Supplemental Materials

DETAILED MATERIALS AND METHODS

Animals

Male Wistar Kyoto (WKY) and male Spontaneous Hypertensive Rats (SHR) [Charles River Laboratories] were obtained at 3-5 weeks of age. SHR are known to be normotensive at birth and begin to become hypertensive at 6-7 weeks of age, plateauing at about 17-20 weeks of age. At 4 weeks of age, their awake systolic pressures by tail cuff average 96.5±6.7 mmHg (n=60) which is equivalent to the pressure of the normotensive WKY rats (95.7±3.9 mmHg, n=60) [1]. The use of all animals was approved by the University of Iowa Institutional Animal Care Use Committee in accordance with institutional guidelines. Animals were anesthetized with isoflurane and sacrificed by decapitation.

Splenocyte, Bone Marrow, and Kidney Cell Isolation and Culture

Spleens were harvested from 3 -5 week old WKY and SHR. A single-cell suspension was created by homogenization in serum free RPMI medium. Erythrocytes were lysed in a hypotonic buffer (150 mM ammonia chloride, 7.2 mM potassium carbonate, 0.6 mM EDTA, pH 7.2). Cells were washed and re-suspended in complete RPMI (10% heat-inactivated fetal bovine serum, 0.1mM non-essential amino acids, 0.1 mM sodium pyruvate, 10 mM Hepes buffer, 100 μg penicillin/ streptomycin, 0.2mM glutamine).

For bone marrow isolation, the femur, tibia, and fibula were collected from both lower extremities. Bones were mechanically disrupted, placed in bone marrow isolation solution (0.2% fetal bovine serum, 1mM EDTA, PBS), and then gently crushed using a mortar and pestle. Isolated bone marrow cells were collected and erythrocytes lysed as per above. Cells were washed and re-suspended in complete RPMI. To determine the effects of nicotinic cholinergic activation, splenocyte and bone marrow cell cultures were incubated in the presence of nicotine (10 μM) or medium for 36-48 hours prior to analysis.

In Vivo Studies

Subcutaneous (SC) osmotic pumps (Alzet) were implanted in 3-5 week old WKY and SHR under isoflurane anesthesia. Pumps infused either saline or nicotine bitartrate (15 mg/kg/day). Animals were sacrificed at the end of 24 hours or 2 weeks. Tissues or cells collected were for immunohistochemistry, confocal microscopy, or flow cytometry.

Flow Cytometry

Splenocytes or bone marrow cells were isolated as described from untreated animals or animals treated with saline or nicotine infusion for 2 weeks. Cells were either stained with monoclonal antibodies immediately or after being cultured for 36-48 hours in the presence or absence of nicotine. Flow cytometry was then performed. Cells (1×10^6) were aliquotted into staining tubes and re-suspended in staining buffer (PBS, 1% appropriate animal sera, 2% FBS). Fluorochrome conjugated antibodies, anti-rat CD3 (BD Biosciences, clone G4.18), anti-rat CD8a (BD Biosciences, clone Ox-8), anti-rat CD161a (BD Biosciences, clone 10/78), anti-rat CD68 (Abd Serotec, clone ED1)] were added at pre-determined working dilutions and splenocytes were incubated 45 minutes at 4^0C. Cells were washed and resuspended in staining buffer for analysis. They were analyzed using a Becton Dickinson LSR II device (Becton Dickinson, Heidelberg, Germany) flow cytometer and data collected and analyzed in FACS Diva Software. For each acquisition, a minimum of 100,000 events were recorded. Cellular debris and necrotic/non-viable cells were excluded by gating.

Immunohistochemistry & Immunofluorescence

CD68 immunohistochemistry was performed similar to previous work [2]. Briefly, paraffin embedded tissues were sectioned (~4 µm) and rehydrated through a series of alcohol and water baths. Antigen retrieval was performed with NxGen Decloaking Chamber™ (Biocare Medical, Concord, CA, USA). Endogenous peroxidase activity was quenched with 3% H_2O_2. A primary mouse monoclonal Ab (#MCA341R, AbD Serotec Company, Raleigh, NC, USA) and a secondary kit (Mouse Envision, DAKO, Carpinteria, CA) was used for the procedure. Tissues were exposed to chromogen (DAB plus, DAKO, Carpinteria, CA), then counterstained with hematoxylin (Surgipath, Leica Biosystems, Buffalo Grove, IL, USA) and dehydrated (series of alcohols and xylene) and coverslipped.

Immunofluorescence was conducted on air dried freshly frozen tissue sections. Sections were fixed in formalin and rinsed with buffer and glycine. Sections were exposed to mouse anti-rat CD68 monoclonal antibody (1:200) and anti-mouse Alexa fluor 555-labeled antibody was used for secondary detection of CD68. Mouse anti-rat CD161a-FITC monoclonal antibody (1:50) was exposed to the tissue sections. Sections were then coverslipped, mounted, and visualized by confocal microscopy within 1-2 days.

Western Blot

Spleen and kidney tissues were homogenized using a RIPA buffer and protein concentrations determined using a bicinchoninic protein assay (Pierce). SDS-PAGE electrophoresis was conducted using 30 micrograms of total protein of each sample. Nitrocellulose membranes were utilized for the transfer of proteins and blocked with 5% bovine serum albumin (BSA) and stained with anti-rat GAPDH (Thermo Fisher Scientific), Anti-rat Very Late Antigen 4 (VLA-4)(LS Biosciences, polyclonal catalog # LS-C192282), anti-rat VCAM-1 (LS Biosciences, clone EPR5047), anti-rat LLT1 (AbCam, clone EPR6584), anti-rat MCP-1 (LS Biosciences, polyclonal, catalog# LS-C104751), anti-rat ICAM-1 (LS Biosciences, polyclonal, catalog# AF583), anti-rat α7nAChR (Alomone Labs, catalog# ANC-007), anti-rat AT1R (Santa Cruz, SC-1173), and anti-rat AT2R (Abcam, clone EPR3876) in 2.5% BSA were used to stain the membranes. Detection was achieved using horseradish peroxidase secondary detection antibodies and chemiluminescent substrate with the use of a PXi4 CCD camera (Synoptics).

Renal Denervation

To assess the role of renal nerves in nicotine mediated renal inflammation, 3-4 week-old SHR and WKY underwent left sided unilateral renal denervation or sham operation on the right side. Renal denervation was accomplished via previous described methods reported by Winternitz et al [3]. In brief, the left or right renal artery was exposed via flank incision and retroperitoneal approach. For effective renal denervation renal artery adventitia was exposed and removed and the renal artery was painted with 20% phenol (wt/vol) in absolute alcohol. Sham operation consisted of flank incisions only.

To assess the effect of nicotinic cholinergic activation, animals were allowed to recover for 1 week and then an osmotic pump was implanted subcutaneously and delivered nicotine (15mg/kg) over the course of 24 hours. At the termination of infusion, tissues were collected and assessed by immunohistochemistry and confocal microscopy as described above.

Nicotinic Cholinergic and Angiotensin Receptors Expression

Spleen and kidney tissues were homogenized and Western blot conducted as described above. After transfer of proteins to nitrocellulose membranes, membranes were probed with antibodies directed against anti-rat α7nAChR (Alomone Labs, catalog# ANC-007), anti-rat AT1R (Santa Cruz, SC-1173), and anti-rat AT2R (Abcam, clone EPR3876). as described above.

RESULTS

Nicotine Induces Inflammatory Cytokine in the Urine of SHR

Nicotine infusion induced elevated levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) ($p<0.001$), monocyte colony stimulating factor (M-CSF)($p<0.001$), regulated on activation, normal T cell expressed and secreted (RANTES) ($p<0.05$), interleukin-2 (IL-2)($p<0.01$), interleukin-6 (IL-6)($p<0.001$), interleukin-12p70 (IL-12p70)($p<0.03$), macrophage inflammatory protein-3a(MIP-3a)($p<0.05$), interleukin-1a (IL-1a)($p<0.03$), interleukin-1b (IL-1b)($p<0.03$), tumor necrosis factor-alpha (TNF-a)($p<0.01$), interleukin-4 (IL-4)($p<0.01$), interleukin-7 (IL-7)($p<0.03$), and interleukin-13 (IL-13)($p<0.03$) (Online Figure I). There appeared to be a slight increase in macrophage inflammatory protein-1a (MIP1a), but failed to reach statistical significance.

Elevation of RANTES, IL-2, IL-1a, IL-1b, MIP-3a, TNF-a, and IL-4 in Renal Homogenates of the Young SHR

After nicotine infusion, IL-2, a T-cell cytokine growth factor, was increased in renal homogenates of the young SHR, compared to saline infused SHR controls ($p<0.05$) and significantly elevated compared to WKY nicotine infused controls ($p<0.001$) (Online Figure II B & F). Although there did not appear to be an increase in RANTES, a cytokine involved in the regulation of T-cell activity, in response to nicotinic cholinergic activation, the levels of RANTES in the saline and nicotine infused kidney were significantly elevated, compared to WKY controls ($p<0.001$) (Online Figure II B & F). Similarly, IL-1a ($p<0.05$), IL-1b ($p<0.05$), MIP-3a ($p<0.001$), TNF-a ($p<0.05$), and IL-4($p<0.05$) were found to be elevated in the saline and nicotine infused SHR, compared to corresponding WKY controls (Online Figure II C, II G, II D, and II H). No significant elevations in GM-CSF, M-CSF, IL-6, IL-12p70, MIP-1a, or IL-12 were found in renal homogenates of the young SHR. Hence, there are significant increases in innate and T-cells adaptive cytokine levels in the SHR.

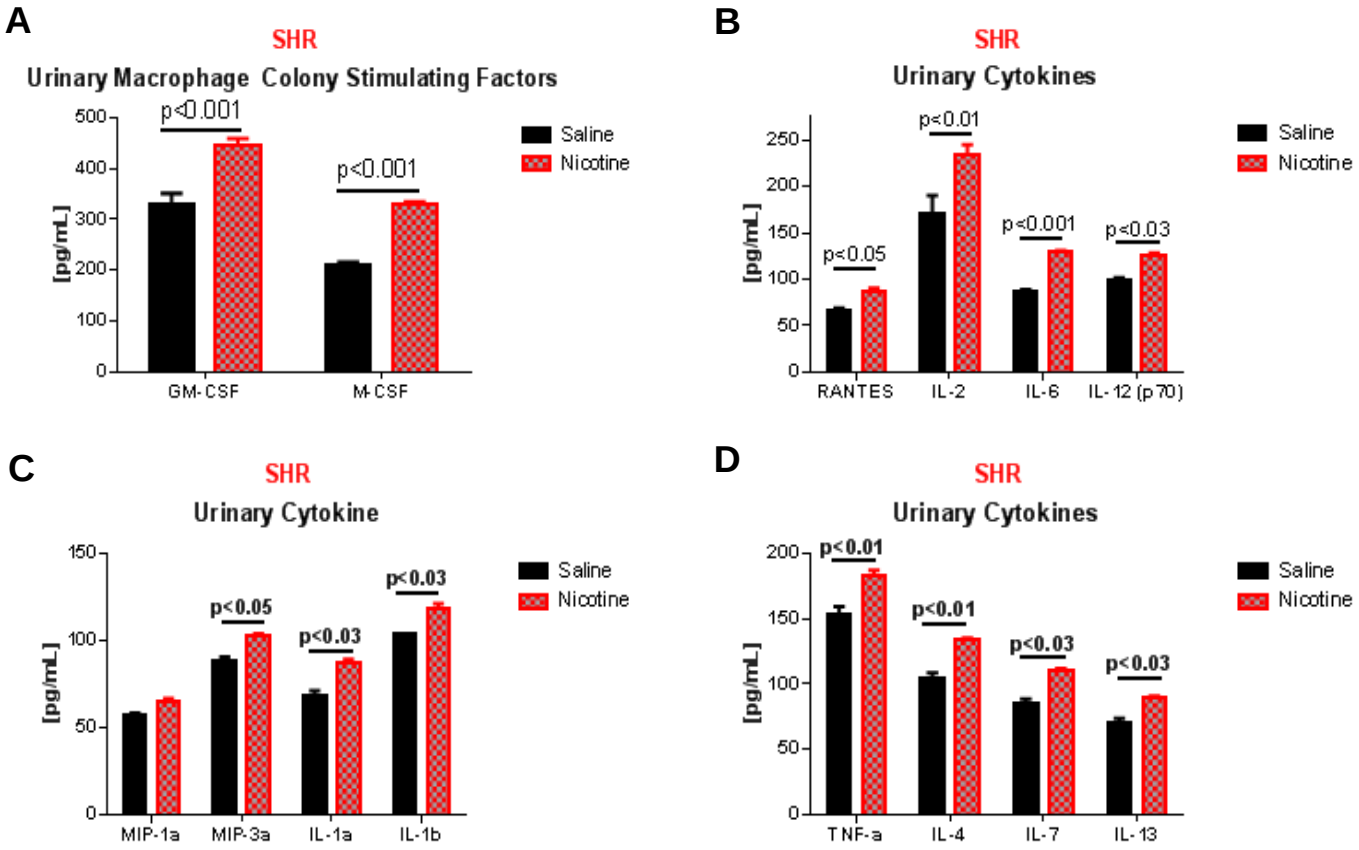
Elevation of Serum RANTES, IL-2, IL-1a, IL-1b, MIP-1a, MIP-3a, and IL-17a in the Young SHR

After nicotinic cholinergic activation, RANTES ($p<0.001$) and MIP-3a ($p<0.05$) were significantly increased in the serum of SHR, compared to saline infused SHR. Interestingly, saline infused SHR also had a significant increase in RANTES, compared to saline infused WKY controls ($p<0.05$) (Online Figure III F). IL-17a was also increased in the serum of nicotine infused SHR, compared to saline infused SHR ($p<0.05$) (Online Figure III H). The innate immune cytokines MIP-1a ($p<0.05$), MIP-3a ($p<0.05$), IL-1a ($p<0.05$), and IL-1b ($p<0.05$) were increased in the serum of the nicotine infused SHR, compared to the corresponding WKY controls ($p<0.05$) (Online Figure III C & G). No significant elevations in GM-CSF, M-CSF, IL-2, IL-6, IL-12p70, or IL-12p70 were found in the young SHR. Hence, the serum of the young SHR shows significant elevations in innate immune cytokines and RANTES, similar to renal homogenates.

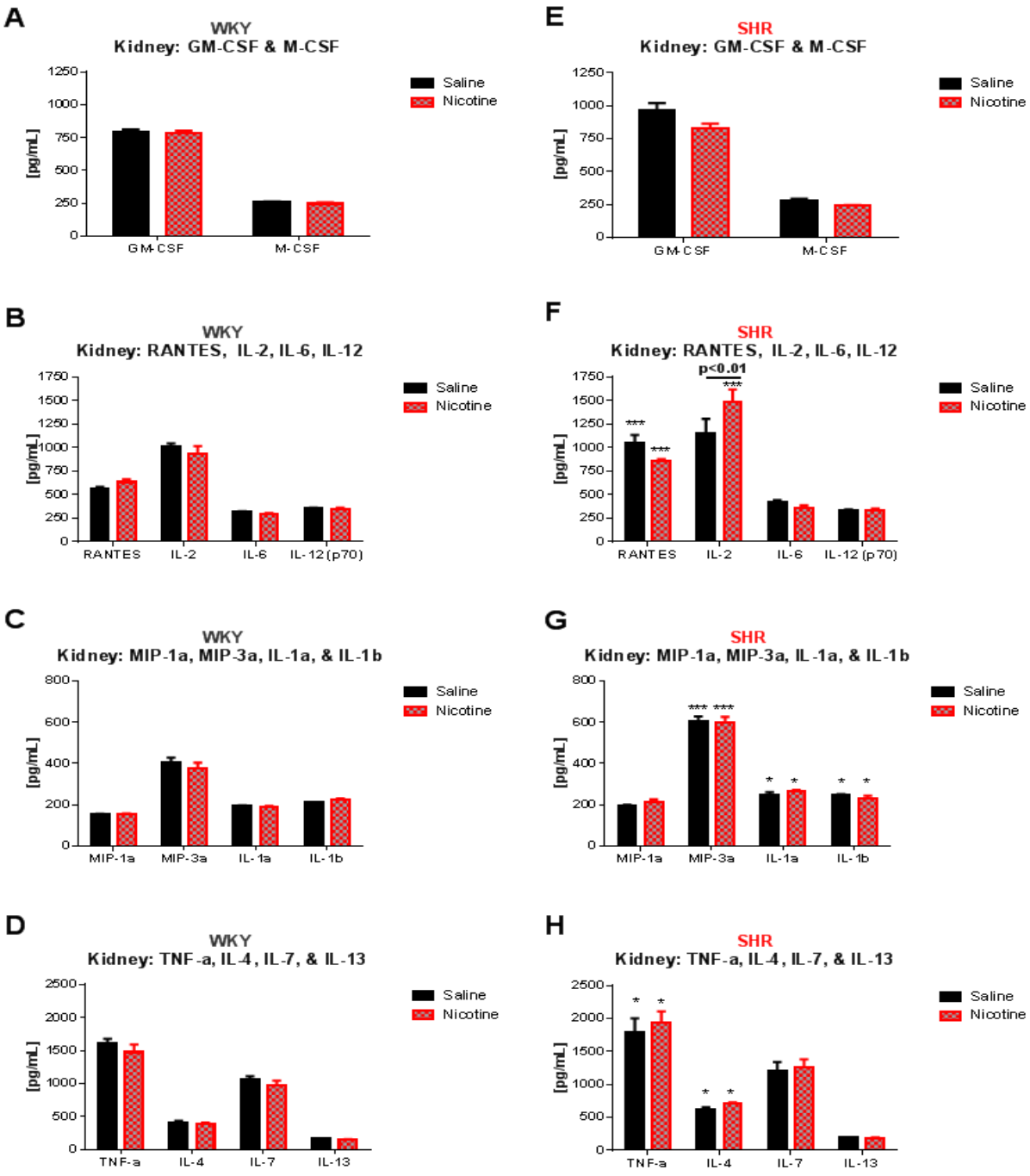
References

1. Andresen MC, Brown AM. Baroreceptor function in spontaneously hypertensive rats. Effect of preventing hypertension. *Circ Res.* 1980;47:829-834.
2. Meyerholz DK, Samuel I. Morphologic characterization of early ligation-induced acute pancreatitis in rats. *Am J Surg.* 2007;194:652-658.
3. Winternitz SR, Katholi RE, Oparil S. Role of the renal sympathetic nerves in the development and maintenance of hypertension in the spontaneously hypertensive rat. *J Clin Invest.* 1980;66:971-978.

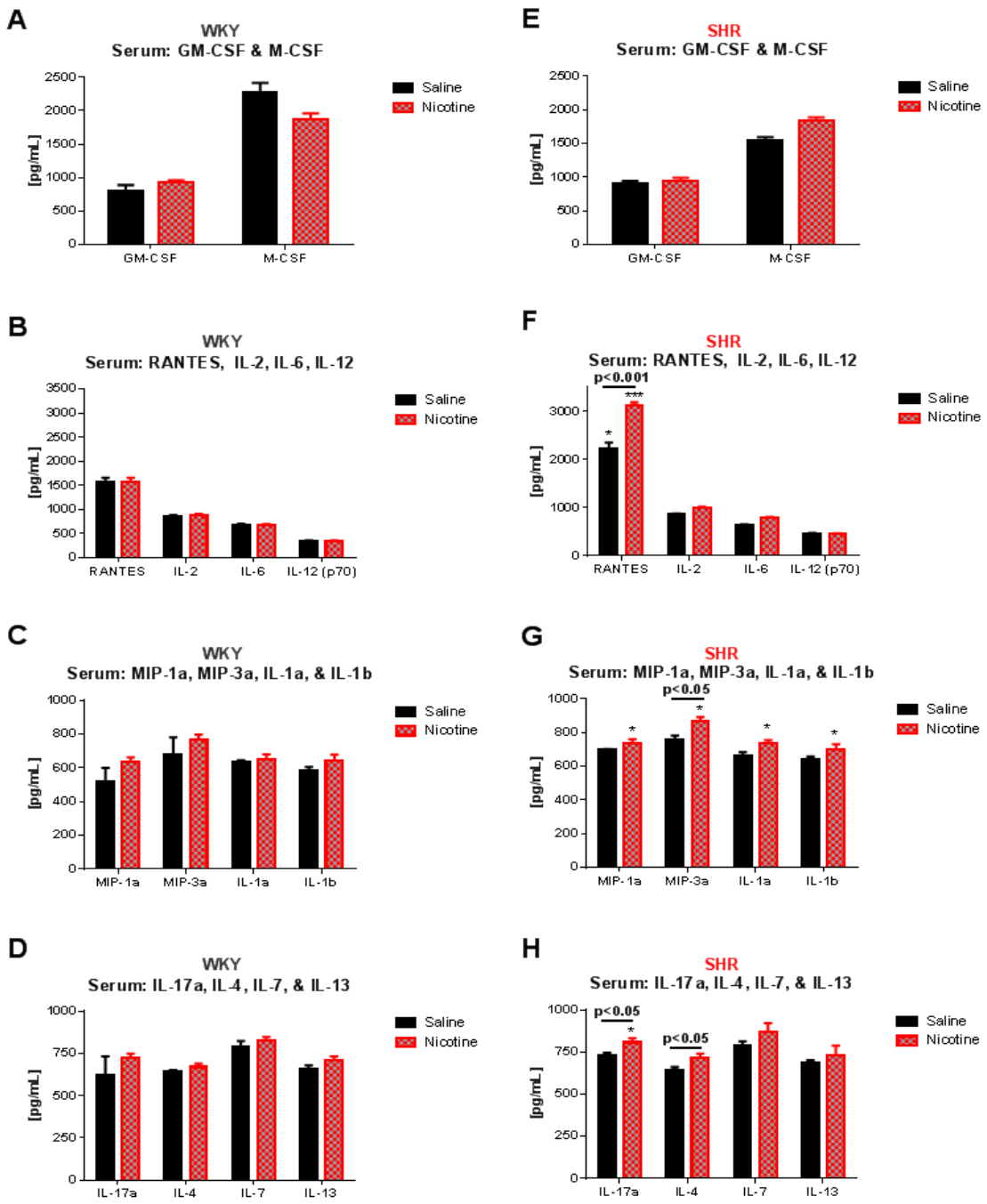
Online Figure I: Effect of Nicotine infusion on Urinary Cytokine Levels. Young (3-5 week old) SHR (n=8) were implanted with osmotic pumps infusing either saline (n=4, black bars) or nicotine (15mg/kg/day) (n=4, red bars) 4 weeks. After infusions were complete urine was collected. Granulocyte-monocyte colony stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-2), macrophage inflammatory protein 1a and 3a (MIP-1a , MIP-3a), interleukin-1a (IL-1a) , interleukin-1b (IL-1b) , tumor necrosis factor alpha (TNF-a), iinterleukin-4 (IL-4) , interleukin-7 (IL-7) , and interleukin-13 (IL-13) were measure by Luminex assay. Error bars represent the standard error of the mean (SEM). P-values as indicated.



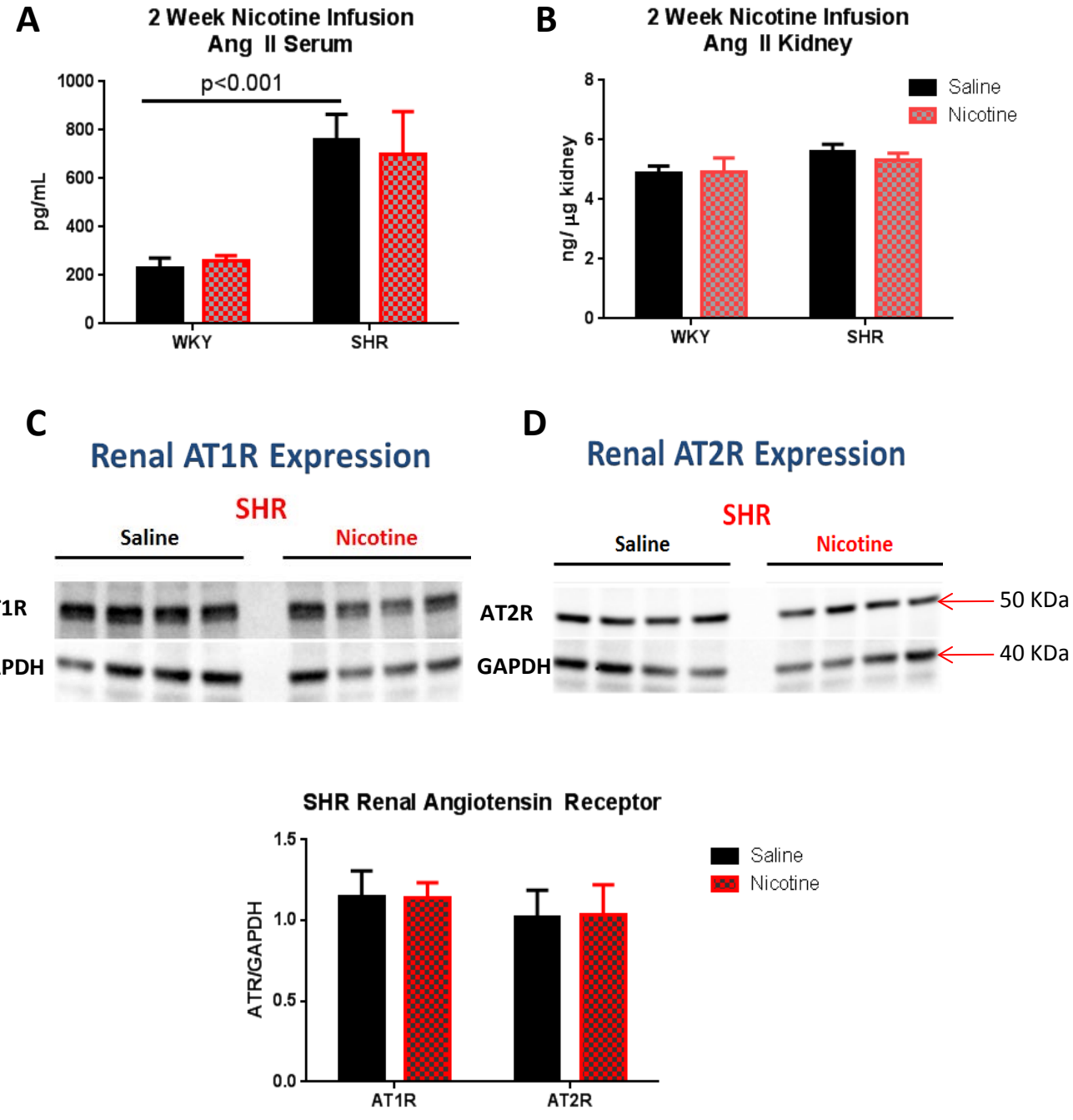
Online Figure II: Effect of Nicotine infusion on Kidney Cytokine Levels. Young (3-5 week old) WKY (n=8) (A-D) and SHR (n=8) (E-H) were implanted with osmotic pumps infusing either saline (n=4) or nicotine (15mg/kg/day) (n=4) 4 weeks. After infusions were complete renal homogenates were prepared and tested for cytokines. Granulocyte-monocyte colony stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-2), macrophage inflammatory protein 1a and 3a (MIP-1a , MIP-3a), interleukin-1a (IL-1a) , interleukin-1b (IL-1b) , tumor necrosis factor alpha (TNF-a), interleukin-4 (IL-4) , interleukin-7 (IL-7) , and interleukin-13 (IL-13) were measure by Luminex assay. Error bars represent the standard error of the mean (SEM). P-values as indicated and * = p<0.05, *** = p<0.001, compared to corresponding WKY controls.



Online Figure III: Effect of Nicotine infusion on Serum Cytokine Levels. Young (3-5 week old) WKY (n=8) (A-D) and SHR (n=8) (E-H) were implanted with osmotic pumps infusing either saline (n=4) or nicotine (15mg/kg/day) (n=4) 4 weeks. Serum was isolated and assayed for cytokines. Granulocyte-monocyte colony stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-2), macrophage inflammatory protein 1a and 3a (MIP-1a , MIP-3a), interleukin-1a (IL-1a) , interleukin-1b (IL-1b), interleukin 17a (IL-17a), interleukin-4 (IL-4) , interleukin-7 (IL-7) , and interleukin-13 (IL-13) were measure by Luminex assay. Error bars represent the standard error of the mean (SEM). P-values based on two-way ANOVA. * = p<0.05, ** * = p<0.001, compared to corresponding WKY controls.



Online Figure IV: Effect of Nicotine on Angiotensin II, AT1 and AT2 Receptors
Young (3 week old) WKY (A, n=8) and SHR (B, n=8) were implanted with osmotic pumps infusing either saline (n=4) or nicotine (15mg/kg/day) (n=4) at 3-4 weeks of age. After 2 weeks of infusion, expression of Ang II was assessed by ELISA in the serum (A) and renal homogenates (B). Levels of AT1R (C), and AT2R (D) were assessed by Western blot in renal homogenates. Nicotine infusion had no effect on serum or renal levels of Ang II. There was also no detectable difference in the expression of AT1R and AT2R in renal homogenates of nicotine infused SHR, compared to saline infusion. Error bars represent the standard error of the mean (SEM). p-values as indicated based on two-way ANOVA.



Online Figure V: Alpha7-nACHR Expression in Spleen and Kidney of Young SHR and WKY
Splenic and renal homogenates were prepared from 3-4 week old untreated WKY (n=3) and SHR (n=3) (A). Splenic and renal homogenates from 6 week old WKY (n=8) and SHR (n=8) that underwent saline (n=4) or nicotine (15mg/kg/day) (n=4) infusion for 2 weeks (B). Homogenates were tested by Western blot for the presence of alpha7-nAChR. Specificity of the antibody was confirmed by incubating the antibody with the n-terminus peptide of rat alpha7-nAChR prior to exposure to the membrane (Pre-adsorbed Antibody). Error bars represent the standard error of the mean (SEM).