CD247 Modulates Blood Pressure by Altering T-Lymphocyte Infiltration in the Kidney

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Abstract—The CD3 ζ chain (CD247), a gene involved in T-cell signaling, has been shown to associate with blood pressure in human genetic studies. To test the functional role of CD247 in hypertension and renal disease, zinc-finger nucleases targeting CD247 were injected into Dahl salt-sensitive (SS/JrHsdMcw) embryos. The resulting 11-bp frameshift deletion in exon 1 of CD247 led to a predicted premature stop codon. Western blotting confirmed the absence of CD247 protein in the thymus, and flow cytometry (n=5–9 per group) demonstrated that the mutant rats (CD247–/) have a >99% reduction in circulating CD3+ T cells compared with littermate controls (CD247+/+). Studies were performed on age-matched, littermate male, CD247+/+ and CD247–/– rats fed a 4.0% NaCl diet for 3 weeks. The infiltration of CD3+ T cells into the kidney after high salt was significantly blunted in CD247–/– (1.4±0.4×10⁵ cells per kidney) when compared with that in the CD247+/+ (8.7±2.0×10⁵ cells per kidney). Accompanying the reduced infiltration of T cells, mean arterial blood pressure was significantly lower in CD247–/– than in CD247+/+ (134±1 versus 151±2 mmHg). As an index of kidney disease, urinary albumin and protein excretion rates were significantly reduced in CD247–/– (17±1 and 62±2 mg/d, respectively) when compared with that in CD247+/+ (49±3 and 121±5 mg/d, respectively). Glomerular and renal tubular damage were also attenuated in the CD247–/–. These studies demonstrate that functional T cells are required for the full development of Dahl salt-sensitive hypertension and indicate that the association between CD247 and hypertension in humans may be related to altered immune cell function. (Hypertension. 2014;63:559-564.)

Kidney

Key Words: hypertension ■ immune system ■ kidney ■ lymphocytes ■ rats

Studies in humans have demonstrated associations between genetic variants and hypertension in genome-wide association studies and other association studies. Many associated genes do not have an obvious link to blood pressure regulation; it is therefore important to explore the mechanisms whereby these genes alter blood pressure to elucidate pathways of disease and develop new approaches for drug therapy. One such gene is CD247 in which a single-nucleotide polymorphism variant in intron 1 is associated with diastolic and systolic blood pressure in hypertensive black and European American subjects. CD247, which encodes the CD3 ζ chain, is involved in the assembly and expression of the T-cell receptor complex and in signal transduction on antigen triggering. Mice with a disruption of the CD3 ζ chain demonstrate a marked reduction in thymocytes and peripheral T cells. Furthermore, a patient with somatic mutations in the CD3 ζ chain was shown to have a reduction in circulating T cells with no change in B cells. In addition, genome-wide association studies have demonstrated an association of variants in CD247 with systemic sclerosis, rheumatoid arthritis, and other autoimmune-related disorders. Although the role of CD247 in immune-related disease seems obvious, the role of this gene in hypertension is not known.

A potential link between CD247, a gene involved in T-cell signaling, and hypertension is provided by many studies that have implicated the immune system in experimental hypertension and renal disease in rats and mice. Moreover, human data are consistent with the observations in animals and indicate that the immune system plays a role in hypertension and renal disease in patients. Recent work in our laboratory has focused on the role of infiltrating immune cells in the kidney in the development of salt-sensitive (SS) hypertension and renal disease in Dahl SS rats. Dahl SS rats develop hypertension, albuminuria, and renal histological damage that are accompanied by a significant increase in infiltrating T lymphocytes and macrophages in the kidney. Interestingly, immunosuppression blocked the infiltration of T cells and macrophages into the kidney and attenuated hypertension and renal damage in Dahl SS rats fed high salt. Because CD247 is involved in T-cell signaling, the present experiments addressed the role of CD247 in the infiltration of immune cells into the kidney in Dahl SS rats fed high salt and the resulting hypertension and renal disease.

Initial experiments examined the infiltration and activation of T lymphocytes in the kidney of Dahl SS rats. Zinc-finger nuclease technology was then used to delete CD247 in the

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Dahl SS genetic background to examine the importance of T-cell activation in the disease response. Experiments were then performed in the CD247 mutant rats to investigate the infiltration of immune cells into the kidney and to assess the hypertensive and renal disease phenotypes.

Methods
All animal procedures were performed at the Medical College of Wisconsin under protocols approved by the Institutional Animal Care and Use Committee. The experimental methods detailing generation of the mutant animals, phenotyping, and statistics are described in the online-only Data Supplement.

Results
A gene expression analysis with real-time polymerase chain reaction was initially performed on RNA isolated from circulating T cells and T cells isolated from the kidneys of 12 Dahl SS rats (4 pooled samples of 3 individual rats each) fed high salt for 3 weeks (Figure 1). The infiltrating T cells in the kidney expressed significantly greater (by 5- to 54-fold) mRNA of inflammatory molecules associated with T-cell signaling. These included proliferation factors such as interleukin-2 and factors associated with T-helper 1 cells, T-helper 2 cells, regulatory T cells, and T-helper 17 cells such as interferon-γ, interleukin-4, interleukin-10, and interleukin-6, respectively. The increased expression of these factors along with other molecules associated with T-cell activity indicates that the infiltrating T cells in the kidney have proliferated and are activated relative to circulating T lymphocytes.

To examine the importance of activation of the infiltrating cells in the Dahl SS disease response, rats with a null mutation in CD247 were generated with zinc-finger nuclease technology. As described in the Methods, DNA sequencing revealed an 11-bp frameshift deletion of bases 154 to 164 in the SS-Cd247<sup>em1Mcwi</sup> mutant (CD247<sup>−/−</sup>) rats. Western blotting experiments demonstrated a deficit of CD247 protein in homogenates of the thymus of CD247<sup>−/−</sup> rats when compared with that in the CD247<sup>+/+</sup> rats (Figure S1A in the online-only Data Supplement). Flow cytometry experiments demonstrated that, in the rat, CD5 is a T-cell marker expressed on the same cells as CD247<sup>−/−</sup> cells in the CD247<sup>+/+</sup> (Figure S1B); in contrast to the CD247<sup>+/+</sup>, CD3 is absent on CD5<sup>+</sup> cells in the CD247<sup>−/−</sup> (Figure S1C). Furthermore, CD5<sup>+</sup> cells in the CD247<sup>−/−</sup> are negative for the T cell receptor α/β chain (Figure S1D and S1E). Together, these data demonstrate the absence of the T-cell receptor complex in the mutant animals. Figure 2 illustrates a flow cytometric analysis of peripheral blood mononuclear cells for CD3<sup>+</sup>, CD5<sup>+</sup>, and CD45R<sup>+</sup> cells (n=5–6 per group). Representative 2-dimensional profiles from CD247<sup>+/+</sup> (Figure 2A and 2C) and CD247<sup>−/−</sup> rats (Figure 2B and 2D) demonstrate the percentage distribution of each cell type in the peripheral blood mononuclear cell population. Those data, along with the peripheral blood mononuclear cell count, were used to assess the total circulating cells (Figure 2E). Total peripheral blood mononuclear cells were reduced by >55% in CD247<sup>−/−</sup> when compared with those in CD247<sup>+/+</sup>. Circulating CD3<sup>+</sup> cells were virtually undetectable in the CD247<sup>−/−</sup> rats, and CD5<sup>+</sup> cells were reduced by 90%. B lymphocytes, as assessed by CD45R<sup>+</sup>, were also significantly reduced in the CD247<sup>−/−</sup> by 28%. At 12 weeks (n=6–8 per group), thymus weight tended to be reduced in the CD247<sup>−/−</sup> (0.30±0.08 g) compared with wild-type (WT) littermates (0.56±0.03 g); body weights (367±10 and 347±6 g) were not different between CD247<sup>−/−</sup> and CD247<sup>+/+</sup> rats, respectively.

As indicated above, CD5, a T-cell-specific marker, was still present on circulating and splenic cells in the mutant rats. To confirm the influence of CD247 deletion on T-cell function, the proliferative response was examined in enriched CD5<sup>+</sup> cells isolated from CD247<sup>+/+</sup> and CD247<sup>−/−</sup> rats. Results of stimulation of cultured CD5<sup>+</sup> cells with concanavalin A for 4 to 6 days are illustrated in Figure S2. The fluorescent peaks illustrated in Figure S2A to S2D, moving from right to left, represent successive generations of live CD5<sup>+</sup> cells. Compared with control cells (Figure S2A), concanavalin A stimulation of CD5<sup>+</sup> cells from WT rats led to a marked proliferative response (Figure S2B), with >80% of the cells dividing at least once. In contrast, cell proliferation of CD5<sup>+</sup> splenocytes obtained from CD247<sup>−/−</sup> rats was virtually absent, averaging ≈1% of the total cells in either control or concanavalin A–stimulated conditions (Figures S2C and S2D, respectively). These experiments illustrate the importance of CD247 in T-cell proliferation. Lacking a functional CD247 gene inhibits the activation and proliferation of CD5<sup>+</sup> cells (T cells) via the T cell receptor, indicating an inability to participate in the inflammatory responses to antigens.

The changes in arterial blood pressure and albumin excretion rate in WT and SS-Cd247<sup>em1Mcwi</sup> littermates during high-salt intake are illustrated in Figure 3. The 24-hour average daily mean arterial pressure (MAP) values measured by telemetry were not different between the CD247<sup>−/−</sup> and CD247<sup>+/+</sup> rats during 7 days of low-salt intake (0.4% NaCl; n=6–8 per group). Blood pressure increased in both groups after the high-salt intake although the increase occurred more rapidly and reached a greater magnitude in the CD247<sup>−/−</sup> rats. The average 24-hour MAP value was significantly increased after 7 days of 4.0% NaCl chow in the WT rats. In contrast, a significant increase in MAP was only observed in CD247<sup>−/−</sup> rats after 18 days of high salt. Moreover, a comparison of blood pressure values between groups indicated significant differences in arterial pressure on 5 of the final 6 days of blood pressure measurement. The albumin excretion rate was elevated in CD247<sup>−/−</sup> rats fed low NaCl compared with that in the CD247<sup>+/+</sup> although the differences did not reach statistical significance. In parallel to the increase in...
blood pressure, albumin excretion rate was increased on days 7, 14, and 21 of high salt in comparison with the low-salt value in the CD247\textsuperscript{+/+} rats. The albumin excretion rate also increased in the CD247\textsuperscript{−/−} rats when NaCl intake was increased, but the albumin excretion rate in the null mutant rats was significantly less than observed in the CD247\textsuperscript{+/+}. No differences in steady-state sodium excretion rate were observed between CD247\textsuperscript{+/+} and CD247\textsuperscript{−/−} rats on the low-salt (1.0±0.1 versus 0.9±0.1 mEq/d) or high-salt diet (14.4±0.6 versus 15.0±0.6 mEq/d).

Blood pressure, albumin excretion rate, and conscious creatinine clearance were measured in a separate set of age-matched CD247\textsuperscript{+/+} and CD247\textsuperscript{−/−} littermates fed the 4.0% NaCl diet and implanted with chronic indwelling femoral arterial catheters (data not shown). Consistent with the studies described above, MAP was significantly lower in the CD247\textsuperscript{−/−} than in the CD247\textsuperscript{+/+} (134±1 versus 151±2 mm Hg; n=8–10 per group) after 3 weeks of 4.0% NaCl diet. As an index of kidney disease, urinary albumin excretion rate was significantly reduced in CD247\textsuperscript{−/−} (17±1 mg/d) when compared with that in WT rats (17±1 versus 49±3 mg/d). Conscious creatinine clearance, as an index of glomerular filtration rate tended to be greater in the CD247\textsuperscript{−/−} rats than in the WT (612±50 versus 466±73 mL/min per gram kidney weight) after 3 weeks of high salt although the difference did not reach statistical significance.

Renal histological changes and infiltration of immune cells into the kidneys are illustrated in Figure 4. The renal histological damage typically observed in Dahl SS fed high salt, including blocked and dilated tubules in the outer medulla, was reduced in the CD247\textsuperscript{−/−} rats (n=4–5 per group; Figure 4A–4F). Histological scoring demonstrated a significant reduction in the glomerular damage index, as well as a reduction in damaged tubules in the outer medulla. Finally, isolation and counting of infiltrating T cells in the kidney showed significantly fewer infiltrating CD3\textsuperscript{+} T cells in the kidneys of the CD247\textsuperscript{−/−} compared with the CD247\textsuperscript{+/+} kidneys after 3 weeks of high salt; the absolute number of CD11b\textsuperscript{+} cells (monocytes and macrophages) in the kidney was not different between groups (Figure 4G).

**Discussion**

Previous studies from our laboratory have indicated an infiltration of T lymphocytes into the kidney of Dahl SS rats fed a high-salt diet. During an immune response, antigen-presenting cells endocytose foreign material, which is presented as antigen to the T-helper cells; T cells expressing the specific
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Hypertension

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The development of hypertension in the Dahl SS rat follows a pattern in which there is an initial increase in MAP during days 1 to 5 of high salt and then a secondary phase that commences after ~10 days of high salt.14,19 The present data indicate that the magnitude of this secondary phase of hypertension in the Dahl SS is significantly attenuated and the initial increase in MAP tended to be lower in the CD247−/− rats. We previously reported a significant blunting of the hypertension throughout the high-salt period in Dahl SS rats lacking both T and B cells.18 The immune system therefore seems capable of altering the SS hypertensive response throughout the period of elevated NaCl intake. The exact mechanisms mediating the hypertensive response are unknown. The present study focused on cells infiltrating the kidney; we interpret the present data to indicate that the renal effects of T cells are mediating the hypertensive response are unknown. The present data indicate that the renal effects of T cells are participating in the observed phenotypic responses. The vascular sensitivity to angiotensin II or other vasoactive molecules in these rats is unknown.

The present experimental approach used a zinc-finger nuclease strategy to mutate CD247. This approach, which has been documented for several other genes,15,24,25 led to an 11-bp frameshift deletion in exon 1 of CD247 and resulted in a pre-mRNA stop codon downstream of the mutation. Phenotyping experiments demonstrated a loss of immunoreactive CD247 protein in the thymus of the mutant rats and a marked reduction in CD3+ cells in the circulation. The reduction of total lymphocytes and CD3+ cells is consistent with observations in a patient with somatic mutations in CD247 and also in a knockout mouse model in which the CD3ζ chain was deleted by homologous recombination.7,26 Although the CD247 mutants have almost no CD3+ cells, the CD247 mutant animals retained hypertension and renal damage.18 The present study permitted us to examine the influence of deletion of a gene associated with human hypertension and to determine the influence of selective T-cell depletion in the Dahl SS because previous studies demonstrated a central role of T cells in angiotensin II–induced hypertension in mice.23 These experiments demonstrate that selective depletion of CD3+ cells blunts Dahl SS hypertension and renal damage, indicating a central role of T cells in the disease response. Considering the significant increase in inflammatory gene expression in infiltrating T cells in the kidneys of Dahl SS, the absence of functionality of CD247 mutant T cells suggests an important role for T-cell signaling in the development of Dahl SS hypertension and renal disease.

Despite the attenuation of SS hypertension, the CD247 mutant rats still developed a significant elevation of arterial blood pressure, albuminuria, and renal damage when dietary NaCl intake was increased. Moreover, despite the large differences in blood pressure and renal damage in the rats fed high NaCl, arterial blood pressure and albumin excretion values were not significantly different between the groups on the low NaCl diet. These data indicate that the infiltration of T cells into the kidney amplifies SS hypertension in Dahl SS rats; the significant elevation in blood pressure and renal damage that develops in the CD247 mutants is apparently because of effects independent of T cells.

T-cell receptor against the antigen are activated and proliferate in a process dependent on the release of a variety of cytokines.19 The present data indicate increased expression of proliferation factors and other cytokines in the infiltrating T cells in the kidney when compared with circulating T lymphocytes. The present study describes the development of a rat in which a null mutation in CD247 was induced in the Dahl SS genetic background using zinc-finger nuclease technology. The genetic deletion of CD247 resulted in the depletion of the cell surface expression of CD3 and the T cell receptor α/β chain and inhibited the ability of the T cells to proliferate. This unique model permitted us to examine the role of T-cell activation in Dahl SS hypertension and renal damage.

Previous studies from our laboratory have indicated that immunosuppressive agents mitigate the development of SS hypertension and renal damage in the Dahl SS rat.14–17 These and similar observations in other animal models of hypertension and renal disease20–23 indicate an important role of the immune system in cardiovascular disease. Potential nonspecific effects of these pharmacological agents may cloud the interpretation of these studies. Moreover, it is difficult to ascertain the importance of a single immune cell type or to determine the time course of disease development with a pharmacological approach. To begin to address specific immune cell types in Dahl SS hypertension, we recently deleted the Rag1 gene in the SS rat; this resulted in a depletion of mature T and B cells and attenuated Dahl SS hypertension and renal damage.18 The present study permitted us to examine the influence of deletion of a gene associated with human hypertension and to determine the influence of selective T-cell depletion in the Dahl SS because previous studies demonstrated a central role of T cells in angiotensin II–induced hypertension in mice.23 These experiments demonstrate that selective depletion of CD3+ cells blunts Dahl SS hypertension and renal damage, indicating a central role of T cells in the disease response. Considering the significant increase in inflammatory gene expression in infiltrating T cells in the kidneys of Dahl SS, the absence of functionality of CD247 mutant T cells suggests an important role for T-cell signaling in the development of Dahl SS hypertension and renal disease.

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The present experimental approach used a zinc-finger nuclease strategy to mutate CD247. This approach, which has been documented for several other genes,15,24,25 led to an 11-bp frameshift deletion in exon 1 of CD247 and resulted in a predicted stop codon downstream of the mutation. Phenotyping experiments demonstrated a loss of immunoreactive CD247 protein in the thymus of the mutant rats and a marked reduction in CD3+ cells in the circulation. The reduction of total lymphocytes and CD3+ cells is consistent with observations in a patient with somatic mutations in CD247 and also in a knockout mouse model in which the CD3ζ chain was deleted by homologous recombination.7,26 Although the CD247 mutants have almost no CD3+ cells, the CD247 mutant animals retained...
a reduced number of circulating CD3+/CD5+ cells compared with CD3+/CD5+ cells in the WT. Proliferation studies of CD5+ splenocytes from both strains demonstrated that CD5+ cells in CD247 mutant animals did not respond to nonspecific activation, indicating the importance of CD247 in T-cell proliferation and activation. Because the infiltrating cells in the kidney are activated relative to circulating T cells, these results indicate that interference with T-cell activation is responsible for the attenuation of the SS disease in the Dahl SS.

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**Disclosures**

Sigma and MCW have a license agreement that could send royalties based on rat sales to the Medical College of Wisconsin.

**References**

Novelty and Significance

What Is New?
- Deletion of CD247 in the Dahl salt-sensitive (SS) genetic background resulted in an almost complete loss of circulating CD3⁺ T lymphocytes, a reduction in infiltration of T lymphocytes into the kidney and an attenuation of SS hypertension and renal damage.

What Is Relevant?
- A genetic variant in CD247 is associated with blood pressure in hypertensive patients. A potential link between CD247, a gene involved in T-cell signaling, and hypertension is provided by a large number of studies that have implicated the immune system in experimental hypertension and renal disease in rats and mice. The present studies demonstrate the important role of CD247 in SS hypertension in rats.

Summary
The CD3ζ chain (CD247), a gene involved in T-cell signaling, has been demonstrated to associate with hypertension in human genetic studies. To test the functional role of the CD3ζ chain in hypertension and renal disease, CD247 was deleted in the Dahl SS rat using a zinc-finger nuclease–mediated approach. Deletion of CD247 in the Dahl SS genetic background resulted in an almost complete loss of CD3⁺ T lymphocytes in affected rats. Compared with control animals, Dahl SS rats lacking CD247 exhibited reduced renal infiltration of T lymphocytes and an attenuation of SS hypertension and renal damage. Because renal infiltration of immune cells has also been observed in hypertensive patients, we speculate that CD247 participates in human disease by mechanisms similar to those described in the Dahl SS rat.
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CD247 Modulates Blood Pressure by Altering T Lymphocyte Infiltration in the Kidney

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DETAILED METHODS

All animal procedures and breeding were performed at the Medical College of Wisconsin under protocols approved by the Institutional Animal Care and Use Committee. The ZFN mutants were generated as previously described.\(^1,2,3\) ZFN constructs specific for the rat CD247 gene were designed, assembled, and validated by Sigma-Aldrich, to target exon 1 of CD247 (NCBI Reference Sequence: NM_001205304.1; target sequence: CTCGCCTGCACTTCTTCAAGtgcaTTCCCAGGAGCAGGTAAGG) where the capital letters are bound by each ZFN monomer, separated by a 5-bp spacer.\(^4\) In vitro-transcribed mRNA encoding the CD247 ZFNs was diluted in microinjection buffer (1 mmol/L Tris, 0.1 mmol/L EDTA, pH 7.4) at a concentration of 10 ng/µL and injected into the pronucleus of 1-cell SS/JrHsdMcwi (SS) rat embryos as described previously.\(^2\) Embryos were injected and transferred to pseudopregnant Sprague Dawley females. At 10 days of age, pups were ear punched, and DNA was extracted and screened for ZFN-induced mutations using the Surveyor Nuclease assay as described previously\(^2\) using PCR primers flanking the target site Cd247_F: 5'-TGTCAGCCACAGAACAAAGC-3' and Cd247_R: 5'-ATACCCAGCTTGCTAC-3'). Among 15 pups born, 8 positive mutant founders were identified, one of which harbored an 11 base pair frameshift deletion of bases 154-164 in exon 1 within the target sequence (TTCAAGTGCAG), resulting in a predicted stop codon within 60 base pairs of the deletion. Previous reports demonstrated that off-target effects of ZFNs are rare and separated from the target locus by backcrossing;\(^3,5\) the founder rats were backcrossed to an SS to establish the SS-Cd247em1Mcwi mutant strain. To minimize possible effects of off-target mutations, multiple separate pairs of heterozygous progeny were intercrossed to generate F2 populations that were used for phenotyping and breeding. Fluorescent genotyping was performed in subsequent generations using the same primers above, with an M13F sequence tag (5'-TGTAAAACGACGGCCAGT-3') added to the 5' end of the CD247_F primer as previously described.\(^6\)

The CD247 mutants are maintained as heterozygous breeders. Experiments were performed on age-matched wild-type (+/+) and homozygous mutant (-/-) littermates (SS-Cd247em1Mcwi). The breeders were maintained on a whole grain diet (Harlan Teklad 3075S, Madison, WI), and weanlings were fed purified AIN-76A rodent diet (Dyets, Inc.; Bethlehem, PA) containing 0.4% NaCl. Within the timeline of these experiments, we noted no differences in survival between the WT and the CD247 mutant rats.

The experimental methods for phenotyping (isolation of infiltrating mononuclear cells from the kidney, histology, immunohistochemistry, flow cytometry, Western blotting, and surgical preparation of the animals) were all performed using methods we previously described.\(^1,7,8,9,10,11\)

Immune Cell Isolation and Flow Cytometry. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.); the kidneys were flushed with heparinized saline, minced with a razor blade, and incubated in a phosphate-buffered solution containing collagenase. Mononuclear cells were separated by centrifugation on Histopaque-1083. In some experiments, infiltrating T cells in the kidney were separated by incubating the mononuclear cells with a rat Pan T cell antibody coupled to magnetic microbeads.
(MACS Rat Pan T Cells Microbeads, Miltenyi Biotec); the T lymphocytes were then isolated with a magnetic column (MACS Separation Columns, Miltenyi Biotec) and counted on a hemocytometer. In other experiments, mononuclear cells were incubated with anti-CD3 (APC-CD3, Becton Dickinson) for T cells, anti-CD5 (FITC-CD5, Biolegend) for T cells, anti-TCR \( \alpha \beta \) (alexa fluor 647-TCR \( \alpha \beta \), Biolegend) for T cells, and anti-CD45R (PE-CD45R, Becton Dickinson) for B cells. In some cases, cell viability was assessed using DAPI or 7-AAD. The cells were analyzed by flow cytometry (FACS Calibur or LSR II, Becton Dickinson) with Cellquest Pro software (Becton Dickinson), FACSDIVA software (Becton Dickinson), or FlowJo software (Tree Star).

**Gene expression analysis.** Wild-type Dahl SS rats fed 4.0% NaCl chow for 3 weeks were utilized for this study (n=12). T cells were isolated from the blood and kidneys as described above. Peripheral and infiltrating T cells from 3 rats were combined separately, resulting in 4 pooled samples of peripheral T cells and 4 pooled samples of infiltrating T cells. RNA was purified from each pooled sample using the RNeasy Plus Mini Kit (QIAGEN). RNA purity was determined on a NanoDrop 2000c (Thermo Scientific). All 260/280 ratios were between 2.00 and 2.10. 400 nanograms of RNA from each pooled sample was converted to cDNA using RT² First Strand Kit (QIAGEN). Relative expression of 84 genes related to T cells was determined via real-time PCR (qPCR) using rat Th1-Th2-Th3 RT² Profiler PCR array plates (QIAGEN) according to manufacturer instructions. qPCR was performed on an ABI 7900HT (Applied Biosystems). Expression data were analyzed using RT² Profiler PCR Array Data Analysis Template v3.3 (SABiosciences).

**T cell proliferation assay.** Age-matched CD247+/+ and -/- rats (n=4/group) maintained on 0.4% NaCl chow were used for this assay. Spleens were minced in DPBS containing 5% heat-inactivated FBS (Atlanta Biologicals) and 2 mM EDTA and passed through a 70 micron cell strainer (BD Falcon) using the plunger from a 1 ml syringe. RBCs were lysed using RBC Lysis Buffer (eBioscience). Splenocytes were passed through a 40 micron cell strainer (BD Falcon) and resuspended in appropriate medium. Splenocytes were enriched for T lymphocytes using Nylon Wool Fiber Columns (Polysciences, Inc) according to the manufacturer’s instructions. After T cell enrichment, the cells were incubated in DPBS containing 5 \( \mu \)M CellTrace Violet reagent (Life Technologies) at a concentration of 3 \( \times \) 10⁵ cells/ml for 20 min at 37°C. Cells were cultured in RPMI 1640 (Life Technologies) containing L-Glutamine, 25 mM HEPES, 100 units/ml penicillin/streptomycin, 100 \( \mu \)M sodium pyruvate, MEM non-essential amino acids, 10% heat-inactivated FBS (Atlanta Biologicals), and 10% T cell growth factor. T cell growth factor was prepared as previously described. Cells were treated at the beginning of culture with 2 \( \mu \)g/ml Concanavalin A (Sigma Aldrich). Control cells were not treated. After 4-6 days, cells were incubated with anti-CD5 for T cells and viability was assessed using 7-AAD. Flow cytometry was performed on a BD LSR II (Becton Dickinson) using FACSDIVA software (Becton Dickinson). Data was later analyzed with FlowJo software (Tree Star, Inc.).

**Western Blot Analysis.** Blotting experiments were performed with 100 \( \mu \)g of total protein obtained from the thymus in a homogenization solution containing 1% Triton-100. Proteins were separated on a 4-15% SDS-PAGE gel, transferred to a nitrocellulose
membrane (Bio-Rad), blocked for 1 hour at room temperature and incubated overnight with a monoclonal anti-CD247 primary antibody (Santa Cruz Biotech) and a monoclonal anti-β-actin (Sigma) at 4°C. The bound primary antibodies were detected with a horseradish peroxidase-labeled secondary antibody (anti-mouse IgG, Thermo Scientific) and visualized by chemiluminescence (HyGLO, Denville Scientific).

**Histological Analysis.** Kidneys were obtained for histological and immunohistochemical analysis using methods previously described. Tissue was fixed in 10% formaldehyde, paraffin embedded (Microm HMP 300), cut in 3 µm sections (Microm HM355S), mounted, and stained with Gomori’s One-Step Trichrome.

**Blood Pressure and Renal Disease Phenotyping.** The influence of an elevated NaCl intake on the development of hypertension and renal damage was assessed in male rats, aged approximately 9 weeks, and instrumented with telemetry transmitters with the catheter implanted in the carotid artery. The rats were permitted to recover for 3 days; blood pressure was then continuously monitored for 7 days while the rats were maintained on the 0.4% NaCl chow and for 21 days following the transition to 4.0% NaCl chow. An overnight urine collection was obtained during the low salt period and after 7, 14, and 21 days of the high salt diet. After three weeks of high salt intake, the rats were deeply anesthetized and the kidneys were flushed and prepared for T cell isolation and counting or for histology and immunohistochemistry. Peripheral blood was obtained from some rats for flow cytometry. A separate set of rats were fed the 4% NaCl chow for three weeks and then implanted with chronic indwelling femoral arterial catheters; arterial blood pressure, urinary creatinine, albumin, and protein excretion rate, and plasma creatinine concentration were assessed in these rats.

**Statistical Analysis.** Data are expressed as the mean ± one standard error. Data were assessed for significance using a t-test, a one-way repeated measures analysis of variance (ANOVA) with a Tukey post-hoc test, or a two-way repeated measures ANOVA with a Holm-Sidak post-hoc test. A probability value of P<0.05 was considered significant.

**Supplemental Reference List**

Expression of zeta but not eta transcripts by rat T cells. *Journal of Immunology*, 1993;151:4705-4717.


Supplemental Figures

**FIGURE S1:** Western blot of CD247 and β-actin protein in thymus homogenates obtained from CD247+/+ and CD247-/- rats (A). Flow cytometric demonstration of CD5 and CD3 in the same subset of CD247+/+ splenocytes (B), and absence of CD3 in CD5+ cells in CD247-/- splenocytes (C). Flow cytometric demonstration of surface expression of TCRαβ in CD5+ splenocytes obtained from CD247+/+ rats (D) and absence of TCRαβ on CD5+ splenocytes obtained from CD247-/- rats (E).
FIGURE S2: Flow cytometric demonstration of cell proliferation in CD5+ splenocytes from CD247+/+ (A,B) and CD247-/- rats (C,D). Representative control (A,C) and Concanavalin A-stimulated cells (B,D). The graph illustrates cell division in control and Concanavalin A-stimulated CD5+ cells from CD247+/+ rats with minimal proliferation in CD247-/- (E). * indicates P<0.05 vs. control.