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ORIGINAL ARTICLE

# Involvement of peptidylarginine deiminase 4 in eosinophil extracellular trap formation and contribution to citrullinated histone signal in thrombi

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## Abstract

**Background:** Extracellular traps formed by neutrophils (NETs) and eosinophils (EETs) have been described in coronary thrombi, contributing to thrombus stability. A key mechanism during NET formation is histone modification by the enzyme PAD4. Citrullinated histones, the product of PAD4 activity, are often attributed to neutrophils. Eosinophils also express high levels of PAD4.

**Objectives:** We aimed to explore the contribution of PAD4 to EET formation.

**Methods:** We performed immunohistological analyses on thrombi, including a large, intact, and eosinophil-containing thrombus retrieved from the right coronary artery using an aspiration catheter and stroke thrombi from thrombectomy retrieval. We studied eosinophils for their capability to form PAD4-dependent EETs in response to strong ET-inducing agonists as well as activated platelets and bacteria.

**Results:** Histopathology and immunofluorescence microscopy identified a coronary thrombus rich in platelets and neutrophils, with distinct areas containing von Willebrand factor and citrullinated histone H3 (H3Cit). Eosinophils were also identified in leukocyte-rich areas. The majority of the H3Cit+ signal colocalized with myeloperoxidase, but some colocalized with eosinophil peroxidase, indicating EETs. Eosinophils isolated from healthy volunteers produced H3Cit+ EETs, indicating an involvement of PAD4 activity. The selective PAD4 inhibitor GSK484 blocked this process, supporting PAD4 dependence of H3Cit+ EET release. Citrullinated histones were also present in EETs produced in response to live *Staphylococci*. However, limited evidence for EETs was found in mouse models of venous thrombosis or infective endocarditis.

**Conclusion:** As in NETosis, PAD4 can catalyze the formation of EETs. Inhibition of PAD4 decreases EET formation, supporting the future utility of PAD4 inhibitors as possible antithrombotic agents.

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KEYWORDS
citrullination, eosinophils, extracellular traps, myocardial infarction, stroke, thrombosis

## 1 | INTRODUCTION

Granulocytes possess a unique mechanism in which they expel their nuclear content into the extracellular space upon stimulation [1]. These so-called extracellular traps (ETs) are a meshwork of DNA, histones, granule proteins including myeloperoxidase (MPO) and antimicrobial peptides, and cytoplasmic proteins including calprotectin. Neutrophil ETs (NETs) have been studied extensively in the past two decades and have been described to play roles in infectious, autoimmune, and thrombotic diseases [2]. Recent evidence suggests a role for NETs in coronary artery disease (CAD) and plaque erosion [3], and pharmacologic approaches that inhibit NET release in CAD [4] show better outcomes [5].

Several stimulants and signaling pathways have been identified to be important for inducing neutrophils to undergo cell death through externalizing their toxic and prothrombotic nuclear content, called NETosis. The enzyme peptidylarginine deiminase 4 (PAD4) is crucial for NETosis by converting positively charged arginyl residues in histones to neutral citrulline, thereby promoting chromatin decondensation. NETosis requires PAD4 [6] and both are involved in acute thrombotic complications of experimental atherogenesis [3]. PAD4-deficient animals are protected from forming thrombi in the venous circulation [7,8] and also have reduced myocardial reperfusion injury–induced tissue damage [9].

Another important granulocyte in circulation is the eosinophil, named as such due to cationic granule proteins that stain brightly with eosin in differential blood cell staining. Eosinophils comprise only 0.5% to 3% of circulating leukocytes in healthy individuals, but can become acutely elevated in cases of allergy or parasitic infections, as well as in certain autoimmune diseases [10] including eosinophilic granulomatosis with polyangiitis, myocarditis, Behçet's disease [11], and Sjögren's syndrome [12]. Eosinophils also possess a unique machinery that allows them to selectively release their granules in a process known as piecemeal degranulation [13]. Here, eosinophils either partially or fully release their granule contents while maintaining their original granule structure. Intact eosinophil granules have also been described on extracellular DNA structures released from activated eosinophils [14].

Eosinophil extracellular traps (EETs) are less well studied, but were recently described to stabilize coronary thrombi after induction with activated platelets [15]. EETs can be distinguished from NETs by the presence of eosinophil-specific proteins found in the cationic granules including major basic protein (MBP) and eosinophil peroxidase (EPX). The first description of EETs showed that rapid reactive oxygen species–dependent release of mitochondrial DNA could occur in eosinophils stimulated with lipopolysaccharides from gram-negative bacteria, with bactericidal activity [16]. Similar to NETs, EETs have been shown to play important roles in the pathogenesis of various diseases in which eosinophilia is attributed, including infection, allergy, and autoimmunity [17].

However, PAD4 dependence for EET release in thrombi is unknown. Answering this question is of relevance, as PAD4 inhibitors are effective in preventing NETosis *in vitro* [18], as well as *in vivo* [19], and may similarly prevent EET release. Upon identification of eosinophils in the thrombus from a patient with atypical STEMI (ST-elevation myocardial infarction) presentation despite low risk factors, we performed a more detailed histologic analysis of the thrombus composition. Within the thrombus, we found not only large amounts of platelets, neutrophils, and NETs but also areas with eosinophils and evidence of EETs. We also performed analysis for the presence of eosinophils and EETs in ischemic stroke thrombi. *In vitro* assays were conducted with eosinophils isolated from healthy donors to investigate whether activated platelets could induce PAD4-mediated EET release and whether this release could be prevented by treatment with the PAD4 inhibitor GSK484. Finally, we examined venous thrombi and endocarditis vegetations from animal models and found limited evidence of EET release overall, despite strong citrullinated histone signal. We thus report PAD4-dependent EET release in a large, intact coronary thrombus, in stroke thrombi, as well as *in vitro*, but sparse evidence of EETs in animal models with H3Cit-prominent thrombi.

## 2 | METHODS

### 2.1 | Ethical approval for human samples

All work with human samples was reviewed and approved by local ethical committees/institutional review boards. Written consent was given by the patient to perform immunohistological analysis of the MI thrombus, which was sent to pathology for diagnostic purposes. Study ethical permission was given by the University Hospital Basel (study number: 685) for the MI thrombus (with patient consent) or by the University of Utah Institutional Review Board (00132234) for ischemic stroke thrombi (no informed consent was required). Blood withdrawal from healthy volunteers was approved by the Ethical Committee of KU/UZ Leuven (study number: S63324) with prior written consent. All volunteers provided written informed consent prior to participating in the study. Eligibility criteria included being 18 years or older, no prior history of thrombotic events, no active infection, and no current use of nonsteroidal anti-inflammatory drugs.

### 2.2 | Thrombus harvest

Thrombus retrieval was performed using a coronary aspiration catheter. The intact thrombus was rinsed with normal saline to remove excess blood and was immediately sent to the Pathology Department

of the University Hospital Basel for further processing. Ischemic stroke thrombi were collected during intracranial endovascular mechanical thrombectomy procedures performed at the University of Utah Health Sciences Center (IRB 00132234).

## 2.3 | Thrombus histology

The MI thrombus was fixed in a 4% formaldehyde solution, embedded in paraffin, and 5 μm-thick sections were cut using a microtome. One section was stained with hematoxylin and eosin (H&E) using standard laboratory protocols for histopathology assessment by the Pathology Department, University Hospital Basel. The remaining sections were analyzed at the Center for Molecular and Vascular Biology, KU Leuven, Belgium. All sections were deparaffinized and rehydrated into aqueous solution prior to immunostaining.

Stroke thrombi were washed in 0.9% saline, fixed overnight in paraformaldehyde, brought through a sucrose gradient prior to embedding in optimal cutting temperature compound, and frozen for cryosectioning. Sections of 10-μm thickness were cut, then fixed in 4% paraformaldehyde, and blocked with 3% donkey serum with 0.5% Tween 20 prior to immunostaining.

### 2.3.1 | Immunostaining

Immunohistochemistry staining was performed according to manufacturer's instructions using the VECTASTAIN ABC-Horseradish Peroxidase kit and Vector VIP substrate. Immunofluorescence staining was performed by first permeabilizing with 0.1% sodium citrate + 0.1% Triton X-100 in PBS and blocking with 3% bovine serum albumin at 37 °C for 1 to 2 hours, followed by incubation with the following primary antibodies overnight at 4 °C: goat antihuman/mouse MPO (AF3667, 1 μg/mL; R&D Systems), rabbit anticitrullinated histone H3 R2+8+17 (ab5103, 0.3-2 μg/mL; abcam), rat anti-Ly6G clone 1A8 (127602, 1 μg/mL; Biolegend), mouse anti-EPX clone AHE-1 (MAB1087, 5 μg/mL; ThermoFisher), mouse antieosinophil MBP antibody, clone BMK13 (CBL419, 5 μg/mL; ThermoFisher), rabbit anti–von Willebrand factor (VWF; A0082, 0.3 μg/mL [Dako]), and mouse anti-CD41 HIP8 clone (303702, 2 μg/mL; Biolegend). After 3 thorough washes with PBS, samples were incubated with their respective AlexaFluor secondary antibodies for 2 hours at room temperature at a concentration of 2 μg/mL, followed by 3 more washes with PBS. Samples were counterstained with 1:10000 diluted Hoechst 33342 (ThermoFisher) and mounted with Fluoromount medium or DAPI (300 nM, Life Technologies) for DNA.

### 2.3.2 | Imaging

All microscopic analysis of EET formation was performed using a Biotek Cytation 5 Cell Imaging Multimode Reader equipped with 4×, 20×, and 60× objectives; CCM camera with fluorescence and pseudocolor brightfield capability; and multiimage stitching capability (Gen5 software). Quantitative analysis was performed on stitched images using ImageJ software and thresholding on the signal of interest compared with total tissue to calculate percent of positively stained areas. All analysis was performed by an investigator blinded to the sample identities. Mouse thrombus mosaics were generated using a Hamamatsu Nanozoomer slide scanner or Zeiss Axioscan Z1 digital slide scanner at the VIB-KU Leuven LiMoNe BioImaging Core. High magnification mouse thrombus images were acquired using a Nikon eclipse Ti-2 confocal microscope equipped with AX camera. Images were processed using the NIS Elements imaging software (version 5.41.01).

Stroke thrombi were imaged using an Olympus IX81, FV300 confocal reflection microscope.

## 2.4 | *In vitro* assays

### 2.4.1 | Eosinophil isolation

Eosinophils were isolated from individual healthy volunteer donors from EDTA-anticoagulated whole blood using the MACSxpress Whole Blood Human Eosinophil Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. This kit involves negative selection for eosinophils with magnetic separation. Eosinophils were counted using an ADVIA 120 Hematology system (Siemens) and aliquots were taken for assessment of eosinophil purity by cytospin and flow cytometry. Cytospins were prepared and fixed with methanol, followed by staining for eosinophilic granules with Congo Red dye (Sigma). Cells were prepared for flow cytometric analysis with surface staining for CD45, CD11b, Siglec-8, CD15, and CD16 and run on a BD FACS Canto II instrument. Siglec-8 positivity, analyzed using FlowJo software (FlowJo LLC), was used to determine eosinophil purity of at least 90% for inclusion of experimental results in *in vitro* experiment analyses.

### 2.4.2 | Induction of EETs

Eosinophils were resuspended in RPMI-1640 (with L-glutamine and without phenol red), supplemented with 10-mM HEPES and fetal calf serum (70 °C heat-inactivated) at a cell density of 300 cells/μL, and plated in CellBind 96-well plates (Corning). A subset of cells was pretreated for 20 minutes with the PAD4 inhibitor GSK484 at a concentration of 10 μM (Sigma-Aldrich). Cells were allowed to settle and adhere for 30 minutes in an incubator at 37 °C and 5% $CO_2$ and then stimulated using 100-nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich), 3-μM ionomycin (Thermo Fisher), or activated platelets as described below. In a separate set of experiments, eosinophils were incubated with a multiplicity of infection of 100 bacteria (*Staphylococcus aureus* laboratory strains Newman or USA300,

coagulase-null USA300, or an infective endocarditis clinical isolate of *S aureus*; or *S epidermidis*) per cell. After 4 hours, cells were fixed overnight using 2% paraformaldehyde containing Hoechst 33342 (Invitrogen) at a concentration of 1 μg/mL.

#### 2.4.3 | Platelet isolation and activation

Platelet-rich plasma (PRP) was first prepared by centrifuging 3.2% citrate-anticoagulated (BD) blood at 175 g for 15 minutes at 22 °C and collecting the supernatant. To isolate platelets, PRP was supplemented with 2-μg/mL $PGI_2$ (Tocris Bioscience) and 0.02-U/mL apyrase (Sigma-Aldrich) and centrifuged at 1000 *g* for 10 minutes at 22 °C. The platelet pellet was subsequently washed in modified Tyrode's HEPES (MTH) buffer (containing 134 mM/L NaCl [VWR], 2.9 mM/L KCl, 0.34 mM/L $Na_2HPO_4$, 1 mM/L $MgCl_2$ [Merck Millipore], 12 mM/L $NaHCO_3$, and 20 mM/L HEPES [Sigma-Aldrich]; pH = 7.4) supplemented with 0.1% glucose (Merck Millipore), 0.35% bovine serum albumin (BSA) (Roche), 0.02-U/mL apyrase (Sigma-Aldrich), and 2-μg/mL $PGI_2$ (Tocris Bioscience). After an additional centrifugation step, the platelet pellet was resuspended to a final concentration of $6 \times 10^8$ platelets/mL in RPMI-1640 (with L-glutamine and without phenol red), supplemented with 10-mM HEPES and fetal calf serum (70 °C heat-inactivated), and allowed to rest for 30 minutes. After 30 minutes, platelet suspensions were stimulated with 0.1-μg/mL collagen (Chrono-log) or 100-mU thrombin (Sigma-Aldrich). Platelets were then incubated at a 200:1 ratio with eosinophils, which had been preplated in CellBind 96-well plates (Corning).

#### 2.4.4 | EET identification and quantification

Fixed plates were washed and permeabilized with sodium acetate buffer containing 0.1% Triton X-100. After blocking with 3% BSA for 1 hour at 37 °C, immunostaining was performed using 0.5-μg/mL citrullinated histone H3 antibody (ab5103, abcam) and anti-EPX antibody diluted 1:200 (Merck Millipore). After secondary antibody incubation at 1-μg/mL concentration (AF488 for H3Cit, AF555 for EPX) at room temperature for 2 hours and 3 washing steps, DNA was counterstained with Hoechst 33342 (Invitrogen). Five images per well were acquired and quantification was performed by an investigator blinded to the identities of the wells for number of H3Cit+ cells, as well as a separate quantification for extracellular DNA structures originating from EPX+ cells.

### 2.5 | Animal models

All animal experimental procedures were performed at KU Leuven in Belgium with prior review and approval of the KU Leuven Ethical Committee on Animal Research (file numbers: P189/2017 and P143/2019). Mice were obtained from an in-house breeding colony of C57BL/6J mice from the KU Leuven Laboratory Animal Center, with stock animals originally purchased from Charles River (France). All mice received 0.1-mg/kg buprenorphine as analgesia prior to surgery and twice daily via subcutaneous injection.

#### 2.5.1 | Inferior vena cava stenosis model of deep vein thrombosis

Thrombosis was induced in 6- to 8-week-old male mice under isoflurane anesthesia by ligation of the infrarenal segment of the inferior vena cava (IVC) using 7/0 polypropylene suture around a 30G spacer that was removed immediately after suture placement, resulting in stenosis as previously described [20]. Side branches were fully ligated and back branches were left untouched. After 48 hours, mice were anesthetized and thrombi collected by dissecting the IVC between the iliac and renal veins. Cervical dislocation was employed as a method of humane killing after thrombus harvest.

#### 2.5.2 | Inflammation-induced infective endocarditis at the aortic valve

Infective endocarditis was induced using a previously reported mouse model developed in Leuven [21]. Briefly, mice under ketamine/xylazine anesthesia received an intravenous injection of *S aureus*, and then, the carotid artery was immediately exposed for insertion of a catheter to the level of the aortic valve. Histamine was infused for 5 minutes to locally activate the endothelium, after which the catheter was removed and the incision sutured for mice to be followed up for 3 days. The aortic valve was isolated after perfusion of saline followed by 4% paraformaldehyde to fix the tissue for histologic analysis. Positive identification of infective endocarditis vegetations were performed by the Brown–Hopps method of Gram staining on tissue sections.

### 2.6 | Statistical analysis

Statistical analyses were performed and figures were made with GraphPad Prism version 9.5.0. All data were analyzed using nonparametric Mann–Whitney tests. Each datapoint represents the quantification of at least 200 cells imaged in duplicate wells from individual donors. The experiments were performed on 4 separate experimental days and combined for presentation and analysis.

## 3 | RESULTS

We examined a large, intact coronary thrombus retrieved from a 44-year-old patient with STEMI and a low TIMI risk score of 1. The thrombus originated from a plaque rupture in the proximal right coronary artery (RCA), and completely occluded the mid RCA segment. In this patient with atypical STEMI presentation, total occlusion of the RCA was evident by angiogram with contrast agent (Figure 1A). Removal of the thrombus via catheterization resulted in restoration of blood flow in the RCA (Figure 1B). Notably, this large thrombus was removed fully intact and measured approximately 2.5 cm in length (Figure 1C). Processing for pathology resulted in fracture of the thrombus into 2 pieces, but the overall structure remains

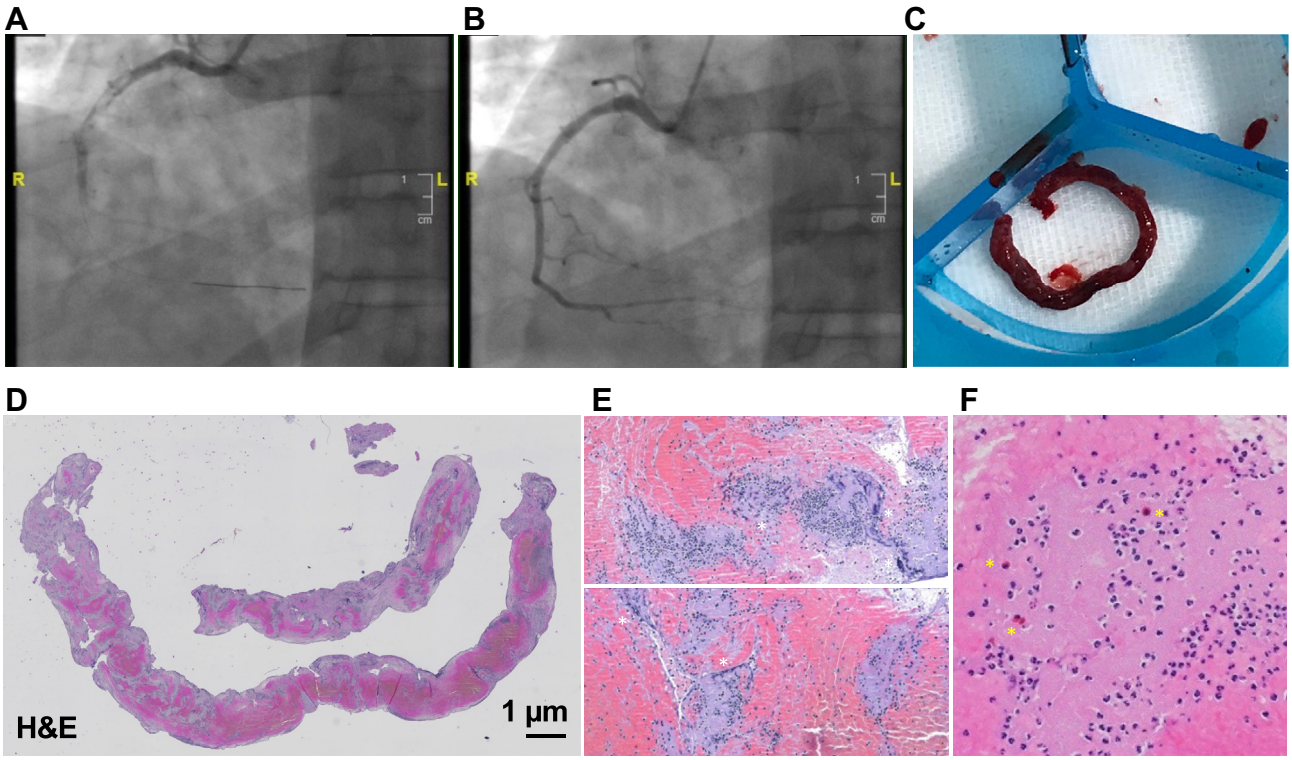


**FIGURE 1** Eosinophils identified in a coronary thrombus from a patient without eosinophilia. (A) Angiogram showing thrombus position within the right coronary artery. (B) Angiogram postthrombus aspiration shows restoration of blood flow. (C) Macroscopic image of entire, intact thrombus. (D) H&E staining on a cross section of the entire thrombus, with (E) magnification on areas showing extensive granulocytes and extracellular DNA (white asterisks) and (F) higher magnification showing positive identification of eosinophils (yellow asterisks) by the Basel University Hospital Pathology Department. H&E, hematoxylin and eosin.

visible. Histopathology analysis of the H&E-stained thrombus revealed part of the thrombus with alternating layers (laminations) of platelets mixed with fibrin (which appear lighter) and darker layers of red blood cells (Figure 1D). Areas of higher cell nucleus density also had associated extracellular nucleic acid patterns (Figure 1E). Numerous neutrophilic and fewer binucleated eosinophilic granulocytes with bright red granulated cytoplasm were entrapped in the lighter layers (Figure 1F). Notably, the patient's differential blood count showed a normal eosinophil count. His past medical history was unremarkable for autoimmune diseases and allergies.

We further performed more in-depth characterization of the thrombus structure using immunostaining with specific antibodies. Clusters of platelets, identified by CD41 staining, and neutrophils, identified by MPO staining, were the predominant cell types throughout the thrombus. Regions of extracellular VWF and citrullinated histone H3 (H3Cit) were found at the interface of areas of high platelet or neutrophil content, respectively (Figure 2A). Eosinophils were also evident in areas of H3Cit positivity, as identified by EPX staining. Higher magnification analysis showed that H3Cit-positive areas comprised both MPO-H3Cit-DNA positivity (Figure 2B) as well as separate areas of EPX-H3Cit-DNA positivity (Figure 2C).

We then queried if this type of H3Cit-positive EET or eosinophil prevalence was present in other arterial thrombi and therefore examined 12 thrombi collected from patients with ischemic stroke through mechanical thrombectomy and previously characterized for presence of NETs [19]. From these, 8 out of 12 clots contained eosinophils. Four of 12 clots had no or very rare eosinophils. Two of the 12 clots had clear H3Cit positivity identified in eosinophils (Figure 3A). Six of the clots had eosinophils either in the vicinity of (Figure 3B) or distinct from H3Cit-positive cellular staining (Figure 3C). From this, we concluded that H3Cit positivity occurs but is not always associated with eosinophil presence in stroke clots, thus providing additional evidence for EETs in arterial thrombi.

EETs being present in fresh, occlusive coronary or cerebral clots are naturally interesting as potential therapeutic targets. Therefore, we aimed to reveal the underlying mechanism leading to EET formation in coronary thrombi. We applied an *in vitro* approach and isolated eosinophils from healthy volunteers. Stimulation with PMA or ionomycin led to robust substantial EETosis together with evidence of histone hypercitrullination (Figure 4A). The underlying molecular mechanism of EET release via stimulation with activated platelets has not yet been studied. The enzyme PAD4 has not only been shown to be involved in the release of ETs from neutrophils [7] but also presents a possible therapeutic target using PAD4 inhibitors [18,22]. We therefore tested the effect of the selective PAD4 inhibitor GSK484 in this *in vitro* assay. The EET-related activation of eosinophils was significantly inhibited with GSK484, indicating a role for PAD4 in this process (Figure 4B, C). In parallel to these nonphysiological agonists,

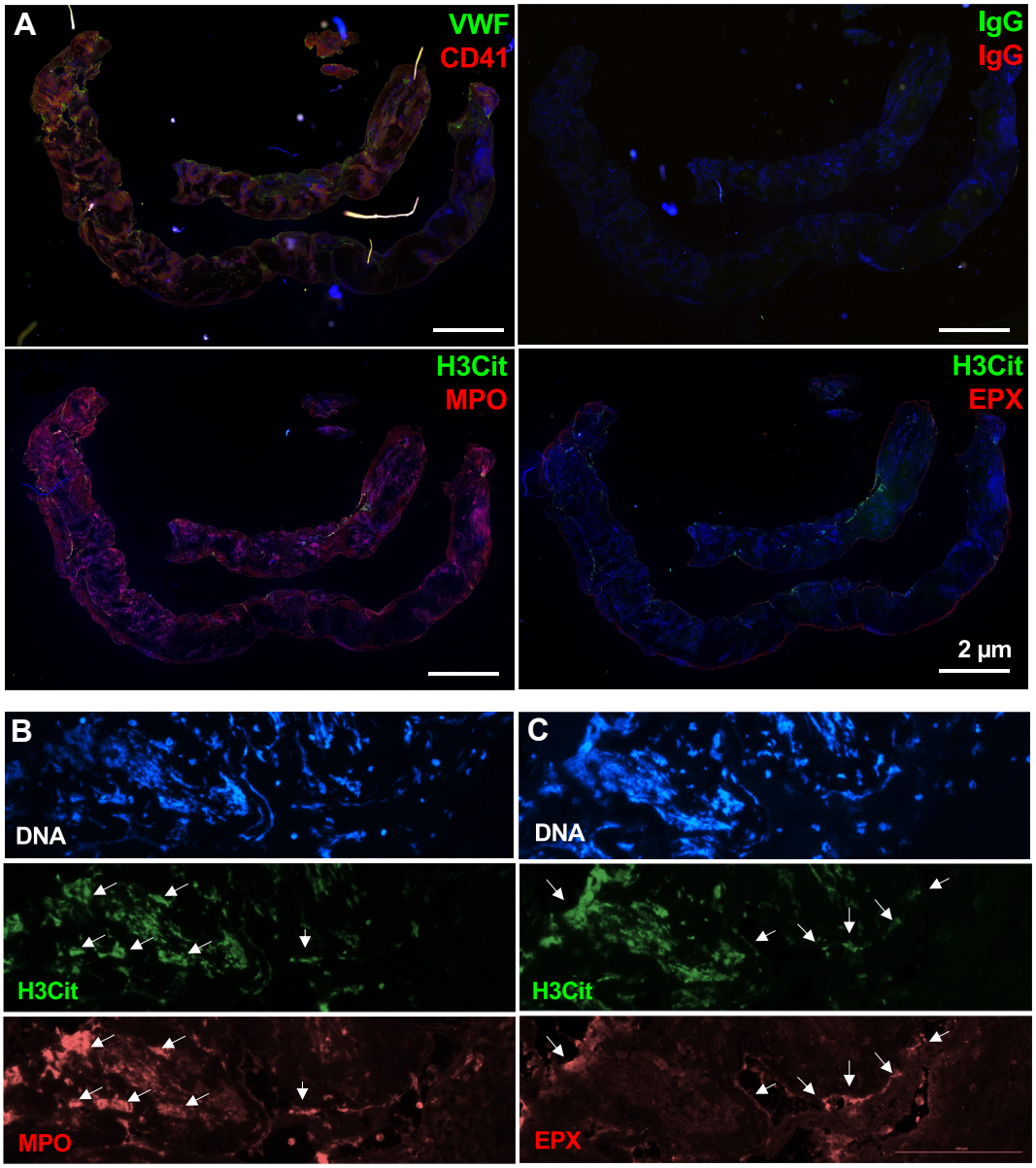


**FIGURE 2** Thrombus characteristics and EETs identified with extracellular H3Cit positivity. Immunostainings at the whole thrombus level (A) showing a platelet- and neutrophil-rich thrombus with distinct areas of VWF and extracellular DNA/citrullinated histones. Representative higher magnification areas of extracellular H3Cit positivity originating from both neutrophils (B) and eosinophils (C). EET, eosinophil extracellular trap; EPX, eosinophil peroxidase; H3Cit, citrullinated histone H3; Ig, immunoglobulin; MPO, myeloperoxidase; VWF, von Willebrand factor.

eosinophils were stimulated with activated platelets to mimic the *in vivo* stimulation likely occurring during plaque rupture and coronary thrombosis in acute coronary syndrome as closely as possible. Washed platelets were preactivated with either collagen or thrombin. Stimulation with activated platelets also led to release of H3Cit+ EETs compared with that with platelet-only controls (Figure 4D), which was also reduced in the presence of PAD4 inhibitors (Figure 4E, F).

We have previously reported the abundant presence of H3Cit+ NETs in animal models of (immuno)thrombosis, including deep vein thrombosis through IVC stenosis [23] and infective endocarditis at the aortic valve [21]. Systemic PAD4 deficiency resulted in a loss of H3Cit signal in IVC stenosis [7], while neutrophil-selective PAD4 deficiency resulted in a substantial but not total reduction in H3Cit signal in infective endocarditis [24]. We therefore performed costainings for neutrophils or eosinophils together with citrullinated histones to see if we could identify any EETs as part of this H3Cit-positive staining. After 48 hours of IVC ligation, a time at which the cellular content of thrombi consists primarily of neutrophils, we observed that the vast majority of H3Cit staining was associated with MPO+ neutrophils and not eosinophils (Supplementary Figure S3A–D). This was confirmed with Ly6G/H3Cit costaining (Supplementary Figure S3C) and absence of colocalized H3Cit/Siglec-F signal. In fact, very few to no eosinophils were identified per thrombus. The H3Cit signal after 48 hours of IVC stenosis thus originates primarily from neutrophils.

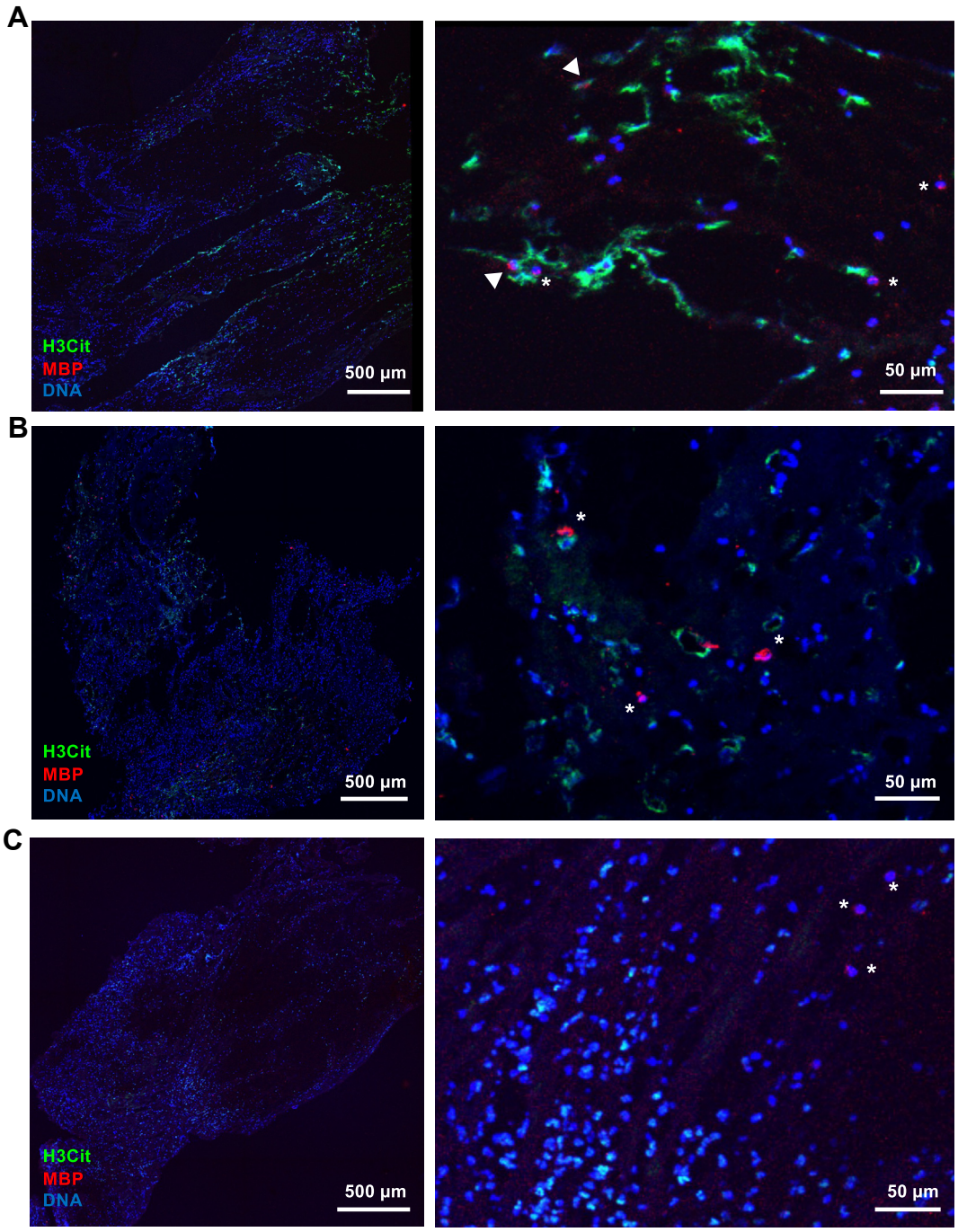


**FIGURE 3** H3Cit-positive eosinophil extracellular traps in stroke thrombi. Representative images showing the patterns identified in ischemic stroke thrombi of extracellular H3Cit-positive EETs and eosinophils in the proximity of H3Cit-positive areas in ischemic stroke thrombi. Identified patterns include H3Cit-positive EETs and eosinophils with H3Cit-positivity in nuclei (panel A; arrowheads), eosinophils in proximity to H3Cit-positive cells (B), and eosinophils distinct from H3Cit-positive areas (C). Asterisks indicate positive identification of eosinophils. MBP, H3Cit, and DNA are indicated in red, green, and blue, respectively. EET, eosinophil extracellular trap; H3Cit, citrullinated histone H3; MBP, major basic protein.

EET release has also been identified in the context of infection. We therefore aimed to identify if PAD4 also contributes to pathogen-induced EET release. In a model of infective endocarditis where we have reported extensive H3Cit positivity [24], very few eosinophils were identified (Supplementary Figure S3E). We investigated whether *Staphylococci* were capable of inducing H3Cit-positive EETs. In contrast to previous reports performed with tissue eosinophils, we found that both *S aureus* and *S epidermidis* induced release of EETs from peripheral blood eosinophils (Supplementary Figure S3F). This was accompanied with H3Cit+ signal both in intact eosinophils and in extracellular structures and was surprisingly most pronounced in *S epidermidis*–treated eosinophils. In comparison, we have recently

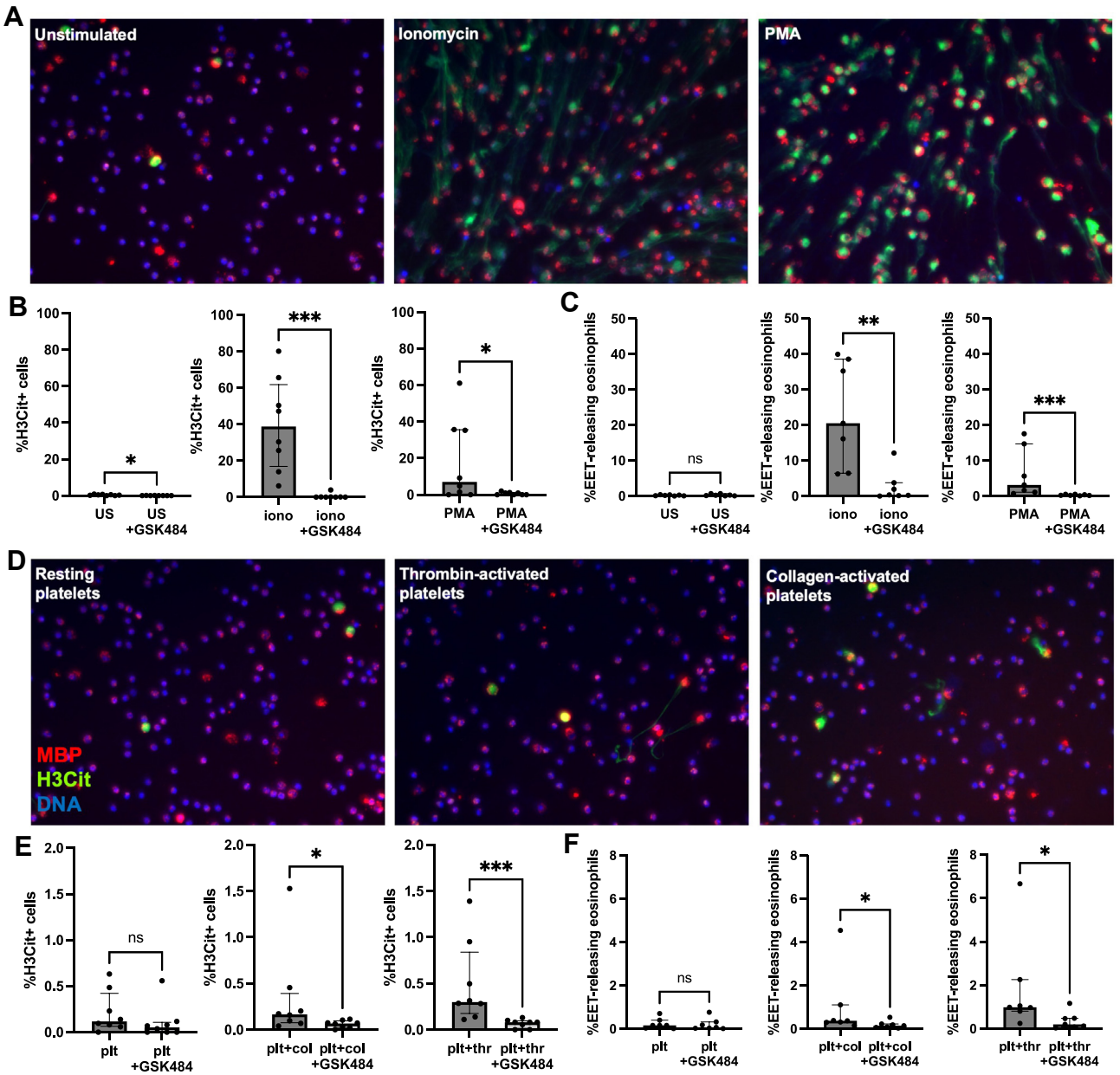


**FIGURE 4** H3Cit-positive eosinophil extracellular trap release is dependent on PAD4 enzyme activity *in vitro*. (A–C) Human eosinophils were isolated and stimulated *in vitro* to undergo EETosis using ionomycin or PMA. Representative images in panel A show H3Cit+ cells and extracellular structures originating from EPX+ cells. H3Cit+ cells (B) and EETs were reduced upon PAD4 inhibition with GSK484 20 minutes prior to stimulation. (D–F) Thrombin-activated or collagen-activated platelets were coincubated with isolated eosinophils to mimic *in vivo* EETosis in thrombotic coronary occlusion. Representative images in panel D show that EETs produced in response to activated platelets are also H3Cit+. H3Cit+ cells (E) and EETs (F) were reduced upon PAD4 inhibition with GSK484 20 minutes prior to stimulation. $n$ = 7-8, Mann–Whitney's $U$ tests. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.EET, eosinophil extracellular trap; EPX, eosinophil peroxidase; H3Cit, citrullinated histone H3; PMA, phorbol-12-myristate-13-acetate.

reported that neutrophils incubated with *S epidermidis* fail to release NETs [24]. The contribution of EETs to cardiovascular diseases involving *S aureus* and *S epidermidis*, such as infective endocarditis where NETs have been identified [25], is an additional avenue to explore in future studies and may be a target for PAD4 inhibition. However, modeling this *in vivo* will be challenging provided our lack of evidence of eosinophils in a relevant mouse model.

## 4 | DISCUSSION

The presence of eosinophils in coronary thrombi is well described [26–29], and hypereosinophilic syndromes are associated with a strongly elevated risk of thrombotic events, including in patients with clonal hematopoiesis of indeterminate potential [30], which has been implicated in cardiovascular disease risk including CAD and stroke

[31] Several studies have aimed to explain how eosinophils may contribute to thrombosis by exposing tissue factor [32,33] and releasing MBP, which can activate platelets [34] and directly inhibit the endothelial anticoagulant protein thrombomodulin [35]. A key study identified eosinophils as important contributors to thrombus formation in the ferric chloride injury–induced model of DVT [33]. Using two distinct eosinophil-deficient mouse lines or by antibody-mediated eosinophil depletion, they saw reduced thrombus size. In dblGATA1 mice, which were also examined in ferric chloride injury to the carotid artery, mice had lower levels of thrombin–antithrombin complexes and less stable thrombi despite similar time to occlusion. Finally, prolonged bleeding time in eosinophil-deficient animals in the tail bleeding model supports a broader role for eosinophils in hemostasis. Whether PAD4 or EET release is also related to TF exposure remains to be explored in future studies.

EPX activity has also been implicated by thrombosis by acting on substrate thiocyanate, resulting in production of the oxidant hypothiocyanous acid (HOSCN), which can activate endothelial cells to produce tissue factor [36]. This distinguishes it from MPO present on NETs. In addition, intact eosinophils have been found within areas of H3Cit positivity in coronary thrombi [37] and can release EETs that contribute to coronary atherothrombosis, promote atherosclerosis, and stabilize thrombosis [15].

ETs can be formed by several other immune cells including neutrophils, mast cells, and macrophages. Studies on molecular mechanisms of ET release in those cell types are incomplete, but may be of clinical relevance for treatment and prevention of thrombosis. In ETs from neutrophils, for example, the enzyme PAD4 catalyzes histone modifications important for NET release [38]. The presence of citrullinated histones and NETs is significantly reduced in neutrophils from PAD4-deficient animals stimulated *ex vivo* for NETosis [6,7]. The impact of specific PAD4 inhibition has not been studied in eosinophils. Given the eosinophil and H3Cit+ EET presence in the freshly harvested thrombi, and their prothrombotic implications, we therefore examined if PAD4 could be sufficient for EET release. This was the case in EET formation induced by PMA, the calcium ionophore ionomycin, and by platelets activated with either thrombin or collagen.

We found H3Cit+ EETs in a fresh coronary thrombus and in ischemic stroke thrombi, and also confirmed *in vitro* inducibility of EETs using activated platelets, mimicking coronary thrombus formation after plaque rupture and a key contributor to stroke development *in vivo*. The platelet-induced induction of EETosis was PAD4-dependent as supported by significant suppression after treatment with the specific PAD4 inhibitor GSK484. We opted to use GSK484 over other GSK inhibitors such as GSK199 for this study due to its lower $IC_{50}$ of 50 nM in a binding assay to the substrate BAEE compared with that for GSK199 (200 nM) [18]. This is likely due to its cyclopropyl group. Here, we observed that the inhibitory effect of GSK484 was stronger for eosinophils than what was reported for primary human neutrophils [18], which is in line with the robust staining for H3Cit in EETs.

This finding may be of therapeutic interest given the fact that EETs stabilize thrombi. PAD4 inhibition likely would inhibit not only NET but also EET release, potentially resulting in a reduced risk of stable coronary thrombi. PAD4 inhibition in a patient with an existing coronary thrombus may also destabilize the thrombus and leave it more accessible to tPA-mediated thrombolysis. PAD4 inhibition is also unlikely to yield increasing bleeding risk, as mice lacking PAD4 systemically have normal blood coagulation and platelet plug formation *in vivo* [7].

However, the PAD4-catalyzed pathway may certainly not be the only one to induce EETs. For NET formation, it is clear that no one factor is solely responsible and that other pathways may be able to supersede PAD4's role. Another pathway via neutrophil elastase, which migrates to the nucleus and cleaves histones to allow chromatin expansion [39], may lead to NETosis independently of PAD4 [40]. In a mouse model of venous thrombosis, NE deficiency or use of a selective NE inhibitor limited neither NET release nor thrombus formation *in vivo*, despite a modest decrease in NET release *in vitro* [41]. Also, as mentioned before, ETs expelled from other cell types may have similar prothrombotic effects through different, so far undetected enzymes and pathways. Therefore, more research is needed to show whether PAD4 inhibition sufficiently suppresses ET formation in arterial thrombi in a manner sufficient to be of therapeutic interest. Furthermore, further investigation into whether this is a phenomenon relevant to venous thrombosis is also warranted. Our current study supports the possibility of PAD4 inhibition in the reduction of not only NET but also EET contribution to thrombosis.

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## AUTHOR CONTRIBUTIONS

K.M. and T.W. designed and supervised the study and wrote the manuscript. K.M., F.D., S.M., M.C., S.V.B., M.S., P.S., and K.G. performed experiments, analyzed data, and critically reviewed the manuscript. R.G., K.G., and T.W. recruited patients and obtained thrombi for histologic analysis.

## DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

## DATA AVAILABILITY

All data generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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## SUPPLEMENTARY MATERIAL

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