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TNFAIP2 Inhibits Early TNFα-Induced NF-κB Signaling and Decreases Survival in Septic Shock Patients

Simone A. Thair[a, c] Elena Topchiy[c] John H. Boyd[c] Mihai Cirstea[c] Catherine Wang[c] Taka-aki Nakada[d] Christopher D. Fjell[c] Mark Wurfel[b] James A. Russell[c] Keith R. Walley[c]

[a]Department of Emergency and Surgery, Stanford University School of Medicine, Stanford, Calif., and [b]Division of Pulmonary and Critical Care Medicine, University of Washington, Harborview Medical Center, Seattle, Wash., USA; [c]Centre for Heart Lung Innovation, St. Paul's Hospital, University of British Columbia, Vancouver, B.C., Canada; [d]Department of Emergency and Critical Care Medicine, Chiba University Graduate School of Medicine, Chiba, Japan

Key Words

Cytokines · Immune response · Sepsis · Septic shock · Tumor necrosis factor α · TNFAIP2 · Nuclear factor-κB · Interleukin-8

Abstract

During septic shock, tumor necrosis factor alpha (TNFα) is an early response gene and induces a plethora of genes and signaling pathways. To identify robust signals in genes reliably upregulated by TNFα, we first measured microarray gene expression in vitro and searched methodologically comparable, publicly available data sets to identify concordant signals. Using tag single-nucleotide polymorphisms in the genes common to all data sets, we identified a genetic variant of the *TNFAIP2* gene, rs8126, associated with decreased 28-day survival and increased organ dysfunction in an adult cohort in the Vasopressin and Septic Shock Trial. Similar to this cohort, we found that an association with rs8126 and increased organ dysfunction is replicated in a second cohort of septic shock patients in the St. Paul's Hospital Intensive Care Unit. We found that TNFAIP2 inhibits NF-κB activity, impacting the downstream cytokine interleukin (IL)-8. The minor G allele of TNFAIP2 rs8126 resulted in greater TNFAIP2 expression, decreased IL-8 production and was associated with decreased survival in patients experiencing septic shock. These data suggest that TNFAIP2 is a novel inhibitor of NF-κB that acts as an autoinhibitor of the TNFα response during septic shock.

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Introduction

Tumor necrosis factor alpha (TNFα) is involved in the early inflammatory response of septic shock and induces a plethora of genes via numerous intracellular signaling pathways. Numerous clinical trials have effectively neutralized TNFα in patients with severe sepsis without improving outcome [1–5]. Conversely, in large numbers of patients on chronic anti-TNFα therapy for inflammatory conditions, it has become evident that a properly functioning TNFα pathway is essential to mount an effective

S.A.T. and E.T. contributed equally to this publication.

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E-Mail karger@karger.com
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Dr. Keith R. Walley
Centre for Heart Lung Innovation
1081 Burrard Street
Vancouver, BC V6Z 1Y6 (Canada)
E-Mail Keith.Walley@hli.ubc.ca

antimicrobial response [6]. We reasoned that genes that are highly upregulated early in the TNFα response may play a clinically important role. We further reasoned that if these genes had clinically important effects, then genetic variants of these genes may be associated with differences in clinical outcome in septic shock. To identify robust signals in genes reliably induced by TNFα, we first measured microarray gene expression and searched methodologically comparable, publicly available data sets to identify concordant signals. Next, in genes reliably induced by TNFα, we genotyped single-nucleotide polymorphisms (SNPs) that tagged groups of SNPs in linkage disequilibrium within genes highly regulated by TNFα. We tested for an association between these tagSNPs and 28-day survival in a cohort of septic shock patients in the Vasopressin and Septic Shock Trial (VASST). This approach identified a gene, *TNFAIP2*, and a genetic variant, rs8126, which allowed us to gain insight into the potential mechanism of its modulatory effect. Specifically, we found that TNFAIP2 inhibits NF-κB activity, impacting downstream cytokines such as interleukin (IL)-8. The minor G allele of *TNFAIP2* rs8126 resulted in greater TNFAIP2 expression, decreased NF-κB activity and IL-8 production and was associated with increased mortality and renal dysfunction in patients experiencing septic shock. We found a concordant association with rs8126 and renal dysfunction in a second independent cohort of septic shock patients. These data suggest that TNFAIP2 is a novel inhibitor of NF-κB that may act to modulate the inflammatory response in septic shock.

Methods

Gene Expression Microarray Measurements

TNFα Induction

HeLa cells were cultured in DMEM with 10% FBS, and treated with or without 10 ng/ml TNFα for 4 h, in biological quadruplicate. RNA was harvested and gene expression was interrogated using the Illumina human HT-12 (v4) expression BeadChip (Genome Quebec Innovation Centre, Montreal, Que., Canada). Data were normalized and fold change was calculated using FlexArray v1.4.1. MIAME compliant data from the HT-12 Illumina gene expression microarray is available at GEO. We then performed a literature search to test for the replication of our results in publically available data sets by using the following criteria in the PubMed search engine: TNF, HELA, MICROARRAY and HUMAN. This resulted in a list of 12 publications (online suppl. table 1; see www.karger.com/doi/10.1159/000437330 for all online suppl. material). Each publication's methods were individually inspected and then selected if they met the following criteria: if the cell type was HeLa, stimulation was with human recombinant TNFα, the stimulation time was 3–5 h and the data files were not encrypted when downloaded from GEO. The search identified 2 publications [7, 8]. Once the data sets were obtained, lists of genes were filtered for genes upregulated compared to controls, as the scope of this study is for highly upregulated genes. We then filtered the lists to include only genes with a fold change >2. An Excel INDEX/MATCH formula was executed to determine which genes were present in all 3 data sets. To confirm the findings, the lists (online suppl. table 2) were then loaded into: http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl. Overlap between data sets generated a Venn diagram (online suppl. fig. 1) and lists of genes represented in each section of the Venn diagram can be found in online supplementary table 3. Genes common to all 3 lists were then selected for genotyping in a large cohort of septic shock patients.

TNFAIP2 siRNA or Overexpression of rs8126 A or G Clones Followed by TNFα Stimulation

TNFAIP2 siRNA (Stealth siRNA Technology, Invitrogen/Life Technologies) was transfected using Lipofectamine 2000 as per the manufacturer's instructions. *TNFAIP2* knockdown was verified using qRT-PCR. Forty-eight hours after siRNA, there was a 97.8% knockdown of *TNFAIP2*. HeLa cells were cultured in DMEM with 10% FBS and were treated with or without 10 ng/ml TNFα for the last 4 h of 48 h of transfection, as *TNFAIP2* is not constitutively transcribed [9, 10] (in biological quadruplicate). For overexpression, HeLa cells were cultured as above and were transfected with *TNFAIP2* overexpression plasmids containing either the A or G allele of TNFAIP2 rs8126 for 48 h (Origene custom plasmids CW100407 and CW101532, Origene). Array analysis was as described above.

Quantitative Real-Time PCR

Quantitative real-time (qRT)-PCR was used to validate *TNFAIP2* gene expression in HeLa cells stimulated with or without 10 ng/ml TNFα for 4 h (n = 4) using the QuantiTect SYBR green PCR kit, and data were collected on the ViiA 7 real-time PCR system. Primer sequences were as follows: TNFAIP2 forward 5′CCCCAATGACATCATCAACA3′ and reverse 5′GCCTCACTGGACAGGAATGT3′; GAPDH forward 5′TGCACCACCAACTGCTTAGC3′ and reverse 5′GGCATGGACTGTGGTCATGA3′.

Patient Cohorts

Vasopression and Septic Shock Trial (VASST) Cohort (Discovery Cohort)

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 [11]. Clinical phenotyping has been described previously [12]. All patients were enrolled within 24 h of meeting the definition of septic shock. DNA was extracted from peripheral blood samples using a QIAamp DNA blood midi kit (QIAgen, Mississauga, Ont., Canada) from 632 patients. Written informed consent was obtained from all patients or their authorized representatives and the trial was approved by all ethics boards of the participating institutions. The research ethics board at the coordinating center (University of British Columbia) approved the genetic analysis.

St. Paul's Hospital Intensive Care Unit (SPHICU) Cohort (Replication Cohort)

All patients admitted to the SPHICU, Vancouver, B.C., Canada, between July 2000 and January 2004 underwent screening.

Two hundred and fourteen Caucasian patients presenting with septic shock upon admission and for whom phenotypic data were available were selected for SNP genotyping. This study was approved by the Institutional Review Board at SPH and the University of British Columbia.

SNP Genotyping

TagSNPs in genes identified in the above microarray studies were selected using a linkage disequilibrium-based method (LD-select [13]) using an r^2 threshold of 0.65 for SNPs with a minor allele frequency of >5%. These tagSNPs were genotyped in all patients of the VASST cohort with available DNA. DNA was extracted from peripheral blood samples using a QIAamp DNA blood midi kit (QIAgen) and genotyped using the Illumina Golden Gate assay (UBC Centre for Molecular Medicine). We then collected dense genotyping of 68 SNPs in the region +/–50,000 bp of *TNFAIP2* in 530 Caucasian VASST patients from the Human Illumina Duo 1.2M SNP chip v3 (Therapeutics Genotyping Core Facility, Toronto, Ont., Canada) [14].

In the SPHICU replication cohort, rs8126 was SNP genotyped in 214 Caucasian patients using a custom Illumina Infinium iSelect BeadChip assay at the Children's Hospital of Philadelphia Research Institute/Center for Applied Genomics.

Quality Control for Genotyping Data

Sample data from Illumina GenomeStudio were imported into Golden Helix SVS software v7.6.4. Genotype data generated with the HapCluster algorithm were selected for analysis [15]. Quality control methods for call rate, cryptic relatedness, gender verification and population structure can be found in Supplemental Methods; these were all performed in SVS unless indicated otherwise.

TNFAIP2 Protein Levels

HeLa cells were cultured in DMEM with 10% FBS and were transfected with TNFAIP2 overexpression plasmids containing either the A or G allele of TNFAIP2 rs8126 for 48 h (Origene custom plasmids CW100407 and CW101532, Origene). Protein levels of TNFAIP2 were assayed by Western blot (5 biological replicates). TNFAIP2 levels were normalized to β-actin as a loading control, and densitometry was calculated using ImageJ v1.46r.

NF-κB Luciferase Assay

HeLa cells were cultured as above. The cells were co-transfected with 0.7 μg pNF-κB luciferase reporter construct (pNFkB-Luc, Clontech) with 0.2 μg of the renilla control plasmid (pRL-TK, Promega) as well as 0.5 μg of one of the following constructs: TNFAIP2 rs8126 A allele, TNFAIP2 G allele or control plasmid (Origene) for a total of 1.4 μg of DNA per 400,000 cells. The pNFkB-Luc is designed with 4 tandem repeats of the NF-κB consensus sequence followed by a TATA-like promoter (pTAL) from the herpes simplex virus thymidine kinase promoter followed by the firefly luciferase gene. Luciferase activity was measured according to the manufacturer's protocol using the Dual Glo luciferase assay (Promega) in biological triplicate.

For co-transfection of *TNFAIP2* siRNA (Stealth siRNA technology, Invitrogen/Life Technologies) and NF-κB luciferase, the luciferase construct was prepared as above, and included the co-transfection of siRNA for a final concentration of 25 nM. Transfection was performed in biological quadruplicate using Lipofectamine 2000 for 48 h and treated for the last 24 h with 10 ng/μl of TNFα in order to induce endogenous *TNFAIP2*. The luciferase assay of all 4 biological replicates included technical duplicates.

IL-8 ELISA

HeLa cells were cultured and transfected as above with *TNFAIP2* siRNA or overexpression vectors using Lipofectamine 2000 for 48 h in biological triplicate. Stimulation with 10 ng/μl of TNFα was then added for the last 24 h prior to the collection of supernatant in the siRNA experiments, and for the last 4 h in the overexpression experiments.

IL-8 ELISAs were conducted according to the manufacturer's protocol (R&D Systems, Minneapolis, Minn., USA).

Statistical Analysis

We used the Kaplan-Meier log-rank test to test for an association between tagSNPs and septic shock survival. The Nyholt correction was applied for multiple tests. In order to prevent spurious associations due to population stratification, all subsequent analysis was limited to Caucasian patients. We then used the Armitage trend test to test for association of the 68 SNPs in *TNFAIP2* with 28-day survival of septic shock. We used the Cox regression analysis in the VASST cohort to correct for potentially confounding variables including age, gender, ancestry, and surgical versus medical diagnostic category. We used a log-rank test to test an rs8126 additive model in the VASST cohort. We then tested for an association between the secondary outcome measures of the days alive and free of organ failure using the Kruskal-Wallis test. We assessed baseline characteristics using the χ^2 test for categorical data and the Kruskal-Wallis test for continuous data, and then reported the median and interquartile ranges. We used the Student t test for comparison between alleles in vitro. Analyses used SPSS v16 (SPSS, Chicago, Ill., USA), the R statistical software package and GraphPad Prism v5.02 (GraphPad, La Jolla, Calif., USA).

Results

TNFAIP2 Discovery Using Microarrays and TagSNP Genotyping

To identify transcripts that are highly expressed and hence represent key genes in the response to TNFα, microarray gene expression analysis was performed on mRNA collected from HeLa cells stimulated with TNFα compared to controls, and then cross-referenced with publically available data sets (online suppl. tables 1, 2). Three genes with a fold change >2 were common to all lists: *TNFAIP2 (B94), NFKBIA (IκBα)* and *NFKB1 (p105)* (online suppl. fig. 1; online suppl. table 3). We therefore genotyped 13 tagSNPs in the 3 genes in the VASST cohort of septic shock patients. Of these tagSNPs, rs8176373 in the *TNFAIP2* gene was significantly associated with survival in the VASST cohort (n = 616, p = 0.0019, Nyholt correction p = 0.018; table 1). qRT-PCR confirmed *TNFAIP2* expression levels (online suppl. fig. 2).

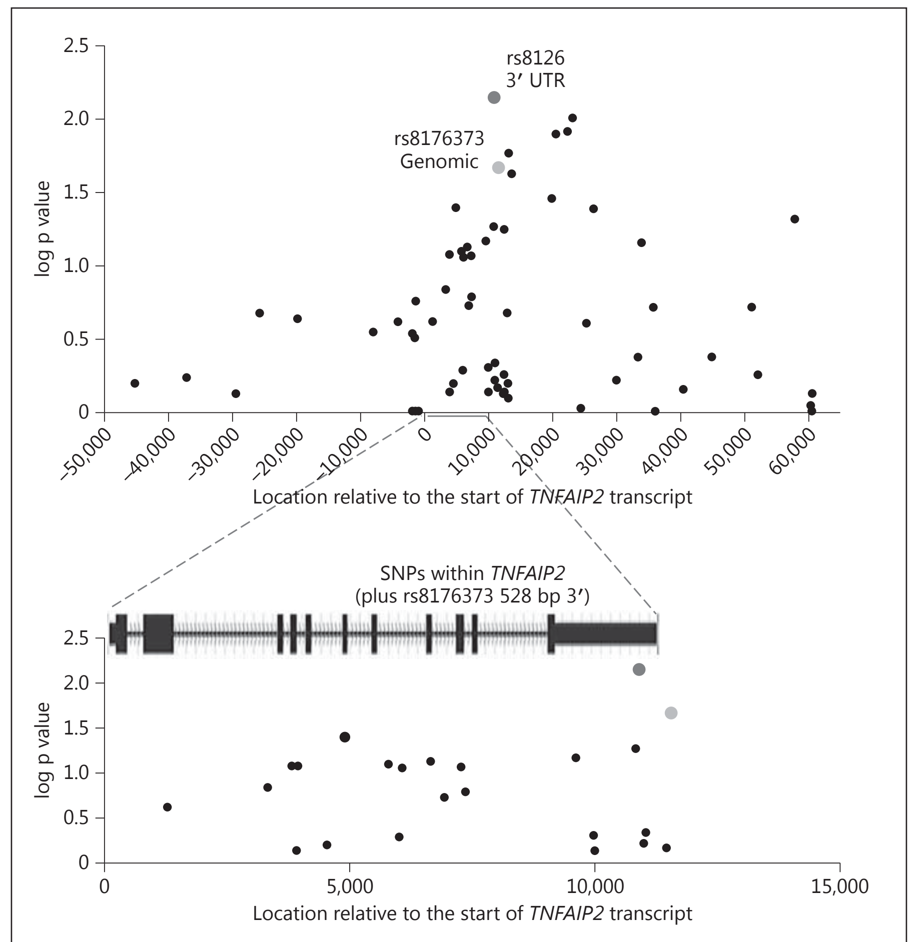

Fig. 1. Armitage trend test of 68 SNPs +/– 50,000 bp of TNFAIP2. Of the 68 SNPs interrogated, rs8126 was the most significantly associated with 28-day survival in 519 patients in the VASST cohort (p = 0.007, Caucasians only). rs8176373 (p = 0.02) is the original discovery tagSNP.

Table 1. log-rank analysis of 13 tagSNPs in 3 genes found by microarray

VASST		All ethnicities (n = 616)			Caucasians only (n = 517)		
Gene	SNP	p value	p value (Nyholt)	p value (HWE)	p value	p value (Nyholt)	p value (HWE)
TNFAIP2	rs8176373	0.0019	0.018	0.326	0.0072	0.063	0.676
TNFAIP2	rs749206	0.005	0.050	0.978	0.024	0.211	0.959
TNFAIP2	rs710100	0.082	0.803	0.380	0.194	1.000	0.173
TNFAIP2	rs3178152	0.221	1.000	0.731	0.303	1.000	0.606
NFKBIA	rs696	0.223	1.000	0.947	0.326	1.000	0.905
NFKBIA	rs3138055	0.395	1.000	0.915	0.258	1.000	0.584
NFKBIA	rs2233514	0.585	1.000	0.102	0.436	1.000	0.053
NFKBIA	rs1957106	0.759	1.000	0.055	0.887	1.000	0.210
NFKB1	rs1599961	0.138	1.000	0.066	0.240	1.000	0.035
NFKB1	rs230521	0.156	1.000	0.329	0.172	1.000	0.102
NFKB1	rs3774934	0.201	1.000	0.299	0.953	1.000	0.569
NFKB1	rs11722146	0.239	1.000	0.489	0.210	1.000	0.171
NFKB1	rs230542	0.397	1.000	0.157	0.395	1.000	0.039

HWE = Hardy-Weinberg equilibrium.

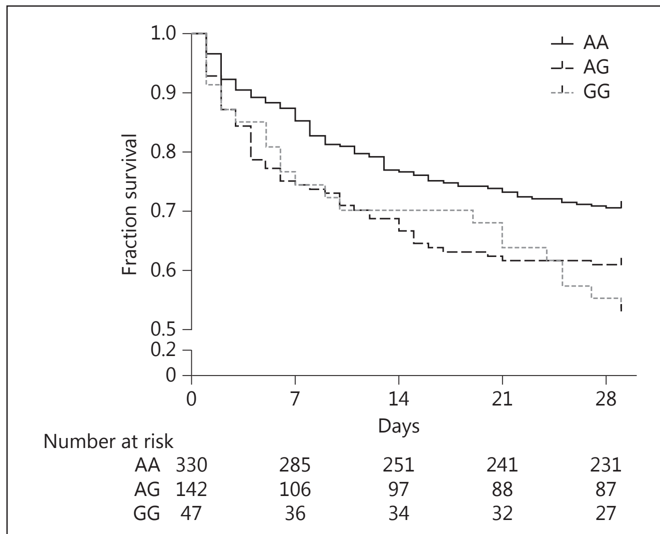

Fig. 2. Kaplan-Meier survival curves for Caucasian patients with septic shock in the VASST cohort by *TNFAIP2* rs8126. Visual inspection suggests a major allele model, where carriage of at least 1 copy of the G allele is detrimental. All subsequent analysis was performed using this model (n = 519, p = 0.0043).

Dense Genotyping Identifies rs8126

In order to elucidate the source of the tagSNP signal, we densely genotyped +/–50,000 bp upstream and downstream of *TNFAIP2*. Of the 68 SNPs interrogated in the region by the Armitage trend test, rs8126 was the most significantly associated with 28-day survival in 519 Caucasian patients in the VASST cohort (p = 0.007; fig. 1). rs8126 SNP is located within 3′-untranslated region (UTR) of TNFAIP2, a region known for its involvement in the regulation of translation and miRNA binding [16].

Septic Shock Patients with the rs8126 G Allele Have Increased Mortality and Organ Failure

VASST Cohort

Patients of the AA genotype of *TNFAIP2* rs8126 had significantly increased 28-day survival compared to patients with the GG genotype (log-rank, p = 0.0043; fig. 2). Visual inspection of the Kaplan-Meier curves suggests a major allele model, whereby carriage of ≥1 copies of the G allele is detrimental, so patients carrying 2 copies of the A (major) allele were grouped and compared to all patients carrying 1 or 2 copies of the G (minor) allele. Henceforth, we compared patients with an AA genotype to those with GG and AG genotypes.

To test for potentially confounding variables such as age, gender and surgical diagnosis, we performed the Cox regression and found that patients who had the GG/AG genotype of *TNFAIP2* rs8126 had significantly decreased survival compared to those with the AA genotype [hazard ratio of death for GG/AG vs. AA genotype: 1.46; 95% confidence interval 1.08–1.96; p = 0.013 (Caucasians only); table 2]. There were no significant differences in the baseline characteristics of the VASST cohort patients when comparing the AA and GG/AG genotypes (table 3). The rs8126 GG/AG genotype patients also experienced significantly fewer days alive and free (therefore more organ dysfunction and a worse outcome) of respiratory (p = 0.008) and renal failure (p = 0.001) as well as renal replacement therapy (p = 0.021) than the AA genotype patients (table 4). These data suggest that the G allele may be detrimental; the patients carrying 1 or 2 of these alleles have more organ dysfunction and increased mortality.

Table 2. 28-day mortality hazard ratios in 519 VASST Caucasian patients with septic shock

	VASST cohort	p value
Age	1.019 (1.008–1.030)	2.0×10^{-4}
Female	1.030 (0.760–1.391)	0.85
Surgical diagnosis	0.853 (0.584–1.213)	0.38
TNFAIP2 rs8126 G allele	1.460 (1.083–1.961)	0.013

Values are shown as a hazard ratio (confidence interval). Cox regression analysis was performed to correct for age, gender and surgical versus medical diagnosis using a major allele model.

SPHICU Cohort

The most significant findings in the VASST cohort were the statistically significant differences in renal dysfunction and the need for renal replacement therapy across genotypes. We therefore tested for replication of this observation in a second cohort of septic shock patients. Similar to the VASST cohort, SPHICU patients with the *TNFAIP2* rs8126 GG/AG genotypes had fewer days alive and free from renal failure (therefore more organ dysfunction and a worse outcome; p = 0.031) and renal replacement therapy (trend: p = 0.071; table 5). There were no differences between genotypes in the baseline prevalence of chronic renal failure [26% (AA) vs. 37% (GG/AG); p = 0.99] and baseline creatinine μmol/l [median and interquartile range: AA 160 (88–266) vs. GG/GA 141 (84–235); p = 0.42]. The survival of AA patients (54.0%) was higher than that of AG/GG patients (47.7%), but this difference was not statistically significant in this smaller replication cohort.

Table 3. Baseline characteristics of VASST Caucasian septic shock patients by TNFAIP2 rs8126 genotype

	AA (n = 330)	GG/AG (n = 189)	p value
Age, years	64 (50–74)	63 (53–72)	0.42
Male gender, %	61.9	62.4	0.23
APACHE II	26 (21–32)	27 (22–32)	0.38
Surgery, %	21.2	22.8	0.50
Pre-existing conditions, %			
Chronic heart failure	7.0	10.6	0.37
Chronic pulmonary disease	17.0	18.5	0.66
Chronic liver disease	11.2	9.5	0.82
Chronic renal failure	10.9	9.5	0.27
Chronic corticosteroid use	20.3	20.6	0.86
Cardiovascular variables on day 1			
Heart rate, bpm	128 (112–141)	125 (108–135)	0.094
Mean arterial pressure, mm Hg	56 (50–62)	55 (49–61)	0.53
Central venous pressure, mm Hg	14 (11–17)	15 (12–18)	0.20
Laboratory variables on day 1			
White blood cell count, $10^3/mm^3$	13.7 (7.4–20.5)	14.1 (8.4–22.4)	0.36
Platelet count, $10^3/mm^3$	152 (87–242)	179 (96–272)	0.31
PaO_2/FiO_2, mm Hg	197 (143–256)	182 (136–246)	0.17
Creatinine, μmol/l	141 (89–248)	170 (93–262)	0.27
Lactate, mmol/l	1.7 (0.8–3.3)	1.9 (1.1–3.8)	0.25
Microbiological results, n (%)			
Culture positive for Gram-negative organism	75 (22.7)	39 (20.6)	0.66
Culture positive for Gram-positive organism	101 (30.6)	58 (30.7)	1.00
Culture positive for any primary site	183 (55.5)	105 (55.6)	0.93

Values are reported as median (IQR) unless otherwise specified. APACHE II = Acute Physiology and Chronic Health Evaluation II.

Table 4. Days alive and free of organ dysfunction and artificial support in VASST Caucasian septic shock patients by TNFAIP2 rs8126 genotype

Parameter	AA (n = 330)	GG/AG (n = 189)	p value
Organ dysfunction			
Cardiovascular	20 (0–24)	13 (0–24)	0.06
Respiratory	4 (0–16)	2 (0–14)	0.008
Renal	24 (7–28)	13 (1–28)	0.001
Hematologic	25 (8–28)	24 (3–28)	0.39
Hepatic	27 (8–28)	23 (3–28)	0.06
Neurologic	17 (0–25)	14 (0–23)	0.07
Artificial organ support			
Vasopressor	20 (0–24)	16 (0–24)	0.07
Ventilator	10 (0–22)	6 (0–19)	0.08
Renal replacement therapy	28 (9–28)	20 (4–28)	0.021

Data are reported as median (IQR).

Table 5. Days alive and free from organ dysfunction and artificial support in SPHICU Caucasian septic shock patients by TNFAIP2 rs8126 genotype

Parameter	AA (n = 126)	GG/AG (n = 88)	p value
Organ dysfunction			
Renal	15 (3–28)	9 (1–24)	0.031
Artificial organ support			
Renal replacement therapy	20 (4–28)	11 (1–28)	0.071

Data are reported as median (IQR).

TNFAIP2 Protein Expression Is Significantly Higher with the rs8126 G Allele

To determine the functionality of rs8126 with regard to the regulation of *TNFAIP2* expression and the subsequent protein expression, clones of the TNFAIP2 overexpression vector expressing either the A or G allele of

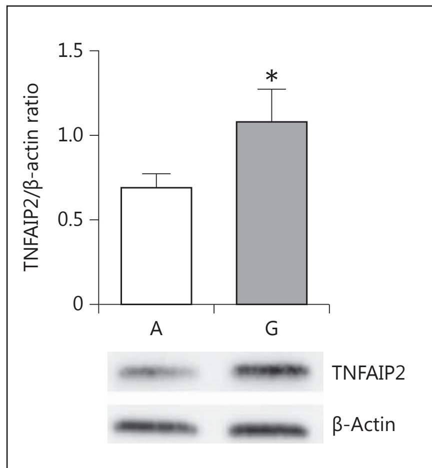

Fig. 3. TNFAIP2 levels are associated with TNFAIP2 rs8126 genotypes. Overexpression of TNFAIP2 clones representing the rs8126 A and rs8126 G alleles in HeLa cells. Protein levels of TNFAIP2 are 1.6-fold greater for the rs8126 G allele (TNFAIP2/β-actin ratio: 1.1) than for the A allele (n = 5; TNFAIP2/β-actin ratio: 0.7). * p = 0.045. A representative image of TNFAIP2 and β-actin Western blots is included.

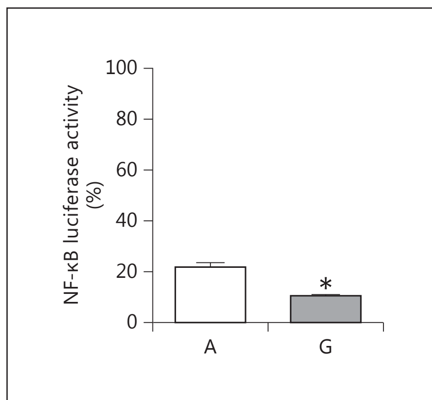

Fig. 4. TNFAIP2 inhibits basal NF-κB luciferase activity with a more pronounced effect in the rs8126 G allele. When co-transfected with the A allele, NF-κB activity is reduced to 22% compared to the control vector ($p_{(A\ to\ control)} = 0.01$), is even further reduced to 10% when the G allele is expressed ($p_{(G\ to\ control)} = 0.009$) and there is a statistically significant difference between the 2 alleles (n = 3). * $p_{(A\ to\ G)} = 0.013$.

TNFAIP2 rs8126 were transfected into HeLa cells for 48 h, and cell lysates were then assayed by Western blot for TNFAIP2. We found that the ratio of the G allele to β-actin was 1.1 whereas the A allele had a ratio of 0.7 and a fold difference of 1.6, suggesting that TNFAIP2 levels are higher when the G allele (minor) is present (5 biological replicates; p = 0.045; fig. 3).

Similar results were obtained after TNFα stimulation (online suppl. fig. 3).

TNFAIP2 Inhibits Basal NF-κB Activity in Unstimulated Cells in an Allele-Specific Manner

In light of the allele-specific differences that we observed for TNFAIP2 protein levels, we co-transfected HeLa cells with either the rs8126 A or G allele of TNFAIP2, along with the NF-κB luciferase reporter to observe NF-κB activity. Both alleles of *TNFAIP2* significantly inhibited NF-κB compared to the control; the A allele inhibited NF-κB activity to 22% ($p_{(A\ to\ control)} = 0.01$) and the G allele inhibited NF-κB activity to 10% ($p_{(G\ to\ control)} = 0.009$) and there was a significant difference between the 2 alleles (3 biological replicates; $p_{(A\ to\ G)} = 0.013$; fig. 4). This suggests that TNFAIP2 inhibits NF-κB signaling (the effect is more pronounced with the rs8126 G allele) and may be a part of the negative feedback loop regulation of NF-κB activation.

TNFAIP2 Silencing in TNFα-Stimulated Cells Upregulates NF-κB Activity, IL-8 mRNA and Protein

Co-transfection of *TNFAIP2* siRNA and NF-κB luciferase resulted in an upregulation of NF-κB luciferase reporter activity (n = 4, p < 0.0001; fig. 5); of note, this is the opposite of the overexpression of TNFAIP2 alleles. IL-8 is a key cytokine in the immune response during septic shock [17], so we looked specifically at the transcription of IL-8 under *TNFAIP2* siRNA conditions in HeLa cells. Based on the luciferase results, we would expect to see an upregulation of IL-8 after transfection with siRNA. We found that IL-8 gene expression was increased by a fold change of 1.925 (p < 0.0001). Similarly, an ELISA of HeLa cell supernatant after siRNA of *TNFAIP2* and TNFα stimulation resulted in a significant increase in IL-8 protein (n = 4, p < 0.00001; fig. 6). Conversely, upon overexpression of the rs8126 A or G allele of *TNFAIP2*, we observed a trend towards inhibition of IL-8 production ($p_{(A\ to\ control)} = 0.036$, $p_{(G\ to\ control)} = 0.90$, $p_{(A\ to\ G)} = 0.031$; online suppl. fig. 4). Interestingly, we found that there was no difference in IL-8 mRNA gene expression (FC = 1.0, p = 0.243).

Discussion

Previous work in our lab and by other study groups has shown that genetic variation within inflammatory genes is associated with outcome in sepsis and septic shock [18–26] as well as with the levels of cytokines [27]. We have shown

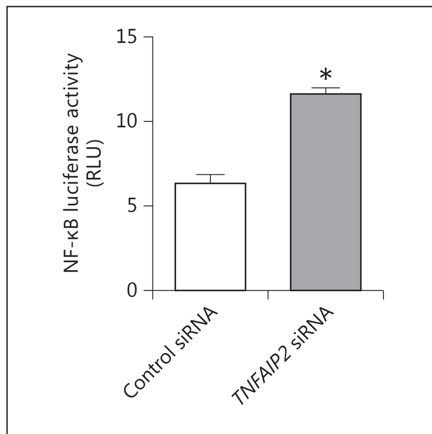

Fig. 5. NF-κB luciferase activity after *TNFAIP2* siRNA transfection. HeLa cells were co-transfected with *TNFAIP2* siRNA and NF-κB luciferase reporter construct for 48 h. The last 24 h included stimulation with 10 ng/μl of TNFα. NF-κB activity increased after transfection with *TNFAIP2* siRNA compared to the control (n = 4). * p < 0.0001.

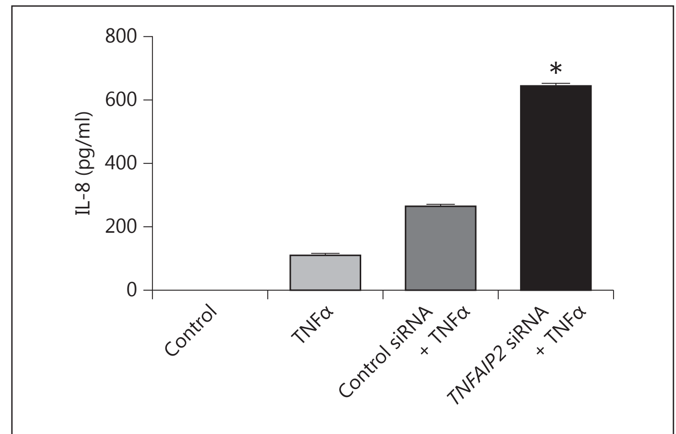

Fig. 6. IL-8 ELISA after siRNA. HeLa cells were transfected with *TNFAIP2* siRNA for 48 h. The last 24 h included stimulation with 10 ng/μl of TNFα. IL-8 ELISA shows that IL-8 protein expression was increased in the supernatant after TNFAIP2 siRNA compared to control siRNA (n = 4). Control and TNFα conditions without any siRNA are also reported. * p < 0.000001.

here that the 3′ UTR SNP rs8126 of TNFAIP2 may functionally regulate the expression of the TNFAIP2 gene. Specifically, the G allele produces increased levels of TNFAIP2 protein in vitro and is associated with higher mortality in septic shock patients. Furthermore, we have found biological plausibility for our clinical genetics association study suggesting that TNFAIP2 is a negative regulator of NF-κB and that this effect is genetically regulated. We cross referenced our gene expression data with published data sets and found three genes with a fold change greater than 2 in HeLa cells stimulated with TNFα common to all data sets *(TNFAIP2, NFKBIA* and *NFKB1)*. We hypothesized that the genes implicated in the microarray would have SNPs associated with outcome in septic shock and that these SNPs would functionally alter signaling. TagSNP genotyping of 13 tagSNPs within the 3 genes showed that rs8176373 of *TNFAIP2* was associated with 28-day survival in the VASST cohort of septic shock patients. In order to elucidate the source of the tagSNP signal, we densely genotyped +/–50,000 bp, upstream and downstream of *TNFAIP2*, and performed the Armitage trend test, which identified rs8126 as the SNP most significantly associated with 28-day survival. Interestingly, rs8176373 is in the 528 bp 3′ of the gene, but rs8126 is located in the 3′ UTR, commonly found to regulate mRNA expression and stability [16].

Based on the discovery of rs8126, we analyzed the Caucasian patients of the VASST cohort by this SNP. The Kaplan-Meier analysis showed a statistically significant difference in 28-day survival, whereby the AA genotype patients had increased survival. This suggests a major allele model. This model would imply that carriage of either 1 or 2 copies of the G allele are detrimental.

To test for potentially confounding variables such as age, gender and surgical diagnosis, we performed the Cox regression using a major allele model and found that patients who had the GG/AG genotype of *TNFAIP2* rs8126 had a significantly decreased survival compared to the AA genotype patients. We also found that the GG/AG genotype patients had fewer days alive and free of organ dysfunction than those with the AA genotype, specifically renal dysfunction and more renal replacement therapy. Furthermore, this effect was replicated when we interrogated renal dysfunction in an independent cohort of septic shock patients (SPHICU), whereby patients with the GG/AG genotype had fewer days alive and free of renal dysfunction than those with the AA genotype. A trend towards fewer days alive and free of renal replacement therapy was also observed.

TNFAIP2, initially discovered as a TNFα-inducible gene, is regulated by NF-κB under most conditions, is induced by retinoic acid in acute promyelocytic leukemia and has been associated with various carcinomas [7, 9, 10, 28–31]. Here, we showed that TNFAIP2 is a negative regulator of NF-κB activity and that the rs8126 G allele potentiates this inhibitory effect. NF-κB plays a central role in regulating the transcription of mediators important in sepsis, and represents a promising therapeutic target in patients with sepsis [32]. Interestingly, another TNFα-

inducible gene, *TNFAIP3 (A20)*, is well known for its upregulation in response to NF-κB signaling for the negative regulation of the NF-κB pathway itself. A20 deubiquitinates RIP at Lys63 and then acts as an ubiquitin ligase to target RIP for degradation [33, 34]. Thus, similar to the known role of A20 in negative regulation of NF-κB signaling, the changes we observed may reflect feedback mechanisms in TNFα superfamily-induced NF-κB signaling. Several approaches have been used to inhibit NF-κB in various models of sepsis [32]. However, the possibility of its use as a therapeutic target is controversial. For example, it was shown that NF-κB inhibition improves survival in endotoxin shock and in a cecal ligation and puncture model of sepsis in combination with antibiotic therapy [35], but, conversely, mice lacking the p50 subunit of NF-κB are unable to effectively clear *Listeria monocytogenes* and are more susceptible to infection with *Streptococcus pneumoniae* [36]. Additionally, enterocyte-specific NF-κB knockout exacerbates sepsis-induced intestinal injury and worsens mortality [37]. Our findings suggest that TNFAIP2 may be one of the several regulatory mechanisms required to modulate NF-κB activity to meet the demands of inflammatory response.

Taken together, our data suggest that the genotype of rs8126 associated with increased mortality in sepsis influences the inhibitory regulation by TNFAIP2 in vitro; clones carrying the G allele of rs8126 make more TNFAIP2, which leads to increased inhibition of NF-κB. We hypothesize that TNFAIP2 could be upregulated in cellular-response settings to negatively regulate the NF-κB pathway and its downstream signaling. We postulate that increased protein levels of TNFAIP2, present when the G allele is expressed, may lead to the excessive inhibition of NF-κB signaling in septic shock, creating a state of immunosuppression due to the lower transcription of cytokines or the lack of proliferation of immune cells. Overexpression of TNFAIP2 results in an inhibition of NF-κB and IL-8 protein, an effect that was reversed after siRNA silencing of *TNFAIP2*. This supports previous data demonstrating that IL-8 transcription is upregulated by TNFα via the NF-κB pathway [38, 39].

This study has several limitations. The analyses of the VASST and SPHICU cohorts were performed post hoc, so the relation of this SNP to outcome or therapy cannot be drawn from this study alone. The discovery that there are significant differences in renal dysfunction between rs8126 genotypes was replicated in the SPHICU cohort, but the association with 28-day survival was not statistically significant. This suggests an organ-specific effect; this justifies further phenotyping and analysis. The Kaplan-Meier curve analysis revealed a major allele model, showing there was very little difference in mortality over time for patients carrying 1 or 2 copies of the detrimental G (minor) allele compared to those carrying 2 copies of the A (major) allele.

We did not measure levels of TNFAIP2 in our septic shock cohort, but the evidence may warrant doing so in a future study. A recent publication found that patients of the CC (GG) genotype of TNFAIP2 rs8126 have an increased risk of squamous cell carcinoma of the head and neck [29]. However, Liu et al. [29] presented luciferase reporter constructs of rs8126 where the C (G) allele made less TNFAIP2, in opposition to our observations; this result could be explained by the differences in the constructs, i.e. we used the full cDNA, but they were only concerned with the 3′ UTR. It is interesting to note that Chen et al. [28] found that silencing *TNFAIP2* reduced the migration and invasion of nasopharyngeal carcinoma HK1 cells, but that knockdown did not affect VEGF. Hence, the migration and metastasis of the carcinomas mediated by VEGF could be via a separate mechanism, suggesting that TNFAIP2 may be pleiotropic in action. Their study did not involve *TNFAIP2* overexpression. In addition, a future study with these experiments, performed in HUVECs or a renal-derived cell line, could enhance the understanding of the tissue-specific action of this mechanism. In order to design a study that would have robust findings, we chose to cross-reference our data with published data before proceeding to genotyping. The low number of genes common to all lists may be due to differences in microarray platform, cell source and passage number, recombinant protein TNFα source, concentration and time period of stimulation, and a recent publication [40] found subpopulations of HeLa cells from ATCC with varying susceptibility to the cytopathic effect of coxsackievirus B3/28. The intersection of these experiments supports the notion that these are, in fact, reliably upregulated genes.

In conclusion, we discovered an association of the A allele of TNFAIP2 rs8126 with increased 28-day survival and organ dysfunction in septic shock patients. Similar to the known role of A20 in the negative regulation of NF-κB signaling, overexpression of TNFAIP2 inhibits NF-κB-driven luciferase activity with significant differences according to rs8126 allele. The changes may reflect feedback mechanisms in TNFα superfamily-induced NF-κB signaling. We found that levels of TNFAIP2 protein differ by genotype and this is supported in the NF-κB luciferase assay of overexpression and knockdown of TNFAIP2. In agreement with these data, cytokine levels of IL-8 are impacted, suggesting that TNFAIP2 is a novel inhibitor of NF-κB.

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