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Using of TLR2 and TLR4 as Biomarker for Detection the Severity of Sepsis

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Abstract*--Our study has been aimed to find the relation between the expression of toll-like receptors 2, 4, level of TNF-α, IL-10 and soluble HLA-DR with the severity bacterial septic syndrome in Iraqi patients. 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 were higher significantly in the all patients (P< 0.05), TNF-α, IL-10 and sHLA-DR serum levels were significantly elevated in the serum of patients with septic syndrome compared with controls (P<0.05) except the level of HLA-DR in SIRS patients there were no differences with a healthy control group. Also the results have been shown, there are significant differences in TLR2 and TLR4 between the stages of septic syndrome and also a positive correlation between TLR 4 and concentration of sHLA-DR. According to the results of our study, we can conclude the possibility of using TLR 2 and TLR 4 expression to determine the severity of sepsis as diagnostic biomarker.*

Key words*--TLR2, TLR4, severity of Sepsis, Soluble HLA-DR, Sepsis biomarker*

I. 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",

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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 NFκB, in which turn to lead the generation of such pro-inflammatory cytokines as TNFα 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 kB (NF-κβ) 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, our study is focused on determining the capability of using TLR 2 and TLR 4 as a biomarker in Iraqi patients with bacterial sepsis syndrome.

II. 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 comparing between them from minor infection to the most severe.

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Cytokine and soluble molecule assay

Serum IL-10, TNF-α and soluble HLA-DR concentrations were determined by ELISA using a commercial human ELISA kit (R&D system Inc, USA) for IL-10 and TNF-α and (MyBioSource Company, USA) for sHLA-DR, 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 TRIzole 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 gene and TLR4 which supplied by (Alpha DNA technologies company), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene (Alpha DNA technologies) (Table 1).

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

Statistical analysis

Analysis of variance (ANOVA) was used for equality of means. Multiple comparisons by LSD (Least significant difference) for testing of equality of means for each pair. LSD test was used for comparing the differences between each group. Data were expressed as mean ±S.E. 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 qRT-PCR For *TLR2* and *TLR4* Genes Designed In Study

Gene	Primer sequences (5'-3')	product
TLR2	**F**: TGTGGATGGTGTGGGTCTTG	943
	R: ATATGCAGCCTCCGGATTGT	
TLR4	**F**:ATATTGACAGGAAACCCCATCCA	300
	R:AGAGAGATTGAGTAGGGGCATTT	
GAPDH	**F**:ATCACTGCCACCCAGAAGACTG	216
	R:AGGTTTTTCTAGACGGCAGGTCAG	

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III. RESULTS

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 (yr)	32 ± 31.36
Male / female	46/39
Sepsis severity	
SIRS	19 (25.3%)
Sepsis	28 (37.6%)
Severe sepsis	17 (22.6%
Septic shock	11 (14.6%)
Infection leads to sepsis	
Urinary tract	24 (32%)
Immune suppression	13 (17.3%)
Wound infection	11 (14.6%)
Neonatal sepsis	10 (13.3%)
Gastrointestinal infection	9 (12%)
Meningitis	5 (6.6%)
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 were significantly high at all stages of septic syndrome compared with controls group $P<0.001$.The level of also high is in all septic syndrome stages when it is compared with its concentration in H.C. Concentration of sHLA-DR was increased significantly in sepsis, septic shock and severe sepsis more than H.C $P<0.001$ while no significant differences shown in its concentration between SIRS patient and healthy controls ($P< 0.789$). The results shown in (Figure 1), (Table 3). Compares the level of TNF-α, IL-10, and sHLA-DR between stages of sepsis shown in (Table 4). The results indicated there no significant differences in the level of TNF-α between stage of infection. As mentioned above IL-10 levels higher in all stages compared with healthy controls, This increase was associated with significant differences in the IL-10

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levels of between the stages of infection (P<0.05). The level of soluble HLA-DR has shown significant differences between stage of sepsis when they were compared (P<0.05).

By using LSD analysis, the differences between the stages of infection were detected. The results indicated the significant differences in levels of IL-10 and soluble HLA-DR occurred between SIRS infection and the other 3 stages (P<0.05), and there are no differences shown in levels between other groups. As shown in (Table 5).

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

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

* *P*<0.001, H.C: Healthy controls, S.E: Standard error

Table 4: Level of TNF-α, IL-10 and soluble HLA-DR in patients with different stages of sepsis.

Severity of sepsis Factor	**SIRS**	**Sepsis**	**Severe sepsis**	**Septic shock**	**Sig**	***P*.value**
No.	19	28	17	11		
TNF-α	52.63 ±6.00	77.09 ± 9.40	78.04 ± 9.98	70.74 ± 10.29	1.655	0.185
IL-10	19.84 ±3.14	61.52 ± 8.45	81.89 ±17.32	72.12 ± 26.41	4.370*	0.007
sHLA-DR	7.31 ±1.37	18.50 ± 1.80	15.08 ± 2.64	15.50 ± 3.29	5.514*	0.002

(*P<0.05)

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Table 5: LSD comparison in level of IL-10 and sHLA-DR between stages of sepsis.

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

*P<0.05

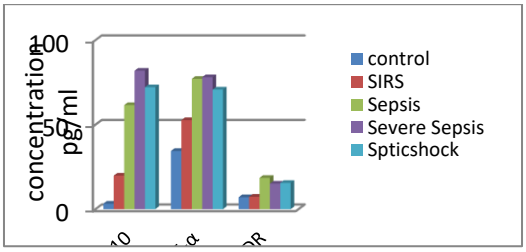

Figure 1: level of IL-10, TNF-α and HLA-DR in different sepsis stages.

Table 6: Fold change of mRNA expression for *tlr2* and *tlr4* gene and differences between stages.

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

*P<0.05

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Expression of TLR2, 4 mRNA in whole blood.

TLR2 and TLR4 mRNA expressions were increased about 1.95 and 2.29 fold, respectively, patients with septic syndrome patients compared with healthy controls. Also, there are a significant variation P<0.05 in the expression among stages, and there is a weak positive correlation between TLR 2 and 4 but this correlation was not significant (Table 6). LSD comparison used to determine the differences between each stage. The results have been shown significant differences in the expression of TLR2 between SIRS and sepsis patients (P<0.05), also, the significant differences were shown in the expression of TLR4 between SIRS patients and both of sepsis and severe sepsis patients. As shown in (Table 7).

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

As shown in (Figure 2), TLR4 mRNA expression in the whole blood of patients are positively correlated with serum sHLA-DR level (R=0.348, P=0.006), whereas no 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).

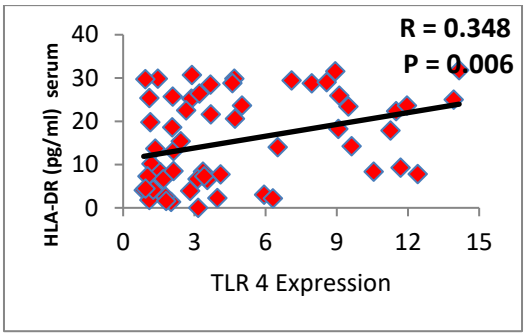

Figure 2: Correlation between TLR4 (r = 0.348; p = 0.006) and sHLA-DR secretion of whole blood in patients with sepsis

Table 7: LSD comparison in level of expression for *tlr2* and *tlr4* gene and differences between stages.

		TLR2		**TLR4**	
Sepsis stage		**Mean ±S.E**	**Sig**	**Mean ±S.E**	**Sig**
SIRS	Sepsis	2.13±0.84	0.015*	3.46±1.23	0.007*
	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

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IV. 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 our study shown the levels of TLR2, TLR4 protein expression has increased in whole blood, high level of cytokines was released after in vitro antigen stimulation in cells and high level of soluble HLA-DR in serum samples of patients with septic syndrome with significant differences between each group. The data were shown receding by the TLR mRNA expression for in whole blood of patients, based on sepsis status: lowest expression of TLR has been shown in SIRS as a first step of infection, followed by septic shock, severe sepsis and sepsis. This result agrees with [18] who have been founded 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. But our results different with Brunialti et al, 2006, they 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 a 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 crucial role in microbial responses indicate that they possibly will 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 following 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 outpatient cases) [23]. In sepsis, a pro-inflammatory phase (SIRS) is provoked to remove the pathogen. The results of study shown an increase level of TNF-α concentration in SIRS patients compared with healthy control (52.63 ± 6.00 vs 34.42 ± 2.07) $P < 0.000$, the level of TNF-α increase within increasing of the severity of sepsis (table 2) with significant difference with the level of concentration in H.C, $P = 0.000$. 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 showed by Kocabas et a.l, 2007 [25]. Dinata et al., 2013 that showed plasma level of TNF-α in severe sepsis patients to be significantly higher than those in sepsis patients [26].

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Our results have been shown that the level of IL-10 in serum increase significantly in SIRS, sepsis, severe sepsis and septic shock compared to a H.C group, 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 related 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 level has been measured and the concentration was increased in all patients significantly compared with control groups except patients $P< 0.05$ with SIRS there were no differences between them and H.C group.

Our results 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 hyperinflammatory [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. Furthermore, 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 ($R = 0.348$, $P < 0.006$). 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

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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 is let to 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, we can consider TLRs act an important role in sepsis development and sepsis-related organ injury via both endogenous ligands and exogenous pathogens, this result came to decrease mortality and the development of infection especially in Iraqi patients and diminishing the time that taken in sepsis diagnosis.

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