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 informative to look at these cells in patients with stage 0 CLL. For this patient group immunological control and dysfunction are probably important factors.

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, IgVH 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. IgVH 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 anemia, 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 CD3⁺ 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 cutoff 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
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+/CD16+/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≤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-70+ patients compared with ZAP-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).
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) compared 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 depending on the median percentage (5.30%) of CD3+/CD16+CD56+ cells. Patients with <5.30% of CD3+/CD16+CD56+ cells showed a longer 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
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-70+ comparing to ZAP-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-70- patients comparing to ZAP-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 I. Median percentage of CD3+/CD56+ cells with intracellular TNF, IFN-γ, IL-4 and IL-10 expression in peripheral blood from CLL patients and healthy controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CLL patients</th>
<th>Healthy controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+CD56-TNF⁺</td>
<td>34.13</td>
<td>69.21</td>
<td>0.001¹</td>
</tr>
<tr>
<td>CD3+CD56-IFN-γ⁺</td>
<td>30.26</td>
<td>47.53</td>
<td>0.007¹</td>
</tr>
<tr>
<td>CD3+CD56-IL-4⁺</td>
<td>4.23</td>
<td>5.38</td>
<td>0.531</td>
</tr>
<tr>
<td>CD3+CD56-IL-10⁺</td>
<td>4.38</td>
<td>1.69</td>
<td>0.01¹</td>
</tr>
</tbody>
</table>

The U Mann-Whitney test was applied for a statistical comparison of the results between CLL patients and healthy controls. ¹p-value of <0.05 was considered statistically significant.

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-70+ and ZAP-70- patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>ZAP-70-positive patients (n=141)</th>
<th>ZAP-70-negative patients (n=159)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+CD56-TNF⁺</td>
<td>31.95</td>
<td>36.09</td>
<td>0.567</td>
</tr>
<tr>
<td>CD3+CD56-IFN-γ⁺</td>
<td>13.08</td>
<td>44.99</td>
<td>0.001²</td>
</tr>
<tr>
<td>CD3+CD56-IL-4⁺</td>
<td>3.54</td>
<td>4.82</td>
<td>0.153</td>
</tr>
<tr>
<td>CD3+CD56-IL-10⁺</td>
<td>5.27</td>
<td>3.61</td>
<td>0.02²</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+CD56-TNF⁺</td>
<td>30.94</td>
<td>35.97</td>
<td>0.431</td>
</tr>
<tr>
<td>CD3+CD56-IFN-γ⁺</td>
<td>16.09</td>
<td>45.09</td>
<td>0.001²</td>
</tr>
<tr>
<td>CD3+CD56-IL-4⁺</td>
<td>2.94</td>
<td>4.83</td>
<td>0.087</td>
</tr>
<tr>
<td>CD3+CD56-IL-10⁺</td>
<td>6.25</td>
<td>3.56</td>
<td>0.001²</td>
</tr>
</tbody>
</table>

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. ²p-value of <0.05 was considered statistically significant.
CD3+/CD16+CD56+ number in BM aspirates. Bone marrow play a role in tumor surveillance (36), next we assessed the of CD3+/CD16+CD56+ cells. Moreover, the percentage of survival times in CLL patient with decreased percentage all survival time. We found a clear tendency towards shorter determination of a worsening of clinical course. 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 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 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 producing 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...
Acknowledgements

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References


3. Damle RN, Wasil T, Fais F, Ghisio F, Valetto A, Allen SL, Buchbinder A, Budman D, Dittmar K, Kolitz J, Lichtman SM, Schubert P, Vinecigueria VP, Rai KR, Ferrarini M and Chiorazzi N: Ig V gene mutation status and CD38 expression 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.


