UNIVERSA MEDICINA

*September-December, 2012* *Vol.31 - No.3*

# Production of tumor necrosis factor-á is increased in urinary tract infections

**Neni Susilaningsih*, Bambang Joni Karjono**, and Ratna Damma Purnawati***

## ABSTRACT

**BACKGROUND**
Urinary tract infection (UTI) is a common source of bacteriemia. The most common cause of UTI is *Escherichia coli (E. coli)*. Tumor Necrosis Factor (TNF)-á gene polymorphism has been reported to be responsible for an excessive production of TNF-á and eventual disruption of pro-inflammatory cytokine regulation. The aim of this study was to compare TNF-á serum levels and TNF-á allele polymorphisms in patients with UTI due to *E.coli* and in non-UTI controls.

**METHODS**
A cross-sectional study was conducted at Dr. Kariadi Central Hospital and the Center for Biomedical Research, Faculty of Medicine, Diponegoro University, Semarang. In 68 patients with UTI the TNF-á serum levels were determined by means of ELISA and compared to those of non-UTI controls (n=55). TNF-á-308G>A gene polymorphism was analyzed by polymerase chain reaction restriction fragment length using the NcoI enzyme. Fragments were visualized on polyacrylamide gel with silver staining.

**RESULTS**
TNF-á serum level in patients with UTI had a median of 8.9 pg/mL, which was significantly higher than the median of 3.7 pg/mL in the control group ($p<0.001$). TNF-á-308G>A gene polymorphisms found in the patient group were G/G=61 (90%), G/A=7(10%) and A/A=0, while in the control group were G/G=48 (87%), G/A=7 (13%) and A/A =0. There was no significant differences ($p=0.578$) in gene polymorphisms between the two groups.

**CONCLUSIONS**
TNF-á serum levels in patients with UTI due to *E. coli* were significantly higher than in non-UTI controls, but for the TNF-á-380 gene polymorphisms no significant difference was found between the two groups. There are presumably more important factors than host genotype that influence UTI pathogenesis.

**Keywords**: Urinary tract infection, *E. coli*, serum TNF-á, TNF-á gene polymorphism

*Department of Histology, Faculty of Medicine, Diponegoro University
**Department of Internal Medicine, Dr. Kariadi Central Hospital/ Faculty of Medicine, Diponegoro University

**Correspondence**
dr. Neni Susilaningsih, M.Si
Department of Histology, Faculty of Medicine, Diponegoro University
Jl. Dr. Sutomo 18 Semarang
Email: nsusilaningsih@gmail.com

*Univ Med 2012;31:167-74*

# *Produksi tumor nekrosis faktor-á meningkat pada penderita infeksi saluran kemih*


## *ABSTRAK*

***LATAR BELAKANG***

*Infeksi saluran kemih (ISK) merupakan sumber tersering bakteremia. Penyebab ISK terbanyak adalah Escherichia coli (E. coli).* Tumor Necrosis factor *(TNF)-á merupakan sitokin penting pada inflamasi akut yang paling sering dihubungkan dengan sepsis. Polimorfisme gen TNF-á bertanggung jawab terhadap produksi TNF-á yang berlebihan dan akhirnya mengacaukan pengaturan kerja sitokin-sitokin serta menyebabkan terjadinya penyakit. Tujuan penelitian ini adalah untuk membedakan kadar TNF-á serum dan polimorfisme gen TNF-á G308A antara penderita ISK karena E. coli dan kontrol non ISK.*

***METODE***

*Penelitian ini adalah cross sectional dilakukan di RSUP Dr. Kariadi dan Center for Biomedical Research FK Undip Semarang. Enam puluh delapan penderita ISK karena E. coli dan 55 kontrol non ISK dukur kadar TNF-á serum dengan metode ELISA. Polimorfisme TNF-á-308 G>A ditentukan dengan metode* polymerase chain reaction restriction fragment length *menggunakan enzim Ncol. Fragmen divisualisasikan pada gel poliakrilamid dan diwarnai dengan* silver.

***HASIL***

*Median kadar TNF-á serum pada penderita ISK karena E.coli didapatkan sebesar 8,9 pg/mL lebih tinggi secara bermakna dibanding kontrol dengan median 3,7pg/mL (p<0,001). Polimorfisme gen TNF-á-308G>A pada kelompok penderita ISK karena E.coli didapatkan G/G=61(90%), G/A=7(10%) dan A/A=0. Kelompok kontrol didapatkan G/G =48 (87%), G/A=7(13%) dan A/A=0. Analisis dengan* Fisher's Exact test *tidak didapatkan perbedaan bermakna (p=0,578) antara penderita ISK karena E.coli dan kontrol.*

***KESIMPULAN***

*Kadar TNF-á serum penderita ISK karena E. Coli lebih tinggi secara bermakna dibanding kontrol non ISK, tetapi tidak didapatkan perbedaan bermakna polimorfisme gen TNF-á antara kedua kelompok.*


***Kata kunci:*** *Infeksi saluran kemih E.coli, TNF-á serum, polimorfisme gen TNF-á*

## INTRODUCTION

Urinary tract infection (UTI) is one of the most frequently encountered bacterial infections, particularly in women.[1] The majority of UTI cases occur in the age range of 16-35 years.[1,2] The female-to-male ratio is 4:1, with 10% of females having UTI annually and 60% having several infections during their lifetime.[1,2] UTI is the most frequent source of bacteriemia.[3] The most frequent cause of UTI is *Escherichia coli* (*E. coli)*, found in 80% to 90% of UTI cases.[4-7]

The body response in UTI is initiated by an immune response at the time of bacterial contact with urothelial cells. Subsequently various bacterial factors (fimbriae, pili and lipopolysaccharide/LPS) interact with epithelial receptors, leading to activation of several pathways. LPS stimulates the phagocytic cells of the innate immune system through interaction with toll-like receptor 4 (TLR-4), resulting in release of cytokines, viz. tumor necrosis factor (TNF)-á, interferron (IFN)-γ, interleukin (IL)-8, IL-1, and IL-6.[7-9]

TNF-á is an important mediator in the acute inflammatory response against Gram-negative bacteria and other infectious microbes, and is responsible for the systemic complications of severe infections. Production of TNF-á is induced by LPS from the cell walls of Gram-negative bacteria. TNF is produced in large amounts in severe infections and causes systemic abnormalities.[10]

TNF-á is a cytokine that is most frequently associated with sepsis or septic shock.[11] Several of the underlying reasons are: 1) TNF-á plasma concentrations increase at the time of sepsis, 2) the increase in TNF-á is associated with increased mortality, 3) administration of exogenous TNF-á in humans and experimental animals induces sepsis and organ failure, and 4) neutralization of TNF-á in experimental animals results in improvement from sepsis and increases survival.[9,12]

Genetic and environmental factors play a role in determining illness in an individual. A person may have been programmed to respond to infection in various ways. Some individuals have a strong immune system and are able to overcome the infection before the appearance of physical signs, whereas others more frequently become ill.[13] The influence of genetic factors on this response to disease has been demonstrated by studies with adopted children and twins. Genetic factors are the major determinants of the tendency towards death from infection. The mortality risk in patients with sepsis is related to gene polymorphisms of TNF-$\alpha$ and TNF-$\beta$.[14,15]

TNF-á gene polymorphism causes variation in TNF-á production. TNF-á gene polymorphism is responsible for overproduction of TNF-á, such as occurs in sepsis and septic shock.[11] One of the identified TNF-á gene polymorphisms is localized in the promoter region at position -308, where the common allele G (guanine) is replaced by the A (adenine) allele, known as TNF-á-308 G>A.[16] TNF-á gene polymorphism in the promoter region at position -308 is reportedly strongly associated with induction of disease, including UTI.[17,18] However, investigations on the TNF-á gene in the promoter region -308G>A in cases of UTI due to *E. coli* have never been reported in Indonesia as well as internationally.

The objective of this study was to compare serum TNF-á levels and TNF-á-308G>A gene polymorphisms in patients with UTI due to *E. coli* and in non-UTI controls.

## METHODS

### Study Design

This was a cross-sectional observational study conducted at Dr. Kariadi Central Hospital and the Cebior Laboratory, Faculty of Medicine, Diponegoro University, from March 2009 until November 2010.

### Study subjects

Patients with UTI were identified on the basis of leukocyturia on urine examination and an *E. coli* count of ≥100.000 colony forming unit (CFU)/mL on bacterial culture of urine. Inclusion criteria were age 14 years and older, presence of *E. coli* infection as evidenced by bacterial culture of urine, and agreeing to become a study subject. Subjects with a past history of urinary tract obstruction (prostate hypertrophy, senile vaginitis), use of instruments in the urinary tract lasting ≥3 days, disturbances of miction (chronic urinary incontinence, etc.), and immunocompromized patients (organ transplantation, renal failure, diabetes mellitus), were excluded from the study. Controls were selected from healthy volunteers not suffering from UTI, based on the absence of leukocyturia and sterile bacterial culture of urine. A total of 68 patients with UTI due to *E. coli* and 55 persons without UTI as controls comprised the subjects of this study.

### Measurement of TNF-á serum levels

Serum was separated from venous blood samples and stored pending measurement of TNF-á concentration by means of ELISA, using the Quantikine® Human TNF-á ELISA Kit

(R&D Systems). Measurements were carried out according to the manufacturer's instructions.

**DNA extraction**

DNA extraction was done by the salt saturation method as described by previous investigators.[19] Blood samples were placed in tubes containing ethylenediamine tetraacetate (EDTA), then 5-10 mL $NH_4Cl$ lysis buffer was added to the tubes, which were left to stand for 10-30 minutes at room temperature. The tubes were centrifuged for 5 minutes at 3000-3500 rpm. The supernatant was discarded, $NH_4Cl$ lysis buffer was added, and the tubes were centrifuged once more. To the white pellets was added 2 ml Telesys, 30-50 µL proteinase K and 100 µl 10% sodium dodecyl sulfate, then the samples were kept at $50^0$C overnight. Subsequently 6 M NaCl was added at 1/3 of the sample volume, and then the sample was shaken and centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred to a new tube, and 100% ethanol was added at twice the sample volume. The DNA in the form of a white substance was taken out and washed in 70% ethanol, left to dry, then dissolved in Tris-EDTA (TE) buffer.

**Analysis of gene polymorphism**

Analysis of TNF-α gene polymorphism was performed by amplification of the TNF-α gene in the promotor region at position -308, using a PCR cycler (Applied Biosystems). The total volume of each sample mixture (20 µl) contained 100 ng DNA, 4 pmol/µL of forward primer and reverse primer, respectively, 0.2 mM dNTP, 1 mM $MgCl_2$ and 0.5 units of the Taq enzyme. The polymerase chain reaction (PCR) cycle consisted of 5 minutes at $95^0$C for initial denaturation, followed by final extension for 7 minutes at $72^0$C. The PCR product of 107 bp was then cut by restriction fragment length polymorphism (RFLP), by incubation at $55^0$C for 18 hours (overnight) with the NcoI restriction enzyme. The cut PCR product was visualized by silver staining on 10% polyacrylamide gel. In individuals homozygous for the -308 (A/A) allele, the PCR product was not cut, resulting in a band on the gel of 107 bp. In contrast, homozygous G/G yielded fragments of 87bp and 20 bp, while heterozygous G/A yielded fragments of 107 bp, 87 bp and 20 bp.[20]

**Data analysis**

Data were analyzed by descriptive statistics to show the frequency distribution of the data. Tests of normality of TNF-α concentrations indicated that the data were non-normally distributed. The Mann Whitney was used for comparing serum TNF-α levels in patients with UTI and in controls. Fisher's exact test was used to compare TNF-α gene polymorphism events in patients with UTI and in controls. All analyses were performed by means of the SPSS program version 11 for Windows, at a significance level of 5%.

**Ethical clearance**

The present study was approved by the Commission on Research Ethics of the Faculty of Medicine, Diponegoro University/Dr Kariadi Central Hospital, Semarang, with ethical clearance No 16/EC/FK/RSDK/2009.

**RESULTS**

This study found that the 68 respondents with UTI due to *E. coli* were in the age range of 16-86 years, while the 55 controls were in the age range of 18-84 years, and a median in the age range of 65-69 years. The group with UTI due to *E. coli* consisted of 46 females (67.6%) and 22 males (32.4%), while the control group had 29 females (52.7%) and 26 males (47.3%). The results of the analysis on age and gender showed no significant difference between the UTI group and the control group (p=0.093).

The subjects with UTI due to *E. coli* were taken from respondents suffering from UTI with leucocyturia and positive bacterial culture of urine (>100.000 CFU/ml). The subjects with UTI were taken from subjects whose urinary bacterial cultures were positive for *E. coli* only

Tabel 1. Distribution of bacterial causes of UTI* (n=68)

| Bacterial species | n | % |
|---|---|---|
| *E. coli* | 51 | 75.0 |
| *E. coli* and *Pseudomonas spp* | 2 | 2.9 |
| *E. coli* and *Enterococcus spp* | 1 | 1.5 |
| *E. coli* and *Staphylococcus aureus* | 8 | 11.8 |
| *E. coli* and *Enterobacter spp* | 6 | 8.8 |

*UTI= urinary tract infection

and for *E. coli* in combination with other bacteria, as seen in Table 1. In 51 (75.0%) subjects the cause of UTI was *E.coli.*

Serum TNF-á measurements yielded a median of 8.9 pg/ml in the group with UTI due to *E. coli* and a median of 3.36 pg/mL in the control group. The Mann Whitney test found significantly higher TNF-á levels in patients with UTI due to *E coli* than in controls ($p<0.001$). The results of the analysis are shown as box plots in Figure 1.

The TNF-α-308G>A gene polymorphism indicated that 7 (10%) of the 68 respondents with UTI and 7 (13%) of the 55 controls had the heterozygous genotype G/A, while the majority of both patients with UTI and controls had the homozygous genotype G/G. No homozygous A/A genotype was found in patients with UTI or in controls (Figure 2). Fisher's exact test showed no significant difference in TNF-α gene polymorphism events in patients with UTI due to *E. coli* and in controls ($p=0.578$) (Table 2).

## DISCUSSION

TNF-á is an important mediator of the acute inflammatory response against Gram-negative bacteria and other infectious microbes, and is responsible for various systemic complications of severe infection. TNF-á production by macrophages is induced by LPS from the cell walls of Gram-negative bacteria. TNF-á is a cytokine most frequently associated with sepsis or septic shock.[10,21]

The results of the present study showed that the TNF-á serum level was higher in the group of patients with UTI due to *E.coli*. This indicates that the inflammation occurring in UTI due to *E. coli* activates TLR4 macrophages and induces TNF-á production.[22] The TNF-á produced plays a role in inducing the activation of integrins in leukocytes and integrin ligands in endothelial cells, so that the leukocytes adhere to the endothelium and subsequently migrate into the extra vascular tissues, where they engage in fighting the microbes.

TNF-á as a potent pro-inflammatory cytokine has been reported to be increased in sepsis. This cytokine is reportedly increased in the urine in UTI (pyelonephritis), and also in the urinary bladder during UTI, particularly at the onset of the disease.[10,23] Sadeghi[24] reported that during bacteriuria in renal transplant patients there was an increase in urinary cytokine content, including TNF-á. The production of TNF-occurs after activation of Toll-like receptors (TLR) by bacterial products, including endotoxins.[9]

Genetic and environmental factors play a role in determining illness in an individual. A person may have been programmed to respond to infection in various ways. Some individuals have a strong immune system and are able to overcome the infection before the appearance of physical signs, whereas others more frequently become ill.[13] Genetic factors are the major

Table 2. Distribution of TNF-α 308G>A gene polymorphisms in UTI* and control groups

| Group | TNF-α-308G>A gene | | | | | | p value |
|---|---|---|---|---|---|---|---|
| | G/G n(%) | | G/A n(%) | | A/A n(%) | | |
| UTI | 61 | (90) | 7 | (10) | 0 | (0) | 0.578 |
| Control | 48 | (87) | 7 | (13) | 0 | (0) | |

*UTI= urinary tract infection

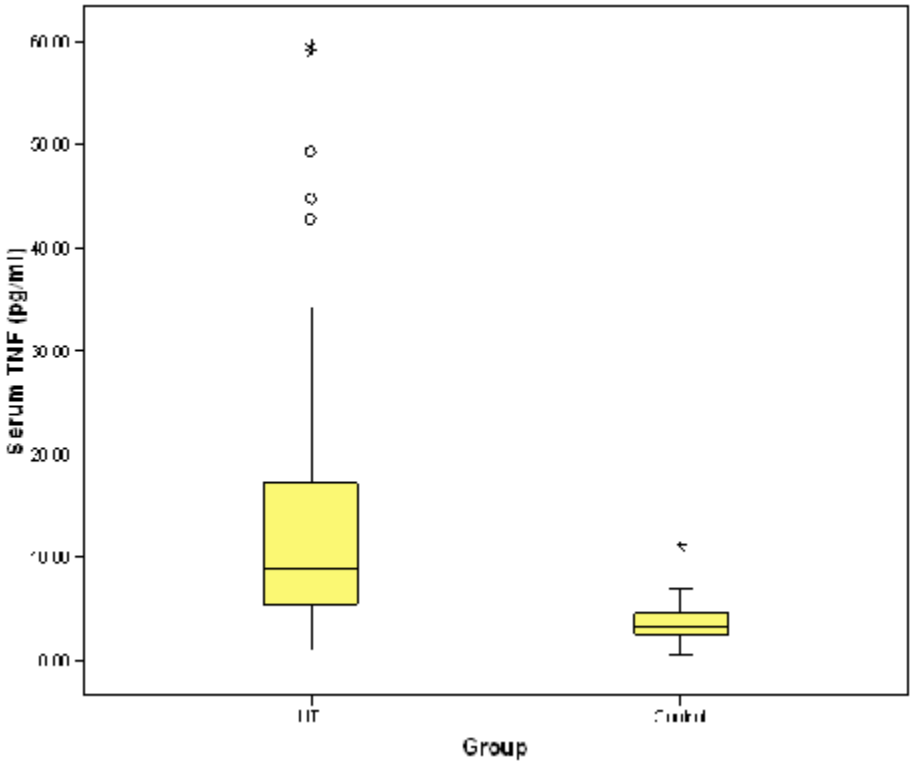


Figure 1. Box plots of TNF-á serum levels. UTI due to *E. coli* (n=68); Non-UTI controls (n=55)

determinants of the tendency towards death from infection. The mortality risk in patients with sepsis is related to gene polymorphism of TNF-α and TNF-β.[14,15]

The present study found the occurrence of TNF-á 308G>A gene polymorphisms, with the G/A genotype present in 7 (10%) of 68 patients with UTI due to *E. coli*, and in 7(13%) of 55 controls, but did not find the homozygous polymorphism allele A/A. TNF-á gene polymorphism causes variation in the production of TNF-á in the defense mechanism of the body

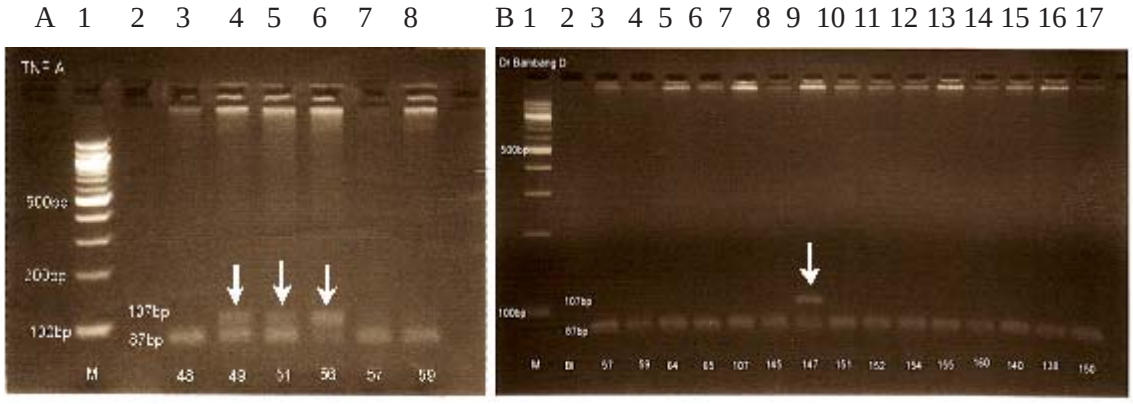


Figure 2. PCR results for TNF-á 308G>A
Lanes A1 and B1= markers, lanes A2 and B2 = blanks.
Lanes A4,5,6 and B9 = heterozygous G/A (sample Nos 49, 51, 56 and 147)
All other lanes = homozygous G/G

against infectious diseases. TNF-á gene polymorphism is responsible for the overproduction of TNF-á and ultimately disrupts the regulation of cytokine activity, thus resulting in disease. The A allele has been reported to increase TNF-á production to a higher degree than the G allele, and the homozygous polymorphism A/A is called a high producer of TNF-á.[11,25]

The homozygous polymorphism G/G was found in dominant numbers, both in patients with UTI due to *E. coli* (90%) and in non-UTI controls (87%), while the homozygous polymorphism A/A was not found in this study. The results of this study showed that there was no significant difference between the group of patients with UTI due to *E. coli* and the non-UTI control group. The genotypic distribution in the TNF-á gene polymorphism was dominated by the G/G polymorphic allele, which is consistent with the various studies conducted by Sallakci,[25] Krasowska[26] in 2005, Moffet et al.[27] in 2005, and the study by Cabantau et al.[28] in Mali in 2006.

A limitation of this study was the small size of the control group in comparison with the UTI group, although no significant difference was found in age range and gender between the two groups. Furthermore, this study was conducted on hospitalized patients, who are thus not representative of non-hospitalized patients.

## CONCLUSIONS

Serum TNF-á level was higher in patients with UTI due to *E. coli* than in non-UTI controls. No significant difference was found for the TNF-á-380 gene polymorphism between patients with UTI due to *E. coli* and controls. Further studies should be done with a better matching between UTI cases and controls, and the inclusion of non-hospitalized patients. There are presumably more important factors than host genotype that influence UTI pathogenesis.

## ACKNOWLEDGEMENTS

We wish to express our gratitude to the Indonesian Ministry of Research and Technology for the funding of this study through the Basic Research Incentives Program *(Program Insentif Riset Dasar)* for 2009-2010. We also thank Prof.dr. Sultana MH Faradz, PhD and staff at the Center for Biomedical Research (Cebior), Faculty of Medicine, Diponegoro University, for their inputs and facilitation of this study.

## REFERENCES

1. Salvatore S, Salvatore S, Cattoni E, Siesto G, Serati M, Sorice P, et al. Urinary tract infections in women. Eur J Obstet Gynecol Reprod Biol 2011;156:131-6.
2. Nicolle LE. Uncomplicated urinary tract infection in adults including uncomplicated pyelonephritis Urol Clin North Am 2008;35:1–12.
3. Bingen-Bidois M, Clermont O, Bonacorsi S, Terki M, Brahimi N, Loukil C, et al. Phylogenetics analysis and prevalence of urosepsis strain of Escherechia coli bearing phatogenecity island like-domains. Infect Immun 2002;70:3216-26.
4. Ronald A. The etiology of urinary tract infection: traditional and emerging pathogens. Am J Med 2002;113 Suppl 1A:S14–9.
5. Nicolle LE. Complicated urinary tract infection in adults. Can J Infect Dis Med Microbiol 2005; 16:349-60.
6. Ronald A. The etiology of urinary tract infection: traditional and emerging pathogens Dis Mon 2003;49:71–82.
7. Lee S-J, Kim SW, Cho Y-H, Yoon MS. Anti-inflammatory effect of an Escherichia coli extract in a mouse model of lipopolysaccharide-induced cystitis. World J Urol 2006;24:33-8.
8. Kucheria R, Dasgupta P, Sacks SH, Khan MS, Sheerin NS. Urinary tract infections: new insights into a common problem. Postgrad Med J 2005;81:83-6.
9. Ramesh G, Kimball SR, Jefferson LS, Reeves WB. Endotoxin and cisplatin synergistically stimulate TNF- production by renal epithelial cells. Am J Physiol Renal Physiol 2007;292: F812–9.
10. Mohkam M, Asgarian F, Fahimzad A, Sharifian M, Dalirani R, Gorgi FA. Diagnostic potential

of urinary tumor necrosis factor-alpha in children with acute pyelonephritis. Iranian J Kidney Dis 2009;3:89-92.
11. Wong HR. Genetics and genomics in pediatric septic shock. Crit Care Med 2012;40:1618-26.
12. Knuefermann P, Remoto S, Baumgarten G, Misra A, Sivasubramanian N, Carabello B, et al. Cardiac inflammation and innate immunity in septic shock. Is there a role for toll like receptors? Chest 2002;121:1329-36.
13. Holmes CL, Russell JA, Walley KR. Genetic polymorphisms in sepsis and septic shock: role in prognosis and potential for therapy. Chest 2003;124:1103-15.
14. Hotchkiss R, Karl I. The pathophysiology and treatment of sepsis. N Engl J Med 2003;348:138-50.
15. Sipahi T, Pocan H, Akar N. Efect of various genetic polymorphisms on the incidence and outcome of sever sepsis. Clin Appl Thromb Hemost 2006;12:47-54.
16. Mossböck G, Renner W, El-Shabrawi Y, Faschinger C, Schmut O, Wedrich A, et al. TNF-á –308 G>A and –238 G>A polymorphisms are not major risk factors in Caucasian patients with exfoliation glaucoma. Mol Vis 2009;15:518-22.
17. Haukim N, Bidwell J, AJP AJPS, Keen LJ, Gallagher G, Kimberly R, et al. Cytokine gene polymorphism in human disease: on-line databases, supplement 2. Genes Immun 2002;3: 313–30.
18. Hollegaard M, Bidwell J. Cytokine gene polymorphism in human disease: on-line databases. Genes Immun 2006;7 Suppl 3:S269–76.
19. Ngestiningsih D, Faradz SMH. Polimorfisme Alel TNF-alfa pada penderita DHF di Semarang. M Med Indones 2006;41:46-51.
20. Moukoko CE, El-Wali N, Saeed OK, Mohamed-Ali Q, Gaudart J, Dessein AJ, et al. No evidence for a major effect of tumor necrosis factor alpha gene polymorphisms in periportal fibrosis caused by Schistosomamansoni infection. Infect Immun 2003;71:5456-60.
21. Knuefermann P, Remoto S, Baumgarten G, Misra A, Sivasubramanian N, Aabello BA, et al. Cardiac inflammation and innate immunity in septic shock. Is there a role for toll like receptors? Chest 2002;121:1329-36.
22. Svanborg C, Bergsten G, Fischer H, Godaly G, Gustafsson M, Karpman D, et al. Uropathogenic Escherichia coli as a model of host-parasite interaction. Curr Opin Microbiol 2006;9:33-9.
23. Engel D, Dobrindt U, Tittel A, Peters P, Maurer J, Gutgemann I, et al. Tumor necrosis factor alpha- and inducible nitric oxide synthase-producing dendritic cells are rapidly recruited to the bladder in urinary tract infection but are dispensable for bacterial clearance. Infect Immun 2006;74:6100-7.
24. Sadeghi M, Daniel V, Naujokat C, Wiesel M, Hergesell O, Opelz G. Strong inflammatory cytokine response in male and strong anti-inflammatory response in female kidney transplant recipients with urinary tract infection. Transpl Int 2005;18:177-85.
25. Sallakci N, Akcurin G, Koksoy S, Kardelen F, Uguz A, Coskun M, et al. TNF-alpha G-308A polymorphism is associated with rheumatic fever and correlates with increased TNF-alpha production. J Autoimmun 2005;25:150-4.
26. Krasowska D, Chodorowska G, Koziol-Montewka M, Ksiazek A, Buraczynska M. The -308 promoter polymorphism in the tumour necrosis factor gene in patients with lichen planus. Acta Derm Venereol 2005;85:400-3.
27. Moffett S, Zmuda J, Oakley J, Beck T, Cauley J, Stone K, et al. Tumor necrosis factor-á polymorphism, bone strength phenotypes, and the risk of fracture in older women. J Clin Endocrinol Metab 2005;90:3491–7.
28. Cabantou S, Doumbo O, Ranque S, Poudiougou B, Traore A, Hou X, et al. Alleles 308A and 238A in the tumor necrosis factor alpha gene promoter do not increase the risk of severe malaria in children with Plasmodium falciparum infection in Mali. Infect Immun 2006;74:7040–2.