

Using of TLR2 and TLR4 as Biomarker of Sepsis Severity Detection

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Abstract

Sepsis syndrome is a complicated clinical dysfunction, which caused by a systemic inflammatory response to bacteria and/or their products. The quantitative real-time PCR technique has been used for measure TLR2 and TLR4 gene expression in whole blood, and ELISA technique has been used for detection of cytokines TNF- α , IL-10 and soluble HLA-DR from 75 septic syndrome cases (nineteen of patients showed symptoms of systemic inflammatory response syndrome (SIRS); twenty eight patients have sepsis, seventeen patients suffered from severe sepsis and eleven patients have septic shock) and 55 healthy controls (HC). TLR2 and TLR4 mRNA expression was high and significant in the all patients ($P < 0.05$). TNF- α , IL-10 and sHLA-DR serum levels were significantly elevated in the serum of patients group with septic syndrome compared with controls ($P < 0.05$). However, the level of HLA-DR in SIRS patients was non-significant in comparing with healthy control group. Furthermore, TLR2 and TLR4 showed that there are significant differences between the severity stages of sepsis. The correlation between TLR 4 with concentration of sHLA-DR was significant and positive. The results of a recent study conclude that the possibility of using TLR 2 and TLR 4 expression to determine the severity of sepsis as diagnostic biomarker.

Keywords: TLR2, TLR4, severity of Sepsis, Soluble HLA-DR, Sepsis biomarker.

الخلاصة

متلازمة الإنتان هو اختلال وظيفي سريري معقد، سببه استجابة التهابية جهازية للبكتيريا و/أو منتجاتها. تم قياس مستوى التعبير الجيني لكل من المستقبلات الشبيهة بالتول 2 و 4 في كامل الدم باستخدام تقنية تفاعل البوليميريز المتسلسل اللحظي الكمي Quantitative Real-Time PCR. ومن خلال تقنية ELISA تم قياس مستوى كل من عامل النخر الورمي الفا والانترولوكين 10 ومستضد كريات الدم البيضاء البشرية-در الذائب في 75 عينة دم من المرضى المصابين بمتلازمة الإنتان (19 مريضاً تم تشخيصهم بمتلازمة الاستجابة الالتهابية الجهازية و 28 مريضاً مصاباً بالانتان (SIRS) ، 17 مريضاً مصاباً بالانتان الحاد و 11 مريضاً مصاباً بالصدمة الانتانية). تمت المقارنة مع 55 شخص كعينات سيطرة. اظهرت النتائج ارتفاع مستوى التعبير الجيني لكل من TLR4 و TLR2 في جميع المرضى مقارنة مع عينات السيطرة ($P < 0.05$). بالإضافة الى الارتفاع الملحوظ في مستويات كل من عامل النخر الورمي الفا و الانترولوكين 10 و مستضد كريات الدم البيضاء البشرية-در الذائب ($P < 0.05$) مقارنة مع مجموعة السيطرة ما عدا مستوى مستضد كريات الدم البيضاء البشرية-در الذائب لم يشكل اي اختلاف ملحوظ في المرضى المصابين بمتلازمة الاستجابة الجهازية مقارنة مع مجموعة السيطرة. اظهرت النتائج ايضا اختلافات واضحة في التعبير الجيني لكل من TLR4 و TLR2 على مستوى كل مرحلة من مراحل الإصابة بمتلازمة الإنتان، كما لوحظ وجود ارتباط موجب بين التعبير الجيني لـ TLR4 و مستوى مستضد كريات الدم البيضاء البشرية-در الذائب في المصل. ومن خلال النتائج الظاهرة، يمكننا الاستخلاص بإمكانية استخدام التعبير في التعبير الجيني للمستقبلات الشبيهة بالتول 2 و 4 كعلامة بايولوجية تشخيصية لشدة الإصابة بمتلازمة الإنتان.

Introduction

Sepsis syndrome is a complicated clinical dysfunction, which caused by a systemic inflammatory response to bacteria and/or their products [1]. Sepsis is an important medical disorder in the 21st century and remain the major reason leads for patients dying in the intensive care unit (ICU) in spite of antibiotic

therapy development and supportive care [2]. Frequently, patients with infections and patients who have a sterile tissue injury which arising from noninfectious sources such, ischemia reperfusion injury, cancer, pancreatitis, or numerous other disorders are most likely to be septic [3, 4].

According to the American College of Chest Physicians and the Society of Critical Care first published Care (ACCP/SCCM) publishing sepsis syndromes could be classified into: systemic inflammatory response syndrome (SIRSS), sepsis, severe sepsis and septic shock [5].

Systemic Inflammatory Response Syndrome is the clinical expression of the acute phase reaction [6]. SIRS is the occurrence of at least two of the following conditions, one of must be abnormal leukocyte count or temperature; Fever or hypothermia, tachycardia, tachypnea, Leukocyte count elevation or depression or >10% immature neutrophils [7]. Severe sepsis defining it as SIRS, clinical signs of an infection site, and end-organ demonstrating insufficient dysfunction or perfusion [8]. Sepsis plus hypotension is defined as "Septic shock", despite adequate resuscitation with fluids, besides the presence of perfusion abnormalities that included; lactic acidosis, oliguria or severe modification in status of mental [7].

The innate immune system is the first defense line against invading pathogens; it is stimulated by the engagement between germline-encoded innate immune receptors "pattern recognition receptors" (PRRs) in response to microbial component [9]. In general, pathogen-associated molecular patterns (PAMPs) are conserved molecular structures known as that are an important in pathogen's life-cycle. However, these PAMPs are detected by the host's germline encoded pattern recognition receptors (PRRs), such Toll like receptors, which are express on the surface of innate immune cells such as macrophages, neutrophils and dendritic cells [10]. The stimulation of innate immune response via PAMPs is aimed to protect the host from infections through removal of pathogenic bacteria [11]. Recently TLR2 and TLR4, are appear to be a highly candidate for involuntary in the immediate immune response to G-ve and G+ve bacteria. The cellular signals cascade of TLR leads to activation of NF κ B, in which turn to lead the generation of such pro-inflammatory cytokines as TNF α and anti-inflammatory cytokines such as IL-10 [12].

TNF- α is unique among inflammatory cytokines and the release of massive amounts of it early in sepsis leads to activation another

pro-inflammatory cytokines and act a role in the conjunction with TNF- α to induction of T-cell apoptosis [13]. IL-10 is one of the most important anti-inflammatory cytokine which acts in the immune response. It is reduced nuclear factor κ B (NF- κ B) nuclear translocation afterward LPS stimulation and stimulates messenger RNA degradation for the pro-inflammatory cytokines [14].

Soluble HLA can act as immune response modulators. sHLA-DR molecules bind to the T-cell receptor, CD4 and CD8 co-receptors, causing stimulation of apoptosis. Soluble HLA-DR are thought to be secreted by circulating antigen presenting cells (APC) and other nonmalignant cells [15, 16].

Because of the complexity of sepsis pathophysiology and involved almost all types of cells, tissues, and organ systems. This study has been aimed to find the relation between the expression of toll-like receptors 2 and 4, level of TNF- α , IL-10 and soluble HLA-DR with the severity bacterial septic syndrome in Iraqi patients. This study is focused on determining the capability of using TLR 2 and TLR 4 as a biomarker in Iraqi patients with bacterial sepsis syndrome.

Materials and Methodologies

Study Design and Population

Seventy-five patients with septic syndrome from different 5 hospitals in Baghdad were studied, among them nineteen patients show symptoms of systemic inflammatory response syndrome (SIRS) twenty eight patients with sepsis, seventeen severe sepsis patients and eleven septic shock patients. The results compared with those measured in 55 volunteers as healthy controls. The age range of patients was from (2 weeks – 92 years). The source of septic syndrome was the urinary tract infection (n = 21), the immune suppression (n = 14), neonatal sepsis (n= 10), Post operation infection (n=10), respiratory infection (n= 9), gastrointestinal infection (n = 6), and meningitis (n = 5).

Sepsis severity

For group comparison, patients have been divided into 4 groups according to severity of infection: SIRS, sepsis, severe sepsis and septic

shock. Comparison between the stages of severity was conducted from minor infection to the most severe.

Cytokine and soluble molecule assay

Serum IL-10, TNF- α (MyBiosource Company, USA) and soluble HLA-DR (R&D system Inc, USA) concentrations were determined by ELISA in accordance with the manufacturer's instructions. The concentrations were calculated through using of the mean optical density of two wells and comparison with a standard curve.

RNA extraction

RNA was extracted by TRIzol provides by (Invitrogen Life Technologies, USA) an efficient method for purifying total RNA from whole blood, and also the procedure of extraction based on the manufacturer's instructions. The concentration of RNA was measured by nano-drop spectrophotometer (Quawell Q5000, USA) and the purity detected by noticing the ratio of optical density (O.D.) at wavelength 260/280.

The mRNA expression of TLR2 and TLR4 was determined through using of (KAPA SYBR FAST one-step qRT-PCR kit, Canada).

RT-PCR was performed using primers specific for *tlr2* and *tlr4* gene with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene which supplied by (Alpha DNA technologies company) (Table 1).

The relative quantitation was calculated from cycle threshold (CT) values. The Ct value of the target genes was normalized (Δ Ct) to the Ct value of the TLR 2, 4 genes of the samples.

Statistical analysis

Data were expressed as mean \pm S.E. Analysis of variance (ANOVA) used to analyze the differences among group means. LSD was used for comparing the least significant difference between groups. Correlations were determined using a Spearman correlation test. P-value was considered significant when it was less than or equal to 0.001 and 0.05. The data were analyzed by the statistical software (SPSS 22.0, SPSS Inc., Chicago, IL, USA).

Table 1: Primers Used InqRT-PCR ForTLR2andTLR4 Genes Designed In Study

Gene	Primer sequences (5'-3')	product
TLR2	F: TGTGGATGGTGTGGGTCTTG	943
	R: ATATGCAGCCTCCGGATTGT	
TLR4	F: ATATTGACAGGAAACCCCAT	300
	CCA	
	R: AGAGAGATTGAGTAGGGGCA	
GAPDH	F: ATCACTGCCACCCAGAAGAC	216
	TG	
	R: AGGTTTTTCTAGACGGCAGG	
	TCAG	

Results and Discussion

Patients'clinical characteristics

Demographic data for the study population are shown in (Table 2).

Healthy controls

The control group has been included fifty five unrelated healthy persons, without signs of any infection or inflammatory disease.

Table 2: Demographic and clinical data of patients.

Patients	
Age	32 \pm 31.36
Male / female	46/39
Sepsis severity	
SIRS	19 (25.33%)
Sepsis	28 (37.33%)
Severe sepsis	17 (22.67%)
Septic shock	11 (14.67%)
Infection leads to sepsis	
Urinary tract	24 (32%)
Immune suppression	13 (17.33%)
Wound infection	11 (14.67%)
Neonatal sepsis	10 (13.33%)
Gastrointestinal infection	9 (12%)
Meningitis	5 (6.67%)
Respiratory infection	3 (4%)
Septic complications	
Acute renal failure	13 (17.3%)
Hepatic failure	3 (4%)
Respiratory failure	3 (4%)

Serum levels of IL-10, TNF- α and sHLA-DR:

TNF- α and IL-10 concentrations revealed that there are highly significant differences ($P < 0.001$) between patient groups and H.C at all severity stages of septic syndrome. sHLA-DR concentrations were increased significantly ($P < 0.001$) in sepsis, septic shock and severe

sepsis groups in comparing H.C. However, the differences between SIRS patients and H.C. were non-significant. TNF- α concentrations were revealed insignificant differences between stage of sepsis severity while IL-10 and sHLA-DR concentrations showed that there are significant differences ($P<0.05$) between them (Table 3).

Due to the significant differences between the severity stages of sepsis of IL-10 and sHLA-

DR, the LSD analysis was used to identify the least significant difference of concentration between them. The significant differences ($P<0.05$) was shown in the concentration of IL-10 and sHLA-DR between SIRS infection and the other 3 stages, while no differences was observed among sepsis, severe sepsis and Septic shock (Table 4).

Table 3: level of IL-10, TNF- α and sHLA-DR in severity stages of septic syndrome

stages of sepsis Severity	No.	IL-10 (Mean \pm S.E)	Sig	TNF- α (Mean \pm S.E)	Sig	sHLA-DR (Mean \pm S.E)	Sig
SIRS	19	19.84 \pm 3.14	76.45*	52.63 \pm 6.00	13.44*	7.31 \pm 1.37	0.07
Sepsis	28	61.52 \pm 8.45	93.92*	77.09 \pm 9.40	34.39*	18.50 \pm 1.80	68.35*
Severe Sepsis	17	81.89 \pm 17.32	68.70*	78.04 \pm 9.98	43.39*	15.08 \pm 2.64	25.32*
Septic Shock	11	72.12 \pm 26.41	36.17*	70.74 \pm 10.29	31.71*	15.50 \pm 3.29	26.04*
H.C	55	3.22 \pm 0.30		34.42 \pm 2.07		7.04 \pm 0.37	

*S.E: Standard error * $P<0.001$

Table 4: LSD comparison in level of IL-10 and sHLA-DR between severity stages of sepsis.

Sepsis stage		IL-10		sHLA-DR	
		Mean \pm S.E	Sig	Mean \pm S.E	Sig
SIRS	Sepsis	41.67 \pm 16.38	0.013*	11.19 \pm 2.78	0.000*
	Severe sepsis	62.05 \pm 18.40	0.001*	7.76 \pm 3.12	0.015*
	Septic shock	52.28 \pm 20.88	0.015*	8.18 \pm 3.54	0.024*
Sepsis	Severe sepsis	20.37 \pm 16.95	0.233	3.42 \pm 2.87	0.237
	Septic shock	10.60 \pm 19.61	0.590	3.00 \pm 3.32	0.370
Severe sepsis	Septic shock	9.76 \pm 21.33	0.649	0.42 \pm 3.61	0.907

* $P<0.05$

Expression of TLR2, 4 mRNA in whole blood

TLR2 and TLR4 mRNA expressions were increased about 1.95 and 2.29 fold in septic syndrome patients' respectively in comparing with healthy controls. Furthermore, there are significant differences in the expression of TLR2 and TLR4 mRNA ($P<0.05$) among the severity stages of sepsis.

In regard to the relationship between TLR 2 and TLR4, the results revealed that there is a

weak (non-significant) and positive correlation between them (Table 5).

LSD was used to determine the differences between each severity stage of sepsis, and the results showed that there is a significant differences in the expression of TLR2 between SIRS and sepsis patients ($P<0.05$). In relation to the expression of TLR4, the significant differences were observed between SIRS patients' and two severity stages of sepsis: sepsis and severe sepsis patients (Table 6).

Table 5: Fold change of mRNA expression for *tlr2* and *tlr4* gene and differences between stages.

Severity stage of sepsis	N	TLR4 (Mean \pm S.E)	sig	P. value	TLR2 (Mean \pm S.E)	Sig	P. Value
SIRS	14	2.29 \pm 0.29			1.95 \pm 0.33		
Sepsis	24	5.76 \pm 0.84	2.92*	0.041	4.09 \pm 0.67	2.81*	0.046
Severe Sepsis	13	5.60 \pm 1.25			3.76 \pm 0.62		
Septic Shock	9	4.71 \pm 1.17			3.38 \pm 0.60		

* $P<0.05$

Table 6: LSD comparison in level of expression for *tlr2* and *tlr4* gene and differences between severity stages of sepsis.

Severity stage of sepsis		TLR2		TLR4	
		Mean ±S.E	Sig	Mean ±S.E	Sig
	Sepsis	2.13±0.84	0.015*	3.46±1.23	0.007*
SIRS	Severe sepsis	1.81 ±0.97	0.068	3.30±1.41	0.023*
	Septic shock	1.43 ±1.07	0.190	2.42±1.56	0.128
Sepsis	Severe sepsis	0.32±0.86	0.710	0.16±1.26	0.900
	Septic shock	0.70 ±.98	0.478	1.04±1.43	0.469
Severe sepsis	Septic shock	0.37 ±1.09	0.730	0.88±1.59	0.580

*P<0.05

Correlation between expression of TLR mRNA and soluble HLA-DR in patients

As shown in Figure 1, TLR4 mRNA expression in the whole blood of patients are correlated significantly and positively with serum sHLA-DR level (R=0.348, P=0.006), whereas non-significant correlation has been shown between the expression of TLR 2 mRNA on whole blood and serum sHLA-DR (R= -0.0157, P=0.904).

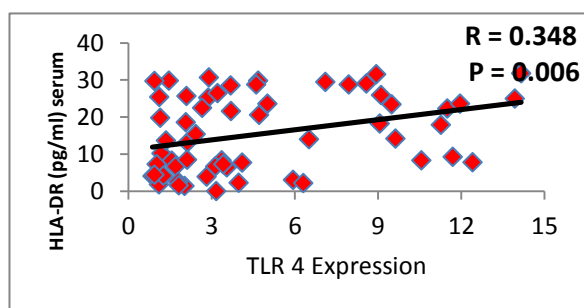


Figure 1: Correlation between TLR4 (r = 0.348; p = 0.006) and sHLA-DR secretion of whole blood in patients with sepsis

Discussion

Because the pathophysiology of sepsis is complex, sepsis remains a main cause which leading to critically ill patient's death, in spite of efforts for patient outcome improvement. Up to now, no magic drugs exist for severe sepsis and septic shock. For detection of sepsis in early time, biomarkers can help doctors distinguish between infections from the host response to inflammation [17].

The results of this study revealed that there is a significant increasing in the expressions levels of TLR2 and TLR4 in whole blood. The other important result is that there are high and

significant levels of IL-10, TNF- α and soluble HLA-DR in the serum of septic syndrome patients. The results of TLR mRNA expression in whole blood of patients showed that: lowest expression of TLR in SIRS was the stage of infection and followed by septic shock, severe sepsis and sepsis. This result agree with [18] who have been found that death and severe infection was associated with low expression of TLR 2 and TLR 4 in whole blood compared with sepsis patients. Akira and Takeda, 2004 reported that TLR2 and TLR4 expression on monocytes has been increased during sepsis and increased attention to it because they are the receptors associated with pathogens of gram-positive and gram-negative products[19]. In addition, Schaaf *et al.*, 2009 found that patients with septic syndrome characterized by increasing TLR2 and TLR4 expression on monocytes compared with controls [20]. Death is associated with down-regulation of TLR2 and CD14 expression on monocytes, which linked in turn with reduced cytokine stimulation. However, the results of this study do not compatible with Brunialti *et al.*, 2006, who noted that there was no variance in the expression of TLR2 and TLR4 between septic syndrome patients and healthy volunteers [21] The reason may be that patients with severe sepsis did not have an increasing in TLRs expression related to patients with less severely injured. Therefore, the detection which supposed that TLRs has an ability to identify endogenous or harmful self-antigens and proposes that their function may possibly not be limited to recognize the extrinsic pathogens [22].

The TLR2 and TLR4 play a crucial role in microbial responses may be occupied in the outcome of human sepsis and its pathophysiology, as well as initial releasing of systemic pro-inflammatory cytokines, elongated cellular hypo-responsiveness to components of bacteria by reducing of cytokine response is supposed to be an important factor in sepsis with limitation of follow capability to mount a suitable inflammatory defense to secondary infections [19, 20, 22].

An interesting report concerning the response of the innate immune system to infection and the role of cytokines in relation to disease severity indicated that serum levels of cytokines were considerably higher in more severe than in mild infections or healthy controls. In general, severe infections develop from mild infections (which comprise the majority of out patient cases) [23]. In sepsis, a pro-inflammatory phase (SIRS) is provoked to remove the pathogen. The results of this study shown that there are a significant increase of TNF- α concentration in SIRS patients more than healthy control and within severity of sepsis. The similar finding, reported by Ghuge *et al.*, 2013 who reported TNF- α level was increased significantly in the patients compared with controls [24] also, same indication has been shown by Kocabas *et al.*, 2007 [25] and by Dinata *et al.*, 2013 who found that plasma level of TNF- α in severe sepsis patients to be significantly higher than those in sepsis patients [26].

The level of IL-10 in this study increased significantly in SIRS, sepsis, severe sepsis and septic shock compared to a H.C group whereas the highest level concentration observed in severe sepsis followed by septic shock, sepsis and finally SIRS. This result was agreed with [27] who reported Sepsis-surviving patients had significant high concentrations of IL-10 in their serum and comparable control group. Lekkou *et al.*, 2004 was reported that elevation of IL-10 levels correlated to the increasing of mortality from septic shock [28]. It was reported that IL-10 is one of important cytokines in the sepsis syndrome pathophysiology and the measurement of cytokines in the patient's serum with severe sepsis showed the IL-10 level enhanced

significantly. The increasing of the IL-10 level in serum was related with the sepsis outcome and death [29]. Del Vecchio *et al.*, 2009 and Chaudhry *et al.*, 2013 have recommended that over expression of IL-10 can stimulate the immunosuppression in bacterial sepsis and increase mortality through preventing bacterial clearance and the production of IL-10 appeared to be controlled mainly at the transcriptional level [30, 31].

The level of IL-10 is also correlated with the severity of septic shock and the high IL-10 level is reported in patients who died from sepsis, in comparison to surviving patients and these results suggested that IL-10 may control the switching from reversible sepsis to late irreversible septic shock [32].

A highly pro-inflammatory response is known to cause an imbalance between pro-inflammatory cytokines such as TNF- α and anti-inflammatory cytokines such as IL-10 leading to the clinical manifestation of sepsis and septic shock [30, 31].

Soluble HLA-DR concentration was increased significantly in all patients compared with control groups except SIRS patients and this agreed with the study of Perry *et al.*, 2004 who founded that high level of soluble HLA-DR was found in septic patients' plasma compared to healthy controls and also high levels of sHLA-DR was noticed in synovial fluid and plasma in hyper-inflammatory [33]. On the other hand, human leukocyte antigen advert in severely injured patients, reducing the levels of both soluble and cellular HLA-DR act as early indicators of an immune deviation that related to severe sepsis development. Further more, the immune alterations of various cell types may be support different kinds of septicemia. Serum sHLA-DR increased in the infections and inflammatory diseases or diseases which results from the activated immune profile [34, 35]. The increasing of sHLA-DR level in septic patients comparing to H.C may indicate to the infection and inflammation response [33].

The molecules of class II HLA are an essential link between the innate and acquired immune system, which is important in prime immune responses for that have been not established immunologic memory. HLA molecules also to be found in serum as soluble forms (sHLA) and

other body fluids and bind to the same physiologic ligands such as the membrane anchored [14]. Despite the increasing support for the serum-sHLA forms act an important role in many disease pathophysiology, but up to the present time, there is no genetic link with specific surface HLA epitopes [36].

Also the data of this study has been shown a positive significant correlation between TLR4 expression and level of sHLA-DR in serum while no correlation has been observed between TLR2 expression and level of sHLA-DR in serum. Therefore, the intracellular molecules of MHC class II may serve as adaptors which encouraging full stimulation of TLR-triggered innate immune responses. The expression of class II major histocompatibility molecules is enhancing of TLR responsiveness to their legend by co-localization in lipid raft domains of the membrane. HLA-DR and TLR2 or TLR4 expression is significantly increased secretion of the antimicrobial peptide comparable with positive cell for TLR2 and TLR4 only. The molecules of MHC class II can act an important role in innate immunity by increasing of antimicrobial effector mechanisms and TLR-mediated cellular activation. Feri *et al.*, 2010 suggested an essential mechanism enhanced the signaling of TLR by co-localization with MHC class II in accumulating plasma membrane rafts instead of direct binding of the ligand to MHC class II [37]. The stimulation of MHC class II expression via TLR agonists isletto effective antigen presentation to activate the adaptive immunity, and as well act as a positive feedback mechanism increasing the mediated responses of TLR [38]

Finally TLR2 and TLR4 act an essential role in contributing to the acute non-septic device dysfunction and moreover speculate on the potential usefulness of inhibition of TLR2 and TLR4 are related to cellular stimulation in improving results from acute illnesses in which the products microbes do not seem to act a pathogenic role [39]. Instead of that, early diagnosis and rapid initial management, such as targeted early treatment, are essential to

improving sepsis outcomes. By taking all these results, TLR 2 and TLR 4 can be used as a diagnostic biomarker of bacterial sepsis syndrome.

Conclusion

Because of the complex pathophysiology of sepsis, and its remaining the main cause which leading to critically ill patient's death. The using of biomarkers can help doctors for detection of sepsis in early time and distinguish between infections from the host response to inflammation. The results of a recent study conclude that the increasing of TLR2 and TLR4 expression in each level of the syndrome can be considered an indication of the severity of the sepsis and also the positive correlation has been observed only between the level of sHLA-DR and expression of TLR4 can act as a good biomarker for early diagnosis of sepsis syndrome.

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References

- [1] Gao, J. W., Zhang, A. Q., Wang, X., Li, Z. Y., Yang, J. H., Zeng, L., and Jiang, J. X., Association between the TLR2 Arg753Gln polymorphism and the risk of sepsis: a meta-analysis. *Critical Care*. 19,1, 416, 2015.
- [2] Angus, D.C. and Van Der Poll, T., Severe sepsis and septic shock. *NEJM*. 369,9,, 840-851,2013.
- [3] Dwyer, J. One boy's death moves state to action to prevent others. *The New York Times*, December 20, 2012.
- [4] ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care

- Medicine Consensus Conference. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Critical Care Medicine*. 20, 864-874,1992.
- [5] Yang, Y. X., and Li, L.. Identification of potential biomarkers of sepsis using bioinformatics analysis. *Exp Ther Med*. 13,5, 1689-1696, 2017.
- [6] Dimcheva, Y., Kalinova, K., Dinkov, D., Georgiev, K., Brahomov, B., andKyazimova, E.,AppendicitisIn Childhood And Systemic Inflammatory Response Syndrome (SIRS). *TJS*. 13,4, 105,2015.
- [7] Vincent, J. L., Definition of sepsis and non-infectious SIRS. *Sepsis and Noninfectious Inflammation*. (2008)
- [8] Das, U., HLA-DR expression, cytokines and bioactive lipids in sepsis. *AMS*. 10,2, 325, 2014.
- [9] Foley, N.M., Wang, J., Redmond, H.P. and Wang, J.H., Current knowledge and future directions of TLR and NOD signaling in sepsis. *Military Medical Research*. 2,1,1 (2015).
- [10] Kumar, H., Kawai, T. and Akira, S., Pathogen recognition by the innate immune system. *International reviews of immunology*. 30,1,16-34, 2011.
- [11] Paredes-Juarez, G. A., de Haan, B. J., Faas, M. M., and de Vos, P., The role of pathogen-associated molecular patterns in inflammatory responses against alginate based microcapsules. *JCR*. 172,3, 983-992, 2013.
- [12] Boomer, J.S., Green, J.M. and Hotchkiss, R.S., The changing immune system in sepsis: is individualized immunomodulatory therapy the answer?. *Virulence*. 5,1, :45-56, 2014.
- [13] Leentjens, J., Kox, M., van der Hoeven, J.G., Netea, M.G. and Pickkers, P., Immunotherapy for the adjunctive treatment of sepsis: from immunosuppression to immunostimulation. Time for a paradigm change?. *AJRCCM*. 187,12,1287-1293, 2013.
- [14] Rebmann, V., Ugurel, S., Tilgen, W., Reinhold, U., and Grosse-Wilde, H., Soluble HLA-DR is a potent predictive indicator of disease progression in serum from early-stage melanoma patients. *IJC*. 100,5, 580-585, 2002.
- [15] Adamashvili, I., Minagar, A., Gonzalez-Toledo, E., Featherston, L., and Kelley, R. E., Soluble HLA measurement in saliva and cerebrospinal fluid in Caucasian patients with multiple sclerosis: a preliminary study. *Journal of neuroinflammation*. 2,1, 13, 2005.
- [16] Cho, S.Y. and Choi, J.H., Biomarkers of sepsis. *Infection and chemotherapy*. 46,1, 1-12, 2014.
- [17] Salomao, R., Martins, P.S., Brunialti, M.K.C., da Luz Fernandes, M., Martos, L.S., Mendes, M.E., Gomes, N.E. and Rigato, O., TLR signaling pathway in patients with sepsis. *Shock*. 30, 7, 73-77, 2008.
- [18] Akira, S., and Takeda, K., Toll-like receptor signalling. *Nature Reviews Immunology*. 4,7, 499-511, 2004.
- [19] Schaaf, B., Luitjens, K., Goldmann, T., van Bremen, T., Sayk, F., Dodt, C., Dalhoff, K. and Droemann, D., Mortality in human sepsis is associated with downregulation of Toll-like receptor 2 and CD14 expression on blood monocytes. *Diagnostic Pathology*. 4,1, 12, 2009.
- [20] Brunialti, M. K. C., Martins, P. S., de Carvalho, H. B., Machado, F. R., Barbosa, L. M., and Salomao, R., TLR2, TLR4, CD14, CD11B, and CD11C expressions on monocytes surface and cytokine production in patients with sepsis, severe sepsis, and septic shock. *Shock* 25,4, 351-357, 2006.
- [21] Tsujimoto, H., Ono, S., Efron, P. A., Scumpia, P. O., Moldawer, L. L., and Mochizuki, H., Role of Toll-like receptors in the development of sepsis. *Shock*. 29,3, 315-321, 2008.
- [22] Droemann, D., Goldmann, T., Tiedje, T., Zabel, P., Dalhoff, K., Schaaf, B., Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respiratory Research*. 6 1, 68,2005.

- [23] Yusa, T., Tateda, K., Ohara, A., and Miyazaki, S., New possible biomarkers for diagnosis of infections and diagnostic distinction between bacterial and viral infections in children. *JIC*. 23, 2, 96-100, 2017.
- [24] Ghuge, G.D., Zine, R and MukundMogrekar., Evaluation of C-reactive protein and tumor necrosis factor- alpha as reliable laboratory markers for early diagnosis of sepsis. *Int J Biol Med Res*. 3,4, 2345-2347, 2012.
- [25] Kocabas, E., Sarikçioğlu, A., Aksaray, N., and Seydaoglu, G., Role of procalcitonin, C-reactive protein, interleukin-6, interleukin-8 and tumor necrosis factor-[alpha] in the diagnosis of neonatal sepsis. *The Turkish journal of pediatrics*. 49, 1, 7, 2007.
- [26] Dinata, K., Runtuuwu, A. L., Mandei, J. M., and Lolombulan, J. H., Correlation between tumor necrosis factor-alpha and septic shock in children. *Paediatrica Indonesiana*. 53,1, 1-5, 2013.
- [27] Nascimento, Daniele C., Paulo H. Melo, Annie R. Pineros, Raphael G. Ferreira, David F. Colón, Paula B. Donate, Fernanda V. Castanheira., "IL-33 contributes to sepsis-induced long-term immunosuppression by expanding the regulatory T cell population." *Nature Communications*. 8, 2017.
- [28] Lekkou, A., Karakantza, M., Mouzaki, A., Kalfarentzos, F. and Gogos, C.A., Cytokine production and monocyte HLA-DR expression as predictors of outcome for patients with community-acquired severe infections. *Clin Diagn Lab Immunol*. 11,1, 161-167, 2004.
- [29] Wu HP, Chen CK, Chung K, Tseng JC, Hua CC and Liu YC., Serial cytokine levels in patients with severe sepsis. *Inflamm Res*. 57,385-93, 2009.
- [30] Del Vecchio, A., Ladisa, G., Laforgia, N., De Felice, C., Presta, G. and Latini, G., Genetic polymorphisms in neonatal sepsis. In *Hematology Meeting Reports*. 2, 10, 31-37, 2009.
- [31] Chaudhry, H., Zhou, J., Zhong, Y., Ali, M.M., McGuire, F., Nagarkatti, P.S. and Nagarkatti, M., Role of cytokines as a double-edged sword in sepsis. *In Vivo*. 27, 6, 669-684, 2013.
- [32] Schulte, W., Bernhagen, J., and Bucala, R., Cytokines in sepsis: potent immunoregulators and potential therapeutic targets—an updated view. *Mediators of inflammation*. 2013,1-16, 2013.
- [33] Perry, S. E., Mostafa, S. M., Wenstone, R., Shenkin, A., and McLaughlin, P. J., HLA-DR regulation and the influence of GM-CSF on transcription, surface expression and shedding. *Int. J. Med. Sci*. 1,3, 126, 2004.
- [34] Verbruggen, L. A., Versaen, H., Rebmann, V., Duquet, W., De Cock, S., Grosse-Wilde, H., and Demanet, C., Soluble HLA-DR levels in serum are associated with therapy and genetic factors in rheumatoid arthritis. *Human immunology*. 63, 9, 758-764, 2002.
- [35] Verbruggen, L.A., Dumarey, N., Velde, H., Rebmann, V., Flament, J., Wayenberge, C., Grosse-Wilde, H. and Demanet, C., Soluble HLA-DR antigen levels in serum correlate with rheumatoid arthritis disease activity and the presence of disease-associated epitopes. *HLA*. 56,5, 436-440, 2000.
- [36] Matalliotakis, I. M., Athanassakis, I., Goumenou, A. G., Neonaki, M. A., Koumantakis, E. E., Vassiliadis, S., & Koumantakis, E. E., The possible anti-inflammatory role of circulating human leukocyte antigen levels in women with endometriosis after treatment with danazol and leuprorelin acetate depot. *Mediators of inflammation*. 10,2, 75-80, 2001.
- [37] Frei, R., Steinle, J., Birchler, T., Loeliger, S., Roduit, C., Steinhoff, D., Seibl, R., Büchner, K., Seger, R., Reith, W. and Lauener, R.P., MHC class II molecules enhance Toll-like receptor mediated

- innate immune responses. *PloS one*. 5,1, 8808, 2010.
- [38] Hertz CJ, Kiertscher SM, Godowski PJ, Bouis DA, and NorgardMV,.. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor. *J Immunol*. 166, 2444–2450,2001.
- [39] Lorne, E., Dupont, H., & Abraham, E ., Toll-like receptors 2 and 4: initiators of non-septic inflammation in critical care medicine?. *ICM*. 36,11, 1826-1835, 2010.