The *NFKB1* Promoter Polymorphism (–94ins/delATTG) Alters Nuclear Translocation of NF-κB1 in Monocytes after Lipopolysaccharide Stimulation and Is Associated with Increased Mortality in Sepsis

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ABSTRACT

Background: Because the nuclear factor-κB (NF-κB) coupled pathway is believed to amplify inflammation prevailing in sepsis, the authors tested the hypotheses that the insertion–deletion polymorphism (–94ins/delATTG) (1) alters nuclear translocation of nuclear factor-κB and activator protein-1 (NF-κB1) in monocytes after lipopolysaccharide stimulation; (2) affects lipopolysaccharide-induced NF-κB1 messenger RNA expression, tumor necrosis factor α concentrations, and tissue factor activity; and (3) may be associated with increased 30-day mortality in patients with sepsis.

Methods: Nuclear translocation of NF-κB1 in monocytes after lipopolysaccharide stimulation from healthy blood donors was performed with immunofluorescence staining (n = 5 each). Lipopolysaccharide-induced NF-κB1 messenger RNA expression was measured with real-time polymerase chain reaction (PCR; n = 60), tumor necrosis factor α concentrations with a multiplexing system kit (n = 60), and tissue factor activity with thromboelastometry (n = 105). In a prospective study, multivariate proportional hazard analysis tested 30-day mortality in patients with sepsis (n = 143).

Methods and Results: The homozygous deletion genotype compared with the homozygous insertion genotype was associated with a nearly twofold increase in nuclear translocation of NF-κB1 (P = 0.001), a threefold difference in NF-κB1

What We Already Know about This Topic

- The nuclear factor-κB and activator protein-1–coupled signaling pathway of the innate immune system is known to amplify and perpetuate inflammatory and coagulatory mechanisms in sepsis
- Genetic variations that alter nuclear factor-κB gene expression could affect pathophysiologic mechanisms and thereby influence mortality in severe sepsis

What This Article Tells Us That Is New

- A functional insertion–deletion polymorphism in the promoter of nuclear factor-κB and activator protein-1 affected expression and regulation of mediators of inflammation and coagulation *in vitro*
- Expression of the same polymorphism was associated with increased 30-day mortality in patients with severe sepsis

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messenger RNA expression (P = 0.001), and a twofold increase in tissue factor expression (P = 0.021). The deletion allele in adults with severe sepsis was tested as an independent prognostic factor for 30-day mortality (hazard ratio, 2.3; 95% CI, 1.13–4.8; P = 0.022). Mortality was 25% for homozygous insertion genotypes but 41% for combined heterozygous deletion/homozygous deletion genotypes (P = 0.034).

Conclusion: The deletion allele of the NFκB1 insertion–deletion (–94ins/delATTG) polymorphism is associated with increased 30-day mortality in patients with severe sepsis and increased reaction of the innate immune system.

THERE is wide variability regarding the outcome in severe sepsis, which in part may be caused by genetic variations.[1–3] A potential candidate for such variations is the gene encoding nuclear factor-κB and activator protein-1 (NF-κB1), because the ubiquitous transcription factor NF-κB1 binds to recognition elements in the promoter regions of several genes encoding the innate immune and the coagulation system. The nuclear factor-κB family comprises five proteins, NF-κB1, NF-κB2, RelA, RelB, and c-Rel. Nuclear factor-κB family members share structural homology with the retroviral oncoprotein v-Rel, resulting in their classification as NF-κB/Rel proteins. The *NFKB1* gene encodes the NF-κB-p105 subunit. NF-κB1 and NF-κB2 proteins are translated as large precursors, p105 and p100, which undergo processing, regulated by the ubiquitin–proteasome pathway, to build the mature nuclear factor-κB subunits, p50 and p52.

In most cells, NF-κB1 is found in the cytoplasm in its inactive form, bound to inhibitory proteins. Many extracellular stimuli, including bacterial lipopolysaccharide, viruses, oxidants, inflammatory cytokines, and immune stimuli, can activate NF-κB1.[4,5] Once activated, it binds to regulatory DNA elements in the promoter regions of inflammatory and immune response genes, such as those encoding proinflammatory cytokines, chemokines, enzymes relevant for inflammation, and adhesion molecules.[6,7] Furthermore, this pathway induces tissue factor in the blood stream and, thus, activates coagulation.[7–10] Consequently, disseminated intravascular coagulation is a common finding during severe sepsis, which in turn can evoke ischemia and multiple organ dysfunction.[8,9] In conclusion, the NF-κB1–coupled pathway is known to amplify and perpetuate inflammatory and coagulatory mechanisms prevailing in sepsis.[10] Accordingly, genetic variations that alter *NFKB* gene expression could impact key mechanisms in sepsis and, therefore, influence mortality in severe sepsis.

Recently, a functional insertion–deletion polymorphism in the promoter of *NFKB1* (–94ins/delATTG in relation to the transcription initiation site or –24.219ins/delATTG in relation to the A as +1 of the initiation codon ATG), which encodes the major isoform of NF-κB1, was reported to be associated with altered *NFKB1* gene expression and an increased risk for ulcerative colitis.[11] Furthermore, we found that the deletion allele influences severity but not mortality of patients suffering from acute respiratory distress syndrome.[12] In conclusion, there are many reasons to suspect that the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism could impact key mechanisms in sepsis and, therefore, influence mortality in severe sepsis.

Accordingly, we prospectively tested the hypotheses that the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism (1) alters nuclear translocation of NF-κB1 in monocytes after lipopolysaccharide stimulation; (2) affects lipopolysaccharide-induced NFκB1 messenger RNA (mRNA) expression, tumor necrosis factor-α (TNFα) concentration, and tissue factor activity; and (3) may be associated with increased 30-day mortality in patients with severe sepsis.[13]

Materials and Methods

Experimental Procedures

Nuclear Translocation of NF-κB1 in Monocytes after Lipopolysaccharide Stimulation Is Dependent on the NFKB1 Promoter Polymorphism (–94ins/delATTG). After ethics committee approval (ethics committee of the University Hospital Essen, Essen, Germany) and written informed consent, venous blood (24 ml) was withdrawn from 10 healthy individuals with the homozygous insertion (II) or deletion (DD) genotype, respectively, and centrifuged at 1800*g* for 20 min using Ficoll density gradient centrifugation tubes (Vacutainer CPT tubes; Becton Dickinson, Franklin Lakes, NJ). Monocytes were resuspended in RPMI 1640 medium (Gibco Products Invitrogen Corporation, Grand Island, NY) containing 5% fetal calf serum (Biochrom AG, Berlin, Germany) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen Corporation, Carlsbad, CA). The cell suspension was added to cell culture tubes, and monocytes were allowed to adhere to the surface of the tubes for 2 h. Finally, the supernatant was discarded, fresh RPMI 1640 medium was added, and the cells were allowed to rest for 48 h (37°C; 5% CO_2 in air) before the experiments. Meanwhile, 12 mm glass plates were coated with 400 μl fibronectin (1 mg/ml; Sigma-Aldrich, St. Louis, MO) inside 24-well plates for 24 h. Afterward, the supernatant was discarded and 500 μl cell suspension (1×10^6 monocytes ml^{-1}) was added. Cells were incubated for 60 min either with lipopolysaccharide (10 μg/ml, serotype 0111:B4, Sigma-Aldrich) or without lipopolysaccharide. After incubation, cells were fixed with ice-cold methanol–acetone (1:1) for 10 min at –20°C. Immunofluorescence staining was performed using a primary NF B rabbit antihuman polyclonal p65 antibody (1:200 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA) followed by a Immunoglobulin G - Alexaflour 568 coupled goat anti-rabbit antibody (1:400 dilution; Molecular Probes, Eugene, OR), as described previously.[14]

An independent investigator, blind for the specific *NFKB1* promoter polymorphism and stimulus applied, processed all immunofluorescence slides. Fluorescence microscopy was performed on a Nikon Eclipse E1000 microscope (Nikon GmbH, Düsseldorf, Germany) using the NIS-Elements F.30.0 imaging software (Laboratory Imaging, Prague, Czech

Republic). All slides were analyzed in a standardized order, that is, a representative image of each quadrant was captured at 20-fold magnification, and nuclear NFκB-positive cells were counted using an Image J software (ImageJ; National Institute of Health, Bethesda, MD).

NF-κB1 mRNA Expression, TNFα Concentration, and Tissue Factor Activity after Lipopolysaccharide Stimulation as Dependent on the NFKB1 Promoter Polymorphism (–94ins/delATTG). After approval by the ethics committee of the University Hospital Essen and written informed consent, venous blood was drawn from the antecubital vein of 105 healthy volunteers. After discarding the first 2 ml, blood was collected in one-tenth volume of citrate (Sarstedt Monovette, Nümbrecht, Germany) and samples were immediately used for the experiments. Subsequently, whole blood samples were incubated with lipopolysaccharide at final concentrations of 0, 25, 75, and 225 μg/ml for 4 h at 37°C. Thereafter, samples were subjected to thromboelastometry, and mRNA and DNA were isolated as described in DNA Genotyping. In addition, TNFα concentration was determined in plasma obtained from whole blood samples after incubation with lipopolysaccharide (225 μg/ml; n = 60).

To further elucidate the mechanism of the lipopolysaccharide-induced activation of coagulation, lipopolysaccharide- and vehicle (NaCl 0.9%)-treated whole blood samples were incubated with the protein synthesis inhibitor cycloheximide (35 μM) and with an inhibitor of tissue factor effects, active site–inhibited factor seven (50 μg/ml), for 30 min, subsequently challenged with lipopolysaccharide (225 μg/ml) or vehicle (NaCl 0.9%) for 4 h, and then subjected to thromboelastometry as recently described.[15,16] To assess the tissue factor concentrations induced by lipopolysaccharide, a standard curve was generated by addition of tissue factor standard to whole blood samples and the resulting clotting time was determined.

Thromboelastometry

Citrate blood samples were subjected to rotational thromboelastometry (Roteg 5™, Pentapharm, Munich, Germany), a modification of the original thromboelastography described in 1948 by Hartert,[17] and coagulation was initiated by recalcification with calcium chloride.[18,19] Clotting times were determined from the thromboelastogram as described in References 18 and 19. Kinetics of clot formations are measured by the clot formation time and the angle α. The maximum amplitude reflects the strength of the clot and is dependent on both the number and function of platelets and their interaction with fibrin.[18,19]

Measurement of TNFα

TNFα plasma levels were determined by using a microsphere-based multiplexing system kit (Invitrogen, Paisley, United Kingdom). As previously shown, the assays were performed according to the manufacturer's protocol and concentrations measured with a Luminex 100 system (Luminex, Austin, TX).[20]

NF-κB1 mRNA Expression

RNA from whole blood (n = 105) was extracted using the RNeasy kit (Qiagen, Hilden, Germany). First-strand complementary DNA was synthesized from 0.6 μg of total RNA. Quantification of NF-κB1 mRNA was performed by using the intron spanning primer (forward primers) 5′-GTGAAGGCCCATCCCATGGT-3′ and (reverse primer) 5′-TGTGACCAACTGAACAATAACC-3′ resulting in a 122-bp fragment. PCR and primers for the housekeeping gene hβ-actin were used as described.[12,21] The real-time PCR reaction was performed as described using the Quantitect SYBR Green Kit (Qiagen).[21,22] A complementary DNA dilution series confirmed a PCR efficiency greater than 95%, which was comparable to the efficiency of hβ-actin. Relative *NF-κB1* mRNA expression was measured by two-step real-time PCR with hβ-actin as internal control and calculated as $2^{-[Ct(NFKB1)-Ct(\beta\text{-Actin})]}$.

DNA Genotyping

Genomic DNA of patients was extracted from whole blood using standard methods (QIAamp, Qiagen). Genotypes of the insertion–deletion polymorphism were determined by pyrosequencing. A 200-bp PCR fragment was amplified using primer *NFKB1*_del/ins_f(5′-ATGGAC CGCATGACTCTATCAG-3′) and biotinylated primer *NFKB1*_del/ins_BIO_r(5′-GGGGCGCGCGTTAG GCGG-3′). PCR was performed at an annealing temperature of 60°C in a 50-μl reaction mixture applying a commercially available PCR master mix (Eppendorf, Hamburg, Germany). Pyrosequencing was performed on a PSQ96 MA (Pyrosequencing, Uppsala, Sweden) using sequencing primer *NFKB1*_del/ins_seq (5′-CGTTCCCCGACCAT-3′). Randomly chosen samples were reanalyzed with a different nucleotide injection order for genotype confirmation.

Materials

Lipopolysaccharide (*Escherichia coli*; serotype 0.111:B4) was obtained from Sigma-Aldrich. Tissue factor standard was obtained from Thrombinoscope BV, Maastricht, Netherlands. Active site–inhibited factor VIIa was a generous gift from Novo Nordisk, Zurich, Switzerland. All other reagents were of analytical grade.

Thirty-day Mortality in Patients with Severe Sepsis Dependent on the NFKB1 Promoter Polymorphism (–94ins/delATTG). This study was reviewed and approved by the ethics committee of the University Hospital Essen. Over a period of 2 yr, 143 patients (93 men and 50 women, mean age: 57 ± 16 yr) admitted to an intensive care unit of the University Hospital of Essen were considered eligible for the study if they fulfilled the criteria for severe sepsis as defined by Bone *et al.*[23] Informed consent was obtained for all patients from the guardian of the patient. Exclusion criteria were age more than 18 yr and no affiliation to the Caucasian ethnicity or no informed consent was obtained. All patients were white Germans of Caucasian ethnicity. Clinical and demographic data on study entry, including Simplified

Table 1. Characteristics of Patients with Severe Sepsis at Baseline

Patient Characteristics At Baseline	II (n = 68) (48%)	ID/DD (n = 75) (52%)	*P* Value
Age (yr; means ± SD)*	56 ± 15	60 ± 16	0.14
Male/Female ‡	46/22	47/28	0.53
BMI (kg/m²; means ± SD)*	27 ± 5	27 ± 6	0.99
Cardiovascular disease	17	12	—
Hemato-oncologic disease	2	4	
Gastrointestinal disease	18	26	
Gastrointestinal cancer	10	12	
Lung disease	7	11	
Lung cancer	2	1	
Urogenital disease	1	3	
Urogenital cancer	6	3	
Skin and fascial infection	3	1	
Trauma	2	2	
CVVHD, % †	53	61	0.41
Mechanical ventilation, % †	100	100	1.00
C-reactive protein (mg/dl; mean ± SD)*	14 ± 10	14 ± 9	0.75
Interleukin 6 (pg/ml; mean ± SD)*	654 ± 1,942	2,622 ± 12,646	0.29
Procalcitonin (ng/ml; mean ± SD)*	15 ± 48	23 ± 48	0.34
SAPS II score (mean ± SD)*	46 ± 17	49 ±± 17	0.23
SOFA score (mean ± SD)*	11 ± 4	13 ± 6	0.015
Gram-positive isolates only, %	44	38	—
Gram-negative isolates only, %	33	32	
Mixed bacterial isolates, %	11	17	
Viral isolates, %	0	0	
Fungal isolates, %	2	2	
Negative cultures, %	10	11	

* Data are presented as means ± SD and *P* value based on Welch *t* test. † *P* values based on Fisher exact tests. ‡ No statistical test has been applied here.

BMI = body mass index; CVVHD = continuous hemofiltration/dialysis; DD = homozygous deletion genotype; II = homozygous insertion genotype; ID = heterozygous deletion genotype; SAPS II = Simplified Acute Physiology Score; SOFA = Sequential Organ Failure Assessment Score.

Acute Physiology Score II[24] and the Sequential Organ Failure Assessment score,[25] were calculated over the first 24 h after the patient met severe sepsis criteria (table 1). All patients were followed up for 30-day mortality. There were no dropouts from the procedures or data lost in any fashion. Patients were treated with a multimodal concept, which included analgosedation, fluid administration, and protective mechanical ventilation, hemodynamic, antibiotic, and diagnostic management. Continuous hemofiltration/dialysis was initiated and technically performed by the Department of Nephrology according to standardized protocols.

DNA was isolated and genotyped for the *NFKB1* promoter polymorphism (–94ins/delATTG) as described in DNA Genotyping.

Data Analysis and Statistics

After incubation, the percentage of nuclear NFκB-positive cells was normalized to the percentage of nuclear NFκB-positive cells in unstimulated controls. The Kolmogorov–Smirnov test and Shapiro–Wilk normality test revealed a normal distribution for nuclear NFκB-positive cells, and the Student *t* test for unpaired samples was used to analyze differences between groups. Semiquantitative analysis was performed using the Graphpad Prism 5 statistics program (Graph Pad Software, San Diego, CA).

To test the effect of the *NFKB1* promoter polymorphism (–94ins/delATTG) on NF-κB1 mRNA expression, TNFα concentration, and tissue factor activity after lipopolysaccharide stimulation, heterozygous deletion (ID) and DD genotypes were combined and then tested against the II genotype, because there were only five volunteers with the DD genotype. Potential deviation from the Hardy–Weinberg equilibrium was tested with the statistic program Excel of Microsoft Office 2010 (Microsoft Deutschland, Unterschleißheim, Germany).

Statistical analyses were performed using two-way ANOVA followed by *post hoc* testing with Bonferroni Holm, respectively, using SPSS 13.0 (SPSS, Chicago, IL) or Graphpad Prism 5.0. Data are presented as mean and standard error or SD of the mean, as indicated. For the determination

Table 2. Cox Regression Analyses in Patients with Severe Sepsis

	Univariate		Multivariate			
			Initial		Restricted	
(Co) Variable	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value
NFKB1 genotype						
II	1	—	1	—	1	—
ID/DD	1.4 (1.03–1.9)	0.039	2.33 (1.11–4.86)	0.025	2.33 (1.13–4.80)	0.022
Sex						
Female	1	—	1	—		
Male	0,87 (0.66–1.4)	0..318	1,41 (0.68–2.96)	0.36		
Age (per 5 yr)	0.99 (0.91–1.09)	0.898	0,97 (0.97–1.02)	0.81		
SAPS II score (per U)	1.03(1.01–1.05)	0.010	1.02(0.99–1.05)	0.28	1.08 (0.98–1.03)	0.665
SOFA score (per U)	1.04(0.99–1.08)	0.132	0.96(0.88–1.10)	0.39		
Requirement of continuous hemofiltration/dialysis						
No	1	—	1	—	1	—
Yes	3.32 (1.53–7.23)	0.003	3.29 (1.30–8.29)	0.12	3.4 (1.38–8.22)	0.008
C-reactive protein concentration (per $\log_{10}$[mg/dl])	0.84 (0.44–1.64)	0.618	0.98 (0.94–1.03)	0.59		
Interleukin 6 concentration (per $\log_{10}$[pg/ml])	2.06 (1.48–2.85)	<0.001	1.0 (1.0–1.0)	0.26	1.0 (1.0–1.0)	0.38
Procalcitonin concentration (per $\log_{10}$[ng/ml])	1.39 (0.96–2.01)	0.085	1.0 (0.99–1.01)	0.42		

Hazard ratio point estimates, 95% CIs, and *P* values (two-sided) from Wald tests are reported.

DD = homozygous deletion genotype; ID = heterozygous deletion genotype; II = homozygous insertion genotype; SAPS II = Simplified Acute Physiology Score; SOFA = Sequential Organ Failure Assessment Score.

of tissue factor concentrations from clotting time, a four-parameter Hill function was used for the curve fit (SigmaPlot Software, San Jose, CA).

The *NFKB1* promoter polymorphism (–94ins/delATTG) genotype distributions were tested for deviations from Hardy–Weinberg equilibrium (exact two-sided *P* value 1.00) in the sepsis cohort. Explorative comparisons by *NFKB1* promoter polymorphism (–94ins/delATTG) genotypes (ID/DD *vs.* II) were performed for several clinical characteristics of the sepsis patients (table 1). ID and DD were combined because of the low frequency of the DD genotype.

The clinical endpoint was survival over the first 30 days dependent on *NFKB1* promoter polymorphism (–94ins/delATTG) genotypes. Survival probabilities were graphically assessed by the Kaplan–Meier method, and the log-rank test was used to evaluate the univariate relationship between *NFKB1* genotypes and clinical outcome. Thereafter, we performed multivariate Cox regression analyses to assess the joint impact of *NFKB1* promoter polymorphism (–94ins/delATTG) genotypes, sex, age, Simplified Acute Physiology Score II, Sequential Organ Failure Assessment score, requirement of continuous hemofiltration/dialysis, C-reactive protein, and interleukin 6 concentration as predictors for the clinical outcome (30-day survival). Model diagnostic of the proportional-hazards assumption for the *NFKB1* promoter polymorphism (–94ins/delATTG) genotypes comprised both graphical and formal investigations—none of which indicated strong evidence for a deviation from the proportional-hazards assumption. The multivariate analyses included two steps with a focus on *NFKB1* promoter polymorphism (–94ins/delATTG) genotypes and 30-day survival. In the initial model, all main effects were simultaneously investigated (table 2). To avoid overfitting, a restricted model with only four variables was assessed afterward using only those predictors with a *P* value ≤0.05 in either the univariate or the initial multivariate comparison (table 2).

CIs were calculated with coverage of 95% (abbreviated 95% CI). All reported *P* values are nominal, two-sided, and we applied a significance level α of 5%.

Results

Nuclear Translocation of NF-κB1 in Monocytes after Lipopolysaccharide Stimulation According to the NFKB1 Promoter Polymorphism (–94ins/delATTG)

After lipopolysaccharide stimulation, the DD genotype was associated with a 5.5-fold (± 2.9 SD) increase in nuclear NFκB-positive cells normalized to controls (incubation with vehicle), compared with a 2.8-fold (±0.9 SD) increase in II genotype (*P* = 0.001, fig. 1).

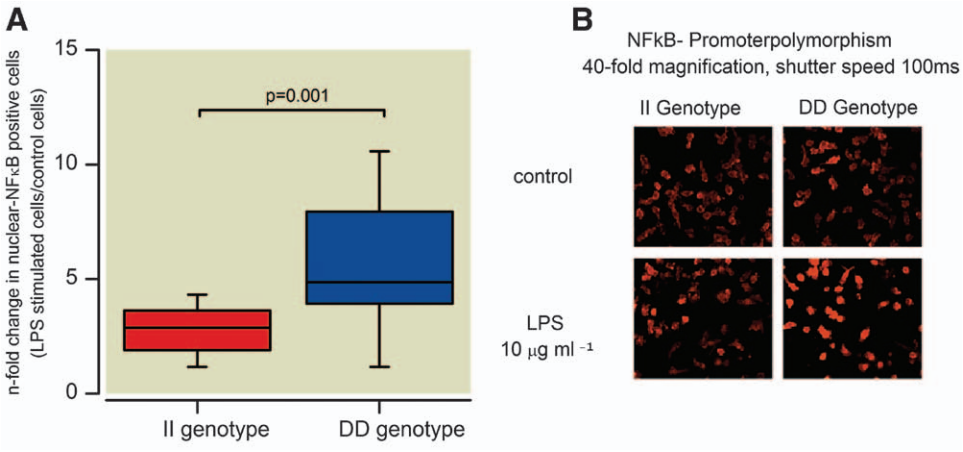

Fig. 1. *A*, The *NFKB1* promoter polymorphism (–94ins/delATTG) alters nuclear translocation of nuclear factor-κB and activator protein-1 (NF-κB1) in monocytes after LPS stimulation. *B*, Nuclear translocation of NF-κB1 in monocytes after lipopolysaccharide stimulation as visualized with immunofluorescence staining. The DD genotype was associated with a twofold increase in nuclear NFκB-positive cells compared with the II genotype. DD = homozygous deletion; II = homozygous insertion; LPS = lipopolysaccharide.

NF-κB1 mRNA Expression, TNFα Concentration, and Tissue Factor Activity after Lipopolysaccharide Stimulation According to the NFKB1 Promoter Polymorphism (–94ins/delATTG)

Lipopolysaccharide-induced Expression of NF-κB1 mRNA. NF-κB1/actin mRNA ratios were determined in the absence and presence of lipopolysaccharide (225 μg/ml). The mRNA for NF-κB1 increased nearly threefold from 0.013 ± 0.025 to 0.034 ± 0.025 (P = 0.002) on lipopolysaccharide stimulation (fig. 2). Moreover, the NF-κB1/actin ratio was markedly associated with genotypes. As shown in figure 2, lipopolysaccharide-induced expression of NF-κB1 was three times as high in the presence of the deletion allele compared with the homozygote II genotype (0.018 ± 0.015 *vs.* 0.051 ± 0.001, P = 0.0001). In the absence of lipopolysaccharide, NF-κB1 expression was not associated with genotypes.

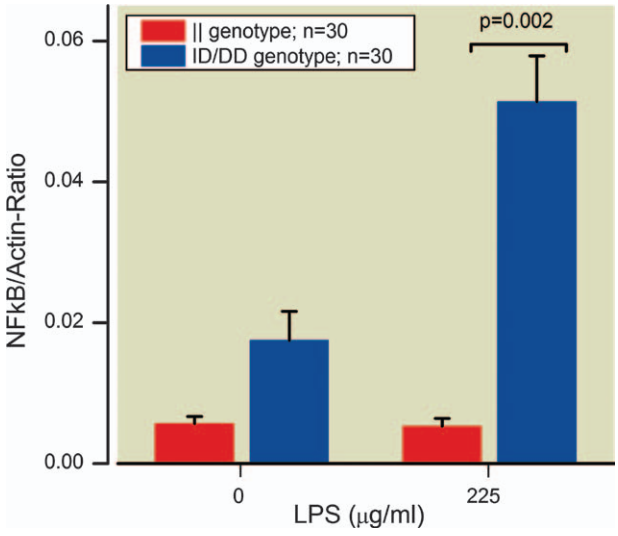

Fig. 2. Effect of the *NFKB1* polymorphism on the expression of the transcription factor NF-κB1 in the absence and presence of lipopolysaccharide. The LPS (225 μg/ml)–induced increase in NF-κB1 messenger RNA was markedly higher in volunteers with the deletion allele (ID/DD) compared with the II genotype. Data are the means ± SEM. DD = homozygous deletion; ID = heterozygous deletion; II = homozygous insertion; LPS = lipopolysaccharide.

Effects of Lipopolysaccharide on Whole Blood Clotting Time. Clotting time was determined in 105 volunteers both in the absence of lipopolysaccharide and after 4 h of incubation with lipopolysaccharide. Lipopolysaccharide shortened the clotting time in a concentration-dependent manner from 612 ± 10 to 325 ± 10 s (25 μg/ml lipopolysaccharide), 282 ± 10 s (75 μg/ml lipopolysaccharide), and 220 s ± 8 s (225 μg/ml lipopolysaccharide), respectively.

Effects of the NFKB1 Insertion–Deletion Polymorphism on Clotting Time. Determination of *NFKB1* genotypes revealed 42 subjects homozygous for the insertion allele, 45 were heterozygous, and 5 were homozygous for the deletion allele. As shown in figure 3, the clotting times of II and combined deletion allele carriers were not different in the absence of lipopolysaccharide (618 ± 17 *vs.* 607 ± 15 s; P = 0.58). After stimulation with lipopolysaccharide, however, the deletion allele overall lipopolysaccharide stimulation concentrations were associated with a 20% lower clotting time compared with the II genotype (25 μg/ml, P = 0.047; 75 μg/ml, P = 0.003; and 225 μg/ml, P = 0.021). Neither maximum clot formation nor clot formation time was associated with genotypes (data not shown).

Mechanism of the Lipopolysaccharide-induced Shortening of Clotting Time. To unravel the mechanism underlying the lipopolysaccharide-induced shortening of clotting time, the effects of the protein synthesis inhibitor cycloheximide and of the tissue factor inhibitor, active site–inhibited factor seven, were determined. Both drugs abolished the effect of lipopolysaccharide on clotting time, demonstrating that

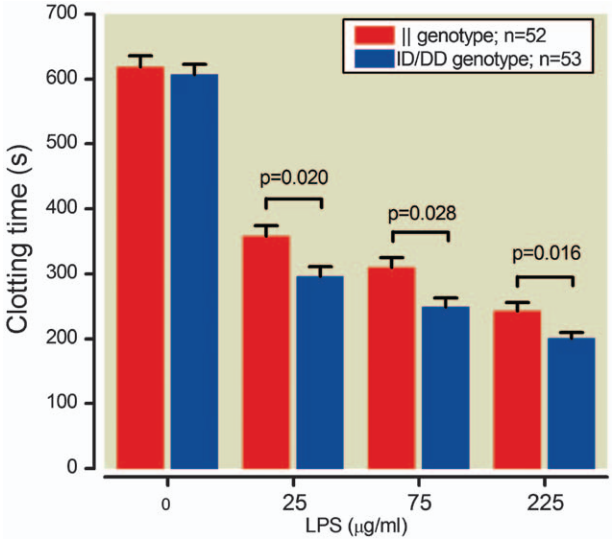

Fig. 3. Effects of the NFKB1 genotypes on whole blood clotting time induced by LPS. Whole blood samples were incubated with LPS (0, 25, 75, 225 μg/ml) for 4 h at 37°C. Thereafter, clotting time was determined by thromboelastometry. LPS evoked a concentration-dependent shortening of clotting time, which was markedly affected by the genotype. Data are shown as means ± SEM, and statistical significances between genotypes are indicated by *P* values. Combined ID/DD genotypes and II genotype. DD = homozygous deletion; ID = heterozygous deletion; II = homozygous insertion; LPS = lipopolysaccharide.

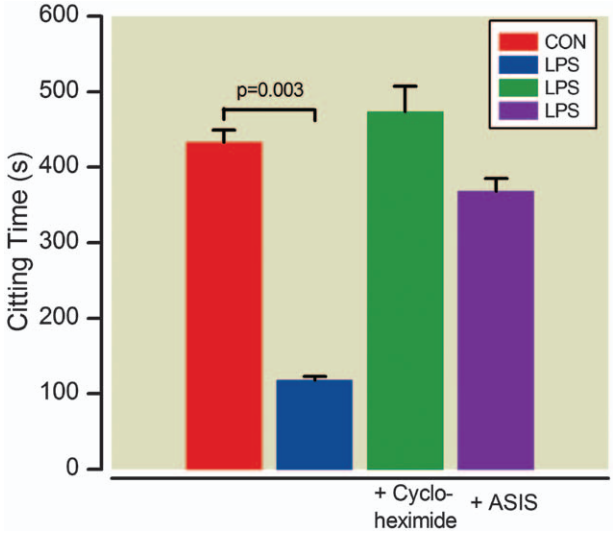

Fig. 4. Influence of protein synthesis inhibition (cycloheximide 35 μM) and tissue factor blockade ASIS (50 μg/ml) on LPS (225 μg/ml)–induced shortening of clotting time. The lipopolysaccharide effect was abolished by protein synthesis inhibition or tissue factor blockade. In the absence of lipopolysaccharide, cycloheximide and active site–inhibited factor seven had no effect on clotting time. Data from six volunteers are the means ± SEM. Statistical significance of the lipopolysaccharide-evoked shortening of clotting time is indicated by *P* values. Controls (CON). ASIS = active site–inhibited factor seven; LPS = lipopolysaccharide.

lipopolysaccharide exerts its effect *via de novo* protein synthesis of tissue factor (fig. 4).

Tissue Factor Concentration and Whole Blood Clotting Time. To determine the biologically active tissue factor concentration responsible for the observed lipopolysaccharide-induced shortening of clotting time, a concentration response curve was constructed using tissue factor standards. As shown in figure 5A, exogenous tissue factor shortened the clotting time over a broad concentration range from 20 fM to 20 pM. Correlation by nonlinear

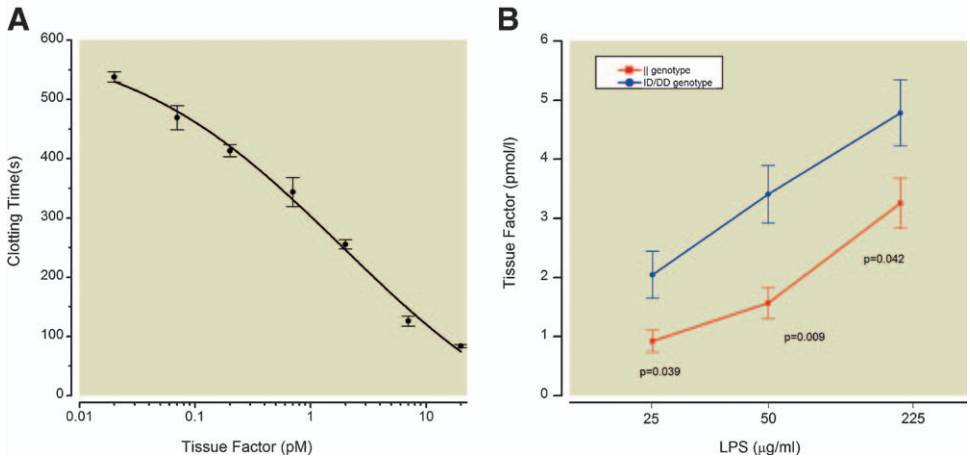

Fig. 5. *A*, Concentration response curve of tissue factor standards on whole blood clotting time. Tissue factor shortened clotting time in a concentration-dependent fashion. Data from six volunteers are the means ± SEM. Clotting time was significantly shortened by tissue factor at all concentrations tested ($P < 0.05$). *B*, Effects of the *NFKB1* polymorphism on the tissue factor concentration in whole blood samples incubated with LPS. Compared with the II genotype, the deletion allele (ID/DD) shifted the dose–response curve to fourfold lower LPS concentrations. Values are depicted as means ± SEM. Statistical significance of lipopolysaccharide-induced shortening of clotting time is indicated by *P* values in the figure. DD = homozygous deletion; ID = heterozygous deletion; II = homozygous insertion; LPS = lipopolysaccharide.

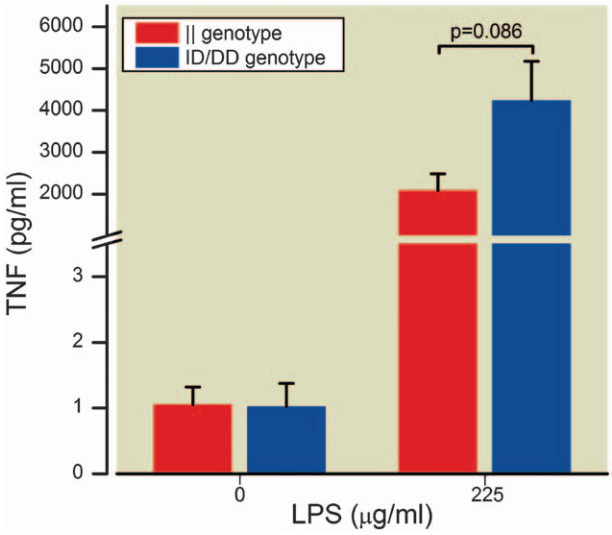

Fig. 6. Effect of the *NFKB1* insertion–deletion polymorphism on the plasma concentration of TNFα. Whole blood samples were incubated with LPS 225 μg/ml or vehicle for 4 h. Thereafter, TNFα was determined in plasma. Data are the means ± SEM, *P* values are given in the figure. II genotype, combined ID/DD genotypes. DD = homozygous deletion; ID = heterozygous deletion; II = homozygous insertion; LPS = lipopolysaccharide; TNF = tumor necrosis factor.

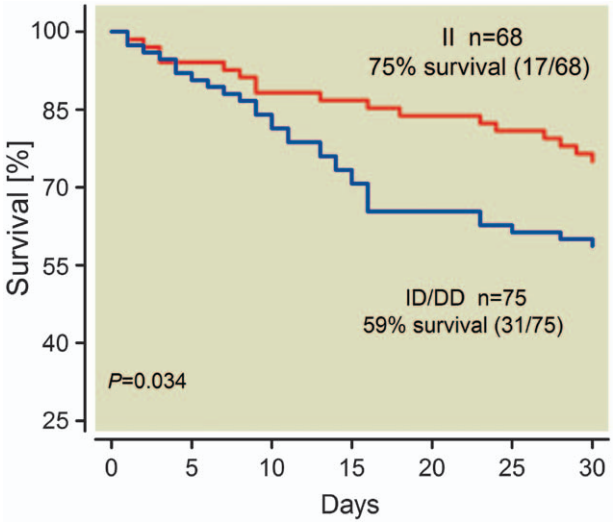

Fig. 7. Kaplan–Meier plot of 30-day mortality of patients with severe sepsis dependent on the *NFKB1* insertion–deletion promoter polymorphism (–94ins/delATTG). Mortality was 25% for II genotypes but 41% for combined ID/DD genotypes (*P* = 0.034). DD = homozygous deletion; ID = heterozygous deletion; II = homozygous insertion.

regression analysis in six experiments using a four-parameter Hill function was 0.98. Figure 5B depicts the effects of the genotypes of the NFKB1 insertion–deletion polymorphism on tissue factor concentration in the presence of lipopolysaccharide as calculated from the standard curve shown in figure 5A. In comparison to the homozygous II genotype, the lipopolysaccharide-induced tissue factor concentration was two- to three-fold increased in the presence of the deletion allele (25 μg/ml, *P* = 0.039; 75 μg/ml, *P* = 0.003; and 225 μg/ml, *P* = 0.021; fig. 5B)

TNFα Plasma Concentration. Because TNFα release is affected by the activity of NF-κB1, we determined the effect of lipopolysaccharide on the concentration of this cytokine (fig. 6). Lipopolysaccharide (225 μg/ml) increased the TNFα concentration more than 1,000-fold (*P* < 0.001). The effect of lipopolysaccharide was not modulated by the genotypes of the *NFKB1* promoter polymorphism, because testing with a two-way ANOVA after *post hoc* testing with Bonferroni Holm revealed a *P* value of 0.08. In addition, in the absence of lipopolysaccharide, TNFα concentrations were not different.

Thirty-day Mortality in Patients with Severe Sepsis According to the NFKB1 Promoter Polymorphism (–94ins/delATTG). Thirty-day mortality was significantly associated with NFKB1 insertion–deletion (–94ins/delATTG) polymorphism genotypes (P = 0.034). Mortality was 25% for II genotypes but 41% for combined ID/DD genotypes (fig. 7). ID/DD genotypes displayed a significantly higher risk for death than patients with homozygous II genotypes (hazard ratio, 1.4; 95% CI, 1.03–1.9; *P* = 0.034).

Multivariate proportional hazard analysis, including sex, age, Sequential Organ Failure Assessment score, Simplified Acute Physiology Score II, procalcitonin, C-reactive protein, or interleucin-6 concentrations, and requirement for continuous hemofiltration/dialysis as covariates revealed the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism as an important and independent prognostic factor for 30-day mortality. ID/DD genotypes had an almost twofold greater risk for death (hazard ratio, 2.3; 95% CI, 1.13–4.8; *P* = 0.022) compared with II genotypes (table 2).

The requirement of continuous hemofiltration/dialysis was also an independent and strong prognostic factor for 30-day survival (hazard ratio, 3.4; 95% CI, 1.38–8.22; *P* = 0.008). Genotype frequencies and the clinical characteristics of the patients grouped by *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism genotypes are displayed in table 1. We found no significant associations of *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism genotypes with sex, age, body mass index, procalcitonin, C-reactive protein, or interleucin-6 concentrations, necessity for continuous hemofiltration/dialysis, type of infection, or primary diagnosis at hospital admission. In contrast, the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism was associated with a genotype-dependent increase of the Sequential Organ Failure Assessment score (*P* = 0.015) (table 1). The deletion allele carriers had higher Sequential Organ Failure Assessment score compared with the II genotype carriers.

Discussion

Our study shows for the first time that the ID/DD genotypes of the *NFKB1* insertion–deletion (–94ins/delATTG)

polymorphism are significantly associated with markedly increased 30-day mortality in patients with severe sepsis. In addition, our study unravels the molecular mechanism by which the D allele may amplify and perpetuate inflammatory mechanism prevailing in sepsis.

Genotypes of the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism turned out to represent both an important and independent prognostic factor for 30-day mortality. The hazard ratio of 2.3 not only suggests that the D allele of the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism has important effects on NF-κB1 expression but also underscores the potential relevance of NF-κB1 expression in severe sepsis. Because wide variability exists regarding the outcome in sepsis,[1,26] part of this variability may be explained by the genetic variation in the promoter of *NFKB1.*

In addition, our results show that the D allele is also associated with initial disease severity, because it was associated with an increased Sequential Organ Failure Assessment score. These findings are in accordance with our previous investigation, showing that the deletion allele of this polymorphism was also associated with increased severity of adult respiratory distress syndrome.[12]

Of note, this study unravels molecular mechanism by which the D allele of the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism seems to amplify and perpetuate inflammatory mechanism prevailing in sepsis, which may consecutively influence severity and mortality of severe sepsis. The DD genotype of the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism is associated with increased nuclear translocation of NF-κB1.

This also suggests that increased nuclear translocation of NF-κB alters the expression of various genes involved in the inflammatory response such as cytokines or the hypoxia inducible factor 1 α. Hypoxia inducible factor 1 α, a well-characterized NF-κB target, is the key regulator linking inflammatory and hypoxic signaling pathways.[27] Therefore, polymorphisms of the NF-κB1 gene play a pivotal role in mediating both an excessive inflammatory response in humans and an induction of the hypoxic signaling. Thus, a variety of pathways are altered, resulting in disturbed vascular tone or cellular adhesion and changes in cellular receptor expression that, in concert, are likely to be associated with an unfavorable outcome.[28]

Because NF-κB1 binds to recognition elements in the promoter regions of inflammatory genes, it can be speculated that the D allele potentially alters the consequences of sepsis *via* the coordinated activation of several inflammatory genes of the innate and adaptive immunity. However, NF-κB1 also influences the coagulation system *via* tissue factor. During sepsis, tissue factor is highly expressed on monocytes, resulting in disseminated intravascular coagulation, ischemia, and organ dysfunction.[29,30] In the current study, we also show for the first time that lipopolysaccharide-induced tissue factor expression was affected by the D allele of the *NFKB1* promoter polymorphism. As the development of disseminated intravascular coagulation, which is mediated by tissue factor expression, is an independent risk factor in sepsis, it can be speculated that the deletion allele may also affect the course of sepsis *via* increased tissue factor expression.

In the current study, whole blood from volunteers was incubated with lipopolysaccharide *in vitro* to investigate the effects of the *NFKB1* promoter polymorphism on toll-like receptor 4 agonist-evoked responses under well-controlled conditions. Specificity of our clotting assay for tissue factor was demonstrated by the use of active site–inhibited factor seven, because the lipopolysaccharide-induced shortening of clotting time was abolished in presence of this inhibitor. We decided to determine clotting time as a measure of tissue factor using thromboelastometry instead of protein, because this functional assay has several important advantages compared with other tissue factor assays.[31,32] The sensitivity of our clotting assay is high, because lipopolysaccharide-induced tissue factor in whole blood samples exceeds the detection limit of the assay more than 100-fold. Furthermore, the functional assay measures biologically relevant tissue factor activity on the surface of intact cells. In contrast, measurements in homogenates, which are commonly used for enzyme-linked immunosorbent assay, also determine the functionally irrelevant intracellular tissue factor.[31,33,34] Moreover, enzyme-linked immunosorbent assay and chromogenic assays, but not a clotting assay, may also detect soluble tissue factor, which is believed to be of minor physiologic relevance.[31,33,34] Tissue factor concentration was calculated from clotting time using a calibration curve obtained by the addition of tissue factor standard to whole blood samples. In theory, this method might be influenced by a direct effect of the *NFKB1* genotypes on platelets or coagulation factors. However, no differences in platelet count or coagulation factor activity was detected, as judged by clot firmness using thromboelastometry.

Because of the study design, the question arises, whether the lipopolysaccharide concentration used in our *in vitro* experiments is of clinical relevance. To study biological effects, lipopolysaccharide has been used in a wide concentration range covering the ng/ml up to the mg/ml range. In most whole blood studies, lipopolysaccharide ranged from μg/ml to mg/ml.[35–38] In contrast, most cell culture studies were performed with lipopolysaccharide concentrations in the ng/ml range,[38–40] that is, concentrations much lower in comparison to whole blood. The use of these different concentration ranges can be explained by the physicochemical properties of the lipopolysaccharide molecule. Lipopolysaccharide, as an amphiphilic molecule in biological fluids, is always bound to other molecules such as peptides and proteins.[41] It is well recognized that human plasma neutralizes lipopolysaccharide up to 6 μg/ml[42] and that lipopolysaccharide diluted in plasma before application is less effective than lipopolysaccharide diluted in crystalloid solution.[43,44] Accordingly, we used 10 μg /ml for the cell culture studies

and 225 μg/ml lipopolysaccharide for the measurements of NFKB1 mRNA expression and TNFα protein concentration in whole blood, respectively. In our study, the EC_{50} for the lipopolysaccharide-induced activation of coagulation was 18 μg/ml. The EC_{50} of lipopolysaccharide in our *ex vivo* assay can also be achieved by intravenous administration of a 2-mg/kg dose of lipopolysaccharide if an intravascular volume of 10% of the body weight is assumed. Notably, this lipopolysaccharide dosage was used in primates,[45,46] and injection of 1 mg lipopolysaccharide has been described in humans.[47]

Limitations of this investigation should also be mentioned. Unrecognized selection bias, inherent to many genetic association studies, cannot ultimately be excluded. Moreover, although all sepsis patients were treated with a rather standardized multimodal regimen, because of the multifactorial nature of this disorder, we cannot exclude that unknown potentially confounding factors exist. Nevertheless, the study population was rather large, and multivariate proportional hazard analysis revealed the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism to be an important and strong independent prognostic factor for survival. This underscores the potential relevance of NF-κB1 expression in severe sepsis, regardless even of mechanisms involved.

In conclusion, after lipopolysaccharide stimulation, the D allele of the *NFKB1* insertion–deletion (–94ins/delATTG) polymorphism is associated with increased nuclear translocation of NF-κB1, boosted coagulation, and increased 30-day mortality in patients with severe sepsis. Accordingly, part of the variability in the outcome may be explained by the genetic variation in the promoter of *NFKB1* and its molecular consequences mediated by altered nuclear translocation of NF-κB1.

References

1. Adamzik M, Frey UH, Möhlenkamp S, Scherag A, Waydhas C, Marggraf G, Dammann M, Steinmann J, Siffert W, Peters J: Aquaporin 5 gene promoter–1364A/C polymorphism associated with 30-day survival in severe sepsis. Anesthesiology 2011; 114:912–7
2. Ma P, Zhu Y, Qiu H, Liu J, Wang Y, Zeng L: Endothelial nitric oxide synthase 894G→T but not -786T→C gene polymorphism is associated with organ dysfunction and increased mortality in patients with severe sepsis. J Trauma 2011; 71:872–7
3. Thair SA, Walley KR, Nakada TA, McConechy MK, Boyd JH, Wellman H, Russell JA: A single nucleotide polymorphism in NF-κB inducing kinase is associated with mortality in septic shock. J Immunol 2011; 186:2321–8
4. Abraham E: NF-κB activation. Crit Care Med 2000; 28:100–4
5. Cao L, Qian LL, Zhu YR, Guo CB, Gong XH, Sun B: Regulation of activity of nuclear factor-κB and activator protein-1 by nitric oxide, surfactant and glucocorticoids in alveolar macrophages from piglets with acute lung injury. Acta Pharmacol Sin 2003; 24:1316–23
6. Blackwell TS, Christman JW: The role of nuclear factor-κB in cytokine gene regulation. Am J Respir Cell Mol Biol 1997; 17:3–9
7. Browder W, Ha T, Chuanfu L, Kalbfleisch JH, Ferguson DA, Williams DL: Early activation of pulmonary nuclear factor κB and nuclear factor interleukin-6 in polymicrobial sepsis. J Trauma 1999; 46:590–6
8. Levi M, van der Poll T, ten Cate H: Tissue factor in infection and severe inflammation. Semin Thromb Hemost 2006; 32:33–9
9. Levi M, Ten Cate H: Disseminated intravascular coagulation. N Engl J Med 1999; 341:586–92
10. Barnes PJ, Karin M: Nuclear factor-κB: A pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997; 336:1066–71
11. Karban AS, Okazaki T, Panhuysen CI, Gallegos T, Potter JJ, Bailey-Wilson JE, Silverberg MS, Duerr RH, Cho JH, Gregersen PK, Wu Y, Achkar JP, Dassopoulos T, Mezey E, Bayless TM, Nouvet FJ, Brant SR: Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. Hum Mol Genet 2004; 13:35–45
12. Adamzik M, Frey UH, Rieman K, Sixt S, Beiderlinden M, Siffert W, Peters J: Insertion/deletion polymorphism in the promoter of NFKB1 influences severity but not mortality of acute respiratory distress syndrome. Intensive Care Med 2007; 33:1199–203
13. Adamzik M, Schaefer S, Frey UH, Becker A, Kreutzer M, Winning S, Frede S, Steinmann J, Fandrey J, Zacharowski K, Siffert W, Peters J, Hartmann M: The NFKB1 promoter polymorphism (94ins/delATTG) alters nuclear translocation of NF-KB1 in monocytes following lipopolysaccharide stimulation and is associated with increased mortality in sepsis. Infection 2011; 39:S101–2
14. Berchner-Pfannschmidt U, Yamac H, Trinidad B, Fandrey J: Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. J Biol Chem 2007; 282:1788–96
15. Hartmann M, Ozlügedik S, Peters J: Thiopental inhibits lipopolysaccharide-induced tissue factor expression. Anesth Analg 2009; 109:109–13
16. Sucker C, Zacharowski K, Thielmann M, Hartmann M: Heat shock inhibits lipopolysaccharide-induced tissue factor activity in human whole blood. Thromb J 2007; 5:13
17. Hartert H: [Thrombelastography, a method for physical analysis of blood coagulation]. Z Gesamte Exp Med 1951; 117:189–203
18. Bischof D, Dalbert S, Zollinger A, Ganter MT, Gantner MT, Hofer CK: Thrombelastography in the surgical patient. Minerva Anestesiol 2010; 76:131–7
19. Ganter MT, Hofer CK: Coagulation monitoring: Current techniques and clinical use of viscoelastic point-of-care coagulation devices. Anesth Analg 2008; 106:1366–75
20. Zacharowski K, Zacharowski PA, Koch A, Baban A, Tran N, Berkels R, Papewalis C, Schulze-Osthoff K, Knuefermann P, Zähringer U, Schumann RR, Rettori V, McCann SM, Bornstein SR: Toll-like receptor 4 plays a crucial role in the immune-adrenal response to systemic inflammatory response syndrome. Proc Natl Acad Sci USA 2006; 103:6392–7
21. Riemann K, Becker L, Struwe H, Nückel H, Dührsen U, Alakus H, Winde G, Neuhäuser M, Rübben H, Schmitz KJ, Wohlschlaeger J, Schmid KW, Siffert W: No association of the NFKB1 insertion/deletion promoter polymorphism with survival in colorectal and renal cell carcinoma as well as disease progression in B-cell chronic lymphocytic leukemia. Pharmacogenet Genomics 2006; 16:783–8
22. Riemann K, Becker L, Struwe H, Rübben H, Eisenhardt A, Siffert W: Insertion/deletion polymorphism in the promoter of NFKB1 as a potential molecular marker for the risk of recurrence in superficial bladder cancer. Int J Clin Pharmacol Ther 2007; 45:423–30
23. Bone RC, Sprung CL, Sibbald WJ: Definitions for sepsis and organ failure. Crit Care Med 1992; 20:724–6
24. Le G, Lemeshow S, Saulnierven-namesF: A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA 1993; 270:2957–63

25. Ferreira FL, Bota DP, Bross A, Mélot C, Vincent JL: Serial evaluation of the SOFA score to predict outcome in critically ill patients. JAMA 2001; 286:1754–8
26. Gu W, Zeng L, Zhang LY, Jiang DP, Du DY, Hu P, Wang HY, Liu Q, Huang SN, Jiang JX: Association of interleukin 4 -589T/C polymorphism with T(H)1 and T(H)2 bias and sepsis in Chinese major trauma patients. J Trauma 2011; 71:1583–7
27. Eltzschig HK, Carmeliet P: Hypoxia and inflammation. N Engl J Med 2011; 364:656–65
28. Kuhlicke J, Frick JS, Morote-Garcia JC, Rosenberger P, Eltzschig HK: Hypoxia inducible factor (HIF)-1 coordinates induction of Toll-like receptors TLR2 and TLR6 during hypoxia. PLoS ONE 2007; 2:e1364
29. Hotchkiss RS, Swanson PE, Freeman BD, Tinsley KW, Cobb JP, Matuschak GM, Buchman TG, Karl IE: Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. Crit Care Med 1999; 27:1230–51
30. Levi M, Keller TT, van Gorp E, ten Cate H: Infection and inflammation and the coagulation system. Cardiovasc Res 2003; 60:26–39
31. Park MS, Martini WZ, Dubick MA, Salinas J, Butenas S, Kheirabadi BS, Pusateri AE, Vos JA, Guymon CH, Wolf SE, Mann KG, Holcomb JB: Thromboelastography as a better indicator of hypercoagulable state after injury than prothrombin time or activated partial thromboplastin time. J Trauma 2009; 67:266–75; discussion 275–6
32. Zacharowski K, Sucker C, Zacharowski P, Hartmann M: Thrombelastography for the monitoring of lipopolysaccharide induced activation of coagulation. Thromb Haemost 2006; 95:557–61
33. Butenas S, Orfeo T, Mann KG: Tissue factor in coagulation: Which? Where? When? Arterioscler Thromb Vasc Biol 2009; 29:1989–96
34. Butenas S, Mann KG: Active tissue factor in blood? Nat Med 2004; 10:1155–6; author reply 1156
35. Nooteboom A, Van Der Linden CJ, Hendriks T: Tumor necrosis factor-alpha and interleukin-1beta mediate endothelial permeability induced by lipopolysaccharide-stimulated whole blood. Crit Care Med 2002; 30:2063–8
36. Nooteboom A, Bleichrodt RP, Hendriks T: Modulation of endothelial monolayer permeability induced by plasma obtained from lipopolysaccharide-stimulated whole blood. Clin Exp Immunol 2006; 144:362–9
37. Ziegler-Heitbrock HW, Wedel A, Schraut W, Ströbel M, Wendelgass P, Sternsdorf T, Bäuerle PA, Haas JG, Riethmüller G: Tolerance to lipopolysaccharide involves mobilization of nuclear factor κB with predominance of p50 homodimers. J Biol Chem 1994; 269:17001–4
38. Pöschl JM, Leray C, Ruef P, Cazenave JP, Linderkamp O: Endotoxin binding to erythrocyte membrane and erythrocyte deformability in human sepsis and *in vitro*. Crit Care Med 2003; 31:924–8
39. Spolarics Z, Siddiqi M, Siegel JH, Garcia ZC, Stein DS, Denny T, Deitch EA: Depressed interleukin-12-producing activity by monocytes correlates with adverse clinical course and a shift toward Th2-type lymphocyte pattern in severely injured male trauma patients. Crit Care Med 2003; 31:1722–9
40. Souter PJ, Thomas S, Hubbard AR, Poole S, Römisch J, Gray E: Antithrombin inhibits lipopolysaccharide-induced tissue factor and interleukin-6 production by mononuclear cells, human umbilical vein endothelial cells, and whole blood. Crit Care Med 2001; 29:134–9
41. Chaby R: Lipopolysaccharide-binding molecules: Transporters, blockers and sensors. Cell Mol Life Sci 2004; 61:1697–713
42. Warren HS, Novitsky TJ, Ketchum PA, Roslansky PF, Kania S, Siber GR: Neutralization of bacterial lipopolysaccharides by human plasma. J Clin Microbiol 1985; 22:590–5
43. Kumwenda ZL, Wong CB, Johnson JA, Gosnell JE, Welch WJ, Harris HW: Chylomicron-bound endotoxin selectively inhibits NF-κB activation in rat hepatocytes. Shock 2002; 18:182–8
44. Harris HW, Johnson JA, Wigmore SJ: Endogenous lipoproteins impact the response to endotoxin in humans. Crit Care Med 2002; 30:23–31
45. Haudek SB, Natmessnig BE, Fürst W, Bahrami S, Schlag G, Redl H: Lipopolysaccharide dose response in baboons. Shock 2003; 20:431–6
46. Haudek SB, Natmessnig BE, Redl H, Schlag G, Hatlen LE, Tobias PS: Isolation, partial characterization, and concentration in experimental sepsis of baboon lipopolysaccharide-binding protein. J Lab Clin Med 2000; 136:363–70
47. Taveira Da Silva AM, Kaulbach HC, Chuidian FS, Lambert DR, Suffredini AF, Danner RL: Brief report: Shock and multiple-organ dysfunction after self-administration of Salmonella endotoxin. N Engl J Med 1993; 328:1457–60