

Innate Immunity SNPs are Associated with Risk for Severe Sepsis after Burn Injury

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Objective: To analyze allelic association with clinical outcome in a cohort of burn patients.

Patients: Two hundred twenty-eight individuals with burns $\geq 15\%$ total body surface area without significant non-burn related trauma who survived >48 hours post-admission were enrolled. One hundred fifty-nine of these patients were analyzed previously.

Methods: Candidate polymorphisms within interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), cellular differentiation marker 14 (CD14) and toll-like receptor 4 (TLR4) were evaluated by logistic regression analysis for association with increased risk for severe sepsis (sepsis plus organ dysfunction or shock).

Results: After adjustment for age, burn size, ethnicity, gender and inhalation injury, alleles at TNF- α (308G, $p=0.013$), TLR4 (+896G, $p=0.027$), IL-6 (174C, $p=0.040$) and CD14 (159C, $p=0.047$) were significantly associated with an increased risk for severe sepsis.

Conclusions: Carriage of variant alleles at immune response genes were associated with increased risk for severe sepsis after burn injury.

Keywords: Allelic association; Burns; CD14; IL-6; Polymorphism; Sepsis; SNP; TLR4; TNF- α

Burn injury remains an important cause of morbidity and mortality worldwide. Infectious complications, including sepsis, septic shock and sepsis-related organ failure, are common among patients with moderate to severe burn injuries. Sepsis accompanied by organ failure carries a 30-50% case-mortality rate, with surviving patients often requiring prolonged and costly medical care.¹⁻⁵

Although intensive research has yielded greater understanding of the pathophysiological processes that follow burn injury and clinical factors that predispose burn victims to severe sepsis and septic shock, we are still unable to predict accurately those patients who are at increased risk for infectious complications.¹ The lack of predictive value provided by demographic and clinical data, as well as a study of mortality risk in adopted children,⁶ suggests that genetic predisposition influences the risk for serious infection and outcome from severe injury. Knowledge of which genes and pathways are important in the host response to severe injury

and infection may provide insight into the molecular etiology of sepsis, facilitating the discovery of novel pharmacologic and therapeutic targets.

Methods

Patient Cohort

Under a protocol approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and Parkland Memorial Hospital, patients admitted to the burn intensive care unit at Parkland Memorial Hospital (Dallas, TX) with burns covering $\geq 15\%$ of their total body surface area (TBSA) were prospectively enrolled between April 1999 and February 2005. In order to remove confounding variables unrelated to burn injury, individuals were excluded if they presented with significant non-burn related trauma (injury severity score ≥ 16), traumatic or anoxic brain injury, or spinal cord injury, or if they failed to survive more than 48 hours post-admission. Sepsis was defined according to the American College of Chest Physicians and the Society for Critical Care Medicine consensus definitions. Severe sepsis

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Table 1. Summary statistics for demographic variables and clinical outcomes following burn injury.

Variable (n=228)	Median/count ¹
Age (years)	35 (21-48)
Male gender	172 (75%)
Ethnicity	
Caucasian	129 (56%)
Hispanic	51 (22%)
African American	41 (18%)
Other	7 (4%)
Percent of total body surface area burned	33 (23-49)
Percent of total body surface area full thickness burn	18 (9-38)
Burn mechanism	
Flame	188 (82%)
Scald	22 (10%)
Electrical	12 (5%)
Other	6 (3%)
Inhalation injury	55 (24%)
Died	40 (18%)
Intensive care unit length of stay (days)	16 (4-32)
Total length of stay (days)	27 (16-48)
Duration of mechanical ventilation (days)	10 (0-22)

¹Continuous data are presented as medians (25th-75th percentiles). Categorical data are presented as number of patients (percentages).

was defined as sepsis temporally accompanied by the need for intravenous vasopressor drug support (excluding dopamine at ≤ 5 $\mu\text{g}/\text{kg}/\text{min}$) to maintain blood pressure (despite adequate fluid resuscitation), along with the presence of perfusion abnormalities or metabolic acidosis ($\text{pH} \leq 7.30$) or the development of organ failure. Sepsis-related organ failure was defined as assignment of a Marshall organ dysfunction score of ≥ 3 (failure) in one or more organs (lung, kidney, liver or blood) within 24 hours prior to or 48 hours following a diagnosis of sepsis.⁷

DNA Isolation and Genotyping

Venous blood samples were collected in EDTA, and genomic DNA was isolated by standard protocols.⁸ Genotypes were determined by pyrosequencing⁹ and TaqMan[®] assays (Applied Biosystems, Inc., Foster City, CA). Details regarding genotyping methods have been published previously.¹⁰

Data Analysis

Data were analyzed with SPSS 14.0 statistical software (SPSS, Chicago, IL.). Categorical variables were compared using a chi-square test, and continuous data were analyzed by the Mann-Whitney U test. Multivariate logistic regression was used to simultaneously evaluate carriage of variant alleles as risk factors for severe sepsis in the context of demographic variables (age, ethnicity and gender) and injury characteristics

(burn size and inhalation injury). Exact p-values are reported for all analyses where $p > 0.001$.

Results

Demographics and Clinical Outcomes

Demographic variables, injury characteristics and clinical outcomes are presented in table 1. The patient cohort was relatively young (median age=35), primarily male (75%) and mostly Caucasian (56%). Forty (18%) of the 228 patients died. Major burn causes were flame (82%), scald (10%) and electrical (5%). Fifty-five (24%) patients developed severe sepsis, defined as sepsis complicated by organ dysfunction or septic shock. Pneumonia was the primary focus in 81% of patients with infection. Patients had relatively few co-morbid medical conditions. Alcohol abuse (43/228, 19%), hypertension (31/228, 13%) and peripheral cardiovascular disease (20/228, 9%) were most common. Pre-existing liver, lung and renal disease were rare (<3%).

Association between Single Nucleotide Polymorphisms (SNP) and Severe Sepsis Risk after Burn Injury

We evaluated allelic associations in the context of other potential risk factors by multivariate logistic regression analysis. After adjustment for age, ethnicity, gender, burn size and inhalation injury, variant alleles at tumor necrosis factor-alpha (TNF- α), toll-like receptor 4 (TLR4), interleukin-6 (IL-6) and cellular differentiation marker 14 (CD14) were significantly

Table 2. Risk factors for the development of severe sepsis after burn injury following adjustment for multiple factors.

Variable (n=228)	Odds Ratio	95% CI Odds Ratio	P-value
Age	1.010	0.991-1.028	0.305
Full-thickness burn size	1.025	1.011-1.040	0.001
Gender (female)	1.726	0.813-3.662	0.155
Inhalation injury	2.714	1.246-5.912	0.012
Race/ethnicity	1.197	0.828-1.729	0.339
CD14 -159C	1.668	1.007-2.763	0.047
IL-1 β -31C	1.272	0.593-2.726	0.537
IL-6 -174C	2.250	1.037-4.884	0.040
TNF- α -308A	2.602	1.229-5.512	0.013
TLR4 +896G	2.943	1.131-7.660	0.027

Odds ratios were determined by multivariate logistic regression. Age and full thickness burn size were analyzed as continuous variables. The odds ratios listed are for each additional year of age or each 1% increase in burn size. Race/ethnicity included the same categories as in table 1: Caucasian (n=129), Hispanic (n=51), African-American (n=41) and other (n=7).

associated with an increased risk for severe sepsis. Adjusted odds ratios (aOR) were 2.60 (95% confidence interval [CI] = 1.23-5.51), 2.94 (95% CI=1.13-7.66), 2.25 (95% CI=1.04-4.88), and 1.67 (95% CI=1.01-2.76) for the TNF- α 308A, TLR4 +896G, IL-6 174C, and CD14 159C alleles, respectively (table 2). The IL-1 β -31 SNP was not significantly associated with severe sepsis risk (p=0.537). Age and burn size were analyzed as continuous variables. Odds ratios represented the risk associated with each increased year of age or percent burn size. Clinical factors that were significantly associated with increased risk included inhalation injury (aOR=2.71, 95% CI=1.25-5.91, p=0.012) and full-thickness (3rd degree) burn size (aOR=1.03, 95% CI=1.01-1.04, p=0.001). Age, ethnicity, gender and pre-existing medical conditions did not significantly affect risk for severe sepsis (table 2).

Discussion

These results provide further evidence that genetic polymorphisms within innate immunity loci are associated with an increased risk for severe sepsis after burn injury. While there has been recent controversy regarding the validity of genetic association data, confidence in our results was strengthened by independent evidence that our candidate SNPs had functional relevance. That is to say, alternate alleles at these SNPs seem to impact protein abundance or function.

For example, studies have suggested that altered TLR4 signaling plays a role in bacterial resistance, as well as the development of sepsis and septic shock.¹¹⁻¹⁴ TLR4 is a trans-membrane protein which initiates a signaling cascade that triggers an innate immune response to endotoxin.^{15,16} A nonsynonymous substitution of guanine in place of adenine (A \rightarrow G) occurs at nucleotide +896 of the human TLR4 mRNA. This transition mutation precipitates substitution of

asparagine with glycine at amino acid residue 299 within the extracellular domain of the protein. Carriers of the G-allele exhibit reduced lipopolysaccharide (LPS) responsiveness, increased risk for septic shock and greater susceptibility to gram-negative sepsis.^{14,17,18} Furthermore, transient transfection experiments in THP-1 cells indicated that the 299Gly allele was able to disrupt TLR4 signaling.¹⁸ Importantly, adenoviral transfection of wild type TLR4 was able to rescue LPS-responsiveness in airway epithelial cells and alveolar macrophages derived from individuals with TLR4 mutations.¹⁸ Finally, associations between altered TLR4 signaling and increased susceptibility or poor outcome have been observed in several inflammatory disease states.¹⁹⁻²¹

CD14 initiates a response to Gram-negative organisms by binding bacterial LPS. Physical contact between CD14-bound LPS, MD-2 and TLR4 initiates signal transduction within macrophages.²²⁻²⁴ A cytosine to thymine (C \rightarrow T) substitution at nucleotide 159 alters Sp1 nuclear protein binding to the CD14 promoter, with higher constitutive and LPS-induced transcription rates for the T-allele.²⁵ The phenotypic impact of this polymorphism is uncertain. One study identified an association between this SNP and risk for myocardial infarction, but a second study did not.^{26,27} Findings have also been conflicting in patients with severe sepsis, with some studies observing no association and another finding a relationship between the T-allele and death from sepsis.^{17,28,29}

TNF- α has been demonstrated to play a major role in the pathogenesis of sepsis and its complications after burn injury.³⁰⁻³³ Furthermore, there is evidence that a guanine to adenine substitution (G \rightarrow A) at nucleotide -308 within the TNF- α promoter affects transcriptional regulation.^{34,35} Carriage of the A-allele at this position has been associated with altered transcription and increased risk for infectious and

inflammatory diseases in a number of clinical settings, including acute graft rejection following renal transplantation, sepsis among trauma patients, acute malaria and death from meningococcal disease.^{34,36-38}

IL-6 is known to be important to many cellular responses. Its functional profile includes pro- and anti-inflammatory activities, regulation of T-cell activation, induction of antibody production, activation of coagulation and stimulation of hematopoiesis.³⁹ Genetic variants within the promoter region have been shown to regulate IL-6 production, and may therefore influence the activity of IL-6 *in vivo*.⁴⁰⁻⁴² A guanine to cytosine (G→C) substitution at nucleotide -174 of the IL-6 promoter has been reported to affect gene transcription and has been associated with a number of conditions in which inflammation is considered to play a role.^{21,43,44}

Allelic associations with severe sepsis were evaluated previously for 159 of the 228 patients described in this report.¹⁰ The present study included patients with ≥15% TBSA burns, compared to patients with ≥20% burns in the previous analysis. Data from the initial investigation generally agreed with the current results, although several interesting differences were apparent. Both studies indicated full-thickness burn size and allele-carriage at TNF-α and TLR4 were important risk factors. In the previous report, inhalation injury was not significantly associated with severe sepsis risk, while in the current analysis, inhalation injury was a strongly associated risk factor (p=0.012). Age and gender exhibited the opposite pattern. Both were significantly associated with increased risk in the original study but not in the current report. In contrast to the original investigation, the analyses we present here showed that carriage of IL-6 -174C and CD14 -159C alleles was significantly associated with increased severe sepsis risk (p=0.040 and 0.047, respectively). Finally, odds ratios for risk alleles at TNF-α and TLR4 were considerably higher in the initial report than those we report here (TNF-α, 4.5 vs 2.6; TLR4, 6.4 vs 2.9). Although if the lower limits of the 95% CI for the odds ratios are compared, the difference is much less dramatic (TNF-α, 1.7 vs 1.2; TLR4, 1.8 vs 1.1).

These differences are likely to be the result of larger sample size and subsequently increased statistical power in the

current report. Narrower 95% CI for the odds ratios reported in the present study support this explanation. However, it is also possible that there was some systematic difference in the 69 additional patients that were included in the present study.

It is possible that reducing the minimum total burn size for inclusion in the study from 20% to 15% TBSA affected the outcome by allowing inclusion of a radically different patient group. However, this seems unlikely, since only eight of 228 patients had <20% TBSA burns, and these cases were clinically unremarkable. To investigate the additional patients further, we compared characteristics of the originally reported sample of 159 patients to the additional group of 69 patients (table 3). As expected, adjustment of eligibility criteria to include patients with smaller burns resulted in smaller median total and full-thickness burn size for the additional group of patients. Median age, gender distribution and rate of severe sepsis were roughly equivalent between the two groups. The mortality rate was higher among the more recent group of patients (21% vs 16%) as were frequencies of the TNF-α and TLR4 risk alleles. Unexpectedly, given the increased incidence of mortality, inhalation injury and carriage of the CD14 C-allele were less frequent among recently enrolled patients.

We still do not know all the genetic and environmental factors that influence severe sepsis risk after burn injury. Because we are unable to adjust for these unknown risk factors, some groups of patients may appear to have spurious results with respect to known risk factors. This effect is expected to be most pronounced among small groups of patients, demonstrating the essential nature of large sample sizes for accurate estimation of effect sizes. When large numbers of patients are evaluated, unmeasured variables tend to be evenly distributed across known risk factors and have a reduced impact on results.

Conclusion

This study provides additional evidence for associations between polymorphisms within innate immunity loci and an increased risk for severe sepsis after burn injury. In addition, when contrasted with an earlier examination of this patient population, these results illustrate the importance of sample size and statistical power in allelic association studies.

Table 3. Characteristics of the original 159 patients compared to the additional 69 patients included in the current study.

Sample	Died	Severe sepsis	Median age	3 rd degree burns	Total burn	Male Gender	Inhalation injury	CD14 C-allele	TNF-α A-allele	TLR4 G-allele
Original report (159)	16%	24%	35	20%	35%	74%	29%	81%	25%	11%
Additional patients (69)	21%	23%	35	15%	29%	76%	22%	74%	32%	13%

Age and gender were equivalent between the two groups. As is evident, the death rate was higher in the recently enrolled patients, despite lower incidence of severe sepsis and inhalation injury. In addition, total and 3rd degree burn size was smaller in the more recent patient group. Although frequency of the CD14 risk allele was lower, risk alleles at both TNF-α and TLR4 were more common among recently enrolled patients.

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