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Assessment of the influence of peripheral blood mononuclear cell stimulation with *Streptococcus pneumoniae* polysaccharides on expression of selected Toll-like receptors, activation markers and Fas antigen in patients with chronic lymphocytic leukemia*

Ocena wpływu stymulacji komórek mononuklearnych krwi obwodowej polisacharydami *Streptococcus pneumoniae* na ekspresję wybranych receptorów Toll-podobnych, markerów aktywacji oraz antygenu FAS u chorych na przewlekłą białaczkę limfocytową

Authors' Contribution:

- A Study Design
- B Data Collection
- C Statistical Analysis
- D Data Interpretation
- E Manuscript Preparation
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Introduction:

Summary

Since 2012, both the 13-valent pneumococcal conjugate vaccine (PCV13) and the 23-valent pneumococcal polysaccharide vaccine (PPV23) have been recommended for pneumococcal infection prevention in patients with chronic lymphocytic leukemia (CLL). Available literature data indicate that leukemic cells may respond to the presence of pathogens through specific Toll-like receptors (TLR).

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Objectives:	The aim of the study was to assess the effect of in vitro PPV23 stimulation of peripheral blood mononuclear cells (PBMCs) on expression of TLR-2, TLR-4, CD25, CD69, and CD95 on the surface of CD3+ T cells and CD19+ B cells in CLL patients.
Methods:	A total of 30 previously untreated patients with CLL, stage 0 according to the Rai classification, were included in the study. PBMCs obtained from each patient were cultured with and without PPV23 antigens. After 24-, 48-, and 72 h cultures, the viable cells underwent labeling with fluorochrome-conjugated monoclonal antibodies, and were analyzed using a flow cytometer.
Results:	Between 24 h and 72 h ($p=0.002$) stimulation with PPV23 and between 48 h and 72 h ($p=0.034$) stimulation with PPV23 the frequencies of CD3+TLR-2+ cells were diminished. Increase of the value of the percentage of CD19+CD69+ cells was observed after 24 h ($p=0.003$), 48 h ($p=0.025$) and 72 h ($p=0.012$) of stimulation with PPV23. Stimulation with PPV23 led to an increase of the percentage of CD19+CD95+ cells after 24 h ($p=0.003$), 48 h ($p=0.015$), and 72 h ($p=0.006$).
Conclusions:	We found that both T and B cells respond to antigens of PPV23 by inducing TLR-dependent pathways, lymphocyte activation and CD95 expression.
Key words:	peripheral blood mononuclear cells • <i>Streptococcus pneumoniae</i> polysaccharides • Toll-like receptors • activation markers • Fas antigen • chronic lymphocytic leukemia
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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in European and North American adult populations and leads to significant immune system dysfunction [24,29]. The dominant clinical presentation in 50% of patients consists of recurrent, often severe, infections [26,29,45]. Infections are the most common cause of deaths in CLL patients, with 60–80% mortality [26]. There are a number of factors causing immune suppression in CLL patients. They include both the specific (humoral and cellular) and innate immunity (dysfunction of the complement system, NK cells, neutrophils and monocytes). The risk of infection is dependent on the duration of the disease and its clinical stage [42]. According to recent data, infectious agents may play an important role in the development of CLL [36]. A particularly frequent pathogen which is isolated from patients with CLL is *Streptococcus pneumoniae* [10,28]. This pathogen is characterized by high virulence and resistance to many classes of antibiotics [45]. The polysaccharide coat is a feature of invasiveness of this microorganism and enables the immune system to avoid components [39,42]. Since 2012, both the 13-valent pneumococcal

conjugate vaccine (PCV13) and the 23-valent pneumococcal polysaccharide vaccine (PPV23) have been recommended for pneumococcal infection prevention in patients with CLL [42].

Correct and appropriately quick reaction to the presence of a pathogen restricts infection and induces a secondary immune response. This leads to effective eradication of the microorganism [43]. Available literature data indicate that leukemic cells may respond to the presence of pathogens through specific Toll-like receptors (TLR) [44]. TLR are common receptors in the body and have a wide spectrum of recognition ligands [2]. The interaction of TLR with an appropriate ligand causes synthesis of pro-inflammatory cytokines and increases expression of histocompatibility complex antigens and co-stimulatory molecules as well as synthesis of interferons and chemokines [2,11]. This interaction induces helper T-cell response type 1 (Th1) but can also lead to the appearance of regulatory T cells (Treg) [11,41]. The B-cell response to the presence of a ligand for the TLR is manifested by the proliferation and expression of co-stimulatory molecules and the secretion of antibodies [11,17,23]. The role of TLR also includes the promotion



of class switching as well as the activation of memory B cells [11,18]. CLL cells' response to TLR ligands can be observed *in vitro*, leading to the increased expression of co-stimulatory molecules, proliferation or apoptosis [14,44].

The influence of the antigens included in the PPV23 on TLR expression on the surface of leukemic cells has not been analyzed yet. In the available literature there can be found only information about the effects of TLR ligands (e.g. immunostimulatory CpG oligonucleotides) on CLL cells [44]. Epidemiological evidence indicates that the stimulation of CLL cells by pathogens contributes to the initiation of the disease or its progression [4]. CLL cells can receive signals from the environment and the TLR may lead to cell activation [8,40]. TLR indirectly contribute to the death of the infected cells. CLL is classified as a disease involving a defect of programmed cell death (19). Leukemic cells are characterized by low expression of CD95 (Fas) [33]. In contrast, *in vitro* spontaneous apoptosis occurs, most likely in the absence of inhibitory factors [35].

The aim of the study was to assess the effect of *in vitro* PPV23 stimulation of peripheral blood mononuclear cells on expression of TLR-2, TLR-4, CD25, CD69, and CD95 on the surface of CD3+ T cells and CD19+ B cells in CLL patients.

MATERIAL AND METHODS

Study group

A total of 30 previously untreated patients with CLL (12 women and 18 men) who were diagnosed in the Outpatient Immunology Clinic of Autonomous Public Clinical Hospital No. 4 in Lublin, Poland, were included in the study. All patients enrolled in the study were in stage 0 according to the Rai classification [30]. None of the patients had been receiving drugs affecting the immune system, none showed any signs of infection (at least 2 months prior to the study) or any signs of autoimmune or allergic disease, and none had received blood transfusions. CLL was diagnosed based on the National Cancer Institute (NCI) International Workshop on CLL (IWCLL) guidelines [16]. The complete blood count, beta-2-microglobulin, and lactate dehydrogenase (LDH) serum concentration, as well as imaging examinations, were conducted using standard diagnostic and radiological laboratory methods. The average age of patients was 64.9±9.1 (median: 65; min: 52; max: 80). This study was approved by the Ethics Committee of the Medical University of Lublin (decision no. KE-0254/227/2010). Written informed consent was obtained from all patients with respect to the use of their blood for scientific purposes.

Examined material

Peripheral blood samples were collected into EDTA tubes and were used to isolate peripheral blood mononuclear

cells (PBMCs) by density gradient centrifugation.

The peripheral blood samples were diluted with 0.9% calcium (Ca^{2+}) and magnesium (Mg^{2+})-free phosphate buffered saline (Biochrome AG, Germany) at a 1:1 ratio. The diluted material was layered onto 3 mL Gradisol L (Aqua Medica, Poland) with specific gravity of 1.077 g/mL, and then centrifuged in a density gradient at 700 g for 20 minutes. The obtained peripheral blood mononuclear cells (PBMCs) were collected with Pasteur pipettes and washed twice with $\text{Ca}^{2+}\text{Mg}^{2+}$ -free PBS for 5 minutes. Subsequently, the cells were suspended in 1 mL of $\text{Ca}^{2+}\text{Mg}^{2+}$ -free PBS, counted with a Neubauer chamber, and their vitality was assessed with trypan blue (0.4% Trypan Blue Solution, Sigma Aldrich, Germany).

After isolation, the cells were cultured for 72 h. Complete culture medium was used for the culture PBMCs consisted of RPMI 1640 (PAA Laboratories, Austria) with 10% human albumin (Baxter, US) and antibiotics: penicillin (100 IU/ml), streptomycin (50 µg/ml) and neomycin (100 µg/ml) (Sigma Aldrich, Germany). Cells were cultured at 37°C in a 5% CO_2 atmosphere. PBMCs obtained from each patient were cultured with PPV23 (PNEUMO 23, Pneumococcal Polysaccharide Vaccine, Sanofi Pasteur, France) and without PPV23 antigens. The cells were stimulated the most effectively with the dose of PPV23 of 100 µl.

After 24-, 48-, and 72 h cultures, following density gradient centrifugation, the viable PBMCs underwent two- or three-color labeling with adequate quantities of monoclonal antibodies, according to the manufacturer's instructions. The isolated suspension of cells was divided into individual test tubes at 1×10^6 /sample and incubated with monoclonal antibodies (MoAbs). 20 µl of antibodies was added to each sample of evaluated cells and incubated at room temperature for 20 minutes. Following incubation, the cells were washed twice with PBS (at 700 g, 5 minutes) and immediately analyzed with the FACSCalibur flow cytometer (Becton Dickinson, US), equipped with an argon laser of 488 nm wavelength. Data acquisition was conducted with the specialist FACS Diva Software 6.1.3 system (Becton Dickinson, US). The data were analyzed with CellQuest Pro (Becton Dickinson, US). CaliBRITE (Becton Dickinson, US) was the calibrating system used to optimize flow cytometer settings. For each sample, data were acquired by routine collection of 30,000 events in the lymphocyte gate (region R1) in a forward-scatter (FSC)/side-scatter (SSC) dot plot. This allowed us to exclude erythrocytes, platelets, dead cells, and cell fragments from the analysis. Labeled cells were recorded based on the created lymphocyte gate. The results were presented as a percentage of CD45+ cells stained with antibody. The following MoAbs conjugated with relevant fluorochromes were used: anti-CD3 PE-Cy5, anti-CD19 PE; anti-CD-69 PE-Cy5, anti-CD95 PE-Cy5, anti-CD25 PE-Cy5, anti-CD45/CD14 FITC, PE, anti-CD3/CD19 FITC/PE (BD Biosciences, US), anti-TLR-2 FITC, and anti-TLR-4 FITC (Abcam, UK).

Table 1. Frequencies of the T CD3+ and B CD19+ cells in PBMCs cultured for 24-72 h in the complete medium with and without PPV23

Marker cells	Stimulation time	With PPV23		Without PPV23	
		Mean±SD	Median (range)	Mean±SD	Median (range)
T CD3+TLR-2+	24h	1.23±0.97%	0.98% (0.4-3.82%)	0.89±0.47%	0.71% (0.26-1.86%)
	48h	0.65±0.46%	0.47% (0.2-1.76%)	0.78±0.47%	0.67% (0.15-1.8%)
	72h	0.29±0.21%	0.25% (0.09-0.73%)	0.72±0.31%	0.65% (0.36-1.38%)
B CD19+TLR-2+	24h	0.41±0.25%	0.34% (0.12-0.99%)	0.40±0.16%	0.44% (0.1-0.66%)
	48h	0.25±0.27%	0.14% (0.05-0.95%)	0.66±0.21%	0.73% (0.13-0.88%)
	72h	0.21±0.32%	0.06% (0-1.13%)	0.65±0.13%	0.67% (0.44-0.83%)
T CD3+TLR-4+	24h	1.56±0.96%	1.34% (0.65-3.95%)	3.75±2.97%	2.92% (0.55-9.17%)
	48h	0.74±0.68%	0.38% (0.08-2.3%)	5.51±3.07%	4.43% (0.77-9.51%)
	72h	0.74±0.68%	0.38% (0.08-2.3%)	5.51±3.07%	4.43% (0.77-9.51%)
B CD19+TLR-4+	24h	0.91±0.40%	0.92% (0.16-1.5%)	2.30±1.23%	2.47% (0.29-4.06%)
	48h	0.64±0.41%	0.48% (0.35-1.76%)	2.65±1.57%	3.06% (0.12-5.01%)
	72h	0.54±0.56%	0.37% (0.02-1.84%)	3.48±0.81%	3.46% (2.32-4.92%)
T CD3+CD69+	24h	30.98±21.45%	32.95% (2.6-58.76%)	4.39±3.64%	2.91% (0.95-10.35%)
	48h	21.23±20%	15.44% (1.23-64.82%)	3.17±3.52%	1.48% (0.41-9.99%)
	72h	17.04±16.47%	15.05% (1.49-46.74%)	3.22±3.10%	2.22% (0.62-10.42%)
B CD19+CD69+	24h	57.74±27.95%	52.13% (9.39-95.29%)	38.05±27.22%	33.41% (4.81-91.48%)
	48h	58.15±30.77%	57.68% (12.56-97.05%)	33.36±25.34%	23.09% (12-91.14%)
	72h	59.03±35.61%	73.42% (7.28-97.41%)	36.75±30.84%	27.52% (7.72-92.16%)
T CD3+CD25+	24h	30.21±17.77%	29.42% (8.24-69.98%)	22.68±15.01%	22.48% (5.76-48.89%)
	48h	28.53±19.00%	31.22% (0.59-68.42%)	22.74±15.40%	23.25% (1.56-47.21%)
	72h	30.44±15.85%	25.61% (10.55-67.49%)	20.70±14.12%	20.68% (5.64-42.18%)
B CD19+CD25+	24h	93.57±8.19%	97.22% (69.45-98.68%)	91.55±7.59%	95.2% (72.97-98.51%)
	48h	82.63±29.74%	92.66% (1.8-98.74%)	80.32±32.51%	94.11% (3.03-98.43%)
	72h	92.43±9.77%	98.11% (74.48-99.72%)	89.96±18.00%	98.44% (39.93-99.49%)
T CD3+CD95+	24h	97.94±3.23%	99.59% (88.85-99.92%)	94.65±6.38%	97.99% (82.64-99.62%)
	48h	82.6±38.02%	99.09% (1.04-99.97%)	78.27±38.71%	98.19% (0.55-99.73%)
	72h	96.13±6.25%	99.10% (81.05-99.87%)	94.35±7.01%	97.85% (79.34-99.76%)
B CD19+CD95+	24h	73.71±29.22%	91.27% (29.1-99.31%)	28.59±18.69%	21.36% (14.1-73.14%)
	48h	74.45±41.07%	97.42% (1.21-99.75%)	26.22±26.06%	21.52% (0.44-83.05%)
	72h	79.76±25.07%	93.17% (33.26-99.91%)	38.78±18.99%	32.64% (14.24-82.86%)

Statistical analysis

Statistical analysis of the results was conducted using Statistica 10 (StatSoft, US). Deviation from normality was evaluated by the Kolmogorov-Smirnov test. Differences between groups were assessed using the Wilcoxon test. Data were expressed as the mean value ± SD, median, minimum and maximum. A value p < 0.05 was considered statistically significant.

RESULTS

The results of *in vitro* PPV23 stimulation of peripheral blood mononuclear cells on expression of TLR-2, TLR-4, CD25, CD69, and CD95 on the surface of CD3+ T cells and CD19+ B cells in CLL patients are shown in Table 1.

Frequencies of TLR-2+ T CD3+ and B CD19+ cells in PBMCs cultured for 24-72 h in complete medium with and without PPV23

Between 24 h and 72 h (p=0.002) stimulation with PPV23 and between 48 h and 72 h (p=0.034) stimulation with PPV23 the frequencies of T CD3+TLR-2+ cells were markedly diminished (Figure 1). After 72 h stimulation with PPV23 the value of the mean percentage of T CD3+TLR-2+ cells was lower in comparison with unstimulated PBMCs (p=0.013, Figure 2). After 48 h stimulation with PPV23 the value of the mean percentage of B CD19+TLR-2+ cells was lower in comparison with unstimulated PBMCs (p=0.008; Figure 3). After 72 h stimulation with PPV23 the value of the mean percentage of B CD19+TLR-2+ cells was lower in comparison with unstimulated PBMCs (p=0.005; Figure 3).



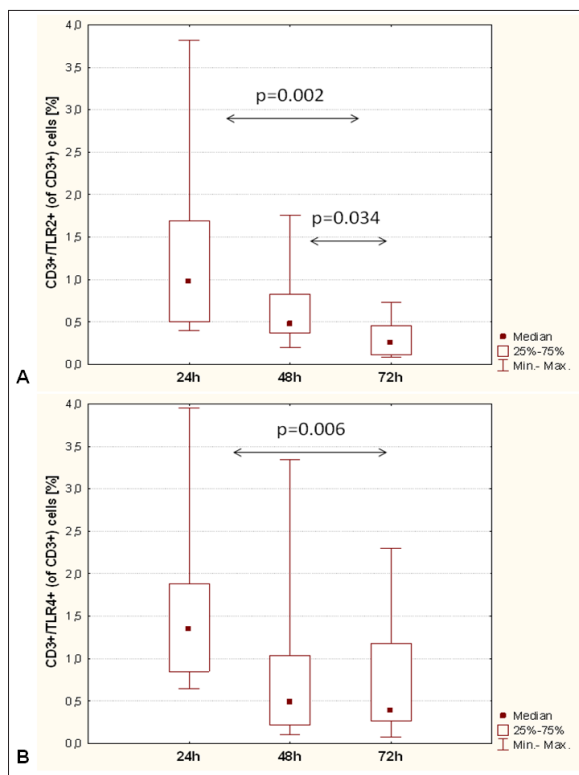


Fig. 1. Frequencies of CD3+/TLR-2+ cells (A) and CD3+/TLR-4+ cells (B) in 24-, 48-, and 72 h culture of PBMCs upon PPV23 stimulation

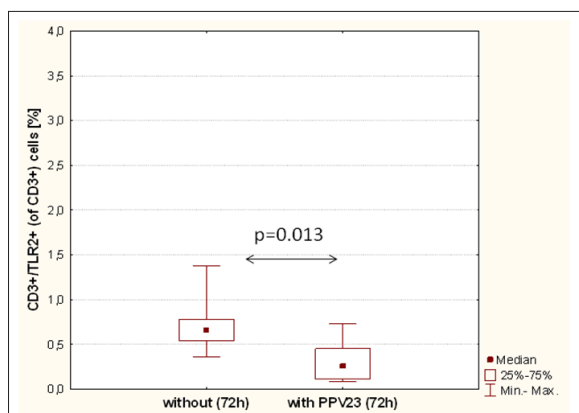


Fig. 2. Frequencies of CD3+/TLR-2+ cells in 72 h culture of PBMCs unstimulated and stimulated with PPV23

Frequencies of TLR-4+ T CD3+ and B CD19+ cells in PBMCs cultured for 24-72 h in complete medium with and without PPV23

Between 24 h and 72 h ($p=0.006$) stimulation with PPV23 the value of the mean percentage of T CD3+TLR-4+ cells was markedly diminished (Figure 1). After 24 h as well as after 72 h stimulation with PPV23 the value of the mean percentage of T CD3+TLR-4+ cells was lower in comparison with unstimulated PBMCs ($p=0.011$, $p=0.004$ respectively; Figure 4).

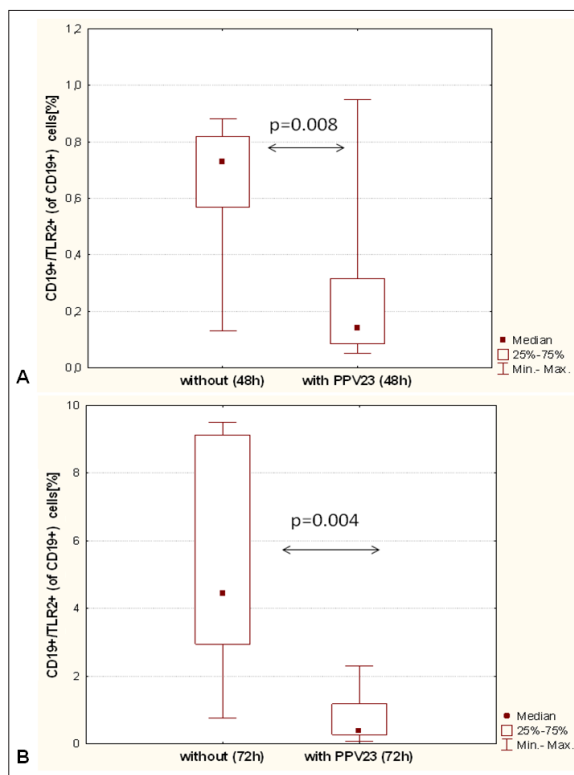


Fig. 3. Frequencies of CD19+/TLR-2+ cells in 48 h (A) and 72 h (B) culture of PBMCs unstimulated and stimulated with PPV23

The value of the mean percentage of B CD19+TLR-4+ cells after 24 h, 48 h and 72 h stimulation with PPV23 was significantly lower in comparison with unstimulated PBMCs ($p=0.005$, $p=0.010$ and $p=0.005$, respectively; Figure 5).

Frequencies of CD69+ T CD3+ and B CD19+ cells in PBMCs cultured for 24-72 h in complete medium with and without PPV23

After 24 h stimulation with PPV23 the value of the mean percentage of T CD3+CD69+ cells was significantly higher in comparison with unstimulated PBMCs ($p=0.004$). Similarly, after 48 h and 72 h stimulation with PPV23 the value of the mean percentage of T CD3+CD69+ cells was higher in comparison with unstimulated PBMCs ($p=0.012$ and $p=0.022$, respectively). A statistically significant increase of the value of the mean percentage of B CD19+CD69+ cells was observed after all tested periods of stimulation with PPV23 in comparison with unstimulated PBMCs: after 24 h ($p=0.003$), 48 h ($p=0.025$) and 72 h ($p=0.012$).

Frequencies of CD25+ T CD3+ and B CD19+ cells in PBMCs cultured for 24-72 h in complete medium with and without PPV23

We found the value of the mean percentage of T CD3+CD25+ cells after stimulation with PPV23 was the

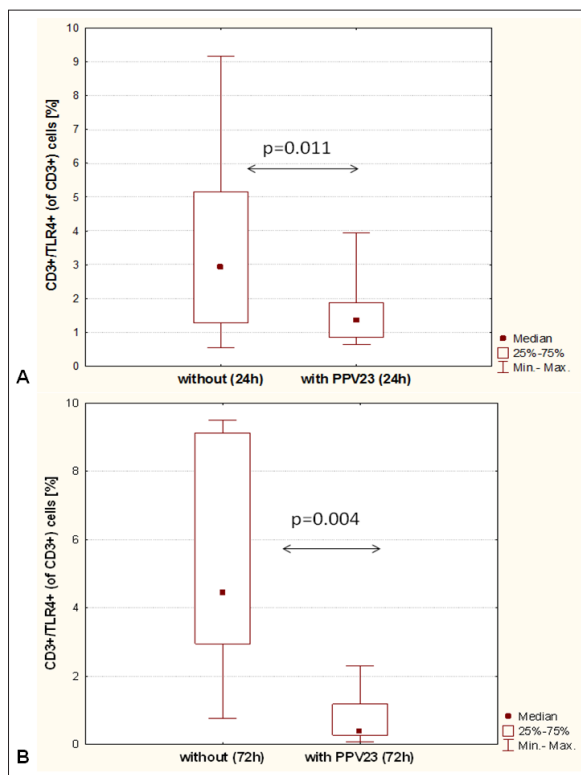


Fig. 4. Frequencies of CD3+/TLR4+ cells in 24 h (A) and 72 h (B) culture of PBMCs unstimulated and stimulated with PPV23

highest after 72 h and was significantly higher in comparison with unstimulated PBMCs ($p=0.010$). After 24 h stimulation with PPV23 the value of the mean percentage of T CD3+CD25+ cells was higher in comparison with unstimulated PBMCs ($p=0.021$). The percentage of the T CD3+CD25+ subpopulation did not differ significantly between stimulated and unstimulated cells which were analyzed after 48 h. A significantly higher value of the mean percentage of B CD19+CD25+ cells in comparison with unstimulated PBMCs was observed after 24 h stimulation with PPV23 ($p=0.003$). The percentage of the B CD19+CD25+ subpopulation did not differ significantly between stimulated and unstimulated cells when analyzed after 48 h and after 72 h.

Frequencies of CD95+ T CD3+ and B CD19+ cells in PBMCs cultured for 24-72 h in complete medium with and without PPV23

We found that the value of the mean percentage of T CD3+CD95+ cells was higher after 24 h ($p=0.003$) and 48 h ($p=0.016$) T CD3+ stimulation with PPV23 in comparison with unstimulated PBMCs but the percentage of the T CD3+CD95+ subpopulation did not differ significantly between stimulated and unstimulated cells when analyzed after 72 h. Between 24 h and 72 h stimulation with PPV23 the value of the mean percentage of B CD19+CD95+ cells showed a significant increase ($p=0.023$). Stimulation with PPV23 was associated with a significant increase of the value of the B CD19+CD95+ cell mean percent-

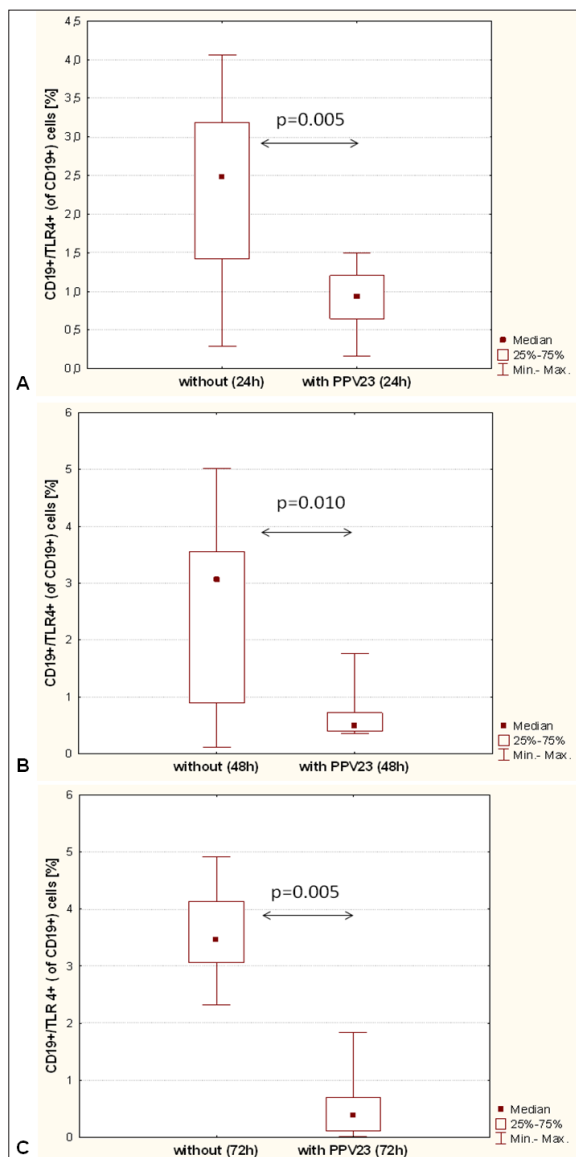


Fig. 5. Frequencies of CD19+/TLR4+ cells in 24 h (A) 48 h (B) 72 h (C) culture of PBMCs unstimulated and stimulated with PPV23

age in comparison with unstimulated PBMCs after 24 h ($p=0.003$), 48 h ($p=0.015$) and 72 h ($p=0.006$).

DISCUSSION

CLL is characterized by increased frequency of infections. Antigens of microorganisms may be involved in the proliferation and accumulation of the leukemic clone [5]. 88% of infections in CLL patients are caused by bacteria, particularly *Streptococcus pneumoniae* [12,19]. The polysaccharide capsule is the main pneumococcal virulence factor, and based on its chemical structure, pneumococci are divided into over 90 different serotypes which differ substantially in their capacity to cause disease [20]. It is already known that the polysaccharide capsule was recognized by pathogen recognition receptors (PRPs) expressed by mac-



rophages: C-type lectin SIGN-R1, MACRO and CD14 [25]. There are no available data reporting the influence of capsule antigens on T and B marker cell expression.

Members of the TLR family have been described as PRPs important in pneumococcal infection. Knapp et al. [22] performed an *in vivo* study and estimated the mRNA for TLR-2 expression in lungs before and after pneumococcal infection. The results showed that after infection, mRNA for TLR-2 increased, but the immune response was also observed in TLR-2-deficient mice [22]. TLR-9 may have significance in the defense against *Streptococcus pneumoniae*, because TLR-9 recognizes bacterial DNA. Experiments showed that protection of pathogens is dependent on cooperation between different TLR [22]. Several studies have revealed that the TLR response to ligands can vary (31). Grandjenete et al. (32) stimulated TLR-9 by CpG ODN, which caused a strong increase in the activation and proliferation of CLL cells. In contrast, stimulation of TLR-7 is characterized by weak cell proliferation [7]. Activation of tumor cells by TLR can cause that cells will be better recognized by the immune system [32].

In the available literature there are no data on research assessing the influence of antigens included in PPV23 on the percentages of B and T cells with TLR expression. In this study we observed a significant reduction of T CD3+TLR-2+ and B CD19+TLR-2+ cells after PPV23 stimulation in comparison to unstimulated cells. The most evident changes in the frequencies of T CD3+TLR-2+ cells were observed after 72 h culture with PPV23. The percentages of B CD19+TLR-2+ cells were reduced after 48 h stimulation. The frequencies of T CD3+TLR-4+ and B CD19+TLR-4+ cells were reduced after PPV23 stimulation. Similar results were obtained by Antosz et al., who evaluated the percentages of B cells with TLR-4 expression after LPS stimulation in CLL patients [2,3]. TLR-4 is activated by pneumolysin during pneumococcal infection or colonization [15]. It has been shown that pneumolysin-TLR4 signaling can induce host cell apoptosis *in vitro* and *in vivo*, which appears to be a protective host response [37]. Mice with a defect of this receptor had an increased mortality rate during *Streptococcus pneumoniae* infection [22].

The stimulation of TLR by ligands can lead to cell activation [8]. B lymphocytes have the ability to produce

IL-6, IL-10, INF- γ and immunoglobulins after TLR agonist stimulation [34]. Leukemic cell activation is dependent on TLR-9 [32]. Activation markers were divided into two groups: the early (CD69+ and CD71+) and late ones (CD25+, CD26+, HLA-DR+) [1,38]. D'Arena et al. [9] observed expression of CD69 antigen on at least 30% of CD19+ B lymphocytes in 52% of CLL patients. Brisslert et al. [6] found that CD25 was present on B cells after stimulation by TLR ligands. Our research estimated the influence of PPV23 on the changes in the percentages of CD69+ B and T cells after 24-, 48- and 72 h culture, and revealed a gradual increase of frequencies of those early activated lymphocytes. De Fanis et al. [10] evaluated percentages of B cells with CD69 expression. The percentages of B CD19+CD69+ cells increased after stimulation with phorbol 12-myristate 13-acetate (PMA) + ionomycin, phytohemagglutinin, or pokeweed mitogen. We obtained similar results, but the increase of activated B and T cell percentages was caused by stimulation with PPV23, which demonstrates that PPV23 may play the role of a polyclonal immune stimulator. The highest increase was observed in the frequencies of activated T cells, probably because we selected patients in stage 0. T cells obtained from CLL patients with less advanced stages may correctly respond to antigen stimulation [31]. Therefore the clinical effect of vaccination with PPV23 may significantly exceed the single prevention of pneumococcal disease and extensively stimulate immunity.

CD95 (Fas) is a molecule belonging to the TNF (tumor necrosis factor) superfamily [21]. Ghamlouch et al. [13] evaluated apoptotic cells which were stimulated with the serum of patients with CLL. The apoptosis was significantly reduced in comparison to the unstimulated control. Our research showed low frequencies of CD19+CD95+ cells before PPV23 stimulation and a significant increase after PPV23 stimulation. Molica et al. investigated the influence of *Staphylococcus aureus* Cowan I (SAC), interleukin 2 and IFN- α on Fas expression on B and T cells in CLL [27]. These authors along with De Fanis et al. obtained similar results to ours, but our research showed a more prominent increase after stimulation [10,27].

In summary, we confirmed that both T and B cells respond to antigens of PPV23 by inducing TLR-dependent pathways, lymphocyte activation and CD95 expression.

REFERENCES

- [1] Amu S., Gjertsson I., Brisslert M.: Functional characterization of murine CD25 expressing B cells. *Scand. J. Immunol.*, 2010; 71: 275-282
- [2] Antosz H., Choroszyńska D.: Negative regulation of Toll-like receptor signalling. *Postępy Hig. Med. Dośw.*, 2013; 67: 339-350
- [3] Antosz H., Sajewicz J., Marzec-Kotarska B., Kocki J., Dmoszyńska A.: Different expression of CD180, CD284 and CD14 receptors on the CD19+ subpopulation of normal and B-CLL lymphocytes. *Folia Histochem. Cytobiol.*, 2009; 47: 593-598
- [4] Arvaniti E., Ntoufa S., Papakonstantinou N., Touloumenidou T., Laoutaris N., Anagnostopoulos A., Lamnissou K., Caligaris-Cappio F., Stamatopoulos K., Ghia P., Muzio M., Belessi C.: Toll-like receptor signaling pathway in chronic lymphocytic leukemia: distinct gene expression profiles of potential pathogenic significance in specific subsets of patients. *Haematologica*, 2011; 96: 1644-1652
- [5] Bertilaccio M.T., Simonetti G., Dagklis A., Rocchi M., Rodriguez T.V., Apollonio B., Mantovani A., Ponzoni M., Ghia P., Garlanda C., Caligaris-Cappio F., Muzio M.: Lack of TIR8/SIGIRR triggers progression of chronic lymphocytic leukemia in mouse models. *Blood*, 2011; 118: 660-669
- [6] Brisslert M., Bokarewa M., Larsson P., Wing K., Collins L.V., Tar-

kowski A.: Phenotypic and functional characterization of human CD25+ B cells. *Immunology*, 2006; 117: 548-557

[7] Chiron D., Bekeredjian-Ding I., Pellat-Deceunynck C., Bataille R., Jego G.: Toll-like receptors: lessons to learn from normal and malignant human B cells. *Blood*, 2008; 112: 2205-2213

[8] Damle R.N., Calissano C., Chiorazzi N.: Chronic lymphocytic leukaemia: a disease of activated monoclonal B cells. *Best Pract. Res. Clin. Haematol.*, 2010; 23: 33-45

[9] D'Arena G., Musto P., Nunziata G., Cascavilla N., Savino L., Pistolesi G.: CD69 expression in B-cell chronic lymphocytic leukemia: a new prognostic marker? *Haematologica*, 2001; 86: 995-996

[10] De Fanis U., Romano C., Dalla Mora L., Sellitto A., Guastafierro S., Tirelli A., Bresciano E., Giunta R., Lucivero G.: Differences in constitutive and activation-induced expression of CD69 and CD95 between normal and chronic lymphocytic leukemia B cells. *Oncol. Rep.*, 2003; 10: 653-658

[11] Dębińska A., Boznański A.: The role of Toll-like receptors in the pathogenesis of allergic diseases - where is the truth? *Postępy Hig. Med. Dośw.*, 2014; 68: 230-237

[12] Francis S., Karanth M., Pratt G., Starczynski J., Hooper L., Fegan C., Pepper C., Valcarcel D., Milligan D.W., Delgado J.: The effect of immunoglobulin VH gene mutation status and other prognostic factors on the incidence of major infections in patients with chronic lymphocytic leukemia. *Cancer*, 2006; 107: 1023-1033

[13] Ghamlouch H., Ouled-Haddou H., Damaj G., Royer B., Gubler B., Marolleau J.P.: A combination of cytokines rescues highly purified leukemic CLL B-cells from spontaneous apoptosis *in vitro*. *PLoS One*, 2013; 8: e60370

[14] Grandjette C., Kennel A., Faure G.C., Béné M.C., Feugier P.: Expression of functional toll-like receptors by B-chronic lymphocytic leukemia cells. *Haematologica*, 2007; 92: 1279-1281

[15] Gray C., Ahmed M.S., Mubarak A., Kasbekar A.V., Derbyshire S., McCormick M.S., Mughal M.K., McNamara P.S., Mitchell T., Zhang Q.: Activation of memory Th17 cells by domain 4 pneumolysin in human nasopharynx-associated lymphoid tissue and its association with pneumococcal carriage. *Mucosal Immunol.*, 2014; 7: 705-717

[16] Hallek M.: Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. *Am. J. Hematol.*, 2013; 88: 803-816

[17] Hartmann G., Krieg A.M.: Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.*, 2000; 164: 944-953

[18] He B., Qiao X., Cerutti A.: CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. *J. Immunol.*, 2004; 173: 4479-4491

[19] Jahrsdorfer B., Mühlhoff L., Blackwell S.E., Wagner M., Poeck H., Hartmann E., Jox R., Giese T., Emmerich B., Endres S., Weiner G.J., Hartmann G.: B-cell lymphomas differ in their responsiveness to CpG oligodeoxynucleotides. *Clin. Cancer Res.*, 2005; 11: 1490-1499

[20] Kamerling J.P.: Pneumococcal polysaccharide: a chemical view. In: *Streptococcus pneumoniae: Molecular Biology and Mechanisms of Disease*. Mary Ann Liebert, Inc. NY, USA, 2000: 81-114

[21] Kaufmann T., Strasser A., Jost P.J.: Fas death receptor signalling: roles of Bid and XIAP. *Cell Death Differ.*, 2012; 19: 42-50

[22] Kowalczyk E., Siednienko J., Matuszyk J.: Regulation of Toll-like receptors-dependent inflammatory response. *Postępy Hig. Med. Dośw.*, 2013; 67: 201-213

[23] Krieg A.M., Yi A.K., Matson S., Waldschmidt T.J., Bishop G.A., Teasdale R., Koretzky G.A., Klinman D.M.: CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*, 1995; 374: 546-549

[24] Linet M.S., Schubauer-Berigan M.K., Weisenburger D.D., Richardson D.B., Landgren O., Blair A., Silver S., Field R.W., Caldwell G., Hatch M., Dores G.M.: Chronic lymphocytic leukaemia: an overview of aetiology in light of recent developments in classification and pathogenesis. *Br. J. Haematol.*, 2007; 139: 672-686

[25] Luczaj-Cepowicz E., Marczuk-Kolada G., Zalewska A., Pawińska M., Leszczyńska K.: Antibacterial activity of selected glass ionomer cements. *Postępy Hig. Med. Dośw.*, 2014; 68: 23-28

[26] Molica S.: Infections in chronic lymphocytic leukemia: risk factors, and impact on survival, and treatment. *Leuk. Lymphoma*, 1994; 13: 203-214

[27] Molica S., Mannella A., Dattilo A., Levato D., Iuliano F., Peta A., Consarino C., Magro S.: Differential expression of BCL-2 oncoprotein and Fas antigen on normal peripheral blood and leukemic bone marrow cells. A flow cytometric analysis. *Haematologica*, 1996; 81: 302-309

[28] Nosari A.: Infectious complications in chronic lymphocytic leukemia. *Mediterr. J. Hematol. Infect. Dis.*, 2012; 4: e2012070

[29] Oscier D., Fegan C., Hillmen P., Illidge T., Johnson S., Maguire P., Matutes E., Milligan D.: Guidelines Working Group of the UK CLL Forum. British Committee for Standards in Haematology. Guidelines on the diagnosis and management of chronic lymphocytic leukaemia. *Br. J. Haematol.*, 2004; 125: 294-317

[30] Rai K.R., Sawitsky A., Cronkite E.P., Chanana A.D., Levy R.N., Pasternack B.S.: Clinical staging of chronic lymphocytic leukemia. *Blood*, 1975; 46: 219-234

[31] Rossmann E.D., Lewin N., Jeddi-Tehrani M., Osterborg A., Mellstedt H.: Intracellular T cell cytokines in patients with B cell chronic lymphocytic leukaemia (B-CLL). *Eur. J. Haematol.*, 2002; 68: 299-306

[32] Rozková D., Novotná L., Pytlík R., Hochová I., Kozák T., Bartunková J., Spisek R.: Toll-like receptors on B-CLL cells: expression and functional consequences of their stimulation. *Int. J. Cancer*, 2010; 126: 1132-1143

[33] Sachanas S., Levidou G., Angelopoulou M.K., Moschogiannis M., Yiakoumis X., Kalpadakis C., Vassilakopoulos T.P., Kontopidou F., Tsrirkinidis P., Dimitrakopoulou A., Kokoris S., Dimitriadou E., Kyrtsonis M.C., Panayiotidis P., Papadaki H., Patsouris E., Korkolopoulou P., Pangalis G.A.: Apoptotic and proliferative characteristics of proliferation centers in lymph node sections of patients with chronic lymphocytic leukemia. *Leuk. Lymphoma*, 2014; 55: 571-582

[34] Seda V., Mraz M.: B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *Eur. J. Haematol.*, 2015; 94: 193-205

[35] Shvidel L., Tadmor T., Braester A., Bairey O., Rahimi-Levene N., Herishanu Y., Klepfish A., Ruchlemer R., Berrebi A., Polliack A., Israeli CLL Study Group: Serum immunoglobulin levels at diagnosis have no prognostic significance in stage A chronic lymphocytic leukemia: a study of 1113 cases from the Israeli CLL Study Group. *Eur. J. Haematol.*, 2014; 93: 29-33

[36] Slager S.L., Benavente Y., Blair A., Vermeulen R., Cerhan J.R., Costantini A.S., Monnereau A., Nieters A., Clavel J., Call T.G., Maynadié M., Lan Q., Clarke C.A., Lightfoot T., Norman A.D., Sampson J.N., Casabonne D., Cocco P., de Sanjosé S.: Medical history, lifestyle, family history, and occupational risk factors for chronic lymphocytic leukemia/small lymphocytic lymphoma: the InterLymph Non-hodgkin Lymphoma Subtypes Project. *J. Natl. Cancer Inst. Monogr.*, 2014; 2014: 41-51

[37] Srivastava A., Henneke P., Visintin A., Morse S.C., Martin V., Watkins C., Paton J.C., Wessels M.R., Golenbock D.T., Malley R.: The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect. Immun.*, 2005; 73: 6479-6487

[38] Starska K., Glowacka E., Kulig A., Lewy-Trenda I., Bryś M., Lewkowicz P.: The role of tumor cells in the modification of T lymphocytes activity - the expression of the early CD69⁺, CD71⁺ and the late



CD25⁺, CD26⁺, HLA/DR⁺ activation markers on T CD4⁺ and CD8⁺ cells in squamous cell laryngeal carcinoma. *Folia Histochem. Cytobiol.*, 2011; 49: 579-592

[39] Szulc-Dąbrowska L., Gieryńska M., Depczyńska D., Schollenberger A., Toka F.N.: Th17 lymphocytes in bacterial infections. *Postępy Hig. Med. Dośw.*, 2015; 69: 398-417

[40] Tomlinson G., Chimalapati S., Pollard T., Lapp T., Cohen J., Camberlein E., Stafford S., Periselneris J., Aldridge C., Vollmer W., Picard C., Casanova J.L., Noursadeghi M., Brown J.: TLR-mediated inflammatory responses to *Streptococcus pneumoniae* are highly dependent on surface expression of bacterial lipoproteins. *J. Immunol.*, 2014; 193: 3736-3745

[41] Voo K.S., Bover L., Harline M.L., Weng J., Sugimoto N., Liu Y.J.: Targeting of TLRs inhibits CD4⁺ regulatory T cell function and activates lymphocytes in human peripheral blood mononuclear cells. *J. Immunol.*, 2014; 193: 627-634

[42] Whitaker J.A., Shanafelt T.D., Poland G.A., Kay N.E.: Room for improvement: immunizations for patients with monoclonal B-cell

lymphocytosis or chronic lymphocytic leukemia. *Clin. Adv. Hematol. Oncol.*, 2014; 12: 440-450

[43] Własiuk P., Tomczak W., Zając M., Dmoszyńska A., Giannopoulos K.: Total expression of HLA-G and TLR-9 in chronic lymphocytic leukemia patients. *Hum. Immunol.*, 2013; 74: 1592-1597

[44] Wolska A., Cebula-Obrzut B., Smolewski P., Robak T.: Effects of Toll-like receptor 7 and Toll-like receptor 9 signaling stimulators and inhibitors on chronic lymphocytic leukemia cells *ex vivo* and their interactions with cladribine. *Leuk. Lymphoma*, 2013; 54: 1268-1278

[45] Ziolkowska E., Wolowiec D., Cebula-Obrzut B., Blonski J.Z., Smolewski P., Robak T., Korycka-Wolowiec A.: Cytotoxic and apoptosis-inducing effects of bendamustine used alone and in combination with rituximab on chronic lymphocytic leukemia cells *in vitro*. *Postępy Hig. Med. Dośw.*, 2014; 68: 1433-1443

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