Nicotine Mediates CD161a+ Renal Macrophage Infiltration and Premature Hypertension in the Spontaneously Hypertensive Rat

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Rationale: Renal inflammation contributes to the pathophysiology of hypertension. CD161a+ immune cells are dominant in the (SHR) spontaneously hypertensive rat and expand in response to nicotinic cholinergic activation.

Objective: We aimed to phenotype CD161a+ immune cells in prehypertensive SHR after cholinergic activation with nicotine and determine if these cells are involved in renal inflammation and the development of hypertension.

Methods and Results: Studies used young SHR and WKY (Wistar-Kyoto) rats. Splenocytes and bone marrow cells were exposed to nicotine ex vivo, and nicotine was infused in vivo. Blood pressures, kidney, serum, and urine were obtained. Flow cytometry, Luminex/ELISA, immunohistochemistry, confocal microscopy, and Western blot were used. Nicotinic cholinergic activation induced proliferation of CD161a+/CD68+ macrophages in SHR-derived splenocytes, their renal infiltration, and premature hypertension in SHR. These changes were associated with increased renal expression of MCP-1 (monocyte chemoattractant protein-1) and VLA-4 (very late antigen-4). LIT1 (lectin-like transcript 1), the ligand for CD161a, was overexpressed in SHR kidney, whereas vascular cellular and intracellular adhesion molecules were similar to those in WKY. Inflammatory cytokines were elevated in SHR kidney and urine after nicotine infusion. Nicotine-mediated renal macrophage infiltration/inflammation was enhanced in denervated kidneys, not explained by angiotensin II levels or expression of angiotensin type-1/2 receptors. Moreover, expression of the anti-inflammatory α7-nAChR (α7-nicotinic acetylcholine receptor) was similar in young SHR and WKY rats.

Conclusions: A novel, inherited nicotinic cholinergic inflammatory effect exists in young SHR, measured by expansion of CD161a+/CD68+ macrophages. This leads to renal inflammation and premature hypertension, which may be partially explained by increased renal expression of LIT-1, MCP-1, and VLA-4. (Circ Res. 2016;119:1101-1115. DOI: 10.1161/CIRCRESAHA.116.309402.)

Key Words: CD161 □ CD68 □ cholinergic □ hypertension □ inflammation □ innate immunity □ integrin □ lectin □ macrophage □ nicotine □ renal □ SHR

Hypertension is a multifaceted disease with many contributing factors. These factors can be categorized into renal, neural, vascular, and immune mechanisms. Classically, elevated sympathetic activity has been thought to contribute to the development of hypertension via direct vascular effects, activation of the renin-angiotensin system, and increased sodium retention. Likewise, inflammatory mechanisms play a role in the development of hypertension. Angiotensin II’s (Ang II) proinflammatory effects were believed to be sufficient to explain the inflammation that accompanies the development of hypertension.

The concept of an anatomic and physiological interaction between the nervous and immune systems has been documented. A major advance in the field was the discovery of the cholinergic anti-inflammatory reflex, which demonstrated that vagal stimulation and cholinergic stimulation with nicotine could suppress the immune response in animal models of sepsis, translating to a mortality benefit. The immunosuppressive effects of nicotinic cholinergic activation were found to be mediated by the α7-nAChR (α7-nicotinic acetylcholine receptor). Interestingly, nicotinic receptors have also been shown to trigger inflammation. Our laboratory
has previously shown that cholinergic activation of splenocytes derived from normotensive WKY (Wistar–Kyoto) rats was anti-inflammatory, whereas activation of splenocytes from prehypertensive SHR (spontaneous hypertensive rats) with nicotine led to an abnormally exaggerated innate inflammatory response to toll-like receptor activation, as measured by increases in both interleukin (IL)-6 and IL-1β.\textsuperscript{13} The anti-inflammatory effect in the WKY was blocked by α-bungarotoxin, a blocker of o7-nAChR. Thus, nicotinic cholinergic activation can induce anti-inflammatory or inflammatory pathways. An imbalance in these pathways could tilt the balance toward excessive inflammation.

There was also an abnormal prevalence and expansion of a CD161a+ immune cell in the splenocytes of young prehypertensive SHR, but not the WKY rats.\textsuperscript{13} These results suggested that nicotine’s abnormal and paradoxical proinflammatory modulation of the CD161a+ immune cell may play a role in the development of hypertension in the SHR.

CD161a was first identified as a marker in human natural killer (NK) cells,\textsuperscript{14} a member of the lectin-like receptor subfamily B, member 1 (NKR-P1A). It is a type II transmembrane C-type lectin that is a member of the NKR-P1 family. CD161a is present as a homodimer and can also be detected on anergic T cells, NK cells, monocyte/macrophages, and effector immune cells (NK and some T cells).\textsuperscript{15} LLT1 (lectin-like transcript-1), another member of the family, was recently identified as a ligand for CD161a. This interaction may play a key role in immunomodulatory functions of Th17 cells, as well as NK and NK T cells.\textsuperscript{16} Given that monocytes/macrophages are known to play an inflammatory role in cardiovascular disease and CD161a+/CD68+ monocytes also play a role in renal transplant rejection, we hypothesized that the expanded CD161a+ immune cell population with nicotine consists of activated monocytes/macrophages and is involved in renal inflammation in the SHR.\textsuperscript{15,17}

The aims of the present study were to (1) characterize the phenotype of CD161a immune cells in the prehypertensive SHR, (2) test the hypothesis that they are prevalent in the bone marrow (BM) of SHR, thus, defining their genetic hematopoietic origin, and (3) determine whether nicotinic cholinergic activation expands that population selectively in the SHR and provokes its renal infiltration, thus, contributing an inflammatory renal component to the hypertensive state of the young prehypertensive SHR. We then tested the dependence of renal migration of immune cells with nicotine on renal innervation, Ang II, and nicotinic cholinergic receptors and defined molecular determinants of this migration and renal inflammation.

### Methods

#### Animals

Male WKY and SHR (Charles River Laboratories) were used at 3 to 5 weeks of age. Blood pressures were measured approximately twice per week via tail-cuff.

#### Splenocyte and Bone Marrow Isolation and Culture

Splenocytes and BM cells were isolated, washed, and resuspended in plain or complete RPMI (10% heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids, 0.1 mmol/L sodium pyruvate, 10 mmol/L Hepes buffer, 100 μg penicillin/streptomycin, 0.2 mmol/L glutamine). Cultures were maintained for 48 hours in the presence or absence of nicotine (10 μM).

#### In Vivo Studies

Subcutaneous osmotic pumps (Alzet model no 2001D or 2002) were implanted in rats anesthetized under isoflurane, and rats were infused with either saline or nicotine bitartrate salt (15 mg/kg/d) for 24 hours or 2 weeks. Nicotine bitartrate salt (nicotine) at the current dose is equivalent to ≈4 mg/kg/d of nicotine base.\textsuperscript{17} Tissues, splenocytes, BM cells, serum, and urine were collected for further analysis. Cells were analyzed with flow cytometry and tissues with immunohistochemistry/immunofluorescence, and Luminex assays were used to assess serum, urine, and renal homogenates.

#### Flow Cytometry

Splenocytes and BM cells were stained with monoclonal antibodies against rat CD3, CD68, CD103, or CD161a. For each acquisition, a minimum of 100 000 events were recorded. Cellular debris and necrotic/nonviable cells were excluded by gating.

#### Immunohistochemistry and Immunofluorescence

CD68 immunohistochemistry was performed similar to previous work.\textsuperscript{18} Immunofluorescence was conducted on air-dried freshly frozen tissue sections. Sections were exposed to mouse anti-rat CD68, and anti-mouse Alexa fluor 555–labeled antibody was used for secondary detection. Tissue sections were exposed to mouse anti-rat CD161a-FITC monoclonal antibody.

#### Cytokines, Markers of Renal Inflammation, Norepinephrine, and Ang II

Serum, renal tissue homogenate, and urine were obtained. Renal tissues were homogenized. Using a Luminex assay, serum, renal homogenate, and urine were tested for the presence of inflammatory cytokines (Bio-Rad, catalog no 171k1001m) or markers of renal damage (Bio-Rad, catalog no LRK000). Serum and renal homogenates were assessed for Ang II by ELISA (Enzo Lifesciences, catalog no 25–0736). Norepinephrine was assayed in renal homogenates by competitive binding ELISA (Eagle Biosciences, catalog no NOU39-K010). Kits were used according to manufacturer’s instructions.

#### Western Blot

Spleen and kidney tissues were homogenized using a RIPA (radioimmunoprecipitation assay) buffer (Abcam, catalog no156034) and protein concentrations determined using a bicitonic protein assay (Pierce). SDS-PAGE electrophoresis was conducted with 4% to 20% gradient polyacrylamide gels. Membranes were exposed to antibodies targeted against GAPDH, VLA-4 (very-late antigen-4), vascular cell adhesion molecule-1 (VCAM-1), LLT1, MCP-1 (monocyte chemoattractant protein-1), intercellular adhesion molecule-1,
α7-nAChR, Ang II receptor-type 1 (AT1R), and Ang II receptor-type 2 (AT2R). Signal detection was accomplished using Amersham ECL Prime Western Blotting Detection Reagent (GE Lifesciences, product no RPN2236).

Unilateral Renal Denervation
Animals were anesthetized with isoflurane and provided analgesia. The left and right renal arteries were directly visualized through a flank incision. Mechanical disruption of the left renal nerve/renal adventitia was completed along with painting of the renal artery with 20% phenol solution.

Nicotinic and Angiotensin Receptor Expression
Western blot was conducted on spleen and kidney tissue for α7-nAChR and kidney tissue for AT1R and AT2R as described earlier.

Statistics
When comparing WKY rats and SHR directly, 2-way ANOVA was used in comparing the effects of nicotine treatment between the 2 strains. Unpaired t test was used when comparing effects of treatments within a single strain and blood-pressure was compared using 2-way ANOVA with repeated measures. $P<0.05$ was considered significant.

Results
Prevalence of CD161a+ Immune Cells in Splenocytes and Bone Marrow of Prehypertensive SHR and Their Expansion In Vivo With Nicotinic Cholinergic Activation
CD161a+ immune cells are significantly more prevalent in, both, the BM ($P<0.001$) and splenocytes ($P<0.001$) of the young SHR, compared with the age-matched WKY controls (Figures 1 and 2). Results were obtained gating on CD3− cells to remove the contribution of T cells, which comprised <1% of the total CD161a+ immune cell population and dendritic cells (CD103+), which were <0.2% of the total cells. In vivo, over a 2-week period of infusion of nicotine, there was significant expansion of CD161a+ immune cells in both the BM and spleen of SHR, and the increases with nicotine were significantly greater in the spleen than in the BM (Figure 2). Corresponding increase with either saline or nicotine in the age-matched WKY were negligible and never exceeded 2%.

Ex Vivo Nicotinic Cholinergic Expansion of CD161a+/CD68+ Macrophages in Prehypertensive SHR
The majority (85%–95%) of immune cells in both the BM and spleen of young (3- to 5-week-old) WKY rats and SHR are CD161a− and CD68−, respectively (Figure 3; upper panels). The exposure of these cells in culture for 48 hours to nicotine resulted in 2 major phenotypic changes. The first was the acquisition of the CD68 macrophage marker by nearly 50% of splenocytes and BM cells of both WKY rats and SHR, while remaining CD161a−. The phenotype of this large CD161a+/CD68+ macrophage population is unclear functionally.

The second and, we think, more meaningful proinflammatory effect of nicotine was the acquisition of both CD161a and CD68 markers by ≈10% of BM cells from both WKY

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Figure 1. Spleen and bone marrow of young prehypertensive SHR have a prevalent CD161a+ immune cell population that is primarily CD68+. Spleen and bone marrow cells were isolated from young (3- to 5-week-old) WKY rats (n=7) and SHR (n=7). Flow cytometry was performed on isolated cells for the presence of CD161a+ immune cells. Upper, Splenocytes; lower, Bone marrow cells. CD161a+ immune cells in the spleen and bone marrow of the young SHR (red bars) are compared with WKY rats (gray bars). Representative histogram plots are presented in the panels to the right. Error bars represent standard error of the mean (SEM) and ***$P<0.001$. SHR indicates spontaneously hypertensive rat; and WKY, Wistar-Kyoto.
rats and SHR and by 10% of SHR-, but not WKY rats-, derived splenocytes (Figure 3, lower panels). Thus, ex vivo nicotine exerts a proinflammatory response with increased CD161a⁺/CD68⁺ macrophages in the central (ie, BM) immune compartment of both the SHR and WKY rats; however, the change that is unique to the SHR is the expansion of the CD161a⁺/CD68⁺ macrophage subpopulation only in the peripheral immune compartment (ie, splenocytes) of the SHR. The ex vivo effect of nicotine on BM cells of WKY rats was not observed in vivo. However, the pronounced ex vivo effect on SHR splenocytes was prominent in vivo, with significantly larger increases than seen in the BM, particularly after nicotine (Figure 2).

Nicotinic Cholinergic Activation Induces Renal Infiltration of CD161a⁺/CD68⁺ Macrophages in Prehypertensive SHR as Early as 24 Hours

Given the ability of nicotine to induce expansion of the CD161a⁺/CD68⁺ macrophages selectively in young SHR splenocytes, we asked whether the in vivo administration of nicotine induced renal infiltration of the CD161a⁺/CD68⁺ macrophages in the young SHR. After 24 hours of subcutaneous administration of nicotine (15 mg/kg/d), there was an increase in the number of CD68⁺ macrophages within the renal cortex and corticomedullary junction of the young SHR that was not seen in the age-matched WKY controls (Figure 4A; P<0.001). Co-localization of CD161a with CD68 could also be seen in macrophages infiltrating the renal medulla (Figure 4B). Blood pressure measurement after nicotine infusion for 24 hours showed no increase in either the young WKY or SHR, compared with saline controls (Figure 4A).

Nicotine Infusion for 2 Weeks Induces Premature Hypertension and Continued Infiltration of CD161a⁺/68⁺ Macrophages Into the Renal Medulla in Young SHR

Nicotine infusion for 2 weeks significantly raised the systolic blood pressure in young SHR from 124±3 to 154±3 mm Hg...
Figure 3. Nicotine selectively induces ex vivo expansion of CD68+ macrophages in splenocytes of young SHR. Splenocyte and bone marrow cells were isolated from 3- to 5-week-old WKY rats (n=4) and SHR (n=4) cultured in the presence or absence of nicotine for 48 hours and analyzed by flow cytometry, staining for CD161a and CD68. Nicotine (orange bars) induces the proliferation of CD161a+/CD68+ macrophages in young SHR-derived splenocytes (Upper) and bone marrow cells (Lower) of both WKY rats and SHR. Representative histogram plots are presented in the panels to the right. Results were compared using 2-way ANOVA. Error bars represent standard error of the mean (SEM) and ***P<0.001. SHR indicates spontaneously hypertensive rat; and WKY, Wistar–Kyoto.
(P<0.003, n=10), whereas no significant increase in systolic blood pressure was noted in the saline-infused SHR (n=10; Figure 5A). Interestingly, the rise in pressure noted in nicotine-infused young SHR occurred into the second week of infusion, after a 3- to 5-day lag period. In contrast, nicotine infusion had no effect on the systolic blood pressure of the WKY rats (Figure 5A). The induction of premature hypertension in response to nicotine infusion in vivo for 2 weeks correlated with a continued infiltration of CD68+ macrophages in the renal cortex and corticomedullary junction of young prehypertensive SHR compared with age-matched WKY controls (Figure 5B). Confocal microscopy confirmed continued increase in infiltration of CD161a+/CD68+ macrophages into the SHR renal medulla (Figure 5C). The presence of CD3+ T cells was negligible in the renal cortex or medulla (data not shown).

Figure 4. Twenty-four-hour nicotine infusion in vivo induces CD161a+/CD68+ macrophage infiltration in renal medulla. Young (3- to 5-week-old) WKY (n=6) and SHR (n=6) were implanted with osmotic pumps infusing either saline (n=3, each strain) or nicotine (15 mg/kg/d; n=3, each strain) for 24 hours. A, In vivo cholinergic activation with subcutaneous nicotine (orange bars) induces expansion/infiltration of CD68+ macrophages in the renal cortex/corticomedullary junction (black and orange arrows) in the prehypertensive SHR, but not WKY, compared with saline infusion (gray bars). Systolic blood pressure (SBP) of each corresponding group is listed under bar graphs and shows that there were no significant differences in (SBP) between groups. B, Confocal images of 2 cells in the renal medulla showing colocalization of CD161a and CD68. Images shown are 63× (40× images are shown in Figure 5C) demonstrate colocalization of CD161 and CD68. Error bars represent standard error of the mean (SEM). ***P<0.001, based on 2-way ANOVA. SHR indicates spontaneously hypertensive rat; and WKY, Wistar–Kyoto.
Renal Inflammation With Nicotinic Cholinergic Activation in SHR

After infusion of nicotine for 2 weeks, there were significant elevations of MCP-1, IL-18, and interferon-γ (IFN-γ) in the kidney in young SHR, compared with saline (Figure 6A–6C). We also noted a significant increase in renal homogenates of IL-17a of saline and nicotine-infused SHR, compared with WKY controls (Figure 6D). Urinary levels of MCP-1, IL-18, and IFN-γ paralleled levels in renal homogenates (Figure 6E and 6F). Serum levels of osteopontin (P<0.03) and clusterin (P<0.001), well documented markers of renal cellular damage, were also significantly elevated in SHR (Figure 6G and 6H, respectively).

The presence of renal inflammation and systemic activation of adaptive immune responses is supported by significantly elevated urinary cytokines, including RANTES (Regulated on Activation-Normal T Cell Expressed and Secreted; Online Figures I, II, and III). Thus, nicotine-mediated renal inflammation was pronounced with increased infiltration of the CD161a+/CD68+ inflammatory macrophages in the young prehypertensive SHR renal medulla.

Mediators of Renal Macrophage Migration

Although nicotine-mediated renal macrophage infiltration leads to renal damage, it was not clear why the CD161a+/CD68+ immune cells hone to the kidney. Based on reports that nicotine led to enhanced VCAM-1 and intracellular adhesion molecule expression21,22 and the fact that CD161a+ is a receptor for LLT1, it was important to ask whether nicotine increased expression of these immune cell adhesion molecules in the kidney of young SHR. There was no increase in VCAM-1 or intracellular adhesion molecule expression by Western blot in renal homogenates of young nicotine-infused SHR or WKY, compared with saline infusion (Figures 6B and 7A). However, there was an increase in the expression of VLA-4 (the immune cell ligand for VCAM-1) in renal homogenates of nicotine-infused SHR (Figure 7C; P<0.05). LLT1 expression was significantly increased in renal homogenates of saline-infused SHR, compared with WKY (P<0.03; Figure 7B), and there was no further increase in LLT1 expression in nicotine-infused SHR, compared with saline infusion.

Renal Denervation Enhances Nicotine-Mediated Renal Macrophage Infiltration

Nicotine can increase central sympathetic outflow, and increased renal sympathetic nerve activity has been reported to possibly play a role in renal inflammation.23,24 Based on this, we asked whether renal sympathetic innervation played a role in nicotine-mediated renal macrophage infiltration. Denervated kidneys in young (3–5 week) SHR actually demonstrated an ≈35% increase in macrophage infiltration after 24 hours.
hours of nicotine infusion, compared with the sham-treated contralateral kidneys in the same animal (Figure 8; \( P < 0.001 \)). Adequate renal denervation was confirmed by norepinephrine analysis, which demonstrated an \( \approx 65\% \) decrease in norepinephrine in the denervated kidney (Figure 8; \( P < 0.001 \)).

**Nicotine Does Not Induce Changes in Ang II or Angiotensin Receptors**

Levels of Ang II and angiotensin AT1 and AT2 receptors were assessed in serum and kidneys of saline and nicotine-infused SHR. There was no increase in Ang II levels in the serum or kidney of nicotine-infused SHR, compared with saline controls (Online Figure IV). However, circulating levels of Ang II were elevated in SHR when compared with age-matched WKY controls (Online Figure IV; \( P < 0.001 \)). There was also no difference in expression of the AT1R or AT2R in the kidneys of nicotine-infused SHR, compared with saline controls.

**\( \alpha_7 \)-nAChR Expression Does Not Differ in Spleen or Kidney of Young SHR**

\( \alpha_7 \)-nAChR has been documented to mediate an anti-inflammatory effect in various models. Although no difference was
Figure 7. Effect of chronic nicotine infusion on renal VCAM-1, ICAM-1, LLT1, and VLA-4 expression in SHR vs WKY. Young (3-week-old) WKY rats (A, n=8) and SHR (B, n=8) were implanted with osmotic pumps infusing either saline (n=4) or nicotine (Continued).
noted in α7-nAChR expression in young prehypertensive SHR, α7-nAChR expression was decreased in 40-week-old hypertensive SHR. As a result, we analyzed the expression of α7-nAChR in the spleen and kidney of young prehypertensive SHR to determine whether decreased expression of this receptor may explain the increased inflammation we find in the SHR in response to nicotinic cholinergic activation. Consistent with previously reported findings, we found no difference in the expression of α7-nAChR in the spleen of 3- to 4-week-old SHR when compared with age-matched WKY controls (Online Figure VA). There was also no difference in the expression of α7-nAChR in the renal homogenates of young SHR, compared with WKY (Online Figure VA). Chronic nicotine infusion also had no

Figure 8. Effect of unilateral renal denervation (RND) on nicotine-mediated renal inflammation and norepinephrine levels. Young (3-week-old) SHR (n=7) and WKY rats (n=3) underwent unilateral renal denervation followed by 24-hour nicotine (15 mg/kg/d) infusion via subcutaneous osmotic pumps. After infusion, kidneys were harvested and analyzed by immunohistochemistry for the presence of CD68+ macrophages in renal cortex/corticomedullary junction (A). Representative fields of view are presented in the panels to the right. B, Norepinephrine (NE) levels were reduced significantly in the denervated kidney. NE levels were assessed by ELISA in the kidney that underwent Sham surgery vs renal denervation. Error bars represent the standard error of the mean (SEM) and *P < 0.01 and **P < 0.001. SHR indicates spontaneously hypertensive rat; and WKY, Wistar–Kyoto.
effect on the expression of α7-nAChR in the young prehypertensive SHR or age-matched WKY (Online Figure VB). Thus, a reduced expression of α7-nAChR may not explain the proinflammatory and prohypertensive effect of nicotinic cholinergic activation in the young SHR. It might, however, sustain the elevated blood pressure once hypertension has developed.

**Discussion**

The central finding of the current study is that an abnormal population of CD161α+ immune cells harbors a significant proinflammatory sensitivity to nicotinic cholinergic activation. This is evidenced by activation and expansion of a CD161α+/CD68+ monocyte/macrophage population in vitro and in vivo, renal infiltration of these macrophages, and the development of hypertension prematurely in young SHR. These CD161α+ immune cells are prevalent in the BM and spleen of the prehypertensive SHR, suggesting that a genetic/hematopoietic abnormality is responsible for this abnormal inflammation. Increased expression of LTL (the specific ligand for CD61αa), MCP-1, and VLA-4 explain the increased renal infiltration of these CD161α+/CD68+ macrophages and the presence of increased renal inflammation. We found no evidence that Ang II, AT1R and AT2R, and α7-nAChR were altered during nicotinic cholinergic activation and, thus, would not explain the proinflammatory response in SHR. Nor did renal denervation reduce the renal macrophage infiltration during the nicotinic cholinergic stimulation in SHR.

The evidence that nicotinic cholinergic activation may induce the development of hypertension in normotensive individuals or in wild-type animal models used as normotensive controls has been debatable. On the contrary, prehypertensive SHR have been shown to develop premature hypertension and oxidative stress after chronic nicotine administration. Our results are consistent with studies demonstrating nicotine-mediated premature development of hypertension and extend the findings of previous studies by demonstrating a cholinergic-mediated proinflammatory mechanism that seems to contribute to hypertension in this model.

**Cholinergic Influence on the Immune System**

When the cholinergic anti-inflammatory reflex was discovered, the proposed mechanistic explanation was elusive, given the presumed lack of parasympathetic nerve fibers into the spleen. However, it was later shown that vagal stimulation indirectly triggered the release of acetylcholine from postsynaptic splenic T cells that were activated by norepinephrine at the nerve terminals of the splenic nerve. These studies advanced our understanding of mechanisms that underlie the neural regulation of immune responses—combining direct innervation and indirect regulation via paracrine secretion of acetylcholine. Essential to understanding the complexity of the neuroimmuno axis is the concept of the neuronal and non-neuronal cholinergic influence on the immune system. The current work demonstrates that the cholinergic influence is through nicotinic cholinergic receptor activation of immune cells and, being ex vivo, can be characterized as non-neuronal. It is likely, as demonstrated in this article, that a combination of direct and indirect neural influences on immune cells are operating to define immune responses.

**Anti- and Proinflammatory Effects of Nicotinic Cholinergic Activation**

The relationship of nicotine with the immune system is dual faceted. In both mice and rats, nicotine has been described as having anti-inflammatory properties. Other reports have documented nicotine’s ability to mediate proinflammatory effects at a range of doses. The nature of the response to nicotine does not seem to be dose-dependent, but rather a reflection of the pathological inflammatory state of the SHR. In vivo and in vitro doses of nicotine bitartrate have anti-inflammatory effects in the WKY, confirming earlier reports by other investigators and our group, while the same doses simultaneously exert a proinflammatory effect in the prehypertensive SHR, as manifested by the expansion of the CD161α+/CD68+ macrophage. Interestingly, we noted ex vivo expansion of CD161α+/CD68+ macrophages in BM and splenocytes of young SHR but only in the BM of WKY in cultures with exposure to nicotine for 48 hours (Figure 3). The possibility exists that, postnatally, the young WKY rats and SHR harbor a proinflammatory potential unmasked by ex vivo nicotine. This propensity may be selectively suppressed in WKY, but not in SHR, and fails to extend to the peripheral immune system over the 2-week period during the in vivo nicotine infusion (Figure 2). During that time span, we noted that the CD68+ macrophages increased significantly in the kidneys of SHR but not WKY (Figure 5), and the blood pressure of SHR increased prematurely in the prehypertensive phase, whereas the WKY remained normotensive (Figure 5). Alternatively, an anti-inflammatory neurohumoral influence of nicotine infusion in vivo may be selectively effective in suppressing the ex vivo proliferation seen in WKY BM, but not in SHR, where the neurohumoral influence may be conversely proinflammatory.

In our earlier study, the anti-inflammatory effect of nicotine in WKY and its proinflammatory effect in SHR were only seen in the inflammatory cytokine response after activation of the innate immune response with toll-like receptors, but nicotine alone had no direct effect on the secretion of cytokines ex vivo in WKY or SHR splenocytes. That study, similar to those of Li et al and Borovikova et al, highlights the importance of the interaction between the innate immune pathways and nicotinic cholinergic pathways.

Studies reporting the prohypertensive effects of nicotine selectively in young prehypertensive SHR, using WKY as controls, have used subcutaneous nicotine pellets that delivered higher concentrations of nicotine base (≤25 mg/kg/d) than the current study. Moreover, higher doses of nicotine base showed selective induction of reactive oxidation/inflammation and enhanced pressor response in SHR, and no inflammatory or hemodynamic effects were seen in normotensive WKY. To the best of our knowledge, this is the first report of nicotine inducing expansion of, both, the CD161α+/CD68+ and CD161α+/CD68+ macrophage populations in the prehypertensive SHR. We conclude that although cholinergic stimulation has anti-inflammatory effects at the current doses of nicotine in the normotensive WKY, there exists an abnormal cholinergic proinflammatory effect in SHR.
Role of Nicotinic Cholinergic Receptors in Immunomodulation

The anti-inflammatory effects of nicotine have been attributed to the activation of the α7-nAChR\(^\text{11}\) and, more recently, to the α4/β2 nicotinic acetylcholine receptors.\(^\text{32}\) Hence, it is possible that differential reduction in the expression of anti-inflammatory nicotinic receptors in the SHR versus WKY could contribute to the inflammatory effects of nicotine observed in SHR. α7-nAChR has been shown to be equally expressed by Western blot in the splenocytes of young SHR, compared with age-matched WKY,\(^\text{25}\) but decreased with advanced age and the development of hypertension.\(^\text{25}\) The decreased expression of α7-nAChR with the development of hypertension and advanced age may be more likely sustaining hypertension than causing it. This is supported by the studies of Ferrari et al\(^\text{33}\) that showed that decreased cell surface expression of α7-nAChR in hypertensive stroke-prone SHR was restored with normalization of blood pressure. Our current study is similar to that of Li et al, in that we confirm the comparable expression of α7-nAChR between the young WKY and SHR, but we also show that nicotinic cholinergic activation induced the development of premature hypertension and inflammatory changes without changing the level of expression of the α7-nAChR in the kidney. Thus, the nicotinic cholinergic-mediated inflammatory response in the SHR cannot be explained on the basis of decreased expression of the anti-inflammatory α7-nAChR.

The subunit composition of the proinflammatory nicotinic receptor in SHR will require further studies. The challenge in using pharmacological blockers is in the lack of specificity of the blockers and the large number of subunits and their various heteromeric and homomeric combinations that form a large number of different nicotinic receptors in various tissues, including the nervous system, skeletal muscle, and immune cells. In our previous work,\(^\text{13}\) we reported the effect of pharmacological blockade with α-bungarotoxin, known to have a greater affinity for α7-nAChR. In those experiments, we had observed that nicotine suppressed the IL-6 release from WKY splenocytes caused by toll-like receptor activation and conversely enhanced significantly the IL-6 release from SHR splenocytes. α-Bungarotoxin prevented the decline in IL-6 release in WKY and did not alter the enhanced IL-6 release in SHR. Thus, the anti-inflammatory response to nicotine was dependent on the α7-nAChR, but the proinflammatory response was not.

Effect of Activation or Deletion of α7-nAChR

The relationship between α7-nAChR expression and hypertension was tested in 2 other experiments reported by Li et al.\(^\text{25}\) One was in α7-nAChR\(^{-/-}\) mice rendered hypertensive by clipping 1 renal artery. Within 8 weeks of surgery, the magnitude of increase in systolic blood pressure was similar in wild-type and α7-nAChR\(^{-/-}\), despite an increase in cytokine levels in the KO mice.

A second experiment was the use of PNU 282987, the agonist of α7 receptors, in 40-week-old hypertensive SHR. The acute intravenous use of the agonist had no effect on arterial pressure or heart rate nor did the chronic administration of PNU for 28 days. Although there was no reduction in blood pressure, there were decreases in several inflammatory cytokines and significant attenuation of end-organ damage. Clearly, α7-nAChR mediates an important anti-inflammatory influence, and its absence exacerbates the end-organ damage by hypertension, without influencing the level of arterial pressure.

Functional Significance of CD161a in the Nicotinic Cholinergic Inflammation

CD161a is inflammatory, and activation of this receptor has been shown to increase inflammatory cytokine secretion.\(^\text{14}\) Our results support an early innate inflammatory role for CD161a\(^\text{+}\) as a marker of inflammatory immune cells that mature into activated CD68\(^\text{+}\) macrophages and play a role in renal inflammation. CD3\(^\text{+}\) (T cells) and CD103\(^\text{+}\) (dendritic cells) immune cells comprised <1% and <2% of the total CD161a\(^\text{+}\) immune cells in the SHR, respectively. Previous studies have identified a role for CD161a\(^\text{+}\) monocytes/macrophages in renal allograft rejection.\(^\text{15}\) Our finding that CD161a\(^\text{+}\)/CD68\(^\text{+}\) macrophages infiltrate the renal parenchyma suggests that there may be a possible autoimmune response to the self kidney. This would be consistent with more recent reports of autoimmunity, playing a potential role in the development of hypertension.\(^\text{36-37}\) The elevated levels of IL-17a in the kidney, urine, and serum in the present study support the possible presence of an autoimmune process that may be marked by renal infiltration of these CD161a\(^\text{+}\)/CD68\(^\text{+}\) macrophages in the early stages.

Mediators of Renal Inflammation

Renal inflammation is a known mechanism for the development of hypertension in several experimental models of hypertension.\(^\text{38-40}\) Data presented here shows how an increase in the renal levels of MCP-1, IL-18, and IFN-γ. These are consistent with previous reports of an increase in the levels of MCP-1 in the SHR, compared with the WKY,\(^\text{41}\) where MCP-1 is a chemotactic factor for recruitment of CD68\(^\text{+}\) monocytes/macrophages. IL-18 induces production of IFN-γ. The recruitment of monocytes and their differentiation into macrophages under cholinergic influence may play a role in the induction or secretion of IL-18 and IFN-γ. As mentioned earlier, the significant elevation of IL-17a suggests that the renal inflammation may represent an autoimmune process, such as nephritis. Consistent with an autoimmune T-lymphocyte response, we found significant elevations of RANTES in the urine, kidney, and serum, suggesting that the local innate immune response in the kidney may be associated with activation of a systemic adaptive T-lymphocyte response.\(^\text{42}\) Macrophage infiltration and the initiation of renal inflammation preceding the development of hypertension is consistent with established models of nephrogenic hypertension.

Role of Adhesion Molecules in Renal Inflammation

Despite the fact that expression of VCAM-1 and intracellular adhesion molecule have been noted to be increased in the presence of nicotine in other studies,\(^\text{43}\) we found no increase in the renal expression of these adhesion molecules in nicotine-infused SHR. Increased expression of MCP-1 and LLT1, on the contrary, in the kidney of the young SHR correlates with and may contribute to the selective recruitment of CD161a\(^\text{+}\)/CD68\(^\text{+}\) immune cells to the kidneys. The role of these cells in the autoimmune reaction to the kidneys is supported by
the increased expression of VLA-4 (the ligand for VCAM-1), which plays a causative role in models of nephritis.\textsuperscript{44,45} Not only is the expression of VLA-4 (the ligand for VCAM-1) and VCAM-1 discordant in the current study, but blocking VLA-4 and not VCAM-1 abrogates renal inflammation in models of nephritis, suggesting a VCAM-1-independent mechanism for the inflammation mediated by the presence of VLA-4.\textsuperscript{44}

**Effects of Sympathetic Activity and Ang II**

Despite the fact that nicotine is known to stimulate central and peripheral sympathetic activity, including the adrenal medulla,\textsuperscript{46,47} unilateral renal denervation failed to abrogate the nicotine-mediated inflammation but, rather, counterintuitively, exaggerated the inflammation. Thus, the proinflammatory effects of nicotinic cholinergic activation that we observed seem to result from autocrine/paracrine activation of nicotinic cholinergic receptors on immune cells.\textsuperscript{48} First, the fact that ex vivo exposure of splenocytes and BM cells to nicotine-induced expansion and maturation of the CD161a\textsuperscript{+}/CD68\textsuperscript{+} inflammatory macrophages independently of innervation argues against the involvement of a neural circuit in the young SHR. Second, the dose of nicotine bitartrate (15 mg/kg/d) used was chosen to avoid induction of synthesis and release of adrenal catecholamines from the adrenal medulla, based on previous studies.\textsuperscript{49} Third, immune cells are known to express cholinergic receptors and the enzymatic proteins required for synthesis and secretion of acetylcholine.\textsuperscript{50} Fourth, previous reports failed to identify a cholinergic parasympathetic innervation of the spleen\textsuperscript{51}; hence, paracrine/autocrine effects of cholinergic agents likely play a role in immunomodulation of splenocytes in vivo.\textsuperscript{52} Finally, since the activation of the renin–angiotensin system pathway is known to activate sympathetic activity and Ang II has been shown by our group to induce proinflammatory immune responses,\textsuperscript{13} we looked at the expression of Ang II, AT1R, and AT2R and found no change in the Ang II, AT1R, or AT2R expression in SHR infused with nicotine, compared with those infused with saline.

**Relevance to Other Models of Hypertension**

The current work is focused on the SHR because of the spontaneous and evolving nature of the rise in pressure over time in this model and its genetic determination, which parallel human essential hypertension. The relevance to other models of hypertension is based on the demonstration of an immunologic process that involves renal inflammation. The role of the immune system has been documented in various models of hypertension, and increased renal macrophage infiltration has also been shown in response to DOCA (deoxycorticosterone acetate) salt- and AngII-induced hypertension in mice.\textsuperscript{39,40} Hence, there is precedence for a similar immunologic process in other models of hypertension. The novel concepts that this model and this study helped us define are (1) the inherent abnormality of the immune system, which is an essential component of the pathologic state, and (2) the proinflammatory contributions of activation of nicotinic cholinergic receptors. Although the importance of drug-induced (angiotensin or DOCA) and renal or neural models of hypertension is unquestionable, our goal was to investigate the cholinergic mediation of proinflammatory and prohypertensive effects in a spontaneous genetic model of hypertension. Although a limitation of the SHR rat model is the difficulty of genetic manipulation, we suggest that this mimics the paramount challenge inherent to the complexity of human hypertension that we are trying to model, with its multiple genetic and environmental components.

**Significance**

The present study specifically identifies an inflammatory CD161a\textsuperscript{+}/CD68\textsuperscript{+} macrophage population that leads to renal inflammation and the development of hypertension. Where other studies have also shown a hematopoietic basis for the development of hypertension in the SHR model, we specifically identify immune cell mediators involved in this process. The neurohormonal expansion of this immune cell population is a novel inflammatory mechanism that implicates the neuroimmuno axis in the development of hypertension, independent of autonomic innervation. This is the first study to document expansion of CD161a\textsuperscript{+}/CD68\textsuperscript{+} and CD161a\textsuperscript{+}/CD68\textsuperscript{+} macrophages in response to activation of nicotinic cholinergic receptors. The importance of this proinflammatory immune regulation in this model of genetic hypertension is heightened by the simultaneous documentation of its absence in the normotensive WKY control. These data implicate an abnormal cholinergic proinflammatory immune response as a contributing mechanism to the development of renal inflammation and an additional renal component to the genetic hypertension.

The current study also identifies LLT1 (the specific ligand for CD161a) as an important chemotactic factor, along with MCP-1, in the renal parenchyma of the SHR for the selective recruitment of CD161a\textsuperscript{+}/CD68\textsuperscript{+} M1 phenotype macrophages. Finally, we implicate the expression of VLA-4 as a mediator of the renal damage affected by the macrophages, similar to other models of renal disease. Of additional interest is the lack of evidence that this proinflammatory nicotinic cholinergic activation in SHR is influenced by Ang II, AT1 or AT2 receptors, α7-nAChR expression, or renal autonomic innervation.

It has become evident that there are several factors that contribute to the link between the autonomic system, the immune system, and hypertension. These include the dependence of hypertension on, both, the adaptive and innate immune systems; the anti-inflammatory cholinergic reflex noted with efferent vagal nerve stimulation and its mediation by the α7-nAChR; the increased sympathetic drive to the BM of SHR, which seems to activate the microglial system in the paraventricular nucleus and induce hypertension; and the abnormalities intrinsic to the immune cells in genetic hypertension that result in a proinflammatory proliferation and migration of macrophages to the kidney on nicotinic cholinergic activation. The distinctive contribution of this work has focused on the last aspect of these interactions.

**Acknowledgments**

We thank Mike Cica for assistance in experiments. We also acknowledge Mr Justin Fishbaugh and the Flow Cytometry Facility staff for use of the equipment and assistance in flow cytometry. Some of the data presented herein were obtained at the Flow Cytometry Facility, which is a Carver College of Medicine/Holden Comprehensive Cancer Center core research facility at the University of Iowa. The facility is funded through user fees and the generous financial support of the
Disclosures

None.

References


Nicotine Mediates CD161a+ Renal Macrophage Infiltration and Premature Hypertension in the Spontaneously Hypertensive Rat
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Circ Res. 2016;119:1101-1115; originally published online September 22, 2016;
doi: 10.1161/CIRCRESAHA.116.309402

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/119/10/1101

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/09/22/CIRCRESAHA.116.309402.DC1

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Supplemental Materials

DETAILED MATERIALS AND METHODS

Animals
Male Wistar Kyoto (WKY) and male Spontaneous Hypertensive Rats (SHR) [Charles River Laboratories] were obtained at 3-5 weeks of age. SHR are known to be normotensive at birth and begin to become hypertensive at 6-7 weeks of age, plateauing at about 17-20 weeks of age. At 4 weeks of age, their awake systolic pressures by tail cuff average 96.5±6.7 mmHg (n=60) which is equivalent to the pressure of the normotensive WKY rats (95.7±3.9 mmHg, n=60) 1. The use of all animals was approved by the University of Iowa Institutional Animal Care Use Committee in accordance with institutional guidelines. Animals were anesthetized with isoflurane and sacrificed by decapitation.

Splenocyte, Bone Marrow, and Kidney Cell Isolation and Culture
Spleens were harvested from 3-5 week old WKY and SHR. A single-cell suspension was created by homogenization in serum free RPMI medium. Erythrocytes were lysed in a hypotonic buffer (150 mM ammonia chloride, 7.2 mM potassium carbonate, 0.6 mM EDTA, pH 7.2). Cells were washed and re-suspended in complete RPMI (10% heat-inactivated fetal bovine serum, 0.1 mM non-essential amino acids, 0.1 mM sodium pyruvate, 10 mM Hepes buffer, 100 µg penicillin/ streptomycin, 0.2 mM glutamine). For bone marrow isolation, the femur, tibia, and fibula were collected from both lower extremities. Bones were mechanically disrupted, placed in bone marrow isolation solution (0.2% fetal bovine serum, 1 mM EDTA, PBS), and then gently crushed using a mortar and pestle. Isolated bone marrow cells were collected and erythrocytes lysed as per above. Cells were washed and re-suspended in complete RPMI. To determine the effects of nicotinic cholinergic activation, splenocyte and bone marrow cell cultures were incubated in the presence of nicotine (10 µM) or medium for 36-48 hours prior to analysis.

In Vivo Studies
Subcutaneous (SC) osmotic pumps (Alzet) were implanted in 3-5 week old WKY and SHR under isoflurane anesthesia. Pumps infused either saline or nicotine bitartrate (15 mg/kg/day). Animals were sacrificed at the end of 24 hours or 2 weeks. Tissues or cells collected were for immunohistochemistry, confocal microscopy, or flow cytometry.

Flow Cytometry
Splenocytes or bone marrow cells were isolated as described from untreated animals or animals treated with saline or nicotine infusion for 2 weeks. Cells were either stained with monoclonal antibodies immediately or after being cultured for 36-48 hours in the presence or absence of nicotine. Flow cytometry was then performed. Cells (1 x 10^6) were aliquotted into staining tubes and re-suspended in staining buffer (PBS, 1% appropriate animal sera, 2% FBS). Fluorochrome conjugated antibodies, anti-rat CD3 (BD Biosciences, clone G4.18), anti-rat CD8a (BD Biosciences, clone Ox-8), anti-rat CD161a (BD Biosciences, clone 10/78), anti-rat CD68 (Abd Serotec, clone ED1]) were added at pre-determined working dilutions and splenocytes were incubated 45 minutes at 4°C. Cells were washed and resuspended in staining buffer for analysis. They were analyzed using a Becton Dickinson LSR II device (Becton Dickinson, Heidelberg, Germany) flow cytometer and data collected and analyzed in FACS Diva Software. For each acquisition, a minimum of 100,000 events were recorded. Cellular debris and necrotic/non-viable cells were excluded by gating.
Immunohistochemistry & Immunofluorescence

CD68 immunohistochemistry was performed similar to previous work. Briefly, paraffin embedded tissues were sectioned (~4 µm) and rehydrated through a series of alcohol and water baths. Antigen retrieval was performed with NxGen Decloaking Chamber™ (Biocare Medical, Concord, CA, USA). Endogenous peroxidase activity was quenched with 3% H₂O₂. A primary mouse monoclonal Ab (#MCA341R, AbD Serotec Company, Raleigh, NC, USA) and a secondary kit (Mouse Envision, DAKO, Carpinteria, CA) was used for the procedure. Tissues were exposed to chromogen (DAB plus, DAKO, Carpinteria, CA), then counterstained with hematoxylin (Surgipath, Leica Biosystems, Buffalo Grove, IL, USA) and dehydrated (series of alcohols and xylene) and coverslipped.

Immunofluorescence was conducted on air dried freshly frozen tissue sections. Sections were fixed in formalin and rinsed with buffer and glycine. Sections were exposed to mouse anti-rat CD68 monoclonal antibody (1:200) and anti-mouse Alexa fluor 555-labeled antibody was used for secondary detection of CD68. Mouse anti-rat CD161a-FITC monoclonal antibody (1:50) was exposed to the tissue sections. Sections were then coverslipped, mounted, and visualized by confocal microscopy within 1-2 days.

Western Blot

Spleen and kidney tissues were homogenized using a RIPA buffer and protein concentrations determined using a bicinchoninic protein assay (Pierce). SDS-PAGE electrophoresis was conducted using 30 micrograms of total protein of each sample. Nitrocellulose membranes were utilized for the transfer of proteins and blocked with 5% bovine serum albumin (BSA) and stained with anti-rat GAPDH (Thermo Fisher Scientific), Anti-rat Very Late Antigen 4 (VLA-4)(LS Biosciences, polyclonal catalog # LS-C192282), anti-rat VCAM-1 (LS Biosciences, clone EPR5047), anti-rat LLT1 (AbCam, clone EPR6584), anti-rat MCP-1 (LS Biosciences, polyclonal, catalog# LS-C104751), anti-rat ICAM-1 (LS Biosciences, polyclonal, catalog# AF583), anti-rat α7nAChR (Alomone Labs, catalog# ANC-007), anti-rat AT1R (Santa Cruz, SC-1173), and anti-rat AT2R (Abcam, clone EPR3876) in 2.5% BSA were used to stain the membranes. Detection was achieved using horseradish peroxidase secondary detection antibodies and chemiluminescent substrate with the use of a PXi4 CCD camera (Synoptics).

Renal Denervation

To assess the role of renal nerves in nicotine mediated renal inflammation, 3-4 week-old SHR and WKY underwent left sided unilateral renal denervation or sham operation on the right side. Renal denervation was accomplished via previous described methods reported by Winternitz et al. In brief, the left or right renal artery was exposed via flank incision and retroperitoneal approach. For effective renal denervation renal artery adventitia was exposed and removed and the renal artery was painted with 20% phenol (wt/vol) in absolute alcohol. Sham operation consisted of flank incisions only.

To assess the effect of nicotinic cholinergic activation, animals were allowed to recover for 1 week and then an osmotic pump was implanted subcutaneously and delivered nicotine (15mg/kg) over the course of 24 hours. At the termination of infusion, tissues were collected and assessed by immunohistochemistry and confocal microscopy as described above.

Nicotinic Cholinergic and Angiotensin Receptors Expression

Spleen and kidney tissues were homogenized and Western blot conducted as described above. After transfer of proteins to nitrocellulose membranes, membranes were probed with antibodies directed against anti-rat α7nAChR (Alomone Labs, catalog# ANC-007), anti-rat AT1R (Santa Cruz, SC-1173), and anti-rat AT2R (Abcam, clone EPR3876). as described above.
RESULTS

Nicotine Induces Inflammatory Cytokine in the Urine of SHR
Nicotine infusion induced elevated levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) (p<0.001), monocyte colony stimulating factor (M-CSF)(p<0.001), regulated on activation, normal T cell expressed and secreted (RANTES) (p<0.05), interleukin-2 (IL-2)(p<0.01), interleukin-6 (IL-6)(p<0.001), interleukin-12p70 (IL-12p70)(p<0.03), macrophage inflammatory protein-3a(MIP-3a)(p<0.05), interleukin-1a (IL-1a)(p<0.03), interleukin-1b (IL-1b)(p<0.03), tumor necrosis factor-alpha (TNF-a)(p<0.01), interleukin-4 (IL-4)(p<0.01), interleukin-7 (IL-7)(p<0.03), and interleukin-13 (IL-13)(p<0.03) (Online Figure I). There appeared to be a slight increase in macrophage inflammatory protein-1a (MIP1a), but failed to reach statistical significance.

Elevation of RANTES, IL-2, IL-1a, IL-1b, MIP-3a, TNF-a, and IL-4 in Renal Homogenates of the Young SHR
After nicotine infusion, IL-2, a T-cell cytokine growth factor, was increased in renal homogenates of the young SHR, compared to saline infused SHR controls (p<0.05) and significantly elevated compared to WKY nicotine infused controls (p<0.001) (Online Figure II B & F). Although there did not appear to be an increase in RANTES, a cytokine involved in the regulation of T-cell activity, in response to nicotinic cholinergic activation, the levels of RANTES in the saline and nicotine infused kidney were significantly elevated, compared to WKY controls (p<0.001) (Online Figure II B & F). Similarly, IL-1a (p<0.05), IL-1b (p<0.05), MIP-3a (p<0.001), TNF-a (p<0.05), and IL-4(p<0.05) were found to be elevated in the saline and nicotine infused SHR, compared to corresponding WKY controls (Online Figure II C, II G, II D, and II H). No significant elevations in GM-CSF, M-CSF, IL-6, IL-12p70, MIP-1a, or IL-12 were found in renal homogenates of the young SHR. Hence, there are significant increases in innate and T-cells adaptive cytokine levels in the SHR.

Elevation of Serum RANTES, IL-2, IL-1a, IL-1b, MIP-1a, MIP-3a, and IL-17a in the Young SHR
After nicotinic cholinergic activation, RANTES (p<0.001) and MIP-3a (p<0.05) were significantly increased in the serum of SHR, compared to saline infused SHR. Interestingly, saline infused SHR also had a significant increase in RANTES, compared to saline infused WKY controls (p<0.05) (Online Figure III F). IL-17a was also increased in the serum of nicotine infused SHR, compared to saline infused SHR (p<0.05) (Online Figure III H). The innate immune cytokines MIP-1a (p<0.05), MIP-3a (p<0.05), IL-1a (p<0.05), and IL-1b (p<0.05) were increased in the serum of the nicotine infused SHR, compared to the corresponding WKY controls (p<0.05) (Online Figure III C & G). No significant elevations in GM-CSF, M-CSF, IL-2, IL-6, IL-12p70, or IL-12p70 were found in the young SHR. Hence, the serum of the young SHR shows significant elevations in innate immune cytokines and RANTES, similar to renal homogenates.

References
Online Figure I: Effect of Nicotine infusion on Urinary Cytokine Levels. Young (3-5 week old) SHR (n=8) were implanted with osmotic pumps infusing either saline (n=4, black bars) or nicotine (15mg/kg/day) (n=4, red bars) 4 weeks. After infusions were complete urine was collected. Granulocyte-monocyte colony stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-2), macrophage inflammatory protein 1a and 3a (MIP-1a, MIP-3a), interleukin-1a (IL-1a), interleukin-1b (IL-1b), tumor necrosis factor alpha (TNF-a), interleukin-4 (IL-4), interleukin-7 (IL-7), and interleukin-13 (IL-13) were measure by Luminex assay. Error bars represent the standard error of the mean (SEM). P-values as indicated.
Online Figure II: Effect of Nicotine infusion on Kidney Cytokine Levels. Young (3-5 week old) WKY (n=8) (A-D) and SHR (n=8) (E-H) were implanted with osmotic pumps infusing either saline (n=4) or nicotine (15mg/kg/day) (n=4) 4 weeks. After infusions were complete renal homogenates were prepared and tested for cytokines. Granulocyte-monocyte colony stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-2), macrophage inflammatory protein 1a and 3a (MIP-1a, MIP-3a), interleukin-1a (IL-1a), interleukin-1b (IL-1b), tumor necrosis factor alpha (TNF-a), interleukin-4 (IL-4), interleukin-7 (IL-7), and interleukin-13 (IL-13) were measure by Luminex assay. Error bars represent the standard error of the mean (SEM). P-values as indicated and * = p<0.05, *** = p<0.001, compared to corresponding WKY controls.
Online Figure III: Effect of Nicotine infusion on Serum Cytokine Levels. Young (3-5 week old) WKY (n=8) (A-D) and SHR (n=8) (E-H) were implanted with osmotic pumps infusing either saline (n=4) or nicotine (15mg/kg/day) (n=4) 4 weeks. Serum was isolated and assayed for cytokines. Granulocyte-monocyte colony stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-12), macrophage inflammatory protein 1a and 3a (MIP-1a, MIP-3a), interleukin-1a (IL-1a), interleukin-1b (IL-1b), interleukin 17a (IL-17a), interleukin-4 (IL-4), interleukin-7 (IL-7), and interleukin-13 (IL-13) were measure by Luminex assay. Error bars represent the standard error of the mean (SEM). P-values based on two-way ANOVA. * = p<0.05, ** = p<0.001, compared to corresponding WKY controls.
Online Figure IV: Effect of Nicotine on Angiotensin II, AT1 and AT2 Receptors

Young (3 week old) WKY (A, n=8) and SHR (B, n=8) were implanted with osmotic pumps infusing either saline (n=4) or nicotine (15mg/kg/day) (n=4) at 3-4 weeks of age. After 2 weeks of infusion, expression of Ang II was assessed by ELISA in the serum (A) and renal homogenates (B). Levels of AT1R (C), and AT2R (D) were assessed by Western blot in renal homogenates. Nicotine infusion had no effect on serum or renal levels of Ang II. There was also no detectable difference in the expression of AT1R and AT2R in renal homogenates of nicotine infused SHR, compared to saline infusion. Error bars represent the standard error of the mean (SEM). p-values as indicated based on two-way ANOVA.

A 2 Week Nicotine Infusion
Ang II Serum

B 2 Week Nicotine Infusion
Ang II Kidney

C Renal AT1R Expression

D Renal AT2R Expression

SHR Renal Angiotensin Receptor
Online Figure V: Alpha7-nAChR Expression in Spleen and Kidney of Young SHR and WKY

Splenic and renal homogenates were prepared from 3-4 week old untreated WKY (n=3) and SHR (n=3) (A). Splenic and renal homogenates from 6 week old WKY (n=8) and SHR (n=8) that underwent saline (n=4) or nicotine (15mg/kg/day) (n=4) infusion for 2 weeks (B). Homogenates were tested by Western blot for the presence of alpha7-nAChR. Specificity of the antibody was confirmed by incubating the antibody with the n-terminus peptide of rat alpha7-nAChR prior to exposure to the membrane (Pre-adsorbed Antibody). Error bars represent the standard error of the mean (SEM).