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RESEARCH

Neutrophils Induce P-Selectin Shedding from Activated Platelets Via Neutrophil Elastase

Yingting Huang[1] · Liqin Ling[1] · Shanshan He[2] · Chaonan Liu[1] · Xunbei Huang[1] · Shuang Wang[1] · Zhiyu Yu[1] · Jing Zhou[1]

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Abstract

It is suggested that neutrophil adhesion to P-selectin (P-sel) on activated platelet can induce P-sel shedding, however, the underlying mechanism remains unclear. This study is to investigate the role of neutrophil in platelet P-sel shedding. Platelets were activated by thrombin, collagen or thrombin receptor agonist peptide (TRAP). Activated platelets were co-cultured with whole blood, peripheral blood mononuclear cells or neutrophils, in the presence or absence of lipopolysaccharide, Pam3CSK4, myeloperoxidase inhibitors, neutrophil elastase (NE) inhibitors, or Latrunculin B (LatB) (inhibiting neutrophil extracellular traps (NETs) formation but not reducing protease release). Samples were collected at 0, 1, 2, and 4 hours. P-sel expression on platelets, soluble P-sel (sP-sel), and soluble NE were assessed. P-sel shedding only occurred on platelets activated by thrombin in the presence of neutrophils (P-sel-positive platelets, 70.0%→11.1%; P-sel intensity fold decrease, 0→0.61; sP-sel fold increase, 0→6.94). This shedding was partially reversed by NE inhibitors and LatB (sP-sel fold increase: 0→3.62; 0→5.18), but accelerated by MPO inhibitors (sP-sel fold increase: 0→17.59). Soluble NE was lower in co-culture of thrombin-activated platelets and neutrophils (110.78→132.75 ng/mL), compared to co-culture of thrombin-activated platelets and LatB-treated neutrophils (144.42→153.33 ng/mL), or compared to co-culture of collagen (or TRAP)-activated platelets and neutrophils (220.39→388.04 ng/mL; 287.47→385.16 ng/mL). Lipopolysaccharide and Pam3CSK4 did not change neutrophil-induced P-sel shedding. Platelet-neutrophil interaction might be mutual. Thrombin-activated platelet promotes neutrophil activation to form NETs; and in turn, neutrophil induces platelet P-sel shedding via concentrated NE in NETs, resulting in irreversible functional downregulation of platelet P-sel-mediated interactions.

Keywords Platelets · Neutrophil · P-selectin · Neutrophil elastase

Introduction

P-selectin (CD62P), a member of calcium-dependent lectins (glycoproteins) family, is produced in megakaryocytes and endothelial cells [1]. It is stored in α-granules of platelets and Weibel-Palade bodies of endothelial cells when the cells are at resting state. When the cells are activated, P-sel rapidly translocates to cell surface, forming membrane P-sel [2]. In addition to serving as a marker of platelet activation, platelet membrane P-sel plays a critical role in leukocyte recruitment and function by mediating their adherence and transmigration to sites of inflammation [3].

Upon activation, platelets rapidly express P-sel on their surface, which binds to P-sel glycoprotein ligand-1 (PSGL-1) on neutrophils, initiating initial adhesion and triggering downstream signaling pathways [4, 5]. This adhesion activates neutrophil integrin Mac-1 (CD11b/CD18), which

Yingting Huang and Liqin Ling contributed equally.

✉ Jing Zhou
zhoujinghuaxi@163.com

[1] Department of Laboratory Medicine, Clinical Laboratory Medicine Research Center, West China Hospital, Sichuan University; Sichuan Clinical Research Center for Laboratory Medicine, No. 37 Guo Xue Alley, Chengdu 610041, Sichuan, China

[2] Key Laboratory of Drug Targeting and Drug Delivery Systems, West China School of Pharmacy, Ministry of Education, Sichuan University, Chengdu 610041, Sichuan, China

subsequently binds to platelet surface receptors (e.g. GPIbα), thereby enhancing intercellular adhesion strength and promoting neutrophil chemotaxis, degranulation, and the release of inflammatory mediators [6]. Meanwhile, activated platelets can induce the formation of neutrophil extracellular traps (NETs) [7]. NETs, composed of DNA, histones, and proteases, contribute to coagulation and thrombosis by trapping pathogens, activating the contact pathway, and promoting thrombin generation—thereby linking inflammation and thrombosis [8]. A positive feedback loop exists between NETs and platelets, particularly evident in cardiovascular disease and sepsis, which amplifies thromboinflammatory responses [9].

Platelets can also bind to peripheral blood mononuclear cells(PBMCs)through P-sel–PSGL-1, leading to upregulated proinflammatory responses and enhanced cytokine secretion [10, 11]. Moreover, activated platelets also secrete various soluble mediators—including CXCL4, IL-1β, TGF-β, reactive oxygen species (ROS), and exosomes to modulate the functions of both neutrophils and PBMCs [10]. Up to now, accumulated data has shown that elevated platelet-leukocyte interactions are closely associated with the severity of thrombo-inflammatory diseases [12, 13], further underscoring the immunoregulatory and prothrombotic roles of platelet–leukocyte interactions.

However, interestingly, although important for the interaction between platelets and leukocytes, P-sel is rapidly shed from activated platelet surface into plasma shortly after activation, becoming soluble P-sel (sP-sel) [14–16]. Unlike P-sel on membrane surface, which forms dimers or oligomers to enhance leukocyte binding, sP-sel exists as a monomer, which is unable to effectively interact with PSGL-1 on leukocytes [17], thus, the overall role of sP-sel in immune responses and hemostasis is not prominent [7].

From an evolutionary perspective, the shedding of P-sel from activated platelets represents a negative feedback mechanism that regulates platelet P-sel-mediated inflammation. It is suggested that neutrophil adhesion to P-sel on activated platelet can induce P-sel shedding, however, the underlying mechanism remains unclear. This study aims to explore the mechanisms of P-sel shedding and its role in modulating platelet-mediated inflammatory responses.

Materials and Methods

Blood Collection

Platelets, leukocytes and red blood cells (RBCs) used in this study were obtained from healthy adult volunteers. Blood was collected into polypropylene tubes containing 0.109 M sodium citrate as an anticoagulant. Samples were processed and isolated in Department of laboratory medicine at West China Hospital, Sichuan University. All procedures were approved by the Ethics Committee of West China Hospital, Sichuan University, and informed consent was obtained from all volunteers.

Preparation of Platelets, Leukocytes, and Whole Blood

Platelet-rich plasma (PRP) was prepared by centrifugation at 200 g for 5 min. The resulting pellet was washed and resuspended in phosphate buffer saline (PBS). For activation, platelets were treated with thrombin (10602400001, Roche, 1 U/mL), collagen (FD034945, Sysmex, 100 µg/mL), or thrombin receptor agonist peptide (TRAP) (CLP0512, Solarbio, 50 µM) for 15 min at 37 °C (P-sel positive platelet was around 70–80% as evidenced by flow cytometry). Peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated from whole blood using Polymorphprep™ (Serumwerk, Germany) through density gradient centrifugation at 500 × g for 30 min at room temperature with slow deceleration. After centrifugation, the layer above Polymorphprep™ contained only peripheral blood mononuclear cells (PBMCs), while the lower layer contained polymorphonuclear leukocytes (mainly neutrophils). Both PBMCs and neutrophils had a purity greater than 90%. Platelets were removed by centrifugation at 200 g for 5 min for whole blood preparation. All procedures were at room temperature.

Co-culture Experiments with Activated Platelets

Activated platelets were co-cultured with whole blood, PBMCs, or neutrophils at 37 °C for 4 h. In some experiments, before co-culturing with activated platelets, neutrophils were pre-treated with neutrophil elastase (NE) inhibitor Sivelestat (HY-17443, MedChemExpress, 1 mM), myeloperoxidase (MPO) inhibitor ABAH (475944-1GM, EMD Millipore, 2 mM), Latrunculin B (LatB) (L5288, Merck Millipore, 5 µM), lipopolysaccharide (LPS) (L2880, Sigma-Aldrich, 100 µg/mL), or Pam3CSK4 (tlrl-pms, InvivoGen, 25 µg/mL) for 1 h. Samples were taken at four time points: 0, 1, 2, and 4 h of co-culture. After centrifugation, the supernatant was collected, and the cell pellets were fixed with 4% paraformaldehyde.

Detection of P-sel on the Platelet Surface

Flow Cytometry Detection of P-sel on Platelets The cell pellets were blocked with 2% Bovine Serum Albumin (BSA) and then stained with CD42b (303934, Brilliant Violet 510™ anti-human CD42b, Biolegend), CD62P antibodies

(304905, PE-anti-human CD62P, Biolegend) and CD45 (304025, PerCP-anti-human CD45, Biolegend) for 1 h at room temperature, protected from light. After washing with PBS buffer, the cells were analyzed using a flow cytometer (BD FACSCanto II, BD Biosciences). At least 50,000 events were analyzed for each sample. Data were analyzed using FlowJo software (FlowJo, LLC) to determine the percentage of CD62P positive platelet (%) and the number of platelet–leukocyte aggregates (PLAs, CD62P positive/PerCP-anti-human CD45 positive).

Chemiluminescence Immunoassay Detection of P-sel on Platelets The expression of platelet P-sel was detected in real-time using a homogeneous chemiluminescence immunoassay instrument (HSCL-5000, Poclight Biotech, China). After sampling at the corresponding time points, the samples were centrifuged and washed with PBS to obtain the cell pellets. Homogeneous chemiluminescence immunoassay is a two-site ("sandwich") chemiluminescence immunoassay based on chemiluminescence (CL) resonance energy transfer (CRET) technology. The acridinium ester (AE) emits a strong CL signal in the presence of H_2O_2. Graphene oxide (GO) serves as an efficient quencher, creating a "signal off" state via CRET between GO and AE-labelled DNA3. Upon sample application, DNA1-coated CD61 antibody, DNA2-coated CD61 antibody, and AE-labelled DNA3 form a sandwich complex, detaching DNA3 from GO and inhibiting CRET. This produces a CD61-CL signal, mimicking quantitative platelet count detection. For P-sel expression on platelets, the same principle is applied using DNA1-coated CD62P antibody and DNA2-coated CD41 antibody. The sandwich complex forms with CD62P-positive platelets, detaching AE-labelled DNA3 from GO and generating a CD62P-CL signal, mimicking quantitative P-sel positive platelet count detection. The CL intensity ratio of CD62P to CD61 is used to represent the P-sel intensity. Due to methodological limitations, chemiluminescence immunoassay could not be used to analyze whole blood samples.

Detection of sP-sel and NE

sP-sel and NE in the supernatants were determined using ELISA kits (Boster Bio, China) according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm using a microplate reader.

Statistics

Data were analyzed using GraphPad Prism 9.5. The normality of the data was assessed using the Shapiro-Wilk test. For normally distributed data, results are presented as mean ± SEM. A two-way ANOVA was conducted to evaluate differences among groups across various time points (0, 1, 2, and 4 h). Tukey's multiple comparisons test was used to analyze differences between groups at each time point, as well as changes from 0 h to 4 h measurements within each group. Spearman correlation analysis was performed to evaluate the relationship between PLAs and the fold increase of sP-sel. Statistical significance was defined as $P < 0.05$.

Results

Platelet P-sel Shedding Occurred in Whole Blood but not in Buffer

Activated by thrombin, after 1 h of co-cuture with whole blood, the percentage of P-sel positive platelets already decreased to a very low level (0 h→1 h, 79.5%→9.7%), while the percentage of P-sel positive platelets co-cultured in buffer did not change significantly over a period of 4 h (0 h→4 h, 74.7%→68.9%) (Fig. 1a). Correspondingly, an increase in sP-sel fold change was already evident at 1 h of co-culture with whole blood (6.77 fold increase), while there was only a slight but nonsignificant increase in sP-sel over a period of 4 h in buffer (1.40 fold increase) (Fig. 1b). At 4 h, compared to the group where platelets co-cultured with buffer, the two parameters were statistically different in the group where platelets co-cultured with whole blood (Fig. 1c, d).

Neutrophil was Involved in Platelet P-sel Shedding

Activated by thrombin, after 1 h of co-culture with neutrophils, the percentage of P-sel positive platelets already decreased to a very low level (0 h→1 h, 70.0%→24.3%), while the percentage of P-sel positive platelets co-cultured with PBMCs did not significantly change until 4 h of co-culture (0 h→4 h, 69.4%→56.4%) (Fig. 2a). Similarly, the fold decrease in CD62P/CD61 ratio was already evident at 1 h when co-cultured with neutrophils (0.28 fold decrease), but not evident until 2 h when co-cultured with PBMCs (0.28 fold decrease) (Fig. 2b). Correspondingly, the fold increase in sP-sel was already evident at 1 h when co-cultured with neutrophils (2.89 fold increase), but not evident until 2 h when co-cultured with PBMCs (2.51 fold increase) (Fig. 2c). At 4 h, compared to the group where platelets co-cultured with buffer or PBMCs, all the above-mentioned parameters were statistically different in the group where platelets co-cultured with neutrophils (Fig. 2d-f).

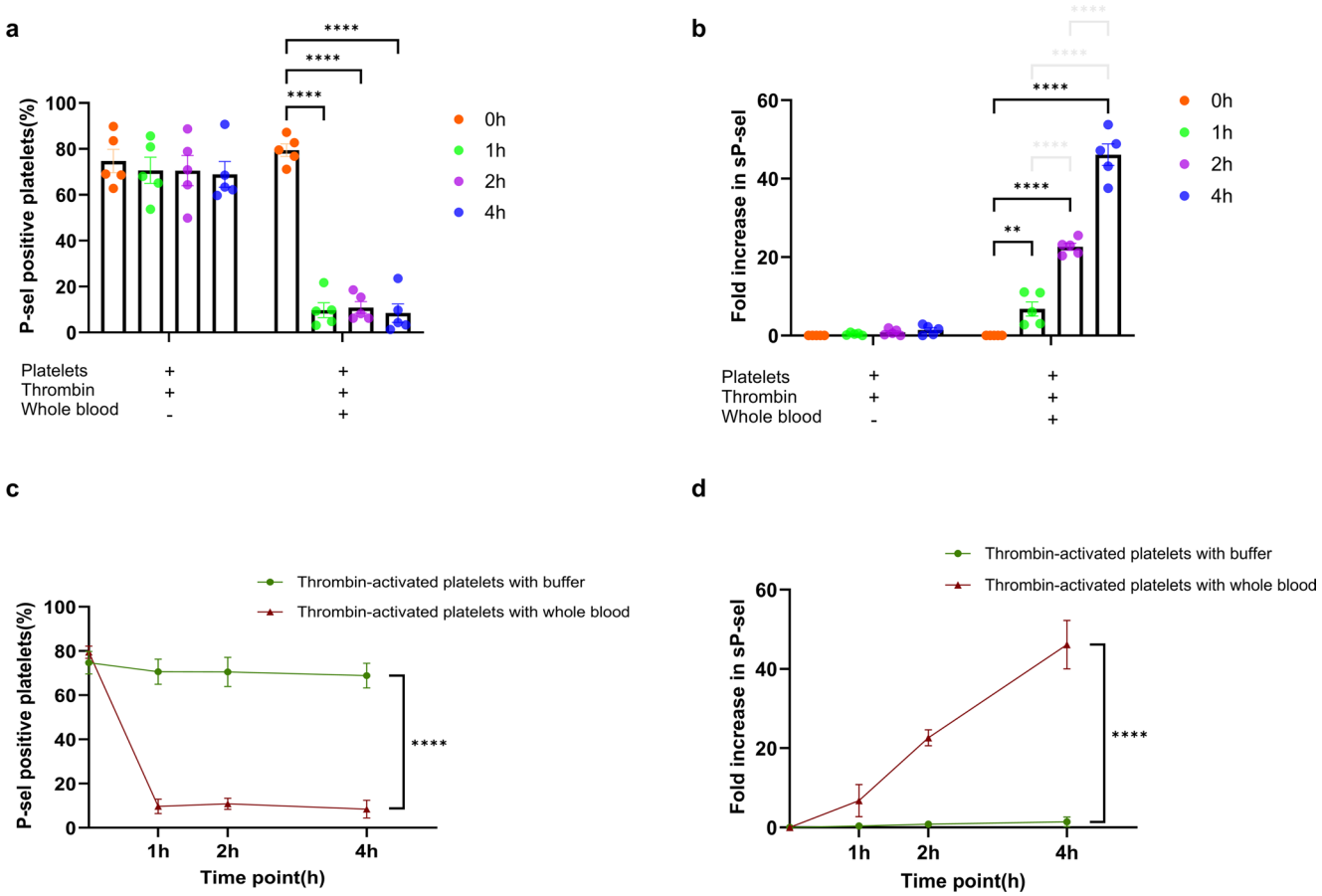

Fig. 1 Thrombin-activated platelets co-cultured with whole blood or buffer **a**, **c** percentage of P-sel-positive platelets ($n=5$); **b**, **d** fold increase in sP-sel levels ($n=5$). Data represent mean ± SEM. $^{**}P<0.01$, $^{****}P<0.0001$.

Neutrophil Induced Thrombin-Activated Platelet P-sel Shedding Via NE

The addition of NE inhibitors partly reversed neutrophils-induced P-sel shedding from thromin-activated platelets, the percentage of P-sel positive platelets did not significantly change until 2 h of co-culture (0 h→2 h, 70.2%→46.4%); while it was already evident at 1 h when co-cultured with neutrophils alone or in the presence of MPO inhibitors (0 h→1 h, 70.0%→24.3% or 73.0%→11.2%) (Fig. 3a). Similarly, the fold decrease in CD62P/CD61 ratio was not evident until 4 h when co-cultured with neutrophils in the presence of NE inhibitors (0.16 fold decrease), while it was already evident at 1 h when co-cultured with neutrophils alone or in the presence of MPO inhibitors (0.28 fold decrease or 0.74 fold decrease) (Fig. 3b). Correspondingly, the fold increase in sP-sel was not evident until 4 h when co-cultured with neutrophils in the presence of NE inhibitors (3.62 fold increase), while it was already evident at 2 h when co-cultured with neutrophils alone (4.18 fold increase) (Fig. 3c). At 4 h, compared to the group where thrombin-activated platelets co-cultured with neutrophils alone or in the presence of MPO inhibitors, all the above-mentioned parameters were statistically different in the group where platelets co-cultured with neutrophils in the presence of NE inhibitors (Fig. 3d-f); compared to the group where thrombin-activated platelets co-cultured with neutrophils alone, fold decrease in CD62P/CD61 ratio and fold increase in sP-sel were different in the group where thrombin-activated platelets co-cultured with neutrophils in the presence of MPO inhibitors (Fig. 3d-f).

Neutrophil Did not Induce P-sel Shedding from Platelets Activated by Collagen/TRAP

Regardless of whether platelets were co-cultured with buffer or neutrophils, collagen-activated platelets exhibited a slight reduction in the percentage of P-sel positive platelets only at 4 h (0 h → 4 h: 68.16% → 47.88% with buffer; 66.76% → 46.28% with neutrophils) (Fig. 4a), meanwhile, no increase in sP-sel was observed in the supernatants (Fig. 4b). Similarly, regardless of being co-cultured with

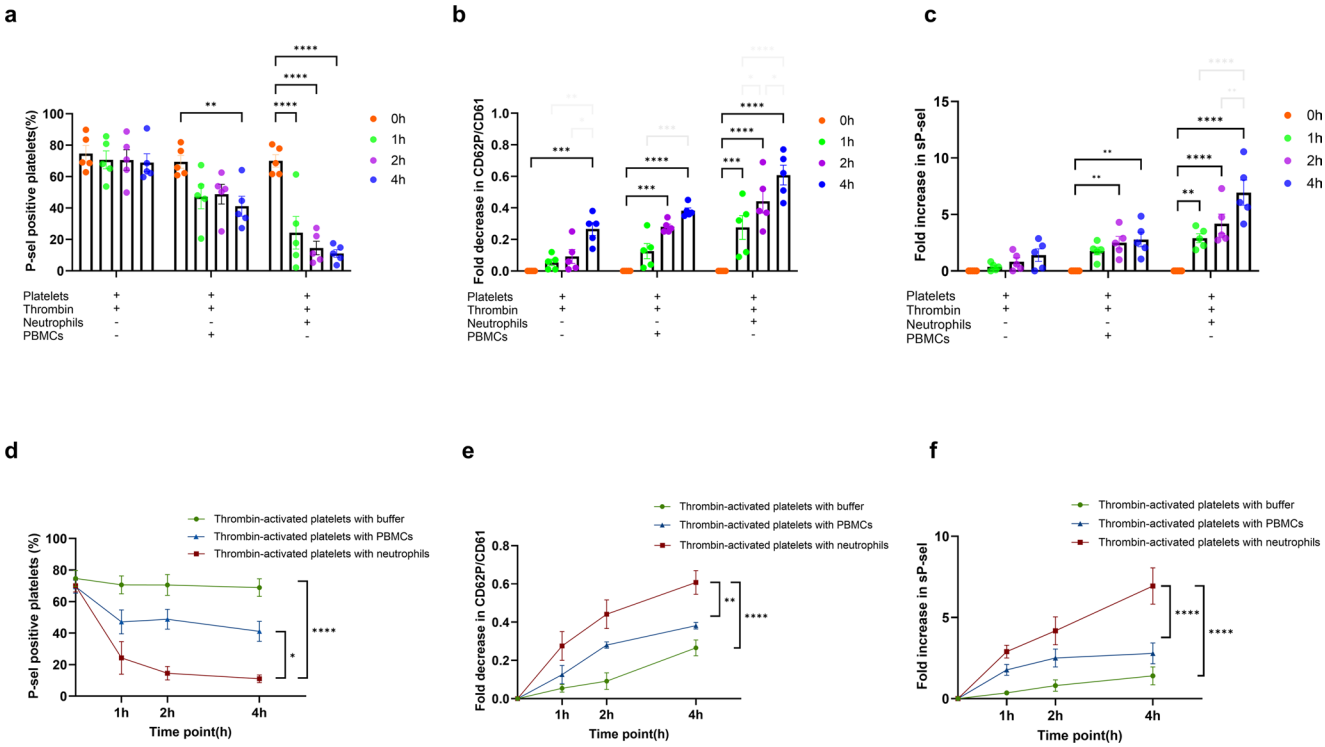

Fig. 2 Thrombin-activated platelets co-cultured with buffer, neutrophils or PBMCs **a**, **d** percentage of P-sel-positive platelets ($n=5$); **b**, **e** fold decrease in CD62P/CD61ratio ($n=5$); **c**, **f** fold increase in sP-sel level ($n=5$). Data represent mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

buffer or neutrophils, TRAP-activated platelets exhibited no significant change in either the percentage of P-sel positive platelets, no increase in sP-sel was observed in the supernatants, either (Fig. 4c and d).

Platelet P-sel Shedding was Negatively Correlated to Increased Soluble NE in Supernatants

Compared to co-culture of thrombin-activated platelets and neutrophils, soluble NE was higher in co-culture of thrombin-activated platelets and LatB-treated neutrophils (110.78→132.75 ng/mL vs. 144.42→153.33 ng/mL) (Fig. 5a), while the P-sel shedding was delayed (the fold decrease in CD62P/CD61 ratio and the fold increase in sP-sel were not evident until 2 h time point) and attenuated (fold decrease in CD62P/CD61 ratio: 0→0.59 vs. 0→0.36.; fold increase in sP-sel: 0→6.94 vs. 0→5.18) (Fig. 5b and c).

Similarly, compared to co-culture of thrombin-activated platelets and neutrophils, soluble NE was much higher in co-culture of collagen (or TRAP)-activated platelets and neutrophils (110.78→132.75 ng/mL vs. 220.39→388.04 ng/mL; or 287.47→385.16 ng/mL) (Fig. 5d and e), while the P-sel shedding almost did not even occur ((Fig. 5f).

Platelet P-sel Shedding was not Correlated To LPS and TLR2 Stimulation of Neutrophils

Compared to untreated group, LPS and/or Pam3CSK4 pretreatment did not result in a significant increase in P-sel shedding. Three groups demonstrated a same pattern of platelet P-sel shedding—the percentage of P-sel positive platelets showed a marked decline as early as 1 h, with no significant differences in the extent of reduction observed at 4 h (Fig. 6a, b).

Discussion

Due to the role of P-sel in mediating platelet-neutrophil interaction, understanding the mechanism of platelets P-sel shedding is essential for studying platelet-mediated inflammatory responses and identifying new biomarkers for thromboinflammation. In this study, we demonstrated that neutrophils promote P-sel shedding from thrombin-activated platelets, evidenced by decreased P-sel positive platelet rate, decreased P-sel intensity, and increased sP-sel concentration. This finding is supported by another notable study which highlights that the presence of PSGL-1, primarily expressed on neutrophils, is essential for P-sel shedding.

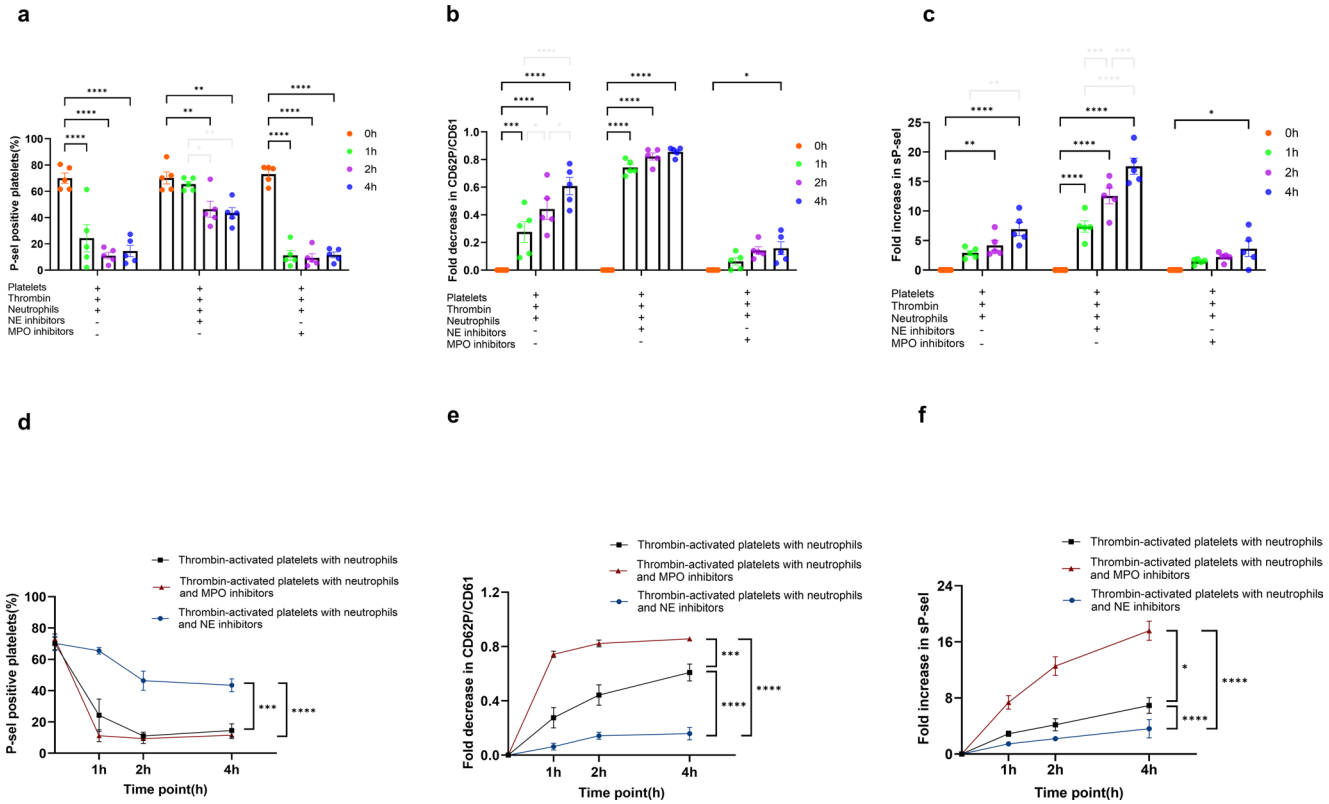

Fig. 3 Thrombin-activated platelets co-cultured with neutrophils in the presence or absence of NE or MPO inhibitors **a**, **d** percentage of P-sel-positive platelets ($n=5$); **b**, **f** fold decrease in CD62P/CD61ratio ($n=5$); **c**, **f** fold increase in sP-sel level ($n=5$). Data represent mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Specifically, P-sel was retained longer on activated platelets in PSGL-1-/- mice compared to wild-type mice [16].

Building on these findings, we further explored the mechanisms by which neutrophils induce P-sel shedding from thrombin-activated platelets. We observed that NE inhibition markedly reduced neutrophil-induced P-sel shedding, suggesting that NE plays a central role in the shedding process. Consistently, purified P-sel has been identified as a direct substrate of NE [18]. We also found that MPO inhibition accelerated neutrophil-induced P-sel shedding, probably because MPO can negatively regulate NE activity through oxidative inactivation via the MPO–H_2O_2–Cl^- pathway, as previously described [19, 20].

However, we also found that platelet P-sel shedding was negatively correlated to increased soluble NE in supernatants, which seems to be contradictory to the findings mentioned above. Considering that NE release may be modulated by NETs—which can sequester NE within the extracellular space in a process known as NETosis, resulting in a locally high-NE-concentration microenvironment—we speculated that the seemingly contradictory results might be attributed to differences in the pattern of NE release. To further explore this structural context, we treated neutrophils with LatB, which inhibits NETosis by disrupting actin polymerization without impairing NE release. LatB treatment led to a partial reduction of P-sel shedding, but an increase of soluble NE level in the supernatant, suggesting that NETosis might play a role in the NE-induced platelet P-sel shedding by concentrating NE within the extracellular space. This can also explain why neutrophils induced P-sel shedding only in thrombin-activated platelets, but not in those activated by TRAP or collagen—when neutrophils were mixed with platelets activated by TRAP or collagen, there might be no sufficient NETosis to concentrate NE, which was evidenced by significant higher soluble NE level in the supernatants. Obviously, platelets should also be retained in NETs so that concentrated NE can work on P-sel shedding, meaning that PLA should be correlated to the extent of P-sel shedding. Indeed, we found that The number of PLAs showed a moderate positive correlation with the fold increase in sP-sel levels ($P=0.0487$) in this study.

Our findings may help to explain why elevated sP-sel has been associated with both pro- and anti-inflammatory effects [21–23]. As sP-sel can originate from both platelet activation (pro-thromboinflammatory) and neutrophil-mediated shedding of platelet P-sel (anti-thromboinflammatory), therefore, solely relying on

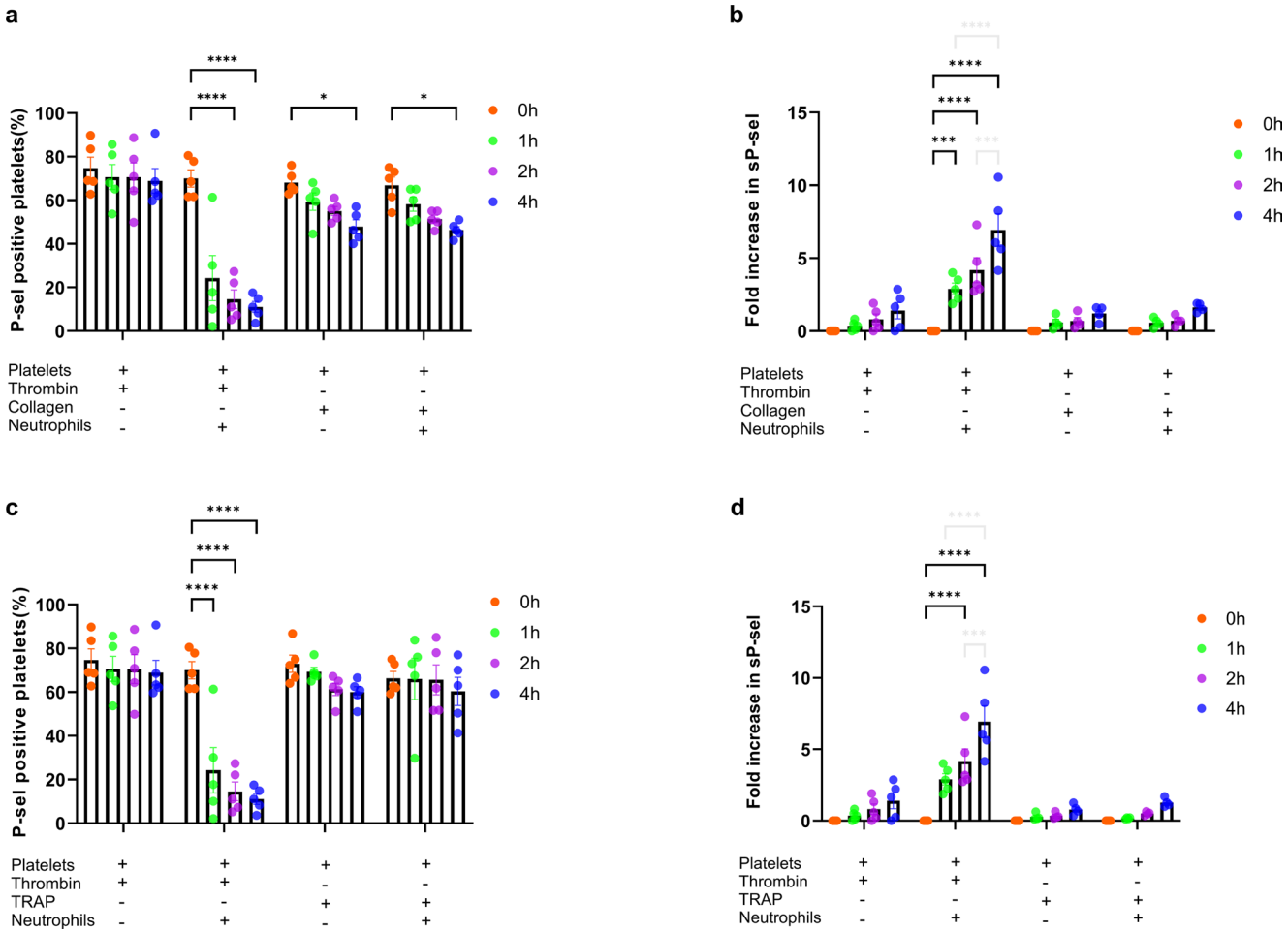

Fig. 4 Platelets activated by different agonists co-cultured with neutrophils **a** percentage of P-sel-positive platelets($n=5$); **b** fold increase in sP-sel level ($n=5$); **c** percentage of P-sel-positive platelets ($n=5$); **d** fold increase in sP-sel level ($n=5$). Data represent mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

sP-sel levels to assess disease activity may be misleading. Endothelial-derived sP-sel adds further complexity to its clinical interpretation. Given the bidirectional interaction between platelets and neutrophils, it may be more appropriate to evaluate sP-sel in conjunction with other markers of platelet-neutrophil interaction. Further studies are warranted to clarify these relationships.

Our findings differ from the in vivo results reported by Dole et al. [16], which demonstrated that in neutrophil elastase deficient animals the P-Selectin was unaffected. This discrepancy may stem from our use of an in vitro human cell system isolating NE's direct effect, whereas in vivo murine models may involve compensatory activity from other serine proteases masking NE's role [18, 24, 25].

Additionally, although previous studies have shown that LPS and Pam3CSK4 enhance neutrophil antimicrobial and proinflammatory responses via the TLR4 and TLR2 pathways respectively [26, 27], our results showed that such stimulation did not further promote P-sel shedding from thrombin-activated platelets.

Conclusions

While previous studies have predominantly focused on the role of platelets in modulating neutrophil activity, our findings provide the first direct evidence that neutrophils, in turn, regulate platelet function through an NE-dependent mechanism. This reciprocal interaction highlights a dynamic regulatory axis between platelets and neutrophils that balances pro- and anti-thromboinflammatory signals under conditions of vascular injury or inflammation. This novel finding offers a new insight into the complicated interplay between platelets and neutrophils, and provides a fresh perspective on the mechanisms underlying platelet P-sel shedding.

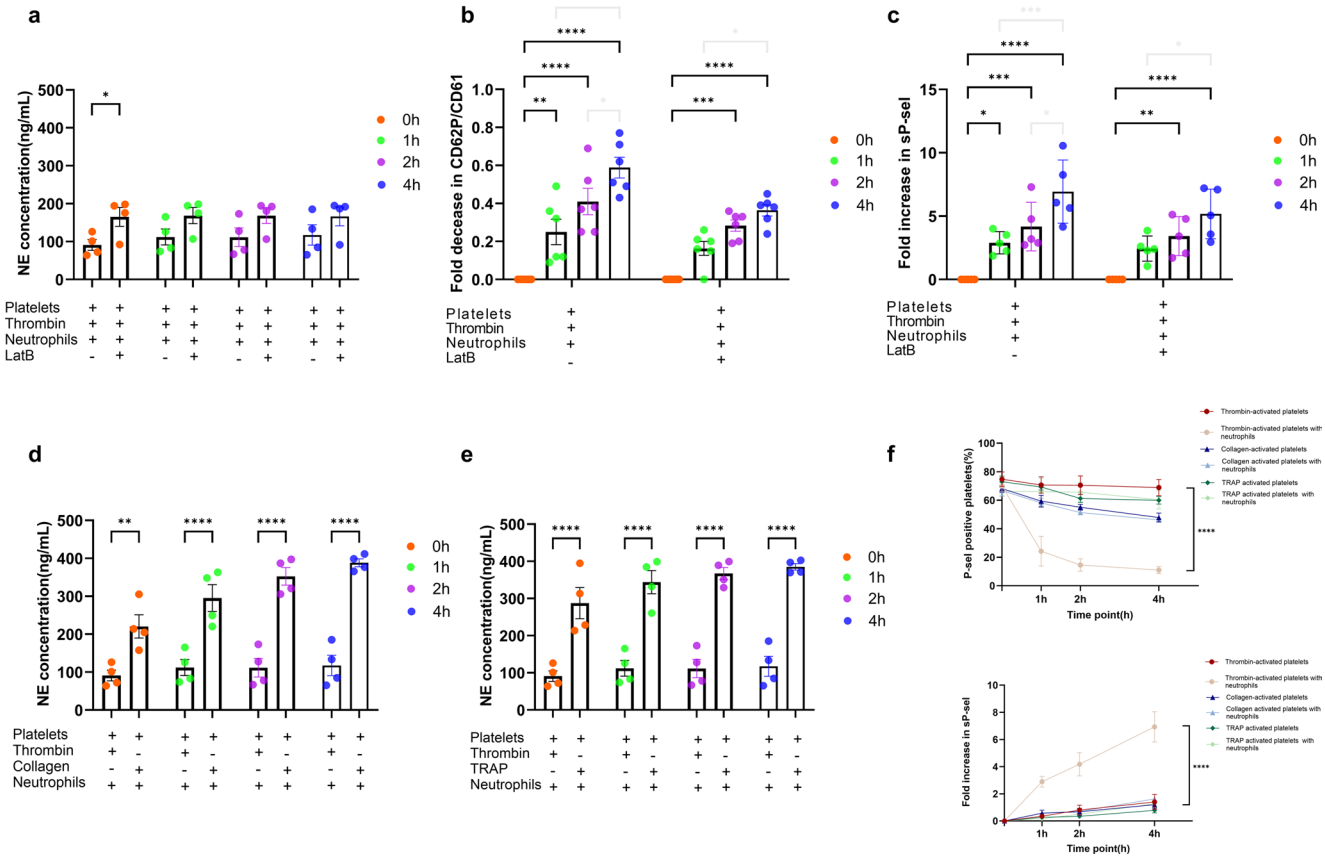

Fig. 5 Differential NE release reflects NET formation in platelet–neutrophil interactions induced by thrombin or mild agonists **a** soluble NE concentrations of thrombin-activated platelets co-cultured with neutrophils with or without LatB ($n=6$); **b** CD62P/CD61 ratio in thrombin-activated platelets co-cultured with neutrophils with or without LatB ($n=6$); **c** Fold increase in sP-sel levels in thrombin-activated platelets co-cultured with neutrophils with or without LatB ($n=5$); **d** soluble NE concentrations of collagen-activated platelets co-cultured with neutrophils ($n=6$); **e** soluble NE concentrations of TRAP -activated platelets co-cultured with neutrophils ($n=4$); **f** the fold decrease in CD62P/CD61 ratio and the fold increase in sP-sel when platelets activated by different agonists co-cultured with neutrophils. Paired t-tests were performed to analyze differences in soluble NE release when platelets were stimulated with various agonists and co-cultured with neutrophils, including comparisons with the latB-treated condition. Data represent mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

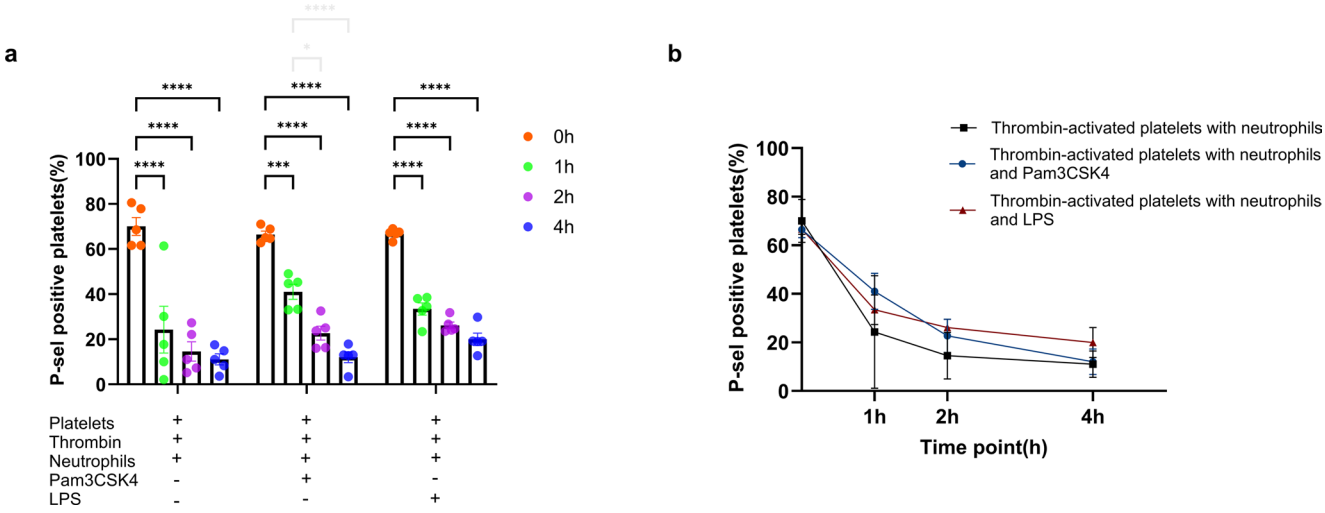

Fig. 6 Effect of LPS and TLR2 stimulation on neutrophil-induced P-selectin shedding Thrombin-activated platelets were co-cultured with neutrophils, or neutrophils pre-treated with LPS and/or Pam-3sck4. **a**, **b** percentage of P-sel-positive platelets ($n=5$). Data represent mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Author Contributions All authors contributed to the study conception and design. Conceptualization: YH, LL. Data curation: LL, YH. Formal analysis: SH, CL. Investigation: LL, SH, YH. Methodology: YH, JZ. Project administration: ZY, SW. Resources: LL, CL. Supervision: YH, LL, SH. Validation: YH, LL, JZ. Visualization: YH, SW, XH. Writing–original draft: YH, JZ. Writing–review & editing: YH, LL. All authors read and approved the final manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of West China Hospital, Sichuan University (ID: 2024－215).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent to Publish Not applicable.

Competing interests The authors declare no competing interests.

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