ORIGINAL ARTICLE

Immune Status Assessment using mRNA gene Expression of New Biomarkers in Children with Sepsis and Septic Shock: Role of G-CSF in Improving the Outcome

[1]Sally A.F. EL-Sahrigy, [2]Tarek A. Abdel Gawad, [1]Azza M.O. Abdel Rahman, [2]Sondos Magdy, [1]Eman A. Mostafa, [1]Rasha M. Hasanin, [1]Ashraf Galal, [1]Hanan M. Hamed, [3]Eman M. Hassan, [3]Amany H. Abdelrahman, [3]Mirhane Hassan

[1]Pediatrics Department, Medical Research. and Clinical Studies Institute, National Research Centre, Cairo, Egypt
[2]Pediatrics Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[3]Clinical and Chemical Pathology Department, Medical Research. and Clinical Studies Institute, National Research Centre, Cairo, Egypt

Key words:
BID1; PD-L1; BCL-2; sepsis; G-CSF

Corresponding Author:
Mirhane Hassan
Clinical and Chemical Pathology Department, Medical Research and Clinical Studies Institute, National Research Centre, Cairo, Egypt.
33 ElBuhouth St, Dokki, Cairo Governorate, Egypt
Postal code 12622
mirhanehassan@gmail.com
Orcid ID 0000-0001-6193-9415

ABSTRACT

Background: *Sepsis is a life- threatening organ dysfunction caused by impaired host response to infection.* ***Objectives:*** *To assess the immune status in children with sepsis and septic shock using mRNA gene expression of 7 biomarkers; Interleukin (IL)-1ß, Tumor necrosis factor (TNF), IL-10, Programmed death protein (PD-1) and Programmed death ligand 1(PD-L1), BID (inhibitory check point), and BCL2 (anti-apoptotic). Secondly, to study the possible influence of G-CSF on the expression of these biomarkers and outcome in septic children.* ***Methodology:*** *This is a case controlled prospective randomized biomarker study including 65 subjects divided into 3 groups. Group 1 comprised 24 septic patients receiving G-CSF in addition to conventional treatment for sepsis. Group 2 included 17 septic patients on conventional treatment. Group 3 control group comprised 24 infants and children matched for age and sex. Two blood samples were withdrawn from each patient at day 1 and day 8. RT-PCR analysis for mRNA gene expression of the studied biomarkers were performed.* ***Results:*** *Group 1 showed significant increase of PD-L1 on day 8 compared to day 1 (p= 0.019) and significant increase of BID1 on day 8 (p= 0.014) and on day 1 (p= 0.08) compared to healthy children. In group 2, IL-10 showed significant decrease on day 8 compared to day 1(p= 0.02). BID1 showed highly significant increase between both days 1 and 8 versus healthy children (p = 0.001, 0.004 respectively). BCL-2 showed significant decrease in serum levels between both days 1 and 8 versus healthy children (p = 0.04 ,0.012 respectively).* ***Conclusion:*** *Upregulation of the apoptotic BID1 and PD-L1 expression with downregulation of IL-10 and BcL2 expression in our septic patients suggests a feature of sepsis-related immunosuppression. G-CSF had no role on the studied biomarkers release and outcome.*

INTRODUCTION

A recent definition of sepsis is dysregulated immunological response to infection associated with some degree of organ dysfunction [1]. Sepsis is the most frequent cause of mortality in intensive care units (ICUs), ranging from 20% for sepsis to 50% for septic shock [2]. In Egypt, mortality rate has reached 42.7% for sepsis [3].

Sepsis initiates a complex immunologic response that varies over time. After a short pro- inflammatory phase, septic patients enter a stage of protracted immune- suppression sometimes called "immune paralysis" [4]. A common feature of sepsis -related immunosuppression is impaired lymphocyte function, and increased release of the inhibitory check-point molecules such as programmed death protein (PD-1) and its ligands [5]. It is manifested clinically by cutaneous anergy, hypothermia, leucopenia, and susceptibility to infection [6].

A prolonged immunosuppression state often replaces the early proinflammatory state of sepsis. As a result of apoptosis, the number of T cells (helper and suppressor) decreases together with reduced response to inflammatory cytokines [7].

Programmed death-1 (PD-1) is a newly defined co-inhibitory receptor primarily present on cell surface of activated CD4 and CD8 T cells, natural killer NK cells, B lymphocytes, macrophages, dendritic cells DC, and monocytes, as well as in hemopoietic and non-

hemopoietic cells. PD-1 plays two opposite roles; it negatively regulates lymphocyte activation and function, thus maintaining the immune tolerance and reducing the harmful immune response. On the other hand, it interferes with the protective immune responses and causes malignant cells development [8].

The inhibitory effect of PD-1 is mediated by its two main ligands; PD-L1 and PD-L2. The former is broadly expressed on hematopoietic cells, including T cells, B cells, dendritic cells, macrophages, endothelial cells, epithelial cells, pancreatic islet cells and fibroblastic reticular cells [9]. Recently increased sPD-1/ sPD-L1 levels may indicate immune dysfunction in patients with severe sepsis and septic shock [10].

In mammalian cells, there are two apoptotic pathways: extrinsic (cell death receptor) and intrinsic (mitochondrial) pathways. The first pathway involves the transfer of death signals through receptors that are located on the cell surface and is mediated by caspase 8 [11]. The intrinsic mitochondrial pathway is regulated by B-cell CCL/lymphoma 2 (Bcl-2) family. This family includes anti-apoptotic and pro-apoptotic proteins. Bcl-2 is an anti-apoptotic protein that provides protection through decreasing the number of lymphocyte apoptosis which in turn is downregulated in sepsis by the induction of the pro-apoptotic member of BcL-2 family; BID1, Bim and Bak which induce mitochondrial outer membrane permeability and the release of apoptogenic factors which leads to caspase 9 activation [12]. Bid is thought to be the link between the extrinsic and the intrinsic pathways of sepsis induced apoptosis [11].

Besides B and T lymphocytes programmed cell death, many other types of immune cells including neutrophils, macrophages are also susceptible to cell death in sepsis. Neutrophils either phagocytose bacteria or form neutrophil extracellular traps (NET's) for clearance of invading organisms [13].

Granulocyte - colony stimulating factor (G-CSF) had shown promise in the treatment of non- neutropenic hosts in animal models [14]. Several clinical trials have been conducted to investigate the effect of G-CSF treatment in neonates and adults with controversial results [2].

In the present study we aimed to assess the immune status in children with sepsis and septic shock using mRNA gene expression of 7 biomarkers; Interleukin (IL)-1ß, Tumor necrosis factor TNF (pro-inflammatory cytokines), IL-10 (anti-inflammatory cytokines), Programmed death protein (PD-1) and Programmed death ligand 1(PD-L1), BID, (check-point inhibitor molecules) and BcL-2 (anti-apoptotic). Secondly, to study the possible influence of G-CSF on the expression of these biomarkers and outcome in septic children.

METHODOLOGY

Study subjects:

Forty-one infants and children suffering from sepsis and septic shock were recruited from Pediatric Intensive Care Unit (PICU), Ain Shams University Hospital. Septic infants and children met two or more of the following criteria; temperature >38°c or <36°c, heart rate >90/minute, respiratory rate >20/min, Paco2 <32mmHg, and white blood cell count >12000/mm2 or<4000/ mm2 or >10% immature bands [15]. According to American Medical Association, 2016, sepsis is defined as life- threatening organ dysfunction caused by dysregulated host response to infection. Septic shock is defined as sepsis with persisting hypotension requiring vasopressors to maintain mean arterial pressure (MAP) ≥ the mean for his age and having serum lactate level>2mmol/L (18mg/dl) despite adequate volume resuscitation [16].

Disease severity was assessed using SOFA score (Sequential Organ Failure Assessment). Organ dysfunction can be identified as an acute change in the total SOFA score ≥ 2 points consequent to infection [17] as reported by Vincent et al[18]

Exclusion Criteria:

Age less than 28 days or more than 16 years. Duration of stay in ICU less than 24 hours, end- stage renal disease with chronic dialysis therapy, end- stage liver disease with evidence of portal hypertension, congenital immunodeficiency.

Studied subjects were divided into:

- **Group 1**: included 24 infants and children suffering from sepsis and septic shock. Their age ranged from 2 months to 8 years. They were 11 females and 13 males. All received rh G-CSF vial (Granulocyte) (300µg/ml) in a dose of 5µg /kg/day subcutaneous injection given as an adjuvant to the conventional treatment protocol [19].
- **Group 2**: included 17 infants and children suffering from sepsis and septic shock. Their age ranged from 2 months to 9 years. They were 9 females and 8 males. They were given only the conventional therapy for sepsis as decided by the PICU doctors.
- **Group 3**: included 24 infants and children age and sex matched collected from outpatient Pediatric clinic, Medical Research Centre of Excellence, NRC.

The study protocol was approved by the ethical committees of the National Research Centre (NRC). Ethical approval number (16317). All infants and children were included in the study after a written consent from their parents or caregivers.

Blood sampling:

5 mL blood were withdrawn under complete aseptic technique from patients and controls, divided into 2 EDTA and one plain vacutainers; for assessment of CBC, chemical parameters and for PCR analysis. Also, heparinized samples were taken for assessment of blood gases.

All the studied cases were subjected to full history taking and complete clinical examination with special emphasis on the clinical signs of sepsis.

Routine laboratory investigations:

Complete blood picture and differential count, Blood gases: Po2, Pco2, Pao2/FIO2. Liver function tests: albumin, SGPT, SGOT. Kidney function tests: blood urea and creatinine. Serum glucose levels. ESR and C-reactive protein. Chest X-ray (postro-anterior and lateral). Blood, sputum, and urine cultures.

Quantification of the mRNA of 7 genes in patients' whole blood by Quantitative Real Time (qPCR)using Rotor Gene Q.

Quantification of the mRNA:

Seven genes involved in the host response to sepsis were chosen as follows:

1. IL-1ß and TNF (pro-inflammatory cytokines).
2. IL-10 (Anti- inflammatory cytokine).
3. PD1 and PD-L1, BID, BcL-2 (Lymphocyte Apoptosis).

Procedure:

Whole blood samples (2ml) were collected in tubes containing EDTA. (For optimal results blood samples were processed within few hours of collection).

Total RNA was extracted from whole blood by QIA amp RNA blood Mini kit according to the manufacturer instruction (Qiagen). RNA quantification and purity was assessed with the Nano drop spectrophotometer.

Reverse transcription into cDNA was done using Quanti- Test Reverse Transcription kit (Qiagen).

mRNA expression assay:

cDNA prepared in a reverse transcription reaction serves as the template for real time PCR analysis using a Quantitect Primer Assay in combination with Quanti Tect SYBER Green PCR Master Mix. QuantiTect Primer Assay consists of a forward and Reverse primer targeting the mRNA of interest (table 1).

Table 1: Gene globe ID of Primer assay used in mRNA gene expression

GeneGlobe ID	Gene name / symbol
QT00021385	IL1B
QT01079561	TNF
QT00041685	IL10
QT00025011	BCL2
QT00077833	BID
QT01005746	PD-1
QT00089761	PD-L1
QT00079247	GAPDH

Quantitative Real time PCR

Initial activation step (DNA polymerase is activated) 15 min at 95° c, then 3 step cycling (Cycle number 40 cycles), followed by denaturation for 15 secs at 94° c, annealing for 30 secs at 55° c, and extension for 30 secs at 70° c.

GAPDH was used as housekeeping gene (reference mRNA) to normalize the expression of mRNAs and calculate the ΔCT value. The relative fold gene expression level was determined using $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis:

Analysis of data was performed using the statistical package SPSS version 15 for Windows (SPSS Inc., Chicago Illinois, USA). Our data was not normally distributed (tested by Kolmogorov-Smirnov and Shapiro-Wilk tests for normal distribution), so they are presented as median and interquartile range (IQR 25th–75th) for quantitative variables. Kruskal Wallis test was performed for comparison between 3 groups with quantitative data followed by Mann-Whitney test when there was significance. The Bivariate Spearman correlation (r) was used to compute the pair-wise associations for a set of variables. P values < 0.05 were considered statistically significant.

RESULTS

All laboratory data of groups1 and 2 patients are presented in table 2 as median and interquartile range (25^{th}- 75^{th} percentile).

Table 2: Laboratory data of Group (1) (n = 24) and Group (2) (n = 17) of PICU patients

Variable	Median (25th-75th IQR) Group (1)	Median (25th-75th IQR) Group (2)
Hemoglobin (Hb) gm/dl	9.75(8.52-10.52)	9.8(10.6 – 11.35)
Total leucocytic count (TLC) 10^3/ul	8.05(5.52-12.07)	7.45(8.9 – 11.4)
Neutrophils	4.9(3.04-6.97)	4.2(5.9 – 7.5)
Lymphocytes	2.35(1.2-3.7)	1.65(2.0 – 3.1)
Platelets (PLT) 10^3 /ul	110.5(54.3-322.5)	41.0(92.0 – 392.0)
C-reactive protein mg/L	12(12-48)	12(48.0 – 96.0)
Blood pH	7.36(7.29-7.4)	7.29(7.35 – 7.4)
p CO2 mmHg	49.75(39.5-61.5)	40.0(48.0 – 66.5)
pO2 mmHg	111(88.5-150)	80.0(96.0 - 140)
Bicarbonate mg/L	23(21-28)	20.15(23.0 – 28.0)
SO2 mmHg	81(74-87)	80.0(88.0 – 90.0
Biochemical indices		
Sodium (Na) meq/L	137.5(124.25-140.75)	133.0(136.0 – 141.5)
Potassium (K) meq/L	4.1(3.6-4.5)	3.1(3.6 – 3.8)
Calcium (Ca) meq/L	8.25(7.8-8.8)	8.35(8.7 – 9.3)
Kidney Function tests		
Urea mg/dl	14(20,5-21.5)	10.0(12.0 – 16.0)
Creatinine mg/dl	0.4(0.3-0.5)	0.3(0.3 – 0.6)
Liver Function tests		
Alanine transaminase(ALT) units/L	14.25(26-57)	9.5(20.0 – 36.0)
Aspartate transaminase (AST) units/L	27(20-43.75)	16.5(20.0 – 31.0)
Albumin gm/dl	2.9(2.6-3)	2.7(3.4 – 3.8)

Levels of studied markers are presented in table 3 as median and interquartile range (25^{th} – 75^{th} percentile). Group 1 showed non-significant increase in median serum levels of the pro-inflammatory cytokines; IL-1B and TNF and anti-inflammatory IL-10 at days 1and 8 compared to healthy controls (HC). The apoptotic markers showed a significant increase in PD-L1 serum level on day 8 compared to day 1 of the study, in addition, there was a significant increase in BID1 on day 8 compared to healthy controls. On the other hand, the anti-apoptotic cytokine BCL2 showed non-significant decrease in days 1 and 8 versus healthy controls (Table 3).

As regards Group (2), no statistical difference was observed in the median serum levels of IL-1B and TNF in days 1 and 8 versus healthy controls. IL-10 showed significant decrease on day 8 of study compared to day 1, BID1 showed significant increase in median levels on days 1 and 8 compared to healthy children. PD-1 and its ligand median levels showed no statistical difference in days 1 and 8 versus healthy controls. On the other hand, BcL-2 showed a significant decrease in the median levels on days 1 and 8 compared to healthy children (Table 3).

Table 3: Comparison of levels of studied markers (median, interquartile range 25^{th} –75^{th} percentile) between day 1 and day 8 in each group of patients.

Variable		Level at day 1 (median, IQR)	Level at day 8 (median,IQR)	Healthy children N = 20	p- value
IL-1ß	Gp 1	1.56 (0.3 – 2.36)	1.06 (0.7 – 1.8)	0.92 (0.58 – 1.78)	0.6^a / 0.7^b / 0.8^c
	Gp 2	1.16 (0.5 – 2.3)	0.66 (0.33 – 1.69)	0.92 (0.58 – 1.78)	0.68^a / 0.7^b / 0.9^c
TNF	Gp 1	0.99 (0.51 – 2.48)	0.87 (0.5 – 1.4)	0.86 (0.5 – 2.18)	0.38^a / 0.9^b /0.8^c
	Gp 2	1.01 (0.78 – 1.8)	0.6 (0.36 – 1.4)	0.86 (0.5 – 2.18)	0.06^a / 0.7^b / 0.09^c
IL-10	Gp 1	2.1 (1.2 - 5.97)	1.85 (0.7 – 3.3)	1.52 (0.1 – 8.6)	0.38^a / 0.4^b /0.8^c
	Gp 2	1.17 (0.46 – 2.37)	0.69 (0.3 – 1.3)	1.52 (0.1 – 8.6)	0.02^a / 0.8^b / 0.9^c
PD-L1	Gp 1	1.056 (0.6 – 4.7)	3.1 (0.5 – 5.1)	2.34 (0.08 – 12.6)	0.019^a /0.5^b /0.6^c
	Gp 2	5.8 (2.4 – 14.3)	3.14 (1.2 – 6.2)	2.34 (0.08 – 12.6)	0.86^a / 0.2^b / 0.39^c
PD-1	Gp 1	0.85 (0.3 – 2.48)	0.54 (0.18 – 2.7)	1.97 (0.09 – 3.7)	0.7^a / 0.8^b /1.00^c
	Gp 2	1.4 (0.46 – 2.6)	1.3 (0.4 – 3.8)	1.97 (0.09 – 3.7)	0.26^a / 0.8^b / 0.8^c
BID1	Gp 1	2.2 (1.1 – 3.9)	3.7 (1.6 – 5.6)	1.00 (0.54 – 1.6)	0.13^a / 0.08^b / 0.014^c
	Gp 2	5.19 (3.7 – 7.00)	6.5 (2.9 – 13.3)	1.00 (0.54 – 1.6)	0.1^a / 0.001^b / 0.004^c
BcL2	Gp 1	0.7 (0.3 – 1.35)	0.8 (0.5 – 1.4)	0.93 (0.76 – 1.5)	0.4^a / 0.25^b /0.35^c
	Gp 2	0.7 (0.4 – 1.1)	0.57 (0.3 – 0.9)	0.93 (0.76 – 1.5)	0.26^a / 0.04^b / 0.012^c

[a] = p-value between median levels at day 1 and day 8 of study. [b]= p-value between median levels at day 1 and levels of healthy children
[c] = p-value between median levels at day 8 and levels of healthy children Gp1= group 1 Gp2= group2

Comparison was done between the three groups using Kruskal Wallis test. Mann Whitney test was performed whenever there was significance to detect which of the two groups were significant.

Non-Survivors showed a significant increase in the median level of BID1 in comparison to healthy controls (Table 4).

Table 4: Comparison of levels of studied markers between survivors, non-survivors and healthy children (median + IQR $25^{th} – 75^{th}$)

Variable	Survivors No. = 21	Non-survivors No. = 10	Healthy children No.= 20	p-value
IL-1ß	1.56 (0.6 – 2.3)	0.6 (0.4 – 3.7)	0.92 (0.58 – 1.8)	0.69
TNF	1.18 (0.58 – 2.1)	0.86 (0.7 – 1.5)	0.86 (0.5 – 2.2)	0.61
IL-10	1.41 (0.97 – 4.2)	2.57 (1.2 – 4.8)	1.5 (0.1 – 8.6)	0.62
PD-L1	1.06 (0.6 – 5.1)	5.74 (2.1 – 13.5)	2.3 (0.1 – 12.6)	0.14
PD-1	0.85 (0.3 – 2.1)	1.83 (0.4 – 7.9)	1.97 (0.1 – 3.7)	0.40
BID1	3.38 (1.2 – 5.2)	3.77 (2.2 – 6.5)*	1.00 (0.5 – 1.6)*	0.027*
BcL2	0.73 (0.4 – 1.3)	0.56 (0.3 – 0.98)	0.9 (0.8 – 1.5)	0.11

A significant correlation was noticed between TNF and IL-1 β. A highly significant one existed between TNF and BID1. Highly significant correlations were present between PD-L1 and both BID1 and PD-1 individually. TLC was significantly correlated with IL-1β, in addition, CRP was highly significantly correlated with the SOFA score (Table 5).

Table 5: Correlation (r) between different studied markers, CRP and TLC and SOFA score in all studied patients (day 1) before start of treatment

Variable	IL-1β	BID-1	PD-L1	PD-1	TNF	BCL_2	IL-10	TLC	CRP	SOFA
IL-1β	_____	0.29	0.33	0.2	0.4*	0.012	0.01	0.4*	-0.19	-0.06
BID-1	0.29	_____	0.67**	0.14	0.45**	0.27	0.012	0.26	0.000	0.3
PD-L1	0.33	0.67**	_____	0.46**	0.13	0.03	-0.10	0.23	-0.025	0.24
PD-1	0.18	0.14	0.46**	_____	0.14	-0.01	0.29	-0.09	-0.3	-0.11
TNF	0.4*	0.45**	0.13	0.14	____	0.30	0.20	0.01	-0.34	0.04
BCL_2	0.012	0.27	0.03	-0.01	0.30	____	0.004	-0.27	-0.17	-0.08
IL-10	-0.001	0.012	-0.10	0.29	0.20	0.004	____	-0.20	-0.15	0.38
TLC	0.4*	0.26	0.23	-0.09	0.01	-0.27	-0.20	____	-0.18	-0.09
CRP	-0.19	0.000	-0.025	-0.3	-0.34	-0.17	-0.15	-0.18	____	0.42**
SOFA	-0.06	0.3	0.24	-0.11	0.04	-0.08	0.38	-0.09	0.42**	

S*= significant correlation at 0.05 level
HS** = significant correlation at 0.01 l

Figure 1 shows the highly significant correlation between BID1 on one hand and TNF (r = 0.45, p = 0.01) and PD-L1 (r = 0.67, p= 0.001)

Figure 2 shows a highly significant correlation between CRP and SOFA score (r = 0.43, p= 0.007).

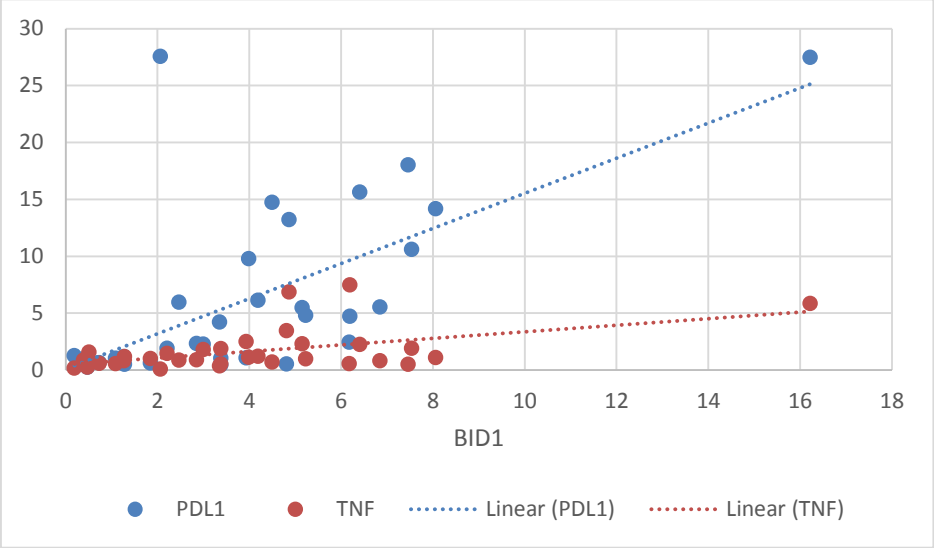

Fig. 1: Correlation between BID1 and TNF and PD-L1

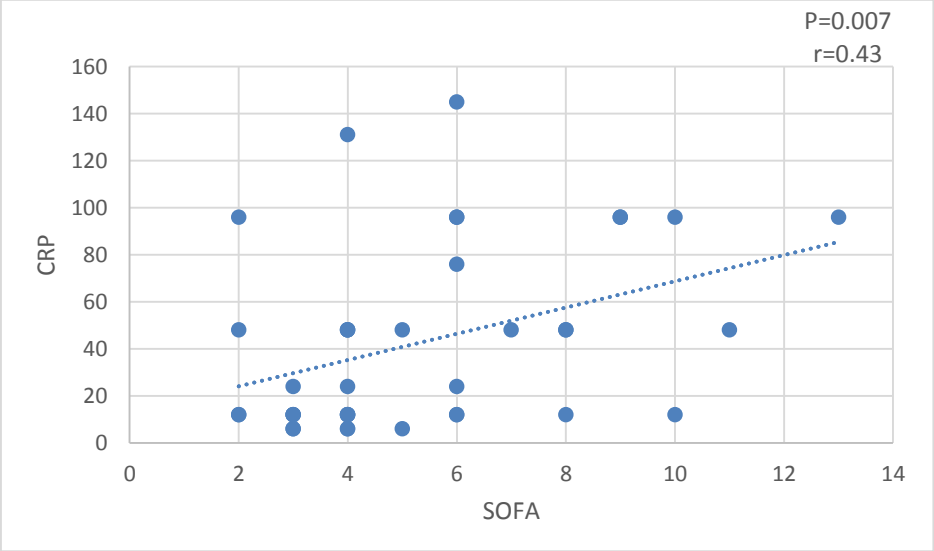

Fig. 2: Correlation between CRP and SOFA score

DISCUSSION

During the past few decades, there were a variety of trials investigating the effect of G-CSF therapy in patients with sepsis. Although the results of many studies revealed an association of G-CSF therapy with significant increase in the reversal rate from infection yet it showed no significant difference in mortality rate[20].

Schefold[21], revealed that immunological biomarkers were needed for guidance of immunotherapy in sepsis and in identification of patients that may benefit from therapy.

In the present work we initiated a trial of G- CSF immunotherapy as an adjuvant to the conventional treatment in one group of our patients (Group1). Studied immunological markers (mRNA gene expression by RT-PCR) were measured on day 1 and re-assessed on day 8.

Our results agreed with Weber et al, [12], and Peronnet et al,[22]. The latter study results showed that pediatric critically ill patients exhibited a pro-apoptotic profile (high expression of BID with low expression of BcL2) in the days following PICU admission like the pattern seen in adult sepsis [22]. BcL2 family of proteins governs cytochrome release through mitochondrial membrane regulation [23]. Toxic stimuli such as hypoxia disrupts the mitochondrial membrane integrity leading to increase in mitochondrial membrane permeability and release of cytochrome C into the cellular cytoplasm. Its cleavage leads to cell apoptosis and death. Moreover, increased expression of programmed- death protein ligand (PD-

LI) was detected in the first day of sepsis in group 1. This result agreed with Liu et al, [24], and Wilson et al, [5]. Jubel et al, [25], pointed out the pivotal role of this apoptotic cytokine in sepsis- induced immunosuppression. Increased expression of apoptotic markers on T lymphocytes would lead to prolonged hypo-inflammatory phase and cellular exhaustion with increased host susceptibility to secondary infection [26].

In addition, group 2 patients showed a significant decrease in the median level of the anti-inflammatory cytokine IL-10 on day 8 compared to day 1. Muszynski et al, [27]reported decreased IL-10 production in children with septic shock who developed persistent nosocomial infection.

At the 8th day, group 1 septic patients who received G-CSF, showed significant upregulation of the apoptotic BID1 and PD-L1 which revealed ongoing apoptotic response despite of treatment (i.e. patient ongoing activity). Researchers conducted clinical trials for sepsis using G-CSF demonstrated increased resolution of infection through increase in the number of polymorphonuclear leucocytes (PML) to enhance pathogen clearance. It increases neutrophil phagocytosis, resolve infections early, decrease the secretion of toxic metabolites and increase the expression of HLA-DR on neutrophils, and monocytes. Hence, they deduced that the use of G-CSF in combination with other immunomodulatory agents may be useful to improve survival rate [28].

Group 2 septic patients who received conventional treatment only showed significant increase in the apoptotic protein BID1 with concomitant decrease in the median level of the anti-inflammatory cytokine IL-10 and the anti-apoptotic cytokine BcL2. Autopsy studies for septic patients proved that apoptosis was the driver of immune cells depletion thus compromising the host ability to clear mild secondary infections and increasing sepsis severity with poor outcome [13]. Therefore, the picture of increased BID1 coupled with decreased IL-10 and BcL2 median levels probably denote ongoing uncontrolled infection which led to a state of immune paralysis and apoptosis.

In the current study, non-survivors showed a significant increase in the median level of BID1 in comparison to healthy children. During sepsis, the myeloid cells and lymphocytes undergo apoptosis with anergy to the other remaining immune cells thus rendering both the innate and adaptive immune responses ineffective and the patient enters chronic immunosuppressive phase and poor outcome [29].

A highly significant correlation existed between BID1 and TNF ($r = 0.45$, $p = 0.01$ (Table 6). Apoptosis is mediated through two pathways: extrinsic and intrinsic pathways. Both pathways proceed through cleavage of caspase-3 which cause DNA fragmentation and cleavage of cytoskeletal and nuclear proteins and cell death [30]. The extrinsic pathway is mediated via the interaction of death ligands Fas and TNFα with their corresponding receptors on the cell surface. The intrinsic pathway is controlled by BcL2 family of proteins, where BID1 is one of its pro-apoptotic proteins that induce apoptosis. BID1 is the protein that links the two pathways of sepsis- induced apoptosis [11].

In addition, a highly significant correlation existed between BID1 and PD-L1. In animal models, sepsis caused an increase in PD-1 expression on T-cells and PD-L1 expression on monocytes which is associated with T-cell apoptosis and death [25]. Liu et al, [24] showed that serum PD-L1 can be used to reflect sepsis severity and outcome.

CONCLUSION

Upregulation of the apoptotic BID1 in both groups and downregulation of IL-10 and BcL2 expression in our septic patients suggests a feature of sepsis-related immunosuppression. G-CSF, in a dose of 5µg /kg/day, seemingly had no effective role in our studied patients.

Limitations and recommendations:

The study was conducted on a relatively small scale and patients were recruited from one center. Large scale of infants and children in a multicenter study is recommended for validation of results. The period of follow up was limited to one week. We recommend to extend the period of follow-up, especially in the group receiving rh G-CSF. Furthermore, a trial with a bigger dose (dose range: 5 to 10 µg /kg/day) might yield more promising outcome.

Acknowledgements

Center of Excellence, National Research Center, Cairo, Egypt. Authors would like to extend their appreciation to patients and their caregivers for their cooperation.

Funding

Funding this research was provided by National Research Centre (Internal Projects, 11010151).

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

List of abbreviation

B-cell CCL/lymphoma 2 (Bcl-2)
Complementary deoxyribonucleic acid (cDNA)
C-reactive protein (CRP)
Erythrocyte sedimentation rate (ESR)
Ethylenediaminetetraacetic acid (EDTA)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Granulocyte - colony stimulating factor (G-CSF)
Intensive care units (ICUs),
Interleukin (IL)
Mean arterial pressure (MAP)
messenger ribonucleic acid (mRNA)

Neutrophil extracellular traps (NET's)
Polymerase chain reaction (PCR)
Programmed death ligand (PDL)
Programmed death protein (PD)
Quantitative Real Time (qPCR)
Serum Glutamic Pyruvic Transaminase (SGPT)
Serum glutamic-oxaloacetic transaminase (SGOT)
SOFA score (Sequential Organ Failure Assessment).
Tumor necrosis factor (TNF)

REFERENCES

1. Arens C, Bajwa SA, Koch C, Siegler BH, Schneck E, Hecker A, Weiterer S, Lichtenstern C, Weigand MA, Uhle F. Sepsis-induced long-term immune paralysis--results of a descriptive, explorative study. Crit Care. 2016;20:93. https://doi.org/10.1186/s13054-016-1233-5
2. Bo L, Wang F, Zhu J, Li J, Deng X. Granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) for sepsis: A meta-analysis. Crit Care. 2011;15(1)R58 https://doi.org/10.1186/cc10031.
3. Khatab, A. A., ElGendy, F. M., Hassan, F. M., El-Hendawy, G. R., & Saleh, N. Y.. Study of fungal infections in pediatric intensive care unit in Menoufiya University Hospital. Menoufia Medical Journal, 2014; 27(1), Article 9. https://doi.org/10.4103/1110-2098.132742
4. Zhang Y, Li J, Lou J, Zhou Y, Bo L, Zhu J, Zhu K, Wan X, Cai Z, Deng X. Upregulation of programmed death-1 on T cells and programmed death ligand-1 on monocytes in septic shock patients. Crit Care. 2011;15(1), R70. https://doi.org/10.1186/cc10059
5. Wilson JK, Zhao Y, Singer M, Spencer J, Shankar-Hari M. Lymphocyte subset expression and serum concentrations of PD-1/PD-L1 in sepsis - pilot study. Crit Care. 2018 Apr 17;22(1):95. https://doi.org/10.1186/s13054-018-2020-2
6. Zhang Y, Zhou Y, Lou J, et al. PD-L1 blockade improves survival in experimental sepsis by inhibiting lymphocyte apoptosis and reversing monocyte dysfunction. Crit Care. 2010;14(6): R220. https://doi.org/10.1186/cc9354
7. Sari MI, Ilyas S. The expression levels and concentrations of PD-1 and PD-L1 proteins in septic patients: A systematic review. Diagnostics. 2022;12(8):2004. https://doi.org/10.3390/diagnostics12082004
8. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: Current researches in cancer. Am J Cancer Res. 2020;10(3):727–742.
9. Gyawali B, Ramakrishna K, Dhamoon AS. Sepsis: The evolution in definition, pathophysiology, and management. SAGE Open Med. 2019;7:2050312119835043. https://doi.org/10.1177/2050312119835043:
10. Sun S, Chen Y, Liu Z, Tian R, Liu J, Chen E, Mao E, Pan T, Qu H. Serum-soluble PD-L1 may be a potential diagnostic biomarker in sepsis. Scand J Immunol. 2021;94(1) https://doi.org/10.1111/sji.13049
11. Chong SJ, Marchi S, Petroni G, Kroemer G, Galluzzi L, Pervaiz S. Noncanonical cell fate regulation by Bcl-2 proteins. Trends Cell Biol. 2020;30(7):537–555. https://doi.org/10.1016/j.tcb.2020.03.004
12. Weber SU, Schewe JC, Lehmann LE, Müller S, Book M, Klaschik S, Hoeft A, Stüber F. Induction of Bim and Bid gene expression during accelerated apoptosis in severe sepsis. Crit Care. 2008;12(5) https://doi.org/10.1186/cc7088
13. Cheng Z, Abrams ST, Toh J, Wang SS, Wang Z, Yu Q, Yu W, Toh CH, Wang G. The critical roles and mechanisms of immune cell death in sepsis. Front Immunol. 2020;11:1918. https://doi.org/10.3389/fimmu.2020.01918
14. Root RK, Lodato RF, Patrick W, Cade JF, Fotheringham N, Milwee S, Vincent JL, Torres A, Rello J, Nelson S, Pneumonia Sepsis Study Group. Multicenter, double-blind, placebo-controlled study of the use of filgrastim in patients hospitalized with pneumonia and severe sepsis. Crit Care Med. 2003;31(2):367–373. https://doi.org/10.1097/01.CCM.0000048629.32625.5D
15. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. Am Coll Chest Physicians/Society Crit Care Med. Chest. 1992;101(6):1644–1655. https://doi.org/10.1378/chest.101.6.1644
16. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL, Angus DC. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315(8):801–810. https://doi.org/10.1001/jama.2016.0287
17. Usman OA, Usman AA, Ward MA. Comparison of SIRS, qSOFA, and NEWS for the early identification of sepsis in the emergency

department. Am J Emerg Med. 2019;37(8):1490–1497 https://doi.org/10.1016/j.ajem.2018.10.058

18. Vincent JL, Opal SM, Marshall JC, Tracey KJ. Sepsis definitions: Time for change. Lancet. 2013;381(9868):774–775. https://doi.org/10.1016/S0140-6736(12)61815-7
19. Sylvester RK. Clinical applications of colony-stimulating factors: A historical perspective. Am J Health Syst Pharm. 2002;59(7 Suppl 2), S6–S12. https://doi.org/10.1093/ajhp/59.suppl_2.S6
20. Cheng AC, Stephens DP, Currie BJ. Granulocyte-colony stimulating factor (G-CSF) as an adjunct to antibiotics in the treatment of pneumonia in adults. Cochrane Database Syst Rev. 2004;(3) CD004400. https://doi.org/10.1002/14651858.CD004400.pub2
21. Schefold JC. Measurement of monocytic HLA-DR (mHLA-DR) expression in patients with severe sepsis and septic shock: Assessment of immune organ failure. Intensive Care Med. 2010;36(11):1810–1812. https://doi.org/10.1007/s00134-010-1965-7
22. Peronnet E, Nguyen K, Cerrato E, Guhadasan R, Venet F, Textoris J, Pachot A, Monneret G, Carrol ED. Evaluation of mRNA biomarkers to identify risk of hospital-acquired infections in children admitted to paediatric intensive care unit. PLoS One. 2016;11(3), e0152388. https://doi.org/10.1371/journal.pone.0152388
23. Patil NK, Bohannon JK, Sherwood ER. Immunotherapy: A promising approach to reverse sepsis-induced immunosuppression. Pharmacol Res. 2016;111:688–702. https://doi.org/10.1016/j.phrs.2016.07.019
24. Liu Q, An L, Qi Z, Zhao Y, Li C. Increased expression of programmed cell death-1 in regulatory T cells of patients with severe sepsis and septic shock: An observational clinical study. Scand J Immunol. 2017;86(5):408–417. https://doi.org/10.1111/sji.12612
25. Jubel JM, Barbati ZR, Burger C, Wirtz DC, Schildberg FA. The role of PD-1 in acute and chronic infection. Front Immunol. 2020;11:487. https://doi.org/10.3389/fimmu.2020.00487
26. Hibbert JE, Currie A, Strunk T. Sepsis-induced immunosuppression in neonates. Front Pediatr. 2018;6:357. https://doi.org/10.3389/fped.2018.00357
27. Muszynski JA, Nofziger R, Greathouse K, Steele L, Hanson-Huber L, Nateri J, Hall MW. Early adaptive immune suppression in children with septic shock: A prospective observational study. Crit Care. 2014;18(4) https://doi.org/10.1186/cc13980
28. Shin J, Jin M. Potential immunotherapeutics for immunosuppression in sepsis. Biomol Ther. 2017;25(6):569–577. https://doi.org/10.4062/biomolther.2017.193
29. Nedeva C, Menassa J, Puthalakath H. Sepsis: Inflammation is a necessary evil. Front Cell Dev Biol. 2019;7:108. https://doi.org/10.3389/fcell.2019.00108
30. Patil NK, Guo Y, Luan L, Sherwood ER. Targeting immune cell checkpoints during sepsis. Int J Mol Sci. 2017;18(11):2413. https://doi.org/10.3390/ijms18112413