Abstract. Dendritic cells (DCs) are the most potent antigen presenting cells, which can stimulate a cellular immune response against malignant tumor cells. Many authors have described the phenomenon of tumor infiltration by dendritic cells and emphasized an immunosuppressive tumor influence on DC function. In the present study, we examined the presence of myeloid CD1c+ (BDCA-1+) dendritic cells and lymphoid/plasmacytoid CD303+ (BDCA-2+) dendritic cells in peripheral blood, lymph nodes and cancer tissue of patients with non-small cell lung cancer (NSCLC). Fifty male patients treated surgically for NSCLC stages I-IIIa without neoadjuvant chemotherapy were included. Employing a multiparameter flow cytometry for CD1c, CD19, CD123 and CD303, we observed an accumulation of immature DCs in the tissues involved in the neoplasmatic process with the predominance of lymphoid/plasmacytoid over myeloid DCs. Moreover, in peripheral blood NSCLC patients had a significantly lower percentage of CD1c+ DCs than healthy donors. Our results suggest that NSCLC cells might hamper the maturation of DCs, thus escaping an efficient immune response.

Introduction

Dendritic cells (DCs) are the most potent antigen presenting cells with an ability to prime both a primary and secondary immune response to tumor cells. Many authors have described the phenomenon of tumor infiltration by dendritic cells. A positive correlation between the grade of tumor tissue infiltration by DCs and a favorable prognosis has been observed (1-5). DCs in tumors might play a stimulating and protective role for effector T lymphocytes. Neoplastic (e.g. non-small cell lung carcinoma) cells are able to induce lymphocyte apoptosis. DCs that infiltrate tumor tissue could prevent (by costimulating molecules and secreted cytokines) tumor-specific lymphocytes from tumor-induced cell death (6). A direct inhibition of tumor proliferation and an induction of tumor cell apoptosis through DCs have also been observed in vitro and in animal models (7-9).

Most authors emphasize an immunosuppressive tumor influence on DCs function. The tumor infiltrating DCs are mainly immature and remain in this stage. This leads to a lack of effective antigen presentation (10-12). Thus, tumor tissue constitutes a ‘black hole’ which attracts and absorbs DCs. Tumor-infiltrating DCs do not migrate to lymphatic nodes after engulfing antigens, but persist in tumor tissue and become apoptotic (13,14). Neoplastic cells secrete many chemokines which attract DCs, and on the other hand, other cytokines, e.g. IL-10, block their physiological migration (15,16). The immunosuppressive tumor influence on DCs is not limited to tumor tissue. Numerous aberrations of circulating dendritic cells, decrease in the blood DCs percentage, and a lack of their maturation have been described in cancer patients (17-19). Proliferating tumor negatively influences DCs through many noxious factors: TGF-β, IL-10, VEGF, IL-4, PGE2, H2O2, NO, soluble IL-12 receptors, complement inhibitors, proteases, gangliosides, hexosamines, α-fetoprotein, fibronectin and phosphatidylserine (16,20-25).

Lung cancer is one of the most frequent neoplasms in the world (26,27). High mortality and an increasing morbidity among men and women makes this cancer a serious economical and social problem, especially in highly developed countries (26).

Since conventional therapies, such as chemotherapy, irradiation and surgery are limited, there is a fervent need for new therapeutical approaches. Immunotherapy, with the use of DCs is becoming of interest for different solid tumors, including NSCLC (28-30). DCs might not only become a useful tool in immunotherapy, but also in the understanding of tumor influence on DCs which could help to explain a tumor’s escape from an immune response.
The aim of our study was to investigate the presence and distribution of CD1c+ and CD303+ dendritic cells in NSCLC patients.

Materials and methods

Patients. Fifty male patients treated surgically for NSCLC, without preoperative chemotherapy, were included in the study. All of them were hospitalized in the Department of Thoracic Surgery at the Medical University of Lublin. The age of the patients ranged from 43 to 73 years (61.27±7.32, median 61). Peripheral blood WBC of the studied group ranged from 4.8 to 19.5 G/L (9.9±3.6, median 9.5). Peripheral blood from 17 healthy men, at the mean age of 58.42±15.04 (median 67), was used as a control. In the healthy donors, peripheral blood WBC was within the normal range between 4 and 10 G/L.

The diagnosis of NSCLC was established by histopathology of tumor samples. Patients were at different stages of the disease: 21% were at stage I, 21% at stage II, and 58% at stage IIIa. Thirty-two percent of tumors were diagnosed as planeo epithelial carcinoma, 29% as carcinoma macrocellulare, 21% as mixed cell carcinoma and 18% adenocarcinoma; 82% were at stage G2, others at G3. Patients were surgically treated according to their disease status. Lobectomy was performed in 21% as mixed cell carcinoma and 18% adenocarcinoma; 82% of tumors were diagnosed as NSCLC.

None of the patients had undergone blood transfusion. Patients with allergic diseases in anamnesis 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.

Samples. Peripheral blood (20 ml) was obtained from all patients. Tumor fragments (without necrotic areas and healthy lung tissue) and draining lymph nodes were taken during surgical treatment. Solid tissue samples of the primary tumor tissue, without necrotic areas and healthy lung tissue, were homogenized using a MediMachine (Dako, Glostrup, Denmark). Mononuclear cells were separated by gradient centrifugation and washed twice in PBS without Ca2+ and Mg2+, containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Germany) and 2 mM EDTA (Sigma-Aldrich). Draining lymph nodes and cancer tissue, without necrotic areas and healthy lung tissue, were taken during surgical treatment. Solid tissue samples were homogenized using a MediMachine (Dako, Glostrup, Denmark). Mononuclear cells were separated by gradient centrifugation and washed twice in PBS without Ca2+ and Mg2+, containing 0.5% BSA and 2 mM EDTA.

Cell immunophenotyping. In each case, cell surface antigens were determined on fresh cells at the time of sample submission. The following directly conjugated monoclonal antibodies were used: mouse anti-human BDCA-1(1CD1c)-FITC (Miltenyi-Biotec, Bergisch Gladbach, Germany), BDCA-2(1CD303)-FITC (Miltenyi-Biotec), CD123-PE (Becton Dickinson, San Jose, CA, USA) and CD19-CyChrome (BD Pharmingen, San Diego, CA, USA). Immunofluorescent staining was prepared according to the manufacturers’ protocols. A class-matched isotype control was used to establish unspecific binding. Cells were collected using a double-color FACSCalibur flow cytometer equipped with a 488-nm argon laser (Becton Dickinson), and analyzed by the CellQuest Software (Becton Dickinson).

A total of 300,000 events were collected. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI staining. BDCA-1 -CD1c marker is also expressed on a subpopulation of CD19+ small resting B lymphocytes. Mononuclear cell analysis region was analysed for CD1c and CD19 staining. CD1c+ B cells were excluded from CD1c+ blood DCs by counter-staining for CD19. CD1c+/CD19+ cells were counted as immature myeloid DCs (31). Then, the mononuclear cell analysis region was analyzed for BDCA-2 -CD303 and CD123 antigens. CD303+/CD123+ cells were considered to be immature lymphoid/plasmacytoid DCs (31). Results are expressed as the percentage of cells in the mononuclear cell gate. Cytometric analysis is shown in Fig. 1. The myeloid/plasmacytoid DCs ratio was calculated by a division of the percentage of CD1c+/CD19+ cells by the percentage of CD303+/CD123+ cells, and this parameter was used for checking any disturbances in a balance between these two subpopulations.

Statistical analysis. The fit of the data to the normal distribution was tested with the Kolmogorov-Smirnov’s test, since the distribution of the data was significantly different from normal Wilcoxon and U Mann-Whitney non-parametric tests. The Statistica 6.0 PL software (Statsoft, Krakow, Poland) was used for statistical analysis and results are shown as median and minimum-maximum values. A survival curve comparison was made with the Mantel-Cox test and is presented as mean values ± standard deviation.

Results

Assessment of the percentage of immature dendritic cells (DCs) in tissues of NSCLC patients. We evaluated the percentage of immature myeloid and lymphoid/plasmacytoid DCs, the number of both DC subpopulations (total DCs) and the ratio of myeloid and plasmacytoid cells in peripheral blood, draining lymph nodes and tumor tissue.

The percentage of myeloid CD1c+CD19+ dendritic cells was the lowest in peripheral blood (0.19%; 0.04-0.61%), but the differences among examined tissues were not statistically significant. The highest percentage of lymphoid/plasmacytoid DCs CD303+/CD123+ was detected in lymph nodes (0.69%, 0.03-18.8%). It was significantly higher (p=0.001) than the DCs content in the peripheral blood (0.23%; 0.05-0.61%), and not significantly higher (p=0.14) than in the tumor tissue (0.4%; 0.06-2.84%). The difference in the DCs content between peripheral blood and tumor tissue was statistically significant (p=0.02). The total number of both DC
subpopulations was the highest in lymph nodes (0.96%; 0.39-19.61%). It was significantly higher (p=0.003) than in peripheral blood (0.47%; 0.25-1.00%), but not significantly (p=0.47) higher in tumor tissue (0.81%; 0.19-4.34%). The difference between peripheral blood and tumor was statistically significant (p=0.04). We observed that the myeloid DCs/plasmacytoid DCs ratio was significantly lower in malignant tissue (tumor and lymph nodes). The ratio of CD1c+/CD19+ myeloid DCs to CD303+CD123+ lymphoid/plasmacytoid DCs was the lowest in lymph nodes: 0.26 (0.04-17.66), and was significantly (p=0.018) different from the peripheral blood ratio: 1.18 (0.47-3.88) and tumor tissue: 0.62 (0.17-6.33) (p=0.03). The

Figure 1. Cytometric analysis of dendritic cells (DCs) in peripheral blood, lymph nodes and tumors of NSCLC patients. The R1 region was gated on live mononuclear leukocytes and the R2 region was set to assess myeloid or lymphoid/plasmacytoid DCs, respectively. (A) Analysis of CD1c+/CD19+ myeloid DCs. (B) Analysis of CD303+/CD123+ lymphoid/plasmacytoid DCs.
difference between peripheral blood and tumor tissue was not statistically significant (p=0.33). The results are summarized in Fig. 2.

**Comparison of CD1c+ or CD303+ dendritic cells in tissues of patients with different histological types of NSCLC.** Between examined tissues there were no significant differences in the percentages of CD1c+/CD19- and CD303+/CD123+ and in the total number of CD1c+/CD19- and CD303+/CD123+ among each histological type of NSCLC, such as planoepithelial carcinoma, carcinoma macrocellulare, adenocarcinoma and mixed cell carcinoma. There was no significant difference in the CD1c+/CD19- and CD303+/CD123+ ratio among different histological types of lung cancer.

**Analysis of patient survival depending on the percentage of CD1c+ and CD303+ dendritic cells in tissues of NSCLC patients.** Patients were divided into two groups based on the values below and above appropriate median percentages of dendritic cells. We concluded that patients with a low percentage of CD1c+ dendritic cells in the tumor survive significantly longer than patients with a high percentage, respectively 42.9±15.9 and 19.2±17.2 months (Fig. 3). Other differences in survival depend on the percentage of CD1c+ and CD303+ dendritic cells in tissues of NSCLC patients.

**Comparison of immature peripheral blood DCs between surgically treated NSCLC patients and healthy donors.** There was a statistically significantly lower (p=0.009) percentage of CD1c+/CD19- and not significantly lower (p=0.41) percentage of CD303+/CD123+ in the study group in comparison with the age-matched control group of healthy men. The total number of myeloid DCs and lymphoid/plasmacytoid DCs was significantly lower in the study group (p=0.04). The myeloid DCs/plasmacytoid DCs ratio was also significantly lower in the study group (p=0.04) in comparison with the healthy donors. The results are summarized in Fig. 4.

**Discussion**

We investigated the presence of both myeloid and plasmacytoid dendritic cells (DCs) in peripheral blood, lymph nodes and tumor tissue of NSCLC patients. DC infiltration of solid
tumors has been described for several solid tumors (1-3). In pancreatic carcinoma, Dallal and associates observed a decreased level of dendritic cells or even their complete absence (32).

The results of our study confirm the findings of these reports, demonstrating the presence of immature DCs in tumor tissue without molecules needed for the induction of an efficient immune response (24-33). We observed the highest frequency of immature lymphoid/plasmacytoid and myeloid dendritic cells in draining lymph nodes, which is in conflict with the physiological status, where mature DCs in lymphoid tissue are able to stimulate the immune system. The presence of so many immature DCs can be explained by cancer development. Many authors have demonstrated a blockade of DCs maturation by the proliferating tumor, e.g. of lung cancer (34-36). Lung cancer cells secreting bombesine-like peptides (BLP) blocked the maturation and function of DCs. BLP also shortened the viability of DCs (35). Other factors considered as inhibitors of DC maturation, such as VEGF, IL-10 and gangliosides are produced by tumor cells (37-39).

It was also shown that DCs that are in direct contact with live cancer cells were not able to stimulate lymphocytes towards the lysis of tumor cells (11,40). Inhibition of DCs maturation and their functional status might constitute one of the tumor escape mechanisms. Tsujitani et al proved that tumor tissue infiltrating DCs do not prevent the spread of tumor invasion, but do prevent nodal involvement (4,41). This confirms clinical observation of tumor growth and metastasis despite the DCs presence in the tumor tissue.

The results of our study suggest that NSCLC also has a disadvantageous influence on DCs. A higher percentage of DCs in tumor tissue seems to prove that cancer tissue acts as a kind of DCs trap. Although DCs migrate to tissues involved in a neoplastic process, their maturation and function are disturbed and DCs are eventually eliminated. These findings seem to be confirmed by the analysis of the survival of patients with a low and high percentage of tumor-infiltrating myeloid dendritic cells. Patients who accumulated CD1c+ DCs in the tumor show shorter survival time than patients with a low percentage of CD1c+ in the tumor. Myeloid dendritic cells seem to be entrapped in cancer tissue and do not migrate to lymph nodes, where they could promote an anti-cancer immune response. Our results are contrary to some authors (1-5), probably due to the fact that we focused on immature dendritic cells.

We noted a prevalence of CD303+/CD123+ lymphoid/plasmacytoid DCs in tumor tissue and lymph nodes. Comparing those tissues to peripheral blood, we found that the ratio of myeloid DCs and lymphoid/plasmacytoid DCs was significantly lower in the majority of our patients and was
even inverse. This fact proves a prevalence of lymphoid/plasmacytoid line DCs in tissues involved in a neoplastic process. As lymphoid/plasmacytoid DCs physiologically stimulate humoral immune responses and immunological tolerance, this might be considered as another tumor-escape mechanism. It seems that in order to guarantee a favorable immunological response, cancer cells may secrete adequate chemotactic substances which can attract a suitable subpopulation of DCs. The presence of examined DC subpopulations was confirmed also in lung tissue non-directly affected by cancer. Demedts et al found that in mononuclear cells isolated from lung 1.18%±0.19 were CD3+/CD19+/CD1c+/HLA-DR+ and 0.57%±0.1 were CD303+/CD123+ (42,43). The percentage of plasmacytoid DCs described in normal lung tissue was comparable to our findings in the tumor, but the percentage of myeloid DCs was higher than in tumor tissue and lymph nodes.

We did not find any significant differences in examined parameters between patients at different stages of NSCLC in contrast to the observations for other cancer types, e.g. breast cancer (3,11).

We also compared the percentage of immature dendritic cells in the peripheral blood of NSCLC patients and healthy donors. We found a statistically lower percentage of DCs among NSCLC patients when compared with the healthy control group. This suggests a systemic influence of cancer on dendritic cells. A similar effect was observed in patients with cancers of the gastrointestinal tract and in patients with breast, lung and head and neck cancer (18,37,44).

A lower dendritic cell count in peripheral blood may be explained by a negative influence of cancer cells on the generation of DCs. Many authors described inhibitory effects on the generation of DCs by substances secreted by cancer cells, e.g. VEGF, IL-10, gangliosides, or PSA (25,34,45).

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We studied not only the absolute amount of DCs in peripheral blood, but also their lymphoid/plasmacytoid and myeloid subpopulations. A decrease in the entire amount of DCs was mainly due to a significant decrease in myeloid DCs, whereas the lymphoid/plasmacytoid subpopulation was significantly higher in the NSCLC patients. Similar results were found by Sakakura et al (46) and Hoffmann et al (47) in squamous cell carcinoma of the head and neck. In summary, our data suggest that the development of NSCLC promotes the generation and survival of lymphoid/plasmacytoid DCs. Thus it may constitute a tumor escape mechanism, since lymphoid/plasmacytoid DCs promote immunological tolerance.

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