ORIGINAL ARTICLE

Prognostic significance of PD-1 expression on peripheral blood CD4+ T cells in patients with newly diagnosed chronic lymphocytic leukemia

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KEY WORDS

chronic lymphocytic leukemia, CD4+ T cells, PD-1, T cells

ABSTRACT

INTRODUCTION Recent studies in a mouse model of chronic lymphocytic leukemia (CLL) demonstrated that inhibition of the programmed death receptor 1 (PD-1)–PD-L1 axis resulted in correction of leukemia-induced CD8+ T cell-related immune dysfunction and protected mice against CLL development. However, it remains unclear whether CLL development and progression can be also associated with CD4+ T cells expressing PD-1.

OBJECTIVES We aimed to analyze whether a quantitative assessment of CD4+PD-1+ T cells performed at the time of diagnosis can have prognostic significance in patients with CLL.

PATIENTS AND METHODS We examined 56 patients with newly diagnosed CLL at different stages of the disease. The quantitative assessment of PD-1-expressing CD4+ T cells was performed in all patients, using multicolor flow cytometry.

RESULTS We demonstrated that CLL patients with an advanced (high and intermediate risk) stage had a significantly higher number of CD4+PD-1+ T cells compared with subjects with low-grade disease. Importantly, we showed that the number of PD-1-expressing CD4+ T cells in the peripheral blood of patients referred for immediate treatment due to the advanced stage of the disease was significantly higher compared with subjects on watchful waiting. Finally, we found that treatment-naive patients with higher numbers of CD4+PD-1+ T cells at baseline showed a significantly shortened time to the first treatment compared with patients with a low number of CD4+PD-1+ T cells.

CONCLUSIONS Our study showed that the quantative assessment of CD4+PD-1+ T cells in peripheral blood using flow cytometry can facilitate prognostication of patients with newly diagnosed CLL.

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INTRODUCTION The past decade has brought significant advances in our understanding of the pathogenesis of chronic lymphocytic leukemia (CLL) and a corresponding increase in the array of possible treatment options. Yet, despite the substantial progress witnessed so far, there is still no cure for CLL.[1] Its final discovery will largely depend on a thorough comprehension of the survival signals and microenvironment that contributes to the establishment of the leukemic clone and enhances its resistance to applied therapies. It is now clear that both intrinsic defects affecting the regulation of programmed cell death (apoptosis) and an altered survival-stimulating microenvironment constitute some of the major pathogenic factors for CLL.[1-3] Therefore, the expansion of the malignant clone is subject not solely to its intrinsic characteristics (such as the expression of antiapoptotic molecules), but also to the delivery of stimulating signals from stromal and immune cells infiltrating neoplastic cells.

TABLE 1 Clinical and molecular characteristics of the study group (n = 56)

Rai stage, number (percentage) of patients	
0	7 (12.5)
I	11 (19.64)
II	24 (41.85)
III	8 (14.28)
IV	6 (10.71)
laboratory tests, median (range)	
WBC, $\times 10^3$	80.9 (9.87–360.1)
lymphocyte, $\times 10^3$	58930 (5580–229000)
hemoglobin, mg/dl	13 (6.6–16)
platelets, $\times 10^3$	160 (32–312)
β_2m, g/l	3.94 (2.094–5357)
LDH, IU/l	221 (173–477)
creatinine, mg/dl	0.91 (0.55–239)
hierarchical cytogenetic subgroup, %	
sole 13q deletion	30.1
normal	34.0
trisomy 12 (no 17p13 or 11q22 deletion)	10.7
11q22 deletion (no 17p13 deletion)	14.3
17p13 deletion	10.7
ZAP70 >30%	30.4
strategy, number of patients	
watchful waiting	30
immune chemotherapy	26
response rate after the treatment, number of patients	
patients with CR response	7
patients with PR response	11
patients with SD response	5

Abbreviations: LDH, lactate dehydrogenase; β_2m, β_2-microglobulin; CR, complete remission; PR, partial remission; SD, standard deviation; WBC, white blood cells

Programmed death 1 (PD-1) is classified as a member of the CD28 costimulatory receptor superfamily. It is expressed on a subset of thymocytes and upregulated on activated T cells, B cells, and myeloid cells.[4,5] PD-1 inhibits T-cell activity by transmitting a negative signal to T cells together with signaling through the T-cell receptor.[6] Recent studies have indicated that PD-1 acts as a negative regulator of the immune system, being also a crucial factor responsible for peripheral tolerance.[7]

B-cell CLL cannot be separated from a T cell-dependent immune dysregulation, which leads to impairment of antitumor and antipathogen responses. Recent studies have reported that T cells from CLL patients show features of immune exhaustion akin in part to those observed in the course of chronic viral infections.[8,9] One of the phenomena symptomatic of immune exhaustion is the upregulation of such surface receptors as PD-1, a molecule responsible for a number of tumor-induced immunosuppressive actions.[10] Importantly, blocking PD-1 with the use of monoclonal antibodies significantly enhanced antitumor effects in both animal models and clinical settings.[11-13] Interestingly, in a mouse model of CLL, McClanahan et al.[14] demonstrated that inhibition of the PD-1–PD-L1 axis resulted in correction of leukemia-induced $CD8^+$ T cell-related immune dysfunction and protected mice from CLL development. On the other hand, Nunes et al.[15] demonstrated the emergence of $CD8^+PD\text{-}1^+$ T cells in CLL patients to be accompanied by an inversion of the CD4:CD8 ratio, a status associated with more aggressive clinical disease. To date, however, it has remained obscure whether a direct assessment of PD-1 expression on T cells (including $CD4^+$ T cells) could facilitate prognostication of patients with newly diagnosed CLL.

The principal aim of the present study was to investigate whether the frequencies of $CD4^+PD\text{-}1^+$ T cells, assessed at the time of diagnosis, could be related to an altered risk of a shortened time to the first treatment. We also evaluated the effect of applied immune chemotherapy on the frequencies of peripheral blood $CD4^+PD\text{-}1^+$ T cells.

PATIENTS AND METHODS

Patients A total of 56 patients (23 men and 35 women) with newly diagnosed B-lineage CLL were enrolled in the study. Their median age at the time of sample collection was 64 years (range, 55–69). We excluded patients with an acute or chronic infection, inflammatory processes, and liver or kidney diseases (creatinine levels above 2.0 mg/dl or creatinine clearance rate below 60 ml/min), or those who received corticosteroids before the start of the treatment course or whose comorbid conditions could possibly require systemic corticosteroids.

The diagnosis of CLL was based on clinical observation, morphological composition of the peripheral blood (flow cytometry to identify the immune phenotype of leukemic cells), bone marrow aspiration and trephine biopsy, and computed tomography from the neck to the pelvis, according to Hallek et al.[16] The characteristics of patients at the time of CLL diagnosis are summarized in TABLE 1.

At the time of diagnosis, patients were graded according to the Rai staging system as follows: low-risk (stage 0), intermediate-risk (stages I and II) and high-risk disease (stages III and IV).[16]

Thirty patients with stable disease did not receive chemotherapy, while 24 patients with progressive disease (including 10 cases at stage 2, with massive lymphadenopathy confirmed by computed tomography) were treated in the Department of Hematology of the Medical University of Bialystok, Białystok, Poland, from 2010 to 2014.

Patients referred for treatment received the FCR therapy: intravenous (IV) fludarabine (25 mg/m^2/d) and cyclophosphamide (250 mg/m^2/d) for 3 days, repeated every 28 days for a total of 6 cycles, and rituximab, 375 mg/m^2 administered by IV infusion on day 1 of the first cycle and 500 mg/m^2 IV on day 1 of the subsequent cycles with premedication (oral acetaminophen and an antihistamine). Prophylaxis for

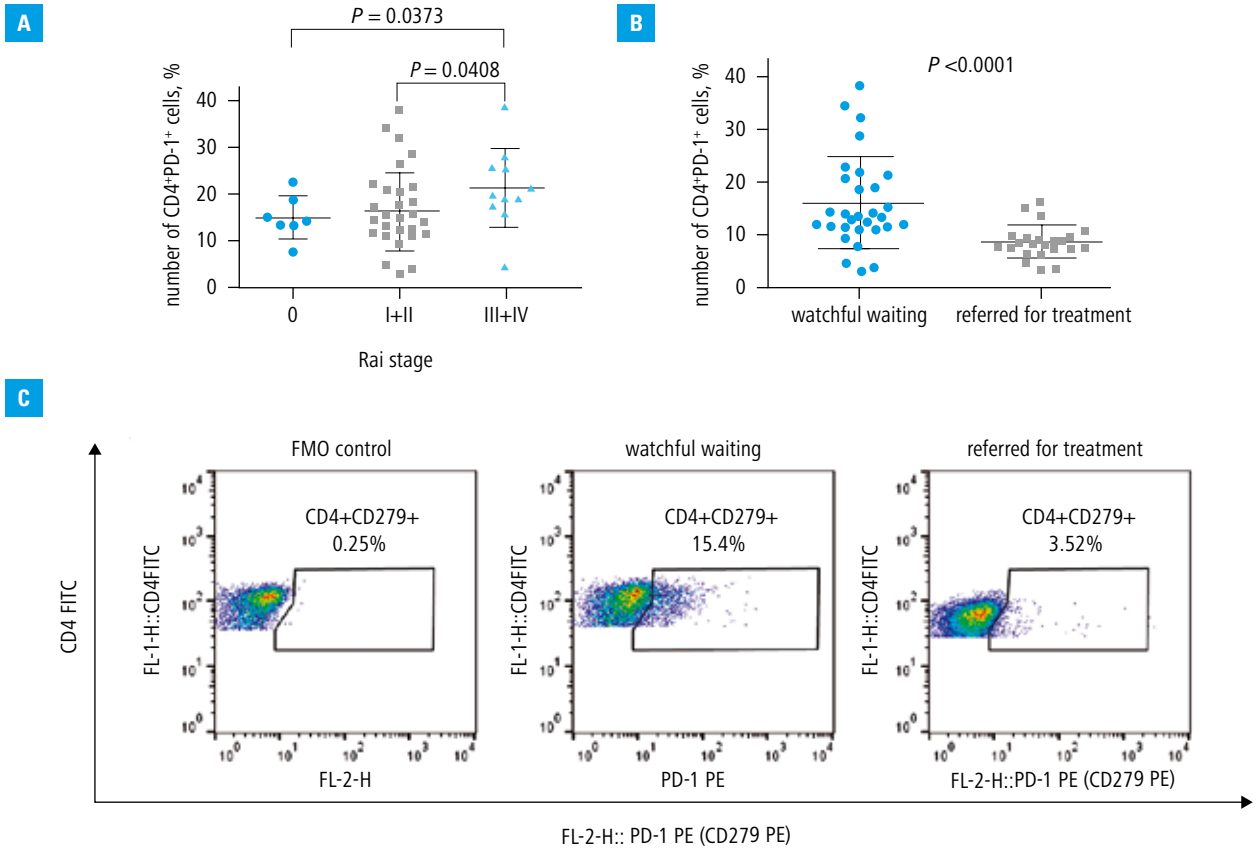

FIGURE 1 Baseline number of CD4⁺PD-1⁺ T cells in patients with chronic lymphocytic leukemia at different stages of the disease (**A**) as well as in patients with stable disease ("wait and watch" strategy) and progressive disease (classified for treatment) (**B**); data are presented as means ± standard deviation; **C** – representative flow cytometry dot plots of CD4⁺PD-1⁺ T cells in patients on watchful waiting and those referred for immediate treatment

Abbreviations: FMO, fluorescence minus one

tumor lysis syndrome (including allopurinol) and prophylactic antimicrobials (sulfametoksazol + trimetoprim and acyclovir) were required in all our patients.

The posttreatment disease status was assessed in all cases by means of regular blood counts, clinical examination, and computed tomography scans following the recommendations of the 2008 International Workshop on Chronic Lymphocytic Leukemia. Complete remissions (CRs) also required confirmation by bone marrow biopsy. The characteristics of CLL patients are listed in **TABLE 1**.

In patients on watchful waiting, the follow-up involved the evaluation of each patient every 3 months until progression of the disease.

All patient samples were collected with the approval of the Ethics Committee at the Medical University of Bialystok. The Committee approved the study protocol and written informed consent was obtained from all patients.

Methods **Flow cytometry** The number of PD-1-expressing CD4⁺ T cells were assessed by means of flow cytometry (FACSCalibur, Becton Dickinson, San Jose, United States) in all studied individuals according to the stain-and-then-lyse-and-wash protocol as previously described.[17] Briefly, 100 µl of EDTA-anticoagulated whole blood was stained with 5 µl of the following murine antihuman monoclonal antibodies CD4 FITC and CD279 PE (all from Becton Dickinson) and incubated for 30 minutes at room temperature, in dark. Thereafter, the cells were lysed with the use of BD FACS Lysing Solution (Becton Dickinson), washed twice with cold phosphate buffered saline (Biomed Lublin, Poland), and fixed with CellFix (Becton Dickinson). Fluorescence-minus-one controls were used for setting compensation and to assure correct gating. At least 250 000 events were acquired. Specimen acquisition was performed using the CellQuest software (Becton Dickinson) and the obtained data were analyzed with the FlowJo v.7.6.5 software (Tree Star, Ashland, Ohio, United States). CD4⁺ T cells were gated as previously described.[17,18]

Statistical analysis A statistical analysis was conducted using GraphPad Prism 6 (GraphPad Software, La Jolla, United States). Categorical variables were analyzed with the Fisher exact test, while continuous variables—with the Mann–Whitney test. The Wilcoxon test was used to compare changes in the number of CD4⁺PD-1⁺ T cells before and after therapy. Kaplan–Meier estimates and the log-rank test were applied to determine differences between the times to initial treatment. The differences were considered statistically significant at a *P*

TABLE 2 Correlations between the number of CD4±PD-1+ T cells and hematological parameters

Parameters	*r*	*P* value
CD4+PD-1+ T cells [%] vs absolute monocyte count	–0.01351	0.9242
CD4+PD-1+ T cells [%] vs absolute lymphocyte count	**0.2778**	**0.0462**
CD4+PD-1+ T cells [%] vs WBC	**0.3273**	**0.0179**
CD4+PD-1+ T cells [%] vs hemoglobin	**–0.2888**	**0.0379**
CD4+PD-1+ T cells [%] vs PLT	–0.1124	0.4324
CD4+PD-1+ T cells [%] vs LDH	0.1501	0.3084
CD4+PD-1+ T cells [%] vs β_2m	0.3137	0.1041
CD4+PD-1+ T cells [%] vs CRP	0.1924	0.1854
CD4+PD-1+ T cells [%] vs APT	–0.05701	0.6941
CD4+PD-1+ T cells [%] vs ALT	0.1809	0.2086
CD4+PD-1+ T cells [%] vs creatinine	–0.06320	0.6595
CD4+PD-1+ T cells [%] vs lymphocyte count on myelogram	0.2128	0.2505
CD4+PD-1+ T cells [%] vs lymphocyte count on trephine biopsy	0.2717	0.2335

Statistically significant correlations are presented in bold.

Abbreviations: ALT, alanine transaminase; APT, aspartate transaminase; CRP, C-reactive protein; PLT, platelet count; others, see TABLE 1

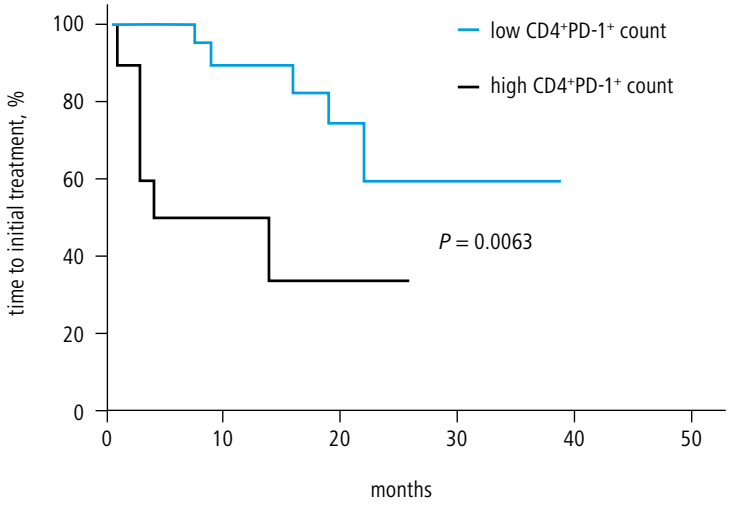

FIGURE 2 Effect of CD4+PD-1+ T cells on the time to first treatment in patients with chronic lymphocytic leukemia (log-rank test)

value of less than 0.05. The results are presented as mean ± standard deviation.

RESULTS CLL patients with advanced high-risk disease (stages III and IV) had a higher number of CD4+PD-1+ T cells (21.37% ±8.58%) compared with subjects with low-risk (stage 0; 14.97% ±4.63%; *P* = 0.0373) and intermediate-risk disease (stages I and II; 16.31% ±8.40%; *P* = 0.0408). Data are shown in FIGURE 1A. In particulara, we showed that the number of PD-1-expressing CD4+ T cells in a group of patients referred for treatment due to the advanced stage of their disease (stages III and IV according to the Rai classification and patients with stage II who had bulky disease confirmed by computed tomography) were significantly higher compared with subjects with low-risk disease on watchful waiting (8.56% ±3.11% vs 15.79% ±8.59%; *P* <0.0001) Data are shown in FIGURE 1B).

We subsequently correlated the number of PD-1-positive CD4+ T cells with a selection of well-known parameters of prognosis and tumor load in CLL (TABLE 2). We demonstrated significantly positive correlations between the number of PD-1-expressing CD4+ T cells and both the level of white blood cell count (*rho* = 0.33; *P* = 0.01) and lymphocytosis (*rho* = 0.28; *P* = 0.04). The correlations of CD4+PD-1+ T cells with hemoglobin levels were negative (*rho* = –0.3, *P* = 0.03). We found no differences in the number of PD-1 T cells in patients with different cytogenetic status: the presence vs absence of del(11), del(12), del(13) or de(l17) (*P* >0.05 for all comparisons).

Next, we analyzed the effect of CD4+PD-1+ T-cell count on prognosis by performing the Kaplan–Meier analysis and the log-rank test. We found that treatment-naive (watchful waiting) patients with CLL with the number of CD4+PD-1+ T cells exceeding 15.79% at baseline (mean of all baseline values in the entire CLL cohort) showed a significantly shortened time to the first treatment compared with CLL patients with lower CD4+PD-1+ T cell numbers (6 months vs 18.5 months, respectively, *P* = 0.006, FIGURE 2).

Finally, no significant differences in the number of PD-1-expressing CD4+ T cells were shown after immune chemotherapy (21.50% ±8.64% before therapy and 19% ±10% after therapy, *P* = 0.6698, FIGURE 3A). Interestingly, patients who achieved complete remission had a significantly lower number of CD4+PD-1+ T cells at baseline compared with subjects who achieved only partial remission (15.66% ±2.94% and 24.68% ±8.76%, respectively, *P* = 0.0431, FIGURE 3B).

DISCUSSION The involvement of T cells in the pathogenesis of CLL has recently gained widespread attention owing to the importance of the constant interplay between neoplastic B cells with the microenvironment substratum and T cells. It is believed that these interactions

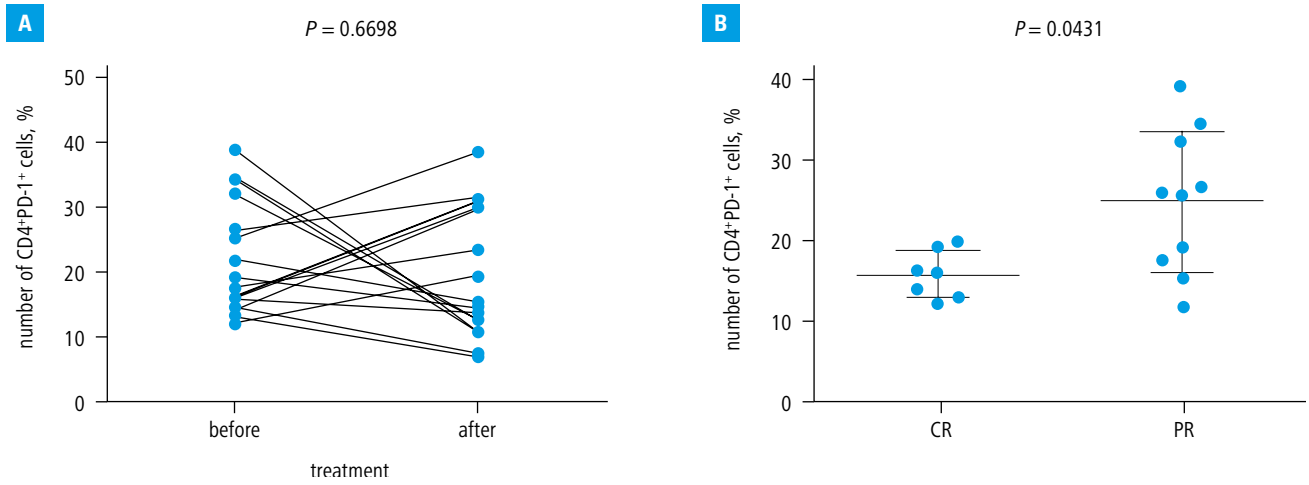

FIGURE 3 Effects of immune chemotherapy on the number of $CD4^+PD\text{-}1^+$ T cells in patients with chronic lymphocytic leukemia (**A**); effect of the baseline number of $CD4^+PD\text{-}1^+$ T cells on the type of response achieved after immune chemotherapy (**B**)
Abbreviations: see **TABLE 1**

modify the clinical course of the disease, predominantly through the regulation of the expansion, differentiation, and survival of CLL B cells. Importantly, this crosstalk may also alter the number, function, and memory phenotype of normal T cells, consequently affecting the amplitude and efficiency of the adaptive immunity in patients with CLL.[19,20]

Although the mechanism by which T cells accumulate in CLL has not been fully elucidated so far, it has been reported that T cells derived from patients with advanced disease were characterized by shorter telomeres, which suggested that these T cells could react in response to the growth of the CLL clone.[21] The above assumption stemmed from the identification of a population of antileukemic T cells, a recent subject of major interest, especially in the context of immunotherapy.[22,23] PD-1 is specifically expressed by germinal center-associated T cells in reactive lymphoid tissue and shows a varying distribution in defined lymphadenopathies.[24,25] Indeed, there is evidence for the prognostic significance of the amount of PD-1-positive tumor-infiltrating lymphocytes, analogous to other tumor microenvironmental components, such as FOXP3-positive tumor-infiltrating lymphocytes in follicular lymphoma (FL) and in classic Hodgkin lymphoma.[26,27]

In the current study, we demonstrated for the first time that the baseline number of PD-1-expressing $CD4^+$ T cells in peripheral blood exceeding 15.79% identifies these CLL patients that are at higher risk of a shorter time to the first treatment. Based on these data, we propose that the use of such easily accessible tool as flow cytometry for assessing the number of $CD4^+PD\text{-}1^+$ T cells in peripheral blood could facilitate the prognostication of patients with newly diagnosed CLL. However, further studies are needed to examine whether such correlations can be identified in larger groups of patients.

To a certain degree, our data are in line with some previous findings on the role of PD-1 in the pathogenesis of malignancies and, more specifically, CLL. PD-1 upregulation, being both a consequence and a marker of immune exhaustion, was postulated to limit the function of tumor- and virus-specific T cells.[28,29] To date, the importance of this mechanism has been corroborated almost exclusively for $CD8^+$ T cells. Based on the current findings, we can now hypothesize that the impairment of an appropriate function of $CD4^+$ T cells could also enhance CLL-induced immunodeficiency. This finding was in part confirmed by the ability of CLL cells to drive the $CD4^+$ T cell repertoire into a more immunosuppressive phenotype.[30]

More importantly, our study confirmed significant associations between the number of $CD4^+PD\text{-}1^+$ T cells and white blood cell count, lymphocytosis, and hemoglobin levels. These findings suggest that there are close relationships between PD-1 expression levels and the severity and prognosis of the disease. On the other hand, it should be noted that our discovery concerning the elevated number of $CD4^+PD\text{-}1^+$ T cells might have been associated to some other confounding variables present in CLL patients. To address these possibilities, we analyzed the number of $CD4^+PD\text{-}1^+$ T cells in the context of possible ongoing infections and other factors responsible for a worse prognosis in CLL patients. For example, we excluded the possibility that the number of $CD4^+PD\text{-}1^+$ T cells might have been related to either C-reactive protein status or cytogenetic status.

Furthermore, gene expression data demonstrated that both FL with a poor response to anti-CD20 therapy and FL with a poor prognosis transforming into diffuse large B-cell lymphoma within 3 years are characterized by an active immune response including high activation state of T cells.[31] In addition, the proliferation of neoplastic cells in FL is known to be conditioned by T cells and T cell-derived cytokines such as interleukin 4 or CLXCL13, another ligand typically expressed by germinal center-associated PD-1 positive helper T cells.[32-34] This microenvironmental dependence in FL ceases with the progression of the disease.[31]

Indeed, our results revealed some significant differences in the baseline number of $CD4^+PD\text{-}1^+$ T cells according to the response achieved after immune therapy: patients with lower levels more frequently achieved complete remission, which is consistent with the previous observations in lymphoma patients. This can suggest that the assessment of $CD4^+PD\text{-}1^+$ T cells at the time of diagnosis can not only predict the time to the first treatment but also, to some extent, the degree of response to applied therapy. This observation, although promising from the clinical perspective, needs to be confirmed in larger studies.

In summary, our data warrant further studies that will explore in detail whether the elevated numbers of circulating $CD4^+PD\text{-}1^+$ T cells in CLL patients constitute a cause or rather a consequence of CLL-related immune dysregulation and exhaustion. However, although the exact mechanism has to be examined in future studies, our findings indicate that the use of flow cytometry to evaluate PD-1 $CD4^+$ T cells in peripheral blood could improve prognostication of treatment-naive patients with newly diagnosed CLL.

Contribution statement MR performed flow cytometry and analyzed data; AE contributed to the study design, analyzed data, performed statistical analyses, and prepared figures; LB contributed to the study design, provided and analyzed clinical data, and drafted the manuscript; EL, IL, JP, and JK provided and analyzed clinical data; PS, MD, AB-L, and JK analyzed clinical and laboratory data; MM designed the study, analyzed data, and wrote the manuscript. All authors approved the final version of the manuscript.

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Znaczenie rokownicze ekspresji receptora PD-1 na powierzchni limfocytów T CD4+ krwi obwodowej u pacjentów z nowo zdiagnozowaną przewlekłą białaczką limfocytową

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SŁOWA KLUCZOWE

limfocyty T, limfocyty T CD4+, przewlekła białaczka limfocytowa, PD-1

STRESZCZENIE

WPROWADZENIE Ostatnie wyniki badań nad przewlekłą białaczką limfocytową (PBL) przeprowadzone na modelu mysim wykazały, że hamowanie osi współdziałania między receptorem zaprogramowanej śmierci 1 (PD-1) i jego ligandem PD-L1 skutkowało naprawą spowodowanej przez białaczkę dysfunkcji immunologicznej limfocytów T CD8+, chroniąc myszy przez rozwojem białaczki. Nie wiadomo jednak, czy rozwój i progresja PBL mogą być również zawiązane z limfocytami T CD4+ z ekspresją PD-1.

CELE Celem badania było ustalenie, czy ocena ilościowa limfocytów T CD4+ PD-1+ dokonana w czasie rozpoznania może mieć znaczenie rokownicze u pacjentów z PBL.

PACJENCI I METODY Analizie poddano 56 pacjentów z nowym rozpoznaniem PBL w różnych stadiach zaawansowania choroby. Ilościowa ocena limfocytów T CD4+ z ekspresją PD-1+ została przeprowadzona u wszystkich badanych pacjentów przy użyciu wielokolorowej cytometrii przepływowej.

WYNIKI Wykazano, że u pacjentów z zaawansowanym (wysokim i pośrednim) stadium PBL występuje znamiennie większa liczba limfocytów T CD4+ z ekspresją PD-1+ we krwi obwodowej w porównaniu z pacjentami z niskim stopniem zaawansowania choroby. Co istotne, wykazano, że liczba limfocytów T CD4+ z ekspresją PD-1 we krwi obwodowej pacjentów zakwalifikowanych do natychmiastowego leczenia ze względu na wysokie stadium zaawansowania choroby była znamiennie wyższa niż u pacjentów zakwalifikowanych do dalszej obserwacji (*watchful waiting*). Wykazano również, że w przypadku pacjentów nieleczonych, u których w momencie diagnozy stwierdzono większą liczbę limfocytów T CD4+PD-1+, czas do pierwszego leczenia był statystycznie znacznie krótszy w porównaniu z pacjentami z niską liczbą limfocytów T CD4+PD-1+.

WNIOSKI Przeprowadzone badanie wykazało, że zastosowanie opartej o cytometrię przepływową ilościowej oceny limfocytów T CD4+PD-1+ we krwi obwodowej może wspomóc postępowanie prognostyczne u pacjentów z nowo zdiagnozowaną PBL.

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