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*See Editorial Commentary, pp 13–15*

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of CXCR3 chemokines in newly diagnosed, treatment-naïve patients with hypertension and in age- and sex-matched, normotensive control subjects by cytometric bead array. Finally, we examined renal expression of a CXCR3 chemokine, interferon (IFN)-inducible T-cell α chemok attractant (I-TAC), in biopsy specimens of patients with hypertensive nephrosclerosis and normotensive control subjects. Because hypertension is a disease process that is tied to aging associated with vascular aging, we tried to elucidate the relationship between hypertension and T-cell immunosenescence by proving the existence of T-cell–driven inflammation in human hypertension.

Methods

Study Participants

Seventy-one newly diagnosed, treatment-naïve, patients with hypertension were prospectively and consecutively enrolled in this study. We recruited patients with hypertension who had a systolic blood pressure $\geq 140$ mm Hg or a diastolic blood pressure $\geq 90$ mm Hg after ≥2 minutes at rest, measured during ≥2 independent visits. Patients with any of the following conditions were excluded from participation: significant valvular heart disease, previous myocardial infarction, unstable angina, congestive heart failure, peripheral vascular disease, malignant debilitating disease, severe respiratory disease, renal failure (creatinine $>1.4$ mg/dL), anemia (hemoglobin <12 g%), history of any cancer, inflammatory disease, and on anti-inflammator medications, clinically significant atrioventricular conduction disturbance, a history of atrial fibrillation or other serious arrhythmia, or malignant hypertension ($>200/140$ mm Hg). Age- and sex-matched healthy control subjects (n=71) were randomly selected, and their health status was evaluated by routine physical examinations, laboratory tests, and radiological examinations. Healthy control subjects, enrolled in the Yonsei Cardiovascular Genome Center, were defined as individuals without a history of hypertension and with a normal baseline blood pressure. Baseline characteristics of both patients with hypertension and normotensive control subjects are summarized in the Table. Blood pressure, body weight, body mass index, and laboratory values, including fasting blood sugar and creatinine, are increased in patients with hypertension. All participants provided informed consent, and study approval was obtained from the Institutional Review Board of Yonsei University College of Medicine.

Surface Phenotype Analysis and Intracellular Cytokine Staining

Peripheral blood mononuclear cells (PBMCs) from anticoagulated blood were isolated by density gradient centrifugation. For surface staining, PBMCs were incubated with directly conjugated monoclonal antibodies for 20 minutes at 4°C. The antibodies used were anti–CD57-FITC, anti–HLA (human leukocyte antigen)-DR-fluorescein isothiocyanate, anti–Fas-fluorescein isothiocyanate, anti–IL-7R-APC (allophycocyanin), anti–IL-17A-PE, and anti–IL-15 fluorochrome staining with anti–CD4-PE-Cy7, anti–CD3-Amcyan, and anti–CD8-APC-Cy7. Cells were fixed and permeabilized using fixation/permeabilization buffer (eBioscience, San Diego, CA) and stained with anti–CD28, anti–CD3, anti–CD4, anti–CD8, anti–IFN-γ, anti–TNF-α (BD Biosciences, San Jose, CA), and anti–CD28-PE (BioLegend, San Diego, CA). The number of lymphocytes showing immunoreactivity to CD3, CD4, and CD8 was counted in 10 high-power fields (≥400). The count was done exclusively in the cortex, and the result was expressed as cell number/height power field.

For chemokine staining, sections were deparaffinized and endogenous peroxidase activity by immersion in methanolic H₂O₂ and blocked with normal rabbit serum for 30 minutes. The sections were stained with rabbit antihuman I-TAC (Abcam Inc, Cambridge, MA) and labeled with biotinylated antirabbit immunoglobulin G for 20 minutes. Sections were incubated with streptavidin–peroxidase complex (Vector, Peterborough, United Kingdom) for 1 hour, followed by incubation with 3,3-diaminobenzidine (Dako, Glostrup, Denmark). All sections were counterstained with hematoxylin.

Immunohistochemical Tissue Staining

Seven renal biopsies from patients diagnosed with hypertensive nephrosclerosis were retrieved from the Department of Pathology, Yonsei University College of Medicine. Primary glomerular or tubulointerstitial lesions were ruled out in all the cases. Nine renal biopsies from patients with essential hematuria, but normal blood pressure, were used as normotensive controls. The reason for biopsy was microscopic hematuria, and there was no evidence of specific glomerular disease in all cases.

Tissues were fixed in 4% paraformaldehyde solution, embedded in paraffin, and sliced into 4-µm thick-sections. The primary monoclonal antibodies used to detect T cells were anti–CD3 (Laboratory Vision, Fremont, CA), anti–CD4 (Cell Marque, Rocklin, CA), and anti–CD8 (Laboratory Vision, Fremont, CA). The number of lymphocytes showing immunoreactivity to CD3, CD4, and CD8 was counted in 10 high-power fields (≥400). The count was done exclusively in the cortex, and the result was expressed as cell number/height power field.

Statistical Analysis

Continuous variables are summarized as a mean±SD. Categorical variables are summarized as a percentage of the group total. Discrete variables are compared using the χ² method, and independent t tests were used for continuous variables. Pearson correlation analysis was used for the simple correlation between continuous variables. All statistical analyses were performed with Statistical Product and Service Solutions 13.0. (SPSS Inc, Chicago, IL).

Results

Immunosenescent CD8+ T Cells Are Increased in Patients With Hypertension

To evaluate the immune phenotype of T cells isolated from patients with hypertension, we compared the surface markers of T cells from 19 patients with hypertension and 19 age- and sex-matched normotensive control subjects. CD28null or CD57+ are well-known markers of immunosenescence in T cells. Our results show a significant increase in both CD28null and CD57+ CD8+ T-cell fractions in patients with hypertension compared...
Table. Clinical Characteristics and Laboratory Findings of Newly Diagnosed, Treatment-Naive Patients With Hypertension and Age- and Sex-Matched Control Subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>HTN (n=71)</th>
<th>Control (n=71)</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>51.6±11.2</td>
<td>51.5±12.2</td>
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<td>Male, n (%)</td>
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<td>35 (49.3%)</td>
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<td>Weight, kg</td>
<td>67.4±13.8</td>
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<td>BMI, kg/m²</td>
<td>25.0±4.2</td>
<td>23.3±2.6</td>
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<tr>
<td>Current smoking, n (%)</td>
<td>8 (14.5%)</td>
<td>18 (25.4%)</td>
<td>0.102</td>
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<tr>
<td>SBP, mmHg</td>
<td>152.5±14.6</td>
<td>113±15.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>97.4±11.6</td>
<td>75.2±8.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>WBC (×10³/μL)</td>
<td>6.5±1.64</td>
<td>5.73±1.50</td>
<td>0.003*</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>14.4±1.9</td>
<td>13.8±1.5</td>
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<tr>
<td>T. chol, mg/dL</td>
<td>200.0±33.2</td>
<td>187.8±30.8</td>
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<td>LDL-C, mg/dL</td>
<td>120.2±30.4</td>
<td>114.8±26.5</td>
<td>0.093</td>
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<td>HDL-C, mg/dL</td>
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<tr>
<td>LDL-C, mg/dL</td>
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<td>114.8±26.5</td>
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<td>FBS, mg/dL</td>
<td>97.4±18.7</td>
<td>84.0±9.4</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.89±0.17</td>
<td>0.69±0.20</td>
<td>&lt;0.001*</td>
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</table>

Values are presented as n (%) or mean±SD. BMI indicates body mass index; DBP, diastolic blood pressure; FBS, fasting blood sugar; HDL-C, high-density lipoprotein cholesterol; HTN, hypertensives; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; T. chol, total cholesterol; TG, triglyceride; and WBC, white blood cell.

*P<0.05 is considered significant.

Renal Infiltration of T Cells in Patients With Hypertension

Previous animal studies revealed that renal infiltration of T cells is associated with the development of hypertension.7,8 We attempted to confirm the histological evidence for T-cell-driven inflammation in patients with hypertension with renal biopsy specimens taken from patients with hypertensive nephrosclerosis and from normotensive control subjects. Immunohistochemical staining revealed that T cells, both CD4+ and CD8+, were more infiltrated in the tubulointerstitial region of patients with hypertensive nephrosclerosis compared with normotensive control subjects (Figure 3).

Circulating Levels of CXCR3 Chemokines Are Increased in Patients With Hypertension

Based on previous renal histological findings, we studied chemokines likely responsible for T-cell infiltration in hyper-tension. Circulating levels of CXCR3 chemokines, which are well-known tissue-homing chemokines for proinflammatory T cells, were assessed in 71 newly diagnosed, treatment-naive patients with hypertension and 71 age- and sex-matched normotensive control subjects. All 3 CXCR3 chemokines measured (MIG, IFN-γ–produced protein 10, and I-TAC) were significantly increased in patients with hypertension (MIG: 125.8±130.0 pg/mL versus 501.9±335.7 pg/mL, P<0.0001; IFN-γ–produced protein 10: 105.8±58.4 pg/mL versus 281.9±103.2 pg/mL, P<0.0001; I-TAC: 112.2±9.1 pg/mL versus 35.4±21.1 pg/mL, P<0.0001; Figure 4). In addition, serum MIG levels were significantly correlated with age (P=0.002).

Renal Expression of Tissue-Homing Chemokines for Proinflammatory T Cells

Finally, we evaluated histological evidence for chemokine expression in renal biopsy specimens. I-TAC, one of the CXCR3 chemokines, was found to be expressed within the

Proinflammatory CD8+ T Cells With Cytotoxic Ability Are Increased in Patients With Hypertension

We examined functional features of T cells from both patients with hypertension and control subjects, to reveal the profiles of cytokine producing ability. Intracellular staining for cytokotic molecules revealed that the fraction of perforin+ or granzyme B+ CD8+ T cell is increased in patients with hypertension (Perforin+ CD8+ T-cell fraction: 12.2±11.9% versus 20.9±12.3%, P=0.017; Granzyme B+ CD8+ T-cell fraction: 31.4±14.2% versus 45.1±12.3%, P=0.001; Figure 2A). Also, a significant increase in the fractions of IFN-γ–producing CD8+ T cells and TNF-α–producing CD8+ T cells was noted in patients with hypertension (IFN-γ CD8+ T-cell fraction: 14.2±6.7% versus 20.5±6.8%, P<0.001; TNF-α CD8+ T-cell fraction: 5.4±5.3% versus 8.5±7.0%, P=0.007). However, there was no difference in IL-17A–producing CD8+ T cells between the 2 groups (Figure 2B). Representative flow cytometry plots are presented for perforin expression and IFN-γ–production from CD8+ T cells (Figure 2C). We also measured levels of circulating granzyme B in 71 treatment-naive patients with hypertension and 71 age- and sex-matched normotensive control subjects. Circulating granzyme B levels were significantly higher in patients with hypertension versus normotensive subjects (91.2±44.7 pg/mL versus 25.0±18.9 pg/mL, respectively, P<0.0001; Figure 2D). In CD4+ T cells, except for perforin, there were no statistically significant differences in intracellular cytokine staining between patients with hypertension and control subjects (Figure 2E and 2F). These data altogether suggest that patients with hypertension display an increased fraction of immunosenescent, proinflammatory, cytotoxic CD8+ T cells.

with control (CD28+null CD8+ T-cell fraction: 29.5±15.5% versus 41.9±13.4%; P=0.003; CD57+ CD8+ T-cell fraction: 28.8±15.7% versus 40.0±12%; P=0.008; Figure 1A). In CD4+ T cells, the CD28+null and CD57+ CD4+ T-cell fractions seemed to be increased in patients with hypertension (CD28+null CD4+ T-cell fraction: 3.5±3.1% versus 4.1±2.9%; P=0.005; Figure 1B). The majority of CD28+null CD8+ T cells were also CD57+ as shown in representative flow cytometry plots (Figure 1C). When immunosenescent T cells are defined as being both CD28+null and CD57+, the increased fraction of CD28+nullCD57+ CD8+ T cells in patients with hypertension compared with controls is more clearly demonstrated (CD28+nullCD57+ CD8+ T-cell fraction: 24.4±15.1% versus 35.4±11.9%; P=0.001; Figure 1D). Other T-cell surface markers, such as HLA-DR, PD-1, FasL, Fas, and IL-7Rα, exhibit no significant differences between patients with hypertension and control subjects (Figure 1E). Also, there was no significant difference in the frequencies of CD25+CD127nullFoxP3+ regulatory T cells in CD4+ T cells between patients with hypertension and normotensive control subjects (4±1.2% versus 4.4±1.1%; P=0.967; Figure 1F).

Values are presented as n (%) or mean±SD. DBP indicates diastolic blood pressure; FBS, fasting blood sugar; HDL-C, high-density lipoprotein cholesterol; HTN, hypertensives; LDI-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; T. chol, total cholesterol; TG, triglyceride; and WBC, white blood cell.

<0.05 is considered significant.
Figure 1. Surface immunophenotyping of T cells from patients with hypertension and control subjects. Immunophenotyping of peripheral blood mononuclear cells from 19 patients with hypertension and 19 age- and sex-matched control subjects was performed. Both CD28null and CD57+ fractions are significantly increased in CD8+ T cells of patients with hypertension (A), but not in CD4+ T cells (B). Representative flow cytometry plots are presented for CD28 and CD57 expression in the CD8+ T-cell population of control subjects and patients with hypertension (C). CD28nullCD57+ fraction is also significantly increased in CD8+ T cells of patients with hypertension compared with controls (D). The percentage of cell population was presented for CD28null, CD57+, CD28nullCD57+, HLA-DR+, and PD-1+ cells, and mean fluorescent intensity (MFI) was presented for FasL, Fas, and IL-7Rα (E). The percentage of T reg, defined by CD25hi CD127lo FoxP3+/CD4+ T cells, showed no significant difference between patients with hypertension and control subjects (F). Each bar represents mean±SD. **P<0.01. HTN indicates hypertension.
Figure 2. Functional characterizations of T cells from patients with hypertension and control subjects. Functional features of T cells were studied by flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) from 19 patients with hypertension and 19 age- and sex-matched control subjects. Intracellular staining of perforin and granzyme B was performed in the CD8+ T-cell population (A) or CD4+ T-cell population (E). PBMCs were stimulated with anti-CD3 antibody, and intracellular cytokine staining of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-17A was performed in the CD8+ T-cell population (B) or CD4+ T-cell population (F). Each bar represents mean±SD. Representative flow cytometry plots are presented for perforin expression or IFN-γ production in CD8+ T cells with gating on CD3+ cells (C). Circulating level of granzyme B was analyzed in sera of 71 newly diagnosed treatment-naive patients with hypertension and 71 age- and sex-matched control subjects (D). *P<0.05; **P<0.01. HTN indicates hypertension.
arterioles, proximal and distal tubules, interstitium, and glomeruli of patients with hypertensive nephrosclerosis. However, in normotensive control subjects, I-TAC was expressed in the arterioles and glomeruli, but not in the proximal and distal tubules. When we compared 7 patients with hypertensive nephrosclerosis with 9 essential hematuria control subjects, more abundant expression of I-TAC in the proximal and distal tubules is clearly demonstrated (proximal tubule: 0% versus 57.1±18.7%, \( P = 0.019 \); distal tubule: 11.1±10.5% versus 71.4±17.1%, \( P = 0.035 \); interstitium: 22.2±13.9% versus 71.4±17.1%, \( P = 0.126 \); Figure 5A). Representative data of I-TAC expression in the renal tubules are presented in Figure 5B.

Discussion

The results from this study demonstrate an increased fraction of immunosenescent, proinflammatory, cytotoxic CD8+ T cell and increased circulating levels of CXCR3 chemokines in patients with hypertension. In patients with hypertensive nephrosclerosis, unlike normotensive control subjects, I-TAC was expressed more abundantly in the proximal and distal tubules. This evidence, along with an increased level of serum granzyme B, supports the increased activity of cytotoxic T cells in human hypertension.

There is a growing body of evidence from animal models supporting a role for T-cell–driven inflammation in hypertension.9–11 Harrison et al9 have suggested a working hypothesis for the mechanism behind this phenomenon. They assert that hypertensive stimuli, such as angiotensin II and high salt, cause a modest elevation in blood pressure, resulting in the development of prehypertension. The development of prehypertension may in turn lead to neointigen formation, stimulating T-cell activation. Activated T cells then enter the kidney and vasculature where T-cell–driven inflammation promotes the entry of other inflammatory cells. These inflammatory cells release cytokines that cause vasoconstriction, arteriolar remodeling, and promote sodium and water absorption, ultimately resulting in the development of clinical hypertension.

In response to initial hypertensive stimuli, tissues may secrete various kinds of inflammatory molecules. Among them, CXCR3 chemokines, such as MIG, IFN \( \gamma \)-induced protein 10, and I-TAC, may be released initiating T-cell–driven inflammation similar to that observed in other systemic inflammatory conditions.12,13 Demonstrating the existence of T-cell–driven inflammation in humans is practically restricted compared with animal models and, realistically, only observational studies are possible. However, we tried to confirm the existence of T-cell–driven inflammation in human hypertension. First, we performed immunologic characterization of T cells from patients with hypertension to determine which subtypes of T cells are involved in elevated blood pressure. Shao et al14 showed a direct role of angiotensin II in the modification of helper T cells into IFN-\( \gamma \)-secreting Th1 cells. In our study, however, changes in the T-cell population were evident in CD8+ T cells, rather than CD4+ T cells. We showed that the fractions of IFN-\( \gamma \) and TNF-\( \alpha \)-producing CD8+ T cells are increased in patients with hypertension. CD28null or CD57+ CD8+ T cells (characteristic of immunosenescence)15,16 are also increased in these patients. These immunosenescent, proinflammatory, and cytotoxic CD8+ T cells might be involved...
in the pathogenesis of T-cell–driven inflammation in human hypertension. In addition, increased levels of serum granzyme B have been reported in various disease states where the pathogenic role of T cells is documented well, such as the acute phase of a viral infection, rheumatoid arthritis, and hypertension. In addition, increased levels of serum granulymph B have been reported in various disease states where the pathogenic role of T cells is documented well, such as the acute phase of a viral infection,17 rheumatoid arthritis,18 and hypertensive nephrosclerosis. HTN indicates hypertension.

We then aimed to elucidate what causes T-cell migration into the kidney, where T-cell–driven inflammation promotes overt hypertension. Increased levels of CXCR3 chemokines may be evidence of T-cell–driven inflammation in human hypertension. Therefore, we measured circulating levels of CXCR3 chemokines in newly diagnosed, treatment-naive patients with hypertension and in age- and sex-matched control subjects. All 3 CXCR3 chemokines we measured were significantly increased in patients with hypertension, suggesting the existence of T-cell–driven inflammation in human hypertension. In addition, serum MIG levels were significantly correlated with age.

Recently, De Miguel et al18 reported that renal infiltration by T cells is associated with the development of hypertension in Dahl salt–sensitive rats with enhanced formation of intrarenal angiotensin II and increased oxidative stress. The suppression of T-cell infiltration with either mycophenolate mofetil or tacrolimus decreased intrarenal angiotensin II and oxidative stress, resulting in the attenuation of hypertension. We sought to determine whether or not there is a significant difference in the renal expression of CXCR3 chemokines, which can cause T-cell infiltration in renal cortex in human hypertension.

In this study, we demonstrate that I-TAC is more abundantly expressed in the proximal and distal tubules of patients with hypertension.

The present study had several potential limitations. First, this is a human observational study, rather than an interventional study, which makes identifying a direct cause and effect relationship difficult. Because of the cross-sectional design of the study, we could not assess the effect of blood pressure control on immunosenescent CD8+ T-cell fractions or level of CXCR3 chemokines. Second, the results from our study population cannot be generalized to represent well-controlled, treated patients with hypertension or patients with mild hypertension. Our subjects were newly diagnosed, treatment-naive patients with hypertension with a high proportion (57.7%) of high-risk hypertensives, defined according to the European Society of Cardiology guideline.39 Third, increased prevalence of obesity in patients with hypertension can be a significant confounding factor in analyzing the immunologic characteristics of human hypertension. However, previous studies regarding the effect of obesity on T-cell subset has shown conflicting results, and there was no significant difference in the frequency of immunosenescent T cell between obese and lean subjects.21,22

Finally, the T-cell immunologic characterization could only be performed in a small population because of the limited availability of PBMCs. Even with these limitations, our findings are still important because these are the first evidence of immune-mediated hypertension in humans.

Previously, there was no definite evidence that T-cell–driven inflammation exists in human hypertension. In the present study, we showed immunologic, serological, and histologic evidence that T-cell–driven inflammation affects hypertension in humans. Hypertension is a well-known phenomenon of vascular aging. We tried to elucidate the relationship between human vascular aging and immune aging by proving the existence of T-cell–driven inflammation in human hypertension.

Perspectives
Recent evidence indicates that T cells are prerequisite for the development of hypertension in animal models. However, the existence or the characteristics of T-cell–driven inflammation in human hypertension remained unclear. In this study, we showed an increased level of T-cell chemokines and an increased fraction of immunosenescent, proinflammatory, cytotoxic CD8+ T cells in patients with hypertension. Renal immunohistochemical staining revealed an increased expression of the T-cell chemokines, I-TAC, in the tubules and interstitium of patients with hypertensive nephrosclerosis. An increased level of serum granzyme B also supports the existence of T-cell–driven inflammation in human hypertension. A more detailed characterization of T-cell–driven inflammation may offer new opportunities for preventing and treating human hypertension.

Sources of Funding
This work was supported by grants (2010-0030075 and 2012-0006516) from the National Research Foundation of Korea (NRF)
and by a grant (2011-0020950) from the Public Welfare & Safety Research Program through NRF funded by the Ministry of Education, Science and Technology, Republic of Korea. This work was also partly supported by the Korea Advanced Institute of Science and Technology Future Systems Healthcare Project from the Ministry of Education, Science and Technology.

Disclosures
None.

References

Novelty and Significance
What Is New?

- Previously, the existence or the characteristics of inflammation in human hypertension has not been well evaluated. This is the first study to reveal immunologic, serological, and histological evidences that T-cell–driven inflammation affects hypertension in humans.

What Is Relevant?

- Because hypertension is a disease process that is tied to aging associated with vascular aging, we tried to elucidate the relationship between hypertension and T-cell immunosenescence by showing the existence of T-cell–driven inflammation in human hypertension. A more detailed characterization of T-cell–driven inflammation may offer new opportunities for preventing and treating human hypertension.

Summary

We demonstrated an increased fraction of immunosenescent, pro-inflammatory, cytotoxic CD8+ T cell and increased circulating levels of C-X-C chemokine receptor type 3 chemokines in patients with hypertension. In patients with hypertensive nephroclerosis, unlike normotensive control subjects, IFN-inducible T-cell α chemoattractant was expressed more abundantly in the proximal and distal tubules. This evidence, along with an increased level of serum granzyme B, supports the increased activity of cytotoxic T cells in human hypertension.
Immunosenescent CD8+ T Cells and C-X-C Chemokine Receptor Type 3 Chemokines Are Increased in Human Hypertension

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_Hypertension._ 2013;62:126-133; originally published online May 28, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.00689

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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