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A Single Nucleotide Polymorphism in NF-κB Inducing Kinase Is Associated with Mortality in Septic Shock

Simone A. Thair,* Keith R. Walley,* Taka-aki Nakada,* Melissa K. McConechy,† John H. Boyd,* Hugh Wellman,‡ and James A. Russell*

We tested the hypothesis that single nucleotide polymorphisms (SNPs) within genes of the NF-κB pathway are associated with altered clinical outcome of septic shock patients. We genotyped 59 SNPs in the NF-κB pathway in a discovery cohort of septic shock patients (St. Paul’s Hospital [SPH], N = 589), which identified the C allele of rs7222094 T/C within MAP3K14 (NF-κB inducing kinase; NIK) associated with increased 28-d mortality (uncorrected p = 0.00024, Bonferroni corrected p = 0.014). This result was replicated in a second cohort of septic shock patients (Vasopressin and Septic Shock Trial [VASST; N = 616]) in which the CC genotype of rs7222094 was associated with increased 28-d mortality (Cox regression: SPH cohort hazard ratio [HR], 1.35; 95% confidence interval [CI], 1.12–1.64; p = 0.002 Caucasian only; and VASST cohort HR, 1.24; 95% CI, 1.00–1.52; p = 0.048 Caucasian only). Patients having the CC genotype of rs7222094 in SPH experienced more renal and hematological dysfunction (p = 0.003 and p = 0.011), while patients of the VASST cohort with the rs7222094 CC genotype showed the same trend toward more renal dysfunction. In lymphoblastoid cell lines, we found the rs7222094 genotype most strongly associated with mRNA expression of CXCL10, a chemokine regulated by NF-κB. Accordingly, we measured CXCL10 protein levels and found that the CC genotype of rs7222094 was associated with significantly lower levels than those of the TT genotype in lymphoblastoid cell lines (p < 0.05) and in septic shock patients (p = 0.017). This suggests that the CC genotype of NIK rs7222094 is associated with increased mortality and organ dysfunction in septic shock patients, perhaps due to altered regulation of NF-κB pathway genes, including CXCL10. The Journal of Immunology, 2011, 186: 2321–2328.
Materials and Methods

Patient cohorts

St. Paul’s Hospital cohort (discovery cohort). All patients admitted to the St. Paul’s Hospital (SPH) Intensive Care Unit (Vancouver, British Columbia, Canada) between July 2000 and January 2004 were screened. Using the current consensus definition (≥ 2 SIRS criteria, known or suspected infection, hypotension unresponsive to fluid resuscitation alone) 601 patients who had septic shock on admission and had DNA available (12, 13). Twelve patients in this cohort had also been enrolled in the Vasopressin and Septic Shock clinical trial (14) and were therefore excluded. Thus 589 patients in total were included in this analysis. This study was approved by the Institutional Review Board at SPH and the University of British Columbia.

VASST cohort (replication cohort). The Vasopressin and Septic Shock Trial (VASST) was a multicenter, randomized, double-blind, controlled trial evaluating the efficacy of vasopressin versus norepinephrine in 779 patients who were diagnosed with septic shock according to the current consensus definition (15). Clinical phenotyping has been described elsewhere (14). All patients were enrolled within 24 h of meeting the definition of septic shock, and DNA was available from 616 patients. The research ethics boards of all participating institutions approved this trial, and written informed consent was obtained from all patients or their authorized representatives. The research ethics board at the coordinating center (University of British Columbia) approved the genetic analysis.

SNP selection and genotyping of patient cohorts

We used Ingenuity IPA (version 8.6, build 93815, content 3003) to identify 19 cytosolic genes in the NF-κB canonical and noncanonical pathway that also had dense resequencing data publicly available (Supplemental Fig. 1) (Seattle SNPs Program for Genomic Applications, http://pga.mbt.washington.edu/; Cardiogenomics, http://cardiogenomics.med.harvard.edu/home; Innate Immunity, http://www.pharmgat.org/; NCI CARD, http://pga.jgi-psf.org/; and SouthWestern PGA, http://pga.swmed.edu/). TagSNPs were identified for genotyping in patient cohorts using a linkage disequilibrium-based tag SNP selection method (16) and using an r² threshold of 0.65 for SNPs with a minor-allele frequency >5% yielding 59 SNPs in 19 cytosolic genes; receptor and their ligands as well as downstream gene targets of NF-κB signaling were excluded (Table I). DNA was extracted from peripheral blood samples using a QIAamp DNA Blood Midi Kit (Qiagen, Mississauga, Ontario, Canada) and genotyped using the Illumina Golden Gate Assay at the McGill University and Genome Quebec Innovation Centre, Montreàl, Queàbec, Canada. Primers for sequencing the region surrounding rs7222094 in 85 CEPH population representatives. The research ethics board at the coordinating center (University of British Columbia) approved the ethical collection and use of DNA samples as well as their genotype information.

Clinical phenotype

The primary outcome was 28-d mortality. Secondary outcomes were days alive and free of organ dysfunction during the first 28 d calculated according to the Brussels criteria (15).

Biological plausibility experiments

Microarray mRNA expression analysis in vitro. Lymphoblastoid DNA from the Coriell Institute was genotyped for rs7222094 in 85 CEPH population samples using Sanger sequencing of the region. Sequencing was performed at the McGill University and Genome Quebec Innovation Centre (Montreàl, Queàbec, Canada). Primers for sequencing the region surrounding rs7222094 are as follows: forward, 5'-GGGTTCCCTATGGAGGAGAG-3'; reverse, 5'-CTGTCCAGCTCTCCAGGTTC-3'. These 85 CEPH population lymphoblastoid cell lines of known genotype for NIK rs7222094 were cultured in RPMI 1640 and subsequently stimulated by the addition of Cytomix (9, 17–19) (2.5 ng/ml of each TNF-α, IL-1β, IFN-γ [R&D Systems] and 12.5 μM CpG [Sigma-Aldrich]) for 24 h. Of the 85 cell lines used for the microarray experiment, 13 carry the minor allele, thus all 13 were interrogated, matched by a random selection of 13 major allele cell lines. Supernatant was collected, and biological duplicate CXCL10 concentrations were measured by ELISA (R&D Systems).

<table>
<thead>
<tr>
<th>Pathway Genes</th>
<th>SNP</th>
<th>Uncorrected p Value</th>
<th>Bonferroni Corrected p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Canonical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYD88</td>
<td>rs2239621</td>
<td>0.443</td>
<td>1.000</td>
</tr>
<tr>
<td>IRAK1</td>
<td>rs1059701</td>
<td>0.070</td>
<td>1.000</td>
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<td>IRAK4</td>
<td>rs4251513</td>
<td>0.839</td>
<td>1.000</td>
</tr>
<tr>
<td>TIRAP</td>
<td>rs786697</td>
<td>0.310</td>
<td>1.000</td>
</tr>
<tr>
<td>MAP3K7</td>
<td>rs1145727</td>
<td>0.712</td>
<td>1.000</td>
</tr>
<tr>
<td>IKKB</td>
<td>rs10958715</td>
<td>0.272</td>
<td>1.000</td>
</tr>
<tr>
<td>RIPK1</td>
<td>rs11242823</td>
<td>0.093</td>
<td>1.000</td>
</tr>
<tr>
<td>MAP2K6</td>
<td>rs12453226</td>
<td>0.066</td>
<td>1.000</td>
</tr>
<tr>
<td>NFKB1A</td>
<td>rs1957106</td>
<td>0.945</td>
<td>1.000</td>
</tr>
<tr>
<td>NFKB1B</td>
<td>rs10775533</td>
<td>0.006</td>
<td>0.335</td>
</tr>
<tr>
<td>NFKBIE</td>
<td>rs2282151</td>
<td>0.012</td>
<td>1.000</td>
</tr>
<tr>
<td>NFKB1</td>
<td>rs730775</td>
<td>0.522</td>
<td>1.000</td>
</tr>
<tr>
<td>RELA</td>
<td>rs1049728</td>
<td>0.669</td>
<td>1.000</td>
</tr>
<tr>
<td>RELB</td>
<td>rs11227247</td>
<td>0.722</td>
<td>1.000</td>
</tr>
<tr>
<td>MKK4</td>
<td>rs230521</td>
<td>0.152</td>
<td>1.000</td>
</tr>
<tr>
<td>MKK1</td>
<td>rs230542</td>
<td>0.627</td>
<td>1.000</td>
</tr>
<tr>
<td>MKK3</td>
<td>rs3774934</td>
<td>0.808</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Noncanonical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAF2</td>
<td>rs10283820</td>
<td>0.349</td>
<td>1.000</td>
</tr>
<tr>
<td>TRAF6</td>
<td>rs2303439</td>
<td>0.152</td>
<td>1.000</td>
</tr>
<tr>
<td>TRAF3</td>
<td>rs503401</td>
<td>0.772</td>
<td>1.000</td>
</tr>
<tr>
<td>TRAF5</td>
<td>rs540386</td>
<td>0.683</td>
<td>1.000</td>
</tr>
<tr>
<td>NIK (MAP3K14)</td>
<td>rs2074293</td>
<td>0.818</td>
<td>1.000</td>
</tr>
<tr>
<td>CHUK</td>
<td>rs1190421</td>
<td>0.050</td>
<td>1.000</td>
</tr>
<tr>
<td>NFKB2</td>
<td>rs1409312</td>
<td>0.442</td>
<td>1.000</td>
</tr>
</tbody>
</table>
| **Table I. Armitage trend test on mortality and SNPs of NF-κB pathway genes in the SPH cohort**
Analyses used SPSS (version 16; SPSS, Chicago, IL), R statistical software, and 28-d mortality in the SPH discovery cohort using an Armitage trend test, as is commonly used for initial discovery surveys in genome-wide association studies (Table I). One SNP emerged as statistically significant after a Bonferroni correction for multiple comparisons. We then tested for replication of this finding in the VASST cohort of septic shock patients. To correct for potentially confounding variables, including age, gender, ancestry, and surgical versus medical diagnostic category, we used Cox regression. We then tested for association between secondary outcome measures of days alive and free of organ failure using Kruskal–Wallis tests to consider and correct for potential confounding variables due to differences at baseline in septic shock patients, we used Cox regression to test an additive model in the SPH septic shock cohort and then used the same analysis in the VASST septic shock cohort.

Patients in the SPH cohort who had the CC genotype of NIK rs7222094 had a significantly increased hazard of 28-d mortality compared with that of patients having the CT or TT genotypes of rs7222094 (hazard ratio [HR], 1.35; 95% confidence interval [CI], 1.12–1.64; p = 0.002 Caucasian only) (Table II). This finding was replicated in the VASST cohort (HR, 1.24; 95% CI, 1.00–1.52; p = 0.048 Caucasian only) (Table II). The results were similar for all patients with ethnicity included as a covariate in a Cox regression model (Fig. 1, Table III).

Similarly, in an unadjusted univariate analysis, the C allele of rs7222094 TC was associated with mortality in SPH (mortality, Caucasian only: TT, 33.8%; CT, 42.4%; CC, 53.7%; p = 0.005; mortality, all ethnicities: TT, 33.8%; CT, 43.2%; CC, 53.0%; p = 0.001), and a similar trend was observed in VASST (mortality, Caucasian only: TT, 26.3%; CT, 34.7%; CC, 38.4%; p = 0.09; mortality, all ethnicities: TT, 27.5%; CT, 33.2%; CC, 40.8%; p = 0.03). Allele frequencies of survivors versus nonsurvivors in both Caucasian and all ethnicities are reported in Table IV.

In the SPH Caucasian cohort, patients having the CC genotype of rs7222094 had greater baseline creatinine concentrations (p = 0.04) and significantly higher PaO2/FIO2 at baseline (p = 0.002) than patients having the CT or TT genotypes of rs7222094 (Table V). The only difference at baseline among VASST Caucasian patients was that patients having the CC genotype of rs7222094 had significantly lower platelet counts than those of patients having the CT or TT genotypes of rs7222094 (p = 0.02) (Table V). Because the VASST cohort was a clinical trial comparing efficacy of vasopressin versus norepinephrine in septic shock, in a secondary analysis we tested for an interaction by logistic regression between NIK rs7222094 and vasopressin treatment in Caucasian patients. We found no significant interaction (interaction statistic p = 0.462).

Results

Twenty-eight–day mortality in septic shock patients

Of the 59 tagSNPs in 19 genes in canonical and noncanonical NF-κB pathways, one SNP, rs7222094 in NIK, was significantly associated with 28-d mortality in the SPH discovery cohort (uncorrected p = 0.00024, Bonferroni corrected p = 0.014) (Supplemental Fig. 1, Table I).

Hardy–Weinberg equilibrium and minor allele frequencies of all SNPs genotyped are presented along with literature based minor allele frequencies in Supplemental Table I. Allele frequencies of rs7222094 differed between ethnic groups within the SPH and VASST cohorts (Supplemental Table II). Therefore, our primary analysis was limited to Caucasians only (SPH, n = 453, VASST, n = 517), and our secondary analysis of all patients included ethnicity as a covariate (SPH, N = 589; VASST, N = 616).

To consider and correct for potential confounding variables due to differences at baseline in septic shock patients, we used Cox regression to test an additive model in the SPH septic shock cohort and then used the same analysis in the VASST septic shock cohort.

Statistical analysis

We assessed baseline characteristics using a χ2 test for categorical data and a Kruskal–Wallis test for continuous data and then reported the median and interquartile ranges. We then tested for association between SNP genotype and 28-d mortality in the SPH discovery cohort using an Armitage trend test, as is commonly used for initial discovery surveys in genome-wide association studies (Table I). One SNP emerged as statistically significant after a Bonferroni correction for multiple comparisons. We then tested for replication of this finding in the VASST cohort of septic shock patients. To correct for potentially confounding variables, including age, gender, ancestry, and surgical versus medical diagnostic category, we used Cox regression. We then tested for association between secondary outcome measures of days alive and free of organ failure using Kruskal–Wallis tests.

A Student t test was performed to test for differences between genetic groups (rs7222094 CC versus TT) of CXCL10 ELISA concentrations. Analyses used SPSS (version 16; SPSS, Chicago, IL), R statistical software package, and GraphPad Prism (version 5.02; GraphPad, La Jolla, CA).

Organ failure in septic shock patients

Patients homozygous for the C allele of rs7222094 in both the SPH and VASST Caucasian cohorts had more organ dysfunction as defined by Brussels criteria compared with that in patients having the CT or TT genotypes of rs7222094 (Table V).

Table II. Hazard ratios of 28-d mortality in Caucasian patients with septic shock (Cox regression)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SPH Cohort (n = 453)</th>
<th>p Value</th>
<th>VASST Cohort (n = 517)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per year</td>
<td>1.006 (1.003–1.009)</td>
<td>0.0036</td>
<td>1.019 (1.008–1.030)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Female</td>
<td>0.9902 (0.670–1.123)</td>
<td>0.530</td>
<td>0.987 (0.717–1.348)</td>
<td>0.930</td>
</tr>
<tr>
<td>Surgical diagnosis</td>
<td>0.8244 (0.600–1.118)</td>
<td>0.220</td>
<td>0.859 (0.583–1.233)</td>
<td>0.420</td>
</tr>
<tr>
<td>NIK rs7222094 C allele</td>
<td>1.35 (1.116–1.635)</td>
<td>0.002</td>
<td>1.24 (1.002–1.524)</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Table III. Hazard ratios of 28-d mortality in patients with septic shock (Cox regression)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SPH Cohort (N = 589)</th>
<th>p Value</th>
<th>VASST Cohort (N = 616)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per year</td>
<td>1.006 (1.003–1.009)</td>
<td>9.0 × 10−4</td>
<td>1.017 (1.008–1.026)</td>
<td>2.0 × 10−4</td>
</tr>
<tr>
<td>Female</td>
<td>0.868 (0.668–1.122)</td>
<td>0.28</td>
<td>1.004 (0.756–1.328)</td>
<td>0.98</td>
</tr>
<tr>
<td>European ancestry</td>
<td>0.962 (0.717–1.290)</td>
<td>0.76</td>
<td>0.870 (0.609–1.272)</td>
<td>0.46</td>
</tr>
<tr>
<td>Surgical diagnosis</td>
<td>0.937 (0.712–1.220)</td>
<td>0.63</td>
<td>0.800 (0.557–1.123)</td>
<td>0.20</td>
</tr>
<tr>
<td>NIK rs7222094 C allele</td>
<td>1.33 (1.126–1.573)</td>
<td>8.0 × 10−4</td>
<td>1.25 (1.030–1.521)</td>
<td>0.024</td>
</tr>
</tbody>
</table>
during the 28-d study period than those of patients having the CT or TT genotypes of rs7222094 (Table VI). Patients of the V ASST cohort with the rs7222094 CC genotype show the same trend toward more renal dysfunction (Table VI). The number of patients affected by each type of organ dysfunction by genotypic group is outlined in Table VII.

In the SPH cohort, patients with the rs7222094 CC genotype also had significantly fewer days alive and free of cardiovascular dysfunction (p = 0.008), with correspondingly fewer days alive and free of vasopressor support (p = 0.01), which was also seen as a trend in the VASST cohort (Table VI). SPH cohort patients also experienced more hepatic and neurologic dysfunction (p = 0.007 and p = 0.006, respectively) (Table VI).

CXCL10 mRNA production by lymphoblastoid cell lines in vitro

The gene with the greatest difference (Δ) in fold change between major (TT) and minor (CC) genotypes was CXCL10 (Δ fold change, 0.67; uncorrected Student t test between groups, p = 0.055) suggesting lower mRNA expression of CXCL10 for the CC genotype compared with that of TT or TC (Supplemental Table III).

CXCL10 protein production by lymphoblastoid cell lines in vitro

Protein levels of CXCL10 were measured in 26 cell lines of known genotype for rs7222094. Cell lines homozygous for the C allele of rs7222094 produced less CXCL10 at both baseline and after inflammatory stimulation than that of cell lines homozygous for the T allele (p = 0.032 and p = 0.050, respectively) (Fig. 2).

CXCL10 protein production in VASST plasma samples

Baseline plasma specimens of a random sample of patients with septic shock from the VASST cohort were assayed in duplicate for CXCL10 by ELISA, and as was found in both the control and stimulated lymphoblastoid cell lines, patients of the CC genotype had significantly lower CXCL10 than that of the TT genotype (p = 0.017) (Fig. 3).

Discussion

We found that patients of the CC genotype of NIK rs7222094 had significantly increased mortality compared with that of patients having the CT or TT genotypes of rs7222094 in two cohorts of patients who had septic shock. Specifically, Caucasian patients in the SPH cohort who had the CC genotype of NIK rs7222094 experienced a significant increase in the hazard of death over the 28 d (HR, 1.35; 95% CI, 1.12–1.64; p = 0.002). This effect was
Table V. Baseline characteristics by NIK genotype rs7222094 for Caucasian patients with septic shock in both SPH and VASST cohorts

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TT (n=133)</th>
<th>CT (n=251)</th>
<th>CC (n=123)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59 (46–71)</td>
<td>62 (48–73)</td>
<td>61 (49–73)</td>
<td>0.34</td>
</tr>
<tr>
<td>APACHE II</td>
<td>25 (20–30)</td>
<td>26 (20–31)</td>
<td>26 (20–33)</td>
<td>0.61</td>
</tr>
<tr>
<td>Surgical percentage</td>
<td>30.2</td>
<td>29.8</td>
<td>33.3</td>
<td>0.79</td>
</tr>
<tr>
<td>Preexisting conditions, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic heart failure</td>
<td>5.8</td>
<td>7.9</td>
<td>4.9</td>
<td>0.54</td>
</tr>
<tr>
<td>Chronic pulmonary disease</td>
<td>17.3</td>
<td>19.9</td>
<td>12.2</td>
<td>0.19</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>3.6</td>
<td>3.1</td>
<td>4.9</td>
<td>0.74</td>
</tr>
<tr>
<td>Chronic corticosteroid use</td>
<td>5.8</td>
<td>6.8</td>
<td>4.9</td>
<td>0.77</td>
</tr>
<tr>
<td>Cardiovascular variables day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>115 (95–130)</td>
<td>114 (95–130)</td>
<td>115 (95–131)</td>
<td>0.86</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>55 (50–60)</td>
<td>55 (50–60)</td>
<td>55 (50–60)</td>
<td>0.86</td>
</tr>
<tr>
<td>Central venous pressure, mm Hg</td>
<td>12 (8–14)</td>
<td>14 (11–17)</td>
<td>14 (11–17)</td>
<td>0.86</td>
</tr>
<tr>
<td>Laboratory variables day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC count, 10⁹/mm³</td>
<td>114 (89–130)</td>
<td>114 (89–130)</td>
<td>114 (89–130)</td>
<td>0.86</td>
</tr>
<tr>
<td>Platelet count, 10⁹/mm³</td>
<td>55 (50–60)</td>
<td>55 (50–60)</td>
<td>55 (50–60)</td>
<td>0.86</td>
</tr>
<tr>
<td>PaO₂/FiO₂, torr</td>
<td>125 (80–194)</td>
<td>125 (80–194)</td>
<td>125 (80–194)</td>
<td>0.86</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>114 (89–130)</td>
<td>114 (89–130)</td>
<td>114 (89–130)</td>
<td>0.86</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>125 (80–194)</td>
<td>125 (80–194)</td>
<td>125 (80–194)</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Values shown are median and interquartile range.

is a chemokine transcribed in response to NF-κB during inflammation and is generally thought to signal in response to canonical pathway stimuli (21, 22). In a recent study by Zarnegar et al. (23), CXCL10 levels were dependent on NIK suggesting noncanonical signaling. CXCL10 is transcribed in response to NF-κB activation and has been shown to be downregulated upon inhibition of NIK (23). The CC genotype of NIK rs7222094 was associated with significantly decreased levels of CXCL10 in supernatant of lymphoblastoid cell lines at baseline and after Cytomix stimulation (p = 0.032 and p = 0.050). Similarly, CXCL10 levels were significantly lower in septic shock patients of the CC genotype (p = 0.017), suggesting a biologically plausible explanation for our observations.

It was interesting to find that the patients with the NIK rs7222094 CC genotype experienced increased mortality while supernatant from cell lines of the same genotype had decreased levels of CXCL10. This effect was replicated when CXCL10 concentration was measured in VASST patient samples. As mentioned previously, CXCL10 is a proinflammatory chemokine released during inflammatory states, such as allograft rejection and infection (24). It is plausible that proinflammatory molecules are necessary to mount an effective immune response during septic shock. CXCL10 is a chemokine that instigates chemotaxis of activated T cells and NK cells (24). We speculate that without enough CXCL10 to drive recruitment of inflammatory cells, it is possible that patients with the CC genotype of NIK rs7222094 have an immunological disadvantage and so have increased mortality of septic shock.

The original characterization of NIK indicated that NIK was a powerful effector of canonical NF-κB signaling under TNF-α and IL-1β stimulation (20). Later studies were unable to confirm this mechanism, and instead the discovery of the noncanonical pathway emerged as subsequent research appeared to distill the two pathways into distinct and separate processes (6, 25–27). Evidence is building suggesting that the IKK–NIK axis is a pivot point for control of both noncanonical and canonical signaling (23, 28, 29). It is possible that the timing of experimental procedures is critical to our understanding of NIK, as many studies that separated the two pathways and excluded NIK from canonical signaling focused on early events (from minutes to less than 2 h) (25, 27). In contrast, many studies find that NIK plays a pivotal role in canonical signaling when evaluating effects at time points of several hours to days (20, 23, 28). The current understanding of the regulation of NIK is that NIK is constitutively transcribed, translated, and degraded via its interaction with TRAF3. However, after degradation of TRAF3 after appropriate stimuli, NIK recruits IKKα to p100, activating IKKα thereby initiating proteosomal degradation of p100 to p52 and consequently translocation of heterodimers to the nucleus (6, 30). It is conceivable that accumulation of NIK over time is a key facet to its mechanism.

Because NIK has been implicated in the host response to infection, we speculate that NIK could modulate the immune response during septic shock. NIK is important in the host response...
to numerous infections including respiratory syncytial virus [with evidence to suggest activation of both noncanonical and canonical signaling (31)], HIV (32), hepatitis B (33), Escherichia coli (34), as well as the response to LPS (35).

Several lines of evidence suggest why polymorphisms of NIK may be predictors of outcome in septic shock. First, NIK stimulates inflammation by upregulating the noncanonical (and possibly the canonical) pathway of NF-κB activation. Second, NIK is required for optimal IgG production by lymphocytes (36). Third, NIK may modulate blood pressure: NIK has a role in the mechanism of action of the calcium channel blocker nifedipine (37), and angiotensin II induces inflammation through NIK activation of the noncanonical NF-κB pathway (38). However, polymorphisms of NIK have not been widely studied. In a survey of 181 SNPs of 17 genes in the SPH cohort of Caucasian patients with a similar trend in V ASST cohorts was performed retrospectively. The association of rs7222094 with mortality, organ dysfunction, and CXCL10 levels does not prove a causal link. Furthermore, CXCL10 was used as a marker for differences in NIK-induced NF-κB signaling. We do not currently know of, nor did we test for, the influence of CXCL10 itself on organ dysfunction or mortality. In view of the large number of genes connected in some way to NF-κB signaling, we chose to limit our analysis to genes of the cytosolic members of the NF-κB pathway, excluding receptors and downstream targets. Therefore, this analysis does not include all potentially functional variants, in particular those recently published after the design of this study (43–45).

In conclusion, we found that NIK rs7222094 was consistently and significantly associated with mortality in two independent cohorts of patients who had septic shock. Patients homozygous for the CC genotype of rs7222094 had increased mortality and also experienced more renal and hematological failure in the SPH cohort of Caucasian patients with a similar trend in VASST Caucasian patients. Furthermore, we found that lymphoblastoid cell lines homozygous for CC of rs7222094 produced less CXCL10 in vitro at baseline and after Cytomix stimulation than that of cell

### Table VI. Days alive and free of organ dysfunction in Caucasian septic shock patients according to NIK rs7222094 genotype

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>VASST Cohort*</th>
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<td>CT (n = 191)</td>
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<td></td>
</tr>
<tr>
<td>Respiratory</td>
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<td>13 (12–15)</td>
</tr>
<tr>
<td>Renal</td>
<td>12 (10–14)</td>
<td>11 (10–13)</td>
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<tr>
<td>Hematologic</td>
<td>15 (17–19)</td>
<td>16 (14–17)</td>
</tr>
<tr>
<td>Hepatic</td>
<td>18 (16–20)</td>
<td>18 (17–20)</td>
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<tr>
<td>Neurologic</td>
<td>19 (17–21)</td>
<td>17 (16–19)</td>
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<tr>
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<tr>
<td>Vasopressor</td>
<td>17 (15–19)</td>
<td>16 (14–17)</td>
</tr>
<tr>
<td>Ventilator</td>
<td>12 (10–14)</td>
<td>10 (9–12)</td>
</tr>
<tr>
<td>Renal replacement therapy</td>
<td>18 (16–20)</td>
<td>17 (15–18)</td>
</tr>
</tbody>
</table>

*Values shown are median and interquartile range.

### Table VII. Caucasian septic shock patients affected by organ dysfunction according to NIK rs7222094 genotype

<table>
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<td>Ventilator</td>
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<td>Renal replacement therapy</td>
<td>62 (44.6)</td>
<td>112 (58.6)</td>
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</table>

NA. The p value was not calculated because all patients in each genotypic group are affected.

Inhibitors of NIK have been synthesized for diseases such as multiple myeloma and other cancers, as anti-inflammatory agents for inflammatory diseases (41), and as a vaccine adjuvant (42). Our data suggest that rs7222094 may be of interest in randomized controlled trials of therapies for septic shock by defining risk categories of patients and perhaps defining response to anti-inflammatory agents.

This study has several limitations. The analysis of the SPH and VASST cohorts was performed retrospectively. The association of rs7222094 with mortality, organ dysfunction, and CXCL10 levels does not prove a causal link. Furthermore, CXCL10 was used as a marker for differences in NIK-induced NF-κB signaling. We do not currently know of, nor did we test for, the influence of CXCL10 itself on organ dysfunction or mortality. In view of the large number of genes connected in some way to NF-κB signaling, we chose to limit our analysis to genes of the cytosolic members of the NF-κB pathway, excluding receptors and downstream targets. Therefore, this analysis does not include all potentially functional variants, in particular those recently published after the design of this study (43–45).

In conclusion, we found that NIK rs7222094 was consistently and significantly associated with mortality in two independent cohorts of patients who had septic shock. Patients homozygous for the CC genotype of rs7222094 had increased mortality and also experienced more renal and hematological failure in the SPH cohort of Caucasian patients with a similar trend in VASST Caucasian patients. Furthermore, we found that lymphoblastoid cell lines homozygous for CC of rs7222094 produced less CXCL10 in vitro at baseline and after Cytomix stimulation than that of cell
lines having the TT genotype of NIK rs7222094 (p = 0.032 and 0.050, respectively). As well, patients who had septic shock in the VASST cohort who were NIK rs7222094 CC genotype had significantly lower plasma levels of CXCL10 than those of the TT genotype (p = 0.017). We speculate that polymorphisms of NIK could be used to predict risk of death from septic shock and to predict response to anti-inflammatory treatment, such as inhibitors of NIK.

Disclosures
S.A.T. has received grant support from MITACs made possible by an industry partnership with Sirus Genomics. J.A.R. holds stock in Sirus Genomics Inc., which has submitted patents owned by the University of British Columbia and licensed to Sirus Genomics that are related to the genetics of vasopressin, NIK, and protein C. The University of British Columbia has also submitted a patent related to the use of vasopressin in septic shock. J.A.R. is an inventor on these patents. J.A.R. has received consulting fees from Ferring, which manufactures vasopressin; from AstraZeneca, which manufactures anti-TNF; and from Sirus Genomics Inc. J.A.R. has received grant support from Sirus Genomics, Novartis, Ferring, and Eli Lilly. J.A.R. has received speaking honoraria from Pfizer and Eli Lilly. K.R.W. holds stock in Sirius Genomics Inc., which has submitted patents owned by the University of British Columbia and licensed to Sirus Genomics that are related to the genetics of vasopressin, NIK, and protein C. The University of British Columbia has also submitted a patent related to the use of vasopressin in septic shock. K.R.W. is an inventor on these patents. K.R.W. has received grant support from Sirus Genomics, H.W. reports serving as director of research and holding shares at Sirus Genomics Inc., which has submitted a patent owned by the University of British Columbia and licensed to Sirus Genomics that is related to the genetics of LNPEP.

References


SUPPLEMENTAL FIGURE 1. NF-κB signaling pathways as dictated by Ingenuity (www.Ingenuity.com). All genes circled in red were genotyped for tag SNPs for a total of 19 genes and 59 SNPs.
**SUPPLEMENTAL TABLE I.**

Hardy–Weinberg Equilibrium (HWE) and Minor Allele Frequencies (MAFs) in the SPH Cohort Caucasian Patients with Septic Shock and as Reported in Bioinformatic Websites.

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<th>SPH major allele</th>
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**SUPPLEMENTAL TABLE II.**
Genotype and Allele Frequencies in All Populations of SPH and VASST cohorts.

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<th>VASST Cohort (n=616) N (%)</th>
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