

# Imbalance in circulatory iNKT, Th17 and T regulatory cell frequencies in patients with B-cell non-Hodgkin's lymphoma

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**Abstract.** T cells are important in B-cell non-Hodgkin's lymphoma immunity, however the function of T cell subsets, including natural killer (iNKT), T helper (Th)17, and T regulatory cells remains to be elucidated. The present study analyzed the frequencies of iNKT, Th17 and T regulatory cells in the peripheral blood of 41 patients with B-cell non-Hodgkin lymphoma at diagnosis, then during and following immunochemotherapy R-CHOP/R-CVP. At lymphoma diagnosis, iNKT and Th17 frequencies were decreased and T regulatory cell frequencies were increased compared with healthy control group. The Th17 cell percentage was lower in patients with a worse prognosis and at a more advanced clinical stage and in contrast, the percentage of T regulatory cells was increased in patients at advanced stages of lymphoma, compared to earlier stages. There was an increase of iNKT and Th17 cells following R-CHOP/R-CVP therapy. In patients that responded, both prior to and following-treatment, percentages of iNKT and Th17 were higher and T regulatory cells were lower compared with patients with subsequent disease progression. Taken together, the results obtained demonstrated the opposing effects of T cell subsets in B-cell lymphoma immunity, with iNKT and Th17 inhibiting and T regulatory cells enhancing tumor growth. These alterations may be caused by malignant B-cells, however there may also be an axis of inverse feedback between T regulatory cells and their interaction with Th17 and iNKT cells.

## Introduction

B-cell non-Hodgkin's lymphomas (B-NHL) are a heterogeneous group of malignancies with different etiopathogenesis, clinical presentation, course, prognosis and response to therapy. Many disorders in the immune system that may influence the tumor growth, development and progression have been described in patients with lymphoma. T-cells are considered to be of key importance in tumor immunity. However, while the role of cytotoxic CD8<sup>+</sup> T cells, CD4<sup>+</sup> Th1 cells and NK cells as the main effector cells with antitumor functions is well established, the functions of more recently discovered T cell populations, like NKT, Th17, and T regulatory cells still remain elusive.

NKT (natural killer T) cells are a subset of T cells sharing the features of T and NK cells. There are three defined NKT cell subtypes. Type I NKT cells (invariant NKT, iNKT) are characterized by canonical T-cell receptor (TCR)  $\alpha$  chain (V $\alpha$ 24J $\alpha$ 18 in humans) and semi-invariant TCR $\beta$  chain (mainly V $\beta$ 11 in humans) that recognize glycolipid  $\alpha$ -galactosylceramide-(GalCer) presented by MHC-like CD1d molecule (1). Type II NKT cells are also CD1d-dependent, but express a more diverse TCR $\alpha$  chain (2). Type III NKT cells (NKT-like) are CD1d-independent and express semiinvariant TCRs. iNKT that are predominant and the best characterized population of NKT cells were found to play an important role in tumor rejection. They exert anti-tumor responses mainly indirectly, by the secretion of Th1-type cytokines, activation and recruitment of other effectors, but can also directly kill CD1d-positive malignant cells in a CD1d-dependent manner (3). iNKT cells were demonstrated to suppress cancer cells growth in several tumor models (4-9). High numbers of tumor-infiltrating or circulating iNKT cells number were associated with improved disease outcome in patients with diverse types of cancer (10-12), including lymphoproliferative malignancies, but the data iNKT cells in patients with B-NHL are limited.

Th17 cells, named after their hallmark cytokine-IL-17, are effector T cells that play a pivotal role in the immune response against extracellular pathogens, they are also involved in the

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pathogenesis of autoimmune and allergic diseases. The studies on their role in cancer has brought divergent results. IL-17 was found to exert anti-tumor functions by recruitment of CD4<sup>+</sup>, CD8<sup>+</sup> T cells, NK cell, neutrophils and dendritic cells to the tumor tissue and by enhancing the NK and cytotoxic T cell activity (13). However it might also promote tumor cell proliferation, angiogenesis, metastasis and invasion. In patients with lung (14) or ovarian cancer (15) the presence of Th17 cell infiltrates in tumor tissue correlated with better prognosis and longer overall survival. Conversely, infiltrations of Th17 cells in tumor microenvironment correlated with poor prognosis in hepatocellular carcinoma (16,17), pancreatic carcinoma (18) and colon cancer (19). In lymphoproliferative diseases, the data are inconsistent. Th17 cells were shown to promote tumor growth and correlate with higher tumor mass in patients with multiple myeloma (MM) (20,21) that probably might be connected the important contribution of inflammation and angiogenesis in pathogenesis of MM, taking into account proinflammatory and proangiogenic properties of IL-17. However in the study of Bryant *et al*, Th17 cell numbers were higher in patients surviving more than 10 years after the diagnosis of MM, than in patients with a shorter survival (22) and Th17 cells seem to play the protective role of in tumor immunity in patients with chronic lymphocytic leukemia (CLL) (23-25) as well as the other types of B-NHL (26), though there is still only a few studies published on this issue.

T regulatory cells (Tregs) are characterized by the expression of transcription factor FoxP and under physiological conditions their main function is maintaining the immune homeostasis by down-regulation of excessive adaptive immune reactivity. Tregs are the most extensively studied population of regulatory cells in cancer diseases. They were shown to promote cancer development and progression by suppressing anti-tumor immune responses, in some, but not all types of malignancies. The correlation between high numbers of Tregs in tumor microenvironment and short overall survival was described in patients with hepatocellular (27), breast (28) and ovarian cancer (29). Conversely, Treg infiltrates correlated with improved prognosis and survival in patients with colorectal and head and neck cancer (30-32) and some types of lymphoma like classical Hodgkin lymphoma or germinal center-like diffuse large B-cell lymphoma (33).

In the present study we analyzed the frequencies of iNKT, Th17 and Treg cells in peripheral blood samples from 41 patients with B-NHL, their interrelationships and the correlations both with disease activity parameters and tumor burden as well as the response to the first-line immunochemotherapy R-CHOP/R-CVP.

## Materials and methods

**Patients and samples.** Peripheral blood samples were taken from 41 consecutive patients (23 male and 18 female) diagnosed with B-NHL and then treated in St. John of Dukla Lublin Region Cancer Center. In the study group there were 29 patients with diffuse large B-cell lymphoma (DLBCL) and 12 patients with indolent NHL (iNHL), including 6 patients with follicular lymphoma (FL) and 6 patients with marginal zone lymphoma (MZL). The median age of patients was 65 years (range: 24-81). Clinical stage of the disease was

established according to the Ann Arbor staging system. Patients with DLBCL were divided into risk groups according to the International Prognostic Index (IPI). Clinical characteristics of the study group are summarized in the Table I.

The control group consisted of 20 age-matched healthy donors (12 male, 8 female, median age 57; range: 36-83). Approval for this study was obtained from the Local Ethics Committee. All patients and donors had given their informed consent. None of the patients had autoimmune disease, and none had ongoing infections at the time of the sample taking or had had infections over the previous 3 months. All the patients received immunochemotherapy (6-8 cycles) with R-CHOP (rituximab, cyclophosphamide, vincristine, doxorubicin, prednisone)-31 patients or R-CVP (rituximab, cyclophosphamide, vincristine, prednisone)-10 patients. The blood samples were taken from all the patients at diagnosis before the start of anti-cancer treatment, before the 3rd cycle and after the completion of the R-CHOP/R-CVP treatment.

**Ethics statement.** This study was approved by the Ethics Committee of the Medical University of Lublin (No. KE-0254/66/2011). Written informed consent was obtained from all patients with respect to the use of their blood for scientific purposes.

**Cell preparation.** Peripheral blood samples were collected into heparinized tubes. Fresh samples were stained within 1-2 h and analyzed directly upon completion of staining process. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation on Biocoll Separating Solution (Biochrom) for 25 min at 400 x g at room temperature. Interphase cells were removed, washed twice, and resuspended in phosphate-buffered saline (PBS).

**Assessment of iNKT cells.** Flow cytometry analysis of iNKT cells was performed using monoclonal antibodies (MoAb) anti-iNKT FITC (anti-V $\alpha$ 24 FITC) and anti-CD3 PE (BD Pharmingen) (as described previously (24)).

**Intracellular IL-17A analysis.** For intracellular IL-17A staining PBMC (2x10<sup>6</sup>/ml) were cultured in RPMI 1640 supplemented with 2 mmol/l L-glutamine, 5% human albumin, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were stimulated with 25 ng/ml of PMA and 1  $\mu$ g/ml of ionomycin (Sigma, Germany) in the presence of BD GolgiStop (BD Pharmingen, USA) for 5 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Flow cytometry analysis of Th17 cells was performed using MoAbs anti-CD4 FITC, anti-CD3 PE and anti-IL-17A PE (BD Pharmingen) (as described previously (24)).

**Analysis of T regulatory (Treg) cells.** The percentage of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg among CD4<sup>+</sup> lymphocytes was determined using the Human Treg Flow kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions.

**Statistical analysis.** Statistical analyses were performed with STATISTICA 10.0 PL and Graphpad Prism 5 (Graphpad Software, Inc.). Differences were considered statistically significant with a P-value  $\leq$ 0.05. The Mann-Whitney U test was applied for statistical comparison of the results between DLBCL, iNHL

Table I. Clinical and laboratory characteristics of the study group.

No of patients	Total number of patients
<b>Sex:</b>	
Male	23
Female	18
<b>NHL subtype</b>	
DLBCL/MCL	29/4
<b>iNHL:</b>	
FL	6
MZL	6
<b>Bone marrow involvement (DLBCL/iNHL):</b>	
Yes	5
No	36
<b>B-symptoms (DLBCL/iNHL):</b>	
Yes	20/5
No	9/7
<b>Stage according to Ann Arbor (DLBCL/iNHL):</b>	
I	0/0
II	4/4
III	10/3
IV	15/5
<b>IPI (DLBCL):</b>	
1	3
2	15
3	6
4	1
Median age (years)	65
<b>Laboratory parameters (DLBCL); median (range);</b>	
WBC (G/l)	6.77 (3.6-13.8)
PLT (G/l)	238 (125-1002)
Hgb (g/dl)	13.1 (0.13-15.3)
LDH (IU/l)	242 (156-971)
<b>Laboratory parameters (iNHL); median (range):</b>	
WBC (G/l)	9.38 (4.45-30.89)
PLT (G/l)	285 (116-876)
Hgb (g/dl)	13 (10.4-15)
LDH (IU)	198 (148-717)
<b>Response to the first line therapy (R-CHOP/R-CVP): DLBCL and Inhl:</b>	
Complete response (CR)	25
Partial response (PR)	11
Progressive disease (PD)	5

patients and HV, as well as between patients in different stages of the disease. The Wilcoxon paired test was used to compare the results before during and after therapy. The Spearman rank correlation coefficient was used in correlation tests.

The percentages of circulatory iNKT, Th17 and T regulatory cells were analyzed depending on the clinical stage of lymphoma according to Ann Arbor, IPI, presence of B-symptoms, bone marrow involvement as well as the response to the first line therapy. They were also correlated with laboratory parameters, such as hemoglobin concentration, white blood cells (WBC), platelets (PLT) counts and lactate dehydrogenase (LDH) activity.

**Results**

*Analysis of iNKT cell percentage.* At lymphoma diagnosis, median percentage of iNKT cells was lower in patients with B-NHL than in healthy donors (0.40% vs. 0.75%, P<0.05). In patients with DLBCL median percentage of iNKT cells was 0.49% and in patients with indolent NHL was 0.37% (Fig. 1). There were no differences in iNKT numbers depending on the clinical stage of lymphoma or IPI (DLBCL). Pre-treatment iNKT cell percentage was higher in patients who subsequently achieved response to immunochemotherapy (0.56%) than in patients with disease progression (0.28%), P<0.01 (Fig. 2).

After the completion of R-CHOP/R-CVP, iNKT cell percentage increased in the whole patients' group (0.65%) comparing to the values before (0.40%) or during treatment (0.35%) (Fig. 3A). In patients with response to R-CHOP/R-CVP, the percentage of iNKT cells was higher than in patients with disease progression (0.48% vs. 0.22%, P<0.01) (Fig. 3B) and similar to the values in control group.

*Analysis of Th17 cell percentage.* Median percentage of Th17 cells in peripheral blood of patients with B-cell NHL was 0.39% and it was significantly lower than in healthy donors (2.95%, P<0.001). In patients with DLBCL the median percentage of Th17 cells was 0.74% and in patients with indolent NHL-0.39% (Fig. 4). Th17 cell percentage was higher in patients with DLBCL with better prognosis (IPI 1/2) than in patients with worse prognosis (IPI 3/4) (0.18% vs. 0.52%, P=0.17) (Fig. 5A) and in patients with iNHL in earlier clinical stages comparing to the advanced ones. Significant difference was noted in between patients with clinical stage II and IV according to Ann Arbor (0.47% vs. 0.12%, P<0.05) (Fig. 5B).

Pre-treatment Th17 cell percentages were higher in patients who subsequently achieved response to the therapy (CR/PR) than in patients with disease progression (0.63% vs. 0.28%, P<0.05) (Fig. 6).

During R-CHOP/R-CVP immunochemotherapy, in the whole patients group there was a gradual increase of the percentage of Th17 that after completion of the treatment was significantly higher than before the therapy (1.57% vs. 0.47%, P<0.05) (Fig. 7A). In patients with response to R-CHOP/R-CVP, the percentage of Th17 was higher than in patients with disease progression (1.17% vs. 0.76%, P<0.05) (Fig. 7B), however it was still lower than in the control group (2.95%).

*Analysis of T regulatory cells.* T regulatory cell percentage was higher in patients with B-NHL compared to healthy volunteers (3.15% vs. 2.1%, P<0.001). In patients with DLBCL,

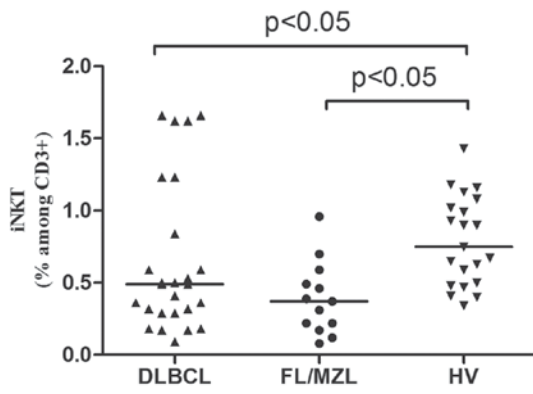


Figure 1. Percentages of iNKT cells in peripheral blood of patients with DLBCL, FL/MZL and healthy volunteers (HV).

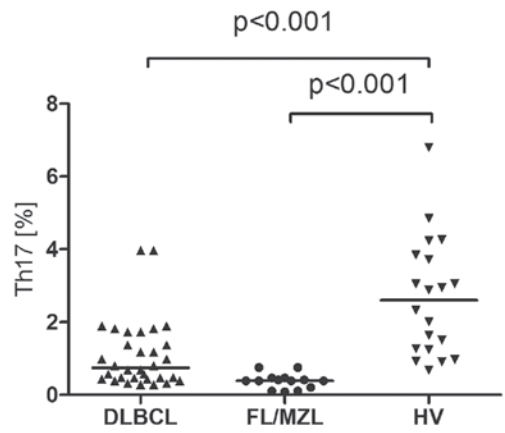


Figure 4. Percentages of Th17 cells in peripheral blood of patients with DLBCL, FL/MZL and healthy volunteers (HV).

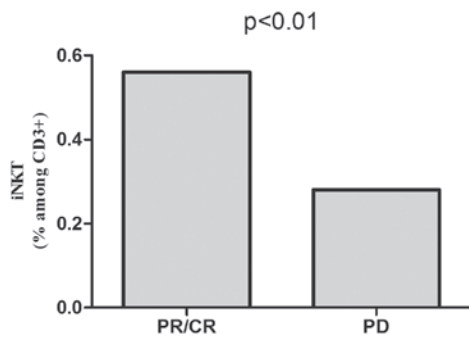


Figure 2. Pre-treatment percentages of iNKT cells in peripheral blood of patients with B-NHL depending on the subsequent response to the first-line immunochemotherapy R-CHOP/R-CVP.

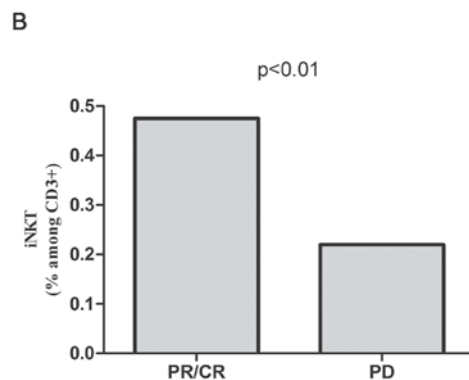
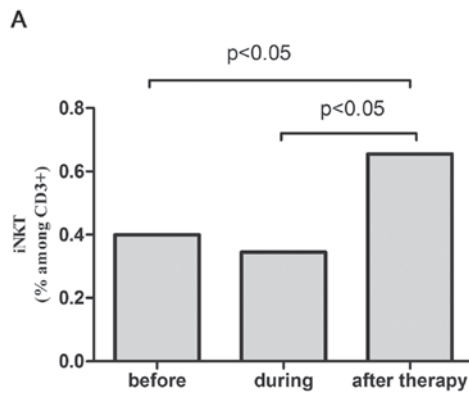


Figure 3. (A) Percentages of iNKT cells in peripheral blood of patients B-NHL before, during and after R-CHOP/R-CVP therapy and (B) iNKT cells percentages after the completion of therapy depending to the achieved response.

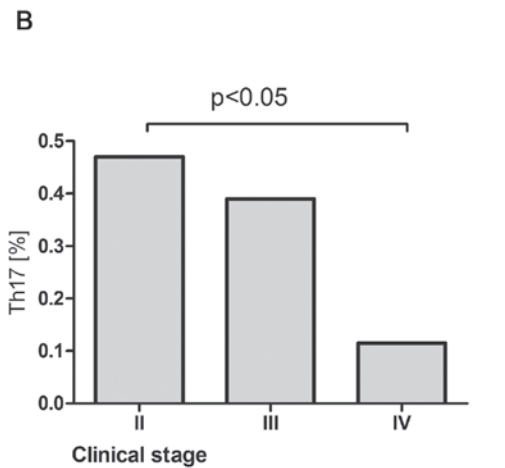
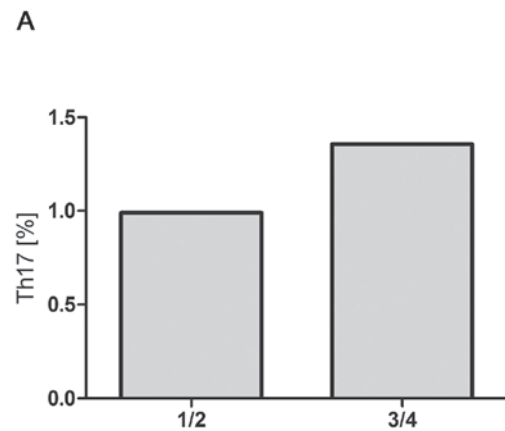


Figure 5. (A) Percentages of Th17 cell in peripheral blood of patients with DLBCL depending on IPI (1/2 vs. 3/4) and (B) in patients with iNHL depending to clinical stage according to Ann Arbor.

Treg percentage was 3,5% and in patients with iNHL it was 3.56% (Fig. 8). In patients with DLBCL with advanced clinical stage (III/IV), the percentage of T regulatory cells was higher as compared to the earlier stages (I/II) (3.95% vs. 3.15%, P<0.05) (Fig. 9).

Pre-treatment T regulatory cell percentage was lower in patients with B-NHL, who subsequently achieved response to the therapy (CR/PR), than in patients with disease progression (3.29% vs. 5.75%, P<0.05) (Fig. 10A). The percentage

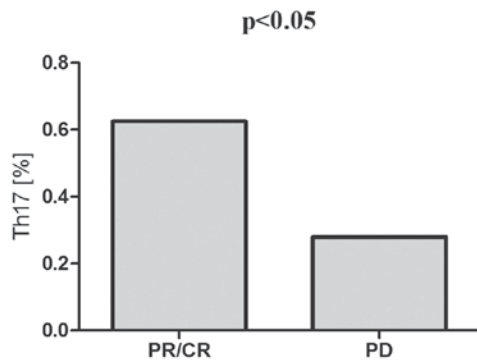


Figure 6. Pre-treatment Th17 cell percentages in patients with B-NHL depending on the subsequent response to first line immunochemotherapy R-CHOP/R-CVP.

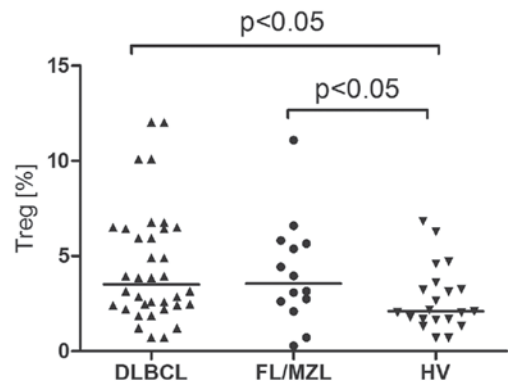


Figure 8. Percentages of T regulatory cells in peripheral blood of patients with DLBCL, FL/MZL and healthy volunteers (HV).

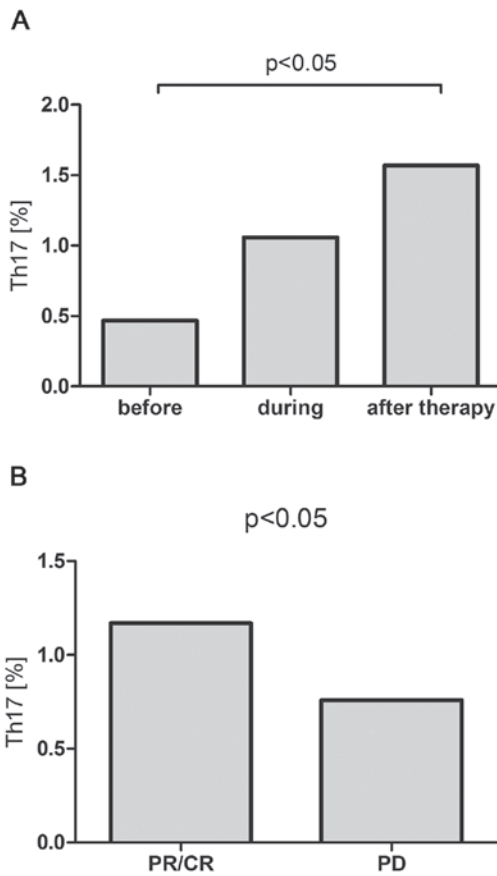


Figure 7. (A) Percentages of Th17 cells in peripheral blood of patients with B-NHL before, during and after R-CHOP/R-CVP therapy and (B) Th17 cells percentages after the completion of therapy depending to the achieved response.

of T regulatory cells did not change significantly during therapy. After treatment, T regulatory cell percentage in DLBCL patients with CR/PR was significantly lower than in patients with disease progression (2.97% vs. 6.13%,  $P < 0.01$ ), but still higher than in control group (2.1%) (Fig. 10B).

**Correlations between Th17 cells, iNKT cells and T regulatory cells.** Both in patients with DLBCL and in patients with iNHL, there was a significant correlation between Th17 cells and iNKT cells ( $R = 0.25$ ;  $P < 0.05$ ;  $R = 0.39$ ;  $P < 0.05$ ,

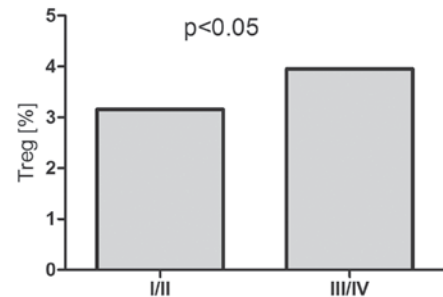


Figure 9. Percentages of T regulatory cells in peripheral blood of patients with DLBCL depending on clinical stage according to Ann Arbor classification.

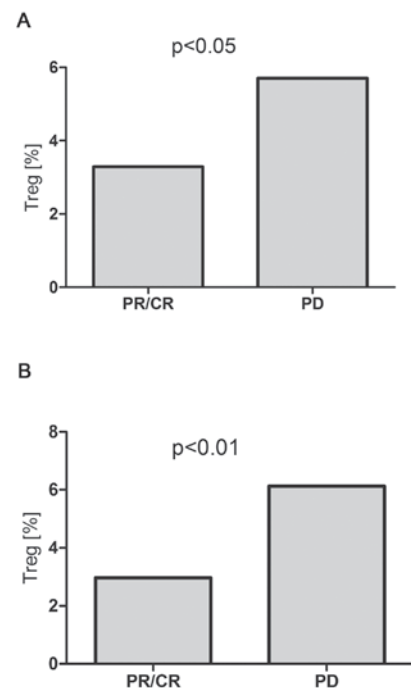


Figure 10. (A) Pre-treatment and (B) after-treatment T regulatory cell percentages in peripheral blood of patients with B-NHL depending on the response to the first line immunochemotherapy R-CHOP/R-CVP.

respectively) and in patients with DLBCL there was also inverse correlation between Th17 cells and T regulatory cells ( $R = -0.242$ ;  $P < 0.05$ ).

## Discussion

Extensive studies showed an ambiguous and divergent role of the immune system in cancer development, on one side protecting but on the other side promoting tumor cell growth. As compared to the solid tumors, the interactions between cancer and immune cells are more complicated in lymphoma where malignant cells themselves are derived from immune system cells and many issues concerning the influence of the immune system on lymphoma development remain unknown. In the present study, circulatory iNKT and Th17 cell percentages in patients with B-NHL at lymphoma diagnosis were decreased, and Treg percentage was increased comparing to the healthy control. Lower Th17 cell numbers and higher Treg numbers were observed in patients with more advanced disease, higher tumor mass and worse prognosis. Corresponding results were obtained in patients with both FL/MZL and DLBCL suggesting similar relationships between studied cell populations in indolent and aggressive types of lymphoma. Presented data indicate for an opposing role of studied cell populations in tumor immunity of B-NHL with iNKT and Th17 protecting from and Tregs promoting tumor growth. These results correspond with accumulated evidence showing iNKT cells as important mediators of antitumor immunity in different types of malignancies including B-NHL. iNKT cells were shown to be essential for the survival of mice against B-cell lymphoma (34). Treatment of lymphoma-bearing mice with potent iNKT cell agonist  $\alpha$ -GalCer or tumor cell vaccine incorporating  $\alpha$ -GalCer resulted in protection from tumor growth and prolonged survival (35,36). Though deficiencies in iNKT numbers and functions correlating with tumor progression was found in patients with diverse type of cancer, including prostate cancer, head and neck cancer, myelodysplastic syndrome and multiple myeloma (37-40), there is only a few data on iNKT cells in patients with B-NHL. Decrease of circulatory iNKT cell percentage was described in patients with hematological malignancies including patients with different types of malignant lymphoma (41). More studies concerned NKT-like cells. Decreased numbers of NKT-like cells in peripheral blood of patients with CLL and DLBCL were found by us and also by the other authors (42-44). Correlation with disease stage and progression indicated for the protective role of NKT-like cells from tumor growth. Further research exploring the role iNKT in B-NHL immunosurveillance would be of significance regarding the potential use of iNKT enhancing strategies in anticancer immunotherapy. Significant correlations between iNKT and Th17 cells frequencies in peripheral blood of patients with B-NHL found in this study suggest their congruous involvement in tumor immunosurveillance. Similarly to iNKT, there is not too much data on Th17 cells in patients with B-NHL, but the results of so far published studies are rather consistent and militate in favor their anti-lymphoma activity. In the recent paper, Lu *et al*, showed the decrease of circulatory Th17 cells in patients with B-NHL, that were lower at the relapse than at lymphoma diagnosis (26). In patients with CLL lower numbers of Th17 in peripheral blood cells correlated with worse prognosis, and there was a also decrease of Th17 cells along with disease progression (23,24). Similarly to the peripheral blood, in the study by Yang *et al* (45), Th17 cell numbers were lower in malignant B-cell lymphoma lymph

nodes than in benign lymph nodes, and peripheral blood and tonsils of healthy individuals. Frequencies of IL-17 producing CD4<sup>+</sup> T cells were lower in patients with FL, MZL and DLBCL compared to MCL, MALT and CLL/SLL (45). In the study of Galand *et al* (46), there was an adverse correlation between IL-17 production by Th17 cells in tumor tissue and tumor burden in mice primary intraocular B-cell lymphoma, suggesting a protective effect of this cell population from tumor development (46).

In opposition to iNKT and Th17 cells, circulatory Treg frequencies were increased in patients with B-NHL compared to healthy control and their higher numbers in more advanced stages of lymphoma suggest a supportive role in tumor development. These data are in line with earlier studies showing increased frequencies of Treg in peripheral blood of patients diagnosed with B-NHL (47,48) that correlated with tumor burden (49). Immunosuppressive effect of Tregs on anti-tumor T-cell responses in lymphoma was demonstrated in several *ex vivo* studies (49-52). The role of T regulatory cells in B-cell lymphoma is, however ambiguous, because Tregs can also inhibit B-cell lymphoma growth in different mechanisms (53,54) and high tumor infiltrating Tregs were found to correlate with good prognosis in patients with B-NHL (55,56). In the present study, except the higher numbers of Tregs in more advanced clinical stages of lymphoma, we have also found an inverse correlation between circulatory Th17 and Treg cell percentages that might result from the effect of malignant B-cells on T cell differentiation-inhibiting Th17 and promoting Tregs. *In vitro* studies revealed that malignant B-cells not only induce the conversion of CD4<sup>+</sup>CD25<sup>-</sup> T cells into Treg cells (47,56), but also skew the balance between Th17 and Treg cells inhibiting Th17 cells and up-regulating Tregs (45). Moreover, in contrast to Th1 and Th2 cells that are irreversibly differentiated, a plasticity exists between Th17 cells and Tregs, so CD25<sup>high</sup>FoxP3<sup>+</sup> Treg might transdifferentiate into Th17 cells and vice versa depending on the presence of lineage-specific polarizing factors (57). In this study there were no differences in circulating iNKT frequencies depending on the tumor mass and we did not observed direct relationship between Tregs and iNKT cells. However lower frequencies of iNKT in the presence of higher frequencies of Tregs might suggest inhibition of iNKT differentiation by Tregs. This suppressive effect of Tregs on iNKT proliferation and functions was therefore demonstrated in *in vitro* studies by Azuma *et al* (58). Activated iNKT cells seem also to modulate both numbers and functions of Tregs (59). Another finding in the present study was an increase of iNKT and Th17 cells after immunochemotherapy. In contrast to the Lu *et al* (26) study, where the numbers of Th17 cells in patients with B-NHL normalized after one or two cycles of chemotherapy, in our study the significant increase was observed after the completion of R-CHOP/R-CVP therapy. In patients with disease progression both iNKT and Th17 cells were significantly lower after therapy than in patients who achieved response, again suggesting possible suppressive effect of tumor on these cell populations. However, higher iNKT and Th17 cell frequencies observed both before and after the therapy in responding patients might also indicate for their important contribution in achieving disease control. Interestingly, Molling *et al* (60), did not find a restoration of iNKT numbers in patients with solid tumors after the therapy like surgery or radiotherapy, but this discrepancy might

result both from different types of malignancy (B-NHL vs. solid tumors) as well as the treatment used (local surgery, radiotherapy vs. systemic immunochemotherapy) (60). In contrast, T regulatory cells percentage was higher before the therapy in patients in whom subsequently disease progression was observed, suggesting their negative impact for treatment results. These data show the potential predictive value of circulatory iNKT, Th17 and Treg in patients with B-NHL.

In conclusion- the results of the present study suggest an opposite role of iNKT, Th17 and Tregs in B-cell lymphoma immunity with iNKT and Th17 inhibiting and Tregs supporting tumor growth. Alterations in studied T cells subsets in peripheral blood of patients with B-NHL might be caused by malignant B-cells, but there might be also an axis of inverse feedbacks between Tregs on one side and Th17 and iNKT cells on the other. Higher baseline frequencies of iNKT and Th17 cells in patients with subsequent response for immunochemotherapy might suggest not only their predictive value but also their supporting role in achieving disease control. Further research on the role of T cells in B-cell lymphoma immunity, involving larger patients' groups and other types of B-cell malignancies would be essential for the understanding B-NHL biology especially in context of introducing novel targeted therapies that were demonstrated to influence T cell populations.

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