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Intracellular cytokine expression in T cells from patients with chronic lymphocytic leukemia

Ekspresja cytokin wewnątrzkomórkowych w limfocytach T u chorych na przewlekłą białaczkę limfocytową

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ABSTRACT

Functional disorders of T lymphocytes play an essential role in abnormal immune response in patients with chronic lymphocytic leukemia. The aim of this study was to assess the profile of cytokines expressed by T cells derived from patients with CLL. We have demonstrated that the intracellular levels of IL-2, IL-4, IFN-γ, TNF, IL-6 and IL-10 were significantly higher in T cells of CLL patients than in healthy donors. Moreover, the percentages of CD4+/CD3+/TNF+, CD4+/CD3+/IFN-γ+, and CD4+/CD3+/IL-2+ cells were significantly higher in ZAP-70-positive patients compared with ZAP-70-negative ones. Likewise, significantly higher percentages of CD4+/CD3+/TNF+, CD4+/CD3+/IFN-γ+ cells were observed in CD38-positive than in CD38-negative cases. What is more, there was a significant difference in median percentage of CD3+/CD4+ cells expressing TNF, IL-4, IFN-γ, IL-2 or IL-6 between patients carrying the 11q22.3 deletion and/or the 17p13.1 deletion and patients without these genetic aberrations. Our results confirm the functional disorders of T cells in CLL and their influence on the clinical course of the disease.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia of adults in the Western World. It is characterized by a heterogenous clinical course with survival ranging from months to several years [1, 2]. In many cases patients do not have any symptoms for at least a few years and the disease is diagnosed during periodical routine laboratory tests. Generally, two schemes of the disease course are adopted: the first is slow and infrequently requires treatment, while the other is fast-growing, with poor prognosis and short survival [3]. The available treatments can often lead to the remission of the disease, but most patients have relapses, which implies that the CLL still remains an incurable disease.

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Chronic lymphocytic leukemia is characterized by excessive proliferation and accumulation of the long-lived morphologically mature but immunologically incompetent malignant-altered B lymphocytes, in blood, bone marrow and peripheral lymphoid organs [1]. The clinical diagnosis of CLL requires an assessment of monoclonal B-cell population in peripheral blood with a minimum threshold of more than 5000 cells/μl [4]. A typical phenotype characteristic of leukemic cells is a co-expression of B lymphocyte antigens (CD19, CD20, CD23) and T cell marker CD5 [5–7].

In chronic lymphocytic leukemia there are many immunological disorders of both the humoral and the cellular immune response that are considered to be involved in disease pathogenesis.

Numerous observations indicate that the long-lived *in vivo* CLL cells quickly undergo spontaneous apoptosis when they are cultured *in vitro* [8]. This suggests that *in vivo*, factors like cytokines significantly affect the activation, proliferation and extended survival [9–11]. Therefore, in this study we have focused on the assessment of cytokines secreted by T cells with a growth-promoting potential, including: tumor necrosis factor (TNF) [12–14], interleukin 2 (IL-2) [15], interleukin 6 (IL-6) [16–18], interferon IFN-γ and interleukin 4 (IL-4) and play a role in the interactions between tumor cells and immune effector cells [19]. Moreover, we analyzed intracellular expression of IL-10 which was found to be associated with CLL progression [20] and IL-12 was considered as an important element of anti-tumor response as well [21].

Patients

Fifty-five newly diagnosed and previously untreated, consecutive, patients with CLL were enrolled for this study. The diagnosis of CLL was made on the basis of NCI-WG criteria [22]. There were 21 women and 34 men, with the median age of 66 years (range 40–83). At the time of diagnosis, patients were staged according to the Rai staging system as follows: stage 0–21 cases, stage 1–15 cases, stage 2–12 cases, stage 3–4 cases and stage 4–3 case. Cytogenetic analysis at the time of testing was available for 36 out of the 55 study patients. The patients were assigned to two groups according to this analysis. The first group consisted of 11 patients who had 11q22.3 deletion and/or 17p13.1 deletion. The second group consisted of the remaining 25 patients without these unfavorable cytogenetic abnormalities.

Control group consisted of 10 age-matched healthy volunteers (HV). The Local Ethical Committee approved the protocol of the study and all the patients signed informed consent.

Monoclonal antibodies and other reagents

The following fluorochrome-conjugated monoclonal antibodies (MoAbs) were used for the detection of intracellular cytokines: IL-12 PE, IL-2 PE, IL-4 PE, TNF PE, IFN-γ PE, IL-10 PE and IL-6 PE (Becton Dickinson, USA). For the identification of cell surface antigens the following antibodies were used: anti-CD3 PerCP, anti-CD4 FITC, (BD, USA). The IgG 1 FITC PE PerCP – conjugate mouse mAbs from Becton Dickinson was used as a negative control. Phorbol 12-mysistate 13-acetate (PMA), ionomycin and brefeldin A (BFA) were purchased from Sigma.

Cell preparation and in vitro stimulation of T cells

All the peripheral blood samples were collected into heparinized tubes and immediately processed. Mononuclear cells were isolated by the density gradient centrifugation on Gradisol L (Aqua Medica, Poland). Interphase cells were removed and washed twice in phosphate-buffered saline (PBS). Cells from every sample were cultured in a complete culture medium (RPMI 1640 supplemented with fetal calf serum 100 IU mL^{-1} penicillin, 100 mg mL^{-1} streptomycin) for 4 h. The cells were stimulated with a mixture containing phorbol myristate acetate (PMA, 25 ng/ml), ionomycin (1 μg/ml). Cytokine secretion was blocked with brefeldin (10 μg/ml). The incubators were set at 37 °C in a 5% CO_2 environment. Moreover, this procedure was also performed on non-activated lymphocytes using only brefeldin A in order to assess the level of residual cytokine synthesis from *in vitro* activation.

Surface and intracellular staining

For surface staining, cultured PBMCs were washed twice in PBS and then incubated with appropriate MoAbs specific for CD3 and CD4 for 30 min at 4 °C. After surface staining, the cells were washed, subsequently fixed and permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences). Afterwards, the cells were incubated with an appropriate amount of MoAbs for intracellular cytokine staining (20 min at 4 °C in the dark). The cells were then washed twice with PBS, and analyzed by flow cytometry.

Flow cytometric analysis

The samples were analyzed by flow cytometry using a Becton Dickinson FACSCalibur instrument. Five data parameters were assessed: linear forward and side scatter (FSC, SSC), FL-1(FITC), FL-2(PE) and FL-3 (PerCP). An acquisition gate was established on their forward and side light scatter properties (R1 region), which included mononuclear cells and excluded dead cells and debris (Fig. 1). The R1 gated events were analyzed for CD3 positive cells (R2). The final dot plots, using R1 and R2 regions, represent the percentage of CD3+/CD4+ cells expressing cytoplasmic cytokines.

For each analysis, 10,000 events were acquired and analyzed using CellQuest Pro software. Isotype-matched antibodies were used to verify the staining specificity and as a guide for setting the markers to delineate positive and negative populations.

Flow cytometric analysis of CD38 and ZAP-70 expression in CLL cells

CLL cells were stained for CD38 antigen and ZAP-70 protein expression (as described previously [23]). The cut-off point for CD38 or ZAP-70 positivity in leukemic cells was ≥20%.

Statistical analysis

The Mann–Whitney U test was applied for statistical comparison of the results between CLL patients and HV, as well as between CLL patients in different stages of the disease.

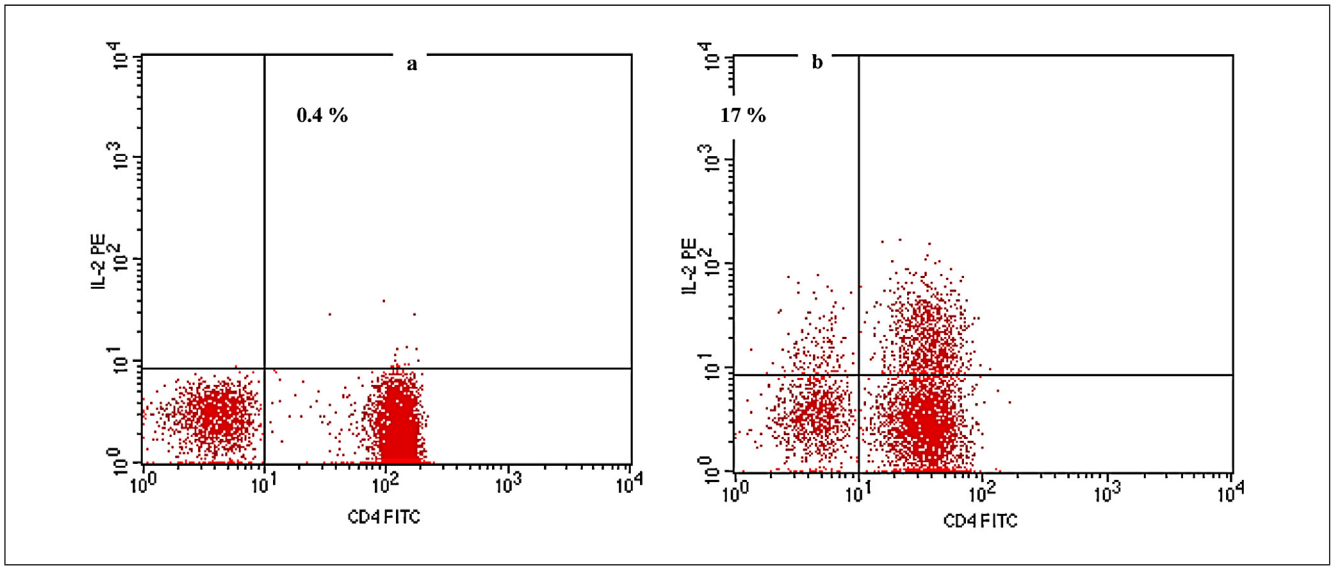

Fig. 1 – The dot plot shows representative date from one CLL patient, illustrating the analysis method for identification of CD4+ T cells expressing appropriately cytokines following three-color staining. (a) The dot plot shows the forward scatter/side scatter (FSC/SSC) distribution and the gate (region R1) used to select lymphocytes for analysis. (b) The R1 gate events were then analyzed for CD3 positive cells (CD3+) were gated (region R2). The dot plot shows the SSC *vs.* CD3PerCP distribution. (c) The final dot plot CD4FITC *vs.* IFN PE, was established by combined gating of events using R1and R2. The number in the upper right quadrant on the dot plot represents the percentage of CD3+/CD4+ cells expressing cytoplasmic IFN

Ryc. 1 – Przykładowy obraz z cytometru przepływowego, przedstawiający metodę analizy komórek T CD4+ wykazujących ekspresję cytokin. (a) Obraz przedstawiający rozkład komórek w zależność od wielkości i ziarnistości (FSC/SSC), region R1 zawiera limfocyty. (b) Z regionu R1 wyodrębniono komórki CD3-pozytywne i oznaczono je jako R2. Obraz przedstawia zależność SSC od CD3PerCP. (c) Końcowy obraz, przedstawiający procent komórek CD3+/CD4+ wykazujących ekspresję IFN-γ, zawiera komórki z rejonów R1 i R2. Komórki w górnym prawym rogu, reprezentują odsetek limfocytów CD3+/CD4+ z cytoplazmatyczną ekspresją IFN-γ

The data were analyzed using STATISTICA 6.0 software for Windows. Differences were considered statistically significant with a p-value ≤ 0.05.

Results

We measured the intracellular cytokines expression of IL-2, IL-4, IFN-γ, TNF, IL-6, IL-10 and IL-12 in T cells of patients with CLL and healthy control group. Representative FACS profiles showing intracellular staining of spontaneous and *in vitro* activated cells are showed in Fig. 2. In CLL patients as well as in healthy control, the percentage of CD4+/CD3+ cells with intracellular cytokine expression in non-activation assays was frequently lower than 1%, comparable with the level of autofluorescence.

The mean percentages of CD3+CD4+ cell positive for IL-2, IL-4, IFN-γ, TNF, IL-6 and IL-10 were significantly higher in

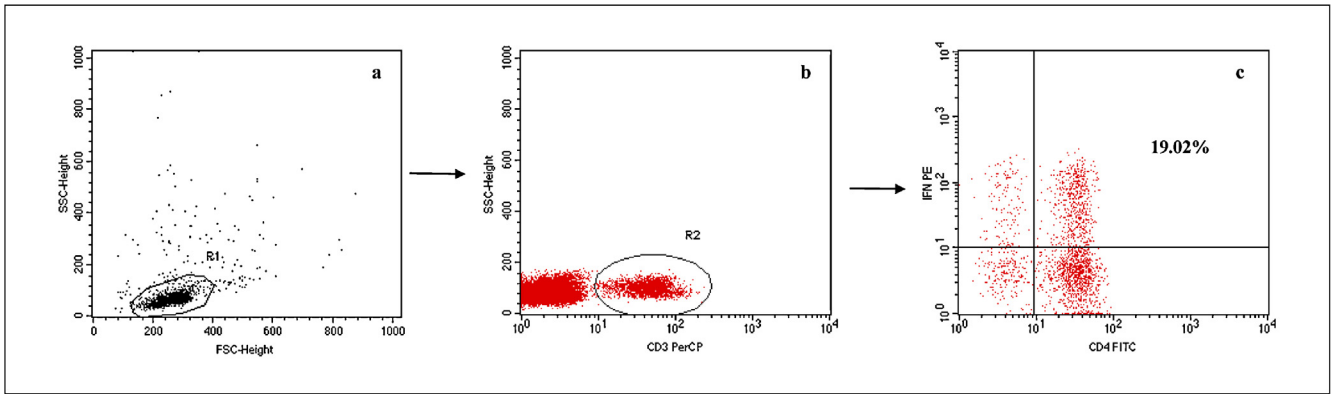

Fig. 2 – Intracellular IL-2 expression in CD3+/CD4+ cells from one representative CLL patent. Expression of intracellular IL-2 without stimulation (a), and with PMA and ionomycin stimulation (b)

Ryc. 2 – Przykładowy obraz przedstawiający odsetek komórek CD3+/CD4+ z wewnątrzkomórkową ekspresją IL-2. Ekspresja IL-2 przed (a) i po stymulacji PMA i jonomycyną (b)

Table I – Median percentage of $CD3^+CD4^+$ cells with intracellular TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 and IL-10 expression in peripheral blood from CLL patients and healthy control
Tabela I – Średni odsetek limfocytów $CD3^+CD4^+$ z wewnątrzkomórkową ekspresją TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 i IL-10 w krwi obwodowej pacjentów z PBL i zdrowych dawców

Variable	CLL	Healthy control	*p*-Value
$CD3^+CD4^+TNF^+$	15.17	5.46	0.003
$CD3^+CD4^+IFN\text{-}\gamma^+$	6.70	4.24	0.002
$CD3^+CD4^+IL\text{-}4^+$	1.46	0.68	0.001
$CD3^+CD4^+IL\text{-}10^+$	1.06	0.88	0.009
$CD3^+CD4^+IL\text{-}6^+$	0.86	0.47	0.001
$CD3^+CD4^+IL\text{-}2^+$	13.85	8.41	0.036
$CD3^+CD4^+IL\text{-}12^+$	1.30	2.24	0.167

The *p*-value was calculated using the *U* Mann–Whitney test (*p*-value of <0.05 was considered statistically significant).
Wartość p obliczono, stosując test *U* Manna–Whitneya ($p < 0.05$ uznano za istotne statystycznie).

CLL patients than in healthy controls ($p < 0.01$) (Table I). However, no significant differences were observed between these groups in the percentage of CD3+CD4+ cells expressing IL-12.

The low-risk group (Rai stage 0) and the intermediate-risk group (Rai stages I and II) CLL patients expressed statistically significant higher levels of IL-4, IFN, TNF, IL-6 and IL-10 than healthy individuals ($p < 0.05$). Moreover, CLL patients in the Rai stages 0-II showed a higher mean expression of IL-10 and IL-6 when compared to the group of patients in more advanced stages of the disease (III–IV) (Table II). However, these differences were not statistically significant.

In our study, the percentages of CD4+/CD3+/TNF+, CD4+/CD3+/IFN-γ+, and CD4+/CD3+/IL-2+ cells were significantly higher in ZAP-70-positive patients compared with ZAP-70-negative patients (Table III). Likewise, significantly higher percentages of CD4+/CD3+/TNF+, CD4+/CD3+/IFN-γ+ cells were observed in CD38-positive than in CD38-negative patients (Table III).

Cytogenetic analysis at the time of testing was available for 46 out of the 55 study patients. There was a significant difference in median percentage of CD3+/CD4+ cells expressing TNF, IL-4, IFN-γ, IL-2 or IL-6 between patients carrying the 11q22.3 deletion and/or the 17p13.1 deletion and patients without these genetic aberrations (Table IV). There were no significant differences in $CD3^+/CD4^+/IL\text{-}10^+$ and $CD3^+/CD4^+/IL\text{-}12^+$ cell percentages between patients with del (11q22.3) or/and del(17p13.1) and patients without these unfavorable genetic aberrations (Table IV). Additionally, the percentage of T lymphocytes expressing all analyzed cytokines was higher in the patient with del(17p13.1) than in the patients with del(11q22.3) (Table V). However, these differences were not statistically significant.

Table II – Median percentage of $CD3^+CD4^+$ cells with intracellular TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 and IL-10 expression in peripheral blood from CLL patients in different stages of disease
Tabela II – Średni odsetek limfocytów $CD3^+CD4^+$ z wewnątrzkomórkową ekspresją TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 i IL-10 w krwi obwodowej pacjentów z PBL w różnych stadiach zaawansowania choroby

Variable	0	I–II	III–IV
$CD3^+CD4^+TNF^+$	15.78	11.55	21.10
$CD3^+CD4^+IFN\text{-}\gamma^+$	6.70	6.54	9.28
$CD3^+CD4^+IL\text{-}4^+$	1.48	1.43	3.16
$CD3^+CD4^+IL\text{-}10^+$	1.07	1.04	1.00
$CD3^+CD4^+IL\text{-}6^+$	0.86	0.87	0.45
$CD3^+CD4^+IL\text{-}2^+$	12.63	13.88	23.99
$CD3^+CD4^+IL\text{-}12^+$	1.47	1.02	NE

Table III – Median percentage of $CD3^+CD4^+$ cells with intracellular TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 and IL-10 expression in peripheral blood from CLL patients with different ZAP-70 and CD38 antigen expressions
Tabela III – Średni odsetek komórek $CD3^+CD4^+$ z wewnątrzkomórkową ekspresją TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 i IL-10 w krwi obwodowej pacjentów z PBL wykazujących różną ekspresją antygenów ZAP-70 i CD38

Variable	ZAP-70- *n* = 32	ZAP-70+ *n* = 23	*p*-Value
$CD3^+CD4^+TNF^+$	43.14	51.55	0.01
$CD3^+CD4^+IFN\text{-}\gamma^+$	16.33	22.28	0.02
$CD3^+CD4^+IL\text{-}4^+$	3.28	3.11	0.14
$CD3^+CD4^+IL\text{-}10^+$	1.55	1.57	0.49
$CD3^+CD4^+IL\text{-}6^+$	1.80	1.01	0.61
$CD3^+CD4^+IL\text{-}2^+$	17.01	26.08	0.01
$CD3^+CD4^+IL\text{-}12^+$	1.4	1.76	0.167
	CD38- *n* = 30	CD38+ *n* = 25	*p*-Value
$CD3^+CD4^+TNF^+$	40.05	49.68	0.014
$CD3^+CD4^+IFN\text{-}\gamma^+$	16.00	19.40	0.035
$CD3^+CD4^+IL\text{-}4^+$	3.45	2.78	0.16
$CD3^+CD4^+IL\text{-}10^+$	1.55	1.58	0.27
$CD3^+CD4^+IL\text{-}6^+$	17.39	20.59	0.052
$CD3^+CD4^+IL\text{-}2^+$	1.08	1.02	0.43
$CD3^+CD4^+IL\text{-}12^+$	1.39	1.94	0.067

The *p*-value was calculated using the *U* Mann–Whitney test (*p*-value of <0.05 was considered statistically significant).
Wartość *p* obliczono, stosując test *U* Manna–Whitneya ($p < 0.05$ uznano za istotne statystycznie).

Discussion

For many years, the main function in anticancer response was attributed to cytotoxic T lymphocytes (CD8+, Tc). Nowadays it is known that T helper lymphocytes (CD4+, Th) play an essential role in the regulation of cell mediated anti-tumor responses while their respective populations may inhibit or promote tumor growth through the cytokine secretion profile. In this study, we have undertaken the assessment of the functional T helper cell function in patients with chronic lymphocytic leukemia, by evaluating their cytokine secretion.

We have measured the intracellular expression of selected cytokines in CLL cells, demonstrating that the levels of IL-2, IL-4, IFN-γ, TNF, IL-6 and IL-10 after *in vitro*

Table IV – Median percentage of $CD3^+CD4^+$ cells with intracellular TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 and IL-10 expression in peripheral blood from CLL patients subdivided according to cytogenetic analysis
Tabela IV – Średni odsetek limfocytów $CD3^+CD4^+$ z wewnątrzkomórkową ekspresją TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 i IL-10 w krwi obwodowej pacjentów z PBL, podzielonych według analizy cytogenetycznej

Variable	Patients with del(11q22.3) or/and del(17p13.1) n = 11	Patients without these genetic aberrations n = 25	*p*-Value
$CD3^+CD4^+TNF^+$	56.38	44.16	0.01
$CD3^+CD4^+IFN\text{-}\gamma^+$	31.12	11.10	0.0004
$CD3^+CD4^+IL\text{-}4^+$	5.71	2.52	0.001
$CD3^+CD4^+IL\text{-}10^+$	1.50	1.52	0.60
$CD3^+CD4^+IL\text{-}6^+$	2.61	0.92	0.002
$CD3^+CD4^+IL\text{-}2^+$	29.61	17.17	0.03
$CD3^+CD4^+IL\text{-}12^+$	0.25	1.79	0.43

The *p*-value was calculated using the *U* Mann–Whitney test (*p*-value of <0.05 was considered statistically significant).
Wartość p obliczono, stosując test U Manna-Whitneya ($p < 0.05$ uznano za istotne statystycznie).

Table V – Median percentage of $CD3^+CD4^+$ cells with intracellular TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 and IL-10 expression in peripheral blood from CLL patients with del (11q22.3) and del(17p13.1)
Tabela V – Średni odsetek limfocytów $CD3^+CD4^+$ z wewnątrzkomórkową ekspresją TNF, IFN-γ, IL-4, IL-6, IL-2, IL-12 i IL-10 w krwi obwodowej pacjentów z PBL z del (11q22.3) i del(17p13.1)

Variable	Patients with del(11q22.3) *n* = 6	Patients with del(17p13.1) *n* = 5
$CD3^+CD4^+TNF^+$	54.43	56.60
$CD3^+CD4^+IFN\text{-}\gamma^+$	24.34	37.91
$CD3^+CD4^+IL\text{-}4^+$	5.79	5.46
$CD3^+CD4^+IL\text{-}10^+$	1.39	1.68
$CD3^+CD4^+IL\text{-}6^+$	2.11	4.11
$CD3^+CD4^+IL\text{-}2^+$	28.00	29.92
$CD3^+CD4^+IL\text{-}12^+$	0.25	1.78

stimulation were significantly higher in CLL than in normal cells. Our results suggest that the production of studied cytokines by T cells may play a role in the maintenance of leukemic cells survival and proliferation.

We have also found significantly higher intracellular IL-2 expression in CD3+/CD4+ T cells from CLL patients than in healthy individuals, which confirms earlier results of Roossmanns et al. who showed a significantly higher level of spontaneous production of IL-2 in patients with leukemia as compared to the control group [24]. In contrast, other investigators such as Gallego et al. detected a significantly lower percentage of CD4+ cells expressing IL-2 after activation with PMA and ionomycin in CLL patients as compared to the control group [25]. The authors confirm the results by the ELISA test. Furthermore, in contrast to the results of the presented study, they found no differences in the level of intracellular cytokines such as TNF and IFN between the study and the control group. These discrepancies might result from the different methods used for the analysis of cytokines expression as well as the heterogeneity of the disease.

In our study we have found no correlation between the stage of the disease and the level of cytokines secreted by T cells. Different results were presented by Hulkkonen et al. [26], who showed variations in the secretion of TNF and IL-6, depending on the stage of the disease. The level of secreted cytokines after stimulation with PMA was significantly lower in the more advanced stages of the disease, according to Binet's classification.

Currently there is a general consensus that the expression of ZAP-70 protein and CD38 antigen by leukemic cells belongs to the important prognostic markers in CLL. In our study, the higher percentage of T cells expressing TNF, IFN-γ and IL-2 in the ZAP-70+ and CD38+ in comparison to ZAP-70– and CD38- group indicates that the production of cytokines is significantly connected with the clinical course of disease; the more aggressive ZAP-70+ or CD38+ CLL cases are characterized by a higher capability for the production of cytokines responsible for disease pathogenesis. What is more, the intracellular cytokine expressions were significantly higher in patients with high-risk cytogenetic aberrations such as deletion of 17p or 11q. Apart from providing insights into the pathogenesis, genomic aberrations identify subgroups of patients with distinct clinical pictures: lymphadenopathy (11q deletion) or resistance to therapy (17p deletion). Deletions at 11q and particularly 17p are associated with rapid disease progression or inferior survival [27]. Genomic aberrations (i.e., 11q and 17p deletion) help to define biological and clinical subgroups. In our study the intracellular cytokine expressions were higher in patients with del(11q22.3) or/and del(17p13.1) than in patients without these unfavorable genetic aberrations. Deletions of 17p and 11q have been associated with unfavorable prognosis. Nevertheless, the deletion of 17p was described as the strongest independent predictor for aggressive behavior, resistance to chemotherapy and early death [27]. In our study we observed a higher intracellular cytokine expression in patients with 17p deletion than in patients with 11q deletion. However, this difference was not statistically significant. In our study the cytogenetic analysis at the time of testing was available for 36 out of the 55 study patients. It should be noted that the group of patients with 11q22.3 deletion or 17p13.1 deletion had only 6 and 5 patients, respectively. We can suppose that future analysis is required in more numerous groups of patients.

CLL patients show much lower percentages of circulating T-lymphocytes than it is observed in normal peripheral blood samples [28]. Although the relative percentage of T cells in the peripheral blood is decreased, the absolute number is frequently increased. Our data suggest that it is very likely that malignant cells, whose number is extremely high in the blood of patients with leukemia, regulate the production of cytokines by T lymphocytes.

In summary, the possible large impairment of T lymphocyte function in the later stages, as compared to the early stages of the disease, may affect cytokine secretion

profile which is involved in the clinical course of chronic lymphocytic leukemia.

Authors' contributions/Wkład autorów

According to order.

Conflict of interest/Konflikt interesu

None declared.

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Ethics/Etyka

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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