

Anti-Neutrophil Cytoplasmic Antibodies Stimulate Release of Neutrophil Microparticles

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ABSTRACT

The mechanisms by which anti-neutrophil cytoplasmic antibodies (ANCA) may contribute to the pathogenesis of ANCA-associated vasculitis are not well understood. In this study, both polyclonal ANCAs isolated from patients and chimeric proteinase 3–ANCA induced the release of neutrophil microparticles from primed neutrophils. These microparticles expressed a variety of markers, including the ANCA auto-antigens proteinase 3 and myeloperoxidase. They bound endothelial cells via a CD18-mediated mechanism and induced an increase in endothelial intercellular adhesion molecule-1 expression, production of endothelial reactive oxygen species, and release of endothelial IL-6 and IL-8. Removal of the neutrophil microparticles by filtration or inhibition of reactive oxygen species production with antioxidants abolished microparticle-mediated endothelial activation. In addition, these microparticles promoted the generation of thrombin. *In vivo*, we detected more neutrophil microparticles in the plasma of children with ANCA-associated vasculitis compared with that in healthy controls or those with inactive vasculitis. Taken together, these results support a role for neutrophil microparticles in the pathogenesis of ANCA-associated vasculitis, potentially providing a target for future therapeutics.

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The anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAVs) include Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), and Churg–Strauss syndrome.^{1,2} These rare diseases are associated with significant mortality and morbidity due to small vessel vasculitis resulting in pauci-immune GN,¹ respiratory tract vasculitis resulting in alveolar hemorrhage, and thrombosis.^{3,4} ANCAs directed against neutrophil antigens, typically proteinase 3 (PR3-ANCA) in WG or myeloperoxidase (MPO-ANCA) in MPA, are found in the sera of most affected patients.⁵ Data from *in vitro* studies,^{6,7} animal models,^{8,9} and clinical observations in humans^{10–12} indicate that ANCAs are directly involved in the pathogenesis of vasculitis. One proposed paradigm of ANCA pathogenesis is that neutrophils, after cytokine priming, are fully activated by ANCAs either in the blood or within lesional tissue and firmly adhere to the

vascular endothelium.^{5–7} These neutrophils degranulate and release numerous cytotoxic mediators provoking endothelial injury and vasculitis.¹³ There are additional mechanisms postulated, including complement activation.¹⁴

Although there is supportive evidence for all of these mechanisms, important unanswered questions concerning the pathogenesis of AAVs remain. These include how ANCAs bind to endothelium independently of ANCA antigens to cause endothelial

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activation¹⁵ even though endothelial cells have not been conclusively demonstrated to produce MPO or PR3,^{16,17} and why patients with AAV have evidence of increased hypercoagulability.^{3,18} Finally, ANCA levels do not always correlate with disease activity,¹⁹ and it is unknown how therapeutic plasma exchange mediates its beneficial effects,^{20,21} as this does not appear to be the result of ANCA removal from the circulation alone. Further understanding of the interaction between ANCAs, leukocytes, endothelium, and coagulation pathways could address some of these important but as yet unexplained observations.

Increased cellular microparticles (MPs) have been described in AAVs,^{22–24} although their pathologic significance in this context is currently unknown. MPs are membrane vesicles released upon activation or apoptosis from various cell types including neutrophils, platelets, and endothelial cells.^{25,26} Loss of phospholipid asymmetry and increased surface expression of phosphatidylserine are crucial events in this process.^{22,25} In children with active vasculitis, we previously demonstrated elevated platelet and endothelial MPs that correlated with disease activity,^{22,27} an observation subsequently confirmed in adults with AAVs.²⁴ In adults with AAVs, Daniel *et al.*²³ observed increased plasma neutrophil microparticles (NMPs) expressing CD66b, although the pathogenic potential of these were not investigated. As they convey various bioactive effectors originating from the parent cells, MPs may exhibit a wide spectrum of biological activities relevant to the pathogenesis of acute vasculitis including participation in inflammation and involvement in hemostatic/thrombotic pathways.²⁸

Because neutrophil activation is a central event in the initiation of vasculitis caused by ANCAs, we hypothesized that stimulation of cytokine-primed neutrophils with ANCAs would result in the release of NMPs, and that these NMPs could be potent mediators of vasculitis and thrombin generation. In this study, we demonstrated for the first time that NMPs carrying adhesion molecules and the ANCA autoantigens MPO and PR3 are increased in the plasma of children with active AAV and vary with disease activity. We show that polyclonal PR3-ANCA and MPO-ANCA from patients and chimeric mouse/human PR3-ANCA directly stimulate cytokine-primed neutrophils to release NMPs. In addition, these NMPs can bind to and activate endothelium via induction of reactive oxygen species (ROS) and generate thrombin.

RESULTS

Patients and Controls

We studied nine children (two male) with a diagnosis of AAV (median age 16 years, range 9–18 years). The nine children had active AAV assessed by a median Birmingham Vasculitis Activity Score (BVAS) of 8 of 63 (3–22 of 63) and evidence of ongoing endothelial injury as detected by an increased median circulating endothelial cell (CEC) count of 136 (16–256) cells/ml and adjunctive laboratory evidence of inflammation as indicated by a median erythrocyte sedimentation rate (ESR)

of 42 (6–155) mm/h and a median C-reactive protein (CRP) of 30 (3–139) mg/L. Four children had inactive AAV (BVAS = 0 of 63 in all), median CEC count of 53 (8–112) cells/ml, median ESR of 11.5 (2–15) mm/h, and median CRP of 7 (5–15) mg/L; and four children had active but ANCA-negative vasculitides (polyarteritis nodosa [PAN] *n* = 3; Kawasaki disease [KD] *n* = 1) with median BVAS of 6 of 63, median CEC count of 75 (40–92) cells/ml, median ESR of 109 (40–126) mm/h, and median CRP of 95 (40–156) mg/L. The clinical features of the vasculitis patients are summarized in Table 1. There were eight sex-matched pediatric healthy controls (two male, six female), median age 9.8 years (range, 2–16 years), with a median CEC count of 24 cells/ml (range, 0–80 cells/ml).

Polyclonal ANCAs and Chimeric Anti-PR3-ANCA Induce Release of NMPs from Primed Neutrophils

The first objective was to determine whether nonpooled ANCAs derived from individual patients with AAV stimulate NMP release from neutrophils. Freshly isolated neutrophils from healthy adult donors were primed with TNF- α 2 ng/ml for 15 minutes and then stimulated with either polyclonal PR3-ANCA or MPO-ANCA derived from patients with AAV or with healthy control polyclonal IgG at a concentration of 200 μ g/ml. NMPs were identified in supernatants using flow cytometry (Figure 1). Of note, these NMPs were negative for the platelet marker CD42a, the monocyte marker CD14, and the endothelial activation marker CD62e, indicating no contamination of our NMP population by MPs from platelets, monocytes, or endothelial cells, respectively (data not shown).

PR3-ANCA, MPO-ANCA, and control IgG had no effect on NMP release from unprimed neutrophils. Furthermore, priming with 2 ng/ml TNF- α did not result in NMP production. In contrast, both PR3-ANCA and MPO-ANCA caused a significant increase in NMP production from primed neutrophils by five- to nine-fold (Figure 2A; *P*=0.04). This effect was not observed with control IgG on primed neutrophils.

To investigate further the specificity and dose-response curve of the PR3-ANCA response, the experiment was repeated this time using 12.5 μ g/ml chimeric IgG1 or 0–20 μ g/ml IgG3 PR3-ANCA. Chimeric IgG3 PR3-ANCA resulted in a dose-dependent NMP release only from TNF- α -primed neutrophils, an effect that was not observed using chimeric control IgG, thus confirming the specificity of NMP release in response to PR3-ANCA (Figure 2, B and C). No differences in total Annexin V⁺ (AnV⁺) MP release were found between the chimeric IgG1 and IgG3 PR3-ANCA at 12.5 μ g/ml (Figure 2B). We then compared the phenotype of NMPs spontaneously released from resting neutrophils with that of the NMPs derived from ANCA stimulation (12.5 μ g/ml anti-PR3 IgG3) or *N*-formyl-methionine-leucine-phenylalanine (fMLP) stimulation (Figure 2D). Chimeric PR3-ANCA-stimulated NMPs had higher expression of active CD11b (*P*=0.04), MPO (*P*=0.01), and PR3 (*P*=0.01), but no significant difference in CD66b (*P*=0.9), as shown in Figure 2D. In contrast, compared with resting NMPs, fMLP-NMPs had comparable expression of

Table 1. Clinical and laboratory features of vasculitis patients

Demographic Data	Active AAV (n=9)	Inactive AAV (n=4)	Other Active Vasculitides (n=4)
Median age, years (range)	16 (9–18)	16 (9–12)	7 (4–10)
Sex, F/M	2/7	1/3	1/4
Classification	WG, n=9	WG, n=4	PAN, n=3 KD, n=1
BVAS (median, range)	8 of 63 (3–22 of 63)	0 of 63 in all	6 of 63 (3–8 of 63)
Organ involvement	ENT, n=6 Orbital lesions, n=3 Renal, n=5 Pulmonary, n=4 Skin, n=9	All in remission	Skin, n=3 Renal, n=1
ANCA, EU/ml (normal range 0–10 EU/ml)	PR3 positivity in n=8, median PR3 295.5 (range 21–530) MPO 62 in a single patient	PR3 negative in n=2 PR3 18, n=1 MPO 45, n=1	Negative in all patients
Laboratory evidence of active disease			
ESR, mm/h (median, range)	42 (6–155)	11.5 (2–15)	109 (40–126)
CRP, mg/L (median, range)	30 (3–139)	7 (5–15)	95 (40–156)
CEC count, cells/ml (median, range)	136 (16–256)	53 (8–112)	75 (40–92)
Treatment, n			
corticosteroids	3	4	3
cyclophosphamide	1	0	0
azathioprine	0	0	0
mycophenolate mofetil	1	4	0

Classification of the vasculitic syndromes was based on the recent EULAR/PRINTO/PRES classification criteria for pediatric vasculitis. KD was identified based on five of six of the American Heart Criteria. Immunomagnetic bead extraction was used for enumeration of circulating endothelial cells. ENT, ear, nose, and throat; EULAR/PRINTO/PRES, European League Against Rheumatism/Pediatric Rheumatology International trials Organisation/Pediatric Rheumatology European Society.

MPO ($P=0.60$), PR3 ($P=0.48$), active CD11b ($P=0.08$), and CD66b ($P=0.9$).

NMPs Bind to Endothelial Cells via CD18

As NMPs generated from ANCA stimulation expressed the β 2-integrin CD18 and the active form of CD11b, it followed that they should have the capacity to bind to endothelial cells via CD18 and intercellular adhesion molecule-1 (ICAM-1) interactions. To investigate this, human umbilical vein endothelial cells (HUVECs) were incubated with NMPs derived after chimeric PR3-ANCA stimulation of neutrophils for 30 minutes at 37°C. NMP binding to HUVECs was measured by flow cytometry after washing the endothelial cells twice and was represented as the percentage of endothelial cells expressing the specific neutrophil markers CD66b, total CD11b, or MPO. NMPs bound to HUVECs: the percentage of HUVECs expressing CD66b was 6.17% (SEM of three experiments, 1.51; Figure 3A); the percentage of HUVECs expressing CD11b was 7.01% (SEM, 1.01; Figure 3B); and the percentage of HUVECs expressing MPO was 13.0% (SEM, 0.79, Figure 3C). Binding of NMPs was blocked using a mAb against CD18 (clone TS1/18), which was added at a concentration of 10 μ g/ml to the HUVECs for 30 minutes prior to the addition of NMPs (Figure 3, A–C). This effect was not observed using an isotype control mAb, confirming CD18 involvement in NMP binding to HUVECs.

NMPs Derived from ANCA Stimulation Upregulate Endothelial ICAM-1 and Increase IL-6 and IL-8 Production

We next investigated the ability of NMPs to cause endothelial activation after binding. HUVEC activation in the presence of NMPs was measured by endothelial surface expression of ICAM-1 and E-selectin using flow cytometry. HUVECs were incubated for 24 hours with NMPs derived from unstimulated (resting) neutrophils, TNF- α -primed neutrophils, IgG1 or IgG3 chimeric PR3-ANCA, or control chimeric IgG-stimulated neutrophils before and after neutrophil priming. Expression of ICAM-1 (CD54) and E-selectin (CD62e) was measured on HUVECs using flow cytometry (Figure 4A). Chimeric IgG1 and IgG3 PR3-ANCA NMPs induced upregulation of ICAM-1 expression (Figure 4, A and B) but had no effect on E-selectin expression (data not shown). In contrast, NMPs derived from supernatants from control experiments (resting NMPs, TNF- α -primed NMPs, or control IgG with or without priming) did not upregulate ICAM-1, consistent with the absence or low levels of NMP production from neutrophils in response to these experimental conditions (Figure 4B). Furthermore infliximab at a concentration of 10 μ g/ml did not abrogate the ability of ANCA-induced NMPs to upregulate ICAM-1, thus excluding any possibility of low-grade endothelial activation derived from TNF- α contamination used in the neutrophil priming step (data not shown).

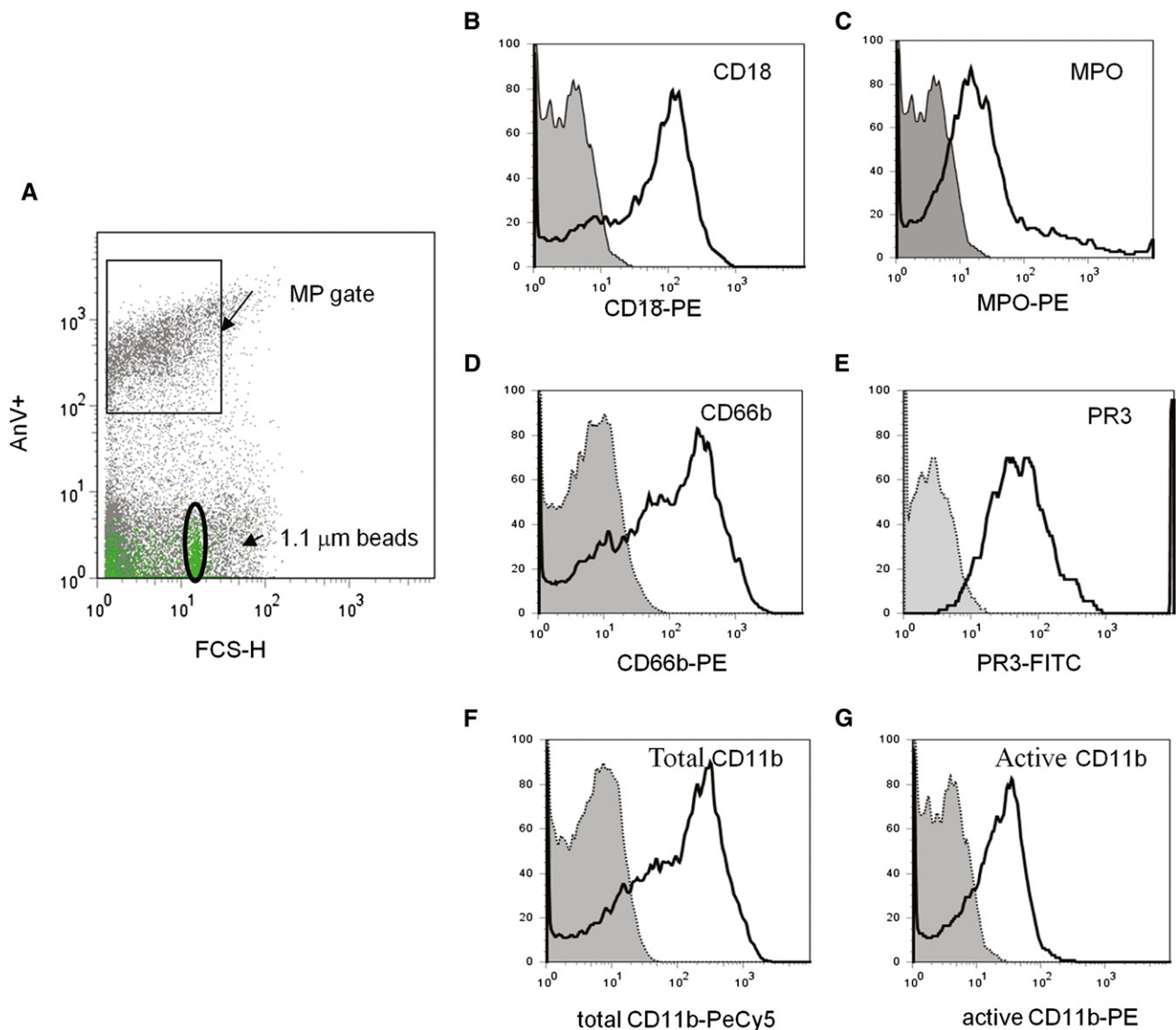


Figure 1. Flow cytometric analysis of NMPs derived from ANCA-stimulated primed neutrophils. (A) Dot plot demonstrating the gating strategy for MPs. Annexin V⁺ MPs derived from stimulation of primed neutrophils from a healthy adult donor with 12.5 μg/ml IgG1 anti-PR3 chimeric antibodies. The NMP gate was defined by forward-scatter characteristics corresponding with a size <1.1 μm and positive annexin V labeling. (B–G) Histograms demonstrating detection of NMP expression of CD18 (B), MPO (C), CD66b (D), PR3 (E), total CD11b (F), and active CD11b (G). The same flow cytometry strategy was used to quantify NMPs in platelet-poor plasma from healthy donors or from patients with AAV.

Filtration of the NMP-containing supernatants using an 0.2-μm filter was performed to investigate whether NMPs would be removed, thus abrogating any potential stimulatory effects of NMPs. Filtration of supernatants containing NMPs derived from chimeric IgG3 PR3-ANCA-stimulated neutrophils removed approximately 95% of the annexin V⁺ NMP events quantified using flow cytometry (Figure 5, A and B) and completely abolished the stimulatory effect of this supernatant on ICAM-1 expression on HUVECs (Figure 5C).

Because we had demonstrated that NMPs bind to endothelium using CD18 and that this can be blocked using

anti-CD18, we investigated the capacity of anti-CD18 to abrogate NMP-induced ICAM-1 upregulation. As shown in Figure 5D, 10 μg/ml of anti-CD18 blocked the ability of NMPs to upregulate ICAM-1, which was not observed with an isotype control antibody.

We then assessed endothelial cytokine production in response to NMP stimulation. Chimeric IgG1 PR3-ANCA NMPs increased EC release of IL-6 (mean 78 pg/ml, SEM 9; versus control 51 pg/ml, SEM 2.7; $P=0.03$); and IL-8 (mean 47 pg/ml, SEM 4; versus control 25.3 pg/ml, SEM 1.1; $P=0.01$; Figure 5, E and F). This response was again attenuated by preincubation

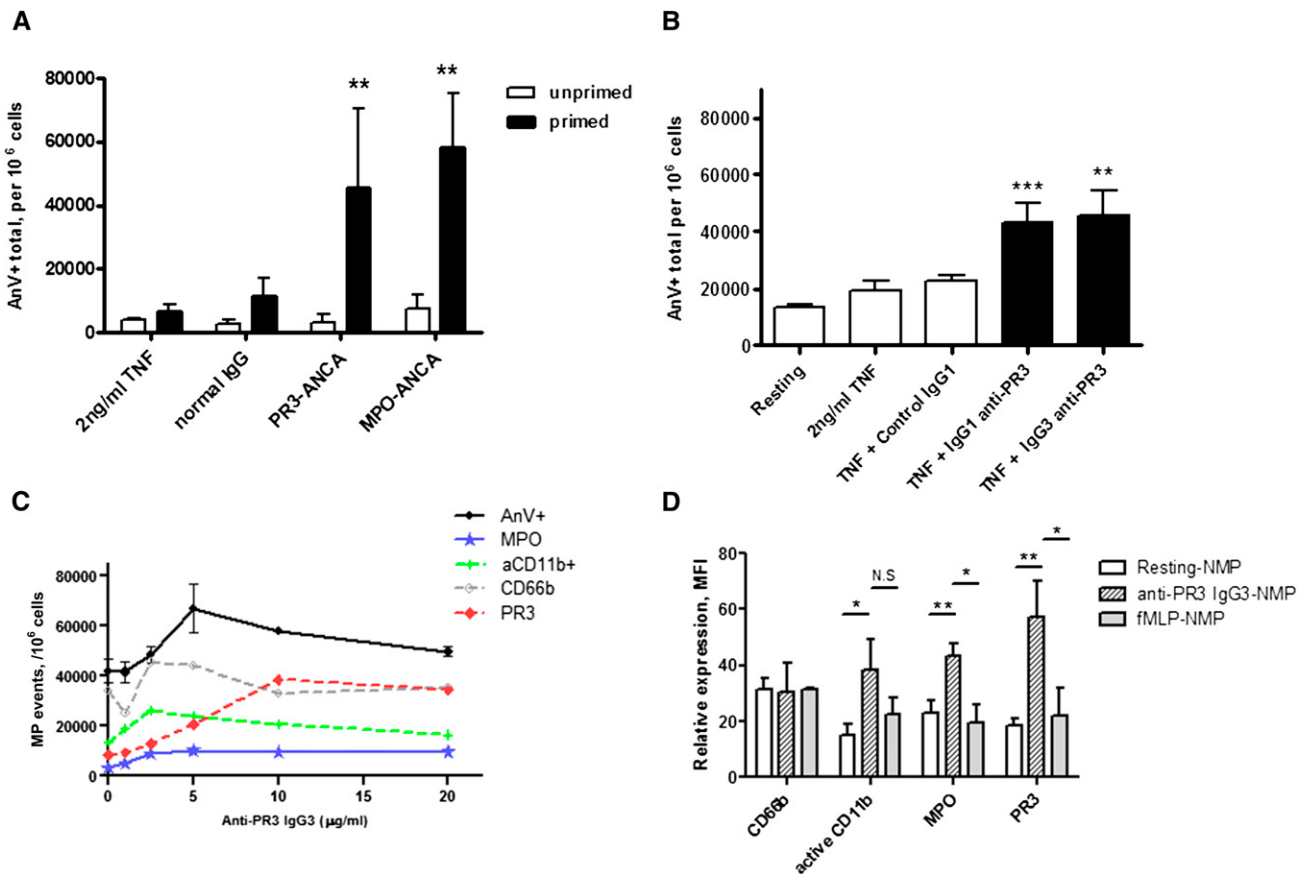


Figure 2. Human polyclonal ANCA and anti-PR3 chimeric ANCA stimulate NMPs release from primed neutrophils. (A) Neutrophils were primed with 2 ng/ml TNF- α for 15 minutes and then treated with 200 μ g/ml polyclonal PR3-ANCA, polyclonal MPO-ANCA, or control polyclonal IgG for 60 minutes. Both PR3-ANCA and MPO-ANCA stimulation of primed neutrophils resulted in a five- or nine-fold (respectively) increase in NMPs, an effect not observed with priming alone or using control polyclonal IgG with or without priming. * $P < 0.05$. (B) This experiment was repeated using 12.5 μ g/ml chimeric IgG1 or IgG3 PR3-ANCA or control chimeric IgG3 antibody for 60 minutes. Chimeric PR3-ANCA again resulted in NMP release from primed neutrophils, an effect not observed with chimeric control IgG3 ($P = 0.24$). There was no difference between IgG1 and IgG3 PR3-ANCA in their ability to induce NMP production ($P = 0.79$). *** $P < 0.001$ and ** $P < 0.01$. (C) Dose-response curve for NMP release in response to chimeric IgG3 PR3 ANCA demonstrated a sharp rise in NMP release from primed neutrophils from a concentration up to 5 μ g/ml, which then plateaued at higher concentrations. Annexin V⁺ NMPs expressed several neutrophil markers including CD18, CD11b, and the ANCA antigens PR3 and MPO. Data are expressed as mean and SEM of three experiments. (D) Comparison of the phenotype of NMPs spontaneously released from resting neutrophils with that of the NMPs derived from ANCA stimulation (anti-PR3 IgG3, 12.5 μ g/ml). PR3-ANCA stimulated NMPs had higher expression of active CD11b ($P = 0.04$), MPO ($P = 0.01$), and PR3 ($P = 0.01$) but no significant difference in CD66b ($P = 0.9$). In contrast, compared with resting NMPs, fMLP-NMP had comparable expression of MPO ($P = 0.60$), PR3 ($P = 0.48$), active CD11b ($P = 0.08$), and CD66b ($P = 0.9$). MFI, median fluorescence intensity.

HUVECs with neutralizing mAb to CD18 or filtration (Figure 5, E and F).

We then performed dose-response experiments comparing NMPs derived from chimeric IgG3 PR3-ANCA stimulation versus NMPs spontaneously released from resting neutrophils. Using endothelial ICAM-1 (CD54) upregulation (Figure 5G) and IL-8 production (Figure 5H) as readouts, we demonstrated higher endothelial activation using NMPs derived from ANCA stimulation in a dose-dependent manner ($P = 0.02$ for ICAM-1; $P = 0.02$ for IL-8 production at the highest NMP concentration), confirming that the lack of endothelial activation from resting NMPs is not only due to low NMP concentration but also

reflects constitutive and functional differences in NMPs derived from ANCA stimulation versus NMPs spontaneously released from resting neutrophils.

NMPs Induce ROS Production by Endothelial Cells

We postulated that NMPs, after binding to endothelial cells, cause upregulation of endothelial ICAM-1 via oxidative stress. To address this hypothesis, we measured endothelial ROS production by measurement of intracellular endothelial ROS levels using 2',7'-dichlorofluorescein (DCF) fluorescence after 1-hour incubation with NMPs. As shown in Figure 6A, compared with controls, NMPs derived from PR3-ANCA

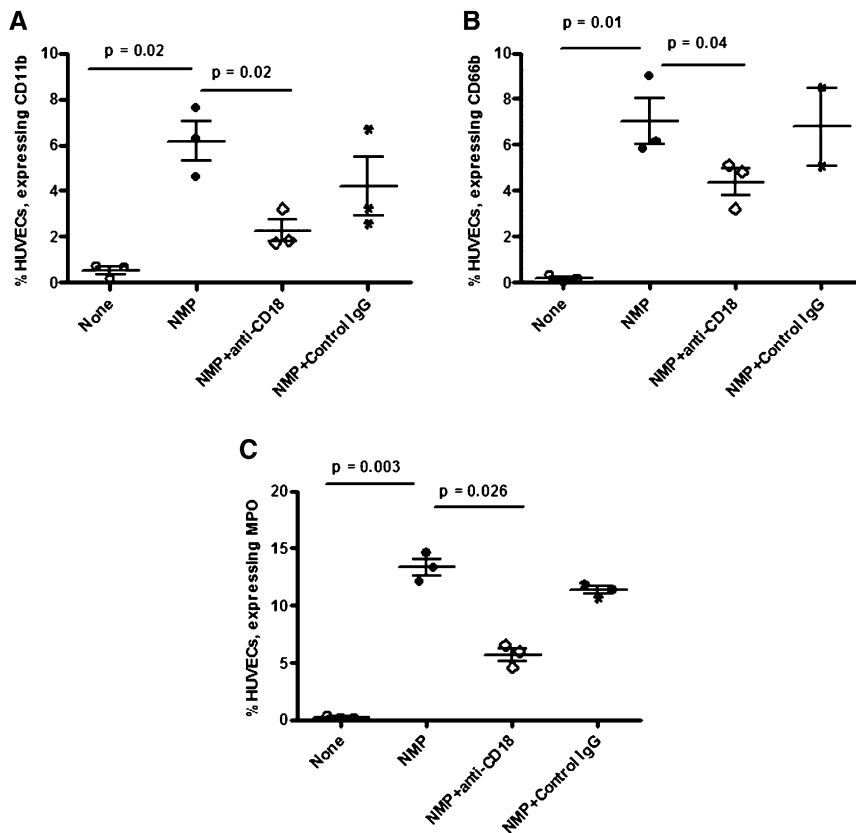


Figure 3. NMPs bind to endothelial cells using CD18. HUVECs were incubated with NMPs for 30 minutes at 37°C. (A–C) After washing the HUVECs twice, NMP binding was measured by flow cytometry and was represented as the percentage of HUVECs expressing specific neutrophil markers CD66b (A), CD11b (B), and MPO (C). This NMP binding to HUVECs was attenuated by addition of a blocking CD18 antibody (A–C).

stimulation of neutrophils significantly increased ROS production as indicated by a rise in the DCF signal (IgG1 anti-PR3 NMP $P=0.013$; IgG3 anti-PR3 NMP $P=0.014$).

Effects of NMPs on Endothelium Are Abrogated by Antioxidants

Having demonstrated NMP-induced ROS production in ECs, we used two different anti-oxidants, the SOD mimetic manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) and a specific NADPH oxidase inhibitor, apocynin, to test their ability to attenuate the effects of NMPs on ICAM-1. Both MnTBAP and apocynin at 3 and 100 μM concentrations, respectively, led to a significant inhibition of NMP-mediated ICAM-1 expression (Figure 6B).

NMPs Generate Thrombin

Because NMPs are rich in phosphatidylserine, we explored their capacity to generate thrombin. We used a fluorometric thrombin generation assay to identify the thrombin-generating potential of NMPs released under different experimental conditions. Figure 7A demonstrates the increased thrombin production

derived from NMPs generated from chimeric anti-PR3 antibodies versus control NMPs. Chimeric IgG1 and IgG3 anti-PR3 induced NMPs exhibited a significantly higher mean peak thrombin of 196 nM (SEM 10.6; $P=0.01$) and 191 nM (SEM 17.2; $P=0.01$), respectively, compared with the resting neutrophil-derived NMP peak thrombin of 116 nM (SEM 4.2); Figure 7B.

NMPs Are Increased in the Plasma of Children with Active AAV

Patients with active AAV had significantly higher total AnV⁺ MPs ($642 \times 10^3/\text{ml}$, range $447 \times 10^3/\text{ml}$ to $1981 \times 10^3/\text{ml}$) than that of children with inactive AAV (total AnV⁺ $237 \times 10^3/\text{ml}$ [$57 \times 10^3/\text{ml}$ to $512 \times 10^3/\text{ml}$]; $P=0.02$; Figure 8A). This was also true for specific NMP markers in the active AAV versus inactive AAV patients: median active CD11b 144 ($21 \times 10^3/\text{ml}$ to $575 \times 10^3/\text{ml}$) versus 19 ($14 \times 10^3/\text{ml}$ to $62 \times 10^3/\text{ml}$), $P=0.03$; median CD66b 79 ($37 \times 10^3/\text{ml}$ to $207 \times 10^3/\text{ml}$) versus 32 ($16 \times 10^3/\text{ml}$ to $48 \times 10^3/\text{ml}$), $P=0.02$; median MPO NMPs 52 ($26 \times 10^3/\text{ml}$ to $207 \times 10^3/\text{ml}$) versus 14 ($3 \times 10^3/\text{ml}$ to $59 \times 10^3/\text{ml}$), $P=0.04$ (Figure 8B). There was no difference in total AnV⁺ MPs between active AAV and active ANCA-negative vasculitides; however, healthy controls and children with ANCA-negative vasculitis had fewer plasma NMPs of all

subtypes (Figure 8B).

DISCUSSION

Our study defines an entirely novel proinflammatory vasculitis amplification mechanism consequent to the interaction of ANCAs with neutrophils. We show for the first time that both polyclonal ANCAs and chimeric PR3-ANCA can induce NMP release from primed neutrophils *in vitro*. These NMPs express the ANCA autoantigens PR3 and MPO and active CD11b and CD18, and differ phenotypically from NMPs spontaneously released from resting neutrophils, because they express higher levels of active CD11b and ANCA autoantigens. They can bind to the endothelium in a CD18-dependent manner and cause the release of endothelial IL-6 and IL-8 and an increase in ICAM-1 largely through induction of endothelial ROS. These NMPs exhibit thrombin-generating capacity and were observed in increased numbers in the plasma of children with AAV. These novel observations provide important insights into the pathogenesis of AAV, and because the removal

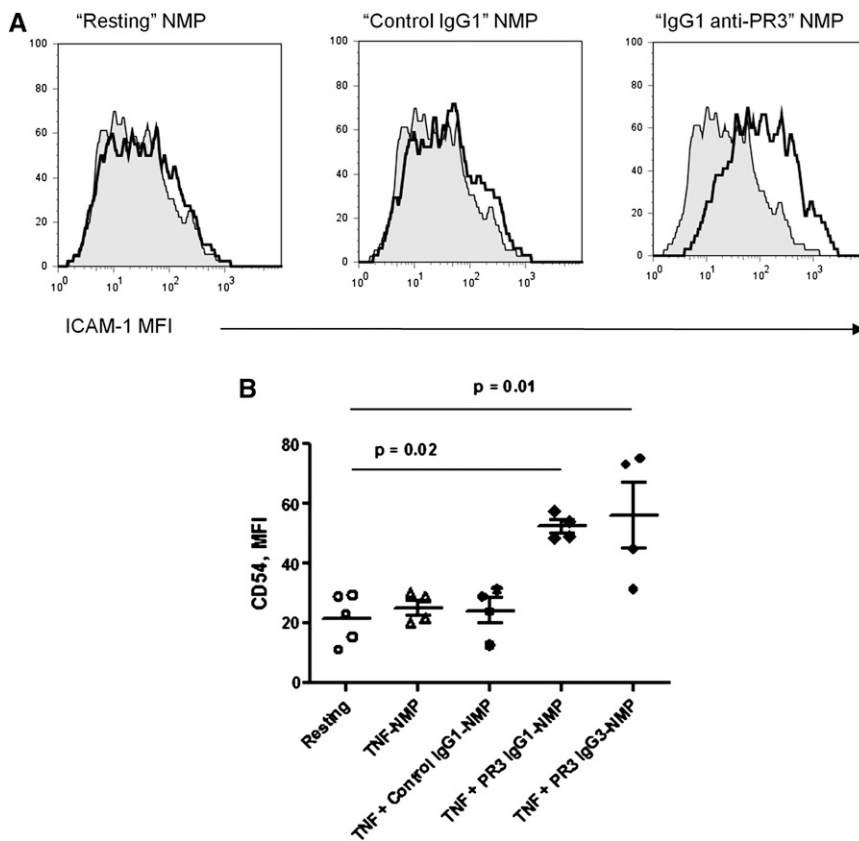


Figure 4. NMPs derived from IgG1 or IgG3 anti-PR3 chimeric antibody-stimulated neutrophils cause upregulation of endothelial ICAM-1 and cytokine production. HUVECs were incubated with NMPs derived from the indicated experimental conditions for 24 hours. The expression of ICAM-1 (CD54) was measured by flow cytometry. (A) Representative flow cytometric profile of ICAM-1 on HUVEC expression after stimulation with IgG3 PR3-ANCA-derived NMPs compared with that of control. (B) ICAM-1 expression on HUVECs increased in response to NMPs derived from stimulation of primed neutrophils using chimeric anti-PR3: summary of three to four experiments.

of NMPs by filtration reversed all of their observed effects, the results of this study could have important therapeutic implications.

MPs are shed membrane vesicles released by various cell types after apoptosis and/or activation and are key protagonists in cardiovascular disease, chronic inflammation, and in hemostatic responses.²⁹ Most studies have focused on the functional properties of endothelial and platelet MPs, with relatively few studies relating to leukocyte-derived MPs. Mesri and Altieri³⁰ demonstrated that overnight incubation of NMPs resulted in activation of HUVECs, providing the first indication that NMPs could play an important role in vascular inflammation. Intriguingly, and consistent with this early report, our data now demonstrate that ANCAs consistently generate large numbers of NMPs rich in phosphatidylserine and expressing several important molecules with functional relevance to the biology of vasculitis. These molecules included the multifunctional leukocyte integrin CD11b/18 ($\alpha_M\beta_2$) in its active form, which conferred avid binding capacity to

endothelium via CD18. NMP binding was likely to be enhanced further through increased endothelial ICAM-1 expression, which was increased via NMP-induced oxidative stress. Notably, NMPs did not upregulate E-selectin, consistent with previous studies demonstrating that sublethal concentrations of hydrogen peroxide cause upregulation of ICAM-1 but not E-selectin.³¹ The signaling pathways responsible for ICAM-1 upregulation in response to oxidative stress are not yet defined but are independent of NF- κ B,³¹ and we demonstrated that this response could be clearly attenuated using antioxidants.

The presence of the ANCA antigens MPO and PR3 on NMPs is also likely to be significant because the binding of NMPs rich in these proteolytic enzymes to endothelium could amplify vascular injury via several mechanisms. These include direct injury to endothelium mediated by proteolytic enzyme activity of PR3 and MPO,³² which can induce endothelial apoptosis, detachment, and IL-8 production.³³ NMP binding could also provide an opportunity for ANCAs to bind endothelial cells despite the fact that endothelial cells are not thought capable of production of the ANCA autoantigens.¹⁷ A similar mechanism has been proposed for soluble MPO, which when bound can be recognized by ANCAs and can enhance complement-dependent cytotoxicity.¹⁴ Notably, NMPs can actively bind C1q,³² and therefore taken together, these observations suggest that

binding of NMPs to endothelium could result in complement activation *in vivo* from both ANCA-dependent and/or -independent mechanisms.

We found that NMPs generated by ANCAs are potent amplifiers of vascular inflammation. NMPs, however, are not always proinflammatory.^{34,35} Dalli *et al.*³⁴ proposed that certain NMP subpopulations may exert an anti-inflammatory role. They generated NMPs by coculture of neutrophils with HUVECs in the presence of fMLP. The NMPs derived from the neutrophils adherent to HUVECs were rich in the anti-inflammatory protein annexin 1, and demonstrated the ability to inhibit further neutrophil adhesion to endothelium.³⁴ Importantly, these anti-inflammatory NMPs represented the non-adherent NMP population generated in this model, and it is possible that proinflammatory NMPs were removed by binding HUVECs and would not have been included in the NMP analysis. However, this study clearly demonstrated that a subpopulation of NMP can be anti-inflammatory, and as such this potential negative feedback pathway would have an important

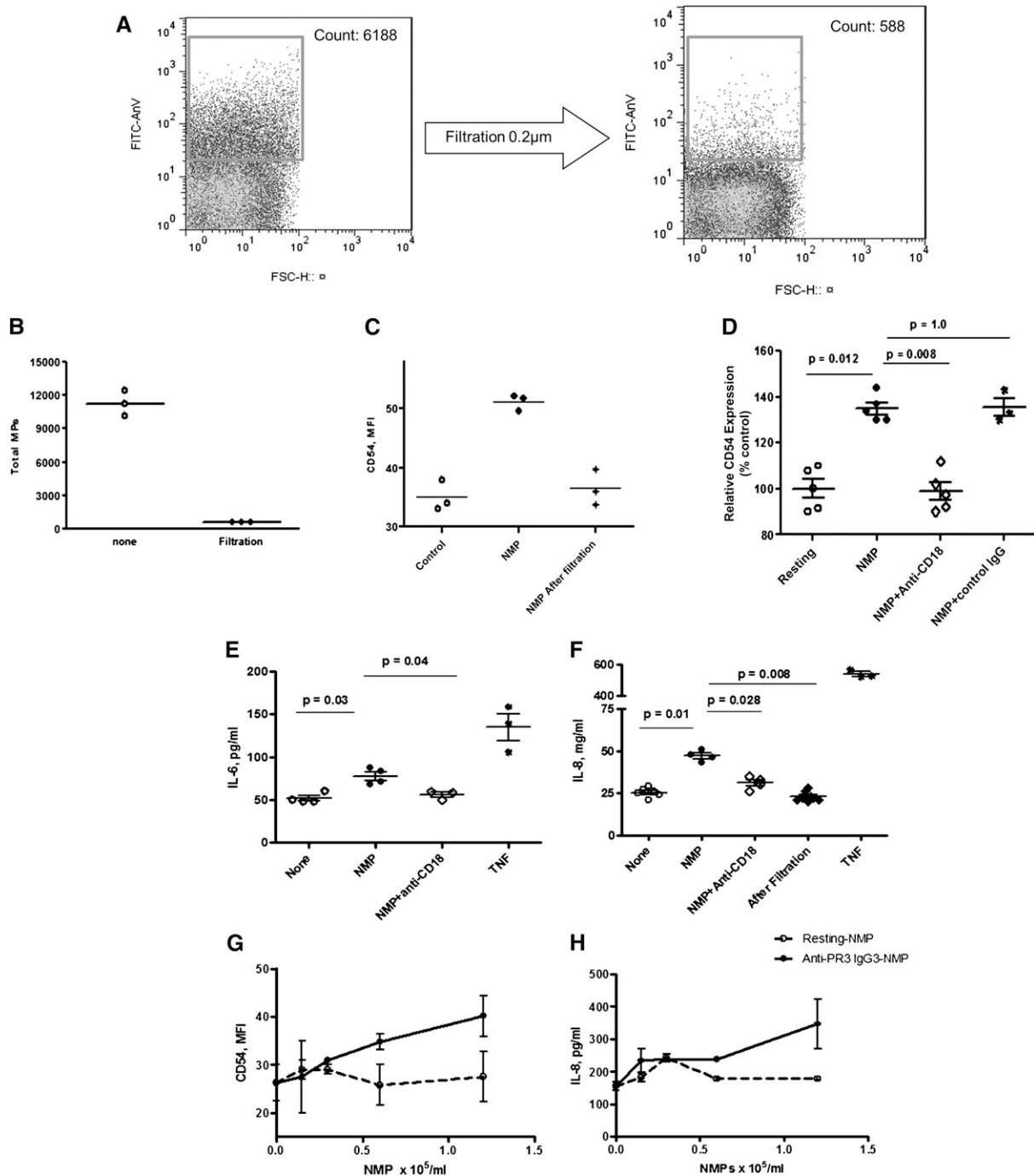


Figure 5. Removal of NMPs by filtration or blockade with anti-CD18 attenuated the stimulatory effects of NMP on HUVECs. (A and B) Representative flow cytometry analysis (A) and summary of three experiments (B) of NMPs derived from supernatants of neutrophils stimulated with IgG1 PR3-ANCA stimulation before and after 0.2- μ m filtration demonstrating 90% removal of annexin V⁺ events. (C and D) Removal of NMPs from supernatants by filtration (0.2 μ m) abolished the effect of the supernatant to upregulate ICAM-1 on HUVECs (C), as did blockade of NMP binding to HUVECs using anti-CD18 (D). (E and F) Chimeric IgG1 PR3-ANCA NMPs caused increased HUVEC release of IL-6 ($P=0.03$) and IL-8 ($P=0.01$); this response was again attenuated by preincubation of HUVECs with neutralizing mAb to CD18 or filtration. Incubation of HUVECs with TNF- α at 100 ng/ml for 24 hours served as a positive control in these experiments. (G and H) Dose-response curves were plotted comparing NMPs derived from chimeric IgG3 PR3-ANCA stimulation versus NMPs spontaneously released from resting neutrophils. Using endothelial CD54 upregulation (G) and IL-8 production (H), there was higher endothelial activation using NMPs derived from IgG3-PR3 ANCA stimulation in a dose-dependent manner ($P=0.02$ for ICAM-1; $P=0.02$ for IL-8 production at the highest NMP concentration).

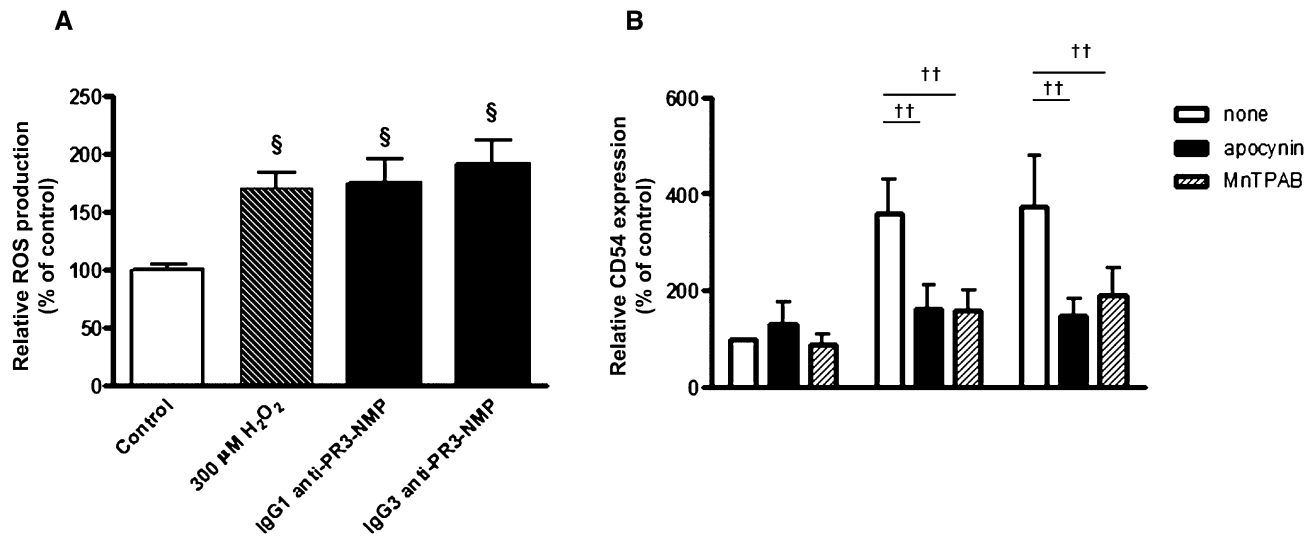


Figure 6. NMPs induce endothelial ROS production, and antioxidants can inhibit the effects of NMP-mediated endothelial activation. (A) HUVECs were loaded with H_2DCFDA in HBSS and exposed to NMPs derived from different experimental conditions (as indicated) or H_2O_2 (positive control). Oxidation-dependent fluorescence of H_2DCFDA was determined in a plate reader at 525 nm, and rates of fluorescence increase were determined over 40 minutes and normalized to untreated, control HUVECs. Both IgG1 and IgG3 PR3-ANCA-derived NMPs caused increased production of ROS in HUVECs ($^{\S}P < 0.01$). (B) HUVECs were pretreated for 1 hour with 100 mM apocynin or 3 μ M MnTPAB, after which the NMPs were added and incubated for an additional 24 hours. Apocynin or MnTPAB inhibited upregulation of ICAM-1 induced by NMPs. Data are presented as mean $\pm$ SE of three experiments. $^{\dagger\dagger}P < 0.05$ versus untreated cells.

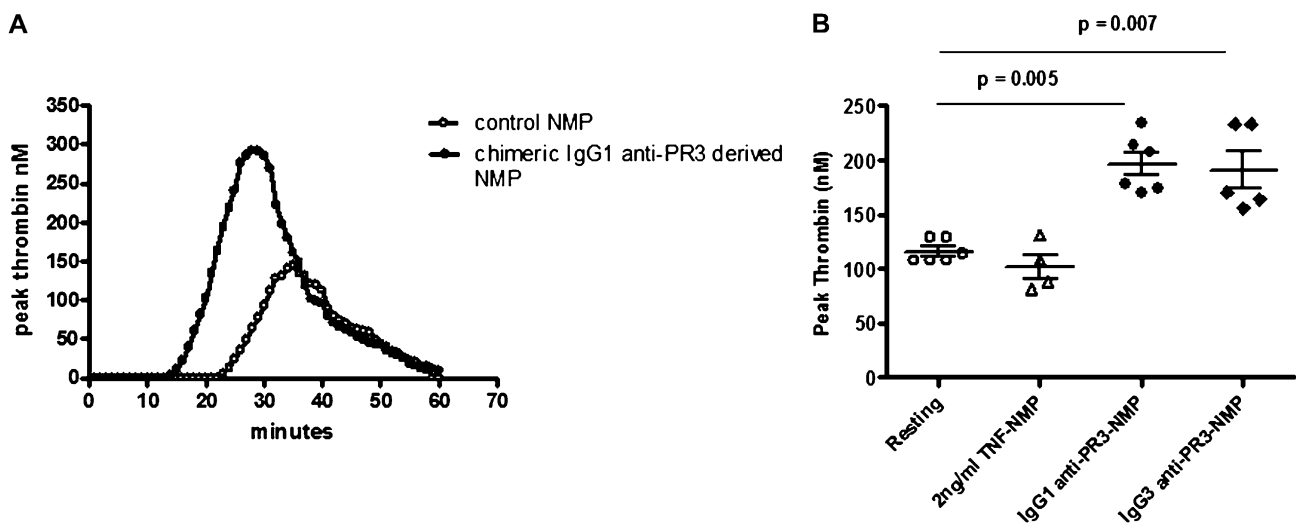


Figure 7. NMPs derived from stimulation of primed neutrophils with PR3-ANCA generate thrombin in MP-depleted human plasma. (A) Representative example of a thrombin-generation assay curve that measures four parameters: lag phase, velocity index, peak thrombin, and area under curve (also referred to as endogenous thrombin potential). (B) Summary of peak thrombin (nM) of the thrombin generation assay: NMPs derived from stimulation of primed neutrophils with PR3-ANCA generated thrombin in MP-depleted human plasma.

physiologic role to control excessive neutrophil adhesion and bystander injury in healthy individuals. Gasser and Schifferli³⁵ also showed that NMPs could exert anti-inflammatory influence on monocytes by suppressing proinflammatory cytokine production and increasing TGF- β secretion. Taken together with our observations, these studies suggest that in disease states such as vasculitis, the balance between proinflammatory

and anti-inflammatory NMPs could be critical as unchecked prolonged vascular inflammation would lead to the injury observed in patients with ANCA vasculitis.

We also demonstrated that NMPs derived from ANCA-stimulated neutrophils have potent thrombin-generating capacity. Thromboembolic disease complicating systemic vasculitis is associated with significant morbidity and mortality.^{3,4}

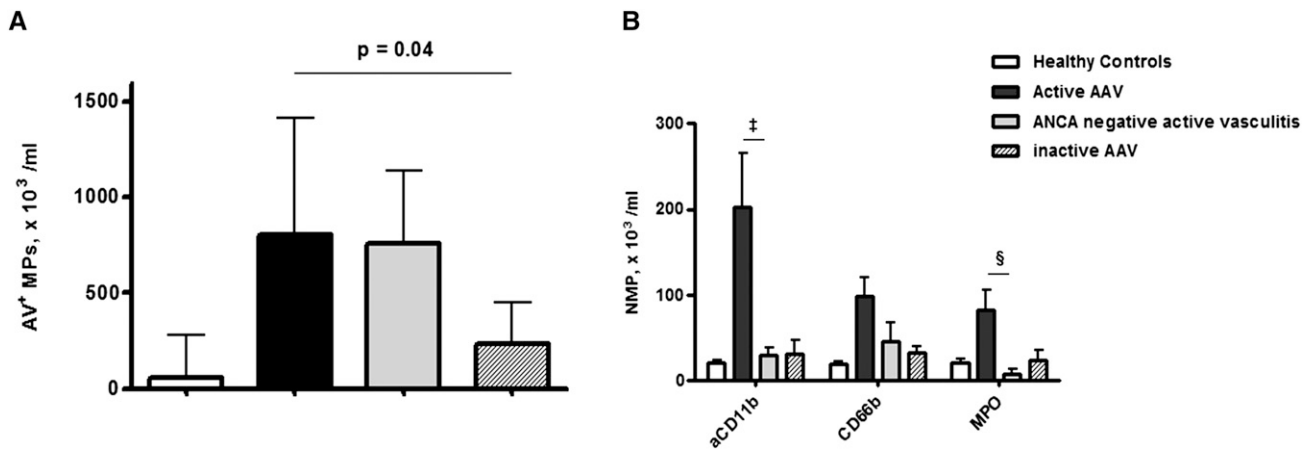


Figure 8. NMPs are increased in plasma of children with active AAV. (A) Patients with active AAV ($n=9$) had significantly higher total AnV⁺ MPs (median $642 \times 10^3/\text{ml}$, range $447 \times 10^3/\text{ml}$ to $1981 \times 10^3/\text{ml}$) than that of children with inactive AAV ($n=4$) (total AnV⁺ $237 \times 10^3/\text{ml}$, range $57 \times 10^3/\text{ml}$ to $503 \times 10^3/\text{ml}$), $P=0.02$. There was no difference in total AnV⁺ MPs between active AAV and active ANCA-negative vasculitides. (B) Breakdown of specific NMP markers in the active AAV versus inactive AAV patients: median active CD11b $144 (21 \times 10^3/\text{ml}$ to $575 \times 10^3/\text{ml})$ versus $19 (14 \times 10^3/\text{ml}$ to $62 \times 10^3/\text{ml})$, $P=0.03$; median CD66b $79 (37 \times 10^3/\text{ml}$ to $207 \times 10^3/\text{ml})$ versus CD66b $32 (16 \times 10^3/\text{ml}$ to $48 \times 10^3/\text{ml})$, $P=0.02$; median MPO NMPs $52 (26 \times 10^3/\text{ml}$ to $207 \times 10^3/\text{ml})$ versus $14 (3 \times 10^3/\text{ml}$ to $59 \times 10^3/\text{ml})$, $P=0.04$. Healthy controls and children with ANCA-negative vasculitis had fewer plasma NMPs of all subtypes. [‡] $P<0.03$ and [§] $P<0.01$.

Recent studies from four different cohorts of patients with AAV strongly suggest an increased occurrence of thromboses associated with these types of vasculitis and that this risk is strongly associated with vasculitis disease activity.^{3,36} Thrombin generated by excess NMPs or other MPs known to be increased in the circulation of patients with AAV^{23,24} in conjunction with endothelial injury,^{27,37} as well as anti-plasminogen antibodies,¹⁸ could significantly contribute to this thrombotic propensity. Further prothrombotic potential of NMPs derived from ANCA stimulation could be mediated via interaction of active CD11b/CD18 on NMPs and platelets. Recently, Plustoka *et al.*³⁸ reported a new prothrombotic pathway initiated by NMPs through the interaction of the integrin CD11b/CD18 and its counter-receptor on platelets, GPIIb/IIIa. This observation taken together with our novel finding that ANCA-derived NMPs cause potent thrombin generation strongly suggest that these NMPs are highly prothrombotic and could contribute to the vasculitic injury associated with AAV.

We confirmed the presence of increased levels of NMPs in the peripheral blood of nine children with active AAV. NMPs expressing a variety of neutrophil markers including active CD11b and ANCA autoantigens were higher in those with active vasculitis than in those with clinically inactive disease (Figure 8B). These preliminary observations warrant further investigation, in particular comparing NMP levels between patients with ANCA-positive and ANCA-negative vasculitides, but suggest a role for NMPs in the pathogenesis of AAV based on these initial studies.

We also demonstrated that filtration removed NMPs and abolished the stimulatory effects on endothelium, attenuating upregulation of ICAM-1 and reducing release of proinflammatory cytokines (Figure 5, A-C and F). The size of the filter

used in this experiment was comparable with that used in therapeutic plasma filtration. The implication of these observations is that NMPs may be important therapeutic targets for plasma exchange in AAV. We have yet to confirm the pathogenic potential of NMPs in animal models and the clinical relevance of our observations in patients; however, these studies are now warranted. Additionally, we have not yet studied the effect of NMPs on endothelium in a flow model, which would provide adjunctive data in this context. Lastly, we have not yet studied the possible role of annexin-1 NMPs in disease activity in AAV, and future detailed proteomic studies of the composition of NMPs derived from ANCA stimulation compared with NMPs spontaneously released from resting neutrophils are warranted.

In summary, our study defines a novel proinflammatory vasculitis amplification mechanism consequent to the interaction of ANCAs with neutrophils. We demonstrate that ANCAs stimulate NMP release from primed neutrophils. These NMPs activate ECs and are thrombogenic. These findings support a possible role for NMPs in the pathogenesis of AAV and as a potential novel therapeutic target.

CONCISE METHODS

Study Population

This research was approved by the Great Ormond Street Hospital National Health Service Trust ethics committee (ethics number 04/Q0508/117). All participants gave written consent to participate. We studied children with AAV attending Great Ormond Street Hospital, London, between October 2008 and June 2010. Inclusion criteria were age <18 years, a diagnosis of AAV confirmed by clinical and/or

histopathologic features and ANCA positivity, and exclusion of secondary causes of the vasculitis. An additional vasculitis disease control group comprised children with ANCA-negative vasculitides: PAN and KD. Control samples were obtained from healthy age- and sex-matched children. Adult healthy controls were members of staff within our laboratory.

Vasculitis subtype was classified using the new classification criteria for pediatric vasculitides by Ozen *et al.*³⁹ for PAN and WG. KD was defined as patients fulfilling at least five of six American Heart Association criteria.⁴⁰ Disease activity was assessed using a modified pediatric BVAS incorporating age-specific laboratory reference ranges, as previously described.^{27,41} Active vasculitis was defined as a score greater than zero for BVAS items attributable to vasculitis that newly appeared or worsened during the preceding 4 weeks and for which other causes such as infection were excluded.^{27,41} The following routine laboratory markers provided adjunctive information related to vasculitis activity: ESR and CRP levels. We also measured CECs with immunomagnetic bead extraction as supporting evidence of endothelial injury due to active vasculitis, using the consensus protocol previously described by ourselves and others.^{27,42}

mAbs and Reagents

Mouse phycoerythrin (PE)-labeled anti-CD54 (ICAM-1), CD62e, CD31, CD11b (ICRF44), PE mouse IgG1 κ isotype control, and FITC-annexin V were from BD Pharmingen. PE-conjugated anti-CD11b activation epitope, CBRM1/5, and anti-CD18 were from eBioscience. FITC-labeled anti-PR3 was from Santa Cruz Biotechnology. PE-labeled anti-MPO was from Invitrogen. PE-conjugated CD66b, neutralizing antibody LEAF purified anti-CD18, and control IgG1 were from BioLegend. Polymorphprep was from Axis-shield. HUVECs and Endothelial Growth Medium-2 Kit were from PromCell. Human IL-6 and IL-8 ELISA kit were from eBioscience. For the thrombin-generation assay, the calcium-fluorogenic substrate reagent was purchased from Pathway Diagnostics.

Stock solutions of fMLP (10 mM), LPS (10 μ g/ml), apocynin (250 mM), and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; 100 mM) were made in DMSO and stored in aliquots at -20°C . MnTBAP (30 mM) was dissolved in H₂O. Reagents were diluted in medium immediately before addition to the cultures. All other reagents were from standard suppliers or as listed in the text.

Polyclonal ANCAs from Patients and Chimeric ANCA

Polyclonal ANCAs from patients with AAV were provided as a gift from the laboratory of Prof. C.O. Savage at the University of Birmingham. IgG was isolated from plasma exchange fluid obtained from ANCA-positive patients or plasma from healthy controls after defibrination.⁴³ These adult patients had active WG or MPA based on clinical and serologic assessments and fulfilled the Chapel Hill Consensus definitions.⁴⁴ Control IgG was isolated from healthy adult volunteers. ANCAs and control IgG were used at 200 μ g/ml based on the results of previous dose-response experiments.⁴³ ANCAs were not affinity purified from total IgG, but ANCA-positive IgG fractions are referred to as PR3-ANCA and MPO-ANCA fractions in this text.

Chimeric ANCAs were generated in Prof. Savage's laboratory using human constant regions of each subclass and a variable region taken

from an mAb directed against PR3.⁴⁵ These chimeric ANCAs were used at a concentration of 12.5 μ g/ml as described previously.⁴⁵ Both polyclonal IgG ANCAs from patients and chimeric ANCAs were confirmed to activate superoxide release⁴⁶ from human neutrophils; in contrast, both control antibodies had no such effect (data not shown).

Neutrophil Separation from Whole Blood and HUVEC Culture

Neutrophils were isolated using Polymorphprep from heparin-anticoagulated blood after informed consent from normal healthy volunteers.

HUVECs were cultured in endothelial basal medium-2 supplemented with 2% FCS and growth factors as supplied by the manufacturer at 37°C under 5% CO₂ in a humidified incubator. For experimental purposes, fully confluent HUVECs at passages 2–5 were preincubated overnight with 0.5% FCS in endothelial basal medium-2 prior to addition of factors and other experimental conditions.

Generation of NMPs from Neutrophils Using ANCAs

All buffers used during MP preparation and FACS were filtered through an 0.2- μ m filter. Freshly isolated neutrophils were resuspended at 5×10^6 cells/ml in RPMI 1640 and primed with 2 ng/ml TNF- α for 15 minutes. This priming step caused externalization of the ANCA autoantigens MPO and PR3 on the cell surface (assessed using flow cytometry; data not shown) but did not result in full neutrophil activation as assessed by superoxide release (data not shown), in agreement with the findings of others.⁴⁶ Primed neutrophils were then treated with either 200 μ g/ml polyclonal MPO or PR3-ANCA derived from patients with AAV, control polyclonal IgG 200 μ g/ml from healthy adult donors, 12.5 μ g/ml chimeric IgG1 or IgG3 PR3-ANCA,⁴⁵ or control chimeric IgG1 or IgG3 antibody with specificity for the 4-hydroxyl-3-nitrophenylacetyl hapten for 60 minutes. In addition, a dose-response curve for NMP generation from primed neutrophils stimulated with chimeric IgG3 PR3-ANCA was plotted over the concentration 0–20 μ g/ml (Figure 2C). Supernatants from experiments were obtained by centrifugation at $5000 \times g$ for 5 minutes to remove cells and large debris, as previously described,⁴⁷ and frozen for future analysis using flow cytometry (see later). A single freeze–thaw cycle did not alter the number, phenotype, or activity of the NMPs (data not shown).

Detection of NMPs Using Flow Cytometry

The optimal flow cytometric gating and labeling of NMPs using fluorochrome-conjugated antibodies was derived using NMPs obtained by stimulating healthy neutrophils using 10 μ M fMLP and 100 ng/ml TNF- α for 1 hour as positive controls, as previously described.³⁰ NMPs were recovered from the supernatants (volume standardized at 1 ml and prepared as described above) by centrifugation at $13,000 \times g$ for 1 hour and identified using flow cytometry. A gate was set on forward scatter, which captured 1.1- μ m latex beads in its upper threshold, as previously described.²² The NMPs were captured within this gate and defined as annexin V⁺ particles coexpressing neutrophil markers MPO, PR3, CD18, or CD11b using appropriate isotype control antibodies for each neutrophil marker with threshold for positivity set at 2%. Because NMP numbers are dependent on the number of neutrophils stimulated in these *in vitro* studies, NMPs were quantitatively expressed as NMP number/ 10^6 neutrophils stimulated. The flow

cytometric gating strategy for NMPs is summarized in Figure 1. This flow cytometry protocol was also used to detect NMP and other MP populations from platelet-poor plasma obtained by centrifugation of whole blood at $5000 \times g$ for 5 minutes twice, derived from vasculitis patients and controls, and MPs in plasma were expressed quantitatively as number/ml of plasma as previously described by our group.^{22,27}

NMPs derived from the supernatants of resting neutrophils are referred to in this study as “resting” NMPs; NMPs derived from PR3-ANCA or MPO-ANCA stimulation are “PR3-ANCA or MPO-ANCA” NMPs; and IgG1 or IgG3 anti-PR3 chimeric ANCA-NMPs are designated “IgG1 or IgG3 anti-PR3” NMPs.

Confirmation That NMPs Are Intact MPs

To confirm that NMPs released from neutrophils by ANCA stimulation were intact MPs (rather than cellular debris), neutrophils were prelabeled with carboxyfluorescein succinimidyl ester (5 μ M; Molecular Probes), a nonfluorescent cell-permeable compound that only fluoresces when exposed to intracellular esterases that are contained within an intact plasma membrane. Thus, NMPs will only fluoresce if they are intact MPs (cytoplasmic fluid contained within an intact plasma membrane).⁴⁸ Supernatants from these experiments were then added to HUVEC monolayers on coverslips for 24 hours before washing with PBS three times to remove nonadherent NMPs, after which cells were fixed in 20% acetone/80% methanol. HUVEC nuclei were stained with 4',6-diamidino-2-phenylindole, and monolayers were visualized using fluorescent microscopy with a Nikon Eclipse E600 microscope with $\times 20$ (NA 0.4) objective, using a Coolsnap digital camera (MediaCybernetics, Bethesda, MD) and the ImagePro 6.0 software (MediaCybernetics). These experiments confirmed that NMPs bound to HUVECs were intact MPs (data not shown).

Assessment of Endothelial Cell Activation

After initial exposure of HUVEC for 6 or 24 hours to washed NMPs from experiments or to filtered culture supernatant with NMPs removed as a control, surface expression of ICAM-1 (CD54) and E-selectin (CD62e) on HUVECs was measured by flow cytometry as previously described.⁴⁷ To investigate the contribution of oxidative stress to endothelial activation, HUVECs were pretreated with the antioxidants 3 μ M MnTBPA or 100 μ M of the NADPH oxidase inhibitor apocynin for 30 minutes before NMP exposure. To exclude any possibility of low-grade endothelial activation derived from TNF- α contamination (used in the neutrophil-priming step prior to stimulation with ANCAs), infliximab (Remicade; Schering-Plough), a chimeric monoclonal anti-TNF- α antibody, at a concentration of 10 μ g/ml equivalent to therapeutic plasma concentrations used in clinical practice⁴⁹ was added before NMP exposure in some experiments.

Supernatants of HUVECs exposed to NMPs for 24 hours were removed and centrifuged at $500 \times g$ for 10 minutes to eliminate cell debris. IL-6 and IL-8 release were quantified using human IL-6 and IL-8 ELISA kits purchased from eBioscience according to the manufacturer's instructions.

Assessment of Endothelial Production of ROS in Response to NMPs

Production of ROS was assessed by using the indicator H₂DCFDA (Molecular Probes); this compound is converted to the fluorescent

DCF after oxidation and intracellular esterase cleavage of the acetate groups.⁵⁰ HUVECs were labeled with 10 μ M H₂DCFDA in HBSS without phenol red for 30 minutes. After washing, HUVECs were returned to HBSS in the presence or absence of NMPs, and changes in fluorescence were measured at excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a fluorescent plate reader.

Capacity of NMPs to Generate Thrombin

For the thrombin-generation assay, NMPs prepared as above were resuspended in 200 μ l control MP-free plasma prepared after MP sedimentation from approximately 50–60 ml plasma from adult healthy volunteers, with 30 μ g/ml corn trypsin inhibitor added to prevent potential *in vitro* contact activation of the coagulation cascade. Subsequently, 40 μ l of MPs suspended in control MP-free plasma was added to the plate well, followed by 50 μ l calcium-fluorogenic substrate reagent (7.5 mmol/L calcium and 0.5 mmol/L carbobenzyloxyGlyGlyArg7amido4methylcoumarin final reagent concentrations). No exogenous tissue factor or phospholipids were added at this stage to ensure that the thrombin generated was solely dependent on MP-related coagulant activity. This procedure is modified from that of Bidot *et al.*⁵¹ The thrombin generated was measured by fluorogenic excitation/emission at 360/460 nm at 1-minute time intervals for 90 minutes against a standard thrombin calibrator in an Optima fluorescence plate reader (BMG). Results are expressed in height of peak thrombin (nM).

Statistical Analyses

All *in vitro* experiments were performed in triplicate unless otherwise stated, and values were presented as mean $\pm$ SEM unless otherwise specified. Statistical differences for *in vitro* experiments between groups were determined by two-way ANOVA, followed by unpaired two-tailed *t* test. Patient demographics and comparisons between groups were summarized as median and range and compared using the Mann–Whitney *U* test. *P* < 0.05 was regarded as significant.

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DISCLOSURES

None.

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