

Induction of Potent Antitumor Immunity by Intratumoral Injection of Interleukin 23–Transduced Dendritic Cells

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Abstract

Dendritic cells (DCs) are potent antigen-presenting cells that play a critical role in priming immune responses to tumor. Interleukin (IL)-23 can act directly on DC to promote immunogenic presentation of tumor peptide *in vitro*. Here, we evaluated the combination of bone marrow–derived DC and IL-23 on the induction of antitumor immunity in a mouse intracranial glioma model. DCs can be transduced by an adenoviral vector coding single-chain mouse IL-23 to express high levels of bioactive IL-23. Intratumoral implantation of IL-23–expressing DCs produced a protective effect on intracranial tumor-bearing mice. The mice consequently gained systemic immunity against the same tumor rechallenge. The protective effect of IL-23–expressing DCs was comparable with or even better than that of IL-12–expressing DCs. IL-23–transduced DC (DC-IL-23) treatment resulted in robust intratumoral CD8⁺ and CD4⁺ T-cell infiltration and induced a specific TH1-type response to the tumor in regional lymph nodes and spleen at levels greater than those of nontransduced DCs. Moreover, splenocytes from animals treated with DC-IL-23 showed heightened levels of specific CTL activity. *In vivo* lymphocyte depletion experiments showed that the antitumor immunity induced by DC-IL-23 was mainly dependent on CD8⁺ T cells and that CD4⁺ T cells and natural killer cells were also involved. In summary, *i.t.* injection of DC-IL-23 resulted in significant and effective systemic antitumor immunity in intracranial tumor-bearing mice. These findings suggest a new approach to induce potent tumor-specific immunity to intracranial tumors. This approach may have therapeutic potential for treating human glioma. (Cancer Res 2006; 66(17): 8887-96)

Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells that exist virtually in every tissue and can both elicit primary and boost secondary immune responses (1–4). Since the identification of their role in immunogenicity (1), tremendous attention has been focused on the critical role of DCs in antitumor immune responses. DCs pulsed with synthetic tumor peptides induce an effective antitumor immune response *in vitro* and after adoptive transfer in mice (3, 5, 6). Murine DCs can efficiently present antigens associated with whole tumor cell lysates to naïve and primed T cells *in vitro* and can

elicit antitumor immunity resulting in tumor regression *in vivo* (7, 8). Furthermore, DCs loaded with acid-eluted tumor peptide (9) or RNA (10, 11) are also effective in inducing immunity against tumors. However, these approaches require identifying tumor-specific peptides or harvesting sufficient tissue from solid human tumors as well as the cumbersome process of *ex vivo* DC pulsing.

An alternative approach to eliciting tumor antigen presentation is *i.t.* delivery of DC to allow tumor antigen capture *in situ* at the tumor site. Intratumoral administration of DCs into *s.c.* growing murine MT-901 breast tumor showed efficient uptake of apoptotic tumor cells by DC. Local injection of DC alone can result in regression of the tumor *in vivo* (12). Intratumoral DC vaccination in intracranial tumor-bearing rats resulted in prolonged survival and immunity to subsequent tumor rechallenge (13). Intratumoral injection with *IL-12* gene–modified DC significantly suppressed the growth of established intradermal tumor and induced a strong antitumor T-cell response (14). After delivery of *p53* gene–transduced DC into established skin tumors, a systemic antitumor effect was induced and tumor growth was significantly inhibited. More importantly, the *i.t.* injection of *p53*–expressing DCs has a greater effect than *s.c.* immunization (15). Furthermore, increased numbers of DC in solid tumor masses led to induction of potent antitumor immunity in animal models (16) and better prognosis in some clinical studies (17).

Interleukin (IL)-23 is a newly discovered cytokine, which consists of a heterodimer of IL-12 P40 subunit and a novel protein, P19. It has been shown that IL-23 acts on memory T cells and DCs directly to promote IL-12 and IFN- γ production *in vitro* (18, 19). Of further importance, IL-23 can act directly on CD8 α^+ DC to promote immunogenic presentation of an otherwise tolerogenic tumor peptide (19). Recently, it has been shown that IL-23–expressing tumor cells exhibit antitumor and antimetastatic functions in *s.c.* colon carcinoma model (20). We recently showed that IL-23–expressing bone marrow–derived neural stem cells display antitumor function in an intracranial glioma model (21).

Based on the essential role of *i.t.* administration of DCs in initiating antitumor immune responses and on the promoting role of IL-23 in the DC presentation of tumor antigen, we hypothesized that *i.t.* delivery of *IL-23* gene–transduced DC may offer an efficient means for triggering immune responses *in situ* within tumors, particularly in intracranial tumors. Therefore, in the present study, we adenovirally transduced bone marrow–derived DCs with IL-23 and studied the antitumor effect of *i.t.* administration of IL-23–expressing DC in an intracranial tumor model. We found that *i.t.* injection with IL-23–transduced DC (DC-IL-23) resulted in significantly prolonged survival in intracranial tumor-bearing mice and induced a strong tumor-specific T-cell immune response. The antitumor effect induced by IL-23–expressing DCs was comparable with or even better than that induced by IL-12–expressing DCs.

Note: J. Hu and X. Yuan contributed equally to this work.

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Furthermore, IL-23-expressing DC-treated survivors were resistant to subsequent intracranial rechallenge with the same tumor.

Materials and Methods

Cell lines and animals. GL26 (murine glioma) and B16-F10 (murine melanoma) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 5×10^{-5} mol/L β -mercaptoethanol, and antibiotics (all from Invitrogen, Gaithersburg, MD), referred to henceforth as CM. GL26-DsRed was a derivative of GL26 and was obtained by stable transfection of red-fluorescent protein into GL26 cells. Six- to eight-week-old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the animal maintenance facility of Cedars-Sinai Medical Center. The animals were used for experiments at 8 to 10 weeks of age. All of the animals used were treated in strict accordance with the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center guidelines.

Generation of bone marrow-derived DCs and transduction with adenoviral vectors. Erythrocyte-depleted mouse bone marrow cells from flushed marrow cavities of femurs and tibias were cultured overnight in CM to remove the adherent macrophages. Nonadherent cells were then placed in fresh CM containing 10 ng/mL granulocyte macrophage colony-stimulating factor and 10 ng/mL IL-4 on day 1. On day 5, DCs were harvested by gentle pipetting and were resuspended at 5×10^6 /mL in CM. Three milliliters of the DC suspension were overlaid onto 3 mL of CM-14.5% (w/v) metrizamide (Sigma, St. Louis, MO) solution in a 15-mL centrifuge tube. The resulting gradient was centrifuged at 2,000 rpm, brake off, 4°C, for 15 minutes. The low-density interface containing the DCs was collected by pipette aspiration. The DCs were rinsed twice with HBSS, enumerated, and resuspended in PBS for adenoviral vector transduction.

Adenoviral vectors bearing genes for single-chain mouse IL-23 (AdIL-23), IL-12 (AdIL-12), LacZ (AdLacZ), or a 2.4-kb noncoding stuffer DNA (AdEmpty) were constructed as previously described (21) and used to transduce DCs with a centrifugal method (22). Briefly, 1×10^6 DCs were suspended in 500 μ L of PBS containing 1% FBS and then mixed with adenoviral vector that was suspended in 500 μ L of PBS containing 1% FBS. A multiplicity of infection (MOI) of 50 was used for all the transductions except for the study to generate similar protein expression level of either IL-23 or IL-12, which had a MOI of 50 for AdIL-12 transduction and a MOI of 20 for AdIL-23 transduction. The mixtures of DCs and viral vectors were centrifuged ($2,000 \times g$ at 37°C) for 2 hours. After the centrifugal transduction process, the DCs were washed twice in PBS and used for subsequent experiments.

Animal studies *in vivo*. C57BL/6 mice (female, 8-10 weeks old) were anesthetized with i.p. ketamine and medetomidine, and stereotactically implanted with 1×10^4 GL26 or GL26-DsRed glioma cells in 2.5 μ L of 1.2% methylcellulose/PBS in the right striatum as previously described (21). Three days after tumor implantation, the mice received three injections (2.5 μ L, 1×10^5 cells) of either adenovirus-transduced DCs, nontransduced DCs, or saline every 7 days with the same coordinates as the tumor implantation. For brain tissue analysis, the mice were euthanized on day 7 after the first i.t. DC injection by intracardiac perfusion-fixation with 4% paraformaldehyde. Animal brains were cut into 40- μ m coronal sections and processed for immunohistochemistry staining. In the tumor rechallenge experiment, surviving animals were inoculated with the same tumor cells on the contralateral side of the brain from the first tumor implantation.

In the lymphocyte depletion experiments, mice were injected i.p. with 0.5 mg of rat anti-mouse CD8 (53-6.7) and anti-CD4 (GK1.5; both from American Type Culture Collection, Rockville, MD) monoclonal antibodies or with normal rat immunoglobulin G (IgG) as control antibody in 200 μ L of PBS 1 day before tumor implantation, once daily for the following 3 consecutive days, and then twice a week. Natural killer (NK) cells were depleted by i.p. injection of 200 μ g of anti-NK1.1 monoclonal antibody (clone PK136, BD PharMingen, San Diego, CA) or IgG2a as control using the same schedule as above. Lymphocyte depletion was confirmed in each depletion experiment by FACScan (Becton Dickinson, San Jose, CA) analysis of peripheral blood.

Flow cytometry. For phenotype analysis of DCs, phycoerythrin- or FITC-conjugated monoclonal antibodies against murine cell-surface molecules (MHC-I, MHC-II, CD11c, CD54, CD80, CD86, and appropriate isotype control; all from BD PharMingen) were used and cytometric analysis was done with a FACScan. For the CD8⁺ and CD4⁺ T-cell infiltration experiment, brain tissues of six mice were pooled and homogenized, followed by Percoll (Amersham Biosciences, Piscataway, NJ) gradient centrifugation to isolate mononuclear cells. The isolated mononuclear cells were stained by FITC-CD4 (clone GK1.5) and phycoerythrin-CD8 (clone 53-6.7) antibodies (BD PharMingen) and then subjected to FACScan analysis.

***In vitro* cytokine release assays.** Lymphoid cells were obtained from the draining lymph node and spleen harvested from each of two mice that had received the second i.t. injection of transduced DCs, nontransduced DCs, or saline 7 days earlier. The spleen or lymph node was pressed through sterile gauze, rinsed, treated for erythrocyte depletion, and rinsed again. These cells (2×10^6) were cocultured with 4×10^5 mitomycin C-treated GL26 or B16-F10 tumor cells in 24-well culture plates for 36 hours. Culture supernatants were collected for measurements of murine IFN- γ and IL-4 release by standard ELISA (BD PharMingen). The lower limit of sensitivity for each assay was 31.3 and 15.6 pg/mL, respectively. In the IL-23 P40 subunit release assay, a sandwich ELISA specific for murine P40 was used as previously described (19). In the IL-23 P19 and IL-12 P70 release assay, sandwich ELISA kits (eBioscience, San Diego, CA) were used according to the instructions of the manufacturer.

Cytotoxicity assays. Splenocytes were harvested as described above and pooled from two mice per group 7 days after the second i.t. injection of transduced DCs, nontransduced DCs, or saline. These cells (5×10^6) were restimulated *in vitro* with 5×10^5 mitomycin C-treated GL26 cells in the presence of 25 IU/mL recombinant human IL-2 for 5 days and used as effector cells in a lactate dehydrogenase (LDH) release assay. GL26 or B16-F10 cells (1×10^4 per well) and serial dilutions of effector cells were incubated in a 96-well U-bottomed plate at 37°C for 5 hours. Supernatants then were analyzed with a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN) according to the instructions of the manufacturer. Results were expressed as the percentage of specific lysis. Statistical comparisons were done with paired Student's *t* test.

Reverse transcription-PCR. Adenoviral vector-transduced and nontransduced DCs were harvested after 24 and 48 hours of transduction. The cell pellets were subjected to total RNA extraction with a RNeasy Mini kit (Qiagen, Valencia, CA). The isolated RNA was reverse transcribed with a Bioscript kit (Bioline, Randolph, MA) and Oligo(dT)₁₂₋₁₈ primer (Invitrogen). The PCR was carried out in a 20- μ L reaction mixture that contained 1 μ L of cDNA as template, specific oligonucleotide primer pairs of P19 subunit (forward, cagcagctctcggaaatc; reverse, tagaactcaggctggcgcac) and β -actin (forward, ggactctatgtgggtgacg; reverse, tacgaccagaggcaccacagg), and Accu-zyme (Bioline). The P19 was concurrently amplified with internal control β -actin in the same reaction tube as previously described (23). The amplified products were identified by agarose gel electrophoresis and ethidium bromide staining.

Trafficking study of i.t. injection of DCs. Three days after intracranial GL26 implantation, animals were randomly divided into four groups and i.t. injected with IL-23- or empty vector-transduced DCs, nontransduced DCs, or saline. Both transduced and nontransduced DCs were labeled with red fluorescent cell linker (PKH26; Sigma) immediately before the i.t. injection. Briefly, the transduced or nontransduced DCs were incubated with PKH26 at a concentration of 2×10^{-6} mol/L at room temperature for 5 minutes, rinsed extensively with HBSS, examined for viability and number by the trypan blue exclusion method, and used for injection. The injected mice were euthanized 24 hours after the injection by intracardiac perfusion-fixation with 4% paraformaldehyde. The ipsilateral deep cervical lymph nodes (draining lymph nodes) were harvested and postfixed at 4°C, embedded in optimum cutting temperature compound, and frozen. Serial 8- μ m sections were cut from these samples with a cryostat and examined under a fluorescent microscope. To quantify the fluorescence-positive cells, images were analyzed at a final magnification of $\times 20$. Four adjacent

sections were analyzed for each animal with the Zeiss AxioVision software (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The numbers of fluorescence-positive cells counted in a field of $\times 20$ magnification were reported as mean \pm SD of the experiment group.

Immunohistochemistry stainings of brain sections. Free-floating 40- μ m sections were treated with 10% donkey serum (Sigma) for 30 minutes at room temperature and then stained with anti-CD4 (rat monoclonal antibody 1:50; BD PharMingen) and anti-CD8 (rat monoclonal antibody 1:100; BD PharMingen) primary antibodies and isotype control antibodies. The primary antibodies were detected with FITC-conjugated donkey anti-rat IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were then counterstained with 4',6-diamidino-2-phenylindole before mounting. The results were evaluated under a confocal microscope. The positively stained cells were quantified by analyzing the images at a final magnification of $\times 20$. Four adjacent sections containing tumor mass were analyzed for each animal with the AxioVision software (Carl Zeiss). The numbers of positively stained cells counted in a field of $20\times$ magnification were reported as mean \pm SD of the experiment group. Comparisons between experiment groups were made with paired Student's *t* test.

Results

DCs can be transduced by an adenoviral vector to express high levels of biologically active IL-23. Using a centrifugal method, we transduced murine bone marrow-derived DCs, which were generated *in vitro* with granulocyte macrophage colony-stimulating factor plus IL-4, with an adenoviral vector carrying a single-chain mouse *IL-23* under the control of murine cytomegalovirus promoter, AdIL-23 (21). Transduction with a LacZ-expressing adenoviral vector at MOI 50 by a centrifugal method, which can efficiently introduce transgene into DC (22), revealed that murine DCs could be adenovirally transduced at $\sim 85\%$ efficiency (data not shown). This is consistent with a previous study by Nishimura et al. (22). After the completion of DC transduction with AdIL-23, the concentration of IL-23 P40 subunit released into the culture medium was measured by ELISA and the mRNA transcription of IL-23 P19 subunit in the cell pellet was detected by reverse transcription-PCR (RT-PCR). As shown in Fig. 1A, accumulation of P40 was observed in cultures of AdIL-23-transduced DCs (DC-IL-23), but not in cultures of non-transduced or AdEmpty-transduced DCs (DC-Empty). RT-PCR showed that P19 mRNA was only detected in the cell pellets that were transduced by AdIL-23, which confirmed that IL-23 expression was only achieved in the DC-IL-23 (Fig. 1B). The IL-23 produced by the DC-IL-23 was confirmed to be biologically active and capable of stimulating IFN- γ production from concanavalin A blast T cells as previously described (data not shown; ref. 19). To examine the effect of adenoviral transduction and exogenous IL-23 expression on the DCs phenotype, various cell-surface molecules were examined by flow cytometry. AdEmpty transduction had little effect on the DC phenotype compared with nontransduced DCs. DC-IL-23 did not differ from DC-Empty or nontransduced DCs, except for increased levels of MHC-I and MHC-II molecules (Table 1).

Intratumoral injections with DC-IL-23 result in prolonged survival in intracranial tumor-bearing mice. To examine whether the DC-IL-23 provided a therapeutic benefit for brain tumor, we established gliomas in C57BL/6 mice with an intracranial implantation of 1×10^4 GL26 cells. Three days after the tumor implantation, mice were randomly divided into four groups. Each group was i.t. injected thrice with either saline, DC-IL-23, DC-Empty, or nontransduced DCs, respectively, at 7-day interval (Fig. 2A). As shown in Fig. 2B, i.t. administration of

DC-IL-23 resulted in significantly prolonged survival compared with DC-Empty or nontransduced DCs. Approximately 80% of the DC-IL-23-treated mice survived beyond an observed period of 120 days. DC-Empty and nontransduced DC treatments led to only 20% of the mice surviving beyond 120 days. All of the saline-treated mice died within 45 days after the GL26 implantation. Statistical analysis revealed that the effect of DC-IL-23 was significantly different from that of DC-Empty or nontransduced DCs ($P = 0.0114$, DC-IL-23 versus DC-Empty; $P = 0.0164$, DC-IL-23 versus nontransduced DC; log-rank test). In addition, the effects of DC-Empty and nontransduced DCs were significantly different from that of saline ($P < 0.0001$, DC-Empty versus saline; $P < 0.0001$, nontransduced DC versus saline; log-rank test). However, there was no significant difference between the DC-Empty and nontransduced DC treatments ($P = 0.9103$; log-rank test). Because IL-23 relates to IL-12 (18) and it has been shown that *IL-12* gene-transduced DC significantly suppressed the growth of established intradermal tumor (14), we sought to compare the

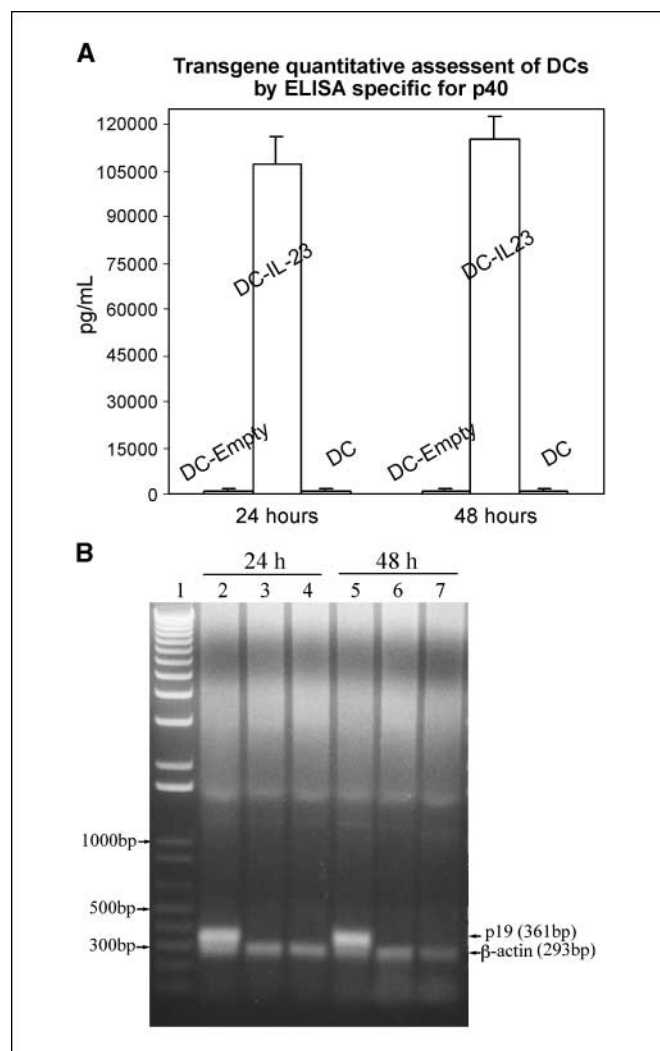


Figure 1. Transgene expression by DCs after adenovirus transduction. A, the accumulation of IL-23 P40 subunit was detected in the culture supernatant of DC-IL-23 with ELISA. Columns, mean of triplicate determinations; bars, SD. B, the mRNA transcription of IL-23 P19 subunit in cell pellets of DC-IL-23 was detected by RT-PCR [lane 1, ladder; lane 2 and 5, DC-IL-23; lane 3 and 6, DC-Empty; lane 4 and 7, nontransduced DCs (DC)].

Table 1. Phenotypic characteristic of adenoviral vector-transduced DCs

	DC	DC-Empty	DC-IL-23
MHC-I	34 (94.22%)*	31 (91.75%)	89 (92.38%) [†]
MHC-II	71 (89.41%)	106 (90.03%)	201 (88.78%) [†]
CD11c	25 (90.75%)	20 (88.41%)	24 (91.17%)
CD54	99 (86.89%)	110 (84.92%)	103 (88.01%)
CD80	131 (89.31%)	128 (90.03%)	142 (87.08%)
CD86	134 (87.93%)	145 (85.78%)	140 (89.36%)

NOTE: DCs generated in same batch were divided into subgroups and used for adenoviral vector transduction or nontransduction control. Adenoviral vector-transduced and nontransduced DCs were stained with antibodies against various cell-surface molecules. The expression of the molecules was analyzed by fluorescence-activated cell sorting and indicated as mean fluorescence intensity. Triplicate samples were analyzed. The percentages of positively stained cells are also shown. The data shown are representative of three independent experiments. *Percentage of positively stained cells.

[†] $P < 0.05$, compared with that of nontransduced or DC-Empty.

antitumor effect of IL-23-expressing DCs with that of IL-12-expressing DCs in the intracranial tumor model. DCs expressing similar transgene protein levels of either IL-23 or IL-12 by adenoviral vector transduction were i.t. injected into intracranial tumor-bearing mice with the same schedule as described in Fig. 2A. The DC-IL-23 treatment resulted in a similar pattern of survival benefit on the tumor-bearing mice as that of the DC-IL-12 treatment (Fig. 2C), but this survival benefit was less than that in Fig. 2B because a lower MOI for AdIL-23 transduction was used in the experiment in Fig. 2C. Statistical analysis revealed that the DC-IL-23 treatment was not significantly different from the DC-IL-12 treatment ($P = 0.1566$, DC-IL-23 versus DC-IL-12; log-rank test), although DC-IL-23 treatment resulted in ~60% survival rate versus ~50% for DC-IL-12. We also compared the antitumor effect of DC transduced by either AdIL-23 or AdIL-12 with the same MOI of 50. The same MOI transduction resulted in a higher transgene protein level in DC-IL-23 than in AdIL-12-transduced DCs (DC-IL-12; ref. 21)⁴ and, consequently, DC-IL-23 showed significantly better antitumor effect than DC-IL-12 ($P = 0.0409$, DC-IL-23 versus DC-IL-12; log-rank test; Fig. 2D).

On the basis of the known ability of IL-23 to induce proliferation of memory T cells, we sought to assess whether the i.t. injection of IL-23-expressing DC could induce enhanced T-cell infiltration in tumor-bearing brains. Seven days after the first i.t. injection of transduced DCs, brain tissues were subjected to homogenization, gradient centrifugation to isolate mononuclear cells, and then immunostaining for flow cytometry. The FACScan analysis revealed that intracranial CD8⁺ and CD4⁺ T-cell infiltration was enhanced in DC-IL-23-treated tumor-bearing brains compared with those of DC-Empty-, nontransduced DC-, and saline-treated tissues (Fig. 3). Stained *in situ* by immunohistochemistry, brain tumors from the mice treated with DC-IL-23 showed robust

infiltration of CD8⁺ and CD4⁺ T cells [Fig. 4A and B (a-h)]. There was negligible CD8⁺ and CD4⁺ T-cell infiltration, however, within tumors from mice treated with saline [Fig. 4A and B (q-t)]. The treatment with DC-Empty and nontransduced DC also resulted in i.t. T-cell infiltration, but the intensity of that infiltration

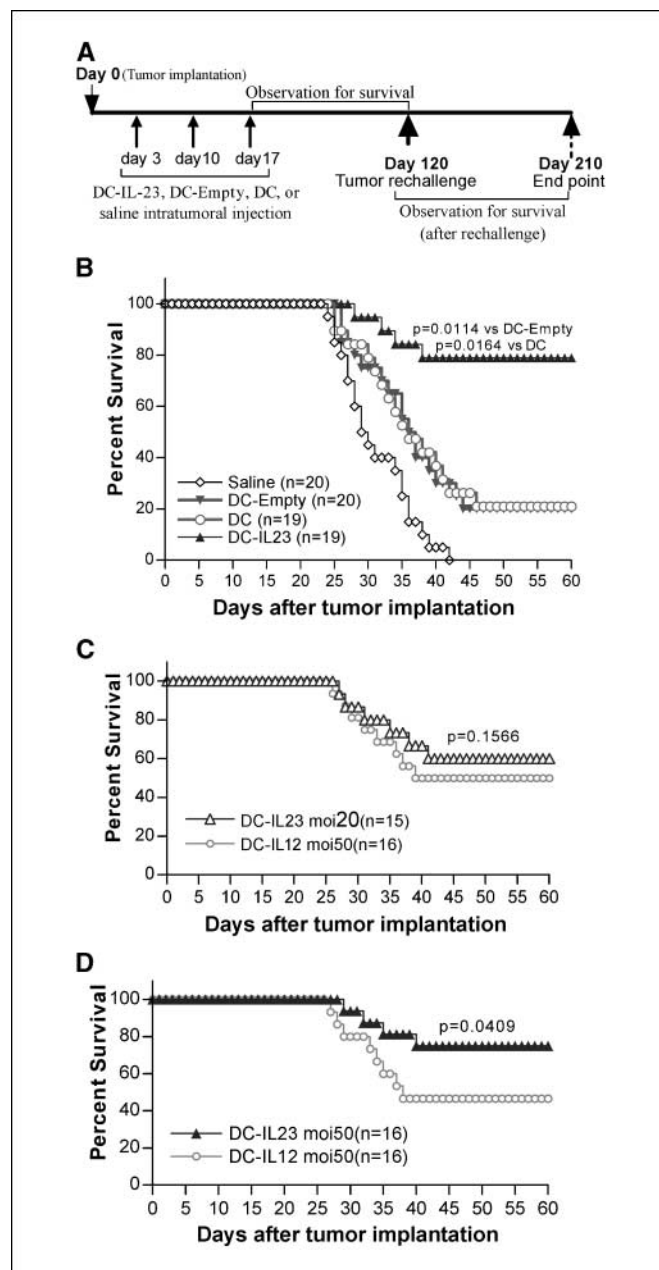


Figure 2. *In vivo* experiments with intracranial tumor model. A, experiment schema shows tumor implantation, DC injection, rechallenge, and survival observation time. B, effect of DC-IL-23 on the experimental glioma in C57BL/6 mice. Kaplan-Meier survival curve of intracranial glioma-bearing C57BL/6 mice that were injected i.t. thrice with DC-IL-23, DC-Empty, nontransduced DCs, or saline. C and D, comparison of the effects of DC-IL-23 and DC-IL-12 on experimental glioma in C57BL/6 mice. Kaplan-Meier survival curve of intracranial glioma-bearing C57BL/6 mice that were injected i.t. thrice with adenoviral vector-transduced DCs that had similar transgene expression levels of either IL-23 or IL-12 (C). Kaplan-Meier survival curve of intracranial glioma-bearing C57BL/6 mice that were injected i.t. thrice with DCs that were transduced with a MOI of 50 by either AdIL-23 or AdIL-12 (D). Representative of two independent experiments.

⁴ Unpublished data.

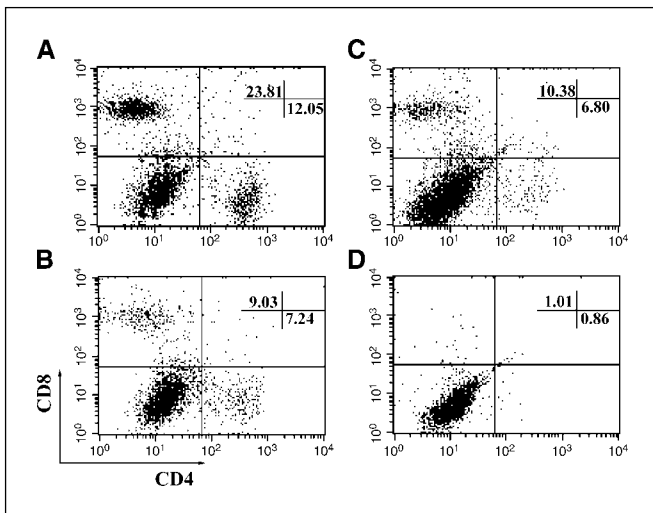


Figure 3. T-cell infiltration within the tumor-bearing brains of transduced DC- and nontransduced DC-injected mice. Fluorescence-activated cell sorting analysis showed the CD8⁺ and CD4⁺ T-cell infiltration in the tumor-bearing brain tissues of mice that were i.t. injected with DC-IL-23 (A), DC-Empty (B), nontransduced DCs (C), and saline (D). DC-IL-23–treated mice showed robust infiltration of CD8⁺ and CD4⁺ T cells compared with that of DC-Empty- and nontransduced DC–treated mice.

was significantly lower than that seen in the DC-IL-23–treated tumors [Fig. 4A and B (*i-p*)]. Semiquantification revealed that there were $\sim 104 \pm 12.75$ CD8 positively stained cells identified in DC-IL-23–treated group as compared with 35 ± 8.21 and 31 ± 4.96 in the DC-Empty- and nontransduced DC–treated groups, respectively. CD4 positively stained cells were $\sim 82.25 \pm 8.48$ in DC-IL-23–treated group as compared with 29.75 ± 13.45 and 28.25 ± 6.85 in the DC-Empty- and nontransduced DC–treated groups, respectively. Both CD8⁺ and CD4⁺ T-cell infiltration reached significant difference ($P < 0.05$) for the DC-IL-23–treated group when compared with either DC-Empty- or nontransduced DC–treated group.

Tumor-specific immune response can be identified after intratumoral implantation of DC-IL-23 into the brains of tumor-bearing mice. Because the above data showed that i.t. injection of DC-IL-23 resulted in prolonged survival and intratumoral T-cell infiltration in the brain tumor-bearing mice, we next asked whether systemic immune responses specific for the tumor were induced by i.t. injection of DC-IL-23. To address this question, deep cervical lymph nodes in the ipsilateral side of the implanted glioma (draining lymph nodes) as well as the spleen were harvested from the tumor-bearing mice 7 days after the second i.t. injection of DC-IL-23. These lymphoid cells were cocultured with mitomycin C–treated GL26 cells *in vitro* and IFN- γ and IL-4 production in the culture supernatant was examined. As shown in Table 2, injection with DC-Empty or nontransduced DCs enhanced tumor-specific IFN- γ production by lymphoid cells harvested from draining lymph nodes and spleen compared with saline injection. More importantly, i.t. injection of DC-IL-23 resulted in greater enhancement of IFN- γ production in response to GL26 restimulation by these lymphoid cells. In contrast, there was no detectable IL-4 increase in spleen and only a slight increase in draining lymph nodes. The IFN- γ was specifically released with GL26 stimulation, but not with syngeneic B16-F10 tumor cells (data not shown). These results suggest that i.t. injected DC-IL-23 can traffic to the draining lymph node and efficiently stimulate

lymphocytes *in situ* to produce IFN- γ . To verify this notion, DC-IL-23, DC-Empty, and nontransduced DCs were labeled with fluorescent linker (PKH26) and injected i.t. into brain tumor-bearing mice, and the ipsilateral deep cervical lymph node (the draining lymph node) was examined 24 hours after the injection. Numerous fluorescence-positive cells were identified in lymph nodes from animals injected with PKH26-labeled adenoviral vector–transduced or nontransduced DCs (Fig. 5). Quantification of the fluorescence-positive cells revealed an average of 25 ± 2 cells per field in all the mice injected with either adenoviral vector–transduced or nontransduced DCs. Spleen cells from the DC-IL-23–treated mice were also analyzed for CTL activity against tumor targets. Figure 6 showed the CTL activity against GL26 cells by spleen cells from mice that received saline compared with those that received adenoviral vector–transduced and nontransduced DCs. Mice that received either transduced or nontransduced DCs showed enhanced CTL activity compared with saline-injected mice. DC-IL-23 injection induced significantly higher CTL activity than did DC-Empty or nontransduced DC injection (Fig. 6A). Statistical analysis revealed that there were significant differences in cytolytic activity between DC-IL-23/GL26 and DC-Empty/GL26, DC/GL26, or saline/GL26 at all three effector-to-target ratios ($P < 0.05$). Significant differences in cytolytic activity were found between saline/GL26 and DC-Empty/GL26 or DC/GL26 only at effector-to-target ratios of 30 and 100. Splenocytes from DC-IL-23–treated mice lacked any apparent CTL activity against the irrelevant tumor cell B16-F10 (Fig. 6B), indicating that the CTL activity was tumor specific.

We also examined whether a memory protective immunity was established by the i.t. injection of DC-IL-23. DC-IL-23–treated survivors were subjected to rechallenge with another intracranial implantation of GL26 (Fig. 1A). All of the rechallenged animals survived beyond day 90 post rechallenge and were tumor-free at the end point of the survival study as verified by H&E staining of brain sections. In contrast, age-matched naïve mice were uniformly susceptible to GL26 glioma challenge and died of brain tumor within 45 days of the challenge.

Involvement of CD8⁺, CD4⁺ T cells and NK cells in the antitumor effect of i.t. injection with DC-IL-23. To identify the population of T cells and NK cells involved in the antitumor immunity induced by DC-IL-23, mice were injected with depleting anti-CD8, anti-CD4, or control rat antibodies for circulating T-cell depletion or with anti-NK1.1 or control mouse antibodies for NK cell depletion. Flow cytometry showed that the injection of antibodies depleted the appropriate cell population by 95% (data not shown). As shown in Fig. 7A, depletion of CD8⁺ T cells greatly impaired the protective effect of DC-IL-23 on intracranial tumor-bearing mice ($P = 0.0013$, CD8 depletion versus control rat IgG; log-rank test). There was no long-term survival observed in the CD8 T-cell–depleted mice. Similarly, but less significantly, the impairment of the protective effect was also observed in the CD4 T-cell–depleted mice ($P = 0.0475$, CD4 depletion versus control rat IgG; log-rank test). In NK cell–depleted mice, DC-IL-23 treatment still had a protective effect on intracranial tumor-bearing mice (Fig. 7B). However, the effect was impaired in these mice compared with the mice that received control mouse IgG ($P = 0.0439$, NK cell depletion versus control mouse IgG; log-rank test). These data indicate that CD8⁺ T cells are essential for the antitumor effect conferred by i.t. injection of DC-IL-23, and that CD4⁺ T cells and NK cells also have roles in the effect.

Discussion

It has been reported that the presence of increased DC numbers within solid tumor masses induces effective antitumor immune responses in animal models (16) and is associated with improved prognosis in clinical patients (17). In the current study, we showed that i.t. administration of DC-IL-23 can induce significant and effective systemic antitumor immune responses that control the growth of experimental intracranial gliomas in immunocompetent mice, suggesting that a gene-modified DC *in situ* vaccine may have a broad therapeutic potential for the management of human brain tumors.

Viral vector-mediated genetic modification of DCs to express immunomodulatory proteins or tumor antigens has been successfully achieved in animal models of tumor treatment (15, 24, 25). Among vectors used for transducing DC, adenoviral vector represents an efficient means of inserting transgenes into DC without detrimental effects on its phenotype or function (22, 26, 27). In this study, we transduced DCs with adenoviral vector

coding single-chain mouse IL-23 using a centrifugal method. A previous study showed that adenoviral infection could induce maturation of DC via a nuclear factor κ B-dependent pathway (28). We found, however, that the adenoviral vector itself had no detrimental effect on the DC phenotype in our experimental settings, which was consistent with a study showing that infection with adenovirus encoding an enhanced green fluorescent protein had little effect on expression of the surface markers of DCs, as well as with findings from Murakami et al.'s (15) report that DC transduced by an adenoviral vector coding p53 protein or control D1312 vector showed no apparent effect on DC differentiation and activation (22). These differences may be due, at least partly, to the methods used for transducing DCs. A centrifugal method, which enhances adenovirus transduction efficiency with a short infection time (22), was used in our study. We also observed that IL-23 adenovirally transduced into DC increased the expression of both MHC class I and class II surface molecules. These phenotypic changes could be due, in part, to the direct effect of

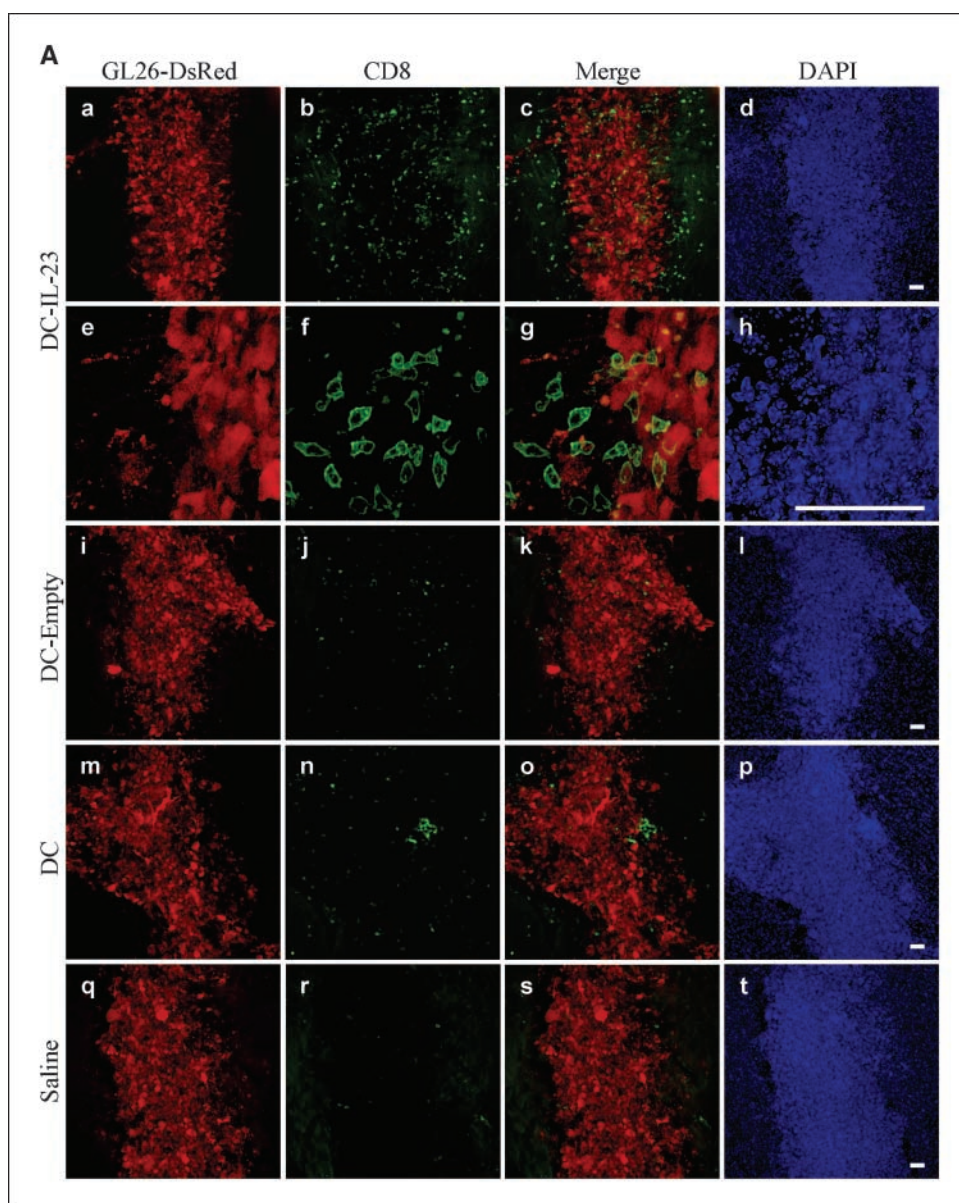


Figure 4. Intratumoral T-cell infiltration after the administration of transduced and nontransduced DCs. *A*, intracranial tumor-bearing mice showed robust infiltration of CD8⁺ T cells within the tumor masses 7 days after the first i.t. injection of DC-IL-23 (*A-H*). Moderate infiltration of CD8⁺ T cells within the tumor masses was also observed in DC-Empty-treated (*I-L*) and nontransduced DC-treated (*M-P*) mice. Saline-treated mice showed negligible CD8⁺ T-cell infiltration in the tumor masses (*Q-T*). Bar, 250 μ m.

IL-23 or, more likely, indirectly through IFN- γ production by contaminating T cells or NK cells. In fact, DCs generated from bone marrow of IFN- γ knockout mice did not show any change in the expression level of MHC class I and class II molecules after adenoviral vector transduction (data not shown).

Treating intracranial glioma-bearing mice with i.t. injection of DC-IL-23 resulted in profound antitumor effects with massive T-cell infiltrations. These data confirm published studies of others (20) as well as our own (21) that found that IL-23 expressed by tumor cells or by neural stem cells showed antitumor effects in *in vivo* colon carcinoma models (20) and brain tumor models (21), respectively. Lymphocytes from spleen and draining lymph nodes of mice treated i.t. with DC-IL-23 displayed heightened levels of IFN- γ secretion as well as CTL activity in a tumor-specific fashion. Indeed, these responses could contribute to the profound *in vivo* antitumor effects that we observed. Moreover, i.t. injection of DC-IL-23 resulted in robust infiltration of CD8⁺ and CD4⁺ T cells within the tumor-bearing brain. These findings

are of particular interest because both cytolytic and noncytolytic tumor-specific, tumor-infiltrating lymphocytes have been shown to mediate potent antitumor effects *in vivo* upon adoptive transfer (29, 30).

Evidence has shown that immature DCs can readily acquire antigen(s) via uptake of apoptotic cells, which in turn elicit MHC class I-restricted CTLs (31, 32). Thus, using flow cytometry, we confirmed in each experiment in the current study that the prepared DCs have an immature phenotype. DCs delivered locally at the tumor site may phagocytose adjacent tumor cells and present tumor antigens *in vivo*, which could be greatly facilitated by the expression of IL-23 that is able to act directly on CD8 α^+ DC to promote immunogenic presentation of tumor antigen. The combination of the tumor antigen presentation ability of DC and the promoting effect of IL-23 on antigen presentation *in situ* within tumor masses may underline the superior antitumor effect of IL-23-expressing DCs. IL-23-expressing DCs showed antitumor activity superior to IL-12-expressing DCs

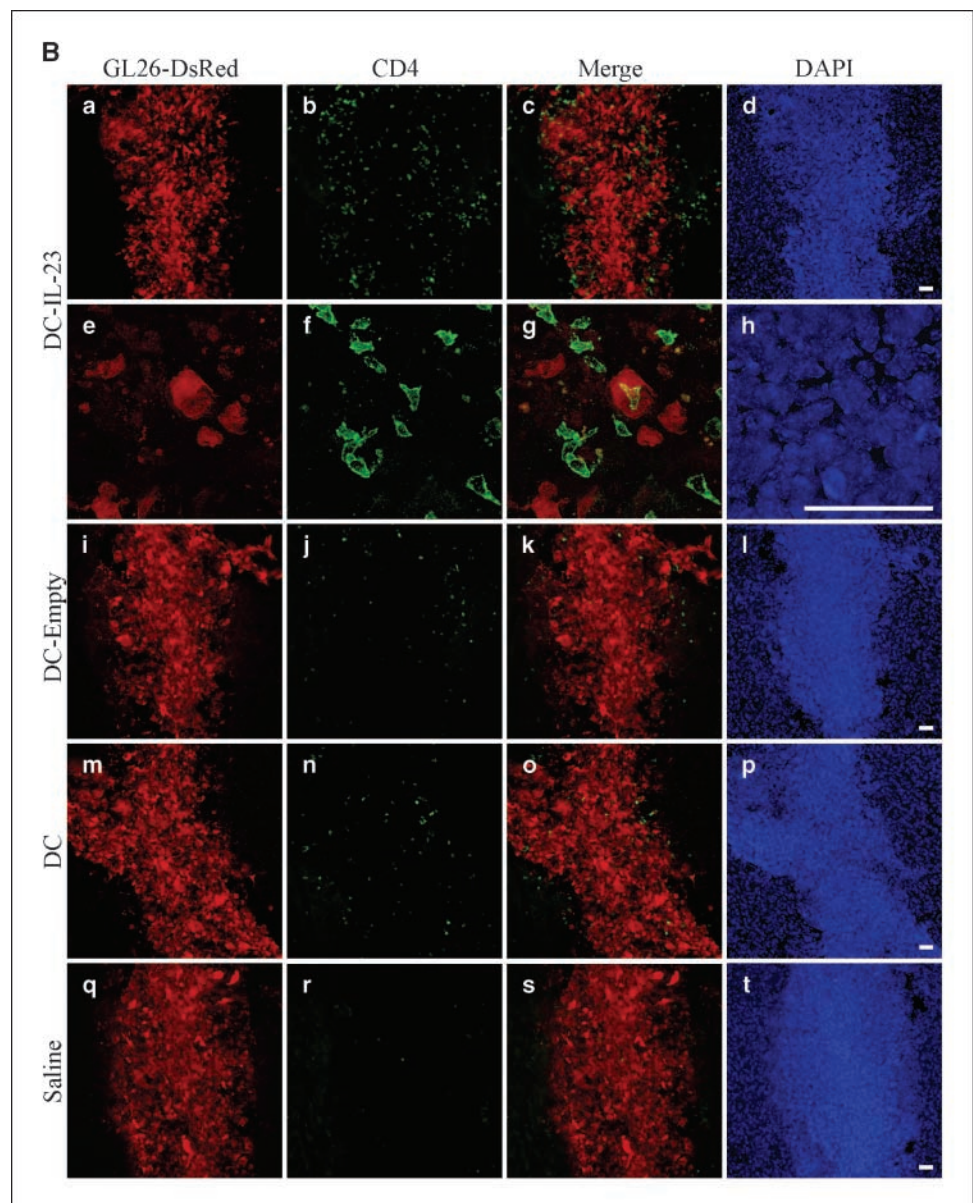


Figure 4 Continued. **B**, intracranial tumor-bearing mice showed robust infiltration of CD4⁺ T cells within the tumor masses 7 days after the first i.t. injection of DC-IL-23 (A-H). Moderate infiltration of CD4⁺ T cells within the tumor masses was also observed in DC-Empty-treated (I-L) and nontransduced DC-treated (M-P) mice. Saline-treated mice showed negligible CD4⁺ T-cell infiltration in the tumor masses (Q-T). Bar, 250 μ m.

Table 2. IFN- γ and IL-4 production by lymphoid cells from draining lymph nodes and spleen

	IFN- γ		IL-4	
	Lymph nodes/spleen		Lymph nodes/spleen	
DC-IL-23	18,096 \pm 172*	8,164 \pm 203*	62 \pm 11 [†]	<16
DC-Empty	2,215 \pm 662 [‡]	798 \pm 35 [‡]	49 \pm 17 [†]	<16
DC	2,307 \pm 104 [‡]	762 \pm 87 [‡]	55 \pm 22 [†]	<16
Saline	104 \pm 9/56	\pm 10	<16/16	<16

NOTE: Draining lymph nodes and spleen were harvested from the tumor-bearing mice 7 days after the second i.t. injection of adenovirus-transduced DCs, nontransduced DCs, or saline. Lymphoid cells were cocultured with mitomycin C-treated GL26 cells *in vitro* for 36 hours, and IFN- γ and IL-4 production (pg/mL) in the culture supernatant was examined by ELISA. The data shown are the mean \pm SD of triplicate samples and are representative of three independent experiments.

* $P < 0.01$, versus DC-Empty and DC.

[†] $P < 0.05$, versus saline.

[‡] $P < 0.01$, versus saline.

when the same MOIs of AdIL-23 and AdIL-12 transduced DCs were injected i.t. When both IL-23-expressing DCs and IL-12-expressing DCs had similar transgene protein levels, IL-23-expressing DCs showed a higher survival rate on tumor-bearing animal. These findings indicate that, under the current experi-

mental settings, DC with AdIL-23 transduction can lead to greater transgene expression and an enhanced antitumor response against intracranial glioma.

Our *in vivo* lymphocyte depletion experiments showed that CD8⁺ T cells and, to a lesser extent, CD4⁺ T cells and NK cells are required for the generation of the antitumor immunity conferred by IL-23-expressing DCs. These data are consistent with our previous findings, which showed that the antiglioma activity of IL-23-expressing neural stem cells was mainly dependent on CD8⁺ T cells, and CD4⁺ T cells and NK cells were also involved in the process (21). However, the data are in contrast to Lo et al.'s study (20). The latter study showed that the antitumor effect of IL-23-expressing tumor cells was independent of CD4⁺ T cells and NK cells. These differences are probably the result of the different tumor models used in the two studies. GL26 glioma was used as an intracranial brain tumor model in the current study whereas Lo et al. used a s.c. colon adenocarcinoma model. The unique environment of the central nervous system with regard to antigen presentation and the differences of immunogenicity among different tumor cell lines may underline the variability in the cellular subsets crucial to an antitumor effect.

It has been reported that IL-23 can activate macrophages to produce proinflammatory cytokines, such as IL-17, which may contribute to autoimmune inflammation of the brain (33–36). In our experimental model, however, we showed that IL-17 could not be detected by mRNA transcription or by protein expression in the brain of DC-IL-23-treated mice (data not shown). We recently reported that IFN- γ mRNA can be greatly elevated in tumor-rechallenged mice that survived previous brain tumor implantation by IL-23-expressing neural stem cell treatment. These rechallenged

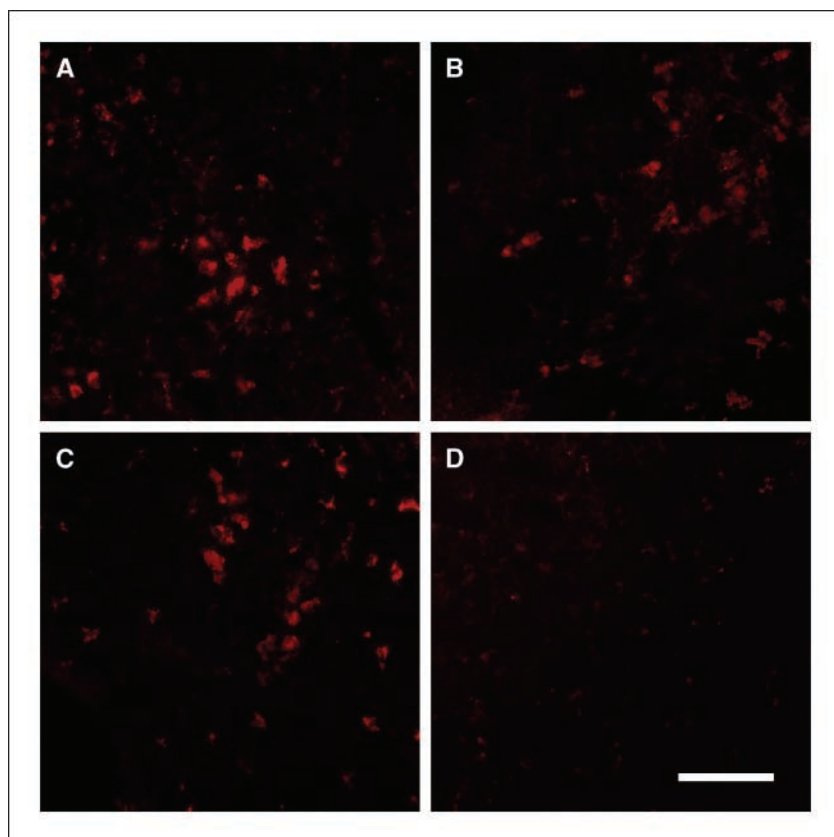


Figure 5. Intratumoral injection of transduced and nontransduced DCs migrated to the draining lymph node. Intracranial tumor-bearing mice were injected i.t. with transduced or nontransduced DCs that were labeled with PKH26. The draining lymph nodes were harvested 24 hours after the injection and were processed for fluorescent microscope examination. Fluorescence-positive cells were observed in the draining lymph nodes harvested from mice injected with DC-IL-23 (A), DC-Empty (B), and nontransduced DCs (C). No fluorescence-positive cell was observed in the draining lymph nodes harvested from mice injected with saline (D). Bar, 250 μ m.

mice showed no demyelination or inflammation changes, as verified by histologic analysis of brain tissue (21). Indeed, in our current study, none of the mice treated with DC-IL-23 showed signs of autoimmune distress (ruffled fur, weight loss, lethargy, or agitation) or histopathologic changes in the brain (data not shown). Thus, although the molecular mechanisms governing the pathways that regulate TH1-type antitumor immunity and autoimmune response by exogenous IL-23 are presently unclear, i.t. injection of IL-23-expressing DCs results in systemic and effective antitumor activity in intracranial tumor-bearing mice with no detectable deleterious side effects.

Taken together, in the current study, we present evidence that i.t. injection with *IL-23* gene-transduced DC is feasible, mediates an effective antitumor immunity that is superior to that observed with DC-Empty or nontransduced DC, or even with DC-IL-12, and is capable of inducing substantial systemic antitumor immune

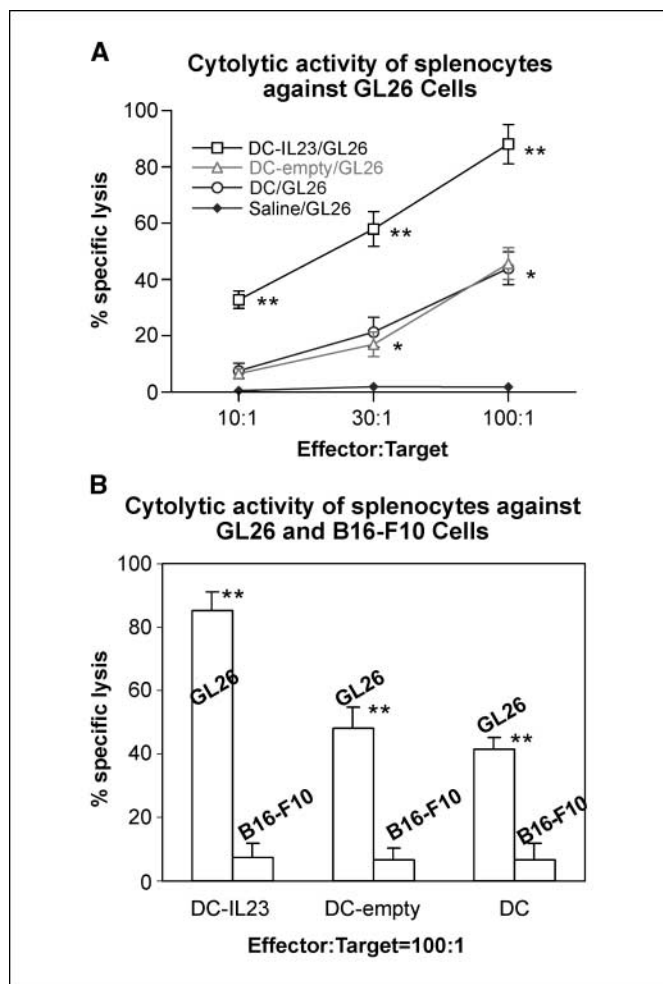


Figure 6. Tumor-specific CTL activity of spleen cells induced by i.t. injection of DC-IL-23. *A*, spleen cells obtained from the tumor-bearing mice 7 days after second i.t. injection of transduced DCs, nontransduced DCs, or saline were restimulated with mitomycin C-treated GL26 cells for 5 days, and the CTL activity of the resultant effector cells was measured against parental GL26 tumor target in a LDH release assay. Representative of three independent experiments. **, $P < 0.05$, compared with either DC-Empty/GL26 or DC/GL26; *, $P < 0.05$, compared with saline/GL26. *B*, spleen cells were obtained and restimulated as described in (*A*) and the CTL activity of the resultant effector cells was measured against GL26 and B16-F10 tumor targets in a LDH release assay. **, $P < 0.05$, compared with B16-F10. Representative of three independent experiments.

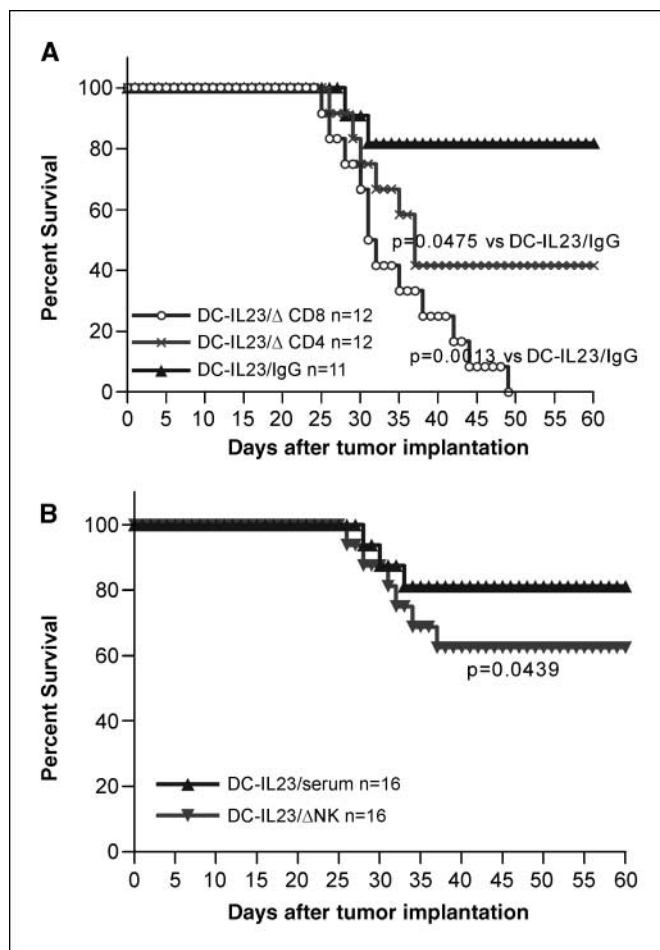


Figure 7. Involvement of CD8⁺, CD4⁺ T cells and NK cells in the antitumor immunity induced by DC-IL-23. *A*, Kaplan-Meier survival curve of intracranial tumor-bearing mice that received anti-CD8, anti-CD4 depleting antibodies or control IgG and were treated with i.t. injection of DC-IL-23. *B*, Kaplan-Meier survival curve of intracranial tumor-bearing mice that received either anti-NK1.1 antibody for NK cell depletion or control IgG and were treated with i.t. injection of DC-IL-23. Representative of three independent experiments.

responses. These data provide a preclinical rationale for IL-23-expressing DC-based brain tumor therapy.

Note: When the present study was finished, Overwijk et al. (37) reported that IL-23 is a potent vaccine adjuvant for the induction of therapeutic, tumor-specific CD8 T-cell response and Bettelli et al. (38) reported that IL-23 is not the differentiation factor for the generation of IL-17-producing T cells, whereas Langowski et al. (39) reported that IL-23 is an important molecular link between tumor-promoting proinflammatory processes and the failure of the adaptive immune surveillance to infiltrate tumors.

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