Activation of Human T Cells in Hypertension
Studies of Humanized Mice and Hypertensive Humans


Abstract—Emerging evidence supports an important role for T cells in the genesis of hypertension. Because this work has predominantly been performed in experimental animals, we sought to determine whether human T cells are activated in hypertension. We used a humanized mouse model in which the murine immune system is replaced by the human immune system. Angiotensin II increased systolic pressure to 162 versus 116 mm Hg for sham-treated animals. Flow cytometry of thoracic lymph nodes, thoracic aorta, and kidney revealed increased infiltration of human leukocytes (CD45+) and T lymphocytes (CD3+ and CD4+) in response to angiotensin II infusion. Interestingly, there was also an increase in the memory T cells (CD3+/CD45RO+) in the aortas and lymph nodes. Prevention of hypertension using hydralazine and hydrochlorothiazide prevented the accumulation of T cells in these tissues. Studies of isolated human T cells and monocytoids indicated that angiotensin II had no direct effect on cytokine production by T cells or the ability of dendritic cells to drive T-cell proliferation. We also observed an increase in circulating interleukin-17A producing CD4+ T cells and both CD4+ and CD8+ T cells that produce interferon-γ in hypertensive compared with normotensive humans. Thus, human T cells become activated and invade critical end-organ tissues in response to hypertension in a humanized mouse model. This response likely reflects the hypertensive milieu encountered in vivo and is not a direct effect of the hormone angiotensin II. (Hypertension. 2016;68:00-00. DOI: 10.1161/HYPERTENSIONAHA.116.07237.)

Key Words: antigens, CD45, dendritic cells, inflammation, lymph nodes, myeloid cells

In the past several years, it has become evident that inflammation contributes to the elevation of blood pressure and end-organ damage in numerous experimental models of hypertension. In mice lacking the recombinase-activating gene (RAG-1−/− mice), which lack all lymphocytes, the hypertensive responses to angiotensin II (Ang II), deoxycorticosterone acetate-salt challenge, norepinephrine, and emotional stress are blunted.1-3 Moreover, these mice are protected against endothelial dysfunction, vascular hypertrophy, and do not develop an increase in vascular superoxide as observed in normal mice. Adoptive transfer of T cells, but not B cells, restores the hypertensive response and vascular dysfunction in RAG-1−/− mice.1 Mice with severe combined immunodeficiency are also protected against hypertension.4 Likewise, deletion of the RAG-1 gene in Dahl salt-sensitive rats blunts their hypertension and renal damage on salt feeding.5 T cells with an effector phenotype infiltrate the kidney and periarteriolar tissues, where they release inflammatory cytokines, including interleukin 17A, interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) that in turn alter renal and vascular function and participate in end-organ damage.6-8 Genetic deletion of these cytokines or treatment with their specific antagonists blunts hypertension and its attendant end-organ dysfunction.

Despite the overwhelming evidence that inflammation and immunity contribute to hypertension in these experimental models, there is a paucity of information to suggest that...
hypothesis or the factors present in the hypertensive milieu lead to T-cell activation in humans. In the present study, we addressed this issue using several approaches. First, we used a unique animal model, the bone/liver/thymus humanized mouse to determine whether Ang II–induced hypertension is associated with T-cell activation and infiltration into key target tissues. Second, we examined the direct effects of Ang II on human T-cell activation and human dendritic cell (DC) function in culture. Finally, we analyzed the phenotype of circulating T cells in humans with and without hypertension.

Methods

Bone/Liver/Thymus Mice

The Vanderbilt University Institutional Animal Care approved all experiments. Mice were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Bone marrow, liver, thymus mice were produced on a NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) background as previously described.9,10 Twelve to 14 weeks after immune reconstitution, osmotic minipumps (Alzet, model 2002) were implanted subcutaneously for infusion of either Ang II (490 ng/kg per minute) or an isotonic vehicle (sodium chloride/acetic acid solution) for sham mice. In some experiments, hypertension was prevented by coadministration of hydralazine and hydrochlorothiazide as previously described.11

Response of Human T Cells and DCs to Ang II

Two experimental approaches were used to examine the direct effects of Ang II on human immune cells. In the first, human T cells were isolated from the blood of normotensive volunteers using magnetic sorting and 10^6 cells were exposed to anti-CD3+–coated culture plates with anti-CD28 (2 μg/mL) added to the media for 3 days in the presence or absence of 0.1 μmol/L of Ang II. Intracellular staining was used to identify cells containing IFN-γ, interleukin (IL)-17A, and forkhead box P3 as previously described.12 As a second approach, human monocytes were differentiated to DCs in the presence of granulocyte/macrophage colony stimulating factor and IL-4 with and without addition of 0.1 μmol/L of Ang II. These cells were then cocultured with CFSE-labeled T cells from the same volunteer at a ratio of 1 DC to 10 T cells.

Studies of Circulating T Cells in Humans

Peripheral blood samples were collected in EDTA from 20 hypertensive patients and 20 healthy volunteers, matched for age, sex, and body mass index. A detailed history of major cardiovascular risk factors for atherosclerosis and characteristics of hypertension were obtained (Table 1). Patient groups did not differ in major cardiovascular risk factors apart from hypertension. Exclusion criteria are listed in the Materials and Methods in the online-only Data Supplement. Office blood pressure was determined in all subjects, and 4 resting measurements were averaged. Twenty-four-hour ambulatory blood pressures were used to confirm hypertension. The Local Research Ethics Committee of Institute of Cardiology at Jagiellonian University approved collection of tissue specimens, and all patients gave written informed consent.

Flow Cytometry

Flow cytometry was performed on single-cell suspensions of thoracic lymph nodes, the right kidney, and the descending thoracic aorta as previously described.12 Human cells were initially identified by antihuman CD45, and subsets were identified using antibodies that specifically react with human cells. Preliminary studies showed that these antibodies did not identify cells in NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice without immune reconstitution. Flow cytometry gates were established using flow minus one as previously described and used in our laboratory (Figure S1 in the online-only Data Supplement).13,14

Statistics

Data in the article are presented as mean±SEM. To compare blood pressure measurements in mice over time, 2-way ANOVA with multiple comparisons was used. To compare numbers of human T cells in various tissues, we used either an unpaired t test to analyze or the nonparametric Mann–Whitney U test when variances between groups were not equal. To show the effect of hypertension on myeloid differentiation, we used 1-way ANOVA to analyze the flow cytometry data. For comparison of the frequency of T-cell subtypes in humans, unpaired T tests were used with a Bonferroni correction. χ² analysis with Bonferroni correction was used to compare the frequency of risk factors between the normotensive and the hypertensive populations. P values reported in the figures and tables represent the adjusted values after multiple testing. (complete description of the methods used is available in the online-only Data Supplement).

Results

Effect of Angiotensin II Infusion on Blood Pressure of Humanized Mice

The blood pressure of humanized mice increased to 162 mm Hg after 2 weeks of Ang II infusion when compared with mice that were exposed to a sham infusion (Figure S2). Coadministration of hydralazine and hydrochlorothiazide reduced blood pressure to 112±0.5 mm Hg.

Effect of Angiotensin II–Induced Hypertension on the Presence of Human T Cells in the Kidney

After 2 weeks of Ang II or sham infusion, flow cytometry was performed to quantify the presence of T-cell activation in the kidney that had not previously undergone the thymus/liver transplant. As shown in the representative flow cytometry dot plots in Figure 1A to 1C and the mean data in Figure 1D to 1G, there was accumulation of total human leukocytes (CD45+ cells), total T cells (CD3+), and several T-cell subpopulations (CD4+ and CD8+) in the kidney. The cells were predominantly composed of CD4+ T cells. There was a nonsignificant trend for an increase in CD8+ T cells. Among the CD3+ and CD4+ T cells, Ang II infusion increased the number of renal CD45RO+ cells indicating an increase in human memory T cells (Table 2). There were relatively few CD69+ T cells in the kidney, and these were not changed by Ang II infusion (Table S1). Prevention of hypertension by coadministration of hydralazine and hydrochlorothiazide completely abrogated the elevation of both CD4+ and CD8+ T cells and the formation of RO+ cells in the kidney (Figure 1; Table 2).

Effect of Angiotensin II–Induced Hypertension on the Presence of Human T Cells in the Aorta

Representative flow cytometry for human T cells in the aorta is shown in Figure 2A through 2C. In contrast to the kidney, relatively few human cells accumulated in the aorta; however, Ang II infusion increased the small numbers of human CD45+ and CD3+, and CD4+, and CD8+ T cells (Figure 2D through 2G) in the aorta compared with sham infusion. Total CD45RO+ memory T cells and CD4+ memory T cells in the aorta were likewise increased by Ang II infusion (Table 2). Prevention of hypertension with hydralazine and hydrochlorothiazide significantly reduced accumulation of CD3+ and CD8+ T cells in the aorta and reduced the presence of total memory T cells and CD4+...
<table>
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<th>Control (n=20)</th>
<th>Hypertension (n=20)</th>
<th>P Value</th>
</tr>
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<td>Female sex, n (%)</td>
<td>11 (55)</td>
<td>11 (55)</td>
<td>NS</td>
</tr>
<tr>
<td>Age, y (±SD)</td>
<td>52.6±12</td>
<td>52.6±11</td>
<td>NS</td>
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<tr>
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<td>100%</td>
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<td>Office blood pressure</td>
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<td>150±19/90±11</td>
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<td>24-h ABPM (systolic mm Hg ± SD)</td>
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<td>143±18</td>
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<td>24-h ABPM (diastolic mm Hg ± SD)</td>
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<td>85±9</td>
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<td>10.8±6</td>
<td>18±9</td>
<td>&lt;0.05</td>
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<td>Aldosterone (after supine position)</td>
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<td>NS</td>
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<tr>
<td>Ever-smoking status (%)</td>
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<td>8 (40)</td>
<td>NS</td>
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<td>0 (0)</td>
<td>NS</td>
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<td>0 (0)</td>
<td>NS</td>
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<tr>
<td>PAD</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Medications (%)</strong></td>
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<tr>
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<tr>
<td>Statins</td>
<td>1 (5)</td>
<td>9 (45)</td>
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</table>

ABPM indicates ambulatory blood pressure; ACE, angiotensin-converting enzyme; BMI, body mass index; CAD, coronary artery disease; DM, diabetes mellitus; EF, ejection fraction; GFR, glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; NS, nonsignificant; PAD, Peripheral arterial disease; TIA, transient ischemic attack; and WBC, white blood cell.
memory T cells. (Figure 2; Table 2). Ang II did not change the few CD69+ T cells in the aorta (Table S1).

Effect of Angiotensin II–Induced Hypertension on the Presence of Human T Cells in Lymph Nodes

The greatest accumulation of human cells observed in the humanized mice was noted in lymph nodes. Representative flow cytometry dot plots are shown in Figure 3A through 3C. Ang II–induced hypertension caused a 3- to 5-fold increase in human CD3+, CD4+, and CD8+ T cells in the murine lymph nodes of this model (Figure 3D through 3G). Ang II also caused a striking increase in the memory CD3+, CD4+, and CD8+/CD45RO+ T cells in the lymph nodes (Table 2). As in the cases of the kidney and aorta, prevention of hypertension abolished the accumulation of total CD3+ cells and both CD4+ and CD8+ T cells (Figure 3; Table 2). Unlike the case of the kidney and aorta, treatment with hydralazine and hydrochlorothiazide paradoxically increased the number of total CD45RO+ memory T cells and CD4+ memory T cells in lymph nodes.

Myeloid Differentiation in Angiotensin II–Treated Humanized Mice

Because of limitations of fluorochromes in our flow cytometry, we were unable to stain for monocyte/macrophages or DCs in the same animals in which we examined T-cell populations; however, one can approximate non–T-cell populations by subtracting the CD3+ population from CD45+ cells. There were nonsignificant trends for increases in these cells in the kidney (Figure 4A) and the aorta (Figure 4B). In the lymph nodes, Ang II infusion caused a significant increase in this population of human cells (Figure 4C). In 1 animal, there was a striking increase in the number of CD3+/CD45+ cells (Figure 4C); however, a significant difference remained between sham- and Ang II–infused mice even after exclusion of this animal.

Table 2. Effect of Ang II and Ang II+Antihypertensive Treatment on Accumulation of Human Memory (CD45RO+) Cells

<table>
<thead>
<tr>
<th></th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ang II</td>
<td>Ang II Hct+Hyd</td>
</tr>
<tr>
<td>Kidney</td>
<td>1443±295</td>
<td>6600±2923*</td>
<td>1227±661†</td>
</tr>
<tr>
<td>Aorta</td>
<td>113±19</td>
<td>333±116*</td>
<td>194±63†</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>443±233</td>
<td>2642±712*</td>
<td>17.736±10.333†</td>
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</tbody>
</table>

n= 4 to 6 per group. Ang II indicates angiotensin II; HctZ, hydrochlorothiazide; and Hyd, hydralazine.

*P<0.05 vs sham.
†P<0.05 vs Ang II.
In selected mice, we examined staining for CD14, a marker of human monocytes and CD83, an activation marker that appears on monocyte-derived DCs (Figure 4D and 4E). There were relatively few of these human myeloid cells in the kidney of Ang II–infused humanized mice, and these were equally distributed between CD14+, CD14+/CD83+, and CD14−/CD83−.
cells. In contrast, in the aorta and lymph nodes, the predominant population of human myeloid cells was CD83+/CD14−.

Direct Effects of Ang II on Human T Cells and DC Function

Our above data suggest that normalizing blood pressure prevents human T-cell activation in vivo, suggesting that Ang II does not directly stimulate T-cell cytokine production. One criticism of these experiments is that the antihypertensive drugs might have off-target effects to inhibit T-cell activation. To further study the direct effect of Ang II on human immune cells, we performed 2 additional experiments. In the first, we analyzed the effect of Ang II (0.1 μmol/L) on the production of IL-17A, IFN-γ, forkhead box P3, and TNF-α in human T cells cultured for 7 days on anti-CD3 plates using intracellular staining. As shown in Figure 5A through 5D, there was no direct effect of Ang II on the production of T(H)1 cytokines or forkhead box P3 by CD3+, CD4+, or CD8+ T cells. Because murine T cells can produce Ang II,15 we considered the hypothesis that endogenous Ang II might affect human T-cell production of cytokines. Coincubation human T cells with ramipril (1 μmol/L), however, had no effect on production of any cytokines measured (data not shown). In another experiment, we cultured human monocytes for 7 days with granulocyte/macrophage colony stimulating factor and IL-4 in the presence and absence of Ang II (0.1 μmol/L). The resultant differentiated DCs were then cultured with CFSE-labeled T cells from the same donor. As shown in Figure 5E and Figure S3, there was no effect of Ang II on the ability of human DCs to drive T-cell proliferation. Moreover, Ang II did not directly stimulate the accumulation of isoketal-protein adducts in human DCs, as evidenced by intracellular staining with the isoketal adduct antibody D11 (Figure 5F).

Inflammatory Profile of Circulating T Cells in Humans With and Without Hypertension

To further characterize the effect of hypertension on T-cell phenotype, we studied 20 normotensive and 20 hypertensive humans, matched for sex, age, and body mass index. Hypertensive patients had significantly higher office blood pressures and both systolic and diastolic blood pressures as measured by ambulatory blood pressure monitoring. Plasma
renin and aldosterone levels were elevated in the hypertensive group compared with the normotensive subjects. As shown in Figure 6A and 6B, the percent of both CD4+ and CD8+/CD45RO+ circulating T cells was greater in the hypertensive patients than in the normotensive controls. Intracellular staining showed that CD4+ T cells of humans with hypertension produce greater amounts of IL-17A than normotensive controls (Figure 6C and 6D). Few circulating CD8 + T cells were positive for IL-17A (data not shown). Intracellular staining for IFN-γ and TNFα revealed that both of these cytokines were increased in CD4+ T cells, and CD8+ T cells of humans with hypertension and were commonly coproduced within the same cells (Figure 6E and 6F).

Discussion

The major finding of the current study is that the hypertensive stimulus of Ang II promotes accumulation of human T cells in the kidney, aorta, and lymph nodes of humanized mice. These cells exhibit an increase in the memory cell marker CD45RO and to a lesser extent the activation marker CD69. In addition, CD3+CD45+ cells, which represent other myeloid cells, are increased in lymph nodes by Ang II infusion. There also seems to be differentiation of human monocytes to activated DCs in hypertensive mice. Finally, we demonstrate that circulating T cells of humans with hypertension exhibit evidence of activation, as indicated by an increased percent of memory T cells and an increase in production of IL-17A and IFN-γ.

Although substantial data support the role of T cells in the genesis of experimental hypertension, there is a paucity of data showing that these cells are activated as a result of human hypertension. Many years ago, Olsen16 noted periarteriolar infiltration of immune cells in humans with hypertension and pointed out that these seemed to be lymphocytes and monocytes. We found that blood levels of IL-17A are 3× higher in diabetic humans with hypertension than in those without hypertension. Herrera et al17 showed that the T-cell suppressing agent mycophenolate mofetil lowers blood pressure in humans with rheumatoid arthritis or psoriasis. Recently, Yoshida et al18 demonstrated that the TNF-α antagonist infliximab lowers blood pressure in patients with rheumatoid arthritis. Of note, the incidence of hypertension is increased in humans with such autoimmune diseases. Youn et al21 showed that humans with hypertension have an increased number of circulating immunosenescent proinflammatory CD8+ T cells compared with age-matched nonhypertensive subjects. These cells produced increased amounts of IFN-γ, TNF-α, and the cytotoxic molecules granzyme B and perforin than CD8+ T cells from normal subjects. Immunohistochemical studies of renal biopsies revealed an increase in both CD4+ and CD8+ T cells in humans with hypertensive nephrosclerosis compared with controls. These authors also observed an increase of CXCR3 cytokines in the sera of hypertensive patients.

Our current studies of circulating T cells from humans with hypertension confirm the findings of Youn et al21 that CD8+ T cells produce large amounts of TNFα and IFN-γ. We also found that CD4+ T cells of hypertensive humans produce increased amounts of IL-17A and IFN-γ. The increased number of T<sub>h</sub>17 cells is compatible with our previous finding that
humans with hypertension and diabetes mellitus have higher circulating levels of IL-17A than patients with diabetes mellitus alone. In the present study, we also found an increase in the percent of circulation memory CD4+ and CD8+ T cells. We recently showed that memory T cells are major sources of IFN-γ and IL-17A in mice with hypertension, and that these cells seem to prime hypertensive responses to repeated challenges with high salt or Ang II. Thus, the finding of increased circulating memory cells might have pathophysiological significance for sustaining hypertension in humans.

The accumulation of human T cells in the kidney of humanized mice is similar to the accumulation of renal T cells observed by Youn et al and parallels similar increases that we and others have observed in mice with experimental hypertension. It is likely that cytokines released from T cells in the kidney promote sodium and volume retention and lead to renal damage. We recently found that IFN-γ modulates phosphorylation and activation of the Na-K-2Cl cotransporter, Na-Cl cotransporter, and Ste20/SPS-1–related proline-alanine-rich kinase in response to long-term Ang II infusion.

In this study, we established an important role of IL-17A and IFN-γ in modulating the expression of Na/H-exchanger isoform 3 and the motor myosin VI in the proximal tubule and in the antinatriuretic and antidiuretic effects of Ang II. The propensity of human T cells to accumulate in the kidney supports the notion that these cells might contribute to human hypertension in a similar fashion.

It is also of interest that we observed a striking increase in human T cells in the thoracic lymph nodes of the humanized mice in response to Ang II infusion. This was paralleled by an
increase in cells bearing the memory cell marker CD45RO. Memory cells persist after an initial expansion of a naïve pool, and it is therefore logical to conclude that the increase in total T cells and memory T cells in the lymph nodes was because of proliferation and expansion in response to Ang II infusion not observed in the sham-infused mice. We cannot exclude the role of chemokines and adhesion molecules that might also have been increased by Ang II in the lymph nodes and kidneys of the humanized mice that could have attracted the human cells to these organs. It is notable that although the prevention of hypertension using hydralazine and hydrochlorothiazide reduced the total number of T cells in the lymph nodes, the number of memory T cells in the lymph nodes increased. This might reflect differences in the effect of hypertension and direct effects of angiotensin II on trafficking of memory cells in secondary lymphoid organs.

Using strict flow minus one gating criteria, we also observed an increase in CD3+ cells lacking either CD4 or CD8 surface markers, particularly in the lymph nodes. We have observed a similar increase in double-negative murine T cells in hypertensive mice. It is conceivable that these double-negative T cells are actually CD4+ cells that have lost surface CD4, as has been observed during intense T-cell stimulation with phorbol esters or myxoma virus.

We have previously proposed that mild elevations in blood pressure, such as observed in prehypertension, lead to formation of neoantigens that activate T cells that exacerbate vascular remodeling and renal dysfunction, leading to further elevations of blood pressure. Our current finding that preventing the initial elevation in blood pressure prevents human T-cell activation is in keeping with this concept. Our results also indicate that Ang II does not directly activate human T cells. We and others have previously shown that T cells contribute to salt-sensitive hypertension in rats and mice. These conditions are associated with suppression of Ang II production, indicating that this octapeptide is not needed for this action of immune cells in hypertension. Our results with pharmacological prevention of hypertension should be viewed with some caution however because pharmacological agents can have off target effects that might directly inhibit T-cell function. As an example, we previously showed that hydralazine potently inhibits the nicotinamide adenine dinucleotide phosphate-oxidase, and thus might prevent formation of immunogenic isoketional-protein adducts independent of its blood pressure–lowering effects. To avoid the confounding effects of such agents, we examined the direct effect of Ang II on both human T cells and human DCs however failed to observe a change in the production of inflammatory cytokines or the T regulatory cell transcription factor forkhead box P3. In preliminary experiments, we also found that norepinephrine did not enhance the ability of DCs to drive T-cell proliferation.

Taken together, we view these findings as suggesting that human immune cells likely take cues from other tissues in vivo to become activated in hypertension. In keeping with this, we recently found that chronic vascular oxidative stress induces activation of both DCs and T cells in mice, likely by generating isoketals within the vessel that are taken up by DCs. Therefore, it is possible that vascular oxidative stress associated with hypertension promotes human T-cell activation.

We encountered several limitations in the present study that precluded in-depth study of these mice. We found that these mice did not tolerate the 2 surgeries needed for implantation of telemetry units for blood pressure measurement and subsequent osmotic minipump placement, and therefore relied on tail cuff measurement of blood pressure. Our studies should also be interpreted with caution because it is unclear how effectively human T-cell receptors interact with mouse adhesion molecules and chemokines. It is possible, for example, that vascular adhesion molecules in humans might more avidly attract activated T cells to vessels than we observed in the humanized mouse. The humanized mouse also presents a limited window of time, ranging from 4 to 12 weeks after reconstitution with human tissues before graft versus host disease develops, and therefore longer-term hypertensive challenges are problematic. In our studies, none of the mice exhibited signs of graft versus host disease, such as weight loss, diarrhea, or hair loss. Because human T-cell expansion did not occur in the sham-infused animals, we do not think that the T-cell activation observed in the Ang II–treated group was because of graft versus host disease.

Perspectives
This is the first study to demonstrate that human T cells respond to a hypertensive stimulus such as Ang II by expanding and infiltrating the kidney and vasculature. Our results might also help explain the striking benefit of blood pressure correction observed in the recent Systolic Blood Pressure Intervention Trial (SPRINT). It is conceivable that intensive blood pressure lowering by any means can prevent activation of human immune cells and therefore reduce cardiovascular inflammation.

Sources of Funding
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Disclosures
None.

References


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Angiotensin II promotes interleukin-17A and CD8+ T cells producing interferon-γ.

The current study shows that human cells are activated in the setting of a membrane-bound NADH oxidase.


Correction of blood pressure prevented T-cell accumulation in the kidney, aorta, and lymph nodes of humanized mice.

Correction of blood pressure prevented T-cell accumulation in the kidney, aorta, and lymph nodes of humanized mice.

Novelty and Significance

What Is New?

angiotensin II–induced hypertension leads to T-cell activation in humanized mice.

Humans with hypertension have an increase in the frequency of circulating memory T cells and an increase in the CD4+ T cells producing interleukin-17A and CD8+ T cells producing interferon-γ.

What Is Relevant?

Animal models have been used as surrogates to study hypertension in humans.

Inflammation is a key component in experimental hypertension, and the current study shows that human cells are activated in the setting of angiotensin II–induced hypertension and in humans with hypertension.

Inflammation has been shown to play a key role in the promotion and maintenance of hypertension in experimental animals and has been indirectly implicated in human hypertension. We provide the first evidence that human T cells are activated by a hypertensive stimulus like angiotensin II. Phenotypic characterization of circulating T cells in humans might provide insight into immune activation in hypertension.

Summary

Inflammation is shown to play a key role in the promotion and maintenance of hypertension in experimental animals and has been indirectly implicated in human hypertension. We provide the first evidence that human T cells are activated by a hypertensive stimulus like angiotensin II. Phenotypic characterization of circulating T cells in humans might provide insight into immune activation in hypertension.

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Activation of Human T Cells in Hypertension: Studies of Humanized Mice and Hypertensive Humans


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Hana A. Itani1*, William G. McMaster, Jr.1,2*, Mohamed A. Saleh1,3, Rafal R. Nazarewicz4, Tomasz P. Mikolajczyk5, Anna M. Kaszuba5,6, Anna Konior5, Aleksander Prejbis6, Andrzej Januszewicz5, Allison E. Norlander1, Wei Chen1, Rachel H. Bonami7, Andrew F. Marshall8, Greg Poffenberger9, Cornelia M. Weyand4, Meena S. Madhur1, Daniel J. Moore8, David G. Harrison1, Tomasz J. Guzik5,10

1 Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN
2 General Surgery, Vanderbilt University Medical Center, Nashville, TN
3 Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt
4 Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA
5 Department of Internal and Agricultural Medicine, Jagiellonian University School of Medicine, Krakow, Poland
6 Department of Hypertension, Institute of Cardiology, Warsaw, Poland
7 Division of Rheumatology, Department of Medicine, Vanderbilt University Medical Center
8 Division of Endocrinology and Diabetes, Department of Pediatrics, Vanderbilt University Medical Center
9 Division of Endocrinology, Department of Medicine, Vanderbilt University Medical Center
10 Institute of Cardiovascular and Medical Sciences, University of Glasgow, Scotland

* Drs. Itani and McMaster contributed equally to this work and should be considered co-first authors

Running Title: Activation of human T cells in hypertension

Address for Correspondence:
David G. Harrison, M.D.
Betty and Jack Bailey Professor of Medicine, Pharmacology and Physiology
Director of Clinical Pharmacology
Room 536 Robinson Research Building
Vanderbilt University
Nashville, TN 37232-6602
Telephone 615-875-3049
Fax 615-875-3297
email: david.g.harrison@vanderbilt.edu
BLT mice: The Vanderbilt University Institutional Animal Care and Use Committee approved all experiments, and all animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Male humanized bone marrow, liver, thymus (BLT) transplant mice were produced on a NSG (NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ) background as previously described, and were purchased from Jackson Laboratories. Approximately 12 weeks after engraftment, reconstitution of the mouse immune system by human cells was confirmed by quantifying circulating human CD45+, CD3+, CD4+, CD8+, B220+, and CD19+ cells using flow cytometry. Mice in which < 25% of the circulating cells were human cells were excluded. To produce hypertension, osmotic minipumps were implanted subcutaneously for infusion of Ang II (490 ng/kg/min). In sham mice, an isotonic vehicle (sodium chloride/acetic acid solution) was infused for 2 weeks. For all surgical procedures, the animals were anesthetized with an intraperitoneal injection of Ketamine/Xylazine (90-120 mg/kg and 10 mg/kg). Ketoprofen (5 mg/kg), administered intraperitoneally pre-operatively, on post-op day 1 and post-op day 2, was used for analgesia. Blood pressure was measured noninvasively via a computerized tail cuff system (Hatteras Inc.) as previously described. Mice were sacrificed at the end of all experiments by CO2 inhalation.

Studied subjects: Subjects were patients of the Department of Hypertension, Institute of Cardiology, undergoing diagnostic evaluations and healthy volunteers. Hypertension diagnosis was based on initial repeated office blood pressure measurements and subsequent ambulatory blood pressure monitoring. The following exclusion criteria were employed: age >70 years old, acute illnesses within 4 weeks, hospitalization within the past 3 months, Life expectancy of < 3 years, history of alcohol/substance abuse, allergic disorders, chronic infections, chronic obstructive pulmonary disease, tuberculosis, hepatitis, pericardial or pleural effusion, ascites, liver disease; (d) Chronic inflammatory/autoimmune conditions, malignancy, myeloproliferative or lymphoproliferative disease within the past 5 years, HIV+; immunization within the past 3 months, pulmonary hypertension, use of systemic or local steroids/immunosuppressive agents within the past 6 months, current or previous use of anti-hypertensive medication, pregnancy, nursing, cardiovascular events within past 12 months, heart failure, BMI > 40, GFR < 60mL/min/1.73 m². Table 1 summarizes key clinical characteristics of the studied groups.

Flow cytometry: For studies of humanized mice, single cell suspensions were prepared from the periaortic lymph nodes, the right kidney and the descending thoracic aorta as previously described. The non-implanted kidney was used for analysis of renal immune cell infiltration. The kidney was mechanically dissociated using a single gentleMACS C tube dissociator system (Miltenyi Biotec) followed by incubation at 37°C for 20 minutes with collagenase D (2 mg/ml) and DNAse I (100 µg/ml) in RPMI 1640 medium with 5% FBS. Periaortic lymph nodes and thoracic aortas with surrounding perivascular fat were minced with fine scissors and digested with 2 mg/ml collagenase D and100 µg/ml DNAse I in RPMI 1640 medium with 5% FBS for 30 minutes at 37°C, with intermittent agitation. Tissue homogenates were filtered through a 70-μm-cell strainer. Kidney samples were subjected to Percoll gradient centrifugation by resuspension in 36% Percoll (Amersham Pharmacia Biotech), gently overlaid onto 72% Percoll, and centrifuged at 1,000 g for 20 minutes at 4°C. Cells were isolated from the Percoll interface and washed in cold PBS. Single-cell suspensions (1×106 cells) were stained for flow cytometry.

To establish gates for each fluorophore, flow minus one samples were run in parallel in each experiment as previously described. For these, cells were stained with all fluorophores except one. Preliminary experiments established that these gates were virtually identical to those
determined using isotype controls. Analysis was performed using BD FACSDiva (BD Biosciences) and FlowJo software. All lymphocyte subpopulations (CD4+, CD8+, CD4–CD8–, CD45RO+ and CD69+) were quantified within the CD3+ gate. Results are expressed as number of cells per kidney, per thoracic aorta, or per 3 lymph nodes.

For analysis of cells from patients, peripheral blood mononuclear cells (PBMC) were isolated by standard gradient centrifugation (Pan Biotech). After isolation, cells were suspended in RPMI with 10% FBS (Gibco, Life Technologies), gentamycin, and L-glutamine (Sigma-Aldrich) whole blood was incubated for 20 minutes at 4°C with fluorescently labeled monoclonal antibodies. For intracellular staining, cells were cultured with Leukocyte Activation Cocktail with BD Golgi Plug (BD Pharmingen) for 4 hours at 37°C in a 5% CO2. Cells were fixed and permeabilized using BD Cytofix/Cytoperm, and were stained for intracellular cytokines for 20 minutes. For the human sample studies, cells were studied on FACSVerse cytometer (BD).

**Materials and reagents used:** Angiotensin II, hydralazine and hydrochlorothiazide were obtained from Sigma-Aldrich in the highest grade available. Anti-CD3 plates were obtained from Corning and anti-CD28 was from BD Pharmingen. Interleukin 4 and GM-CSF were obtained from Miltenyi Biotech. Carboxyfluorescein succinimidyl ester (CFSE) was from ThermoFisher. Osmotic minipumps were obtained from Alzet (model 2002). Antibodies used for flow cytometry in the humanized mice studies were as follows: peridinin chlorophyll protein–cyanin-5.5–conjugated (PerCP-Cy5.5) anti-CD45 mouse antibody (BD Biosciences, clone 30-F11), allophycocyanin–Hilite-7–conjugated (APC-H7) anti-CD45 human antibody (BD Biosciences, clone 2D1), Phycoerythrin (PE) anti-CD3 human (BD Biosciences, clone UCHT1), Brilliant Violet 510–conjugated (BV510) anti-CD4 human antibody (BD Biosciences, clone SK3), Brilliant Violet 450–conjugated (BV450) anti-CD8 human antibody (BD Biosciences, clone RPA-T8), Fluorescein isothiocyanate (FITC) anti-CD45RO human antibody (BD Biosciences, clone UCHL1), Allophycocyanin (APC) anti-CD69 human (BD Biosciences, clone FN50), Phycoerythrin (PE) anti-CD45 human (eBiosciences, clone 2D1), Fluorescein isothiocyanate (FITC) anti-CD14 human antibody (miltenyibiotec, clone TÜK4), Brilliant Violet 510–conjugated (BV510) anti-CD83 human antibody (BD Biosciences, clone HB15e), FITC-conjugated isoketal adduct antibody (D11), APC anti-foxP3 human antibody (BD Biosciences), FITC anti-IFN-γ human antibody (BD Biosciences, catalogue #: 502505), and BV510 anti-IL-17A human antibody (BD Biosciences, Clone N49-653). For live/dead staining and cell counting purposes, 7-aminoactinomycin-D (7-AAD) (BD Biosciences, catalogue #: 559925) and 50 microliters of CountBright™ Absolute Counting Beads (Life Technologies, catalogue #: C36950) were added to the cells just prior to analysis on a BD FACSCanto II system for precise quantification of cells in the respective organs.

For studies of circulating human cells we employed anti-CD3-PerCP (Clone SK7), anti-CD4-APC (Clone RPA-T4), anti-CD8-APC-H7 (Clone SK1) and anti-CD45RO-PE (Clone UCHL1) from Biolegend. Antibodies for intracellular staining included anti-IL-17-PE (clone N49-653), anti-TNFα-PE (Clone 6401.1111) and anti-IFNγ-FITC (clone B27), all from BD Pharmingen.

**Statistics:** Data in the manuscript are presented as mean ± standard error of the mean. To compare blood pressure measurements over time, two-way ANOVA with multiple comparisons was employed. To compare numbers of human T cells in various tissues we used either an unpaired T test to analyze or the non-parametric Mann Whitney test when variances between groups were not equal. To show the effect of hypertension on myeloid differentiation, we used one-way ANOVA to analyze the flow cytometry data. For comparison of the frequency of T cell subtypes in humans, unpaired T tests were employed with a Bonferroni correction. Chi square
analysis with Bonferonni correction was employed to compare the frequency of risk factors between the normotensive and hypertensive populations. P values reported in the figures and tables represent the adjusted values after multiple testing.


Table S1: Effect of ang II and ang II + anti-hypertensive treatment on accumulation of CD69+ RO+ cells.

<table>
<thead>
<tr>
<th>Organ analyzed</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ang</td>
<td>Ang II</td>
</tr>
<tr>
<td>Kidney</td>
<td>4 ± 1</td>
<td>4 ± 2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Aorta</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lymph Nodes</td>
<td>12 ± ND</td>
<td>49 ± ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

N = 4-6 per group, * p < 0.05 vs sham. ND = not detected
Figure S1: Fluorescence minus one (FMO) used to establish gating for CD45+, CD3+, CD4+, and CD8+ cells.
Figure S2: Humanized mice response to Ang II. The blood pressure of humanized mice increased from 110 to 162 mmHg by day 13/14 of Ang II infusion (490 ng/kg/min). Data were analyzed using two-way ANOVA, n=6 per group.
Figure S3: Dot plots of CFSE labeled CD4$^+$ and CD8$^+$ T cells co-cultured with allogeneic DCs derived from monocytes. DCs were produced by exposure of human monocytes to GM-CSF and IL-4 for 7 days in the presence or absence of ang II, 100 nM/liter. The resultant DCs were then co-cultured with CFSE-labeled T cells from the same subject for 7 days at a ratio of 1 DC to 10 T cells. The bar graph on the right shows proliferated cells in the presence or absence of ang II.