Sex Differences in T-Lymphocyte Tissue Infiltration and Development of Angiotensin II Hypertension

Dennis P. Pollow, Jennifer Uhrlaub, Melissa J. Romero-Aleshire, Kathryn Sandberg, Janko Nikolich-Zugich, Hedwenn L. Brooks,* Meredith Hay*

See Editorial Commentary, pp 237–239

Abstract—There is extensive evidence that activation of the immune system is both necessary and required for the development of angiotensin II (Ang II)–induced hypertension in males. The purpose of this study was to determine whether sex differences exist in the ability of the adaptive immune system to induce Ang II–dependent hypertension and whether central and renal T-cell infiltration during Ang II–induced hypertension is sex dependent. Recombinant activating gene-1 (Rag-1–/–) mice, lacking both T and B cells, were used. Male and female Rag-1–/– mice received adoptive transfer of male CD3+, CD8+, and regulatory Foxp3+–CD4+ T-cell subsets identified that renal lymphocyte infiltration was significantly increased in males versus both control and Ang II–infused animals (P<0.05). Immunohistochemical staining for CD3+–positive T cells in the subfornical organ region of the brain was increased in males when compared with that in females. These results suggest that female Rag-1–/– mice are protected from male T-cell–mediated increases in Ang II–induced hypertension when compared with their male counterparts, and this protection may involve sex differences in the magnitude of T-cell infiltration of the kidney and brain. (Hypertension. 2014;64:384-390.) ● Online Data Supplement

Key Words: angiotensin II ■ hypertension ■ kidney ■ sex characteristics ■ subfornical organ ■ T-lymphocytes

Emerging clinical and experimental data suggest that inflammation, and adaptive immunity in particular, is an important contributor to the development of hypertension.1,2 Angiotensin II (Ang II)–induced hypertension has been shown to involve inflammatory mechanisms in the peripheral vasculature, the kidney, and the central nervous system.3–6 Experimental studies have provided extensive evidence that the activation of the immune system is both necessary and required for the development of Ang II–induced hypertension in males. In male mice deficient for the recombinant activating gene-1 (Rag-1–/–), which lack both B and T cells, increases in blood pressure (BP) after Ang II infusion are significantly attenuated when compared with wild-type mice.3,5 When T cells were transferred back into the male Rag-1–/––deficient mice (adoptive transfer), the hypertensive effects of Ang II were restored. Target organ lymphocyte infiltration is thought to contribute to the development of hypertension in males. Renal infiltration of lymphocytes is known to be associated with increases in BP in Ang II–dependent and salt-sensitive hypertension.6–10 Recent studies support an essential role for the central nervous system and subfornical organ (SFO) in the induction and maintenance of Ang II–dependent hypertension, which is associated with peripheral activation of lymphocytes and tissue infiltration.11,12

To date, however, there is limited information on the role of the immune system in the development of hypertension in females. Sex-specific differences in the development of hypertension are well documented.12–14 It has been proposed that 17β-estradiol delays or prevents the onset of cardiovascular disease and hypertension and may function to keep women cardiovascularly younger than men of the same age. Similar observations have been made in experimental models of cardiovascular regulation and hypertension.15,16 The underlying mechanisms involved in the relative protection of females from hypertension involve multiple end organs and systems,
including the peripheral vasculature, renal function, central regulation of sympathetic outflow, and likely include the adaptive immune system.19–21

Both the kidney and the brain are known to be important in the development of Ang II–dependent hypertension. The SFO has dense angiotensin type 1 receptor expression and innervates the paravascular neurons of the periventricular nucleus, which are known to be involved in the regulation of sympathetic outflow and BP. Important to the present study, the SFO is richly endowed with sex steroid receptors, including estrogen receptors α and β.22 Previously, we have demonstrated that estrogen receptor-α are expressed in SFO neurons, and it has been shown that 17β-estradiol alters the physiological responses of SFO neurons to Ang II.23

The purpose of the present study was to determine whether the sex of the Rag-1–/– host affects the ability of male T cells to restore the magnitude of the Ang II–induced hypertension. Furthermore, we investigated whether T-cell infiltration into the kidney and brain was affected by the sex of the Rag1–/– host after adoptive transfer of male T cells and Ang II infusion.

Methods

Detailed descriptions of the animals, methods, and statistics can be found in the online-only Data Supplement. All methods were approved by the University of Arizona Animal Care and Use Committee (Figure 1).

Results

No Sex Differences Were Observed in Basal Systolic BP and Heart Rate in Rag-1–/– Mice

Baseline systolic BP (SBP) values prior to male T-cell adoptive transfer and Ang II infusion were not significantly different between male and female Rag-1–/– mice (Table 1). Mean heart rate between groups was also similar at baseline.

No Sex Differences Were Observed in the Magnitude of Ang II–Induced Hypertension in T-Cell–Deficient Rag-1–/– Mice

Ang II infusion significantly increased SBP in both male and female Rag-1–/– mice; however, there were no sex differences in the magnitude of the hypertension (Rag1–/– vs Ang II, Δ22.1±5 versus CD3M–→Rag1–/– → M+Ang II, Δ18±4 mm Hg; P<0.05 versus baseline; Figure 2A). Ang II infusion did not significantly change heart rate in either sex (Figure 2B).

Sex Differences Were Observed in Ang II–Induced Hypertension in Rag-1–/– Mice After Adoptive Transfer of Male T Lymphocytes

As reported previously,1,3 adoptive transfer of male T cells into male Rag-1–/– mice significantly augmented the Ang II–induced increase in SBP (Rag1–/–→M+Ang II, Δ22.1±5 versus CD3M–→Rag1–/–→M+Ang II, Δ23.7±7 mm Hg; P<0.05; Figure 2A). In contrast, there were no significant differences in the Ang II–induced SBP response after adoptive transfer of male T cells into female Rag-1–/– mice (Rag1–/–→F+Ang II, Δ18±4 versus CD3M–→Rag1–/–→F+Ang II, Δ13.7±7 mm Hg; Figure 2A). There were no sex differences in heart rate in the Rag-1–/– mice after adoptive transfer of male T cells and Ang II infusion (Figure 2B).

Sex Differences in Renal T-Cell Infiltration Were Independent of Ang II Infusion in Rag-1–/– Mice After Adoptive Transfer of Male T Lymphocytes

After adoptive transfer of male T cells into male and female Rag-1–/– mice, kidney and brain tissues were analyzed via flow cytometry to determine whether there were sex differences in the degree of T-cell infiltration in the presence and absence of Ang II infusion. The number of CD3+, CD3CD4+, CD3CD8+, or CD3CD4Foxp3+ T cells found in the spleen (Figure 3A–3D) was similar among all groups, demonstrating that T-cell engraftment after adoptive transfer was similar between the sexes.

Basal renal T-cell infiltration for all T-cell subtypes measured was greater in the male when compared with the female Rag-1–/– host after adoptive transfer of male T cells (Figure 3E–3H); however, Ang II infusion had no effect on renal T-cell infiltration in either sex.

Flow cytometric analysis from whole-brain homogenates demonstrated a trend for greater T-cell infiltration in male when compared with that in female Rag-1–/– mice; however, this trend did not reach significance (Figure 4A–4D). Furthermore, similar to the kidney, there was no effect of Ang II on the number of brain infiltrating T cells.

Sex Differences Were Observed in the Ang II–Induced Inflammatory Response in Rag-1–/– Mice After Adoptive Transfer of Male T Lymphocytes

Ang II–induced renal inflammation contributes to the hypertensive response in male mice and is markedly attenuated in females.8,9,18 To determine whether altered T-cell function contributes to the reduction in Ang II–induced renal inflammation in females, we measured renal expression...
of the inflammatory cytokines interleukin-2, tumor necrosis factor-α, and monocyte chemoattractant protein-1 using quantitative real-time polymerase chain reaction in male and female Rag-1−/− mice (Table 2). Renal expression of interleukin-2, tumor necrosis factor-α, and monocyte chemoattractant protein-1 was significantly upregulated by Ang II infusion in CD3+/→ Rag1−/−M when compared with that in Rag1−/−M. Conversely, there were no differences in the expression of these cytokines between CD3+/→ Rag1−/−F and Rag1−/−F after 14-day Ang II infusion, suggesting that during Ang II infusion, T-cell–dependent renal inflammation is attenuated in females and may contribute to the female resistance against Ang II–induced hypertension.

Sex Differences Were Observed in T-Cell Infiltration Into the SFO of Rag-1−/− Mice After Adoptive Transfer of Male T Cells and Ang II Infusion

Activation of inflammatory processes within the SFO contributes to maintaining Ang II–induced hypertension.24–26 These inflammatory processes, such as Ang II–induced production of reactive oxygen species, are inhibited by activation of estrogen receptors.27,28 Immunohistochemical techniques were used to determine whether sex differences exist in T-cell infiltration into the SFO of Rag-1−/− mice after adoptive transfer of male T cells and Ang II infusion. Photomicrographic visualization demonstrated that the degree of CD3+–positive staining in the SFO was greater in male when compared with that in female Rag-1−/− mice after adoptive transfer of male T cells and Ang II infusion (Figure 4E and 4F).

Sex Differences in Ang II–Induced Glomerular Hypertrophy Were Independent of T Cells in Rag-1−/− Mice

Glomerular hypertrophy is an early physiological adaptation of the kidney during hypertension.29 Thus, we compared glomerular areas in periodic acid Schif–stained renal sections to determine whether sex differences exist in the susceptibility of the Rag-1−/− host to glomerular hypertrophy after adoptive transfer of male T cells and Ang II infusion (Figure 5A–5F). Quantification via ImageJ software revealed that the glomerular area was significantly greater in male when compared with that in female Rag-1−/− mice at baseline (Figure 5G). Ang II infusion caused significant glomerular hypertrophy in both males and females. The glomerular area was greater in the male when compared with that in the female Rag-1−/− host after adoptive transfer of male T cells and Ang II infusion; however, adoptive transfer of male T cells had no effect on the magnitude of the glomerular hypertrophy induced by Ang II in either sex.

Discussion

The results of this study identify a sex difference in the ability of the adaptive immune system to facilitate Ang II–induced hypertension. The major findings of the present study are (1) male CD3+ T-cell adoptive transfer facilitates a significantly greater Ang II–induced increase in SBP in male when compared with that in female Rag-1−/− mice; (2) renal T-cell infiltration after T-cell adoptive transfer is significantly greater in male versus female Rag-1−/− mice; however, this effect was independent of Ang II infusion; and (3) After adoptive transfer, there was a tendency for increased T-cell infiltration in whole-brain homogenates of male versus female Rag-1−/− mice and examination of the SFO region identified higher numbers of T cells in males versus females. These results suggest that the prohypertensive effects of male T cells on Ang II–induced hypertension are inhibited in the female host and that reduced T-cell infiltration of the kidneys and brain could protect females against the hypertensive actions of Ang II.

Emerging evidence about the importance of the adaptive immune system in the development of hypertension has been supported from several different laboratories and models of hypertension. Guzik et al3 were among the first to report that male mice lacking T lymphocytes have a reduced hypertensive response to both Ang II and deoxycorticosterone acetate-salt. Furthermore, these studies demonstrated...
that adoptive transfer of T cells, but not B cells, could restore the hypertensive effects of Ang II and deoxycorticosterone acetate-salt. Similar results on the role of lymphocytes in Ang II–induced hypertension have also been reported by others; however, all of these studies were performed in male animals.8,9 The present study confirmed and expanded these results by comparing the role of T cells in Ang II–induced hypertension in both sexes. We found that in male Rag-1–/– mice, adoptive transfer of male CD3+ T cells markedly enhanced the hypertensive response to a 14-day infusion of Ang II (490 ng/kg per minute) when compared with their response in the absence of T cells. Similar to what has been previously reported, this suggests that the presence of T cells is essential for the full development of hypertension in male mice. However, in females, the effect of T cells was absent. The mechanism responsible for the protection of females from the prohypertensive effect of T cells is currently unknown.

Previously, we reported that there are sex differences in the ability of Ang II to induce an increase in sympathetic outflow.28,30 After 14 days of Ang II infusion, the drop in mean arterial pressure during ganglionic blockade with hexamethonium was greater in males than in females, suggesting that the higher levels of hypertension in males were a result of larger increases in sympathetic outflow in response to Ang II infusion. Marvar et al31 have recently suggested that an increase in sympathetic outflow during Ang II infusion may only be directly responsible for an initial modest increase in BP, and that the formation of neoantigens induced by this rise in BP causes the activation and infiltration of T cells to the kidney and vasculature, subsequently increasing cytokine release and promoting the genesis of severe hypertension. In the current study, the T-cell–dependent BP response exhibited by males supports this 2-phase hypothesis. The hypertensive response to Ang II in both female groups was similar to that in T-cell–deficient males, suggesting that the observed protection in females from the T-cell–dependent severe hypertension may be the result of a lack of neoantigen formation or T-cell activation. Additional studies are necessary to examine this hypothesis further.

**Figure 3.** Effect of angiotensin II (Ang II) infusion on splenic and renal T-lymphocyte infiltration into male and female Rag-1–/– mice. Similar splenic infiltration of adoptively transferred CD3+ (A), CD3+CD4+ (B), CD3+CD4–Foxp3+ (C), and CD3–CD8+ (D) T lymphocytes at the time of euthanasia indicates equal engraftment occurred in both sexes. Male mice demonstrated greater renal infiltration of CD3+ (E), CD3+CD4+ (F), CD3–CD4+Foxp3+ (G), and CD3–CD8+ (H) T lymphocytes when compared with females at the time of euthanasia, and this sex difference was not affected by Ang II infusion. *P<0.05.

**Table 2.** Ang II–Induced Renal Cytokine mRNA Expression in Male and Female Rag-1–/– Mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Male (Rag1–/– + Ang II)</th>
<th>Male (Rag1–/– + Ang II)</th>
<th>Female (Rag1–/– + Ang II)</th>
<th>Female (Rag1–/– + Ang II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>1.00±0.15</td>
<td>2.87±0.5*</td>
<td>1.00±0.19</td>
<td>0.68±0.27</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.00±0.33</td>
<td>2.08±0.33*</td>
<td>1.00±0.16</td>
<td>0.99±0.25</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.00±0.21</td>
<td>2.44±0.36*</td>
<td>1.00±0.34</td>
<td>0.72±0.25</td>
</tr>
</tbody>
</table>

Ang II indicates angiotensin II; IL-2, interleukin-2; MCP-1, monocyte chemoattractant protein; Rag, recombinant activating gene; and TNF-α, tumor necrosis factor-α. *P<0.05 vs Rag-1–/– + Ang II.
Lymphocyte infiltration of the kidney has also been linked with the development of hypertension.8–10,32,33 It has been suggested that the infiltration of T cells into the kidney in salt-sensitive hypertension, Ang II–induced hypertension, and in the spontaneous hypertensive rat results in increased renal inflammation, including increased renal cytokine and reactive oxygen species production. Subsequent inhibition of the inflammatory response with the immunosuppressive drug, mycophenolate mofetil, reduced hypertension and renal T-cell infiltration. Importantly, most of these studies investigating the role of renal T-cell infiltration in the development of hypertension were conducted in male animals. However, a recent study by Tipton et al35 investigated sex differences in renal T-cell infiltration in the spontaneous hypertensive rat. They reported that lymphocytes are necessary for the development of hypertension in the spontaneous, and that there is a significant difference in the T-cell profile in the kidneys between males and females. In these studies, females had greater numbers of CD8+ and T regulatory cells than males, whereas males had greater numbers of CD4+ and Th17 T-cell infiltration. After adoptive transfer of male T cells, the male Rag-1–/– host exhibited significantly greater basal infiltration of renal CD4+, CD8+, and Foxp3+ T cells when compared with the female host, despite similar BP between the sexes. Although we did not detect greater T-cell infiltration after Ang II infusion in either male or female Rag-1–/– mice, it is possible that Ang II increased the number of activated T cells and that sex difference in the number of activated T cells contributed to the augmented hypertensive response in the male when compared with that in the female host.

The observed sex differences in renal T-cell infiltration are likely to be influenced by sex differences in the host hormonal milieu. On transfer of male T cells into the female host, the cells become exposed to an ovarian hormone milieu. Thus, the potential for an immunogenic response exists and the female host receiving male T cells could reject the transferred cells because of the presence of the male H-Y antigen. However, fluorescence-activated cell sorter analysis of splenic T-lymphocyte infiltration confirmed that the transfer of male T cells into female Rag-1–/– recipients was well tolerated and did not generate an immunogenic response, at least during the course of the 5-week experiment. In addition, the pretransfer environment of the donor mouse can

Figure 4. Effect of angiotensin II (Ang II) infusion on whole-brain and subfornical organ (SFO) T-lymphocyte infiltration into male and female Rag-1–/– mice. Whole-brain homogenate CD3+ (A), CD3+CD4+ (B), CD3+CD4+Foxp3+ (C), and CD3+CD8+ (D) T-lymphocyte infiltration did not differ between sex or among treatment groups. Representative images of CD3+–stained SFO sections demonstrate attenuated infiltration in female (E and F) when compared with that in male (G and H) mice. Slices shown for each sex are from 2 different animals.

Figure 5. Effect of angiotensin II (Ang II) infusion on renal morphological changes in male and female Rag-1–/– mice with and without male T-cell adoptive transfer. Representative images of periodic acid Schiff–stained renal sections from (A) CD3M→Rag1–/–, (B) Rag1–/–+Ang II, (C) CD3M→Rag1–/–+F+Ang II, (D) CD3M→Rag1–/–+M, (E) Rag1–/–+M+Ang II, and (F) CD3M→Rag1–/–+M+Ang II. G, Quantification shows that Ang II infusion induced significant glomerular hypertrophy in both males and females; however, this effect was independent of the presence of T cells. *P<0.05 vs CD3M→Rag1–/–, same sex; #P<0.05 vs female, same group.
significantly affect the recipient’s hypertensive response. Because of the known prohypertensive properties of male T cells in the genesis of Ang II hypertension, the inability of male T cells to generate a full hypertensive response in female recipients strongly suggests that female resistance to Ang II–induced hypertension involves inhibition of T-cell–mediated processes. The mechanisms responsible for preventing T-cell–mediated Ang II hypertension in females are unknown but are likely to involve hormonal differences between the sexes.

In several different experimental models, Reckelhoff et al [12–13] have shown that testosterone is an important mediator of hypertension and renal injury in males, and that castration slows the onset of hypertension and related renal damage. Recently, a series of studies has offered convincing support for the hypothesis that androgens act in the kidney to increase 20-HETE, thereby activating an inflammatory cascade that alters renal vascular reactivity and results in the development of hypertension. [14, 15] In our studies, adoptively transferred T cells in male Rag-1–/– mice infiltrated the kidney in the absence of Ang II or hypertension. On the basis of above-mentioned studies into the effects of androgen on 20-HETE production, we hypothesize that our observed sex differences in basal renal T-cell infiltration could be because of a higher baseline inflammation, induced by higher testosterone levels in males, which thus serve as a signal for T-cell trafficking and predisposing them to renal tissue T-cell infiltration. Additional studies are needed to clarify the role of circulating androgens in renal T-cell infiltration.

We also observed sex differences in T-cell trafficking into the brain, specifically into the SFO. The mechanisms responsible for the sex differences in T-cell infiltration of the SFO are unknown. In previous studies of Ang II–induced hypertension in mice, we have shown that central infusion of 17β-estradiol attenuates Ang II–induced hypertension in both males and ovariectomized females, [16, 17] suggesting that 17β-estradiol acting on the brain can inhibit Ang II–induced increases in sympathetic outflow and hypertension. We have also shown that 17β-estradiol inhibits Ang II–induced increases in SFO reactive oxygen species. [18] Thus, differences in circulating 17β-estradiol levels may be one potential mechanism underlying the protection of females from T-cell infiltration into the SFO. Additional studies are needed to characterize the effects of 17β-estradiol on T-cell infiltration into the kidney and SFO, and its ability to facilitate Ang II hypertension.

Perspectives

The present studies suggest that understanding the role of the adaptive immune system in the development of hypertension requires studies be conducted in both male and female animal models. Of the multiple factors that are known to contribute to the development of hypertension, the sex of the subject being studied must be taken into consideration. The clear sex differences that were observed in these studies highlight that translation of our understanding of the underlying physiology of hypertension to the development of new antihypertensive therapies for both men and women will require ongoing physiological studies to include data from both sexes.

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Disclosures

None.

References

20. Maric C, Xu Q, Sandberg K, Hinajosa-Laborde C. Age-related renal disease in female Dahl salt-sensitive rats is attenuated with 17 beta-estradiol


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### Novelty and Significance

**What Is New?**

- There is growing evidence for a critical role of the adaptive immune system in the genesis of hypertension in males. We demonstrate for the first time that the ability of the adaptive immune system to induce hypertension is blunted in females and coincides with a reduction in T lymphocyte infiltration in the kidney.

**What Is Relevant?**

- Females are protected against the genesis of hypertension before menopause; however, the mechanisms responsible for this protection are unclear.

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**Summary**

Females are protected from the hypertensive properties of T lymphocytes when compared with males. Targeting these cells may prove to be an effective treatment against hypertension.
Sex Differences in T-Lymphocyte Tissue Infiltration and Development of Angiotensin II

Hypertension

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SEX DIFFERENCES IN T-LYMPHOCYTE TISSUE INFILTRATION AND DEVELPMENT OF ANGIOTENSIN II HYPERTENSION

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Animals
Rag-1-/- mice on the B6-Ly5.1 background, with a genetic deletion of the recombinase-activating gene and lacking both T and B cells were used in this study and purchased from The Jackson Laboratory. Six animal groups were studied including male (M) and female (F) Rag-1-/- mice treated with Ang II (Rag1-/-M+Ang II, n=7; Rag1-/-F+Ang II, n=7) and Rag-1-/- mice treated with or without Ang II three weeks after adoptive transfer of male CD3 T cells (CD3M-Rag1-/-M, n=7; CD3M-Rag1-/-M+Ang II, n=8; CD3M-Rag1-/-F, n=7; CD3M-Rag1-/-F+Ang II, n=8). Male B6-Ly5.1 (National Cancer Institute) mice were used as donors for the T cell adoptive transfer. Mice were housed in standard polypropylene cages placed in a temperature- and humidity-controlled facility. The mice were maintained in a 12:12-h light-dark cycle (6:00 AM to 6:00 PM) and were fed normal (0.25%) NaCl mouse chow with water available ad libitum. All methods were approved by the University of Arizona Animal Care and Use Committee.

Experimental Protocol
To determine if there are sex differences in the T cell-dependent genesis of hypertension, male and female Rag-1-/- mice received adoptive transfer of male CD3+ T cells three weeks prior to 14 days of Ang II infusion (490ng/kg/min). Control groups received Ang II or T cells alone. Systolic blood pressure (SBP) and heart rate (HR) were measured immediately prior to adoptive transfer of T cells, immediately prior to Ang II infusion and again 14 days following Ang II infusion (Figure 1). Animals were sacrificed after 14 days of Ang II infusion. Tissues from four animals per group were used for flow cytometry. Kidneys and brains from the remaining animals in each group were removed and processed for immunohistochemistry.

Adoptive Transfer of Purified T cells
Adoptive transfer of T cells was performed 3 weeks before Ang II infusion, similarly to the protocol described by Guzik et al. Total splenocytes were isolated from B6-Ly5.1 congenic male mice using a pan T cell isolation kit (Miltenyi Biotech) and negative magnetic separation (AutoMACS), yielding sterile and highly enriched CD3+ T cells. The purity of these was confirmed to be >95% by flow cytometry before injection. Immediately after the cell isolation, 7.5 x 10^6 cells were resuspended in 200 microliters of sterile phosphate buffered saline (PBS), passed through a 70 µm filter, and injected into male and female Rag-1-/- mice via tail vein. T cell engraftment was assessed in blood on day 8 post-transfer, prior to further treatment, and deemed successful as defined by an average of 1.5± 0.4 x 10^6 T cells in the spleen of Rag-1-/- mice at the final harvest.

Blood Pressure Measurements and Angiotensin II Infusion
BP and HR were monitored non-invasively via tail cuff (MC4000, Hatteras Instruments)
prior to T cell adoptive transfer, immediately prior to beginning the Ang II infusion and 14 days post Ang II infusion.

**Osmotic pump implantation.** Three weeks following T cell adoptive transfer, mice were anesthetized with inhalational isoflurane and implanted subcutaneousy on the back with osmotic minipumps (model 1002, Alzet) containing Ang II (Sigma Chemical) at a concentration sufficient to allow an infusion rate of 490 ng/kg/min.

**Flow Cytometry**
Spleen, kidney and brain from each group (n=4) were minced and digested with Accutase (eBioscience) for 30 min at 37 °C. Organs were passed through a 40-μm mesh screen to prepare a single-cell suspension for analysis (spleen) or to disassociate the tissue (kidney and brain). Lymphocytes from kidney and brain were isolated over a 30%/70% percoll gradient. Cells were stained with surface antibodies overnight at +4 °C and then with a live/dead discriminator dye (Life Technologies) followed by fixation and permeabilization with the Foxp3 Staining Buffer Set (eBioscience) and intracellular staining with Foxp3 antibody. Lymphocyte counts were determined using Count Bright Absolute counting beads (Life Technologies) for kidney and brain or extrapolated from a complete blood count differential collected on a Hemavet 950LV (Drew Scientific Group) for spleen. Flow cytofluorometric (FCM) data was acquired on a BD Fortessa instrument using BD FACS Diva software (Becton Dickinson) and analysis was performed using FlowJo software (Tree Star).

**Immunoperoxidase staining in brain tissues**
Brains from three males and three females were fixed in 4% paraformaldehyde in 0.1 M PBS for one week. A total of 10 frozen, 30 μM subfornical organ (SFO) sections were analyzed from the females and a total of 7 SFO sections were analyzed from the males. Free floating sections were permeabilized with 0.3% H2O2 for one hour, blocked with 5% goat serum with 0.3% Triton X-100 for 2 hours and incubated with primary CD3+ antibody for 48 hours (rabbit, 1:200, Calbiochem PC630). Sections were incubated with biotinylated anti-rabbit IgG (1:300, Vector Laboratories BA-100) followed by ABC complex for 30 minutes (Vectastain Elite kit, Vector Laboratories) and reaction with diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) for 5-15 minutes in Tris, pH 7.7. Sections were washed thoroughly, mounted onto gelatin-coated slides, air-dried overnight and coverslipped for analysis.

**RNA isolation and real-time quantitative PCR**
RNA isolation and real-time quantitative PCR experiments were performed as previously described. Briefly, RNA was isolated from one kidney per mouse (Qiagen RNeasy Mini Kit, cat #74101) according to manufacturer instructions. A DNase (Qiagen, cat #79254) incubation was performed to remove potential DNA contamination. Nanodrop ND 1000 spectrophotometer (Wilmington, DE) was used to quantify RNA. Real Time PCR experiments were performed on a RotorGene RG3000 (Qiagen). 2.5μg RNA was reverse transcribed. Resulting cDNA was diluted to 8ng/μl. Primer sequences were as follows: IL-2 forward: aaagggctctgacaacacattt, reverse: agggcttgtagatgatgc; MCP-1 forward: caagaaggaatgggtccaga, reverse: agaccttaggcagatgagc; TNF-α
forward: cttgtgcctctcttttgc, reverse: accgtagggcgattacagt. Expression levels of the genes of interest were normalized to dynactin mRNA after running samples in parallel.

**Glomerular Area Determination**
Kidneys were fixed in 4% paraformaldehyde for 48 hours, embedded in paraffin and sectioned (5 μm). Sections were stained by the University of Arizona Histology Lab following a standard Periodic Acid Schiff (PAS) protocol. To minimize the potential impact of measuring glomeruli on non-identical planes, 15 cortical images per slide were captured at 200x magnification, resulting in the analysis of 45-55 glomeruli per slide, or approximately 200 glomeruli per treatment group. The mean area (μm²) of each glomerular profile was measured by manually tracing the minimal convex polygon surrounding the glomerular capillary tuft and calculating its area by computerized morphometry using ImageJ software (NIH). All imaging was conducted in a blinded fashion.

**Statistics**
Data are expressed as means ± SEM. Differences between groups were assessed by Tukey’s multiple comparison test and Graph Pad Prism Software v5.0 (GraphPad Software Inc, La Jolla, CA). Paired t test was used to analyze pre- and post-treatment blood pressures within groups. Significance was defined as p<0.05.
Supplemental References
