CD70 Exacerbates Blood Pressure Elevation and Renal Damage in Response to Repeated Hypertensive Stimuli


Methods and Results: We imposed repeated hypertensive challenges using either Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME)/high salt or repeated angiotensin II stimulation in mice. During these challenges effector memory T cells (TEm) accumulated in the kidney and bone marrow. In the L-NAME/high-salt model, memory T cells of the kidney were predominant sources of interferon-γ and interleukin-17A, known to contribute to hypertension. L-NAME/high salt increased macrophage and dendritic cell surface expression of CD70 by 3- to 5-fold. Mice lacking CD70 did not accumulate TEm cells and did not develop hypertension to either high salt or the second angiotensin II challenge and were protected against renal damage. Bone marrow–residing TEm cells proliferated and redistributed to the kidney in response to repeated salt feeding. Adoptively transferred TEm cells from hypertensive mice homed to the bone marrow and spleen and expanded on salt feeding of the recipient mice.

Conclusions: Our findings illustrate a previously undefined role of CD70 and long-lived TEm cells in the development of blood pressure elevation and end-organ damage that occur on delayed exposure to mild hypertensive stimuli. Interventions to prevent repeated hypertensive surges could attenuate formation of hypertension-specific TEm cells. (Circ Res. 2016;118:1233-1243. DOI: 10.1161/CIRCRESAHA.115.308111.)

Key Words: adaptive immunity ▪ bone marrow ▪ immunologic memory ▪ inflammation ▪ interferon ▪ interleukin 17A ▪ kidney

During the past several years, emerging evidence has demonstrated that the adaptive immune system plays an important role in the pathogenesis of hypertension.1 In several experimental models of hypertension, there is accumulation of T cells in the kidney that release inflammatory cytokines, such as interleukin (IL)-17A and interferon (IFN)-γ, which promote renal and vascular dysfunction.2 We and others have shown that these cytokines contribute to salt and volume retention by the kidneys and stimulate vascular production of reactive oxygen species and promote vascular stiffening.3-6 In keeping with this, mice deficient in IL-17A and IFN-γ are protected against the antidiuretic and antinatriuretic effects of angiotensin II (ang II).2,3 A cardinal feature of adaptive immunity is immunologic memory, which provides protection against repeated antigenic exposure. On initial antigen presentation, the classical T-cell immune response is characterized by stimulation of naive T cells to proliferate and form effector T cells.7 The majority of effector T cells ultimately die, but a few remaining cells become long-lived memory T cells. Some of

Original received December 2, 2015; revision received March 15, 2016; accepted March 17, 2016. In February 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.68 days.

From the Division of Clinical Pharmacology, Department of Medicine (H.A.I., L.X., M.A.S., J.W., B.L.D., J.D.F., A.K., K.R.M., A.E.N., W.C., M.S.M., D.G.H.) and Division of Infectious Diseases (M.A.P., S.A.M.), Vanderbilt University Medical Center, Nashville, TN; Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt (M.A.S.); Laboratory of Cardiovascular Pharmacology, Department of Pharmacology, Faculty of Medical Sciences, University of Campinas, Campinas, Sao Paulo, Brazil (N.R.B.); Department of Physiology and Hypertension and Renal Center, School of Medicine, Tulane University, New Orleans, LA (R.S., L.G.N.); and Departments of Biomedical Sciences (K.E.B.) and Pathology and Laboratory Medicine (K.E.B.), Cedars-Sinai Medical Center, Los Angeles, CA.

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.115.308111/-/DC1.

Correspondence to David G. Harrison, MD, Division of Clinical Pharmacology, Room 536, Robinson Research Bldg, Vanderbilt University, Nashville, TN 37232. E-mail david.g.harrison@vanderbilt.edu

© 2016 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.115.308111
these return to secondary lymphoid organs such as lymph nodes and the spleen and are referred to as central memory (T<sub>CM</sub>) cells, which are characterized by the surface markers CD44<sup>hi</sup>/CD62L<sup>hi</sup>/CCR7<sup>+</sup>. Others remain in the periphery as effector memory (T<sub>EM</sub>) cells bearing the surface markers CD44<sup>hi</sup>/CD62L<sup>lo</sup>/CCR7<sup>−</sup>. A third recently identified subset are resident memory (T<sub>RM</sub>) cells, which are CD44<sup>hi</sup>/CD62L<sup>lo</sup>/CD103<sup>+</sup>/CD69<sup>+</sup>. The presence of CD69 inhibits the sphingosine-1 phosphate receptor and keeps activated T<sub>RM</sub> cells within specific tissues. Formation of memory cells requires the interaction of CD27 on T cells with CD70 on activated antigen-presenting cells (APCs). This costimulator interaction is analogous to that of T-cell CD28 with the B7 ligands, which is required for naïve T-cell activation. Mice lacking either CD27 or CD70 fail to develop memory T cells on antigen rechallenge.<sup>4,5</sup>

We hypothesized that the development of memory T cells in response to an initial hypertensive stimulus could sensitize the host to the development of hypertension in response to subsequent modest stimuli that would otherwise not raise blood pressure. In this study, we show that repeated hypertensive stimuli promote accumulation of T<sub>EM</sub> cells in the kidney and bone marrow and that these cells are primarily responsible for production of IFN-γ and IL-17A. This study illustrates a previously undefined role of CD70 in the genesis of hypertension in response to rather mild, repeated hypertensive challenges.

### Methods

**Animals and Blood Pressure Measurement**

Wild-type (WT), and IFN-γ--deficient mice (IFN-γ<sup>−/−</sup>) on a C57Bl/6J background were purchased from Jackson Laboratories (Bar Harbor, ME). CD70-deficient mice (CD70<sup>−/−</sup>) on a C57Bl/6J background were obtained from Dr Ross Kedl (University of Colorado). Mice were provided regular chow and water ad libitum. At 12 weeks of age, male mice were randomly selected for treatment.

Mice were euthanized at the end of all experiments by CO<sub>2</sub> inhalation. All animal procedures were approved by Vanderbilt University Institutional Animal Care and Use Committee, and mice were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals.

**Statistics**

Data are expressed as mean±SEM. Blood pressures were analyzed by ANOVA for repeated measures. For comparisons of experiments involving a 2x2 design 2-way ANOVA was used. When individual comparisons were made within this 2x2 design, Newman–Keuls or Holm–Sidak post hoc tests were used. When variances between groups were unequal, a Mann–Whitney comparison followed by a Bonferroni correction was used. For complete description of methods, please see the Online Data Supplement.

### Results

**Repeated Hypertensive Stimuli Induce Formation of Effector Memory T Cells That Reside in the Kidney and Bone Marrow**

To test the hypothesis that repeated hypertensive stimuli lead to formation of memory T cells and their accumulation in critical tissues such as the kidney, we used an experimental model that involves an initial exposure of the nitric oxide synthase inhibitor N<sup>ω</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.5 mg/mL) for 2 weeks, followed by a 2-week washout and subsequent high-salt diet (4% NaCl) feeding for 3 weeks (Figure 1A). This model permits repeated hypertensive challenges without surgical interventions and recapitulates salt-sensitive hypertension that is common in humans.

Flow cytometry analysis of single-cell homogenates from kidneys of these mice revealed a dramatic increase in both CD4<sup>+</sup> and CD8<sup>+</sup> memory cells within the kidney (Figure 1B–1D). In contrast, CD4<sup>+</sup> primary effector T cells were not increased by exposure to L-NAME/high salt. Exposure to either L-NAME or high-salt diet alone caused only a modest increase in the number of memory T cells. Surface staining with CD62L indicated that L-NAME followed by high-salt diet caused a progressive increase in both CD4<sup>+</sup> and CD8<sup>+</sup> memory T<sub>EM</sub> cells (Figure 1E and 1F), but did not change the small number of CD62L<sup>hi</sup> T<sub>CM</sub> nor T<sub>RM</sub> cells (Online Figure IA) present in the kidney. It has been reported that a subpopulation of memory T cells express the surface receptor Ly-6C<sup>+</sup>.<sup>10</sup> In keeping with this, we found that 40% of CD4<sup>+</sup> and 80% of CD8<sup>+</sup> memory T cells in the kidney are Ly-6C<sup>+</sup>. The renal CD8<sup>+</sup>/Ly-6C<sup>+</sup> T<sub>EM</sub> population was markedly increased by the L-NAME/high-salt protocol (Online Figure IB). There was no increase in memory T cells in the aorta or spleen of L-NAME/high-salt mice (Online Figure IC–IF).

**Effector Memory Cells Are the Major Source of IFN-γ and IL-17A in the Kidney of Hypertensive Mice**

Data from our group and others indicate that cytokines such as IL-6, IFN-γ, and IL-17A affect renal tubular function, modulating expression, and activity of the sodium/chloride cotransporter, the sodium potassium chloride cotransporter, and the sodium hydrogen exchanger 3.<sup>3</sup> Mice lacking IFN-γ and IL-17A are likewise partly protected from ang II–induced hypertension and have preserved natriuresis in response to a sodium challenge during ang II infusion, further emphasizing a role of these cytokines in altering renal function in hypertension.<sup>1</sup> We therefore sought to determine if T<sub>EM</sub> cells within the kidney play a role in production of these cytokines in hypertension. Intracellular staining indicated that IFN-γ production increased significantly in both CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells in hypertensive mice compared with mice fed a normal diet. In contrast, IFN-γ production was minimal in CD44<sup>+</sup> primary effector T cells and was not changed by the L-NAME/hypertensive challenge (Figure 2A and 2B). In a similar fashion, we found that renal CD44<sup>+</sup> memory T cells are predominantly responsible for IL-17A production in this hypertensive model (Figure 2C and 2D). Thus, among CD4<sup>+</sup>...
and CD8⁺ T cells in the kidney, memory T cells are predominantly responsible for production of these injurious cytokines.

**CD70 Is Increased on APCs and Participates in Memory T-Cell Formation and Hypertension**

The interaction of CD70 on APCs with CD27 on T cells plays a critical role in the formation of memory T cells, and in particular CD8⁺ memory T cells.⁸,¹² Using a gating strategy described by Jakubzick et al.,¹³ which discriminates between dendritic cells (DCs) and macrophages (Figure 3A), we found that L-NAME/high salt increases CD70 expression by 5- and 3-fold on macrophages and DCs, respectively (Figure 3B and 3C). To further define a role of the CD70 and CD27 in hypertension, we used radiotelemetry to monitor blood pressure in WT and CD70⁻/⁻ mice during the L-NAME/high-salt protocol. In WT mice, systolic pressure increased to 134±3 mm Hg during L-NAME administration and returned to baseline levels during the subsequent washout period (Figure 3D). This exposure to L-NAME caused WT mice to become salt sensitive, such that the subsequent addition of 4% salt to their diet increased systolic pressure to 147.5±9 mm Hg. In contrast, although the increase in systolic pressure caused by L-NAME in CD70⁻/⁻ mice was similar to that observed in WT mice, the hypertension induced by subsequent salt administration was markedly attenuated (Figure 3D). Because memory T cells are a major source of IFN-γ, we examined the hypertensive response to the L-NAME/high-salt protocol in IFN-γ⁻/⁻ mice. Similar to CD70⁻/⁻ mice, mice lacking IFN-γ failed to develop hypertension during the high-salt feeding phase of this protocol. Of note, the accumulation of TEM cells within the kidney was completely prevented in mice lacking CD70 and likewise attenuated in IFN-γ⁻/⁻ mice (Figure 3E–3G).

We have recently demonstrated a role for isoketal-adducted peptides in DCs immunogenicity.¹⁴ In this study, we found that L-NAME increased the presence of isoketal-protein adducts in DCs and this persisted during subsequent high-salt feeding (Online Figure IIA). We have also shown that noradrenaline stimulates formation of these adducts in DCs.¹⁵ In keeping with this, we found that both the L-NAME and high salt increased the low- to high-frequency ratio of heart rate variability, a parameter of sympathetic tone (Online Figure IIB). Thus, APCs promote hypertension not only by providing the initial stimulus of isoketal formation but also...
by increasing expression of CD70, which facilitates memory T-cell formation.

**Role of Memory T Cells in Glomerular and Tubular Injury**

In additional experiments, we sought to define how memory T cells and IFN-γ modulate renal damage. Exposure of WT mice to the L-NAME/high-salt protocol caused a marked increase in albuminuria and renal mRNA levels of neutrophil gelatinase–associated lipocalin. In contrast, CD70−/− and IFN-γ−/− mice were completely protected against the development of albuminuria (Figure 4A) and did not exhibit increases in renal neutrophil gelatinase–associated lipocalin mRNA expression (Figure 4B), indicating that IFN-γ producing memory cells promote glomerular and tubular injury. Likewise, CD70−/− and IFN-γ−/− mice had less renal fibrosis than WT mice after the L-NAME/high-salt protocol as detected by Masson Trichrome staining (Figure 4C). It has been proposed that IFN-γ can stimulate angiotensinogen production and promote sodium reabsorption within the renal tubule. We therefore examined kidney and urinary angiotensinogen and creatinine concentration and found no difference between WT, CD70−/−, and IFN-γ−/− mice (Online Figure IIIA and IIIB).

**T EM Cells Reside in the Bone Marrow and Are Responsive to High-Salt Feeding**

It has recently been appreciated that after an initial antigenic exposure, T EM cells do not simply circulate, but persist in a quiescent state in the bone marrow for months in the mouse and decades in humans. In keeping with this, we observed that there was a 1.8-fold increase in CD4+ T EM cells and a 3-fold increase in CD8+ T EM cells in the bone marrow after the L-NAME/high-salt exposure compared with control mice (Figure 5A and 5B). Bone marrow memory T-cell maintenance and survival are dependent on stromal cell production of IL-7 and IL-15. We did not observe a change in expression of either of these within the bone marrow of hypertensive mice (Online Figure IV). In contrast to T EM cells, there was no increase in the number of T CM or T RM cells present in the bone marrow (Figure 5A and 5C; Online Figure VA). In keeping with previous reports,10 bone marrow–residing T EM cells expressed Ly-6C and in the case of CD8+ T cells, were markedly increased by the L-NAME/high-salt challenge (Online Figure VB). Bone marrow–derived T EM cells exist largely in a quiescent state,10 and we found that relatively few bone marrow T EM cells were positive for the proliferation marker Ki-67, and this was not changed by the hypertensive challenge (Online Figure VC and VD).

**Figure 2.** Subsets of T cells within the kidney producing interferon (IFN)-γ and interleukin (IL)-17A. Intracellular staining was performed on renal-infiltrating memory T cells, at baseline or in response to Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME)/high salt. Data were obtained on cells studied in Figure 1D. A and B, Representative flow cytometry dot plot showing CD4+ and CD8+ memory T cells producing IFN-γ and (C and D) IL-17 by intracellular staining. Data are expressed as mean±SEM (n=9–15 per group). P values for the effect of L-NAME/high salt as calculated by 1-way ANOVA is shown. **P<0.001, ***P<0.001, ****P<0.001 vs normal diet.

Because repeated antigenic exposure has been shown to reactivate bone marrow–residing T EM cells, we sought to determine if these cells might exhibit salt sensitivity. To perform these experiments, we adoptively transferred 0.5×10⁶ T EM cells of CD45.2 mice that had undergone the L-NAME/high-salt protocol to recipient CD45.1 mice (Figure 5D). In recipient mice fed a normal diet for the ensuing 3 weeks, these adoptively transferred T EM cells were detected in the bone marrow and to a lesser extent in the spleen but not in the kidney (Figure 5E). In recipient mice fed a high-salt diet, there was expansion of T EM cells in the bone marrow (Figure 5F). Importantly, there was also marked accumulation of these adoptively transferred T EM cells in the kidney in response to high-salt feeding (Figure 5G). We observed a modest increase in the number of T EM cells in the spleen, but these were relatively few compared with the bone marrow and kidney (Figure 5H). Of note, bone marrow T EM cells from control mice fed a normal diet were not detectable in the spleen, bone marrow, or kidney of recipient mice (Figures 5F–5H).

In additional experiments, we exposed WT mice to the L-NAME/high-salt protocol followed by 3 weeks of normal feeding. We observed that there was a 1.8-fold increase in CD4+ T EM cells and a 3-fold increase in CD8+ T EM cells in the bone marrow after the L-NAME/high-salt exposure compared with control mice (Figure 5A and 5B). Bone marrow memory T-cell maintenance and survival are dependent on stromal cell production of IL-7 and IL-15. We did not observe a change in expression of either of these within the bone marrow of hypertensive mice (Online Figure IV). In contrast to T EM cells, there was no increase in the number of T CM or T RM cells present in the bone marrow (Figure 5A and 5C; Online Figure VA). In keeping with previous reports, bone marrow–residing T EM cells expressed Ly-6C and in the case of CD8+ T cells, were markedly increased by the L-NAME/high-salt challenge (Online Figure VB). Bone marrow–derived T EM cells exist largely in a quiescent state, and we found that relatively few bone marrow T EM cells were positive for the proliferation marker Ki-67, and this was not changed by the hypertensive challenge (Online Figure VC and VD).
diet. The mice were then re-exposed to a second high-salt challenge (Figure 6A). Radiotelemetry recording of blood pressure revealed that these animals maintain salt sensitivity for as long as 3 months after the initial L-NAME exposure, as reflected by an increase in blood pressure to 140 mm Hg (Figure 6B). A striking finding in these animals was that there was a further increase in CD4+ and CD8+ TEM cells in the kidney in response to this second salt challenge (Table 1), and a concomitant decrease in the bone marrow–residing TEM cells (Table 2). Taken together with our adoptive transfer experiments, these data suggest that bone marrow–residing TEM cells are salt sensitive, and redistribute to the kidney on salt feeding.

**Induction of Memory T Cells in Ang II–Induced Hypertension**

In additional experiments, we also investigated a potential role of T-cell memory in ang II–induced hypertension. WT mice were implanted with an osmotic pump for infusion of either ang II (490 ng/kg per minute) or vehicle for 2 weeks, followed by a 2-week washout period (Figure 7A). The mice were then implanted with a second osmotic minipump for infusion of low dose of ang II (140 ng/kg per minute), and blood pressure was monitored by radiotelemetry. As shown in Figure 7B, WT mice that initially received a sham infusion had minimal increase in blood pressure in response to this low dose of ang II. In contrast, mice that had previously received high-dose ang II exhibited an increase in blood pressure to 160 mm Hg. CD70−/− mice developed a modest hypertensive response during the first 2 weeks of high-dose ang II infusion, however, had essentially no increase in blood pressure during the subsequent infusion of low-dose ang II (Figure 7B). During this 2-week exposure to high-dose ang II, there was a markedly blunted increase in CD8+ TEM cells and no change in CD4+ TEM cells in the kidneys of CD70−/− mice (Online Figure VIA). Of note, there was a modest increase in renal CD44lo cells, which represent a primary effector population and in double-negative T cells (Online Figure VIB and VIC). Consistent with our findings in the L-NAME/high-salt model, in WT mice that had previously received high-dose ang II, there was almost a 3-fold increase in CD4+ and CD8+ TEM cells in the kidney (Figure 7C) and bone marrow (Figure 7D) compared with mice that had previously received a sham infusion. In contrast, there was no increase in renal or bone marrow TEM cells of CD70−/− mice at the end of this high-dose/low-dose ang II protocol. Thus, previous exposure to ang II primes severe hypertension in response to a subsequent normally subpressor dose of this octapeptide and leads to accumulation of TEM cells in the kidney and bone marrow. This memory response to ang II is dependent on CD70.
Discussion

In this study, we show that TEM cells formed during an initial hypertensive challenge enhance sensitivity to a second mild hypertensive challenge, leading to elevations in blood pressure and renal damage that does not occur in the absence of these cells. Our data are compatible with the paradigm illustrated in Online Figure VII, in which TEM cells accumulate in the kidney and mediate renal dysfunction and sensitize the host to both salt-sensitive and ang II–induced hypertension. These TEM cells can be long lived and can potentially sensitize the host to repeated hypertensive stimuli encountered clinically, such as recurrent episodes of emotional stress, catecholamine surges in sleep apnea, or repeated bouts of excess sodium intake.

To mimic salt-sensitive hypertension, which is common in humans, we used a model of L-NAME followed by high-salt feeding. We found that this form of hypertension is associated with the accumulation of memory T cells in the kidney and bone marrow and that these cells are major sources of IL-17A and IFN-γ, which we and others have shown to be important in mediating hypertension and its end-organ damage.\(^1\)\(^6\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\) The formation of memory T cells in hypertension was dependent on CD70, and we found that mice lacking this costimulatory ligand, or lacking IFN-γ did not demonstrate salt-induced hypertension after L-NAME exposure. To confirm these findings, we used a second model in which we initially exposed mice to a 2-week infusion of a pressor dose of ang II (490 ng/kg per minute). This also sensitized the mice to develop severe hypertension on re-exposure to a second low dose of ang II that normally does not raise blood pressure. As in the case of the L-NAME/high-salt model, TEM cells accumulated in the kidney and bone marrow, and mice lacking CD70 were protected against the second exposure to ang II. Thus CD70, and by inference its role in promoting formation of memory T cells, plays a critical role in 2 forms of hypertension.

The development of salt sensitivity after exposure to L-NAME is of interest because the loss of nitric oxide (NO) might promote formation of isoketal-protein adducts, which we have previously shown to be immunogenic when formed in DCs.\(^1\)\(^4\) In this previous work, we demonstrated that isoketal-protein adducts have many features of neoantigens, in that they drive memory T-cell proliferation and cytokine production. NO rapidly reacts with lipid peroxy-radicals\(^1\)\(^2\)\(^3\) and in doing so can limit formation of lipid oxidation products, such as isoprostanes and isoketals. Indeed in this study, we found isoketal adducts in DCs were increased after L-NAME and were further increased after exposure to salt. This sequence of events could, therefore, provide repeated neoantigen exposure leading to expansion of memory T cells. Of note, many conditions linked to hypertension, such as diabetes mellitus, obesity, and hypercholesterolemia are associated with loss of endothelial NO production, and in some cases an increase in the endothelial production of the strong oxidant peroxynitrite.\(^2\)\(^3\) The administration of L-NAME thus mimics aspects of

Figure 4. Parameters of renal injury in response to N\(^\omega\)-nitro-L-arginine methyl ester hydrochloride (L-NAME)/high salt. A, Glomerular injury was assessed by quantifying 24-hour urinary excretion of albumin in wild-type (WT), CD70\(^+/−\), and interferon (IFN)-γ\(^+/−\) mice. B, Neutrophil gelatinase–associated lipocalin (NGAL) mRNA was detected by quantitative real-time polymerase chain reaction. C, Masson Trichrome staining was used to detect renal fibrosis. Data are expressed as mean±SEM, (A) n=5 to 6, (B and C) n=6 to 11 per group. P values analyzed by 1-way ANOVA; *P<0.1, **P<0.01; ***P<0.0001, and ****P<0.0001. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.
endothelial dysfunction observed in these common diseases. Myeloid-derived DCs, such as those studied in this article, are formed when monocytes traverse the vascular endothelium, and the interaction with either NO or peroxynitrite producing endothelial cells might modulate formation of intracellular isoketal adducts within the DCs and thus lead to formation of salt-sensitive hypertension. Accordingly, Kopkan et al have shown that endothelial nitric oxide synthase–deficient mice develop hypertension on salt feeding in a fashion analogous to our mice that had previously received L-NAME.

Related to the above, we recently showed that sympathetic innervation is important for formation of isoketal adducts in dendritic cells of the kidney, and that this is prevented by renal denervation. In this previous study, we showed that DCs possess adrenergic receptors and that norepinephrine can directly stimulate formation of isoketal adducts in these cells. In our current study, both L-NAME and L-NAME/high salt increased the low frequency:high frequency ratio of heart rate variability, reflecting increased sympathetic tone. It is therefore conceivable that in addition to a loss of endothelial NO, increased sympathetic outflow could stimulate formation of these immunogenic adducts in DCs.

An interesting finding in this study is that the L-NAME/high-salt protocol increases surface expression of CD70 on macrophages and dendritic cells within the kidney. T-cell activation requires not only engagement of the T-cell receptor but also costimulation. In the case of naive T cells, a common costimulatory event involves interaction of CD28 on T cells with the B7 ligands CD80 and CD86 on APCs. We have previously shown that blockade of this interaction with Abatacept prevents ang II and deoxycorticosterone acetate salt hypertension. In addition to this interaction, the interplay between CD70 on APCs and CD27 on T cells plays a critical role in formation of memory T cells. An important result of this study was that mice lacking CD70 do not develop salt sensitivity after L-NAME exposure. These animals also do not accumulate memory T cells in the kidney and seem to be protected against the development of albuminuria reflecting preserved glomerular function. These effects are likely because of the absence of injurious cytokines in mice lacking CD70, and indeed were mimicked in IFN-γ–deficient mice.

We also used a model of high-dose/low-dose ang II as a means to study a potential role of immunologic memory in WT and CD70−/− mice. These studies confirmed that there is an immunologic memory response to a second dose of ang II that is normally subpressor, and that this response is absent in mice that are unable to generate TEM cells. It is of interest that CD70−/− mice develop modest hypertension during the initial 2-week infusion of high-dose ang II, and during this time exhibited an increase in primary effector T cells.

Figure 5. Bone marrow effector memory T cells exhibit salt sensitivity. Mice received the Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME)/high-salt protocol as in Figure 1. Representative flow cytometry dot plot showing CD4+ and CD8+ effector memory (TEM) cells in the bone marrow (A). Summary data of the absolute numbers of indicated T-cell types at baseline or in response to L-NAME, high salt, and L-NAME/high-salt challenges in CD4+ and CD8+ are shown in bone marrow (B and C). Experimental paradigm (D). Bone marrow TEM cells from donor CD45.2 mice fed either a normal diet or the L-NAME/high-salt protocol were adoptively transferred to recipient CD45.1 mice. Recipient mice were then fed either normal diet (E) or high-salt diet for 3 weeks. Representative plots of CD45.2 TEM cells from mice exposed to the L-NAME/high-salt protocol to recipient CD45.1 mice fed either normal or high salt are shown in E–H. Representative plots for adoptively transferred CD45.2 TEM cells in the bone marrow (F), kidney (G), and spleen (H) are shown. Examples are representative of 3 separate experiments. Data are expressed as means±SEM (n=5–9 per group). P values for the effect of L-NAME/high salt as calculated by 1-way ANOVA are shown. **P<0.01, ****P<0.0001.
and double-negative T cells in their kidneys. It is possible that these cells contribute to the initial high-dose response to ang II. In keeping with this, we previously showed that either Abatacept or genetic deletion of B7 ligands, which block co-stimulation of naive T cells, prevents the acute effect of ang II.27 Thus, taken with our current results, it is possible that the acute 2-week response to high-dose ang II depends on a different population of immune cells when compared with the recall response a much lower dose of this hormone.

It is likely that the renal accumulation of memory T cells that produce IFN-γ and IL-17A plays an important role in the development of salt sensitivity. We recently found that these cytokines modulate renal tubular sodium transporters, including the sodium chloride cotransporter, the sodium potassium chloride cotransporter, and the sodium hydrogen exchanger-3.3 Likewise, Garcia et al21 demonstrated a blunted hypertensive response to chronic aldosterone infusion in IFN-γ−/− mice compared with WT animals. Satou et al11 have shown that IFN-γ modulates production of angiotensinogen by proximal tubular cells. This leads to intrarenal production of ang II, which in turn activates Na+ transport in the distal nephron. In addition to these changes in renal sodium transport, it is likely that these cytokines promote frank renal injury. We observed less renal damage as reflected by albuminuria, neutrophil gelatinase–associated lipocalin expression, and fibrosis in mice lacking either CD70 or IFN-γ. In keeping with this, Marko et al20 showed that mice lacking the IFN-γ receptor are protected against tubulointerstitial damage, have lower neutrophil gelatinase–associated lipocalin mRNA and exhibited preserved glomerular filtration rates when given ang II.

Table 1. Memory T Cells in the Kidney After Normal Diet or L-NAME With Either 1 or 2 High-Salt Challenges

<table>
<thead>
<tr>
<th></th>
<th>Normal Diet</th>
<th>L-NAME+HS1</th>
<th>L-NAME+HS1+HS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8 Tem</td>
<td>2.4±0.6 (29.1±6)</td>
<td>8.4±1.5** (36.6±2.5)*</td>
<td>23.1±4***, ††† (68.8±1.9)***, †††</td>
</tr>
<tr>
<td>CD8 Tcm</td>
<td>0.5±0.14 (12.8±2.1)</td>
<td>1.5±0.8 (17.1±2.8)</td>
<td>5.3±0.8 ***, ††† (15.9±0.8)</td>
</tr>
<tr>
<td>CD4 Tem</td>
<td>4.7±0.8 (68.3±3.8)</td>
<td>12±1.3*** (67.6±3.8)</td>
<td>18±2.3*** (74.4±2.1)</td>
</tr>
<tr>
<td>CD4 Tcm</td>
<td>0.5±0.2 (8.5±1.7)</td>
<td>0.3±0.3 (8.1±1.4)</td>
<td>1.6±0.2†† (6.8±0.5)</td>
</tr>
</tbody>
</table>

Data are mean±SEM×10^3 (n=5–20 per group). Parentheses denote the percent of total CD8+ or CD4+ T cells for the various populations. P values for the effect of N-ω-nitro-L-arginine methyl ester hydrochloride (L-NAME)/high salt as calculated by 1-way ANOVA are shown. HS1 and HS2, respectively, refer to the first and second high-salt challenges. HS indicates high salt; T cm, central memory; and Tem, effector memory.

*P<0.05 vs ND, **P<0.01 vs ND, ***P<0.001 vs ND, ****P<0.0001 vs ND.
†P<0.01 vs HS1, ††P<0.001 vs HS1, †††P<0.0001 vs HS1.

Table 2. Memory T Cells in the Bone Marrow After Normal Diet or L-NAME With Either 1 or 2 High-Salt Challenges

<table>
<thead>
<tr>
<th></th>
<th>Normal Diet</th>
<th>L-NAME+HS1</th>
<th>L-NAME+HS1+HS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8 Tem</td>
<td>0.6±0.1 (10±0.6)</td>
<td>1.4±0.2*** (19.8±0.9)***</td>
<td>1.0±0.01† (19.8±1.0)**</td>
</tr>
<tr>
<td>CD8 Tcm</td>
<td>2.4±0.27 (50±1.4)</td>
<td>3.7±0.4* (56±1.5)</td>
<td>2.9±0.1 (58.7±1.3)**</td>
</tr>
<tr>
<td>CD4 Tem</td>
<td>1.5±0.4 (48.3±3.9)</td>
<td>2.6±0.7*** (64.3±1.2)**</td>
<td>1.7±0.2 (62.9±1.0)*</td>
</tr>
<tr>
<td>CD4 Tcm</td>
<td>0.4±0.04 (34.4±1.4)</td>
<td>0.4±0.2 (26.5±1.0)***</td>
<td>0.14±0.01 (5.3±0.5)***, †††</td>
</tr>
</tbody>
</table>

Data are mean±SEM×10^3 (n=5–20 per group). Parentheses denote the percent of total CD8+ or CD4+ T cells for the various populations. P values for the effect of N-ω-nitro-L-arginine methyl ester hydrochloride (L-NAME)/high salt as calculated by 1-way ANOVA are shown. HS1 and HS2, respectively, refer to the first and second high-salt challenges. HS indicates high salt; T cm, central memory; and Tem, effector memory.

*P<0.05 vs ND, **P<0.01 vs ND, ***P<0.001 vs ND, ****P<0.0001 vs ND.
†P<0.01 vs HS1, ††P<0.001 vs HS1, †††P<0.0001.
salt–sensitive rats, T cells accumulate in perivascular regions and near glomeruli and are associated with renal fibrosis. We previously demonstrated that IL-17A stimulates collagen production by murine fibroblasts in a p38 mean arterial pressure kinase–dependent fashion, perhaps explaining how T EM cells that produce this cytokine might promote renal collagen as observed in Figure 4. Thus, the production of IFN-γ and IL-17A by memory CD4+ and CD8+ T cells likely mediates renal dysfunction, renal damage, and ultimately hypertension.

This study further supports the concept that the kidney is an important site of immune activation in hypertension. Recently, we showed that renal denervation prevents formation of isoketal adducts in dendritic cells not only in the kidney but also in the spleen during ang II–induced hypertension. We also showed that renal denervation prevents accumulation of memory cells in the kidney and reduces inflammatory cells in vessels outside of the kidney. Taken together with our current results, these findings suggest that cells activated in the kidney can migrate to other sites, such as the spleen, vasculature, and the bone marrow to mediate systemic inflammation. Findings such as these might help to explain the frequency of vascular disease among patients with chronic kidney disease.

An interesting observation in this study is that T EM cells accumulate in the bone marrow of mice with hypertension. Likewise, adoptively transferred T EM cells from the bone marrow of L-NAME/high-salt–treated mice accumulated in the bone marrow of recipient mice, and expanded on salt feeding of the recipients. This result is compatible with the recent recognition that TEM cells can reside in the bone marrow in a quiescent state for prolonged periods and can be reactivated by a repeated antigenic challenge. It is interesting to note that several recent lines of evidence support the concept that bone marrow cells contribute to hypertension. As examples, we demonstrated that transplant of bone marrow from hypertension-prone LNK−/− mice markedly enhances hypertension in recipient WT mice. Likewise, Santisteban et al recently showed that bone marrow transplantation from normal rats to spontaneously hypertensive rats reduces hypertension in the latter and attenuates both peripheral and central nervous system inflammation.

A caveat to our observations is that the number of T cells detected by flow cytometry represent the recovered cells.
and are unlikely a precise estimate of the cells present in the kidney, vessels, or bone marrow in vivo. These experiments involve tissue homogenization, gradient centrifugation, and exclusion of dead cells and selection of single cells by flow cytometry. The latter 2 steps are necessary to avoid artificial identification of cells. Thus, there is almost certainly loss of a substantial number of immune cells before a final estimate is made. We therefore caution that although our reported ratios of naive and memory cells and the relative differences in various hypertensive states are correct, the precise number of cells in various tissues are likely much higher than reported.

Our findings likely have clinical relevance. The recent Systolic Blood Pressure Intervention Trial (SPRINT) showed that strict blood pressure control is superior to modest blood pressure lowering.35 Long-lived hypertension-specific TEM cells in the bone marrow and secondary lymphoid organs could place humans at risk for enhanced blood pressure elevation and renal damage in response to stimuli-like salt, stress, or ang II for many years after an initial challenge. Thus, rigorous treatment of hypertension and efforts to prevent these repeated hypertensive exposures might be necessary to prevent reactivation of these cells and limit their injurious effects. Likewise it is known that transient hypertension during pregnancy places women at risk for cardiovascular events later in life via mechanisms that are poorly understood.54,35 It is interesting to speculate that such an insult could lead to formation of long-lived TEM cells that can persist for decades and promote vascular and renal disease on reactivation.

Sources of Funding
This work is supported by National Institutes of Health grants R01HL039006, P01HL058000, P01HL095070, P01GM015431, R01HL108701, R01HL105294, VITA award HSHN2628201400010C and the Strategically Focused Research Network Award from the American Heart Association. Dr Itani was funded by the T32 GM007569 and is the recipient of a National Institute of Health Ruth L. Kirschstein Individual National Research Service Award (1F32HL124972-01).

Disclosures
None.

References


CD70 Exacerbates Blood Pressure Elevation and Renal Damage in Response to Repeated Hypertensive Stimuli


_Circ Res._ 2016;118:1233-1243; originally published online March 17, 2016;
doi: 10.1161/CIRCRESAHA.115.308111

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
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/118/8/1233

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/03/17/CIRCRESAHA.115.308111.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

CD70 Exacerbates Blood Pressure Elevation and Renal Damage in Response to Repeated Hypertensive Stimuli

Hana A. Itani¹, Liang Xiao¹, Mohamed A. Saleh¹,³, Jing Wu¹, Mark A. Pilkinton², Bethany L. Dale¹, Natalia R. Barbaro⁴, Jason D. Foss¹, Annet Kirabo¹, Kim R. Montaniel¹, Allison E. Norlander¹, Wei Chen¹, Ryosuke Sato⁵, L. Gabriel Navar⁵, Simon A. Mallal², Meena S. Madhur¹, Kenneth E. Bernstein⁶, and David G. Harrison¹

Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA¹. Vanderbilt University Medical Center, Division of Infectious Diseases². Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.³ Department of Pharmacology, Faculty of Medical Sciences, Laboratory of Cardiovascular Pharmacology, University of Campinas, Campinas, Sao Paulo, Brazil.⁴ Department of Physiology and Hypertension and Renal Center, School of Medicine, Tulane University, New Orleans, LA⁵, Department of Biomedical Sciences and Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, California, USA.⁶

Running Head: T cell memory in hypertension

Address for Correspondence:

David G. Harrison, M.D.
Betty and Jack Baily Professor of Medicine and Pharmacology
Director of Clinical Pharmacology
Room 536 Robinson Research Building
Vanderbilt University
Nashville, TN 37232-6602
Telephone: 615-875-3049
Fax: 615-875-3049
email: david.g.harrison@vanderbilt.edu
METHODS

Animals and blood pressure measurement: Wild type, and interferon-gamma deficient mice (IFN-γ−/−) on a C57Bl/6J background were purchased from Jackson Laboratories (Bar Harbor, Maine). CD70-deficient mice (CD70−/−) on a C57Bl/6J background were obtained from Dr. Ross Kedl (University of Colorado). Mice were provided regular chow and water ad libitum. At 12 weeks of age, male mice were randomly selected for treatment. The animals initially received L-NAME (0.5mg/ml, Abcam 120136) in the drinking water for two weeks. LNAME was then stopped and the mice were allowed a two-week washout period, and then fed a high salt diet (4% NaCl, Teklad TD.92034) for three weeks. Other mice received a normal diet, two-weeks of L-NAME, or three weeks of high salt diet as controls. Blood pressure was measured invasively using radio-telemetry as previously described.1, 2 After telemetry implantation, mice were allowed to recover for 10 days prior to starting L-NAME/high salt protocol. In another model, mice received infusion of ang II (490 ng/kg/min) via osmotic minipumps for two weeks, followed by a two-week washout and then a second infusion of angiotensin II at 140 ng/kg/min.

For adoptive transfer experiments, donor CD45.2 mice underwent the L-NAME/high salt protocol described above and TEM cells were isolated from the bone marrow using cell sorting. Approximately 0.5 X 10^6 cells were then injected retro-orbitally into CD45.1 recipients. One half of these mice were then fed a 4% NaCl diet for the ensuing 3 weeks and the other half remained on normal mouse chow. As an additional control, TEM cells from the bone marrow of CD45.2 mice fed a normal diet were transferred to CD45.1 recipients and these recipients were fed a high salt diet for 3 weeks.

Mice were sacrificed at the end of all experiments by CO2 inhalation. All animal procedures were approved by Vanderbilt University Institutional Animal Care and Use Committee (IACUC), and mice were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals.

Flow cytometry: Single cell suspensions of kidneys were prepared as follows. Briefly, kidneys were mechanically dissociated using a single gentleMACS C tube dissociator system (Miltenyi) followed by incubation at 37°C for 20 min with collagenase D (2 mg/ml) and DNAse I (100 µg/ml) in RPMI 1640 medium with 5% FBS. Kidney homogenates were filtered through a 70 mm cell strainer. The resultant cell suspensions were subjected to Percoll gradient centrifugation as described previously,3, 4 and the enriched cells were washed and stained LIVE/DEAD® Fixable Violet dead cell stain (Invitrogen) and the following antibodies: Brilliant Violet 510 (BV510)-conjugated anti-CD45 antibody (BioLegend), peridinin chlorophyll protein-cyanin-5.5 (PerCP-Cy5.5)-conjugated anti-CD3 antibody (BioLegend), phycoerythrin-cyanin-7(PE-Cy7)-conjugated anti-CD8 antibody (BioLegend), allophycocyanin-Hilite-7 (APC-H7)-conjugated anti-CD4 antibody (BD Biosciences), APC-conjugated anti-CD44 antibody (BD Biosciences), PE-conjugated anti-CD62L antibody (BD biosciences), BV510-conjugated anti-LY-6C antibody (BD Biosciences), and FITC conjugated anti-Ki-67 antibody (eBioscience). Flow cytometry was performed on a BD FACS Canto II™ system and data analysis was performed using BD FACSDiva software (BD Biosciences). Gates were set using fluorescence minus one (FMO) controls. All lymphocyte subpopulations (CD4+, CD8+) were quantified within the CD45+CD3+ gate. The absolute number of infiltrating cells of each type was calculated by dividing the number of each population by the number of live cells obtained during flow cytometry and then multiplying by the total number of live cells counted on a
hemocytometer before staining (using trypan blue exclusion or counting beads). Results were expressed as number of cells per kidney. Intracellular staining for IL-17A or IFN-γ was performed as previously described. Briefly, 1x10^6 kidney cells were resuspended in RPMI medium supplemented with 5% FBS and stimulated with 2 µl of BD Leukocyte Activation Cocktail (ionomycin and phorbol myristic acetate (PMA) along with the golgi inhibitor, brefeldin A) at 37°C for 5 hours. Surface staining was performed as described above followed by intracellular staining using Fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ antibody (eBioscience) or PE conjugated anti-IL-17A as previously described. For studies of bone marrow cells, tibias and femurs of mice were flushed using the RPMI media and cells stained as above.

**Measurements of renal injury:** Neutrophil gelatinase-associated lipocalin (NGAL) mRNA was assessed by quantitative real-time PCR and normalized to GAPDH mRNA. Urinary albumin from 24-hour urine samples was determined with ELISA kits from Exocell. At the end of the L-NAME/high salt protocol, mice were placed in metabolic cages for 24 hours for acclimatization followed by 24 hours for urine collection. All concentrations were multiplied by total urine volume to obtain the daily excretion rate.

**Statistics:** Data are expressed as mean ± standard error of the mean. Blood pressures were analyzed by ANOVA for repeated measures. For comparisons of experiments involving a 2x2 design two-way ANOVA was used. When individual comparisons were made within this 2x2 design, Newman-Keuls or Holm-Sidak post-hoc tests were employed. When variances between groups were unequal, a Mann Whitney comparison followed by a Bonferroni correction was used.
Online Figure I: Comparison of various populations of memory T cells in the kidney (A, B) aorta (C and D) and spleen (E and F). $T_{RM}$ = resident memory T cells, $T_{EM}$ = Effector memory T cells, $T_{CM}$ = Central memory T cells. N = 5-8. Data were analyzed by ANOVA. ** < 0.01, *** <0.001.
Online Figure II: Effect on L-NAME and L-NAME/high salt on DC isoketal-adduct formation and heart rate variability. (A) Splenic DCs were stained for isoketal adducts and analyzed by flow cytometry. (B) Telemetry recordings of blood pressure were analyzed for the ratio of low frequency to high frequency heart rate variability. N = 4 – 11. Data were analyzed by ANOVA. * < 0.05. ** < 0.01, *** < 0.001.
Online Figure III: Effect of L-NAME/high salt on urinary angiotensinogen and creatinine excretion. (A) Urinary concentrations of angiotensinogen (UAGT) and (B) Creatinine (UCre) were measured by ELISA among the three groups of WT, CD70−/−, and IFN−γ−/− mice (n=7 per group). Data are expressed as means ± SEM.
Online Figure IV: mRNA expression levels of interleukin 15 and 7 within the kidney and bone marrow of mice exposed to normal diet or the L-NAME/high salt protocol. N = 4-9.
Online Figure V: Effector memory T cells in the bone marrow. Panel A compares effector (TEM) and resident (TRM) cells in the bone marrow of mice that received either a normal diet or L-NAME/high salt protocol. Panel B shows bone marrow Ly-6C+ TEM cells. Representative flow cytometry dot plots (C) and mean data (D) of bone marrow (BM) TEM cells stained for Ki-67. n= 5 -8 for each group. * < 0.05. ** < 0.01, **** <0.0001.
Online Figure VI: Effect of two-week ang II infusion on blood pressure and renal T cell infiltration. WT and CD70\(^{-/-}\) mice were infused with either angiotensin II (490 ng/kg/min) or vehicle for two weeks. The accumulation of renal CD4\(^{+}\) and CD8\(^{+}\) TEM cells and primary effector T cells is shown in panels A and B. Panel C shows presence of double negative (DN) cells in the kidneys of WT and CD70\(^{-/-}\) mice. n= 5-8 for each. Groups were compared using a Mann-Whitney non-parametric analysis with a Bonferroni correction for multiple comparisons. * < 0.05.
Online Figure VII: Proposed model for memory T cell formation in hypertension. Hypertensive stimuli such as L-NAME/high salt or angiotensin II promote formation of neoantigens that are presented by DCs to naïve T cells. The latter proliferate and form CD44\textsuperscript{high} effector memory T cells that accumulate in the kidney and produce cytokines such as IL-17A and IFN-\(\gamma\), which alter renal sodium and volume reabsorption and promote hypertension. Long-lived effector memory T cells also reside in the bone marrow and likely can respond to additional hypertensive challenges.
REFERENCES


