Delivery of Interferon-α Transfected Dendritic Cells into Central Nervous System Tumors Enhances the Antitumor Efficacy of Peripheral Peptide-Based Vaccines

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ABSTRACT

We evaluated the effects, on immunity and survival, of injection of interferon (IFN)-α-transfected dendritic cells (DC-IFN-α) into intracranial tumors in mice immunized previously with syngeneic dendritic cells (DCs) pulsed either with ovalbumin-derived CTL or T helper epitopes. These immunizations protected animals from s.c. challenge with ovalbumin-expressing M05 melanoma (class I+ and class II-negative). Notably, antiovalbumin CTL responses were observed in animals vaccinated with an ovalbumin-derived T helper epitope but only after the mice were challenged with M05 cells. This cross-priming of CTL was dependent on both CD4+ and CD8+ T cells. Because we observed that s.c., but not intracranial, tumors were infiltrated with CD11c+ DCs, and because IFN-α promotes the activation and survival of both DCs and T cells, we evaluated the combinational antitumor effects of injecting adenoviral (Ad)-IFN-α-engineered DCs into intracranial M05 tumors in preimmunized mice. Delivery of DC-IFN-α prolonged survival. This was most notable for animals prevaccinated with both the CTL and T helper ovalbumin epitopes, with 60% (6 of 10) of mice (*versus* 0 of 10 of control animals) surviving for >80 days after tumor challenge. DC-IFN-α appeared to persist longer than mock-transfected DCs within the intracranial tumor microenvironment, and DC-IFN-α-treated mice exhibited enhanced levels of ovalbumin-specific CTL in draining cervical lymph nodes. On the basis of these results, we believe that local expression of IFN-α by DCs within the intracranial tumor site may enhance the clinical efficacy of peripheral vaccine approaches for brain tumors.

INTRODUCTION

Although initial studies on T-cell responses against human tumors have focused on the preclinical and clinical evaluation of class I-presented epitopes recognized by CD8+ CTL (reviewed in Ref. 1), a number of recent studies, including our own (2), clearly support the important role of CD4+ T cells in the induction of antitumor CD8+ CTLs (2–5). Using ovalbumin-based models, it has been demonstrated that induction of an effective long-term CTL response is dependent on the generation of antigen-specific CD4+ T cell help (6, 7). In the case of class II-deficient tumor cells, tumor-specific CD4+ T-cell responses occur via cross-presentation mechanisms that may concurrently promote specific CTL responses (3).

Both primary and metastatic brain tumors are able to overcome host immune defenses through a variety of mechanisms, many of which have become increasingly well characterized over the past decade (8). Immunological tolerance of brain tumors may result in part from the paucity of specialized antigen-presenting cells (APCs) such as dendritic cells (DCs) infiltrating these sites, which would limit the induction of specific immunity within the central nervous system (CNS). Furthermore, with the exception of inflammatory situations (9), CNS-derived DCs appear to inhibit rather than promote T-cell proliferation (10), suggesting that endogenous CNS DCs may be responsible for maintaining a state of organ-associated ignorance or anergy within the inflamed CNS. This immunosuppressed state is likely mediated by immunosuppressive factors, such as transforming growth factor-β2 (11) and soluble Fas ligand (CD95L; Ref. 12), among others, that are elaborated by both normal and neoplastic brain tissues (13).

However, this "immunologically privileged" status of the brain is not absolute. Delivery of interferon (IFN)-γ in the CNS- and CNS-tumor-immunological environment results in a remarkable up-regulation of MHC class II on tumor-infiltrating APCs (14) and enhanced recruitment of antigen-specific T cells (15), providing a rationale for site-specific modulation of the CNS microenvironment by cytokine-based (*i.e.*, IFN-α) immunotherapy. Of note, local transgene delivery of type-1 IFNs within the CNS tumor site induces significant anti-CNS tumor immunity in preclinical models (16, 17). The various biological properties of type-1 IFNs on DCs include maturation (18, 19) and initiation of cross-priming of CD8+ T cells against viral antigens (20). IFN-α-transduced tumor cells also activate and promote the survival of tumor-specific CD8+ CTLs *in vivo* (21, 22). Such intralesional therapeutic strategies would be arguably most effective when applied in combination with tumor-specific immunization strategies capable of increasing frequencies of circulating antitumor T cells that might be recruited successfully into intracranial tumor sites.

In the present study, we observed that peripheral DC-ovalbumin T helper peptide-based vaccines minimally impacted intracranial M05 growth and animal survival, unless IFN-α gene-transduced DCs were coordinately delivered into the intracranial tumor sites. Although comparable efficacy was not observed for the corresponding combinational therapy using IFN-α gene-transduced fibroblasts, we believe that the potentiation of therapeutic effects occurs via mechanisms associated with improved or sustained DCs-mediated cross-presentation of tumor antigens to T cells *in situ*.

MATERIALS AND METHODS

Animals. Female 6–8-week-old C57BL/6 mice, C57BL/6 background transgenic mice line expressing enhanced green fluorescent protein (EGFP) C57BL/6-TgN(ACTbEGFP)1Osb (EGFP-Tg mouse hereafter) were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were handled under aseptic conditions in microisolator cages within the Central Animal Facility at the University of Pittsburgh per an Institutional Animal Care and Use Committee-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell Lines and Culture. The ovalbumin cDNA-transfected B16 melanoma cell line M05 (H-2b) was kindly provided by Dr. Louis Falo III (University of Pittsburgh, Pittsburgh, PA). M05 was chosen as a model cell line in these studies because the model tumor antigen ovalbumin encodes well-defined CD4+ and CD8+ T-cell epitopes (6). The EL4 lymphoma (H-2b) cell line and TIB81 fibroblast line were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in complete medium [RPMI

1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all of the reagents were from Life Technologies, Inc., Grand Island, NY)] in a humidified incubator in 5% CO_2 at 37°C.

Generation of DCs *in Vitro* from Bone Marrow. The procedure used in this study was described previously (23). Briefly, C57BL/6 or EGFP-Tg C57BL/6 mouse-derived bone marrow cells were cultured in complete medium supplemented with 1000 units/ml recombinant murine granulocyte/macrophage colony-stimulating factor and recombinant murine interleukin 4 (Schering-Plough, Kenilworth, NJ) at 37°C in a humidified, 5% CO_2 incubator for 7 days. DCs were then isolated at the interface of 14.5% (w/v) metrizamide (Sigma, St. Louis, MO) in complete medium discontinuous gradients by centrifugation. DCs typically represented >90% of the harvested population of cells based on morphology and expression of the CD11b, CD11c, CD40, CD54, CD80, CD86, and class I and class II MHC antigens (data not shown).

Viral Vectors. The mock adenoviral vector Ad-ψ5 and the adenoviral vector encoding mouse IFN-α gene (Ad-IFN-α) were produced and provided by the University of Pittsburgh Cancer Institute's Vector Core Facility as reported previously (24).

Adenoviral Transfection of DCs. Five million (day 7 cultured) DCs were infected with Ad-ψ5 or Ad-IFN-α at a MOI of 50 as reported previously (24). After 48 h, infected DCs were harvested and analyzed for their phenotype and function. Culture supernatants were also collected for measurement of mouse IFN-α production using a species-specific IFN-α ELISA kit (Research Diagnostics Inc., Flanders, NJ). The level of IFN-α expression from 1×10^6 IFN-α-transfected mouse DCs was determined to be ~50 ng/48 h *versus* <60 pg for control Ad-ψ5-infected DCs.

Peptides and Immunization. The H-2K^b-restricted OVA$_{257-264}$ (SIINFEKL) and H-2IA^b-restricted OVA$_{265-280}$ (TEWTSSNVMEERKIKV) peptides (6) were synthesized using an automated solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA) by the protein synthesis facility at the University of Pittsburgh Cancer Institute, purified by reverse-phase high-performance liquid chromatography, and validated by mass spectroscopy. The experimental designs are summarized in the Experimental Schema. Day 7 DCs were pulsed with 10-μM OVA$_{257-264}$ and/or 10-μM OVA$_{265-280}$ peptides for 4 h at 37°C as described previously (23). Cells were then washed twice with HBSS, with animals receiving injections of 5×10^5 peptide-pulsed DCs in 0.1 ml HBSS s.c. on days −14 and −7.

Tumor Challenge. As shown in the Experimental Schema, in the s.c. model, preimmunized animals were challenged s.c. on day 0 with 1×10^5 M05 cells in the right flank. In the intracranial model, immunized animals received an intracranial injection of 1×10^4 M05 cells as described previously (23). Briefly, using a 10-μl Hamilton syringe, 1×10^4 M05 cells suspended in 2 μl of PBS were stereotactically injected through an entry site at the bregma 2 mm to the right of the sagittal suture and 3 mm below the surface of the skull of anesthetized C57BL/6 mice using a Kopf stereotactic frame (Kopf Instruments, Tujunga, CA). Some animals bearing intracranial tumors also received an intratumoral injection with 1×10^5 adenovirally transfected DCs in the same

location on day 5 after the intracranial tumor challenge. For animals bearing s.c. tumors, antitumor responses were assessed based on comparative longitudinal measurements of tumor areas. For animals bearing intracranial tumors, the animals were monitored daily after treatment for the manifestation of any pathological signs associated with elevated intracranial pressure, such as hemiparesis, loss of appetite, or any altered grooming habits. Affected animals were sacrificed by CO_2 inhalation. Representative animals in each treatment cohort were sacrificed at selected time points to obtain tissues (brain tumors and lymphoid organs) for immunological analyses.

T-Cell Depletion Experiments. On days −4, −1, and 2 after tumor inoculation, mice received i.p. injections containing 50 μl of ascitic fluid of anti-CD4 (GK1.5 hybridoma; American Type Culture Collection), anti-CD8 (53–6.72 hybridoma; American Type Culture Collection), or PBS. The efficiency of specific subset depletions was validated by flow cytometry analysis of splenocytes, using phycoerythrin-conjugated anti-CD4 and anti-CD8 monoclonal antibodies (PharMingen). In all of the cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Microscopic Analyses of the Tumor Tissues. For immunohistochemical analyses of DC-infiltration, animals injected with tumor cells intracranial or s.c. were sacrificed on day 14 after tumor inoculation. Whole brains bearing intracranial tumors and s.c tumors were fixed for 2 h in 4% paraformaldehyde (in PBS), and then cryoprotected in 30% sucrose in PBS before being shock-frozen in liquid nitrogen-cooled isopentane. Five-μm frozen sections were then made on a cryostat. After air-drying, sections were stained with FITC-conjugated antimouse CD11c (clone HL3; BD Biosciences, San Diego, CA) or isotype controls. Sections were also counterstained with 2 mg/ml Hoechst 33258 (Sigma) for 3 min. Neighboring sections were also stained with H&E. The sections were then mounted in Vectashield H1000 (Vector Laboratories Inc., Burlingame, CA) and observed using a Nikon Eclipse E800 microscope equipped with a cooled charge-coupled device color camera.

To assess the fate and function of DCs injected into the intracranial tumor site, day 7 bone marrow-derived DCs from EGFP-Tg mice were generated and infected with Ad-ψ5 or Ad-IFN-α, as indicated above. Forty-eight h later, 1×10^6 control or virally infected DCs were injected into day 10 intracranial M05 tumors established in syngeneic C57BL/6 mice. After 5 additional days, whole brains were harvested and fixed with 4% paraformaldehyde. For background staining, Cell Tracker Red CMTPX (C-34552; Molecular Probes, Eugene, OR) was used at a concentration of 5 μM for 20 min. Sections (1 mm) were imaged for EGFP-DCs, using a two-photon microscope comprising a titanium-sapphire ultrafast tunable laser system (Coherent Mira Model 900-F), Olympus Fluoview confocal scanning electronics, an Olympus IX70 inverted system microscope, and custom-built input-power attenuation and external photomultiplier detection systems. Single-plane image acquisition used two-photon excitation at 850 nm with Olympus water-immersion objectives (×20 UApo 0.7NA, ×40 UApo 1.15NA, and ×60 UplanApo 1.2NA). Emission filters (Chroma, Brattleboro, VT) comprised a HQ535/50m filter (green emission), a 565dclp dichroic mirror, and a HQ610/75m filter (red emission).

Scheme 1. DC s.c. vaccines: DCs loaded with K^b-restricted OVA$_{257-264}$ (a class I epitope) and/or I-A^b-restricted OVA$_{265-280}$ (a class II epitope). (DCs, dendritic cells.)
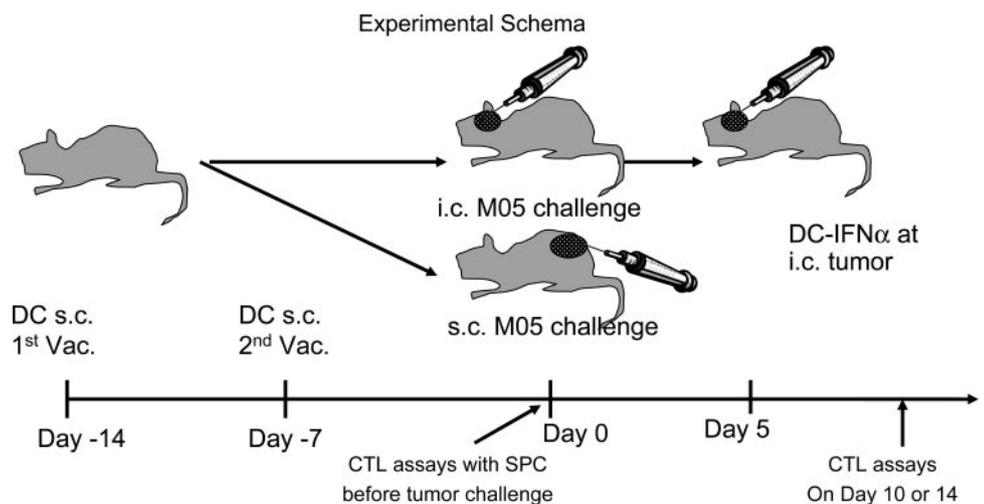
T Helper and CTL Activity Assays. Spleens or cervical lymph nodes were resected, and single-cell suspensions were cultured at 2×10^6 cells/ml with 2 μg/ml OVA$_{257-264}$ or OVA$_{265-280}$ in presence of 10 units/ml human interleukin 2 (Chiron, Emeryville, CA), 50 μM 2-mercaptoethanol (Sigma), and 50 μM N^G mono-methyl-$_L$-arginine (Cyclopss, Salt Lake City, UT) in 24-well plates (Corning, Corning, NY) for 5 days. T helper activity was determined by IFN-γ secretion levels measured by specific ELISA (BD PharMingen, San Diego, CA). Specific CTL activity was determined in 4 h ^{51}Cr-release assays against control or peptide-pulsed EL4 target cells, as described previously (25).

Statistical Analysis. Survival estimates and median survival times were determined using the method of Kaplan and Meier. Survival data were compared using a log-rank test. Comparative growth of s.c. tumors and T-cell responses were compared by Student's t test for two samples with unequal variances. Statistical significance was determined at the <0.05 level.

RESULTS

Immunization with an Ovalbumin-Derived MHC Class II-Restricted Epitope Protects Mice from s.c. But Not Intracranial Challenge with the Ovalbumin$^+$ M05 Melanoma. In our initial experiments, we investigated the ability of peripheral prophylactic vaccination with DCs pulsed with ovalbumin CTL and/or T helper epitopes to promote protective immunity against M05 melanoma s.c. or intracranial challenge. The M05 cell line expresses both the H-2K^b and D^b class I alleles, but does not express MHC class II molecules, even after *in vitro* treatment with IFN-γ or 3 weeks of *in vivo* growth (data not shown). Syngeneic wild-type C57BL/6 mice were immunized twice s.c. on a weekly schedule with bone marrow-derived DCs pulsed with either K^b-restricted peptide epitope OVA$_{257-264}$ (ovalbumin CTL epitope), I-A^b-restricted peptide epitope OVA$_{265-280}$ (ovalbumin T helper epitope), or both peptides. Control animals received injections of DCs that had not been pulsed with synthetic peptides. The mice were subsequently challenged with M05 melanoma cells s.c. or intracranial 14 days after their initial vaccination. Fig. 1A demonstrates that preimmunization of mice with the DC/ovalbumin T helper peptide vaccine resulted in complete protection against s.c. MO5 challenge, and that this regimen was as effective as

preimmunizing mice with the DC/K^b-restricted OVA$_{257-264}$ CTL epitope, despite the class II-negative phenotype of the M05 melanoma. As expected, vaccines containing both epitopes also protected the animals, whereas nonpulsed DCs did not. In contrast, the survival of animals receiving intracranial tumor challenge was not prolonged by prior immunization with the ovalbumin T helper epitope, although vaccination with the ovalbumin CTL epitope prolonged survival when compared with the control group ($P < 0.0001$; Fig. 1B). Prior vaccination with both the CTL and T helper peptides did not provide a significant survival advantage beyond that observed for animals vaccinated with the ovalbumin CTL epitope alone ($P = 0.3927$).

Paucity of DC Infiltration in Intracranial Tumors. The striking contrast in protection against s.c. *versus* intracranial M05 challenge in mice that had received prevaccinations with the I-A^b-restricted epitope (Fig. 1) led us to investigate more closely the corresponding tumor microenvironments for immune cell infiltration. Although pre-immunization with the I-A^b- or K^b-binding epitope does not allow the growth of measurable s.c. tumors, we compared s.c. *versus* intracranial tumors that are growing in animals preimmunized with no peptides (Fig. 2, A and B) or with mock I-A^b-binding epitope for intracranial tumors (Fig. 2C). Whereas a significant level of CD11c$^+$ DC infiltration was observed for s.c. tumors 14 days after tumor challenge (Fig. 2A), these cells were rare or absent in intracranial tumor lesions (Fig. 2, B and C), suggesting that considerable differences may exist between these sites with regard to antigen presentation and the cross-priming of tumor-reactive T cells. Notably, very few CD4+ or CD8+ T cells were identified in either the s.c or intracranial tumors in the conditions tested (data not shown). H&E staining (Fig. 2, D–F) demonstrated tumor tissues with high cellularity levels with no major necrotic or inflammatory lesions identified.

T-Cell Dependence of Vaccine-Induced Protection against M05 Tumors. We investigated the individual contribution of CD4$^+$ and CD8$^+$ T cells in the protective impact of prevaccination in the s.c. M05 model. C57BL/6 mice were treated with depleting antibodies against CD4 or CD8 or with control PBS before (days -4 and -1) and shortly after (day 2) s.c. M05 tumor challenge. Antibody treat-
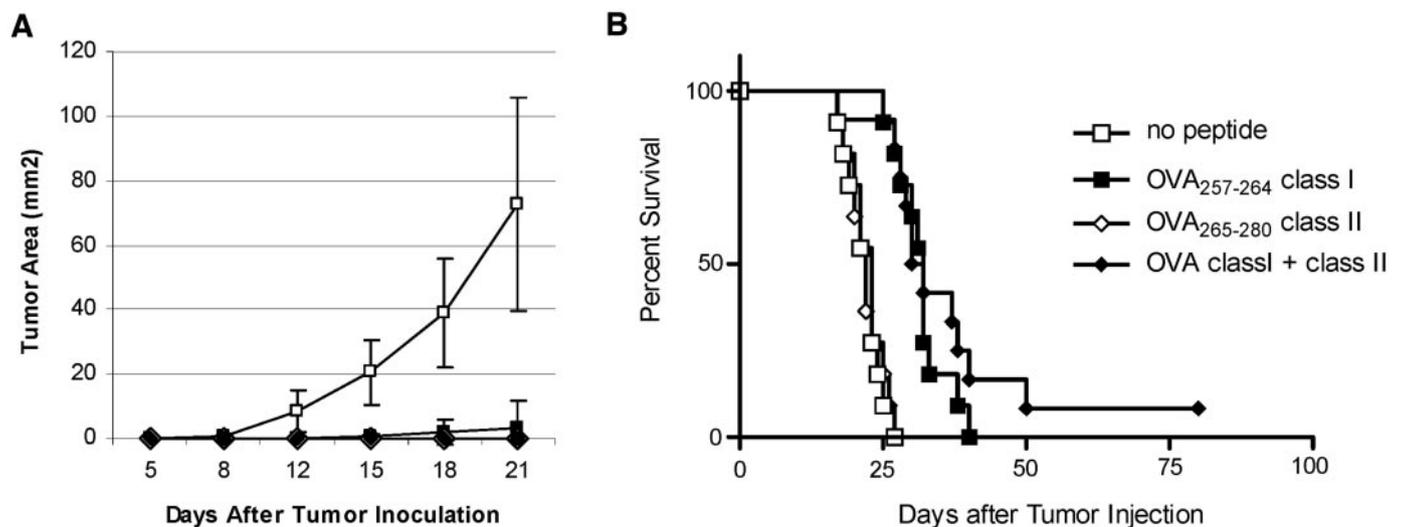

Fig. 1. Ovalbumin T helper peptide-based vaccines promote protective immunity against s.c. but not intracranial-injected M05 melanomas. C57BL/6 mice were immunized s.c. with syngeneic bone marrow-derived DCs loaded with the K^b-restricted OVA$_{257-264}$ (■), I-A^b-restricted OVA$_{265-280}$ ([diafo]), or both OVA$_{257-264}$ and OVA$_{265-280}$ (♦), or no (□) peptides twice on days -14 and -7. On day 0, 1 week after the secondary immunization, vaccinated animals received s.c. (A) or intracranial (B) injections with 1×10^5 or 1×10^4 M05 (ovalbumin$^+$, class I$^+$, or class II-negative) cells, respectively. Tumor area was monitored for s.c. tumors (A), whereas the survival rate was assessed in the case of intracranial tumors (B). In the data presented in A, $n = 6$ in all of the groups, and the results from one of three independent experiments with similar results are shown. The significance of differences in the tumor size on day 21 (Student's t test): OVA$_{265-280}$ *versus* no peptide, $P = 0.0039$; OVA$_{257-264}$ plus OVA$_{265-280}$ *versus* no peptide, $P = 0.0039$; and OVA$_{257-264}$ *versus* no peptide, $P = 0.0074$. In the data presented in B, $n = 10$ in all of the groups. Significance of differences (log-rank test): OVA$_{257-264}$ *versus* no peptide, $P < 0.0001$; OVA$_{265-280}$ *versus* no peptide, $P = 0.9711$; OVA$_{257-264}$ plus OVA$_{265-280}$ *versus* no peptide, $P < 0.0001$; and OVA$_{257-264}$ plus OVA$_{265-280}$ *versus* OVA$_{257-264}$, $P = 0.3927$. *Bars,* ±SD.
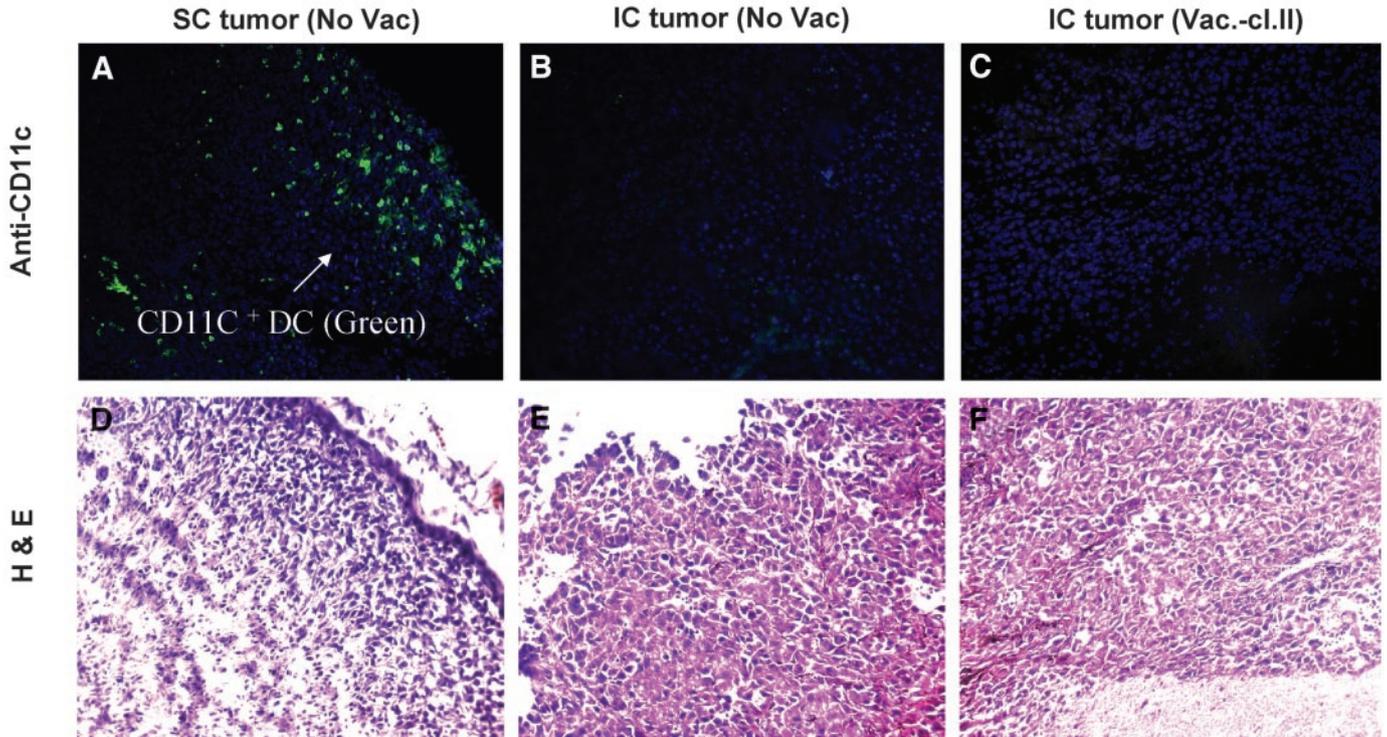
Fig. 2. Immunohistochemical analyses reveals extensive infiltration of CD11c$^+$ DCs in s.c. tumors but not intracranial tumors. Animals bearing s.c. or intracranial tumors were sacrificed on day 14 after the tumor inoculation. The tumors were frozen in OCT compound, thin sectioned (5 μm), and stained with a monoclonal antibody against mouse CD11c and with Hoechst 33,258 to visualize cell nuclei (*A–C*) or H&E (*D–F*). CD11c$^+$ cell infiltration is depicted for s.c. tumors resected from a nonimmunized animal (*A* and *D*) for intracranial tumors obtained from a nonimmunized (*B* and *E*) or an I-A^b-restricted OVA$_{265-280}$ immunized animal (*C* and *F*). (IC, intracranial; DC, dendritic cell)

ment resulted in depletion of >95% of the respective T-cell subsets as monitored by FACScan analysis of splenocytes (data not shown). Anti-CD4 monoclonal antibody treatment completely abrogated the protective effect of vaccination with the I-A^b-restricted OVA$_{265-280}$, indicating the critical role of CD4$^+$ T cells in facilitating or mediating M05 tumor rejection (Fig. 3). Similarly, depletion of CD8$^+$ T cells virtually ablated the antitumor effect in mice that were preimmunized with the I-A^b-restricted OVA$_{265-280}$. These results indicate that both CD4$^+$ and CD8$^+$ T cells are essential for rejection of M05 tumor challenge.

Cross-Priming of Antiovalbumin CTLs After Tumor Rejection in Mice Prevaccinated with a DC/Ovalbumin T Helper Epitope Vaccine. Rejection of class II-negative M05 tumors in mice vaccinated with the ovalbumin T helper epitope combined with the observed infiltration of DCs into s.c. tumors led us to hypothesize that ovalbumin-specific CD4$^+$ T cells may have facilitated the cross-priming of protective antitumor CTLs *in vivo*. To address this possibility, we evaluated ovalbumin-specific, splenic CTL responses in vaccinated mice before and after s.c. challenge with M05 melanoma cells. As demonstrated in Fig. 4A, only mice vaccinated with the K^b-restricted OVA$_{257-264}$ peptide displayed specific CTL responses before M05 challenge. Interestingly, those animals immunized with DCs pulsed with the I-A^b-restricted OVA$_{265-280}$ T helper epitope that subsequently rejected a s.c. challenge with M05 melanomas developed strong antiovalbumin CTL responses that were similar in magnitude to those observed in mice vaccinated with DCs loaded with the synthetic CTL epitope (Fig. 4B). Preimmunization with the I-A^b-restricted OVA$_{265-280}$ appeared critical for CTL induction in this model, because control mock-immunized animals did not develop specific CTL after M05 challenge. Additional control experiments indicate that there is no cryptic (H-2^b) CTL epitope embedded within the T helper peptide (data not shown) and that *in vivo* primed CD4$^+$

T cells secrete IFN-γ in response to the OVA$_{265-280}$ peptide (Fig. 4C). This suggests that the constitutive antitumor (*i.e.,* antiovalbumin) T helper response in prevaccinated mice facilitates the consequent cross-priming of protective/therapeutic antitumor (including antio-
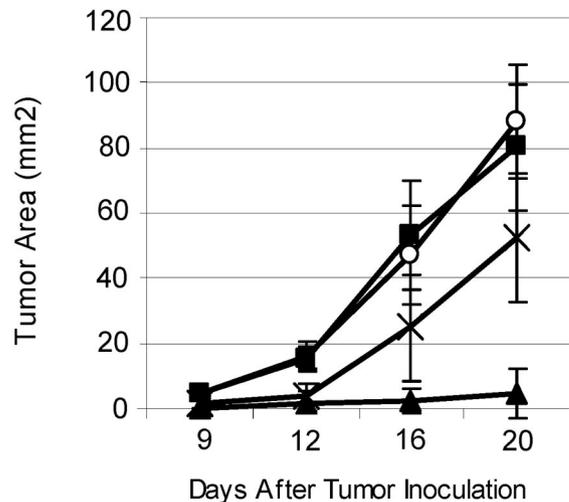

Fig. 3. CD4$^+$ and CD8+ T cells are required for protection against s.c. challenge with M05 melanoma in mice preimmunized with the class II (I-A^b) -restricted OVA$_{265-280}$ epitope. C57BL/6 mice were preimmunized s.c. twice with DCs loaded with I-A^b-restricted OVA$_{265-280}$ or control nonpeptide-pulsed DCs (○). Mice preimmunized with OVA$_{265-280}$ received subsequent i.p. injections of antimouse CD4 (■) or CD8 (*crosses*) monoclonal antibodies or with HBSS (▲) on days −4, −1, and 2 after the s.c. challenge with 1×10^5 M05 cells. Depicted are the results of one of three independent experiments performed, each yielding comparable data. Each treatment group contained 5 mice. The statistical significance of differences in the tumor size (Student's *t* test) on day 20: CD8 depletion *versus* no depletion, $P = 0.010$; CD4 depletion *versus* no depletion, $P = 0.002$; CD8 depletion *versus* no peptide, $P = 0.159$; and CD4 depletion *versus* no peptide, $P = 0.362$. *Bars*, ±SD.
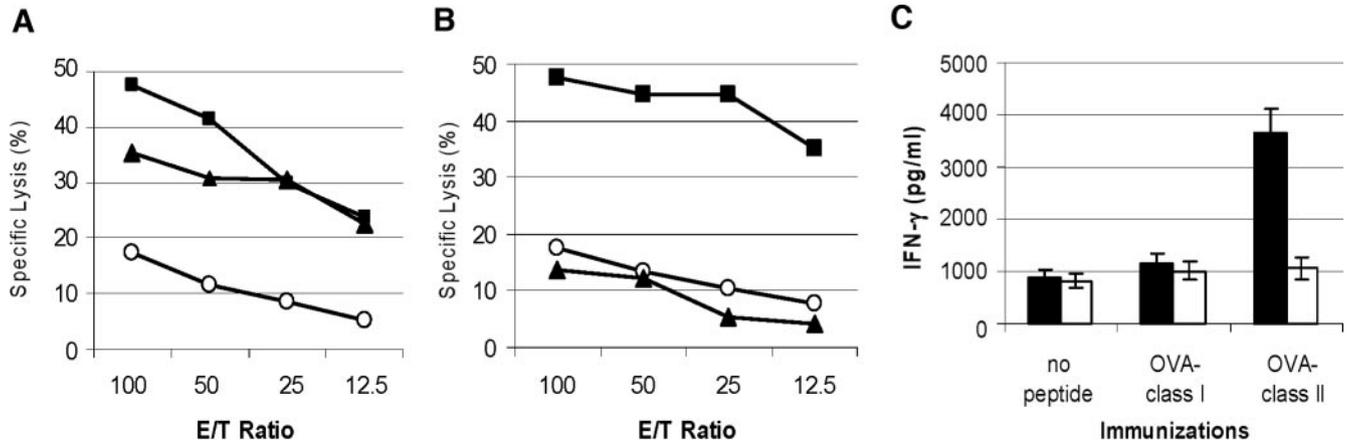
Fig. 4. Mice immunized with the I-A^b-restricted OVA$_{265-280}$ develop specific antiovalbumin helper T lymphocyte and CTL responses during the rejection of s.c. M05 tumors. C57BL/6 mice received two cycles of immunization with either unpulsed DCs (○), DCs pulsed with the class I (K^b) -restricted OVA$_{257-264}$ (■), or DCs pulsed with the class II (I-A^b) -restricted OVA$_{265-280}$ (▲). *A and C,* immunized animals were sacrificed on day 14 after the secondary immunization. *B,* immunized animals received 1×10^5 M05 cells s.c. in the flank on 14 days after the secondary immunization and then sacrificed on day 28 after the secondary immunization (*i.e.,* on day 14 after the s.c. tumor challenge), and splenocytes were harvested. Splenocytes were stimulated *in vitro* with OVA$_{257-264}$ (for *A* and *B*) or with OVA$_{265-280}$ (*C*) for 5 days in the presence of 10 units/ml human interleukin-2. IFN-γ production was measured using specific ELISA (*C*), and cytotoxic activity of the stimulated splenocytes was measured in a standard 4 h ^{51}Cr-release assay against control or OVA$_{257-264}$ peptide-pulsed EL4 target cells (*A* and *B*). Specific lysis against control (nonpulsed) EL4 cells was uniformly <10% (data not shown). *Bars,* ±SD. (DC, dendritic cell; Th, T helper)

valbumin) CTLs developed after tumor challenge in this cohort of animals.

Intratumoral Injection of IFN-α cDNA-Transfected DCs Potentiates the Efficacy of Peripheral Ovalbumin Peptide-Based Immunizations. The striking contrast that we observed in the ability of ovalbumin T helper peptide vaccines to elicit protective immunity against s.c. *versus* intracranial M05 tumors and the improved prognosis of mice exhibiting tumors that were infiltrated by CD11c+ DCs led us to hypothesize that delivery of activated APCs into the intracranial tumor site might improve the clinical outcome of vaccinated, then tumor-challenged mice by enhancing the cross-priming of antitumor T cells *in situ.* On the basis of the known biological properties of IFN-α [*i.e.,* enhancement of tumor-specific CTL activation and survival (22) and support of DC-mediated cross-priming of exogenous antigens (20)], we hypothesized that IFN-α produced locally within the brain tumor microenvironment by DCs might additionally enhance the potency of vaccine-induced antitumor T cells in this model.

DCs were generated and infected with control Ad-ψ5 or Ad.IFN-α adenovirus *in vitro,* before being injected into the intracranial M05 tumor lesion of prevaccinated mice 5 days after tumor implantation (Fig. 5). As noted earlier in Fig. 1*B,* even in the absence of intratumoral DC injection, a prolongation of survival was seen in intracranial tumor-bearing animals immunized with the ovalbumin CTL, but not the ovalbumin T helper, peptide-based vaccine. In contrast, intracranial M05-bearing animals that received intratumoral injections of DC-IFN-α and were preimmunization with ovalbumin T helper-based vaccines displayed statistically significant prolongation of survival when compared with nonpreimmunized animals ($P = 0.0174$), with 1 of 10 animals treated with this combinational therapy surviving for longer than 80 days. DC-IFN-α injection into the intracranial tumor site also promoted the enhanced survival of animals that had been prevaccinated with either the ovalbumin CTL epitope alone or with both ovalbumin epitopes, resulting in long-term survival (>80 days) in 4 of 10 and 6 of 10 animals, respectively (Fig. 5*B*). These data support the notion that modulation of the intracranial tumor microenvironment with DC-IFN-α enhances the efficacy of peptide-based immunization strategies in a combinational immunotherapy approach.

Intratumoral Injection of DC-IFN-α Enhances Specific CTL Responses in the Draining Cervical Lymph Nodes. To determine whether intratumoral delivered DC-IFN-α enhanced the cross-priming of ovalbumin-specific CTLs *in situ,* cervical lymph nodes were

isolated from differentially treated mice 10 days post-DC injection intratumoral and analyzed for specific CD8+ T cell responses against the K^b-restricted CTL epitope OVA$_{257-264}$ *in vitro.* Cervical lymph nodes cell suspensions were cultured in the presence of OVA$_{257-264}$ peptide and low-dose (10 IU/ml) interleukin 2 for 5 days before performance of CTL assays, using control or peptide-pulsed (H-2^b) EL4 cells as target cells (Fig. 6). In animals prevaccinated with the ovalbumin CTL epitope, a moderate increase of OVA$_{257-264}$-specific killing was observed if DC-ψ5 cells had been injected intratumorally (Fig. 6*A*). Peripheral immunization with the ovalbumin T helper epitope (Fig. 6*B*) or mock-immunization with nonpulsed DCs (Fig. 6*C*) did not induce any response over the background level, consistent with the lack of prolongation of survival associated with these regimens noted previously in Fig. 5. Notably, intratumoral injections of either control DC-ψ5 or syngenic fibroblasts engineered to secrete IFN-α (at levels comparable with DC-IFN-α) did not enhance ovalbumin-specific CTL activity above that observed for the peripheral immunization regimens. In contrast, there was a remarkable increase in anti-OVA$_{257-264}$ CTL activity in animals treated with intratumoral delivery of DC-IFN-α cells that had been prevaccinated with ovalbumin CTL or ovalbumin T helper epitope-based vaccine (Fig. 6, *A* and *B*). Delivery of DC-IFN-α also appeared to induce a slight increase in ovalbumin-specific CTL reactivity in animals that had received mock peripheral vaccines (Fig. 6*C*). These data support the notion that modulation of the intracranial tumor microenvironment with DC-IFNα, but not just delivery of IFN-α by gene-modified fibroblasts, facilitates the cross-priming of tumor-reactive CTL in cervical lymph nodes, which is illustrated most clearly in the case of animals prevaccinated with the ovalbumin T helper epitope.

IFN-α Transfection Promotes the Survival of DCs Injected into the Intracranial Tumor Microenvironment. We next assessed the impact of IFN-α gene insertion on the distribution of viability of intratumoral injected DCs. DCs were generated from the bone marrow of EGFP-Tg (H-2^b) mice, infected with no virus, control Ad-ψ5 or Ad-IFN-α, and then injected intratumoral into mice bearing established day 14 intracranial M05 tumors. Five days later, animals were sacrificed, and EGFP$^+$ DCs were observed using two-photon microscopy. As shown in Fig. 7, numerous EGFP$^+$ (green) DCs were detected in the intracranial tumor lesion if the injected DCs had been infected with Ad-IFN-α before transfer (Fig. 7, *A* and *B*). EGFP$^+$ cells were observed around the needle track and distributed throughout the
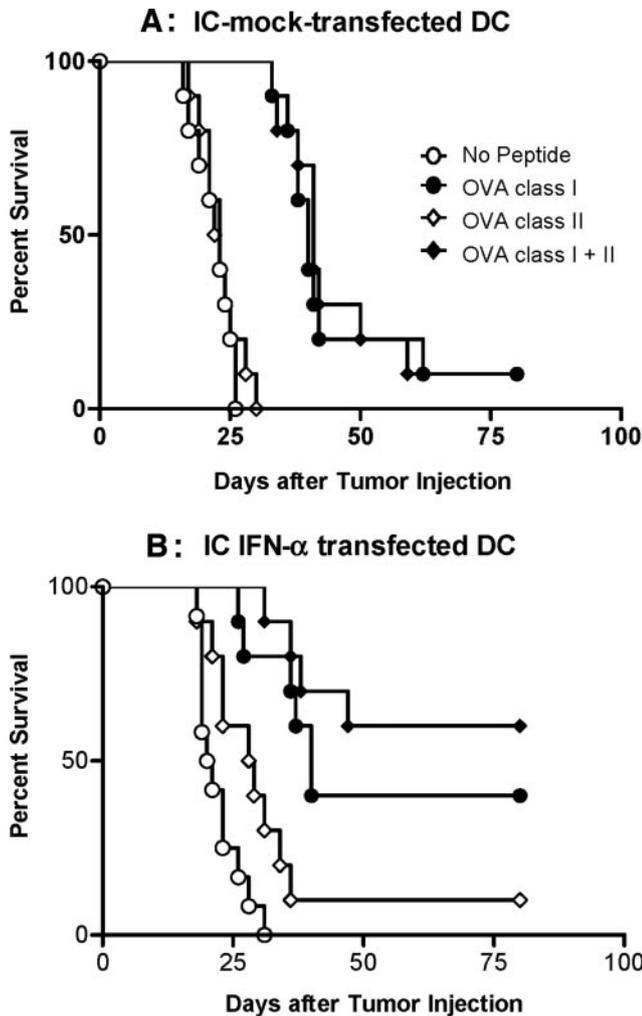
A: IC-mock-transfected DC

B: IC IFN-α transfected DC

Fig. 5. I.t. injection of DC-IFN-α enhances the efficacy of vaccines with I-A^b-restricted OVA$_{265–280}$ and K^b-restricted OVA$_{257–264}$"tumor" epitopes. C57BL/6 mice received two cycles of s.c. preimmunizations with either the K^b-restricted peptide epitope OVA$_{257–264}$ (SIINFEKL, ●), the I-A^b-restricted peptide epitope OVA$_{265–280}$ (TEWTSS-NVMEERKIKV, [diafo]), or both epitopes (◆). Control animals received nonpulsed DCs only (○). Animals received intratumoral injection with mock-transfected DCs (A) or IFNα-transfected DCs (B); n = 10 in all of the groups. Significance of differences (Log-rank test): (A) OVA$_{257–264}$ *versus* no peptide, $P < 0.0001$; OVA$_{265–280}$ *versus* no peptide, $P = 0.5830$; OVA$_{257–264}$ plus OVA$_{265–280}$ *versus* no peptide, $P < 0.0001$; and OVA$_{257–264}$ plus OVA$_{265–280}$ *versus* OVA$_{257–264}$, $P = 0.7330$. (B) OVA$_{257–264}$ *versus* no peptide, $P < 0.0001$; OVA$_{265–280}$ *versus* no peptide, $P = 0.0174$; OVA$_{257–264}$ plus OVA$_{265–280}$ *versus* no peptide, $P < 0.0001$; and OVA$_{257–264}$ plus OVA$_{265–280}$ *versus* OVA$_{257–264}$, $P = 0.3458$. (IC, intracranial)

tumor. Tissue sections of corresponding cervical lymph nodes also demonstrated the presence of EGFP$^+$ cells, suggesting that adoptively transferred DCs were also capable of migrating from the intracranial tumor site/injection site to the cervical lymph nodes.[8] In marked contrast, tumors injected with uninfected or Ad-ψ5-infected DCs contained few EGFP$^+$ cells (Fig. 7, *C* and *D*, respectively). A tumor that did not receive EGFP-DC injection and a normal brain tissue demonstrate background staining only for the tumor or normal brain cells (red signals, Fig. 7, *E* and *F*, respectively). When taken together with the functional data presented in Fig. 6, IFN-α transfection of DCs appears to promote the survival and cross-priming of intra-tumorally

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[8] K. Kuwashima, H. Sato, T. Sakaida, M. Hatano, T. Tsugawa, J. Dusak, W. Fellows-Mayle, K. Okada, G. Papworth, S. Watkins, A. Gambotto, I. Pollack, W. Storkus, and H. Okada. Delivery of interferon-α transfected DCs into central nervous system tumors enhances the efficacy of peripheral immunizations with cytokine-gene transfected tumor cell vaccines in an apoptosis-inducing ligand-dependent manner, manuscript in preparation.

injected DCs both within the intracranial tumor site and the tumor-draining cervical lymph nodes.

DISCUSSION

In this study, we demonstrated differential protection afforded to mice receiving peripheral immunizations with syngeneic DC/peptide vaccines, dependent on the site of subsequent tumor inoculation. In a s.c. tumor model, preimmunization of mice with DCs loaded with ovalbumin T helper peptide vaccines results in the effective cross-priming of CD8$^+$ T cells capable of directly recognizing the class I$^+$, class II-negative M05 melanoma. In contrast, such prevaccination minimally impacted M05 tumors inoculated intracranial, unless the established tumor was subsequently injected with IFN-α gene-transduced DCs, resulting in successful cross-priming of ovalbumin-specific CTLs.

Rejection of MHC class II-negative s.c. tumors by immunization with a MHC class II-restricted epitope has been described previously, with the cross-priming of T cells by tumor-associated antigen-presenting cells suggested as a key underlying mechanism (3). A recent study has indicated additionally that imprinting of memory function requires specific CD4$^+$ T helper cells interacting with DCs (26). The remarkable contrast observed in intracranial *versus* s.c. M05 tumors after immunization with the H-2IA^b-restricted ovalbumin-derived epitope in our study led us to hypothesize that the paucity of DCs infiltrating intracranial tumor sites might represent a major limitation to the consequent cross-priming of specific T cells reactive against intracranial tumors. Despite the different endpoints that were used in evaluating the CNS *versus* s.c. tumors, statistical analyses between treatment groups in each anatomical location revealed clearly that immunizations with the ovalbumin T helper epitope induced significant antitumor responses against s.c. tumors but not intracranial tumors.

Although DCs and/or other professional APCs of the DC lineage play important roles in the cross-presentation of tumor antigens, their maturation stage is an important factor in determining whether cross-priming or cross-tolerance occurs after the T-cell contact with DCs (reviewed in Ref. 27). With regard to the status of APCs in the CNS, a recent report demonstrated that CD11c$^+$ DCs isolated from the brain of mice with experimental autoimmune encephalomyelitis exhibit a maturational phenotype similar to immature bone marrow-derived DC or splenic DCs (10). Furthermore, these DCs appear unable to prime naïve T cells and, in fact, were observed to inhibit T cell proliferation (10). APCs obtained from the CNS tumors have also been reported to be dysfunctional (28). Indeed, the microenvironment of the CNS may induce endogenous brain APCs cells to become tolerogenic; and this may also explain our data indicating that even *ex vivo* activated, (nontransfected) DCs might not be able to function as stimulatory APCs within the intracranial microenvironment after their adoptive transfer. It has been suggested previously that transforming growth factor-β, interleukin 10, and tumor necrosis factor-related apoptosis inducing ligand may all contribute to the inhibitory or hypostimulatory characteristics of brain-derived DCs (10).

Nevertheless, the critical role of endogenous brain APCs for priming and restimulation of antitumor T effector cells has been demonstrated in a congeneic mouse model (29). H-2K^{d+} brain APCs cross-primed CTLs against the H-2K^d-restricted CW3$_{170–179}$ epitope derived from CW3$^+$ murine gliomas lacking H-2K^d molecules (29). In our model, in contrast, very few tumor-infiltrating CD11c$^+$ APCs, CD4$^+$, or CD8$^+$ T cells were detected in the intracranial M05 melanoma and, perhaps accordingly, only weak levels of functional anti-OVA$_{257–264}$-reactive CTLs were detectable in the cervical lymph node without peripheral immunizations. The paucity of vital, stimu-
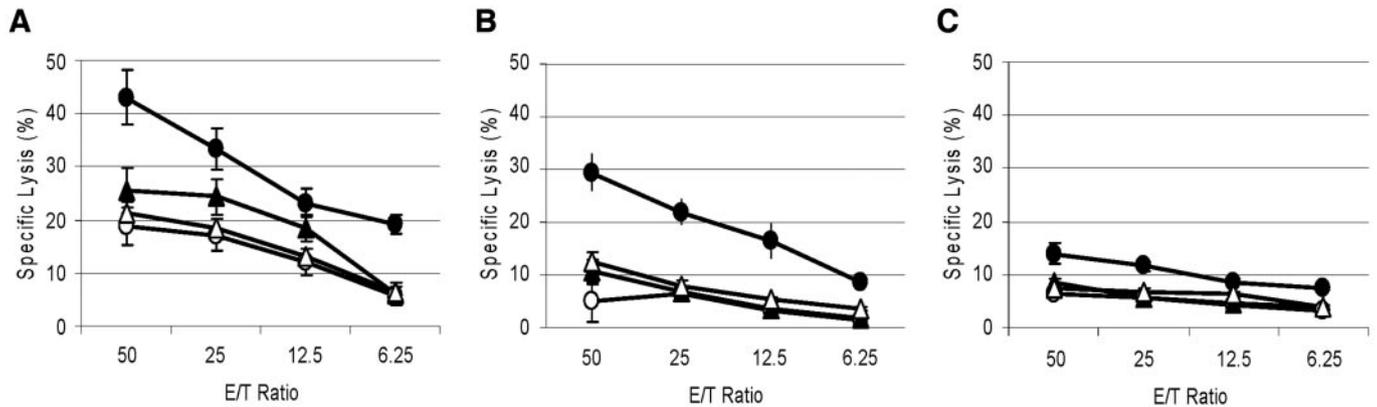
Fig. 6. I.t. injection of DC-IFNα enhances specific CTL reactivities in the cervical lymph nodes of animals that also received peripheral vaccines. Animals preimmunized with either the K^b-restricted peptide epitope OVA$_{257-264}$ (*A*), the I-A^b-restricted peptide epitope OVA$_{265-280}$ (*B*), or adjuvant DCs only (*C*) received intracranial injections of M05 tumors. On day 5 after the intracranial tumor challenge, animals also received either IFN-α transfected DC (●), mock-transfected DCs (▲), syngeneic fibroblasts transfected with Ad-IFN-α (△), or PBS (○). Animals were then sacrificed on day 15 after the intracranial tumor challenge (*i.e.,* 10 days after the DC injection), and cervical lymph node cells were *in vitro* stimulated with the K^b-restricted peptide epitope OVA$_{257-264}$ for 5 days. These cells were subjected to CTL assays using EL4 cells pulsed with OVA$_{257-264}$ as the target cells and nonpulsed EL4 cells as control cells. As the specific lysis values against nonpulsed EL4 cells were constantly below 8%, data were not included in the presented figures. Each value represents the triplicate determinations for each group. Statistical significance at effector *versus* target ratio 50:1 (Student's *t* test); $P < 0.05$ for DC-IFN-α *versus* any other control group in *A, B,* and *C. Bars,* ±SD. (DC, dendritic cell)

latory APCs infiltrating the progressive tumor in our model may reflect the situation observed typically for human malignant brain tumors, where both a paucity of infiltrating DCs and poor clinical prognosis are characteristic of this disease. We have reported that some DCs in the 9L rat glioma undergo apoptotic cell death and that hyaluronic acid, which is abundant in the extracellular matrix of the CNS and CNS tumors, induces nitric oxide production by DCs and the consequent demise of these APCs (30). In accordance with these observations, in the current study, even intratumoral injection of *ex vivo* activated nontransfected, DCs in the brain did not dramatically enhance the therapeutic efficacy associated with vaccine-induced systemic T-cell responses. Although a recent study using a rat 9L glioma and syngeneic bone marrow derived DCs demonstrated that intratumoral DC injection induced specific T-cell reactivity against 9L, the level of response was rather modest (31). Our experiments using adoptively transferred EGFP-Tg DCs demonstrated that very few of these APCs could be detected within the intracranial tumor lesion 5 days after their injection. Although it is possible that the injected (non-IFN-α transfected) EGFP-Tg DCs may have migrated away from the intracranial tumor site; if this did occur, these APCs were ineffective at mediating the cross-priming of ovalbumin-specific T cells within the cervical lymph node. Rather, we anticipate that these injected DCs undergo tumor-induced apoptosis *in situ*, a hypothesis that we are currently evaluating using terminal deoxynucleotidyl transferase-mediated nick end labeling imaging techniques.

There has been some controversy as to whether tumor cells themselves have to metastasize lymphoid organs to (directly) induce specific antitumor CTL responses (32). However, in the current study, a dramatic enhancement in the priming of ovalbumin-specific CTLs in the cervical lymph node occurred only after intratumoral DC-IFNα injection in combination with peripheral ovalbumin T helper peptide-based vaccination, suggesting that DC migration to the cervical lymph nodes and that cross-presentation of tumor-antigens is critical in this system. Interestingly, IFN-α has been reported to markedly enhance the migratory capacity of human skin-derived DCs (33) and monocyte-derived DCs both *in vitro* and *in vivo* through up-regulation of C-C chemokine receptor-7 expression (34), suggesting that genetic modification of DCs with the IFN-α expression vector may also promote DC migration.

Ex vivo adenoviral transfection of DCs with IFN-α cDNA dramatically improved the durability and the apparent function of these

APCs after intratumoral injection. It is unlikely that this is because of adenovirus-induced DCs maturational effects, because DC-ψ5 controls did not promote cross-priming of antiovalbumin T-cell responses. Our preliminary data on the phenotype of mouse DCs transfected with Ad-IFN-α indicate slight up-regulation of CD40, CD86, and H-2IA^b when compared with Ad-ψ5-transfected DC control cells (data not shown). This indicates that *cis*-production of IFN-α may contribute to the promotion of DC survival and prolonged antigen-presenting function. It is known that tumor cells suppress the functionality (35) and vitality of DCs (30, 36) via diverse mechanisms. Mature DCs are reported to be more resistant than immature DCs to the killing effects of CD95L or tumor necrosis factor-related apoptosis inducing ligand and to express higher levels of the caspase 8 inhibitory protein (37). Type I IFNs are known to promote maturation of DCs and to activate T-effector cells (38); however, the precise mechanisms by which IFN-α transfection protects DCs from tumor-induced death has yet to be elucidated.

Whereas recent studies demonstrated that IFN-α promotes cross-priming by increasing the expression of the peptide transporter TAP-1 in DCs (39), and the critical role of type I IFNs in cross-priming of antigen-specific CTLs has been directly demonstrated using IFN-α/β receptor-deficient mice (20), our results are the first demonstration that ectopic expression of IFN-α by DCs may promote their ability to cross present tumor-associated antigens to CD8$^+$ T cells *in situ*. Our data also indicate that addition of the H-2IA^b-restricted OVA$_{265-280}$ T helper epitope to vaccines enhances both ovalbumin-specific CTL generation and antitumor efficacy of combinational approaches using DC-IFN-α delivery. CD4$^+$ T cells may not directly act on CD8$^+$ CTL precursors, at least initially, but rather on DCs that cross-present tumor-associated antigens to CD8$^+$ T cell precursors (5).

DCs injected into tumors might directly induce tumor cell death, thereby generating tumor cell apoptotic bodies that serve as a substrate for the cross-presentation of tumor-antigens to T cells (40). In our *in vitro* data, although nontransfected and mock-transfected DCs exhibited a moderate direct cytotoxicity against M05 tumors, IFN-α gene insertion did not appear to significantly enhance the antitumor killing capacity of DCs. In addition, IFN-α gene-transfected fibroblasts did not demonstrate an antiproliferative or cytotoxic effect on M05 tumor cells *in vitro*, suggesting that the expression of the IFN-α transgene may have more dominantly promoted the vitality and stim-
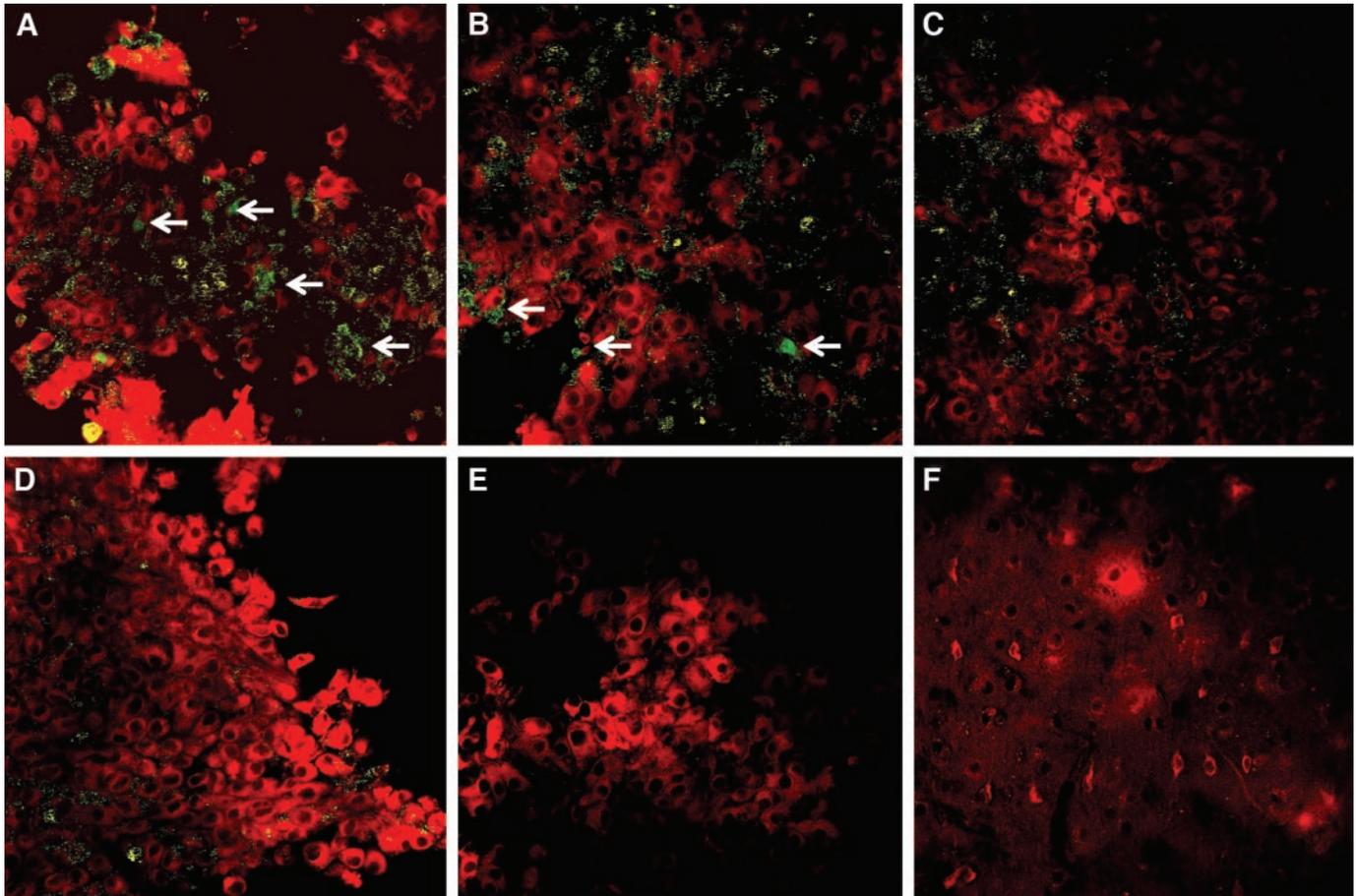
Fig. 7. IFN-α gene transfection leads to a persistent presence of EGFP DCs that were injected into the intracranial M05 tumors. Mice bearing intracranial M05 tumors received intratumoral injections of EGFP-Tg mice-derived DC that are as follows: *A–B,* transfected with Ad-IFN-α; *C,* transfected with a control Ad-ψ5 or (*D*) nontransfected; *E,* a MO5 tumor with no injection; and *F,* a normal brain. The animals were sacrificed on day 5 after DC injection, and the presence of DC was observed using multiphoton microscopy. Original magnification, ×400. *Green* fluorescence signals pointed by *arrows* indicate EGFP DCs, whereas *red* fluorescence signals indicate background staining with Cell Tracker Red CMTPX (Molecular Probes). The *yellow* color prevalent in images of the M05 tumors is caused by laser interactions with concentrated areas of melanin pigment within the tumor and is not indicative of the colocalization of the two fluorescent markers used in the study. This is shown additionally by the total absence of this *yellow* appearance in normal brain tissue (*F*).

ulatory functions of the gene-modified DCs and had little direct effect on M05 tumor growth *in situ.*[8]

We used the intracranial M05 melanoma as a model of metastatic brain tumors, which are common and devastating (frequently lethal; Ref. 41) complications in melanoma patients. This may be because of the failure of adjuvant immunotherapies otherwise effective in preventing the systemic recurrence of melanoma to prevent relapses in the CNS (41). Clearly, CNS relapse is a major obstacle that must be overcome before cancers can be cured by any means.

Data from our studies suggests that delivery of DC-IFN-α directly into intracranial tumors may be a suitable strategy for enhancing the efficacy of peripherally delivered therapeutic vaccines. Thus far, we have been able to achieve long-term survival in ~60% of animals bearing intracranial M05 tumors, using the current protection/early stage therapeutic protocol. Whereas this response rate should be improved on in consideration of prospective clinical trial designs, it is important to note that the B16-derived M05 tumor is considered extremely aggressive and has proven challenging in the setting of even s.c. treatment models. In this regard, we would envision that DC-based combination gene therapies integrating IFN-α and other cytokines such as interleukin 23, which promotes the activation of T helper-1 type memory T cells (42), may additionally promote clinically favorable vaccine-induced immune responses against intracranial tumors in tumor-bearing hosts.

ACKNOWLEDGMENTS

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