Ann Rheum Dis 000 (2025) 1–15

Contents lists available at ScienceDirect

Annals of the Rheumatic Diseases

journal homepage: https://www.sciencedirect.com/journal/annals-of-the-rheumatic-diseases

Antiphospholipid syndrome

CD40-CD40L inhibition attenuates platelet-neutrophil interaction and neutrophil extracellular trap release in primary antiphospholipid syndrome

Stavros Naoum[1,2], Charilaos Spyropoulos[2], Andriani Angelopoulou[3], Harikleia Gakiopoulou[4], Michalis Katsimpoulas[5], Vassilis G. Gorgoulis[3,5,6,7], Petros P. Sfikakis[1], Konstantinos Ritis[8], Ioanna-Evdokia Galani[5], Konstantinos Kambas[2], Maria G. Tektonidou[1,*]

[1] *First Department of Propaedeutic Internal Medicine, School of Medicine, National and Kapodistrian University of Athens, Joint Academic Rheumatology program, Athens, Greece*
[2] *Laboratory of Molecular Genetics, Department of Immunology, Hellenic Pasteur Institute, Athens, Greece*
[3] *Molecular Carcinogenesis Group, Department of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Athens, Greece*
[4] *First Department of Pathology, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece*
[5] *Biomedical Research Foundation Academy of Athens, Athens, Greece*
[6] *Ninewells Hospital and Medical School, University of Dundee, Dundee, UK*
[7] *Molecular and Clinical Cancer Sciences, Manchester Cancer Research Centre, Manchester Academic Health Sciences Centre, University of Manchester, Manchester, UK*
[8] *First Department of Internal Medicine and Laboratory of Molecular Hematology, Department of Medicine, Democritus University of Thrace, Alexandroupolis, Greece*

ARTICLE INFO

Article history:
Received 19 September 2025
Received in revised form 19 December 2025
Accepted 20 December 2025

ABSTRACT

Objectives: Neutrophil extracellular traps (NETs) are increasingly recognised for their role in primary antiphospholipid syndrome (PAPS) pathogenesis. Herein, we examined underlying mechanisms driving NET formation and the thrombogenic effects of NETs in patients with PAPS, along with potential inhibitors.

Methods: We examined NET release in PAPS, asymptomatic antiphospholipid autoantibodies (aPLs) carriers, and healthy controls (HCs) as well as NET-bound proteins by immunofluorescence, immunoblotting, quantitative polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA). We assessed platelet-neutrophil aggregates by flow cytometry (CD61/CD66b staining) and autophagy using LC3B immunofluorescence and immunoblotting. Immunofluorescence was performed in paraffin-embedded kidney, skin ulcer, and endoarterial thrombus tissues from patients with PAPS. Suppression of NET release via CD40-CD40L inhibition was tested in *in vitro* and venous thrombosis mouse models.

Results: Neutrophils from patients with PAPS exhibited increased NET release compared with asymptomatic aPL carriers and HCs. NETs from patients with PAPS expressed tissue factor (TF) that induced thrombin generation in platelet-poor plasma from HCs. aPL induced *in vitro*

*Correspondence to Dr. Maria G. Tektonidou, First Department of Propaedeutic Internal Medicine, School of Medicine, National and Kapodistrian University of Athens, Laiko Hospital, Athens, Greece.

E-mail addresses: mtektonidou@gmail.com, mtektonidou@med.uoa.gr (M.G. Tektonidou).

Konstantinos Kambas and Maria G. Tektonidou are senior authors.

Handling editor Josef S. Smolen.

https://doi.org/10.1016/j.ard.2025.12.012

intracellular TF expression in HC neutrophils. TF-expressing NETs were abundant in the kidney, skin ulcer, and endoarterial thrombus tissues from patients with PAPS, and colocalised with fibrinogen. NET release in PAPS neutrophils was driven by aPL-mediated platelet activation, subsequent platelet-neutrophil interaction, and autophagy induction. CD40-CD40L blockade reduced platelet activation, autophagy, and NET formation in patients with PAPS. In a mouse model, inhibition of CD40-CD40L reduced neutrophil-platelet aggregates and myeloperoxidase (MPO)-DNA complexes in mouse peripheral blood, and the presence of NETs in the formed thrombi.

Conclusions: Targeting the platelet-neutrophil/autophagy/TF-expressing NETs axis by CD40-CD40L inhibition attenuates thromboinflammation in PAPS and should be explored as a potential therapeutic target.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- The involvement of neutrophil extracellular traps (NETs) in thromboinflammation pathways in primary antiphospholipid syndrome (PAPS) is gaining increased attention, but there are still unanswered questions concerning the mechanisms driving NET release and thrombogenic effects of NETs, as well as potential inhibitors.

WHAT THIS STUDY ADDS

- NETs from patients with PAPS are decorated with bioactive tissue factor and induce thrombin generation.
- Tissue factor (TF)-expressing NETs are abundant in kidney, skin ulcer, and endoarterial thrombus tissues from patients with PAPS, and colocalised with fibrinogen.
- NET release in neutrophils from patients with PAPS is driven by antiphospholipid antibody-mediated platelet activation, subsequent platelet-neutrophil interaction, and autophagy induction.
- CD40-CD40L blockade in *in vitro* cultures reduces platelet activation, autophagy, and NET formation in patients with PAPS. In a mouse model, inhibition of CD40-CD40L reduces neutrophil-platelet aggregates and MPO-DNA complexes in mouse peripheral blood and the presence of NETs in the formed thrombi.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- These findings provide compelling proof-of-concept data that targeting the CD40-CD40L pathway can disrupt platelet-neutrophil interactions and mitigate thromboinflammation in PAPS.
- CD40-CD40L inhibition can represent a promising therapeutic target in PAPS. Its role should also be explored in other thromboinflammation-associated conditions.

INTRODUCTION

Antiphospholipid syndrome (APS) is a complex, systemic autoimmune disorder with a poorly understood pathophysiology. APS is characterised by recurrent arterial and venous thrombosis, microvascular manifestations, and pregnancy morbidity in the presence of antiphospholipid autoantibodies (aPLs), namely lupus anticoagulant (LA), anticardiolipin (aCL), and anti-β2 glycoprotein I (anti-β2GPI) antibodies [1]. Although earlier studies of APS pathogenesis were mainly focused on coagulation and fibrinolysis disruption processes, an interplay between thrombotic mechanisms and innate immune responses is increasingly recognised [2–9]. The involvement of neutrophil extracellular traps (NETs) in thromboinflammation pathways in primary APS (PAPS) is gaining increased attention [2], but there are still unanswered questions concerning the mechanisms driving NET release and thrombogenic effects of NETs, as well as potential inhibitors.

NETs decorated with bioactive tissue factor (TF), a main initiator of the extrinsic blood coagulation cascade, have been demonstrated in inflammatory conditions [eg, sepsis, acute myocardial infarction, and antineutrophil cytoplasmic antibody (ANCA) vasculitis] [10–12] and in systemic lupus erythematosus (SLE), a systemic autoimmune disorder often co-occurring with APS [13]. However, the expression of bioactive TF on NETs from patients with PAPS needs to be further elucidated.

A key intracellular mechanism in the regulation of NET generation is autophagy [11,14], a homeostatic mechanism for the elimination of harmful or damaged intracellular components. NET release has been shown to be mediated by increased levels of basal autophagy in SLE [13], but its role in the release of NETs in PAPS remains unexplored. Platelet-neutrophil complexes (PNCs), resulting from the interaction between neutrophils and activated platelets, have been shown to induce neutrophil autophagy and NET formation [15].

Interactions between platelets and neutrophils are mediated by P-selectin ligand and P-selectin, CD40 ligand (CD40L) and CD40, intracellular adhesion molecule 2 (ICAM-2) and integrin $\alpha L\beta 2$, and triggering receptor expressed on myeloid cells 1 (TREM-1) ligand (TREM-1L) and TREM-1 on platelets and neutrophils, respectively. It has been recently reported that PNCs might enhance the APS IgG-mediated NET release in a P-selectin-dependent manner [16]. CD40L enhances P-selectin activity on platelets and Mac-1 expression on neutrophils [17]. CD40-CD40L pathway acts as an amplifier of thromboinflammation pathways [18–20], and its inhibition has been examined as a targeted therapy in cardiovascular disease [21,22].

Herein, we aimed to examine: (1) NET release in patients with PAPS and asymptomatic aPL-positive individuals, (2) thrombogenicity of NETs through TF expression on NETs in both circulating neutrophils and organ tissues of patients with PAPS, (3) the role of aPL in platelet activation and subsequent platelet-neutrophil interaction for the generation of NETs, (4) autophagy as a potential mediator of NET release, and (5) the effect of inhibition of the CD40-CD40L axis as a mediator of the platelet-neutrophil cross-talk, both *in vitro* and in an APS murine model.

METHODS

In total, 30 patients fulfilling the classification criteria for thrombotic PAPS [1,23], 11 age- and sex-matched healthy controls (HCs), and 5 asymptomatic aPL individuals with positive aPL but without clinical criteria for APS (median duration of aPL positivity: 7 [6-9] years) were included in this study. None fulfilled the European Alliance of Associations of

Rheumatology/American College of Rheumatology (EULAR/ACR) classification criteria for SLE [24]. Additionally, sera from a disease control group that did not have underlying autoimmune disorders or aPL positivity were collected: 7 patients with acute arterial thrombosis (3 with acute stroke and 5 with ST-segment elevation acute myocardial infarction [STEMI]) and 8 patients with acute venous thrombosis; 1 patient had simultaneous arterial (stroke) and venous thrombosis. Participant characteristics and inclusion/exclusion criteria are presented in the Supplementary Table S1. None was on glucocorticoid or immunosuppressive treatment. The study was approved by the Ethics Committee of Laiko General Hospital (protocol number 398/31-05-2021) in compliance with the Declaration of Helsinki. All participants provided written informed consent. aPL quantification and aPL profile were determined as described in Supplementary Methods.

Venous blood samples were collected in EDTA and heparin-containing tubes for the study of neutrophils and NETs, in sodium citrate tubes for the study of platelets and isolation of platelet-poor plasma (PPP), and in serum-separating tubes for serum isolation. Polymorphonuclear neutrophils (PMNs) were isolated using Percoll for stimulation and inhibition studies. To investigate the bioactivity of NET-bound TF, thrombin ELISA was carried out on isolated NET structures from patients with PAPS. To inhibit the formation of NETs, isolated neutrophils were pretreated with the early-stage autophagy inhibitor SC79, whereas for the inhibition of CD40-CD40L interaction the inhibitor DRI-C21045 (at C = 10 μM) was used. Total IgG fractions were isolated and purified from 6 patients with triple aPL +ve PAPS and 2 HCs for the *in vitro* stimulation of neutrophils and platelets. NETs were visualised by confocal microscopy using immunofluorescence. MPO/DNA complex ELISA was carried out on isolated NET structures for the quantification of NET release. *TF* mRNA expression was assessed by quantitative real-time PCR. Neutrophil lysates and NET proteins were isolated for the study of TF and microtubule-associated protein 1 light chain 3B (LC3B) expression, using western blotting. Peripheral blood samples in heparin were used for the study of platelet-activated neutrophil aggregates, using flow cytometry. For *in vivo* CD40-CD40L inhibition, we utilised a thrombotic APS murine model, through inferior vena cava (IVC) ligation, as previously described [25]. More details on these methods are provided in Supplementary Methods.

Moreover, archival formalin-fixed, paraffin-embedded kidney (n = 5), skin ulcer (n = 4), and endoarterial thrombus (n = 2) tissues from patients with PAPS, along with kidney samples from the healthy parenchyma from 2 individuals who underwent nephrectomy for renal masses diagnosed as oncocytomas (used as 'healthy' controls) and a skin biopsy from 1 HC were examined by immunofluorescence microscopy to detect neutrophils, NETs and NET-bound TF, or fibrinogen. We also examined as disease controls, formalin-fixed paraffin-embedded kidney biopsies from 4 patients with acute renal thrombotic microangiopathy (2 patients with haemolytic uremic syndrome, 1 with thrombotic thrombocytopenic purpura, and 1 with Hemolysis, Elevated Liver enzymes and Low Platelets syndrome), a skin ulcer biopsy from a patient with diabetes mellitus, and endoarterial thrombus tissues retrieved during thrombectomy from 2 patients with acute femoral artery atherothrombosis. None of these patients fulfilled criteria for APS or any other autoimmune disorder, nor did they have positive aPL.

A more detailed description of the Methods is presented in Supplementary Methods.

Statistical analysis

For continuous variables, Wilcoxon (paired t-test) and Mann-Whitney (unpaired t-test) were used, after implementing the Shapiro-Wilk normality test. For comparison of more than 2 groups, 1-way analysis of variance (ANOVA, Tukey's multiple comparison test) with Bonferroni post hoc or Kruskal-Wallis was applied. Data analysis was performed using GraphPad Prism v9.0 software (www.graphpad.com). Data are presented as mean ± SD. Statistical significance was considered at $P < .05$.

RESULTS

Higher NET release in patients with PAPS compared with HCs and asymptomatic aPL individuals

Neutrophils from patients with PAPS demonstrated higher spontaneous NET release than neutrophils from age- and sex-matched HCs and asymptomatic aPL carriers, as assessed by immunofluorescence staining in *ex vivo* neutrophils (Fig 1A,B) and MPO-DNA complex ELISA in isolated NET structures (Fig 1C). In a 3-month follow-up, no significant difference was observed in the *ex vivo* generation of NETs in patients with PAPS (Fig 1D).

No correlation was demonstrated between NET release and any specific aPL type, isotype, or titres, or venous or arterial thrombosis history in patients with PAPS (Supplementary Fig Sl). Given that phosphatidylserine/prothrombin (aPS/PT) autoantibodies have been associated with elevated thrombotic risk and their presence can help identify individuals at higher risk [26] beyond the criteria aPL (aCL, anti-β2GPI, and LA), we measured aPS/PT IgG and IgM titres in isolated sera from all patients with PAPS included in the study (n = 30). aPS/PT antibodies did not correlate with NET release (Supplementary Figure S1K).

Overall, these findings suggest that patients with PAPS have persistently higher spontaneous NET release than HCs and asymptomatic aPL individuals.

NETs from patients with PAPS are decorated with bioactive TF

Using immunofluorescence staining, we observed that NETs from 9 of 30 patients with PAPS expressed TF (Fig 1E). These findings were verified by immunoblotting on isolated NET proteins. TF expression in immunoblotting was significantly higher in patients who also demonstrated TF expression by immunofluorescence staining (Fig 1F).

We further assessed the ability of NET-bound TF from patients with PAPS to generate thrombin, using *in vitro* stimulations of PPP from HCs with isolated NET structures. We found that NET structures isolated from PAPS neutrophils induced thrombin generation in PPP from HCs, as assessed by thrombin ELISA assay. This effect was dependent on NET-bound TF, since TF inhibition with an anti-TF neutralising antibody abrogated the generation of thrombin. Interestingly, NETs from patients with PAPS with no prominent TF expression did not induce thrombin generation (Fig 1G).

TF-bearing NETs and fibrinogen are present in arterial thrombi, skin ulcer, and kidney tissues from patients with PAPS

To gain further insight into the role of NETs in thrombus development and organ injury in PAPS, we examined the presence of neutrophils and NETs in acute arterial thrombi, skin ulcer, and kidney biopsies from patients with PAPS, in

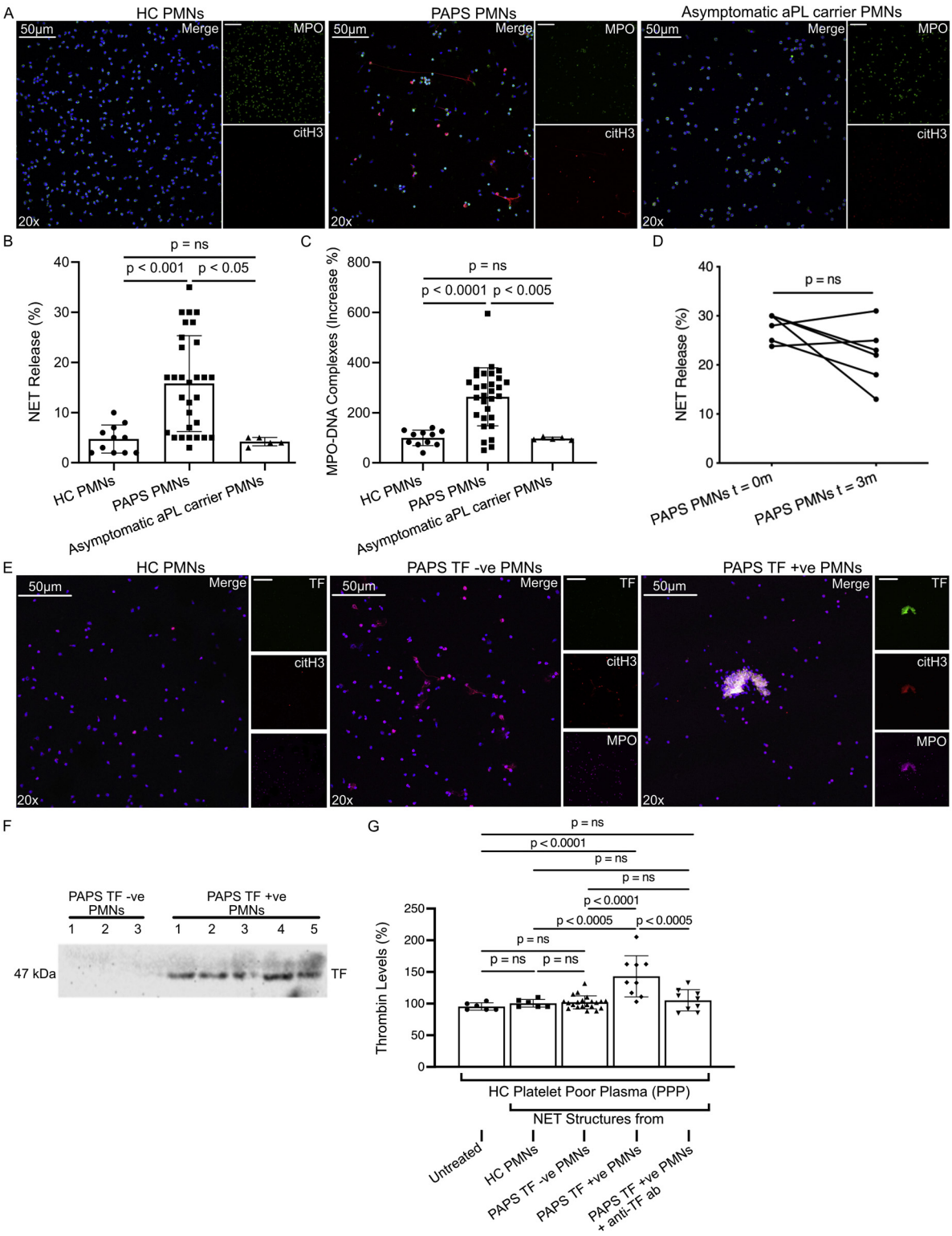

Figure 1. Increased spontaneous NET release is observed in neutrophils from patients with PAPS. (A) NET release by isolated peripheral neutrophils from patients with PAPS (n = 30) compared with HCs (n = 11) and asymptomatic aPL carriers (n = 5). Representative data from 1 of 30 patients with PAPS, 1 of 11 HCs, and 1 of 5 asymptomatic aPL carriers are shown. (B) Quantification of NET release in (A). (C) MPO-DNA complex enzyme-linked immunosorbent assay (ELISA) in isolated NET structures from neutrophils in (A). (D) NET release in neutrophils from patients with PAPS at t = 0 and 3 months later. (E) Expression of TF on NETs released by isolated peripheral neutrophils from patients with PAPS compared with HCs. Representative data from 2 out of 30 patients with PAPS are shown. (F) TF expression in purified NET proteins from isolated peripheral neutrophils from patients with PAPS (n = 8). (G) Thrombin generation levels in HC platelet-poor plasma treated with NET structures from patients with PAPS (n = 30) and HCs (n = 6). Representative data from 1 of 4 independent experiments. Green: (A) MPO, (E) TF, red: (A, E) citH3, magenta: (E) MPO, blue: (A, E) DAPI/DNA. For (B, C, G), statistical analysis was performed using ordinary 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For (D), statistical analysis was performed using the Wilcoxon signed-rank test for paired samples. For (B, C, G), bars represent mean values ± SD, and each dot represents a patient. ANOVA, analysis of variance; citH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAPS, primary antiphospholipid syndrome; TF, tissue factor.

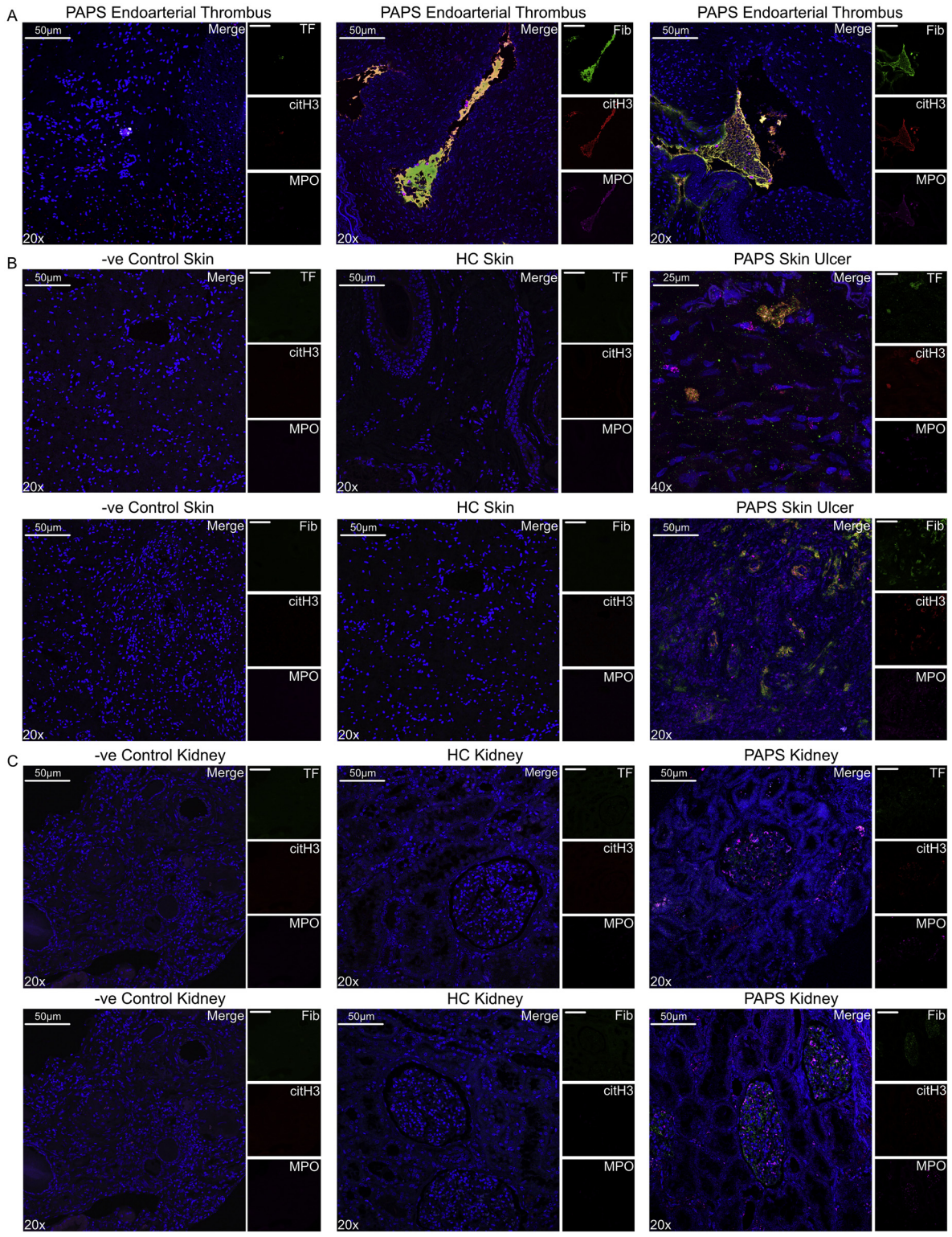

Figure 2. TF-bearing NETs and fibrinogen are identified in organised endoarterial thrombi, skin ulcer tissues, and kidney biopsies from patients with PAPS. (A) TF-bearing NETs and fibrinogen in an organised arterial thrombus from a patient with PAPS. Representative data from 1 of 2 patients. (B) TF-bearing NETs and fibrinogen in skin ulcer biopsies from patients with PAPS compared with skin biopsies from HCs. −ve control: secondary antibody control. Representative data from 1 of 4 patients. (C) TF-bearing NETs and fibrinogen in renal biopsies from patients with PAPS with renal involvement compared with kidney biopsies from apparently HCs (without autoimmune disorders). −ve control: secondary antibody control. Representative data from 1 of 5 patients. Green: TF/fibrinogen (Fib), red: citH3, magenta: MPO, blue: DAPI/DNA. ANOVA, analysis of variance; citH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAPS, primary antiphospholipid syndrome; TF, tissue factor.

comparison with skin and renal biopsies from individuals without a diagnosis of autoimmune disorder, serving as controls. Neutrophils and NETs were present in the arterial thrombi (Fig 2A) and skin ulcer biopsies from patients with PAPS (Fig 2B). NETs were often colocalised with TF and fibrinogen in all patients (Fig 2A,B). In the case of arterial thrombi, NETs were found within the organised thrombus in the arterial lumen, but also in the thrombi of the recanalised lumen (Fig 2A). In biopsies from skin ulcers, NETs and neutrophils were present, both inside the thrombotic vessels and in the perivascular space. However, they were also present in the reticular dermis, to a lesser extent. Skin biopsies obtained from HCs did not demonstrate any presence of neutrophils or NETs (Fig 2B).

Neutrophils and NETs were also observed in kidney biopsy tissues from patients with PAPS with renal involvement. Specifically, NETs colocalised with TF or fibrinogen were detected in the glomerular capillaries, in the peritubular capillaries, the tubulointerstitial tissue close to the Bowman's capsule, and within thrombosed arterioles, even when intact neutrophils were not visible (Fig 2C). Conversely, neutrophils and NETs were not detected in kidney biopsies from the healthy parenchyma of nephrectomy tissues (used as HC tissues) (Fig 2C).

Moreover, we used endoarterial thrombus tissues from patients with peripheral artery atherothrombosis (n = 2), kidney biopsies from patients with renal thrombotic microangiopathy (TMA, n = 4), and 1 skin ulcer biopsy from a patient with diabetes as disease control biopsies. We identified TF-expressing NETs (Supplementary Fig S2A) colocalised with fibrinogen (Supplementary Fig S2B) in endoarterial thrombus and renal TMA biopsies. NETs were also present in the skin biopsy of the patient with diabetes but were not colocalised with TF or fibrinogen (Supplementary Fig S2A,B).

Taken together, these findings indicate the presence of TF-bearing NETs, colocalised with fibrinogen, in endoarterial thrombi, skin, and kidney biopsy specimens, suggesting their involvement in the thromboinflammation process in target tissues/organs among patients with PAPS.

aPL are required for the intracellular expression of TF in neutrophils, activation of platelets, and subsequent NET release in PAPS

To test whether the observed difference in the presence of NET-bound TF in neutrophils of patients with PAPS is attributed to the capacity of aPL to induce the expression of intracellular TF in neutrophils, we treated HC neutrophils with isolated IgGs, which were isolated from 6 patients with triple-positive PAPS. We observed that IgGs from patients with PAPS with prominent expression of TF on their *ex vivo* NETs induced higher TF mRNA expression and TF protein levels, than IgGs from patients without prominent TF expression on their NETs (Fig 3A-C).

Neither IgGs isolated from patients with PAPS, nor PAPS sera were able to induce NET release directly in HC neutrophils, as assessed by immunofluorescence staining and MPO-DNA complex ELISA (Fig 3D,E). We further examined a disease control group of aPL-negative patients with acute arterial and/or venous thrombosis (n = 16). *In vitro* treatment of HC neutrophils with sera from the above patients has been shown to induce intracellular upregulation of TF expression (in 5 of 8 patients with arterial thrombosis and 4 of 8 patients with venous thrombosis) (Supplementary Figure S3A), but not NET formation as assessed by both immunofluorescence staining and MPO-DNA complex ELISA (Supplementary Fig S3A-C).

Studies have shown that activated platelets are involved in NET formation in infections and cardiovascular disease [10,27–30]. To assess the ability of PAPS platelets to induce NET release, we treated neutrophils from HCs with *ex vivo*-isolated platelets from patients with PAPS or heterologous HC platelets. We observed significantly increased NET release in HC neutrophils treated with platelets from patients with PAPS, compared with HC neutrophils treated with heterologous HC platelets or PAPS sera (Fig 3F-H). PAPS platelets did not induce TF expression in HC neutrophils (Fig 3I).

Considering that the presence of platelet-neutrophil aggregates is one of the most accurate markers of platelet activation, we investigated their levels in our patients. We observed increased platelet-neutrophil aggregates in the peripheral blood of patients with PAPS, compared with HCs, expressed as CD61/CD66b double-positive (+ve) events by flow cytometry (Fig 3J, K). The spontaneous NET release observed in neutrophils from patients with PAPS was strongly correlated with CD61/CD66b double +ve events (Fig 3L). Checking for correlations between CD61/CD66b double +ve events and PAPS clinical characteristics, the only association found was with the presence of venous thrombosis (Fig 3M, Supplementary Fig S4). CD61/CD66b double +ve events did not correlate with aPS/PT autoantibodies either (Supplementary Fig 4I). Moreover, we verified the presence of platelets on *ex vivo*-isolated neutrophils from patients with PAPS by immunofluorescence confocal microscopy (Fig 3N).

Prompted by these findings, we investigated whether aPL are involved in platelet-neutrophil interactions. To reproduce this hypothesis *in vitro*, we examined platelet-neutrophil aggregates in HC neutrophils treated with PAPS IgG-pretreated platelets. Increased CD61/CD66b double +ve events were observed in HC neutrophils treated with PAPS IgGs-pretreated platelets, compared with HC neutrophils treated with control IgG-pretreated platelets or untreated HC neutrophils (Fig 4A,B).

Furthermore, we treated HC platelets with either isolated IgGs from patients with PAPS, demonstrating TF-expressing NETs or not. Subsequently, these platelets were introduced to HC neutrophils, pretreated with the same PAPS IgGs. Pretreatment of platelets with either IgGs from patients with PAPS expressing or not TF on NETs induced NET release in HC neutrophils, compared with controls (Fig 4C,D). However, NET-bound TF was observed only when HC neutrophils were pretreated with IgGs from patients with NET-bound TF-expressing PAPS, but not with IgGs from patients with TF-negative PAPS or HCs (Fig 4C,D). Importantly, TF presence in these *in vitro* stimulated HC neutrophils was functional, since isolated NET structures were able to induce thrombin generation in PPP from HCs in a TF-dependent manner (Fig 4E).

Overall, aPL play a dual role by inducing TF expression in neutrophils and activating platelets with subsequent NET release in PAPS.

Autophagy in PAPS neutrophils is induced by their interaction with activated platelets and mediates NET release

Autophagy is a key mechanism in the regulation of NET generation and release [14]. Autophagy-mediated release of NETs has been described in patients with SLE with active disease [13]. Herein, we investigated autophagy in neutrophils from patients with PAPS and its association with NET release. We observed that neutrophils from patients with PAPS demonstrated higher basal autophagy levels than HCs, as assessed by

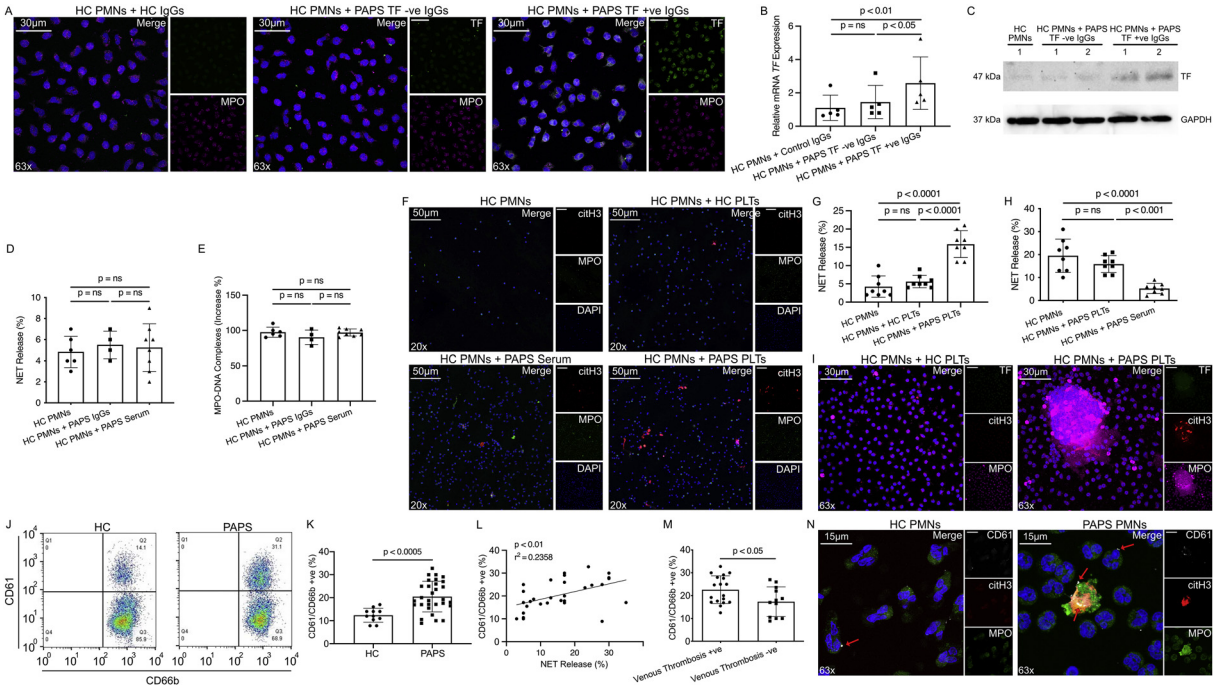

Figure 3. Platelets from patients with PAPS induce NET release in neutrophils. (A) Intracellular expression of TF in isolated peripheral neutrophils from HCs treated with HC IgGs (n = 2), IgGs from TF −ve (n = 3) or TF patients with +ve PAPS (n = 3). Representative data from 1 of 4 experiments. (B) Relative mRNA *TF* expression by neutrophils in (A). (C) TF expression in isolated peripheral neutrophils incubated with purified PAPS IgGs from TF −ve (n = 2) and TF +ve (n = 2) patients. (D) Quantification of NET release in isolated peripheral neutrophils from HCs (n = 6) treated *in vitro* with purified IgGs (n = 4) or sera (n = 8) from patients with PAPS. (E) MPO-DNA complex enzyme-linked immunosorbent assay (ELISA) in isolated NET structures from neutrophils in (D). (F) NETs released by isolated peripheral neutrophils from HCs treated with PAPS platelets (n = 8) or heterologous HC platelets (n = 8). Representative data from 1 of 4 experiments. (G) Quantification of NET release in (F). (H) Quantification of NET release by *ex vivo*-isolated peripheral neutrophils from patients with PAPS (n = 8) compared with *in vitro* treatment of HC neutrophils with the corresponding platelets or sera from these patients. Representative data from 1 of 4 experiments. (I) TF −ve NETs released by isolated peripheral neutrophils from HCs treated with PAPS platelets (n = 8) or heterologous platelets from HCs (n = 8). Representative data from 1 of 4 experiments. (J) CD61/CD66b flow cytometry dot plots in whole blood from patients with PAPS (n = 30) and HCs (n = 11). Representative data from 1 of 30 patients with PAPS. (K) Quantification of CD61/CD66b double +ve events in (I). (L) Correlation of NET formation in patients with PAPS (Fig 1B) with CD61/CD66b double +ve events. (M) CD61/CD66b double +ve events in patients with PAPS with venous thrombosis (n = 18) compared with patients without (n = 12). (N) Representative immunofluorescence images of *ex vivo*-isolated peripheral neutrophils from HC (n = 6) and PAPS (n = 6) patients, stained for the platelet marker CD61. Green: (A,I) TF, (F,N) MPO, red: (F,I) citH3, magenta: (A,I) MPO, white: (N) CD61, blue: (A,F,I,N) DAPI/DNA. For (B), statistical analysis was performed using repeated-measures 1-way analysis of variance (ANOVA) with Bonferroni's post hoc test. For (D,E,G, H), statistical analysis was performed using ordinary 1-way ANOVA followed by Tukey's multiple comparisons test. For (K,M), statistical analysis was performed using the Mann-Whitney U test. For (L), statistical analysis was performed using the Pearson (r) correlation test. For (B,D,E,G,H,K,M), bars represent mean values ± SD, and each dot represents a patient. ANOVA, analysis of variance; citH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAPS, primary antiphospholipid syndrome; TF, tissue factor.

immunofluorescence staining using LC3B, a major autophagy protein demonstrated in punctuated structures (puncta) (Fig 5A). These findings were quantified by immunoblotting for the lipidated form of LC3B (LC3B-II) and further verified by calculating the integrated optical density (IOD) ratio of LC3B-II to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig 5B, C).

To further elucidate the involvement of autophagy in the formation of NETs, we pretreated isolated *ex vivo* PAPS neutrophils with SC79, a pan-AKT phosphorylator molecule, which acts as an early-stage autophagy inhibitor. Pretreatment of PAPS neutrophils with SC79 significantly attenuated basal autophagy levels (Fig 5D upper) and the subsequent release of NETs (Fig 5D lower, E, F), as assessed by immunofluorescence staining and MPO-DNA complex ELISA in isolated NET structures.

To investigate whether the increased autophagy levels observed in PAPS neutrophils are induced by their interaction with activated platelets, we treated HC neutrophils with HC platelets that had been pretreated with isolated PAPS IgGs. We observed that platelets pretreated with PAPS IgGs induced autophagy, in contrast to platelets pretreated with IgGs isolated from HC serum, as shown using immunofluorescence staining and immunoblotting of LC3B (Fig 5G,H).

These findings suggest that NET release in patients with PAPS is correlated with increased basal autophagy in neutrophils, which is mediated by aPL-activated platelets.

CD40-CD40L axis inhibition attenuates NET release and thrombin generation in PAPS

Evidence has shown that several neutrophil and platelet receptor pairs drive platelet-neutrophil interactions, including the P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1), the adhesion molecule ICAM2 and integrin $\alpha L\beta 2$, known as lymphocyte function-associated antigen 1 (LFA-1), the TREM-1L and TREM-1, and the CD40 ligand (CD40L/CD154) and CD40 [21]. We performed *in vitro* studies using inhibitors for LFA-1 − ICAM-2 (Lifitegrast), TREM-1 − TREM-1L (VJDT), and CD40-

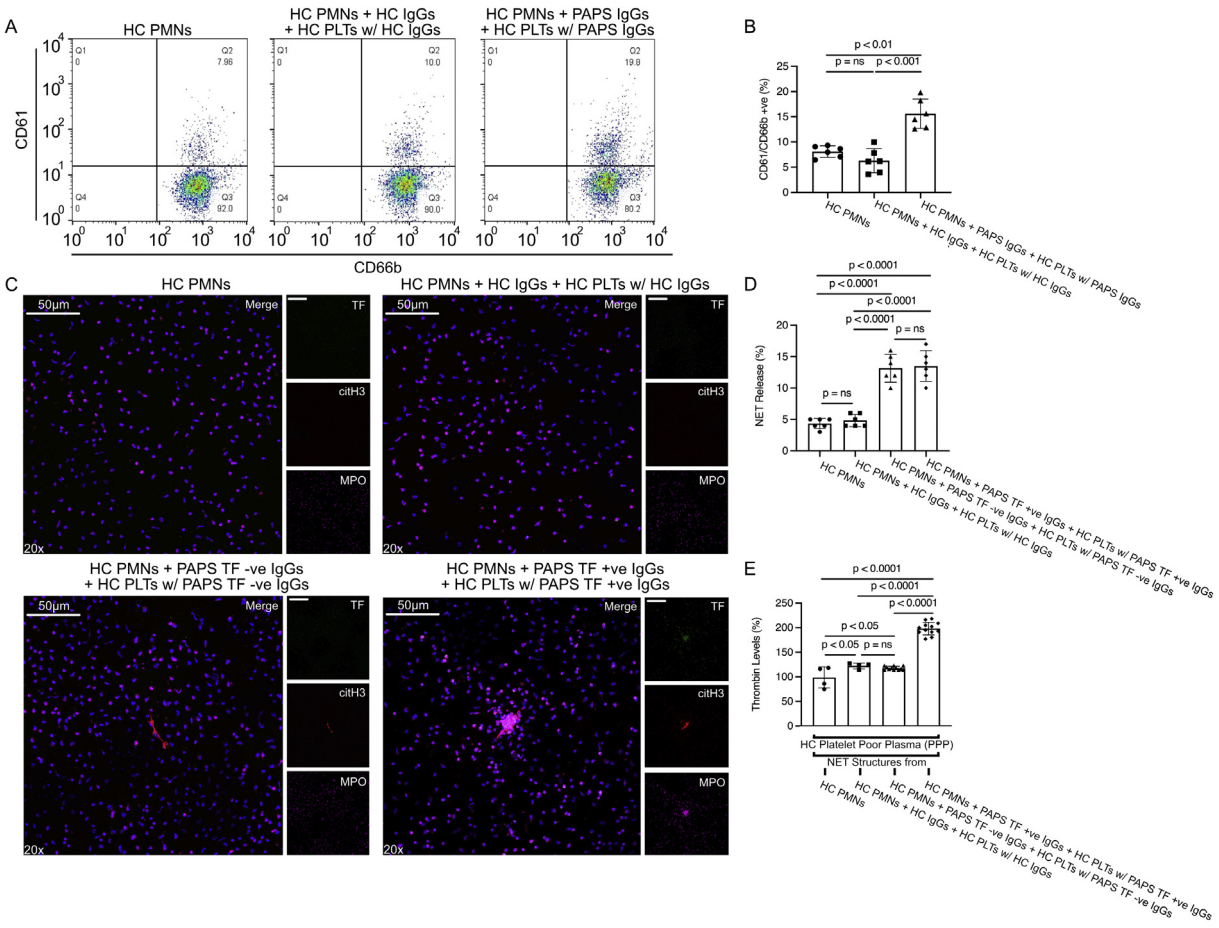

Figure 4. aPL require platelets to induce NET release. (A) CD61/CD66b flow cytometry dot plots in isolated peripheral neutrophils from HCs, stimulated with isolated HC platelets pretreated with purified HC or PAPS IgGs (n = 6). Representative data from 1 of 6 experiments. (B) Quantification of CD61/CD66b double + ve events in (A). (C) NETs released by isolated peripheral neutrophils from HCs pretreated with purified IgGs from TF −ve (n = 3) and + ve (n = 3) patients with PAPS, followed by stimulation with isolated HC platelets pretreated with IgGs from the same patients. Representative data from 1 of 3 experiments. (D) Quantification of NET release in (C). (E) Thrombin generation levels in HC platelet-poor plasma treated with NET structures from neutrophils in (C). Green: CD61, red: citH3, magenta: MPO, blue: DAPI/DNA. For (B,D,E), statistical analysis was performed using ordinary 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For (B,D,E), bars represent as mean values ± SD, and each dot represents a patient. ANOVA, analysis of variance; citH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAPS, primary antiphospholipid syndrome; TF, tissue factor.

CD40L (DRI-C21045); we did not examine the P-selectin and PSGL-1 interaction inhibition, since it has been previously examined in APS [16]. Among the above, only the CD40-CD40L inhibition could reduce NET release in HC neutrophils treated with PAPS IgG-activated platelets (Supplementary Fig S5A). Thus, we decided to extend our study of CD40-CD40L inhibition in PAPS.

Pretreatment of HC neutrophils with the CD40-CD40L inhibitor significantly reduced NET release induced by PAPS IgG-activated platelets, as assessed by immunofluorescence staining and MPO-DNA complex ELISA in isolated NET structures (Fig 6A-C). Moreover, pretreatment of HC neutrophils with DRI-C21045 resulted in a significant decrease in CD61/CD66b double + ve events, compared with neutrophils stimulated with PAPS IgG-activated platelets in the absence of pretreatment (Fig 6D). Importantly, inhibition of the CD40-CD40L interaction reduced the generation of thrombin in PPP from HCs, following stimulation with isolated NET structures from these cultures, as assessed by thrombin ELISA (Fig 6E).

To verify these findings, we repeated these experiments with platelets isolated from patients with PAPS. CD40-CD40L interaction blockade significantly reduced NET release in HC neutrophils induced by *ex vivo*-isolated PAPS platelets, as assessed by immunofluorescence staining and MPO-DNA complex ELISA in isolated NET structures (Fig 6F-H). Moreover, pretreatment of HC neutrophils with DRI-C21045 resulted in decreased CD61/CD66b double + ve events (Fig 6I).

Furthermore, we investigated the role of the CD40-CD40L axis in the process of autophagy. Pretreatment of HC neutrophils with DRI-C21045 significantly attenuated basal autophagy levels induced by PAPS IgG-stimulated platelets, as assessed by immunofluorescence staining and immunoblotting of LC3B (Supplementary Fig S5B,C).

CD40-CD40L axis inhibition alters thrombus composition in an in vivo venous thrombosis model

To validate our findings *in vivo*, we employed a murine model of venous thrombosis by performing flow restriction of the IVC in wild type (WT) mice, in combination with administration of PAPS IgGs (Fig 7A). CD40-CD40L inhibition in this model was mediated by a specific anti-CD40L antibody bearing LALA mutations similar to the second-generation anti-CD40L antibodies used in clinical practice [31]. CD40-CD40L inhibition significantly reduced platelet-neutrophil aggregates and

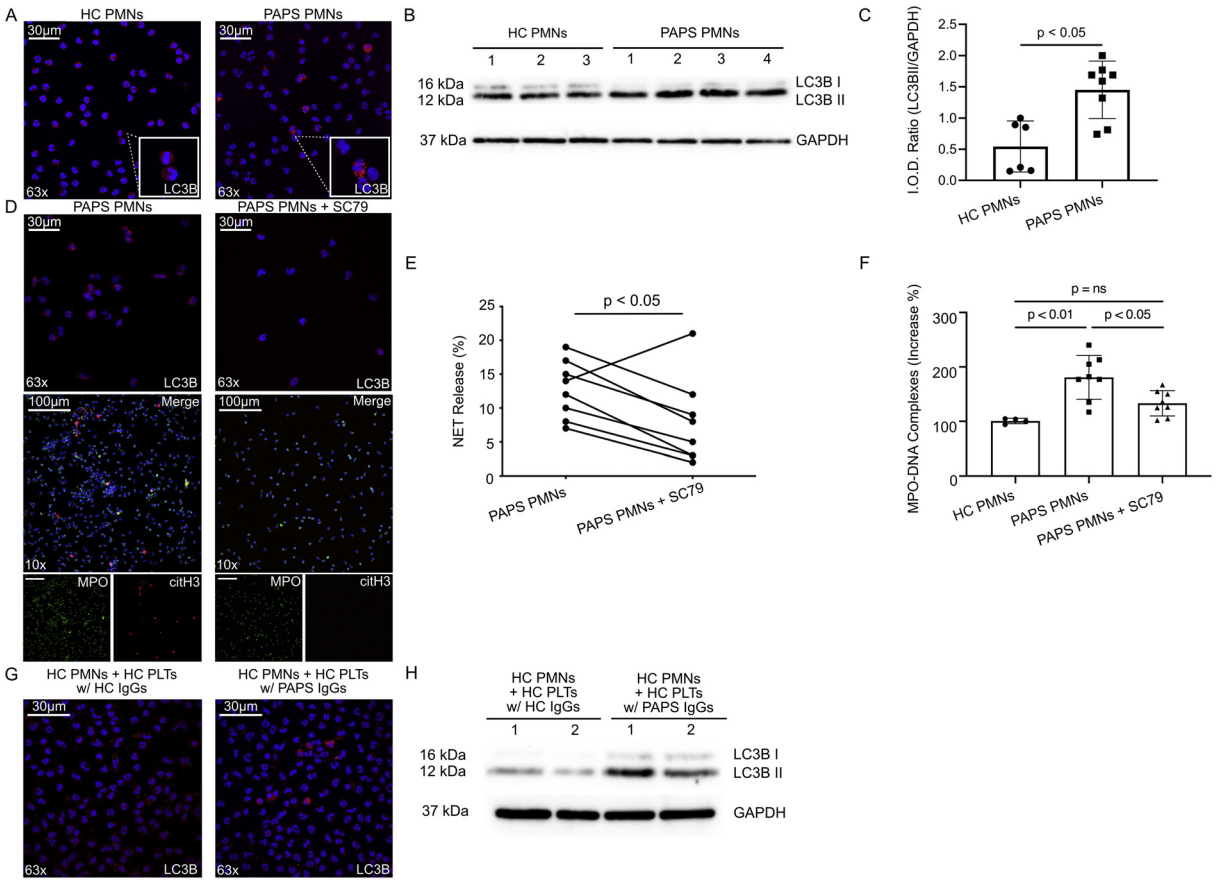

Figure 5. Increased basal autophagy levels observed in PAPS neutrophils are induced by their interaction with activated platelets and mediate NET release. (A) Detection of LC3B-positive puncta on autophagosomes in isolated peripheral neutrophils from patients with PAPS compared with HCs. Representative data from 1 of 30 patients. (B) Quantification of the nonlipidated and lipidated forms of LC3B, LC3B-I and LC3B-II, in isolated total proteins from patients with PAPS (n = 4) compared with HCs (n = 3). (C) Integrated optical density (IOD) in isolated total proteins from (B). Representative data from 1 of 2 independent experiments. (D) Basal autophagy levels (upper) and NET release (lower) in *ex vivo* PAPS patient neutrophils compared with neutrophils pretreated with SC79 inhibitor (n = 8). (E) Quantification of NET release in (D). (F) MPO-DNA complex enzyme-linked immunosorbent assay (ELISA) in isolated NET structures from neutrophils in (D). (G) Detection of LC3B-positive puncta on autophagosomes in isolated peripheral neutrophils from HCs treated *in vitro* with purified PAPS IgG-pretreated platelets. (H) Quantification of the non-lipidated and lipidated forms of LC3B, LC3B-I, and LC3B-II in isolated total proteins from neutrophils in (G). Green: (C—lower) MPO, red: (A, C—upper, F) LC3B, (C—lower) citH3, blue: (A,C,F) DAPI/DNA. For (E), statistical analysis was performed using the Wilcoxon signed-rank test for paired samples. For (F), statistical analysis was performed using ordinary 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For (C and F), bars represent mean values ± SD, and each dot represents a patient. ANOVA, analysis of variance; citH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAPS, primary antiphospholipid syndrome; TF, tissue factor.

the subsequent NET release in anti-CD40L-treated animals compared with saline or isotype IgG-treated mice, as assessed by flow cytometry in whole blood (Supplementary Fig S6A and Fig 7B) and MPO-DNA complex ELISA in mouse sera (Fig 7C), respectively. Accuracy of MPO-DNA complex ELISA for murine samples was verified on isolated PMA/ionomycin-induced NETs from murine neutrophils (Supplementary Figure S6B). Interestingly, CD40-CD40L inhibition significantly reduced NET presence in murine thrombi, as assessed by immunofluorescence staining (Fig 7D,E). Thrombus size and weight were not statistically different (Supplementary Fig S6C,D), whereas mouse weight was similar in all study groups (Supplementary Fig S6E).

Overall, the CD40-CD40L axis is implicated in the platelet-neutrophil interactions, as CD40-CD40L blockade inhibits platelet-neutrophil interaction, the subsequent autophagy-mediated release of NETs, and finally the generation of thrombin. Additionally, inhibition of the CD40-CD40L axis *in vivo* altered thrombus composition in an APS murine model by significantly reducing NET presence.

DISCUSSION

In this study, to our knowledge, we describe that *ex vivo* neutrophils from patients with PAPS can release TF-decorated NETs, which are able to generate thrombin in a TF-dependent manner, and that aPL play a dual role by inducing intracellular TF expression in neutrophils, and activating platelets, with subsequent platelet-neutrophil interaction, autophagy induction, and NET release. Most importantly, we demonstrate for the first time that CD40-CD40L blockade inhibits this platelet-neutrophil interaction and attenuates NET release both *in vitro* and *in vivo*.

More specifically, we observed that *ex vivo* peripheral blood neutrophils from patients with PAPS exhibit increased spontaneous release of NETs, compared with HCs and asymptomatic aPL-positive individuals, which was persistent in a 3-month follow-up. Studies have shown TF expression on NETs in some inflammatory conditions [10,12,13] and TF expression and procoagulant activity on monocytes from patients with PAPS [32]. A previous study showed that aPL-induced complement activation led to TF expression in neutrophils [33]; however, to our

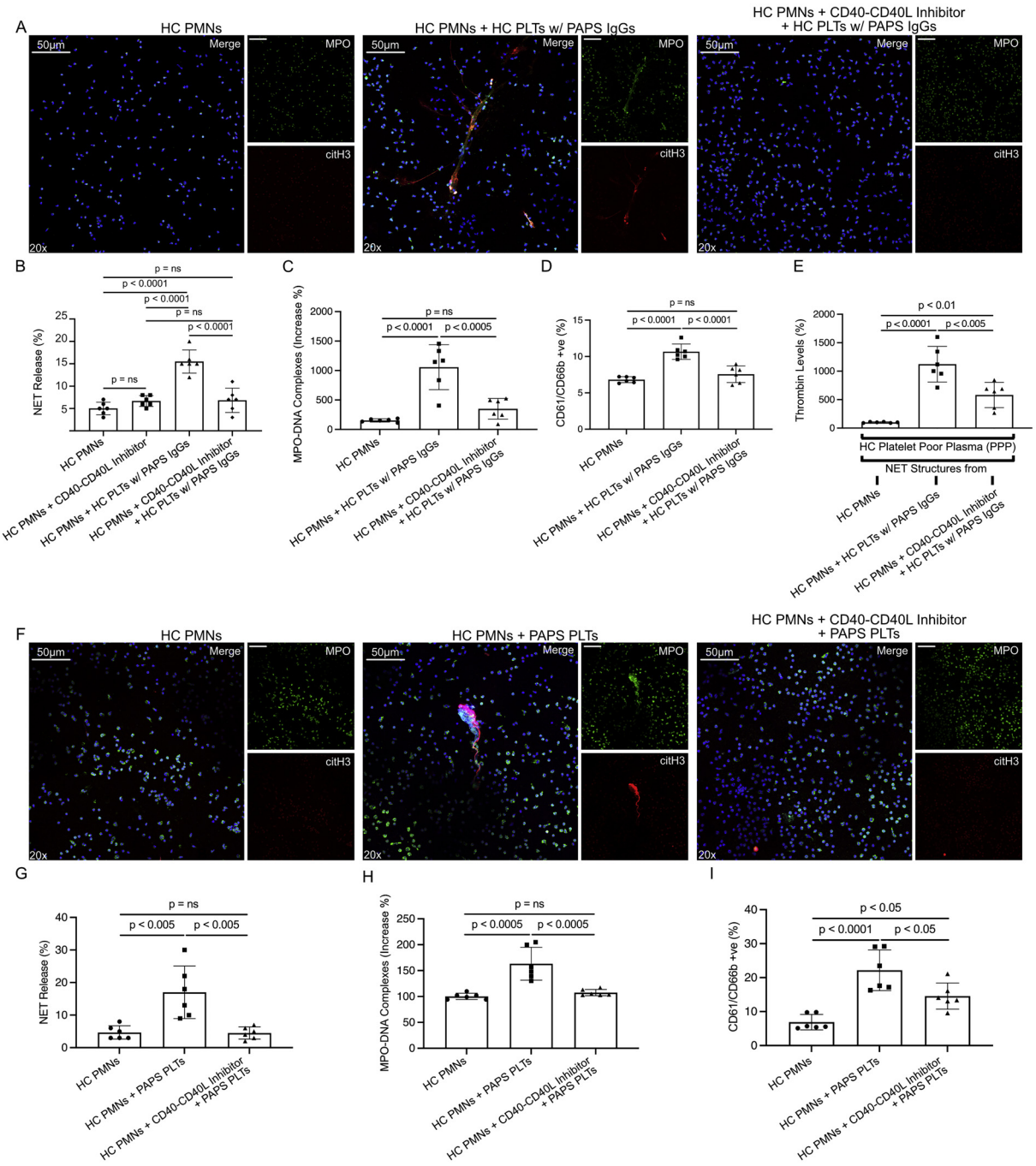

Figure 6. CD40-CD40L blockade attenuates NET formation in PAPS. (A) NETs released by isolated peripheral neutrophils from HCs stimulated with HC platelets pretreated with PAPS IgGs, with or without pretreatment with the CD40-CD40L interaction inhibitor DRI-C21045. Representative data from 1 of 6 experiments. (B) Quantification of NET release in (A). (C) MPO-DNA complex enzyme-linked immunosorbent assay (ELISA) in isolated NET structures from neutrophils in (A). (D) Quantification of CD61/CD66b double +ve events in (A). (E) Thrombin generation levels in HC platelet-poor plasma treated with NET structures from neutrophils in (A). (F) NETs released by isolated peripheral neutrophils from HCs treated with PAPS platelets (n = 6), compared with neutrophils pretreated with the CD40-CD40L interaction inhibitor DRI-C21045, prior to the addition of PAPS platelets. Representative data from 1 of 6 experiments. (G) Quantification of NET release in (F). (H) MPO-DNA complex enzyme-linked immunosorbent assay (ELISA) in isolated NET structures from neutrophils in (F). (I) Quantification of CD61/CD66b double +ve events in (F). Green: MPO, red: citH3, blue: DAPI/DNA. For (B-E,G-I), statistical analysis was performed using ordinary 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For (B-E,G-I), bars represent mean values ± SD, and each dot represents a patient. ANOVA, analysis of variance; citH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAPS, primary antiphospholipid syndrome; TF, tissue factor.

knowledge, no studies reported on the externalisation of TF in APS. Here, to our knowledge, we showed for the first time that *ex vivo* neutrophils from patients with PAPS externalise bioactive TF on released NETs.

Although NET release was observed in all patients with PAPS, not all patients demonstrated TF-decorated NETs. However, the thrombogenicity of NETs was TF-dependent. In previous studies, thrombin generation was enhanced by isolated neutrophils and cell supernatants from patients with PAPS [33,34], but not from isolated NETs. We also found that intracellular TF expression in neutrophils is induced by aPL. Notably, not all aPL from patients with PAPS were able to induce TF expression in neutrophils *in*

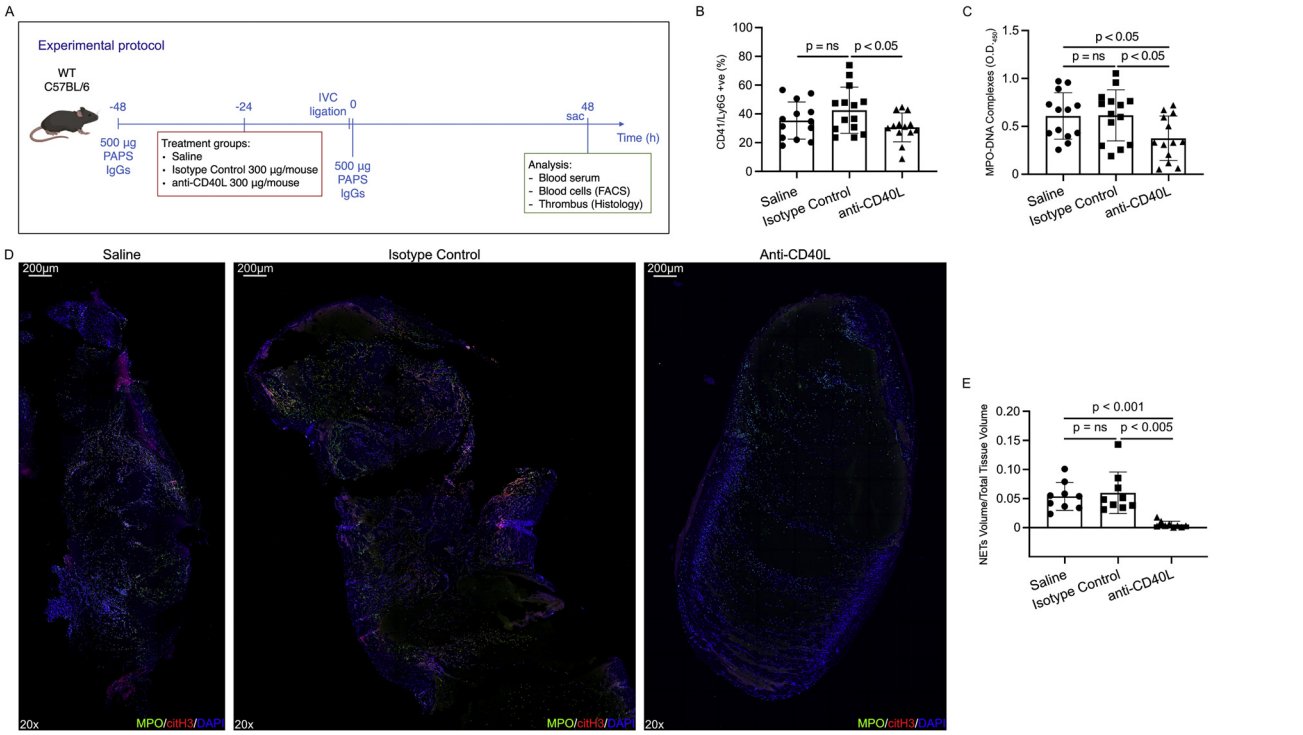

Figure 7. CD40-CD40L blockade alters thrombus composition in an experimental mouse model of PAPS. (A) Schematic representation of the experimental induction of PAPS in mice. C57BL/6 mice received 2 intraperitoneal (i.p.) doses of 500 μg isolated PAPS IgGs at a 48-hour interval. Treatment [saline (vehicle control), isotype control antibody (300 µg/mouse), or anti-CD40L antibody (300 µg/mouse)] was administered i.p. between the 2 PAPS IgG injections. Prior to the second PAPS IgG administration, mice underwent IVC ligation, and were humanely euthanised 48 hours later. (B) Quantification of platelet-neutrophil aggregates from mice in (A), determined as the % of CD41/Ly6G +ve cells by flow cytometry. (C) MPO-DNA complex enzyme-linked immunosorbent assay (ELISA) in isolated serum samples from mice in (A). (D) Presence of NETs, defined as MPO/citH3 +ve structures, in isolated thrombi from mice in (A). (E) Quantification of NET presence in thrombi from (D). Green: MPO, red: citH3, blue: DAPI/DNA. For (B,C,E), statistical analysis was performed using ordinary 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For (B,C,E), bars represent mean values ± SD. For (E), NET quantification was defined as the ratio NET volume/total tissue volume. IVC, inferior vena cava; ANOVA, analysis of variance; citH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAPS, primary antiphospholipid syndrome; TF, tissue factor.

vitro. Only aPL from patients who demonstrated TF expression in *ex vivo* neutrophils and NETs were able to induce *in vitro* TF expression. Moreover, sera from other thrombotic disorders (deep vein thrombosis, acute myocardial infarction, and stroke patients) were able to induce TF expression intracellularly in HC PMNs but were not able to induce NET release and externalisation of TF. These findings are in agreement with previous studies, which demonstrated that sera from patients with STEMI induce TF expression but do not induce NET release directly in PMNs [10].

The role of TF and NETs in thromboinflammation at the tissue level (eg, kidneys) has been demonstrated in an aPL-induced renal thrombotic microangiopathy mouse model [35]. However, their presence *in situ* was not previously investigated in tissue biopsies from patients with PAPS. Cutaneous manifestations in patients with APS may vary, from livedo reticularis to skin ulcers or necrosis, whereas kidney involvement in the form of APS nephropathy has been well-recognised in the context of both PAPS and SLE/APS [36]. We have shown here, for the first time, the presence of TF-decorated NETs and deposited fibrinogen in the proximity of NETs, in organ tissues from patients with PAPS, such as arterial thrombi and thrombosed vessels from skin ulcer biopsies. In kidney biopsies, TF-decorated NETs and fibrinogen were found in the glomerular capillaries and peritubular capillaries, but also in the tubulointerstitial compartment close to the Bowman's capsule, suggesting a possible involvement in the ongoing periglomerular scarring and tubular atrophy, in addition to TMA lesions [35]. Interestingly, a recent RNA-sequencing study on archival kidney biopsies from patients with PAPS showed higher expression of NET-related genes in PAPS vs control kidney samples [37]. We also found the presence of TF-decorated NETs and deposited fibrinogen in kidney biopsies of patients with renal TMA and in thrombus tissue from patients with peripheral artery atherothrombosis. These findings further support the importance of NETs as a mechanism in thromboinflammation and atherothrombosis [38].

We further investigated the intracellular pathways underlying NET release. Neutrophils and platelets interact minimally in the circulation in normal conditions, while the platelet-neutrophil cross-talk is important in the process of haemostasis and thrombosis, and is even implicated in the clearance of pathogens [39–44]. Circulating PNCs have been associated with thrombosis in individuals with acute coronary syndromes, and activated platelets were the main inducers of NET release in these patients [10]. In the context of APS, anti-β2GPI aPL interact with Fcγ-receptor IIa on the platelet surface and activate them [45]. Moreover, the release of platelet factor 4 (PF4) has been shown to promote the formation of highly antigenic PF4-complexed β2GPI, and subsequent platelet activation [46]. A previous study has demonstrated that platelets from patients with APS display a proinflammatory and procoagulant profile, including enhanced TF expression and participation in platelet-leukocyte aggregates

[47]. Platelet TF expression has been further characterised in APS, with TF-positive platelets and TF-positive platelet-leukocyte aggregates reinforcing the idea that platelet-driven thromboinflammation is itself a key contributor to APS thrombosis [48]. Additionally, a recent publication reported that platelets enhance the capacity of aPL to induce NET formation in neutrophils [16]. In the present study, we showed that patients with PAPS exhibit increased PNCs compared with HCs, and they are significantly correlated with the *ex vivo* NET release observed in neutrophils; PNCs also associate with the presence of venous thrombosis. We found for the first time that isolated *ex vivo* platelets alone from patients with PAPS can activate neutrophils and promote the formation of NETs.

Moreover, we showed that neutrophils in PAPS have increased basal autophagy levels, compared with HCs, and that increased autophagy was responsible for NET release. The use of a pan-AKT phosphorylator (autophagy inhibitor) halted this process, even in *ex vivo* PAPS neutrophils. We also observed that activated platelets that trigger NET release in patients with PAPS, induce autophagy in neutrophils of HCs.

Considering the role of platelets and their interaction with neutrophils in inducing thrombogenic NET release, targeting platelets or the platelet-neutrophil interaction can be a potential treatment strategy in APS. Platelet activation can be eliminated via platelet cyclooxygenase activity inhibition with aspirin, or via Toll-like receptor 7 inhibition with hydroxychloroquine [49]. Aspirin, in combination with vitamin K antagonists (with an INR 2-3), is one of the management options for thrombotic APS (with low level of evidence) [50], but it may increase the risk of bleeding. The platelet-neutrophil interaction in the blood and endothelium involves several receptor–ligand pairs, including P–selectin–P–selectin glycoprotein ligand, GPIbα–macrophage 1 antigen (MAC–1 and αMβ2), GPIIbIIIa–MAC–1 through fibrinogen, TREM-1 ligand and TREM-1, and CD40–CD40L [21]. CD40-CD40L interaction enhances neutrophil recruitment through upregulation of Mac-1, while CD40 has been shown to stimulate autophagy [51,52]. Performing functional validation assays using a CD40-CD40L interaction inhibitor, we found that CD40-CD40L blockade *in vitro* could eliminate platelet activation, autophagy, and NET formation in PAPS.

Most importantly, in our murine APS model, administration of a neutralising anti-CD40L antibody, inhibited platelet-neutrophil aggregation and subsequent NET release and significantly altered thrombus composition by significantly diminishing NET presence. It has already been established in several high-impact studies [53,54] that NETs are critical for the formation of arterial and venous thrombi in mice. A potential explanation for the lack of thrombus size reduction in our experiments might be that, as NET presence enhances thrombus stability through fibrin structure alterations and inhibits thrombus resolution [55], the chosen time point of analysis (48 hours after IVC ligation) was short to observe thrombus resolution, and we might be able to document thrombus size differences at a later experimental time point.

Treatments targeting CD40-CD40L pathway have been examined in systemic autoimmune diseases, mainly SLE [56–58]. First-generation anti-CD40L antibodies have been associated with increased thromboembolic risk, resulting from platelet activation triggered by FcγRIIa stimulation. However, the antibody used in our study has modifications in the Fc region, similar to the new anti-CD40L antibodies containing a modified Fc with low FcγRIIa binding. Trials of second-generation anti-CD40-CD40L antibodies have been recently completed, and others are currently taking place in patients with SLE and lupus nephritis [31,56–58].

A prior pioneering study demonstrated increased NET release in *ex vivo* neutrophils from patients with PAPS [34]. This study found an association between NET release and anti-β2GPI IgG positivity [34], a finding not observed in our study, which could be possibly due to a lack of power to detect possible associations. The study above showed that IgG purified from patients with APS stimulated the release of NETs from control neutrophils. In our experimental setting, we observed that serum or purified IgGs from patients with PAPS alone cannot induce NET release in neutrophils isolated from HCs, which could be due to different experimental settings, such as the neutrophil isolation method (presence/absence of platelets). In addition, our study group (patients with PAPS, asymptomatic aPL carriers, and HCs) consisted solely of White Europeans, limiting the generalisability of our findings to other ethnic groups. A multiethnic study with a larger sample size can lead to greater precision and higher power to detect true associations. Most patients with PAPS in our study were high-risk patients, with 63% experiencing recurrent thrombotic events, and 83% having LA positivity, 63% triple aPL positivity, and about 50% high IgG antiβ2GPI and aCL titres. These characteristics confirm a well-characterised APS population, and also help to better understand the pathophysiology of patients with high recurrence rates, who can benefit from potential new treatment targets.

In summary, our results showed a TF-dependent thrombogenicity of NETs and identified platelets as indispensable components of NET release in PAPS, while this process was mediated intracellularly by autophagy. Our data support a dual role of aPL, involving the intracellular TF expression in neutrophils and the activation of platelets for the generation of thrombogenic NETs. Inhibition of the platelet-neutrophil interaction through CD40-CD40L attenuated APS thrombogenicity both *in vitro* and *in vivo*. We also demonstrated the presence of TF-expressing NETs in endoarterial thrombi and microthrombi in skin ulcer and kidney biopsies. These findings provide additional evidence for intercepting NET-induced thromboinflammation in tissue damage in microvascular APS, a newly classified entity by the 2023 ACR/EULAR classification criteria for APS, including APS nephropathy, livedoid vasculopathy/skin ulcers, and alveolar haemorrhage [1]. These data may help in identifying potentially new therapeutic targets but also support repositioning of existing drugs, such as anti-CD40L antibodies in patients with refractory thrombotic APS, anticoagulation-resistant microvascular APS manifestations, or even other thrombotic disorders with prominent platelet-neutrophil interaction.

Competing interests

MT reports funding provided by National and Kapodistrian University of Athens and a relationship with National and Kapodistrian University of Athens. The other authors declare no competing interests.

CRediT authorship contribution statement

Stavros Naoum: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Charilaos Spyropoulos:** Writing – review & editing, Formal analysis. **Andriani Angelopoulou:** Writing – review & editing, Formal analysis. **Harikleia Gakiopoulou:** Writing – review & editing, Formal analysis. **Michalis Katsimpoulas:** Writing – review & editing, Formal analysis, Methodology. **Vassilis G. Gorgoulis:**

Writing – review & editing, Formal analysis. **Petros P. Sfikakis:** Writing – review & editing, Resources. **Konstantinos Ritis:** Writing – review & editing, Investigation, Conceptualization. **Ioanna-Evdokia Galani:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Konstantinos Kambas:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Maria G. Tektonidou:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Acknowledgements

We would like to thank the Light Microscopy Unit of Hellenic Pasteur Institute for providing the facilities for confocal microscopy. Additionally, we would like to thank Konstantinos Lazaridis for providing isolated and purified IgGs from serum samples, Evangelos Balafas for assisting in mouse surgery procedures, and Mrs. Anna Agapaki for preparing murine biopsies.

Contributors

SN conducted experiments, analysed the data and drafted the manuscript. CS and AA conducted experiments and analysed the data. HG contributed to biopsy samples acquisition and data interpretation. MK contributed to animal study design and conducted animal surgery experiments. VGG contributed to the design of experiments and data interpretation. PPS contributed to data acquisition and data interpretation. KR contributed to study design, design of experiments, and data interpretation. I-EG contributed to animal study design, conducted experiments, data analysis, data interpretation, and drafted the manuscript. KK conceived and supervised the project, contributed to study design, design of experiments, experiments supervision, data analysis, data interpretation, and drafted the manuscript. MGT conceived and supervised the project, contributed to study design, study funding, recruitment, sample collection, and clinical data extraction from patients and healthy individuals, data analysis, data interpretation, and drafted the manuscript. KK and MGT act as guarantors for the overall content. All authors critically revised the manuscript and approved its final version.

Funding

This work was supported by Special Account for Research Funding (E.L.K.E., grant number 11123), Medical School, National and Kapodistrian University of Athens, Athens, Greece.

Patient consent for publication

Not applicable.

Ethics approval

The study was approved by the Ethics Committee of Laiko General Hospital (protocol number 398/31-05-2021) and was conducted according to Declaration of Helsinki principles. All participants provided written informed consent.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

The data that support the findings of this study are available from the corresponding author, MGT, upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.12.012.

Orcid

Maria G. Tektonidou: http://orcid.org/0000-0003-2238-0975

REFERENCES

[1] Barbhaiya M, Zuily S, Naden R, Hendry A, Manneville F, Amigo MC, et al. The 2023 ACR/EULAR antiphospholipid syndrome classification criteria. Arthritis Rheumatol 2023;75(10):1687–702. doi: 10.1002/art.42624.
[2] Knight JS, Branch W, Ortel TL. Antiphospholipid syndrome: advances in diagnosis, pathogenesis, and management. BMJ 2023;380:e069717. doi: 10.1136/bmj-2021-069717.
[3] Giannakopoulos B, Krilis SA. The pathogenesis of the antiphospholipid syndrome. N Engl J Med 2013;368(11):1033–44. doi: 10.1056/NEJMra1112830.
[4] De Groot PG, de Laat B. Mechanisms of thrombosis in systemic lupus erythematosus and antiphospholipid syndrome. Best Pract Res Clin Rheumatol 2017;31(3):334–41. doi: 10.1016/j.berh.2017.09.008.
[5] Corban MT, Duarte-Garcia A, McBane RD, Matteson EL, Lerman LO, Lerman A. Antiphospholipid syndrome: role of vascular endothelial cells and implications for risk stratification and targeted therapeutics. J Am Coll Cardiol 2017;69(18):2317–30. doi: 10.1016/j.jacc.2017.02.058.
[6] Tektonidou MG. Cardiovascular disease risk in antiphospholipid syndrome: thrombo-inflammation and atherothrombosis. J Autoimmun 2022;128:102813. doi: 10.1016/j.jaut.2022.102813.
[7] Salet DM, Bekkering S, Middeldorp S, van den Hoogen LL. Targeting thromboinflammation in antiphospholipid syndrome. J Thromb Haemost 2023;21(4):744–57.
[8] Raschi E, Borghi MO, Tedesco F, Meroni PL. Antiphospholipid syndrome pathogenesis in 2023: an update of new mechanisms or just a reconsideration of the old ones? Rheumatology (Oxford) 2024;63(Suppl I):SI4–SI13.
[9] López-Pedrera C, Pérez-Sánchez C, Tektonidou MG. Towards precision medicine in antiphospholipid syndrome. Lancet Rheumatol 2025;7(8):e576–89.
[10] Stakos DA, Kambas K, Konstantinidis T, Mitroulis I, Apostolidou E, Arelaki S, et al. Expression of functional tissue factor by neutrophil extracellular traps in culprit artery of acute myocardial infarction. Eur Heart J 2015;36(22):1405–14. doi: 10.1093/eurheartj/ehv007.
[11] Kambas K, Mitroulis I, Apostolidou E, Girod A, Chrysanthopoulou A, Pneumatikos I, et al. Autophagy mediates the delivery of thrombogenic tissue factor to neutrophil extracellular traps in human sepsis. PLOS ONE 2012;7(9):e45427. doi: 10.1371/journal.pone.0045427.
[12] Kambas K, Chrysanthopoulou A, Vassilopoulos D, Apostolidou E, Skendros P, Girod A, et al. Tissue factor expression in neutrophil extracellular traps and neutrophil derived microparticles in antineutrophil cytoplasmic antibody associated vasculitis may promote thromboinflammation and the thrombophilic state associated with the disease. Ann Rheum Dis 2014;73(10):1854–63. doi: 10.1136/annrheumdis-2013-203430.
[13] Frangou E, Chrysanthopoulou A, Mitsios A, Kambas K, Arelaki S, Angelidou I, et al. REDD1/autophagy pathway promotes thromboinflammation and fibrosis in human systemic lupus erythematosus (SLE) through NETs decorated with tissue factor (TF) and interleukin-17A (IL-17A). Ann Rheum Dis 2019;78(2):238–48. doi: 10.1136/annrheumdis-2018-213181.
[14] Remijsen Q, Kuijpers KW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. Cell Death Differ 2011;18(4):581–8. doi: 10.1038/cdd.2011.1.
[15] Sennett C, Pula G. Trapped in the NETs: multiple roles of platelets in the vascular complications associated with neutrophil extracellular traps. Cells 2025;14(5):335. doi: 10.3390/cells14050335.
[16] NaveenKumar SK, Tambralli A, Fonseca BM, Yalavarthi S, Liang W, Hoy CK, et al. Low ectonucleotidase activity and increased neutrophil-platelet

aggregates in patients with antiphospholipid syndrome. Blood 2024;143 (12):1193–7. doi: 10.1182/blood.2023022097.
[17] Liu Q, Zhu W, Wen X, Da Y. The role of platelet–neutrophil interactions in driving autoimmune diseases. Immunology 2025;175(1):1–15.
[18] Henn V, Slupsky JR, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 1998;391(6667):591–4. doi: 10.1038/35393.
[19] Cognasse F, Duchez AC, Audoux E, Ebermeyer T, Arthaud CA, Prier A, et al. Platelets as key factors in inflammation: focus on CD40L/CD40. Front Immunol 2022;13:825892. doi: 10.3389/fimmu.2022.825892.
[20] Barbar S, Noventa F, Rossetto V, Ferrari A, Brandolin B, Perlati M, et al. A risk assessment model for the identification of hospitalized medical patients at risk for venous thromboembolism: the Padua Prediction Score. J Thromb Haemost 2010;8(11):2450–7. doi: 10.1111/j.1538-7836.2010.04044.x.
[21] Kaiser R, Escaig R, Erber J, Nicolai L. Neutrophil-platelet interactions as novel treatment targets in cardiovascular disease. Front Cardiovasc Med 2022;8:824112. doi: 10.3389/fcvm.2021.824112.
[22] Daub S, Lutgens E, Münzel T, Daiber A. CD40/CD40L and related signaling pathways in cardiovascular health and disease-the pros and cons for cardioprotection. Int J Mol Sci 2020;21(22):8533. doi: 10.3390/ijms21228533.
[23] Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4 (2):295–306. doi: 10.1111/j.1538-7836.2006.01753.x.
[24] Aringer M, Costenbader K, Daikh D, Brinks R, Mosca M, Ramsey-Goldman R, et al. 2019 European League Against Rheumatism/American College of Rheumatology classification criteria for systemic lupus erythematosus. Arthritis Rheumatol 2019;71(9):1400–12. doi: 10.1002/art.40930.
[25] Payne H, Brill A. Stenosis of the inferior vena cava: a murine model of deep vein thrombosis. J Vis Exp 2017;130:56697. doi: 10.3791/56697.
[26] Vandevelde A, Chayoua W, de Laat B, Moore GW, Musiał J, Zuily S, et al. Added value of antiphosphatidylserine/prothrombin antibodies in the workup of thrombotic antiphospholipid syndrome: communication from the ISTH SSC Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies. J Thromb Haemost 2022;20(9):2136–50. doi: 10.1111/jth.15785.
[27] Ott I, Neumann FJ, Gawaz M, Schmitt M, Schömig A. Increased neutrophil-platelet adhesion in patients with unstable angina. Circulation 1996;94 (6):1239–46. doi: 10.1161/01.cir.94.6.1239.
[28] Tuttle HA, Davis-Gorman G, Goldman S, Copeland JG, McDonagh PF. Platelet-neutrophil conjugate formation is increased in diabetic women with cardiovascular disease. Cardiovasc Diabetol 2003;2:12. doi: 10.1186/1475-2840-2-12.
[29] Petzold T, Zhang Z, Ballesteros I, Saleh I, Polzin A, Thienel M, et al. Neutrophil "plucking" on megakaryocytes drives platelet production and boosts cardiovascular disease. Immunity 2022;55(12):2285–2299.e7. doi: 10.1016/j.immuni.2022.10.001.
[30] Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med 2007;13(4):463–9. doi: 10.1038/nm1565.
[31] Ramanujam M, Steffgen J, Visvanathan S, Mohan C, Fine JS, Putterman C. Phoenix from the flames: Rediscovering the role of the CD40-CD40L pathway in systemic lupus erythematosus and lupus nephritis. Autoimmun Rev 2020;19(11):102668. doi: 10.1016/j.autrev.2020.102668.
[32] Cuadrado MJ, López-Pedrera C, Khamashta MA, Camps MT, Tinahones F, Torres A, et al. Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. Arthritis Rheum 1997;40(5):834–41. doi: 10.1002/art.1780400509.
[33] Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, et al. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. J Immunol 2006;177(7):4794–802. doi: 10.4049/jimmunol.177.7.4794.
[34] Yalavarthi S, Gould TJ, Rao AN, Mazza LF, Morris AE, Núñez-Álvarez C, et al. Release of neutrophil extracellular traps by neutrophils stimulated with antiphospholipid antibodies: a newly identified mechanism of thrombosis in the antiphospholipid syndrome. Arthritis Rheumatol 2015;67(11):2990–3003. doi: 10.1002/art.39247.
[35] Seshan SV, Franzke CW, Redecha P, Monestier M, Mackman N, Girardi G. Role of tissue factor in a mouse model of thrombotic microangiopathy induced by antiphospholipid antibodies. Blood 2009;114(8):1675–83. doi: 10.1182/blood-2009-01-199117.
[36] Tektonidou MG. Antiphospholipid syndrome nephropathy: from pathogenesis to treatment. Front Immunol 2018;9:1181. doi: 10.3389/fimmu.2018.01181.
[37] Tektonidou MG, Verrou KM, Gakiopoulou H, Manoloukos M, Lembessis P, Hatzis P, et al. Kidney whole-transcriptome profiling in primary antiphospholipid syndrome reveals complement, interferons and NETs-related gene expression. Rheumatology (Oxford) 2024;63(11):3184–90. doi: 10.1093/rheumatology/keae397.
[38] d'Alessandro E, Becker C, Bergmeier W, Bode C, Bourne JH, Brown H, et al. Thrombo-inflammation in cardiovascular disease: an expert consensus document from the third Maastricht Consensus Conference on Thrombosis. Thromb Haemost 2020;120(4):538–64. doi: 10.1055/s-0040-1708035.
[39] Lisman T. Platelet–neutrophil interactions as drivers of inflammatory and thrombotic disease. Cell Tissue Res 2018;371(3):567–76. doi: 10.1007/s00441-017-2727-4.
[40] Darbousset R, Thomas GM, Mezouar S, Frère C, Bonier R, Mackman N, et al. Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. Blood 2012;120(10):2133–43. doi: 10.1182/blood-2012-06-437772.
[41] Deppermann C, Kubes P. Platelets and infection. Semin Immunol 2016;28 (6):536–45. doi: 10.1016/j.smim.2016.10.005.
[42] Jenne CN, Kubes P. Platelets in inflammation and infection. Platelets 2015;26 (4):286–92. doi: 10.3109/09537104.2015.1010441.
[43] Ed Rainger G, Chimen M, Harrison MJ, Yates CM, Harrison P, Watson SP, et al. The role of platelets in the recruitment of leukocytes during vascular disease. Platelets 2015;26(6):507–20. doi: 10.3109/09537104.2015.1064881.
[44] Pircher J, Engelmann B, Massberg S, Schulz C. Platelet-neutrophil crosstalk in atherothrombosis. Thromb Haemost 2019;119(8):1274–82. doi: 10.1055/s-0039-1692983.
[45] Hollerbach A, Müller-Calleja N, Ritter S, Häuser F, Canisius A, Orning C, et al. Platelet activation by antiphospholipid antibodies depends on epitope specificity and is prevented by mTOR inhibitors. Thromb Haemost 2019;119 (7):1147–53. doi: 10.1055/s-0039-1685453.
[46] Sikara MP, Routsias JG, Samiotaki M, Panayotou G, Moutsopoulos HM, Vlachoyiannopoulos PG. {beta}2 Glycoprotein I ({beta}2GPI) binds platelet factor 4 (PF4): implications for the pathogenesis of antiphospholipid syndrome. Blood 2010;115(3):713–23. doi: 10.1182/blood-2009-03-206367.
[47] Capozzi A, Manganelli V, Riitano G, Recalchi S, Truglia S, Alessandri C, et al. Tissue factor over-expression in platelets of patients with anti-phospholipid syndrome: induction role of anti-β2-GPI antibodies. Clin Exp Immunol 2019;196(1):59–66. doi: 10.1111/cei.13248.
[48] Camera M, Brambilla M, Lonati PA, Becchetti A, Grossi C, Nallio K, et al. Mechanisms sensitive to different drugs mediate a pro-adhesive and prothrombotic platelet phenotype in anti-phospholipid syndrome. Haematologica 2026;111(1):219–29. doi: 10.3324/haematol.2024.287133.
[49] Scherlinger M, Richez C, Tsokos GC, Boilard E, Blanco P. The role of platelets in immune-mediated inflammatory diseases. Nat Rev Immunol 2023;23 (8):495–510. doi: 10.1038/s41577-023-00834-4.
[50] Tektonidou MG, Andreoli L, Limper M, Amoura Z, Cervera R, Costedoat-Chalumeau N, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. Ann Rheum Dis 2019;78(10):1296–304. doi: 10.1136/annrheumdis-2019-215213.
[51] Jin R, Yu S, Song Z, Zhu X, Wang C, Yan J, et al. Soluble CD40 ligand stimulates CD40-dependent activation of the β2 integrin Mac-1 and protein kinase C zeda (PKCζ) in neutrophils: implications for neutrophil-platelet interactions and neutrophil oxidative burst. PLOS ONE 2013;8(6):e64631. doi: 10.1371/journal.pone.0064631.
[52] Portillo JA, Okenka G, Reed E, Subauste A, Van Grol J, Gentil K, et al. The CD40-autophagy pathway is needed for host protection despite IFN-Γ-dependent immunity and CD40 induces autophagy via control of P21 levels. PLOS ONE 2010;5(12):e14472. doi: 10.1371/journal.pone.0014472.
[53] Jiménez-Alcázar M, Rangaswamy C, Panda R, Bitterling J, Simsek YJ, Long AT, et al. Host DNases prevent vascular occlusion by neutrophil extracellular traps. Science 2017;358(6367):1202–6. doi: 10.1126/science.aam8897.
[54] von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 2012;209(4):819–35. doi: 10.1084/jem.20112322.
[55] Thålin C, Hisada Y, Lundström S, Mackman N, Wallén H. Neutrophil extracellular traps: villains and targets in arterial, venous, and cancer-associated thrombosis. Arterioscler Thromb Vasc Biol 2019;39(9):1724–38. doi: 10.1161/ATVBAHA.119.312463.
[56] Shock A, Burkly L, Wakefield I, Peters C, Garber E, Ferrant J, et al. CDP7657, an anti-CD40L antibody lacking an Fc domain, inhibits CD40L-dependent

immune responses without thrombotic complications: an *in vivo* study. Arthritis Res Ther 2015;17(1):234. doi: 10.1186/s13075-015-0757-4.

[57] Furie RA, Bruce IN, Dörner T, Leon MG, Leszczyński P, Urowitz M, et al. Phase 2, randomized, placebo-controlled trial of dapirolizumab pegol in patients with moderate-to-severe active systemic lupus erythematosus. Rheumatology (Oxford) 2021;60(11):5397–407. doi: 10.1093/rheumatology/keab381.

[58] Chamberlain C, Colman PJ, Ranger AM, Burkly LC, Johnston GI, Otoul C, et al. Repeated administration of dapirolizumab pegol in a randomised phase I study is well tolerated and accompanied by improvements in several composite measures of systemic lupus erythematosus disease activity and changes in whole blood transcriptomic profiles. Ann Rheum Dis 2017;76 (11):1837–44. doi: 10.1136/annrheumdis-2017-211388.