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Targeted inhibition of Axl receptor tyrosine kinase ameliorates anti-GBM-induced lupus-like nephritis

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Abstract

Glomerulonephritis (GN) is a typical lesion in autoantibody and immune complex disorders, including SLE. Because the Gas6/Axl pathway has been implicated in the pathogenesis of many types of GN, targeting this pathway might ameliorate GN. Consequently, we have studied the efficacy and mechanism of R428, a potent, selective Axl inhibitor, in the prevention of experimental anti-GBM nephritis. Axl upregulation was investigated with Sp1/3 siRNA in the SV40-transformed mesangial cells. For Axl inhibition, a daily dose of R428 (125 mg/kg) or vehicle was administered orally. GN was induced with anti-GBM sera. Renal disease development was followed by serial blood urine nitrogen (BUN) determinations and by evaluation of kidney histology at the time of sacrifice. Axl-associated signaling proteins were analyzed by Western blotting and inflammatory cytokine secretion was analyzed by Proteome array. SiRNA data revealed the transcription factor Sp1 to be an important regulator of mesangial Axl expression. Anti-GBM serum induced severe nephritis with azotemia, protein casts and necrotic cell death. R428 treatment diminished renal Axl expression and improved kidney function, with significantly decreased BUN and glomerular proliferation. R428 treatment inhibited Axl and significantly decreased Akt phosphorylation and renal inflammatory cytokine and chemokine expression; similar effects were observed in anti-GBM antiserum-treated Axl-KO mice. These studies support a role for Axl inhibition in glomerulonephritis.

1. Introduction

Renal involvement occurs in most patients with systemic lupus erythematosus (SLE) and is one of the most damaging organ manifestations of the disease [1]. Lupus nephritis often causes end-stage kidney failure due to glomerular and tubular inflammation that is associated with massive cellular proliferation [2]. Current therapies are insufficiently

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effective and outcomes following immunosuppression induction are unsatisfactory. Although a new generation of biological agents is currently under development, the long-term beneficial and adverse effects of such agents remain unknown. Consequently, more effective drugs with favorable safety profiles are urgently needed [3, 4]. In particular, molecule-specific approaches offer hope for more effective and safe treatment.

Axl belongs to the TAM (Tyro-3, Axl, and Mer) family of receptor tyrosine kinases (RTKs). TAM RTKs are important for regulating cell proliferation/survival, cell adhesion and migration, and inflammatory cytokine release [5, 6]. Genetic and experimental alteration of TAM receptor function influences a number of disease states, including lupus-like autoimmune disease [7, 8]. Gas6 is a common ligand for the TAM receptors and the only ligand that activates Axl. The Gas6/Axl pathway is highly regulated in cancer, autoimmunity, and nephritis. Gas6-dependent signaling through Axl leads to phosphorylation of MAPKs/ERKs and PI3kinase/Akt, which promote cellular survival and proliferation. Although Gas6 and Axl are generally not detected in the healthy kidney, they are strongly upregulated in both mice and humans at sites of inflammation [9–11]. Using global knockout mice, a critical role for the Gas6/Axl pathway in mouse models of nephritis and streptozotocin-induced diabetic nephropathy has been demonstrated [12–14]. Loss of Gas6 protects mice against mesangial cell proliferation and glomerular hypertrophy and improves proteinuria and survival in anti-Thy1.1-induced rat nephritis [12]. However, because Gas6 is an important coagulation factor and the coagulation cascade is activated in severe human and experimental nephritis [15], it is unclear whether Gas6 deletion ameliorates nephritis by TAM-independent suppression of coagulation or by a TAM-dependent anti-inflammatory effect. Therefore, targeting Axl offers a clearer mechanism of action and a potentially safer approach than targeting Gas6 to suppress nephritis.

Studies of a murine anti-GBM model of nephritis have provided important information about the roles of cellular and molecular mediators in lupus nephritis [16]. Using this model, we have shown that Axl-deficient mice develop less renal Akt phosphorylation, Bcl-xl upregulation, inflammation, and azotemia and have a significantly improved survival rate, as compared to similarly treated WT and Mer-KO mice [14]. These observations suggest that Gas6/Axl pathway inhibitors may ameliorate renal disorders.

R428 (also called BGB324) is the most selective small molecule inhibitor of Axl reported to date and is the first kinase inhibitor to be intentionally designed to target Axl [17]. It inhibits Axl in an ATP-competitive manner with low nanomolar activity ($IC_{50}=14$ nM) and displays strong selectivity (>40-fold greater activity towards Axl as compared to Mer, Tyro3 and other RTKs). Holland et al showed that R428 blocks Axl-dependent events, including Akt phosphorylation and cell survival and proliferation [18]. R428-treated vascular smooth muscle cells contain less PI3K and pAkt than control cells [19]. Pharmacologic studies have revealed favorable R428 tissue levels after oral administration of this drug [18], which was the first Axl inhibitor to progress to clinical trials [20].

We previously showed that Axl-KO mice developed significantly less Akt activation and milder kidney inflammation after anti-GBM injection compared to similar treated WT-control mice [14]. In this study, we tested the therapeutic efficacy of R428 in the prevention

of experimental nephritis. Mice treated with R428 daily showed significant preservation of kidney function and decreased inflammatory cytokine production as compared to the untreated WT nephritic mice. Inhibition of Axl led to reduced Akt phosphorylation and glomerular cellular proliferation.

2. Materials and methods

2.1. Reagents and animals

Anti-Axl antibody was purchased from R&D Systems (Minneapolis, MN). All other antibodies were purchased from Cell Signaling Technology (Boston, MA). R428 was purchased from Selleck Chemicals (Houston, TX) and prepared in 0.5% hydroxypropylmethylcellulose (Sigma-Aldrich, St. Louis, MO) with 0.1% Tween-80 (Fisher Scientific, Pittsburgh, PA). WT C57BL/6J (B6) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). Axl-KO mice were on B6 background and housed under pathogen-free conditions at the animal facilities of the University of Cincinnati. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

2.2. Cell culture

SV40-mesangial cells were purchased from ATCC (Manassas, VA) and cultured according to the manufacturer's protocol. Cells were maintained in base medium (3:1 mixture of DMEM and Ham's F12 medium) with 5% FBS. For mithramycin A (MTA, purchased from Sigma-Aldrich, St. Louis, MO) treatment, 5×10^5 cells per well were plated in 24-well plates and cultured with MTA for 9 hrs. For siRNA treatment, 1×10^6 cells per well were plated in 6-well plates incubated with premade siRNA for Sp1 (Specificity protein) and/or Sp3 (Thermo Fisher Scientific, Waltham, MA) for two days.

2.3. R428 treatment and anti-GBM-induced lupus nephritis

Mice were divided into three groups (WT, WT-R428, and Axl-KO). R428 (125mg/kg/day) or vehicle was orally administered at day -2. All three groups were then iv injected with 7.5 ml/kg of anti-GBM sera prepared from sheep. The severity of nephritis was followed by measuring BUN with a Urea Nitrogen Direct kit (Stanbio Laboratory, Boerne, TX). Animals were euthanized at days 7 and 14 post-injection. Kidney samples were collected and embedded in OCT medium and snap frozen in liquid nitrogen. Sections (4 μ m) were processed for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining. Sections were examined in a blinded fashion. The glomeruli were screened and the severity of GN was graded on a 0–4 scale as previously described [21]. Briefly, 0, normal; 1, mild increase in mesangial cellularity and matrix; 2, moderate increase in mesangial cellularity and matrix, with thickening of the GBM; 3, substantial increase in the thickness and irregularity of the GBM; and 4, segmental necrosis, crescents, and hyalinized end-stage glomeruli.

2.4. Western blot analysis

For cell cultures, cells were lysed in the tissue culture plate with RIPA buffer (Santa Cruz Biotechnology, Dallas, TX, USA) and collected into centrifuge tubes. For kidney tissues,

cortex samples were cut into small pieces (<1mm) and homogenized in RIPA buffer with proteinase and phosphatase inhibitors (Roche, Indianapolis, IN, USA). Proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore, Billerica, MA, USA) and incubated with primary antibodies (rabbit antibodies against mouse cell signaling molecules as indicated in figures), and subsequently with secondary anti-rabbit IgG antibodies labeled with HRP, both in blocking buffer (1% dry milk in buffer). Proteins were visualized with enhanced chemiluminescence (Fisher Scientific, Hampton, NH, USA).

2.5. Immunofluorescence staining

Mouse kidney sections (4 µm) were fixed in methanol and blocked with 5% BSA. Anti-mouse Abs (as indicated in the figure legends, 1:100 dilution) were added and incubated with sections. Secondary Abs were added after wash. Images were acquired using a Leica DMi8 fluorescent microscope (Buffalo Grove, IL) and analyzed with the LAX S software developed by Leica Microsystems Inc.

2.6. Proteome array analysis

The cytokine profile of kidney cortex was measured using the Proteome Profiler Array from R&D Systems according to the manufacturer's guidelines. In brief, kidney cortex was separated and homogenized in PBS containing proteinase and phosphatase inhibitors. Samples were then incubated overnight at -70°C, followed by 10 minutes centrifugation (10,000 × g) to remove particles. A mouse cytokine array membrane was blocked for one hour at room temperature and incubated with a detecting antibody/sample mix overnight at 4°C. The membrane was then washed and incubated with Streptavidin-HRP. Cytokine protein levels were visualized with Chemi Reagent Mix.

2.7. Statistical analysis

Western blot data were analyzed using ImageJ software (NIH, Bethesda, MD, USA). Intensity differences between groups were tested using the Mann-Whitney *U* test. Data are shown as median with interquartile range. All statistical analyses were carried out using Microsoft Excel or Prism software (GraphPad, San Diego, CA, USA). Results were considered significant at **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

3. RESULTS

3.1. Transcription factor Sp1, but not Sp3 promotes Axl expression in mesangial cells

The promoter region of Axl in human colorectal cell lines has been systematically characterized [22]. Several mechanisms were revealed to regulate human Axl gene expression, including methylation and the transcription factors Sp1 and Sp3 [22, 23]. We subsequently analyzed the promoter region of mouse Axl (Fig. 1). Eight of 17 methylation sites overlapped with transcription factor binding sites. We also revealed transcription factor-binding sites (including sites binding Sp1/Sp3) that were similar to those reported in human cancer cell lines. We consequently evaluated Sp1/Sp3 driven Axl expression. MTA, a selective Sp1 inhibitor, binds to GC-rich regions, where it displaces Sp1 [24]. As expected, MTA treatment diminished Sp1 expression in a dose-dependent manner in SV40-transformed mesangial cells (Fig. 2A). The decreased Sp1 expression correlated positively

with decreased Axl expression. To confirm the essential role of Sp transcription factors in promoting Axl expression, siRNA targeting Sp1 and Sp3 were introduced into the mesangial cell culture and incubated for 9 hours. Western results showed that Sp1 targeting inhibited Axl expression in mesangial cells, while inhibition of Sp3 alone or both Sp1 and Sp3 had no effect on Axl expression (Fig. 2B). Taken together, Sp1, but not Sp3, upregulates Axl in mesangial cells and that Sp3 inhibits Axl expression in the absence of Sp1.

3.2. R428-treatment ameliorates kidney inflammation in anti-GBM nephritis

R428 was the first kinase inhibitor to be intentionally designed to target Axl. It inhibits Axl in an ATP-competitive manner in the low nanomolar range [17]. R428 has been studied in the treatment of cancer development, but had never been tested as a treatment of kidney disease. To investigate the therapeutic role of R428 in the development of anti-GBM nephritis, mice were given R428 orally two days before anti-GBM injection and kidney disease progression was compared to that in vehicle-treated WT control mice. The previously described anti-GBM antiserum-induced glomerulonephritis-associated increase in renal Axl (Fig. 3B) [14, 25] was strongly suppressed by treatment with R428 (Fig. 3B).

R428 treatment also significantly blunted the rise in BUN (Fig. 3A) and decreased the number of glomerular and tubular protein casts (Fig. 3C) that developed in anti-GBM-injected WT mice. However, BUN remained higher in R428-treated WT nephritic mice than in Axl-KO nephritic mice, indicating that prolonged and/or complete inhibition of Axl may be required for maximal kidney protection. Although the reduced severity of nephritis in R428-treated mice could have also been due to a decreased deposition of nephrotoxic immune complexes, the amount of linear glomerular capillary deposition of sheep anti-mouse GBM antibodies was identically strong in all three experimental groups (Fig. 4A, top row). The subsequent deposition of immune complexes indicative of the endogenous response to sheep IgG as detected with an anti-mouse IgG antibody was essentially the same between R428-treated and untreated Axl-KO and WT groups (Fig. 4A bottom row).

3.3. Enhanced kidney function in R428-treated mice is associated with reduced glomerular proliferation

Previous studies using Gas6-KO and Axl-KO mice have shown a pathological role for Gas6/Axl in anti-GBM nephritis and streptozotocin-induced diabetic nephropathy [12, 14, 26]. Loss of either the ligand or the receptor protected against mesangial cell proliferation and glomerular hypertrophy. To determine whether Axl inhibition with R428 might reduce glomerular cell proliferation, we examined this by staining the kidneys of R428-treated mice, Axl-KO mice, and WT mice for the proliferation-associated protein PCNA during the early phase of experimental nephritis. Consistent with our previous data [14], nuclear PCNA on day 7 was increased to a considerably greater extent in the glomeruli of anti-GBM-treated WT than Axl-KO mice and was partially suppressed in anti-GBM-treated WT mice by R428 (Fig. 4B). However, numbers of glomerular PCNA-positive cells were still significantly higher in anti-GBM-injected, R428-treated WT mice than in anti-GBM-injected Axl-KO mice (Fig. 4B). This suggests that a higher dose or increased frequency of R428 treatment is required to inhibit Axl sufficiently to maximally suppress glomerular cell proliferation.

3.4. Axl inhibition by R428 results in decreased Akt phosphorylation

Gas6/Axl pathway activation promotes cell survival, migration, and proliferation [27]. These effects are likely mediated by Gas6/Axl-induced activation of the MAPK/ERK and PI3K signaling pathways. While the MAPK/ERK pathway typically results in Axl-mediated proliferation, the Axl/PI3K/Akt pathway has been linked to multiple downstream pathways that converge on increased cell survival [5]. Because our previous study showed decreased Akt phosphorylation in Axl-KO mice compared to WT nephritic mice, but no difference in MAPK activity [14], we reevaluated the Akt pathway and explored the ERK1/2 pathway in R428-treated nephritic mice and compared the results with Axl-KO and WT nephritic mice. In accord with previous results, Akt phosphorylation was significantly elevated in the kidney of WT mice when anti-GBM was injected compared to the WT (control serum-injected) mice. Deficiency of Axl led to a significant decrease in Akt activity (Fig. 5A and C). Consistent with this, R428 treatment significantly decreased Akt phosphorylation. Total Akt levels were not altered in all three groups of nephritic mice, although injection of anti-GBM enhanced Akt expression (Fig. 5C). In contrast to Akt, ERK phosphorylation was similar in all three groups of nephritic mice (Fig. 5B and C). These results indicate the greater importance of the Axl-mediated PI3K/Akt pathway than the ERK pathway in anti-GBM nephritis.

3.5. Decreased cytokine/chemokine production in the kidney of nephritic mice treated with R428

Local complement activation and cytokine and chemokine production contribute to neutrophil infiltration and inflammatory damage in nephritic mice [28]. Because Axl inhibition by R428 reduced inflammatory cytokine release, in a dose-dependent manner, in primary tumor lysates [18], we evaluated whether R428 would also suppress inflammatory cytokine and chemokine expression, as well as complement activation in anti-GBM-induced nephritis. Forty cytokines and chemokines were measured with a proteome profiler array from lysates of the kidney cortex samples from all groups of experimental mice. Seven cytokine/chemokines were upregulated after anti-GBM injection and R428-treatment significantly diminished this increase in two of them (Fig. 6), including SDF-1, which attracts and activates lymphocytes, and IL-8 (also called KC in mice), which attracts and activates neutrophils, which contribute to glomerular destruction [29]. Infiltration of neutrophils and lymphocytes plays a major role in early and developmental renal damage upon inflammation, respectively.

4. Discussion

Our observations show the importance of Axl in the pathogenesis of anti-GBM nephritis, identify Sp1 as a critical transcription factor in the upregulation of Axl in mesangial cells, and show that the Axl inhibitor, R428, suppresses disease progression in murine anti-GBM nephritis. We have additionally shown that Axl activation of the Akt signaling pathway, which promotes cell survival, migration and proliferation, is a likely mechanism that promotes proliferative glomerulonephritis and that R428 inhibits this pathway, as well as chemokine- and complement-dependent mechanisms that promote neutrophil-mediated glomerular damage.

Previous studies have suggested that the only Axl ligand, Gas6, might be a useful therapeutic target in inflammatory glomerular disease. However, Gas6 is also a ligand for Mer, which has a protective effect in glomerular disease [30]. Furthermore, Gas6 acts as a coagulation factor and as a regulator of thrombosis and other biological processes [31]. Although Gas6 inhibition is beneficial in glomerulonephritis, chronic Gas6 inhibition might cause bleeding and anemia by inhibiting platelet aggregation [31]. Previous evidence showed that the coagulation cascade is activated in severe human and experimental glomerulonephritis [15] made it unclear whether Gas6 deletion ameliorated glomerulonephritis through Axl-independent suppression of coagulation or through an Axl-dependent anti-inflammatory effect. Although our data do not eliminate the possibility that the anti-thrombotic activity of Gas6 inhibition contributes to its beneficial effects in nephritis, our studies with Axl-deficient mice and R428-treated mice demonstrate that Gas6 activation of Axl is an important mechanism by which it contributes to the pathogenesis of glomerular disease.

These considerations suggest that direct and specific inhibition of Axl, with a small molecule inhibitor, such as R428, is likely to be a better therapeutic approach than Gas6 inhibition for suppression of glomerulonephritis. R428 is the first kinase inhibitor to be intentionally designed to target Axl and is the most selective small molecule inhibitor of this receptor tyrosine kinase. R428 is very stable in vivo, has a long plasma half-life (13 hours at 75 mg/kg), and distributes effectively to tissue (Fig. 3 and [18]). All of these features contribute to its potential usefulness as a therapeutic.

Our observations with R428-treated mice and Axl-deficient mice also increase understanding of the mechanisms involved in glomerulonephritis pathogenesis. Mesangial cell proliferation characterizes many forms of both experimental and human glomerular disease, including IgA nephropathy, lupus nephritis, diabetic nephropathy, and other forms of membranoproliferative glomerulonephritis [32]. Expression of Gas6 and Axl is dramatically increased in Thy1.1 and anti-GBM glomerulonephritis in parallel with the degree of glomerular proliferation [14, 25] and Gas6 has been reported to be one of the switches leading to mesangial proliferation. Consistent with this, loss of Gas6 protects rats against mesangial cell proliferation and glomerular hypertrophy and improves proteinuria and survival in anti-Thy1.1-induced nephritis [12, 25].

Although we previously reported decreased glomerular hypertrophy in Axl-KO nephritic mice compared to WT nephritic mice [14], the Gas6/Axl signaling pathways involved in glomerular hypertrophy were poorly characterized. One potential mechanism was increased mesangial cell survival and proliferation through the PI3K/Akt and/or MAPK/ERK pathways. Consistent with our previous findings in Axl-deficient mice with nephritis, R428 treatment decreased both Akt activation and glomerular proliferation in anti-GBM injected WT mice. In contrast, MAPK and ERK did not differ significantly in the experimental groups studied; this suggests that Axl inhibition suppresses glomerular mesangial proliferation by suppressing the Akt, rather than the MAPK/ERK signaling pathway. One caveat to this conclusion, however, is that our observations were made with whole kidneys, in which multiple pathways and cell subsets could influence the inflammation-induced MAPK and ERK activation seen in Fig. 5. Indeed, the ERK pathway is activated in anti-

GBM nephritis (Fig. 5C, control lane vs. experimental nephritis lane). Upregulation of the ERK pathway was also reported by others in the similar model [33, 34]. Although the ERK pathway is not targeted by Axl deficiency, or by R428-mediated-Axl inhibition, targeting the ERK pathway would likely ameliorate anti-GBM nephritis. It also remains to be seen whether R428 inhibits the MAPK and/or ERK pathways in ex vivo cultures of purified mesangial cells, where there would be no potential confounding factors caused by the presence of multiple cell types. Nevertheless, our observations and the importance of the Akt/mTOR pathway in Gas6-mediated mesangial and glomerular hypertrophy in diabetic nephropathy [13] make us favor the view that R428 treatment decreases glomerular hypertrophy by inhibiting Akt-induced mTOR activation.

It is worth noting that of the 7 cytokine/chemokines detected by the protein array, two of them, IL-8 and SDF-1, were significantly decreased by R428 treatment. These two cytokines are known to play an important role in kidney inflammation in the anti-GBM nephritis model [30, 35–37]. The increased levels of IL-8 in WT nephritic mice are consistent with our previously published observations [30]. Expression of intact C5 and/or its C5a fragment (which are not distinguished by the array that we used) were also significantly decreased by R428-treated group (Fig. 6). This may ameliorate disease, because treatment with the anti-C5 antibody, eculizumab, has been shown to have beneficial effects in lupus nephritis [38]. IL-1ra is an additional protein whose expression is decreased by R428 treatment and Axl deficiency; however, IL-1ra has anti-inflammatory, rather than pro-inflammatory effects. Expression of the other cytokine/chemokines that were detected by our array was either unchanged or too weak to evaluate (data not shown).

Axl is undetectable in the kidney under normal conditions, but is upregulated in association with renal inflammation. The mechanisms for Axl upregulation in the inflamed kidney are still unknown. However, studies of malignant cells have shown Axl regulation at the transcriptional level. Based on this, we analyzed the 5'-UTR of the mouse Axl gene and compared it to that of the human Axl gene. Similar elements of transcriptional regulation were identified that showed similarities between human and mouse Axl, including methylation and transcription factor-binding sites (Sp1/3, P300, and AP1) (Fig. 1 and [27]). The Sp zinc-finger transcription factor Sp1, but not Sp3, upregulated Axl in SV40 transformed mesangial cell lines (Fig. 2). Additional transcription factors, while not functionally validated here, are required for the overexpression of Axl in mesangial cells.

Transcription factors are not the sole regulator of Axl at the protein, translational, and transcriptional levels. DNA methylation levels of Axl are implicated in disease and are inheritable [27, 39]. Post-transcriptional regulation has been shown to be mediated through miRNA binding of the 3'-UTR of Axl and appears to be conserved: the miR34a and miR-199a/b target sequences of the Axl 3'-UTR are identical between mouse and human [23]. Consistent with the importance of miR34a in regulating Axl expression during inflammatory conditions, nephritis is associated with significantly lower renal miR34a expression (Shao WH, unpublished data). Further studies are needed to elucidate all of these mechanisms in vivo.

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Abbreviations

GN	glomerulonephritis
Gas6	growth-arrest-specific protein 6
BUN	blood urine nitrogen
SLE	systemic lupus erythematosus
TAM	(Tyro-3, Axl, and Mer) family
RTKs	receptor tyrosine kinases
B6	C57BL/6J
MTA	mithramycin A
H&E	hematoxylin and eosin
PAS	periodic acid-Schiff

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- R428 treatment improves kidney function in murine lupus nephritis.
- R428-mediated Axl inhibition decreases Akt phosphorylation.
- Sp1 is an important regulator of mesangial Axl expression.

Figure 1. Possible 5'-UTR regulation of Axl expression in mouse

The 5'-flanking region of 2400 bp upstream of Axl was retrieved from *ensembl.org*. Transcription factor binding sites in the Axl promoter 5' UTR were analyzed using the transcription factor binding site software PROMO (version 3.0.2) [40, 41] with maximum 15% dissimilarity. Sites of CCWGG methylation are marked with blue arrows.

Figure 2. Regulation of Axl expression by Sp1/3 in mouse kidney

A. SV40-mesangial cells were treated with the indicated range of concentrations of mithramycin (MTA). Expression levels of Axl and Sp1 were analyzed by Western blot. **B.** SV40-mesangial cells were treated with siRNAs targeting Sp1, Sp3, or both Sp1 and Sp3. Expression levels of Axl, Sp1, and Sp3 were analyzed by Western blot. β -actin was used as a loading control for both A and B. Data shown were representative of three repeats.

Figure 3. R428 improves kidney function in nephritic mice

Experimental nephritis was induced in WT and Axl-deficient mice as described. One group of WT mice was inoculated with R428 2 days before anti-GBM injection. Control WT mice and Axl-deficient mice were injected with vehicle buffer. **A.** BUN levels. Data are representative of 3 independent experiments and expressed as mean $\pm$ SEM. *p<0.05, **p<0.01, WT versus WT-R428; #p<0.05, WT-R428 versus Axl-KO. **B.** Western blotting confirmed the suppression of Axl expression in the kidney in all groups 7 days after the injection. **C.** Kidney samples were sectioned and stained with H&E and PAS. Arrows show protein casts in the capsules. Images are representative of the 3 treatment groups. Tissues were examined terminally for glomerulonephritis score. **p<0.01, WT nephritic group versus WT-R428 and Axl-KO groups.

Figure 4. R428 reduces glomerular cell proliferation in nephritic mice

R428 was administered and nephritis was induced as previously described [30]. **A.** Kidney sections were stained with FITC-conjugated anti-sheep (top) and PE-conjugated anti-mouse (bottom) IgG Abs. Relative immunofluorescent intensity was quantified at the bottom. **B.** Kidney sections were stained for PCNA (left). Images were taken and evaluated (right). Proliferation data were quantified and compared using two-way ANOVA with repeated measures. * $p < 0.05$.

Figure 5. Decreased Akt phosphorylation in R428-treated nephritic mice

Kidney samples were homogenized and prepared in RIPA buffer. Phosphorylation status of Axl signaling pathway molecules Akt and ERK1/2 was analyzed by Western blotting. Representative blots are shown at the bottom. Densitometry analysis to quantify the ratio of phosphorylated over total signaling protein is shown at the top. Total amounts of protein loading were indicated by β -actin. The Mann-Whitney U test was used to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Experimental versus WT-control; ### $p < 0.001$, WT nephritic versus Axl-KO and WT (R428) nephritic.

Figure 6. R428 decreases cytokine/chemokine expression in nephritic kidneys

Cortexes of NTS-injected and vehicle-injected mice were cut into <1 mm pieces, homogenized in 1× PBS with protease/phosphatase inhibitors, and frozen overnight at −80°C. Samples were then centrifuged and protein concentration was determined. Tissue lysates were then incubated with the R&D Systems Proteome Profiler Array according to the manufacturer's guidelines. The density of each dot was quantified with ImageJ software. Experiments were repeated twice. Values are the median and interquartile range.

*** $p < 0.001$, NTS-treated versus untreated control; ### $p < 0.001$, WT-NTS versus Axl-KO and WT(R428), Mann-Whitney U test.