iNKT cell percentage is decreased in patients with chronic lymphocytic leukemia and correlates inversely with the clinical stage and negative prognostic factors

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Abstract

Invariant NKT (iNKT) cells are CD1d-restricted T cells which express invariant T-cell receptor (TCR) incorporating Vα24 chain paired with Vβ11 chain and NK cell markers. Human Vα24$^+$NKT cells play crucial roles in various immune responses including autoimmune and antitumor responses. Little is known however about the role of iNKT in patients with CLL. We investigated iNKT cells numbers in peripheral blood of 60 untreated patients with CLL and 20 healthy individuals matched for age. Analysis of Vα24 surface expression on CD3$^+$ T cells revealed significantly lower median percentage of iNKT cells in peripheral blood of CLL patients than in healthy donors. Moreover iNKT percentages decrease along with disease progression, and adversely correlate with negative prognostic factors. These results suggest an important role of iNKT cells in the development and progression of CLL, as well as their potential prognostic significance.

Key words: *chronic lymphocytic leukemia, invariant NKT cells, ZAP-70, CD38.*

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Introduction

Chronic lymphocytic leukemia (CLL), which is the most common type of leukemia in the Western world is characterized by the proliferation and accumulation of monoclonal CD19$^+$CD5$^+$ B cells in the blood, bone marrow and peripheral lymphoid organs. The etiology of CLL remains unknown. The development of disease is not dependent on environmental factors, because no connection was found between the occurrence of CLL and exposure to pesticides, ionizing radiation or electromagnetic or other known carcinogens.

Immune system disorders are one of the characteristics in the clinical course of chronic lymphocytic leukemia. It is believed that they not only play an important role in the development of the disease, but are also responsible for phenomena such as increased susceptibility to infections, autoimmune complications, and incidence of secondary malignancies [1]. Abnormalities in the immune system in patients with CLL are complex and affect both humoral immunity (mainly in the form of hypogammaglobulinemia and reduced production of antibodies in response to antigens), as well as cellular immunity involving granulocytes, monocytes, dendritic cells, T cells and NK cells [2]. It was recently demonstrated that in addition to conventional T and NK cells, an important role in tumor immunity, plays a third type – natural killer T (NKT) cells [3].

Natural killer T (NKT) cells are unique subpopulation of T cells that share the properties of NK cells and T lymphocytes [4]. Unlike conventional CD4$^+$ and CD8$^+$ T

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cells that recognize peptide antigens, NKT cells respond to glycolipid antigens in the context of MHC class I-like antigen presenting molecule CD1d [5, 6]. Natural killer T cells are heterogeneous population composed of different subsets. A large majority of NKT cells express an invariant T cell receptor (TCR), Vα14 in mice and Vα24 in humans, and they are termed as invariant NKT (iNKT) or type I NKT [7]. A second type of NKT cells, so-called type II NKT with a more diverse TCR repertoire was also identified both in mice and humans [8]. iNKT cells are the most studied and best characterized NKT cell population. α-galactosyloceramide (α-GalCer) a potent agonist for iNKT cells, originally derived from marine sponge, has been used as a synthetic glycolipid ligand, both in research on their properties as well as in iNKT-targeted immunotherapy [9]. iNKT activation by α-GalCer results in rapid production of large amounts of both Th1 (IFN-γ) and Th2 (IL-4) cytokines [10]. Natural killer T cells may serve different functions in the immune system, on the one hand, by secretion of many cytokines and chemokines, on the other hand by direct cytotoxic effects [11]. Through the influence of other immune cells they may enhance or inhibit the immune response [12]. This variability of function results from the diversity of NKT cell subpopulations and the variable profile of the released cytokines depending on the type of antigen that stimulates TCR. Natural killer T cells are involved in protection against a number of microorganisms, and play a dual role in autoimmune diseases, controlling some of them, while exacerbating others [4]. During last years there has been significant progress in understanding the biology of glycolipid antigen presentation and the possible role of NKT cells in tumor immunity [13].

The aim of the presented study was to assess the potential role of iNKT cells in the development and progression of CLL by the analysis of their percentages in peripheral blood of CLL patients in relation to clinical and laboratory parameters of disease activity and prognostic factors.

Material and methods

Patients and samples

Peripheral blood (PB) samples were obtained from 60 untreated patients diagnosed with CLL between 2002 and 2008 (29 men and 31 women). The median age of patients was 66 years (ranging from 48 to 85 years). CLL diagnosis was based on a clinical examination, morphological and immunological criteria [14]. At the time of diagnosis, patients were staged according to the Rai staging system [15] as follows: stage 0 (19 patients), stage 1 (10 patients), stage 2 (14 patients), stage 3 (9 patients) and stage 4 (8 patients). All blood samples were taken after the diagnosis of CLL and before the start of any anti-cancer therapy. The control group consisted of 20 healthy volunteers (8 men and 12 women aged from 26 to 65 years, median age of 57 years). PB samples were collected into heparinized tubes and immediately processed. The study was approved by the Local Ethical Committee.

Assessment of iNKT cells

Flow cytometry analysis of iNKT cells was performed on fresh PB samples stained with anti-iNKT FITC (*anti-Vα24* FITC) (BD Pharmingen) and anti-CD3 PE (BD Pharmingen). During analysis, $iNKT^+/CD3^+$ population was determined. A standard, whole-blood assay with erythrocyte cell lysis was used for preparing the PB specimens. The samples were analyzed by flow cytometry directly after preparation. For data acquisition and analysis, a FACSCalibur instrument (BD) with CellQuest software (BD) was used. Negative controls were always used by omitting MoAbs as well as by incubating the cells with mouse Ig of the same isotype as MoAb conjugated with FITC or PE (BD Pharmingen). The percentage of positive cells was measured from a cut-off set using isotype matched nonspecific control antibody. iNKT cells were analyzed within gated $CD3^+$ T lymphocytes. Dot plots, illustrating the analysis metod for the identification of $iNKT^+/CD3^+$ cells are show in Figure 1.

Statistical analysis

Differences between two groups were assessed using the U Mann-Whitney test, comparisons among three or more groups were done with the Kruskal-Wallis test. The Spearman rank correlation coefficient was used in correlation tests. Statistica 7.0 PL software was used for all statistical procedures. Differences were considered statistically with p-value ≤ 0.05.

Results

The results of the study showed that the median percentage of iNKT cells within $CD3^+$ cells was significantly lower in peripheral blood of patients with CLL (0.57%) than in healthy subjects from control group (0.80%; $p = 0.039$). There was also a significant decrease in median iNKT cell numbers in CLL patients in stages 3-4 according to Rai (0.37%) as compared to patients in stage 0 (1.35%; $p = 0.042$; Kruskal-Wallis test) (Figure 2).

Next, we analyzed the relationship between the percentage of iNKT cells within $CD3^+$ lymphocytes and selected laboratory parameters associated with disease stage and activity, including: the number of peripheral blood leukocytes and lymphocytes, hemoglobin concentration, platelet count, the concentration of β_2-microglobulin, lactate dehydrogenase, immunoglobulins: IgG, IgA, IgM. We found significant inverse correlations between the percentage of iNKT cells and the count of peripheral blood WBC ($R = –0.378$, $p = 0.035$) lymphocytes ($R = –0.471$,

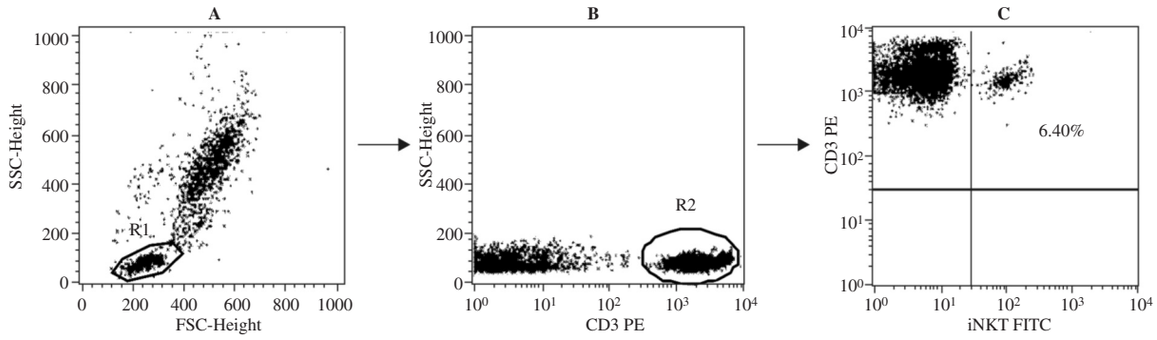

Fig. 1. The dot plots show representative data illustrating the analysis method for identification of iNKT cells among CD3+ T lymphocytes. **A** – the dot plot shows the forward scatter/side scatter (FSC/SSC) distribution and the gate (region R1) used to select lymphocytes for analysis. **B** – the R1 gated events were then analyzed for CD3 PE staining, and positive cells (CD3+) were gated (region R2). **C** – the final dot plot CD3 PE vs. iNKT FITC was established by combined gating of events using R1 and R2. The number in the upper right quadrant in the dot plot represents the percentage of iNKT+/CD3+ cells (6.40%)

p = 0.046) and concentration of β_2-microglobulin (R = –0.479; p = 0.041). Then we assessed the relationship between the percentage of iNKT cells in peripheral blood of CLL patients and the expression of ZAP-70 protein in leukemic cells and expression of CD38 antigen on their surface. The percentage of iNKT cells within CD3+ lymphocytes was significantly higher in ZAP-70-negative patients (0.73%) than in ZAP-70-positive ones (0.35%; p = 0.045) (U Mann Whitney test; Figure 3). There was also an inverse correlation between iNKT cell percentage and the number of CD19+/CD5+ lymphocytes expressing ZAP-70 (p = 0.008; Spearman rank correlation; Figure 4). The representative flow cytometric dot plots illustrating the percentage of iNKT cells within CD3+ lymphocytes in ZAP-70+ and ZAP-70– patients with CLL are shown on Figure 5.

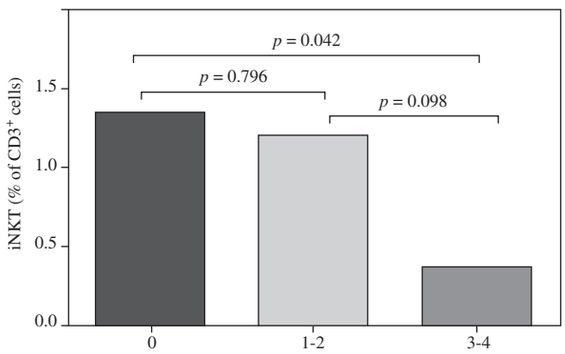

Fig. 2. Comparison of iNKT cell percentages in patients with CLL in Rai stages: 0, 1-2, 3-4

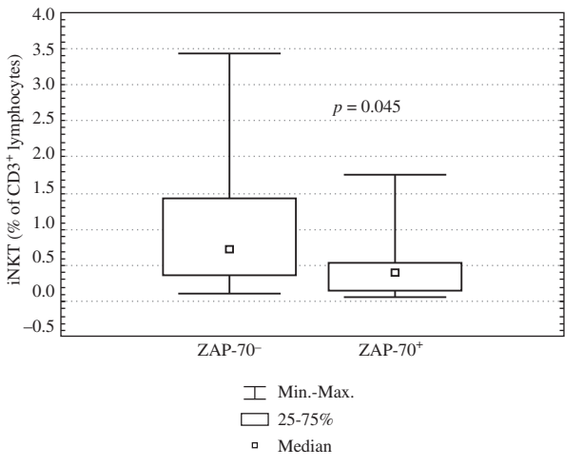

Fig. 3. Percentage of iNKT cells within CD3+ lymphocytes in ZAP-70– and ZAP-70+ patients with CLL

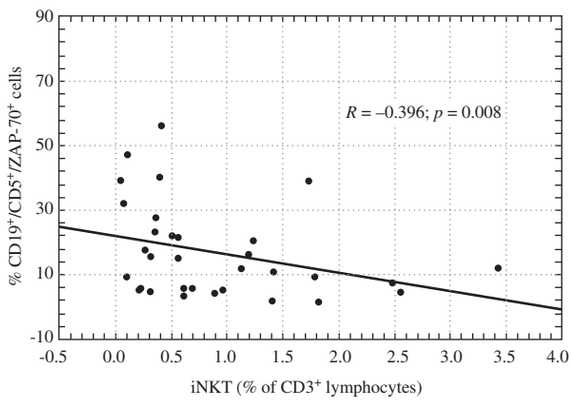

Fig. 4. Correlation between the percentage of iNKT cells within CD3+ lymphocytes and CD19+/CD5+ B cells expressing ZAP-70

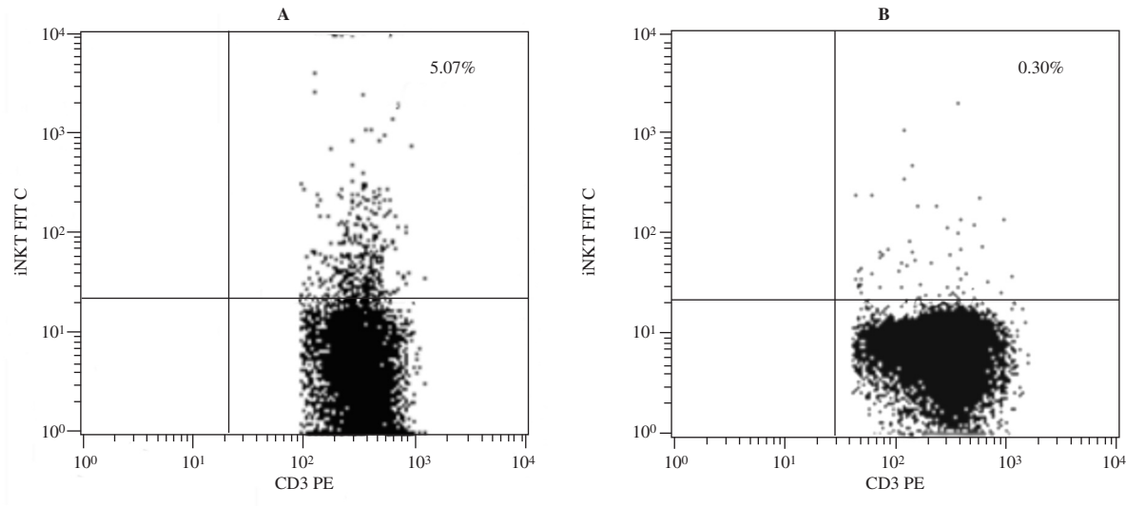

Fig. 5. Flow cytometric dot plots illustrating the percentage of iNKT cells within CD3$^+$ lymphocytes in ZAP-70$^+$ (A) and ZAP-70$^-$ patients with CLL (B)

Analysis of the iNKT cell numbers in CLL patients depending on the expression of CD38 antigen on leukemic cells, showed that the percentage was significantly higher in CD38-negative patients (0.70%) comparing to CD38-positive ones (0.32%, $p = 0.008$; U Mann Whitney test; Figure 6). There was also an inverse correlation between iNKT percentage and the number of CD19$^+$/CD5$^+$ expressing CD38 ($p = 0.008$; Spearman rank correlation; Figure 7).

Discussion

Recent studies revealed that iNKT cells mediate direct cytotoxicity and exert adjuvant effects on anti-tumor immunity by activating other cytotoxic lymphocytes through Th1 cytokines cascade [11]. These data relate however mainly to solid tumors [3, 16, 17], whereas the issue of iNKT cells in CLL remains unexplored. In this study, we found that the percentage of iNKT cells within CD3$^+$ lymphocytes in peripheral blood of CLL patients was

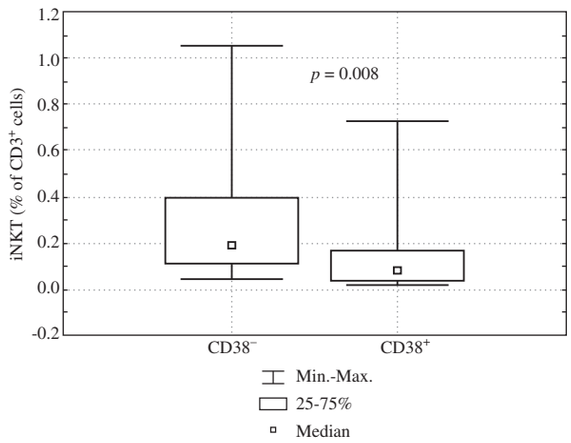

Fig. 6. Percentage of iNKT cells within CD3$^+$ lymphocytes in CD38$^-$ and CD38$^+$ patients with CLL

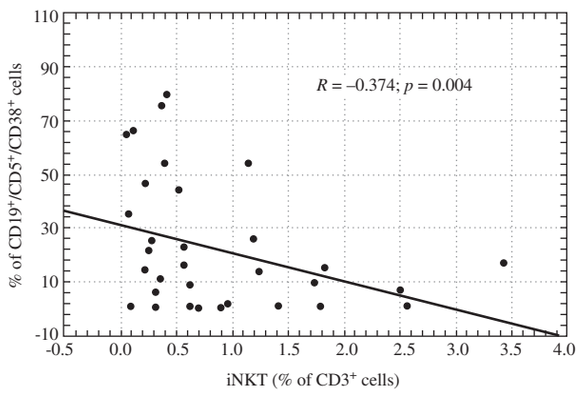

Fig. 7. Correlation between the percentage of iNKT cells within CD3$^+$ lymphocytes and CD19$^+$/CD5$^+$ B cells expressing CD38

significantly lower comparing to healthy donors. In the available literature there is no data on the evaluation of iNKT cells in patients with CLL. In the one published study, Fais *et al*., evaluated the expression of CD1a antigen on leukemic cells, and noted a decreased percentage of iNKT cells (< 0.1%) in patients with CLL, but the number of six patients was too small to obtain significant statistical data [18]. In patients with different types of solid tumors (colorectal, breast, kidney and prostate cancer, melanoma, head and neck cancer), both the reduction of absolute number of circulating iNKT cells and their percentage among T cells has been shown [3, 19, 20]. Observations from the studies in solid tumors suggest that the reduction in iNKT cells in peripheral blood of cancer patients may result from their abnormal proliferation, death or the accumulation in tumor tissue. In patients with multiple myeloma and with prostate cancer impaired iNKT cell function was observed [17, 21] caused probably by the influence of tumor cells. Normal function of iNKT cells could be restored *ex vivo*, after incubation with antigen presenting cells (APC) stimulated with α-GalCer, suggesting that the observed defects are at least partially reversible [16, 21].

Next, we have shown that the percentage of iNKT cells is significantly decreased in patients with advanced clinical stages compared to early ones, suggesting their participation in the processes of CLL progression. There is no published data on the relationship between the number of iNKT cells and the stage of CLL and the results in patients with solid tumors seem to be divergent. The first published studies that demonstrated a reduction in the number of iNKT cells in patients with melanoma [16] and prostate cancer [17], included only patients in advanced clinical stages. Further studies on various types of solid tumors did not show any relationship between tumor stage and the number of iNKT cells [3, 22, 23]. The study of Molling *et al*., covering a group of 120 patients with colorectal cancer, breast cancer, renal cancer, melanoma and head and neck cancer showed that the number of circulating iNKT cells was less than half comparing to healthy subjects. These results were independent of the type of cancer or the tumor mass and have not improved after achieving remission of the disease [3]. The authors suggest that the reduction in iNKT cells is not dependent on the clinical stage, but is rather as a risk factor for cancer development. Analyzing the results of the present study in comparison to the studies on solid tumors, one should remember that in patients with leukemia, different results can be expected. Cancer cells are present in the peripheral blood and can accumulate iNKT cells around them, however they can have also an adverse effect on inhibiting the function and proliferation of iNKT cells.

Further analysis showed a significant inverse correlation between the percentage of iNKT cells and leukocytosis and lymphocytosis which indicates an adverse effect of the tumor mass on iNKT cell numbers. In addition, peripheral blood lymphocytosis is an independent poor prognosis factor in CLL. Among the serum markers assessed, negative correlation was observed between β_2-microglobulin level and the percentage of iNKT cells. β_2-M has an independent prognostic value in patients with CLL [28]. Serum levels of this protein correlate with clinical stage of CLL, the degree of bone marrow infiltration by leukemic cells and tumor burden [29].

Recently, new prognostic factors were determined in CLL including mutational status of immunoglobulin heavy chain variable region (IgVH) genes, cytogenetic abnormalities, expression of ZAP-70 protein in leukemic cells and expression of CD38 antigen on their surface [24-27]. In the present study, the percentage of iNKT cells was significantly lower in patients ZAP-70^+ as compared to ZAP-70^- and in $CD38^+$ patients when compared with $CD38^-$, which indicates that a smaller percentage of iNKT cells at CLL diagnosis is associated with a more aggressive clinical course of disease. Letestu and colleagues presented data on the evaluation of conventional and new prognostic factors in 339 CLL patients in Binet stage A and found that only four parameters had independent prognostic value, including thymidine kinase, β_2-M, lymphocytosis and expression of CD38 antigen [28]. In our study, the percentage of iNKT cell correlated with lymphocytosis, serum β_2-M and the percentage of leukemic cells expressing CD38, which indicates their prognostic significance. Lower percentage of iNKT cells in patients with a higher percentage of CLL cells expressing CD38 or ZAP-70 appears to confirm the relationship between a more aggressive form of the disease and a greater degree of the innate immune system impairment.

In conclusion, obtained results suggest an important role in iNKT cells in the development and progression of chronic lymphocytic leukemia. Their significance highlights the fact that in the available literature one can find no data regarding the evaluation of iNKT cells in CLL patients. Presented preliminary results indicate the need for further studies involving larger groups of patients, supplemented by an assessment of iNKT cell function that are important not only because of their possible use as a prognostic factor, but primarily in the immunotherapy of chronic lymphocytic leukemia.

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