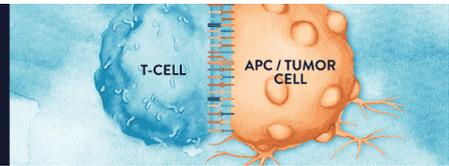


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Cutting Edge: Multiple Autoimmune Pathways in *kd/kd* Mice¹

Wayne W. Hancock,* Tsai-Lung Tsai,[†] Michael P. Madaio,[‡] and David L. Gasser^{2†}

The kidney disease (kd) mutation was transferred to a C57BL/6 (B6) background by selection for closely linked microsatellite markers. The resulting congenic strain, B6.kd, was mated with partners homozygous for targeted mutations of CD4, CD8, CD28, IL-2, recombina-activating gene-1 (Rag-1), ICAM-1, or β_2 -microglobulin. In most of the resulting double mutants, kidney disease occurred as readily and as severely as in the B6.kd controls, although disease occurred somewhat less frequently in age-matched CD28^{-/-} kd/kd mice. Immunohistology demonstrated a predominance of macrophages in the lesions of B6.kd and most of the double mutants, with the remaining cells consisting of T cells and variable numbers of NK cells. In Rag-1^{-/-} kd/kd, ~50% of infiltrating cells were macrophages, and ~50% were NK cells. These results suggest that the initial lesion caused by the mutant gene is intrinsic to the kidney and that the immune response that subsequently occurs can involve any one of several different cellular compositions. The Journal of Immunology, 2003, 171: 2778–2781.

The kidney disease (*kd*)³ mutation that occurred spontaneously in a colony of CBA/CaH mice (1) provides an interesting model of autoimmune disease. Mice homozygous for this mutation are apparently healthy for at least the first 10 weeks of life but then develop polyuria and polydipsia. Proteinuria can be demonstrated by urinalysis, and death from renal failure usually occurs by 8 mo. Histological examination at early time points reveals a mononuclear cell infiltration and tubular dilatation in cortical areas, with further dilatation progressing with time over larger areas (2, 3). This disease was initially thought to resemble nephronophthisis, which is also known as medullary cystic disease, and in which the fundamental defect appears to be production of an abnormal tubular basement membrane (TBM) (1, 4).

Many cases of nephronophthisis in humans are inherited, and genes have been identified on chromosomes 2q13 (5), 9q22–31 (6), 3q22 (7) and 1p36 (8) that cause forms of this

disease that are inherited recessively. Two loci associated with autosomal dominant forms of nephronophthisis were identified on chromosomes 1q21 (9) and 16p12 (10). Although nephronophthisis in humans may or may not have an immunological basis, the evidence for an autoimmune component in *kd/kd* mice is strong. After transferring bone marrow cells from CBA/CaH-*kd* donors to lethally irradiated, thymectomized CBA/CaH recipients, Neilson et al. (2) observed disease in the recipients that was nearly as extensive as that of *kd/kd* controls. By injecting spleen and lymph node cells i.v. into recipient mice and challenging the recipients in one footpad with antigenic extracts, these investigators showed that the Ag-reactive cells in this disease are H2K restricted, CD8 positive, and directed against a TBM Ag (11). Adoptive transfer of the latter cells under the kidney capsules of cyclophosphamide-pretreated CBA/CaH mice also induced this renal pathology (11), although the initial cellular events leading to a breakdown in immunologic tolerance were not determined.

During experiments on the positional cloning of the *kd* gene, we identified mice with recombinant haplotypes in which crossover events had occurred very near the gene for this disease (12). This enabled us to transfer the *kd* gene to the C57BL/6 (B6) background by selecting for microsatellite markers that are closely linked to the disease gene. To our knowledge, the CBA/CaH-*kd* strain no longer exists, so the B6.kd strain described here may be the only living carrier of this mutant gene. The evidence that B6.kd carries the same mutant gene as CBA/CaH-*kd* is quite strong. Genetic mapping experiments conducted by crossing the CBA/CaH-*kd* strain with an unrelated strain, CAST, found no recombination between *kd* and the microsatellite markers D10Mit-225, -184, -108, -54, and -55 of 539 backcross and 435 F₂ progeny (12). In the recombinant haplotype, this block of CBA/CaH-*kd*-derived genes is associated with adjacent CAST-derived microsatellite alleles, thus enabling the transfer to the B6 background. The derivation of the B6.kd strain has also made it possible to develop doubly mutant lines in which both the *kd/kd* genotype and a knockout genotype of interest have been placed on the B6 background so as to assess the contributions of specific cell types to pathogenesis.

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³ Abbreviations used in this paper: kd, kidney disease gene; TBM, tubular basement membrane; BUN, blood urea nitrogen; Rag, recombina-activating gene.

Materials and Methods

Mice

The mouse designated No. 1137 in a previous report had a recombinant haplotype, with a mutant *kd* allele obtained from a CBA/CaH-*kd* grandparent and a series of closely linked microsatellite alleles from a CAST grandparent (12). This mouse was mated with a C57BL/6J (B6) partner and backcrossed to the B6 strain for 10 generations by selecting for the recombinant chromosome allele of the CAST donor. Mice homozygous for the recombinant chromosome are designated B6.*kd*. Mice with targeted mutations for CD4 (B6.129S6-*Cd4^{tm1Knu}*), CD8 (B6.129S2-*Cd8a^{tm1Mak}*), CD28 (B6.129S2-*Cd28^{tm1Mak}*), IL-2 (B6.129P2-*IL2^{tm1Hor}*), ICAM-1 (B6.129S7-*Icam1^{tm1Bay}*), recombinase-activating gene-1 (*Rag-1*; B6.129S-*Rag1^{tm1Mom}*), and β_2 -microglobulin (B6.129P2-*B2m^{tm1Unc}*) were obtained from The Jackson Laboratory (Bar Harbor, ME). Doubly mutant mice were obtained by mating B6.*kd* mice with the appropriate knockout partners, mating the F₁ progeny with one another, and selecting F₂ individuals of the desired doubly mutant genotypes. In all cases, the B6.*kd* mice that had reached at least the N4 generation before these matings were initiated. In all of these combinations, the targeted mutant had the B6 background.

Evaluation of nephritis

Kidneys were bisected, fixed in formalin, and paraffin-embedded; 4- μ m sections through the longitudinal axis of each kidney were stained with H&E. The severity of disease in the glomerular, interstitial, and vascular compartments was graded on a 0–4+ scale involving scoring of biopsy features by one observer (M. Madaio), who was blinded to the origin of the specimens, according to previously described methods (13). Because mild symptoms are occasionally seen in nonmutant controls, mice were considered positive for nephritis only if the score was at least 2+; this excludes mice in which there were small pockets of 5–10 mononuclear cells. Blood urea nitrogen levels were determined in sera from individual mice using a commercial kit (Sigma-Aldrich, St. Louis, MO).

Immunohistology

Cryostat sections of snap-frozen kidneys (≥ 5 /group) were analyzed in a blinded manner. Sections were stained by immunoperoxidase using mAbs (BD Pharmingen, San Diego, CA) directed against all leukocytes (CD45), T cells (TCR- $\alpha\beta$), and subsets (CD4, CD8), macrophages (F4/80, CD11b), NK cells (DX5), B cells (CD19, IgM), neutrophils (Gr1), activated caspase-3, and 2 markers of immune activation, IL-2R (CD25) and inducible costimulatory molecule (ICOS), as described (14).

Results and Discussion

Similar phenotype of B6.*kd* and CBA/CaH-*kd* mice

Because all previous studies with the *kd* mutation used the CBA/CaH-*kd* strain, it was important to establish the phenotype of B6.*kd* mice. As shown in Table I, mice with the *kd/kd* genotype on the B6 background were evaluated at various ages. None of these congenic mice demonstrated a positive phenotype before 75 days of age, and nearly all were positive by 120 days of age. An example of a positive phenotype is shown in Fig. 1*a*. The BUN levels in B6 control mice were below 50 mg/dl, and B6.*kd* mice in the early stages of disease had similar values.

Table I. Disease phenotypes in mutant and control strains

Genotype	No. Positive/Total Dissected		
	Days 75–99	Days 100–120	Day >120
B6	ND ^a	0/3	0/11
B6. <i>kd</i> ^b	1/11	13/19	34/37
<i>CD4^{-/-}kd/kd</i>	1/4	1/4	22/26
<i>CD8^{-/-}kd/kd</i>	ND	8/10	13/17
<i>CD28^{-/-}kd/kd</i>	ND	3/19	41/62
<i>IL-2^{-/-}kd/kd</i>	4/9	2/2	ND
<i>Rag-1^{-/-}kd/kd</i>	4/8	7/7	21/27
<i>ICAM-1^{-/-}kd/kd</i>	0/2	1/2	23/23
<i>$\beta_2m^{-/-}$kd/kd</i>	0/2	2/2	8/15

^a ND, Not done.

^b The B6.*kd* mice in this table were from at least the eighth generation of backcrossing.

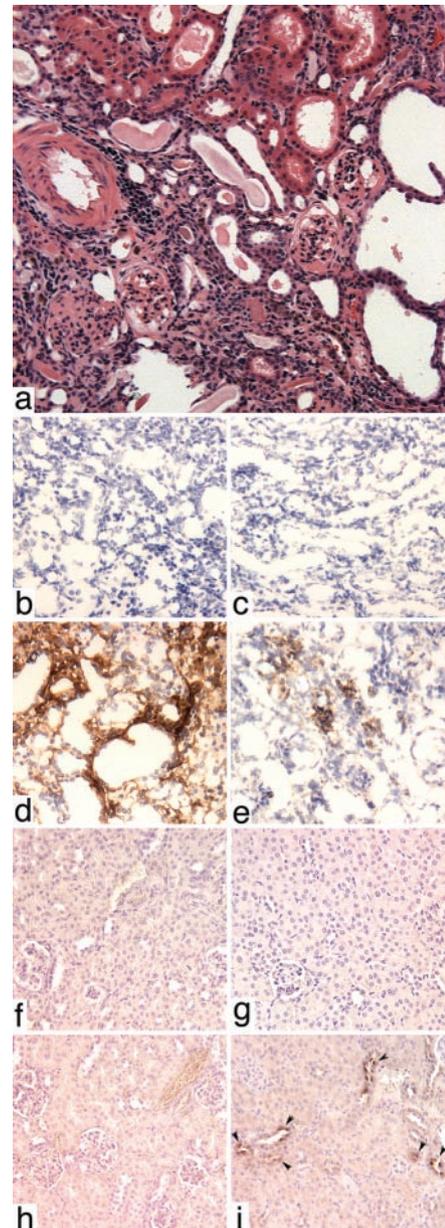


FIGURE 1. Histological and immunopathologic features of renal disease in B6.*kd* mice. *a*, Severe interstitial inflammation and cystic dilatation with proliferative glomerulonephritis in a B6.*kd* mouse at 184 days of age, near end-stage renal disease (H&E-stained paraffin section). *b–e*, Immunohistological analysis of the cellular infiltrates in the kidneys of a *RAG-1^{-/-} kd/kd* mouse at 150 days of age, showing lack of staining with an isotype control mAb (*b*), absence of TCR+ T cells (*c*), but marked infiltration by CD11b⁺ macrophages (*d*) and DX5⁺ NK cells (*e*) (cryostat sections, hematoxylin counterstain). *f–i*, Evaluation of the onset of tubular injury and apoptosis using a mAb to activated caspase-3, showing lacking of staining of a kidney from a 73-day-old control B6 mouse using control mAb (*f*) or anti-activated caspase-3 mAb (*g*), whereas a kidney from a 78-day-old B6.*kd* mouse shows lack of staining with a control mAb (*h*), but focal tubular labeling (*i*; arrowheads) for activated caspase-3. Immunohistological data are in each case representative of five kidneys examined from each group at the specified time point; all panels are $\times 250$ original magnification.

After 30 wk of age, these levels frequently exceeded 120 mg/dl (data not shown). Because the CBA/CaH-*kd* strain is no longer available, a precise comparison could not be made between these two strains. However, a comparison of our results with

those in previous reports (1–3) suggests that there are no significant differences between the two strains in their disease phenotype.

Infiltrating leukocytes in B6.kd kidneys are mainly macrophages and CD4⁺ T cells

In agreement with Sibilac et al. (3) who studied CBA/CAH-*kd* mice, we found macrophages to be the most abundant cell type in the infiltrate. Most of the infiltrating T cells were CD4⁺ and included markers of immune activation (IL-2R and ICOS expression).

Lack of requirement of CD4, CD8, β_2 -microglobulin, IL-2, ICAM-1, or Rag-1 for disease

As shown in Table I, all of the groups of mice in which the *kd/kd* genotype was present along with one of the targeted mutations developed kidney disease. The histological appearance in each group was indistinguishable from that observed in B6.*kd* mice, mice at more advanced ages developing extensive infiltration and tubular dilatation, as well as a highly elevated BUN (data not shown). None of the *IL-2^{-/-} kd/kd* mice survived beyond 120 days; therefore, comparisons can be made only in the two younger age ranges. The results in Table II demonstrate that the *CD4^{-/-} kd/kd* double mutants lacked CD4⁺ T cells as expected, the *CD8^{-/-} kd/kd* mice lacked CD8⁺ T cells as expected, and the *Rag-1^{-/-} kd/kd* mice lacked both CD4⁺ and CD8⁺ T cells as expected (15).

Absence of CD28 delays but does not prevent the occurrence of disease

In the two age groups tested, mice with the *CD28^{-/-} kd/kd* genotype were significantly less susceptible to development of renal disease than B6.*kd* mice; these included the 100- to 120-day range ($\chi^2 = 11.09$, $p < 0.005$) and in the age group above 120 days ($\chi^2 = 7.84$, $p < 0.01$). This provides evidence that the immune response is not irrelevant to the progression of this disease and suggests that costimulation by CD28 is involved (16).

Multiple autoimmune pathways, with or without T cells, can be involved in this disease

In the B6.*kd* mice, the most abundant kidney-infiltrating cells were macrophages, with a good representation of CD4⁺ T cells. In the *CD4^{-/-} kd/kd* double mutants, there were no CD4⁺ leukocytes, but there were numerous CD8⁺ cells. Very few NK cells were present in the B6.*kd* mice or most of the double mutants, but ~50% of the infiltrating cells in the *Rag-1^{-/-} kd/kd* double mutants were NK cells (Table II and Fig. 1, *b–e*).

T cell immunity appears to be unnecessary for manifestation of this kidney disease

The finding that *kd/kd* mice deficient in *Rag-1* develop the kidney disease as readily and as severely as B6.*kd* controls was most unexpected, inasmuch as it was expected from previous work that functional T cells would be required for development of disease in this animal model. The observation that this response consists almost entirely of macrophages and NK cells suggests that the trigger for this response may be oxidative stress and the exposure of phosphatidylserine (17, 18). If so, this would have similarities to the trigger for systemic lupus erythematosus (19).

How to reconcile our observations with previous reports remains an unresolved issue. Because multiple autoimmune pathways can be involved in this disease, the lesions observed in bone marrow chimeras by Kelly et al. (11) were most likely caused by a mechanism different from that which is active in *Rag-1^{-/-} kd/kd* double mutants. In the 75- to 99-day age range, a somewhat higher proportion of the *Rag-1^{-/-} kd/kd* double mutants had disease than B6.*kd* controls. This was of borderline significance ($\chi^2 = 3.997$), so larger numbers should be examined. If this difference were confirmed, it would support the conclusion of Neilson et al. (2) that this disease is influenced by regulatory T cells.

The *ICAM-1^{-/-} kd/kd* double mutant was studied, given that it has been reported that anti-ICAM-1 mAb localizes to inflammatory sites in the kidneys of *kd/kd* mice, and causes a significant reduction in leukocyte infiltration (20). However, our results suggest that ICAM-1 is not required for disease progression.

Staining with Ab to activated caspase-3

Because our results suggested that some defect occurred in the kidney before the initiation of an immune response, we looked for evidence of apoptosis in the kidney tubules of young B6.*kd* mice that do not have significant leukocyte infiltration, using an Ab directed against activated caspase-3. As shown in Fig. 1, *f–i*, staining for activated caspase-3 was evident in both proximal and distal tubules of a 78-day-old mouse, which suggests that the onset of apoptosis has occurred in these cells. To a lesser extent, this also occurred in the kidneys of 57-day-old but not 32-day-old mice (not shown).

Table II. Composition of leukocytic infiltrates in mutant mice at ≥ 200 days^a

	B6. <i>kd</i>	CD4 ^{-/-} <i>kd/kd</i>	CD8 ^{-/-} <i>kd/kd</i>	Rag-1 ^{-/-} <i>kd/kd</i>
CD45 ⁺	Diffuse infiltrate with peritubular aggregates 61.6 \pm 8.8 cells/HPF	Diffuse infiltrate with peritubular aggregates 58.2 \pm 7.3 cells/HPF	Diffuse infiltrate with peritubular aggregates 64.5 \pm 9.2 cells/HPF	Diffuse infiltrate with peritubular aggregates 31.2 \pm 4.6 cells/HPF**
T cells	10–20% of infiltrate	10–20% of infiltrate	10–20% of infiltrate	Negative
CD4	10–20% of infiltrate	Negative	10–20% of infiltrate	Negative
CD8	1–5% positive	10–20% of infiltrate	Negative	Negative
IL-2R	1–5% positive	<1% positive	1–5% positive	Negative
M ϕ	>75% leukocytes	>75% leukocytes	>75% leukocytes	~50% leukocytes
NK	~10% leukocytes	~10% leukocytes	~10% leukocytes	~50% leukocytes
ICOS	5–10% of infiltrate	~10–20% of infiltrate	1–5% of infiltrate	Negative

^a Assessed in three to four kidneys/group; HPF, high power field; CD45⁺ cells determined in six consecutive fields/sample (mean \pm SD); **, $p < 0.01$ vs infiltrates in each of the other three groups listed (t test); additional semiquantitative assessment <1%, 1–5%, 5–10%, 10–20%, 20–50%, 50–75%, or >75%.

The kd/kd mouse provides a unique autoimmune disease model

If the mutant phenotype in this model is intrinsic to the kidney, previous results demonstrating that the disease can be transferred from CBA/CaH-*kd* bone marrow donors to lethally irradiated CBA/CaH recipients (2) suggest that activated cells of an unknown class may have been present in the donor inocula. The fact that disease was not transferred in the reciprocal direction may suggest that the molecular defect in the *kd/kd* recipients could be alleviated by a secreted product of the inoculated cells. Although the *kd/kd* genotype is associated with a normal state of health for at least the first 75 days of life, our results suggest that apoptosis is already occurring in renal tubular epithelial cells by 57 days of age. This may lead to externalization of a signal molecule such as phosphatidylserine on the outer leaflet of the plasma membrane of the apoptotic cells, which is recognized by macrophages (17). Cytokines secreted by the macrophages would attract both NK cells and T cells. The T cells may recognize additional determinants, such as a TBM component of ~56 kDa (2, 21). Tubular dilatations could be caused by the release of cytokines. By combining the *kd/kd* genotype with various additional targeted deficiencies on a common B6 background, individual components of this process can be demonstrated more clearly than has previously been the case with CBA/CaH-*kd* mice. What is especially interesting about this model is that there is apparently an anti-self immune response which occurs after phagocytosis of the apoptotic cells. Mechanisms must normally be in place to prevent this occurrence, or inappropriate inflammatory responses would routinely occur whenever apoptotic cells are engulfed. This may include the production of anti-inflammatory cytokines or a failure to express costimulatory molecules (22, 23). The *kd/kd* mouse model may be very useful in gaining information about why these mechanisms sometimes break down.

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