Online-Only Materials and Methods

Animal care and use

C57Bl/6 mice (stock# 000664) and mT/mG Cre-reporter mice on C57Bl/6 background (stock# 007676) were purchased from The Jackson Laboratory (Bar Harbor, ME). Col1α2-CreER^T mice (backcrossed into C57Bl/6 background) were a gift from Dr. Arjun Deb (University of North Carolina, Chapel Hill). This transgenic mouse was originally created by Zheng B. and colleagues at University of Texas M.D. Anderson Cancer Center. [1] RelA-floxed (RelA f/f) mice containing loxP sites in intron-4 and -8 of the *RelA* gene were generated in our laboratory [2] and backcrossed 8 times into C57Bl/6 background. Briefly, a gene targeting vector containing loxP sites and a neomycin positive-selection cassette was electroporated into 129B6F1 mouse embryonic stem cells. After drug selection, cell colonies that survived were screened for homologous recombination at the *RelA* locus. Clones that had incorporated the loxP sequences were then injected into C57Bl/6 blastocysts. The resultant chimeras were crossed with FLPeR mice to remove the neomycin cassette from the mutant *RelA* allele. Two clones were generated, of which one was backcrossed with C57Bl/6 mice to segregate the FLP1 transgene and to establish the RelA-flox line. The RelA f/f mice developed normally, were fertile and expressed RelA at a level similar to wild-type mice.

To characterize the specificity of CreER^T recombinase activity in Col1α2-CreER^T mice, mT/mG mice were mated with a Col1α2-CreER^T transgenic. The resultant progeny, mT/mG; Col1α2-CreER^T- and mT/mG; Col1α2-CreER^T+, was administered 1 mg tamoxifen per day for 10 consecutive days. Tissues were isolated two weeks after the last injection for identification of cells that underwent Cre-mediated recombination.

For hypothesis testing, RelA f/f mice were mated with Col1α2-CreER^T mice to generate RelA f/f; Col1α2-CreER^T– and RelA f/f; Col1α2-CreER^T+ transgenic animals. Tamoxifen (1 mg/day) was administered to both genotypes via i.p. injections for 10 consecutive days. Mice harboring Col1α2-CreER^T were then classified as RelA conditional knockout (RelA-CKO) while those without Col1α2-CreER^T were considered as RelA wild-type (RelA-WT). RelA-WT and RelA-CKO mice were infused with saline (sham) or angiotensin II (Ang II) 3 weeks after the last tamoxifen injection or their aortas were isolated for characterization by flow cytometry and qRT-PCR.

Tamoxifen (Sigma) was dissolved in 10 % ethanol and 90 % corn oil to make a 10 mg/ml stock solution for administration via i.p. injections. Both male and female mice were utilized in the experiments. Mice were 3-4 weeks old at the start of tamoxifen treatment and 9-12 weeks old at the time the Ang II infusion experiments were performed.

Ang II was delivered at a rate of 2,500 ng/kg/min for 7 days via subcutaneous Alzet osmotic minipump (Durect Corporation) while saline-infused animals were used as controls. Ang II was synthesized by University of Texas Medical Branch (UTMB) Peptide Synthesis Core or purchased from Sigma. All animal experiments performed as part of this study were approved by the UTMB Institutional Animal Care and Use Committee.

Mouse genotyping

The genotype of the transgenic mice was determined by PCR. DNA was isolated from tail snips using phenol:chloroform extraction and ethanol precipitation. Col1α2-CreERT genotyping was performed using the following primers: 5'- GCGGTCTGGCAGTAAAAACTATC-3' and 5'-GTGAAACAGCATTGCTGTC ACTT- 3'. The resultant product of 540 bp appeared only in

CreER^{T}+ samples. PCR reaction for detection of RelA-flox allele was performed with the following primers: **RelAFlx F**, 5'-TGCAAACAGACCTCCTTTGT CTTGA- 3', and **RelAFlx R**, 5'-TCCTGAGACCAGACTCCTCCTCC- 3'. These primers provided a 450 bp product if the RelA-flox allele was present or 270 bp product if a WT allele was present. To detect the present of the RelA KO allele, following primers were used: **5-8Δ F**, 5'- GCCGGCCAGGCAGCTCTTAC- 3', and **5-8Δ R**, 5'- GGCCAGTCACCATGGCCAGC- 3'. A 335 bp product appeared only when RelA-flox allele had underwent recombination. In addition, to detect the WT *RelA* alleles, following primers were used: **7-8 F**, 5'- ACACTGCCGAGCTCAAGATC- 3', and **7-8 R**, 5'-AGCTGCATGGAGACTCGAAC- 3'. These primers provided a 425 bp product when WT *RelA* allele was present.

Ultrasonography

Abdominal aortas of mice, from diaphragm to the renal arteries, were imaged using Vevo2100 ultrasound imaging system (VisualSonics, Toronto, Canada) at baseline and during Ang II infusion (day 6). Mice were sedated with 1 % isoflurane in a supine position during the imaging process. *In vivo* ultrasound imaging allowed us to determine changes in aortic dilation and to visualize dissections during Ang II infusion. Aortic diameter measurements were made in triplicate at the site of maximum dilation in the supra-renal aorta of each mouse.

Flow cytometry

At experiment termination, mice were euthanized in accordance with the animal use protocol and American Veterinary Medical Association guidelines, and whole aortas were isolated and periadventitial fat was removed under a dissecting scope. Aortas were then minced into small pieces and incubated in a digestion buffer containing 0.6 U/ml Liberase Blendzyme (Roche), 50 µg/ml Elastase (Sigma), and 0.1 % BSA in DMEM/F12 base media. Aortic tissue was digested for 1-1.5 hours at 37° C on a vertical rotator. After complete digestion, cells were filtered through a 100 µm cell strainer and washed with FACS buffer (0.5 % BSA and 0.02 % NaN3 in DMEM). RBCs were removed using a lysis buffer from Qiagen. All antibodies were purchased from eBioscience unless stated otherwise. The Fc receptors on leukocytes were blocked with anti-CD16/32 antibody. Fluorochrome-conjugated antibodies against surface antigens were then added, including anti-CD45 (30-F11), anti-CD31 (390), anti-PDGFRα (APA5), anti-CD90.2 (53-2.1), anti-NK1.1 (PK136), anti-CD49b (DX5), anti-CD45R (RA3-6B2), anti-Ly6G (1A8, BD Pharmigen), anti-CD11b (M1/70), anti-F4/80 (BM8), anti-Ly6C (HK1.4) and incubated for 30 minutes. Cells were washed twice, fixed and permeabilized (eBioscience solution) and then incubated with fluorochrome-conjugated antibodies against intracellular antigens including anti-αSMA (1A4, Sigma) and anti-RelA (D14E12, Cell Signaling) for 30 minutes. At the end of the incubation period, cells were washed again and lightly fixed in 0.5 % PFA solution before being analyzed with BD LSRFortessa cell analyzer. Data were evaluated using FlowJo software. Dead cells and debris, as defined by a high forward scatter, were excluded from analysis [3].

Immunohistochemistry (IHC)

After aortic perfusion with PBS to remove all circulating blood cells, supra-renal abdominal aortas were isolated, embedded in optimal cutting temperature (OCT) compound and sectioned (8 µm thickness) using a cryostat. Aortic cross-sections were blocked with 5 % goat serum before being incubated with primary antibodies overnight at 4° C. Antibodies utilized were rabbit anti-phospho-Ser536-RelA (Cell Signaling), rabbit anti-RelA (Santa Cruz), rabbit anti-IL6 (eBioscience), rat anti-MCP-1 (Abcam) rabbit anti-αSMA (Abcam) and rabbit anti-PDGFRα (eBioscience). AlexaFluor-568 conjugated goat anti-rabbit or AlexaFluor-488 conjugated goat anti-rat secondary antibodies (ThermoFisher Scientific) were utilized for detection via immunofluorescence. To detect phospho-Ser536-RelA, the signal was amplified via an avidin-

biotin kit (PK6101, Vector labs) and visualized with DAB chromogen. Secondary antibody-only negative controls were used to determine specificity of the immunostaining. Images were obtained using DXM1200F Nikon Digital Camera attached to a Nikon Eclipse 80i microscope. LSM 510 Meta UV confocal microscope was used for analysis of αSMA and PDGFRα immunostaining.

Histological characterization

Formalin-fixed tissue sections of abdominal aortas were stained with Hematoxylin and Eosin using standard procedures to determine changes in cellular composition. Images were captured at 200x magnification and adventitial area was quantified using ImageJ software (NIH). The adventitia was defined as the area between external elastic lamina and tunica externa. The adventitial area from three different aortic cross-sections per mouse was analyzed and averaged, and n = 5-7 abdominal aortas were evaluated in each group.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from whole aortas using Trizol reagent (Sigma) and quantified with a Nanodrop apparatus (Thermo Scientific). Samples with 260/280 ratio >1.6 were used for further analysis. One µg RNA was reverse transcribed using Superscript III Reverse Transcriptase System (Invitrogen) and diluted 1:2 with DEPC-treated water. Real-time PCR reactions utilizing iQ SYBR Green Supermix (Bio-Rad) were run in triplicates in a CFX96 Real-Time Detection System (Bio-Rad). 18s rRNA was used as the internal control. Primers used in the real-time reactions are listed in Supplementary Table SI. Relative mRNA abundance was calculated using the $\Delta\Delta$CT method.

Blood pressure measurements

Blood pressure measurements were made as previously described [3]. Briefly, arterial blood pressure and heart rate were recorded using a non-invasive tail-cuff method (Kent Scientific, Torrington, CT) on at least 4 consecutive days at baseline and during Ang II infusion. Mice were acclimated to the tail-cuff inflation for several days prior to osmotic minipump implantation. Measurements are reported as mean ± SEM.

Data analysis

Data are reported as mean ± SEM. Statistical analyses were performed using SigmaPlot 12.0 (SYSTAT software, San Jose, CA). Student's t-test was used to analyze differences between two groups. One-way RM ANOVA on Ranks was utilized to determine differences between multiple groups over time followed by Tukey's post-hoc test for group-wise comparison. Fisher's exact test was performed on AAA data set. $P < 0.05$ was considered to be significant.

REFERENCES

(1) Zheng B, Zhang Z, Black CM, de CB, Denton CP. Ligand-dependent genetic recombination in fibroblasts : a potentially powerful technique for investigating gene function in fibrosis. *Am J Pathol* 2002 May;160:1609-17.

(2) Ijaz T, Wakamiya M, Sun H, Recinos A, III, Tilton RG, Brasier AR. Generation and characterization of a novel transgenic mouse harboring conditional nuclear factor-kappa B/RelA knockout alleles. *BMC Dev Biol* 2016;16:32.

(3) Ju X, Ijaz T, Sun H, Ray S, LeJeune W, Lee C, Recinos A, III, Guo DC, Milewicz DM, Tilton RG, Brasier AR. Interleukin-6-signal transducer and activator of transcription-3 signaling mediates aortic dissections induced by angiotensin II via the T-helper lymphocyte 17-interleukin 17 axis in C57BL/6 mice. *Arterioscler Thromb Vasc Biol* 2013 33:1612-21.