Original Contribution

SNPS IN THE CYTOKINE GENES IN ASSOCIATION WITH THEIR FUNCTIONAL ACTIVITY IN SEPSIS

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ABSTRACT

This study investigated the functionality of the -1082(A/G) polymorphism in the promoter of the IL-10 gene and +16974 (A/C) SNP in 3'UTR of the IL-12B gene in association with the cytokine production from stimulated PBMC in relation with sepsis. A group of 33 patients in ICU and 99 healthy volunteers were included. The SNP in IL-12B gene was detected by restriction assay using Taq I enzyme. The amplification refractory mutation system PCR was used for IL-10 -1082(A/G) detection. The results for IL-10 - 1082 SNP demonstrated that AA genotype was associated with the lower IL-10 production in LPS- or PHA-stimulated PBMC. Carriage of at least one copy of IL-10 - 1082 allele G in sepsis patients as well as in healthy controls resulted in significant increase in IL-10 production from stimulated PBMC. Sepsis patients with AG/GG genotypes of IL-10 -1082 produced significantly higher IL-10 from stimulated PBMC. The presence of "C" allele in IL-12B gene correlated with strongly decreased production of IL-12p40 of stimulated PBMC from sepsis patients. In conclusion it was demonstrated that the induced production of IL-12 and IL-10 depended on polymorphisms in corresponding genes and physiological status.

Key words: sepsis, PBMC, IL-12, IL-10, SNP

INTRODUCTION

The evidence that primary responses in SIRS/sepsis are mediated by cytokines has led to various investigations of these mediators in sepsis prognosis, progression or treatment. Regulatory mechanisms to counterbalance the production of proinflammatory and antiinflammatory cytokines are also present (1-3). IL-12 and IL-10 contribute to these regulatory processes with their immunomodulatory properties.

IL-10 has beneficial anti-inflammatory properties. On the other hand an excess of IL-10 has been reported to induce immunosuppression in bacterial sepsis and increased mortality (4, 5). The IL-10 gene has been mapped to chromosome 1q31-32, and three single nucleotide polymorphisms (SNPs)

at -1082(A/G), -819(C/T), -592(C/A) bp upstream from the transcription start site have been described and their influence on gene expression shown (6,7).

Interleukin-12 (IL-12) is a heterodimeric proinflammatory and immunoregulatory cytokine, composed of two disulphide-bounded polypeptide chains- p35 and p40 that is critical to the orchestration of cell - mediated immune responses in both the innate and adaptive immune systems. The complete genomic sequence analysis of the IL-12 gene encoding its p40 subunit (IL-12B) identified a SNP at position +16974 (A/C) in the 3'-untranslated region (UTR) of IL-12B (8).

It has been reported that this polymorphism influences cytokines expression and IL-12p40 production (9, 10). In our previous study, we also demonstrated that this association depended on the stimuli used and, in particular, that the presence of the allele "A" correlated with increased IL-12p40 production by normal human PBMC stimulated with C3bgp. (11)

As has been previously demonstrated

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the clinical outcome of sepsis appears to correlate with the overall balance of production of IL-12 and IL-10 from stimulated PBMC at the onset of sepsis (12). Hence, much interest has focused upon the differences in genes expressing these cytokines between healthy and systemic immune response/sepsis conditions.

In particular, this study has addressed whether genetic polymorphisms as single nucleotide polymorphism within IL-10 and IL-12B genes might influence the level of expression, and therefore the overall immune response in sepsis.

MATERIALS AND METHODS

Patients

Thirty–three (33) consecutively admitted adult patients meeting the criteria for sepsis with a verified infection in one or more major organs were included. The sepsis group contained 16 women and 17 men from Bulgaria, aged 16-85 years. The control group, for IL-10 study, included 53 healthy volunteers, 37 women and 16 men, aged 18-71 years and for IL-12 study - 99 healthy volunteers, 64 women and 35 men, aged 18-71 years, all from Bulgaria.

Severe sepsis was defined according to the definitions set by the 1992 American College of Chest Physicians/Society of Critical Medicine Consensus Committee (13), as manifested by two or more of the following conditions: fever or hypothermia (T≥38 °C or ≤35.50°C); tachycardia (heart rate≥90 beats/minute); tachypnea (respiration rate≥20 breath/min or pCO_2<32mmHg); and leukocytosis or leukopenia (WBC≥2.109 G/l or ≤4.109 G/l) or 10% immature forms, during the preceding 24 hours, in the presence of a documented source of infection and at least one organ dysfunction, throughout their stay in the intensive care unit (ICU). Patients were excluded if they were <16 yrs old; with severe medical conditions, caused by other illnesses (myocardial infarct, liver cirrhosis, metastatic tumors, etc.); or had received immunosuppressive or immunostimulating drugs.

The Ethics Review Board of the Faculty of Medicine, Trakia University, approved the study. All patients and healthy volunteers involved gave written informed consent for the study.

Isolation of human PBMC and culture

The PBMC were isolated from the healthy donors and patient's peripheral blood by centrifugation on a Histopaque-1077 gradient. The $1x10^6$ cells were then cultured in sterile polystyrene flasks containing 2 ml RPMI 1640 media (Sigma-Aldrich Inc., St Louis, USA) serum free, supplemented with penicillin G (100 U/ml), gentamycin (10 µg/ml) and L-glutamine (0.3 mg/ml) for 24 hours. The following triggering/activating agents were added to the culture flasks: 30µg lipopolysaccharide (LPS) from *E. coli*; 20µg phytohaemagglutinin (PHA); 30µg C3-binding glycoprotein (C3bgp) and 30µg pokeweed mitogen (PWM). The culture lasted for 24 hours. At the end of this period supernatants were collected and stored at -80^0C until use. All stimulating agents except C3bgp were purchased from Sigma-Aldrich Inc., St Louis, USA, as well as other reagents for cell isolation and culturing. C3bgp was isolated as described elsewhere (14). C3bgp and culture reagents were tested for lipopolysaccharide contamination with Limulus amebocyte lysate "E-Toxate" Multiple test (Sigma-Aldrich Inc., St Louis, USA). The endotoxin level in all cases was less than 10pg/ml.

Cytokine determination

The amounts of IL-10 and IL-12 were determined in culture supernatant using commercially available ELISA kits purchased from BioSource, International, Belgium, following the manufacturer's instructions. Colour reaction developed was measured in as OD units at 450 nm on an ELISA reader (Rosys Anthos 2010, Austria). The concentration of cytokines was expressed in pg/ml using the ELISA kit standards.

DNA extraction and genotyping

Blood specimens were collected in tripotassium ethylenediaminetetraacetic acid (EDTA) sterile tubes. Genomic DNA was extracted using a GFX genomic blood DNA purification kit (Amersham Biosciences, UK) and stored at - 20°C until use.

Genotyping for the -1082 A/G polymorphism in promoter region of the IL-10 gene was performed with amplification refractory mutation system - polymerase chain reaction (ARMS- PCR) methodology as previously described with modifications (15).

Briefly, 5' primers specific for the IL-10 -1082 allele were used in combination with a generic 3' primer: IL-10G (CCTATCCCTACTTCCCCC), IL-10A (CCTATCCCTACTTCCCCT) and IL-10 generic 3' primer (AGCAACCACTCC

TCGTCGCAAC). PCR amplification was carried out in 10 μl volumes containing GeneAmp 10xPCR buffer, 0.25 U of AmpliTaq Gold polymerase, 2.5 mmol/l MgCl2, 100 μmol/l of each dNTP, 0.4 μmol/l of each primer (primers and reagents were supplied by Applied Biosystems, USA), and 0.1- 0.5 μg of genomic DNA. Amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems,Warrington, UK). After the initial incubation step (95°C for 10 min), PCR was performed for 30 cycles: 30 sec at 94°C, 1 min at 60°C, and 1 min at 72°C. A final extension step of 7 min at 72 °C completed the reaction. The PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Genotyping of +16974 A/C SNP in the 3'UTR of the IL-12B was performed by amplification of 1046bp fragment containing polymorphic site, using the forward primer 5'-ATTTGGAGGAAAAGTGGAAGA-3' and the reverse primer 5'–AATTTCATGTCCTTAGCCATA–3' (16). PCR amplification was carried out in 20 μL volumes containing GeneAmp 10x PCR buffer, 0.25 U of AmpliTaq Gold polymerase, 3.0 mmol/l MgCl$_2$, 100 μmol/l of each dNTP, 0.4 μmol/l of each primer (primers and reagents were supplied by Applied Biosystems, USA), and 0.1- 0.5 μg of genomic DNA. Amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems). After the initial incubation step (95°C for 10 min), PCR was performed for 30 cycles: 1 min at 94°C, 1 min at 54.3 °C, and 2 min at 72 °C. A final extension step of 7 min at 72 °C completed the reaction. Amplified products (10 μL) were digested using 10 units of TaqI (Amersham-Pharmacia, Amersham, U.K) per reaction for 4 h at 65°C. Digested products were electrophoresed on a 2% agarose gel and were visualised by ethidium bromide. The 16974 C allele yields two fragments, 906 bp and 140 bp, respectively. Ethidium bromide and agarose were supplied by Sigma Chemical Co. The100 bp DNA marker ladder was supplied by Amersham-Pharmacia, Biotech.

Statistical analysis

Allele and genotype frequencies were calculated by direct counting. The statistical significance of differences was tested by chi-square method. Fisher's exact tests were applied if the expected frequency was less than 5. In comparing the genetic risk of cases and controls and the inducibility of cytokines two genotype classes were considered for analysis: homozygous genotype AA was compared with genotypes AG/GG and AA genotype was compared with genotypes AC/CC for -1082A/G SNP in IL-10 gene and +16974A/C SNP IN IL-12B, respectively.

The goodness of fit to Hardy-Weinberg equilibrium, calculating the expected frequencies of each genotype and comparing them with the observed values for patients and healthy controls, was performed using a chi-square test.

Logistic regression method was used to estimate odds ratios (OR), expressed with their 95% confidence intervals (95% CI) for disease susceptibility and sepsis outcome in relation to the cytokine polymorphism. Differences in cytokine production of PBMC between patients and control subjects were analysed using the Mann- Whitney U test. The results are presented as mean +/- standard error (SE) and +/- standard deviation (SD). In all cases p<0.05 was considered significant.

RESULTS

Allele and genotype distribution of IL-10-1082 (A/G) and +16974 (A/C) in 3'UTR of the IL-12B in healthy subjects versus patients

Genotype distribution and allele frequencies of the IL-10-1082 (A/G) and IL-12 +16974 (A/C) bi-allelic polymorphisms in patients with severe sepsis and healthy controls are shown on **Table 1**.

The IL-10 –1082 genotype distribution differed among patients with severe sepsis and healthy controls. The AA homozygous genotype was found more frequently in sepsis patients (51.5 %), compared with controls (23%). Homozygous subjects had a 3.6302 - fold increased risk of developing sepsis (OR = 3.6302; 95% CI: 1.4211-9.2735; p = 0.005869).

The allele frequency of the -1082 SNP was quite different for the group of sepsis patients than healthy controls. Patients with sepsis had significant elevation of IL-10 -1082 allele A, compared with controls (74.2% versus 52.8%; p = 0.005) and the OR=2.57; 95% CI, 1.31– 5.03.

The IL-12B genotype (+16974 AA; AC; CC) distribution showed no differences between sepsis patients and healthy controls, demonstrated that the SNP had no significance in susceptibility to sepsis.

Table 1. *Genotype distribution and allele frequency of IL-10 -1082 (A/G) and IL-12B (A/C) among sepsis patient (SP) and healthy controls (HC).*

	Genotypes n (%)			Allele frequency n (%)	
IL-12B +16974 A/C	**AA**	**AC**	**CC**	**A allele**	**C allele**
Total group of sepsis patients, n = 33	21 (64%)	11 (33 %)	1(3 %)	53 (80.3 %)	13 (19.7 %)
Healthy controls, n = 99	59 (60 %)	33 (33 %)	7 (7 %)	151 (76 %)	47 (24 %)
IL-10 – 1082 A/G	**AA**	**AG**	**GG**	**A allele**	**G allele**
Total group of sepsis patients, n = 33	17 **(51.5%)**[1]	15 (45.5%)	1 (3 %)	49 **(74.2 %)**[2]	17 (25.8 %)
Healthy controls, n = 53	12 (23 %)	32 (60 %)	9 (17 %)	56 (52.8 %)	50 (47.2 %)

[1]*OR = 3.6302, 95% CI 1.42-9.27; p=0.00587 (SP vs HC)*
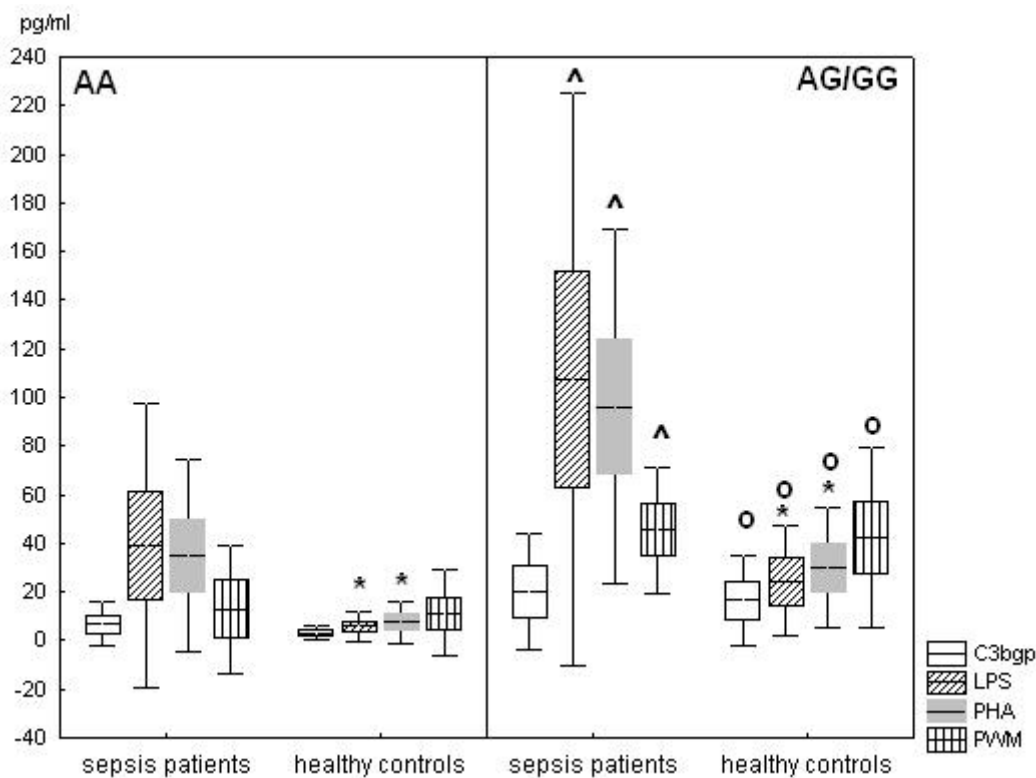[2]*OR = 2.5735, 95% CI 1.32-5.03; p=0.00511 (SP vs HC)*

Figure 1. *IL-10 produced by cultured PBMC in healthy donors and sepsis patients in relation to -1082 promoter polymorphism. Isolated PBMC from healthy donors and sepsis patients were cultured for 24 h at 37°C with C3-binding glycoprotein (C3bgp), lipopolysaccharide (LPS), phytohemagglutinin (PHA) and pokeweed mitogen (PWM).The quantity of IL-10 was determined in culture supernatants by ELISA test. The middle lines; the boxes and the whiskers correspond to mean value, SE and SD respectively.*

* p<0.05, sepsis patients versus healthy controls with the same genotype
^ p<0.05, AA versus AG/GG healthy controls
° p<0.05, AA versus AG/GG sepsis patients

IL-10 production from stimulated PBMC

An association between genotype and inducible IL-10 production in healthy subject and sepsis patients depends on the stimuli used. Results for -1082 SNP are presented in **Figure 1**. PBMC from AG/GG genotype produced significantly increased quantity of IL-10 compared to genotype AA after stimulation with LPS, PHA and PWM in healthy controls. Differences between PBMC from AA and AG/GG genotype, stimulated with C3bgp did not reach any significance.

Sepsis patients' PBMC produced lower

quantity of IL-10 compared to healthy controls' PBMC with the same genotype after all stimuli used; however significant decrease in IL-10 production were observed in sepsis patients after LPS and PHA stimulation. Sepsis patients carrying G allele produced significantly higher IL-10 compared to AA genotype sepsis patients after PBMC stimulation with all stimuli used: C3bgp, LPS, PHA and PWM.

IL-12 production from stimulated PBMC

Results for IL-12 production from cultured PBMC in relation to +16974 polymorphism in

IL-12B gene are presented in **Figure 2**. The mean concentration of IL-12p40 production after stimulation with C3bgp was higher in individuals carrying the AA genotype - 1757 pg/ml, compared to genotype AC/CC-875 pg/ml (p=0.01). These results indicated that the presence of AA genotype correlated with increased production of IL-12p40 of human PBMC after stimulation with C3bgp. We also found decreasing IL-12 p40 production after stimulation of PBMC with LPS and PHA for AC/CC genotypes, but the difference was not significant.
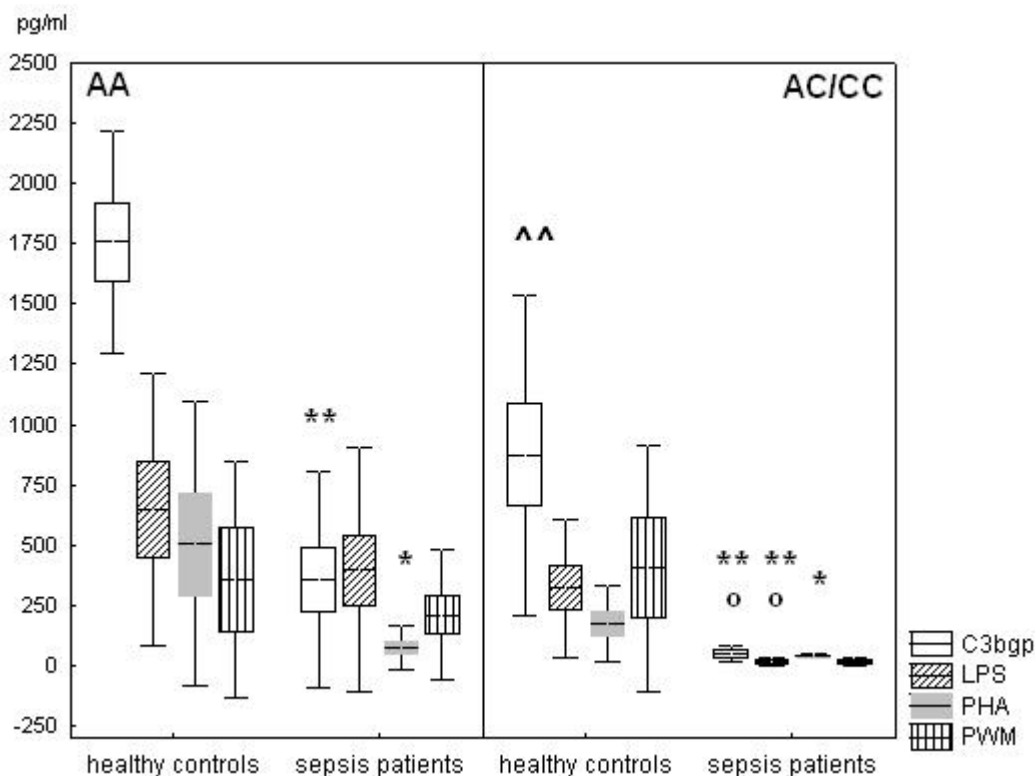

Figure 2. IL-12 produced by cultured PBMC in healthy donors and sepsis patients in relation to +16974 (A/C) SNP in 3'UTR of the IL-12B gene. Isolated PBMC from healthy donors and sepsis patients were cultured for 24 h at 37°C with C3-binding glycoprotein (C3bgp), lipopolysaccharide (LPS), phytohemagglutinin (PHA) and pokeweed mitogen (PWM).The quantity of IL-12 was determined in culture supernatants by ELISA test. The middle lines; the boxes and the whiskers correspond to mean value, SE and SD respectively.

* p<0.05, sepsis patients versus healthy controls with the same genotype
^^ p<0.01, AA versus AC/CC healthy controls
° p<0.05, AA versus AC/CC sepsis patients

As seen in **Fig.2**, IL-12B genotypes influence the production of IL-12p40 in sepsis patients more than in healthy controls. Sepsis patients' PBMC produced lower quantity of IL-12 compared to healthy controls' PBMC with the same genotype after all stimuli used, however significant decrease in IL-12 production were observed in sepsis patients after C3bgp, LPS and PHA stimulations. Sepsis patients carrying C allele produced significantly lower IL-12 compared to AA genotype sepsis

patients after PBMC stimulation with C3bgp and LPS.

DISCUSSION

The role of an individual's genetic background and predisposition for the individual immune response is determined at least by genes encoding immunomodulatory cytokines. Studies on the genomic polymorphisms of cytokine genes that

obviously contribute to sepsis and its sequela have been conducted (17, 18). Most reports have focused on inflammatory cytokine, particularly TNF- promoter polymorphisms and its association with susceptibility to severe sepsis and the sepsis outcome (19, 20). As previous data suggested the IL-10 -1082 promoter polymorphism was independently associated with the observed differences in IL-10 production in vitro. This led us to study the functional significance of this polymorphism using PBMC, isolated from sepsis patients and healthy donors and stimulated with both monocyte (LPS and C3bgp) and lymphocyte (PHA and PWM) activators.

Our experimental results demonstrated that the SNP at -1082 in the IL-10 promoter region is associated with induced IL-10 production in PBMC from severe sepsis patients and healthy controls and this association is dependent on the stimuli used. Generally the AA genotype was related to lower production of IL-10 after stimulation of PBMC from both healthy and sepsis groups. G allele in hetero and homozygous normal subject revealed significantly increased inducibility of IL-10 production after stimulation with LPS and PHA. Our data are supported by those of Turner et al. (6) who found that the IL-10 -1082A allele was associated with lower in vitro IL-10 production by Con A stimulated PBMC from normal subject.

Studies performed with severe sepsis patients suggested the importance of functional activity of the immune cells for induced cytokine production. The genotype dependency of IL-10 production after PBMC stimulation was also observed in sepsis patients group. Significant increase of IL-10 production was measured in AG/GG genotype compared to AA, after stimulation with C3bgp, PHA, LPS and PWM. All data demonstrate that the cytokine production of stimulated PBMC depends on the patient's genotype as well as on the physiological status of the patients: sepsis patients produced significantly lower quantity of IL-10 after stimulation compared to healthy subjects. We suppose that the low production of IL-10 from monocyte/macrophage cell line in the onset of infection may be a reason for production of high quantity of proinflammatory cytokine and development of sepsis.

We have shown that the presence of A allele at -1082 in IL-10 promoter region is associated with severe sepsis in a case control study. Patients with severe sepsis have a significant elevation of IL-10-1082 allele A, compared with controls (74.2% vs. 52.8%, p=0.005) and the individuals with genotype AA and AG have 6.54-fold more relative risk for severe sepsis than individuals with GG genotype.

In our previous study we demonstrated that higher IL-12 and lower IL-10 production after PBMC stimulation with one monocyte (C3bgp or LPS) and one lymphocyte (PHA or PWM) agent significantly correlated with favourable outcome of sepsis (12). The results in this study demonstrated that the SNP at 16974 in the IL-12 3'UTR region is associated with induced IL-12 production in PBMC from severe sepsis patients and healthy controls and this association is strongly dependent on the stimuli used. Generally, the AA genotype was related to higher production of IL-12 after stimulation of PBMC from both healthy and sepsis groups. C allele in hetero and homozygous normal subject revealed significantly lower inducibility of IL-12 production after stimulation with C3bgp. Moreover, this dependency is striking for sepsis group and obvious after stimulation with C3bgp and LPS. We suppose that the suppressed production of IL-12 from monocyte/macrophage cell line in sepsis patient may be due to immune paralysis development of sepsis. Moreover, the data from genotype and allele frequencies between control and sepsis groups demonstrated no significance in susceptibility to severe sepsis.

CONCLUSION

The A allele of the -1082 (A/G) SNP in the interleukin-10 gene promoter is strongly associated with lower stimulated interleukin-10 production and increased susceptibility to severe sepsis.

Sepsis patients carrying C allele of the +16974 (A/C) SNP in 3'UTR of the IL-12B gene produced significantly lower IL-12 compared to AA genotype sepsis patients after PBMC stimulation with C3bgp and LPS. SNP in 3'UTR of the IL-12B gene has impact on cytokine production and probably does not play a role in susceptibility to sepsis.

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