# Kallikrein genes are associated with lupus and glomerular basement membrane–specific antibody–induced nephritis in mice and humans

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**Immune-mediated nephritis contributes to disease in systemic lupus erythematosus, Goodpasture syndrome (caused by antibodies specific for glomerular basement membrane [anti-GBM antibodies]), and spontaneous lupus nephritis. Inbred mouse strains differ in susceptibility to anti-GBM antibody–induced and spontaneous lupus nephritis. This study sought to clarify the genetic and molecular factors that may be responsible for enhanced immune-mediated renal disease in these models. When the kidneys of 3 mouse strains sensitive to anti-GBM antibody–induced nephritis were compared with those of 2 control strains using microarray analysis, one-fifth of the underexpressed genes belonged to the kallikrein gene family, which encodes serine esterases. Mouse strains that upregulated renal and urinary kallikreins exhibited less evidence of disease. Antagonizing the kallikrein pathway augmented disease, while agonists dampened the severity of anti-GBM antibody–induced nephritis. In addition, nephritis-sensitive mouse strains had kallikrein haplotypes that were distinct from those of control strains, including several regulatory polymorphisms, some of which were associated with functional consequences. Indeed, increased susceptibility to anti-GBM antibody–induced nephritis and spontaneous lupus nephritis was achieved by breeding mice with a genetic interval harboring the kallikrein genes onto a disease-resistant background. Finally, both human SLE and spontaneous lupus nephritis were found to be associated with kallikrein genes, particularly *KLK1* and the *KLK3* promoter, when DNA SNPs from independent cohorts of SLE patients and controls were compared. Collectively, these studies suggest that kallikreins are protective disease-associated genes in anti-GBM antibody–induced nephritis and lupus.**

## Introduction

Immune-mediated nephritis is an important pathogenic determinant in SLE and Goodpasture syndrome (anti–glomerular basement membrane [anti-GBM] disease). In spontaneous lupus nephritis, both Ab-mediated and Ab-independent mechanisms lead to renal pathology (1–4). In particular, anti-DNA and antiglomerular Abs, as well a few other specificities, have been implicated in the pathogenesis of lupus nephritis, in both mice and humans (1–8). A useful experimental tool for dissecting out the molecular mechanisms leading to immune-mediated nephritis in lupus and Goodpasture disease is the experimental anti-GBM Ab–induced glomerulonephritis (AIGN) model, wherein the transfer of anti-GBM Abs elicits glomerulonephritis (GN) with reproducible kinetics. Although the specificities of the inciting Abs may

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**Nonstandard abbreviations used:** AIGN, anti-GBM Ab–induced glomerulonephritis; B6, C57BL/6; BK, bradykinin; GBM, glomerular basement membrane; GN, glomerulonephritis; OR, odds ratio.

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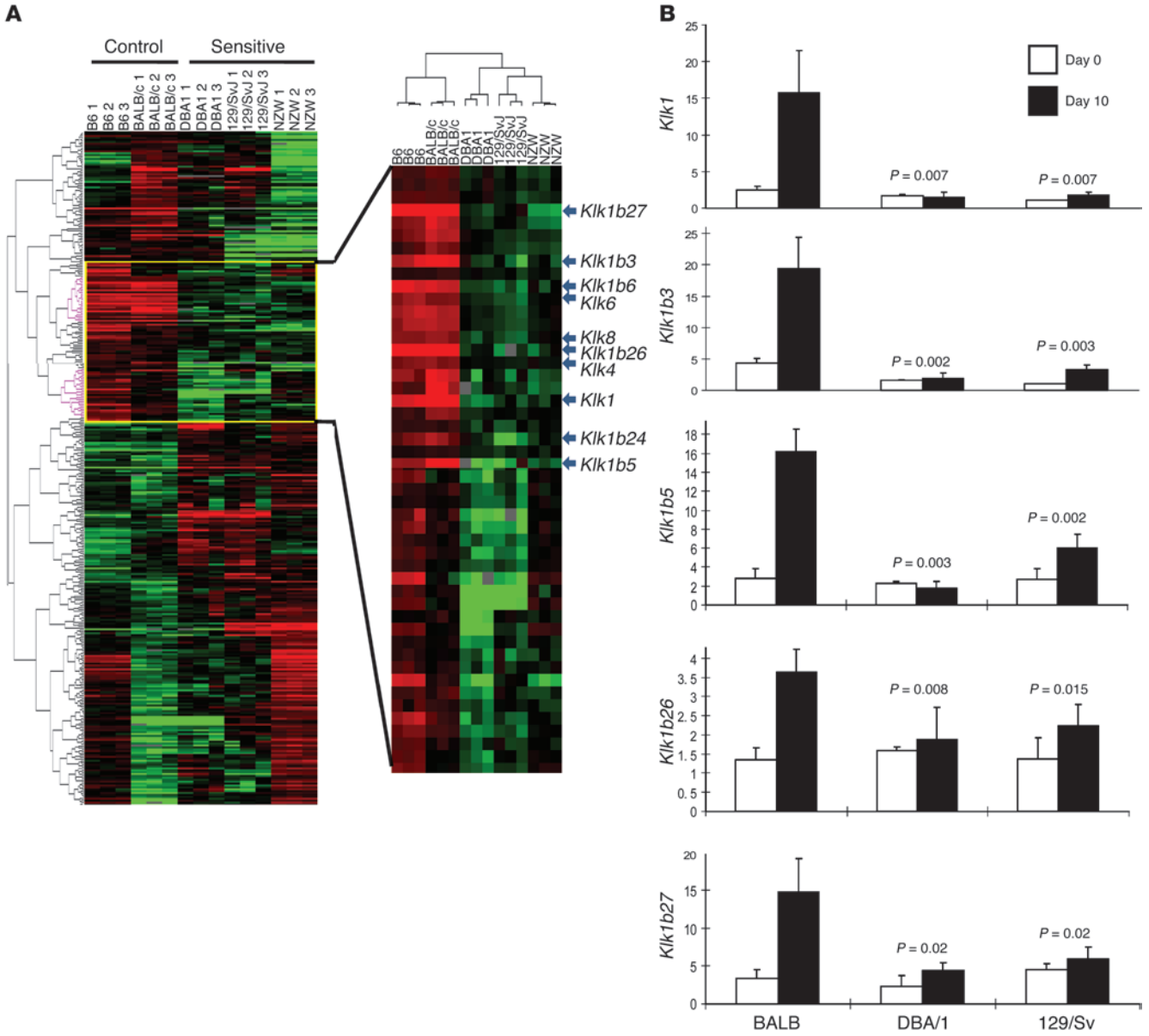


**Figure 1**
Strain-dependent gene expression differences in the renal cortex in AIGN. (**A**) Anti-GBM disease was induced in 3 disease-sensitive strains (DBA1, NZW, and 129/SvJ) and 2 control strains (BALB/c and B6), after which renal cortex RNA was analyzed using DNA microarrays on day 10 of disease (i.e., 5 days after injection of anti-GBM Abs). Three biological replicates were included for each strain. The left panel shows that a total of 360 gene transcripts were differentially expressed between the study strains (>2 fold, $P < 0.001$). The right panel (a higher-magnification view of the boxed region on the left) shows a cluster of gene transcripts that were increased in all control strains but not in the AIGN-sensitive strains (>2-fold difference, $P < 0.001$), including 10 *Klk* genes. (**B**) Renal cortex gene expression differences in *Klk1*, *Klk1b3*, *Klk1b5*, *Klk1b26,* and *Klk1b27* were confirmed by real-time PCR in the indicated strains, before (day 0) and after (day 10) anti-GBM Ab challenge. Each bar represents the mean of 6 samples. Similar changes were seen between B6 and NZW mice (data not shown). *P* values pertain to comparisons with BALB/c day 10 values. Error bars denote SD.

differ in experimental AIGN and spontaneous lupus nephritis, the downstream pathogenic cascades that lead to disease in the 2 scenarios appear to be shared, as reviewed recently (9).

Hence, the AIGN experimental model may be a useful tool for dissecting out the molecular and genetic basis of lupus nephritis. Notably, of more than 20 inbred mouse strains challenged with anti-GBM Abs, severe renal disease was noted in only 5 strains, including DBA/1, NZW, and 129/SvJ (10–12). Coincidentally, the latter 2 strains are known to develop spontaneous lupus nephritis (13–16). We had previously reported that the strain differences in AIGN susceptibility cannot be simply attributed to differences in systemic immune response (to the administered rabbit anti-mouse GBM Abs) or to differences in Th1 skewing (10, 11). On the other hand, the degree to which differences in renal-intrinsic

**Table 1**
Several kallikrein gene messages were significantly upregulated in the kidneys of B6 and BALB/c mice compared with NZW, DBA1, and 129/SvJ mice, upon anti-GBM Ab challenge

| Gene | Accession no. | Average signal intensity | | | | | Fold change[A] | *P*[A] |
|---|---|---|---|---|---|---|---|---|
| | | B6 | BALB/c | NZW | 129/SvJ | DBA1 | | |
| *Klk1b3* | NM_008693 | 36,397 | 26,179 | 16,453 | 9,715 | 7,056 | 2.6 | <0.001 |
| *Klk1b27* | NM_020268 | 26,608 | 22,255 | 10,360 | 7,185 | 5,164 | 2.9 | <0.001 |
| *Klk1b26* | NM_010644 | 17,694 | 17,596 | 7,907 | 7,932 | 5,602 | 2.3 | <0.001 |
| *Klk1b5* | NM_008456 | 14,546 | 15,007 | 3,608 | 3,847 | 4,286 | 3.7 | <0.001 |
| *Klk1* | NM_010639 | 12,779 | 13,115 | 5,079 | 4,682 | 3,300 | 2.8 | <0.001 |
| *Klk1b24* | NM_010643 | 269 | 129 | 77 | 43 | 58 | 3.0 | <0.001 |
| *Klk1b8* | NM_008457 | 246 | 350 | 47 | 62 | 35 | 6.2 | <0.001 |
| *Klk4* | NM_019928 | 7 | 103 | 1 | 1 | 3 | 30 | <0.001 |
| *Klk6* | NM_010639 | 19,269 | 28,119 | 9,375 | 13,804 | 17,533 | 2.0 | <0.001 |

All values are normalized fluorescence intensity. [A]Fold change and *P* values were calculated by comparing B6 and BALB/c (pooled, $n = 6$) with the other 3 strains (pooled, $n = 9$).

processes may contribute to the observed strain differences in AIGN susceptibility remains to be elucidated.

Given the possibility that renal-intrinsic differences may be contributory, we undertook a microarray-based transcriptomic analysis of the renal cortex from 3 AIGN-sensitive strains and 2 control strains, after anti-GBM challenge. Surprisingly, we found that a significant fraction of the differentially expressed genes that distinguish the nephritis-sensitive strains from the control strains belong to the kallikrein (*Klk*) gene family. Importantly, this gene complex is encoded within an interval on chromosome 7 that had previously been associated with spontaneous lupus nephritis (14–21). Kallikreins constitute a multigene family of serine esterases with a wide spectrum of biological functions (22–39). These reported functions include the regulation of inflammation, apoptosis, redox balance, and fibrosis within the kidneys, as well as local blood pressure. In further genetic and functional studies, we demonstrate that *Klk* genes are renoprotective in immune-mediated renal disease and may constitute important disease susceptibility genes for experimental anti-glomerular Ab–induced nephritis as well as spontaneous lupus nephritis in mice and in humans.

## Results

Displayed in Figure 1 are all genes that were significantly upregulated or downregulated (at least 2-fold difference, $P < 0.001$) in the strains that were highly sensitive to AIGN disease (i.e., NZW, DBA/1, and 129/SvJ) compared with either of the control strains (C57BL/6 [B6] or BALB/c), following challenge with anti-GBM serum. Though several strain-specific gene differences were also noted within this panel of genes, a subset of 50 genes within this panel were consistently downregulated in all 3 of the highly disease-sensitive strains compared with the control strains (shown enlarged on the right, Figure 1A). Intriguingly, 10 of these genes belonged to the kallikrein (*Klk*) family, with the highest expression levels and differences being noted in *Klk1*, *Klk1b3*, *Klk1b5*, *Klk1b26*, and *Klk1b27*, as summarized in Table 1. In addition to the *Klk* genes displayed in Table 1 (all of which were found to be different between the 2 sets of strains at $P < 0.001$), a few other *Klk* genes, notably *Klk1b9* and *Klk1b21*, exhibited similar expression differences between the disease-sensitive strains and the control strains, though these differences did not reach statistical *P* values of 0.001 (data not shown). Hence, in total, 12 *Klk* genes were underexpressed in the kidneys of AIGN-sensitive strains, following anti-GBM challenge, compared with the control strains.

Next, renal *Klk* gene expression was examined before and after induction of AIGN, using real-time PCR as an orthogonal approach. Real-time PCR analyses validated the above microarray results, indicating that whereas the B6 and BALB/c control strains successfully upregulated *Klk* following anti-GBM Ab challenge, the DBA/1, 129/SvJ, and NZW strains were ineffective at doing so (Figure 1B). In contrast, the basal, predisease levels of renal *Klk* were similar in all strains (Figure 1B). These differences were also confirmed at the protein level by Western blot analysis of renal cortex samples from these 5 strains, as illustrated for *Klk1* (Figure 2A and data not shown). Parallel differences in Klk enzymatic activity were also noted in urine samples from the same mice following anti-GBM Ab challenge (Figure 2B). Thus, whereas the BALB/c control strain exhibited a robust increase in urinary Klk activity 10–14 days after challenge, in the AIGN-sensitive strains DBA/1 and 129/SvJ, Klk was not significantly upregulated over the same period (Figure 2B).

Given that Klk upregulation in the kidneys and urine of anti-GBM Ab–challenged mice correlated well with the subdued nephritis noted in the B6 and BALB/c control strains, we next asked whether Klk might have a disease-protective role in immune nephritis. Kallikreins act through the generation of bradykinins (BKs), which in turn exert their biological effects by binding BK (BK B1 and B2) receptors on various cells (25, 26). Selective receptor blockade using pharmacological inhibitors further indicated that the biological effects of BK (and Klk) were mediated by the BK B2 receptor, since blocking this receptor aggravated proteinuria, azotemia, and GN following anti-GBM Ab challenge in BALB/c mice (Figure 3, A–D). Importantly, the mice in which BK B2 receptor was blocked exhibited significantly more severe GN compared with the other groups of mice (average GN score of 2.1 versus 0.2, $P < 0.001$, as partly illustrated in Figure 3, C and D). Similar differences were noted when the BK B2 receptor blockers were administered to anti-GBM–challenged B6 mice (data not shown). Conversely, the administration of BK dampened the severity of anti-GBM disease in 129/SvJ mice, which otherwise develop severe AIGN following the experimental insult (Figure 3E and data not shown).

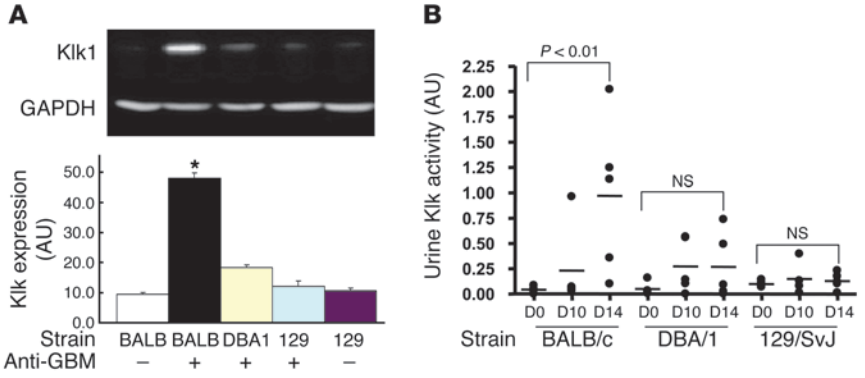


**Figure 2**

The differential renal expression of *Klk* in the AIGN-sensitive versus control strains was confirmed at the protein level. BALB/c, DBA1, and 129/SvJ mice were subjected to AIGN. (**A**) Fourteen days after anti-GBM challenge, kallikrein protein expression was assayed in the renal cortex by Western blotting, using a rabbit anti–mouse Klk1 Ab. The bar chart below ($n = 3$ mice per group) shows Klk expression normalized to GAPDH (AU). *$P < 0.001$, compared with all other study groups. (**B**) Twenty-four-hour urine samples collected from these mice on days 0, 10, and 14 after anti-GBM insult were also assayed for kallikrein enzymatic activity, using the synthetic chromogenic substrate HD-Val-Leu-Arg-pNA (S-2266), as detailed in Methods. Similar differences in renal and urinary kallikrein levels were noted between B6 and B6.*Sle3*$^z$ mice (data not shown). Error bars in **A** denote SD. In **B**, each dot represents data from a single mouse, and the horizontal bars denote arithmetic group means.

With respect to the 3 AIGN-sensitive strains, DBA/1, 129/SvJ, and NZW, it is already known that several genomic intervals in the latter 2 strains (including loci on chromosomes 1 and 7) also contribute to spontaneous lupus nephritis (13–16). Indeed, the entire *Klk* gene complex is encoded within a lupus susceptibility interval on chromosome 7. The NZW-derived lupus susceptibility locus on chromosome 7, *Sle3*$^z$ (which includes the *Klk* gene complex), has previously been introgressed onto the B6 genome as a congenic interval, and this locus had already been shown to facilitate development of spontaneous lupus nephritis (17–19). Through recursive backcrossing of B6.*Sle3*$^z$ congenic mice to B6 parents and microsatellite-assisted selection, we generated B6.*Sle3*$^z_{157–158}$ recombinants harboring the NZW-derived *Klk* gene complex within a 4-Mb interval, with termini at *D7mit157* and *D7mit158*, as diagramed in Figure 4A.

Importantly, B6.*Sle3*$^z$ mice and the newly generated B6.*Sle3*$^z_{157–158}$ congenic recombinants both displayed heightened sensitivity to AIGN, marked by elevated proteinuria and severe nephritis (Figure 4, B–D). Moreover, the renal cortex of both these congenics failed to efficiently upregulate *Klk* following anti-GBM Ab challenge, compared with the B6 controls (Figure 4E), as assessed by real-time PCR. Taken together with the functional data presented above, these findings suggest that the *z* allele of *Klk* positioned within the *Sle3*$^z_{157–158}$ subinterval may harbor important culprit genes for the heightened experimental anti-GBM disease (and spontaneous lupus nephritis) seen in NZW (and related strains of) mice.

Five of the most differentially expressed *Klk* genes, *Klk1*, *Klk1b3*, *Klk1b5*, *Klk1b26*, and *Klk1b27* were sequenced (GenBank accession numbers EU597301–EU597324). *Klk4* and *Klk1b8*, though differentially expressed, were not studied further because of their relatively low expression levels in all strains (Table 1). Several strain-specific differences were noted in the promoter regions of the 5 sequenced genes, as summarized in Table 2. The B6 and BALB/c *Klk* genes were almost identical, while the *Klk* genes from the AIGN-sensitive strains NZW and DBA/1 were closely related to each other, as illustrated by the phylogenetic trees in Figure 5A and the promoter region sequence exemplified in Figure 5B. The *Klk* genes from the 129/SvJ strain clustered more closely with the B6/BALB/c genes in some cases, and with the NZW/DBA/1 genes in others; despite repeated attempts, we could not amplify some of the critical regions of the 129/SvJ *Klk1b26* gene (Table 2). In contrast to the promoter regions, no sequence differences were noted in the coding regions or the 3′ untranslated regions of these *Klk* genes, when the 5 strains were compared (data not shown).

Some of the observed strain-specific promoter sequence differences in *Klk* fell within putative transcription factor binding sites. In the promoter region of *Klk1b3*, for example, SNPs were noted in potential transcription factor binding sites for PBF, TFIID, HoxD9, TCF-4E, NF-S, NF-E, and LBP-1, which distinguished the AIGN-sensitive strains from the controls. In particular, the GGCTT[C→G]AAAAT SNP in the promoter region of *Klk1b3* is predicted to abrogate TFIID binding. Importantly, promoter-luciferase studies indicated that sequence differences in the promoter region of *Klk1b3* may contribute in part to the reduced expression of this gene in the disease-sensitive strains (Figure 5C). Given that more than 200 sequence variations have been noted in the regulatory regions of the *Klk* genes (Table 2), each difference has to be systematically evaluated for its potential functional relevance.

As in murine lupus, the orthologous human interval encompassing the *KLK* genes on human chromosome *19q13* has also been implicated in human SLE susceptibility in previous genome scans (40–43). To ascertain whether *KLK* might also be a culprit gene in human SLE, we examined several SNPs in the renal-expressed *KLK* genes encoded within this interval, specifically human *KLK1*, *KLK5*, *KLK6*, and *KLK7*. We first genotyped a set of German SLE patients ($n = 340$) and a set of controls matched for ethnicity, age, and sex ($n = 400$). As detailed in Table 3, association to two *KLK1* SNPs, *rs1054713* (a synonymous coding SNP) and *rs2740502* (an intronic SNP), but not to the nonsynonymous substitution *rs5517*, was observed, with the strongest associations being noted in SLE patients with nephritis (*KLK1* SNP, *rs2740502*; $P = 0.007$ compared with non-nephritic SLE patients and $P = 0.01$ compared with healthy controls). A weak association was also observed for SNP *rs1897604* located in *KLK5*; however, this SNP was not in Hardy-Weinberg equilibrium in the controls (data not shown). No disease association was detected for any of the other *KLK* genes analyzed in this preliminary study.

To validate these preliminary associations, we genotyped 6 more cohorts of patients, including additional European, European-American, and Korean patients with SLE, as detailed in Tables 4 and 5. For the *KLK1* SNP *rs2740502*, we could replicate the genetic association to lupus nephritis compared with controls in the German and Italian samples but not in the remaining patient sets (Table 5). Likewise, the German SLE patients exhibited association

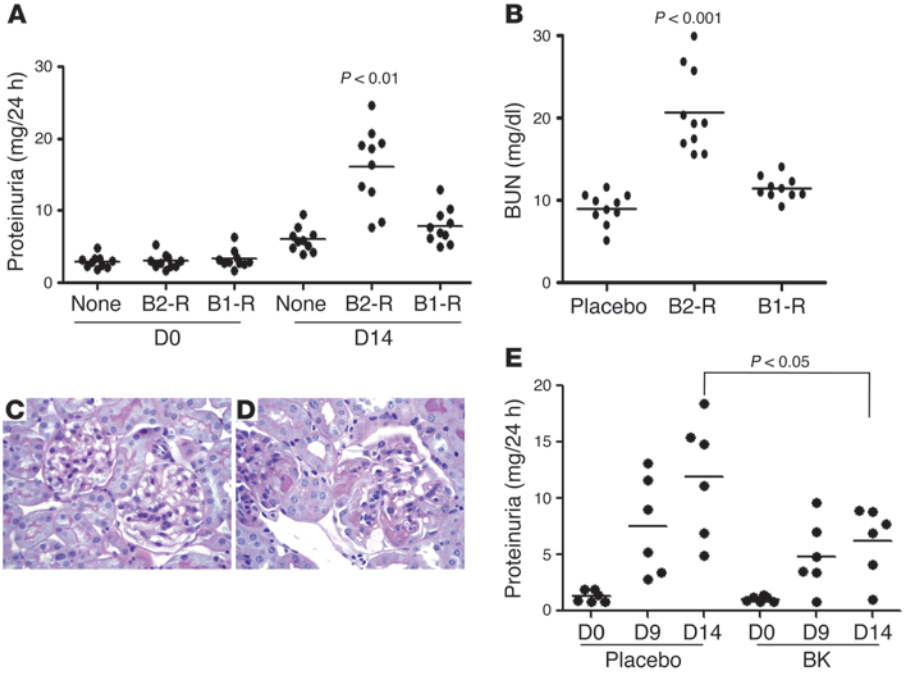


**Figure 3**
Impact of BK receptor blockade or activation on the severity of AIGN. BALB/c mice were treated with BK receptor antagonists (B1 receptor blocker H158 [B1-R] or B2 receptor blocker H157) or PBS vehicle alone as placebo (None), using osmotic pumps, for the 14-day duration of an anti-GBM challenge and phenotyped for proteinuria (**A**), azotemia (**B**), and GN (**C**, B1-R blocked; and **D**, B2-R blocked). Original magnification, ×400. (**E**) Conversely, the administration of BK into 129/SvJ mice using osmotic pumps ameliorated disease after anti-GBM challenge, compared with mice treated with vehicle placebo. In **A**, **B**, and **E**, each dot represents data from a single mouse, and the horizontal bars denote arithmetic group means.

to another KLK1 SNP, *rs5516* (Table 4). Due to likely heterogeneity in haplotype distribution and potential differences in linkage disequilibrium between the analyzed SNPs and functional SNPs in the different ethnic groups, we were unable to perform a Mantel-Haenszel metaanalysis with these data sets.

For further independent validation, we gained access to the genotype data for SNPs of the entire KLK region typed in 689 SLE patients and 3,718 healthy controls from the International Consortium for Systemic Lupus Erythematosus Genetics (SLEGEN) and 595 SLE patients from the UCSF Lupus Genetics Project cohort, both of which had been assembled for genome-wide association studies. Although the sets of SNPs spanning the entire KLK locus utilized in these studies were not completely overlapping, 56 SNPs were, and these were examined further for disease association. The strongest association with SLE was again noted to SNPs close to *KLK1*, in an intergenic region bordered at the centromeric end by *KLK1* and *KLK15* and at the telomeric end by *KLK3* and *KLK2* (Figure 6A and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI36728DS1). Additional associations to SNPs in *KLK4* promoter, *KLK5*, *KLK7*, *KLK11*, and *KLK12* were also uncovered when cases with nephritis were compared with cases without nephritis (Figure 6B and Supplemental Table 2). Next, we defined the haplotype blocks across the *KLK* locus (Figure 6A) and examined them for association to SLE. Again, the haplotype blocks harboring the promoter region of *KLK3* (block 1) displayed the strongest association, followed by the haplotype containing *KLK8* to *KLK11* genes (block 8) (Figure 6C and Supplemental Table 3). However, when only the patients with nephritis were considered as cases, the association to the *KLK3* promoter region was weaker; instead, the haplotypes harboring the *KLK4* promoter, *KLK5*, and the *KLK8–KLK11* block yielded stronger association. Of note, the 2 strongest associations observed corresponded to haplotypes with significantly lower frequencies in patients, reflecting a protective effect conferred by the KLK polymorphism.

Overall, these 3 sets of independent findings support a likely genetic association of the human *KLK1* gene and the *KLK3* promoter region with lupus and lupus nephritis.

## Discussion

AIGN is an experimental tool that shares downstream molecular cascades with spontaneous lupus nephritis, as recently reviewed (9). Over the past decade, the roles of about 25 different molecules (including various complement proteins and TLR ligands, FcR, B7/CD28/CTLA4, LFA1/ICAM1, P-selectin, TNF-α, IL-1, IL-6, IL-12, IL-18, IFN-γ, M-CSF, PDGF, MCP-1, and NO) have been directly assessed (using gene knockouts or by deliberate experimental modulation of the molecules) in 2 disease settings — anti-GBM disease and lupus nephritis. Importantly, the effects of each of these molecules were consistent in both disease settings (9). In other words, molecules documented to influence the progression of experimental anti-GBM disease also impacted the development of lupus nephritis in the same direction. Hence, although experimental anti-GBM nephritis and spontaneous lupus nephritis differ in the nature of the inciting Abs and the localization of the immune deposits, pathology in both settings may be mediated by a shared network of downstream molecular pathways, including complement- and FcR-dependent activation of resident renal cells and infiltrating leukocytes, T cell help, proinflammatory mediators initially, and profibrotic molecules later in disease (9). In the present study, we have identified kallikrein as an additional molecule that appears to impact both diseases concordantly.

Of more than 20 inbred strains tested for AIGN susceptibility, DBA/1, 129/SvJ, and NZW are particularly sensitive to disease in this model. Though the genetic and molecular origins of increased disease susceptibility may vary among these strains, it is remarkable that all 3 strains shared approximately 50 genes that were underexpressed in their renal cortex during disease, compared with other strains. Even more remarkable is the finding that one-fifth of these 50 genes belonged to the same family

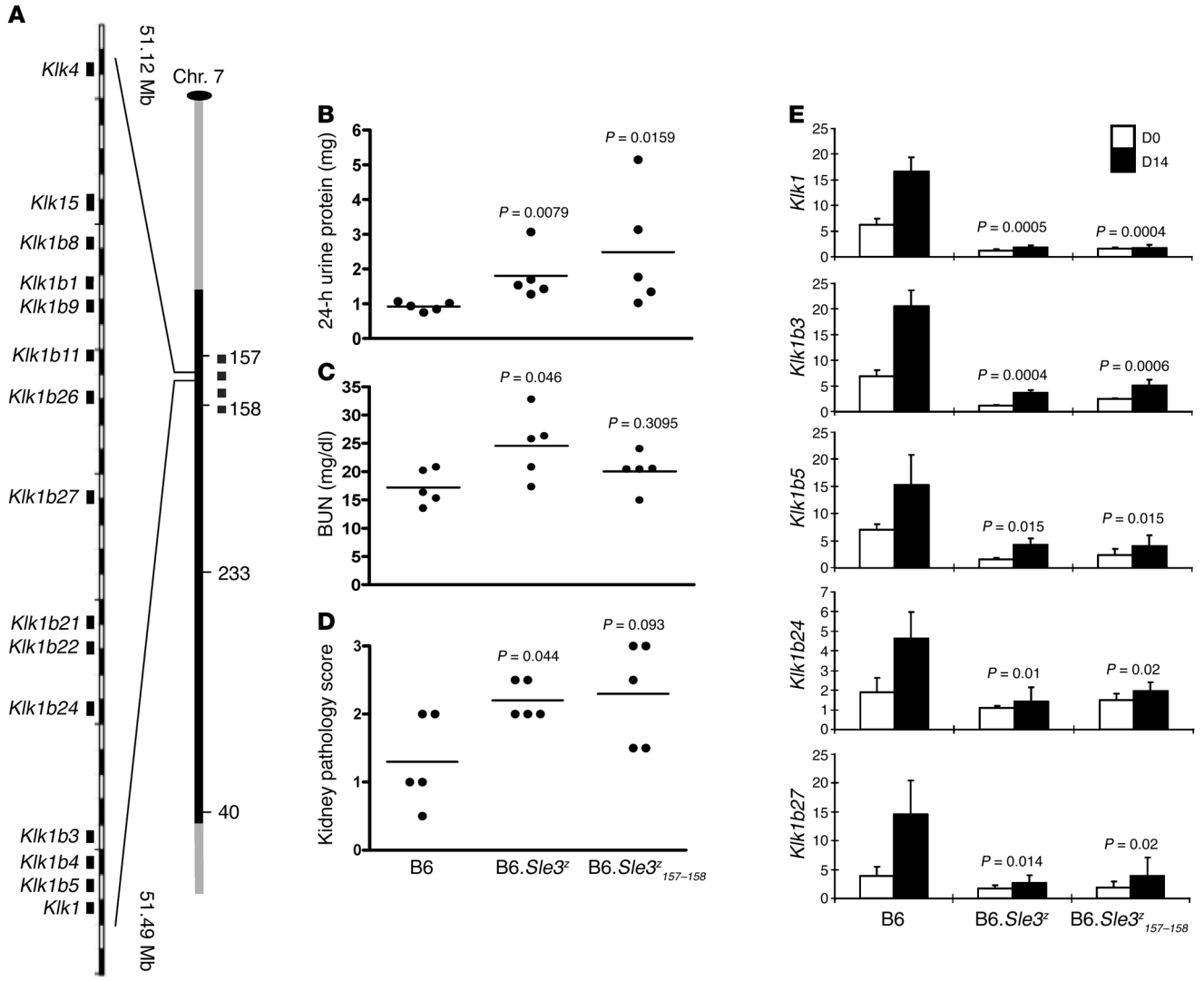


**Figure 4**

The *Sle3*$^{z}$ locus, particularly the *Sle3*$^{z}_{157–158}$ subinterval on chromosome 7, may be responsible for the reduced *Klk* and enhanced nephritis susceptibility seen in NZW mice. (**A**) Shown are the *Sle3*$^{z}$ lupus susceptibility interval on chromosome 7 (Chr. 7; black denotes the interval derived from NZM2410/NZW; gray denotes B6 origin); the 4-Mb subinterval spanning *D7mit157* to *D7mit158* (denoted by the dashed line on right); and the cluster of *Klk* genes harbored within the indicated subinterval. The numbers on the right refer to the positions of respective microsatellite markers (e.g., 157 represents *D7mit157*). Shown also are the 24-hour urine protein excretion profiles (**B**), blood urea nitrogen (BUN) (**C**), GN pathology score (**D**), and renal *Klk* message levels (**E**), 14 days after anti-GBM challenge of B6, B6.*Sle3*$^{z}$, and B6.*Sle3*$^{z}_{157–158}$ congenics ($n$ = 5 each). The data shown in **B**–**D** were reproduced in at least 2 additional experiments. In the second study, for example, the B6.*Sle3*$^{z}_{157–158}$ congenics exhibited significantly higher 24-hour protein levels in urine ($P < 0.045$) and GN score ($P < 0.013$) and more severe tubulo-intersitial disease ($P < 0.001$), compared with the B6 control (data not plotted). All statistical comparisons were made with the respective B6 controls, using the Mann-Whitney $U$ test. In **B**–**D**, each dot represents data from a single mouse, and the horizontal bars denote arithmetic group means. Error bars in **E** denote SD.

of genes, the kallikreins. This strain difference in *Klk* gene expression may originate from polymorphisms in the *Klk* genes themselves or they may be the consequence of yet other candidate genes in the AIGN-susceptible strains. The finding that a similar renal *Klk* expression profile was recapitulated in B6 mice bearing a 4-Mb congenic interval harboring the NZW allele of *Klk* (Figure 4) suggests that the observed strain differences are likely to originate from sequence differences in the *Klk* locus itself. The observation that the AIGN-sensitive strains have *Klk* sequences that are related to each other but diverge from the *Klk* sequences of the 2 control strains, B6 and BALB/c, offers further evidence that the strain differences in renal Klk upregulation must be intrinsically encoded within the *Klk* genetic locus.

Though many sequence polymorphisms in the promoter regions of the *Klk* genes were uncovered in this study, with several falling within transcription factor binding sites, the functional relevance of these regulatory sequence differences to the observed expression differences warrants systematic study. At the very least, our completed studies indicate that sequence polymorphisms within the promoter region of *Klk1b3* may be contrib-

**Table 2**
Sequence polymorphisms in the promoter region of the mouse *Klk* genes

| Gene | position | B6 and BALB/c | 129/SvJ | DBA1 and NZW |
|---|---|---|---|---|
| *Klk1b3* | –70 to –80 | – | TGTCAGGGAGG | TGTCAGGGAGG |
| | –157 | G | A | A |
| | –163 | C | G | C |
| | –191 | G | A | A |
| | –205 | A | G | G |
| | –275 | G | A | G |
| | –287 | T | G | G |
| | –298 | C | T | T |
| | –301 | T | C | C |
| | –423 | T | A | A |
| | –539 | C | T | T |
| | –607 | C | G | G |
| | –734 | T | G | G |
| | –735 to –736 | – | T | GG |
| | –822 | G | A | G |
| | –885 | G | T | G |
| | –912 | C | C | T |
| | –973 | T | C | C |
| | –1,004 | A | A | – |
| | –1,095 to –1,096 | GG | AA | AA |
| | –1,167 | A | T | T |
| | –1,170 | G | G | A |
| | –1,171 | A | G | G |
| | –1,249 | T | C | T |
| | –1,262 | A | G | G |
| | –1,271 | A | G | G |
| | –1,274 | C | G | G |
| | –1,317 | A | G | G |
| | –1,368 | G | A | A |
| | –1,426 | C | T | T |
| | –1,430 to –1,433 | – | TCGA | TCGA |
| | –1,446 | C | C | – |
| | –1,477 | G | A | G |
| *Klk1b5* | –516 | A | A | G |
| | –657 | C | A | A |
| | –706 | T | T | C |
| | –866 | G | G | A |
| *Klk1b26* | –144 | C | N/A | T |
| | –153 | T | N/A | C |
| | –158 | T | N/A | C |
| | –162 | A | N/A | C |
| | –192 | A | N/A | G |
| | –341 | C | N/A | T |
| | –734 to –753 | TATTTTATTTTATTTTATTT | N/A | – |
| | –1,034 | A | N/A | G |
| | –1,282 | C | N/A | A |
| *Klk1* | –170 | G | G | A |
| | –202 | G | T | G |

uting to the observed expression differences in this gene, when B6.*Sle3*$^{z}$/NZW kidneys are compared with the B6 control (Figure 5B). Alternatively, the entire *Klk* gene complex may be differentially regulated in the 2 haplotypes by polymorphic regulatory regions located within or perhaps even outside this gene complex. Further sequencing of both haplotypes and *Klk* allele–specific knock-in studies are warranted to obtain definitive evidence that the observed *Klk* polymorphisms are indeed responsible for the strain-specific phenotypic differences.

Having established that the reduced Klk observed in the AIGN-sensitive strains is genetically encoded by the *Klk* locus, we next examined whether reduced Klk may be responsible for the increased severity of immune-mediated nephritis seen in these susceptible strains. This indeed appears to be the case, given that subduing Klk action (using B2 receptor blockers) rendered BALB/c control mice AIGN susceptible, while the administration of BK ameliorated nephritis in AIGN-susceptible 129/SvJ mice (Figure 3). These findings resonate well with previous genetic and pharmacological studies that also reveal a renoprotective role for Klk and BKs in nephritis following other insults, including hypertension, ischemic stroke, salt imbalance, and diabetes (26–35). Previous studies suggest that Klk may be playing 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 (25, 26, 31, 36–39).

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. Though it is formally possible that additional genes within the *Sle3*$^{z}$ interval may be contributing to the increased susceptibility to lupus nephritis associated with this locus (14, 17–19), the observation that a recombinant congenic harboring a 4-Mb interval spanning the *Klk* locus confers susceptibility to experimental immune nephritis (Figure 4) suggests that the *Klk* genes are likely to be major players in spontaneous lupus nephritis as well. Efforts are underway to evaluate whether long-term modulation of Klk levels in vivo can impact the severity of spontaneous lupus nephritis.

The corresponding interval on human chromosome *19q13* bearing the *KLK* gene complex has also been implicated in human SLE susceptibility in previous genome scans, particularly in patients of European descent (40–43). The observation that SNPs in *KLK1* show significant association to lupus nephritis in European-descent SLE patients suggests that *KLK* gene polymorphisms may also influence human lupus nephritis, at least in some ethnic groups. The observed heterogeneity among different populations in some of our earlier studies (as summarized in Tables 4 and 5) may be due to haplotype differences among the populations. However, the larger and more comprehensive SNP and haplotype analysis conducted in the SLEGEN and UCSF studies clearly validates the existence of a disease locus in *KLK1*. It is intriguing that most of the disease-associated SNPs are located in the regulatory region between *KLK1* and *KLK3*, with these genes being transcribed in opposite orientations to each other (Figure 6A). This region possesses many regulatory elements sensitive to steroid hormones extensively studied in prostate cancer, which have been shown to affect *KLK3* (prostate-specific antigen) transcription. Incidentally, the murine *Klk* genes that are

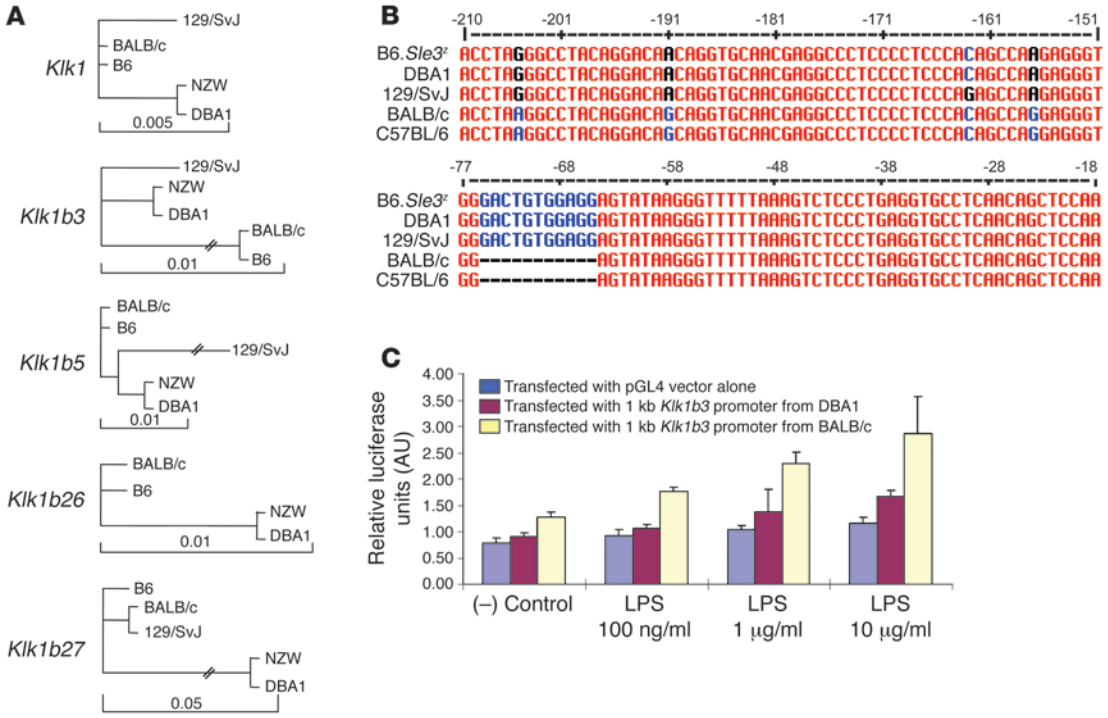


**Figure 5**

Sequence analysis of the 5′-regulatory regions of *Klk* genes reveals nucleotide polymorphisms that distinguish the AIGN-sensitive strains from the control strains. When 2 kb of the 5′-regulatory regions of *Klk1*, *Klk1b3*, *Klk1b5*, *Klk1b26*, and *Klk1b27* from the indicated strains were sequenced, several SNPs/deletions were identified, as detailed in Table 2 (GenBank accession numbers EU597301–EU597324). (**A**) Phylogenetic analysis revealed the sequence of the AIGN-sensitive strains to be closely related, compared with the sequences of the 2 control strains. Bars represent the fraction of sequence variation. (**B**) Part of the nucleotide sequence of the *Klk1b3* promoter (up to 200 bp upstream of transcription start site) from the different study strains indicated. Note that the B6.*Sle3*$^{z}$ strain bears the NZW allele at *Klk*. (**C**) One kilobase of the promoter region of *Klk1b3* from both BALB/c and DBA/1 strains was inserted into the pGL4 luciferase vector and transfected into COS-7 cells, and luciferase activity was assayed 24 hours later, as detailed in Methods. Each bar represents the median of triplicate values, and the error bars denote SD. Cells transfected with vectors carrying the BALB/c-derived *Klk1b3* promoter showed significantly increased luciferase activity compared with cells with vectors bearing the DBA/1 promoter or vector alone ($P < 0.01$). Similar differences were noted when the B6 *Klk1b3* promoter was compared with the B6.*Sle3*$^{z}$ *Klk1b3* promoter (data not shown).

most differentially expressed in disease-prone kidneys (as listed in Table 1) are most homologous to human *KLK1* and *KLK3*, raising the interesting possibility that regulatory polymorphisms shared by the two species could be at play in dictating reduced kallikrein production, which could potentially lead to enhanced nephritis in mice and patients with lupus.

In follow-up studies, it would be important to study SLE patients with clearly defined and graded nephritis (as determined using uniformly applied diagnostic criteria across different patient sets originating from different collaborative groups) to further validate the association to *KLK1/KLK3*, to elucidate any corresponding promoter polymorphisms, and to define the functional consequences of any such polymorphisms. Interestingly, familial clustering of reduced renal Klk expression as well as African American hypertensive patients with reduced renal Klk have been reported (44, 45). Given that African American ethnicity and coexisting hypertension are both risk factors for severe lupus nephritis (46, 47), it would be important to establish the potential role of *KLK* gene polymorphisms in driving disease severity in human SLE. Along these lines, there is some evidence that promoter polymorphisms in human *KLK1* may be associated with reduced renal and urinary Klk, as well as with hypertension and nephritis (48, 49).

Whereas some lupus susceptibility genes may impact the adaptive arm of the immune system, others appear to augment innate immunity (50–52). The present study uncovers yet another class of lupus susceptibility genes — those that may potentially modulate end-organ disease. Besides their central relevance to the genetics of lupus nephritis, these findings have additional clinical implications. Monitoring urinary kallikreins as a marker of renal disease in lupus and developing kallikrein-based therapeutics for modulating renal lupus may be future directions worthy of pursuit.

## Methods

*Mice and AIGN.* BALB/c, DBA/1, 129/SvJ, B6, and NZW mice were purchased from The Jackson Laboratory. B6.*Sle3*$^{z}$ congenic mice, described previously (17, 18), were bred in-house. Recursive backcrossing of B6.*Sle3*$^{z}$ mice to B6 parents and microsatellite-assisted selection were used to generate B6.*Sle3*$^{z}_{157–158}$ recombinants harboring the NZW-derived *Klk* gene complex within a 4-Mb interval, with termini at *D7mit157* and *D7mit158*. All mice were maintained in a specific pathogen–free colony. Two- to 3-month-old females were used for all studies. To induce nephritis, we first sensitized mice on day 0 with rabbit IgG (250 μg/mouse, i.p.), in adjuvant, as described previously (10, 11). On day 5, the mice were challenged i.v. with rabbit anti-GBM Ig (200 μg per 25 g body weight, in a 300-μl volume).

**Table 3**
Association analysis of KLK SNPs in German SLE patients and controls

| *KLK* gene | SNP | Allele | Allele frequencies[A] Cases | Reference group | $\chi^2$ | *P*[B] |
|---|---|---|---|---|---|---|
| **SLE/total[C] vs. healthy controls** | | | | | | |
| *KLK1* | *rs5517* | C | 0.325 | 0.321 | 0.025 | 0.874 |
| *KLK1* | *rs1054713* | G | 0.694 | 0.64 | 3.833 | **0.050** |
| *KLK1* | *rs2740502* | C | 0.385 | 0.346 | 1.94 | 0.164 |
| *KLK5* | *rs1897604*[D] | C | 0.33 | 0.275 | 4.441 | **0.033** |
| *KLK5* | *rs268908* | A | 0.873 | 0.854 | 0.981 | 0.322 |
| *KLK5* | *rs2569522* | T | 0.63 | 0.603 | 0.965 | 0.326 |
| *KLK6* | *rs1654537* | G | 0.345 | 0.34 | 0.033 | 0.855 |
| *KLK7* | *rs1701924*[D] | C | 0.346 | 0.345 | 0.001 | 0.977 |
| **SLE/nephritis vs. SLE/no nephritis** | | | | | | |
| *KLK1* | *rs5517* | T | 0.716 | 0.654 | 1.006 | 0.316 |
| *KLK1* | *rs1054713* | G | 0.757 | 0.658 | 2.503 | 0.114 |
| *KLK1* | *rs2740502* | C | 0.5 | 0.326 | 7.271 | **0.007** |
| *KLK5* | *rs1897604*[D] | T | 0.706 | 0.672 | 0.292 | 0.589 |
| *KLK5* | *rs268908* | A | 0.914 | 0.871 | 0.974 | 0.324 |
| *KLK5* | *rs2569522* | T | 0.662 | 0.61 | 0.672 | 0.412 |
| *KLK6* | *rs1654537* | A | 0.712 | 0.65 | 0.909 | 0.341 |
| *KLK7* | *rs1701924*[D] | C | 0.348 | 0.346 | 0.002 | 0.968 |
| **SLE/nephritis vs. healthy controls** | | | | | | |
| *KLK1* | *rs5517* | T | 0.716 | 0.679 | 0.425 | 0.514 |
| *KLK1* | *rs1054713* | G | 0.757 | 0.64 | 3.822 | **0.051** |
| *KLK1* | *rs2740502* | C | 0.5 | 0.346 | 6.563 | **0.010** |
| *KLK5* | *rs1897604* | C | 0.294 | 0.275 | 0.117 | 0.732 |
| *KLK5* | *rs268908* | A | 0.914 | 0.854 | 1.941 | 0.164 |
| *KLK5* | *rs2569522* | T | 0.662 | 0.603 | 0.993 | 0.319 |
| *KLK6* | *rs1654537* | A | 0.712 | 0.66 | 0.743 | 0.389 |
| *KLK7* | *rs1701924* | C | 0.348 | 0.345 | 0.003 | 0.957 |

[A]The allele frequencies were determined by SNP-typing an average of 722 normal control alleles, 508 SLE patient alleles, 260 non-nephritic SLE patient alleles, and 70 nephritic SLE patient alleles. [B]Significant associations are indicated in bold. [C]Total includes patients with and without nephritis. [D]These SNPs were not in Hardy-Weinberg equilibrium in controls.

The source and preparation of the anti-GBM serum/Ig have been detailed previously (10, 11). Twenty-four-hour urine samples were collected from experimental mice on days 0, 10, and 14, using metabolic cages, with free access to drinking water. Urinary protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Thermo Scientific). Serum was collected on days 0, 10, and 14 for measurement of blood urea nitrogen (BUN), using a urea nitrogen kit (Sigma-Aldrich). Animals were sacrificed on day 10 or 14, and the kidneys were processed for histopathological examination by light microscopy, as detailed previously (10, 11).

*Microarray and real-time PCR analysis*. Kidneys collected on day 10 of experimental nephritis were used for gene expression analysis by microarray. Total RNA was isolated from renal cortex using RNeasy (QIAGEN) and quality-checked using an Agilent Bioanalyzer (Agilent Technologies). Sentrix Mouse-6 Whole Genome Expression BeadChips (Sentrix Mouse-6 v1_1; Illumina) were used for the microarray analysis, according to the manufacturer's instructions. Microarray data were extracted using BeadStudio v3.1, background-subtracted, and normalized using a cubic spline algorithm. Genes differentially expressed between groups were identified using the Illumina custom error model implemented in BeadStudio. Genes were considered significantly differentially expressed when *P* values were less than 0.001 and the change was greater than 2-fold. Supervised hierarchical clustering of transcripts that were differentially expressed between the groups was performed. Data were median centered; in Figure 1A, green represents expression below median; red represents above-median expression; and gray represents missing data.

Renal cortex *Klk* gene expression differences were validated by quantitative RT-PCR using validated TaqMan assays (Applied Biosystems) and Mm00834006_g1 for *klk1*, Mm01203825_gH for *klk1b3*, Mm00833453_g1 for *klk1b5*, Mm01702809_m1 for *klk1b26*, and Mm00834759_gH for *klk1b27*. Transcription of eukaryotic 18S rRNA (assay ID Hs99999901_s1) was used as an internal control.

*Generation of rabbit anti-mouse Klk1 Ab*. Rabbit Abs against mouse Klk1 were developed by Abgent, using a peptide sequence from mouse Klk1, EKNSQPWQVAVYRFTKYQC, conjugated to keyhole limpet hemocyanin (KLH). The peptide was used to immunize rabbits (5 mg per rabbit, 3 injections administered 21 days apart). Sera obtained from the rabbits 2 months following the primary immunization reacted strongly with recombinant mouse Klk1 protein (titer, >1:4,000). Immune rabbit sera were harvested 2 weeks after the third injection, protein G purified, aliquoted, and stored at –80°C.

*Assaying Klk protein*. BALB/c, DBA1, and 129/SvJ mice were subjected to AIGN, as described above. Mice (3 per group) were sacrificed on day 0 or 14. Renal cortex was homogenized in lysis buffer (25 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 2 mM EDTA) containing 1:100 protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 4°C for 30 minutes. The supernatants were stored at –80°C. Protein concentrations were determined by Lowry's method. These homogenates were subjected to Western blot analysis using the custom-generated rabbit anti–mouse Klk1 Ab (1:1,000; described above) and chemiluminescence detection (Pierce, Thermo Scientific). Urinary kallikrein enzymatic activity was assayed using the synthetic chromogenic substrate HD-Val-Leu-Arg-pNA (S-2266). Briefly, 50 μl of urine was added to 50 μl of assay buffer (0.2 M Tris-HCl, pH 8.2, containing 300 μg/l soybean trypsin inhibitor and 375 μg/l EDTA) and incubated at 37°C for 30 minutes. Then, 50 μl of S-2266 was added and incubated at 37°C for 3 hours, and the absorbance was read at 405 nm. A standard curve was constructed using purified kallikrein (human urinary kallikrein [HUK]; Calbiochem), and the optical densities were converted to activity units.

*In vivo studies using BK agonists and antagonists*. Two- to 3-month-old female BALB/c mice were divided into 3 groups (10 mice per group), and subjected to anti-GBM Ab challenge. The mice in the first 2 groups were infused with either kinin B2 receptor antagonist H157 (HOE140 or icatibant) or B1 receptor antagonist H158, using subcutaneous osmotic minipumps (DURECT; dosage, 0.74 μg/h) from day 3 to day 14. The third group was infused with PBS vehicle as control. Urine and sera were collected on days 0 and 14 for proteinuria and BUN assessment. All mice were sacrificed on day 14, and kidneys were scored for pathology. For the in vivo agonist studies, 129/SvJ mice were subjected to anti-GBM disease, as described above. One group received BK (Sigma-Aldrich; 0.5 μg/h released by osmotic minipumps, from day 3 to day 14), while the other group received PBS. All mice were phenotyped for disease as detailed above.

*Amplification and sequencing of the 5′-flanking region of the Klk genes*. Two-kilobase fragments of the 5′-flanking region of various *Klk* genes were sequenced after amplifying with the following primers: *Klk1*-F: 5′-AATCACCTC-TACCACCCAGTAC-3′, *Klk1*-R: 5′-TTGGAGCTGTAGAGGCTCCTG-3′,

**Table 4**
Genotypic and allelic association of *KLK1* rs5516 in 6 additional sets of SLE patients with nephritis (cases) and healthy controls

| Population | | CC | CG | GG | *P* | OR (CI) | Allele G | Allele C | *P* | OR (CI) |
|---|---|---|---|---|---|---|---|---|---|---|
| Germany | Cases (40) | 7.50% | 27.50% | 65.00% | 0.010 | CG: 0.97 (0.26–3.61) | 78.80% | 21.20% | **0.008** | 2.10 (1.18–3.92) |
| | Controls (352) | 12.50% | 47.40% | 40.10% | | GG: 2.70 (0.78–9.37) | 63.80% | 36.20% | | |
| European-American | Cases (579) | 11.74% | 41.97% | 46.29% | 0.446 | CG: 0.84 (0.63–1.13) | 67.27% | 32.73% | 0.991 | 1.00 (0.87–1.14) |
| | Controls (3089) | 10.49% | 44.51% | 45.00% | | GG: 0.92 (0.69–1.23) | 67.25% | 32.75% | | |
| Argentina | Cases (136) | 4.40% | 41.20% | 54.40% | 0.040 | CG: 2.68 (1.08–6.63) | 75.0% | 25.0% | **0.028** | 1.42 (1.03–1.98) |
| | Controls (367) | 11.70% | 40.90% | 47.40% | | GG: 3.05 (1.24–7.47) | 67.8% | 32.2% | | |
| Spain | Cases (125) | 16.80% | 47.20% | 36.00% | 0.180 | CG: 0.67 (0.38–1.19) | 59.6% | 40.4% | 0.089 | 0.78 (0.59–1.05) |
| | Controls (569) | 11.20% | 46.90% | 41.80% | | GG: 0.58 (0.32–1.04) | 65.3% | 34.7% | | |
| Italy | Cases (92) | 10.90% | 57.70% | 30.40% | 0.149 | CG: 1.27 (0.57–2.85) | 59.8% | 40.2% | 0.194 | 0.79 (0.55–1.15) |
| | Controls (216) | 11.10% | 47.20% | 41.70% | | GG: 0.75 (0.32–1.75) | 65.3% | 34.7% | | |
| Korea[A] | Cases (296) | 4.40% | 35.10% | 60.50% | 0.140 | CG: 1.79 (0.94–3.39) | 78.00% | 22.00% | 0.122 | 1.19 (0.95–1.51) |
| | Controls (785) | 7.8% | 34.8% | 57.5% | | GG: 1.86 (1.00–3.47) | 74.80% | 25.20% | | |

[A]Hardy-Weinberg equilibrium, $P = 0.037$. Significant associations are indicated in bold.

*Klk1b26*-F: 5′-CAGAGAGAATCATAAAAGAGTGC-3′, *Klk1b26*-R: 5′-AATACCCTCATTCTCTCCATAGT-3′, *Klk1b3*-F: 5′-AGGTTTACCATGAAAGAGTTAGG-3′, *Klk1b3*-R: 5′-TTGGAGCTGTTGAGGCACCTC-3′, *Klk1b5*-F: 5′-GTCACCTCTAGCACCCAATGG-3′, *Klk1b5*-R: 5′-CTGTCCAGGAGCTGCAGGCT-3′, *Klk1b27*-F: 5′-AGAAAGACTCCTGGAAGAGTGG-3′, and *Klk1b27*-R: 5′-GTGAACTTGGAGCTGTTGAGGA-3′. The DNA sequences of these PCR fragments were analyzed on an ABI 3100 sequence analyzer (Applied Biosystems) and deposited into GenBank (accession numbers EU597301–EU597324). Based on the sequence information, phylogenetic trees were constructed using publicly available software (Protein Information Resource, http://pir.georgetown.edu/pirwww/search/multialn.shtml).

*Promoter function studies*. One kilobase of the promoter sequence upstream of *Klk1b3* was PCR amplified from B6, BALB/c, DBA/1, and NZW/B6.*Sle3*$^{z}$ mice using the primers 5′-CCGGTACCGCCACCAAGCTTAACCCTGA-3′ and 5′-CCCTCGAGGCTTGGAGCTGTTGAGGCAC-3′, cloned separately into pGL4.10[luc2] luciferase reporter vector (Promega), and sequence verified. COS-7 cells ($2 \times 10^5$ to $5 \times 10^5$ cells in 96-well plates) were transfected with 0.1 μg of pGL4.10[luc2]-promoter luciferase construct or vector using PolyFect (QIAGEN), together with 0.01 μg of an internal control plasmid, pGL4.73[hRluc/SV40] (Promega). Cells were lysed after 48 hours of LPS stimulation, using 1× passive lysis buffer (Promega) and assayed for luciferase activity, according to the manufacturer's instructions. Each construct was measured in at least 8 replicates. The results were normalized against *Renilla* luciferase control and presented as median values.

*Human KLK genetics*. Several independent sets of cases and controls were used in this study, originating from Germany, Italy, Spain, Argentina, the United States, Mexico, and Korea (53). The European sets and the Latin American sets have been previously described (54, 55). All patients fulfilled American College of Rheumatology criteria for the classification of SLE (56). All human studies were approved by the Central Ethical Review Board, Sweden, and the local ethical review boards of Instituto de Biomedicina y Parasitología López Neyra; Sanatorio Parque; Hospital Carlos Haya; Hospital for Rheumatic Diseases, South Korea; University of Hannover; University of Eastern Piedmont; U.O.C. di Reumatologia Azienda Ospedaliera San Camillo-Forlanini; Oklahoma Medical Research Foundation; University of Alabama at Birmingham; Feinstein Institute for Medical Research; Medical University of South Carolina; and Hammersmith Hospital. For the initial analysis, SNPs on human renal-expressed *KLK* genes *KLK1*, *KLK5*, *KLK6,* and *KLK7* were selected from the HapMap-CEU genotype data (http://www.hapmap.org) using Haploview version 3.2 (Broad Institute of MIT and Harvard), and were genotyped using protocols described earlier (55). Differences between patients and controls

**Table 5**
Genotypic and allelic association of *KLK1* rs2740502 in 6 additional sets of SLE patients with nephritis (cases) and healthy controls

| | | CC | CG | GG | *P* | OR (CI) | Allele C | Allele G | *P* | OR (CI) |
|---|---|---|---|---|---|---|---|---|---|---|
| Germany | Cases (35) | 25.70% | 48.60% | 25.70% | 0.036 | CG: 1.831 (0.79–4.23) | 50.0% | 50.0% | **0.010** | 1.89 (1.12–3.19) |
| | Controls (364) | 12.40% | 44.50% | 43.10% | | CC: 3.49 (1.31–9.31) | 34.6% | 65.4% | | |
| European-American | Cases (579) | 17.27% | 48.53% | 34.20% | 0.277 | CG: 1.09 (0.90–1.33) | 41.50% | 58.50% | 0.113 | 1.11 (0.97–1.26) |
| | Controls (3089) | 15.09% | 47.94% | 36.97% | | CC: 1.24 (0.95–1.61) | 39.10% | 60.90% | | |
| Argentina | Cases (134) | 19.40% | 41.00% | 39.60% | 0.057 | CG: 0.85 (0.55–1.31) | 39.9% | 60.1% | 0.242 | 1.19 (0.88–1.60) |
| | Controls (365) | 11.50% | 48.80% | 39.70% | | CC: 1.69 (0.95–3.03) | 35.9% | 64.1% | | |
| Spain | Cases (119) | 14.30% | 48.70% | 37.00% | 0.915 | CG: 0.96 (0.63–1.49) | 38.7% | 61.3% | 0.693 | 0.94 (0.70–1.27) |
| | Controls (567) | 15.70% | 48.70% | 35.60% | | CC: 0.88 (0.48–1.62) | 40.0% | 60.0% | | |
| Italy | Cases (93) | 12.90% | 47.30% | 39.80% | 0.023 | CG: 0.59 (0.35–0.99) | 36.6% | 63.4% | **0.008** | 0.63 (0.44–0.90) |
| | Controls (259) | 21.60% | 52.50% | 25.90% | | CC: 0.39 (0.18–0.81) | 47.9% | 52.1% | | |
| Korea[A] | Cases (296) | 5.10% | 35.80% | 59.10% | 0.223 | CG: 1.21 (0.91–1.61) | 23.00% | 77.00% | 0.833 | 1.02 (0.81–1.29) |
| | Controls (785) | 7.00% | 31.10% | 61.90% | | CC: 0.76 (0.42–1.38) | 22.50% | 77.50% | | |

[A]Hardy-Weinberg equilibrium, $P = 0.003$. Significant associations are indicated in bold.

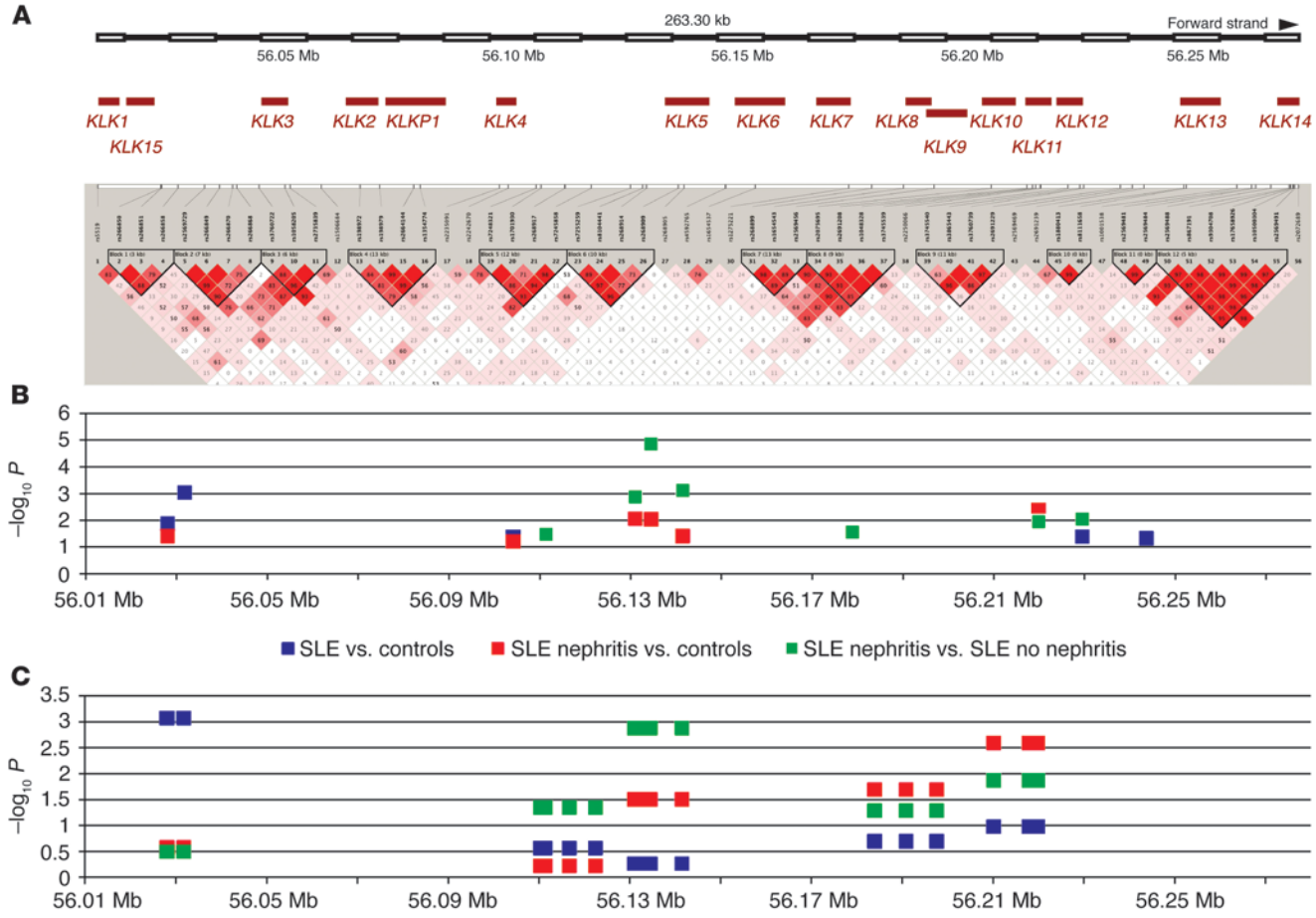


**Figure 6**
KLK association in SLE patients — second validation study. (**A**) The Haploview plot shows the genotyped markers in the KLK locus, from *KLK1* at the centromeric limit until *KLK14* at the telomeric end, as well as the linkage disequilibrium between them measured by the D prime coefficient. Blocks were defined by the solid spine method implemented in Haploview version 4.1. Dataset: UCSF ($n$ = 595 SLE patients) plus SLEGEN ($n$ = 689 SLE patients and $n$ = 3,718 controls). (**B**) In the indicated numbers of SLE patients and healthy controls, 56 KLK SNPs were tested for disease association using logistic regression analysis, with the phenotype "SLE" as the outcome variable (shown in blue). To examine whether the risks conferred by the KLK polymorphisms were influenced by the presence of nephritis, we tested the KLK SNPs for association, considering the phenotype "nephritis" as the outcome variable. Red indicates significant differences compared with controls; green indicates significant differences between cases with nephritis and cases without nephritis calculated by a metaanalysis, in order to control for heterogeneity among the contributing clinical centers. (**C**) The observed linkage disequilibrium blocks across the *KLK* locus were tested for haplotype association, using both omnibus and haplotype-specific association statistics (T test) as implemented in PLINK. Shown are significant haplotype associations when SLE patients were compared with controls (blue), when patients with lupus nephritis were compared with controls (red), and the case-only analysis (green). Besides the SNPs indicated in **B** and the blocks indicated in **C**, none of the other SNPs/blocks shown in **A** showed significant disease associations. For a larger version of this figure, see Supplemental Figure 1.

were statistically analyzed using $\chi^2$ test. Odds ratio (OR) with 95% CI was calculated using StatsDirect software for allelic differences and using UNPHASED software (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) for genotypic differences.

*Validation of KLK associations using SLEGEN and UCSF datasets*. We extracted the genotype data for the human KLK locus (Chr19:56014125-56277428) from 2 different genome-wide association studies (GWASs) conducted recently in SLE patients of European ancestry (57, 58). The patients and controls included in those studies, as well as their contributing centers and the genotyping platforms used, have been described in detail in the original publications. All patients fulfilled the American College of Rheumatology's revised criteria for classification of SLE (56) and were matched to healthy controls by sex, age, and ethnic origin (i.e., only individuals of European ancestry were included). From the GWAS performed by the SLEGEN, we obtained data for 63 *KLK* SNPs genotyped in 689 cases and 3,718 controls. From the UCSF Lupus Genetics Project, we added 595 patients who had genotype data for 113 *KLK* SNPs, of which 56 were also present in the SLEGEN dataset. Hence, in total, we had access to genotype data for 56 *KLK* SNPs pertaining to 1,284 (689 SLEGEN plus 595 UCSF) SLE patients and 3,718 controls, drawn from the 2 GWAS studies.

*Statistics*. For the murine studies, Student's $t$ test (1-tailed) was used for statistical analysis, unless otherwise indicated. Error bars shown in figures represent standard deviations. For the human genetic studies, statistical analysis was performed using Haploview version 4.1 (59) and PLINK version 1.04 (60). We first verified that all the SNPs/alleles were concordant in terms of positive or negative strand. Then, quality control filters were applied to

remove individuals and SNPs with more than 5% of data missing, differential missing rate between cases and controls ($P < 0.05$), significant deviation from Hardy-Weinberg equilibrium in controls ($P < 0.001$), or a minor allele frequency of less than 1%. After this filtering step, we had 56 SNPs in 1,241 cases and 3,664 controls, with a total genotyping rate of 0.996054. Clinical data regarding the presence or absence of nephritis was available for 1,122 patients, of whom 319 had nephritis (28.4%).

In this filtered sample, we tested the allelic frequencies for significant association by logistic regression analysis using the phenotype "SLE" as the outcome variable. To examine whether the risk conferred by the KLK polymorphism on SLE was influenced by the presence of nephritis, we also performed the analysis considering the phenotype "nephritis" as the outcome variable. OR and 95% CI limits (L95, lower limit; U95, upper limit) were calculated controlling by the covariate "sex." We also performed a case-only $\chi^2$ test to determine whether there were significant differences in allelic frequencies between the subsets of patients with and without nephritis. Since the patient samples had originated from different centers, combined ORs were estimated by Cochran-Mantel-Haenszel metaanalysis, including a Breslow-Day test for homogeneity of the OR between centers. Multiple testing was corrected by adjusting the *P* values using the false discovery rate (FDR) control.

Finally, we examined the linkage disequilibrium (LD) structure of the region in Haploview, and the observed blocks were tested for haplotype association. The data were analyzed using the omnibus test and haplotype-specific association statistics (T test) as implemented in PLINK. The case/control omnibus test is an H – 1 degree of freedom test, where H is the number of different haplotypes. We compared the haplotype frequencies in patients with SLE versus controls, patients with lupus nephritis versus controls, and patients with lupus nephritis versus patients without nephritis.

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