SUPPLEMENTAL MATERIAL

Materials and Methods

Mice

Seven weeks old male C57BL/6 (stock # 000664) and apolipoprotein A knockout mice (stock # 002052) mice were obtained from The Jackson Laboratory (US). All mice were given water and a minimal phytoestrogen diet[1] (2016 Teklad Diet; Harlan Laboratories, Indianapolis, IN) *ad libitum*. The mice were used for various studies at 8 weeks of age. All protocols were approved by University of Virginia Animal Care and Use Committee.

B cell depletion and experimental model of mouse AAA

The B cell depleting antibody clone 18B12 (IgG2a isotype) and the isotype control antibody (clone 2B8) are obtained from Biogen Idec, Inc, (Cambridge, MA). The antibodies were diluted in PBS at 1 mg/ml concentration and 250 µl of the diluted antibody was injected to the mice intraperitoneally (IP) or intravenously (IV). Seven days following antibody treatment, the mice were sacrificed and B cell depletion in various tissues was examined using flow cytometry. To find out the effect of B cell depletion on AAA formation, control or anti-CD20 antibodies were injected IP or IV at seven days before and seven days after the induction of AAA using the elastase perfusion model. Similarly, in the AngII model, mice were injected with control or anti-CD20 antibodies via IV at 7 days before the osmotic pump implantation, and 7 and 21 days after the pump implantation.

Elastase perfusion model of murine AAA:

The elastase perfusion model was performed as described by Johnston et al.[2] Following adequate anesthesia with inhaled isoflurane, a midline laparotomy was performed with reflection of the peritoneal contents superiorly. The abdominal aorta was isolated from the level of the left renal vein to the iliac artery bifurcation. Once the lateral tissue was dissected from the aorta branches were cauterized or ligated with 10-0 nylon suture. The maximal aortic diameter was measured *via* video micrometry (Leica Microsystems, Heerbrugg, Switzerland). The aorta was occluded with a 5-0 silk suture distally followed by proximal occlusion below the level of the renal veins. An aortotomy was made with a 30-gauge needle, using external compression the residual blood was evacuated from the aorta via the aortotomy. The aorta was cannulated with 0.033" polyethylene catheter (Braintree Scientific, Braintree, MA) and perfused with purified porcine elastase solution (Sigma-Aldrich, St. Louis, MO) diluted in 0.9% sodium chloride for 5 minutes. An infusion pump (Kent Scientific, Torrington, CT) was utilized to provide uniform flow delivery. Aortic turgor and aortic wall digestion were evaluated *via* visual inspection and ballotability. Following perfusion, the catheter was removed, the elastase solution was evacuated, and the aortotomy was closed with a single simple stitch using 10-0 nylon suture. The aortic ligation sutures were then untied. Typically, after the perfusion, we record about 50% expansion in aorta diameter. The distal extremities were inspected for adequate perfusion and the aorta was examined for the presence of thrombus. The peritoneal contents were returned to their anatomical position and the skin was closed in two layers: a running 6-0 polyglactin for the abdominal fascia followed by interrupted 6-0 polypropylene sutures for skin closure. Mice received buprenorphine IP for analgesia.

At the time of harvest (14 days of elastase perfusion), mice were anesthetized with inhaled isoflurane, and a midline laparotomy was made with dissection of the infrarenal abdominal aorta from the level of the left renal vein to the iliac bifurcation. Once dissected, the aorta was photographed, and the maximal aortic diameter was measured with video micrometry (Leica Microsystems, Heerbrugg, Switzerland). Blood was collected with a 25g needle from the inferior vena cava. The aorta was then harvested from below the level of the left renal vein to

the aortotomy closure for tissue analysis. Generally, at the time of harvest, any extra connective tissue present was removed from the abdominal aorta, and the elastase perfused segment of aorta was harvested excluding the sites of temporary ligatures and aortotomy. For flow cytometry and gene expression analysis, care was taken not to include any small lymph nodes present near to the aorta.

AngII infusion-induced AAA:
Calculation and dissolution of AngII, osmotic pump filling and AngII infusion-induced AAA was performed as described by Daugherty and Lu et al[3, 4]. In this well-established model of experimental aneurysm induction, 8 weeks old male ApoE KO were infused with AngII (1000 ng/kg/min) *via* Alzet® osmotic pumps (Model 2004) for 4 weeks. Control animals were undergone sham surgery and placement of saline carrying osmotic pumps. Implantation was completed following injection of intraperitoneal anesthesia (ketamine (60 mg/kg)/xylazine (8 mg/kg)), followed by removing hair nape of the animal's neck, disinfecting and drying, making a small incision, minimal tunneling posteriorly to accommodate osmotic pumps. All incisions were undergone staple closer, and the animals were allowed to recover for 4 weeks. Following the end of the study period, animals were sacrificed with CO2 inhalation. Next, a small cut was made in the right atrium and the mice were perfused with 50 ml of 1x PBS solution containing 5 mM EDTA through the left ventricle. Subsequently, the whole aorta was excised from the root to the iliac bifurcation, fixed in 4% paraformaldehyde and imaged to study of abdominal aortic aneurysms. In this study, three groups of mice were used: (1) Control antibody treated and infused with saline, n= 5, (2) Control antibody treated and infused with AngII, n=6 and (3) Anti-CD20 antibody treated and infused with AngII, n=5.

Immunohistochemistry
The aortas were perfused with saline before harvesting, and stored in Zinc Formalin for 24 hours, followed by storing in 70% Ethanol for another 24 hours. Subsequently, the aortic tissues were imbedded in paraffin and 10 µm sections were cut. Verhoeff-Van Gieson (VVG) staining on aortic sections was performed to stain the elastin layers. For immunohistochemistry, following antibodies were used: F4/80 Antibody (clone Cl:A3-1, AbD serotec, Raleigh, NC); rat anti-mouse Ly-6B.2 antibody (clone 7/4, AbD serotec, Raleigh, NC); Rat anti-mouse CD45R (clone RA3-6B2, BD Pharmingen, San Jose, CA); anti-CD3 antibody (sc-1127, Santa Cruz Biotechnology, Inc., Dallas, TX); Anti-FOXP3 antibody (ab54501, Abcam) and CD20 antibody (sc-7735, Santa Cruz Biotechnology, Inc.). Whole molecule control primary antibodies of Rat IgG (31933), Goat IgG (31245) and Rabbit IgG (31235) were from ThermoFisher Scientific (Waltham, MA).

Multiple antigen labeling was performed using ImmPACT DAB Peroxidase (HRP) Substrate, (SK-4105) and ImmPACT SG Peroxidase (HRP) Substrate (SK-4705) obtained from Vector Laboratories, Burlingame, CA. Briefly, after antigen retrieval using Citric Acid based antigen unmasking solution (Vector Laboratories, Burlingame, CA), aortic sections were blocked using Donkey serum and Avidin-Biotin kit (SP-2001, Vector Laboratories), primary antibody was applied and incubated overnight at 4 ºC. After washing the primary antibody, biotinylated secondary antibody was applied and incubated at room temperature for 1 hour, then washed and incubated with diluted ABC (PK-6100, Vector Laboratories). After washing again, the sections were stained with ImmPACT DAB for 10 to 15 minutes and excess DAB was removed by washing the sections in water. Subsequently, the sections were blocked and stained with second primary antibody similar to the first one. Finally, the sections were mounted on VectaMount AQ Aqueous Mounting Medium (H-5501, Vector Laboratories) and images were acquired on Nikon Eclipse Ti –U inverted microscope and NIS-Elements Br microscope imaging software. Immunofluorescence staining of aortic sections was performed using standard protocol. The stained sections were mounted using VECTASHIELD Mounting Medium with

DAPI (H-1200, Vector Laboratories) and confocal images were acquired on Zeiss LSM 700 using 405, 488 and 633 nm lasers and ZEN software.

Quantification plasma IgM and IgG

Plasma samples from WT mice were collected 14 days after inducing AAA via elastase perfusion. Total IgM and IgG levels in plasma collected from mice were quantified using Mouse IgM ELISA Ready-SET-Go (Cat# 88-50470-22) and Mouse IgG total ELISA Ready-SET-Go (Cat# 88-50400-22) from eBioscience (San Diego, CA), respectively.

Quantification of plasma cytokines and chemokines

Plasma samples from WT mice were collected 14 days after inducing AAA via elastase perfusion. MILLIPLEX® Multiplex Assay kit for quantification of mouse plasma IL-2, IL-4, IL-5, IL-10, IL-17, IL12-p70, IFNγ, IL-1β, IL-6, TNFα, RANTES, MIP-1α, MIP-1β, IP-10, IL-13, Eotaxin, KC, MIP-1, MIP-2 and MIG were obtained from EMD Millipore (Massachusetts, US) and run on a Luminex 100 IS System as described by the manufacturer.

Flow cytometry

The aortic tissues were harvested at 14 days following elastase perfusion, digested with an enzymatic cocktail and stained with fluorescent dye conjugated antibodies as described before by Meher et al.[5] Briefly, elastase perfused aortas were carefully collected from mice under a dissection microscope. Extra connective tissues were removed from the abdominal aorta, and the elastase perfused segment of aorta was harvested excluding the sites of temporary ligatures and aortotomy. Care was taken not to include any small lymph nodes present near to the aorta. The isolated aortas were individually digested with an enzymatic cocktail of collagenase type I (1000 U/ml), collagenase type XI (400 U/ml), Hyaluronidase type I-s (125 U/ml) and DNase (60 U/ml) and stained with flow antibodies. Thus, one aorta constitute one flow sample. Following antibodies were used: Purified anti-mouse CD16/32 (clone 93, Biolegend), PE/Cy7 anti-mouse CD3ε (clone 145-2C11, Biolegend), Brilliant Violet 785™ anti-mouse CD4 Antibody (cloneRM4-5, Biolegend), PE rat anti-mouse Foxp3 (clone MF23, BD Pharmingen), APC/Cy7 anti-mouse CD45 (clone 30-F11, Biolegend), Alexa Fluor 488 anti-mouse/human CD45R/B220 (clone RA3-6B2, Biolegend), PerCP/Cy5.5 anti-mouse CD19 (clone 6D5, Biolegend), Alexa Fluor 647 anti-mouse CD5, clone 53-7.3, Biolegend), PE/Cy7 anti-mouse IgM Antibody (clone RMM-1, Biolegend), Brilliant Violet 650™ anti-mouse IgD Antibody (clone 11-26c.2a, Biolegend), Brilliant Violet 650™ anti-mouse CD317 (BST2, PDCA-1) Antibody (clone 927, Biolegend), Alexa Fluor® 647 anti-IDO1 Antibody and clone 2E2/IDO1, Biolegend). Dead cells were discriminated using LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Cat# L34955, Invitrogen). After staining cell suspensions with live/dead stain and cell surface antibodies, the cells were fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Cat# 554715, BD Biosciences, San Jose, CA) or fixed with 4% paraformaldehyde for 15 min at room temperature, washed with FACS buffer (PBS containing 2% FBS and 0.05% Sodium Azide) and permeabilized using BD Permeabilization Solution. The cells were stained with intracellular antibodies before adding CountBright Absolute Counting Beads (Molecular Probes) and running on the 16 color Flow cytometer machine Becton Dickinson LSRFortessa (equipped with laser lines 488nm, 405nm, 561nm, and 640nm) located in the Flow Cytometry Core Facility at University of Virginia. Fluorescent minus one (FMO) controls were used for each antibody in the experiment. Since the number of CD45+ lymphocyte is quite variable among the groups, while acquiring events in flow cytometer, we acquired 50,000 live cells. For counting of cells, 5100 CountBright absolute counting beads were added to 400 µl of cell suspension, and identified in Forward and Side-scatter gating.

Measurement of tryptophan and kynurenine *via* Mass Spectrometry

Plasma Tryptophan (Trp) and Kynurenine (Kyn) were quantified as described before[6]. Fifty microliters of water and 20 µl of d5-Tryptophan (d5-Trp, internal standard) were added to 50 µl of mouse plasma. The plasma proteins were removed by precipitation with 25 µl of 5-Sulfosalicylic acid and centrifugation at 17,000 x g for 5 min. The resulting clear supernatant was used for mass spectrometry analysis. Trp, Kyn and d5-Trp were profiled in positive ion mode a Prominence UFLC Liquid Chromatography System (Shimadzu) coupled to a 4000 QTRAP Mass Spectrometrer (AB Sciex) fitted with an electrospray ionization source. The ion spray voltage was set to 5.5kJ and the source temperature was set to 100°C. The mass spectrometer was tuned for each individual analyte for optimal detection, and establishment of multiple reaction monitoring (MRM). The first quadrupole selected the protonated ions at mass to charge ratios (m/z): 205.082 (Trp), 209.101 (Kyn), and 210.089 (d5-Trp). Nitrogen was used as the standard collision gas to produce the highest intensity fragments selected by the third quadrupole (m/z): 188.1 (Trp), 191.0 (Kyn), and 150.1 (d5-Trp). Standard curves of Trp, Kyn, and d5-Trp were created over the range of 10nM-100uM using dilutions of each of the purified compounds (Sigma). The column used to separate the metabolites was a 50 x 2.1mm 5µm Discovery C18 (Supelco). The column was loaded and eluted with a binary solvent system comprised of mobile phase A (5mM ammonium formate in water, pH 3.5) and mobile phase B (100% acetonitrile). The mobile phase was pumped at 0.2 mL/min. The loading and elution of the column started with a linear gradient of 5% solvent B to 80% B over 2.5 minutes. 80% solvent B was held for 1.5 minutes, followed by a 10 second linear gradient to 5% solvent B, where it held for another 2 minutes to the end of the program. Analyst software (version 1.6, AB Sciex) was used to process the raw LC-MS data and calculate the peak integration of each highest intensity ion.

Real-time RT PCR

The aortas were harvested as described in 'Flow cytometry'. After harvest, aortas were perfused with PBS-EDTA and frozen in liquid nitrogen and stored in -80 ºC. At the time of preparation, the aortas were grinded using a sterile mortar pestle and transferred to TRIzol Reagent (Cat# 15596-026, ThermoFisher Scientific, Grand Island, NY). Subsequently, cDNA was synthesized and gene expression was quantified as described previously by Meher et al [7, 8].

Coculture of pDC and B cell

pDCs and B cells were isolated from spleens of 8 to 10 weeks old male C57BL/6 mice using MACS column and Plasmacytoid Dendritic Cell Isolation Kit (Cat. No. 130-107-093) and Pan B Cell Isolation Kit (Cat. No. 130-095-813) from Miltenyi Biotech (San Diego, CA). ODN 1826 and Co-ODN 1826 were obtained from Invivogen (San Diego, CA). Soon after isolation, pDCs were cultured for 2 days in with 0.2 µM of ODN 1826 or co-ODN 1826 in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 1x Antibiotic-Antimycotic (Cat. No. 15240062), 1x MEM Non-Essential Amino Acids Solution (Cat. No. 11140050), 1x Sodium Pyruvate (Cat. No. 11360070) and 55 µM 2-Mercaptoethanol (Cat. No. 21985023) from ThermoFisher Scientific (Waltham, MA) at 37 ºC in a humidified incubator supplied with 5% CO2. After 2 days of incubation, the culture medium was completely removed and splenic B cells were added at various concentrations. The coculture was continued for 1 days, and thereafter, the cells were collected, washed in PBS, stained with fixable live/dead marker and stained for CD317, B220, CD19 and IDO, run on a flow cytometer and analyzed as described in 'Flow cytometry' section of the Supplemental material. Separately, ODN 1826 stimulated mouse splenocytes were used to prepare FMO controls such as CD317 FMO, B220 FMO, CD19 FMO and IDO FMO to develop gates for flow data analysis.

Statistical analysis

The programs GraphPad Prism 5 and Excel were used for data analysis and preparing graphs. Two groups are compared using t-test, whereas, multiple groups were compared using one-way ANOVA and multiple t-tests were used to determine the significant difference in aneurysm pathology between two groups. Typically, before determining significant differences between or among the groups, column statistics was applied on the data points of each group in the GraphPad Prism. As recommended by Prism, D'Agostino-Pearson normality test was performed. If the P value is not significant (>0.05), a parametric test (unpaired t-test assuming both populations have the same SD) was applied to determine significant differences between the groups. If the P value is significant (<0.05), non-parametric t-test (Mann-Whitney test) was applied. In the multiple comparisons, if a significant difference was found among the groups, pair of groups compared using a parametric or non-parametric t-test which was again based on the values obtained from the normality test and indicated in figure legends. Statistical analyses are provided in each figure legend. Differences between the groups were considered significant when p-value is <0.05. p-values >0.05 were indicated in the graphs.

References

1. Lu G, Su G, Zhao Y, Johnston WF, Sherman NE, Rissman EF, Lau C, Ailawadi G, Upchurch GR, Jr. Dietary phytoestrogens inhibit experimental aneurysm formation in male mice. J Surg Res. 2014;188:326-338
2. Johnston WF, Salmon M, Su G, Lu G, Stone ML, Zhao Y, Owens GK, Upchurch GR, Jr., Ailawadi G. Genetic and pharmacologic disruption of interleukin-1beta signaling inhibits experimental aortic aneurysm formation. Arterioscler Thromb Vasc Biol. 2013;33:294-304
3. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest. 2000;105:1605-1612
4. Lu H, Howatt DA, Balakrishnan A, Moorleghen JJ, Rateri DL, Cassis LA, Daugherty A. Subcutaneous Angiotensin II Infusion using Osmotic Pumps Induces Aortic Aneurysms in Mice. J Vis Exp. 2015
5. Meher AK, Johnston WF, Lu G, Pope NH, Bhamidipati CM, Harmon DB, Su G, Zhao Y, McNamara CA, Upchurch GR, Jr., Ailawadi G. B2 cells suppress experimental abdominal aortic aneurysms. Am J Pathol. 2014;184:3130-3141
6. Forteschi M, Sotgia S, Assaretti S, Arru D, Cambedda D, Sotgiu E, Zinellu A, Carru C. Simultaneous determination of aromatic amino acids in human blood plasma by capillary electrophoresis with UV-absorption detection. J Sep Sci. 2015;38:1794-1799
7. Meher AK, Sharma PR, Lira VA, Yamamoto M, Kensler TW, Yan Z, Leitinger N. Nrf2 deficiency in myeloid cells is not sufficient to protect mice from high-fat diet-induced adipose tissue inflammation and insulin resistance. Free Radic Biol Med. 2012;52:1708-1715
8. Kadl A, Meher AK, Sharma PR, Lee MY, Doran AC, Johnstone SR, Elliott MR, Gruber F, Han J, Chen W, Kensler T, Ravichandran KS, Isakson BE, Wamhoff BR, Leitinger N. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. Circ Res. 2010;107:737-746