SPANDIDOS PUBLICATIONS

Natural killer-like T CD3+/CD16+CD56+ cells in chronic lymphocytic leukemia: Intracellular cytokine expression and relationship with clinical outcome

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Received May 5, 2010; Accepted June 22, 2010

DOI: 10.3892/or_00000924

Abstract. The clinical significance of NK-like T CD3+/CD16+CD56+ cells in chronic lymphocytic leukemia (CLL) is still a subject of controversy. There are few previous descriptions that this cell population can be qualitatively or quantitatively deficient in CLL patients. In the present study we investigated the clinical value of CD3+/CD16+CD56+ cells as predictors of disease progression. We assessed the frequencies of CD3+/CD16+CD56+ cells by the flow cytometry in a group of 300 CLL patients. The percentage of CD3+/CD16+CD56+ cell population expressed as the percentage of CD3+ lymphocyte compartment showed an inverse correlation with ZAP-70 and CD38. Additionally, the CD3+/CD16+CD56+ showed an inverse correlation with LPL/ADAM29 ratio. Likewise, the ability of these cells to cytokine expression correlated with ZAP-70 expression. A positive correlation between percentage CD3+/CD16+CD56+ cells and TFS was found. The decreased percentage of these cells was associated with higher death risk in CLL patients. Furthermore, the percentage of CD3+/CD16+CD56+ cells was significantly decreased in patients who showed progression of disease. This study suggests that assessment of CD3+/CD16+CD56+ cells may be helpful in determining a worsening of clinical course. Monitoring of these cell numbers and function may provide useful information for determining disease activity. Especially, it could be intormative to look at these cells in patients with stage 0 CLL. For this patient group immunological control and dysfunction are probably important factors.

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Key words: CLL, CD3+CD16+CD56+, NK-like T cells, ZAP-70, CD38

Introduction

Chronic lymphocytic leukemia (CLL) which is the most common leukemia of adults in Western countries still remains incurable. It follows an extremely variable course with survival from months to decades. Studies on the novel prognostic factors in CLL are essential for improvement of criteria for starting the cytostatic treatment. Besides Rai or Binet staging system, some chromosomal aberrations, in particular concerning 11q and 17p, IgV_H mutational status, as well as CD38 and ZAP-70 expression, are currently considered as the most important prognostic factors as to progression-free and overall survival (1). The issue of defining of unfavorable prognostic factors which may give reason for the early beginning, i.e. before the meeting of the currently admitted criteria, of cytostatic treatment, needs further studies.

Current prognostic factors (i.e. IgV_H mutational status, ZAP-70, CD38) concentrate on the intrinsic abnormalities of the malignant B cell clone (2-4). However, it became clear that transformation and progression of tumors is not an independent process but it is controlled by their interactions with the tumor microenvironment (5). Probably interactions between the leukemic cells and native immune system could have an important influence on disease progression. There are three main lymphocyte subsets that play important roles in the regulation of immune responses to pathogens as well as to tumors: conventional T lymphocytes, natural killer (NK) cells and the third lymphocyte subset referred as natural killer T (NKT) cells (6). It has been shown that T cell dysfunctions account for pathological conditions such as increased susceptibility to infections, autoimmune hemolytic anaemia, and hypo-gammaglobulinemia, commonly noted in CLL patients. The changes in number and functions of T lymphocytes may support the 'microenvironment' that sustains the malignant B cells clone, delays their apoptosis and may contribute to the pathogenesis and progression of the disease (7-9). Likewise, the phenotypic and functional abnormalities found in NK cells, such as defective cytotoxic activity, might allow the appearance and maintenance of leukemic B cells accumulation. A dilutional effect of leukemic B cells and intrinsic

functional defects of NK cells might be responsible for a low NK activity in most patients with CLL (10). However, the studies examining NKT cell number and function in B-CLL patients are limited (10,11). Guven *et al* (10) reported the possibility of expanding the $CD3^+CD56^+$ cell population, from peripheral blood mononuclear cells of CLL patients. They found that these cells have cytolytic capacity and may be a feasible clinical strategy for controlling CLL.

The population of $CD3^+CD56^+$ cells was initially described in 1986 by Lanier *et al* (12) and Schmidt *et al* (13) and further characterized by Ortaldo *et al* (14) who showed that $CD3^+CD56^+$ cells are capable of lysing a large group of tumor cells in a non-MHC-restricted manner. Later, $CD3^+CD56^+$ subgroup was analyzed in more detail and called NKT (15). The CD56 antigen is typically expressed by NK cells. T cells expressing CD56 marker are not classical invariant NKT (iNKT) cells but are a broader group of T lymphocytes that conform to the original definition of NKT cells, which was T lymphocytes coexpressing T cell antigen receptor (TCR) and NK cell markers (16,17). NK receptor (NKR)-positive T cells possess dual innate and adaptive immune functions displaying properties of both T and NK cells capable of both MHC-restricted and MHC-unrestricted cytotoxicity and cytokine production. The human iNKT (cells binding CD1d-α-Galcer and expressing Vα24-Jα18 paired with Vß11) and other T cells expressing NKR (other than CD56) are found almost exclusively within the $CD56^+$ T cell population (16). $CD56^+$ T cells can be activated by TCR ligation or in response to cytokines in the microenvironment and stress-inducible proteins present on target cells (18-20). $CD3^+CD56^+$ cells can contribute to the activation and regulation of T cells, B cells, and other cells of the adaptive immune system via the production of cytokines. Upon activation, $CD56^+$ T cells can rapidly produce Th1-type and Th2-type cytokines (18). Activities of $CD3^+CD56^+$ cells are regulated by inhibitory KIR and CD94 molecules (21,22). This cell population can be highly expanded *in vitro* by the addition of the cytokines (IFN-γ, IL-1, IL-2) and anti-CD3 monoclonal antibody. These $CD3^+CD56^+$ cells have been termed cytokine-induced killer cells (CIK cells) and it was shown that they possess enhanced cytotoxicity against various tumor cells (23). In contrast to NK-like T cells that are *de novo*, CIK cells are generated following *in vitro* culturing (24,25). $CD3^+CD56^+$ have also been named as Natural T (NT) cells (16,26). In our study NK-like T cells have generally been used.

The aim of our study was to examine the role of $CD3^+/CD16^+CD56^+$ (NKT-like) cells in the pathogenesis and clinical course of CLL. We investigated possible associations between $CD3^+/CD16^+CD56^+$ cells and poor prognostic factors, both established ones, such as: CD38 antigen or ZAP-70 protein (zeta-associated protein of 70 kD) expression (2-4) as well as new ones, such as lipoprotein lipase (LPL) and metalloproteinase ADAM29 gene expression (27,28). To our knowledge, this is the first study of this cell population investigating the relationship between the peripheral blood $CD3^+/CD16^+CD56^+$ cells and clinical outcome in newly diagnosed patients with CLL. In the current study, for the first time, we present this cell population frequencies in the CLL bone marrow.

Materials and methods

Patients and samples. Peripheral blood (PB) and bone marrow (BM) specimens were obtained from 300 untreated CLL patients diagnosed between September 1997 and December 2008 (187 men and 113 women). The median age of patients was 66 years (ranging from 32 to 87 years). CLL diagnosis based on a clinical examination, morphological and immunological criteria (29). At the time of diagnosis, patients were staged according to the Rai staging system (30) as follows: stage 0 (92 cases), stage 1 (57 cases), stage 2 (69 cases), stage 3 (40 cases) and stage 4 (42 cases). One hundred and thirty-one patients (43.7%) with stable disease did not receive chemotherapy, and 169 patients (56.3%) with progressive disease required treatment. The patients cohort was divided into three groups: patients with Rai stage 0 (92 cases), stage 1-2 (126 cases) and stage 3-4 (82 cases). The control samples consisted of PB from 20 healthy volunteers (8 men and 12 women aged from 33 to 66 years, median age of 58 years) and BM from 10 healthy donors. PB and BM samples were collected into heparinized tubes and immediately processed. The study was approved by the Local Ethics Committee. Peripheral blood and bone marrow samples were obtained from the patients and healthy volunteers after informed consent.

Assessment of $CD3^+/CD16^+CD56^+$ cells. Immunophenotypic analysis is a key adjunctive tool for diagnosis of chronic lymphocytic leukemia and is typically performed by flow cytometry (29). The primary purpose of such analysis is to look for the immunophenotypic pattern that is characteristic for leukemic cells in CLL. In our study the standard diagnostic flow cytometric analysis included also monoclonal antibodies (MoAbs) anti-CD3 FITC/$CD16^+$CD56 PE/CD45 PerCP (BD Biosciences) which permitted simultaneous assessment of T ($CD3^+$) lymphocytes and NK ($CD16^+CD56^+$) cells. During analysis, $CD3^+/CD16^+CD56^+$ population was determined. A standard, whole-blood assay with erythrocyte cell lysis was used for preparing the PB or BM specimens. The samples were analyzed by flow cytometry directly after preparation. For data acquisition and analysis, a FACS Calibur instrument (BD) with CellQuest software (BD) was used. For each analysis 10,000 events were acquired and analyzed. The percentage of positive cells was measured from a cut-off set using isotype matched non-specific control antibody. $CD3^+/CD16^+CD56^+$ T cells were analyzed within gated $CD3^+$ T lymphocytes. Dot plots, illustrating the analysis method for the identification of $CD3^+/CD16^+CD56^+$ cells are shown in Fig. 1.

Analysis of ZAP-70 expression in CLL cells. All PB samples were stained for ZAP-70 protein expression. We used a modification of a previously described method for flow cytometric examination of ZAP-70 protein expression (31). A cut-off point for ZAP-70 positivity in leukemic cells was ≥20%.

Detection of CD38 expression. Flow cytometry analysis of CD38 antigen expression was performed on fresh PB samples, as described previously (32). CLL cells were

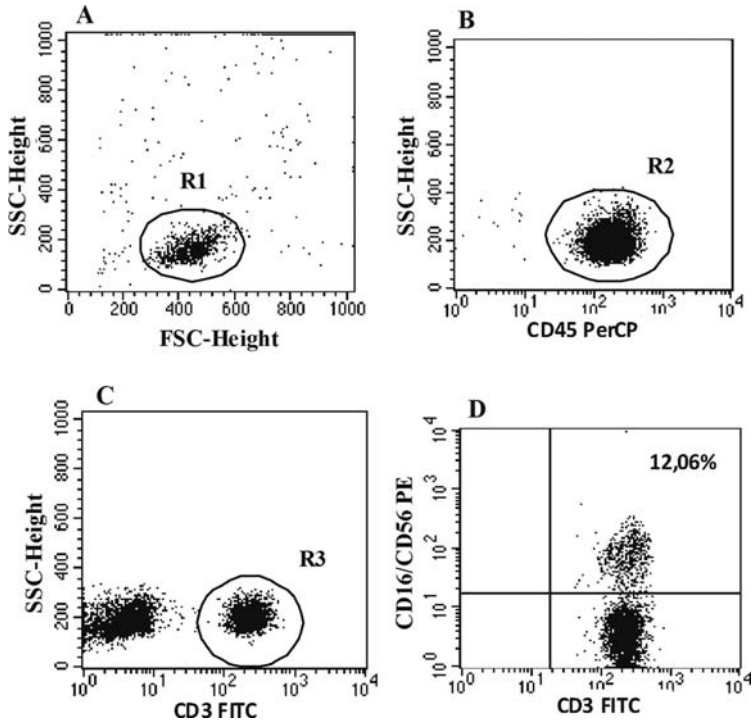

Figure 1. The dot plots show representative data illustrating the analysis method for identification of $CD3^+/CD16^+CD56^+$ cells among $CD3^+$ T lymphocytes following three-color staining. (A) The dot plot shows the forward scatter/side scatter (FSC/SSC) distribution and the gate (region R1) used to select lymphocytes for analysis. (B) The R1 gated events were then analyzed for CD45 PerCP staining, and positive cells ($CD45^+$) were gated (region R2). (C) The dot plot shows the SSC vs. CD3 FITC distribution. The dot plot, CD3FITC vs. SSC was established by combined gating of events using R1 and R2. $CD3^+$ cells were thus identified as cells in R3. (D) The final dot plot CD3 FITC vs. $CD16^+CD56$ PE was established by combined gating of events using R1, R2 and R3. The number in the upper right quadrant in the dot plot represents the percentage of $CD3^+/CD16^+CD56^+$ T cells.

considered CD38-positive when ≥20% of them expressed the membrane antigen.

Analysis of LPL and ADAM29 expression in CLL cells. Peripheral blood samples from 100 CLL patients were stained for LPL and ADAM29 expression. We used a previously described method for flow cytometric examination of LPL and ADAM29 expression (33).

Intracellular IFN-γ, TNF, IL-10 and IL-4 staining. In 25 CLL patients we examined an intracellular IFN-γ and IL-4 expression by $CD3^+CD56^+$ cells. Intracellular TNF analysis was performed on fresh PB or BM samples, as described previously (34). MoAbs used for analyses included anti-CD3 FITC (BD PharMingen), anti-CD56 CyChrome (BD PharMingen) and anti-TNF PE (BD Biosciences), anti-IFN-γ PE (BD Biosciences), anti-IL-4 (BD Biosciences) and anti-IL-10 PE (BD PharMingen). In the experiment, the mean percentages of $CD3^+CD56^+$ cells with intracellular cytokine expression were analyzed.

Statistical analysis. Differences between two groups were assessed using the U Mann-Whitney test. The Wilcoxon paired test was used to compare the results in peripheral blood and bone marrow. The Spearman rank correlation coefficient was used in correlation tests. The Kaplan-Meier method was used for drawing survival curves, and results were compared using the log-rank test. The treatment free survival (TFS) was calculated from the date of diagnosis to the date of first treatment. We used Statistica 7.0 PL software for all statistical procedures. Differences were considered statistically at $p \leq 0.05$.

Results

Percentage of $CD3^+/CD16^+CD56^+$ cells in CLL patients and healthy controls. The percentage of $CD3^+/CD16^+CD56^+$ cells within $CD3^+$ T lymphocytes was significantly decreased in patients with CLL in comparison to healthy donors (5.30 vs. 7.61%, p=0.01). The frequency of $CD3^+/CD16^+CD56^+$ cells decreased with the stage of disease. The median percentage of $CD3^+/CD16^+CD56^+$ cells in stage 0 was 6.49%, 5.01% in stages 1-2 and 4.41% in stages 3-4 according to Rai classification (Fig. 2). No significant difference was noted between the percentage of $CD3^+/CD16^+CD56^+$ cells in CLL patients and those from healthy donors after adjusting for age and sex.

Relationship between $CD3^+/CD16^+CD56^+$ cells and prognostic factors. We found an inverse correlation between the percentage of $CD3^+/CD16^+CD56^+$ cells and the proportion of $CD19^+/CD5^+$ cells expressing ZAP-70 (R=-0.271; p=0.006). The percentage of $CD3^+/CD16^+CD56^+$ cells was significantly higher in $ZAP\text{-}70^-$ patients compared with $ZAP\text{-}70^+$ ones (5.96 vs. 4.72%, p=0.003) (Fig. 3). Similarly, there was a tendency for higher frequencies of $CD3^+/CD16^+CD56^+$ cells in patients who were CD38 negative (5.51 vs. 4.74%, p=0.005) (Fig. 4).

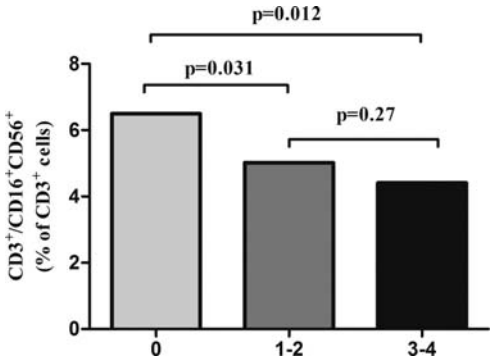

Figure 2. The percentage of $CD3^+/CD16^+CD56^+$ cells decreases with the stage of CLL. The median percentage of $CD3^+/CD16^+CD56^+$ cells was compared between groups of CLL patients at different stages (according to the Rai classification).

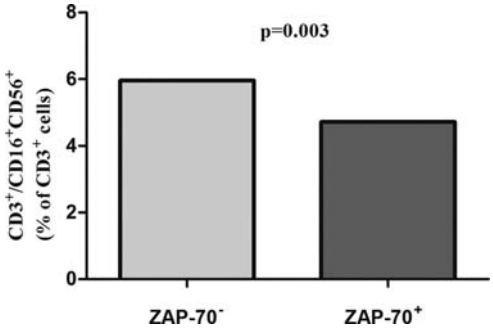

Figure 3. The median percentage of $CD3^+/CD16^+CD56^+$ cells within $CD3^+$ lymphocytes in ZAP-70-positive and ZAP-70-negative patients. The CLL population was considered ZAP-70 positive when at least 20% of $CD19^+/CD5^+$ cells were positive for the ZAP-70, as assessed by flow cytometry.

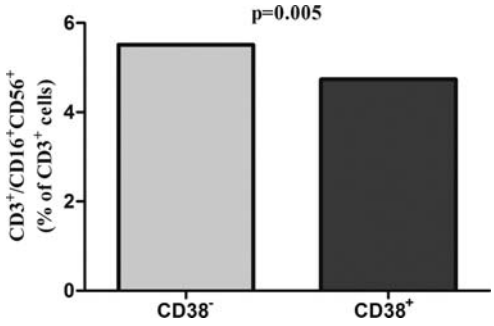

Figure 4. The median percentage of $CD3^+/CD16^+CD56^+$ cells in CD38-positive and CD38-negative patients. The CLL population was considered CD38 positive when at least 20% of $CD19^+/CD5^+$ cells were positive for the CD38, as assessed by flow cytometry.

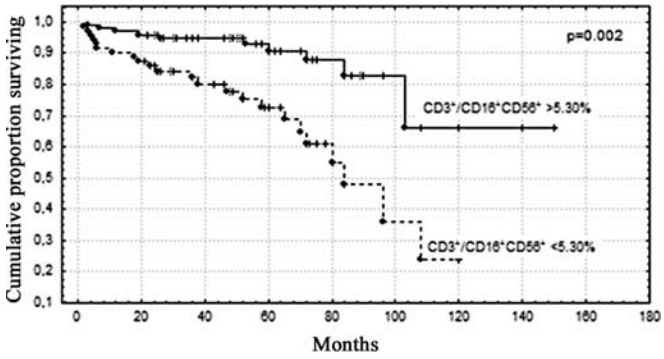

Figure 5. Probability of survival from the date of diagnosis compared among CLL subgroups with less or greater than 5.30% of $CD3^+CD16^+CD56^+$ cells; 5.30% is an equivalent of the median percentage of NK-like T cells analyzed within $CD3^+$ lymphocytes.

In the present study we found a significant positive correlation between LPL and ZAP-70 expression (R=0.261; p=0.037) as well as between LPL and CD38 expression (R=0.227; p=0.032). The mean fluorescence intensity of LPL staining in $CD19^+/CD5^+$ cells was lower in ZAP-70-negative patients (median, 25.65 MFI) comparing to ZAP-70-positive ones (median, 37.49 MFI) (p=0.007). The median LPL/ADAM29 MFI ratio in ZAP-70-positive patients was 1.5 and in ZAP-70-negative patients was 0.7 (p=0.004). We observed an inverse correlation between the percentage of $CD3^+/CD16^+CD56^+$ cells and the LPL/ADAM29 ratio (R=-0.231; p=0.04).

Relationship between $CD3^+/CD16^+CD56^+$ cells and treatment-free survival (TFS) and overall survival. The percentage of $CD3^+/CD16^+CD56^+$ cells was lower in the patients requiring treatment at diagnosis than in patients who did not (5.85 vs. 7.84%, p=0.013). In patients who died after the measurement, the percentage of $CD3^+CD16^+CD56^+$ cells in peripheral blood assessed at diagnosis was significantly lower than in patients who survived (3.39 vs. 6.50%, p=0.029).

We divided patients into two groups using the median percentage of $CD3^+/CD16^+CD56^+$ cells (5.30%) as a cut-off. Fig. 5 displays curves of overall survival of CLL patients

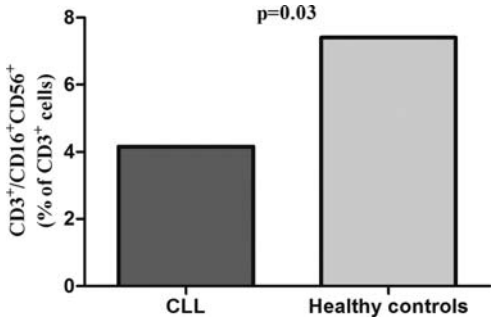

Figure 6. The NK-like T cell median percentage within $CD3^+$ lymphocytes in bone marrow from CLL patients and healthy controls.

depending on the median percentage (5.30%) of $CD3^+/CD16^+CD56^+$ cells. Patients with <5.30% of $CD3^+/CD16^+CD56^+$ cells showed a shorter overall survival (27 months) compared to the patients with >5.30% of $CD3^+/CD16^+CD56^+$ cells (54 months) (p=0.002). Patients with <5.30% of $CD3^+/CD16^+CD56^+$ cells had a shorter TFS (14 months) than those with >5.30% of $CD3^+/CD16^+CD56^+$ cells (43 months) (p=0.001).

Next, the analysis of survival curves in both groups was performed only in early stages (Rai stage 0). However, due

Median percentage of $CD3^+CD56^+$ cells with intracellular TNF, IFN-γ, IL-4 and IL-10 expression in peripheral blood from CLL patients and health controls.

Variable	CLL patients	Healthy controls	P-value
$CD3^+CD56^+TNF^+$	34.13	69.21	0.001[a]
$CD3^+CD56^+IFN\text{-}\gamma^+$	30.26	47.53	0.007[a]
$CD3^+CD56^+IL\text{-}4^+$	4.23	5.38	0.531
$CD3^+CD56^+IL\text{-}10^+$	4.38	1.69	0.01[a]

The U Mann-Whitney test was applied for a statistical comparison of the results between CLL patients and healthy controls. [a]p-value of <0.05 was considered statistically significant.

to the lack of deceased cases, only the assessment of TFS was possible. In the group with <5.30% of $CD3^+/CD16^+CD56^+$ cells, TFS was significantly shorter (19 months) in comparison with the patients with higher percentage of $CD3^+/CD16^+CD56^+$ cells (49 months) (p=0.005).

Among CLL patients treated with chemotherapy, partial or complete remission was achieved in 92 patients. In non-responding patients, the percentage of $CD3^+/CD16^+CD56^+$ cells was significantly lower than in patients in remission (median, 4.81 vs. 5.33%, p=0.023) or when compared with healthy donors (4.81 vs. 7.61%, p=0.002).

Percentage of $CD3^+/CD16^+CD56^+$ cells in bone marrow of CLL patients. We next assessed the NK-like T cells number in BM aspirates from CLL patients. We observed a significantly lower percentage of $CD3^+CD16^+CD56^+$ cells in BM in comparison to PB (4.16 vs. 5.30%, p=0.005). The percentage of $CD3^+CD16^+CD56^+$ cells in BM from CLL patients was significantly lower in comparison to BM from healthy donors (4.16 vs. 7.40%, p=0.03) (Fig. 6).

Intracellular cytokine expression. We examined the production of IFN-γ, TNF, IL-4 and IL-10 by $CD3^+CD56^+$ cells in CLL patients. Following PMA stimulation the majority of NK-like T cells produced IFN-γ or TNF, and only a minority produced IL-4 or IL-10 (Table I). Decreased percentages of $CD3^+CD56^+$ cells with intracellular expression of TNF and IFN-γ in CLL patients in comparison with normal controls were found (p=0.001 and p=0.007, respectively). The percentage of $CD3^+CD56^+$ cells with intracellular expression of IL-10 was significantly higher in CLL patients when compared with healthy volunteers (p=0.01). No statistically significant differences in the proportions of $CD3^+$ $CD56^+$ cells expressing IL-4 between CLL patients and normal controls were found (Table I). Likewise, no significant difference in the percentages of $CD3^+CD56^+$ cells producing these cytokines between PB and BM was detected.

When we divided the patients into ZAP-70-positive and ZAP-70-negative ones, significantly lower proportions of $CD3^+CD56^+$ cells expressing IFN-γ were present in $ZAP\text{-}70^+$ comparing to $ZAP\text{-}70^-$ patients (p=0.001). Likewise, a tendency for higher proportions of $CD3^+CD56^+$ cells expressing TNF in ZAP-70-negative patients when compared to ZAP-70-positive ones was noted (Table II). On the contrary, percentages of $CD3^+CD56^+$ cells expressing IL-10 were significantly lower in $ZAP\text{-}70^-$ patients comparing to $ZAP\text{-}70^+$ ones (p=0.001) (Table II).

Discussion

It is known that patients suffering from hematopoietic malignancies are under immunosuppressive conditions induced by increasing tumor cells and administration of chemotherapy. A previous study showed a reduced number of

Table II. Median percentage of $CD3^+CD56^+$ cells with intracellular TNF, IFN-γ, IL-4 and IL-10 expression in peripheral blood and bone marrow from $ZAP\text{-}70^+$ and $ZAP\text{-}70^-$ patients.

Variable	ZAP-70-positive patients (n=141)	ZAP-70-negative patients (n=159)	P-value
Peripheral blood			
$CD3^+CD56^+TNF^+$	31.95	36.09	0.567
$CD3^+CD56^+IFN\text{-}\gamma^+$	13.08	44.99	0.001[a]
$CD3^+CD56^+IL\text{-}4^+$	3.54	4.82	0.153
$CD3^+CD56^+IL\text{-}10^+$	5.27	3.61	0.02[a]
Bone marrow			
$CD3^+CD56^+TNF^+$	30.94	35.97	0.431
$CD3^+CD56^+IFN\text{-}\gamma^+$	16.09	45.09	0.001[a]
$CD3^+CD56^+IL\text{-}4^+$	2.94	4.83	0.087
$CD3^+CD56^+IL\text{-}10^+$	6.25	3.56	0.001[a]

The CLL population was considered ZAP-70 positive when at least 20% of $CD19^+/CD5^+$ cells were positive for ZAP-70, as assessed by flow cytometry. The p-value was calculated using the U Mann-Whitney test. [a]p-value of <0.05 was considered statistically significant.

peripheral Vα24$^+$ NKT cells in the patients with a variety of hematopoietic malignancies in comparison with that in healthy donors (35). However, there has been few previous descriptions that NKT cells can be qualitatively or quantitatively deficient in CLL patients. Fais *et al* (11) suggested that number of NKT cells in CLL patients is low. Guven *et al* (10) reported the possibility of expanding of CD3$^+$CD56$^+$ cell population, from peripheral blood mononuclear cells of CLL patients. The expansion rates were lower for the patients with progressive disease (10). The present study demonstrated the reduction of the percentage of CD3$^+$/CD16$^+$CD56$^+$ cells in CLL patients. In our study, we determined a lower percentage of CD3$^+$/CD16$^+$CD56$^+$ cells within CD3$^+$ T lymphocytes in CLL patients in comparison to healthy donors. A decreased frequency of these cells may represent an important mechanism responsible for disease progression. It is noteworthy that CLL patients with unfavorable prognosis showed lower percentages of CD3$^+$/CD16$^+$CD56$^+$ cells. We have found, that a smaller size of CD3$^+$/CD16$^+$CD56$^+$ cell population was associated with more advanced Rai stage at diagnosis as well as ZAP-70 expression. Additionally, the percentage of this cell population expressed as a percentage of the CD3$^+$ lymphocyte compartment was higher in CD38-negative patients compared with CD38-positive patients. Moreover, we found an inverse correlation between the percentage of CD3$^+$/CD16$^+$CD56$^+$ cells and the LPL/ADAM29 ratio. Recent reports indicated that a combination of the LPL/ADAM29 ratio with ZAP-70 expression can be used as a surrogate marker for IgV_H mutational status (27,28).

These results suggest a potential role of CD3$^+$/CD16$^+$CD56$^+$ cells as a factor determining disease activity that has to be evaluated in further studies. However, in the present study it can be supported by the strong correlation between the percentage of CD3$^+$/CD16$^+$CD56$^+$ cells and treatment free survival that suggests a potential use of this parameter as a predictor of TFS. Differences in TFS connected with the percentage of CD3$^+$/CD16$^+$CD56$^+$ cells were recorded both in all patients group as well as in the patients in early stage of CLL. Furthermore, the crucial correlation between the frequencies of CD3$^+$/CD16$^+$CD56$^+$ cells was linked with overall survival time. We found a clear tendency towards shorter survival times in CLL patient with decreased percentage of CD3$^+$/CD16$^+$CD56$^+$ cells. Moreover, the percentage of CD3$^+$/CD16$^+$CD56$^+$ cells was significantly decreased in patients who showed progression of disease. This study suggests that CD3$^+$/CD16$^+$CD56$^+$ cells may be helpful in determining a worsening of clinical course.

As bone marrow NKR$^+$ T cells have been postulated to play a role in tumor surveillance (36), next we assessed the CD3$^+$/CD16$^+$CD56$^+$ number in BM aspirates. Bone marrow examination is not generally essential for the diagnosis of CLL, it is important for determination of the course of cytopenias and evaluation of the response to therapy (37). CLL bone marrow is invariably infiltrated with leukemic cells, and the extent of infiltration correlates with the clinical stage and prognosis (37-39). It can support a diagnosis of CLL showing atypical morphology and exclusively allows the assessment of the infiltration type (diffuse vs. non-diffuse) (37). In addition to hematopoietic progenitor cells, bone marrow contains phenotypically and functionally mature T and NK cells (36). The presence of such cells may be important in fighting malignancy, as both cell types are known to play a role in the attack on cancerous cells (36). Recent findings suggest that NKT cells facilitate the conversion of immunosuppressive myeloid-derived suppressor cells (MDSCs) into immunogenic antigen-presenting cells (APCs), eliciting successful antitumor immunity (40). In our study, percentages of CD3$^+$/CD16$^+$CD56$^+$ cells analyzed within CD3$^+$ lymphocytes were significantly lower in bone marrow than in peripheral blood. Decreased number of bone marrow CD3$^+$/CD16$^+$CD56$^+$ cells in CLL patients may reflect an immune escape mechanism that contributes to the progression of disease.

Not only NKT-like cell number might be important in determining the clinical outcome of CLL patients, but also their cytokine expression pattern. In addition to aberrant frequencies, CD56$^+$ T cells from CLL patients also appear dysfunctional in terms of cytokine expression. Following PMA stimulation we found decreased percentage of CD3$^+$CD56$^+$ cells with intracellular IFN-γ and TNF expressions in CLL patients compared to healthy controls. Changes in the cytokine network could sustain the expansion of the leukemic B-cell clone. The explanation of abnormalities in the circulating non-malignant immune cells of CLL patients offers important insights into the biology of the disease. In the current study, ZAP-70-positive patients showed lower percentages of CD3$^+$CD56$^+$ cells with IFN-γ or TNF expression. These cytokines are necessary for potentiation of the cytotoxic activity of T cells and NK cells (41), and decreased synthesis of these cytokines is the potential reason of impaired cytotoxic function. Furthermore, both IFN-γ and TNF possess direct cytotoxic and cytostatic activity toward tumor cells and are involved in anticancer response (42,43). A reduced number of CD3$^+$CD56$^+$ cells expressing intracellular IFN-γ or TNF might represent a risk factor for the progression of disease. Different data exist on IFN-γ production by NKT cells. Some investigators showed that invariant NKT cells in cancer patients are functionally impaired and produce less IFN-γ (44) while others established that iNKT cells are functionally normal (45) and have a preserved production of IFN-γ (46). We also examined IL-4 and IL-10 expression by CLL CD3$^+$CD56$^+$ cells. In the patients population the percentage of CD3$^+$ CD56$^+$ cells with intracellular IL-10 expression was higher than in healthy individuals. The combination of IL-4 and IL-10 is able to suppress the generation of IFN-γ producting cells (47). These result suggests that tracking the frequency and function of NKT-like cells could contribute to the understanding of disease pathogenesis. Because CD56$^+$ T cells can produce Th1-type and Th2-type cytokines (18) together with NK cell activities, downregulation in this cell population can deteriorate various immune signaling cascades in CLL patients. The functional and proportional changes in CD56$^+$ T cells may be influenced by CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs). Recently, enhanced frequencies of Tregs in CLL patients when compared to healthy volunteers, have been detected. The progressive increase of Tregs was noted in advanced stage of disease (48). This special type of cells is involved in functional downregulation of T, NK or NKT

gs can suppress the proliferation, cytokine release oxic activity of NKT cells (49). In our study we did not analyze regulatory T cells with intracellular component stained for Foxp3. However, we found an inverse correlation between percentage of $CD3^+/CD16^+CD56^+$ T cells population and $CD4^+CD25^{high}$ regulatory T cells (data not shown).

$CD3^+/CD56^+CD16^+$ cell population is heterogeneous phenotypically and functionally, so it is not possible to draw direct conclusions with clinical behavior without more detailed phenotyping. However, these results suggest that monitoring of $CD3^+/CD16^+CD56^+$ cells number (which can be assessed easily in clinical routine practice as a part of diagnostic flow cytometry analysis) and function may provide useful information for determining disease activity/progression. For the stage 0 CLL patient group evaluation of the immunological control and dysfunction probably is of special importance.

Acknowledgements

We would like to thank Ms. Agata Surdacka for her excellent technical support in this study. This study was supported by State Committee for Scientific Research (Poland) (grant numbers N N402082834 and N N402084234).

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