

Inflammation, Myocardial Dysfunction, and Mortality in Children With Septic Shock: An Observational Study

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Received: 24 June 2013 / Accepted: 14 September 2013 / Published online: 4 October 2013
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Abstract We aimed to investigate whether nuclear factor *kappa*-B activation, as evaluated by gene expression of its inhibitor (I- κ B α) and cytokine serum levels, was associated with myocardial dysfunction and mortality in children with septic shock. Twenty children with septic shock were prospectively enrolled and grouped according to ejection fraction (EF) <45 % (group 1) or EF \geq 45 % (group 2) on the first day after admission to the pediatric intensive care unit. No interventions were made. In the first day, patients from group 1 ($n = 6$) exhibited significantly greater tumor necrosis factor- α (TNF- α) and interleukin (IL)-10 plasma levels. However, I- κ B α gene expression was not different in both groups. Mortality and number of complications were significantly greater in group 1. Patients who died had greater plasma concentrations of TNF- α . In conclusion, TNF- α and IL-10 are involved in myocardial dysfunction accompanying septic shock in children, and TNF- α is associated with mortality.

Keywords Sepsis · Echocardiography · Nuclear factor *kappa*-B · Pediatric critical care

Septic shock is the leading cause of morbidity and mortality in intensive care units worldwide [25]. It is accompanied by myocardial dysfunction in 40–44 % of adults

and \leq 80 % of children [7, 29]. Myocardial dysfunction is associated with the severity of the systemic inflammatory response syndrome (SIRS) after infection and is the major cause of death in children with septic shock [7, 18]. Infections can trigger SIRS by activation of the nuclear factor *kappa*-B (NF- κ B), which is the main transcription factor that regulates the expression of genes encoding inflammatory mediators [12]. These mediators have been implicated in the genesis of sepsis-related myocardial dysfunction [18, 26, 27], which may contribute to the development of multiple-organ dysfunction syndrome [8, 25, 29]. In addition, greater NF- κ B activation has been observed in adults with septic shock who died [1, 4]. However, the association between NF- κ B activation, sepsis-related myocardial dysfunction, and mortality has not been investigated in children. Improved knowledge on the pathogenesis of organ dysfunction associated with sepsis may lead to novel therapeutic approaches. Thus, the objective of this study was to investigate whether NF- κ B activation, as evaluated by gene expression of its inhibitor, I- κ B α , and cytokine serum levels, was associated with myocardial dysfunction and mortality in children with septic shock.

Methods

Ethics Statement

This is a prospective, longitudinal, observational cohort study, approved by our Institutional Review Board, Research Ethics Committee of the Hospital das Clinicas, Ribeirao Preto Medical School, University of Sao Paulo (process no. 6060/2005). Written informed consent was obtained from the patients' parents or caregivers.

Some preliminary results were presented at the 6th World Congress on Pediatric Critical Care (2011, Sydney, Australia) in a poster.

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Patients

All patients admitted consecutively during an 18-month period to the pediatric intensive care unit (PICU) of Hospital das Clinicas, Ribeirao Preto Medical School, University of Sao Paulo, with a diagnosis of septic shock, according to internationally accepted criteria, were eligible for the study [11]. Exclusion criteria were as follows: pre-existing congenital heart disease or heart surgery, previous use of cardiotoxic or immunosuppressant drugs, pre-existing coronary artery disease, human immunodeficiency virus infection, newborns, and parental request. All patients were treated according to standardized protocols [6]. No interventions were made.

Data Collection

Clinical and demographic data and outcomes were collected from medical records. PRISM (pediatric risk of mortality) score was calculated within 12 h of PICU admission [28]. Total inotropic support was estimated by a modified inotropic score calculated as follows: doses of dopamine + dobutamine + milrinone $\times 10$ + epinephrine $\times 100$ + norepinephrine $\times 100$ [10]. Heart rate, respiratory rate, and systolic blood pressure were adjusted to age and sex. Echocardiography was performed daily starting within 24 h of PICU admission.

Blood Sampling

Arterial or venous blood samples were collected into ethylene diamine tetraacetic acid vacuum tubes within the first 24 h of PICU admission. Plasma was separated and stored at -70 °C. The pellet containing blood cells was lysed with a solution of ammonium bicarbonate plus ammonium chloride and centrifuged at 3,000 rpm for 15 min at 4 °C. The supernatant was discarded, and the remaining pellet of RNA, DNA, and protein was resuspended in phosphate-buffered solution and Trizol LS (Invitrogen, Carlsbad, CA), and stored at -70 °C.

Assessment of NF- κ B Activation

Because NF- κ B activation induces the production of its own inhibitor (I- κ B α), I- κ B α gene expression reflects NF- κ B activity within the nucleus [5]. Thus, NF- κ B activity was assessed by the relative gene expression of I- κ B α by real-time quantitative polymerase chain reaction (7500 Real Time PCR System; Applied Biosystems, Foster City, CA) using a Taqman prime for human I- κ B α (reference: Hs_00153283_m1) and GUS- β (Applied Biosystems) as

endogenous control. RNA was purified using the chloroform/isopropanol/ethanol method, quantified in a spectrophotometer (Eppendorf BioPhotometer Plus; Eppendorf AG, Hamburg, Germany) and had its quality assessed in 1.2 % agarose gel electrophoresis. Complementary DNA was synthesized with a commercially available kit (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems) according to manufacturer's instructions. I- κ B α relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method as previously described [21]. The calibrator was mRNA from seven healthy volunteers.

Because NF- κ B is the main transcription factor that regulates the expression of genes encoding cytokines, its activity was also evaluated by plasma concentrations of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-10 measured by enzyme-linked immunosorbent assay (ELISA) (DuoSet Elisa Development Systems; R&D Systems, Minneapolis, MN). Values lower than the lower limit of detection were arbitrarily considered as equal to 0.01 pg mL $^{-1}$ for analysis.

Assessment of Myocardial Function

Myocardial function was assessed by biochemical and echocardiographic parameters. Patients were divided into two groups according to ejection fraction (EF) <45 % (group 1 [moderately or severely decreased LV function]) or EF ≥ 45 % (group 2 [preserved or mildly decreased LV function]) within 24 h after PICU admission according to American Echocardiographic Society guidelines [19]. All patients underwent transthoracic bidimensional echocardiography evaluation (Phillips SONOS 5500 [Phillips Electronics North America, Andover, MA] or HP Sonos 2500 [Hewlett-Packard, Palo Alto, CA]), which was performed daily, starting within 24 h of PICU admission, by one of two echocardiographers with at least 10 years of experience. Left ventricle (LV) status was assessed by ventricle volumes and Teicholz EF. End-systolic and -diastolic LV volumes were estimated by long- and short-axis measurements. Stroke volume was calculated as the difference between end-systolic and -diastolic LV volumes. Cardiac output was estimated by multiplying the calculated stroke volume by the heart rate. Cardiac index (CI) was obtained by dividing cardiac output by body surface area (BSA). Systolic index (SI) was obtained by dividing stroke volume by BSA. End-diastolic LV volume (EDLVV) was also adjusted to BSA.

B-type natriuretic peptide (BNP) was measured in plasma samples using a semiautomated ELISA kit (Triage BNP; Biosite, San Diego, CA), and cardiac troponin I (cTnI) plasma concentration was measured using a

semiautomated sandwich immunoassay with fluorescent detection (miniVIDAS; bioMérieux Vitek, Marcy l’Etoile, France).

Statistical Analysis

Results were expressed as median (total range) or counts (percentage). Comparisons between groups were made using Mann–Whitney U test or Student *t* test for continuous variables, according to data distribution, and Fisher’s exact test for categorical variables. Correlations between continuous variables were assessed by linear regression models with analysis of residuals. For all tests, logarithmic transformation was applied to IL-6, IL-10, TNF- α , and leukocyte count to normalize data distribution. Because corticosteroid use could be a potential confounder and our sample size would not allow a multivariate analysis, we performed *post hoc* tests comparing inflammatory markers between patients who did and did not receive corticosteroids before enrollment in the study. Statistical software packages Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA) and SPSS 16 (SPSS, Chicago, IL) were used. A *p*-value <0.05 was considered significant.

Results

Demographic and Epidemiologic Data

Twenty patients with septic shock were enrolled from May 2007 to November 2009. They were studied for a median of 5.5 (range 2–15) days. 6 patients (30 %) had EF <45 % (group 1), and 14 patients (70 %) had EF \geq 45 % (group 2). Demographic and epidemiologic data are listed in Table 1. There were no differences regarding sex, age, body weight, time between first symptoms and PICU admission, or PRISM score between groups. The main diagnoses and the

Table 1 Demographic and epidemiologic data of children with septic shock according to LVEF on the first day of ICU admission

Demographic and epidemiologic data	Group 1 (EF <45 % [n = 6])	Group 2 (EF \geq 45 % [n = 14])	<i>p</i>
Male sex (%)	3 (50)	8 (57)	1.00
Age (months)	45 (9.7–175)	33 (2.3–215)	0.56
Body weight (kg)	17.2 (6.0–40)	10.8 (2.2–113)	0.71
Days from first symptom to PICU admission	2 (1–9)	4 (1–6)	0.38
PRISM score	12 (6–20)	11.5 (0–25)	0.90

Values shown are number (proportion) or median (range)
 EF ejection fraction (Teicholz), PICU pediatric intensive care unit, PRISM pediatric risk of mortality, LVEF left ventricle ejection fraction

most frequently isolated etiologic agents are listed in Table 2.

Echocardiographic and Hemodynamic Parameters

There was no difference between groups in median ED-LVV (45 [range 33–58] mL.m⁻² in group 1 vs. 59 [range 17–93] mL.m⁻² in group 2, *p* = 0.34), median CI (2.9 [range 1.5–4.5] mL.min⁻¹.m⁻² in group 1 vs. 4.3 [range 0.8–5.9] mL.min⁻¹.m⁻² in group 2, *p* = 0.46), or median SI (18.9 [range 7.4–28.1] mL.m⁻² in group 1 vs. 29.8 [range 8.3–45.1] mL.m⁻² in group 2, *p* = 0.15) (Fig. 1) on the first day of PICU admission. Echocardiograms were performed for a median of 4.5 (range 1–13) days in all patients. Among the patients from group 1, EF increased to >45 % after a median of 5 (range 3–7) days of PICU admission. Median arterial lactate on the first day after PICU admission was significantly greater in patients from group 1 (3.9 [range 1.5–28] mmol.L⁻¹ in group 1 vs. 1.5 [range 0.9–6.3] mmol.L⁻¹ in group 2, *p* = 0.02). However, there were no differences in inotropic score, amount of fluids received, central venous pressure, heart rate, respiratory rate, systolic blood pressure, urine output, or serum creatinine between groups (data not shown).

Table 2 Main diagnoses and etiologic agents of children with septic shock according to LVEF

Diagnoses and etiologic agents	Group 1 (EF = 45 % [n = 6])	Group 2 (EF \geq 45 % [n = 14])
Main diagnoses		
Pneumonia	4	9
Meningoencephalitis	1	1
Toxic shock syndrome	1	2
Infectious diarrhea	1	1
Skin and subcutaneous tissue infections	0	2
Central venous catheter-related infection	0	1
Main etiologic agents		
<i>Staphylococcus aureus</i>	1	3
<i>Pseudomonas aeruginosa</i>	0	3
<i>Streptococcus pneumoniae</i>	1	2
<i>Acinetobacter baumannii</i>	0	2
<i>Candida albicans</i>	0	2
<i>Haemophilus influenzae</i>	0	1
<i>Enterococcus faecalis</i>	0	1
<i>Klebsiella pneumoniae</i>	0	1
<i>Enterobacter cloacae</i>	1	0
<i>Neisseria meningitidis</i>	0	1

Values shown are counts
 EF ejection fraction (Teicholz), LVEF left ventricle ejection fraction

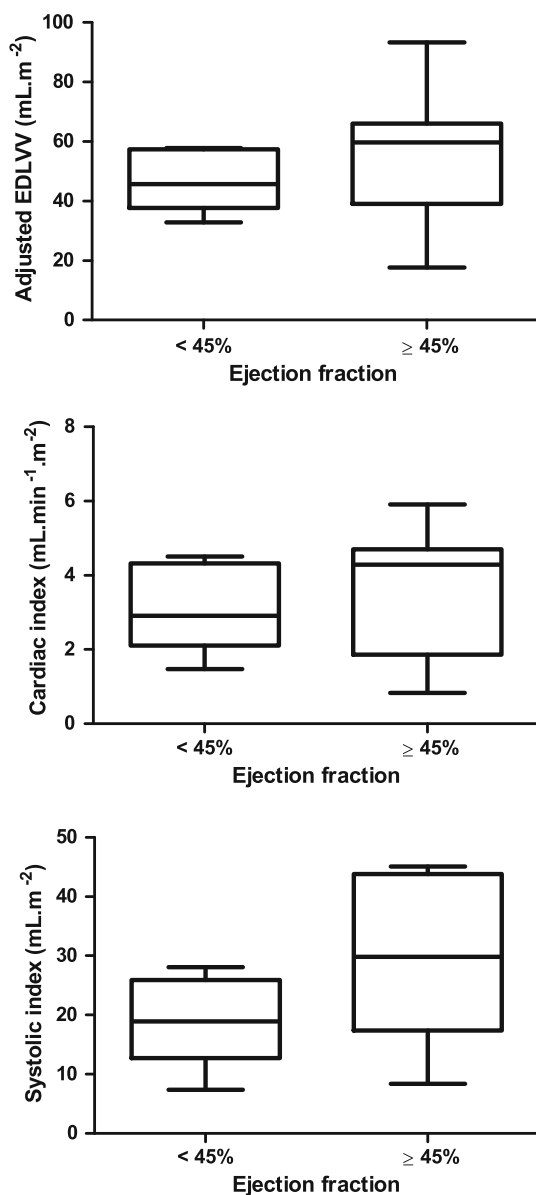


Fig. 1 Echocardiographic variables according to EF on first admission day ($n = 18$): Adjusted EDLVV ($p = 0.34$), CI ($p = 0.46$), and SI ($p = 0.015$). The central line is the median

Cardiac Markers

Within the first 24 h after PICU admission, median cTnI plasma levels were 0.01 (range 0.01–2.6) ng.mL^{-1} in group 1 and 0.01 (range 0.01–4.5) ng.mL^{-1} in group 2 ($p = 0.82$), and median BNP plasma levels were 308 (range 31–597) pg.mL^{-1} in group 1 and 200 (range 5–1,680) pg.mL^{-1} in group 2 ($p = 0.90$).

Inflammatory Markers

Median TNF- α (52.9 [range 12.6–314] vs. 8.0 [range 0.01–76] pg.mL^{-1} , $p = 0.01$) and IL-10 (4.9 [range

1.9–298] vs. 4.5 [range 2.2–9.3] pg.mL^{-1} , $p = 0.04$) plasma levels were significantly greater in patients from group 1 within the first 24 h after PICU admission. However, no differences were observed between groups in IL-6 plasma levels (1,071 [range 0.7–3,216] vs. 9.3 [range 0.5–6,417] pg.mL^{-1} , $p = 0.15$) or I- $\kappa\text{B}\alpha$ mRNA expression (4.2 [range 0.5–11.2] vs. 2.2 [range 0.2–15.3], $p = 0.56$) (Fig. 2).

A significant positive correlation was observed between I- $\kappa\text{B}\alpha$ mRNA relative expression and leucocyte count ($r^2 = 0.25$, $p = 0.02$) and polymorphonuclear cell count ($r^2 = 0.40$, $p = 0.003$) (Fig. 3). Nevertheless, there was no association between I- $\kappa\text{B}\alpha$ mRNA relative expression and IL-6, IL-10, and TNF- α plasma levels or between mononuclear cell and platelet counts. I- $\kappa\text{B}\alpha$ mRNA relative expression was also not significantly associated with time between disease onset and admission to the PICU.

Post Hoc Analysis: Corticosteroid Use

On the first day of PICU admission, all patients from group 1 (100 %) and 8 patients from group 2 (57 %) had already received hydrocortisone for catecholamine-refractory shock before inclusion in the study. Interestingly, patients who received corticosteroids before entry in the study had greater TNF- α (38.0 [range 0–314.4] vs. 8.7 [range 0–49.8] pg.mL^{-1} , $p = 0.03$), IL-6 (609.0 [range 0.8–6416.8] vs. 1.3 [range 0.5–946.6] pg.mL^{-1} , $p = 0.01$), and IL-10 (15.9 [range 2.2–297.9] vs. 4.1 [range 1.9–5.0] pg.mL^{-1} , $p = 0.02$) plasma levels compared with those who did not receive corticosteroids on the first day after PICU admission. However, no differences in I- $\kappa\text{B}\alpha$ mRNA expression were found between them (4.0 [range 0.1–11.2] vs. 3.0 [range 0.6–15.3], $p = 0.63$).

Outcomes

Complications occurred more frequently in group 1 (4 [range 3–7] vs. 2 [range 0–5] complications/patient, $p = 0.03$) (Table 3). The main sites of secondary infections in all patients were lung ($n = 3$), mediastinum ($n = 1$), digestive tract ($n = 1$), and subcutaneous tissue ($n = 2$). Overall mortality was 30 %; it was significantly greater in group 1 (66 vs. 14 %, $p = 0.03$). All deaths were caused by multiple organ failure. Patients who died had greater plasma concentrations of TNF- α (56.1 [range 10.8–314.4] vs. 8.8 [0–76.2] pg.mL^{-1} , $p = 0.01$). However, there were no significant differences between survivors and nonsurvivors in plasma concentrations of IL-6 (1196.3 [range 1.5–3216.2] vs. 11.4 [range 0.5–6416.8] pg.mL^{-1} , $p = 0.07$), plasma concentrations of IL-10 (43.0 [range 2.2 to 297.9] vs. 4.5 [range 1.9 to 129.7] pg.mL^{-1} , $p = 0.29$), and mRNA expression of

Fig. 2 Plasma levels of TNF- α ($p = 0.04$), IL-6 ($p = 0.15$), and IL-10 ($p = 0.04$) and I- κ B α mRNA relative quantification ($p = 0.56$) according to EF. The central line is the median

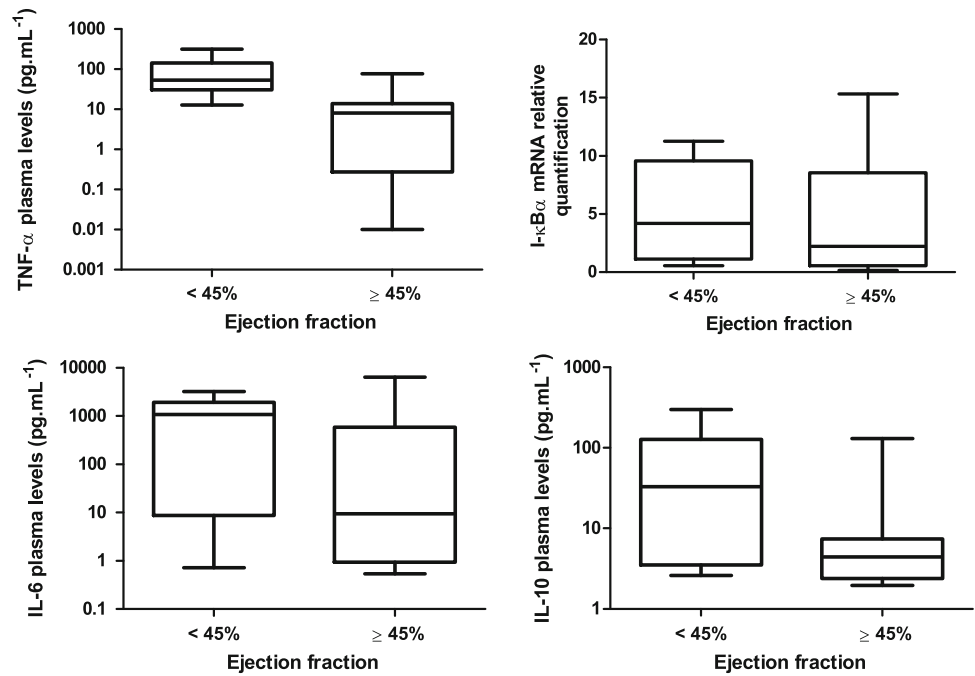
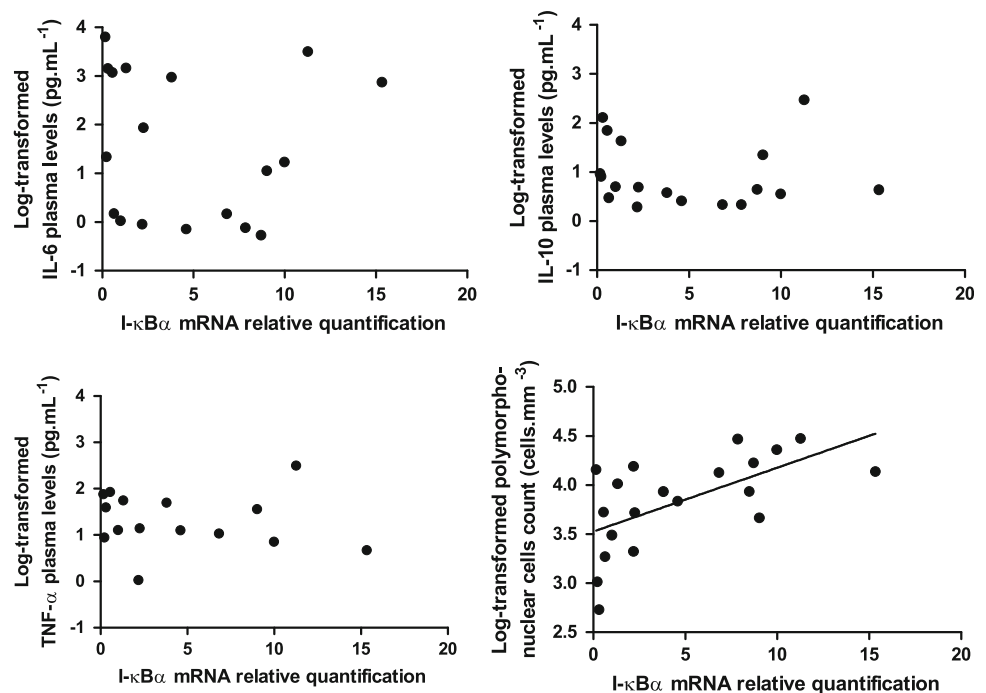


Fig. 3 Correlations between I- κ B α mRNA relative quantification and log-transformed plasma levels of IL-6, IL-10, and TNF- α (all nonsignificant) as well as polymorphonuclear cell count ($r^2 = 0.40$, $p = 0.003$)



I- κ B α (3.0 [range 0.5–11.2] vs. 3.4 [range 0.1–15.3], $p = 1$).

Discussion

We have shown that children with moderate-to-severe myocardial dysfunction (EF <45 %) associated with septic shock had greater plasma levels of TNF- α and IL-10, more

complications, and greater mortality compared with those with preserved or mildly decreased LV function (EF \geq 45 %). Moreover, patients who died had greater plasma concentrations of TNF- α . However, there was no significant difference in I- κ B α mRNA expression between patients with and without moderate-to-severe myocardial dysfunction or between survivors and nonsurvivors. In addition, plasma levels of cytokines did not correlate with I- κ B α mRNA expression.

Table 3 Complications of children with septic shock according to LVEF on the first day of ICU admission

Complication	Group 1 (EF <45 % [n = 6])	Group 2 (EF ≥45 % [n = 14])
Acute kidney failure	5 (83)	5 (35)
ARDS	4 (66)	4 (28)
Steroid use for refractory shock	6 (100)	8 (57)
Seizures	2 (33)	3 (21)
Intracranial hemorrhage or ischemia	2 (33)	0
Intravascular disseminated coagulation	6 (100)	5 (35)
Secondary infection	2 (33)	5 (35)
Other complications ^a	1 (16)	3 (21)
Death	4 (66)	2 (14)

Values shown are number (percentage)

EF ejection fraction (Teicholz), ARDS acute respiratory distress syndrome

^a Lung abscess, diabetes insipidus, pulmonary hypertension, and necrosis of extremities

Although the study groups had different EF, there were no significant differences regarding other echocardiographic parameters of myocardial performance or cardiac biochemical markers, such as cTnI and BNP plasma levels, probably because the patients were already on inotropic support at the time of assessment. Nevertheless, arterial lactate levels were significantly greater in patients from group 1. In accordance with adult data [24], myocardial dysfunction was fully reversible in survivors with normalization of EF after 3–7 days of initiation of treatment.

Conflicting results have been reported regarding the serum cytokine profile in patients with septic shock. It has been showed that TNF- α and IL-1 β were responsible for the depression of myocardial cell contractility in vitro induced by human septic shock serum [18]. In contrast, serum from septic shock adults that induced a decrease of cardiomyocyte contractility in vitro had high levels of IL-6, IL-8 and IL-10; however, the concentrations of TNF- α and IL-1 β were not increased [16]. IL-6 isolated from serum obtained from children with meningococcal septicemia impaired in vitro myocardial contractility [26]. TNF- α added to IL-6 had negative inotropic activity in vitro after prolonged exposure (>48 h) [27]. Our data add to the evidence that TNF- α and IL-10 are involved in myocardial contractile dysfunction associated with septic shock. Moreover, we observed greater levels of TNF- α , IL-6, and IL-10 in patients with catecholamine-refractory shock, and there was an association between TNF- α levels and mortality. In children with septic shock, serum levels of TNF and IL-10 measured at admission to the PICU were related to the severity of organ failure and mortality [13]. Taken

together, these findings suggest that inflammation is associated with disease severity and outcome of children with septic shock. Interestingly, experimental studies have showed that in addition to the systemic release of cytokines, cardiomyocytes can also generate cytokines locally in the heart in response to Gram-positive sepsis and lipopolysaccharide administration, which could aggravate sepsis-induced myocardial dysfunction [14, 17, 20]. However, evaluation of cytokine production by cardiomyocytes is not clinically feasible.

Increased NF- κ B activation, assessed by electrophoretic mobility shift assay (EMSA), has been reported in adults with septic shock who died [1, 4]. In children with sepsis, flow cytometric analysis showed a greater percentage of monocytes/macrophages and polymorphonuclear cells exhibiting NF- κ B activity compared with controls. Moreover, a positive correlation has been observed between the percentage of cells exhibiting NF- κ B activity and serum levels of IL-6 and IL-10 [15]. In contrast, NF- κ B activity, as evaluated by gene expression of its inhibitor, I- κ B α , was not associated with myocardial dysfunction or mortality in our study. In theory, I- κ B α mRNA quantification would be a more accurate marker of the transcriptional strength of NF- κ B [5]. EMSA, which visualizes the binding of NF- κ B to synthetic oligonucleotides, does not necessarily reflect the transcriptional power of NF- κ B because it can be modified after NF- κ B DNA binding by phosphorylation or interaction with cofactors [5]. However, several substances can influence I- κ B α mRNA levels, such as corticosteroids, which lead to increased transcription of the gene for I- κ B α [2]. Because most patients (14 of 20) had received corticosteroids before entry in the study, this may have altered I- κ B α gene expression.

Although studies on anti-inflammatory agents for sepsis have shown protective effects in animal models [3, 9, 31, 32], clinical trials have found inconsistent results [30]. Nevertheless, the efficacy of anti-inflammatory agents in sepsis appears to be related to sepsis-associated risk of death [23]. Clinical trials have been performed with the use of a polyclonal antibody against TNF, and preliminary results suggest that it may be beneficial in patients with severe sepsis and shock or evolving organ failure [22, 23]. This could be a therapeutic option for children with severe myocardial dysfunction at high risk of death. Further studies are needed to elucidate this option.

As a limitation, the correlation between I- κ B α mRNA expression and NF- κ B protein could not be studied. Nevertheless, quantifying the NF- κ B protein would not have provided information on the degree of NF- κ B activity within the nucleus. In addition, our limited sample size does not allow us to draw any definite conclusion, and larger studies are urgently needed.

Conclusion

In conclusion, TNF- α and IL-10 are involved in myocardial dysfunction and TNF- α is associated with mortality in children with septic shock. Anti-inflammatory agents, such as TNF inhibitors, may be a therapeutic option for these patients.

Acknowledgments Funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processes 2004/01441-1 and 2008/05067-8.

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