

Direct Antitumor Activity of Interferon-Induced Dendritic Cells of Healthy Donors and Patients with Primary Brain Tumors

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1. Introduction

Dendritic cells (DCs) are well known for their capacity to induce adaptive antitumor immune response through their unique ability to uptake, processing and presenting antigens (Ags), and tumor-specific T cell activation. In addition, cytokines produced by dendritic cells are able to regulate the direction and strength of immune response, activate the cytotoxic cells (NK-, NKT-cells) and participate in the coordination of the humoral immune response (Melief, 2008; Banchereau et al., 2000).

An increasing number of reports evidenced that besides this role, DCs may display additional antitumor effects. Indeed, DCs in vitro can inhibit proliferation and provide a direct cytotoxic effect on tumor cells. In this, human monocyte-derived DCs might exert antitumor activity through multiple TNF family members (i.e. TNF- α , lymphotoxin- α 1 β 2, FasL, TRAIL), as well as perforin and/or granzyme (Wesa & Storkus, 2008; Chauvin & Josien, 2008).

Direct tumor cell killing by DCs themselves appear to be highly important since involves immediate presentation of tumor-associated Ags in the context of MHC molecules for recognition by cognate T cells, inducing a specific immune response. Importantly, pleiotropy in DC mechanisms of cytotoxicity allows DCs to overcome the resistance of tumor cells that are heterogeneous with regard to their sensitivity to the various death pathways. A number of evidence suggests that the direct antitumor effect of DCs is not purely in vitro phenomenon, and is implemented in vivo. First, DCs are present in the tumors, and their higher content correlates with a more favorable prognosis (Becker 1999). Second, intra-tumoral injection of intact DC (not loaded with tumor antigen) has been shown to correlate with reduced tumor growth and even regression (Becker et al., 2001; Ehtesham et al., 2003). Finally, it is shown that the intra-tumoral introduction of DCs improves the effectiveness of chemotherapy, which may be due to synergistic effects of cytostatics and DCs (Vanderheyde et al., 2004).

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Most studies on antitumor activity of DCs in humans were performed with myeloid DCs isolated from peripheral blood or generate *in vitro* from peripheral blood monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). Cytotoxic activity of these DCs was found to be stronger after treatment with type I interferons (IFN) or IFN γ (Liu et al., 2001; Fanger et al., 1999). More recently, monocyte-derived DCs generated in the presence of GM-CSF and IFN α instead of IL-4 were described. It is also known that IFN α -DCs are characterized by a higher expression of some molecules (TRAIL, FasL), which may mediate the cytotoxic/cytostatic activity of DCs (Chauvin & Josien, 2008). Indeed, we have demonstrated that LPS-activated IFN α -DCs inhibit the growth of tumor cell line HEp-2 more efficiently than IL4-DCs (Leplina et al., 2010). However, the antitumor potential of DCs generated in the presence of IFN α , remain virtually unexplored.

Of great relevance is also an issue on safety of DC cytotoxic activity in cancer settings. Recently, we found that in patients with malignant gliomas IFN α -DCs generated *in vitro* are able to activate Th1 cells and induce an antitumor immune response (Leplina et al., 2007a). Nevertheless, these DCs exhibit several phenotypic and functional features, such as the moderate delay of differentiation/maturation and low capacity to induce the IFN γ -producing T-cells in mixed lymphocyte culture (MLC) (Leplina et al., 2007b). Given the data on the development of immune insufficiency and monocyte dysfunctions in patients with malignant brain tumors (Khonina et al., 2002) the study of various functions of DCs in this pathology is important not only in terms of understanding pathogenesis of the disease, but also to rationale the therapy with dendritic cells. In this article we investigated the cytotoxic potential of human monocyte-derived DCs generated under replacement of IL-4 with IFN α , and compared cytostatic/cytotoxic activities of IFN α -induced DCs in healthy donors and patients with brain tumors.

2. The text of the article

2.1 Materials and methods

2.1.1 Patients

The study was held in 32 healthy volunteers and 37 patients with brain tumors (21 men and 16 women; from 21 to 71 years; median age 37 years). Patients' group included 20 patients with histologically verified glioblastoma (Grade IV), 8 - with astrocytoma (Grade III) and 9 - with angioreticuloma, fibrillary-oligodendrocytic astrocytoma or meningioma (Grade I-II). All studies were performed after receiving a written informed consent.

2.1.2 *In vitro* differentiation and maturation of DCs

Peripheral blood mononuclear cells (MNCs) were obtained by density gradient centrifugation (Ficoll-Paque, Sigma-Aldrich) of heparinized whole blood samples. Dendritic cells were generated by culturing of plastic-adherent MNC fraction in 6-well plates (Nunc, Denmark) in RPMI-1640 medium (Sigma-Aldrich), supplemented with 0,3 mg/ml L-glutamine, 5 mM HEPES buffer, 100 μ g/ml gentamicin and 5% fetal calf serum (FCS, Sigma-Aldrich), in the presence of recombinant human (rh) GM-CSF (40 ng/ml, Sigma-Aldrich) and rhIFN- α (Roferon-A, 1000 U/ml, Roche, Switzerland) for 4 days (IFN α -DCs) or with rhGM-CSF (40 ng/ml) and IL-4 (40 ng/ml, Sigma-Aldrich) for 5 days (IL4-DCs). The resulting immature DCs were further exposed with 10 μ g/ml lipopolysaccharide (LPS E.colli 0114: B4, Sigma-Aldrich) into IFN α -DC and IL4-DC cultures for additional 24h

and 48h, respectively. For some experiments DC supernatans generated from LPS-activated IFN α -DCs were collected. The viability of obtained IFN α -DCs or IL4-DCs determined by Trypan blue exclusion was more than 93-95% in all cases.

2.1.3 Cell lines

Tumor cell lines used in this study included leukemia cell line Jurkat (T- lymphoblast cell leukemia) and solid tumor-derived cell lines: epithelial cells of human larynx carcinoma HEp-2 and glioblastoma U-87 were purchased from American Type Culture Collection (Manassas, VA). All cell lines were of human origin, mycoplasma free and were grown under standard cell culture conditions.

2.1.4 Cytotoxicity assay

Generated IFN α -DCs were tested for their cytotoxic activity against various tumor cell lines including Jurkat, HEp-2 and U-87. Before coculture, target cells were labeled with [3 H]thymidine (1 μ Ci/well) for 18 h at 37°C, washed and placed at 10 4 /well in 96-well tissue culture plates in RPMI-1640 medium containing 10% FCS. Cell-free supernatants from DC cultures (30%, v/v) or different numbers of effector cells (DCs) were added to tumor cells at effector:target (E:T) ratios of 10:1, 20:1 and 40:1. In some experiments DCs were pre-incubated for 1 h with the following fusion proteins: rhTNFR1/TNFRSF1A Fc chimera (10 μ g/ml), rhFas/TNFRSF6/CD95 Fc chimera (10 μ g/ml), and rhTRAIL R2/TNFRSF10B Fc chimera (10 μ g/ml; all reagents from R & D Systems, USA). After 18 h of culture cells were harvested and thymidine incorporation was measured on a Liquid Scintillation beta-Counter (Packard Instrument, Meriden, CT). Percentage of cytotoxicity was calculated by the formula:

$$[1 - (\text{cpm in cocultures of tumor and effector cells or DC supernatans} / \text{cpm in tumor cell cultures})] \times 100\%.$$

2.1.5 Cytostatic assay

Cytostatic activity of DCs was evaluated by their ability to suppress the proliferation of tumor line cells (HEp-2 and U-87). For this, the target cells (10 3 /well) were incubated for 48 h in 96-well plates alone and in the presence of effector cells at E:T ratios about 10:1, 20:1 and 40:1. Cell proliferation was measured by [3 H]thymidine incorporation (1 μ Ci/well for last 24 h). The percentage of cytostatic activity was calculated by the formula:

$$[1 - (\text{cpm in cultures with effector cells} / \text{cpm in control cultures})] \times 100\%.$$

2.1.6 Apoptosis detection

To determine the level of apoptosis, HEp-2 tumor cells were preliminary stained with vital dye CFSE (2 mM, Molecular probes, USA) for 15 min, then washed in RPMI-1640/10% FCS and incubated in 96-well tissue culture plates (10 4 /well) in the presence of IFN α -DCs at a ratio 10:1 for 18 hours. The number of cell divisions was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, USA) on channel FL1 (CFSE fluorescence) with the emission of 517 nm. The level of apoptosis was detected by DNA intercalating dye 7-AAD (Calbiochem, Israel). Results were expressed as a percentage of positive cells to the total cell number in the region studied.

2.1.7 TNF α production

DC-free supernatants collected as describe above were measured for soluble TNF α by ELISA using a commercial kit (R & D Systems, USA) according to the manufacturer's recommendations.

2.1.8 Statistical analysis

The data were expressed as mean \pm SE. Statistica 6.0 software for Windows (StatSoft Inc. USA) was used for analysis of data. Statistical comparisons were performed using the nonparametric Mann-Whitney U test. P-values < 0,05 indicate significant differences.

2.2 Results

2.2.1 Cytotoxic activity of donor IFN α -DCs and DC supernatants against tumor cell lines

First, we assessed whether in vitro generated mature IFN α -DCs could lyse tumor cell lines. IFN α -DCs in our study possessed significant dose-dependent cytotoxic activity against [3 H]thymidine-labeled tumor cell lines HEP-2 and Jurkat. As illustrated in Fig.1A, Jurkat cells were lysed more efficiently at all E:T cell ratios. The most pronounced differences in cytotoxic activity of IFN α -DCs against Jurkat and HEP-2 were observed at a E:T ratio of 20:1 (35,17 \pm 5,6% and 16,44 \pm 4,01 %, respectively; $P_U=0,027$). High cytotoxic activity of IFN α -DCs was also manifested when human glioblastoma U-87 cells were used as targets (Fig.1B). In this case, the cytotoxic potential of DCs at 20:1 was two-fold higher than with HEP-2 cells (38,6 \pm 8,3%; $P_U=0,049$). Taken together, these data indicate that i) mature IFN α -DCs mediated significant antitumor cytotoxic activity, effective at various E:T cell ratios, and ii) the cytotoxic activity of IFN α -DCs against tumor cell lines Jurkat and U-87 unlike HEP-2 cells was considerably higher.

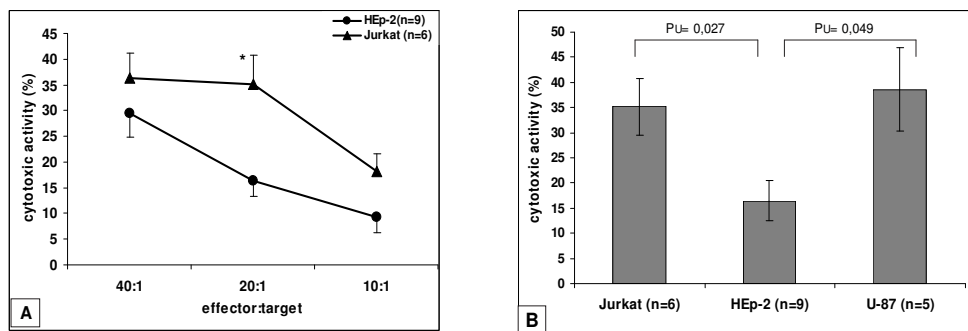


Fig. 1. Cytotoxic activity of donor IFN α -DCs against Jurkat, Hep-2, and U-87. A) The average values of IFN α -DC cytotoxic activity against tumor cell lines Jurkat and HEP-2 are presented. Effector cells (DCs) and [3 H]thymidine-labeled target cells (Jurkat and HEP-2) were cultured for 18 h at ratios indicated. B) Cytotoxic activity of IFN α -DCs against Jurkat, HEP-2 and U-87 tumor cells at E:T ratio of 20:1. Results are shown as mean \pm SE of triplicate values. * - $P_U < 0,05$ - between HEP-2 and Jurkat at E:T ratio of 20:1 (U - Wilcoxon's test, Mann-Whitney).

To examine the possible mechanisms underlying the cytotoxic activity of IFN α -DCs against tumor cells, we evaluated the cytotoxic activity associated with DC culture-conditioned medium. Contrary to DCs themselves, IFN α -DC culture-conditioned medium lacked cytotoxic activity or had a low ability to lyse tumor cells. Indeed, supernatants of DC cultures added to targets at 30% (v/v) were unable to lyse HEp-2 cells, and had some cytotoxic activity against Jurkat cell line ($13,0 \pm 1,7\%$) (Fig.2). These results showed that mediators of DC-associated antitumor activity are more likely cell membrane-bound molecules but not secreted proteins.

2.2.2 Induction of apoptosis of tumor cell line HEp-2 by IFN α -DCs

To investigate whether DC killer activity involved the induction of apoptosis, we next analyzed cell cycle in tumor cells HEp-2 pre-labeled with vital dye CFSE (Fig.3). Co-culturing of CFSE-labeled HEp-2 cells with IFN α -DCs resulted in significant increase in the level of apoptosis detected in tumor cells. In addition, the cultivation of tumor cells with IFN α -DCs was accompanied by a decrease in number of cycling tumor cells (S + G2M phases of the cell cycle). These results showed that DCs in vitro can efficiently induce death of tumor cells using an apoptotic mechanism.

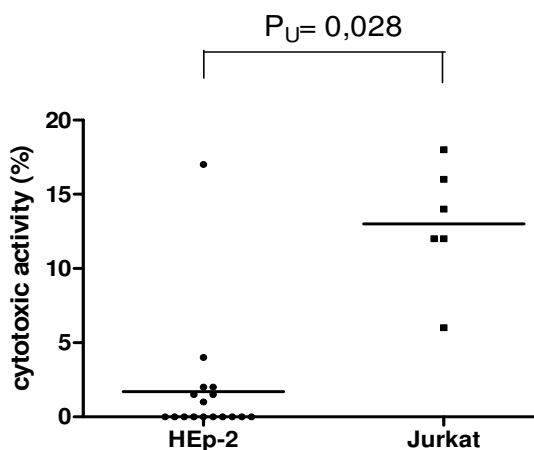


Fig. 2. Cytotoxic activity of DC culture-conditioned medium against Jurkat and HEp-2. The figure represents individual values of cytotoxic activity mediated by supernatants from cultures of healthy donor IFN α -DCs against tumor cell lines HEp-2 (n = 17) and Jurkat (n = 6). Jurkat cells and HEp-2 cells (10^4 cells/well) were labeled with [3 H]thymidine and incubated with DC culture-conditioned medium (30%, v/v) for 18 hours.

2.2.3 Growth inhibition effect of IFN α -DCs on tumor cell lines HEp-2 and U-87

These data result in the suggestion that the cytotoxic activity of IFN α -DCs is conditioned by induction of apoptosis in tumor cells and that, along with a cytotoxic effect, IFN α -DCs apparently could block cell cycle in tumor cells, thereby providing cytostatic effect. Indeed, analysis of IFN α -DCs impact on the proliferation of tumor cells (Fig. 4) revealed a pronounced antiproliferative effect exerted by donor DCs against the cell line HEp-2.

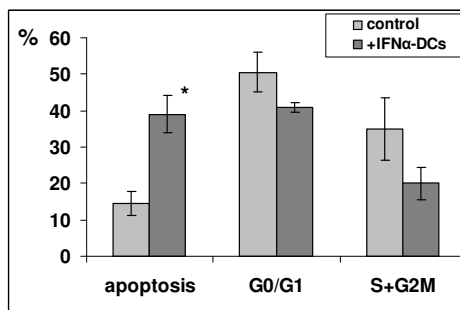


Fig. 3. Effect of IFN α -DCs from healthy donors on the cell cycle in HEP-2. The figure shows the relative content (%) of CFSE-labeled HEP-2 cells in cell cycle phases in the absence of DCs (control; n= 4) and in co-cultures with IFN α -DCs (n= 4) for 18 hours at E:T ratio of 10:1. The data are presented as M \pm SE (%). * - $P_U < 0,01$ (U - Wilcoxon's test, Mann-Whitney).

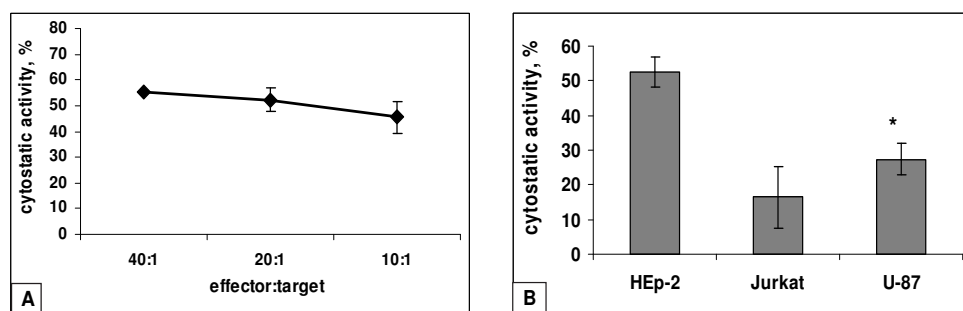


Fig. 4. Tumor-inhibiting activity of IFN α -DCs from healthy donors against HEP-2, Jurkat, and U-87. **A**) The graph shows the mean values (M \pm SE) of cytotostatic activity of IFN α -DCs against HEP-2 tumor cells (n = 8). Effector cells (DC) and target cells were cultured at ratios indicated for 24 hours, followed by the introduction of [3 H]thymidine for 24 hours. **B**) Cytostatic activity rendered by IFN α -DCs against HEP-2 (n = 8), Jurkat (n = 7) and U-87 (n = 5) in E:T ratio of 20:1. * - $P_U < 0,05$ - between the cytotostatic activity of DCs vs HEP-2 and U-87 (U - Wilcoxon's test, Mann-Whitney).

Importantly, DCs mediated potent inhibitory activity (45,4 \pm 6,24%) even at a low E:T cell ratio (10:1). Moreover, IFN α -DCs also suppressed the proliferation of glioblastoma cell line U-87. However, in this case inhibition was almost two-fold lower, accounting for 27,4 \pm 4,4% at E:T ratio of 20:1 vs 52,4 \pm 4,4% in HEP-2 cultures ($p < 0,05$). Thus, in our study IFN α -DCs were found to be cytostatic for tumor cell lines. Comparative analysis of cytotoxic and cytotostatic activity mediated by IFN α -DCs showed no correlations between the level of DC cytotoxicity and their ability to inhibit the proliferation of HEP2 ($r_s = 0,21$; $p = 0,7$), U-87 ($r_s = 0,5$; $p = 0,28$) and Jurkat ($r_s = 0,33$; $p = 0,5$) tumor cell line. The lack of such a relationship was also indicated by the fact that in cultures of U-87 dendritic cells displayed the highest cytotoxic effect while their cytotostatic effects were only moderate. Contrary, in cultures of HEP-2 DCs had a relatively low cytotoxic effect and pronounced anti-proliferative activity.

2.2.4 Role of TNF α , FasL and TRAIL in cytotoxic activity of IFN α -DCs

To get inside into the mechanism that could be responsible for DC tumoricidal activity, we have investigated the role of key molecules involved in the apoptosis pathway. Cytotoxic activity of DCs is attributed to the expression of some proapoptotic molecules such as TRAIL, FasL, perforin, granzymes A and B, TNF- α , lymphotoxin- α 1, β 2 (Chauvin & Josien, 2008). To further characterized the molecular mechanisms by which HEp-2 cell death results from interaction with DCs, we studied the effect of some soluble receptors at the DC-mediated cytotoxic activity. As evident from Fig. 5, pretreatment of IFN α -DCs with TNFR1:Fc resulted in almost complete neutralization of DC cytotoxic activity, whereas pretreatment with soluble forms of TRAIL-R2:Fc and Fas:Fc did not followed by suppression of DC killer activity.

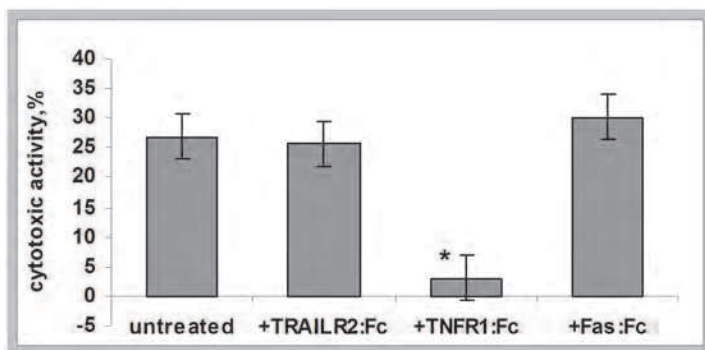


Fig. 5. Neutralization of DC cytotoxic function by soluble forms of R:Fc, specific for TNF-family ligands. [3 H]thymidine-labeled tumor cells HEp-2 (10^4 cells/well) were incubated for 18 hours with IFN α -DCs (at E:T ratio of 20:1) pre-treated for 1 h with TRAIL-R2:Fc fusion protein (10 μ g/ml; n = 6), or TNFR1:Fc fusion protein (10 μ g/ml; n = 6), or Fas:Fc fusion protein (10 μ g/ml; n = 6). Data are presented as mean (M \pm SE) of cytotoxic activity of IFN α -DCs vs HEp-2. * - $P_U < 0,01$ - between intact DCs and DCs treated with TNFR1:Fc (U - Wilcoxon's test, Mann-Whitney).

Thus, our data suggest that lysis of HEp-2 cells is not related with TRAIL- and FasL-mediated cytotoxicity but occurs with the involvement of TNF α molecules, since blocking of TNF α /TNFR1 binding leads to almost full suppression of DC killer activity. Apparently, the involvement only a single of three described mechanisms of DC cytotoxicity is due to resistance of tumor cells HEp-2 to TRAIL- and FasL-mediated apoptosis and determines relatively low cytotoxic activity of DCs against HEp-2 cells compared to Jurkat and U-87 which are sensitive to FasL- and TRAIL-mediated apoptosis (Röhn et al., 2001; Hoves et al., 2003).

2.2.5 Cytotoxic activity of donor IL4-DCs in compared with IFN α -DCs

Since we proposed IFN α -DCs may have a more pronounced antitumor activity than DCs generated with GM-CSF and IL-4, we then investigated whether cytotoxic and cytostatic activities of these two types of LPS-activated DC were distinct. As seen in Fig.6, IFN α -DCs possessed the higher ability to lyse leukemia cells Jurkat (Fig. 6A) and comparable cytotoxic activity in HEp-2 cultures (Fig.6B). However, IFN α -DCs were found to be more effective in suppressing the growth of tumor cell line HEp-2 than IL4-DCs ($45 \pm 6\%$ vs $29 \pm 7\%$, respectively, at E:T ratio of 10:1, $P_U < 0,05$).

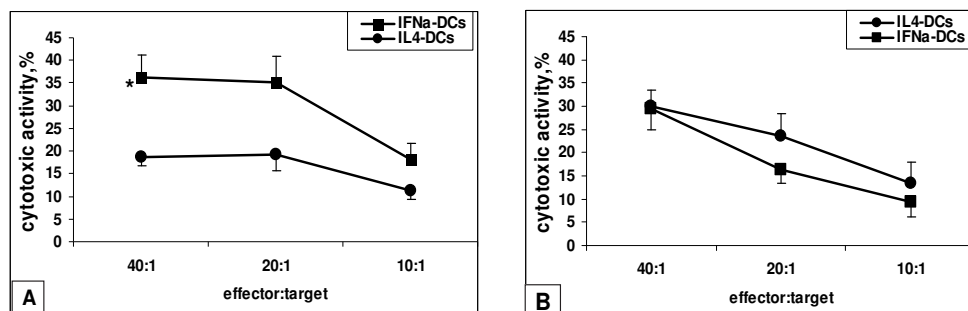


Fig. 6. Cytotoxic activity of IFN α -DCs and IL4-DCs of healthy donors against tumor lines Jurkat (A) and HEp-2 (B). Data are presented as mean (M \pm SE) of cytotoxic activity. Effector cells (donor IFN α -DCs and IL4-DCs) were incubated with target cells (3 H)thymidine-labeled tumor cell lines Jurkat and HEp-2) at ratios indicated for 18 h. * - $P_U < 0,05$ - between IFN α -DCs and IL4-DCs against Jurkat (U - Wilcoxon's test, Mann-Whitney).

2.2.6 Cytotoxic activity of patient IFN α -DCs vs HEp-2

While donor DCs were found to be tumoricidal, evaluation of the cytotoxic activity of DCs generated in vitro from peripheral blood of brain glioma patients revealed they were significantly less cytotoxic against HEp-2 cells (Fig.7A). The decrease of cytotoxic activity was manifested at all E:T ratios which were analyzed. At the same time assessment of patient DC killer activity at E:T ratio of 20:1 (n=37) allowed to reveal significant heterogeneity for DC cytotoxic potential in patients with brain tumors (Fig.7B). Indeed, in 25 patients (67%) cytotoxic activity was completely absent, whereas remained relatively unaltered in another 12 patients (32%).

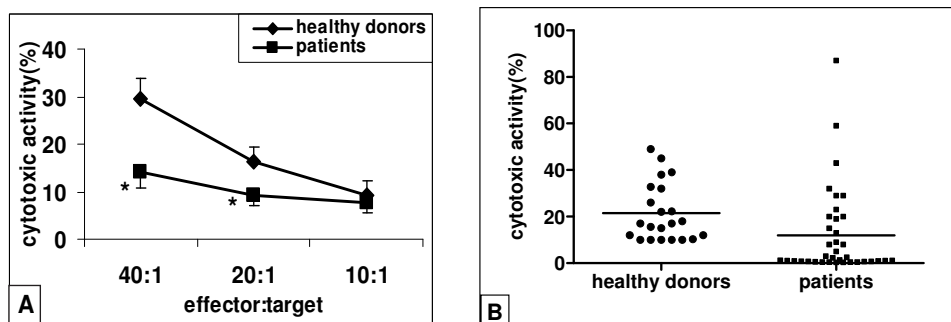


Fig. 7. Cytotoxic activity of IFN α -DCs of patients with brain tumors against HEp-2. **A)** Effector cells (donor and patient IFN α -DCs) were cultured with (3 H)thymidine-labeled target cells (HEp-2) for 18 h at ratios indicated. Results are shown as the mean \pm SE of DC cytotoxic activity. * - $P_U < 0,01$ - between donor and patient DCs (U - Wilcoxon's test, Mann-Whitney) at E:T ratio of 40:1 and 20:1. **B)** Individual values of cytotoxic activity mediated by IFN α -DCs of healthy donors and brain tumor patients against HEp-2 are presented. Effector cells (DC) were cultured with (3 H)thymidine-labeled target cells (HEp-2) for 18 h at ratio of 20:1.

Analysis of patients according to the degree of tumor malignancy demonstrated that the decrease in cytotoxic activity of DCs was typical for patients with high grade (III-IV) gliomas while patients with low grade (I-II) intracerebral gliomas were characterized by unaltered cytotoxic activity (Table 1).

E:T ratio = 20:1	Donors (n=22)	Patients with brain tumors	
		Grade I-II (n=9)	Grade III-IV (n=28)
M ± S.E	21,5 ± 2,6	22,56 ± 5,64	4,75 ± 1,95
Median	17,0	20,0	0
LQ-UQ	10,3- 32,0	13,0- 29,0	0- 4,0

Table 1. Cytotoxic activity of IFN α -DCs in patients with low and high grade glioma

Effector cells (IFN α -DCs) were generated from peripheral blood of healthy donors and patients with low grade (I-II) and high grade (III-IV) gliomas and cultured with [3 H]thymidine-labeled target cells (HEp-2) for 18 h at E:T ratio of 20:1. The average values (M \pm SE), Median and interquartile range (from low to upper quartile, LQ-UQ) of cytotoxic activity are presented.

Figure 8 shows the individual examples of cytotoxic activity of IFN α -DCs of patients with Grade I-II (n=3) and III-IV (n=3) brain tumors.

2.2.7 Survival rates of patients with intact and reduced levels of IFN α -DC cytotoxic activity vs HEp-2 cells

Considering that the degree of malignancy is predictive factor of patient survival, we further questioned about the survival rates of patients with intact and reduced levels of DC cytotoxic activity against HEp-2 cells (Fig. 9). The criterion for division into such groups was the lower quartile range of cytotoxic activity mediated by donor IFN α -DCs against HEp-2 cells (LQ=10,3%).

Patients with decreased cytotoxic activity of IFN α -DCs (< 10,3%, 1 patient with Grade II and 15 patients with Grade III-IV) differed by a lower survival rate compared with patients of the opposite group. For example, a median of survival in patients with low DC cytotoxic activity was about 13 months, and in the group with unchanged cytotoxic activity of DCs all patients (5 patients with Grade I-II and 4 patients with Grade III) were followed alive.

2.2.8 Growth inhibition effect of patient IFN α -DCs on tumor cell line HEp-2

Next, we investigated whether DCs of patients with brain tumors could inhibit the growth of HEp-2 cells (Table 2). While donor DCs possessed the marked cytostatic activity, IFN α -DCs of patients with intracerebral gliomas were found to be incapable of suppressing the proliferation of HEp-2. Moreover, the addition of DCs led to 3-fold increased tumor cell proliferation. The index of DC impact ranged from 0,5 to 7,2, averaging about 3,06 \pm 0,4. It should be noted that such a stimulatory effect of DCs on HEp-2 cell growth was detected both in patients with high grade III-IV (3,08 \pm 0,65; n = 19) and low grade I-II (3,37 \pm 1,49; n = 8) tumors, and unlike cytotoxic activity, was independent on the degree of malignancy.

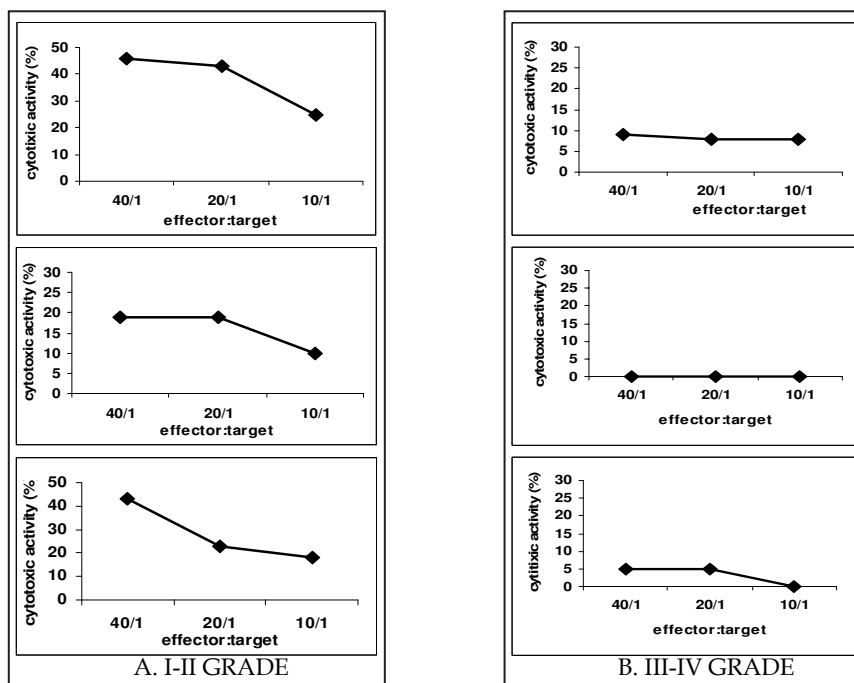


Fig. 8. Cytotoxic activity of IFN α -DCs of individual patients. Figure represents the individual values of cytotoxic activity of IFN α -DCs generated in vitro from peripheral blood of tumor patients against HEP-2. Effector cells (DCs) and [3 H]thymidine-labeled HEP-2 cells were co-cultured for 18 h at ratios indicated. Percentage of cytotoxicity was calculated as follows: [1 - (cpm in cultures with target and effector cells / cpm in control cultures without effector cells)] \times 100%.

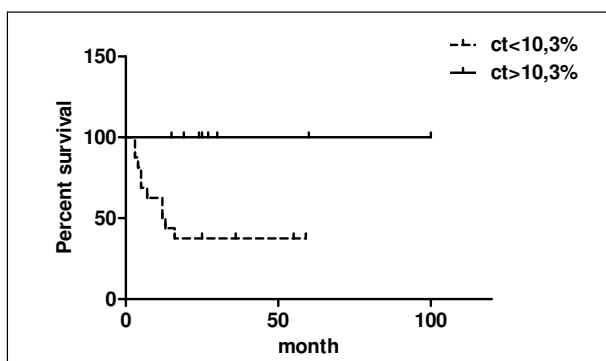


Fig. 9. Survival rates of patients with brain tumors based on the level of DC cytotoxic activity. Dotted line: cytotoxic activity of DCs of patients is below (<10,3%) donor lower quartile values. Solid line: cytotoxic activity of patient DCs is above 10,3%.

The table represents the individual values of indexes of IFN α -DC impact on proliferation of tumor cell line HEp-2. For this, effector cells (DCs) and targets were cultured at 20:1 for 24 hours, followed by the introduction of [3 H]thymidine for the next 24 hours. The index of DC impact was calculated by the formula: cpm in cultures with target and effector cells / cpm in control cultures without effector cells. * - $P_U < 0,01$ between donors and patients (U - Wilcoxon's test, Mann-Whitney).

Patients	Diagnosis	DC cytostatic activity (Indexes of DC impact)
P 1, female, 60 years	Grade 1	5,6
P 2, female, 71 years	Grade 1	6,2
P 3, male, 53 years	Grade 1	0,9
P 4, female, 35 years	Grade 2	7,2
P 5, male, 42 years	Grade 2	3,4
P 6, female, 38 years	Grade 2	0,8
P 7, female, 36 years	Grade 2	0,5
P 8, female, 35 years	Grade 2	0,5
M \pm SE (n=8)		3,37 \pm 1,49
P 9, male, 56 years	Grade 3	5,0
P 10, male, 25 years	Grade 3	3,5
P 11, male, 46 years	Grade 3	0,5
P 12, male, 29 years	Grade 3	2,3
P 13, female, 24 years	Grade 3	3,2
P 14, male, 32 years	Grade 4	3,1
P 15, female, 54 years	Grade 4	5,2
P 16, male, 48 years	Grade 4	3,2
P 17, male, 53 years	Grade 4	2,4
P 18, male, 41 years	Grade 4	2,4
P 19, male, 34 years	Grade 4	1,8
P 20, male, 24 years	Grade 4	1,4
P 21, male, 38 years	Grade 4	0,8
P 22, male, 47 years	Grade 4	2,3
P 23, female, 45 years	Grade 4	6,9
P 24, female, 58 years	Grade 4	6,5
P 25, female, 60 years	Grade 4	6,9
P 26, female, 71 years	Grade 4	0,8
P 27, female, 57 years	Grade 4	2,0
M \pm SE (n=19)		3,08 \pm 0,645
Patients (n=27)		3,06 \pm 0,4*
Healthy donors (n=14)		0,4 \pm 0,04

Table 2. Effect of IFN α -DCs of patients with brain tumors on the proliferation of HEp-2 cells

2.2.9 Cytotoxic activity of patient IFN α -DCs vs U-87

In the next experiments, we investigated cytotoxic potential of patient IFN α -DCs towards TRAIL-sensitive tumor line U-87. Interestingly, we found no decrease in killer activity of patient DCs in this case. Furthermore, IFN α -DCs of patients with malignant tumors (Grade III-IV), unable to lyse HEp-2 cells, were highly cytotoxic against U-87 cells compared with healthy donors (Table 3). Further experiments demonstrated the ability of patient DCs to inhibit the proliferation of U-87 cell line which is also more expressed in patients than in donors ($46,6 \pm 7,5$ and $27,4 \pm 4,4\%$, respectively; $P_U < 0,01$). In this, we found a strong positive correlation between cytotoxic and cytostatic activities ($r_s = 0,89$; $p = 0,001$). Thus, impairment of cytotoxic and cytostatic activity of patient DCs was only revealed against HEp-2 cells.

Patients	Diagnosis	DC cytotoxic activity (%)	DC cytostatic activity (%)
P 1, male, 36 years	Grade 4	76	54
P 2, male, 24 years	Grade 4	55	28
P 3, female, 69 years	Grade 4	39	40
P 4, female, 42 years	Grade 3	60	44
P 5, female, 71 years	Grade 3	57	40
P 6, female, 54 years	Grade 4	60	43
P 7, female, 46 years	Grade 4	56	12
P 8, male, 48 years	Grade 4	80	89
P 9, male, 24 years	Grade 3	69	69
Patients (n=9)		61,3 \pm 4,1*	46,6 \pm 7,5*
Healthy donors (n=5)		38,6 \pm 8,3	27,4 \pm 4,4

Table 3. Cytotoxic/cytostatic activity of DCs in patients with brain tumors against U-87. The table represents the individual and average values ($M \pm SE$) of cytotoxic and cytostatic activities of patient and donor DCs. Cytotoxicity was measured by coculturing of DCs and [3 H]thymidine-labeled U-87 cells for 18 h at 20:1. For cytostatic activity evaluation, DCs and U-87 cells were cultured at 20:1 for 24 hours, followed by the introduction of [3 H]thymidine for 24 hours. * - $P_U < 0,01$ between donors and patients (U - Wilcoxon's test, Mann-Whitney).

2.2.10 TNF α production by donor and patient IFN α -DCs

Since the cytotoxic activity of DCs against HEp-2 cells was related with TNF-mediated apoptosis, we further compared the ability of DCs of patients with malignant gliomas (Grade III-IV) and donors to produce TNF α . The concentration of TNF α was evaluated in 4-

day cultures of LPS-activated IFN α -DCs. As follows from Table 4, supernatants from patient DC cultures differed little from healthy donor culture-conditioned medium by the level of TNF α production. A slight decrease in production of TNF α was a tendency, which had no statistical significance.

Patients	Diagnosis	TNF α (pg/ml)
P 1, male, 39 years	Grade 4	856
P 2, female, 46 years	Grade 3	767
P 3, male, 35 years	Grade 3	800
P 4, male, 46 years	Grade 3	914
P 5, male, 24 years	Grade 4	918
P 6, female, 43 years	Grade 4	693
P 7, female, 38 years	Grade 2	256
P 8, male, 52 years	Grade 4	486
P 9, male, 68years	Grade 3	710
Patients (n=9) M \pm SE		711 \pm 82
Healthy donors (n=11) M \pm SE		824 \pm 59

Table 4. TNF α concentrations in cultures of donor and glioma patient IFN α -DCs. The table represents the individual and average values (M \pm SE) of TNF α concentrations in culture supernatants of IFN α -DCs generated in vitro from peripheral blood of patients with malignant gliomas and healthy donors.

2.3 Discussion

The ability of DCs generated in vitro to inhibit the growth of human tumor cell lines and lyse tumor cells was first demonstrated by Chapoval (Chapoval et al., 2000). Thereafter, spontaneous cytotoxicity mediated by DCs without any stimulation was also described by other authors (Vanderheyde et al. 2004; Yang et al., 2001; Manna & Mohanakumar, 2002; Joo et al., 2002 ; Janjic et al., 2002), which revealed that the tumoricidal potential of DCs generated in the presence of GM-CSF and IL-4 was mediated by effector molecules such as FasL (Yang et al., 2001), TNF (Manna & Mohanakumar, 2002; Joo et al., 2002), lymphotoxin- α 1, β 2 (Lu et al., 2002) or TRAIL (Liu et al., 2001). The cytolytic properties of cultured human monocyte-derived DCs are enhanced by certain activation stimuli, such as LPS (Chapoval et al., 2000; Manna & Mohanakumar, 2002). While myeloid DCs being treated with IFN- γ exhibited upregulation of intracellular TRAIL and increased cytotoxic potential (Liu et al., 2001), study of antitumor activity of DCs generated in the presence of IFN α were not performed previously. In this study, we reported the novel data on cytostatic/cytotoxic activities of LPS-activated IFN α -DCs generated in vitro from peripheral blood monocytes of healthy donors and patients with brain tumors.

We report here that LPS-activated IFN α -DCs can lyse both NK-sensitive (Jurkat lymphoma cells) and NK-resistant (HEp-2, U-87) tumor cell lines. Such a cytotoxic effect requires cell contact, since the supernatants of IFN α -DCs either lack or possess the poor cytotoxic activity. Using HEp-2 tumor cells as targets, we revealed that DCs appear to promote the apoptosis and suppress cell cycle in tumor cells, thus having a cytostatic effect. Cytostatic

activity was also confirmed by the tumor growth/proliferation inhibiting capacity realized by IFN α -DCs. When compare the cytotoxicity of IFN α -DCs and IL4-DCs, we revealed that IFN α -DCs expressed the higher ability to kill Jurkat tumor cells as well as comparable with IL4-DCs cytotoxic activity in HEP-2 cultures.

Since the highest cytotoxic activity of IFN α -DCs was manifested in U-87 and Jurkat tumor cell cultures, which are reported to be sensitive to TRAIL-induced apoptosis (Lee et al., 2003; Panner et al., 2005; Siegelin et al., 2009), it is reasonable to assume that this cytotoxicity could be due to the stimulative effect of IFN α on TRAIL expression (Riboldi et al., 2009). Indeed, as a further corroboration of the suggested cytotoxic capacity, our data demonstrated that LPS-activated IFN α -DCs were found to contain significantly higher amounts of cells expressing membrane-bound TRAIL compared with IL4-DCs (data not shown). Apparently, these data could also explain a higher cytotoxic activity of IFN α -DCs on Jurkat tumor cells.

There are a very few data on sensitivity of HEP-2 cells to the cytotoxic effect of DCs mediated by TNF family molecules. The tumoricidal activity was not mediated by FasL/Fas or TRAIL/TRAILR2 systems, whereas TNF α /TNFR1 blocking completely abolished the ability of DCs to lyse HEP-2 cells. Thus, tumor cell line HEP-2 cells can be considered as resistant to FasL- and TRAIL-induced apoptosis, but sensitive to cytotoxicity triggered by TNF α /TNFR1 pathway. At that, the fact that anti-TNF α antibody almost completely decreased cytotoxicity, while DC culture-conditioned medium containing quite high concentrations of TNF α lacked lytic activity (Leplina et al., 2007b), further implies that membrane-bound, not the soluble form, of TNF α partially contributes to the effect.

Our results are consistent with the reported data on TNF α expression by HEP-2 cells (Paland et al., 2008), as well as the resistance of this tumor cell line to FasL-induced apoptosis (Morton & Blaho, 2007). The lack of sensitivity HEP-2 cells to TRAIL/TRAILR2-induced death explains the absence of distinction in the cytotoxic activity of IFN α -DCs and IL4-DCs against HEP-2 cells.

Since we revealed no correlations between cytotoxic and cytostatic effects of IFN α -DCs on HEP-2 cells, then one can believe that cytotoxicity and cytostasis could be mediated by distinct mechanisms. Similar tendency was observed for IL4-DCs (Vanderheyde et al., 2004), where the authors have shown that LPS-stimulated IL4-DCs possess TNF α -associated cytostatic activity unrelated with cytotoxicity. Indeed, IL4-DCs inhibited the growth of modified Jurkat cells deficient in caspase-8 or overexpressing Bcl-2. On the other hand, supernatants of IL4-DCs suppressed the proliferation of non-modified Jurkat cells, but showed no cytotoxic activity. Evidently, cytostatic and cytotoxic activities of IFN α -DCs are also implemented through independent mechanisms. This hypothesis could be confirmed by the fact that a higher cytotoxic activity of IFN α -DCs against U-87 and Jurkat cells was associated with less pronounced DC cytostatic activity on these lines compared with HEP-2. Investigation of the effector functions of IFN α -DCs in patients with intracerebral gliomas revealed impaired ability of these cells to lyse HEP-2 tumor cells. Importantly, such an impairment of DC cytotoxicity was identified mainly in patients with high grade (III-IV) brain tumors, while in low grade (I-II) tumors DCs were quite effective killers. Furthermore, patients with intact DC cytotoxic activity had a higher survival rate than patients with reduced killer activity. In addition, patient DCs regardless of tumor histology showed no cytotoxic activity against HEP-2 cells, whereas both cytotoxic and cytostatic activities of DCs against U-87 cells were found to be enhanced.

According to the data, glioblastoma cells U-87 are resistant to cytotoxicity mediated by TNF α (Sawada et al., 2004), and sensitive to TRAIL- (Knight et al., 2001) and Fas-induced

apoptosis (Choi et al., 2004). As we have found, DC cytotoxicity on HEP-2 cells likely engaged TNF/TNFR1 pathway than TRAIL or FasL effects. Given these facts, the high cytotoxic activity of patient DCs against U-87 cells and dramatic decline of such activity against HEP-2 tumor cells could indicate a defect in TNF α -related mechanism of DC cytotoxicity in patients with malignant gliomas.

Our facts seemed to be important in several aspects. First, the defect in cytotoxic activity of DCs may be of interest in diagnosis and prognosis, since the expression of cytotoxic activity is associated with tumor malignancy and survival rates. Second, this phenomenon is of interest from the pathogenetic point of view. Malignant brain tumors induce a weak antigen-specific response due to tumor-induced immunosuppression, as well as localization of the tumor in the immunologically privileged brain tissues (Parney et al., 2000). We have previously shown that patient IFN α -DCs are characterized by intact antigen-presenting function, capable of activating T cells to produce Th1 cytokines and induce proliferation of T lymphocytes to antigens of tumor cell lysates (Chernykh et al., 2009). Then impairment of DC effector functions may inhibit the early induction of antigen-specific immune response being yet another reason for infringement of anti-tumor protection in patients with malignant brain tumors.

We can also assume that if tumoricidal potential of DCs is disturbed, cytoreductive therapy (radio-chemotherapy) becomes especially important, both in regard of direct elimination of tumor cells and release of tumor antigens required to start specific immune response.

However, whether IFN α -DCs could effectively destroy primary glioma cells and does this ability is disrupted in patients with malignant gliomas remains to find. Most of glioblastoma cell lines and primary cells gliomas are absolutely resistant to cytotoxic effect of certain proapoptogenic molecules, such as TRAIL (Eramo et al., 2005) and TNF. At that, the effective destruction of tumor lines by DCs requires the interaction of separate molecules. Besides, it is shown that some proapoptogenic molecules could induce the expression of receptors for another mediators of apoptosis. Such as, TNF α can induce the expression of Fas and sensitize glioma cells to FasL/Fas-mediated apoptosis (Weller et al., 1994).

The further elucidation of the DC cytotoxic/static activity mechanisms and the possible role of the defect of DC cytotoxic properties in patients with gliomas as well as the studies on correlation between the antitumor activity of IFN α -DCs and clinical outcomes could probably explain the different sensitivity of cancer patients to the treatment, and justify new immunotherapeutic approaches to the treatment of malignant brain gliomas.

3. Conclusion

The capacity of IFN α -DCs to lyse tumor cell lines and inhibit their proliferation has been investigated. LPS-activated IFN α -DCs of healthy donors were shown to have dose-dependent cytotoxic and cytostatic activity against various tumor lines through the induction of apoptosis and arrest of cell cycle. DCs lysed both TRAIL-sensitive (Jurkat cells) and TRAIL-resistant (HEP-2) cells, and cytotoxic activity against HEP-2 line was mediated through the TNF-TNFR1 pathway. In contrast to healthy donors, DCs of patients with malignant glioma failed to inhibit growth, but stimulated proliferation of HEP-2 cells. In addition, patient DCs had significantly reduced cytotoxic activity against HEP-2 cells. Patients with decreased cytotoxic activity were characterized by significantly lower survival since defect of cytotoxic activity was associated with high-grade glioma. The defective cytotoxic activity of DCs noted against HEP-2 cells was not revealed against glioblastoma U-

87 line. The data obtained suggest that defect of antitumor activity of patient DCs may have diagnostic and prognostic significance. However, whether IFN α -DCs could effectively destroy primary glioma cells and does this ability play role in pathogenesis of brain glioma remains to be clarified.

4. Acknowledgment

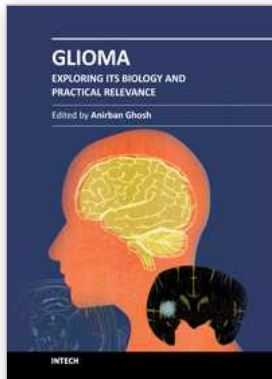
We are grateful to our patients for their courage and faith in us.

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Glioma - Exploring Its Biology and Practical Relevance

Edited by Dr. Anirban Ghosh

ISBN 978-953-307-379-8

Hard cover, 486 pages

Publisher InTech

Published online 02, November, 2011

Published in print edition November, 2011

The title 'Glioma - Exploring Its Biology and Practical Relevance' is indicative of its content. This volume contains 21 chapters basically intended to explore glioma biology and discussing the experimental model systems for the purpose. It is hoped that the present volume will provide supportive and relevant awareness and understanding on the fundamental advances of the subject to the professionals from any sphere interested about glioma.

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Olga Leplina, Tamara Tyrinova, Marina Tikhonova, Ekaterina Shevela, Vyacheslav Stupak, Sergey Mishinov, Ivan Pendyurin, Mikhail Sadovoy, Alexander Ostanin and Elena Chernykh (2011). Direct Antitumor Activity of Interferon-Induced Dendritic Cells of Healthy Donors and Patients with Primary Brain Tumors, Glioma - Exploring Its Biology and Practical Relevance, Dr. Anirban Ghosh (Ed.), ISBN: 978-953-307-379-8, InTech, Available from: <http://www.intechopen.com/books/glioma-exploring-its-biology-and-practical-relevance/direct-antitumor-activity-of-interferon-induced-dendritic-cells-of-healthy-donors-and-patients-with->

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