Reduced Toll-Like Receptor 4 Expression in Children with Asymptomatic Bacteriuria

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Toll-like receptor (TLR) 4 is essential for the defense against infection with gram-negative pathogens, but reduced TLR4 expression has not been linked to altered disease susceptibility in humans. In mice, Tlr4 controls the mucosal response to Escherichia coli urinary tract infections. Inactivation of mouse Tlr4 causes an asymptomatic carrier state resembling asymptomatic bacteriuria (ABU). The present study compared neutrophil TLR4 expression levels between children with ABU (n = 17) and age-matched control subjects (n = 24), and significantly lower levels were detected in the patients with ABU. We also found elevated levels of the TLR4 adaptor protein TRIF and reduced levels of the TLR4-inhibitor SIGIRR in the patients with ABU, but MyD88 and TRAM levels were not significantly altered. Altered TLR4 and adaptor protein expression might impair TLR4 signaling and explain the weak mucosal response to urinary tract infection in patients who develop ABU rather than symptomatic disease.

Urinary tract infections (UTIs) are so common and so complex that a molecular basis for disease susceptibility may appear unlikely. Yet there are marked individual differences in susceptibility, as shown by the clinical manifestations of disease [1]. Some patients develop severe, sometimes life-threatening infections, but in at least 1% of all UTI cases bacteria establish a carrier state called “asymptomatic bacteriuria” (ABU) [2, 3]. The difference in disease severity is partly due to the virulence properties of the infecting Escherichia coli strain, but a lack of virulence factors alone does not explain why patients with ABU develop persistent infection without symptoms [4, 5]. The present study proposes that ABU might develop as a result of reduced Toll-like receptor (TLR) 4 expression.

Determinants of host susceptibility have been identified in the murine UTI model [4, 6]. Innate immunity controls susceptibility to acute infection, and TLR4 signaling is especially essential for the mucosal defense, whereas adaptive immunity is less important [7–9]. The crucial role played by Tlr4 in the urinary tract defense was first implied in studies in C3H/HeJ mice [9]; due to a point mutation in the Toll/interleukin (IL)–1 receptor (TIR) domain, Tlr4 signaling is inactivated in these mice [10], which develop an asymptomatic carrier state resembling human ABU [9]. Tlr4−/− mice develop a similar ABU-like state, suggesting that abrogated TLR4 signaling might protect the host from symptomatic infection [11].

TLR signaling is modified by adaptor proteins such as TIR domain–containing adaptor inducing interferon [IFN]–β (TRIF), TIR domain–containing adaptor inducing IFN-β–related adaptor molecule (TRAM), myeloid differentiation factor 88 (MyD88), and TIR domain–containing adaptor protein (TIRAP) [12–15]. In
Table 1. Characteristics of patients with asymptomatic bacteriuria (ABU).

<table>
<thead>
<tr>
<th>Group, patient&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diagnosis</th>
<th>Recurrence</th>
<th>Reflux&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Blood sampling</th>
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<tr>
<td></td>
<td>Index infection</td>
<td>Age at index infection, years</td>
<td>Age at recurrent infection, years</td>
<td>Result Grade ABU</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>ABU</td>
<td>&lt;1</td>
<td>NA</td>
<td>...</td>
</tr>
<tr>
<td>2</td>
<td>ABU</td>
<td>7</td>
<td>NA</td>
<td>...</td>
</tr>
<tr>
<td>3</td>
<td>ABU</td>
<td>12</td>
<td>NA</td>
<td>...</td>
</tr>
<tr>
<td>4</td>
<td>ABU</td>
<td>9</td>
<td>+ 2</td>
<td>...</td>
</tr>
<tr>
<td>5</td>
<td>ABU</td>
<td>1</td>
<td>NA</td>
<td>...</td>
</tr>
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<td>...</td>
</tr>
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<td>6</td>
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<td>...</td>
</tr>
<tr>
<td>12</td>
<td>ABU</td>
<td>4</td>
<td>-</td>
<td>...</td>
</tr>
<tr>
<td>13</td>
<td>ABU</td>
<td>&lt;1</td>
<td>NA</td>
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<td>Secondary ABU</td>
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</tr>
<tr>
<td>14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Py</td>
<td>&lt;1</td>
<td>Py (many)</td>
<td>&lt;1 and older</td>
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<td></td>
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<tr>
<td>15</td>
<td>Py</td>
<td>5</td>
<td>ABU</td>
<td>6</td>
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</tr>
<tr>
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<td>Py</td>
<td>1</td>
<td>Cy</td>
<td>7</td>
</tr>
<tr>
<td>17</td>
<td>Cy</td>
<td>4</td>
<td>Cy (many)</td>
<td>&lt;5</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>E. coli</td>
<td>&lt;1</td>
<td>ABU</td>
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<tr>
<td></td>
<td>sepsis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>19&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Py</td>
<td>3</td>
<td>Py (many)</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>20&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>1</td>
<td>Py</td>
<td>1</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Py</td>
<td>5</td>
<td>ABU</td>
<td>7</td>
</tr>
<tr>
<td>22</td>
<td>ABU</td>
<td>2</td>
<td>Cy</td>
<td>2</td>
</tr>
<tr>
<td>23&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ABU</td>
<td>4</td>
<td>-</td>
<td>...</td>
</tr>
<tr>
<td>24</td>
<td>Py</td>
<td>&lt;1</td>
<td>ABU</td>
<td>&lt;1</td>
</tr>
<tr>
<td>25</td>
<td>Py</td>
<td>&lt;1</td>
<td>ABU</td>
<td>1</td>
</tr>
<tr>
<td>26&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Py</td>
<td>9</td>
<td>ABU</td>
<td>+ 4</td>
</tr>
<tr>
<td>27&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Cy/ABU</td>
<td>7</td>
<td>ABU</td>
<td>7</td>
</tr>
<tr>
<td>28</td>
<td>Py</td>
<td>4</td>
<td>ABU</td>
<td>4</td>
</tr>
</tbody>
</table>

NOTE. Cy, cystitis; Py, pyelonephritis.

<sup>a</sup> All patients were infected with *Escherichia coli* except patient 3, who was infected with *Klebsiella oxytoca*. Patients 1, 6, 10, 14, 20, 24, and 25 were infected with different strains at different times (patient 6 with *E. coli*, *K. oxytoca*, and group B streptococci; patients 1, 14, and 20 with *E. coli* and enterococci; and patients 10, 24, and 25 with Proteus species and *E. coli*).

<sup>b</sup> Some patients were examined by voiding cystourethrography, which revealed reflux (+) or no reflux (−). If a patient was not investigated by this procedure, the result was considered to be not available (NA).

<sup>c</sup> Unilateral renal aplasia.

<sup>d</sup> Status after splenectomy.

<sup>e</sup> Hydroureter and ureterocele.

<sup>f</sup> Nephrectomy due to reflux nephropathy.

<sup>g</sup> Unilateral hydronephrosis.

<sup>h</sup> Bilateral duplex, unilateral heminephrectomy.

<sup>i</sup> Mild incontinence.
TLR4 and Urinary Tract Susceptibility

Figure 1. Reduced Toll-like receptor (TLR) 4 expression in patients with asymptomatic bacteriuria (ABU) (P), compared with that in age-matched control subjects (C), despite a lack of sequence variation. A, Comparison of TLR4 expression between patients with ABU (purple) and age-matched control subjects (green), as shown by flow cytometry (P = .003, for the comparison of the groups by the 2-tailed Mann-Whitney U test; P = .039, for the paired analysis of each patient with his or her age-matched control subject by the paired t test). Table 3 also demonstrates that the patients with ABU had significantly lower TLR4 expression than did the age-matched control subjects. B, Genomic organization of TLR4 and positions of genetic variants in the coding region, found in one control subject and shown with a star. LLR, lucine-rich region; TIR, Toll/interleukin-1 receptor domain.

addition, TLR4 signaling is negatively regulated by several proteins, including single immunoglobulin domain–containing IL-1 receptor–related molecule (SIGIRR), which blocks TLR4 signaling by sequestering IL-1 receptor–associated kinase (IRAK) and tumor necrosis factor (TNF) receptor–associated factor (TRAF) 6 [16–19]. The TLR4 adaptor proteins were recently shown to influence the mucosal response to UTI [11]. Trif−/− and Tram−/− mutant mice responded poorly to P fimbriated E. coli, and Myd88−/− mice developed ABU with type 1 fimbriated E. coli, indicating that ABU might result from variant adaptor protein expression.

In light of these results, we hypothesized that reduced TLR4 expression might contribute to the low mucosal response in children who develop ABU instead of symptomatic UTI. In addition to investigating this possibility, we also examined whether variant TLR4 adaptor protein and SIGIRR expression might contribute to the unresponsiveness in these patients.

METHODS

Patients and control subjects. We enrolled 27 children and 1 young adult with a history of ABU (18 females and 10 males; median [range] age at blood sampling, 6.5 [1–20] years). They were followed up by the same pediatric nephrologist at the Department of Pediatric Nephrology at Lund University Hospital. The diagnosis of ABU was based on at least 3 consecutive urine cultures with the same bacterial strain (>10^5 cfu/mL of urine) in a patient with no symptoms of UTI and no increase in C-reactive protein level. Thirteen patients had no evidence of prior symptomatic UTI and were defined as having primary ABU (5 males and 8 females; median [range] age at blood
saponin solution followed by FITC-conjugated anti-rabbit sec-
human MyD88, TRIF, and TRAM at a ratio of 1:100 in 0.1%
with 0.1% saponin solution and incubated with rabbit anti–
antibodies from Biosite (rabbit anti–human polyclonal TRAM,
pression of 3 TLR4 adaptor proteins was determined using
scribed above for TLR4 staining.
with rabbit anti-SIGIRR polyclonal antibody (Biosite) as de-
antibody signal. SIGIRR expression was determined by staining
expression was determined after subtraction of the secondary
alysis on a FACSCalibur device. Adaptor protein expression was consid-
ered to be the value remaining after subtraction of the sec-
ondary antibody control.
DNA sequencing. Genomic DNA was isolated from pe-
ipheral blood using the QIAamp DNA Blood Midi Kit (QIA-
gen) and stored at 4°C. Exons 1 (4158–4417 bp), 2 (8414–
8580 bp), and 3 (12,239–15,625 bp) of the TLR4 locus
GenBank accession number AF177765) were amplified and sequenced using oligonucleotide primers as described elsewhere [20]. The BigDye Terminator (version 3.1; Applied Biosystems) was used, and sequences were resolved using ABI model 3100 automater DNA sequencer. Patient sequences were base called and multialigned with the control sequences by use of
the programs PolyPhred and Phrap, from the University of
Washington Genome Center [21]. The entire coding region in
exon 2 of the gene for TRIF (TRIF) was amplified and sequenced using specific oligonucleotide primers (table 2). Dye
terminator chemistry was used, and sequences were resolved using ABI model 373 and 377 machines. Trace files were opti-
themally assembled using the programs PolyPhred and Phrap
[21] and analyzed manually using the program Consed [22].

Real-time reverse-transcription polymerase chain reaction
(RT-PCR). RNA was extracted using the RNeasy Mini Kit
(QIAGEN) and stored at −80°C. RNA was treated with DNaseI,
and cDNA was obtained with SuperScriptIII with oligo-dT (In-
vitrogen). TaqMan RT-PCR was done on an Applied Biosystems
Sequence Detection System 7000 device, using reagents from
Applied Biosystems.

Statistical analysis. Protein expression in patients and pedi-
atic control subjects was compared by the 2-tailed Mann-
Whitney U test and the paired t test, whereas RNA levels were
compared using the unpaired t test. Analysis was done using
GraphPad InStat for Windows (version 3.06; GraphPad
Software).

| Table 3. Toll-like receptor (TLR) 4 expression in patients with asymptomatic bacteriuria (ABU) and in control subjects. |
|---|---|---|
| Group | TLR4 | P* |
| Patients with ABU | 33.2 (4.3–74.3) | .003a |
| Control subjects | | |
| Pediatric | 56.1 (19.2–87.6) | >.05b |
| Adult | 51.2 (7.5–139.0) | |

NOTE. Data are mean (range) values.

a Two-tailed Mann-Whitney U test.

b For the comparison between the patients with ABU and the pediatric control subjects.

c For the comparison between the pediatric and adult control subjects.
Figure 2. Elevation of Toll/interleukin-1 receptor (TIR) domain–containing adaptor inducing interferon-β (TRIF) in patients with asymptomatic bacteriuria (ABU), compared with that in age-matched control subjects, despite a lack of sequence variation. A, Comparison of TRIF expression between patients with ABU (purple) and age-matched control subjects (green), as shown by flow cytometry ($P = .046$, 2-tailed Mann-Whitney U test). Table 4 also demonstrates that TRIF expression differed significantly between the patients with ABU and the age-matched control subjects and additionally shows that the expression of TIR domain–containing adaptor inducing interferon-β–related adaptor molecule, or TRAM, and myeloid differentiation factor 88 did not. B, Genomic organization of TRIF and positions of genetic variants in the coding region and in the intron upstream of the coding region. PRR, proline-rich region; SNP, single-nucleotide protein.

RESULTS

Reduced TLR4 protein expression in children with ABU. Because $Tlr4^{-/-}$ mice develop an ABU-like state, we examined whether patients with ABU have lower TLR4 expression levels than do pediatric control subjects. Neutrophil TLR4 expression was quantified by flow cytometry after staining with a polyclonal rabbit anti–human TLR4 antibody specific for the extracellular domain. Samples were available from 17 patients with ABU and 24 control subjects (figure 1A). TLR4 expression was significantly lower in the patients ($P = .003$, for the comparison of the groups by the 2-tailed Mann-Whitney U test; $P = .039$, for the paired analysis of each patient with his or her age-matched control subject by the paired $t$ test) (figure 1). TLR4 expression was further analyzed in relation to the history of UTI. The patients with primary ABU had lower TLR4 expression levels (median, 22.6; range, 4.3–42.3) than did the patients with secondary ABU (median, 45.0; range, 18.1–74.3) ($P = .0004$, for primary ABU vs. pediatric control subjects by the 2-tailed Mann-Whitney U test, whereas $P = .307$ for secondary ABU and pediatric control subjects), but there was no difference in TLR4 expression levels between pediatric and adult control subjects (table 3).
Figure 3. Reduced single immunoglobulin domain–containing interleukin (IL)–1 receptor–related molecule (SIGIRR) expression in patients with asymptomatic bacteriuria (ABU), compared with that in age-matched control subjects. A, Comparison of SIGIRR expression between patients with ABU (purple) and age-matched control subjects (green), as shown by flow cytometry ($P = .0005$, 2-tailed Mann-Whitney $U$ test). Table 5 also shows that SIGIRR expression was significantly lower in the patients with ABU than in the age-matched control subjects. B, Correlation analysis showing an inverse relationship between Toll-like receptor (TLR) 4 and Toll/IL-1 receptor domain–containing adaptor inducing interferon-$
abla$ adaptord protein (TRIF) expression ($r = -0.04$; $P = .034$).

**ABU not due to polymorphisms in TLR4.** The human TLR4 gene encodes an extracellular, a transmembrane, and a cytoplasmic domain (figure 1B) [20, 23]. The extracellular domain combines with microbial ligands, whereas the cytoplasmic TIR domain controls signaling through the adaptor proteins MyD88, TIRAP, TRAM, and/or TRIF.

TLR4 DNA sequences were obtained from 28 children with ABU and 14 healthy control subjects. On the basis of analogy to the C3H/HeJ mouse, the patient DNA sequences were first examined for mutations in the TIR domain, but no sequence variation was detected. Exons 1 and 2 were monomorphic and agreed with the TLR4 consensus sequence (GenBank accession number AF177765). However, the known single-nucleotide polymorphisms (SNPs) at aa 299 and 399 of exon 3 were detected in 1 control subject. This allelic variant, TLR4B, has a reported frequency of 7% in white persons [20]. The rare heterozygous missense mutations in TLR4, which have been associated with systemic meningococcal disease [24], were not found, and no other mutations were observed. Thus, TLR4 sequence variation in the coding region does not explain the reduced TLR4 protein expression in patients with ABU.

**TLR4 protein expression and renal scarring.** Because acute
pyelonephritis plays an important role in the development of renal scarring and because the children with secondary ABU had prior symptomatic UTI episodes, we examined the extent of renal scarring in the ABU group. Three (37.5%) of the 8 patients with secondary ABU who had been examined had developed renal scarring, compared with none in the primary ABU group. TLR4 expression appeared to vary depending on the presence or absence of renal scarring, with higher TLR4 expression in patients with renal scarring (mean, 53.4; ) than in ABU patients without scarring (mean, 34.3; ) (figure 4). Age-matched comparison revealed that 12 of 15 patients with ABU had significantly lower SIGIRR expression levels ( ) than did the pediatric control subjects (table 5). Age-matched comparison revealed that 12 of 15 patients with ABU had lower SIGIRR expression levels than did their corresponding age-matched control subject. No correlation between TLR4 and SIGIRR expression was found.

TLR4 adaptor protein expression in children with ABU. Because mice carrying deletions in the TLR4 adaptor proteins develop a carrier state resembling ABU, we quantified TRIF, TRAM, and MyD88 expression. The patients with ABU had significantly higher TRIF expression levels than did the pediatric control subjects (mean, 139.0; P = .02) (figure 2A and table 4). Age-matched comparison revealed that 11 of 17 patients with ABU had higher TRIF expression levels than did the corresponding age-matched control subject. In contrast, the expression of TRAM and MyD88 did not differ between patients and control subjects. There was a significant inverse correlation between TLR4 and TRIF expression (r = −0.04; P = .03) (figure 3B) but no significant correlation between TLR4 expression and the other adaptors.

ABU not due to polymorphisms in the gene for TRIF(TRIF). TRIF DNA sequences were obtained from 12 of the children with ABU and 11 healthy control subjects. The human TRIF gene forms a protein of 712 aa, with a TIR domain and 2 proline-rich regions (figure 2B). We identified 4 different polymorphisms in exon 2. SNP1 involved aa 3 (9T/A, counting from the start codon in NC_000019, position 63), and SNP2 was between the TIR domain and the second proline-rich region, at aa 556 (1668C/T); neither caused an amino acid change. SNP3 and SNP4 were outside the coding region but inside exon 2, at 2146 bp and 2227 bp, respectively. Four additional SNPs were detected in the intron, upstream of exon 2 (figure 2B), but the SNPs did not affect potential transcription factor binding sites, on the basis of the TFSEARCH database (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html).

The frequency of TRIF polymorphisms was compared between the patients with ABU and the control subjects. SNP1 (9A/G) was found in 58% (7/12) of the patients and in 46% (5/11) of the control subjects but was not linked to ABU (P > .05). There was no apparent difference between children with primary or secondary ABU. Linkage disequilibria were detected between the SNPs at −98 and −97 and among the SNPs at −345, 1668, and 2227 (data not shown).

SIGIRR protein expression in children with ABU. We hypothesized that variant SIGIRR expression might influence the TLR4-dependent response in patients with ABU. SIGIRR is thought to inhibit lipopolysaccharide (LPS) signaling by attenuating the recruitment of MyD88, IRAK, and TRAF6 to TLR4 through TIR-TIR domain interactions between SIGIRR and TLR4. SIGIRR expression on neutrophils was quantified using flow cytometry, after staining with polyclonal rabbit anti-human SIGIRR antibody (figure 3). The patients with ABU had significantly lower SIGIRR expression levels (P = .0005) than did the pediatric control subjects (table 5). Age-matched comparison revealed that 12 of 15 patients with ABU had lower SIGIRR expression levels than did their corresponding age-matched control subject. No correlation between TLR4 and SIGIRR expression was found.

Lower expression of TLR4 mRNA in children with ABU. TLR4, TRIF, MyD88, and SIGIRR expression was quantified by real-time RT-PCR. Neutrophil RNA was available from 13 patients with ABU and 7 age-matched control subjects (figure 4 and table 6). The patients with ABU had significantly lower TLR4 mRNA levels (mean [range], 0.72 [0.41–1.28]) than did the control subjects (mean [range], 1.20 [0.25–2.50]) (P = .046; unpaired t test).

TLR4 protein expression and bacteriuria. Urine cultures were obtained at the time of blood sampling (figure 5). In the secondary ABU group, patients with bacteriuria had significantly higher TLR4 expression levels than did patients with sterile urine (P < .05). In the primary ABU group, TLR4 expression was low, regardless of bacteriuria (P > .05).

### Table 4. Toll/interleukin-1 receptor (TIR) domain–containing adaptor inducing interferon (IFN)–β adaptor protein (TRIF), TIR domain–containing adaptor inducing IFN-β–related adaptor molecule (TRAM), and myeloid differentiation factor 88 (MyD88) expression in patients with asymptomatic bacteriuria (ABU) and in age-matched control subjects.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Patients with ABU</th>
<th>Pediatric control subjects</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIF</td>
<td>139.0 (34.5–315.1)</td>
<td>85.1 (12.4–171.5)</td>
<td>.02</td>
</tr>
<tr>
<td>TRAM</td>
<td>107.9 (22.0–262.9)</td>
<td>101.7 (39.8–269.2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>MyD88</td>
<td>100.5 (9.3–197.3)</td>
<td>84.3 (9.9–168.5)</td>
<td>&gt; .05</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean (range) values.

* Two-tailed Mann-Whitney U test.

### Table 5. Single immunoglobulin domain–containing interleukin-1 receptor–related molecule (SIGIRR) expression in patients with asymptomatic bacteriuria (ABU) and in age-matched control subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>SIGIRR</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with ABU</td>
<td>14.9 (8.2–63.3)</td>
<td>.0005</td>
</tr>
<tr>
<td>Pediatric control subjects</td>
<td>21.9 (12.5–35.5)</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean (range) values.

* Two-tailed Mann-Whitney U test.
DISCUSSION

The present study proposes that low TLR4 expression may lead to asymptomatic bacterial carriage. Patients with UTI were selected for study, because Tlr4−/− mice develop an asymptomatic carrier state resembling human ABU [9]. We found reduced TLR4 protein and mRNA expression in children with ABU, compared with that in age-matched control subjects without UTI. All of the patients had detectable amounts of TLR4 on their neutrophils, suggesting that the function of TLR4 was attenuated but not lost. Our results support the hypothesis that reduced TLR4 expression protects the urinary tract from the consequences of inflammation and promotes the development of an asymptomatic carrier state.

Earlier studies focused mainly on bacterial virulence as a determinant of disease severity in the urinary tract [25, 26]. The virulent, uropathogenic E. coli strains express a number of virulence factors more efficiently than do the ABU strains [4, 25]. For example, P fimbriae are expressed by almost all pyelonephritis isolates and promote the establishment of bacteriuria and the mucosal attack [27]. ABU strains rarely express P fimbriae or other virulence factors, and, thus, the lack of symptoms can be attributed, in part, to the low virulence of those strains. The present study proposes the existence of an additional level of control of disease severity, through the innate immune response. We propose that individuals who have reduced TLR4 expression might run a reduced risk of developing symptomatic UTI as a result of abrogated inflammation. Consistent with the hypothesis, children with primary ABU had the lowest TLR4 levels. Most of the patients with secondary ABU had experienced prior symptomatic infections, suggesting that their TLR4 expression had been intact at that time and that they had been able to respond to the uropathogenic E. coli strains that activate TLR4 signaling. In those patients, TLR4 expression might have been down-regulation by a tolerance-related mechanism, whereas the patients with primary ABU might have had a regulatory defect. The mechanisms underlying reduced TLR4 expression may thus differ between the patients with primary and secondary ABU.

We obtained further indications of a difference between the primary and secondary ABU groups when TLR4 expression was related to bacteriuria at the time of blood sampling. In the secondary ABU group, patients with bacteriuria had significantly higher TLR4 expression than did patients with sterile urine, but TLR4 expression did not reach control levels. This indicates that the secondary ABU group responded to infection, even though their TLR4 expression was suppressed. The patients with primary ABU, in contrast, had low TLR4 expression when they had bacteriuria, suggesting that their ability to respond was impaired. Preliminary results showed that neutrophils from patients with primary and secondary ABU differ in the in vitro response to LPS and uropathogenic E. coli. Polymorphonuclear neutrophils from patients with ABU showed a weaker response than did those from the age-matched control subjects, reflecting their low TLR4 phenotype, and the patient with primary ABU had the lowest response. This observation needs to be confirmed in further studies.

DNA sequencing showed that the TIR domain of TLR4 was intact, unlike the that of the C3H/HeJ mouse. This was expected, because the point mutation in the TIR domain does not reduce surface expression but does abolish signaling. There was also no evidence that TLR4 sequence variation explained the reduced TLR4 expression. The patients with ABU did not carry the Asp299Gly polymorphism, which alters the extracellular domain of TLR4, and its cosegregating polymorphism Thr399Ile. Many studies have attempted to link these polymorphisms to disease susceptibility and endotoxin hyporesponsiveness, but, according to Arbour et al. [28] and Erridge et al. [29], heterozygous individuals have intact LPS recogni-

![Figure 4. Reduced Toll-like receptor (TLR) 4 mRNA expression in patients with asymptomatic bacteriuria (ABU), compared with that in age-matched control subjects. Data are mean ± SD values (P = .046, unpaired t test). See also table 6.](https://academic.oup.com/jid/article-abstract/196/3/475/805893)
Figure 5. Differing Toll-like receptor (TLR) 4 expression in the secondary asymptomatic bacteriuria (ABU) group depending on the presence or absence of bacteriuria at blood sampling. The patients with bacteriuria had significantly higher TLR4 levels than those who had sterile urine (P<.05). In contrast, TLR4 expression did not vary significantly in patients with primary ABU with bacteriuria at blood sampling. Data are mean ± SD values.

Given that the structural genes were intact but mRNA levels were reduced, the results suggest that expression but not the structure of the protein is modified. This is interesting, because there have been many attempts to link polymorphisms in the TLR4 gene with disease—mostly without success—and opens the door for studies of other mechanisms of TLR4 regulation.

Studies in the murine model of UTI have shown that the TLR4 adaptor proteins further regulate the innate host defense against UTI, and loss of TRIF, TRAM, and MyD88 function has been shown to facilitate bacterial persistence [11]. Consistent with these results, changes in adaptor protein expression were detected in the ABU group. TRIF expression was significantly higher in patients with ABU than in pediatric control subjects. The functional consequence of this finding is unclear, but there was an inverse correlation between TLR4 and TRIF expression in individual patients. It may be speculated that low TLR4 expression may cause TRIF accumulation when this adaptor is not consumed by the activation of TLR4 signaling. Furthermore, high TRIF levels might serve to maintain a defense against P fimbriated E. coli. The host defense against P fimbriated E. coli in the mouse has been shown to rely on TLR4 and the TRIF/TRAM adaptors [11].

SIGIRR is a negative regulator of TLR4 signaling. We had expected to find elevated SIGIRR levels in the patients, because inhibition of TLR4 might have explained their unresponsiveness. A study by Xiao et al. [30] has shown that SIGIRR plays an important role in the innate response of the colonic mucosa, where it controls homeostasis and protects the epithelia from overreacting to commensals and their products. The present study suggests that patients with ABU might compensate for low TLR4 function by suppressing SIGIRR expression, thus allowing for optimal function of the few TLR4 molecules that are expressed. Further studies will have to evaluate what role SIGIRR plays in the defense against UTI.

This is the first attempt to link reduced TLR4 expression to asymptomatic carriage in patients. Most studies of low TLR4 expression have addressed the phenomenon as LPS tolerance in vitro. Mouse macrophages/monocytes show a reduced TLR4 response and a reduction in surface expression of the TLR4/MD-2 complex when stimulated for the second time with LPS [31]. Although the urinary tract epithelium lacks CD14 and LPS alone is a poor host response activator, type 1 fimbriated E. coli can deliver an LPS signal to the mucosa in the absence of CD14. Neutrophils have CD14 on their surface, unlike urinary tract epithelium, so it is possible that the low TLR4 expression seen in patients with ABU reflects repeated exposure to LPS. Several studies have proposed a correlation between sepsis and hyporesponsiveness to LPS, but the mechanisms are not fully understood. Unlike patients with ABU, patients with sepsis have elevated levels of TLR4, but, despite this increase, they do not release higher amounts of TNF-α and IL-8 [32, 33]. It is possible that the reduced TLR4 response in ABU is a form of LPS tolerance, even though it differs from the endotoxin tolerance seen in patients with sepsis [34].

Mucosal TLR4 responses require sophistication, because TLR4 must distinguish gram-negative pathogens from the commensal gram-negative microflora. In asymptomatic carriers, the mucosa appears to remain undisturbed; however, in symptomatic patients, uropathogenic bacteria break the inertia of the
barrier and trigger inflammation. We have previously shown that uropathogens are recognized by epithelial TLR4, albeit through mechanisms involving specific virulence factors such as P fimbriae rather than LPS [11]. The activated epithelial cells then recruit the antibacterial defense with the side effect of mucosal inflammation and disease. The present study predicts that the host can protect from inflammation by suppressing TLR4 expression and by modulating the adaptor protein expression levels. Reduced rather than absent TLR4 expression is likely to serve the dual function of protecting the mucosa while retaining a sufficient part of the systemic defense against severe gram-negative infections, including acute pyelonephritis.

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References