause of disease burden worldwide (1). Based on clinical presentation, myocardium injury biomarkers, electrocardiography characteristics, and the extent of myocardium injury, CAD is generally classified as stable CAD and acute coronary syndrome (ACS), which included unstable angina and acute myocardial infarction (AMI). Stable CAD is primarily caused by lumen stenosis caused by atherosclerotic plaque and subsequent oxygen demand-supply mismatch, while ACS is the rupture of vulnerable plaque and subsequent occlusive thrombosis formation and myocardial necrosis (2).

However, the exact mechanism underlying the formation, progression, and rupture of plaque is unclear. The current well-established mechanism of this process involves lipid-driven inflammation (3). Monocyte-derived macrophages are one type of the key inflammatory cells within the plaque and participated in each stage of atherosclerosis plaque formation (4). The rapid development of high-throughput omics technology, such as genomics, transcriptomics, proteomics, and metabolomics, provides new insights into the mechanisms and biomarkers of CAD. For instance, peripheral RNA expression differed significantly between CAD patients and normal control (5) and within CAD patients (6).

There has been rich evidence in studies on biomarkers for diagnosis and risk stratification of coronary heart disease. The validated biomarkers are related to different pathophysiological processes of coronary heart disease, including myocardial injury, altered myocardial stress, inflammation, and vascular endothelial dysfunction. For example, cardiac troponin T (cTnT) and cardiac troponin I (cTnI) demonstrate myocardial tissue specificity (7) and are released into the blood when myocardial tissue suffers damage, leading to an elevated concentration level in peripheral blood (8). Likely, a heart-type fatty acid-binding protein is released into peripheral blood during AMI, and studies confirm its prognostic value for patients with suspected ACS and negative cardiac troponin test results (9, 10). The natriuretic peptide family is a set of typical biomarkers associated with myocardial stress. In 2017, B-type natriuretic peptide (BNP) and N-terminal pro-B-type natriuretic peptide (NT-proBNP) were recommended for the diagnosis, evaluation, and management of heart failure patients by AHA (11). Also, a recent study shows that BNP and NT-proBNP can guide risk stratification in patients with coronary heart disease (12). The soluble suppressor of tumorgenicity 2 (sST2) is a typical biomarker associated with inflammation and is proved to be an independent risk factor for long-term all-cause death in a stable coronary disease cohort (13). Endothelin, converted from its relatively stable primer big endothelin-1, has a strong constrictive effect on the coronary arteries, leading to endothelial dysfunction (14, 15). Research evidence shows that big endothelin–1 has the ability to predict long–term prognosis for both stable coronary heart disease and acute myocardial infarction (16, 17).

However, previous studies mainly focused on the biomarkers associated with the occurrence of CAD, while the biomarkers associated with the progression of CAD remain lacking.

Weighted correlation network analysis (WGCNA), proposed by scholars Zhang and Horvath, is an efficient and accurate bioinformatics method for analyzing microarray data (18). WGCNA analysis divides genes into several modules based on the similarity of gene expression profile and identifies the gene module and corresponding hub genes that are highly correlated with the clinical trait of interest.

WGCNA methods have been successfully applied to identify hub genes in many diseases, including cardiovascular disease (19), cancer (20), psychological disease (21), etc.

However, the primary objective of WGCNA, like most traditional bioinformatics methods, is to distinguish a disease state from a normal state or to diagnose the disease state rather than the "predisease" state (22). In other words, WGCNA may fail to accurately predict the early onset of disease before its development. To overcome this limitation, Chinese scholars Chen et al. proposed the dynamic network biomarker (DNB) theory based on the dynamic features of molecules within the biological system (23). Compared with traditional bioinformatics methods such as WGCNA, the DNB method mainly aims to diagnose the "predisease state," which can detect the early warning signals and achieve early diagnosis before the onset of disease. The DNB method has been successfully used to detect the critical transition point and key molecules in many pathological process (22, 24–26). We hypothesize that the combined use of both WGCNA and the DNB method will contribute to the identification of robust biomarkers to predict disease deterioration.

The current study aims to explore novel gene biomarkers as early warning signals of AMI by analyzing the gene expression profile of peripheral blood monocytes in different stages of CAD based on transcriptomic sequencing technology, combined with both WGCNA and DNB methods.

MATERIALS AND METHODS

Study Design and Participants

The study design is shown in **Figure 1**. We collected blood samples from patients who agreed to provide blood samples from June 2011 to March 2015 at Fuwai Hospital. Eligible patients had symptoms indicating CAD and were receiving elective coronary angiography. We excluded patients with rheumatic heart disease, organic heart diseases and cardiomyopathy, severe liver and renal dysfunction, severe infectious diseases, malignant tumors, immune system and connective tissue diseases, and metabolic diseases, including hyperthyroidism and Cushing syndrome. Fasting blood samples and demographic information were collected at Fuwai Hospital after obtaining informed consent. Specifically, blood sample of patients in the AMI group were collected when they were rehospitalized due to ischemia symptoms. Sampling was performed prior to angiography in the fasting status. We randomly selected 8 patients with stable coronary heart disease and angiography–confirmed intermediate lesion (IML) (visual stenosis 50%–70%), as well as 8 age– and sex–matched patients with normal coronary angiography (NCA) and patients with AMI. AMI was diagnosed according to the third universal diagnosis of acute myocardial infarction, with cardiac biomarker (primarily cTn) elevation above 99% upper reference limit with ischemia symptoms, new–onset ischemic ST–T segment change or left bundle branch block, and Q wave formation. Patients in the IML group had objective evidence of ischemia such as chest pain and other myocardial ischemia symptoms or positive exercise stress test results. In recent years, the coronary physiology evaluation method fractional flow reserve (FFR) is the "gold standard" for identifying lesions with physiological significance. Recent studies have also confirmed that a high proportion of intermediate lesions, despite diameter stenosis <80%, may cause myocardial ischemia as detected by FFR. In addition, patients with intermediate lesions varied significantly in their long–term prognosis despite a similar degree of lesion stenosis. Therefore, the current study enrolled patients with intermediate lesions to represent the disease stage of "stable coronary heart disease." The study protocol complied with the Declaration of Helsinki and was approved by the ethics committee of Fuwai Hospital (No. 2012–431).

Peripheral Blood Monocyte Isolation, RNA Isolation, and Sequencing

Fasting blood samples were collected in the morning before the angiography procedure. Leukocytes were isolated by centrifugation, and monocytes were isolated by using EasySep™ Human CD14 Positive Selection Kit (#18058, Stemcell, USA) following the manufacturer's instructions. RNA was extracted from leukocytes by using TRIzol® Reagent (#15596018, Invitrogen, USA) and frozen at −80°C. The purity of RNA was assessed by NanoPhotometer Spectrophotometers (IMPLEN, USA), and the quantity and quality of RNA were assessed by Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Assay Kits.

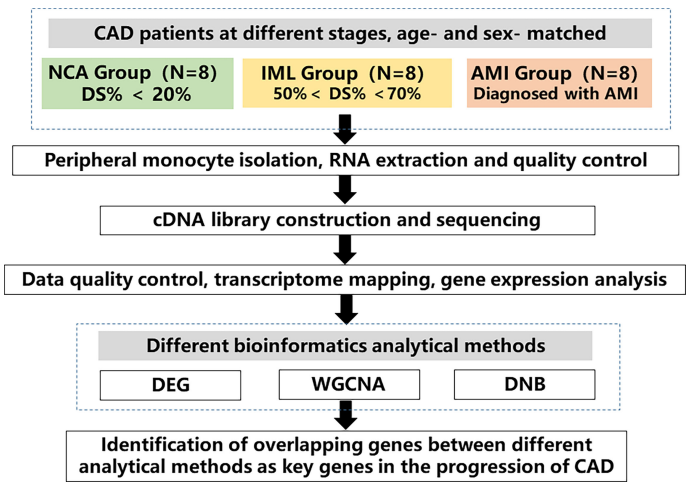

FIGURE 1 | Study flow chart. A total of 24 age– and sex–matched coronary artery disease (CAD) patients at different disease stages were enrolled, which included 8 patients with normal coronary angiography (NCA), 8 patients with intermediated coronary lesion (IML), and 8 patients with acute myocardial infarction (AMI). Peripheral monocytes were isolated and RNA was extracted. Transcriptomic sequencing was performed, and various analytical methods were used to analyze gene expression profile across different groups. The overlapping genes between different analytical methods were identified as key genes in the progression of CAD. CAD, coronary artery disease, NCA, normal coronary angiography, IML, intermediate coronary lesion, AMI, acute myocardial infarction, DEG, differential expressed genes, WGCNA, weighted gene correlation network analysis, DNB, dynamic network biomarker.

A total of 3 μg of qualified RNA was used to construct the cDNA library. mRNA was purified from total RNA using poly–T oligo–attached magnetic beads and fragmented with NEBNext First Strand Synthesis Reaction Buffer (5×). The sequencing was performed by Annoroad Gene Technology Corporation (Beijing) by using the Illumina Novaseq S2 platform (Illumina, USA) with the PE–150 module.

Data Processing

Basic data processing included three major steps: data quality control, transcriptome mapping, and gene expression analysis. Raw data were filtered by the Cutadapt software to remove low–quality sequencing reads and generate high–quality data (clean reads). The human reference database and annotation files were downloaded from the ENSEMBL database (version Homo_sapiens.GRCh38.91.chr). The clean reads were then aligned to the reference genome by using HISAT2 v2.1.0 software.

Fragments per kilobase per million mapped fragments (FPKM) were calculated to assess gene expression level and used for subsequent further analysis. The algorithm for calculating FPKM is as follows:

$$FPKM = \frac{10^3 * F}{NL/10^6}$$

where F is the number of reads mapped to the gene, N is the total number of mapped reads or fragments, and L is the gene length.

Differential Expression Analysis and GO Enrichment Analysis

Differentially expressed gene (DEG) analysis was performed by using DESeq2 R packages, and genes with fold change ≥1.5 or fold change ≤0.67 and an adjusted p–value <0.05 were identified as differentially expressed genes. The volcano plot was used to visualize differentially expressed genes between groups and was generated by the ggplot packages. GO enrichment analysis was used to investigate the biological function of differentially expressed genes, which was performed and visualized on the Metascape website (https://metascape.org/gp/index.html#/main/step1) (27). The parameters were set as follows: min overlap of 3, p–value cutoff of 0.01, and minimum enrichment factor of 1.5. The top 20 enriched pathways were selected for visualization.

WGCNA Analysis

The WGCNA analysis was performed by using the R package "WGCNA" (28), which mainly included three steps: construction of a gene coexpression network, identify gene modules, and gene module–clinical trait correlation analysis. The expression matrix was constructed with the original FPKM. We included genes with FPKM greater than zero in more than 8 samples. The soft–threshold power was chosen by the pickSoftThreshold function. The gene network was constructed by the blockwiseModules function, and the parameters were set as follows: minModuleSize = 30, reassignThreshold = 0, and mergeCutHeight = 0.25. The corresponding eigengenes were obtained to summarize the expression profile of each gene module. The association between each gene module and clinical trait was performed by using the Pearson's correlation method, which correlate the module eigengenes with each clinical trait. Genes with absolute gene modulemembership > 0.8 and genetraitsignificance > 0.2 were identified as hub genes (29).

DNB Analysis

A detailed theoretical foundation and computational algorithm for DNB analysis were described previously (23). Based on the DNB theory, the progression of the disease is not smooth but abrupt (**Figure 2**), and there is a critical transition point after which the system shifts abruptly from one state to another. According to this transition point, the disease progression process can be divided into three stages: "normal state," which is a relatively stable state where the disease undergoes gradual and slow change, "predisease state," which is the limit of the normal state just before the transition to the disease state, and the "disease state," which is another relatively stable state and is usually irreversible to the normal state. Based on the DNB

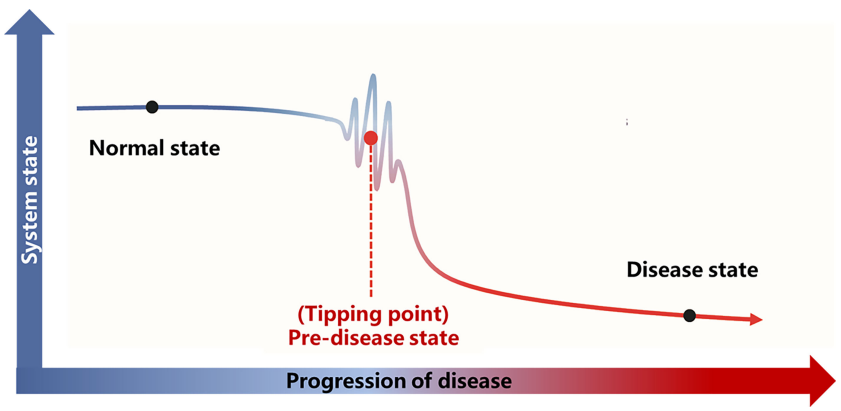

FIGURE 2 | Illustration of DNB theory. Based on the DNB theory, there is a critical transition point (tipping point) during the progression of the disease. The disease progression process can be divided into three stages according to the tipping point: the normal state, the predisease state, and the disease state. The abbreviations are the same as above.

theory, there exists a group of molecules (genes, proteins or metabolites, etc.) in the predisease state, which can be used for predicting disease. This group of molecules characterizes the dynamic features of the underlying system and are termed as DNB and satisfies the following three criteria:

1. The average standard deviations (SDs) of DNB molecules increase drastically.
2. The average Pearson's correlation coefficients (PCCs) of DNB molecules drastically increase in the predisease state.
3. The average PCCs of molecules between DNB molecules and non–DNB molecules drastically decrease.

The above three criteria can which can be represented as a composite index (CI):

$$CI = \frac{SD_d \text{x} PCC_d}{PCC_O}$$

whereas SD_d is the average standard deviation (SD) for molecules inside the DNB module, PCC_d is the average Pearson's correlation coefficient (PCC) in absolute value for molecules inside the module, and PCC_o is the average PCC in absolute value for molecules between DNB and non–DNB. The CI is expected to increase abruptly and significantly before the critical transition to the disease state and can serve as an early warning signal.

Statistical Analysis

SPSS 26.0 was used for statistical analysis. Continuous variables were expressed as mean ± standard deviation, and comparisons between groups were performed by analysis of variance. Categorical variables were expressed as frequency (percentage) using the chi–square test or Fisher's exact test for comparison between groups. The difference was considered statistically significant when the bilateral P value was less than 0.05.

RESULTS

Clinical Characteristics of the Recruited Patients

The baseline characteristics of the 8 IML patients and 8 gender–and–age–matched NCA and AMI patients are demonstrated in **Table 1**. Among the 24 patients, 17 (70.83%) were men, with a median age of 64 years. Additionally, no significant difference was found in terms of age, gender, and body mass index (BMI) between the different groups. As expected, CAD patients had a significantly higher rate of family history of CAD and hyperlipidemia, and more tended to be current smokers, but the difference was not statistically significant.

Differential Gene Expression Analysis of Different Phases of CAD Patients

Gene expression profiles differed significantly across groups. As shown in **Figure 3**, there were a total of 192 DEGs between the NCA and IML groups, 2,269 DEGs between the AMI and NCA groups, and 385 DEGs between the AMI and IML groups. To analyze the potential biological function of DEGs, the online analytic tool Metascape was used, and differential genes were uploaded on the website. The top enriched pathways of the DEGs between the IML group and the NCA group were associated with cell adhesion, cell migration, and cell response to inflammation, the top enriched pathways of the DEGs between the AML group and the NCA group were associated with cellular response to stress and cellular cytokine production, and the top enriched pathways of the DEGs between the AML group and the IML group were associated with mRNA metabolic process, cellular signal transduction, and cellular response to growth factor stimulus (**Supplementary Figures S1–S3**).

WGCNA Analysis

The soft threshold of 8 was used to construct the scale–free network (**Figure 4**). Nine modules were identified based on average hierarchical clustering and dynamic tree clipping (**Figure 5**). The number of genes in each gene module is shown in **Table 2**.

To identify the genes associated with the progression of atherosclerosis, we evaluated the association between each gene module and clinical trait by calculating the module significance (MS) for each module–trait correlation (**Figure 6**). Disease stage is a trichotomous variable that indicates NCA, IML, and AMI. The pink module had the highest positive association with disease stage (r^2 = 0.64, p = 8e−04), followed by the turquoise module (r^2 = 0.55, p = 0.005).

Genes within each gene module, as well as the corresponding module membership and gene significance, are shown in

TABLE 1 | Baseline characteristics across groups.

	NCA group (*N* = 8)	IML group (*N* = 8)	AMI group (*N* = 8)	*p*–value
Age (years)	59.75 ± 8.71	63.38 ± 9.04	63.25 ± 8.29	0.741
Male (%)	5 (62.50)	6 (75.00)	6 (75.00)	1.000
BMI (kg/m^2)	24.75 ± 2.13	22.72 ± 2.28	26.00 ± 3.31	0.062
Diabetes (%)	2 (25.00)	1 (12.50)	3 (37.50)	0.837
Hypertension (%)	6 (75.00)	5 (62.50)	4 (50.00)	0.866
Dyslipidemia (%)	5 (62.50)	5 (62.50)	7 (87.50)	0.866
Family history of CAD (%)	0 (0.00)	7 (87.50)	1 (12.50)	0.557
Current smoker (%)	1 (12.50)	5 (62.50)	3 (37.50)	0.171
Alcohol (%)	4 (50.00)	1 (12.50)	2 (25.00)	0.402
LVEF (%)	64.95 ± 6.70	65.12 ± 4.36	58.43 ± 9.76	0.136

NCA, normal coronary angiography, IML, intermediate lesion, AMI, acute myocardial infarction, BMI, body mass index, CAD, coronary artery disease, LVEF, left ventricular ejection fraction.

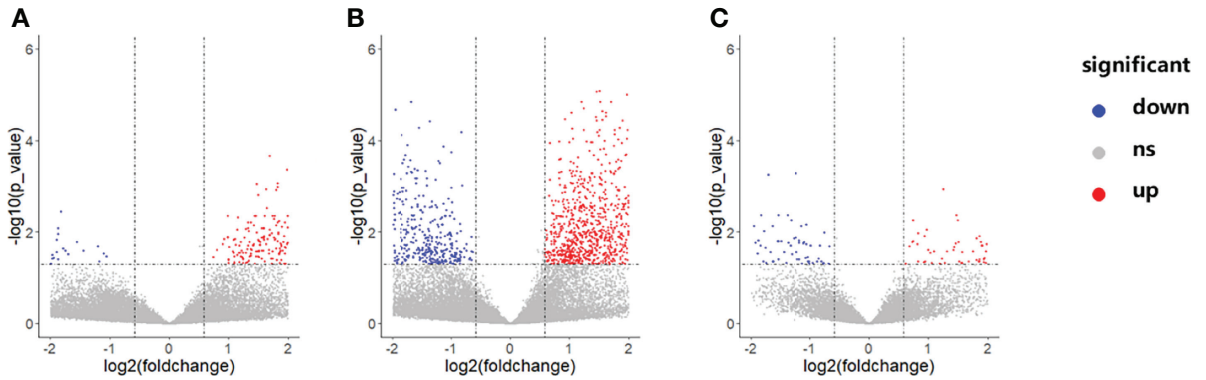

FIGURE 3 | Volcano plot of differentially expressed genes between the IML and NCA groups **(A)**, AMI and NCA groups **(B)**, as well as AMI and IML group **(C)**. The abbreviations are the same as above.

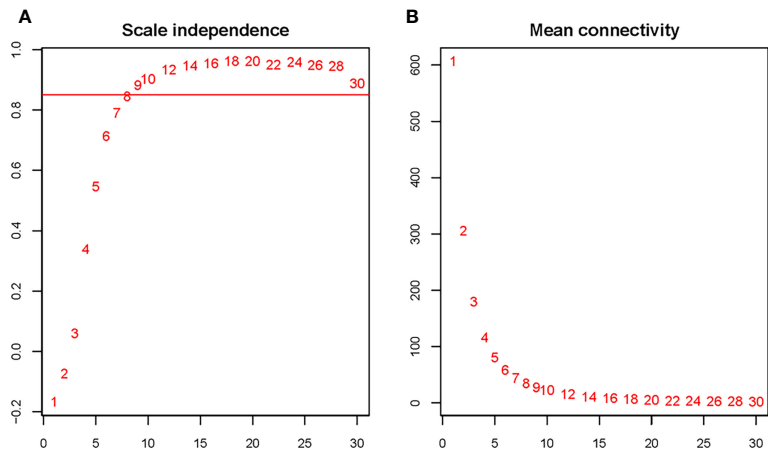

FIGURE 4 | Determination of the soft threshold for the WGCNA analysis. Analysis of the scale–free index for various soft–threshold powers **(A)** and mean connectivity **(B)** for various soft–threshold powers. The abbreviations are the same as above.

Supplementary Table S1. The top 3 GO–BP–enriched pathways in pink module are endoplasmic reticulum organization, regulation of alpha–beta T cell, and peptidyl–serine phosphorylation (Figure not shown). The top enriched pathways in the turquoise module are associated with cell response to inflammatory stimuli, lymphocyte activation, cytokine production, and the apoptotic signaling pathway (**Supplementary Figure S4**), which has been reported to be closely associated with the atherosclerotic process.

The scatter plot for gene significance and module membership for the above gene modules are shown in **Supplementary Figure S5**.

DNB Genes

The average DNB score for each group is shown in **Figure 7**. The average DNB score of the IML group is greater than that of the NCA and AMI groups, which indicates that the critical transition point of atherosclerosis is at the IML stage. This is also consistent with the pathophysiological process of atherosclerosis progression in clinical practice.

There are a total of 103 DNB genes (**Supplementary Table S2**). The top 3 GO–BP enrichment pathways included regulation of activation of Janus kinase activity, regulation of cell adhesion, and negative regulation of proteolysis (**Supplementary Figure S6**).

Genes Potentially Associated with CAD progression

To identify robust biomarkers that may serve as early warning signals of AMI, we analyzed the common genes (overlapping genes) between differentially expressed gene sets and DNB gene sets and identified the SGPP2 genes that may play key roles in the process of atherosclerosis progression (**Figure 8**).

We also analyzed the overlapping genes between DNB gene sets and WGCNA gene sets and identified the following 12 genes: DAZAP2, INSIG1, CD82, OLR1, ARL6IP1, LIMS1, CCL5,

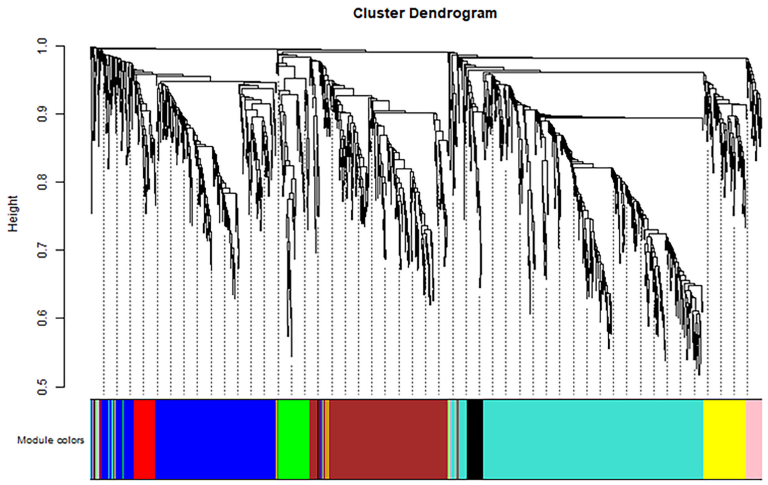

FIGURE 5 | Clustering dendrogram of all differentially expressed genes based on dissimilarity, with an assigned color for each gene module.

TABLE 2 | The number of genes and median MM and GS in each gene module.

Gene module	No. of genes	MM	GS
Gray	12	0.540	0.296
Turquoise	575	0.812	0.441
Blue	367	0.761	0.380
Brown	330	0.803	0.681
Yellow	118	0.854	0.613
Green	77	0.844	0.599
Red	57	0.832	0.430
Black	50	0.829	0.290
Pink	40	0.796	0.508

MM, module membership; GS, gene significance.

CDK7, HBP1, PLAU, SELENOS, and DNAJB6. Gene expression levels are expressed in the violin plot in **Figure 9**.

DISCUSSION

CAD remains the leading cause of disease burden worldwide. Elevated LDL–c levels are the well–established principal risk factor for the onset and progression of CAD. However, a substantial number of patients still suffer from adverse cardiovascular events despite the reduction of LDL–c level to 40 mg/dl or less (30, 31), suggesting additional "residual cardiovascular risks" are yet to be explored.

In the microenvironment of coronary atherosclerotic plaques, the main type of immune cell is the macrophage, which is differentiated from peripheral blood monocyte. In atherogenic conditions, circulation monocytes sense changes in the microenvironment and adopt specific gene expression in response. These monocytes are then recruited to the subintimal layers of the artery wall and differentiated into macrophages. Therefore, studying the gene expression of peripheral blood monocytes in different stages of CAD through RNA–sequencing will help to reveal the underlying mechanism.

Disease progression is a complex nonlinear process, which is not necessarily "smooth" but "abrupt" (23). There is usually a drastic change during disease progression, which causes the critical transition from the normal state to the disease state (**Figure 2**).

Traditional bioinformatics methods primarily compare the molecular characteristics between disease and normal state and poorly detect the predisease state, or accurately predict the onset/deterioration of disease before its occurrence, due to the similarity of molecular characteristics between the pre–disease state and the normal state. To overcome this limitation, a novel computational algorithm, dynamic network biomarkers, was proposed to detect the critical transition point in the complex biological process, such as the progression of atherosclerosis in our study. The DNB theory was based on solid nonlinear dynamic system theory and was reported to serve as an early–warning signal prior to disease deterioration.

Several of these genes have been reported to be closely associated with the onset or progression of atherosclerosis, further demonstrating the robustness of our conclusion.

C–C motif chemokine ligand 5 (CCL5) encodes a 68–amino acid chemokine, which functions as a chemoattractant for blood immune cells and the natural ligand for the chemokine receptor chemokine (C–C motif) receptor 5 (CCR5). CCL5 is involved in a wide range of inflammatory processes, including advanced atherosclerosis and myocardial reperfusion injury. CCL5 has been detected in atherosclerosis plaque (32). CCR5 deficiency reduces the development of diet–induced atherosclerosis in mice (33). Inhibition of CCL5 reduces myocardial reperfusion in atherosclerosis mice (34).

Oxidized low–density lipoprotein receptor 1 (OLR1) encodes a low–density lipoprotein receptor, which binds, internalizes, and degrades oxidized low–density lipoprotein. The previous meta–analysis demonstrated a significant association between OLR1 gene polymorphisms and CAD risk (35). OLR1 promotes endothelial dysfunction by inducing pro–atherogenic signaling

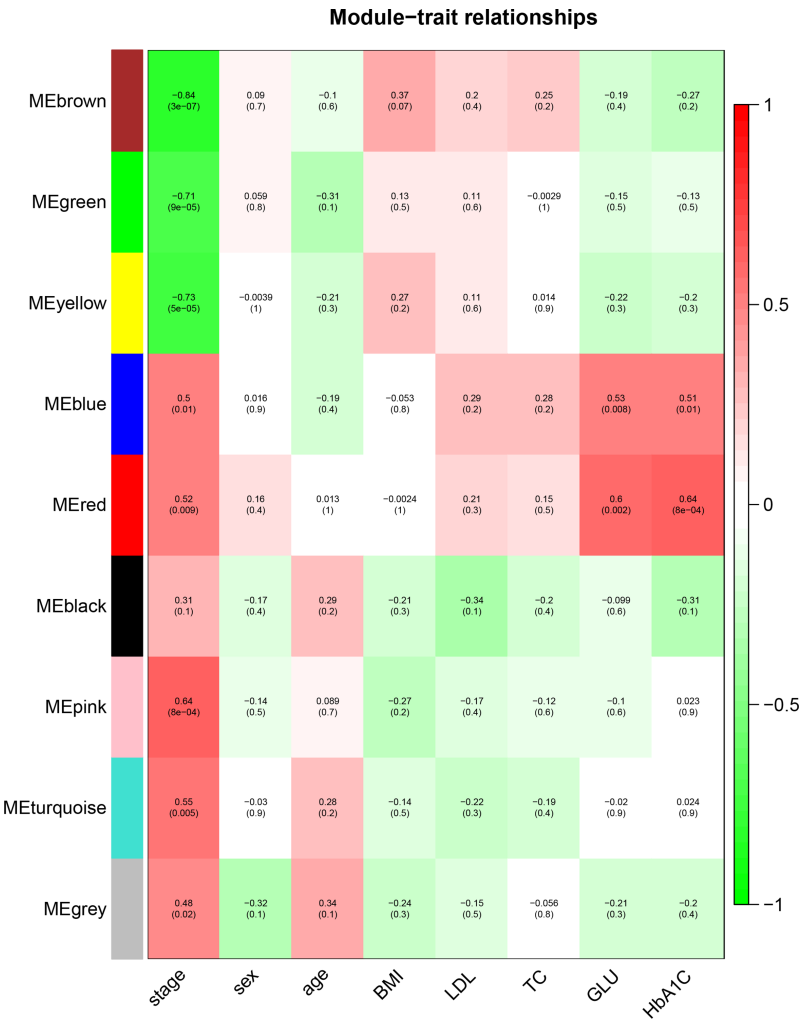

FIGURE 6 | Identification of the gene module associated with clinical traits. Heatmap of the correlation between the module eigengenes and clinical traits of CAD patients. Disease stage is a trichotomous variable (NCA, IML, and AMI). The number in each cell is the correlation coefficient (the corresponding *p*–value). The abbreviations are the same as above.

via the endothelial uptake of oxidized LDL (oxLDL), which contributes to the initiation, progression, and destabilization of atheromatous plaques (36). In addition to its expression in endothelial cells, OLR1 is also expressed in immune cells, including macrophages, lymphocytes, and neutrophils, further implicating this receptor in multiple aspects of atherosclerotic plaque formation. In conclusion, OLR1 holds promise as a novel diagnostic and therapeutic target for atherosclerosis and CHD.

Plasminogen activator, urokinase (PLAU) encodes a secreted serine protease that converts plasminogen to plasmin. PLAU has been implicated in a broad spectrum of biological and pathological processes, including chemotaxis, cell adhesion, migration and growth, fibrinolysis, proteolysis, angiogenesis, inflammation, and neointima formation (37). PLAU has also been reported to be associated with atherosclerosis plaque formation and AMI: macrophage–specific overexpression of the PLAU gene accelerated atherosclerosis, coronary artery occlusions, and premature death in ApoE$^{-/-}$ mice (38). PLAU has already been reported to be closely associated with AMI: an SNP rs4065 of the PLAU gene is associated with AMI risk in the Chinese Han population (39). Taken together, the above evidence suggests the PLAU gene as a novel therapeutic target for the treatment of atherosclerosis.

Several genes have been reported to be involved in metabolic or inflammatory processes and, therefore, may potentially be involved in the pathophysiological process of atherosclerosis. However, this hypothesis still needs to be demonstrated in future studies.

Sphingosine–1–phosphate phosphatase 2 (SGPP2) is differentially expressed between both the AMI and NCA groups, as well as the AMI and IML groups. SGPP2 encodes sphingosine–1–phosphate phosphatase 2, which can degrade sphingosine 1–phosphate (S1P) to produce sphingosine. SGPP2 is expressed in human umbilical vein endothelial cells

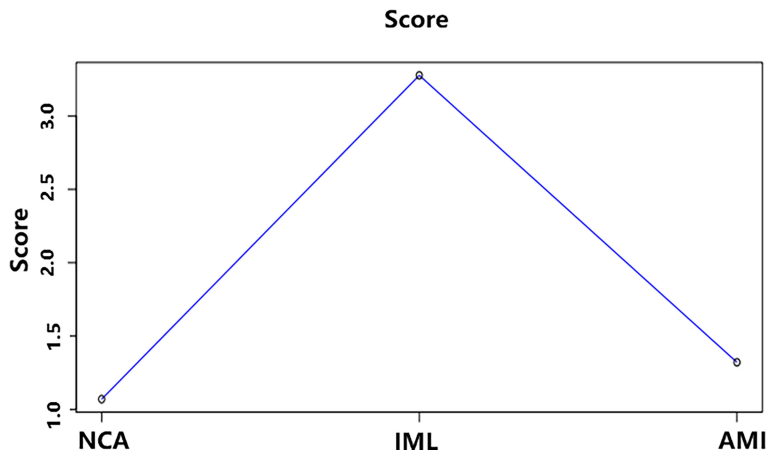

FIGURE 7 | The average DNB score and corresponding parameters for the three groups. *x*–axis represents the three groups, and *y*–axis represents the average DNB score for each group.

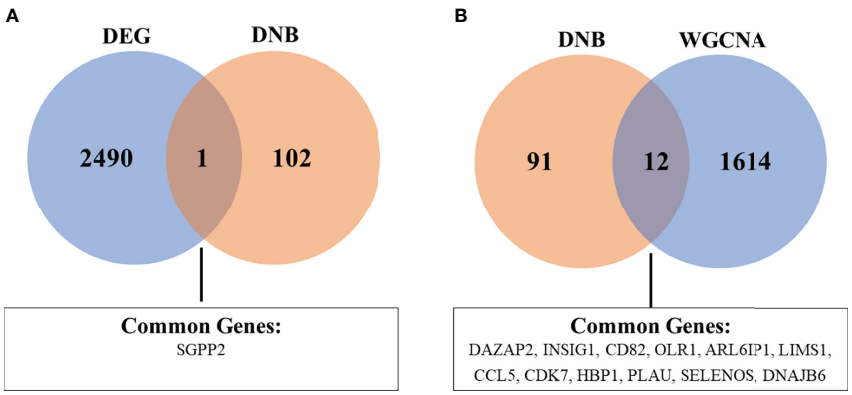

FIGURE 8 | Venn diagram showing the common genes between DEG and DNB **(A)**, as well as DNB and WGCNA analyses **(B)**. The abbreviations are the same as above.

and neutrophils (40). The results of previous studies suggest that the *SGPP2* gene may be involved in the onset and progress of atherosclerosis. The *SGPP2* gene affects the endothelial barrier function *via* altering the expression of interleukin 1–β (IL–1β) (40) in endothelial cells. IL–1β is a proinflammatory cytokine that can induce endothelial cell inflammation and destroy the endothelial barrier function (41). In addition, SGPP2 is involved in the inflammatory response process: SGPP2 knockout mice showed reduced expression of proinflammatory factors, including IL–6, TNF–α, and IL–1β, molecular mechanisms, and inhibition of inflammation–induced signal transducer and activator of transcription 3 (STAT–3) signal pathway activation related. The activation of the STAT–3 signaling pathway plays an important role in the process of macrophage inflammation and polarization (42). Endothelial barrier dysfunction, inflammatory cell infiltration, and release of inflammatory factors are the key factors leading to plaque progression and rupture.

Insulin–induced gene 1 (INSIG1) gene encodes an endoplasmic reticulum membrane protein that regulates cholesterol metabolism, lipogenesis, and glucose homeostasis in various tissues (43). INSIG1 acts as a negative regulator of cholesterol biosynthesis by mediating the retention of the SCAP–SREBP complex in the endoplasmic reticulum, thereby blocking the processing of sterol regulatory element–binding proteins (SREBPs) (44). INSIG1 gene single nucleotide polymorphisms were associated with coronary heart disease risk in the Chinese Han population (45). Knockdown of INSIG1 resulted in a significant reduction of cholesterol efflux to HDL (46). INSIG1 variation may contribute to statin–induced changes in plasma TG in a sex–specific manner (47).

CD82 encodes a membrane glycoprotein, a member of the transmembrane 4 superfamily. The primary research area of CD82 is tumors, and CD82 has been recognized as a tumor metastasis suppressor gene (48). CD82 inhibits pathological angiogenesis. Endothelial cells CD82 knockout enhanced the migration and invasion capabilities of endothelial cells (49). CD82 also plays a key role in the regulation of endothelial–monocyte interactions, which include monocyte recruitment and

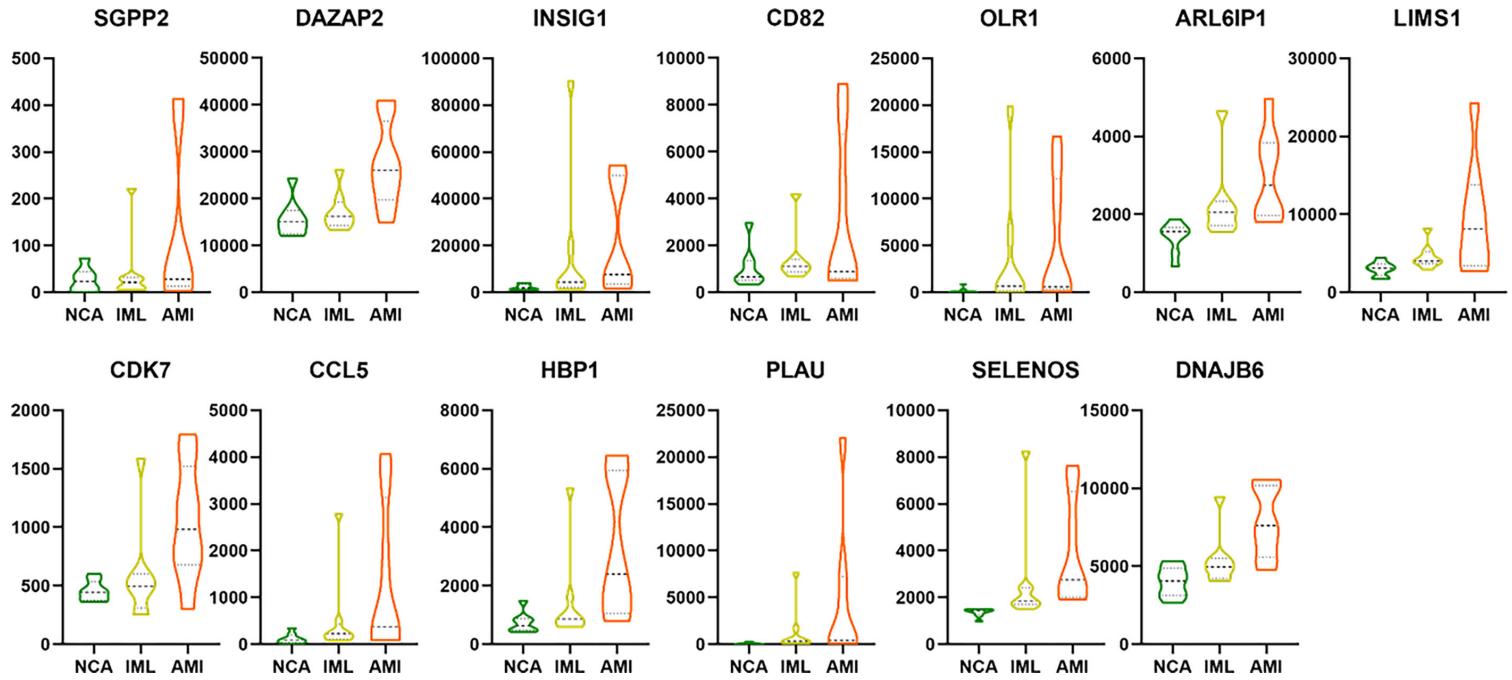

FIGURE 9 | The violin plot showing the gene expression level of the 13 overlapping genes between DEG/WGCNA and DNB analyses. The abbreviation.

migration (50). The above studies suggest that CD82 may affect atherosclerotic plaque formation, which requires validation in future studies.

Selenoprotein S (SELENOS) encodes a transmembrane protein, which is involved in the degradation process of misfolded proteins in the ER and may also have a role in inflammation control. SELENOS has been reported to be associated with the risk of diabetes: genetic polymorphisms of SELENOS genes are associated with diabetes risk in the Chinese population (51, 52). The serum source of SELENOS is primarily from hepatocytes, and the serum level of SELENOS was associated with the risk of DM and its macrovascular complications (53). Given that SELENOS is closely associated with inflammation, oxidative stress, as well as glucose metabolism (53), the above evidence indicates SELENOS played a key role in the pathophysiology process of AS, which requires future validation.

The biological function and their roles in atherosclerosis progression for the other genes, including DAZAP2, ARL6ip, CDK7, LIMS, HBP1, and DNAJB6, remain to be investigated. The current study has several limitations: Firstly, sample size of the current study was limited, and 8 samples were tested using RNA–seq for each group. Setting up biological replicates is necessary to eliminate errors in sequencing studies, and sequencing technique or statistical tools cannot fully eliminate biological variability. One recent study recommended no fewer than 6 biological replicates should be included in a single group in RNA–seq study (54). Also, to compensate for this deficiency, the DNB algorithm was applied. One advantage of the DNB algorithm is that it can identify the critical transition stage even based on small samples of high–throughput data (55). The first publication proposed and demonstrated the validity of the DNB algorithm by detecting the "early warning signals" prior to acute lung injury in carbonyl chloride inhalation–induced acute lung injury, based on 2–5 samples of lung tissue transcriptomic data at each sampling period (23). Secondly, since the current study was a retrospective study, future studies are required to validate whether the key genes in our study can predict adverse events in a prospective cohort, which is an ongoing project. Thirdly, since the current study was based on high–throughput sequencing data, quantitative validation of the gene expression level is required based on the qPCR method. Finally, basic studies are needed to explore the biological functions of the key genes identified in our study.

CONCLUSION AND FUTURE PROSPECTS

In conclusion, based on the peripheral blood mononuclear cell transcriptome sequencing data from patients at different disease progression stages of coronary artery disease, combined with traditional DEG analysis, WGCNA analysis, and novel DNB methods, the current study identified a total of 13 genes that may play key roles involved in the progression of atherosclerotic plaque and provides new insights for early warning biomarkers and potential underlying mechanisms underlying the progression of CAD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE166780.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Fuwai Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CS and ZQ carried out the sequence data analysis, participated in the sequence alignment, and drafted the manuscript. LC and JG created the method of the DNB analysis. SY, XB, CW, RZ, LJ, and QL participated in the design of the study and performed the statistical analysis. RF and KD conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.879657/full#supplementary-material

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Elevated serum C1q is an independent predictor of high residual platelet reactivity in CAD patients receiving clopidogrel therapy

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Background: Inflammation increases the risk of thrombosis in coronary artery disease (CAD) patients and affects the antiplatelet efficacy of clopidogrel. C1q interacts with platelets to activate platelets and induce thrombosis by participating in and regulating the inflammatory response. Whether C1q affects adenosine diphosphate (ADP)-induced platelet reactivity during clopidogrel therapy was unclear and our study aimed to explore the issue.

Method: We enrolled 1,334 CAD patients receiving clopidogrel therapy and evaluated the association between C1q level and high residual platelet reactivity (HRPR) using logistic regression and restricted cubic spline (RCS). HRPR was defined as ADP-induced maximum amplitude (MA_{ADP}) > 47 mm plus ADP-induced platelet aggregation (ADP_i) < 50%.

Results: A total of 516 patients (38.7%) performed HRPR. The frequency of HRPR increases with the increase in C1q level (26.3%, 38.4%, 43.2%, and 46.7% for the 1st to 4th quartile of C1q). The result of multivariate logistic regression demonstrated elevated C1q as an independent predictor for HRPR (2^{nd} quartile: OR = 1.722, 95% CI 1.215–2.440; 3^{rd} quartile: OR = 2.015, 95% CI 1.413–2.874; 4^{th} quartile: OR = 2.362, 95% CI 1.631–3.421, compared to the 1st quartile). RCS depicted the nonlinear relationship between C1q and HRPR risk (*p* for non-linear < 0.05).

Conclusion: The current research is the first to explore the association of C1q and ADP-induced platelet reactivity and to demonstrate elevated C1q as an independent risk factor for HRPR in CAD patients during clopidogrel therapy.

KEYWORDS

clopidogrel, complement C1q, platelet activity, thromboelastography, percutaneous coronary intervention, coronary artery disease

Introduction

As the leading cause of death worldwide, cardiovascular diseases represent more than 40% of all deaths among Chinese (1). CAD, usually caused by atherosclerosis, is one of the most severe cardiovascular diseases, which often results in myocardial dysfunction and/or organic lesions. Percutaneous coronary intervention (PCI) is the major revascularization strategy for CAD patients with clinical indications for improving current myocardial blood supply and long-term outcomes. Dual antiplatelet therapy (DAPT) consisting of aspirin and oral P2Y12 inhibitors represents a guideline-recommended cornerstone of secondary prevention in patients undergoing PCI (2).

Clopidogrel, one of the earlier P2Y12 inhibitor agents to be applied (3), is still widely applied currently in peri-interventional treatment and long-term DAPT after PCI among patients with stable coronary artery disease (SCAD) (2, 4), especially on those at relatively lower risk. Clopidogrel possibly decreases bleeding risk compared with ticagrelor and prasugrel (5, 6) in acute coronary syndrome, especially in elderly patients (7). The antiplatelet efficacy of clopidogrel is influenced by multiple factors like drug interactions, genetic polymorphisms, and clinical and biological factors (8). As a result, a clinically significant proportion of patients treated with recommended doses of clopidogrel cannot the display desired antiplatelet response (9). Previous studies had demonstrated that HRPR during clopidogrel therapy is associated with adverse ischemic events, particularly in early stent thrombosis (10).

Complement component C1q is the recognition molecule of the classical activation pathway, which plays a critical role in defending against invading pathogens, maintenance of immunologic tolerance, and modulation of inflammatory responses (11). Serum level of C1q had been proposed as a biomarker for diagnosis and assessing activity in certain diseases (12). The classical complement pathway is involved in the regulation of atherosclerosis progression and exerts dual effects in different stages (13), and both low and high C1q plasma levels can be potential risk factors for CAD (14–16). Previous research had indicated that inflammation induces plaque rupture and coronary thrombus formation (17), and influences ADP-induced platelet aggregation during clopidogrel treatment. C1q and classical pathway also interact with platelets and coagulation factors to promote hemostasis and thrombotic processes by participating in and regulating inflammatory response (16, 18), and activation of the classical pathway was observed in acute coronary thrombi (19). However, whether C1q affects the antiplatelet efficacy of clopidogrel remains unknown. The present investigation collected clinical data from SCAD patients who had undergone elective PCI to compare the differences in clinical characteristics between individuals with and without ADP-induced HRPR and aimed to explore the contribution of C1q on ADP-induced HRPR during clopidogrel therapy.

Methods

Study design and patients

A cross-sectional study was carried out to explore the association between C1q and ADP-induced HRPR during clopidogrel therapy. Study data were derived from a prospectively collected database, which was created in the Twelfth Department of Cardiology, Beijing Anzhen Hospital, Capital Medical University. We consecutively recruited 1,404 SCAD patients undergoing elective PCI prospectively and evaluated their platelet function using thromboelastography (TEG) from January 2019 to December 2019. All the subjects were Chinese Han population of northern China. All participants were required to receive a loading dose (300 mg) of clopidogrel at least 12 h before PCI or receive a maintenance dose (75 mg, once daily) for at least 5 days before PCI. The following were the exclusion criteria (1) age <18 years old, (2) an abnormal baseline platelet count of <50 × 109/L, or >400 × 109/L, (3) taking other drugs known to affect platelet function, (4) intolerant to DAPT therapy consisting of aspirin and clopidogrel [e.g. BARC type 3 to 5 bleeding (20) or drug allergy], (5) presence of acute heart failure, (6) presence of acute or chronic infections, (7) known history of rheumatic immune diseases, (8) with neoplastic diseases, and (9) severely damaged renal or/and liver function (estimated glomerular filtration rate < 30 ml/min/1.73 m2, alanine aminotransferase > 2.5 times the normal upper limit). The details of inclusion and exclusion criteria are shown in Figure 1.

Demographic, clinical, and laboratory information

We collected and documented every patient's information according to the unified standard process. Data entry clerks collected demographic, clinical, and laboratory information from Beijing Anzhen Hospital's electronic medical record management system and constructed a database. Demographic variables consisted of gender and age. Clinical variables included heart rate, blood pressure, body mass index (BMI), smoking history, and previous medical history. BMI was calculated as weight (kg)/[height(m)]^2. Patients with BMI >30 kg/m^2 were classified as obese population. Patients' smoking status was self-reported, which was grouped into current (smoking within the last 3 months), former (quitted for more than 3 months before admission), and never smokers. Hypertension was defined as the previous history, use of antihypertensive medication, or current diagnosis (≥140/90 mmHg more than two times on different days). Diabetes was diagnosed according to the previous history of diabetes, current diagnosis based on the guidelines (21), or those who received a prescription of either an oral hypoglycemic agent or insulin. Patients with dyslipidemia were defined as those with total cholesterol (TC) > 6.2 mmol/L, high-density

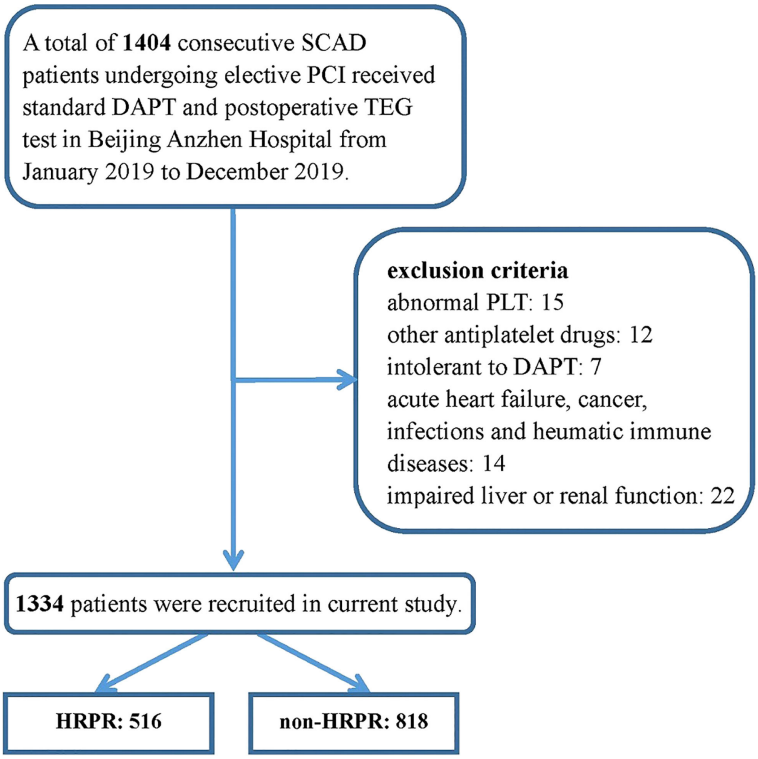

FIGURE 1
Inclusion and exclusion in the study population.SCAD, stable coronary artery disease; PCI, percutaneous coronary intervention; DAPT, dual antiplatelet therapy; TEG, thromboelastography; PLT, platelet count.

lipoprotein cholesterol (HDL-C) < 1.0 mmol/L, low-density lipoprotein cholesterol (LDL-C) > 4.1 mmol/L, or triglyceride (TG) > 2.3 mmol/L. Cerebrovascular disease included cerebral bleeding or ischemic attack. The history of revascularization was established based on patients' medical records.

All of the patients included in our study were examined in the same way. The peripheral venous blood sample was drawn after at least 8 h of fasting. Laboratory data included routine blood indicators [hemoglobin (Hb), platelet count (PLT), and white blood cell count (WBC)], lipid profiles (HDL-C, LDL-C, TC, and TG), glycemic parameters [glycosylated hemoglobin A1c (HbA1c) and fasting blood glucose (FBG)], uric acid (UA), creatinine (CR), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), high-sensitivity C-reactive protein (hs-CRP), and complement C1q, which were obtained from the Department of Laboratory Medicine of Beijing Anzhen Hospital based on standard procedure. We used an online tool (http://ckdepi.org/equations/gfr-calculator/) to get an estimated glomerular filtration rate (eGFR) based on Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. We used a biochemical analyzer to test serum C1q based on the operation process and quality control criteria suggested by the instrument's instruction (Hitachi-7600, Tokyo, Japan).

Assessment of platelet aggregation

We used the LEPU TEG System (CFMS, Beijing, China) to assess ADP-induced platelet aggregation, the detection principle of which was described in detail in previous literature (22, 23). Samples of fasting blood were taken from the patients on the following morning after PCI and processed within 2 h. The equipment adds 1 ml of heparinized blood into a vial containing kaolin, and then transfers 500μl of activated blood to a vial containing heparinase in order to neutralize heparin. Next, the equipment immediately adds 360 μl of neutralized blood into a cup coated with heparinase and measure the maximum amplitude of thrombin-induced clot strength ($MA_{thrombin}$) by the TEG System. Then, the equipment adds 340 μl of heparinized blood into a cup with activator F and reptilase to measure the maximum amplitude of fibrin cross-linked clot without platelet (MA_{fibrin}). Finally, the equipment adds 340 μl of heparinized blood to a cup coated with nonheparinase in the presence of the activator F and ADP (2 μmol) to generate a whole blood cross-linked clot with activated platelet and measure its max strength (MA_{ADP}). MA_{ADP} reflects the peak intensity of clot developed by ADP in a heparinized whole blood sample, which represents the aggregation capacity of platelet and

fibrin induced by ADP. The aggregation capacity of platelet and fibrin induced by thrombin and the aggregation capacity of only fibrin induced by activator F is evaluated by the maximum amplitude of thrombin-induced clot strength ($MA_{thrombin}$) and of fibrin clot strength (MA_{fbrin}), respectively. The efficacy of clopidogrel in inhibiting ADP-induced platelet aggregation was assessed using ADPi, which was calculated as $ADP_i = \frac{(MA_{ADP} - MA_{Fibrin})}{(MA_{Thrombin} - MA_{Fibrin})} \times 100\,\%$. ADP-induced HRPR during clopidogrel treatment was defined as MAADP > 47 mm plus ADP-induced platelet inhibition rate < 50% (24, 25).

Statistical methods

We presented continuously normally or non-normally distributed variables by the mean and standard deviation (SD) or by the median and interquartile range (IQR), and presented categorical variables by number and percentage. Normality distribution was assessed using graphical methods and the Shapiro–Wilk test. To compare differences between groups with low and high responses to clopidogrel, p-values were determined using the Student's t-test or Mann–Whitney test for continuous normally or non-normally distributed variables and using the chi-square test for categorical variables. C1q level was divided into quartiles, and we detected baseline characteristics' differences among patients with different quartiles of C1q using analysis of variance, Kruskal–Wallis test, or chi-square test when appropriate. Chi-square test, Student's t-test, and Mann–Whitney test were used to multiply assess differences in ADP-induced platelet aggregation between the reference group (the lowest quartile of C1q) and each higher level using a Bonferroni-corrected significance level of p = 0.05/3. We calculated the odds ratio (OR) and 95% confidence interval (CI) for HRPR in each C1q quartile using univariate logistic regression analysis. In order to evaluate the effect of C1q on a continuous scale, we additionally constructed models including continuous C1q. Furthermore, we included a series of confounders that were picked based on clinical judgment, previous literature, and statistical significance, in multivariable regression models while correcting for confounders (1) Model 1: adjusted for age and sex (female); (2) Model 2: adjusted for variables included in Model 1 and smoking status (current smoker), and medical history of diabetes and hypertension; (3) Model 3: adjusted for variables included in Model 2 and laboratory data including PLT, WBC, Hb, LDL-C, HDL-C, eGFR, and hs-CRP]. To reduce the risk of distorted model, we additionally calculated the variance inflation factor (VIF) for each covariate to test data multicollinearity. All models met our criteria of nonmulticollinearity with VIF less than 5 (Supplementary Table 1). A linear trend test across quartiles was performed by assigning medians to each quartile of C1q as a continuous variable in these models. We also used restricted cubic splines (RCSs) fitted for univariate and multivariable regression models with three knots at the 25th, 50th, and 75th percentiles to flexibly model and visualize the nonlinear relation between C1q and HRPR risk. We set the median of C1q's first quartile as the reference. A likelihood ratio test comparing the RCS model with a model including only a linear term was used to test for potential non-linearity.

A two-tailed p-value of < 0.05 was considered significant. All statistical analyses were performed using SPSS Statistics 26.0 (SPSS, Inc., Chicago, IL, USA) and R (v. 4.0.5).

Results

Baseline characteristics

After screening according to inclusion and exclusion criteria, a total of 1,334 participants were consecutively recruited in this study, of whom 516 patients (38.7%) had low response to clopidogrel. All covariate data of interest are complete. Baseline characteristics are presented using stratification based on low and high ADP-induced platelet aggregation in Table 1. Patients with HRPR are older and have a higher proportion of female patients, compared to the controls. The proportion of current or former smokers in patients with HRPR is lower, which reaches borderline statistical significance (p = 0.053). Patients with HRPR are more likely to have a medical history of hypertension (p = 0.053) and diabetes and have lower levels of WBC, Hb, and eGFR as well as higher levels of PLT, LDL-C, HDL-C, hs-CRP, and C1q (178.49 ± 31.11 mg/L vs. 168.56 ± 31.57 mg/L, p < 0.001) at baseline laboratory data.

Correlations between C1q and other variables

A total of 1,334 participants were split into four quartiles based on C1q: Q1 (0, 151.1], Q2 (151.1, 168.1], Q3 (168.1, 191.9], and Q4 (191.9, ~). Table 2 shows covariates of interest and detects differences in patients with different C1q quartiles. Correlations between individual covariates of interest and correlations between C1q and TEG parameters were assessed using Pearson test or Spearman test as appropriate. Figure 2 shows statistically significant correlation (p-value < 0.05) and correlation coefficient r. C1q has weak but significant positive associations with female patients, history of hypertension, WBC, PLT, TC, LDL-C, hs-CRP, and MA_{ADP}, while correlating negatively with age and ADP_i.

TEG parameters in patients with different C1q levels

Figure 3 displays monotonic variation of increased MA_{ADP} and decreased ADP_i with the increase of C1q level (median value of MA_{ADP} in each quartile: 39.60 mm, 42.85 mm, 45.10 mm, and

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TABLE 1 Baseline characteristics in patients with and without HRPR.

Variables	Overall	Patients without HRPR	Patients with HRPR	*p*
N (%)	1,334	818 (61.3%)	516 (38.7%)	-
Clinical data				
Female, *n* (%)	361 (27.1%)	158 (19.3%)	203 (39.3%)	<0.001
Age (years), mean ± SD	61.28 ± 9.53	60.35 ± 9.656	62.75 ± 9.130	<0.001
BMI (kg/m^2), mean ± SD	25.93 ± 6.05	25.82 ± 3.20	26.10 ± 8.86	0.417
Obesity, *n* (%)	125 (9.4%)	71 (8.7%)	54 (10.5%)	0.276
HR (bpm), mean ± SD	69.55 ± 7.64	69.61 ± 7.61	69.45 ± 7.69	0.703
SBP (mmHg), mean ± SD	129.04 ± 15.86	128.43 ± 15.91	130.00 ± 15.75	0.079
DBP (mmHg), mean ± SD	75.35 ± 10.99	75.55 ± 10.92	75.03 ± 11.10	0.399
Smoking				0.053
Smoker, *n* (%)	343 (25.7%)	228 (27.9%)	115 (22.3%)	
Ex-smoker, *n* (%)	92 (6.9%)	51 (6.2%)	41 (7.9%)	
Never-smokers, *n* (%)	899 (67.4%)	539 (65.9%)	360 (69.8%)	
Medical history				
Hypertension, *n* (%)	828 (62.1%)	491 (60.0%)	337 (65.3%)	0.053
Diabetes, *n* (%)	470 (35.2%)	267 (32.6%)	203 (39.3%)	0.013
Dyslipidemia, *n* (%)	638 (47.8%)	394 (48.2%)	244 (47.3%)	0.754
Previous cerebrovascular disease, *n* (%)	115 (8.6%)	65 (7.9%)	50 (9.7%)	0.269
Previous PCI, *n* (%)	384 (28.8%)	250 (30.6%)	134 (26.0%)	0.071
Previous CABG, *n* (%)	31 (2.3%)	17 (2.1%)	14 (2.7%)	0.453
Biochemical data				
WBC (×10^9/L), mean ± SD	6.72 ± 1.89	6.88 ± 1.95	6.45 ± 1.75	<0.001
Hb (g/L), mean ± SD	140.52 ± 15.97	143.11 ± 15.68	136.42 ± 15.57	<0.001
PLT (×10^9/L), mean ± SD	212.71 ± 3.86	205.88 ± 53.82	223.55 ± 52.18	<0.001
ALT (mmol/L), median [IQR]	20.00 [15.00–29.00]	21.00 [15.00–29.00]	20.00 [15.00–30.00]	0.194
AST (mmol/L), median [IQR]	21.00 [18.00–25.00]	21.00 [18.00–25.00]	21.00 [17.25–26.00]	0.697
ALB (g/L), mean ± SD	42.31 ± 3.59	42.31 ± 3.45	42.32 ± 3.79	0.973
TC (mmol/L), mean ± SD	4.01 ± 0.98	3.94 ± 0.96	4.11 ± 1.01	0.003
LDL-C (mmol/L), mean ± SD	2.34 ± 0.80	2.30 ± 0.79	2.40 ± 0.82	0.027
HDL-C (mmol/L), mean ± SD	1.09 ± 0.25	1.07 ± 0.24	1.11 ± 0.26	0.027
TG (mmol/L), mean ± SD	1.62 ± 1.19	1.65 ± 1.29	1.59 ± 1.01	0.411
FBG (mmol/L), median [IQR]	5.65 [5.06–6.96]	5.63 [5.01–7.00]	5.72 [5.12–6.91]	0.097
HbA_1C (%), median [IQR]	6.20 [5.80–7.00]	6.20 [5.70–6.90]	6.30 [5.80–7.10]	0.059
UA (μmol/L), mean ± SD	348.66 ± 86.62	350.05 ± 82.79	346.47 ± 92.41	0.474
Creatinine (μmol/L), mean ± SD	71.70 ± 18.24	72.29 ± 17.15	70.77 ± 19.82	0.152
eGFR (ml/min/1.73 m^2), median [IQR]	94.57[85.90–101.23]	95.09 [86.93–102.21]	93.41 [84.46–99.96]	0.001
Hs-CRP (mg/L), median [IQR]	1.18 [0.50–2.93]	1.05 [0.44–2.61]	1.4300 [0.69–3.47]	<0.001
C1q (mg/L), mean ± SD	172.41 ± 31.75	168.56 ± 31.57	178.49 ± 31.11	<0.001
TEG parameter				
MA_{ADP} (mm), median [IQR]	43.45 [34.98–51.30]	37.10 [29.40–41.70]	53.70 [49.80–58.40]	<0.001
ADPi (%), mean (SD)	37.15 ± 23.60	50.49 ± 19.38	15.99 ± 10.66	<0.001

HRPR, high residual platelet reactivity; PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft; BMI, body mass index; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; PLT, platelet count; Hb, hemoglobin; WBC, white blood cell count; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; HbA_1C, hemoglobin A_1C; UA, uric acid; CR, creatinine; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MA_{ADP}, maximum amplitude of ADP-induced clot strength; ADP_i, ADP-induced platelet inhibition rate.

46.40 mm; mean value of ADP_i in each quartile: 39.28%, 38.74%, 35.71%, and 34.75%). Additionally, patients with higher C1q level were more likely to perform HRPR (Q1: 26.1%, Q2: 38.4%, Q3: 43.8%, and Q4: 46.4%). MA_{ADP} and frequency of HRPR in each higher C1q quartile significantly increase compared to the reference group (all p-values ≤ 0.001), but the significant difference in ADP_i is present only between the lowest and the highest quartile of C1q (p = 0.011).

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TABLE 2 Covariates of interest in patients with different C1q levels.

Variables	Overall	Q1 (334)	Q2 (333)	Q3 (333)	Q4 (334)	*p*
Female, *n* (%)	361 (27.1%)	51 (15.3%)	81 (24.3%)	104 (31.2%)	125 (37.4%)	<0.001
Age (years), mean ± SD	61.28 ± 9.53	62.87 (9.35)	62.18 (9.47)	60.31 (9.39)	59.75 (9.58)	<0.001
Smoking						0.154
Current smoker, *n* (%)	343 (25.7%)	93 (27.8%)	88 (26.4%)	83 (24.9%)	79 (23.7%)	
Ex-smoker, *n* (%)	92 (6.9%)	22 (6.6%)	25 (7.5%)	31 (9.3%)	14 (4.2%)	
Never-smokers, *n* (%)	899 (67.4%)	219 (65.6%)	220 (66.1%)	219 (65.8%)	241 (72.2%)	
Hypertension, *n* (%)	828 (62.1%)	189 (56.6%)	204 (61.3%)	218 (65.5%)	217 (65.0%)	0.066
Diabetes, *n* (%)	470 (35.2%)	109 (32.6%)	119 (35.7%)	124 (37.2%)	118 (35.3%)	0.657
WBC ($\times 10^9$/L), mean ± SD	6.72 ± 1.89	6.26 ± 1.73	6.74 ± 1.93	6.76 ± 1.89	7.11 ± 1.90	<0.001
Hb (g/L), mean ± SD	140.52 ± 15.97	140.84 ± 15.97	139.77 ± 15.49	139.76 ± 15.82	141.72 ± 16.56	0.320
PLT ($\times 10^9$/L), mean ± SD	212.71 ± 3.86	193.41 ± 50.83	209.76 ± 51.93	219.37 ± 50.82	228.33 ± 55.64	<0.001
TC (mmol/L), mean ± SD	4.01 ± 0.98	3.75 ± 0.85	3.92 ± 1.02	4.04 ± 0.87	4.33 ± 1.07	<0.001
LDL-C (mmol/L), mean ± SD	2.34 ± 0.80	2.15 ± 0.76	2.246 ± 0.78	2.36 ± 0.72	2.58 ± 0.89	<0.001
HDL-C (mmol/L), mean ± SD	1.09 ± 0.25	1.09 ± 0.24	1.07 ± 0.26	1.094 ± 0.26	1.08 ± 0.25	0.661
eGFR (ml/min/1.73 m^2), median [IQR]	94.57 [85.90–101.23]	95.52 [86.91–101.75]	94.40 [86.54–100.69]	94.49 [84.19–101.70]	94.33 [85.82–101.00]	0.834
hs-CRP (mg/L), median [IQR]	1.18 [0.50–2.93]	0.61 [0.30–1.58]	1.11 [0.4450–2.6500]	1.39 [0.62–3.08]	1.78 [0.86–4.12]	<0.001
C1q (mg/L), mean ± SD	172.41 ± 31.75	136.03 ± 11.34	159.74 ± 5.10	179.23 ± 7.21	214.60 ± 22.57	<0.001
MA_{ADP} (mm), median [IQR]	43.45 [34.98–51.30]	39.55 [32.33–48.10]	42.80 [33.30–50.95]	45.00 [37.00–52.65]	46.60 [37.48–54.73]	0.012
ADP_i (%), mean ± SD	37.15 ± 23.60	39.34 ± 21.73	38.61 ± 24.54	36.02 ± 23.71	34.62 ± 24.12	0.033
HRPR, *n* (%)	516 (38.7%)	88 (26.3%)	128 (38.4%)	144 (43.2%)	156 (46.7%)	<0.001

HRPR, high residual platelet reactivity; PLT, platelet count; Hb, hemoglobin; WBC, white blood cell count; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MA_{ADP}, maximum amplitude of ADP-induced clot strength; ADP_i, ADP-induced platelet inhibition rate.

Effect of elevated C1q and other factors on antiplatelet therapy with clopidogrel

C1q quartile was set as an ordered categorical variable, which was included in a univariate logistic regression model. The results show that higher C1q level significantly increases risk of HRPR during clopidogrel therapy compared with the lowest quartile (Q2: OR = 1.745, 95% CI 1.257–2.425, p = 0.001; Q3: OR = 2.130, 95% CI 1.537–2.951, p < 0.001; Q4: OR = 2.450, 95% CI 1.771–3.390, p < 0.001). We constructed three additional multivariate logistic regression models (as described previously) to adjust for confounding variables. As Table 3 shows, elevated C1q level remains to be an independent risk factor of HRPR during clopidogrel therapy (all p of each quartile in models 1, 2, and 3 < 0.05). When C1q was included as a continuous covariate in the logistic regression model, crude odds ratio and adjusted odds ratio were greater than 1. Furthermore, significant upward trends are observed regardless of univariate or multivariate logistic regression models (all p for trend < 0.05). In Table 4, we provided some of the ORs of all variables included in the multivariate analysis. After the multiple regression analysis, age, diabetes, and high PLT were independent factors associated with HRPR.

Restrictive cubic spline

We constructed restrictive cubic spline models to flexibly visualize the relationship between the risk of HRPR during clopidogrel therapy and serum C1q on a continuous scale with or without correcting for covariates. In Figure 4, inverted L-shaped RCS curve indicates a non-linear relationship (p for non-linear < 0.05). The crude and adjusted odds ratios for HRPR (the median of C1q's first quartile was set as reference value: 138.7 mg/L) rise with the increase of C1q until approximately 180 mg/L of C1q, then the curves are relatively gentle and the slope changes little.

Discussion

We reported the following novel findings based on the present cross-sectional study. Serum level of C1q is correlated with ADP-induced platelet aggregation assessed by TEG parameters in CAD patients receiving clopidogrel therapy and elevated C1q was proved to be an independent risk factor for HRPR induced by ADP during clopidogrel treatment. Besides these, there exist weak but significant associations between C1q

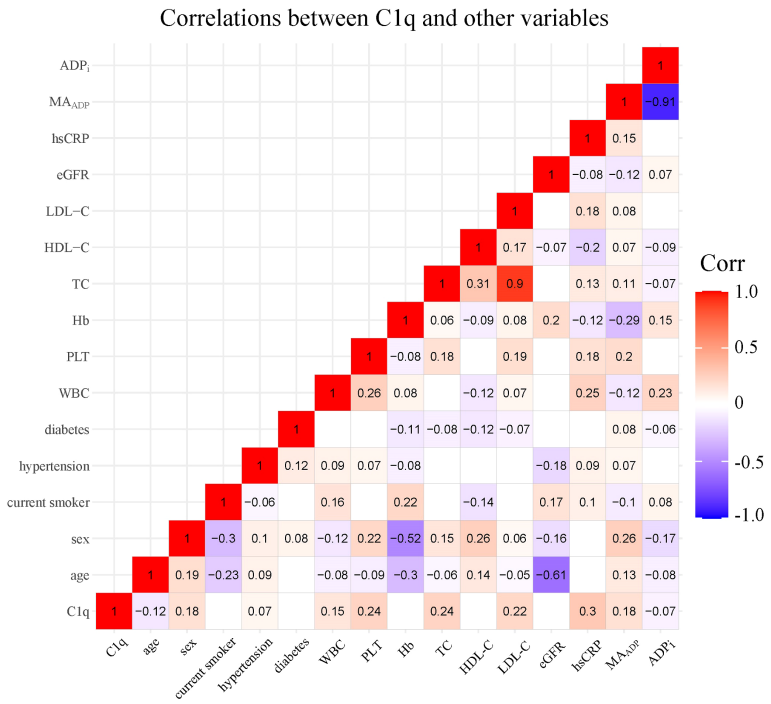

FIGURE 2
Correlations between C1q and covariates of interest. The number and color in each box represented correlation coefficient for two covariates corresponding to the box, of which blank indicated non-statistically significant association. PLT, platelet count; Hb, hemoglobin; WBC, white blood cell count; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MA$_{ADP}$, Maximum amplitude of ADP-induced clot strength; ADP$_i$, ADP-induced platelet inhibition rate.

and several HRPR-related factors detected in current and previous studies. Previous studies had demonstrated that the occurrence of HRPR significantly increases the risk of thrombotic and ischemic events in CAD patients (26, 27), which might suggest the potential predictive value of C1q in predicting certain adverse cardiovascular events. Based on the above findings, C1q emerges as a convenient, accessible, and reliable predictor for HRPR induced by ADP during clopidogrel treatment.

Antiplatelet therapy represents the cornerstone of secondary prevention for avoiding coronary artery thrombosis in patients with CAD. Although novel potent P2Y12 receptor antagonists including prasugrel and ticagrelor had already been widely applied in clinical practice, clopidogrel is still indispensable in many clinical scenarios due to its advantages in lower bleeding risk, lower economic burden, and better medication adherence (2, 28). As a prodrug, clopidogrel exerts its effect after being metabolized by cytochrome P450 (CYP450). There is a higher incidence of HRPR in east Asians, due to the higher prevalence of the CYP2C19 loss-of-function alleles (29), and the incidence in the current study population is 38.7%. The genetic factor is a crucial, but not a unique factor affecting antiplatelet treatment with clopidogrel (30), and the routine detection of HRPR-related genotypes increases healthcare costs. Therefore, it is essential to investigate other potential risk factors of HRPR. Contemporary biomedical literature supports that inflammation may play an important role in the progression of atherosclerosis and thrombosis initiated by rupture of atherosclerotic plaques, and a previous study found that inflammatory biomarkers could influence ADP-induced platelet aggregation assessed by platelet function test during clopidogrel treatment (31). As a major participant in innate immune responses and inflammatory processes, the complement system is also involved in the regulation of atherosclerosis. C1q can induce cytokines and enhance NLRP3 inflammasome to promote atherosclerosis development by initiating a classical pathway (32) while retarding the formation of the necrotic core in plaques via promoting cholesterol efflux from macrophage foam cells and downregulating their apoptosis (33). Despite evidence that C1q and downstream complement components in the classical pathway interact with platelets to activate platelets and induce thrombosis (18), no former studies had explored the effect of C1q on antiplatelet therapy with clopidogrel.

TEG is a dynamic observation of the blood coagulation process by dynamic measurement of clot strength. MA$_{ADP}$ represents the peak clot strength in citrated or heparinized

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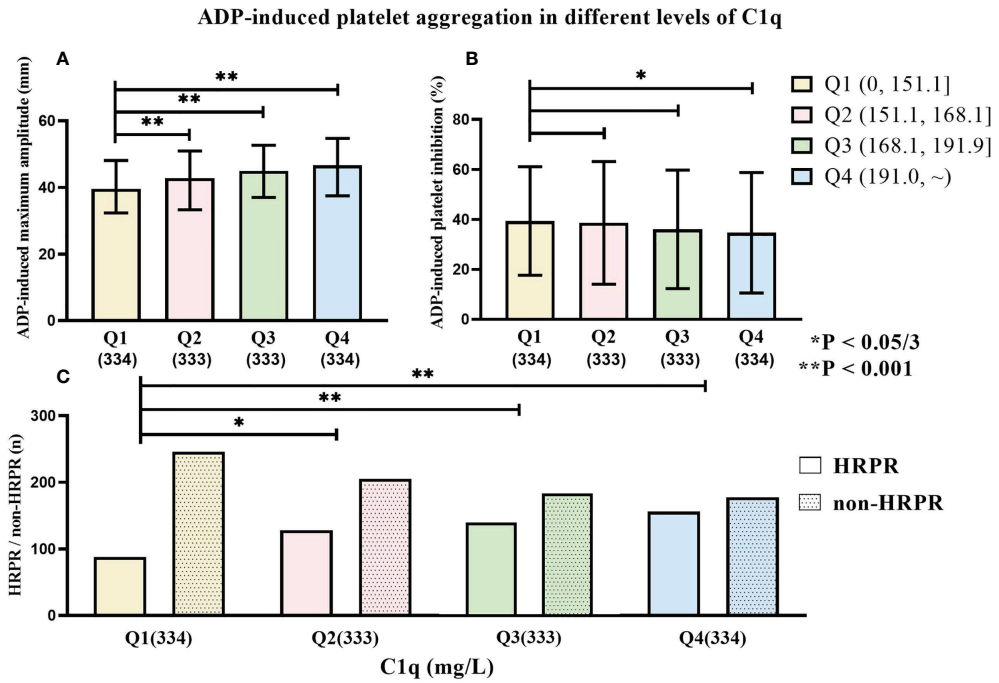

	Q1(334)	Q2(333)	Q3(333)	Q4(334)
MA_{ADP} (mm), median [IQR]	39.55 [32.33-48.10]	42.80 [33.30-50.95]	45.00 [37.00-52.65]	45.00 [37.00-52.65]
ADPi (%), mean ± SD	39.34±21.73	38.61±24.54	36.02±23.71	34.62±24.12
HRPR, n (%)	88 (26.3%)	128 (38.4%)	144 (43.2%)	156 (46.7%)

FIGURE 3
(A) ADP-induced platelet inhibition rate (ADP_i), **(B)** the maximum amplitude of ADP-induced clot strength (MA_{ADP}), and **(C)** the frequency of HRPR in patients with different C1q quartile. * means "P < 0.05/3". ** means "P < 0.001".

whole blood samples with the addition of moderate ADP (23), which is used to evaluate the aggregation capacity of both platelet and fibrin induced by ADP. The current consensus sets MA_{ADP} > 47 mm as a predictive factor of ischemic events in post-PCI patients with DAPT (34). The efficacy of clopidogrel in reducing ADP-induced platelet aggregation is assessed using ADP_i, which is calculated according to the formula: $ADP_i = \frac{(MA_{ADP} - MA_{Fibrin})}{(MA_{Thrombin} - MA_{Fibrin})} \times 100\,\%$. We established a composite criterion

TABLE 3 Odds ratio (OR) and 95% CI for HRPR in each C1q quartile.

C1q, mg/L	Non-HRPR	HRPR	Crude OR (95% CI)	OR (95% CI) in model 1	OR (95% CI) in model 2	OR (95% CI)[1] in model 3
C1q	-	-	1.010 (1.006–1.014)	1.009 (1.005–1.013)	1.009 (1.005–1.013)	1.009 (1.005–1.014)
Q1, ≤151.1	246	88	-	-		-
Q2, (151.1, 168.1]	205	128	1.745 (1.257–2.425)	1.678 (1.199–2.349)	1.668 (1.191–2.337)	1.722 (1.215–2.440)
Q3, (168.1, 191.9]	189	144	2.130 (1.537–2.951)	2.047 (1.459–2.872)	2.030 (1.446–2.851)	2.015 (1.413–2.874)
Q4, >191.9	178	156	2.450 (1.771–3.390)	2.286 (1.626–3.214)	2.279 (1.619–3.207)	2.362 (1.631–3.421)
p for trend[2]			<0.001	<0.001	<0.001	<0.001

[1]Odds ratio (OR) and 95% CI for HRPR in each C1q quartile were calculated using univariate and multivariable logistic regression. Model 1 was adjusted for age, sex (female), smoking status (current smoker), and medical history of diabetes and hypertension. Model 2 was adjusted for variables included in Model 1, smoking status (current smoker), and medical history of diabetes and hypertension. Model 3 was adjusted for variables included in Model 2 and laboratory data including PLT, WBC, Hb, LDL-C, HDL-C, eGFR, and hs-CRP. [2]Test for trend based on variable containing median value for each quintile. HRPR, high residual platelet reactivity; OR, odds ratio; CI, confidence interval; PLT, platelet count; Hb, hemoglobin; WBC, white blood cell count; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein.

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TABLE 4 ORs (and 95% CIs) for HRPR of other variables.

variables	OR (95% CI) in model 1	OR (95% CI) in model 2	OR (95% CI) in model 3
Age	1.026 (1.013–1.039)	1.026 (1.013–1.040)	1.023 (1.008–1.040)
Sex	2.212 (1.708–2.863)	2.236 (1.706–2.932)	1.372 (0.991–1.898)
Current smoker	-	1.117 (0.840–1.486)	1.184 (0.880–1.592)
Diabetes	-	1.235 (0.971–1.571)	1.290 (1.004–1.658)
Hypertension	-	1.056 (0.830–1.345)	1.092 (0.849–1.405)
PLT	-	-	1.006 (1.004–1.009)
WBC	-	-	0.807 (0.749–0.870)
Hb	-	-	0.984 (0.975–0.993)
LDL-C	-	-	1.101 (0.943–1.285)
HDL-C	-	-	1.084 (0.654–1.797)
eGFR	-	-	0.999 (0.990–1.008)
hsCRP	-	-	1.023 (0.996–1.052)

ORs (and 95% CIs) for HRPR of other variables were calculated using univariate and multivariable logistic regression. HRPR, high residual platelet reactivity; OR, odds ratio; CI, confidence interval; PLT, platelet count; Hb, hemoglobin; WBC, white blood cell count; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein.

that defined patients with a MA_{ADP} of >47 mm plus an ADP_i of <50% as patients with HRPR based on previous literature (24, 25). Our findings show that serum C1q is significantly positively related to MA_{ADP} as well as negatively related to ADP_i. Compared to MA_{ADP}, ADP_i shows a much weaker association with C1q, which is demonstrated by a smaller correlation coefficient and only one significant difference of ADP_i between the lowest and the highest quartile of C1q. At the same time, HRPR risk exhibits an inverted L-shaped curve pattern with an increase in serum C1q.

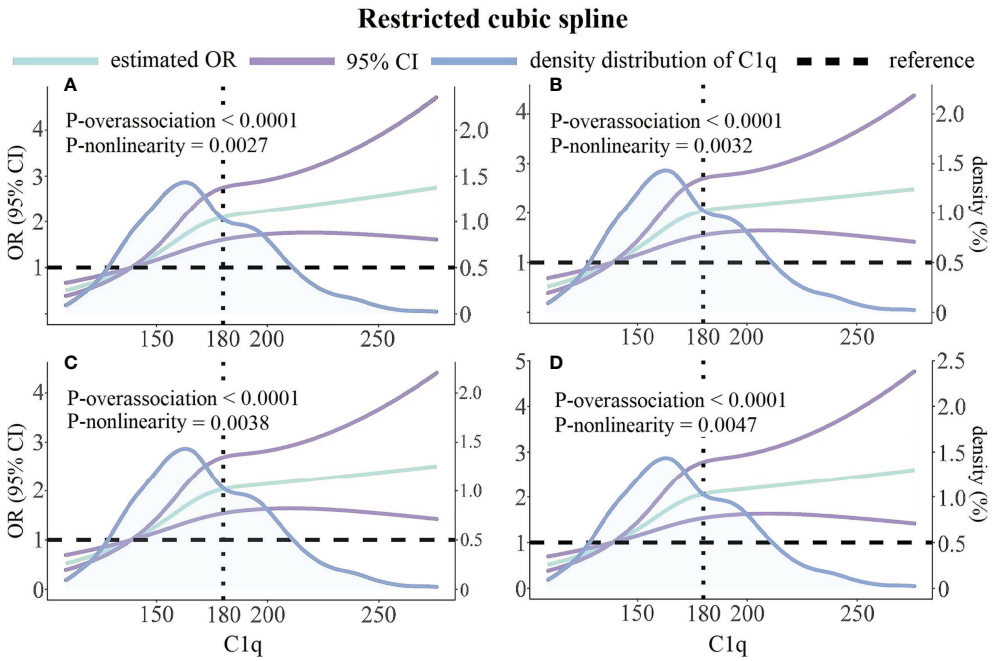

FIGURE 4
Unadjusted and multivariable-adjusted ORs and 95% CIs for HRPR according to C1q and the density distribution curve of C1q. **(A)** Unadjusted model. **(B)** Adjusted for variables included in Model 1 as Table 3 described. **(C)** Adjusted for variables included in Model 2 as Table 3 described. **(D)** Adjusted for variables included in Model 3 as Table 3 described. Green solid lines represent ORs, with purple lines representing the 95% CIs. Black horizontal dotted lines represent the references corresponding to an OR of 1.0. Blue solid lines describe density distribution of C1q. Reference point is the median of C1q's first quartile with three knots placed at the 25th, 50th and 75th percentiles of C1q. OR, odds; CI, confidence interval.

The explanations for current findings are unclear but may be multifactorial. Consistent with previous literature (30, 35), current research identified several HRPR-related factors, including aging, sex, worse renal function, and diabetes. The correlations of C1q with some of these factors might partially explain our findings. Despite adjusting for potential mediator variables and confounders, elevated C1q is still an independent indicator of platelet aggregation assessed by TEG parameters, which implies that complement components themselves affected ADP-induced platelet activity in CAD patients treated by clopidogrel. As a prodrug, clopidogrel needs to be metabolized by CYP450 to execute its antiplatelet function. Elevated inflammatory cytokines such as interleukin-6 (IL-6) and the tumor necrosis factor-alpha (TNF-α) downregulate the expression and activity of CYP450 in the state of inflammation (36, 37). We propose a hypothetical explanation that C1q may cause pharmacokinetic effects on CYP2C19-mediated clopidogrel metabolism by participating in the inflammatory process. Previous evidence that C1q enhances the release of inflammatory cytokines including TNF-α and IL-6 might partially support our hypothesis (38, 39). P-glycoprotein, a transmembrane transporter encoded by the ABCB1 gene, can expel drugs (including clopidogrel) from the cell interior. ABCB1 polymorphisms and abnormal activity of P-glycoprotein affect clopidogrel's absorption and transport, and TRITON-TIMI 38 trail (40) had detected that the ABCB1 3435 TT genotype attenuates platelet inhibition and increases the risk of recurrent ischemic events in acute coronary syndrome patients receiving clopidogrel treatment. In the field of oncology, some literature had found that WNT/β-catenin canonical signaling pathway positively regulates the ABCB1 gene expression in tumor cells (41). Naito et al. (42) provided evidence demonstrating complement C1q as an activator of Wnt signaling. These existing findings suggest a plausible mechanism that elevated C1q affects clopidogrel absorption and transport via activating Wnt signaling to promote p-glycoprotein expression. Besides these, C1q binding to C1q receptors (gC1qR/p33 and cC1qR) expressed on the platelet surface can enhance platelet aggregation and activation (18). After ADP activating platelets via binding to P2Y12 and P2Y1 receptors, platelet degranulation and the release of P-selectin from alpha granules are upregulated. P-selectin localized on the platelet membrane binds P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes to recruit leukocytes, which enhances the pro-inflammatory and pro-thrombotic effects of leukocytes (43). In vitro experiments had found that clopidogrel downregulates ADP-induced P-selectin expression and platelet–leukocyte adhesion (44). C1q binding to C1q receptors can promote the release of P-selectin, then initiate downstream events (18), which is another possible mechanism. Stable thrombosis is formed by platelets and a cross-linked fibrin network. Coagulation is a complex cascade involving activation of the coagulation system, activation of platelet, and formation of cross-linked fibrin, all steps of which interact with each other. The effects of C1q on thrombosis are not restricted to platelet activation and aggregation and involve cross-linking of fibrin. As to why MA_{ADP}, a TEG parameter evaluating aggregation capacity of both platelet and fibrin, has a stronger association with C1q than ADP_i, one explanation may be that C1q directly or indirectly affects cross-linking of fibrin. Considering the above, despite these findings we detected, the exact mechanism remains speculation based on previous research.

In this study, we had tried to avoid effects on C1q level of identifiable inflammation by just recruiting stable CAD patients and setting a series of exclusion criteria. Among the study population, elevated C1q significantly promotes ADP-induced thrombosis measured by TEG parameters and independently predicted the risk of HRPR during clopidogrel therapy. Our findings call for more attention to C1q when physicians formulate antiplatelet prescriptions and evaluate future thrombosis risk for SCAD patients undergoing PCI.

Several limitations of the current study should be acknowledged. Our study was an observational analysis derived from a small single-center sample, which might cause potential bias and limit the extrapolation of our conclusions. Second, we were unable to record some related factors, such as patients' HRPR-related genotypes, due to limited resources. Third, our cross-sectional study had not analyzed post-discharge follow-up adverse cardiovascular events, which limit clinical application of current findings. We plan to investigate whether C1q could increase the risk of thrombotic events via affecting ADP-induced platelet activity in our future, follow-up study, when we complete a protocol for a detailed, comprehensive study. Fourth, TEG is based on an in vitro coagulation process and not the gold standard for HRPR, which might not make an entirely accurate evaluation of the actual in vivo platelet aggregation. Fifth, both TEG parameters and other laboratory information were measured at baseline only once. Sixth, due to technical limitations in our Department of Laboratory Medicine, we evaluated platelet function by only measuring platelet aggregation. We plan to use more methods to evaluated platelets' other functions during clopidogrel therapy in future basic research. Finally, the biological mechanism underlying our conclusion remains unclear, and the detailed mechanism needs further investigation.

Conclusion

The current study firstly indicated that elevated C1q promotes TEG-simulated platelet aggregation induced by ADP in CAD patients during clopidogrel therapy and elevated C1q is an independent risk factor of clopidogrel HRPR. Our finding implied that elevated C1q is a novel, accessible, and reliable clinical biomarker used for risk assessment of clopidogrel HRPR and to help guide the use of antiplatelet treatment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Beijing Anzhen Hospital, Capital Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

ZZ designed the study, analyzed the data, and drafted the manuscript. MM and XH analyzed the data and drew the figures. KH and TS collected the data from the electronic medical system and constructed the database. SY and YZ designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.969984/full#supplementary-material

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Postoperative neutrophil-lymphocyte ratio predicts unfavorable outcome of acute ischemic stroke patients who achieve complete reperfusion after thrombectomy

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Purpose: Only approximately half of anterior circulation large vessel occlusion (LVO) patients receiving endovascular treatment (EVT) have a favorable outcome. The aim of this study was to explore the association of dynamic inflammatory markers (i.e., neutrophil to lymphocyte ratios, NLR, measured at different times after EVT) as well as other potential influencing factors with unfavorable outcome among acute ischemic stroke (AIS) patients who achieved complete reperfusion after EVT.

Methods: Patients treated with EVT for LVO between January 2019 to December 2021 were prospectively enrolled. Complete reperfusion was defined as modified thrombolysis in cerebral infarction (mTICI) grade 3. A modified Rankin scale at 90 days (mRS90) of 3–6 was defined as unfavorable outcome (i.e., futile reperfusion). A logistic regression analysis was performed with unfavorable outcome as a dependent variable. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were then used to determine the diagnostic values of NLR and other relevant factors.

Results: 170 patients with complete reperfusion (mTICI 3) were included in this study. Unfavorable outcome was observed in 70 (41.2%). Higher NLR within 24h (p=0.017) and at 3-7d (p=0.008) after EVT were an independent risk factors for unfavorable outcome at 3 months. In addition, older age, higher NIHSS scores, poor collaterals, and general anesthesia were independent predictors of unfavorable outcomes. When accounting for NLR, the diagnostic efficiency improved compared to conventional characteristics.

Conclusion: Our findings suggest that advanced age, increased stroke severity, poor collaterals, general anesthesia, and NLR are independent predictors for an unfavorable clinical outcome following complete reperfusion after EVT. Neuroinflammation may merit particular attention in future studies.

KEYWORDS

acute ischemic stroke, endovascular treatment, NLR, inflammation, prognosis, reperfusion

Introduction

Endovascular treatment (EVT) has proven to be effective in improving the outcome of patients with acute ischemic stroke (AIS) due to large vessel occlusion (LVO) within the anterior circulation (1). The benefits of EVT has been largely attributed to the higher rates of successful revascularization compared with thrombolysis therapy (1). The modified thrombolysis in cerebral infarction (mTICI) score of 2b and above has traditionally been considered successful reperfusion (2). Recent clinical trials have shown that EVT achieves TICI 2b-3 reperfusion in more than 85% of AIS patients (3–5). Even when achieving successful reperfusion, AIS patients with unfavorable outcome are reported in up to 48.7% (6). Thus, identification of predictors of futile reperfusion have been hotly debated in recent years. Prompt identification of patients at increased risk of adverse outcome such as futile reperfusion, cerebral infarction volumes, ischemic reperfusion (I/R) injury and/or neuroinflammation may help target patients who deserve close attention and timely treatments (7).

In previous studies, some risk factors, such as higher initial National Institute of Health Stroke Scale (NIHSS) score, older age, longer time from onset to treatment, and infarct growth despite successful reperfusion, were reported to be associated with futile reperfusion after EVT (8–11). However, these studies largely focused on successful reperfusion (mTICI 2b-3), rather than complete reperfusion (mTICI 3). A recent systematic review and meta-analysis indicates mTICI 3 is associated with superior outcome and better safety profiles than mTICI 2b (12). Therefore, identifying predictors of futile complete reperfusion could yield crucial value to optimize patient management or inform future research endeavors exploring novel adjunct therapies. Previously, Van Horn et al. (13) proposed clinical and image indicators of poor clinical outcome despite TICI 3 reperfusion. However, they did not observe serological or other postoperative predictors, which may provide further valuable information.

Inflammatory and immune responses play key roles in ischemic stroke pathophysiology, treatment effects, and outcomes. The neutrophil to lymphocyte ratio (NLR) integrates information about both nonspecific neutrophil-driven inflammation and more targeted lymphocyte immune regulation (14). Emerging evidence indicates that post-stroke immune responses can affect the neurovascular interface, causing reperfusion injury and symptomatic intracranial hemorrhage (15). Compared with previously reported serologic biomarkers, such as matrix metalloproteinase-9, tenascin-C, and thioredoxin (16, 17), NLR is easily accessible and routinely examined in clinical practice. Thus, it may serve as a more practical tool to predict outcomes for AIS patients. A recent meta-analysis analyzed NLR values for admission and post-operation separately and found that NLR was closely related to the prognosis of AIS patients (18). Previous studies have shown that NLR is a predictor of functional outcome in patients with AIS and occurrence of intracranial hemorrhage (5, 19–22), but the role of dynamic NLR in predicting outcomes for patients who achieve complete reperfusion after EVT is unknown. The aim of this study was to explore the association of dynamic inflammatory markers (i.e., NLR measured at different times after EVT) as well as other potential influencing factors with unfavorable outcome among AIS patients who achieved complete reperfusion after EVT.

Materials and methods

Patient population

In this retrospective single-center study, we evaluated 443 consecutive patients who underwent EVT for acute LVO within the anterior circulation from a prospectively collected database from January 2019 to December 2021. All patients were treated according to the latest clinical guidelines in a "real-world" setting.

The inclusion criteria of the present study were as follows: (1) age ≥18 years; (2) AIS due to LVO within the anterior circulation; (3) complete reperfusion (defined as mTICI 3); (4) known admission National Institutes of Health Stroke Scale (NIHSS) and ASPECTS; (5) known mRS90 days after EVT The exclusion criteria were as follows: (1) pre-mRS ≥3; (2) NIHSS ≤5; (3) ASPECTS ≤5; (4) incomplete data. Figure 1 shows the flow chart of the patient selection process. This study was approved by the institutional review board, and the need for written informed consent was waived based on the study's retrospective design, de-identified data, and minimal patient risk.

Baseline characteristics and image analysis

Patient demographics and clinical data were assessed, including age, sex, comorbidities (hypertension [HTN], diabetes mellitus [DM], coronary artery disease [CAD], etc.), presentation NIHSS, stroke type according to the Trial of Org 10172 in Acute Stroke Treatment classification (TOAST), intravenous thrombolysis, anesthesia type, time from onset-to-reperfusion (OTR), etc. Routine blood tests including glucose level and lipid panel (triglyceride [TG], total cholesterol [TC], high-density lipoprotein [HDL], low-density lipoprotein [LDL]) were measured at admission. Neutrophil and lymphocyte levels were collected at admission (neutrophil1, lymphocyte1 and NLR1), within 24h (neutrophil2, lymphocyte2 and NLR2) and at 3-7d (neutrophil3, lymphocyte3 and NLR3) after EVT (7).

Imaging data included presentation ASPECTS from non-contrast computed tomography (NCCT) and occlusion site from initial CTA and confirmed with digital subtraction angiography (DSA). Collateral status was evaluated using the American Society of Interventional and Therapeutic Neuroradiology/Society of Interventional Radiology (ASITN/SIR) collateral grading system and collateral status was dichotomized into poor (ASITN/SIR 0–2) or good (ASITN/SIR 3–4) (23). Reperfusion status was evaluated using the modified Thrombolysis in Cerebral Infarction (mTICI) score. CT images were analyzed by an experienced radiologist (>5 years of experience) and DSA images were analyzed by an experienced neuroradiologist (>10 years of experience).The angiographic result was assessed on the final DSA image series and was classified according to the mTICI scale; complete reperfusion was defined as mTICI 3.

Statistical analysis

Participants were classified into two groups by 90-day mRS (mRS90): mRS90 0-2 vs 3-6 (13). Continuous variables are presented as median (interquartile range [IQR]) due to their skewed distribution. Categorical variables are presented as frequency and percentage. The non-parametric Wilcoxon test was used to compare group differences for continuous variables, and chi-square test or Fisher exact test was used for categorical variables as appropriate.

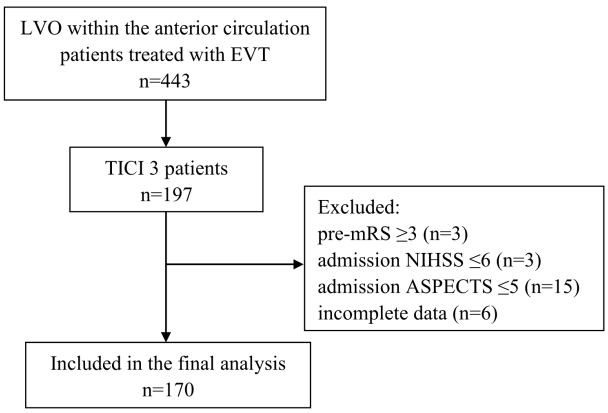

FIGURE 1
Flow chart demonstrating the number (n) of patients included in the analysis. ASPECTS, Alberta Stroke Program Early CT Score; LVO, large vessel occlusion; mRS, modified Rankin scale; EVT, endovascular treatment; NIHSS, National Institutes of Health Stroke Scale; TICI, Thrombolysis in Cerebral Infarction.

All tests were 2-tailed, and the level of significance was set at p<0.05. Multivariable analysis was performed with a logistic regression model including those factors with a *p*-value of ≤0.05. Subsequently, a backward stepwise selection was applied to identify the parsimonious model restricted to the most relevant prognostic factors; a significance level of 0.05 for a variable to stay in the model was chosen. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for unfavorable outcome. Data were analyzed using IBM SPSS Version 23.0 software (IBM Corporation, Armonk, New York, USA). The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were then used to determine the diagnostic values of these factors to predict unfavorable outcome.

Results

There were 197/443 (44.5%) patients who underwent EVT for an anterior circulation large vessel occlusion with complete reperfusion (mTICI 3). The inclusion criteria were met by 170 patients (Figure 1).

The median age of the patients was 66.0 (IQR, 58.8-74.3) years and 115 (67.6%) were male. One hundred (58.8%) patients had a favorable clinical outcome at 3 months. Compared to patients with a favorable outcome, those with unfavorable outcomes were more likely to be older (p<0.001). Patients with DM (p=0.019) or CAD (p=0.015) were also more likely to have unfavorable outcomes. Patients in the unfavorable group had higher NIHSS scores (p<0.001) on admission, less collateral compensation (p<0.001), and were more likely to undergo general anesthesia (p<0.001). Regarding serology, there were significant differences in glucose (p=0.041), NLR1 (p<0.001), NLR2 (p<0.001), and NLR3 (p<0.001). However, there were no significant differences in lipid panel (p>0.05) (Table 1).

Multivariable logistic regression analysis showed that higher NLR within 24h or at 3-7d after EVT independently increased the odds of unfavorable outcome at 3 months (OR=1.070, 95% CI 1.012 to 1.131, p=0.017; OR=1.129, 95% CI 1.032 to 1.236, p=0.008). In addition, older age, higher NIHSS scores, poor collaterals, and general anesthesia were also significant determinants of poor functional outcome (Table 2). We calculated the diagnostic values for the conventional model alone (Model 1, including age, DM, CAD, NIHSS, good collaterals and glucose) and conventional model with NLR parameters (Model 2 [convention model plus NLR1], Model 3 [convention model plus NLR2], and Model 4 [convention model plus NLR3]). The AUCs for each model were 0.864, 0.872, 0.872 and 0.883, respectively.

Discussion

In this study, we confirm that futile reperfusion is a common phenomenon in anterior circulation stroke patients achieving complete reperfusion, with a rate of 41.2%. This result is consistent with a previous study (13). Additionally, several variables, including older age, higher NIHSS score, poor collaterals, general anesthesia, NLR within 24h, and NLR during 3-7d after EVT were independent predictors for unfavorable clinical outcome at 3-month follow-up. When NLR is combined with a model of conventional determinants of outcome, the diagnostic efficiency is improved. Results of the current study could provide future targets to improve outcomes for patients with futile reperfusion, such as controlling the inflammatory cascade and EVT selection considerations.

The inflammatory cascade has been implicated in the ischemic process at different stages of stroke, during which brain damage and repair coexist (24, 25). Inflammatory activity is likely initiated to clear damaged tissue and promote synapse reconstruction *via* cytokines released by immune cells. However, continuous inflammatory activity beyond the acute stage may aggravate injury and hamper repair (26). Within the first hours after reperfusion, neutrophils are among the first cells to penetrate hypoxic tissue; they can cause damage to the blood brain barrier and contribute to injury of surrounding tissues (27). NLR is seen as a systemic marker of subclinical inflammation, and an increased ratio is of prognostic value in several disorders. Thus, perioperative NLR values likely reflect the state of inflammation in stroke patients.

Even when achieving complete reperfusion (i.e., mTICI 3), a substantial proportion of AIS patients still do not achieve satisfactory outcomes. Neuroinflammation may help to explain futile reperfusion, and NLR is an easily measurable parameter in routine clinical practice. Thus, we focus on this marker to illustrate the association of inflammation with prognosis in this special group of patients. In multivariable analyses, 24 hour and 3-7 day NLR are significantly associated with outcome whereas admission NLR is not, suggesting that postoperative neuroinflammation may be strongly associated with unfavorable outcome at 90 days. Aly et al. (7) also found that lower NLR at 3–7 days rather than on admission is associated with successful reperfusion and an independent predictor of favorable clinical outcomes. Besides, Chen et al. also concluded that day 1 NLR is better than admission NLR for predicting AIS patients outcome after reperfusion therapy with a large clinical population (28). A current meta-analysis (18) also mentioned that delayed NLR (postoperative NLR) has a better prognostic utility than admission NLR because of larger standard mean difference for good functional outcomes. This could be due to underlying pathophysiology; as lymphocyte would enter into ischemic area of the brain 1-2 days after initial cerebral ischemia, and subsequently combined with other pro-inflammatory cytokine to further aggravate the damage (29). Therefore, postoperative NLR may improve prognostication for functional outcomes (15). In another study examining interleukin-6 (IL-6) as a marker of inflammation, high IL-6 levels at 24 hours were associated with futile

TABLE 1 Patient demographics, clinical characteristics, blood test and procedure information.

Characteristics	Total (n = 170)	Favorable outcome (mRS90 0-2) (n = 100)	Unfavorable outcome (mRS90 3-6) (n = 70)	P value
Demographics				
Age, median (IQR)	66.0 (58.8-74.3)	64.0 (56.0-70.8)	71.0 (64.8-78.0)	<0.001[a]
Male, n (%)	115 (67.6)	68 (68.0)	47 (67.1)	0.906[b]
HTN, n (%)	111 (65.3)	61 (61.0)	50 (71.4)	0.160[b]
DM, n (%)	49 (28.8)	22 (22.0)	27 (28.6)	0.019[b]
Dyslipidemia, n (%)	43 (25.3)	23 (23.0)	20 (28.6)	0.411[b]
CAD, n (%)	42 (24.7)	18 (18.0)	24 (24.3)	0.015[b]
AF, n (%)	47 (27.6)	27 (27.0)	20 (28.6)	0.822[b]
Smoking, n (%)	63 (37.1)	36 (36.0)	27 (38.6)	0.733[b]
Drinking, n (%)	58 (34.1)	36 (36.0)	22 (31.4)	0.536[b]
Stroke history, n (%)	31 (18.2)	14 (14.0)	17 (24.3)	0.087[b]
Antiplatelet use, n (%)	36 (21.2)	17 (17.0)	19 (27.1)	0.111[b]
Anticoagulation use, n (%)	15 (8.8)	10 (10.0)	5 (7.1)	0.518[b]
Clinical characteristics				
Intravenous thrombolysis, n (%)	62 (36.5)	37 (37.0)	25 (35.7)	0.864[b]
NIHSS, median (IQR)	15 (12-19)	13 (10-16)	18 (14-21)	<0.001[a]
ASPECT score, median (IQR)	9 (8-9)	9 (8-10)	8 (7-9)	0.242[a]
Occlusion site, n (%)				
ICA	61 (35.9)	32 (32.0)	29 (41.4)	0.189[b]
MCA-M1	87 (51.2)	57 (57.0)	30 (42.9)	
MCA-M2	22 (12.9)	11 (11.0)	11 (15.7)	
Right side, n (%)	76 (44.7)	49 (49.0)	27 (38.6)	0.211[b]
Good collaterals, n (%)	70 (41.2)	57 (57.0)	13 (18.6)	<0.001[b]
TOAST, n (%)				
LAA	66 (38.8)	44 (44.0)	22 (31.4)	0.274[c]
Cardioembolic	100 (58.8)	54 (54.0)	46 (65.7)	
Others	4 (2.4)	2 (2.0)	2 (2.9)	
Blood test				
Glucose, mmol/L, median (IQR)	7.28 (5.99-9.52)	6.91 (5.93-9.16)	8.11 (6.10-10.46)	0.041[a]
TG, mmol/L, median (IQR)	1.18 (0.78-1.73)	1.17 (0.78-1.67)	1.20 (0.79-1.74)	0.804
TC, mmol/L, median (IQR)	4.42 (3.71-4.92)	4.43 (3.80-4.90)	4.42 (3.59-5.00)	0.689
HDL, mmol/L, median (IQR)	1.15 (0.99-1.38)	1.14 (0.95-1.37)	1.16 (1.01-1.38)	0.358
LDL, mmol/L, median (IQR)	2.63 (2.07-3.33)	2.69 (2.26-3.40)	2.59 (1.97-3.25)	0.210
Neutrophil1, 10^9/L, median (IQR)	6.75 (6.14-8.85)	6.01 (4.90-8.28)	7.35 (4.57-9.80)	0.184[a]
Lymphocyte1, 10^9/L, median (IQR)	1.34 (0.93-1.84)	1.43 (1.00-1.90)	1.26 (0.74-1.73)	0.080[a]
NLR1, median (IQR)	4.95 (2.66,7.59)	4.17 (2.65-6.99)	5.85 (2.63-10.67)	0.044[a]
Neutrophil2, 10^9/L, median (IQR)	7.74 (5.96-10.29)	7.31 (5.45-8.96)	8.57 (6.81-11.39)	0.001[a]
Lymphocyte2, 10^9/L, median (IQR)	1.05 (0.69-1.39)	1.19 (0.85-1.47)	0.80 (0.48-1.23)	<0.001[a]
NLR2, median (IQR)	7.51 (4.60-13.26)	6.82 (4.21-9.45)	10.87 (6.39-19.00)	<0.001[a]
Neutrophil3, 10^9/L, median (IQR)	6.23 (4.80-8.32)	5.65 (4.63-7.23)	7.53 (5.74-8.95)	<0.001[a]
Lymphocyte3, 10^9/L, median (IQR)	1.22 (0.87-1.57)	1.34 (0.96-1.70)	1.04 (0.77-1.35)	<0.001[a]
NLR3, median (IQR)	5.25 (3.40-7.94)	4.29 (2.77-6.69)	6.39 (4.47-10.64)	<0.001[a]
Procedure				
General anesthesia, n (%	35 (20.6)	15 (15.0)	20 (28.6)	0.031[b]
OTD, min, median (IQR)	262.5 (150.0-384.8)	279.0 (151.0-408.0)	244.5 (148.5-351.5)	0.369[a]
PTR, min, median (IQR)	36.5 (24.8-51.0)	33.0 (23.0-48.8)	39.0 (25.8-553.5)	0.094[a]
OTR, min, median (IQR)	428.0 (330.3-574.3)	446.0 (332.5-593.0)	418.5 (322.8-553.5)	0.585[a]

[a]Analysed by Mann-Whitney U test; [b]Analysed by Chi-square test; [c]Analysed by Fisher's exact test;
HTN, hypertension; DM, diabetes mellitus; AF, atrial fibrillation; CAD, coronary artery disease; NIHSS, National Institutes of Health Stroke Scale; ASPECT, Alberta Stroke Program Early CT score; ICA, internal carotid artery; MCA, middle cerebral artery; TOAST, the Trial of Org 10172 in Acute Stroke Treatment classification; LAA, large artery atherosclerosis; NLR, neutrophil to lymphocyte ratio; OTR, time of onset to recanalization; mTICI, the modified Thrombolysis in Cerebral Infarction score; IQR, interquartile rang;
Neutrophil1, Lymphocyte1 and NLR1 were measured at admission;
Neutrophil2, Lymphocyte2 and NLR2 were measured within 24h after EVT;
Neutrophil3, Lymphocyte3 and NLR3 were measured at 3-7d after EVT.

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TABLE 2 Independent predictors of unfavorable outcome despite complete reperfusion.

Variable	Model 1		Model 2		Model 3		Model 4	
	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
Age	1.059 (1.018-1.102)	0.004*	1.064 (1.021-1.108)	0.003*	1.052 (1.009-1.096)	0.017*	1.047 (1.005-1.092)	0.029*
DM	1.905 (0.654-5.549)	0.237	2.151 (0.725-6.385)	0.168	2.077 (0.691-6.241)	0.193	2.231 (0.737-6.752)	0.156
CAD	1.339 (0.483-3.713)	0.575	1.341 (0.484-3.716)	0.573	1.174 (0.408-3.379)	0.766	1.211 (0.418-3.512)	0.725
NIHSS	1.246 (1.121-1.385)	<0.001*	1.249 (1.120-1.391)	<0.001*	1.231 (1.104-1.372)	<0.001*	1.226 (1.098-1.367)	<0.001*
Good collaterals	0.161 (0.066-0.392)	<0.001*	0.166 (0.068-0.406)	<0.001*	0.158 (0.063-0.396)	<0.001*	0.135 (0.052-0.353)	<0.001*
Glucose	0.946 (0.817-1.096)	0.461	0.931 (0.801-1.082)	0.350	0.924 (0.790-1.080)	0.320	0.955 (0.822-1.110)	0.548
General anesthesia	4.507 (1.514-13.412)	0.007*	4.256 (1.423-12.733)	0.010*	4.211 (1.351-13.130)	0.013*	4.444 (1.434-13.768)	0.010*
NLR1	–	–	1.072 (0.995-1.154)	0.066	–	–	–	–
NLR2	–	–	–	–	1.070 (1.012-1.131)	0.017*	–	–
NLR3	–	–	–	–	–	–	1.129 (1.032-1.236)	0.008*

OR, odds ratio; CI, confidence interval; DM, diabetes mellitus; CAD, coronary artery disease; NIHSS, National Institutes of Health Stroke Scale; NLR, neutrophil to lymphocyte ratio
NLR1 were measured at admission;
NLR2 were measured within 24h after EVT;
NLR3 were measured at 3-7d after EVT.
*P<0.05.

reperfusion after adjusting for confounders (30). When NLR1, NLR2, and NLR3 were respectively added into our models for predicting outcomes, the efficiency improved compared to the model with conventional predictors alone (Figure 2). Therefore, postoperative neuroinflammation may be a promising and modifiable target for future research to improve outcomes for patients who may otherwise experience futile reperfusion.

In addition to NLR, some other factors, such as age, baseline NIHSS score, and collateral status also influenced final outcomes after complete reperfusion. Older age is a widely-accepted risk factor for futile reperfusion, and the benefit of EVT is known to decrease with advanced age possibly attributed to increased frailty (31). The NIHSS score closely reflects the stroke severity and has been previously associated with outcomes after EVT (32). General anesthesia is more often utilized in critically ill patients in our practice, and these patients tend to benefit less from EVT. In practices where general anesthesia is routinely used, it is unclear whether this signal would persist given data supporting its non-inferiority compared to conscious sedation in EVT (33). Lastly, collateral circulation is known to help sustain viable cerebral tissue prior to successful reperfusion (34, 35), which could explain the reason why patients with good collaterals are more likely to have a favorable outcome. These various factors can provide interventionalists with new perspectives toward preventing futile reperfusion.

There are limitations to our study, principally related to bias inherent to the study design. We also did not continuously measure serologic markers through 90 days after EVT. Intercurrent complications such as infection which is common in severe stroke leading to an increase in neutrophil and NLR cannot be ruled out.

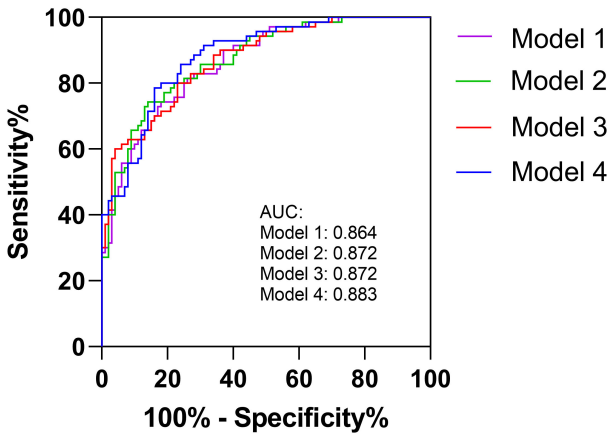

FIGURE 2
The AUCs for conventional model (Model 1) and conventional model with NLR parameters (Model 2, Model 3 and Model 4).

Conclusions

Even with technically complete reperfusion, several factors are strongly associated with unfavorable outcomes. Our findings suggest that advanced age, increased stroke severity, poor collaterals, general anesthesia, and NLR post-EVT are independent predictors of an unfavorable clinical outcome following complete reperfusion. Neuroinflammation likely merits further attention in future studies.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YF, XB and WL contributed to the initial idea for this study. WC, XX, and FY finished the study design. JL, PG, BY, YM, FC and LJ were consulted about the clinical issues. ZF, QT, and XG contributed to the original draft. TW, AS, AAD, RWR, QM XB, YC, YW and JC were responsible for the revision of the draft. YF and XB contributed equally to this article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Platelet-monocyte aggregates: molecular mediators of thromboinflammation

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Platelets, key facilitators of primary hemostasis and thrombosis, have emerged as crucial cellular mediators of innate immunity and inflammation. Exemplified by their ability to alter the phenotype and function of monocytes, activated platelets bind to circulating monocytes to form monocyte-platelet aggregates (MPA). The platelet-monocyte axis has emerged as a key mechanism connecting thrombosis and inflammation. MPA are elevated across the spectrum of inflammatory and autoimmune disorders, including cardiovascular disease, systemic lupus erythematosus (SLE), and COVID-19, and are positively associated with disease severity. These clinical disorders are all characterized by an increased risk of thromboembolic complications. Intriguingly, monocytes in contact with platelets become proinflammatory and procoagulant, highlighting that this interaction is a central element of thromboinflammation.

KEYWORDS

monocyte-platelet aggregates, thromboinflammation, antiplatelet therapy, P2Y12 inhibitor, inflammatory diseases, atherosclerosis

Introduction

Platelets interact and bind to monocytes by a variety of mechanisms, including attachment of platelets to monocytes via platelet P-selectin and monocyte PSGL1, the release of platelet granules containing chemokines and cytokines, and shedding of platelet-derived microvesicles. These interactions result in the upregulation of monocyte proinflammatory surface markers (e.g., CD40), migration (CD11b/CD18), and procoagulant tissue factor (TF), a principal initiator of coagulation. In addition, monocytes exposed to platelets secrete proinflammatory cytokines (TNF-α, MCP-1, IL-1β) and exhibit a proinflammatory transcriptome. Furthermore, platelets skew monocyte and macrophage differentiation towards a proatherosclerotic phenotype.

Our review covers how platelets affect monocytes in inflammatory diseases, and we present recent findings on potential therapeutic strategies to target the platelet-monocyte proinflammatory axis in thromboinflammation.

Platelets in hemostasis and thrombosis

Derived from megakaryocytes, platelets are small, anucleate cells circulating in the blood for seven to ten days. While crucial to hemostasis and thrombosis, an immunomodulatory effector role for platelets is increasingly apparent (1). Under physiological conditions, circulating platelets become activated when they come in

contact with subendothelial collagen following vascular injury. Exposed collagen attaches to von Willebrand factor (VWF) - a large globular multimeric glycoprotein - which then unfolds to a string-like structure. Platelets adhere to tethered VWF via the glycoprotein (GP) Ib-IX-V receptor complex (2) or directly via collagen through the GPVI receptor. Interaction of platelets with collagen and VWF results in platelet activation as shown by changes in the platelet cytoskeleton and release of cytokines, chemokines, and growth factors stored in alpha and dense granules (3). Adenosine diphosphate (ADP) derived from platelet dense granules and thromboxane A2 (TXA2) produced by activated platelets out of arachidonic acid, and other mediators including epinephrine and thrombin generated on the platelet membrane amplify and maintain the initial platelet response by recruiting and activating additional platelets. This ultimately leads to activation of the integrin complex GPIIb/IIIa, a key receptor for platelet adhesion, aggregation, and thrombus stabilization (4). As a result, activated platelets adhere to the vessel wall and form a thrombus, thereby preventing excessive blood loss at the injury site.

In addition to local platelet activation required for thrombosis and wound healing, platelets have effector roles in systemic inflammatory conditions. Platelet activation has been observed in sepsis (5–9), autoimmune disorders (10–14), and chronic proinflammatory conditions, including hyperlipidemia (15–17), atherosclerosis (18–22), and cardiovascular disease (22–26). Resultantly, patients with these diseases express elevated levels of circulating proinflammatory cytokines and chemokines and are also prone to thromboembolic complications (17, 27–29).

The role of monocytes in thromboinflammation

Monocytes originate in the bone marrow and constitute a subpopulation of approximately 10% of all peripheral blood leukocytes. Monocytes are key mediators of innate immunity. They phagocytose and present antigens, secrete chemokines and cytokines, and can terminally differentiate into different macrophage and dendritic cell subtypes that reside in the extravascular tissues (30, 31).

Based on the expression of surface markers CD14 and CD16, circulating monocytes are traditionally classified as either classical ($CD14^{++}$ $CD16^{-}$), intermediate ($CD14^{+}$ $CD16^{+}$), or nonclassical ($CD14^{+}$ $CD16^{++}$) subpopulations that exhibit different functional properties (32). While classical monocytes are phagocytic, $CD16^{+}$ monocytes are often upregulated in systemic infections, constituting the major cytokine source (33).

The interaction of platelets with monocytes has emerged as a key mechanism connecting thrombosis and inflammation. In support of a proinflammatory and procoagulant platelet effector function, monocyte-platelet aggregates (MPA) are elevated in multiple thromboinflammatory diseases and correlate with disease severity (34). To be able to bind to monocytes, platelets need to become activated (35, 36).

Platelet activation during inflammation

Inflammatory mediators such as complement factors (37), interleukin-6 (IL-6) (38), IL-8 (39), and tumor necrosis factor-alpha (TNF-α) (40) secreted from activated immune and vascular cells contribute to platelet activation by enhancing their adhesion properties to endothelial cells, elevating collagen-induced aggregation, and enforcing release of TXA_2.

Under inflammatory conditions, platelet-activating agonists such as TXA_2, ADP, and thrombin bind to heterotrimeric G protein-coupled receptors (GPCR) expressed on the platelet surface. GPCR consist of different transmembrane spanning G proteins (G_S, G_i, G_q, and $G_{12/13}$) that are associated with platelet receptors including the ADP receptors $P2Y_1$ (G_q) and $P2Y_{12}$ (G_i), the thrombin receptors PAR1 (G_q, $G_{12/13}$) and PAR4 (G_q, $G_{12/13}$), and the TXA_2 receptor (TP; G_q, $G_{12/13}$) (41). Upon ligand binding, GPCR induce receptor-specific interconnected signaling pathways that lead to platelet alpha and dense granule release and activation of GPIIb/IIIa (inside-out signaling) (42).

Pathways of platelet-monocyte interaction in inflammation

Following platelet activation, P-selectin (CD62P) stored in alpha granules translocates to the platelet surface. Platelet P-selectin binds to glycoprotein ligand 1 (PSGL1) which is constitutively expressed on the surface of monocytes. This initial engagement of platelets with monocytes is strengthened by platelet CD40L binding to monocyte CD40, platelet GPVI binding to extracellular matrix metalloprotease inducer (EMMPRIN, CD147) (43) and platelet GPIb attaching to monocyte CD11b/CD18 (MAC-1) (44). Fibrinogen-mediated binding of platelet GPIIb/IIIa to MAC-1 has also been discussed (45) but appears to play only a minor role in the formation of MPA (46). In addition to platelet and monocyte receptor binding, platelets attract monocytes via chemokines and cytokines released from alpha and dense granules, including soluble CD40L (sCD40L) (47, 48), CXCL4 (platelet factor 4) (49), and CCL5 (50). Platelet-derived extracellular vesicles (PEV) including platelet microparticles, microvesicles, and exosomes are tiny circular fragments shed from the platelet membrane, and have been shown to regulate monocyte properties (51–53).

Interaction of platelets with monocytes by direct cell-to-cell contact and platelet-derived mediators facilitates the transition of monocytes to a proinflammatory and procoagulant phenotype. In the following sections, we will outline the different mechanisms by which platelets modify monocytes, and summarize how these mechanisms contribute to inflammation and thrombosis in the clinical setting (**Figure 1**).

Activated platelets modify the phenotype of circulating monocytes

Monocyte subtypes as defined by their expression of surface markers CD14 and CD16 can be modified by platelet interaction:

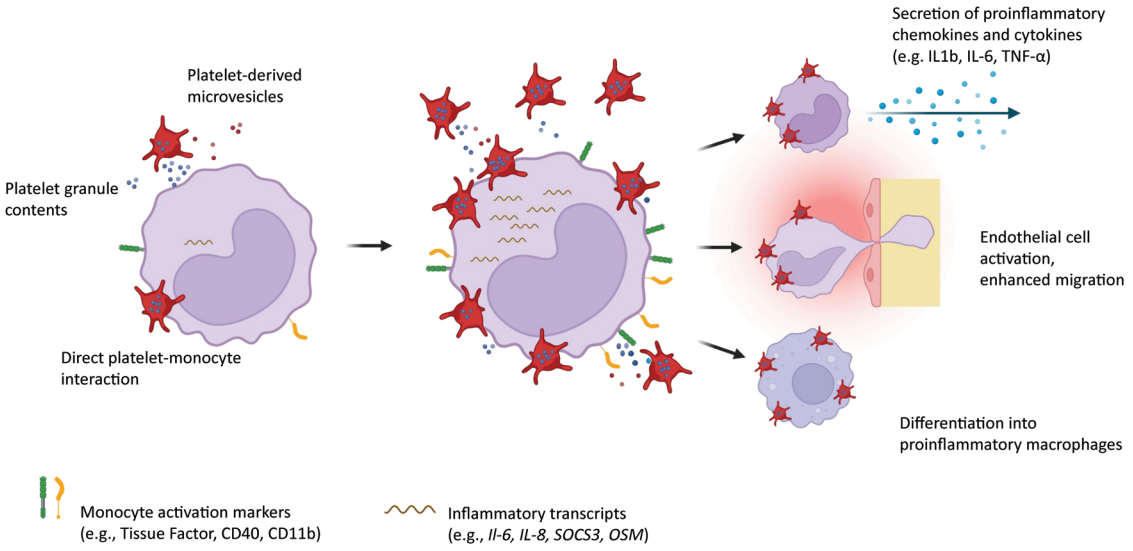

FIGURE 1
Platelets interact with circulating monocytes by direct attachment onto the monocyte surface, by release of alpha and dense granules containing chemokines and cytokines, and by shedding of microvesicles. Monocytes in turn upregulate inflammation markers on their surface, overexpress proinflammatory transcripts, secrete chemokines and cytokines, show enhanced migration potential, and differentiate into proinflammatory macrophages. Created with BioRender.com

Platelets appear to preferentially bind to CD16-bearing monocytes (16, 54). When bound to platelets, classical ($CD14^{++}CD16^{-}$) human monocytes upregulate surface CD16 - likely via platelet-derived transforming growth factor-β (TGF-β) inducing cyclooxygenase 2 (COX-2) upregulation and prostaglandin E2 synthesis in monocytes (55–57). High levels of antigen presenting-related molecules and secretion of proinflammatory cytokines and chemokines characterize these monocyte populations. In support of a proinflammatory platelet effector role, intermediate monocytes are expanded in various inflammatory disorders (34). Several other monocyte activation markers linked to atherosclerosis and inflammation are higher expressed on monocytes attached to platelets than monocytes without adhered platelets, including CD11b/CD18, CD40, PSGL1, HLA-DR, CD86, CD54, and CCR2 (34, 58).

Findings from several studies support a platelet-mediated proinflammatory monocyte phenotype (59). For example, activated platelets adhering to monocytes via the P-selectin PSGL1 axis in combination with alpha granule-released, CD40L, CXCL4 and CCL5 induce expression and secretion of monocyte chemoattractant protein-1 (MCP), IL-8, TNF-α, IL-1β, and IL-6 (50). Another proinflammatory mediator secreted by activated platelets is beta (β)-2 microglobulin which has been shown to induce CD16+ monocytes and augment monocyte inflammatory cytokine secretion (60, 61). β-2 microglobulin is also a component of the physiologic plasma compartment. Hence, the composition and interplay of platelet granule-derived and plasma molecules within the close monocyte-platelet interaction may shape the monocyte outcome rather than single molecular mediators. For example, β2 microglobulin and platelet TGF-β exert their effects on the same monocyte receptor but through different downstream signaling cascades, leading to different (proinflammatory vs. pro-reparative) monocyte phenotypes (61).

Intriguingly, monocytes attached to platelets have increased TF expression on their surface (62), resulting in thrombin-mediated fibrin generation and clot formation (63). Under physiologic conditions, TF is expressed on monocytes in an encrypted (inactive) form but can be decrypted (activated) into its procoagulant isoform upon proinflammatory stimuli (64). Activated platelets induced rapid TF upregulation on monocytes (65). This is mediated by platelet-bound P-selectin and does not require *de novo* protein synthesis (65) albeit incubation of monocytes with soluble P-selectin or platelets over several hours has been shown to induce TF (*F3*) gene expression (66). Additionally, soluble P-selectin shed from activated platelets contributes to the formation of procoagulant MV (67), emphasizing the contribution of platelets to coagulation activation.

Exposure of circulating monocytes to platelets also affects and modulates their subsequent differentiation into tissue-resident macrophages and dendritic cells: For example, platelets - via direct P-selectin-PSGL1-mediated cell-cell interaction - induced maturation of monocytes into antigen-presenting dendritic cells (68). In a murine sepsis model, activated platelets polarized monocytes toward proinflammatory M1 macrophages (69, 70). This reprogramming was initiated as soon as platelets attached to the monocyte surface and could not be altered later on during the differentiation progress (69). Hence, depending on the underlying pathogenesis, platelets shape the monocyte effector function in circulation and define the fate of macrophage recruitment into extravascular tissues. Platelet-mediated macrophage polarization plays an important role in chronic inflammatory diseases such as atherosclerosis and will be outlined further in the upcoming section.

In summary, activated platelets are required to accelerate monocyte-driven inflammation and thrombosis, two tightly interconnected pathogenic mechanisms that profoundly impact on cardiovascular disease (CVD) and beyond.

Monocyte-platelet interaction in atherosclerosis and cardiovascular disease

Atherosclerosis is the underlying pathology in most cases of CVD including myocardial infarction (MI), stroke, and peripheral artery disease (PAD) (71). Atherosclerosis is a chronic non-resolving inflammatory disease characterized by the formation of lipid-rich calcified plaques within the arterial wall of large and medium-sized vessels due to transmigration and accumulation of activated proinflammatory immune cells consisting of macrophages and T cells. Rupture or erosion of an unstable atherosclerotic plaque results in life-threatening thromboembolic events.

While low MPA levels of around 5%–20% are a normal phenomenon observed in healthy populations (54, 72), their rise is indicative of inflammatory processes and is associated with atherothrombotic complications: Patients with PAD and CVD and/or cardiovascular risk factors, including diabetes mellitus (DM), arterial hypertension and hyperlipidemia exhibit elevated levels of circulating MPA (36, 47, 54, 73–75). Elevated levels of MPA in patients with acute MI were further increased in patients that developed in-hospital adverse events (74).

Circulating monocytes in CVD and other chronic inflammatory disorders are characterized by elevated expression of CD40, a member of the TNF receptor superfamily (76). Proinflammatory cytokines including IFN-γ, IL-1, and TNF-α induce upregulation of monocyte CD40, and monocytes attached to platelets have higher CD40 expression on their surface (34, 77). Importantly, CD40 is a receptor for CD40L which is on the surface of and a marker for activated platelets (48). CD40L-CD40 interaction does not only stabilize MPA but also enhances monocyte migration into the arterial wall (19, 78). CD40-activated macrophages then secrete inflammatory cytokines and matrix metalloproteinases, thereby contributing to plaque destabilization and rupture. In a murine atherosclerosis model, CD40 deficient mice had lower MPA and platelet-mediated leukocyte-endothelium interactions resulting in decreased plaque formation (19). In response to CD40 binding, platelet CD40L is shed from activated platelets into the circulation, which further augments platelet activation (79–81). Notably, platelet-derived CD40L contributes to P-selectin-mediated TF upregulation on monocytes (82, 83). TF expressing macrophages, as well as elevated levels of circulating TF, have been observed in patients with atherosclerosis (84, 85), metabolic cardiovascular risk factors (86–88), and in acute coronary syndrome (89–91).

CXCL4 is one of the most abundant chemokines released from alpha granules upon platelet activation (92). Importantly, CXCL4 has been detected in human carotid atherosclerotic plaques and positively correlates with lesion grade and presence of clinical symptoms (19, 93). The pathogenic role of CXCL4 in accelerating atherosclerosis is mediated, in part, by augmenting the arrest of monocytes on endothelial cells in conjunction with CCL5 (RANTES) (94).

Platelets not only induce attachment and facilitate transmigration of monocytes into the subendothelial space (95) and into atherosclerotic lesions (20) but also shape the subsequent macrophage phenotype: Monocytes exposed to platelet-derived CXCL4 differentiate into proinflammatory M4-type macrophages, which have been suggested as crucial contributors to plaque rupture in coronary artery disease (96). *In vitro* studies and murine models of atherosclerosis demonstrated that oxidized low-density lipoprotein (oxLDL) generated under chronic inflammatory conditions promoted platelet-monocyte interaction with subsequent monocyte extravasation and foam cell formation (16). Foam cells are lipid-laden macrophages that are a major constituent of atherosclerotic plaques (97).

Findings from our group confirm that platelets drive atherogenesis by inducing proinflammatory monocytes and macrophages: We reported that platelet competent atherogenic $Ldlr^{-/-}$ mice had increased monocyte surface expression of the adhesion receptor CD11b and had higher expression of inflammatory transcripts *CCL2*, *IL6*, and *CD11b* relative to platelet-depleted mice. Furthermore, in the presence of platelets, plaque macrophages were skewed towards a proinflammatory phenotype as defined by upregulation of *SOCS3,* which promoted proinflammatory cytokine production of IL-6, IL-1b, and TNF-α (20). Consistently, *SOCS3* mRNA expression of whole blood correlated with platelet activity and MPA formation in a clinical dataset of women with MI and in patients with symptomatic lower extremity atherosclerosis (20).

Platelets have been shown to also activate dendritic cells (DC) via the P-selectin-PSGL1 axis, thereby contributing to atherosclerosis progression in hyperlipidemic mice (98). This is mediated by toll-like receptor 4 (TLR4) signaling pathways, leading to enhanced secretion of inflammatory cytokines, T cell communication, and adhesion and migration properties of platelet-bearing DC (98).

In addition to physical contact and secretion of granule contents, platelets can modulate monocyte and macrophage responses by shedding of PEV into the circulation. There is evidence that PEV (via P-selectin) adheres to monocytes, thereby inducing cytokine and TF production, and contributing to atherothrombosis in CVD (99). Interestingly, P-selectin-positive and fibrinogen-positive PEV were elevated in patients six months after acute MI (100). Procoagulant PEV may also contribute to monocyte-endothelial interaction, thereby promoting atherosclerosis initiation and progression (101, 102).

Genetic RNA sequencing of platelets from CVD patients allows the identification of new mediators in MPA formation and monocyte reprogramming: Platelets from patients with symptomatic PAD were enriched with *myeloid-related protein-14* (*MRP-14*) mRNA and protein. MRP-14 augmented the expression of P-selectin, thereby enhancing MPA (21). Elevated serum levels of MRP14 were found in PAD patients with incident cardiovascular and limb events, underscoring a clinically important role for this protein (21).

Monocyte-platelet interaction in autoimmune disorders

Patients with autoimmune diseases such as Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are at higher risk of developing atherosclerotic and thromboembolic complications (103, 104). Again, elevated levels of MPA in patients with vasculitis, rheumatoid arthritis, and SLE highlight the tight interconnection between inflammation and atherothrombosis (58, 105–108). Monocytes attached to platelets exhibited higher levels of proinflammatory CD54, CD16, CD86, and HLA-DR in SLE patients (58). Additionally, procoagulant microvesicles shed from activated platelets have been implicated in inducing proinflammatory monocytes in RA and SLE (51, 106, 109).

In RA patients, activated platelets attaching to intermediate CD16+ monocytes contributed to elevated MPA formation and inflammatory cytokine secretion via CD147 signaling (106). High platelet activation and platelet-leukocyte aggregation were associated with enhanced TF-dependent global coagulation activation in SLE patients (108).

Monocyte-platelet interaction in infection and inflammation

In addition to a hyperreactive platelet phenotype and a heightened incidence of thromboembolic complications, elevated levels of circulating MPA were a prominent clinical finding in patients with severe COVID-19 (110–113). Platelet-monocyte interaction in COVID-19 was strongly associated with monocyte TF expression and global coagulation activation as shown by elevated fibrinogen and D-dimers (112). Interestingly, upregulation of P-selectin-dependent monocyte TF expression could be reproduced when monocytes from healthy donors were co-cultured with platelets from COVID-19 patients, once more underlining the proinflammatory effector role for platelets in COVID-19 (112).

In dengue virus infection, platelets are activated - evidenced by increased P-selectin and release of CD40L and cytokines- upon direct contact with the virus. As a consequence, elevated MPA can accelerate the monocyte inflammatory response (114–116). In an experimental cerebral malaria model, platelet-derived CXCL4 drove monocyte cytokine production in *Plasmodium berghei* infected mice (117).

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection (118) and is characterized by platelet activation and platelet-monocyte interaction (119). Elevated levels of circulating MPA were associated with higher morbidity and mortality and correlated with thromboembolic complications in patients with sepsis (120, 121).

While the majority of studies clearly show a proinflammatory role for MPA, platelets exhibited anti-inflammatory properties on the macrophage phenotype in a murine endotoxemia model of severe septic shock (122). These seemingly divergent findings indicate that the effector role of platelets is complex and depends on the underlying clinical disease and severity. More studies are warranted to delineate and better understand the divergent functions of MPA.

Interaction of platelets with neutrophils and lymphocytes

In addition to monocyte-platelet interaction, formation of neutrophil-platelet aggregates (NPA) represents another intercellular connection linking thrombosis and inflammation. Similar to MPA, elevated circulating NPA have been observed in CVD (123), PAD (124), in COVID-19 (125), and have been linked to an enhanced risk of deep venous thrombosis (126). Platelets – via the P-selectin-PSGL1 axis – activate and induce neutrophils to release neutrophil-extracellular traps (NETs) into circulation (127). NETs consist of DNA, histones, and neutrophile-derived enzymes, most importantly myeloperoxidase, and constitute the most crucial platelet-mediated effect on neutrophils to promote thrombosis in inflammation (128). Since NPA and MPA are found to be simultaneously elevated in proinflammatory conditions (129), activated platelet induce a prothrombogenic state via both leukocyte subpopulations by enhancing their respective inflammatory response *in vivo*. In contrast, while the magnitude of circulating lymphocyte-platelet aggregates does not change in inflammatory, thrombotic, and atherosclerotic diseases, platelet-lymphocyte interaction has been delineated to exert a specific role in cancer: For example, platelets attenuated T cell activity in cancer patients ex vivo (130), and promoted tumor progression via suppression of CD8 T cells in murine cancer models (131).

In summary, platelets differentially modulate leukocyte activity in response to the underlying disease, with MPA and NPA being important contributors in thromboinflammatory diseases.

Can platelet inhibitors prevent MPA formation?

Targeting MPA has come into focus as an interesting therapeutic strategy. Interrupting the P-selectin-PSGL1-axis offers therapeutic potential in preventing (athero)thrombosis as shown in preclinical models (132–134). Importantly, since P-selectin is also present on activated endothelial cells (135), using an anti-P-selectin antibody allows to attenuate microvascular inflammation in addition to preventing proinflammatory platelet-monocyte interaction.

However, the synthetic P-selectin inhibitor PSI-697 failed to decrease MPA in healthy smokers (136). So far, among P-selectin inhibitors, only crizanlizumab was approved in 2019 (137) and received a conditional marketing authorization by the European Medicines Agency (EMA) in 2020 (138) for the prevention of pain crises in sickle cell disease. In this clinical setting, crizanlizumab is used to prevent sickle erythrocytes to adhere to and activate P-selectin expressing endothelial cells (139). Due to unpublished results of the STAND trial, fate is currently unclear (140).

Recently, a phase 2 clinical trial investigated the effects of crizanlizumab on patients hospitalized with moderate COVID-19 which is associated with endothelial dysfunction and vascular inflammation. Here, crizanlizumab therapy was associated with significantly decreased plasma levels of prothrombin fragments and thrombin-antithrombin complexes, both laboratory markers for coagulation activation (141). Subsequently, the effectiveness of crizanlizumab in preventing adverse clinical outcomes in hospitalized COVID-19 patients is being investigated in a large international phase 4 randomized clinical trial (142).

While PSGL1 and P-selectin inhibitors directly interfere with platelet-monocyte interaction as well as with leukocyte and erythrocyte attachment onto endothelial cells, most therapeutics that are in clinical use block platelet signaling pathways upstream of the release of alpha granule contents like P-selectin. Targeting the ADP-activated $P2Y_{12}$ pathway effectively reduces MPA in several clinical studies: In patients with acute MI, atherosclerotic vascular disease, and cardiovascular risk factors including diabetes mellitus, intake of $P2Y_{12}$ inhibitor therapy was associated with MPA reduction *in vivo* and ex vivo (73, 75, 143, 144). Importantly, the $P2Y_{12}$ inhibitor ticagrelor resulted in reduced MPA and dampened myocardial inflammation (as shown by increased FGD-uptake and lower cardiac ejection fraction) in acute MI patients (75). Moreover, $P2Y_{12}$ blockade reduced monocyte TF expression (145, 146) and attenuated circulating levels of IL-8, TNF-α, and CCL2 in an experimental human model of systemic inflammation (147). In patients with acute coronary syndrome, the $P2Y_{12}$ inhibitor clopidogrel diminished circulating levels of proinflammatory TNF-α and C-reactive protein (144). Of note, pneumonia patients had significantly reduced plasma IL-6 under ticagrelor compared to patients taking placebo and needed less supplemental oxygen (148). In contrast, in non-critically ill patients with COVID-19, $P2Y_{12}$ blockade in addition to heparin compared to heparin alone did not result in increased odds of improvement in organ support-free days (142) and – equally to COX-1 inhibitors - only had a low likelihood of improving the number of organ support-free days in critically-ill COVID-19 patients when compared to patients without platelet inhibitors (149). Accordingly, depending on the underlying inflammatory condition, the effects of platelet inhibitors on defined clinical outcomes may vary.

COX-1 inhibition with aspirin did not lower MPA assessed *in vitro* and following 1-week of low-dose aspirin in healthy volunteers ex vivo (54). This might be due to aspirin not directly interfering with GPCR signaling-induced alpha degranulation and P-selectin expression. While aspirin therapy was insufficient to decrease MPA in acute stroke patients, it attenuated MPA ex vivo when whole blood from healthy volunteers was stimulated with thrombin receptor activator peptide 6 (TRAP-6) (150). Different outcomes of MPA under aspirin seem to depend on the measurement methods. Aspirin did not reduce mortality linked to sepsis in healthy elderly patients (151). Importantly, in a murine model of severe bacteremia, aspirin even enhanced inflammation and was associated with higher mortality (122), indicating a potential detrimental effect in severe sepsis.

Clinical data on the effect of PAR1 inhibitors on platelet-monocyte interaction are scarce. PAR1 inhibition attenuated MPA formation in whole blood stimulated with the TXA_2 analog U-46619, and consequently, monocyte attached to platelets had less surface expression of CD40 and TF (129). In another in-vitro analysis, incubation of whole blood from healthy donors with PAR1 inhibitors prevented TRAP-6 - but not PAR4 - or collagen-related peptide (CRP)-induced MPA formation, emphasizing that the efficacy of platelet inhibitors is dependent on the activation pathway (152). There is conflicting data on the effect of GPIIb/IIIa inhibitors on MPA with some studies even showing paradoxically elevated MPA levels under eptifibatide (153, 154).

In addition to blocking cell surface receptors, platelet activation can also be targeted by intracellular inhibition of phosphodiesterases (PDE). As a result, intracellular levels of cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP) accumulate, which eventually disrupts the rearrangement of the actin cytoskeleton required for platelet activation and granule release (155). While the PDE inhibitor dipyridamole did not attenuate thrombin- or collagen-stimulated monocyte-platelet aggregates *in vitro* (156), patients under combined treatment with aspirin and dipyridamole following a transitory ischemic attack (TIA) had lower MPA when exhibiting dipyridamole responsiveness in a platelet function test in contrast to patients that were identified as dipyridamole non-responders (157).

Platelet GPIb and its ligand MAC-1 on leukocytes are newer potentially therapeutic targets (158). Interestingly, inhibiting this interaction resulted in impaired thrombus formation and delayed thrombosis in murine models (158). In a phase I study on healthy volunteers, GPIb-binding anfibatide, a snaclet (snake C-type lectins) purified from snake venom, showed antithrombotic efficacy without affecting haemostasis (159). Clinical trials are anticipated to investigate the clinical efficacy of GPIb and MAC-1 inhibitors in attenuating thrombosis linked to inflammation.

In conclusion, targeting platelet-monocyte interactions is likely to be beneficial to various inflammatory diseases. Among platelet inhibitors, directly targeting platelet P-selectin or preventing its release from alpha granules by upstream blockade of $P2Y_{12}$ have been shown to be the most potent strategies to attenuate MPA. Further clinical studies would be welcome to evaluate the effectiveness of antiplatelet therapy-mediated MPA decrease on clinical outcomes within the different inflammatory diseases.

Potential therapies targeting MPA are summarized in (**Figure 2**).

Measurement of monocyte-platelet interactions

Measuring monocyte-platelet aggregates in whole blood by flow cytometry is a straightforward and commonly used method to assess ex vivo markers of thromboinflammation in clinical studies. However, since there is no clear consensus on the best methodological approach, various preanalytical variables such as blood drawing (venous puncture or access via central venous catheter), sample handling (e.g., temperature, transportation,

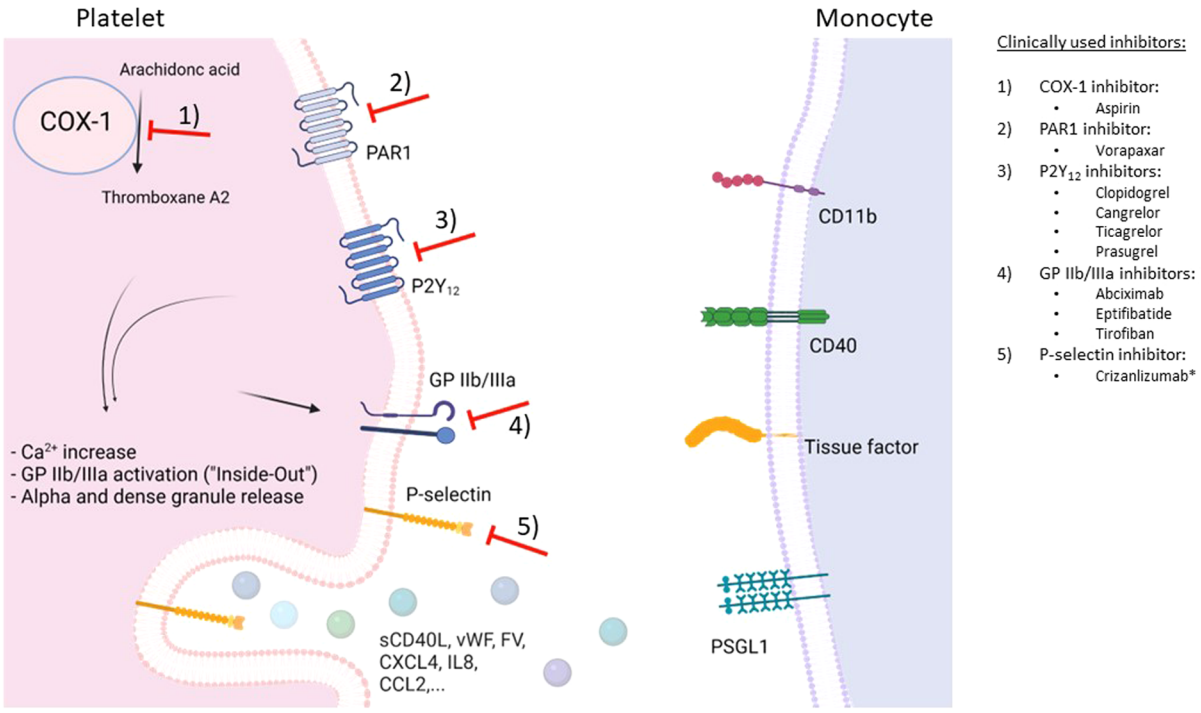

FIGURE 2
Schematic overview of potential therapeutic targets to prevent monocyte-platelet interactions. Depicted are G protein-coupled receptors (GPCR) associated with platelet receptors $P2Y_{12}$ and PAR1 as well as GPIIb/IIIa receptors on the platelet surface, and cyclooxygenase-1 (COX-1)-mediated production of platelet-activating thromboxane A2. Following platelet activation, many interconnected pathways result in intracellular calcium increase, activation of GPIIb/IIIa, and release of alpha and dense granule contents. P-selectin (released from alpha granules) translocates to the platelet surface and binds to P-selectin glycoprotein ligand-1 (PSGL1) on the monocyte surface, resulting in monocyte-platelet aggregates (MPA). In turn, monocytes become proinflammatory and procoagulant. *has received a conditional marketing authorization by the European Medicines Agency in 2020. Figure created with BioRender.com

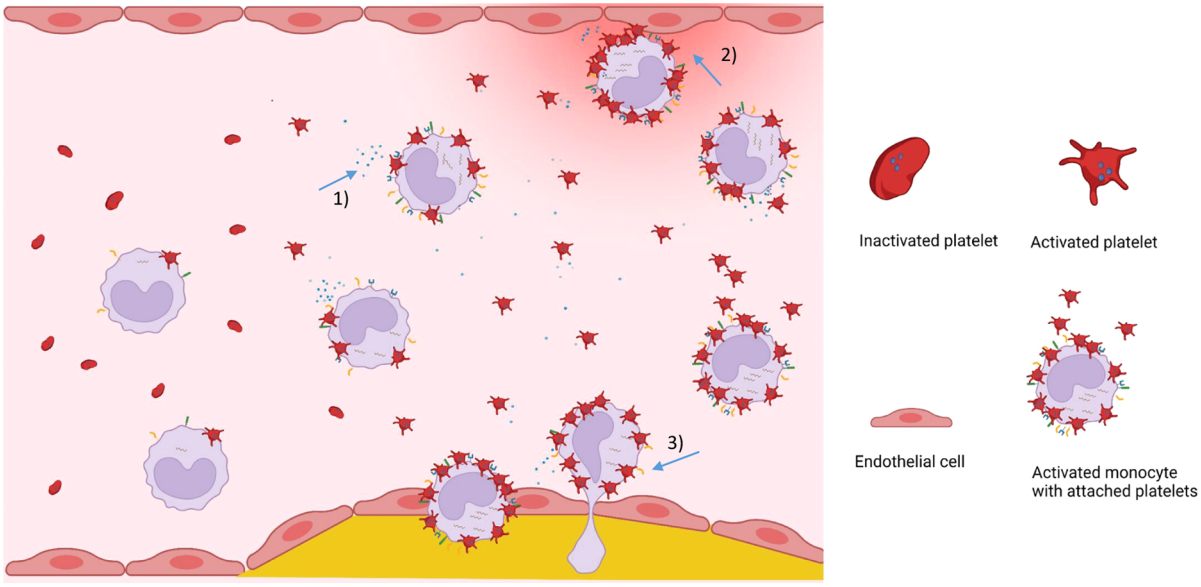

FIGURE 3
Visual summary on the role of MPA in cardiovascular disease. Plasma levels of circulating monocyte-platelet aggregates are increased in inflammatory diseases. Platelet-induced proinflammatory monocytes secrete inflammatory cytokines and chemokines (1), attach to and activate endothelial cells (2), show enhanced transendothelial migration potential (3), and differentiate into proinflammatory macrophages that contribute to the progression of atherosclerosis. Figure created with BioRender.com

storage), and processing (centrifugation configuration) may lead to incongruent results (72, 160). When interpreting MPA data, it is therefore important to know the experimental set-up and techniques applied for analysis. The following cornerstones need to be considered when measuring MPA by flow cytometry: To avoid any artificial platelet activation, blood collection should always be performed by direct venous puncture without a tourniquet and after an initial discard. The type of anticoagulant (e.g., heparin, EDTA, or sodium citrate) in the collection tube may affect the magnitude of monocyte-platelet aggregate levels (160). Following staining with monocyte and platelet antibodies, blood can be fixed, lysed and stored at 4°C for up to 24 h (160). Monocytes are identified by forward and side scatter properties and by CD14 (and CD16) expression. For platelet labeling, antibodies binding to constitutively expressed platelet surface glycoproteins, e.g., CD41, CD42a, CD42b, or CD61, can be used. MPA are quantified as the percentage of monocytes positive of a platelet marker within the gated monocyte population. Alternatively, multispectral imaging flow cytometry combining flow cytometry and imaging data provides additional information on platelet binding to individual monocytes (161).

Conclusions

Accumulating evidence demonstrates that activated platelets induce a proinflammatory monocyte phenotype, affecting the inflammatory response in acute and chronic inflammation. Mediated by direct platelet-monocyte interactions and platelet-derived cytokines, chemokines, and shedding of procoagulant extracellular vesicles. As a consequence, monocytes release inflammatory cytokines into circulation, become procoagulant, and differentiate into proatherogenic macrophages (**Figure 3**). Targeting platelet effector cell properties – and hence monocyte-driven inflammation and coagulation – appears to be a promising strategy in inflammatory settings beyond CVD.

Author contributions

CR: drafted the first version of the manuscript. TB and JB: critically revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Jeffrey Berger is PI for the NIH-funded ACTIV4a trial investigating P2Y12 inhibitors in patients hospitalized with COVID-19.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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