Lack of Association between the *Tlr4* (*Lps^d*/*Lps^d*) Genotype and Increased Susceptibility to *Escherichia coli* Bladder Infections in Female C3H/HeJ Mice

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ABSTRACT **Toll-like receptor 4 is thought to have a primary role in host defense against *Escherichia coli* bladder colonization, based on mouse models of urinary tract infection using C3H/HeJ female mice. This strain carries a point mutation in the *Tlr4* gene, which renders the mice unresponsive to lipopolysaccharide (LPS) and thus limits the bladder inflammatory response and infection resolution. The importance of *Tlr4* as the sole genetic determinant of resistance or susceptibility can be questioned, however, by the observation that C3H/HeOuJ female mice with a functional Tlr4 do not effectively resolve *E. coli* bladder infections. The present study further examined this inconsistency by investigating the association of *Tlr4 Lps^d* and *Lps^n* alleles with bladder infection susceptibility by using genetic crosses of C3H/HeJ mice with *Tlr4* (*Lps^n*/*Lps^n*) or (*Lps^n*/*Lps^d*) mice. Heterozygous offspring of C3H/HeJ (*Lps^d*/*Lps^d*) × BALB/cAnN (*Lps^n*/*Lps^n*) mice successfully resolved bladder infections induced by a uropathogenic *E. coli* strain, while heterozygous mice from a C3H/HeJ (*Lps^d*/*Lps^d*) × C3H/HeOuJ (*Lps^n*/*Lps^n*) cross had severe infections. A backcross of C3H/HeJ (*Lps^d*/*Lps^d*) with (BALB/cAnN × C3H/HeJ)F$_1$ (*Lps^n*/*Lps^d*) produced mice that were either resistant or susceptible to *E. coli* bladder infections and had *Lps^d*/*Lps^d* or *Lps^n*/*Lps^d* *Tlr4* genotypes. The *Lps^d*/*Lps^d* or *Lps^n*/*Lps^d* genotypes were present in individual mice with unresolved bladder infections, and the *Lps^d*/*Lps^d* genotype was found in infection-resistant mice. These results indicate that at least one gene other than *Tlr4* strongly influences susceptibility to *E. coli* bladder infections in C3H/HeJ mice.**

IMPORTANCE **We have previously demonstrated that mouse strains with either a functional or nonfunctional Tlr4 were not able to resolve induced *Escherichia coli* bladder infections and that a chromosomal site distinct from *Tlr4* was associated with an inability to resolve bladder infections in C3H/HeJ mice. The present study has further investigated the relevance of Tlr4 in bladder infection resolution by defining the *Tlr4* alleles present in offspring of genetic crosses of C3H/HeJ mice with infection-resistant and -susceptible inbred strains. The results of these experiments showed that mice with a normal Tlr4 on different genetic backgrounds were not able to clear *E. coli* bladder infections and that animals with a defective Tlr4 could successfully resolve infections. These results strongly imply the presence of a gene other than in *Tlr4* as an important genetic determinant of infection resistance/susceptibility in C3H/HeJ and other inbred mouse strains used in mouse models of infectious diseases.**

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The importance of Tlr4 in host defense against *Escherichia coli* urinary tract infection (UTI) has been extensively studied in mouse models where the course of infection and immune responses can be studied in animals with normal and defective *Tlr4* alleles (1, 2). Mice from the C3H/HeJ strain have a point mutation in the *Tlr4* gene that makes Tlr4-bearing cells unresponsive to lipopolysaccharide (LPS), and the animals are unable to clear *E. coli* from the bladder up to 14 days after intravesical inoculation (2, 3). Because uroepithelial cells in C3H/HeJ mice do not secrete proinflammatory cytokines normally initiated through the Tlr4, there is a markedly reduced neutrophil influx into the infected bladder. Mouse strains with a functional Tlr4 such as BALB/cAnN, C3H/HeN, and C57BL/6 resolve similarly induced infections within several days and exhibit pyuria (4) and inflammatory cells in the bladder wall (3). These studies imply that a defective Tlr4 is the primary reason for failure of C3H/HeJ mice to resolve *E. coli* bladder infections.

In contrast to the studies cited above, further investigations with C3H/HeJ mice and the closely related C3H/HeOuJ strain have raised the question of whether *Tlr4* is the sole genetic determinant of bladder infection susceptibility in mouse models of *E. coli* UTI. First, we observed that Tlr4-normal, C3H/HeOuJ female mice failed to resolve induced *E. coli* bladder infections in spite of an intense bladder inflammatory response, indicating that one or more genes other than *Tlr4* play a significant role in resistance to infection (3). Second, an investigation into the inheri-

TABLE 1 Bladder infection intensities and *Tlr4* genotypes of female inbred mice and hybrids following intravesical inoculation with *E. coli*

Inbred mouse strain or hybrid[a]	No. of mice tested	*Tlr4* genotype	CFU in bladder[b]
Inbred			
BALB/cAnN	10	Lps^n/Lps^n	70 (1.84 ± 0.20) ABC
C3H/HeJ	6	Lps^d/Lps^d	533,921 (5.73 ± 0.60) AD
C3H/HeOuJ	6	Lps^n/Lps^n	489,778 (5.69 ± 0.20) BD
Hybrid			
C3H/HeJ × BALB/cAnN	15	Lps^n/Lps^d	44 (1.64 ± 0.09) EC
C3H/HeJ × C3H/HeOuJ	7	Lps^n/Lps^d	248,427 (5.40 ± 0.04) E

[a] Female mice from specific inbred strains (BALB/cAnN, C3H/HeJ, and C3H/HeOuJ) and hybrids bred from these strains [(C3H/HeJ × BALB/cAnN) and (C3H/HeJ × C3H/HeOuJ)].

[b] Female mice were inoculated intravesically with 1×10^8 *E. coli* 1677 and euthanized 10 days later to assay for the total number of CFU in the bladder. Values are geometric means with mean $\log_{10}$ (CFU/mg of tissue) ± standard errors of the means in parentheses. Statistical analysis was conducted in two steps using SAS/STAT software, version 9.1 (SAS Institute, Inc.). The log-transformed CFU data were first analyzed by analysis of variance to estimate variability of values within and between experimental groups and then by Fisher's protected least significant differences test to determine significant differences between groups. *P* values less than 0.05 were considered statistically significant. For comparisons between groups indicated by the letters A, B, and E, *P* is <0.001. For comparisons between groups indicated by the letters C and D, *P* is >0.05.

tance of susceptibility to *E. coli* bladder infection using C3H/HeJ, BALB/cAnN, and (C3H/HeJ × BALB/cAnN)F_1 mice showed that infection susceptibility was a recessive trait (5). Furthermore, statistical analysis indicated that one or more genes in C3H/HeJ mice were responsible for their inability to clear bladder infections. Third, in a genetic linkage analysis using BALB/cAnN and C3H/HeJ mice, we identified a quantitative trait locus (QTL), *Becis1*, that was significantly associated with the inability of C3H/HeJ female mice to resolve *E. coli* bladder infections (6). The location of *Becis1* was estimated to be at 29.0 cM on chromosome 4, which is near the *Tlr4* locus at 33.0 cM, suggesting that two distinct genes may contribute to host defense against bladder infection.

The present studies have sought to determine whether the defective *Tlr4* allele present in C3H/HeJ mice could be consistently associated with increased susceptibility or resistance to *E. coli* bladder infections in mice that are homozygous or heterozygous for the defective and normal alleles in different genetic backgrounds. To assess the relationship between the *Tlr4* genotype and resistance or susceptibility to severe *E. coli* bladder infection in female mice, two sets of experiments investigated *E. coli* bladder infection intensities in mice with various combinations of the normal and defective *Tlr4* alleles, Lps^n and Lps^d, respectively. The first experiments utilized inbred mice that were homozygous for Lps^n or Lps^d or hybrids that were heterozygous Lps^n/Lps^d. A subsequent study investigated the association between the *Tlr4* genotype and infection phenotype in a backcross population where animals carried the Lps^d/Lps^d or Lps^n/Lps^d genotypes in combination with other genes present in the parental strains. All experiments measured bladder infection intensities 10 days after intravesical inoculation with 2×10^8 uropathogenic *E. coli* cells of strain 1677 using a protocol (7) approved by the Animal Use and Care Committee of the University of Wisconsin School of Medicine and Public Health. *E. coli* strain 1677 was isolated from a woman with a febrile UTI. Strain 1677 belongs to the B2 phylotype and has the O6 serotype and many virulence genes and markers commonly associated with uropathogenic *E. coli*, such as type 1 and p fimbriae and the genes *iha*, *hlyA*, *sat*, *fyuA*, *iutA*, *ompT*, *chuA*, *yjaA*, *malX*, and *traT*.

The results in Table 1 show clear differences in the abilities of the BALB/cAnN, C3H/HeJ, C3H/HeOuJ strains and hybrids of these mice to resolve *E. coli* bladder infections, implying that the *Tlr4* Lps^d allele is not the primary genetic factor in infection susceptibility. Female BALB/cAnN mice (Lps^n/Lps^n) were able to effectively resolve their bladder infections by 10 days after inoculation, while C3H/HeJ (Lps^d/Lps^d) mice remained severely infected at this time point. Mice that were Lps^n/Lps^d on a background of BALB/cAnN and C3H/HeJ alleles were also resistant to infection, suggesting that the *Tlr4* Lps^n allele confers protection. The importance of a functional Tlr4 can be questioned, however, by the failure of C3H/HeOuJ (Lps^n/Lps^n) mice to effectively resolve infections. Moreover, females from a cross between C3H/HeJ (Lps^d/Lps^d) and C3H/HeOuJ (Lps^n/Lps^n) mice were also not able to effectively resolve *E. coli* bladder infections within 10 days. Thus, there are very likely to be alleles of genes other than *Tlr4* that significantly lower resistance to *E. coli* bladder infection in both of these C3H strains.

The relevance of the normal and defective *Tlr4* alleles in resolution of *E. coli* bladder infections was further investigated in female mice from a backcross of C3H/HeJ with (BALB/cAnN × C3H/HeJ)F_1. These backcross mice were either heterozygous for BALB/cAnN and C3H/HeJ alleles or homozygous for C3H/HeJ alleles at individual loci, including *Tlr4*. All mice were inoculated with *E. coli* and assessed for bladder CFU 10 days later as described above. The *Tlr4* genotypes of mice with less than 1,000 or greater than 100,000 CFU/mg of tissue were analyzed for associations between *Tlr4* and infection intensity. Using the single nucleotide polymorphism that distinguishes the Lps^n and Lps^d *Tlr4* alleles (8), we genotyped 140 backcross mice for the *Tlr4* normal and defective alleles by a previously described method (9). Genomic DNA was prepared from the spleen of each of mouse with the Gentra Puregene tissue kit (Qiagen). A DNA fragment covering the SNP region was generated by PCR using forward (5′ GCTTTCACCTCTGCCTTCAC3′) and reverse (5′ATAACCTTCCGGCTCTTGTG3′) primers (Integrated DNA Technologies); RedTaq polymerase (Sigma, Chemical Co.); and 35 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 45 s with final extension at 72°C for 10 min. The PCR product was purified with the Wizard PCR cleanup system (Promega). To differentiate the Lps^n and Lps^d alleles, the reaction product was digested with Hsp92II restriction endonuclease (New England Biolabs), followed by electrophoresis on a 2.5% agarose gel. The restriction enzyme cleaves DNA with the Lps^d allele to yield two fragments distinguishable from the undigested DNA with the Lps^n allele. These results are presented in Table 2. Twenty-one of the backcross mice had severe bladder infections 10 days after inoculation with *E. coli*. Six of these animals were Lps^n/Lps^d heterozygous and had infection intensities

TABLE 2 Bladder infection intensities and *Tlr4* genotypes of C3H/HeJ female backcross mice following intravesical inoculation with *E. coli*

Backcross mice tested[a]	No. of mice tested	*Tlr4* genotype[b]	CFU in bladder[c]
(BALB/cAnN × C3H/HeJ) × C3H/HeJ	15	Lps^d/Lps^d	468,814 (5.67 ± 0.10) AB
(BALB/cAnN × C3H/HeJ) × C3H/HeJ	6	Lps^n/Lps^d	322,775 (5.51 ± 0.30) AC
(BALB/cAnN × C3H/HeJ) × C3H/HeJ	48	Lps^d/Lps^d	76 (1.88 ± 0.07) BCD
(BALB/cAnN × C3H/HeJ) × C3H/HeJ	71	Lps^n/Lps^d	32 (1.51 ± 0.06) BCD

[a] Female mice from a backcross of an infection-susceptible C3H/HeJ mouse to infection-resistant (BALB/cAnN × C3H/HeJ)F_1.

[b] Mice were genotyped to determine if they were homozygous or heterozygous for the *Tlr4*-normal (Lps^n) and *Tlr4*-defective (Lps^d) alleles, as defined by a single nucleotide polymorphism in the alleles.

[c] Female mice were inoculated intravesically with 1×10^8 *E. coli* cells and euthanized 10 days later to assay for the total number of CFU in the bladder. Values are geometric means with mean $\log_{10}$ (CFU/mg of tissue) ± standard errors of the means in parentheses. Statistical analysis was conducted in two steps with SAS/STAT software, version 9.1 (SAS Institute, Inc.). The log-transformed CFU data were first analyzed by analysis of variance to estimate variability of values within and between experimental groups and then by Fisher's protected least significant differences test to determine significant differences between groups. *P* values less than 0.05 were considered statistically significant. For comparisons between the groups indicated by the letters A and D, *P* is >0.05. For comparisons between the groups indicated by the letters B and C, *P* is <0.001.

that were equivalent to those of either the C3H/HeJ or C3H/HeOuJ strains shown in Table 1. All of the remaining 119 backcross mice resolved their bladder infections as effectively as BALB/cAnN mice. Of these infection-resistant mice, 48 were Lps^d/Lps^d homozygous, and 71 were Lps^n/Lps^d heterozygous. The results showing successful resolution of infections in mice with two copies of the Lps^d allele are thus inconsistent with those from C3H/HeJ mice shown in Table 1.

A functional Tlr4 receptor, as specified by the *Tlr4* Lps^n allele, has been proposed to play a central role in host defense against *E. coli* bladder infections in inbred strains of mice through initiation of local inflammatory responses (1, 10). These studies have noted impaired infection resolution in C3H/HeJ (Lps^d/Lps^d) mice compared to successful resolution in C3H/HeN (Lps^n/Lps^n) mice. Several of the findings reported here, however, question whether *Tlr4* is the gene solely responsible for determining host resistance to bladder infection. First, C3H/HeOuJ (Lps^n/Lps^n) mice are comparable to C3H/HeJ (Lps^d/Lps^d) mice in their inability to resolve *E. coli* bladder infections. Second, when the *Tlr4* Lps^n allele was introduced into C3H/HeJ by a cross with C3H/HeOuJ, the hybrid mice are unable to clear infections. This observation is in contrast to results obtained in a cross of BALB/cAnN (Lps^n/Lps^n) mice with C3H/HeJ (Lps^d/Lps^d) mice in which the hybrid mice successfully resolved bladder infections. Third, heterozygous Lps^n/Lps^d mice from a C3H/HeJ backcross to (C3H/HeOuJ × BALB/cAnN)F_1 mice had severe *E. coli* bladder infections similar to C3H/HeJ mice or the (C3H/HeOuJ × C3H/HeJ) hybrid. Fourth, homozygous Lps^d/Lps^d backcross mice were able to successfully resolve induced *E. coli* bladder infections. Thus, there is strong evidence that alleles of a gene or genes other than *Tlr4* strongly affect resistance or susceptibility to *E. coli* bladder infections in C3H/HeJ, C3H/HeOuJ, and BALB/cAnN mice.

One model to reconcile findings in this study would be one in which a UTI-associated gene with at least two alleles is present in C3H/HeJ, C3H/HeOuJ, and BALB/cAnN mice. An allele conferring resistance to *E. coli* bladder infections would be homozygous in the BALB/cAnN strain. An allele associated with an impaired ability to resolve infections would be homozygous in C3H/HeJ, C3H/HeOuJ, and (C3H/HeOuJ × C3H/HeJ) mice. Because heterozygous offspring of a cross between BALB/cAnN and C3H/HeJ mice were able to effectively clear induced *E. coli* bladder infections, infection resistance would be considered a dominant trait. The infection-susceptible allele in this model would be expected to be homozygous in C3H/HeJ backcross mice with severe bladder infections, while at least one copy of the infection-resistant allele from the BALB/cAnN parental strain would be in backcross mice who successfully resolved infections.

Although the UTI-associated gene proposed in the above model is not currently known, our previous genetic studies have identified the *Becis1* QTL as significantly associated with unresolved bladder infections in C3H/HeJ mice (6). This locus maps to chromosome 4 at 29 cM, and the as yet unidentified gene inferred by the *Becis1* QTL is the most likely candidate to account for the results in the present study. The *Becis1* gene is very likely different from *Tlr4* based on chromosome location and the inconsistent associations of the *Tlr4* Lps^d allele with high bladder infection intensities. In addition, there are no currently defined genes near *Becis1* that are directly involved with either innate or adaptive immune responses or appear to be related to host factors affecting bladder colonization by *E. coli*. The gene inferred by *Becis1* will thus need to be defined and characterized.

Another candidate gene to consider in explaining the current results is *Tlr11*, which has been noted to play an important role in host defense against *E. coli* UTIs in mice (11). As currently defined, however, *Tlr11* is thought to be located on chromosome 14 and does not coincide with the *Becis1* QTL site. A recent report on the evolution of the Toll-like receptor gene family has proposed that mouse *Tlr11* should be renamed *Tlr12* based on similarities between the *Tlr11* and *Tlr12* sequences (12). If the two genes are identical or alleles of a single gene, then *Tlr11/12* would be located at the current site of *Tlr12* on chromosome 4. The *Tlr12* gene has not been genetically mapped to a specific site but is syntenic on chromosome 4 (13), and physical mapping places the gene at bp 128292690 to 128295863 (13). The physical mapping would thus indicate a chromosomal location at approximately 61 cM and thus not near either *Becis1* or *Tlr4*.

The present study has investigated the significance of *Tlr4* in resolution of *E. coli* bladder infections in C3H/HeJ mice or mice derived from genetic crosses with C3H/HeJ mice. Although C3H/HeJ (Lps^d/Lps^d) mice have been used as a model to study host defense mechanisms against *E. coli* UTIs and have shown the importance of innate immune responses in infection resolution, the results presented here and in other studies support the view that *Tlr4* is not the sole determinant of resistance or susceptibility to *E. coli* bladder infections in mice. Rather, there is good evidence that another gene on chromosome 4 has alleles that strongly influence whether an induced *E. coli* bladder infection will be successfully resolved.

These studies have important implications for investigations using C3H/HeJ mice to study the role of Tlr4 in resolution of infections in organ systems other than the urinary tract. For example, comparisons between C3H/HeJ and Tlr4-normal strains have provided model systems with which to study host defense mechanisms against *Neisseria meningitidis* bacteremia (14), *Haemophilus influenzae* pulmonary infections (15), and lethal *Salmonella enterica* serovar Typhimurium infection (16). Although the defective Tlr4 likely plays a role in susceptibility to bacterial infections in models using C3H/HeJ mice, the studies reported here strongly indicate the presence of multiple genetic factors in host defense against infections caused by *E. coli* and, potentially, other types of bacteria. This view is consistent with the multigenic nature of susceptibility or resistance to infections documented in several mouse models (17). Thus, it is important to consider genes acting individually or together with *Tlr4* when using C3H/HeJ mice in models of infectious diseases.

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