CD11c\(^+\) and CD123\(^+\) Dendritic Cell Subsets in Peripheral Blood of Lung Cancer Patients

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Dendritic cells (DCs) play a central role in antitumor immune responses. Recent studies however have emphasized an immunosuppressive tumor influence on DCs in various types of cancer. We evaluated the percentages of myeloid and plasmacytoid related DCs in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Myeloid CD11c\(^+\) DCs (mDC) and plasmacytoid CD123\(^+\) DCs (pDC) cells were assessed by Flowcytometry in peripheral blood of twenty untreated lung cancer patients (13 NSCLC and 7 SCLC) and 15 healthy subjects. Lower percentages of pDCs and mDCs were found in patient with NSCLC and SCLC as compared to controls, with significant value only between NSCLC patients and controls (\(P=0.001\) and \(P=0.000\) respectively). The percentages of pDCs and mDCs subsets were significantly lower in patient with SCLC than NSCLC (\(P=0.013\) and \(P=0.005\) for pDCs and mDCs respectively). Our results suggest that NSCLC and SCLC might hamper the maturation of DCs, thus escaping an efficient immune response.

DCs represent a system of hematopoietic cells that are rare but ubiquitously distributed throughout the peripheral tissues. Their journey starts in the bone marrow where, at least, two DC lineages have been identified so far, namely the conventional myeloid-related DCs and the newly defined lymphoid/plasmacytoid related DCs (Nestle \textit{et al.}, 2001). These DCs are circulating in an immature form in the human blood, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) that can be identified on the basis of phenotypic markers and different function (Shortman & Liu, 2002). In particular mDCs express CD11c marker and they require granulocyte macrophage colony stimulating factor (GM-CSF) for growth and functions such as antigen uptake, T-cell activation and secretion of interleukin (IL)12 and (IL)18. pDCs express CD123 marker, they are dependent on IL3 for survival and they produce high levels of interferon (IFN)-\(\alpha\) (Shortman & Liu, 2002; MacDonald \textit{et al.}, 2002). Both populations migrate to the blood and become immature DCs, which wander through tissues where they monitor the invading pathogens (Lotze, 2001). 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 (Tsuge \textit{et al.}, 2000; Ivan \textit{et al.}, 2007).

Lung cancer is one of the most frequent neoplasm in the world (Radzikowska \textit{et al.}, 2002; Hill \textit{et al.}, 2003). High mortality and an increasing morbidity among men and women make this cancer a serious economical and social problem, especially in highly developed countries (Hill \textit{et al.}, 2003). Immunotherapy, with the use of DCs is becoming of interest for different solid tumors, including NSCLC (Iwashita \textit{et al.}, 2003; Hirschowitz \textit{et al.}, 2004). 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 (Krawczyk \textit{et al.}, 2007). The important role of DCs in cancer is underscored by number of reports in which the presence of dendritic cells in tumor tissues was associated with good clinical prognosis of disease (Lotze, 2001). However in recent years several groups have described defective
functions of DCs in tumor-bearing mice in cancer patients (Almand, 2001; Wojas et al., 2004). These findings call our attention to circulating population of dendritic cells. In this pilot study we evaluated the percentage of myeloid- and plasmacytoid-related DCs in peripheral blood of patients with non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

**Subjects and Methods**

The study population comprised of 13 NSCLC (10 males and 3 females) and 7 SCLC patients (5 males and 2 females) with mean age, 52.9±11.04 years from the National Cancer Institute- Cairo University. The histopathology of lung cancer type was done for all patients. Laboratory tests, chest X-rays, computed tomography, ultrasonography, and bronchoscopy were used for the evaluation of the lung cancer stage and type of disease manifestation. Stages from I to IIIA were specified as local disease, whereas stages IIIB and IV were specified as generalized lung cancer (Table 1).

All patients had advanced cancer with no prior chemotherapy. 15 healthy age-matched volunteers served as controls. None of the patients had signs of infection at the time of investigation and none had been taking drugs of known influence on the immune system. None of the patients had undergone blood transfusion. Patients with allergic diseases were excluded from the study. The research protocol was approved by the Ethics Committee of the National Cancer Institute Cairo University. Informed consent was obtained from each individual. All peripheral blood samples were homogeneously collected in the morning using EDTA.

<table>
<thead>
<tr>
<th>Total number of patients:</th>
<th>20</th>
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<tbody>
<tr>
<td>Age (years) (mean ±SD, range):</td>
<td>52.9±11.04 (38-66)</td>
</tr>
<tr>
<td>Pathological classification</td>
<td></td>
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<tr>
<td>- NSCLC (Localized clinical stage) e.g.:</td>
<td>13 (10 males and 3 Females)</td>
</tr>
<tr>
<td>Squamous cell carcinoma Grade 2:</td>
<td>2</td>
</tr>
<tr>
<td>Squamous cell carcinoma Grade 3:</td>
<td>5</td>
</tr>
<tr>
<td>Adenocarcinoma Grade 2:</td>
<td>4</td>
</tr>
<tr>
<td>Undifferentiated*:</td>
<td>2</td>
</tr>
<tr>
<td>- SCLC (metastasis clinical stage):</td>
<td>7 (5 males and 2 Females)</td>
</tr>
</tbody>
</table>

NSCLC= Non small cell lung cancer, SCLC= Small cell lung cancer, *=undifferentiated and unclassified NSCLC.

**Direct Immunofluorescence Staining of Whole Blood Cells**

Circulating pDCs and mDCs DCs subsets were enumerated and quantified using a four color flowcytometry (Becton Dickinson, BD, USA). All monoclonal antibodies (mAbs) were purchased from Becton Dickinson (BD Biosciences Pharmingen, Italy).

Whole blood samples were collected using EDTA tubes. Each sample (0.05 ml) was directly stained with the following monoclonal antibodies; anti CD11c-APC, anti-CD123-PE (Anti–IL-3Rα) and Lineage-FITC cocktail (lin 1) which is composed of {anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and anti-CD56}. Peripheral blood dendritic cells and basophils were distinguished from other leucocytes by their lack of staining with lin 1. Relevant isotype controls (mAbs) were used. Tubes were mixed gently, and incubated for 15 min in the dark, at room temperature (RT). To each tube 2 mL of 1X FACSTM Lysing solution were added, tubes were vortexed and incubated for 10 min at RT. The mixture was centrifuged at 300 Xg for 5 minutes. Cells were washed with 1 mL of 1X phosphate-buffered saline (PBS) and analyzed.

**Flowcytometric Analysis and Data Acquisition**

Dendritic cells were analyzed using a FACSCalibur Flowcytometer (Becton Dickinson) equipped with 488-nm argon laser. An acquisition gate was established based on forward scatter (FSC) and SSC that included
both the lymphocyte and monocyte populations (mononuclear cells) and excluded dead cells and debris. Each measurement contained 50,000 total events (R1). These events were displayed in a second dot plot to identify lineage-negative (R2 gate). To define DCs subsets (mDC and pDC), contour plots of CD11c versus Lineage-FITC cocktail and CD123 versus Lineage-FITC cocktail were created respectively. Figure 2.

### Statistical Analysis

Results presented as mean values ± standard deviation, median and minimum-maximum values, unless otherwise stated. Comparisons between groups were analyzed using one-way ANOVA test and Tekuk's test. $P<0.05$ was considered significant.

### Results

#### Enumeration of DC Subset in NSCLC

Circulating pDCs and mDCs were counted in all subjects. A statistically significant reduction in percent of pDCs was found in patients when compared to healthy controls. The median value ± SD of pDCs in patients was $0.895 ± 0.385$ (range 0.360–1.460) versus $1.004 ± 0.306$ (range 0.660–1.610) in healthy subjects ($P=0.002$). Similar results were obtained with mDCs (median ± SD 0.388 ±0.196 (range 0.160–0.720) vs.0.480±0.075 (range 0.390–0.620) $P=0.000$. There was no significant difference in the pDCs and mDCs ratio among different histological types of NSCLC (Fig. 1).

#### Enumeration of DC Subsets in SCLC

pDCs and mDCs percentages were lower in patients with SCLC when compared to localized disease NSCLC. In SCLC the median value of pDCs and mDCs was $0.455 ± 0.195$ (range 0.280–0.840), and $0.191% ± 0.025$ (range 0.140–0.230) respectively versus $1.0040% ± 0.196$ (range 0.280–0.840) and $0.480±0.075$ (range 0.390–0.620) $P=0.000$. There was no significant difference in the pDCs and mDCs ratio among different histological types of NSCLC (Fig. 1).

#### Comparison between NSCLC and SCLC

When the localized NSCLC and metastatic SCLC were compared with healthy controls, significant differences were found between healthy controls and the metastatic group in both pDCs and mDCs % ($P=0.001$ and $P=0.000$ respectively) but not between healthy controls and the localized group ($P=0.175$ and $P=0.662$ respectively) (Fig. 1). The percentages of DCs subsets were also lower in SCLC than in NSCLC patients with significant value in percentage of pDCs and mDCs $P=0.013$ and $P=0.005$ respectively.

**Figure 1.** Comparison of number (%) of plasmacytoid (top) and myeloid (bottom) DCs percent between NSCLC and SCLC and control.

The Flowcytometric analysis of two representative patients of NSCLC and SCLC are shown in Figure 2.
CD11c<sup>+</sup> and CD123<sup>+</sup> DC Subsets in Peripheral Blood of Lung Cancer Patients

Figure 2. A representative Flowcytometry of Plasmacytoid and Myeloid DCs in one of the normal (A), NSCLC (B) and SCLC (C) subjects respectively. Each quadrant is composed of four regions; UL, UR, LL and LR areas. Each subject has 5 square figures as follows:

1- Separation of lymphocytes (LL), monocytes and granulocytes.
2- Isotype control Mouse IgG 1 FITC, (lower left corner).
3- mDC (CD11c-APC) versus pDCs (CD123-PE) (Double positive in upper right corner; R3)
4 and 5- Contour plot of CD11c-APC versus Lineage-FITC cocktail and CD123-PE versus Lineage-FITC cocktail respectively (Single positive for pDCs or mDCs). The R1 and R3 region were set to assess plasmacytoid or myeloid DCs, respectively. Lineage-negative (R2 gate).

Discussion

The role of DCs in the initiation and control of innate and adaptive immune responses is well documented. In recent years there has been an increased interest in the evaluation of DCs subsets in peripheral blood, since an improvement in the enumeration methods has been obtained (Vuckovic, et al., 2004). Several methods for identifying and counting DCs have been proposed (Jankowska, et al., 2008).

Lung cancer has the highest mortality rate amongst men and women in western countries (Beadsmoire & Screaton, 2003). Non-small-cell lung cancer (NSCLC), which includes adenocarcinoma, squamous cell carcinoma, and giant cell carcinoma, comprises the majority of cases and is refractory to conventional therapeutic modalities (Beadsmore & Screaton, 2003). Chemotherapy and radiotherapy applied to that type of cancer are of low efficacy (Kim et al., 2005; Wisnivesky, et al., 2005). The lack of a standard therapy has prompted study of the development of novel therapies for the treatment of lung cancer. The discovery of dendritic cells (DCs) as professional antigen-presenting cells (APCs) opened up new...
possibilities for their use in the development of cancer vaccines (Cranmer, et al., 2004; Soruri & Zwirner, 2005; Yannelli et al., 2005).

Dendritic cells (DCs) are the first protecting barrier against different pathogens (viruses, bacteria and neoplasms cells). Immature myeloid- and lymphoid dendritic cells possess ability to phagocytose and present antigens to lymphocytes. They have also ability to produce IL-12, which is also known as natural killer cell stimulatory factor or cytotoxic lymphocyte maturation (Krawczyk, et al., 2005). The presence of functional, competent DCs is critical for effective anti-tumor control and for the success of cancer immunotherapy. There is ample evidence of inadequate function of these cells in tumor-bearing hosts (Almand, 2001). Tumor cells may produce several growth factors and cytokines able to affect hematopoiesis, differentiation and accumulation of effector cells at the site of neoplastic lesion (Almand, 2001).

In the present study we directly evaluate and quantify circulating blood DCs subsets in lung cancer patients using a whole blood and a flowcytometric analysis and it is considered to be a simple assay with a very precise counting (Vuckovic, et al., 2004). The use of this technique for DCs quantification combines three key advantages: (I) mAbs that bind to antigens expressed on circulating DCs, (II) Precise cellular quantification and (III) a whole blood “Lyse/No Wash” flowcytometric protocol to eliminate the potential cell loss during washing steps. In this way, the single assay provides accurate and reproducible DCs quantification and eliminates the problem of variability related to the method. Sciarra, et al. (2007) affirmed that this assay provides new methodological guidelines that could lead to the global standardization of DCs counting for physiological, diagnostic and prognostic application in clinical practice.

In the present study, we investigated differences in plasmacytoid and myeloid DCs percentages circulating in peripheral blood of lung cancer patients. We found that in all examined groups the percentages of lymphoid DCs was significantly higher than that of myeloid DCs. The ratios were always 2/1 respectively. We found noteworthy unchanged ratio but decrease in percentage of both populations of DCs in NSCLC and SCLC patients. On the contrary, Almand et al. (2001) found increased production of immature myeloid cells in cancer patients and these cells were able to directly inhibit antigen-specific T-cell responses. Gabrilovich et al., (1997) isolated DCs from the peripheral blood of patients with breast cancer and they demonstrated significantly reduced ability to cluster and to stimulate immune responses by this population of DCs. We found significant differences in both populations of DCs between lung cancer patients and healthy donors. The higher percentage of plasmacytoid DCs in NSCLC patients compared to SCLC patients may result from different types of cancer in the examined subjects (Wojas, 2002; Bergeron, et al., 2006).

In study done by Dallal and associates on 2002, they observed decreased level of dendritic cells or even their complete absence in pancreatic cancer. The same was reported by Della Bella, et al. (2003) in breast cancer patients in particular, the myeloid CD11c subpopulation of DCs which was dramatically reduced.

The results of present study confirmed the findings of these reports, demonstrating the presence of low percentage of DCs subset in both types of lung cancer and no significant difference in the CD123+ and CD11c+ ratio among different histological types of Non
Small lung cancer and this results are similar to Jacek, et al., (2008). Jacek and associates noted a prevalence of CD303+/CD123+ myeloid/plasmacytoid DCs in tumor tissue and lymph nodes. They comparing those tissues to peripheral blood, they found that the ratio of myeloid DCs and lymphoid/plasmacytoid DCs was significantly lower in the majority of their patients and was even inverse (Jacek, et al., 2008). This fact proves a prevalence of lymphoid/plasmacytoid line DCs in tissues involved in a neoplasmatic 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. In pancreatic carcinoma, Coussens & Werb, (2002) noted that in most cancer patients the inflammation process coexists with the development of neoplasm, which could result in a greater release of immunomodulatory factors. There is now enough evidence that defective DC function is an important mechanism of tumor escape from the immune system control (Almand, 2001).

Our observation presented in this paper is a pilot study on the quantitative analysis of dendritic cells and allows us to conclude that the amount of DCs in peripheral blood of lung cancer patients may correspond to the type of neoplasm. It faces us with the necessity to expand the examined groups and to include the other pathological conditions. The further study should be aimed at comparisons of the stage, duration and localization of neoplasm with the number of dendritic cells.

References


