Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II–Induced Hypertension

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ABSTRACT

**Rationale:** Inflammation and adaptive immunity plays a crucial role in the development of hypertension. Angiotensin II and likely other hypertensive stimuli activate the central nervous system and promote T cell activation and end-organ damage in peripheral tissues.

**Objective:** To determine if renal sympathetic nerves mediate renal inflammation and T cell activation in hypertension.

**Methods and Results:** Bilateral renal denervation (RDN) using phenol application to the renal arteries reduced renal norepinephrine (NE) levels and blunted angiotensin II induced hypertension. Bilateral RDN also reduced inflammation, as reflected by decreased accumulation of total leukocytes, T cells and both CD4⁺ and CD8⁺ T cells in the kidney. This was associated with a marked reduction in renal fibrosis, albuminuria and nephrinuria. Unilateral RDN, which partly attenuated blood pressure, only reduced inflammation in the denervated kidney, suggesting that this effect is pressure independent. Angiotensin II also increased immunogenic isoketal-protein adducts in renal dendritic cells (DCs) and increased surface expression of costimulation markers and production of IL-1α, IL-1β, and IL-6 from splenic dendritic cells. NE also dose dependently stimulated isoketal formation in cultured DCs. Adoptive transfer of splenic DCs from angiotensin II-treated mice primed T cell activation and hypertension in recipient mice. RDN prevented these effects of hypertension on DCs. In contrast to these beneficial effects of ablating all renal nerves, renal afferent disruption with capsaicin had no effect on blood pressure or renal inflammation.

**Conclusions:** Renal sympathetic nerves contribute to dendritic cell activation, subsequent T cell infiltration and end-organ damage in the kidney in the development of hypertension.

**Keywords:**
T cells, dendritic cells, norepinephrine, isoketals, sympathetic nerves, lymphocyte, immunology.

**Nonstandard Abbreviations and Acronyms:**
RAG-1  recombination activating gene 1  
RDN  renal denervation  
DC  dendritic cell  
IL  interleukin  
IFN-γ  interferon-γ  
VCAM-1  vascular cell adhesion molecule 1  
ICAM-1  intercellular adhesion molecule 1  
MCP-1  monocyte chemotactic protein 1  
RANTES  regulated on activation, normal T cell expressed and secreted  
eNOS  endothelial nitric oxide synthase  
Klf4  Kruppel-like factor 4  
CCR7  C-C chemokine receptor type 7
INTRODUCTION

Inflammation, and in particular adaptive immunity, contributes to the development of hypertension. Recombination activating gene 1 deficient (RAG-1-/-) mice lacking lymphocytes are relatively resistant to hypertension caused by angiotensin II, norepinephrine, or deoxycorticosterone acetate-salt challenge.1, 2 Similar protective effects have also been observed in mice with severe combined immune deficiency and in Dahl salt-sensitive rats with deletion of the RAG-1 gene.3,4 Adoptive transfer of T cells to RAG-1-/- mice restores hypertension and end-organ dysfunction in response to hypertensive stimuli.1, 2 T lymphocytes and other inflammatory cells accumulate in the kidneys and vasculature during hypertension and produce cytokines such as interleukin-17A (IL-17A), interferon-γ (IFN-γ) and tumor necrosis factor α, which in turn lead to vascular remodeling and renal sodium retention, augmenting blood pressure elevation.1, 4 The mechanisms underlying T cell activation are not yet fully understood.

T cell activation requires antigen presenting cells, in particular dendritic cells (DCs) and the phenomenon of costimulation.5 We have previously shown that DCs from hypertensive mice have increased surface expression of the costimulatory B7 ligands (CD80 and CD86), suggestive of DC maturation and activation.6 Pharmacological blockade or genetic deletion of CD80/CD86 prevents T cell activation in response to angiotensin II and reverses both angiotensin II and DOCA-salt hypertension.6 We recently demonstrated that proteins oxidatively modified by highly reactive γ-ketoaldehydes (isoketals) are formed in hypertension and accumulate in DCs, leading to T cell activation and proliferation.7

The central nervous system orchestrates much of the inflammation caused by angiotensin II and likely other hypertensive stimuli. Inhibition of sympathetic outflow by AV3V lesioning or by deletion of the NADPH oxidase subunit p22phox in the subfornical organ reduces hypertension and T cell activation, while activation of sympathetic outflow by deletion of the extracellular superoxide dismutase in the subfornical organ enhances T cell activation.2, 8, 9 The mechanisms underlying this link between the central nervous system and T cell activation are however, not well understood. In keeping with this, immune cells possess adrenergic receptors, which have been implicated in both pro- and anti-inflammatory responses.10-12

Renal denervation has been employed as a potential treatment for hypertension for several decades.13 Some, but not all, animal studies and clinical trials indicate that renal sympathectomy effectively reduces blood pressure and is expected to slow the progression of chronic renal disease.14, 15 Independent of its therapeutic efficacy in humans, renal denervation provides a useful platform on which to study the relationship between sympathetic nerve stimulation and immune activation. A potential benefit of renal denervation is reduction of renal inflammation, perhaps by reducing dendritic cell activation and ultimately formation of effector T cells that secrete pro-inflammatory cytokines. In keeping with this, a recent study by Mathis et al. showed that renal denervation reduces albuminuria and renal cortical monocyte-chemoattractant protein expression in mice with experimental systemic lupus erythematosus.16 In the present study, we tested the hypothesis that renal sympathetic nerves modulate renal inflammation and T cell activation in hypertension and sought to understand mechanisms underlying a potential anti-inflammatory role of renal denervation. We determined the efficacy of renal denervation in both preventing and reversing hypertension and examined the effect of both renal efferent and afferent nerves in modulating renal inflammation.

METHODS

The Institutional Animal Care and Use Committee of Vanderbilt University approved all animal protocols. A detailed description of the materials and methods can be found in the Online Data Supplement.
RESULTS

Effects of renal denervation on catecholamine content and hypertension.

In initial studies, we examined the efficacy of renal artery phenol application in producing renal denervation. Norepinephrine content was markedly decreased in denervated kidneys compared with the sham treated kidneys (Figure 1A). In contrast, renal epinephrine, which is largely derived from the adrenal glands, was not altered by phenol application. Western blots for tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis, confirmed successful denervation (Figure 1B). We also confirmed that this technique does not interrupt innervation of adjacent lymph nodes and the adrenal gland, as verified by the neuronal marker β3 tubulin expression and catecholamine content in lymph nodes and adrenal glands respectively (Online figure I).

The hypertensive response to angiotensin II, assessed by radiotelemetry, was markedly blunted in mice that had previously undergone bilateral renal denervation. In sham-operated mice, systolic pressure increased to 163 ± 4 mmHg in response to angiotensin II. This was reduced to 127 ± 4 mm Hg in mice that had undergone bilateral renal denervation (Figure 1C). Likewise, diastolic pressure was reduced from 131 ± 5 to 111 ± 5 mm Hg (p<0.001) by renal denervation (Figure 1D).

Effects of renal denervation on renal inflammation and T-Cell activation in Angiotensin II–induced hypertension.

To examine the effect of denervation on renal inflammation, we prepared single cell suspensions of renal homogenates and performed flow cytometry. As apparent in Figure 2A and in the mean data, angiotensin infusion increased the presence of all leukocyte subsets, including total leukocytes, identified by CD45, total T cells and both CD4+ and CD8+ T cells. Renal denervation prevented the increased renal accumulation of leukocytes in response to angiotensin II infusion (Figure 2B-G). Of note, the number of memory T cells, as defined as the CD44high, was also increased by angiotensin II, and this was normalized by renal denervation. Renal denervation has recently been reported to have beneficial effects on vascular inflammation in ApoE−/− mice. In keeping with this, we also found that renal denervation reduced aortic infiltration of total leukocytes, total T cells, CD4+ T cells, and CD8+ T cells (Online figure II).

To confirm our flow cytometry results and to localize inflammatory cells in the kidney, CD3+ and F4/80+ cells in the kidney were visualized using immunohistochemistry. T cells were increased in both the renal cortex and medulla by angiotensin II-infusion (Figure 3A-B) and this was prevented by renal denervation. Monocyte/macrophages, as identified by F4/80 staining, were also increased by angiotensin II infusion, and were reduced in the renal medulla by denervation. Further flow cytometry studies using a gating strategy recently described by Jakubzick et al18 showed that the majority of these F4/80+ cells were monocytes and not macrophages (Online figure III). Taken together, these data indicate that sympathetic innervation plays a major role in mediating renal inflammation in the angiotensin II–induced hypertension.

We further examined the effect of renal denervation on renal injury in hypertension. Angiotensin II infusion caused collagen deposition in both the renal cortex and medulla (Figures 3A-B), as evidenced by Masson’s Trichrome staining. This was almost completely prevented by renal denervation. Increased superoxide production was observed in numerous cell types of the kidney in hypertensive mice, but predominantly in vascular endothelial cells. This was attenuated by renal denervation (Figure 3C). Angiotensin II infusion induced both albuminuria and nephrinuria in sham operated mice, and these were also reduced by denervation (Figures 3D and 3E).
Local effects of sympathetic nerves on renal inflammation in a unilateral renal denervation model.

To determine if renal nerves directly contribute to inflammation or if renal inflammation is predominantly due to high blood pressure, we performed unilateral renal denervation. Using this approach, both kidneys are exposed to a similar pressure, but only one is denervated. Unilateral renal denervation also reduced the hypertensive response to a two-week infusion of angiotensin II, albeit not as effectively as bilateral denervation (146 ± 5 vs. 164 ± 3 mmHg, p < 0.05) (Figure 4A). Interestingly, significantly fewer total leukocytes (Figure 4B), lymphocytes (Figure 4C) and both CD4+ and CD8+ T cells (Figure 4E and F) accumulated in the denervated kidney as compared to the innervated kidney. Combining with the results from bilateral denervation, this suggests that there is a significant proinflammatory effect of sympathetic innervation, independent of pressure elevation (Online figure IV).

Effect of renal denervation on chemoattractant and adhesion molecules.

The homing of inflammatory cells to an affected tissue depends on local levels of adhesion and chemoattractant molecules. We therefore employed PCR to detect mRNA levels of selected proteins known to attract immune cells. Hypertension caused a striking increase in the vascular cell adhesion molecule 1 (VCAM1), intercellular cell adhesion molecule 1 (ICAM1), monocyte chemotactic protein 1 (MCP1) and RANTES. Renal denervation significantly attenuated, and in the case of VCAM1 and RANTES, completely prevented this response (Figure 5 A-D). Nitric oxide has anti-inflammatory properties, and we found that mRNA for the endothelial NO synthase (eNOS) was markedly reduced by angiotensin II-induced hypertension and this was partly corrected by renal denervation (Figure 5E). An important transcription factor that modulates eNOS transcription is Klf4. Hypertension was associated with a striking reduction of Klf4, and this was completely prevented by renal denervation (Figure 5F).

We considered the hypothesis that renal denervation increases renal blood flow, as eNOS and Klf4 are known to be shear responsive. We therefore measured renal blood flow using MRI in angiotensin II infused mice subjected to unilateral renal denervation surgery and found no significant change in renal perfusion caused by denervation (Online figure V).

Effect of renal denervation on dendritic cell activation in Angiotensin II–induced hypertension.

T cell activation is dependent on antigen presenting cells, and in particular DCs. These cells present antigen and provide co-stimulation to initiate T cell proliferation and activation. Upon maturation, DCs increase expression of CD80 and CD86. We therefore performed additional studies to determine if renal denervation affects DC activation and their ability to promote hypertension. Subtypes of splenic DCs were characterized according to the surface markers I-Ab, CD11c, CD11b, and B220 (Online figure VI). Angiotensin selectively increased CD80 and CD86 expression by approximately 26% and 15% in CD11b+/CD11c+ DCs, and this was prevented by renal denervation. (Figure 6A and 6B).

We have recently found that isoketal-protein adducts are formed in hypertension. These accumulate in DCs and promote DC activation, causing DCs to express co-stimulatory molecules, produce cytokines and stimulate T cell proliferation.7 We performed additional studies using flow cytometry to detect intracellular isoketal-protein adducts in sham-operated and renal denervation mice. As we previously reported, splenic CD11c+/CD11b+ DCs from hypertensive mice robustly accumulated isoketal adducts, and renal denervation prevented this (Figure 6C). One explanation for these findings is that renal sympathetic nerves stimulate formation of isoketal adducts in the kidney, perhaps directly in DCs. To address this, we examined isoketal adducts in the DCs within the kidney shortly after the onset of angiotensin II infusion. In sham mice not receiving angiotensin II, only 25% of DCs stained positively for isoketal-protein adducts. In contrast, nearly 80% of the DCs isolated from kidneys 5 days after the onset of angiotensin II contained isoketal-adducted proteins. Renal denervation markedly reduced these (Figure 6D).
Activated DCs produce cytokines that influence the inflammatory milieu, in large part by guiding T cell polarization. To examine if renal denervation alters DC cytokine production, splenic DCs were cultured in RPMI for 24 hours and cytokines released into the media were measured using Luminex. Angiotensin II infusion increased splenic DC IL-1α, IL-1β and IL-6 production two- to six-fold, and these increases were completely prevented by bilateral renal denervation (Figure 6E-G). Other cytokines, including IL-12, IL-23, GM-CSF, and TGF-β were not altered by renal denervation (Online Table I).

Role of activated dendritic cells in priming hypertension and effect of renal denervation.

To further determine if renal innervation affects the ability of DCs to promote hypertension and renal inflammation, we performed adoptive transfer experiments. Mice underwent renal denervation or sham surgery as described above, and ten days later underwent infusion of angiotensin II (490 ng/kg/min) for two weeks. 1 X 10^6 splenic DCs were isolated from these animals and injected by tail vein into recipients. Ten days later, the recipients were treated with low dose angiotensin (140 ng/kg/min) for two weeks. This experimental paradigm is illustrated in figure 7A. DCs from mice with intact renal nerves markedly augmented the hypertensive response to this generally subpressor dose of angiotensin II (Figure 7B,C). In contrast, DCs from denervated animals did not alter the hypertensive response to low dose angiotensin II. Flow cytometry revealed that adoptive transfer of DCs from angiotensin II–infused mice with intact renal nerves increased renal accumulation of total leukocytes, CD3+ cells and both CD4+ and both CD8+ T cells in response to low dose angiotensin II. These values were reduced by 30 to 50% in mice that had received DCs from mice with renal denervation (Figure 7D-H). These data illustrate an important contribution of DCs to hypertension, and show that this is modulated by the sympathetic nervous system. Role of Afferent Renal Nerves in Angiotensin II–induced Hypertension.

Activation of renal afferent nerves has been reported to produce hypertension in response to calcineurin19, renal failure 20 and renal injury 21. To examine a role of afferent renal nerves in modulating inflammation, we applied capsaicin to the renal arteries. This markedly reduced the afferent nerve marker calcitonin gene related peptide in the renal pelvis, but had no effect on renal norepinephrine levels (Online Figure VIIA and VIIB). Disruption of the afferent nerves using this approach did not alter either the hypertension (Online Figure VIIIC) or renal inflammation (Online Figure VIID-H) caused by angiotensin II.

Effect of renal denervation after the onset of hypertension.

In clinical trials, renal denervation is usually performed after the onset of hypertension. To mimic this clinical scenario, we infused angiotensin II for 4 weeks in mice, and performed renal denervation after 2 weeks. Other mice underwent a sham denervation procedure at two-week angiotensin II infusion. As shown in Figure 8A and B, renal denervation after the onset of hypertension lowered blood pressure by about 15 mmHg, compared to that observed in mice undergoing sham denervation, during the ensuing two weeks. This value was significantly less that the blood pressure reduction observed when denervation was performed at the onset of angiotensin II infusion (149 ± 4 mmHg vs 129 ± 3 mmHg, p < 0.001). Of note, this 4-week protocol resulted in substantially more leukocytes in the kidney than observed after two weeks of angiotensin II, but renal denervation substantially reduced total leukocytes and all T cell subsets as demonstrated by flow cytometry (Figure 8C-F).

Direct effect of sympathetic stimuli in isoketal adduct formation in dendritic cells.

To examine if sympathetic stimuli directly affect dendritic cell function in hypertension, adrenergic receptor expression in splenic DCs was quantified using real time PCR (Online figure VIIIA), and further confirmed by Western blot (Online figure VIIIB). We found that DCs contain mRNA for α1D, α2A, α2B, α2C, β1 and β2 adrenergic receptors. In most cases, adrenergic receptors were down regulated by angiotensin II
infusion, with the exception of β2 adrenergic receptors, which were increased. To further determine if adrenergic signaling contributes to DC activation, bone marrow derived DCs were cultured with norepinephrine, and isoketals were measured by flow cytometry one week later. Norepinephrine dose-dependently increased isoketal-protein adducts in those cells. A similar response was observed with DCs obtained from β2 receptor-deficient mice (On figure 8C-D). In contrast to the effects of norepinephrine, neuropeptide Y, another sympathetic nerve transmitter, had no effect on DC isoketal-protein content (Online figure VIIIIE).

Potential role of CCR7 in homing of DCs from the kidney to secondary lymphoid organs.

Our data regarding the effect of renal denervation on splenic DC function and isoketal content are compatible with the hypothesis that these cells are initially activated in the kidney and migrate to secondary lymphoid organs such as the spleen, where they interact with T cells to promote T cell activation (Figure 8G). To gain further insight into this hypothesis, we performed additional studies on C-C chemokine receptor type 7 (CCR7)-deficient mice. CCR7 is employed by DCs to allow homing to secondary lymphoid organs. The hypertensive response to angiotensin II was identical to that observed in wild-type mice (Online figure IXA). Likewise, DCs in the kidney demonstrated an increase in CD80 and CD86 (Online figure IXB), however, there was no effect of angiotensin II infusion on these DC surface markers in the spleen (Online Figure IXC). Angiotensin infusion failed to induce inflammatory cell infiltration in the kidneys of CCR7−/− mice (Online Figure IXC to IXF). However, memory CD8+ T cells were increased (Online Figure IXH).

DISCUSSION

In the present study, we show that the renal sympathetic nerves play an important role in activation of adaptive immunity in hypertension. Renal denervation attenuates the hypertension caused by chronic angiotensin infusion and prevents T cell infiltration and subsequent renal injury. Renal sympathetic outflow also promotes DC maturation as defined by up-regulation of co-stimulatory molecules and augmented proinflammatory cytokine production during angiotensin II infusion, and all of these changes are reversed by renal denervation. In accordance with these, DC adoptive transfer experiments further indicate that renal nerves indeed contribute to DC activation, which in turn promote T cell activation and hypertension. Our data also indicate that efferent but not afferent renal nerves mediate these effects.

Before our present study, the interplay between renal sympathetic nerves and inflammation was poorly defined. As in prior studies, we found that hypertension is associated accumulation of leukocytes, T cells and both CD4+ and CD8+ T cells in the kidney. In recent studies, we have shown that T cells that infiltrate the kidney in hypertension have an effector memory phenotype. Importantly, renal denervation not only reduces the total number of immune cells in the kidney, but also memory T cells. We have found that activated T cells in hypertension produce IL-17A, TNF α and IFN-γ, and we and others have shown that these affect vascular and renal function. For example, prolonged exposure of renal proximal tubular cells to IFN-γ enhances production of angiotensinogen by renal epithelial cells, which modulates tubular sodium transport. In keeping with this concept, we have recently shown that angiotensin II infusion increases phosphorylation of the Na-K-2Cl cotransporter the NaCl cotransporter and Ste20/SPS-1 related proline-alanine rich kinase in WT mice but not in IFN-γ−/− mice. In this study, we also found evidence that IL-17A and IFN-γ modulate abundance of Na/H-exchanger isoform 3 and the motor myosin VI during angiotensin II infusion. It is therefore likely that the reduction of effector memory T cells in the kidney contributes to the anti-hypertensive effect of renal denervation.

The homing of immune cells to sites of inflammation is mediated by local increases in adhesion and chemoattractant molecules. In keeping with this, we found that angiotensin II-infusion increases renal expression of VCAM-1, ICAM-1, MCP-1 and RANTES, and that these are all attenuated by renal
denervation. In contrast, angiotensin II-induced hypertension is associated with a marked decrease in eNOS mRNA expression, and this is completely prevented by denervation. NO has myriad anti-inflammatory effects on the endothelium, reducing expression of VCAM1, MCP1, RANTES, ICAM1 and monocyte adhesion. In many cases, this has been attributed to modulation of transcription factors such as NF-κB, AP1 and CREB. In this regard, the Klf4 modulates endothelial function by promoting eNOS expression, reducing VCAM1 expression and exerting anti-thrombotic effects. Thus, the enhancement of eNOS and Klf4 expression could explain at least a part of the anti-inflammatory effects of renal denervation.

A critical finding in this study is that renal denervation reduces DC activation and the ability of these cells to convey hypertension. Recently, we showed that isoketal-adducted proteins accumulate in DCs and seem to act as neo-antigens which lead to T cell activation and ultimately hypertension. In the present study, we find that isoketals accumulate in DCs within the kidney within 5 days of angiotensin II infusion, and that this is prevented by renal denervation. In accord with our prior study, we found that adoptive transfer of DCs from an angiotensin II-infused mice to naïve recipients markedly enhances the hypertensive response to a generally subpressor dose of angiotensin II. We now demonstrate that this pro-hypertensive effect of DCs is eliminated if the donor mouse has undergone renal denervation. When DCs process antigens, they undergo a maturation step involving increased surface expression of the B7 ligands CD80 and CD86, which we have shown are essential for development of hypertension. Importantly, denervation reduces DC expression of these co-stimulatory molecules and also markedly reduces DC production of the pro-inflammatory cytokines IL-1α, IL-1β, IL-2 and IL-6. These cytokines skew T cells to T H1 and T H17 phenotypes, and thus promote inflammation.

Our present studies also define mechanisms by which sympathetic nerves affect DC function. We found that DCs express numerous adrenergic receptors, and that β2 receptors are increased during angiotensin II infusion. Long-term exposure of DCs to norepinephrine in vitro dose-dependently increased isoketal-adduct formation in DCs. Our data also indicate that β2 receptor signaling is not involved in this process as the increase in DC isoketal-adducts was unchanged in mice lacking this receptor. We also provide evidence that angiotensin II-induced hypertension causes a fairly striking increase in vascular superoxide production in the kidney, and that this is prevented by renal denervation. It is therefore possible that ROS such as hydrogen peroxide or peroxynitrite produced by vascular cells could diffuse into DCs and lead to lipid oxidation and isoketal formation. Our data exclude a role of neuropeptide Y in this process.

It is possible that many of the beneficial effects of renal denervation are simply related to a decrease in blood pressure and the resultant decrease in pressure-induced damage in the kidney. In an effort to separate the effects of pressure and sympathetic denervation, we employed unilateral denervation. In this setting T cell accumulation was reduced in the denervated, but not in the innervated kidney. These findings suggest that innervation per se, rather than pressure elevation, participates in the renal inflammatory response in hypertension. We cannot completely exclude a role of pressure, as vascular stretch is known to increase production of reactive oxygen species and MCP1, VCAM1 and IL-6 expression. We have previously found that anti-hypertensive treatment with hydralazine and hydrochlorothiazide prevents vascular inflammation, however these agents have off-target effects that directly affect immune responses. Data from prior studies by our group and others suggest that various hypertensive stimuli such as angiotensin II and salt promote modest increases in blood pressure that are augmented by inflammation in a feed-forward fashion. It is likely that sympathetic innervation plays an important intermediate role in such interplay.

A striking finding in this study is that renal denervation affected the function of DCs in the spleen. One explanation for this is that DCs arising from the kidney migrate to the spleen, where they can activate T cells that promote systemic inflammation (Figure 8G). Our data in CCR7−/− mice are compatible with this concept. CCR7 is employed by DCs as a homing marker that allows their transmigration to secondary lymphoid organs. In CCR7−/− mice, we continued to observe DC activation in the kidney, but not in the

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spleen, suggesting that their movement from the kidney to the spleen might be defective. These studies must be interpreted with caution, as CCR7 is also critical for T cell homing to secondary lymphoid organs. Nevertheless, findings in these animals suggest an interplay between the kidney and spleen, and perhaps other sites of immune activation.

There has been substantial debate as to the role of renal afferent nerves in hypertension. Intrarenal injection of phenol in rats leads to sustained hypertension and increased hypothalamic norepinephrine production, thought secondary to afferent nerve activity. Circulating calcitonin related peptide level, reflecting afferent nerve activity, is elevated in this model. Likewise, activation of renal afferent nerves enhances hypertension in response renal wrapping. In contrast, activation of sensory nerves has been proposed to buffer the increase in blood pressure caused by angiotensin II. Sensory afferents could conceivably lead to reflex activation of cells within the spleen, as observed in the present study. Our data using capsaicin renal sensory denervation suggest that afferent nerves have little role in blood pressure regulation of blood pressure or immune cell activation in response to chronic angiotensin II infusion, but do not discount a role of these in the hypertension caused by other stimuli.

An important issue related to the present study is the clinical benefit of renal denervation in humans. The SYMPLICITY HTN-1 and 2 trials reported a striking lowering of blood pressure by catheter based radiofrequency renal nerve ablation with sustained reductions near 30 mmHg. The Global SYMPLICITY registry confirmed a similar effect on office blood pressure. These studies however were not sham-controlled or blinded. Questions were raised regarding study design and the patients included in these trials. In contrast, the SYMPLICITY HTN-3 trial, which included sham controls and was blinded, showed essentially the same lowering of blood pressure in the sham-treated and denervation-treated patients. It has been suggested that the benefit observed in the earlier trials were due to the Hawthorne effect, regression of blood pressure values to a mean and placebo effects. In contrast, a recent reanalysis of the SYMPLICITY HTN-3 trial suggested that a greater number of catheter ablations correlated with greater blood pressure lowering and that non-African Americans responded more to this procedure. Another issue is that long-standing hypertension in humans, with attendant changes in renal and vascular function, might not be responsive to renal nerve ablation. Indeed, we found that the anti-hypertensive effect of denervation was less pronounced when applied after the onset of hypertension than when denervation was performed prior to the onset of angiotensin II infusion. Despite this, we observed a substantial anti-inflammatory effect of renal denervation performed after the onset of hypertension. It is therefore possible that the anti-inflammatory effects of renal denervation might not be reflected by simple measures of blood pressure, but might have long-term benefit on renal function or non-renal cardiovascular events.

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DISCLOSURES
None.
REFERENCES


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FIGURE LEGENDS

**Figure 1:** Renal denervation reduces sympathetic drive in the kidney and attenuates angiotensin II-induced hypertension. A: Mice underwent phenol application to one renal artery. Three week later, catecholamines were extracted from the innervated and denervated kidney homogenates and analyzed by HPLC. (n=4 in both groups) B: Western blot showing tyrosine hydroxylase (TH) in innervated (I) and denervated kidneys. C and D: Effect of renal denervation on the hypertensive response to 2 weeks angiotensin II infusion (490 ng/kg/min). RDN indicates renal denervation. Data in panels A and B were analyzed by paired t tests and data in panels C and D with two-way ANOVA with repeated measurements, n=10 and 8 in each group. *P < 0.05, **P < 0.01, ***P < 0.001

**Figure 2:** Effects of bilateral renal denervation on renal leukocyte and T cell infiltration. Mice underwent bilateral renal denervation and one week later had osmotic minipumps for angiotensin II infusion implanted. Two weeks later, kidneys were harvested for flow cytometry. Representative flow cytometry of kidneys from mice with sham surgery or bilateral renal denervation are shown in panel A. Live singlet cells were gated for total leukocytes (CD45+), total T cells (CD3+), CD4+ and CD8+ T cells. CD44 expression was examined in CD4+ and CD8+ T cells. Fluorescence-minus-one (FMO) controls for CD44hi cells are shown as dotted lines. Mean data are shown for total leukocytes (B), T cells (C to E), and memory T cell accumulation (F and G) in response to angiotensin II was examined in either sham-treated or denervated kidneys. Data were analyzed using two-way ANOVA, n=5 to 10 in each group. *P < 0.05, **P < 0.01.

**Figure 3:** Bilateral renal denervation prevents renal injury in angiotensin II-induced hypertension. Mice were treated as in figure 2. T cells were identified using anti-CD3+ monocyte/macrophages using anti-F4/80 and collagen was identified using Massons Trichrome in the renal cortex and medulla (A and B, n=6 per group). DHE fluorescence was visualized at 530-560 nm and relative fluorescence intensity was quantified (C, n = 4 per group). Twenty-four urine collections were analyzed for albumin content and nephrin (D and E, n=6 per group). Data in panels A to C were analyzed using one-way ANOVA and data in D and E were analyzed using two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 4:** Local anti-inflammatory effect of unilateral renal denervation. Mice underwent unilateral renal denervation by application of phenol to the left renal artery. Systolic blood pressure in mice after two weeks of angiotensin II infusion measured by tail cuff (A, n=8 for Sham and 7 in both unilateral and bilateral RDN). Infiltration of total leukocytes (CD45+), T cells (CD3+), and both CD4+ and CD8+ T cells, in the innervated and denervated kidneys was determined by flow cytometry (B-E, n = 6 per group). Data in panel A was analyzed using one-way ANOVA. Data in panels B-E were analyzed using paired t test, n=6. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 5:** Effect of renal denervation on expression of genes involved in vascular function and inflammation. Mice underwent renal denervation as in figure 1 and subsequently received angiotensin II-infusion. Levels of renal mRNA for the designated genes were determined using real-time quantitative PCR. Data were analyzed with two-way ANOVA, n=6 to 8. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 6:** Renal denervation prevents dendritic cell activation, isoketal adduct formation, and cytokine production in response to angiotensin II infusion. Dendritic cell activation was determined by measuring the expression of co-stimulatory molecules CD80 (A), CD86 (B) and intracellular isoketal-protein adducts (C). Shown are representative histograms and mean data. Isoketal adducts were also measured in DCs isolated from kidneys from mice that received angiotensin II infusion for 5 days (D). DCs were isolated from spleen and cultured in vitro for 24 hour. IL-1α, IL-1β and IL-6 released in medium were measured by Luminex (E to G). The black dotted lines in histograms indicate fluorescence-minus-one (FMO) controls for antibodies against CD80, CD86 and isoketals. Data were analyzed with one-way ANOVA.
ANOVA, n=6 per group in flow cytometry and n=5 to 8 in cytokine measurements. *P < 0.05, **P < 0.01, ***P < 0.001

**Figure 7:** Renal denervation prevents formation of pro-hypertensive/pro-inflammatory dendritic cells. Panel A shows the experimental protocol. DCs were obtained from either sham or bilateral renal denervation (RDN) mice after two weeks of angiotensin (Ang) II infusion, and 1×10^6 cells were adoptively transferred to naive recipient mice. Ten days after DC adoptive transfer recipient mice received low-dose angiotensin II (140 ng/kg/min). Systolic and diastolic pressures were measured using telemetry (B and C). Data are analyzed with two-way ANOVA with repeated measurements. Infiltration of total leukocytes (CD45^+), T cells (CD3^+), and both CD4^+ and CD8^+ T cells in the kidneys of recipient mice were analyzed with flow cytometry(D-H). n= 5 to 7 per group. Data are analyzed with two-way ANOVA, *P < 0.05, **P < 0.01.

**Figure 8:** Effect of renal denervation on blood pressure and renal inflammation in established hypertension. Mice received angiotensin II (490 ng/kg/min) infusion for 4 weeks, and bilateral renal denervation or sham surgery were performed after two weeks. Systolic blood pressure was measured by tail cuff (n=6 per group) and by telemetry for at baseline, and after two, three and four weeks of angiotensin II infusion (n=5 per group). Dotted lines indicate the time when renal denervation (RDN) or sham surgeries were performed. Renal infiltration of total leukocytes (CD45^+), T cells (CD3^+ panel D), and both CD4^+ (E) and CD8^+ T cells (F) were quantified with flow cytometry (n=6 per group). Blood pressures were compared using two-way ANOVA with repeated measures. Data in panels C-F were analyzed with t test, *P < 0.05, **P < 0.01. G: Proposed model of the effect of renal sympathetic nerves on activation of adaptive immunity in hypertension.
Novelty and Significance

What Is Known?

- Sympathetic outflow contributes to T cell activation in hypertension.
- In hypertension, proteins oxidatively modified by highly reactive \(\gamma\)-ketoaldehydes accumulate in dendritic cells. These are immunogenic and lead to activation of adaptive immunity and end-organ damage.
- Renal denervation lowers blood pressure in animals with experimental hypertension and in some studies of hypertensive humans.

What New Information Does This Article Contribute?

- Renal denervation attenuates renal inflammation in angiotensin II induced hypertension.
- Renal sympathetic nerves and norepinephrine cause DC activation and isoketal accumulation.
- Renal denervation prevents DC and T cell activation in response to chronic angiotensin II infusion.

This study shows that the kidney is a major site of immune activation in hypertension and that this is mediated by efferent sympathetic nerves and their release of norepinephrine. Renal denervation reduces activation of dendritic cells and ultimately T cells and thus prevents both renal and vascular inflammation. Our findings may help explain why renal denervation has pleiotropic systemic effects.
Figure 1

(A) Levels of norepinephrine and epinephrine in innervated and denervated kidneys. Significantly lower levels in denervated kidneys for both norepinephrine (**p<0.01) and epinephrine.

(B) Western blot analysis showing TH (62kDa) and GAPDH (37kDa) expression in innervated and denervated kidneys. TH expression is higher in innervated kidneys (I) compared to denervated kidneys (D) (**p<0.01).

(C) Systolic blood pressure (BP) over time with Ang II infusion. Significant increase in BP in RDN group compared to Sham (**p<0.01).

(D) Diastolic BP over time with Ang II infusion. Significant difference in BP between Sham and RDN groups (*p<0.05).

** p<0.01 vs. sham surgery

* p<0.05 vs. sham surgery
Figure 6

A

Vehicle

CD80+
55.2%

Ang II

CD80+
68.7%

Ang II + RDN

CD80+
51.7%

B

CD86+
19.9%

CD86+
23.0%

CD86+
16.4%

C

isoketals

isoketals

isoketals

D

Isoxetals in DCs from kidney %

Vehicle Ang Ang+RDN

*** *

E

IL-1α pg per 10^6 cells

Vehicle Ang Ang+RDN

* *

F

IL-1β pg per 10^6 cells

Vehicle Ang Ang+RDN

*** *

G

IL-6 pg per 10^6 cells

Vehicle Ang Ang+RDN

* *
Figure 7

A. Schematic representation of the experimental setup involving Sham/RDN donors, adoptive transfer of splenic DCs, and Naïve recipients. Ang II infusion at 490 ng/kg/min or vehicle for 14 days, telemetry implant for 7-10 days, and Flow Cytometry for analysis.

B. Graph showing changes in recipients' systolic blood pressure over the course of Ang II infusion. DC donors: ○ Intact renal nerves + Veh. □ Renal denervated + Veh.

C. Graph showing changes in recipients' diastolic blood pressure over the course of Ang II infusion. DC donors: ○ Intact renal nerves + Ang. □ Renal denervated + Ang.


E. Bar graph showing CD45+ cells × 10^5 per kidney for Sham and RDN.

F. Bar graph showing CD3+ cells × 10^5 per kidney for Sham and RDN.

G. Bar graph showing CD8+ cells × 10^5 per kidney for Sham and RDN.

H. Bar graph showing CD4+ cells × 10^4 per kidney for Sham and RDN.
Figure 8

Panel A: Systolic BP (mmHg) over time with Ang II infusion. 
- Baseline, 1 week, 2 week, 3 week, 4 week

Panel B: Systolic BP (mmHg) with Ang II infusion.
- Baseline, 2 week, 3 week, 4 week

Panel C: CD45+ cells x10^5 per kidney.
- Sham vs. RDN

Panel D: CD3+ cells x10^4 per kidney.
- Sham vs. RDN

Panel E: CD4+ cells x10^4 per kidney.
- Sham vs. RDN

Panel F: CD8+ cells x10^4 per kidney.
- Sham vs. RDN

Panel G: Hypertensive stimuli leading to severe hypertension.
- Brain, Sympathetic nerve activity, Kidney, Severe Hypertension
- Isoketal formation and DC activation, Renal inflammation and damage
- Homing of activated T cells, Other end-organ damage
Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II-Induced Hypertension

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Supplemental Material

Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II–Induced Hypertension

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Detailed Materials and Methods

Animals: All mice were obtained from Jackson Laboratories on a C57Bl/6 background. In the case of the β2AR-/– mice, these were outbred from β1/β2AR+− mice. At 3 months of age, mice were randomly selected for renal denervation or sham surgery. For renal denervation, mice were anesthetized by intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) and the renal arteries were visualized via flank incisions. The renal nerves were ablated by encircling the renal artery with a 5-0 suture soaked with 10% phenol in ethanol. In sham-operated mice, normal saline was applied rather than phenol. In some experiments, unilateral denervation was performed by applying phenol to only the left renal artery. To selectively disrupt renal afferent nerves, a similar surgical approach was used except except capsaicin (33 mmol/L) dissolved in 5% Tween 80, 5% ethanol and 90% normal saline was applied to the renal artery for 15 minutes as described recently. Blood pressure was measured either invasively using telemetry or non-invasively using an automated tail-cuff system as previously described. Hypertension was induced by the infusion of angiotensin II (490 ng/kg/min) via osmotic minipumps for two weeks to four weeks. At study termination, mice were euthanized by exposure to CO2. One week later, osmotic minipumps were implanted subcutaneously for infusion of angiotensin II (490 ng/kg/min) or vehicle for 2 weeks unless otherwise indicated. In subsets of mice, telemetry units were implanted for measurement of blood pressure. After at least one week recovery from telemetry implant, blood pressure was recorded for 10 minutes every hour for the duration of the experiments (i.e. three days prior to osmotic minipump implantation and until the end of angiotensin II infusion at Day 14). At the end of each experiment, mice were sacrificed with CO2 inhalation and the chest was rapidly opened and the superior vena cava sectioned. A catheter was placed in the left ventricular apex and the animals were perfused at a physiological pressure with KrebsHepes buffer until the effluent from the vena cava was cleared of blood. The Institutional Animal Care and Use Committee approved all experimental protocols.

Material The antibodies and fluorophores were purchased from Biolegend (San Diego, CA) included: 7-AAD for live/dead staining; BV510-conjugated anti-CD45 (30-F11); APC-conjugated anti-CD4 (GK1.5); APC/Cy7-conjugated anti-CD8 (53-6.7); PE/Cy7-conjugated anti-CD3 (145-2C11); FITC-conjugated CD44 (IM7); PE-conjugated anti-CD80 (16-10A1); BV421-conjugated anti-CD86 (GL-1); PE/Cy7-conjugated anti-I-Aβ (AF6-120.1); APC/Cy7-conjugated anti-CD11c (Bu15), APC-conjugated anti-CD11b (M1/70), FITC-conjugated anti-B220 (RA3-6B2). An Alexa 488 tagged single-chain antibody that has been previously described was used for detecting intracellular isoketal adducts D11. Primary antibodies for Western blot included rabbit polyclonal antibodies anti-tyrosine hydroxylase (AB152 from Millipore), rabbit monoclonal anti-d2 adrenergic receptor (ab151727), anti-β1 adrenergic receptor (ab3442), anti-β2 adrenergic receptor(ab182136, all from Abcam) and anti-GAPDH (sc-32233, from Santa Cruz Biotechnology). Enzyme immunoassay kits for urinary albumin and nephrin were purchased from Exocell (Philadelphia, PA). Enzyme immunoassay kit for calcitonin gene related peptide was fromCayman Chemical (Ann Arbor, MI). All primers and probes for real time PCR (NOS3; Mm00435217_m1, Klf4; Mm00516104_m1, MCP-1/Ccl2: Mm00441242_m1, VCAM-1: Mm01320970_m1, ICAM-1: Mm00516023_m1 gene expression assays as well as a GAPDH endogenous control) were from Applied Biosystems. Norepinephrine and neuropeptide Y were purchased from Sigma Aldrich (St Louis, MO).
Measurements of Albuminuria/Nephrinuria: Albumin and nephrin were measured by ELISA from 24 hour urine samples as described previously. All concentrations were multiplied by total urine volume to obtain the daily excretion rate.

Catecholamines: After euthanasia, the kidneys and adrenal glands were freeze-clamped and pulverized by a mortar and pestle chilled in liquid nitrogen. Norepinephrine and epinephrine contents were measured by high-performance liquid chromatography via electrochemical detection as previously described.

Calcitonin Gene Related Peptide: With kidneys harvested immediately after euthanasia, the renal pelvis using a dissecting microscope. Tissues were homogenized in 1M acetic acid and CGRP was measure according to the protocol provided in the ELISA kit.

Western Blot Analysis and Real-time PCR: Western blotting was performed using antibodies against tyrosine hydroxylase, α2b, β1 and β2 adrenergic receptors, and GAPDH. Goat anti-rabbit and goat anti-mouse secondary polyclonal antibodies were employed. Western blots were quantified by densitometry. Levels of endothelial nitric oxide synthase (eNOS), transcription factor Kruppel-like factor 4 (Klf4), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), regulated on activation normal T cell expressed and secreted (RANTES), and monocyte chemotactic protein 1 (MCP-1) mRNA were measured using TaqMan real-time PCR.

Cytokine Detection: DCs were positively selected from the spleen using an autoMACS separator and CD11c magnetic beads (Miltenyi Biotec). The purity of these was confirmed to be >95% by flow cytometry. Splenic CD11c+ cells were placed in 96-well plates at a density of 5x10^5 per well and cultured in 200 μL RPMI1640 medium supplemented with 10% fetal bovine serum for 24 hours. Cytokines released into the medium were quantified via a Luminex assay. We have previously confirmed that this kit does not select mouse macrophages.

Confocal Microscopy: For superoxide detection, kidneys were rapidly removed after euthanasia, immersed in optimal cutting temperature (OCT) media and frozen in dry ice. Thirty μm sections were obtained and used for detecting superoxide by DHE as described previously. Sections were imaged using confocal microscopy with an excitation wavelength of 405 nm and an emission wavelength of 530-560 nm. For β3 tubulin staining, lymph nodes adjacent to the kidneys were carefully isolated, and 10-μm-thick frozen sections were prepared. Images were obtained using a primary anti-β3 tubulin antibody and the Alexa 488 goat anti-rabbit secondary antibody.

Dendritic Cell Adoptive Transfer: One million splenic DCs were obtained from mice that had undergone either sham surgery or bilateral renal denervation and subsequent angiotensin II or vehicle infusion for two weeks. These were suspended in 200 μL PBS, and adoptively transferred to naïve mice by tail vein injection. Telemetry transmitters were implanted in the recipient mice one week before adoptive transfer, and low dose (140 ng/kg/min) angiotensin II infusion were initiated 10 days later.

Preparation of Bone Marrow Derived Dendritic Cells: Bone marrow cells from C57BL/6 and β2AR−/− mice were cultured in 6-well plates at a concentration of 0.5×10^6 cells/2.5 ml in the presence of GMCSF and IL-4 as described previously. Norepinephrine (1 – 3 μmol/L) was added to the medium at the beginning of culture, and supplemented on day 3 and day 6. In other experiments neuropeptide Y was added to achieve a final concentration of 100 nmol/L.

Immunohistochemistry: Five micron sections were obtained from formalin fixed, paraffin embedded kidneys. Collagen was visualized by Masson Trichrome blue staining, and immunohistochemistry was used to detect CD3+ and F4/80+ cells in the kidney as previously described.
Magnetic Resonance Imaging: Relative renal blood flow (rRBF) and relative renal blood volume (rRBV) were measured by dynamic susceptibility contrast magnetic resonance imaging (DSC-MRI) with the administration of iron oxide nanoparticles as described. Six mice with unilateral renal denervation and two-week angiotensin II infusion were used in this experiment. One or two days before of scanning, a catheter was placed in the jugular vein, and externalized from the interscapular region for administration of contrast agent. Mice were anesthetized (isoflurane 1.5-2%) and scanned on a Varian 7T MRI system using a Doty 38 transmit/receive coil. Body temperature was maintained at 37° during the scan. An oblique axial plan through both kidneys was chosen. A dynamic susceptibility-weighted gradient-echo sequence was applied during an iv injection of monocrystalline iron oxide nanoparticles (MION, 6mg/kg), which was administered rapidly in less than one second. A dynamic series of 600 images (~1s/volume) was acquired up to 10 minutes. \( R_2 \) imaging was performed using a multiple spin echo sequence with refocusing pulses of 180 (repetition time=2500ms, 16 echoes, 4 averages) before and after MION injection. The averaged \( \Delta R_2 \) across voxels of each kidney was used to represent rRBV, while peak amplitude/area from the relative DSC signal intensity time curve of each kidney was used to represent rRBF (sec\(^{-1}\)).

Statistics: Data in the manuscript are expressed as mean ± SEM. For telemetry and tail cuff blood pressure measurements over time, two-way ANOVA with repeated-measures was employed, followed with a Bonferroni post hoc test when significance was indicated. To compare the effect of renal denervation on renal inflammation, two-way ANOVA was used as indicated. Because of differences in variation between groups, we employed a non-parametric rank sum test with a Bonferroni correction for comparisons of cytokine release by DCs. P values are reported in the figures and a value less than 0.05 was considered significant.
References:
**Supplemental Table I: Effect of Renal Denervation on Cytokine Production in DCs**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sham (n=5)</th>
<th>Ang II (n=8)</th>
<th>Ang II + RDN (n=8)</th>
</tr>
</thead>
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<tr>
<td>IL-2</td>
<td>0.65±0.06</td>
<td>1.87±0.42 *</td>
<td>1.28±0.35</td>
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<td>IL-12 p40</td>
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<td>0.22±0.18</td>
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<tr>
<td>IL-23</td>
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<td>672.2±245.6</td>
<td>377.6±60.62</td>
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<tr>
<td>GM-CSF</td>
<td>13.9±3.46</td>
<td>76.04±35.65</td>
<td>13.65±3.60</td>
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<tr>
<td>TGFβ1</td>
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<tr>
<td>TGFβ3</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

In pg per 10^8 cells. *p<0.05 vs. Sham.

IL: interleukin
GM-CSF: granulocyte-macrophage colony-stimulating factor
TGF: transforming growth factor
Online Figure I: Effects of renal denervation on adjacent lymph nodes and adrenal glands. The neuronal marker beta 3 tubulin was visualized in the lymph nodes adjacent to the kidneys of mice by confocal microscopy (A). Norepinephrine and epinephrine content in adrenal glands were measured by HPLC (B). Data were analyzed using unpaired t tests, n=5 in each group. No statistical significant difference was detected between groups.
Online Figure II: Effect of renal denervation on aortic inflammation in response to angiotensin II. Mice underwent bilateral renal denervation or sham surgery and then received angiotensin II (490 ng/kg/min) for 14 days. Aortas were harvested for flow cytometry and total leukocytes (A), T cells (B) and CD4+ and CD8+ T cells (C and D) quantified using flow cytometry. Data were analyzed using one-way ANOVA, n=5 in each group. *P < 0.05, **P < 0.01, ***P<0.001.
Online Figure III: Gating strategy for detection of macrophages, dendritic cells and monocytes from spleen and kidney.
Online Figure IV: Correlation between systolic pressure and renal T cell infiltration in mice after sham, unilateral (uniRDN) and bilateral renal denervation (biRDN). Mice underwent either sham surgery, unilateral or bilateral renal denervation and subsequently received either vehicle (Veh) or angiotensin II (Ang) infusion for two weeks. In unilateral denervated mice, the innervated (in) and denervated (de) kidneys are plotted separately. Flow cytometry was used to quantify total T cells (left panel), CD4⁺ and CD8⁺ T cells (middle and right panel).
Online Figure V: Effect of renal denervation on relative renal blood flow in mice with unilateral renal denervation after two-week angiotensin II infusion. Renal MRI was performed as described in the supplemental method. Data were analyzed with paired t test, n=5. *P < 0.05, **P < 0.01.
Online Figure VI: Effects of renal denervation on activation of other antigen presenting cells in the spleen in mice with angiotensin II infusion.
Online Figure VII: Ablation of afferent renal nerves does not prevent hypertension or renal inflammation. Selective ablation of afferent renal nerves by capsaicin treatment was validated by measurements of calcitonin gene related peptide (CGRP) (A). Renal norepinephrine and epinephrine contents were measured by HPLC as described in the methods (B). Systolic blood pressure was measured using the tail cuff method (C, n=8 for each group.) Flow cytometry of single cell homogenates was used to quantify renal infiltration of leukocytes and lymphocytes in the kidney (D-H). Data for panels A and B (n=5 per group) were analyzed using paired t test. Data in panel C was measured using two-way ANOVA. Data in panels E – H were analyzed by unpaired t tests.
Online Figure VIII: Effects of sympathetic signaling on isoketal-protein adduct formation in dendritic cells. DCs were magnetically isolated from spleens of mice that received vehicle or angiotensin II for two weeks. mRNA expression of adrenergic receptor (AR) subtypes were measured by realtime PCR (n=3). Protein levels of α2, β1 and β2 adrenergic receptors in DCs were determined by Western blot and mouse brain was used as a positive control (B). DCs were derived from bone marrow of either wild type or β2AR−/− mice using GM-CSF and IL-4, and cultured with norepinephrine in vitro for 7 days (n=5 per group). In other experiments (E) wild type bone marrow-derived DCs were cultured in the presence of neuropeptide Y (n=4 per group). Isoketal-protein adducts were quantified by flow cytometry. Data in panels A and E were analyzed with t test. Data in panel D were analyzed using two-way ANOVA.
Online Figure IX: Effect CCR7 deficiency on hypertension, dendritic cell maturation and renal inflammation. CCR7−/− mice received angiotensin II (490 ng/kg.min) for two weeks. Blood pressure was measured by tail cuff (A). Flow cytometry was used to determine surface expression of CD80 and CD86 on DCs from the kidney (B) and spleen (C) and the renal presence of total leukocytes (D), T cells (E to G), and memory T cells (H and I). Data were analyzed using t tests, n=5 in each group. *P < 0.05.