**Abstract**—Recent studies have emphasized a role of adaptive immunity, and particularly T cells, in the genesis of hypertension. We sought to determine the T-cell subtypes that contribute to hypertension and renal inflammation in angiotensin II–induced hypertension. Using T-cell receptor spectratyping to examine T-cell receptor usage, we demonstrated that CD8+ cells, but not CD4+ cells, in the kidney exhibited altered T-cell receptor transcript lengths in Vβ3, 8, 1, and 17 families in response to angiotensin II–induced hypertension. Clonality was not observed in other organs. The hypertension caused by angiotensin II in CD4−/− and MHCII−/− mice was similar to that observed in wild-type mice, whereas CD8−/− mice and OT1xRAG-1−/− mice, which have only 1 T-cell receptor, exhibited a blunted hypertensive response to angiotensin II. Adoptive transfer of pan T cells and CD8+ T cells but not CD4+/CD25− cells conferred hypertension to RAG-1−/− mice. In contrast, transfer of CD4+/CD25+ cells to wild-type mice receiving angiotensin II decreased blood pressure. Mice treated with angiotensin II exhibited increased numbers of kidney CD4+ and CD8+ T cells. In response to a sodium/volume challenge, wild-type and CD4−/− mice infused with angiotensin II retained water and sodium, whereas CD8−/− mice did not. CD8−/− mice were also protected against angiotensin-induced endothelial dysfunction and vascular remodeling in the kidney. These data suggest that in the development of hypertension, an oligoclonal population of CD8+ cells accumulates in the kidney and likely contributes to hypertension by contributing to sodium and volume retention and vascular rarefaction. *(Hypertension. 2014;64:1108-1115.)* ◆ Online Data Supplement

**Key Words:** adaptive immunity ■ angiotensin II ■ diuresis ■ natriuresis ■ receptors, antigen, T-cell

Hypertension affects 30% of adults in Western populations and predisposes to myocardial infarction, stroke, heart failure, and renal failure.1,2 Despite the prevalence of this disease, the cause of hypertension in most adults is unknown and perturbations of the central nervous system, the kidney, and the vasculature have been implicated. Recently, our laboratory and others have demonstrated a role of inflammation and the adaptive immune system in the genesis of hypertension.3,4 Several studies have shown that RAG-1−/− mice, which lack lymphocytes, are partly protected against hypertension caused by angiotensin II, deoxycorticosterone acetate–salt challenge, and norepinephrine. Adaptive transfer of T cells restores hypertension in these animals. Very recently, Mattson et al3 have shown that T cells play a role in hypertension in Dahl salt-sensitive rats. Recent data suggests that T cells release cytokines that mediate sodium retention in the kidney and vasoconstriction.4 Our laboratory has previously found that both CD4+ and CD8+ T cells accumulate in aortic perivascular fat of hypertensive mice. CD4+ T regulatory cells have been shown to be protective against hypertension and end-organ damage.6 Despite these findings, there is little information on the relative importance of CD4+ and CD8+ T cells and whether hypertension is dependent on expansion of specific T-cell clones. The purpose of this investigation is to determine the T-cell subtypes involved in the genesis of hypertension. We hypothesized that T cells in the kidney and vasculature would exhibit altered T-cell receptor (TCR) usage in response to angiotensin II–induced hypertension. We found that CD8+ but not CD4+ T cells in the kidney exhibited altered T-cell receptor usage. We examined the role of CD4+ and CD8+ T cells using mice lacking these subtypes and mice lacking CD8+ T cells was protected against hypertension. Furthermore, CD8+ T cells seem to modulate renovascular remodeling and promote the antinatriuretic and antiduressive effects of angiotensin II.

**Materials and Methods**

**Animals Studied**

Hypertension was produced in wild-type (WT), RAG-1−/−, CD4+−, CD8−/− and MHCII−/−, and OT1xRAG-1−/− male mice by infusion of

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angiotensin II (490 ng/kg per minute) for 2 weeks. Blood pressure was measured both invasively using telemetry and noninvasively using the tail cuff method as previously described. Flow cytometry, vascular reactivity in mesenteric arteries, sodium and volume challenge experiments, and kidney vascular morphology analyses are described in detail in the online-only Data Supplement.

**TCR Analysis**

TCR Vβ spectratyping was performed using polymerase chain reaction with a forward primer for each of the 24 TCR Vβ genes and a constant reverse primer for the Cβ gene labeled with fluorescein amidite on the 5' end (Table S1 in the online-only Data Supplement). TCR deep sequencing was performed in spleen, mesenteric, and renal CD8+ T cells as described in the Methods in the online-only Data Supplement and in the online-only Data Supplement.

**Results**

**Effect of Hypertension on TCR Clonality**

T-cell activation occurs on presentation of antigenic peptides by major histocompatibility complexes, leading to proliferation of T-cell clones that are specific for individual antigens. The Vβ region of the TCR gene exhibits enormous diversity, and analysis of this region can provide insight into adaptive immune responses. The presence of a dominant transcript length for a given Vβ family is indicative of clonal expansion and provides insight into the antigenic repertoire responsible for T-cell activation. Therefore, we performed spectratyping analysis of CD4+ and CD8+ T cells isolated from the spleen, mesenteric vascular arcade, and the kidneys of mice treated with vehicle or angiotensin II. In the spleen both CD4+ and CD8+ TCR Vβ families exhibited a Gaussian distribution in both sham and angiotensin II–treated mice, indicative of a relatively naïve pool of T cells (Figure 1A and 1B). Greater TCR transcript length variability was exhibited in mesentery (Figure 1C and 1D) and kidney (Figure 2A and 2B). In many Vβ families, in both kidney and mesentery, there was considerable variation in the spectratyping profiles regardless of the presence or absence of angiotensin II. To determine whether the transcript length distribution was consistently altered across mice that received angiotensin II and to detect the presence of dominant transcript lengths, Dirichelet and MaGiK analyses were used. TCR spectratyping profiles from the spleen and mesenteric vasculature

**Figure 1.** Sample T-cell receptor Vβ family spectratype profiles from spleen and mesenteric vasculature. Mice were made hypertensive by infusion of angiotensin II (Ang II; 490 ng/kg per minute) for 14 days. CD4+ and CD8+ T cells were isolated from the spleen or mesentery, mRNA isolated and reverse transcribed to cDNA. Polymerase chain reaction was performed using 24 different Vβ primers and a single fluorescein amidite–labeled Cβ primer. The resulting fragment profiles were visualized using Peak Scanner software from Applied Biosystems. **A**, Spleen sample distributions for each CD4+ Vβ family from sham and Ang II–treated mice. **B**, Spleen sample distributions from CD8+ Vβ families. **C**, Mesentery sample distributions from CD4+ Vβ families. **D**, Mesentery sample distributions from CD8+ Vβ families. Vβ families not shown were undetectable.
CD4+ and CD8+ cells, as well as kidney CD4+ cells, revealed no statistical differences when comparing sham with angiotensin II–treated mice (n=8–10 per group). In contrast, angiotensin II resulted in the presence of dominant transcript lengths in kidney T-cell CD8+ Vβ3, 8.1, and 17 families (Figure 2C–2E).

In additional studies, we used deep sequencing of CD8+ TCR to provide further insight into potential clones involved in the hypertensive response. In kidney CD8+ T cells, deep sequencing revealed 4341±514 unique TCR sequences in sham mice and 3522±1049 unique TCR sequences in mice treated with angiotensin II (n=5 per group). The most commonly shared clonotypes among angiotensin II–treated mice included 3 clonotypes, which were shared by 4 of 5 angiotensin II–treated mice and 2 that were shared by 3 of 5 angiotensin II–treated mice (Table). These clonotypes were not present in multiple sham mice. Notably, the clone with amino acid sequence CASSDNTEVFF observed in angiotensin II–treated mice exists in the Vβ8.1 family and is identical to the dominant transcript length observed by spectratyping (Figure 2D). We found that these unique sequences were also present in the spleen, but their frequencies were low and not different between sham and angiotensin II–treated mice. The sequences shared by angiotensin II–treated mice in the kidney were not present in the mesentery.

Role of T-Cell Subtypes in Hypertension

The specific skewing of CD8+ T cells, rather than CD4+ cells was surprising. We, therefore, performed additional experiments to examine the roles of these cells in hypertension. We infused angiotensin II for 14 days into WT, CD4−/−, and CD8−/− mice. Both systolic and diastolic blood pressures were significantly increased in WT and CD4−/− mice (Figure 3A and 3B). In contrast, the angiotensin II–induced increase in blood pressure was blunted in CD8−/− mice (Figure 3A and 3B). MCHII−/− mice, an alternate model of CD4+ deficiency, also exhibited increased blood pressure in response to angiotensin II (Figure 3A and 3B).

To determine whether an alternate model of hypertension is also dependent on CD8+ T cells, we induced deoxycorticosterone acetate–salt hypertension in WT, CD8−/−, and CD4−/− mice. The increase in blood pressure in response to deoxycorticosterone acetate–salt challenge was significantly reduced in CD8−/− mice when compared with either CD4−/− or WT mice (Figure S1), similar to angiotensin II–induced hypertension.

To determine whether mice with an altered TCR phenotype are resistant to hypertension, we infused angiotensin II or
vehicle in OT1xRAG-1−/− mice, which possess a transgene for a TCR specific for ovalbumin peptide OVA257-264 presented on MHC I. These mice are on a RAG-1−/− background and, therefore, cannot generate endogenous TCRs. The angiotensin II–induced increases in both systolic and diastolic blood pressures were blunted in OT1xRAG-1−/− mice when compared with those observed in WT, CD4−/−, and MHCII−/− mice but were similar to the pressures observed in CD8−/− mice (Figure 3A and 3B).

To gain further insight into T-cell subtypes in hypertension, adoptive transfer experiments were performed. As previously reported, angiotensin II had only a modest effect on blood pressure in RAG-1−/− mice, whereas adoptive transfer of pan T cells from WT mice that had received angiotensin II for 2 weeks increased blood pressure to ≈160 mm Hg in the recipient RAG-1−/− mice (Figure 3C). Adoptive transfer of CD4+/CD25− cells from angiotensin II–treated mice into RAG-1−/− mice had no effect on blood pressure (Figure 3C). In contrast, adoptive transfer of CD8+ cells caused a striking elevation in blood pressure to >180 mm Hg (Figure 3C). As previously reported, adoptive transfer of CD4+/CD25+ T regulatory cells to WT mice on day 9 of angiotensin II infusion lowered blood pressure (Figure 3D).

Leukocyte Accumulation in Kidney and Vasculature

Flow cytometry was used to determine whether the angiotensin II alters the T-cell subtypes present in the kidney. Angiotensin II infusion caused a 3-fold increase in both CD8+ and CD4+ T cells in the kidney (Figure 4A and 4B). To determine whether CD8+ T cells orchestrate angiotensin II–induced accumulation of leukocytes in the vasculature aortas form WT and CD8−/− mice were also analyzed. The accumulation of CD45+ leukocytes was greater in WT when compared with that in CD8−/− mice (Figure S2).

Role of CD8+ and CD4+ T Cells on Responses to Sodium and Volume Challenge

To determine the effect of T-cell subsets on sodium and volume retention, studies were performed in mice that received an intraperitoneal injection of normal saline equal to 10% of their body weight at various times before and during angiotensin II infusion. At baseline (day 1), mice excreted >80% of this challenge in 4 hours, independent of their T-cell populations (Figure 4C). In WT mice, angiotensin II infusion induced a modest retention of volume and NaCl when compared with baseline values that persisted through day 10 (Figure 4C–4E).

Table. Kidney CD8+ T-Cell Clonotypes Shared by Angiotensin II–Treated Mice

<table>
<thead>
<tr>
<th>CDR3 Sequence</th>
<th>% Clone Frequency in Angiotensin II–Treated Mice</th>
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<tbody>
<tr>
<td></td>
<td>Vβ</td>
</tr>
<tr>
<td>CASSLGTANTGQLYF</td>
<td>5.1</td>
</tr>
<tr>
<td>CASSDNTEVF</td>
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</tr>
<tr>
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<td>11</td>
</tr>
<tr>
<td>CASSFSSGNTLYF</td>
<td>13</td>
</tr>
<tr>
<td>CASGDGHDQTYF</td>
<td>8.2</td>
</tr>
</tbody>
</table>

*Clone not present.

Figure 3. Role of T-cell subtypes in angiotensin II (Ang II)–induced hypertension. Wild-type (WT, n=4), CD4−/− (n=5), CD8−/− (n=5), MHCII−/− (n=6), and OT1xRAG-1−/− (n=10) mice received Ang II (490 ng/kg per minute) for 15 days via osmotic minipumps. Blood pressure was monitored by telemetry, and values represent day/night averages for each group during the past 3 days of infusion. A, Baseline systolic (top) and diastolic blood pressures (bottom). B, Fourteen-day systolic (top) and diastolic blood pressures (bottom). C, Adoptive transfer of T cells into RAG-1−/− mice. RAG-1−/− mice received Ang II via osmotic minipump starting at day 0 and at day 10 received no cells, pan T cells, CD4+/CD25−, or CD8+ T cells from WT mice (n=7–9 per group) that had previously received Ang II for 2 weeks. Blood pressure was measured by tail cuff. D, Adoptive transfer of CD4+/CD25+ into WT mice. Both groups received Ang II starting at day 0 and at day 9 received no cells or CD4+/CD25+ T cells from untreated WT mice (n=5 per group). P values for blood pressure were determined using repeated measures ANOVA and a Student–Newman–Keuls post hoc test. D indicates day; and N, night.
In CD4−/− mice, volume and NaCl retention was similar to that in WT mice on day 2 but on day 5; CD4−/− mice excreted only ≈20% of the volume and NaCl challenge (Figure 4C–4E). In contrast, CD8−/− mice were protected against urinary volume and NaCl retention at all times after the onset of angiotensin II infusion (Figure 4C–4E). Taken together, these data illustrate that CD8+ T cells play a critical role in the antidiuretic and antinatriuretic responses to angiotensin II.

Effect of CD8+ T Cells on Renal Vascular Remodeling in Hypertension

To determine whether inflammatory T cells mediate vascular remodeling in the kidney during hypertension, we examined renal vascular volume using micro-computed tomographic angiography (Figure 5A). Total kidney and cortical vascular volumes were decreased with angiotensin II in WT and CD4−/− mice but was persevered in CD8−/− mice (Figure 5B and 5C). Notably, a decrease in vessels <25 μm in diameter, represented by a loss of blue pseudocolor, seems to account for the decrease in vascular volume with angiotensin II (Figure 5A). Medullary vascular volume was decreased with angiotensin II in WT but not in CD4−/− or CD8−/− mice (Figure 5D). To characterize alterations in small arterioles further, α actin staining was used. In these small vessels, media:lumen ratio was increased in WT and CD4−/− mice but not in CD8−/− mice (Figure 5E and 5F).

Role of T-Cell Subtypes in Vascular Reactivity

To examine vascular reactivity, we studied second- and third-order mesenteric arteries, which are involved in modulation of vascular resistance, in a wire myograph preparation. Endothelium-dependent relaxations in response to acetylcholine were impaired in vessels of WT and CD4−/− mice when compared with arteries of sham-infused WT mice. In contrast, angiotensin II did not impair endothelium-dependent relaxation in vessels of CD8−/− mice (Figure 6A). Endothelium-independent relaxations were not altered (Figure 6B).

Discussion

In the current study, we sought to determine the nature of T cells involved in the genesis of hypertension by examining clonality in various organs involved in hypertension and by examining the role of T cell subtypes. Spectratyping of TCRs revealed the development of dominant transcript lengths in kidney CD8+ T-cell Vβ3, 8.1, and 17 families after angiotensin II infusion. Deep sequencing of renal CD8+ cells revealed 5 sequences that were shared by angiotensin II–treated mice. Mice that lacked CD8+ T cells or endogenous TCRs exhibited blunted pressor responses to angiotensin II. Adoptive transfer of pan T cells or CD8+ T cells induced hypertension in RAG-1−/− mice infused with angiotensin II–treated mice. Mice that lacked CD8+ T cells were protected against angiotensin II–induced endothelial dysfunction, renal salt and water retention, and vascular rarefaction. These data suggest that an oligoclonal population of CD8+ T cells accumulates in the kidney and that CD8+ T cells promote sodium and volume retention and arterial rarefaction resulting in overt hypertension.

To determine whether the T-cell response in the kidney is monoclonal, oligoclonal, or nonspecific, we used TCR spectratyping. During the T-cell development in the thymus, recombination of the variable (V), junctional (J), and diversity (D) regions of the TCR β chain occurs. In addition, terminal deoxynucleotidyl transferase facilitates random insertion of nucleotides at the V/D and D/J junctions, ultimately leading to enormous TCR diversity and specificity.14 In naïve pools of T cells, this results in a Gaussian distribution of TCR gene...
transcript lengths. Spectratyping allows analysis of TCR transcript lengths and detection of non-Gaussian distributions. The presence of TCR dominant transcript lengths strongly suggests clonal expansion.9 Such analyses have revealed clonal expansion in the context of infection15 and autoimmune disease.16 More recently, this has also been observed in models of atherosclerosis17 and obesity.18,19 We found that angiotensin II infusion resulted in dominant TCR transcript lengths in CD8+ T cells within the Vβ3, 8.1, and 17 TCR families in the kidney, while not altering the distributions within CD4+ cells or either CD4+ or CD8+ T cells in the spleen or mesenteric vasculature. Although perturbations of both the vasculature and the kidney have been shown to be involved in hypertension, the accumulation of a unique population of CD8+ T cells in the kidney suggests that this might be a primary site for immune activation in hypertension.

To gain further insight into TCRs involved in hypertension, we also used deep sequencing of kidney, spleen, and mesentery CD8+ TCRs. This approach provides a complete overview of the amino acid sequences of the TCR CDR3 region, which is the region of the TCR that binds to antigen.7,8 We found that 5 CD8+ TCR sequences in the kidney were shared by multiple angiotensin II–treated mice. Of note, the CASSDNTEVFF clone is of the same transcript length as the dominant length observed within the Vβ8.1 family identified by spectratyping. Importantly, these sequences were not shared with sham mice. These sequences were not found in the mesenteric vasculature and found in both sham and angiotensin II–treated mice in the spleen. The presence of these sequences in the spleens of both sham and angiotensin II–treated mice suggests that these sequences exist in the naïve pool of T cells in all animals and that unique sequences are mobilized to the kidney in hypertension. The presence of TCR sequences shared by angiotensin II–treated mice exclusively in the kidney together with the skewing of selected TCR Vβ family transcript lengths in the kidney suggests that the kidney might represent a site of neoantigen formation in hypertension.

Figure 5. Effect of T-cell subtypes on the kidney vascular structure. A, Representative computed tomographic models of the kidney vasculature in sham and angiotensin II (Ang II)–treated mice. Quantification of (B) total vascular volume (mm3), (C) cortex vascular volume, and (D) medulla vascular volume (n=3–5 per group). E, α actin staining for renal cortical arterioles in wild-type (WT), CD4−/−, and CD8−/− mice. F, Renal arterioles <25 mm in diameter and arteries >25 mm were quantified by planimetry (n=4 per group). P values determined by 2-way ANOVA.

Figure 6. Influence of T-cell subtypes in angiotensin II (Ang II)–induced vascular dysfunction. Mice received sham or Ang II infusions for 2 weeks as in Figure 1. Second- and third-order mesenteric vessels were mounted in wire myographs and preconstricted with phenylephrine (1 μM). A, Endothelium-dependent relaxations to acetylcholine and (B) endothelium-dependent independent relaxations to sodium nitroprusside (SNP) were then determined and compared using ANOVA for repeated measures (n=4–7 per group).
In unusual instances, such as T-cell leukemia, a single TCR clone can account for ≤50% of the total TCR usage. In contrast, we found that clones shared among hypertensive mice represent <0.2% of all CD8+ TCR transcripts in the kidney. This is similar to shared clonotypes of islet infiltrating T cells in mice with type 1 diabetes mellitus. The relatively low frequency of unique TCR clonotypes in hypertensive mice is consistent with a common pattern of inflammation where specific T-cell clones are first activated, followed by infiltration of other T cells unrelated to the initial clone that then contribute to the inflammatory response. In addition, in a region of inflammation, there may be development of other antigens, because of protein modifications that lead to the activation of T cells other than the originally stimulated clones. This phenomenon is often referred to as epitope spreading, which can occur in response to either foreign proteins in infectious disease or self-proteins in the context of autoimmune disease. In addition, depending on the topology of the MHC complex and antigen peptide public or private T-cell responses can occur. If the MHC/peptide complex has distinct features, a single T-cell clone is likely to recognize this complex across mice and this phenomenon is referred to as a public response. In contrast, if the MHC/peptide complex is relatively featureless, a private response can occur, where the TCR clones vary from one animal to the next. In summary, recruitment of non-specific T-cell clones, epitope spreading, and public versus private T-cell response may explain the generally low frequencies of unique TCR clonotypes in the kidneys of hypertensive mice. The precise nature of potential neoantigens involved in hypertension is an important topic for future study.

Because we observed skewing of CD8+ TCR in the kidney, we performed additional studies to examine the role of these cells in hypertension. Interestingly, we found the hypertensive response to angiotensin II was blunted in CD8−/− but not in CD4−/− mice. We also found that mice lacking MHCIIC, which are also deficient in CD4+ cells, were not protected against hypertension. These data indicate that not only is there alteration of CD8+ TCR sequences but also that these cells likely contribute to the elevation of blood pressure in response to angiotensin II. Importantly, Youn et al recently demonstrated that proinflammatory immunosenescent CD8+ cells are increased in humans with hypertension.

It is possible that the renal inflammation promotes inflammation in other organs. In keeping with this, we found that total leukocyte accumulation in the aorta of CD8−/− mice with angiotensin II treatment was less than that of WT mice. One explanation for this is the reduced systemic pressure in the CD8−/− mice. In a recent study, we found that lowering blood pressure with hydralazine and hydrochlorothiazide markedly reduced vascular inflammation in response to chronic angiotensin II infusion. As we observed that hypertension is associated with alterations in TCR receptor usage in the kidney, we sought to determine whether mice with an altered TCR repertoire would be protected against hypertension. The pressor response in OT1xRAG-1−/− mice, which do not posses endogenous TCR, is similar in magnitude with that observed in RAG-1−/− mice. This supports the concept that endogenous TCR are required for the development of overt hypertension. If the T-cell response in hypertension were nonspecific, these mice would be expected to develop hypertension in a similar manner to WT mice. We used OT1xRAG-1−/− mice, as OT1 mice, despite a heavily skewed TCR repertoire, can still generate endogenous TCR, whereas OT1xRAG-1−/− mice cannot.

Hypertensive stimuli, such as angiotensin II, have an antinatriuretic effect, promoting an increase in intravascular volume. This is then followed by an increase in systemic vascular resistance or systemic autoregulation, which is accompanied by alterations in vascular tone, arterial remodeling, and vascular rarefaction. Our current findings suggest that CD8+ T cells act as an intermediate in angiotensin II–induced endothelial dysfunction, vascular rarefaction, and sodium and volume retention, key processes in the development of hypertension. Importantly, 25% of the cardiac output is directed to the kidneys. The loss in renal cortical arterioles and the resultant increase in systemic vascular resistance likely contributes to hypertension. In addition, it is conceivable that decreased kidney blood flow may contribute to enhanced sodium and water reabsorption, also leading to increased blood pressure.

Our studies should not be interpreted as indicating that there is no role of CD4+ T cells in hypertension. The development of memory CD8+ T cells depends on CD4+ cell help. In addition, CD8+ T regulatory cells also exist and can exert anti-inflammatory effects in a similar manner to CD4+ T regulatory cells. We found greater numbers of both CD4+ and CD8+ T cells in the kidneys of hypertensive mice. Recent reports from our laboratory have also shown that CD4+ and CD8+ T cells accumulate in the aortas of hypertensive mice. In RAG-1−/− mice, transfer of CD4+ cells from which CD25+ cells had been depleted (thus excluding most T regulatory cells) did not raise blood pressure. Transfer of CD8+ cells alone caused a greater elevation in blood pressure than pan T cells, suggesting that CD4+ cells play a protective role. Similarly, blood pressure in CD4−/− mice was greater than that of WT mice in the deoxycorticosterone acetate–salt experiments. Importantly, CD4−/− mice lack CD4+ regulatory cells, which are protective in hypertension. In accordance with this observation, we found that adoptive transfer of CD4+CD25+ (primarily T regulatory cells) decreased blood pressure in WT animals. These findings are consistent with ample evidence for inhibition of CD8+ T cells by CD4+ regulatory cells. Taken together, these studies confirm a unique prohypertensive role of CD8+ T cells.

**Perspectives**

Our data suggest that CD8+ T cells have an important role in hypertension and provide evidence that there is an oligoclonal response of these cells in the kidney. These cells help coordinate alterations in renal and vascular function that ultimately lead to hypertension and end-organ dysfunction caused by hypertensive stimuli. One explanation for these results is that unique neoantigens develop in the hypertensive kidney and are presented in the context of MHCII. Our findings allow new insight into understanding hypertension and potentially provide new therapeutic directions for the treatment in this disease and its end-organ damage.

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Disclosures
None.

References

Novelty and Significance

What Is New?
• T cells have been shown to play a role in the development of hypertension, this study demonstrates that a specific subset of CD8+ T cells play a critical role in hypertension.
• These CD8+ T cells have altered T-cell receptors, suggesting an immune response to a neoplastic in kidney.

What Is Relevant?
• The identification of this subset of CD8+ T cells provides insight into the development of high blood pressure and a potential target for future treatment.

Summary
In the development of hypertension, a subset of CD8+ T cells act to decrease small blood vessel number and size in the kidney and contribute to the retention of salt and water, causing an increase in blood pressure.
Oligoclonal CD8+ T Cells Play a Critical Role in the Development of Hypertension

Daniel W. Trott, Salim R. Thabet, Annet Kirabo, Mohamed A. Saleh, Hana Itani, Allison E. Norlander, Jing Wu, Anna Goldstein, William J. Arendshorst, Meena S. Madhur, Wei Chen, Chung-I. Li, Yu Shyr and David G. Harrison

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OLIGOCLONAL CD8+ T CELLS PLAY A CRITICAL ROLE IN THE DEVELOPMENT OF HYPERTENSION

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Running Head: CD8+ T cells and Hypertension

Supplemental Figures: 2

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MATERIALS AND METHODS

Animals studied: Wild type, RAG-1\(^{-/-}\), CD4\(^{-/-}\), CD8\(^{-/-}\) and MCHII\(^{-/-}\) mice all on a C57Bl/6 background, were obtained from Jackson Laboratories. OT1xRAG-1\(^{+/-}\) mice were obtained from Taconic. To produce hypertension, osmotic minipumps were implanted for infusion of angiotensin II (490 ng/min/kg) for two weeks. DOCA-salt hypertension was produced as previously described.\(^1\) Blood pressure was measured both invasively using telemetry and noninvasively using the tail-cuff method, as previously described.\(^2\) When telemetry was employed, mice were allowed to recover for ten days before the osmotic minipumps implants were inserted.

Following sacrifice, the chest was rapidly opened and the superior vena cava sectioned. A catheter was placed in the left ventricular apex and the animals were perfused at a physiological pressure with Krebs-Hepes buffer until the effluent from the vena cava was cleared of blood. For studies of renal histology or vascular morphology, this perfusion with buffer was followed by perfusion with 4% formaldehyde. The Institutional Animal Care and Use Committee approved all experimental protocols.

Reagents and Materials employed: Isolation kits for pan-T cells, CD4\(^{+}\) and CD8\(^{+}\) cells were from either Miltenyi Biotec or Invitrogen. Reagents used for flow cytometry included: Pacific Blue cell viability stain; FITC anti-CD45; AmCyan-CD45; PerCP Cy5.5 anti-CD3e; APC Cy7 anti-CD4; PE-Cy7 anti-CD8a. These were obtained from Becton Dickinson. Percoll was obtained from GE Healthcare. All other reagents were obtained in the highest grade possible from Sigma.

Isolation and Analysis of leukocytes: Spleens, aortas, mesenteric vascular arcades (excluding lymph nodes) and kidneys were digested using collagenase type XI (125 U/ml), collagenase type I-S (450 U/ml), and hyaluronidase I-S (60 U/ml) dissolved in 20 mM Hepes-PBS buffer containing calcium and magnesium for 30 min at 37°C. The tissues were further dispersed using repeated pipetting and the resultant homogenate was passed through a 70-µm sterile filter, yielding single-cell suspensions. To enrich kidney samples for leukocytes percoll gradient centrifugation was employed. Cells were washed with PBS buffer and resuspended in 2 ml 40% Percoll. Resuspended cells were layered on top of 2 ml of 60% Percoll, the resultant gradient centrifuged at 2400 RPM for twenty minutes. The uppermost layer of the gradient, containing fat and non-cellular material not removed by the filter was discarded and cells in the lower portion of the gradient were counted and used for flow cytometry and cell isolation.

Polymerase chain reactions (PCR): For PCR analysis of T cell V\(\beta\) subtypes, single cell suspensions from spleen, mesenteric vascular arcade and kidney were obtained as described above. CD4\(^{+}\) and CD8\(^{+}\) cells were isolated using kits from Miltenyi Biotec following manufacturer’s directions. Total RNA from CD4\(^{+}\) and CD8\(^{+}\) cells was isolated using the RNEasy Mini Kit (Qiagen). One hundred ng (kidney and mesentery T cells) or 1 µg (spleen T cells) of RNA was used for reverse transcriptase cDNA synthesis (iScript cDNA synthesis kit, BioRad).
**T cell Vβ Receptor Spectratyping Analysis:** PCR was employed using a forward primer for 1 of the 24 TCR Vβ genes and a constant reverse primer for the Cβ gene labeled with FAM on the 5' end (primer sequences shown in Table S1). T cell receptor Vβ family nomenclature consistent with Wilson et al. is used throughout.³ PCR reactions were carried out as follows: 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 72°C and a final 10 min at 72°C. Amplified products were then subjected to high-resolution capillary electrophoresis on an Applied Biosystems 3730 at the Vanderbilt Genome Sciences Resources Core (VANTAGE).

TCR length profiles were analyzed using Applied Biosystems Peak Scanner software and the area under each peak at a given transcript length was divided by the total area under all peaks to determine the relative frequency of each individual transcript length.⁴, ⁵

**T cell Receptor Deep Sequencing:** TCRβ CDR3 regions were amplified and sequenced by Adaptive Biotechnologies Corp. (Seattle, WA) from a subset of cDNA samples from spleen, mesentery and kidney resident CD8+ T cells. Briefly, multiplex PCR amplified all possible rearranged TCRβ CDR3 regions using 35 forward primers specific for all Vβ gene segments and 13 reverse primers for all Jβ segments. The primers contain at their 5' ends the universal forward and reverse sequences compatible with the Illumina HiSeq cluster station solid-phase PCR system. The HiSeq system generates 60 base-pair reads, which cover the entire CDR3 length sequencing from the J to the V region. The amplification and sequencing protocols have been previously described in detail.⁶, ⁷

**Adoptive transfer:** In some studies, either pan-T cells, CD4+/CD25−, + or CD8+ cells were isolated using negative selection from the spleens of mice previously treated for two weeks with angiotensin II. These cells were then resuspended in 200 µL of saline and injected by tail vein into RAG-1−/− mice that had previously received angiotensin II for ten days. To determine the role of T regulatory cells CD4+/CD25+ cells were isolated from spleens of non-treated wild type mice and injected into wild type mice that had received angiotensin II for 9 days. When total T cells were used approximately 10⁷ cells were infused. For adoptive transfer of either CD4+/CD25− or CD8+ cells, we infused 5 X 10⁶ cells.

**Mesenteric vascular reactivity:** Second and third order mesenteric vessels were examined using a wire myograph technique and isometric tension recordings as previously described.⁸ Cumulative dose responses to acetylcholine and sodium nitroprusside were examined following preconstruction with phenylephrine (1 µM/L).

**Sodium and volume challenge experiments:** To examine the ability of mice to excrete a volume and salt challenge at baseline and in the setting of angiotensin II infusion, animals were briefly anesthetized with isoflurane in a specialized chamber and then injected intraperitoneally with a volume of normal saline (pre-warmed to 37°C) equal to 10% of their body weight. The mice awoke immediately following this. Urine in the bladder was eliminated by mild supra-pubic compression and the mice then were placed in a metabolic chamber for the subsequent 4 hours. The volume of urine was recorded and urinary sodium and chloride excretion was measured and expressed as a percent of the saline previously injected. These experiments were performed at 2, 5 and 10 days following onset of angiotensin II infusion. Urinary volume, sodium and chloride were measured as previously described.⁹
Analysis of kidney vascular morphology: Following sacrifice, mice were perfused with 5 ml of PBS with 10 IU/ml heparin sulfate via the left cardiac ventricle. This was followed by perfusion with approximately 10 ml of the radiopaque contrast agent Microfil MV-122 (Flowtech, Inc., Carver, MA). The aorta and vena cava were clamped at the end of perfusion to maintain the perfused volume during curing and the entire carcass was held at 4°C overnight to promote curing of the Microfil. Both kidneys were excised the following day and placed in 10% neutral buffered formalin. The Microfil-perfused vasculature of each kidney was imaged and analyzed by microCT using a µCT50 (Scanco Medical AG, Brüttisellen, Switzerland). Cross-sectional images of the entire kidney were acquired with an isotropic voxel-size of 5.0 µm in a 10.24 mm field-of-view using X-ray source settings of 55 kVp and 200µA without beam filtering, 1000 projections per rotation, and 700 msec integration per projection. Image acquisition required approximately 2.5 hours per kidney.

The entire kidney was selected by manually contouring the outer edge of the kidney in 50-300 slice increments and using an automated ROI interpolation algorithm included in the Scanco Evaluation software. The cortex was defined as the region within 600 µm of the renal capsule and the medulla was defined the volume interior this region. Three-dimensional vascular renderings were created by segmenting the soft tissue from contrast perfused vasculature using a grey-scale threshold of 260 and Gaussian noise filter with Sigma of 2.3 and Support of 4. Renal, cortical and vascular volumes were determined using a voxel counting method.

For analysis of renal arteriolar media lumen ratios, perfused, fixed kidneys were sliced into 5 µm sections and stained for alpha smooth muscle actin to identify renal arterioles. This stain does not identify capillaries or veins. Likewise, vessels in the renal medulla do not stain positively for actin. Sections from the renal cortex were visualized on a computer screen and the inner and outer diameter of the vascular media was planimetered from more than 15 arterioles less than 25 µm in diameter. The difference between these two values was taken to be the medial area and the inner diameter the lumen area.

Statistics: Data in the manuscript are expressed as the means ± SEM. Comparisons of blood pressure over time were made using one-way ANOVA for repeated measures, followed by a Student Newman Keuls post hoc test when significance was indicated. To compare the effect of angiotensin II on different groups of mice, one- or two-way ANOVA was employed, as indicated. TCR spectratyping data was analyzed using the Dirichelet distribution and the MaGiK analysis. The MaGiK analysis is a statistical technique developed to identify alterations in TCR spectratyping profiles and dominant transcript lengths. This analysis is highly rigorous and resistant to type I errors as it requires a greater than 3 standard deviation difference when comparing experimental group to controls to be considered statistically different. P values are reported in the figures.
REFERENCES


Table S1. T cell Receptor primer sequences

<table>
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<th>Primer</th>
<th>Sequence</th>
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<td>Vβ2</td>
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**Figure S1:** Effect of T cell subtypes on DOCA-salt hypertension. Mice underwent uninephrectomy, implantation of a pellet of desoxycorticosterone (DOCA) and were administered 1% salt in drinking water. A) Systolic and B) Diastolic Blood pressures were measured using telemetry and compared using repeated measures ANOVA. n = 4-6 per group.
**Figure S2:** Leukocyte accumulation in aortas of wild type and CD8\(^{-/-}\) mice. Aortas from mice treated with angiotensin II (Ang II) for 2 weeks were digested and leukocyte accumulation was determined using flow cytometry with a FITC anti-CD45 antibody. A) Sample flow cytometry plots and B) Absolute number of CD45+ leukocytes per aorta. \(n = 4\text{-}5\) per group.