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The lupus susceptibility gene kallikrein down-modulates antibody-mediated glomerulonephritis

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Abstract

Sle3 is an NZM2410/NZW-derived lupus susceptibility interval on murine chromosome 7, that is associated with spontaneous lupus nephritis, and also anti-GBM induced glomerulonephritis. The tissue kallikrein gene cluster is located within the *Sle3* interval and constitutes potential candidate genes for this locus. We have recently reported that renal kallikrein expression was up-regulated by anti-GBM antibody challenge in a strain-specific manner and that it was significantly under-expressed in the anti-GBM sensitive strains, including B6.*Sle3*. Further sequencing and functional studies reported previously provided evidence that kallikreins could constitute disease genes in lupus. In the present report, we have used an adenoviral vector to deliver the *klk1* gene to B6.*Sle3* congenics to directly test if kallikreins might have a protective effect against anti-GBM induced nephritis. Our data shows that *klk1* gene delivery ameliorated anti-GBM induced nephritis in B6.S*le3* congenics. Taken together with previous studies, these findings indicate that kallikreins play an important protective role in autoantibody-initiated glomerulonephritis, and could constitute potential candidate genes for anti-GBM induced and spontaneous lupus nephritis.

Keywords

Kallikrein; anti-GBM; glomerulonephritis; adenovirus; lupus

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production and immune complex (ICs) formation (1-7). Immune-mediated nephritis caused by deposition of pathogenic autoantibodies and ICs in the glumeruli is the leading cause of mortality and morbidity in this disease. The studies of spontaneous lupus nephritis (SLN) in mouse models and experimental anti-GBM disease have provided

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valuable insights into the underlying mechanisms of human lupus nephritis (8-14). The experimentally induced anti-GBM model has proven to be a particularly useful approach for studying end-organ susceptibility to immune-mediated damage (9-12). Our previous studies have revealed that NZW mice exhibit increased susceptibility to experimental anti-GBM induced glomerulonephritis (EAG), compared to C57BL/6 (B6) (10). Instead of performing a traditional mapping study to identify the responsible genetic loci, we took advantage of the fact that several NZW/NZM2410-derived "*Sle*" susceptibility loci had already been bred onto the normal B6 background as congenic intervals (13-25). One such lupus susceptibility interval is *Sle3*^{z}, on chromosome 7 (19, 22, 26). Unlike the other B6.*Sle* congenic strains tested, B6.*Sle3*^{z} mice (bearing the NZM2410/NZW-derived "z" allele of *Sle3* on the relatively normal B6 background) exhibit increased susceptibility to EAG compare with B6 (27). Further fine mapping using recombinant sub-congenics bearing progressively narrower subintervals of *Sle3* revealed the susceptibility genes to be located between *D7mit157* and *D7mit158* on chromosome 7 (27), an interval harboring the kallikrein cluster of genes. Microarray and real-time PCR studies indicated that several EAG susceptible strains (such as 129/svJ, NZW and DBA/1) as well as the B6.*Sle3* congenic strain had significantly reduced renal expression of kallikreins, compared to B6 and BALB/c controls, following anti-GBM challenge. Furthermore, sequence comparison of several *klk* genes indicated that nephritis-prone mouse strains and patients with lupus nephritis possessed different *klk* alleles, compared to controls (27).

The above studies suggested that kallikreins may be renoprotective in immune-mediated nephritis. Indirect evidence for this was provided by demonstrating that bradykinins (which are generated by kallikreins) can be renoprotective, while bradykinin receptor blockade aggravated anti-GBM induced nephritis (27). The previous studies did not address if kallikreins themselves could modulate disease when deliberately administered to nephritis-susceptible mice. In this communication, we directly test if systemic delivery of kallikreins is renoprotective against autoantibody-induced nephritis, using B6.*Sle3*^{z} congenic mice as the disease model.

MATERIAL AND METHODS

Construction and preparation of recombinant adeno-klk1

The recombinant adenoviral Ad-GFP vector (AdEasy™ vector system, Stratagene, USA) was used for making the Ad-m*klk1* construct, following the vendor's instructions. Briefly, the mouse *klk1* gene coding region (786 bp) was PCR amplified from the B6 strain using the following primers: forward *5′-AGC GTC GAC ACC ATG AGG TTC CTG ATC-3′*, reverse *5′-AGC CTC GAG GTC ATT TTC AGC CAT AG-3′*, and inserted into the multi-cloning site of pShuttle-IRES-hrGFP vector. Once constructed, the shuttle vector was linearized with Pme I and co-transformed into BJ5183 competent cells together with pAdEasy-1, a supercoiled viral DNA plasmid. Transformants were selected for kanamycin resistance, and recombinants containing m*Klk1* insert were subsequently identified by restriction digestion. Once a recombinant was identified, it was produced in bulk using the recombination-deficient XL10-Gold strain. Purified recombinant Ad-m*klk1* plasmid DNA was digested with Pac I to expose its inverted terminal repeats (ITR), and then used to transfect AD-293

cells, in which deleted viral assembly genes are complemented in vivo. Ad-m*klk1* was amplified and purified from these cells, and the titer of recombinant virus was measured by plaque assays. The Ad-GFP vector was used as a control.

Animal studies

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.*Sle3*^{z} is a congenic stain bearing the lupus susceptibility interval, *Sle3*^{z} (15, 19, 26). All mice were maintained in a specific pathogen-free colony. 2-3 month old females were used for all studies. To induce EAG, 10 mice from each strain were sensitized on day 0 with rabbit IgG (250 μg/mouse, i.p.), in adjuvant. On day 3, mice of each strain were randomly divided into two groups of 5 mice each. One group received recombinant Ad-*mklk1* virus via tail vain injection (1×10^7 plaque-forming units per mouse) and another group receive the same dose of Ad-GFP vector as control. On day 5, all mice were challenged (i.v.) with rabbit anti-GBM IgG (200 μg per 25 g of body weight). Twenty-four-hour urine and serum samples were collected from all mice on days 0, 7, 14 and 21, for measuring proteinuria, serum BUN and kallikrein activity. All animals were sacrificed on day 21, and the kidneys were processed for histo-pathological examination by light microscopy. Five mice were included in each experimental group.

Detection of Klk1 expression in serum by Western blotting

Serum samples were collected from each experimental mouse at day 0, 7, 14 and 21. Sera were diluted 1:10 with PBS and protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL). 10 ug of serum protein from each sample was subjected to SDS-PAGE and transferred to nylon membrane for western blot analysis using a rabbit anti-mouse kallikrein-1 antibody (1:1000), as described (27). Immunoreactivity was detected by chemiluminescence (Pierce).

Detection of urine kallikrein excretion by enzymatic activity assay

24-hour urine samples were collected from each mouse using metabolic cages on days 0, 7, 14, and 21. Total urinary kallikrein enzymatic activity was measured using the synthetic chromogenic substrate HD-Val-Leu-Arg-pNA (S-2266), as described by Moodely et al (28). Briefly, 50 ul of mouse urine sample was added to 50 ul of assay buffer (0.2M Tris-HCl, pH 8.2, containing 300ug/l SBTI and 375ug/l EDTA) and incubated at 37°C for 30 min. Then, 50 ul of S-2266 was added and incubated for 3 h at 37°C. The plate was read at 405 nm. A standard curve was constructed using purified human urinary kallikrein (HUK, Calbiochem). The amount of total protein in the urine was measured using the BCA Protein assay kit (Pierce).

Assessing renal disease

Twenty-four-hour urine samples were collected from experimental mice on days 0, 7, 14 and 21, using metabolic cages, with free access to drinking water. Urinary protein concentration was determined using the Coomassie Plus protein assay kit (Pierce). Serum was collected on day 14, for measuring blood urea nitrogen (BUN), using a urea nitrogen kit (Sigma-Aldrich, St. Louis, MO). Three-micrometer sections of formalin-fixed, paraffin-embedded kidney

tissues were cut and stained with H&E and periodic acid-Schiff. All sections were examined in a blinded fashion, for any evidence of pathology in the glomeruli, tubules, or interstitial areas, as described before (12). The severity of glomerulonephritis (GN) was graded on a 0-4 scale as follows: 0, normal; 1, mild increase in mesangial cellularity and matrix; 2, moderate increase in mesangial cellularity and matrix, with thickening of the GBM; 3, focal endocapillary hypercellularity with obliteration of capillary lumina and a substantial increase in the thickness and irregularity of the GBM; and 4, diffuse endocapillary hypercellularity, segmental necrosis, crescents, and hyalinized end-stage glomeruli. The severity of tubulointerstitial nephritis (TIN) was graded on a 0-4 scale, based on the extent of tubular atrophy, inflammatory infiltrates, and interstitial fibrosis, as detailed previously (29).

Statistics

Results are expressed as mean ± sem. Data were analyzed using the unpaired Student's t test or ANOVA, with the use of GraphPad Prism 5.0 software (San Diego, CA). Values were considered significantly different if $P < 0.05$.

RESULTS

Expression of kallikrein in B6 and B6.*Sle3*^{z} mice after *klk1* gene delivery

Following intravenous injection of an adenovirus encoding the mouse *klk1* gene, recombinant kallikrein levels in mouse sera and tissues were assayed by Western blot using a rabbit anti-m*Klk1* antibody. All B6 and B6.*Sle3*^{z} mice receiving Ad-m*klk1* expressed significantly elevated kallikrein levels in their blood and liver, but not in the kidneys or spleen (Fig. 1 and data not shown), compared to the control mice. Figure 1A shows a representative Western blot. *Klk1* expression reached its peak on day 7 (i.e., 5 days after Ad-m*klk1* injection) and then started to decrease. The expression of *Klk1* in mouse serum was still detectable on the day of sacrifice (day 21). Mice receiving Ad-GFP vector control did not have detectable kallikrein expression in their serum. Kallikrein excretion was also detected in the urine of B6.*Sle3*^{z} mice receiving Ad-m*klk1* injection on days 7, 14 and 21 (3.35±2.23, 13.38±6.86 and 7.44±4.42 ug/24hr, respectively), compared to the vector control group (0.69±0.43, 0.49±0.3, and 0.95±0.66 ug/24hr, respectively) (*P* values: 0.03, 0.007, 0.05, respectively), as summarized in Figure 1B.

Effect of kallikrein gene delivery on proteinuria and serum BUN

As expected, the B6.*Sle3*^{z} mice in the control group (receiving Ad-GFP vector injection) developed high levels of proteinuria on day 14 (19.2±2.1 mg/24hr) and day 21 (9.7±2.5 mg/24hr), after anti-GBM Ab challenge. In contrast, B6.*Sle3*^{z} mice in the *klk1* gene delivery group (receiving Ad-m*klk1* injection) showed significantly reduced proteinuria on day 14 and day 21 (3.2±0.6 mg/24 hr and 2.6±0.3 mg/24hr, respectively) compared to the vector control group ($P<0.001$, n=5), as diagramed in Figure 2A. There was no significant increase in proteinuria in un-challenged B6 mice receiving the adenoviral vector (data not shown). Likewise, on day 14 after anti-GBM challenge, the serum BUN levels in B6.*Sle3*^{z} mice injected with Ad-GFP control vector reached 33.9±4.0 mg/ml. However, B6.*Sle3*^{z} mice treated with recombinant Ad-m*klk1* showed significantly lower serum BUN (18.5±4.6

mg/ml) compared to the vector control mice (P=0.004), as shown in Figure 2B. Unchallenged B6 mice treated with Ad-GFP or Ad-m*klk1* showed low levels of serum BUN, which was similar to the levels in klk1-treated B6.*Sle3*^{z} mice (data not shown).

Effect of kallikrein gene delivery on renal histology

Histological analysis of kidneys confirmed that the *klk* gene delivery attenuated histological GN induced by anti-GBM challenge (Figure 3). B6.*Sle3*^{z} mice receiving Ad-GFP vector showed moderate to severe GN with a score of 2.7±0.5, whereas the Ad-*klk1* injected B6.*Sle3*^{z} mice exhibited ameliorated glomerular disease with a GN score of 1.4±0.2 (P=0.02) (Figure 3A). The marked GN in the control group was also accompanied by significant glomerular proliferation and crescent formation, as exemplified by representative slides depicted in Figure 3B. Collectively, the above functional readouts and histological findings indicate that the deliberate administration of kallikreins systemically has the capacity to dampen autoantibody-initiated glomerulonephritis. In terms of the proteinuria, BUN and renal pathology, the kallikrein-treated mice did not differ significantly from unmanipulated mice (i.e., not challenged with anti-GBM Abs) (10-12).

DISCUSSION

The EAG experimental model of immune-mediated nephritis has emerged as a useful tool for dissecting out the molecular and genetic basis of lupus nephritis (9). The finding that B6.*Sle3*^{z} congenic mice are more sensitive to EAG than the B6 controls has fueled studies aimed at identifying potential renal-intrinsic factors that may govern this phenotype (27). One key molecular difference that clearly distinguishes the EAG sensitive strains from the resistant strains is their renal kallikrein expression levels upon anti-GBM Ab challenge (27). Interestingly, the *klk* gene cluster is located within the *Sle3* lupus susceptibility interval on murine chromosome 7, with interesting sequence polymorphisms, raising the possibility that these could be the genes responsible for the observed renal phenotypes in this strain. The impaired renal expression of kallikreins in B6.*Sle3*^{z} and other EAG sensitive strains suggests that *klk* may play a protective role against nephritis (27). These earlier findings resonate well with previous genetic and pharmacological studies that also reveal a renoprotective role for kallikreins and bradykinins in nephritis following other types of insults (30-38).

In the present study, we offer the first demonstration that deliberate kallikrein gene delivery can indeed protect mice against anti-GBM induced nephritis. After systemic delivery of adenovirus containing mouse *klk1*, immunoreactive mouse klk1 was detected in the serum and urine of Ad-*klk1* injected B6 and B6.*Sle3*^{z} mice, indicating that recombinant mouse *Klk1* (most likely of serum origin) was being excreted from the kidneys. Importantly, the *klk1* gene transfer markedly reduced urinary protein, serum BUN levels and glomerular inflammation in the anti-GBM challenged B6.*Sle3*^{z} mice, demonstrating that kallikreins can indeed be renoprotective in antibody-mediated nephritis. Though only Klk1 was used in this study, it is likely to be representative of all kallikreins given that the genes in this family are evolutionarily conserved and have 67-91% primary sequence identity (43). A second rationale for using Klk1 relates to the fact that *Klk1* gene transfer has previously been demonstrated to successfully ameliorate renal disease in other animal models (35).

As reported by others, kallikreins may be exerting a protective role in nephritis by modulating several different parameters, including local blood pressure, the inflammatory milieu, redox balance and/or signaling within various cell types (39-44). Kallikreins and kinins have also been shown to improve renal function by increasing the glomerular filtration rate and renal blood flow (31, 45). Anti-GBM Abs directed to antigens on the glomerular basement membrane can trigger a rapid inflammatory reaction characterized by accumulation of polymorphonuclear leukocytes and mononuclear cells (46). It has been well documented that anti-GBM Ab induced nephritis is associated with oxidative stress in the form of ROS, which in turn can activate MAPKs, particularly p38 MAPK and JNK, and also induce apoptosis (47-52). In this context, it is worth noting that others have reported that kallikreins and kinins could also act as anti-oxidant and anti-inflammatory agents through the suppression of oxidative stress, monocyte/macrophage recruitment and renal cell apoptosis (34, 53). Others have observed that the activation of the kinin B2 receptor by kallikreins and kinins can mobilize intracellular Ca2+, enhance intracellular nitric oxide synthase (eNOS) activity and thus nitric oxide (NO) production (34, 35, 42, 53). The increased bioavailability of NO could in turn counter oxidative stress, MAPK activation and ongoing inflammation (34-37, 40-53). Hence, the renoprotective effect of kallikreins in Ab-mediated nephritis could potentially have been mediated through a wide variety of molecular mechanisms operating in parallel.

It is also worth considering the interplay of kallikreins/kinins with another multi-molecular enzyme system, the renin-angiotensin system. It is now apparent that both these systems interface at multiple levels (54-58). Both angiotensin and kinin peptides are metabolized by many different shared peptidases, including prolylcarboxypeptidases. This interaction, along with the roles of angiotensin converting enzyme, cross talk between bradykinin and angiotensin action, and the opposing effects of activation of the ANG II receptors 1 and 2 support a hypothesis that these two systems might counterbalance each other. Finally, there is evidence that ACE inhibitors, which are also therapeutically effective in lupus nephritis, could function in part by potentiating the effect of bradykinin and its analogs on B2 receptors independently of blocking peptide metabolism. Hence, some of the documented impacts of the renin-angiotensin system on nephritis need to be re-evaluated in terms of how they might impact kallikrein/kinin activity.

The observation that the NZW-derived *Sle3*^{z} lupus-susceptibility locus confers susceptibility to spontaneous lupus nephritis raises the interesting possibility that polymorphisms in *Klk* may also confer susceptibility to spontaneous lupus nephritis. Resonating well with this observation in the mouse is the finding that SNPs in human *KLK* genes may also be associated with lupus nephritis in patients (27). Efforts are in progress to evaluate if long-term modulation of Klk levels in vivo can impact the severity of spontaneous lupus nephritis. The current study provides significant insights regarding the role of the kallikrein/kinin system in mediating protection against antibody-mediated nephritis. These studies also raise the interesting possibility that different end-organs may harbor an arsenal of molecules with anti-inflammatory and/or disease modulating potential as safeguards against immune-mediated disease.

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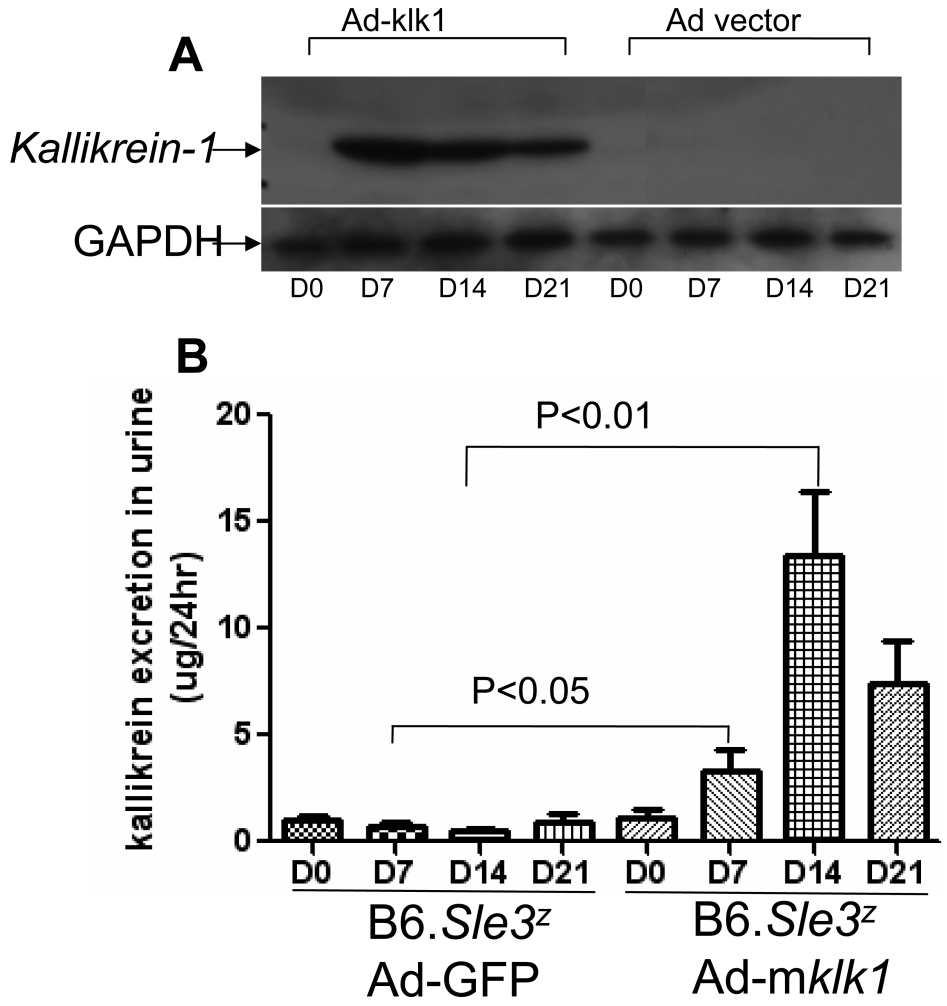

Figure 1. The expression and excretion of recombinant mouse klk1 protein after Ad-m*klk1* gene delivery

(A)Anti-GBM disease was induced in B6.*Sle3*^{z} mice with or without *klk1* gene delivery. Serum was collected on days 0, 7, 14 and 21 from each mouse and *klk1* protein expression was gauged using Western blot. Five biological replicates were included in each group. The left panel shows that high *klk1* expression was detected from the mice with *klk1* gene transfer (Ad-m*klk1*) on day 7 after anti-GBM challenge, and the levels persisted until day 21. The right panel depicted the serum Klk levels from mice receiving Ad-GFP vector as control.

(B)Kallikrein level in urine was determined using a kallikrein enzymatic activity assay. *Klk1* gene transferred mice (Ad-m*klk1*) showed increased kallikrein excretion from day 7 (right panel). No kallikrein activity was detected in the control group of mice (Ad-GFP).

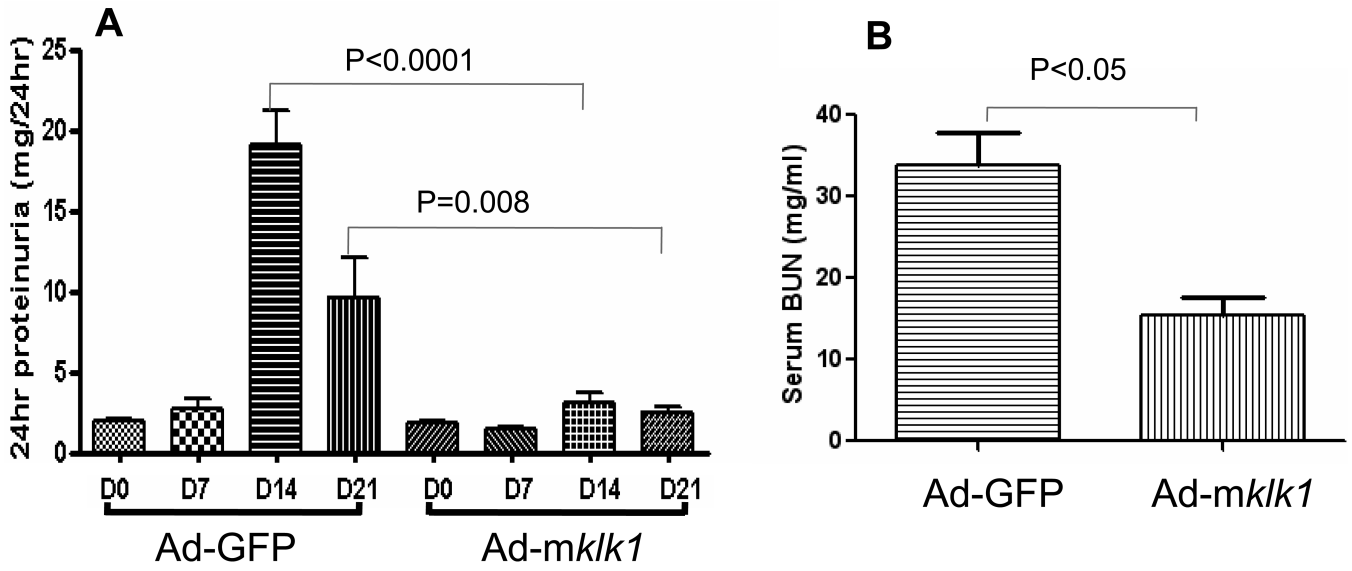

Fig 2. Urinary protein and blood urea nitrogen levels in B6.*Sle3*^{z} mice after kallikrein gene delivery

(A)Total urinary protein levels in Ad-m*klk1* and Ad-GFP vector treated mice were assayed. Urine samples were collected on days 0, 7, 14 and 21 and urine protein was determined by ELISA. Kallikrein gene delivery attenuated anti-GBM induced proteinuria. Values are expressed as mean ± SEM (N = 5 per group).

(B)Blood urea nitrogen (BUN) levels were gauged after adenoviral gene delivery. Blood was collected on day 14 after anti-GBM challenge (i.e., 12 days after *klk1* gene transfer). Kallikrein administration reduces anti-GBM induced BUN. Values are expressed as mean ± SEM (N = 5 per group).

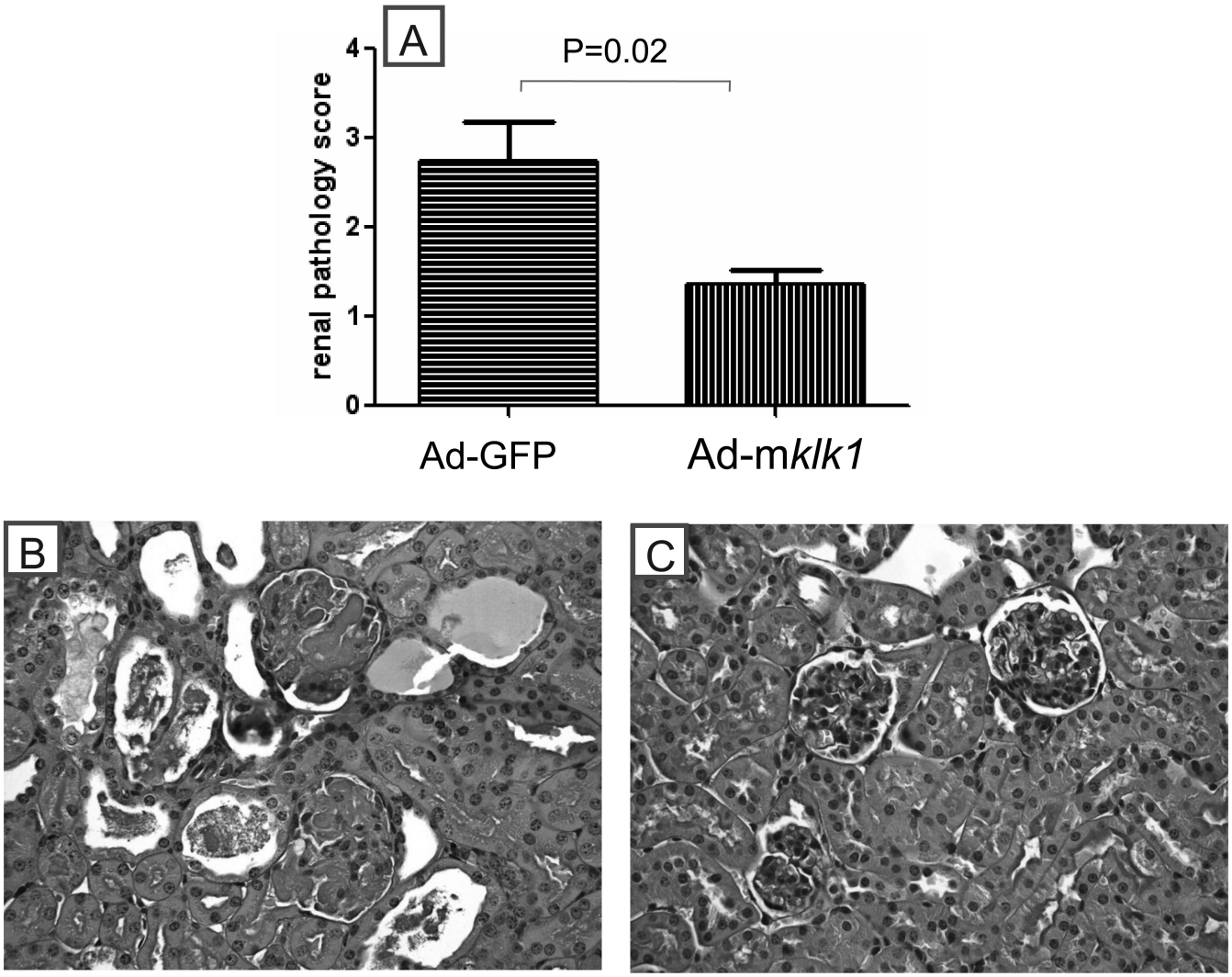

Figure 3. Effect of *klk1* gene delivery on renal pathology in anti-GBM challenged B6.*Sle3*^{z} mice

(A)Kidney specimens (collected on day21) were examined by light microscopy for evidence and grade of GN, using the WHO classification scheme. Bars represent means ± SEM; n=7 mice per group.

(B)Light-microscopic views of periodic acid-schiff-stained renal sections from Ad-vector treated B6.*Sle3*^{z} mice on day 21 after anti-GBM challenge. The mice in this group exhibited typical features of anti-GBM nephritis, including glomerular proliferation, crescent formation, intracapillary hypercellularity with obliteration of capillary lumen, and tubular dilatation with casts. Shown figure is representative of sections from at least four mice in each study group. Shown are sections of the renal cortex; no differences were seen in the renal medulla (data not shown).

(C)Anti-GBM challenged B6. *Sle3*^{z} mice receiving Ad-m*klk1* gene transfer showed essentially normal renal histology. Shown figure is representative of sections from at least four mice in each study group.