TLR4 and TNF-\(\alpha\) polymorphisms are associated with an increased risk for severe sepsis following burn injury

R C Barber, C C Aragaki, F A Rivera-Chavez, G F Purdue, J L Hunt, J W Horton

**Context:** Sepsis, organ failure, and shock remain common among patients with moderate to severe burn injuries. The inability of clinical factors to identify at-risk patients suggests that genetic variation may influence the risk for serious infection and the outcome from severe injury.

**Objective:** Resolution of genetic variants associated with severe sepsis following burn injury.

**Patients:** A total of 159 patients with burns \(\geq 20\%\) of their total body surface area or any smoke inhalation injury without significant non-burn related trauma (injury severity score (ISS) \(\geq 16\)), traumatic or anoxic brain injury, or spinal cord injury and who survived more than 48 h post-admission.

**Methods:** Candidate single nucleotide polymorphisms (SNPs) within bacterial recognition (TLR4 +896, CD14 −159) and inflammatory response (TNF-\(\alpha\) −308, IL-1\(\beta\) −31, IL-6 −174) loci were evaluated for association with increased risk for severe sepsis (sepsis plus organ dysfunction or septic shock) and mortality.

**Results:** After adjustment for age, full-thickness burn size, ethnicity, and gender, carriage of the TLR4 +896 G-allele imparted at least a 1.8-fold increased risk of developing severe sepsis following a burn injury, relative to AA homozygotes (adjusted odds ratio (aOR) 6.4; 95% confidence interval (CI) 1.8 to 23.2). Carriage of the TNF-\(\alpha\) −308 A-allele imparted a similarly increased risk, relative to GG homozygotes (aOR = 4.5; 95% CI 1.7 to 12.0). None of the SNPs examined were significantly associated with mortality.

**Conclusions:** The TLR4 +896 and TNF-\(\alpha\) −308 polymorphisms were significantly associated with an increased risk for severe sepsis following burn trauma.

Burn trauma offers certain advantages for the study of such associations. Notable among these is the highly quantifiable nature of the injury, in terms of burn depth and extent (burn size). The simple expression of burn size as a percentage of total body surface area (TBSA) adjusts for individual variation in body mass, enabling the direct comparison of patients. In addition, associated smoke inhalation can be diagnosed accurately. Finally, the typical burn patient is relatively young and free from comorbidity prior to the burn injury.

Identification of genetic polymorphisms that predispose burn patients to the most severe manifestations of infection would allow early targeting of high risk individuals for aggressive or novel treatment, potentially improving their clinical outcome. Eventually, rapid genetic screening of patients may enable physicians to tailor treatment to the individual, rather than the injury.

Selection of candidate single nucleotide polymorphisms (SNPs) was based upon location within a biologically relevant locus and evidence of a functional effect. Candidate SNPs were located within genes responsible for microbial recognition (TLR4 and CD14), as well as cytokine loci that are known to mediate the inflammatory response (TNF-\(\alpha\), IL-1\(\beta\), and IL-6). In addition, candidate SNPs had at least one report of an effect upon protein function or abundance or a previous report of an association with an inflammation or immune response related phenotype.

**Methods**

**Study design and data collection**

Patients admitted to the burn intensive care unit (BICU) unit at Parkland Memorial Hospital (Dallas, TX) with any smoke inhalation injury or with \(\geq 20\%\) TBSA burns were prospectively enrolled between April 1999 and December 2003, under a protocol approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and Parkland Memorial Hospital that waived the requirement for informed consent. In order to remove confounding variables that were unrelated to burn injury, individuals were excluded if they presented with significant non-burn related trauma (injury severity score (ISS) \(\geq 16\)), traumatic or anoxic brain injury, spinal cord injury, or if they failed to survive more than 48 h post-admission.

Clinical data were recorded daily and stored in a computerised database; concurrent with admission to the

**Abbreviations:** aOR, adjusted odds ratio; BICU, burn intensive care unit; ISS, injury severity score; LPS, lipopolysaccharide; PCR, polymerase chain reaction; SNPs, single nucleotide polymorphisms; TBSA, total body surface area
DNA isolation and genotyping
Venous blood samples were collected into EDTA and genomic DNA was isolated by standard protocols.24 Fragments containing each of the SNPs were amplified from genomic DNA by polymerase chain reaction (PCR) using Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN). All amplifications were carried out in a PTC 200 thermal cycler (MJResearch, Watertown, MA) using a thermal profile, reaction conditions, and primer sequences that were optimised for each SNP (table 1). All the genotypes examined in this study were determined by Pyrosequencing (Pyrosequencing AB, Westborough, MA). Pyrosequencing is a DNA sequencing technique that is based upon the detection of pyrophosphate, which is released in direct proportion to the number of incorporated nucleotides during DNA synthesis. The energy retained by pyrophosphate was utilised in an enzymatic cascade to cleave a targeted substrate and produce visible light. The quantity of light produced was measured and genotypes were resolved using PSQ 96 SNP Software, v 1.2 AQ. Each SNP was assayed with a specific primer sequence (table 1), which enabled the scoring of heterozygotes and alternate homozygotes with equal reliability.25 In addition, a representative sample of genotypes at each SNP was confirmed by restriction fragment length polymorphism and/or sequence analysis (data not presented).

Quantification of sepsis severity
Sepsis was defined according to the American College of Chest Physicians/Society of Critical Care Medicine consensus definitions. Severe sepsis was defined as sepsis that was temporally accompanied by the need for intravenous vasopressor drug support (excluding dopamine at ≤5 μg/kg/min) to maintain blood pressure (despite adequate fluid resuscitation) along with the presence of perfusion abnormalities, or metabolic acidosis (pH ≤7.30) or the development of respiratory, renal, hepatic, or haematological failure. Specifically, a patient was considered to have sepsis related organ failure if a Marshall organ dysfunction score of ≥3 (failure) was assigned in one or more organs concurrent with, or within 48 h of, the diagnosis of sepsis.26 Mild sepsis was defined as sepsis without any evidence of organ dysfunction or the need for intravenous vasopressor drug support to maintain blood pressure. Inhalation injury was defined as a history of being burned in a closed, smoke filled space, with or without positive bronchoscopy and acute respiratory distress syndrome (bilateral fluffy infiltrates in the absence of congestive heart failure, P:F<200, and the need for more than 96 h of mechanical ventilation, independent of airway protection).

Data analysis
Data analysis was performed using SPSS 10.1 (SPSS, Chicago, IL) and SAS 8.02 (SAS Institute, Cary, NC) statistical software. Descriptive statistics included counts and percentages for categorical variables and medians with associated 25th and 75th quartiles for continuous data. Categorical data were compared using the χ2 test, while continuous data were compared by Mann-Whitney U. Best sets multiple logistic regression was used to simultaneously evaluate the effects of multiple variables as risk factors for severe sepsis. Actual p values are reported for all analyses. The adjusted odds ratios (aORs) obtained from the regression analysis are presented with their associated 95% confidence intervals. Due to the low allele frequencies, homozygous carriers of the TNF-α −308A, TLR4 +896G, and IL-6 −174C-alleles were grouped with heterozygotes for analysis. Alternate homozygotes at the more common IL-1β −31 and CD14 −159 SNPs were analysed separately from heterozygotes. The IL-1β and CD14 SNPs were analysed both ways and the results did not differ (data not presented).

RESULTS
Demographics and clinical outcomes
Between April 1, 1999 and December 31, 2003, 159 patients admitted to the BICU at Parkland Memorial Hospital, Dallas, TX with ≥20% TBSA burns or any inhalation injury were enrolled. A subgroup of these patients was included in a previous report concerning the clinical features of sepsis and organ failure after burn trauma.24 Demographic and clinical outcome data are summarised in table 2 and described briefly below. Of the 159 patients, 25 (16%) died. The major causes of injury were flame (63%) or scalding (21%).

Table 1  Polymerase chain reaction (PCR) primer sequences, PCR amplification conditions, and lengths for each of the candidate SNP amplifiers

<table>
<thead>
<tr>
<th>Locus</th>
<th>Amplimer length</th>
<th>Primer sequences</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α −308</td>
<td>107 bp</td>
<td>PCR (For): AACGAATAGTTTTGAGGGGCAT  PCR (Rev): TCCCTGCTCCTCCATCCGC  Pyrosequencing (Rev): GCCTGAACCCCGTCC</td>
<td>One cycle: 95˚C, 4 min; 35 cycles: 95˚C, 30 s, 60˚C, 30 s, 74˚C, 30 s, one cycle: 74˚C, 6 min</td>
</tr>
<tr>
<td>IL-6 −174</td>
<td>111 bp</td>
<td>PCR (For): CGCTAGCGCTAATGAC  PCR (Rev): CGGTGGGGCTTGGATTTGAA  Pyrosequencing (Rev): CCCTGTTTGTGTCGTGC</td>
<td>One cycle: 95˚C, 4 min; 35 cycles: 95˚C, 45 s, 63˚C, 30 s, 74˚C, 45 s, one cycle: 74˚C, 6 min</td>
</tr>
<tr>
<td>IL-1β −31</td>
<td>222 bp</td>
<td>PCR (For): CCCCCATCTCTCTTCAAAGGC  PCR (Rev): AAGAGAGAGAGAAGAGAATAATGC  Pyrosequencing (Rev): TCCCTGCTGTTTITA</td>
<td>One cycle: 95˚C, 4 min; 35 cycles: 95˚C, 30 s, 55˚C, 30 s, 74˚C, 30 s, one cycle: 74˚C, 6 min</td>
</tr>
<tr>
<td>CD14 −159</td>
<td>193 bp</td>
<td>PCR (For): ATACCTCCTTTCCTCACACC  PCR (Rev): GCCCTTCCCTCTCTTGGGA  Pyrosequencing (Rev): TCAGGGAGGGGG</td>
<td>One cycle: 95˚C, 4 min; 35 cycles: 95˚C, 30 s, 64˚C, 30 s, 74˚C, 30 s, one cycle: 74˚C, 6 min</td>
</tr>
<tr>
<td>TLR4 +896</td>
<td>193 bp</td>
<td>PCR (For): ATGCTACGTTTTGCTCCTTGGG  PCR (Rev): CACTTACCAGGGGAAAAAT  Pyrosequencing (Rev): ACAATTTAAAAGTCAATTA</td>
<td>One cycle: 95˚C, 4 min; 35 cycles: 95˚C, 60 s, 55˚C, 60 s, 74˚C, 60 s, one cycle: 74˚C, 6 min</td>
</tr>
</tbody>
</table>

Pyrosequencing primer sequences are also presented.
Clinical risk factors for and outcome from severe sepsis

Of the 159 patients, 36 (23%) developed severe sepsis, defined as sepsis complicated by organ dysfunction or septic shock. Of these 36 patients with severe sepsis, 79% had evidence of a clinically relevant Gram-negative, or a mixed Gram-negative/Gram-positive infection (table 3). One patient with severe sepsis had candidiasis and two patients had infections of unknown characteristics. In the majority (63%) of patients, pneumonia was the cause of severe sepsis. Patients had relatively few comorbid medical conditions, with alcohol abuse (n = 28, 18%) and hypertension (n = 23, 14%) being reported most commonly. Pre-existing liver, lung, and renal disease was uncommon (<1%). Age, burn size, and gender were associated with severe sepsis.

Association between SNPs and outcome of burn injury

Unadjusted analysis

Genotype frequencies for the candidate SNPs among patients with and without severe sepsis are shown in table 3. The TNF-α −308A allele was associated with an increased risk for severe sepsis by unadjusted analysis. Patients who were carriers of the A-allele at TNF-α −308 had a 41% risk (16/39) for severe sepsis versus a 17% risk (20/120) for patients homozygous for the wildtype G-allele (p = 0.002). The unadjusted relative risk for severe sepsis associated with carriage of the TNF-α −308 A-allele was 3.47 (95% CI 1.56 to 7.73). Similarly, carriers of the TLR4 +896G allele had a slight, but non-significant increased risk (unadjusted relative risk = 2.46, 95% CI 0.88 to 6.90) for severe sepsis by unadjusted analysis (p = 0.08). Seven (39%) of the 18 G-allele carriers developed severe sepsis, versus 29 (21%) of the 141 AA homozygotes. Data for the remaining SNPs (CD14, IL-1β, and IL-6), which were not significantly associated with outcome, are included in table 4.

Logistic regression

In order to evaluate the candidate SNPs in the context of other potential risk factors, we performed a best sets multivariate logistic regression. Adjusting for age, burn size, ethnicity, and gender, carriage of the TNF-α −308 A-allele and the TLR4 +896 G-allele was significantly associated with an increased risk for severe sepsis. The aORs were 4.47 (95% CI 1.67 to 11.96) and 6.41 (95% CI 1.77 to 23.17) for the TNF-α −308 A-allele carriers developed severe sepsis, versus 29 (21%) of the 120 patients who were homozygous for the wildtype G-allele (p = 0.002). The unadjusted relative risk for severe sepsis associated with carriage of the TNF-α −308 A-allele was 3.47 (95% CI 1.56 to 7.73). Similarly, carriers of the TLR4 +896G allele had a slight, but non-significant increased risk (unadjusted relative risk = 2.46, 95% CI 0.88 to 6.90) for severe sepsis by unadjusted analysis (p = 0.08). Seven (39%) of the 18 G-allele carriers developed severe sepsis, versus 29 (21%) of the 141 AA homozygotes. Data for the remaining SNPs (CD14, IL-1β, and IL-6), which were not significantly associated with outcome, are included in table 4.

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Table 5  Risk factors for the development of severe sepsis after burn injury following adjustment for multiple factors

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>OR</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α −308 A-allele carriage</td>
<td>4.47</td>
<td>1.67 to 11.96</td>
<td>0.008</td>
</tr>
<tr>
<td>TLR4 +896 G-allele carriage</td>
<td>6.41</td>
<td>1.77 to 23.17</td>
<td>0.0013</td>
</tr>
<tr>
<td>Age ≥50 years</td>
<td>5.74</td>
<td>1.99 to 16.55</td>
<td>0.0001</td>
</tr>
<tr>
<td>Full-thickness burn ≥30% TBSA</td>
<td>4.62</td>
<td>1.79 to 11.88</td>
<td>0.0002</td>
</tr>
<tr>
<td>Female gender</td>
<td>3.84</td>
<td>1.47 to 10.00</td>
<td>0.0055</td>
</tr>
</tbody>
</table>

Odds ratios were determined by multivariate logistic regression using a “best sub-sets” model. This analytical model selected the most parsimonious set of prognostic factors that best described the risk for severe sepsis.

Table 6  The frequency of severe sepsis and mortality following burn injury in high versus low clinical risk groups

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Low clinical risk (n = 76)</th>
<th>High clinical risk (n = 83)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe sepsis</td>
<td>8 (11%)</td>
<td>28 (34%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Death</td>
<td>3 (4%)</td>
<td>22 (27%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The adverse outcomes of severe sepsis and death were more common among patients in the high clinical risk group. Clinical risk groups were stratified using age ≥50 years of age and/or full thickness burn size ≥30% TBSA as cut points.

Stratification on clinical risk

We hypothesised that the associations between the candidate SNPs and severe sepsis might have been influenced by the degree of clinical risk. Previous analysis of a large dataset determined that age ≥50 years and full-thickness burn size ≥30% TBSA identified patients at increased risk for death.23 Patients were categorised according to the presence or absence of these clinical risk factors (full thickness burn size ≥30% TBSA and/or age ≥50 years). The risk for severe sepsis and death according to clinical risk stratification is presented in table 6. A total of 76 (48%) patients were younger than 50 years of age with full-thickness burns covering less than 30% TBSA; these patients constituted the low risk group. Some 74 (47%) patients were either ≥50 years old or had ≥30% TBSA full-thickness burns and nine (6%) patients were both ≥50 years of age with ≥30% TBSA full-thickness burns. Therefore, a total of 83 patients were categorised into the high risk group. The association between A-allele carriage at the TNF-α 896 SNP and severe sepsis was similar in both the high and low risk groups (Mantel-Haenszel χ² = 0.05; p = 0.821). However, the increased risk for severe sepsis associated with the TLR4 +896 G-allele was limited to the low risk group (Mantel-Haenszel χ² = 8.04; p = 0.005). The unadjusted relative risk for the TLR4 +896 G-allele among low and high risk patients was 7.44 and 0.99, respectively, versus 2.25 and 2.26 for the TNF-α −308 A-allele (fig 1).

DISCUSSION

These results provide the first evidence that genetic polymorphisms within innate immunity loci are associated with an increased risk for severe sepsis after burn injury. Carriage of the TNF-α −308 A- or the TLR4 +896 G-allele was associated with an increased risk for developing severe sepsis. The increase in risk associated with carriage of variant alleles at these SNPs was comparable to the risk associated with traditional clinical factors such as age and full thickness burn size.

While there has been recent controversy regarding the validity of genetic association data, confidence in the legitimacy of our results was strengthened by independent evidence that the TLR4 +896 and TNF-α −308 SNPs have functional relevance. That is to say, alternate alleles at these SNPs seem to impact protein abundance or function. For example, recent studies have suggested that altered TLR4 signalling plays a role in bacterial resistance as well as the development of sepsis and septic shock.26–29 TLR4 is a transmembrane protein that initiates a signalling cascade that triggers an innate immune response to endotoxin.30–31 In mammals, TLR4 initiates a signal upon activation by CD14-bound lipopolysaccharide (LPS) that is transmitted through a series of adapter molecules and protein kinases resulting in nuclear translocation of NF-κB and a subsequent cascade of inflammatory cytokines and chemokines.32–35 The non-synonymous adenine to guanine (A→G) transition at nucleotide +896 of the human TLR4 mRNA that occurs within the extracellular domain of the TLR4 protein results in the substitution of asparagine with glycine at amino acid residue 299 (Asp299Gly). Carriers of the G-allele at this SNP exhibit reduced LPS responsiveness as well as an increased risk for septic shock and susceptibility to Gram-negative sepsis.26–29,36 Furthermore, transient transfection experiments in THP-1 cells indicated that the 299Gly allele was able to disrupt TLR4 signalling.37 Importantly, adenoviral transfection of a wild-type TLR4 construct was able to rescue LPS responsiveness in airway epithelial cells and alveolar macrophages derived from individuals with TLR4 mutations.38 Finally, associations between altered TLR4 signalling and a number of inflammatory disease states have been reported.11–18

Similarly, evidence of a functional effect exists for the TNF-α −308 SNP. Importantly, TNF-α has been demonstrated to play a major role in the pathogenesis of sepsis and its complications after burn trauma.39–42 Furthermore, there is evidence that the guanine to adenine substitution (G→A) at nucleotide −308 within the TNF-α promoter affects transcriptional regulation of TNF-α.3–4 Carriage of the less common A-allele at this position has been associated with unfavourable clinical outcomes and increased risk of acquiring several infectious and inflammatory diseases in a number of clinical settings.4,40–45

It was of interest that carriage of variant alleles at TLR4 and TNF-α have a seemingly opposite impact upon the amplitude of the endotoxin response. While carriage of the TLR4 +896G allele appears to decrease the intensity of the LPS response signal,46 the TNF-α −308A allele is associated with an increased rate of TNF-α transcription.47 In order to further investigate the effects of the TLR4 and TNF-α SNPs upon risk for severe sepsis, patients were stratified into low

![Figure 1](https://example.com/figure1.png)
and high clinical risk groups using two established clinical risk factors: full thickness burn size ≥30% TBSA and/or age ≥50 years. A Mantel-Haenszel test was used to evaluate interaction between clinical risk and carriage of variant alleles at the candidate SNPs. Interestingly, the test for homogeneity between high and low clinical risk groups was significant for the TLR4 +896 G-allele, indicating that the clinical risk status significantly impacted the effect of TLR4 +896 G-allele carriage. Carriage of the TLR4 G-allele was significantly associated with an increased risk for severe sepsis among low risk patients only. The biological implication of this observation may be that reduced toll pathway signalling by TLR4 +896 G-allele carriers fails to control local infection in cases of lower clinical risk and that these uncontrolled local infections proceed to become systemic. Alternatively, when the clinical risk is sufficiently severe, stimulation of the endotoxin response through TLR4 is strong enough to overcome a genetic predisposition for reduced toll pathway signalling.

In the case of the TNF-α SNP, the higher level of transcription that has been ascribed to the −308 A-allele appears to increase risk for severe sepsis in all cases. However, there is an important caveat to this reasoning: the Mantel-Haenszel results could have been statistically spurious. Stratification of our relatively small patient sample into clinical risk groups increased this possibility by effectively decreasing power. In addition, the results were the result of subgroup analysis, which is well known to inflate the likelihood of type I (false positive) error. In addition, although we adjusted for ethnicity in our logistic regression, unmeasured genetic differences in this diverse cohort may have contributed to the risk for severe sepsis in unappreciated ways. Allelic frequencies of many polymorphisms are known to vary across ethnic groups and this variation may either reduce statistical power by generating undetected heterogeneity within the genomic background or produce spurrous results if clinically relevant traits are in linkage disequilibrium with one or more candidate SNPs by chance. Further investigation with a much larger, preferably geographically distinct, patient population and/or the development of sophisticated statistical models will be required to fully resolve these issues.

In conclusion, this study provides strong evidence regarding the association of the TNF-α −308 promoter and TLR4 +896 coding region polymorphisms with an increased risk for severe sepsis following burn trauma. Furthermore, the independent predictive value of A-allele carriage at the −308 position in the TNF-α promoter and G-allele carriage at TLR4 +896 appear to impart the same risk as traditional clinical factors.

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Conflict of interest: none declared.

REFERENCES

TLR4 and TNF-α SNPs and severe sepsis risk


Retraction statement

The authors of the following manuscript Ninis VN, Kylick P, Kandemir M, Daely E, Tolun. High Frequency of 9T and CFTR Mutations in Children with Idiopathic Bronchiectasis. J Med Genet 2003;40:530–5, are retracting it because the polythymidine track genotype data are not correct. Recently the authors repeated the genotyping or 17 of the subjects to check whether the reported genotypes were correct and found out that they were not. At the time of submission of the manuscript, the authors were very confident of the data, since they had employed two independent methods for the genotyping of all subjects. However subsequently the authors were prompted to the recheck Vasiliki N Ninis results and have been unable to confirm them. The authors regret that we did not find out prior to publication.