

# CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cell numbers in peripheral blood are correlated with higher tumor burden in patients with diffuse large B-cell lymphoma

Iwona Hus<sup>1\*</sup>, Elżbieta Starosławska<sup>2\*</sup>, Agnieszka Bojarska-Junak<sup>3</sup>,  
Aneta Dobrzyńska-Rutkowska<sup>2</sup>, Agata Surdacka<sup>3</sup>, Paulina Wdowiak<sup>3</sup>,  
Magdalena Wasiaak<sup>3</sup>, Maria Kusz<sup>1</sup>, Anna Twardosz<sup>2</sup>, Anna Dmoszyńska<sup>1</sup>,  
Jacek Roliński<sup>3</sup>

<sup>1</sup>Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, Poland

<sup>2</sup>I Department of Chemotherapy and Radiotherapy, Regional Oncology Center in Lublin, Lublin, Poland

<sup>3</sup>Department of Clinical Immunology, Medical University of Lublin, Poland

\*Equal contribution of both authors

**Abstract:** Diffuse large B-cell lymphoma is the commonest histological type of malignant lymphoma, and remains incurable in many cases. Developing more efficient immunotherapy strategies will require better understanding of the disorders of immune responses in cancer patients. NKT (natural killer-like T) cells were originally described as a unique population of T cells with the co-expression of NK cell markers. Apart from their role in protecting against microbial pathogens and controlling autoimmune diseases, NKT cells have been recently revealed as one of the key players in the immune responses against tumors. The objective of this study was to evaluate the frequency of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells in the peripheral blood of 28 diffuse large B-cell lymphoma (DLBCL) patients in correlation with clinical and laboratory parameters. Median percentages of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> were significantly lower in patients with DLBCL compared to healthy donors (7.37% vs. 9.01%,  $p = 0.01$ ; 4.60% vs. 5.81%,  $p = 0.03$ ), although there were no differences in absolute counts. The frequency and the absolute numbers of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells were lower in advanced clinical stages than in earlier ones. The median percentage of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells in patients in Ann Arbor stages 1–2 was 5.55% vs. 3.15% in stages 3–4 ( $p = 0.02$ ), with median absolute counts respectively 0.26 G/L vs. 0.41 G/L ( $p = 0.02$ ). The percentage and absolute numbers of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells were significantly higher in DLBCL patients without B-symptoms compared to the patients with B-symptoms, (5.51% vs. 2.46%,  $p = 0.04$ ; 0.21 G/L vs. 0.44 G/L,  $p = 0.04$ ). The percentage of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells correlated adversely with serum lactate dehydrogenase ( $R = -0.445$ ;  $p < 0.05$ ) which might influence NKT count. These figures suggest a relationship between higher tumor burden and more aggressive disease and decreased NKT numbers. But it remains to be explained whether low NKT cell counts in the peripheral blood of patients with DLBCL are the result of their suppression by the tumor cells, or their migration to affected lymph nodes or organs. (*Folia Histochemica et Cytobiologica* 2011; Vol. 49, No. 1, pp. 183–187)

**Key words:** diffuse large B-cell lymphoma, anti-tumor immunity, CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells

**Correspondence address:** I. Hus, Department of Hematooncology, Medical University of Lublin, Staszica Str. 11, 20–081 Lublin, Poland; tel.: (+ 48 81) 534 54 68; fax: (+ 48 81) 534 56 05; e-mail: iwonach.hus@gmail.com

## Introduction

Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of malignancies derived from lymphoid system cells. They follow different clinical courses and prog-

noses depending on the type of malignant cells, the degree of their differentiation, and their location in lymphoid structures. B-cell lymphoma comprises about 86% of all lymphomas, with diffuse large B-cell lymphoma (DLBCL) being the commonest histological type. Immunochemotherapy with monoclonal antibodies is nowadays the most effective method of NHL therapy [1, 2], although in many cases the disease remains incurable. The key to developing new, more efficient immunotherapy strategies is better understanding of the disorders of immune responses in cancer patients. In malignant lymphomas, interactions between tumor cells and immune cells are different and much more complex than in solid tumors, since malignant cells themselves belong to the immune system. In recent years, new populations of T helper cells have been described, and their role in tumor immunity is still only partly understood. NKT cells constitute a unique population of T cells with the co-expression of NK cell markers such as NK1.1. (NKR1P1C; CD161) and Ly49 [3–5]. In contrast to CD4 and CD8 T cells, NKT cells recognize lipid antigens in the context of the major histocompatibility complex (MHC) class I-like antigen presenting molecule CD1d [6, 7]. Upon TCR activation, they rapidly produce large amounts of both pro- and anti-inflammatory cytokines, such as: IL-2, IFN- $\gamma$ , TNF $\alpha$  and IL-4 [8]. NKT cells are divided into two populations, invariant NKT cells (iNKT, type I NKT) which express invariant TCR using V $\alpha$ 14-J $\alpha$ 281  $\alpha$ -chain in mice and V $\alpha$ 24-J $\alpha$ Q in humans, and variant NKT (type II NKT) with a more diverse TCR repertoire. NKT cells have been shown to play a role in numerous immune responses, including protection against microbial pathogens [3, 9] and control of autoimmune diseases [3]. Moreover, iNKT cells have been recently revealed to be one of the key players in immune responses against tumors. CD3<sup>+</sup>/CD16<sup>+</sup>/CD56<sup>+</sup> T cells are not classical invariant NKT cells, but are a broader group of T cells matching the original definition of NKT cells [4, 5]. Since there is only limited data on the role of NKT-like cells in malignant lymphomas, the objective of this study was to evaluate the frequency of NKT-like cells in the peripheral blood of DLBCL patients in correlation with clinical and laboratory parameters.

## Material and methods

### Patients and samples

Peripheral blood (PB) samples were collected from 28 patients diagnosed with DLBCL between August 2008 and February 2010 (13 men and 15 women). The samples were taken at diagnosis, before the start

of any anti-cancer therapy. The median age of patients was 64 years (range 36 to 80 years). At the time of diagnosis, patients were assessed according to the Ann Arbor classification as follows: stage 1 — three patients, stage 2 — six patients, stage 3 — seven patients, stage 4 — 12 patients. B-symptoms were present in 12 patients. Control samples of PB were obtained from 20 healthy volunteers (8 men and 12 women aged from 33 to 66 years, median age 58 years). PB samples were collected into heparinized tubes and immediately processed. The study was approved by the Local Ethical Committee. Peripheral blood samples were obtained from the patients and healthy volunteers after informed consent.

### Assessment of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells

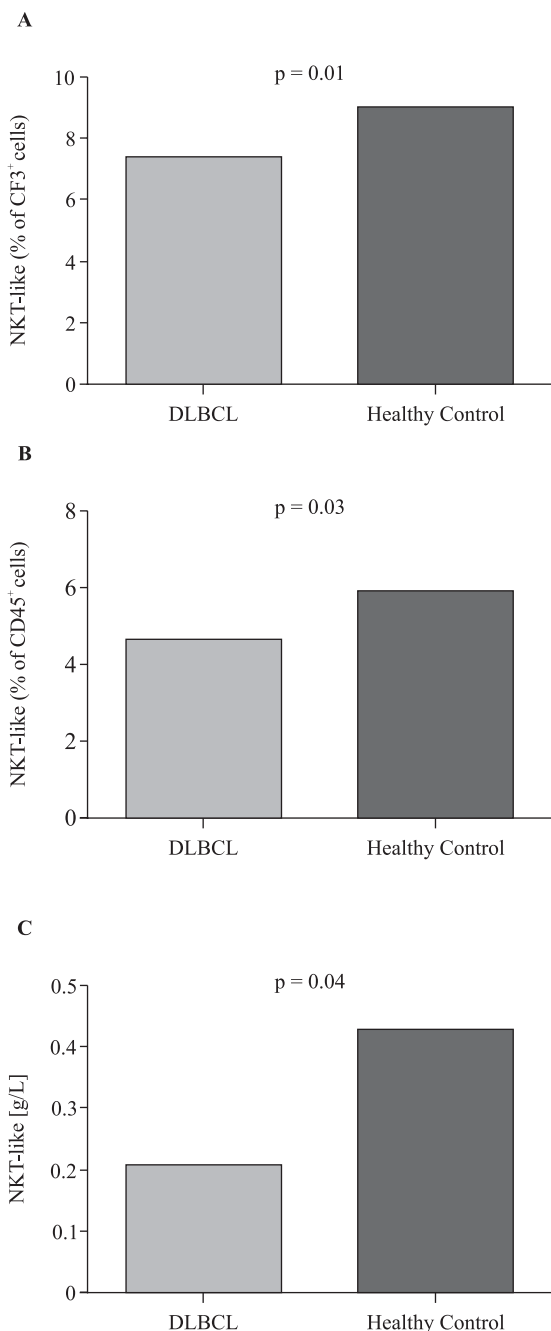
Percentages of NKT-like cells were evaluated with flow cytometry using monoclonal antibodies (MoAbs) anti-CD3 FITC/CD16<sup>+</sup>CD56 PE/CD45 PerCP (BD Biosciences), which allowed for simultaneous assessment of T (CD3<sup>+</sup>) lymphocytes and NK (CD16<sup>+</sup>CD56<sup>+</sup>) cells. During analysis, the CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> population was determined. A standard, whole-blood assay with erythrocyte cell lysis was used to prepare the PB samples. 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. 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 nonspecific control antibody. CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> T cells were analyzed within gated CD45<sup>+</sup> cells as well as CD3<sup>+</sup> T lymphocytes.

### Statistical analysis

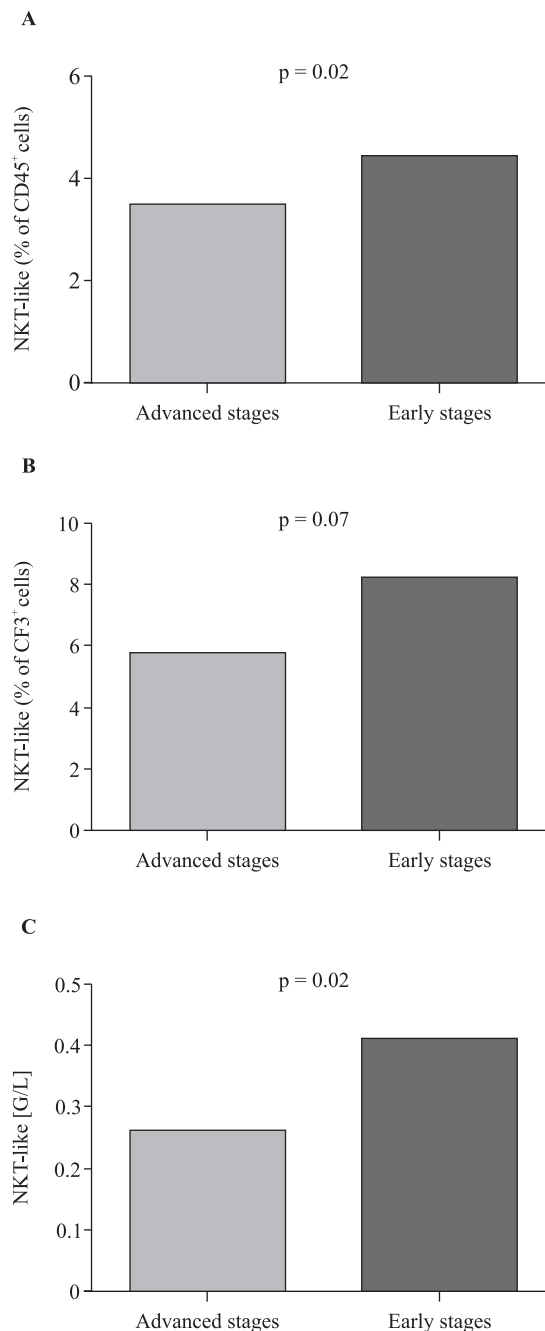
The statistical significance of the observed differences was deduced by means of the Mann–Whitney U test. Spearman rank test was used to assess correlation between the variables. We used Statistica 7.0 PL software for all statistical procedures. Differences were considered as statistically significant when the *p* value was  $\leq 0.05$ .

## Results

Median percentages of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells within CD3<sup>+</sup> T lymphocytes and within CD45<sup>+</sup> cells were significantly lower in patients with DLBCL compared to healthy donors (7.37% vs. 9.01%, *p* = 0.01; 4.60% vs. 5.81%, *p* = 0.03). Median absolute count of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells was also lower in DL-BCL patients compared to the healthy controls (0.2 G/L vs. 0.43 G/L; *p* = 0.04) (Figures 1A–C).



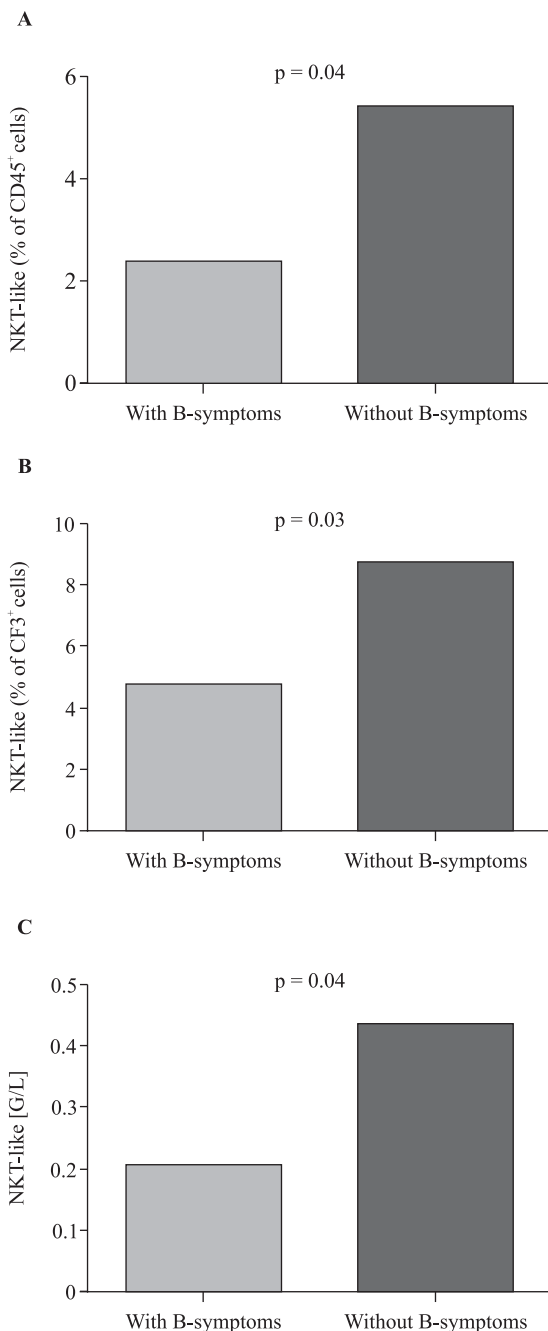
**Figure 1.** NKT-like (CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup>) numbers in patients with DLBCL and healthy controls



**Figure 2.** NKT-like (CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup>) numbers in patients in early and advanced stages of disease

The frequency of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells decreased with the stage of disease. The median percentage of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells within CD3<sup>+</sup> cells was 8.14% in patients in stages 1 and 2 and 5.81% in stages 3 and 4 (p = 0.07). Within CD45<sup>+</sup> cells, the frequencies were 5.55% and 3.15%, respectively (p = 0.02). Median absolute count of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells was also lower in more advanced stages compared to earlier ones (0.26 G/L vs. 0.41 G/L; p = 0.02) (Figures 2A–C).

The percentage of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells was significantly higher in DLBCL patients without B-symptoms compared to patients with B-symptoms, both within CD3<sup>+</sup> cells (8.70% vs. 4.89%; p = 0.03), and within CD45<sup>+</sup> cells (5.51% vs. 2.46%; p = 0.04). Median absolute count of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells was also significantly lower in the patients with B-symptoms (0.21 G/L vs. 0.44 G/L; p = 0.04) (Figures 3A–C).



**Figure 3.** NKT-like (CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup>) numbers in DLBCL patients without B-symptoms and patients with B-symptoms

Percentage of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells correlated adversely with lactate dehydrogenase ( $R = -0.445$ ;  $p < 0.05$ ).

## Discussion

Hopes for an improved prognosis for cancer patients are associated with cellular immunotherapy, which involves the elimination of tumor cells using the patient's own immune mechanisms. Recent studies have

demonstrated encouraging preclinical results of NKT cell based anti-cancer immunotherapy. Activated iNKT cells may destroy tumor cells, both directly and indirectly. Directly, they exert a cytotoxic effect through expression of effector molecules that induce cell death, such as perforin, FasL and TRAIL. Indirectly, through the secretion of cytokines, especially Th1 and cell-to-cell contact, they stimulate innate and acquired immune cell types involved in the anti-cancer response [10–12]. Experimental studies have shown that presented in the context of CD1d, synthetic molecule  $\alpha$ Gal-Cer strongly stimulates both the immediate and direct anti-tumor effects of human and murine iNKT cells [13, 14]. NKT cell numbers are decreased in the peripheral blood of patients with solid tumors such as melanoma, prostate or lung cancer [15–18], but opinions on their activity diverge, with some authors showing impaired [17, 19, 20], and others normal, function [15, 18].

In our study, we found lower frequencies of NKT cells in peripheral blood patients with DLBCL, but absolute counts of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> were comparable to the healthy controls. Molling et al. in a study group of 120 cancer patients found on average 47% fewer iNKT cells compared to healthy subjects [21], regardless of the type of tumor or tumor mass. The number of NKT cells did not increase after removal of the tumor by surgery or radiotherapy [21]. The authors concluded that a reduced number of circulating NKT cells secreting IFN- $\gamma$ /V $\alpha$ 24/V $\beta$ 11 could therefore be a risk factor for developing cancer rather than a result of the presence of a tumor. In contrast, we noted significantly lower counts of CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> in DLBCL patients in more advanced clinical stages, which indicates that higher tumor burden might influence NKT count. Moreover, the CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cell percentage was lower in patients with poor prognostic factors like the presence of B-symptoms and adversely correlated with LDH serum level, which indicates a relationship between a more aggressive form of the disease and a decrease of NKT numbers.

It is still to be explained whether low NKT cell counts in peripheral blood of patients with DLBCL results from their suppression by the tumor cells or their migration to affected lymph nodes or organs. Yoneda et al. found a relationship between NKT cell numbers and tumor mass. They found decreased V $\alpha$ 24+NKT cell numbers in patients with hematopoietic malignancies, including malignant lymphoma. All studied NHL patients were previously treated with chemotherapy, and NKT cells were decreased only in patients with progressive disease, not in patients in remission. Such conflicting data might result from the small number and diversity of patient groups, but

also from different methods of NKT cell evaluation. Our study measured CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells, but both Molling et al. and Yoneda et al. evaluated V $\alpha$ 24+NKT cells. It seems that different subpopulations of NKT might play distinct roles in tumor immunity [22]. Renukaradhya et al. showed that type I NKT cells protect, and type II NKT cells suppress, innate anti-tumor responses to a B-cell lymphoma [23]. Yoneda et al. postulated that CD4–CD8–V $\alpha$ 24+NKT cells are more dominantly involved in activating tumor immunity [22]. It is entirely possible that the function of immune cells might differ according to differing types of lymphoma.

Results of preclinical studies suggest a potential use of NKT cells in patients with lymphoma. Bagnara et al. demonstrated in a SCID mouse model that adoptive transfer of expanded *ex vivo* NKT cells together with  $\alpha$ -GalCer resulted in reduction of tumor size (inoculated C1R-CD1d lymphoma cell line) [24]. Guven et al. showed that CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup> cells from peripheral blood of patients with chronic lymphocytic leukemia could be easily expanded in *in vitro* cultures and that these cells had cytotoxic activity [25]. However, these results are only preliminary, so it will be of great importance to determine the function of distinct NKT subsets in malignant lymphoma.

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