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Increased Adhesive Potential of Antiphospholipid Syndrome Neutrophils Mediated by β2 Integrin Mac-1

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Objective. While the role of antiphospholipid antibodies in activating endothelial cells has been extensively studied, the impact of these antibodies on the adhesive potential of leukocytes has received less attention. This study was undertaken to investigate the extent to which antiphospholipid syndrome (APS) neutrophils adhere to resting endothelial cells under physiologic flow conditions and the surface molecules required for that adhesion.

Methods. Patients with primary APS (n = 43), patients with a history of venous thrombosis but negative test results for antiphospholipid antibodies (n = 11), and healthy controls (n = 38) were studied. Cells were introduced into a flow chamber and perfused across resting human umbilical vein endothelial cells (HUVECs). Surface adhesion molecules were quantified by flow cytometry. Neutrophil extracellular trap release (NETosis) was assessed in neutrophil-HUVEC cocultures.

Results. Upon perfusion of anticoagulated blood through the flow chamber, APS neutrophils demonstrated increased adhesion as compared to control neutrophils under conditions representative of either venous (n = 8; $P < 0.05$) or arterial (n = 15; $P < 0.0001$) flow. At the same time, APS neutrophils were characterized by up-regulation of CD64, CEACAM1, β_2-glycoprotein I, and activated Mac-1 on their surface (n = 12–18; $P < 0.05$ for all markers). Exposing control neutrophils to APS plasma or APS IgG resulted in increased neutrophil adhesion (n = 10–11; $P < 0.0001$) and surface marker up-regulation as compared to controls. A monoclonal antibody specific for activated Mac-1 reduced the adhesion of APS neutrophils in the flow-chamber assay ($P < 0.01$). The same monoclonal antibody reduced NETosis in neutrophil–HUVEC cocultures ($P < 0.01$).

Conclusion. APS neutrophils demonstrate increased adhesive potential, which is dependent upon the activated form of Mac-1. In patients, this could lower the threshold for neutrophil–endothelium interactions, NETosis, and possibly thrombotic events.

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune condition of unknown cause and is defined by the presence of circulating antiphospholipid antibodies (aPLs; anticardiolipin, anti–β_2-glycoprotein I [anti-β_2GPI], or lupus anticoagulant [LAC]) (1). The morbidity and mortality of APS are significant, as patients carry a markedly increased risk of thrombotic events (especially stroke and deep vein thrombosis) and pregnancy loss (2). Beyond these disease-defining events, patients with APS may also develop cytopenias, heart valve damage, nephropathy, and cognitive dysfunction, among other complications (3). While it has long been recognized that circulating leukocytes play some role in the pathophysiology of APS, the impact of neutrophils has only come to light in the past few years (4). Our group and others have shown that APS neutrophils are prone to the exaggerated release of neutrophil extracellular traps (NETs), prothrombotic tangles of DNA, and microbicidal proteins released from dying neutrophils

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(5). At the same time, at least some APS blood does not degrade NETs normally (6). Indeed, dismantling NETs with deoxyribonuclease (7) and preventing NET release (NETosis) via activation of adenosine receptors (8) has proven effective in murine models of APS. In further support of neutrophil hyperactivity in APS, our group has demonstrated that the APS neutrophil transcriptome is characterized by the up-regulation of a number of meta-groups, including a cellular defense node that includes L-selectin and P-selectin glycoprotein I among other adhesion molecules (9).

Beyond neutrophils, both animal models and descriptive studies of patients have demonstrated signs of smoldering endothelial activation in APS. For example, tissue factor activity is increased in carotid homogenates from mice treated with antiphospholipid antibody (10), which correlates with increased leukocyte–endothelium interplay (11). In keeping with the latter concept, antagonizing either E-selectin or P-selectin (the key selectins expressed on the endothelium) is protective against thrombosis in mice; the same is true for strategies blocking the endothelial integrin ligands vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 (ICAM-1) (12,13). One study has suggested that down-regulation of endothelial nitric oxide synthase by aPLs may be another important factor in increased leukocyte–endothelium interplay (14). Beyond these in vivo data, there is robust evidence in vitro that aPLs can activate endothelial cells to express tissue factor and adhesion molecules (15,16). Mechanistically, NF-κB, p38 MAPK, and Krüppel-like factors have all been implicated in antiphospholipid antibody–mediated activation of endothelial cells (17–19), demonstrating how aPLs may co-opt pathways normally associated with more "authentic" activating stimuli.

Mac-1 is a heterodimeric β2 integrin especially expressed by myeloid-lineage cells. In its activated state, Mac-1 mediates cell–cell interactions by engaging a variety of surface molecules, including the endothelium-expressed glycoprotein ICAM-1. In this study, we focused on leukocytes and especially neutrophils (rather than on the endothelium) and how they contribute to heterotypic adhesive interactions relevant to APS. We studied blood samples from APS patients as well as control leukocytes conditioned with either APS plasma or APS IgG. We characterized leukocyte adhesion to resting endothelial cells under physiologic flow conditions. We also considered key adhesion molecules, including Mac-1, on the surface of APS neutrophils and explored their role in not just adhesion, but also in NETosis.

MATERIALS AND METHODS

Human subjects. Patients were recruited from rheumatology and hematology clinics at the University of Michigan (Supplementary Tables 1–3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41057/abstract). All 43 patients diagnosed as having APS fulfilled the clinical and laboratory criteria for APS according to the updated international consensus (Sydney) classification criteria (1). None of the patients met American College of Rheumatology revised criteria for systemic lupus erythematosus (20). Of the patients with APS, some were classified as having "obstetric APS" if they had no prior history of vascular thrombosis but did have APS-associated obstetric complications as defined by the Sydney classification criteria (i.e., ≥3 unexplained, consecutive spontaneous pregnancy losses; or ≥1 unexplained fetal deaths at ≥10 weeks of gestation; or ≥1 preterm deliveries of a morphologically normal infant before 34 weeks of gestation due to severe preeclampsia, eclampsia, or features consistent with placental insufficiency) (1). Eleven patients with a history of unprovoked venous thrombosis (VT) but with negative test results for aPLs, were also recruited (Supplementary Table 4, available at http://onlinelibrary.wiley.com/doi/10.1002/art.41057/abstract); many of these patients had genetic risk factors for VT, such as factor V Leiden heterozygosity as detailed in Supplementary Table 4.

Thirty-eight healthy controls were recruited through a posted flyer; exclusion criteria included history of a systemic autoimmune disease, active infection, and pregnancy. All 38 controls were screened for IgG anti-β_2GPI and all had negative test results. Blood samples were collected by phlebotomist-performed venipuncture, and serum was prepared by standard methods and stored at –80°C until used. IgG, IgM, and IgA anti-β_2GPI, as well as IgG and IgM anticardiolipin antibodies, were determined by multiplex assay on a BioPlex 2200 System (Bio-Rad). LAC was tested according to published guidelines (21). This study was reviewed and approved by the University of Michigan Institutional Review Board. Written informed consent was obtained from all participants prior to inclusion.

Preparation of human IgG. IgG was purified from human serum samples with a Protein G–Agarose kit according to the instructions of the manufacturer (Pierce). Briefly, serum was diluted in IgG binding buffer and passed through a protein G–agarose column at least 5 times. IgG was eluted with 0.1*M* glycine. This solution was neutralized with 1*M* Tris followed by overnight dialysis against phosphate buffered saline (PBS) at 4°C. After passage through a 0.2μ filter, IgG purity was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. IgG concentrations were quantified by bicinchoninic acid protein assay (Pierce). IgG preparations were confirmed to be free of endotoxin contamination as determined by a chromogenic endotoxin quantification kit (Pierce).

Human neutrophil purification. For neutrophil preparation, blood samples were collected into sodium citrate tubes by standard phlebotomy techniques. The anticoagulated blood was then fractionated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). Neutrophils were 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.

In vitro flow adhesion assays. For all flow chamber experiments, blood was collected into citrate tubes. A parallel-plate flow chamber (PPFC) with straight gaskets forming the flow channel (GlycoTech) was then used for in vitro flow adhesion assays. Briefly, a single straight gasket was placed over a human umbilical vein endothelial cell (HUVEC) monolayer cultured on a glass coverslip (22) and vacuum-sealed to the flow deck to form the bottom adhesion substrate of the chamber.

For some experiments, "leukocytes" were prepared by mixing together the buffy coat and RBCs (after discarding plasma). In other cases, "neutrophils" were prepared by retrieving the neutrophil–RBC pellet that remained after Ficoll gradient separation. For these leukocyte and neutrophil experiments, cells were always brought back to their original blood volume with flow buffer (PBS with calcium and magnesium with 1% bovine serum albumin [BSA]). A total of 2 ml of whole blood, leukocytes, or neutrophils was introduced into the chamber from an inlet reservoir via a programmable syringe pump (KD Scientific). For low-shear experiments, samples were perfused across the HUVEC monolayer using a laminar flow profile. The wall shear rate (WSR; γw) was fixed by adjusting the volumetric flow rate (Q) through the channel according to the following equation:

$$Q = \frac{\gamma h^2 w}{6}$$

where h is the channel height (127 μm) and w is the channel width (0.25 cm). The height of 127 μm and WSR of 200 seconds^{-1} were chosen to approximate the flow profile within veins and venules. Low-shear samples were perfused over HUVECs for 5 minutes. For high-shear experiments, pulsatile flow was used in the horizontal PPFC as described previously (22). Specifically, samples were perfused over HUVEC monolayers in pulsatile flow at a WSR of 1,000 seconds^{-1} for 15 minutes (23–25). The flow time was chosen to ensure the same volume of blood passed through the chamber as for laminar/low-shear experiments (22). At the end of the prescribed flow time, flow buffer was added to the PPFC to flush out nonadherent cells.

Ten images per sample were collected along the length of the flow chamber using a Nikon TE2000S inverted microscope with a digital camera (Photometrics CoolSNAP EZ with a Sony CCD sensor). Results were imaged and analyzed using NIS-elements analysis software and ImageJ. The adherent cells were normalized to the control cells examined on the same day so as to minimize variation attributable to different batches of HUVECs.

For experiments involving the pretreatment or conditioning of control leukocytes, the buffy coat/RBC sample was incubated at 37°C for 1 hour with plasma before washing again with flow buffer. For blocking experiments, anti–Mac-1 (20 μg/ml, clone CBRM1/5) antibody or isotype control was also included during the incubation.

Flow cytometry studies. For all flow cytometry experiments, blood samples were collected into citrate tubes and immediately processed. Fc blocking of cells (in whole blood) was carried out using Human TruStain FcX (BioLegend), according to the instructions of the manufacturer. Subsequently, cells (still in whole blood) were stained with specific antibodies for 30 minutes on ice, followed by immediate lysis of RBCs and fixation of leukocytes using eBioscience 1-step Fix/Lyse Solution. Samples were analyzed on an LSRFortessa cell analyzer (BD Biosciences) and ZE5 cell analyzer (Bio-Rad). Further data were analyzed with FlowJo software (Tree Star). Specific primary antibodies were against apolipoprotein H (ABS162; EMD Millipore), CD15 (W6D3; BioLegend), CD16 (3G8; BioLegend), CEACAM1 (283340; R&D Systems), CD64 (10.1; BioLegend), activated lymphocyte function–associated antigen 1 (LFA-1) (m24; BioLegend), activated Mac-1 (CBRM1/5; BioLegend), and CD62L (DREG-56; BioLegend). We also used eBioscience Fixable Viability Dye eFluor 506 and secondary antibody Alexa Fluor 680 AffiniPure donkey anti-rabbit IgG (heavy and light chains) (711-625-152; Jackson ImmunoResearch). For leukocyte conditioning experiments, the sample was spiked with increasing concentrations of either APS or control IgG (10 μg/ml or 100 μg/ml), or the citrated plasma of the sample was discarded and replaced with heterologous control or APS plasma and incubated for 1 hour at 37°C before staining and flow analysis.

Toll-like receptor 4 (TLR-4) and complement inhibition. Anticoagulated control blood was preincubated with 20 μ*M* TLR-4 inhibitor (TAK-242) or 10 μ*M* C5a receptor antagonist (W-54011) (both from Cayman Chemical) for 30 minutes. The sample was then spiked with IgG as described above and incubated for 1 hour at 37°C.

Quantification of NETosis. Neutrophils were labeled with CytoTrace Red CMTPX (5 μ*M*; AAT Bioquest) according to the instructions of the manufacturer and resuspended in RPMI medium supplemented with 0.5% BSA and 0.5% fetal bovine serum (all from Gibco). Neutrophils (1.5×10^5 cells/well) were then incubated in 48-well plates with a preestablished monolayer of HUVECs at 37°C. Samples were additionally treated with 100 μg/ml APS IgG or control IgG in the presence of anti–Mac-1 (20 μg/ml; clone CBRM1/5) or isotype control. After 3 hours, Sytox green (ThermoFisher Scientific) was added to a final concentration of 0.2 μ*M* and incubated for an additional 10 minutes. Fluorescence was quantified at excitation and at emission wavelengths of 485 nm and 520 nm, respectively, using a BioTek Cytation 5 Cell Imaging Multi-Mode Reader. Representative images were captured by the BioTek Cytation 5 reader's 20× objective lens.

Statistical analysis. Data analysis was conducted using GraphPad Prism software, version 7. Normally distributed data were analyzed by *t*-tests, while skewed data were assessed by the Mann-Whitney test. Analysis of variance (ANOVA) with appropriate correction for multiple comparisons was also used where appropriate. For each panel of data, the specific statistical test is

indicated in the figure legend. *P* values less than 0.05 were considered significant.

RESULTS

Demonstration of increased adhesion under flow by APS neutrophils. Utilizing anticoagulated whole blood collected from patients with primary APS or matched healthy volunteers (Supplementary Tables 1–3, available at http://onlinelibrary.wiley.com/doi/10.1002/art.41057/abstract), we tested leukocyte adhesion to unactivated/resting early-passage HUVECs in a PPFC assay. Representative images of leukocyte adhesion in the PPFC assay are shown in Figure 1A. Compared to control blood, we observed increased adhesion of APS leukocytes under high-shear (1,000 seconds^{-1}) pulsatile flow conditions, as might be found in arteries or the arterioles (Figure 1B). Similar results were observed when blood was passed through the chamber under lower-shear (200 seconds^{-1}) laminar flow, as would be found in the venous system (Figure 1B). If the increased adhesion were being driven by factors inherent to the leukocytes themselves, we reasoned that a similar phenotype would be observed if plasma (along with the cytokines and autoantibodies that might activate the HUVECs) were discarded. Indeed, isolated APS leukocytes, in the absence of plasma, still adhered in exaggerated manner to HUVECs (under both high- and low-shear flow conditions) (Figure 1C). Finally, we removed not just plasma, but also peripheral blood mononuclear cells by spinning the blood through a density gradient. Again, we observed increased adhesion of neutrophils to HUVECs as compared to controls (Figure 1D). In summary, these data reveal that leukocytes, and specifically neutrophils, demonstrate increased adhesion to unstimulated HUVECs in the context of various flow profiles. The phenotype persisted even after plasma was discarded, which is consistent with an inherent role for neutrophils in the adhesive interaction.

Up-regulation of adhesion molecules on the surface of APS neutrophils. In an effort to understand what seemed to be an inherent increase in APS neutrophil adhesion, we evaluated

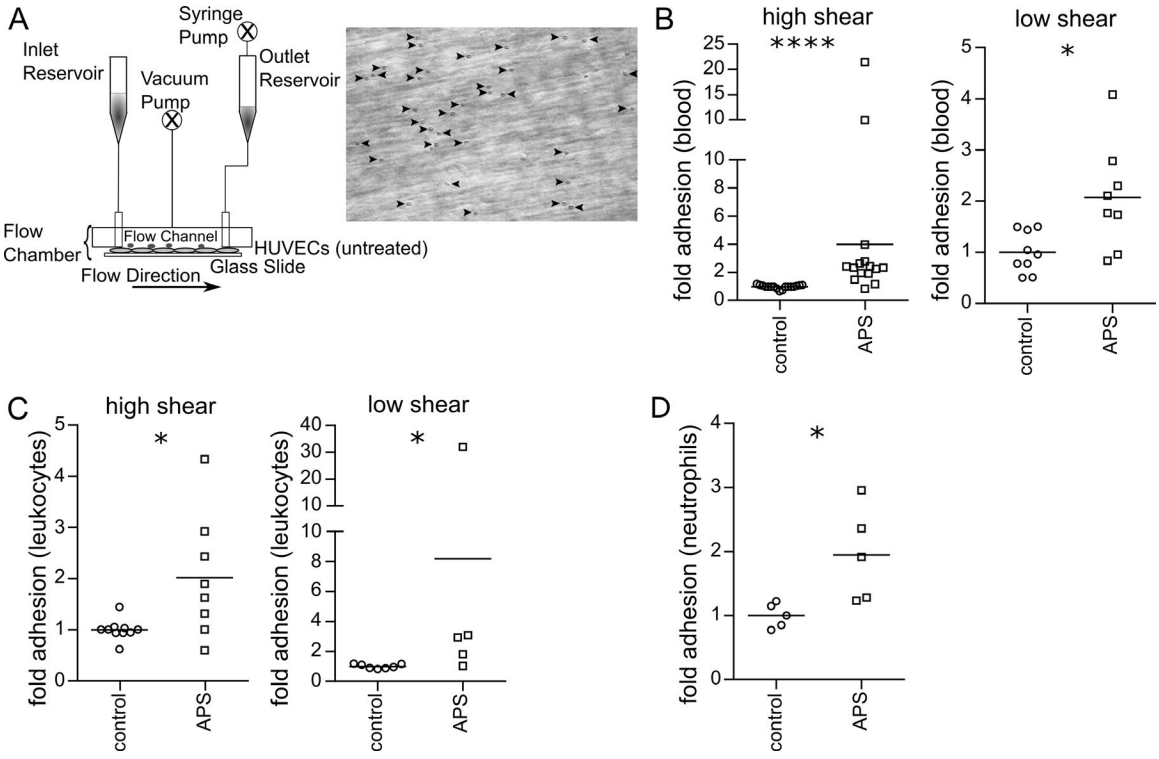

Figure 1. Antiphospholipid syndrome (APS) neutrophils showing increased adhesion in multiple flow profiles. Adhesion was measured under either pulsatile high-shear (1,000 seconds^{-1}) conditions or laminar low-shear (200 seconds^{-1}) conditions. **A**, Schematic illustration of the parallel-plate flow chamber (left) and a representative image (right) showing adhesion of APS leukocytes (**arrowheads**). Original magnification × 20. **B**, Perfusion of anticoagulated whole blood samples from healthy controls (n = 18 for high shear and n = 9 for low shear) or patients with APS (n = 15 for high shear and n = 8 for low shear) through the flow chamber and quantification of adherent cells. **** = $P < 0.0001$ versus controls, by Mann-Whitney test; * = $P < 0.05$ versus controls, by *t*-test. **C**, Perfusion of isolated and resuspended (in flow buffer; plasma discarded) control leukocytes (n = 10 for high shear and n = 7 for low shear) or APS leukocytes (n = 8 for high shear and n = 5 for low shear) through the flow chamber and quantification of adherent cells. * = $P < 0.05$ versus controls, by Mann-Whitney test. **D**, Perfusion of isolated and resuspended (in flow buffer) control neutrophils (n = 5) or APS neutrophils (n = 5) through the flow chamber and quantification of adherent cells. * = $P < 0.05$ versus controls, by *t*-test. In **B**–**D**, symbols represent individual samples; horizontal lines show the mean. HUVECs = human umbilical vein endothelial cells.

the surface expression of various adhesion molecules on the neutrophil surface (Figure 2A). As ICAM-1 is known to be expressed even by resting HUVECs, we reasoned that β2 integrin family members (which are well known to interact with ICAM-1) might be up-regulated on APS neutrophils, thus mediating the increased adhesion. While we observed no difference in the activated form of β2 integrin LFA-1 (Figure 2B), the activated form of another β_2 integrin, Mac-1, was robustly up-regulated on the surface of APS neutrophils (Figure 2C). An evaluation of other potential markers of neutrophil activation revealed no significant difference in CD62L (L-selectin), but did reveal up-regulation of both CD64 and CEACAM1 (Figures 2D–F). Interestingly, autoantigen β_2GPI was also present at increased levels on the surface of APS neutrophils (Figure 2G). In summary, these data demonstrate increased expression of activated Mac-1, but not activated LFA-1, on the neutrophil surface, which correlates with the up-regulation of other neutrophil activation markers such as CD64 and CEACAM1.

Dependence of APS IgG–mediated up-regulation of Mac-1 on neutrophils on TLR-4 and complement anaphylatoxin receptors. Previous work by our group has demonstrated that NETosis can be triggered from control neutrophils by incubation with either APS serum or APS IgG (5). In the present study we explored whether adhesion molecules were also up-regulated by similar treatment (Figure 3A). When we "conditioned" control blood cells with APS plasma, we did not find increased expression of activated LFA-1 on the surface of neutrophils (Figure 3B). In contrast, there was a striking increase in surface expression of activated Mac-1 (Figure 3C). We also found evidence of shedding of CD62L from neutrophils and up-regulation of both CD64 and CEACAM1 (Figures 3D–F). β_2GPI was measured, but was not significantly up-regulated (Figure 3G). We then conditioned control blood with IgG purified from patients with primary APS; under these conditions we observed up-regulation of activated Mac-1 on neutrophils (Figure 4A), along with shedding of CD62L (Figure 4B). Having previously observed that APS IgG–mediated NETosis is dependent on TLR-4, we assessed that same pathway in the context of Mac-1 activation. Indeed, the TLR-4 signaling inhibitor TAK-242 prevented APS IgG from up-regulating activated Mac-1 on neutrophils (Figure 4C). We reasoned that we might also find a role for the complement cascade in neutrophil activation. When blood was treated with a C5a receptor inhibitory antibody, up-regulation of activated Mac-1 on neutrophils was blunted (Figure 4D). In summary, these data together indicate

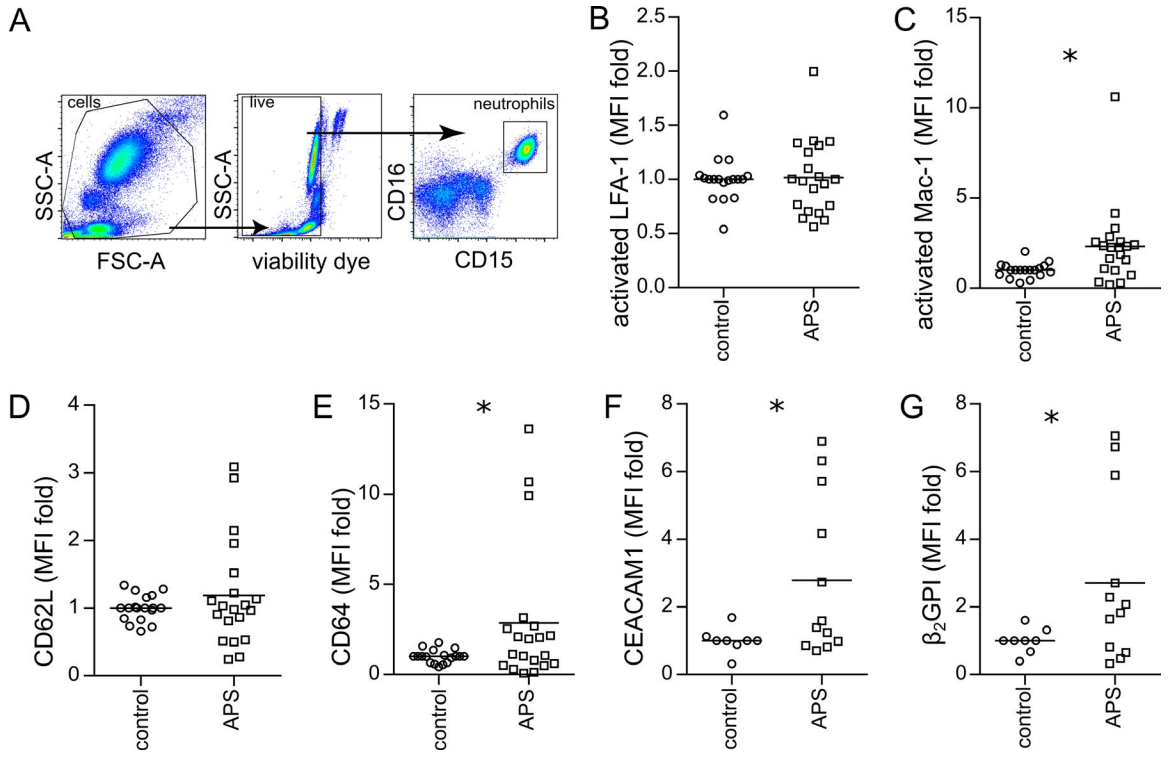

Figure 2. Increased expression of activated Mac-1 and other adhesion molecules on antiphospholipid syndrome (APS) neutrophils. Flow cytometry was performed after treating anticoagulated whole blood samples with fluorescently labeled antibodies. Mean fluorescence intensity (MFI) was normalized to controls run in the same batch. **A**, Cell sorting plots showing gating strategy for identification of neutrophils in whole blood. **B–G**, Up-regulation of activated lymphocyte function–associated antigen 1 (LFA-1) (**B**), activated Mac-1 (**C**), CD62L (**D**), CD64 (**E**), CEACAM1 (**F**), and β_2-glycoprotein I (β_2GPI). (**G**). In **B**–**E**, n = 18 for control samples and n = 20 for APS samples. In **F** and **G**, n = 8 for control samples and n = 12 for APS samples. In **B**–**G**, symbols represent individual samples; horizontal lines show the mean. * = $P < 0.05$ versus controls, by *t*-test. Differences in **B** and **D** were not significant.

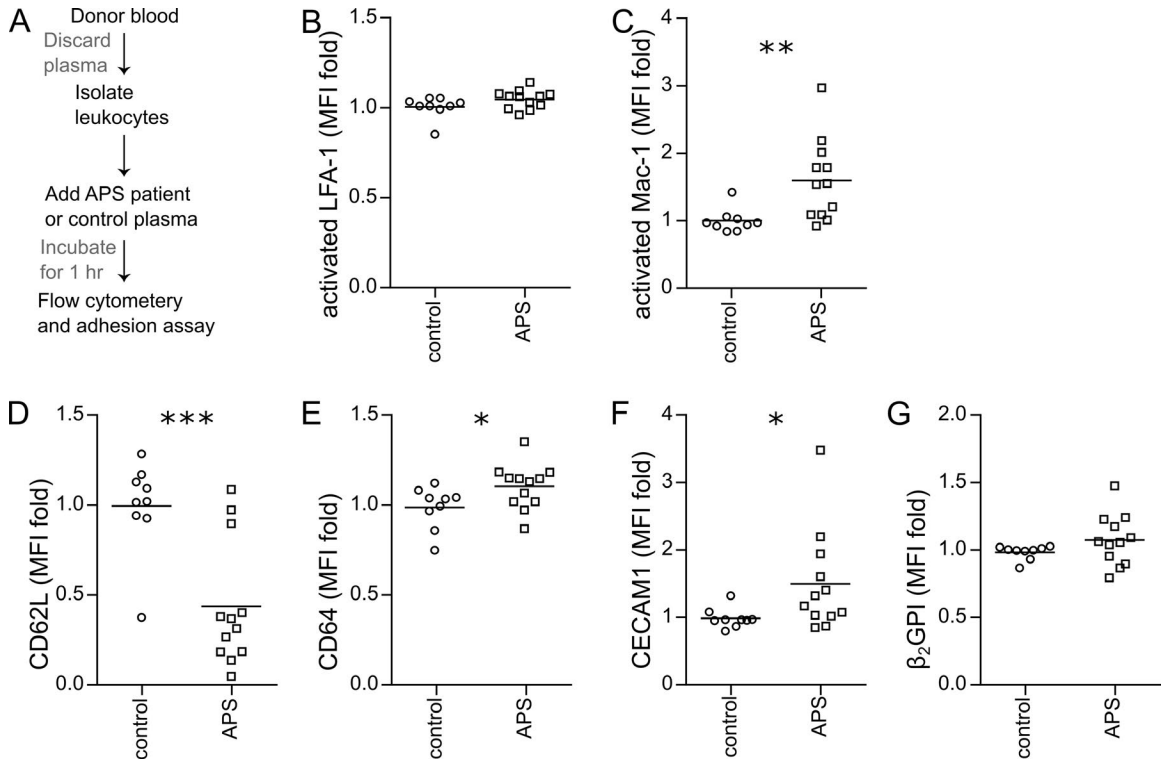

Figure 3. Increased expression of activated Mac-1 and other adhesion molecules when control neutrophils are conditioned with APS plasma. **A**, Conditioning of control leukocytes with heterologous control plasma or APS plasma and incubation with fluorescently labeled antibodies. MFI was normalized to controls run in the same batch. **B**, No increased expression of activated LFA-1 on the neutrophil surface (differences not significant, by *t*-test). **C**, Increase in surface expression of activated Mac-1. ** = $P < 0.01$ versus controls, by *t*-test. **D**, Shedding of CD62L from neutrophils. *** = $P < 0.001$ versus controls, by *t*-test. **E**, Up-regulation of CD64. * = $P < 0.05$ versus controls, by *t*-test. **F**, Up-regulation of CEACAM1. * = $P < 0.05$ versus controls, by Mann-Whitney test. **G**, Up-regulation of β_2GPI (differences not significant, by *t*-test). In **B**–**G**, symbols represent individual samples (n = 9 for controls and n = 12 for APS); horizontal lines show the mean. See Figure 2 for definitions.

that control neutrophils up-regulate activated Mac-1 in response to conditioning with either APS plasma or APS IgG and that this up-regulation requires TLR-4 and the C5a receptor.

Requirement of activated Mac-1 for increased adhesion of APS neutrophils. Having found that APS plasma up-regulates Mac-1 on the surface of control neutrophils, we reasoned that this up-regulation might be directly responsible for increased neutrophil adhesion. Indeed, APS plasma–treated cells, but not control plasma–treated cells, demonstrated increased adhesion under both high-shear and low-shear flow conditions (Figure 5A). Furthermore, a monoclonal antibody specific for the activated form of Mac-1 effectively neutralized adhesion in the context of conditioning with APS plasma, but had no effect in the setting of control plasma (Figure 5B). To determine whether the ability of plasma to stimulate cell adhesion was unique to patients with APS or whether the phenotype might extend to any patient with a history of thrombosis, we recruited 11 patients with a history of unprovoked VT but with test results negative for aPLs (Supplementary Table 4, available at http://onlinelibrary.wiley.com/doi/10.1002/art.41057/abstract).

When compared to plasma samples from healthy controls, plasma samples from the VT cohort triggered no increase in cell adhesion (Figure 5C). Similar to this finding, conditioning neutrophils with plasma from the VT cohort did not alter levels of activated Mac-1, CD62L, or CD64 (Figures 5D–F) on the neutrophil surface. Finally, we asked whether the increased cell adhesion triggered by APS plasma might be limited to patients with a history of "thrombotic APS" (i.e., at least 1 documented arterial, venous, or small vessel thrombotic event). Interestingly, we observed increased adhesion whether the plasma was collected from patients with "thrombotic APS" or patients with purely "obstetric APS" (Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41057/abstract). In summary, these data demonstrate that antagonizing the activated form of Mac-1 is sufficient to reduce APS-relevant adhesion to levels seen in controls.

Requirement of activated Mac-1 for NETosis by APS neutrophils bound to endothelial cells. Given evidence by our group and others (5,26) that NETosis proceeds most efficiently upon cell adhesion, we investigated whether the aforementioned antibody targeting activated Mac-1 might mitigate NETosis.

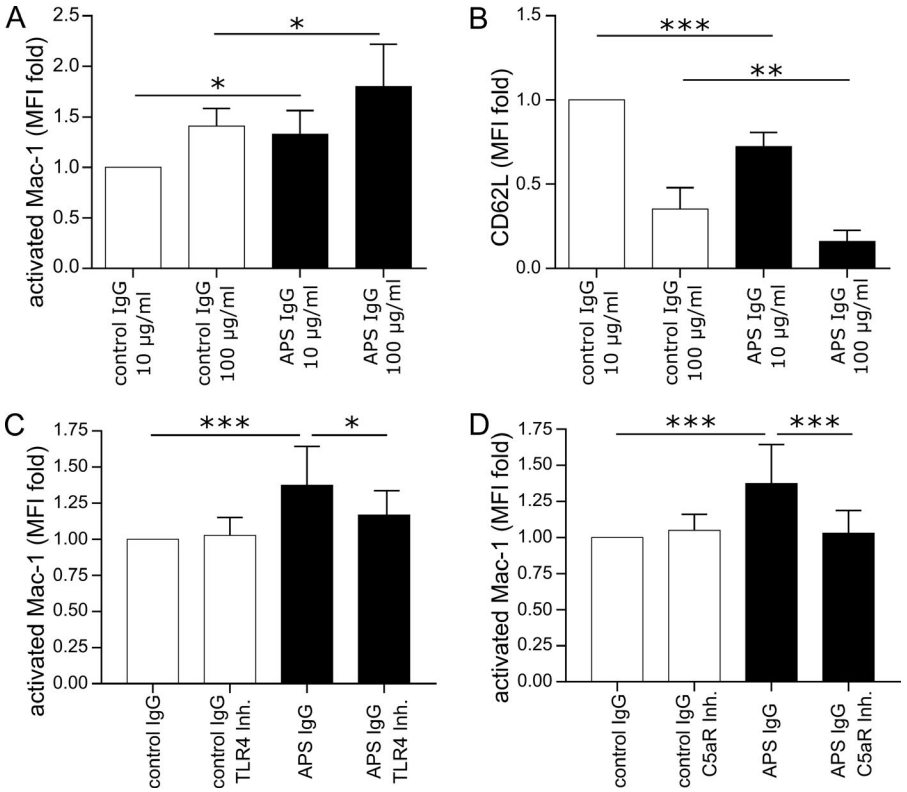

Figure 4. Increased expression of activated Mac-1 on control neutrophils occurring in a Toll-like receptor 4 (TLR-4)– and complement-dependent manner through exposure to purified APS IgG. Control leukocytes were treated with control or APS IgG as indicated. Activated Mac-1 and CD62L were quantified by flow cytometry. **A**, Up-regulation of activated Mac-1. * = $P < 0.05$ by one-way analysis of variance (ANOVA) with correction for multiple comparisons by the Holm-Sidak method (n = 4 independent experiments). **B**, Shedding of CD62L. ** = $P < 0.01$; *** = $P < 0.0001$ by one-way ANOVA with correction for multiple comparisons by the Holm-Sidak method (n = 4 independent experiments). **C**, Control leukocytes treated with control or APS IgG (100 µg/ml) in the presence or absence of TLR-4 inhibitor (Inh.). Activated Mac-1 was quantified by flow cytometry. * = $P < 0.05$; *** = $P < 0.001$ by one-way ANOVA with correction for multiple comparisons by the Holm-Sidak method (n = 8 independent experiments). **D**, Control leukocytes treated with control or APS IgG (100 µg/ml) in the presence or absence of C5a receptor (C5aR) inhibitor. Activated Mac-1 was quantified by flow cytometry. *** = $P < 0.001$ by one-way ANOVA with correction for multiple comparisons by the Holm-Sidak method (n = 7 independent experiments). Values are the mean ± SEM. See Figure 2 for other definitions.

To test this, we adhered vital dye–labeled neutrophils to resting HUVECs and then tracked NETosis in real time via the loss of vital dye and the local release of decondensed DNA (Figure 6A). As compared to isotype treatment, the Mac-1 monoclonal antibody significantly neutralized NETosis in response to APS IgG, but not phorbol myristate acetate (Figure 6B). In summary, these data demonstrate that inhibition of the activated form of Mac-1 can neutralize NETosis, at least in the context of APS.

DISCUSSION

While there are many studies characterizing the activated endothelium in APS (11,15), comparatively little is known about the adhesive nature of circulating cells (27). In this study we examined the adhesive potential of APS leukocytes and, particularly, neutrophils. We found enhanced adhesion of APS neutrophils to resting HUVECs irrespective of the flow conditions (Figures 1B and C). Notably, this functional increase in adhesion was observed in the context of up-regulated adhesion molecules on the neutrophil surface, including CD64, CEACAM1, and the activated form of Mac-1 (Figure 2). These findings did not extend to patients with a history of unprovoked VT and negative test results for aPLs (Figures 5C–F), suggesting that these results may represent relatively unique features of APS. Of note, all flow experiments were performed in the presence of RBCs, which are known to stabilize leukocyte adhesion, thereby more closely modeling conditions observed in vivo (28).

In addition to thrombosis of arteries, veins, and small vessels, another hallmark of APS is pregnancy-related morbidity. There is evidence that obstetric complications of APS have distinct pathophysiology as compared to thrombotic APS (29); however, recent data have suggested that up to 60% of patients who begin with a diagnosis of "obstetric APS" will eventually develop a thrombotic event (30). While the cohort tested here was relatively enriched for patients with thrombotic complications, we identified and tested 8 patients with a history of only obstetric morbidity (see definition in Methods). Interestingly, these patients with obstetric APS

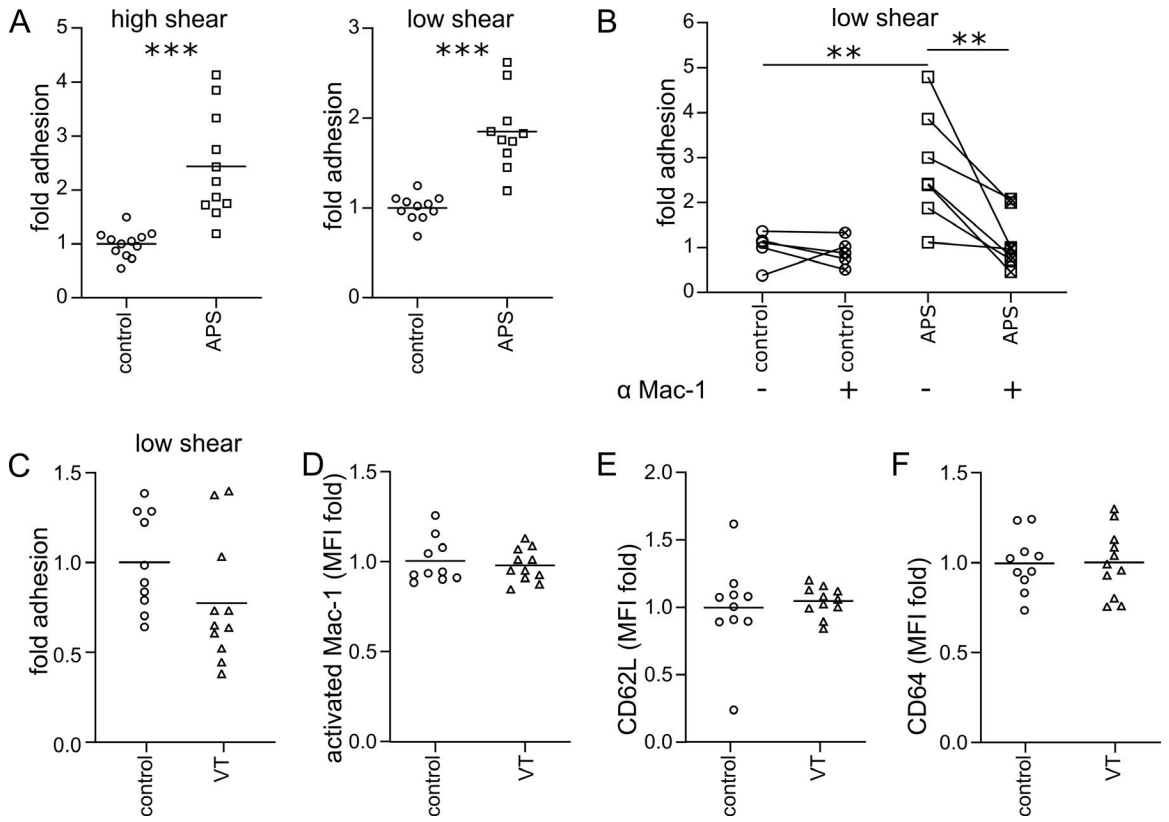

Figure 5. Increased adhesion of APS leukocytes mediated by activated Mac-1. Control leukocytes were incubated with heterologous control or APS plasma, resuspended in flow buffer, and perfused through the flow chamber. Adherent cells were quantified. **A**, Increased adhesion of APS plasma–treated cells. *** = $P < 0.001$ versus controls, by *t*-test. **B**, Conditions similar to those in **A**, except with the addition of a blocking antibody for activated Mac-1 to some samples. ** = $P < 0.01$ by one-way analysis of variance (ANOVA) with correction for multiple comparisons by the Holm-Sidak method. **C**, Conditions similar to those in **A**, except that control leukocytes were incubated with heterologous control plasma or plasma from patients with a history of unprovoked venous thrombosis (VT) and negative test results for antiphospholipid antibody. The leukocytes were then resuspended in flow buffer and perfused through the flow chamber. Adherent cells were quantified. **D**–**F**, Conditions similar to those in Figure 3, except that control leukocytes were conditioned with heterologous control plasma or VT plasma and incubated with fluorescently labeled antibodies, followed by quantification of the MFI fold change in activated Mac-1 (**D**), CD62L (**E**), and CD64 (**F**) in VT plasma–conditioned cells relative to controls run in the same batch. Differences in **C**–**F** were not significant, by *t*-test. Symbols represent individual samples; horizontal lines indicate the control value (set as 1). See Figure 2 for other definitions.

were indistinguishable from patients with a history of thrombotic events in terms of neutrophil adhesion (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41057/abstract). We hope that further research of disease models will allow us to understand the extent to which activated Mac-1 may be a direct mediator of thrombotic (versus obstetric) pathophysiology.

In our previous study, we found that inhibition of TLR-4 signaling could mitigate APS IgG–mediated NETosis (5). This is in addition to other studies demonstrating that TLR-4 deficiency protected mice from APS in vivo, and that neutrophil TLR-4 supported phagocytosis and reactive oxygen species production by APS neutrophils (10,31). We demonstrated that the TLR-4 inhibitor TAK-242 prevented APS IgG from up-regulating activated Mac-1 on neutrophils (Figure 4C). These data support further investigation of TLR-4 signaling as a potential therapeutic target in APS. Similar to the TLR-4 pathway, complement contributes to neutrophil activation in many contexts. Here we demonstrated that inhibition of C5a receptor attenuates the up-regulation of activated Mac-1 on APS IgG–stimulated neutrophils (Figure 4D). These data are consistent with previous studies demonstrating that C5a receptor contributes to up-regulation of Mac-1 (32,33).

Despite extensive CD62L shedding in control neutrophils conditioned with APS plasma (Figure 3D), CD62L shedding was not detected at a significant level in neutrophils freshly isolated from patients with APS (Figure 2D). One possibility is that the patient neutrophils have had time to up-regulate CD62L expression, thereby effectively compensating for shedding. In support of this idea, CD62L was up-regulated at the gene level in our recent transcriptomic profiling of APS neutrophils (9). Alternatively, it is possible that neutrophils that have shed CD62L in vivo are strongly activated to the point that they have preferentially left circulation, thereby being unavailable for our analysis. Interestingly, we also detected increased surface expression of the APS autoantigen β_2GPI on the surface of APS neutrophils by flow cytometry

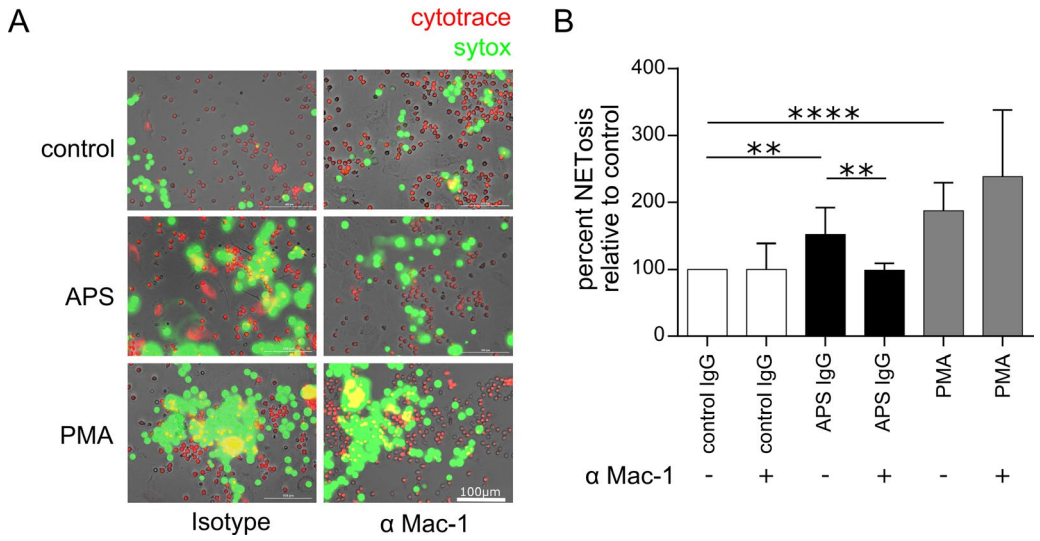

Figure 6. Necessary activation of Mac-1 for regulation of antiphospholipid syndrome (APS) IgG–mediated neutrophil extracellular trap release (NETosis). **A**, Fluorescence intensity showing live cells (stained with CytoTrace Red) and extracellular DNA (stained with Sytox green) in samples stimulated with control IgG (top), APS IgG (middle), or phorbol myristate acetate (PMA; bottom). Bars = 100 μm. **B**, Quantification of NETosis as measured by fluorescence intensity of Sytox green in cells stimulated as indicated in the presence or absence of antibodies to Mac-1. Bars show the mean ± SEM. ** = $P < 0.01$; **** = $P < 0.0001$ by one-way analysis of variance with correction for multiple comparisons by the Holm-Sidak method (n = 4 independent experiments).

(Figure 2G), which is in line with our 2017 study demonstrating a 4.5-fold increase in β_2GPI gene expression in APS neutrophils (9). It should be noted that at least one other group has demonstrated similar β_2GPI surface and expression phenotypes in circulating APS monocytes (34,35).

Blocking experiments demonstrated that the adhesion of APS neutrophils is at least partially mediated by activated Mac-1 (Figure 5B). Interestingly, we also showed that APS IgG–mediated NETosis was Mac-1 dependent (Figure 6). This latter finding is similar to a study by Neeli et al, who showed that Mac-1 was required for both hypercitrullination of histones and NETosis in response to lipopolysaccharide (LPS) stimulation (26). Of note, both LPS and APS IgG activate neutrophils via TLR-4 (5). Taken together, the data presented in this study reveal a previously unknown role for activated Mac-1 in the adhesion and NETosis of APS neutrophils.

In the general population, numerous study findings have suggested a link between Mac-1, neutrophils, and endothelium in thrombotic vascular diseases (36–38). For example, significant up-regulation of Mac-1 by neutrophils has been detected at the time of myocardial infarction and for up to 1 week after the event (39). In another study, neutrophils from myocardial infarction patients displayed enhanced adhesion to endothelial cells ex vivo, which could be reduced by blocking Mac-1 (40). In patients with acute ischemic stroke, there was significant up-regulation of neutrophil Mac-1 immediately after the event, and persisting into the subacute phase of the stroke (41). In patients with venous thromboembolism, increased adhesive potential of neutrophils was associated with a higher rate of recurrence (42). As indicated in Supplementary Table 1 (http://onlinelibrary.wiley.com/doi/10.1002/art.41057/abstract), the average time from last thrombotic event to blood collection for patients with APS included in this study was approximately 4.5 years. One might hypothesize that up-regulation of activated Mac-1 is detected only acutely (i.e., at the time of thrombosis) for persons in the general population, but remains undetected in patients with APS, which is potentially consistent with the life-long anticoagulation that such patients require. To further explore this question, it will be necessary to build longitudinal APS cohorts and study them alongside cohorts from the general population.

Beyond activated Mac-1, we also observed consistent up-regulation of CD64 on the surface of APS neutrophils (Figure 2E). This is somewhat reminiscent of studies of patients with sickle cell disease. Sickle cell neutrophils demonstrate increased levels of CD64 and increased adhesion to endothelial cells, with some evidence that CD64 directly contributes to the adhesion (43,44). Future studies should investigate whether this surface molecule, typically thought of as an IgG receptor, might also play a role in APS neutrophil adhesion. CEACAM1 (CD66a) expression was also consistently up-regulated on APS neutrophils (Figure 2F). Interestingly, there are studies to suggest that signaling through CEACAM1 (and potentially other CEACAM family members) results in activation of Mac-1 and increased adhesion to endothelial cells (45–48). At the same time, recent reports (predominantly in mice) have suggested that CEACAM1 may have inhibitory functions, protecting against neutrophil hyperactivity and neutrophil-mediated tissue damage. For example, CEACAM1 protected against ischemic stroke by inhibiting matrix metalloproteinase 9 (49,50). CEACAM1-deficient mice also formed larger carotid thrombi in a ferric chloride injury model, suggesting that CEACAM1 may inhibit arterial thrombus (51). Thus, this very interesting molecule seems to warrant further study in APS.

In conclusion, our study has revealed a novel role for activated Mac-1 in regulating APS neutrophils and NETosis, and hints at a role for Mac-1 in APS pathophysiology. While Mac-1 can be considered as a therapeutic target in APS, mutations in CD11b are a well-recognized risk factor for lupus (52), and many, but not all, mouse studies have suggested that CD11b deficiency has the potential to exacerbate autoimmunity (52–55). Also, since Mac-1 binds to a variety of ligands, selective inhibition of specific Mac-1 adhesive interactions could emerge as a potential therapeutic strategy. For example, one proof-of-concept study has demonstrated that targeted inhibition of the Mac-1–CD40L interaction improved bacterial clearance and survival in a polymicrobial model of sepsis (56). Another innovative approach has involved the utilization of small-molecule Mac-1 agonists. These agonists tend to induce an intermediate-affinity conformation in Mac-1 (57), which may permit neutrophil adhesion, with less potential for endothelial damage. Indeed, a partial Mac-1 agonist not only protected MRL/lpr mice from end-organ injury, but also enhanced endothelium-dependent vasorelaxation and thereby demonstrated an overall vasoprotective effect (58). Taken together, these study findings indicate that targeting Mac-1 might indeed be feasible and emphasize the need for future research in patients with APS.

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 submitted for publication. 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. Sule, Kelley, Bockenstedt, Eniola-Adefeso, Knight.

Acquisition of data. Sule, Kelley, Gockman, Yalavarthi, Vreede, Banka.

Analysis and interpretation of data. Sule, Kelley, Gockman, Yalavarthi, Vreede, Banka, Eniola-Adefeso, Knight.

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