T Regulatory Lymphocytes Prevent Aldosterone-Induced Vascular Injury

Daniel A. Kasal, Tlili Barhoumi, Melissa W. Li, Naoki Yamamoto, Evguenia Zdanovich, Asia Rehman, Mario F. Neves, Pascal Laurant, Pierre Paradis, Ernesto L. Schiffrin

Abstract—Aldosterone mediates actions of the renin-angiotensin-aldosterone system inducing hypertension, oxidative stress, and vascular inflammation. Recently, we showed that angiotensin II–induced hypertension and vascular damage are mediated at least in part by macrophages and T-helper effector lymphocytes. Adoptive transfer of suppressor T-regulatory lymphocytes (Tregs) prevented angiotensin II action. We hypothesized that Treg adoptive transfer would blunt aldosterone-induced hypertension and vascular damage. Thirteen to 15-week–old male C57BL/6 mice were injected intravenously at 1-week intervals with \(3\times10^9\) CD4+CD25+ cells (representing Treg) or control CD4+CD25− cells and then infused or not for 14 days with aldosterone (600 µg/kg per day, SC) while receiving 1% saline to drink. Aldosterone induced a small but sustained increase in blood pressure (\(P<0.001\)), decreased vasodilatory responses to acetylcholine by 66% (\(P<0.001\)), increased both media:lumen ratio (\(P<0.001\)) and media cross-sectional area of resistance arteries by 60% (\(P<0.05\)), and increased NADPH oxidase activity 2-fold in aorta (\(P<0.001\)), kidney and heart (\(P<0.05\)), and aortic superoxide production. As well, aldosterone enhanced aortic and renal cortex macrophage infiltration and aortic T-cell infiltration (all \(P<0.05\)), and tended to decrease Treg in the renal cortex. Treg adoptive transfer prevented all of the vascular and renal effects induced by aldosterone. Adoptive transfer of CD4+CD25− cells exacerbated aldosterone effects except endothelial dysfunction and increases in media:lumen ratio of resistance arteries. Thus, Tregs suppress aldosterone-mediated vascular injury, in part through effects on innate and adaptive immunity, suggesting that aldosterone-induced vascular damage could be prevented by an immunomodulatory approach.

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Key Words: adaptive ■ innate immunity ■ inflammation ■ oxidative stress ■ blood pressure ■ remodeling ■ endothelial dysfunction

The renin-angiotensin (Ang)-aldosterone system (RAAS) plays an important role in the homeostasis of blood pressure (BP).1 Ang II acts in part by causing vasoconstriction or, indirectly, by increasing blood volume through enhanced renal sodium and water reabsorption, the latter directly but also via the release of aldosterone from the adrenal glands, which, through binding to mineralocorticoid receptors (MRs), increases renal sodium and water reabsorption, contributing to BP rise. MR is expressed in several nonrenal tissues such as blood vessels,2 and thus, aldosterone could also regulate BP by direct vascular actions.3–6

Ang II participates in the development of hypertension by increasing peripheral vascular resistance, at least in part by causing endothelial dysfunction and vasoconstriction, remodeling, oxidative stress, and inflammation. Aldosterone mediates some of the Ang II–induced vascular effects. Ang II–induced hypertension and vascular injury in rats were reduced by MR antagonist treatment in rats.7–9 Aldosterone infusion indeed caused endothelial dysfunction and vascular remodeling, oxidative stress, and inflammation in rats10–12 and mice.13

Innate and adaptive immunity appear to be involved in Ang II–induced hypertension and vascular damage.14–17 A role for regulatory T lymphocytes (Tregs), which are suppressors of the innate and adaptive immune responses,18–20 has been suggested in Ang II–induced BP elevation and vascular damage. Ang II induced a decrease in Treg, and adoptive transfer of Treg prevented Ang II–induced BP rise and vascular damage.21–22 Innate and adaptive immunity may also be involved in aldosterone-induced vascular damage. Aldosterone induced perivascular monocyte/macrophage infiltration in the heart13 and in mesenteric arteries.13

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From the Lady Davis Institute for Medical Research (D.A.K., T.B., M.W.L., N.Y., E.Z., A.R., P.P., E.L.S.) and Department of Medicine (E.L.S.), Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montreal, Quebec, Canada; State University of Rio de Janeiro (D.A.K., M.F.N.), Rio de Janeiro, Brazil; Université d’Avignon et des Pays de Vaucluse-Avignon (T.B., P.L.), Avignon, France.

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induced endothelial dysfunction and vascular oxidative stress were decreased in mcsf

which have a low monocyte/macrophage number in the vessel wall.13 Guzik et al23 showed that deoxycorticosterone acetate (DOCA)/salt–induced hypertension and superoxide production in the aorta were blunted in rag–/– mice deficient in T and B lymphocytes, and adoptive transfer of T but not B cells restored the mineralocorticoid effects. Furthermore, aldosterone activates T cells in vitro.24 However, it is unknown whether Tregs play a role in aldosterone-induced hypertension and vascular damage.

To test the hypothesis that Tregs play a role in aldosterone-induced vascular damage, we determined whether Treg adoptive transfer could prevent aldosterone-induced endothelial dysfunction and vascular remodeling, oxidative stress, and inflammation in mice. In addition, we determined whether aldosterone would decrease Treg.

Methods
Additional materials and methods are mentioned in the online Data Supplement (please see http://hyper.ahajournals.org).

Animals
Thirteen to 15-week–old male C57BL/6 mice (Harlan Laboratories, Chicago, IL) mice were injected intravenously via the tail vein twice, at 1-week intervals, with PBS or 3×10^6 CD4^+CD25^+ (Treg) or CD4^+CD25^- cells, and then infused with aldosterone (600 μg/kg per day) for 14 days using ALZET osmotic minipumps (Durect, Chicago, IL) mice were injected intravenously via the tail vein twice, with PBS or 3

Nox1, Nox2, Nox4, and vascular cell adhesion molecule (VCAM)-1 expression in the aorta and monocyte/macrophage (MOMA-2) and T lymphocyte (CD3) infiltration in the aorta and kidney, and Treg (Foxp3) infiltration in the kidney were determined by immunofluorescence microscopy on 5-μm–thick cryostat sections. Details of the protocol are presented in the online Data Supplement.

Endothelial Function and Vessel Mechanics
Second-order branches of mesenteric arteries (ID 150–250 μm) were dissected and mounted on a pressurized myograph and endothelial function and vessel mechanics determined as described previously.13 Details of the protocol are presented in the online Data Supplement.

NADPH Oxidase Activity and Generation of Superoxide
NADPH oxidase activity was determined by lucigenin chemiluminescence,13 and superoxide production was examined by dihydroethidium staining,23 as described previously. Details of the protocol are presented in the online Data Supplement.

Immunofluorescence Microscopy
Nox1, Nox2, Nox4, and vascular cell adhesion molecule (VCAM)-1 expression in the aorta and monocyte/macrophage (MOMA-2) and T lymphocyte (CD3) infiltration in the aorta and kidney, and Treg (Foxp3) infiltration in the kidney were determined by immunofluo-
NO was the major mediator of the vasodilator response in all of the groups in this size vessel in this experimental paradigm. Vasodilatory responses to sodium nitroprusside were similar in all of the groups (Figure 2D).

Adoptive Transfers of Treg Prevented Aldosterone-Induced Mesenteric Artery Hypertrophic Remodeling
Aldosterone-treated mice exhibited a 60% greater media:lumen ratio and a 57% larger media cross-sectional area of mesenteric arteries compared with control (Figure 2E and 2F). This effect was prevented by adoptive transfer of Treg but not CD4+CD25− cells. In addition, adoptive transfer of CD4+CD25− cells exacerbated aldosterone-induced increase in media cross-sectional area, causing an additional 28% increase compared with aldosterone-treated rats receiving sham lymphocyte transfer.

Adoptive Transfer of Treg Prevented Aldosterone-Induced Oxidative Stress
Aldosterone infusion increased NADPH oxidase activity 2-fold in aorta, heart, and renal cortex (Figure 3A through 3C). Adoptive transfer of Treg but not CD4+CD25− cells prevented these effects. Adoptive transfer of CD4+CD25− cells exacerbated aldosterone-induced increase in NADPH oxidase activity in aorta, resulting in 24% higher activity level than in aldosterone-infused mice not receiving lymphocyte transfer (Figure 3A). Aldosterone increased superoxide as measured by dihydroethidium fluorescence in vascular smooth muscle cells and perivascular cells of aorta, which was abrogated by adoptive transfer of Tregs but not CD4+CD25− cells (Figure 3D). To better understand the mechanism involved in changes in NADPH oxidase activity described above, the expressions of Nox1, Nox2, and Nox4 were determined by immunofluorescence in the aorta. No significant change in the expression of the different Nox family homologues was found because variability was large (Figure S3). However, aldosterone qualitatively increased the level of expression of Nox2 and Nox4. Because NADPH activity was higher, this may represent increased activity of either of these 2 latter Nox family members.

Adoptive Transfer of Treg Prevented Aldosterone-Induced Inflammatory and Immune Cell Tissue Infiltration
Aldosterone increased 2.2-fold the level of VCAM-1 expression in aorta, which was not prevented by adoptive transfers of Treg and was enhanced 1.5-fold by CD4+CD25− cells (Figure 4A). Aldosterone increased 4.6-fold monocyte/macrophage-specific antigen MOMA-2 immunostaining in adventitia and periadventitial fat of aorta, which was prevented by adoptive transfer of Treg and exaggerated by CD4+CD25− cells by 30% (Figure 4B). Aldosterone treatment increased aortic vascular and perivascular T cell infiltration 4.5-fold. This effect was prevented by adoptive transfer of Treg and enhanced 2.7-fold by adoptive transfer of CD4+CD25− cells (Figure 4C).

To determine whether T reg infiltration is modulated by aldosterone infusion, the number of Foxp3+ cells was quantified by immunofluorescence in the renal cortex and me-
Aldosterone-induced oxidative stress was prevented by T-regulatory lymphocyte (Treg) adoptive transfer. NADPH oxidase activity in aorta (A), heart (B), and renal cortex (C) and dihydroethidium staining revealing superoxide generation in aorta (D) were determined in the same groups as in Figure 1. A through C. Data are means ± SEM; *P < 0.05 and **P < 0.001 vs Ctrl, †P < 0.05 and ††P < 0.001 vs PBS, ‡‡P < 0.001 vs Treg, and †††P < 0.001 vs Aldo, with n = 6 to 9. D, Representative dihydroethidium-stained sections of aorta from 4 to 5 mice per group are presented.

Discussion
An association among the RAAS, hypertension, vascular damage, and chronic low-grade inflammation has been suggested by numerous experimental and clinical studies. Innate and adaptive immunities have been shown to play a role in hypertension and associated cardiovascular disease.15–17 The present study extends this notion by demonstrating that Treg adoptive transfer prevents aldosterone-induced vascular damage in a BP-independent fashion.

The observation that adoptive transfer of Treg prevented the vascular damage and monocyte/macrophage and T lymphocyte infiltration associated with aldosterone infusion underlines the importance of the role of innate and adaptive immunity in RAAS-induced hypertension and vascular damage. We showed previously the participation of the innate immune response in Ang II- and DOCA/salt–induced hypertension and vascular damage and aldosterone-induced vascular injury when these were blunted in mice deficient in macrophages.13,25,26 The importance of adaptive immune responses was demonstrated by Guzik et al,23 who showed that Ang II- and DOCA/salt–induced hypertension and vascular damage were blunted in rag−/− mice deficient in T and B lymphocytes and effects restored by adoptive transfer of T but not B cells. More recently, we and others showed that adoptive transfer of Treg prevents Ang II–induced infiltration of monocyte/macrophages and T cells and, thus, innate and adaptive immune responses.21,27 Interestingly, Ang II might activate, at least in part, adaptive immunity by decreasing the number of Tregs, thus reducing their suppressive effects. Indeed, we showed that Ang II decreased the number of Tregs in the renal cortex,21 and Matrougui et al22 reported that Ang II–induced apoptosis of Tregs. The results of the present study suggest that both the innate and adaptive immune responses are involved in aldosterone-induced vascular injury, because aldosterone induced monocyte/macrophage and T lymphocyte infiltration, which was prevented by adoptive transfer of Tregs. As in the case of Ang II, aldosterone tended to reduce the number of Tregs in the renal cortex, which could contribute to activate the adaptive immune response. Importantly, adoptive transfer of CD4+CD25− T lymphocytes not including Treg) exacerbated most of the aldosterone effects, including the increases in mesenteric artery media cross-section, oxidative stress, VCAM-1, and monocyte/macrophage, as well as T-cell infiltration, which supports the hypothesis that aldosterone activates immune responses.

Aldosterone-induced endothelial dysfunction and vascular remodeling and inflammation are mediated through genera-
tion of oxidative stress that could be related to infiltrating macrophages and T cells, because both oxidative stress and immune cell infiltration were prevented by T regulatory lymphocyte (Treg) adoptive transfer. Macrophages express both MR and Ang II type 1 receptor, whereas T cells abundantly express Ang II type 1 receptor, but MR at low levels, and both cell types express NADPH oxidase. Aldosterone induces oxidative stress in macrophages in vitro and in vivo in apoE−/− mice. Macrophages express a local renin-Ang system that may contribute to target organ damage. Local production of Ang II may participate in aldosterone-induced oxidative stress in macrophages and vascular cells. Keidar et al showed that aldosterone-induced oxidative stress in macrophages was completely inhibited only when both MR and
Ang-converting enzyme or Ang II type 1 receptor were inhibited. Guzik et al.\textsuperscript{13,21} showed that DOCA/salt-induced superoxide production was blunted in \textit{rag} \textsuperscript{-/-} mice and restored by adoptive transfer of T but not B cells, suggesting that T cells could be involved in aldosterone-induced oxidative stress, because DOCA acts through MR.\textsuperscript{22} T lymphocytes also express a local renin-Ang system that could contribute to aldosterone-induced oxidative stress.\textsuperscript{20} Aldosterone induced superoxide production in the vascular wall, which could be attributed at least partly to infiltrating macrophages and T cells, because aldosterone-induced superoxide production in vascular smooth muscle cell was prevented by adoptive transfer of Treg and blunted in mice deficient in macrophages.\textsuperscript{13,21} Whether superoxide produced by infiltrating macrophages or T cells or by vascular smooth muscle cells is able to interact with endothelium-derived NO and to contribute to endothelial dysfunction\textsuperscript{22} remains to be determined. The quantification of expression of Nox1, Nox2, and Nox4 in the aorta by immunofluorescence showed qualitative increases of Nox2 and Nox4 in response to aldosterone. Because NADPH oxidase activity was enhanced by aldosterone, the increase may occur in part as a result of upregulation of Nox2 and Nox4. However, the mechanisms of regulation of NADPH oxidase activity by aldosterone with or without adoptive transfer of Treg or CD4\textsuperscript{+}CD25\textsuperscript{-} cells remain to be clarified.

Although we found previously that Ang II–induced VCAM-1 expression in the aorta was prevented by Treg adoptive transfer,\textsuperscript{3} Treg adoptive transfer did not prevent aldosterone-induced VCAM-1 expression in the aorta. Aldosterone-induced VCAM-1 expression in mesenteric arteries was blunted in \textit{mcsfOpf} \textsuperscript{-/-} mice with reduced macrophages when compared with control wild-type mice.\textsuperscript{13} Treg may fail to affect the action of aldosterone on VCAM-1 expression.

The kidney could represent one of the sites of action of Treg that could participate in the regulation of inflammation and immunity-induced BP rise.\textsuperscript{21} As in the case of Ang II, aldosterone tended to decrease Treg and increased monocyte/macrophage infiltration in the renal cortex, which were reversed by Treg adoptive transfer. However, aldosterone induced only modest increases in BP. Accordingly, the kidney may represent one of the sites of action of Treg that could play an important role in the regulation of vascular inflammation beyond BP regulation.

Perspectives

The present study demonstrates that enhanced suppressor Treg prevent aldosterone-induced endothelial dysfunction, vascular remodeling, and oxidative stress, extending previous findings on the effects of Treg on Ang II effects. Treg may be implicated in mechanisms that modulate the degree to which the RAAS induces hypertension, vascular injury, and innate and adaptive immune responses. Several studies in animal models and humans have shown that inhibition of the RAAS used to control hypertension and vascular damage is associated with a decrease in the inflammatory state. Platten et al.\textsuperscript{13} showed that blocking Ang-converting enzyme or Ang II type 1 receptor suppressed autoreactive T-helper 1 and T-helper 17 cells and promoted Treg in a model of autoimmunity.

Taken together, these observations suggest that it could be possible to develop immunomodulatory approaches to treat hypertension and associated vascular injury. Loss-of-function studies are required to confirm the role of Treg in the pathogenesis of Ang II– and aldosterone-induced hypertension and vascular damage.

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Disclosures

None.

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T regulatory lymphocytes prevent aldosterone-induced vascular injury

Daniel A. Kasal1,3,*; Tlili Barhoumi1,4,*; Melissa W. Li1; Naoki Yamamoto1; Evguenia Zdanovich1; Asia Rehman1; Mario F. Neves3; Pascal Laurant4; Pierre Paradis1; and Ernesto L. Schiffrin1,2

1Lady Davis Institute for Medical Research, and 2Department of Medicine, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montreal, Canada; 3State University of Rio de Janeiro, Brazil; 4Université d'Avignon et des Pays de Vaucluse-Avignon, France

*Both authors contributed equally to this work.

Running title: Treg blunt aldosterone vascular effects

Correspondence to:
Ernesto L. Schiffrin MD, PhD
Sir Mortimer B. Davis-Jewish General Hospital, #B-127,
3755 Cote Ste-Catherine Road,
Montreal, Quebec, Canada H3T 1E2
Ph.: (514) 340-7538; Fax: (514) 340-7539
Email: ernesto.schiffrin@mcgill.ca
Http://www.ladydavis.ca/en/ernestoschiffrin
Expanded Methods

Isolation of Treg and CD4+CD25- cells

CD4+CD25+ cells (Treg) were isolated from 2 spleens of 10 to 12-week old male C57BL/6 mice through CD4+ T cell negative selection followed by a CD25+ T cell positive selection of splenocytes as per manufacturer’s instructions using the using EasySep® Mouse CD4+ T Cell Pre-Enrichment and CD25 Positive Selection Kits (Stem Cell Technologies, Vancouver, BC, Canada). CD4+CD25- cells not retained by the CD25+ positive selection were collected and used as control cells for the adoptive transfer. This produced enough cells for one injection of Treg and CD4+CD25+ cells. Purity of Treg and CD4+CD25- cells was determined by flow cytometry as follows. Isolated cells (10^6) were resuspended in PBS containing 2% fetal bovine serum (Fluorescence Activated Cell Sorting (FACS) Buffer). Cells were blocked with mouse BD Fc-Block™ (1:200, clone 2.4G2, BD Biosciences, Mississauga, ON, Canada) for 30 minutes at 4°C to avoid non-specific binding to mouse Fc-gamma receptors, stained for 1 hour at 4°C with APC-conjugated rat anti-mouse CD4 antibody (1:2000, clone RM4-5, BD Biosciences), and with PE-conjugated anti-mouse CD25 antibody using Mouse CD25 Positive Selection Kits (Stem Cell Technologies), washed with FACS Buffer, fixed and permeabilized using the Foxp3 Staining Buffer Set as per manufacturer’s instructions (eBioscience, San Diego, CA), and stained with FITC-conjugated rat anti-mouse Foxp3 antibody (1:1000, clone FJK-16s, eBioscience) for 1 hour at 4°C. Cells were washed, resuspended with FACS Buffer, and analyzed using FACSCalibur™ flow cytometer (BD Biosciences) and FCS Express V3 software (De Novo Software, Los Angeles, CA).

Endothelial function and vessel mechanics

Second-order branches of the mesenteric arterial tree (internal diameter between 150–250μm) were dissected and mounted on a pressurized myograph as previously described. Briefly, vessels were equilibrated for 60 min at 45mmHg intraluminal pressure in warmed Krebs solution (pH 7.4) containing (mmol/l): 120 NaCl, 25 NaHCO3, 4.7 KCl, 1.18 KH2PO4, 1.18 MgSO4, 2.5 CaCl2, 0.026 EDTA and 5.5 glucose, bubbled with a mixture of 95% air–5% CO2. Media and lumen diameters were measured by a computer-based video imaging system (Living Systems Instrumentation, Burlington, Virginia, USA). Endothelium-dependent and -independent relaxation was assessed by measuring the dilatory responses to cumulative doses of acetylcholine (Ach, 10⁻⁹ to 10⁻⁴mol/L) or sodium nitroprusside (SNP, 10⁻⁸ to 10⁻⁴mol/L), respectively, in vessels precontracted with norepinephrine (NE, 5 x 10⁻⁵ mol/L). To evaluate the contribution of nitric oxide (NO) to the vascular response, the dose-response curve to Ach was determined before and after a 30-minute preincubation with the NO synthase inhibitor N°-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴ mol/L). Thereafter, vessels were perfused with Ca²⁺-free Krebs solution containing 10 mmol/l EGTA for 30 min to eliminate vascular tone. Media and lumen diameter were measured at 3, 10, 20, 30, 40, 45, 60, 80, 100, 120 and 140 mmHg intraluminal pressures. Media cross-sectional area, media/lumen ratio, and stress and strain were calculated as previously described.
**Immunofluorescence microscopy**

Nox1, Nox2, Nox4 and VCAM-1 expression in the aorta and monocye/macrophage (MOMA-2) and lymphocyte T (CD3) infiltration in the aorta and kidney and Treg (Foxp3) infiltration in the kidney were determined by immunofluorescence microscopy on 5-µm-thick cryostat sections. Sections were air-dried for 30 min. and then fixed for 5 min in a mixture of acetone:methanol (1:1) at -20°C (for VCAM-1), in 4% paraformaldehyde for 20 min at RT (for CD3, Foxp3, MOMA-2, Nox2, and Nox4) or in ice cold acetone for 10 min at RT (for Nox1). Thereafter, sections were washed with PBS containing 0.1% Tween-20 (PBST) twice for 10 min followed by a wash with PBS. Sections were blocked with PBST containing 10% normal goat serum (for Nox1 and VCAM-1) or PBS containing 20% fetal bovine serum, 1% bovine serum albumin and 0.4% triton (for CD3, Foxp3, MOMA-2, Nox2 and Nox4), for 1 h at RT and then incubated with a rat anti-VCAM-1 (1:100, Abcam, Cambridge, MA), a rat anti-macrophage-specific antigen MOMA-2 (1:50, Abcam), a rabbit anti-CD3 polyclonal (1:200, Dako Canada, Burlington, ON, Canada), a rabbit anti-Foxp3 (1:500, Abcam), a goat anti-Nox1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a rabbit anti-Nox2 (1:50, kindly provided by Dr. Mark Quinn, University of Montana, MT), a rabbit anti-Nox4 (1:200, Abcam) antibodies in blocking solution overnight at 4°C. The sections were washed 3 times with PBST and incubated with Alexa Fluor® 555 goat anti-rat, Alexa Fluor® 488, 555 or 568 goat anti-rabbit or Alexa Fluor® 555 donkey anti-goat antibodies (1:400 for Nox4 and 1:200 for all the other antibodies, Invitrogen Corp., Carlsbad, CA, USA) and then washed 3 times with PBST and mounted with Vectashield® Hardset™ with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were captured using a fluorescent microscope Leica DM2000 (Leica Microsystems, Richmond Hill, ON, Canada) and analyzed with Image J software (National Institute of Mental Health, Bethesda, Maryland).
References


Figure S1. Flow cytometry profile of T regulatory lymphocytes (Treg) and CD4^+CD25^− cells. Cells were separated using CD4^+ T cell negative selection followed by CD25^+ T cell positive selection of splenocytes, stained with APC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD25 and FITC-conjugated anti-mouse Foxp3 antibodies, and analyzed by flow cytometry. Cells were gated as lymphocytes in the Side Scatter (SSC)/Forward Scatter (FSC) plot, and lymphocytes were gated as CD4^+ cells in the SSC/CD4 plot. Gated CD4^+ cells were characterized for CD4^+CD25^+ (right panels), CD4^+Foxp3^+ (upper panels) and CD4^+CD25^+Foxp3^+ (upper right panel) in the Foxp3/CD25 plot. Representative flow cytometry results are presented. The % of CD4^+ cells in lymphocytes, % of CD25^+ cells in CD4^+ cells, % of Foxp3^+ cells in CD4^+ cells and % of CD4^+CD25^+Foxp3^+ cells in CD4^+ cells are presented. Data are means ± SD from three independent experiments.
Figure S2. Diastolic blood pressure was not significantly changed by any of the treatments. Diastolic blood pressure (DBP) was determined by telemetry in control mice (Ctrl) and in mice infused with aldosterone (Aldo) and pretreated with PBS, Treg or CD4^+CD25^- cells. Mean 24-h SBP data are presented. Data are presented as means ± SEM. n = 5-6 mice.
Figure S3. Nox1, Nox2 and Nox4 expression in aorta. Immunofluorescence was used to determine the expression level of Nox1 (in yellow), Nox2 (in yellow) and Nox4 (in red) in the aorta of control mice (Ctrl) and in mice infused with aldosterone (Aldo) and pretreated with PBS, Treg (CD4^+CD25^+) or CD4^+CD25^- cells. Elastin autofluorescence and the nuclear stain DAPI are shown in green and blue, respectively. Representative elastin autofluorescence, DAPI nuclear staining and Nox immunofluorescence merged images are shown. Data are means ± SEM, n = 3.
Figure S4. Adoptive transfer of Treg counteracts aldosterone-induced decrease in Treg infiltration in the renal cortex. Immunofluorescence (in red) was used to determine the number of Treg in renal cortex (n = 4) in control mice (Ctrl) and in mice infused with aldosterone (Aldo) and pretreated with PBS, Treg (CD4^+CD25^+) or CD4^+CD25^- cells. DAPI nuclear staining is shown in blue. Representative DAPI nuclear staining, Foxp3 immunofluorescence and merged images are shown.
Figure S5. Treg infiltration in the renal medulla. Immunofluorescence (in red) was used to determine the number of Treg infiltration in the renal medulla in control mice (Ctrl) and in mice infused with aldosterone (Aldo) and pretreated with PBS, Treg (CD4+CD25+) or CD4+CD25− cells. DAPI nuclear staining is shown in blue. Representative Foxp3 immunofluorescence and DAPI nuclear staining (in blue) merged images are shown. Data are means ± SEM, n = 3-4.
Figure S6. Aldosterone-induced monocyte/macrophage infiltration in the renal cortex was prevented by Treg adoptive transfer. Immunofluorescence (in red) was used to determine monocyte/macrophage infiltration in renal cortex (n = 4) in control mice (Ctrl) and in mice infused with aldosterone (Aldo) and pretreated with PBS, Treg (CD4^+CD25^+) or CD4^+CD25^- cells. DAPI nuclear staining is shown in blue. Representative DAPI nuclear staining, MOMA-2 immunofluorescence and merged images are shown. The arrows pointed at the same glomerulus in the DAPI, MOMA-2 and Merged panels.
Figure S7. Monocyte/macrophage infiltration in the renal medulla. Immunofluorescence (in red) was used to determine the number of the monocyte/macrophage infiltration in the renal medulla in control mice (Ctrl) and in mice infused with aldosterone (Aldo) and pretreated with PBS, Treg (CD4⁺CD25⁺) or CD4⁺CD25⁻ cells. DAPI nuclear staining is shown in blue. Representative MOMA-2 immunofluorescence and DAPI nuclear staining (in blue) merged images are shown. Data are means ± SEM, n = 3-4.
Figure S8. T lymphocyte infiltration in the kidney. Immunofluorescence (in red) was used to determine the number of T lymphocyte infiltrating the renal cortex (A) and medulla (B) in control mice (Ctrl) and in mice infused with aldosterone (Aldo) and pretreated with PBS, Treg (CD4^+CD25^-) or CD4^+CD25^- cells. DAPI nuclear staining is shown in blue. Representative DAPI nuclear staining and Foxp3 immunofluorescence merged images are shown. Data are means ± SEM, n = 3-4.