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**Differences in dendritic cells maturation and differentiation upon intravenous, intraperitoneal and subcutaneous administration of EL-4 lymphoma cells in mice**

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Дендритные клетки (ДК) как антиген – презентирующие (АПК) клетки играют ключевую роль в первичном иммунном ответе. В последние годы было показано нарушение функции ДК у пациентов с опухолями, а также у мышей, развивших опухоль. Цель работы заключалась в изучении эффектов лимфомы на дифференцировку и созревание ДК, а именно факторов опухолевого микроокружения и интравации опухолевых клеток, т.е. проникновение в кровеносные и лимфатические сосуды. В качестве модели использовалась линия EL-4. Опухолевые клетки вводились мышам линии C57BL/6 интраперитонеально, подкожно и внутривенно. Изучая иммунофенотип ДК и АПК, а также биопсийный материал подкожной опухоли, легких, печени и селезенки, было показано, что противоопухолевый иммунный ответ существенно зависит от пути введения опухоли.
Ключевые слова: дендритные клетки; антиген-презентирующие клетки; EL-4 лимфома; путь введения, маркеры дифференцировки.

Dendritic cells (DC) are key APC for the initiating of primary immune responses. However, in recent years, several groups have shown the defective function of DC in tumor-bearing mice and in cancer patients. Our aim was to study the effects of lymphoma on DC differentiation and maturation and to check the input of such factors as tumor microenvironment and intravasation of tumor cells. Lymphoma cells were administrated via different routes and DC phenotype was investigated. EL-4 lymphoma model was used, and tumor cells were administered intraperitoneally, subcutaneously and intravenously. Bone marrow derived DC and antigen-presenting cells (APC) obtained from mice spleen were examined by flow cytometry, and immunohistochemistry of subcutaneous lymphoma, lungs, livers and spleens was performed. Intravenous administration of lymphoma cells induced suppression of DC differentiation and maturation. We have shown the significant decrease of the IAb, CD80, CD86, CD11b and CD11c expression in the bone marrow derived DC and a decrease of IAb level in the spleen APC after intravenous administration of lymphoma cells. Activation of DC differentiation was observed in experimental groups with the subcutaneous and intraperitoneal EL-4 administration; expression of CD40 and CD86 increased substantially in spleen APC in these groups. The obtained data show that anti-tumor immune response differs significantly depending on the route of tumor cell administration.
Key words: dendritic cells; antigen-presenting cells; EL-4 lymphoma; route of administration; differentiation markers.

Dendritic cells are key APC for the initiating of primary immune responses. They play a central role in antitumor immunity by taking up tumor antigens and stimulating antigen-specific T-cells [1]. In recent years, several groups have shown the defective function of DC in tumor-bearing mice and in cancer patients [2-8]. The major finding of these studies was the lack of expression of co-stimulatory molecules and activation markers, such as CD40, CD80, CD86, CD83 and others, in tumor-associated DC, consistent with the phenotype of immature, nonactivated DC [9-13]. A population of tumor-infiltrating DC isolated from rats with colon cancer were defective in their antigen-presenting function. Most of these cells did not express B7, an essential co-stimulatory signal for T cells [11]. In agreement with these reports, DC function was impaired in patients with breast, head and neck and lung cancer. The defective DC function in cancer patients was associated with the dramatic decrease of competent DC in the peripheral blood and with accumulation of cells lacking markers of mature cells [14].

Our aim was to study effects of lymphoma on DC differentiation and maturation and to check the input of such factor as tumor microenvironment and intravasation of tumor cells. The EL-4 lymphoma model was used and tumor cells were administrated intraperitoneally, subcutaneously and intravenously. The level of DC maturation was studied with the help of a panel of antigens for mature DC.

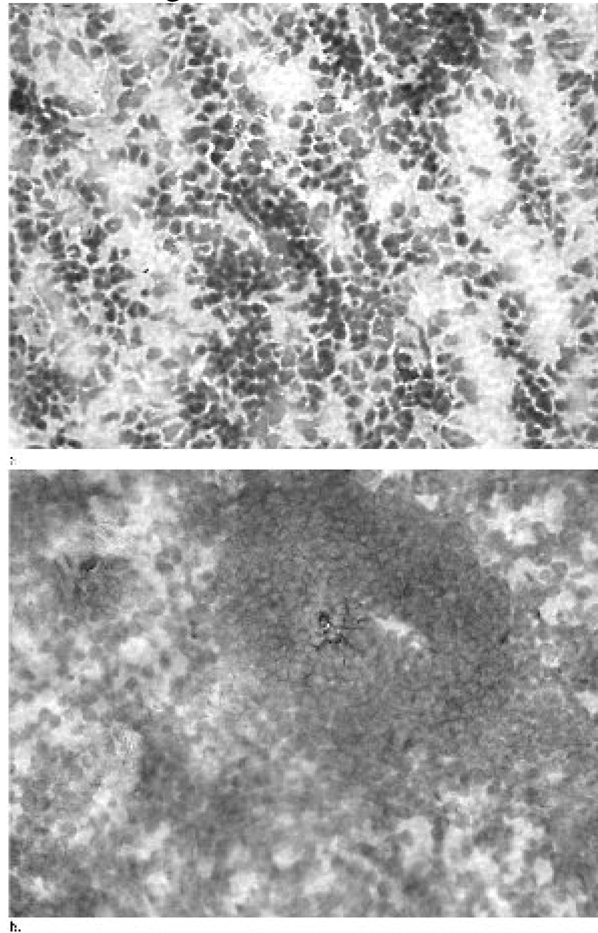

Fig 1. Immunohistochemistry of spleen using anti-NLDC-145 mAbs: a – control group, b – group with IV lymphoma cells administration.

Materials and methods

Mice

Male C57BL/6 mice were obtained from Taconic (Germantown, NY, USA), acclimatized for at least two weeks, and used at the age of 6-8 weeks. Mice were maintained under a 12 h light/dark cycle at a temperature of 20-22 °C. Food and water

were available ad libitum. All animal procedures were approved by the Animal Care and Use Committee of the University of Pittsburgh Medical Center.

### Tumor Cell Line

The syngeneic to C57BL/6 mice EL-4 lymphoma cells (chemically induced thymic lymphoma cell line) were cultured in RPMI 1640 complete medium (with 10% heatinactivated FBS, 0.1 mg/ml gentamicin, 0.1 mM nonessential amino acids, 1 mM sodium piruvate, 1% L-glutamine (Gibco BRL, USA)), at 37 °C in 5% CO2. The cell line was mycoplasma-free.

### Generation of Bone Marrow Derived Dendritic Cells

Mouse bone marrow cells were obtained from flushed marrow cavities. Red blood cells were removed by lysis with Red blood cell lysing buffer (Sigma, UK). Bone marrow cells were further depleted of B-and T-cells by incubation with anti-B220, anti-CD4, and anti-CD8 monoclonal antibodies plus rabbit complement for 1 h at 37 °C. The cells were triple washed in RPMI 1640 complete medium, cultured in 4 ml RPMI 1640 complete medium with 1000 U/ml recombinant mouse GM-CSF and 1000 U/ml recombinant mouse IL-4 (Pepro Tech, USA) at 1 • 106 cells/well in 6-well plates. On the 4th day cytokines were added once again.

### Isolation of APC from Mouse Spleen

Mouse spleens were homogenized in RPM11640 complete medium under sterile conditions and then filtered through a 70 μm nylon filter (Becton Dickinson & Co, USA). Single-cell suspensions were treated with red blood cell lysing buffer (Sigma, USA) for 5 min on ice to remove erythrocytes and washed in RPM11640 complete medium at 1400 rpm at 10°C for 7-10 min. The obtained suspension was used as a source of antigen-presenting cells.

### Flow Cytometry

DC and APC were washed in PBS, counted and at 3 • 105 cells/tube were suspended in FACScan buffer (PBS + 0.1% bovine serum albumin, Sigma, USA, and 0.1% sodium azide, Sigma, USA), centrifugated at 1400 g for 7 min, and double stained for 30 min with the following monoclonal antibodies (mAb):

DC with anti-IAb, CD86, CD80, CD40, CD11c and CD11b (PharMingen, USA), spleen APC with anti-IAb, CD86 and CD11c (PharMingen, USA);

And were compared with the appropriate isotype-matched controls (PharMingen, USA). Samples were fixed in 2% formaldehyde and analyzed on the FACScan (Becton Dickinson, USA).

### Immunohistochemistry

Immunohistochemistry of tumor tissue, lungs, livers and spleens was performed using 4 μm cryostat sections. After application of the Avidin/Biotin blocking kit SP-2001 (Vector Laboratories, USA), CD11c (PharMingen, USA) and NLDC-145 (Serotec, USA) mAb were used. Twice washed in PBS, the sections were incubated for 30 minutes with biotinylated secondary mouse anti-rat antibodies (Jackson ImmunoResearch Laboratories Inc., USA). The color reaction was developed for 7 minutes using the peroxidase chromogen kit (AEC, BioMega Corp., USA). All sections were slightly counter-stained with hematoxylin. The number of positive cells per tissue sections was determined semiquantatively: 0 indicated a completely clean field with no positive staining, + indicated weak, but definitely positive staining of scattered cells, ++ indicates positive staining, with at least 50% of the cells being positive, ++++ indicates strongly positive staining and +++ indicates a staining intensity between + and +++.

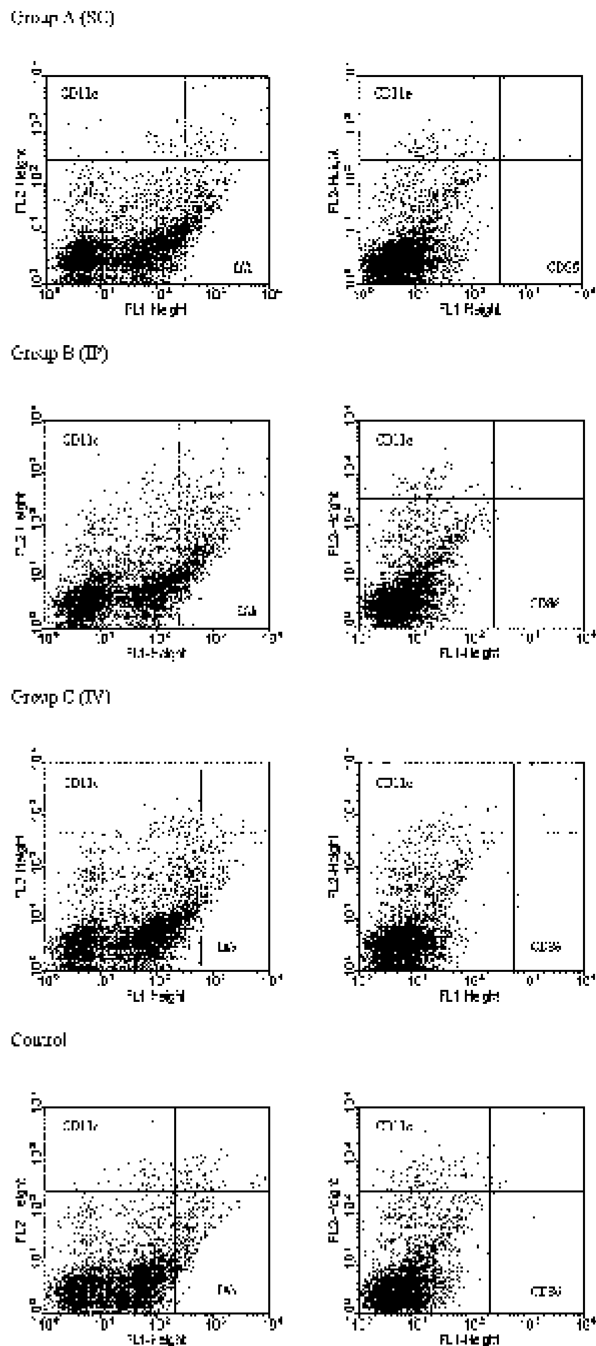


Fig 2. Dot-plots from double staining of murine spleen APC (IAb and CD86 markers with CD11c). Cells in upper left quadrant are positive for CD11c, cells in lower right quadrant are positive for IAb and CD86 accordingly. Cells in upper right quadrant are double stained

Experimental Design

To detect effects of route of tumor administration on function and phenotype of spleen DC three groups of mice were studied; each group consisted of six animals. Group A was injected intraperitoneally (IP), group B-subcutaneously (SC) into the shaved right flank, and group C-intravenously (IV) in tale vein, each group with 105 EL-4 lymphoma cells in total volume of 0.2 ml in PBS. Four intact mice were used as the control. 3 animals per group were sacrificed on the 7th and 14th day of lymphoma cell administration, and immunohistochemistry of lungs, livers and spleens and tumor tissue (in case of SC transplantation) was performed. The APC were obtained from spleens and stained with labeled mAb for flow cytometry. The DC were obtained from the bone marrow (from femur and tibia) on the 14th day of lymphoma cell administration, cultivated and marked

for flow cytometry on the 7th day of culture. The experiment was duplicated for improved accuracy.

Statistical analysis

Results are given as mean ± standard deviation. Differences between experimental and control values were evaluated by Student's t-test.

Results

We have observed disseminated lymphoma growth upon intravenous administration, local growth – upon subcutaneous and partially circumscribed growth – upon intraperitoneal injection. After SC injection EL-4 lymphoma cells disseminated in derma and SC lymph nodes. Mice died on 24th – 28th day after SC injection and without any distant metastases were being observed; the tumor was encapsulated and located in the place of injection. Upon IP administration of lymphoma cells, ascites was developed and tumor growth was circumscribed within abdominal cavity and mesenteric lymph nodes. IV administration lead to the generalizing of the process, EL-4 cells were delivered with blood flow to all the organs and tissues. On the 24th day after injection we observed lymphoma's infiltration in mesenteric, inguinal and axillary lymph nodes, 10 – 20% of lymphoma cells in spleen, and tumor formation in the liver and, in some cases, in the kidneys.

Table 1

Results of immunohistochemistry of tumor and organs

| Organs, route of the lymphoma administration | Markers | | | |
|---|---|---|---|---|
| | CD11c | | NLDC-145 | |
| | 7th day of lymphoma growth | 14th day of lymphoma growth | 7th day of lymphoma growth | 14th day of lymphoma growth |
| Spleen: | | | | |
| control | +++ | +++ | ++ | ++ |
| SC | ++−+ | 0 | ++ | + |
| IP | +++ | + | ++ | −++ |
| IV | ++−+ | ++ | ++ | ++ |
| Lung : | | | | |
| control | 0 | 0 | 0 | 0 |
| SC | 0 | 0 | 0 | 0 |
| IP | + | 0 | 0 | 0 |
| IV | +− | 0 | 0 | 0 |
| Liver: | | | | |
| control | 0 | 0 | 0 | 0 |
| SC | 0 | 0 | 0 | 0 |
| IP | 0 | 0 | 0 | 0 |
| IV | + | + | 0 | 0 |
| Lymph nodes: | | | | |
| control | +− | ++ | +−− | −++ |
| SC | - | + | - | + |
| IP | - | + | - | 0 |
| IV | - | + | - | 0 |
| Tumor: | | | | |
| SC | + | 0 | 0 | 0 |

Notes: 0 – completely clean field with no positive staining; + - weak, but definitely positive staining; ++ - positive staining; +++ - results between ++ and ++++; ++++ - strongly positive staining; - - no data.

The results of the immunohistochemistry showed the increase of the CD11c expression on the 7th day after lymphoma cell injection in the spleen at SC and IV administration, in the lungs at IP and IV administration and in the liver at IV administration (see Table 1). On the 14th day CD11c expression was decreased in the spleen and lymph nodes in all the experimental groups irrespective of the route of administration, and again increased in the liver upon IV administration in comparison with the control. CD11c+ cells were revealed in tumor tissues in group with the SC administration on the 7th day but were undetectable on the 14th day (see Table 1). These data suggest that possible activation of DC as immune response to the allogeneic antigens was eliminated with the growth of the tumor.

NADC-145 expression in the spleen on the 7th day after EL-4 transplantation was at the control level. On the 14th day expression of the NLDC-145 increased in the spleen in the groups with IV and IP administration and decreased in the group with the SC administration. Furthermore, on the 14th day expression of the NLDC-145 on the APC surface decreased in the lymph nodes in all the studied groups in comparison with the control irrespective of the route of lymphoma cells administration (see Table 1, Fig. 1). Expression of the NLDC-145 on the cell surface was not revealed in liver, lungs and tumor.

The flow cytometry data showed considerable decrease of IAb level in the APC at the 7th day after the IV lymphoma cell administration in comparison with the control. Expression of the IAb was close to control levels (expression on the cell surface of the intact animals) upon SC and IP routes of administration (see Table 2, Fig. 2).

Table 2

Results of flow cytometry of murine spleen APC (on the 7th day of lymphoma administration)

*** $P < 0.001$ as compared to control.

| Route of lymphoma administration | Expression of markers on the spleen APC | |
|---|---|---|
| | $IA^b$ | CD86 |
| Control | 13.68 ± 1.07 | 0.18 ± 0.06 |
| SC | 15.43 ± 2.03 | 0.21 ± 0.04 |
| IV | 6.65 ± 0.80 *** | 0.16 ± 0.03 |
| IP | 17.54 ± 2.62 | 0.27 ± 0.06 |

In all the groups phenotype of BMDC differed substantially from that of intact animals (see Table 3). the dramatic decrease of the IAb, CD80, CD86, CD11b and CD11c expression on the surface of the BMDC was detected in the group with IV EL-4 injection, the level of these markers' expression was significantly lower than in the control group (see Table 3). On the contrary, 42% increase of CD86 and 73% increase of CD40 expression was observed upon the SC lymphoma cell administration. In addition, 35% increase of CD40 expression was shown in the group with IP injection of lymphoma cells (see Table 3). Expression of all studied markers (IAb, CD80, CD86, CD40, CD11b and CD11c) after IV administration differed significantly from those after SC and IP administration ($P < 0.001$). The obtained data suggest that route of lymphoma cells administration determines the tumor effects on differentiation and maturation of DC.

Table 3

Results of flow cytometry of the cultivated BMDC (on the 14th day after the lymphoma administration)

| Route of lymphoma administration | Expression of markers on DC (in %) | | | | | |
|---|---|---|---|---|---|---|
| | $IA^b$ | CD86 | CD80 | CD40 | CD11b | CD11c |
| Control | 42.9 ± 1.5 | 28.4 ± 2.2 | 28.1 ± 3.9 | 15.4 ± 2.3 | 38.1 ± 1.9 | 51.8 ± 1.5 |
| SC | 48.6 ± 1.7* | 40.2 ± 1.4* | 31.3 ± 1.2 | 26.7 ± 2.9* | 31.0 ± 1.5* | 50.7 ± 1.5 |
| IV | 21.5 ± 1.1* | 15.4 ± 1.8* | 17.7 ± 2.0* | 14.9 ± 1.5 | 17.3 ± 1.0* | 31.0 ± 2.2* |
| IP | 49.4 ± 2.1* | 32.9 ± 3.0 | 31.1 ± 2.3 | 20.8 ± 1.3* | 39.4 ± 2.9 | 56.4 ± 1.6* |

* P < 0.05 as compared to control.

Discussion

Though dendritic cells are able to induce a cytotoxic T-lymphocyte reaction against tumors, there are a number of mechanisms by which tumor cells can avoid detection and destruction by the immune system: down-regulation of HLA class I expression on the surface of tumor cells; down-regulation of tumor antigen expression; lack of costimulatory molecules on tumors cells and others [15]. Some of the tumor-derived factors, such as vascular endothelial growth factor (VEGF) [16-17], transforming growth factor-beta (TGF-в) [18], cyclooxygenase-2 [19], IL-6 [20], IL-10 [21-22], and others are able to down-regulate the production and maturation of DC or to induce DC apoptosis. More aggressive tumors induce higher levels of DC apoptosis and, therefore, more significant inhibition of antigen recognition, processing, and presentating by DC, which are necessary for the initiation and maintenance of an effective anti-tumor immune response [23].

Site of the origin has been described as a prognostic factor that determines clinical outcome in some tumors, such as T-cell lymphomas, an anaplastic large cell lymphoma, cutaneous diffuse large B-cell lymphoma and others [24-26]. At the same time, the route of lymphoma cell administration determines the patterns of EL-4 growth and lymphoma process manifestation. Lymphoma cells disseminate predominantly to the skin and subcutaneous lymph nodes upon subcutaneous administration, disseminate to mesenteric lymph nodes and abdominal organs upon intraperitoneal administration and spread effectively to all organs with blood upon intravenous injection.

The obtained data showed that effects of lymphoma cells on the DC maturation and function depended on the route of tumor cell administration. We have observed the significant decrease of the IAb, CD80, CD86, CD11b and CD11c expression on the surface of the BMDC and decrease of IAb level in the spleen APC after IV administration of lymphoma cells.

It was found that DC express MHC class II molecules, and, thus, can effectively stimulate primary T-lymphocyte response [27]. Decrease of IAb expression may point to the impaired antigen-presenting function of DC.

CD80 (B7-1) and CD86 (B7-2), ligands for CD28 and CTLA-4, are costimulation molecules in the DC-T-cell interaction [28]. Low level of B7 molecules expression on DC surface in patients with cancer, however, may play a certain role in tumor evasion of host defences [1]. CD40 regulates DC differentiation, maturation, and antitumor activity. CD40-CD40L signaling plays an essential role in stimulating of DC-mediated IL-12 secretion and cytotoxicity, which induce cell-mediated tumor immunity [29]. Therefore,

DC differentiation and maturation were down-regulated upon IV lymphoma cells administration. On the other hand, DC differentiation was activated in groups with the SC and IP EL-4 administration. The observed activation may indicate the start of immune response that can be connected with more favorable microenvironment or conditions for DC to contact with tumor cells and to initiate further immune reactions. DC inhibition shown upon IV administration of the same tumor can be explained by the more aggressive and generalized disease process as lymphoma cells spread freely with blood and can disseminate to all organs. Thus, intravascular location of the lymphoma cells leads to the inhibition of DC maturation, failure of immune surveillance and, therefore, worse clinical prognosis of the lymphoma process. Our findings correlate with the work of Ferreri et al. [30]. In his study of intravascular lymphoma, characterized by predominant growth of neoplastic cells within the lumina of blood vessels, he showed that patients with the disease limited to the skin ('cutaneous variant') exhibited a significantly better outcome and improved survival.

The CD11c is usually expressed at high density on the DC surface and is considered to be a marker of bone marrow derived DC [27]. The slight increase of CD11c expression on the 7th day after lymphoma cell transplantation was probably associated with the DC activation, which was eliminated with the growth of the tumor by the 14th day. Increase of the number of NLDC-145+ cells in the spleen on the 14th day after IP and IV transplantation could be related to the increase of macrophages that also express NLDC-145 [27].

In summary, our results demonstrate that tumor effects on the DC differentiation, function and phenotype depend on the route of lymphoma transplantation. Intravenous administration of lymphoma cells induced significant down-regulation of DC maturation. On the other hand, activation of DC differentiation was observed in groups with the subcutaneous and intraperitoneal EL-4 administration. These data show that anti-tumor immune response differs significantly depending on the route of tumor cells administration.

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