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Renin-Angiotensin System Blockade Is Renoprotective in Immune Complex–Mediated Glomerulonephritis

Shunhua Guo, Jolanta Kowalewska, Tomasz A. Wietecha, Masayuki Iyoda, Li Wang, Kenneth Yi, Min Spencer, Miriam Banas, Sanda Alexandrescu, Kelly L. Hudkins, and Charles E. Alpers

Department of Pathology, University of Washington School of Medicine, Seattle, Washington

ABSTRACT

Blockade of the renin-angiotensin system is renoprotective in a variety of chronic nephropathies, but the direct effect of such treatment in active, immune complex–mediated glomerulonephritis is unknown. This study investigated the short- and long-term effects of an angiotensin-converting enzyme inhibitor (enalapril) and an angiotensin II type 1 receptor blocker (losartan) in thymic stromal lymphopoietin transgenic (TSLPtg) mice, which develop mixed cryoglobulinemia and severe cryoglobulinemia-associated membranoproliferative glomerulonephritis. Enalapril and losartan each reduced hypertension, proteinuria, glomerular extracellular matrix deposition, and mesangial cell activation in TSLPtg mice. These renoprotective effects were not observed with hydralazine treatment, despite a similar antihypertensive effect. Treatment with enalapril or losartan also decreased renal plasminogen activator inhibitor-1 in TSLPtg mice, assessed by immunohistochemistry and quantitative real-time reverse transcriptase–PCR. None of the treatments affected immune complex deposition or macrophage infiltration. Overall, enalapril- and losartan-treated TSLPtg mice survived significantly longer than untreated TSLPtg mice. These studies demonstrate that angiotensin blockade may provide renoprotective benefits, independent of its BP-lowering effect, in the treatment of active immune complex–mediated glomerulonephritis.

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Effective treatment for most forms of glomerulonephritis remains an elusive goal. In cases of immune complex–mediated glomerulonephritis, specific treatment options are often limited to immunosuppressive agents such as glucocorticoids and cytotoxic agents, which have the dual burdens of limited efficacy and multiple severe toxicities. An exception is the case of membranoproliferative glomerulonephritis (MPGN) consequent to cryoglobulinemia associated with longstanding hepatitis C virus (HCV) infection. In that setting, antiviral therapy directed at the underlying HCV infection, typically IFN based, can cause remission of cryoglobulinemia and the MPGN if HCV viremia is eradicated.[1] Unfortunately, this occurs in only a minority of patients with this disorder, and other efficacious therapies either have not been identified or have been tested in only a small number of patients.

In recent years, blockade of the renin-angiotensin system (RAS) with angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II type 1 receptor blockers (ARB) has shown compelling renoprotective effects in chronic renal diseases of humans and animal models.[2,3] Most experimental studies of RAS blockade have used chronic nephropathy models resulting from diabetes,[4,5] hypertension,[6,7] nephrotoxicity,[8] reduction of renal

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Correspondence: Dr. Charles E. Alpers, Department of Pathology, University of Washington, 1959 NE Pacific Avenue, Box 357470, Seattle, WA 98195. Phone: 206-598-6409; Fax: 206-543-6678; E-mail: calp@u.washington.edu

mass,[6,9] or chronic renal injury subsequent to acute mesangiolysis induced in rats by anti–Thy-1 antibody[10,11] or subsequent to establishment of an immune complex–induced glomerulonephritis.[12,13] Similarly, the efficacy of RAS blockade in human kidney diseases has been best demonstrated in patients with diabetic nephropathy,[14,15] hypertension,[16,17] or chronic kidney disease (CKD) arising from multiple causes.[18–20] These studies largely focused on general effects of RAS blockade on renal function and proteinuria and did not focus on active immune complex–induced renal disease. ACEI was tested in an active immune complex deposition model of glomerulonephritis in nonhuman primates.[13] That study, in which BP was not measured, failed to demonstrate a benefit during acute injury but showed structural improvements in glomeruli when ACEI commenced subsequent to the termination of immune complex stimulus. We address this limited information base by examining the effect of ACEI and ARB in a unique mouse model of immune complex–mediated MPGN.

We characterized a mouse model of cryoglobulinemia-associated MPGN resulting from overexpression of thymic stromal lymphopoietin (TSLP), a cytokine that causes abnormalities in B cell development.[21,22] TSLP transgenic (TSLPtg) mice develop mixed cryoglobulinemia and a systemic inflammatory disease that involves the kidney, lung, spleen, liver, and skin. These mice develop renal disease that closely resembles human cryoglobulinemia-associated MPGN in which the glomeruli show extensive subendothelial and mesangial immune deposits, marked macrophage influx, mesangial cell proliferation, and mesangial matrix expansion.[21] The disease is fully established at 50 d of age in females and at 120 d of age in males.

In this study, female TSLPtg and wild-type (WT) mice were treated for 4 and 8 wk and male TSLPtg and WT mice were treated for 12 and 24 wk with enalapril (ACEI) or losartan (ARB), and additional groups of female mice were treated with hydralazine. Both enalapril and losartan but not hydralazine treatment resulted in remarkable and rapid amelioration of this immune complex–mediated glomerulonephritis, pointing to potentially new uses for these therapeutic agents in the treatment of humans with similar types of glomerulonephritis.

RESULTS

Enalapril and Losartan Reduced Glomerular Matrix Deposition

TSLPtg mice showed marked enlargement of the glomerular tuft area (GTA) and hypercellularity (Figure 1). Neither enalapril nor losartan altered the overall glomerular cell number. TSLPtg mice had significant expansion of mesangial matrix that progressed from 4- to 8-wk time points in females and from 12- to 24-wk time points in males (Figures 1 and 2). At each time point, enalapril and losartan treatment significantly decreased mesangial matrix expansion, whereas TSLPtg mice treated with hydralazine for 4 and 8 wk showed similar mesangial matrix expansion as untreated TSLPtg mice (percentage of silver-stained area/GTA: females at 8 wk: TSLPtg untreated 24.8 ± 2.0, hydralazine 24.2 ± 1.4, enalapril 15.4 ± 2.2, losartan 16.2 ± 1.2, WT 9.6 ± 1.5; males at 24 wk: TSLPtg untreated 26.9 ± 2.4, enalapril 18.1 ± 1.7, losartan 20.6 ± 1.6, WT 12.7 ± 2.5; $P < 0.01$ *versus* matched untreated or hydralazine-treated TSLPtg mice).

Type IV collagen, an important component of glomerular extracellular matrix, was used as a second measure of matrix expansion. There was an increase in type IV collagen deposition comparable in extent and amount to increased silver staining matrix in TSLPtg mice (Figures 1 and 2). The deposition of type IV collagen in glomeruli decreased significantly in enalapril- or losartan- but not hydralazine-treated TSLPtg groups (percentage of type IV collagen staining area/GTA: females at 8 wk: TSLPtg untreated 16.7 ± 0.9, hydralazine 17.1 ± 0.5, enalapril 10.7 ± 1.0, losartan 12.5 ± 1.5, WT 7.4 ± 0.9; males at 24 wk: TSLPtg untreated 18.3 ± 3.5, enalapril 13.4 ± 1.3, losartan 14.3 ± 1.2, WT 8.1 ± 0.5; $P < 0.05$ *versus* matched untreated or hydralazine-treated TSLPtg mice). There was no alteration of type IV collagen distribution in treated *versus* untreated WT mice.

Enalapril and Losartan Reduced Glomerular Mesangial Cell Activation

Glomerular α-smooth muscle actin (α-SMA) expression, an indicator of mesangial cell activation, was dramatically increased in the TSLPtg mice. Treatment with enalapril or losartan but not hydralazine significantly decreased mesangial α-SMA expression (Supplemental Figures 1 and 2).

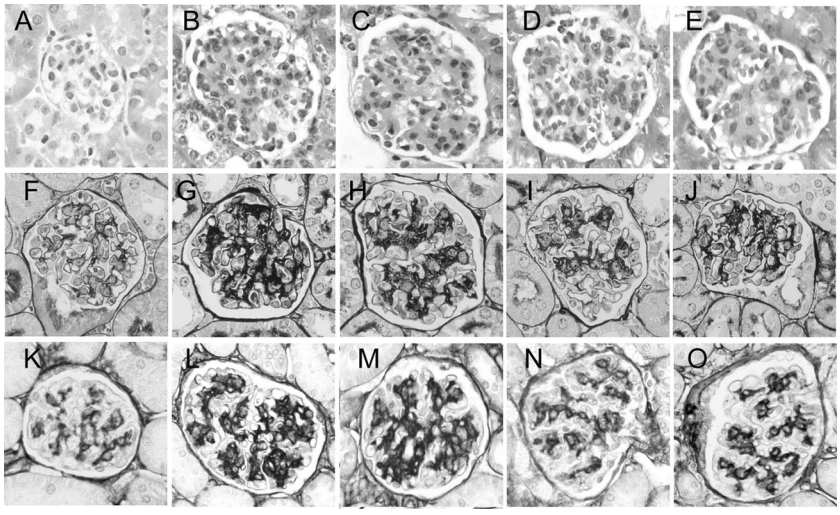

Figure 1. Representative photographs of glomeruli with hematoxylin and eosin (A through E), silver methenamine (F through J), and type IV collagen (K through O) immunohistochemistry staining of 8-wk groups of untreated WT (A, F, and K) and TSLPtg mice (B, G, and L), and hydralazine-treated (C, H, and M), enalapril-treated (D, I, and N), or losartan-treated (E, J, and O) TSLPtg mice. Magnification, ×400.

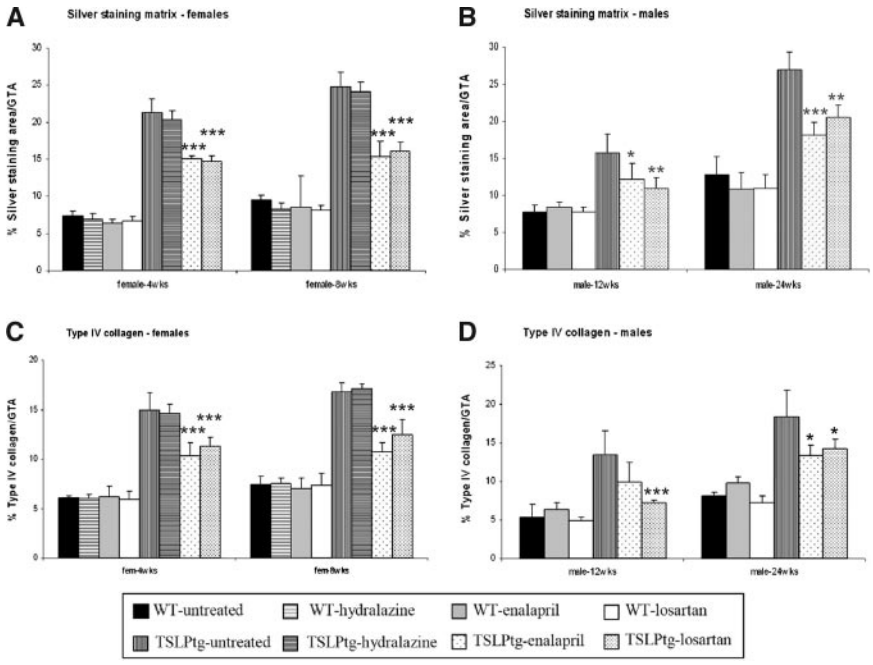

Figure 2. Morphometric analysis of glomerular silver staining matrix and type IV collagen immunohistochemistry staining in untreated and hydralazine-, enalapril-, or losartan-treated WT and TSLPtg mice of female 4- and 8-wk and male 12- and 24-wk groups. Data are means ± SEM ($n = 4$ to 6 in each group). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ *versus* matched untreated TSLPtg control.

Enalapril and Losartan Decreased Renal Plasminogen Activator Inhibitor-1 Expression

TSLPtg mice express high plasminogen activator inhibitor-1 (PAI-1) mRNA levels in the kidney (Figure 3). Enalapril decreased PAI-1 mRNA expression levels in both 8- and 24-wk treatment groups (fold increase of PAI-1 mRNA expression relative to WT control: females at 8 wk: TSLPtg untreated 3.3 ± 0.4, enalapril 1.4 ± 0.4, losartan 1.8 ± 0.6; males at 24 wk: TSLPtg untreated 4.5 ± 0.5, enalapril 2.3 ± 0.5, losartan 3.4 ± 0.5; $P < 0.05$ enalapril groups *versus* matched untreated TSLPtg mice). Losartan-treated groups had a trend toward decreased expression of PAI-1 mRNA, but the differences compared with untreated TSLPtg control were not statistically significant.

In parallel, glomerular PAI-1 protein was markedly increased in TSLPtg mice (Figures 4 and 5). Both enalapril and losartan treatment decreased glomerular PAI-1 protein expression (percentage of PAI-1 staining area/GTA: females at 8 wk: TSLPtg untreated 3.4 ± 0.1, enalapril 1.9 ± 0.1, losartan 2.3 ± 0.1, WT control 1.3 ± 0.1; males at 24 wk: TSLPtg untreated 3.9 ± 0.1, enalapril 2.7 ± 0.1, losartan 2.6 ± 0.1, WT control 1.4 ± 0.2; $P < 0.05$ *versus* matched untreated TSLPtg control).

Enalapril and Losartan Did not Change Renal Ig and Complement Deposition and Macrophage Infiltration

Immunofluorescence showed that TSLPtg mice had massive deposition of IgG, IgM, and, to a lesser extent, IgA and complement C3 in glomeruli. Treatment with hydralazine, enalapril, and losartan did not change the extent or intensity of staining of deposited Ig and complement C3 (Figure 6 and Supplemental Figure 3). Electron microscopy showed that the untreated and treated TSLPtg groups had similarly extensive electron-dense immune deposits in subendothelial and mesangial area, as described previously (data not shown).[21]

TSLPtg mice had prominent monocyte/macrophage infiltration in the glomerular tuft as assessed by Mac-2 staining. Hydralazine, enalapril, and losartan treatment did not significantly change the extent of monocyte/macrophage influx (Supplemental Figures 1 and 2).

Enalapril and Losartan Did not Change Glomerular Cell Proliferation and Apoptosis

In TSLPtg mice, the number of glomerular Ki-67–expressing cells was increased significantly compared with WT controls. Hydralazine, enalapril, and losartan treatment did not change the number of Ki-67–expressing cells in TSLPtg and WT mice (Ki-67–expressing cells/50 glomeruli: females at 8 wk: TSLPtg untreated 26.6 ± 6.6, hydralazine 28.3 ± 3.1, enalapril 26.2 ± 1.8, losartan 27.6 ± 1.5, WT untreated 18.8 ± 1.4; males at 24 wk: TSLPtg untreated 31.2 ± 2.8, enalapril 31.8 ± 3.6, losartan 30.2 ± 2.6, WT untreated 22.8 ± 3.1). As assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, TSLPtg mice had few apoptotic cells in glomeruli (two to three positive cells in 50 glomeruli) with only rare scattered TUNEL-positive cells in the interstitium. Hydralazine, enalapril, and losartan treatment did not change the number of apoptotic cells in glomeruli and interstitium (data not shown).

Proteinuria Was Markedly Reduced by Enalapril and Losartan but not by Hydralazine Treatment

Urine albumin excretion increased dramatically in untreated TSLPtg mice (Figure 7). Hydralazine-treated TSLPtg mice showed a similar level of massive proteinuria. Both enalapril and losartan markedly reduced albumin excretion in TSLPtg mice (urine albumin-creatinine ratio [μg/mg]: females at 8 wk: TSLPtg untreated 156.8 ± 42.4, hydralazine 161.6 ± 30.8, enalapril 66.9 ± 17.4, losartan 56.8 ± 12.0, WT untreated 27.2 ± 4.9; males at 24 wk: TSLPtg untreated 107.3 ± 13.4, enalapril 66.9 ± 7.5, losartan 60.0 ± 12.0, WT untreated 30.0 ± 5.9; $P < 0.05$ *versus* matched untreated and hydralazine treated TSLPtg mice). There was no difference in serum creatinine levels among the various study groups (data not shown).

BP was Effectively Decreased by Enalapril, Losartan, and Hydralazine

The systolic BP increased progressively from 4 to 8 wk and from 4 to 24 wk in female and male TSLPtg mice, respectively, as compared with age-matched WT controls. Treatment with hydralazine, enalapril, and losartan reduced BP in TSLPtg mice and WT mice at every time point, but the decrease of BP in WT mice is less than in TSLPtg mice (Figure 8). Enalapril and losartan had similar antihypertensive effects in males, but in the 4- and 8-wk female TSLPtg mice, systolic BP of the enalapril group was lower than that of the losartan group but was similar to that of the hydralazine group (BP of TSLPtg 4 wk [mmHg]: untreated 123 ± 4, hydralazine 88 ± 6, enalapril 82 ± 5; losartan 104 ± 4; TSLPtg 8 wk: untreated 131 ± 4, hydralazine 94 ± 5, enalapril 90 ± 2, losartan 110 ± 4; $P < 0.05$ losartan groups *versus* matched enalapril or hydralazine groups).

Enalapril and Losartan Increased the Long-Term Survival of TSLPtg Mice

Observation of the 24-wk groups of male TSLPtg and WT mice demonstrated that untreated male TSLPtg mice had a high mortality rate (58% at 24 wk), similar to previous studies.[21] In contrast, enalapril- and losartan-treated male TSLPtg mice had much lower mortality rates (8% in enalapril group and 17% in losartan group at 24 wk of treatment; Figure 9). Treatment of WT mice had no effect on survival.

Systemic Effects of Enalapril and Losartan

All TSLPtg mice had circulating cryoglobulins, whereas WT mice did not. Treatment with enalapril and losartan did not change circulating cryoglobulin levels (data not shown).

In TSLPtg mice, manifestations of systemic cryoglobulinemia and B cell defects included marked increase of the weight of spleen and lung as a result of increased cellularity or inflammation. Hydralazine, enalapril, and losartan treatment did not change spleen weights. Lung weights tended to decrease with enalapril and losartan treatment, although only the decrease of lung weights in the 12-wk losartan group and the 24-wk enalapril group reached statistical significance (Supplemental Figure 4). In contrast, hydralazine-treated TSLPtg mice had similar lung weights as untreated TSLPtg control. The weights of kidney and liver were not different among groups (data not shown).

Histologic evaluation of the liver showed portal leukocyte infiltration as described previously,[21,23] which was unchanged by treatment with hydralazine, enalapril, or losartan (Table 1). The lung lesion in TSLPtg mice is characterized by marked perivascular and peribronchiolar leukocyte infiltration (Figure 10). Hydralazine-treated TSLPtg mice showed a similar level of lung inflammation. Both enalapril and losartan treatment resulted in decreased leukocyte infiltration in the lung with more open bronchioli and alveoli (Figure 10, Table 1).

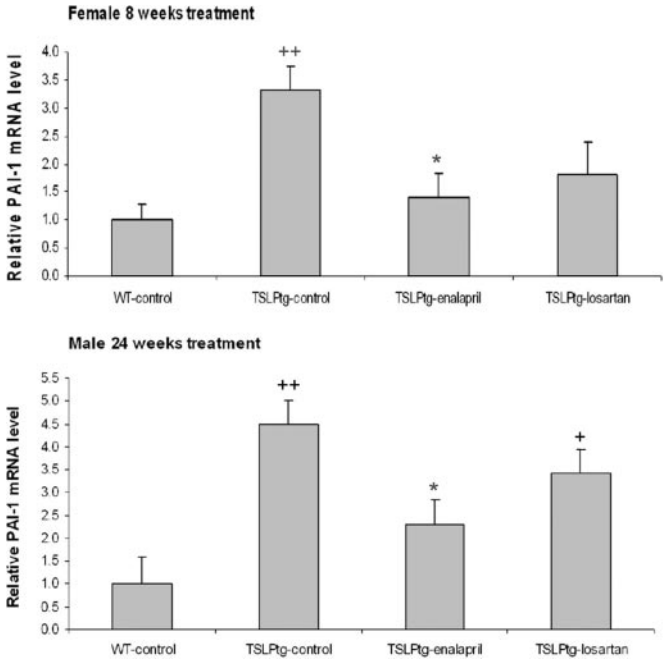

Figure 3. Kidney PAI-1 mRNA expression was reduced by enalapril and losartan treatment as assessed by quantitative real-time RT-PCR. The fold change of the expression level of PAI-1 gene in TSLPtg mice compared with WT mice was normalized to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Data are fold change (means ± SEM) of PAI-1 mRNA expression relative to WT control ($n = 4$ in WT control and $n = 6$ in untreated and treated TSLPtg groups). $^{++}P < 0.01$, $^{+}P < 0.05$ *versus* matched WT control; $^{*}P < 0.05$ *versus* matched untreated TSLPtg control.

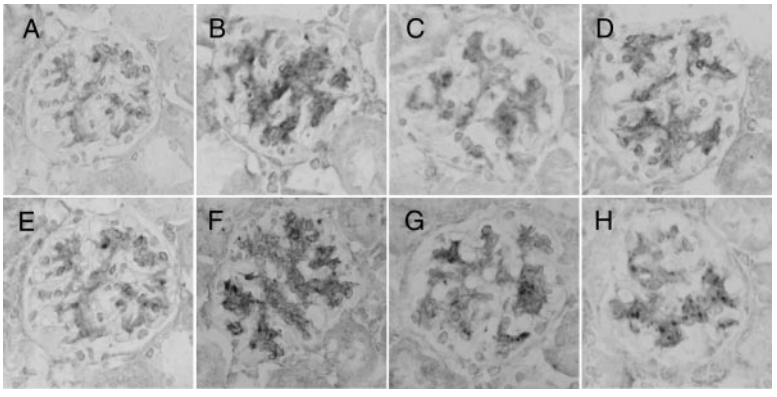

Figure 4. Representative photographs of glomerular PAI-1 immunohistochemistry staining of female 8-wk groups (A through D) and male 24-wk groups (E through H) of untreated WT (A and E), untreated TSLPtg (B and F), and enalapril-treated (C and G) and losartan-treated (D and H) TSLPtg mice. Magnification, ×400.

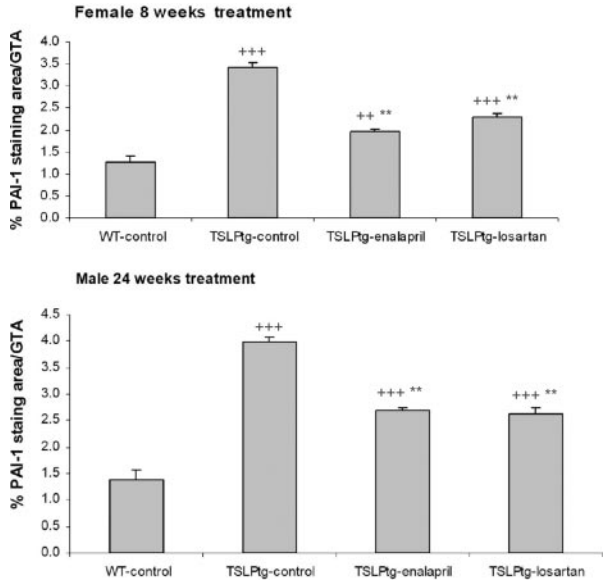

Figure 5. Morphometric measurements of glomerular PAI-1 protein expression of female 8-wk groups and male 24-wk groups. Untreated TSLPtg control mice have markedly increased glomerular PAI-1 protein expression in both 8-wk females and 24-wk males compared with WT mice of the same age. Enalapril and losartan treatment decreased glomerular PAI-1 expression in TSLPtg mice. Data are means ± SEM ($n = 4$ in WT control, $n = 5$ in untreated and treated TSLPtg groups). $^{+++}P < 0.001$, $^{++}P < 0.01$ *versus* WT control; $^{**}P < 0.01$ *versus* untreated TSLPtg control.

DISCUSSION

The value of inhibition of the RAS, whether by ACEI or by ARB, has been established in clinical trials of patients with most forms of CKD, including diabetic nephropathy and hypertensive kidney disease. In this study, we demonstrated that these agents also provide a clear and continuous benefit in ameliorating the glomerular injury consequent to persistent, ongoing immune complex deposition. These benefits are both structural (diminished extracellular matrix [ECM] accumulation and reduced activation of mesangial cells) and functional (decreased proteinuria). The renal and systemic benefits confer reduced mortality in cryoglobulinemic mice. In contrast, despite a comparable lowering of BP, hydralazine was not effective in ameliorating glomerular injury and reducing proteinuria. These findings, in aggregate, suggest that RAS blockade can be effective as a treatment for active glomerulonephritis in humans, in addition to their established benefits in CKD.

The findings underscore the specific renoprotective effects of RAS blockade that are independent of any benefit conferred by reduction in BP. Although hydralazine, enalapril, and losartan showed marked lowering of BP in the TSLPtg mice throughout the treatment course, only enalapril and losartan achieved significant structural and functional improvement of renal lesions. Treatment did not significantly affect the levels of circulating cryoglobulins or the extensive immune complex deposition or complement fixation in glomeruli, all components of the process by which the glomerular injury was initiated. Our studies do not allow us to conclude that the survival benefit in the ACEI- and ARB-treated mice was due to a specific effect of RAS blockade independent of effects on systemic BP lowering, because a needed control group of hydralazine-treated mice with lowered BP was not done for the survival study component of this experiment.

Because the improvement of MPGN was similar in mice that were treated either with an ACEI or a blocker targeting the AT1R, the benefit is not due to interruption of activities of angiotensin II consequent to engagement of other receptors (*e.g.*, AT2R) or blockade of other activities attributable to ACE such as degradation of bradykinin. It is well established that angiotensin II acting *via* AT1R triggers vasoconstriction and aldosterone release and mediates proinflammatory and profibrogenic effects that perpetuate progressive kidney injury.[24,25] Angiotensin II activates mesangial cells, induces the expression of TGF-β1 and PAI-1, and thus promotes ECM accumulation.[25] Our study demonstrates that treatment of TSLPtg mice with enalapril or losartan, possibly acting through each of these mechanisms, effectively reduces glomerular ECM expansion and mesangial cell activation, largely abolishing the amplification phase of MPGN after injury was initiated.

Decreased degradation of ECM is also an important factor in the expansion of

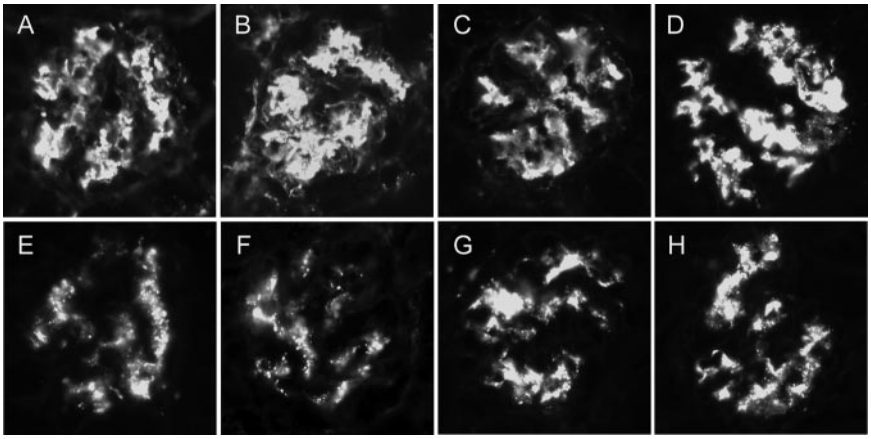

Figure 6. Kidney immunofluorescence study of untreated and hydralazine-, enalapril-, or losartan-treated TSLPtg mice. Photos are representative glomerular immunofluorescence staining of deposited IgG (A through D) and complement C3 (E through H) of female 8-wk groups of untreated TSLPtg mice (A and E) and hydralazine-treated (B and F), enalapril-treated (C and G), and losartan-treated (D and H) TSLPtg mice. Magnification, ×400.

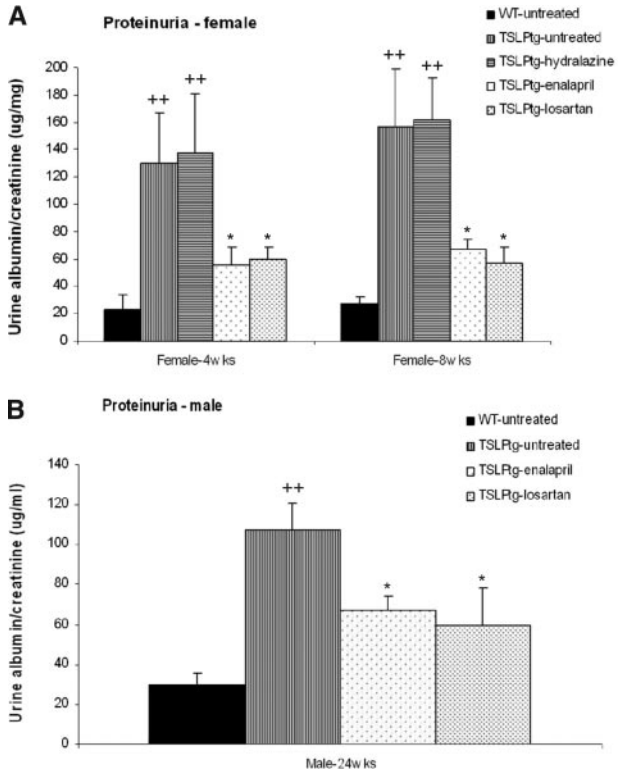

Figure 7. (A and B) Proteinuria of 4- and 8-wk females of untreated WT and TSLPtg mice and hydralazine-, enalapril-, or losartan-treated TSLPtg mice (A), and 24-wk males of untreated WT and TSLPtg mice and enalapril- or losartan-treated TSLPtg mice (B). Proteinuria was measured as urine albumin (μg)-creatinine (mg) ratio. Untreated TSLPtg mice have marked increased urine albumin excretion compared with WT mice. The proteinuria level of hydralazine-treated TSLPtg mice is similar to that of untreated TSLPtg mice. Proteinuria was significantly decreased in enalapril- or losartan-treated TSLPtg mice. Data are means ± SEM ($n = 4$ to 6 per group). $^{*}P < 0.05$ *versus* matched untreated TSLPtg control; $^{++}P < 0.01$ *versus* WT control.

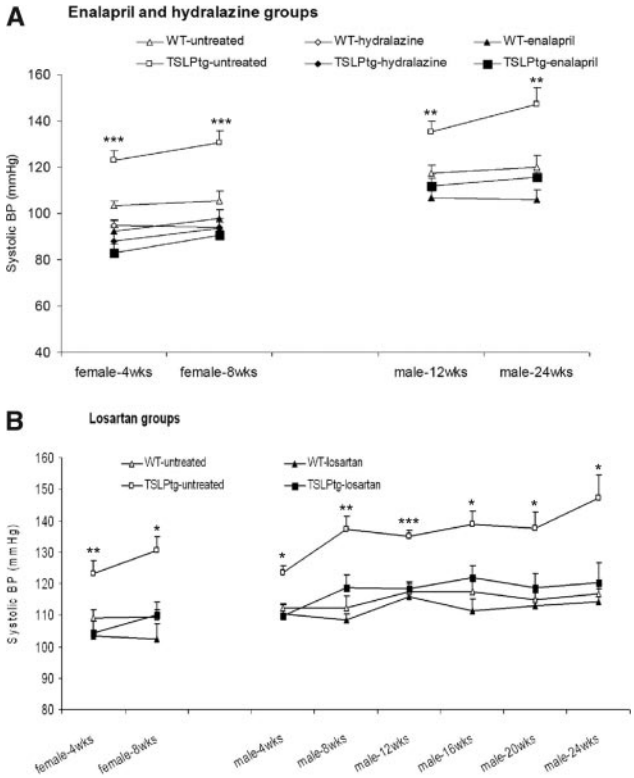

Figure 8. Time course of systolic BP in untreated and hydralazine-, enalapril- or losartan-treated wild type and TSLP transgenic mice. BP of each mouse at each time point is the mean of 6 to 10 measurements. Every group had 4 to 6 mice. Data are mean ± SEM (mmHg). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ hydralazine-, enalapril- or losartan-treated TSLPtg mice *versus* matched untreated TSLPtg control.

mesangial matrix and glomerulosclerosis. PAI-1 is an important factor in the maintenance of the delicate balance between ECM synthesis and degradation; its activities on plasmin and other proteases limit matrix degradation. Angiotensin induces PAI-1, and, conversely, angiotensin blockade decreases PAI-1 expression and decreases sclerosis.[9] Both enalapril and losartan decreased renal mRNA and protein expression of PAI-1 in TSLPtg mice, likely contributing to the reduction in matrix accumulation.

There is increasing evidence that angiotensin II has immunomodulatory effects.[26,27] Some immunomodulatory effects of RAS blockade likely contributed to the improvement of the systemic well-being of TSLP mice. Enalapril- and losartan-treated TSLPtg mice had lower mortality, which we attribute in part to reduction of renal injury and part to the reduction of the severe lung inflammation that occurs in these mice. In contrast, hydralazine-treated TSLPtg mice showed a similar degree of lung inflammation as untreated TSLPtg mice. This immunomodulatory effect of RAS blockade, not yet identified more specifically, may be organ specific because improvement in the concurrent hepatitis that occurs in the TSLPtg model was not observed using any of the treatment modalities.[28]

In summary, we demonstrated that treatment with enalapril and losartan resulted in a dramatic attenuation of MPGN and had a beneficial effect on long-term survival in mice with cryoglobulinemia-associated glomerulonephritis. The long-term effects of RAS blockade have been well established in a variety of chronic injury models; what is surprising is the speed and efficacy of this effect in glomerulonephritis, where there is active, ongoing deposition of immune reactants. The beneficial effects included reduced accumulation of glomerular ECM, decreased mesangial cell activation, decreased expression of PAI-1, reduced proteinuria, and decreased mortality. We conclude that RAS blockade has the potential to be a useful therapeutic strategy in

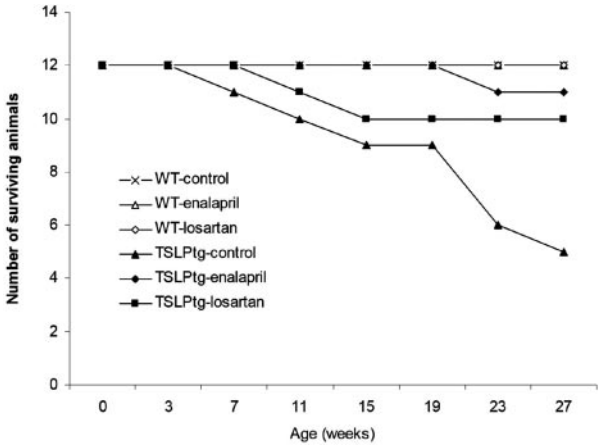

Figure 9. Survival curves of 24-wk male TSLPtg and WT mouse groups. At the start point, all groups had 12 male mice. Treatment groups started enalapril or losartan treatment at 3 wk of age and continued for 24 wk. Seven of 12 untreated TSLPtg mice died in this duration, whereas one of 12 enalapril-treated TSLPtg mice and two of 12 losartan-treated TSLPtg mice died in this duration, suggesting enalapril and losartan treatment markedly improved survival in TSLPtg mice. They did not show an impact on survival of WT mice in this time frame.

the treatment of at least some types of immune complex–mediated glomerulonephritis in humans.

CONCISE METHODS

Animals and Experimental Design

The experimental protocol was approved by the Animal Care Committee of the University of Washington. TSLPtg mice on a C57BL/6 background have been characterized previously.[21,29,30]

Pups weaned at age 3 wk were randomly assigned to the following treatment groups: (*1*) Enalapril treatment groups: enalapril (Sigma-Aldrich, St. Louis, MO) 125 mg/L in drinking water, a dosage equal to 30 mg/kg body wt per d; (*2*) losartan treatment groups: losartan (Merck, Whitehouse Station, NJ) 100 mg/L in drinking water, equal to 25 mg/kg body wt per d; (*3*) hydralazine treatment groups: hydralazine (Sigma-Aldrich) 200 mg/L in drinking water, equal to 50 mg/kg body wt per d; (*4*) control groups: normal drinking water. Each group had four to eight TSLPtg and WT littermates each except for the 24-wk groups, which had 12 mice of each genotype for assessment of the long-term survival effect of ACEI and ARB treatment. Hydralazine treatment continued for 4 and 8 wk in females. Enalapril and losartan treatment continued for 4 or 8 wk in females and for 12 or 24 wk in males until they were killed. Different time frames for treatment of females and males were based on previous observations that although male and female mice develop an identical pattern of glomerular injury in this model, the effects of gender are manifested by a greater rapidity in onset and progression of disease in females as compared with males, with females typically having overt disease by age 30 d and developing the full manifestations of disease by day 50. In contrast, the onset of disease is slower in males, typically evident by day 50, and may take upwards of 120 d to be fully manifested. At the time the disease fully manifests, the renal and systemic manifestations of the cryoglobulinemic process are indistinguishable in males and females.

BP Measurement

BP was measured by CODA6 noninvasive tail-cuff system (Kent Scientific, Torrington, CT). Mice were kept in a quiet and warm environment in this procedure. The first five measuring cycles were acclimation cycles, and these measurements were not recorded. BP of each mouse at each time point is the mean of 6 to 10 subsequent measurements. For female mice, BP was measured at 4 and 8 wk of treatment. For males, BP of losartan groups was measured at 4, 8, 12, 16, 20, and 24 wk of treatment and of enalapril groups at 12 and 24 wk of treatment.

Analysis of Kidney Function

Before the mice were killed, spot urine was collected from each mouse. Blood was collected by retro-orbital bleeding after anesthetization. Urine albumin was measured by ELISA using the Albuwell kit (Exocell, Philadelphia, PA), and urine creatinine was measured using the Creatinine Companion kit (Exocell). Proteinuria was calculated as the urine albumin-creatinine ratio. Serum creatinine was measured using HPLC.

Serum Cryoglobulin Evaluation

Serum cryocrit was measured by aliquotting 100 μl of serum into Natelson blood collecting glass tubes (Chase Scientific Glass, Rockwood, TN). After 1 wk at 4°C, the heights of cryoprecipitates were measured and expressed as a percentage of total serum heights.

Table 1. Semiquantitative grading of lung and liver inflammation[a]

Parameter	TSLPtg-Untreated	TSLPtg-Hydralazine	TSLPtg-Enalapril	TSLPtg-Losartan
Lung inflammation				
female 8 wk	2.8 ± 0.4	2.7 ± 0.3	1.8 ± 0.4[b]	2.0 ± 0.7[b]
male 24 wk	2.7 ± 0.5	–	1.3 ± 0.5[c]	1.7 ± 0.5[b]
Liver inflammation				
female 8 wk	1.5 ± 0.6	1.5 ± 0.8	1.3 ± 0.5	1.4 ± 0.6
male 24 wk	1.5 ± 0.6	–	1.5 ± 0.6	1.3 ± 0.5

[a]Data are means ± SEM (n = 4 to 5 in each group).
[b]$P < 0.05$, [c]$P < 0.01$ *versus* matched untreated TSLPtg control mice.

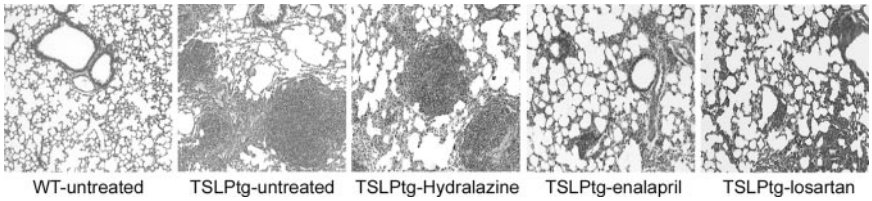

Figure 10. Representative lung sections with hematoxylin and eosin staining of 8-wk female groups of untreated WT and TSLPtg mice, and hydralazine-, enalapril-, or losartan-treated TSLPtg mice. Magnification, ×100.

Tissue Collection and Histologic Study

Kidney, spleen, liver, and lung were collected when the mice were killed. Tissues were fixed, embedded, and stained with hematoxylin and eosin and methenamine silver as described previously.[21] Portions of kidney were snap-frozen and stored at −80°C for RNA extraction and for immunofluorescence studies. Tissue for electron microscopy was fixed in half-strength Karnovsky's solution and processed as described previously.[21,31]

Immunofluorescence

Acetone-fixed frozen kidney sections were incubated with fluorescein-conjugated antibodies against mouse IgG, IgM, IgA, and complement C3 (all from Cappel Pharmaceuticals, Aurora, OH) as described previously.[21,30] In a blinded manner, the glomerular fluorescence intensity was scored semiquantitatively (0, negative; 1, weak; 2, moderate; 3, strong). For every sample, 15 glomeruli were counted and a mean score was calculated.

Immunohistochemistry

The immunohistochemistry protocols for type IV collagen, α-SMA, macrophage marker Mac-2, and PAI-1 have been described previously.[21,29] Cellular proliferation was assessed with a rat monoclonal anti-Ki-67 antibody (Dako, Carpinteria, CA). Apoptosis was evaluated by TUNEL using Apoptag apoptosis kit (Chemicon, Temecula, CA) as described previously.[9]

Quantitative Real-Time PCR

Total kidney RNA was extracted from frozen kidney tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). Real-time PCR was performed using Taqman gene expression assay kits for the primers and probes of mouse PAI-1 and glyceraldehyde-3-phosphate dehydrogenase gene (Applied Biosystems, Foster City, CA). The fold change of the expression level of PAI-1 gene in TSLPtg mice compared with WT mice was normalized to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Analytical Methods and Statistical Analysis

Morphometry study for GTA and the glomerular area occupied by silver staining or stained by antibodies to type IV collagen, α-SMA, Mac-2, and PAI-1 were quantified as described previously.[29,30] Glomerular cell proliferation and apoptosis were assessed by counting the number of Ki-67– and TUNEL-positive cells in 50 random glomeruli. Liver and lung tissue sections stained with hematoxylin and eosin were assessed for evidence of inflammation. Liver and lung inflammation was scored semiquantitatively on a scale of 0 to 3: 0, no inflammation; 1, mild; 2, moderate; 3, severe with regard to the extent and density of leukocytic infiltration.

All data are expressed as mean ± SEM. Statistical analysis of the data for multiple groups was performed by ANOVA with Tukey-Kramer multiple comparisons test using the InStat program (version 3.0; Intuitive Software for Science, San Diego, CA). $P < 0.05$ was considered significant.

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DISCLOSURES

None.

REFERENCES

1. Kamar N, Rostaing L, Alric L: Treatment of hepatitis C-virus-related glomerulonephritis. *Kidney Int* 69: 436–439, 2006
2. Wolf G, Butzmann U, Wenzel UO: The renin-angiotensin system and progression of renal disease: From hemodynamics to cell biology. *Nephron Physiol* 93: P3–P13, 2003
3. Tylicki L, Larczynski W, Rutkowski B: Renal protective effects of the renin-angiotensin-aldosterone system blockade: from evidence-based approach to perspectives. *Kidney Blood Press Res* 28: 230–242, 2005
4. Wilkinson-Berka JL, Gibbs NJ, Cooper ME, Skinner SL, Kelly DJ: Renoprotective and anti-hypertensive effects of combined valsartan and perindopril in progressive diabetic nephropathy in the transgenic (mRen-2)27 rat. *Nephrol Dial Transplant* 16: 1343–1349, 2001
5. Zheng F, Zeng YJ, Plati AR, Elliot SJ, Berho M, Potier M, Striker LJ, Striker GE: Combined AGE inhibition and ACEi decreases the progression of established diabetic nephropathy in B6 db/db mice. *Kidney Int* 70: 507–514, 2006
6. Yu C, Gong R, Rifai A, Tolbert EM, Dworkin LD: Long-term, high-dosage candesartan suppresses inflammation and injury in chronic kidney disease: Nonhemodynamic renal protection. *J Am Soc Nephrol* 18: 750–759, 2007
7. Izuhara Y, Nangaku M, Inagi R, Tominaga N, Aizawa T, Kurokawa K, van Ypersele de Strihou C, Miyata T: Renoprotective properties of angiotensin receptor blockers beyond blood pressure lowering. *J Am Soc Nephrol* 16: 3631–3641, 2005
8. Kramer AB, van der Meulen EF, Hamming I, van Goor H, Navis G: Effect of combining ACE inhibition with aldosterone blockade on proteinuria and renal damage in experimental nephrosis. *Kidney Int* 71: 417–424, 2007
9. Ma LJ, Nakamura S, Aldigier JC, Rossini M, Yang H, Liang X, Nakamura I, Marcantoni C, Fogo AB: Regression of glomerulosclerosis with high-dose angiotensin inhibition is linked to decreased plasminogen activator inhibitor-1. *J Am Soc Nephrol* 16: 966–976, 2005
10. Nakamura T, Obata J, Kimura H, Ohno S, Yoshida Y, Kawachi H, Shimizu F: Blocking angiotensin II ameliorates proteinuria and glomer-

ular lesions in progressive mesangioproliferative glomerulonephritis. *Kidney Int* 55: 877–889, 1999
11. Asai M, Monkawa T, Marumo T, Fukuda S, Tsuji M, Yoshino J, Kawachi H, Shimizu F, Hayashi M, Saruta T: Spironolactone in combination with cilazapril ameliorates proteinuria and renal interstitial fibrosis in rats with anti-Thy-1 irreversible nephritis. *Hypertens Res* 27: 971–978, 2004
12. Ruiz-Ortega M, Gomez-Garre D, Liu XH, Blanco J, Largo R, Egido J: Quinapril decreases renal endothelin-1 expression and synthesis in a normotensive model of immune-complex nephritis. *J Am Soc Nephrol* 8: 756–768, 1997
13. Hebert LA, Birmingham DJ, Mahan JD, Cosio FG, Dillon JJ, Sedmak DD, Shen XP, McAllister C: Effect of enalapril therapy on glomerular accumulation of immune complexes and mesangial matrix in experimental glomerulonephritis in the nonhuman primate. *Am J Kidney Dis* 30: 243–252, 1997
14. Keane WF, Brenner BM, de Zeeuw D, Grunfeld JP, McGill J, Mitch WE, Ribeiro AB, Shahinfar S, Simpson RL, Snapinn SM, Toto R: The risk of developing end-stage renal disease in patients with type 2 diabetes and nephropathy: The RENAAL study. *Kidney Int* 63: 1499–1507, 2003
15. Imai E, Ito S, Haneda M, Chan JC, Makino H: Olmesartan reducing incidence of endstage renal disease in diabetic nephropathy trial (ORIENT): Rationale and study design. *Hypertens Res* 29: 703–709, 2006
16. Iino Y, Hayashi M, Kawamura T, Shiigai T, Tomino Y, Yamada K, Kitajima T, Ideura T, Koyama A, Sugisaki T, Suzuki H, Umemura S, Kawaguchii Y, Uchida S, Kuwahara M, Yamazaki T: Renoprotective effect of losartan in comparison to amlodipine in patients with chronic kidney disease and hypertension: A report of the Japanese Losartan Therapy Intended for the Global Renal Protection in Hypertensive Patients (JLIGHT) study. *Hypertens Res* 27: 21–30, 2004
17. Wright JT Jr, Bakris G, Greene T, Agodoa LY, Appel LJ, Charleston J, Cheek D, Douglas-Baltimore JG, Gassman J, Glassock R, Hebert L, Jamerson K, Lewis J, Phillips RA, Toto RD, Middleton JP, Rostand SG: Effect of blood pressure lowering and antihypertensive drug class on progression of hypertensive kidney disease: Results from the AASK trial. *JAMA* 288: 2421–2431, 2002
18. Brenner BM: Remission of renal disease: Recounting the challenge, acquiring the goal. *J Clin Invest* 110: 1753–1758, 2002
19. Ruggenenti P, Perna A, Gherardi G, Garini G, Zoccali C, Salvadori M, Scolari F, Schena FP, Remuzzi G: Renoprotective properties of ACE-inhibition in non-diabetic nephropathies with non-nephrotic proteinuria. *Lancet* 354: 359–364, 1999
20. Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. The GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). *Lancet* 349: 1857–1863, 1997
21. Taneda S, Segerer S, Hudkins KL, Cui Y, Wen M, Segerer M, Wener MH, Khairallah CG, Farr AG, Alpers CE: Cryoglobulinemic glomerulonephritis in thymic stromal lymphopoietin transgenic mice. *Am J Pathol* 159: 2355–2369, 2001
22. Astrakhan A, Omori M, Nguyen T, Becker-Herman S, Iseki M, Aye T, Hudkins K, Dooley J, Farr A, Alpers CE, Ziegler SF, Rawlings DJ: Local increase in thymic stromal lymphopoietin induces systemic alterations in B cell development [erratum appears in *Nat Immunol* 8: 780, 2007]. *Nat Immunol* 8: 522–531, 2007
23. Kowalewska J, Muhlfeld AS, Hudkins KL, Yeh MM, Farr AG, Ravetch JV, Alpers CE: Thymic stromal lymphopoietin transgenic mice develop cryoglobulinemia and hepatitis with similarities to human hepatitis C liver disease. *Am J Pathol* 170: 981–989, 2007
24. Ruster C, Wolf G: Renin-angiotensin-aldosterone system and progression of renal disease. *J Am Soc Nephrol* 17: 2985–2991, 2006
25. Wolf G: Renal injury due to renin-angiotensin-aldosterone system activation of the transforming growth factor-beta pathway. *Kidney Int* 70: 1914–1919, 2006
26. De Albuquerque DA, Saxena V, Adams DE, Boivin GP, Brunner HI, Witte DP, Singh RR: An ACE inhibitor reduces Th2 cytokines and TGF-beta1 and TGF-beta2 isoforms in murine lupus nephritis. *Kidney Int* 65: 846–859, 2004
27. Weidanz JA, Jacobson LM, Muehrer RJ, Djamali A, Hullett DA, Sprague J, Chiriva-Internati M, Wittman V, Thekkumkara TJ, Becker BN: ATR blockade reduces IFN-gamma production in lymphocytes in vivo and in vitro. *Kidney Int* 67: 2134–2142, 2005
28. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, Gyarmati D, Aye T, Campbell DJ, Ziegler SF: Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol* 6: 1047–1053, 2005
29. Taneda S, Hudkins KL, Cui Y, Farr AG, Alpers CE, Segerer S: Growth factor expression in a murine model of cryoglobulinemia. *Kidney Int* 63: 576–590, 2003
30. Muhlfeld AS, Segerer S, Hudkins K, Carling MD, Wen M, Farr AG, Ravetch JV, Alpers CE: Deletion of the fcgamma receptor IIb in thymic stromal lymphopoietin transgenic mice aggravates membranoproliferative glomerulonephritis. *Am J Pathol* 163: 1127–1136, 2003
31. Alpers CE, Hudkins KL, Pritzl P, Johnson RJ: Mechanisms of clearance of immune complexes from peritubular capillaries in the rat. *Am J Pathol* 139: 855–867, 1991

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