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TOLL-LIKE RECEPTORS (TLR) 2, 3, AND 4 GENE POLYMORPHISMS IN CRITICALLY ILL PATIENTS

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Abstract – Considering that TLR2, TLR3 and TLR4 play very important roles in inflammatory processes, the question arises whether the presence of polymorphisms in these genes is associated with susceptibility to sepsis. The aim of this study was to examine the association of *TLR2*, *TLR3* and *TLR4* polymorphisms with clinical characteristics and outcome of critically ill patients. A follow-up study was conducted on 121 critically ill Caucasian Serbian patients. Five polymorphisms in *TLR*s, *TLR2* (rs5743708), *TLR3* (rs3775291, rs5743312) and *TLR4* (rs4986790, rs4986791) were genotyped by real-time PCR. Investigated polymorphisms in *TLR2* and *TLR4* were not associated with the clinical characteristics and outcome of critically ill patients. *TLR3* rs3775291 polymorphism was associated with patient's outcome (p=0.018). Patients with sepsis and a *TLR3* rs3775291-mutated genotype had a four-fold higher mortality rate compared to wild type and heterozygous carriers. Multivariate regression analysis showed that age, sex and *TLR3* rs3775291 polymorphism are independent variables of the outcome of critically ill patients. For the first time, our preliminary findings indicate the role of TLR3 with MyD88 independent signaling and its polymorphism (*TLR3* rs3775291) in sepsis and survival in critically ill Serbian patients.

Key words: TLR polymorphisms; critically ill patients; sepsis; trauma

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INTRODUCTION

Sepsis is the leading cause of mortality and morbidity in intensive care units (Martin et al., 2003). Repair of severe tissue injury (traumatic or non-traumatic) is a complex, dynamic and interactive physiological, cellular, biochemical, and molecular process. This process involves coordinated recruitment, proliferation, and intracellular communications amongst multiple cell types, including inflammatory cells, local and mobilized distant stem cell/progenitor cell populations and vascular endothelial cells. The most serious complication of major injury is the sequential dysfunction of vital organs (Multiple Organ Dysfunction Syndrome, MODS), which is usually associated with severe sepsis (Wang, 2005). Therefore, the pre-

vention of sepsis is fundamental in the treatment of trauma patients. Genetic variants, in particular single nucleotide polymorphisms (SNP) could be critical determinants of inter-individual differences of the inflammatory response and clinical recovery of critically ill patients (Schroder and Schumann, 2005; Jiang, 2005). Variations in the genes and their associated differences in response to injury may participate in the development of new genetic diagnostic and therapeutic interventions that can improve the outcomes and recovery of patients with severe trauma.

Any cause that leads to infection, stress and tissue damage with the delivery of danger signals is recognized by the immune system according to the accepted "danger model" of the immune response. Cells of the immune system are activated by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Inflammation occurs as the primary response to the presence of PAMPS and DAMPs that are recognized by the pattern recognition receptors (PRRs). PRRs begin the production of inflammatory mediators that can alter the function of many tissues and organs (Medzhitov and Janeway, 2002; Figdor et al., 2002; Underhill, 2007). Toll-like receptors (TLRs) represent the most important PRR group. TLR ligation can trigger intracellular signals that lead to phenotypic and functional changes in cells that are recognized appropriate ligands by these receptors (Kawai and Akira, 2010). The existence of 10 TLRs types is confirmed in humans. TLRs recognize and bind a wide spectrum of PAMPs (Barbalat et al., 2011).

TLR2, TLR3 and TLR4 play an important role in inflammatory processes in different pathological conditions, including severe sepsis (Verstak et al., 2007). TLR2 is involved in the recognition of a wide spectrum of Gram-positive and Gram-negative bacterial molecules, fungi, parasites and viruses (Akira et al., 2006). TLR4 is essential for the recognition of bacterial lipopolysaccharide (LPS). TLR4 interacts with three different extracellular proteins: the LPS binding protein (LBP), CD14 and myeloid differentiation protein 2 (MD-2) (Kawai and Akira, 2010). Because of its activation, MyD88- and TRIF-dependant pathways are activated, which leads to inflammatory cytokine induction (Kawai and Akira, 2009). TLR3 is the only TLR that does not use the MyD88-dependent pathway and TLR3 recognizes double-stranded RNA (Barbalat et al., 2011). Although the activation of an innate immune response by PAMPs has been reported to contribute to hyper-inflammation and organ injury during sepsis, many aspects of sepsis immunopathogenesis need further elucidation. It is possible that the presence of SNPs in TLR genes could compromise their function and contribute to complex phenotypes, including severe sepsis. In a previous paper by our group, we showed a significant association of $CD14_{159}$ polymorphism with the type of infectious microorganism in critically ill patients (Surbatovic et al., 2010). Considering that TLR2, TLR3, and TLR4 play very important roles in inflammatory processes through different signaling pathways, the question arises whether the presence of SNPs in these genes is associated with the underlying cause of sepsis, the type of infectious microorganisms and the outcome of critically ill patients. In addition, associations of these SNPs with the development of secondary sepsis in trauma patients were analyzed. Analysis of the SNPs of the above genes could be important in defining the pathogenetic mechanisms and potential therapeutic targets in these patients.

The aim of this study was to examine the polymorphisms of TLR genes with different signaling pathways and the association of these polymorphisms with outcome, the underlying cause of sepsis (pancreatitis, peritonitis) or type of infectious microorganisms (Gram-positive, Gram-negative, mixed) in critically ill Serbian patients. In addition, associations of these TLR polymorphisms with the development of secondary sepsis in trauma patients were analyzed.

MATERIALS AND METHODS

Ethics statements

This study was approved by the Ethics Committee of the Military Medical Academy, Belgrade, according

to the Helsinki Declaration from 2008. Informed consents were obtained from all individuals involved in the study or from a first-degree relative.

Study group and samples

The study group consisted of 121 critically ill patients with severe sepsis and/or trauma on admission to the surgical intensive care unit (ICU) at the Military Medical Academy (Belgrade, Serbia) from July 2010 to May 2012. The Simplified Acute Physiology Score II (Le Gall et al., 1993), Acute Physiology and Chronic Health Evolution II score (Knaus et al., 1985), and Sequential Organ Failure Assessment score (Moreno et al., 1999) 24 h after ICU admission were calculated and recorded. The patients' demographic and clinical characteristics are shown in Table 1. Determination of trauma severity was performed using the Injury Severity Score (ISS). ISS was determined using the Abbreviated Injury Scale. The majority of trauma patients were casualties from motor vehicle accidents with blunt and/or penetrating trauma. All patents with trauma with or without the development of sepsis had a similar severity of trauma. Sepsis patients entered the study if they met the following criteria (according to the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference): documented or suspected infection plus the presence of systemic inflammatory response syndrome and sepsis-associated organ dysfunction, hypotension, hypoperfusion (hyperlactatemia >2mmol/L). Exclusion criteria included age below 18 years, pregnancy, severe chronic respiratory disease, severe chronic liver disease, malignancy, use of high-dose immunosuppressive therapy, and AIDS. To lower potential confounding influences due to different ethnic backgrounds, only Caucasians of the Serbian population were enrolled in this study. Informed consent was obtained from subjects or from their legal surrogates before enrollment. The follow-up period was one year. The demographic and clinical characteristics of critically ill patients divided into subgroups according to the cause of critically status and present of sepsis are shown in Table 2. The 80 critically ill patients had severe sepsis without trauma. Of 41 trauma patients, 20 developed secondary sepsis. Peripheral blood samples were collected from the patients and stored at -20^{0}C, until DNA isolation.

DNA isolation and genotyping

DNA was isolated using the GeneJet Genomic DNA Purification kit according to the manufacturer's instructions (Fermentas, St. Leon-Rot, Germany). Polymorphisms in TLR2, TLR3 and TLR4 genes were determined by the real-time PCR method, using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster city, CA, USA). Obtained genotypes were analyzed using SDS 7500 Software (Applied Biosystems). Details on used TaqMan Assays are available upon request.

Statistical analysis

Statistical analysis was performed using SPSS software, version 20.00 (SPSS Ins., Chicago, IL, USA). Contingency tables were analyzed by the chi-square test or Fisher's exact test when appropriate. For survival analysis, Kaplan-Meier curves were used and compared by the log-rank test. Finally, because variables are inter-related, multivariate regression analysis stepwise method was performed to assess the independent variables that may explain the outcome of critically ill patients. The probability of F was used to select the variables to be included in the model, the variables with p-values <0.05 were entered and variables with p-values >0.10 were removed from the model. All reported p values were two-sided and considered as significant if were less than 0.05.

RESULTS

Genotype and allele distribution in TLR gene polymorphisms

The distribution of analyzed polymorphisms in TLR genes in the group of critically ill Caucasian Serbian patients is presented in Table 3. the percentage of alleles for the investigated *TLR2* polymorphism was similar. On the other side, the distribution of wild type C and G alleles for *TLR3* polymorphisms

Table 1. Demographic and clinical characteristics of critically ill patients.

	Critically ill patients n=121
Age (years)	56 ± 19
Sex, n (%)	
Male	76(63)
Female	45(37)
Simplified Acute Physiology Score II, mean ± SD	56.70 ± 9.10
Acute Physiology and Chronic Health Evolution II score, mean ± SD	23.07 ± 3.20
Sequential Organ Failure Assessment score, mean ± SD	7.20 ± 2.56
Reason for ICU admission, n (%)	
Severe sepsis without trauma	80 (74)
Severe trauma (ISS 27.8 ± 9.8)	41 (26)
Cause of sepsis, n (%)	
Pancreatitis	17 (17)
Peritonitis	63 (63)
Trauma	20 (20)
Blood culture, n (%)	
Sterile	45 (37)
Mixed	49 (41)
Mixed + fungi	4 (3)
Gram-positive	16 (13)
Gram-negative	5 (4)
Fungi	2 (2)
Outcome, n (%)	
Death	57 (47)
Survival	64 (53)
Mortality, n (%)	
< 28 days	39 (68)
< 90 days	14 (24)
> 90 days	2 (4)
> 12 months	2 (4)

Data are expressed as mean ± SD or absolute number (percentage). ISS, Injury Severity Score; ICU, Intensive Care Unit.

rs5743312 and rs3775291 were higher compared to mutated T and A alleles, respectively.

The polymorphism prevalence in the subgroups of critically ill patients is presented in Table 4. There were no significant differences in genotype frequencies for all SNPs studied among the analyzed subgroups. It should be noted that only patients with *TLR3* (rs5743312)-mutated genotype had sepsis with peritonitis as the underlying cause. On the other hand, patients with trauma and mutation in *TLR3* rs3775291 polymorphism (10% of trauma patients with sepsis) developed sepsis.

Associations of TLR gene polymorphisms with clinicopathological variables

Associations of TLR gene polymorphisms with clinicopathological variables are presented in Table 5. *TLR3* rs3775291 was associated with patient's out-

Table 2. Demographic and clinical characteristics of critically ill patients divided in subgroups.

	Patients with sepsis/pancreatitis (n=17)	**Patients with sepsis/peritonitis (n=63)**	**Patients with trauma (n=21)**	**Patients with trauma and sepsis (n=20)**	p value
Age (years)	52 ± 16	63 ± 17	54 ± 19	39 ± 16	*, #, §§§, ††
Sex, n (%)					
Male	12 (71)	29 (46)	17 (81)	18 (90)	°°
Female	5 (29)	34 (54)	4 (19)	2 (10)	
Blood cultures, n (%)					°°
Sterile	11 (64)	13 (21)	21 (100)	13 (65)	
Mixed	4 (24)	32 (51)	0 (0)	0 (0)	
Mixed with fungi	2 (12)	2 (3)	0 (0)	0 (0)	
Gram-positive	0 (0)	11 (17)	0 (0)	5 (25)	
Gram-negative	0 (0)	4 (6)	0 (0)	1 (5)	
Fungi	0 (0)	1 (2)	0 (0)	1 (5)	
Outcome, n (%)					
Death	7 (41)	40 (64)	4 (19)	6 (30)	°°
Survival	10 (59)	23 (36)	17 (81)	14 (70)	
Mortality, n (%)					
< 28 days	4 (57)	28 (70)	4 (100)	3 (50)	
< 90 days	1 (14)	11 (27)	0 (0)	2 (33)	
< 3 > 12 months	2 (29)	0 (0)	0 (0)	0 (0)	
> 12 months	0 (0)	1 (3)	0 (0)	1 (17)	

Data are expressed as mean ± SD or absolute number (percentage). °° $p < 0.01$ between groups one way ANOVA. * $p < 0.05$, patients with sepsis pancreatitis *vs* patients with sepsis peritonitis. # $p < 0.05$, patients with sepsis pancreatitis *vs* patients with trauma and sepsis. §§§ $p < 0.0001$, patients with sepsis peritonitis vs. patients with trauma and sepsis. †† $p < 0.01$, patients with trauma and sepsis *vs* patients with trauma without sepsis.

come (p=0.018). A significant tendency toward association between *TLR3* rs3775291 polymorphism and the type of pathogen detected in the blood was noticed (p=0.078). No significant associations were observed between the other TLR gene polymorphisms and analyzed variable characteristics in the critically ill patients (Table 5). Patients with sepsis and *TLR3* rs3775291-mutated genotype had a four-fold higher mortality rate compared to the wild type and heterozygous carriers.

Survival analysis

A significant decrease in overall survival was observed in critically ill patients with mutated genotype in *TLR3* rs3775291 polymorphism compared to patients with wild type and heterozygous genotype (p=0.029, log-rank test) (Fig. 1). There were no significant differences in overall survival for the other studied TLR gene polymorphisms (data not shown).

Multivariate regression analysis

Multivariate regression analysis according to the stepwise model was used to assess the independent variables that can affect outcome in sepsis (survival) (Table 6). The variables entered in the model as independent variables were age, sex and all investigated TLR polymorphisms. In critically ill patients as the dependent variable, the significantly independent

Table 3. Distribution of TLRs genes polymorphisms in the group of critically ill patients.

Gene/SNP	Genotype	N (%)	Allele	N (%)
	CC (*wt*)	15 (12)	C	105 (43)
TLR2	CT (*het*)	75 (62)		
rs3804099	TT (*mut*)	31 (26)	T	137 (57)
	CC (*wt*)	91 (75)	C	210 (87)
TLR3	CT (*het*)	28 (23)		
rs5743312	TT (*mut*)	2 (2)	T	32 (13)
	GG (*wt*)	54 (45)	G	160 (66)
TLR3	GA (*het*)	52 (43)		
rs3775291	AA (*mut*)	15 (12)	A	82 (34)
	CC (*wt*)	108 (89)	C	229 (95)
TLR4	CT (*het*)	13 (11)		
rs4986791	TT (*mut*)	0 (0)	T	13 (5)
	AA (*wt*)	108 (89)	A	229 (95)
TLR4	AG (*het*)	13 (11)		
rs4986790	GG (*mut*)	0 (0)	G	13 (5)

Wt/het/mut-wild type/heterozygote/mutated

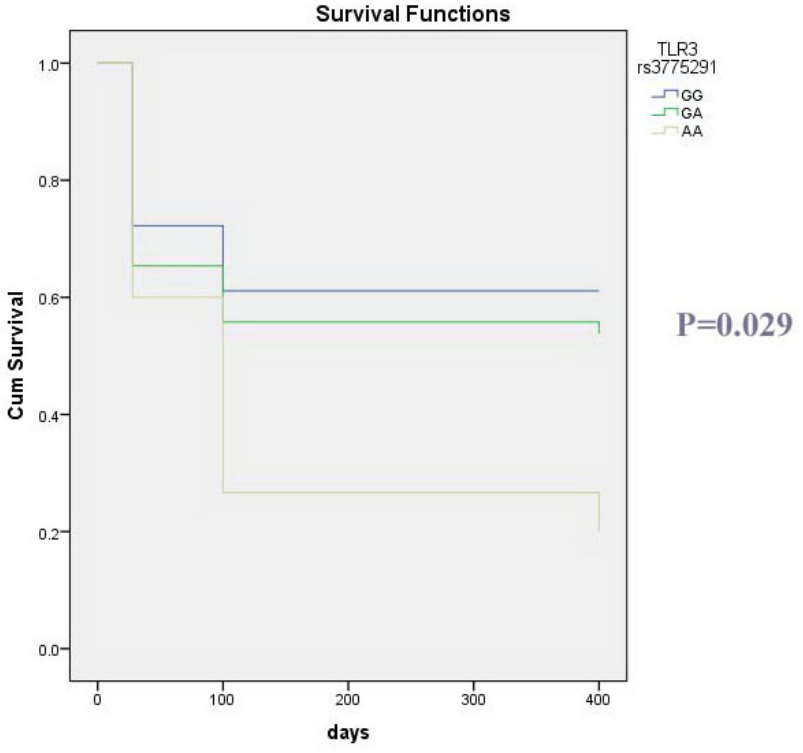

Fig. 1. Kaplan-Meier curve of overall survival of critically ill patients' dependent of TLR3 rs3775291 polymorphisms compared by the long-rank test.

Table 4. Distribution of TLRs genes polymorphisms in the subgroups of critically ill patients.

Gene/SNP genotype	Patients with sepsis/pancreatitis (n = 17)	Patients with sepsis/ peritonitis (n = 63)	Patients with trauma without sepsis (n = 21)	Patients with trauma and sepsis (n = 20)	p value
TLR2 (rs3804099), n (%)					
CC (*wt*)	3 (18)	6 (10)	2 (9)	4 (20)	
CT (*het*)	8 (47)	43 (68)	13 (62)	11 (55)	NS
TT (*mut*)	6 (35)	14 (22)	6 (29)	5 (25)	
TLR3 (rs5743312), n (%)					
CC (*wt*)	13 (77)	48 (76)	15 (71)	15 (75)	
CT (*het*)	4 (23)	13 (21)	6 (29)	5 (25)	NS
TT (*mut*)	0 (0)	2 (3)	0 (0)	0 (0)	
TLR3 (rs3775291), n (%)					
GG (*wt*)	10 (59)	26 (41)	9 (43)	9 (45)	
GA (*het*)	3 (18)	28 (45)	12 (57)	9 (45)	NS
AA (*mut*)	4 (23)	9 (14)	0 (0)	2 (10)	
TLR4 (rs4986791), n (%)					
CC (*wt*)	14 (82)	57 (91)	19 (91)	18 (90)	
CT (*het*)	3 (18)	6 (9)	2 (9)	2 (10)	NS
TT (*mut*)	0 (0)	0 (0)	0 (0)	0 (0)	
TLR4 (rs4986790), n (%)					
AA (*wt*)	14 (82)	58 (92)	19 (91)	17 (85)	
AG (*het*)	3 (18)	5 (8)	2 (9)	3 (15)	NS
GG (*mut*)	0 (0)	0 (0)	0 (0)	0 (0)	

Data are expressed as an absolute number (percentage). NS, non-significant; statistical analysis performed by χ^2 test. *Wt/het/mut- wild type*/heterozygote/mutated

variables were age, sex and *TLR3* rs3775291 polymorphism.

DISCUSSION

To the best of our knowledge, this is the first report of an association between *TLR3* polymorphism and survival in critically ill patients that indicates the role of *TLR3* polymorphisms in sepsis. The general major observation in this study is that critically ill Serbian patients with *TLR3* rs3775291-mutated genotype had a significant decrease in overall survival compared to patients with wild type and heterozygous genotype. The obtained results make it possible to establish a link between a specific genotype in the TLR3 gene and factors of tissue with injury and/or infection, which, in future, would be used for implementing molecular-genetic diagnostic procedures for individual treatment of critically ill patients and predicting outcome of the disease.

Sepsis begins with the production of mediators that lead to MODS with high mortality rate. The immune pathogenesis of sepsis is very complex (Hotchkiss and Karl, 2003). The immune system uses a naive and adaptive immune system to respond to danger signals. Cells of naive immunity recognize PAMPs and DAMPs by PRRs and activate a cascade of preformed plasma proteins and immune cells such as monocytes, macrophages, dendritic cells, neutrophils and lymphocytes. The fast production of pro-inflammatory mediators

Table 5. Association of TLR polymorphisms with sex diagnosis, blood cultures, mortality and outcome of critically ill patients.

	N	TLR2 (rs3804099) *wt/het/mut*	TLR3 (rs5743312) *wt/het/mut*	TLR3 (rs3775291) *wt/het/mut*	TLR4 (rs4986791) *wt/het/mut*	TLR4 (rs4986790) *wt/het/mut*
Sex						
Male	76	9/46/21	56/19/1	34/35/7	70/6/0	69/7/0
Female	45	6/29/10	35/9/1	20/17/8	38/7/0	39/6/0
p^*		NS	NS	NS	NS	NS
Diagnosis						
Sepsis pancreatitis	17	3/8/6	13/4/0	10/3/4	14/3/0	14/3/0
Sepsis peritonitis	63	6/43/14	48/13/2	26/28/9	57/6/0	58/5/0
Trauma with sepsis	20	4/11/5	15/5/0	9/9/2	18/2/0	17/3/0
Trauma	21	2/13/6	15/6/0	9/12/0	9/12/0	9/12/0
p^*		NS	NS	NS	NS	NS
Blood culture						
Sterile	45	5/25/15	30/15/0	21/23/1	39/6/0	39/6/0
Mixed	49	7/36/6	37/10/2	20/21/8	44/5/0	45/4/0
Mixed with fungi	4	1/2/1	4/0/0	2/0/2	4/0/0	4/0/0
Gram positive	16	2/8/6	13/3/0	8/5/3	15/1/0	15/1/0
Gram negative	5	0/2/3	5/0/0	2/3/0	4/1/0	3/2/0
Fungi	2	0/2/0	2/0/0	1/0/1	2/0/0	2/0/0
p^*		NS	NS	0.078	NS	NS
Outcome						
Death	57	8/39/10	41/15/1	21/24/12	49/8/0	49/8/0
Survival	64	7/36/21	50/13/1	33/28/3	59/5/0	59/5/0
p^*		NS	NS	**0.018**	NS	NS
Mortality						
< 28 days	39	6/27/6	25/13/1	15/18/6	35/4/0	35/4/0
< 90 days	14	0/10/4	12/2/0	6/4/4	12/2/0	12/2/0
< 3 > 12 months	2	1/1/0	2/0/0	0/1/1	1/1/0	1/1/0
> 12 months	2	1/1/0	2/0/0	0/1/1	1/1/0	1/1/0
p^*		NS	NS	NS	NS	NS

N total number of patients. NS, non-significant; statistical analysis performed by χ^2 test. [a] Age according to median value of 56 years. *p<0.05 are presented in bold.

is usually related to a simultaneous production of anti-inflammatory mediators in order to maintain homeostasis in the body. Inflammatory mediators implement mechanisms that will lead to either recovery or death of the organism by mechanisms of sepsis. TLR signaling plays an important role in initiation of the inflammatory response in sepsis (Akira et al., 2001). Based on the primary structure, TLRs can be divided into several subfamilies, each of which recognizes a similar PAMP: subfamily TLR1, TLR2 and TLR6 recognizes lipids and TLR7, TLR8 and TLR9 recognize nucleic acids. However, the main division of TLRs was performed according to their position in the cell and the corresponding PAMP ligands. The group TLR1, TLR2, TLR4, TLR5, and TLR6 was expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 were located in intracellular compartments such as the endoplasmic reticulum, endosomes, lysosomes, and endolysosomes (Underhill, 2007). Although TLRs are mainly expressed in immune cells, phagocytes and antigen-presenting cells, more studies have shown that they are also expressed in the cells of different tissues, including cells of the adrenal glands, gastrointestinal tract, brain, and kidney (Doi et al., 2009). Prolonged and excessive activation of TLRs and their signaling cascades contributes to the pathogenesis of sepsis. Regarding all presented data, sep-

Table 6. Multivariate regression analysis (stepwise model) with outcome of sepsis (survival) as dependent variables in critically ill patients.

	B	p	F (p)
Outcome of sepsis (survival)			
(Constant)	2.149		
Age	-0.013	0.000	
Sex	0.218	0.006	10.959
TLR3 rs3775291	-0.139	0.012	(0.000)
$R^2 = 0.353$			

pB-parameter estimate; F-Fisher test.

sis could be seen as a TLR-mediated dysregulation of the immune system following pathogen invasion in which the careful balance between inflammatory and anti-inflammatory responses is vital.

Susceptibility and response to infection is, in part, heritable. There is evidence that genetic factors may be relevant and important determinants for interindividual differences in susceptibility to infection (Arcaroli et al., 2005; Kumpf and Schumann, 2008). The essential role of TLRs in inflammation initiation makes them interesting candidates for genetic analysis. We have found that one quarter of our critically ill patients (28) had *TLR2* rs3804099-mutated genotype, 12 patients had *TLR3* rs3775291-mutated genotype, and only 2 patients had *TLR3* rs5743312-mutated genotype. In 3 patients, we detected the simultaneous presence of *TLR2* rs3804099- and *TLR3* rs3775291-mutated genotypes. Regarding the presence of genotypes in the investigated cohort of critically ill Serbian patients, the percentage showed that alleles of *TLR2* are equally distributed, while wild type C and G alleles of investigated *TLR3* polymorphisms were more frequent compared to mutated T and A alleles, respectively. Significant differences in the distribution of alleles of both investigated *TLR4* polymorphisms were detected as well. Song and colleagues (2011) showed that in the Chinese Han population with sepsis, a lower minor allele frequency (0.32) was detected than in our population (0.57). *TLR4* rs4986791 and rs4986790 mutations were found within approximately 5-10% of the total population and were first identified by Arbour et al. (2000) in studies describing these SNPs' association to a blunted response to inhaled LPS in humans. It has been reported that mutated genotypes of *TLR4* rs4986791 and rs4986790 SNPs were associated with increased susceptibility to Gram-negative bacterial infection (Arbour et al., 2000). In contrast to these data, in our study there were no mutated genotypes of both investigated *TLR4* polymorphisms.

There are several studies of the role of functionally relevant *TLR* polymorphisms in sepsis and trauma. TLR2 and TLR4 are expressed in human adrenals (Lee et al., 2002) and suggest that both receptors may be involved in the hypothalamic-pituitary-adrenal axis function with a significant role in the development of immunosuppressed conditions in sepsis. Zacharowski et al. (2006) showed that TLR4 is a major mediator in the crosstalk between the innate immune system and the endocrine stress response in an animal model of systemic inflammatory response syndrome. TLR4 is involved in the signaling of both exogenous and endogenous danger signals and is the prototype of TLR involved in sepsis. Additionally, TLR4 is involved in hemorrhage shock signaling in the interaction with High-Mobility Group Box-1 (HGMB-1), ischemia-reperfusion signaling, toxic challenge signaling, tissue trauma, burn, and wound repair signaling (Castellheim et al., 2009). TIRAP/Mal is an important adaptor molecule for intracellular signaling of both TLR2 and TLR4 (Fitzgerald et al., 2001). Kumpf et al. (2010) showed that the presence of *TLR4* mutations (rs4986791 and rs4986790) in combination with *TIRAP/Mal* variants resulted in a significant increase in the risk of severe infection. Aberrant functioning of the TLR/

CD14 pathway of innate immunity changes the risk of infectious complications in patients with severe trauma. It was shown that the *TLR2* T-16934A TA genotype increased the risk of a Gram-positive infection and SIRS trauma patients (Bronkhorst et al., 2013). The same authors showed that TLR4 variation seemed unrelated to the outcome of sepsis. This result in *TLR4* polymorphism is in line with the *TLR4* polymorphisms in our cohort of critically ill patients. Additionally, in agreement with the literature and our data are the results of Jessen et al. (2007) who showed no correlation exists between severity or outcome of sepsis and TLR4. TLR2 signaling plays a critical role in orchestrating the innate immune response and the development of sepsis (Arcaroli et al., 2005; Hoth et al., 2007). Upregulation of *TLR2* gene expression was observed in trauma (Hoth et al., 2007), which might be a mechanism for injury that primes the innate immunity (Peterson et al., 2007). *TLR2* rs5743704 and rs5743708 polymorphisms could affect transmembrane signaling (Bochud et al., 2003) and be associated with susceptibility to microbial infections, such as Gram-negative sepsis (Woehrle et al., 2008). The results of Chen et al. (2011) indicate that *TLR2* rs3804099 polymorphism is associated with sepsis morbidity rate in patients with major trauma and might be used as relevant risk estimates for the development of sepsis and MOD. In addition, the same author reported the clinical relevance of *TLR2* rs1898830 and rs7656411 polymorphisms to sepsis. The results of *TLR2* rs3804099 polymorphism in our cohort of critically ill patients are not in accordance with presented literature data, since any associations for *TLR2* polymorphism were not observed in our study. Additionally, gender might be one of the factors influencing susceptibility to sepsis in trauma patients. Male gender has been shown to be associated with an increased risk for sepsis in some studies (Chen et al., 2011; Eachempati et al., 1999). Our results also revealed that 19 of the 28 critically ill patients with a variant homozygous genotype of *TLR2* rs3804099 were males. It is known that TLR2, although regulating the activation of immune cells by a wide range of pathogens, is a PRR mainly responsible from Gram-positive bacteria (Jiang et al., 2000). However, literature data indicate that the predominant pathogens inducing traumatic sepsis are Gram-negative bacteria, although a mixed infection often occurs in patients with trauma (Jiang, 2009). In line with these data, in our patients mixed infection as the most frequent (41%) was detected, while Gram-positive bacteria were detected in 13% of patients and Gram-negative bacteria in 4% of patients. This might be a reason why the *TLR2* polymorphisms are relatively less associated with sepsis and posttraumatic complications in our study.

To our knowledge, this is the first study that investigates *TLR3* polymorphisms in sepsis. We showed a decreased overall survival in critically ill patients with *TLR3* rs3775291-mutated genotype. *TLR3* rs3775291 is located in exon 4 and is a missense mutation (G > A, Leu412Phe) resulting in a functionally impaired receptor. In addition, multivariate regression analysis showed that age, sex and *TLR3* rs3775291 polymorphisms were independently associated with survival in critically ill Serbian patients. Furthermore, a potentially interesting finding is that only trauma patients with *TLR3* rs3775291-mutated genotype (5% of all trauma patients) developed sepsis. These results could indicate that functional TLR3 and TLR3-mediated signaling is necessary for an adequate response in the prevention of sepsis development in trauma patients. On the other hand, all critically ill patients with *TLR3* rs5743312-mutated genotype had sepsis with peritonitis as the underlying cause. This result could indicate that the normal function of TLR3 with MyD88-independent signaling has a potential role in an adequate response to injury and infections in the peritoneum in humans. In literature, there are no data about the role of *TLR3* in sepsis and the susceptibility to develop sepsis in patients with trauma. Chen et al. (2011) showed that *TLR9* polymorphisms rs187084 and rs352162 might be used to provide relevant risk estimates for the development of sepsis and MODs in patients with major trauma. These data together with our results about *TLR3* polymorphism indicate that endosomal TLRs that sense different intracellular pathogens and intracellular damage that use a different signaling cascade have a potential role in the de-

velopment of severe inflammation and sepsis in patients with trauma and severe surgery. In addition, nonpathogenic factors, such as ischemia and hypoxia from hypoperfusion occurring immediately after trauma, may also lead to the development of sepsis and MODS (Soreide, 2009). Ferreira et al. (2013) presented the interesting fact that PPARγ activation impaired TLR responses by inhibiting the expression of MyD88 and preventing a systemic inflammatory response. Considering these data, the important finding is that PPARγ-2 12Ala allele frequency in healthy Serbs is in the same range as in other Caucasians (Lukic et al., 2013).

The potential limitations of the current study include the relatively small sample size and this study can be considered as preliminary results. However, a major limitation in this approach is the potential for stratification when inappropriate patient-control matching occurs. The only population studied was Serbian, and therefore the results may not be generalized to other populations. Nevertheless, because we enrolled a highly selected and clinically clear cohort of critically ill patients, we could confidently interpret our results. However, the *TLR3* polymorphisms were negatively associated with survival period, and this indicates the need for further study of the association of the cytokine level and signaling cascade induced by these TLRs in critically ill patients.

In conclusion, the present study has provided evidence that *TLR3* rs3775291polymorphism might be used as a predictor of outcome in critically ill patients with sepsis, and it has shown a negative impact of mutated genotype on the survival of critically ill patients. It suggests that MyD88-independent signaling may exert an important role in the pathogenesis of sepsis. *TLR3* rs3775291 polymorphism may be a useful marker to identify patients with a high risk of developing sepsis and high risk for lethal outcome, but it needs to be validated in larger prospective studies. Early genotyping may prove to be helpful in the future in identifying critically ill patients at risk of severe sepsis and in identifying trauma patients at risk of infection/sepsis. Further steps are needed to elucidate the host response pathway and new approaches in sepsis trial design that take into account patient heterogeneity and phase of the immune response.

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Authors' contributions

Atia Elikalny and Katarina Zeljić performed the experiments and contributed to the preparation of the paper. Maja Surbatović and Dragan Djordjević treated patients, collected all clinical data for patients and their blood samples. Zvonko Magić contributed to the study design and supervised the experimental part of the study. Biljana Božić designed the study, performed all result analysis including statistical analysis, prepared the paper, and supervised the study.

Conflict of interest disclosure

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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