Supplemental Materials

Supplemental Methods

RNA extraction and real-time quantitative PCR

Total RNA was extracted from the kidneys or cells according to the manufacturer's instructions of the RNeasy Plus Mini kit (Cat#: 74136, Qiagen) or RNeasy Micro kit (Cat#: 74004, Qiagen). RNA was reverse transcribed with a high capacity cDNA reverse transcriptase kit (Cat#: 4368813, Invitrogen) and real time quantitative PCR (RT-qPCR) was performed with SYBR (Cat#: 4367663) or Taqman assay (Cat#: 4370074) from Invitrogen. Taqman primers and probes were used for IL-1β, TNF-α, IFN-γ, CCL5, NGAL, KIM-1, Collagen I (Col I), Fibronectin (FN), A20, NF-kB1, NF-kB2, CD40, CD80, and CD86. SYBR primers used for IL-17a were purchased from Qiagen (Cat#: PPM03023A) and the primer for β-MHC, sense: 5′- gcaggcggaacaagacaac -3′; antisense: 5′- gctcggcattcatctcctc -3′.

Periodic acid–Schiff staining and kidney injury score

Kidneys were harvested and fixed with 10% paraformaldehyde. The tissues were embedded in paraffin, and 5-μm sections were cut and stained with periodic acid–Schiff. Renal pathology including loss of brush borders, tubule dilatation, and cast formation was scored on a 0~4 scale. 0, <5%; 1, 5–25%; 2,26–50%; 3, 51–75%; and 4,>75% in both kidney cortex and medulla. The observer was blinded to the experimental groups.

Urinary Albumin measurement

Mice were placed into metabolic cages for 24 hours with free access to food and water. Urine was collected and centrifuged 3000 rpm for 3 minutes. The clear urine was used quantitate albumin per instructions of the Albuwell kit (Cat#: 1011, Exocell), and of the albumin concentration was multiplied by the 24-hour urine volume.

Masson's trichrome staining

Hearts were fixed in 10% neutral buffered formalin and processed for staining. After deparaffinization, slides were processed for masson's trichrome staining by using a kit from ThermoFisher Scientific (Cat#: 87020). After mounting, 5-8 images were randomly captured from each section, and positive staining signals were analyzed using ImageJ 1.38 (NIH, USA). Percentages of the fibrotic area were averaged for each animal and then across each group.

Histopathological analysis

Hearts were fixed in 10% neutral buffered formalin and processed for staining. Briefly, the tissue samples were dehydrated and embedded in paraffin. After deparaffinization, thin sections (5 μm) were processed for CD3 staining. After antigen retrieval, the sections were blocked in 1% BSA for 1 hour and then incubated with CD3 antibody (Cat#: RM-9107-S, Lab Vision) at 4°C overnight. After washing off the primary antibody, sections were incubated with HRP-conjugated secondary antibody (Cat#: K4003, Dako) at room temperature for 1 hour. Following three washes with PBS, the sections were incubated with 3,3'-diaminobenzidine chromogen substrate resulting in positive brown staining of the target protein. The nuclei were counterstained with Mayer's hematoxylin before dehydration and mounting. Positive staining signals were counted by an observer. Positive cells from each section were averaged for each animal and then compared between experimental groups.

Cell preparations and flow cytometry

Following Ang II infusion, the spleens or renal lymph nodes were harvested and digested into single cell suspensions. For the T cell panel, cells were stained with fluorescently-labeled anti-CD45 (Cat#:103149,

BioLegend), anti-CD3 (Cat#:100308, BioLegend), anti-CD4 (Cat#:100557, BioLegend), anti-CD8 (Cat#:100734, BioLegend), anti-CD62L (Cat#:104436, BioLegend), and anti-CD44 (Cat#:17-0441-82, Invitrogen) and subjected to flow cytometric analysis. In the DC panel, cells were stained with fluorescently-labeled anti-CD45 (Cat#:103138, BioLegend), anti-CD3 (Cat#:100355, BioLegend), anti-CD19 (Cat#:115543, BioLegend), anti-NK1.1(Cat#:108749, BioLegend), anti-MHCII (Cat#:107626, BioLegend), anti-CD11c (Cat#:117308, BioLegend), anti-CD40 (Cat#:124622, BioLegend), anti-CD80 (Cat#:104731, BioLegend), and anti-CD86(Cat#:105031, BioLegend) prior to analysis. Representative flow plots were chosen to reflect the means from the summary data. The numbers shown on the representative flow plots are exact percentages for the samples shown.

Cell culture and treatment

Splenocytes were harvested from 12-week old naïve WT and DC ACT mice. Cells were isolated with the mouse CD11c positive selection kit (Cat#: 18780A, STEMCELL), and seeded into 6 well plates. The culture media for the DCs was RPMI-1640 (Cat#: R-7638, Sigma) containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine. The cells were stimulated with or without Angiotensin II (AngII, 100nM) for 12 hours. Cells were collected for RNA extraction and analyzed by real-time QPCR.

ELISA

After 10 days of AngII infusion, the blood of the mice was collected and centrifuged 6000 rpm for 10 minutes in microtainer tubes (Cat#: VT365967, VWR international). The supernatant was collected to determine the serum level of TNF-α following kit instructions (Cat#: 887324-22, ThermoFisher Scientific).

Immunoblotting

Kidney tissues were lysed and subsequently sonicated in RIPA buffer that contained 250 μM phenylmethanesulfonyl fluoride (PMSF), 2 mM EDTA, and 5 mM dithiothrietol (DTT) (pH 7.5). Protein concentrations were determined by the use of Coomassie reagent. 50 μg of protein for each sample was denatured in boiling water for 10 min, then separated by SDS-PAGE, and transferred onto PVDF membranes. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBST) for 1 h, followed by overnight incubation with the primary antibody. After washing with TBST, blots were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody and visualized using Enhanced Chemiluminescence (ECL, Cat#: 32106, Invitrogen). The blots were quantitated by using ImageJ 1.38 (NIH, USA). Primary antibodies were as follows: goat anti-Collagen I antibody (Cat#: 1310-01, SouthernBiotech), and rabbit anti- GAPDH (Cat#: 2118L, Cell Signaling Technology), rabbit anti-NHE3 antibody (Cat#: SPC-400D, Stressmarq Biosciences INC.), rabbit anti-α-ENaC antibody (Cat#: SPC-403D, Stressmarq Biosciences INC.), rabbit anti-β-ENaC antibody (Cat#: SPC-404D, Stressmarq Biosciences INC.), rabbit anti-γ-ENaC antibody (Cat#: SPC-405D, Stressmarq Biosciences INC.), rabbit anti-NCC antibody (Cat#: NBP1-44270, Novus Biologicals.), rabbit anti-NKCC2 antibody (Cat#: 38436S, Cell Signaling Technology), and rabbit anti- GAPDH (Cat#: 2118L, Cell Signaling Technology).

Urinary Sodium measurement

Urines were collected in the metabolic cages and analyzed using the M429 Flame Photometer for measurement. The sodium amounts were quantitated, and results were tabulated as the percentage of the sodium load originally injected in the saline challenge experiment.

Assessment of Renal Blood Flow

Mice were anesthetized with 2% isoflurane, and a catheter was inserted into the left jugular vein for the administration of basal fluids and Ang II. A small incision was made on the right flank to expose the kidney, and a noncannulating ultrasonic flowmeter interfaced with a 5 mm V-shaped probe was placed around the right renal artery (MA0.5PSB and

TS420 Flowmeter; Transonic Systems, Ithaca, NY). Mice were allowed to stabilize for 30 minutes before measurements were started. Vascular reactivity of the renal circulation was assessed by injecting increasing doses of Ang II (0.03, and 0.3 μg/kg) as previously described[1] into the internal jugular vein while renal blood flow (RBF) was continuously monitored. Results are reported as the percent change in RBF from baseline value determined just before injection.

Lipid Peroxidation (MDA) Assay

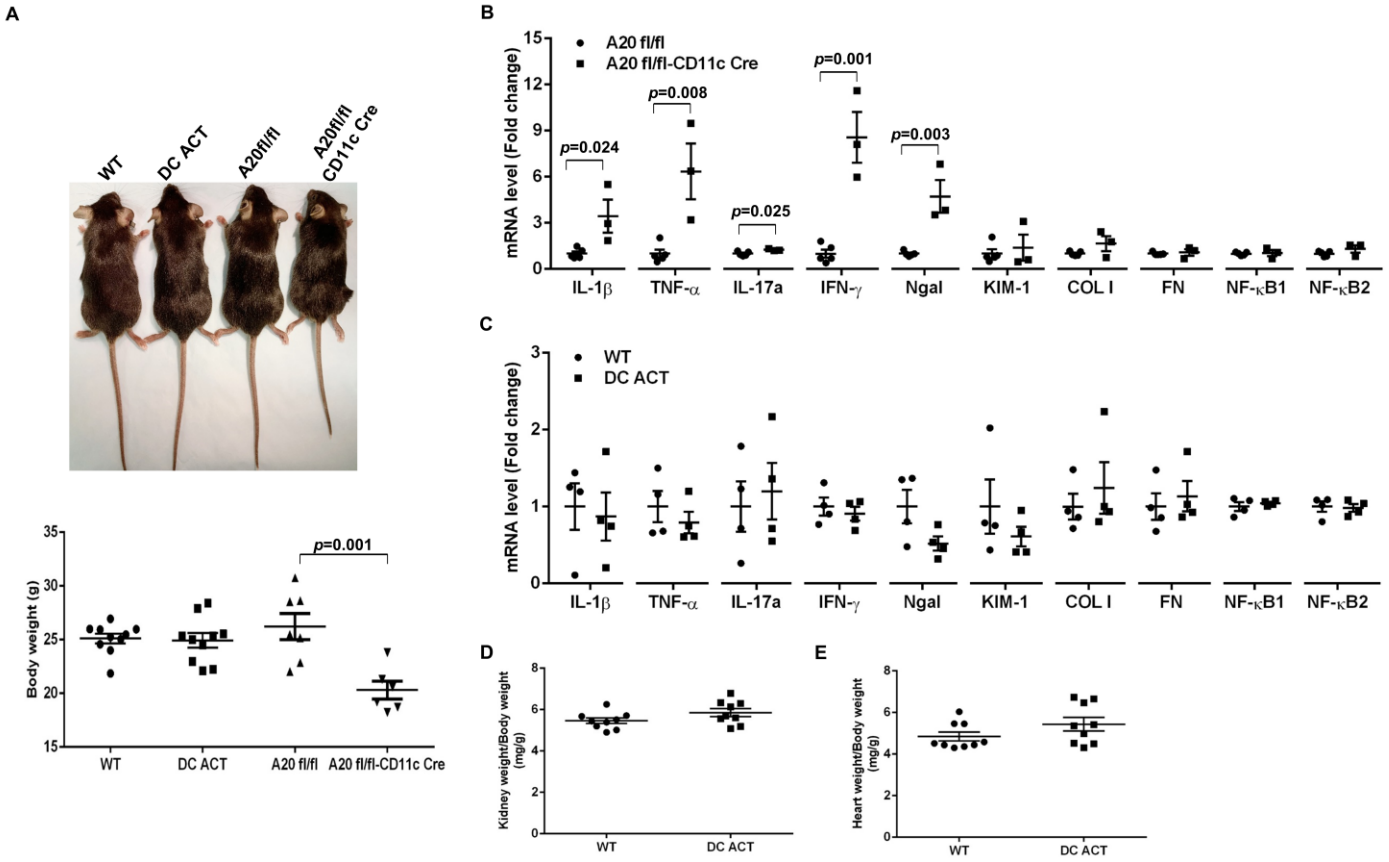
After 4 weeks of Ang II infusion, the blood of the mice was collected and centrifuged 6000 rpm for 10 minutes in the microtainer tubes (Cat#: VT365967, VWR international). The serum was collected for and the MDA levels quantitated per kit instructions (Cat#: MAK085, Millipore Sigma).

Statistical analysis.

The values of each parameter within a group are expressed as the mean ± the standard error of the mean (SEM). For the blood pressure measurement experiments, comparisons between the groups were performed using the two-way ANOVA with repeated measures. For comparisons between two groups with normally distributed data, statistical significance was assessed using an unpaired student' t-test. For comparisons between more than two groups with normally distributed variables, the one-way ANOVA was employed. In these cases, we adjusted for multiple comparison using the Tukey's multiple comparison test. Normality was determined using the Shapiro-Wilks and the Kolmogorova-Smirnov tests. For comparisons between groups with non-normally distributed variables, the Mann-Whitney *U* test was employed. Graphpad Prism was used for statistical analysis. Sample sizes were chosen based on previous studies using our hypertension model.

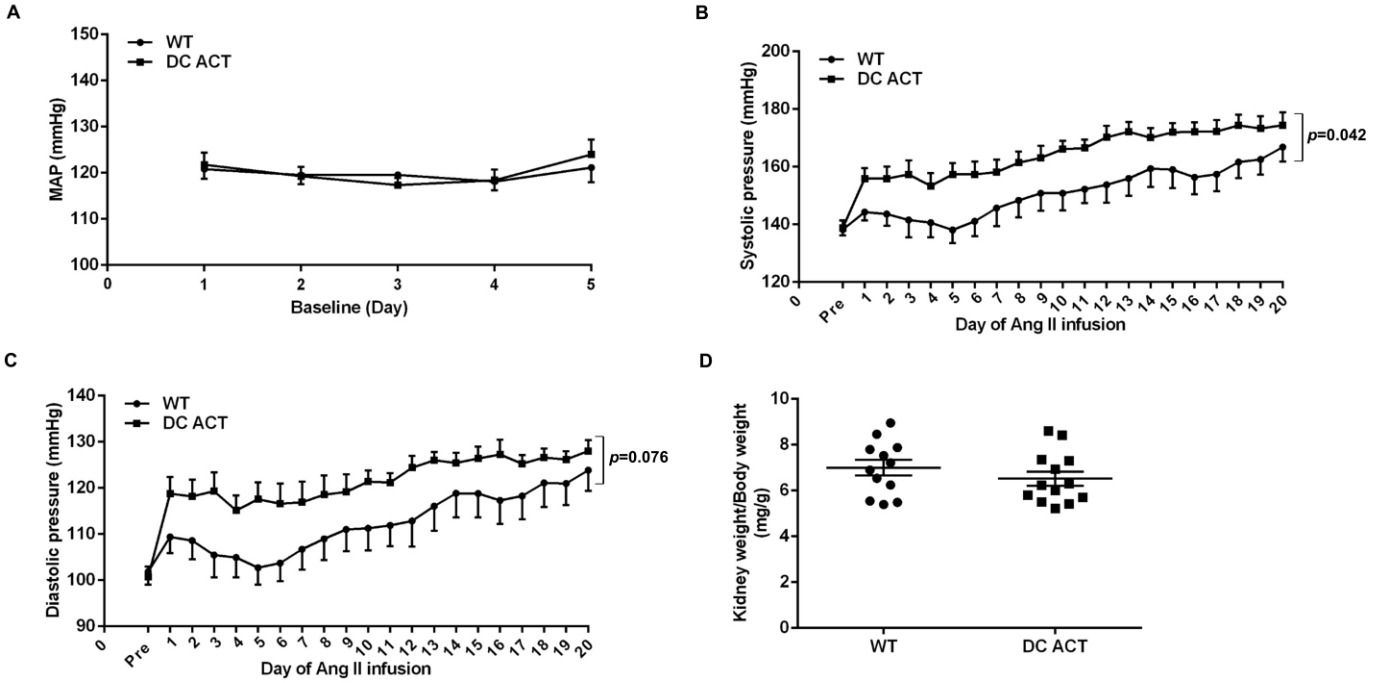
Supplemental Figures

Online Figure I



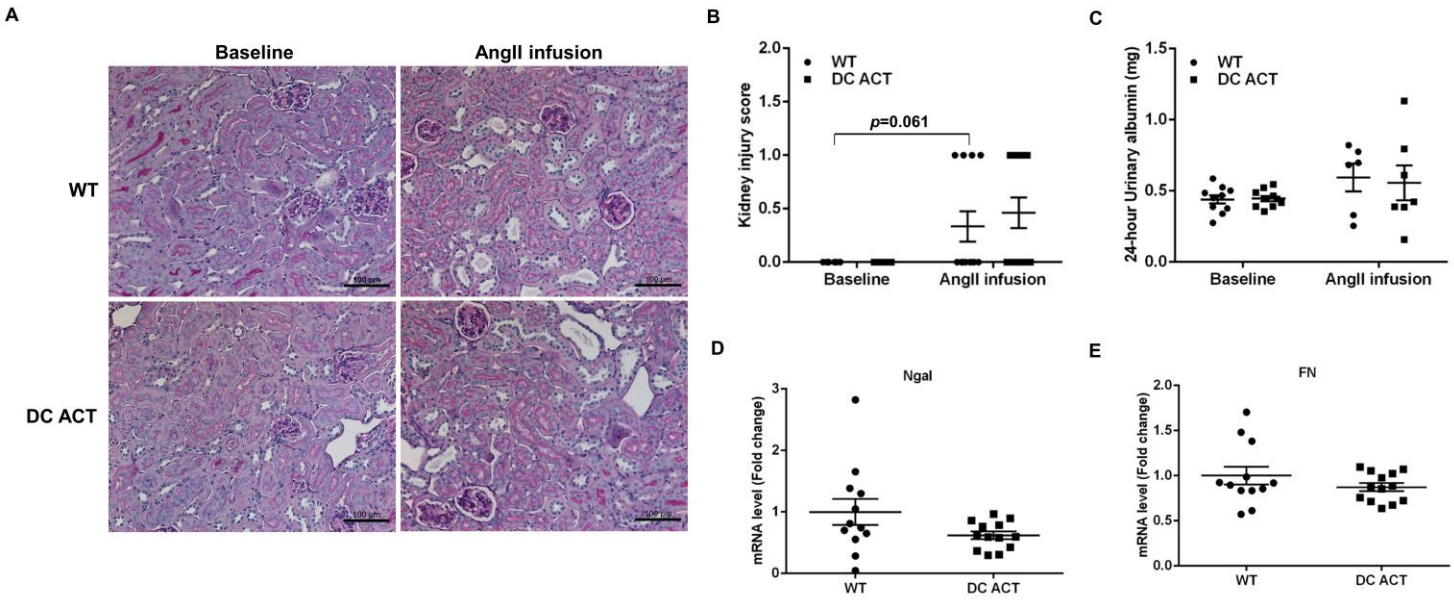
Online Figure I. Baseline physiological parameters of the DC ACT mice. (A) The body weights of the mice at baseline. A20fl/wt ("WT"); *Cd11c*-Cre^{+} A20$^{flox/wt}$ ("DC ACT"). (B-C) The baseline renal mRNA levels for indicated cytokines, kidney injury, and fibrosis makers, and NF-κB subunits in (B) the A20$^{flox/flox}$ and *Cd11c*-Cre^{+} A20$^{flox/flox}$ cohorts, and (C) the WT and DC ACT mice. (D) The ratios of heart weight/body weight (mg/g) in the WT and DC ACT groups at baseline. (E) The ratios of kidney weight/body weight (mg/g) in the WT and DC ACT groups at baseline. N=4-10 mice/group. Data are mean ± SE.

Online Figure II



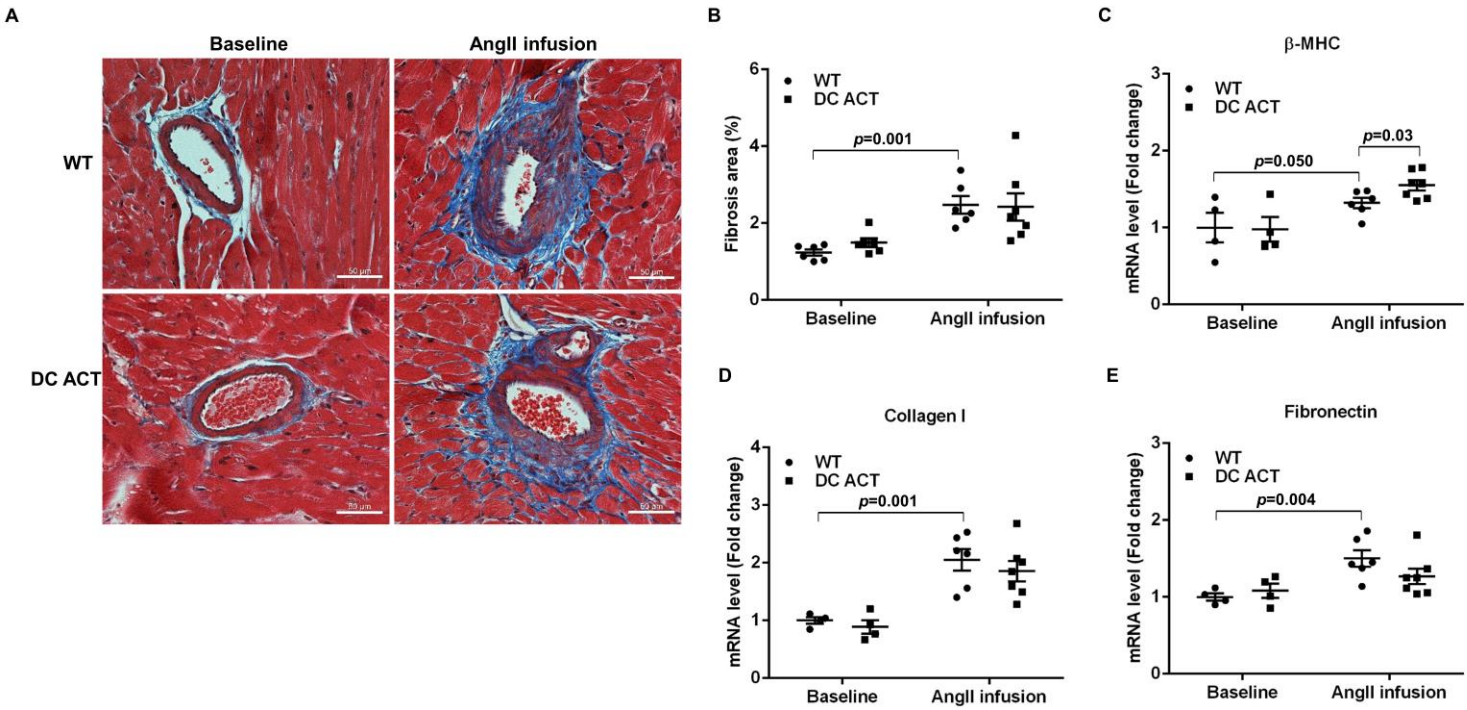
Online Figure II. A20 in CD11c-expressing myeloid cells limits angiotensin (Ang) II-induced hypertension. Blood pressures measured by radio-telemetry. Wild-type ("WT"), circles. *Cd11c*-Cre^{+} A20$^{flox/wt}$ ("DC ACT"), squares. (A) Mean arterial pressures measured in the experimental groups at baseline. N=8 mice/group. (B) Systolic blood pressures of the groups at baseline ("pre") and during chronic Ang II infusion. *, $p< 0.05$ vs. WT. N=8 mice/group. (C) Diastolic blood pressures at baseline ("pre") and during chronic Ang II infusion. N=8 mice/group. (D) The ratio of kidney weight/body weight (mg/g) in the WT and DC ACT groups after 28 days of Ang II infusion. N=12-13 mice/group. Data are mean ± SE.

Online Figure III



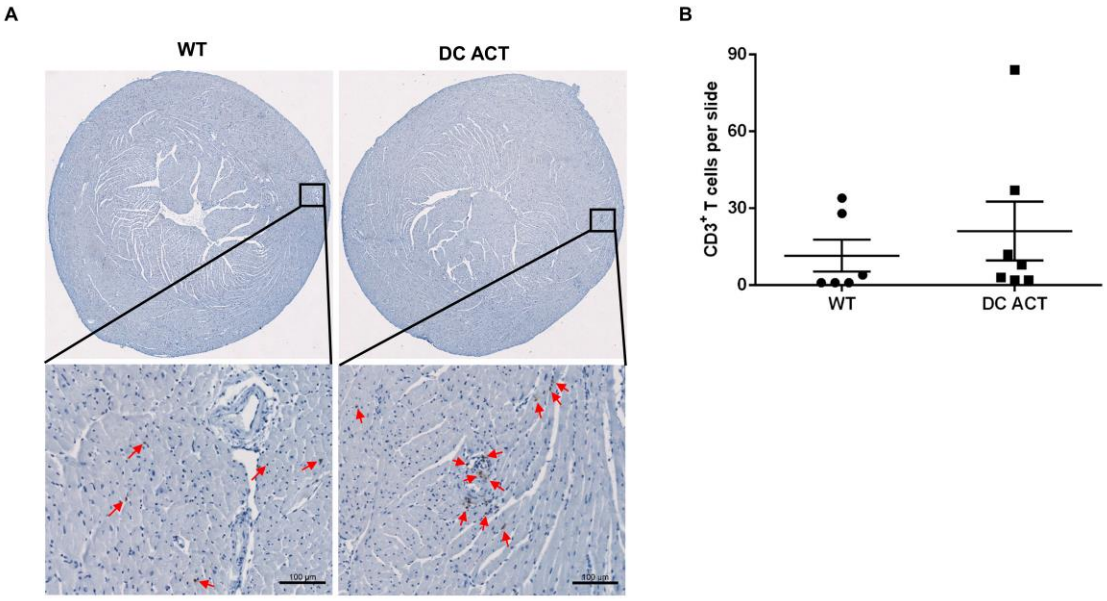
Online Figure III. Deletion of A20 in CD11c^{+} myeloid cells does not influence renal injury during Ang II-induced hypertension. At baseline and day 25 of Ang II infusion, urine was collected for analysis. Kidneys were harvested for analysis at baseline and day 28 of Ang II infusion. (A) Representative images of PAS-stained sections of kidneys from WT and DC ACT mice at baseline and following Ang II infusion. (B) Summary kidney injury scores. (C) 24-hour urinary albumin excretion at baseline and following Ang II infusion. (D-E) Renal mRNA levels for (D) Ngal and (E) fibronectin (FN) in kidneys of Ang II-infused WT and DC ACT animals. N=4-13 mice/group. Data are mean ± SE.

Online Figure IV



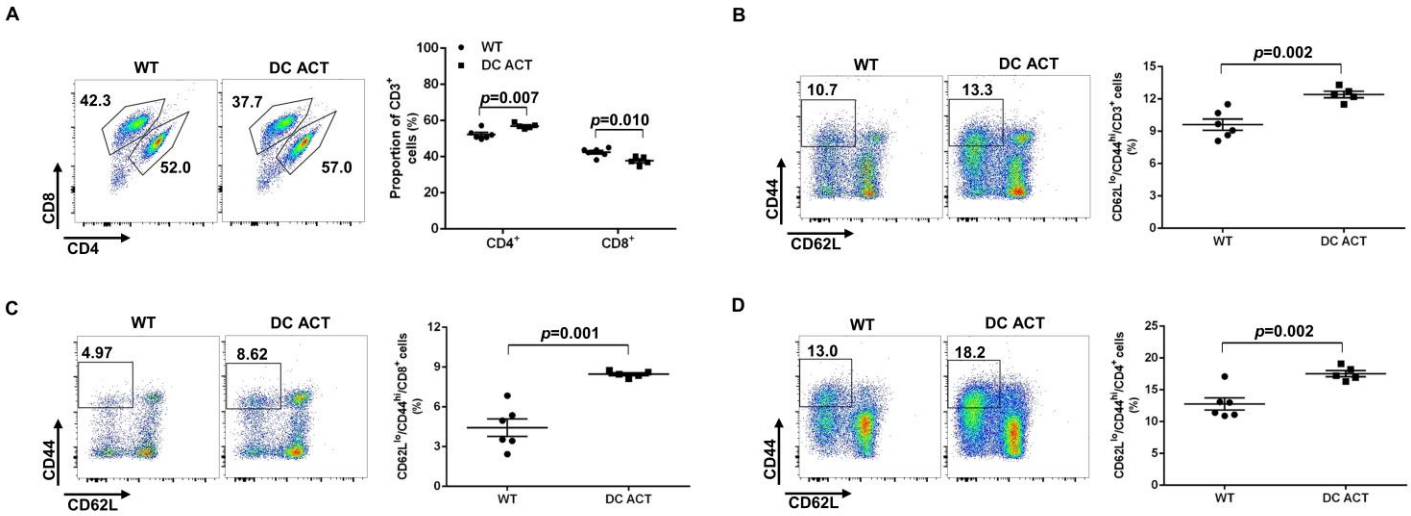
Online Figure IV. A20 in CD11c^{+} myeloid cells protects against Ang II-induced cardiac hypertrophy. Hearts were harvested from WT and DC ACT mice at baseline and following 28 days Ang II infusion. (A) Representative images of Masson's trichrome-stained sections of hearts from WT and DC ACT mice. (B) Blinded quantification of the fibrotic areas. (C-E) mRNA levels for (C) β-MHC, (D) Collagen I (Col I), and (E) fibronectin (FN) in WT and DC ACT hearts. N=4-7 mice/group. Data are mean ± SE.

Online Figure V



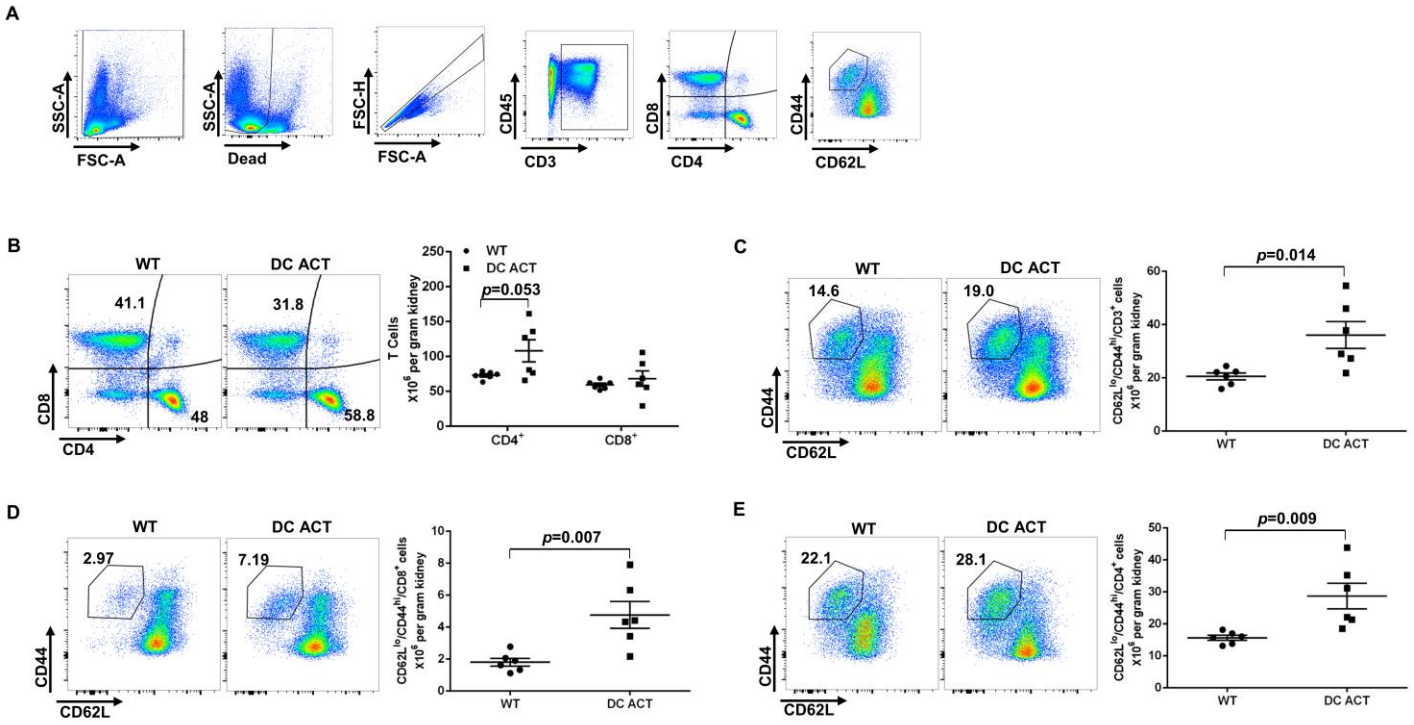
Online Figure V. T cell accumulation in the heart during Ang II induced-hypertension. After 10 days of Ang II infusion, hearts were harvested and stained for CD3. (A) Representative images of CD3-stained heart sections. (B) Number of the CD3-positive cells per section. N=6-7 mice/group. Data are mean ± SE.

Online Figure VI



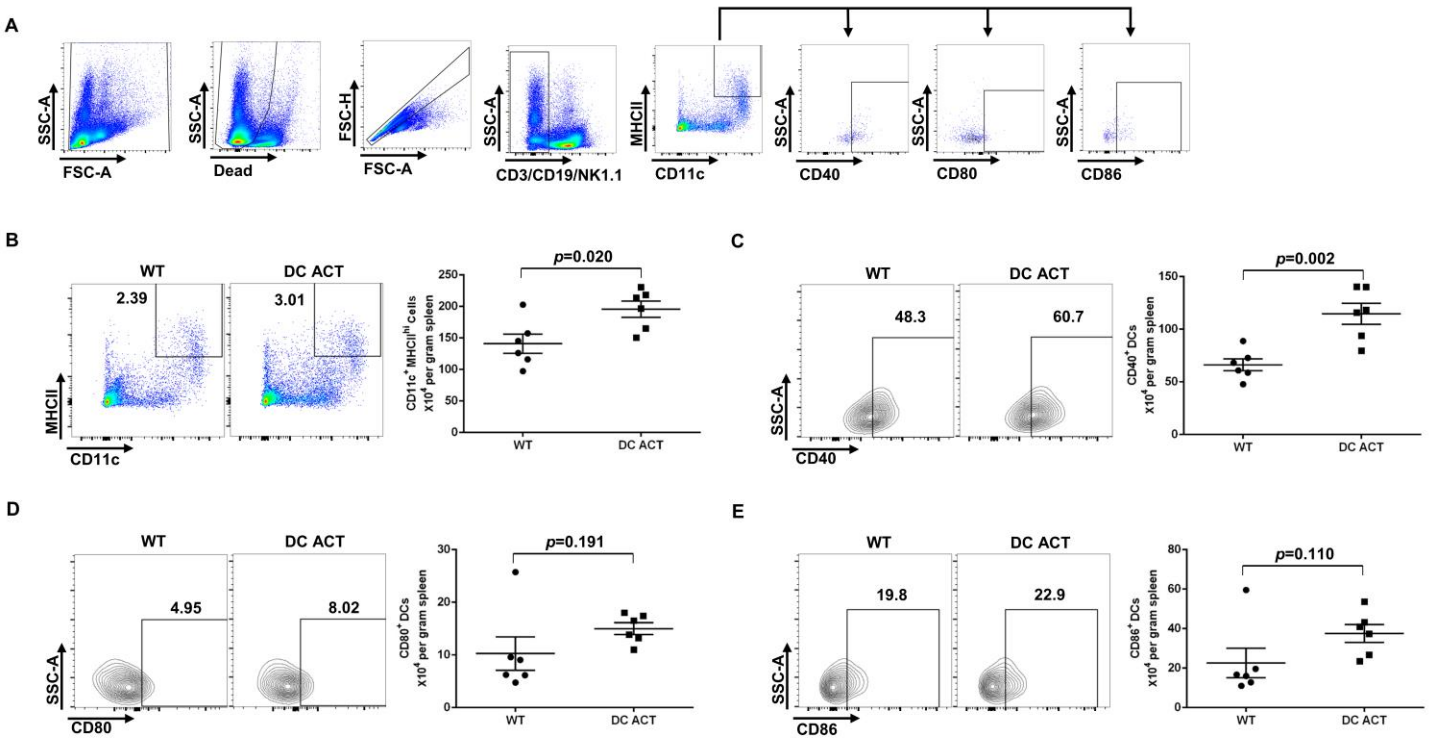
Online Figure VI. A20 in CD11c$^+$ myeloid cells inhibits T cell activation in the renal lymph node during hypertension. Flow cytometric analysis of renal lymph node cells after 4 weeks of Ang II-induced hypertension. (A) Representative flow plots and proportions of the CD4$^+$ and CD8$^+$ T lymphocytes from WT and DC ACT groups. (B-D) Representative flow plots and proportions of the CD44^{hi}CD62L^{lo} effector memory cells among (B) CD3$^+$ T lymphocytes, (C) CD4$^+$ T cells, and (D) CD8$^+$ T cells from WT and DC ACT mice. N=5-6 mice/group. Data are mean ± SE.

Online Figure VII



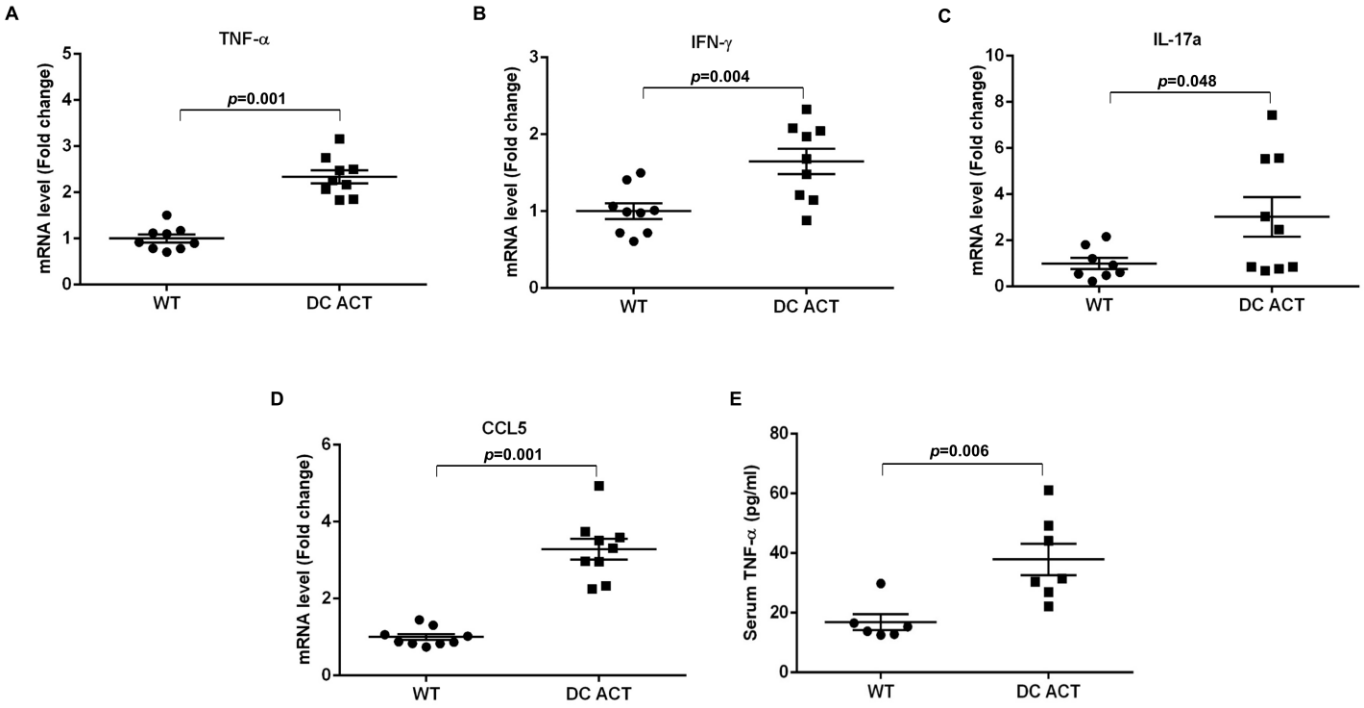
Online Figure VII. A20 in CD11c$^+$ myeloid cells inhibits T cell activation in the spleen during hypertension. Flow cytometric analysis of splenocytes after 10 days of Ang II-induced hypertension. (A) Gating strategy for parsing T cell populations in splenocytes. (B) Representative flow plots and absolute number of the CD4$^+$ and CD8$^+$ T lymphocytes from WT and DC ACT groups. (C) Representative flow plots and absolute number of the CD44^{hi}CD62L^{lo} CD3$^+$ T lymphocytes from WT and DC ACT groups. (D) Representative flow plots and absolute number of the CD44^{hi}CD62L^{lo} CD8$^+$ T cells from WT and DC ACT cohorts. (E) Representative flow plots and absolute number of the CD44^{hi}CD62L^{lo} CD4$^+$ T cells from WT and DC ACT mice. N=6 mice/group. Data are mean ± SE.

Online Figure VIII



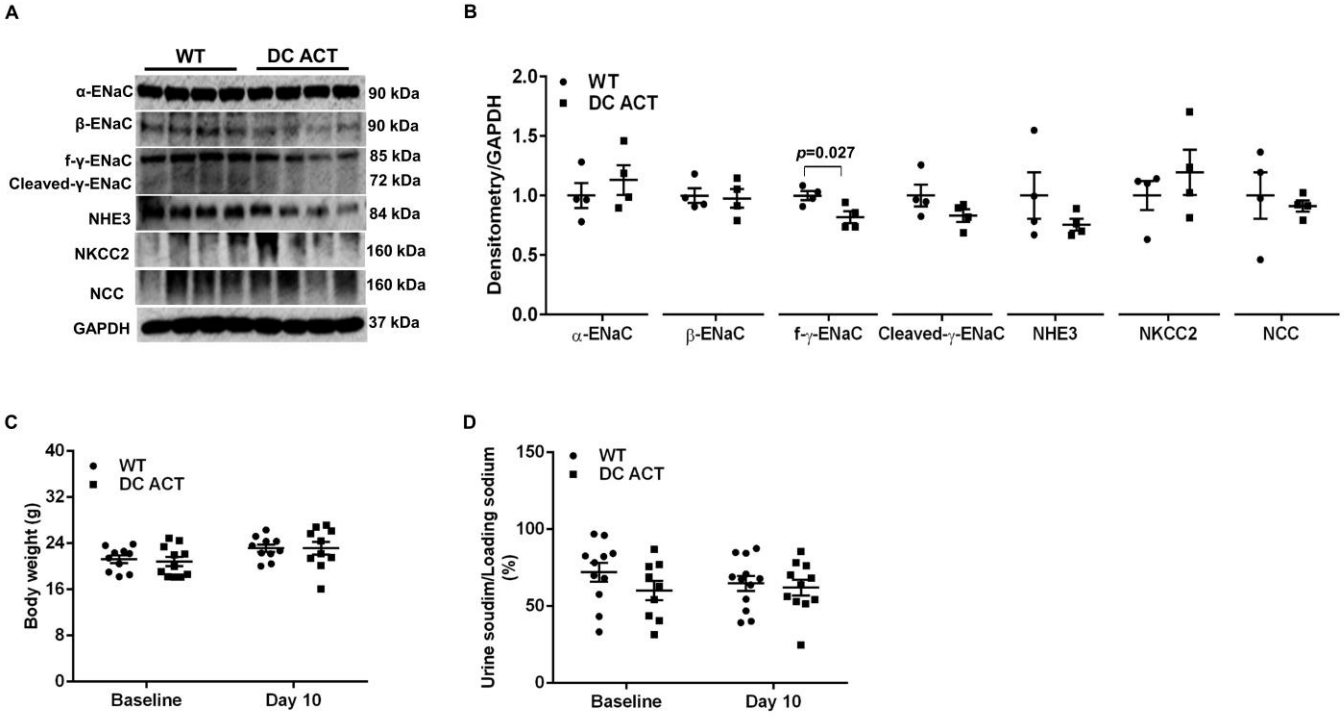
Online Figure VIII. A20 suppresses DC activation in the spleen during Ang II-dependent hypertension. Assessment of expression of co-stimulatory molecules CD40, CD80 and CD86 on $CD11c^{+}$ $MHCII^{hi}$ DC isolated from the spleen after 10 days of Ang II. (A) Gating strategy. (B) Representative flow plots and absolute number of $CD11c^{+}MHCII^{hi}$ Cells the from WT and DC ACT groups. (C-E) Representative flow plots and absolute numbers of (C) $CD40^{+}$, (D) $CD80^{+}$, and (E) $CD86^{+}$ DCs from WT and DC ACT spleens. N=6 mice/group. Data are mean ± SE.

Online Figure IX



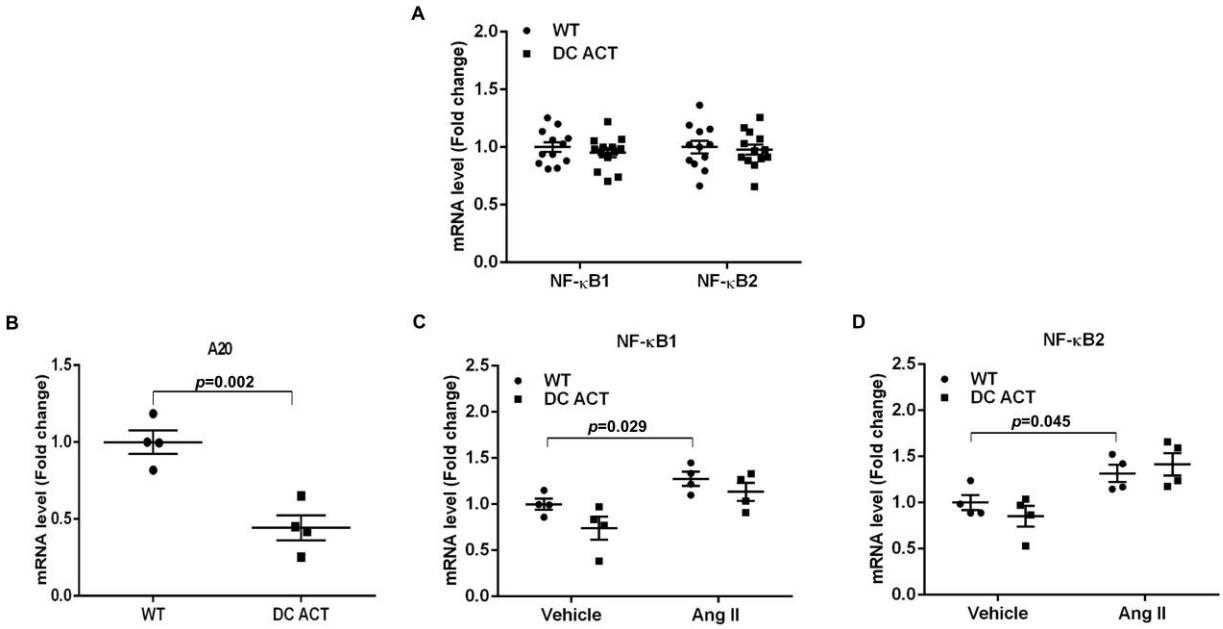
Online Figure IX. Pro-hypertensive cytokines are upregulated in kidney and circulation from Ang II-infused DC ACT mice. After Ang II infusion, the kidneys were harvested and the serum was collected for analysis. (A-D) Renal mRNA levels for TNF-α, (B) IFN-γ, (C) IL-17A, and (D) CCL5. (E) Serum level of TNF-α. N=6-9 mice/group. Data are mean ± SE.

Online Figure X



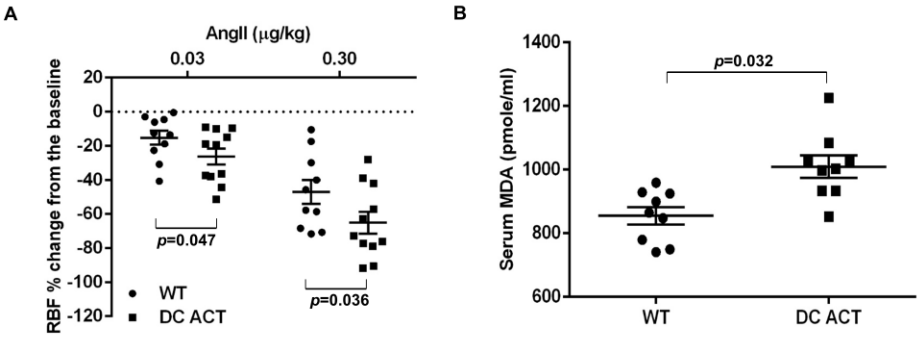
Online Figure X. Additional parameters for saline challenge test in WT and DC ACT cohorts. (A) The renal protein levels of α-ENaC, β-ENaC, γ-ENaC, NHE3, NKCC2, and NCC at baseline. (B) Densitometry values of blots normalized to GAPDH. (C-D) WT and DC ACT mice were challenged with an i.p. bolus of the saline equivalent of 10% of their body weights and placed in metabolic cages for urine collection. (C) Body weights of the mice in the saline challenge study. (D) Ratio of urinary sodium to injected sodium. N=4-13 mice/group. Data are mean ± SE.

Online Figure XI



Online Figure XI. A20 does not regulate NF-κB mRNA expression. (A) In kidneys from Ang II infused animals, mRNA levels for NF-κB subunits were measured by qPCR. (B-D) CD11c+ splenocytes from either WT or DC ACT mice were isolated and treated with or without Ang II (100nM) for 12 hours. Cells were then harvested for real-time QPCR analysis. (B) Relative mRNA expression for A20 without treatment to validate the deletion of A20. (C) Relative mRNA expression for NF-κB1. (D) Relative mRNA expression for NF-κB2. N=4 wells/group. Data are mean ± SE.

Online Figure XII



Online Figure XII. DC ACT mice have a higher renal vasoconstrictor responses and serum MDA in response to Ang II infusion. (A) Percentage reduction in renal blood flow compared to the baseline values following injection with 0.03 μg/kg or 0.3 μg/kg Ang II. (B) The serum levels of the MDA in the Ang II-infused WT and DC ACT mice.

Supplemental References

1. Sparks MA, Stegbauer J, Chen D, Gomez JA, Griffiths RC, Azad HA, Herrera M, Gurley SB, Coffman TM. Vascular type 1a angiotensin ii receptors control bp by regulating renal blood flow and urinary sodium excretion. *J Am Soc Nephrol*. 2015;26:2953-2962