Peptidylarginine Deiminase Inhibition Reduces Vascular Damage and Modulates Innate Immune Responses in Murine Models of Atherosclerosis

Jason S. Knight, Wei Luo, Alexander A. O’Dell, Srilakshmi Yalavarthi, Wenpu Zhao, Venkataraman Subramanian, Chiao Guo, Robert C. Grenn, Paul R. Thompson, Daniel T. Eitzman, Mariana J. Kaplan

Rationale: Neutrophil extracellular trap (NET) formation promotes vascular damage, thrombosis, and activation of interferon-α–producing plasmacytoid dendritic cells in diseased arteries. Peptidylarginine deiminase inhibition is a strategy that can decrease in vivo NET formation.

Objective: To test whether peptidylarginine deiminase inhibition, a novel approach to targeting arterial disease, can reduce vascular damage and inhibit innate immune responses in murine models of atherosclerosis.

Methods and Results: Apolipoprotein-E (ApoE)−/− mice demonstrated enhanced NET formation, developed autoantibodies to NETs, and expressed high levels of interferon-α in diseased arteries. ApoE−/− mice were treated for 11 weeks with daily injections of Cl-amidine, a peptidylarginine deiminase inhibitor. Peptidylarginine deiminase inhibition blocked NET formation, reduced atherosclerotic lesion area, and delayed time to carotid artery thrombosis in a photochemical injury model. Decreases in atherosclerosis burden were accompanied by reduced recruitment of netting neutrophils and macrophages to arteries, as well as by reduced arterial interferon-α expression.

Conclusions: Pharmacological interventions that block NET formation can reduce atherosclerosis burden and arterial thrombosis in murine systems. These results support a role for aberrant NET formation in the pathogenesis of atherosclerosis through modulation of innate immune responses. (Circ Res. 2014;114:947-956.)

Key Words: atherosclerosis ■ immunology ■ interferon-α ■ neutrophils ■ protein-arginine deiminase ■ thrombosis

Neutrophils, along with other inflammatory cells, infiltrate murine atherosclerotic plaques.1-4 Furthermore, an intriguing role has recently been suggested for neutrophil extracellular trap (NET) formation in this process.5-6 NETs are proinflammatory, antimicrobial structures consisting of extracellular chromatin decorated with granular and cytoplasmic proteins, such as myeloperoxidase (MPO), neutrophil elastase, and cathelicidin-LL37 (or the murine ortholog plasmic proteins, such as myeloperoxidase (MPO), neutrophil elastase, and cathelicidin-LL37 (or the murine ortholog cathelicidin-related antimicrobial peptide [CRAMP]).7,8 In addition to causing direct organ and endothelial toxicity,9,10 NETs stimulate plasmacytoid dendritic cells (pDCs) to release interferon-α (IFN-α),11-13 a cytokine with recognized proatherogenic properties.14-18 Through profound effects on platelet and coagulation factor activation, NETs can also promote clotting, as recently recognized in deep vein thrombosis models.19-22
block NET formation and modulate SLE disease activity.26 Furthermore, in that model, CI-aminde led to a striking abrogation of vascular abnormalities attributable to SLE, including endothelial dysfunction, abnormal vascular repair, and arterial thrombosis.26 Given recent evidence that, similar to SLE, atherosclerosis is exacerbated by both NET formation and IFN-α production,5,6,16 we tested whether PAD inhibition might mitigate atherosclerosis in the apolipoprotein-E (Apoe)−/− murine model. We found a striking improvement in atherosclerosis, which our evidence suggests is at least partially attributable to abrogation of NET formation and local IFN-α production.

Methods
An expanded Materials and Methods section is available in the Online Data Supplement.

Mice and Drug Treatment
C57BL/6 control and Apoe−/− (B6.129P2-Apoe+/−/−/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The Apoe−/− Sll−/− mice, which also have a knockout of the type I IFN receptor, have previously been generated and described by us.18 N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, or CI-aminde,28 was synthesized as previously described.29 Unless otherwise specified, mice were treated with either CI-aminde (10 mg/kg/d) or an equal volume of PBS (vehicle) by daily subcutaneous injection, beginning at 7 weeks and through euthanasia.26.27 Mice were fed high-fat chow (42% from fat) beginning at 8 weeks and until euthanasia. For in vitro experiments, CI-aminde was used at a concentration of 200 μmol/L.

Quantification of Atherosclerosis
Processing and quantification were performed as previously described.30,31 Briefly, arterial trees were stained with Oil Red O to quantify the atherosclerotic surface area occupied in the aortic arch, brachiocephalic trunk, common carotid arteries, and subclavian arteries. Furthermore, paraffin-embedded arteries were sectioned through the aortic sinus and stained with hematoxylin and eosin; the lipid-rich region of the intima (containing foam cells and cholesterol clefts) was quantified in cross-section as a percentage of total intimal area.

Induction of Carotid Artery Thrombosis by Photochemical Injury
This was performed as described previously.15 Briefly, rose bengal dye (Fisher Scientific, Pittsburgh, PA) was injected into the tail vein (50 mg/kg in PBS), and a 1.5-mW green light laser was applied to the carotid artery injury from a distance of 6 cm, and the vessel was monitored until occlusive thrombosis occurred.

Neutrophil Isolation and Neutrophil Assays
Bone marrow neutrophils were isolated as described.32 NET quantification and immunofluorescence microscopy was as previously described by us.15 The generation of H₂O₂ by neutrophils was quantified as described.33

Quantification of L-Selectin Shedding
In some cases, samples were preincubated with TAPI-0 (100 μg/mL) or CI-aminde (200 μmol/L) for 30 minutes, before stimulation with phorbol-12-myristate-13-acetate (100 nmol/L) for another 30 minutes. Cells were then stained with fluorochrome-labeled antibodies to L-selectin (BioLegend, San Diego, CA) and Ly-6G (BD Pharmingen, San Jose, CA), and analyzed by flow cytometry. The hydroxamic acid based L-selectin shedding inhibitor KD-IX-73–4 (TAI-0) was purchased from Peptides International (Louisville, KY).34,35

Clearance of Lipids From Serum
Lipids were removed by Cleanascite Lipid Removal Reagent (Biotech Support Group, Monmouth Junction, NJ).

Enzyme-Linked Immunosorbent Assay and Multiplex Assay
Commercial enzyme-linked immunosorbent assays for murine anti-double-stranded DNA (dsDNA) antibodies and total immunoglobulin G were performed according to manufacturer instructions (Alpha Diagnostic, San Antonio, TX). In house enzyme-linked immunosorbent assays for anti-NETs and anti-CRAMP are described in Methods in the Online Data Supplement. A multiplex assay for 5 cytokines (IFN-γ, IL-2, IL-4, IL-5, and TNF-α) was with a MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA).

Quantitative Polymerase Chain Reaction
RNA isolation and quantitative PCR were performed as described.36 RNA Integrity Number (RIN) was >7 for all included samples. Statistical significance was determined by comparing groups of ΔΔCt values using a 2-tailed Student t test, and ΔΔCt values were then determined by comparing the averages of the 2 groups.

Quantification of Lipids
Lipids were directly assayed using reagents for cholesterol (No. 3313018), triglycerides (No. 3034658), and high-density lipoproteins (No. 3034569), all from Roche Diagnostics.

Aortic Sinus Immunostaining
Primary antibodies were to citrullinated histone H3 (Abcam), Ly-6G (BD Pharmingen), F4/80 (Abcam), and MPO (Dako).

Western Blotting
Protein was prepared from dissected aortas using TriPure Isolation Reagent. Primary antibodies were specific to citrullinated histone H3 (Abcam), Ly-6G (BD Pharmingen), F4/80 (Abcam), and MPO (Dako).

Detection of Neutrophil-Platelet Aggregates
This was similar to what has been previously described,13 with neutrophil-platelet aggregates (Ly-6G+CD61+) quantified in fresh heparinized blood.

Neutrophil Depletion
Neutrophils were depleted as described.4 In brief, depletion was with intraperitoneal injection of monoclonal antibody 1A8 (BioXCell, West Lebanon, NH). Mice were specifically treated with 100 μg of the antibody every other day from weeks 8 to 18. The control antibody 2A3 was also from BioXCell.

Statistical Analysis and Oversight
Unless otherwise indicated, results are presented as the mean and standard error of the mean (SEM), and statistical analysis was performed using GraphPad Prism software version 5. All protocols were approved by the Committee on Use and Care of Animals of the University of Michigan.

Results
PAD Inhibition With CI-aminde Reduces Atherosclerosis and Arterial Thrombosis in Apoe−/− Mice
Apoe−/− mice were treated with either CI-aminde (10 mg/kg per day) or an equal volume of PBS (vehicle) by daily
subcutaneous injection, beginning at 7 weeks and through euthanasia at 18 weeks (n=10/group). Mice were fed high-fat chow beginning at 8 weeks and until euthanasia. After 11 weeks of exposure to Ci-amidine, atherosclerotic lesion area was significantly reduced when compared with vehicle-treated mice by analysis of en face oil red O lesion area (Figure 1A and 1B), as well as by quantification of cross-sectional atherosclerotic lesion area (Figure 1C). There was a statistically significant correlation between the 2 methods of analysis (for en face versus cross-sectional, linear regression P=0.0296). Furthermore, mice treated with Ci-amidine displayed prolongation of time to thrombosis in a photochemical injury model (Figure 1D). There was a statistically significant inverse correlation between lesion area and time to thrombosis (Figure 1E).

In a separate experiment, we fed Apoe<sup>−/−</sup> mice high-fat chow from 8 to 22 weeks, and started Ci-amidine treatment at 18 weeks. Under these conditions, there was no statistical difference in atherosclerotic lesion area at 22 weeks (P=0.89 with n=10 mice per group). Taken together, these results indicate that pharmacological inhibition of PADs can significantly reduce both atherosclerosis and arterial thrombosis in Apoe<sup>−/−</sup> mice when initiated as a preventive strategy.

**Apoe<sup>−/−</sup> Mice Demonstrate Enhanced NET Formation and Develop AutoAbs to NETs**

To address the mechanisms by which Ci-amidine mitigates vascular damage, we explored whether NET formation was accelerated in Apoe<sup>−/−</sup> mice. Indeed, Apoe<sup>−/−</sup> NETs displayed typical morphology and contained established protein markers of NETs including citrullinated histone H3 (H3-Cit) and MPO (Figure 2A and 2B). We then tested whether Apoe<sup>−/−</sup> serum could stimulate NET formation (Figure 2C). Neutrophils isolated from young Apoe<sup>−/−</sup> mice have a low rate of NET formation (<1%) in the absence of serum. Although serum from control C57BL/6 mice and young Apoe<sup>−/−</sup> mice did not stimulate NET formation, serum from aged, high-fat chow-fed Apoe<sup>−/−</sup> mice was significantly stimulatory (Figure 2C). This stimulation was not abrogated by lipid depletion (Figure 2C). These findings suggest that circulating factors in mice with atherosclerotic lesions are stimulatory to NET formation, independent of lipid content.

Increased titers of autoantibodies (autoAbs) have previously been demonstrated in Apoe<sup>−/−</sup> mice, as well as in humans with idiopathic atherosclerosis. To investigate this observation in more detail, and to better understand its relevance to NETs, we tested whether immunoglobulin G isolated from Apoe<sup>−/−</sup> mice could recognize NETs, as determined by immunofluorescence microscopy and enzyme-linked immunosorbent assay. Serum immunoglobulin G from aged mice bound NETs at a dilution that gave undetectable binding with serum from younger Apoe<sup>−/−</sup> mice (Figure 2D and 2E). CRAMP, the murine ortholog of human LL37, is known to be externalized in NETs. LL37/CRAMP has been implicated in triggering autoimmune response in SLE, and to play a prominent role in atherosclerosis development in murine systems. When Apoe<sup>−/−</sup> mice were fed a high-fat chow diet, they developed increased titers of anti-CRAMP autoAbs (Figure 2F). Overall, these results suggest that Apoe<sup>−/−</sup> mice develop an immune response directed at autoantigens externalized in the NETs, reminiscent of what has been previously described in SLE models.

**IFN Expression Is Upregulated in Atherosclerotic Lesions**

Several groups have shown that NETs signal through toll-like receptors to stimulate IFN-α production by pDCs in SLE, a concept also recently suggested for atherosclerosis. By quantitative PCR, we determined the expression of type I and
II IFNs (IFN-α and IFN-γ, respectively), as well as a panel of IFN-responsive genes, in aortic tissue. We confirmed enhanced expression of the IFN-α gene, as well as several of the IFN-responsive genes, in aortic arches from older mice fed high-fat chow, as compared with younger mice (Figure 3A). In contrast, there were no significant differences in these genes when comparing spleens isolated from the same mice (data not shown).

We also detected markers of NETs in the aortic arches of older mice fed high-fat chow, as compared with younger mice. By Western blotting, H3-Cit protein was significantly

**Figure 3. Interferon expression and histone citrullination are upregulated in atherosclerotic lesions.** A, Apoe−/− mice were placed on high-fat chow beginning at 8 weeks of age. RNA was isolated from aortic arches of 8- and 18-week-old Apoe−/− mice. Fold change in gene expression was calculated for 18-week-old mice, relative to 8-week-old mice (n=5). Mean and SEM are plotted. *P<0.05, **P<0.01, and ***P<0.001; P values that did not reach significance are indicated. B, Protein was prepared from aortic arches of the indicated Apoe−/− mice. Protein from 5 mice per group was pooled and 20 μg of total protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before Western blotting with the indicated antibodies. C, Low magnification view of an atherosclerotic lesion with a phase-contrast image showing intima (I), media (M), and adventitia (A). DNA is stained blue, and MPO is stained green, with an overlay to the far right. Scale bar=100 μm. D, A higher magnification view of the media/adventitia interface shows an MPO-positive cell in more detail. Extracellular MPO (green) juxtaposed with decondensed DNA (blue) is seen (red arrowhead); in the middle, the DNA channel is shown in grayscale to improve contrast. Scale bar=20 μm.
upregulated (Figure 3B). Furthermore, by immunofluorescence of aortic sinus plaques, MPO-positive cells could be detected infiltrating the media and adventitia (Figure 3C). In many cases, the MPO-positive cells demonstrated nuclear decondensation and extracellular MPO, where the MPO staining overlapped with DNA (Figure 3D), a staining pattern consistent with NETs. Overall, these observations indicate that diseased arteries are associated with enhanced neutrophil netting and local IFN responses, with a time course and localization that parallels the development of atherosclerosis.

**Cl-Amidine Abrogates NET Formation**

Because Apoe−/− mice displayed evidence of enhanced NET formation and IFN responses, we tested the impact of PAD inhibition on these parameters. First, we demonstrated in vitro that Cl-amidine was relatively specific for NET inhibition. Although Cl-amidine robustly blocked NET formation by neutrophils (Figure 4A), neither H2O2 production (Figure 4B) nor L-selectin shedding (Figure 4C) was affected. When administered in vivo, Cl-amidine treatment did not alter weight (Figure 5A), and lipid profile was also largely unchanged, except for a slight (3.8 mg/dL) increase in HDL levels with Cl-amidine treatment, although L-selectin shedding was significantly reduced in mice treated with Cl-amidine (Figure 5B). Although NET formation was significantly reduced in mice treated with Cl-amidine (Figure 5C), the regulation of neutrophil adherence molecules was not affected based on quantification of neutrophil-platelet aggregates (Figure 5D). Furthermore, with Cl-amidine treatment, no significant changes were detected in circulating levels of TNF-α, IFN-γ, IL-4, anti-CRAMP, or total immunoglobulin G (Figure 5E, and data not shown). In summary, Cl-amidine had a relatively specific effect on NET formation, without targeting other neutrophil functions, or autoantibody titers. Combined with previous work by us and others with this agent,26,27 we propose that Cl-amidine primarily impacts the immune system through abrogation of NET formation.

**PAD Inhibition Reduces the Recruitment of Netting Neutrophils to the Media and Adventitia of Apoe−/− Aortic Sinus Lesions**

To further understand the mechanism by which PAD inhibition reduces atherosclerosis, we analyzed the inflammatory infiltrates of aortic sinus arterial lesions. Although F4/80 (macrophage) staining highlighted areas of the intima bordering on the vessel lumen, a distinctly different pattern was observed for Ly-6G (neutrophil) and H3-Cit (netting neutrophils) staining, which highlighted cells clustering in the media and at the media-adventitia interface (see Figure 6A for representative staining). Quantification of these areas revealed a significant decrease in both H3-Cit- and Ly-6G-positive cells when mice were treated with Cl-amidine (Figure 6B–D). Furthermore, there was a significant reduction in F4/80-positive area within the intima of Cl-amidine–treated mice (Figure 6E), although H3-Cit staining did not colocalize with this region.

Importantly, the number of H3-Cit–positive cells correlated directly with lesion area (Figure 6C). In contrast, neither Ly-6G–positive cells nor F4/80-positive staining showed a statistically significant correlation (data not shown). These data demonstrate that Cl-amidine can reduce the infiltration of macrophages into the intima, and of netting neutrophils into the media and adventitia.

Given that NETs are recognized promoters of IFN-α production in SLE,11–13 as well as atherosclerosis,5,16 we predicted that reduced NET formation in arterial lesions would also lead to downregulation of IFN-α expression. Indeed, by quantitative PCR, we found a 5-fold repression of the IFNA gene with Cl-amidine treatment (Figure 6F). This repression was not seen in the spleens of the same animals (data not shown) nor was it seen for the IFNG gene. The only IFN-responsive gene to show a slight trend toward repression was MX1 (the tested gene most selective for IFN-α as compared with IFN-γ),26 although this did not reach statistical significance (Figure 6F). Cl-amidine also downregulated H3-Cit protein by Western blot in the same samples for which quantitative PCR was performed (Figure 6G). To summarize, PAD inhibition represses IFN-α synthesis, probably by blocking NET formation.

**Cl-Amidine Does Not Protect Against Atherosclerosis in Neutropenic or in Type I IFN Receptor–Deficient Mice**

It has previously been shown that neutrophil depletion with an anti–Ly-6G antibody protects against atherosclerosis in

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**Figure 4. Cl-amidine abrogates neutrophil extracellular trap (NET) formation, but not H2O2 production or L-selectin shedding.** A, Bone marrow neutrophils were isolated from 8-week-old Apoe−/− mice. Neutrophils were stimulated with phorbol-12-myristate-13-acetate (PMA) in the presence or absence of 200 μmol/L Cl-amidine, and NET formation was quantified by immunofluorescence microscopy. B, Cl-amidine treatment does not alter H2O2 production. Apoe−/− bone marrow neutrophils were stimulated with PMA in the presence of inhibitors as indicated. The PMA-stimulated sample was arbitrarily set at 100% H2O2 production; statistical comparisons are to this group. DPI=NADPH oxidase inhibitor. C, Cl-amidine treatment does not alter L-selectin shedding. Neutrophils were stimulated with PMA in the presence of inhibitors as indicated. Surface staining was then with anti–Ly-6G (to confirm the identity of neutrophils) and anti-L-selectin, before analysis by flow cytometry. Data are presented as the percentage of Ly-6G+ that are also L-selectin+. All experiments were repeated at least 3 times. **P<0.01; ***P<0.001.
Apoe\(^{-/-}\) mice.\(^4\) Here, we hypothesized that if Cl-amidine were primarily providing its protective effects by inhibiting neutrophil function, then Cl-amidine would mitigate atherosclerosis after neutrophil depletion. Using a published treatment regimen,\(^4\) Apoe\(^{-/-}\) mice were administered either a control or anti-Ly-6G antibody, from 8 to 18 weeks. Mice were at the same time treated with Cl-amidine or vehicle, as above, from 7 to 18 weeks. With this regimen, Ly-6G-positive neutrophils remained effectively depleted at 18 weeks of age (Figure 7A). Furthermore, there was a strong trend toward reduction in atherosclerosis with anti-Ly-6G treatment (Figure 7B, compare the first and second conditions). Importantly, in the background of neutrophil depletion, Cl-amidine did not provide any further protection (Figure 7B, compare the second and third conditions).

Our group has previously shown that atherosclerosis is reduced in Apoe\(^{-/-}\) mice that also carry a mutation in the type I IFN receptor gene.\(^3\) Similar to neutropenic mice, these Apoe\(^{-/-}\) ifnat\(^{-/-}\) mice were not protected by treatment with Cl-amidine (Figure 7C). In summary, Cl-amidine does not protect against atherosclerosis in the background of neutrophil depletion or type I IFN receptor deletion, suggesting that Cl-amidine likely acts through a neutrophil-based pathway, such as NET formation, and the induction of type I IFN responses in the artery.

Discussion

Recent studies have observed the infiltration of netting neutrophils into the atheromatous lesions of mice.\(^4\)–\(^6\) Indeed, in murine systems, depletion of either whole neutrophils or the NET component CRAMP can protect against atherosclerosis,\(^4\)–\(^6\) whereas treatment with exogenously prepared CRAMP–DNA complexes can accelerate disease.\(^5\) Netting neutrophils can also be detected in the blood of patients with severe coronary atherosclerosis,\(^3\) as well as in the atherosclerotic plaques themselves.\(^40\) Furthermore, in human plaques, PAD4 has been observed deaminating fibrinogen to generate a novel rheumatoid arthritis autoantigen.\(^41\) Although the cellular sources of this PAD4 have not been explored,\(^41\) neutrophils are a prime candidate. Our group recently showed that PAD inhibition reduces NET formation, alters markers of autoimmunity, and potently mitigates vascular damage in a murine model of SLE,\(^26\) a disease process that is highly dependent on type I IFNs like IFN-\(\alpha\).\(^4\) Although disruption of PAD activity has been considered in a model of venous thrombosis,\(^4\) it has not been evaluated in a pure model of arterial damage or atherosclerosis.

We now report that Apoe\(^{-/-}\) mice are protected from atherosclerosis when treated with the PAD inhibitor Cl-amidine. We also show that PAD inhibition abrogates NET release, mitigates arterial type I IFN responses, and reduces the number of netting neutrophils that infiltrate the media and adventitia of atheromatous lesions. The fact that we did not see further protection with Cl-amidine in neutropenic mice, or in mice lacking the type I IFN receptor, suggests that the protective effects of Cl-amidine are primarily through downregulation of neutrophil and IFN pathways. And because Cl-amidine specifically targets NET formation, but not other neutrophil functions,\(^2\) we have now demonstrated in vivo a causative role for neutrophil netting in the development of murine atherosclerosis. Importantly, previous studies by our group and others have not identified a role for PAD inhibition in modulation of the phenotype and function of other immune cells, including lymphocytes and natural killer cells.\(^2\)–\(^7\)

Cl-amidine prolonged the time to carotid thrombosis in these mice, which could be related to mitigation of vascular disease burden since an inverse correlation between lesion area and time to thrombosis was observed. Furthermore, previous work indicates that NETs infiltrate and stimulate venous and arterial thrombosis through effects on platelets and coagulation factors,\(^13\)–\(^2\) it is therefore plausible that the decrease in
Prothrombotic phenotype was attributable, at least in part, to impaired neutrophil function. Indeed, in venous injury models, neutrophil PAD4 has been found to be critical for clots to form in vivo. Our results newly support an important role for neutrophil posttranslational modifications (deimination) and chromatin decondensation (NET formation) in thrombus formation in animal models of atherosclerosis. This has potentially important clinical implications, given the well-recognized role of arterial thrombosis in atherosclerosis-mediated tissue ischemia.

Our group and others have shown that type I IFNs are proatherogenic and have pleiotropic deleterious effects in the vasculature. Furthermore, depletion of IFN-producing pDCs reduces plaque area and macrophage recruitment, and protein–DNA complexes derived from NETs activate pDCs to accelerate atherosclerosis in Apoe−/− mice. Indeed, both NETs and CRAMP have been detected at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP have been detected at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerotic lesions, whereas depletion of either pDCs or CRAMP protects against atherosclerosis. A notable difference in our study is that, as has also been reported by others, neutrophils were detected in the media and adventitia at the luminal surface of early atherosclerosis.
Although CI-amidine was effective as a preventive strategy for plaque formation, significant changes were not seen if started once atheroma formation was well underway. This is in line with previous work demonstrating a particular role for neutrophils in the early stages of murine atherosclerosis. Future studies should continue to address, in various murine and human systems, whether more targeted PAD inhibition might have therapeutic effects in reversing atherosclerosis rather than preventing it. Furthermore, given the well-recognized association between inflammatory disorders and atherosclerosis, this preventive data could still have significant clinical implications for the subset of patients known to be prone to accelerated atherosclerosis.

Whether the modest increase in HDL induced by CI-amidine could have played a role in plaque inhibition is unclear and will require further study. We previously reported that Apoe−/− mice that lack type I IFN receptor signaling display increases in HDL. As such, it is possible that the effect of CI-amidine on HDL quantity is through abrogation of proinflammatory cellular responses mediated by type I IFNs.

In conclusion, a possible therapeutic intervention for humans, pharmacological PAD inhibition, significantly decreases atherosclerosis burden and mitigates arterial thrombosis in the Apoe−/− model. Future studies should focus on more specific PAD4 inhibition by chemical and genetic strategies, as well as different dosing strategies for already described PAD inhibitors like CI-amidine. Other areas needing investigation include the potential implications of atheroma-derived PAD activity beyond NET formation, and the different circulating factors—such as autoAbs, inflammatory cytokines, and activated platelets—that may predispose neutrophils toward netting. Overall, our observations further support the notion that innate immune responses, specifically NETs and type I IFNs, play prominent roles in the pathogenesis of atherosclerosis. These pathways should be further studies as therapeutic targets to mitigate atherosclerosis and thrombotic risk in the general population.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Neutrophil depletion protects against atherosclerosis in mice.
- Neutrophil extracellular traps (NETs), chromatin-based structures released by neutrophils to capture and kill pathogens, are highly stimulatory to the immune system, sometimes with deleterious consequences.
- NETs have been implicated in vascular damage in other diseases, such as systemic lupus erythematosus (SLE).
- Peptidylarginine deiminase (PAD) inhibition is an effective, and relatively specific, means for blocking NET formation by neutrophils.

What New Information Does This Article Contribute?

- Atherosclerosis-prone Apoe−/− mice have evidence of accelerated NET formation, as well as an autoimmune response to NETs.
- PAD inhibition not only blocks NET formation but also significantly protects against both atherosclerosis and arterial thrombosis, strongly suggesting a causative role for NET formation in atherosclerosis.
- NETs stimulate type I interferon production in murine atherosclerosis, a process that is effectively blocked by PAD inhibition.

Neutrophils infiltrate atherosclerotic plaques and, in mice, neutrophil depletion has been shown to protect against atherosclerosis. Neutrophils, and in particular NET formation, have been implicated in the vascular damage of other inflammatory diseases, such as SLE, possibly through the stimulation of type I IFN production and endothelial cell cytotoxicity. Here, we tested whether pharmacological blockade of NET formation by inhibition of PAD enzymes could protect against atherosclerosis. Indeed, Apoe−/− mice were protected from both atherosclerosis and arterial thrombosis when NET formation was prevented with a PAD inhibitor. Blocking NETs also led to downregulation of type I interferons in diseased arteries. Importantly, PAD inhibition was not effective in the setting of neutrophil depletion or interferon receptor mutation, thereby implicating both pathways in NET-mediated arterial damage. This article adds to the existing literature by strongly arguing for a causative role of NETs in atherosclerosis. Furthermore, and in contrast to neutrophil depletion or genetic mutation, NETs were inhibited here by a pharmacological approach that might someday be applicable to humans. Future studies should further assess these pathways as therapeutic targets to mitigate atherosclerosis and thrombotic risk in the general population.
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SUPPLEMENTAL METHODS

Mice and drug treatment. C57BL/6 control and \( Apoe^{\text{\textsuperscript{\textcircled{c}}}} \) (B6.129P2-ApoelmtUnc/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The \( Apoe^{\text{\textsuperscript{\textcircled{c}}}} \) \( \text{Ifn}\alpha\beta r^{-/-} \) mice, which also have a knockout of the type I IFN receptor, have previously been generated and described by us.\(^1\) Mice were bred and housed in a specific pathogen-free barrier facility. N-\(\alpha\)-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, or Cl-amidine,\(^2\) was synthesized as previously described.\(^3\) Unless otherwise specified, mice were treated with either Cl-amidine (10 mg/kg/day) or an equal volume of phosphate-buffered saline (PBS) by daily subcutaneous injection, beginning at 7 weeks of age and through euthanasia at 18 weeks of age. Mice were fed high-fat chow (Harlan Teklad TD.88137, 42% from fat, Haslett, MI) beginning at 8 weeks of age and until euthanasia. Venous blood was collected at regular intervals after starting treatment with Cl-amidine, and serum was stored at -80°C until processing. For \textit{in vitro} experiments, Cl-amidine was used at a concentration of 200 µM, including a 30-minute pretreatment in Locke’s solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 0.1% glucose, and 10 mM HEPES buffer pH 7.3).

Quantification of atherosclerosis. Processing and quantification were performed as previously described.\(^4,5\) After euthanasia with intraperitoneal pentobarbital (100 mg/kg), the vasculature was perfused with normal saline, followed by formalin, with a 25-gauge needle inserted into the left ventricle at a rate of 1 ml/min. Carcasses were fixed in formalin for at least 24 hours, before meticulous dissection of the arterial tree. Trees were stained with Oil Red O to quantify the atherosclerotic surface area occupied in the aortic arch, brachiocephalic trunk, right and left common carotid arteries, and right and left subclavian arteries. Quantification was with Metamorph Premier software (Molecular Devices, Sunnyvale, CA), and lesion area was expressed as a percentage of total surface area examined. As internal validation, paraffin-embedded arteries were sectioned through the aortic sinus and stained with hematoxylin and eosin; the lipid-rich region of the intima (containing foam cells and/or cholesterol clefts) was quantified in cross section as a percentage of total intimal area.

Induction of carotid artery thrombosis by photochemical injury. As described previously,\(^1\) mice were subjected to photochemical injury of the right carotid artery by rose bengal dye. After pentobarbital anesthesia as above, the right carotid artery was isolated under a dissecting microscope. A Doppler flow probe (Transonic Systems, Ithaca, NY) was applied, and rose bengal dye (Fisher Scientific, Pittsburgh, PA) was injected into the tail vein (50 mg/kg in PBS). A 1.5-mW green light laser (540 nm; CVI Melles Griot, Albuquerque, NM) was applied to the
desired site of injury from a distance of 6 cm, and the vessel was monitored until occlusive thrombosis occurred, defined as flow cessation for at least 10 minutes.

**Neutrophil isolation.** Bone marrow neutrophils were isolated as described. Bone marrow was flushed from femurs and tibias, and total cells were spun on a discontinuous Percoll gradient (52%, 69%, 78%) at 1500 x g for 30 minutes. Cells were collected from the 69%-78% interface, and RBCs were lysed with RBC Lysis Buffer (Multi-species) following manufacturer’s instructions (eBioscience, San Diego, CA). Cells were >95% Ly-6G-positive by flow cytometry, and had the typical nuclear morphology by microscopy (data not shown).

**NET quantification and microscopy.** A protocol similar to what we described previously was followed. 1.5 x 10⁵ neutrophils were seeded onto coverslips coated with 0.001% poly-L-lysine (Sigma, St. Louis, MO) and incubated in RPMI-1640 supplemented with L-glutamine, 2% BSA, and 10 mM HEPES buffer. In experiments with serum stimulation, incubation was for 4 hours at 37°C. Stimulation with phorbol-12-myristate-13-acetate (PMA 100 nM, Sigma) was for 6-12 hours. For immunofluorescence, cells were fixed with 4% paraformaldehyde without permeabilization. DNA was stained with Hoechst 33342 (Invitrogen, Grand Island, NY). Protein staining was with rabbit polyclonal antibodies to MPO (Dako, Carpinteria, CA), neutrophil elastase, or citrullinated histone H3 (both from Abcam, Cambridge, MA), and FITC-conjugated anti-rabbit IgG (SouthernBiotech, Birmingham, AL). When 1% control or Apoe⁻/⁻ serum was used for staining, detection was with Texas Red-conjugated anti-mouse IgG (Invitrogen). Images were collected with an Olympus microscope (IX70) and a CoolSNAP HQ2 monochrome camera (Photometrics, Tucson, AZ) with Metamorph Premier software. Image overlays and background correction were with Metamorph, and the recorded images were loaded onto Adobe Photoshop for further analysis. NETs (decondensed extracellular DNA co-staining with one of the aforementioned protein markers) were manually quantified by two blinded observers, and digitally recorded to prevent multiple counts. The percentage of NETs was calculated by averaging 5-10 fields per sample.

**Quantification of H₂O₂ generation by neutrophils.** The generation of H₂O₂ was quantified, as described. H₂O₂ secretion from bone marrow neutrophils stimulated with 100 nM PMA for 1 hour was assayed after pre-treatment with either Cl-amidine or 25 µM diphenyleneiodonium (DPI, Tocris Bioscience, Bristol, United Kingdom). H₂O₂ production was detected by a colorimetric assay, adding 50 µM Amplex Red reagent (Invitrogen) and 10 U/ml horseradish
peroxidase (Sigma) to the culture media. Absorbance was measured at 560 nm and linearity was assured with an H₂O₂ standard curve. The detection limit of the assay was 0.625 nM H₂O₂.

**Quantification of L-selectin shedding.** EDTA-anticoagulated blood was collected and RBCs were lysed in RBC Lysis Buffer as above. In some cases, samples were preincubated with TAPI-0 (100 µg/ml) or Cl-amidine (200 µM) for 30 min, before stimulation with PMA (100 nM) for another 30 min. Cells were then stained with fluorochrome-labeled antibodies to L-selectin (BioLegend, San Diego, CA) and Ly-6G (BD Pharmingen, San Jose, CA), and analyzed by flow cytometry. The hydroxamic acid based L-selectin sheddase inhibitor KD-IX-73-4 (TAPI-0) was purchased from Peptides International (Louisville, KY).⁹,¹⁰

**Clearance of lipids from serum.** Lipids were removed by Cleanascite Lipid Removal Reagent (Biotech Support Group, Monmouth Junction, NJ) according to manufacturer’s instructions. The protocol removed >80% of total cholesterol and triglycerides.

**ELISA and multiplex assay.** Commercial ELISAs for murine anti-double-stranded DNA (dsDNA) antibodies and total IgG were performed according to manufacturer’s instructions (Alpha Diagnostic, San Antonio, TX). For other ELISAs, recombinant CRAMP protein was purchased from AnaSpec, while total NET protein was prepared by inducing Apoe⁻/⁻ bone marrow neutrophils with 100 nM PMA for 12 hours, collecting supernatants, and then precipitating protein with 80% acetone. Protein was diluted to 100 ng/ml, and 100 µl of the solution was used to coat high-binding EIA/RIA 96-well plates (Corning Incorporated, Troy, MI). Plates were blocked with 10% FBS in PBS, then incubated with experimental mouse serum diluted 1:100. Secondary and developing reagents were biotinylated anti-mouse IgG (B7264, Sigma), HRP-conjugated streptavidin (7100-05, SouthernBiotech), and 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Invitrogen). Final absorbance was measured at 450 nm with a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). A multiplex assay for five cytokines (IFN-γ, IL-2, IL-4, IL-5, and TNF-α) was with a MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA) following manufacturer’s instructions.

**Quantitative polymerase chain reaction (PCR).** RNA isolation and quantitative PCR were performed as described.¹¹ RNA was isolated from either spleens or dissected aortas using TriPure Isolation Reagent (Roche) according to manufacturer’s instructions. RNA Integrity Number (RIN) was >7 for all included samples. cDNA was synthesized using MMLV RT (Invitrogen) and 1 µg of RNA using a MyCycler thermocycler (Bio-Rad, Hercules, CA).
Quantitative PCR was with SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY) according to manufacturer’s instructions, and carried out using an ABI PRISM 7900HT (Applied Biosystems). Murine primer sequences were (5’ to 3’, with forward primer listed first):

**IFNA** = ATGGTAGRCTCTGTGCTTTTCT and AGGGCTCTCCAGAYTCTGCTTG;

**MCP1** = AGGTCCCTGTGACTCTTCTG and TCTGGACCCATTCTTTCTTG;

**MX1** = GATCCGACTCCTACCTCCAGATGG and CATCTCAGTGGTAGTCAACC;

**IRF7** = TGCTGTGAGACTGGCTAT and TCCAAGCTCCGGCTAAGT;

**IP10** = ATCATCCCTCGAGACTGTACAT and ATTCTTGCTTGGCGAGTTAC;

**ISG15** = CAGAAGCAGACTCTCAAATT and AGACCTCATATATGGTGGCTGT;

**IFNG** = AGCGGCTGACTGAACTCAGATTGTA and GTCACAGTTTTCCAGCTGTAGGG

**ACTB** = TGGAATCCTGTGGCATCCTGAAAC and TAAAACGCAGCTCAGTAACAGTG.

Ct values were normalized to the housekeeping gene **ACTB** to determine ΔCt. Statistical significance was determined by comparing groups of ΔCt values using a 2-tailed Student’s t-test, and ΔΔCt values were then determined by comparing the averages of the two groups. Data is presented as relative fold change by the formula $2^{\Delta\Delta \text{Ct}}$.

**Quantification of lipids.** Serum was prepared at the time of sacrifice and lipids were directly assayed using reagents for cholesterol (No. 3313018), triglycerides (No. 3034658), and HDL (No. 3034569), all from Roche Diagnostics, according to manufacturer’s instructions. Calculated LDL was determined with the formula, LDL = total cholesterol minus HDL minus (triglycerides/5).

**Aortic sinus immunostaining.** Tissue was fixed with formalin and embedded in paraffin; 5-micron sections were prepared. For immunohistochemistry, deparaffinization and rehydration were with standard xylene-to-ethanol washes. Heat-induced epitope retrieval was achieved by boiling samples for 30 minutes in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Samples were blocked in PBS with 1% BSA and either 10% FBS or 10% normal goat serum, depending on the secondary antibody. Primary antibodies were to citrullinated histone H3 (Abcam), Ly-6G (BD Pharmingen), and F4/80 (Abcam), while the secondary antibodies were HRP-conjugated anti-rabbit Ig (Amersham Biosciences, Pittsburgh, PA) and HRP-conjugated
anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). Color change was detected with the DAB-Plus Substrate Kit (Invitrogen). For immunofluorescence experiments, the primary antibody was to MPO (Dako) and the secondary antibody was FITC-conjugated anti-rabbit IgG (SouthernBiotech). DNA was stained with Hoechst 33342 (Invitrogen). Publicly-available ImageJ software (National Institutes of Health) was used for area determination and quantification of each section.

**Western blotting.** Protein was prepared from dissected aortas using TriPure Isolation Reagent according to manufacturer’s instructions. Protein was resolved by 15% SDS-PAGE under denaturing conditions and then transferred to a 0.45-micron nitrocellulose membrane. Primary antibodies were specific to citrullinated histone H3 (Abcam) and α-tubulin (Sigma). Detection was with HRP-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies (Jackson ImmunoResearch) and Western Lightning Plus-ECL (PerkinElmer, Waltham, MA). Images were captures with an Omega Lum C detector and densitometry was with UltraQuant software (Aplegen, Pleasanton, CA).

**Detection of neutrophil-platelet aggregates.** This was similar to what has been previously described.¹ Neutrophil-platelet aggregates (Ly-6G+CD61+) were quantified in fresh heparinized (50 units/ml) blood after 30 minutes of staining with APC-conjugated anti-Ly-6G and FITC-conjugated anti-CD61 (eBioscience). Flow cytometry was on a Becton Dickinson FACSCalibur 3, and analysis was with FlowJo (Tree Star, Inc., Ashland, OR).

**Neutrophil depletion.** Neutrophils were depleted as described.¹² Depletion was with intraperitoneal injection of monoclonal antibody 1A8 (BioXCell, West Lebanon, NH). Mice were specifically treated with 100 μg of the antibody every other day from weeks 8 to 18 of age. The control antibody 2A3 was also from BioXCell.

**Statistical analysis and oversight.** Unless otherwise indicated, results are presented as the mean and standard error of the mean (SEM), and statistical analysis was performed using Student’s t-test in GraphPad Prism software version 5. All protocols were approved by University of Michigan’s Committee on Use and Care of Animals.
REFERENCES


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