Interplay between circulating von Willebrand factor and neutrophils: implications for inflammation, neutrophil function, and von Willebrand factor clearance

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Abstract

von Willebrand factor (VWF) plays a critical role in hemostasis, and emerging evidence suggests its involvement in inflammation. Our study aimed to investigate the interaction between circulating plasma VWF and neutrophils (polymorphonuclear cells [PMN]), elucidate the fate of VWF after binding, and explore its impact on neutrophil behavior. Neutrophils were isolated from the whole blood of healthy volunteers, and their interaction with plasma VWF was examined *ex vivo*. Immunofluorescence imaging revealed an enhanced binding of VWF to neutrophils following stimulation with inflammatory agents (PMA, TNFα, and IL-8) and exposure to shear forces, highlighting a previously unknown interaction. Furthermore, immunofluorescence images demonstrated increased co-localization of VWF with the early endosome marker EEA1 and the late endosome marker Rab7 over time, indicating the uptake of VWF by neutrophils subsequent to the binding. This was supported by a significant decrease in VWF antigen levels in the supernatant of cells after stimulation. Moreover, stimulated neutrophils exposed to purified plasma-derived VWF exhibited elevated expression of neutrophil surface markers CD45 and CD66b, indicative of altered neutrophil function related to cell adhesion, migration, and phagocytosis. These findings suggest that VWF binding can modulate neutrophil function, potentially influencing their role in immune responses and inflammation. In summary, our study provides novel insights into the complex interplay between VWF and neutrophils, shedding light on the multifaceted roles of VWF in inflammation. Importantly, we have identified neutrophils as potential cellular mediators involved in the clearance of VWF from circulation, introducing a novel mechanism for VWF removal.

Introduction

von Willebrand factor (VWF), a large multimeric plasma glycoprotein, plays a crucial role in primary and secondary hemostasis, where it facilitates platelet adhesion and aggregation at the site of vascular injury and stabilizes coagulation factor VIII (FVIII) in circulation.[1-4] However, beyond its established role in hemostasis, VWF has been found to play a multifaceted, complex role in inflammation that still needs further investigation.[5,6] Previous studies have suggested that VWF may modulate inflammation through both intracellular and extracellular pathways. Intracellularly, VWF has been shown to initiate the biogenesis of endothelial cell storage organelles called Weibel-Palade bodies (WPB), which contain inflammatory mediators such as P-selectin, angiopoietin-2 (Ang2), interleukin 6 (IL-6), interleukin 8 (IL-8), and CXCL1.[7-9] This intrinsic ability of VWF to influence WPB formation suggests an indirect mechanism of inflammation modulation. Extracellularly, VWF, when immobilized or anchored to the endothelium, directly interacts with leukocytes, including polymorphonuclear leukocytes (PMN, or known as neutrophils) and monocytes. The interaction of VWF with leukocytes involves binding to receptors such as P-selectin glycoprotein ligand-1 (PSGL-1) for rolling adhesion and Mac-1 (αMβ2 integrin [CD11b/CD18]) for stable adhesion.[10] Notably, the interaction between VWF and PMN has been shown to enhance PMN recruitment and transmigration across the endothelium, thereby contributing to the initiation and propagation of inflammation.[6,11-13]

Furthermore, earlier in our laboratory, immunofluorescence (IF) staining of isolated neutrophils from healthy volunteers

and from a patient with von Willebrand disease (VWD) (who exhibited an accelerated VWF clearance in plasma) surprisingly displayed VWF staining that was even stronger after the patient received therapeutic plasma-derived (pd) VWF concentrates. This observation suggested a potential interaction between unbound plasma (circulating) VWF and neutrophils.[14] Additional *ex vivo* investigations using endothelial colony-forming cells (ECFC) derived from the VWD patient demonstrated malformed WPB, impairing the recruitment of inflammatory cargoes and associated downstream signaling pathways. Transcriptome analysis revealed the overexpression of neutrophil-activating chemoattractants, including IL-6, IL-8, and CXCL-1, in patient-derived ECFC compared to healthy volunteers.[14] These findings led us to propose that the overexpression of these chemoattractants could trigger neutrophil activation, increased binding of VWF to neutrophils, and consequent accelerated VWF uptake. Consequently, this previous study prompted the hypothesis that in addition to endothelium-anchored VWF, unbound (circulating) VWF might also interact with neutrophils, potentially contributing to VWF clearance from circulation, which forms the focus of our current investigation.

In this study, our objectives were to investigate the interaction of unbound plasma VWF with resting and stimulated neutrophils under both static and flow conditions. Additionally, we aimed to explore the internalization and fate of VWF following its interaction with human neutrophils by examining VWF trafficking within neutrophils. Furthermore, we sought to assess the effects of purified pdVWF/FVIII concentrate binding on the function and behavior of human neutrophils.

Methods

Isolation of human neutrophils

Whole blood (5-10 mL) was collected from healthy volunteers in EDTA tubes (Sarstedt, Germany) after obtaining informed consent, in compliance with the principles of the Declaration of Helsinki and the ethical guidelines of Germany. Neutrophils were isolated using the EasySep™ Direct Human Neutrophil Isolation Kit (StemCell Technologies, Germany). Cell number and viability were assessed with the NucleoCounter® NC-202™ (ChemoMetec, Denmark), and cell purity was confirmed by flow cytometry.

Flow cytometry

Neutrophils were washed in phosphate-buffered saline, resuspended in staining buffer, and stained with fluorochrome-conjugated antibodies (anti-CD66b-FITC, anti-CD45-APC, anti-CD16-PE) or isotype controls (Miltenyi Biotec, Germany). Analysis was performed using a Navios EX Flow Cytometer (Beckman Coulter, Germany) and FlowJo software.

von Willebrand factor binding to neutrophils upon activation

Neutrophils suspended in autologous plasma were either left untreated or stimulated with phorbol myristate acetate (PMA) (10 ng/mL), TNFα (5 ng/mL), or IL-8 (0.3-0.7 ng/mL) for 15 minutes (min) at 37°C. To test VWF binding via the Mac-1 receptor, neutrophils were treated with neutrophil inhibitory factor (NIF; 400 ng/mL) for 30 min before PMA stimulation. VWF binding was assessed by IF microscopy.

von Willebrand factor binding to neutrophils upon exposure to shear forces

Neutrophils were exposed to shear flow using the Bioflux 200 instrument (Fluxion Biosciences). Cells were resuspended in RPMI medium with pdVWF/FVIII (Haemate, CSL Behring, Germany) or recombinant VWF (VONVENDI®, Baxalta) and incubated under static or shear conditions (10 dyne/cm²) for 30 min at 37°C. Cells were fixed and analyzed by IF microscopy.

von Willebrand factor internalization and trafficking

VWF internalization was studied by co-localizing VWF with early (EEA1) and late (Rab7) endosomal markers using IF microscopy. Neutrophils were fixed immediately or after 60 or 180 min of incubation. Additional experiments involved exposing neutrophils to shear stress, followed by incubation and subsequent IF analysis.

Immunofluorescence microscopy

Cells were fixed with formalin, permeabilized, and stained with primary antibodies against VWF, FVIII, CD11b, EEA1, and Rab7, followed by fluorescent secondary antibodies. Imaging was done with an Apotome.2 microscope (Carl Zeiss, Germany), and VWF signals and co-localization were quantified using ZEN 2.6 software.

Measurement of von Willebrand factor content in supernatants

VWF levels in supernatants from resting and stimulated neutrophils were measured. After isolation, neutrophils were resuspended in autologous supernatant, stimulated with IL-8 (0.7 ng/mL), PMA (10 ng/mL), or TNFα (5 ng/mL) for 15 min at 37°C, and VWF levels were compared with supernatants without cells.[15]

Purification of plasma-derived von Willebrand factor/coagulation factor VIII concentrates

The pdVWF/FVIII concentrate (Haemate) was purified by size-exclusion chromatography (ÄKTA pure 25 system, Cytiva) and protein purity was confirmed by SDS-PAGE.

Western blotting

Neutrophils were stimulated with IL-8 in the presence of autologous VWF, lysed, and subjected to western blotting using rabbit anti-human VWF antibodies.[16] Neutrophils, stimulated

with IL-8 in the presence of FITC-labeled VWF (Invitrogen, USA), were lysed and analyzed by gel electrophoresis.

Effects of von Willebrand factor on neutrophil characteristics

Neutrophils were stimulated with IL-8 or shear forces in the presence of pdVWF/FVIII and analyzed for surface proteins by flow cytometry. Neutrophil extracellular traps (NET) formation was assessed with and without pdVWF/FVIII.

Statistical analysis

Data significance was evaluated using the unpaired Student's *t* test (GraphPad Prism 8.0.1). Results are presented as mean ± standard error of the mean (SEM), with $P \leq 0.05$ considered statistically significant.

Results

Highly viable and purified neutrophils were isolated from whole blood

The viability of isolated cells, determined by AO/DAPI on an automated cell counter, was between 99% to 100% for each isolation (Figure 1Ai). Flow cytometric analysis confirmed the purity of >95% of the isolated neutrophils each time (Figure 1B). It was confirmed that >95% of the isolated neutrophils were still viable after 180 min of incubation (Figure 1Aii). In addition, >90% of cells were non-apoptotic after 180 min, as determined by the level of CD16 shedding, a marker of neutrophil apoptosis (Figure 1C).

Neutrophils showed enhanced interaction with plasma von Willebrand factor upon activation *ex vivo*

IF images showed increased binding of plasma VWF to neutrophils after stimulation with the inflammatory modulators PMA, TNFα, and IL -8 and activation by shear forces, assessed by visual inspection and evaluation of VWF signal intensity (by measuring VWF mean intensity values [MIV] of at least 50 cells from 3 independent experiments). Cells stimulated with TNFα and PMA in the presence of autologous plasma VWF exhibited a significantly increased average MIV of VWF signal. The MIV values were 494.8±23.9 and 517.3±29.6, respectively, compared to 313±10.9 in resting neutrophils (Figure 2A). Similarly, when neutrophils were

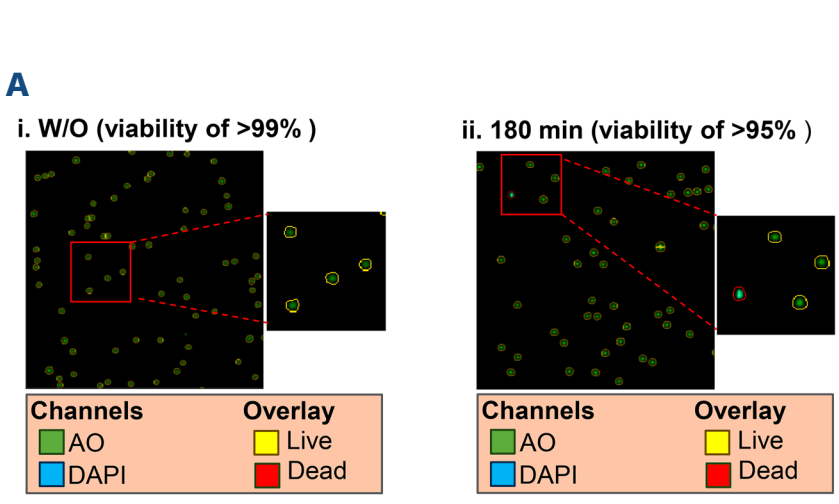

Figure 1. Viability verification and flow cytometry analysis of purified neutrophils from whole blood. (A) Viability assessment of isolated neutrophils without incubation (W/O) or after 180 minutes (min) of incubation using the NucleoCounter® NC-202™. Viability was determined by acridine orange (AO) and DAPI staining for live and dead cells, respectively. (B) Multicolor flow cytometry analysis confirming the purity of the cells (>95%). Neutrophils were analyzed directly after isolation using antibodies against specific surface receptors, including CD16, CD66b, and the leukocyte marker CD45, along with respective isotype controls. The gated $CD45^+$ population demonstrated a typical neutrophil content ($CD66b^+CD16^+$) above 95%. The histograms depict neutrophils stained with anti-CD66b-FITC (red), anti-CD16-PE (green), and anti-CD45-APC (blue), incorporating isotype controls (gray). (C) Assessment of apoptotic cells by evaluating CD16 receptor shedding after 180 min of incubation at 37ºC/5% CO_2 (ii), compared to cells analyzed immediately after isolation (W/O; i). The histograms display neutrophils stained with anti-CD66b-FITC (red), anti-CD16-PE (blue), and isotype controls (gray).

treated with IL-8 concentrations of 0.3, 0.5, and 0.7 ng/mL, in the presence of autologous plasma VWF, there was a notable increase in VWF MIV. The respective MIV values were 534.4±21.1, 700.4±57.9, and 819.8±58.4, compared to 313.5 ±9.1 for untreated cells (P≤0.0001) (Figure 2B). Remarkably, the co-localization of VWF and CD11b, a constituent of the Mac-1 receptor, significantly intensified following the stimulation of neutrophils. This observation implies that VWF binds to the Mac-1 receptor upon interacting with neutrophils (Figure 2C). Further analysis, including NIF treatment, confirmed the binding of VWF to neutrophils through interaction with the Mac-1 receptor. NIF blocks the leukocyte Mac-1 (CD11b/CD18), inhibiting adhesion to its ligands. Our results demonstrated that neutrophils treated with NIF exhibited no detectable VWF signals and showed a significant reduction in CD11b signals. This confirms the effective inhibition of Mac-1 by NIF and its interference with VWF binding (Figure 2D).

Similarly, our results showed an increase in VWF MIV when neutrophils were exposed to shear flow (10 dyne/cm^2) in the presence of spiked pdVWF/FVIII, with a MIV of 443.3±19.9 for shear-exposed cells *versus* a MIV of 297.5±8.4 for unexposed cells (Figure 3A). Our supplemental experiments in which the isolated neutrophils were exposed to shear forces (10 dynes/cm^2) in the presence or absence of pdVWF/FVIII resulted in VWF MIV of 364.0±25.3 and 256.2±7.2, respectively (Figure 3B). This result confirmed that the increased VWF signal intensity was due to enhanced binding of the spiked pdVWF/FVIII and precluded possible synthesis of VWF by the neutrophils when exposed to shear forces.

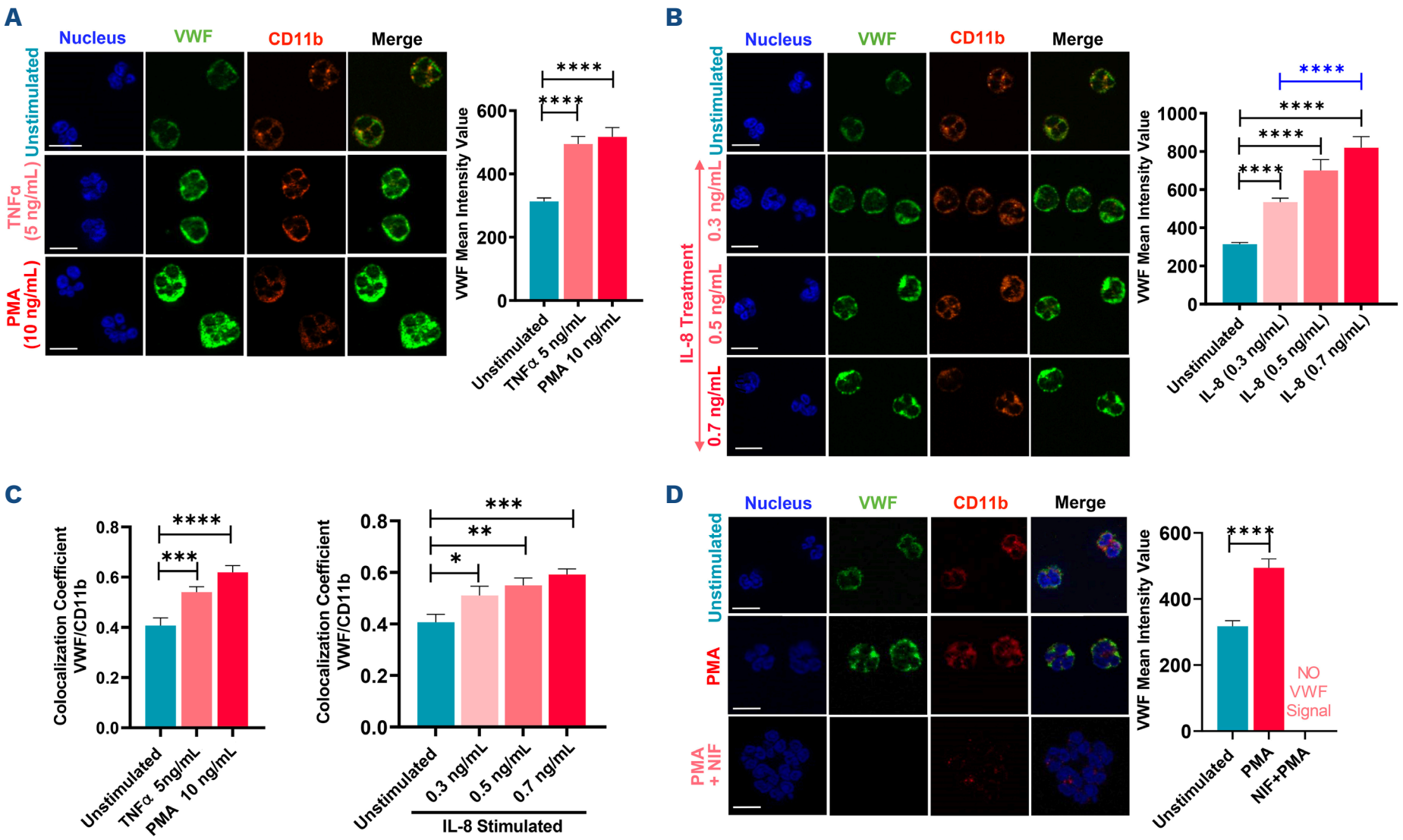

Figure 2. Analysis of von Willebrand factor interaction with resting and activated neutrophils using immunofluorescence microscopy. (A) Immunofluorescence (IF) microscopy images of unstimulated neutrophils (control, upper row) and neutrophils stimulated with TNFα (5 ng/mL) or phorbol myristate acetate (PMA) (10 ng/mL). The bar graph presents von Willebrand factor (VWF) mean intensity values (MIV) for unstimulated and stimulated neutrophils, which is calculated from at least 50 cells in 3 independent experiments. (B) IF microscopy images of unstimulated neutrophils and neutrophils stimulated with different concentrations of interleukin-8 (IL-8) (0.3 ng/mL, 0.5 ng/mL, or 0.7 ng/mL). The bar graph demonstrates an increase in VWF MIV with increasing IL-8 concentrations, quantified by VWF signals from at least 50 cells in 3 independent experiments. (C) Co-localization co-efficients of VWF and CD11b (a component of Mac-1 receptor [αMβ2 integrin]) determined from at least 25 regions of interest (25 cells) in 3 independent experiments. (D) IF microscopy images of unstimulated neutrophils (control, upper row), neutrophils stimulated with PMA (10 ng/mL), as well as neutrophils stimulated with PMA (10 ng/mL) in presence of neutrophil inhibitory factor (NIF; 400 ng/mL). The bar graph presents VWF MIV for unstimulated and stimulated neutrophils, which is calculated from at least 50 cells in 3 independent experiments. IF images show VWF (green), CD11b (red), and nucleus (DAPI, blue). The merge channel displays VWF-CD11b overlay. Scale bar: 10 μm. Statistical significance: ****P≤0.0001, ***P≤0.001, **P≤0.01, *P≤0.05.

Similarly, we observed binding of rVWF when the neutrophils are exposed to shear force, demonstrated by an increase in VWF MIV in cells exposed to shear in presence of rVWF, compared with unexposed cells or absence of any VWF (Figure 3C). Furthermore, as anticipated, we detected the co-localization of VWF with factor VIII (Figure 3D).

Neutrophils internalize von Willebrand factor after binding

IF images of neutrophils revealed that co-localization of VWF with the early endosome marker EEA1 and the late endosome Rab7 increased with incubation time, suggesting uptake of VWF into neutrophils after binding.

The IF image analysis of neutrophils isolated from whole blood and incubated directly for up to 180 min under static conditions showed that the Pearson coefficient of co-localization of VWF/EEA1 increased from 0.02±0.01 at time 0 to 0.13±0.01 after 60 min and to 0.24± 0.01 after 180 min (P≤0.0001) (Figure 4A). Similarly, the Pearson coefficient of VWF/Rab7 increased from 0.14±0.01 at time 0 to 0.24±0.01 after 60 min and 0.27±0.01 after 180 min (P≤0.0001) (Figure 4B).

Comparable results were obtained when neutrophils were first exposed to shear flow in the presence of pdVWF/FVIII concentrates for 30 min and then incubated for 30 or 60 min. Analysis of the Pearson co-localization coefficient of VWF and EEA1 showed an increase from 0.11±0.01 at time 0 to 0.20±0.02 and 0.27±0.02 after 30 and 60 min, respectively (P≤0.0001) (Figure 4C). Similarly, the co-localization coefficient of VWF and Rab7 showed an increase from 0.13±0.01 at time 0 to 0.15±0.02 and 0.29±0.02 after 30 and 60 min, respectively (Figure 4D).

Furthermore, we demonstrated that the VWF:Ag concentration in the plasma supernatant of IL -8 (0.7 ng/mL), TNFα (5 ng/mL), and PMA (10 ng/mL)-stimulated neutrophils was

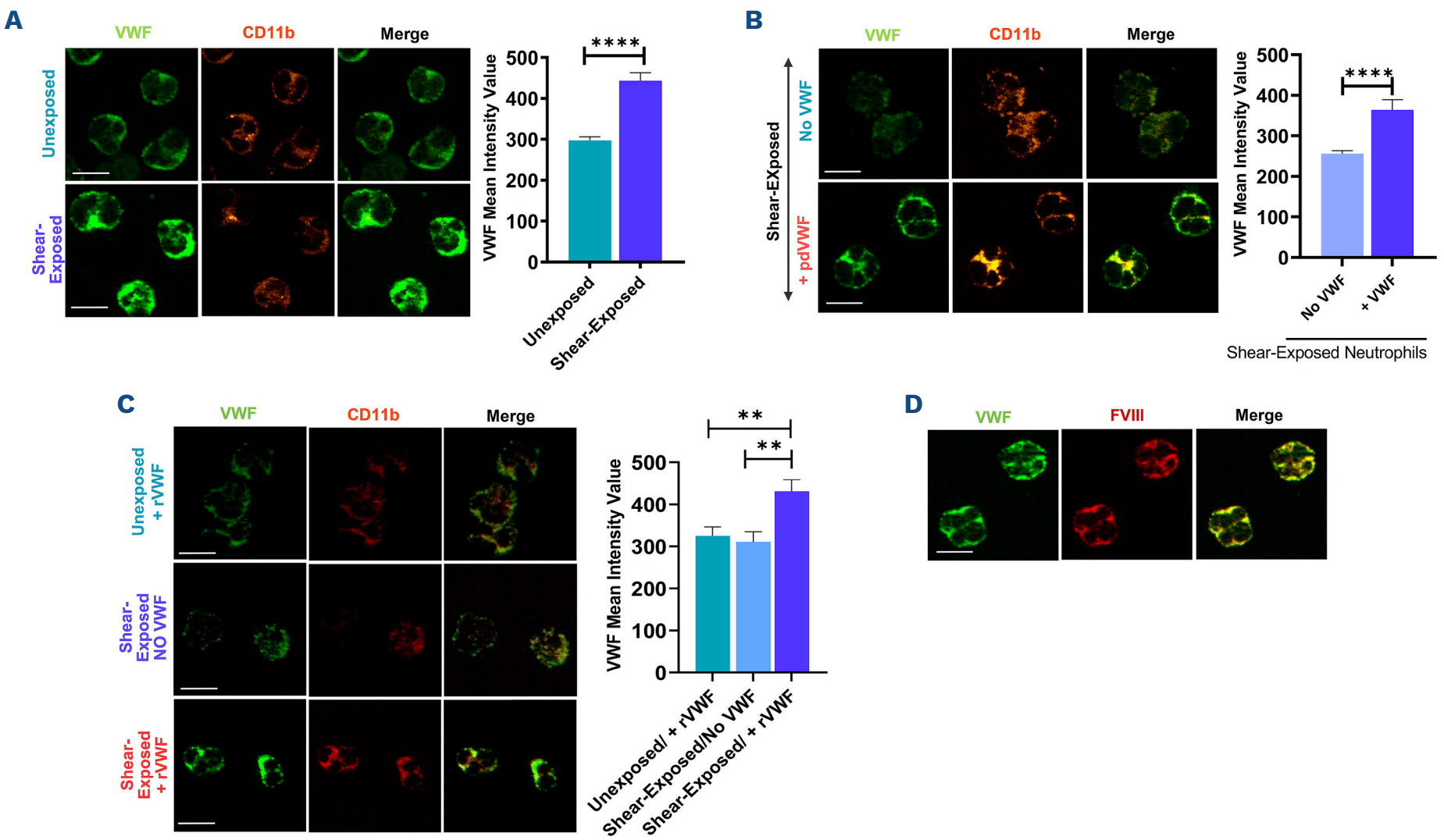

Figure 3. Investigating impact of shear flow on von Willebrand factor interaction with neutrophils using immunofluorescence microscopy. (A) Immunofluorescence images representing von Willebrand factor (VWF) binding to neutrophils exposed to shear flow (10 dyne/cm², lower row) compared to unexposed/resting cells (upper row). The bar graph shows measured VWF mean intensity values (MIV) associated with unexposed and exposed neutrophils to shear flow, which was measured from at least 50 cells in 3 independent experiments. (B) Immunofluorescence images of VWF staining in neutrophils exposed to shear flow in the presence and absence of plasma-derived (pd) VWF/coagulation factor VIII (FVIII). The bar graph illustrates VWF MIV for exposed neutrophils with and without pdVWF/FVIII. Measurements were taken from at least 50 cells in 3 independent experiments for each condition. (C) Immunofluorescence images representing recombinant VWF (rVWF) binding to neutrophils exposed to shear flow (10 dyne/cm², lower row) compared to unexposed/resting cells (upper row), as well as shear-exposed cells in absence of VWF (middle row). The bar graph shows measured VWF MIV associated with unexposed and exposed neutrophils to shear flow, which was measured from at least 50 cells in 3 independent experiments. (D) Immunofluorescence image demonstrating co-localization of VWF and FVIII on neutrophils. Immunofluorescence images show VWF (green), CD11b (red), and nucleus (DAPI, blue). The merge channel displays VWF-CD11b overlay. Scale bar: 10 μm. Statistical significance: ****P≤0.0001, **P≤0.01.

reduced by an average of 2.6%, 2%, and 2.4% ($P \leq 0.001$), respectively compared to the supernatant of resting cells and the negative control (supernatant deprived of cells) (Figure 5A).

von Willebrand factor was degraded after uptake by neutrophils

Western blotting of lysates of resting neutrophils and IL-8 (0.7 ng/mL)-stimulated neutrophils (both incubated for 0, 60, and 180 min) revealed degraded VWF fragments around 20 and 70 kDa in addition to the intact full-length VWF (250 kDa). The strength of the bands corresponding to VWF degradation was more pronounced in stimulated cells (relative to intact VWF) than in resting cells (Figure 5B). Stimulation of neutrophils with IL-8 (0.7 ng/mL) in the presence of fluorescently labeled purified pdVWF/FVIII and subsequent gel electrophoresis yielded VWF fragments of the same size. This confirmed that VWF present in cell supernatants is internalized and degraded by neutrophils (Figure 5C).

von Willebrand factor binding to neutrophils affects the surface expression of their functional proteins

We observed increased expression of CD45 and CD66b on the surface of neutrophils exposed to shear flow and cells stimulated with IL-8 in the presence of purified pdVWF/FVIII compared with cells without VWF in the cell suspension. The surface proteins CD45 and CD66b are essential markers of neutrophil function (cell adhesion, migration, and phagocytosis), and their expression levels may therefore be used to assess the activation and function of these cells. Flow cytometric analysis showed a significant increase in the mean fluorescence intensity (MFI) average of CD66b and CD45 markers in shear-exposed neutrophils in the presence of VWF (an increased MFI of 35.3±5.66% and 29.7±7.37%, respectively) compared with those in the absence of VWF ($P \leq 0.001$ and $P \leq 0.01$, respectively) (Figure 6A).
Similarly, we observed an increase in MFI average of 27.9±10.12% and 15.2±3.69%, respectively, for the surface

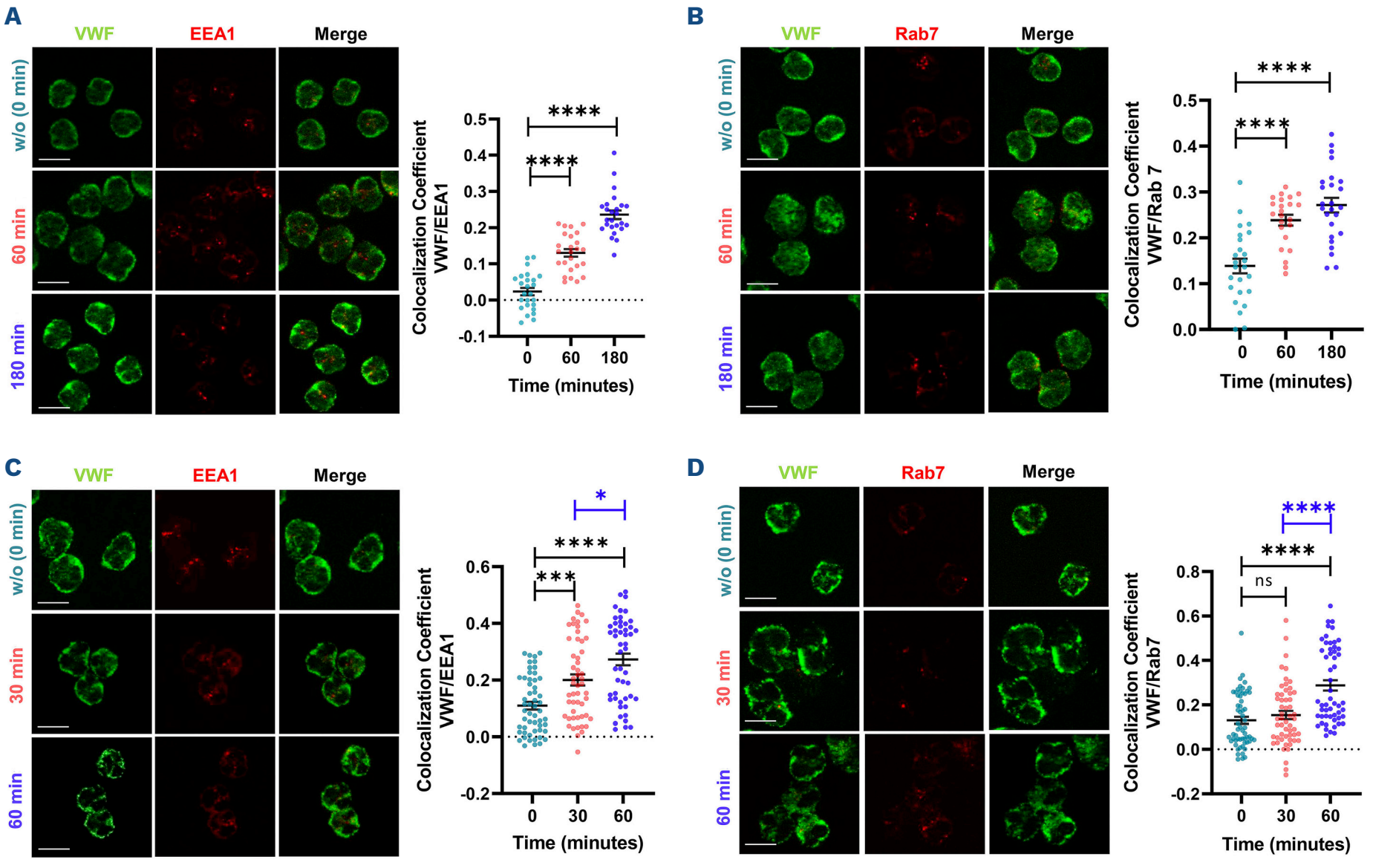

Figure 4. Intracellular trafficking of von Willebrand factor after interaction with neutrophils in static and shear flow conditions. (A, B) Immunostaining of neutrophils with von Willebrand factor (VWF) (green), EEA1 (early endosome marker, red in A), and Rab7 (late endosome marker, red in B) at different time points (0, 60, and 180 minutes [min]) following their interaction with VWF under static conditions. Bar graphs show Pearson's co-localization coefficients of VWF/EEA1 and VWF/Rab7 over time, which was calculated from at least 25 cells of 2 independent experiments, each with 3 replicates (N=6). (C, D) Immunostaining of neutrophils with VWF (green), EEA1 (red in C), and Rab7 (red in D) at different time points (0, 30, and 60 min) following their interaction with VWF under flow conditions (10 dyne/cm^2 for 30 min). Bar graphs display Pearson's co-localization coefficients of VWF/EEA1 and VWF/Rab7 over time, which was measured from at least 50 cells of 2 independent experiments, each with 3 replicates (N=6). Scale bar 10 μm. Statistical significance: ****$P \leq 0.0001$, ***$P \leq 0.001$, *$P \leq 0.05$; not significant (NS): $P > 0.05$.

proteins CD66b and CD45 on the surface of IL -8-stimulated cells in the presence of purified pdVWF/FVIII compared with those stimulated in the absence of VWF ($P \leq 0.05$, and $P \leq 0.01$, respectively) (Figure 6B).

Moreover, we observed that VWF promotes the formation of NET following the stimulation of isolated neutrophils with 25 ng/mL PMA, both in the presence and absence of VWF, across different incubation times of 60, 120, 180, and 240 min. According to the literature, NETosis occurs in distinct stages: initially, neutrophils exhibit larger but still segmented nuclei (around 60 min); this is followed by the disintegration of nuclei and chromatin decondensation (120-180 min); the late stages of NETosis (180-240 min) are characterized by the presence of cloudy and shooting cells, indicating that neutrophils have fully expelled their DNA; and finally, cells undergo lysis (240 min), where everything appears degraded.[17-20] The most significant differences between cells stimulated in the presence of VWF and those in its absence were observed after 120 and 180 min. After 120 min, cells stimulated in the presence of VWF showed a greater number of cells with disintegrated nuclei compared to those stimulated in the absence of VWF. Additionally, after 180 min, a greater proportion of cells stimulated in the presence of VWF entered the NETosis phase (about 50% of cells showing a cloudy appearance and 50% a shooting form) compared to those in the absence of VWF (Figure 7).

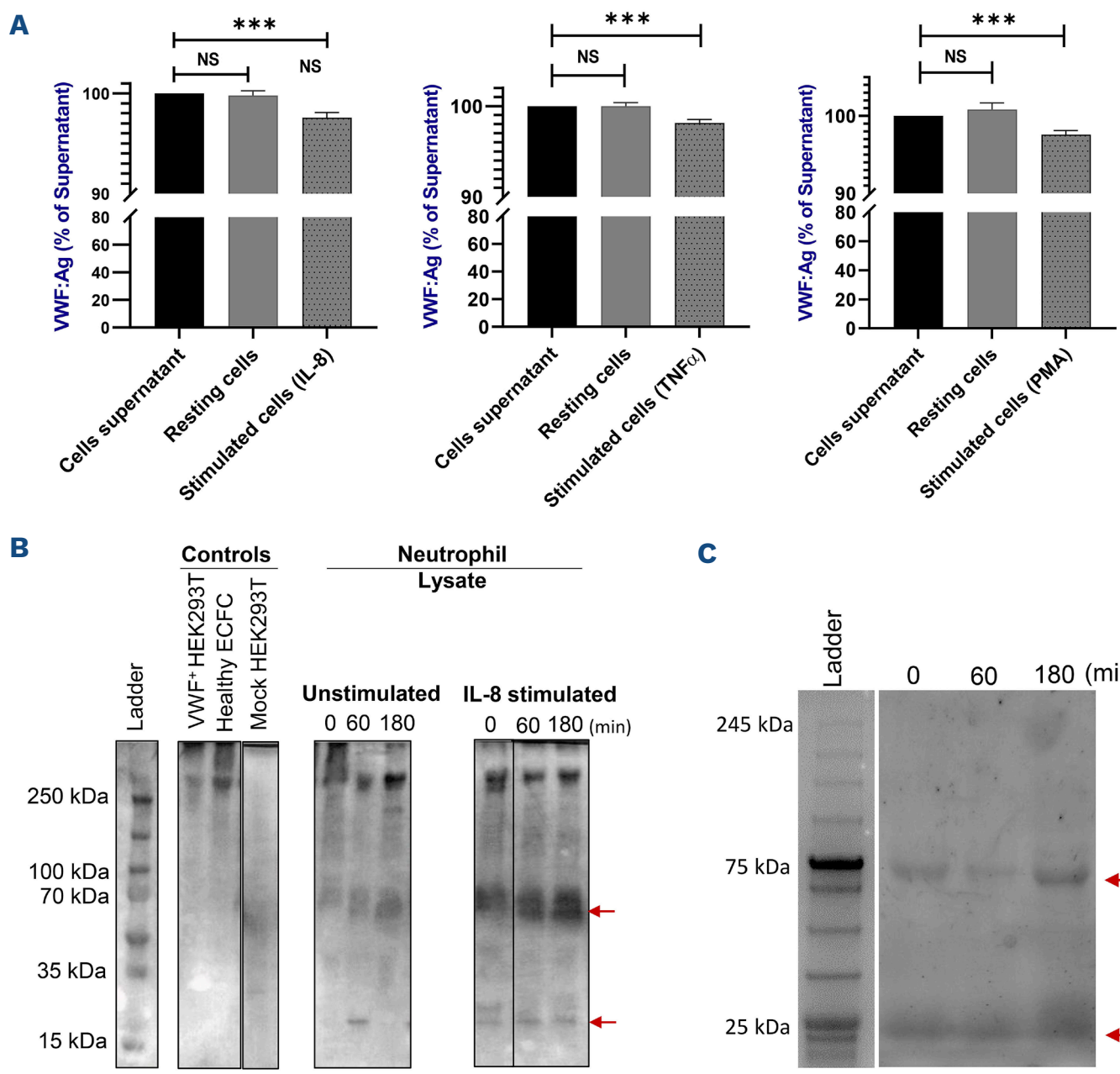

Figure 5. Evaluation of von Willebrand factor quantity in neutrophil supernatant and von Willebrand factor integrity in neutrophil lysates. (A) Comparison of von Willebrand factor antigen (VWF:Ag) levels in different supernatants to confirm VWF uptake by neutrophils. The left bar represents VWF:Ag value in supernatant without cells (negative control), set as 100%. The middle and right bars show mean values of VWF:Ag levels measured in supernatants of resting cells and cells stimulated with interleukin-8 (IL-8) (0.7 ng/mL), TNFα (5 ng/mL), and phorbol myristate acetate (PMA) (10 ng/mL) (N=6 for each), respectively. Statistical significance: ***$P \leq 0.001$; not significant (NS): $P > 0.05$. (B) Western blot analysis of cell lysates, including resting neutrophils and IL-8 (0.7 ng/mL)-stimulated neutrophils (incubated for 0, 60, and 180 minutes [min]), along with positive controls (lysate cells producing recombinant VWF, transfected HEK293T cells, and cells producing endogenous VWF, healthy endothelial colony-forming cells [ECFC]) and a negative control (mock HEK293T cells). Both resting and stimulated neutrophil lysates revealed degraded VWF fragments around 20 and 70 kDa in addition to intact full-length VWF (250 kDa). (C) Gel electrophoresis of lysates from neutrophils stimulated with IL-8 (0.7 ng/mL) in the presence of fluorescently labeled purified VWF. Cells were lysed either immediately after stimulation with IL-8 in the presence of labeled VWF or incubated for an additional 60 or 180 min.

Discussion

While VWF is widely recognized as a critical component in hemostasis, emerging evidence highlights its multifaceted involvement in vascular inflammation.[2,5,21] One proposed function of VWF in inflammation is its ability to recruit leukocytes to the site of injury when it is anchored onto the endothelium as bound VWF.[6,10] Bound VWF onto endothelium acts as an adhesive molecule, directly binding to leukocyte receptors (Mac-1 and P-selectin), facilitating leukocyte adhesion and migration. VWF also indirectly promotes the release of pro-inflammatory mediators by activating platelets.[22] VWF also influences the release of inflammatory modulators by triggering the biogenesis WBS

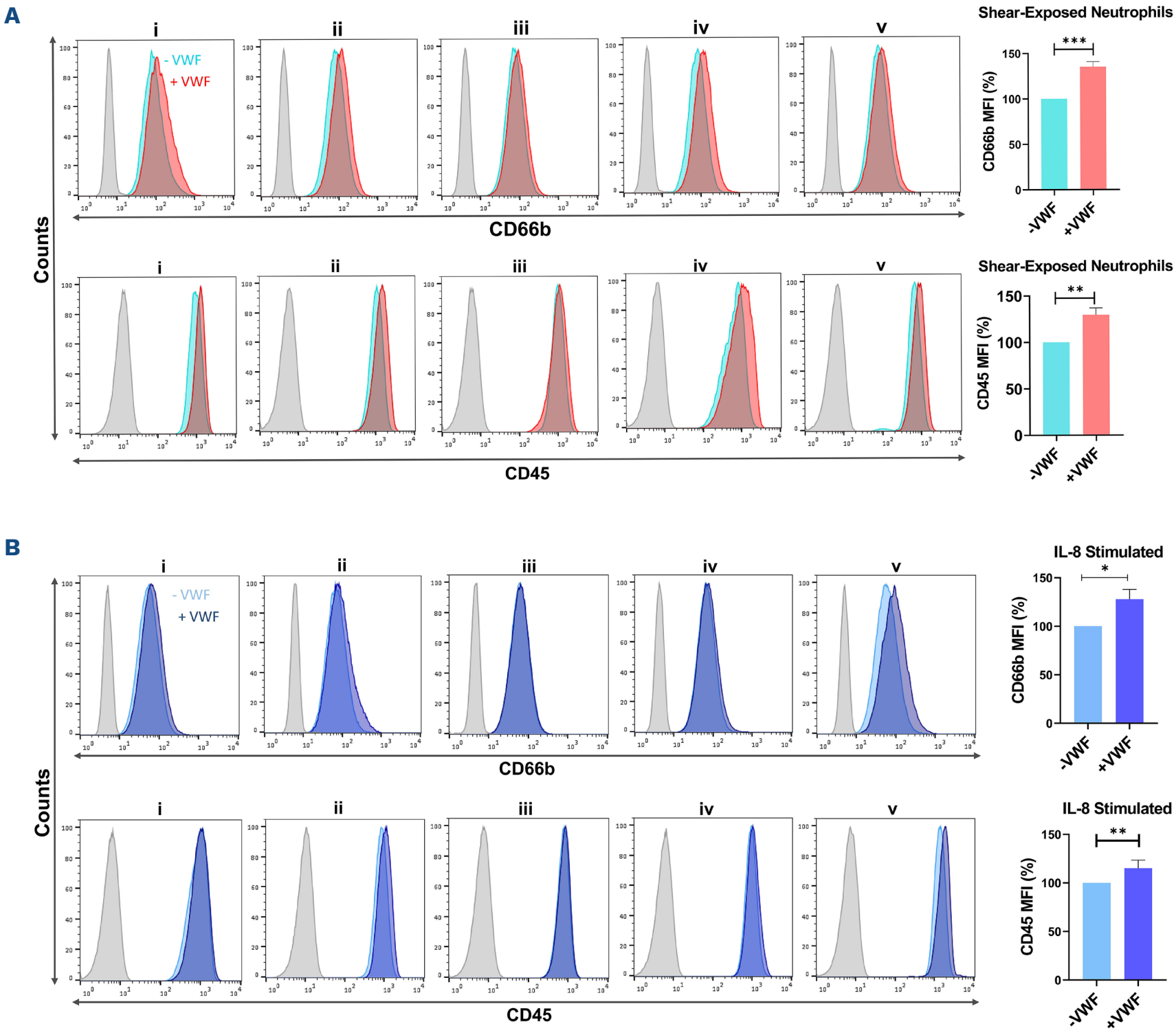

Figure 6. Effect of purified von Willebrand factor on neutrophils by evaluating the expression of surface markers. (A) Flow cytometry analysis demonstrates increased expression of surface markers CD66b (FITC) and CD45 (APC) on shear-exposed neutrophils in the presence of von Willebrand factor (VWF) compared to experiments without VWF. The histograms depict the expression levels of CD66b and CD45 on shear-exposed neutrophils isolated from 5 different healthy individuals (i-v) in the absence (light green) or presence of VWF (red), with isotype controls (grey) incorporated. The bar graph shows the CD66b and CD45 mean fluorescent intensity (MFI) in neutrophils exposed to shear flow in the absence or presence of VWF, determined from 5 different experiments. (B) Flow cytometry analysis shows increased expression of surface markers CD66b (FITC) and CD45 (APC) on IL-8-stimulated neutrophils in the presence of VWF compared to experiments without VWF. The histograms display the expression levels of CD66b and CD45 on stimulated neutrophils isolated from 5 different healthy individuals (i-v) in the absence (light blue) or presence of VWF (marine blue), incorporating isotype controls (grey). The bar graph shows the CD66b and CD45 MFI in neutrophils stimulated with interleukin-8 (IL-8) in the absence or presence of VWF, determined from 5 different experiments. Statistical significance: ****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

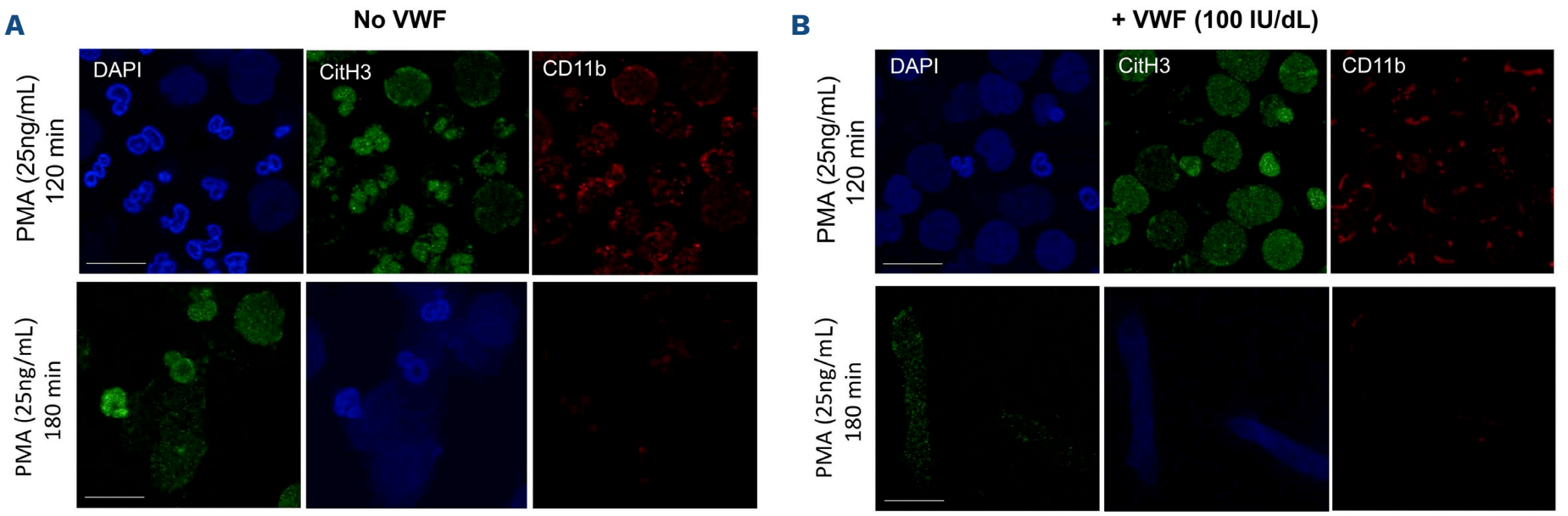

Figure 7. Effect of plasma-derived von Willebrand factor/coagulation factor VIII concentrate on neutrophil extracellular traps. Isolated neutrophils were stimulated with 25 ng/mL phorbol myristate acetate (PMA) for 120 and 180 minutes (min), in the presence or absence of plasma-derived von Willebrand factor/coagulation factor VIII (pdVWF/FVIII) concentrate (A, B). The figure presents results for these time points, reflecting the late stages of NETosis. At 120 min, neutrophils exhibit disintegrated nuclei and chromatin decondensation. By 180 min, the cells show characteristic NETosis features, including cloudy and shooting cells, indicating complete expulsion of DNA. After stimulation, cells were fixed and subjected to immunofluorescence staining using CitH3 antibody, CD11b, and DAPI to visualize neutrophil extracellular trap (NET) formation. Scale bar: 20 μm.

that contain cytokines, chemokines, and adhesion molecules.[7,14,23-26]

In our current study, we gained new insights into VWF's involvement in inflammation by investigating the interaction between plasma VWF (unbound) and neutrophils. We observed that unbound VWF interacts with neutrophils, resulting in enhanced expression of functional neutrophil surface proteins that indicate cell activation. We also demonstrated that neutrophils could internalize and degrade plasma VWF, suggesting their contribution to its removal from circulation. To our knowledge, our study is the first to investigate the interaction between plasma VWF and neutrophils under flow conditions, as well as in the presence of inflammatory molecules such as PMA, TNFα, and IL-8. Our analysis provides evidence that VWF binds to neutrophils through the Mac-1 receptor. Our findings complemented the previous work by Pendu *et al.*, who demonstrated the attachment of bound (onto endothelium) VWF to integrin receptors on the surface of PMN. We extended these findings by showing that unbound plasma VWF can also bind to integrin receptors. The β2 integrins are exclusively expressed on the surface of leukocytes, including neutrophils.[27] β2 integrins, particularly αMβ2 (Mac-1, CD11b/CD18), are crucial in the innate immune response, mediating interactions with endothelium, phagocytosis, degranulation, and cytokine production.[28-31] Integrins are typically found in an inactive state on the cell surface, but their activation occurs when agonists, such as chemokines or cytokines, bind to their receptors, triggering a switch to an active state.[28] Integrins exist as heterodimers, with the ability to adopt different conformations, each with distinct affinities for ligands: a low-affinity bent conformation, an intermediate-affinity extended-closed conformation, and a high-affinity extended-open conformation.[32,33] Recent research suggests that multiple integrin conformations can co-exist on the cell surface.[32] Additionally, mechanical forces exerted by blood flow can activate β2 integrins.[27] Our study revealed that a shear flow of 10 dynes/cm^2 resulted in the interaction between plasma VWF and neutrophils, and led to enhanced internalization. This shear flow value falls within the physiological range observed in arterial blood flow (10-20 dynes/cm^2).[34] These findings suggested that even under normal physiological conditions, plasma VWF can bind to and be internalized by neutrophils, potentially facilitated by shear flow-induced activation of β2 integrins. Furthermore, activating neutrophils with cytokines (e.g., IL8 and TNFα) enhanced the interaction between VWF and neutrophils, potentially further modulating this interaction. Neutrophils employ multiple endocytosis mechanisms to internalize proteins, including clathrin-mediated endocytosis.[35-38] Our study specifically investigated the internalization of VWF by neutrophils using this mechanism. This process involves the binding of protein to cell surface receptors, internalization of the protein into clathrin-coated vesicles, the fusion of the vesicles with early endosomes (marked by EEA1) or late endosomes (marked by Rab7), as well as lysosomes, resulting in protein degradation. Our findings revealed an increasing co-localization of VWF with EEA1 and Rab7 over time, indicating the uptake of VWF by neutrophils, which was further enhanced under flow conditions. While our study focused on clathrin-mediated endocytosis, it is important to acknowledge that neutrophils may also utilize other endocytosis mechanisms for VWF internalization. Further, we confirmed the capability of neutrophils to uptake VWF by conducting experiments to assess VWF antigen levels in the supernatant of both

resting and IL-8-stimulated neutrophils, as well as in supernatants without cells (used as a negative control). Significantly, we observed a decrease in VWF antigen levels in the supernatant of IL-8-activated neutrophils compared to the control groups. Furthermore, through protein analysis using western blotting, we demonstrated that VWF undergoes degradation upon binding to neutrophils. In this study, we observed VWF degradation products of 20 and 70 kDa, differing from the fragments produced by myeloid proteases as reported by Raife *et al.*[39] The discrepancy between our VWF degradation products and those reported by Raife *et al.* may stem from different experimental setups and the cumulative effect of multiple proteases in our cell lysates. Raife *et al.* examined each enzyme separately, while our approach reflects an aggregated impact. Future proteomic analysis will help clarify these differences and determine the sequence of VWF fragments produced.

Survival of VWF in circulation is approximately 12 hours, but the mechanisms regulating VWF clearance from plasma are not fully elucidated. It is well-established that macrophages significantly contribute to VWF removal from circulation. Recent studies have identified various cell types and surface receptors involved in VWF clearance, including sinusoidal endothelial endocytic receptors stabilin-2 and CLEC4M, the hepatocyte receptor ASGPR, and macrophage receptors LRP1 and Siglec-5.[29,40-52] Our current study provides evidence for an additional novel cellular mechanism, highlighting the potential role of neutrophils in the clearance of plasma VWF. This mechanism, although likely one of several, underscores the multifaceted nature of VWF clearance processes. It remains to be elucidated whether neutrophils contribute to steady-state VWF clearance or primarily act during inflammatory responses to modulate VWF levels and mitigate thrombotic risk. Neutrophils' capacity to internalize and process proteins like albumin and fibrinogen during inflammation underlines their potential role in plasma homeostasis.[53,54] Further studies are needed to confirm their specific involvement in VWF clearance. The specific VWF domains or motifs responsible for the binding of circulating VWF to neutrophils are not yet understood. Identifying these domains is a key area for future research. Additionally, we speculate that VWF variants observed in VWD patients might influence the interaction between VWF and neutrophils, potentially affecting VWF uptake. Moreover, the impact of a complete absence of VWF (type 3 VWD) on neutrophil behavior and function remains unclear and warrants further investigation. In our study, we also observed that activated neutrophils, whether exposed to shear forces or stimulated with IL-8, exhibited increased expression of surface receptors CD45 and CD66b in the presence of VWF compared to when VWF was absent in the cell suspension. This finding suggests that VWF binding to neutrophils may modulate neutrophil receptor expression, possibly through the regulation of inside-out signaling pathways. To further elucidate the mechanisms by which VWF binding to neutrophils affects downstream signaling pathways, we are currently performing whole-transcriptome RNA sequencingof neutrophils in our laboratory. This analysis will provide valuable insights into the alterations in gene expression and the underlying molecular mechanisms involved. CD45 and CD66b are receptors on neutrophils involved in immune cell activation, migration, adhesion, and effector functions.[55-59] Our study showing increased CD45 and CD66b expression on stimulated neutrophils in the presence of VWF suggests VWF's impact on neutrophil function and provided additional evidence for its role in inflammation. Furthermore, the current study demonstrated the impact of plasma VWF on promoting NET formation.

Notably, a recent study found that VWF binding to macrophages triggers downstream MAP kinase signaling, NF-κB activation, and the production of pro-inflammatory cytokines and chemokines, thereby promoting monocyte chemotaxis. This study demonstrated a significant role for VWF in modulating macrophage function.[60]

Considering our results demonstrating the impact of VWF-neutrophil interaction on neutrophil behaviour, this may have implications in inflammatory diseases and the pathogenesis of thrombosis. Among the few studies reporting the impact of VWF on inflammation, Hillgruber *et al.* showed massive accumulation of VWF in skin biopsies of patients suffering from immune complex (IC)-mediated vasculitis (ICV). To clarify the impact of VWF on cutaneous inflammation, they induced experimental ICV in mice treated with VWF-blocking antibodies or in $VWF^{-/-}$ mice. Interference with VWF led to significant inhibition of the cutaneous inflammatory response, highlighting VWF's role incutaneous neutrophil recruitment. Anti-VWF treatment successfully blocked cutaneous inflammation. Similarly, further investigations are needed to assess the impact of VWF-neutrophil interaction on other pathological conditions such as thrombosis.

In conclusion, our study offers novel insights into the interplay between free circulating plasma VWF and neutrophils, shedding light on the impact of this interaction on neutrophil functions and highlighting the role of VWF in inflammation. Additionally, the findings of our study demonstrate the involvement of neutrophils in the clearance of VWF from circulation, suggesting their potential significance in enhancing therapeutic VWF concentrate preparations with an extended half-life for the effective treatment of patients with VWD.

Disclosures

No conflicts of interest to disclose.

Contributions

AK performed the experimental work and wrote the paper. HY designed the study, supervised the study, interpreted the data, and wrote the paper. JM evaluated and interpreted flow cytometry data and edited the manuscript. NSH and SUR contributed with VWF purification and edited the manuscript. JO provided advice, evaluated data, reviewed and edited the manuscript.

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Data-sharing statement

For original data, please contact the corresponding author.

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