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Original Article

Calcium ionophore-activated platelets induce eosinophil extracellular trap formation

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ABSTRACT

Background: Platelets play a modulatory role in inflammatory response by secreting a vast array of granules and disintegrating into membrane-bound microparticles upon activation. The interplay between eosinophils and platelets is postulated to be implicated in the pathology of allergic airway inflammation. In this study, we investigated whether activated platelets can induce eosinophil extracellular trap (EET) formation, a cellular process by which activated eosinophils release net-like DNA fibers.

Methods: Platelets were stimulated with the calcium ionophore, A23187, and the platelet agonists, thrombin and adenosine diphosphate (ADP). Platelet cultures were fractionated into conditioned medium (CM) and pellet, which were then overlaid on eosinophils to examine EET formation.

Results: The CM and pellet from A23187-activated platelets stimulated eosinophils to generate EET, whereas those from thrombin- or ADP-activated platelets failed to induce such generation. The EET-inducing activity of the A23187-activated platelet culture was linearly proportional to the number of activated platelets. Interestingly, while EET formation induced by the direct stimulation of eosinophils with A23187 was NADPH oxidase (NOX)-dependent, EET formation induced by A23187-activated platelets was NOX-independent and significantly inhibited by necroptosis pathway inhibitors.

Conclusions: Activated platelets and their products may induce EET formation, thereby potentiating their role in eosinophilic airway inflammation.

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Introduction

Eosinophils are terminally differentiated granulocytes morphologically characterized by the presence of bilobed nuclei and cytoplasmic granules packed with preformed cytotoxic proteins. Eosinophils are considered prominent inflammatory cells that cause tissue damage in allergic conditions such as asthma.[1,2] Such damage is principally mediated by degranulation and eosinophil extracellular trap (EET), a new strategy for ready-to-use granules released from activated eosinophils.[3–5] EET has a highly viscous web-like structure decorated with DNA, histones, and granules that are released from lytic eosinophils in a stepwise manner.[6,7] Relative to neutrophil extracellular traps (NET), which were first demonstrated as a network of extracellular fibers released by activated neutrophils,[8] knowledge of EET is limited, specifically with regard to triggers, signaling pathways, interactions with other immune cells, and roles in the pathogenesis of inflammatory diseases.[9,10] In particular, only few EET triggers have been identified to date, including immunoglobulins, sputum autoantibody, complement factor, platelet activating factor (PAF), lysophosphatidylserine, phorbol myristate acetate (PMA), and the calcium ionophore A23187,.[6,9,11–13] The latter three triggers appear to be the most potent at inducing EET formation, and appears to accompany the loss of membrane integrity and cell vitality in vitro.

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Further identification of EET-triggering factors or contexts will help to better understand their involvement and role in eosinophil-associated diseases including bronchial asthma.

Platelets are anucleated cell fragments generated from megakaryocytes in bone marrow. Beyond their well-recognized role in the formation of hemostatic clots and obstructive thrombi, platelets are increasingly acknowledged for their importance in innate immune responses and inflammation.[14] Upon activation, platelets expose negatively charged phospholipids, secrete a wide variety of granular proteins (>300) and small molecules, mobilize adhesion molecules to the surface, such as P-selectin (also known as CD62P), shed platelet-derived microparticles (PMPs), and interact with the endothelium and other immune cells to form aggregates.[15,16] The products generated during these cellular processes are manifested as markers of activated platelets. A range of immune-related functions exerted by platelets involve an interplay with leukocytes.[17,18] One functional consequence of platelet–leukocyte interactions is NET formation induced by activated platelets.[19–21] The components released by active platelets, such as soluble mediators and PMPs, have been demonstrated to activate the platelet-mediated NET process in both contact-dependent and contact-independent manners.[22] Whether platelets exert activity similar to that of eosinophils is poorly investigated.

Several clinical and experimental studies pertinent to allergic asthma have demonstrated the interplay between platelets and eosinophils in asthma pathology.[23–31] When both cells are activated, increased adherence of platelets to eosinophils has been reported.[32] We hypothesized that activated platelets stimulate EET formation, a functional sequela of their intimate interactions. Accordingly we investigated whether EET formation is generated in response to platelets activated by classical platelet agonists, thrombin and adenosine diphosphate (ADP), and A23187, all of which are known to be potent triggers of NET formation.[33]

Methods

Study subjects

Study subjects were recruited from the Department of Allergy and Clinical Immunology at Ajou University Hospital in Suwon, South Korea, and they had typical symptoms, increased airway hyperresponsiveness, and reversible airway obstruction for the diagnosis of asthma. Atopy status was assessed on the basis of at least one positive result on skin-prick tests with common inhaled allergens. Serum total IgE was measured using the ImmunoCAP system (Thermo Fisher Scientific, Witham, MA, USA). All study subjects were on anti-asthmatic medications, including inhaled corticosteroids and long-acting beta 2 agonists. No one had used systemic corticosteroids for four weeks prior to recruitment. Asthmatic subjects, who had any comorbid disease affecting asthma outcomes or current smokers, were excluded. Clinical characteristics of the study subjects, including peripheral eosinophil counts and asthma severity, were summarized in Supplementary Table 1. This study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). Blood samples were collected from asthmatic patients, who provided informed consent.

Materials

The eosinophil isolation kit was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Ficoll–Paque was obtained from GE Healthcare (Chicago, IL, USA). The calcium ionophore (A23187), thrombin, ADP, PMA, NADPH oxidase inhibitor (diphenyleneiodonium chloride, DPI), prostaglandin E1 (PGE1), human serum, a pan-caspase inhibitor (zVAD-fmk), RIP1 inhibitor (necrostatin-1, Nec1s), RIP3 inhibitor (GSK'872), and MLKL inhibitor (necrosulfonamide, NSA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The PAD4 inhibitor, GSK484, and its inactive control, GSK106, were purchased from Cayman Chemical (Ann Arbor, MI, USA). The flow cytometry sub-micron particle size reference kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant platelet factor (PF)-4 and anti-PF-4 antibodies were purchased from PeproTech (Rocky Hill, NJ, USA).

Isolation of peripheral blood eosinophils and platelets

The platelets were isolated under minimally activated condition. Peripheral blood was centrifuged at 200×*g* for 20 min without brake in room temperature, and fractionated into platelet-rich plasma, buffy coat, and red blood cells. Platelet-rich plasma was collected and centrifuged to eliminate contaminated red and white blood cells. The supernatant was mixed with an equal volume of HEP buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, and 5 mM EGTA, pH 7.4), and PGE1 was added to the mixture to a final concentration of 1 μM. Platelets were pelleted by centrifugation at × 800 g for 20 min with no brake and washed twice with wash buffer [10 mM sodium citrate, 150 mM NaCl, 1 mM EDTA, and 1% (w/v) dextrose, pH 7.4]. The platelets were resuspended in Tyrode's buffer (12 mM $NaHCO_3$, 137 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, and 3 mg/mL BSA). To isolate eosinophils, the buffy coat and red blood cell layers, were diluted with DPBS, stacked on a Ficoll–Paque PREMIUM solution (1.078 g/mL; GE Healthcare, Chicago, IL, USA), and centrifuged at 700×*g* at 4 °C for 20 min. After the RBCs were lysed, eosinophils were isolated by negative selection using an eosinophil isolation kit (Miltenyi Biotec), according to the manufacturer's protocol. The purities of the eosinophils and platelets were >95%, as analyzed by Diff-Quick staining (Sysmex, Kobe, Japan) and microscopic observation, respectively.

Measurement of EET formation

Isolated platelets were resuspended in the culture medium, RPMI1640 containing 0.1% fetal bovine serum (FBS) in 15-mL conical tubes, and activated with 2 μM A23187, 0.05 U/mL thrombin, or 20 mM ADP for 2 h at 37 °C. Activated platelet cultures were fractionated to conditioned medium (CM) and pellet by centrifugation at 800×*g* for 20 min. Eosinophils were seeded on 96-well plates at 1×10^5 cells/well for the plate reader assay or poly-L-lysine coated 8-well chamber culture slides (SPL, Pochen, Korea) at 0.5×10^5 cells/well for ICC. CM and pellet from activated platelet cultures were overlaid on eosinophils a at 1:1 (v/v) ratio and incubated for 4 h at 37 °C to induce EET formation. Plate reader assays were performed in the presence of 1 μM SYTOX Green, a membrane-impermeable DNA-intercalating dye (Invitrogen Life Technologies, Carlsbad, CA, USA). Fluorescence intensity was monitored every 30 min-intervals for 4 h using a hybrid multi-mode reader (BioTek, Winooski, VT, USA). For ICC, the cultures were fixed with 4% PFA, permeabilized, and stained with phosphate-buffered saline (PBS) containing 0.1% saponin, 5% human serum, and antibodies. The antibodies used for ICC included anti-PF4 (Abcam), anti-H3Cit (Abcam), anti-major basic protein 1 (MBP1) (Atlas Antibodies AB, Stockholm, Sweden), and anti-CD41 (Abcam). The DNA was stained with Hoechst33342 (Sigma–Aldrich). The secondary antibodies were conjugated with Alexa 488 (Thermo Fisher Scientific) for MBP1 and anti-CD41 antibodies, and Alexa 594 (Thermo Fisher Scientific) for anti-H3Cit and anti-PF4 antibodies. Isotype-matched mouse IgG and rabbit IgG were used as control antibodies (Sigma–Aldrich). Images were

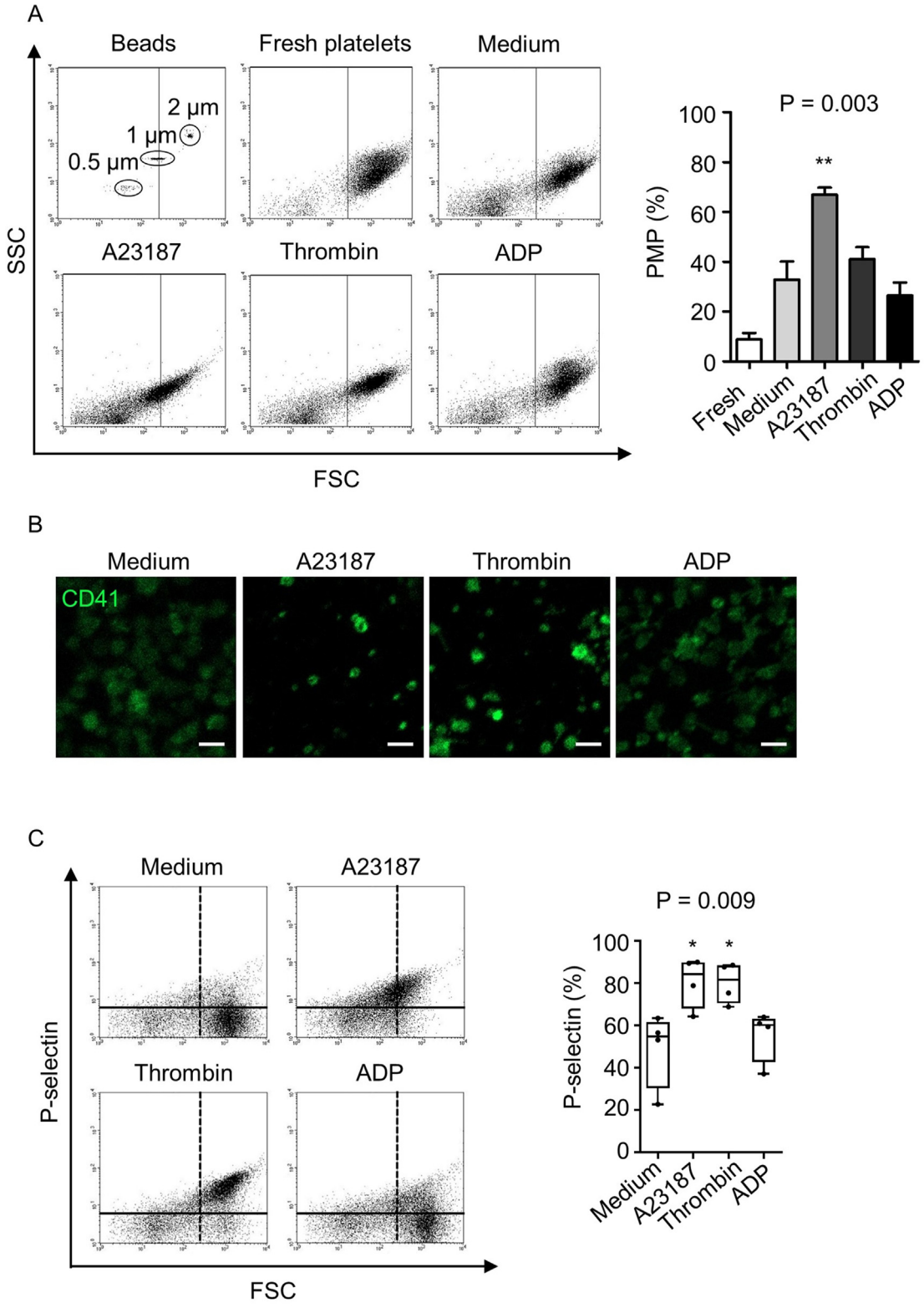

Fig. 1. A23187 and platelet agonists activate platelets in quantitatively and qualitatively different manners. Freshly isolated platelets from human peripheral blood were stimulated with 2 μM A23187, 0.05 U/mL thrombin or 20 mM ADP for 2 h. **A,** PMPs were gated using fluorescent beads of standard size and enumerated as particles less than 1 μm in diameter. A representative dot plot is shown in the left panel and PMP production is expressed as the mean ± SEM of eight independent experiments in the right panel. The P value was obtained using the one-way ANOVA with the Bonferroni's post hoc analysis (**P < 0.01 compared with Medium). **B,** Activated platelets were examined for CD41 expression. The results are representative of two independent experiments. Scale bars represent 5 μm. **C,** P-selectin expression is shown as a representative dot plot and a histogram. The vertical dotted line indicates a diameter of 1 μm. P-selectin expression is presented as the median and ranges of four independent experiments. The P value was obtained using the nonparametric Kruscal–Wallis test with the Bonferroni's post hoc analysis (*P < 0.05 compared with Medium).

visualized using a ZEISS LSM 800 confocal microscope (ZEISS, Oberkochen, Germany).

Flow cytometry

Gates for platelets and PMPs were established on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using a blend of size-calibrated fluorescent beads (flow cytometry submicron particle size reference kit) with sizes of 0.5, 1, and 2 μm. Fresh and activated platelets were analyzed for P-selectin and CD41 expression using APC-conjugated anti-P-selection (Abcam), FITC-conjugated anti-CD41 (Abcam), and isotype-matched antibodies (Abcam).

Statistical analysis

All data were analyzed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). When the number of independent experiments was 6 or more, comparisons between groups were performed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc analysis. If the number of independent experiments was less than 6, the difference between groups was evaluated using nonparametric Kruskal Wallis test followed by post-hoc analysis using Mann–Whitney's U test. Differences with a p-value <0.05 were considered statistically significant. The results are expressed as mean ± SEM.

Results

Platelets are activated by A23187 and thrombin agonists in quantitatively and qualitatively different manners

Platelets were isolated under minimally activated condition, as described in the Materials and Methods. Production of PMPs, which represent activated platelets ex vivo,[15] was measured. Platelets were gated by FSC and SSC parameters following calibration with fluorescence-labeled beads of size standards 0.5, 1, and 2 μm, assigning particles <1 μm to PMPs. Freshly isolated platelets had a minimum number of PMPs, indicating minimal platelet preactivation (Fig. 1A). When platelets were incubated in culture medium containing 0.1% FBS for 2 h, PMPs increased with a concomitant decline in the platelet population, which was demonstrated to occur even in the absence of activation, as previously described.[34] When platelets were stimulated with 2 μM A23187, PMP levels were elevated (66.9 ± 2.7% vs 32.8 ± 6.9%). In contrast, thrombin and ADP caused slight augmentation (41.0 ± 4.6%) and reduced PMPs (26.5 ± 4.9%), respectively. When activated platelet cultures were immunostained for CD41, which is known to be present in both platelets and PMPs,[35,36] all platelets and PMPs were CD41-positive, regardless of the presence or absence of agonists, as revealed by ICC (Fig. 1B) and flow cytometry (Supplementary Fig. 1). Platelets of typical size (2–3 μm in diameter), which were predominantly observed in the absence of activation, were markedly reduced following treatment with A23187, which might be due to their disintegration into small fragments that were hardly visible by microscopic observation (Fig. 1B). Thrombin also caused loss of cell integrity, but to a markedly lesser extent than A23187, whereas ADP caused little disintegration (Fig. 1B). These results were consistent with those of a previous study in which the ionophore, ionomycin, yielded extracellular vesicles (EVs), including PMPs, at a seven-fold higher level than thrombin, which was three-fold more potent than ADP.[37] Thrombin was also demonstrated to marginally generate PMPs compared to a more potent agonist.[38] In contrast to PMP generation, A23187 and thrombin were equally efficacious at inducing the expression of P-selectin in platelets and PMPs (Fig. 1C). Collectively, these data suggest that the two classical platelet agonists and the ionophore, A23187, activate platelets in quantitatively and qualitatively different manners.

EET formation is induced by the CM and pellet from A23187-, but not by thrombin- or ADP-, activated platelet cultures

We proceeded to determine whether agonist-activated platelets could induce EET formation. After platelets were treated with the three agonists for 2 h, the platelet cultures were fractionated into CM and pellet by conventional centrifugation. The pellet was washed once with culture medium and resuspended in fresh culture medium. CM and pellet were added to the eosinophil suspension at 1:1 (v/v) and incubated for 4 h. The platelet-to-eosinophil ratio was adjusted to 1000:1, which reflects the typical ratio of the two leukocytes in blood. EET formation was monitored either by ICC for H3Cit or DNA staining with SYTOX Green. EET was induced when eosinophils were stimulated with both CM and pellet from A23187-activated platelet cultures, but neither CM nor pellet from thrombin- or ADP-activated platelets could induce EET (Fig. 2A, B). The EET-forming activity of CM from A23187-activated platelet cultures was comparable to that of the pellet (Fig. 2C). EET induced by A23187-activated platelet cultures did not co-localize with platelets, PMPs or their aggregates (Fig. 2A), suggesting that EET does not require direct contact between eosinophils and platelets. EET induced by the A23187-activated platelet cultures was accompanied by MBP1 punctate foci in the DNA net (Fig. 3), which is a characteristic feature of EET.[6,11] Importantly, when eosinophils were stimulated with CM and pellet from increasing numbers of platelets activated in the presence of A23187, EET formation was linearly proportional to the ratio of platelets to eosinophils (Fig. 4A, B). H3Cit-positive cells were significantly increased in response to CM and pellet from A23187-activated platelet cultures at high ratios of platelets to eosinophils (500:1 and 1000:1) and tended to be dose-dependent in general (Fig. 4C). These data support that EET formation results from the activated platelet cultures rather than the A23187 remnant. The medium washes of tubes containing A23187 without platelets did not induce EET formation (Supplementary Fig. 2), aligning with the observations from previous studies that employed a similar experimental setting.[39,40] As thrombin- and ADP-activated platelets did not induce EET formation, we further examined the EET-inducing activity of several inflammatory mediators that can activate platelets, eosinophils or both. Although eosinophil-activating cytokines, such as IL-5, GM-CSF, and TSLP, and known platelet-activators, such as thrombin, collagen, LPS, TNF-α, and PAF, were tested, none of these mediators alone (data not shown) or combined induced EET formation (Supplementary Table 2).

PMP fraction in pellet from A23187-activated platelet cultures retain EET-forming activity

Pellet from A23187-activated platelet cultures contained heterogenous particles ranging from platelets to PMP in size (Fig. 1A). As PMPs mediate platelet functions,[41] we asked whether PMPs had the EET-forming activity. We fractionated the heterogenous particles using 0.8 μm pore sized-filter. Flow cytometry analysis demonstrated the efficient fractionation of PMPs, greatly eliminating platelets of typical size. The PMP fraction had a significant EET-forming activity, albeit slightly lower than unfractionated pellet (Supplementary Fig. 3), indicating that PMPs retain the EET-forming activity.

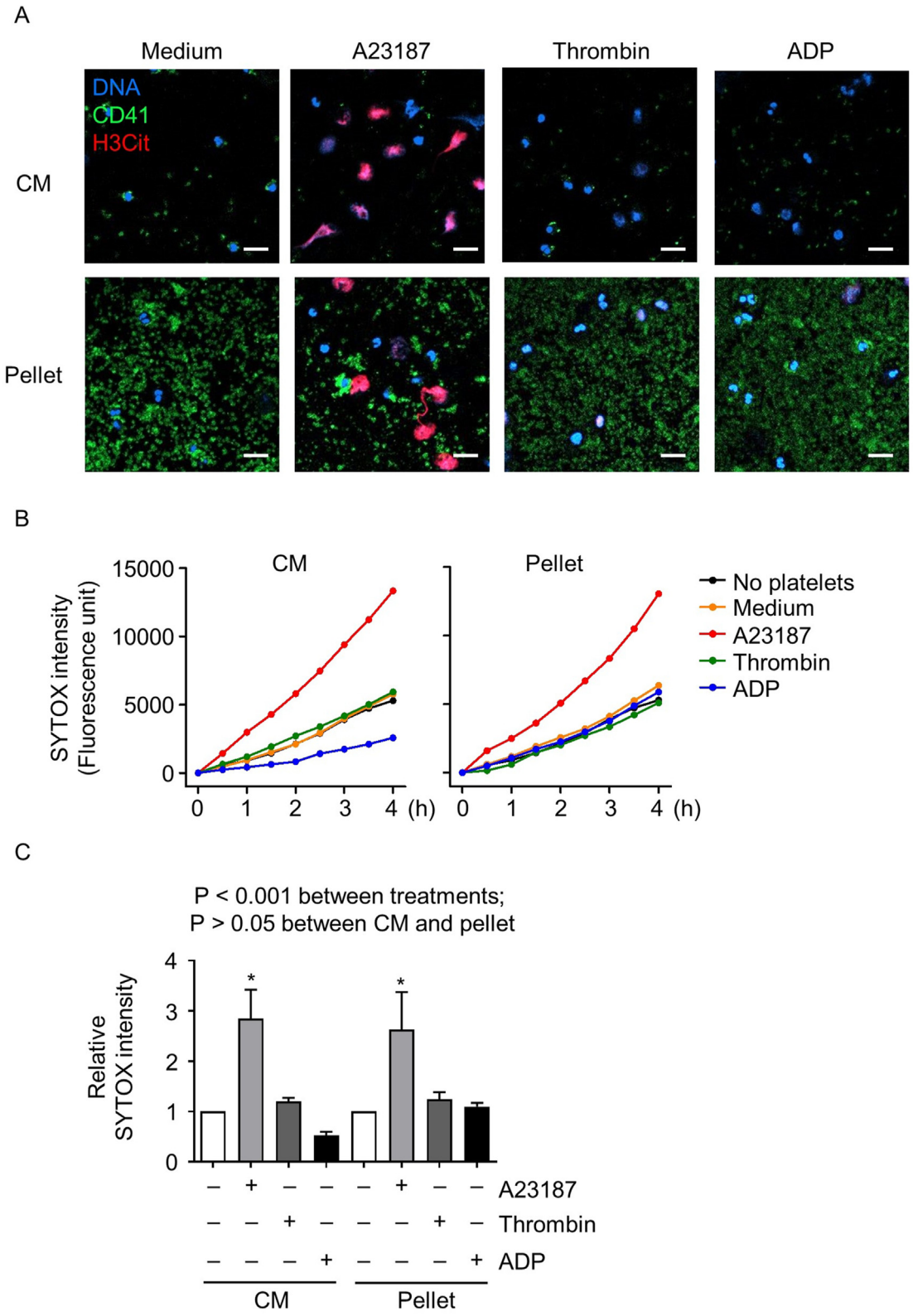

Fig. 2. EET formation is induced by A23187-, but not thrombin- or ADP-, activated platelets. CM and pellet from platelet cultures that were activated with A23187, thrombin, or ADP for 2 h were overlaid on eosinophils and incubated for 4 h. The platelet-to-eosinophil ratio was adjusted to 1000:1. **A,** Cells were stained with CD41 (green), H3Cit (red), and DNA (blue). Scale bars represent 20 μm. **B and C,** Eosinophils stimulated with CM and pellet from activated platelet cultures were analyzed for EET formation in the presence of SYTOX Green. The results are representative of EET formation and are expressed as SYTOX intensity every 30 min **(B)**. EET formation was determined as the relative SYTOX intensity [SYTOX intensity in the presence of platelet agonists at 4 h/SYTOX intensity in the presence of medium at 4 h]. The results are presented as mean ± SEM of four independent experiments. The P value was obtained using the two-way ANOVA for the treatments and fractions with the Bonferroni's post hoc analysis (*P < 0.01 compared with other treatments) **(C)**.

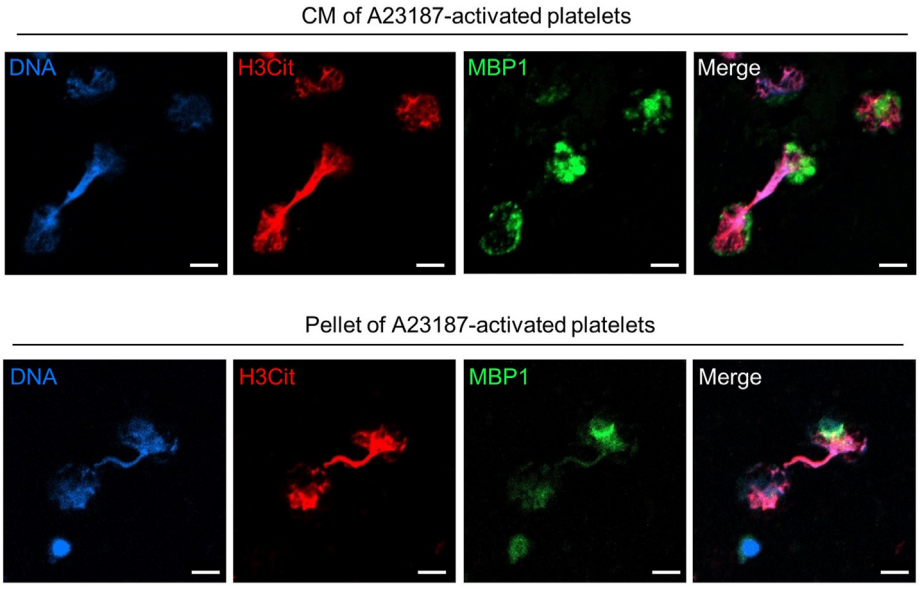

Fig. 3. MBP1 and H3Cit are included in activated platelet-induced EET. Eosinophils were seeded on 8-chamber slides and stimulated with CM or pellet from A23187-activated platelet cultures for 4 h, and stained for MBP1 (green), H3Cit (red), and DNA (blue). The slides were imaged using a ZEISS LSM 800 confocal microscope. The scale bars represent 10 μm.

Platelet factor-4 co-localizes with EET induced by CM from A23187-activated platelet cultures

Among the molecules released upon platelet activation, PF-4 accounts for approximately 25% of the α-granule content.[42] As PF-4 is known to be a potent inducer of NET formation,[21,43] we addressed its role in EET formation. ICC analysis revealed no staining using the isotype-matched Ab (data not shown). PF-4 was minimally detected in the absence of EET, which was observed when eosinophils were stimulated with CM and pellet from medium-activated platelet cultures (Fig. 5). Strikingly, PF-4 co-localized with EET induced by CM from A23187-activated platelet cultures. In contrast, when eosinophils were stimulated with pellet from A23187-activated platelet cultures, PF-4 positivity was sparsely detected, but did not co-localize with EET. Thus, PF-4 preferentially associated with the formation of EET induced by CM. To determine whether PF-4 played an active role in CM-induced EET formation, eosinophils were stimulated with recombinant PF-4 or incubated with CM in the presence of a neutralizing PF-4 antibody. Recombinant PF-4 did not induce EET formation in the absence or presence of platelets, and neutralizing the PF-4 antibody did not prevent CM-induced EET formation (Supplementary Fig. 4). These results suggest that PF-4 might be an effect rather than the cause of CM-induced EET formation, highlighting the differential roles of PF-4 in NET and EET formation.

EET formation by A23187-activated platelet cultures is dependent on PAD4 but independent of NADPH oxidase

To assess the reliability of NADPH oxidase dependency, PMA was used, which has been previously demonstrated to induce EET[13] and NET formation in a completely NADPH oxidase-dependent manner.[44] Consistently, PMA-induced EET formation was completely inhibited by DPI, an NADPH oxidase inhibitor (Fig. 6A), confirming that PMA-induced EET is strictly NADPH oxidase-dependent. When eosinophils are stimulated with A23187, EET formation is completely[6] or partially[11] inhibited by DPI. In the present study, A23187-induced EET formation was partially inhibited by DPI (Fig. 6A). In contrast, EET formation by CM and pellet from A23187-activted platelet cultures was completely insensitive to DPI (Fig. 6B). Of note, DPI promoted EET formation from A23187-activated platelets, while it inhibited EET formation from A23187-stimulated eosinophils. We are currently unaware of the different modes of action of DPI. However, the findings imply that A23187 and activated platelets acting on eosinophils initiate EET formation through separate signaling pathways, and strongly suggest that in our experiments, activated platelets do not trigger EET formation through the leftover A23187 following activation of platelets. We also investigated the dependence of activated platelet-driven EET formation on PAD4, which is essential for A23187-induced EET formation.[11] A23187- and PMA-induced EET formation was inhibited by GSK484, a PAD4 inhibitor, but not by GSK106, an inactive analog of GSK484[45] (Fig. 6C). Similarly, EET formation by CM and pellet from A23187-activated platelet cultures was considerably inhibited by GSK484 (Fig. 6D). Thus, PAD4 was required for activated platelet-induced EET formation. Of note, PMA-induced EET formation was considerably inhibited by the PAD4 inhibitor (Fig. 6C), which is in marked contrast to the finding that PMA-induced NET formation was not affected by the inhibitor,[44] suggesting a difference in the utilization of PAD4 between PMA-induced EET and NET formation.

EET formation by the CM and pellet from A23187-activated platelet cultures occurs via a necroptotic pathway

Adhesion-induced eosinophil cytolysis occurs via the necroptosis pathway.[13] The necroptosis pathway is necessary for platelet-induced NET formation.[46] Therefore, we examined the involvement of the necroptotic pathway in EET formation induced by A23187-activated platelets. All the experiments were performed in the presence of z-VAD-fmk, a pan-caspase inhibitor. When eosinophils were directly stimulated with A23187, EET formation was weakly but significantly blocked by the RIP3 inhibitor (GSK'872) and the MLKL inhibitor (NSA) (Fig. 7A). When eosinophils were

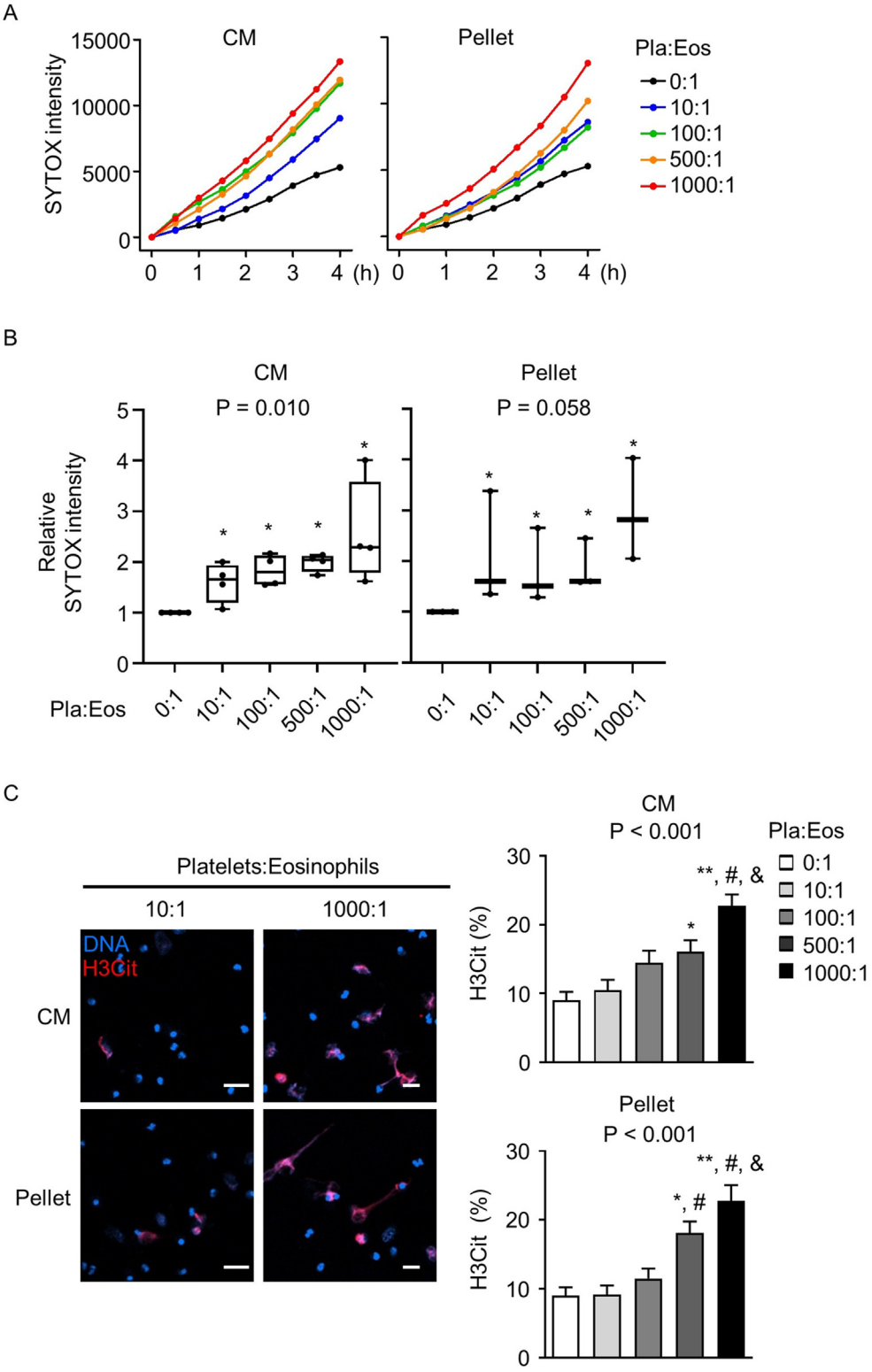

Fig. 4. A23187-activated platelets induce EET formation in proportion to the platelet numbers used. A fixed number of eosinophils was overlaid with CM or pellet from cultures of increasing numbers of platelets that were activated by 2 μM A23187 for 4 h and examined for EET formation. The results are representative of SYTOX intensity **(A)** and relative SYTOX intensity at 4 h and are expressed as median and ranges of three to four independent experiments The P value was obtained using the nonparametric Kruscal–Wallis test with post hoc Mann–Whitney's U test (*P < 0.05 compared with no-platelet group) **(B)**. **C,** Activated eosinophils were stained for H3Cit (red) and DNA (blue). H3Cit-positive cells were counted in 12 high-power fields from four independent experiments. H3Cit positivity was calculated by dividing the number of H3Cit-positive cells by that of DNA-stained cells and is expressed as mean ± SEM. The P value was obtained using the one-way ANOVA with the Bonferroni's post hoc analysis (*P < 0.05, **P < 0.01 compared with no-platelet group; #, P < 0.05 compared with 100:1 group; &, P < 0.05 compared with 500:1 group). Scale bars represent 20 μm. Pla, platelets; Eos, eosinophils.

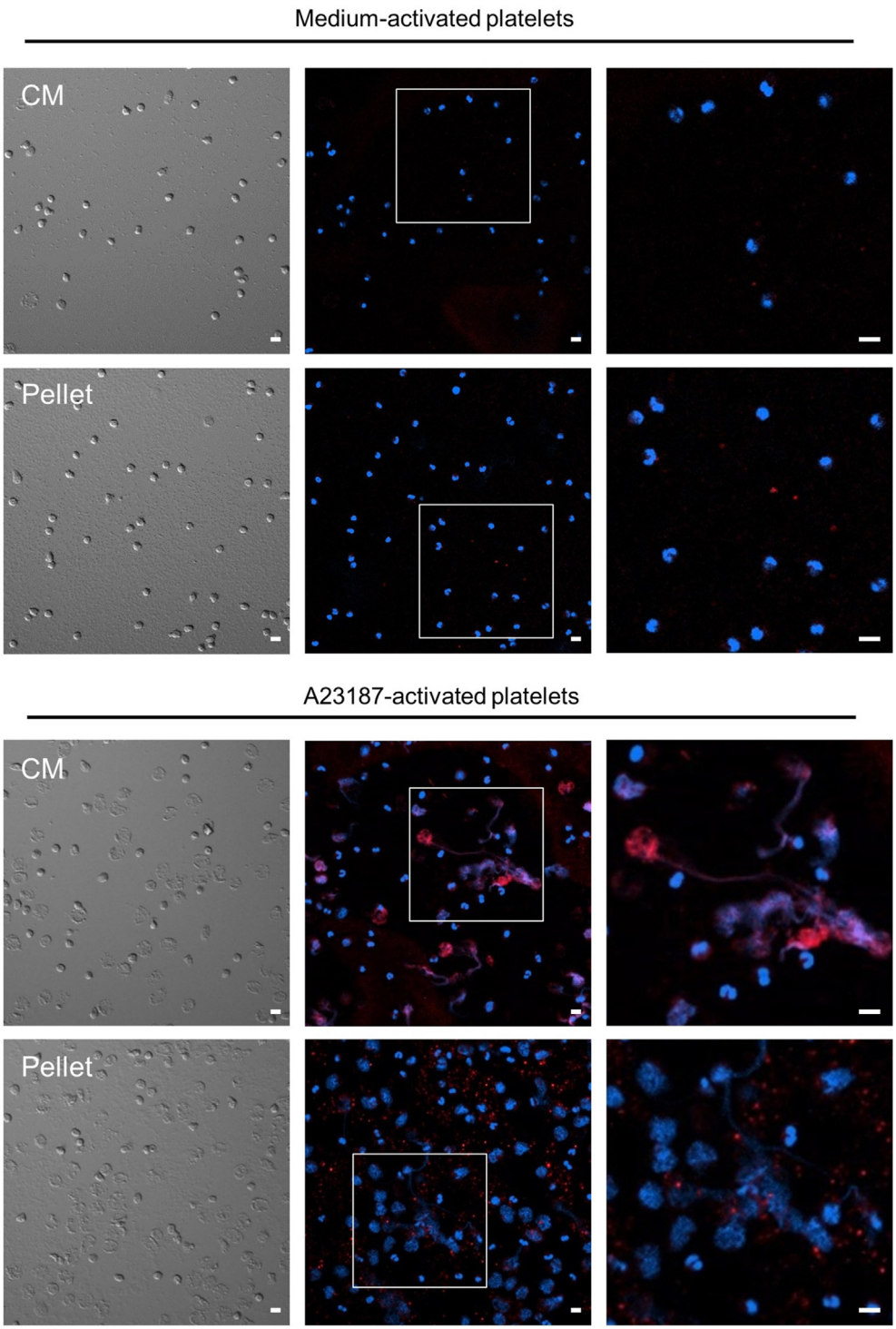

Fig. 5. PF-4 preferentially co-localizes with EET induced by CM, but not pellet, from the A23187-activated platelet cultures. Eosinophils were stimulated with CM or pellet from medium- or A23187-activated platelet cultures for 4 h and stained with PF-4 (red) and DNA (blue). The insets indicate magnified images of the boxes in the middle. The results are representative of two independent experiments. The scale bars represent 10 μm.

stimulated by either pellet or CM from A23187-stimulated platelet cultures, EET was strongly inhibited by Nec1s, GSK'872, or NSA in combined with z-VAD-fmk (Fig. 7B). Of note, in our study, z-VAD-fmk alone markedly enhanced SYTOX intensity in eosinophils. These results suggest that EET formation induced by A23187-activated platelets occurs through a pathway involving at least RIP3 and MLKL.

Discussion

Platelets drive NET formation in the presence of classical platelet agonists and inflammatory mediators,[20,21,47–49] and NET in turn modulates neutrophil and platelet functions in the pathology of infectious and non-infectious diseases.[22] Multifaceted links between platelets and eosinophils are demonstrated to be involved in

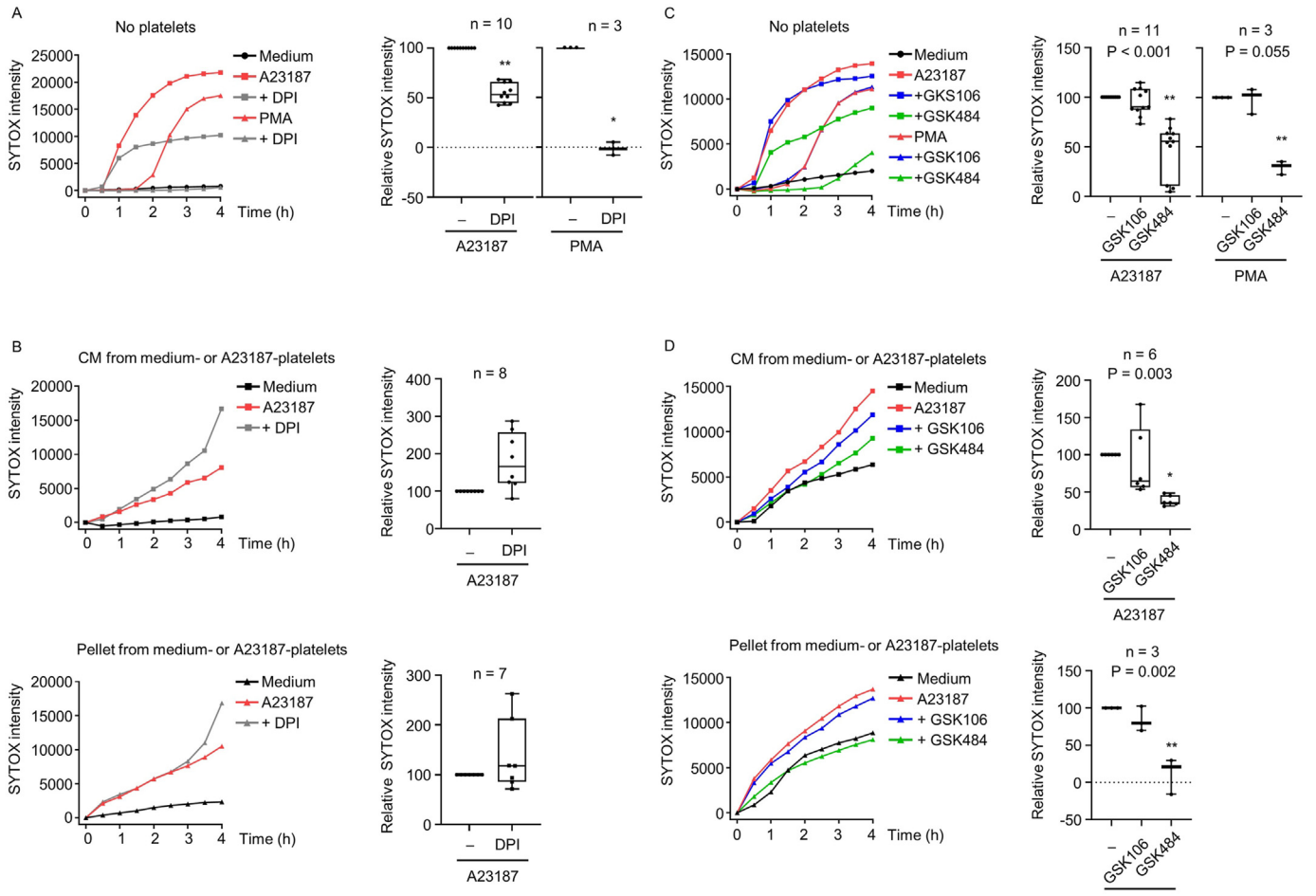

Fig. 6. EET formation induced by A23187-activated platelets is independent of NADPH oxidase, but dependent on PAD4. **A and C**, Freshly-isolated eosinophils were stimulated with 1 μM A23187 or 10 ng/mL PMA in the absence or presence of 10 μM DPI **(A)** or 10 μM GSK484 **(C)**. The results are representative of SYTOX intensity (left) and the relative SYTOX intensity at 4 h and are expressed as median and ranges (right) (**P < 0.01 in Bonferroni's post hoc analysis). **B and D,** CM and pellet from A23187-activated platelet cultures were overlaid with eosinophils in the absence or presence of DPI **(B)** or GSK484 **(D)**. The results are representative of SYTOX intensity (left) and the relative SYTOX intensity at 4 h and are expressed as median and ranges (right) (*P < 0.05, **P < 0.01 in Bonferroni's post hoc analysis).

the pathophysiology of allergic inflammation.[26,28,31,50] Therefore, such relationship could be assumed to exist between the two leukocytes, as found in platelet-driven NET formation, accounting for the role of platelets in eosinophilic inflammation. In the present study, EET formation was induced when platelets were activated by A23187. Both CM and pellet from A23187-activated platelet cultures retained EET-triggering activity. In contrast, platelets activated by the classical platelet agonists thrombin and ADP could not induce EET formation. Although the identity of the EET-triggering activity remains unclear, these results suggest that one functional consequence of the interactions between the two cell types may represent EET formation. Activated platelets (by calcium ionophore) could induce EETs formation, perpetuating eosinophilic airway inflammation in asthma.

Notably, A23187 itself is a known trigger of EET formation, raising the possibility that A23187-stimulated platelets contain residual A23187, which can directly induce EET formation. EET formation might be attributed to the activated platelets rather than a direct effect of the remnant A23187 on eosinophils owing to reasons. Herein, the EET forming activity of CM and pellet from A23187 (2 μM)-activated platelet cultures was proportional to the number of platelets used (Fig. 4). Upon addition of A23187 to platelet cultures, it is incorporated into membrane-bound structures owing to its highly lipid-soluble nature,[51] causing vigorous disintegration of the membrane structures. Such incorporation would lead to a lower amount of A23187 in CM, and a greater amount in the pellet with increasing numbers of platelets. In this circumstance, the use of an increasing number of platelets would yield less EET formation in CM and greater EET formation in the pellet. Based on our findings, this result was not achieved, showing that CM and pellet elicit the greatest EET formation at the highest platelet-to-eosinophil ratio. Second, EET formation induced by A23187-activated platelet cultures was not sensitive to DPI, unlike EET formation which was directly induced by A23187 (Fig. 6). The dependency of the number of platelets and DPI-insensibility clearly demonstrate that A23187-activated platelets rather than A23187 itself provide EET forming activity.

An array of evidence unequivocally demonstrates that several classical platelet agonists or other physiological agents released from activated platelets mediate and/or induce NET formation. These agents include thrombin, ADP, collagen, thromboxane A2 (TXA2), leukotriene B4, PAF, PF-4, β-defensin 1, and HMBG1.[18,21,52] LPS-activated platelets induce NET formation through TLR4-dependent signaling.[19] The ligation of adhesion molecules between the two cell types is also necessary for NET formation, including P-selectin and GPIb on platelets and P-selectin glycoprotein ligand 1 and $\alpha_M\beta_2$ on neutrophils.[20,21] Whether these soluble mediators and adhesion molecules

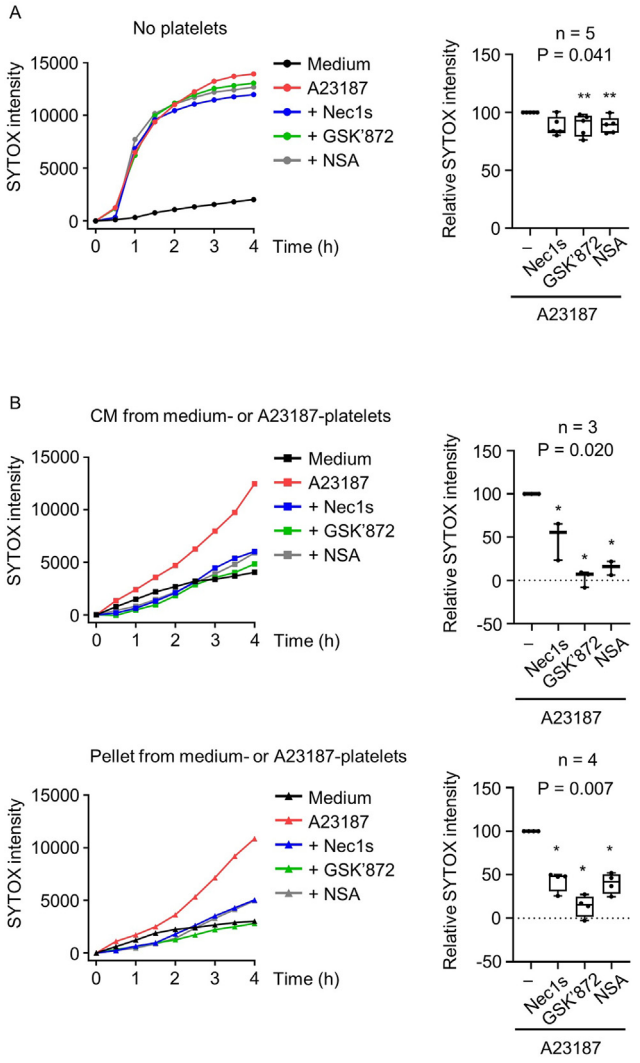

Fig. 7. EET formation induced by A23187-activated platelets is blocked by necroptosis pathway inhibitors. **A,** Freshly-isolated eosinophils were stimulated with 1 μM A23187 in the presence of necroptosis pathway inhibitors, 50 μM zVAD-fmk, 10 μM Nec1s, 20 μM GSK'872, and 1 μM NSA. The results are representative of SYTOX intensity (left) and the relative SYTOX intensity at 4 h and are expressed as median and ranges (right) (**P < 0.01 in post hoc Mann–Whitney's U test following Kruskal–Wallis test). **B,** CM and pellet from A23187-activated platelet cultures were overlaid with eosinophils in the absence or presence of necroptosis inhibitors. The results are representative of SYTOX intensity (left) and the relative SYOX intensity at 4 h and are expressed as median and ranges (right) (*P < 0.05 in post hoc Mann–Whitney's U test following Kruskal–Wallis test).

derived from activated platelets induce EET formation remains largely unknown. A previous study demonstrated that thrombin-activated platelets, but not the CM of platelet cultures, induce EET formation in vitro in a P-selectin-dependent manner.[53] However, we could not detect EET formation induced by both CM and pellet of thrombin- and ADP-activated platelets (Fig. 2). This discrepancy between the previous and our studies is unclear, owing to the similar in vitro experimental conditions, including nearly the same numbers and ratios of the two cell types used, and the identical methodology for the analysis of EET formation. Under the pathophysiological conditions of asthma, a wide variety of inflammatory mediators that can stimulate eosinophils or platelets or both are present in inflamed tissues. Thrombotic mediators might act on activated eosinophils to induce EET formation. However, we failed to identify the potential inflammatory stimuli that could trigger EET formation (Supplementary Table 2). As a very limited repertoire of inflammatory mediators is known to induce EET formation compared with those that trigger NET formation,[33] EET formation occurs under markedly narrower ranges of physiological context, and is thus more strictly regulated in vivo than NET formation.

Depending on the activating stimuli, platelets display significant differences in their markers or phenotypes.[37,54] Platelets stimulated by ionomycin or A23187 produce more EVs, such as PMPs and exosomes, and more heterogeneity and amounts of proteins in EVs than those stimulated by LPS or a combination of thrombin and collagen. Therefore, ionophore-activated platelet-derived EVs are expected to manifest enhanced platelet activation. Based on our results, despite comparable levels of P-selectin expression in each group, platelets activated by A23187 produced significantly more PMPs than those activated by thrombin or ADP (Fig. 1). Differential platelet activation may thus be linked to stimulus-dependent induction of EET formation. Furthermore, the induction of EET formation is thought to require strong and complex signals delivered by agents such as A23187 and PMA, thereby presumably providing a state of full activation. As both EET and NET are found in induced thrombi in an experimental model,[53] EET formation may require a factor or context that is absent in vitro settings.

In summary, A23187-activated platelets were demonstrated to induce EET formation; however, the classical platelet agonists, thrombin and ADP, which are known to be efficacious at inducing NET formation, cannot induce EET formation. Thus, depending on the nature of the activation signal, activated platelets can induce EET formation. Activated platelet-induced NET formation has been associated with coagulation in patients with sepsis and chronic vascular disorders.[18] EET is found along with NET in thrombi of a mouse model.[53] In fact, EET has been implicated in diverse eosinophilic diseases.[9] Moreover, EET promotes type 2 inflammation and mucus production, and is inversely proportional to lung function in allergic asthma.[4,39,55,56] Owing to the variety of changes in platelet function in allergic airway inflammation, platelet-dependent EET might plays a crucial role in the pathophysiology of eosinophilic lung disorders, such as severe eosinophilic asthma and eosinophilic granulomatosis with polyangiitis (EGPA).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2022.12.002.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

MSS, H-SP, and IYC designed the study. MSS, CK, HSC, H-SP and IYC wrote the manuscript. MSS, HJK, IB, D-HL, YC, and HSC performed experiments, and collected data. All authors read and approved the final manuscript.

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