Intra-tumoral dendritic cells increase efficacy of peripheral vaccination by modulation of glioma microenvironment

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Pilot data showed that adding intratumoral (IT) injection of dendritic cells (DCs) prolongs survival of patients affected by glioblastoma multiforme (GBM) treated by subcutaneous (SC) delivery of DCs. Using a murine model resembling GBM, we investigated the immunological mechanisms underlying this effect. C57BL6/N mice received brain injections of GL261 glioma cells. Seven days later, mice were treated by 3 SC injections of DCs with or without 1 IT injection of DCs, DC maturation, induced by pulsing with GL261 lysates, was necessary to develop effective immune responses. IT injection of pulsed (pDC), but not unpulsed DCs (uDC), increased significantly the survival, either per se or in combination with SC-pDC (P < .001 vs controls). Mice treated by IT-pDC plus SC-pDC survived longer than mice treated by SC-pDC only (P = .03). Injected pDC were detectable in tumor parenchyma, but not in cervical lymph nodes. In gliomas injected with IT-pDC, CD8+ cells were significantly more abundant and Foxp3+ cells were significantly less abundant than in other groups. Using real-time polymerase chain reaction, we also found enhanced expression of IFN-γ and TNF-α and decreased expression of transforming growth factor-beta (TGF-β) and Foxp3 in mice treated with SC-pDC and IT-pDC. In vitro, pDC produced more TNF-α than uDC; addition of TNF-α to the medium decreased the proliferation of glioma cells. Overall, the results suggest that IT-pDC potentiates the anti-tumor immune response elicited by SC-pDC by pro-immune modulation of cytokines in the tumor microenvironment, decrease of Treg cells, and direct inhibition of tumor proliferation by TNF-α.

Keywords: dendritic cells, glioma, intratumoral vaccination, tumor microenvironment

Strategies for cancer immunotherapy critically rely on the use of dendritic cells (DCs) for antigen presentation in peripheral lymph nodes, where CD8+ T lymphocytes are instructed to initiate a cytolytic antitumor response.1 Such a response, however, has to develop in a tumor microenvironment that is strongly characterized by the presence of diverse immunosuppressive factors, allowing the tumor to escape immune surveillance:2 brain tumors provide an excellent example of this scenario.3 Several clinical experiences have been reported for DC immunotherapy of malignant gliomas and specifically of glioblastoma multiforme (GBM), the most frequent and aggressive of primary brain tumors.4 Results in terms of safety have been satisfactory but clinical efficacy, especially when treating relapses of GBM, can certainly be increased.5 The production of different immune suppressive cytokines, particularly transforming growth factor-beta (TGF-β), and the presence of CD4 + CD25 + Foxp3+ T cells (T-regulatory cells, Treg) has been correlated to evidence of low or decreased efficacy of DC immunotherapy in preclinical models and in patients.6–9 Thus, combined strategies linking peripheral immune responses to a modification of the tumor microenvironment are desirable in order to improve the clinical potential of DC immunotherapy.

Direct intratumoral (IT) injection of unpulsed DCs (uDC) has been attempted in different tumors, including GBM:10–17 the results may encourage further investigations on the molecular and cellular
modifications that the local presence of DCs implies for cancer biology. We have done this in a murine model of malignant glioma obtained by brain injection of GL261 cells, studying the effects of IT injection of pulsed DC (pDC) with tumor lysate in established GL261 tumors. Results suggest that IT-DCs do not migrate to cervical lymph nodes and increase survival significantly in mice when combined with peripheral vaccination.

This enhanced efficacy, therefore, may be due to direct, significant changes in the tumor microenvironment, which are modulated by IT-pDC combined with the increased recruitment of specific T cells induced by SC-pDC. These data may have clinical implications for future clinical trials in patients affected by GBM and malignant gliomas.

**Material and Methods**

**Cell Cultures**

GL261 cells were grown in DMEM (EuroClone), 20% fetal bovine serum, L-glutamine, and penicillin/streptomycin. Immature DCs were prepared from the bone marrow (BM) of C57BL6/N mice and from C57BL/6-TgN(ActbEGFP) transgenic mice, both syngeneic to GL261 cells. Briefly, mice were sacrificed and BM was flushed from the femur and tibia. Cells were cultured in 6-well plates for 5 days in Iscove’s medium (Sigma Aldrich, St. Louis, MO) in the presence of 5 ng/mL rmGM-CSF and 5 ng/mL rmIL-4 (LI StarFISH, Milan, Italy). After 3 days, 70% of the medium was replaced with fresh medium containing GM-CSF and IL-4. For cytokine assays, culture supernatants were collected on day 5 (immature DC) or on day 6 after pulsing with tumor lysate (mature DC). Concentrations of IL-6 (R&D Systems), TNF-α, and IFN-γ (T1/Th2 Kit, CBA system, Becton Dickinson, San Diego, CA) were defined according to manufacturer’s instructions.

DC phenotype was characterized using the anti-mouse CD11c, CD80, antibodies CD86, MHC class I (H2b), class II (Ia), and CD40 (Pharmingen, San Diego).

**T-cell Monitoring**

The spleen and lymph nodes were analyzed for T-cell subsets using anti-mouse CD3-FITC, CD4-PE-Cy5, CD8-PE, and CD25-FITC, all from Pharmingen and FoxP3-PE, from eBioscience (San Jose, CA). FACS analyses were conducted on an FACSCalibur flow cytometer (Becton Dickinson).

Ipsilateral or contralateral cervical or axillary lymph nodes constituted four separate groups. Two or three lymph nodes from each group were removed from each mouse (n = 4 for each group) at each time point (ie, day 15, 24, and 31 after GL261 cells injection). Cell suspensions were prepared from pooled lymph nodes from each group by gentle mechanical disaggregation and counted. The number of viable cells per group of lymph nodes was determined using trypan blue exclusion and ranged from 3 to 6 million cells. The total number of lymphocytes in the different groups of lymph nodes obtained from treated mice was comparable with that of control mice.

Cell staining for phenotypic analysis was carried out as described by the eBioscience protocol.

Briefly, 1 million cells were surface stained with anti-mouse CD4-PE-Cy5 and anti-mouse CD25-FITC and subsequently with anti-mouse FoxP3-PE (EJF-16s) or isotype control. About 500 000 cells were stained for CD3/CD8 (co-staining).

**DC Labeling and Loading**

DCs from C57BL6/N were labeled by superparamagnetic iron oxide nanoparticles (Endorem; Guebert, Genova) before pulsing with GL261 lysate. On day 5, Endorem (15 μg/mL) was added to the culture medium and incubated for 24 h at 37°C, 5% CO2. Higher concentrations of Endorem induced nonspecific maturation of DCs (data not shown). On day 6 after labeling, DCs were loaded using tumor lysate obtained by sonication of 1 × 10⁷ GL261 cells. DCs were pulsed as described by Ashley et al.¹⁹ Cell surface markers such as MHC class I and II, CD80, CD86, CD40 (Pharmingen) were evaluated on labeled DCs before (uDC + Endorem) and after pulsing (pDC + Endorem) and compared with unlabeled DCs.

**DC Vaccinations**

A total of 100 C57BL6/N mice received brain injections of 1 × 10⁵ GL261 cells (coordinates with respect to the bregma: 0.7 mm posterior, 3 mm left lateral, 3.5 mm deep, into the nucleus caudatum). On day 7 after tumor implantation, mice were treated by subcutaneous (SC) injections of 1 × 10⁶ pDC (3 injections, each spaced 1 week) apart with or without one IT injection of 2 × 10⁵ pDC or uDC, respectively (IT injection was performed once, at the same time of the first SC injection). DCs were injected into the tumor using the same stereotactic coordinates. For the survival studies, DCs injected were GFP positive and experimental groups were as follows: PBS only as control (n = 15); IT-uDC (n = 10); IT-pDC (n = 10); SC-pDC (n = 31); IT-uDC plus SC-pDC (n = 10); IT-pDC plus SC-pDC (n = 14). For monitoring studies, DCs injected IT or SC alone were GFP or Endorem positive to track the potential migration toward peripheral lymph nodes, while DCs SC injected for the other combination of treatments were obtained from naive mice to allow the identification of potential IT-DC migration toward the lymph nodes.

**Real-Time Polymerase Chain Reaction**

RNA for real-time polymerase chain reaction (RT-RCR) from paraffin-embedded material was extracted using reagents included in the Absolutely RNA FFPE kit (Stratagene, San Diego, CA). cDNA was synthesized from the RNA using oligo(dT) and M-MLV Reverse Transcriptase (Life Technologies). PCR was performed.
using a GeneAmp 5700 Sequencer Detector (Applied Biosystems, CA). Gene-specific oligonucleotide probes were included in the Assay on Demand Kit (Applied Biosystems). Amplification values obtained for each gene used were normalized to the endogenous control β-2-microglobulin.

**Histology and Immunohistochemistry**

A representative number of brain and lymph nodes from each group were embedded in OCT, snap frozen in cold isopentane, and stored in liquid nitrogen or post-fixed in 4% paraformaldehyde for paraffin embedding. Frozen sections were analyzed using anti-CD8 antibodies (Novocastra Laboratories), whereas paraffin-embedded sections were analyzed with rat monoclonal IgG1 anti-CD3, anti-CD4 (1:20, Novocastra Laboratories); and anti-Foxp3 (eBioscience) antibodies.

**Quantification of Tumor Infiltrating Lymphocytes by Immunohistological Analysis**

The absolute cell number of CD8+/−, CD4+/−, and Foxp3+ total infiltrating lymphocytes (TILs) were calculated in 5 independent high power fields corresponding to 0.2 mm² for a total of 1 mm² of tumor area for each sample. Positive cells were counted only within the tumor area (TILs) with the exclusion of peritumoral lymphocytes. Two representative mice for each treatment group at both time points have been compared: IT-pSC plus SC-pDC, SC-pDC, and control on day 15 and 24 after tumor implantation. Results have been expressed as mean number of positive cells ± SD for each group. The positive rates were counted thrice manually from the photographs by two observers (M.R. and P.L.P.). Student’s t-test was performed for evaluating the significance of data. Statistical significance was determined at the <.05 level.

**Statistical Analysis**

Survival estimates and median survivals were determined using the Kaplan–Meier method. Student’s t-test was used for calculating the significance of data. Statistical significance was determined at the <.05 level and investigated using MedCalc, version 9.3.

**Results**

**IT Injection of pDC Increases Survival of Mice Bearing GL261 Glioma**

The anti-tumor efficacy of IT injection of DCs was evaluated per se or in combination with SC injection of DCs in immune-competent C57BL6/N mice with established GL261 tumors. Kaplan–Meier analysis of survival is shown in Fig. 1A and B and in Supplementary Material, Table S1. All controls (tumor-bearing mice treated with PBS) died by day 33. Treated mice were monitored for 200 days. SC-uDCC injection had no therapeutic effect and mice survived as long as controls. IT-uDCC injection had a minor effect on survival, as only 10% of mice survived the tumor challenge (P < .02). IT injection of pDC, however, extended survival to 30% of the mice (P < .001 vs control).

Treatment with SC-pDC led to survival of 50% of the mice (P < .001 vs control): combining IT-uDCC left unchanged the anti-tumor efficacy of SC-pDC. In contrast, combination with IT-pDC increased survival to 78% of the mice. The survival rate of IT-pDC plus SC-pDC mice was significantly higher than that of control mice (P < .0001) and, interestingly, of SC-pDC mice (P = .03).

These data demonstrate that DC pulsing with tumor lysate is necessary to develop an immune-based, effective anti-tumor response in the GL261 model of glioma and that IT injection of pDC is effective per se and significantly increases the efficacy of SC-pDC.

**After Injection into Brain Gliomas, DCs Remain Viable and Active**

As a first step in the study of the effects of IT DCs, we investigated their fate by monitoring the distribution of Endorem-loaded or GFP-positive DCs injected into the tumor mass. DC labeling by Endorem (see Materials and Methods) did not change significantly the expression of relevant maturation markers, as evaluated by flow cytometry, in comparison with GFP or naïve DC (Supplementary Material, Fig. S1A, C, and D). Histological staining with anti-GFP antibody revealed the presence of DC-GFP in the tumor a mass of vaccinated IT-pDC plus SC-pDC mice and of direct interaction between DC-GFP and tumor cells (Fig. 2A and B).

Prussian Blue staining showed Endorem-loaded DCs in the tumor bulk and evidence of an interaction with T cells (Fig. 2C and D). These studies also suggested that transplanted DCs were viable for 1 week after IT injection. Analysis of cervical lymph nodes in 8 mice 1 week after DC injection, however, failed to detect Endorem-positive (n = 4 mice) or GFP-positive DCs (n = 4 mice). The persistence of pDC into the tumor, their interaction with tumor infiltrating CD3+ lymphocytes (Fig. 2D), and the absence of DC in regional lymph nodes all suggest that DCs did not migrate a long distance from their injection site into the brain, implying that their biological activity was mostly mediated in situ.

**Increase of CD8+ Cytotoxic Cells and Decrease of Treg in Gliomas of IT-pDC Vaccinated Mice**

Tumor infiltrating lymphocytes were characterized by immunohistochemistry performed 15 and 24 days after implantation of GL261 cells. Quantitative evaluation of the data is summarized in Table 1: the statistical analysis comparing treated groups and controls showed significant differences.

At both time points we found a significant increase of CD8+ T cells in tumors treated with IT-pDC plus SC-pDC compared with SC-pDC only (P < 10^-8) or
controls \( (P < 10^{-11}) \). On day 15 a significant decrease of CD4\(^+\) cells in tumor was present in IT-pDC plus SC-pDC compared with SC-pDC \( (P < .01) \) or with control \( (P < 10^{-14}) \). The differences in TIL composition were more evident on day 24 after tumor implantation. In particular, controls showed very few CD3\(^+\) and CD8\(^+\) T cells compared with treated tumors. Foxp3\(^+\) cells appeared significantly less in tumors from mice treated with IT-pDC plus SC-pDC than SC-pDC only \( (P = .01 \text{ on day } 15; P < 10^{-6} \text{ on day } 24) \) or controls \( (P = .05) \). Interestingly, no significant difference was observed in the Foxp3 expression among SC-pDC or control groups. Examples of histological evaluation are shown in Figs. S2 and S3 (Supplementary Material).

The distribution of TILs was characteristic on day 24 after GL261 cell injection (Supplementary Material, Fig. S3). Interestingly, while control and SC-pDC mice showed several CD4\(^+\)Foxp3\(^+\) cells preferentially dispersed in the tumor parenchyma, IT-pDC plus SC-pDC mice showed a peculiar perivascular distribution of the CD4\(^+\)Foxp3\(^+\) cells.

Tumor diameters were measured at both time points in all mice investigated (4 for each group). Mice injected IT with pDC exhibited delayed tumor growth, compared with control and SC-pDC mice, in agreement with survival data (Table 1).

**IT DCs Modulate the Glioma Microenvironment by Decreasing TGF-\( \beta \) and Increasing TNF-\( \alpha \) and IFN-\( \gamma \) Expression**

To characterize the effect of IT injection of pDC on the tumor microenvironment, we investigated the expression of key molecules for immune suppression (TGF-\( \beta \), Foxp3) or activation (IFN-\( \gamma \) and TNF-\( \alpha \)) by RT-PCR on paraffin-embedded tumors from treated and control mice. These mice were the same also investigated by immunohistochemistry (Supplementary Material, Figs. S2 and S3). We found that in mice treated by SC-pDC only compared with those treated by IT-pDC plus SC-pDC TGF-\( \beta \) expression was 2.2 \( (P = .009, \text{ day } 15) \) and 1.6-fold higher \( (P = .01 \text{ day } 24) \), respectively (Fig. 3A), and Foxp3 expression was 2.6 \( (P = .007 \text{ day } 15) \) and 1.2-fold \( (P = .03 \text{ day } 24) \) higher (Fig. 3B). IFN-\( \gamma \)-expression, on the contrary, was 2-fold higher in IT-pDC plus SC-pDC than in SC-pDC only \( (P = .03 \text{ on day } 15; P = .002 \text{ on day } 24) \).

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**Fig. 1.** IT injection of pDC enhances anti-tumor efficacy of peripheral administration of DC. Kaplan–Meier survival curves represent the percentage of surviving animals over time (in days). Mice received \( 1 \times 10^5 \) GL261-glioma cells IT on day 0 and were treated with three SC injections of \( 1 \times 10^6 \) pDC spaced 1 week apart and/or one IT injection of \( 2 \times 10^5 \) GL261 Lysate-LPDC or UDC on day 7 after tumor implantation. The experimental conditions were: control mice received PBS \( (n = 15, \text{ reported in [A] and [B]}) \); (A) IT-pDC \( (n = 10; P = .001 \text{ vs PBS}) \); IT-uDC \( (n = 10; P = .02 \text{ vs PBS}) \); IT-uDC plus SC-pDC \( (n = 10; P < .001 \text{ vs PBS}) \); SC-uDC \( (n = 10, P = .2) \); (B) SC-pDC \( (n = 31, P < .0001 \text{ vs PBS}) \); IT-pDC plus SC-pDC \( (n = 14, P < .0001 \text{ vs PBS}) \). We divided the survival curves for clarity. Control curves are indeed the same. In Supplementary Material, Table S1, all \( P \)-values for treatment groups were reported vs control and vs each other group.
Fig. 2. Intra-tumoral detection of pDC labeled by GFP or Endorem. DC-GFP can interact with tumor cells (A) and contact them with long spiky arms (B). Histological evaluations using Prussian Blue staining revealed iron in DC loaded with Endorem ex vivo 1 week after IT injection (C). Loaded DC can interact with tumor infiltrating, CD3+ lymphocytes (D).

Table 1. Quantitative analysis of TILs by immunohistochemistry

<table>
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<tr>
<th>Treatment</th>
<th>Tumor size (mm)a</th>
<th>Tumor infiltrating lymphocytesb</th>
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<td>CD3</td>
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<td>34.5 ± 3.7****</td>
</tr>
<tr>
<td>SC-pDC (15 days)</td>
<td>2</td>
<td>19.7 ± 3.0***</td>
</tr>
<tr>
<td>Control (15 days)</td>
<td>3</td>
<td>13 ± 3.2</td>
</tr>
<tr>
<td>IT-pDC + SC-pDC (24 days)</td>
<td>3</td>
<td>29.5 ± 3.9***</td>
</tr>
<tr>
<td>SC-pDC (24 days)</td>
<td>5</td>
<td>19.9 ± 2.9**</td>
</tr>
<tr>
<td>Control (24 days)</td>
<td>6</td>
<td>15.3 ± 3.3</td>
</tr>
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</table>

aMajor tumor diameter from a representative mouse for each group.
bAverage ± standard deviation of positive cells in 5 areas/mouse per group (10 areas/group). From each group two representative mice were analyzed (number of positive cells/5 different 40× HPF).

****p < 10^-12; ***p < 10^-8; **p < 10^-4; *p < .05.

(Fig. 3C) and TNF-α was 3.8-fold higher on day 15 (P = .03) and 1.5-fold on day 24 (P = .07) (Fig. 3D).

The cytokine concentration was also evaluated by RT-PCR on frozen tumors from the same groups of mice on day 15 and 24: results confirmed the expression patterns described above (data not shown).

**TNF-α Released by Mature DCs Decreases Proliferation of GL261 Glioma Cells**

The to gain hints on the direct contribution of IT-pDC to tumor microenvironment, we studied cytokine production of freshly isolated BM-derived DC-GFP. For this, we used conditioned media from immature DCs (ie, DC cultured for 5 days in the presence of GM-CSF and IL-4) and mature DCs (ie, 24 hours after pulsing with tumor lysate). Maturation was defined according to phenotypic analysis by flow cytometry, considering significantly increased expression of MHC class I and II, co-stimulatory molecules, and CD40 (Supplementary Material, Fig. S1A). Three key cytokines produced by mature DCs were considered: TNF-α, IFN-γ, and IL-6.20–23 pDCs produced more TNF-α, but not IFN-γ or IL-6, than uDCs (Fig. 3D). The same evaluation was performed on immature and mature naïve DCs loaded with Endorem: the concentration of secreted cytokines was similar (data not shown). To define the effect of TNF-α, IFN-γ, and IL-6 on tumor proliferation, we cultured GL261 cells for 24 hours in the presence of “physiological” (100 or
500 pg/mL) or “supra-physiological” (10 or 100 ng/mL) concentrations of each of these cytokines. The MTT assay showed a significant reduction of GL261 proliferation only at “supra physiological” concentrations of IL-6 (100 ng/mL; \( P = .001 \)) and IFN-\( \gamma \) (\( P < .001 \)), but not in the presence of 100 pg/mL. TNF-\( \alpha \)–treated cells, however, showed a significant reduction in GL261 proliferation also at “physiological” concentrations, suggesting that TNF-\( \alpha \) is very effective at inhibiting GL261 growth in vitro and, possibly, in vivo (Fig. 4B).

**SC-pDCs Induce the Generation of GL261-specific Effector Lymphocytes**

To characterize the direct effects of treatment on T-cell function, the spleen and draining lymph nodes were harvested, counted for total number of cells, and monitored by flow cytometry 15 and 24 days after tumor implantation.

Splenocytes for in vitro cytotoxicity studies were monitored before and after prestimulation with GL261 in controls (\( n = 2 \) on day 15, \( n = 2 \) on day 24), IT-pDC plus SC-pDC (\( n = 4 \) on day 15, \( n = 3 \) on day 24), SC-pDC only (\( n = 4 \) on day 15, \( n = 3 \) on day 24), and one healthy mouse (Table 2). This stimulation resulted in an expansion of CD3\(^+\)/CD8\(^+\) and CD4\(^+\)/CD25\(^2\) T cells in vaccinated mice compared with control mice. In IT-pDC plus SC-pDC, CD8\(^+\) T cells increased from 13.2 \( \pm \) 8.2 to 39.9 \( \pm \) 9.8 after prestimulation (\( P = .005 \), 15 days; \( P = .002 \), 24 days). In contrast, on day 15, TNF-\( \alpha \) expression was significantly higher in IT-pDC plus SC-pDC–treated mice than in SC-pDC–treated mice (\( P = .005 \), 15 days; \( P = .07 \), 24 days). Relative expression of cytokines in vaccinated mice was compared with controls.
of mice treated with IT-pSC plus SC-pDC vs SC-pDC only is not dependent on the generation of more effective immune responses at the periphery.

Monitoring of lymphocytes was performed on cervical and axillary lymph nodes obtained from the same mice and from mice on day 31 after tumor implantation. On day 15 and 24, in the cervical and axillary lymph nodes of both groups of treated mice, T cells were at similar levels. In fact, we observed a significant increase of CD3+CD8+ and CD4+CD252 T cells, and a significant decrease of CD4+CD25+ when compared with controls (Supplementary Material, Tables S2 and S3).

Overall, we can conclude that IT-pDC are not effective to potentiate the peripheral immune reaction.

**Time-Dependent Increase of CD4 + CD25 + Foxp3+ T Cells in the Spleen and Draining Lymph Nodes of Tumor-Bearing Mice**

To study the peripheral subpopulation of Treg, we evaluated by flow cytometry and RT-PCR the expression of Foxp3 on CD4 + CD25+ T cells isolated from the spleen, cervical, and axillary lymph nodes ipsilateral and contralateral to the tumor of control, IT-pDC plus SC-pDC and SC-pDC mice at different time points.

In Table 2, we reported the characterization of CD4 + CD25+ splenocytes from treated and control mice, monitored before and after prestimulation. A significant decrease of CD4 + CD25+ T cells was evident at different time points in IT-pDC plus SC-pDC mice: from 9.8 ± 1.6 to 6.0 ± 1.4 (P = .007) on day 15 and from 11.6 ± 1.2 to 5.5 ± 2.1 (P = .01) on day 24. Splenocytes from SC-pDC only-treated mice had a similar decrease: from 12.2 ± 0.4 to 8.5 ± 1.7 (P = .005) on day 15 and from 12 ± 1 to 6.8 ± 1.6 (P = .008) on day 24.

Control mice showed a significant increase of CD4 + CD25+ T cells when compared with treated mice (P = .007 or P = .05 IT-pDC plus SC-pDC vs control; P = .05 or P = .01 SC-pDC vs controls on day 15 or 24, respectively). Differences between the two groups of treated mice were not significant. Similarly, ipsilateral, but not contralateral, lymph nodes of control mice showed a time-dependent increase of CD4 + CD25+ T cells (Supplementary Material, Tables S2 and S3, and data not shown).
Table 2. Flow cytometry monitoring of GL261-specific T cells from splenocytes of controls and vaccinated mice before and after GL261 prestimulation on day 15 and 24 after tumor injection

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<th>Treatment</th>
<th>% CD3^+ /CD8^+</th>
<th>% CD4^+ CD25^-</th>
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The Treg fraction was monitored in healthy mice, controls, IT-pDC plus SC-pDC- and SC-pDC- vaccinated mice (n = 4 for each group and time point, including those reported in Table 2 and Supplementary Material, Table S2) by flow cytometry. Foxp3 expression in CD4+ CD25+ cells isolated on day 15, 24, and 31 from cervical lymph nodes and from spleens, and on day 31 from axillary lymph nodes. Tumor cells were implanted in the left hemisphere; however, Treg cells were evaluated in both ipsilateral and contralateral cervical and axillary lymph nodes. No significant differences were observed between right and left lymph nodes of treated (SC-pDC or IT-pDC plus SC-pDC) and healthy mice, whereas in control mice Treg percentage was significantly higher in ipsilateral cervical lymph nodes (not shown).

As shown in Fig. 5, a time-dependent, significant increase in the percentage of Foxp3+ cells was detectable in cervical lymph nodes after tumor implantation. In treated mice, the percentage of Treg decreased significantly when compared with controls. The P-values for each time point of treated vs control or healthy mice are reported in Supplementary Material, Table S4. A similar trend was confirmed by evaluating Foxp3 expression by RT-PCR in CD4+ CD25+ immunoselected T cells from the spleen and lymph nodes on day 24 and 31 (Supplementary Material, Fig. S5B and C).

The Treg fraction monitored by flow cytometry Foxp3 expression on CD4+ CD25+ cells isolated from axillary lymph nodes and from spleens of healthy mice, controls, and IT-pDC plus SC-pDC vaccinated mice was reported in Supplementary Material, Fig. S5A.

Discussion

A number of studies have shown that DC immunotherapy of cancer, and specifically of gliomas, has an interesting therapeutic potential but that important limitations are created locally by cytokines produced by the tumor that may induce T-cell anergy and amplification of the Treg fraction.24 Thus, together with the identification of relevant neoplastic targets for DC “vaccination”, the reversal of tumor-mediated immune suppression appears to be of paramount importance for the success of DC-based immunotherapy of cancer.25 Different strategies have been explored to locally modify tumor immune suppression: eg, the use of antisense oligonucleotides to quench expression of one of the most important of immunosuppressive cytokines, TGF-β.6,26,27 Yamanaka et al. in particular, have found that patients with both intratumoral and intradermal administration of DC (n = 7) had a longer survival time than the patients with intradermal administration only (n = 11; P = .043).16 Our results support the idea that IT delivery of DC may have increased anti-tumor efficacy by creating an IT environment more favorable to the development of T-cell-mediated immune responses.

This is demonstrated by the prolonged survival of mice with GL261 malignant gliomas treated by IT-pDC only or in combination with SC-pDC. Histological, quantitative analysis shows that in these mice the number of CD8+ T cells infiltrating the tumor is significantly higher and the number of immunosuppressive Treg cells is significantly lower. At the
molecular level, in agreement with this, the amount of TGF-β and Foxp3 decreases and that of IFN-γ increases.

Several evidences support the concept that Foxp3 is a necessary and sufficient marker for mouse Treg expansion and function. Ectopic Foxp3 expression has been shown to drive Treg function, lack of Foxp3 has been correlated with a lack of Treg cells. Thus, the decrease of Foxp3 in our model, as evaluated by quantitative histological evaluation and by RT-PCR within tumors, indicates a decreased number of functionally active Tregs.

We believe that these results are mostly mediated by the DCs acting locally and surviving for several days after injection, as we could not find any evidence of DCs migrating from intracranial tumors to cervical or axillary lymph nodes. On the contrary, using similar experimental conditions (ie, DC manufacturing, number of cells injected, animal strain), we previously found that SC injection in the flanks was followed, 1 week later, by evidence of migrating, labeled DCs to axillary draining lymph nodes. Previous data have proposed that, after intracranial injection, DC may migrate to cervical lymph nodes. One interesting observation, possibly explaining this discrepancy with our data, suggests that only DCs that are injected into the cerebrospinal fluid, but not into brain parenchyma, are actually able to reach lymph nodes in the vicinity. Thus, our stereotaxic coordinates for DC injection may not favor their migration to the lymph nodes, implying that their biological activity is mostly mediated in situ.

For this reason, we do not find an increased efficacy at the periphery of IT-pDC plus SC-pDC over SC-pDC only, when monitoring T cells in the spleens and draining lymph nodes and their cytotoxic effects in vitro. The SC injection of DGs induces a systemic anti-tumor effect and is effective in eliciting T-cell responses and creating a convergence of immune response elements to the tumor site. IT-pDC can modulate the tumor microenvironment and potentiate the anti-tumor response, significantly increasing survival in combination with SC-pDC and exerting different functions on tumor biology.

Together with temporary release of pro-inflammatory cytokines by DCs, it is also conceivable that DCs initiate some dialogue with surrounding microglia, a possibility not explored by our experiments but suggested by previous evidence showing that under inflammatory conditions a fraction of CD11b+ microglial cells may express the DCs marker CD11c. Other data also showed that DCs may amplify T-cell–mediated immune responses in the CNS. The role of DCs in the CNS, however, has also been reported as an immunosuppressive one, with DCs inhibiting activation of T cells. Interestingly, however, such an inhibitory role was played by DCs showing a “phenotype similar to immature BM-derived DC.” This draws attention to the central role played by DC maturation in raising effective T-cell responses against gliomas in the experiments we have reported. In particular, the use of IT-uDC only led to survival of 10% of the mice and the addition of IT-uDC to SC-pDC was ineffective. This is a relevant issue, as several reports have described the effects of IT injection of DC in glioma models in rodents. The studies, however, DC were not loaded with tumor homogenates or induced to maturation by in vitro exposure to appropriate cytokines, rather they were co-injected with irradiated glioma cells or transduced to overexpress IL-23. IT injection of DCs in other tumors has also been performed but had to be supplemented by other treatments aimed at enhancing antigen presentation, like apoptosis of tumor cells in situ obtained by tumor irradiation, hyperthermia, or photodynamic therapy. Thus, we propose that DC maturation, which in our system is obtained by loading DCs ex vivo with a tumor lysate, is a critical requisite for developing effective anti-tumor responses by IT injection of DCs. Observations showing that infiltration of malignant melanomas by mature DCs, defined by expression of the maturation marker LAMP, is significantly correlated to prolonged survival, providing indirect confirmation of our claim.

Our results support the idea that IT-pDC may contrast tumor immune suppression favoring T-cell activation, as demonstrated by the significant presence of CD8+ TILS and by significant increased levels of IFN-γ on day 24 after tumor injection, ie, 10 days after IT “vaccination”. TNF-α, on the contrary, was highly expressed on day 15 (8 days after IT “vaccination”) and less on day 24, which is consistent with the limited life span of DCs injected IT.

High levels of TNF-α produced by pDC were confirmed in vitro and, most importantly, we showed that those levels of TNF-α could decrease the in vitro proliferation of GL261 GBM cells. As a consequence, we also propose that pDC may have a TNF-mediated anti-tumor effect that may amplify that of CD8+ cells that have been primed peripherally by SC-pDC “vaccination”. Interestingly, combined expression of IFN-γ and TNF-α may stimulate expression by local astrocytes of the CXCR3 cytokine fractalkine: fractalkine may amplify anti-tumor immune responses by involving not only CD8+ T cells but also NK cells. Although not investigated in our studies, this defines an interesting scenario, as the crosstalk between DC and NK cells has an important role in the coordination of innate and adaptive immune responses.

These findings may have translational relevance for clinical applications of DC immunotherapy of gliomas and possibly other solid tumors. IT injection of immune DC has already been performed, for instance, in metastatic cancers and malignant gliomas with intriguing results. Furthermore, an inverse relationship has been demonstrated between the degree of local immune suppression in GBM, measured as the amount of TGF-β expressed locally, and the success of DC immunotherapy. Thus, our findings encourage the clinical use of IT-pDC, to be delivered (stereotaxically or through the use of Rickam catheters) in parallel to peripheral treatment by SC-pDC, as a promising tool to enhance the efficacy of DC immunotherapy in malignant gliomas and other solid tumors.
Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

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References


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