Materials and Methods

Animals. WT C57BL/6J mice were obtained from The Jackson Laboratory. DPPI-, NE-, NEPR3, NECGPR3-deficient mice were generated as previously described[1-3] and backcrossed to C57BL/6J (The Jackson Laboratory) for more than 10 generations. Microsatellite genotyping showed that DPPI-deficient mice were 99.2% congenic with C57BL/6J, NE- and NEPR3-deficient mice were 100% congenic with C57BL/6J; NECGPR3-deficient mice were generated by intercrossing CG-deficient mice with NEPR3-deficient mice after verifying that CG-deficient mice were 100% congenic with C57BL/6J by microsatellite genotyping. All WT and protease-deficient mice were kept in standard pathogen-free environment and have access to standard mouse chow and water ad libitum (but no antibiotics at any point in their lifetime). They appear healthy, well groomed and have normal life expectancy and fertility. All animal experiments were performed in compliance with federal laws and in strict accordance with the guidelines established by the Division of Comparative Medicine at Washington University in St. Louis. The animal protocol is subjected to annual review and approval by The Animal Studies Committee of Washington University.

Human tissues. Human abdominal aorta tissue specimens were obtained from consented patients at time of elective surgery through a protocol approved by the Institutional Review Board (IRB) at Washington University School of Medicine. Sera were obtained from consented individuals through a protocol approved by the IRB at Washington University School of Medicine. The excess human tissues used in these studies were provided anonymously by Dr. Robert Thompson (Washington University School of Medicine).

Murine elastase-induced AAA disease model. AAA was induced as previously described.[4] AAA is defined as an overall increase in aortic diameter (AD) greater than 100% compared to pre-elastase perfusion. In adoptive transfer studies, purified bone marrow derived neutrophils (10e7) were injected i.v. immediately after elastase perfusion and on day 1 post surgery according to previously published protocol.[4] Some mice were injected with DNase1 (20 U i.v. and 100 U i.p.) twice a day from days 0-5 or days 4-9. Control group received heat inactivated DNase1 as the same dose and schedule. To deplete plasmacytoid dendritic cells (pDCs), mice were injected i.p. with a pDC depletion antibody (0.5 mg/mouse, PDCA-1 clone # JF05-IC2.41, Miltenyi Biotec) on day -2 and day -1 prior to elastase perfusion. To block the *in vivo* activity of type I interferons (IFNs), mice were injected i.p. with a monoclonal antibody specific for the IFNα receptor 1 (IFNAR-1) (0.25 mg/mouse, MAR1-5A3, generously provided by Dr. Kathleen Sheehan, Washington University School of Medicine) immediately after elastase perfusion and again on days 2 and 4. Mouse IgG1, κ and rat IgG2b served as isotype controls for anti-IFNAR-1 and anti-PDCA-1, respectively.

Generation of DNA-CRAMP complexes. Genomic DNA was isolated from the bone marrow of male WT C57BL/6 mice. Cells were lysed with lysis buffer containing 1mol/L Tris buffer (pH 8.0), 0.25M EDTA, 4 mol/L sodium chloride, 1% Triton X-100 and 2 μg/mL of proteinase K). Cell lysate was extracted with phenol:chloroform and genomic DNA was precipitated with 100% ethanol. The pellet was washed in 70% ethanol, resuspended in PBS and treated with RNase followed by another round of phenol:chloroform extraction and ethanol precipitation. CRAMP was purchased from Bachem (Torrance, CA). Scrambled CRAMP (sCRAMP) was purchased from Innovagen (Lund, Sweden). To generate genomic DNA-CRAMP complexes, 40 μg of DNA was mixed with 200 μg of CRAMP (or sCRAMP) and the mixture was incubated on ice for 1 h and injected i.p. immediately after elastase perfusion and closure of laparotomy. Control mice were injected with either 40 μg of genomic DNA or 200 μg of CRAMP.

Immunohistochemistry. Verhoeff–van Gieson (VVG) staining and elastin degradation scoring system were performed on frozen cross-sections of mouse aortas using a scale of 1-4 as previously described: 1, < 25% degradation; 2, 25–50% degradation; 3, 50–75% degradation; and 4, > 75% degradation.[5] Macrophages, $CD3^+$ T cells and pDCs were stained with a primary biotinylated anti–Mac-3 antibody (1:200 dilution; Cat # CL8943B; Cedarlane Laboratories), biotinylated anti-CD3 antibody (1:100 dilution; Cat # 553060; BD Pharmingen Biosciences) and anti-Siglec-H antibody (1:100 dilution; Cat # MCA4647GA; AbD Serotec). Human AAA tissues were embedded in OCT compound, sectioned at 9 μm and fixed in 4% paraformaldehyde. After blocking, slides were incubated with an antibody to LL-37 (1:200 dilution; Cat # SC-166770; Santa Cruz Biotechnology) or CD303 (1:200 dilution; Cat # 130-097-923; Miltenyi Biotec). IHC was performed in consecutive sections to compare the staining pattern between LL-37 and CD303. All sections were counterstained with 1% methyl green. All images were acquired with QCapture software on a Nikon Eclipse microscope. The positive cells were quantified using ImageJ software (http://rsb.info.nih.gov/ij) at low magnification. Data presented were derived from 6-9 cross sections that spanned the entire abdominal aorta, with 4-6 aortas per genotype or treatment.

Immunofluorescence. Immunofluorescence was performed on frozen cross-sections of tissues collected from day 2 or day 14 mouse aortas and human AAA tissues as previously described.[6] Briefly, cross sections of aortic tissues (9μm) were fixed in 4% paraformaldehyde, blocked in 8% BSA in PBS and incubated with the primary antibodies: biotinylated anti-mouse CD3 (1:100 dilution; Cat # 553060; BD Pharmingen Biosciences), anti-mouse IFNγ (1:100 dilution; Cat # 559065 BD Pharmingen Biosciences), anti-Histone H2B (1:100 dilution; Cat # SC-8651; Santa Cruz Biotechnology), anti-mouse MPO (1:100 dilution; Cat # HM1051BT; Hycult Biotech), anti-CRAMP (1:100 dilution; Cat # SC-21579; Santa Cruz Biotechnology); anti-mouse Siglec-H (1:100 dilution; Cat # MCA4647GA; AbD Serotec); CD303 (1:200 dilution; Cat # 130-097-923; Miltenyi Biotec), anti-CD85g (1:100, Cat # GTX119457; GeneTex, Inc.) followed by the appropriate rhodamine red- or FITC-conjugated secondary antibody (1:100-1:200; Jackson ImmunoResearch Laboratories). Nuclei were counterstained with DAPI.

Flow cytometric analysis of pDCs. Aortas from elastase-perfused mice were harvested on day 14 and digested with collagenase IV (291 U/ml, Worthington Biochemical Corporation) and dispase (0.625 U/ml, BD Biosciences) at 37°C for 1 h. The cells were passed through a 70μm cell strainer to remove debris and single cell suspensions were analyzed by flow cytometry. For pDC confirmation, $B220^+Siglec\text{-}H^+$ cells that expressed low to moderate level of CD11c and high level of PDCA1 were identified as pDCs. For quantitative analysis of pDCs, cells obtained from 3 aortas per treatment condition were pooled and stained with the following surface markers: B220, CD11c, CD45, Siglec-H and analyzed by flow cytometry. The number (events) of $CD45_+$ leukocytes was enumerated by flow cytometry. $CD45^+$ cells that expressed mid-low CD11c, B220 and Siglec-H were identified as pDCs. The absolute number of pDCs was calculated using the following equation: total number of $CD45^+$ events x % of $CD45^+CD11c^{lo}B220^+Siglec\text{-}H^+$ pDCs (expressed as a decimal) ÷ 3. Single cell suspensions from mice injected with isotype control or anti-PDCA1 mAb on days -1 and -2 (N = 3 mice per treatment group) were also prepared from various organs (spleen, lymph nodes, bone marrow, peripheral blood) on day 1 post elastase perfusion. Cells were stained with various surface markers including: CD19-FITC (Cat #09654, BD PharMingen), CD4-PE (Cat # 3100408, BioLegend), CD8α-PerCP (Cat # 100731, BioLegend), Gr1-FITC (Cat # 553127, BD Pharmingen), F4/80-PerCP (Cat # 123126, BioLegend), CD11b-APC (Cat # 17-0112-82, eBioscience), CD11b-AlexaFluor 488 (Cat # 101217, BioLegend), mSIGLEC-H (Cat # MCA4647GA, AbD Serotec), CD11c-PE (Cat # 553802, BD Pharmingen), CD317 (PDCA1)-PerCP (Cat # 46-3172-80, eBioscience),

CD45R/B220-APC (Cat # 553092, BD Pharmingen) and analyzed by flow cytometry. Mϕ/MON: macrophages/monocytes that are F4/80$^+$, CD11b (high), and CD11c-

*IFNγ immunofluorescence image analysis***.** Cross sections of aortic tissues (9μm) were fixed in 4% paraformaldehyde, blocked in 8% BSA in PBS and incubated with a biotinylated anti-CD3 antibody (1:100 dilution; Cat # 553060; BD Pharmingen Biosciences) followed by FITC-conjugated streptavidin and an anti-mouse IFNγ (1:100 dilution; Cat # 559065 BD Pharmingen Biosciences) followed by rhodamine-conjugated anti-rat secondary antibody. All images were visualized on a Nikon Eclipse fluorescence microscope and acquired with QCapture software using the same exposure time. Merged and single color images were loaded into ImageJ software (http://rsb.info.nih.gov/ij) for analysis. Threshold color of all images was set to the same hue, saturation and brightness. Using the brightness to filter the picture, positively stained areas were isolated by increasing the contrast between the color and background. This facilitates the selection of regions of interest (ROIs) by allowing ROIs to be selected easily with the wand tool. Double positive (CD3$^+$IFNγ$^+$) cells were selected. The ROIs were measured on the unfiltered images, normalized to the positive area and presented as integrated optical density (IntDen) and presented as percentage of normalized WT activity in aorta cross-sections, which was set at 100%. Data were obtained from 6-8 fields per section and 6-9 sections per aorta, 4-5 aortas per treatment.

In situ zymography. Gelatinase activity was measured using fluorescein conjugated gelatin as a substrate.[6] In brief, 9 μm unfixed frozen sections were incubated in DQ Gelatin (0.1 mg/mL; Cat # 12054; Invitrogen Molecular Probes) for 3 h at room temperature. For negative control, slides were incubated in the presence of 25 mmol/L EDTA. All zymographic images were visualized on a Nikon Eclipse fluorescence microscope using identical shutter conditions and acquired with QCapture software. The images were analyzed by ImageJ software, and gelatinase activity was quantified as mean integrated density and presented as percentage of normalized WT activity in aorta cross-sections, which was set at 100%. Data were calculated from 6-9 cross-sections per aorta, with 4-5 aortas per treatment.

Real time PCR. Total RNA was purified from human aorta homogenates using RNeasy Midi Kit (Qiagen). cDNA was generated with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per manufacturer's protocol. Target mRNA was quantified by real time PCR assay with TaqMan Gene Expression Master mix (Applied Biosystems) and TaqMan primers and probes for human *GAPDH* (Hs02758991_g1) (Applied Biosystems) and human *IFNA2* (Hs00265051_s1) (Applied Biosystems). Samples were assayed with the 7500 Real-Time PCR System and analyzed using System Software (Applied Biosystems). mRNA expression of target gene was normalized to *GAPDH* expression and expressed as fold differences relative to the lowest positive *IFNA2* signal, which was set at 1.

In vitro NET stimulation. Neutrophils were isolated from bone marrow as previously described.[7] Isolated neutrophils were seeded on Thermanox plastic coverslips (Cat # 174950, Thermo Fisher Scientific Inc.) or 5 mm round glass coverslips (Cat # 101413-528, VWR), placed in 24 well plates (75,000 cells/well) and incubated for 1h at 37ºC to allow adherence to coverslips. The following activating agents were used: LPS (10 mg/L, Cat # L2762, Sigma-Aldrich) or C5a (100 mg/L, Cat # 2150-C5-025/CF, R&D Systems Inc.). After 30 min of stimulation, cells were fixed with 4% paraformaldehyde in PBS overnight and the DNA was stained with Sytox green (Cat # S7020, Invitrogen). NETs were visualized on a Nikon Eclipse fluorescence microscope and low magnification images (40x) were acquired with QCapture software on non-overlapping

random images (7-11 separate fields per coverslips, derived from 3 wells per condition and genotype). NETs were manually identified on acquired images as Sytox-positive structures emanating from neutrophils with an overall length at least twice as long as the cell diameter,[8] and expressed as percentage of neutrophils with released DNA. Each experiment was repeated at least 3 times.

Cytokine analysis. Cytokine concentrations in human serum samples were measured using the Cytometric Bead Arrays for human IFNα (Cat # 560379, BD Biosciences**)** and human IFNγ (Cat # 560111, BD Biosciences) according to the manufacturer's protocols.

Statistical analysis. Comparisons between multiple groups (≥ 3) were performed by one-way ANOVA. Equality of variance assumption was tested and Bonferroni's correction for multiple comparisons was performed. The sample size (number of animals per genotype/treatment) chosen is based on means and variances in similar experiments in this mouse model of AAA for detection of differences between experimental groups at an alpha level of 0.05 and a statistical power of 0.80, assuming a 2-sided test. F test was used to compare variances within each group of data and the difference in variances was found to be not significant between groups. For the analysis of human *IFNA2* mRNA levels in aortic tissues and IFNα levels in human sera, the results were categorized as positive (detectable) or negative (not detectable) according to the respective methods of detection. The categorical data were analyzed by chi square test.

References

1. Pham CT and Ley TJ. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. *Proc Natl Acad Sci U S A*. 1999;96:8627-32.
2. Belaaouaj A, McCarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN and Shapiro SD. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med*. 1998;4:615-8.
3. Schreiber A, Pham CT, Hu Y, Schneider W, Luft FC and Kettritz R. Neutrophil Serine Proteases Promote IL-1beta Generation and Injury in Necrotizing Crescentic Glomerulonephritis. *J Am Soc Nephrol*. 2012;23:470-82.
4. Pagano MB, Bartoli MA, Ennis TL, Mao D, Simmons PM, Thompson RW and 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.
5. Zhou HF, Yan H, Bertram P, Hu Y, Springer LE, Thompson RW, Curci JA, Hourcade DE and Pham CT. Fibrinogen-specific antibody induces abdominal aortic aneurysm in mice through complement lectin pathway activation. *Proc Natl Acad Sci U S A*. 2013;110:E4335-44.
6. Zhou HF, Yan H, Cannon JL, Springer LE, Green JM and Pham CT. CD43-Mediated IFN-gamma Production by CD8+ T Cells Promotes Abdominal Aortic Aneurysm in Mice. *J Immunol*. 2013;190:5078-85.
7. Adkison AM, Raptis SZ, Kelley DG and 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.
8. Farley K, Stolley JM, Zhao P, Cooley J and Remold-O'Donnell E. A serpinB1 regulatory mechanism is essential for restricting neutrophil extracellular trap generation. *J Immunol*. 2012;189:4574-81.