

Expression of TLR4 on Blood Monocytes Correlates with Severity of Ischemic Stroke

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ABSTRACT

Background: Accumulating evidence suggests that Toll-like receptors (TLRs) are important players in the pathophysiology of ischemic stroke (IS). In most cases of IS, endogenous ligands binding to TLRs activate monocytes to secrete pro inflammatory cytokines. It remains elusive as to whether there is any correlation between a National Institutes of Health Stroke Scale (NIHSS) score with TLR2/TLR4 expression on the blood monocytes and pro-inflammatory/anti-inflammatory cytokines in IS.

Methods: We used flow cytometry in 68 consecutive IS patients and 78 controls without IS to assess the TLR2/TLR4 expression on peripheral blood monocytes. Serum IL-1 β , IL-6, IL-10 cytokine levels were measured using ELISA.

Results: At baseline, there were no differences in baseline blood monocytes counts, serum IL-1 β and IL-10 levels between IS patients and controls. Serum concentrations of IL-1 β and IL-6 were significantly increased in IS patients after 10 days. Serum concentrations of IL-1 β were positively correlated with CD14⁺CD284⁺ (%) ($r=0.64$; $p<0.05$), CD14⁺CD284⁺($\times 10^3/\mu\text{L}$) ($r=0.51$; $p<0.05$), CD14⁺HLADR⁺CD284⁺ (%) ($r=0.43$; $p<0.05$) and CD14⁺HLADR⁺CD284⁺ ($\times 10^3/\mu\text{L}$) ($r=0.43$; $p<0.05$) at day 10. NIHSS at day 10 was positively correlated with serum concentrations of IL-1 β ($r=0.69$; $p<0.05$), with CD14⁺CD284⁺ (%) ($r=0.63$; $p<0.05$) and CD14⁺CD284⁺($\times 10^3/\mu\text{L}$) ($r=0.51$; $p<0.05$) in IS patients. There were no significant correlations between NIHSS with IL-6 and IL-10 levels and TLR2 expression on the monocytes.

Conclusions: In human IS, upregulation of TLR4 expression on peripheral blood monocytes and IL-1 β production could be one of the peripheral mechanisms of brain inflammatory injury.

Keywords: TLR4, TLR2, Monocytes, Ischemic, Stroke

INTRODUCTION

Ischemic stroke (IS) is one of the leading causes of morbidity and mortality around the world. The mechanism underlying brain injury caused by cerebral ischemia and reperfusion remains very complex, but an inflammatory response has been found to be one of the main factors leading to brain damage in IS [1,2]. Accumulating evidence suggests that Toll-like receptors (TLRs) TLR2 and TLR4 are more important than other TLRs in the pathologic progression of cerebral ischemia and reperfusion [3,4]. TLR2 and TLR4 play crucial roles in modulating inflammatory response caused by cerebral ischemia and reperfusion via linking to their endogenous ligands, respectively [5,6]. Moreover, TLR4 polymorphisms were found to be associated with IS [7] and TLR2 and TLR4 expression on monocytes was associated with poor outcome in IS patients [8,9]. Early studies in rats described the arrival of circulating monocytes to capillaries

and venules of brain ischemic areas as early as 4 h after IS onset [10]. These monocytes are of supreme immunologic relevance because they are the main effectors of innate immunity through the expression of TLRs [11]. Existing studies in mice have shown a remarkably rapid activation of peripheral immunity after experimental IS [12]. Interestingly, as early as 6 h after middle cerebral artery occlusion, activated

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splenocytes secreted significantly enhanced levels of the pro-inflammatory cytokines (TNF- α , IL-1, IL-6) and also the anti-inflammatory IL-10 [12]. In most cases of IS, endogenous ligands binding to TLRs activate monocytes/macrophages to secrete IL-1 β and the level of IL-1 β mRNA was found to be decreased in the TLR2 and TLR4 deficient mice which suffered from thromboembolic IS [13]. IL-6 has been mainly secreted by astrocyte and microglia during the course of cerebral ischemia/reperfusion and it was demonstrated that the neurological impairments and the level of IL-6 were both improved more apparently in TLR4 deficient mice than in wild-type mice [14].

It remains elusive as to whether there is any correlation between monocyte numbers in peripheral blood, TLR2/TLR4 expression on the monocytes and pro-inflammatory/anti-inflammatory cytokines in IS. Elucidation of this question is required to judge the need of immunomodulatory therapies in IS and to define specific immune targets that might translate into a better clinical outcome.

MATERIALS & METHODS

Study subject

This study was a hospital-based case-control study conducted in patients attending the affiliated hospitals of Chita Medical Academy (Chita, Russia) from January 2013 to December 2017. We prospectively studied 68 consecutive IS patients with a National Institutes of Health Stroke Scale (NIHSS) score on admission by a certified neurologist. Patients were evaluated at a median of 180 min (interquartile range, 120-350) of stroke onset. Age and sex matched subjects without a history of cerebrovascular disease ($n=78$) served as control. They were recruited from community samples or hospital employees.

A full physical examination of all the subjects was carried out, and data involving smoking habits, body mass index (BMI), hypertension, diabetes mellitus, and dyslipidemia were recorded. The BMI was calculated by using the formula: body weight in kilograms divided by height in meters squared. Systolic and diastolic blood pressure was measured twice at an interval of 30 min using the automated oscillometric device. An average value of the two readings was considered. Exclusion criteria included a history of infection, use of steroids or immune suppressants within the preceding 3 months. We excluded those patients with possible concomitant infections by carefully looking for signs and symptoms of infectious diseases at the admission.

All patients underwent a brain CT scan or MRI on admission and were transferred and managed in the Stroke Unit according to the 2013 "Guidelines for the Early Management of Patients with Acute Ischemic Stroke" [15]. Neurological deficiency was assessed daily using the NIHSS until day 7. Patients were categorized according to NIHSS severity in

mild (NIHSS ≤ 8), moderate or severe (NIHSS ≥ 9) stroke [15].

The study was approved by the local ethics committee at Chita Medical Academy, Russia, and all participants or their legal representatives signed a written informed consent.

Laboratory analyses

Blood samples were collected between 8:00 AM and 9:00 AM at days 1, 10, and 21 after admission in IS patients and once in Control group patients.

Flow cytometry

The phenotype of monocytes was analyzed immediately after blood removal. Blood samples (100 μ l) were transferred into polystyrene FACS tubes and incubated with mouse anti-human CD14 V500 (BD Bioscience, San Jose, CA), mouse anti-human TLR2 PE-Cy7 (eBioscience, San Diego, CA), mouse anti-human TLR4 FITC (IMGEX, San Diego, CA), and mouse anti-human HLA-DR PerCP-Cy5.5 conjugated (BD Bioscience, San Jose, CA) antibodies. After 30 min at room temperature, 3 ml of FACS lysing solution (FACS Lysing Solution, BD Pharmingen) was added for additional 10 min. Then, the samples were centrifuged at 400 g for 5 min and washed twice with 4 ml phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (FACS buffer). Immediately after the supernatants were removed, the cells were diluted in 300 μ l FACS buffer and were then subjected to flow cytometry with the Cytomics FC-500 flow cytometer (Beckman Coulter Inc. Miami, USA). CXP Cytometer software (Beckman Coulter Inc. Miami, USA) was used for cytometry analysis. Corresponding mean fluorescence intensity (MFI) values were obtained for each expression. The monocyte population was defined by gating CD14+ cells in the corresponding forward and side scatter scan. From each sample, a minimum of 20,000 monocytes was measured.

Cytokines

Serum IL-1 β , IL-6, IL-10 cytokine levels were measured using an enzyme immunoassay kits (Vector-Best, Novosibirsk, Russia) for the quantitative determination of the cytokines in serum according to the manufacturer's protocol.

Statistical Analysis

The distribution of categorical variables was expressed as numbers and percentages. As the variables were non-normally distributed, they are presented as the median and interquartile range (IQR). For statistical comparison of more than two groups, one-way ANOVA was conducted using Bonferroni's post-hoc test. The unpaired *t*-tests were used to compare only two groups. Correlations were calculated with the Spearman Rank correlation coefficient. All tests were performed using the Statistica v. 6 software packages (StatSoft, Tulsa, OK, USA). $p < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

The main characteristics of the patients and the control groups are presented in (Table 1) Among 68 patients who presented with acute ischemic stroke, forty-six patients (67.6 %) had mild IS (defined as IHSS ≤ 8), and the remaining 22 patients had moderate or severe (NIHSS ≥ 9) stroke. Demographic

characteristics, as well as a rate of arterial hypertension, diabetes, hyperlipidemia, atrial fibrillation, smoking status, blood pressure, and laboratory characteristics, were not significantly different between the two IS groups. Nevertheless, the IS groups were characterized by higher levels of systolic and diastolic blood pressure, blood glucose and CRP than the Control group.

Table 1. Demographics, risk factors and clinical data of the study population.

	Mild IS (NIHSS ≤ 8) N=46	Moderate and Severe IS (NIHSS ≥ 9) N=22	Control group N=78	P ¹	P ²
Age years, mean $\pm$ standard deviation	61.4 $\pm$ 5.5	62.1 $\pm$ 5.8	62.8 $\pm$ 6.3	0.451	0.631
Male, n (%)	27 (58.7 %)	12 (54.5 %)	45 (57.8 %)	0.551	0.625
Smokers, n (%)	17 (36.9 %)	9 (40.1 %)	19 (24.4 %)	0.248	0.465
Hypertension, n (%)	25 (54.3 %)	14 (63.6 %)	29 (37.2 %)	0.808	0.616
Diabetes mellitus, n (%)	12 (26.1 %)	7 (31.8 %)	12 (15.4 %)	0.126	0.276
Hyperlipidemia, n (%)	21 (45.6 %)	12 (54.5 %)	35 (44.9 %)	0.801	0.919
Atrial fibrillation, n (%)	5 (10.9 %)	4 (18.2 %)	1 (1.3 %)	0.071	0.183
Prior TIA or stroke, n (%)	2 (4.3 %)	3 (13.6 %)	0	0.057	0.146
History of ischemic heart disease, n (%)	8 (17.4 %)	6 (27.3 %)	8 (10.3 %)	0.096	0.106
Antiplatelet treatment, n (%)	8 (17.4 %)	9 (40.1 %)	8 (10.3 %)	0.124	0.307
Anticoagulant treatment, n (%)	3 (6.5 %)	3 (13.6 %)	1 (1.3 %)	0.059	0.144
Statin treatment, n (%)	17 (36.9 %)	12 (54.5 %)	24 (30.8 %)	0.369	0.661
Body temperature, °C	36.4 $\pm$ 0.3	36.4 $\pm$ 0.4	36.5 $\pm$ 0.2	0.088	0.999
Systolic blood pressure, mmHg	159.5 $\pm$ 16.7	163.6 $\pm$ 18.1	141.7 $\pm$ 10.8	<0.001	0.360
Diastolic blood pressure, mmHg	91.4 $\pm$ 8.9	94.2 $\pm$ 11.5	84.2 $\pm$ 7.1	<0.001	0.289
Glucose levels, mg/dL	137.1 $\pm$ 42.3	134.9 $\pm$ 48.2	116.9 $\pm$ 21.7	0.003	0.849
Leukocytes, X10 ³ /mL	8.9 $\pm$ 3.2	9.2 $\pm$ 3.5	8.5 $\pm$ 2.7	0.563	0.727
Monocyte count, X /mL	574 $\pm$ 45	585 $\pm$ 61	582 $\pm$ 75	0.755	0.405
Platelets, X10 ³ /mL	257.9 $\pm$ 44.7	255.3 $\pm$ 46.8	226.3 $\pm$ 51.1	0.001	0.826
CRP, mg/dL	5.7 $\pm$ 2.5	6.1 $\pm$ 3.1	2.4 $\pm$ 1.9	<0.001	0.570
IL-1 β (pg/ml) median (IQR)	0 (0-0)	0 (0-0)	0(0-0)		
IL-6 (pg/ml) median (IQR)	4.78(4.03-6.45)	9.26(4.39-20.11)	1.74(1.05-3.31)		
IL-10 (pg/ml) median (IQR)	3.04 (0-4.13)	1.96(0.76-8.03)	0(0-0)		
NIHSS, median (IQR)					
Admission (day 1)	4.2(2.1-7.8)	12.4 (9.7 - 16.1)	0	<0.001	<0.001
Day 10	3.2 (1.9 - 6.4)	8.1 (3.5 - 11.2)	0	<0.001	<0.001

P¹: ANOVA between the patients with mild IS (NIHSS ≤ 8), moderate + severe IS (NIHSS ≥ 9) and Control groups;

P²: comparison between the patients with mild IS (NIHSS ≤ 8) and moderate + severe IS (NIHSS ≥ 9)

Monocytes and serum IL-1 β , IL-6 and IL-10 levels cytokine levels

There were no differences in baseline monocytes counts (Table 1) and serum IL-1 β and IL-10 levels (Table 2) between IS patients and controls at baseline. IL-6 concentrations were higher in patients with moderate and severe IS (9.26, IQR 4.39-20.11) than in mild IS patients (4.78, IQR 4.03-6.45) and control subjects (1.74, IQR 1.05-

3.31). Serum concentrations of IL-1 β and IL-6 were significantly increased in IS patients after 10 days (Table 3).

Correlation between monocyte numbers in peripheral blood, TLR2/TLR4 expression on the monocytes and IL-1 β , IL-6 and IL-10 levels cytokine levels

Expression of TLR2, TLR4 on monocytes of peripheral blood in IS patients and Control group is presented in (Table 2) and

Table 2. Expression of TLR2, TLR4 on monocytes of peripheral blood in IS patients and Control group (Day 10).

Study groups	IS Group(n=68)		IS Group (n=68)	Control Group (n=78)
	Mild IS (NIHSS ≤8) (n=46)	Moderate and Severe IS (NIHSS ≥9) (n=22)		
CD14+ (%)	7.41 (4.92 - 7.77) ⁴	11.02 (9.71 - 11.45) ^{2,3}	8.65 (7.6 - 10.7) ⁶	5.95 (5.13 - 6.55)
CD14+ (x10 ³ /μL)	6.97 (6.84 - 7.43) ⁴	12.2 (9.42 - 13.61) ^{2,3}	8.49 (6.96 - 11.63) ⁶	4.55 (4.31 - 5.68)
CD14+ CD282+ (%)	33.14 (29.31 - 42.73)	42.91 (42.74 - 43.81) ^{1,3}	39.55 (32.6 - 43.8) ⁶	22.6 (21.83 - 26.75)
CD14+ CD282+ (x10 ³ /μL)	2.31 (2.01 - 3.07) ⁴	4.97 (4.03 - 5.28) ^{2,3}	3.64 (2.2 - 4.87) ⁶	1.16 (0.94 - 1.61)
CD14+ CD284+ (%)	38.1 (32.1 - 38.6) ⁴	36.2 (36.7 - 51.1) ³	37.6 (34.6 - 51.6) ⁷	25.41 (22.34 - 31.37)
CD14+ CD284+ (x10 ³ /μL)	2.69 (2.49 - 2.75) ⁵	3.98 (3.45 - 6.91) ^{2,3}	2.88 (2.69 - 3.84) ⁷	1.32 (1.07 - 1.42)
CD14+ HLADR+ (%)	92.1 (91.8 - 94.6)	95.13 (94.1 - 95.5)	94.5 (91.87-95.47)	93.25 (91.5 - 94.81)
CD14+ HLADR+ (x10 ³ /μL)	6.42 (6.31 - 7.01) ⁴	11.25 (8.94 - 12.91) ^{2,3}	8.07 (6.41 - 10.67) ⁶	4.31 (4.06 - 5.27)
CD14+ HLADR+ 282+ (%)	27.3 (26.4 - 30.1)	43.11 (42.52 - 44.76) ^{1,3}	36.85 (28.7 - 44.45) ⁷	22.75 (20.41 - 26.62)
CD14+ HLADR+ 282+ (x10 ³ /μL)	1.2 (1.71 - 2.81) ⁴	4.21 (3.79 - 4.85) ^{2,3}	3.45 (1.78 - 4.17) ⁷	0.98 (0.82 - 1.42)
CD14+ HLADR+ 284+ (%)	30.6 (29.3 - 38.1) ⁵	34.84 (33.25 - 47.72) ³	34.11 (29.62 - 48.6) ⁶	25.65 (22.51 - 27.14)
CD14+ HLADR+ 284+ (x10 ³ /μL)	2.23 (1.98 - 2.48) ⁵	3.09 (2.97 - 6.03) ^{2,3}	2.58 (2.15; 3.06) ⁶	1.13 (0.93 - 1.31)
MFI	168.1 (133.4; 246.1) ^{1,4}	120.52 (98.54 - 138.49)	337.75 (244.5 - 446) ⁶	124.03 (95.14 - 153.21)

The variables are presented as median and interquartile range (IQR):

p^1 – $p<0.05$; p^2 – $p<0.001$ – comparison between the patients with mild IS (NIHSS ≤8) and moderate +severe IS (NIHSS ≥9);

p^3 – $p<0.001$ – comparison between the patients with mild IS (NIHSS ≤8) and Controls;

p^4 – $p<0.05$; p^5 – $p<0.001$ – comparison between the patients with moderate + severe IS (NIHSS ≥9) and Controls;

p^6 – $p<0.05$; p^7 – $p<0.001$ – comparison between the patients with IS and Controls

Table 3. Serum IL-1 β , IL-6 and IL-10 in IP patients and Control group.

	Mild IS (NIHSS $\leq$ 8) (n=46)		Moderate and Severe IS (NIHSS $\geq$ 9) (n=22)		Control group n=78
	Day 1	Day 10	Day 1	Day 10	Day 1
IL-1 β (pg/ml)	0 (0-0)	3.05(0-8.85) ²	0 (0-0)	2.39(0-13.8)	0(0-0)
IL-6 (pg/ml)	4.78(4.03-6.45) ¹	7.09(4.33-17.51) ²	9.26(4.39-20.11) ¹	11.68(5.6-85.9) ²	1.74(1.05-3.31)
IL-10 (pg/ml)	2.04 (0-4.13)	2.17(0.41-4.64)	1.96(0.76-8.03)	2.14(1.98-2.31)	1.89 (0.13-5.24)

The variables are presented as median and interquartile range (IQR):

p^1 – $p < 0.05$ – comparison between the IS patients and Controls on Day 1

p^2 – $p < 0.05$ – comparison between serum IL of the IS patients on days 1 and 10

and serum concentrations of IL-1 β , IL-6 and IL-10 in IP patients and Control group are presented in (Table 3).

Serum concentrations of IL-1 β were positively correlated with CD14⁺CD284⁺(%) ($r=0.64$; $p<0.05$) (Figure 1), CD14⁺CD284⁺($\times 10^3/\mu\text{L}$) ($r=0.51$; $p<0.05$) (Figure 2), CD14⁺HLADR⁺CD284⁺ (%) ($r=0.43$; $p<0.05$) (Figure 3) and CD14⁺HLADR⁺CD 284⁺ ($\times 10^3/\mu\text{L}$) ($r=0.43$; $p<0.05$) (Figure 4) at day 10. There was no significant correlation between serum concentrations of IL-1 β and TLR2 expression on the monocytes in both IS and control groups. There were no significant correlations between NIHSS with IL-6 and IL-10 levels and TLR2 expression on the monocytes.

Correlation between NIHSS with TLR2/TLR4 expression on the peripheral blood monocytes, IL-1 β , IL-6, and serum IL-10 levels cytokine levels at day 10

At day 10, NIHSS was positively correlated with serum concentrations of IL-1 β ($r=0.69$; $p<0.05$) (Figure 5), with CD14⁺CD284⁺(%) ($r=0.63$; $p<0.05$) (Figure 6), with CD14⁺CD284⁺($\times 10^3/\mu\text{L}$) ($r=0.51$; $p<0.05$) (Figure 7), with CD14⁺HLADR⁺CD284⁺ (%) ($r=0.19$; $p<0.05$) (Figure 8) and with CD14⁺HLADR⁺CD284⁺ ($\times 10^3/\mu\text{L}$) ($r=0.18$; $p<0.05$) (Figure 9). No significant correlations were found between NIHSS and TLR2 expression on the monocytes. Likewise, no significant correlations were observed between NIHSS and IL-6/IL-10 levels.

DISCUSSION

The results of this study suggest that activated TLR4 signaling on peripheral blood monocytes may possibly have some role in the pathophysiology of IS. A number of observations support this conclusion: (1) Baseline TLR4 expression levels on blood monocytes were similar in IS and in control subjects without IS; (2) Rises in TLR4 expression on blood monocytes in IS patients at day 10 were positively correlated with serum IL-1 β levels; (3) Higher expression of TLR4 on blood monocytes was associated with poor functional outcome in IS patients.

Growing data suggest that IS alters the phenotype of the peripheral immune system; therefore, a better understanding of this response could provide new insights into IS pathophysiology. Our observation goes in agreement with the previous study reporting the independent association between increased expression of TLR4 in monocytes and poor outcome after IS in humans [16]. Moreover, previous experimental studies also showed that TLR4-deficient mice had smaller infarctions and less inflammatory response after IS [17] and that the brain damage caused by IS prime mechanisms that signal through TLR4 [18], even so poor outcome in IS was not associated with TLR2 in adjusted models [19].

In this study, we showed that peripheral blood monocytes with TLR4 expression were significantly increased at day 10 after IS and that these laboratory changes were positively correlated with the levels of inflammatory cytokine IL-1 β and NIHSS. In IS pathophysiology, both central and peripheral inflammatory alterations occur immediately after cerebral infarction, including mobilization of immature pro-inflammatory Ly6C(hi)CD43(lo) monocytes that acutely infiltrate the ischemic tissue reaching the core of the lesion [19]. In human studies, IS triggered an increased number of total monocytes in the blood circulation; while the number of classical monocyte subsets (CD14⁺⁺CD16⁻), significantly increased in blood circulation, whereas the number of the non-classical monocyte subsets (CD14⁺CD16⁺⁺) decreased [20]. While most studies implicating the bone marrow as the source of monocytes examined the accumulation of monocyte-derived cells at target sites several days after the tissue inflammation, the recent important study using models of myocardial ischemia has revealed that the spleen represents the dominant reservoir for monocytes during early stages of inflammation [21]. Furthermore, the reduced number of monocyte subsets in the spleen was found to be strongly correlated with their increased number in the ischemic brain [22]. Recently, Hsi-Min Hsiao et al. [23] demonstrated that splenic monocyte-derived IL-1 β orchestrates critical steps of acute ischemia-reperfusion injury. It has also been shown that TLR4 activation in monocytes may lead to the blood-brain

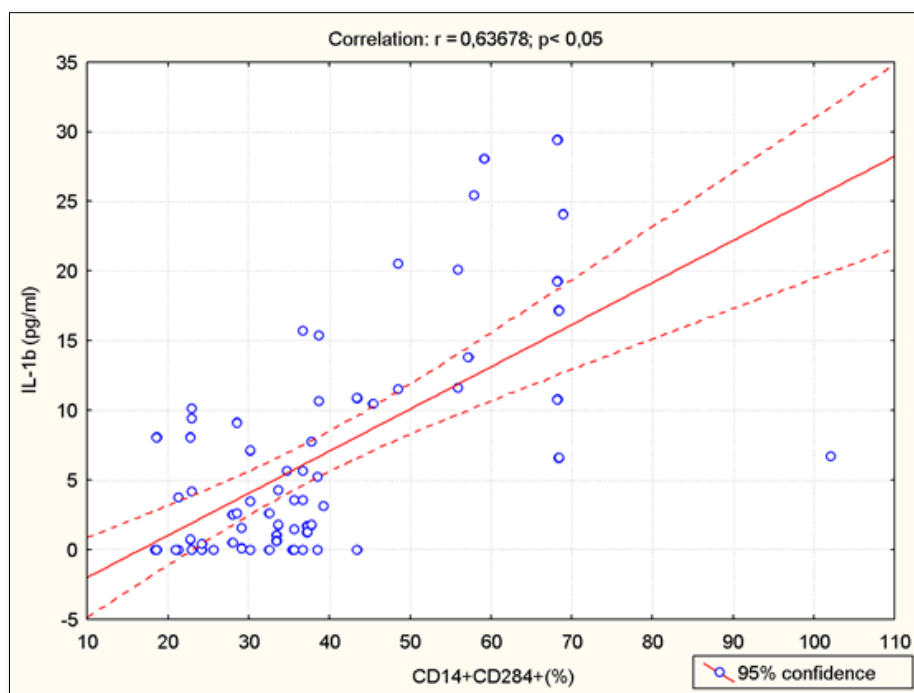


Figure 1. Correlation between serum concentrations of IL-1 β and CD14⁺CD284⁺(%) at day 10.

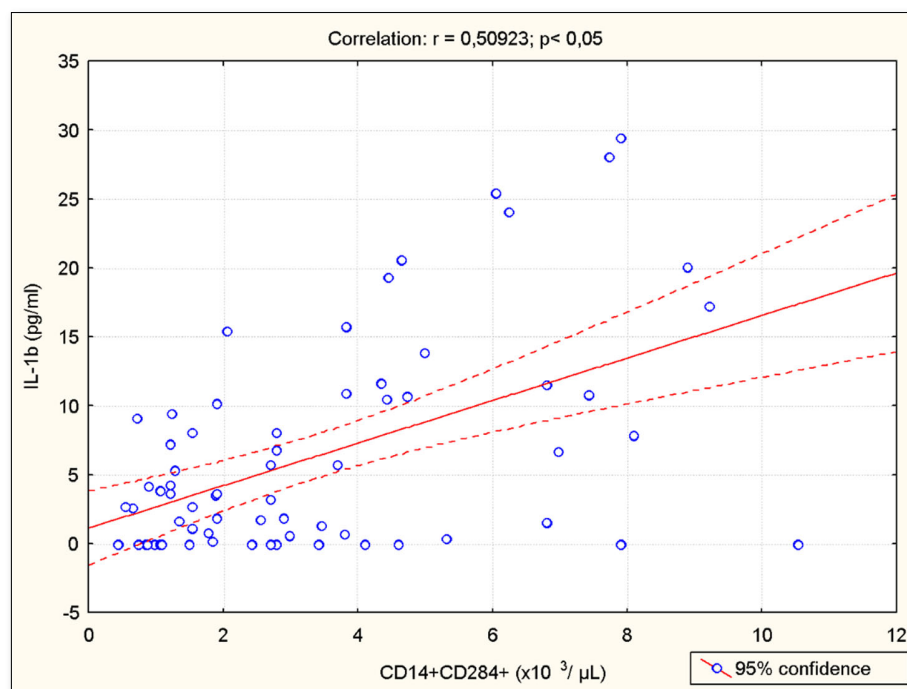


Figure 2. Correlation between serum concentrations of IL-1 β and CD14⁺CD284⁺($\times 10^3/\mu\text{L}$) at day 10.

barrier (BBB) leakage through upregulation and release of proinflammatory cytokines [24] and blood monocytes that are stimulated with the TLR4 agonist can cross the BBB in response to CCL19 [25].

Therefore, our observation could possibly be in agreement with the hypothesis that peripheral blood monocytes with upregulated TLR4 expression may lead to secondary injury when they penetrate the BBB, consequently aggravating brain damage and neural functional deficits [26]. Recently, the first

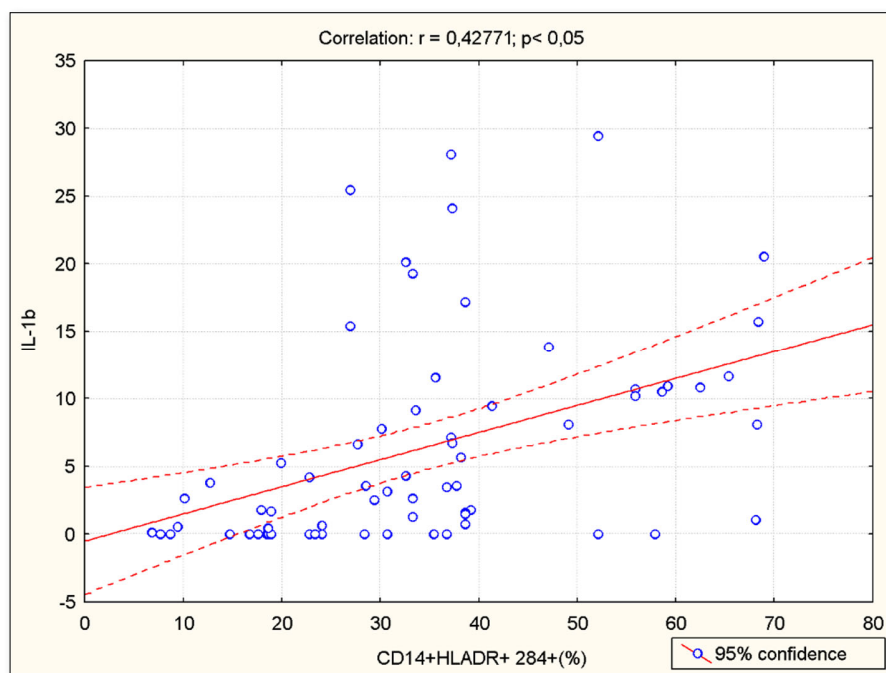


Figure 3. Correlation between serum concentrations of IL-1 β and CD14⁺HLADR⁺CD284⁺(%) at day 10.

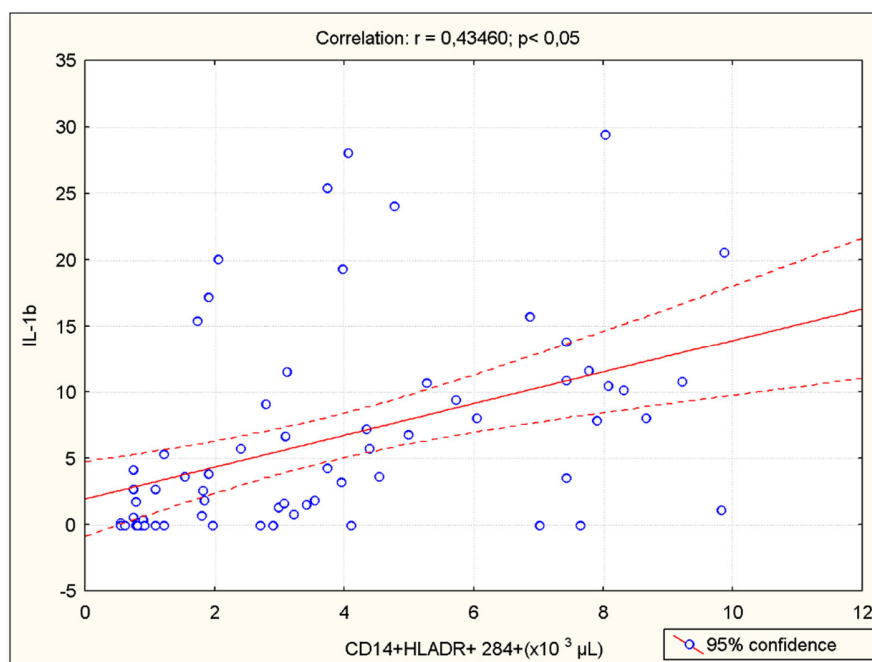


Figure 4. Correlation between serum concentrations of IL-1 β and CD14⁺HLADR⁺CD284⁺ ($\times 10^3 / \mu\text{L}$) at day 10.

evidence that peripheral immune cells accumulate over 14 days post-stroke at sites of secondary neurodegeneration, triggering microglia activation and progressive neuronal loss, has been provided in one experimental study [27]. The migration of monocytes through the BBB is regulated by CC chemokine receptor 2 (CCR2), which is upregulated on

endothelial cells in the ischemic area [28]. Approximately two weeks after IS, the factors released by ischemic neurons, including ATP and Fas ligand, induce morphological changes in local microglia transmigrated peripheral monocytes to promote their more pro-inflammatory M1 phenotype [29]. The M1 phenotype is characterized by the release of

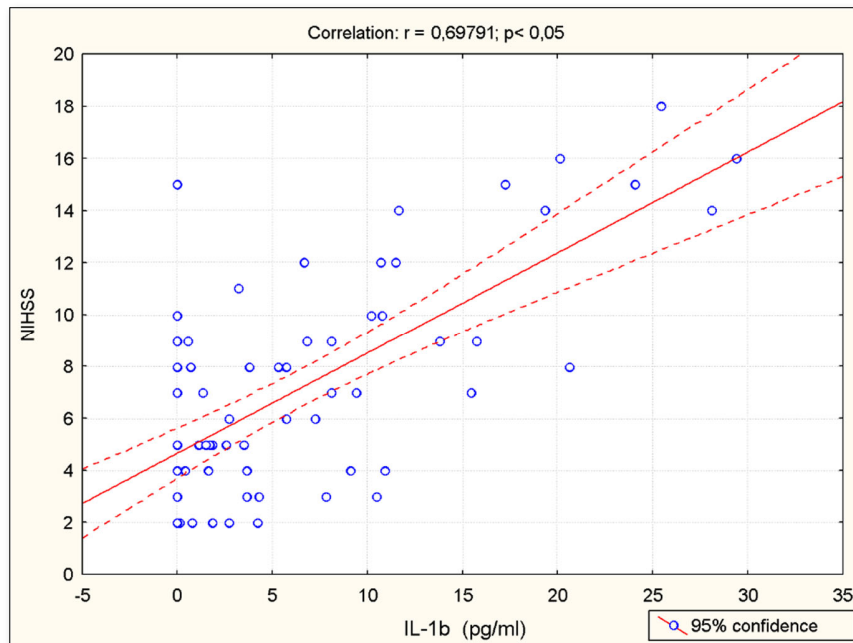


Figure 5. Correlation between NIHSS with serum concentrations of IL-1 β at day 10.

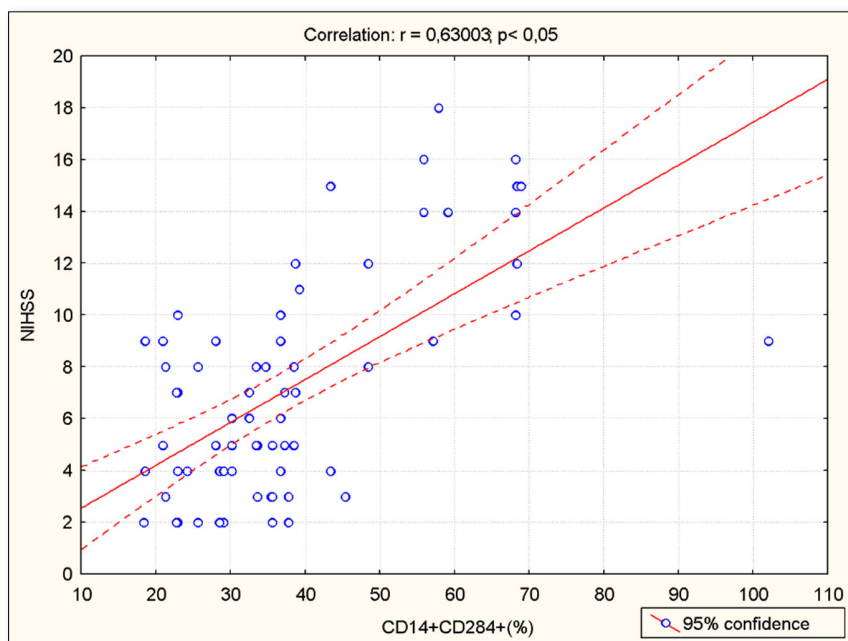


Figure 6. Correlation between NIHSS with CD14⁺CD284⁺(%) at day 10.

inflammatory mediators such as TNF α and IL-1 β and reactive oxygen species (ROS) [30]. It was also experimentally demonstrated that activated TLR4 monocytes are able to increase ROS production by upregulating expression of inducible nitric oxide synthase and IL-1 β [31]. Moreover, these monocytes are the primary source of IL-1 β [32] and it has been clinically confirmed that blocking IL-1 β receptor may improve long-term outcomes after IS [33]. In our study,

there was a strong positive correlation between serum IL-1 β , TLR4 expression on peripheral blood monocytes and NIHSS at day 10 after IS. A well-recognized detrimental action of IL-1 β in cerebral ischemia is the induction of adhesion molecules, thus stimulating the infiltration of circulating immune cells and the release or synthesis of other proinflammatory mediators [34].

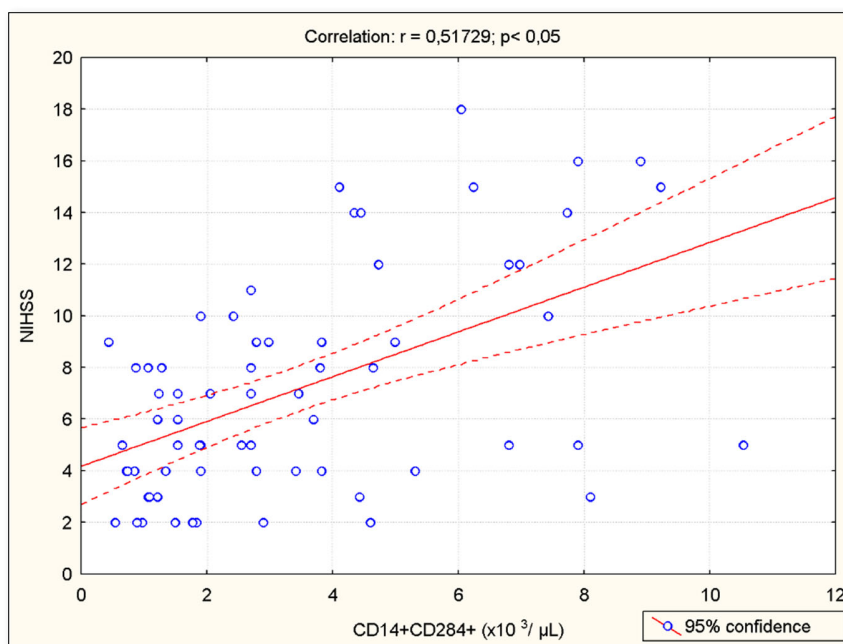


Figure 7. Correlation between NIHSS with $\text{CD14}^+\text{CD284}^+(\times 10^3/\mu\text{L})$ at day 10.

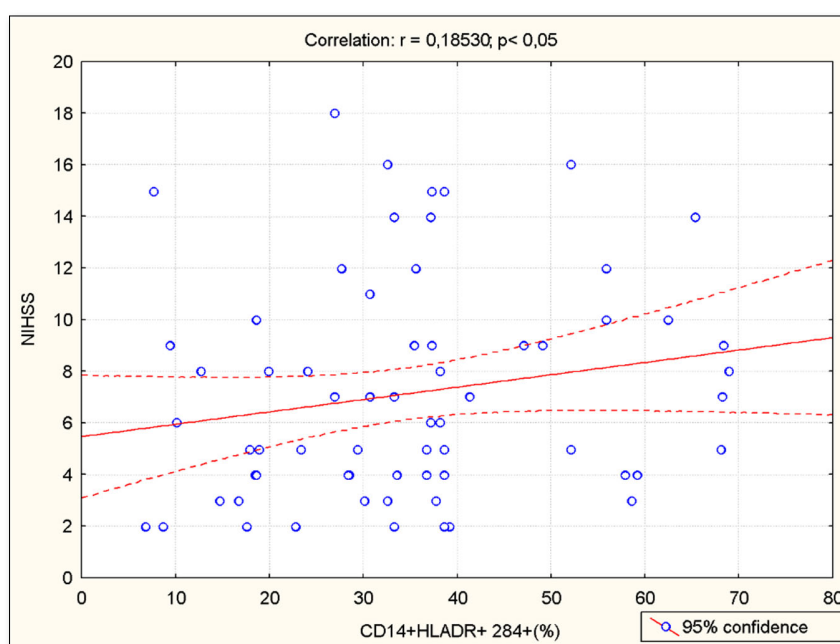


Figure 8. Correlation between NIHSS with $\text{CD14}^+\text{HLADR}^+\text{CD284}^+(\%)$ at day 10.

Interestingly, although serum IL-6 concentrations were higher in our IS patients than in the control subjects after 10 days, we did not observe any correlation between IL-6 levels and TLR4 expression on blood monocytes. Previously, one small clinical study showed that serum IL-6 correlated significantly with infarct size measured by MRI at multiple time points [35]. On the contrary, another study found an inverse relationship between the levels of serum IL-6 and neurological impairment and infarct size three months after IS

[36], suggesting that elevated IL-6 may be suggestive of neuroprotection rather than damage. Interestingly, IL-1 β , but not IL-6 has been specifically associated with neurotoxic effects after ischemia-reperfusion-induced brain injury [37,38].

In summary, coincident with other researches [5], we show that upregulation of TLR4 expression on peripheral blood monocytes could be one of the peripheral mechanisms of

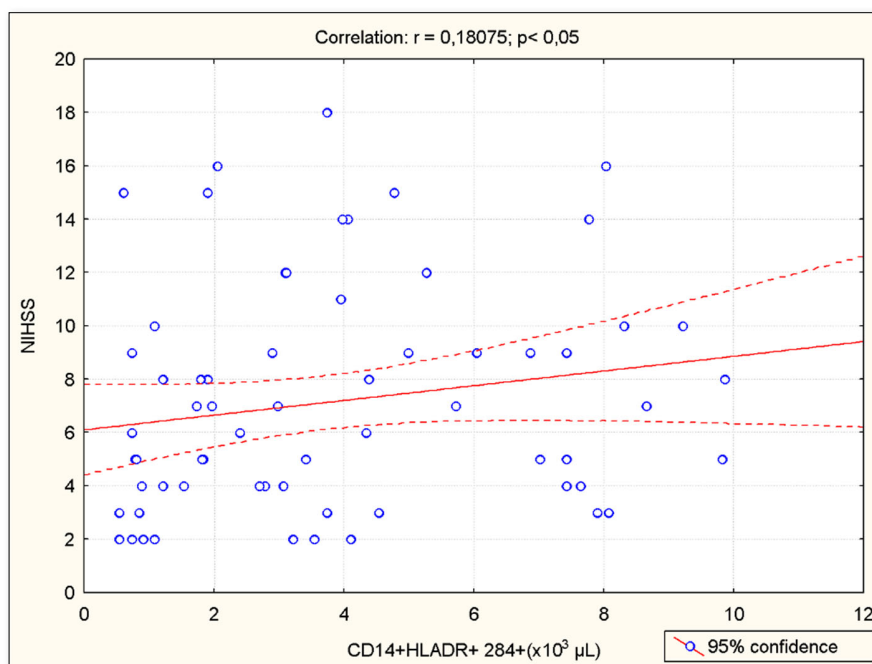


Figure 9. Correlation between NIHSS with CD14⁺HLADR⁺CD284⁺ ($\times 10^3/\mu\text{L}$) at day 10.

inflammatory injury after IS. Inhibiting harmful immune responses of peripheral monocytes may possibly result in the development of “immunomodulatory strategies” for the better adjunct therapies of ischemic stroke.

STATEMENT OF ETHICS

The study was approved by the local ethics committee at Chita Medical Academy, Russia, and all participants or their legal representatives signed a written informed consent.

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The manuscript represents the authors' own work, without external finding.

AUTHOR CONTRIBUTIONS

Krohaleva YuA, Strambovskaya NN, Kuznik BI, contributed to the implementation of the research and to the analysis of the results, Magen E contributed to the design of the research and to the writing of the manuscript.

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