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ORIGINAL STUDY

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The role of tumor cells in the modification of T lymphocytes activity — the expression of the early CD69$^+$, CD71$^+$ and the late CD25$^+$, CD26$^+$, HLA/DR$^+$ activation markers on T CD4$^+$ and CD8$^+$ cells in squamous cell laryngeal carcinoma. Part I

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Abstract: The role of interactions between tumor cells and autologous immunocompetent cells, the impact on the modulation of the activity of T CD4$^+$ and CD8$^+$ lymphocytes, as well as the influence on the regulation and determination of antitumor cellular immune response in patients with head and neck squamous cell carcinomas (HNSCC) is not completely clear. The aim of this study was to analyze early and late activation antigens expression on T cells subpopulations modified under the influence of the presence of cancer cells to investigate the regulatory mechanisms of the local cellular immune response in carcinoma of the larynx. Cytofluorymetric analysis of the early (CD69$^+$, CD71$^+$) and late activation markers (CD25$^+_{high}$, CD26$^+$, HLA/DR$^+$) expression on T CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells subpopulations in mixed cellular cultures of freshly isolated tumor cells (MLTMC) and non-cancerous normal epithelial cells (MLNCC) with immunocompetent cells was performed in 55 cases of squamous cell laryngeal carcinoma. The whole peripheral blood concentrations of IL-10 and IFN-γ in 21 h and 72 h of experiments were also measured by ELISA. The relationships between the activation markers expression depending on the type of cells used in co-cultures, as well as the level of secreted cytokines, were investigated. Our work has revealed a statistically significant dependence of cytofluorymetric results on the presence of TMC or NCC in mixed cellular cultures. Increased expression of CD69$^+$, CD71$^+$ and CD25$^+_{high}$, CD26$^+$, HLA/DR$^+$ antigens on T CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells was higher in MLTMC cultures, in comparison with MLNCC. We demonstrated negative significant relationships of IFN-γ and IL-10 secretion with regard to CD4$^+$CD69$^+$, CD8$^+$CD69$^+$, CD4$^+$CD71$^+$, CD8$^+$CD71$^+$ antigens expression in 21 h of experiments without mitogenic stimulation. Furthermore, this study revealed negative significant relationships of IFN-γ secretion with regard to CD4$^+$HLA/DR$^+$ and CD8$^+$HLA/DR$^+$ as well as between IL-10 concentration and CD4$^+$HLA/DR$^+$ in trials without PHA stimulation. Our findings have confirmed a key role for tumor cells in determining the function of T cells involved in the immunological processes and impact of neoplastic cells on modulating the activity of T CD4$^+$ and CD8$^+$ lymphocytes in laryngeal carcinoma. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 4, pp. 579–592*)

Key words: squamous cell laryngeal carcinoma, T cells activation markers, regulatory cytokines

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Introduction

The role of immune cells in the course of neoplastic disease, including cancers of the head and neck region, is not completely clear.

Despite the lack of direct evidence that cells of the immune system can protect against the development and progression of cancer, clinical observations and experimental studies suggest their activity in the response against tumor cells of different origins. Unfortunately, little is known about the pathomechanisms and mutual interactions between tumor cells and the autologous immune cells i.e. the impact of neoplastic cells in modulating the function of cells involved in the immunological processes, the activity of T CD4$^+$ and CD8$^+$ lymphocytes and the regulation and determination of antitumor cellular immune response in patients with head and neck squamous cell carcinomas (HNSCC), in particular with carcinoma of the larynx. Detailed knowledge about the regulatory mechanisms influencing the final result of the immune response to tumor antigens, understanding of the interactions of cells involved in immunological processes ⇔ cancer cells, as well as indications of the immunological parameters associated with the clinical and morphological features of neoplastic infiltration, may therefore be important in determining the invasiveness of tumor lesions, and thus in assessing the clinical course, choice of optimal treatment and prognosis in patients with HNSCC. Precise knowledge of the immune status of a patient with cancer may also be one of the prognostic factors taken into consideration in selecting an appropriate model of postoperative care i.e. an indication of the extent of follow-up carried out in terms of postoperative early detection of cancer recurrence.

During the activation of T cells at their surface, the activation marker CD (cluster of differentiation) appears. Depending on the duration of activation response after the stimulus, CD markers can be divided into early stimulation (CD69$^+$ and CD71$^+$) and late activation (CD25$^+$, CD26$^+$, HLA/ /DR$^+$) antigens [1].

CD69$^+$ antigen (also called induced activator molecule, AIM, MLR3) occurs mainly in stimulated T cells, but also on B lymphocytes and macrophages. CD69$^+$ marker has the characteristics of pectin and it is involved in activating signal transduction, leading to the synthesis of various cytokines, including IL-2 and IFN-γ and the receptor for IL-2 (IL-2R) [1, 2]. CD69$^+$ is the earliest marker that is expressed on stimulated immune cells 2–4 hours after TCR/CD3 receptor stimulation [1, 2]. CD71$^+$ antigen, which occurs in proliferating cells and macrophages, is also the receptor for transferrin and the iron transporting protein. It covalently links the chain of the TCR receptor on T cells and plays a role in signal transduction pathways. CD25$^+$ antigen is one of three receptors for IL-2 (IL-2R), known as receptor α, occurring in activated T and B lymphocytes and monocytes [1]. Mitogenic or antigen stimulation of T cell causes the appearance of CD25$^+$ on the surface of immunocompetent cells [3]. CD26$^+$ (dipeptidyl peptidase IV) is a surface protein that exhibits the characteristics of a protease. It occurs constitutively in various cell types involved in immunological processes i.e. T cells, B, and monocytes. CD26$^+$ appears about 48 hours after activation on T cells. The function of this molecule is to enhance lymphocyte activation. It stimulates the activation of T cells depending on CD3 and CD2 antigens, tyrosine phosphorylation of proteins involved in signal transduction after stimulation of TCR/CD3 receptor. CD26$^+$ marker substrates are proline-containing peptides such as growth factors, chemokines, neuropeptides, and vasoactive peptides. It also acts in the phenomenon of apoptosis, playing an important role in tumor development [4, 5]. HLA/DR$^+$ antigen is expressed on B cells, macrophages and it occurs during the later stages of T lymphocytes and NK cells activation. Increased expression of HLA/DR$^+$ appears about 24 hours after stimulation [1].

The aim of this study was to analyze the activation antigens expression (early activation markers: CD69$^+$, CD71$^+$ and late activation markers: $CD25^+_{high}$, CD26$^+$, HLA/DR$^+$) on T CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells subpopulations modified under the influence of the presence of cancer cells to demonstrate the interactions between tumor cells and the autologous cells of the immune system as well as to investigate the regulatory mechanisms of the local cellular immune response in squamous cell carcinoma of the larynx.

Material and methods

Tissue samples, histological classification and morphological features. For this study of archival tissue samples, we used paraffin-embedded tissues of surgically resected specimens from 55 patients (53 men, two women, aged 48–83 years, mean age 58.3 ± 9) treated for squamous cell laryngeal carcinoma. The criteria for participation in this study were as follows: 1. histologically confirmed diagnosis of carcinoma planoepitheliale; 2. primary surgical resection without receiving prior immuno-, radio- or chemotherapy; 3. absence of distant metastases. The lesions were assessed according to the criteria of the International Union Against Cancer (UICC-TNM 2009) for head and neck carcinomas within three days of tissue collection [6]. In this study, 25.4% (14/55) of all tumors were classified as pT2 stage, 38.2%

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(21/55) as pT3, and 36.4% (20/55) as pT4. Nodal stages were histologically assessed as pN0 in 70.9% (39/55) of cases, as pN1 in 12.7% (7/55), as pN2 in 7.3% (4/55), and as pN3 in 9.1% (5/55) of cases. Histological differentiation was estimated as G1 in 7.3% (4/55), as G2 in 80% (44/55) and G3 in 12.7% (7/55) of cases of laryngeal carcinoma. The control group consisted of 51 (41 men, ten women, aged 48–84 years, mean age 57.4 ± 5) healthy volunteers. The criteria for volunteers were a negative history for autoimmune diseases, metabolic and other chronic lesions, as well as lack of immunosuppressive treatment, radio- or chemotherapy in the past. Blood and tissue samples were collected and tested for all analysis from all 55 patients and 51 healthy volunteers.

Non-cancerous/cancerous epithelium cells isolation. After radical laryngectomy, the surgical tissue specimens were excised aseptically immediately after operation from at least four tumor sites: two from the tumor centre and two from the tumor margin and two sites of normal non-cancerous laryngeal epithelium (as far as possible away from the tumor) of the same tumor patients. Fragments of tissue were washed with PBS to remove contaminated blood and inserted in RPMI 1640 medium (Biomed, Poland) supplemented with antibiotics streptomycin/penicillin/gentamycin 1% v/v (Sigma, Aldrich, Germany). The whole procedure was performed on an ice plateau. Briefly, tissue specimens were cut with a surgical knife and minced with a scalpel. This was done in RPMI 1640 medium (Biomed, Poland) supplemented with antibiotics streptomycin/penicillin/gentamycin 2% v/v (Sigma, Aldrich, Germany). Fragments of tissues were then washed three times with Hanks solution (Biomed Lublin, Poland). Next, the tumor and normal epithelial pieces were digested overnight (for 18 h) in Nunc Petri-dishes with 0.16 mg/mL hyaluronidase (Sigma, Aldrich, Germany), 0.55 mg/mL collagenase (Sigma, Aldrich, Germany) and antibiotics streptomycin/penicillin/gentamycin 1% v/v (Sigma, Aldrich, Germany) at 37°C, 5% CO_2 (Cellstar Incubator). The digested tissues were pressed gently through a 50-μm (mesh) sieve (Sigma, Aldrich, Germany) with RPMI 1640 medium (Biomed, Poland). Subsequently, the suspension was washed three times with PBS (without Mg^{2+} and Ca^{2+}) for 20 min at 8°C by centrifugation in a MPW-350R centrifuge at 1,800 rpm/500 rcf and poured over by dyspase solution 2.4 U/mL, incubated for 30 min at 37°C and resuspended in 1 mL PBS. The concentration of cells was estimated using a microscope and Bürker's chamber. To get rid of the apoptotic and necrotic cells, the columns of a Magnetic Cell Sorting Separator MACS (Miltenyi Biotec, Germany) and a Dead Cell Removal Kit was used. Cells were resuspended at a concentration of 1×10^5 cells/mL in RPMI 1640 medium. The isolated cells i.e. tumor marginal cells (TMC) and non-cancerous normal epithelial cells (NCC), were collected immediately after the procedure.

FACS analysis of early and late activation antigens on T CD4⁺ and CD8⁺ lymphocytes. Our investigations were performed with the approval of the Ethical Committee of the Medical University of Lodz, Poland and the National Science Council, Poland (No RNN/15/03/KN). Blood was collected directly before premedication into pyrogen free Heparin Li-tubes (final concentration 10 U/mL) and resuspended at a concentration of 1×10^6 cells/mL in RPMI 1640 medium (Biomed, Poland) supplemented with antibiotics streptomycin/penicillin/gentamycin 1% v/v (Sigma, Aldrich, Germany). Next, blood was incubated in 24-well flat-bottomed plates (Nunc Corp., Roskilde, Denmark) in a final volume of 0.2 mL (per well) and collected after 24 h at 37°C, 5% CO_2 (Cellstar Incubator). The experiences with the use of mitogenic stimulation with 5 μg of PHA (phytohemagglutinin) in cultures were also performed. For immunostaining, the following conjugated antibodies were used: anti-CD4 FITC labeled (clone RPA-T4), anti-CD4 PE (SK3), anti-CD8 PE (RPA-T8), anti-CD69 APC (L78), anti-CD71 APC (M-A712), anti-CD25 PE (2A3), anti-CD26 PE (L272) and anti-HLA-DR APC (L243), all provided by BD Pharmingen. 100 ml of blood was mixed and incubated for 30 min. at room temperature with appropriate quantities of antibodies or isotype controls. Erythrocyte contamination was eliminated by the addition of lysing solution (BD Bioscience) into the samples. After a short incubation and rinsing, the samples were fixed with 1% paraformaldehyde and analyzed by flow cytometry (FACSCalibur TM, CELLQuestTM software; BD Bioscience). The cell analysis and gates were restricted to lymphocytes in dot-plot. The results were expressed as mean fluorescence intensity (MFI) of the labeled surface antigens or percent positive $CD4^+$ or $CD8^+$ cells.

ELISA for IL-10 and IFN-γ measurement. Blood was collected directly before premedication (blood samples were collected before tumor samples) into pyrogen free Heparin Li-tubes (final concentration 10 U/mL). Peripheral blood mononuclear cells were checked and counted for viability using the trypan blue staining method. Next, whole blood was resuspended at a concentration of 1×10^6 cells/mL in RPMI 1640 medium and incubated in a 96-well plate in a final volume of 0.1 mL (per well). The supernatants of cultures were collected after 21 h and 72 h at 37°C, 5% CO_2 (Cellstar Incubator). Concentration of IL-10 and IFN-γ was assayed using the immunoenzymatic method ELISA using Human IL-10 (sensitivity 2 pg/mL) and IFN-γ (1 pg/mL) ELISA SET BD Opt EIA (San Diego, CA, USA). The samples' absorbance was read on an ELx808 reader at the wavelength 450 nm (BioTek Instruments, Winooski, VT, USA). The experiences with the use of mitogenic stimulation with 5 μg of PHA (phytohemagglutinin) in cultures were also performed.

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Mixed cellular cultures formation. The experiments *in vitro* were performed in the following cellular cultures of immunocompetent cells of whole blood (L — lymphocytes) with both freshly isolated tumor cells (TMC) and non-cancerous epithelial cells (NCC) (at a ratio 10:1): MLTMC — mixed autologous lymphocytes and tumor marginal cells; MLNCC — mixed autologous lymphocytes and non-cancerous cells; $MLTMC_{PHA}$ — mixed autologous lymphocytes and tumor marginal cells with mitogenic stimulation; $MLNCC_{PHA}$ — mixed autologous lymphocytes and non-cancerous cells with mitogenic stimulation.

Statistical analysis of data. None of the parameters recorded in material studied passed tests for being normally distributed (Kolmogorov–Smirnov test), and they were analyzed with nonparametric analysis of variance by ranks (Kruskal–Wallis test, Spearman test) and within group differences by *post hoc* analysis with Mann–Whitney U test and Dunnett correction for multiple comparisons. A value of $p \leq 0.05$ was considered statistically significant. All data was analyzed using STATISTICA version 9.0 (StatSoft, Poland).

Results

The activation antigens expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells subpopulations in the studied group and the control group. The early activation antigens ($CD69^+$ and $CD71^+$) and the late activation markers ($CD25^+_{high}$, $CD26^+$, HLA/DR^+) expression

In the studied group, mean values of $CD4^+$ and $CD8^+$ activation antigens expression on T cells subpopulations, measured as a percentage of T cells with positive expression, were lower compared to the control group. The mean values were (% ± SEM): 34.6 ± ± 7.16 for $CD3^+CD4^+$ and 30.2 ± 5.82 for $CD3^+CD8^+$ in patients with laryngeal squamous cell carcinoma, and 40.6 ± 7.17 for $CD3^+CD4^+$ and 31.0 ± 4.75 for $CD3^+CD8^+$ in the control group. We observed significant differences in $CD3^+CD4^+$ activation antigen expression between these two groups ($p = 0.01$). $CD4^+$/ $/CD8^+$ ratio in the study group and the control group was: 1.2 (34.6:30.2) and 1.4 (40.6:31.0), respectively. Statistically significant differences of white blood cell count (WBC) in patients with laryngeal carcinoma and control groups were not noted. WBC ratio in the analyzed groups was $8.5 \pm 2.6 \times 10^3/\mu L$ in the study group and $7.8 \pm 3.4 \times 10^3/\mu L$ in the control group.

The evaluation of $CD69^+$ and $CD71^+$, the early activation antigens and $CD25^+_{high}$, $CD26^+$, HLA/DR^+, the late activation markers expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells demonstrates the presence of significant differences between the studied group and the control group in experiments without mitogenic stimulation. Patients with laryngeal carcinoma were characterized by significantly higher values of the average expression of $CD8^+CD69^+$ ($p = 0.002$), $CD4^+CD71^+$ ($p = 0.01$) and $CD8^+CD71^+$ ($p = 0.05$). In addition, our research also confirmed significantly lower average expression of $CD4^+CD26^+$ ($p = 0.04$) and $CD8^+CD26^+$ ($p = 0.02$), as well as higher values of the average expression of $CD4^+HLA/DR^+$ ($p = 0.05$) in the studied group compared to the control group. Similar relationships concerned the early and the late activation antigens expression in experiments with mitogenic stimulation. Patients with laryngeal tumors were characterized by significantly lower values of the average expression of $CD8^+CD26^+_{PHA}$ ($p = 0.03$) and higher values of the average expression of $CD8^+CD26^+_{PHA}$ ($p = 0.03$) and $CD4^+HLA/DR^+_{PHA}$ ($p = 0.02$), compared to the control group. The mean expressions of the early and the late activation markers and the statistical test results are shown in Table 1.

To check whether the early activation antigens ($CD69^+$ and $CD71^+$) and the late activation markers ($CD25^+_{high}$, $CD26^+$, HLA/DR^+) expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells subpopulations can be modified *in vitro* under the influence of the presence of cancer cells, mixed cellular cultures were performed. Our study confirmed the interactions between tumor cells and the autologous cells of the immune system in the group of laryngeal squamous cell carcinomas. The mean expressions of the early and the late activation markers with regard to the cell types used in mixed cellular cultures (MLTMC and MLNCC) are shown in Tables 2 and 3.

The activation antigens expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells subpopulations in the mixed cellular cultures in experiments without mitogenic stimulation

Data analysis showed a statistically significant increase in the average expression of the early activation antigens when isolated epithelial tumor marginal cells (TMC), as well as normal epithelial cells of the larynx (NCC), were added to blood in the trials without stimulation. Increased expression of $CD69^+$ and $CD71^+$ antigens on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells was higher in the experiments with tumor cells (MLTMC), in comparison with the cultures of non-cancerous cells (MLNCC). The following statistically significant differences between mean values of expression of early markers on T cells subpopulations and in the analyzed cell cultures were found: increased expression of $CD4^+CD71^+$ in mixed cellular cultures MLTMC ($p = 0.02$) and MLNCC ($p = 0.02$), as well as an increase in the expression of $CD8^+CD71^+$ in MLTMC cultures ($p = 0.05$) and MLNCC ($p = 0.01$).

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Table 1. Activation antigens expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in the studied and the control group

Activation antigens	Studied group % ± SEM	Control group % ± SEM	p	Studied group % ± SEM	Control group % ± SEM	p
Early antigens	Without mitogenic stimulation			With mitogenic stimulation		
$CD4^+CD69^+$	11.1 ± 1.91	5.4 ± 1.07	NS	32.9 ± 3.6	25.5 ± 5.42	NS
$CD8^+CD69^+$	15.1 ± 2.25	5.7 ± 0.71	0.002	45.4 ± 4.08	28.2 ± 5.22	0.02
$CD4^+CD71^+$	6.4 ± 1.31	2.1 ± 0.55	0.01	27.9 ± 3.64	21.3 ± 5.09	NS
$CD8^+CD71^+$	4.3 ± 0.89	1.6 ± 0.36	0.05	22.8 ± 2.72	18.9 ± 3.96	NS
Late antigens	Without mitogenic stimulation			With mitogenic stimulation		
$CD4^+CD25^+_{high}$	10.4 ± 1.4	11.7± 1.98	NS	14.9 ±1.4	14.9 ± 1.98	NS
$CD8^+CD25^+_{high}$	9.9 ± 1.14	9.4 ± 3.00	NS	13.0 ± 1.83	10.1 ± 2.82	NS
$CD4^+CD26^+$	0.3 ± 0.05	0.5 ± 0.07	0.04	4.6 ± 0.94	5.8 ± 1.00	NS
$CD8^+CD26^+$	20.8 ± 2.35	30.6 ± 2.72	0.02	26.5 ± 2.54	36.8 ± 3.51	0.03
$CD4^+HLA/DR^+$	25.4 ± 2.17	18.4 ± 1.54	0.05	34.9 ± 2.31	25.6 ± 2.19	0.02
$CD8^+HLA/DR^+$	38.4 ± 2.05	34.9 ± 2.78	NS	45.2 ± 2.35	42.6 ± 2.53	NS

Table 2. Early activation antigens expression on $CD3^+CD^{4+}$ and $CD3^+CD8^+$ T cells in mixed cellular cultures

Activation antigens	Cellular culture	% ± SEM	Cellular culture	% ± SEM
Early antigens	Without mitogenic stimulation		With mitogenic stimulation	
$CD4^+CD69^+$	L MLNCC MLTMC	12.2 ± 2.53 14.2 ± 3.69 14.7 ± 4.57	L MLNCC MLTMC	25.3 ± 4.61 31.0 ± 5.51 32.5 ± 6.03
$CD8^+CD69^+$	L MLNCC MLTMC	18.2 ± 3.69 20.3 ± 5.02 24.6 ± 6.15	L MLNCC MLTMC	36.8 ± 5.3 44.5 ± 6.96 44.0 ± 7.13
$CD4^+CD69^+$	L MLNCC MLTMC	8.4 ± 1.79 19.2 ± 4.20 19.3 ± 4.93	L MLNCC MLTMC	25.2 ± 5.17 32.9 ± 6.78 31.7 ± 6.62
$CD8^+CD69^+$	L MLNCC MLTMC	6.5 ± 1.19 14.6 ± 4.67 16.0 ± 5.14	L MLNCC MLTMC	21.9 ± 3.48 25.9 ± 5.04 24.8 ± 5.02

No significant differences between the mean expression of early activation markers comparing cultures with isolated epithelial tumor marginal cells and normal epithelial cells of the larynx (MLTMC vs. MLNCC) in the experiments without stimulation were noted.

The evaluation of $CD25^+_{high}$, $CD26^+$, HLA/DR^+, late activation markers expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells demonstrates the presence of a significant increase of values of the average expression when TMC and NCC cells were added in trials without mitogenic stimulation. MLNCC mixed cultures were characterized by a significantly higher level of $CD8^+CD25^+$ antigens expression ($p = 0.04$), compared to cultures of T $CD3^+CD8^+$ cells. Similar relationships concerned the $CD8^+CD25^+$ activation antigens in experiments with tumor cells MLTMC ($p = 0.03$). No significant differences between the mean expression of the late activation markers comparing cultures with isolated epithelial tumor marginal cells and normal epithelial cells of the larynx (MLTMC vs. MLNCC) in the trials without stimulation were noted. The mean expressions of the activation markers with regard to experiments without mitogenic stimulation and the statistical test results are shown in Figures 1A and 1B.

The activation antigens expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells subpopulations in the mixed cellular cultures in experiments with mitogenic stimulation

Our data demonstrates a statistically significant increase in the average expression of the early activation markers when isolated epithelial tumor margin-

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Table 3. Late activation antigens expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in mixed cellular cultures

Activation antigens	Cellular culture	% ± SEM	Cellular culture	% ± SEM
Early antigens	Without mitogenic stimulation		With mitogenic stimulation	
$CD4^+CD25^+_{high}$	L MLNCC MLTMC	11.0 ± 2.29 9.9 ± 2.79 10.9 ± 2.82	L MLNCC MLTMC	14.4 ± 2.16 13.8 ± 3.5 12.9 ± 3.48
$CD8^+CD25^+$	L MLNCC MLTMC	1.7 ± 1.33 2.0 ± 1.19 2.1 ± 1.20	L MLNCC MLTMC	4.9 ± 1.49 5.5 ± 2.35 5.1 ± 1.78
$CD4^+CD26^+$	L MLNCC MLTMC	17.8 ± 3.37 20.0 ± 5.66 22.1 ± 8.3	L MLNCC MLTMC	22.3 ± 3.96 19.5 ± 6.09 20.2 ± 7.37
$CD8^+CD26^+$	L MLNCC MLTMC	5.5 ± 1.74 4.9 ± 1.61 6.2 ± 2.1	L MLNCC MLTMC	6.2 ± 1.65 4.5 ± 1.14 5.7 ± 1.34
$CD4^+$HLA/DR$^+$	L MLNCC MLTMC	28.3 ± 3.43 25.4 ± 4.04 24.4 ± 4.39	L MLNCC MLTMC	33.3 ± 5.45 33.0 ± 5.06 30.8 ± 5.5
$CD8^+$HLA/DR$^+$	L MLNCC MLTMC	37.3 ± 3.5 35.8 ± 4.65 39.7 ± 4.07	L MLNCC MLTMC	42.7 ± 3.56 44.7 ± 4.01 45.2 ± 3.91

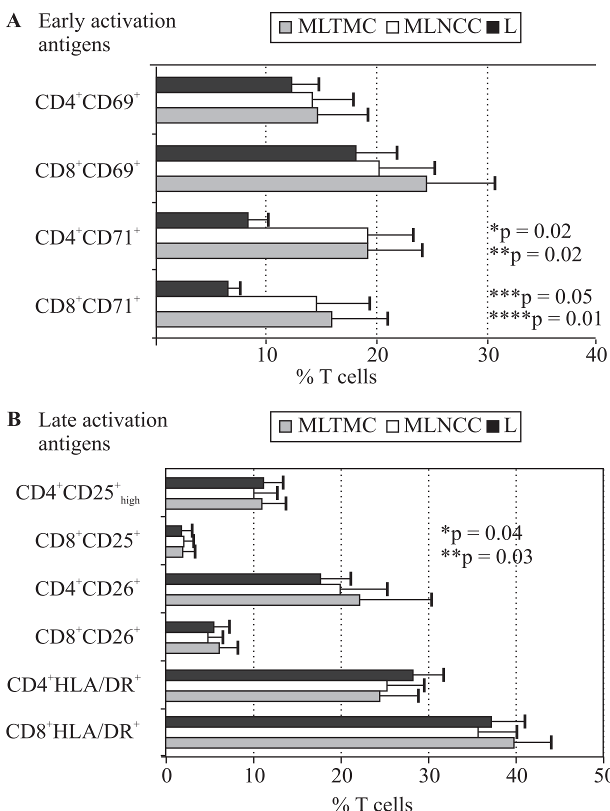

Figure 1. Mean expressions of activation markers and statistical test results with regard to various types of cells used in experiments without mitogenic stimulation. (**A**) Increased expression of $CD4^+CD71^+$ in mixed cellular cultures MLNCC (*p = 0.02) and MLTMC (**p = 0.02), as well as an increase in the expression of $CD8^+CD71^+$ in MLNCC (***p = 0.01) and MLTMC cultures (****p = 0.05) were demonstrated. (**B**) Increased expression of $CD8^+CD25^+$ in MLNCC (*p = 0.04) and in MLTMC (**p = 0.03) cultures was noted

al cells (TMC), as well as normal epithelial cells of the larynx (NCC), were added to blood in the experiments with mitogenic stimulation. Increased expression of $CD25^+_{high}$ and $CD26^+$ as well as HLA/DR$^+$ antigens on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells was higher in the mixed cultures with tumor cells ($MLTMC_{PHA}$), in comparison with the cultures of non-cancerous cells ($MLNCC_{PHA}$). We discovered statistically significant differences between mean values of $CD8^+CD71^+$ antigen expression on $CD8^+$ T cells subpopulation and in mixed cellular cultures $MLTMC_{PHA}$ ($p = 0.02$). Our results also indicated a significant difference between $CD8^+CD71^+$ on $CD3^+CD8^+$ T cells and in $MLNCC_{PHA}$ cultures ($p = 0.04$). No significant differences between the mean expression of early activation markers comparing cultures with isolated epithelial tumor marginal cells and normal epithelial cells of the larynx ($MLTMC_{PHA}$ vs. $MLNCC_{PHA}$) in the experiments with stimulation were noted.

In contrast, the evaluation of $CD25^+_{high}$, $CD26^+$, HLA/DR$^+$, the late activation markers expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells demonstrates no statistical changes of values of the average expression when TMC and NCC cells were added in trials with mitogenic stimulation. Nor were significant differences between the mean expression of the late activation markers comparing cultures with isolated epithelial tumor marginal cells and normal epithelial cells of the larynx ($MLTMC_{PHA}$ vs. $MLNCC_{PHA}$) noted. The mean expressions of the activation markers with regard to experiments with mitogenic stimulation and the statistical test results are shown in Figures 2A and 2B.

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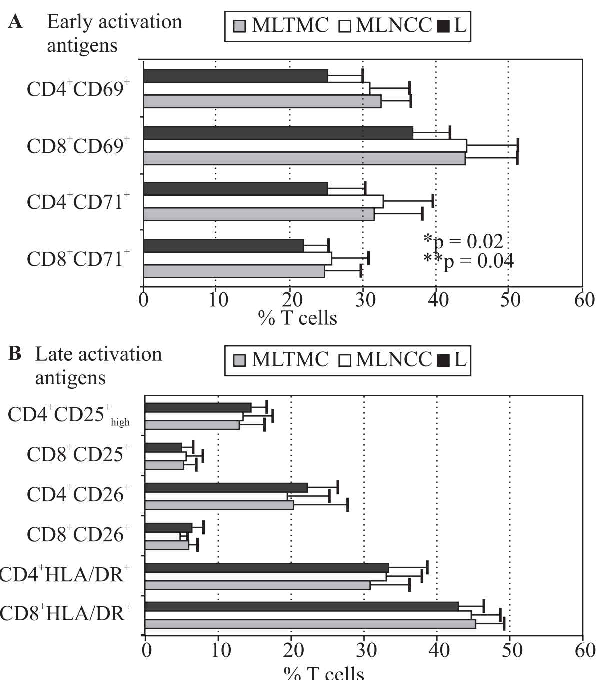

Figure 2. Mean expressions of activation markers and statistical test results with regard to various types of cells used in experiments with mitogenic stimulation. (**A**) Increased expression of $CD8^+CD71^+$ in mixed cellular cultures MLNCC (*p = 0.02) and MLTMC (**p = 0.04) were demonstrated. (**B**) No significant differences in the late activation markers expression for mixed cellular cultures were disclosed

Cytokine IL-10 and IFN-γ secretion in the studied group and the control group

We assessed cytokine IL-10 and IFN-γ secretion in whole peripheral blood at two time points (21 h and 72 h of incubation), in trials without and after mitogenic stimulation. The average concentrations of secreted cytokines in the studied group and the control group were compared. Our results indicated a significant difference of IFN-γ level in 21 h culture with PHA ($p = 0.02$) between these two groups. The supernatants from patients with laryngeal carcinoma were characterized by a significantly lower level of IFN-γ, compared to the group of volunteers. No other significant differences in experiments were noted. However, we noticed a trend towards IFN-γ lower secretion in patients with laryngeal carcinoma, both in the trials without and with stimulation, as well as towards lower production of IL-10 after stimulation. The mean cytokine concentrations in the studied groups and the statistical test results are shown in Figures 3A and 3D.

To check whether the early activation antigens ($CD69^+$ and $CD71^+$) and the late activation markers ($CD25^+_{high}$, $CD26^+$, HLA/DR^+) expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells subpopulations could be associated with IL-10 and IFN-γ secretion, we rated the cytofluorymetric assessment against their concentra-

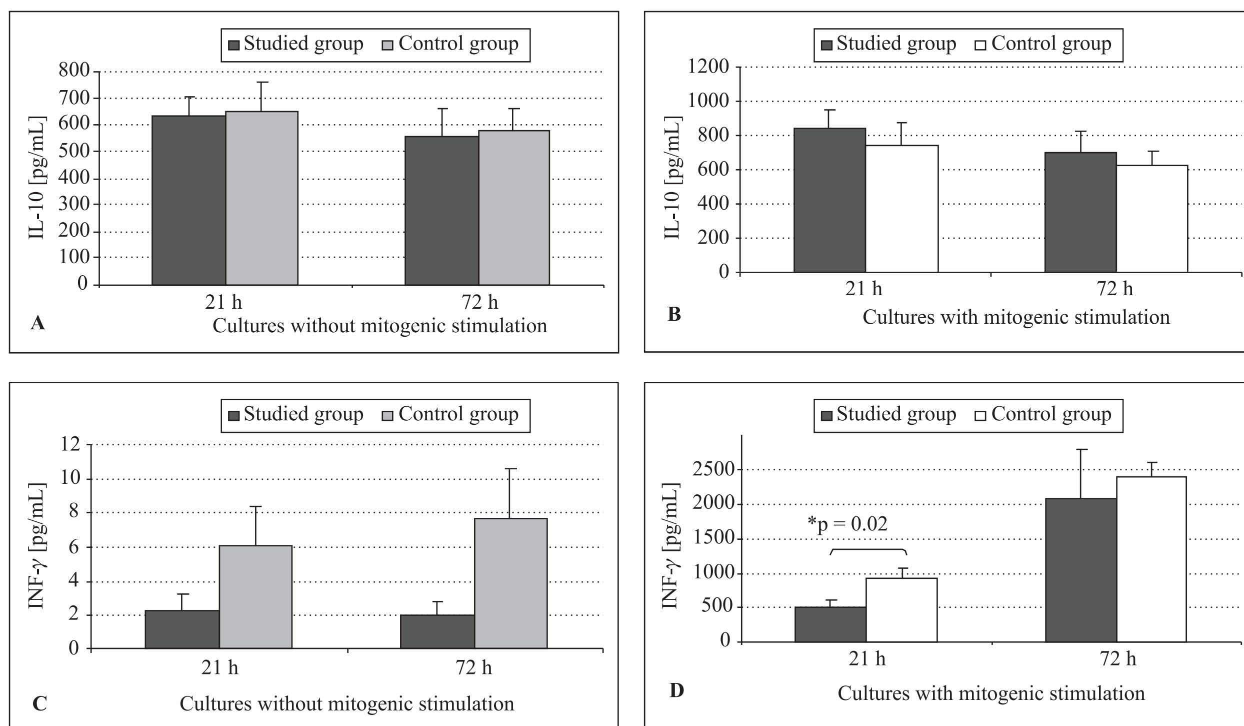

Figure 3. Mean cytokine production in the studied group and the control group. (**A–C**) No significant differences in experiments were noted, although a trend towards IFN-γ lower secretion in patients with laryngeal carcinoma, both in the trials without and with stimulation, as well as a trend towards IL-10 lower production after stimulation, were noticed. (**D**) A significant difference of IFN-γ level in 21 h culture with mitogenic stimulation (*p = 0.02) between the studied group and the control group was noted

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tions. Our study confirmed the presence of correlations between the analyzed activation markers expression and the immunoregulatory cytokine level in the group of laryngeal squamous cell carcinomas.

The relationships between the activation antigens expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells subpopulations and cytokine secretion

Our data demonstrates the presence of negative significant relationships of IFN-γ secretion with regard to the early activation antigens expression: $CD4^+CD69^+$, $CD8^+CD69^+$, $CD4^+CD71^+$, $CD8^+CD71^+$ in 21 h of experiments, as well as $CD4^+CD69^+$ in 72 h of incubation, in trials without mitogenic stimulation. In subjects with higher early activation antigens expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells subpopulations, lower IFN-γ concentrations were detected. We also noted a negative relationship between both $CD69^+$ and $CD71^+$ status on T $CD3^+CD4^+$ cells and IL-10 concentration in 21 h of incubation without mitogenic stimulation. Patients with carcinomas characterized by the highest early activation antigens expression on $CD4^+$ subpopulation were found to demonstrate lower IL-10 concentration. Moreover, in all cases of laryngeal carcinomas studied, the evaluation of cytokine expression disclosed the presence of significant positive relationships between $CD4^+CD69^+_{PHA}$, $CD8^+CD69^+_{PHA}$, $CD4^+CD71^+_{PHA}$, $CD8^+CD71^+_{PHA}$ early antigens expression and IFN-γ secretion in 21 h of experiments when stimulated with PHA. In patients with higher early activation antigens expression on $CD3^+CD4^+_{PHA}$ and $CD3^+CD8^+_{PHA}$ T cells subpopulations, higher IFN-γ concentrations were noted. In addition, we confirmed significant negative relationships between $CD4^+CD69^+_{PHA}$, $CD8^+CD69^+_{PHA}$, $CD4^+CD71^+_{PHA}$ activation markers and IL-10 concentrations in 21 h of incubation when stimulated. In contrast, the evaluation of the early activation markers expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells demonstrates the presence of significant positive relationships between $CD8^+CD69^+_{PHA}$, $CD4^+CD71^+_{PHA}$, $CD8^+CD71^+_{PHA}$ early antigens expression and IL-10 secretion in 72 h of trials with mitogenic stimulation. Patients with carcinomas with the highest activation markers expression demonstrated higher IL-10 secretion. The statistical test results concerning the early activation antigens relationships with cytokine production are shown in Table 4.

Our data demonstrates a statistically significant increase in the average expression of the late activation markers in the experiments without mitogenic stimulation. The presence of negative significant relationships of IFN-γ secretion with regard to $CD4^+HLA/DR^+$ and $CD8^+HLA/DR^+$ in 21 h of experiments and $CD8^+HLA/DR^+$ activation markers expression in 72 h of incubation was noted. Increased expression of HLA/DR^+ antigens on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells correlated with IFN-γ lower production. We also discovered a statistically significant

Table 4. Statistical test results concerningearly activation antigens' relationships with IFN-γ and IL-10 production

Cytokines	Without mitogenic stimulation				
	t	$CD4^+CD^+$	$CD8^+CD69^+$	$CD4^+CD71^+$	$CD8^+CD71^+$
IFN-γ	21 h	r = –0.67 p < 0.001	r = –0.41 p = 0.02	r = –0.55 p = 0.002	r = –0.56 p = 0.001
	72 h	r = –0.76 p = 0.02	r = –0.59 NS	r = –0.23 NS	r = –0.31 NS
IL-10	21 h	r = –0.42 p = 0.02	r = –0.31 NS	r = –0.37 p = 0.04	r = –0.34 NS
	72 h	r = –0.11 NS	r = –0.21 NS	r = –0.52 NS	r = –0.37 NS
With mitogenic stimulation					
IFN-γ	21 h	r = 0.49 p = 0.006	r = 0.51 p = 0.004	r = 0.5 p = 0.006	r = 0.46 p = 0.01
	72 h	r = –0.06 NS	r = 0.33 NS	r = 0.4 NS	r = 0.55 NS
IL-10	21 h	r = –0.44 p = 0.01	r = –0.35 p = 0.05	r = –0.36 p = 0.04	r = –0.25 NS
	72 h	r = –0.05 p = 0.89	r = –0.7 NS	r = 0.85 p = 0.004	r = 0.7 p = 0.03

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negative correlation between mean values of HLA/ /DR$^+$ antigen expression on CD4$^+$ T cells subpopulation and IL-10 secretion in 21 h of incubation, in experiments without mitogenic stimulation.

In addition, the evaluation of CD25$^+_{high}$, CD26$^+$, HLA/DR$^+$ activation markers expression on CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells demonstrates the presence of statistical changes of values of the average production of regulatory cytokines, in trials with PHA stimulation. In all laryngeal carcinomas cases studied, the evaluation of cytokine expression disclosed the presence of significant positive relationships between CD4$^+$CD26$^+_{PHA}$ early antigens expression and IFN-γ secretion in 21 h of experiments when stimulated with PHA. In patients with higher CD26$^+$ activation antigens expression on T CD3$^+$CD4$^+$ cells subpopulations, higher IFN-γ concentrations were noted. In addition, we confirmed significant negative relationships between CD4$^+$ CD25$^+_{highPHA}$ activation marker and IFN-γ concentrations in 72 h of incubation when stimulated. Patients with laryngeal carcinomas with the highest CD4$^+$ CD25$^+_{highPHA}$ activation markers expression demonstrated lower IFN-γ secretion. The statistical test results concerning the late activation antigens relationships with cytokine production are shown in Table 5.

Discussion

In experimental studies in which the epithelial tumor cells of laryngeal carcinoma, as well as the normal epithelial cells of the larynx, were added to blood culture, a significant increase in the expression of early activation antigens i.e. CD4$^+$CD71$^+$ and CD8$^+$CD71$^+$ as well as the lack of significant differences for late stimulation markers on T cells, in addition to CD8$^+$CD25$^+$ was confirmed. Confirmation of higher expression of the early activation antigens, and a significant reduction in the expression of the late activation markers on T cells in patients with squamous cell carcinoma of the larynx, compared to the group of volunteers, may indicate the cellular immune response fading and the occurrence of dysfunction in regulatory immune mechanisms in patients with cancer. The declining activity of T CD4$^+$ and CD8$^+$ cells and progressive dysfunction of regulatory mechanisms in laryngeal squamous cell carcinoma is also evidenced by no significant differences in the expression of the late activation markers in experimental studies in which the epithelial tumor cells of laryngeal cancer were added to blood culture. The results showed that in the initial phase of immune response associated with the appearance of tumor antigens, the activity of T lymphocytes, both CD4$^+$ and CD8$^+$, is intense.

The observed phenomenon may be related to the mobilization of immune mechanisms which are designed to prepare the cells involved in immunological processes for the appropriate antitumor response. It is linked, among others, with increased expression of MHC class I and MHC class II molecules, which remarkably enhance antigen presentation for T cells, activation of T lymphocytes that recognize an antigen, differentiation of T cells towards cytotoxic lymphocytes, and activation of CD8$^+$ lymphocytes and

Table 5. Statistical test results concerning early activation antigens' relationships with IFN-γ and IL-10 production

Cytokines	Without mitogenic stimulation						
	t	CD4$^+$CD25$^+_{high}$	CD8$^+$CD25$^+$	CD4$^+$CD26$^+$	CD8$^+$CD26$^+$	CD4$^+$HLA/DR$^+$	CD8$^+$HLA/DR$^+$
IFN-γ	21 h	r = 0.04 NS	r = 0.05 NS	r = 0.28 NS	r = –0.01 NS	r = –0.55 p = 0.002	r = –0.43 p = 0.02
	72 h	r = 0.4 NS	r = 0.01 NS	r = 0.07 NS	r = –0.48 NS	r = –0.59 NS	r = –0.73 p = 0.04
IL-10	21 h	r = –0.25 NS	r = 0.07 NS	r = –0.03 NS	r = 0.03 NS	r = –0.44 p = 0.01	r = –0.34 NS
	72 h	r = –0.6 NS	r = 0.05 NS	r = –0.09 NS	r = –0.3 NS	r = 0.05 NS	r = –0.21 NS
Without mitogenic stimulation							
IFN-γ	21 h	r = –0.06 NS	r = –0.29 NS	r = 0.79 p < 0.001	r = 0.06 NS	r = 0.16 NS	r = 0.04 NS
	72 h	r = –0.73 p = 0.02	r = –0.34 NS	r = 0.54 NS	r = –0.33 NS	r = 0.64 NS	r = 0.5 NS
IL-10	21 h	r = –0.24 NS	r = 0.07 NS	r = 0.01 NS	r = –0.14 NS	r = –0.16 NS	r = –0.29 NS
	72 h	r = –0.35 NS	r = 0.36 NS	r = 0.64 NS	r = –0.04 NS	r = 0.16 NS	r = 0.21 NS

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NK cells, which have the ability to direct inhibition of tumor cell proliferation and cytolytic activity against the tumor cells. It is also connected with induction of cytokines expression that stimulate the activity of Tc and Th1 lymphocytes, NK cells and interleukins which inhibit the functions of Th2 cells [3, 7–11]. The observed decrease in the percentage of T lymphocytes, both CD4+ and CD8+, reduced expression of the late activation markers, and no significant differences in expression of activation antigens in most mixed cellular cultures, points to progressive disability and decrease in the effectiveness of antitumor immune mechanisms, increased with longer duration of tumor cell interactions. Nor can we ignore the role of the suppressive effects of tumor cells on the functions of immune cells. This can lead to the phenomenon of anergy and immunological tolerance in cancer patients, and it points to the importance of regulatory CD4+ lymphocytes, demonstrating an immunosuppressive effect on effector cells [8–21].

Analysis of the relationships of the early and the late antigens expression on T CD4+ and CD8+ cells to the level of cytokines in peripheral blood also suggest complex disorders of regulatory mechanisms of the immune response. These observations are confirmed by the recorded negative correlations between the measured parameters, which indicate the absence of increased expression of the activation markers, despite the increased secretion of IFN-γ in the analyzed cultures and the need to non-specifically stimulate the immunocompetent cells, conditioning the existence of the positive correlations between the parameters of the cellular immune response. No effect of IFN-γ, which in normal conditions increases the expression of MHC molecules on the APC cells and enhances antigen presentation to lymphocytes Th, increases Tc lymphocytes cytotoxicity as well as induces expression of cytokines such as IL-1, IL-6, TNF, and thus stimulates the activity of T CD4+ and CD8+ cells, confirms the immunosuppressive role of tumor cells on T lymphocytes and indicates a defect in the immune response [3, 7–11].

In the literature, it has been difficult to find publications in which the expression of activation antigens on cells involved in immunological processes in squamous cell carcinoma of the larynx have been analyzed in detail. The results of the analysis of the early and the late antigens activation in the study group and the control group, and the findings of experiments with cultures of isolated tumor cells, do not have their counterparts in the literature. This is new scientific information in the field of immunopathology of squamous cell carcinoma of the larynx, which cannot be directly compared to data obtained by other researchers. A few publications have evaluated the expression of activation markers on the immune system cells in the group of cancers of the head and neck region. Most of these, however, concerned the analysis of tumor infiltrating cells (TIL) and lymphocytes present in regional lymph nodes and cells of different origin such as monocytes and dendritic cells, less frequently cells in the bloodstream. This does not allow direct comparisons of results obtained in patients with laryngeal cancer [13, 22–29]. In addition, the findings show large discrepancies in the assessment of the T cells activity in various types of head and neck cancers, and lead to a different conclusion regarding the expression of activation markers on T lymphocytes in patients with neoplastic disease [22–29]. This indicates the necessity of an individual approach to the immunological phenomena observed in a particular type of cancer, in order to properly interpret the results. Only a few selected publications, whose authors have adopted a similar panel of activation markers on subpopulations of T lymphocytes and similar research methods, were used.

It should be emphasized that the results of the cited publications were related to heterogeneous groups of cancers of the head and neck region, which are characterized by a different biology. This must have affected the findings obtained by investigators and the final conclusions.

Despite these limitations, studies on the pathogenesis and immunopathology of head and neck carcinomas clearly indicate the existence of complex disorders in the regulatory mechanisms of immune cellular response as well as the suppression and decreased activity of T cells in cancer patients [22–29]. One reason for the ineffectiveness of the mechanisms of immune cell response to tumor antigens may be the reduced rate and/or reduction in the number and activity of T CD4+ lymphocytes, also confirmed in this study, which has an impact on the function and activity of T CD8+ cells. CD4+ T cells play a key role in initiating and sustaining the immune responses directed against the tumor cells with T CD8+ lymphocytes. CD4+ T cells are important in preventing anergy of cytotoxic lymphocytes and are involved in the formation of memory CD8+ subpopulation, as well as stimulating macrophages and eosinophils present in the tumor stroma [30, 31]. In light of the role of T CD4+ lymphocytes in the activation of antitumor mechanisms, reducing the activity of this subpopulation of T cells results in decreased activity and abnormal cytotoxic T CD8+ cell function, but also of other cells involved in immunological processes such as CD56+ NK cells and macrophages, actively involved in the cellular response [22–29]. Evidence of suppression of

the immune response and reduction in the cytotoxic activity of defense mechanisms directed against tumor antigens, is provided by the diminished expression of surface molecules (perforins, granzymes, FasL receptors) on $CD8^+$ T cells and $CD56^+$ NK cells confirmed in studies by other authors [22]. As mentioned earlier in this discussion, the phenomenon of apoptosis of T $CD4^+$ and $CD8^+$ cells occurring in cancer patients, repopulation of lymphocytes from naïve cells as well as the immune response determined by the activity of memory cells are the resultant of immune mechanisms in cancer disease [8, 12, 23, 24, 28]. A detailed assessment of T $CD4^+$ and $CD8^+$ lymphocytes, including memory cells, naïve cells and Treg lymphocytes, was not analyzed in the studied group of squamous cell carcinoma of the larynx. However, in the context of the results and to further analyze the findings of experiments and clinical observations, it seems necessary to give a brief overview of the importance of shifts in T-cell subtypes, which impact the activity of the examined populations.

In the literature, researchers have emphasized the need for diversification of $CD4^+$ subpopulation in $CD4^+CD45RO^-CD27^+$ (naďve $CD4^+$ T cells) phenotype cells and the memory T cells ($CD4^+CD45RO^+$), to obtain objective conclusions about the activity and the number of lymphocytes circulating in patients with cancer [25, 32].

Kuss et al. [25] showed significant reductions in both studied subsets of $CD4^+$ T lymphocytes, both CD4 $CD4^+CD45RO^-CD27^+$ and $CD4^+CD45RO^+$ in patients with cancers of the head and neck. The same authors, performing research in a different group of patients, have also shown significant reductions in the number of $CD4^+$ T cells, while also highlighting the presence of characteristic shifts in the subtypes of these cells, i.e. a significant reduction in the number of $CD4^+CD45RO^-CD27^+$ and an increase in subpopulations of memory cells in the whole blood of patients with HNSCC [32]. Also important in determining the activity and the number of cells involved in immunological processes is differentiation of T $CD8^+$ cells subpopulations in the $CD8^+CD45RO^-CD28^+$ (naïve $CD8^+$ T cells) phenotype, the memory lymphocytes ($CD8^+CD45RO^+CD28^+$) and the effector cells ($CD8^+CD28^-$), the most important subpopulation in the anticancer defense mechanisms.

Tsukishiro et al. [29] showed a decrease of $CD8^+CD45RO^-CD28^+$ T cells subpopulation and an increase in the number of memory cells and the effector lymphocytes in the peripheral blood of patients with head and neck cancers. They found, however, a much higher percentage of apoptotic cells in a subpopulation of $CD8^+CD28^-$ compared to $CD8^+CD45RO^-CD28^+$, which resulted in a reversal of the ratio of $CD8^+CD45RO^-CD28^+/CD8^+CD28^-$. The observed phenomenon causes that in the peripheral blood dominate the less mature and active cells, which are recruited from naïve T cells. In addition, severe apoptosis and decrease in the percentage of $CD8^+CD28^-$ effector cells, which have the ability to cancer cells lysis and IFN-γ production in response to the presence of tumor antigens and exhibit high expression of granzyme B, explain the tumor progression. The results presented in a few publications regarding the evaluation of the expression of activation markers on T $CD4^+$ and $CD8^+$ cells in carcinomas of the head and neck region, show large discrepancies in assessing the activity of immune cells [22, 33, 34]. Differences in the conclusions reached may be associated with the aforementioned different methodologies, but also there have been insufficient studies in heterogeneous groups of head and neck cancers of different origin.

Bose et al. [22] analyzed the expression of $CD69^+$ molecules on isolated peripheral blood T cells in patients with cancers of the head and neck region and confirmed the reduced activity of immune cells, assessed in a 96 hour experience, even after stimulation with phytohemagglutinin (PHA). Aarstad et al. [33] also assessed the presence of selected activation antigens on T cells circulating in patients with HNSCC and the relationship of markers with the effectiveness of anticancer immunological mechanisms. The researchers did not find significant differences in the expression of activation markers examined in the study group and control group. Vidal-Rubino et al. [34], who evaluated the presence of $CD69^+$ molecules on T cells in regional lymph nodes with histologically confirmed lymphonodulitis reactiva, also found no significant differences in the expression of $CD69^+$ antigen on immunocompetent cells in patients with head and neck carcinomas, compared to the expression of this marker in normal structured lymph nodes in the control group.

In the literature, we found no publications featuring an analysis of the expression of HLA/DR^+ on circulating blood cells involved in immunological processes in HNSCC cancers. Most of the articles concerned the analysis of the activity of the marker on cancer cells isolated from tumor or cell lines and assessing correlation of coexpressed HLA/DR^+ on both tumor cells and immune cells, mainly on tumor infiltrating lymphocytes (TIL) [16, 35–39]. Evaluation of HLA/DR^+ on these cell types was not studied in this group of squamous cell carcinoma of the larynx, but the results presented in the literature clearly indicate the interactions and the effect of tumor cells on the

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expression of HLA/DR$^+$ activation antigens on cells of the immune system, and thus on the mechanisms of anticancer activity. Meissner et al. [37] demonstrated the relationship between the expression of MHC class II antigens on the tumor cell lines and the activity of CD4$^+$ T lymphocytes, induced by the presence of cancer antigens. However, the authors observed a lack of expression of MHC class II molecules until at 86% of cell lines, even in experiments after the induction of IFN-γ. Chikamatsu et al. [16] confirmed the relationship of HLA/DR$^+$ expression on CD4$^+$ T cells with increased secretion of IFN-γ measured in mixed cultures of immune cells with autologous tumor cells. Gomatos et al. [35] analyzed the expression of HLA/DR$^+$ on TIL and confirmed the expression of the molecules in 48.6% of laryngeal cancer cases and also showed no presence of the late activation marker on lymphocytes in the control group. The authors also pointed to a relationship of HLA/DR$^+$ antigen expression with Bax$^+$/Bcl$^-$ phenotype of both immune system cells, as well as the laryngeal cancer cell lines. Dworacki et al. [40] also confirmed the dependence of ability of T CD3$^+$CD8$^+$ lymphocytes to infiltrate tumor tissues and the activity of CD3$^+$HLA//DR$^+$ cells on the coexistence of HLA class I antigens on laryngeal cancer cells. The authors noted a higher percentage of CD3$^+$HLA/DR$^+$ TIL lymphocytes in tumors that express HLA/DR$^+$. Sikorska et al. [41] also found a correlation between the expression of HLA/DR$^+$ on the cancer cells and TIL cells (CD45RO$^+$) and the ability to infiltrate the tumor front in squamous cell carcinoma of the larynx.

The presented results confirm the importance of not only MHC class I antigens, but also MHC class II molecules on tumor cells in the antigens presentation and activation of CD8$^+$ T cells, as well as the CD4$^+$ subpopulation, which also plays an important role in the antitumor response. This is demonstrated by the studies of genetic and structural changes that lead to disturbances in expression of MHC class I molecules and MHC class II antigens on tumor cells and indicate the relationship of these aberrations with a reduced activity of effector cells and the phenomenon of 'tumor escape' against the defense mechanisms of cellular immune response [35–38, 42–45].

In assessing the activity of immune cells, the regulatory role of Treg cells with immunosuppressive properties is also important. Several investigators have confirmed the role of these cells in promoting tumor development and progression of head and neck region carcinomas [12–21, 26, 46]. It should be emphasized that the activity of immune cells is also a result of immunological mechanisms regulated by immunosuppressive regulatory Treg cells (CD4$^+$CD25^{+high}Foxp^{3+}), which exhibit inhibitory properties in particular in relation to CD8$^+$ and CD4$^+$. Regulation of immune response dependent on the suppressive Treg cells, which may be more than 30% of the subpopulation of CD4$^+$ T cells, cannot remain without influence on the activity of T CD4$^+$CD25$^-$ and CD8$^+$ cells [12, 18, 46, 47].

In the literature, the results of research on Treg activity in various cancers of the head and neck region clearly show an increase in the percentage of these cells, from 15.2% to as much as 25–30% of CD4$^+$ cells in patients with HNSCC, compared to the control group, for which a percentage of this subpopulation was 5–10% [12, 18, 46, 47]. Badoual et al. [48] analyzed the activity of T cells infiltrating the tumor stroma and the relationship between subpopulations of T lymphocytes in cancers of the head and neck region and showed a significant advantage of the number of activated T CD4$^+$CD25$^+$ cells over the regulatory CD4$^+$CD25$^+_{high}$Foxp^{3+} lymphocytes and lymphocytes showing the expression of CD4$^+$CD69$^+$ phenotype. They also observed a positive correlation between the occurrence of the number of CD4$^+$CD25$^+$ and CD4$^+$CD69$^+$ and did not show such a connection with the regulatory phenotype lymphocytes. It should be noted, however, that the regulatory cells also undergo apoptosis, which affects their activity, and indirectly their function, as well as immunological mechanisms involving other peripheral blood cells active in immune processes in cancer. Schaefer et al. [18] demonstrated in their study that Treg cells CD4$^+$CD25^{+high} in patients with cancers of the head and neck region underwent apoptosis to a much greater extent than CD4$^+$CD25$^-$, which showed a diminished ability to bind V annexin. Other authors also showed increased sensitivity of T regulatory cells to apoptosis, compared to CD4$^+$CD25$^-$ lymphocytes.

The researchers point to the phenomenon of preferential Treg movement to the tumor tissue as one of the escape mechanisms from programmed death. This implies that the circuit is dominated by less mature Treg cells, recruited from naïve CD4$^+$ T cells (CD4$^+$CD45RO$^-$CD27$^+$) or from active, highly differentiated cells that have lost the ability to migrate, as well as their helper or cytotoxic properties [18, 47]. Most authors have confirmed, however, an increased number and increased activity of CD4$^+$CD25^{+high} Treg cells, both in the tumor infiltrating lymphocytes population TIL and circulating blood cells, which to the greatest extent determines the development, progression and higher degree of invasiveness of neoplastic infiltration in patients with tumors of various origins, including patients with head and neck carcinomas [12–17, 19–21, 45–47].

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