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Dendritic cell subsets in neoplastic tissue and peripheral blood of laryngeal cancer patients: Relation with grade and stage of the disease

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Abstract. Despite improvements in immunotherapy, little is known about dendritic cell (DC) subpopulations naturally occurring in the laryngeal cancer tissue (LCT) and peripheral blood (PB) of untreated laryngeal cancer (LC) patients. The purpose of the present study was to evaluate mature, immature myeloid and plasmacytoid DCs in the LCT and PB of patients with various stages and grades of squamous cell carcinoma of the larynx (n=66) and PB of healthy donors (n=20), and to explore the correlation of the percentages of the DCs to clinical parameters. The percentage of DCs in LCT and PB was determined using monoclonal antibodies and flow cytometry. It has been revealed that DCs accumulate in LCT in comparison with their content in PB. The myeloid to lymphoid/plasmacytoid DC ratio was higher in LCT compared to PB. It was found that in cases of poorly-differentiated LC, there were higher percentages of lymphoid/plasmacytoid DCs in LCT in comparison to their content in the PB. Moreover, in the blood of patients with T4 cancers we found significantly lower percentages of myeloid DCs in comparison to individuals with T1 neoplasms. The percentage of myeloid DCs infiltrating cancer tissue positively correlated with the T stage. In patients with no metastases in the lymph nodes, PB contained less mature DCs but higher amount of myeloid DCs compared to LCT. Alteration of the DC proportion in LC patients may result in the development of immunotolerance.

Introduction

Laryngeal cancer is one of the most commonly occurring malignant cancer of the head and neck region, with squamous cell carcinoma as the predominant histological type, in Poland and in other countries (1,2). High mortality rates and constantly increasing morbidity, mainly among males, constitutes a serious socioeconomic problem (2). Equally disturbing is the observed increasing prevalence of this type of cancer in younger patients and its constantly high mortality rates and increasing morbidity especially among men. In spite of their constant development, the classical therapeutic methods, such as surgical procedures or radiotherapy are not sufficiently effective in prolonging the survival time of cancer patients and improving their quality of life (3). Therefore, the increasing morbidity and unsatisfactory effects of treatment, especially in cases of advanced laryngeal carcinoma, have prompted researchers to develop new more effective therapy schemes including immunotherapy with the use of dendritic cells (DCs) (4).

There have been a significant number of studies on DCs over the last two decades. DCs have been analyzed for their biology (5,6), the role they play in the pathogenesis of diseases in humans (7-9), their protective function against cancers, their anticancer activity (10) and their application in therapy (11-14). The progress in research on DCs, which has been motivated by hope that these cells may be used in therapies for human diseases, has resulted in a relatively wide knowledge of DCs. Nevertheless, the results of the studies on the number and role of particular DC subpopulations naturally occurring in cancer patients are still ambiguous.

The aim of this study was to assess the pathological influence of larynx cancer on DCs, by means of analyzing a percentage of immature myeloid and lymphoid DCs and mature DCs in the peripheral blood and neoplastic tissue in male patients with laryngeal cancer. A detailed analysis was applied to assess the relation between the particular subpopulations of DCs and the clinical stage of the disease, tumours mass, the presence of the glandular metastases and the degree of histological malignancy. It is believed that an ongoing neoplastic process may disturb the maturation and the functioning of DCs. The hereby discussed cells constitute a perfect material to examine the mechanism that enables cancer to escape the immune response. This is the first study that provides a detailed analysis of the DCs in such a large group of male laryngeal squamous cell carcinoma patients.

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Key words: laryngeal cancer, mature dendritic cells, immature dendritic cells, myeloid dendritic cells, plasmacytoid dendritic cells

Materials and methods

Patients. Sixty-six male patients surgically treated for primarily diagnosed squamous cell carcinoma of the larynx, without preoperative chemotherapy or radiotherapy, were included in the study. All of them were hospitalized in the Department of Otolaryngology at the Medical University of Lublin within the last 5 years. Patient characteristics are presented in Table I.

Peripheral blood from 20 healthy male donors, at the mean age of 49.57±17.59 (median 56), was used as a control. In patients and healthy donors peripheral blood white blood cells were within the normal range between 4 and 10 G/L. The diagnosis of squamous cell carcinoma of the larynx was established by histopathology of tumour samples. Patients were at different stages and grades of the disease (Table I) and were surgically treated according to their disease status: partial laryngectomy was performed in 34 (51.5% of the cases) and total laryngectomy in 32 individuals (48.5% of the cases).

None of the patients and controls had signs of infection at the time of investigation and for a month before surgery none had been taking drugs of known influence on the immune system. None of the patients or healthy donors had undergone blood transfusion. Persons with a history of allergic diseases were excluded from the study. The research protocol was approved by the Ethics Committee of the Medical University of Lublin and all patients gave written informed consent.

Table I. Patient characteristics.

Patient characteristics	n (%)
Total number of patients	66
Age (years), mean ± SD median	59.87±13.23 (60)
Grading[a]	
G1	13 (19.70)
G2	31 (46.97)
G3	22 (33.33)
T classification[b]	
T1	10 (15.15)
T2	14 (21.21)
T3	24 (36.36)
T4	18 (27.27)
Lymph node metastasis	
Yes	23 (34.85)
No	43 (65.15)
Distant metastasis	
Yes	0 (0)
No	66 (100)
Site of tumour	
Supraglottic	32 (48.48)
Glottic	25 (37.89)
Transglottic	9 (13.63)

[a]Histological differentiation of surgically resected tumour; [b]the size of the tumour and whether it has invaded nearby tissue.

Sample collection. Peripheral blood samples were collected in sterile heparinised tubes in the amount of 10 ml from all patients and controls. Tumour fragments (without necrotic areas) were taken during surgery and immersed in 0.9% NaCl solution (Polfa, Lublin, Poland). Immediately after sampling they were exposed to further processing.

Isolation of peripheral blood mononuclear cells. Mononuclear cells were separated from peripheral blood by gradient centrifugation over Gradisol-L (Aqua Medica, Poland) and washed twice in PBS (phosphate-buffered saline) (PAA, Pasching, Austria) without Ca^{2+} and Mg^{2+}, containing 0.5% BSA (bovine serum albumin) (Sigma, Germany) and 2 mM EDTA (Sigma-Aldrich, Germany).

Isolation of cancer tissue mononuclear cells. Cancer tissue, without necrotic areas, was taken during surgical treatment. Samples were homogenized using a Medimachine (Dako, Glostrup, Denmark). Mononuclear cells were separated by gradient centrifugation and washed twice in PBS without Ca^{2+} and Mg^{2+}, containing 0.5% BSA and 2 mM EDTA.

Immunophenotyping of the cells. The cell surface antigens in each case were determined on fresh cells at the time of sample submission. The following directly conjugated monoclonal antibodies were used: mouse anti-human BDCA-1 (CD1c)-FITC (Miltenyi-Biotec, Bergisch Gladbach, Germany), BDCA-2 (CD303)-FITC (Miltenyi-Biotec), CD123-PE (Becton- Dickinson, Franklin Lakes, NJ, USA), CD19-PE-Cy5 (BD Pharmingen, San Diego, CA, USA). For the the estimation of DCs maturation marker, we used the following combination of antibodies: anti-CD83 FITC/anti-CD1a PE/anti-HLA-DR PE-Cy5, all of them from BD Pharmingen. Immunofluorescence staining was prepared according to the manufacturer's protocols. Class matched isotype control was used to establish non-specific binding. Cells were collected using a three-color FACSCalibur flow cytometer equipped with 488-nm argon laser (Becton-Dickinson) and analyzed with CellQuest and FlowJo 7.5 software. We collected 300,000 total events. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI staining. The mononuclear cell analysis region was analyzed for CD1c and CD19 staining. $CD1c^+$ B cells were excluded from $CD1c^+$ blood dendritic cells by counterstaining for CD19. $CD1c^+/CD19^-$ cells were counted as immature myeloid DCs. Next, the mononuclear cell analysis region was analyzed for CD303 and CD123 antigens. $CD303^+/CD123^+$ cells were counted as immature plasmacytoid DCs. Representative sample analysis is shown in Fig. 1. Results are expressed as percentage of the cells in the mononuclear cell population.

Statistical analysis. Kruskal-Wallis ANOVA, Mann-Whitney U, Spearman rank correlation and Wilcoxon non-parametric tests, and Statistica 9 PL software were applied to statistical analysis. The results are presented in the form of mean percentage values ± standard deviation, as well as medians and minimum-maximum percentage values of the cells with the expression of a given antigen.

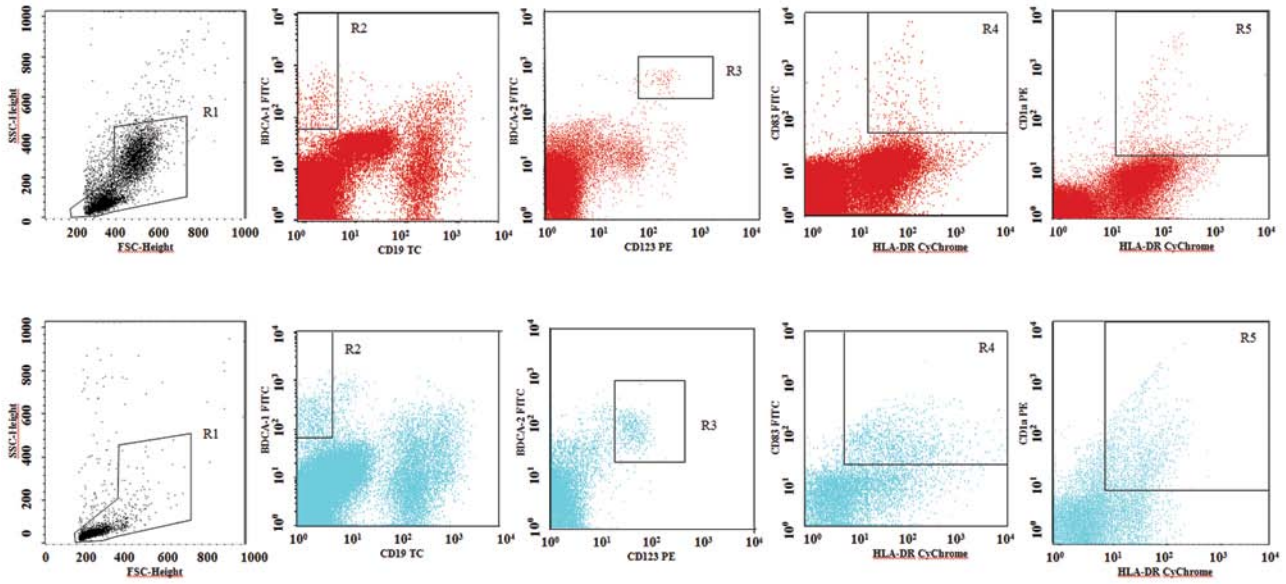

Figure 1. Cytometric analysis of dendritic cells (DCs) in peripheral blood (upper images) and tumors (lower images) of laryngeal squamous cell cancer patients. The R1 region was gated on live mononuclear leukocytes, the R2 region was set to assess myeloid DCs, the R3 region, lymphoid/plasmacytoid DCs, the R4 region, mature DCs and the R5 region, immature DCs.

Results

Immature lymphoid/plasmacytoid and myeloid, and mature dendritic cells in peripheral blood (PB) of patients with laryngeal cancer and healthy individuals. The percentage of immature CD1a$^+$/CD83$^+$/HLA-DR$^+$ DCs in PB of healthy donors was lower than in the group of patients with laryngeal cancer (0.23±0.37; median, 0.05%; 0.02-0.79% vs. 0.23±0.16; median, 0.24%; 0.01-0.62%). The percentage of mature CD1a$^-$/CD83$^+$/HLA-DR$^+$ DCs in PB of healthy donors amounted to 0.18±0.20% (median, 0.18%; 0.02-0.79%) and in patients with laryngeal cancer, 0.31±0.43% (median, 0.18%; 0.02-2.23%). The mature to immature DCs ratio amounted to 2.29±3.43 (median, 0.75; 0.25-7.42) in the control group and 1.43±1.77 (median, 0.77; 0.10-7.14) in the study group. An analysis of myeloid CD1c$^+$(BDCA-1$^+$)/CD19$^-$ DCs revealed that the mean percentage values in the control and study group were quite similar (0.35±0.27%; median, 0.26%; 0.05-1.14% vs. 0.42±0.37%; median, 0.32%; 0.03-1.67%, respectively). The myeloid to lymphoid/plasmacytoid DCs ratio amounted to 1.11±1.03 (median, 0.66; 0.16-3.46) in the control group and 2.14±6.01 (median, 1; 0.05-50) in the study group. The study of percentages of lymphoid/plasmacytoid CD303$^+$(BDCA-2$^+$)/CD123$^+$ DCs in PB of healthy donors amounted to 0.39±0.27% median, 0.34%; 0.13-1.26% and did not differ in comparison to patients with laryngeal cancer (0.50±0.71%; median, 0.34%; 0.02-4.78%). The statistical significance was not observed in the presented cases.

Immature lymphoid/plasmacytoid and myeloid, and mature dendritic cells in tumour tissue (LCT, laryngeal cancer tissue) of patients with laryngeal cancer. The percentage values of immature CD1a$^+$/CD83$^+$/HLA-DR$^+$ DCs in LCT amounted to 0.24±0.22% (median, 0.16%; 0.03-0.63%); mature CD1a$^-$/CD83$^+$/HLA-DR$^+$ DCs, 0.74±0.75% (median, 0.30%; 0.07-1.75%); myeloid CD1c$^+$(BDCA-1$^+$)/CD19$^-$ DCs, 0.41±0.48% (median, 0.20%; 0.01-1.85%); and lymphoid/plasmacytoid CD303$^+$(BDCA-2$^+$)/CD123$^+$ DCs, 1.32±2.44% (median, 0.36%; 0.01-10.35%). The mature to immature DCs ratio amounted to 1.67±1.09 (median, 2.14; 0.43-3.01) and the myeloid to lymphoid/plasmacytoid DCs ratio was 3.93±12.94 (median, 0.56; 0.01-54).

Immature lymphoid/plasmacytoid and myeloid, and mature dendritic cells in PB and LCT of patients with laryngeal cancer. The sum of lymphoid/plasmacytoid and myeloid DCs was significantly higher in LCT of patients in comparison to the sum of these cells' percentages in PB (P=0.002, Fig. 2A). Our study also revealed that the percentage of lymphoid/plasmacytoid DCs was significantly higher in LCT in comparison to their content in PB of patients suffering from laryngeal cancer (P=0.005, Fig. 2B). A myeloid to lymphoid/plasmacytoid DCs ratio was higher in LCT than in PB (P=0.013, Fig. 2C) and the percentage of mature DCs in LCT was statistically significantly higher than in PB (P=0.044, Fig. 2D).

Immature lymphoid/plasmacytoid and myeloid, and mature dendritic cells in PB and LCT of patients with different types of laryngeal cancer according to the neoplastic grading (G1, G2, G3). The percentages of the DC subsets and ratios in different types of laryngeal cancer according to the neoplastic grading are presented in the Table II. The most interesting findings are presented on the diagrams (Fig. 3).

Moreover, our study revealed that the percentage of lymphoid/plasmacytoid DCs was significantly higher in LCT in comparison to their content in the PB in patients suffering from G3 laryngeal cancer (P=0.014). Thus, myeloid to lymphoid/plasmacytoid DCs ratio was lower in LCT than in PB (P=0.022) of individuals with poorly-differentiated laryngeal cancer. What is more, the percentage of mature DCs in LCT was statistically significantly higher than in PB (P=0.040) in cases of laryngeal cancer in the intermediate grade (G2).

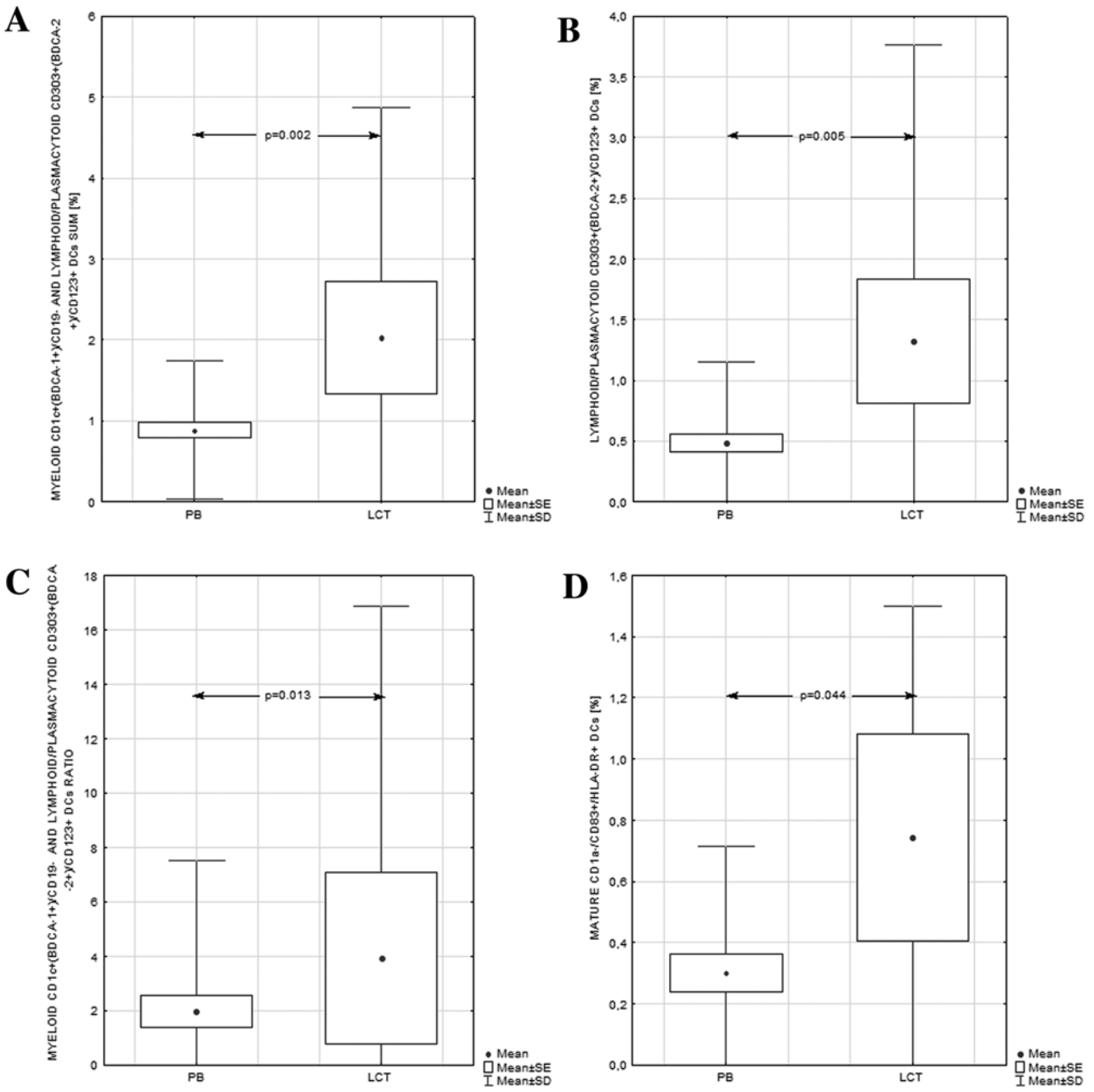

Figure 2. Immature lymphoid/plasmacytoid, myeloid, and mature dendritic cells in peripheral blood (PB) and in laryngeal cancer tissue (LCT) of the study group. Dot, median; box, 25-75 percentiles; whiskers, minimum and maximum. (A) The sum of lymphoid/plasmacytoid and myeloid DCs in PB and in LCT. (B) The percentage of lymphoid/plasmacytoid DCs in LCT and in PB. (C) The myeloid to lymphoid/plasmacytoid DCs ratio in LCT and in PB. (D) The percentage of mature DCs in LCT and in PB.

Immature lymphoid/plasmacytoid and myeloid, and mature dendritic cells in PB and LCT of patients with different T stages of laryngeal cancer according to the TNM staging system. To conduct an analysis of immature, mature, lymphoid/plasmacytoid and myeloid dendritic cells in PB and LCT of patients with different T stages of laryngeal cancer according to the TNM staging system, we divided the patients according to the size of the tumour and whether it has invaded nearby tissue, in group I (T1 and T2) and group II (T3 and T4). The results are presented in Table III.

In the blood of patients with T4 cancers we found considerably lower percentages of myeloid DCs in comparison to individuals with T1 neoplasms (P=0.019). The percentage of myeloid DCs in infiltrating cancer tissue positively correlated with the T stage (Pearson correlation, r=0.85, P=0.009; Fig. 4).

Immature, mature, lymphoid/plasmacytoid and myeloid dendritic cells in PB and LCT of patients with different N stages of laryngeal cancer according to the TNM staging system. There are four main lymph node stages in cancer of the larynx, but for our investigations, we divided the patients into two subgroups. In group I there were no lymph nodes containing cancer cells (N0) and in group II there were metastases in lymph nodes (N1, N2, N3). There were no statistically significant differences between both groups (Table IV).

Table II. Percentages and ratios of dendritic cell subsets in peripheral blood and laryngeal cancer tissue of patients with different types of laryngeal cancer according to the three-tier neoplastic grading (G1, G2, G3).

	G1			G2			G3			P-value		
Source of the dendritic cells (DCs)	Min-Max	Mean ± SD	Median	Min-Max	Mean ± SD	Median	Min-Max	Mean ± SD	Median	G2 vs. G3	G1 vs. G3	G2 vs. G3
Peripheral blood DCs												
Myeloid	0.04-1.51	0.29±0.37	0.17	0.03-0.35	1.58±0.44	0.35	0.03-0.44	1.67±0.54	0.41	0.029	0.014	NS
Lymphoid/plasmacytoid	0.02-1.23	0.29±0.31	0.17	0.02-0.90	4.78±0.58	0.34	0.03-0.49	1.85±0.57	0.48	NS	0.042	NS
Myeloid to lymphoid/plasmacytoid ratio	0.37-7.00	1.47±1.56	0.94	0.05-8.03	50.0±2.71	1.16	0.52-2.53	10.30±1.55	0.93	NS	NS	NS
Mature	0.02-0.21	0.08±0.08	0.04	0.02-0.49	2.23±0.34	0.18	0.02-0.42	1.21±0.41	0.27	0.021	0.038	NS
Immature	0.01-0.39	0.16±0.17	0.09	0.01-0.18	0.62±0.24	0.25	0.12-0.11	0.40±0.24	0.21	NS	NS	NS
Mature to immature ratio	0.10-2.00	0.74±0.74	0.47	0.23-1.76	6.25±1.53	0.85	0.17-2.43	7.14±1.78	0.83	NS	NS	NS
Laryngeal cancer tissue DCs												
Myeloid	0.10-0.50	0.22±0.16	0.17	0.01-0.49	1.71±0.43	0.26	0.07-0.72	1.85±0.61	0.46	NS	NS	NS
Lymphoid/plasmacytoid	0.09-0.43	0.20±0.16	0.14	0.01-1.51	5.11±0.86	0.32	0.17-4.23	10.35±3.54	1.44	NS	0.049	0.046
Myeloid to lymphoid/ plasmacytoid atio	0.42-1.11	0.84±0.37	1.00	0.10-17.70	54.0±6.97	0.56	0.01-0.34	0.71±0.35	0.33	NS	NS	NS
Mature	0.06-0.09	0.08±0.02	0.08	0.30-0.75	1.75±1.13	1.35	0.11-0.13	0.38±0.26	0.30	NS	NS	NS
Immature	0.05-0.16	0.09±0.06	0.06	0.07-0.24	0.63±0.33	0.29	0.03-0.25	0.39±0.21	0.21	NS	NS	NS
Mature to immature ratio	0.41-0.45	0.43±0.02	0.44	2.14-0.50	3.02±2.43	2.14	0.59-0.06	0.71±0.65	0.65	NS	NS	NS

Myeloid cells are $CD1c^+$(BDCA-1^+)/$CD19^-$; lymphoid/plasmacytoid, $CD303^+$ (BDCA-2^+)/$CD123^+$; mature, $CD1a^-$/$CD83^+$/HLA-DR^+; immature, $CD1a^+$/$CD83^+$/HLA-DR^+. NS, not significant.

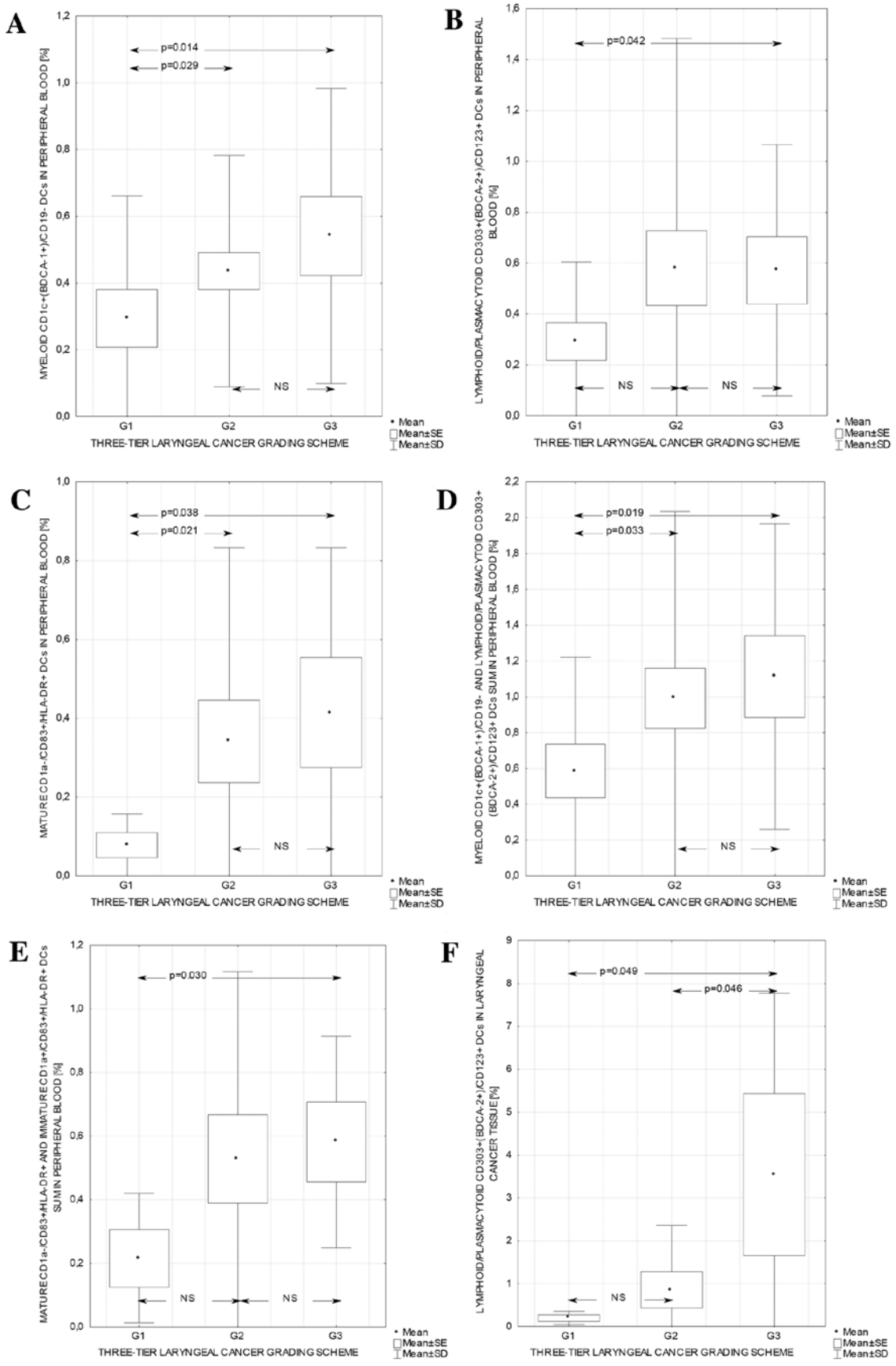

Figure 3. Immature lymphoid/plasmacytoid and myeloid, and mature dendritic cells in peripheral blood (PB) and laryngeal cancer tissue (LCT) of patients with different types of laryngeal cancer according to the neoplastic grading (G1, G2, G3). Dot, median; box, 25-75 percentiles; whiskers, minimum and maximum; NS, not statistically significant difference. (A) The percentages of myeloid $CD1c^+(BDCA\text{-}1^+)/CD19^-$ DCs, (B) the percentages of lymphoid/plasmacytoid $CD303^+(BDCA\text{-}2^+)/CD123^+$ DCs, (C) the percentages of mature $CD1a^-/CD83^+/HLA\text{-}DR^+$ DCs, (D) the sum of the percentages of myeloid and lymphoid/plasmacytoid DCs, (E) the sum of the percentages of mature and immature DCs in PB, and (F) the percentages of lymphoid/plasmacytoid DCs in LTC of patients with different grades of the squamous cell carcinoma of the larynx according to the three-tier laryngeal cancer grading scheme.

Table III. Percentages and ratios of dendritic cell subsets in peripheral blood and laryngeal cancer tissue of patients with different types of laryngeal cancer according to the TNM staging system (group I, T1+T2; group II, T3+T4).

	Group I (T1+T2)			Group II (T3+T4)			Group I vs. Group II
Source of the dendritic cells (DCs)	Min-Max	Mean ± SD	Median	Min-Max	Mean ± SD	Median	P-value
Peripheral blood DCs							
Myeloid	0.11-1.51	0.49±0.35	0.38	0.03-1.58	0.41±0.40	0.34	NS
Lymphoid/plasmacytoid	0.02-2.96	0.51±0.68	0.36	0.03-2.03	0.43±0.44	0.35	NS
Myeloid to lymphoid/plasmacytoid ratio	0.28-50.00	4.26±11.54	1.27	0.09-7.00	1.31±1.38	0.85	NS
Mature	0.02-0.42	0.16±0.12	0.14	0.02-2.23	0.29±0.55	0.14	NS
Immature	0.01-0.62	0.25±0.18	0.25	0.01-0.50	0.23±0.18	0.24	NS
Mature to immature ratio	0.10-2.00	0.85±0.59	0.76	0.24-6.25	1.60±1.91	0.77	NS
Laryngeal cancer tissue DCs							
Myeloid	0.10-0.50	0.27±0.21	0.20	0.01-1.71	0.47±0.56	0.29	NS
Lymphoid/plasmacytoid	0.09-5.11	1.99±2.72	0.14	0.01-3.04	0.59±0.96	0.17	NS
Myeloid to lymphoid/plasmacytoid ratio	0.10-1.11	0.49±0.54	0.26	0.38-54.00	7.05±17.67	0.71	0.018
Mature	1.27-1.44	1.35±0.07	1.35	0.07-1.75	0.71±0.91	0.30	0.041
Immature	0.57-0.69	0.63±0.05	0.63	0.14-0.58	0.29±0.25	0.16	0.032
Mature to immature ratio	2.04-2.24	2.14±0.10	2.14	0.44-3.02	1.87±1.31	2.14	NS

Myeloid cells are $CD1c^{+}$(BDCA-1^{+})/$CD19^{-}$; lymphoid/plasmacytoid, $CD303^{+}$ (BDCA-2^{+})/$CD123^{+}$; mature, $CD1a^{-}$/$CD83^{+}$/HLA-DR^{+}; immature, $CD1a^{+}$/$CD83^{+}$/HLA-DR^{+}. NS, not significant.

Table IV. Percentages and ratios of the dendritic cell subsets in peripheral blood and laryngeal cancer tissue of laryngeal cancer patients with different lymph node stages according to the TNM staging system (group I, N0; group II, N1+N2+N3).

	Group I (N0)			Group II (N1+N2+N3)			Group I vs. Group II
Source of the dendritic (DCs)	Min-Max	Mean ± SD	Median	Min-Max	Mean ± SD	Median	P-value
Peripheral blood							
Myeloid	0.03-1.58	0.46±0.39	0.35	0.04-1.30	0.38±0.33	0.34	NS
Lymphoid/plasmacytoid	0.02-2.96	0.50±0.61	0.37	0.03-0.64	0.33±0.21	0.35	NS
Myeloid to lymphoid/plasmacytoid ratio	0.09-50.00	2.94±8.61	1.00	0.53-3.75	1.25±0.96	0.86	NS
Mature	0.02-2.23	0.25±0.48	0.14	0.02-0.37	0.19±0.15	0.19	NS
Immature	0.01-0.62	0.22±0.16	0.23	0.01-0.50	0.30±0.21	0.37	NS
Mature to immature ratio	0.10-6.25	1.34±1.59	0.78	0.24-4.00	1.13±1.43	0.68	NS
Laryngeal cancer tissue							
Myeloid	0.01-0.50	0.20±0.16	0.17	0.12-1.71	0.73±0.66	0.44	NS
Lymphoid/plasmacytoid	0.01-5.11	1.05±1.82	0.36	0.02-3.04	0.78±1.28	0.17	NS
Myeloid to lymphoid/plasmacytoid ratio	0.10-1.11	0.61±0.42	0.39	0.48-54.00	12.13±23.48	0.71	NS
Mature	0.07-1.75	0.87±0.81	0.83	0.09-1.79	0.94±1.20	0.94	NS
Immature	0.14-0.63	0.38±0.26	0.37	0.17-0.68	0.43±0.36	0.43	NS
Mature to immature ratio	0.44-3.02	1.94±1.08	2.14	2.04-2.24	2.14±0.14	2.14	NS

Myeloid cells are $CD1c^{+}$(BDCA-1^{+})/$CD19^{-}$; lymphoid/plasmacytoid, $CD303^{+}$ (BDCA-2^{+})/$CD123^{+}$; mature, $CD1a^{-}$/$CD83^{+}$/HLA-DR^{+}; immature, $CD1a^{+}$/$CD83^{+}$/HLA-DR^{+}.; NS, not significant.

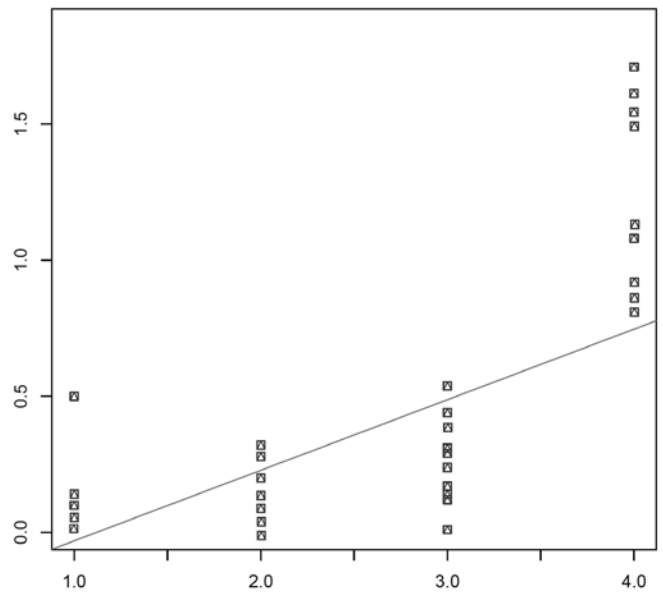

Figure 4. Diagram presenting the correlation between the percentages of myeloid dendritic cells in laryngeal cancer tissue and the T stages of laryngeal cancer according to the TNM staging system (r=0.85, p=0.009).

In patients with no metastases in the lymph nodes, the blood contains less mature DCs (P=0.047) but higher amount of myeloid DCs (P=0.010) than tumour tissue, resulting in a higher ratio of myeloid to lymphoid/plasmacytoid DCs (P=0.046).

Discussion

Interactions between DCs and growing cancer have been widely studied in order to analyze the participation of DCs the in immune response against cancer cells, and to observe the immunosuppressive influence of cancer cells on DCs (15). Many studies suggest that DCs play a significant role in the anticancer immune response (16). It has been immunohistochemically proven (17) that in cancers of the head and neck region, a small infiltration by DCs is accompanied by the presence of activated lymphocytes surrounding the DCs. In tongue cancer (18), the infiltration of the neoplastic tissue by $CD1^+$ cells is associated with a better prognosis. It has also been observed that the number of infiltrating DCs in glottic cancer is positively correlated with a better prognosis (19), while the analysis of the squamous cell carcinoma of the oral cavity does not show such correlation (20).

By focusing on squamous cell carcinoma of the larynx, and by examining a large and homogeneous group of patients, our study provides new insights into the current state of knowledge in this issue. We investigated the presence of DC subsets in peripheral blood and tumour tissue of 66 patients in various histological grades and clinical stages of the disease.

DC infiltration of solid tumours has been described for several neoplasms (18,21,22). Qin *et al* (23), similarly to us, found that DCs are highly expressed in laryngeal carcinoma tissue and are related to the process of carcinogenesis. DCs may therefore act as important indicators of laryngeal carcinoma prognosis. Wang *et al* (24) reported that tumour RNA-loaded DCs can significantly activate cytotoxic T lymphocytes (CTLs) and the antitumour specific CTLs can both induce antitumour specific immune response against laryngeal carcinoma *in vitro* and inhibit the growth of the implanted tumour *in vivo*. Probably the high amount of DCs detected in the tumour microenvironment accumulates there in order to stimulate antitumour immunity.

Impaired balance between mature and immature myeloid cells is one of the hallmarks of cancer. There is increasing evidence that progressive tumour growth is associated with accumulation of immature myeloid cells. These cells are involved in suppression of both $CD8^+$ and $CD4^+$ T cell responses (25). A positive correlation between the T stage and the amount of myeloid DCs described in our study is in agreement with previous reports.

Li *et al* (26) analysed the number of CD1a and CD83 positive DCs in cervical lymph nodes obtained from the hypopharyngeal and laryngeal carcinoma patients. They found that the amount of DCs was significantly greater in non-metastatic lymph nodes than in metastatic ones. In the metastatic lymph nodes, CDla positive DCs were predominantly detected in the cancer nest, whereas mature dendritic cells staining for CD83 antigen were prominent in the peritumour area. Our research, focused on PB and LCT, revealed that both mature and immature DCs occur in higher amounts in the tumour than in the blood. In metastatic cancer cases, the blood of our patients contained less mature DCs than LCT. We did not proceed with an analysis of DCs in lymph nodes but our results seem to conform with those described by Li *et al* (26), because the small amount of mature DCs in the blood may be due to their accumulation in the metastatic lymph nodes and peritumour area. The results of our study also confirm the findings of aforementioned and several other reports (27,28), demonstrating the presence of immature DCs in tumour tissue without molecules needed for the induction of an efficient immune response leading to their maturation.

Moreover, Hirooka *et al* (29) concluded that the percentages of myeloid DCs were significantly lower in cancer patients than in healthy volunteers, but our findings are not convergent with theirs. Laryngeal cancer seems to affect the amount of DCs in peripheral blood mononuclear cell subsets. We noticed that only the myeloid to lymphoid/plasmacytoid DCs ratio was higher in LCT than in PB.

We proceeded with a detailed analysis of DCs subsets in both PB and LCT and found that in cases of poorly-differentiated laryngeal cancers, there were higher percentages of lymphoid/plasmacytoid DCs in LCT in comparison to their content in the PB. There are a few studies presenting the relation of DCs and cancer grade (G1, G2 and G3) and such research has to be conducted in order to determine the role of DCs in different grades of cancer. Moreover, in the blood of patients with T4 cancers we found significantly lower percentages of myeloid DCs in comparison to individuals with T1 neoplasms. The percentage of myeloid DCs infiltrating cancer tissue positively correlated with the T stage.

Analysing the DCs subsets, Sakakura *et al* (4) found that the percentage of myeloid, but not plasmacytoid DCs,

is significantly lower in patients with squamous cell carcinoma of the head and neck compared to healthy donors. The aforementioned authors however, did not consider different grades and stages of laryngeal cancer. We did not observe any differences in PB of the control group and laryngeal cancer patients. However our data suggest, similarly to those reported by Whiteside *et al*, that cancers located in the head and neck region, including the larynx, are generally well-infiltrated with mononuclear leukocytes (30), consisting largely of T lymphocytes, and dendritic cells (18).

DCs play critical roles in the initiation of immune responses that, depending on the microenvironment and DC subtype (e.g., myeloid vs. lymphoid), can support either Th1- or Th2-type immune responses (31). T-helper cells play an important role in the destruction of squamous cell carcinoma of head and neck through necrosis and apoptosis, a process that includes activation of $CD8^+$ lymphocytes (32-34). They may be assisted by $S\text{-}100^+$DCs, which are also active in the presentation of tumor antigens to $CD4^+$ lymphocytes (35). A decrease in the proportion of circulating myeloid DCs has been also observed in patients with head and neck squamous cell carcinoma (36) and may be related to the presence of the tumour. Human plasmacytoid dendritic cells are present in solid tumour tissue and metastatic cervical lymph nodes in head and neck squamous cell carcinoma. Thiel *et al* (37) showed that classical plasmocytoid DC functions are heavily disturbed in the tumour microenvironment. In our patients with no metastases in the lymph nodes, PB contained less mature DCs but higher amount of myeloid DCs than LCT. Günther *et al* (38) revealed that the density of dendritic cells in rectal cancer is not a prognostic factor for metachronous distant metastasis and, therefore, cannot serve as a selection parameter for adjuvant therapy. On the other hand, there are reports about the association of carcinoma progression with a reduced frequency of blood DCs (39).

The results presented in this study allow us to conclude that DCs play an important role in tumour immunity against laryngeal carcinoma. The phenotypic antigens of DCs may constitute important indices with which the prognosis of laryngeal cancer can be predicted. Laryngeal cancer tissue is able to attract DCs by numerous cytokines which increase taxis of DCs toward the tumour, and others which simultaneously block further physiological migration of DCs. Despite the observation of disturbed circulation of DCs in other cancers, in our research statistically significant differences in PB among healthy donors and cancer patients were not noticed. The myeloid to lymphoid/plasmacytoid DC ratio was higher in the tumour tissue than in the peripheral blood and the aforementioned relationships are the most considerable in moderately- and poorly-differentiated cases of laryngeal cancer and may be related to the disturbances of plasmacytoid DC functions in the tumour microenvironment. Moreover, in tumour tissues of less advanced cases, higher percentages of mature DCs were noticed in comparison to more advanced carcinomas, but there was a strong correlation between the amount of myeloid DCs in LTC and the T stage. All these observations bring new insights to laryngeal cancer influence on human immunity and support the idea that laryngeal cancer is one of the most immunogenic solid tumours.

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