Original Article

B6.Rag1 Knockout Mice Generated at the Jackson Laboratory in 2009 Show a Robust Wild-Type Hypertensive Phenotype in Response to Ang II (Angiotensin II)

Anika Seniuk, Jonas L. Thiele, Andra Stubbe, Philipp Oser, Alva Rosendahl, Marlies Bode, Catherine Meyer-Schwesinger, Ulrich O. Wenzel, Heimo Ehmke

See Editorial Commentary, pp xxx–xxx

Abstract—A key finding supporting a causal role of the immune system in the pathogenesis of hypertension is the observation that *RAG1* knockout mice on a C57Bl/6J background (B6.Rag1$^{-/-}$), which lack functional B and T cells, develop a much milder hypertensive response to Ang II (angiotensin II) than control C57Bl/6J mice. Here, we report that we never observed any Ang II resistance of B6.Rag1$^{-/-}$ mice purchased directly from the Jackson Laboratory as early as 2009. B6.Rag1$^{-/-}$ mice displayed nearly identical blood pressure increases monitored via radiotelemetry and hypertensive end-organ damage in response to different doses of Ang II and different levels of salt intake (0.02%, 0.3%, and 3% NaCl diet). Similarly, restoration of T-cell immunity by adoptive cell transfer did not affect the blood pressure response to Ang II in B6.Rag1$^{-/-}$ mice. Full development of the hypertension-resistant phenotype in B6.Rag1$^{-/-}$ mice appears to depend on the action of yet unidentified nongenetic modifiers in addition to the absence of functional T cells. **(*Hypertension*. 2020;75:00-00. DOI: 10.1161/HYPERTENSIONAHA.119.13773.)** ● **Online Data Supplement**

Key Words: angiotensin II ■ blood pressure ■ hypertension ■ immune system ■ mice

In 2007, Guzik et al[1] reported a resistance to Ang II (angiotensin II)–induced hypertension in mice unable to generate mature T and B lymphocytes due to a disrupted recombination-activating gene 1 (*RAG1*). Male Rag1$^{-/-}$ mice on a C57Bl/6J background (B6.Rag1$^{-/-}$) developed much milder hypertension in response to a continuous subcutaneous administration of Ang II at a rate of 490 ng/min per kg than control C57Bl/6J mice. Adoptive transfer of T cells, but not B cells, fully restored the hypertensive response to Ang II. This seminal observation strongly supported a significant and causal role of T cells in the pathogenesis of hypertension and stimulated a surge of studies investigating immunologic aspects of hypertension and hypertensive end-organ damage.[2–4] Since the initial observation, further studies from David Harrison's laboratory,[5,6] as well as from several independent laboratories, confirmed the significantly reduced blood pressure increase during chronic Ang II infusion in B6.Rag1$^{-/-}$ using radiotelemetry,[7] tail cuff plethysmography,[8,9] or indwelling femoral artery catheterization.[10] A reduced blood pressure response was also seen in *RAG1*-deficient rats.[11]

Recently, Ji et al[12] reported that the resistance to Ang II–induced hypertension observed in B6.Rag1$^{-/-}$ mice purchased from the Jackson Laboratory before 2015 was lost in B6.Rag1$^{-/-}$ mice purchased in 2015/2016. Ji et al suggested that this phenotypic change was induced by spontaneous mutations, which occurred in the inbred Jackson Laboratory B6.129S7-Rag1^{tm1Mom}/J mouse strain since 2009. Here, we report that we have never observed any resistance to Ang II–induced hypertension in strictly inbred B6.Rag1$^{-/-}$ mice directly imported from the Jackson Laboratory as early as in 2009.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Mice

Male B6.129S7-Rag1^{tm1Mom}/J (B6.Rag1$^{-/-}$) mice were directly imported from the Jackson Laboratory in 2009 and kept strictly inbred in pathogen-free individually ventilated cage housing in our animal facility. Control male C57Bl/6J mice were also obtained from the Jackson Laboratory via Charles River–the exclusive licensed breeder of Jackson mice in Germany.

After transfer to the laboratory, mice were kept in single housing in sterilized cages with filter cover. All procedures (daily health check, feeding, surgery, injections) were performed under sterile conditions in a work bench (Thermo Scientific Heraeus Clean Bench HERAguard HPH 12). Each mouse had free access to gamma-irradiated normal standard food (ROD 16-R, LASvendi, containing 0.3% NaCl) and sterilized tap water. The 12-hour light/dark cycle was

Received July 26, 2019; first decision August 9, 2019; revision accepted December 9, 2019.

From the Institute of Cellular and Integrative Physiology (A. Seniuk, J.L.T., A. Stubbe, P.O., C.M.-S., H.E.) and Third Department of Medicine (A.R., M.B., U.O.W.), University Medical Center Hamburg, Germany; and German Center for Cardiovascular Research, Partner Site Hamburg/Kiel/Lübeck (A. Seniuk, A.R., U.O.W., H.E.).

The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.119.13773.

Correspondence to Heimo Ehmke, Institute of Cellular and Integrative Physiology, University Medical Center Hamburg, 20246 Hamburg, Germany. Email ehmke@uke.de

synchronized in all mice. All animal procedures have been approved by the local animal committee and were in accord with the national and institutional animal care guidelines.

Genotyping

Mice were genotyped by standard polymerase chain reaction using the following primers: 5′-gaggttccgctacgactctg-3′, 5′-ccggacaagtttttcatcgt-3′, and 5′-tggatgtggaatgtgtgcgag-3′. Polymerase chain reaction products were separated by gel electrophoresis on 1.5% agarose gel. The expected wild-type band ran at 474 bp, the mutant band at 530 bp.

Radiotelemetric Blood Pressure Measurements

Mice with a minimum body weight of 23 g were anesthetized by intraperitoneal application of 120 mg ketamine and 16 mg xylazine/kg. Telemetric transmitters (PhysioTel PA-C10; Data Sciences International) were implanted subcutaneously, with the sensing tip placed in the aorta via the left carotid artery. After 10 days of recovery from surgery, interventions and recordings (Dataquest A.R.T. software for acquisition and analysis) were started.

Vascular Relaxation Response

Thoracic aortae immediately dissected from euthanized mice were carefully removed from fat and connective tissue and cut into 2 pieces of 4-mm length each for contraction force measurements in a wire myograph system (Kent Scientific). Vessels were kept in buffer (in mmol/L: NaCl 100, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH2PO_4$ 1, glucose 11, indomethacin 0.01) under gentle fumigation with 95% O_2, 5% CO_2 at a pretension of 1.1 g and 3x conditioned with 80 mmol/l KCl. After precontraction with 2 µmol/L PGF2α (prostaglandin F2α; tris salt; Sigma Aldrich), relaxation response to increasing concentrations of acetylcholine chloride (Sigma Aldrich) or nitroglycerin (1 mg/mL Nitrolingual infus.; Pohl Boskamp) was measured as described previously.[13] Data were analyzed with Chart v4.0 software (ADInstruments) and calculated in percentage of the maximum contraction force. For each mouse, the mean relaxation response was calculated from the relaxation response curves of 2 pieces of the aorta.

Histopathologic Analysis

After organ harvesting and fixation in 4% phosphate-buffered formalin solution, paraffin-embedded hearts, kidneys, and thoracic aortae were sectioned and stained by our mouse pathology facility. In Elastica van Gieson–stained aortic rings, the area of the tunica media aortae, defined by the inner and outer lamina elastica, was quantified by FIJI software. Cardiac fibrosis was assessed by determination of the average score of 10 visual fields of a Picro-Sirius Red–stained ventricular section per animal, whereby the score zero refers to no fibrosis and the score 2 indicates massive fibrosis. Scoring was performed by 2 blinded investigators.

Adoptive T-Cell Transfer

Single-cell suspensions from the spleens of C57Bl/6J donor mice were purified by indirect magnetic cell sorting (Pan T Cell Isolation Kit II, mouse, MACS; Miltenyi Biotech). After flow cytometric control of purity, 10^7 T cells diluted in 100 µL 0.9% NaCl solution or 100 µL 0.9% NaCl solution (sham treatment) were injected into the tail vein of B6.Rag1$^{-/-}$ mice. For investigation of the effects of Ang II infusion after adoptive T-cell transfer, mice underwent the injection procedure 4 weeks before implantation of Ang II–containing osmotic minipumps.

Ang II Infusion

Ang II was infused at different rates (170, 490, or 1500 ng/min per kg) for 14 days via subcutaneously implanted osmotic minipumps (Alzet, model 1002).

Salt Diet

To investigate the influence of salt intake, mice received a modified special diet (Altromin C1036), containing either 0.02% sodium as NaCl (low salt) or 3% sodium as NaCl (high salt), beginning 14 days before the start of the Ang II infusion protocol.

Isolation of Leukocytes From Aorta, Kidney, and Blood

The thoracic aortas including the adventitia, as well as kidneys and blood, were taken from euthanized mice. Cardiac blood was taken and mixed with 1.6 mg/mL EDTA and centrifuged at 4000*g* for 15 minutes. Aortas were first rinsed with 2 mmol/L EDTA and 2% fetal calf serum, cut into small pieces and digested as described.[1,14] Briefly, aorta dissects were incubated for 45 minutes at 37°C and 300 rpm in digestion medium (PBS with 240 U/mL collagenase type I, 125 U/mL collagenase type XI, 60 U/mL hyaluronidase type I, and 1.20 U/mL DNAse; Sigma Aldrich, Saint Louis, MO). After digestion, the cells were passed through a 70-µM cell strainer to generate a single-cell suspension. Kidneys were minced and incubated in digestion medium (RPMI 1640 medium containing 10% fetal calf serum, 1% HEPES, 1% penicillin/streptomycin, 0.4 mg/mL collagenase D, and 0.1 mg/mL DNAse) at 37°C for 45 minutes. To obtain a single-cell suspension, kidneys were then dissociated using the gentleMACS Dissociater and centrifuged at 300*g* at 4°C for 8 minutes. Percoll gradient (37% Percoll; GE Healthcare, Chalfont St. Giles, Great Britain) centrifugation was performed at 500*g* at room temperature for 20 minutes to further purify the cells. After lysis of erythrocytes in the cellular part of blood and the single-cell suspensions of kidneys with ammonium chloride, cells were filtered over 40-µm meshes, washed again, and resuspended in PBS for staining and flow cytometry. For determining absolute cell numbers, a defined volume of nonstained cell suspensions was taken and separately stained.

Flow Cytometry

For flow cytometry, fluorochrome-conjugated antibodies were used: cluster of differentiation (CD) 45 (30-F11), CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), γδ T cell receptor (GL3), NK1.1 (PK136), and CD19 (6D5; Biolegend, San Diego, CA). LIVE/DEAD staining (Invitrogen Molecular Probes, Carlsbad, CA) was used to exclude dead cells during flow cytometry. For cell count, CD45 (30-F11) antibody and counting beads were used (Life Technologies Europe, Bleiswijk, the Netherlands). Samples were acquired on a Becton and Dickinson LSRII System (Becton Dickinson, Heidelberg, Germany) using the Diva software. Data analysis was performed with FlowJo (Tree Star, Inc, Ashland, OR). After gating for single cells by forward scatter width and forward scatter area followed by gating for living cells, leukocytes isolated from the aorta, blood, and kidneys were determined via CD45 surface staining. Absolute cell numbers were determined by measuring a defined amount of counting beads with comeasuring the CD45$^+$ cells in flow cytometry. Those CD45$^+$ cell numbers were related to the used tissue weight.

Statistical Analysis

Data were analyzed with GraphPad Prism 7 and are shown as mean±SEM whereby n represents the number of animals or specimen. Statistical analysis was performed by 1- or 2-way ANOVA, if not stated otherwise. $P<0.05$ was considered statistically significant.

Results

B6.Rag1$^{-/-}$ Mice Are Not Protected From Ang II–Induced Hypertension and End-Organ Damage

Confirmation of *RAG1* deficiency in B6.Rag1$^{-/-}$ mice was routinely performed by polymerase chain reaction detection of the knockout band, by the assessment of reduced spleen weight and by the assessment of mature CD3$^+$ lymphocyte depletion in homogenized kidney cell solutions by flow cytometry (Figure S1 in the online-only Data Supplement). After confirmation of *RAG1* deficiency in B6.Rag1$^{-/-}$ directly imported from the Jackson Laboratory in 2009, we infused Ang II in B6.Rag1$^{-/-}$ mice. Unexpectedly, despite

adhering to the original experimental protocol described by Guzik et al,[1] we found comparable blood pressure increases in B6.Rag1$^{-/-}$ mice and C57Bl/6J controls in response to Ang II infusions at a rate of 490 ng/min per kg (Figure 1A; Table). Examination of end-organ damage also revealed no significant differences in endothelial function, vascular morphology, and myocardial hypertrophy (Figure 1B through 1E), except for a partial protection from Ang II–mediated cardiac fibrosis in B6.Rag1$^{-/-}$ mice (Figure 1F).

Tissue-Specific Flow Cytometric Analysis of Cell Populations

Earlier studies have described an increased T-cell infiltration in vascular[1] and renal tissue[15] from Ang II–treated mice leading to vascular dysfunction and the development of hypertension. We, therefore, determined relative and absolute numbers of pan-T lymphocytes (CD3-positive CD45), T-helper cells (CD4-positive CD45), cytotoxic T cells (CD8-positive CD45), and γδ-T cells (γδ–T-cell receptor–positive CD45) in kidneys (Figure 2A; Figure S3A) and aortas (Figure 2B; Figure S3A) derived from B6.Rag1$^{-/-}$ and C57Bl/6J control mice after 14 days of either Ang II infusion (490 ng/min per kg) or sham treatment by flow cytometry (for gating strategy, see Figure S2). As expected, neither CD3, CD4, CD8, nor γδ-T cells could be detected in B6.Rag1$^{-/-}$ mice, independent of Ang II treatment. In C57Bl/6J controls, relative and absolute numbers of CD3, CD4, CD8, and γδ-T cells remained unaffected during the Ang II treatment. Identical findings for absolute cell number were obtained for pan-B lymphocytes (CD19-positive CD45; Figure 2). While the number of infiltrating natural killer cells (NK1.1-positive CD45) appears to be increased in untreated B6.Rag1$^{-/-}$ mice, it was not augmented by Ang II treatment in B6.Rag1$^{-/-}$ or C57Bl/6J control mice (Figure 2). natural killer T cells were not detected in B6.Rag1$^{-/-}$ mice. Flow cytometric analysis of the circulating blood yielded similar results for all cell populations (Figure S4).

Effect of Different Doses of Ang II

To test whether the absence of Ang II resistance in our B6.Rag1$^{-/-}$ mice might be related to a dose-dependent effect, we investigated the blood pressure responses to Ang II infused at a 3-fold lower (170 ng/min per kg; Figure 3A; Table) or 3-fold higher (1.500 ng/min per kg; Figure 3B; Table) dose. This dose range causes elevations of circulating Ang II from modest to a high pathophysiologic range.[16] Mean arterial pressure was unaffected by the low-dose Ang II infusion in both genotypes. In response to the high Ang II dose infusion, mean arterial pressure increased more rapidly and to a higher level than during the original protocol but without any significant difference between B6.Rag1$^{-/-}$ and control C57Bl/6J mice.

Effect of Salt Diet

The salt content of the diet fed in the 2007 study demonstrating the blood pressure differences between B6.Rag1$^{-/-}$ mice and C57Bl/6J controls in response to 490 ng/kg Ang II infusion was not reported. Since salt intake has been recently identified as a major modifier of immune responses,[17–21] we assessed whether differences in salt intake might be responsible for the discrepant

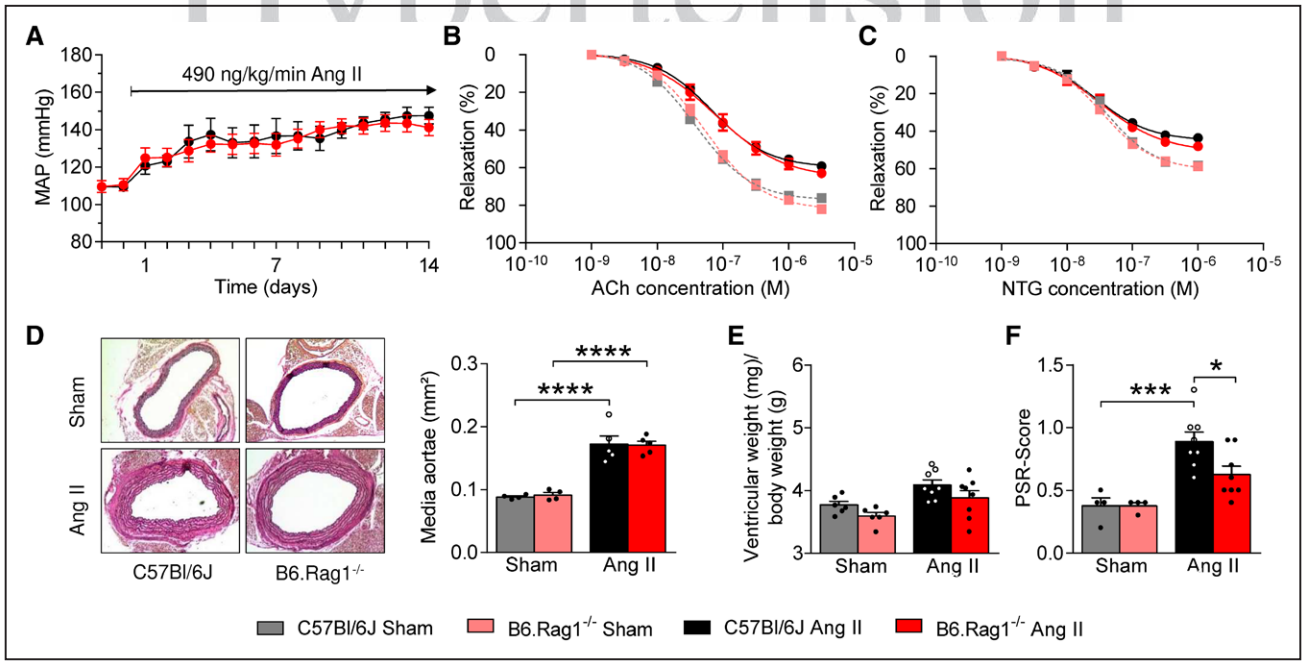

Figure 1. B6.Rag1$^{-/-}$ mice are not protected against Ang II (angiotensin II)–mediated hypertension and hypertensive end-organ damage. **A**, Telemetric blood pressure recordings in sham- or Ang II–infused C57Bl/6J (black circles) and B6.Rag1$^{-/-}$ (red circles) mice. Depicted are mean night values (7 PM to 7 AM); no statistically significant differences; 2-way ANOVA; n=7 to 8. **B** and **C**, Effect of sham or Ang II infusion (490 ng/kg per min) on endothelium-dependent (acetylcholine [Ach]) and endothelium-independent (nitroglycerin [NTG]) vasodilatation of aortic rings from C57Bl/6J (gray squares and black circles) and B6.Rag1$^{-/-}$ (light red squares and red circles) mice; no statistically significant differences; 2-way ANOVA; n=5 to 6. **D**, Representative aortic sections (**left**) and quantification of tunica media hypertrophy in the aortic wall (**right**) of sham- or Ang II–infused (490 ng/kg per min) C57Bl/6J (black bars) and B6.Rag1$^{-/-}$ (red bars) mice. ****P<0.0001, 1-way ANOVA; n=4 to 5. **E** and **F**, Ventricular weight/body weight ratios and myocardial fibrosis in sham- or Ang II–infused (490 ng/kg per min) C57Bl/6J (black bars) and B6.Rag1$^{-/-}$ (red bars) mice. ***P<0.001, *P<0.05, 1-way ANOVA; n=4 to 8. All data are expressed as mean±SEM. MAP indicates mean arterial blood pressure; and PSR, Picro-Sirius Red.

Table. Steady-State MAP Responses to Different Doses of Ang II and Dietary Salt Intake

Ang II Dose, ng/min per kg	Diet	MAP, mm Hg		See in Figure
		C57Bl/6J	B6.Rag1$^{-/-}$	
490	Normal salt (0.3% NaCl)	146±4	143±4	Figure 1A
170	Normal salt (0.3% NaCl)	117±6	114±2	Figure 3A
1500	Normal salt (0.3% NaCl)	145±6	157±9	Figure 3B
490	Low salt (0.02% NaCl)	140±4	145±3	Figure 3C
490	High salt (3% NaCl)	159±2	158±3	Figure 3D
1500	Normal salt (0.3% NaCl)		150±8	Figure 4
			140±5*	

Depicted are means±SEM of night values (7 PM to 7 AM) from continuous telemetric recordings in C57Bl/6J and B6.Rag1$^{-/-}$ averaged over the last 4 d of the Ang II infusion. Ang II indicates angiotensin II; and MAP, mean arterial blood pressure.

*B6.Rag1$^{-/-}$ mice after adoptive T-cell transfer.

phenotypes. Dietary salt depletion (0.02% NaCl; Figure 3C; Table) and dietary salt excess (3% NaCl; Figure 3D; Table) paired with the infusion of Ang II at a rate of 490 ng/min per kg elicited a significant increase in mean arterial pressure in both dietary conditions. High salt intake resulted in a stronger elevation of mean arterial pressure than low salt intake. Importantly, no significant differences in the Ang II–dependent blood pressure responses could be discerned between B6.Rag1$^{-/-}$ and C57Bl/6J control mice under both dietary conditions. In line, assessment of end-organ damage did not reveal a protection of B6.Rag1$^{-/-}$ in comparison to C57Bl/6J control mice under these conditions (Figures S5A through S5F and S6A through S6F).

Effect of Adoptive T-Cell Transfer in B6.Rag1$^{-/-}$

To test for a possible prohypertensive function of T cells, we compared the blood pressure responses to high-dose Ang II treatment (1.500 ng/min per kg) in B6.Rag1$^{-/-}$ mice after T-cell transfer or sham treatment. Mean arterial blood pressure increased to similar levels during the Ang II infusion in B6.Rag1$^{-/-}$ mice with and without adoptive T-cell transfer (Figure 4; Table).

Discussion

Shortly after the seminal observation that B6.Rag1$^{-/-}$ mice are protected from hypertension,[1] we started a project aimed at dissecting the mechanisms underlying the attenuated Ang II response in B6.Rag1$^{-/-}$ mice. Unexpectedly, we found that *RAG1* deficiency did not affect the development of Ang II–induced hypertension in mice carrying deletions in candidate genes. We, therefore, decided to reproduce the original experiments in B6.Rag1$^{-/-}$ mice as closely as possible, including the identical experimental protocol, experimental techniques, and animal source as published.[1] The average blood pressure increases and end-organ damage achieved by us in the control C57Bl/6J mice were similar to those reported by Guzik et al, consistent with a successful replication of their original experimental conditions. Nonetheless, we never observed any resistance to Ang II–induced hypertension or hypertensive end-organ damage in B6.Rag1$^{-/-}$ mice.

The absence of resistance to Ang II–mediated hypertension in B6.Rag1$^{-/-}$ mice was recently also reported by Ji et al[12] from Kathryn Sandberg's group. The authors found identical blood pressure responses to Ang II infusions at rates of 200 and 490 ng/kg per min in B6.Rag1$^{-/-}$ mice and control C57Bl/6J mice receiving a salt intake corresponding to our normal-salt diet. Based on our inability to induce different blood pressure responses in B6.Rag1$^{-/-}$ mice and control C57Bl/6J mice in this dose range, we further increased the Ang II dose, but even the administration of a high Ang II dose, which causes Ang II plasma concentrations in an upper pathophysiological range,[16] did not result in different blood pressure elevations between B6.Rag1$^{-/-}$ mice and control C57Bl/6J mice in our hands.

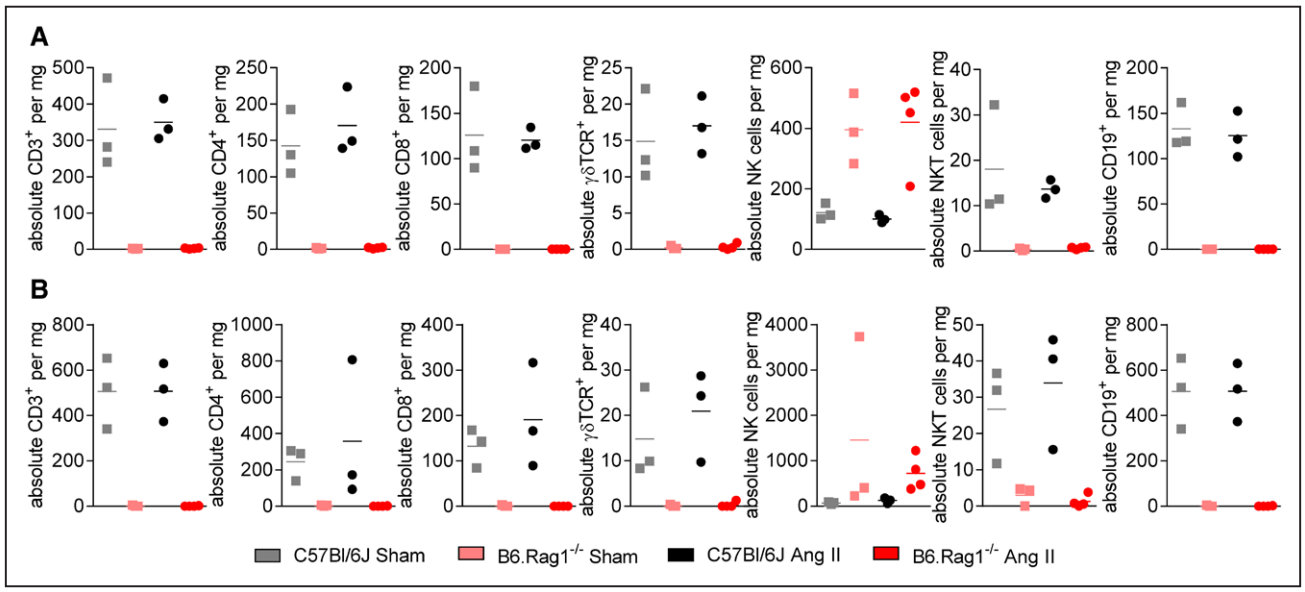

Figure 2. C57Bl/6J and B6.Rag1$^{-/-}$ mice do not show augmented angiotensin II–mediated infiltration of immune cells into renal and aortic tissue. **A** and **B**, Determination of absolute numbers of T-cell, natural killer (NK)-cell, and B-cell subpopulations by flow cytometric measurements in renal (**A**) and aortic (**B**) tissue from sham- or angiotensin II–infused (490 ng/kg per min) C57Bl/6J (gray squares and black circles) and B6.Rag1$^{-/-}$ (light red squares and red circles) mice; no statistically significant differences between sham and angiotensin II treatment within each genotype, 1-way ANOVA; n=3 to 4 CD indicates cluster of differentiation; and TCR, T cell receptor.

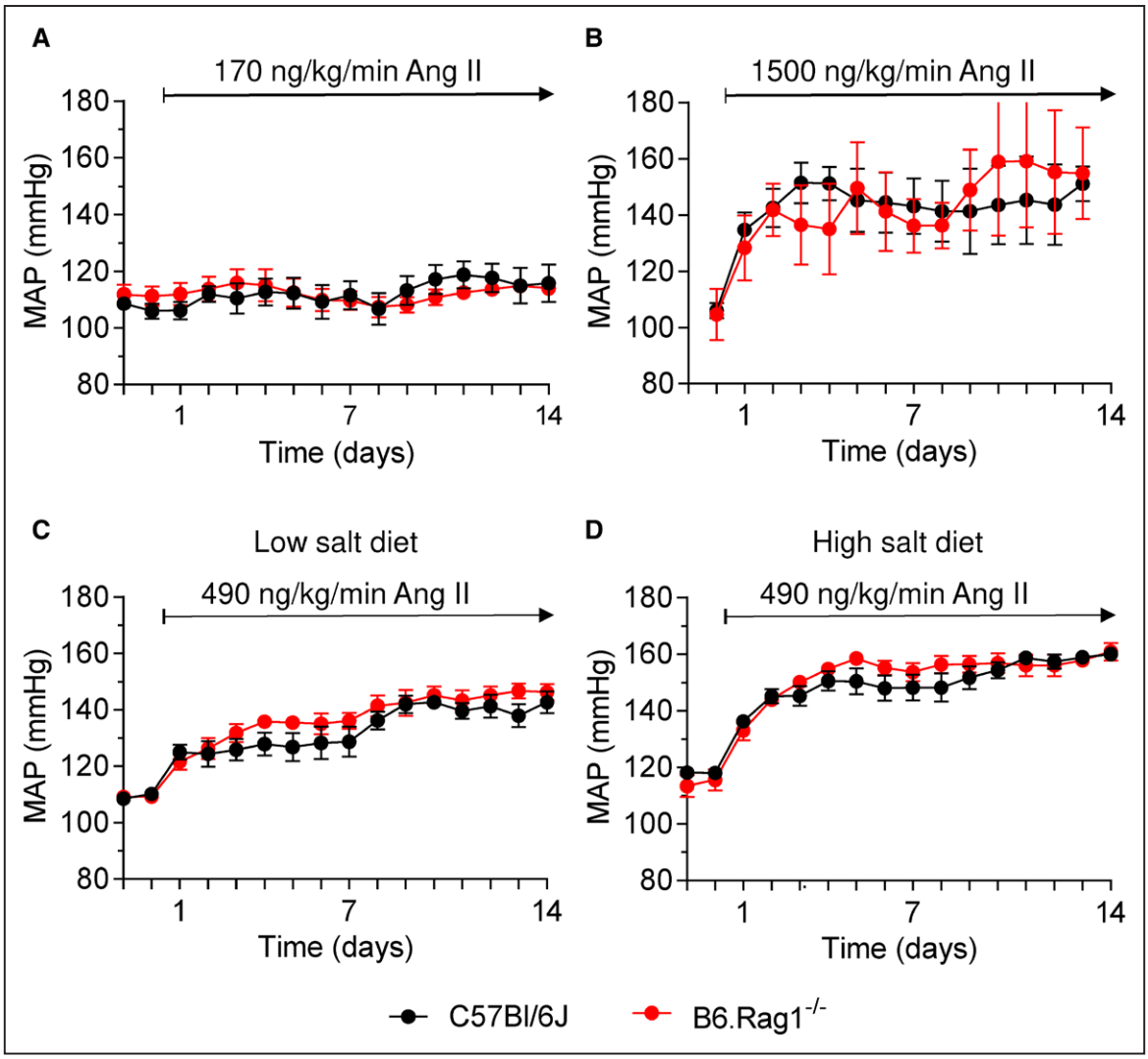

Figure 3. Effect of different doses of Ang II (angiotensin II) and different levels of salt intake on the blood pressure responses in C57Bl/6J and B6.Rag1$^{-/-}$ mice. Depicted are mean night values (7 PM to 7 AM) from continuous telemetric recordings in C57Bl/6J (black circle) and B6.Rag1$^{-/-}$ (red circle) mice on a normal-salt diet (0.3% NaCl) treated with a low-dose Ang II infusion (**A**), on a normal-salt diet (0.3% NaCl) treated with a high-dose Ang II infusion (**B**), on a low-salt diet (0.02% NaCl) treated with a medium-dose Ang II infusion (**C**), or on a high-salt diet (3% NaCl) treated with a medium-dose Ang II infusion (**D**). Means±SEM; no statistically significant differences between genotypes; 2-way ANOVA; n=5 to 8. MAP indicates mean arterial blood pressure.

Accumulating evidence indicates that excessive dietary intake of salt can lead to an inadequate activation of the immune system through a variety of pathways.[4,22] We, therefore, also varied the salt intake in our study by a factor of 150 from almost salt-free to extremely high values. Again, we found almost identical effects of Ang II on blood pressure, endothelial function, vascular structure, cardiac hypertrophy, and cardiac fibrosis in B6.Rag1$^{-/-}$ mice and control C57Bl/6J mice at low and high salt intake. Consistent with these findings, a restoration of T cells by adoptive T-cell transfer also did not augment the blood pressure response in B6.Rag1$^{-/-}$ mice in the present study.

The reasons for the lack of resistance to Ang II in B6.Rag1$^{-/-}$ mice observed here as well as in the study of Ji et al[12] remain unclear. A pathogenic role of various immune cell populations, in particular of T cells, in the development of hypertensive end-organ damage in experimental Ang II hypertension has been demonstrated by numerous independent studies.[2–4] Ji et al purchased their mice from Jackson Laboratory in 2015 and 2016. Since the same laboratory consistently observed blunted Ang II–mediated hypertensive responses, which could be fully restored to wild-type levels following the adoptive transfer of T cells in B6.Rag1$^{-/-}$ mice purchased earlier than 2016 from the Jackson Laboratory,[7] the authors suggested that spontaneous mutations might have occurred in the Jackson B6.129S7-Rag1^{tm1Mom}/J strain between 2012 and 2015, masking the protective effect of the lack of T cells. Such a presumed genetic drift, however, most likely does not account for the lack of Ang II resistance in our B6.Rag1$^{-/-}$ mice, as we purchased the mice 6 years earlier than Ji et al.[12] Additionally, to the timely aspect, it seems unlikely that mutations should have occurred in 2 independent lines, each leading to an exact normalization of the Ang II blood pressure response B6.Rag1$^{-/-}$ mice.

We, therefore, rather think that the full development of the hypertension-resistant phenotype in B6.Rag1$^{-/-}$ mice depends on the action of yet unidentified nongenetic modifiers in addition to the absence of functional T cells. The composition of

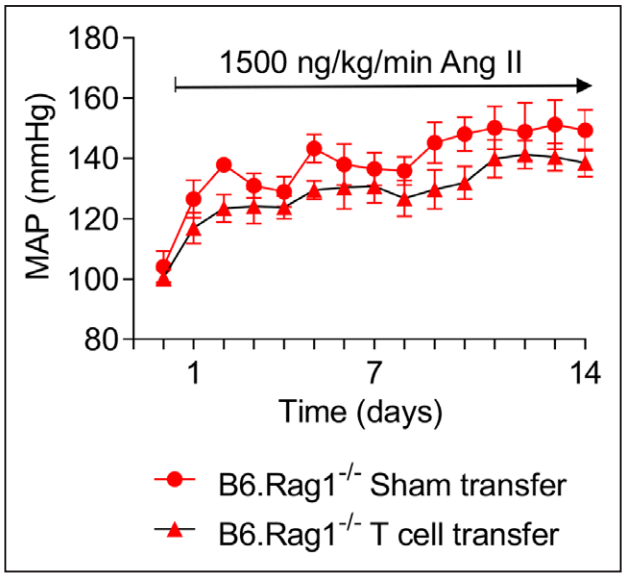

Figure 4. Effect of restoration of T cells by adoptive T-cell transfer on the blood pressure responses in B6.Rag1$^{-/-}$ mice. Depicted are mean night values (7 PM to 7 AM) from continuous telemetric recordings in B6.Rag1$^{-/-}$ mice on a normal-salt diet (0.3% NaCl) either with (red triangle) or without (red circle) injection of 10^7 donor T cells 4 wk before the commencement of a high-dose angiotensin infusion. Means±SEM; no statistically significant differences between genotypes; 2-way ANOVA; n=4 to 6. Ang II indicates angiotensin II; and MAP, mean arterial blood pressure.

the experimental diets, in addition to the sodium content, can have major effects on the development of hypertension and end-organ damage. We contacted the Jackson laboratory and were informed that the diet used there has not changed since 2007. The diet administered in our present study is almost identical to the one used by the Jackson laboratory, but other laboratories may feed their mice other diets. In addition, epigenetic changes and fetal programming effects may affect immunologic mechanisms.[23,24] For example, housing conditions such as ambient noise have been shown to significantly impact on the immune system and the feto-maternal cross talk.[25] Finally, recent studies have shown that the microbiome exerts a major influence on the immune system. Specifically, the gut microbiome has a major impact on the induction of T_H17 cells and thereby influences the development of salt-dependent hypertension.[26] The composition of the microbiome is mainly determined by environmental influences.[27] Our mice have always been kept strictly pathogen-free in the breeding units, as well as in the laboratory. Hence, differences in the microbiome in between laboratories or within one laboratory over the years may critically modulate Ang II resistance in B6.Rag1$^{-/-}$ mice and might additionally explain the lack of an inflammatory response in aortic and renal tissue in the present study. A recent study reported that C57Bl/6 mice transferred as embryos into wild mice dams (so-called wildlings) develop a microbiome throughout the body that is almost identical to that of wild mice.[28] This natural microbiome profoundly altered the immune landscape of the spleen, blood, and of immunologically important epithelial barrier sites. Most importantly, wildlings but not C57Bl/6 mice harboring a laboratory microbiome phenocopied the results of 2 clinical trials on immunotherapy. Accordingly, it would be of utmost interest to further investigate the contribution of immunologic mechanisms to the development of arterial hypertension in these wildings.

Perspectives

Hypertension is the predominant cardiovascular risk factor of our Western society. Pathogenic concepts, which contribute to the development of hypertension, are essential for defining new treatment strategies for this frequent and detrimental condition. The concept of a T-cell involvement in the pathogenesis of hypertension and in the development of end-organ damage is, therefore, compelling, as it opens new therapeutic avenues. However, in light of the varying experimental results between laboratories, the involvement of T and B lymphocytes in the development of hypertension needs to be revisited in a collaborative effort between different laboratories. Using the same B6.Rag1$^{-/-}$ strain and control littermates, the confounding involvement of nongenetic factor(s) might be identified by comparing the mouse microbiome, DNA methylation sites, or other nongenetic confounders with the responsiveness of B6.Rag1$^{-/-}$ mice to Ang II in between the contributing laboratories.

Acknowledgments

A. Seniuk designed the research, performed and supervised most of the experiments, analyzed the data, and wrote and edited the paper; J.L. Thiele performed and analyzed experiments; A. Stubbe and P. Oser performed and analyzed experiments; A. Rosendahl conducted the flow cytometric analysis; M. Bode conducted the flow cytometric analysis; C. Meyer-Schwesinger performed experiments and edited the paper; U.O. Wenzel designed the research and edited the paper; H. Ehmke designed the research and wrote and edited the paper.

Sources of Funding

This study was supported by SFB 1192.

Disclosures

None.

References

1. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C, Harrison DG. Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J Exp Med*. 2007;204:2449–2460. doi: 10.1084/jem.20070657
2. Norlander AE, Madhur MS, Harrison DG. Correction: the immunology of hypertension. *J Exp Med*. 2018;215:719. doi: 10.1084/jem.2017177301022018c
3. Rodriguez-Iturbe B, Pons H, Johnson RJ. Role of the immune system in hypertension. *Physiol Rev*. 2017;97:1127–1164. doi: 10.1152/physrev.00031.2016
4. Wenzel U, Turner JE, Krebs C, Kurts C, Harrison DG, Ehmke H. Immune mechanisms in arterial hypertension. *J Am Soc Nephrol*. 2016;27:677–686. doi: 10.1681/ASN.2015050562
5. Trott DW, Thabet SR, Kirabo A, Saleh MA, Itani H, Norlander AE, Wu J, Goldstein A, Arendshorst WJ, Madhur MS, et al. Oligoclonal CD8+ T cells play a critical role in the development of hypertension. *Hypertension*. 2014;64:1108–1115. doi: 10.1161/HYPERTENSIONAHA.114.04147
6. Wu J, Thabet SR, Kirabo A, Trott DW, Saleh MA, Xiao L, Madhur MS, Chen W, Harrison DG. Inflammation and mechanical stretch promote aortic stiffening in hypertension through activation of p38 mitogen-activated protein kinase. *Circ Res*. 2014;114:616–625. doi: 10.1161/CIRCRESAHA.114.302157
7. Ji H, Zheng W, Li X, Liu J, Wu X, Zhang MA, Umans JG, Hay M, Speth RC, Dunn SE, et al. Sex-specific T-cell regulation of angiotensin II-dependent hypertension. *Hypertension*. 2014;64:573–582. doi: 10.1161/HYPERTENSIONAHA.114.03663
8. Pollow DP, Uhrlaub J, Romero-Aleshire M, Sandberg K, Nikolich-Zugich J, Brooks HL, Hay M. Sex differences in T-lymphocyte tissue infiltration and

development of angiotensin II hypertension. *Hypertension*. 2014;64:384–390. doi: 10.1161/HYPERTENSIONAHA.114.03581
9. Uchida HA, Kristo F, Rateri DL, Lu H, Charnigo R, Cassis LA, Daugherty A. Total lymphocyte deficiency attenuates angII-induced atherosclerosis in males but not abdominal aortic aneurysms in apoE deficient mice. *Atherosclerosis*. 2010;211:399–403. doi: 10.1016/j.atherosclerosis.2010.02.034
10. Vital SA, Terao S, Nagai M, Granger DN. Mechanisms underlying the cerebral microvascular responses to angiotensin II-induced hypertension. *Microcirculation*. 2010;17:641–649. doi: 10.1111/j.1549-8719.2010.00060.x
11. Mattson DL, Lund H, Guo C, Rudemiller N, Geurts AM, Jacob H. Genetic mutation of recombination activating gene 1 in dahl salt-sensitive rats attenuates hypertension and renal damage. *Am J Physiol Regul Integr Comp Physiol*. 2013;304:R407–R414. doi: 10.1152/ajpregu.00304.2012
12. Ji H, Pai AV, West CA, Wu X, Speth RC, Sandberg K. Loss of resistance to angiotensin II-induced hypertension in the Jackson laboratory recombination-activating gene null mouse on the C57BL/6J background. *Hypertension*. 2017;69:1121–1127. doi: 10.1161/HYPERTENSIONAHA.117.09063
13. Rudolph TK, Wipper S, Reiter B, Rudolph V, Coym A, Detter C, Lau D, Klinke A, Friedrichs K, Rau T, et al. Myeloperoxidase deficiency preserves vasomotor function in humans. *Eur Heart J*. 2012;33:1625–1634. doi: 10.1093/eurheartj/ehr193
14. Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med*. 2006;203:1273–1282. doi: 10.1084/jem.20052205
15. Crowley SD, Frey CW, Gould SK, Griffiths R, Ruiz P, Burchette JL, Howell DN, Makhanova N, Yan M, Kim HS, et al. Stimulation of lymphocyte responses by angiotensin II promotes kidney injury in hypertension. *Am J Physiol Renal Physiol*. 2008;295:F515–F524. doi: 10.1152/ajprenal.00527.2007
16. Gonzalez-Villalobos RA, Seth DM, Satou R, Horton H, Ohashi N, Miyata K, Katsurada A, Tran DV, Kobori H, Navar LG. Intrarenal angiotensin II and angiotensinogen augmentation in chronic angiotensin II-infused mice. *Am J Physiol Renal Physiol*. 2008;295:F772–F779. doi: 10.1152/ajprenal.00019.2008
17. Kleinewietfeld M, Manzel A, Titze J, Kvakan H, Yosef N, Linker RA, Muller DN, Hafler DA. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature*. 2013;496:518–522. doi: 10.1038/nature11868
18. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, Regev A, Kuchroo VK. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature*. 2013;496:513–517. doi: 10.1038/nature11984
19. Binger KJ, Gebhardt M, Heinig M, Rintisch C, Schroeder A, Neuhofer W, Hilgers K, Manzel A, Schwartz C, Kleinewietfeld M, et al. High salt reduces the activation of IL-4- and IL-13-stimulated macrophages. *J Clin Invest*. 2015;125:4223–4238. doi: 10.1172/JCI80919
20. Mattson DL. Immune mechanisms of salt-sensitive hypertension and renal end-organ damage. *Nat Rev Nephrol*. 2019;15:290–300. doi: 10.1038/s41581-019-0121-z
21. Chessa F, Mathow D, Wang S, Hielscher T, Atzberger A, Porubsky S, Gretz N, Burgdorf S, Gröne HJ, Popovic ZV. The renal microenvironment modifies dendritic cell phenotype. *Kidney Int*. 2016;89:82–94. doi: 10.1038/ki.2015.292
22. Wenzel UO, Bode M, Kurts C, Ehmke H. Salt, inflammation, IL-17 and hypertension. *Br J Pharmacol*. 2019;176:1853–1863. doi: 10.1111/bph.14359
23. Liang M. Epigenetic mechanisms and hypertension. *Hypertension*. 2018;72:1244–1254. doi: 10.1161/HYPERTENSIONAHA.118.11171
24. Morton JS, Cooke CL, Davidge ST. In utero origins of hypertension: mechanisms and targets for therapy. *Physiol Rev*. 2016;96:549–603. doi: 10.1152/physrev.00015.2015
25. Solano ME, Kowal MK, O'Rourke GE, Horst AK, Modest K, Plösch T, Barikbin R, Remus CC, Berger RG, Jago C, et al. Progesterone and HMOX-1 promote fetal growth by CD8+ T cell modulation. *J Clin Invest*. 2015;125:1726–1738. doi: 10.1172/JCI68140
26. Wilck N, Matus MG, Kearney SM, Olesen SW, Forslund K, Bartolomaeus H, Haase S, Mähler A, Balogh A, Markó L, et al. Salt-responsive gut commensal modulates TH17 axis and disease. *Nature*. 2017;551:585–589. doi: 10.1038/nature24628
27. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, Costea PI, Godneva A, Kalka IN, Bar N, et al. Environment dominates over host genetics in shaping human gut microbiota. *Nature*. 2018;555:210–215. doi: 10.1038/nature25973
28. Rosshart SP, Herz J, Vassallo BG, Hunter A, Wall MK, Badger JH, McCulloch JA, Anastasakis DG, Sarshad AA, Leonardi I, et al. Laboratory mice born to wild mice have natural microbiota and model human immune responses. *Science*. 2019;365:eaaw4361. doi: 10.1126/science.aaw4361

Novelty and Significance

What Is New?

- Mice unable to generate mature T and B lymphocytes because of a disrupted recombination-activating gene 1 purchased directly from the Jackson Laboratory as early as 2009 are not protected from experimental hypertension, regardless of the Ang II (angiotensin II) dose used, the salt diet implemented, or of the restoration of T cells.

What Is Relevant?

- Even in the complete absence of T and B cells, Ang II can cause arterial hypertension and hypertensive end-organ damage to the same extent as in wild-type animals with an intact immune system.

Summary

Our results hint at a strong, as yet unidentified nongenetic modifier, which determines the relevance of T cells in the development of hypertension and hypertensive end-organ damage and which may also be relevant for other immunologically modulated phenotypes.