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

Jason S. Knight¹, Wei Luo², Alexander A. O’Dell¹, Srilakshmi Yalavarthi¹, Wenpu Zhao³, Venkataraman Subramanian⁴, Chiao Guo², Robert C. Grenn¹, Paul R. Thompson⁴, Daniel T. Eitzman², and Mariana J. Kaplan³

¹Rheumatology; ²Cardiology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI; ³Systemic Autoimmunity Branch, Intramural Research Program, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, and; ⁴Department of Chemistry, The Scripps Research Institute, Jupiter, FL.

Running title: PAD Inhibition Protects Against Atherosclerosis

Subject codes:
[96] Mechanism of atherosclerosis/growth factors,
[134] Pathophysiology of atherosclerosis
[172] Arterial thrombosis

Address correspondence to:
Dr. Jason S. Knight
University of Michigan
Department of Internal Medicine
Division of Rheumatology
5520 MSRB 1
1150 W. Medical Center Dr.
SPC 5680
Ann Arbor, MI 48109-5680
Tel: 734-936-3257
Fax: 734-763-4151
jsknight@umich.edu

Dr. Mariana J. Kaplan
Systemic Autoimmunity Branch
Intramural Research Program
National Institute of Arthritis and Musculoskeletal and Skin Diseases
National Institutes of Health
10 Center Dr, 10/6D47C
Bethesda, MD 20892-1560
Tel: 301-496-0517
Fax: (301) 402-0765
mariana.kaplan@nih.gov

In December 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 11.66 days.

DOI: 10.1161/CIRCRESAHA.114.303312
ABSTRACT

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

Objective: To test whether PAD 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 PAD inhibitor. PAD 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: Pharmacologic 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.

Keywords: Atherosclerosis, peptidylarginine deiminase, neutrophil extracellular trap, interferon alpha, polymorphonuclear neutrophils activation, immunology, thrombosis

Nonstandard Abbreviations and Acronyms:
- autoAb  autoantibody
- H3-Cit  citrullinated histone H3
- IFN  interferon
- MPO  myeloperoxidase
- NET  neutrophil extracellular trap
- PAD  peptidylarginine deiminase
- pDC  plasmacytoid dendritic cell
- SLE  systemic lupus erythematosus
INTRODUCTION

Neutrophils, along with other inflammatory cells, infiltrate murine atherosclerotic plaques.\textsuperscript{1-4} Further, an intriguing role has recently been suggested for neutrophil extracellular trap (NET) formation in this process.\textsuperscript{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 orthologue CRAMP).\textsuperscript{7, 8} In addition to causing direct organ and endothelial toxicity,\textsuperscript{9, 10} NETs stimulate plasmacytoid dendritic cells (pDCs) to release interferon alpha (IFN-α),\textsuperscript{11-13} a cytokine with recognized proatherogenic properties.\textsuperscript{14-18} Through profound effects on platelet and coagulation factor activation, NETs can also promote clotting, as recently recognized in deep vein thrombosis models.\textsuperscript{19-22}

NETs contain deiminated (citrullinated) histones, and there is evidence that histone deimination by peptidyl arginine deiminase 4 (PAD4) plays a fundamental role in NET formation. Indeed, \textit{PAD4}/-/- mice do not form NETs, while chemical inhibition of PAD4 can also abrogate NET formation by human neutrophils in vitro and mouse neutrophils in vivo.\textsuperscript{23, 24} Cl-amidine is a haloacetamidine-based PAD inhibitor, which preferentially targets PAD4 over PAD2.\textsuperscript{25} Previous work from our group and others has suggested that neutrophils are the primary inflammatory/immune cell affected by this highly-specific PAD inhibitor.\textsuperscript{26, 27} Indeed, we recently showed that NET formation plays a pathogenic role in a model of murine systemic lupus erythematosus (SLE), where treatment with Cl-amidine can block NET formation and modulate SLE disease activity.\textsuperscript{26} Further, in that model, Cl-amidine led to a striking abrogation of vascular abnormalities attributable to SLE, including endothelial dysfunction, abnormal vascular repair, and arterial thrombosis.\textsuperscript{26} Given recent evidence that, similar to SLE, atherosclerosis is exacerbated by both NET formation and IFN-α production,\textsuperscript{5, 6, 16} we tested whether PAD inhibition might mitigate atherosclerosis in the apolipoprotein-E (\textit{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

Detailed methodology is provided in the online Supplemental Methods.

Mice and drug treatment.
C57BL/6 control and \textit{Apoe}^-/- (B6.129P2-\textit{Apoe}tm1Unc/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The \textit{Apoe}^-/- \textit{Ifnar}^-/- mice, which also have a knockout of the type I IFN receptor, have previously been generated and described by us.\textsuperscript{28} N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, or Cl-amidine,\textsuperscript{28} was synthesized as previously described.\textsuperscript{29} 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. This dose of Cl-amidine has been published previously.\textsuperscript{26, 27} Mice were fed high-fat chow (42% from fat) beginning at 8 weeks of age and until euthanasia. For in vitro experiments, Cl-amidine was used at a concentration of 200 µM.

Quantification of atherosclerosis.
Processing and quantification were performed as previously described.\textsuperscript{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. Further, 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.**

This was performed as described previously. 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. NET quantification and immunofluorescence microscopy was as previously described by us. The generation of H$_2$O$_2$ by neutrophils was quantified, as described.

**Quantification of L-selectin shedding.**

In some cases, samples were preincubated with TAPI-0 (100ug/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).

**Clearance of lipids from serum.**

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

**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). In house ELISAs for anti-NETs and anti-CRAMP are described in Supplemental Methods. 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).

**Quantitative polymerase chain reaction (PCR).**

RNA isolation and quantitative PCR were performed as described. RNA Integrity Number (RIN) was >7 for all included samples. 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.

**Quantification of lipids.**

Lipids were directly assayed using reagents for cholesterol (No. 3313018), triglycerides (No. 3034658), and HDL (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) and α-tubulin (Sigma).

**Detection of neutrophil-platelet aggregates.**

This was similar to what has been previously described, with neutrophil-platelet aggregates (Ly-6G+CD61+) quantified in fresh heparinized blood.
Neutrophil depletion.
Neutrophils were depleted as described. 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 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.

RESULTS

PAD inhibition with Cl-amidine reduces atherosclerosis and arterial thrombosis in Apoe−/− mice.

Apoe−/− mice were treated with either Cl-amidine (10 mg/kg/day) or an equal volume of PBS (vehicle) by daily subcutaneous injection, beginning at 7 weeks of age and through euthanasia at 18 weeks (n=10/group). Mice were fed high-fat chow beginning at 8 weeks of age and until euthanasia. After 11 weeks of exposure to Cl-amidine, atherosclerotic lesion area was significantly reduced when compared to vehicle-treated mice by analysis of en face oil red O lesion area (Figure 1A-B), as well as by quantification of cross-sectional atherosclerotic lesion area (Figure 1C). There was a statistically significant correlation between the two methods of analysis (for en face vs. cross-sectional, linear regression p=0.0296). Furthermore, mice treated with Cl-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−/− mice high-fat chow from 8 to 22 weeks of age, and started Cl-amidine treatment at 18 weeks of age. In this context, 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 pharmacologic inhibition of PADs can significantly reduce both atherosclerosis and arterial thrombosis in Apoe−/− mice when initiated as a preventive strategy.

Apoe−/− mice demonstrate enhanced NET formation and develop autoAbs to NETs.

To address the mechanisms by which Cl-amidine mitigates vascular damage, we explored whether NET formation was accelerated in Apoe−/− mice. Indeed, Apoe−/− NETs displayed typical morphology and contained established protein markers of NETs including citrullinated histone H3 (H3-Cit) and MPO (Figure 2A-B). We then tested whether Apoe−/− serum could stimulate NET formation (Figure 2C). Neutrophils isolated from young Apoe−/− mice have a low rate of NET formation (<1%) in the absence of serum. While serum from control C57BL/6 mice and young Apoe−/− mice did not stimulate NET formation, serum from aged, high-fat chow-fed Apoe−/− 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 autoAbs have previously been demonstrated in Apoe−/− 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 IgG isolated from Apoe−/− mice could recognize NETs, as determined by immunofluorescence microscopy and ELISA. Serum IgG from aged mice bound
NETs at a dilution that gave undetectable binding with serum from younger Apoe−/− mice (Figures 2D-E). CRAMP, the murine orthologue 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−/− mice were fed a high-fat chow diet, they developed increased titers of anti-CRAMP autoAbs (Figure 2F). Overall, these results suggest that Apoe−/− 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 (IRGs), in aortic tissue. We confirmed enhanced expression of the IFN-α gene, as well as several of the IRGs, in aortic arches from older mice fed high-fat chow, as compared to 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 to younger mice. By western blotting, H3-Cit protein was significantly upregulated (Figure 3B). Further, 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.

Since 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. While 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, when compared to vehicle treatment (Figure 5B). While 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). Further, with Cl-amidine treatment, no significant changes were detected in circulating levels of TNF-α, IFN-γ, IL-4, anti-CRAMP, or total IgG (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 (autoAb) titers. Combined with previous work by us and others with this agent, 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. While 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). Further, 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 no co-localize 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 five-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 IRG to show a slight trend toward repression was MX1 (the tested gene most selective for IFN-α as compared to IFN-γ),38 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 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 following 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 of age. Mice were at the same time treated with Cl-amidine or vehicle, as above, from 7 to 18 weeks of age. With this regimen, Ly-6G-positive neutrophils remained effectively depleted at 18 weeks of age (Figure 7A). Further, there was a strong trend toward reduction in atherosclerosis with anti-Ly-6G treatment (Figure 7B, compare the 1st and 2nd conditions). Importantly, in the background of neutrophil depletion, Cl-amidine did not provide any further protection (Figure 7B, compare the 2nd and 3rd 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.18 Similar to neutropenic mice, these Apoe−/− Ifnar−/− 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 while 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,39 as well as in the atherosclerotic plaques themselves.40 Further, in human plaques, PAD4 has been observed deiminating fibrinogen to generate a novel rheumatoid arthritis autoantigen.41 While 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-α. While disruption of PAD activity has been considered in a model of venous thrombosis, 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 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 Cl-amidine’s protective effects are primarily through downregulation of neutrophil and IFN pathways. And, since Cl-amidine specifically targets NET formation, but not other neutrophil functions, 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 NK cells.

Cl-amidine prolonged the time to carotid thrombosis in these mice, which could be related to mitigation of vascular disease burden, as an inverse correlation between lesion area and time to thrombosis was observed. Further, previous work indicates that NETs infiltrate and stimulate venous and arterial thrombosis through effects on platelets and coagulation factors; 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. Further, 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, while 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 the media/adventitia interface, and were only rarely found along the luminal surface of lesions. It should be noted that we quantified cells at a later time point than some other studies, and did not utilize live image capture; as such, cells transiently associated with the endothelial surface could have been lost during processing for immunohistochemistry. It is certainly possible that neutrophils and NETs play important roles both in the initiating events of atherogenesis like endothelial damage, and in later events like the IFN-mediated recruitment of macrophages to developing lesions; indeed, a reduction in arterial macrophage infiltration was evident after Cl-amidine treatment.

While Cl-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. Further, 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 Cl-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 Cl-
amidine on HDL quantity is through abrogation of proinflammatory cellular responses mediated by type I IFNs.

In conclusion, a possible therapeutic intervention for humans, pharmacologic 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 Cl-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 studied as therapeutic targets to mitigate atherosclerosis and thrombotic risk in the general population.

ACKNOWLEDGEMENTS
Microscopy was performed in the Center for Live Cell Imaging (CLCI) at the University of Michigan. This work utilized the Chemistry Core of the Michigan Diabetes Research and Training Center funded by DK020572 from the National Institute of Diabetes and Digestive and Kidney Diseases.

SOURCES OF FUNDING
This work was supported by the National Institutes of Health (NIH) through PHS grant HL088419 (to MJK). JSK was supported by a Rheumatology Research Foundation Rheumatology Scientist Development Award. PRT is supported by NIH grants GM079357 and CA151304. The authors have declared that no conflict of interest exists. This article was prepared while WZ and MJK were employed at the University of Michigan. The opinions expressed in this article are the authors’ own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

DISCLOSURES
The authors have no conflicts of interest to disclose.

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DOI: 10.1161/CIRCRESAHA.114.303312


DOI: 10.1161/CIRCRESAHA.114.303312


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FIGURE LEGENDS

Figure 1. PAD inhibition with Cl-amidine reduces atherosclerosis and arterial thrombosis in Apoe−/− mice. Apoe−/− mice exposed to high-fat chow were treated with vehicle or Cl-amidine from 7 to 18 weeks of age (n=10/group). A, Atherosclerotic lesions were quantified in arterial trees following en face Oil Red O staining. B, Representative arterial trees from vehicle- (left) and Cl-amidine-treated (right) mice. C, Atherosclerosis was also scored by quantifying the lipid-rich region of the intima (containing foam cells and/or cholesterol clefts) in cross section as a percentage of total intimal area. D, Carotid artery thrombosis was induced by photochemical injury, and time to occlusion was determined. E, Correlation between arterial lesion area and time to carotid occlusion (n=20); the best fit-line and 95% confidence intervals are plotted. For panels A, C, and D, boxes represent the median, 25th percentile, and 75th percentile, while whiskers delineate the minimum and maximum values. *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Apoe−/− mice demonstrate enhanced NET formation and develop autoAbs to NETs. A and B, Representative immunofluorescence staining of nonpermeabilized Apoe−/− neutrophils for H3-Cit (A) and MPO (B) (both green). DNA is stained blue. Scale bars=10 microns. C, Bone marrow neutrophils were isolated from 8-week-old Apoe−/− mice. Neutrophils were incubated in the presence of 10% serum from the indicated mice for 4 hours (n=5 mice per group). ***p<0.001. D, Apoe−/− NETs were fixed and incubated with 1% serum from 18-week-old Apoe−/− mice (top) or 8-week-old Apoe−/− mice (bottom). Detection of bound antibodies was with Texas-Red-conjugated anti-IgG; DNA is stained blue. Scale bars=50 microns. E, NET proteins were prepared as described in Methods and used to coat plates for the ELISA. OD index normalizes data to the average value for C57BL/6 mice. Box-and-whisker plots show data for 8 mice per group, with boxes representing the median, 25th percentile, and 75th percentile; whiskers delineate the minimum and maximum values. **p<0.01; ***p<0.001. F, An ELISA for anti-CRAMP was performed as described in Methods. Some Apoe−/− mice were placed on high-fat chow (HF) beginning at 8 weeks of age; others remained on regular chow (reg). OD index normalizes data to the average value for control mice. Mean and SEM are plotted, with n≥8 per group. *p<0.05 and ***p<0.001 when compared to C57BL/6 control mice.

Figure 3. Interferon expression and histone critullination 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 SDS-PAGE 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 microns. 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 panel, the DNA channel is shown in grayscale to improve contrast. Scale bar=20 microns.

Figure 4. Cl-amidine abrogates 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 PMA in the presence or absence of 200 µM 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 is 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.

**Figure 5.** PAD inhibition abrogates NET formation and alters anti-NET autoAb profiles in Apoe−/− mice. High-fat chow-fed Apoe−/− mice were treated with vehicle or Cl-amidine from 7 to 18 weeks of age. A, Body weight was recorded at the indicated time points. B, Serum was collected at 18 weeks of age and total cholesterol, triglycerides, and HDL were determined by direct measurement; LDL was calculated. *p<0.05; no other comparison of vehicle vs. Cl-amidine was significant. C, Bone marrow neutrophils were isolated at 18 weeks of age and stimulated with PMA. **p<0.01. D, Neutrophil-platelet aggregates (NPA) were determined in blood at 18 weeks of age. NPA were defined as events positive for both Ly-6G and CD61. The percentage is calculated relative to total Ly-6G-positive cells. E, Serum cytokine levels were measured by multiplex assay; ns=not significant. For all panels, n=10 per group. For panels A, C, D, and E, the mean and SEM are plotted. For panel B, boxes represent the median, 25th percentile, and 75th percentile; whiskers delineate the minimum and maximum values.

**Figure 6.** PAD inhibition reduces the recruitment of netting neutrophils to the media and adventitia of Apoe−/− aortic sinus lesions. A, Aortic sinuses from the 18-week-old mice presented in Figure 1 were sectioned and stained by immunohistochemistry for NETs (H3-Cit), neutrophils (Ly-6G), and macrophages (F4/80). Representative staining is shown, with a neutrophil and H3-Cit-rich infiltrate at the interface between the intima and the media/adventitia (arrows). The inset shows a relatively intact H3-Cit-positive cell with polymorphonuclear morphology (black arrowhead) as well as a cell with decondensed morphology (red arrowhead). Scale bar=250 microns. B, Quantification of H3-Cit-positive cells in the media/adventitia. C, Correlation between the number of H3-Cit-positive cells and arterial lesion area; the best fit-line and 95% confidence intervals are plotted. D and E, Quantification of Ly-6G-positive cells in the media/adventitia (D), and F4/80-positive area in the intima (E). For B, D, and E, n=10 per group, and mean and SEM are plotted. *p<0.05; ***p<0.001. F, PAD inhibition reduces IFN-α expression in the aortic arch of Apoe−/− mice. RNA was prepared from aortic arches at 18 weeks of age. The data is expressed as fold change (positive value=activation, and negative value=repression) for Cl-amidine-treated mice relative to vehicle-treated mice (n=10 per group). *p<0.05; no other Cl-amidine-versus-vehicle comparison reached statistical significance. G, Protein was prepared from aortic arches at 18 weeks of age. Each lane represents protein pooled from 5 similarly treated mice, with H3-Cit and α-tubulin detected by western blotting. Data is plotted as the ratio of H3-Cit density to α-tubulin density for each sample. The western blot portion of the experiment was performed twice with similar results.

**Figure 7.** Cl-amidine does not protect against atherosclerosis in neutropenic mice. A, Apoe−/− mice exposed to high-fat chow were treated with vehicle or Cl-amidine from 7 to 18 weeks of age as indicated. Mice were also treated with either anti-Ly-6G or a control antibody from 8 to 18 weeks of age as indicated. At 18 weeks of age, peripheral blood was collected and anti-Ly-6G-positive cells were determined by flow cytometry as a percentage of total circulating leukocytes. B, Mice were treated as in Panel A, and atherosclerotic lesions were quantified in arterial trees by en face Oil Red O staining. C, Apoe−/− Ifnαβr−/− mice lack the type I IFN receptor. These mice were exposed to high-fat chow, and were treated with vehicle or Cl-amidine from 7 to 18 weeks of age as indicated. Atherosclerotic lesions were quantified in arterial trees by en face Oil Red O staining. For all experiments, box-and-whisker plots show data for 10 mice per group, with boxes representing the median, 25th percentile, and 75th percentile; whiskers delineate the minimum and maximum values. *p<0.05; **p<0.01; ns=not significant. One p value that approaches significance is denoted.
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 deminase (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<sup>−/−</sup> 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 pharmacologic blockade of NET formation by inhibition of PAD enzymes could protect against atherosclerosis. Indeed, Apoe<sup>−/−</sup> 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 paper adds to the existing literature by strongly arguing for a causative role of NETs in atherosclerosis. Further, and in contrast to neutrophil depletion or genetic mutation, NETs were inhibited here by a pharmacologic 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.
Figure 1

[Graphs and images showing statistical comparisons and relationships between variables such as arterial lesion area, lipid-rich content, and time to occlusion.]
Figure 3

A

fold change (18- vs. 8-wk)

IFNA
MCP1
MX1
IRF7
IP10
ISG15
IFNG

* 0.12
* 0.10

B

8-wk arch
18-wk arch

H3-Cit

IFNG

C

D
Figure 7

A

% Ly-6G positive

control / vehicle
Ly-6G / vehicle
Ly-6G / Cl-am

B

arterial lesion area (%)

control / vehicle
Ly-6G / vehicle
Ly-6G / Cl-am

C

arterial lesion area (%)

vehicle
Cl-amidine

* 0.0810

**

ns
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 and Mariana J Kaplan

Circ Res. published online January 14, 2014;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2014/01/14/CIRCRESAHA.114.303312

Data Supplement (unedited) at:
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SUPPLEMENTAL METHODS

Mice and drug treatment. C57BL/6 control and Apoe\(^{-/-}\) (B6.129P2-Apoemtm1Unc/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The Apoe\(^{-/-}\) 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 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).³⁹,¹⁰

**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** = ATGGCTAGRCTCTGTGCTTTTCT and AGGGCTTCCAGAYTTCTGCTCTG;

**MCP1** = AGGTCCCTGTCATGCTTCTG and TCTGGACCATTCTTTCTTG;

**MX1** = GATCCGACTCTCATTCCAGATGG and CATCTCAGTGTTAGTCAACCC;

**IRF7** = TGCTGTGTTGGAGACTGCTAT and TCCAAGCCTCCGGCTAAAGT;

**IP10** = ATCGTCTGCTGAGACTGCTAT and TATTCTTGCTTCCGGCGATTAC;

**ISG15** = CAGAAGCAGACTCTCTTAATT and AGACCTCATATATGTTGCTG;

**IFNG** = AGCGGCTGACTGAACTCAGATTGTA and GTCACAGTTTTCAGCTGTATAGGG

**ACTB** = TGGAATCCTGTGGCATCCTGAAAC and TAAAACGCAGCTCAGTAACAGTCCG.

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 $2^{\Delta\Delta C_t}$.

**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. 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. 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.
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