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Research Article **Nephrology**

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Studies in humans and animal models indicate a key contribution of angiotensin II to the pathogenesis of glomerular diseases. To examine the role of type 1 angiotensin (AT_1) receptors in glomerular inflammation associated with autoimmune disease, we generated MRL-*Fas*$^{lpr/lpr}$ (*lpr*) mice lacking the major murine type 1 angiotensin receptor (AT_{1A}); *lpr* mice develop a generalized autoimmune disease with glomerulonephritis that resembles SLE. Surprisingly, AT_{1A} deficiency was not protective against disease but instead substantially accelerated mortality, proteinuria, and kidney pathology. Increased disease severity was not a direct effect of immune cells, since transplantation of AT_{1A}-deficient bone marrow did not affect survival. Moreover, autoimmune injury in extrarenal tissues, including skin, heart, and joints, was unaffected by AT_{1A} deficiency. In murine systems, there is a second type 1 angiotensin receptor isoform, AT_{1B}, and its expression is especially prominent in the renal glomerulus within podocytes. Further, expression of renin was enhanced in kidneys of AT_{1A}-deficient *lpr* mice, and they showed evidence of exaggerated AT_{1B} receptor activation, including substantially increased podocyte injury and expression of inflammatory mediators. Administration of losartan, which blocks all type 1 angiotensin receptors, reduced markers of kidney disease, including proteinuria, glomerular pathology, and cytokine mRNA expression. Since AT_{1A}-deficient *lpr* mice had low blood pressure, these findings suggest that activation of type 1 angiotensin receptors in the glomerulus is sufficient to accelerate renal injury and inflammation in the absence of hypertension.

Introduction

The renin-angiotensin system (RAS) has profound effects on blood pressure and vascular reactivity (1). Angiotensin II, acting through type 1 angiotensin (AT_1) receptors, contributes to the pathophysiology of diverse disorders from progressive renal disease to cardiac hypertrophy to atherosclerosis (2–4). In addition, recent studies have suggested that AT_1 receptors may contribute to disease pathogenesis through direct effects on cellular immune responses and inflammation. For example, angiotensin II triggers T lymphocyte proliferation through a calcineurin-dependent pathway during the alloimmune response (5). Furthermore, blockade of the AT_1 receptor or targeted deletion of the dominant murine AT_1 receptor isoform, AT_{1A}, prolongs allograft survival in rodent models (4, 5). In contrast, actions of AT_1 receptors may have protective effects in other disorders. For instance, in a model of obstructive uropathy, the absence of AT_1 receptors on hematopoietic cells is associated with enhanced levels of renal fibrosis (6). While most of the actions of angiotensin II to promote inflammation and tissue injury are mediated by the AT_1 receptor, activation of AT_2 receptors has been linked to enhanced chemokine expression in the kidney (7).

In autoimmune glomerulonephritis, previous studies suggest that blockade of the RAS has beneficial effects. MRL/MpJ-*Fas*$^{lpr/lpr}$ (*lpr*) mice develop florid autoimmune disease resembling human SLE including autoantibodies, arthritis, and proliferative glomerulonephritis (8, 9). Treatment of *lpr* mice with angiotensin-converting enzyme (ACE) inhibitors or AT_1 receptor blockers reduces glomerular injury and albuminuria (10, 11). Together, these data suggest that angiotensin II may contribute to the pathogenesis of autoimmune nephritis. In humans with SLE, the contribution of the RAS to the development of renal disease is less clear. Although lower serum ACE levels have been associated with reduced severity of renal disease in some studies (12, 13), this has not been a universal finding (14). Case series suggest a benefit of ACE inhibition in patients with established lupus nephritis (15). However, there are

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: ACE, angiotensin-converting enzyme; AT_1, type 1 angiotensin (receptor); BUN, blood urea nitrogen; CFP, cyan fluorescent protein; JGA, juxtaglomerular apparatus; *lpr*, *MRL-Fas*$^{lpr/lpr}$; *lpr*-KO, *MRL-Fas*$^{lpr/lpr}$*Agtr1a*$^{-/-}$; MCP-1, monocyte chemoattractant protein-1; RAS, renin-angiotensin system; ssDNA, single-stranded DNA; TCA-3, T cell activation gene-3.

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Table 1
Lymphoid organ parameters in *lpr* and *lpr*-KO mice at 12 weeks of age

		Left axillary lymph node	Spleen	Thymus
Weight (g)	*lpr*	47 ± 12	196 ± 19	94 ± 6
	lpr-KO	52 ± 12	188 ± 22	103 ± 10
$CD3^+$ $B220^+$ cells (%)	*lpr*	56.3 ± 2.8	28.2 ± 2.6	15.4 ± 3.5
	lpr-KO	54.0 ± 3.4	26.1 ± 2.9	7.8 ± 2.9
$CD4^+$ $CD25^+$ cells (%)	*lpr*	2.0 ± 0.2	4.5 ± 0.7	5.5 ± 0.8
	lpr-KO	2.3 ± 0.4	4.1 ± 0.2	5.5 ± 0.6

no randomized trials of ACE inhibitors or angiotensin receptor blockade in patients with lupus nephritis.

Thus, previous studies suggest a role for the RAS in regulating inflammatory responses, but its role is complex. Accordingly, the objective of the present studies was to use a genetic approach to investigate the role of AT_1 receptors in the development of autoimmune disease. To this end, we generated an *lpr* mouse line deficient in the major murine AT_1 receptor, AT_{1A}, and characterized its clinical features. Surprisingly, we found enhanced mortality and more aggressive kidney disease in *lpr* mice lacking AT_{1A} receptors. This acceleration of renal disease is caused by activation of glomerular AT_{1B} receptors. These experiments therefore demonstrate for what we believe is the first time that activation of AT_1 receptors in the glomerulus can directly promote inflammatory injury in the kidney independent of elevated blood pressure.

Results

Accelerated mortality in MRL-$Fas^{lpr/lpr}$ mice lacking AT_{1A} receptors. To examine the role of AT_1 receptors in modulating autoimmune disease, we generated MRL-*$Fas^{lpr/lpr}$Agtr1a*$^{-/-}$ (*lpr*-KO) mice lacking AT_{1A} receptors. Compared with those of MRL-*$Fas^{lpr/lpr}$Agtr1a*$^{+/+}$ (*lpr*) controls, average weights for lymph nodes, spleen, or thymus were very similar at 12 weeks of age. In addition, the absence of AT_{1A} receptors did not significantly affect the cell-surface phenotypes of crucial cells in these lymphoid organs (Table 1), including the population of the double-negative $CD4^-CD8^-CD3^+B220^+$ cells, which are characteristic of *lpr* mice. However, as shown in Figure 1, survival was significantly reduced in the *lpr*-KO animals compared with the *lpr* group. The mean duration of survival was 117 ± 4 days in the *lpr*-KO group compared with 162 ± 11 days in the *lpr* group ($P = 0.001$). Also, blood pressure levels in *lpr*-KO mice (129 ± 2 mmHg) were modestly but significantly lower than those of the *lpr* controls (137 ± 2 mmHg; $P = 0.001$).

Urinary albumin excretion is increased in *lpr* mice lacking AT_{1A} receptors. Kidney disease is the major cause of death in *lpr* mice. Similarly to the situation with patients with lupus nephritis, proteinuria is a useful index of renal disease activity in *lpr* mice. Therefore, we compared urinary albumin excretion in *lpr* and *lpr*-KO mice at 12 weeks of age, a time point in the disease before there is appreciable mortality in either group. As shown in Figure 2, urinary albumin excretion was significantly higher in the *lpr*-KO mice (77 ± 14 μg/24 h) than in *lpr* controls (39 ± 4 μg/24 h; $P < 0.02$).

Absence of AT_{1A} receptors increases the severity of kidney structural injury in lpr mice. To determine whether the increased albuminuria in *lpr*-KO mice is associated with enhanced renal injury, kidneys were systematically examined for the presence of pathological changes in the glomerular, vascular, and interstitial compartments. As shown in Table 2, the severity of renal pathological abnormalities was significantly increased in *lpr*-KO animals compared with *lpr* controls ($P = 0.001$). Typical of MRL-*$Fas^{lpr/lpr}$* mice at this age, glomerular lesions in the 12-week-old *lpr* group were relatively mild (Figure 3A and Table 2) (16). In contrast, kidneys from *lpr*-KO mice showed severe pathological findings in glomeruli including hypercellularity, focal necrosis, mesangial expansion, and glomerulosclerosis (Figure 3B and Table 2). While the interstitial regions of kidneys from *lpr* mice contained very few inflammatory cells (Figure 3C and Table 2), mononuclear cell infiltrates focused primarily around the vasculature were prominent in kidneys from *lpr*-KO animals (Figure 3D and Table 2). Finally, whereas vascular structures were largely preserved in kidneys from *lpr* controls, abnormal vascular architecture was commonly observed in the *lpr*-KO mice characterized by sclerosis and/or stenosis of arteries and arterioles (Figure 3, E–G). To determine whether enhanced renal pathology was associated with alterations in kidney function, we measured blood urea nitrogen (BUN) levels in 16-week-old *lpr* and *lpr*-KO groups. By this time point, a number of the more severely affected *lpr*-KO animals had succumbed. Nonetheless, BUN levels (54 ± 7 mg/dl) were still significantly higher in *lpr*-KO mice than in *lpr* controls (40 ± 4 mg/dl) ($P = 0.03$).

Expression of inflammatory mediator genes is increased in kidneys of lpr mice lacking AT_{1A} receptors. We used the RNAse protection assay to measure mRNA levels of a panel of pertinent inflammatory mediators in the kidney. Typically, there is minimal expression of these mediators in kidneys of normal mice (17). As shown in Figure 4, A–D, expression of mRNAs for several cytokines and chemokines was detected in kidneys from *lpr* controls. Expression of a number of these mRNAs, including IFN-γ, RANTES, monocyte chemoattractant protein-1 (MCP-1), and T cell activation gene-3 (TCA-3), was significantly augmented in kidneys from the *lpr*-KO animals.

In human patients with lupus nephritis, TGF-β RNA levels in the urinary sediment correlate with disease activity (18). Moreover, several lines of evidence indicate that angiotensin II may promote progression of chronic kidney disease by stimulating production of TGF-β (19, 20). Surprisingly, as demonstrated in Figure 4, E and F, TGF-β mRNA expression was significantly increased in kidneys of *lpr*-KO mice compared with those of *lpr* controls ($P = 0.02$).

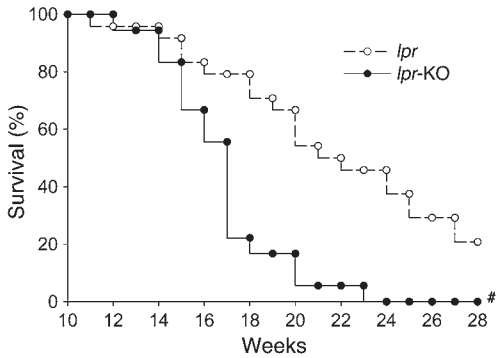

Figure 1
Reduced survival in *lpr* mice lacking AT_{1A} receptors. #The mean duration of survival was significantly reduced in *lpr*-KO mice (117 ± 4 days) compared with *lpr* controls (162 ± 11 days; $P = 0.001$). $n = 24$ for *lpr* (10 males and 14 females) group; $n = 18$ *lpr*-KO group (8 males and 10 females).

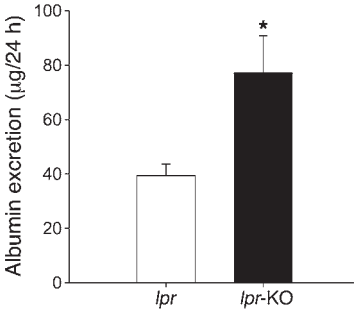

Figure 2
Elimination of AT_{1A} receptors augments albuminuria in MRL-*Fas*$^{lpr/lpr}$ mice. At 12 weeks of age, 24-hour urinary albumin excretion was significantly higher in *lpr*-KO mice (77 ± 14 μg/24 h) than in *lpr* controls (39 ± 4 μg/24 h; *$P < 0.02$). $n = 18$ for *lpr* group (9 males and 9 females); $n = 21$ for *lpr*-KO group (11 males and 10 females).

Autoimmune injury in extrarenal tissues is not enhanced in lpr-KO mice. Autoimmune disease in MRL-*Fas*$^{lpr/lpr}$ mice affects multiple organ systems in addition to the kidney (8). To determine whether elimination of AT_{1A} receptors affected the severity of autoimmune damage in other systems, we also assessed the extent of pathological injury in extrarenal tissues including hearts, joints, and skin from 12-week-old mice. There were minimal and equivalent pathological changes in hearts from *lpr* and *lpr*-KO mice (data not shown). Similarly, by 12 weeks of age, none of the mice had developed clinically apparent autoimmune skin lesions. To characterize the severity of arthritis, knees were harvested, decalcified, fixed, stained, and systematically scored for fibrosis and mononuclear cell infiltrates as previously described (21). Both groups demonstrated a low level of inflammation in the joint, but the degree of injury was not different between the *lpr* and *lpr*-KO groups (2.4 ± 0.6 vs. 1.3 ± 0.3 AU; P = NS). Thus, at 12 weeks of age, when we noted a marked exaggeration of kidney pathology in *lpr*-KO mice compared with *lpr* controls, only minimal and equivalent levels of pathology could be detected in extrarenal tissues commonly affected in this model.

Another feature of the *lpr* lymphoproliferative phenotype is the development of circulating autoantibodies. However, as shown in Figure 5, the experimental groups had similar levels of anti–single-stranded DNA (anti-ssDNA) and anti-dsDNA activity.

Accelerated renal disease in AT_{1A} receptor–deficient lpr mice is not due to differences in blood pressure. To determine whether the differences in blood pressure levels contributed to the discrepant severity of kidney disease between *lpr* and *lpr*-KO mice, we treated groups of *lpr* mice with hydralazine (37 mg/kg/d) starting after 4 weeks of age. Blood pressure levels in the *lpr* group treated with hydralazine ($n = 12$) were significantly lower than in untreated *lpr* controls (132 ± 2 vs. 137 ± 2 mmHg; $P < 0.04$) and not different from those of *lpr*-KO animals (132 ± 2 vs. 129 ± 2 mmHg; P = NS). Mean survival of *lpr* mice treated with hydralazine was significantly greater than that of the *lpr*-KO group (165 ± 12 vs. 117 ± 4 days; $P = 0.001$) and was similar to that of the *lpr* control group (162 ± 11 days; P = NS). Likewise, urinary albumin excretion (46 ± 5 μg/24 h) and the extent of renal pathology in the *lpr* group treated with hydralazine were comparable to that of *lpr* controls (8.3 ± 1.9 AU; P = NS) and substantially attenuated compared with that of *lpr*-KO mice (13.1 ± 1.0; $P = 0.06$).

Transplantation with bone marrow from AT_{1A} receptor–deficient donors does not affect survival of lpr mice. The transfer of normal MRL-MpJ bone marrow into MRL-*Fas*$^{lpr/lpr}$ mice attenuates autoimmune disease in the bone marrow recipients (22, 23), confirming a primary role for immune dysregulation in the pathogenesis of *lpr* disease. To determine whether the accelerated disease in the *lpr*-KO mice is due to direct consequences of AT_{1A} receptor deficiency on immune cell function, we transplanted bone marrow from *lpr*-KO (MRL-*Fas*$^{lpr/lpr}$*Agtr1a*$^{-/-}$) or *lpr* (MRL-*Fas*$^{lpr/lpr}$*Agtr1a*$^{+/+}$) donors into irradiated *lpr* (MRL-*Fas*$^{lpr/lpr}$*Agtr1a*$^{+/+}$) recipients. With this strategy, we compared outcome in *lpr* bone marrow chimeras, lacking AT_{1A} receptors *only* in cell lineages derived from bone marrow, to that of *lpr* controls with normal expression of AT_{1A} receptors in all tissues.

Using Southern blot analysis, we confirmed that the extent of engraftment with AT_{1A} receptor–deficient bone marrow was more than 98% (Figure 6A). Survival of *lpr* mice transplanted with bone marrow from *lpr* donors (230 ± 11 days) was significantly prolonged compared with that of nontransplanted nonirradiated *lpr* controls (162 ± 11 days; $P < 0.0001$). However, as shown in Figure 6B, this was virtually identical to the mean survival of *lpr* animals transplanted with *lpr*-KO bone marrow lacking AT_{1A} receptors (234 ± 8 days; P = NS) (Figure 6B). Thus, the absence of AT_{1A} receptors on immune cell lineages derived from bone marrow does not account for the accelerated mortality in *lpr*-KO mice.

Renin expression is enhanced in lpr mice lacking AT_{1A} receptors. Because the absence of AT_{1A} receptors from bone marrow–derived cells does not appear to explain the accelerated disease in *lpr*-KO mice, we considered the possibility that systemic consequences of AT_{1A} receptor deficiency might play a role. On other genetic backgrounds, the absence of AT_{1A} receptors is associated with enhanced renin expression and hypertrophy of the juxtaglomerular apparatus (JGA), presumably due to the absence of short-loop feedback inhibition of renin mediated by AT_1 receptors (24, 25). As generation of renin is a key rate-limiting step determining the activity of the RAS, the increase in renin in AT_{1A} receptor–deficient mice leads to increases in circulating levels of angiotensin II (26). To assess the extent of RAS activity, we compared renin mRNA expression in kidneys of *lpr* and *lpr*-KO mice at 12 weeks of age. Renin mRNA levels were increased more than 3-fold in kidneys from *lpr*-KO mice compared with those of *lpr* controls (333 ± 56 vs. 100 ± 17 AU; $P = 0.001$). To confirm that the increase in renin mRNA levels was accompanied by an increase in renin protein, we performed immunostaining for renin in sections of renal cortex ($n \geq 12$ mice per group; >300 glomeruli scored per group). Using standard methods (27) and as illustrated in Figure 7, the number of glomeruli with detectable staining for renin protein in the JGA was significantly higher in the *lpr*-KO mice (127 out of 419 glomeruli: 30.3%) than in controls (59 out of 328 glomeruli: 18.0%; $P < 0.0001$ by χ^2). In contrast, aldosterone levels in the blood were similar in the *lpr* and *lpr*-KO groups (327 ± 87 vs. 370 ± 24 pg/ml;

Table 2
Histopathologic injury scores for kidneys from *lpr* and *lpr*-KO mice at 12 weeks of age

Group	Glomeruli	Interstitium	Vascular damage	Total
lpr	5.6 ± 0.9	0.6 ± 0.1	2.4 ± 0.3	8.0 ± 1.1
lpr-KO	7.8 ± 0.7[A]	1.3 ± 0.1[B]	5.3 ± 0.4[C]	13.1 ± 1.0[D]

[A]$P = 0.05$; [B]$P = 0.0002$; [C]$P = 0.0001$; [D]$P = 0.001$ vs. *lpr*. $n = 19$ *lpr* mice (9 males and 10 females) and 24 *lpr*-KO mice (14 males and 10 females).

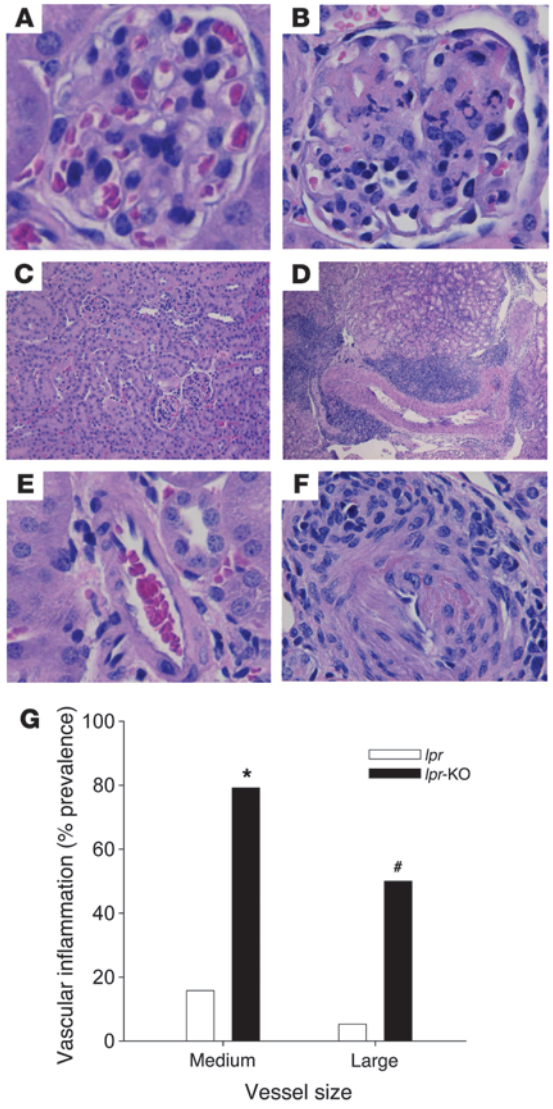

Figure 3
Kidney injury is augmented in *lpr*-KO mice. High-power views of representative glomeruli from H&E-stained sections of kidneys from *lpr* (**A**) and *lpr*-KO (**B**) mice at 12 weeks of age. Original magnification, ×600. Typical of MRL-*Fas*$^{lpr/lpr}$ mice at this age, glomerular pathology in the *lpr* group was relatively mild. In contrast, glomeruli from *lpr*-KO mice showed severe pathology including hypercellularity, focal necrosis, mesangial expansion, and glomerulosclerosis. Representative low-power views of kidneys from *lpr* (**C**) and *lpr*-KO (**D**) mice. Original magnification, ×200. There was minimal interstitial infiltration in kidneys from *lpr* mice (**C**). In contrast, robust mononuclear cell infiltrates were observed in kidneys from *lpr*-KO animals (**D**), especially in perivascular regions. Typical small renal arteries from *lpr* (**E**) and *lpr*-KO (**F**) mice depict preservation of normal structure in the *lpr* control (**E**), with abnormal vascular architecture in the *lpr*-KO mice (**F**) characterized by inflammation and reactive hyperplasia with thickening and sclerosis of the arterial wall. Original magnification, ×600. (**G**) Prevalence of vascular pathology in *lpr* and *lpr*-KO kidneys comparing medium and large vessels $^{*}P < 0.001$ and $^{\#}P = 0.002$ versus *lpr* by χ^2 test. $n = 19$ for *lpr* group (9 males and 10 females); $n = 24$ for the *lpr*-KO group (14 males and 10 females).

P = NS), consistent with previous reports in nonautoimmune mouse strains showing that AT_{1A} receptor deficiency did not alter basal aldosterone levels (28, 29).

Activation of glomerular AT_1 receptors accelerates renal disease in lpr-KO mice. In view of the elevated renin levels in *lpr*-KO mice, we considered the possibility that systemic activation of the RAS through receptors other than AT_{1A} might be driving more severe renal disease and enhanced mortality in these animals. Such effects might be mediated by the second AT_1 receptor isoform, AT_{1B}, which is fully expressed in AT_{1A} receptor–deficient mice (30). Previous studies have suggested that within the kidney, the AT_{1B} receptor is primarily expressed in the glomerulus (30–32), so that activation of glomerular AT_{1B} receptors could potentially have an impact on glomerular damage and proteinuria in our model. Therefore, to confirm expression of the AT_{1B} receptor in the kidney in our own experiments, we quantitated renal expression for *Agtr1b* using real-time PCR. Modest levels of AT_{1B} mRNA expression could be detected in RNA extracted from whole kidneys, and there were no significant differences in levels of AT_{1B} mRNA expression between the *lpr*-KO mice (1.31 ± 0.17 AU) and the *lpr* controls (1.00 ± 0.14 AU; P = NS).

To further characterize the pattern of expression of the AT_{1B} receptor within the kidney, we compared levels of AT_{1B} receptor mRNA from whole kidney and isolated glomeruli from wild-type mice. AT_{1B} mRNA levels in the glomerulus were nearly 4 times higher those in whole kidney extract (1.39 ± 0.70 vs. 0.37 ± 0.21 AU), consistent with previous studies suggesting predominant renal expression of the AT_{1B} receptor in the glomerulus (30–32). To further evaluate AT_{1B} mRNA expression in various glomerular cell populations, we used FACS to isolate glomerular podocytes or endothelial cells from respective lines of transgenic mice expressing cyan fluorescent protein (CFP) specifically in podocytes (*nephrin*-CFP) or GFP specifically in endothelial cells (*Flk1*-GFP). AT_{1B} mRNA levels in these freshly sorted cells were compared with those in a mouse mesangial cell line. Using this approach, we found expression of AT_{1B} receptors in sorted cell populations enriched for podocytes (4.39 ± 2.68 AU), but AT_{1B} expression was not detectable in the freshly isolated glomerular endothelial cells or cultured mesangial cells.

To examine the role of the glomerular AT_{1B} receptor in promoting kidney injury, we treated *lpr*-KO animals with losartan, a potent and specific blocker of both the AT_{1A} and AT_{1B} receptor isoforms, and evaluated its effects on the course of renal disease. Losartan treatment was initiated at 4 weeks of age, after renal development is complete but before significant renal injury becomes evident in the MRL/*lpr* model. Treatment of *lpr*-KO mice with losartan for 8 weeks caused a marked reduction in albuminuria from 233 ± 135 μg/24 h in *lpr*-KO mice receiving water alone to 46 ± 4 μg/24 h in *lpr*-KO mice treated with losartan ($P < 0.007$). Indeed, the level of albuminuria in the *lpr*-KO mice given losartan was no different than that of untreated *lpr* controls (39 ± 4 μg/24 h; P = NS). Consistent with its effects on albuminuria, treatment with the AT_1 receptor blocker also reduced the extent of glomerulosclerosis in *lpr*-KO mice by nearly 40% from 12% ± 2% to 21% ± 4% ($P = 0.03$). However, losartan did not affect interstitial mononuclear cell infiltration or the severity of vascular pathology.

Based on our finding that renal expression of AT_{1B} receptors is primarily confined to glomerular podocytes, we examined the extent of podocyte injury in kidneys from the experimental groups (n = 10 *lpr*-KO mice; n = 11 *lpr* controls) by immunostaining for

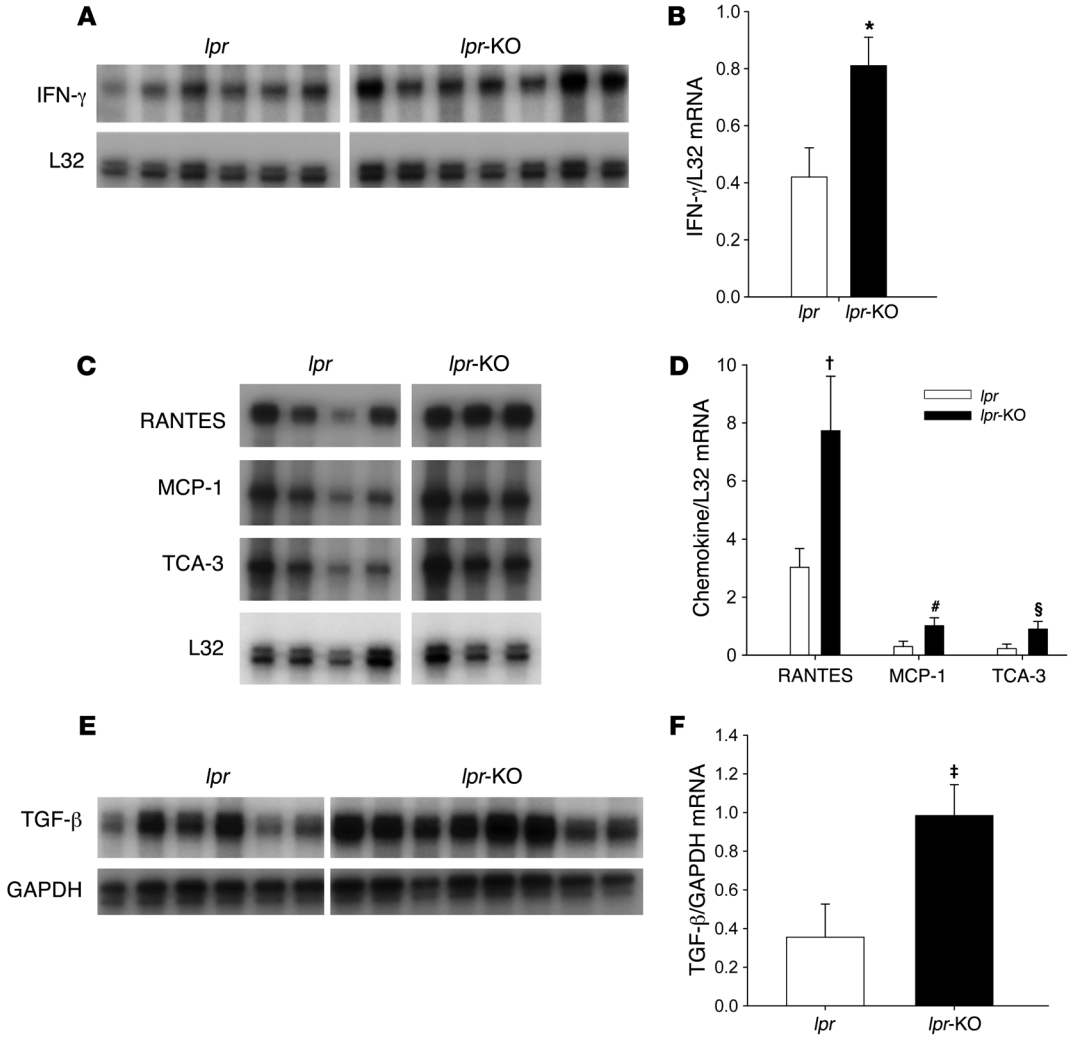

Figure 4
Enhanced mRNA expression for cytokines and chemokines in kidneys from *lpr*-KO mice. (**A**) Levels of mRNA for IFN-γ and housekeeping gene L32 assessed by RNAse protection assay (RPA) in male kidneys at 12 weeks of age; the lanes were run on the same gel but were noncontiguous. (**B**) Plot of the corresponding analysis by densitometry; mRNA levels for IFN-γ were significantly increased in *lpr*-KO mice. **P* < 0.02 versus *lpr* controls. *n* ≥ 6 per group. (**C**) mRNA levels for selected chemokines and the housekeeping gene L32 assessed by RPA; the lanes were run on the same gel but were noncontiguous. (**D**) Corresponding plot of the densitometry analysis; levels of mRNA for RANTES, MCP-1, and TCA-3 were all significantly increased in kidneys from *lpr*-KO mice. †*P* = 0.03, §*P* = 0.04, and #*P* = 0.05 versus *lpr* controls. *n* ≥ 4 per group. (**E**) Kidney mRNA expression for TGF-β and the housekeeping gene GAPDH; the lanes were run on the same gel but were noncontiguous. (**F**) Corresponding densitometry analysis; TGF-β mRNA expression was significantly increased in kidneys of *lpr*-KO mice. ‡*P* = 0.02 versus *lpr*. *n* ≥ 6 per group.

desmin. Desmin is normally expressed in mesangial cells but not in podocytes. However, expression in podocytes is triggered by injury, and it has therefore been used as a reliable marker of podocyte damage (33, 34). As can be seen in Figure 8, modest and equivalent levels of desmin staining were seen in the glomerular mesangial cells from the kidneys in each experimental group, confirming the technical adequacy of staining. On the other hand, desmin staining in podocytes was detected in only 1 of 11 kidneys from *lpr* mice (9.1%). In contrast, robust podocyte staining for desmin was seen in 8 of 10 *lpr*-KO kidneys (80%; $P < 0.002$ by Fisher's exact test). Thus, podocyte damage was a major feature of glomerular disease in 12-week-old *lpr*-KO mice but was only rarely seen in *lpr* controls.

Glomerular AT_1 receptors augment expression of inflammatory mediators in the kidney. To determine whether blockade of the AT_{1B} receptor affected expression of key inflammatory mediators associated with progression of lupus nephritis, we again performed RNAse protection assay on the kidneys from the *lpr*-KO mice treated with vehicle or losartan. Expression of IFN-γ was dramatically reduced in the kidneys from 0.15 ± 0.03 AU in the vehicle group to 0.04 ± 0.02 AU in the losartan-treated animals ($P < 0.02$) despite the persistence of renal mononuclear cell infiltrates in this group. In addition, losartan significantly attenuated expression of TGF-β in *lpr*-KO animals (1.15 ± 0.05 vs. 1.79 ± 0.22 AU; $P < 0.01$), suggesting that a major pathway for stimulation of TGF-β expression in this setting is mediated by activation of the glomerular AT_{1B} receptor.

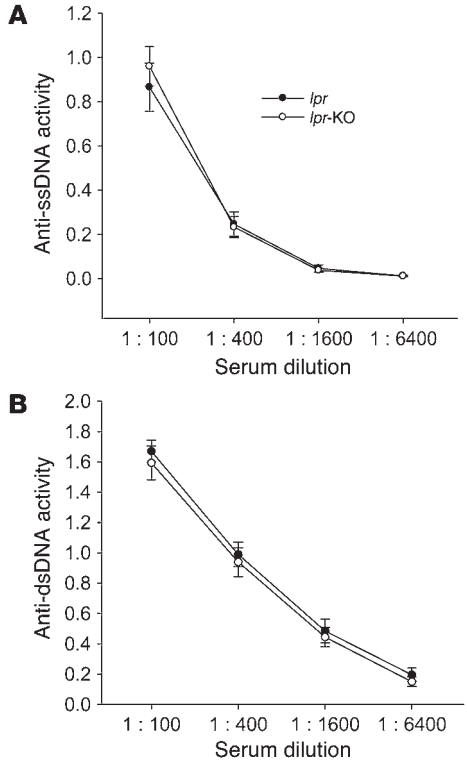

Figure 5
IgG anti-DNA antibody activity in serum from MRL-*Fas*$^{lpr/lpr}$ experimental groups. (**A**) Anti-ssDNA and (**B**) anti-dsDNA antibody activity was determined by ELISA on serum from MRL-*Fas*$^{lpr/lpr}$ mice lacking AT_{1A} receptors (*lpr*-KO) and *lpr* controls at 12 weeks of age. $n = 20$ for the *lpr* group (10 males and 10 females); $n = 24$ for the *lpr*-KO mice (14 males and 10 females).

Discussion

Abnormal activation of the RAS contributes to the pathogenesis of a range of disorders including hypertension, congestive heart failure, and diabetic kidney disease (35–37). Pharmacological and genetic studies have demonstrated that activation of AT_1 receptors by angiotensin II is a key pathway driving pathology in these disorders (35–38). Along with the AT_1 receptor's effects on blood pressure levels and fluid homeostasis, recent studies have suggested that AT_1 receptors may also modulate immune and inflammatory responses (5, 39, 40). Moreover, these proinflammatory actions may contribute to end-organ damage in disorders such as hypertension and coronary disease (4, 40, 41), as well as immunological processes such as transplant rejection (5, 42). Thus, we were interested in exploring the role of this ligand-receptor pathway in autoimmune disease, focusing on effects in immune-mediated glomerulonephritis.

Previous studies have shown that administration of ACE inhibitors or angiotensin receptor blockers attenuates autoimmune disease in murine lupus models, reducing the severity of glomerular damage (10, 11, 43). Accordingly, we anticipated that elimination of AT_{1A} receptors from the MRL-*fas*$^{lpr/lpr}$ background would be protective. Yet we found that autoimmune nephritis was significantly accelerated by AT_{1A} receptor deficiency. This was manifested by early mortality with increased proteinuria and severe kidney pathology. Despite the marked worsening of renal disease and consequent early mortality, the extent of lymphadenopathy was not affected and the number of abnormal "double-negative" lymphocytes characteristic of this model was similar in AT_{1A}-deficient animals and *lpr* controls. Similarly, elimination of AT_{1A} receptors did not affect autoantibody levels or the extent of autoimmune injury in extrarenal tissues including skin, heart, and joints.

To determine whether this unexpected result was due to altered function of autoimmune lymphocytes, we transplanted bone marrow from *lpr* or *lpr*-KO donors into syngeneic *lpr* recipients. The mean survival of *lpr*-KO→*lpr* bone marrow chimeras lacking AT_{1A} receptors only in hematopoietic cell lineages was virtually identical to that of *lpr*→*lpr* transplanted controls with normal expression of AT_{1A} receptors. We therefore concluded that the absence of AT_{1A}

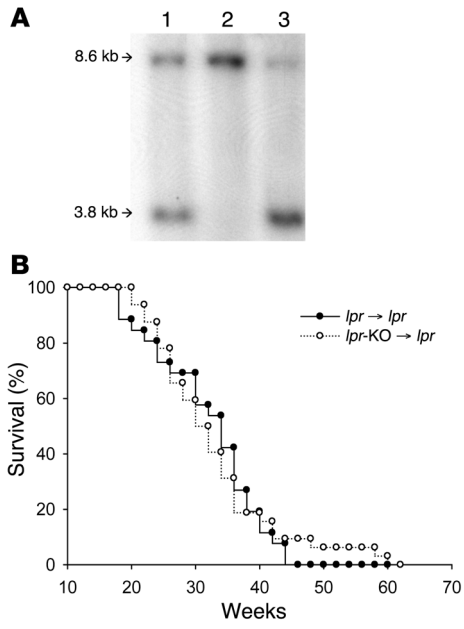

Figure 6
Absence of AT_{1A} receptors on bone marrow–derived cells does not account for the accelerated mortality of *lpr*-KO mice. (**A**) Southern blot analysis of genomic DNA isolated from splenocytes, digested with *BamH1*, and hybridized with a DNA probe that distinguishes the 8.6-kb fragment generated from the wild-type *Agtr1a* allele and the 3.8-kb fragment identifying the targeted allele (1). Samples: lane 1, nontransplanted MRL-*Fas*$^{lpr/lpr}$*Agtr1a*$^{+/-}$ control; both bands are present with equal intensities. Lane 2, *lpr*→*lpr*, an *lpr* (MRL-*Fas*$^{lpr/lpr}$*Agtr1a*$^{+/+}$) mouse transplanted with *lpr* bone marrow; only the wild-type band is observed. Lane 3, *lpr*-KO→*lpr*, an *lpr* mouse transplanted with bone marrow from an *lpr*-KO mouse (MRL-*Fas*$^{lpr/lpr}$*Agtr1a*$^{-/-}$); the 3.8 kb band predominates, indicating that the majority of cells in the sample (98.3% by densitometry) come from the donor. (**B**) Survival of MRL/*lpr* mice transplanted with wild-type versus *Agtr1a*$^{-/-}$ bone marrow. Survival of *lpr* mice ($n = 26$; 12 males, 14 females) transplanted with bone marrow from *lpr* donors was virtually identical to the mean survival of *lpr* animals ($n = 32$; 17 males, 15 females) transplanted with *lpr*-KO bone marrow lacking AT_{1A} receptors (230 ± 11 versus 234 ± 8 days; P = NS).

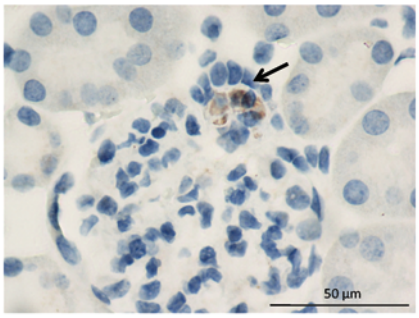

Figure 7
Renin protein immunostaining in the JGA of a kidney from an *lpr*-KO mouse. Kidney sections from *lpr* and *lpr*-KO mice were stained for renin using a specific antibody as described. The number of glomeruli with detectable staining for renin protein in the JGA was significantly higher in the *lpr*-KO mice ($n \geq 12$ mice per group; 127 out of 419 glomeruli: 30.3%) versus controls (59 out of 328 glomeruli: 18.0%; $P < 0.0001$). Photomicrograph shows a representative glomerulus with positive staining for renin. Arrow points to renin-containing juxtaglomerular cells. Original magnification, ×100.

receptors on lymphocytes or other cell lineages derived from bone marrow does not explain the enhanced severity of disease in the *lpr*-KO mice. Since previous studies by our group and others have suggested that activation of AT_1 receptors can stimulate cellular immune responses (5, 41), it is likewise notable that the isolated absence of AT_{1A} receptors from immune cell populations did not prolong survival of *lpr* mice in this setting. Taken together, these findings suggest that effects of AT_1 receptors on lymphocytes do not contribute significantly to either augmenting or attenuating the severity of this autoimmune disease.

Since an enhanced cellular immune response is not the mechanism driving accelerated disease in *lpr*-KO mice, we next considered the possibility that other systemic consequences of AT_{1A} receptor deficiency might play a role. Previous studies have shown that genetic deletion of AT_{1A} receptors is associated with marked stimulation of renin expression (24) due to the absence of AT_{1A} receptor signaling in the juxtaglomerular cells, with resulting attenuation of short-loop feedback inhibition of renin release (24, 25). Increased renin expression in AT_{1A} receptor–deficient mice leads to increases in circulating levels of angiotensin II (26). Indeed, we found that renin mRNA expression was increased more than 3-fold and JGA renin protein was significantly augmented in the *lpr*-KO animals, indicating significant activation of the RAS and suggesting that stimulation of residual elements of the system might be a mechanism promoting exaggerated kidney disease.

Of the 2 classes of angiotensin receptors, AT_1 receptors mediate most of the classical functions of the RAS in physiology and disease, whereas AT_2 receptors generally oppose or attenuate the actions of AT_1 receptors (38, 44). The *lpr*-KO mice lack the major murine AT_1 receptor, AT_{1A}, but expression of the minor isoform, AT_{1B}, is preserved in these animals. In the kidney, it has been reported that AT_{1B} receptor expression is largely limited to the glomerulus (31). We have confirmed the predominance of AT_{1B} expression in the glomerulus and have shown that this expression is primarily concentrated in podocytes. Thus, the AT_{1A}-deficient mouse line provides a fortuitous model wherein renal AT_1 receptor expression is limited to the glomerulus. Accordingly, we postulated that the systemic activation of the RAS seen in *lpr*-KO mice promotes aggressive kidney disease by stimulating the residual glomerular AT_{1B} receptors.

To test this directly, we administered a potent and specific AT_1 receptor antagonist (losartan) to *lpr*-KO mice and assessed effects on the severity of kidney disease. In *lpr*-KO mice, blockade of glomerular AT_{1B} receptors with losartan dramatically reduced proteinuria and markedly attenuated renal structural damage. Interestingly, treatment with losartan did not reduce the interstitial mononuclear cell infiltrates, consistent with previous reports that these lymphoid aggregates in the kidney may be an indicator of the *lpr* lymphoproliferative phenotype rather than a marker for the severity of kidney damage (11). Taken together, these data suggest that activation of glomerular AT_{1B} receptors is a powerful pathway promoting functional injury in autoimmune nephritis, and this mechanism likely explains the accelerated glomerular disease seen in the untreated *lpr*-KO mice. More broadly, these data identify potent interactions between autoimmunity and RAS activation to augment glomerular pathology.

An important role for AT_1 receptors in the pathogenesis of kidney diseases has been well documented, but the specific pathways linking the AT_1 receptor to the development of proteinuria and structural injury have been difficult to precisely define in vivo. In other settings, AT_1 receptor–mediated systemic hypertension is thought to be a major contributor to proteinuria and the progression of kidney disease (37). However, an effect on blood pressure levels is clearly not a factor in *lpr*-KO mice since they do not have hypertension; in fact, their blood pressure levels are significantly lower than those of the *lpr* controls. It has also been suggested that stimulation of AT_1 receptors in the glomerular microcirculation contributes to proteinuria and structural damage by increasing glomerular hydrostatic pressures (45, 46) due to the predominant constriction of efferent arterioles caused by angiotensin II (45–47). AT_{1B} receptors are expressed in afferent but *not* efferent arterioles (30, 32). In the blood-perfused juxtamedullary nephron preparation, activation of AT_{1B} receptors in isolation causes a predomi-

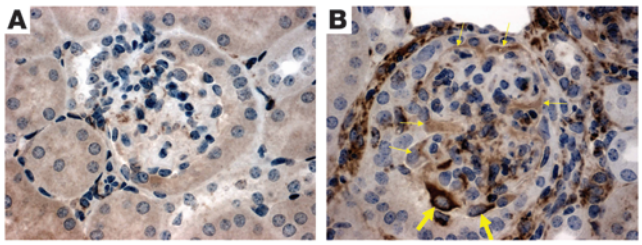

Figure 8
Enhanced podocyte injury in kidneys from *lpr*-KO mice. Immunostaining for desmin, a marker of podocyte injury, was carried out in sections of kidneys from *lpr* ($n = 11$; 7 males and 4 females) and *lpr*-KO mice ($n = 10$; 6 males and 4 females). (**A**) A representative glomerulus from an *lpr* mouse shows no staining for desmin in podocytes, with very focal staining for desmin in the mesangial cell cytoplasm (1+ to 2+). (**B**) In the representative glomerulus from an *lpr*-KO mouse, the podocytes are hyperplastic, with positive staining for desmin in the cytoplasm varying in intensity from 1+ to 2+ (thin arrows) to 3+ (thick arrows). The mesangial cell cytoplasm is also strongly positive for desmin (3+). Very focal staining is also noted in some parietal epithelial cells and in interstitium, probably reflecting increased numbers of fibroblasts. Positive staining from desmin in podocytes was found in 80% of *lpr*-KO kidneys versus 9% of *lpr* control kidneys ($P < 0.002$). Original magnification, ×40.

nant constriction of the afferent arteriole (30, 32) which would be expected to reduce glomerular hydrostatic pressure. These data suggest that a maladaptive alteration in glomerular hemodynamics mediated by activation of AT_{1B} receptors in isolation would be an unlikely cause of augmented proteinuria and kidney pathology in *lpr*-KO mice, especially in view of the low systemic blood pressure levels in these animals. However, we did not measure glomerular pressures in our experiments and it is possible that the character of this response described in the ex vivo system might be different in the intact animal.

In the absence of an apparent hemodynamic cause for accelerated nephritis in *lpr*-KO mice, it follows that these effects must be a direct consequence of activation of AT_{1B} receptors in specific glomerular cell populations. Our studies show that within the glomerulus, AT_{1B} receptors are primarily expressed in podocytes, a key constituent of the glomerular filter. Moreover, recent studies have established a central role for podocyte dysfunction in the pathogenesis of a variety of kidney diseases (48–50). Experiments in cultured podocytes suggest that AT_1 receptors regulate critical functions including proliferation and apoptosis (51–54). Thus, we suggest that accelerated glomerular disease in *lpr*-KO mice is a direct consequence of AT_{1B} receptor activation in podocytes and is clearly independent of effects of angiotensin II on systemic blood pressure. Moreover, using immunostaining for desmin, we found clear evidence for exaggerated podocyte injury in *lpr*-KO mice.

Enhanced expression of cytokines in the kidney has been suggested to play a key role in the pathogenesis of autoimmune nephritis (55, 56). For example, tissue expression of IFN-γ is significantly upregulated in *lpr* mice (57) and studies using knockout mice support a causal role for IFN-γ in disease pathogenesis (56, 58–60). Similarly, TGF-β expression in the kidney is thought to be a final common pathway leading to the development of structural damage and fibrosis in a range of glomerular diseases (61–63). In our studies, expression of both IFN-γ and TGF-β were markedly increased in kidneys of *lpr*-KO mice compared with those of *lpr* controls. This enhanced expression was significantly ameliorated by treatment with losartan. These findings suggest that activation of glomerular AT_1 receptors, presumably in podocytes, is sufficient to markedly augment expression of key inflammatory cytokines implicated in the pathogenesis of autoimmune glomerulonephritis. These proinflammatory actions are independent of effects on peripheral lymphocytes, which do not express AT_{1B} receptors, and therefore appear to be a direct consequence of glomerular AT_1 receptor activation.

In summary, genetic deletion of AT_{1A} receptors from the MRL-*Fas*$^{lpr/lpr}$ background accelerates mortality, increases proteinuria, and augments the severity of kidney pathology. Activation of residual populations of glomerular AT_{1B} receptors in podocytes is the mechanism responsible for the more severe renal disease in AT_{1A} receptor–deficient *lpr* animals. These studies clearly define for the first time the potent capacity for cellular actions of glomerular AT_1 receptors in isolation to promote proteinuria, to stimulate proinflammatory cytokine expression, and to induce structural injury in vivo in a manner that is independent of systemic hypertension or other hemodynamic perturbations. Conversely, even in the absence of hypertension, blockade of these glomerular receptors provides powerful protection against kidney damage. Based on the action of renal AT_1 receptors to induce kidney damage in autoimmune nephritis, blockade of the RAS with ACE inhibitors or ARBs may be useful in protecting the kidney in patients with lupus or other autoimmune disorders.

Methods

Mice. All mice were bred and maintained in pathogen-free animal barrier facilities at the Durham VA Medical Center under local and NIH guidelines. All animal studies were approved by the Institutional Animal Care and Use Committee, Durham Veterans Affairs Medical Center (Durham, North Carolina, USA). MRL/MpJ and MRL/MpJ-*Tnfrsf6*^{lpr} (MRL/*lpr*) mice were purchased from The Jackson Laboratory. Mice with a targeted deletion of the AT_{1A} receptor gene locus (*Agtr1a*) were generated as previously described (1). *Agtr1a*$^{+/-}$ MRL/MpJ mice were generated through repeated backcrosses for more than 10 generations. Successive intercrosses with MRL/*lpr* animals yielded groups of *Agtr1a*$^{+/+}$ (*lpr*) and *lpr*-KO littermates used for our experiments. 24 *lpr* (10 males and 14 females) and 18 *lpr*-KO mice (8 males and 10 females) were maintained indefinitely for long-term survival assessment. For disease progression studies at 12 weeks of age, there were 19 *lpr* mice (9 males and 10 females) and 24 *lpr*-KO mice (14 males and 10 females).

Evaluation of lymphoid organs. 24 hours following the urine collection, the 12-week-old animals were anesthetized, blood was collected via intracardiac puncture, and the left kidney, spleen, thymus, and left axillary lymph node were removed for analysis as described below. Blood samples were centrifuged for 6 minutes at 3,800 *g*. The sera were stored at –80°C. Spleen, thymus, and lymph node tissues were weighed and placed in ice-cold RPMI media with 10% heat-inactivated FCS for flow cytometry. Portions of kidney and heart tissue were snap-frozen and stored at –80°C for extraction of RNA. The remainder of the kidney was fixed in 10% buffered formalin.

Assay of lymphocyte populations. Single-cell suspensions were prepared from spleens, thymuses, and lymph nodes of *lpr* and *lpr*-KO animals (n = 8 per group) isolated as above. Cells (1×10^6 /ml) were washed, rested for 15 minutes in RPMI containing 10% FCS to reduce nonspecific binding, and resuspended in PBS containing 2% FCS and 0.02% sodium azide. The cells were then stained in 200 μl volumes at 4°C using predetermined optimal concentrations of antibodies. All antibodies and isotype controls were purchased from BD Biosciences. The antibodies used were as follows: PE-labeled anti-CD3; FITC-labeled anti-CD4; Cy5-labeled anti-CD8; FITC-labeled anti-B220/CD45R; PE-labeled NK1.1; PE-labeled F4/80; PE-labeled anti-CD25; and PE-, FITC-, and Cy5-labeled isotype antibodies. After final washing, cells were fixed in PBS containing 2% formalin and analyzed within 72 hours. Analyses were performed on a FACSCalibur (BD Biosciences) at the Flow Cytometry Facility at Duke University.

Measurement of autoantibody levels. Anti-DNA antibody levels were determined by ELISA as previously described using, as antigen, photobiotinylated ssDNA and dsDNA added to streptavidin-coated plates (64, 65). For this purpose, the ssDNA and dsDNA were first biotinylated according to the manufacturer's instructions (Vector Laboratories). Immulon 2HB microtiter plates (ThermoLab) were coated with Streptavidin (Sigma-Aldrich) at 5 μg/ml in 0.1 M phosphate buffer, pH 9.0, 100 μl/well, and incubated overnight at 4°C. After 3 washes with PBS, biotinylated DNA at 0.2 μg/ml was added to wells and incubated at room temperature for 1 hour. Serum diluted 1:100 in PBS-Tween (PBS containing 0.5% of Tween-20) was added in duplicate and incubated for 1 hour. Then, 100 μl of peroxidase-conjugated goat anti-mouse IgG (heavy and light chains; Sigma-Aldrich) diluted 1:1000 in PBS-Tween was added, and after 1 hour incubation, followed by 200 μl of the substrate containing 0.015% of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich) in 0.1 M citrate buffer, pH 4.0, and 0.01% hydrogen peroxide for color development. After 35 minutes incubation, OD380 was determined with a microtiter plate reader (Molecular Dynamics).

Bone marrow cell transplantation. Bone marrow cells were obtained by flushing the femurs and tibiae of 1-month-old donor *lpr* or *lpr*-KO mice with RPMI 1640 medium supplemented with 10% FCS as described (66). After counting, cells were then resuspended in PBS at 4×10^7 cells/ml. Recipient

lpr mice were lethally irradiated with 10.5 Gy using a cesium irradiator (67). Within 4 hours after irradiation, the mice were injected intravenously via tail vein with 10^7 cells (66–68). To avoid immunologic responses due to the H-Y antigen, male recipients received male bone marrow cells and females received female bone marrow cells. Mortality was recorded daily. At least 26 viable transplants were performed in each group.

Detection of donor chimerism. To confirm repopulation of the recipient lymphoid organs with donor bone marrow–derived cells, Southern blot analysis was performed on DNA from recipient splenocytes as previously described (68). In brief, splenocytes were harvested from recipients 8 weeks following bone marrow transplantation. The splenocytes were resuspended in sterile rbc lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 130 mM EDTA) and incubated for 4 minutes at room temperature to remove red blood cells as described (69), then washed in PBS to yield purified mononuclear cells. DNA was then extracted from the mononuclear cells, digested with *Bam*HI, and analyzed by Southern blot analysis as described (1). Insertion of the *Agtr1a*$^{-/-}$ targeting vector into the locus introduced another *BAM*HI site, so that the targeted allele could be identified by the presence of a diagnostic 3.8-kb *Bam*HI fragment using an *Agtr1a* probe as reported elsewhere (1). The extent of bone marrow chimerism was determined by densitometric analysis of the autoradiogram.

Biochemical measurements for kidney injury. At 12 weeks of age, urine was collected from mice individually housed in specially designed metabolic cages with free access to tap water. Urine was collected for 24 hours and stored at –80°C. Urinary albumin concentration was determined using an indirect competitive ELISA according to the manufacturer's instructions (Albuwell M; Exocell). BUN levels were quantitated using a Vitros 250 Chemical Analyzer (Ortho Clinical Diagnostic).

Histopathological evaluations. Fixed kidney tissues were embedded in paraffin, sectioned, and stained with H&E. All of the tissues were examined by a pathologist (P. Ruiz) without knowledge of the experimental groups. The kidney sections were graded based on the presence and severity of abnormalities in glomeruli, tubules, vessels, and interstitium. As previously described (16, 70), the severity of renal pathological abnormalities was graded using a semiquantitative scale, in which 0 represented no abnormalities, and 1+, 2+, 3+, and 4+ represented mild, moderate, moderately severe, and severe abnormalities, respectively. An overall histologic score for each kidney was obtained by summing the individual grades for the glomeruli, tubules, and interstitium, plus 1 point for the presence of vascular damage or arterial stenosis (64). Percentage glomerulosclerosis is defined as the number of glomeruli with evidence of sclerosis divided by the total number of glomeruli in the section. For the analysis of knee joints, knees were dissected from the right hind limb and fixed in 10% buffered formalin. The joints were decalcified, paraffin-embedded, and stained with H&E. Joints were then evaluated for proliferation of the synovial lining and stroma cells, fibrosis of the synovial membrane, and presence of lymphocytes, macrophages, and granulomas. Grading was performed using a 4-point semiquantitative scale as described (21, 64).

RNase protection assays. Total cellular RNA was extracted from kidney tissues using the RNeasy kit (QIAGEN), according to the manufacturer's instructions and was stored in RNase-free water at –80°C. To detect cytokine mRNA, commercially available multiprobe template sets (RiboQuant; BD Biosciences) were labeled with [α-32P]UTP (PerkinElmer) according to manufacturer's instructions and then diluted to a concentration of 300,000 cpm/μl of hybridization buffer. All reagents used in probe synthesis were obtained from BD Biosciences (45004K; In Vitro Transcription Kit). The following probe sets were used: *MCK-1* (IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, IFN-γ), *mCK-3b* (LT, TNF, IL-6, IFN-γ, IFN-β, TGF-β1, TGF-β2, TGF-β3, MIF), and *mCK-5c* (Ltn, RANTES, MIP-1b, MIP-1a, MIP-2, IP-10, MCP-1, TCA-3, eotaxin). RNase protection assays were performed using the RNase Protection Assay Kit (45014K; BD Biosciences) and following the protocol suggested by the manufacturer. Samples were then heat blocked (95°C) for 3 minutes and run on acrylamide gels. Gels were covered with Saran Wrap (SC Johnson) and dried under vacuum at 80°C for 45 minutes. The dried gels were placed on film in a cassette with an intensifying screen and stored at –80°C. The exposure time ranged from 2 hours (for the housekeeping genes) to 7 days (for faint bands). Films were developed and scanned and bands were analyzed as a ratio of target RNA/housekeeping gene control using the Scion Image for Windows program, version 4.02.

Tail-cuff blood pressure measurements. Systolic blood pressure levels were determined in conscious mice by the noninvasive computerized tail-cuff method after 2 weeks of daily training, which involved placing the mouse into the blood pressure–measuring apparatus for acclimation. This method was validated previously and correlates well with direct measurements of intraarterial pressure (1). Data were recorded for 2 weeks. Measurements with SD of more than 30 mmHg for systolic blood pressure levels were not accepted.

Real-time RT-PCR for renin and AT_{1B} receptor. Relative levels of mRNA for renin were determined by real-time RT-PCR with the ABI Prism 7700 Sequence Detection System (PE Biosystems) as described previously (71). Kidneys were harvested, and total RNA was isolated using an RNeasy Mini Kit per the manufacturer's instructions (QIAGEN). The nucleotide sequences of the PCR primers and their fluorogenic probes have been published previously (71). Real-time RT-PCR amplifications were performed in a 96-well plate in the ABI Prism 7700 sequence detector (PE Biosystems) as previously described (71). A standard plasmid containing a DNA fragment for the renin gene was used as an external control, and amplification of the β-actin gene was used as an endogenous control. For each experimental sample, the amounts of the target and of the endogenous control were determined according to the appropriate standard curves. Relative levels of mRNA for *Agtr1b* were similarly determined using the TaqMan Primer Probe Set for *Agtr1b* (Applied Biosystems) with 18S ribosome as endogenous control.

Measurements of serum aldosterone. At 16 weeks of age, blood was drawn from experimental animals by terminal cardiac puncture and stored at –70°C. Aldosterone concentrations were then determined using the Coat-A-Count Radioimmunoassay Procedure (Diagnostic Products).

FACS separation of glomerular cell populations for RT-PCR. Kidneys were harvested from transgenic mice with *nephrin*-CFP (expressed in podocytes) or *Flk1*-GFP (GFP expressed in endothelial cells) transgenes (72, 73). Glomeruli were isolated by sequential passing through 105- and 71-micron sieves. Single-cell suspensions were obtained by enzymatic digestion (0.2% trypsin, 0.2% collagenase A, 0.1% EDTA in PBS) at 37°C for 1 hour on a shaker. After incubation, cells were washed by repeating centrifugation and PBS exchanges. Before sorting, 7-aminoactinomycin was added to exclude dead cells. Cells were then sorted into positive and negative fractions for respective genetic markers by FACS (BD FACSAria; BD Biosciences). Sorting was carried out using low pressure (20 psi) with excitation wavelengths of 407 nm (CFP) and 488 nm (GFP) and emission detection filters of 450/40 and 530/30, respectively. The positive fractions were centrifuged, and cells were resuspended in TriZOL for preparation of mRNA. Mesangial cells were obtained from a mouse mesangial cell line (74). In separate experiments, mRNA from kidney cortex and isolated glomeruli was used. mRNA was reverse transcribed to cDNA, and expression of *Agtr1b* and *Hprt* (housekeeping gene) was determined by real time PCR as described above. Changes in expression between groups were calculated using the ΔΔCt method (Applied Biosystems) and correlated to glomerular expression. Each real-time PCR reaction had a starting cDNA quantity of at least 2 ng. Expression was measured in 3 or more independent preparations per group.

Immunohistochemistry: renin. Renal tissue from 12-week old mice was fixed in formalin and embedded in paraffin. Paraffin-embedded mouse kidney

sections (3 μm) were immunostained by the immunoperoxidase technique using an automatic robot system (Dako), which allowed all slides to be exposed to reagents and antibody for an identical incubation time. Mounted tissue sections were sequentially incubated with normal blocking rabbit serum, primary antibody (rabbit anti-renin polyclonal antibody provided by Tadashi Inagami, Vanderbilt University, Nashville, Tennessee, USA) at 1:4,000 concentration, detected with a rabbit HRP-Polymer kit (Biocare Medical) using DAB (0.1% 3,3′-diaminobenzidine tetrahydrochloride) (Sigma-Aldrich) to visualize peroxidase activity. In each kidney section, the glomeruli were scored as positive or negative for renin staining localized to juxtaglomerular cells (27). 328 glomeruli from 12 male *lpr* mice and 419 glomeruli from 12 male *lpr*-KO mice were scored without knowledge of the experimental groups (M.C. Prieto-Carrasquero).

Immunohistochemistry: desmin. Three-micron–thick sections were stained using an avidin-biotin immunoperoxidase technique with polyclonal antibodies against desmin (1:100) (Dako) after antigen retrieval in high pH water bath for 20 minutes. DAB was used as a detection system, and sections were counterstained with hematoxylin. Sections were then scored for presence or absence of desmin staining in podocytes by a pathologist masked to experimental conditions. (n = 11 *lpr* mice, 7 males and 4 females; n = 10 *lpr*-KO mice, 6 males and 4 females).

Antihypertensive therapy. To control for the effects of blood pressure, a separate group of AT_{1A}-receptor wild-type *lpr* mice were treated after 4 weeks of age with hydralazine (Sigma-Aldrich), approximately 37 mg/kg/day in the drinking water (n = 12; 6 males and 6 females). In a subsequent study, we treated *Agtr1a*$^{-/-}$-MRL/*lpr* mice with water or 30 mg/kg body weight/d of losartan provided as a gift by Merck. Animals were treated from 4 weeks of age until 12 weeks of age (n = 23 for the vehicle-treated group, 11 males and 12 females; n = 25 for the losartan-treated group, 11 males and 14 females).

Statistics. The values for each parameter within a group are expressed as the mean ± SEM. For comparisons between groups, statistical significance was assessed using an unpaired 2-tailed Student's t test for normally distributed data. For nonparametric analyses, a Mann-Whitney U test was used. For comparison of proportions of histologic specimens in the experimental groups demonstrating vascular injury, podocyte injury, and juxtaglomerular renin staining, a χ^2 or Fisher's exact test was used as stated in the text.

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