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Mediators and molecular pathways involved in the regulation of neutrophil extracellular trap formation mediated by activated platelets

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ABSTRACT

In addition to being key elements in hemostasis and thrombosis, platelets amplify neutrophil function. We aimed to gain further insight into the stimuli, mediators, molecular pathways, and regulation of neutrophil extracellular trap formation mediated by human platelets. Platelets stimulated by lipopolysaccharide, a wall component of gram-negative bacteria, Pam3-cysteine-serine-lysine 4, a mimetic of lipopeptide from gram-positive bacteria, *Escherichia coli*, *Staphylococcus aureus*, or physiologic platelet agonists promoting neutrophil extracellular trap formation and myeloperoxidase-associated DNA activity under static and flow conditions. Although P-selectin or glycoprotein IIb/IIIa were not involved, platelet glycoprotein Ib, neutrophil cluster of differentiation 18, and the release of von Willebrand factor and platelet factor 4 seemed to be critical for the formation of neutrophil extracellular traps. The secretion of these molecules depended on thromboxane A_2 production triggered by lipopolysaccharide or Pam3-cysteine-serine-lysine 4 but not on high concentrations of thrombin. Accordingly, aspirin selectively inhibited platelet-mediated neutrophil extracellular trap generation. Signaling through extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, and Src kinases, but not p38 or reduced nicotinamide adenine dinucleotide phosphate oxidase, was involved in platelet-triggered neutrophil extracellular trap release. Platelet-mediated neutrophil extracellular trap formation was inhibited by prostacyclin. Our results support a role for stimulated platelets in promoting neutrophil extracellular trap formation, reveal that an endothelium-derived molecule contributes to limiting neutrophil extracellular trap formation, and highlight platelet inhibition as a potential target for controlling neutrophil extracellular trap cell death. ***J. Leukoc. Biol.*** **99: 000–000; 2016.**

Introduction

NETs are webs of histone-modified nuclear material and proteins that include elastase and MPO extruded from activated neutrophils during the inflammatory response [1]. NETs exhibit antimicrobial functions by trapping and killing extracellular pathogens in the blood and tissues during infection [1–3]. NETs were found in preeclampsia [4], small-vessel vasculitis [5], systemic lupus erythematosus [6], and thrombosis [7], suggesting that they may also have a role in the pathogenesis of several noninfectious inflammatory diseases.

Platelet–neutrophil interactions are critical to NET induction during sepsis mediated by gram-negative bacteria [8, 9]. NETs released into the vasculature ensnare bacteria from the bloodstream and prevent dissemination, a phenomenon that is decreased after platelet depletion or the disruption of integrin-mediated platelet–neutrophil binding [8]. Platelets also seem to be relevant mediators of NETs both during bacterial infections and in sterile inflammatory conditions, such as in different experimental models of acute lung injury [10, 11]. Moreover, human thrombi of patients with myocardial infarction mainly consist of activated platelets, neutrophils, and NETs in close proximity to platelets [12]. In addition, human platelets stimulated with TRAP or ADP have been shown to induce NETs, suggesting that classic agonists of platelet activation might also trigger NET production [10, 12].

Abbreviations: AA = arachidonic acid, ASA = acetylsalicylic acid, HNE = human neutrophil elastase, LT = leukotriene, MPO = myeloperoxidase, NET = neutrophil extracellular trap, NETosis = NET cell death, Pam3CSK4 = Pam3-Cys-Ser-Lys4, PF4 = platelet factor 4, PGI_2 = prostacyclin, PI = propidium iodide, PSGL-1 = P-selectin glycoprotein ligand 1, ROS = reactive oxygen species, TMB = tetramethylbenzidine, TRAP = thrombin receptor activating peptide, TX = thromboxane, vWF = von Willebrand Factor

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The mediators and molecular pathways involved in human platelet-mediated NET generation remain poorly defined. In this sense, engagement between adherent neutrophils and activated platelets (stimulated by LPS or plasma from septic patients) was shown to initiate NET release through β2-integrin–mediated platelet–neutrophil engagement [8] and requires platelet TXA_2 formation and MEK signaling in neutrophils [10]. In addition, activated platelets present high mobility group box 1 protein to neutrophils and commit them to autophagy and NET generation [12].

In this study, we aimed to gain a deeper insight into the stimuli, mediators, and molecular pathways involved in the production of NETs mediated by human platelets.

MATERIALS AND METHODS

Reagents

ASA, purified LPS derived from *Escherichia coli* O111:B4, AA, ADP, PI, thymus DNA, diphenyl iodonium, LT-B4, poly-L-lysine, and dihydrorhodamine 123 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pam3CSK4 was obtained from InvivoGen (San Diego, CA, USA). TMB was from Thermo Scientific (Waltham, MA, USA). Micrococcal nuclease was purchased from Roche Diagnostics (Mannheim, Germany). PGI_2 and a TXB_2 ELISA kit were obtained from Cayman Chemical (Ann Arbor, MI, USA). A human CXCL4/PF4 ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). TO-PRO 3, SYBR Gold, anti-rabbit Alexa 488, and anti-rabbit Alexa 546 Abs were obtained from Invitrogen (Carlsbad, CA, USA). Recombinant human PF4 and Abs against human PF4 were obtained from PeproTech (Rocky Hill, NJ, USA). Myeloperoxidase, rabbit anti-HNE Abs, and LTC4 were obtained from Calbiochem–Merck Millipore (Darmstadt, Germany). mAbs against CD18–FITC and unlabeled vWF were obtained from Immunotech (Marseille, France). The mAb against MPO-FITC was from BioLegend (San Diego, CA, USA). Blocking Abs against human TLR2 (clone T2.5) and TLR4 (HTA125) were purchased from eBioscience (San Diego, CA, USA). The mouse mAb anti-human P-selectin (CLB/thromb/6) and eptifibatide were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CD42b (GPIb) blocking Abs, FITC, and unlabeled irrelevant immunoglobulin IgG1 were purchased from BD Biosciences (San José, CA, USA). Rabbit anti-human vWF was obtained from Dako (Glostrup, Denmark, and Carpinteria, CA, USA). Inhibitors (U 0126, Ly 294002, SB 203580, and PP1) were purchased from Biomol (Plymouth Meeting, PA, USA).

Bacterial culture and preparation

Escherichia coli DH5 α and *Staphylococcus aureus* bacteria were purchased from Invitrogen and American Type Cell Culture 6538, respectively. The bacteria were cultured at 37°C under aerobic conditions in Luria Bertani (*E. coli*) or medium composed of tryptone, yeast extract, and sodium chloride (*S. aureus*).

Isolation of neutrophils

Neutrophils were isolated from the peripheral blood of healthy donors as previously described [13]. This study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and approved by the institutional ethics committee. All patients provided written, informed consent for the sample collection and subsequent analysis. Cell suspensions contained >98% neutrophils and 10–15% of $CD61^+$ cells. The cells were resuspended in RPMI 1640 supplemented with FBS (0.5%).

Preparation of platelets

Blood was drawn directly into plastic tubes containing 3.8% sodium citrate. Platelets were isolated as previously described [14] and were resuspended in RPMI 1640 supplemented with FBS (1%) at 2.4×10^8/ml. Platelets were incubated with LPS, Pam3CSK4, *E. coli*, or *S. aureus* bacteria or the indicated platelet agonist for 30 min at 37°C and then transferred to neutrophils.

NET formation assay under static conditions

In all experiments, NET formation was visualized by immunofluorescence microscopy and quantified by evaluation of the DNA released in the supernatants by fluorometry or ELISA.

Immunofluorescence assays for static conditions

Nonstimulated or activated platelets (as described above) were transferred to neutrophils (5×10^5/ml) adhered on poly-L-lysine–treated coverslips and placed in 24-well, flat-bottom plates in a humidified incubator at 37°C with CO_2 (5%) for 1 h [10]. Then, the cells were fixed with paraformaldehyde (1%), permeabilized with 0.1%Triton, and blocked with goat serum. Cells were then stained with mouse anti-human MPO–FITC Abs (1:20) and rabbit anti-HNE (1:1000) or the corresponding IgG controls. For HNE staining Alexa Fluor 546–labeled goat anti-rabbit secondary Abs, DNA was stained with TO-PRO 3. Mounted specimens were analyzed by confocal fluorescence microscopy using a FV-1000 microscope (Olympus, Tokyo, Japan) equipped with a Plapon ×60/NA1.42 objective.

Quantification of extracellular DNA

Nonstimulated or activated platelets were transferred to adhered neutrophils in 24-well, flat-bottom plates without coverslips. The plates were placed in a humidified incubator at 37°C with CO_2 (5%) for 1 h. DNA released from neutrophils during NETosis was digested with micrococcal nuclease (500 mU/ml) for 15 min. EDTA (5 mM) was added to stop the nuclease activity [15]. Supernatants were collected, centrifuged and DNA was measured in the supernatants using SYBR Gold in a fluorometer (BioTek Instruments, Winooski, VT, USA). The calibration curve was constructed using thymus DNA of a known concentration.

HNE–DNA complexes ELISA

Quantification of HNE–DNA complexes were performed as previously described [10]. Briefly, 96-well plates were coated with 5 μg/ml anti-HNE Abs overnight at 4°C. After washing 3 times, 20 μl of sample was added to the wells with 80 μl incubation buffer containing a peroxidase-labeled anti-DNA mAb (Cell Death ELISAPLUS, Roche Diagnostics, dilution 1:25). The plate was then incubated for 2 h, shaken at 300 rpm at room temperature. After 3 washes, 100 μl of peroxidase substrate (ABTS; Thermo Scientific) was added. Absorbance at 405 nm wavelength was measured after 20 min incubation at room temperature in the dark. Values for soluble HNE–DNA complexes were expressed as their fold increase in absorbance above the control.

MPO activity

After NET induction, medium was removed and then NETs were digested with micrococcal nuclease, as described above. MPO activity was measured in supernatants by adding TMB, and absorbance was read at 450 nm after stopping the reaction with sulfuric acid. The calibration curve was constructed using purified MPO of a known concentration [16].

NET formation in a flow chamber assay

Evaluation of NET formation under flow was evaluated as previously described [9]. Briefly, neutrophils (1×10^7/ml) were loaded onto a plasma-coated chamber slide and allowed to adhere at 37°C in a CO_2 (5%) atmosphere for 45 min. After removing plasma, different concentrations of resting or activated platelets were warmed at 37°C for 30 min before perfusing through the flow chamber (GlycoTech, Gaithersburg, MD, USA) at 10 μl/min (shear force, 1 dyne/cm^2) for 5 min.

Immunofluorescence assays for flow conditions

The cells were fixed with 1% paraformaldehyde, blocked, stained with an anti-HNE Ab or the corresponding IgG control, washed, stained with a secondary Ab and PI (2 μg/ml), and mounted on slides. Images for NET evaluation were taken and analyzed by confocal fluorescence microscopy.

Measurement of TXB_2, vWF, and PF4 release

After platelet stimulation, the reaction was stopped by the addition of ice-cold EDTA (2 mM) and ASA (500 μM) for TXB_2 detection and with PGI_2 (75 nM) for PF4 and vWF. The samples were centrifuged, and the supernatants were stored at −80°C until assayed. TXB_2, vWF, and PF4 levels in the supernatants were measured using commercial ELISA kits for TXB_2 and PF4 and as previously described for vWF [14].

Flow cytometry studies

For CD18 expression, neutrophils were stimulated for 60 min, fixed, and stained with FITC-conjugated CD18 or IgG1. For extracellular vWF, stimulated platelets were incubated with an Ab against vWF or IgG1 and then stained with a secondary Ab conjugated with Alexa 488. For ROS generation, neutrophils were stimulated and incubated with dihydrorhodamine (5 μM) for 60 min at 37°C. The samples were analyzed on a FACSCalibur flow cytometer using Cellquest software (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

The results are expressed as means ± SEM and were analyzed by 1-way ANOVA followed by the Newman-Keuls multiple comparison test to determine significant differences between groups. P values <0.05 were considered statistically significant.

RESULTS

Platelet activation by LPS or Pam3CSK4 triggers NET formation

The contribution of platelets to NETosis triggered by bacteria has been previously inferred using human platelets stimulated with LPS, a TLR4 agonist and a major component of the gram-negative bacteria cellular wall [8, 9, 12]. Because there are no reports of the role of gram-positive bacteria in platelet-mediated NETosis, we analyzed the effect of Pam3CSK4, a TLR1/TLR2 agonist and a synthetic mimetic of the main lipopeptide wall component of gram-positive bacteria [17]. Confocal microscopy studies showed that the addition of nonstimulated platelets to the neutrophils did not trigger NET formation (**Fig. 1A**). However, platelets that were prestimulated with LPS or Pam3CSK4 induced the generation of NETs (Fig. 1A). To discriminate between the contribution of TLR-activated platelets and the direct effect of TLR agonists on neutrophils, we stimulated neutrophils with similar concentrations of each compound in the absence of platelets. As shown in Figure 1B and C, both LPS and Pam3CSK4 induced NETs in a concentration-dependent manner; however, the NET quantity was significantly lower than those generated in the presence of platelets (Fig. 1B and C). Of note, in agreement with previous reports [10], around 25% of the donor samples failed to produce NETs upon neutrophil stimulation without platelets. The observation that similar results were obtained when HNE–DNA complexes (which are only present in NETs) were evaluated (Fig. 1D), confirmed that the release of extracellular DNA was associated with NET formation and not with any other possible cell death mechanisms. Moreover, morphologic analysis of neutrophils labeled with PI or acridine orange, showed that neither platelets stimulated with LPS nor those stimulated with Pam3CSK4 induced neutrophil apoptosis or necrosis (data not shown). MPO that decorates NETs retains its enzymatic activity [16]. To evaluate whether platelet-mediated NETosis also influences MPO activity, the enzymatic activity was measured after micrococcal nuclease digestion of NETs using a standard TMB peroxidase activity assay. Figure 1E shows that, although neutrophil stimulation with LPS or Pam3CSK4 increased the MPO activity compared with basal levels, it was greater in the presence of stimulated platelets.

When platelets were preincubated with a blocking anti-TLR4 or anti-TLR2 Ab, washed, and then stimulated with the TLR ligands, the amount of released DNA was reduced to those observed with nonstimulated platelets (Fig. 1F), confirming a specific response mediated by the interaction of LPS and Pam3CSK4 with platelet TLR4 and TLR2, respectively. Control IgG did not modify NET release.

To determine whether potentiation mediated by platelets was specific for platelets activated by the cell wall components or whether it was also triggered by the bacteria, platelets were stimulated with different concentrations of *E. coli* and *S. aureus*, as a gram-negative and gram-positive bacteria, respectively. Similar to LPS and Pam3CSK4, both bacteria induced NET formation, and it was potentiated in the presence of platelets (Fig. 1G and H).

Using a flow chamber system, we observed that the perfusion of nonstimulated platelets did not result in NETs (**Fig. 2A**). In contrast, LPS- or Pam3CSK4-treated platelets avidly induce the release of DNA (Fig. 2A). Again, the direct stimulation of neutrophils with LPS or Pam3CSK4 without platelets triggered a reduced degree of NETosis (Fig. 2A). Interestingly, although NETosis was observed after 5 min of platelets perfusion, in the static model, neutrophils required at least 30 min of incubation with stimulated platelets to release DNA (Fig. 2B). NET generation was induced in a platelet number–dependent manner (Fig. 2C). Although in the static model, a minimum of 1.25×10^8 platelets/ml was required to observe a potentiated effect, under flow conditions, that number decreased to 0.1×10^8 platelets/ml (Fig. 2D).

Together, these findings indicate that platelets significantly promote NET formation in response to TLR4 and TLR2 activation by gram-negative and gram-positive bacteria or their wall components.

Platelet activation by classic platelet agonists also induces NET formation

NETs develop not only during sepsis but also during sterile inflammation, conditions in which several platelet agonists are synthesized, released, or both [4–7]. Thus, we next explored the effect of human platelet activation by physiologic agonists in NET generation. The stimulation of platelets with ADP, collagen, thrombin, or AA promoted NET formation (**Fig. 3A**). Remarkably, among all of the agonists, AA was the strongest stimulus and the only one that triggered NETs without platelets by direct neutrophil activation (Fig. 3A). The metabolism of AA in leukocytes occurs mainly through the lipoxygenase enzyme

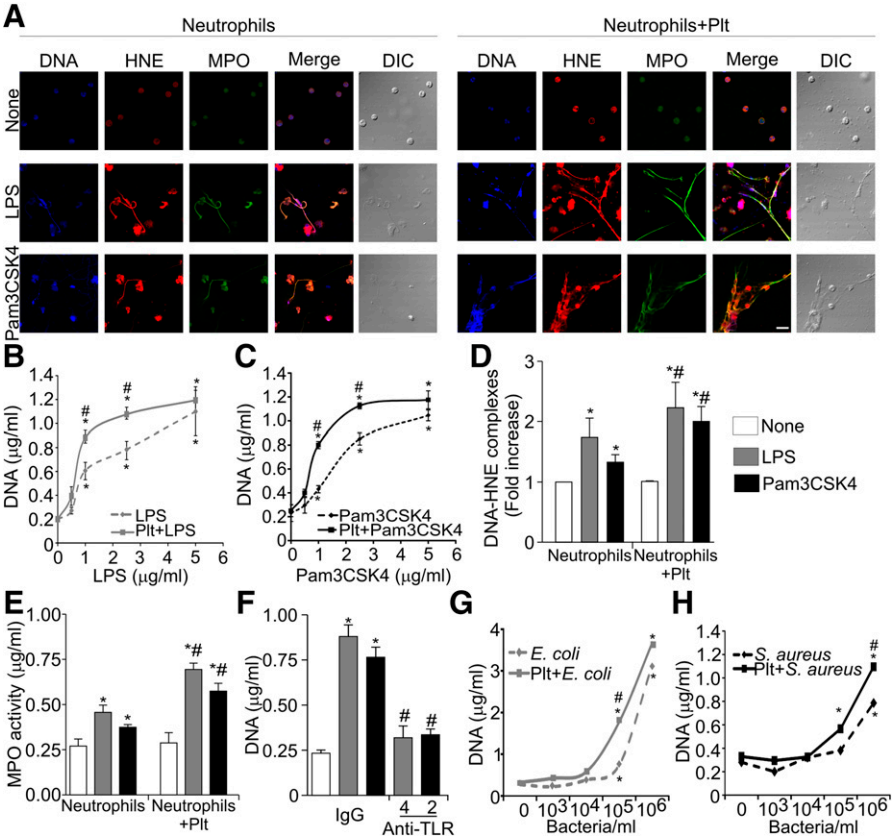

Figure 1. NET formation triggered by platelets. (A) Neutrophils (5×10^5/ml) were incubated for 60 min with platelets (Plt) (2.4×10^8/ml) that were prestimulated with LPS (1 μg/ml) or Pam3CSK4 (1 μg/ml) for 30 min. The cells were then fixed; permeabilized; stained with TO-PRO 3 for DNA (blue), the specific markers anti-human neutrophil elastase (HNE, red), and anti-MPO (green); and visualized by confocal fluorescence microscopy ($n = 10$). Original magnification, ×60 (scale bar, 20 μm). Neutrophils were incubated with different concentrations of LPS (B) or Pam3CSK4 (C), and then, DNA was quantified in the supernatants by fluorometry ($n = 10$). HNE–DNA complexes (D) and MPO activity (E) were measured in supernatants. (F) Plt (2.4×10^8/ml) were preincubated with anti-TLR4 or 2 Abs and stimulated with LPS (1 μg/ml) or Pam3CSK4 (1 μg/ml), and then, DNA was quantified in the supernatants by fluorometry ($n = 4$). (G and H) Neutrophils were incubated with *E. coli* (G) or *S. aureus* stimulated Plt (H), and then, DNA was quantified in the supernatants by fluorometry ($n = 4$). $^*P < 0.05$ vs. none. $\#P < 0.05$ vs. LPS, Pam3CSK4, *E. coli*, or *S. aureus* without Plt or IgG of the same agonist.

that leads to the generation of LTs [18]. For this reason, we analyzed the effect of LTB4 and LTC4 on NETosis. We found that LTB4 is a potent inducer of NETs, whereas LTC4 failed to trigger NETs (Fig. 3B). Moreover, NET induction by AA or LTB4 was suppressed by the pretreatment of neutrophils with Ly29311, an antagonist of the LTB4 receptor, which strongly supports the role of LTB4 in NETosis (Fig. 3B). Moreover, activation of neutrophils with AA increased NET-associated MPO activity and HNE–DNA complexes, and it was further increased in presence of platelets (Fig. 3C and D).

Although the perfusion of AA over immobilized neutrophils induced NET formation under flow conditions, the amount of released DNA using AA-stimulated platelets was greater (Fig. 3E). Together, these data indicate that NET generation is promoted after human platelet activation induced by TLR2 and TLR4 agonists and by physiologic platelet agonists under static or flow conditions.

Platelet and neutrophil membrane receptors involved in NET formation

We next studied whether platelet-induced NETosis was mediated by soluble or membrane factors. For this purpose, platelets stimulated with LPS, Pam3CSK4, or AA were centrifuged, and then neutrophils were stimulated with platelet supernatants. **Figure 4A** shows that although the supernatants of the stimulated platelets promoted NETs, it was to a lesser degree than the NETosis induced by whole platelet suspensions (supernatant and cells together). Interestingly, stimulated platelets without the supernatants (centrifuged after stimulation and resuspended in RPMI 1640) also induced NETs, indicating that both soluble and membrane-bound platelet components are required for NET generation.

To analyze the contribution of cell–cell interactions, we first evaluated the role of P-selectin, the main platelet cell adhesion molecule that mediates neutrophil–platelet interactions [19]. When stimulated platelets pretreated with the anti-P-selectin Abs were added to the neutrophils, the NET quantity was similar to that of the samples without Abs, indicating that P-selectin does not mediate platelet-induced NETs (Fig. 4B). However, this Ab effectively suppressed the formation of thrombin-induced platelet–neutrophil heterotypic aggregates (data not shown).

GPIIb/IIIa and GPIb/V/IX are the major platelet surface receptors involved in platelet activation. Blocking GPIIb/IIIa with the integrin antagonist eptifibatide did not modify NET generation. However, the preincubation of platelets with a blocking Ab against GPIb significantly decreased NET formation triggered by LPS, Pam3CSK4, or AA (Fig. 4B). Because it was reported that platelets bind to neutrophils in response to LPS or TRAP via β_2-integrin (CD18) [8, 11], we also analyzed the participation of this integrin. We found that neutrophil activation by LPS, Pam3CSK4, or AA augmented CD18 expression on the neutrophil membrane surface (Fig. 4C). Additionally, the blockade of this integrin significantly inhibited the release of DNA triggered by platelets stimulated by any agonist (Fig. 4D). Thus, GPIb on platelets and CD18 on neutrophils appear to be key receptors involved in platelet-mediated NETosis.

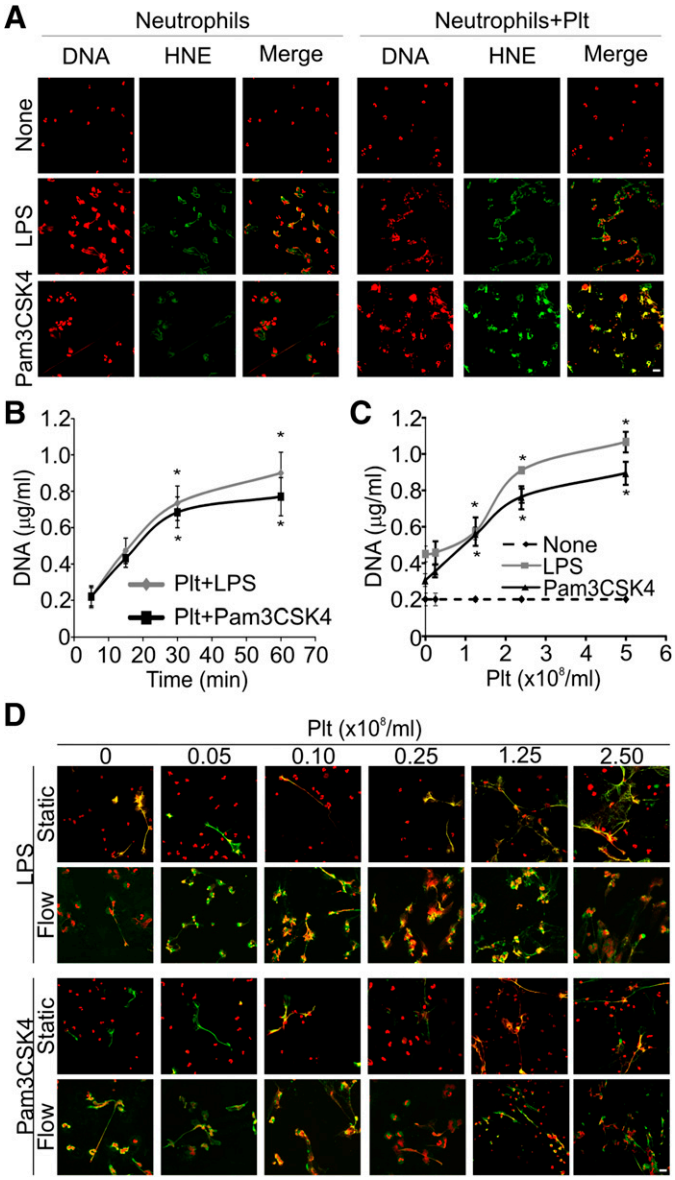

Figure 2. Platelet-mediated NET formation under static and flow conditions. (A) NETs were formed under shear forces in vitro with LPS- or Pam3CSK4-stimulated platelets (Plt). The cells were fixed, stained, and visualized by confocal fluorescence microscopy ($n = 4$). (B) LPS- or Pam3CSK4-stimulated Plt were incubated with neutrophils under static conditions for different times, and then, DNA was quantified in the supernatants by fluorometry ($n = 4$). (C and D) Different platelet concentrations were prestimulated with LPS or Pam3CSK4 and incubated with neutrophils. The DNA released under static conditions was measured by fluorometry (C), and NETs formed under both static and flow conditions were visualized by confocal microscopy (D). Original magnification, ×60 (scale bar, 20 μm) ($n = 4$). *$P < 0.05$ vs. 5 min or without Plt.

Molecules involved in platelet-mediated NET formation

Having demonstrated that supernatants from LPS-, Pam3CSK4-, or AA-stimulated platelets generated NETs, we next aimed to identify which mediators were involved. Because GPIb appeared to be required for platelets to trigger NETosis (Fig. 4B), we examined the role of vWF, the main ligand of this GP [20]. Platelet activation by all 3 platelet agonists resulted in vWF release and its binding to the platelet membrane (**Fig. 5A** and **B**). In addition, the blocking of vWF markedly reduced platelet-mediated NET formation (Fig. 5C), further suggesting a role of vWF in this process.

After activation, platelets express or secrete a wide variety of molecules that can modulate neutrophil responses. Among them, PF4 both activates leukocytes and exerts antimicrobial activity [21]. The stimulation of platelets with LPS, Pam3CSK4, or AA resulted in PF4 release (Fig. 5D). DNA release was markedly prevented after PF4 blocking (Fig. 5E), whereas the stimulation of neutrophils with human recombinant PF4 resulted in NET generation (Fig. 5F). Taking together, these results strongly suggest an important role for PF4 in NET generation.

Considering that the generation of TXA_2 mediates NET formation triggered by LPS-activated platelets [10], we next confirmed and extended the effect of TXA_2 on NET formation mediated by LPS-, Pam3CSK4-, and AA-stimulated platelets. The activation of platelets with each of these molecules resulted in the generation of TXB_2, the stable metabolite of TXA_2 (Fig. 5G). In addition, when platelets were preincubated with ASA (an inhibitor of TXA_2 generation), NET formation was suppressed (Fig. 5H). To our knowledge, the presence of TXA_2 receptor in neutrophils has not been described previously. Thus, it is conceivable that TXA_2 produced by platelets induces the release of other mediators responsible for triggering NETs. To address this hypothesis, we next measured the release of vWF and PF4 in platelets preincubated with ASA. As shown in Figures 5I and J, ASA completely abrogated the vWF and PF4 release by LPS-, Pam3CSK4-, or AA-activated platelets. In contrast, the stimulation of ASA-treated platelets with a high thrombin concentration (1 U/ml), which triggers activation independent of TXA_2, resulted in the release of DNA (0.65 ± 0.09 μg/ml), vWF, and PF4 (2.5 ± 0.12- and 2.7 ± 0.3-fold increase vs. control, respectively; $n = 3$).

Signaling pathways involved in platelet-mediated NET formation

Although the molecular bases governing NETs are still not completely elucidated, activation of the NADPH oxidase and the RAF/MEK/ERK axis appears to be relevant for PMA-induced NET formation [22, 23]. To determine whether these transduction signals were also involved in platelet-mediated NETosis, we initially evaluated ROS formation (signaling downstream NADPH activation). Although platelets stimulated with LPS, Pam3CSK4, or AA failed to trigger ROS, a moderate but significant increase in ROS levels was observed in neutrophils activated by TLR ligands or AA (**Fig. 6A**). However, the preincubation of both cell types with diphenyl iodonium, an inhibitor of NADPH oxidase, did not modify the DNA release (Fig. 6B–D), suggesting that ROS are not involved in platelet-triggered NETosis. On the other hand, the individual or combined treatment of neutrophils or platelets with U0126, a specific inhibitor of ERK, reduced NET production (Fig. 6B–D). A similar degree of inhibition was also observed when both cell types were preincubated with PP1, an inhibitor of Src

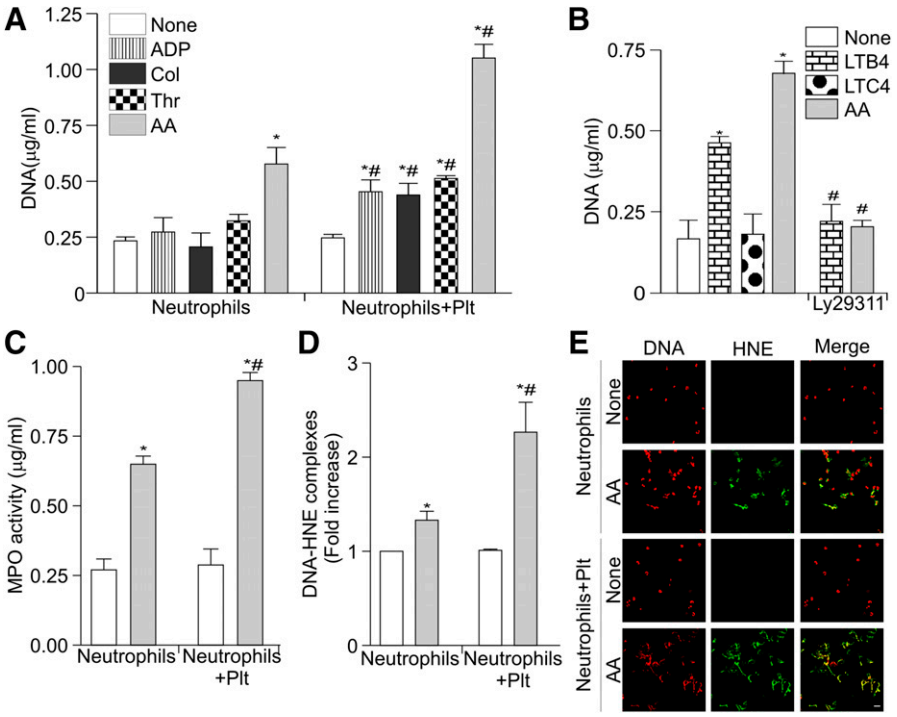

Figure 3. Platelet activation by classic platelet agonists induces NET formation. (A) Neutrophils (5×10^5/ml) were incubated for 60 min with platelets (Plt) (2.4×10^8/ml) that were prestimulated with ADP (20 μM), collagen (col; 10 μg/ml), thrombin (Thr; 0.05 U/ml), or AA (100 μM) for 30 min. or with leukotriene (LT)B4 (10^{-6} M) or C4 (10^{-6} M) in the presence or absence of the LTB4 antagonist Ly29311 (10^{-6} M) (B). DNA was quantified in the supernatants by fluorometry ($n = 10$). Neutrophils were incubated with AA-stimulated Plt and NET-associated MPO activity (C) and DNA–HNE complexes (D) were measured in supernatants. (E) NET formation occurred under shear forces in vitro with AA-stimulated Plt. The cells were fixed and stained with PI for DNA (red) and the specific marker anti-HNE (green) and were visualized by confocal fluorescence microscopy. Original magnification, ×60 (scale bar, 20 μm) ($n = 4$). *$P < 0.05$ vs. none. #$P < 0.05$ vs. same agonist without Plt or AA or LTB4 without Ly29311.

pathways (Fig. 6B–D). The PI3K pathway is involved only in neutrophils because preincubation of neutrophils but not platelets with Ly294002 significantly reduced NET formation (Fig. 6B–D). Interestingly, the blockade of P38 in any cellular type did not show significant effects (Fig. 6B–D). Thus, signaling through ERK, PI3K, and Src kinases, but not P38 or NADPH, participates in platelet-triggered NET release.

Effect of endothelial-derived platelet inhibitor on platelet-triggered NET formation

The endothelium is the cellular substrate for vascular NET formation [8, 24]. Because an endothelial-derived molecule, such as PGI_2, physiologically regulates platelet activation [25], we evaluated the effect of this molecule on platelet-mediated NETosis. Platelets were preincubated with PGI_2, before activation with LPS, Pam3CSK4, or AA. The pretreatment of platelets with this inhibitor resulted in a marked decrease of NET formation under static (**Fig. 7A**) or flow conditions (Fig. 7B), highlighting the role of this physiologic platelet activation modulator on platelet-triggered NETosis.

DISCUSSION

In this study, we extended the knowledge about the role of platelets in NET formation. Human platelet activation with components of both gram-negative (LPS) and gram-positive (Pam3CSK4) bacteria results in NET production via binding to the TLR4 and TLR2 receptors, respectively. Moreover, activation of platelets with *E. coli* or *S. aureus* also promoted NET generation. Platelet-mediated NET formation occurs under static or flow conditions. In accordance with a more-physiologic setting, the time for NET formation, as well as the number of platelets, under flow conditions was less than that required under static conditions. Platelet-mediated NETosis seems not to be restricted to platelet activation by bacteria because TRAP stimulation also resulted in the release of neutrophil DNA [10]. In addition, while this manuscript was in preparation, Maugeri et al. [12] showed that collagen or ADP platelet challenge resulted in NET formation. Our results are in agreement with those observations and further show that platelet stimulation with AA also triggered NETosis. Moreover, among all of the platelet agonists, including LPS and Pam3CSK4, AA was the most potent.

The observation that stimulated platelets or their supernatants triggered NET formation indicated that platelet membrane surface and soluble components are able to induce NETosis. The interaction of platelets with neutrophils has been attributed mainly to the cross-talk between P-selectin and its counter-receptor PSGL-1 [19]. Platelet stimulation by Pam3CSK4 or AA results in the expression of P-selectin [14]. However, controversial results have been shown regarding the exposure of this molecule triggered by LPS [14, 26]. We now show that blocking P-selectin did not modify NET production. Similar findings were reported by Maugeri et al. [12]. Moreover, they also showed that recombinant P-selectin was not able to induce NETs. These data indicate that NETosis triggered by platelets, either by bacterial components or platelet agonists, is a P-selectin–independent phenomenon. In contrast, NET formation in mice appears to be dependent on the presence of P-selectin in platelets and neutrophil PSGL-1 or Mac-1 [27, 28]. The differences regarding the role of P-selectin and PSGL-1 could be associated with an in vivo effect of P-selectin, not included in our study, or to the different species. We also found that platelet-stimulated NET formation depends on the platelet GPIb and the

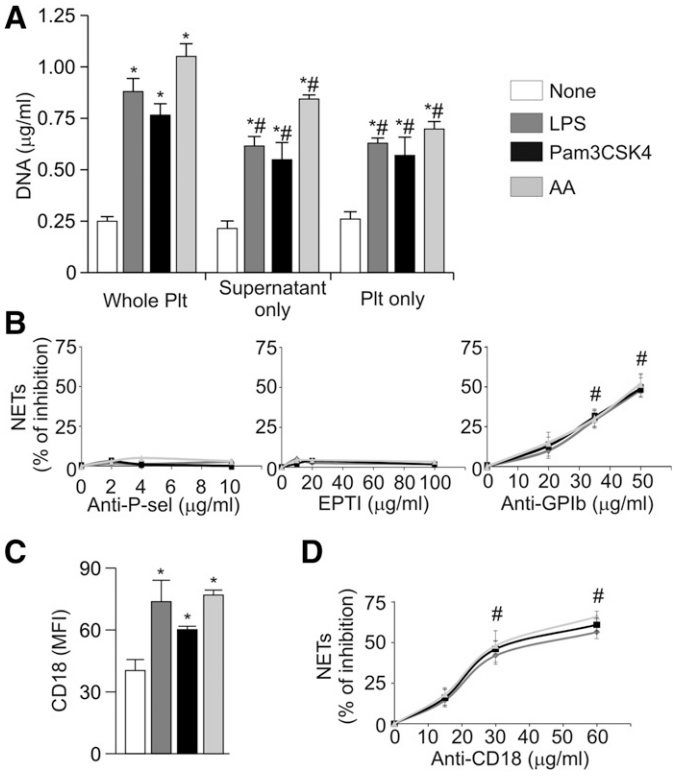

Figure 4. Receptors involved in platelet-mediated NET formation. (A) Platelets (Plt; 2.4×10^8/ml) were stimulated with LPS (1 μg/ml), Pam3CSK4 (1 μg/ml), or AA (100 μM) for 30 min, and then, whole Plt (Plt + supernatant), supernatant only, or Plt only (without supernatant) were coincubated with neutrophils (5×10^5/ml) for 60 min, and DNA was quantified in the supernatants by fluorometry ($n = 4$). (B) Plt were preincubated with the corresponding IgG control, anti–P-selectin (anti-P-sel), anti-GPIb Ab, or eptifibatide (EPTI); washed; stimulated with LPS (1 μg/ml), Pam3CSK4 (1 μg/ml), or AA (100 μM); and coincubated with neutrophils for 60 min, and then, DNA was quantified in the supernatants ($n = 4$). (C) Neutrophils were stimulated with LPS (1 μg/ml), Pam3CSK4 (1 μg/ml), or AA (100 μM), and then CD18 expression was measured by flow cytometry. (D) Neutrophils were preincubated with the corresponding IgG control or an anti-CD18 Ab, washed, and coincubated with resting or activated Plt, and then DNA was quantified in the supernatants ($n = 4$). $*P < 0.05$ vs. none. $\#P < 0.05$ vs. whole Plt or IgG of the same agonist.

neutrophil β2-integrin. In this context, the cooperation between this receptor/counterreceptor system has been considered another major event involved in sustaining platelet–neutrophil adhesion [29, 30]. Although a murine model of lung injury blocking the platelet GPIIb/IIIa decreased NET levels and tissue damage [10], our in vitro data showed that in human platelets, this GP does not appear to participate in platelet-mediated NETosis because the preincubation of platelets with eptifibatide did not prevent NET production.

We concluded that vWF, PF4, and TXA_2 released from platelets are soluble mediators that orchestrate NET production based on 3 observations: 1) platelet stimulation with LPS, Pam3CSK4, or AA triggered the synthesis and/or secretion of vWF, PF4, and TXA_2; 2) inhibition of their action markedly decreased NET formation; and 3) human recombinant PF4 was a strong NET inducer.

GPIb in platelets and PSGL-1 and β2-integrin in neutrophils are vWF receptors [31]. We found that blocking GPIb and β2-integrin prevented NET generation. Thus, it is conceivable that, upon being released, vWF may act as a bridge that brings platelets and neutrophils into proximity. Previous studies in a mice model of deep vein thrombosis have shown that strings of citrullinated histone 3 colocalizes with vWF in the thrombi, suggesting that vWF released from endothelial cells and platelets and NETs form a mutually supportive network that contributes to vWF A1 domain activation and to the growth and stabilization of a venous thrombus [32]. Our data give further support to this hypothesis and reveal for the first time, to our knowledge,

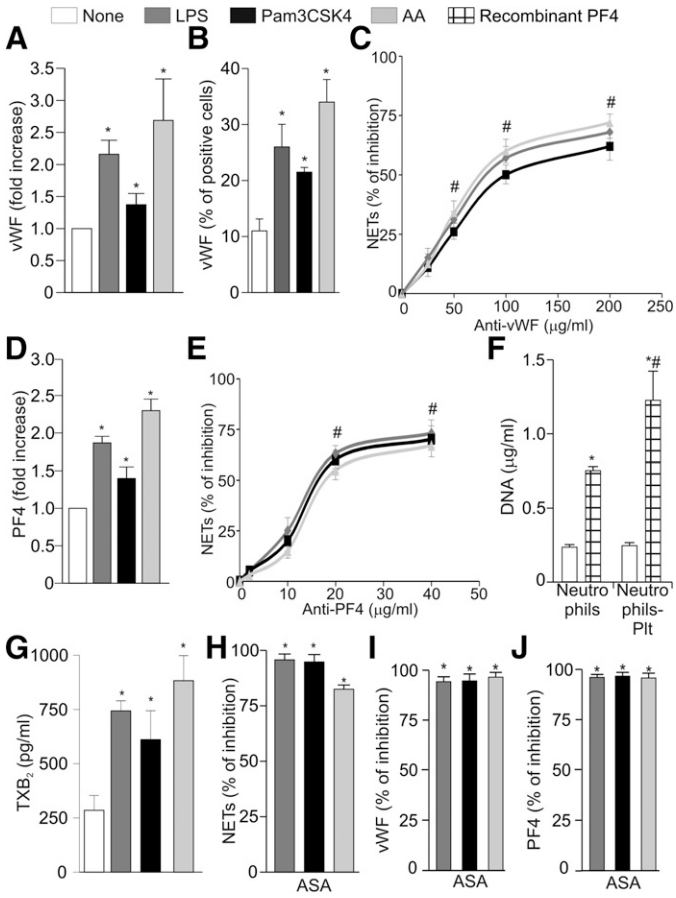

Figure 5. Molecules involved in NET formation mediated by platelets. vWF release (A) and platelet surface-bound vWF (B) were measured in LPS (1 μg/ml)-, Pam3CSK4 (1 μg/ml)-, or AA (100 μM)-stimulated platelets by ELISA and flow cytometry, respectively. (C) Plt were incubated with an anti-vWF Ab or the corresponding IgG control and washed before stimulation and incubation with neutrophils, and DNA was measured by fluorometry. (D) PF4 release was assayed in resting or stimulated Plt. Neutrophils were incubated with Plt pretreated with an anti-PF4 Ab (E) or the corresponding IgG control or recombinant PF4 (1.5 μg/ml)-stimulated Plt (F), and DNA was measured ($n = 4$). (G) Thromboxane (TXB_2) release was measured in resting or stimulated Plt by ELISA. (H) Plt preincubated or not with ASA (1 mM) was stimulated with LPS, Pam3CSK4, or AA, incubated with neutrophils, and then, DNA was quantified in the supernatants by fluorometry. vWF (I) and PF4 (J) release were measured in stimulated Plt preincubated with ASA (1 mM). $*P < 0.05$ vs. none. $\#P < 0.05$ vs. IgG of the same agonist or neutrophils.

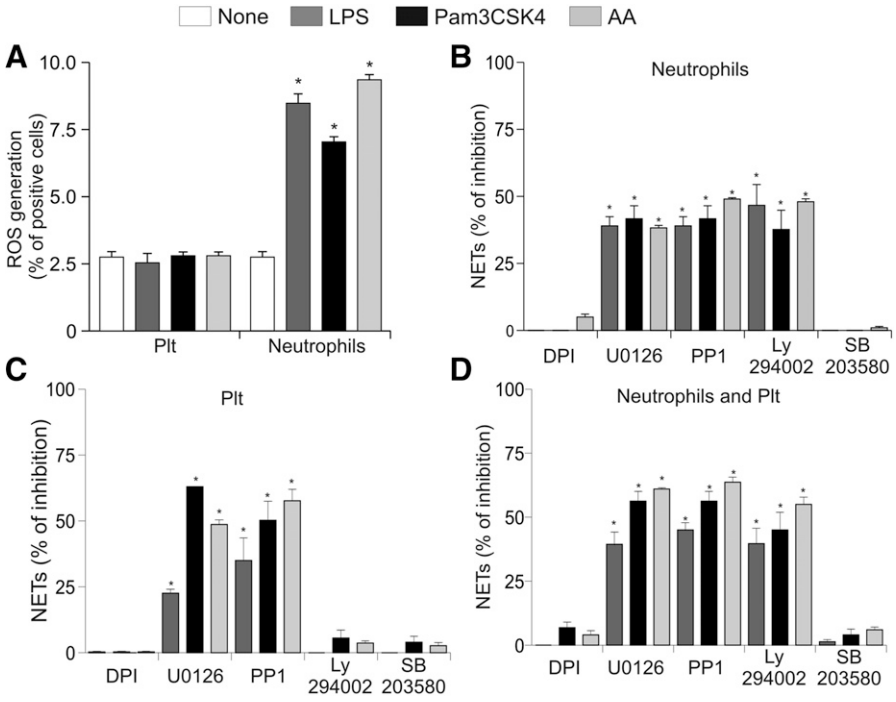

Figure 6. Signaling pathways involved in platelet-mediated NET formation. (A) Plt and neutrophils were stimulated with LPS (1 μg/ml), Pam3CSK4 (1 μg/ml), or AA (100 μM), and then, ROS generation was measured by flow cytometry. Neutrophils (B), Plt (C), or both (D) were pretreated with an inhibitor of NADPH (diphenyl iodonium [DPI], 10 μM), ERK (U0126, 10 μM), Src (PP1, 5 μM), PI3K (Ly294002, 5 μM), or P38 (SB203580, 25 μM), and then, DNA was measured by fluorometry ($n = 4$). *$P < 0.05$ vs. none. The concentrations of the inhibitors were selected from pilot studies and were the minimal amount that completely suppressed phosphorylation of the specific target proteins (data not shown).

a functional role for human platelet-derived vWF. Moreover, it was reported that circulating vWF binds and immobilizes extracellular DNA released from neutrophils acting as a linker for leukocyte adhesion to the endothelium, supporting leukocyte extravasation and inflammation [33]. Thus, vWF circulating or released from endothelial cells, platelets, or both appear to have relevant roles in different stages of platelet-mediated NET formation.

The role of platelet-derived chemokines, such as PF4 and RANTES, in NETosis has been demonstrated recently in a neutrophil- and platelet-dependent mouse model of ventilator-induced lung injury [11]. Our results give further support to the role of PF4 in NETosis triggered by human platelets and also demonstrate that this chemokine is released upon platelet stimulation with TLR4 or TLR2 ligands.

The presence of TXA_2 receptor in neutrophils has not been described; thus, considering that, once released into the platelet cytoplasm, AA is rapidly metabolized to TXA_2, a strong autocrine and paracrine platelet activator, it could be argued that TXA_2 produced by platelets accounts for the release of vWF and PF4, which, in turn, trigger NET formation. In fact, this hypothesis appears to be the case for platelet agonists that induce α-granule secretion in a TXA_2-dependent manner. Using ASA-treated platelets, which prevent TXA_2 formation, we found that vWF and PF4 release and NET formation were inhibited in platelets challenged by LPS, Pam3CSK4, or AA. In contrast, and despite the fact that TXA_2 was not synthesized, the release of vWF, PF4, and NETs was normal in platelets activated by a high-thrombin concentration.

We also demonstrated that LTB4 is a NET inducer. Although LTs are mainly produced by leukocytes, the transcellular metabolism of AA may occur from platelets to neutrophils that are in close contact [34]. Therefore, in addition to being directly produced by stimulated neutrophils, LTB4 can be generated during the cross talk of platelets and neutrophils in the process of NET formation. Moreover, this effect could be more relevant under the therapeutic use of ASA, where AA could be exclusively metabolized by the lipoxygenase enzyme of neutrophils. In fact, AA stimulation of ASA-treated platelets did not completely prevent NET formation. Notably, the application of LTB4 in the skin induces NET formation and the synthesis of interleukin-17, which is involved in the pathogenesis of psoriasis [35]. Whether platelets contribute to this process is a relevant issue that remains to be investigated.

Although the signaling pathways mediating NET formation are still not completely elucidated, the activation of NADPH and the MAPK–ERK pathways appears to be relevant, at least for NETs induced by PMA-activated neutrophils [23]. In contrast with these observations, we found that the inhibition of NADPH did not interfere with NET formation induced by LPS-, Pam3CSK4-, or AA-stimulated platelets, suggesting that ROS are not mediators of NETs under these conditions. However, ERK, Src, or PI3K activation is required for platelet-mediated NET induction.

Intravascular NETs are firmly attached to the vessel walls of liver sinusoids [8]. Here, we demonstrated that the preincubation of platelets with PGI_2 down-regulated NET production under static and flow conditions, which suggests that the preservation of endothelial antiplatelet activity would be critical to the prevention of excessive NET formation.

In conclusion, our data show that platelets activated by TLR2 and TLR4 ligands as well as classic platelet agonists trigger NETosis under static or flow conditions. The orchestrated action between platelet GPIb and neutrophil CD18 and the secretion of

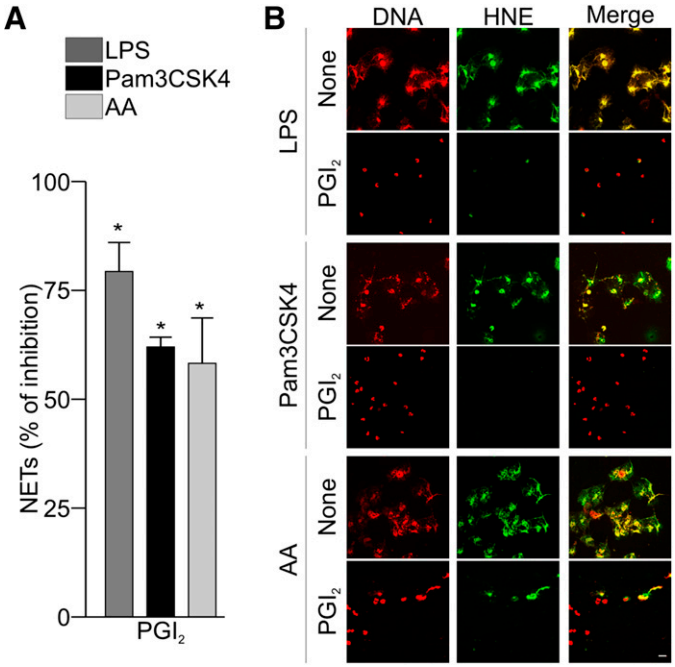

Figure 7. Effect of endothelial-derived platelet inhibitor on platelet-triggered NET formation. Plt preincubated for 1 min with PGI_2 (3 nM) were then stimulated with LPS (1 μg/ml), Pam3CSK4 (1 μg/ml), or AA (100 μM) and DNA (A) was quantified in the supernatants by fluorometry ($n = 4$), or NET induction (B) occurred under shear forces in vitro by the infusion of resting or stimulated Plt. The cells were then fixed, stained, and visualized by confocal fluorescence microscopy. Original magnification, ×60 (scale bar, 20 μm) ($n = 4$). *$P < 0.05$ vs. none.

TXA_2, PF4, and vWF mediate the release of these structures. Furthermore, platelet-induced NET formation is tightly regulated by endothelial-derived PGI_2 and is selectively controlled by ASA-induced platelet inhibition. The signaling pathways involved in NET formation mediated by platelets include activation of ERK, PI3K and Src kinases. Our results highlight that platelets are relevant contributors to host-defense cellular responses.

AUTHORSHIP

A.C. conducted the experiments, interpreted the data, and drafted the manuscript. T.K., L.R., V.I.-L., R.G.P., S.N., and L.P.D. performed the experiments. R.M.G. interpreted the data and revised and edited the manuscript. M.S. conceived of the study and supervised the research, interpreted the data, and wrote the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

REFERENCES

1. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science* **303,** 1532–1535.
2. Urban, C. F., Reichard, U., Brinkmann, V., Zychlinsky, A. (2006) Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell. Microbiol.* **8,** 668–676.
3. Narasaraju, T., Yang, E., Samy, R. P., Ng, H. H., Poh, W. P., Liew, A. A., Phoon, M. C., van Rooijen, N., Chow, V. T. (2011) Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am. J. Pathol.* **179,** 199–210.
4. Gupta, A. K., Hasler, P., Holzgreve, W., Gebhardt, S., Hahn, S. (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum. Immunol.* **66,** 1146–1154.
5. Kessenbrock, K., Krumbholz, M., Schönermarck, U., Back, W., Gross, W. L., Werb, Z., Gröne, H. J., Brinkmann, V., Jenne, D. E. (2009) Netting neutrophils in autoimmune small-vessel vasculitis. *Nat. Med.* **15,** 623–625.
6. Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C., Gregorio, J., Meller, S., Chamilos, G., Sebasigari, R., Riccieri, V., Bassett, R., Amuro, H., Fukuhara, S., Ito, T., Liu, Y. J., Gilliet, M. (2011) Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci. Transl. Med.* **3,** 73ra19.
7. Fuchs, T. A., Brill, A., Duerschmied, D., Schatzberg, D., Monestier, M., Myers, D. D., Jr., Wrobleski, S. K., Wakefield, T. W., Hartwig, J. H., Wagner, D. D. (2010) Extracellular DNA traps promote thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 15880–15885.
8. McDonald, B., Urrutia, R., Yipp, B. G., Jenne, C. N., Kubes, P. (2012) Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe* **12,** 324–333.
9. Clark, S. R., Ma, A. C., Tavener, S. A., McDonald, B., Goodarzi, Z., Kelly, M. M., Patel, K. D., Chakrabarti, S., McAvoy, E., Sinclair, G. D., Keys, E. M., Allen-Vercoe, E., Devinney, R., Doig, C. J., Green, F. H., Kubes, P. (2007) Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat. Med.* **13,** 463–469.
10. Caudrillier, A., Kessenbrock, K., Gilliss, B. M., Nguyen, J. X., Marques, M. B., Monestier, M., Toy, P., Werb, Z., Looney, M. R. (2012) Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J. Clin. Invest.* **122,** 2661–2671.
11. Rossaint, J., Herter, J. M., Van Aken, H., Napirei, M., Döring, Y., Weber, C., Soehnlein, O., Zarbock, A. (2014) Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. *Blood* **123,** 2573–2584.
12. Maugeri, N., Campana, L., Gavina, M., Covino, C., De Metrio, M., Panciroli, C., Maiuri, L., Maseri, A., D'Angelo, A., Bianchi, M. E., Rovere-Querini, P., Manfredi, A. A. (2014) Activated platelets present high mobility group box 1 to neutrophils, inducing autophagy and promoting the extrusion of neutrophil extracellular traps. *J. Thromb. Haemost.* **12,** 2074–2088.
13. Lapponi, M. J., Carestia, A., Landoni, V. I., Rivadeneyra, L., Etulain, J., Negrotto, S., Pozner, R. G., Schattner, M. (2013) Regulation of neutrophil extracellular trap formation by anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.* **345,** 430–437.
14. Rivadeneyra, L., Carestia, A., Etulain, J., Pozner, R. G., Fondevila, C., Negrotto, S., Schattner, M. (2014) Regulation of platelet responses triggered by Toll-like receptor 2 and 4 ligands is another non-genomic role of nuclear factor-kappaB. *Thromb. Res.* **133,** 235–243.
15. Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., Zychlinsky, A. (2007) Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **176,** 231–241.
16. Parker, H., Albrett, A. M., Kettle, A. J., Winterbourn, C. C. (2012) Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. *J. Leukoc. Biol.* **91,** 369–376.
17. Aliprantis, A. O., Yang, R. B., Mark, M. R., Suggett, S., Devaux, B., Radolf, J. D., Klimpel, G. R., Godowski, P., Zychlinsky, A. (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* **285,** 736–739.
18. Raulf, M., König, W. (1991) Modulation of leukotriene generation from human polymorphonuclear granulocytes by polychlorinated biphenyls (PCB). *Immunology* **73,** 485–490.
19. Vandendries, E. R., Furie, B. C., Furie, B. (2004) Role of P-selectin and PSGL-1 in coagulation and thrombosis. *Thromb. Haemost.* **92,** 459–466.
20. Clemetson, K. J. (1983) Platelet membrane glycoprotein I: structure and function. The domain of glycoprotein I involved in the von Willebrand receptor. *Blood Cells* **9,** 319–329.
21. Yount, N. Y., Yeaman, M. R. (2004) Multidimensional signatures in antimicrobial peptides. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 7363–7368.
22. Hakkim, A., Fuchs, T. A., Martinez, N. E., Hess, S., Prinz, H., Zychlinsky, A., Waldmann, H. (2011) Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat. Chem. Biol.* **7,** 75–77.

23. Keshari, R. S., Verma, A., Barthwal, M. K., Dikshit, M. (2013) Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. *J. Cell. Biochem.* **114,** 532–540.
24. Fuchs, T. A., Brill, A., Wagner, D. D. (2012) Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **32,** 1777–1783.
25. Cines, D. B., Pollak, E. S., Buck, C. A., Loscalzo, J., Zimmerman, G. A., McEver, R. P., Pober, J. S., Wick, T. M., Konkle, B. A., Schwartz, B. S., Barnathan, E. S., McCrae, K. R., Hug, B. A., Schmidt, A. M., Stern, D. M. (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **91,** 3527–3561.
26. Zhang, G., Han, J., Welch, E. J., Ye, R. D., Voyno-Yasenetskaya, T. A., Malik, A. B., Du, X., Li, Z. (2009) Lipopolysaccharide stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway. *J. Immunol.* **182,** 7997–8004.
27. Sreeramkumar, V., Adrover, J. M., Ballesteros, I., Cuartero, M. I., Rossaint, J., Bilbao, I., Nácher, M., Pitaval, C., Radovanovic, I., Fukui, Y., McEver, R. P., Filippi, M. D., Lizasoain, I., Ruiz-Cabello, J., Zarbock, A., Moro, M. A., Hidalgo, A. (2014) Neutrophils scan for activated platelets to initiate inflammation. *Science* **346,** 1234–1238.
28. Etulain, J., Martinod, K., Wong, S. L., Cifuni, S. M., Schattner, M., Wagner, D. D. (2015) P-selectin promotes neutrophil extracellular trap formation in mice. *Blood* **126,** 242–246.
29. Pluskota, E., Woody, N. M., Szpak, D., Ballantyne, C. M., Soloviev, D. A., Simon, D. I., Plow, E. F. (2008) Expression, activation, and function of integrin $\alpha_M\beta_2$ (Mac-1) on neutrophil-derived microparticles. *Blood* **112,** 2327–2335.
30. Simon, D. I., Chen, Z., Xu, H., Li, C. Q., Dong, Jf., McIntire, L. V., Ballantyne, C. M., Zhang, L., Furman, M. I., Berndt, M. C., López, J. A. (2000) Platelet glycoprotein ibα is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J. Exp. Med.* **192,** 193–204.
31. Pendu, R., Terraube, V., Christophe, O. D., Gahmberg, C. G., de Groot, P. G., Lenting, P. J., Denis, C. V. (2006) P-selectin glycoprotein ligand 1 and β2-integrins cooperate in the adhesion of leukocytes to von Willebrand factor. *Blood* **108,** 3746–3752.
32. Brill, A., Fuchs, T. A., Savchenko, A. S., Thomas, G. M., Martinod, K., De Meyer, S. F., Bhandari, A. A., Wagner, D. D. (2012) Neutrophil extracellular traps promote deep vein thrombosis in mice. *J. Thromb. Haemost.* **10,** 136–144.
33. Grässle, S., Huck, V., Pappelbaum, K. I., Gorzelanny, C., Aponte-Santamaría, C., Baldauf, C., Gräter, F., Schneppenheim, R., Obser, T., Schneider, S. W. (2014) von Willebrand factor directly interacts with DNA from neutrophil extracellular traps. *Arterioscler. Thromb. Vasc. Biol.* **34,** 1382–1389.
34. Antoine, C., Murphy, R. C., Henson, P. M., Maclouf, J. (1992) Time-dependent utilization of platelet arachidonic acid by the neutrophil in formation of 5-lipoxygenase products in platelet-neutrophil co-incubations. *Biochim. Biophys. Acta* **1128,** 139–146.
35. Keijsers, R. R., Hendriks, A. G., van Erp, P. E., van Cranenbroek, B., van de Kerkhof, P. C., Koenen, H. J., Joosten, I. (2014) In vivo induction of cutaneous inflammation results in the accumulation of extracellular trap-forming neutrophils expressing ROR-γt and IL-17. *J. Invest. Dermatol.* **134,** 1276–1284.

KEY WORDS:
platelet factor 4 · von Willebrand factor · TLR agonists · thromboxane A_2