Decreased Toll-like receptor 4 and CD11b/CD18 expression on peripheral monocytes of hypertensive patients correlates with a lesser extent of endothelial damage—a preliminary study.

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Research Article

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Abstract

Background

Low-grade chronic inflammation is recognized to contribute to the physiopathology of arterial hypertension. Therefore, this study aimed to assess the pro-inflammatory phenotype of peripheral monocytes of hypertensive patients by analyzing Toll-like receptor 4 (TLR4) and CD11b/CD18 surface expression. In the second part, the influence of phenotypic alterations of monocytes on the endothelial status reflected by circulating endothelial cells (CECs) was evaluated.

Patients

The study involved thirty patients with mild hypertension (MH) and thirty subjects with resistant hypertension (RH). The control group included thirty-three age and sex-matched normotensive volunteers.

Results

Reduced TLR4 and CD11b/CD18 surface expression was found in MH and RH patients compared to normotensive volunteers. In addition, the percentage of monocytes co-expressing TLR4 and CD11b/CD18 decreased with the clinical severity of the disease. A statistically significant correlation between TLR4 and CD18 expression was observed in MH patients. Decreased TLR4 surface expression was inversely associated with plasma TNF-α levels in RH patients. A decreased TLR4 surface expression in MH patients and losing CD11b/CD18 on cell membrane in RH patients correlated with a lower number of CECs.

Conclusion

Our preliminary study showed for the first time that hypertension of varying severity is accompanied by phenotypic changes in monocytes, manifested by reduced surface expression of both TLR4 and CD11b/CD18. This phenotypic feature may allow monocytes to downregulate inflammatory response and limit vascular damage. Our study opens a new unexplored area of research on the anti-inflammatory function of monocytes in hypertension.

1. Introduction

Hypertension is the most common chronic disease and the primary cause of heart failure, stroke, chronic kidney disease, and mortality in the Western world [1, 2]. In recent years, a lot of effort has been put into understanding the exact mechanisms underlying essential hypertension. The strong correlation between hypertension and popular biomarkers of inflammation observed in many clinical investigations indicates that abnormal immune system activation is involved in this pathology [3–5]. Initially, it has seemed that low-grade systemic inflammation is a consequence of hypertension, but recent reports suggest that this
process can be a causal factor [6, 7]. Resistant hypertension is a clinically distinct subgroup defined by the failure to achieve blood pressure control on optimal dosing of at least three antihypertensive medications of different classes, including a diuretic [8]. Previous studies have demonstrated that patients with resistant hypertension have higher arterial stiffness and impaired endothelial function compared to normotensive and mild to moderate subjects [9]. Endothelial dysfunction is closely related to inflammation, accompanied by excessive adhesion of leukocytes to the vessel wall and the liberation of various endothelial damaging molecules, such as chemokines, reactive oxygen species, and metalloproteases. Endothelial damage is manifested by its vasoconstrictive, prothrombotic, and pro-inflammatory phenotype, which promotes the development of arterial hypertension [10, 11]. Our previous studies confirmed endothelial damage reflected by an increased number of circulating endothelial cells (CECs) in hypertension of various clinical severity [12]. CECs represent mature endothelial cells detached from blood vessels due to multiple mechanisms, including apoptosis, mechanical injury, weakening of intracellular connection, and endothelial structure injury caused by cytokines/proteases [13, 14]. The number of CECs, therefore, reflects the condition of the endothelium and can be used to assess the extent of blood vessel injury. This work is a continuation of those previous studies in which we extended the earlier issue of endothelial injury by attempting to clarify the role of inflammatory cells in this process.

We focused on toll-like receptors (TLRs), a large family of innate immune receptors that may constitute an essential link in leukocyte-endothelial interactions [15–17]. TLRs, broadly distributed in the immune cells, play a critical role in innate and adaptive immune responses. Among the eleven identified in humans so far, a unique role is assigned to the TLR4, whose increased expression has been shown in conditions associated with chronic low-grade inflammation, such as atherosclerosis, diabetes, and rheumatoid arthritis [18–20]. TLR4 is crucial in activating monocytes and their adhesion to the vascular endothelium [21]. Recent studies have shown that activation of TLR4 induces intracellular signaling cascades, resulting in surface expression of CD11b/CD18 integrin [21–23]. The CD11b/CD18 integrin (also known as Mac-1) is a heterodimer of αM (CD11b) and β2 (CD18) subunits. The binding of CD11b/CD18 to intracellular adhesion molecule 1 (ICAM-1) expressed on blood vessels triggers monocyte adhesion and subsequent transmigration, which, if exaggerated, may promote endothelial injury [24, 25]. In addition, signaling from TLR4 activates the NK-κB pathway through several adaptor molecules and regulates cytokine expression, such as IL-6 and TNF-α [26, 27]. These factors facilitate the recruitment and attachment of circulating leukocytes to the vascular endothelium.

The exact role of monocytes and TLR4-dependent mechanisms described above is not fully understood in the pathogenesis of arterial hypertension. For this reason, the present study aimed to evaluate the expression of TLR4 and CD11b/CD18 on the surface of peripheral monocytes of patients with mild and resistant hypertension. In the second part, we analyzed how the observed monocyte phenotypic alterations affect endothelial status reflected by the number of CECs.

2. Material And Methods

2.1 Patients
The study was performed in accordance with the principles of the Declaration of Helsinki, and the investigational protocol was approved by the Local Bioethical Committee of Poznan University of Medical Sciences (no. 163/17). The study was carried out in a group of hypertensive patients (38 men and 22 women) aged 21–73 (mean age 55.57 ± 12.91) who had been admitted to the Department of Hypertension at the University of Medical Sciences in Poznan. The control group consisted of 33 normotensive blood donors of Regional Blood Centre in Poznan (25 men and 8 women), aged 27–61 (mean age: 41.87 ± 6.99), with no symptoms and/or signs of cardiovascular disease. Written informed consent was obtained from all participants. All patients underwent laboratory, and physical examination, including blood pressure (BP) measurements, performed three times at rest, in a supine position, using a validated upper-arm blood pressure monitor (Omron 705IT). Based on the detailed interview and a clinical examination, the patients were divided into two groups: patients with mild hypertension (MH), including twenty men and ten women (mean age 52.87 ± 13.55), and with resistant hypertension (RH) comprising eighteen men and twelve women (mean age 58.27 ± 11.85). Resistant arterial hypertension was recognized when, despite using at least three antihypertensive agents (including a diuretic) in maximum doses, it was impossible to achieve the target values of arterial blood pressure lower than 140/90 mmHg. The exclusion criteria were as follows: secondary hypertension, white coat hypertension, myocardial infarction and revascularization within six months before the study, stroke and transient ischemic attack (TIA) within six months before the study, congestive heart failure with grade III-IV according to New York Heart Association grading, chronic kidney disease (eGFR < 30 ml/min), addiction to alcohol and psychotropic substances, smoking, active cancer, or diabetes. The demographics and clinical characteristics of the control and study subjects are given in Table 1.
Table 1
Demographic and clinical characteristics of control and hypertensive groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 33)</th>
<th>MH (n = 30)</th>
<th>RH (n = 30)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age(years)</td>
<td>41.87 ± 6.99</td>
<td>52.87 ± 13.55</td>
<td>58.27 ± 11.85b</td>
<td>NS</td>
</tr>
<tr>
<td>Gender F/M(n)</td>
<td>8/25</td>
<td>10/20</td>
<td>12/18c</td>
<td>NS</td>
</tr>
<tr>
<td>BMI ((kg/m²)</td>
<td>25 ± 4</td>
<td>28 ± 5</td>
<td>30 ± 6b</td>
<td>0.020</td>
</tr>
<tr>
<td>WBC(10⁹ /L)</td>
<td>6.06 (5.21–7.05)</td>
<td>7.04 (5.68–8.89)</td>
<td>6.95 (5.69–8.60) a</td>
<td>0.029</td>
</tr>
<tr>
<td>NEUT(10⁹ /L)</td>
<td>3.08 (2.49–3.71)</td>
<td>4.27 (3.29–5.71)*</td>
<td>4.42 (3.55–5.85)* Δa</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MONO(10⁹ /L)</td>
<td>0.57 (0.44–0.67)</td>
<td>0.46 (0.34–0.53)</td>
<td>0.45 (0.28–0.67)a</td>
<td>NS</td>
</tr>
<tr>
<td>LYMPH(10⁹ /L)</td>
<td>2.17 (1.81–2.45)</td>
<td>1.64 (1.39–2.37)*</td>
<td>1.82 (1.37–2.15)* a</td>
<td>0.026</td>
</tr>
<tr>
<td>PLT (10⁹ /L)</td>
<td>228 (200–278)</td>
<td>215 (173–267)</td>
<td>224 (170–272)a</td>
<td>NS</td>
</tr>
<tr>
<td>RBC (10¹² /L)</td>
<td>4.97 (4.87–5.20)</td>
<td>4.80 (4.44–5.13)</td>
<td>4.60 (4.34–5.07)a</td>
<td>NS</td>
</tr>
<tr>
<td>HGB (mmol/L)</td>
<td>15 ± 1.09</td>
<td>12.5 ± 2.42</td>
<td>14.67 ± 3.21b</td>
<td>NS</td>
</tr>
<tr>
<td>NLR</td>
<td>1.52 (1.13–1.83)</td>
<td>2.38 (1.71–3.73)*</td>
<td>2.74 (2.18–3.52) *Δ a</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>PLR</td>
<td>110 ± 38.72</td>
<td>129.5 ± 51.74</td>
<td>129.8 ± 42.04b</td>
<td>NS</td>
</tr>
<tr>
<td>CECs</td>
<td>50 (17–78)</td>
<td>126 (67–198)*</td>
<td>113 (64–223)a</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

**Antihypertensives**

<table>
<thead>
<tr>
<th>Antihypertensives</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop diuretics (%)</td>
<td>-</td>
<td>23</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Thiazide-like diuretics (%)</td>
<td>-</td>
<td>37</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme (ACE) inhibitors (%)</td>
<td>-</td>
<td>47</td>
<td>53</td>
<td>-</td>
</tr>
<tr>
<td>Angiotensin II (Ang II) type 1 (AT1) receptor blockers (%)</td>
<td>-</td>
<td>20</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>Calcium channel blockers (%)</td>
<td>-</td>
<td>47</td>
<td>77</td>
<td>-</td>
</tr>
<tr>
<td>Parameter</td>
<td>Control (n = 33)</td>
<td>MH (n = 30)</td>
<td>RH (n = 30)</td>
<td>( p ) value</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------</td>
<td>------------</td>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Beta-blockers (%)</td>
<td>-</td>
<td>20</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Mineralocorticoid Receptor Antagonists (%)</td>
<td>-</td>
<td>0</td>
<td>43</td>
<td>-</td>
</tr>
</tbody>
</table>

\( ^{(a)} \) Results shown as median and interquartile range and analyzed using Kruskal-Wallis test with Dunn’s multiple comparisons; \( ^{(b)} \) Results shown as mean ± SD and analyzed using ANOVA test with Turkey’s multiple comparisons; \( ^{(c)} \) Fischer’s exact test was used for categorical data comparison. NS—not statistically significant, BMI—body mass index, WBC—white blood cells, NEUT—neutrophils, MONO—monocytes, LYMH—lymphocytes, PLT—blood platelets, RBC—red blood cells, HGB—hemoglobin, NLR—neutrophils/lymphocytes ratio, PLR—platelet/lymphocytes ratio, CECs—circulating endothelial cells.

\(* \) MH/RH group vs. control group, \(^{\Delta} \) RH group vs. MH group

### 2.2 Sample collection

Blood samples were drawn in the early morning, from the arms of MH and RH patients, in the recumbent position after 10 minutes of rest. Two neutral vacuum tubes of blood were collected from each patient into ethylenediaminetetraacetic acid (EDTA) anticoagulant. After 30 minutes, the first EDTA test tube was centrifuged at 3,000 rpm for 15 minutes, and the obtained plasma was stored at a temperature of -80°C until all assays were performed. The second EDTA test tube was used for peripheral blood mononuclear cells (PBMCs) isolation.

### 2.3 Laboratory analysis

#### 2.3.1 PBMCs isolation

PBMCs were obtained from whole venous blood using Ficoll-Histopaque® density gradient centrifugation, following the method described by Böyum [28]. Briefly, 2.5 mL of blood was gently layered over 2 mL of Histopaque solution (Sigma-Aldrich, St. Louis, MO), then centrifuged at 1300 rpm for 30 min. The white band of mononuclear cells was collected and washed three times using phosphate-buffered saline (PBS) through centrifugation at 950 rpm for 10 min. PBMCs were suspended in a cryoprotective medium supplemented with 10% fetal bovine serum and 5% DMSO and frozen to -80° degrees.

#### 2.3.2 Direct immunofluorescence using flow cytometry to assess the expression of TLR4 and CD11b/18 molecules on the surface of cells.

PBMC were unfreezing and washed using PBS through centrifugation at 1500 rpm for 5 minutes. The obtained cell pellet was suspended in the saline and labeled with a cocktail of directly conjugated pre-
diluted monoclonal antibodies (mAbs) provided by Becton Dickinson (Franklin Lakes, NJ). Aliquots $1 \times 10^6$ PBMC were labeled with 60µL of APC-Cy™7 mouse anti-human CD45 mAb (catalog no.:557833), PerCP mouse anti-human CD14(catalog no.:340585), PE mouse anti-human TLR4 mAb (catalog no.: 564215), APC mouse anti-human CD11b/Mac-1 mAb (catalog no.: 550019), FITC mouse anti-human CD18 mAb (catalog no: 555923). After incubation for 15 min at room temperature, antibodies-antigen connections were fixed, and residual erythrocytes were lysed with 500 µL lysing solution (Becton Dickinson, Franklin Lakes, NJ, catalog no.:349202) for 10 min, followed by twice washing in 3 mL PBS and immediate flow cytometric analysis. Monocyte analyses were performed using forward and side scatter parameters combined with CD14-positive and CD45-positive stained cells. Single-parameter histograms were then generated to identify TLR4, CD11b, and CD18 expression on total monocytes. Appropriately conjugated isotype controls were used to eliminate non-specific staining (Fig. 1). Electronic color compensation was used to exclude any overlapping emission spectra, and each directly conjugated mAb was separately analyzed to ensure that the appropriate fluorescent signals appeared in a single detector filter. When all parameters were set, 10,000 of the gated CD14+/CD45+ events were acquired for analysis. The surface receptor expression was measured as the mean fluorescence intensity (MFI).

2.3.3 Reverse transcription and real-time qPCR (RT-qPCR) for TLR4 gene expression in PBMCs

Total RNA was extracted from PBMCs with GeneMATRIX universal RNA purification kit, according to the manufacturer's instructions (EURX, Poland, catalog no.: E3598). The concentration of total RNA was detected using an ultraviolet spectrophotometer.

cDNA was synthesized by random primer reverse transcription using an iScript™ advanced cDNA synthesis kit (Bio-Rad, Hercules, CA, catalog no.:1725038). The housekeeping gene TATA box binding protein (TBP) was used as an endogenous reference gene [29]. Primer-BLAST was used for detection to confirm the specificity of primer sequences. The primer sequences for the TLR-4 gene were as follows: 5'-GGGAGACACAGATGGCTGGGA-3' (forward) and 5'CAAGGAGCATTGCCCAACAGGA-3' (reverse); the primer sequences for TBP were: 5'-GTGACCCAGCAGCATCAGT-3' (forward) and 5'- AACCAGAACCTTGGCCTG – 3' (reverse). Primer sequences for TLR4 and reference gene TBP were synthesized by the Institute of Biochemistry and Physics (Polish Academy of Sciences, Poland). Real-time qPCR was performed with Azure-Cielo Real-Time apparatus (Azure Biosystems, Dublin, CA) using the SsoAdvanced Universal SYBR® Green Supermix. (Bio-Rad, Hercules, CA,catalog no.: 1725272). The PCR reaction was conducted with a total volume of 20 µL containing 10 µL of SsoAdvanced Universal SYBR® Green Supermix., 2 µL of cDNA template, 1 µL of 10 µmol L$^{-1}$ each forward and reverse primer, and 6 µL of RNase-free H$_2$O. All qPCR was conducted at 95°C for 30 s, then 40 cycles of 95°C for 5 s and 60°C for 30 s. The specificity of the reaction was verified by melt curve analysis. Relative TLR4 mRNA concentrations were normalized to the corresponding TBP internal control and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3.4 Cytokine assay
Serum TNF-α (assay sensitivity: 1 pg/mL) and IL-6 (assay sensitivity: 0.3 pg/mL) concentrations were determined using a commercially available PicoKine ELISA kit (Boster Bio, Pleasanton, CA, catalog no.: EK0525, EK0411), according to the manufacturer’s instructions. Cytokines concentrations were calculated using the mean optical density of two wells and comparison with a standard curve.

2.3.5 Statistical analysis

The statistical analysis was conducted using GraphPad Prism software 6.0 (GraphPad Software, San Diego, CA). The normality of quantitative variables was tested using the Kolmogorov-Smirnov or Shapiro-Wilk test. Any parameter not following the normal distribution was presented as a median and interquartile range and analyzed using the non-parametric Mann-Whitney test. Categorical data and proportions were compared using Chi-square or Fisher's exact test, as appropriate. Normally distributed, continuous variables were presented as a mean and standard deviation and analyzed using the Student’s t-test. Multiple group comparisons were performed by one-way analysis of variance or the Kruskal-Wallis test. The Pearson or the Spearman correlation coefficient was used to test the strength of any association between different variables. In all cases, P value ≤ 0.05 was considered significant. The relationship between TLR4, CD11b, and CD18 surface expression and endothelial status was evaluated using multiple regression analysis.

3. Results

3.1 Demographic and clinical characteristics of control and hypertensive groups

No difference in age was observed between the control and the MH and RH groups (Table 1). Both groups with hypertension showed a statistically significantly higher value of the BMI index (Table 1). Blood morphology showed a statistically significant increase in the WBC count in both groups of hypertensive patients, which was the highest in the RH group. In both the MH and RH groups, a significant increase in the NEUT was observed with a simultaneous decrease in the LYMPH compared to the control group (Table 1). For this reason, the NLR increased significantly in both groups of hypertensive patients, reaching the highest value in the RH group. Other parameters, such as MONO, PLT, RBC count, and HGB concentration, did not differ statistically in the groups of patients with hypertension compared to the control (Table 1). There was also no statistical change in the value of the PLR between MH and RH and the control subjects (Table 1).

3.2 The surface TLR4 and CD11b/18 expression on blood monocytes

The percentage of TLR4-expressing monocytes and the MFI levels of TLR4 evaluated by flow cytometry decreased significantly in patients with hypertension, reaching the lowest values in patients with RH (Table 2). The percentage of monocytes that separately expressed the CD18 or CD11b receptor on their surface in the MH and RH groups was similar to that in the control group (Table 2). However, the MFI
levels of CD11b and CD18 were significantly lower in both groups of patients with hypertension than in control (Table 2).

### Table 2
Constitutive surface expression of TLR4, CD11b, CD18 on blood monocytes

<table>
<thead>
<tr>
<th>% Cells</th>
<th>Control</th>
<th>MH</th>
<th>RH</th>
<th>Cell Surface Expression (MFI)</th>
<th>Control</th>
<th>MH</th>
<th>RH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>73.1 ± 8.9</td>
<td>61.3 ± 16*</td>
<td>60.7 ± 11.7*</td>
<td>1143 ± 976</td>
<td>814 ± 253*</td>
<td>781 ± 124*</td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>89.4 ± 4.1</td>
<td>84.5 ± 13.3</td>
<td>87.1 ± 8.7</td>
<td>5413 ± 1733</td>
<td>3177 ± 1219*</td>
<td>3284 ± 1157*</td>
<td></td>
</tr>
<tr>
<td>CD18</td>
<td>98.6 ± 1.5</td>
<td>97.4 ± 5.2</td>
<td>97.7 ± 4.2</td>
<td>6339 ± 257</td>
<td>4636 ± 373*</td>
<td>5078 ± 278*</td>
<td></td>
</tr>
</tbody>
</table>

TLR4, CD11b, and CD18 levels were determined on monocytes by flow cytometry. Results present the mean percentage ± SD of cells expressing a particular antigen and mean fluorescence index (MFI) ± SD as a measure of the particular antigen expression density. Results shown as mean ± SD were analyzed using ANOVA test with Turkey's multiple comparisons. * statistically significant difference compared to the control group (p ≤ 0.05).

Moreover, the percentage of monocytes with TLR4⁺CD11b⁺ (MH: 47.1 ± 16.4%, RH: 47.2 ± 13.3% versus control: 60.8 ± 10%; p < 0.0001) and TLR4⁺CD18⁺ immunophenotype (MH: 66.6 ± 15.2%, RH: 65.7 ± 10.5% versus control: 76.7 ± 7.7%; p = 0.0003) was significantly reduced in both groups of patients with hypertension (Fig. 1, Fig. 2).

A significant positive correlation between TLR MFI and CD18 MFI was also found (r = 0.519, p = 0.009) in MH group (Fig. 3).

### 3.3 TLR4 mRNA relative expression in PBMCs.

The TLR4 gene expression in PBMCs from MH and RH patients was examined. The results showed that the TLR4 mRNA relative expression did not significantly differ in both groups of hypertensive patients compared to the control (MH: 1.062 ± 0.807, RH: 1.240 ± 0.881 versus control: 1 ± 0.689; p = 0.652) (Fig. 4).

### 3.4 Serum IL-6 and TNF-α concentration

In both groups of patients with arterial hypertension, the concentrations of TNF-α and IL-6 showed a tendency to increase, which did not reach statistical significance compared to the control (Table 3).

### Table 3
The plasma concentration of TNF-α and IL-6 in control and hypertensive groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>MH</th>
<th>RH</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/mL)</td>
<td>43.68 (27.01–109.7)</td>
<td>56.76 (36.63–106.6)</td>
<td>70.94 (34.18–156.2)</td>
<td>0.353</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>7.62 (5.85–13.85)</td>
<td>10.42 (7.17–15.79)</td>
<td>13.05 (7.22–17.27)</td>
<td>0.293</td>
</tr>
</tbody>
</table>
Results shown as median and interquartile range and analyzed using Kruskal-Wallis test with Dunn's multiple comparisons.

$p \leq 0.05$ was considered statistically significant.

A significant negative correlation between TNF-α concentration and TLR4 MFI was observed in RH group ($r = -0.486, p = 0.022$) (Fig. 5).

### 3.5 The association between TLR4 and CD11b/18 surface expression on monocytes and endothelial status reflected by CECs

In the first step, univariate analysis was performed to determine the association between antigens surface expression and endothelial injury reflected by CECs in each group of hypertensive patients. In the next step, multivariate linear regression analysis was performed to determine TLR4 and CD11b/CD18 as independent factors potentially influencing endothelial status measured by the number of CECs. Univariate analysis revealed that TLR4 correlated positively with CECs number in MH group. In RH group, CD11b and CD18 surface expression correlated positively with CECs number (Table 4). In a multivariate analysis, only CD11b and CD18 expression was associated with an increased number of CECs in RH group, independently of other factors considered, including the age, BMI, systolic and diastolic blood pressure, WBC, NEUT, LYMPH, MONO and NLR index (Table 4).

<table>
<thead>
<tr>
<th>CECs</th>
<th>MH</th>
<th>Univariate analysis</th>
<th>Multiple regression</th>
<th>RH</th>
<th>Univariate analysis</th>
<th>Multiple regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>0.446</td>
<td>0.025*</td>
<td>0.315</td>
<td>0.208</td>
<td>0.364</td>
<td>-</td>
</tr>
<tr>
<td>CD11b</td>
<td>-0.225</td>
<td>0.280</td>
<td>-</td>
<td>0.581</td>
<td>0.006*</td>
<td>0.016*</td>
</tr>
<tr>
<td>CD18</td>
<td>-0.045</td>
<td>0.834</td>
<td>-</td>
<td>0.472</td>
<td>0.030*</td>
<td>0.037*</td>
</tr>
</tbody>
</table>

Results shown as Spearman’s rank correlation coefficient. Multiple regression analysis models for significant correlations from the univariate analysis, adjusted for age, BMI, systolic blood pressure, diastolic blood pressure, WBC, NEUT, LYMPH, MONO, NLR. *$p \leq 0.05$.

### 4. Discussion
Many previous studies have demonstrated that inappropriate immune system activation is involved in the pathogenesis of hypertension [3–5]. Nonetheless, the exact mechanisms triggered by inflammatory cells to promote vasoconstriction and, thus, increase in blood pressure are still being revealed. One assumes endothelial damage caused by activated inflammatory cells via several mechanisms, including chemotaxis, enhanced adhesion to the vessel wall, and liberation of various cytokines and reactive oxygen species [10, 11, 30, 31]. While the participation of lymphoid-derived immune cells in the pathogenesis of hypertension has been well described in the literature [32–34], the mechanisms by which myeloid-derived innate immune cells contribute to blood pressure elevation remain an active area of investigation.

Monocytes, whose pro-inflammatory phenotype we tried to assess by analyzing the surface expression of TLR4, was the main interest of this study. TLR4 plays a pivotal role in orchestrating inflammation, mainly by regulating the expression of an array of pro-inflammatory cytokines [26, 27]. Results of some studies using the animal model have demonstrated that hypertension is accompanied by increased expression of TLR4 in non-immune cells across the vascular, renal, and nervous systems [35–40]. Some others have indicated that silencing or inhibiting TLR4 using specific anti-TLR4 antibodies resulted in lower blood pressure and improved cardiac function in hypertensive rats [35, 39, 41, 42]. These findings support the involvement of TLR4 in the development and progression of multiple end-organ damage in hypertension. However, peripheral tissue inflammation results from cross-talk between the non-immune cells and the immune system. Only activated leukocytes can adhere to the vascular endothelium and infiltrate various organs, causing unfavorable morphological and functional changes. It means that knowledge of the phenotypic and behavioral alterations within inflammatory cells will provide complete insight into these interactions underlying the development of hypertension and its complications. Although TLR4 has been shown to be involved in promoting the inflammatory response, its status in inflammatory cells in hypertension is poorly understood. The number of studies involving humans is limited, and those using animal models are unreliable due to the species and tissue specificity of TLR4 [43]. Our study evaluating TLR4 expression in peripheral monocytes of hypertensive patients brings new knowledge about the role of the TLR4-mediated inflammatory response of monocytes in the complex systemic inflammatory response in hypertension.

Contrary to expectations, we have shown that both the percentage of TLR4-expressing monocytes and the surface expression of TLR4 receptors are significantly reduced in hypertensive patients. However, the level of TLR4 expression did not differ depending on the clinical severity of the disease. This result may suggest that this change in TLR4 expression appears in the early stage of hypertension and remains relatively constant despite worsening clinical symptoms. Our results are inconsistent with those obtained by Simundic et al., who have demonstrated that patients with non-controlled hypertension exhibit higher TLR4 monocyte expression than well-controlled [44]. These discrepancies are difficult to explain and may be due to differences in patient characteristics. The authors recruited smokers whom we, in turn, excluded from the observation. The proportions of women and men in the analyzed cohort of hypertensive patients in both studies were also different. In the present work, males predominated in each group, while in the authors’ analysis, the number of females and males was relatively equal. Since sex and smoking have
been shown to influence TLR4 expression, these factors should be treated with caution when designing studies and interpreting results [45–47]. Although in the same study, the authors state that elevated expression of TLR4 on monocytes of patients with uncontrolled hypertension indicates their involvement in promoting the inflammatory response, this conclusion may be incorrect. The authors did not compare their results with the healthy group, so they could not correctly assess the trend of the observed alterations. The higher monocytic TLR4 expression found by the authors in patients with uncontrolled hypertension compared to the well-controlled group does not exclude that in both cases, it may remain lower than in healthy individuals, as we have shown in our studies.

Marketou et al. found elevated TLR4 mRNA expression in peripheral monocytes of non-diabetic hypertensive patients [48]. Our research also measured TLR4 mRNA expression in peripheral blood mononuclear cells (PBMCs) isolated from hypertensive patients. This expression remains constant regardless of disease severity and does not change compared to healthy control. Since PBMCs include lymphocytes, monocytes, and dendritic cells, this experiment does not provide information on the level of TLR4 mRNA expression in monocytes alone. We can only conclude that the overall TLR4 gene expression is not significantly perturbed in PBMCs during hypertension. The study does not answer whether the observed decrease in TLR4 surface expression on monocytes is related to the downregulation of the TLR4 gene. However, it has been shown that the flow cytometry results for various TLRs are not always consistent with mRNA expression levels. Decreased levels of protein surface expression were often observed with unchanged or even increased corresponding transcript levels [49, 50]. It may result from a post-transcriptional regulatory mechanism, which involves downregulating the TLR4 membrane receptor through internalization or shedding [51–54].

Our study is the first in which a simultaneous analysis of surface TLR4 and CD11b/CD18 expression was performed in hypertensive patients. This approach is supported by reports indicating TLR4-dependent synthesis of CD11b/CD18 [21–23]. Lee et al. demonstrated that activation of TLR4 resulted in increased expression of Mac-1 on monocytes in a concentration- and time-dependent manner. Moreover, monocyte adhesion to HUVEC was significantly inhibited by pre-treatment with an anti-TLR4 antibody [21]. Thus, it is suggested that TLR4 signaling in monocyte might be a major contributor to the initiation of monocyte recruitment. Our research, in which we observed the behavior of CD11b/CD18 analogous to TLR4, may provide further evidence of a strong relationship between these two molecules. Surface integrin expression decreased regardless of the clinical severity of hypertension. Moreover, this decline was significantly correlated with a decrease in TLR4 expression in mild hypertension.

The decrease in TLR4 expression and the accompanying depletion of CD11b/CD18 on the monocytes of hypertensive patients indicates an anti-inflammatory rather than a pro-inflammatory phenotype of these cells. Interestingly, the percentage of monocytes co-expressing TLR4 and CD11b/CD18 decreased with the severity of the disease.

This result was unexpected, so we tried to find its explanation based on the existing literature reports. The phenomenon of reduced expression of TLR4 was first described as a mechanism responsible for
bacterial lipopolysaccharides (LPS) tolerance in monocytes previously challenged with LPS [55–57]. LPS acts as a proto-endotoxin and contributes to the inflammatory cascade by binding to the CD14/TLR4/MD2 receptor complex in many cell types, but primarily to monocytes and dendritic, macrophages, and B cells [57]. LPS signaling by TLR4 leads to the activation of intracellular signaling that promotes the expression of inflammatory genes, which stimulate the acute and sustained defense of the host [57, 58]. Exaggerated and uncontrolled pro-inflammatory signaling triggered by TLR4 during infection can lead to sepsis, septic shock, and death [59]. However, LPS-induced internalization of TLR4 follows immune stimulation and acts as an essential immune-homeostatic response that prevents overactivation of the inflammatory response and septic complications [57, 60]. Depletion in TLR4 receptors desensitizes cells to stimuli and effectively inhibits the pro-inflammatory signaling pathway.

Further studies have shown that this negative control of TLR4 surface expression not only appears in microbial infections but may also be a universal mechanism triggered in response to increased inflammation of various etiologies. Reduced expression of TLR4 on peripheral monocytes was observed in autoimmune diseases, acute pancreatitis, or chronic kidney diseases [61–65]. Krejsek et al. observed TLR4 down-regulation on monocytes of patients undergoing coronary artery bypass grafting, an invasive procedure associated with activating an inflammatory response [66]. The concept of the crucial role of TLR4 in suppressing inflammation is supported by recent studies which indicate that TLR4 deficiency in monocytes favors their differentiation into an anti-inflammatory M2-like phenotype, characterized by a reduced release of pro-inflammatory cytokines [67].

A similar mechanism is assumed in hypertensive patients, in whom a decrease in the surface expression of TLR4 may be a response to enhanced inflammation, manifested in our studies by an increase in the neutrophil count and the NLR index. Recent reports indicate that the increasing number of neutrophils may have an immunomodulatory effect on other immune cells, including monocytes, revealing their anti-inflammatory phenotype [68–71]. Cytokines released by neutrophils and monocytes during prolonged inflammation can cause hyporesponsiveness of inflammatory cells similar to that induced by bacterial endotoxins.

TNF-α plays a specific role in this process [72, 73]. According to the mechanism proposed by Tsai et al., by binding to its receptor on the monocytes, TNF-α activates an intracellular signaling pathway that decreases TLR4 surface expression [73]. This finding suggests that TNF-α and possibly other cytokines secreted by inflammatory cells may serve as negative regulators of TLR4 expression leading to the silencing of inflammatory response. Higher TNF-alpha concentration that correlated with reduced TLR4 surface expression in our research can be proof of this phenomenon. Down-regulation of TLR4 induced by rising cytokine concentration may consequently lead to a gradual decrease in their level based on negative feedback. This mechanism may explain the lack of significant differences in the concentration of TLR-4-dependent cytokines: IL-6, and TNF-alpha in the blood of hypertensive patients, despite the evidence of inflammation reflected by a high NLR index.
Our research shows that observed phenotypic alterations of monocytes are associated with a decreased extent of endothelial damage. This relationship was particularly pronounced in patients with resistant hypertension, in whom lower expression of CD11b and CD18 was an independent indicator of endothelial improvement. Disclosure of this cause-and-effect axis only in resistant hypertension may result from more severe inflammation, which faster and more effectively triggers anti-inflammatory compensatory processes that limit tissue and organ damage. The observed decrease in CD11b/CD18 expression on the surface of monocytes will cause their reduced adhesion to the endothelium, resulting in a lesser extent of vascular damage.

We are aware of the limitations of our research. The number of patients is relatively small, which requires verification of our results on a larger population in further studies. The results obtained may have been influenced by the blood pressure-lowering drugs taken by the patients. Therefore, the aim of further research should be the evaluation of TLR4 and CD11b/CD18 expression in patients with newly diagnosed hypertension. If our conclusions are confirmed, it is crucial to determine the exact molecular mechanisms responsible for the decrease in TLR4 and CD11b/CD18 surface expression. It is also essential to establish the impact of these phenotypic features on the behavior of monocytes themselves and other cellular effectors of the immune response.

5. Conclusions

Our preliminary study showed for the first time that hypertension of varying severity is accompanied by phenotypic changes in monocytes, manifested by reduced surface expression of both TLR4 and CD11b/CD18. This phenotypic feature may allow monocytes to downregulate inflammatory response and limit vascular damage. Our study opens a new unexplored area of research on the anti-inflammatory function of monocytes in hypertension. Such knowledge will provide a broader insight into the inflammatory mechanisms in the pathogenesis of hypertension and may also point to a new strategy involving the therapeutic manipulation of monocytes.

Declarations

AUTHOR CONTRIBUTIONS

STATEMENTS AND DECLARATIONS

This work was supported by Poznan University of Medical Sciences [grant numbers: 502-14-02228370-10617].

The authors have no competing interests to declare that are relevant to the content of this article.

Approval was obtained from the ethics committee of Poznan University of Medical Sciences. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Informed consent was obtained from all individual participants included in the study.

AVAILABILITY OF DATA AND MATERIALS

Data are available from the authors upon reasonable request.

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References


**Figures**
Figure 1

The percentage of TLR4⁺CD11b⁺ monocytes in MH and RH groups.

Results shown as mean ± SD were analyzed using ANOVA test with Turkey's multiple comparisons.

*statistically significant difference compared to the control group ($p \leq 0.05$).
Figure 2

The percentage of $\text{TLR}4^+\text{CD18}^+$ monocytes in MH and RH groups.

Results shown as mean ± SD were analyzed using ANOVA test with Turkey's multiple comparisons.

*statistically significant difference compared to the control group ($p \leq 0.05$).
Figure 3

The correlation between TLR4 MFI and CD18 MFI in the MH group.
Figure 4

TLR4 mRNA relative expression in PBMCs of MH and RH group.

Results shown as mean ± SD were analyzed using ANOVA test with Turkey's multiple comparisons.
Figure 5

The correlation between TNF-α concentration and TLR4 MFI in the RH group.