T-Cell Mineralocorticoid Receptor Controls Blood Pressure by Regulating Interferon-Gamma

Xue-Nan Sun,* Chao Li,* Yuan Liu, Lin-Juan Du, Meng-Ru Zeng, Xiao-Jun Zheng, Wu-Chang Zhang, Yan Liu, Mingjiang Zhu, Deping Kong, Li Zhou, Limin Lu, Zhu-Xia Shen, Yi Yi, Lili Du, Mu Qin, Xu Liu, Zichun Hua, Shuyang Sun, Huiyong Yin, Bin Zhou, Ying Yu, Zhiyuan Zhang, Sheng-Zhong Duan

Rationale: Hypertension remains to be a global public health burden and demands novel intervention strategies such as targeting T cells and T-cell–derived cytokines. Mineralocorticoid receptor (MR) antagonists have been clinically used to treat hypertension. However, the function of T-cell MR in blood pressure (BP) regulation has not been elucidated.

Objective: We aim to determine the role of T-cell MR in BP regulation and to explore the mechanism.

Methods and Results: Using T-cell MR knockout mouse in combination with angiotensin II–induced hypertensive mouse model, we demonstrated that MR deficiency in T cells strikingly decreased both systolic and diastolic BP and attenuated renal and vascular damage. Flow cytometric analysis showed that T-cell MR knockout mitigated angiotensin II–induced accumulation of interferon-gamma (IFN-γ)–producing T cells, particularly CD8+ population, in both kidneys and aortas. Similarly, eplerenone attenuated angiotensin II–induced elevation of BP and accumulation of IFN-γ–producing T cells in wild-type mice. In cultured CD8+ T cells, T-cell MR knockout suppressed IFN-γ expression whereas T-cell MR overexpression and aldosterone both enhanced IFN-γ expression. At the molecular level, MR interacted with NFAT1 (nuclear factor of activated T-cells 1) and activator protein-1 in T cells. Finally, T-cell MR overexpressing mice manifested more elevated BP compared with control mice after angiotensin II infusion and such difference was abolished by IFN-γ–neutralizing antibodies.

Conclusions: MR may interact with NFAT1 and activator protein-1 to control IFN-γ in T cells and to regulate target organ damage and ultimately BP. Targeting MR in T cells specifically may be an effective novel approach for hypertension treatment. (Circ Res. 2017;120:1584-1597. DOI: 10.1161/CIRCRESAHA.116.310480.)

Key Words: hypertension ■ inflammation ■ interferon-gamma ■ mineralocorticoid receptor ■ T-cell

Hypertension continues to severely endanger public health.1,2 Prolonged elevation of blood pressure (BP) leads to damage of target organs such as kidneys, blood vessels, and the heart, representing a major risk factor for cardiovascular diseases. Stringent control of BP, on the contrary, lowers cardiovascular events and death.1 However, BP is poorly controlled in a large number of hypertensive individuals and new approaches are in urgent need.3

The importance of T cells and T-cell–derived cytokines in hypertension has been recognized in the past decade.3 Mice lacking T cells and B cells are resistant to hypertension and vascular dysfunction induced by angiotensin II (AngII) or deoxycorticosterone acetate/salt, whereas replenishment of T cells, but not B cells, restores the hypertension phenotypes.4 Later data suggest that functional T cells are required for the pathogenesis of hypertension. Blockade or deletion of CD80 and CD86, the costimulatory molecules required for T-cell
activation, attenuates AngII- or deoxycorticosterone acetate/salt-induced hypertension and vascular damage in mice.\(^7\) Deletion of CD247, a component of the T-cell receptor complex, blunts BP elevation and renal damage in Dahl salt-sensitive rats.\(^8\) Moreover, CD8 deficiency attenuates AngII-induced hypertension and renal and vascular damage in mice.\(^9\) On activation and infiltration into target organs, T cells secret cytokines such as interferon-gamma (IFN-\(\gamma\)), interleukin 17A, and tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) that may orchestrate injuries to these organs and contribute to hypertension. Therefore, regulation of T cells and cytokines derived from these cells may be a feasible strategy for intervention of hypertension.

Mineralocorticoid receptor (MR) is an important drug target in the cardiovascular system and MR antagonists have been used to treat hypertension.\(^10,11\) Particularly, MR antagonists (spironolactone and eplerenone) are highly effective for patients with primary hypertension or resistant hypertension.\(^12\) MR is expressed at high level in renal epithelial cells and MR antagonists are also diuretics.\(^10\) However, clinical data indicate that the antihypertensive effects of MR antagonists are not primarily attributable to their impacts on electrolyte and fluid homeostasis.\(^13\) It is essential to delineate the underlying mechanisms, which may lead to better usage of currently available MR antagonists and facilitate the design of new agents to target MR and treat hypertension.

Recent studies have begun to unveil the cellular and molecular mechanisms how MR functions in BP regulation although they are still not completely deciphered. MR deficiency in vascular smooth muscle cells lowers BP in aged or AngII-challenged mice, likely through regulation on vascular oxidative stress and L-type calcium channel Cav1.2 that in turn controls vascular myogenic tone and contraction.\(^14\) Overexpression of MR in endothelial cells promotes vascular contractile response and increases BP in mice,\(^15\) but deletion of MR in these cells do not affect BP.\(^16,17\) Myeloid MR deficiency either blocks uninephrectomy/deoxycorticosterone acetate/salt-induced hypertension\(^18\) or slightly increases BP in mice treated with NG-nitro-L-arginine methyl ester/AngII.\(^19\) However, the function of T-cell MR in BP regulation has not been explored.

In this study, we aimed to determine the role of T-cell MR in BP regulation and delineate the mechanisms. We first examined the impacts of T-cell MR deficiency on BP, using T-cell MR knockout (TMRKO) mice in combination with AngII-induced hypertension. Then, we analyzed the effects of TMRKO on AngII-induced renal and vascular damage as well as T-cell populations, particularly IFN-\(\gamma\)-producing T cells, in kidneys and blood vessels. Subsequently, we explored the possibility that MR regulated IFN-\(\gamma\) in T cells utilizing in vitro system. Finally, we further confirmed the role of MR/IFN-\(\gamma\) axis in BP regulation in vivo using T-cell MR overexpressing (TMROV) mice in conjunction with IFN-\(\gamma\)-neutralizing antibodies.

**Methods**

**Animals**

TMRKO mice were generated by crossing MR\(^{\text{floxed}}\) mice\(^20\) to CD4-Cre mice.\(^21\) MROV\(^{\text{floxed}}\) mice harboring floxed stop codon and mouse MR coding sequence driven by CMV (cytomegalovirus) enhancer/chicken \(\beta\)-actin promoter were generated by Shanghai Model Organisms Center, Inc (Shanghai, China). MROV\(^{\text{floxed}}\) mice were bred to CD4-Cre mice to obtain TMROV mice. Wild-type C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China).

All animal studies were approved by the Institutional Review and Ethics Board of Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine and the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.
BP Measurements
Minipumps (2004D; Alzet, Cupertino, CA) were implanted subcutaneously in 11- to 12-week-old mice to deliver AngII (750 ng/kg per minute) or vehicle (sodium chloride).24,25 For Tail-cuff BP measurements, mice were infused with AngII or vehicle for 2 weeks and BP was measured using BP-2000 Blood Pressure Analysis System (Visitech Systems, Napa Place Apex, NC).25 For radiotelemetry, pressure transmitters (PA-C10; Data Sciences International, New Brighton, MN) were embedded in mice as described previously,26 and minipumps were implanted 7 days later to infuse AngII or vehicle for 4 weeks. For IFN-γ-neutralizing experiments, AngII or vehicle was delivered through minipumps for 3 weeks; anti-mouse IFN-γ (BP0055, clone XMG1.2; Bio X Cell, West Lebanon, NH; 16.7 mg/kg per injection) was administrated intraperitoneally once every 3 days starting from 3 days before until the end of experiments.8

Histological Analyses
Paraformaldehyde-fixed tissue samples were embedded in paraffin and 7-µm sections were stained with hematoxylin and eosin or 0.1% picросirus red.24 For immunofluorescence staining, sections were treated for antigen retrieval, blocked with 5% normal goat serum, and then incubated with primary antibodies at 4°C overnight, followed by incubation with fluorochrome-conjugated secondary antibodies (A-11007; Life Technologies/Thermo Fisher Scientific, Bartlesville, OK) at 37°C for 1 hour. Finally, the slides were mounted with antifade reagent containing DAPI (4,6-diamidino-2-phenylindole; P36941; Life Technologies). The following primary antibodies were used: Mac2 (14-5301-81; eBioscience, San Diego, CA), CD4 (550280; BD Biosciences, San Jose, CA), and CD8 (558733; BD Biosciences).

Measurement of Albuminuria
Mice were placed in metabolic cages, and urine was collected for 12 hours. Albumin levels in the 12-hour urine samples were measured using ELISA kit (Exocell Inc, Philadelphia, PA) according to the manufacturer’s protocol.

Analysis of Vasorelaxation
Mesenteric arteries were dissected and mounted on a myograph system (Danish MyoTechnology, Aarhus). The vessels were first precontracted with phenylephrine. Acetylcholine dose–response curves were then performed, and the results were expressed as percentage of precontraction.24,26

Measurement of Tissue Superoxide
Renal and aortic superoxide was measured by dihydroethidium and high-performance liquid chromatography assay as described previously.27 Briefly, renal tissues (30 mg) were finely ground in liquid nitrogen, transferred into Krebs-HEPES buffer containing 50 µM dihydroethidium, and incubated at 37°C for 30 minutes. Subsequently, tissue suspension was homogenized in 300-µL cold methanol. After determination of protein concentration, the homogenate was centrifuged and the supernatant was analyzed using an high-performance liquid chromatography instrument (e2695; Waters Corporation, Milford, MA). dihydroethidium, 2-hydroxyethidium, and ethidium were separated using a C-18 reverse-phase column (250×4.6 mm, 5 µm; Phenomenex, Torrance, CA) at a flow rate of 1 mL/min. Ethidium and 2-hydroxyethidium were detected with a fluorescence detector (emission wavelength: 580 nm; excitation wavelength: 480 nm). The content of 2-hydroxyethidium was normalized to protein concentration. For aortas, five 2-mm thoracic aortic rings were used for the measurements.

Quantitative Real-Time-Polymerase Chain Reaction
Total RNA was extracted using TRizol reagent (15596026; Life Technologies), and reverse transcription was performed using PrimeScriptTM RT reagent Kit (RR037A; Takara, Shiga, Japan). Quantitative real-time polymerase chain reaction (RT-PCR) was performed on an ABI7900HT (Applied Biosystems/Thermo Fisher Scientific, Bartlesville, OK) using SYBR Green Mix (Applied Biosystems). Relative expression of each gene was determined by normalizing to GAPDH for aorta and kidney samples, and ml19 or 18s for T cells.

Flow Cytometry
For kidneys and aortas, tissues were minced and placed in HBSS containing 1.5 mg/mL Collagenase II ( Worthington, Lakewood, NJ), 1.5 mg/mL Collagenase IV ( Worthington), and 60 U/mL DNase I ( AppliChem, Lochem, Darmstadt). Samples were first mechanically dissociated using gentleMACS Dissociator system (Miltenyi Biotec, Bergisch Gladbach, Germany) and then filtered through 70-µm cell strainer ( BD Biosciences) to obtain single cell suspensions. Kidney samples were subjected to Percoll (17089102; GE, Marlborough, MA) gradient centrifugation before flow cytometric analysis.28 Spleens were directly crushed on cell strainers to obtain single cell suspensions. Red blood cells were lysed before flow cytometric staining. 7-Aminoactinomycin D (559925; BD Biosciences) was added into cell suspensions before flow cytometry detection. Samples were analyzed using BD FACSAria II or ACEA NovoCyte ( ACEA Bioscience, Inc, San Diego, CA).

The following antibodies were used: Fc block (553142, BD Biosciences), CD45-PE-Cy7 (25-0451-81, eBioscience), CD3-PE (12-0031-81, eBioscience), CD3-APC (56-0032-80, eBioscience), CD4-FITC (11-0043-82, eBioscience), CD4-eFluor450 (48-0042-80, eBioscience), CD8-APC (17-0081-80, eBioscience), γ-DNase I (73-0731-81, eBioscience), TNF-α-eFluor450 (48-7321-80, eBioscience), CD11b-FITC (11-0112-82, eBioscience), Ly6G-APC (560599, BD Biosciences), C-X-C motif Chemokine (CXCR3)-eFluor450 (48-4801-80, eBioscience), CD44-APC (17-0441-82, eBioscience), and CD62L-FITC (11-0621-82, eBioscience).

Cell Isolation, Culture, and Treatment
Splenic CD8+ T cells were isolated using negative selection kit (18953A; Stem Cell, Vancouver, Canada) and cultured in RPMI 1640 (Life Technologies) containing 10% fetal bovine serum (04-001-1ACS; Biological Industries, Kibbutz Beit-Haemek, Israel) and 2% penicillin–streptomycin. T cells were activated by purified anti-CD3 antibodies (2 µg/mL, 14-0031-85; eBioscience) that were coated on plates. Jurkat cells (clone E6-1; ATCC, Manassas, VA) were cultured in RPMI 1640 (Life Technologies) containing 10% fetal bovine serum (Biological Industries) and 1% penicillin–streptomycin.

Lentivirus Preparation and Infection
Mouse MR-flag was subcloned to pHAGE plasmid and then used to transfect 293FT cells together with lentivirus packaging plasmids using Lipofectamine 2000 (Life Technologies). Lentiviruses were harvested, filtered through 0.22-µm filters, and condensed by ultracentrifugation. Splenic CD8+ T cells and Jurkat cells were infected with lentivirus before further treatments.

Chromatin Immunoprecipitation Assay and Coimmunoprecipitation
Chromatin Immunoprecipitation experiments were performed using EZ-ChIP kit (17–371; Millipore, Darmstadt, Germany) according to the manufacturer’s protocol. Mouse anti-FLAG antibodies (M208; Abmart, Berkeley Heights, NJ; or F3165, Sigma) were used for immunoprecipitation, and normal mouse IgG was used as a negative control. DNA was isolated for QRT-PCR analysis. Primer sequences for amplifying the IFN-γ promoter are listed in Online Table I.

For coimmunoprecipitation, Jurkat cells were lysed in IP lysis buffer containing 50 mMol/L Tris HCl pH 7.4, 150 mMol/L NaCl, 1 mMol/L EDTA, 1% NP-40, and protease inhibitor cocktail (P8340; Sigma). One milligram of the total lysate was incubated with anti-Flag antibodies and protein A/G beads (sc-2003; Santa Cruz Biotechnology Inc, Santa Cruz, CA) overnight. The immunocomplex was subjected to Western blotting analysis with antibodies against cFos (Sc-52; Santa Cruz Biotechnology Inc), Jun (9165; Cell Signaling Technology, Beverly, MA), and NFAT1 (nuclear factor of activated T-cells 1; Santa Cruz Biotechnology Inc). 2% of total protein was used as input.

Statistical Analysis
Data were presented as mean±SEM and analyzed using Prism (Graph Pad Software). Multiple comparisons were tested by 2-way ANOVA with Bonferroni post-tests. Pair-wise comparisons were tested by Student t test. Results were considered significantly different if P≤0.05.
Results

T-Cell MR Deficiency Attenuates Angiotensin II–Induced Hypertension in Mice

Previous data have suggested that T cells possess MR. Flow cytometry analysis detected MR expression in T cells (Online Figure 1A). Recombination of MR gene was detected in spleen, thymus, lymph node, and isolated T cells, but not in other tissues such as kidney, heart, aorta, and bone marrow of TMRKO mice (Online Figure 1B); QRT-PCR analysis and Western blotting revealed efficient deletion of MR in T cells isolated from TMRKO mice (Online Figure IC and ID). Flow cytometry results showed that TMRKO did not affect T-cell development in thymus (Online Figure IE and IF). There was no difference in the percentage of CD4+ or CD8+ T cells in spleen or peripheral blood between littermate control (LC) and TMRKO mice (Online Figure IG through II).

To determine the roles of T-cell MR in BP regulation, we induced hypertension in LC and TMRKO mice by AngII infusion and monitored BP using both noninvasive tail cuff and radiotelemetry. Tail-cuff BP measurements revealed that TMRKO mice had slightly lower systolic and diastolic BP than their LC counterparts at baseline without AngII infusion (Figure 1A and 1B). Much bigger difference in both systolic BP and diastolic BP were observed between LC and TMRKO mice after 2 weeks of AngII infusion (Figure 1A and 1B). Radiotelemetry BP monitoring confirmed markedly lower BP in TMRKO mice than in LC mice in the whole process from pretreatment (baseline) to 4 weeks after AngII infusion (Figure 1C and 1D). Particularly, TMRKO mice manifested drastically blunted BP elevation compared with LC mice during AngII infusion, with systolic BP averagely 46 mmHg lower and diastolic BP 40 mmHg lower (Figure 1C and 1D). There was no difference in the heart rate between LC and TMRKO mice (Online Figure II).

T-Cell MR Deficiency Attenuates AngII-Induced Renal Damage

Renal dysfunction is a major manifestation of hypertension and conversely a significant contributor to BP elevation in both clinical and experimental settings. We therefore investigated the effects of T-cell MR deficiency on renal damage in the model of AngII-induced hypertension. We first examined structural changes. Hematoxylin and eosin staining illustrated that 4 weeks of AngII infusion caused glomerular hypertrophy, which was completely prevented in TMRKO mice (Figure 2A and 2B). Picrosirius red staining demonstrated less accumulation of CD4+ or CD8+ T cells in spleen, thymus, lymph node, and isolated T cells, but not in other tissues such as kidney, heart, aorta, and bone marrow of TMRKO mice (Online Figure II). QRT-PCR results demonstrated that AngII-induced expression of inflammatory genes including vascular cell adhesion molecule 1 and monocyte chemotactic protein 1, both of which was attenuated in kidneys of TMRKO mice (Figure 2I). AngII caused an increase of superoxide in kidneys as revealed by high-performance liquid chromatography analysis, and this was significantly reduced in TMRKO mice (Figure 2J and 2K). Direct dihydrothidium staining also suggested much less superoxide in kidney samples of TMRKO mice compared with LC mice (Online Figure III).

T-Cell MR Deficiency Mitigates AngII-Induced Accumulation of IFN-γ–Producing T Cells in Kidneys

Infiltration of T cells into kidneys has been observed in hypertension and considered as an important contributor to renal damage. The impacts of T-cell MR deficiency on T-cell accumulation in kidneys were examined using flow cytometry (gating strategy shown in Online Figure IV). Both CD4+ T cells and CD8+ T cells were substantially increased in kidneys by AngII infusion, and the increase was notably attenuated in TMRKO mice (Figure 3A and 3B). As a result, TMRKO mice had fewer CD4+ T cells and CD8+ T cells in kidneys than LC mice (Figure 3A and 3B). Immunofluorescence staining also demonstrated less accumulation of CD4+ T cells and CD8+ T cells in glomeruli of TMRKO mice after AngII infusion (Online Figure V).

T-cell–derived IFN-γ has been shown to play crucial roles in BP regulation. We then explored how T-cell MR deficiency affected IFN-γ–producing T cells in kidneys. Flow cytometry analysis (gating strategy shown in Online Figure IV) illustrated that AngII increased the percentage and number of CD4+ IFN-γ+ T cells in kidneys and the upregulation was significantly mitigated in TMRKO mice compared with LC mice (Figure 3C and 3D). A profound increase of CD8+ IFN-γ+ T cells was induced by AngII in kidneys of LC mice (Figure 3E and 3F). Notably, this increase was almost completely blocked by T-cell MR deficiency and resultantly TMRKO mice had substantially less accumulation of CD8+ IFN-γ+ T cells in kidneys compared with LC mice after AngII infusion (Figure 3E and 3F). Therefore, T-cell MR deficiency may have influenced IFN-γ–producing T cells, particularly the CD8+ IFN-γ+ subpopulation, to affect renal damage in AngII-induced hypertension. Interestingly, flow cytometric analysis revealed that T-cell MR deficiency also attenuated the accumulation of CD4+ IFN-γ+ T cells and CD8+ IFN-γ+ T cells in the spleen after AngII infusion (Online Figure VI). We did not detect any difference in TNFα–producing T cells in kidneys between TMRKO mice and LC mice (Online Figure VII). Similar to the results in TMRKO mice, eplerenone significantly lowered BP and decreased CD8+ IFN-γ+ T cells in the kidney and spleen of AngII-infused mice (Online Figure VIII).

Effector memory T cells (T_em) are predominant sources of IFN-γ in kidneys under hypertensive stimuli. We further tested whether T-cell MR deficiency affected T_em. Flow
Figure 1. T-cell mineralocorticoid receptor (MR) deficiency attenuates angiotensin II (Ang II)–induced hypertension in mice. A and B, Noninvasive tail-cuff monitoring of systolic blood pressure (BP) (A) and diastolic BP (B) of littermate control (LC) and T-cell MR knockout (TMRKO) mice infused with vehicle (0.9% NaCl) or AngII for 2 wk (n=6–7). C and D, Telemetric monitoring of systolic BP (C) and diastolic BP (D) of LC and TMRKO mice infused with vehicle or AngII for 4 wk (n=7–9). Daytime (D) trough and nighttime (N) peak BP data were recorded for 3 d at baseline and during each week after AngII infusion. *P<0.05, **P<0.01, ***P<0.001. LC indicates littermate control; and TMRKO, T-cell MR knockout.
Figure 2. T-cell mineralocorticoid receptor (MR) deficiency attenuates angiotensin II (Ang II)–induced renal damage. A, Representative hematoxylin and eosin staining of kidney sections. Arrows indicate glomeruli. B, Quantification of glomerular areas (n=7). C, Representative picrosirius red staining of kidney sections. Fibrotic tissues stained red. D, Quantification of renal fibrotic areas (n=7). E, Assessment of 12-h urinary albumin excretion (n=4–5). F, QRT-polymerase chain reaction (PCR) analysis of genes associated with renal injury (n=7). G, Representative flow cytometric analysis of CD11b+F4/80+ macrophages in kidney samples. H, Quantification of CD11b+F4/80+ macrophages (n=4–5). I, QRT-PCR analysis of inflammatory gene expression in kidneys (n=7). J, Representative high-performance liquid chromatography tracings of 2-hydroxyethidium (2-HE+) in kidney samples. K, Quantification of relative 2-HE+ content (n=4–5). Mice were infused with vehicle or AngII for 4 wk. All scale bars, 50 μm. *P<0.05, **P<0.01, ***P<0.001. LC indicates littermate control; MCP, monocyte chemotactic protein; NGAL, neutrophil gelatinase-associated lipocalin; OPN, osteopontin; TMRKO, T-cell MR knockout; and VCAM, vascular cell adhesion molecule.
Cytometric analysis illustrated that TMRKO mice had significantly less CD4+ TEm (CD44high CD62Llow) and CD8+ TEM in kidneys compared with LC mice after AngII infusion (Online Figure IX). TMRKO decreased CD8+ TEM but not CD4+ TEM in the spleen (Online Figure IX).

Dihydroethidium staining suggested that IFN-γ increased superoxide generation in human kidney-2 cells and mouse bone marrow–derived macrophages (Online Figure X), whereas activated CD8+ T cells from TMRKO mice and those from LC mice generated comparable amounts of superoxide (Online Figure XI), suggesting that TMRKO affected superoxide accumulation through IFN-γ–mediated effects.

**T-Cell MR Deficiency Attenuates AngII-Induced Vascular Damage**

Hypertension also causes vascular damage that conversely contributes to elevation of BP. We therefore studied the effects of T-cell MR deficiency on vascular damage in the model of AngII-induced hypertension. We first examined structural changes. Hematoxylin and eosin staining illustrated that 4 weeks of AngII infusion caused hypertrophy of aortas, which was mitigated in TMRKO mice (Figure 4A and 4B). Picrosirius red staining demonstrated that AngII-induced fibrosis of aortas was significantly reduced in TMRKO mice compared with LC mice (Figure 4C and 4D). We then examined functional changes. AngII infusion impaired endothelium-dependent relaxation of mesenteric arteries responding to acetylcholine and T-cell MR deficiency evidently blunted such impairment (Figure 4E). As a result, mesenteric arteries of TMRKO mice exhibited significantly improved endothelium-dependent vasodilatation compared with those of LC mice after AngII infusion (Figure 4E).

Inflammation and oxidative stress also play important roles in mediating vascular damage. Immunofluorescence staining illustrated much less accumulation of Mac2-positive macrophages in the aortic wall of TMRKO mice compared with LC mice after AngII infusion (Figure 4F and 4G). Gene
Figure 4. T-cell mineralocorticoid receptor (MR) deficiency attenuates angiotensin II (Ang II)–induced vascular damage. A, Representative hematoxylin and eosin staining of aortic sections. B, Quantification of aortic wall thickness (n=6–8). C, Representative picrosirius red staining of aortic sections. Fibrotic tissues stained red. D, Quantification of aortic fibrotic areas (n=8). E, Acetylcholine dose–response curves of mesenteric arteries (n=6–7). F, Representative immunofluorescence staining of Mac2 in aortic sections. G, Quantification of Mac2-positive cells (n=5–6). H, QRT-polymerase chain reaction (PCR) analysis of CD68 gene expression in aortas (n=6–8). I, QRT-PCR analysis of RANTES (regulated upon activation normal T-cell expressed and secreted) and monocyte chemotactic protein (MCP)-1 gene expression in aortas (n=6–8). J, Representative high-performance liquid chromatography tracings of 2-hydroxyethidium (2-HE+) in aortic samples. K, Quantification of relative 2-HE+ content (n=4–5). Mice were infused with vehicle or AngII for 4 wk. All scale bars, 50 μm. *P<0.05, **P<0.01, ***P<0.001. LC indicates littermate control; and TMRKO, T-cell MR knockout.
expression of CD68 was markedly suppressed in aortas of TMRKO mice (Figure 4H). Expression of inflammatory genes including RANTES (regulated upon activation normal T-cell expressed and secreted) and monocyte chemotactic protein 1 was also decreased in aortas of TMRKO mice (Figure 4I). AngII caused increase of superoxide in the aortic wall as revealed by high-performance liquid chromatography analysis, and this was significantly reduced in TMRKO mice (Figure 4J and 4K). Direct dihydroethidium staining also suggested much less superoxide in aortic samples of TMRKO mice compared with LC mice (Online Figure XII).

**T-Cell MR Deficiency Mitigates AngII-Induced Accumulation of IFN-γ-Producing T Cells in Aortas**

Infiltration of T cells in the vasculature has also been observed in hypertension and contributes to vascular damage. We therefore examined the impacts of T-cell MR deficiency on the accumulation of T cells, particularly IFN-γ-producing T cells, in aortas using flow cytometry (gating strategy shown in Online Figure IV). Similar to the results we observed in the kidneys, the number of CD4+ T cells and that of CD8+ T cells were both significantly lower in thoracic aortas of TMRKO mice than in those of LC mice after AngII infusion (Figure 5A and 5B). TMRKO mice had notably fewer CD4+ IFN-γ+ T cells in thoracic aortas than LC mice after AngII infusion (Figure 5C and 5D). The number of CD8+ IFN-γ+ T cells in thoracic aortas was also significantly decreased in TMRKO mice and this was to a larger extent than the decrease of CD4+ IFN-γ+ T cells (Figure 5E and 5F).

Similar to its effects on renal cells, IFN-γ also increased superoxide generation in mouse vascular smooth muscle cells as illustrated by dihydroethidium staining (Online Figure XIII).

![Figure 5. T-cell mineralocorticoid receptor (MR) deficiency mitigates angiotensin II (Ang II)-induced accumulation of interferon-gamma (IFN-γ)-producing T cells in aortas. A, Representative flow cytometric analysis of T cells in thoracic aortas. B, Quantification of CD4+ T cells and CD8+ T cells (n=4; each 2 thoracic aortas were pooled as 1 sample). C, Representative flow cytometric analysis of CD4+ IFN-γ+ T cells in thoracic aortas. D, Quantification of CD4+ IFN-γ+ T cells (n=4). E, Representative flow cytometric analysis of CD8+ IFN-γ+ T cells in thoracic aortas. F, Quantification of CD8+ IFN-γ+ T cells (n=4). *P<0.05, **P<0.01. LC indicates littermate control; and TMRKO, T-cell MR knockout.](http://circres.ahajournals.org/doi/10.1161/CIRCRESAHA.117.310979)
Figure 6. Mineralocorticoid receptor (MR) regulates interferon-gamma (IFN-γ) expression in CD8+ T cell. A, Representative flow cytometric analysis of CD8+ IFN-γ+ subpopulation in splenic CD8+ T cells from littermate control (LC) or T-cell MR knockout (TMRKO) mice. The cells were either not stimulated (unstimulated) or stimulated with anti-CD3 antibodies for 48 h. B, Quantification of CD8+IFN-γ+ T cells exemplified in A. C, QRT-polymerase chain reaction (PCR) analysis of IFN-γ in CD8+ T cells from LC and TMRKO mice. D, Representative flow cytometric analysis of CD8+IFN-γ+ subpopulation in wild-type splenic CD8+ T cells infected with control lentivirus (Ctrl) or MR-Flag overexpressing lentivirus (MROV) for 48 h. E, Quantification of CD8+IFN-γ+ T cells exemplified in D. F, (Continued)
Figure 6 Continued. QRT-PCR analysis of IFN-γ in control or MROV CD8+ T cells. G, Chromatin immunoprecipitation assays of IFN-γ promoter using wild-type splenic CD8+ T cells infected with MR-Flag overexpressing lentivirus for 48 h. The cells were then stimulated with anti-CD3 antibodies for 36 h. H, Coimmunoprecipitation assays in Jurkat cells infected with MR-Flag overexpressing lentivirus. \( ^{\text{P}<0.05, \quad ^{\text{P}<0.01, \quad ^{\text{P}<0.001.}} \) NFAT1 indicates nuclear factor of activated T-cells 1; and ns, not significant.

MR Regulates IFN-γ Expression in T Cells

Our in vivo data suggested that T-cell MR deficiency decreased IFN-γ-producing T cells, particularly the CD8+ IFN-γ+ subpopulation, in kidneys and aortas in the setting of AngII-induced hypertension. Previous data have indicated that CD8+ T cells play a more important role than CD4+ T cells during AngII-induced hypertension.\(^{9}\) We next addressed whether MR directly regulated IFN-γ in CD8+ T cells. After stimulation with anti-CD3 antibodies, the percentage of IFN-γ+ cells was substantially lower in splenic CD8+ T cells from TMRKO mice than in those from LC mice (Figure 6A and 6B). Similar results were observed in renal CD8+ T cells (Online Figure XIV). QRT-PCR results further demonstrated downregulation of IFN-γ expression in splenic CD8+ T cells from TMRKO mice (Figure 6C). Conversely, overexpression of MR in wild-type splenic CD8+ T cells using MR-Flag lentivirus increased the percentage of IFN-γ+ subpopulation and gene expression of IFN-γ under the stimulation of anti-CD3 antibodies (Figure 6D through 6F). Similarly, renal CD8+ T cells from TMROV mice (Online Figure XVA and XB) had a larger IFN-γ+ subpopulation than those from corresponding LC mice (Online Figure XVC and XVD). Moreover, aldosterone, an endogenous agonist of MR, increased the percentage of IFN-γ+ cells and the expression of IFN-γ in wild-type splenic CD8+ T cells after stimulation with anti-CD3 antibodies (Online Figure XVI). Consistent with our in vivo data, neither deficiency nor overexpression of MR affected TNFα expression in splenic CD8+ T cells (Online Figure XVII).

We did not identify any MR response element in the promoter region of IFN-γ and therefore postulated that MR might interact with other transcription factors to regulate the expression of IFN-γ. NFAT1 and AP-1 are essential regulators of IFN-γ expression and are thus 2 major candidates.\(^{34-36}\) To test our hypothesis, wild-type splenic CD8+ T cells were infected with MR-overexpressing lentivirus and antibodies against Flag tag was used to perform chromatin immunoprecipitation assays. No enrichment of MR-Flag was detected at a site that bound to NFAT1 alone or Yin Yang 1/AP-1/2 (Figure 6G, primers 1 and 2). Instead, such enrichment was identified at 2 sites that bound to both NFAT1 and AP-1 (Figure 6G, primers 3 and 4). Coimmunoprecipitation assays demonstrated interaction between MR and NFAT1, as well as interaction between MR and cFos and cJun, the 2 subunits of AP-1 (Figure 6H). These results together suggested that MR bound to both NFAT1 and AP-1 to regulate IFN-γ expression in T cells.

T-Cell MR Overexpression Exacerbates AngII-Induced Hypertension Through IFN-γ in Mice

Finally, we directly tested whether IFN-γ was responsible for the impacts of MR on BP control in vivo. TMROV mice and corresponding LC mice were infused with AngII and treated with antibodies against mouse IFN-γ. Tail-cuff BP monitoring showed that TMROV mice had much higher systolic and diastolic BP than LC mice >3 weeks of AngII infusion (Figure 7A and 7B), consistent with the phenotype of lower BP we observed in the TMRKO mice. When anti–IFN-γ antibodies were administrated, BP was decreased in both LC and TMROV mice and the difference between the 2 genotypes was abolished (Figure 7A and 7B), indicating that the effects of T-cell MR overexpression on BP was dependent on the regulation of IFN-γ by MR in T cells.

Discussion

The cellular and molecular mechanisms how MR functions to regulate BP have remained incompletely understood although MR antagonists have been used to treat primary hypertension and resistant hypertension for a long time. Through the current study, we demonstrated that T-cell MR deficiency...
amplified and conversely T-cell MR overexpression exacerbated AngII-induced hypertension. Mechanistically, MR directly regulated IFN-γ in T cells, reflecting the effects of T-cell MR on accumulation of IFN-γ–producing T cells, particularly CD8+ T cells, on target organ damage, and ultimately on BP regulation.

Our data support that MR blockade in T cells may be a feasible strategy to treat hypertension. Our results suggested that the BP-lowering effects of MR blockade by eplerenone might be mediated by its impacts on T cells. More importantly, we showed that T-cell–specific MR deficiency was sufficient to lower both systolic and diastolic BP in a mouse model of AngII-induced hypertension. Moreover, mitigated renal and vascular damages were observed in TMRKO mice with AngII infusion. Conversely, T-cell–specific MR overexpression elevated BP in AngII-treated mice. It is plausible to target MR in T cells specifically using strategies such as chimeric major histocompatibility complex class II that binds to T-cell receptor and nanoparticles that target T cells. Such novel approaches may help to reduce side effects of global administration of MR antagonists and to increase their efficacy for hypertension treatment. Intriguingly, TMRKO decreased but TMROV did not affect BP at baseline, suggesting a permissive role of T-cell MR in the maintenance of BP.

CD8+ T cells may have played a more prominent role than CD4+ T cells in mediating the function of MR in hypertension. Several lines of evidence suggest that CD8+ T cells have more influence on BP regulation than CD4+ T cells. Deficiency of CD8, but not CD4, attenuates AngII-induced hypertension and target organ damage in mice. More CD8+ IFN-γ+ T cells, but not CD4+ IFN-γ+ T cells, have been detected in peripheral blood of hypertensive patients than normotensive control subjects. Finally, activated CD8+ T cells produce more IFN-γ than CD4+ T cells. Our results showed more drastic reduction of CD8+ IFN-γ+ T cells than CD4+ IFN-γ+ T cells in both kidneys and blood vessels of AngII-treated TMRKO mice, implying that CD8+ IFN-γ+ T cells were the primary subpopulation mediating the impacts of T-cell MR on BP regulation. Our data further suggested that these IFN-γ–producing cells affected by TMRKO were T EM in kidneys. It has been previously demonstrated that T cells migrate from spleen to target organs such as the kidney and aorta to cause organ damage and BP elevation. Therefore, the CD8+ IFN-γ+ T-cell population affected by TMRKO was presumably originated from the spleen.

We have identified an MR/IFN-γ signaling axis that mediates the effects of T cells in BP regulation. Although previous studies have suggested that MR may be involved in polarization toward T helper 17 cells and regulatory T cells and that dendritic cells may mediate MR’s impacts on T cells, the direct function of T-cell MR and the molecular mechanisms have been unclear. We reported here that MR directly regulated IFN-γ in T cells. Interestingly, this was through interactions with both NFAT1 and AP-1, 2 crucial transcription factors for controlling IFN-γ secretion in activated T cells. Neither NFAT1 nor AP-1 separately mediated the effects of MR, consistent with the cooperative nature of these 2 transcription factors in regulating functions of T cells. In line with the in vitro observations, much less IFN-γ–producing T cells accumulated in kidneys and blood vessels of TMRKO mice after AngII infusion, likely contributing to the suppressed inflammation and superoxide production, the attenuated target organ damage, and the lowered BP. Moreover, blockade of IFN-γ signaling with neutralizing antibodies abolished the effects of TMROV on BP elevation in mice, further supporting the importance of the MR/IFN-γ axis in BP regulation.

This study has provided evidence to substantiate the roles of T-cell–derived IFN-γ in BP regulation. Previous data showed secretion of IFN-γ, but not of TNFα or interleukin 17A, from CD8+ T cells was affected by AngII treatment in mice, suggesting the importance of IFN-γ in AngII-induced hypertension. However, different results were reported on the impacts of IFN-γ signaling on BP. IFN-γ knockout mice exhibit lower BP than control mice in a hypertension model induced by uninephrectomy/aldosterone/salt. In models of AngII-induced hypertension, IFN-γ knockout mice have either lower or similar BP compared with control mice. Different genetic background and different dose of AngII may be responsible for the discrepancy. Our results showed that TMRKO mice had decreased expression of IFN-γ in T cells and lower BP in response to AngII and that IFN-γ–neutralizing antibodies tempered hypertension of TMROV mice, supporting a prohypertensive role of T-cell–derived IFN-γ.

IFN-γ may mediate the influence of T-cell MR on hypertension through modulating inflammation and oxidative stress in target organs. T-cell–derived IFN-γ has been established as an inflammatory cytokine although its anti-inflammatory properties have also been realized. Under hypertensive stimuli, activated T cells, particularly CD8+ T cells, migrate into target organs and produce IFN-γ, which causes inflammation in kidneys and blood vessels. On the contrary, our data showed that IFN-γ induced superoxide generation in renal cells and vascular cells, indicating that activated T cells might increase the production of superoxide in kidneys and blood vessels by secretion of IFN-γ. TMRKO mice manifested reduced inflammation and oxidative stress in parallel with decreased accumulation of IFN-γ–producing T cells in both kidneys and blood vessels after AngII infusion. It is conceivable that T-cell–derived IFN-γ mediates the effects of T-cell MR on inflammation and oxidative stress in target organs, contributing to BP regulation.

Taken all together, T-cell MR plays essential roles in BP regulation and target organ damage during hypertension, likely through its regulation of IFN-γ in T cells, particularly CD8+ T cells. These data have unveiled novel functions of T-cell MR and provided mechanistic insights on how T-cell MR regulates BP. These findings support that targeting MR in T cells specifically is an appealing strategy to treat hypertension with more efficiency and less side effects.

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Disclosures
None.

References


T-Cell Mineralocorticoid Receptor Controls Blood Pressure by Regulating Interferon-Gamma

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Administration of eplerenone in mice
Eplerenone was mixed in regular rodent chow (2g/kg, SLAC Laboratory Animal Co., Shanghai, China) and administrated 3 days before implantation of AngII mini-pumps until the end of experiments to deliver a dosage of approximately 200mg/kg/day.

Cell culture
Human kidney-2 (HK-2) cells were cultured in DMEM (Life Technologies/Thermo Fisher Scientific, Bartlesville, Oklahoma) containing 20% fetal bovine serum and 1% penicillin-streptomycin.

Bone-marrow-derived macrophages (BMDMs) were induced from bone marrow cells as described before. Briefly, bone marrow cells were obtained from femur and tibia of C57BL/6 mice and cultured in media containing 30% L929-conditioned media and 10% heat-inactivated FBS for 7 days. BMDMs were used for experiments on day 8.

Vascular smooth muscle cells (VSMCs) were isolated from aortas with enzyme digestion method as previously described. VSMCs were maintained in DMEM containing 20% fetal bovine serum and 1% penicillin-streptomycin.

Renal CD8+ T cells were isolated using CD8a MicroBeads (130-049-401, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

For experiments with aldosterone treatment, regular FBS in culture medium were replaced by 10% Charcoal/Dextran-treated FBS (Hyclone, GE Healthcare Life Sciences, Pittsburgh, Pennsylvania). CD8+ T cells were incubated with aldosterone (1nM, A9477, Sigma, St. Louise, Missouri) for 24 hours before stimulation with anti-CD3 antibodies.

Histological analyses
For dihydroethidium (DHE) staining in tissues, frozen sections were washed in PBS for 5 minutes and then incubated with 2x10^-6 M DHE (D11347, Life Technologies) in the dark at 37°C for 30 minutes. Fluorescent signals were captured using a fluorescent microscope (IX73, Olympus, Tokyo). Mean fluorescence intensity for each section was determined by a blinded investigator using ImageJ software.

For DHE staining in cells, HK-2, BMDMs, and VSMCs were starved for 1 hour before IFNγ (315-05, PепроTech, Rocky Hill, New Jersey) was added into the media. After simulation for indicated time, DHE (10μM) were added and incubated for 30 minutes. The cells were washed with cold 1XPBS and fluorescent signals were captured using a fluorescent microscope (IX73, Olympus, Tokyo).
Fluorescence

For DHE staining of CD8⁺ T cells, DHE (10μM) were added into the media and incubated for 30 minutes. The cells were washed with cold 1XPBS and fluorescent signals were analyzed using ACEA NovoCyte (ACEA Bioscience. Inc., San Diego, California).
Online Figure I. Generation of TMRKO mice. (A) Flow cytometric analysis of MR expression in CD8⁺ T cells. (B) PCR analysis of genomic DNA isolated from various tissues of LC and TMRKO (KO) mice. LN, lymph node; CD4⁺, CD4⁺ T cells; CD8⁺, CD8⁺ T cells; BM, Bone marrow. (C) QRT-PCR analysis of MR gene expression in CD4⁺ and CD8⁺ T cells isolated from spleens of LC and TMRKO mice. (D) Western blotting analysis of MR expression in T cells from LC and TMRKO mice. (E) Representative plots of flow cytometric analysis of T cells in thymus. (F) Quantification of percentage of different T cell subsets in thymus measured by flow cytometry. DP, double positive (CD4⁺CD8⁺) T cells. DN, double negative (CD4⁻CD8⁻) T cells. (G) Representative plots of flow cytometric analysis of T cells in spleens. (H) Quantification of percentage of CD4⁺ and CD8⁺ T cells in spleens measured by flow cytometry. (I) Representative plots of flow cytometric analysis of T cells in blood. (J) Quantification of percentage of CD4⁺ and CD8⁺ T cells in blood measured by flow cytometry. For A and D, representative results of 3 independent experiments are shown. For C, F, H, and J, n = 5. N.S., not significant. *P<0.05.
Online Figure II

Online Figure II. T cell MR deficiency does not affect heart rate in mice. Telemetric monitoring of heart rate in littermate control (LC) and T cell MR knockout (TMRKO) mice before and after angiotensin II (AngII) infusion (n=7-9).

Online Figure III

Online Figure III. T cell MR deficiency decreases accumulation of AngII-induced superoxide in kidneys. (A) Representative dihydroethidium (DHE) staining of kidney sections. (B) Quantification of DHE fluorescent intensity (n=4-5). Mice were infused with vehicle or AngII for 4 weeks. Scale bar, 50μm. *P<0.05, ***P<0.001.
Online Figure IV. Gating strategy for flow cytometric analysis of T cell subsets in kidney and aorta samples. Tissue suspension was prepared and labeled with various antibodies. CD45 was used in conjunction with SSC to identify CD45$^+$ SSC$^{low}$ cells, which included T cells. After doublet exclusion, T cells were selected as CD45$^+$ CD3$^+$. CD4$^+$ and CD8$^+$ T cell subsets were identified by corresponding antibodies. IFN$\gamma$-producing T cells were further stratified as CD4$^+$ IFN$\gamma^+$ and CD8$^+$ IFN$\gamma^+$ subsets. TNF$\alpha$-producing T cells were similarly analyzed using antibody against TNF$\alpha$. 
Online Figure V. T cell MR deficiency prevents T cell infiltration in glomerulus of kidney with AngII infusion. A. CD4^+ T cell infiltrated in glomerulus of mice with AngII 28 days. B. Quantification of CD4^+ T cell infiltrated in glomerulus of mice with AngII 28 days (n=7-8). C. CD8^+ T cell infiltrated in glomerulus of mice with AngII 28 days. D. Quantification of CD8^+ T cell infiltrated in glomerulus of mice with AngII 28 days (n=6-7). *P<0.05, **P<0.01.
Online Figure VI. T cell MR deficiency mitigates AngII-induced accumulation of IFNγ-producing T cells in spleen. A. Representative flow cytometric analysis of CD4+ IFNγ+ T cells in spleen samples. B. Quantification of CD4+ IFNγ+ T cells (n=4). C. Representative flow cytometric analysis of CD8+ IFNγ+ T cells in spleen samples. D. Quantification of CD8+ IFNγ+ T cells (n=5). *P<0.05, **P<0.01, ***P<0.001.
Online Figure VII. T cell MR deficiency does not affect AngII-induced accumulation of TNFα-producing T cells in kidneys. **A.** Representative flow cytometric analysis of CD4⁺ TNFα⁺ T cells in kidney samples. **B.** Quantification of CD4⁺ TNFα⁺ T cells (n=4). **C.** Representative flow cytometric analysis of CD8⁺ TNFα⁺ T cells in kidney samples. **D.** Quantification of CD8⁺ TNFα⁺ T cells (n=4). ns: not significant, **p<0.01, ***p<0.001.
Online Figure VIII. Eplerenone attenuates AngII-induced hypertension and decreases IFNγ expression in CD8+ T cells. A and B. Noninvasive tail cuff monitoring of systolic blood pressure (A) and diastolic blood pressure (B) of C57BL/6 mice without AngII infusion (WT), with AngII infusion (WT+AngII), or with AngII infusion and eplerenone chow (WT+AngII+Epl). C. Representative flow cytometric analysis of CD4+ T cells in kidney samples. D. Quantification of CD4+ IFNγ+ T cells in kidney samples (n=4-5). E. Representative flow cytometric analysis of CD8+ T cells in kidney samples. F. Quantification
of CD8⁺ IFNγ⁺ T cells in kidney samples (n=4-5). G. Representative flow cytometric analysis of CD4⁺ T cells in spleen samples. H. Quantification of the percentage of CD4⁺ IFNγ⁺ T cells in spleen samples (n=4-5). I. Representative flow cytometric analysis of CD8⁺ T cells in spleen samples. J. Quantification the percentage of CD8⁺ IFNγ⁺ T cells in spleen samples (n=4-5).
Online Figure IX. T cell MR deficiency mitigates accumulation of memory effector T (T_{EM}) cells in kidneys and spleens. (A) Representative flow cytometric analysis of CD4+
T_{EM} cells in kidney samples. (B) Quantification of CD4^{+} T_{EM} cells (CD4^{+} CD44^{high} CD62L^{low}) in kidney samples (n=4-7). (C) Representative flow cytometric analysis of CD8^{+} T_{EM} cells in kidney samples. (D) Quantification of CD8^{+} T_{EM} cells (CD8^{+} CD44^{high} CD62L^{low}) in kidney samples (n=4-7). (E) Representative flow cytometric analysis of CD4^{+} T_{EM} cells in spleen samples. (F) Quantification of CD4^{+} T_{EM} cells in spleen samples (n=4-7). (G) Representative flow cytometric analysis of CD8^{+} T_{EM} cells in spleen samples. (H) Quantification of CD8^{+} CD44^{high} CD62L^{low} T cells in spleen samples (n=4-7). Mice were infused with vehicle or AngII for 4 weeks. ns: not significant, *P<0.05, **P<0.01, ***P<0.001.
Online Figure X. IFNγ stimulates superoxide production in HK-2 cells and macrophages. (A) DHE staining of HK-2 cells without or with IFNγ (20ng/ml) stimulation for 6 hours. Scale bar, 100μm. (B) DHE staining of bone marrow derived macrophages (BMDMs) without or with IFNγ (40ng/ml) stimulation for 2 hours. Scale bar, 50μm. Representative results of 3 independent experiments are shown.

Online Figure XI. MR deficiency does not affect superoxide production in CD8+ T cells. (A) Representative flow cytometric analysis of DHE staining of CD8+ T cells isolated from LC and TMRKO mice without or with stimulation by anti-CD3 antibodies. (B) Quantification of the mean fluorescence intensity of DHE in CD8+ T cells (n=5). ns: not significant, ***P<0.001.
Online Figure XII. T cell MR deficiency decreases accumulation of AngII-induced superoxide in aortas. (A) Representative dihydroethidium (DHE) staining of aortic sections. (B) Quantification of DHE fluorescent intensity (n=4-5). Mice were infused with vehicle or AngII for 4 weeks. Scale bar, 50μm. *P<0.05.

Online Figure XIII. IFNγ stimulates superoxide production in vascular smooth muscle cells. DHE staining of vascular smooth muscle cells (VSMCs) without or with IFNγ (20ng/ml) stimulation for 6 hours. Scale bar, 100μm. Representative results of 3 independent experiments are shown.

Online Figure XIV. T cell MR deficiency decreases IFNγ expression in renal CD8⁺ T cells. (A) Representative flow cytometric analysis of CD8⁺ T cells isolated from kidneys of LC and
TMRKO mice. The cells were treated with anti-CD3 antibodies for 48 hours. (B) Quantification of the percentage of CD8⁺ IFNγ⁺ cells (n=3). **P<0.01.
Online Figure XV

(A) Schematic illustration of the strategy to generate TMROV mice. CAG promoter: CMV enhancer/chicken β-actin promoter. (B) QRT-PCR analysis of MR gene expression in cells or tissues isolated from LC and TMROV mice. (C) Representative flow cytometric analysis of CD8 T cells isolated from kidneys of LC and TMRKO mice. The cells were treated with after anti-CD3 antibodies for 48 hours. (D) Quantification of the percentage of CD8 T cells (n=4). ns: not significant, **P<0.01, ***P<0.001.

Online Figure XVI

(A) Representative flow cytometric analysis of splenic CD8 T cells isolated from wild type mice.
The cells were pretreated with ethanol (Vehicle) or aldosterone (Aldo, 1nM) for 24 hours and then stimulated with anti-CD3 antibodies for 48 hours in the presence of Aldo. (B) Quantification of the percentage of CD8⁺ IFNγ⁺ cells exemplified in (A) (n=4). (C) QRT-PCR analysis of IFNγ in CD8⁺ T cells treated the same way as in (A) (n=4). *P<0.05, **P<0.01.
Online Figure XVII. T cell MR does not influence TNFα expression in CD8+ T cells. A. Representative flow cytometric analysis of CD8+ TNFα+ subpopulation in splenic CD8+ T cells from LC or TMRKO mice. The cells were either not stimulated (unstimulated) or stimulated with anti-CD3 antibody for 48 hours. B. Quantification of CD8+ TNFα+ T cells exemplified in (A) (n=4). C. QRT-PCR analysis of TNFα in CD8+ T cells from LC and TMRKO mice (n=4). D. Representative flow cytometric analysis of CD8+ TNFα+ subpopulation in splenic CD8+ T cells infected with control lentivirus (Ctrl) or MR overexpressing lentivirus (MROV) for 48 hours. E. Quantification of CD8+ TNFα+ T cells exemplified in (D) (n=5). F. QRT-PCR analysis of TNFα in control or MROV CD8+ T cells (n=5). ns: not significant, **P<0.01, ***P<0.001.
Supplemental Tables

Online Table I

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Supplemental References
