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The percentage of iNKT cells among other immune cells at various clinical stages of laryngeal cancer*

Wartości odsetkowe komórek iNKT na tle innych komórek układu odpornościowego w różnych stadiach zaawansowana raka krtani

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Invariant natural killer T (iNKT) cells constitute a small population of immune cells that share functional and phenotypic characteristics of T lymphocytes and NK cells. Due to their involvement in specific and non-specific immune responses, iNKT cells may represent an important component of antitumor and anti-infectious immunity.

Material and methods: Using flow cytometry, we analyzed the percentages of iNKT cells as well as T and B lymphocytes in peripheral blood of 50 laryngeal cancer patients at various clinical stages in comparison to healthy controls (n=15). Moreover, we determined the expression of CD25, CD69 and CD95 antigens on T lymphocytes.

Results: The percentage of CD4+/CD3+ T lymphocytes in the controls was higher than in laryngeal cancer patients, both with early and late stages of the disease. The percentage of CD8+/CD3+ T lymphocytes in healthy controls was lower than in patients with early and late clinical stages of laryngeal cancer. Patients with advanced laryngeal cancer showed a lower percentage of iNKT cells and higher frequencies of T regulatory cells (Tregs) than the controls. Advanced clinical stages of laryngeal cancer are associated with impaired activation of lymphocytes.

Conclusions: Our study confirmed that laryngeal cancer cells exert a strong suppressor effect on the immune system of the host. This is reflected by a decrease in the percentage of iNKT cells that are capable of cancer cell elimination, and a concomitant increase in the percentage of Tregs. However, further studies are needed in order to explain the underlying mechanisms of immunosuppression and understand interactions between immune and cancer cells.

Keywords: laryngeal cancer • iNKT cells • regulatory T cells • activation markers

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Introduction

Laryngeal cancer is the most prevalent malignancy of the head and neck. It is markedly more frequent in men than in women, being a significant cause of mortality in male patients [15]. Squamous cell carcinoma is the most frequent histological type of laryngeal cancer [16]. Although management of laryngeal cancer is individualized, it is generally based on surgical treatment and radiotherapy, and combined treatment is implemented at advanced clinical stages [14]. Despite continuous progress and improvement in conventional methods of treatment, the therapeutic outcomes still remain unsatisfactory [6,17]. Available evidence suggests that future research should center on the immune system and its role in the etiopathogenesis and outcome of cancer. Understanding of mechanisms through which various components of the immune system are involved in pathogenesis of laryngeal cancer can lead to development of an efficient immunotherapy, which could serve as an adjuvant for classic treatment modalities.

Natural killer T (NKT) cells constitute a subpopulation of T cells that share both functional and phenotypic characteristics of T lymphocytes and natural killer (NK) cells [23]. "Classic" human NKT cells (type I), also referred to as invariant NKT (iNKT) cells, are characterized by expression of T cell receptors (TCRs) with conservative α (Vα24-Jα18) and β (Vβ11) chains [3]. iNKT cells recognize endogenous and exogenous lipid and glycolipid antigens presented by CD1d expressed on APC [3, 13, 23]. Along with T lymphocytes and NK cells, they play important role in antitumor immunity. iNKT cells show direct cytotoxicity, expressing molecules that induce cell death, such as Fas/FasL and TRAIL; moreover, they can release perforin. Furthermore, iNKT cells indirectly modulate the antitumor response, being involved in activation of many other immune cells, such as NK cells, cytotoxic T lymphocytes and dendritic cells [13]. All these cells are characterized by reactivity with α-galactosylceramide (α-GalCer; KRN7000) [3,13]. Dendritic cells, presenting α-GalCer on CD1d, stimulate early release of an array of cytokines (IFN-γ, IL-4, -2, -5, -6, -10, -13, TNF, TGF-β and GM-CSF) from iNKT cells [2,3,8,13,18,23]. The early release of large amounts of IFNγ is reflected by recruitment of NK cells and activation of their antitumor response, as well as by cytotoxic reaction of CD8+ T lymphocytes that recognize complexes of tumor antigens with MHC-1 molecules. iNKT cells can constitute an early source of IL-4, and due to their ability to rapid reaction can support the Th2 response and synthesis of IgE [3]. Moreover, iNKT cells were observed to inhibit the activity of such immunosuppressive components as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages, thus counteracting the state of immunosuppression that is frequently observed in a tumor microenvironment [5,19]. iNKT cells are postulated to constitute a significant component of the immune system during both antitumor and anti-infectious responses, and can be involved in the etiopathogenesis of autoimmune conditions and allergies [8,25,27].

The aim of this study was to determine the percentage of iNKT cells within a pool of all immune cells present in peripheral blood of patients with various clinical stages of laryngeal cancer and healthy volunteers. Moreover, we analyzed the degree of lymphocyte activation in these groups, determining the expression of the activation markers CD69, CD25 and CD95.

Material and methods

Participants

The study included material from laryngeal patients who were treated at the Department of Otolaryngology and Laryngological Oncology, Medical University of Lublin, between 2012 and 2013. A total of 50 patients (40 men and 10 women) aged between 45 and 77 years (median age: 60 years) were enrolled. Based on the TNM classification, the patients were classified as having stage I (n=4), stage II (n=13), stage III (n=22) or stage IV laryngeal cancer (n=11). The control group consisted of 15 healthy volunteers (12 men and 3 women) between 43 and 82 years of age (median age: 61 years).

None of the enrolled individuals had undergone a blood transfusion, suffered from infection, and had been taking antibiotics or other drugs with a known influence on the immune system for a month before the examination. Persons with a history of allergic diseases were excluded from the study. The protocol of the study was approved by the Local Bioethical Committee at the Medical University of Lublin.

Peripheral blood samples (15 ml) from the basilic vein were collected by venipuncture using sterile, sodium hep-

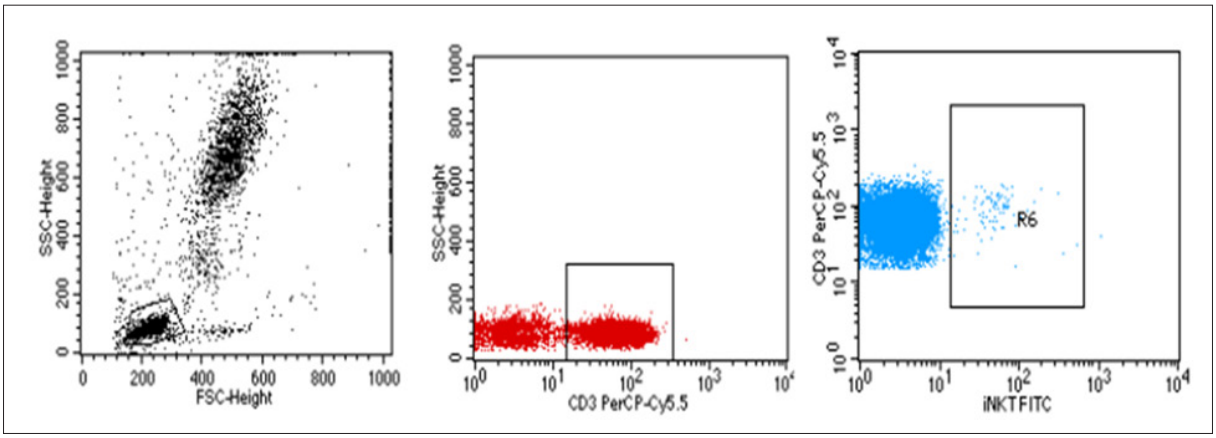

Fig. 1. Example of cytometric analysis of iNKT cells

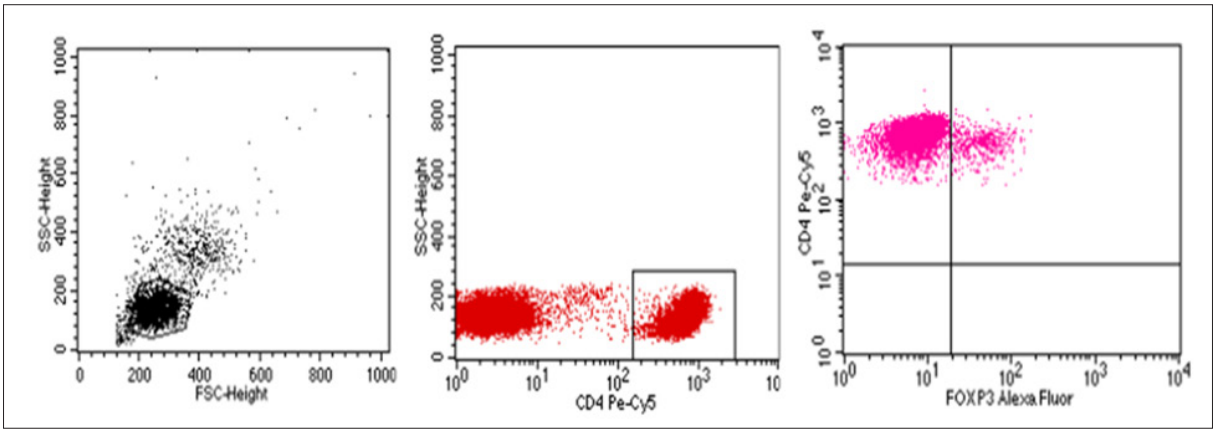

Fig. 2. Example of cytometric analysis of regulatory T lymphocytes

arin-treated tubes (20 units per ml of blood), and used for cytometric analyses.

Immunophenotyping of peripheral blood cells

The samples for cytometric analyses were prepared from freshly obtained peripheral blood incubated with a set of monoclonal antibodies: anti-CD3 FITC, anti-CD3 PECy5, anti-CD4 FITC, anti-CD8 PE, anti-CD19 PE, anti-iNKT FITC, anti-CD69 PECy5, anti-CD25 PECy5, anti-CD95 PECy5, and anti-CD3 FITC/anti-CD16 PE/anti-CD56PE (BD Pharmingen, United States). The samples were deprived of erythrocytes by addition of lysing solution (FACS Lysing Solution, Becton Dickinson, United States). The immunophenotype of peripheral blood cells was determined with a FACSCalibur flow cytometer (Becton Dickinson, United States) equipped with an argon laser emitting at 488 nm. The results were analyzed with CellQuest Pro software (Becton Dickinson, United States) (Figure 1).

Isolation of mononuclear cells

Peripheral blood was diluted with buffered physiological saline without magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions (PAA Laboratories GmbH, Austria) in a 1:1 ratio, and built up with Gradisol L (Aqua-Med, Poland). After 20-min centrifugation in a density gradient, the interphase mononuclear cells were collected, washed twice in PBS without Ca^{2+} or Mg^{2+}, and used for further analyses.

Identification of regulatory T cells

CD4+/CD25+/FoxP3+ regulatory T cells were identified with the Human Treg Flow Kit (FOXP3 Alexa Fluor 488/CD4 PE-Cy5/CD25 PE; BioLegend), in line with the manufacturer's instructions. First, the surface antigens were labeled with anti-CD4-PE-Cy5 and anti-CD25-PE antibodies. After incubation and washing out excess unbound antibodies, the cells were subjected to fixation and permeabilization with buffers included in the kit. Subsequently, the intracellular marker FoxP3 was labeled with anti-FoxP3 Alexa Fluor 488 antibody, with murine IgG1-Alexa Fluor 488 used as an isotypic control. After incubation and washing out excess unbound antibodies, the cells were subjected to cytometric analysis (Figure 2).

Statistical analysis

Statistical analysis was conducted with Statistica 7.1 PL software (StatSoft, United States). The fractions of iden-

tified cells were expressed as medians and ranges. The Mann-Whitney U-test and Kruskal-Wallis test were used for intergroup comparisons. The differences were considered significant at $p<0.05$.

Results

Due to the relatively large differences in results and small number of patients with the earliest clinical stages of laryngeal cancer, the participants were divided into two groups: with early disease (stages I and II, n=17 patients) and with highly advanced laryngeal cancer (stages III and IV, n=33 patients).

The analyzed groups did not differ in terms of the percentage of CD3+ T lymphocytes, which was 66.99% (39.91-79.58%) in healthy individuals and 72.13% (56.54-75.26%) and 69.73% (15.99-90.47%) in patients with early and late clinical stages of laryngeal cancer, respectively (Figure 3). The percentage of CD19+ B lymphocytes in patients with advanced stages of laryngeal cancer (6.04%, 1.66-20.9%) was significantly lower than in the controls (9.26%, 4.5-17.59%; p=0.0028). In contrast, the percentage of CD19+ B lymphocytes in individuals with early clinical stages of the disease (9.59%, 5.58-15.23%) did not differ significantly when compared with the remaining groups (Figure 4). The percentage of CD4+/CD3+ T lymphocytes in the controls (75.81%, 65-89.57%) was significantly higher than in laryngeal cancer patients, both with early (55.94%, 39.1-65.56%; p=0.0037) and late stages of the disease (65.93%, 39.75-84.95%; p=0.00099) (Figure 5). The percentage of CD8+/CD3+ T lymphocytes in healthy controls (11.79%, 2.78-23.84%) was significantly lower than in patients with early (36.72%, 25.91-48.95%; p=0.0027) and late clinical stages of laryngeal cancer (28.21%, 2.64-53.59%; p=0.000097) (Figure 6). The analyzed groups did not differ significantly in terms of NK cell percentages, being 12.56% (6.3-24.16%) in healthy volunteers and 13.33% (8.32-15.07%) and 11.9% (3.05-25.19) in patients with early and late clinical stages of laryngeal cancer, respectively (Figure 7). Patients with advanced laryngeal cancer showed a significantly lower percentage of iNKT cells than the controls (0.08%, 0-0.44% vs. 0.23%, 0.06-0.94; p=0.00046). In contrast, the percentage of iNKT cells in persons with early clinical stages of the disease (0.13%, 0.08-0.32%) did not differ significantly compared with the remaining groups (Figure 8). We revealed that the percentage of regulatory T lymphocytes in patients with advanced stages of laryngeal cancer (6.91%, 2.14-15.75) was significantly higher than in persons with earlier stages of the disease (3.94%, 2.53-4.18; p=0.008) and in the controls 4.67% (2.42-8.58; p=0.0043) (Figure 9).

Moreover, we analyzed the degree of activation in effector cells. Therefore, we determined the expression of the activation markers CD69, CD25 and CD95 on CD4+/CD3+ and CD8+/CD3+ T lymphocytes. The percentage of CD4+/CD3+/CD69+ cells was found to be significantly higher in patients with advanced laryngeal cancer than in healthy controls (1.03%, 0.31-3.83% vs. 0.81%, 0.29-1.68%; p=0.04) (Figure 10). The percentage of these cells in individuals with early stages of laryngeal cancer was 0.98% (0.52-1.42%) and did

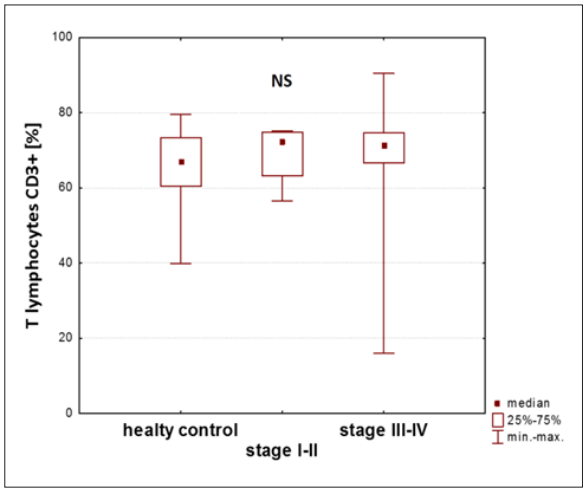

Fig. 3. Percentage of lymphocytes T CD3+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

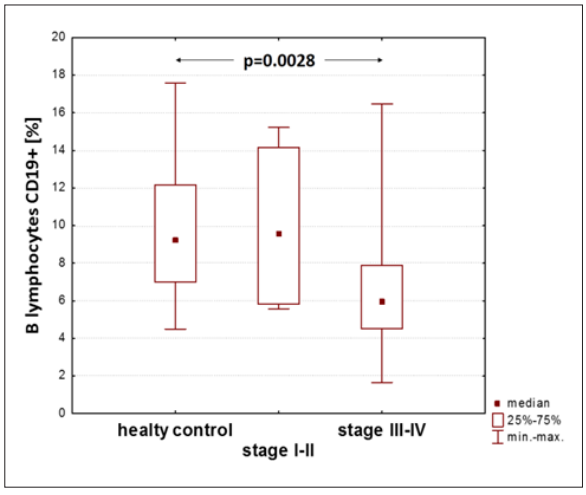

Fig. 4. Percentage of lymphocytes B CD19+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

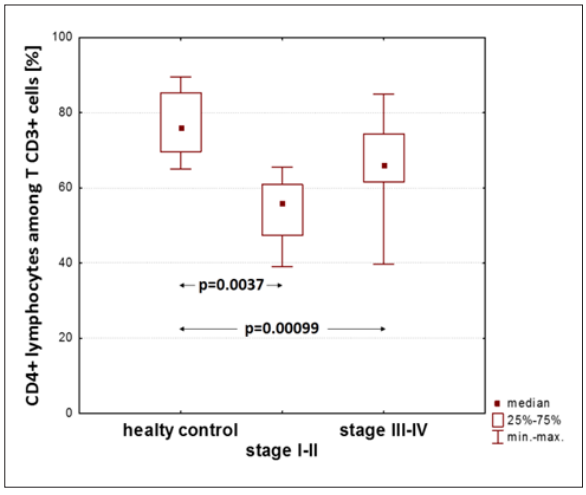

Fig. 5. Percentage of lymphocytes Th CD3+/CD4+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

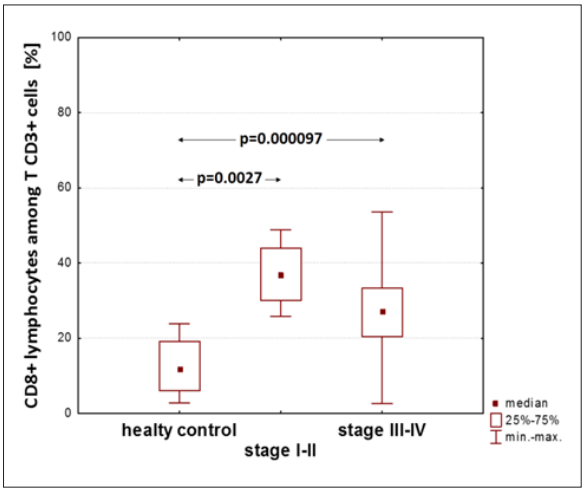

Fig. 6. Percentage of lymphocytes Tc CD3+/CD8+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

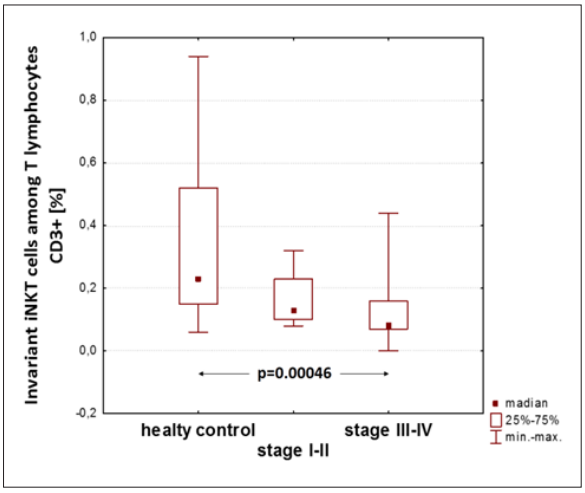

Fig. 7. Percentage of iNKT+/CD3+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

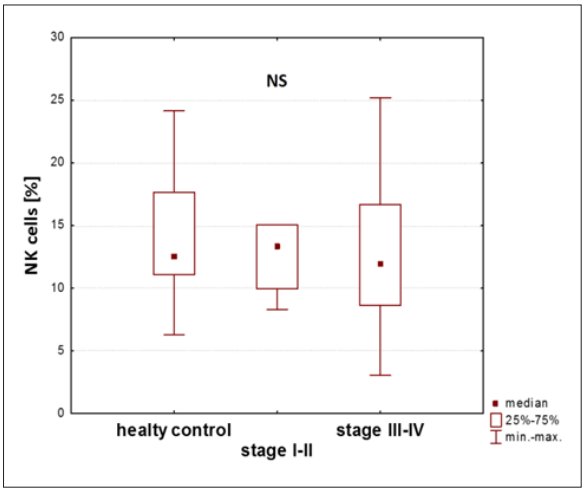

Fig. 8. Percentage of NK cells (CD3-/CD16+/CD56+) in patients with early and late clinical stages of laryngeal cancer and in healthy controls

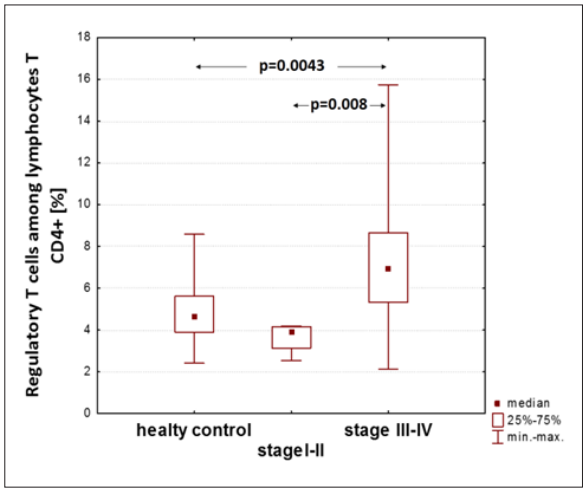

Fig. 9. Percentage of lymphocytes Treg CD4+/CD25+/FoxP3+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

not differ significantly when compared to the remaining groups. The percentage of CD8+/CD3+/CD69+ lymphocytes was significantly lower in the controls (0.51%. 0.13-1.36%) than in patients with early (11.23%, 8.9-15.1%; p=0.0027) and late stages of laryngeal cancer (6.095%, 0.18-20.35; p=0.0087) (Figure 11). The percentage of CD4+/CD3+/CD25+ T lymphocytes in patients with advanced laryngeal cancer was 16.48% (2.33-52.37%) and was significantly lower than in the controls (28.86%, 17.15-34.85%; p=0.045) (Figure 12). The percentage of these cells in persons with early laryngeal cancer (14.25%, 4.05-38.09%) did not differ significantly from the fractions documented in the remaining groups. The fraction of CD8+/CD3+/CD25+ T lymphocytes in the control group (0.87%, 0.29-1.54%) was found to be significantly lower than in patients with early laryngeal cancer (11.54%, 8.1-18.96%; p=0.0027), while the relevant fraction in individuals with advanced laryngeal cancer (7.34%, 0.13-24.84%) did not differ significantly from those documented in the other groups (Figure 13). The percentage of CD4+/CD3+/CD95+ cells in patients with advanced laryngeal cancer was 74.05% (44.63-95.25) and proved significantly higher than in healthy controls (56.31%, 49.01-76.47%; p=0.00026) (Figure 14). Also the percentage of these cells in patients with early laryngeal cancer (85.84%, 64.66-94.66) was significantly higher than in the control group (p=0.0069) (Figure 14). The percentage of CD8+/CD3+/CD95+ lymphocytes was the highest in individuals with early laryngeal cancer (92.22%, 89.42-94.52%); this value was significantly higher than in both patients with advanced disease (55.45%, 4.91-95.97; p=0.0027) (Figure 15) and healthy controls (27.53%, 15.79-37.58%; p=0.00026) (Figure 15).

Discussion

The hereby documented distribution of immune cells in peripheral blood of laryngeal cancer patients pointed to a predominance of cell response, a crucial element of cancer control. Although the percentages of all CD3+ T lymphocytes did not differ significantly between laryngeal cancer patients and the controls, a decrease in the percent-

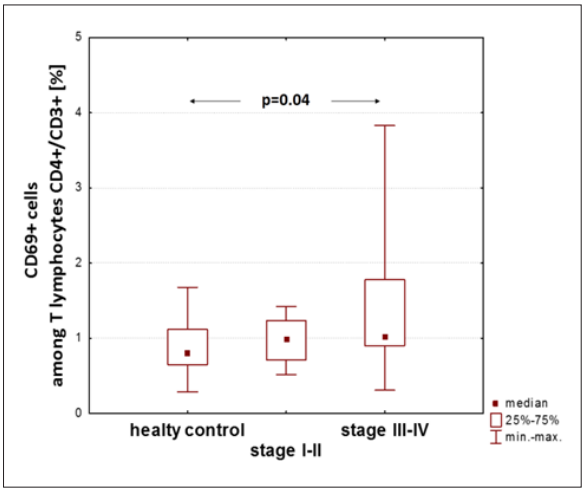

Fig. 10. Percentage of lymphocytes CD4+/CD3+/CD69+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

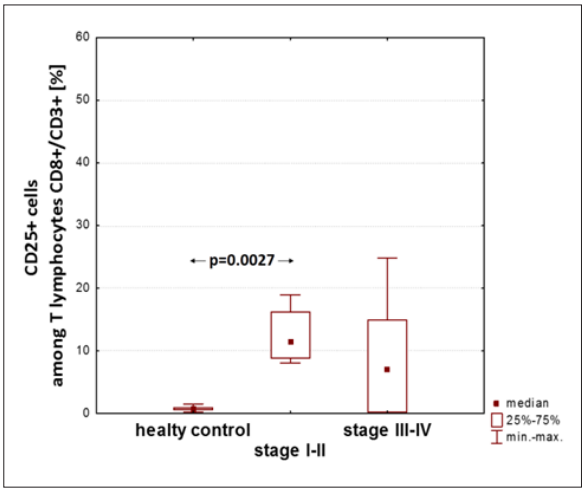

Fig. 13. Percentage of lymphocytes CD8+/CD3+/CD25+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

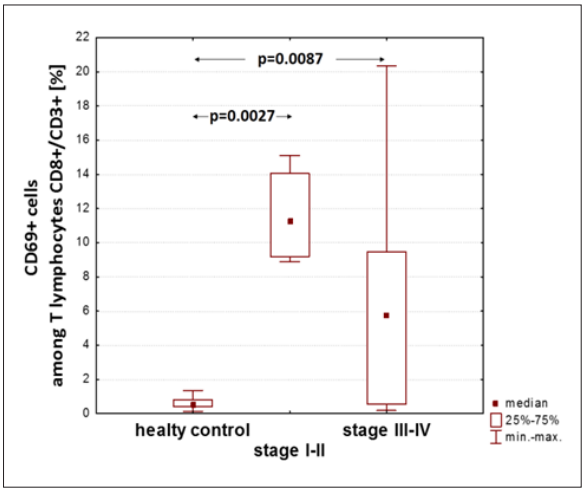

Fig. 11. Percentage of lymphocytes CD8+/CD3+/CD69+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

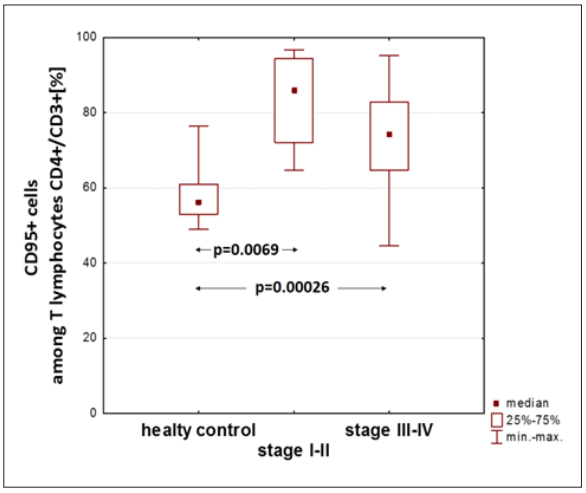

Fig. 14. Percentage of lymphocytes CD4+/CD3+/CD95+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

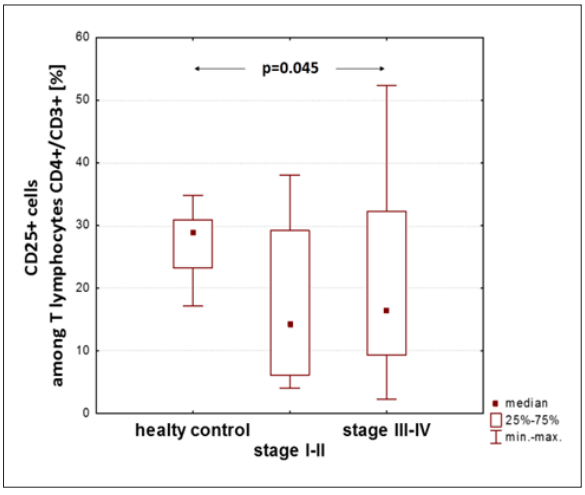

Fig. 12. Percentage of lymphocytes CD4+/CD3+/CD25+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

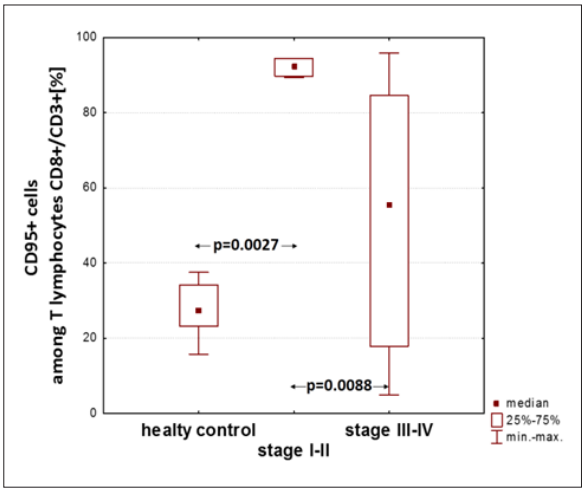

Fig. 15. Percentage of lymphocytes CD8+/CD3+/CD95+ in patients with early and late clinical stages of laryngeal cancer and in healthy controls

age of CD4+/CD3+ helper T lymphocytes was observed in the former group along with an increase in the fraction of cytotoxic CD8+/CD3+ T lymphocytes. Noticeably, the percentage of NK cells potent at spontaneous killing of cancer cells did not differ significantly between the patients and the controls, and did not change proportionally to the clinical degree of laryngeal cancer.

The subpopulation of iNKT cells in laryngeal cancer patients was found to be smaller than in healthy individuals, and decreased proportionally to the severity of the disease. A decrease in the percentage of iNKT cells was previously reported by Molling et al. [9] in patients with squamous carcinoma of the head and neck; furthermore, these authors revealed that a decreased fraction of iNKT cells is associated with shorter survival [9]. A decrease in the percentage of iNKT cells in the peripheral blood was also documented in patients with other malignancies (rectal, breast, renal, prostate and lung cancers, malignant melanoma, chronic lymphocytic leukemia) [10,11,12,28].

A number of hypotheses have been proposed to explain the underlying mechanism of the decrease in the percentage of iNKT cells in cancer patients. One potential reason is impaired proliferation and activation of these cells. Also release of suppressor compounds from neoplastic tissue, which affect survival of iNKT, was postulated. According to another hypothesis, the decreased percentage of iNKT cells in peripheral blood may result from accumulation thereof in neoplastic tissue. Finally, some authors have explained the functional impairment of iNKT cells as a consequence of decreased expression of CD1d of dendritic cells [7,26]. According to Tahir et al. [24], the functional impairment of iNKT cells documented in prostate cancer patients probably reflects the effect of cancer cells. Motohashi et al. [12] observed a decreased number of NKT cells in peripheral blood of patients with primary lung cancer, but did not document functional alterations of these cells.

We analyzed the percentage of regulatory T lymphocytes. Although we did not find a direct correlation between the percentage of these cells and the number of iNKT cells, the former subpopulation was found to be higher in laryngeal cancer patients than in healthy controls and increased proportionally to the severity of the disease. Similar findings have been reported by other authors [4,22]. In contrast, the percentage of iNKT cells in laryngeal cancer patients was lower than in the controls, and these cells were virtually absent in persons with advanced clinical stages of the disease.

Our findings regarding T cell activation suggest that early stages (I and II) of laryngeal cancer are associated with strong activation of CD8+ T lymphocytes, as the fraction of these cells expressing CD69 and CD25 was markedly higher in the patients than in the controls. The percentage of these cells in persons with advanced clinical stages of laryngeal cancer was still elevated but lower than at the early stages ($p<0.05$ in Kruskal-Wallis test for all the activation markers of CD8+/CD3+ cells). The pattern of lymphocyte T CD4+ activation was not as evident, however. The above-mentioned results are in line with those obtained by Starska et al., who confirmed the implication of early and late activation antigen expression on CD4+ and CD8+ T lymphocytes in clinicomorphological parameters of the tumor, especially TFG total score and depth of invasion, and their importance as indicators of the invasive phenotype of laryngeal carcinoma [20,21].

Our hereby reported findings point to strong activation of the immune system and its involvement in the antitumor response at early clinical stages of laryngeal cancer. However, at the advanced stages of the disease, the efficiency of the immune system is considerably reduced due to increasing activity of immunosuppressive factors. This suppressor effect is reflected by a decrease in the percentage of cytotoxic cells and impaired activation thereof, especially CD4+/CD3+/CD95+ and CD8+/CD3+/CD95+ cells. Apoptosis is one mechanism of cancer cell elimination, and interaction between CD95 and its ligand induces this process in neoplastic tissue [1,29]. Therefore, a decreased fraction of CD95+ cells may correspond to reduced cytotoxic potential of T lymphocytes.

In conclusion, our study confirmed that laryngeal cancer cells exert a strong suppressor effect on the immune system of a host. This is reflected by a decrease in the percentage of iNKT cells that are capable of cancer cell elimination, and a concomitant increase in the percentage of Tregs. However, further studies are needed in order to explain the underlying mechanisms of the immunosuppression and understand the interactions between immune and cancer cells. Understanding of factors that are responsible for immunosuppression in laryngeal cancer could constitute a basis for immunotherapy aimed at elimination or at least inhibition of these factors.

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