TOLL-LIKE RECEPTOR KINETICS IN SEPTIC SHOCK PATIENTS: A PRELIMINARY STUDY

O. PIAZZA[1], G. PULCRANO[2], P.L. FIORI[3], R. TUFANO[4], M. LONARDO[4], F. ROSSANO[2] and M.R. CATANIA[2]

[1]Department of Medicine and Surgery, University of Salerno, Italy; [2]Department of Cellular and Molecular Biology and Pathology, Federico II University of Naples, Italy; [3]Department of Biomedical Sciences, University of Sassari, Italy; [4]Department of Anesthesiology and Intensive Care, Federico II University of Naples, Italy

The aim of this study is to evaluate some inflammatory parameter changes in septic shock patients and their possible correlation with clinical outcome, in particular when continuous veno-venous hemofiltration (CVVH) treatment is required. Considering the objective difficulty in enrolling this kind of patient, a preliminary study was initiated on seventeen septic shock patients admitted to a medical and surgical ICU. The mRNA expression of Toll-like receptor (TLR)-1, TLR-2, TLR-4, TLR-5, TLR-9, TNFα, IL-8 and IL-1β was assessed, the plasmatic concentrations of IL-18, IL-2, IL-10 and TNFα were measured on the day of sepsis diagnosis and after 72 h. In those patients who developed acute renal failure unresponsive to medical treatment and who underwent CVVH treatment the same parameters were measured every 24 h during CVVH and after completion of the treatment. On sepsis diagnosis, gene expression of TLRs was up-regulated compared to the housekeeping gene in all the patients. After 72 h, in 35% of the patients a down-regulation of these genes was found compared to day 1, but it was not associated with a reduction of cytokine serum levels or improved clinical signs, better outcome or reduced mortality. After high volume hemofiltration treatment, cytokine serum levels and TLR expression were not significantly modified. In conclusion, considering the not numerous number of cases, from our preliminary study, we cannot certainly correlate TLR over-expression in septic shock patients with severity or outcome scores.

Sepsis is among the commonest causes of death in intensive care units and modern therapies have only modest success in reducing sepsis-related mortality (1). This lack of progress has been attributed to several possible causes, among which there is the incomplete understanding of the host response to sepsis.

The pathogenesis of sepsis involves complex interactions between the host and the infectious microorganism. Toll-like receptors (TLRs) recognize preserved patterns of microbial structures. Thirteen TLRs (TLR1 to TLR13) have been identified. TLRs bind and become activated by different ligands, which are located on different types of organisms or structures. For instance, TLR2, 4,5,6, and 11 are activated by bacterial membrane components, such as lipopolysaccharide, lipoprotein, and peptidoglycan; TLR5 recognizes bacterial flagellin; TLR7, 8, and 9 bind bacterial and viral RNA and DNA. The interaction between the TLR and its ligand results

Mailing address: Prof. Maria Rosaria Catania,
Department of Cellular
and Molecular Biology and Pathology,
Federico II University of Naples,
Via S. Pansini 5, 80131 Naples, Italy
Tel +39 081 7464577 Fax +39 081 7463015
e-mail: mariarosaria.catania@unina.it

in the activation of innate immunity through the induction of antimicrobial activity and the production of inflammatory cytokines, and in the generation of adaptive immunity through the induction of antigen presenting molecules (major histocompatibility complex, MHC, class II), costimulatory molecules and specific cytokines on antigen presenting cells (2). Inappropriate activation of TLRs has been shown to contribute to various human pathologies, including bacterial sepsis, autoimmune and neurodegenerative diseases, and cancer.

With regard to sepsis, the protective and damaging host responses are part of the same process since the inflammatory response that is aimed at controlling the infectious process also underscores many of the pathophysiological events of sepsis.

In septic shock patients, the inflammatory response is excessive, for example when the offending microbial toxin is highly potent, when the microorganism is very aggressive, when the microbial burden is high, or when there is a genetic predisposition, resulting in large amounts of effector molecules in response to a modest insult (3, 4). TLRs on one hand are essential for early detection of pathogens: mutations in the genes encoding TLRs and downstream signal transducing molecules influence innate immune responses and increase susceptibility to many infectious diseases (5). On the other hand, TLRs can cause excessive inflammation after uncontrolled stimulation: Salomao et al. (6) hypothesized that the TLR9 blockade could be a potential strategy for the treatment of human sepsis.

Any association between TLR expression and regulation of sepsis is complex: different TLRs respond to different microbial products to induce different transcription factors, leading to the expression of several genes with several clinical consequences.

As sepsis is characterized by excessive release of proinflammatory cytokines, the non-specific elimination of cytokines and other inflammatory mediators from the bloodstream could improve the clinical condition of a septic patient. In the past two decades, animal experiments have shown that hemofiltration significantly improves survival rates in sepsis (7). Continuous venovenous hemofiltration (CVVH) is a technique of renal replacement therapy in critically ill patients, that may partially attenuate the inflammatory process by removal of cytokines. The reduction of inflammatory cytokine serum levels after CVVH may influence TLR expression, either by negative or positive feedback.

The aim of this study is to evaluate inflammatory parameter changes during septic shock and their correlation to the severity of clinical presentation. Considering the objective difficulty in enrolling this kind of patients, a preliminary study was initiated on seventeen septic shock patients admitted to a medical and surgical ICU.

An indicative observational study was performed and the mRNA expression of genes involved in innate immune response (TLR 1-2-4-5-9, TNFα, IL-1β, IL8) was assessed and correlated to severity scores and outcome. In addition, the effect of continuous veno-venous hemofiltration (CVVH) treatment, performed when acute renal failure was present, on up/down regulation of gene expression and inflammatory molecular patterns, was evaluated.

MATERIALS AND METHODS

Study population

The study group included 17 septic shock patients admitted to the Intensive Care Unit of the University Hospital Federico II of Naples, Italy, during a 12-month study period. Sepsis was diagnosed according to the International Sepsis Definitions Conference (8).

Patients under 18 and over 80 years or with heart failure (NYHA III-IV), liver failure (Child C), immunosuppression or medullar aplasia were excluded from the study. All patients were evaluated using the Simplified Acute Physiologic Score (SAPS) III on admission day, and Sequential Organ Failure Assessment (SOFA) during all the period of hospitalization. Five of the 17 patients were affected by acute renal failure that required continuous veno-venous hemofiltration (CVVH). Clinical characteristics of the study population are shown in Table I. Eighteen unrelated healthy volunteers without signs of inflammatory diseases served as control group (9 males and 9 females, mean age 27.5±2.6 yrs).

Study protocol

The study was approved by the local Hospital Ethics Committee.

Blood samples were obtained from septic patients and healthy volunteers to measure plasmatic cytokine concentrations and to evaluate TLR and cytokine gene expression. The blood samples were taken once from the healthy volunteers, on the day of septic shock diagnosis

Table I. *Clinical characteristics of the study population.*

Case	Gender	Age	Origin of Sepsis	Microbiology	SAPS3	SOFA	CVVH	Length of stay (days)	Outcome
1	F	47	Pulmonary	Pseudomonas aeruginosa	65	7	no	39	died
2	M	59	Bacteraemia	Acinetobacter baumanii	57	6	no	23	died
3	M	74	Pulmonary	Candida albicans	67	10	no	14	died
4	M	67	Pulmonary	Candida albicans	67	10	no	11	died
5	F	74	Bacteraemia	Staphylococcus haemolyticus	60	5	no	21	died
6	M	80	Abdominal	Staphylococcus hominis	65	10	no	12	died
7	F	24	Bacteraemia	Pseudomonas aeruginosa	47	5	no	90	died
8	M	17	Bacteraemia	Staphylococcus haemolyticus	77	10	no	30	died
9	F	66	Catheter related infection	Pseudomonas aeruginosa	77	8	no	35	survived
10	F	57	Bacteraemia	Pseudomonas aeruginosa	59	12	no	21	died
11	M	56	Pulmonary	Klebsiella pneumoniae	71	9	no	19	died
12	F	77	Pulmonary	Staphylococcus aeurus	91	7	no	5	died
13	M	45	Necrotizing Fasciitis	Klebsiella pneumoniae	57	8	yes	34	died
14	M	80	Pulmonary	Candida albicans	69	13	yes	13	died
15	F	78	Pulmonary	Candida tropicalis	51	6	yes	20	died
16	M	80	Pulmonary	Streptococcus pneumoniae	72	11	yes	45	died
17	F	80	Bacteraemia	Klebsiella pneumoniae	56	16	yes	21	survived

and after 72 hours from septic shock patients not receiving CVVH, and every 24 hours in septic patients receiving CVVH.

Blood samples, obtained by venipuncture, were collected in Vacutainer tubes with EDTA as anticoagulant and immediately transferred to the laboratory for RNA extraction and cytokine assays or frozen and stored at -20°C until tested.

CVVH procedure

CVVH was performed with the Aquamax HF12 hemofilter (membrane area of $1.2m^2$, filter internal diameter of 200 microns) and the Aquarius machine (Edwards Lifesciences, Switzerland) was used for the study. A double lumen venous catheter was used for femoral or subclavian access. A blood flow rate of 200mL/h was set and the filtration rate was 35mL/kg/h. Bicarbonate-buffered replacement fluids were used, added in the post-dilutional phase. The extracorporeal circuit was anti-coagulated by continuous unfractionated heparin infusion according to an individual patient-adjusted anticoagulation regimen.

Cytokine assay

The plasma levels of IL-18, IL-2, IL-10 and TNFα in blood samples from septic shock patients and the control group were measured by immunoassays. IL-18 levels were quantified by MBL ELISA kit (MBL, Naka-ku Nagova, Japan), while IL-2, IL-10 and TNFα levels were assessed using R&D ELISA kit (R&D Systems Minneapolis MN, USA), according to the manufacturer's instructions.

All assays were performed in duplicate wells and results were considered valid if replicated differed by <2-fold.

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Table II. *Sequence accession number, probes and primer pairs used in PCR and RT-PCR experiments.*

Gene	Accession number	Primer pair and probe
TLR-1	NM_003263	FW 5'- gggtcagctggacttcagag RW 5'- aaaatccaaatgcaggaacg PROBE 5'- tttgcccatccaaaattagc
TLR-2	NM_003264	FW 5'-ggggttgaagcactggacaat RW 5'-tcctgttgttggacaggtca PROBE 5'- cctccaatcaggcttctctg
TLR-4	NM_138557	FW 5'-tgagcagtcgtgctggtatc RW 5'-cagggcttttctgagtcgtc PROBE 5'- gtggagaagaccctgctcag
TLR-5	NM_003268	FW 5'-ggaaccagctcctagctcct RW 5'-aagagggaaaccccagagaa PROBE 5'- gggcctcctgcagacatata
TLR-9	NM_017442	FW 5'-cagcagctctgcagtacgtc RW 5'-aaggccaggtaattgtcacg PROBES 5'- cacttcttccaaggcctgag
TNF-α	NM_000594	FW 5'-tccttcagacaccctcaacc RW 5'-aggccccagtttgaattctt PROBE 5'- gattcaggaatgtgtggcct
IL-1β	NM_000576	FW 5'-gggcctcaaggaaaagaatc RW 5'-ttctgcttgagaggtgctga PROBE 5'- aagcccactctacagctgga
IL-8	NM_000584	FW 5'-tagcaaaattgaggccaagg RW 5'-aaaccaaggcacagtggaac PROBE 5'- tctggcaaccctagtctgct
β-ACT1	NM_001101	FW 5'- ggacttcgagcaagagatgg RW 5'- agcactgtgttggcgtacag PROBE 5'- ctcttccagccttccttcct

RNA extraction and cDNA preparation

Gene expression of *TLR-1, TLR-2, TLR-4, TLR-5, TLR-9, TNFα, IL-1β* and *IL-8* were measured on the day of sepsis diagnosis and after 72 h or after 24, 48 and 72 h of hemofiltration treatment.

Total RNAs were extracted from 200 μl of blood sample using TRI REAGENT BD (Sigma), following the manufacturer's instructions and were treated with 2U of RNase-free DNAse (Promega, Charbonnières, France)

to ensure the absence of genomic DNA contamination. Total RNAs were quantified before cDNA synthesis by measuring adsorbance at A_{230} using a Biophotometer (Eppendorf) and their integrity was verified on denaturing agarose gel.

First strand cDNAs were synthesized from 250 ng of total RNA using AMV reverse transcriptase (Sigma Aldrich). Briefly, 250 ng of total RNA and 1 μL of 10 mM of random hexamer primers were added to each tube

and incubated at 70°C for 10 min to denature secondary structures. Then, reaction buffer, 1 μl of 10 mM dNTPs and 20 U of AMV were added to the mixture and the reaction was carried out for 50 min at 42°C. RNase H was added to the mixture after reaction to remove total RNA prior to real-time PCR assays.

Real-time PCR

Real-time PCR assays were performed in a 48-well plate with the MiniOpticon (Bio-rad). For each gene, reactions were performed in 25 μL of reaction mixture containing 5 μl of cDNA obtained from 250 ng of total RNA, 10 μl of TaqMan Master Mix (Sigma Aldrich), 300 nM of specific primers and 150 nM of probe reported in Table II. PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 20 sec, 57°C for 20 sec and 72°C for 20 sec. Expression levels for each gene were normalized with actin gene expression (b-ACT1) and presented as number of transcripts per 1,000 copies of control housekeeping gene. Three parallel experiments under identical conditions for each isolate were performed.

Statistical analysis

Data were expressed as mean values ± standard deviation. Statistical significance of differences was determined by the paired or unpaired two-tailed Student's t test. Differences were considered statistically significant for p<0.05.

RESULTS

Seventeen septic shock patients were included in this study. The mean age was 62.41 ± 19.62 yrs, SAPS III at ICU admission was 65.18 ± 10.79, SOFA at sepsis diagnosis was 9 ± 2.98. Fifteen (88%) patients died and two (12%) survived. Clinical characteristics of the study population are shown in Table I.
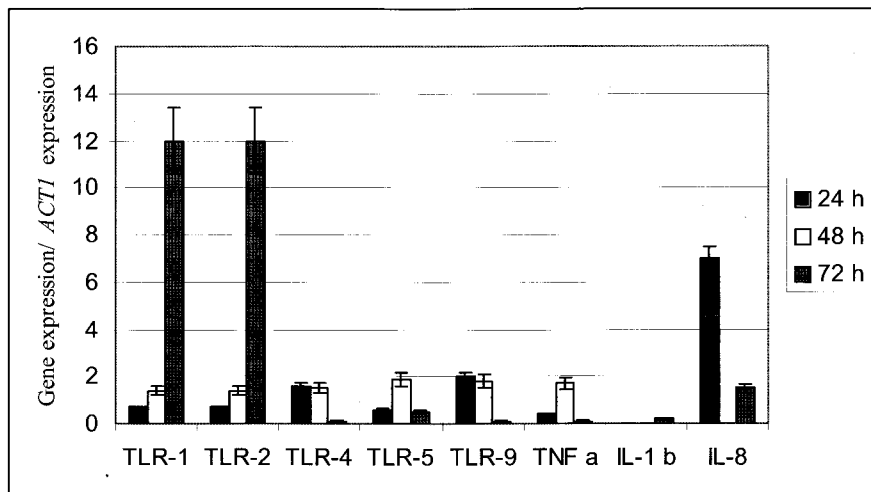
On sepsis diagnosis, gene expression of TLR-1, TLR-2, TLR-4, TLR-5, TLR-9, TNFα, IL1 β, IL8 was measured and resulted up-regulated in comparison with the housekeeping ACT1 gene (Fig. 1A).

After 72 hours in six of the seventeen (35%) patients there was a down-regulation of these genes, compared to day 1, but it was not associated with a reduction of cytokine serum levels or with improved clinical signs, better outcome or reduced mortality (Fig. 1B).

In those patients (five out of seventeen) who developed acute renal failure and required CVVH, gene expression of TLR-1, TLR-2, TLR-4, TLR-5, TLR-9, TNFα, IL1 β, IL8 was measured after 24, 48 and 72 h of hemofiltration treatment and we observed no down-regulation of gene expression after 24, 48 and 72 h of hemofiltration treatment compared to
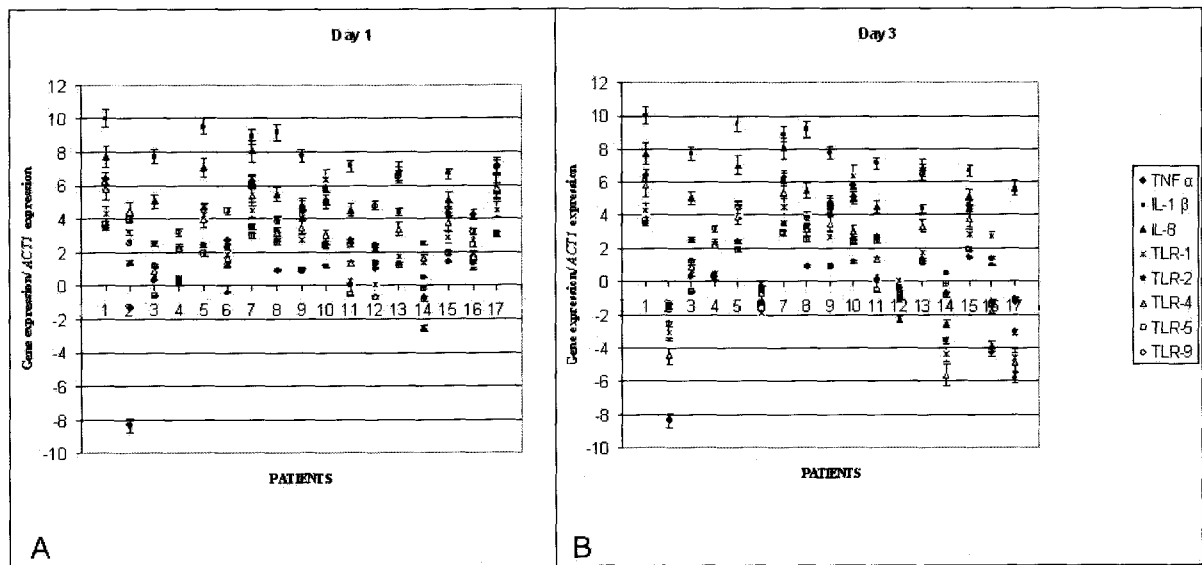
Fig. 1. Gene expression of TLR-1, TLR-2, TLR-4, TLR-5, TLR-9, TNFα, IL1 β and IL8 compared to the ACT1 housekeeping gene for 17 patients on day of sepsis diagnosis (Panel A) and after 72 h (Panel B). Experiments were performed in triplicate and error bars were reported for each value. On X-axis the 17 patients are reported. On Y-axis each gene expression values normalized with ACT1 gene expression are reported.

Fig. 2. *Gene expression of TLR-1, TLR-2, TLR-4, TLR-5, TLR-9, TNFα, IL1 β and IL8 after 24, 48 and 72 h of hemofiltration treatment compared to expression on day one of sepsis diagnosis. Experiments were performed in triplicate and error bars were reported for each value. On X-axis the eight genes analyzed are reported. On Y- gene expression values normalized with ACT1 gene expression axis are reported.*
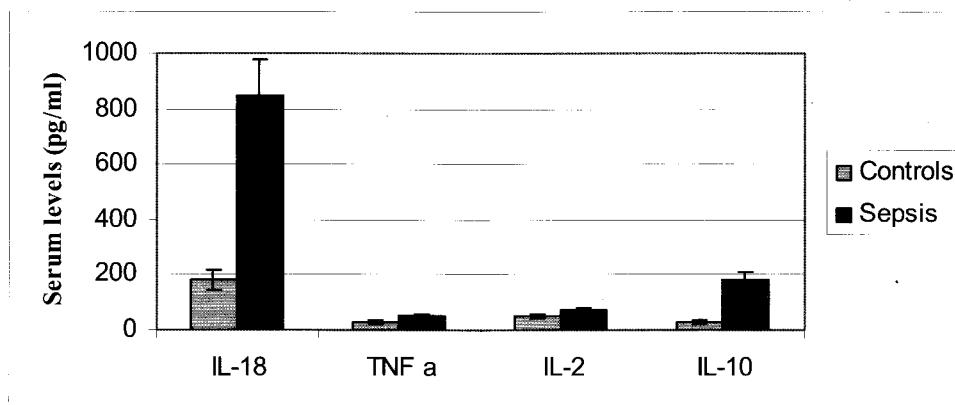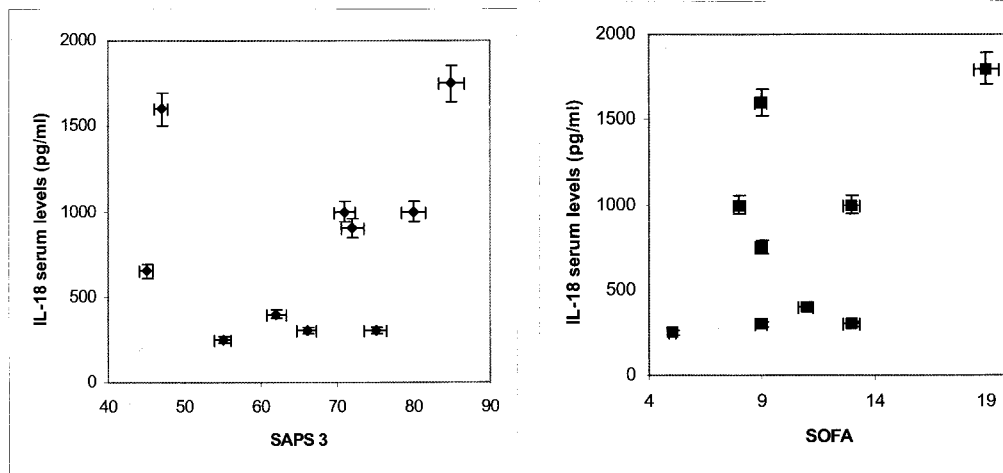

Fig. 3. *IL-18, TNFα, IL-2 and IL-10 serum levels in sepsis patients compared to serum levels of healthy volunteers. Experiments were performed in duplicate and error bars were reported for each value. Serum levels were significatively different between control and sepsis patients with P values of 0.02.*

day one, and no correlation between gene expression (Fig. 2) and cytokine serum levels was found.

At sepsis diagnosis, IL-18 serum levels were significantly higher in sepsis patients compared to healthy volunteers (862±588.8 pg/ml *vs* 182.8±35.59, p<0.02). IL-2, TNFα and IL-10 plasma levels were higher in septic patients than in healthy volunteers, but this difference was not significant (Fig. 3)

IL-18, IL-2, TNFα, IL-10 serum levels did not correlate with severity scores (SAPS 3 and SOFA) and no significant difference between survivors and non-survivors was found in the first three days of hospitalization, even if patients who survived had lower IL-18 serum levels on day 3 than those who died, p>0.05 (Fig. 4).

No correlation was found between cytokine

Fig. 4. *IL-18 serum levels in sepsis patients correlated with severity scores (SAPS 3 and SOFA). IL-18 measurements were performed in duplicate and error bars were reported for each value. Serum levels were not significatively different between patients who survived and those who died with P values > 0.05.*
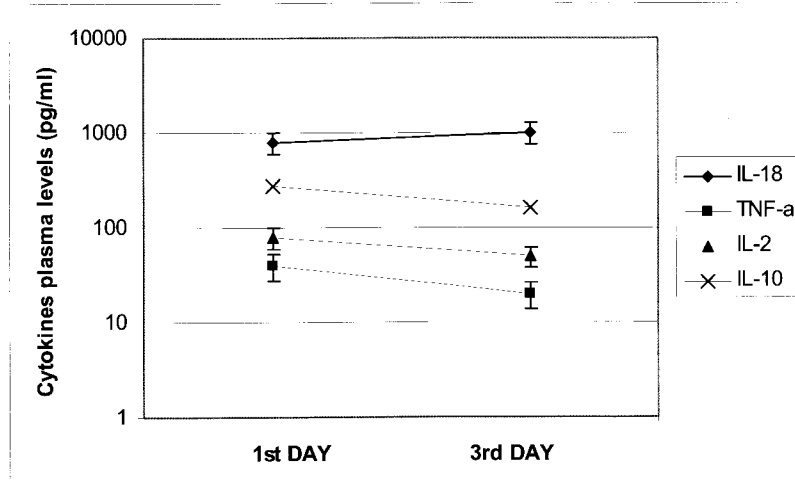
Fig. 5. *Il-18, TNF-alpha, IL-2 and IL-10 plasma concentrations checked on admission and after 72 h of hemofiltration. Measurements of cytokine levels were performed in duplicate and error bars were reported for each value. Serum levels were not significatively different between 1st day and 3rd day with P values > 0.05.*

serum levels and the type of isolated microorganism (Gram positive, Gram negative, fungi).

There were no differences in plasma cytokine concentrations between septic patients who did not develop acute renal failure and those who were treated by CVVH. In patients treated by CVVH, cytokine concentrations did not significantly decrease after 72 h of hemofiltration (Fig. 5).

DISCUSSION

The role of TLRs in the pathogenesis of sepsis is of great interest. TLRs recognize specific pathogen-associated molecular patterns and activate inflammatory responses with beneficial or dangerous effects on the host (9).

With these premises, it was reasonable to suppose

that in septic shock an increased expression of TLRs should be evident and a predominance of one class of TLRs over the others should be noted, in respect to the infectious agent involved.

In the present paper, no correlation was found between TLR expression and the type of isolated microorganism (Gram positive, Gram negative, fungi): we found an up-regulation of gene expression of all the TLR genes studied, TLR-1, TLR-2, TLR-4, TLR-5 and TLR-9.

The up-regulation of TLRs in critically ill patients might be related to several factors, not only to the microorganisms involved in sepsis, but also in the attempt of keeping organ functions alive. TLRs are indeed important in maintaining tissue homeostasis, preventing apoptosis and limiting the extent of damage to initial injury. A protective effect of TLRs in inflammatory diseases has been suggested. In a mouse model of intestinal injury, Fukata et al. (10) showed that the response to bacteria through TLRs is essential for the proper repair of the epithelium. In the colon, steady-state activation of TLRs by commensal bacteria is important for epithelial barrier function through the strengthening of tight junctions and induction of cytoprotective factors (11-13). In the colon, liver, and central nervous system, TLRs regulate the compensatory proliferation of parenchymal cells after injury (14-18).

As expected, cytokine serum levels were not correlated with the type of isolated microorganism: this in not surprising because TLR signaling results in activation of MAP kinases and NF-kB and release of inflammatory cytokines, irrespective of which TLR is activated (19).

To date the efficacy of CVVH, as routinely performed, is still controversial as a method of removing inflammatory mediators to improve septic patient outcome. In our series, after 72 h of high volume hemofiltration cytokine serum levels did not significantly decrease and no correlations between CVVH and TLR expression were found.

According to our data, the standard CVVH procedure does not have a substantial impact on cytokine plasma levels, perhaps due to the low cut-off point of the conventional hemofilter membrane.

Prophylactic hemofiltration in the absence of evidence for renal injury has been shown to be ineffective in trauma patients (20) and in patients with septic shock without renal dysfunction (21). Several studies have attempted to address the question of whether the choice of continuous renal replacement therapy (CRRT) affects patient outcome. However, observational and prospective studies (22, 23) comparing intermittent hemodialysis with CRRT, could not demonstrate an impact of CRRT on all-cause mortality or the recovery of renal function. Kellum et al. (24) performed a meta-analysis concluding that, after adjustment for study, quality and severity of illness, mortality was lower in patients treated with CRRT than in those treated by dialysis.

The lack of effect on cytokine serum levels prevented us from any consideration regarding the direct effect of CVVH on TRL expression and outcome improvement.

In our preliminary study, the limited number of patients does not allow us to obtain conclusive data, however there is space for one possible consideration: TLR over-expression, in septic shock patients, may be due to the dual function of TLRs: protection from infection and control of tissue homeostasis. Both functions depend on the recognition of pathogens and commensal microorganisms, respectively. This dual function may explain why TLR-induced gene products, such as inflammatory cytokines, are involved in both host and tissue repair responses.

Our future studies enrolling a higher number of patients, will concern the understanding of which of the two TLR functions evolves first and how TLR genetic polymorphisms influence patients' outcome, giving us new perspectives for the management of sepsis.

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