Supplemental Material

Detailed Methods

Mice

C57BL/6 *Agtr1a*$^{flox/flox}$ mice were previously generated[1] and were crossed in 2 steps of breeding with mice harboring *Cre recombinase* under the control of the CD4 promoter[2] to generate *CD4 Cre*$^{+}$ *Agtr1a*$^{flox/flox}$ (*T cell KO*) mice. To increase the susceptibility to kidney injury, *T cell KO* mice from the C57Bl/6 strain were crossed with *Agtr1a*$^{flox/flox}$ mice from the 129/SvEv strain to yield the [C57BL/6 x 129/SvEv]F_1 *T cell KO* and *T cell WT* (CD4 Cre^{-} *Agtr1a*$^{flox/flox}$) littermates for our experiments. To confirm selective *CD4 Cre* expression in lymphoid tissues, membrane-targeted *tdTomato (mT)*/*membrane-targeted EGFP (mG)* mice with *loxP* sites flanking the membrane-targeted *dtTomato* cassette followed by an N-terminal membrane-tagged version of *EGFP* from the Jackson Laboratory were crossed with the CD4 *Cre* recombinase transgenic lines. *mT/mG* mice normally express red fluorescence protein in all tissues. When *Cre* is present, the *mT* cassette is deleted, triggering expression of the membrane-targeted *EGFP*.[3] T-bet$^{-/-}$ mice on the C57/BL6 background were purchased from Jackson Laboratory and backcrossed to the 129/SvEv strain for 6 generations to increase susceptibility to kidney damage. Then, T-bet heterozygotes were intercrossed to yield the *T-bet KO* and *WT* littermates for our experiments. Mice were bred and maintained in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facilities at the Durham VA Medical Center according to National Institutes of Health guidelines. All of the animal studies were approved by the Durham Veterans' Affairs Medical Center Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals had free access to standard rodent chow and water. Eight- to 12-week-old male mice and littermate controls were used for experiments.

Model of angiotensin II-dependent hypertension

Experimental animals underwent left nephrectomy followed 1 week later by implantation of a pressure-sensing catheter (TA11PA-C10, Transoma Medical) via the left common carotid artery as described.[4] After allowing 7 days for reestablishment of diurnal blood pressure variation, baseline blood pressure measurements were recorded for 3 days by radiotelemetry (Transoma) in conscious unrestrained animals. Then an osmotic mini-pump (ALZET model, 2004) was implanted subcutaneously to infuse Ang II (1000ng/kg/min; Sigma; n≥11 mice per group) or saline (n=4 or 5 mice per group) continuously for 28 days. Blood pressure measurements continued for 3 weeks of saline or Ang II infusion. On day 25, the mice were placed into metabolic cages, and urines were collected for 24 hours. Urinary concentration of albumin were measured in individual samples using specific ELISA kits for mouse albumin (Exocell) as previously described.[5] Urine creatinine concentrations were measured with a picric acid-based method using a kit (Exocell).

Histological Analysis

At the end of the 28-day experimental period, blood samples were collected, and the heart and kidney were harvested and weighed. Weighing the hearts provides separate confirmation that chronic Ang II-infusion has increased the blood pressure in these animals. Serum creatinines were quantitated by the Animal Clinical Chemistry and Gene Expression Laboratory at University of North Carolina, Chapel Hill, using a colorimetric assay kit. Portions of kidney were fixed and pathological analysis was performed as we have described previously [6]. Briefly, to assess macrophage and T-lymphocyte infiltration in the kidneys, sections were stained with anti-

F4/80 (#MCA497G, Serotec) and anti-CD3 (clone SP7, Laboratory Vision) antibodies, respectively, according to manufacturer's instructions. On each section, 20 randomly selected fields were then scored in a blinded fashion for the presence of >20 macrophages per high-powered field or scored via computerized morphometric analysis. The severity of perivascular T-cell infiltrates was also scored in a blinded fashion on the basis of a previously established method by assigning renal vessels to the following tertiles: (1) mild, indicating 0 to 10 T cells, (2) moderate, indicating infiltrates containing 11 to 20 T cells, or (3) severe, indicating infiltrates with >20 T cells.[6] To stain glomerular podocytes on kidney sections, we employed a WT1 antibody (Santa Cruz) at a 1:400 dilution per a previously established method.[7] WT1-positive podocytes in each glomerular cross-section were then enumerated with a blinded approach, scoring 15-20 glomeruli per mouse, 6-8 mice per group. Tubular epithelial injury and tubular cast formation were scored by a pathologist (P.R.) masked to experimental conditions using a semi-quantitative scoring system as follows: 0 – none, 1 – mild (1-10% of parenchyma involved), 2 – moderate (11-30%), and 3 – severe (>30%).[8]

Harvest of mouse kidney tubular cells

In order to measure gene expression for CCL5 in kidney tubular cells, primary mouse renal tubular cells were obtained and cultured by following a previously described method.[9] Briefly, mice were anesthetized and flushed with 5 mL ice-cold HBSS. Renal cortices were dissected visually and sliced into small pieces. The fragments were transferred through two layers of nylon sieves (pore size 125 µm and 106 µm). After sieving, mouse tubular fragments were selected and seeded in collagen pre-coated flasks (Corning) with Dulbecco's modified Eagle's/Ham's F12 (DMEM-F12) containing 10% heat inactivated FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The plate was incubated in a standard humidified incubator equipped with 5% CO_2. The medium was changed two days later, and maintained every other day until the cell monolayer reached a confluency of 90%. Immunostaining against megalin (SC-16478, Santa Cruz) confirmed the presence of tubular cells.

Evaluation of lymphocyte populations

At the conclusion of the Ang II-infusion protocol, the thymus, spleen, and kidney were collected for analysis. Single cell suspensions of splenocytes and thymocytes were prepared as described previously,[5, 10] counted, and resuspended in FACS buffer for flow cytometry analysis or in R10 complete medium for *in vitro* culture experiments. To generate single cell suspensions from kidney, kidneys were minced with a scissors, digested with type 1 collagenase for 40 minutes at 37 degrees Celsius, and then passed through a 40µM strainer. Flow cytometry was then performed as previously described[5, 10] using the following antibodies: PE-labeled anti-CD3; PE-Cy5-labeled anti-CD4; FITC-labeled anti-CD8; APC-labeled anti-CD19; PE-labeled anti-Thy1; PerCP-Cy5.5-labeled anti-CD45; PE-labeled anti-CD4; APC-labeled anti-CD3; PerCP-Cy5.5-labeled CD160 (NK T cell); APC-labeled anti-CD25 (all BD Bioscience); FITC-labeled anti-PD-1; PE-labeled anti-TIM-3 (both eBioscience); FITC-labeled anti-F4/80 (Serotec); and corresponding isotype antibodies. After final washing, splenocytes and thymocytes labeled for phenotyping analysis were fixed in PBS containing 4% paraformaldehyde and analyzed within 72 hours. Cells labeled for gene expression analysis were suspended in R10 (splenocytes / thymocytes) or DMEM with 10% FBS (kidney cell suspensions) without fixation, sorted immediately for enumeration and RNA extraction. In the kidney cell suspensions, anti-CD45 expression was used to parse mononuclear cells from renal parenchymal cells. Analyses were performed on a FACSCalibur (BD Biosciences) at the Flow Cytometry Facility at Duke University.

Isolation of RNA and Realtime PCR

Total RNA was isolated from individual cells or tissues by using the RNeasy Mini Kit according to manufacturer's instructions. RNA expression levels were then determined for interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-1β (IL-1β), interleukin-4 (IL-4), monocyte chemoattractant protein 1 (MCP-1), CCL5 (regulated on activation, normal T expressed and secreted), neutrophil gelatinase-associated lipocalin **(**NGAL), CD4, CD8, T-bet, and GATA-3 using TaqMan primers (Applied Biosystems) in real-time PCR, as described previously [4]. RNA expression levels were determined for the AT_{1A} receptor (forward 5'-GCTTGGTGGTGATCGTCACC-3' and reverse 5'-GGGCGAGATTTAGAAGAACG-3' and GAPDH (forward 5'-TCACCACCATGGAGAAGGC-3' and reverse 5'-GCTAAGCAGTTGGTGGTGCA-3') using SYBR Green (Bio-Rad) in real-time PCR.

Microarray Analysis of T cells Infiltrating the Kidney

$CD4^+$ T cells were isolated from the kidneys of Ang II-infused *T cell WT* and *KO* experimental animals via fluorescent cell sorting as described above. RNA was then harvested as above and reverse-transcribed to cDNA, which was subjected to quantitative RT-PCR in a 92-gene Taqman Mouse Immune Response Array (Applied Biosystems; n=3 per group). The TaqMan Mouse Immune Array is a 384-well microfluidic card based system. The primers and probe for each assay are preloaded and dried onto the designated duplicate wells. Taqman cards were run on the Applied Biosystems 7900HT real-time PCR. The master mix for each array card was prepared as following: 60 µL of cDNA (20ng total input RNA/ µL) and an equal amount of TaqMan Universal Master mix (Applied Biosystems) were added into a master tube. Each reaction was mixed by pipetting and was dispensed into loading wells on the Taqman low density array (TLDA) card. The final volume in each TLDA well is approximately 1 µL. The cards were centrifuged twice at 300 × *g* for 1 min and placed into the PCR system. The following cycling conditions were used for the TLDA reactions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min.

Cell proliferation assays

For CFSE experiments, splenic lymphocytes were obtained as described above, labeled by CFSE (Renovar) at a concentration of 2µg/ml, and then seeded into 96-well plates at a density of 5×10^3/well, according to a previously published protocol.[11] Wells were pre-coated with 0.1 µg/ml anti-CD3ε antibody (No Azide/Low Endotoxin, BD Bioscience) or vehicle. After culture at 37°C for 72 hours, cells from individual wells were collected, stained with PE-labeled anti-CD4 (Bioscience), and processed for flow cytometric analysis. Proliferation was measured as the geometric Mean Fluorescence Index (MFI), or 100 X the ratio of the geometric mean channel fluorescence in stimulated cells over that in unstimulated cells, as previously described.[12]

Mixed lymphocyte assays were conducted as previously described [13] using irradiated BALB/c ($H\text{-}2^d$) stimulators to activate (C57BL/6 x 129/SvEv)F_1 ($H\text{-}2^b$) lymphocytes in culture for 72hrs followed by pulsing with 0.5 µCi of [^{3}H]thymidine per well for the final 20 hours in culture. Each sample was conducted in quadruplicate and is representative of 3 separate experiments. Values are expressed as specific counts per minute with counts from responder wells only subtracted from counts with both stimulators and responders.

For chronic T cell receptor stimulation assays, splenic lymphocytes were harvested as above and cultured for 7 days on plates pre-coated with vehicle or anti-CD3ε 0.5µg/ml (BD Bioscience). Ang II 1µM (Sigma) or vehicle was added to the culture daily. Media was changed on day 4. Following 7 days, RNA was harvested from the cultures and analyzed for gene expression of IFN-γ and TNF-α as above. Data were collected from duplicate wells for each condition with 3 animals in each experimental group.

ELISPOT Assay for Interferon-γ

ELISPOT plates were coated with anti-IFN-γ capture Ab (4 μg/ml; BD) overnight. The plates were then blocked for 90 minutes and washed with sterile PBS. Splenic lymphocytes were placed in each well ($5x10^5$ cells/well) with vehicle or anti-CD3 Ab for 24 h at 37 deg C. After washing, biotinylated rat anti-mouse IFN-γ detection Ab (4 μg/ml) was added overnight. After washing with PBS/Tween, streptavidin-horseradish peroxidase was added for 90 minutes. The plates were developed using 3-amino-9-ethylcarbazole (AEC) mixed in 0.1 M sodium acetate plus 12ml H_2O_2.[14] The resulting spots were counted on a computer-assisted ELISPOT image analyzer.

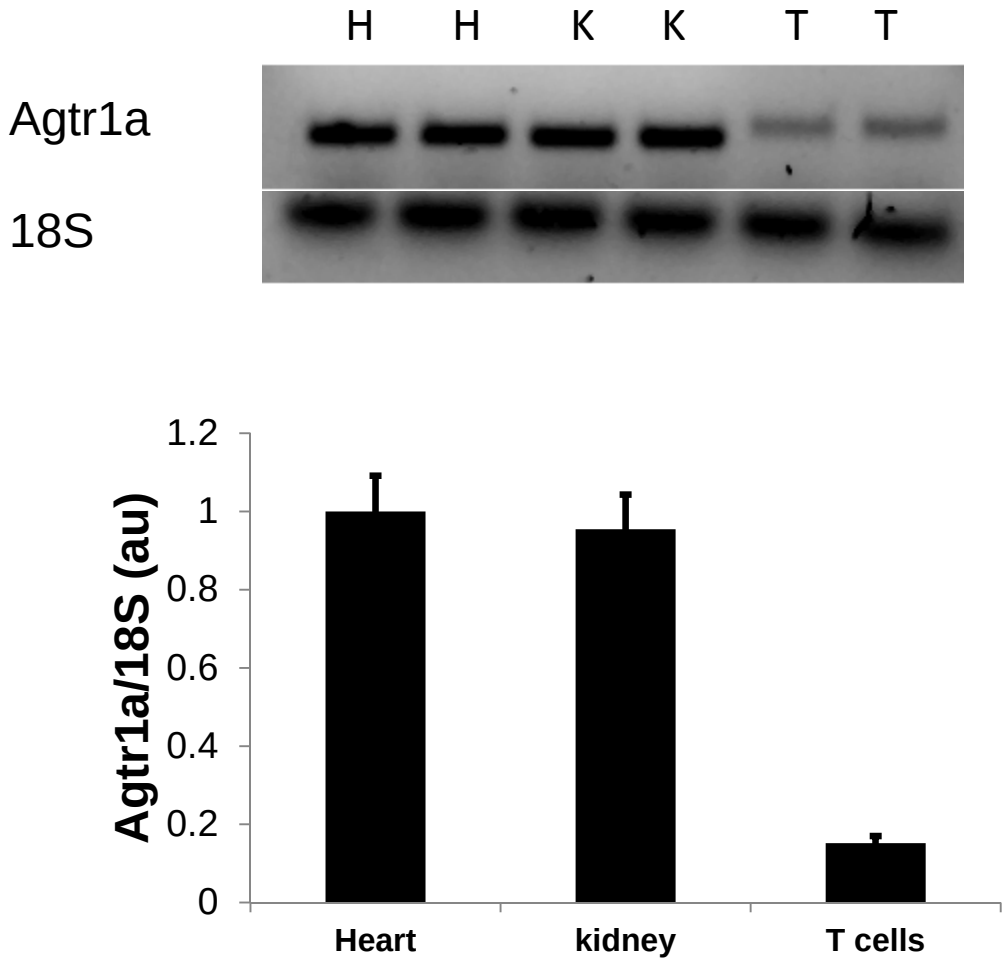
Cytokine ELISA

Splenic lymphocytes were obtained from both *T cell KO* and *WT* mice at the end of the Ang II infusion protocol as above. Cytokines were quantified in the media after the stated time of co-culture of the lymphocytes with anti-CD3ε (NA/LE, BD) or isotope antibody. ELISA assays targeting IFN-γ, TNF-α, and IL-4 were performed with specific kits from Invitrogen according to manufacturer's instructions.

Statistics

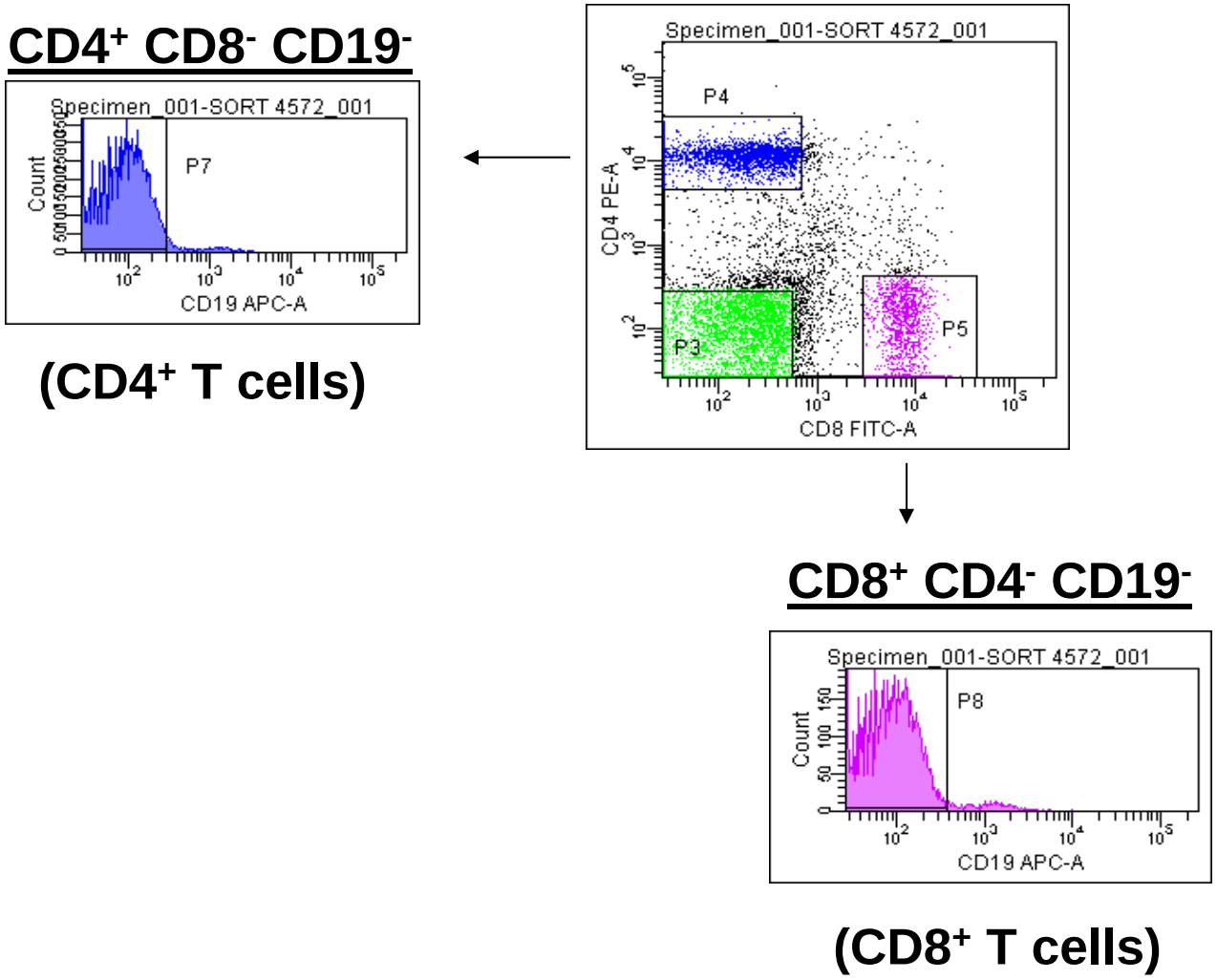
The values of each parameter within a group are expressed as the mean ± the standard error of the mean (SEM). For comparisons between groups with normally distributed data, statistical significance was assessed using ANOVA followed by unpaired student's t test. For comparisons between groups with non-normally distributed variables, the Mann Whitney U test was employed. For comparison within groups, normally distributed variables were analyzed by a paired t test, whereas non-normally distributed variables were analyzed by the Wilcoxon signed rank test. For the comparison of proportions of renal vessels in the experimental groups surrounded by T lymphocyte infiltrates, a chi square test or Fisher's Exact test was used, depending on the category frequencies.

Online Figure I



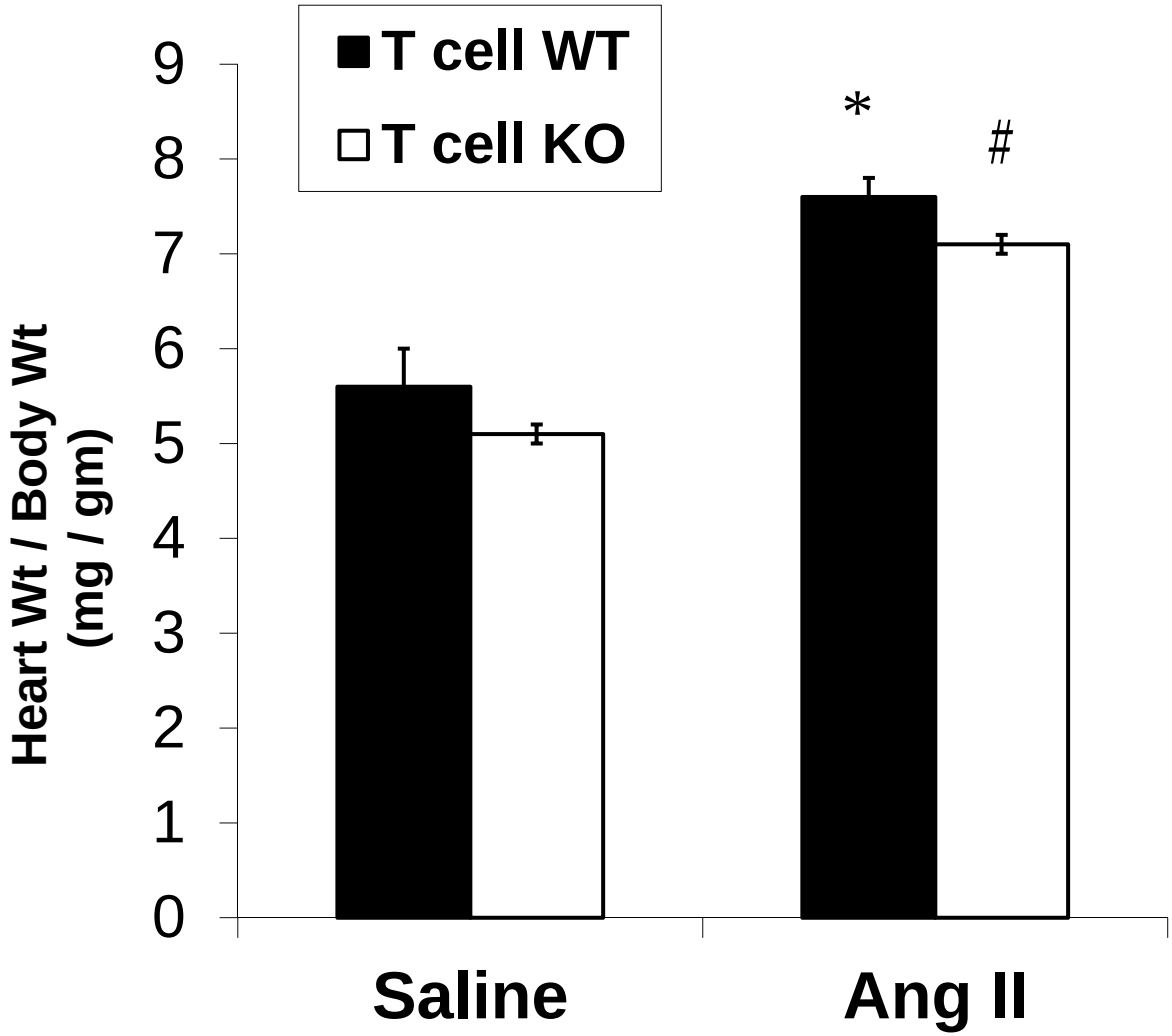
Online Figure I. Detection of AT_{1A} receptor on T lymphocytes. *Agtr1a* mRNA expression measured by Real-time PCR in heart (H), Kidney (K), and T lymphocytes (T) with representative bands on top and summary data on bottom**.**

Online Figure II



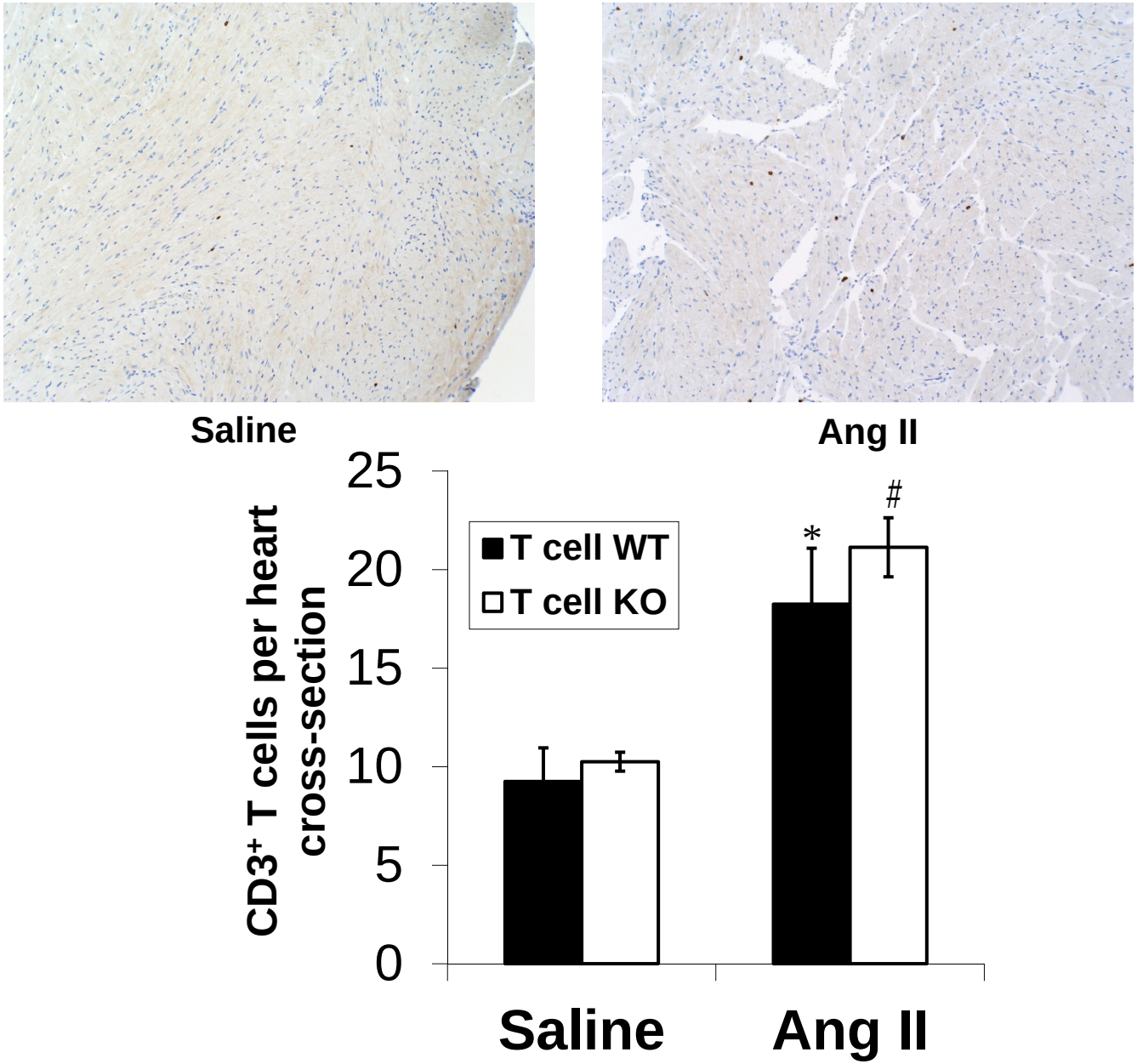
Online Figure II. Isolation of CD4⁺ and CD8⁺ T cells for assessment of cell-specific AT_{1A} receptor deletion in *T cell KO* mice. Diagram of workflow for fluorescent cell sorting of splenic T lymphocytes.

Online Figure III



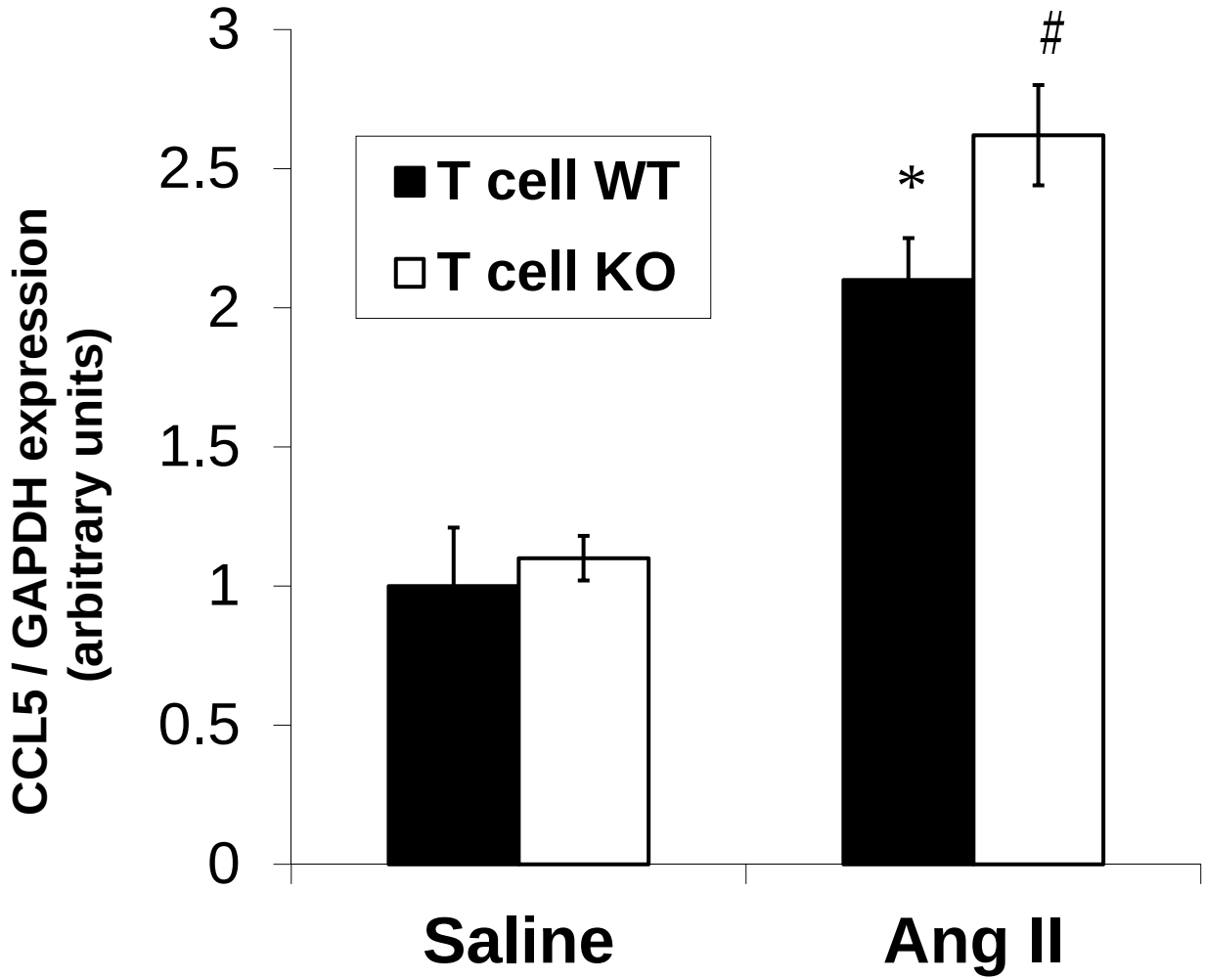
Online Figure III. Ang II induces significant cardiac hypertrophy in *T cell WT* and *KO* animals. Cardiac hypertrophy indices were obtained by calculating the ratio of heart weight (mg) to body weight (g) at day 28 of saline (n=4) or angiotensin II (n=11-12) infusion. **P* = 0.0002 vs. Saline *T cell WT*; $^{\#}P < 0.0001$ vs. Saline *T cell KO*.

Online Figure IV



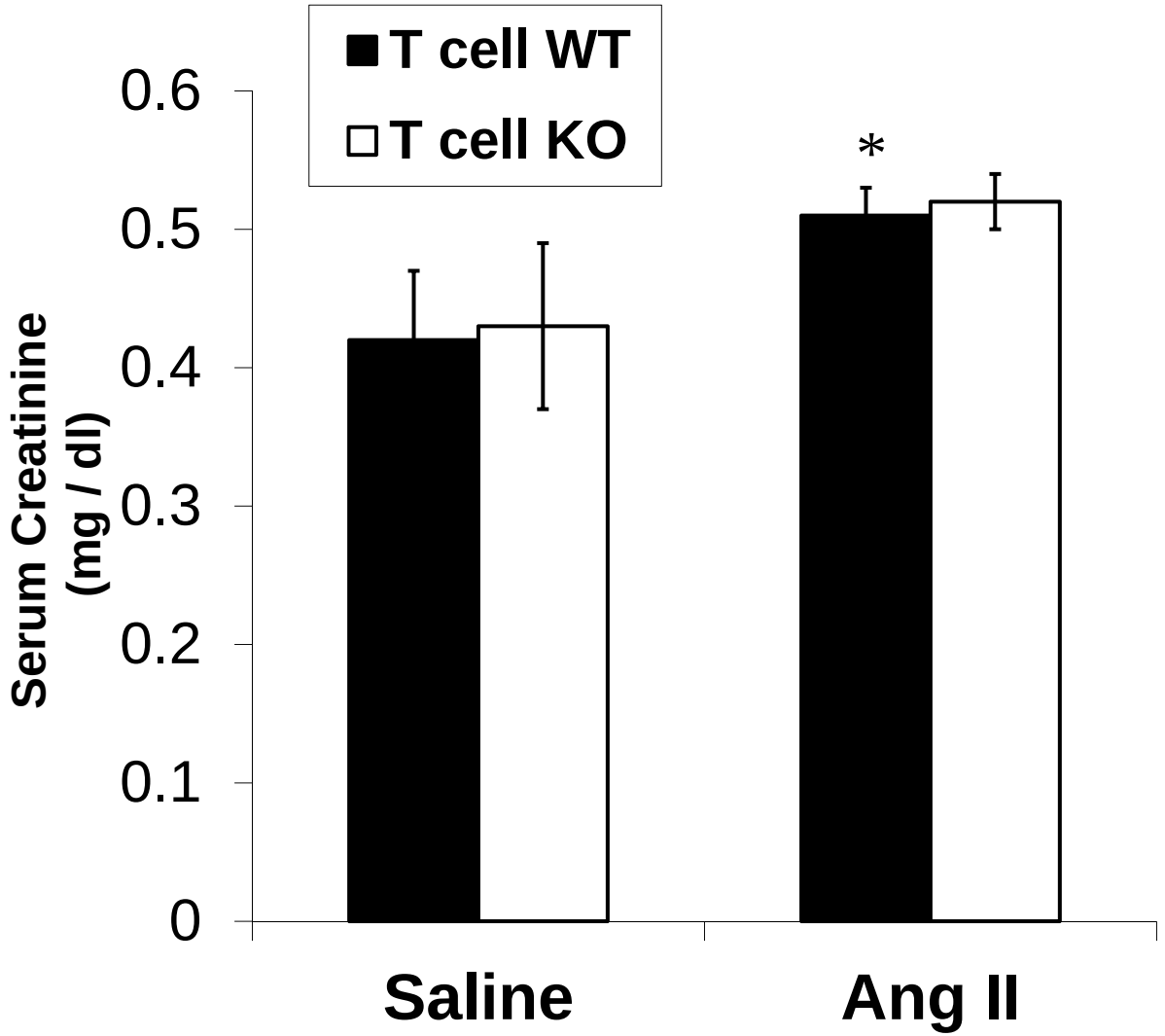
Online Figure IV. T cell infiltration into heart is increased following Ang II. $CD3^+$ T cells were manually counted with a blinded method on stained sections of heart after 28 days of saline (n=4) or angiotensin II (n≥11) infusion. T cell counts were similar in Ang II-infused *T cell WT* and *KO* hearts. Representative heart sections are shown on top with T lymphocytes staining brown. **P* = 0.099 vs. Saline *T cell WT*; $^{\#}P$ = 0.002 vs. Saline *T cell KO*.

Online Figure V



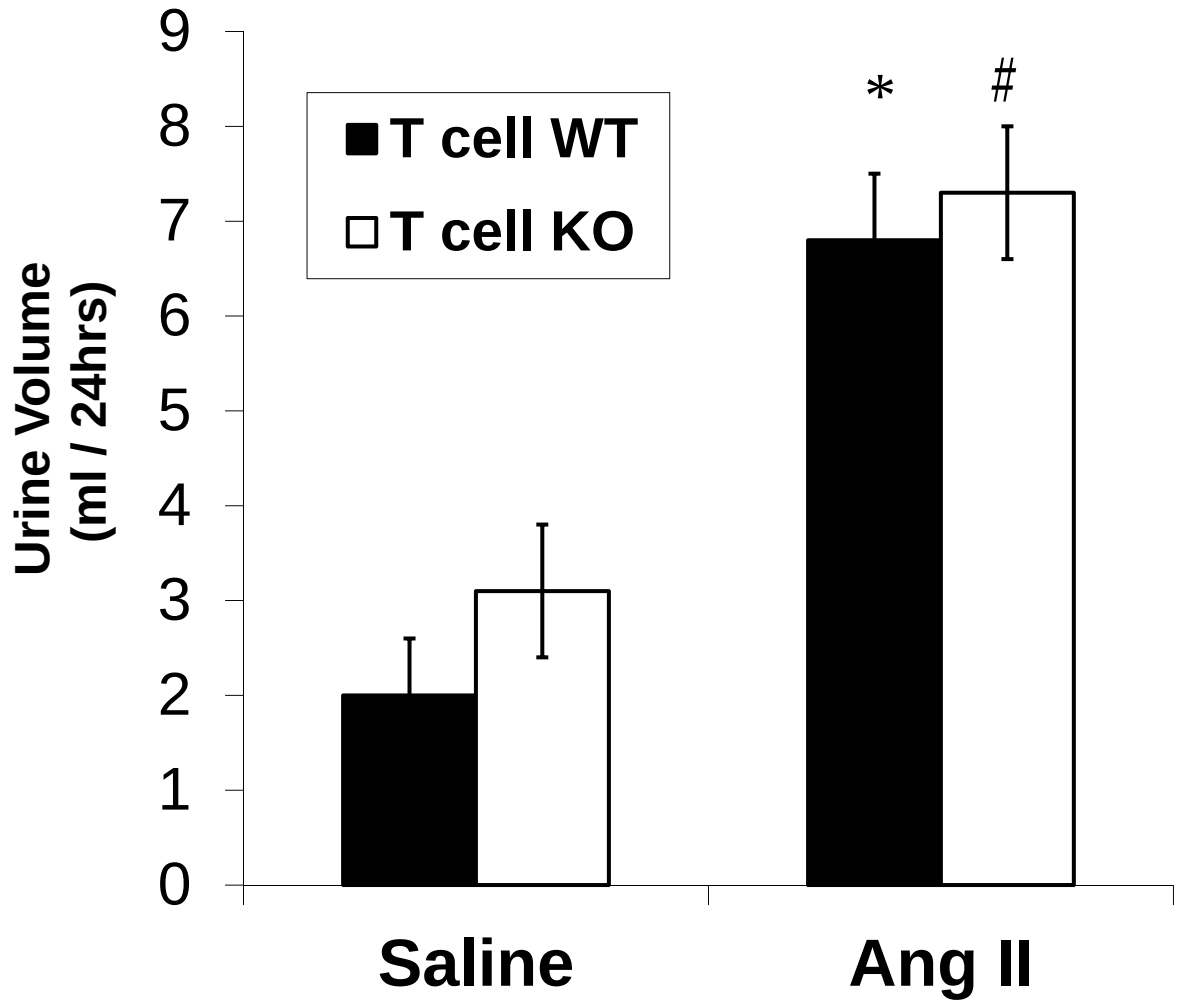
Online Figure V. Ang II upregulates cardiac expression of T cell chemokine CCL5. CCL5 mRNA expression was measured in *T cell WT* and *KO* hearts following saline or Ang II infusion. **P* = 0.002 vs. Saline *T cell WT*; $^{\#}P$ = 0.0002 vs. Saline *T cell KO*, *P* < 0.06 vs. Ang II *T cell WT*

Online Figure VI



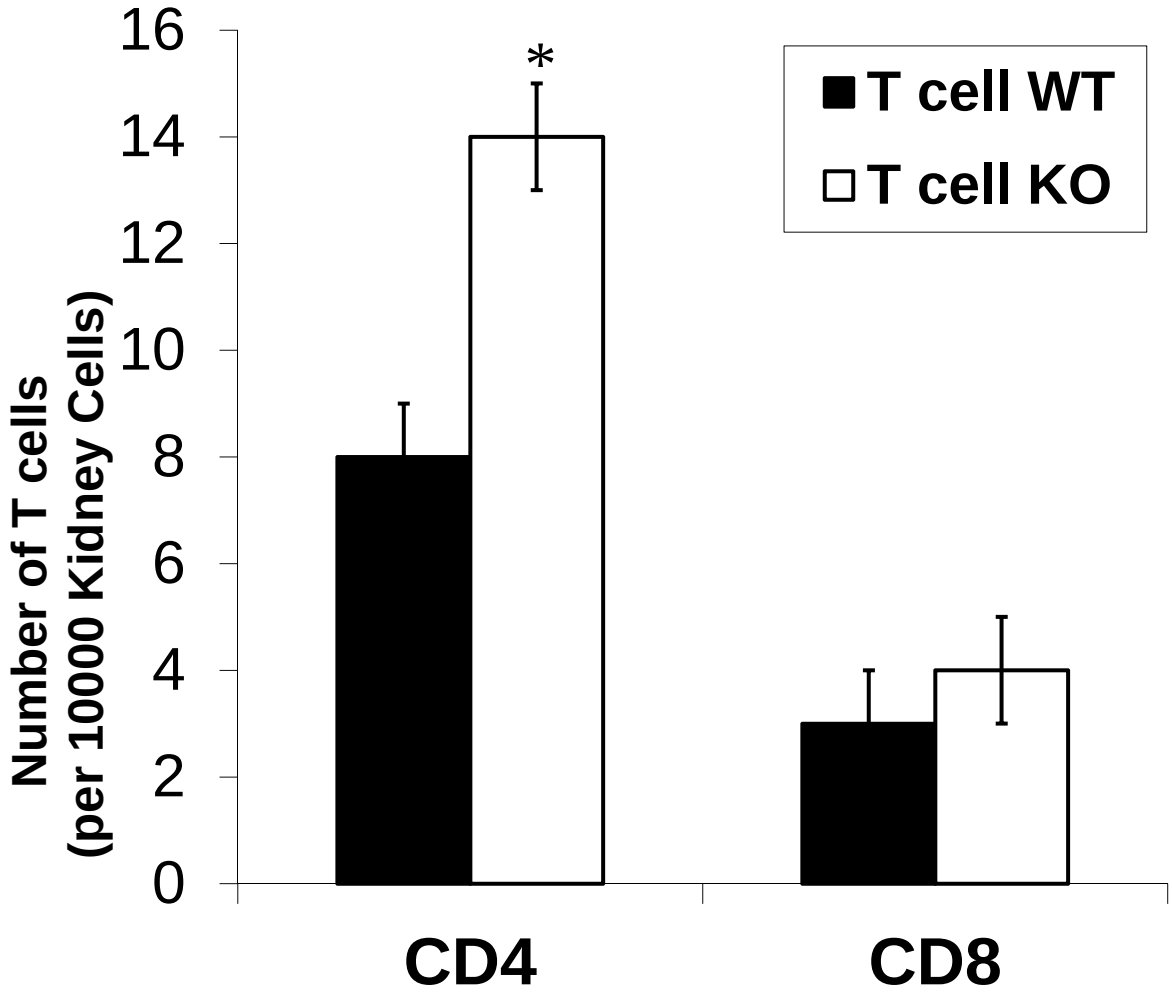
Online Figure VI. Ang II causes rise in serum creatinine in experimental groups. Serum creatinines were measured at day 28 of saline (n=3-4) or angiotensin II (n=14-15) infusion. **P* = 0.07 vs. Saline *T cell WT*.

Online Figure VII



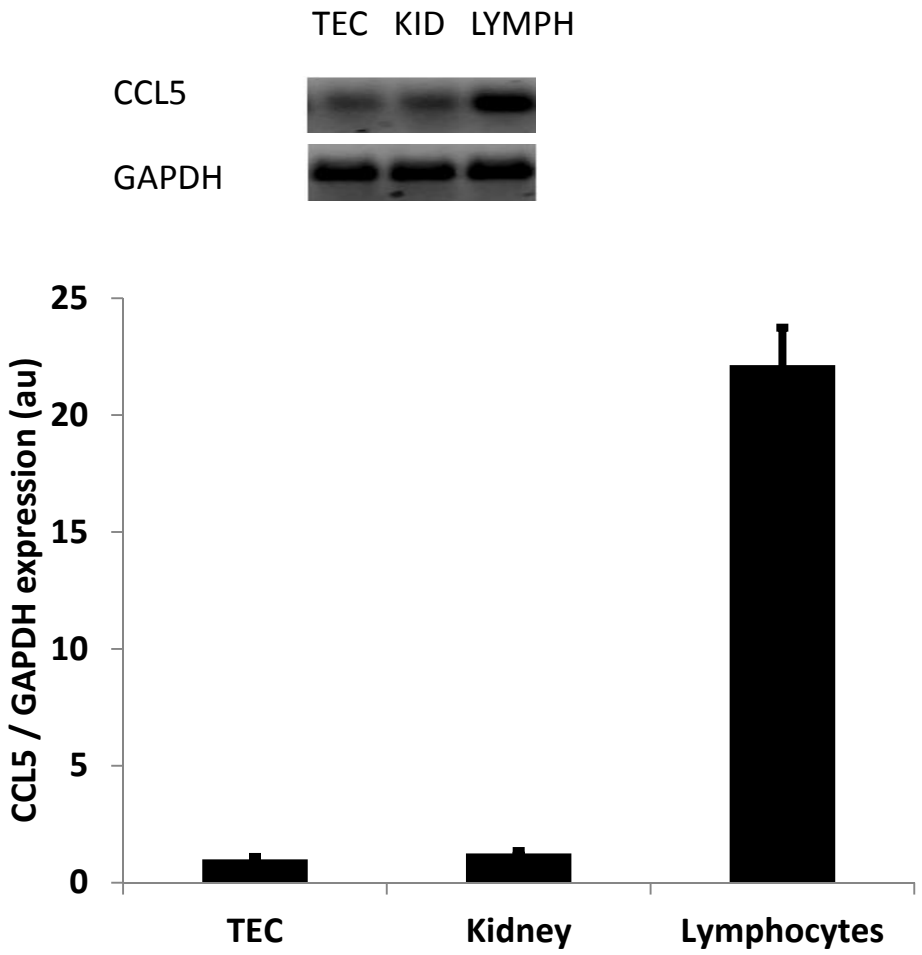
Online Figure VII. Ang II induces increase in urine volumes in *T cell WT* and *KO* mice. Urine volumes were measured at day 25 of saline (n=4) or angiotensin II (n≥13) infusion. **P* = 0.003 vs. Saline *T cell WT*; $^{\#}P$ = 0.01 vs. Saline *T cell KO*.

Online Figure VIII



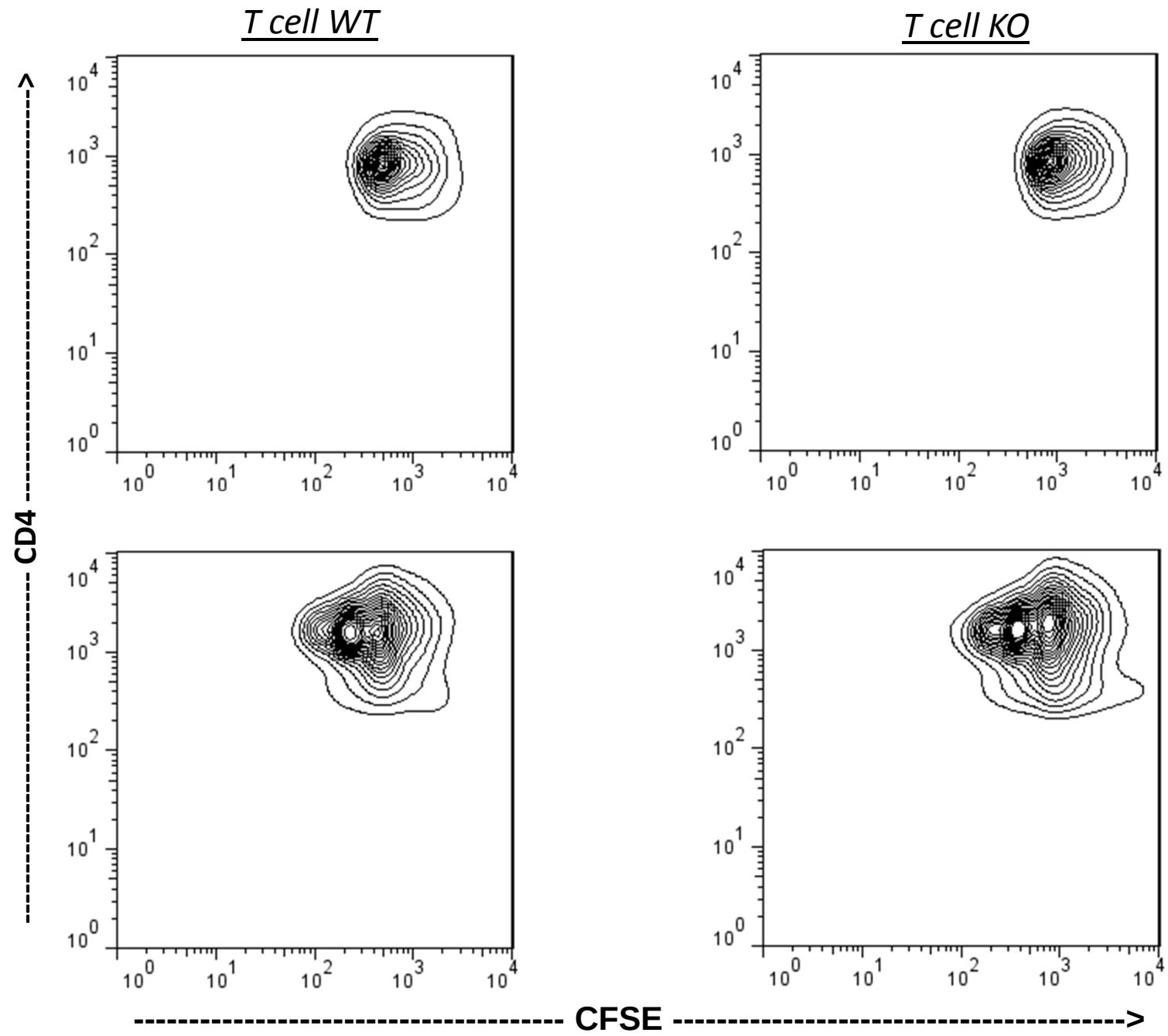
Online Figure VIII. *T cell KO* mice have increased numbers of CD4$^+$ T lymphocytes in the kidney following 4 weeks of Ang II infusion. Kidneys from Ang II-infused animals were digested with collagenase to single cell suspensions. T lymphocytes were then isolated through fluorescent cell sorting as illustrated in Online Figure I, and the numbers of CD4$^+$ and CD8$^+$ T cells were counted. *$P < 0.04$ vs. *T cell WT.*

Online Figure IX



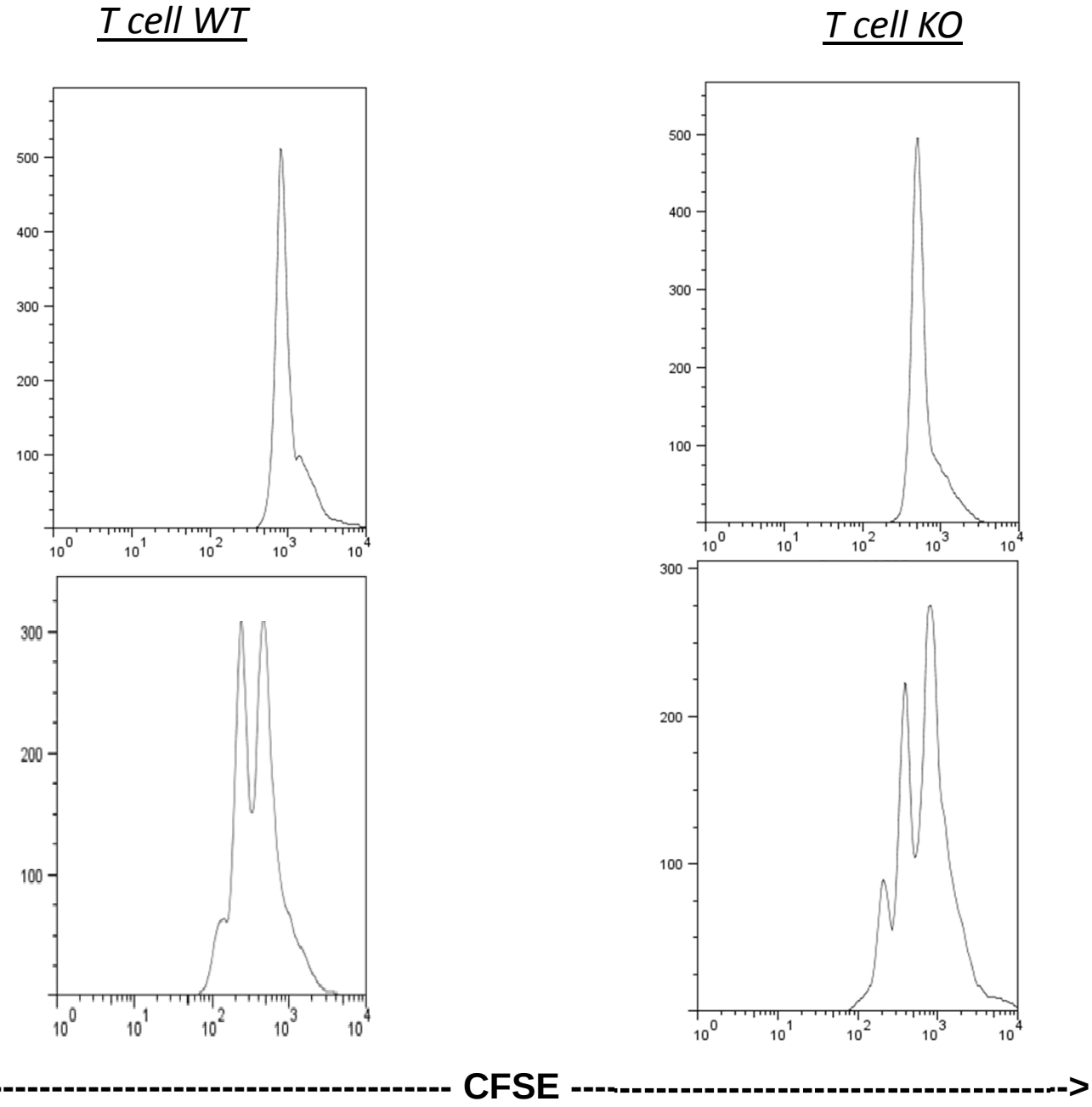
Online Figure IX. CCL5 is expressed in kidney tubular epithelium but at lower levels than in lymphocytes. mRNA expression of CCL5 in freshly isolated wild-type kidney tubular epithelial cells (TEC), whole kidney (KID), and lymphocytes (LYMPH) with representative gel on top and summary data on bottom.

Online Figure XA



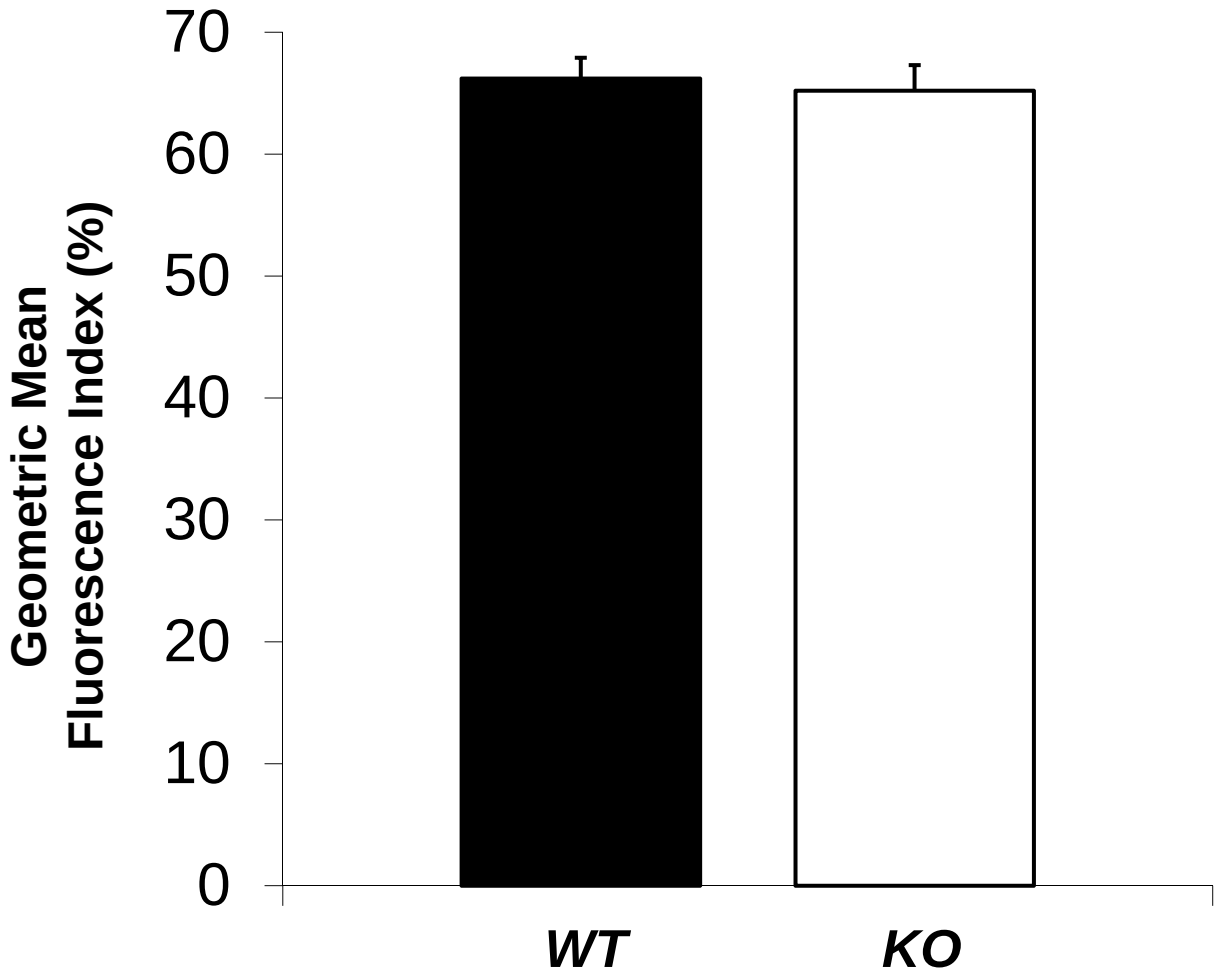
Online Figure XA, Contour plots of proliferative response measured by CFSE method in splenic $CD4^+$ T cells from *T cell WT* and *KO* mice in absence ("Resting") and presence ("Stimulated") of T cell receptor activation (n=3).

Online Figure XB



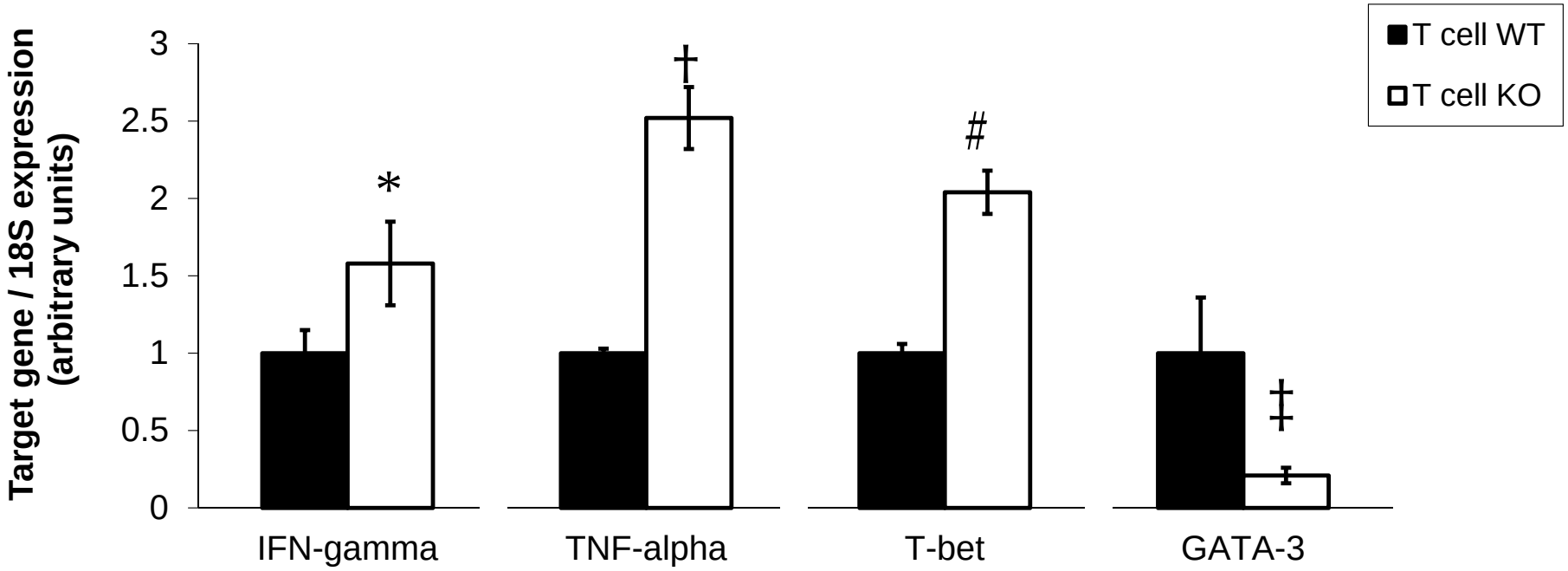
Online Figure XB, Same representative CFSE proliferation data from Figure XA displayed as histograms to reveal cellular division peaks. The $CD4^+$ T cells from both groups manifested 1 full division and initiated a second division during 72hr of T cell receptor stimulation with anti-CD3ε.

Online Figure XC



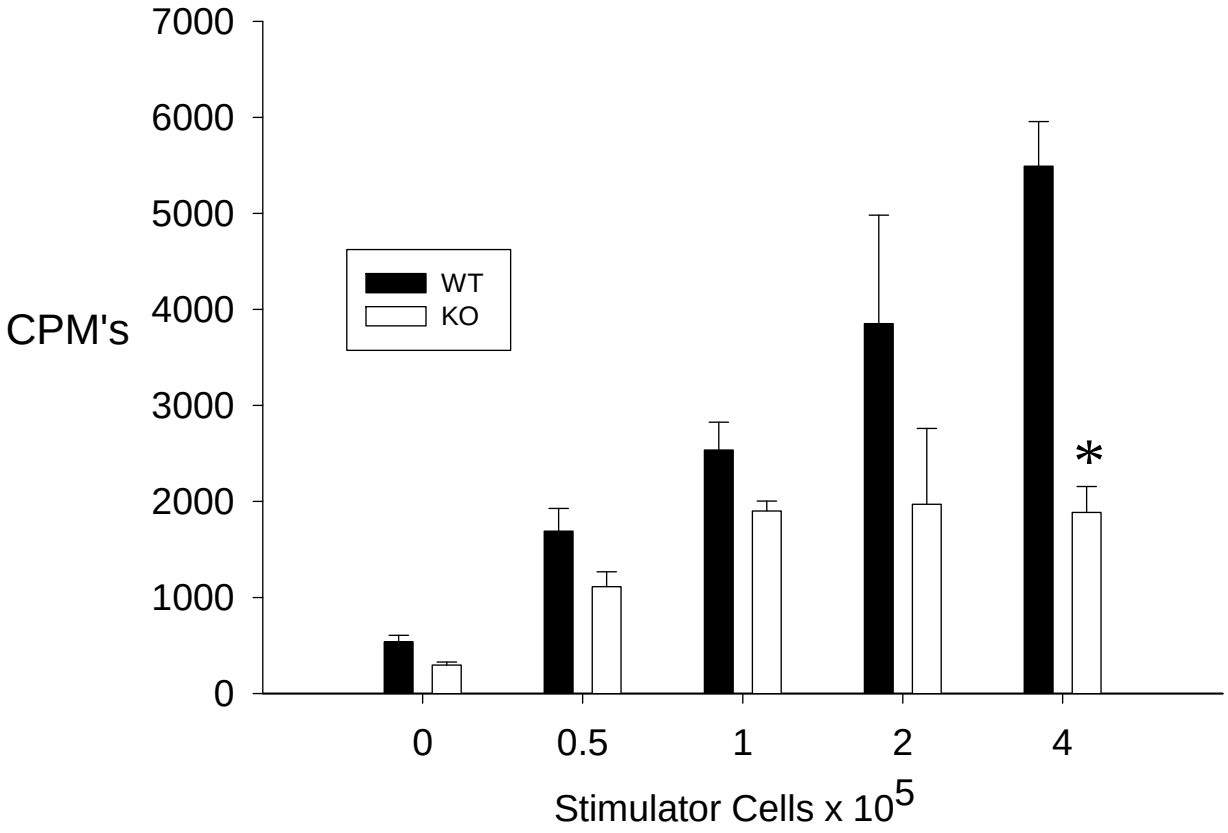
Online Figure XC, Summary data of geometric mean fluorescence index for $CD4^+$ T lymphocytes from *T cell WT* and *KO* animals [100 x (mean fluorescence of stimulated cells / mean fluorescence of resting cells)] calculated from 3 independent experiments.

Online Figure XI



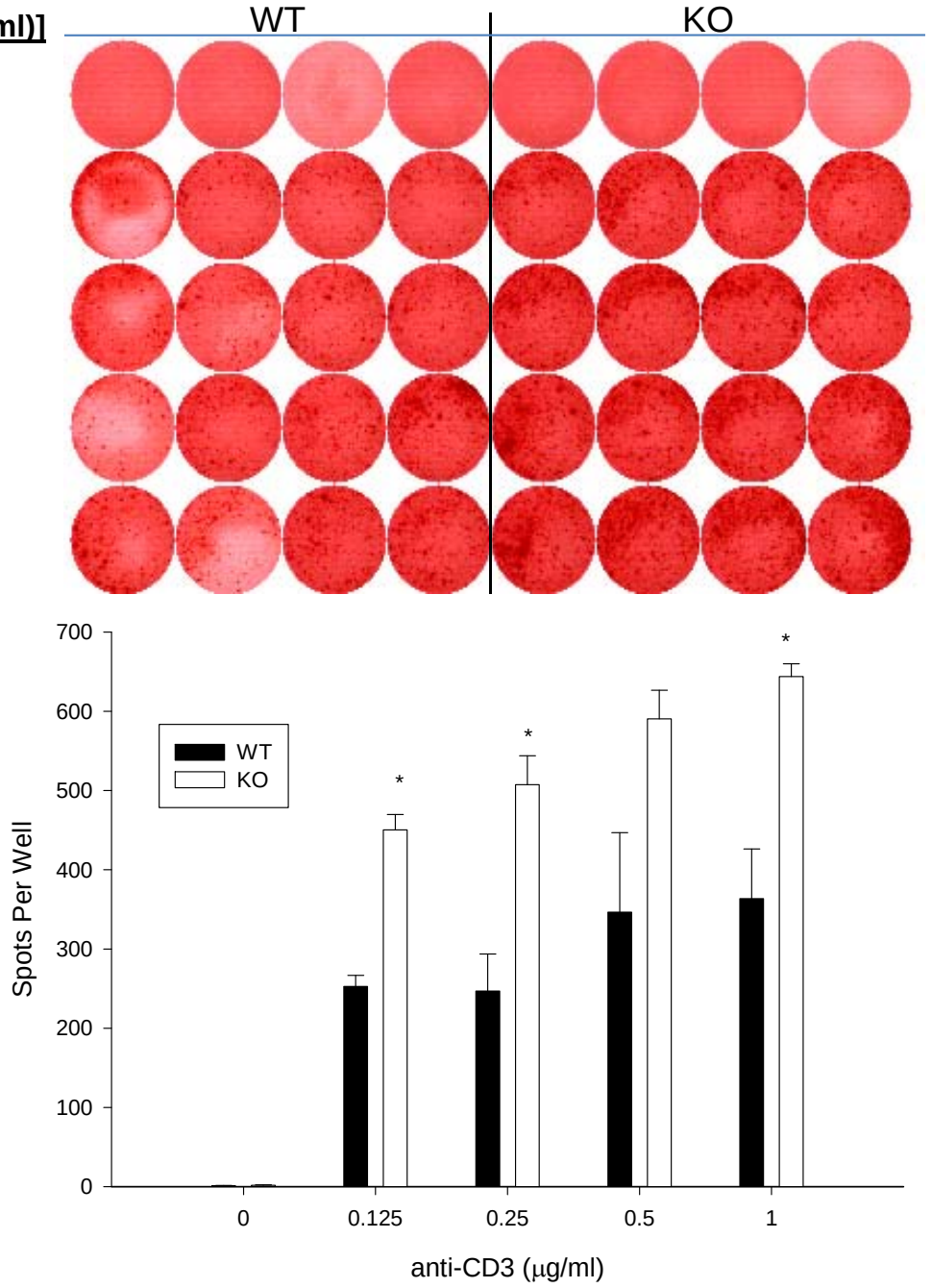
Online Figure XI, Expressions of IFN-γ, TNF-α, T-bet, and GATA-3 mRNA in $CD4^+$ T cells isolated from the kidneys of Ang II infused-*T cell WT* and *KO* mice. **P* = 0.03 vs. *T cell WT*; †*P* < 0.0002 vs. *T cell WT*; #*P* = 0.0003 vs. *T cell WT;* ‡*P* < 0.02 vs. *T cell WT.*

Online Figure XII



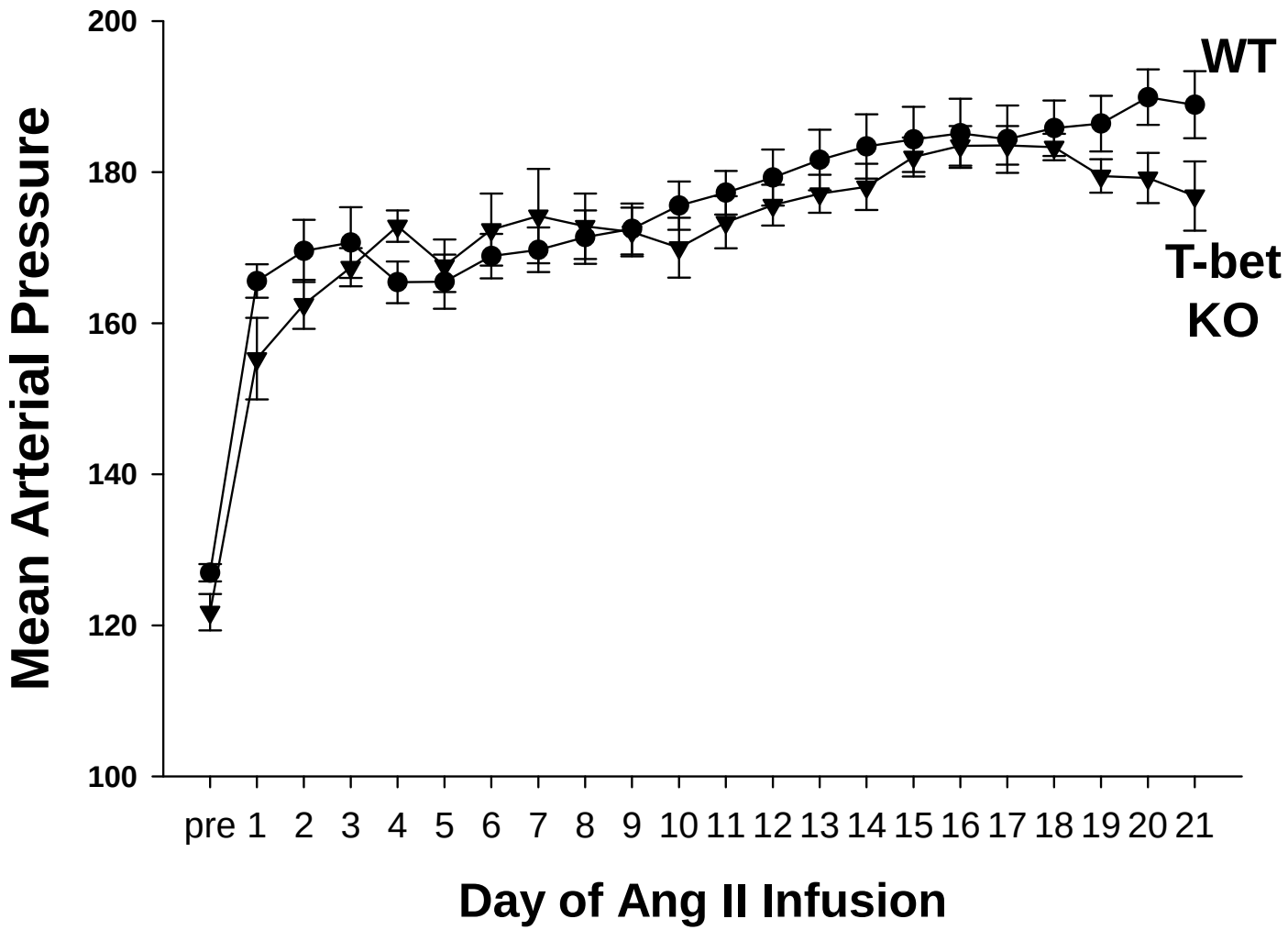
Online Figure XII. Mixed Lymphocyte Assay. Proliferative response as measured by thymidine incorporation in naïve (C57BL/6 x 129/SvEv)F_1 WT or *Agtr1a*$^{-/-}$ KO lymphocytes stimulated by irradiated splenocytes (Stimulators) from BALB/c (H-2^d) mice. "CPM's"= counts per minute. $^*P$ = 0.0005 vs. *WT.*

Online Figure XIII



Online Figure XIII. IFN-γ ELISPOT Assay. Frequency of IFN-γ-producing lymphocytes identified by ELISPOT following T cell receptor stimulation with incremental doses of anti-CD3ε. * *P* < 0.006 vs. *WT.*

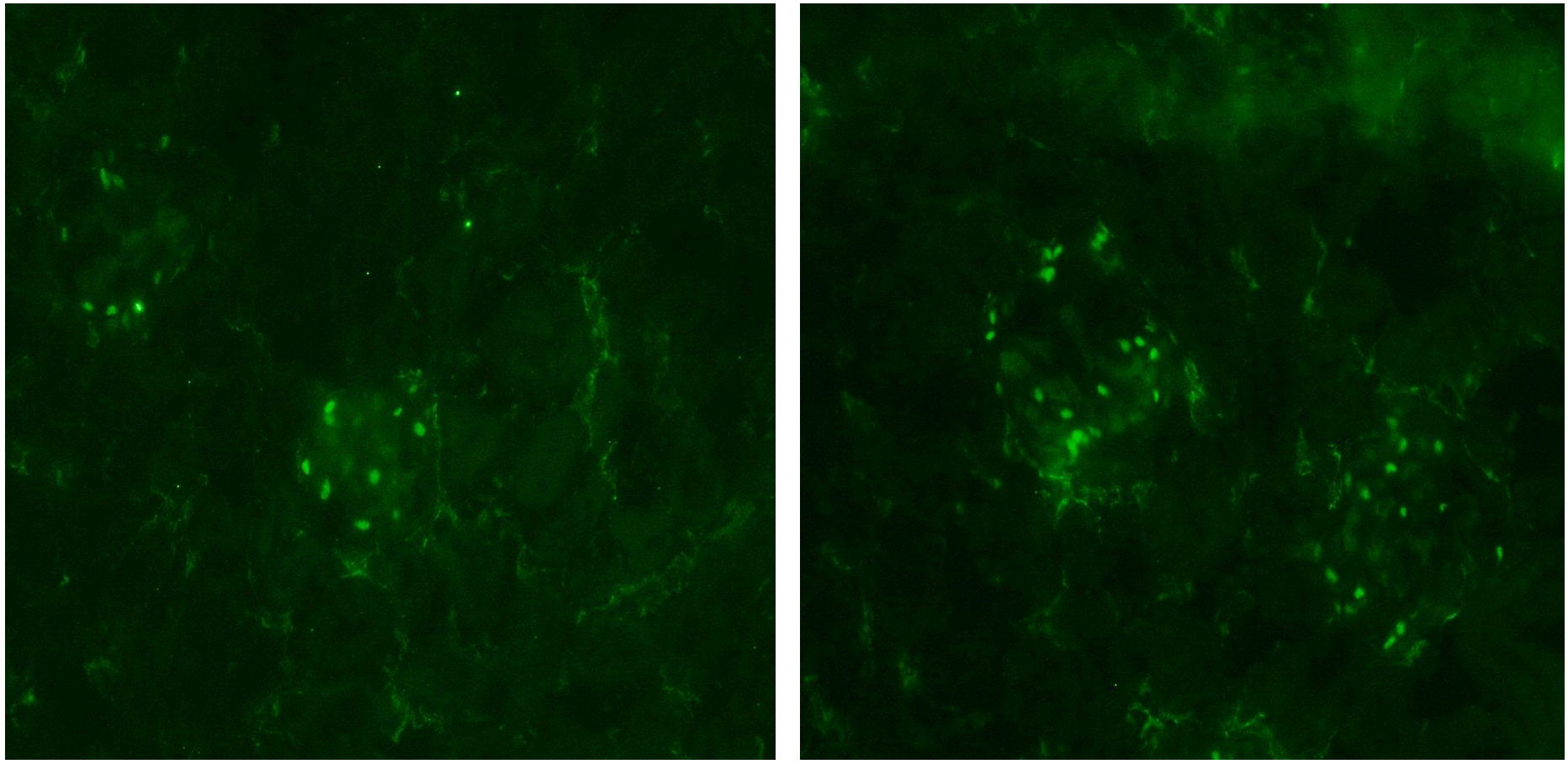
Online Figure XIVA



Online Figure XIV. T-bet deficiency ameliorates hypertensive kidney damage. A, Blood pressures measured by radiotelemetry in uni-nephrectomized *WT* and *T-bet KO* mice at baseline and during chronic Ang II infusion.

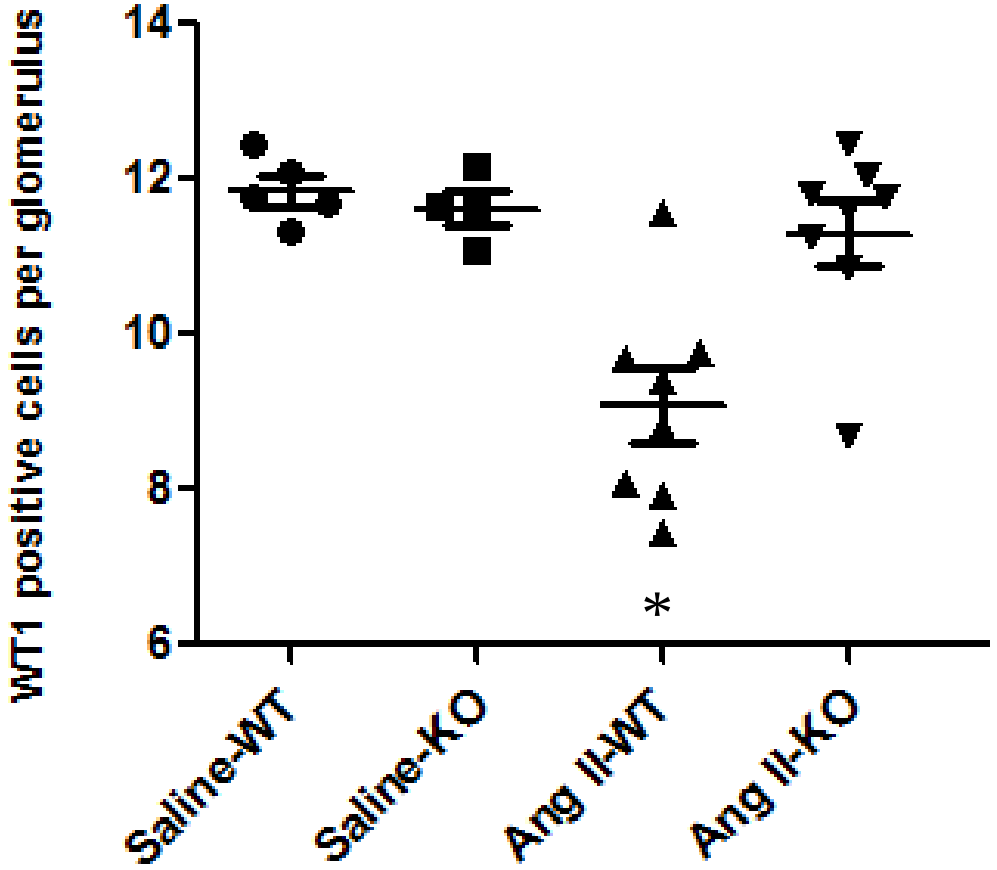
Online Figure XIVB

XIVC



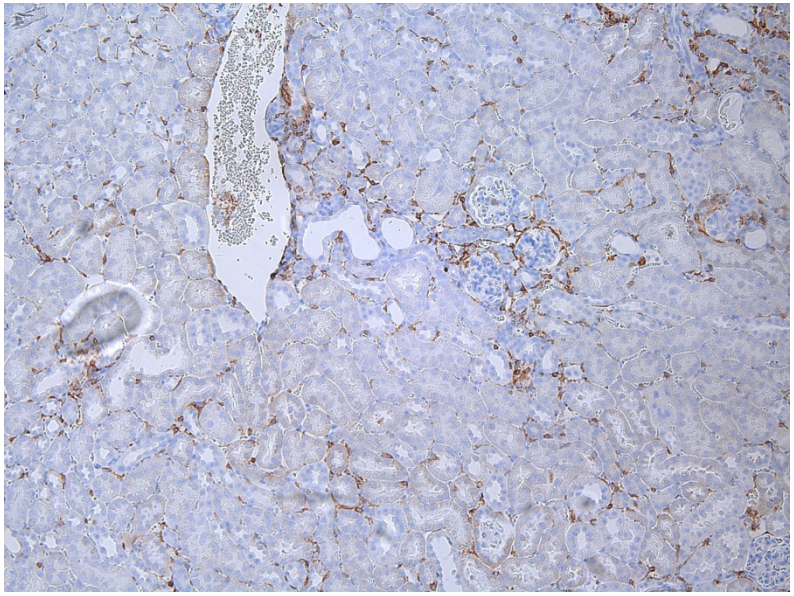
Online Figure XIVB-C, Staining of glomerular podocytes with WT1 following 4 weeks of Ang II. Podocytes stain bright green. Representative images of **(B)** *wild-type* and **(C)** *T-bet KO* glomeruli. (Magnification 40X)

Online Figure XIVD

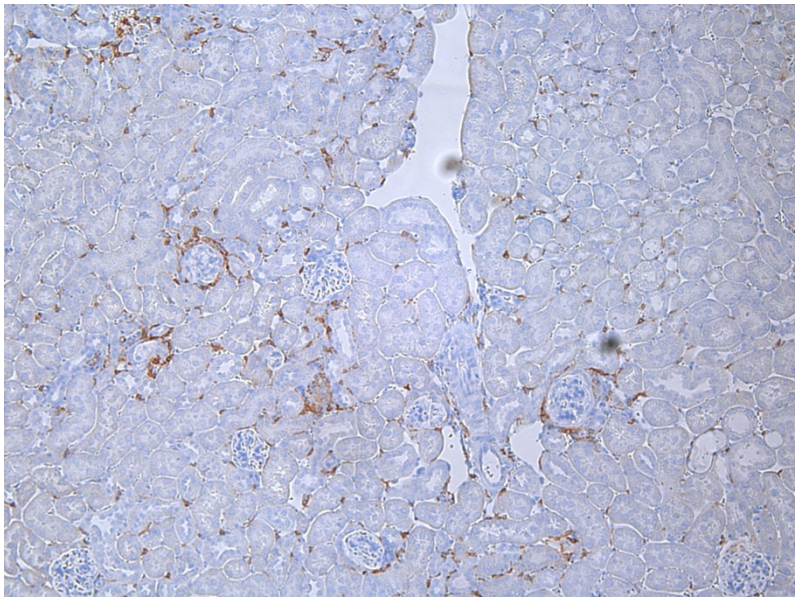


Online Figure XIVD, Number of podocytes per glomerulus in WT and T-bet KO ("KO") kidneys scored in blinded fashion following 4 weeks of Ang II (8 mice per group) or saline (4-5 mice per group). **P*<0.003 vs. Ang II-KO, *P* = 0.0009 vs. Saline-WT.

Online Figure XIVE

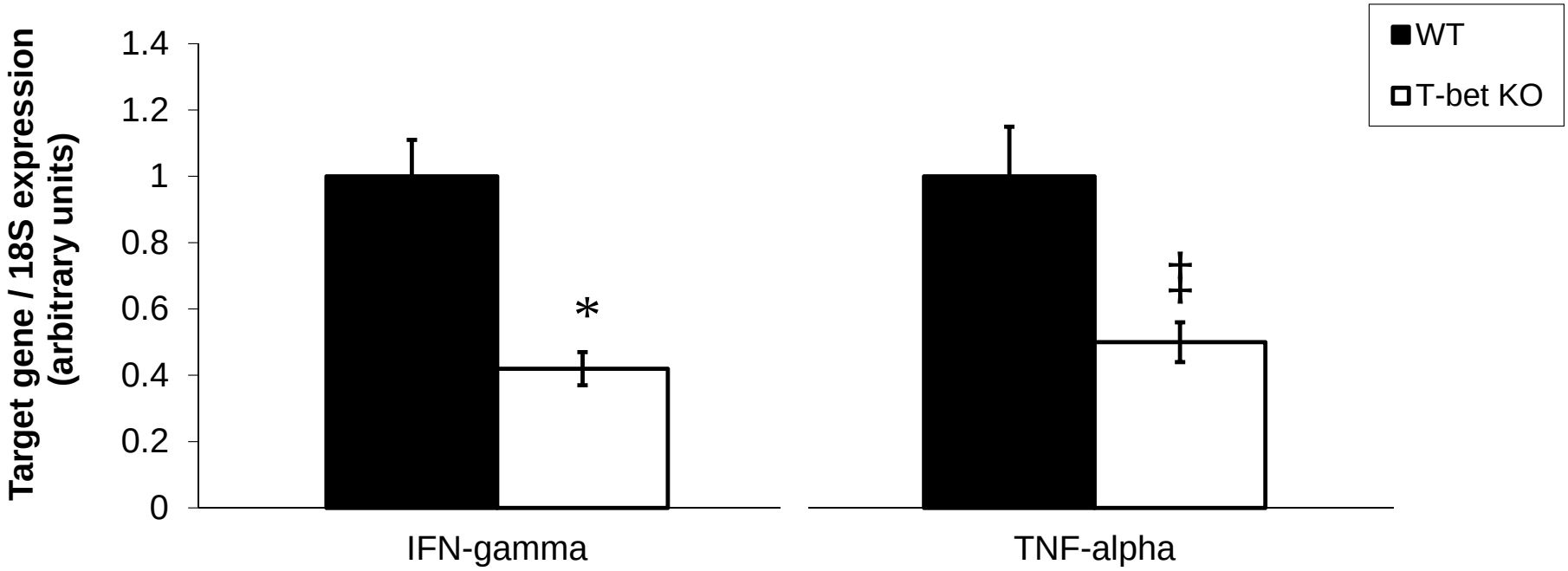


XIVF



Online Figure XIVE-F, T-bet-deficiency blunts Ang II-induced macrophage accumulation in kidney. F4/80^{+} macrophage stains in representative kidneys from Ang II-infused **(E)** *WT* and **(F)** *T-bet KO* mice.

Online Figure XIVG



Online Figure XIVG, mRNA expressions of IFN-γ and TNF-α in *WT* and *T-bet KO* kidneys following Ang II. **P* < 0.0002 vs. *WT*; ‡*P* < 0.005 vs. *WT*.

Online Table I. Genes with expression altered by >1.5 fold in $CD4^+$ T cells isolated from kidneys of Ang II-infused *T cell WT* and *KO* animals. P-values are given for differences achieving at least borderline significance ($P \leq 0.06$). "Increased" indicates increased in *T cell KO* group.

Gene (Increased > 1.5 fold)	Fold	*P* value
Cd28	2.45094	0.006
Cd38	15.41931	0.004
Cd3e	2.69821	0.01
Cd4	1.77440	
Cxcr3	4.85454	0.02
Lrp2	13.92237	
Stat4	2.56744	0.0003
Nfkb1	1.67520	
Gene (Decreased > 1.5 fold)	Fold	
Ccr2	0.51323	
H2-Eb1	0.37701	
Il1b	0.70825	
Il2ra	0.00018	0.06
Pgk1	0.05688	
Ptprc	0.42347	
Smad3	0.42259	
Stat1	0.11633	
Stat3	0.28100	
Stat6	0.18664	
Tnfrsf18	0.40810	

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