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# The interleukin-6 G(-174)C polymorphism and the ex vivo interleukin-6 response to endotoxin in severely injured blunt trauma patients

## Abstract

The aim of this study was to evaluate whether the -174 G/C promoter polymorphism of the interleukin-6 (IL-6) gene is associated with the ex vivo, whole blood IL-6 response to endotoxin with the development of severe sepsis in severely injured, blunt trauma patients. Patients with a severe trauma and an injury severity score of 16 were included in the study. The IL-6 -174 G/C promoter

polymorphism was determined by real-time polymerase chain reaction (PCR) assay using specific fluorescence-labelled hybridisation probes. Whole blood of the patients was stimulated with endotoxin and the IL-6 concentrations were measured by ELISA. There was no association between the IL-6 -174 genotypes and the ex vivo, stimulated IL-6 response: 25% of the patients developed severe sepsis later in the clinical course. These patients had higher IL-6 concentrations following whole blood stimulation on day 1 ($p = 0.046$) after the trauma than patients with uncomplicated post-traumatic recovery. The difference was even more significant on day 2 after the trauma ($p = 0.02$). High IL-6 responses in a whole blood stimulation assay with endotoxin on days 1 and 2 after a trauma are associated with severe post-traumatic sepsis. Genotyping for the IL-6 -174 G/C polymorphism does not allow early identification of trauma patients with a high, ex vivo IL-6 synthesis capacity.

## Full text

### INTRODUCTION

Major surgery and severe blunt trauma induce a generalized inflammatory reaction. Subsequently, sepsis and multiple organ failure develop in some patients, but not in others. The inflammatory response involves a complex pattern of humoral and cellular responses and the expression of related factors is thought to be governed by genetic variabilities [1-4].

Cytokines are integral components of the host immune response to trauma or infection. In particular, tumor necrosis factor, as well as interleukin-6 (IL-6), are thought to play a pivotal role. IL-6, a cytokine with pro- and anti-inflammatory properties mediates the acute phase response and triggers the synthesis of acute phase proteins in the liver [5, 6]. High plasma IL-6 concentrations are associated with fever, leukocytosis, activation of coagulation factors and catabolism [7].

In surgical patients, IL-6 reflects the extent of the surgical or accidental trauma and therefore has been suggested for routine monitoring of multiple trauma patients [8, 9]. Patients undergoing laparoscopic cholecystectomy had lower IL-6 levels than a control group undergoing conventional laparotomy [10]. Attenuated IL-6 levels were associated with an enhancement of respiratory function, a shorter duration of hospital stay and a reduced consumption of

analgesics [10]. Sepsis and shock cause an increase in IL-6 plasma levels [11-13], and anti-IL-6 antibodies were protective against a lethal challenge with *E. coli* or tumor necrosis factor-alpha in an animal model of sepsis[14]. TNF-alpha and IL-6 are known to be of prognostic value in predicting the severity and outcome of sepsis [15-17]. In a subgroup of septic patients defined by IL-6 plasma levels greater than 1,000 pg/ml, TNF antibodies proved beneficial in a retrospective analysis [18], but not in a prospective study setting [19]. Moreover, in severely injured and septic patients, impairment of host defense mechanisms, which are accompanied by a diminished whole blood cytokine response to endotoxin and reduced monocytic HLA-DR expression, have been described [20-25].

Recently, Fishman and colleagues discovered a polymorphic site within the IL-6 promoter region, 174 base pairs upstream from the start of the coding region [26]. The polymorphism consisting of a G/C exchange was reported to affect IL-6 synthesis. The C allele was associated with remarkably lower IL-6 production in a reporter gene assay stimulating transfected HeLa cells with IL-1 or lipopolysaccharide. Also, in fasting healthy individuals, significant differences in the basal IL-6 plasma levels were found, with highest values for carriers of the GG genotype, intermediate values for heterozygotes, and lowest values in C homozygotes. However, this influence of the G/C polymorphism on gene expression has been challenged by Terry and colleagues [27].

It was the aim of the present study to examine whether the *ex vivo* IL-6 synthesis by whole blood on various days after a severe trauma is associated with the IL-6 -174 G/C polymorphism.

## METHODS

This study was approved by the local ethics committee. Fifty-seven Caucasian trauma patients with an injury severity score (ISS) [28] of 16 were included. Only patients without preexisting chronic diseases, not receiving medication, and without penetrating injuries were included. All patients were admitted to the intensive care unit due to the severity of the trauma. Injuries to various body regions (head and neck, face, thorax, abdomen, extremities, skin) were classified using the Abbreviated Injury Scale (AIS) [28]. The clinical course was followed prospectively in all patients. Severe sepsis was diagnosed according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee [29]. The systemic inflammatory

response syndrome (SIRS), which in addition to infection, defines the clinical entity sepsis [29], is present in almost all patients in the immediate post-traumatic period. Therefore, we focused on severe sepsis; *i.e.* the criteria of sepsis and organ failure such as hypoperfusion and/or renal failure had to be present. Moreover, the diagnosis of severe sepsis was not made before day 5 following the trauma, as previously described [4]. The multiple organ failure score (MOF-Score) was calculated daily according to Goris *et al.* [30]. All patients were ventilated mechanically within the first 24 hours after injury. Patients requiring surgical intervention received standard surgical care and postoperative intensive care.

### Ex vivo *endotoxin-induced IL-6 production by whole blood*

A human whole blood assay was used as previously described [23]. Briefly, whole blood samples of the patients were taken on days 1, 2, 4, 6, 8, and 14 after admission to the ICU. After mixing the blood 1:1 (v/v) with RPMI 1640 medium (Gibco BRL, Karlsruhe, Germany), endotoxin (*Salmonella friedenau*, a kind gift from Dr. H. Brade, Forschungszentrum Borstel, Borstel, Germany), in a final concentration of 100 ng/ml was added. The incubation lasted for 4 hours at 37° C/5% $CO_2$ and was followed by centrifugation. The supernatants were stored at - 20° C until determination of IL-6 plasma levels was performed by means of ELISA (Endogen, Bonn, Germany). The detection limit of the ELISA was 30 pg/ml.

### *Genotyping for the IL-6 -174 G/C polymorphism*

Genomic DNA was isolated from blood cells using a standard procedure (QIAmp Bloodkit, Quiagen, Hilden, Germany). Twenty to 50 ng DNA were used for the real-time PCR. The real-time PCR was carried out on a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). Real-time polymerase chain reaction (PCR) assays with specific fluorescence-labelled hybridisation probes were used, as reported recently for the IL-6 polymorphism [31]. 0.5 mumol/L of the primers (sense: 5'-AAATTCTAGCCTGTTAATCTGGTC, antisense: 5'-ATCCCACATTTGATAAATCTTTGT) as well as 0.2 mumol/L of the detection probe specific for the T allele (5'-TGTGTCTTGCCATGCTAAAGGACG) and the anchor probe (5'-CGACCTAAGCTGCACTTTTCCCCCTA) were used. The PCR contained 1 mul reaction buffer (LightCycler DNA master hybridization probes 10C buffer, 1.75 mmol/L, Roche Diagnostics), in a total volume of 10 muL. Sixty PCR cycles were carried out. A PCR cycle consisted of denaturation (95° C for 0 sec, 20°

C/s), annealing at 57° C for 10 sec, and extension (72° C for 25 sec). Based on the derivative melting curves (-(dF/dT) *versus*. T, F: fluorescence, T: temperature), a sample was classified as the IL-6 genotype GG, CG or CC.

### *Statistical analysis*

If not otherwise mentioned, data are expressed as mean ± the standard error of the mean (SEM). Analysis of the *ex vivo* IL-6 synthesis according to the three IL-6 -174 genotypes GG, CG, and CC, was performed using the Kruskal-Wallis H test. The Wilcoxon Mann-Whitney test was used to test for differences in the *ex vivo* IL-6 synthesis capacity of septic and non-septic patients. A p value < 0.05 was used to reject the null hypothesis. Statistical analysis was performed using the SPSS for Windows, Release 10.0.7 (SPSS Inc. 2000) computer program.

## RESULTS

The allele frequency was 48% for C and 52% for G. Eleven (19.3%) patients were C homozygous, 33 (57.9%) were heterozygous (CG), and 13 (22.8%) individuals carried the GG genotype.

Demographic data of the patients are given in Table 1. Differences in the AIS were only found for the face region. Neither ISS values, maximal MOF scores nor the percentage of survivors differed significantly among the three groups.

IL-6 levels after endotoxin stimulation of whole blood on various days after the trauma are given in Table 2. No significant differences were found between carriers of the IL-6 -174 genotypes.

Fourteen out of 57 patients (25%) developed severe sepsis during the post-traumatic course. All septic patients had positive blood cultures (2 patients with *Enterococcus*, 1 patient with *Klebsiella/Enterobacter*, 1 with *Klebsiella pneumoniae*, 1 with *Escherichia coli*, 1 with *Klebsiella oxytoca*, 1 with *Pseudomonas aeruginosa*, 4 with *Staphylococcus epidermidis*, and 4 with *Staphylococcus aureus*.) One patient had two isolates.

On days 1 and 2 after the trauma, patients who developed a severe sepsis had higher IL-6 values in the whole blood stimulation assay than patients with an

uncomplicated post-traumatic course. Table 3 shows the IL-6 levels in patients with and without severe sepsis.

## DISCUSSION

Westendorp *et al.* [2] provided convincing evidence that *ex vivo* production of TNF and IL-10 is influenced by genetic factors. They found a variation of 60% (TNF) and 75% (IL-10), on the basis of heritability alone. Subsequently, polymorphisms in the TNF gene were identified and their role in host defense and *ex vivo* cytokine production was studied. The -308 promoter polymorphism of the TNF gene was associated with the susceptibility and outcome of sepsis in some studies [32, 33], while other authors did not confirm these findings [34]. Furthermore, an NcoI restriction fragment length polymorphism of the TNF gene has been shown to be associated with development and outcome of sepsis [3, 4].

In our study of patients suffering from severe trauma, no association could be found between the IL-6 -174 genotypes and the *ex vivo* IL-6 synthesis on various days after the trauma. The number of C homozygotes in our study was quite low. However, we studied the IL-6 response in each patient at 6 different time-points so that a minimum of 66 samples (in the group of C homozygotes), and a maximum of 198 samples (in the group of CG carriers) was analysed for the association with the IL-6 -174 genotypes. The finding of our study questions the role of the IL-6 -174 polymorphism for the IL-6 synthesis by human cells. Fishman *et al.* [26] reported that basal IL-6 concentrations in fasting healthy subjects differed according to the IL-6 -174 genotypes. Similarly, they found a significantly higher IL-6 response upon stimulation with IL-1 and LPS for the C allele compared with the G allele.

The lower IL-6 synthesis of the C allele was attributed to the creation of a binding site for the transcription factor NF-1. NF-1 is a suppressor of gene transcription [35] in HeLa cells, which were used for the reporter gene assays [26]. This result was challenged by Terry *et al.* [27]. In their study, differences in the transcriptional regulation of IL-6 gene expression by the IL-6 -174 G and C allele were observed when comparing ECV304 with HeLa cells. This may be, at least partly, due to different effects of the transcription factor NF-1 in various cell types [36]. Therefore, it cannot be ruled out that the effect of this polymorphism in physiological systems may differ greatly from *in vitro* situations.

Fishman *et al.* [26] also reported an association of the IL-6 -174 genotypes and the IL-6 plasma levels of fasting healthy volunteers with the highest values in G homozygotes, intermediate values found in GC heterozygotes, and the lowest IL-6 plasma levels in subjects homozygous for the C allele. Since IL-6 is produced by a variety of cells [7], many more cell types may contribute to the IL-6 levels measured in the plasma compared to IL-6 levels produced by blood cells only in our experimental setting. A cell-type-specific effect of the IL-6 -174 polymorphism and the related binding site for transcription factor NF-1 is one possible explanation for the findings of our study.

Additionally, it has to be taken into consideration that the regulation of IL-6 synthesis may not depend on one promoter polymorphism only. There is some evidence from *in vitro* assays with ECV304 cells which suggests a rather complex process for IL-6 transcription. Terry *et al.* [27] studied four promoter polymorphisms of the IL-6 gene, at positions -597, -572, -373, and -174. This group found that IL-6 gene transcription in ECV304 cells is regulated by a complex interaction of various polymorphic sites. The functional relevance of these polymorphisms for IL-6 production in human cells has not yet been proven. So far, only the -174 promoter polymorphism was found to affect IL-6 levels in humans [26].

*Ex vivo* cytokine synthesis in whole blood is characterized by a high reproducibility [37]. However, several factors have to be taken into account which can differ among trauma patients, such as the concentration of circulating endotoxin and lipopolysaccharide binding protein as well as the endotoxin-neutralising capacity. Also, monocyte CD14 [38], soluble CD14 [39] and Toll-like receptors [40, 41], as well as polymorphisms of these genes [42-45] can affect the IL-6 response to endotoxin. Several studies found lower cytokine levels after endotoxin challenge in patients with trauma/haemorrhage [20, 22, 23] or sepsis [21, 24] than in healthy control subjects. Surgical trauma caused a decrease in IL-6 and TNF levels in a whole blood stimulation assay compared to preoperative values [46]. In this patient group, a dissociation between the IL-6 levels in peripheral blood and the IL-6 concentrations after *ex vivo* stimulation of whole blood was observed. Patients with high systemic levels had low stimulated IL-6 and TNF concentrations and *vice versa* [47]. As has been shown previously, patients with severe sepsis had significantly higher IL-6 values after endotoxin activation in whole blood assays in the early period after trauma than patients with an uncomplicated recovery [47-49]. This finding suggests that the

*ex vivo* IL-6 response to endotoxin in the early days after the trauma may have a predictive value for the development of severe sepsis in the later course.

CONCLUSION

In conclusion, the IL-6 -174 G/C promoter polymorphism is not associated with *ex vivo* IL-6 synthesis following severe blunt trauma. Assessment of the *ex vivo* IL-6 synthesis could possibly serve for the identification of patients at high risk of severe sepsis, who may benefit from early prophylactic measures.

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# Figures

**Table 1**
**Demographic and clinical characteristics of the trauma patients**

| IL-6 –174 genotype | GG | GG | CC |
|---|---|---|---|
| Age (years) | 38.7 ± 17.8 | 38.0 ± 4.0 | 38.4 ± 3.6 |
| Sex (male/female) | 10/3 | 17/16 | 9/2 |
| ISS (points) | 27 ± 9 | 28 ± 2 | 24 ± 2 |
| AIS (points) head/neck | 2.5 ± 1.4 | 1.2 ± 0.3 | 2.1 ± 0.5 |
| AIS (Points) face | 1.4 ± 1.6 | 0.5 ± 0.2 | 0.0 ± 0.0* |
| AIS (points) thorax | 1.9 ± 1.9 | 2.2 ± 0.4 | 1.4 ± 0.6 |
| AIS (points) abdomen | 0.8 ± 1.5 | 2.0 ± 0.4 | 1.5 ± 0.6 |
| AIS (points) extremities | 2.4 ± 1.4 | 2.6 ± 0.2 | 2.9 ± 0.2 |
| AIS (points) skin | 0.1 ± 0.3 | 0.5 ± 0.2 | 0.4 ± 0.3 |
| MOF-Score (maximum) | 5 ± 2 | 6 ± 3 | 5 ± 2 |
| Non-survivors/ Survivors | 0 (0%)/13 (100%) | 5 (15%)/28 (85%) | 1 (9%)/10( 91%) |

* p = 0.03.

**Table 2**
**IL-6 concentrations (ng/ml) after *ex vivo* stimulation of whole blood according to the IL-6 –174 G/C genotypes**

| | IL-6 -174 genotypes | | |
|---|---|---|---|
| Days after trauma | CC (n = 11) | CG (n = 33) | GG (n = 13) |
| 1 | 10.4 ± 3.6 | 5.0 ± 1.0 | 9.6 ± 3.4 |
| 2 | 8.0 ± 4.1 | 6.5 ± 1.1 | 13.5 ± 5.8 |
| 4 | 14.0 ± 5.8 | 8.5 ± 1.7 | 14.0 ± 5.0 |
| 6 | 12.0 ± 2.0 | 9.3 ± 2.0 | 16.0 ± 6.4 |
| 8 | 11.8 ± 2.9 | 8.4 ± 1.3 | 16.9 ± 7.2 |
| 14 | 15.1 ± 6.8 | 10.0 ± 2.7 | 13.4 ± 5.4 |

**Table 3**
**IL-6 concentrations (ng/ml) after *ex vivo* stimulation of whole blood of trauma patients with and without development of severe sepsis**

| Days after trauma | Severe sepsis (n = 14) | Uncomplicated course (n = 43) | p-value |
|---|---|---|---|
| 1 | 11.2 ± 3.6 | 6.0 ± 1.2 | 0.046 |
| 2 | 9.4 ± 1.6 | 8.3 ± 2.4 | 0.02 |
| 4 | 11.1 ± 2.8 | 10.8 ± 2.4 | 0.35 |
| 6 | 9.0 ± 2.4 | 12.5 ± 2.5 | 0.34 |
| 8 | 11.4 ± 3.8 | 11.5 ± 2.6 | 0.99 |
| 14 | 10.9 ± 4.2 | 12.3 ± 2.9 | 0.69 |

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osteoclast, estrogen receptor, SERM, cytokines, apoptosis.

## Interleukin-10 and cAMP-elevating agents cooperate to induce suppressor of cytokine signaling-3 via a protein kinase A-independent signal

Sara Gasperini, Luca Crepaldi, Federica Calzetti, Luana Gatto, Chiara Berlato, Flavia Bazzoni, Akihiko Yoshimura, Marco A. Cassatella
SOCS-3, PGE, IL-10, PBMC, neutrophils, PKA.

## Different patterns of interleukin-1alpha and interleukin-1beta expression in organs of normal young and old mice

Moshe Hacham, Shmuel Argov, Rosalyn M. White, Shraga Segal, Ron N. Apte
IL-1alpha, IL-1beta, organ cytokine expression, young/old age, homeostasis.

## The combination of the interleukin-1alpha (IL-1alpha-889) genotype and the interleukin-10 (IL-10 ATA) haplotype is associated with increased interleukin-10 (IL-10) plasma levels in healthy individuals

Sanna Kilpinen, Heini Huhtala, Mikko Hurme
interleukin-1, interleukin-10, gene polymorphism, plasma.

## The interleukin-6 G(-174)C polymorphism and the ex vivo interleukin-6 response to endotoxin in severely injured blunt trauma patients

Michael Heesen, Udo Obertacke, F. Ulrich Schade, Brunhilde Bloemeke, Matthias Majetschak
interleukin-6, endotoxin, genetic polymorphism, polymerase chain reaction, whole blood, critical illness.

## Comparison of leptin- and interleukin-6-regulated expression of the rPAP gene family: evidence for differential co-regulatory signals

Daniël Broekaert, Sven Eyckerman, Delphine Lavens, Annick Verhee, Wim Waelput, Joël Vandekerckhove, Jan Tavernier
forskolin, IL-6, leptin, rPAP/Reg gene family, signal transduction.

## Mice lacking alpha2-macroglobulin show an increased host defense against Gram-negative bacterial sepsis, but are more susceptible to endotoxic shock

Tino Hochepied, Fred Van Leuven, Claude Libert
TNF, shock, acute phase, inflammation, alpha2-macroglobulin.

## Tumor necrosis factor-alpha mRNA stability in human peripheral blood cells after lipopolysaccharide stimulation

Lucile Baseggio, Carole Charlot, Jacques Bienvenu, Pascale Felman, Gilles Salles
tumor necrosis factor, mRNA stability, lipopolysaccharide, tristetraprolin.

## Caspase-1 levels in biological fluids from patients with multiple sclerosis and from patients with other neurological and non-neurological diseases

Diego Franciotta, Gianvito Martino, Elisabetta Zardini, Roberto Furlan, Roberto Bergamaschi, Maira Gironi, Alessandra Bergami, Giovanna Angelini, Fabrizio De Benedetti, Patrizia Pignatti, Gianna Moscato, Vittorio Cosi
asthma, cerebrospinal fluid, juvenile idiopathic arthritis, sputum, synovial fluid, THP-1 cells.

## Divergent effects of tumor necrosis factor-alpha and lymphotoxin-alpha on lethal endotoxemia and infection with live Salmonella typhimurium in mice

Edi Dharmana, Monique Keuter, Mihai G. Netea, Ineke C.M.M. Verschueren, Bart Jan Kullberg
tumor necrosis factor, lymphotoxin, salmonella typhimunium, knock-out mice.

## Th1/Th2 cytokine balance and nitric oxide in cerebrospinal fluid and serum from patients with multiple sclerosis

María del Carmen Rodríguez-Sáinz, Silvia Sánchez-Ramón, Clara de Andrés, Margarita Rodríguez-Mahou, M Angeles Muñoz-Fernández

cytokine, nitric oxide, multiple sclerosis, cerebrospinal fluid, oligoclonal bands, Igs.

## Retinoids, cisplatin and interferon-alpha in recurrent or metastatic cervical squamous cell carcinoma: clinical results of 2 phase II trials

Anne-Chantal Braud, Laurence Gonzague, François Bertucci, Dominique Genre, Jacques Camerlo, Gwenaelle Gravis, Anthony Goncalves, Vincent Moutardier, Frédéric Viret, Dominique Maraninchi, Patrice Viens

all-trans-retinoic acid, 13 Cis retinoic acid, interferon-alpha, cervical squamous cell carcinoma.

## Circulating macrophage colony stimulating factor as a marker of tumour progression

Raymond S. McDermott, Laurent Deneux, Véronique Mosseri, Jacques Védrenne, Krishna Clough, Alain Fourquet, José Rodriguez, Jean-Marc Cosset, Xavier Sastre, Philippe Beuzeboc, Pierre Pouillart, Susy M. Scholl

CSF-1, tumour progression, metastasis, de-differentiation.

## The effect of interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) on granulocyte macrophage-colony stimulating factor (GM-CSF) production by neuronal precursor cells

J. Benjamin Dame, Nasser Chegini, Robert D. Christensen, Sandra E. Juul

IL-1beta, TNF-alpha, GM-CSF, neuron, mRNA, protein.

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