CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Innate Immune Cells Are Regulated by Axl in Hypertensive Kidney

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The balance between adaptive and innate immunity in kidney damage in salt-dependent hypertension is unclear. We investigated early renal dysfunction and the influence of *Axl*, a receptor tyrosine kinase, on innate immune response in hypertensive kidney in mice with lymphocyte deficiency (*Rag1*^{-/-}). The data suggest that increased presence of CD11b⁺ myeloid cells in the medulla might explain intensified salt and water retention as well as initial hypertensive response in *Rag1*^{-/-} mice. Global deletion of *Axl* on *Rag1*^{-/-} background reversed kidney dysfunction and accumulation of myeloid cells in the kidney medulla. Chimeric mice that lack *Axl* in innate immune cells (in the absence of lymphocytes) significantly improved kidney function and abolished early hypertensive response. The bioinformatics analyses of *Axl*-related gene-gene interaction networks established tissue-specific variation in regulatory pathways. It was confirmed that complement C3 is important for *Axl*-mediated interactions between myeloid and vascular cells in hypertensive kidney. In summary, innate immunity is crucial for renal dysfunction in early hypertension, and is highly influenced by the presence of *Axl*. (*Am J Pathol* 2018; 188: 1794–1806; <https://doi.org/10.1016/j.ajpath.2018.04.013>)

The kidney is an important target of hypertension-induced organ injury, because it plays a significant role in the regulation of fluid and electrolyte balance as well as blood pressure (BP). The immune system is proved to regulate angiotensin II (AngII) or deoxycorticosterone-acetate (DOCA) and salt types of hypertension, specifically via T lymphocytes on vascular dysfunction in *Rag1*^{-/-} mice.¹ A recent report² shows that B lymphocytes also promote pathologic vascular remodeling and AngII-induced hypertension. However, deficiency of AngII type 1 receptor on T cells significantly exaggerates kidney injury in hypertension, suggesting some protective roles of lymphocytes.³ Pathophysiological mechanisms involved in the T-lymphocyte-mediated regulation of the kidney and arteries in hypertension are still unclear.

Innate immunity [mast cells, eosinophils, basophils, neutrophils, dendritic cells, macrophages, and natural killer (NK) cells] has also been shown to affect kidney function directly or via activation of lymphocytes in hypertension.⁴ The growth arrest-specific gene 6 (*Gas6*) and its receptor

tyrosine kinase (*Axl*) are known to control innate immune cell functions.⁵ *Gas6* and *Axl* contribute to DOCA-salt hypertension.^{6,7} *Axl* in bone marrow cells improves early kidney dysfunction, whereas lack of *Axl* in T cells prevents vascular remodeling in the late phases of DOCA-salt hypertension.^{8,9} A complement pathway [complement 3 (C3)] was among one of the major proinflammatory mediators that was down-regulated in chimeras with *Axl* depletion in bone marrow cells in hypertensive kidney or after vascular injury.^{8,10} In this study, we focused on

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exploring the role of innate immunity in early hypertension and the influence of *Axl* on innate immune cells in hypertensive kidney.

Materials and Methods

Animals

Axl wild-type (*Axl*^{+/+}) and *Axl* knockout (*Axl*^{-/-}) male mice were used from our colony. Breeding pairs of B6.129S7-Rag1^{tm1Mom/J} (*Rag1*^{-/-}) and B6.SJL^{PtpcaPep3b/}BoyJ (B6.SJL) mice and C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Double wild-type (*Axl.Rag1*^{+/+}) and double knockout (*Axl.Rag1*^{-/-}) mice were generated in an intercross between heterozygous *Axl.Rag1*^{+/-} littermates (Supplemental Figure S1, A and B). Gene presence or deletion was confirmed by two-step genotyping using DNA oligonucleotides (Integrated DNA Technologies, Skokie, IL): *Axl* (forward: 5'-AGAAGGGGTAGATGAGG-3', forward: 5'-ACCGCTTCCTCGTGCTTTA-3', and reverse: 5'-GCCGAGGTATAGGCTGTC-3') and *Rag1* (forward: 5'-GAGGTTCCGCTACGACTCTG-3', forward: 5'-TGGATGTGGAATGTGTGCGAG-3', and reverse: 5'-CCGGACAA-GTTTTTCATCGT-3'). Animal facility was controlled by the 12-hour light/dark cycle (lights on at 6 AM, and lights off at 6 PM). Mice had free access to chow and water. The studies were conducted on the basis of guidelines from the NIH and the American Heart Association for the *Guide for the Care and Use of Laboratory Animals*,¹¹ and they were approved by the University of Rochester Animal Care Committee (Rochester, NY).

Isolation of Immune Cells from Peripheral Tissues

CD4⁺ T lymphocytes were isolated from spleens and lymph nodes of *Axl*^{+/+} mice with CD4⁺ T-cell enrichment kit (Miltenyi Biotech, Bergisch Gladbach, Germany) with negative magnetic sorting (AutoMACS), as before.⁹ Donor bone marrow cells were collected from tibia and femur bones of the *Rag1*^{-/-}, *Axl.Rag1*^{-/-}, and B6.SJL mice, as shown before.^{8,9} Peripheral blood or kidneys from mice after adoptive transfer or bone marrow transplant were collected as before.^{8,9}

Adoptive Transfer of the CD4⁺ T Cells into *Rag1*^{-/-} and *Axl.Rag1*^{-/-} Mice

Rag1^{-/-} or *Axl.Rag1*^{-/-} mice were adoptively transferred with donor *Axl*^{+/+} CD4⁺ T cells [6×10^6 in 0.2 mL sterile phosphate-buffered saline (PBS)] via tail vein injections (Supplemental Figure S2). *Axl*^{+/+} mice were used as controls. Successful repopulation after 3 weeks was confirmed by flow cytometry with CD4⁺ antibody in peripheral blood.

Bone Marrow Transplant of the *Rag1*^{-/-} or *Axl.Rag1*^{-/-} Bone Marrow Cells

Bone marrow transplants were done between CD45.2⁺ donors (*Rag1*^{-/-} or *Axl.Rag1*^{-/-} mice) and CD45.1⁺ recipients (B6.SJL). Recipient mice were irradiated (9.0 Gy) to ablate the host bone marrow in RS2000 irradiator (Rad Source Technologies, Inc., Buford, GA). Within 3 to 4 hours after irradiation, the recipient mice were injected with donor-derived cells (6×10^6 in 0.2 mL sterile PBS) via tail vein. Control chimeras received CD45.1⁺ BM cells after irradiation (Supplemental Figure S3, A and B). Repopulated cells were confirmed by flow cytometry (CD45.1⁺, CD45.2⁺, and CD3⁺ antibody cocktail) in peripheral blood after 6 weeks.

Isolation of Primary Smooth Muscle Cells from *Axl* Mice

Mouse aortic smooth muscle cells (MASMCs) were isolated from *Axl*^{+/+} and *Axl*^{-/-} mice, as described previously.^{10,12} MASMCs were used at 70% to 80% confluence of passages from three to five in experiments.

Migration of Smooth Muscle Cells

MASMC migration was measured using a Boyden chamber assay, as described previously.¹³ Cells were serum starved with 3% Dulbecco's modified Eagle's medium for 24 hours. Control medium (3% serum Dulbecco's modified Eagle's medium) or medium containing C3 (0.01, 0.1, or 1.0 µg/mL in Dulbecco's modified Eagle's medium) alone or combination with Gas6 (100 nmol/L in Dulbecco's modified Eagle's medium) was placed in the lower chamber. A polyvinylpyrrolidone-free polycarbonate membrane coated with collagen was placed over the bottom wells. Total number of 10,000 cells was placed into the upper chamber and incubated for 6 hours at 37°C in 95% air/5% CO₂. Migrated cells to the lower side of the Boyden chamber were fixed and stained using a Diff-Quik staining set (VWR, Radnor, PA). Positive cells were quantified in a blind manner (S.N.B., G.J.D., and V.A.K.) using MCID image software (MCID Elite version 6.0; Imaging Research, Toronto, Canada).

Biochemistry Analysis of Complement 3 in *Axl* Mice

Mice were deeply anesthetized, and blood was collected in EDTA-coated collection tubes via heart puncture. Plasma was immediately separated in a centrifuge (Clinical 50; VWR) and stored at -80°C until used as described.¹² Livers from *Axl* mice were snap frozen in liquid nitrogen and, later, were sonicated on ice in a cell lysis buffer (1×; Cell Signaling Technology, Danvers, MA) with protease inhibitor (1:1000; Sigma, St. Louis, MO) for 3- to 5-second bursts three times. Homogenates were centrifuged at $8,050 \times g$ for 10 minutes at 4°C (accuSpin Micro17R; Thermo Fisher Scientific, Waltham, MA). Bradford protein

assay (Bio-Rad, Hercules, CA) was performed, and equal amounts of protein were separated on 8% SDS gels. Proteins were transferred to nitrocellulose membrane and blocked in 5% milk-PBS for 1 hour at room temperature, followed by overnight incubation in 2% bovine serum albumin–PBS–Tween containing C3 (1:1000; Novus Biologicals, Littleton, CO) and glyceraldehyde-3-phosphate dehydrogenase (1:1000; Cell Signaling Technology) antibodies. Blots were washed in PBS–Tween and incubated with secondary horseradish peroxidase–conjugated rabbit antibodies for 2 hours at room temperature. Protein was visualized using enhanced chemiluminescence. Quantifications of protein levels were assessed through densitometry analyses with ImageJ software version 1.46r (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) and expressed as the ratio of the target proteins/loading control. Total C3 level in the plasma from *AxI* mice was quantified using enzyme-linked immunosorbent assay kit by following manufacturer's instructions (GenWay, San Diego, CA) using a plate reader (Victor²; Wallac, Winooski, VT).

DOCA and Salt Mouse Model of Hypertension

Mice were challenged with DOCA-salt mouse model of hypertension that was described before, with minor changes.⁶ Specifically, mice were anesthetized with an i.p. cocktail of ketamine and xylazine (130 and 9 mg/kg, respectively). An incision was made to expose the left kidney, which was ligated and removed. At the time of surgery, a 75-mg DOCA pellet (21-day release; Innovative Research of America, Sarasota, FL) was placed subcutaneously in a lateral area on the back of mice, and mice were provided with regular chow and 1% NaCl drinking ad libitum. The control animals were uninephrectomized (Nephx) and kept on regular chow and water. To alleviate postoperative pain, mice were treated with i.p. analgesics buprenorphine HCl (0.1 mg/kg) and flunixin meglumine (120 mg/kg). In the pilot studies, it was found that post-surgical pain regimen with opioid analgesic (buprenorphine) with flunixin had no effect on survival and/or development of 6-week DOCA-salt hypertension compared with combination of the flunixin and topical analgesic (bupivacaine, 1 mg/kg) in *AxI*^{+/+} mice (Supplemental Figure S4). Systolic BP was measured 1 week after the surgery using noninvasive tail-cuff method plethysmography (Visitech Systems, Apex, NC), as before.⁶

Ultrasound Analysis of the Hypertensive Kidneys

High-resolution ultrasound system (Vevo2100; FUJIFILM Visual Sonics, Toronto, Canada) was applied to evaluate hypertensive kidneys and renal artery hemodynamics, as previously reported.¹² Mice were anesthetized with isoflurane and monitored to maintain heart rate >500 beats/min during measurements. Three-dimensional imaging of the right kidneys was captured with the Vevo2100. Kidney

volume, vascularity, fluid, and analyzed renal artery hemodynamics were calculated using VevoLab analysis software version 1.6.0 (FUJIFILM VisualSonics, Toronto, Canada). Reconstruction of the kidney vascularity and in-kidney fluid movie (Supplemental Movie S1) were done using Amira 3D software release 5.5 (FEI, Waltham, WA).

Evaluation of the Kidney Functions in Mice

Mouse urine was collected in Nalgene (North Las Vegas, NV) diuresis cages for 24 hours, as reported.⁸ Microalbumin Blue 580 assay¹⁴ was used, and concentrations of microalbumin were measured in mouse urine. Twenty-four-hour albumin was also calculated in urine volume per body weight (mg/mL per g). The University of Rochester Clinical Laboratories processed samples for Na⁺ and Cl[−] (mmol/L) in mouse urine. Na⁺ and Cl[−] microequivalents (mEq) were calculated in urine for 24 hours.

Flow Cytometry

Four-color BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) was used for detection of the CD4⁺ T cells (CD4–fluorescein isothiocyanate; eBioscience, Waltham, MA) after adoptive transfer experiments or repopulated cells by cocktail of antibodies (CD45.1–fluorescein isothiocyanate, 1:500; CD45.2-phosphatidylethanolamine (PE), 1:500; and CD3-APC, 1:100; eBioscience) in chimeric mice in peripheral blood. Five major subsets of the immune cells were detected using 12-color LSRII flow cytometer (BD Biosciences), as reported.⁸ The cells were incubated with a cocktail of CD45.2-PE (1:500; eBioscience), CD3-APC (1:200; eBioscience), CD19-PE-CYC (1:500; eBioscience), CD11b-PE-CY5.5 (1:500; BD Bioscience), CD11c-PE-TXR (1:500; Invitrogen, Carlsbad, CA), and NK1.1-APC-CY7 (1:100; BioLegend, San Diego, CA) antibodies at room temperature for 30 minutes. Cells were washed and resuspended in FACS buffer. Compensation controls were stained with single antibodies. FlowJo software version 7.6.3 (FlowJo LLC, Ashland, OR) was used for flow cytometry analyses.

Immunohistochemical Evaluation of the Kidneys from Hypertensive Mice

Experimental and control mice were perfusion fixed with 10% paraformaldehyde, and kidneys were processed as before.^{6,8} Kidney cross sections were incubated with 3% H₂O₂ to block endogenous peroxidase activity. Antigen retrieval was performed with a Decloaker buffer (pH = 6.0; Biocare, Pacheco, CA), and high temperature was applied in the following protocols. A rat anti-mouse Mac-2 (1:15,000; Cedarlane, Burlington, ON, Canada) antibody was applied for 60 minutes at room temperature, which was subsequently stained with rabbit anti-mouse T-cell Ig and mucin domain 1 (TIM-1; 1:5000; Thermo Fisher Scientific) overnight at 4°C. A mouse anti-mouse CD45.2 (1:100;

Pharmingen, San Jose, CA) antibody was applied at 37°C for 60 minutes, which was followed by rabbit anti-mouse TIM-1 (1:5000; Thermo Fisher Scientific) overnight at 4°C. A rabbit anti-mouse C3 (1:2000; Novus Biologicals) was incubated overnight at 4°C. A rabbit anti-Axl (1:500; Abcam, Cambridge, UK) antibody was incubated for 60 minutes at room temperature. Peroxidase-binding sites (Mac-2, CD45.2, C3, and Axl) were verified with 3,3'-diaminobenzidine (Dako, Santa Clara, CA) after application of the rat-on-mouse, mouse-on-mouse, or rabbit-on-mouse horseradish peroxidase—polymer (Biocare). The alkaline-phosphatase—binding sites (TIM-1) were recognized by Fast Red (Vulcan Red; Biocare) after rabbit-on-rodent or mouse-on-mouse alkaline-phosphatase-polymer (Biocare). All double-labeled slides were counterstained with methyl green or hematoxylin, as before.^{9,10,12} SPOT INSIGHT FireWire camera (Diagnostic Instruments, Sterling Heights, MI) was used to capture kidney sections. Size and contrast of the images were uniformly adjusted (Adobe Photoshop CS3 version 10.0; Adobe, San Jose, CA) to meet the journal guidelines. Positively stained cells were analyzed in a blind manner (G.J.D. and V.A.K.) by using ImagePro Analyzer version 6.2.1 (Media Cybernetics, Rockville, MD) in three mice, as before.¹⁰ A percentage of positive cells (brown versus pink staining) was determined in relationship to negative cells (green color) by defined kidney area (medulla versus cortex).

Bayesian Network Models of Causal Gene-Gene Interactions for Axl

Gene regulatory networks were reconstructed using bayesian network models.^{15,16} The bayesian networks are directed acyclic graphs, in which the edges of the graph are defined by conditional probabilities that characterize the distribution of states of each gene given the state of its parents. The network topology defines a partitioned joint probability distribution over all genes in a network. Bayesian network models from human^{17,18} and mouse studies were constructed on the basis of genetics and gene expression data generated from multiple tissues from multiple previously published studies, each involving hundreds of individuals.^{18–22} In addition, the Genome-Scale Integrated Analysis of Gene Networks in Tissues networks,²³ including adipose, aorta, artery, blood vessel, kidney, macrophage, mast cell, monocyte, mononuclear phagocyte, NK cell, and nephron, were also combined in the analyses. Pathway analysis was performed using KEGG, REACTOME, and BIOCARTA to highlight the potential effect of the *Axl* and its partners within the gene-gene interaction network using Fisher's exact test, followed by multiple testing using the Q value approach.²⁴

Statistical Analysis

Data are represented as means $\pm$ SEM. JMP software version 12.0.0 (SAS, Cary, NC) was used for statistical analyses.

Differences between two groups were determined by unpaired *t*-test. For more than two experimental groups, one-way analysis of variance was applied, followed by post hoc comparisons (*t*-test). *P* < 0.05 was regarded as significant.

Results

Lack of Lymphocytes Has No Effect on Renal Dysfunction in Early DOCA-Salt Hypertension

Despite previous data on protection from DOCA-salt and AngII-induced hypertension in *Rag1*^{−/−} mice,¹ it was recently reported that systolic BP significantly increased in *Rag1*^{−/−} compared with wild-type B6 mice after 1 week of DOCA-salt.⁹ To further investigate this time-dependent discrepancy, focus was given to assessing kidney functions at 1 week of DOCA-salt in B6 and *Rag1*^{−/−} mice (Figure 1). As expected, B6 and *Rag1*^{−/−} mice had similar increases in systolic BP (approximately 30 mmHg) compared with their baseline or Nephx value (Figure 1A). Both genotypes showed elevated albumin in urine after DOCA-salt versus Nephx (Figure 1B). Fluid intake was significantly increased in *Rag1*^{−/−} mice, whereas urine volume increased in both genotypes after DOCA-salt (Supplemental Table S1). Urine Na⁺ and Cl[−] mEq were significantly increased after DOCA-salt versus Nephx in both genotypes (Supplemental Figure S5A). However, urine Na⁺ and Cl[−] mEq were lower in *Rag1*^{−/−} versus B6 after DOCA-salt (Supplemental Figure S5A). An advanced three-dimensional imaging revealed slightly higher kidney volumes *in vivo* versus kidney weights *ex vivo* relative to body weights after DOCA-salt in both genotypes (Supplemental Movie S1 and Figure 1, C and D). *Rag1*^{−/−} mice were manifested by accumulation of twice as much fluid in the medullary region compared with B6 after DOCA-salt (Figure 1, C and E). Blood flow velocity in the renal artery was similar between genotypes after DOCA-salt (Supplemental Table S2). Thus, mice lacking lymphocytes have pronounced impairment in renal function and are not protected from initial phase of DOCA-salt hypertension.

Rag1^{−/−} Mice Increase Kidney Accumulation of Myeloid Cells and Medulla Damage after DOCA-Salt

A higher amount of innate versus adaptive immune cells has previously been shown in the kidneys after 1 week of DOCA-salt.⁸ As expected, *Rag1*^{−/−} mice had low numbers of CD45⁺ or CD11b⁺ cells, whereas CD3⁺ lymphocytes were below detection levels in the blood compared with Nephx B6 mice (Figure 2A). DOCA-salt significantly reduced numbers of circulating leukocytes in B6 mice, whereas these cells remained at low levels in *Rag1*^{−/−} mice (Figure 2A). In contrast, comparable numbers of CD45⁺ and CD11b⁺ cells were found in kidneys from both genotypes after Nephx (Figure 2B).

Figure 1 Lack of T lymphocytes does not prevent the development of deoxycorticosterone-acetate (DOCA) salt hypertension and renal dysfunction. **A:** Systolic blood pressure (BP) in C57BL/6J (B6) and *Rag1*^{-/-} mice after uninephrectomy (Nephx) or DOCA and 1% NaCl in drinking water for 1 week. Open bars show baseline values. Closed bars show mice after 1 week. **B:** Twenty-four hour urine concentration of albumin relative to body weight in B6 and *Rag1*^{-/-} mice. **C:** Three-dimensional (3D) images of kidney representing vasculature and renal fluid retention after 1 week of DOCA-salt. **D:** Relative kidney weight (black bars) versus kidney volume (white bars) changes in B6 and *Rag1*^{-/-} mice. **E:** Percentages of the kidney vasculature (black bars) and renal fluid (white bars) on the basis of 3D kidney imaging in B6 and *Rag1*^{-/-} mice. Data are expressed as the means $\pm$ SEM (**A**, **B**, **D**, and **E**). $n = 4$ to 6 per group (**A**, **B**, **D**, and **E**). * $P < 0.05$ versus Nephx, B6; [†] $P < 0.05$ versus Nephx, *Rag1*^{-/-}; [‡] $P < 0.05$ versus DOCA, B6.

There was significant increase in total kidney CD45⁺ cells that was reflected by a threefold increase in CD11b⁺ numbers in kidneys from *Rag1*^{-/-} versus B6 mice after DOCA-salt (Figure 2A). There was no difference in CD11c⁺ or NK1.1, and CD19⁺ cells mirrored CD3⁺ profiles in both blood and kidneys across groups (Supplemental Figure S6). Histologic analyses of kidneys demonstrated redistribution of tissue macrophages (Mac-2⁺) from cortex (low in *Rag1*^{-/-} versus high in B6) (Supplemental Figure S7A) to medulla (Figure 2, C and D). Quantifications showed significantly more Mac-2⁺ in *Rag1*^{-/-} versus B6 mice after DOCA-salt (Figure 2, C and D). We also found that tubular damage marker (TIM-1) was uniformly distributed in medulla and cortex in B6 mice after DOCA-salt (Figure 2C and Supplemental Figure S7C). In contrast, *Rag1*^{-/-} mice exhibited reduced TIM-1⁺ areas that were closer to the clusters of Mac-2⁺ cells after DOCA-salt (Figure 2, C and D). Our data suggested an increased myeloid immune cell relocation to the kidney medulla or extravasation, which might explain renal dysfunction in mice lacking lymphocytes after DOCA-salt.

Global Deletion of *Axl* on *Rag1*^{-/-} Background Reverses Kidney Dysfunction after DOCA-Salt

Chimeric mice with depletion of *Axl* improved DOCA-salt hypertension, with low CD11b⁺ cells in kidneys.⁸ *Axl* immunoreactivity was uniformly distributed in kidneys from B6 mice after 1 week of DOCA-salt (Supplemental Figure S1C). In contrast, *Rag1*^{-/-} mice had much stronger cellular *Axl*⁺ staining in tubulointerstitial area after DOCA-salt (Supplemental Figure S1C). There was only slight decline in BP elevation of *Axl.Rag1*^{-/-} compared with *Axl.Rag1*^{+/+} littermates after DOCA-salt (Figure 3A). Fluid intake and urination in *Axl.Rag1*^{-/-} was similar to that in *Rag1*^{-/-} after 1 week of DOCA-salt (Supplemental Table S1). However, there was a significant increase in renal vascularity and similar kidney fluid in *Axl.Rag1*^{-/-} after DOCA-salt (Figure 3B). Unlike *Rag1*^{-/-} after DOCA-salt, Na⁺ and Cl⁻ mEq in urine were normalized in *Axl.Rag1*^{-/-} compared with *Axl.Rag1*^{+/+} littermates after DOCA-salt (Supplemental Figure S5B). Improvements of kidney function were also reflected by comparable Mac-2⁺ between *Axl.Rag1* genotypes as well as more uniform

Figure 2 Innate immune cells infiltrate the kidney in response to 1 week of deoxycorticosterone-acetate (DOCA) salt in mice without lymphocytes. Immune cells isolated from blood and kidneys from C57BL/6J (B6) and *Rag1*^{-/-} mice after 1 week of uninephrectomy (Nephx) or DOCA-salt by flow cytometry. **A:** Numbers of CD45⁺ cells, CD3⁺ cells, and CD11b⁺ cells in the blood. **B:** Numbers of CD45⁺ cells, CD3⁺ cells, and CD11b⁺ cells in the kidney. **C:** Representative images of the double-stained [Mac-2 and T-cell Ig and mucin domain 1 (TIM-1)] medulla regions of the kidneys from B6 and *Rag1*^{-/-} mice after DOCA-salt. Nephx controls are shown in insets. Mac-2⁺ cells are brown (black arrows). TIM-1⁺ staining is pink (white arrows). Counterstain is green. **D:** Quantitative analysis of Mac-2 and TIM-1 expression in the medullary region of the kidney. Open bars show mice after Nephx. Closed bars show mice after DOCA-salt. Data are expressed as the means ± SEM (**A**, **B**, and **D**). *n* = 3 per group (**A**, **B**, and **D**). **P* < 0.05 versus Nephx, B6; †*P* < 0.05 versus Nephx, *Rag1*^{-/-}; ††*P* < 0.05 versus DOCA, B6. Scale bar = 100 μm (C).

distribution of TIM-1⁺ in medulla (Figure 3, C and D). However, low Mac-2⁺ cells with equal TIM-1⁺ in the cortex in *Axl.Rag1*^{-/-} were the same as in *Rag1*^{-/-} after DOCA-salt (Supplemental Figure S7, B and D). Global deletion of *Axl* on *Rag1*^{-/-} background is critical in reversing kidney dysfunction and accumulation of myeloid cells in the medulla.

Deletion of Axl in Innate Immune Cells Prevents Early DOCA-Salt Hypertension

The contribution of Axl signals in T cells versus non-lymphoid lineages was investigated in early stage of DOCA-salt by adoptive transfer of Axl^{+/+} CD4⁺ T cells to *Rag1*^{-/-} or *Axl.Rag1*^{-/-} (Supplemental Figure S2). The hypertensive responses and kidney vascularity were similar across Axl^{+/+}, CD4⁺ T cells → *Rag1*^{-/-}, or CD4⁺ T cells → *Axl.Rag1*^{-/-} mice (Supplemental Figure S2). Adoptive transfer of the CD4⁺ lymphocytes had no effect on fluid intake or urination in *Rag1*^{-/-} versus *Axl.Rag1*^{-/-} and Axl^{+/+} mice (data not shown). These findings suggest that

CD4⁺ T lymphocytes have little effect on kidney dysfunction after DOCA-salt.

To gain insight into the role of Axl in innate immune cells in early kidney damage, B6.SJL recipients (CD45.1⁺) were repopulated with bone marrow cells (CD45.2⁺ and lacks lymphocytes) from *Rag1*^{-/-} (expressed Axl) or *Axl.Rag1*^{-/-} mice (Supplemental Figure S3). Chimeric mice with no lymphocytes and Axl in innate immune cells only (*Axl.Rag1*^{-/-} → B6.SJL) averted BP increase and significantly increased kidney vascularity compared with chimeras without lymphocytes (*Rag1*^{-/-} → B6.SJL) or complete immune system (B6.SJL → B6.SJL) (Figure 4, A and B). There was a trend toward a smaller percentage of kidney fluid accumulation (*P* = 0.10) in *Axl.Rag1*^{-/-} → B6.SJL chimeras (Figure 4B), but fluid homeostasis or renal artery velocity remained the same across chimeras 1 week after DOCA-salt (Supplemental Tables S1 and S2). Similar to *Axl.Rag1*^{-/-}, there was significant increase in urine Na⁺ and Cl⁻ mEq in *Axl.Rag1*^{-/-} → B6.SJL versus *Rag1*^{-/-} → B6.SJL after DOCA-salt (Supplemental Figure S5, B and C). As confirmed by the flow cytometry in peripheral blood

Figure 3 Kidney inflammation and dysfunction reduces on genetic deletion of *Axl* in *Rag1*^{-/-} mice after 1 week of deoxycorticosterone-acetate (DOCA) salt. **A:** Systolic blood pressure (BP) in *Axl.Rag1*^{+/+} and *Axl.Rag1*^{-/-} littermates after 1 week of uninephrectomy (Nephx) or DOCA-salt. Open bars show baseline values. Closed bars show mice after 1 week. **B:** Quantitative analyses of the percentages of the kidney vasculature (black bars) and renal fluid (white bars) on the basis of three-dimensional (3D) kidney imaging. **C:** Representative images of the double-stained [Mac-2 and T-cell Ig and mucin domain 1 (TIM-1)] medulla regions of the kidneys from *Axl.Rag1*^{+/+} and *Axl.Rag1*^{-/-} littermates after DOCA-salt. Nephx controls are shown in insets. Mac-2⁺ cells are brown (black arrows). TIM-1⁺ staining is pink (white arrows). Counterstain is green. **D:** Quantitative analysis of Mac-2 and TIM-1 expression in the medullary region of the kidney in *Axl.Rag1* littermates. White bars show mice after Nephx. Black bars show mice after DOCA-salt. Data are expressed as the means $\pm$ SEM (**A**, **B**, and **D**). $n = 3$ to 7 per group (**A**, **B**, and **D**). * $P < 0.05$ versus Nephx, *Axl.Rag1*^{+/+}; † $P < 0.05$ versus Nephx, *Axl.Rag1*^{-/-}. Scale bar = 100 μ m (**C**).

(Supplemental Figure S3B), there were no donor (CD45.2⁺) cells in kidneys from CD45.1⁺ B6.SJL $\rightarrow$ B6.SJL chimeras (Figure 4, C and D). Repopulation of the CD45.2⁺ bone marrow without lymphocytes showed significant decline in CD45.2⁺ cells in medulla from *Axl.Rag1*^{-/-} $\rightarrow$ B6.SJL versus *Rag1*^{-/-} $\rightarrow$ B6.SJL after DOCA-salt (Figure 4, C and D). TIM-1 expression was identical in chimeras with or without lymphocytes but tended to be lower ($P = 0.08$) in *Axl.Rag1*^{-/-} $\rightarrow$ B6.SJL after DOCA-salt (Figure 4D). Together, these findings suggest that innate immune cells promote early kidney dysfunction and DOCA-salt hypertension. Furthermore, *Axl*-dependent signals in myeloid cells are responsible for fluid and salt retention and medulla damage, with reduced kidney vasculature in early hypertension.

Axl Is a Critical Regulator of C3 in Hypertensive Kidneys by Autocrine and Paracrine Mechanisms

Our previous experiments suggested *Axl* importance in multiple cell lineages in development of vascular disease, and the *C3* is highly regulated by *Axl*.^{6,8,10} For an unbiased

discovery of cellular and molecular mechanisms of *Axl*, *Axl* regulatory gene-gene interaction networks were constructed using bayesian network models (Figure 5A). These models were based on genetic variation and gene expression data from multiple tissues in human and mouse studies, each involving hundreds of individuals.^{16,19,25,26} The analyses revealed nine interactions appearing in at least two tissue-specific gene regulatory networks, including *GADD45B*, *EMP3*, *AEBP1*, *C1R*, *EFEMP2*, *FBN1*, *FGF2*, *ITGB5*, and *PIK3R1* (Figure 5A). There were >30 enriched signaling pathways related to the *Axl* interacting partners, with focal adhesion and complement and coagulation cascades being the most significant (Supplemental Table S3). The *C3/C1R* component of the complement pathway was one of the most significant in relation to overall *Axl* gene-gene network (Supplemental Table S3 and Figure 5A). A tissue specificity was found in *Axl* gene-gene networks, and *C3* appeared in *Axl*-specific network in aorta, but not in immune cells, nephron, or adipose networks (Figure 5B and Supplemental Figure S8). Liver and myeloid cells mainly produce *C3*, and SMCs are possible secondary sources of *C3*.^{27,28} A comparable *C3* protein expression was detected in livers and

Figure 4 Deoxycorticosterone-acetate (DOCA) salt hypertension is prevented by depletion of *Axl* in nonlymphoid hematopoietic cells. **A:** Systolic blood pressure (BP) in wild-type chimeras (B6.SJL → B6.SJL), chimeras that express *Axl* but lack lymphocytes in hematopoietic lineage (*Rag1*^{-/-} → B6.SJL), and chimeras that lack lymphocytes and *Axl* in hematopoietic lineage (*Axl.Rag1*^{-/-} → B6.SJL) after 1 week of DOCA-salt. White bars show baseline values. Black bars show mice after 1 week. **B:** Quantitative analyses of the percentages of the kidney vasculature (black bars) and renal fluid (white bars) on the basis of three-dimensional (3D) kidney imaging in chimeras. **C:** Representative images of the double-stained [CD45.2 and T-cell Ig and mucin domain 1 (TIM-1)] medulla regions of the kidneys from chimeras. CD45.2⁺ cells are brown (black arrows). TIM-1⁺ staining is pink (white arrows). Counter-stained cells are green (arrowheads). **D:** Quantitative analysis of CD45.2 and TIM-1 expression in the medullary region of the kidney across chimeras. Black bars show B6.SJL → B6.SJL. Dark gray bars show *Rag1*^{-/-} → B6.SJL. Light gray bars show *Axl.Rag1*^{-/-} → B6.SJL. Data are expressed as the means ± SEM (**A**, **B**, and **D**). *n* = 3 to 7 per group (**A**, **B**, and **D**). **P* < 0.05 versus B6.SJL → B6.SJL; †*P* < 0.05 versus *Rag1*^{-/-} → B6.SJL. Scale bar = 50 μm (**C**).

blood plasma from *Axl*^{+/+} and *Axl*^{-/-} mice at baseline (Supplemental Figure S9). There was significant increase in C3 immunoreactivity in tubulointerstitial area of kidney medulla from *Rag1*^{-/-} versus B6 mice after DOCA-salt (Figure 5C). The lowest C3 expression was observed after *Axl* depletion in *Axl.Rag1*^{-/-} or *Axl.Rag1*^{-/-} → B6.SJL after DOCA-salt (Figure 5C). Previous studies showed that C3 is responsible for exaggerated growth of vessels and migration of SMCs or adventitial fibroblasts in spontaneously hypertensive rats.^{29–31} Genetic depletion of *Axl* significantly impaired the abilities of MASMCs to migrate toward increased concentration of C3 or 20% serum (Figure 5D). Also, MASMC migration was unaffected by combining the *Axl* ligand, Gas6, with C3 in *Axl*^{+/+} or *Axl*^{-/-} MASMCs (Figure 5D). These findings suggest that *Axl* is critical for immune cell-mediated increase in C3 in kidney medulla and regulates C3-directed migration via autocrine and paracrine mechanisms.

Discussion

A major finding of this study is that the myeloid cells are critical for the initial kidney damage (versus a long-term antigen presentation to lymphocytes) in DOCA-salt

hypertension. In fact, depletion of lymphocytes in *Rag1*^{-/-} resulted in extravasation of myeloid cells to the kidney medulla, a shift toward fluid and salt retention in early DOCA-salt hypertension. A global deletion of *Axl* on *Rag1*^{-/-} background reduced accumulation of myeloid cells in the medulla and normalized intrarenal fluid, with an increase in salt excretion and kidney vascularity after DOCA-salt. Adoptive transfer of wild-type CD4⁺ T lymphocytes into *Rag1*^{-/-} or *Axl.Rag1*^{-/-} mice had minimal effect on early kidney dysfunction and DOCA-salt hypertension. Generation of chimeric mice with *Axl* depletion in innate immune cells in the absence of lymphocytes prevented DOCA-salt hypertension and reduced accumulation of the immune cells, fluid, and salt retention in the medulla, with an increase in kidney vascularity. The bioinformatics analyses revealed tissue specificity of *Axl*-dependent regulatory partners and inflammatory mediators. It was confirmed that complement C3 pathway is crucial for paracrine and autocrine *Axl*-mediated inflammatory mechanisms in hypertension. Taken together, *Axl*-dependent signaling in macrophage-like cells is likely the primary cause of renal dysfunction in early hypertension, whereas CD4⁺ T cells that express *Axl* may control vascular remodeling as hypertension advances (Figure 6).

Figure 5 Paracrine and autocrine interactions between *Axl* and complement C3 (C3) in salt-dependent hypertension. **A:** A bayesian gene-gene interaction analysis of *Axl* from 11 tissues from human and mouse cells. **B:** A bayesian gene-gene interaction analysis of *Axl* in aorta. Black circles highlight C3 and its proximity to *Axl* in the networks. **C:** Representative images of the C3 staining (brown; arrows) of medulla regions of the kidneys in *Rag1*^{-/-} and *Axl.Rag1*^{-/-} mice after deoxycorticosterone-acetate salt. **Insets:** Wild-type (B6) and *Axl.Rag1*^{-/-} → B6.SJL chimera. Counterstained cells are green. Bar graph shows a quantitative analysis of C3 expression in the medullary region of the kidney across experimental mice. **D:** *Axl* is important for C3-mediated migration of mouse aortic smooth muscle cells from *Axl*^{+/+} (black bars) or *Axl*^{-/-} (white bars). Data are expressed as the means $\pm$ SEM (**C** and **D**). $^*P < 0.05$ versus *Rag1*^{-/-}; $^\dagger P < 0.05$ versus *Axl*^{+/+} serum 3%; $^\ddagger P < 0.05$ versus *Axl*^{+/+}; $^\S P < 0.05$ versus *Axl*^{+/+} growth arrest-specific gene 6 (Gas6; 100 nmol/L); $^\P P < 0.05$ versus *Axl*^{+/+} Gas6. Scale bar = 50 μm (**C**).

Figure 6 A proposed model on the role of *Axl* in immune cells during salt-dependent hypertension. Blood pressure changes are shown by a black line. A time in weeks is shown on the x axis. Light gray highlighted area and the black arrow show contribution of *Axl* in macrophage (M ϕ)–like cells to renal dysfunction in early hypertensive response (white arrow). Dark gray highlighted area and the gray arrow represent regulation of vascular remodeling by *Axl*-expressing CD4⁺ T lymphocytes during the late phase of hypertension (white arrow).

Seminal studies in *Rag1*^{−/−} mice showed that lack of T cells prevented vascular dysfunction and BP increase after AngII or DOCA-salt¹; AngII activates AngII type 1 receptors in T lymphocytes and is important for BP increase.^{32–34} However, mice lacking *AT1R* in T cells had exaggerated renal damage in response to hypertensive challenge.³ This study raised the concern of inhibiting the renin-angiotensin system in lymphocytes and potential protective role of T cells in hypertension.³⁵ Accelerated intimal thickening in response to vascular injury was reported in *Rag1*^{−/−} and *Rag2*^{−/−} mice, and reintroduction of the adaptive immune arm or inhibition of C3 or interferon- γ reduced vascular remodeling.^{36–39} Protective roles for lymphocytes in early renal or vascular dysfunction might be attributed to overactive innate immunity in genetically modified models, like *Rag1*^{−/−} mice. In the current study, reintroduction of the CD4⁺ T cells into *Rag1*^{−/−} mice had no effect on early BP increases or kidney dysfunction after DOCA-salt. In contrast, antigen-presenting dendritic cells stimulate adaptive immune response and exert tissue damage via activated lymphocytes in hypertension.⁴⁰ Macrophages were also implicated in renal injury in rats after DOCA-salt.^{41,42} This study suggests that myeloid cells can directly affect kidney function during onset of salt-dependent hypertension. There was a significant accumulation of CD11b⁺ (but not CD11c⁺) cells and redistribution of the Mac-2⁺ cells from cortex to the medulla in *Rag1*^{−/−} versus wild-type mice after 1 week of DOCA-salt. Such Mac-2⁺ cell redistribution resulted in greater focal damage of the kidney medulla with salt and fluid retention. Decreased urine NaCl in *Rag1*^{−/−} suggests an acceleration of the nephron's damage and fluid retention, as further suppression of urine Na⁺ found after 3 weeks of DOCA-salt by others.⁴³ Deletion of *Axl* in *Rag1*^{−/−} or lack of *Axl* in

innate immune cells (without lymphocytes) normalized NaCl mEq in urine after 1 week of DOCA-salt in the experiments. Similar to these observations, recent studies showed that a hypertonic micromilieu in the kidney medulla drives dendritic cells into macrophage-like cells with inhibition of the antigen processing and activation of antigen-specific T cells.^{44,45} The advanced imaging techniques revealed a greater capacity of a mouse kidney to retain fluids compared with a rat, which could explain a modest (approximately 40 mmHg) increase in BP in a mouse versus a rat (approximately 80 mmHg) after DOCA-salt.⁴⁶ Taken together, these results suggest that macrophage-like cells are the primary contributors to initial kidney dysfunction via salt and fluid retention in hypertension.

Innate and adaptive immunity has been implicated in human hypertension.⁴⁷ However, it is clinically challenging to determine how imbalance between innate and adaptive immune arms is affecting high BP in patients. Overactivated innate immunity might be significant because the higher neutrophil/lymphocyte ratio increased risks of hypertension in humans.⁴⁸ A recent report in hemodialysis patients and patients with chronic kidney disease showed significant elevation of soluble *Axl*, an extracellular domain of the receptor, in plasma.⁴⁹ The authors speculated that a high plasma soluble *Axl* level reflects an increased activity of proinflammatory cells.⁴⁹ Inflammation is a well-accepted catalytic risk factor for cardiovascular disease and chronic kidney disease.⁵⁰ These findings are significant for hypertensive kidney damage because of documented up-regulation of *Axl* in kidneys from salt-sensitive hypertensive rats.⁵¹ Taken together, these data suggest that *Axl* signaling in myeloid cells promotes early renal damage in salt-induced hypertension.

Current challenges include identification of the key molecules that regulate interactions between immune cells and other cell types in promoting kidney pathology in hypertension. The bioinformatics analyses emphasize a diversity of responses of the *Axl* partners and downstream pathways. However, C3 is a part of the innate immune system that initiates the complement cascade in response to foreign bodies.⁵² Another important function of C3 is chemotaxis by attracting macrophages toward antigens and helping to present the antigen to T cells.⁵³ Consistent with our previous findings on C3 mRNA in *Axl* chimeric mice,¹⁰ global or innate immune compartmental deletion of *Axl* resulted in reduction of C3 expression in renal medulla, whereas *Rag1*^{−/−} exhibited highest levels of C3 after DOCA-salt. Others have shown that addition of C3 has enhanced proliferation and migration of SMCs from spontaneously hypertensive rats.^{29–31} Recent findings support a promigratory role of C3 for SMCs downstream to *Axl* in response to vascular injury.¹² Thus, these data suggest that *Axl* mediates interactions between myeloid and vascular cells by increase in C3 in hypertensive kidney, which warrants future investigations.

Indirect assessment of BP is a potential limitation of studies in hypertensive mice. However, we previously

validated the tail-cuff plethysmography method by radiotelemetry in mice.⁵⁴ The published 6-week time course of DOCA-salt in *Rag1*^{-/-} mice⁹ also supports the current findings at 1-week time point after DOCA-salt. A recent report suggested genetic instability of *Rag1*^{-/-} mice from the Jackson Laboratory that resulted in variability in AngII-induced hypertension.⁵⁵ As referred,⁹ our laboratory established *Rag1*^{-/-} and *Axl.Rag1*^{-/-} colonies before the genetic drift, with comparable BP responses to DOCA-salt in the current and published studies.^{6,8,9}

These studies highlight an important role for Axl-dependent myeloid cells in the renal dysfunction associated with early hypertension. However, there are many interesting questions that remain. In particular, detailed studies of the roles of Axl in leukocytes and monocyte differentiation in hypertensive kidney and better characterization of the myeloid cells are needed. Functionally, myeloid cells have been characterized in several disease states into M1 and M2 phenotypes, which have distinct proinflammatory versus regulatory functions, respectively.⁵⁶ These cells exhibit functional plasticity and could coexist in mixed phenotypes that support organ- and disease-specific immune response.⁵⁷ It will be interesting in future studies to assess the subsets of myeloid cells and proinflammatory cytokines expressed by the myeloid cells that infiltrate the kidney in the early phase of hypertension. The current findings in chimeric animals as well as published results of competitive bone marrow repopulation⁹ support a direct effect of Axl on myeloid cell differentiation. Expression of Axl has been shown to vary across tissue-specific macrophages with preference to mucosal macrophages in lungs.⁵⁸ The authors also showed that granulocyte-macrophage colony-stimulating factor, but not macrophage colony-stimulating factor, induced Axl expression *in vitro* in bone marrow-derived macrophages. Therefore, future *in vivo* studies targeting Axl in myeloid cells will be required to identify major factors and the pathways that interact with Axl and promote renal-specific subpopulation(s) of macrophages in hypertension.

We conclude that myeloid cells are important for initial kidney damage in DOCA-salt hypertension. The idea of the protective roles for lymphocytes in early renal or vascular damage might be attributed to an overactive innate immune arm in genetically modified models, like *Rag1*^{-/-} mice. An accelerated renal dysfunction was found by a compensated extravasation of myeloid cells to the kidney medulla (opposite to a kidney cortex) in early hypertension in *Rag1*^{-/-} mice. Therefore, antigen presentation to lymphocytes is reduced because of high salt concentration in the renal medulla and could be secondary versus direct renal injury exerted by innate immune cells in hypertension. Axl is an important gene that promotes infiltration of myeloid cells and salt and fluid retention. Lack of Axl in myeloid cells (without lymphocytes) significantly increased kidney vascularity and abolished early hypertensive response, in part, by decreasing C3 expression. Future research will

help in developing new anti-inflammatory approaches in preventing end-organ damage in hypertension.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.04.013>.

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