Adoptive Transfer of Type 1 CTL Mediates Effective Anti–Central Nervous System Tumor Response: Critical Roles of IFN-Inducible Protein-10

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Abstract

The development of effective immunotherapeutic strategies for central nervous system (CNS) tumors requires a firm understanding of factors regulating the trafficking of tumor antigen–specific CTLs into CNS tumor lesions. Using C57BL/6 mice bearing intracranial (i.c.) ovalbumin-transfected melanoma (M05), we evaluated the efficacy and tumor homing of i.v. transferred type 1 or 2 CTLs (Tc1 or Tc2, respectively) prepared from ovalbumin-specific T-cell receptor–transgenic OT-1 mice. We also tested our hypothesis that intratumoral (i.t.) delivery of dendritic cells that had been transduced with *IFN-α* cDNA (DC-IFN-α) would enhance the tumor-homing and antitumor effectiveness of adoptively transferred Tc1 via induction of an IFN-γ-inducible protein 10 (IP-10). *In vitro*, DC-IFN-α induced IP-10 production by M05 and enhanced the cytolytic activity of Tc1. *In vivo*, i.v. transferred Tc1 trafficked efficiently into i.c. M05 and mediated antitumor responses more effectively than Tc2, and their effect was IP-10 dependent. I.t. injections of DC-IFN-α remarkably enhanced the tumor homing, therapeutic efficacy, and *in situ* IFN-γ production of i.v. delivered Tc1, resulting in the long-term survival and persistence of systemic ovalbumin-specific immunity. These data suggest that Tc1-based adoptive transfer therapy may represent an effective modality for CNS tumors, particularly when combined with strategies that promote a type 1 polarized tumor microenvironment. (Cancer Res 2006; 66(8): 4478-87)

Introduction

We have recently identified glioma-associated antigen–derived CTL epitopes (1, 2) and developed glioma vaccine clinical trials for patients with glioma (3). One major challenge for these vaccines, however, is the significant suppression of the immune system in patients with progressive malignant gliomas (reviewed in ref. 4). Although active immunizations with glioma-associated antigen vaccines require the residual antitumor immune competence of the host, recent studies suggest that greater clinical efficacy may be obtained through the adoptive transfer of tumor-reactive, *ex vivo*–activated autologous T lymphocytes, particularly when combined with nonmyeloablative but lymphodepleting chemotherapeutic regimens (5, 6). This strategy may be particularly suitable for patients with malignant gliomas because the clinical use of chemotherapeutic agents is rapidly becoming standard of care in these patients (7, 8).

To further optimize such strategies for central nervous system (CNS) tumors, we require a better understanding of how the adoptively transferred T cells may be best recruited into CNS tumor sites. We have previously shown that intratumoral (i.t.) injections of dendritic cells that had been transduced with *IFN-α* cDNA (DC-IFN-α) *ex vivo* enhance the effectiveness of peripheral vaccinations by promoting the cross-presentation of tumor antigens in the draining cervical lymph nodes (CLN; ref. 9). We also hypothesized that i.t. delivery of DC-IFN-α would promote the tumor homing and cytotoxic activities of antitumor effector T cells, potentially through the induction of various cytokines and chemokines involved in the recruitment of T cells into inflammatory sites (10). In particular, IFN-α and IFN-γ induce the production of the potent chemokine IP-10 from a variety of cells, including astrocytes (11) and dendritic cells (12–14). Type 1 memory/activated T cells express the CXCR3 chemokine receptor, which binds IP-10 and allows activated type 1 T cells to migrate in response to IP-10 gradients (15, 16). Moreover, IFN-α up-regulates CXCR3 expression on type 1 T cells (17).

To address the role of type 1 cytokines and IP-10 in tumor homing and to test the therapeutic effectiveness of adoptively transferred T cells against CNS tumors, in the present study, we used mice bearing intracranial (i.c.) ovalbumin-transfected M05 melanoma lesions and adoptive transfer of ovalbumin-reactive Tc1 or Tc2 cells isolated from syngeneic OT-1 mice (18, 19). We show for the first time that i.v.-injected ovalbumin-specific Tc1 are superior to Tc2 in trafficking into i.c. M05 lesions and mediate a potent therapeutic response. Furthermore, i.t. delivered DC-IFN-α enhanced the tumor trafficking and therapeutic effects of i.v. Tc1 infusion in an IP-10-dependent manner. Our data indicate that this combinational therapeutic approach may prove efficacious in the treatment of established CNS tumors.

Materials and Methods

Animals. C57BL/6 (H-2^b) and C57BL/6-background Thy1.1 (B6.PL-$Thy1^a$/CyJ) mice (6-10 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). Ovalbumin-specific T-cell receptor (TCR) transgenic OT-1 mice (C57BL/6-background, Thy1.2) were purchased from Taconic (Germantown, NY). IP-10-deficient mice (12) were kindly provided by Dr. Andrew D. Luster. Animals were handled in the Animal Facility at the University of Pittsburgh per an Institutional Animal Care and Use Committee–approved protocol.

Cell lines. Mouse (H-2^b) B16 and ovalbumin-transfected B16 (M05) melanoma cell lines were kindly provided by Dr. Louis Falo, Jr. (University of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Pittsburgh, Pittsburgh, PA). These cell lines were maintained in mouse complete medium [RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mmol/L L-glutamine (all reagents from Life Technologies, Inc., Grand Island, NY)] in a humidified incubator in 5% CO_2 at 37°C.

***In vitro* generation of bone marrow dendritic cells and adenoviral transduction.** C57BL/6 mouse–derived bone marrow cells were generated as previously described (20). Briefly, bone marrow cells were cultured in complete medium supplemented with 1,000 units/mL recombinant murine granulocyte macrophage colony-stimulating factor, recombinant murine interleukin-4 (mIL-4; Cell Sciences, Canton, MA), 100 μmol/L nonessential amino acids, 55 μmol/L 2-mercaptoethanol, and 1 mmol/L pyruvate for 7 days.

Adenoviral vectors encoding *mIFN-α* gene (Ad-IFN-α) and the control Ad-ψ5 were produced as reported previously and provided by the University of Pittsburgh Cancer Institute Vector Core Facility (9, 21, 22). Dendritic cells were transduced with Ad-ψ5 or Ad-IFN-α at a multiplicity of infection of 50, as reported previously (9, 22). After 24 hours, adenoviral-transduced cells were harvested.

Generation of ovalbumin-specific $CD8^+$ effector T cells. Tc1 and Tc2 for adoptive transfer were prepared from OT-1 mice using the methods described previously (23). Briefly, for preparation of Tc1, naive $CD8^+$ T cells were isolated from OT-1 mice and cultured (2×10^5 T cells/mL) with mitomycin C–treated ovalbumin$_{257-264}$ peptide-pulsed splenocytes as antigen-presenting cells (6×10^5/mL) in the presence of recombinant human IL-2 (rhIL-2; 20 units/mL; R&D Systems, Minneapolis, MN), rmIL-12 (100 units/mL; Cell Sciences), and anti-mIL-4 monoclonal antibody (mAb; 200 units/mL; R&D Systems). Tc2 cells were generated from naive $CD8^+$ OT-1 T cells stimulated with ovalbumin-pulsed antigen-presenting cells in the presence of rhIL-2 (20 units/mL), rmIL-4 (200 units/mL), and anti-IFN-γ mAb (20 μg/mL; Cell Sciences). The cells were incubated for 4 days.

CTL and triple-cell cytolytic assay. A total of 1,200 ^{51}Cr-labeled target M05 or B16 cells were mixed with varying numbers of effector cells with or without dendritic cells in round-bottom microplates. ^{51}Cr release in supernatants was measured after 20 hours of incubation at 37°C as previously described (9).

Therapy of i.c. M05-bearing mice with i.t dendritic cell delivery and i.v. adoptive transfer with Tc1 or Tc2. Preparation of i.c. tumor-bearing mice was done as previously described (20). Briefly, 5×10^3 M05 cells 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 mice using a stereotactic frame. Some animals bearing i.c. tumors also received an i.t. injection with 1×10^5 adenovirally transduced dendritic cells in the same location on day 5 following tumor challenge. On day 6 (and on day 12 in some experiments), mice received i.v. injections with 2×10^6 to 7.5×10^6 Tc1 or Tc2. The animals were monitored daily after treatment for the manifestation of any pathologic signs.

Isolation of brain-infiltrating lymphocytes. Mice were sacrificed by CO_2 asphyxia, then perfused through the left cardiac ventricle with PBS. Brains were enzymatically digested (24, 25) and cells from each brain were resuspended in 70% Percoll (Sigma, Saint Louis, MO), overlaid with 37% and 30% Percoll, then centrifuged for 20 minutes at $500 \times g$. Enriched brain-infiltrating lymphocyte (BIL) populations were recovered at the 70% to 37% Percoll interface.

Flow cytometry. Single-cell suspensions from spleens, CLNs, or BILs were stained with FITC-anti-CD8α (BD PharMingen, San Diego, CA) and phycoerythrin (PE)-labeled ovalbumin-specific tetramer (Beckman Coulter, Inc., Fullerton, CA). Additional analyses were done using PE-Cy5-anti-CD8β (Caltag Laboratories, Burlingame, CA), FITC-anti-H-$2K^b$, FITC-anti-Thy1.2, PE-anti-CD62L, FITC-anti-CD44, PE-anti-CD8α (all of these reagents were from BD PharMingen), rabbit anti-CXCR3 polyclonal antibody (Zymed Laboratories, South San Francisco, CA), and FITC-goat anti-rabbit Ig-specific polyclonal antibody (BD PharMingen).

For intracellular IFN-γ staining, cells were surface-stained with PE-anti-CD8α, washed, fixed, and then permeabilized with Cytofix/Cytoperm buffer (BD PharMingen) before staining with anti-IFN-γ (BD PharMingen). Samples were examined by flow cytometry (Coulter EPICS cytometer, Beckman Coulter).

Cytokine release assay. Tc1 and Tc2 were harvested, washed, and restimulated with antigen-presenting cells loaded with the ovalbumin peptide for 72 hours. Culture supernatants were assessed for mIFN-γ and mIL-4 using specific ELISA kits (BD PharMingen and eBioscience, Inc., San Diego, CA, respectively). IP-10 productions from tumor cells were also examined by a mIP-10 ELISA kit (R&D Systems).

Statistical analysis. Survival data were compared using a log-rank test. Comparative number of BILs and T-cell responses were analyzed by Student's *t* test for two samples with unequal variances.

Results

Phenotypic characterization of ovalbumin-specific Tc1 and Tc2. In addition to their type 1–polarized cytokine production profiles, Tc1 exhibited decreased CD62L and increased CD44 expression levels compared with freshly isolated naïve OT-1-derived $CD8^+$ T cells, suggesting that *in vitro* stimulated Tc1 were activated, early effector memory cells (refs. 26, 27; Supplementary Fig. S1). Tc1 cells also expressed a higher level of CXCR3, a receptor for IP-10 (15, 16), than Tc2, suggesting that Tc1 expressed a favorable phenotype toward potential migration to the inflammatory site in response to IP-10 (Supplementary Fig. S1).

M05 cells produce IP-10 in response to IFNs and dendritic cells. We tested the ability of M05 to produce IP-10 under various culture conditions, including responses to IFN-α or IFN-γ and to dendritic cells. M05 cultured in complete medium produced 2.08 ± 0.020 ng IP-10/1×10^5 cells/24 hours, with this level significantly up-regulated by addition of IFN-γ or IFN-α to cultures (Fig. 1*A*). When M05 cells were cocultured with DC-IFN-α or mock-transduced DC-ψ5, DC-ψ5 up-regulated IP-10, and, likewise, DC-IFN-α induced comparatively even higher levels of IP-10 production from M05 (Fig. 1*B*). We also examined the effect of cell-free, culture-conditioned medium from DC-ψ5 or DC-IFN-α on M05 production of IP-10 (Fig. 1*B*). Importantly, these dendritic cells did not produce high levels of IP-10 in the conditioned medium (0.10-0.15 ng/L $\times 10^5$ cells). As predicted based on our previous data demonstrating that both DC-ψ5 and DC-IFN-α produce IFN-γ and IFN-α *in vitro* (22), conditioned medium from both DC-ψ5 and DC-IFN-α enhanced IP-10 production from M05, with DC-IFN-α promoting higher levels than DC-ψ5, suggesting that DC-derived cytokines, such as IFN-α and IFN-γ, may play major roles for the induction of IP-10 from tumor cells *in vitro*.

DC-IFN-α enhances the cytolytic activity and IFN-γ production of Tc1 *in vitro*. To test our hypothesis that delivery of DC-IFN-α into the tumor microenvironment would enhance the antitumor functions of Tc1, we designed *in vitro* experiments measuring the specific cytotoxic activity of Tc1 against radiolabeled target M05 in the absence or presence of DC-IFN-α (Fig. 1*C*). Tc1 efficiently lysed M05 targets in the absence of dendritic cells. However, addition of DC-IFN-α, but not DC-ψ5, significantly enhanced the lysis of M05, suggesting that transgene-derived IFN-α bolsters CTL activity mediated by Tc1. Control B16 targets were not lysed by Tc1, demonstrating the specificity of the antiovalbumin Tc1.

We next addressed whether DC-IFN-α could also promote Tc1 secretion of IFN-γ in the presence of tumor *in vitro*. As depicted in Fig. 1*D*, M05 remarkably suppressed IFN-γ expression from day 1 Tc1 when compared with Tc1 cultured alone, suggesting the activity of suppressive tumor-derived substances (28). In contrast, DC-IFN-α seemed to enhance IFN-γ production by Tc1, supporting

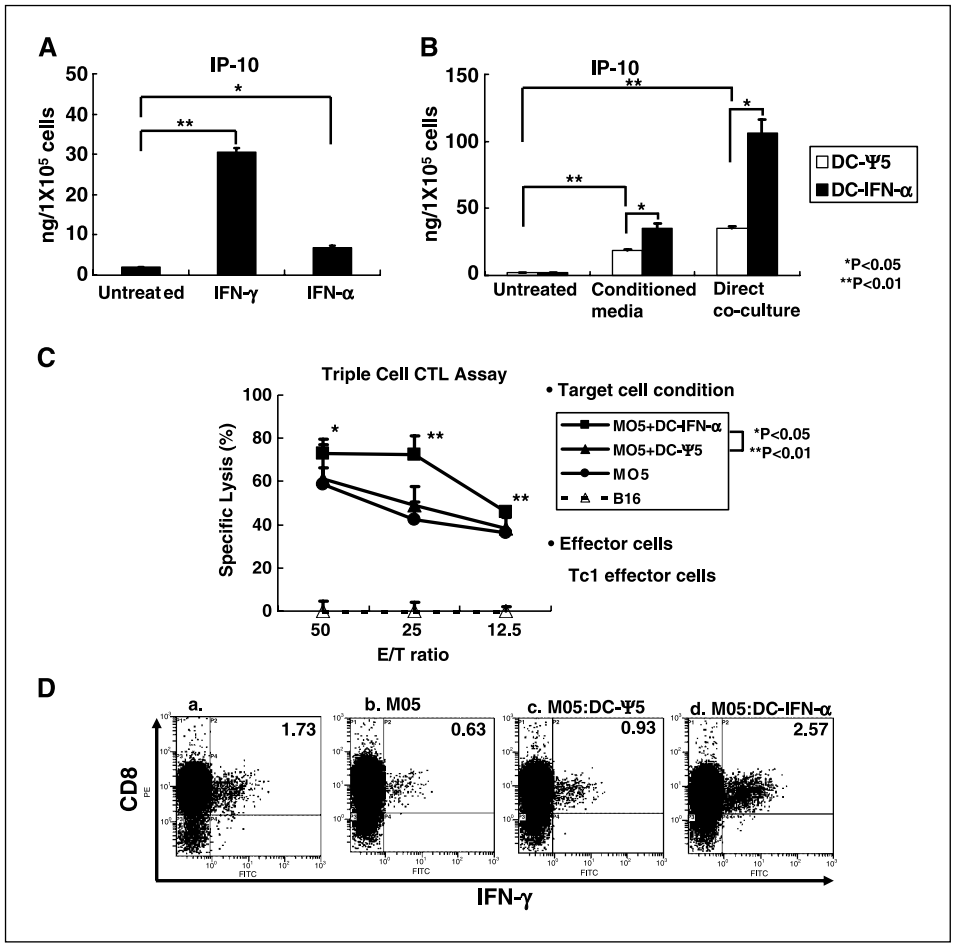


Figure 1. DC-IFN-α induce IP-10 production from M05 cells and enhance the type 1 CTL function of Tc1 *in vitro*. *A,* aliquots of M05 cells were cultured in the presence or the absence of 1,000 units/mL IFN-γ or 1,000 units/mL IFN-α for 24 hours. *B,* aliquots of M05 cells were cultured in the presence or absence of conditioned medium from cultures of DC-ψ5 or DC-IFN-α for 24 hours, or were directly cocultured with DC-ψ5 or DC-IFN-α for 24 hours (the M05/dendritic cell ratio was 1:2). Mouse IP-10 expression from *in vitro* cultured M05 cells was measured by ELISA. *Columns,* mean of culture triplicates; *bars,* SD. Representative of three experiments. *C,* triple-cell CTL assay. ^{51}Cr-labeled M05 or parental B16 cells were cocultured with day 3 Tc1 effector cells in the absence or presence of DC-ψ5 or DC-IFN-α for 20 hours. The ratio of target tumor cell to dendritic cell was 2:1. *Points,* mean specific lyses of culture triplicates; *bars,* SD. Representative of three experiments. *D,* intracellular IFN-γ production by day 1 Tc1 cocultured with M05 tumor cells in the absence or presence of DC-ψ5 or DC-IFN-α. Aliquots of Tc1 cells (1×10^6/mL) were cultured with no tumor cells (*a*), with M05 tumor cells (1×10^6/mL; *b*), with M05 cells and 1×10^5/mL DC-ψ5 (*c*), or 1×10^5/mL DC-IFN-α (*d*) for 24 hours.

type 1–promoting effects of DC-IFN-α with the potential to induce IP-10 production within a tumor-associated microenvironment. In addition, IFN-γ induced expression of H-2K^b on M05 (data not shown), suggesting that type 1 inflammatory microenvironments might enhance the recognition of M05 by Tc1.

I.v. transferred Tc1 cells traffic into i.c. tumor lesions and are therapeutic, with their efficacy enhanced by i.t. delivery of DC-IFN-α. Based on our *in vitro* data indicating that DC-IFN-α promotes IP-10 secretion from M05 and M05-specific lysis by Tc1 (Fig. 1), we evaluated the therapeutic efficacy of a combinational therapy implementing (*a*) i.v. infusion of anti-ovalbumin Tc1 and (*b*) i.t. injection of DC-IFN-α. As shown in Fig. 2*A*, one i.v. infusion of Tc1 on day 6 after i.c. M05 tumor inoculation significantly prolonged the survival of mice when compared with untreated mice ($P < 0.0001$). Moreover, i.t. injection of DC-IFN-α further prolonged the survival of mice receiving a single day 6 i.v. infusion of Tc1 when compared with mice treated with i.t. delivered DC-ψ5 and one i.v. infusion of Tc1 ($P = 0.0018$). In marked contrast, a combinational therapy using day 6 transfer of antiovalbumin Tc2 was far less effective (Fig. 2*A* and *B*; $P = 0.0139$ for combined Tc2 i.v. versus Tc1 i.v.). In addition, in contrast to Tc1 therapy, i.t. DC-IFN-α delivery did not prolong the survival of mice when compared with Tc2 therapy only ($P = 0.1894$ for i.v. Tc2 versus i.t. DC-IFN-α + i.v. Tc2).

These results prompted us to characterize the efficiency of tumor infiltration by the adoptively transferred T cells, because differential recruitment of these effector cells could explain the contrasting therapeutic indices of these therapies. Mice bearing i.c. M05 received i.t. DC-IFN-α and i.v. transfers of 2×10^6 Tc1 or Tc2 cells. On day 3 after the adoptive transfer, BILs were isolated and analyzed by flow cytometry. As Fig. 2*C* shows, a high percentage (32%) of BILs gated for lymphocytes are CD8$^+$ cells recognizing by an H-2K^b/ovalbumin peptide-specific tetramer following Tc1 infusion (Fig. 2*C, left*), whereas very few (5.9%) CD8$^+$/tetramer$^+$ events were identified in BILs following Tc2 infusion (Fig. 2*C, right*), suggesting that Tc1 possess a superior capacity versus Tc2 to traffic into i.c. M05 tumor sites. The total number of ovalbumin-reactive CD8$^+$ BILs from mice receiving i.t. DC-IFN-α and i.v. Tc1 was also remarkably higher than mice receiving i.t. DC-IFN-α and i.v. Tc2 (Fig. 2*D*). These results indicated that i.v. adoptive transfer of Tc1 is an effective treatment for i.c. M05 tumors, particularly when combined with i.t. delivery of DC-IFN-α cells. In contrast, Tc2 failed to infiltrate i.c. M05 tumors. As both Tc1 and Tc2 exhibited similar levels of lytic activity against M05 *in vitro* (data not shown), the striking therapeutic efficacy of Tc1 for treatment of i.c. M05 tumors may thus be largely attributed to the efficient homing of Tc1 into i.c. M05 tumors.

Roles of i.t. injected DC-IFN-α in recruitment of Tc1. We next sought to discern a level of mechanistic insight into how i.t. DC-IFN-α delivery enhances the therapeutic potential of Tc1-based adoptive transfer in mice bearing i.c. M05 tumors to suggest potentially optimized protocols.

First, we examined whether an intensified i.v. cell transfer regimen of Tc1 would further improve therapeutic efficacy. As

shown in Fig. 3*A*, two i.v. Tc1 infusions, given on days 6 and 12, when combined with a single i.t. DC-IFN-α delivery, remarkably enhanced the therapeutic efficacy of this regimen when compared with a control group of mice receiving i.v. Tc1 infusions and i.t. DC-ψ5 ($P = 0.0039$). Seven of 10 mice treated with i.t. DC-IFN-α and two Tc1 infusions survived longer than 80 days, whereas only 1 of 10 mice receiving i.t. DC-ψ5 and two Tc1 i.v. infusions survived long-term (i.e., >80 days). None of untreated, control mice survived longer than 27 days (Fig. 3*A*).

To evaluate whether i.t. DC-IFN-α delivery promoted the tumor-homing abilities of adoptively transferred ovalbumin-reacting Tc1 rather than ovalbumin-specific T cells primed in recipient mice, we characterized BILs obtained from mice receiving i.v. Tc1 and i.t. delivery of DC-IFN-α or DC-ψ5. Although i.t. delivery of DC-ψ5 attracted ovalbumin-reactive $CD8^+$ cells when compared with BILs obtained from mice treated with i.v. Tc1 only (22.66% for i.t. DC-ψ5 plus i.v. Tc1 group versus 15.08% for i.v. Tc1 group, data not shown), i.t. injection of DC-IFN-α remarkably enhanced the homing of ovalbumin-reactive $CD8^+$ T cells when compared with mice receiving i.t. DC-ψ5 and i.v. Tc1 (Fig. 3*B, top*). The use of congeneic mice allowed us to evaluate whether BILs were of donor (Thy1.2) or recipient (Thy1.1) origin (Fig. 3*B, bottom*). By gating for $CD8^+$ populations, the vast majority of ovalbumin-tetramer$^+$ events, in both the DC-ψ5 and DC-IFN-α cotreatment groups, represented $Thy1.2^+$ donor cells. Based on flow cytometric data, we determined absolute numbers of ovalbumin-reactive $CD8^+$ subpopulations for Thy1.2-positive (i.e., donor-derived) cells (Fig. 3*C*). Our results indicated that dendritic cell–mediated i.t. delivery of IFN-α enhances the trafficking of principally the adoptively transferred, ovalbumin-reactive $CD8^+$ T cells into i.c. tumor sites. The extent of Tc1 proliferation in CLN cells and BILs from i.t. DC-IFN-α–treated group was similar to that observed in groups receiving i.t. DC-ψ5 or no i.t. treatment (Supplementary Fig. S2), suggesting that DC-IFN-α may not directly influence the proliferation rate but rather enhance accumulation of adoptively transferred Tc1 cells into brain tumor sites and draining CLNs.

Next, isolated BILs were evaluated for their intracellular IFN-γ expression in response to the ovalbumin-peptide. I.t. DC-IFN-α delivery increased the numbers of IFN-γ-producing $CD8^+$ BILs when compared with i.t. delivery of DC-ψ5 (Fig. 3*D*). These results suggest that i.t. DC-IFN-α delivery promoted a type 1 (i.e., IFN-γ expressing) tumor microenvironment.

Ovalbumin-reacting Tc1 cells traffic preferentially into ovalbumin-positive tumors over ovalbumin-negative tumors in the brain. It was recently shown that adoptively transferred $CD8^+$ T cells traffic indiscriminately and ubiquitously to both antigen-positive and antigen-negative tumors while mediating antigen-specific tumor destruction as the result of specific T-cell triggering within tumor sites (29). We examined whether the homing of ovalbumin-reactive $CD8^+$ T cells into i.c. tumors depends on specific target antigen expression by the tumor cells. Mice bearing i.c. parental B16 (ovalbumin-negative) or ovalbumin-transfected M05 tumors were treated with or without one i.v. Tc1 infusion, and consequent BILs isolated from these mice were analyzed by flow cytometry. Adoptive transfer of Tc1 increased the live lymphocyte population in the B16-BILs, but this increase was less pronounced than that observed in the M05-BILs (Fig. 4*A, top*). Although similar percentages of gated BILs expressed the CD8 and were stained with ovalbumin-specific tetramers in the B16 and M05 lesions following i.v. Tc1

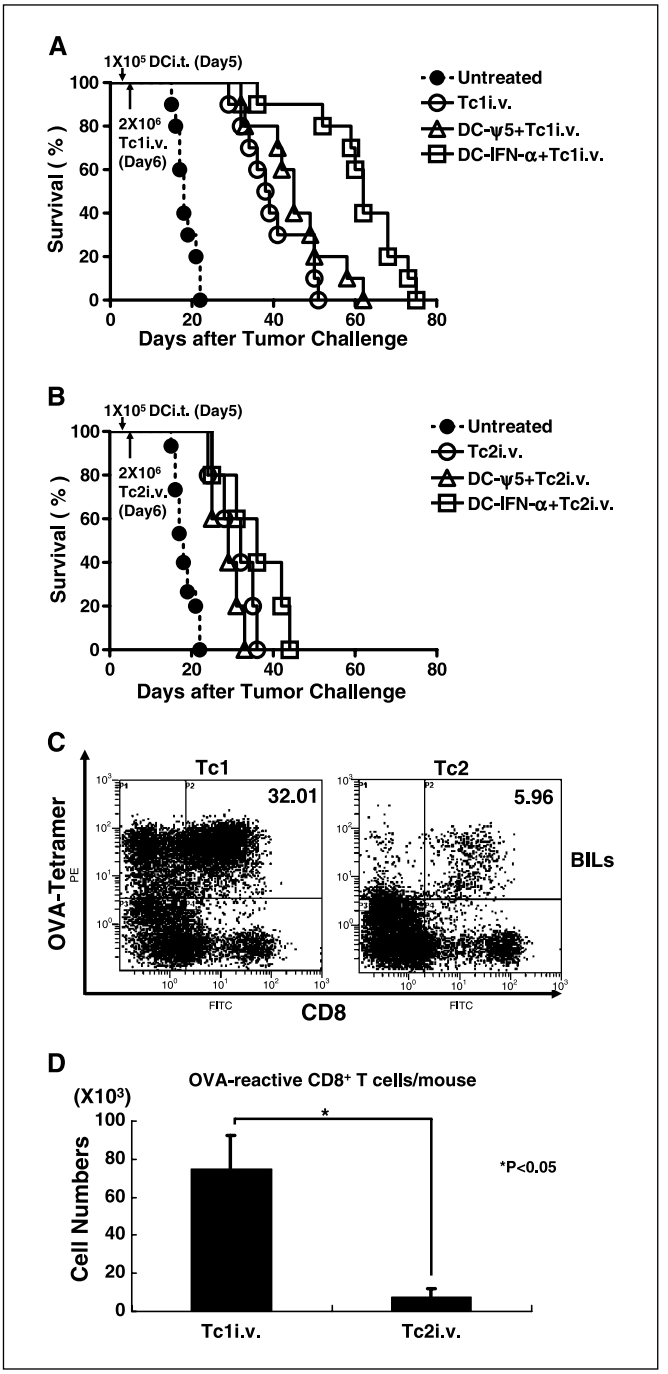
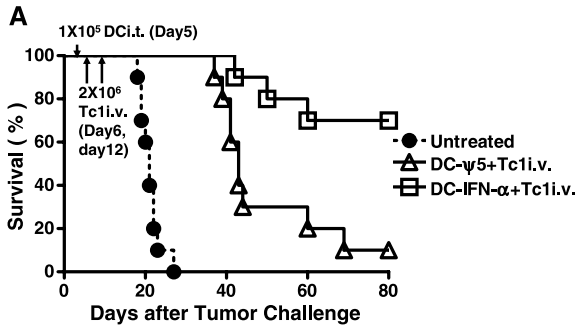
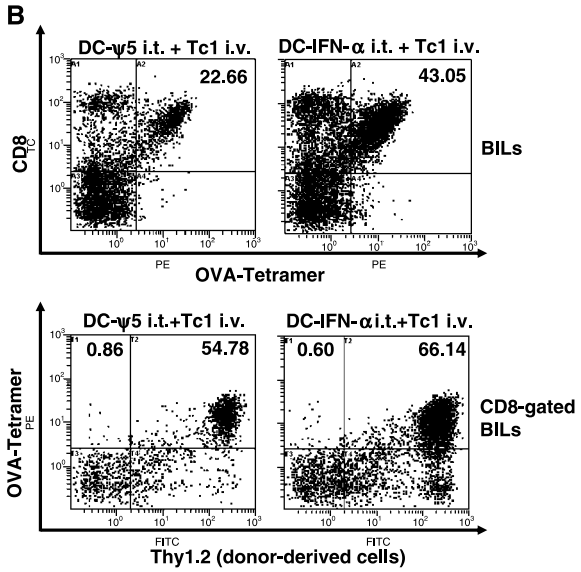
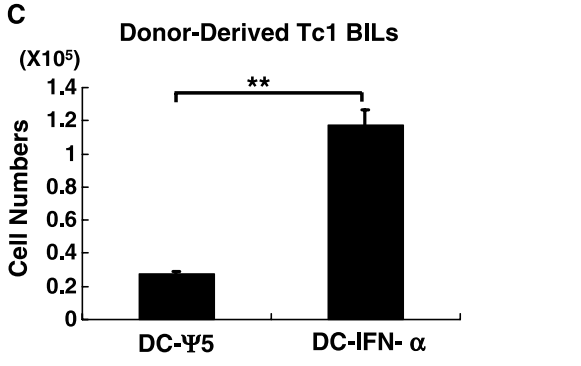
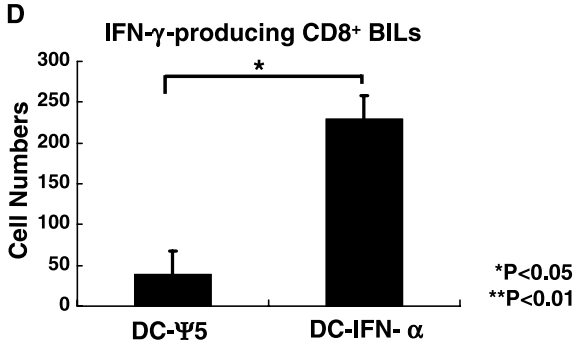


Figure 2. Tc1 cells are superior to Tc2 cells in trafficking to i.c. M05 tumors and mediate antitumor therapeutic responses. *A* and *B,* mice bearing day 5 M05 in the brain received either one of the following combinations: i.t. delivery of DC-IFN-α followed by i.v. adoptive transfer of 2×10^6 Tc1 (*A: n* = 10 per group) or Tc2 (*B: n* = 5 per group) on day 6 (□); i.t. DC-ψ5 followed by i.v. adoptive transfer of 2×10^6 Tc1 (*A*) or Tc2 (*B*; △); i.v. adoptive transfer of 2×10^6 Tc1 (*A*) or Tc2 (*B*; ○); or untreated (●). Significance of differences: *A* (log-rank test), $P < 0.0001$ for untreated versus i.v. Tc1; $P = 0.0018$ for i.t. DC-ψ5+ i.v. Tc1 versus i.t. DC-IFN-α+ i.v. Tc1; and $P < 0.0001$ for i.v. Tc1 versus i.t. DC-IFN-α+ i.v. Tc1; *B* (log-rank test), $P = 0.0003$ for untreated versus i.v. Tc2; $P = 0.1894$ for i.v. Tc2 versus i.t. DC-IFN-α+i.v. Tc2; and $P = 0.0658$ for i.t. DC-ψ5+ i.v. Tc2 versus i.t. DC-IFN-α+ i.v. Tc2. It was also noted that Tc1 therapy was superior to Tc2; $P = 0.0139$ for Tc2 i.v. versus Tc1 i.v. *C,* BILs from mice treated with i.t. DC-IFN-α and i.v. Tc1 (*left*) or Tc2 (*right*) were harvested 3 days after adoptive transfer and stained with FITC-anti-CD8 and PE-H-$2K^b$-$ovalbumin_{257-264}$–specific tetramer. Lymphocyte-gated populations are depicted. *D,* numbers of ovalbumin-reactive $CD8^+$ T cells in BILs per mouse. *Columns,* mean of four mice; bars, SD. Representative of three experiments.









transfer (Fig. 4*A, bottom*), M05 tumors contained significantly higher numbers of total BILs and ovalbumin-reactive $CD8^+$ T cells than B16 tumors (Fig. 4*B* and *C*). These results suggest that trafficking of ovalbumin-reactive $CD8^+$ T cells into the i.c. tumor site was, at least in part, dependent on ovalbumin expression by the tumor cells.

IP-10 plays a critical role in the recruitment of Tc1 into i.c. M05 tumors. Our data presented in this study thus far show that (*a*) M05 tumor cells express IP-10, which can be further induced by IFN-α, IFN-γ, or by cultured dendritic cells; (*b*) Tc1 that express IFN-γ efficiently infiltrate i.c. M05 tumors; and (*c*) i.t. injected DC-IFN-α enhance tumor infiltration by Tc1 and promotes IFN-γ production from BILs. These data prompted us to address the potential role of IP-10 in the observed outcomes of this combinational therapy. To determine the specific role of IP-10, anti-IP-10-neutralizing mAb was administered to i.c. M05 tumor-bearing mice that also received combination therapy. As depicted in Fig. 5*A*, neutralization of IP-10 completely abrogated therapeutic efficacy, whereas mice treated with isotype control IgG continued to benefit from this modality. Moreover, flow cytometric analyses of BILs revealed that administration of anti-IP-10 mAb virtually ablated tumor infiltration by ovalbumin-reactive $CD8^+$ cells, compared with mice treated with isotype control IgG (Fig. 5*B*). Total numbers of BILs and ovalbumin-reactive $CD8^+$ BILs per mouse also decreased remarkably after injection of anti-IP-10 mAb (Fig. 5*C* and *D*). Collectively, these results indicate that IP-10 plays a critical role in the recruitment of ovalbumin-reactive Tc1 into brain tumor sites.

I.v. Tc1 infusion in combination with i.t. DC-IFN-α induces long-term memory responses in recipient mice. It has been shown that passive immunizations with adoptively transferred T cells is capable of yielding long-lasting, antitumor memory responses in other tumor models (19, 30, 31). To examine whether i.c. tumor-bearing mice treated with i.t. injection of DC-IFN-α and i.v. transferred Tc1 exhibit long-term antitumor immunity, survivors in experiments in Fig. 3*A* were rechallenged with 5×10^3 M05 cells i.c. on day 80 after the initial tumor inoculation. As shown in Fig. 6*A*, six of seven rechallenged mice exhibited protection and long-term survival through the end of the observation period (130 days after initial tumor challenge), with no signs of tumor growth upon necropsy (data not shown). Control untreated mice all died within 30 days after receiving i.c. M05 challenge, indicating that protective immunity to a lethal i.c. tumor challenge was established in mice previously treated with the combinational Tc1-based therapy.

Figure 3. Local delivery of DC-IFN-α into the i.c. tumor site enhances the antitumor effectiveness and tumor homing of IFN-γ-producing Tc1 effector cells. *A,* mice bearing day 5 M05 in the brain received either one of the following combinations: i.t. delivery of DC-IFN-α (□) or i.t. DC-ψ5 (△) on day 5 followed by i.v. adoptive transfer of 2×10^6 Tc1 on days 6 and 12 (*A: n* = 10 per group); or untreated (●). Significance of differences (log-rank test): $P < 0.0001$ for untreated versus i.t. DC-ψ5+ i.v. Tc1; $P = 0.0039$ for i.t. DC-ψ5+ i.v. Tc1 versus i.t. DC-IFN-α+ i.v. Tc1. *B-D,* mice bearing day 11 M05 in the brain received i.t. delivery of 1×10^5 DC-IFN-α or DC-ψ5 followed by i.v. adoptive transfer of 2×10^6 Tc1. BILs were harvested 3 days after the adoptive transfer and stained with PE-H-$2K^b$-ovalbumin$_{257\text{-}264}$–specific tetramer, PE-Cy5-CD8, and FITC-anti-Thy1.2, and then analyzed by flow cytometry for (*B, top*) $CD8^+$ and/or ovalbumin-tetramer$^+$ populations in the lymphocyte-gated populations; (*B, bottom*) $Thy1.2^+$ and/or ovalbumin-reactive populations within $CD8^+$-gated populations; (*C*) numbers of donor-derived (Thy1.2) Tc1 cells in BILs per mouse; and (*D*) numbers of IFN-γ-producing $CD8^+$ T cells in BILs per mouse calculated based on intracellular staining of IFN-γ.

Finally, surviving animals on day 130 were sacrificed; then BILs, CLN cells, and splenocytes were harvested for further analyses. Although there were no macroscopically visible tumor nodules in brains derived from these mice, flow cytometric analyses of BILs revealed elevated numbers of ovalbumin-reactive $CD8^+$ T cells when compared with BILs obtained from control untreated M05-bearing mice (Fig. 6*B, left* and *right*). We also detected a small population of donor-derived (Thy1.2), ovalbumin-reactive $CD8^+$ cells in BILs obtained from these long-term survivors (data not shown), suggesting that adoptively transferred Tc1 cells were not depleted even 118 days after i.v. transfer. In the experiments depicted in Fig. 3*A*, there was a single mouse that survived for 80 days following the initial i.t. DC-ψ5 and i.v. Tc1 treatment. Although this mouse survived the rechallenge with i.c. M05 until day 130, there was a small visible tumor in the rechallenged hemisphere of the brain upon necropsy (data not shown). Perhaps due to the presence of this tumor, BILs from this mouse showed a higher percentage of ovalbumin-reactive $CD8^+$ cells than mice treated with i.t. DC-IFN-α and i.v. Tc1 (Fig. 6*B, middle*).

To evaluate the status of long-term, antitumor T-cell memory responses in these mice, we evaluated the cytotoxic ability of splenocytes and draining CLN cells against M05 targets following *in vitro* restimulation with the ovalbumin peptide for 6 days. Both splenocytes (Fig. 6*C*) and CLN cells (Fig. 6*D*) derived from mice treated with i.t. DC-IFN-α and i.v. Tc1 therapy exhibited significantly higher cytotoxic activities against M05 when compared with the identical cell types obtained from control mice at effector-to-target ratios of 50 and 25 for splenocytes and at all effector-to-target ratios evaluated for CLN cells (Fig. 6*C* and *D*). Taken together, the combination of i.v. infusion of Tc1 and i.t. delivery of DC-IFN-α mediates effective anti-i.c. tumor therapeutic responses and provides long-term memory responses.

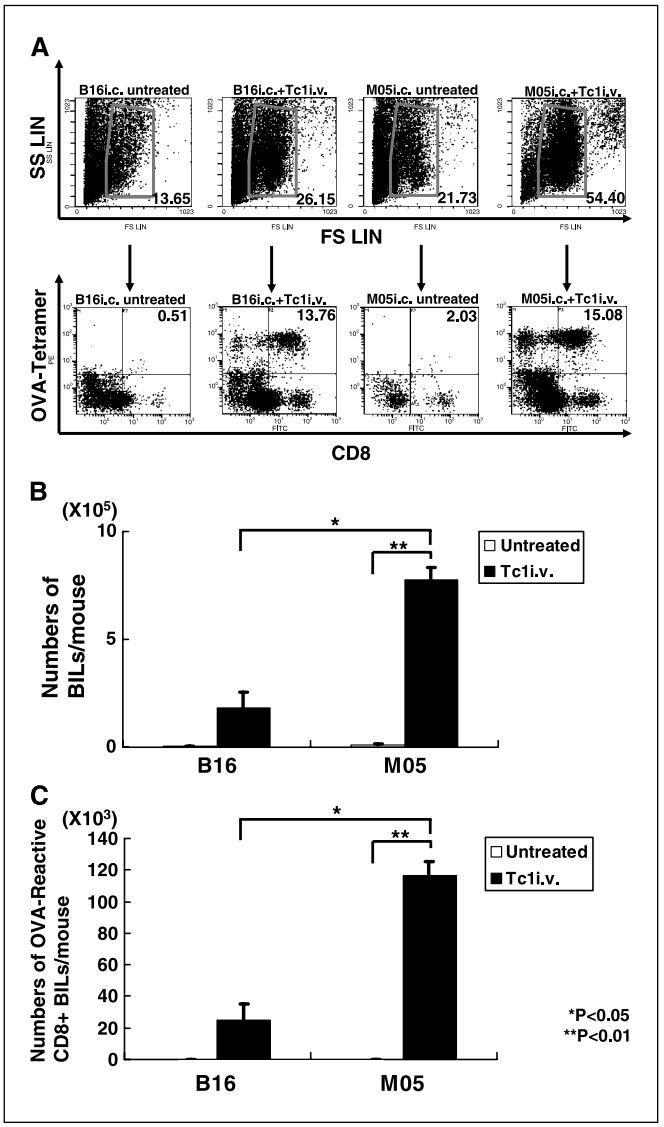


Figure 4. Tumor homing of ovalbumin-reactive $CD8^+$ T cells was dependent on ovalbumin expression on tumor cells. *A,* mice bearing day 12 i.c. B16 or M05 received i.v infusions with 5×10^6 Tc1. Mice were sacrificed 3 days later and BILs were harvested and stained with FITC-anti-CD8 and PE-H-$2K^b$-$ovalbumin_{257\text{-}264}$–specific tetramer. *Top,* percentages of gated populations in the total BILs. *Bottom,* percentages of ovalbumin-reactive $CD8^+$ T cells within the gated populations. *FS LIN,* forward scatter linear scale. *SS LIN,* side scatter linear scale. *B,* numbers of live BILs per mouse. *C,* numbers of ovalbumin-reactive $CD8^+$ T cells were calculated based on flow cytometric data.

Discussion

The most significant finding in the current study is that efficient trafficking of antitumor T effector cells into i.c. tumor lesions may be achieved by adoptive transfer of tumor antigen–specific CTLs with a type 1 phenotype (i.e., Tc1), in concert with the delivery of a stimulus for IP-10 production within the tumor site. This strategy has clinical relevance for the treatment of patients with malignant gliomas, as human glioma cells can produce high levels of IP-10, a CXC chemokine that chemoattracts type 1 T cells and monocytes, following stimulation by type 1 cytokines, such as IFN-α, IFN-γ, and tumor necrosis factor (TNF)-α (32, 33).

In contrast to cells of the Tc2 phenotype, Tc1 clearly supported efficient homing to experimental i.c. M05 tumors. This property of Tc1 may be attributed to their high-level expression IFN-γ (an inducer of IP-10). In addition to IFN-α and IFN-γ, dendritic cell–conditioned medium induced remarkable levels of IP-10 production from M05 cells *in vitro*, suggesting that dendritic cells secrete potent inducers of IP-10. We have shown in our recent study (22) that dendritic cells transduced with Ad-ψ5 or Ad-IFN-α produce high levels of IFN-α, IFN-γ, and IL-12p70. Therefore, we believe that these cytokines play supporting roles in the induction of high levels of IP-10 production from M05 cells in our model system.

IFN-γ also induces the up-regulation of MHC class I (H-$2K^b$) expression on M05 cells, which facilitates Tc1 recognition of target tumor cells (34). Indeed, adoptive transfer of *ex vivo* activated tumor-infiltrating lymphocytes (TIL) in melanoma patients has been shown to up-regulate MHC class I expression within target tumor tissues, presumably via the elaboration of high local concentrations of IFN-γ by adoptively transferred TILs (5). In this regard, in our study, DC-IFN-α promoted IFN-γ production from Tc1, both *in vitro* and *in vivo*, and enhanced the cytolytic activity of Tc1 against M05 targets. IFN-α also up-regulates MHC class I expression on cells in the CNS (34) and promotes the survival of T cells (35), including antitumor CTLs (36). Thus, i.c. injections of DC-IFN-α seems to represent an attractive approach that promotes both type 1 effector responses and antigen presentation within the tumor microenvironment.

Recent studies suggested that tumor expression of indoleamine 2,3-dioxygenase (IDO), which is the major enzyme involved in the catabolism of tryptophan by the kynurenine pathway, may

compose one major mechanism for tumor immune escape (37, 38) by inhibiting the functions of and inducing apoptotic death of antitumor T cells within the tumor microenvironment (39). As IFN-α (40) and IFN-γ (41) are known to induce IDO, we sought to determine whether DC-IFN-α induces IDO expression and increases apoptotic death of Tc1 cells in our model by reverse transcription-PCR analyses of the IDO-encoding gene *Indo* in M05 cells and flow cytometric analyses of Tc1 cells. As shown in our Supplementary Fig. S3, although IFN-γ induced *Indo*-mRNA expression in M05 cells, we did not observe either induction of *Indo* message or increase of Annexin V^+ apoptotic Tc1 cells by addition of DC-IFN-α, suggesting the IDO-mediated immunosuppression was not an issue in our particular model. Nevertheless, the potential for IDO-mediated immunosuppression will have to be monitored in our future clinical translation of similar approaches.

With regard to the roles of dendritic cell as the vehicle for the transgene expression, syngeneic fibroblasts transduced with Ad-IFN-α and elaborating similar amounts of IFN-α did not enhance the Tc1-mediated antitumor effects (data not shown), suggesting that transgene-derived IFN-α promotes antigen-presenting function of dendritic cells rather than merely direct effects on M05 tumor cells. Nevertheless, addition of *IP-10* gene delivery has been shown to improve the efficacy of cancer gene therapy using IL-12 (42), and, thus, it is intriguing to test whether delivery of IP-10 to the CNS tumor site can also enhance the therapeutic effects of Tc1 therapy.

Our current study clearly indicates that IP-10 plays a critical role in the homing of Tc1 into CNS tumors. Although there are at least three CXCR3 ligands, namely IP-10, monokine induced by IFN-γ (Mig), and IFN-inducible T-cell α-chemoattractant (43), it is becoming clear that all three exhibit unique expression patterns *in vivo*; additional experiments using neutralizing antibody and gene-targeted mice support the concept that these three chemokines may mediate nonredundant functions *in vivo*. In the CNS, antibody neutralization of IP-10 has been shown to block the recruitment of autoimmune effector T cells into the CNS in experimental allergic encephalitis (44) and murine viral-induced neurologic disease (11). Interestingly, anti-Mig antibody treatment has little effect on disease severity, suggesting that of these two CXCR3 ligands, IP-10 may play a more dominant role in recruiting type 1 "antigen experienced" T cells into the CNS. Furthermore, IP-10 has been also shown to inhibit angiogenesis (45), which may have played a role in the antitumor effects observed in our current study.

With regard to the optimal activation status and phenotype of adoptively transferred T cells for treatment of cancers, a recent study suggested that central memory cells generated *ex vivo* using rIL-15 are superior to effector memory T cells in mediating recall responses *in vivo* when combined with tumor-antigen vaccination and exogenous IL-2 (46). Also, early effector cells have been

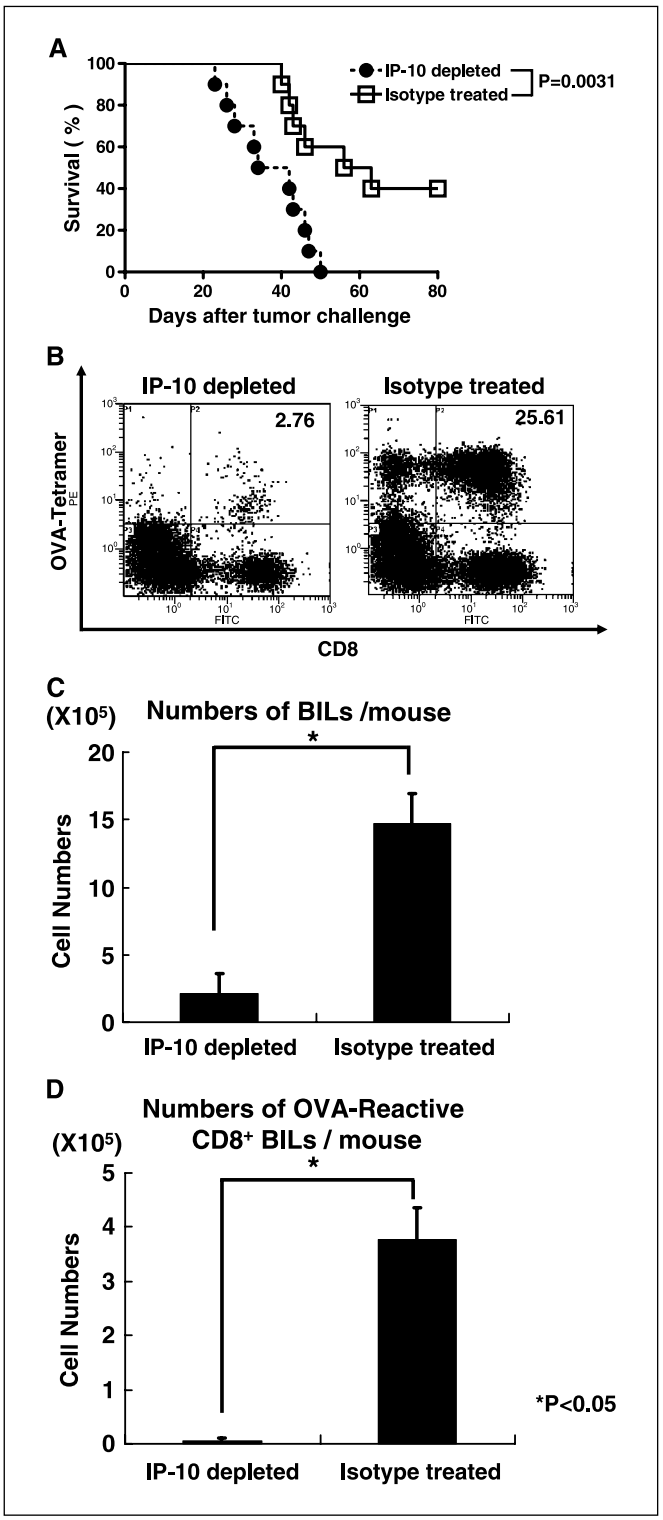


Figure 5. IP-10 played a critical role in the recruitment of Tc1 effector cells to the brain tumor site. *A,* mice bearing i.c. M05 tumors received i.p. injections of antimouse IP-10 (250 μg on day 4 and 100 μg on days 5, 6, 7, and 8) and i.v. adoptive transfer of 3×10^6 Tc1 on day 5 (●). On the same day, mice received i.t. 1×10^5 DC-IFN-α admixed with 50 μg antimouse IP-10 mAb. Control animals received the same amounts of hamster IgG throughout the course (□). Significance of differences between the two groups (log-rank test): $P = 0.0031$. *B,* IP-10-deficient mice bearing day 7 i.c. M05 received i.v. adoptive transfer of 3×10^6 Tc1 cells and anti-mouse IP-10 i.p. (250 μg on day 6 and 100 μg on days 7, 8, and 9). On the same day (day 7), 50 μg anti-mouse IP-10 was i.t. coinjected with 1×10^5 DC-IFN-α. BILs were harvested 3 days after adoptive transfer and stained with FITC-anti-CD8 and PE-H-$2K^b$-ovalbumin$_{257\text{-}264}$–specific tetramer. Lymphocyte-gated populations were analyzed for CD8 and ovalbumin-tetramer reactivity. *C* and *D,* numbers of BILs per mouse (*C*) and ovalbumin-reactive $CD8^+$ BILs per mouse (*D*) in IP-10-depleted mice versus isotype IgG-treated mice. *Columns,* mean of three mice; *bars,* SD. Representative of three experiments.

shown to be more effective than differentiated effector cells (47). The Tc1 used in our study were generated over a short-term *in vitro* culture period (4 days) and were actively proliferating following i.v. transfer, independent of i.t. dendritic cell injections and IFN-α delivery (Supplementary Fig. S2). Further studies are warranted to determine the optimal activation status of adoptively transferred Tc1 for therapy of tumors located in the CNS.

In our i.c. M05 tumor model, expression of the target antigen ovalbumin by tumor cells was required for efficient trafficking of ovalbumin-reactive Tc1 into i.c. tumor sites. Our results are in contrast to a recent study demonstrating that adoptively transferred $CD8^+$ T cells traffic indiscriminately to both antigen-positive and antigen-negative s.c. tumors while mediating antigen-specific tumor destruction as the result of lesion-specific T-cell recognition (29). This may reflect relatively limited entry of nonspecific immune cells into the CNS tumor environment due to unique mechanical structures inherent to the CNS, such as the presence of the blood-brain barrier (48). Our study suggests that delivery of tumor antigen–specific effector T cells may be particularly important for successful immunotherapy of CNS tumors.

Although the persistence of the infused cells has been suggested to be the most critical factor correlating with clinical responsiveness (49), it is interesting that in our current study, the combinational therapy using passive transfer of Tc1 and i.t. delivery of DC-IFN-α resulted in systemic long-term protective responses against M05 tumor cells. CLN cells derived from long-term surviving mice showed particularly potent anti-ovalbumin CTL responses, suggesting that CLNs serve as the primary draining lymph nodes for CNS tumors (25). Data in our current study extend our understanding on the roles of CLNs in the long-lasting anti-CNS tumor immunity, in addition to cross-priming of CNS-tumor antigen-specific T cells (9, 21, 22).

With regard to the mechanisms underlying Tc1-mediated induction of long-term antitumor immune responses, in an M05 lung tumor model, Tc1-derived IFN-γ not only induced elevated levels of lung-derived chemokines including IP-10 *in vivo,* but also increased the local accumulation of a variety of host-derived, activated immune cell populations within the tumor site (18). Moreover, both recipient and donor T cell–derived IFN-γ played a significant role in the long-term response mediated by Tc1 adoptive transfer therapy (18, 19). In our brain tumor models, although low numbers of BILs obtained from treated mice did not allow a detailed analyses of host-derived immune cell populations, our data showing the induction of long-term immunity against CNS tumors warrant further examination of the roles that host-derived type 1 cytokines, such as IFN-γ and TNF-α, might play in the observed antitumor activities (19).

Attempts to translate the recent conceptual and technological advances in the fields of adoptive transfer–based immunotherapy to bedside treatment regimens of human malignant glioma is compelling; however, this task will most certainly be hampered by the challenges of isolating or enriching sufficient numbers of glioma-associated antigen-specific effector T cells from this patient population. A better approach might be to genetically modify T cells so that they expressed a defined specificity for the target tumor cell, a strategy uniquely suited for adoptive transfer therapies (reviewed in refs. 50, 51). The cDNAs encoding *TCRs* have been cloned from tumor-reactive T cell lines and efficiently transferred into peripheral blood lymphocytes using viral delivery methods (52–54). TCR-modified lymphocytes have shown the ability to recognize and kill tumor cells in an antigen-specific

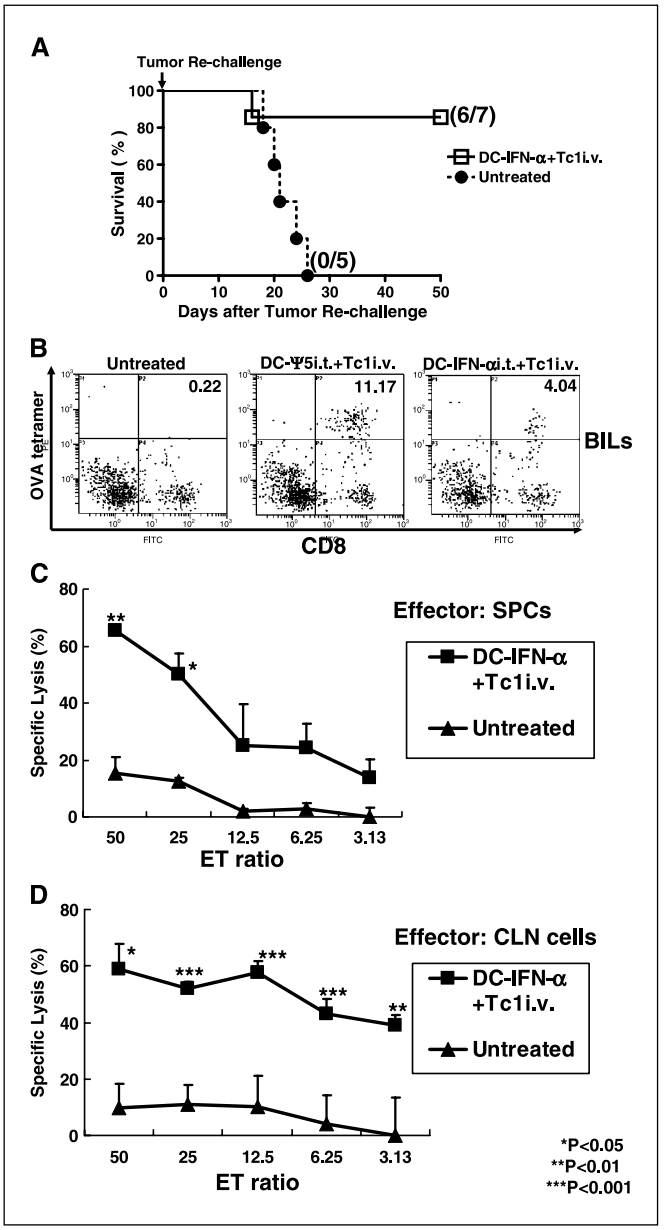


Figure 6. Tc1 therapy in combination with i.t. DC-IFN-α injection induced long-term memory responses in the host mice. *A,* mice that survived for 80 days following i.t. DC-IFN-α and i.v. Tc1 in Fig. 3*A* were rechallenged with 5×10^3 M05 in the contralateral hemisphere of the brain (□). As controls, naïve mice received 5×10^3 M05 in the brain (●). Survival was monitored. *B,* BILs were harvested from mice surviving at 50 days after tumor rechallenge (130 days after initial tumor challenge) and stained with FITC-anti-CD8 and PE-H-$2K^b$-ovalbumin$_{257-264}$–specific tetramer. BILs from control untreated mice bearing day 10 M05 tumor (*left*), one mouse that survived 130 days with i.t. DC-ψ5 and i.v. Tc1 (*middle*), and mice that had received i.t. DC-IFN-α and i.v. Tc1 (*right*) were analyzed for $CD8^+$ and ovalbumin-reactive populations. *C* and *D,* CTL assays. Aliquots of splenocytes or CLN cells (4×10^5/mL) obtained from mice treated with indicated regimens were stimulated with ovalbumin-pulsed antigen-presenting cells (1.2×10^6/mL) for 6 days. Then, ^{51}Cr-labeled M05 cells were cocultured with responder splenocytes (*SPC; C*) or CLN cells (*D*) for 20 hours. *Points,* mean of specific lyses in culture triplicates; *bars,* SD. Representative of three experiments.

manner. Therefore, TCR-modified autologous lymphocytes can be generated in culture and adoptively transferred into patients for whom antitumor T cells of a given specificity are poorly represented within their circulating immune repertoire.

In conclusion, our data supports the utility of combinational therapies incorporating antigen-specific Tc1-adoptive transfer therapy and i.t. delivery of DC-IFN-α as an innovative treatment option for patients with CNS tumors. Importantly, this type of immunotherapeutic approach may also be applied to cancers located in other organs (55).

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