FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 60, No. 3, 2022
pp. 203–214

ORIGINAL PAPER

VIA MEDICA

Predictive role of NKCD56^{bright} cells in monitoring the progression of chronic lymphocytic leukemia during treatment

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Abstract

Introduction. Standard treatment for chronic lymphocytic leukemia (CLL) has experienced a dramatic change over the last few years. Until recently, CLL was treated using chemoimmunotherapy (CIT) with anti-CD20 monoclonal antibodies. Even though novel agents such as BTKi (Bruton Tyrosine Kinase inhibitor) and BCL2 inhibitors are the standard of care in most therapeutic settings, CIT still has its place in CLL treatment. Interestingly, little is known about its effects on the immune system of patients with CLL. Contrary to the reduction of the number of CLL cells during CIT administration, little attention has been paid to the cellular microenvironment, the evaluation of which during treatment may provide additional information about the course of the disease and prognosis and therefore was set as the aim of this study.

Material and methods. Flow cytometry was used to evaluate the phenotypes of different populations and subpopulations of lymphocytes in the peripheral blood (PB) of 20 patients with CLL before, during, and after CIT.

Results. During the CIT with R-FC (Rituximab, Fludarabine, and Cyclophosphamide) and R-B (Rituximab, Bendamustine) regimens, the sizes of the assessed populations and subpopulations of lymphocytes were dramatically reduced. Twenty-eight days after the first course of treatment, the exponential decrease of CLL cells was observed, and their number had declined to the median level of 10% of the numbers observed before the treatment. T cells, NK cells, NK-CD56^{dim}, NKT-like, and NKT-like CD56^{dim} also decreased exponentially. After the second treatment course, a decline in the numbers of T, NK, NKCD56^{dim}, NKT-like, and NKT-like CD56^{dim} cells was observed, which were stable until the sixth treatment course. However, the number of NKT-like CD56^{bright} cells decreased to the third course of treatment and then increased. The number of CLL cells in peripheral blood correlated with the number of NKCD56^{bright} cells, influencing the treatment response.

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Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0023
ISSN 0239-8508, e-ISSN 1897-5631

www.journals.viamedica.pl/folia_histochemica_cytobiologica

Conclusions. Upon CIT, the reduction of CLL cells is accompanied by shifts in immune cell populations, T, NK, and NKT-like cells. Monitoring changes of those cell populations in the peripheral blood may serve as an important predictive and prognostic indicator. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 3, 203–214)*

Keywords: chronic lymphocytic leukemia; chemoimmunotherapy; NKCD56^{bright} cells; NKT-like CD56^{dim} cells; flow cytometry; prognosis

Introduction

Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by the accumulation of pathological B lymphocytes in the peripheral blood (PB), bone marrow, and lymphoid organs. CLL is characterized by the occurrence of many clones of neoplastic cells that constantly evolve during the disease [1]. While certain factors such as genomic changes in CLL cells were shown to play a vital role in its pathogenesis, the influence of external factors such as direct CLL cells' interactions in the microenvironment has not been fully elaborated, despite their important role, as they allow CLL cells to avoid immune surveillance [2, 3]. Key features of CLL are defects in the immune system and the ability of CLL cells to evade immune defenses and induce immunosuppression, resulting in increased susceptibility to infections and disease progression. Many factors in the microenvironment promote proliferation, survival, and resistance of CLL cells. Moreover, the importance of the microenvironment in CLL is demonstrated by the clinical effects of new drugs, which disrupt the interaction between neoplastic cells and the tissue microenvironment [4–6]. The complex architecture of the microenvironment of circulating CLL cells in the PB, both before and during the treatment of CLL patients, has not yet been fully described. Previous studies have focused on individual populations of PB cells after the end of treatment and have included the assessment of activation disorders and depletion of T lymphocytes [7–9] and emphasized the impaired function of natural killer (NK) cells in CLL patients [10, 11]. The role of T cells in defense against CLL and CLL progression and chemoimmunotherapy (CIT) has been extensively studied [12]. Less is known about the role of NK cells and natural killer T (NKT-like) cells in this leukemia, and data on NK/ /NKT-like cell alterations in CLL and cancers are contrasting [11, 13]. NK cells, which constitute approximately 4–15% of PB lymphocytes, can be divided into two subsets, dim and bright, according to CD56 surface density expression [14]. Dim (CD3–/CD56+^{dim}) NK cells are cytolytic and comprise more than 90% of CD3–/CD56+ NK cells, whereas bright (CD3–/ /CD56+^{bright}) NK cells play immunoregulatory function generally *via* cytokine production. Bright (CD3–/CD56$^{+bright}$) NK cells, which lack perforin granules, display homing receptors required for migration to secondary lymph nodes [15]. NKT-like (CD3+/ /CD56+) cells constitute a unique subset of T cells that lie at the interface between innate and adaptive immunity with the ability to induce antitumor effects. They represent a small but distinctive population of T lymphocytes, constituting approximately 0.02–0.2% of human circulating T cells. NKT-like cells possess cytotoxic capabilities but are primarily considered to have an essential regulatory function *via* the secretion of large amounts of pro- or anti-inflammatory cytokines upon activation, thereby resulting in amplification or dampening of the immune responses [13].

NKT-like cells do not represent a homogeneous population but do not differentiate into CD56 bright and dim cells. Comprehensive research into circulating immune cells and their relationship to CLL cells before and during treatment could contribute to a new look at the complexity of immunology of CLL. We have characterized the populations and subpopulations of immune cells that shape the CLL microenvironment in the PB, along with CLL cells in 20 patients. In this work, we analyzed the phenotypes and levels of lymphocytes in patients before, during, and after CIT using the Rituximab (R) with Bendamustine (B) and R, Fludarabine (F), and Cyclophosphamide (C) regimens, trying to understand the long-term changes associated with CIT that may affect them, the interaction between different subtypes of lymphocyte subpopulations, and the potential implications for the immune system, the course of the disease and response to treatment.

Materials and methods

Patients and samples. The study group consisted of 21 patients (13 men and 8 women) diagnosed with CLL. Twenty patients were included in the analysis, as one died during treatment. The mean age of the patients was 66 years (range: 35 to 82 years).

Five patients in the study group were relapsed patients formerly treated with at least one line of CIT using R. Three patients had previously received one treatment regimen, one had received two treatment regimens, and one had received four treatment regimens (Table 1). The median interval between treatment courses in patients receiving R was 32 months. Ten healthy donors/volunteers (median age 61 years, range: 42 to

91 years) were included as controls. The disease was diagnosed in the Flow Cytometry Laboratory at the Maria Sklodowska-Curie National Research Institute of Oncology in Warsaw, Poland, based on flow cytometry (FCM) criteria of PB involvement, as previously described [1, 16] and/or histopathological examination of lymph nodes according to the 2016 revision of the World Health Organization (WHO) lymphoma classification and new European Society for Medical Oncology (ESMO) diagnostic recommendations [1, 17]. Detailed clinical characteristics of the patients are presented in Table 1. All patients met the criteria for treatment inclusion according to the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) [18]. Nine patients, relatively young (under 60–65 years of age) or those without other significant diseases, in good general condition, were given F (25 mg/m^2 during the day) and C (250 mg/m^2 per day) for the first 3 days in the intravenous or oral form. The remaining 11 elderly comorbid patients in a worse general condition were given B intravenously (90 mg/m^2 during the day) for 2 days each cycle. 375 mg/m^2 of R was administered intravenously to both groups on day 0 of cycle one, followed by 500 mg/m^2 for the subsequent 5 cycles on day one.

The median treatment courses were 6 (range, 4–6). If the neutrophil count and PLT were too low, then subsequent treatment courses were delayed at the treating physician's discretion. Granulocyte colony-stimulating factor (G-CSF) was administered during treatment as recommended by the treating physician. Dose reductions of F and C, but not R, occurred if the patient experienced Grade 3 or 4 prolonged hematological toxicity, infection, or organ failure. In three patients, due to neutropenia, treatment was discontinued on the 4th treatment cycle (patient no. 16) and on the 5th cycle (patient no. 17), and patient no. 5 discontinued treatment after the 5th cycle, for personal reasons. Response to treatment was assessed according to the National Cancer Institute-sponsored Working Group (NCI-WG) criteria for CLL [19]. All CLL patients have given written informed consent for using PB for research purposes, per the Declaration of Helsinki. The study was approved by the local ethics committee of the National Institute of Oncology No. 8/2020.

Table 1. Characteristics of clinical and laboratory parameters in twenty patients with CLL

Patient number	Age [years]/ sex	WBC (G/L)	Treatment	Treatment response	Previous treatment
1	35/m	19.05	6 R-FC	CR	
2	72/m	59.4	6 R-B	PR	
3	62/m	12.37	6 R-B	CR	
4	69/m	7.31	6 R-B	CR	R-FC
5	49/m	316.74	5 R-FC	PR	
6	73/m	119.21	6 R-B	CR	R-CVP
7	49/f	29.27	6 R-FC	PR	
8	58/f	163.64	6 R-B	PD	B, R chlorambucil, R-CVP, C, CHOP, R-CHOP, R-B
9	59/f	13.21	6 R-FC	CR	
10	70/m	16.47	6 R-B	CR	
11	66/m	38.87	6 R-FC	CR	
12	67/f	62.14	6 R-B	CR	
13	70/f	29.01	6 R-B	CR	
14	72/f	96.06	6 R-B	NR	R-B, R
15	67/m	34.01	6 R-B	CR	
16	48/m	137.77	4 R-B	PR	R-FC
17	50/m	11.55	5 R-FC	CR	
18	72/f	16.45	6 R-FC	CR	
19	56/m	84.42	6 R-FC	CR	
20	67/f	169.05	6 R-FC	CR	

Abbreviations: CR — complete remission; f — female; m — male; NR — no response; PD — progressive disease; PR — partial remission; R-B — Rituximab + Bendamustine; R-CHOP — Rituximab + Cyclophosphamide + Doxorubicin hydrochloride + Vincristine + Prednisolone; R-CVP — Rituximab + Cyclophosphamide + Vincristine + Prednisolone; R-FC — Rituximab + Fludarabine + Cyclophosphamide; WBC — white blood cells.

Flow cytometry (FC). Absolute numbers and percentages of lymphocyte populations were determined in the whole peripheral blood. The PB was aspired into tubes for EDTA twice, both before and after treatment initiation. The PB was then incubated with the mixture of conjugated antibodies [CD19 FITC (clone SJ25C1), CD3 PerCP (SK7), CD56 Pe-Cy7 (NCAM16.2), CD5 APC (L17F12); Becton-Dickinson (BD), San Jose, CA, USA] for 15 min in the dark, at room temperature. Red blood cells were lysed with 2 mL of Facial Action Coding System (FACS) lysing solution (diluted 1:10 with distilled water) and washed with phosphate-buffered saline. The samples prepared in this way were determined using flow cytometry. Analysis was conducted no later than 30–60 min from the start of the procedure. The number and percentage of the CLL and immune cell populations were expressed as a number of cells per unit volume (cells/μL) and calculated by multiplying the percentage of FC-derived cells by the number of lymphocytes from complete blood counts measured by the SYSMEX automated hematology analyzer (SYSMEX CANADA, INC., Mississauga, Canada). The following cell types were immunophenotyped: CLL cells (CD19+/CD5+), normal B cells (CD19+/CD5–), T cells (CD3+/CD56–) or (CD5+/CD56–), NK cells (CD3–/ /CD56^{+dim}) and (CD3–/CD56$^{+bright}$) and NKT-like cells (CD3+/ /CD56^{+dim}) and (CD3+/CD56$^{+bright}$). The analysis was performed using a BD FACSCanto™ II Flow Cytometer. Flow cytometry data were analyzed using BD FACSDiva™ software v. 6.1.3. Positive expression was defined using negative control antibody staining to determine negative/positive cut-off points and these were consistently used in all samples.

Statistical analysis. During the treatment of patients with CLL, the mean number of lymphocyte populations with the corresponding standard deviation (SD) was calculated. Apart from the small fractions of NKCD56$^{+bright}$ and NKT-like CD56$^{+bright}$ lymphocytes, there was an approximately exponential decrease in their concentration compared to the number before treatment. For individual patients, the number of CLL cells was correlated with the number of other cell categories. Then, the mean values of the calculated correlation coefficients in the group of patients distinguished by the treatment effects assessed as mentioned according to the NCI-WG criteria for CLL were compared [19]. The slope coefficients (linear regression coefficients, LRC) between the number of cells in consecutive treatment courses were also calculated for each patient. SAS software v. 9.4 (SAS Institute Inc., Cary, NC, USA) was used to perform statistical calculations.

Standard assessment of the course of treatment. Standard evaluation of the course of treatment was performed as follows: the number of specified cell populations was transformed as a function of x log (x + 1), *e.g.* the number of CLL cells (log CLL). Adding 1 was because all numbers are less than 1, and log (x) would produce negative numbers. Moreover, after the third, fourth, and fifth courses of treatment, most patients had a low number of B cells, including CLL cells. The straight line equation (y = ax + b) was calculated from the log values of the cells after successive treatment courses, and the coefficient "a" at x, the slope coefficient, was taken as a measure of the change in a specific subpopulation of lymphocytes in the PB during treatment.

Results

Analysis of the number of immune cells before and during treatment according to the R-FC (Rituximab, Fludarabine, and Cyclophosphamide) and R-B (Rituximab and Bendamustine) protocols

To assess the effect of CIT on the immune system, the phenotype, and the number of different populations and cell subpopulations in twenty patients with CLL. The data were obtained and analyzed before and after every course of CIT (Fig. 1). Besides the small fractions of NKCD56^{bright} and NKT-like CD56^{bright} cells, the remaining cells showed an approximately exponential decline in cell numbers compared to pre-treatment.

After the first treatment course, there were significant decreases in the number of all 9 assayed cell populations and lymphocyte subpopulations compared to pre-treatment levels, with a relatively high degree of variability in the group of studied patients. After the first course of treatment with the R-B and R-FC regimens, log CLL decreased quickly. CLL cells showed an extreme decline to median levels of 10% compared to count before therapy. The numbers of T cells, NK cells, NKCD56^{dim}, NKT-like, and NKT-like CD56^{dim} also decreased exponentially as a percentage of pre-treatment G/L (relative number), though to a lesser extent. After the second course of treatment, a decrease in the relative number of T lymphocytes, NK cells, NKCD56^{dim}, NKT-like, and NKT-like CD56^{dim} was observed, which remained constant until the sixth course (Fig. 2). The number of T lymphocytes decreased during treatment with R-B and R-FC regimens, but with no apparent differences between the two treatment regimens (median R-B and R-FC were 1 and 0.8 respectively). In the case of NKT-like CD56^{bright} cells, a decrease in their number to the third course was observed, while in the following three treatment courses, their number slightly increased and then decreased. The relative number of NKCD56^{bright} cells decreased to the second course and then increased with each subsequent treatment course. These observations indicate the directions of changes in the lymphocyte peripheral blood populations during treatment.

Response to treatment of CLL patients in the context of changes in CLL and NKCD56^{bright} cell counts

Response to treatment was assessed according to the NCI-WG criteria for CLL [19]. The numbers of

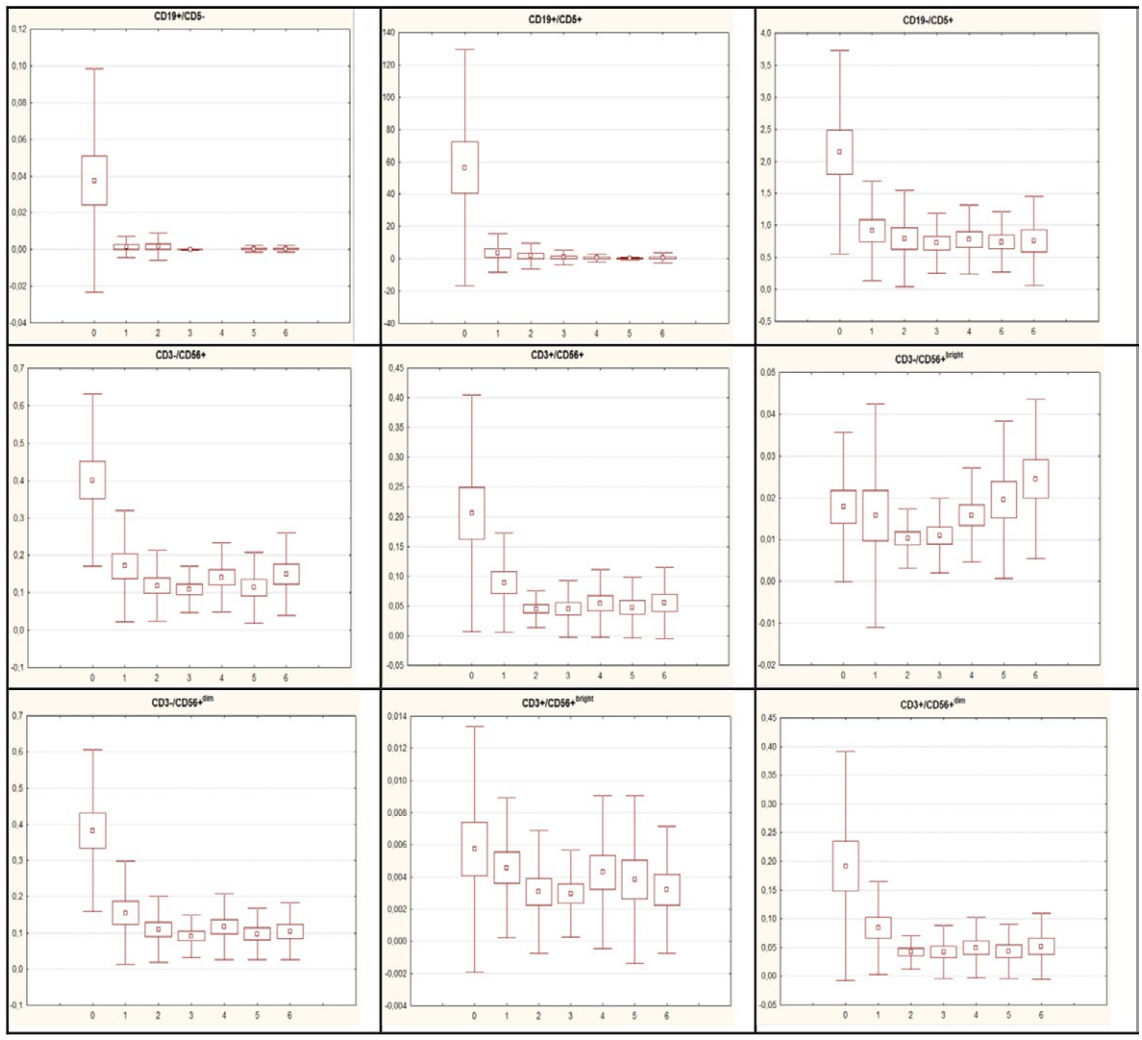

Figure 1. Lymphocyte subtypes and absolute numbers of the lymphocyte subgroups shown were determined by flow cytometry on peripheral blood samples from twenty patients with chronic lymphocytic leukemia (CLL) before, during, and after chemoimmunotherapy (CIT). Results are presented as means and standard deviations with minimal and maximal values and 25–75% quantiles: range of variation indicates that approximately 70% of data fall within this range.

CLL, T, B, NK, NKT-like, and NKCD56^{dim} cells in the patients' PB were compared with the response to treatment. There were no differences in the absolute number of T lymphocytes, NK cells, NKCD56^{dim}, NKCD56^{bright}, and NKT-like CD56^{bright} cells before therapy between patients and healthy individuals. However, a higher total number of NKT-like cells and NKT-like CD56^{dim} cells was demonstrated in CLL patients. The number of cell populations and subpopulations during treatment were then analyzed for each CLL patient up to and including the fourth course. Then it was checked whether the number of individual cell populations is related to the NCI-WG response criteria to treatment [19]. It was observed that the faster decline in CLL cell counts up to the fourth treatment course was more common in patients whose response to treatment was assessed as partial response (PR) or progressive disease (PD). NKCD56^{bright} cell counts behaved similarly, with the fastest decline in patients with PR or PD.

To analyze in more depth the relationship between the microenvironment of immune cells and the number of CLL cells during CIT, possible correlations between the number of NKCD56^{brigh} cells and the number of CLL cells depending on the response to treatment were examined. During the treatment of patients with

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0023
ISSN 0239-8508, e-ISSN 1897-5631

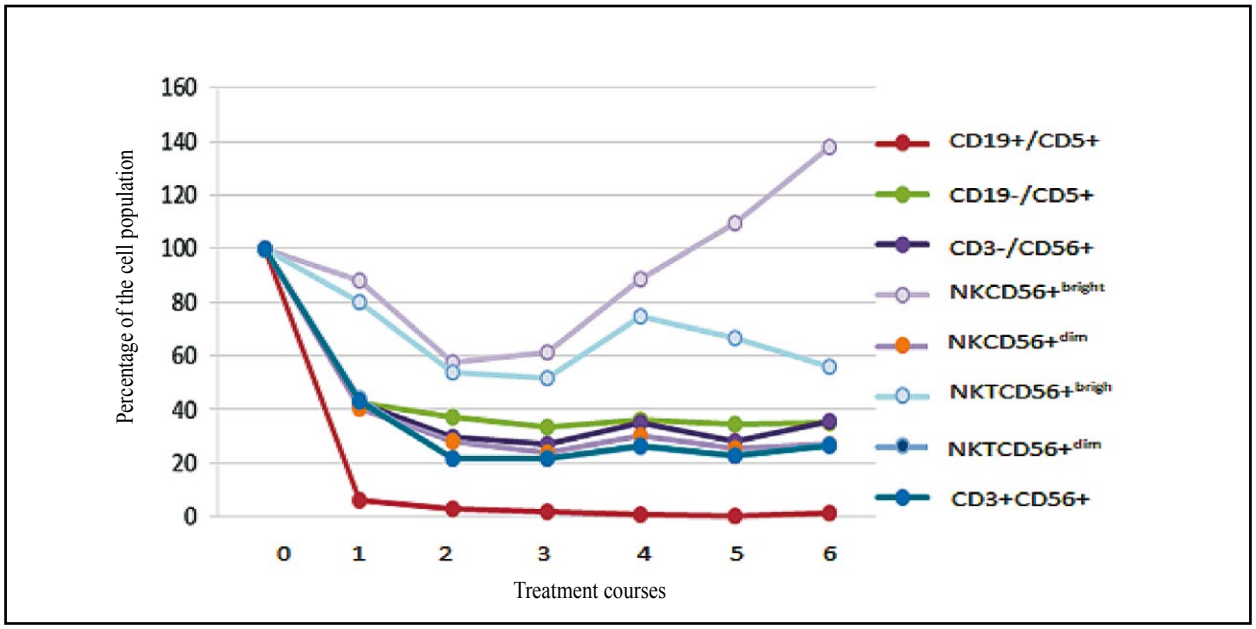

Figure 2. The mean number of cell populations and subpopulations in subsequent treatment courses of CLL patients as a percentage of pre-treatment G/L values (compare with Fig. 1).

CLL, the mean number of lymphocyte populations was assessed together with possible correlations between the number of CLL cells and the number of remaining lymphocyte populations from the first treatment course to the end of treatment. A correlation was demonstrated between the number of CLL cells and the number of NKCD56^{bright} cells depending on the response to treatment expressed as complete remission CR/PR/PD. Significant differences of CR < PR < PD were obtained for the correlation of the number of CLL cells with the number of NKCD56^{bright} cells when all treatment courses from the first course to the end of treatment were taken into account ($p = 0.0046$; Table 2). Correlation analysis presented in Table 2 and data are shown in Fig. 3 demonstrate that changes in NKCD56^{bright} cell counts indicate patients who are more likely to process into complete or partial remission in the investigated group of patients.

Assessment of patients' treatment

Treatment of patients was carried out in six courses except for patients no. 5, 16, and 17 (Table 1). After each treatment course, the number of individual populations and subpopulations of cells was analyzed and compared with the number before the start of the treatment. The slope factor was calculated for each patient and called the Linear Regression Coefficient (LRC) between the treatment course number from 0 (baseline data) up to course number 4, inclusive, and the log transposed numbers of the distinguished cell populations.

LRC (the rate of decline in the number of the specified categories of lymphocytes) were called treatment evaluation indexes. The best treatment results were achieved when the LRC for the transposed CLLs cells were as small as possible (mostly negative) and for the remaining categories of lymphocytes as small as possible the least negative or positive. LRCs for subsequent patients are listed in Table 3.

Analysis of the relationship between the relative number of NKCD56^{bright} cells and response to treatment in patients with CLL

NKCD56^{bright} cell count was defined and presented as absolute levels relative to pretreatment for each patient (Fig. 4). A correlation was demonstrated between the number of CLL cells and the number of NKCD56^{bright} cells depending on the response to treatment expressed as complete remission CR/PR/PD.

Discussion

Standard treatment of CLL has experienced a dramatic change over the last few years. Until recently, CLL was treated using CIT with anti-CD20 monoclonal antibodies. Patients received more or less intensive CIT. The CIT regimens are selected on the basis of patient fitness and are suitable for those without the high–risk markers of *17p* deletion or *TP53* mutations, who typically have a poor prognosis and are largely resistant to CIT regimens [20, 21]. Currently, patients with *17p* deletion or *TP53* mutation. are mostly treated

Table 2. Correlations between the number of CLL cells in the peripheral blood and the number of T cells and lymphocyte subpopulations from the first treatment course to the end of treatment

Treatment response		CR	PR	PD	F	P
T cells	**mean**	0.147	0.225	0.411	0.19	0.8319
	SD	0.412	0.600	.		
NK cells	**mean**	0.198	–0.104	0.372	0.49	0.6192
	SD	0.549	0.722	.		
NKCD56+^{bright}	**mean**	–0.325	–0.141	0.908	**7.69**	**0.0046**
	SD	0.233	0.518	.		
NKCD56+^{dim}	**mean**	0.274	–0.103	0.125	0.73	0.4989
	SD	0.514	0.699	.		
NKT cells	**mean**	0.429	0.002	–0.044	1.51	0.2509
	SD	0.495	0.427	.		
NKTCD56+^{bright}	**mean**	0.252	–0.049	–0.239	0.76	0.4824
	SD	0.550	0.521	.		
NKTCD56+^{dim}	**mean**	0.416	0.032	0.005	1.03	0.3800
	SD	0.541	0.430	.		

Abbreviations: CR — complete remission; PD — progressive disease; PR — partial remission.

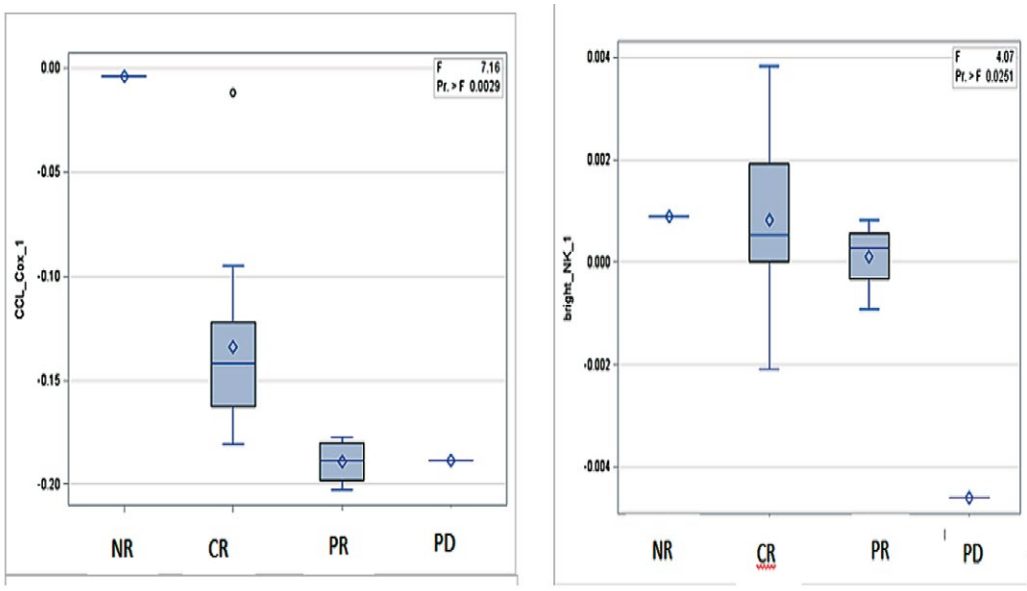

Figure 3. Treatment response of CLL patients and changes in CLL cells' and NKCD56^{bright} cells' counts. Abbreviations: CR — complete remission; NR — no response; PD — progressive disease; PR — partial remission.

with novel agents, including BTK inhibitors, BCL2 inhibitors, and PI3K (phosphoinositide 3-kinases) inhibitors [17, 22, 23]. CLL is an incurable disease, therefore, most patients relapse on or after treatment with these drugs and require multiple lines of therapy. Our study presents and discusses changes in the immune system during treatment with the most common treatment regimens for CLL, namely R-FC and R-B. Although treatment of patients with CLL with these regimens has been carried out for many years, there is still little knowledge about its effects on the immune system, especially on the subpopulation of NKT-like and NK lymphocytes. For many years, therapeutic approaches have focused on suppressing the number of CLL cells to reduce the tumor burden. Apart from the reduction in the number of CLL cells, little attention has been paid to the CLL microenvironment, whose evaluation during treatment may provide additional information about the course of the disease and its prognosis. There is growing evidence that immune

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0023
ISSN 0239-8508, e-ISSN 1897-5631

Table 3. Log transformed G/L + 1 ratios for individual cell categories in twenty patients with CLL determined during treatment

Number	the slope coefficient (a)							
	CLL	**T**	**NK**	**NK CD56^{bright}**	**NK CD56^{dim}**	**NKT**	**NKT CD56^{bright}**	**NKT CD56^{dim}**
1	–0.2130	–0.0173	–0.3106	0.0001	0.0072	0.0006	0.0001	0.1242
2	–0.4334	0.0585	0.2037	0.0016	0.0170	0.0024	0.0016	0.8679
3	–0.1010	–0.0629	–0.5820	0.0000	–0.0625	–0.0038	0.0000	–0.4761
4	–0.0688	–0.0572	–0.2577	0.0000	–0.0215	–0.0006	0.0000	–0.0824
5	–0.5390	–0.1110	–0.2366	0.0001	–0.0329	0.0005	0.0001	0.0905
6	–0.4084	–0.1387	–0.3579	–0.0025	–0.0323	–0.0176	–0.0025	–1.1237
7	–0.3350	–0.0387	–0.1606	0.0011	–0.0214	–0.0312	0.0011	–0.6806
8	–0.4978	–0.0924	–0.1453	–0.0004	–0.0244	–0.0120	–0.0004	–0.9984
9	–0.1235	–0.0261	–0.1405	–0.0007	–0.0170	–0.0265	–0.0007	–0.8937
10	–0.2196	–0.0225	–0.2631	–0.0001	–0.0300	–0.0003	–0.0001	–0.0359
11	–0.3084	–0.0661	–0.4243	–0.0002	–0.0337	–0.0439	–0.0002	–1.5446
12	–0.3653	–0.0219	–0.1349	–0.0002	–0.0088	–0.0160	–0.0002	–1.0167
13	–0.2716	–0.0099	–0.2013	–0.0027	–0.0190	–0.0311	–0.0027	–1.2516
14	–0.2080	–0.0384	–0.1345	–0.0001	–0.0104	–0.0049	–0.0001	–0.3813
15	–0.2829	–0.0445	–0.1504	0.0003	–0.0163	–0.0434	0.0003	–1.1179
16	–0.4421	–0.0654	–0.2630	0.0000	–0.0281	–0.0163	0.0000	–0.9031
17	–0.1326	–0.0613	–0.4917	–0.0002	–0.0424	–0.0255	–0.0002	–0.8857
18	–0.0052	0.0026	–0.0166	0.0003	–0.0004	0.0096	0.0003	0.4591
19	–0.3779	–0.0657	–0.1885	–0.0006	–0.0266	–0.0066	–0.0006	–0.6622
20	–0.4557	–0.1280	–0.2465	0.0004	–0.0179	0.0035	0.0004	0.4349

The linear regression coefficient (LRC) is called the slope of the log line was established as described in Materials and methods. The equation of the line $y = ax + b$, where the coefficient 'a' determines the angle of inclination of the plot of the linear function to the x-axis.

function is associated with treatment response. Thus, knowledge of the effects of CIT on the immune system may be the key to their successful use in patients.

Few publications have reported on the complex relationship between the CLL clone/s and the immune system [24, 25]. The immune microenvironment's interactions with CLL cells play an important role since they allow the CLL cells to escape from immune surveillance and promote their growth and/or survival. These interactions could result in abnormalities in the number and function of both T and NK lymphocytes that encounter CLL cells in the peripheral blood of patients with CLL, which are crucial for disease course and its progression, and distant treatment outcomes [26–29]. Until recently, two commonly used treatment regimens for CLL were R-FC and R-B. R-FC was the standard treatment for previously untreated young CLL patients with high overall response rates of 44% and a median progression-free survival of almost 52 months [30]. The R-B treatment regimen was associated with fewer hematologic complications and infections than R-FC treatment. However, treatment with this regimen also decreases the number of CD4+ cells, delaying their regeneration [31].

Our study did not find any differences in the impact of FC-R and B-R regimens on T-cell subpopulations. According to the literature, the choice of cytotoxic agents is essential because Fludarabine has immunosuppressive effects, while Cyclophosphamide may have immunostimulatory effects. Fludarabine and/or Cyclophosphamide, which are very effective treatment regimens for CLL, may interfere with the therapeutic strategy of T-cell activation. Previous analysis of peripheral blood samples from CLL patients before

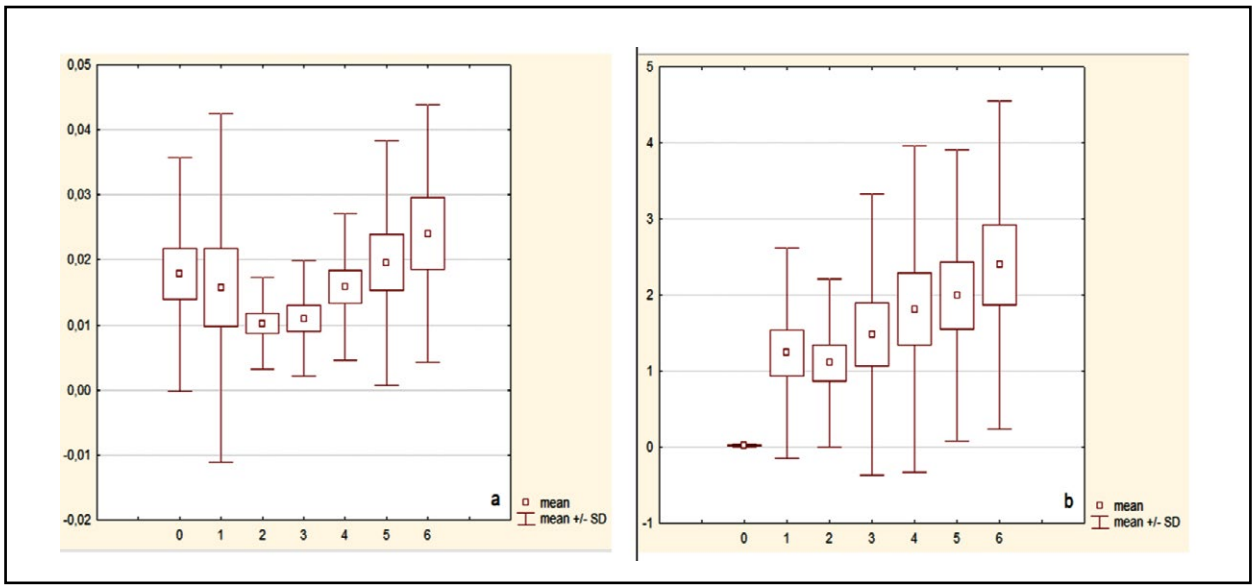

Figure 4. NKCD56^{bright} cells after treatment of CLL patients with R-FC and R-B regimens. Absolute numbers were determined by flow cytometry on peripheral blood samples collected before, during, and after CIT. Data are shown as absolute numbers (a) or relative to matched levels before CIT (b). Abbreviation: X-axis — number of treatment courses.

and during Fludarabine/Cyclophosphamide therapy showed a rapid and sustained reduction in the number of tumor cells, but also suppression of the number of CD4+ and CD8+ cells [32].

The effect of B

Bendamustine on specific lymphocyte subsets, particularly CD4+ lymphopenia, is often severe and may persist for a prolonged period (> 3 years) after treatment [31].

An important aim of the current study was to compare the number of T cells, NK cells, and NKT-like cells in patients with CLL and healthy controls, and further analyze the subpopulations of lymphocytes after up to six courses of CIT. No description of such systematic observations was found in the literature. We observed in patients with CLL before treatment that there is an increase in the total number of T cells, NK cells, and NKT-like cells in the PB in parallel with the increase in CLL cells. This observation has long been reported in the literature, where many abnormalities of immune function have been presented in patients with CLL [33–38]. The study of the potential association of the analyzed lymphocytes' subpopulations (T, NK, NKT-like) with CLL cells was indirect, *i.e.*, it concerned the quantitative analysis of the above-mentioned lymphocyte subpopulations in the PB before and during CIT. Our results thus indicate that CLL is accompanied by an increased or decreased number of normal B lymphocytes, NKT-like cells, and NKT-like CD56^{dim} cells compared with healthy individuals, without determining whether this condition is a cause or effect of the CLL. Studies determining the relationship of the number of lymphocyte subpopulations to prognosis, as well as dynamic analyses of changes in the number of these lymphocytes throughout the disease, have additional value. During treatment with R-FC and R-B regimens, we found decreases in all lymphocyte populations and subpopulations, including T cells, B cells, and NK cells, confirming that CIT affects the immune system and its cells. Previous studies have primarily documented the sensitivity of T cells to Fludarabine [39, 40]. It has also been described that NK cells were relatively preserved after treatment with the R-FC regimen [40].

Comparative data on CD4+ lymphocyte recovery after R-B versus R-FC treatment have not been published, but the evidence discussed above suggests that CD4+ lymphocyte recovery after RB treatment may take longer compared to R-FC [41]. Our study showed that in patients with CLL, the number of CLL cells in the PB decreased after each course of treatment. The decline in the number of CLL cells was so rapid that the number of CLL cells in peripheral blood had to be transformed into a logarithmic scale and simple equations calculated to make statistical inferences. On this basis, the LRC was calculated. The calculation method used assessed the quality of treatment and provided a basis for evaluating the prognosis after only the fourth course of treatment. In addition to profound changes in lymphocyte populations during treatment, patients with CLL showed a decrease in T cells, NK cells, NKCD56^{dim}, NKT-like cells, and NKT-like

CD56^{dim} cells by the second course of treatment, which remained constant until the sixth course of treatment. NKT-like CD56^{bright} cells showed a decrease in their number up to the third course, while their number increased slightly in the following three courses of treatment. NKCD56^{bright} cells behaved differently, with the total count decreasing until the second course and then increasing with each subsequent course of treatment. Therefore, in the context of changes in the number of NKCD56^{bright} cells and the clinical response to treatment, it seems important to systematically monitor patients up to the fourth course of treatment, as this is the time point, at which an increase in the number of these cells occurs, and may be vital in distinguishing such patients who will be observed to have PR or relapse despite the absence of other laboratory changes. Because NK cells are thought to be critical immune effectors for disease control and defense against pathogens, we analyzed in detail the number of NKCD56^{bright} cell populations that increased after each successive course of CIT. We observed that NKCD56^{bright} cell labeling might be a parameter for predicting treatment progression. A correlation of CLL cell counts with NKCD56^{bright} cell counts was observed during the treatment of patients with CLL. The ratio between the number of CLL cells and the remaining population and subpopulations was evaluated from the start to the end of treatment. We found that this ratio can be used to assess the treatment outcome of patients numerically, however, it does not depend on the number of CLL cells before treatment, which has not been described before.

In addition to the clinical evaluation of the therapy characterized as CR, PR, and PD, equally important is the numerical evaluation of the treatment outcome, which could be a secondary summary evaluation also based on the response defined as CR/PR/PD. This may be of practical importance, as determining the number of CLL cells in G/L and NKCD56^{bright} cells after each course are sufficient to calculate the correlation coefficient. In addition, the lower number of CLL cells remaining in peripheral blood after each treatment course, the higher NKCD56^{bright} cells count can be indicated. It can be speculated that NKCD56^{bright} cells can migrate from lymph nodes or other organs into blood [42–44].

In conclusion, the results show that treatment with R-FC and R-B regimens, which until recently were the standard of care for CLL patients, induces changes in the peripheral blood levels of T lymphocytes, NK cells, and NKT-like cells. Changes in the immune system accompany the treatment-induced reduction of the number of CLL cells; therefore, it is possible to monitor the effects of drugs on these cells closely during the treatment. Quantifying the lymphocytes' (sub)populations by flow cytometry during treatment may have prognostic significance. NKCD56^{bright} cells may be a prognostic clue and provide a marker to assess the treatment's efficacy.

Authors' contributions

Katarzyna Blachnio and Jerzy Kawiak designed the study and wrote the manuscript. Grzegorz Rymkiewicz contributed to the diagnosis of chronic lymphocytic leukemia. Beata Grygalewicz, Renata Woroniecka, Jolanta Rygier, and Barbara Pienkowska-Grela performed cytogenetic tests and contributed to the preparation of the manuscript. All authors agree to be accountable for the work.

Conflict of interest

The authors declare no conflicts of interest.

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Submitted: 17 May, 2022
Accepted after reviews: 18 August, 2022
Available as AoP: 1 September, 2022