

Relevance of Mutations in the TLR4 Receptor in Patients With Gram-Negative Septic Shock

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Background: Septic shock remains a significant health concern worldwide, and despite progress in understanding the physiological and molecular basis of septic shock, the high mortality rate of patients with septic shock remains unchanged. We recently identified a common polymorphism in toll-like receptor 4 (TLR4) that is associated with hyporesponsiveness to inhaled endotoxin or lipopolysaccharide in humans.

Methods: Since TLR4 is a major receptor for lipopolysaccharide in mammals and gram-negative bacteria are the prevalent pathogen associated with septic shock, we investigated whether these specific *TLR4* alleles are associated with a predisposition to a more severe disease outcome for patients with septic shock. We genotyped

91 patients with septic shock as well as 73 healthy blood donor controls for the presence of the *TLR4* Asp299Gly and *TLR4* Thr399Ile mutations.

Results: We found the *TLR4* Asp299Gly allele exclusively in patients with septic shock ($P = .05$). Furthermore, patients with septic shock with the *TLR4* Asp299Gly/Thr399Ile alleles had a higher prevalence of gram-negative infections.

Conclusion: Mutations in the TLR4 receptor may predispose people to develop septic shock with gram-negative microorganisms.

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DESPITE SIGNIFICANT advances in understanding the molecular basis of sepsis and its associated immunologic response, sepsis remains a problem worldwide and is associated with a high mortality. Annually, septic shock, the most severe form of sepsis, causes the death of more than 100 000 people in the United States.^{1,2} Gram-negative bacteria are the most common pathogens associated with bacterial infections in diseases such as meningitis. Endotoxin or lipopolysaccharide (LPS), the main component of the cell wall of gram-negative bacteria, has been shown to elicit an inflammatory response that mimics all of the features described in septic shock.³ In vivo and in vitro, LPS activates macrophages and monocytes, as shown by increased secretion of cytokines such as tumor necrosis factor (TNF) α , interleukin (IL) 1, IL-6, and IL-8.^{4,5} Activation of an innate immune mechanism causes proinflammatory cytokine release to be magnified to dangerous levels, resulting in hypotension, organ failures, and even death in a significant number of patients.^{1,2} A likely target for novel therapeutic interven-

tion is the initial recognition of pathogens and the initiation of an early innate immune response.

The molecular events leading to cell recognition and response to LPS are becoming more clearly defined. Recently, the toll family of proteins, which consists of at least 10 type 1 transmembrane receptor proteins, has been identified.⁶ The toll proteins share a highly homologous cytoplasmic domain, similar to the IL-1 receptor, a very short transmembrane domain, and an extracellular portion consisting of a various number of leucine-rich repeats.⁷ Toll-like receptor 4 (TLR4), in particular, has been shown to be a major LPS recognition receptor.⁸ In vivo, mouse strain C3H/HeJ (which has a proline-to-histidine substitution at amino acid 712 of *TLR4*) and C57BL/10ScCR (which has a deletion of the *TLR4* locus and does not express *TLR4* RNA) are both hyporesponsive to LPS.⁹ In addition, macrophages and B cells from *TLR4*^{-/-} mice are unable to mount a cellular response to LPS.^{10,11} In vitro data from transfection of mammalian cell lines further proved that TLR4 is able to enhance the LPS responsiveness of cells, a process augmented by CD14 and leading to activation of tran-

SUBJECTS AND METHODS

STUDY SUBJECTS

The patient samples were collected as part of a multicenter study conducted in 7 academic adult intensive care units in France between March 1, 1996, and November 30, 1997. The protocol was approved by the institutional review board of Cochin University Hospital, Paris, France. Informed consents were obtained from control subjects and patients or their relatives.

The control group comprised 73 healthy unrelated white blood donors to the Cochin University Hospital blood bank; 42 of the controls had been previously genotyped for a study on TNF- α polymorphisms.¹⁵ An additional 31 healthy blood donors were collected as control subjects for this study. The septic shock group, defined by the criteria of the consensus conference,¹⁶ included 91 intensive care unit (ICU) patients. Of these patients, 88 had been included in a previously published study on the effect of TNF- α polymorphism on septic shock,¹⁵ and 3 patients were entered into the study after June 1997.

To be eligible for enrollment, the patients with septic shock had to be white and to have 6 of the following inclusion criteria of septic shock within a 12-hour period: (1) clinical evidence of infection; (2) hyperthermia (temperature $>38^{\circ}\text{C}$) or hypothermia (temperature $<35.6^{\circ}\text{C}$); (3) tachycardia (heart rate >90 beats/min); (4) tachypnea (respirations 120/min) or need for mechanical ventilation; (5) use of vasopressor to maintain systolic blood pressure higher than 90 mm Hg, or hypotension, defined as systolic blood pressure less than 90 mm Hg for more than 30 minutes or a decrease in systolic blood pressure of more than 40 mm Hg from previously established values for more than 30 minutes (hypotension had to be present at enrollment and refractory to an intravascular volume challenge of at least 500 mL); and (6) evidence of inadequate organ function or perfusion within 12 hours of enrollment, as manifested by at least 1 of the following syndromes (previously described): acute deterioration of the patient's mental status; arterial hypoxemia ($\text{PaO}_2/\text{fraction of inspired oxygen} <280$); plasma lactate concentration above the normal range or metabolic acidosis; oliguria; and disseminated intravascular coagulation. The exclusion criteria were the following: (1) age greater than 80 years; (2) cardiac failure (class III or IV); (3) liver insufficiency (Child

class C); (4) bone marrow aplasia (white blood cell count $<500/\mu\text{L}$); or (5) immunosuppression (positive human immunodeficiency virus serologic result, current immunosuppressive therapy including corticosteroids [equivalent prednisone >0.5 mg/kg per day], or cancer).

Patients were followed up throughout their stays in the ICU. Age, sex, primary site of infection, infection-related organisms, and severity indexes including Simplified Acute Physiology Score (SAPS II),¹⁷ which uses a total of 15 variables to assess the severity of ICU patients, and Organ System Failure score,¹⁸ were collected at each patient's entry into the ICU. All the samples and information used in the study were coded and patient confidentiality was preserved according to the guidelines for studies of human subjects.

MUTATION DETECTION

To determine the *TLR4* genotype of the samples with respect to residues 299 and 399, the genomic DNA was amplified with primers (forward primer: 5'-tctagaggcctgtg-caatt-3'; reverse primer, 5'-tgaaaactcacttattgttcaa-3') that span the region containing the 2 polymorphisms. Polymerase chain reaction conditions were as follows: 5-minute initial denaturation at 95°C , followed by 30 cycles (95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute). After polymerase chain reaction amplification, fragments were purified (Gel Purification Kit; Qiagen, Valencia, Calif) and sequenced. The sequence of the genomic DNA from each subject was determined with an automated DNA sequencer (Model 377; Perkin Elmer, Norwalk, Conn). The sequence analysis was performed blinded to the phenotype of the study subjects.

STATISTICAL ANALYSIS

The statistical analysis was designed to determine whether specific mutations in the *TLR4* gene were associated with several factors related to septic shock. We used a Fisher 1-sided exact test (consistent with our hypothesis) to determine whether specific mutations of the *TLR4* gene occurred more frequently in cases than controls.¹⁹ Similar comparisons were made between *TLR4* allele type and disease characteristics among those with septic shock. A Mann-Whitney test was used to determine the significance of continuous variables.¹⁹

scription factors nuclear factor- κB and activator protein 1.¹² These findings indicate that *TLR4* is a recognition molecule for gram-negative pathogens and specifically LPS.

The cloning of *TLR4* and its identification as a primary LPS receptor in mammals prompted us recently to investigate whether mutations in *TLR4* would modulate the response to LPS in humans. Using a reliable model of LPS-induced airflow obstruction,¹³ we demonstrated that specific mutations in *TLR4* (Asp299Gly and Thr399Ile) are associated with a diminished airway response to inhaled LPS in normal human volunteers.¹⁴

Since mice that contain mutations in *TLR4* are hyporesponsive to LPS and are also more susceptible to gram-negative bacterial challenge, we hypothesized that the mutations in human *TLR4* associated with hypore-

sponsiveness to inhaled endotoxin would also enhance the susceptibility of humans to gram-negative sepsis. To investigate this hypothesis, we studied the distribution of mutations in *TLR4* in healthy blood donors and in patients with septic shock. Our findings indicate that, although the overall frequency of *TLR4* mutations was similar in both populations, within the septic shock group patients with *TLR4* mutations had more severe disease and an increased frequency of gram-negative infections. In addition, mutations of *TLR4* involving only the 299 residue occurred exclusively in those with septic shock, and all of the patients with this isolated mutation had gram-negative septic shock. These findings indicate that mutations of the *TLR4* receptor may predispose individuals to gram-negative septic shock.

Table 1. Characteristics of the Study Subjects*

	Septic Shock	Blood Donors
No. of subjects	91	73
Age, mean (SD), y	57.7 (15.0)†	36.9 (11.6)
Sex, No. (%) male	63 (69.2)	51 (69.9)
<i>TLR4</i> Asp299Gly/Thr399Ile, No. (%)	6 (6.6)	8 (11.0)
<i>TLR4</i> Asp299Gly, No. (%)	5 (5.5)‡	0
<i>TLR4</i> mutant total, No. (%)	11 (12.1)	8 (11.0)

**TLR4* indicates toll-like receptor 4.

† $P < .001$.

‡ $P = .05$.

RESULTS

We studied 91 patients with septic shock and 73 healthy control subjects. The control subjects were on average younger (mean, 36.9 years; SD, 11.6 years) than the patients (average age, 57.7 years; SD, 15.0 years). The sex was evenly distributed between both study populations. Eleven patients (12%) and 8 control subjects (11%) had either the 299 or the 399 mutation in *TLR4* (**Table 1**), a frequency similar to what we reported previously.¹⁴ When the populations were tested for occurrence of the *TLR4* mutation (Asp299Gly/Thr399Ile), we observed a similar prevalence of the double mutation Asp299Gly/Thr399Ile in both the patients with septic shock and the control group (Table 1). However, the *TLR4* Asp299Gly mutation in the absence of a cosegregating mutation at residue 399 was found to occur only in the septic shock group. Importantly, 5 patients with septic shock (5.5%) had the Asp299Gly *TLR4* mutation, while this occurred in none of the control subjects ($P = .05$) (Table 1).

Among the patients with septic shock, we investigated the relationship between specific *TLR4* alleles and clinical characteristics of the disease process. While no significant differences were observed for age, sex, or treatment site (medical or surgical ICU) (data not reported), infections due to gram-negative organisms ranged from about 35% for patients carrying the wild-type *TLR4* locus or the *TLR4* Asp299Gly/Thr399Ile allele to 80% in patients with only the *TLR4* Asp299Gly allele ($P = .06$; **Table 2**). When we looked at gram-negative organisms occurring with/without polymicrobial infections, the numbers increased to 50% in the *TLR4* wild-type group and to 100% in patients with the *TLR4* Asp299Gly allele ($P = .04$; Table 2), whereas the percentage remained unchanged for patients carrying the *TLR4* Asp299Gly/Thr399Ile mutation.

We next investigated the relationship between the different alleles of *TLR4* and the primary sites of infection (**Table 3**). Although we found that meningitis occurred more frequently in those with both the *TLR4* Asp299Gly and the Thr399Ile mutations, the number of study subjects was small, and this association was no longer evident when the analysis was limited to patients with gram-negative sepsis. Since our in vitro data had suggested that the mutation at residue 299 had a more severe effect on *TLR4* function,¹⁴ we tested whether the different *TLR4* mutations had an effect on disease severity as measured by SAPS II, Organ System Failure score, and

mortality (**Table 4**). No significant differences were observed between the *TLR4* genotype and SAPS II values or mortality. While patients with the *TLR4* Asp299Gly allele had a higher Organ System Failure value than the other groups (Table 4), the difference was not significant ($P = .24$).

Of note, the only patient who was homozygous for the *TLR4* Asp299Gly allele in our study population was a 15-year-old girl who died of *Escherichia coli* pyelonephritis. This patient was admitted to the ICU with altered mental status, fever (temperature, 41°C), and abdominal pain. She was found to have pyelonephritis and was treated with fluids and appropriate antibiotics. Despite these treatments, she developed a dramatic septic shock and multiorgan failure (adult respiratory distress syndrome, anuria, refractory shock) and died at day 3 of hospitalization. Subsequent bacteriologic analysis showed that the antibiotic treatment was appropriate for a multisensitive *E coli* that was cultured from her blood and urine.

Previously, the -308 TNF- α polymorphism, TNF2, had been shown to be associated with sepsis in this population.¹⁵ We therefore investigated whether the presence of the -308 TNF- α polymorphism altered the relationship between *TLR4* polymorphisms and septic shock. In the patient population, the values for the TNF2 polymorphism ranged from 0% in the patients with the *TLR4* Asp299Gly mutation to 42.5% in the patients with wild-type *TLR4* ($P = .07$). Stratifying the septic subjects by those with ($n = 32$) and without ($n = 59$) the TNF2 (-308 TNF- α polymorphism), we did not observe any novel associations between the *TLR4* alleles and the type or severity of septic shock.

COMMENT

Our findings demonstrate that mutations in the human *TLR4* gene appear to be associated with an increased risk for septic shock and a higher prevalence of gram-negative bacterial infection. These findings suggest that mutations in the *TLR4* gene, which impair its responsiveness to LPS, could lead to an increased risk of subsequent bacterial infections, analogous to the in vivo findings in the mouse strain C3H/HeJ.²⁰ This hypothesis could explain our finding that the higher percentage of carriers for the *TLR4* polymorphisms may be associated with a higher risk of developing gram-negative sepsis in the patient population. Even though the patient and control populations differed significantly in age (see Table 1), the difference in polymorphism frequency is not age related, and the occurrence of the *TLR4* Asp299Gly polymorphism exclusively in the patient population indicates an association of specific *TLR4* mutations with septic shock. It is important to note that in previous studies the *TLR4* Asp299Gly polymorphism occurred solely in combination with the mutation at residue 399.¹⁴ The septic shock population used in this study constitutes the first example, to our knowledge, of a white population in which the *TLR4* Asp299Gly mutation occurs regularly in the absence of a second mutation at residue 399. Moreover, 1 case in our study, that of a 15-year-old girl homozygous for the *TLR4* Asp299Gly allele, who died

Table 2. Association of *TLR4* Genotype With Certain Bacteria in Study Subjects With Septic Shock*

	No. (%)			
	Wild-Type	Mutant	299/399	299 Only
No. of subjects	80	11	6	5
GNB with/without polymicrobial infection	40 (50.0)	7 (63.6)	2 (33.3)	5 (100.0)†
GNB alone	28 (35.0)	6 (54.5)	2 (33.3)	4 (80.0)
GPB	20 (25.0)	2 (18.2)	2 (33.3)	0
Yeast	2 (2.5)	1 (9.1)	1 (16.7)	0
Unknown	18 (22.5)	2 (18.2)	2 (33.3)	0
Others	1 (1.2)	0	0	0

*This table presents data for all bacterial infections in the study subjects. If infected with multiple organisms, a study subject may appear in more than one type of infection. *TLR4* indicates toll-like receptor 4; GPB, gram-positive bacteria; GNB, gram-negative bacteria.

†*P* = .04.

Table 3. Association of *TLR4* Genotype With Specific Disease in Study Subjects With Septic Shock*

	No. (%)			
	Wild-Type	Mutant	299/399	299 Only
No. of subjects	80	11	6	5
Respiratory	32 (40.0)	4 (36.4)	2 (33.3)	2 (40.0)
GI	17 (21.2)	2 (18.2)	1 (16.7)	1 (20.0)
Blood	8 (10.0)	1 (9.1)	0	1 (20.0)
Cutaneous/skin	2 (2.5)	1 (9.1)	1 (16.7)	0
Urinary tract	2 (2.5)	1 (9.1)	0	1 (20.0)
Meningitis (CSF)	3 (3.8)	2 (18.2)	2 (33.3)†	0
Other	9 (11.2)	0	0	0
Unknown	7 (8.8)	0	0	0

**TLR4* indicates toll-like receptor 4; GI, gastrointestinal tract; and CSF, cerebrospinal fluid.

†*P* = .04.

Table 4. Association of *TLR4* Genotype With Survival and Severity of Disease in Study Subjects With Septic Shock*

	Wild-Type	Mutant	299/399	299 Only
No. of subjects	80	11	6	5
Survival, No. (%)	36 (45.0)	5 (45.5)	3 (50.0)	2 (40.0)
OSF score, mean (SD)	2.9 (1.0)	3.2 (1.3)	2.8 (0.8)	3.6 (1.8)
SAPSII score, mean (SD)	54.6 (19.8)	58.2 (18.6)	59.2 (16.8)	57.0 (22.5)
TNF α -308 (TNF2), No. (%)	34 (42.5)	2 (18.8)	0	2 (40.0)

**TLR4* indicates toll-like receptor 4; OSF, Organ System Failure; SAPSII, Simplified Acute Physiology Score; and TNF, tumor necrosis factor.

of *E coli* pyelonephritis, illustrates the potentially detrimental effects of the *TLR4* Asp299Gly mutation in a homozygous state. It is possible that the increased severity of the 299 mutation in terms of impairing protein function, as shown by our previous in vitro analysis,¹⁴ causes a more severe effect in carriers for this mutation alone, making these patients more susceptible to bacterial infection.

The increased occurrence of the *TLR4* Asp299Gly genotype in the septic patients suggests that mutations of the *TLR4* receptor enhance the susceptibility to gram-negative sepsis because of a decrease in endotoxin responsiveness. These in vivo findings confirm our in vitro studies that suggest that the mutations at residues 299 and 399 interrupt LPS signaling.¹⁴ In this previous study, we demonstrated that the *TLR4* Asp299Gly allele and, to a much lesser degree, the *TLR4* Thr399Ile allele caused

THP-1 cells to be hyporesponsive to LPS in culture. The difference in mutation severity shown in vitro could potentially explain why the *TLR4* Asp299Gly allele is more frequent in this septic shock patient population than the *TLR4* Thr399Ile allele alone.

Since TLR4 is a transmembrane protein, it is not surprising to find a putative signal peptide at the N-terminus, presumably responsible for proper trafficking to the cell membrane.⁸ This raises the possibility that sequence variants in the extracellular domain of TLR4 can disrupt trafficking of this receptor to the cell membrane and enhance proteolysis.²¹ Mutations of this kind could give rise to a range of phenotypes from almost normal to completely unresponsive, depending on the severity of the trafficking defect, which would indirectly affect receptor density on the cell surface. Potential other effects of the mutant TLR4 receptor include decreased ligand binding or changes in the

receptor conformation that could impair signal transduction, once the ligand is bound. In the absence of any structural information on the extracellular domain of the TLR4 receptor, the potential structural effect of the TLR4 mutation remains unclear, although the immunostaining of the airway epithelial cells suggests an effect on receptor density.¹⁴

Another finding in this study is the high prevalence of gram-negative infections in patients with TLR4 receptor mutations. In addition to all patients with TLR4 receptor mutations having a higher prevalence of gram-negative infections, the 5 patients carrying the TLR4 Asp299Gly variant had solely gram-negative infections. Although this finding may not be statistically significant in a larger patient population, it could suggest that the increased severity of the TLR4 Asp299Gly could more severely attenuate the immune response to gram-negative infections among these individuals, thereby increasing the susceptibility to infections. Since TLR4 and LPS binding is an important first step in the defense against some gram-negative bacteria, TLR4 plays a crucial role in mediating the cellular response to recognize and eliminate these pathogens. The correlation between a potential lack of receptor function and an increased susceptibility to infection is analogous to what is seen in the TLR4^{-/-} mice¹¹ and the C3H/HeJ strain, which are both hyporesponsive to LPS but more susceptible to gram-negative infections.²⁰

The -308 TNF- α polymorphism and the TLR4 Asp299Gly polymorphism appear to be independent predictors of septic shock. While the -308 TNF- α polymorphism was found to occur more frequently among those with septic shock and found to be associated with enhanced mortality,¹⁵ in the current study we found that the TLR4 polymorphisms were associated with gram-negative septic shock. The absence of an interaction between these genotypes suggests that the effect of these polymorphisms on sepsis may be additive. Future investigations examining the interaction between several biologically relevant polymorphisms may provide important information to risk stratify patients with life-threatening infections.

In summary, we show a potential link between mutations in the TLR4 receptor and gram-negative septic shock. This preliminary finding warrants further studies with a larger patient population to determine whether some of the findings, such as a more severe disease phenotype, increased presence of gram-negative bacteria in particular, will reach statistical significance.

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