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ORIGINAL PAPER

Mortality in adult intensive care patients with severe systemic inflammatory response syndromes is strongly associated with the hypo-immune *TNF* −238A polymorphism

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Abstract The systemic inflammatory response syndrome (SIRS) is associated with activation of innate immunity. We studied the association between mortality and measures of disease severity in the intensive care unit (ICU) and functional polymorphisms in genes coding for Toll-like receptor 4 (TLR4), macrophage migratory inhibitory factor (MIF), tumour necrosis factor (TNF) and lymphotoxin-alpha (LTA). Two hundred thirty-three patients with severe SIRS were recruited from one general adult ICU in a tertiary centre in the UK. DNA from patients underwent genotyping by 5′ nuclease assay. Genotype was compared to phenotype. Primary outcome was mortality in ICU. Minor allele frequencies were TLR4 +896G 7%, MIF 173C 16%, TNF −238A 10% and LTA +252G 34%. The frequency of the hypoimmune minor allele TNF −238A was significantly higher in patients who died in ICU compared to those who survived (p=0.0063) as was the frequency of the two haplotypes LTA +252G, TNF −1031T, TNF −308G, TNF −238A and LTA +252G, TNF−1031T, TNF−308A and TNF−238A (p=0.0120 and 0.0098, respectively). These findings re-enforce the view that a balanced inflammatory/anti-inflammatory response is the most important determinant of outcome in sepsis. Genotypes that either favour inflammation or its counter-regulatory anti-inflammatory response are likely to influence mortality and morbidity.

Keywords Systemic inflammatory response syndrome · Toll-like receptor 4 · Macrophage migration inhibitory factor · Polymorphism (genetics) · Tumour necrosis factor alpha · Severe sepsis

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Introduction

The systemic inflammatory response syndrome (SIRS) is associated with an activation of the innate immune system and can occur after the release of endogenous intracellular or membrane-bound molecular signals (e.g. following pancreatitis, polytrauma, surgery or ischaemia–reperfusion injury) or after exposure to a variety of exogenous pathogen associated molecular patterns (PAMPs; Leaver et al. 2007; Sutherland and Russell 2005). Sepsis is defined by the presence of SIRS in the context of presumed or proven infection. Both SIRS and sepsis can lead to an indistinguishable clinical phenotype involving multiple organ dysfunction syndrome (Bone et al. 1992a).

Approximately 29% of intensive care unit (ICU) admissions in the UK are due to severe sepsis. ICU mortality for these patients in 2004 was 31% and hospital mortality was 45% (Harrison et al. 2006). Up to 75% of patients labelled as having severe sepsis are culture negative and yet have a similar associated mortality (Rangel-Frausto et al. 1995).

The innate immune response to PAMPs and endogenous pro-inflammatory molecules involves interaction or modulation of pattern recognition receptors (PRRs) in the host (Leaver et al. 2007; Sutherland and Russell 2005). Those involved in the recognition of lipopolysaccharide (LPS) include Toll-like receptor 4 (TLR4), CD14, LPS-binding protein and MD-2. Together, these molecules constitute the LPS receptor complex (Bosshart and Heinzelmann 2007). TLR4 expression can be modulated by macrophage migration inhibitory factor (MIF; Roger et al. 2001b). Its interaction with LPS leads to a series of intracellular signalling events involving interleukin-1 receptor-associated kinase 1 and a variety of adapter proteins. This signalling cascade leads to production of the pro-inflammatory cytokines responsible for SIRS (for example TNF-α), and anti-inflammatory cytokines associated with the compensatory anti-inflammatory response syndrome (CARS) (Osuchowski et al. 2006).

Both SIRS and CARS can be influenced by genetic variation (Holmes et al. 2003). We chose a candidate gene approach, which directly tests the effects of genetic variants of a potentially contributing gene in an association study. This was performed to investigate phenotype–genotype associations in ICU patients with SIRS and organ dysfunction caused by either infectious or non-infectious triggers.

TLR4 is a transmembrane receptor for LPS. A single nucleotide polymorphism (SNP) in the TLR4 gene, resulting in an aspartate (Asp) to glycine (Gly) substitution at amino acid 299, induces hypo-responsiveness to endotoxin in humans (Arbour et al. 2000). MIF is a pro-inflammatory cytokine that up-regulates TLR4 expression (Roger et al. 2001a,b). A guanine (G) to cytosine (C) substitution at position −173 in the MIF gene increases immune responsiveness (De Benedetti et al. 2003; Donn et al. 2002; Donn et al. 2001; Renner et al. 2005).

The inflammatory cytokines lymphotoxin alpha (LTA) and tumour necrosis factor alpha (TNF-α) are intimately involved in the inflammatory process orchestrated by innate immunity. TNF-α is secreted by macrophages in response to both infectious and non-infectious stimuli. It causes apoptosis in many cell lines, activates neutrophils, and has effects on endothelial function, the liver (induction of acute phase proteins), and lipid metabolism (Locksley et al. 2001). In contrast to TNF-α, LTA is expressed and released by lymphocytes. TNF-α and LTA form the integrated signalling network necessary for efficient innate and adaptive immune responses (Ware 2005). A number of SNPs in the LTA/TNF region have been identified including LTA +252*A/G and TNF −238*G/A, which may play a role in regulating transcription levels of either or both genes (Bayley et al. 2004; Knight et al. 2004).

We performed a single-centre genetic association study to assess the influence of these polymorphisms on various measures of outcome in ICU patients with severe sepsis/SIRS. We found a strong association between the hypoimmune TNF −238A allele and mortality in ICU.

Materials and methods

Inclusion criteria

Patients with severe sepsis/SIRS were recruited from the Southampton General Hospital ICU after fully informed assent from their closest relatives. The inclusion criteria were the presence of SIRS as defined by the presence of three or more SIRS criteria (tachycardia, tachypnoea or mechanical ventilation, abnormal white cell count or abnormal body temperature) plus evidence of at least one organ dysfunction (hypoxaemia, hypotension, acidosis, oliguria, thrombocytopenia, coagulopathy or reduced Glasgow coma score) (Bone et al. 1992b; Levy et al. 2003) at any point after their admission to ICU. This study was approved by the Southampton & South West Hants Local Research Ethics Committee.

Primary outcome measure

Our primary outcome measure was the association between the four SNPs identified in genes encoding TLR-4 (+896A/G, rs4986790), MIF (−173G/C, rs755622), LTA (+252A/G, rs909253) and TNF-α (−238G/A, rs361525) and ICU mortality. In addition further SNPs around the TNF locus were analysed in order to carry out haplotype analysis (−1031T/C, rs1799964; −308G/A, rs1800629). Secondary outcomes included hospital mortality, ICU length of stay, Acute Physiology Age and Chronic Health Evaluation II (APACHE II) score (Knaus et al. 1985), daily Sequential Organ Failure Assessment (SOFA) scores (Vincent et al. 1996) and microbiological evidence of infection.

Genotyping

Blood samples were collected in EDTA tubes and stored at −18°C. DNA was extracted using published methods (Miller et al. 1988). DNA was genotyped by 5′ nuclease assay (Taqman™; de Kok et al. 2002; Livak 1999).

Table 1 ICU mortality by demographics

	ICU outcome		Hospital outcome		
	Survived	Died	Survived	Died	Total
Mean age (range)	58 (18–95)	64 (26–90)	56 (18–84)	67 (26–95)	59 (18–95)
Diagnosis (%)					
Sepsis	69 (73.4)	25 (26.6)	58 (61.8)	36 (38.2)	94 (43.7)
Pancreatitis	11 (91.7)	1 (8.3)	10 (83.3)	2 (16.7)	12 (5.6)
Other surgical	40 (90.9)	4 (9.1)	36 (81.8)	8 (18.1)	44 (20.5)
Other medical	51 (78.5)	14 (21.5)	43 (66.2)	22 (33.8)	65 (30.2)
Total	171 (79.5)	44 (20.5)	147 (68.4)	68 (31.6)	215 (100)
Mean APACHE score	19.3	23.9	18.8	23.3	20.2
Mean SOFA score	6.56	11.3	6.51	9.61	7.48

Statistical analysis and power calculation

Using chi-square analysis, Fisher's exact test or *t* test (SPSS Version 14) as appropriate, genotypes were compared to phenotypes. A $p<0.05$ (two-tailed) was considered significant. As there may be more than one functional SNP within the TNF-LTA region and/or the tested SNPs may not be directly causal, but are in linkage disequilibrium with another causal SNP(s), analysis of TNF-LTA SNP haplotypes was undertaken using Haploscore (Reilly et al. 2002). This uses score statistics to test associations between haplotypes and both dichotomous and quantitative traits and allows for adjustment for non-genetic covariates.

The software 'PS' was used to calculate power (Dupont and Plummer 1990). With our sample size, genetically determined increase in risk would need to be in the order of magnitude of 2.7–3.1 to be detected with a power of 80% and a $p\leq0.05$.

Results

A total of 233 adults were recruited (mean age, 59 years) by four students between May 2000 and October 2005 out of a total of 4,964 patients admitted during this period. There was no formal screening log. Eighteen patients were excluded (five insufficient DNA, six readmissions, three non-Caucasian, two contaminated DNA, one transferred from another ICU and one incomplete phenotypic data). A total of 215 patients remained for analysis. This includes 94 patients recruited as part of a previous smaller study (Child et al. 2003). ICU mortality was 20.5%, and hospital mortality was 31.6%. Both mortality statistics are significantly lower than those reported in severe sepsis and probably reflect our decision to include patients with SIRS and end organ dysfunction but in whom infection was neither proven nor suspected (Brun-Buisson 2000); (Harrison et al. 2006). Patient demographics are shown in Table 1.

Minor allele frequencies were TLR4 +896G 7%, MIF −173C 16%, TNF −238A 10% and LTA +252G 34%. Genotype frequencies (Table 2) were similar to previous reports in the Caucasian population and observed Hardy–Weinberg equilibrium.

There was an association between TNF −238 genotype and ICU mortality (Fisher's exact test=6.43, $p=0.033$; Table 3). When TNF −238A allele frequency and mortality in ICU were compared, the association was even greater (Pearson chi-square=7.46, $p=0.0063$; Table 4). Haplotype analysis for the TNF-α polymorphisms was carried out using Haploscore (Table 5). Nine haplotypes at >0.5% frequency were predicted to account for 99% of the total haplotypes in the study population. A positive haploscore refers to death in ICU and a negative haploscore to survival.

Table 2 Genotype frequency

Gene	Homozygous wild type	Heterozygous	Homozygous mutant type	Minor allele frequencies (%)[a]
TNF-α −238	162 GG	28 GA	5 AA	10
LTA +252	88 AA	88 AG	25 GG	34
TLR4 +896	182 AA	25 AG	2 GG	7
MIF −173	133 GG	62 GC	1 CC	16

[a] Minor allele frequencies calculated from genotype frequencies in total cohort.

Table 3 TNF-238 Genotype frequency and mortality from severe SIRS

TNF-238 genotype	Died in ICU (*N*=39) Genotype frequency (*N*)	Survived ICU (*N*=156) Genotype frequency (*N*)
GG	0.72 (28)	0.86 (134)
GA	0.20 (8)	0.13 (20)
AA	0.08 (3)	0.01 (2)

$p<0.033$, Fisher's exact test, 3×2 analysis of genotype frequencies and mortality

Table 4 TNF-238 Allele frequency and mortality from severe SIRS

TNF −238 allele	Died in ICU Allele frequency (*N*)	Survived ICU Allele frequency (*N*)
A	0.18 (14)	0.08 (24)
G	0.82 (64)	0.92 (288)*

*$p<0.0063$, Pearson chi-square, 2*df*, analysis of allele frequencies and mortality

Two haplotypes (ht 8 and 9), which both carry the TNF −238A allele, showed strong association with death in ICU (Hap-score=2.511, $p=0.012$ and Hap-score=2.582, $p=0.00981$, respectively) but are present at a very low frequency (1.6% and 0.5%, respectively). There were no other significant associations found.

Discussion

This is the first study to demonstrate a significant association between the TNF −238A polymorphism and outcome in severe sepsis/SIRS. This SNP is known to be strongly associated with psoriasis (Rahman et al. 2006), and in this clinical context, previous studies using reporter assays have shown that this polymorphism reduces TNF-α gene transcription (Kaluza et al. 2000). In addition, when peripheral blood mononuclear cells from these patients are stimulated, with either T-cell mitogens or streptococcal antigens, significantly less TNF-α is seen in plasma than in controls (Kaluza et al. 2000), suggesting that this SNP has functional significance. The sequence of base pairs on the TNF promoter gene between positions −238 and −254 is known to contain the binding site for a repressor of TNF-α gene transcription (Fong et al. 1995). It is possible that the TNF −238A polymorphism enhances the affinity of this repressor protein for its binding site resulting in lower plasma TNF-α levels.

The results of a previous study (Gordon et al. 2004) contradict ours. They found no association between the TNF −238A allele and mortality in severe sepsis. This may be because the mortality impact of the pro-/anti-inflammatory cytokine balance is greater in our cohort whose phenotype is broader than that previously studied (Westendorp et al. 1997; Docke et al. 1997).

Haplotype analysis showed significant associations between haplotypes 8 (LTA +252G, TNF-1031T, TNF-308G and TNF-238A) and 9 (LTA +252G, TNF-1031T, TNF-308A and TNF-238A) and ICU mortality (*p* values, 0.0098 and 0.0120, respectively). This may reflect the result of a functional influence of the TNF-238A polymorphism or represent an important interaction between a wider range of different polymorphisms that exist in linkage disequilibrium. It should be noted that there is a very low frequency of carriers of these haplotypes. Much larger studies will be required to accurately assess the significance of haplotype variation in this gene region. Furthermore, LTA and TNF-α are homologous, and their genes are in significant linkage disequilibrium (Locksley et al. 2001) with each other and also with the extensive HLA locus. Consequently, any observed genotype–phenotypic associations at the TNF-LTA locus could instead represent an effect due to TNF, LTA or indeed other genes in the HLA class III region.

The traditional view of severe sepsis/SIRS implies that PAMPs interact with host PRRs to elicit an innate immune response. A principle PRR is the LPS receptor complex described above. Downstream signalling consequences of this cell surface interaction include gene transcription and the production of pro-inflammatory cytokines, principal amongst these being TNF-α. If hyper-stimulation leads to hyper-inflammation, then not only pathogen eradication but

Table 5 TNF-LTA haplotypes versus ICU outcome

Haplotype	Genotype				Hap-Freq	Hap-Score	*p* value
	LTA +252	TNF −1031	TNF −308	TNF −238			
ht1	G	T	G	G	0.194	−1.0041	0.315
ht2	A	T	G	G	0.432	−0.8524	0.394
ht3	A	C	G	G	0.130	−0.6540	0.513
ht4	G	T	A	G	0.132	0.0407	0.968
ht5	A	C	G	A	0.058	1.2034	0.229
ht6	A	T	G	A	0.017	1.6411	0.101
ht7	A	T	A	G	0.011	1.8521	0.064
ht8	G	T	G	A	0.016	2.5114	0.012
ht9	G	T	A	A	0.006	2.5825	0.010

Haploscore analysis using ICU outcome. A positive score denotes increased risk of death in ICU and a negative score a lower risk

also host damage might occur. In this way, an unbalanced innate immune response may predispose to both organ dysfunction and an increase in morbidity and mortality.

However, studies have suggested that down-regulated innate immune responses, including B- and T-cell apoptosis, reduced inflammatory cytokine production in response to challenge, and a shift toward type-2 helper T cells are detrimental (Ertel et al. 1995; Hotchkiss et al. 1999; Lederer et al. 1999; O'Sullivan et al. 1995). This study re-emphasises the importance of a balanced inflammatory response to any given insult and its importance in determining outcome.

The analysis of the TLR4 +896 polymorphism failed to replicate our previous observation that it was associated with an increased risk of mortality in patients with severe SIRS based on a sub-population of this cohort. This may result from the possibility that the original observation was a type I error. However, given the size of the current cohort and the relatively low frequency of this polymorphism in the general population, we cannot make any definite conclusions as to the role of this polymorphism in the outcome of severe SIRS.

Potential limitations should be addressed. Survivor bias may have occurred due to death of the sickest patients before recruitment, and this might underestimate any association. Multiple comparisons were performed, increasing the chance of type I error; nevertheless, ICU mortality was the a priori outcome measure, and secondary measures of disease severity such as APACHE II and SOFA were inter-related, making strict correction for multiple comparisons less relevant. Nonetheless, correcting for the number of SNPs analysed (n=4), the association of TNF-α −238 allele frequency with ICU mortality remains significant (Pc=0.0252); however, our findings require replication in other patient cohorts.

No other significant associations were found between genotypes and secondary outcomes such as hospital mortality, disease severity scores or microbiology. Although our patient cohort was one of the larger groups in studies of its kind, it still may be under powered. As previously discussed, a genetically determined increase in risk would need to be quite large, in the order of magnitude of 2.7–3.1, to be detected. The lack of other associations may reflect an effect too small to demonstrate with this sample size.

This study should be repeated in another centre, with larger numbers. In particular, any further investigations should use agreed methods to interpret the results of multiple association studies. To assess the temporal relationship of TNF-α levels to mortality in sepsis, whole-blood TNF-α assays could be taken on sequential days. In addition, wider markers could be taken for TNF to assess the extent of linkage disequilibrium in the region. This could contribute to the mapping of any etiologically significant SNP.

Conclusion

Identifying genetic markers associated with outcome in severe SIRS could aid the development of novel therapeutic agents, help identify high risk patients and target expensive new therapies (e.g. recombinant activated protein C) to those ICU patients at greatest risk. The results of this study support the notion that genetic factors may predispose to worse outcomes in severe SIRS, as highlighted in the recent predisposing conditions, insult, response and organ dysfunction staging of sepsis.

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Conflict of interest statement The authors declare that they have no conflict of interest.

Contributions VJP, IAY and JWH designed the study. TC, NJAC, DKM, SMN, MCKP and KdeCG recruited patients. TC, NJAC, DKM, SNM, MR-Z and IAY performed genotyping. VJP, TC, SB and IAY undertook statistical analysis. VJP, TC and JWH drafted the manuscript.

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