

Intracranial Paracrine Interleukin-2 Therapy Stimulates Prolonged Antitumor Immunity That Extends Outside the Central Nervous System

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Summary: To explore the potential efficacy of local cytokine delivery against tumors in the central nervous system (CNS), C57BL6 mice were simultaneously given intracranial injections of tumor challenge and of irradiated B16F10 melanoma cells transduced to secrete interleukin-2 (IL-2). Intracranial IL-2 therapy generated antitumor responses capable of extending the survival of animals that received simultaneous intracranial tumor challenge either locally or at distant sites in the brain. Nontransduced melanoma cells had little effect. Animals that survived intracranial IL-2 therapy and tumor challenge showed prolonged survival compared with controls when challenged with a second tumor dose 70 days after initial treatment. In addition, animals that rejected intracranial tumors were also protected from tumor growth upon rechallenge at sites outside the CNS (i.e., subcutaneous tumor challenge). Conversely, identical or 10-fold larger doses of IL-2–transduced cells administered by subcutaneous injection failed to generate protection against intracranial tumor challenges. Elimination of T-cell and natural killer (NK) subsets using gene knockout mice and antibody-depletion techniques demonstrated that NK cells were most important for the initial antitumor response, whereas CD4⁺ T-cells were not necessary. These studies demonstrate that local IL-2 therapy in the brain not only generates an immediate local antitumor immune response, but also establishes long-term immunologic memory capable of eliminating subsequent tumor challenges within and outside of the CNS. Furthermore, the antitumor response to paracrine IL-2 in the brain differed significantly from that in the flank, suggesting that the intrinsic CNS cells involved in initiating immunity within the brain have different cytokine requirements from their peripheral counterparts. **Key Words:** Brain tumor—Immunotherapy—Immunologic memory—Interleukin-2—Melanoma.

The presence of distinct antigens or the overexpression of normal antigens on tumor cells is necessary for the development of cancer vaccines. Normal cells, which either do not express these antigens or express them at

much lower levels, would thus be spared by antitumor immune responses (1). Tumor-specific antigens have been described for human cancers such as melanoma and breast cancer (2), encouraging efforts to stimulate immune responses against them. Unfortunately, although nearly a dozen melanoma antigens specifically recognized by T-cells have been identified (3), melanoma cells are still able to avoid immune destruction in most in-

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stances (4). Because the generation of an effective anti-tumor immune response requires both the presence of foreign antigen and a costimulatory molecule or signal, tumor cells displaying tumor antigens may avoid immune detection because of the absence of appropriate costimulation. Thus, antitumor immune responses might be potentiated by more effective local (paracrine) delivery of costimulatory molecules. The identification and cloning of genes encoding cytokines has made this technically feasible. Tumor cells genetically engineered to secrete one of a variety of cytokines (e.g., IL-2, IL-4, IL-7, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), and others reviewed elsewhere (1)) will both display tumor-specific antigens and secrete a costimulatory molecule, and they have been shown to stimulate effective immune responses against tumors outside the central nervous system.

Efforts to elicit similar antitumor immune responses in the brain have been less successful. One potential explanation is the limited access of immune cells to the central nervous system (CNS). However, whereas trafficking of immune cells across the blood-brain barrier is tightly regulated, the brain is not immunologically isolated. Systemic paracrine therapy with GM-CSF, IL-3, and IL-6 (5,6) offers modest protection against tumor challenges in the brain. In addition, activated T-cells are known to enter the CNS to perform antigen surveillance and generate inflammatory reactions (7,8). We (5) and others (9) have shown that local immunotherapy can be used to control initial tumor growth in animal brain tumor models. The delivery of IL-2 directly to the brain bypasses the blood-brain barrier (BBB) and provides a high concentration of a potent immunoregulatory molecule at the site of the tumor, allowing local control over tumor growth (9).

Whereas local control of brain tumors is important for increasing survival, both primary and metastatic brain tumor patients harbor tumor deposits that are separate from the primary lesion. For immunotherapy to have a sustained effect, the antitumor response must extend beyond the local tumor site. In these experiments, we show that the antitumor immune response generated by intracranial paracrine IL-2 therapy using transduced melanoma cells induces immunologic memory capable of protecting animals from subsequent intracranial and extracranial tumor challenges. Furthermore, through experiments in antibody-depleted and gene-knockout mice, we define the lymphocyte subsets that are stimulated in the brain to establish the antitumor immunity. This information regarding the priming of immune responses within the brain may be useful in designing future immunotherapy strategies for brain tumors.

RESEARCH DESIGN AND METHODS

Common Methods and Materials

Tumor Cell Lines and Animals

Six- to 12-week-old C57BL6 female mice were obtained from Harlan (Indianapolis, IN). B16F10 melanoma cells were obtained from the DCT Tumor Repository (National Cancer Institute, Frederick, Maryland) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and penicillin/streptomycin inside humidified incubators supplied with 5% carbon dioxide. B16F10 cells were transduced with the murine IL-2 gene using the replication-defective MFG retroviral vector as previously described (10). The amount of cytokine produced by transformed B16F10 tumor cells was quantified regularly using a standard enzyme-linked immunosorbent assay (ELISA) technique (Endogen, Cambridge, MA). The total amount of IL-2 secreted in vitro by the IL-2-transduced B16F10 melanoma cells (B16F10/IL-2) was 68–92 ng/10⁶ cells/day. Cultured tumor monolayers were harvested with trypsin and resuspended in DMEM before injection. The wild-type tumor challenges given were not irradiated. The transduced tumor cells used to deliver IL-2 (and appropriate nontransduced controls) were rendered replication-incompetent by irradiation with 5000 cGy prior to injection into the brain. Transduced tumor cells injected into the flank received 13,780 cGy (¹³⁷Cs source cell irradiator for brain and flank injections, Gammacell model #62 irradiator, Nordin International, Kanata, Ontario, Canada). Previous studies had shown that IL-2-transduced B16F10 cells exposed at least 5000 cGy are rendered replication-incompetent but maintain their metabolic viability and continue to secrete cytokine in vivo for up to 5 days (11). The higher radiation dose for flank tumors was chosen to ensure that the larger tumor inoculum (10⁶ cells) was replication-incompetent. This dose of radiation does not interfere with the cells' ability to secrete IL-2.

Intracranial Injections

Mice were anesthetized with a 0.1-mL intraperitoneal injection of an anesthetic solution containing ketamine hydrochloride 2.8 mg/mL, xylazine 0.3 mg/mL, and 1.6% ethyl alcohol diluted in 0.9% NaCl solution. For stereotactic intracranial injections of tumor cells, the surgical site was shaved and prepared with 70% ethyl alcohol and iodine solution. After a midline incision, a 1-mm burr hole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were placed in a stereotactic frame and cells were

delivered by a 26 gauge needle to a depth of 3 mm during a period of 3 min. The total volume of injected cells was 5–10 μ L. The needle was removed, the site irrigated with sterile 0.9% NaCl solution, and the skin sutured closed with 4.0 vicryl.

Previous work from our laboratory has demonstrated that stereotactic intracranial injection of 100 wild-type B16F10 melanoma cells results in a large tumor at the injection site that is uniformly fatal, with a median survival time between 16 and 18 days after injection (5). Spread of tumor throughout the CNS is often seen as well. Injection of a larger number of replication-competent wild-type B16F10 cells commonly causes rapid, widespread intracranial disease, analogous to carcinomatous meningitis (5). Because this is a relatively rare clinical entity, the smaller dose of 100 cells was selected to provide a better model of localized intracranial disease.

Toxicity trials demonstrated that a dose of 75,000 irradiated, IL-2-secreting B16F10 cells was the maximally tolerated intracranial dose of immunotherapy in our model (5). Therefore, this dose was used in all experiments investigating intracranial IL-2 therapy.

Intracranial IL-2 Delivery Efficacy Experiments

The fundamental design of the experiments consisted of 2 control groups and 1 experimental group. All three groups received a live intracranial tumor challenge. One control group received an intracranial injection of the tumor challenge and cell culture media. A second control group, the antigen control group, received an intracranial injection of the tumor challenge injected simultaneously via the same needle with 75,000 nontransduced, irradiated B16F10 melanoma cells, designed to confirm that an irradiated tumor cell bolus does not generate an anti-tumor immune response. The experimental IL-2 group received the tumor challenge with a simultaneous coinjection of 75,000 irradiated B16F10/IL-2. Animals that survived 70 days after an initial tumor challenge are referred to as long-term survivors. These animals were then used in subsequent experiments to demonstrate the long-term protective effects that are established after intracranial IL-2 therapy.

Flank Injections for Tumor Challenge and Vaccination

The left flank region just posterior to the forelimb was shaved and prepared with iodine-containing solution. Animals were given a single subcutaneous flank injection of 50,000 wild-type (nonirradiated) B16F10 mel-

noma cells. Dose escalation trials determined that large tumors uniformly developed at the injection site of naïve animals challenged with 50,000 wild-type B16F10 melanoma cells in the flank (data not shown). Mice receiving flank vaccination with IL-2-transductants received subcutaneous injections of either 75,000 or 10^6 irradiated B16F10/IL-2 cells.

Natural Killer Cell Depletion

Antibodies were used to deplete natural killer (NK) cells (anti-NK antibody, PK136, ATCC, Rockville, MD) in vivo using standard techniques. Animals received 0.1-mL intraperitoneal injections of depleting antibodies three times in the first week and once per week thereafter. On the day prior to the scheduled week 3 injection, a randomly selected animal was killed along with a healthy control. Spleens and intestinal lymph nodes were harvested, and the percentage of lymphocyte subset depleted was analyzed using FACScan. If the percentage of depletion was >99%, then the tumor challenge was given on the following day. If the depletion was <99%, then the antibody depletion was continued until 99% depletion was reached.

CD4 and CD8 Knockout Animals

C57BL6 Mice with selective deletion of either the CD4 or CD8 gene locus were kindly provided by Dr. Tak Mak (Amgen Institute, Toronto, Canada). These animals were housed and bred separately from healthy animals. At the completion of the experiments, sample groups of both CD4 and CD8 knockouts (subsequently referred to as: CD4^{-/-}, CD8^{-/-}) were killed and the absence of the appropriate lymphocyte subset confirmed by FACScan.

Animal Monitoring

Animals were monitored for signs of clinical neurotoxicity such as hemiparesis or lethargy. In previous experiments in our laboratory, moribund or paralyzed animals died uniformly within 12–24 h; therefore, in these experiments, animals in this condition were humanely killed. Animals were routinely autopsied to determine the cause of death.

Statistical Analysis

Survival was the primary endpoint in all experiments with intracranial tumor challenges. In experiments with flank tumor challenges, the presence of a discrete subcutaneous tumor nodule was the endpoint. The distribu-

tion of the intervals until death (or development of a flank mass) were estimated using the method of Kaplan and Meier (12). Comparison of survival between groups was made using Kaplan-Meier, log-rank, and Wilcoxon analysis (12). Calculations were made using the JMP 3.1 statistical program (SAS Institute, Cary, North Carolina).

Experimental Design

Efficacy of Intracranial Vaccination With B16F10/IL-2 Against a Simultaneous Ipsilateral or Contralateral Intracranial Tumor Challenge With B16F10 Melanoma Cells

To determine whether a protective effect is seen when irradiated B16F10/IL-2 and the wild-type tumor challenge were coinjected and whether the tumor challenge and B16F10/IL-2 therapy can be separated spatially, five groups of mice (n = 11–12 per group) were tested. A standard control group received tumor challenge in the left cerebral hemisphere. An ipsilateral experimental group received irradiated B16F10/IL-2 and wild-type tumor challenge as a coinjection in the left hemisphere. The contralateral experimental group received wild-type tumor challenge in the left cerebral hemisphere and irradiated B16F10/IL-2 in the right hemisphere simultaneously. Antigen controls both ipsilateral and contralateral to the tumor challenge were performed. Survival was assessed.

Efficacy of Systemic Vaccination With B16F10/IL-2 Against Intracranial Tumor Challenge With B16F10 Melanoma

To test whether subcutaneous vaccination with IL-2–transductants could protect against intracranial tumor challenge, mice received either 75,000 or 10⁶ nonreplicating B16F10/IL-2–transductants in the flank and simultaneous intracranial tumor challenge (n = 8–10 per group). Antigen control groups were treated with identical doses of irradiated nontransduced tumor cell boluses in the flank. Survival was assessed.

Efficacy of Intracranial B16F10/IL-2 Therapy in Establishing Immunologic Memory Capable of Protecting Against Subsequent Intracranial Tumor Challenges

To determine whether the protective effect of paracrine B16F10/IL-2 therapy in the brain was transient or long-term, the following set of experiments was performed. Long-term survivors were generated from the

above-described intracranial B16F10/IL-2 efficacy model. These long-term survivors (n = 22) were then rechallenged with an additional tumor dose 70 days after the original injection of B16F10/IL-2–transductants and the initial tumor challenge; no further therapy with IL-2–transductants was given. At the time of the second tumor challenge, a group of previously untreated mice were also injected with an identical tumor challenge and served as a control group (n = 39). This paradigm was performed in 3 distinct experiments with survival as the primary endpoint. Each experiment was analyzed separately and then the three experiments were combined to generate a single composite Kaplan-Meier survival curve.

In the first experiment, a separate group of long-term survivors (n = 5) was rechallenged with cell culture media rather than a second dose of wild-type tumor. The purpose of this was to confirm that long-term survivors dying of tumor after the rechallenge were not suffering late recurrence of the initial tumor challenge that had been injected 70 days previously.

Efficacy of Intracranial B16F10/IL-2 Therapy in Establishing Immunologic Memory Capable of Protecting Against Subsequent Systemic Tumor Challenges

To determine whether the long-term protection developed after intracranial therapy with B16F10/IL-2–transductants was localized to the CNS or extended outside the brain, a group of long-term survivors was generated as previously described. These long-term survivors (n = 9), along with a group of previously untreated animals (controls, n = 8), were challenged with 50,000 wild-type tumor cells in the flank. The long-term survivors were rechallenged 70 days after their intracranial B16F10/IL-2 therapy. The endpoint of this experiment was the development of a clear flank tumor.

Determination of the Lymphocyte Subsets Important for the Antitumor Immune Response Generated by B16F10/IL-2–Transductants

To determine which lymphocyte subsets were important for the initial antitumor immune response, intracranial injections of a) wild-type tumor only, b) wild-type tumor plus nontransfected irradiated tumor cells (antigen controls), and c) wild-type tumor plus irradiated B16F10/IL-2 were performed in healthy mice, NK-depleted mice (NK⁻), CD8 knockout mice, and CD4 knockout mice. Because of the limited number of animals, a distinct antigen control group in the CD4 knockouts was not

performed. Survival analysis of these 11 groups of mice (3 normal, 3 NK⁻, 3 CD8^{-/-}, and 2 CD4^{-/-} n = 9–15 per group) was performed.

Histologic Analysis of B16F10/IL-2 Injection Site

Three mice were killed on days 7 and 14 after intracranial injection of wild-type B16F10 melanoma (standard tumor challenge) and irradiated B16F10/IL-2 cells. Brains were removed immediately and fixed in 4% buffered paraformaldehyde. After adequate fixation, brains were serially sectioned in the coronal plane, and submitted in their entirety for routine histologic sectioning and staining with hematoxylin and eosin (H&E). Slides were examined for the location, size, and viability of tumor at the injection site, as well as for the presence and composition of any inflammatory infiltrate at the injection site or other CNS location. Reactive changes were assessed in the white matter, cortical gray matter, and hippocampus.

RESULTS

Intracranial Vaccination With B16F10/IL-2 Protects Against a Simultaneous Ipsilateral or Contralateral Intracranial Tumor Challenge With B16F10 Melanoma Cells

We have previously shown that intracranial B16F10/IL-2 can provide protection from a simultaneous tumor challenge given at the same location (5). To determine whether the protective effect of B16F10/IL-2 extended to tumor challenges at a different site, mice were treated with injections of B16F10/IL-2-transductants in one cerebral hemisphere with simultaneous tumor challenge at the same site or in the opposite cerebral hemisphere (Figure 1).

All 12 animals in the untreated control group died from the tumor challenge within 22 days (median survival 20). As expected, survival was prolonged in animals receiving simultaneous B16F10/IL-2 therapy at the same site as the tumor challenge (median survival 61 days; 5 of 11 (45%) animals surviving 70 days (termed long-term survivors); $p < 0.0001$ vs. untreated controls). Interestingly, a simultaneous intracranial injection of B16F10/IL-2 in the right cerebral hemisphere protected animals from a contralateral B16F10 wild-type tumor challenge in the left cerebral hemisphere (4 of 12 mice (33%) were long-term survivors, median survival 31 days, $p < 0.0001$ vs. untreated controls). Median survival was not significantly different between the group of animals receiving B16F10/IL-2 ipsilateral and those receiving

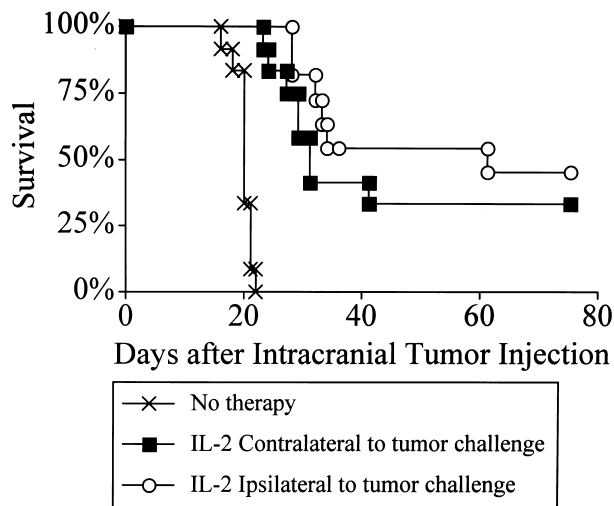


Figure 1. Mice receive an intracranial B16F10 melanoma tumor challenge and simultaneous therapy with irradiated IL-2-transduced B16F10 melanoma cells. A prolongation in survival is seen both in mice receiving the IL-2-transductants and tumor challenge in the same cerebral hemisphere (○, median survival 61 days, $p < 0.0001$ versus controls) and in mice in which the IL-2-producing cells and tumor challenge have been given in opposite sides of the brain (■, median survival 31 days, $p < 0.0001$ versus control). Untreated control animals (×) had a median survival of 20 days. 33% of the animals with IL-2 therapy contralateral to the tumor challenge and 45% of those with IL-2 therapy ipsilateral to the tumor challenge were long-term survivors (>70 days). The ipsilateral and contralateral IL-2 groups were not significantly different. Appropriate controls for tumor antigenicity were performed.

ing it contralateral to the tumor challenge. The two antigen control groups had median survivals of 26 days (contralateral to tumor challenge) and 22 days (ipsilateral to tumor challenge) with no long-term survivors.

Efficacy of Systemic Vaccination With B16F10/IL-2 Against Intracranial Tumor Challenge With B16F10 Melanoma

Mice injected subcutaneously with either 75,000 or 10^6 irradiated B16F10/IL-2-transductants were unable to reject a simultaneous tumor challenge in the brain (Figure 2). Survival of the B16F10/IL-2 treated animals did not differ significantly from that of animals treated with nontransduced irradiated B16F10 cells or from untreated controls.

Intracranial B16F10/IL-2 Therapy Establishes Immunologic Memory Capable of Protecting Against Subsequent Intracranial Tumor Challenges

A group of long-term survivors was generated in the B16F10/IL-2 model. These mice were then rechallenged

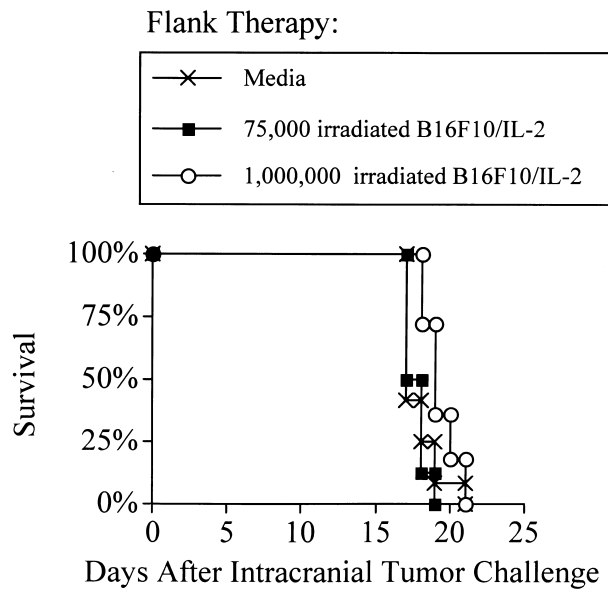


Figure 2. Paracrine IL-2 therapy in the flank with either the same dose used intracranially (75,000 transductants, ■) or with a dose 13 times greater (1,000,000 transductants, ○) failed to provide any protection from simultaneous intracranial tumor challenge. Control animals vaccinated only with media (x) had similar survival after intracranial tumor challenge to mice receiving subcutaneous IL-2 therapy.

intracranially with a second dose of wild-type tumor cells 70 days after the intracranial B16F10/IL-2 therapy. This complete, two-step experiment was performed on 3 separate occasions. A composite Kaplan-Meier curve is presented (Figure 3). Previously untreated control mice had a median survival of 17.5 days (n = 39, 1/39 mice

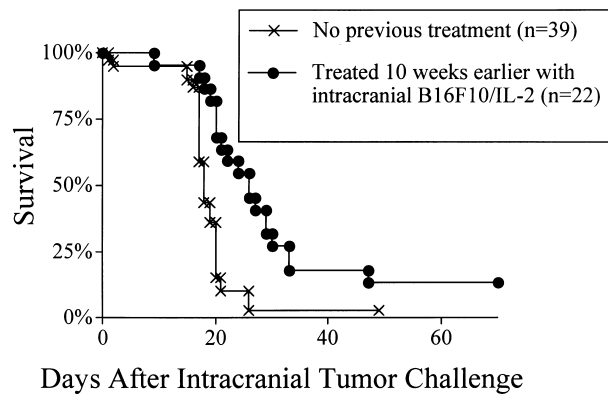


Figure 3. Long-term survivors of intracranial IL-2 therapy and tumor challenge were rechallenged in three separate experiments. A combined Kaplan-Meier curve for all three experiments is presented. Mice surviving 70 days after intracranial IL-2-transductant therapy and tumor challenge who then receive a second tumor challenge survive significantly longer (●, n = 22, median survival = 25 days, interquartile range 19–31 days, 13% long-term survivorship) than previously untreated control mice receiving an identical intracranial tumor challenge (x, n = 39, median survival = 17.5 days, interquartile range 16–19 days, p = 0.0001).

(2.5%) survive 70 days after tumor challenge, interquartile range 16–19 days). In contrast, the animals who had previously received intracranial B16F10/IL-2 therapy and survived 70 days had a significantly prolonged median survival of 25 days after the second tumor challenge, with 3 of 22 animals (14%) surviving an additional 70 days after their second tumor challenge (n = 22, interquartile range 19–31 days, p < 0.001). In one of the three experiments, a separate group of the long-term survivors were rechallenged on day 70 with blank media rather than wild-type tumor to control for late regrowth of the original tumor challenge. All of these animals survived an additional 70 days.

Intracranial B16F10/IL-2 Therapy Establishes Immunologic Memory Capable of Protecting Against Subsequent Systemic Tumor Challenges

Long-term survivors of intracranial B16F10/IL-2 (n = 9) underwent a flank challenge with B16F10 wild-type cells. All controls (n = 8) developed large, palpable flank tumors by day 16 (Figure 4). In contrast, 75% of the long-term survivors of intracranial IL-2-transductants therapy were able to reject the flank tumor challenge and were tumor-free on day 60 (p < 0.0001).

Initial Antitumor Response Generated by Intracranial IL-2 Therapy Involves Natural Killer Cells But Not CD4⁺ T-cells

Natural killer cells were essential for the initial antitumor response after B16F10/IL-2 therapy (Figure 5). NK-depleted mice that received B16F10/IL-2 therapy had a mild prolongation in survival (median survival =

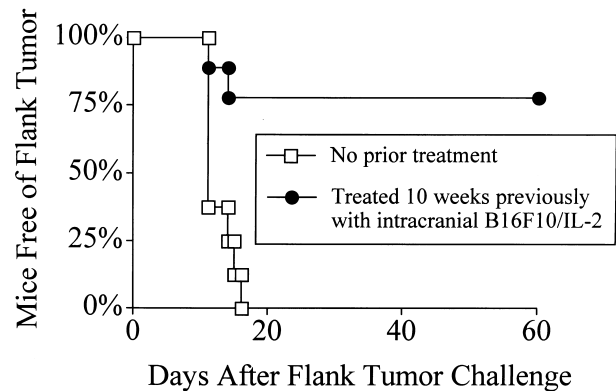


Figure 4. Animals who are long-term survivors of intracranial IL-2-transductant therapy and simultaneous intracranial tumor challenge (●) can resist a second tumor challenge given in the flank 10 weeks later (75% tumor-free at day 70); previously untreated control animals (□) uniformly grow flank tumors in less than 20 days (p < 0.0005).

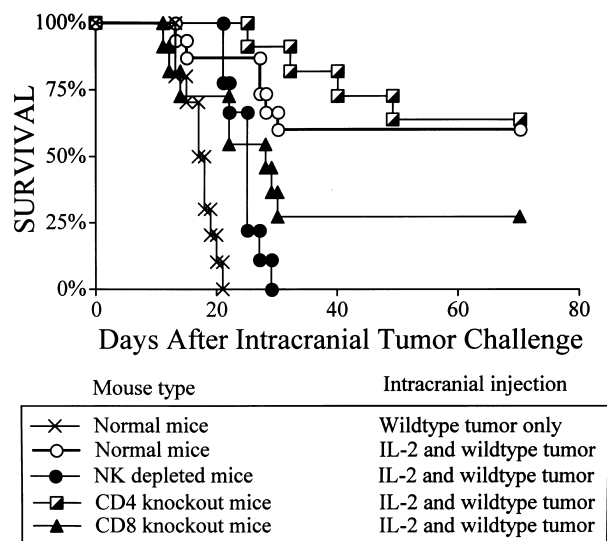


Figure 5. Normal mice (○, median survival not reached at 70 days, $p < 0.001$ versus control) and CD4 knockout mice (□, median survival not reached at 70 days, $p < 0.001$ vs. control) can resist intracranial tumor challenge when simultaneous IL-2-transductant therapy is performed. CD8 knockout mice have a significantly prolonged survival as well (▲, median survival 27 days, $p = 0.005$, 27% long-term survival vs. control). The CD8 knockout mice trended toward a shorter survival in comparison with the normal mice (○) who also received IL-2-therapy ($p = 0.0774$). NK-depleted animals (●, median survival 25 days, $p < 0.001$, 0% long-term survival) have a small response, but all animals succumb to tumor challenge as do all untreated controls (x, median survival 17.5 days). Normal mice and CD4 knockout mice receiving IL-2 therapy survived significantly longer than NK-depleted animals ($p < 0.01$).

25 days; $n = 9$, $p < 0.001$ vs. control). However, intracranial tumors eventually developed in all NK-depleted mice, and the mice died soon after the immunologically competent control animals (median survival = 17.5 days; $n = 10$).

CD8^{-/-} mice that were treated with intracranial B16F10/IL-2-transductant therapy (median survival = 27 days, 27% long-term survivors; $n = 11$) survived significantly longer than immunologically competent control animals ($p = 0.005$). Their response to intracranial IL-2 was mildly decreased in comparison to immunocompetent animals (60% alive at 70 days; $n = 15$) receiving identical intracranial IL-2 therapy, but this did not reach statistical significance ($p = 0.0774$).

CD4 cells were not necessary for the initial antitumor response. CD4 knockout animals (63% alive at 70 days; $n = 11$) had survival nearly identical to immunologically competent animals (60% alive at 70 days; $n = 15$) after intracranial IL-2 therapy. Both competent mice ($p < 0.001$) and CD4 knockout mice ($p < 0.001$) receiving IL-2 therapy had significantly prolonged survival compared to the immunocompetent controls receiving only

media and tumor challenge and compared to NK-depleted mice receiving intracranial IL-2 therapy.

Histologic Analysis of B16F10/IL-2 Injection Site

Microscopic examination of H&E stained whole-brain sections of mice killed at day 7 after injection of IL-2-transductants and tumor challenge was performed (Figure 6). Scattered aggregates of melanoma cells (measuring up to 2 mm in greatest dimension) at injection sites centered in the region of the deep caudate/putamen and septal nuclei were seen. The majority of tumor cells were degenerating, dyscohesive, and associated with an inflammatory infiltrate composed of lymphocytes and macrophages. Occasional neutrophils were present. The brain surrounding the injection site showed reactive and degenerative changes, with scattered lymphocytes. A mild leptomeningeal chronic inflammatory infiltrate was present. Distant brain structures including hippocampus, cortical gray matter, and white matter showed no significant reactive changes.

At day 14, tumor sizes were larger, measuring up to 4 mm in greatest dimension, and centered in the region of the deep caudate/putamen and septal nuclei. One of the tumors extended into the lateral ventricle. Tumors contained solid masses of viable melanoma as well as smaller regions of peripheral tumor degeneration. Degenerating peripheral tumor was distinct from the foci of central tumor necrosis, and was associated with a sur-

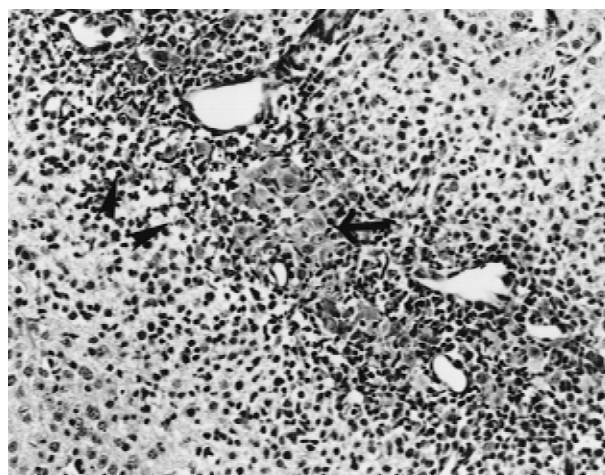


Figure 6. The brains of mice who received intracranial coinjection of B16F10 wild-type tumor and nonreplicating B16F10 IL-2-secreting transductants were examined 14 days after the injection. Microscopic examination of H&E-stained sections of involved brain revealed that residual viable neoplastic cells (arrow) were often surrounded by a significant lymphocytic infiltrate (arrowheads). In the other regions, degenerating tumor cells could be seen with surrounding macrophages and lymphocytes.

rounding lymphocytic infiltrate as well as occasional macrophages. Rare neutrophils were seen. Brain surrounding the tumor contained scattered lymphocytes in regions adjacent to tumor degeneration. Only rare leptomeningeal lymphocytes were noted at day 14. No evidence of distant CNS toxicity was seen.

DISCUSSION

These studies demonstrate that intracranial B16F10/IL-2 therapy delivered by replication-deficient, IL-2-transduced tumor cells can elicit an antitumor response that protects animals from simultaneous tumor challenges either at the same site or at distant sites within the brain. Furthermore, intracranial paracrine IL-2 therapy established immunologic memory that protected animals from tumor rechallenge in both the brain and outside the CNS. We also showed that natural killer cells are most important for the initial antitumor response, whereas CD4⁺ T-cells are not required initially. Paracrine IL-2 therapy in the flank with identical or higher doses of transduced cells does not protect animals from intracranial challenge. This suggests that the CNS is more sensitive to IL-2 stimulation. Intrinsic CNS cells may play a key role and may have different cytokine requirements than their peripheral counterparts.

Natural killer cells and perhaps CD8⁺ T-cells are involved in the antitumor immune response generated after intracranial paracrine IL-2 therapy in this model using poorly antigenic B16F10 melanoma. The initial antitumor response requires NK cells, as no NK-depleted (NK⁻) animal survived more than 27 days after simultaneous tumor challenge. The NK⁻ animals, however, did mount a small response compared with controls. This suggests that whereas NK cells are very important for the initial response, they are not the only cells involved. In systemic models of paracrine IL-2 therapy, NK cells have also been important for the immune response (13).

The role of CD8⁺ cytotoxic T-lymphocytes in this model is less clear. Animals lacking CD8 cells still mounted a significant initial antitumor immune response. However, CD8^{-/-} mice had a nonsignificant trend ($p = 0.0774$) toward diminished antitumor response in comparison with immunologically competent animals who received IL-2 therapy. CD8⁺ cells play an important role in antitumor responses seen after paracrine IL-2 therapy outside the CNS (flank models) (5). It appears from this work, however, that the response to IL-2 in the CNS is distinct from that in the flank, so data obtained outside the CNS may not be generalizable to the intracranial response. In addition, B16F10 melanoma expresses only low levels of major histocompatibility complex (MHC) I.

This could limit the role for CD8⁺ cells, particularly if cytokines that normally upregulate MHC I are not expressed at high levels within the CNS. In other models of IL-2 delivery in our laboratory using polymeric IL-2 delivery, CD8⁺ cells have not been necessary for a strong antitumor response (Hanes et al, personal communication). Thus, further studies will determine the degree to which CD8⁺ cells participate in the intracranial antitumor immune response seen after intracranial IL-2.

CD4⁺ T helper cells are not required for the initial intracranial antitumor response; this is also true in models using paracrine B16F10/IL-2 therapy outside the CNS (14). Exogenous paracrine B16F10/IL-2 delivery may bypass the role usually filled by CD4 cells, secreting cytokines such as IL-2, which act upon cytotoxic T-cells and macrophages (14). An exogenous source for the appropriate costimulatory molecules, such as IL-2, makes CD4 cells unnecessary initially. Although the CD4 cells are not required for the initial response, they may still be primed during the initial immune reaction. Immunohistologic studies demonstrate that CD4⁺ cells are present in the lymphocytes that infiltrate the tumor bed (6). CD4⁺ cells have been shown to be important for the generation of memory immune responses in other tumor vaccine models. Preliminary data in our model suggests that CD4⁺ cells may be required to mount the memory response, even though they are not required for the initial response.

These studies and others demonstrate that systemic (flank) vaccination with IL-2-secreting B16F10 cells does not protect animals from an intracranial tumor challenge with wild-type B16F10 cells, regardless of the timing of the vaccination relative to that of the CNS tumor challenge. No protection from an intracranial tumor challenge was afforded by a simultaneous subcutaneous vaccination (Figure 2), by one given 7 days before tumor challenge (6), or by one given 14 days before tumor challenge (5). In models of antitumor immunity outside the CNS, a 2-week delay affords maximal antitumor immunity (14). Additionally, flank vaccination doses 10-fold greater than effective intracranial doses were ineffective against intracranial tumor challenge. Interestingly, the B16F10/IL-2-transductants and tumor challenge could be separated in the brain, but if the transductants were moved outside the CNS, the protective effect was lost. Thus it appears that for tumor within the CNS, the brain is the preferred site for paracrine IL-2 delivery.

There are several potential explanations why intracranial IL-2 therapy generates an antitumor response whereas flank therapy with equivalent doses is ineffective. Intrinsic CNS cells may have cytokine requirements distinct from their peripheral counterparts, and thus gen-

erate a more potent response to IL-2. We have described an analogous discordance in response to paracrine cytokine therapy between the brain and the flank using GM-CSF. Granulocyte macrophage colony-stimulating factor–transductants produce an antitumor response when given in the flank but not in the brain (5), also suggesting that a different population of cells is involved at the two sites. Because the effector cells involved (NK and possibly CD8⁺ lymphocytes) are similar in both flank and brain responses to IL-2, the differences in response to IL-2 may be caused by the cells involved in priming the immune response (13). A key cell in priming antitumor immunity outside the CNS is the dendritic cell. Therapy in the flank with a dendritic cell stimulant, GM-CSF, promotes a strong antitumor response (10), whereas GM-CSF therapy in the brain is ineffective (5). This suggests that circulating antigen-presenting cells such as dendritic cells cannot enter the CNS easily and that an intrinsic CNS cell may be involved.

Microglia—both perivascular (bone marrow-derived) and parenchymal (intrinsic to the CNS)—may perform antigen presentation in the brain. They are known to prime mixed lymphocyte reactions (15–17). Furthermore, local production of IL-2 and gamma-interferon in the brain has been shown to cause activation of microglia and an influx of CD4- and CD8-positive lymphocytes (18). In this model, IL-2 may stimulate intrinsic CNS cells to present antigen and prime lymphocytes. This would explain why the IL-2–transductants and wild-type tumor challenge could be given at separate sites in the brain, but the antitumor immune response was lost if the IL-2–transductants were delivered outside the CNS.

An alternate explanation for the differing effects of systemic and intracranial vaccination with IL-2 is that vaccination in the CNS may activate a larger percentage of lymphocytes with specific homing receptors to the CNS, facilitating the entry into the brain of cells primed against the tumor antigens. Homing of activated lymphocytes into various tissues has been demonstrated (19–22). The site of antigen encounter and subsequent activation of the lymphocyte determines its homing potential (23). Specific homing receptors, for instance, have been identified on the surfaces of lymphocytes that guide them to the gut mucosa (24–26), peripheral lymph nodes (27–30), or skin (31,32). Recently a novel cell adhesion molecule, telencephalin, that is expressed only in the CNS has been shown to bind to peripheral T-cells and other lymphocytes (33). It is possible that when IL-2 therapy is delivered in the CNS, a larger percentage of the total pool of activated, circulating immune cells may have a homing receptor on their surface that facilitates reentry into the CNS.

The decision to use a model of melanoma in the brain rather than a primary brain tumor such as a glioma was made for several reasons. First, melanoma, like primary human brain tumors, is poorly immunogenic. This contrasts with experimental glioma lines such as the rat 9L glioma that can be immunogenic. Second, clinically significant metastatic brain tumors are far more common than primary brain tumors. Preclinical strategies for metastatic brain tumors will have a larger patient population for clinical trials than strategies for malignant gliomas. In addition, of all malignant tumors, melanoma has the highest propensity to metastasize to the brain. In autopsy studies, 70–90% of patients with melanoma have tumors in their brain. Third, metastatic brain tumors are multifocal (more than one lesion) in as many as 50% of cases. When multiple lesions are present, local treatment strategies such as surgery cannot always be employed. In these cases, systemic therapies including immunotherapy take on heightened importance.

Of melanoma models, the murine B16F10 melanoma is most appropriate for these studies. First, B16F10 is a poorly immunogenic tumor, making it a rigorous model for testing immune-based antitumor strategies. Secondly, B16F10 expresses low levels of MHC class I but not MHC class II, and its MHC I expression can be upregulated by gamma-interferon (10). Thus, the B16F10 tumor could be recognized and killed by cytotoxic T-lymphocytes, but it cannot present antigen to CD4⁺ cells, because this presentation is MHC class II restricted. Third, it is rapidly and uniformly fatal if untreated; this is equally true for metastatic brain tumors in humans.

The final consideration in defining the model was the timing of the tumor challenge relative to the vaccination. Simultaneous tumor challenge rather than treatment of an established tumor was chosen to maximize the sensitivity of the model. Had the vaccine failed to protect animals against simultaneous tumor challenge, no further exploration would be warranted. Future studies will show whether this strategy can be used to treat established macroscopic disease. Nevertheless, this sort of strategy has potential clinical applications because of the long-term protection that is established. In clinical practice, more than 50% of patients with brain metastasis have one or two lesions that can be completely surgically resected. However, these patients can suffer recurrence because of microscopic tumor foci present in surrounding functional brain or because of reseeding of the brain with subsequent tumor emboli. A surveillance memory for late recurrence and distant metastasis could provide protection to these patients. Thus, the potential exists to treat a large number of cancer patients with these types of immune-based therapies.

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REFERENCES

- Pardoll DM. Paracrine cytokine adjuvants in cancer immunotherapy. *Annual Rev Immunol* 1995;13:399-415.
- Cox A, Skipper J, Chen Y, et al. Identification of a peptide recognized by five melanoma-specific human cytotoxic T-cell lines. *Science* 1994;264:716-9.
- Van Pel A, van der Bruggen P, Coulie PG, et al. Genes coding for tumor antigens recognized by cytolytic T lymphocytes. *Immunological Reviews* 1995;145:229-50.
- Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas (Apo-1/CD95) ligand: Implications for tumor immune escape. *Science* 1996;274:1363-6.
- Thompson RC, Pardoll DM, Jaffee EM, et al. Systemic and local paracrine cytokine therapies using transduced tumor cells are synergistic in treating intracranial tumors. *J of Immunother* 1996;19:405-13.
- Sampson JH, Archer GE, Ashley DM, et al. Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8⁺ cell-mediated immunity against tumors located in the "immunologically privileged" central nervous system. *Proc Natl Acad Sci USA* 1996;93:10399-404.
- Wucherpfennig K. Autoimmunity in the CNS: Mechanisms of Antigen Presentation and Recognition. *Clinical Immunology and Immunopathology* 1994;72:293-306.
- Hickey WF, Hsu BL, Kimura H. T-lymphocyte entry into the central nervous system. *J Neurosci Res* 1991;28:254-60.
- Glick RP, Lichter T, Kim TS, Ilangovan S, Cohen EP. Fibroblasts genetically engineered to secrete cytokines suppress tumor growth and induce antitumor immunity to a murine glioma in vivo. *Neurosurgery* 1995;36:548-55.
- Dranoff G, Jaffee E, Lazenby A, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U.S.A.* 1993;90:3539-43.
- Jaffee EM, Lazenby A, Meurer J, et al. Use of murine models of cytokine-secreting tumor vaccines to study feasibility and toxicity issues critical to designing clinical trials. *Journal of Immunotherapy with Emphasis on Tumor Immunology* 1995;18:1-9.
- Zar J. *Biostatistical analysis* Englewood Cliffs, New Jersey: Prentice-Hall; 1984.
- Karp SE, Farber A, Salo JC, et al. Cytokine secretion by genetically modified nonimmunogenic murine fibrosarcoma. Tumor inhibition by IL-2 but not tumor necrosis factor. *J Immunol* 1993;150:896-908.
- Fearon ER, Pardoll DM, Itaya T, et al. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell* 1990;60:397-403.
- Williams KJ, Ulvestad E, Cragg L, Blain M, Antel JP. Induction of primary T cell responses by human glial cells. *J Neurosci Res* 1993;36:382-90.
- Williams KC, Dooley NP, Ulvestad E, et al. Antigen presentation by human fetal astrocytes with the cooperative effect of microglia or the microglial-derived cytokine IL-1. *J Neurosci* 1995;15:1869-78.
- Cash E, Rott O. Microglial cells qualify as the stimulators of unprimed CD4⁺ and CD8⁺ T lymphocytes in the central nervous system. *Clin Exp Immunol* 1994;98:313-8.
- Tjuvajev J, Gansbacher B, Desai R, et al. RG-2 glioma growth attenuation and severe brain edema caused by local production of interleukin-2 and interferon-gamma. *Cancer Res* 1995;55:1902-10.
- Butcher EC. The regulation of lymphocyte traffic. *Curr Top Microbiol Immunol* 1986;128:85.
- Springer TA. Adhesion receptors of the immune system. *Nature* 1990;346:425.
- Yednock TA, Rosen SD. Lymphocyte homing. *Adv Immunol* 1989;44:313.
- Picker LJ. Mechanisms of lymphocyte homing. *Curr Opin Immunol* 1992;4:277.
- Kantele A, Kantele JM, Savilahti E, et al. Homing potentials of circulating lymphocytes in humans depend on the site of activation: Oral, but not parenteral, typhoid vaccination induces circulating antibody-secreting cells that all bear homing receptors directing them to the gut. *J Immunol* 1997;158:574-9.
- Berlin C, Berg EL, Briskin MJ, et al. a-4,b-7 integrin mediates lymphocyte binding to the mucosal vascular adhesion MAdCAM-1. *Cell* 1993;74:185.
- Erle DJ, Briskin MJ, Butcher EC, Garcia-Pardo A, Lazarovits AI, Tidswell M. Expression and function of the MAdCAM-1 receptor, integrin a-4,b-7, on human leukocytes. *J Immunol*. 1994;153:517.
- Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. Role of a-4 integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol* 1994;152:3282.
- Camerini D, James SP, Stamenkovic I, Seed B. Leu-8/TQ1 is the human equivalent of the MEL-14 lymph node homing receptor. *Nature* 1989;342:78.
- Kishimoto JK, Jutila MA, Butcher EC. Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. *Proc Natl Acad Sci USA* 1990;87:2244.
- Jutila MA. Leukocyte traffic to sites of inflammation. *APMIS* 1992;100:191.
- Kansas GS. Structure and function of L-selectin. *APMIS* 1992;100:287.
- Berg EL, Yoshino T, Rott LS, et al. The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. *J Exp Med* 1991;174:1461.
- Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC. ELAM-1 is an adhesion molecule for skin homing T-cells. *Nature* 1991;349:796.
- Tian L, Yoshihara Y, Mizuno T, Mori K, Gahmberg CG. The neuronal glycoprotein telencephalin is a cellular ligand for the CD11a/CD18 leukocyte integrin. *J Immunol* 1997;158:928-36.
- Hara I, Nguyen H, Takechi Y, Gansbacher B, Chapman PB, Houghton AN. Rejection of mouse melanoma elicited by local secretion of interleukin-2: implicating macrophages without T cells or natural killer cells in tumor rejection. *Int J Cancer* 1995;61:253-60.
- Cavallo F, Giovarelli M, Gulino A, et al. Role of neutrophils and CD4⁺ T lymphocytes in the primary and memory response to non-immunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene. *J Immunol* 1992;149:3627-35.
- Iwanuma Y, Kato K, Yagita H, Okumura K. Induction of tumor-specific cytotoxic T lymphocytes and natural killer cells by tumor cells transfected with the interleukin-2 gene. *Cancer Immunol Immunother* 1995;40:17-23.
- Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* 1994;264:961-5.

38. Lampson LA. Interpreting MHC class I expression and class I/class II reciprocity in the CNS: Reconciling divergent findings. *Microsc Res Tech* 1995;32:267–85.
39. Lassmann H, Zimprich F, Vass K, Hickey WF. Microglial cells are a component of the perivascular glia limitans. *J Neurosci Res* 1991;28:236–43.
40. Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science* 1988;239:290–2.
41. Fontana A, Fierz W, Wekerle H. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature* 1984;307:273.
42. Wong GHW, Bartlett PF, Clark-Lewis I, Battye F, Schrader JW. Inducible expression of H-2 and Ia antigens on brain cells. *Nature* 1984;310:688.
43. Fontana A, Erb P, Pircher H, Zinkernagel RM, Weber E, Fierz W. Astrocytes as antigen-presenting cells. II. Unlike H-2K-dependent cytotoxic T cells, H-2Ia-restricted T cells are only stimulated in the presence of interferon-gamma. *J Neuroimmunol* 1986;12:15.
44. Nikcevich KM, Gordon KB, Tan L, et al. IFN-gamma-activated primary murine astrocytes express B7 costimulatory molecules and prime naive antigen-specific T cells. *J Immunol* 1997;158:614–21.
45. Sedgwick JD, Mossner R, Schwender S, ter Meulen V. Major histocompatibility complex-expressing nonhematopoietic astroglial cells prime only CD8⁺ T lymphocytes: astroglial cells as perpetuators but not initiators of CD4⁺ T cell responses in the central nervous system. *J Exp Med* 1991;173:1235–46.
46. Wakimoto H, Abe J, Tsunoda R, Aoyagi M, Hirakawa K, Hamada H. Intensified antitumor immunity by a cancer vaccine that produces granulocyte-macrophage colony-stimulating factor plus interleukin-4. *Cancer Research* 1996;1828–33.