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Release of Neutrophil Extracellular Traps by Neutrophils Stimulated With Antiphospholipid Antibodies

A Newly Identified Mechanism of Thrombosis in the Antiphospholipid Syndrome

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Objective. **Antiphospholipid antibodies (aPL), especially those targeting β_2-glycoprotein I (β_2GPI), are well known to activate endothelial cells, monocytes, and platelets, with prothrombotic implications. In contrast, the interaction of aPL with neutrophils has not been extensively studied. Neutrophil extracellular traps (NETs) have recently been recognized as an important activator of the coagulation cascade, as well as an integral component of arterial and venous thrombi. This study was undertaken to determine whether aPL activate neutrophils to release NETs, thereby predisposing to the arterial and venous thrombosis inherent in the antiphospholipid syndrome (APS).**

Methods. **Neutrophils, sera, and plasma were prepared from patients with primary APS (n = 52) or from healthy volunteers and characterized. No patient had concomitant systemic lupus erythematosus.**

Results. **Sera and plasma from patients with primary APS had elevated levels of both cell-free DNA and NETs, as compared to healthy volunteers. Freshly isolated neutrophils from patients with APS were predisposed to high levels of spontaneous NET release. Further, APS patient sera, as well as IgG purified from APS patients, stimulated NET release from control neutrophils. Human aPL monoclonal antibodies, especially those targeting β_2GPI, also enhanced NET release. The induction of APS NETs was abrogated with inhibitors of reactive oxygen species formation and Toll-like receptor 4 signaling. Highlighting the potential clinical relevance of these findings, APS NETs promoted thrombin generation.**

Conclusion. **Our findings indicate that NET release warrants further investigation as a novel therapeutic target in APS.**

Antiphospholipid syndrome (APS) is an autoimmune disease of unknown cause associated with elevated titers of antiphospholipid antibodies (aPLs), which predispose to arterial and venous thrombosis, as well as fetal loss (1). While first described in association with systemic lupus erythematosus (SLE) (2), APS is now well recognized to also exist as a primary autoimmune syndrome, with thrombosis and pregnancy loss as its cardinal manifestations, and with other associated features including thrombocytopenia, livedo reticularis, cognitive dysfunction, seizure disorder, and renal vasculopathy (1). Importantly, APS is relatively unique among prothrombotic diatheses, in that it clearly predisposes to both arterial and venous events. There is no cure for APS, and current treatments focus on suppressing coagulation, rather than targeting the underlying pathophysiology (3).

Clinically significant aPLs recognize both thrombin and β_2-glycoprotein I (β_2GPI), with antibodies to

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the latter having more uniform clinical testing and better understood downstream signaling pathways (1). β_2GPI, a cationic lipid-binding protein produced by liver, endothelial cells, monocytes, and trophoblasts, circulates at high levels in plasma (50–200 μg/ml) (4,5). While some interesting recent studies have suggested that β_2GPI has specific and important roles in innate immunity (6), the function of this abundant plasma protein remains largely unknown.

It has been suggested that anti-β_2GPI aPLs promote thrombosis by engaging the β_2GPI protein on cell surfaces and thereby activating cells, resulting in increased tissue factor expression and release of proinflammatory cytokines such as tumor necrosis factor (7–9). These processes have been extensively studied in endothelial cells and monocytes, where annexin A2 and Toll-like receptor 4 (TLR-4) function as important coreceptors (7–9), and in platelets, where aPL/β_2GPI associate with and signal through apolipoprotein E receptor 2 (10). Despite extensive study of the aforementioned cell types, the interaction of aPLs with the most abundant leukocyte in human blood, the neutrophil, has only rarely been considered (11–16). The limited studies that do exist suggest that aPLs can directly activate neutrophils, as measured by enhanced granule release, oxidative burst, and interleukin-18 production (11,12), with possible amplification of this activation by complement C5a (14,15).

With emerging recognition of links between neutrophils and thrombosis in nonautoimmune conditions (17,18), there is now a compelling reason to further explore aPL–neutrophil interplay. Neutrophil extracellular trap (NET) release, a form of neutrophil cell death that results in the externalization of decondensed chromatin decorated with granule and nuclear proteins (19), has recently been recognized as an important mediator of pathologic thrombosis. NETs form an integral part of venous thrombi in animals and humans (17,20), with NET-derived proteases activating the coagulation cascade, and the NET structure serving as scaffolding for clot assembly (21,22). Activated neutrophils and resultant NETs are also known to damage the endothelium (23,24) and have been recognized as potential mediators of atherosclerosis and arterial thrombosis (25–27). We therefore sought to determine whether NET release might be a mechanism by which aPL–neutrophil interplay predisposes to thrombotic events in APS.

MATERIALS AND METHODS

Human subjects. Patients were recruited from Rheumatology and Hematology clinics at the University of Michigan or from the Department of Immunology and Rheumatology at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. All patients met the laboratory criteria for APS according to the updated Sydney classification criteria (28). If patients had only thrombocytopenia and/or hemolytic anemia (n = 6), they were classified as having APS according to the classification criteria of Alarcón-Segovia et al (29). All remaining patients with primary APS (n = 46) met the full Sydney classification criteria. Importantly, no APS patient in our primary cohort met the American College of Rheumatology (ACR) criteria for SLE (30); the only exceptions were the patients explicitly labeled as having secondary APS in the serum-stimulation experiments. This study was reviewed and approved by the Institutional Review Boards from both institutions, and all patients gave their written informed consent.

The Michigan population was predominantly Caucasian (European American) and the Mexico population predominantly Hispanic (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract). Despite this disparity, we found no difference between the Michigan and Mexico populations in any of the NET/neutrophil assays described in this study (data not shown). Healthy volunteers were recruited by advertisement at the University of Michigan; this control population was predominantly Caucasian (see Supplementary Table 1).

Blood was collected by phlebotomist venipuncture into standard serum or 3.2% sodium citrate tubes. Serum and plasma were prepared by standard methods and stored at −80°C until ready for use. Levels of anti-β_2GPI IgG, IgM, and IgA, as well as anticardiolipin IgG and IgM, were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Inova Diagnostics) according to the manufacturer's instructions. Lupus anticoagulant (LAC) levels were determined with a LAC/1 screening reactant and a confirmatory LAC/2 test according to published guidelines (31). Supplementary Table 1 displays the demographic and clinical characteristics of the patients included in the study.

Reagents. Human monoclonal aPLs were a generous gift from Drs. Pojen Chen (David Geffen School of Medicine, University of California, Los Angeles) and Barton Haynes (Duke University School of Medicine, Durham, NC) and have been described previously (32). Monoclonal antibodies and other purified reagents were determined to be free of endotoxin contamination via use of a chromogenic endotoxin quantification kit, used according to the recommendations of the manufacturer (Pierce). Other reagents are described within the individual assay protocols below.

Quantification of cell-free DNA and myeloperoxidase (MPO)–DNA complexes. Cell-free DNA was quantified in serum and plasma using a Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen) according to the manufacturer's instructions. MPO–DNA complexes were quantified similarly to what has been previously described (33). This protocol used several reagents from the Cell Death Detection ELISA kit (Roche). First, a high-binding 96-well enzyme immunoassay/radioimmunoassay (EIA/RIA) plate (Costar) was coated overnight at 4°C with anti-human MPO antibody (0400-0002; Bio-Rad), diluted to a concentration of 5 μg/ml in coating buffer (Cell Death kit). The plate was washed 3 times with wash buffer (0.05% Tween 20 in phosphate buffered saline [PBS]), and then blocked with 1% bovine serum albumin (BSA) in PBS for 90 minutes at room temperature. The plate was again washed 3 times before incubating overnight at 4°C with 10% serum or

plasma in the aforementioned blocking buffer. The plate was washed 5 times, and then incubated for 90 minutes at room temperature with 1× anti-DNA antibody (horseradish peroxidase [HRP]–conjugated; Cell Death kit) diluted in blocking buffer. After 5 more washes, the plate was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Invitrogen) followed by a 2*N* sulfuric acid stop solution. Absorbance was measured at a wavelength of 405 nm with a Synergy HT Multi-Mode Microplate Reader (BioTek). Data were normalized to an in vitro–prepared NET standard, which was included on every plate.

Cell isolation. Citrated blood from patients or healthy volunteers was fractionated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) to separate peripheral blood mononuclear cells (PBMCs) from neutrophils. Neutrophils were then further purified by dextran sedimentation of the red blood cell (RBC) layer, before lysing residual RBCs with 0.2% sodium chloride. Neutrophil preparations were >98% pure as confirmed by both flow cytometry and nuclear morphology.

Quantification of NETs by immunofluorescence. A protocol similar to what has been described previously was followed (34). Briefly, 1.5×10^5 neutrophils were seeded onto coverslips coated with 0.001% poly-L-lysine (Sigma) and then incubated in RPMI 1640 supplemented with L-glutamine. Experiments were done in the absence of fetal bovine serum or human serum unless explicitly stated otherwise. Neutrophils were stimulated with a variety of reagents including phorbol myristate acetate (PMA) (20 n*M*; Sigma), lipopolysaccharide (LPS) from *Escherichia coli* (100 μg/ml; Sigma), and various human antibodies at 10 μg/ml. Tested inhibitors included diphenyleneiodonium (DPI) (20 μ*M*; Tocris Bioscience), TLR-4 inhibitor TAK-242 (5–10 μ*M*; Millipore), anti–TLR-2 (clone T2.5) (10–25 μ*M*; eBioscience), anti–TLR-4 (HTA125) (10–25 μ*M*; eBioscience), polymyxin B (50 μg/ml; Sigma), and anti-CD11b (clone M1/70) (10 μg/ml; BioLegend). Neutrophils were stimulated for 2–3 hours at 37°C. For immunofluorescence-based quantification, cells were fixed with 4% paraformaldehyde without permeabilization. DNA was stained with Hoechst 33342 (Invitrogen). Protein staining was performed with a rabbit polyclonal antibody to neutrophil elastase (ab21595; Abcam) and fluorescein isothiocyanate–conjugated anti-rabbit IgG (SouthernBiotech).

Images were obtained using an Olympus microscope (IX70) and an HQ^2 CoolSNAP monochrome camera (Photometrics) with Metamorph Premier software. Image overlay and background correction were performed with Metamorph, and the recorded images were loaded into Adobe Photoshop for further analysis. NETs (decondensed extracellular DNA costaining with neutrophil elastase) were quantified by 2 observers under blinded conditions, with the results digitally recorded to prevent multiple counts; the percentage of NETs was calculated by averaging 5–10 40× fields per sample. To quantify extracellular DNA, incubation was performed essentially as above, but with 1×10^5 cells/well in 96-well tissue culture black plates with clear bottoms (Costar). After incubation, 1× reagent from a Quant-iT PicoGreen dsDNA Assay Kit was mixed 1:1 with the culture medium directly in the incubation plate. After 5 minutes at room temperature, fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm in a Synergy H1 Hybrid Plate Reader (BioTek). When visualized by immunofluorescence microscopy, the only demonstrable staining after this short incubation period was of extracellular DNA; no intact nuclear DNA could be visualized. Representative images were captured with an Olympus microscope (IX70) and a HQ^2 CoolSNAP monochrome camera with Metamorph Premier software.

Identification of low-density granulocytes (LDGs). LDGs were identified and quantified by flow cytometry as previously described (35). Briefly, PBMCs were isolated from citrated blood by density centrifugation (Ficoll-Paque Plus). Residual RBCs were lysed with hypotonic saline, and cells were then resuspended in flow buffer consisting of PBS supplemented with 1% BSA and 1% horse serum. LDGs were identified by their characteristic appearance on forward- and side-scatter plots. LDGs were consistently $CD10^{high}CD14^{low}CD15^{high}$.

IgG purification. IgG was purified from APS or control sera with a Protein G–Agarose kit according to the manufacturer's instructions (Pierce). Briefly, serum was diluted in IgG binding buffer and passed through a protein G–agarose column at least 5 times. IgG was then eluted with 0.1*M* glycine and neutralized with 1*M* Tris. This was followed by overnight dialysis against PBS at 4°C. IgG purity was verified with Coomassie staining, and concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce) according to the manufacturer's instructions.

$F(ab')_2$ generation. $F(ab')_2$ fragments were generated and purified from total IgG fractions from healthy controls or APS patients with a Pierce $F(ab')_2$ Preparation Kit, according to the manufacturer's instructions.

Anti-β_2GPI depletion with purified β_2GPI. High-binding EIA/RIA plates were coated overnight at 4°C with 10 μg/ml purified β_2GPI (US Biologicals) diluted in coating buffer from a Cell Death Detection ELISA kit. Plates were then washed with 0.05% Tween 20 in PBS, and blocked with 1% BSA in PBS for 3 hours at room temperature. APS total IgG fractions (10 μg/ml) were added to the plates and incubated overnight at 4°C with gentle shaking. As a negative control, APS total IgG fractions were added to wells coated with BSA (mock depletion). After overnight incubation, unbound APS IgG was removed from the plates, sterile filtered, and used for stimulation of neutrophils.

Western blotting. Cells were lysed by resuspending in radioimmunoprecipitation assay buffer (50 m*M* Tris pH 7.4, 150 m*M* NaCl, 2 m*M* EDTA, 1% Triton X, and a Roche protease inhibitor cocktail pellet) on ice for 1 hour. After spinning to remove debris, protein concentration was measured with a BCA Protein Assay Kit according to the manufacturer's instructions. Samples were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under denaturing conditions, and transferred to a 0.45μ nitrocellulose membrane. Primary antibodies were directed against β_2GPI (A80-142A), annexin A2 (ab178677; Abcam), and β-actin (ab8227; Abcam). HRP-conjugated anti-goat or anti-rabbit secondary antibodies (Jackson ImmunoResearch) and Western Lightning Plus ECL (PerkinElmer) were used for detection. Images were captured with an Omega Lum C detector, and densitometry was performed using UltraQuant software (Aplegen).

Immunofluorescence. Neutrophils (1.5×10^5) were seeded onto coverslips coated with 0.001% poly-L-lysine and fixed with 4% paraformaldehyde. In some experiments, cells were then permeabilized with 0.1% Triton X for 15 minutes at room temperature. Blocking was with 1% BSA. Antibodies to β_2GPI and myeloperoxidase (A0398, Dako) were used as primary antibodies. Appropriate fluorochrome-conjugated secondary antibodies were from SouthernBiotech. DNA was stained with Hoechst 33342. An Olympus microscope (IX70) was used for imaging.

Flow cytometry. β_2GPI expression on the cell surface of control neutrophils and monocytes was determined by flow cytometry, using a protocol similar to what has been described previously (4). Freshly isolated control neutrophils and PBMCs were stained with rabbit polyclonal anti-human β_2GPI (ABS162; Millipore), as well as anti-human CD10 (BioLegend) as a neutrophil marker or anti-human CD14 (BioLegend) as a monocyte marker. Staining was for 30 minutes at 4°C. After washing, cells were fixed in 2% paraformaldehyde before analysis with a CyAn ADP Analyzer (Beckman Coulter). Further data analysis was done in FlowJo.

Quantification of neutrophil H_2O_2 production. The generation of H_2O_2 was quantified, essentially as described previously (36). Briefly, H_2O_2 production was detected by a colorimetric assay, with 50 μM Amplex Red reagent (Invitrogen) and 10 units/ml HRP (Sigma) added to the culture medium. Absorbance was measured at 560 nm, and linearity was assured with an H_2O_2 standard curve. The data were plotted with the no-inhibitor PMA condition set to 100%, after subtracting for background H_2O_2 production from unstimulated cells.

Thrombin generation assay. Thrombin generation was measured as previously described (37). Platelet-poor plasma (PPP) was prepared by centrifugation of citrated blood at 1,500*g* for 10 minutes at room temperature. Control neutrophils (1×10^5) were added to 35-μl aliquots of PPP from healthy volunteers or patients in wells of a 96-well black Costar plate. Where indicated, PPP was treated with the following enzymes or antibodies before the addition of neutrophils: DNase I (Pulmozyme [dornase alfa]) (20 μg/ml; Genentech), HTF-1 (murine monoclonal antibody against tissue factor) (10 μg/ml; BD Biosciences), or APS IgG monoclonal antibodies (10 μg/ml). The sample volume was brought to a final volume of 50 μl by the addition of PBS.

After incubation of neutrophils in plasma for 30 minutes at 37°C, coagulation was initiated by the addition of 50 μl of reaction buffer containing 15 mM $CaCl_2$ and 1 mM Z-Gly-Gly-Arg-AMC (Bachem). Thrombin generation was then monitored using a Technothrombin TGA thrombin generation assay (Technoclone). Thrombin generation profiles were analyzed using Technothrombin TGA software (Technoclone). Delta values were calculated by subtracting the baseline condition (either plasma alone or plasma supplemented with IgG) from the experimental condition of interest (for example, plasma and neutrophils). Plasma samples from patients treated with either warfarin or heparin-based anticoagulants were not included in this analysis.

Statistical analysis. Unless otherwise indicated, results are presented as the mean ± SEM. GraphPad Prism software version 6 was used for data analysis. Data sets were tested for Gaussian distribution by D'Agostino-Pearson omnibus normality test. Pairs of data with a Gaussian distribution were analyzed by unpaired *t*-test, while nonparametric data were assessed by

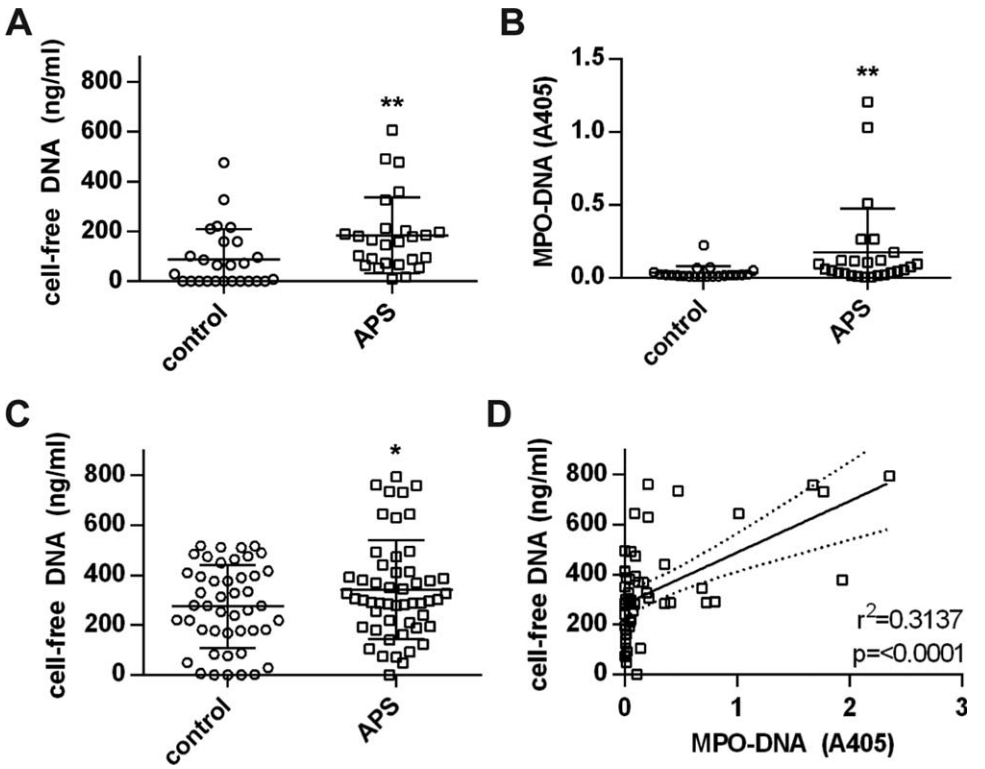

Figure 1. Elevated levels of cell-free DNA and neutrophil extracellular traps (NETs) in the circulation of patients with primary antiphospholipid syndrome (APS). **A,** Levels of cell-free DNA in the plasma of patients with APS or healthy controls (n = 26 per group). **B,** Levels of circulating NETs in the same plasma samples, measured by myeloperoxidase (MPO) DNA enzyme-linked immunosorbent assay (n = 21 controls and 26 patients with APS). **C,** Levels of cell-free DNA in serum samples from APS patients (n = 52) or healthy controls (n = 46). In **A–C**, symbols represent individual samples; horizontal and vertical lines show the mean ± SD. **D,** Correlation between cell-free DNA and circulating NETs (MPO–DNA complexes) in APS sera (n = 52). * = $P < 0.05$; ** = $P < 0.01$, versus control.

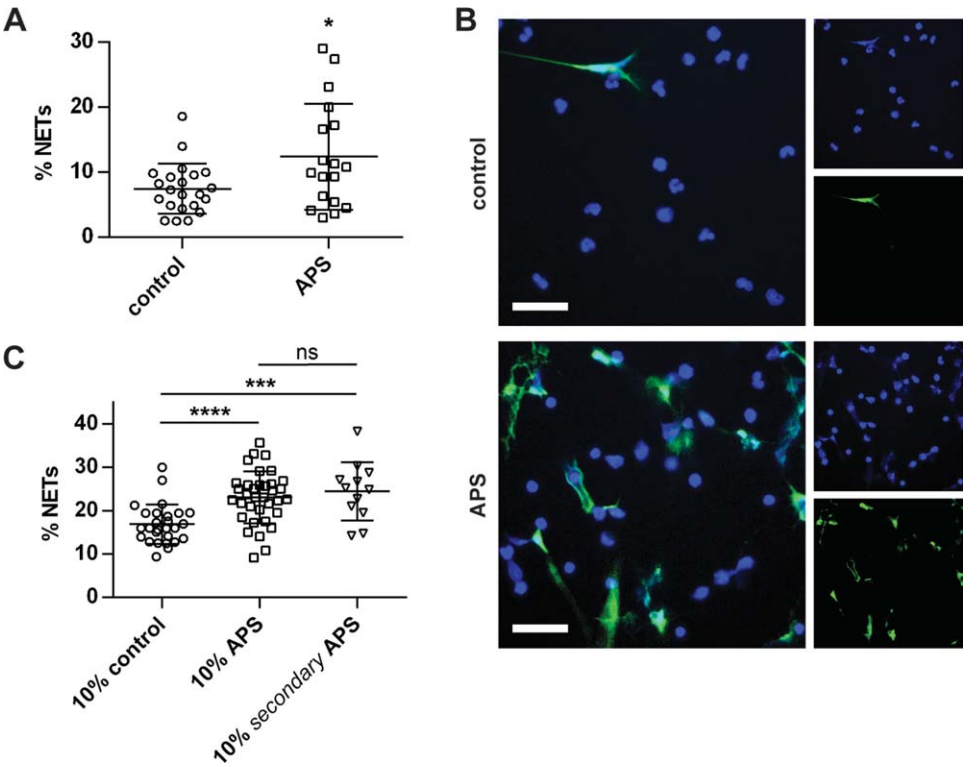

Figure 2. Enhanced neutrophil extracellular trap (NET) release in neutrophils from patients with primary antiphospholipid syndrome (APS). **A,** NET release, determined by immunofluorescence microscopy, from freshly isolated neutrophils from healthy controls (n = 22) or APS patients (n = 18) that were seeded onto coverslips and incubated in serum-free media for 2 hours. **B,** Representative immunofluorescence microscopy of control and APS neutrophils. Blue indicates DNA; green indicates neutrophil elastase. Bars = 25μ. **C,** NET release, determined by immunofluorescence microscopy, from control neutrophils that were treated with 10% serum from heterologous healthy controls or APS patients for 2 hours. In **A** and **C**, symbols represent individual samples; horizontal and vertical lines show the mean ± SD (n = 27 controls, n = 35 patients with APS, and n = 12 patients with secondary APS). $* = P < 0.05$; $*** = P < 0.001$; $**** = P < 0.0001$. NS = not significant.

Mann-Whitney test. Correlations were tested by Pearson's correlation coefficient. *P* values less than 0.05 were considered significant, unless stated otherwise.

RESULTS

Given previous and extensive links between NETs and both human and murine lupus (34,36,38–41), we focused on patients with primary APS for the key experiments of this study. All 52 patients with primary APS met the Sydney laboratory criteria, while 46 fulfilled both the Sydney criteria (28) and the criteria of Alarcón-Segovia et al (29). Four patients had thrombocytopenia and 2 had Evans' syndrome as their sole clinical manifestation, and therefore only fulfilled the clinical criteria of Alarcón-Segovia et al (28,29). Importantly, none of the patients with primary APS met the ACR criteria for SLE (30). The 52 patients had an array of serologic positivity, clinical manifestations, and medication usage, as shown in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract. No patient included in this study had experienced a new thrombotic event in the 3 months prior to blood collection.

Increase in cell-free DNA and NETs in the circulation of patients with APS. We first measured levels of cell-free DNA and NETs in the plasma of APS patients (n = 26). These levels are elevated in other disease processes with a prothrombotic diathesis, such as sepsis (37), thrombotic microangiopathy (42), small vessel vasculitis (33), and cancer (43). As compared to healthy controls, we found plasma from patients with APS to have increased levels of both cell-free DNA (Figure 1A) and NETs (Figure 1B), with the latter detected with an ELISA for MPO–DNA complexes (33).

In general, serum samples contain more cell-free DNA than plasma (44), which is probably the result of the in vitro clot that forms during serum preparation. Despite this potential background of nonspecific DNA, others have had success measuring NETs in diseased sera, for example in antineutrophil cytoplasmic antibody–

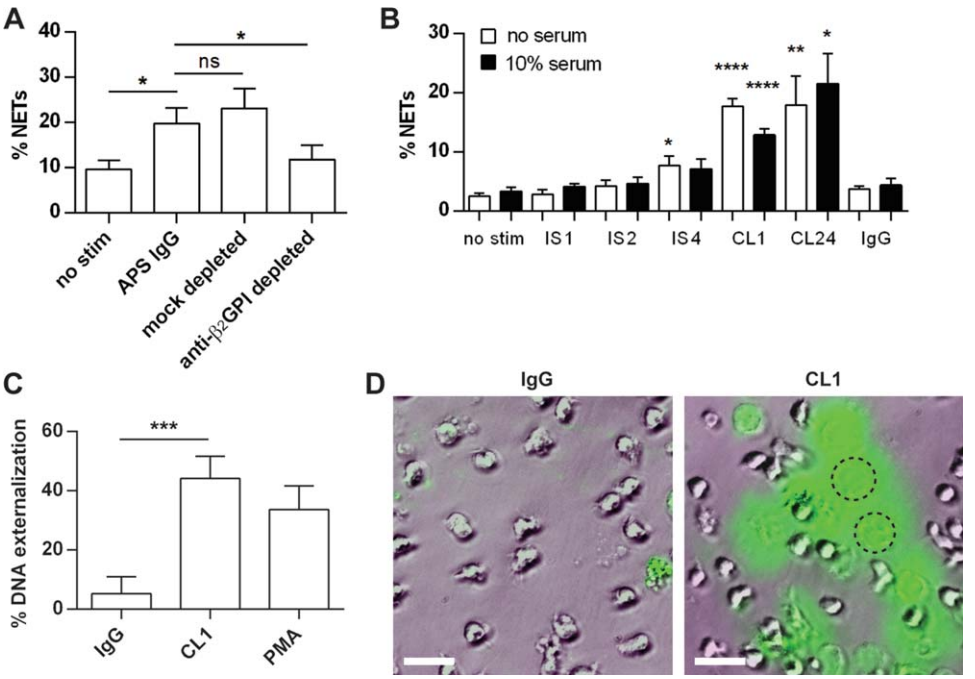

Figure 3. Effect of anti–β_2-glycoprotein I (anti-β_2GPI) IgG on the release of neutrophil extracellular traps (NETs) by neutrophils. **A,** NET release, determined by immunofluorescence microscopy, from antiphospholipid syndrome (APS) IgG samples. Five APS IgG samples were pooled, and then depleted of anti-β_2GPI IgG using purified β_2GPI protein. Control neutrophils were stimulated with IgG (10 μg/ml) as indicated for 3 hours. $* = P < 0.05$. NS = not significant. **B,** NET release from neutrophils stimulated with the indicated antibodies. Control neutrophils were treated with purified monoclonal antiphospholipid antibodies (aPL; 10 μg/ml) for 3 hours in the presence or absence of 10% autologous serum. IS4, aPL, CL1, and CL24 are known to bind β_2GPI, while IS1 and IS2 do not. $* = P < 0.05$; $** = P < 0.01$; $**** = P < 0.0001$, versus control IgG in the presence or absence of serum. **C,** Fluorescence intensity (corresponding to extracellular DNA) in control neutrophils stimulated with control IgG, CL1, or 20 n*M* phorbol myristate acetate (PMA). After 2 hours, PicoGreen was added to the culture, and fluorescence intensity was measured. Results were normalized to cells permeabilized with 0.1% Triton. $*** = P < 0.001$. In **A–C**, bars show the mean ± SEM of at least 5 independent experiments. **D,** Representative live cells stained with PicoGreen as described in **C**. In the sample stimulated with CL1, expanded cell remnants (encircled areas) are surrounded by a halo of DNA (green). Bars = 25μ.

associated vasculitis (33). Given our access to more serum samples from APS patients (n = 52) than plasma, we also tested the levels of cell-free DNA and NETs in these samples. Indeed, we found a significant increase in cell-free DNA in APS sera as compared to control sera (Figure 1C). Further, cell-free DNA showed a statistically significant correlation with circulating NETs in these samples (Figure 1D), demonstrating that the cell-free DNA is at least partially neutrophil derived. In summary, APS patients have elevated levels of circulating cell-free DNA and NETs as compared to healthy controls, even between thrombotic episodes, suggesting that their neutrophils are predisposed toward NET release.

Spontaneous release of NETs by neutrophils from patients with primary APS. From a subset of the APS patients, we isolated neutrophils for analysis of NET release. Indeed, APS neutrophils demonstrated enhanced spontaneous NET release as compared to controls (Figures 2A and B). Of note, these neutrophils were isolated by a typical Ficoll protocol (see Materials and Methods) and would therefore be considered of "normal" density. Specifically, they would not meet the criteria for the LDGs that have been described at increased numbers in lupus patients (35), and that are known to undergo exaggerated NET release (34). To be as definitive as possible on this point, we tested patients with primary APS for the presence of circulating LDGs. In contrast to lupus patients, LDGs were only detected at low levels in patients with primary APS, similar to healthy controls (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract).

The assay of NET release described above (Figures 2A and B) was performed in the absence of specific in vitro stimulation, suggesting that the primary stimulus for NET release was provided in vivo before neutrophil isolation. Indeed, there was a positive correlation between this in vitro NET release and levels of circulating MPO–DNA

complexes in vivo (see Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract). Further supporting the notion that this predisposition toward NET release was determined by circulating factors, we were able to induce NET formation in control neutrophils by incubation with sera from patients with primary APS (Figure 2C). We found similar stimulation when treating neutrophils with sera from patients with secondary APS (Figure 2C). The clinical characteristics of these patients with secondary APS (all of whom had SLE) are shown in Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract. In summary, APS neutrophils are predisposed to release NETs, an effect that can be replicated by incubating control neutrophils with the sera of APS patients.

Release of NETs by neutrophils stimulated with anti-β_2GPI IgG. While it is likely that multiple factors contribute to the ability of APS sera to promote NET release, we were especially interested in whether aPL may play a specific role in the process, a concept supported by the interaction of aPL with other cell types such as monocytes, endothelial cells, and platelets (7,9,10), as well as the limited studies to date in neutrophils (11,12). Indeed, we found a positive correlation between anti-β_2GPI IgG and circulating MPO–DNA complexes in APS patients (see Supplementary Figure 3A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract). Further, positive testing for LAC was associated with higher levels of MPO–DNA (see Supplementary Figure 3B), as was anticardiolipin IgG (see Supplementary Figure 3C) (although the trend for the latter was not statistically significant). In contrast, aPL IgM and IgA did not correlate with circulating MPO–DNA (see Supplementary Figure 3). Finally, there was a clear trend for higher levels of circulating MPO–DNA in patients who were "triple-positive" for anti-β_2GPI, anticardiolipin, and LAC, as compared to patients who were double- and single-positive (Supplementary Figure 3E); the difference between triple-positive and single-positive was statistically significant.

Based on the data described above, we selected 5 patients with anti-β_2GPI IgG (all triple-positive) and 5 healthy controls, and isolated their total IgG fractions. The IgG from the APS patients significantly stimulated NET release as compared to the healthy controls. This effect was independent of the presence of serum (see Supplementary Figure 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract]) and could be abrogated by specifically depleting the anti-β_2GPI fraction (Figure 3A and Supplementary Figure 5 [http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract]). Further, the stimulation persisted even when the Fc region of APS IgG was removed. Specifically, APS F(ab′)$_2$ fragments showed similar activity in NET formation assays as the parent APS IgG (see Supplementary Figure 6 [http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract]).

To further test whether this effect could be mediated by anti-β_2GPI IgG, we used several well-characterized human aPL IgG monoclonal antibodies. Two of the antibodies, IS1 and IS2, are known to bind phospholipids in a β_2GPI-independent manner (32), and 3 antibodies, IS4, CL1, and CL24, are β_2GPI-dependent antibodies (32). While IS1 and IS2 were associated with minimal stimulation of NET formation, the other 3 antibodies all promoted significant NET release (Figure 3B). Similar to the total IgG fraction experiments described above, this effect was independent of human serum (Figure 3B). To gauge the potency of this NET release by another technique, we quantified extracellular DNA with PicoGreen, a relatively cell-impermeable reagent that specifically fluoresces when associated with dsDNA. Using this technique, we saw similar quantities of externalized DNA when neutrophils were treated with the anti-β_2GPI monoclonal antibody CL1, as compared to the well-recognized and robust NET stimulator PMA (Figure 3C). By microscopy, we confirmed that intact, unstimulated neutrophils demonstrated little fluorescence with PicoGreen staining (Figure 3D). In summary, anti-β_2GPI antibodies promote NET release, both in total IgG fractions and as human monoclonal antibodies.

Detection of β_2GPI on the neutrophil surface. We had originally hypothesized that an exogenous source of β_2GPI protein (for example, from human serum) would be necessary for anti-β_2GPI–mediated NET release. However, with both APS IgG fractions and anti-β_2GPI monoclonal antibodies, this was not the case (Figure 3 and Supplementary Figure 4). We therefore investigated whether β_2GPI protein might be detectable in freshly isolated control neutrophils, as were used for the stimulation experiments described above. By Western blotting, we were able to detect β_2GPI in the purified neutrophils, at a level that was actually higher than that detected in total PBMCs (Figure 4A). This was in contrast to the endothelial cell and monocyte coreceptor for aPL/β_2GPI, annexin A2 (9,45), which was not detectable in neutrophils (Figure 4A). We also characterized neutrophil β_2GPI by immunofluorescence microscopy. Punctate β_2GPI was detectable on unpermeabilized neutrophils, with no significant change in the staining pattern with detergent (0.1% Triton) permeabilization (Figure 4B). This was in contrast to the cytoplasmic granule protein neutrophil elastase, which was detectable only with permeabilization (Figure 4B).

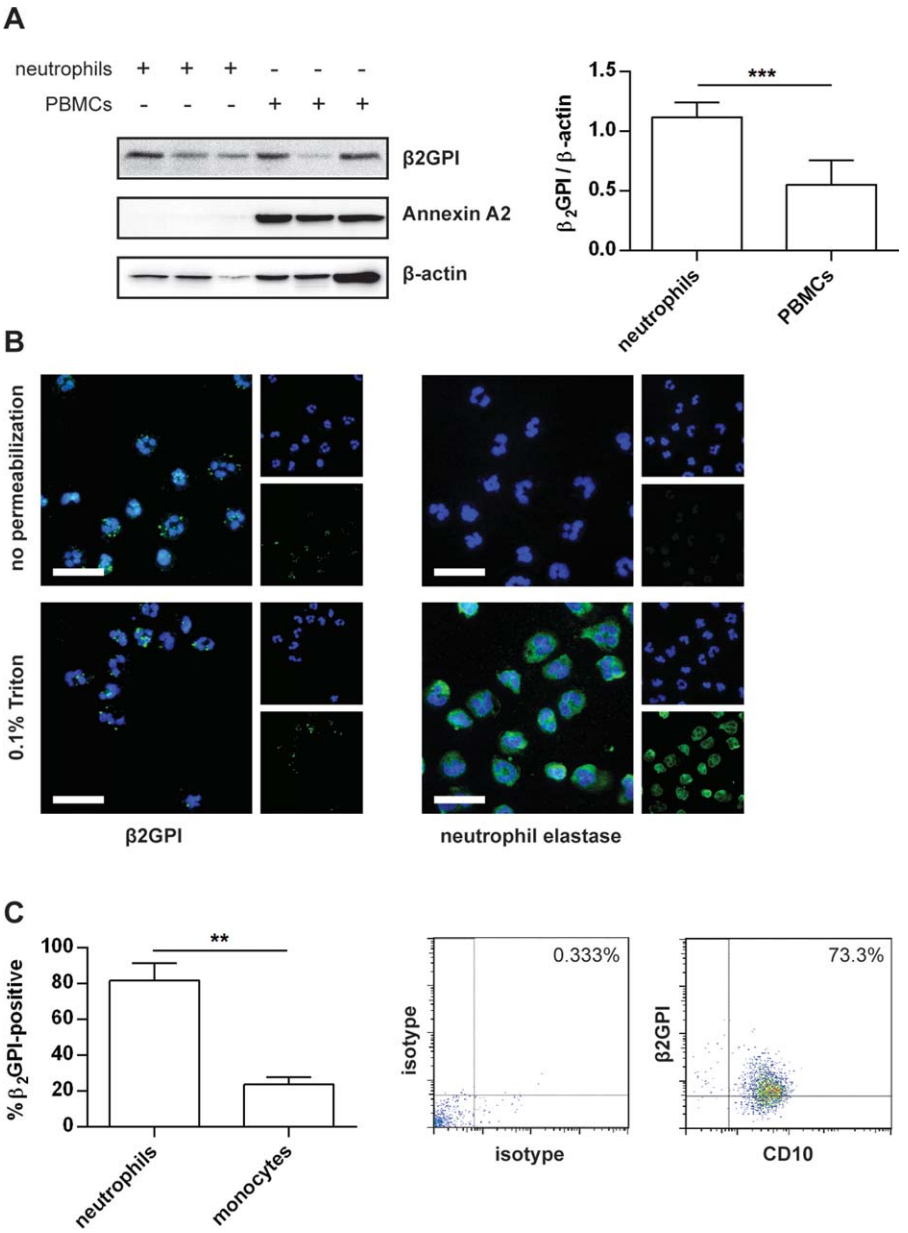

Figure 4. β_2-glycoprotein I (β_2GPI) on the neutrophil surface. **A,** Left, Western blot of β_2GPI, annexin A2, and β-actin in neutrophils and peripheral blood mononuclear cells (PBMCs) isolated from healthy controls. Total protein extracts were prepared by detergent lysis. Western blotting was repeated twice with similar results. Right, Densitometric quantification, expressed in arbitrary units. Bars show the mean ± SEM (n = 6, including 3 samples for each group that are not shown on the Western blot). *** = $P < 0.001$. **B,** Representative images of staining for β_2GPI and neutrophil elastase. Neutrophils were isolated from healthy controls and allowed to adhere to coverslips. Cells were then immediately fixed with paraformaldehyde, and in some cases permeabilized with detergent (0.1% Triton). Green indicates β_2GPI or neutrophil elastase; blue indicates DNA. Bars = 25μ. **C,** Left, Percentage of β_2GPI-positive cells in neutrophils and monocytes. Neutrophils and monocytes were identified by forward/side scatter, and additionally confirmed to be CD10 positive and CD14 positive, respectively. The percentage of β_2GPI-positive cells was then determined. Bars show the mean ± SEM (n = 6 healthy controls for each group). ** = $P < 0.01$. Right, Representative neutrophil histograms, demonstrating that the majority of CD10-positive cells were also positive for β_2GPI.

To further confirm this finding, we quantified levels of neutrophil-surface β_2GPI by flow cytometry. Indeed, we were able to detect β_2GPI on at least 80% of circulating neutrophils (Figure 4C), which was higher than the percentage of β_2GPI-positive monocytes, by both our analysis (Figure 4C) and the work of others

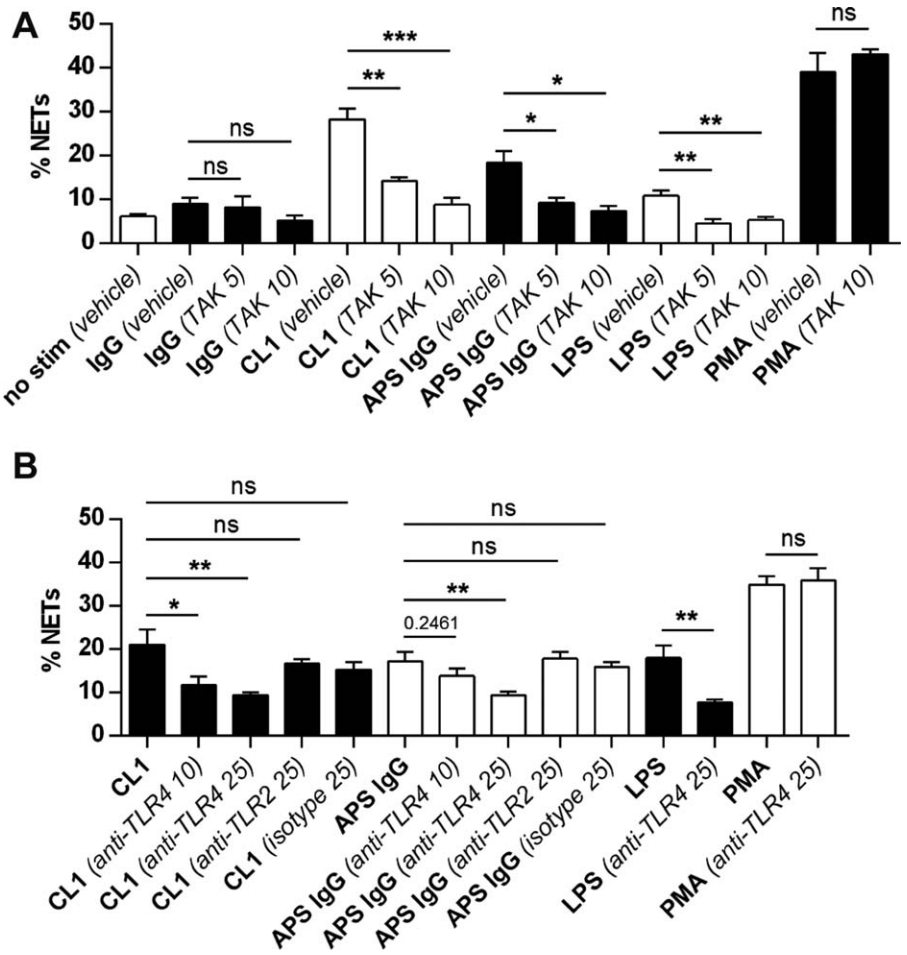

Figure 5. Blocking of antiphospholipid antibody (aPL)–mediated neutrophil extracellular trap (NET) release by inhibition of Toll-like receptor 4 (TLR-4) but not TLR-2. **A,** NET release, determined by immunofluorescence microscopy, from control neutrophils that were stimulated with control human IgG, an anti–β_2-glycoprotein I monoclonal antibody (CL1), or pooled IgG from 5 patients with antiphospholipid syndrome (APS) (all 10 μg/ml) for 3 hours. Stimulation with lipopolysaccharide (LPS; 100 ng/ml) and phorbol myristate acetate (PMA; 20 n*M*) served as controls. Some samples were pretreated with TAK-242, a TLR-4 inhibitor, at 5 μM or 10 μM, as indicated. **B,** NET release, determined by immunofluorescence microscopy, from control neutrophils that were stimulated as indicated (with the concentrations described in **A**) for 3 hours. Some samples were pretreated with anti–TLR-4, anti–TLR-2, or isotype, at 10 μM or 25 μM, as indicated. Bars show the mean ± SEM (n = 5 independent experiments per condition). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. NS = not significant.

(4). Further, the percentage of β_2GPI-positive neutrophils was not significantly different in APS patients as compared to healthy controls (see Supplementary Figure 7, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract). In summary, β_2GPI is present on the surface of neutrophils, where it can potentially mediate anti-β_2GPI binding.

Dependence of aPL-mediated NET release on reactive oxygen species (ROS) and TLR-4. ROS are generated during NET formation, and their blockade has been shown to prevent many (46), but not all (47,48), forms of NET release. When the aforementioned aPL monoclonal antibodies were tested in an H_2O_2 production assay, a similar pattern was seen as for NET formation, with IS1 and IS2 yielding minimal activity, and the other 3 monoclonal antibodies demonstrating robust stimulation (see Supplementary Figure 8A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract). A similar result was seen when control neutrophils were stimulated with the total IgG fractions described above, with APS IgG stimulating more H_2O_2 production than control IgG (Supplementary Figure 8B). We also investigated whether aPL-mediated NET release could be prevented by an inhibitor of NADPH oxidase, and consequently ROS formation. Indeed, treat-

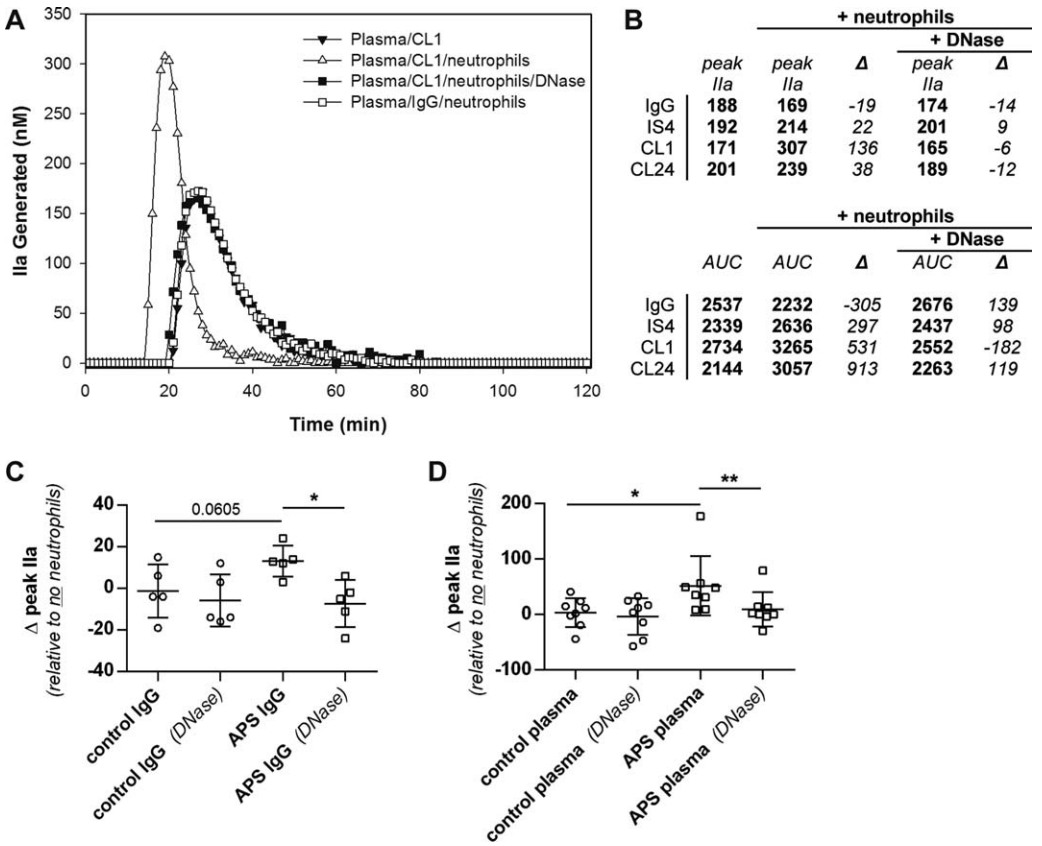

Figure 6. Stimulation of thrombin generation by purified antiphospholipid antibodies (aPL), as well as plasma from patients with antiphospholipid syndrome (APS), in a neutrophil- and DNA-dependent manner. **A,** Representative thrombin (IIa) generation plot demonstrating enhanced generation when neutrophils are exposed to platelet-poor plasma (from a healthy control) supplemented with aPL CL1 (10 μg/ml). The effect is not seen with plasma alone or plasma supplemented with control IgG. The effect is disrupted by DNase treatment. **B,** Representative data demonstrating that control plasma supplemented with anti–β_2-glycoprotein I (anti-β_2GPI) monoclonal antibodies promotes neutrophil-mediated thrombin generation. Delta values were calculated relative to the baseline condition (in this case, plasma supplemented with IgG but not neutrophils). Data are representative of 3 experiments, all with similar results. **C,** Thrombin generation in control plasma that was supplemented with total IgG (10 μg/ml) isolated from 5 healthy controls or 5 patients with primary APS (all anti-β_2GPI IgG positive) and then mixed with control neutrophils alone or neutrophils and DNase. **D,** Thrombin generation in plasma from healthy controls or patients with primary APS that was mixed with control neutrophils alone or neutrophils and DNase. In **C** and **D**, symbols represent the average of 3 independent assays in individual samples; horizontal and vertical lines show the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$. AUC = area under the curve.

ment with DPI potently inhibited NET release (see Supplementary Figure 8C).

In other cell types, such as monocytes and endothelial cells, TLR-4 has been implicated as an important mediator of aPL stimulation (8,9), while knockout of TLR-4 has been shown to protect against APS-like disease in mouse models (49). Further, one study has shown that aPL activation of neutrophils (albeit without testing NET release) can be mediated by TLR-4 (12). In this study, we found that a chemical TLR-4 inhibitor, TAK-242, abrogated both NET formation (Figure 5A) and H_2O_2 production (Supplementary Figure 9 [http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract]) in response to anti-β_2GPI monoclonal antibodies as well as total APS IgG fractions. A similar inhibition of NET formation was seen with an anti–TLR-4 blocking antibody, but not with anti–TLR-2 or isotype control (Figure 5B). All antibodies used for stimulation were free of endotoxin as measured by *Limulus* amebocyte lysate assay. Further, aPL-mediated stimulation remained effective in the presence of the known endotoxin inhibitor polymyxin B (see Supplementary Figure 10, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract). In summary, both ROS formation and TLR-4 engagement are important for aPL-mediated NET release, in contrast to PMA-stimulated NET formation, which is TLR-4 independent. Also, TLR-4 signaling here was not mediated by endotoxin, which is consistent with

the work of others studying the interplay between aPL and TLR-4 in monocytes and endothelial cells (8,9).

Other groups have shown that when neutrophils are cultured in vitro on poly-L-lysine–coated coverslips (as was done for many of the stimulation experiments here), β2 integrin engagement is an important part of achieving full neutrophil activation and NET release (50). Indeed, blockade of the β2 integrin Mac-1 with an anti-CD11b monoclonal antibody prevented NET release in response to not just aPL, but also PMA (Supplementary Figure 11 [http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract]). This result emphasizes that parallel pathways may be necessary to achieve full activation, and suggests that homotypic and heterotypic cellular interactions should be carefully considered when aPL–neutrophil interplay begins to be studied in vivo.

Enhancement of thrombin generation by purified aPL, as well as APS patient plasma, in a neutrophil- and DNA-dependent manner. The above findings are especially important when considered in the context of the arterial and venous thrombotic events that affect APS patients. To investigate whether aPL-mediated NET release has prothrombotic potential, we used a thrombin generation assay (37). We first found that thrombin generation is enhanced when control plasma supplemented with anti-β_2GPI monoclonal antibodies is incubated with control neutrophils (see Figure 6A for representative data for CL1, and Figure 6B for representative numerical data for the 3 anti-β_2GPI monoclonal antibodies known to stimulate NET release). Importantly, neutrophil-mediated thrombin generation could be attenuated by treatment with recombinant human DNase (Figures 6A and B), strongly indicating that the effect is dependent on NET formation and not up-regulation of surface molecules such as tissue factor. Similarly, the addition of APS total IgG fractions to control plasma resulted in enhanced thrombin generation, which could also be abrogated by DNase treatment (Figure 6C and Supplementary Figure 12A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract).

We were also interested in assessing APS patient plasma for its effects on neutrophil-dependent thrombin generation. When APS patients were treated with warfarin, their plasma uniformly failed to generate thrombin in the presence of neutrophils (see Supplementary Figure 13, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract), presumably secondary to the depletion of thrombin and other vitamin K–dependent factors in this context. This absence of thrombin generation was despite NET release being active in these samples (data not shown). We were able to identify 8 APS plasma samples that had been isolated from patients who were not being treated with either warfarin or a heparin-based anticoagulant. Four of these patients had anti-β_2GPI IgG, while 7 had anticardiolipin IgG. Similar to the aPL-supplemented control plasma samples (Figures 6A–C), APS patient plasma was a significant trigger of NET-mediated thrombin generation (Figure 6D and Supplementary Figure 12B), an effect that could again be abrogated by DNase treatment. Importantly, DNase treatment in the absence of neutrophils had no effect on baseline thrombin generation (data not shown). In summary, aPL stimulate neutrophils to release NETs, which then promote thrombin generation. In this model, thrombin generation can be prevented by either DNase treatment or depletion of clotting factors with warfarin.

Correlation of circulating NETs with a history of arterial thrombosis. As discussed above, circulating NET levels correlated with both anti-β_2GPI IgG levels and the presence of a LAC phenotype (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract). We also investigated whether circulating NETs and NET release could be predicted by clinical variables including history of specific events (Supplementary Table 3 [http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract]) and medications (Supplementary Table 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.39247/abstract]). Indeed, we found a positive correlation between history of arterial thrombosis and circulating cell-free DNA–NETs ($P = 0.04$). While other trends existed (for example, fewer circulating NETs in patients with a history of pregnancy morbidity), no other correlation reached statistical significance.

DISCUSSION

We have shown for the first time that neutrophils from patients with APS are predisposed to exaggerated NET release. This effect seems to predominantly depend on circulating aPL, since both purified IgG fractions and anti-β_2GPI monoclonal antibodies can promote NET release. Somewhat surprisingly, we found that the stimulation was not dependent on the addition of an exogenous source of β_2GPI (either in serum or as a purified protein). This is likely explained by the presence of β_2GPI on the surface of freshly isolated neutrophils. Whether β_2GPI is produced by neutrophils or simply acquired in circulation is unknown, although its presence on the neutrophil surface is not explained by up-regulation of phosphatidylserine, since isolated neutrophils were consistently negative for detectable annexin V binding (data not shown). Simi-

larly, we did not find the monocyte/endothelial receptor for β_2GPI, annexin A2, on the neutrophil surface.

We found a positive correlation in APS patients between circulating levels of NETs and both anti-β_2GPI IgG and LAC positivity (as well as triple-positivity for anti-β_2GPI, anticardioipin, and LAC). This was despite all patient samples having been collected outside of acute thrombotic episodes. We speculate that NET levels would increase even further at the time of APS-related events, as is known to happen in the general population for arterial disease (25), deep vein thrombosis (17), and microscopic thrombosis (42). Confirmation of this in APS will require longitudinal investigation. While our in vitro work strongly supports the notion of a role of aPL in directly promoting NET release, the higher levels of circulating NETs may also be partially explained by a recent study demonstrating an impaired ability of APS sera to degrade NETs in vitro (16), replicating a phenotype first described in SLE (38). That study did not, however, measure circulating NET levels, nor was the interaction between intact neutrophils and aPL assessed (16).

A compelling and unusual feature of APS is that it predisposes to *both* arterial and venous thrombosis; this is in contrast to most thrombotic risk factors, which promote one or the other. As mentioned above, NETs themselves have also been associated with arterial (25–27,51), as well as venous (17,20), events. Additionally, there is emerging evidence of an important role of NETs in cancer-associated thrombosis (43), another disease process associated with both arterial and venous vascular disease (52). Whether aPL–neutrophil interplay has a role in APS pregnancy morbidity is unknown, although it is interesting to note that exaggerated NET release has been seen in non-autoimmune patients with preeclampsia (53). NETs have also been linked to thrombotic microangiopathy, for example in thrombotic thrombocytopenic purpura (42), a process that replicates many features of the catastrophic form of APS. Although this study was not designed or powered to comprehensively study clinical correlations, we did interestingly find an association between the history of arterial events and circulating NETs. This finding awaits confirmation in larger and independent cohorts.

Mechanistically, aPL-mediated NET release is dependent on the production of ROS. Such dependence has been seen in most (46), but not all (47,48), forms of NET release. This could have implications for the interplay between oxidative stress, neutrophils, and aPL, with oxidative stress having previously been shown to be a risk factor for modifications of β_2GPI that promote aPL–β_2GPI interaction (54). We additionally found that aPL activation of neutrophils was at least partially dependent on TLR-4. Numerous studies have shown TLR-4 signaling to be important for aPL activation of both monocytes and endothelial cells (8,9), while TLR-4 mutation protects against APS-like disease in animals (49). Indeed, our demonstration of this pathway in neutrophils is complementary to previous work showing synergy between LPS and aPL in neutrophil activation (12). As infections are well recognized to up-regulate neutrophil TLR-4 (55), one can envision a scenario in which NET release smolders at a low level in patients between events, before being dramatically activated in the setting of an infection (two-hit hypothesis).

To fully understand these pathways, NET release will need to be studied in vivo using experimental models of APS. For example, aPL have been shown to indirectly activate neutrophils through the complement cascade and the well-recognized neutrophil stimulator C5a (14,15). We believe that in vitro aPL-mediated NET release is independent of C5a, as neither total IgG fractions nor anti-β_2GPI monoclonal antibodies were dependent on the presence of serum (and exogenous complement) for neutrophil activation. Further, APS patient sera promoted NET release even when heat-inactivated (data not shown). However, with the recognition that phagocytes themselves may generate and activate complement components (56), we cannot completely rule out some type of auto-amplification loop here.

We believe these data have implications for the prothrombotic diathesis inherent to APS. Neutrophils stimulated with aPL promote thrombin generation, an effect that could be attenuated by DNase treatment, but not treatment with an anti–tissue factor antibody. We also found that plasma isolated from APS patients treated with warfarin generated no detectable thrombin, despite still promoting NET release. Similarly, heparin can dismantle NETs (17), and prevent NET/histone-mediated platelet activation (57). We therefore postulate that these traditional APS treatments function downstream of NET release.

Regarding other therapies, aspirin, which already has a role as an adjuvant agent in APS when arterial manifestations are present, has been shown to prevent NET release (58). Antimalarial medications also have a role in both the criteria and noncriteria manifestations of APS (3). With evidence that antimalarials interact with, and modulate cellular responses to, extrinsic DNA (59), one wonders if their protective role in APS may be partially attributed to their interaction with NETs. In terms of novel therapeutics, peptidylarginine deiminase inhibitors, which prevent NET release, have shown therapeutic benefits in murine SLE (36), atherosclerosis (27), and inflammatory arthritis (60), although they have yet to be tested in APS. Further, DNase has a protective role in animals in both arterial (stroke and myocardial infarction) (61), and venous models (20).

Whether exogenous DNase will ever be a practical means of disrupting circulating NETs is unclear, although Fc fusion proteins have been described (62).

Overall, this study clearly demonstrates that aPL can activate neutrophils to release NETs and hints that these circulating NETs contribute to thrombotic events. NET release should be further assessed in experimental models, with an eye toward more targeted approaches to therapy for APS patients.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Knight had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Yalavarthi, Gould, Liaw, Knight.
Acquisition of data. Yalavarthi, Gould, Rao, Mazza, Morris, Núñez-Álvarez, Hernández-Ramírez, Bockenstedt, Liaw, Cabral, Knight.
Analysis and interpretation of data. Yalavarthi, Gould, Rao, Liaw, Cabral, Knight.

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