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Full Length Article

The differential formation and composition of leukocyte-platelet aggregates induced by various cellular stimulants

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ABSTRACT

Background: Leukocyte-platelet aggregates comprise a pathogenic link between hemostasis and immunity, but the prerequisites and mechanisms of their formation remain not understood.
Aims: To quantify the formation, composition, and morphology of leukocyte-platelet aggregates in vitro under the influence of various cellular activators.
Methods: Phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS), thrombin receptor-activating peptide (TRAP-6), and adenosine diphosphate (ADP) were used as cellular activators. Flow cytometry was utilized to identify and quantify aggregates in whole human blood and platelet-rich plasma. Cell types and cellular aggregates were identified using fluorescently labeled antibodies against the appropriate cellular markers, and cell activation was assessed by the expression of appropriate surface markers. For confocal fluorescent microscopy, cell membranes and nuclei were labeled. Neutrophil-platelet aggregates were studied using scanning electron microscopy.
Results: In the presence of PMA, ADP or TRAP-6, about 17–38 % of neutrophils and 61–77 % of monocytes formed aggregates with platelets in whole blood, whereas LPS did not induce platelet aggregation with either neutrophils or monocytes due the inability to activate platelets. Similar results were obtained when isolated neutrophils were added to platelet-rich plasma. All the cell types involved in the heterotypic aggregation expressed molecular markers of activation. Fluorescent and electron microscopy of the aggregates showed that the predominant platelet/leukocyte ratios were 1:1 and 2:1.
Conclusions: Formation of leukocyte-platelet aggregates depends on the nature of the cellular activator and the spectrum of its cell-activating ability. An indispensable condition for formation of leukocyte-platelet aggregates is activation of all cell types including platelets, which is the restrictive step.

1. Introduction

Interaction of platelets and leukocytes is a crucial mechanism that links hemostasis and the immune system [1–5]. In addition to their essential roles in hemostasis and thrombosis, platelets also modulate innate and adaptive immune responses, playing a significant role in inflammation and infection [6–8]. Platelets can influence immunity both indirectly through secreted mediators and through direct physical interactions with various types of leukocytes. This interaction results in intercellular adhesion, leading to the formation of heterotypic cellular aggregates circulating in the bloodstream and deposited at the sites of injury and/or inflammation [9,10].

Leukocyte-platelet aggregates primarily form through the interaction between adhesive P-selectin expressed on activated platelets

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[11,12] and P-selectin glycoprotein ligand-1 (PSGL-1) constitutively expressed on lymphoid and myeloid cells, including neutrophils and monocytes [13]. While the P-selectin/PSGL-1 interaction is sufficient for supporting leukocyte-platelet adhesion, activated Mac-1 integrin molecules (αMβ2 or CD11b/CD18) on neutrophils and monocytes significantly contribute to leukocyte-platelet aggregation, likely through binding to glycoprotein 1b on platelets [14–16]. Alternatively, activated Mac-1 on leukocytes can form a bridge with the activated platelet integrin αIIbβ3 through bivalent fibrinogen [3]. The engagement of platelets with leukocytes can also be strengthened by membrane-associated CD40L on platelets interacting with CD40 on leukocytes [5,17]. Other intercellular binding interactions, although less strong and physiologically relevant, have the potential to support leukocyte-platelet adhesion [2–4,18–20].

The adhesion of platelets and leukocytes significantly impacts the pathophysiological processes underlying immune reactions and thrombosis [7,8]. Upon binding to neutrophils, platelets modulate their functions and contribute to inflammatory reactions following injury or infection by promoting phagocytosis, cytokine release, trans-endothelial migration, oxidative burst through the generation of superoxide anions, and the formation of neutrophil extracellular traps [21–23]. Platelets interacting with monocytes can enhance their pro-inflammatory activity, including differentiation into macrophages, formation of foam cells in atherosclerotic plaques, adhesion to endothelial cells, and cytokine release [24,25]. Platelets can also adhere to lymphocytes, but, unlike binding to neutrophils and monocytes, lymphocyte-platelet aggregates are not associated directly with inflammatory thrombosis and primarily modulate the adaptive immune response [26]. It is known from the literature that platelet-lymphocyte aggregates can be formed and include approximately 3 % of circulating lymphocytes. Platelet conjugation was most common among large (monocyte-sized) lymphocytes. Platelet activation by ADP slightly increased platelet–T-cell conjugation (up to ~5 %), mainly due to T-cytolytic (Tc) cells, but markedly elevated platelet–natural killer (NK)-cell conjugation (up to ~20 %). The LPS stimulation (for 8 h) caused a marked increase of the expression of the B-cell activation marker CD86, but did not enhance platelet–B-cell aggregation; platelet activation by ADP or B-cell activation by LPS did not significantly enhance platelet–B-cell aggregation [27–29].

Beyond their pathogenic importance, leukocyte-platelet aggregates serve as markers for thrombotic and inflammatory diseases, such as cardiovascular diseases, acute lung inflammation, infections (including COVID-19), acute ischemic stroke, post-stroke venous thrombosis, liver diseases, HIV infection, myeloproliferative disorders, type 1 diabetes, and other autoimmune and inflammatory processes, as previously described and reviewed [30–38].

An increased presence of leukocyte-platelet aggregates in the blood has been demonstrated as a sensitive marker of platelet activation [39,40] and holds prognostic and/or diagnostic value in numerous diseases [41]. Despite the diverse pathophysiological roles and clinical importance of leukocyte-platelet aggregates, the prerequisites and conditions of their formation remain not fully understood. Given the complex multifactorial nature and pathogenesis of inflammation and thrombosis, with numerous bioactive compounds released into the bloodstream, it remains unclear which cellular activators and mechanisms specifically promote the formation of leukocyte-platelet aggregates and whether the effects of well-known cellular stimulants can be differential. It is also uncertain whether the pre-activation of both platelets and leukocytes is necessary for their heterotypic aggregation or if one cell type, when initially activated, induces activation in the other type of cells upon adhesion. Other understudied aspects of the interactions between inflammatory leukocytes and platelets include the qualitative characterization of the cells within the aggregates and the relative quantities of the aggregated cell types.

To address these questions, we conducted a study of leukocyte-platelet aggregates formed in vitro under the influence of various cellular activators, using flow cytometry. We also characterized the morphology and quantified the composition of cellular aggregates using fluorescence and scanning electron microscopy.

2. Materials and methods

2.1. Collection and processing of human blood samples

All procedures involving human subjects received approval from the Ethical Committee of Kazan Federal University (protocol #27, dated December 28, 2020). Written informed consent was obtained from healthy subjects enrolled in the study. For in vitro model experiments, blood was collected from 56 donors, comprising 23 (41 %) men and 33 (59 %) women, aged 21 to 55 years (average 25 ± 4 years). Only subjects who had not taken anticoagulants or non-steroidal anti-inflammatory drugs within 2 weeks prior to blood drawing were included in the study. Blood collection and handling adhered to approved guidelines and followed standard pre-analytical requirements. Venous blood was drawn into vacutainers containing 3.8 % trisodium citrate (Vacuette, Greiner Bio-one, USA), mixed 9:1 by volume. One portion of whole citrated blood samples was used to study the formation of leukocyte-platelet aggregates under various experimental conditions (see below). The second portion was used for neutrophil isolation (see below). The third portion was centrifuged (200 *g*, 10 min, room temperature) to obtain platelet-rich plasma (PRP) as a source of platelets. All samples were utilized within 4 h after blood collection.

2.2. Formation of leukocyte-platelet aggregates in whole blood

200 μl of whole citrated blood was diluted 5-fold with Ca^{2+}- and Mg^{2+}-free HBSS (KCl 5.33 mM, NaCl 138 mM, Na_2HPO_4 0.3 mM, KH_2PO_4 0.44 mM, $NaHCO_3$ 4 mM, D-glucose 5.6 mM) as recommended [13]. The mixture was then incubated with one of the following cellular activators: i) 100 nM phorbol-12-myristate-13-acetate (PMA, Millipore Sigma, USA) for 10 min at room temperature; ii) 20 μM adenosine 5′-diphosphate (ADP, Millipore Sigma, USA) for 15 min at room temperature; iii) 50 μM thrombin-receptor activating peptide (TRAP-6, Millipore Sigma, USA) for 15 min at room temperature; iv) 100 ng/ml lipopolysaccharide from *Escherichia coli* O26:B6 (LPS, Millipore Sigma, USA) for 50 min at 37 °C. The agonist concentrations and incubation times/temperatures used were selected based on the literature [13,42–44]. To prevent P-selectin-mediated cellular aggregation, inclacumab, an anti-human P-selectin blocking antibody was used (MedChemExpress, USA). The formation of leukocyte-platelet aggregates was assessed using flow cytometry, as described below.

2.3. Isolation of neutrophils and formation of neutrophil-platelet aggregates in platelet-rich plasma

Neutrophils were isolated from 5 ml of fresh citrated blood by centrifugation at 500 *g* for 35 min at room temperature on the Lympholyte-Poly Cell Separation Media (Cedarlane, Canada) at a volume ratio of 1:1 for the separation medium to blood. Following centrifugation, the opaque ring containing neutrophils was collected, and the cells were washed three times with Tyrode's buffer (4 mM HEPES; 135 mM NaCl; 2.7 mM KCl; 2.4 mM $MgCl_2$; 3.3 mM NaH_2PO_4; 5.6 mM D-glucose; 0.3 % bovine serum albumin, pH 7.4) by centrifugation for 10 min at 300 *g*. The number, purity and morphology of neutrophils were assessed using a hemocytometer, flow cytometry, and scanning electron microscopy, respectively. To model the formation of heterotypic neutrophil-platelet aggregates, isolated neutrophils were added to PRP at a physiological ~1:60 neutrophil/platelet ratio (0.8×10^6 neutrophils and 48×10^6 platelets in a sample). The mixture was incubated with the same cellular activators (PMA, ADP, TRAP-6, LPS) and under the same conditions described for the whole blood. For the control, a corresponding volume of Tyrode's buffer was added to PRP instead of a neutrophil suspension. The formation of neutrophil-platelet aggregates

was assessed with flow cytometry.

2.4. Cell labeling for flow cytometry

The following cell-type-specific markers were labeled with corresponding fluorescent monoclonal antibodies: CD45 (all leukocytes), CD14 (monocytes), CD16 (neutrophils), CD3 (lymphocytes), and CD41 (platelets). Anti-human CD45 antibodies were labeled with fluorescein isothiocyanate (FITC) or allophycocyanin (APC) (BD Biosciences, USA). Anti-human CD16 antibodies were labeled with phycoerythrin (PE) or a tandem fluorochrome composed of APC and coupled to the cyanine dye Cy7™ (APC-CyTM7) (BD Biosciences, USA). Anti-human CD14 antibodies were labeled with PE or APC (BD Biosciences, USA). Anti-human CD41 antibodies were labeled with peridinin chlorophyll protein-cyanine 5.5 (PerCP/Cyanine5.5) or FITC (Biolegend, USA). Anti-human CD3 antibodies were labeled with FITC (Beckman Coulter, USA).

To reveal cell activation, the samples were analyzed for the expression of the following markers: CD62p (P-selectin) on platelets, activated CD11b (αM subunit of the αMβ2 or Mac-1 integrin) on neutrophils, CD142 (tissue factor) on monocytes, and CD69 or CD25 on lymphocytes. In particular, the following labels were used: for activated platelets, anti-human CD62p antibodies labeled with FITC or PE or PerCP/Cyanine5.5 (BD Biosciences, USA); for activated neutrophils, anti-human CD11b antibodies (against the αM chain of the activated integrin αMβ2) labeled with APC (Biolegend, USA); and for activated monocytes, anti-human CD142 (tissue factor) labeled with APC (Biolegend, USA); for activated lymphocytes, anti-human CD69 antibodies labeled with PE (BD Biosciences, USA) and anti-human CD25 antibodies labeled with PE (Beckman Coulter, USA).

2.5. Sample preparation for flow cytometry

The samples were either untreated or activated with various stimulants. 1 ml of whole blood or PRP (~200 μl containing 48×10^6 platelets) supplemented with isolated neutrophils (~80 μl containing 0.8×10^6 neutrophils) and diluted up to 1 ml with Tyrode's buffer, was mixed with 5 μl of a corresponding commercial monoclonal antibody. The mixture was then incubated for 10 min at room temperature for all antibodies, except for the anti-CD142 antibody, for which the incubation time was 50 min at 37 °C in the dark. After incubation, the samples were fixed with excess of 1 % paraformaldehyde (1:15 by volume) for 10 min. This was followed by centrifugation at 200 *g* for 10 min and re-suspension in HBSS buffer without Ca^{2+} and Mg^{2+}, pH 7.4. After washing and repeated centrifugation, the cells were re-suspended in 200 μl of the same buffer. For whole blood samples, red blood cells (RBCs) were lysed using the ACK lysing buffer (Thermo Fisher Scientific, USA) or BD FACS lysing solution (Becton Dickinson, USA) before analysis. The lysis buffer was added to the re-suspended sample at a volume ratio of 1:20 and incubated for 5 min. After RBC lysis, the samples were washed twice in HBSS, followed by centrifugation at 200 *g* for 10 min at room temperature, and re-suspended in 200 μl of HBSS.

2.6. Flow cytometry and identification of neutrophil-platelet and monocyte-platelet aggregates

Flow cytometry was conducted using either a FACSCalibur (Beckman Dickinson, USA) or CytoFlex (Beckman Coulter, USA) instrument, and data analysis was carried out using FlowJo software (Beckman Dickinson, USA). 1500 monocytes, 5000 neutrophils, and 5000 platelets were analyzed for each measurement. The identification of neutrophil-platelet and monocyte-platelet aggregates in whole blood using flow cytometry is illustrated in Fig. S1. Signals were initially discriminated based on their forward scatter (FSC) and side scatter (SSC) characteristics. White blood cells were identified as CD45-positive (CD45+) events (Fig. S1A). These CD45+ events were then separated into two population based on granularity, with the higher granularity identified as neutrophils and the lower granularity as monocytes. Neutrophils were further quantified/defined as CD16+ signals (neutrophil-specific marker) (Fig. S1B), and monocytes were quantified/defined as CD14+ signals (monocyte-specific marker) (Fig. S1E). Within each of these gates, CD41+ events (platelet-specific marker) were detected to reveal neutrophil-platelet (CD16+/CD41+) (Fig. S1C), monocyte-platelet (CD14+/CD41+) (Fig. S1F) aggregates formed in the blood upon activation with PMA. Notably, platelet-monocyte aggregates formed two sub-fractions (Fig. S1F), which may comprise lymphocytes aggregated with platelets or reflect platelet-monocyte aggregates with distinct size and granularity [45]. In the absence of PMA, >95 % of neutrophils (Fig. S1D) and 98 % monocytes without platelets (Fig. S1G) were observed. Using the fluorescence of the combined CD16+/CD41+ and CD14+/CD41+ signals, the neutrophil-platelet and monocyte-platelet aggregates were quantified as the percentage of total CD16 neutrophil signals or CD14 monocyte signals, respectively, that also stain for the platelet-specific marker CD41+. The same gating strategy was employed to identify and quantify neutrophil-platelet aggregates in PRP mixed with isolated neutrophils. Lymphocytes were gated as CD45+ or CD3+ signals. Within each of these gates, CD41+ events were detected to reveal and quantify lymphocyte-platelet aggregates formed in the blood upon activation with PMA, LPS, ADP, or TRAP (Figs. S6 and S7).

2.7. Fluorescence microscopy

200 μl of whole blood was diluted 5-fold with Ca^{2+}- and Mg^{2+}-free HBSS buffer and then incubated at room temperature with 100 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, USA) for 10 min. Sample fixation and red blood cell (RBC) lysis were performed as described in the section entitled "Sample preparation for flow cytometry". After washing, samples were re-suspended in 200 μl HBSS and stained with a DNA-specific dye, DAPI (4′,6-diamidino-2-phenylindole, Biolegend, USA), and a cell membrane-specific dye, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, ThermoFisher, USA), following the manufacturers' instructions. Subsequently, the sample was placed in a cell-imaging dish (35 mm × 10 mm, Eppendorf) and visualized using a DMi8 Inverted LED Fluorescence THUNDER Microscope (Leica, Germany) with an HC PL APO 63×/1.40 OIL objective lens.

2.8. Scanning electron microscopy

Isolated neutrophils were added to platelet-rich plasma (PRP) in the absence (control) or presence of 100 nM phorbol-12-myristate-13-acetate (PMA) and incubated for 10 min. The neutrophil/platelet ratio was approximately equal to physiological counts in human blood (~1:60), and the cells were suspended to a final volume of 600 μl in Tyrode's buffer. The mixed cells were fixed in 2 % glutaraldehyde in saline for 90 min at room temperature, with a cells/fixative volume ratio of 1:15. Subsequently, the fixed cells were pelleted followed by re-suspension in 600 μl of Tyrode's buffer and layered on a polycarbonate filter with a 0.4-μm pore size at 320 g for 2 min (with fast acceleration and slow deceleration). The samples were rinsed three times with phosphate-buffered saline (pH 7.4) for 20 min, dehydrated in ascending concentrations of ethanol, immersed in hexamethyldisilazane, and dried overnight. Finally, a thin film of gold palladium was layered on the samples using a sputter coater Quorum Q 150 T ES (Quorum, Lewes, UK), and micrographs were taken with a scanning electron microscope (Merlin, Carl Zeiss, Germany).

2.9. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 software package (GraphPad Software, USA). The normality of data distribution was assessed using the D'Agostino-Pearson test. Pairwise statistical differences were determined using either the Student's *t*-test

(parametric) or Mann-Whitney *U* test (nonparametric). For multiple comparisons, statistical differences were assessed using one-way ANOVA with the post-hoc Tukey's test. The level of statistical significance was set at 95 % ($p < 0.05$). Results are presented as a median and interquartile range (IQR; 25th, 75th percentile), unless otherwise indicated.

3. Results

3.1. Formation of neutrophil-platelet and monocyte-platelet aggregates in vitro under various experimental conditions

To mimic diverse cell activation mechanisms in inflammatory and thrombotic conditions, we studied the formation of heterotypic blood cell aggregates under the influence of several cellular stimulants. These included PMA, which activates protein kinase C present in all human cell types [46,47]; LPS, a mediator of bacterial inflammation [48]; TRAP-6, a thrombin receptor-activating peptide targeting the widely expressed PAR1 receptor [49]; and ADP, the agonist of ubiquitously expressed purinergic receptors [50]. Leukocyte-platelet aggregates were defined as the percentage of neutrophils or monocytes 'co-expressing' the platelet-specific marker CD41.

Incubating citrated blood with PMA induced the formation of aggregates between platelets and monocytes or neutrophils. Approximately 38 % of neutrophils (Fig. 1A) and 74 % of monocytes (Fig. 1B) were found within the CD41+ heterotypic aggregates, representing a ~38–46-fold increase compared to the untreated control (Table S1).

In contrast, the addition of LPS failed to induce the formation of both neutrophil-platelet and monocyte-platelet aggregates compared to the control untreated blood samples (Fig. 1C, D; Table S1), which is examined and explained further below. As a positive control for LPS, we applied a combination of LPS and PMA, which caused the increased formation of aggregates compared to LPS alone with the numbers similar to those induced by PMA alone (Figs. S8, S9).

An increase in neutrophil-platelet (17 %) and monocyte-platelet (61 %) aggregates was observed after incubation of the blood samples with ADP (Fig. 1E, F; Table S1). Incubation of blood samples with TRAP-6 also drastically increased the formation of aggregates between neutrophil-platelet (24 %) and monocyte-platelet (77 %) aggregates (Fig. 1G, H; Table S1). Notably, with all the stimulants, except for LPS, a statistically significant higher occurrence of monocyte-platelets versus neutrophil-platelet aggregates was observed.

To model the formation of neutrophil-platelet aggregates in the absence of erythrocytes, isolated neutrophils were introduced into platelet-rich plasma with addition of the same cellular activators. Flow cytometry revealed that under the influence of PMA, approximately 87 % of neutrophils were incorporated into aggregates with platelets (Fig. 2, Table S2). In contrast to PMA, the presence of LPS resulted in only 4 % of neutrophils being part of small and scanty CD41+ cellular aggregates, which was insignificantly higher than the control (~1 %) without LPS ($p = 0.92$). The addition of ADP led to the inclusion of a median 23 % fraction of neutrophils in the aggregates, while the introduction of TRAP-6 resulted in 75 % of neutrophils being present in neutrophil-platelet aggregates (Fig. 2, Table S2). The results obtained indicate strong neutrophil-platelet adhesion in blood plasma in the presence of PMA, ADP, and TRAP-6, whereas LPS induced the formation of very few neutrophil-platelet aggregates, similar to the control without the addition of cellular activators.

For comparison with myeloid leukocytes, we followed formation of lymphocyte-platelet aggregates under the same conditions. The percentage of lymphocytes involved in the aggregates with platelets reached a maximum of ~20 % and the expression of the markers of lymphocyte activation was quite small ranging within 0.2–5 % in response to PMA, LPS, ADP or TRAP-6 (Figs. S6, S7). The results show that under the conditions applied, the formation of lymphocyte-platelet aggregates is relatively weak compared to the formation of neutrophil/monocyte-platelet aggregates.

3.2. Differential incorporation of platelets into homo- and heterotypic cellular aggregates induced by various stimulants

Aggregated platelets were identified and quantified as CD41+ signals with high granularity, absent in untreated blood samples and appearing only after treating a blood sample with a cellular stimulant (Fig. S2; Table S3). Both homo- and heterotypic cellular aggregates were detected and analyzed based on the absence or presence of leukocyte-specific CD45+ signals in the gate of aggregated CD41+ platelets. The fractions of CD41+ platelets incorporated into aggregates with CD45+ leukocytes before (control) and after the addition of PMA, LPS, ADP, TRAP-6 are shown in Fig. 3. Under the effect of PMA, about 10 % median platelet fraction was incorporated into aggregates compared to 0.4 % in control ($p < 0.0001$) (Fig. 3A, Table S3). Approximately 64 % of the aggregated platelets were part of neutrophil-platelet aggregates, 14 % were incorporated into monocyte-platelet aggregates, and 22 % comprised platelets aggregated with each other or with unidentified cells or large particles (Fig. 3B). Almost no aggregated platelets (~0.3 %) were detected in blood before or after treatment with LPS (Fig. 3C, D, Table S3). After adding ADP, about 3 % of total platelets (versus 0.2 % in control, $p < 0.001$) were found within aggregates (Fig. 3E, Table S3), of which 43 % were in neutrophil-platelet aggregates, 34 % aggregated with monocytes, and 23 % formed unidentified aggregates (Fig. 3F), presumably homotypic platelet aggregates or large micron-size structures containing platelet-derived microparticles. TRAP-6 induced the formation of leukocyte-platelet aggregates involving about 7 % of the platelet population (versus 0.2 % in control, $p < 0.0001$) (Fig. 3G, Table S3). 25 % of the aggregated platelets were associated with monocytes, 50 % were found in neutrophil-platelet aggregates, and 25 % were in unidentified aggregates, likely comprising homotypic aggregates between platelets or other unidentified platelet-containing aggregates (Fig. 3H).

3.3. Proportion of activated platelets within neutrophil-platelet and monocyte-platelet aggregates

Platelet activation was assessed by the surface expression of P-selectin, which enables activated platelets to adhere to leukocytes through the interaction with PSGL-1 constitutively expressed on the surface of all leukocytes [51]. Accordingly, a very high fraction of P-selectin-expressing platelets was found within neutrophil-platelet (Fig. 4) and monocyte-platelet (Fig. S3) aggregates formed in blood in the presence of PMA, ADP, and TRAP-6, when compared to the untreated control (Figs. 4 and S3, Table S4); there was a very strong direct rank correlation ($r = 0.98$, $p < 0.0001$) between the expression of P-selectin and the amount of the heterotypic platelet aggregates. The fraction of platelets expressing P-selectin reached 100 % in all the blood samples treated with PMA, which contained both neutrophil-platelet (Fig. 4B, C; Table S4) and monocyte-platelet aggregates (Fig. S3B, C; Table S4). Upon addition of TRAP-6, a median 77 % fraction of platelets within neutrophil-platelet aggregates expressed P-selectin (Fig. 4K, L; Table S4), while in the monocyte-platelet aggregates, the fraction of activated platelets was 99 % (Fig. S3K, L; Table S4). ADP caused the expression of P-selectin on the surface of 43 % platelets within neutrophil-platelet aggregates (Fig. 4H, I; Table S4) and in 77 % platelets within monocyte-platelet aggregates (Fig. S3H, I; Table S4). Unlike the other stimulants, LPS caused neither platelet activation nor (co-) aggregation compared to the untreated control blood samples with zero activated platelets (Figs. 4E, F and S3E, F, Table S4). With all the efficient activators applied, the fraction of activated platelets was higher in monocyte-platelet aggregates than in neutrophil-platelet aggregates (Table S4).

To validate the role for P-selectin-PSGL-1 binding, we formed platelet-leukocyte aggregates in whole blood in the presence of

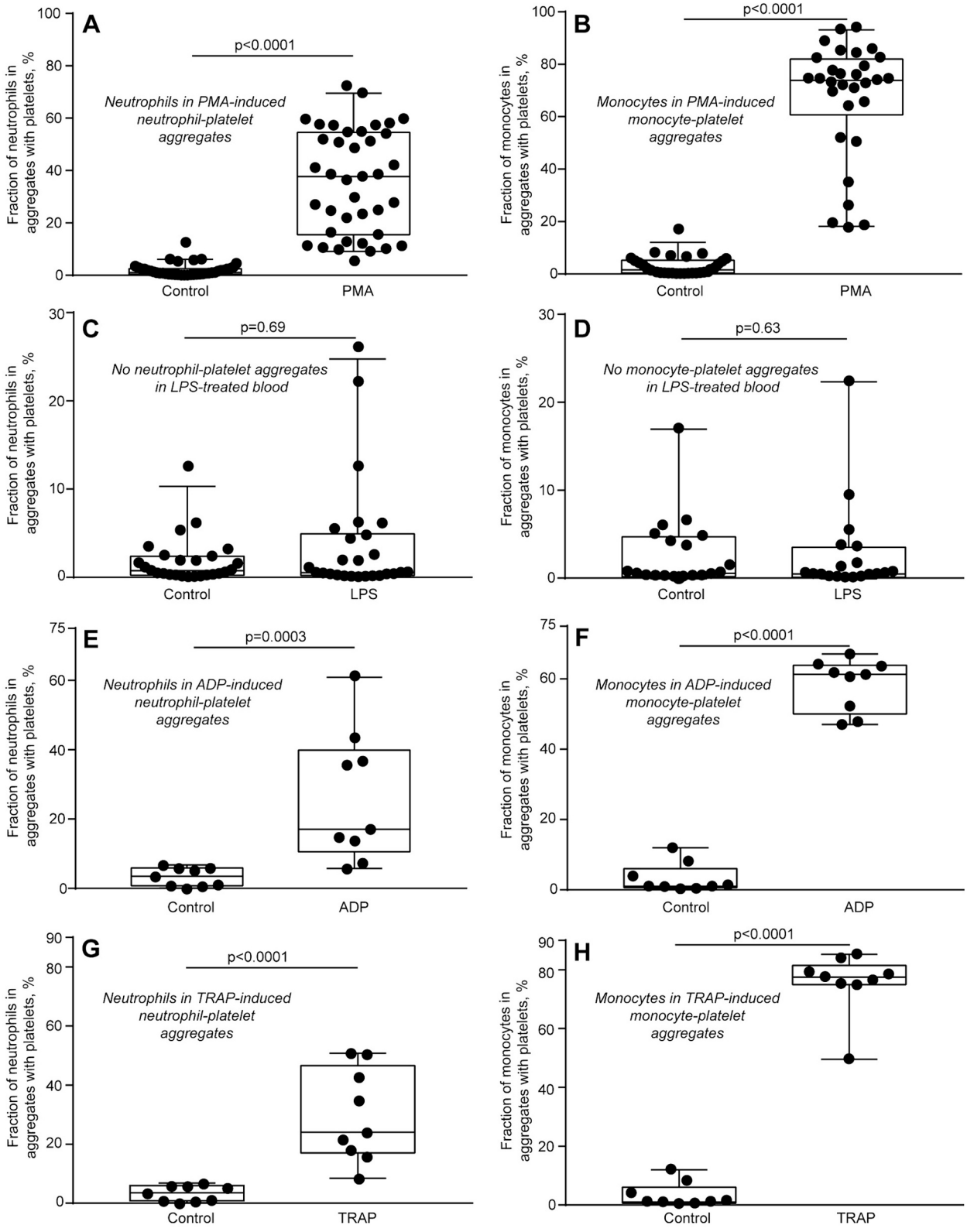

Fig. 1. Formation of neutrophil-platelet and monocyte-platelet aggregates in normal whole blood under various experimental conditions. Using flow cytometry, the blood samples were analyzed before (control) and after addition of 100 nM PMA (A, B), 100 ng/ml LPS (C, D), 20 μM ADP (E, F), and 50 μM TRAP-6 (G, H). Quantities of neutrophil-platelet aggregates were determined as percentage of double CD16+/CD41+ events in the corresponding CD16+ neutrophil gates (A, C, E, G). Quantities of monocyte-platelet aggregates were determined as percentage of double CD14+/CD41+ events in the corresponding CD14+ monocyte gates (B, D, F, H). The raw data points for each independent blood sample ($n \geq 9$) are overlaid with a median and interquartile interval between the 25th and 75th percentiles (boxes) as well as between the 5th and 95th percentiles (Mann-Whitney *U* test).

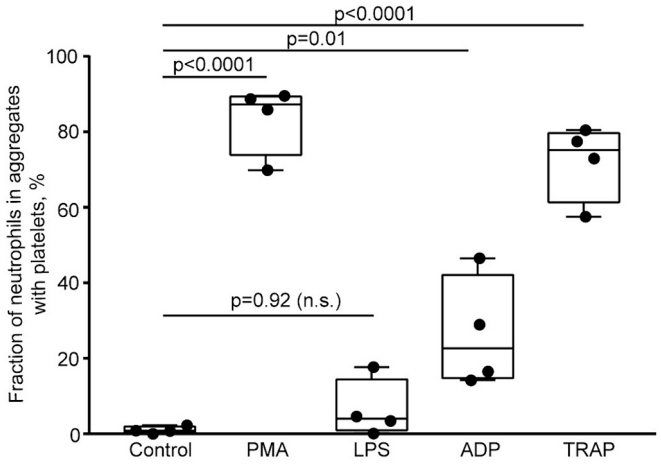

Fig. 2. Formation of neutrophil-platelet aggregates after addition of isolated neutrophils to platelet-rich plasma in the absence (control) or presence of various cellular stimulants (100 nM PMA, 100 ng/ml LPS, 20 μM ADP, and 50 μM TRAP-6). The results are presented as fractions of CD16+ neutrophils within gates containing double CD41+/CD16+ events, reflecting neutrophil-platelet aggregates. Designations: n.s. – not significant. The raw data points for each independent blood plasma sample and an autologous neutrophil preparation ($n = 4$) are overlaid with a median and interquartile interval between the 25th and 75th (boxes) as well as between the 5th and 95th percentiles (Mann-Whitney U test compared to control).

inclacumab, an anti-human P-selectin antibody. The results show that inclacumab completely prevented the formation of the platelet-neutrophil aggregates induced by TRAP as shown in Fig. S4. This result is in line with the earlier data obtained in P-selectin blocking experiments [52,53] as well as in P-selectin-knockout mouse models [54,55], altogether indicating that P-selectin is crucial for formation of platelet-neutrophil aggregates.

3.4. Proportion of activated neutrophils and monocytes within leukocyte-platelet aggregates

In the control untreated samples, the fraction of neutrophils expressing active CD11b comprised only ~0.3 %, which was found in the CD16+ gate of non-aggregated neutrophils (Q1) in the absence of CD16+/CD41+ aggregates (Q2) (Fig. 5B, C; Table S5). In the PMA-activated samples, the fraction of neutrophils expressing the active integrin Mac-1 comprised as much as 65 %, which was revealed within neutrophil-platelet aggregates (Q2) and was significantly different compared to the untreated control ($p = 0.001$) (Fig. 5D, E; Table S5).

Similarly, in the control untreated samples, the fraction of monocytes expressing tissue factor (TF or CD142) comprised only 1 %, which was found in the CD14+ gate of non-aggregated monocytes (Q1) because there were no CD14+/CD41+ aggregates (Q2) (Fig. S5B, C; Table S5). In contrast, in the PMA-activated blood samples, the fraction of monocytes expressing tissue factor (TF or CD142) comprised about 94 %, which was revealed within monocyte-platelet aggregates (Q2) and was significantly higher compared to the untreated control ($p = 0.03$) (Fig. S5D, E; Table S5).

3.5. LPS causes activation of neutrophils but not platelets, which prevents neutrophil-platelet aggregation in the presence of LPS

LPS is recognized as a potent neutrophil stimulant, triggering neutrophil activation through Toll-like receptors [56,57]. However, data on the effects of LPS on platelets have been controversial [28]. In our experiments, LPS, unlike other cellular stimulants used in this study, induced neither leukocyte-platelet nor homotypic platelet aggregates (Figs. 1–4, Tables S1-S3). To elucidate the mechanism underlying the inability of LPS to induce leukocyte-platelet aggregation, we separately studied the activating effects of LPS (100 ng/ml, 50 min, 37 °C) on neutrophils and platelets, either in whole blood or in a mixture of isolated neutrophils and platelet-rich plasma. As assessed by the expression of the active Mac-1 (CD18/CD11b), neutrophils were strongly activated by LPS, with a median fraction of CD11b + cells rising up to 91 % compared to 1.4 % in the control untreated blood samples (Fig. 6A–C; Table S6). In the same blood samples, LPS did not cause any platelet activation, as assessed by the expression of P-selectin, with a median fraction of activated platelets remaining almost unchanged before (0.2 %) and after (0.8 %) treatment with LPS (Fig. 6D–F, Table S6).

The differential effects of LPS on neutrophils and platelets were confirmed in the mixture of isolated neutrophils and platelet-rich plasma, with PMA used as a positive control (Fig. 6G, H). PMA caused the formation of neutrophil-platelet aggregates with strong activation of both neutrophils and platelets. In the presence of LPS, very few aggregates containing activated neutrophils were observed, but no activated platelets were detected. These results highlight the necessity for platelet activation for the adhesion of neutrophils and platelets.

3.6. Morphological characterization of leukocyte-platelet aggregates and quantification of their composition

To visualize heterotypic blood cell aggregates in a fluorescent microscope and distinguish the cell types, leukocyte nuclei were stained with the DNA-specific DAPI, and the platelet (and leukocyte) plasma membrane was labeled with DiI, a membrane-specific fluorescent dye. Following 10 min after the addition of PMA to whole blood, neutrophils and monocytes attached to platelets could be visualized (Fig. 7A–D). Microscopy of platelet-leukocyte aggregates showed that the prevailing and roughly equal neutrophil/platelet and monocyte/platelet ratios were 1:1 and 1:2 for both types of heterotypic aggregates.

High-resolution scanning electron microscopy offered a more detailed morphological characterization of isolated neutrophils and platelets forming heterotypic aggregates after activation (Fig. 8A–C). In Fig. 8A, one neutrophil is shown with membrane outgrowths (pseudopodia or filopodia) attached to five platelets, each displaying membrane protrusions known as filopodia - a characteristic feature of activated platelets. In addition to single neutrophils, aggregates consisting of three or more neutrophils bound to each other and aggregated with platelets were observed (Fig. 8B, C). Occasionally, homotypic platelet aggregates were seen alongside the aggregated neutrophils (not shown). Analysis of several tens of neutrophil-platelet aggregates revealed the numbers of individual neutrophils and platelets within these aggregates, presented as a distribution of the neutrophil-to-platelet ratios (Fig. 8D). Fitting the experimental distribution with a bimodal Gaussian function revealed that the peak positions corresponding to the most probable neutrophil/platelet ratios in the heterotypic aggregates were 1:2 and 1:1, consistent with the values obtained through fluorescence microscopy.

For comparison, we estimated the neutrophil/platelet and monocyte/platelet ratios in aggregates using the flow cytometry data and compared it with the numbers obtained in morphological studies. To do so, we calculated the fraction of platelet events (CD41+) within the neutrophil-platelet aggregates gate (CD41+/CD16+) and calculated the fraction of neutrophil events (CD16+) in the same neutrophil-platelet aggregates gate (CD16+/CD41+) (see Figs. 4B and 5D). Then we calculated the ratio between CD41+ and CD16+ events in the aggregates. The same approach was used to count the ratio of monocyte-platelet aggregates (see Figs. S3B and S5D). The results show that the most likely neutrophil/platelet ratios are 1:2 (~20 %) and 1:1 (~60 %), and the prevalent monocyte/platelet ratio is 1:1 (~80 %), which is consistent with the microscopy findings.

4. Discussion

Leukocyte-platelet aggregates formed in the blood reflect the

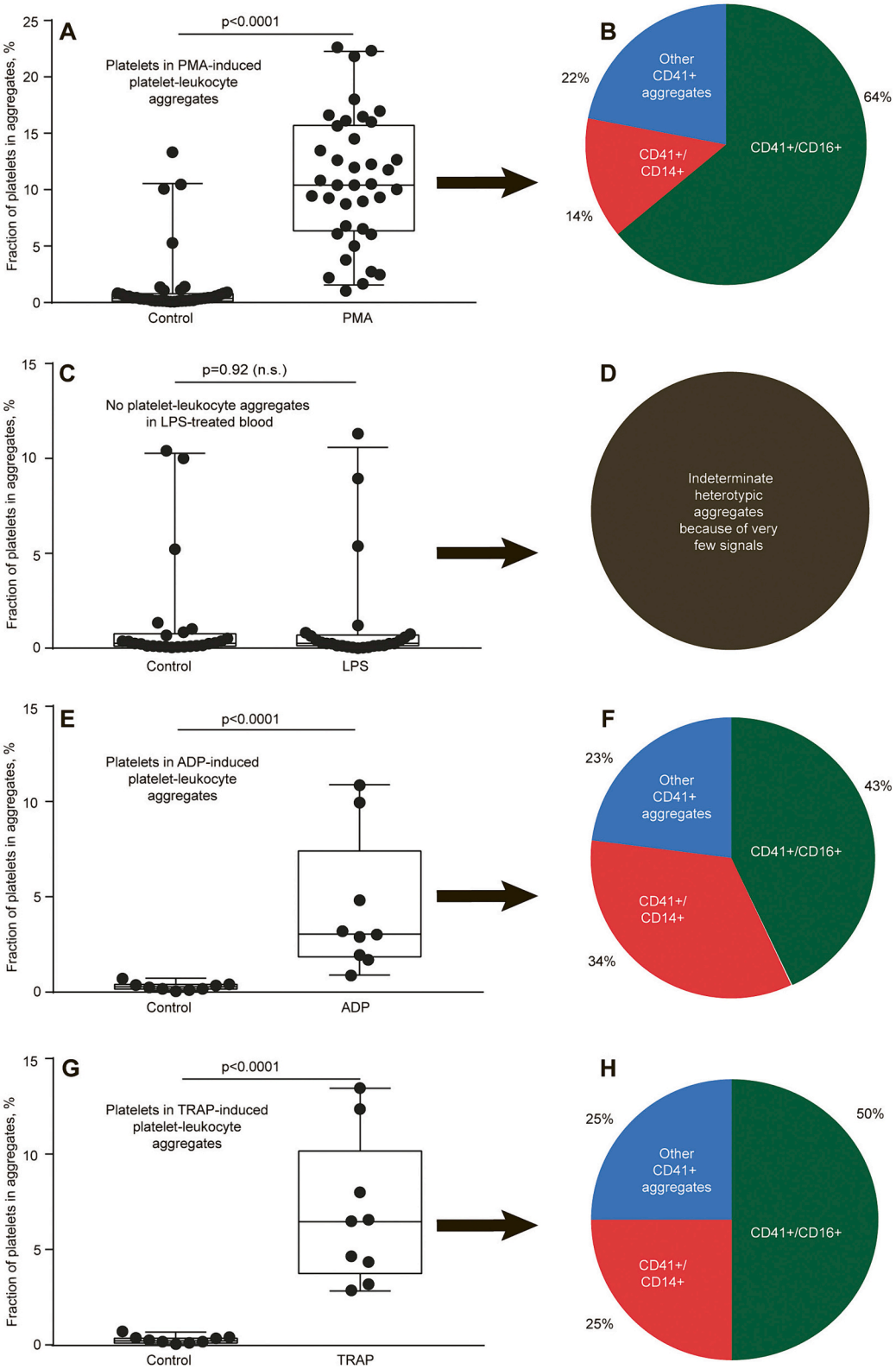

Fig. 3. Differential incorporation of platelets into various types of cellular aggregates in the blood samples treated with different cellular stimulants. (A, C, E, G) Fractions of CD41+ platelets in whole blood incorporated into aggregates with CD45+ leukocytes before (control) and after addition of 100 nM PMA (A), 100 ng/ml LPS (C), 20 μM ADP (E) and 50 μM TRAP-6 (G). The raw data points for each independent blood sample ($n \geq 9$) are overlaid with a median and interquartile interval between the 25th and 75th (boxes) as well as between the 5th and 95th percentiles (Mann-Whitney U test). (B, D, F, H) Distribution of platelets in PMA- (B), LPS- (D), ADP- (F), and TRAP-6-treated (H) blood samples between aggregates with neutrophils (CD41+/CD16+), monocytes (CD41+/CD14+), and other CD41+ platelet aggregates interacting either with each other or with unidentified cells or large particles. In B, D, F, H the total number of aggregated CD41+ platelets was taken as 100 %.

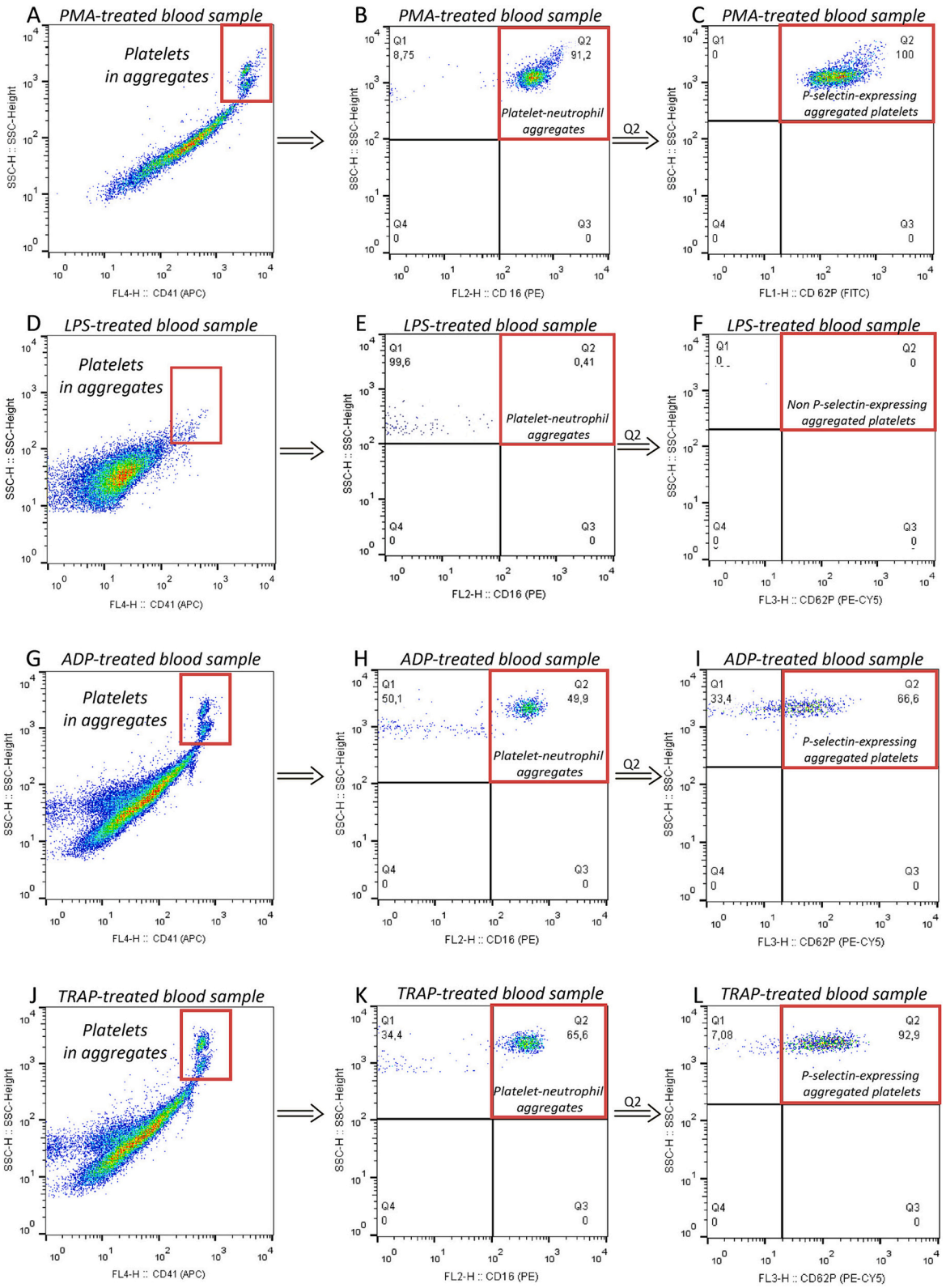

Fig. 4. Characteristic dot-plots showing expression of P-selectin on platelets involved in formation neutrophil-platelet aggregates in whole blood. (A–C) Flow cytometry of a blood sample treated with 100 nM PMA, showing the gating of CD41+ aggregated platelets (A), CD16+/CD41+ neutrophil-platelet aggregates (B), and CD62p+, i.e. P-selectin-expressing platelets, in Q2 (C). (D–F) Flow cytometry of a blood sample treated with 100 ng/ml LPS, showing the gating of CD41+ aggregated platelets (D), CD16+/CD41+ neutrophil-platelet aggregates (E), and the lack of CD62p + platelets in Q2 (F). (G-I) Flow cytometry of a blood sample treated with 20 μM ADP, showing the gating of CD41+ aggregated platelets (G), CD16+/CD41+ neutrophil-platelet aggregates (H), and CD62p + platelets in Q2 (I). (J-L) Flow cytometry of a blood sample treated with 50 μM TRAP-6, showing the gating of CD41+ aggregated platelets (J), CD16+/CD41+ neutrophil-platelet aggregates (K), and CD62p+in Q2 (L). The numbers of aggregated platelets shown in A, D, G, and J were taken as 100 %.

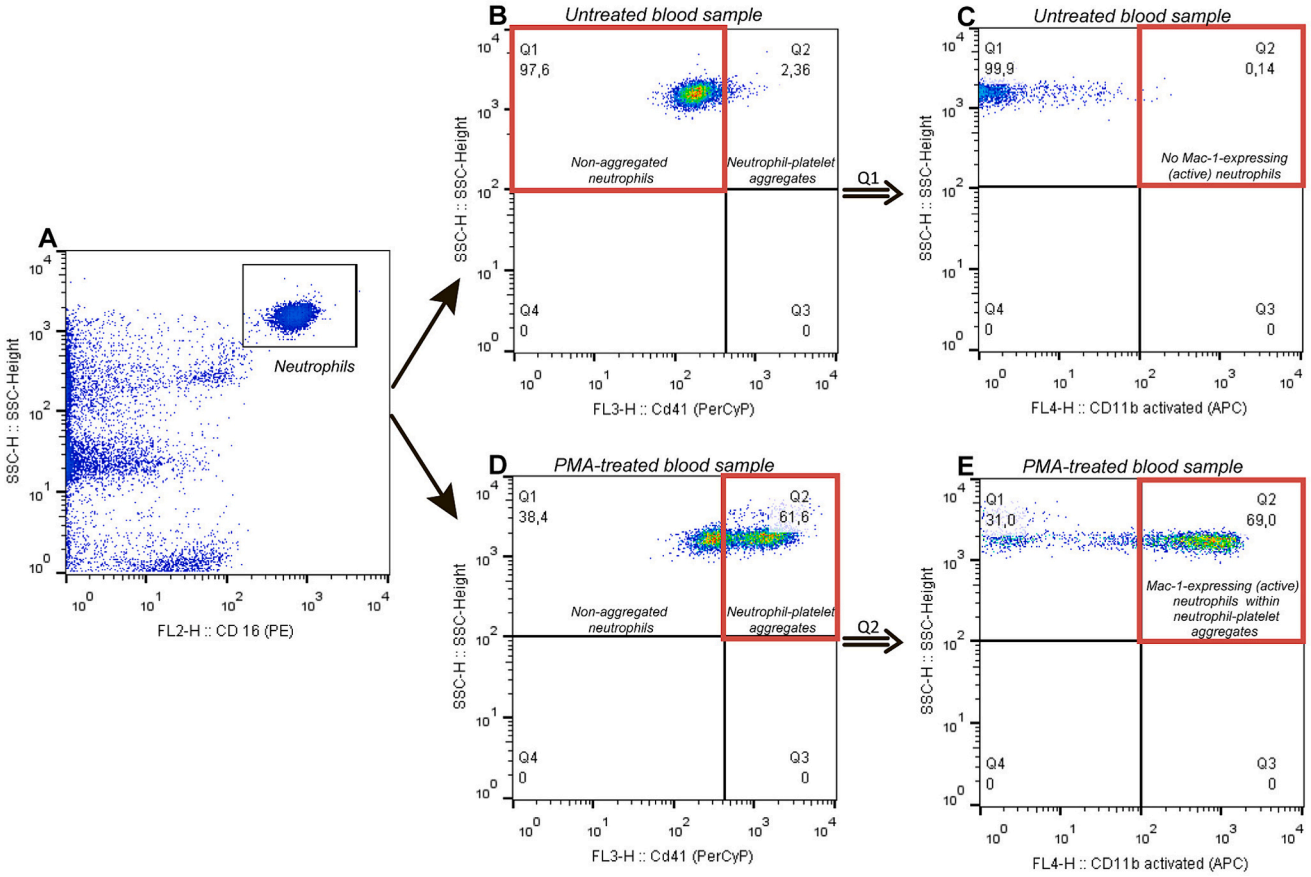

Fig. 5. Activated neutrophils within aggregates with platelets identified by the expression of the active integrin Mac-1 (CD11b). (A-E) Characteristic dot-plots showing activated neutrophils (CD11b+) within neutrophil-platelet aggregates formed in a PMA-treated whole blood sample. (A) Gating of CD16+ neutrophils. (B) Dominant non-aggregated neutrophils (Q1) and (C) no CD11b + neutrophils (Q2) interacting with platelets within the CD41+/CD16+ gate shown in B in the untreated blood sample. (D) Double CD41+/CD16+ signals (Q2) showing formation of neutrophil-platelet aggregates in the PMA-treated blood sample. (E) Fraction of activated neutrophils expressing the active Mac-1 (CD11b+) (Q2) within the CD41+/CD16+ gate of neutrophil-platelet aggregates shown in D in the PMA-treated blood sample. The total number of CD16+ neutrophils gated in A was taken as 100 % for B and D. Yet, the total number of non-aggregated neutrophils in B (Q1) was taken as 100 % for C, and the total number of neutrophil–platelet aggregates in D (Q2) was taken as 100 % for E.

pathological activation of blood cells, followed by the expression of adhesive proteins that mediate cell-cell adhesion [58]. Despite the importance of this phenomenon for biology and medicine, the conditions and mechanisms of leukocyte-platelet binding remain not fully understood. Here, we conducted a systematic study aimed at exploring the differential formation and composition of leukocyte-platelet aggregates induced by various cellular stimulants. Additionally, we investigated the mechanisms of intercellular adhesion in the presence of the widely used activators, such as PMA, LPS, ADP, and TRAP-6.

In the presence of PMA, about 38 % of neutrophils and 74 % of monocytes are involved in aggregates with platelets in whole blood (Fig. 1, Table S1), and 87 % of isolated neutrophils are found within neutrophil-platelet aggregates (Fig. 2, Table S2). Additionally, PMA induced expression of cell activation markers, including P-selectin on platelets, Mac-1 on neutrophils, and TF on monocytes within the heterotypic aggregates (Figs. 4, 5, S3, S5). These results are attributed to the ability of PMA to activate protein kinase C [59] in all cell types followed by activation of the αIIbβ3 integrin and expression of P-selection on the platelet surface [60]. These data are consistent with the main interaction between platelets and leukocytes being mediated by the binding of platelet P-selectin to PSGL-1, constitutively present on neutrophils and monocytes [61]; therefore, the blockage of P-selectin with inclacumab completely prevents the formation of platelet-leukocyte aggregates induced by TRAP (Fig. S4). The results obtained are also in line with strong platelet adhesion to leukocytes being mediated by the binding of the active integrin Mac-1 to platelet GPIb or to fibrinogen associated with the active platelet integrin αIIbß3 [62].

The presence of ADP in whole blood leads to the incorporation of 17 % neutrophils and 61 % monocytes into aggregates with platelets (Fig. 1, Table S1), and 23 % of isolated neutrophils form aggregates with platelets (Fig. 2, Table S2). It is noteworthy that all cell types involved in ADP-induced leukocyte-platelet aggregation express molecular markers of activation (Figs. 4, 5, S3, Table S4). These numbers obtained suggest that ADP is quite functional in the formation of leukocyte-platelet aggregates, although it is a somewhat weaker cellular activator compared to PMA, as ADP leads to approximately half the expression of P-selectin on platelets (Fig. 4, Table S4). The neutrophil-activating ability of ADP is mediated via ligand-gated ion channels (P2X) or G-protein-coupled receptors (P2Y) [50]. Monocytes are also sensitive to the stimulating effect of ADP, leading to increased expression of TF on the surface of monocytes [63]. Simultaneously, ADP is recognized as a platelet agonist that interacts with the purinergic receptors P2Y1, P2Y12, and P2X1, resulting in the activation of the αIIbβ3 integrin and an enhanced platelet ability to bind fibrinogen [64]. Importantly, platelet activation by ADP is followed by secretion from storage granules and pronounced expression of P-selectin, necessary to form stable aggregates with both neutrophils and monocytes via PSGL-1 [65–69]. ADP also induces platelet shape change, Ca^{2+} influx, and intracellular mobilization of Ca^{2+}, along with the inhibition of stimulated adenylyl cyclase activity [70].

The addition of TRAP-6 results in the inclusion of 24 % neutrophils and 78 % monocytes in aggregates with platelets in whole blood (Fig. 1, Table S1), and as much as 75 % of isolated neutrophils form neutrophil-

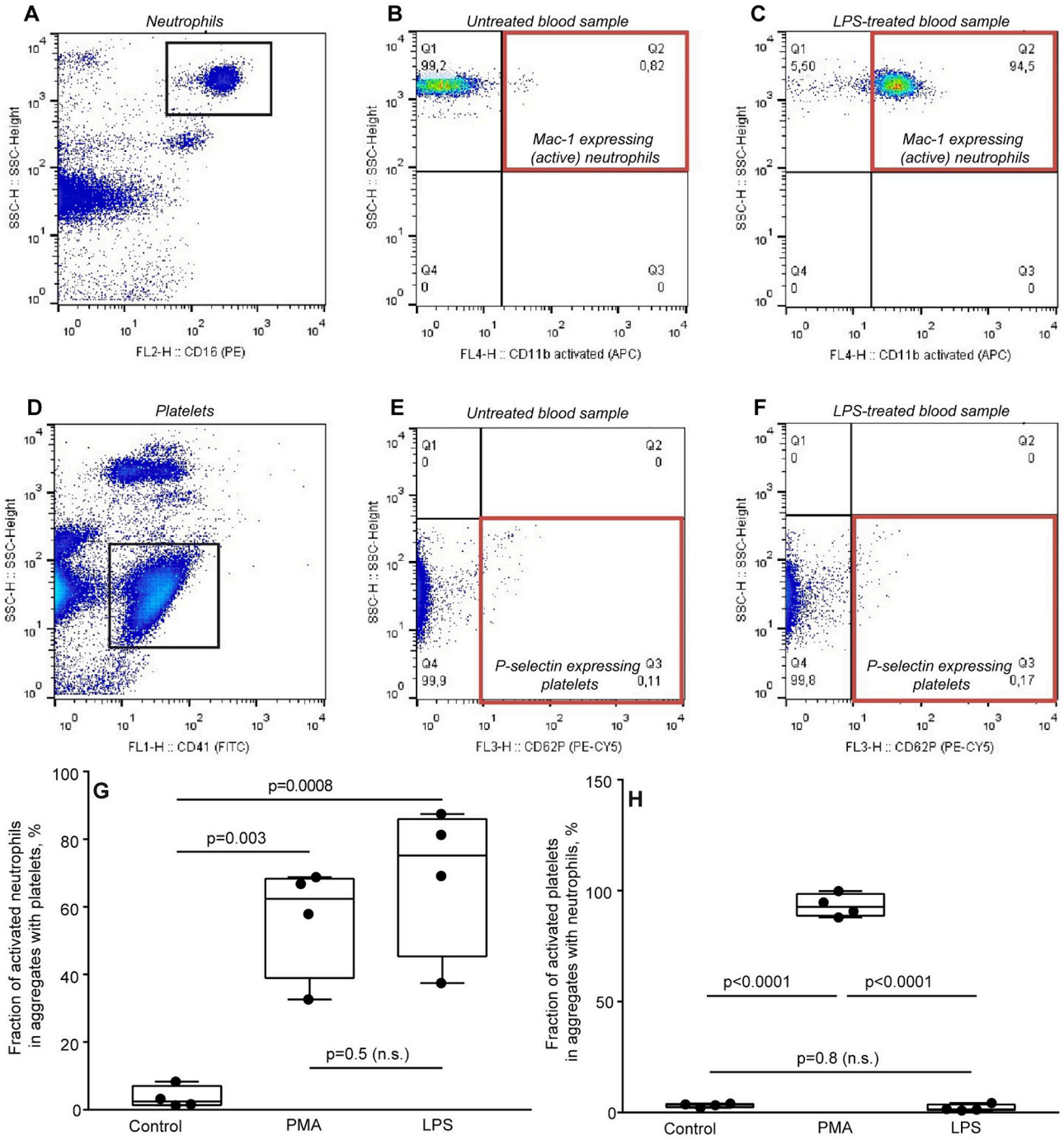

Fig. 6. LPS causes activation of neutrophils but not platelet activation, which leads to the lack of neutrophil-platelet aggregates in the presence of LPS. (A–F) Characteristic dot-plots showing the activating effect of LPS on neutrophils, but no platelet activation in an LPS-treated *whole blood sample*. (A) Gating of CD16+ neutrophils. (B) No activated Mac-1 (CD11b) expressed by neutrophils (Q2) in a control untreated blood sample. (C) A large fraction of CD11b + neutrophils expressing activated Mac-1 (Q2) within the gate of CD16+ neutrophils shown in A in the LPS-treated blood sample. (D) Gating of CD41+ platelets. (E) Almost no CD62p + P-selectin-expressing platelets (Q3) in the untreated control blood sample. (F) Almost no P-selectin expressing platelets (Q3) in the LPS-treated blood sample. The corresponding numbers of CD16+ neutrophils in A and CD41+ platelets in D were taken as 100 %. (G, H) The activating effect of LPS on *isolated neutrophils* without formation of neutrophil-platelet aggregates *in platelet-rich plasma*. (G) Fractions of activated neutrophils (CD16+) assessed by the expression of activated Mac-1 (CD16+/CD11b+) within a gate containing double CD16+/CD41+ events, reflecting neutrophil-platelet aggregates formed in the presence of LPS or PMA. (H) Fractions of activated platelets expressing P-selectin (CD41+/CD62p+) within the gate containing double CD16+/CD41+ events, reflecting almost no formation of neutrophil-platelet aggregates in the presence of LPS in contrast with PMA (positive control). Notably, in the presence of LPS, unlike PMA, the number of neutrophil-platelet aggregates analyzed in G and H was almost as small as in the untreated control samples (not shown). Designations: n.s. – non significant. The raw data points for each independent blood plasma sample and autologous neutrophil preparation (n = 4) are overlaid with a median and interquartile interval between the 25th and 75th, as well as between the 5th and 95th percentiles (ordinary one-way ANOVA test with Tukey's multiple comparisons post hoc test).

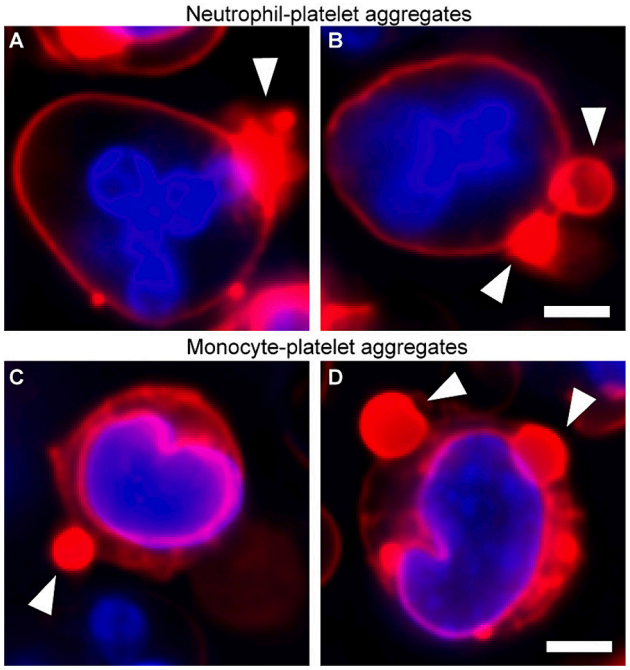

Fig. 7. Fluorescence microscopy of leukocyte-platelet aggregates. After stimulation of whole blood with 100 nM PMA followed by lysis of erythrocytes and fixation with paraformaldehyde, cells were labeled with DiI (plasma membrane, *red*) and DAPI (nucleus, *blue*). In both types of the cellular aggregates that contained platelets and neutrophils (A, B) or platelets and monocytes (B, C) the prevailing leukocyte/platelet ratios were 1:1 (A, C) or 1:2 (B, D). White arrows indicate platelets. Scale bars = 3 μm.

platelet aggregates (Fig. 2, Table S2). Based on the results obtained, the stimulants employed in this study have the following order of their relative ability to induce leukocyte-platelet aggregation in blood: PMA (100 nM) > TRAP-6 (50 μM) > ADP (20 μM) > LPS (100 ng/ml), suggesting that protein kinase C activation (caused by PMA and TRAP-6) is the mechanism that leads to more intense heterotypic cellular aggregation. TRAP-6 induces the activation of platelets, monocytes, and neutrophils through protease-activated receptor PAR1, which has a number of important functional consequences: strong P-selectin expression on platelets [49], Mac-1 activation on neutrophils [71], and TF upregulation in monocytes [72].

Unlike PMA, ADP, and TRAP-6, exposure of normal blood to LPS in our experiments resulted in no discernable leukocyte-platelet aggregates compared to the control (Fig. 1, Table S1). This was generally true for isolated neutrophils added to platelet-rich plasma in the presence of LPS, showing only an insignificant increase in the number of neutrophil-platelet aggregates (Fig. 2, Table S2). The explanation for these mostly negative results lies in the fact that LPS can induce an inflammatory response by activating neutrophils and monocytes [56,57], but it has a relatively weak or no stimulating effect on platelets [28,73]. Under the experimental conditions applied, there were no signs of LPS-induced platelet activation (Figs. 4, 6, and S3, Tables S4, S6), while neutrophils responded to LPS by strongly expressing active Mac-1 (Fig. 6, Table S6). The ineffective, yet existing, formation of a small number of heterotypic cellular aggregates in the absence of platelet activation can be attributed to relatively weak intercellular interactions, such as the binding of the junctional adhesion molecule (JAM3) on platelets to the integrin Mac-1 activated on leukocytes in the presence of LPS [74,75]. Another conceivable mechanism of feeble platelet binding to monocytes in the presence of LPS may be related to the interaction of CD14 on the surface of monocytes and TLR4 expressed on the surface of non-activated platelets in response to LPS [28].

LPS is a pro-inflammatory molecule that activates many cell types via TLR4 and the receptor-signal protein complex, including TLR4, MD2, CD14, and MyD88 [76,77]. Numerous studies have presented contradictory results regarding the effect of LPS on platelet activation and the formation of leukocyte-platelet aggregates [for reviews see 28,78–81]. Some studies report the inability of LPS to induce platelet activation [73,82,83], while others suggest an activating effect [77,78,84]. Under some conditions, LPS significantly increased platelet activation only if combined with sub-threshold concentrations of collagen or ADP [85]. Several conceivable explanations for this discrepancy exist. The structural diversity of LPS obtained from different bacterial strains might play a major role, given the diversity of the O-antigen, which can result in distinct cellular responses [86–88]. Unlike the LPS strain O26:B6 used in our study, [89] utilized LPS from different bacterial strains (O157:H7, O103:H2, O111:HN, O121:H19, and O111:B4), all of which caused a substantial increase in the formation of monocyte-platelet and neutrophil-platelet aggregates in whole blood. Another critical methodological difference is that the observed effect took place after incubating LPS with blood for 4 h, whereas in our experiments, the incubation time was much shorter (50 min). Regardless of the reason for the lack of LPS-induced platelet activation and heterotypic cellular aggregation under our experimental conditions, it is evident that the formation of leukocyte-platelet aggregates requires activation of both platelets and leukocytes. Notably, if platelets are activated initially, they cause secondary activation and recruitment of leukocytes [90,91], but not vice versa. From this standpoint, platelets emerge as the restrictive factor for aggregation with leukocytes because all the platelet stimulants studied activate leukocytes, whereas not all leukocyte agonists activate platelets. Therefore, the level of leukocyte-platelet aggregates in blood can be used as a quantitative measure of in vivo platelet activation [92]. Circulating aggregates of monocytes and neutrophils with platelets have been considered the most sensitive marker of platelet activation in vivo, even compared with the surface expression of platelet P-selectin [30,39].

Several papers have utilized scanning electron microscopy to visualize leukocyte and platelet aggregates, describing their morphological features [93–96]. While these studies provided valuable insights, quantitative analyses of the composition of leukocyte-platelet aggregates were lacking. To address this gap, we employed both fluorescence and electron microscopy to quantify the composition of these aggregates, with the result that the most frequent neutrophil-platelet ratios were 1:2 and 1:1, similar to the platelet/leukocyte ratios calculated from the flow cytometry data. Given that the heterotypic neutrophil-platelet aggregates were formed at a physiological leukocyte/platelet ratio of ~1:60 (i.e., in the presence of a large excess of platelets), the resulting ratios within aggregates again suggest that the limiting factor for aggregation with leukocytes is the fraction of activated platelets. This ratio is probably limited by the surface density of the interacting adhesion molecules and steric hindrance for their binding due to the complex and non-uniform topology of cell plasma membranes. The fewer platelets in the aggregates with neutrophils also indicate that homotypic platelet-platelet aggregation is more likely.

There are a number of limitations of this study, one being that aggregation of blood cells was induced in static conditions, while the formation and composition of platelet-leukocyte aggregates may be somewhat different in dynamic conditions, either with stirring flow [10,13,24,45]. Our experimental conditions implied one concentration of each cellular agonist, while dose-response studies would provide more comprehensive insights into the potencies and mechanisms involved. Room temperature is not physiological, but it was used for agonists known to induce rapid platelet activation, while longer incubations at 37 °C were employed for agonists with slower kinetics or those targeting leukocyte activation [44,43,97].

In summary, as inflammatory and thrombotic diseases are linked to an elevated presence of leukocyte-platelet aggregates in the blood, the interaction between activated platelets and various types of leukocytes emerges as an important pathogenic mechanism with clinical

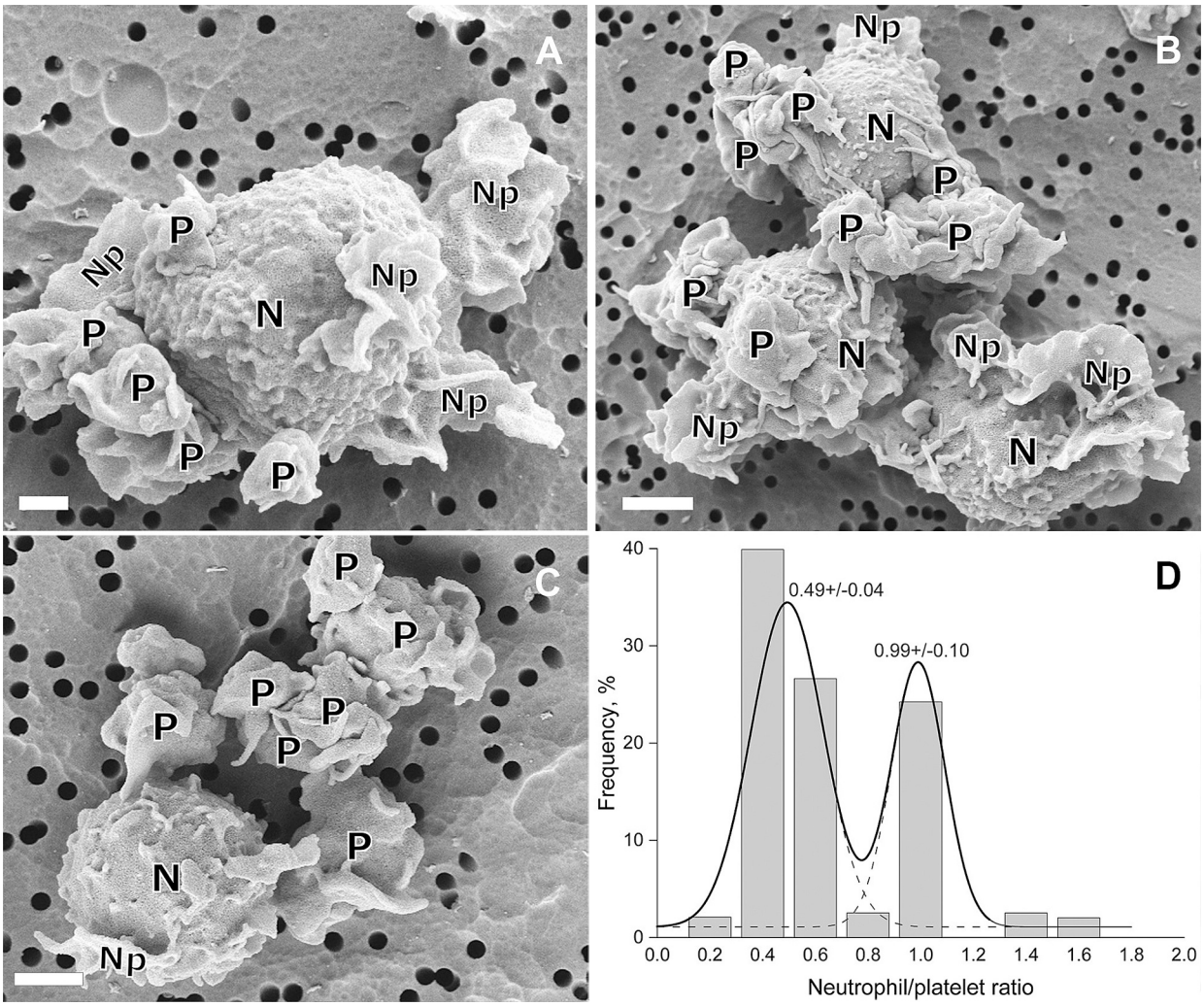

Fig. 8. Representative scanning electron micrographs of neutrophil-platelet aggregates and quantification of their composition. Isolated neutrophils were added to platelet-rich plasma in the presence of 100 nM PMA. Cellular aggregates were settled down on a polycarbonate filter with a 0.4-μm pore size, processed and subjected to scanning electron microscopy (see Materials and methods). (A–C) Neutrophil-platelet aggregates of various sizes. Designations: *N* – neutrophil, *P* – platelet, *Np* – neutrophil pseudopods. Scale bars: for A – 1 μm; for B and C – 2 μm. (D) Distribution of neutrophil/platelets ratios within neutrophil-platelet aggregates ($n = 76$) visualized by scanning electron microscopy and fitted with a bimodal Gaussian function. Centroids of the two peaks correspond to the most likely neutrophil/platelets ratios of 1:2 and 1:1.

implications.

CRediT authorship contribution statement

Alina D. Peshkova: Project administration, Writing – original draft, Methodology, Investigation. **Shakhnoza M. Saliakhutdinova:** Writing – original draft, Methodology, Investigation. **Khetam Sounbuli:** Methodology, Investigation. **Yuliya A. Selivanova:** Methodology, Investigation. **Izabella A. Andrianova:** Visualization, Methodology, Data curation. **Alina I. Khabirova:** Methodology, Investigation. **Rustem I. Litvinov:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **John W. Weisel:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2024.109092.

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