HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2017 August 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2016 August ; 36(8): 1660–1669. doi:10.1161/ATVBAHA.116.307786.

Neutrophil proteases promote experimental abdominal aortic aneurysm via extracellular trap release and plasmacytoid dendritic cell activation

Huimin Yan[#], **Hui-Fang Zhou**[#], **Antonina Akk**[#], **Ying Hu**, **Luke E. Springer**, **Terri L. Ennis**, and **Christine T.N. Pham**

John Cochran VA Medical Center, Saint Louis, Missouri USA; the Department of Medicine, Division of Rheumatology and the Department of Surgery, Section of Vascular Surgery, Washington University School of Medicine, Saint Louis, Missouri, USA

[#] These authors contributed equally to this work.

Abstract

Objective—We previously established that neutrophil-derived dipeptidyl peptidase I (DPPI) is essential for experimental abdominal aortic aneurysm (AAA) development. Since DPPI activates several neutrophil serine proteases (NSPs), it remains to be determined whether the AAA-promoting effect of DPPI is mediated by NSPs.

Approach and Results—Using an elastase-induced AAA model, we demonstrate that absence of two NSPs, neutrophil elastase and proteinase 3, recapitulates the AAA-resistant phenotype of DPPI-deficient mice. DPPI and NSPs direct the *in vitro* and *in vivo* release of extracellular structures termed neutrophil extracellular traps (NETs). Administration of DNase1, which dismantles NETs, suppresses elastase-induced AAA in WT animals and in DPPI-deficient mice reconstituted with WT neutrophils. NETs also contain the cathelicidin-related antimicrobial peptide (CRAMP) that complexes with self-DNA in recruiting plasmacytoid dendritic cells (pDCs), inducing type I interferons (IFNs) and promoting AAA in DPPI-deficient mice. Conversely, depletion of pDCs or blockade of type I IFNs suppresses experimental AAA. Moreover, we find an abundance of LL-37, the human orthologue of CRAMP, in the vicinity of pDCs in human AAA tissues. Increased type I IFN mRNA expression is observed in human AAA tissues and circulating IFNα is detected in approximately 50% of the AAA sera examined.

Conclusions—These results suggest that neutrophil protease-mediated NET release contributes to elastase-induced AAA through pDC activation and type I IFN production. These findings increase our understanding of the pathways underlying AAA inflammatory responses and suggest that limiting NET, pDC and type I IFN activities may suppress aneurysm progression.

Keywords

abdominal aortic aneurysm; neutrophil extracellular traps; neutrophil proteases; type I interferons; plasmacytoid dendritic cells

Address correspondence to: Christine T.N. Pham, 660 South Euclid Avenue, Campus Box 8045, Saint Louis, MO 63110. Telephone: 314-362-9043. Fax: 314-454-1091. cpham@dom.wustl.edu.

c) Disclosures: None

Introduction

AAA is a complex disease associated with male gender, advanced age, hypertension, hypercholesterolemia, coronary artery disease, atherosclerosis, and cigarette smoking.[1] Open surgical repair of large AAA is an effective option in preventing death from rupture; however, it can be associated with high post-operative mortality (up to 6%).[2] Endovascular repair carries lower post-operative mortality risk but does not lead to increased long-term survival among older patients.[3] Given the uncertainties that still pertain to endovascular repair, medical treatment to slow progression of AAA represents an attractive alternative.

Although the precise mechanisms that initiate and perpetuate AAA are still under active investigation, studies suggest that they are likely multifactorial, involving an interplay between genetic predisposition,[4] environmental and mechanical stimuli,[5, 6] matrix metalloprotease (MMP)-mediated structural damage,[7] and complex inflammatory process.[8] Whether inflammation is a cause or consequence of aortic wall damage is still being debated. However, a recent prospective study supports a causal role of inflammation in AAA progression.[9] Yet, despite overwhelming evidence of an inflammatory process in AAA, anti-inflammatory therapies aimed at some targets identified in preclinical models have not resulted in consistent AAA stabilization in clinical studies.[10] Therefore, further research is needed to identify and validate additional pathways that participate in aneurysmal process.

Using an elastase-induced model of AAA, we previously reported that dipeptidyl peptidase I (DPPI) plays a critical role in the development of aneurysm.[11] DPPI is required for the expression of active neutrophil serine proteases (NSPs) neutrophil elastase (NE), cathepsin G (CG), and proteinase 3 (PR3) in mature neutrophils.[12-14] Absence of DPPI protects mice against elastase-induced AAA, due in part to diminished recruitment of neutrophils to the aortic wall.[11] We also established that initiation of the aneurysmal process requires complement activation, releasing anaphylatoxin C5a that attract neutrophils to the aortic wall.[15, 16] It is generally understood that once recruited to a site of inflammation, neutrophils undergo constitutive apoptosis and are rapidly cleared by phagocytic macrophages. However, there is increasing evidence that during this brief period, neutrophils stimulate a network of immune cell types that together can direct a chronic pathological response.[17] Activated neutrophils form NETs, which were first described as an innate immune response to bacterial infection.[18] NETs are also released in response to non-infectious, endogenous stimuli such as C5a.[19] We hypothesize that, in elastase-induced AAA, complement activation generates C5a that stimulates neutrophils recruited to the aortic wall to release NETs, propagating the inflammatory responses and culminating in eventual AAA.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement

Results

NETs are present in elastase-perfused murine aortic tissues

A recent report suggests that NETs are present in human AAA tissues and associated with experimental AAA in the context of *Porphyromonas gingivalis* infection.[20] Although NETs are implicated, whether they are formed in response to endogenous stimuli and their pathogenic mechanisms in AAA are unknown. We used the elastase-induced AAA model to examine whether NETs were associated with aneurysmal development, independent of infection. In this model a dilute solution of porcine elastase was transiently instilled (for 5 min) into the isolated segment of the infrarenal abdominal aorta on day 0 and AAA development was assessed at day 14.[21] NET formation was examined on day 2, during the peak neutrophil recruitment to abdominal aortic wall post-elastase perfusion.[11] We observed extensive NET formation in the elastase-perfused WT aorta, as assessed by immunostaining with myeloperoxidase (MPO), histone, and DNA (Figure 1A). NETs localized mainly to the adventitia, in areas of neutrophil accumulation as previously observed[11] and corroborating the findings in human AAA.[20] Perfusion with heat-inactivated elastase, which did not promote AAA,[21] did not support NET formation in aortic tissues (Figure 1B). Concomitant administration of DNase1, which dismantled NETs,[22, 23] also largely abrogated NET formation (Figure 1B). NETs were mostly absent in DPPI-deficient aorta, confirming our previous report that DPPI is required for neutrophil recruitment in elastase-induced AAA (Figure 1B).[11] Previous studies suggest that NSPs are critical for NET formation *in vitro* and *in vivo*.[24] In agreement with these studies we observed significantly less NET release in the abdominal aortic tissues on day 2, following elastase perfusion of mice deficient in all 3 NSPs (NECGPR3) (Figure 1B).

To corroborate these *in vivo* findings and further quantify NETs, we purified resting bone marrow neutrophils and stimulated them *in vitro* with C5a, a complement degradation product that is essential for elastase-induced AAA.[15] C5a stimulated equivalent release of NETs compared with lipopolysaccharide (LPS, Figures 1C-D). DPPI- and NECGPR3-deficient neutrophils exposed to C5a or LPS for 30 min generated significantly less NETs, compared with stimulated WT neutrophils (Figures 1C-D, $p < 0.001$). Despite previous reports describing an essential role for NE in NET release[24, 25] we observed that NE-deficient neutrophils showed no “NETing” defect when stimulated with C5a or LPS (Figure S1). On the other hand the combined absence of NE and PR3 resulted in a NET defect similar to that observed with the NECGPR3-deficient neutrophils (Figure S-I). Lastly, we found that WT NETs generated in response to C5a or LPS also contained DNA-associated cathelicidin-related antimicrobial peptide (CRAMP), the murine orthologue of human LL-37,[26] while stimulated DPPI- and NECGPR3-deficient neutrophils released no or minimal amount of CRAMP (Figure 1E).

Neutrophil proteases and NETs actively contribute to AAA development

Having established that DPPI and NSPs are required for optimal NET formation *in vivo* and *in vitro* we next evaluated the contribution of neutrophil proteases and protease-mediated NET release to elastase-induced AAA: Following elastase perfusion there was an immediate dilatation of the abdominal diameter (AD) of approximately 75%, remaining stable up to 7

days, after which there was rapid increase in AD.[21] AAA was routinely assessed on day 14 and defined as an increase in AD of more than 100% over the pre-perfused diameter.[11, 15, 21] We observed no significant difference in the immediate post-perfusion dilatation between genotypes (Figure 2A). We confirmed that DPPI deficiency significantly suppressed AAA development ($p < 0.001$ compared to WT) (Figure 2A) , corroborating our published results.[11] The overall increase in AD of NECGPR3-deficient mice was similarly reduced ($p < 0.001$), confirming the importance of NSPs to aneurysm development (Figures 2A). Histologic analysis of aortic sections revealed preservation of the elastic lamellae (Figure 2B) and reduced inflammatory cell infiltrates, specifically Mac-3^+ macrophages, in DPPI- and NECGPR3-deficient mice (Figure 2C). To determine which NSPs were required for AAA development, we examined mice with different loss-of-function mutations. Mice deficient in NE developed AAA at WT level while NEPR3-deficient animals developed much smaller AAA ($p < 0.001$) (Figure 2D), suggesting that NE and PR3 are essential for the AAA phenotype.

To evaluate the role of NETs in AAA we treated elastase-perfused WT mice with DNase1. DNase1 treatment suppressed AAA development in WT animals ($p < 0.001$ compared to untreated mice), confirming that NETs directly contribute to aneurysm formation (Figure 2E). Heat inactivation abolished the effect of DNase1 on AAA (Figure 2E). In addition, delaying DNase1 treatment until day 4-post-elastase perfusion, after the peak of neutrophil recruitment,[11] resulted in no suppression of AAA suggesting that neutrophils and extracellular trap components released during the acute response following elastase perfusion were essential for triggering the downstream inflammatory cascade (Figure S-II). To further confirm that NSP-mediated NET release contributed to the aneurysmal process, we adoptively transferred WT neutrophils (10e7) into DPPI-deficient mice following a regimen that has previously been shown to restore susceptibility to AAA in DPPI-deficient mice.[11] While WT neutrophils promoted aneurysm in DPPI-deficient mice[11] NECGPR3-deficient neutrophils did not, confirming the importance of NSPs downstream of DPPI in AAA development (Figure 2E). Concomitant administration of DNase1 abolished the ability of WT neutrophils to induce AAA in DPPI-deficient mice while inactivated DNase1 (iDNase1) had no effect (Figure 2E). These results strongly support the concept that NSP-mediated NETs contribute to AAA genesis.

DNA-CRAMP complexes contribute to elastase-induced AAA

Several NET components have been shown to mediate tissue injuries and stimulate autoimmune responses.[27] Studies suggest that CRAMP, the mouse homologue of human LL-37 facilitates immune recognition of self-DNA by promoting access to and activation of pDCs, inducing type I IFNs that in turn stimulate T cell activation.[28, 29] Indeed, we detected an abundance of extracellular CRAMP in day 2 elastase-perfused WT aortas while DPPI-deficient aortas were devoid of NETs and CRAMP (Figure 3A). To further evaluate the role of DNA-CRAMP complexes in AAA development, we treated DPPI-deficient mice with exogenous DNA-CRAMP complexes. The administration of exogenous DNA-CRAMP complexes promoted aneurysm development DPPI-deficient mice while neither CRAMP nor DNA administered individually was effective (Figure 3B). Moreover, administration of DNA-scrambled CRAMP (sCRAMP) to DPPI-deficient mice had no effect on aneurysm

size, suggesting a specific role for CRAMP and arguing against the fact that administration of exogenous DNA-CRAMP complexes initiates AAA development through a secondary inflammatory response (Figure 3B).

pDCs and type I IFNs propagate the inflammatory responses in AAA

Although the role of DNA-CRAMP complexes in pDC activation may be assumed from previous studies,[28, 29] their pathogenic mechanism in AAA development has yet to be confirmed. To this end, we first established the presence of pDCs in aortic sections from elastase-perfused mice using Siglec-H as a specific marker. We found a temporal increase in Siglec-H$^+$ cells in WT but not in DPPI-deficient mice (Figures 3C-D). Multicolor flow cytometry further revealed that Siglec-H$^+$ cells in WT aortic tissues expressed B220, CD11c (low), and PDCA1, confirming that they were indeed pDCs (Figure S-IIIA). In elastase-perfused DPPI-deficient mice, exogenous administration of DNA-CRAMP complexes significantly increased recruitment of Siglec-H$^+$ cells to the abdominal aorta (Figure 3E). Moreover, these Siglec-H$^+$ cells expressed IFNα (Figure 3F), supporting the notion that DNA-CRAMP complexes in NETs amplify the recruitment and activation pDCs *in vivo*.

The role of pDCs in aneurysm development was further analyzed through depletion. The anti-PDCA1 monoclonal antibody (mAb) that has been shown to specifically deplete pDCs[30] was administered to naïve WT mice on days -2 and -1 prior to elastase perfusion. Flow cytometry of cells obtained from spleen, lymph nodes, bone marrow and peripheral white blood cells harvested 24 h after elastase perfusion confirmed that anti-PDCA1 mAb did not deplete additional major cell types that may upregulate PDCA1 expression upon surgical manipulation and elastase perfusion (Figure S-IIIB). On the other hand anti-PDCA1 mAb administration led to significant suppression of AAA development ($p < 0.01$ compared to isotype control) (Figure 4A) and pDC recruitment to abdominal aortic tissues (WT elastase-perfused mice, 481 pDCs/aorta; WT elastase-perfused + anti-PDCA1, 40 pDCs/aorta; WT elastase-perfused + isotype control, 1,202 pDCs/aorta) (Figure 4B). pDC depletion also abrogated CD3$^+$ T cell recruitment to the aorta (Figure 4C-D) and significantly lower MMP (gelatinase) activity detected by *in situ* zymography (Figure 4E-F).

As activated pDCs can produce large amount of type I IFNs,[31] we next sought to inhibit their *in vivo* activity with an IFN-α receptor 1 (IFNAR-1) specific mAb that has been shown to block IFN-α activity.[32] The administration of anti-IFNAR-1 mAb significantly attenuated elastase-induced AAA $p < 0.01$ compared to no treatment and isotype control) (Figure 5A) and reduced but did not completely abrogate T cell recruitment (Figure 5B). We previously showed that activated T cells in elastase-induced AAA elaborated IFNγ.[33] Consistent with these results we found that IFNAR-1 blockade largely suppressed IFNγ production by T cells (Figures 5C-D) and significantly reduced MMP activity (Figure 5E). Taken together, these results suggest that type I IFNs released by pDCs stimulate T cells to produce IFNγ, the activity of which is essential for MMP activity in elastase-induced AAA.[33]

pDCs and type I IFNs are observed in human AAAs

Although expression of IFNγ is widely recognized in human AAA[33-35] presence of type I IFNs has not been described. We first examined human AAA tissues for the presence of the

NET component LL-37, the human orthologue of CRAMP.[28] We detected abundant expression of LL-37, some of which localized to the vicinity of $CD303^+$ (a marker for human pDCs) cells (Figure 6A) and could be seen taken up inside $CD303^+$ cells (Figure S-IV). Non-AAA aortic tissues were negative for both LL-37 and CD303 staining (Figure 6A). To further confirm the identity of pDCs in human AAA tissues, we showed that $CD303^+$ cells also stained positive for CD85g (Ig-like transcript 7, ILT7) (Figure 6B), a marker specifically expressed on human pDCs.[36] Activated pDCs produce type I IFNs. Indeed real time PCR revealed increased incidence of *IFNA2* mRNA expression in AAA tissues (47% in AAA tissues vs. 11% in non-AAA tissues, $p = 0.0479$) (Figure 6C). More strikingly, we detected circulating low levels of IFNα in a number of AAA patients (6 out of 13) while no circulating IFNα was detectable in non-AAA sera (Figure 6D, $p = 0.0122$). In contrast, we found very low levels of circulating IFNγ in the same samples but no significant difference between non-AAA and AAA sera (Figure 6E) and low correlation ($r = 0.0899$) between IFNα and IFNγ levels in the serum of AAA patients (Figure 6F).

Discussion

NETs are implicated in the pathogenesis of several inflammatory diseases, including rheumatoid arthritis,[37] atherosclerosis,[29, 38] systemic lupus erythematosus,[39] anti-neutrophil cytoplasmic antibody-associated vasculitis,[40] chronic obstructive pulmonary disease,[41] and types 1 and 2 diabetes [42], just to name a few. However, their mechanism of action in AAA pathogenesis remains largely unexplored. We establish that NETs require DPPI and active NSPs while DNase1 dismantles NETs *in vivo*, suppressing elastase-induced AAA development. DNA-CRAMP complexes released by neutrophils during this “NETing” process amplify pDC recruitment, inducing IFNα production and promoting AAA in DPPI-deficient mice. Depletion of pDCs or blockade of type I IFN activity suppresses the aneurysmal process. In addition to previously described NET components,[20] LL-37 and pDCs are observed in human AAA tissues as well as increased type I IFN expression. Circulating IFNα is also detected in some AAA sera but not non-AAA controls, suggesting that this cytokine may identify a specific subset of AAA patients.

NSPs are major constituents of neutrophil azurophil granules. In 2004 Brinkman *et al.* reported that NSPs (NE specifically) participate in the generation of NETs.[18] NETs are composed of extracellular DNA, histone, and several granule proteins including MPO, NSPs, and the antimicrobial peptide LL-37 that complexes with self-DNA to activate pDCs,[28] inducing the release of type I IFNs which in turn stimulate the immune responses.[43] Our *in vitro* and *in vivo* data revealed that the absence of NSPs, which are activated by DPPI,[12, 13] largely abrogated NET formation *in vitro* and *in vivo*, and suppressed AAA. While our results confirmed the *in vivo* contribution of DPPI (and active NSPs) to NET production previously suggested by *in vitro* studies[14] we found that NE deficiency alone did not abrogate NETs in response to C5a/LPS *in vitro* nor suppress AAA development *in vivo* despite previous reports suggesting a critical role for this protease in NET formation.[24, 25] It is thought that NE translocation to the nucleus, where it partially degrades specific histones and promotes chromatin decondensation, represents an essential step in NET formation.[24, 25] Recent studies, however, suggest that NE is dispensable for NET formation in mice,[44] corroborating our findings. And while the precise role of PR3 is not well defined, it is

present in NETs[45] and increased circulating levels of PR3 are closely associated with augmented NET formation in patients with type 1 diabetes.[46] Although the dominant proteolytic activity in NETs is attributed to NE, cleavage sites corresponding to PR3 and CG are also evident.[47] Along this line, we found that NE and PR3 combined deficiencies largely abrogated *in vivo* NET formation and significantly attenuated aneurysm development. These results are consistent with a recent study showing that Apolipoprotein E/PR3/NE-deficient mice had smaller atherosclerotic plaques that were devoid of NETs.[38] Taken together, these data infer that, in addition to NE, PR3 participates in NETs and NET-dependent inflammatory responses in AAA. However, the exact mechanism by which PR3 promotes NET formation remains to be determined. We also establish that NECGPR3-deficient neutrophils were inefficient at promoting AAA and DNase1 administration suppressed AAA development in DPPI-deficient mice reconstituted with WT neutrophils, confirming that NSPs and neutrophil protease-mediated NET release is required for the aneurysmal process. Although delayed DNase1 treatment did not confer protection against AAA development in the mouse, these results should be considered in the context of the model wherein the aorta is subjected to a single insult (elastase perfusion on day 0). In contrast, human AAA growth proceeds in a discontinuous, staccato manner, [48] suggesting that repeated episodes of insults over time lead to eventual tissue degeneration and aneurysmal dilation. Indeed, there is evidence of ongoing NET formation in human AAA,[20] even in the late stages of disease (i.e. large aneurysms), suggesting that controlling neutrophil extracellular trap formation may limit aneurysm progression clinically.

Several studies have shown that self-DNA-LL-37/CRAMP complexes released by neutrophils during NET formation act as unique trigger of the immune responses. DNA-LL-37/CRAMP complexes may drive pDCs to release type I IFNs that initiate the inflammatory cascade in atherosclerosis.[29] DNA-containing NETs may also directly prime macrophages for cytokine release.[38] Our data suggest that during the aneurysmal process DNA-CRAMP complexes released along with NETs may be taken up by pDCs, triggering their activation, inducing type I IFN production and driving the inflammatory responses. Indeed, administration of exogenous DNA-CRAMP complexes rendered the DPPI-deficient mice more susceptible to AAA. Although we could not exclude the possibility that NETs directly activated macrophages in elastase-induced AAA, the fact that pDC depletion abrogated T cell recruitment/activation, MMP activity, and aneurysm development strongly suggests that NET-dependent priming of pDCs is required for AAA formation in this model. pDCs secrete large amounts of type I IFNs in response to viruses.[31] In addition, chronic activation of pDCs and increased synthesis of type I IFNs in the absence of viral infection also promote immunity and autoimmunity.[49] We demonstrate herein that blockade of IFNα activity using an IFNAR-1 blocking antibody also abrogated T cell recruitment/activation, MMP activity and suppressed AAA, thus validating the contribution of pDCs and revealing a requirement for type I IFNs in this preclinical model. Others and we have previously established a role for IFNγ in experimental AAA[33, 34] and systematic review suggests that IFNγ is upregulated in large human AAAs.[35] The role of pDCs and type I IFNs in AAA, however, is unknown. We examined human AAA tissues and found an abundance of the NET component LL-37 localizing in close proximity to $CD303^{+}CD85g^{+}$ pDCs. We also found that approximately 50% of human AAA tissues examined harbored detectable levels

of *IFNA2* mRNA expression. In addition, circulating IFNα was detectable only in AAA sera while low but equivalent levels of IFNγ were found in both non-AAA and AAA sera examined. Whether circulating IFNα level may be used as a specific biomarker that predicts AAA behavior/progression will require larger prospective studies. Nonetheless, we show that the results are highly significant despite the small sample size (n = 13-15), suggesting that type I IFNs merit further investigation as additional players in AAA pathogenesis..

In conclusion, our studies demonstrate that neutrophil protease-mediated NET release in response to an endogenous stimulus (such as C5a) contributes to elastase-induced AAA. We propose that NET-mediated pDC recruitment/activation and production of type I IFNs promote a cell-mediated immune response, perpetuating the inflammatory cascade in aneurysm development. We present evidence that pDCs and type I IFNs are found in human AAA tissues and circulating IFNα is detected in a subset of patients with AAA. These findings increase our understanding of the pathways underlying the mechanisms of AAA inflammation and provide additional targets that may be antagonized to limit or suppress aneurysm progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

a) Y.H., H.-F.Z, A.A., Y.H., L.S., T.E. performed experiments and analyzed data; Y.H., H.-F.Z., A.A., and C.P. contributed to manuscript writing; C.P. conceived the overall direction of the studies. The authors thank Dr. Kathleen Sheehan for the generous gift of anti-IFNα receptor 1 (IFNAR-1) mAb and Dr. Robert Thompson for providing anonymous discarded human tissues.

b) Source of Funding: This work was supported in part by the VA Merit Review Award 1I01 BX002714 and NIH grants AR067491, AI051436, HL073646 and bridge funding from the Department of Medicine at Washington University in St. Louis. Experimental support was also partially provided by the Hybridoma Center of the Rheumatic Diseases Core Center (NIH P30AR048335). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations

AAA	abdominal aortic aneurysm
AD	aortic diameter
CG	cathepsin G
CRAMP	cathelicidin-related antimicrobial peptide
DNA	deoxyribonucleic acid
DNase1	deoxyribonuclease 1
DPPI	dipeptidyl peptidase I
IFN	interferon
IFNAR-1	interferon alpha receptor 1

LL-37	human cathelicidin peptide, a 37 amino acid sequence starting with two leucines
mAb	monoclonal antibody
MMP	metalloprotease
MPO	myeloperoxidase
NE	neutrophil elastase
NET	neutrophil extracellular trap
NSP	neutrophil serine protease
pDC	plasmacytoid dendritic cell
PDCA1	plasmacytoide dendritic cell antigen 1
PR3	proteinase 3

References

1. Lederle FA, Nelson DB, Joseph AM. Smokers' relative risk for aortic aneurysm compared with other smoking-related diseases: a systematic review. J Vasc Surg. 2003; 38:329–34. [PubMed: 12891116]
2. Mortality results for randomised controlled trial of early elective surgery or ultrasonographic surveillance for small abdominal aortic aneurysms. The UK Small Aneurysm Trial Participants. Lancet. 1998; 352:1649–55. [PubMed: 9853436]
3. Lederle FA, Freischlag JA, Kyriakides TC, Matsumura JS, Padberg FT Jr. Kohler TR, Kougias P, Jean-Claude JM, Cikrit DF, Swanson KM. Long-term comparison of endovascular and open repair of abdominal aortic aneurysm. N Engl J Med. 2012; 367:1988–97. [PubMed: 23171095]
4. Hinterseher I, Tromp G, Kuivaniemi H. Genes and abdominal aortic aneurysm. Ann Vasc Surg. 2011; 25:388–412. [PubMed: 21146954]
5. Jin J, Arif B, Garcia-Fernandez F, Ennis TL, Davis EC, Thompson RW, Curci JA. Novel mechanism of aortic aneurysm development in mice associated with smoking and leukocytes. Arterioscler Thromb Vasc Biol. 2012; 32:2901–9. [PubMed: 23042818]
6. Hoshina K, Sho E, Sho M, Nakahashi TK, Dalman RL. Wall shear stress and strain modulate experimental aneurysm cellularity. J Vasc Surg. 2003; 37:1067–74. [PubMed: 12756356]
7. Thompson RW, Holmes DR, Mertens RA, Liao S, Botney MD, Mecham RP, Welgus HG, Parks WC. Production and localization of 92-kilodalton gelatinase in abdominal aortic aneurysms. An elastolytic metalloproteinase expressed by aneurysm-infiltrating macrophages. J Clin Invest. 1995; 96:318–26. [PubMed: 7615801]
8. Kuivaniemi H, Platsoucas CD, Tilson MD 3rd. Aortic aneurysms: an immune disease with a strong genetic component. Circulation. 2008; 117:242–52. [PubMed: 18195185]
9. Folsom AR, Yao L, Alonso A, Lutsey PL, Missov E, Lederle FA, Ballantyne CM, Tang W. Circulating Biomarkers and Abdominal Aortic Aneurysm Incidence: The Atherosclerosis Risk in Communities (ARIC) Study. Circulation. 2015; 132:578–85. [PubMed: 26085454]
10. Lindeman JH. The pathophysiologic basis of abdominal aortic aneurysm progression: a critical appraisal. Expert Rev Cardiovasc Ther. 2015; 13:839–51. [PubMed: 26028299]
11. Pagano MB, Bartoli MA, Ennis TL, Mao D, Simmons PM, Thompson RW, Pham CT. Critical role of dipeptidyl peptidase I in neutrophil recruitment during the development of experimental abdominal aortic aneurysms. Proc Natl Acad Sci U S A. 2007; 104:2855–60. [PubMed: 17301245]

12. Adkison AM, Raptis SZ, Kelley DG, Pham CT. Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. J Clin Invest. 2002; 109:363–71. [PubMed: 11827996]
13. Pham CT, Ivanovich JL, Raptis SZ, Zehnbauer B, Ley TJ. Papillon-Lefevre syndrome: correlating the molecular, cellular, and clinical consequences of cathepsin C/dipeptidyl peptidase I deficiency in humans. J Immunol. 2004; 173:7277–81. [PubMed: 15585850]
14. Sorensen OE, Clemmensen SN, Dahl SL, Ostergaard O, Heegaard NH, Glenthoj A, Nielsen FC, Borregaard N. Papillon-Lefevre syndrome patient reveals species-dependent requirements for neutrophil defenses. J Clin Invest. 2014; 124:4539–48. [PubMed: 25244098]
15. Pagano MB, Zhou HF, Ennis TL, Wu X, Lambris JD, Atkinson JP, Thompson RW, Hourcade DE, Pham CT. Complement-dependent neutrophil recruitment is critical for the development of elastase-induced abdominal aortic aneurysm. Circulation. 2009; 119:1805–13. [PubMed: 19307471]
16. Zhou HF, Yan H, Stover CM, Fernandez TM, Rodriguez de Cordoba S, Song WC, Wu X, Thompson RW, Schwaeble WJ, Atkinson JP, Hourcade DE, Pham CT. Antibody directs properdin-dependent activation of the complement alternative pathway in a mouse model of abdominal aortic aneurysm. Proc Natl Acad Sci U S A. 2012; 109:E415–22. [PubMed: 22308431]
17. Muller I, Munder M, Kropf P, Hansch GM. Polymorphonuclear neutrophils and T lymphocytes: strange bedfellows or brothers in arms? Trends Immunol. 2009; 30:522–30. [PubMed: 19775938]
18. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science. 2004; 303:1532–5. [PubMed: 15001782]
19. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ. 2009; 16:1438–44. [PubMed: 19609275]
20. Delbosc S, Alsac JM, Journe C, Louedec L, Castier Y, Bonnaure-Mallet M, Ruimy R, Rossignol P, Bouchard P, Michel JB, Meilhac O. Porphyromonas gingivalis participates in pathogenesis of human abdominal aortic aneurysm by neutrophil activation. Proof of concept in rats. PloS One. 2011; 6:e18679. [PubMed: 21533243]
21. Thompson RW, Curci JA, Ennis TL, Mao D, Pagano MB, Pham CT. Pathophysiology of abdominal aortic aneurysms: insights from the elastase-induced model in mice with different genetic backgrounds. Ann N Y Acad Sci. 2006; 1085:59–73. [PubMed: 17182923]
22. Hakkim A, Furnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, Herrmann M, Voll RE, Zychlinsky A. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A. 2010; 107:9813–8. [PubMed: 20439745]
23. Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, Toy P, Werb Z, Looney MR. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. J Clin Invest. 2012; 122:2661–71. [PubMed: 22684106]
24. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol. 2010; 191:677–91. [PubMed: 20974816]
25. Metzler KD, Goosmann C, Lubojemska A, Zychlinsky A, Papayannopoulos V. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. Cell Rep. 2014; 8:883–96. [PubMed: 25066128]
26. Soehnlein O, Wantha S, Simsekyilmaz S, et al. Neutrophil-derived cathelicidin protects from neointimal hyperplasia. Sci Transl Med. 2011; 3:103ra98.
27. Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. Cell Death Differ. 2011; 18:581–8. [PubMed: 21293492]
28. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, Cao W, Wang YH, Su B, Nestle FO, Zal T, Mellman I, Schroder JM, Liu YJ, Gilliet M. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature. 2007; 449:564–9. [PubMed: 17873860]

29. Doring Y, Manthey HD, Drechsler M, et al. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. Circulation. 2012; 125:1673–83. [PubMed: 22388324]
30. Blasius AL, Giurisato E, Cella M, Schreiber RD, Shaw AS, Colonna M. Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. J Immunol. 2006; 177:3260–5. [PubMed: 16920966]
31. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. Nat Immunol. 2004; 5:1219–26. [PubMed: 15549123]
32. Sheehan KC, Lai KS, Dunn GP, Bruce AT, Diamond MS, Heutel JD, Dungo-Arthur C, Carrero JA, White JM, Hertzog PJ, Schreiber RD. Blocking monoclonal antibodies specific for mouse IFN-alpha/beta receptor subunit 1 (IFNAR-1) from mice immunized by in vivo hydrodynamic transfection. J Interferon Cytokine Res. 2006; 26:804–19. [PubMed: 17115899]
33. Zhou HF, Yan H, Cannon JL, Springer LE, Green JM, Pham CT. CD43-Mediated IFN-gamma Production by CD8+ T Cells Promotes Abdominal Aortic Aneurysm in Mice. J Immunol. 2013; 190:5078–85. [PubMed: 23585675]
34. Xiong W, Zhao Y, Prall A, Greiner TC, Baxter BT. Key roles of CD4+ T cells and IFN-gamma in the development of abdominal aortic aneurysms in a murine model. J Immunol. 2004; 172:2607–12. [PubMed: 14764734]
35. Golledge AL, Walker P, Norman PE, Golledge J. A systematic review of studies examining inflammation associated cytokines in human abdominal aortic aneurysm samples. Dis Markers. 2009; 26:181–8. [PubMed: 19729799]
36. Cao W, Rosen DB, Ito T, Bover L, Bao M, Watanabe G, Yao Z, Zhang L, Lanier LL, Liu YJ. Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production. J Exp Med. 2006; 203:1399–405. [PubMed: 16735691]
37. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, Friday S, Li S, Patel RM, Subramanian V, Thompson P, Chen P, Fox DA, Pennathur S, Kaplan MJ. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med. 2013; 5:178ra40.
38. Warnatsch A, Ioannou M, Wang Q, Papayannopoulos V. Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. Science. 2015; 349:316–20. [PubMed: 26185250]
39. Carmona-Rivera C, Zhao W, Yalavarthi S, Kaplan MJ. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. Ann Rheum Dis. 2015; 74:1417–24. [PubMed: 24570026]
40. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, Grone HJ, Brinkmann V, Jenne DE. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009; 15:623–5. [PubMed: 19448636]
41. Grabcanovic-Musija F, Obermayer A, Stoiber W, Krautgartner WD, Steinbacher P, Winterberg N, Bathke AC, Klappacher M, Studnicka M. Neutrophil extracellular trap (NET) formation characterises stable and exacerbated COPD and correlates with airflow limitation. Resp Res. 2015; 16:59.
42. Wong SL, Demers M, Martinod K, Gallant M, Wang Y, Goldfine AB, Kahn CR, Wagner DD. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. Nat Med. 2015; 21:815–9. [PubMed: 26076037]
43. Hervas-Stubbs S, Perez-Gracia JL, Rouzaut A, Sanmamed MF, Le Bon A, Melero I. Direct effects of type I interferons on cells of the immune system. Clin Cancer Res. 2011; 17:2619–27. [PubMed: 21372217]
44. Martinod K, Witsch T, Farley K, Gallant M, Remold-O'Donnell E, Wagner DD. Neutrophil elastase-deficient mice form neutrophil extracellular traps in an experimental model of deep vein thrombosis. J Thromb Haemost. 2015; 14:551–8. [PubMed: 26712312]
45. Sangaletti S, Tripodo C, Chiodoni C, Guarnotta C, Cappetti B, Casalini P, Piconese S, Parenza M, Guiducci C, Vitali C, Colombo MP. Neutrophil extracellular traps mediate transfer of cytoplasmic

neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. Blood. 2012; 120:3007–18. [PubMed: 22932797]

46. Wang Y, Xiao Y, Zhong L, Ye D, Zhang J, Tu Y, Bornstein SR, Zhou Z, Lam KS, Xu A. Increased neutrophil elastase and proteinase 3 and augmented NETosis are closely associated with beta-cell autoimmunity in patients with type 1 diabetes. Diabetes. 2014; 63:4239–48. [PubMed: 25092677]
47. O'Donoghue AJ, Jin Y, Knudsen GM, Perera NC, Jenne DE, Murphy JE, Craik CS, Hermiston TW. Global substrate profiling of proteases in human neutrophil extracellular traps reveals consensus motif predominantly contributed by elastase. PloS One. 2013; 8:e75141. [PubMed: 24073241]
48. Kurvers H, Veith FJ, Lipsitz EC, Ohki T, Gargiulo NJ, Cayne NS, Suggs WD, Timaran CH, Kwon GY, Rhee SJ, Santiago C. Discontinuous, staccato growth of abdominal aortic aneurysms. J Am Coll Surg. 2004; 199:709–15. [PubMed: 15501110]
49. Swiecki M, Colonna M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. Immunol Rev. 2010; 234:142–62. [PubMed: 20193017]

Highlights

- Neutrophil proteases mediate the release of neutrophil extracellular traps (NETs) in elastase perfused murine abdominal aorta.
- The NET component cathelicidin-related antimicrobial peptide (CRAMP) complexed to autologous DNA triggers plasmacytoid dendritic cells (pDCs) to release type I interferons, propagating the inflammatory cascade through T cell and macrophage activation.
- NET degradation and type I interferon blockade attenuate elastase-induced AAA.
- NETs, pDCs and type I interferons are expressed in human AAA tissues and merit further investigation as potential therapeutic targets.

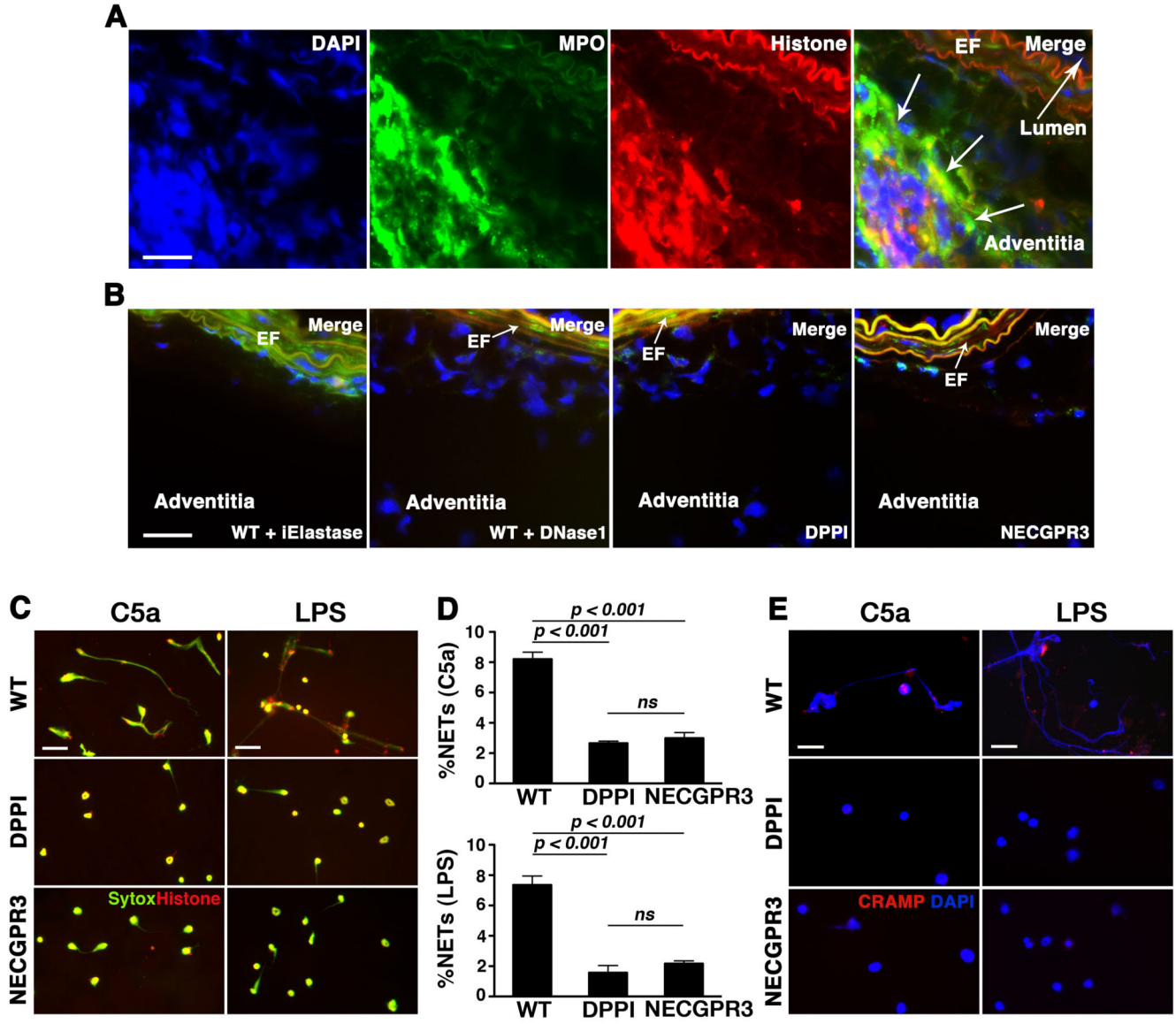

Figure 1. Neutrophil proteases promote NET release in vivo and in vitro

(A) WT mice were transiently perfused with elastase on day 0. On day 2 their aortas were harvested and stained for DNA (DAPI, blue), MPO (green), and histone (red). NETs localized mainly to the adventitia, where neutrophils accumulated (arrows). (**B**) Heat-inactivated elastase (iElastase) did not promote NET release; concomitant injections of DNase1 following elastase perfusion also largely abrogated NETs; DPPI- and NECGPR3-deficient mice exhibited a marked defect in NET formation. (**C**) Bone marrow-derived neutrophils from WT, DPPI- and NECGPR3-deficient mice were stimulated with C5a or LPS for 30 min and stained for DNA (Sytox green) and histone (red). (**D**) Quantification of NETs (mean ± SEM derived from 3 separate experiments). (**E**) Stimulated WT NETs also contained CRAMP (red) while DPPI- and NECGPR3-deficient neutrophils lacked both NETs and extracellular CRAMP. Scale bars = 25 μm. EF, elastic fibers are autofluorescent.

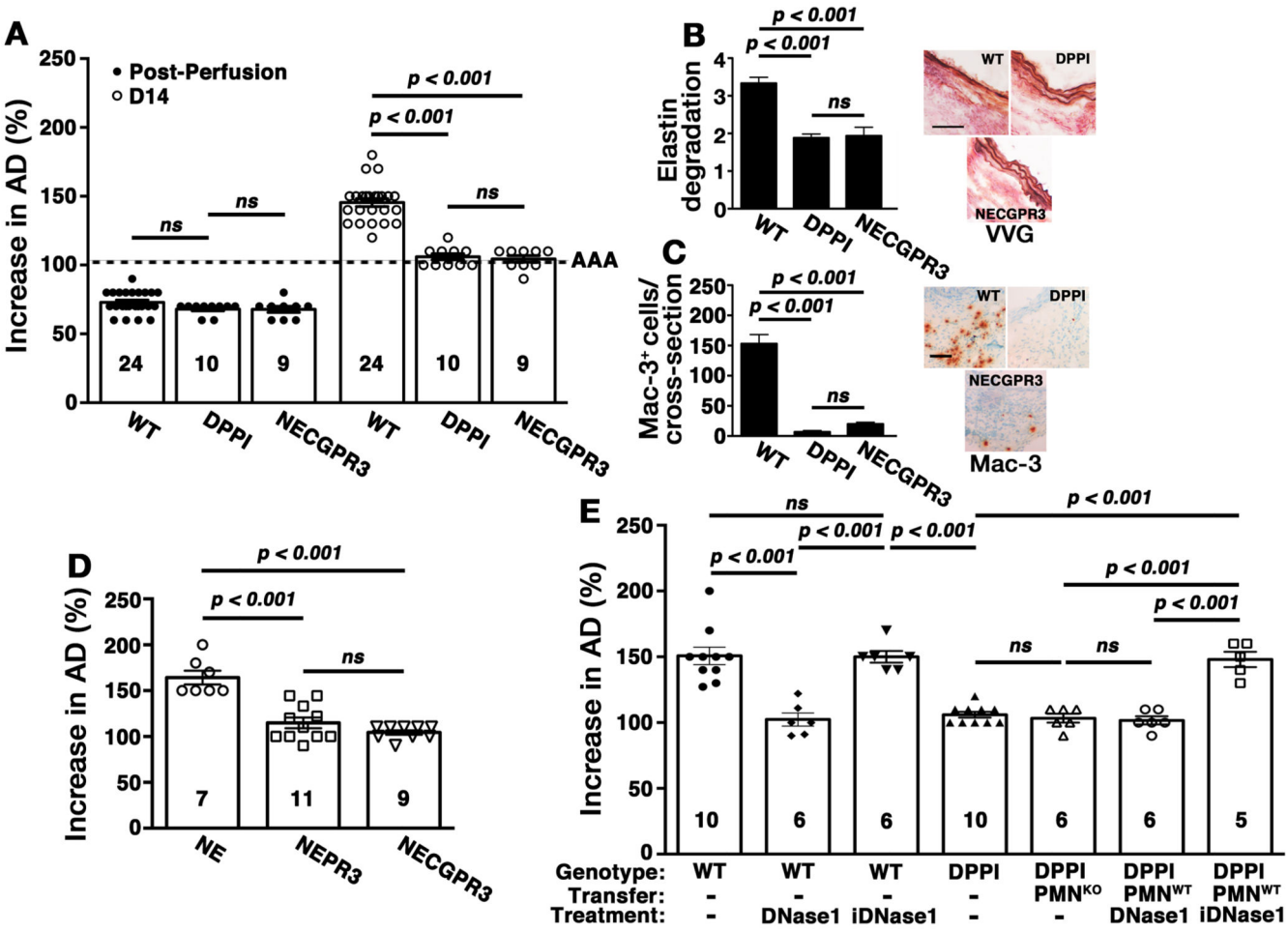

Figure 2. NSPs and NETs contribute to AAA development

(**A**) Mice were transiently perfused with elastase on day 0. There was no difference in the immediate post-perfusion aortic diameter (AD) between genotypes. AD on day 14 was defined as an increase in AD of greater than 100% compared with the AD measured prior to elastase perfusion. Absence of DPPI or NSPs (NECGPR3-deficient mice) significantly suppressed aneurysm development. Values represent mean ± SEM. The number of animals per genotype/treatment is indicated in each column. Suppression of aneurysm development was accompanied by relative preservation of elastic fibers, as assessed by elastin degradation with VVG staining (**B**), and inflammatory cell (Mac-3^{+}) recruitment (**C**). N = 5 aortas per genotype, 6-9 cross sections per aorta. Scale bars = 100 μm (**D**) Absence of NE and PR3 (NEPR3), but not NE alone, suppressed aneurysm development. (**E**) DNase1 treatment (days 0-5) also suppressed AAA development in WT mice while heat-inactivated DNase1 (iDNase1) had no effect. Adoptive transfer of NECGPR3-deficient neutrophils (PMN^{KO}, 10e7 on days 0 and 1) failed to fully reconstitute the AAA phenotype in DPPI-deficient mice. Concomitant DNase1 injections (days 0-3) also prevented DPPI-deficient mice from developing aneurysm following adoptive transfer of WT neutrophils (PMN^{WT}, 10e7 on days 0 and 1) while heat-=inactivated DNase1 (iDNase1) had no effect on WT neutrophil reconstitution and AAA development in DPPI-deficient mice.

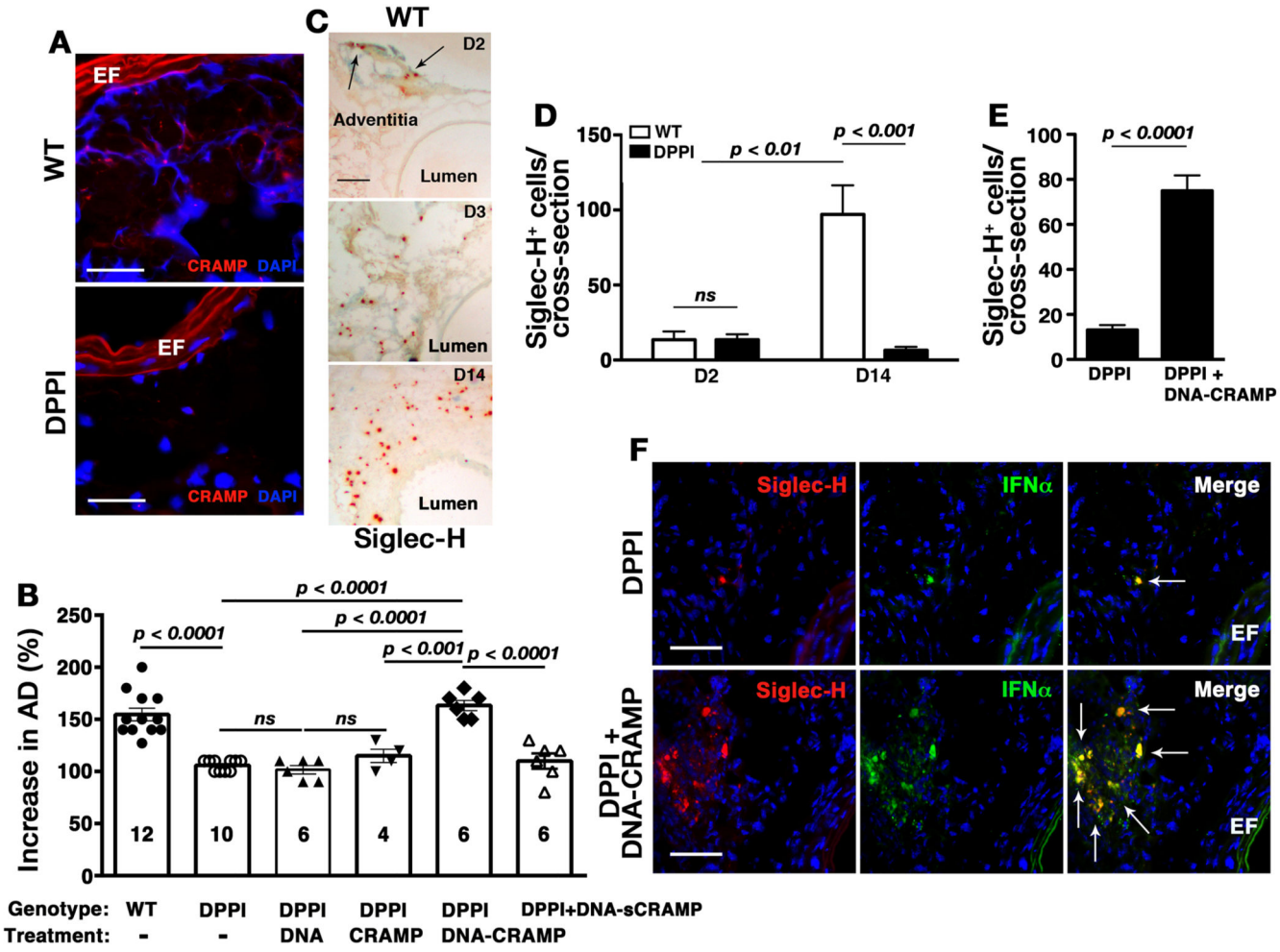

Figure 3. DNA-CRAMP complexes promote AAA via pDC

(**A**) WT and DPPI-deficient mice were perfused with elastase on day 0 and aortas were examined on day 2 for presence of extracellular CRAMP (red) associated with DNA (DAPI, blue). (**B**) Exogenous DNA-CRAMP complexes, but not DNA, CRAMP or DNA-(scrambled) sCRAMP complexes, promoted AAA in DPPI-deficient animals. AD was measured on day 14. Values represent mean ± SEM. (**C**) The number of Siglec-H$^+$ cells (arrows) in abdominal aortic wall (adventitia) increased over time in elastase-perfused WT animals but not in DPPI-deficient animals (**D**). (**E**) Administration of exogenous DNA-CRAMP complexes to DPPI-deficient mice led to a significant increase in the number of Siglec-H$^+$ cells recruited. (**D**, **E**) N = 4-5 aortas per genotype/treatment, 6-9 cross sections per aorta. (**F**) Day 14 aortas were stained for Siglec-H (red) and IFNα (green). Most Siglec-H$^+$ cells expressed IFNα (arrows) and the colocalization appeared yellow in merged images. Scale bars = 25μm (**A**), 100 μm (**C**), 50 μm (**F**). EF, elastic fibers.

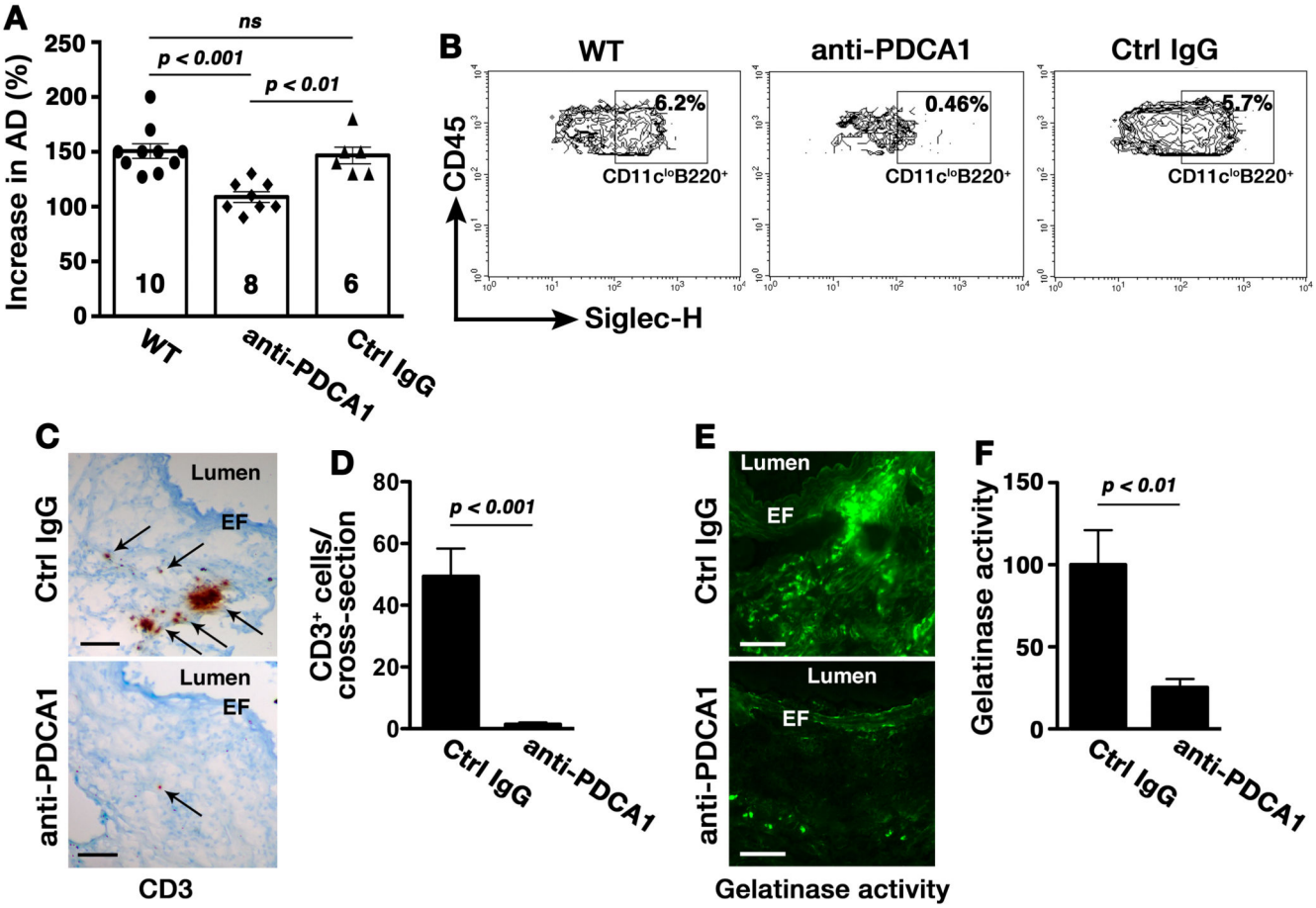

Figure 4. pDCs are required for T cell recruitment and inflammatory response in AAA

(**A**) Administration of anti-PDCA1 mAb suppressed elastase-induced AAA. AD was measured on day 14. Values represent mean ± SEM. (**B**) Aortas from elastase-perfused WT mice without treatment or treated with anti-PDCA1 mAb or isotype control (IgG Ctrl) mAb were harvested on day 14 and processed as detailed in Materials and Methods. The number of $CD45^+$ leukocytes (events) was enumerated by flow cytometry. $CD45^+$ cells that expressed mid to low CD11c, B220 and mSiglec-H were identified as pDCs. The absolute number of pDCs was calculated using the following equation: total number of $CD45^+$ events × percent of $CD45^+CD11c^{lo}B220^+Siglec\text{-}H^+$ pDCs (expressed as a decimal) ÷ 3 (animals). pDC depletion largely abrogated $CD3^+$ T cell recruitment in elastase-perfused aortas on day 14 (**C-D**) and markedly inhibited MMP (gelatinase) activity, as detected by *in situ* zymography (**E-F**). Gelatinase activity was analyzed by ImageJ and presented as percentage of normalized WT activity, which was set at 100% (**F**). (**B**, **D**, **F**) N = 4-5 aortas per treatment, 6-9 cross sections per aorta. Scale bars = 100 μm

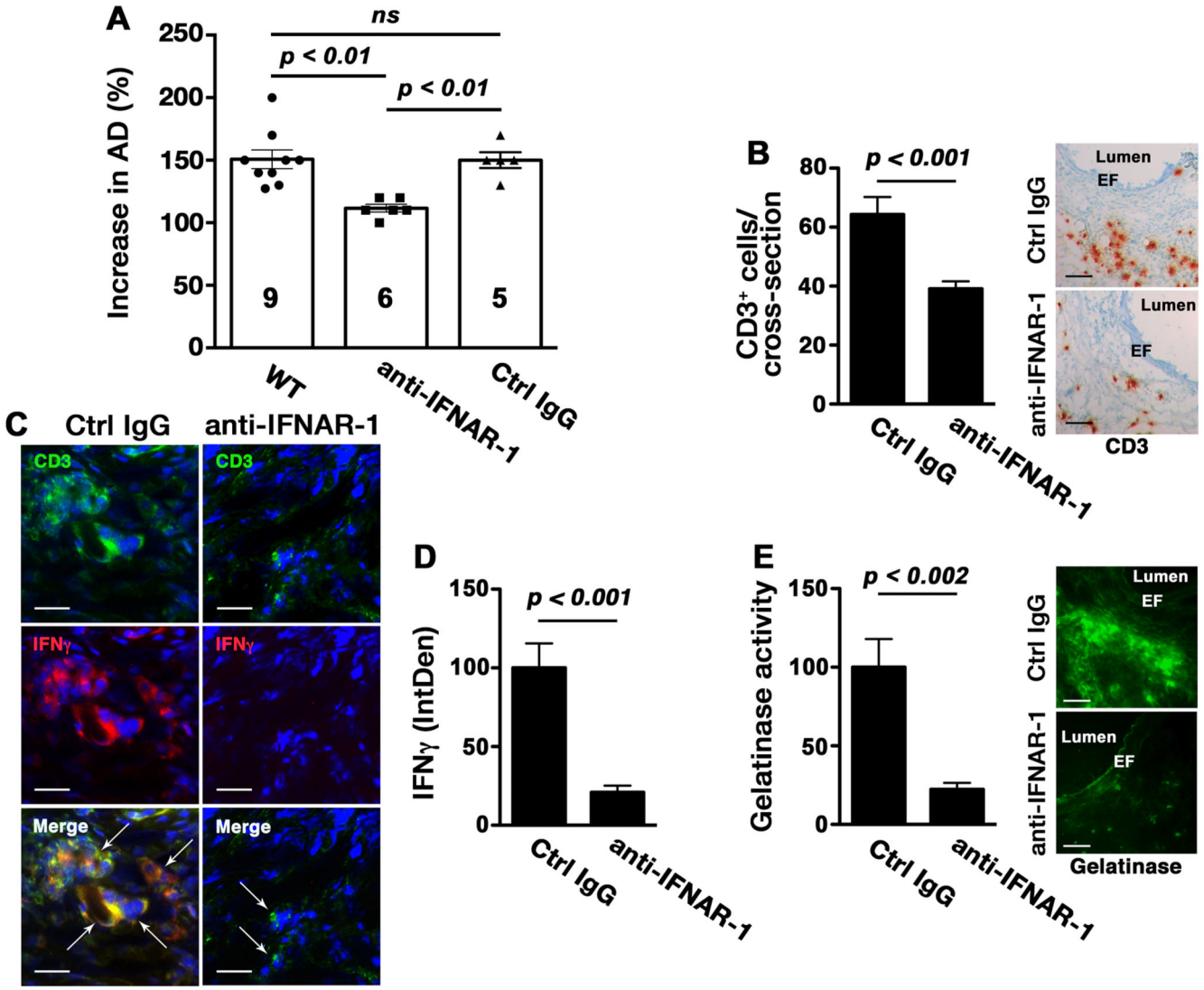

Figure 5. Blockade of type I IFN activity suppresses AAA development

(**A**) WT mice were perfused with elastase on day 0 and administered anti-IFNAR-1 antibody immediately after laparotomy and again on days 2 and 4. AD was measured on day 14. Values represent mean ± SEM. (**B**) IFNAR-1 blockade suppressed but did not completely abrogate $CD3^+$ T cells recruitment. (**C**) Day 14 aortas were harvested and stained for $CD3^+$ T cells (arrows, green) and IFNγ (red). Co-localization appeared yellow. (**D**) Cellular level of IFNγ in $CD3^+$ cells was analyzed using ImageJ and presented as integrated optical density (IntDen), which was normalized to the intensity of WT sections that was set at 100%. (**E**) IFNAR-1 blockade also inhibited gelatinase activity, as detected by *in situ* zymography. (**B**, **D**, **E**) N = 5-6 aortas per treatment, 6-9 cross sections per aorta. EF, elastic fibers. Scale bars = 100 μm (**B, E**); 25 μm (**C**)

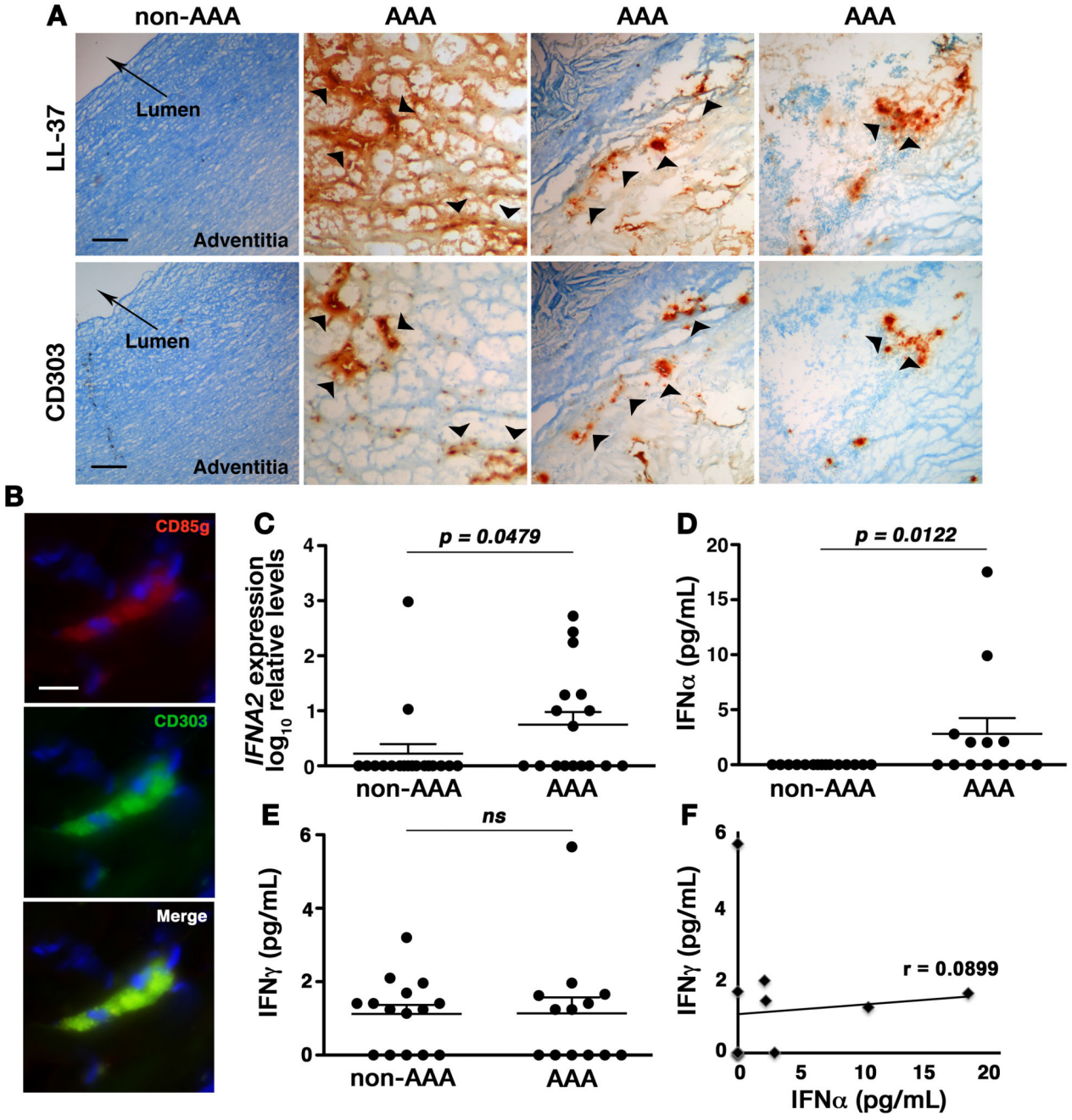

Figure 6. pDCs and type I IFNs are detected in individuals with AAA

(**A**) Non-AAA and AAA tissues (n = 3) were examined for LL-37 and CD303 in consecutive sections. LL-37 localized to the vicinity of $CD303^+$ cells (arrowheads). (**B**) $CD303^+$ cells (green) co-localized with CD85g (red), confirming their identity as pDCs. (**C**) Expression of *IFNA2* in human aortic tissues was detected by real time PCR (non-AAA = 17; AAA = 18). IFNα (**D**) and IFNγ (**E**) levels in serum were determined by Cytokine Bead Array assay

(non-AAA = 15; AAA = 13). (**F**) There was low correlation between IFNα and IFNγ levels (r = 0.0899). Scale bars = 250 μm (**A**); 10 μm (**B**)