

A Conjugate of a Tumor-Targeting Ligand and a T Cell Costimulatory Antibody To Treat Brain Tumors

Ute Gawlick,[†] David M. Kranz,[‡] Victor D. Schepkin,[§] and Edward J. Roy^{*,†}

Neuroscience Program and Department of Biochemistry, University of Illinois, Urbana, Illinois 61801, and Center for Molecular Imaging, University of Michigan, Ann Arbor, Michigan 48109. Received April 9, 2004; Revised Manuscript Received July 22, 2004

T cell immunotherapy is a potential strategy for the treatment of brain tumors because it offers a high degree of specificity, the ability to extravasate into solid tumors, and the potential for eliciting a long-term protective immune response. Various approaches have been developed to overcome T cell immune tolerance to cancer, including the use of cytokines and bispecific antibodies. T cell stimulation with the proinflammatory cytokine IL-12 can elicit antitumor immunity. T cell activation can be increased using bispecific antibodies against activating molecules on the surface of T cells and a tumor antigen. We studied the effects of systemic IL-12 administration in combination with a conjugate of an anti-CD28 antibody and a ligand for the folate receptor. The high affinity folate receptor is expressed on endogenously arising choroid plexus tumors of SV11 mice, which are transgenic for large T antigen under the control of the SV40 promoter. SV11 mice are immunocompetent, yet immunologically tolerant to large T antigen expressed by choroid plexus tumors. MRI analysis showed that the administration of IL-12 and anti-CD28 Fab/folate significantly slowed tumor growth. Proliferating CD8⁺ T cells were found in choroid plexus tumors of treated animals. Treatment of animals with IL-12 + anti-CD28 Fab/folate prolonged survival compared to IL-12 alone. Cytokine treatment combined with tumor-targeted costimulation may be a useful adjunct treatment.

INTRODUCTION

One goal of immunotherapy against cancer is to induce a response that will lead to cytotoxic T lymphocytes (CTLs) with specificity for the tumor. A major obstacle in the activation of antitumor T cells is the process involving self-tolerance to tumor epitopes, a consequence of several processes, including clonal deletion of higher affinity self-reactive T cells during thymic development and exposure to antigens in a noninflammatory context. T cell activation and proliferation usually requires at least two signals: one in the form of the interaction of the peptide/major histocompatibility complex (pepMHC) on the antigen-presenting cell with the T cell antigen receptor (TCR) on the T cell, the other the engagement of the costimulatory receptor CD28 on the T cell with its ligand B7 (1, 2). Increasing evidence suggests that the dependence of CTL activation on CD28 costimulation is related to surface levels of the pepMHC antigen, affinity of the TCR:pepMHC interaction, and duration of the cell to cell interaction (3–5). Immunogenic tumors may elicit a T cell response to a viral peptide presented by class I MHC or to an antigen expressed at higher levels that are sufficient to overcome self-tolerance (6). In some cases, where there is a higher affinity TCR (e.g. with K_D values below 1 μ M), tumors can be eradicated in the apparent absence of ligation of CD28 (7). In other cases, nonimmunogenic tumors can stimulate a CTL response when they are transfected with B7, the ligand for CD28 (8).

Hence, strengthening the CD28 signal would be expected to increase activation of CTLs, particularly those with lower affinity TCRs. The approach described here is to strengthen the activating signals received by T cells by targeting a costimulatory receptor with an antibody conjugated to a tumor-selective ligand.

In addition to CD28 costimulation, IL-12 has emerged as a third signal for CTL activation and a potent cancer therapeutic (9). IL-12 is a proinflammatory cytokine that can reverse antigen specific tolerance by affecting signaling through B7 expressed by antigen presenting cells (10). Exogenous IL-12 has been demonstrated to be effective in mice for the elimination of P815 tumors in DBA/2 mice (11). Furthermore, the coadministration of soluble IL-12 with peptide pulsed dendritic cells induced peptide specific CTLs and protection in mice challenged with P815 tumor cells (12). Tumor cell lines that are otherwise nonimmunogenic were rejected when transfected with the IL-12 gene (13).

To combine the effects of IL-12 and stimulation through CD28 in the generation of an antitumor CTL response, the costimulatory signals should be linked to the recognition of tumor antigens by the TCR. The high affinity folate receptor (FR) ($K_D = 1$ nM) has been used as a target because it is expressed at high levels on several types of cancer. Although the low affinity folate binding protein ($K_D = 1–100$ μ M) is widely expressed on normal tissues, the high-affinity folate receptor is more selectively expressed on normal tissues and tumors. FR is expressed on over 90% of ovarian carcinomas, choroid plexus tumors, and ependymomas (14, 15). In the present studies, folate was conjugated to an anti-CD28 antibody in order to link signaling through CD28 to the engagement of tumor cells. In this approach, activation of the T cell would require recognition by the TCR of pepMHC antigens expressed by the same tumor cell.

* Address correspondence to: Edward J. Roy, Department of Biochemistry, University of Illinois, 600 S. Mathews Ave., Urbana, IL 61801. e-roy@uiuc.edu. Phone: 217-333-3375. Fax: 217-244-5858.

[†] Neuroscience Program, University of Illinois.

[‡] Department of Biochemistry, University of Illinois.

[§] University of Michigan.

FR is expressed on human choroid plexus tumors and ependymomas, and it is expressed in murine tumors of a similar origin. SV11 mice are transgenic for large T antigen (Tag) of simian virus 40 (SV40) on a C57BL/6 background (16–18). With the large T antigen (Tag) gene under the SV40 promoter, these mice develop endogenous choroid plexus tumors, with some level of Tag expression in the thymus. Tag of SV40 is processed into several specific peptides that are presented by class I MHC and these pepMHC antigens can activate CTLs (19). In the mouse, Tag has been shown to contain four peptide epitopes that serve as targets for CTLs that interact with the H2^b MHCs that are found in C57BL/6 mice and SV11 mice (20). Epitope V is immunorecessive while epitopes I, II/III, and IV are immunodominant. Tolerance to Tag epitopes in SV11 mice has been demonstrated by the failure of CTL to develop after immunization with Tag transformed cells or Tag expressed by a recombinant vaccinia virus (rVV) (21). In SV11 mice receiving adoptively transferred C57BL/6 splenocytes, epitope IV specific CTLs are implicated as the chief mediators of immunity against Tag-expressing choroid plexus tumors (21). SV11 mice are tolerant to Tag epitopes, either due to deletion of T cells in the thymus or a mechanism of peripheral tolerance following high levels of expression of Tag in tumors (22). Nevertheless, a population of low avidity T cells specific for epitope V of the T antigen does exist in SV11 mice and can be expanded *ex vivo* by stimulating splenocytes with cells that express large T antigen (23).

In an attempt to stimulate a T cell response against endogenous choroid plexus tumors in SV11 mice, we treated mice with a combination therapy of systemic IL-12 and anti-CD28/Folate bispecific conjugate. We found that anti-CD28 Fab/Folate treatment synergizes with systemic IL-12 to increase survival of choroid plexus tumor bearing animals. MRI analysis revealed that the treatment strongly inhibited tumor growth over a 21 day period. Flow cytometry and immunohistochemistry of the tumor revealed activated T cells consistent with a role of CTLs in the therapeutic immune response.

MATERIALS AND METHODS

Mice. SV11 heterozygous mice transgenic for SV40 large T antigen (obtained from Dr. T. A. Van Dyke, University of North Carolina, Chapel Hill, NC) were mated with C57BL/6 females (obtained from Jackson Laboratory, Bar Harbor, ME). Genotyping was done as previously described using PCR for large T antigen gene (24). Seven days prior to experiments, animals were placed on folate free chow in order to reduce competition of endogenous folate with the anti-CD28 Fab/Folate. RAG/TCR mice are RAG-1 knockout mice transgenic for 2C T cell (7). Animals were housed in animal facilities at the University of Illinois, and all studies were approved by the Institutional Animal Care and Use Committee.

Reagents and Cells. PV-1 hybridoma was obtained from Dr. Jeff Bluestone (University of California, San Francisco). Recombinant murine IL-12 was obtained from Dr. Stan Wolf (Genetics Institute, Cambridge, MA). *N*- α -Pteroyl-*N*- ϵ -(4'-fluoresceinthiocarbamoyl)-L-lysine (PLF) purchased from T. P. McAlinden (25). PV-1 IgG was biotinylated at a concentration of 1 mg/mL using biotinamidocaproate *N*-hydroxysuccinimide ester following standard procedures. Anti-TCR Fab/Folate was made in our laboratory as previously described (26). Antibodies and fluorophores used were CY-labeled anti-CD8, CY-labeled anti-CD3, FITC-labeled anti-CD25, PE-labeled anti-hamster IgG (Pharmingen); rat anti-CD8 IgG (Lein-

co); biotinylated goat anti-rat IgG mouse adsorbed (Vector), anti-BrdU (Becton-Dickinson); SA-594 and SA-488 (Molecular Probes). Flow cytometry was performed at the Flow Cytometry Facility at the University of Illinois. Primary splenocytes were obtained from spleens of euthanized animals and dissociated through wire mesh. Red blood cells were depleted using lysis buffer (0.14 M NH₄Cl, 0.017 M Tris, pH 7.2) and cells were washed and counted for experiments. Tumor cells lines KB (human) and EL-4 (mouse H-2^b) were maintained in folate free RPMI (Gibco). Culture media contained 10% heat inactivated FCS (Hyclone), 1.3 mM L-glutamine, 50 mM 2-mercaptoethanol, 100 unit/mL penicillin (Gibco), and 100 units/mL streptomycin (Gibco). Cells were grown at 37 °C in 5% CO₂ in a humidified incubator.

Preparation of Anti-CD28 Fab/Folate. Anti-CD28 IgG antibody was purified from ascites fluid generated by the hybridoma PV-1 (27). Anti-CD28 IgG was purified by lipid removal, ammonium sulfate precipitation, DEAE column chromatography, and protein A column chromatography. Fab fragments were produced by 24 h incubation with papain conjugated beads (Pierce) at 37 °C on a shaker. Fab fragments were purified over a protein A column, and purity was examined by 10% SDS-PAGE. Anti-CD28 Fab fragments were concentrated to 1 mg/mL and covalently linked to folic acid using the standard EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) reaction at a molar ratio of 50 folates per one Fab fragment. Labeled fragments were purified over a size exclusion column, dialyzed into phosphate-buffered saline (PBS), and concentrated to 1 mg/mL.

Anti-CD28 Fab/Folate Flow Cytometry Inhibition Assays. To assess binding of anti-CD28 Fab/folate preparations to the FR, a competitive inhibition assay was performed. KB cells were incubated with various concentrations of anti-CD28 Fab/folate at 37 °C for 20 min in 0.01% PBS-BSA. The fluorescent folic acid analogue, *N*- α -pteroyl-*N*- ϵ -(4'-fluoresceinthiocarbamoyl)-L-lysine, PLF, was added at the indicated concentrations to the cells and incubated at 37 °C for an additional 20 min. To assess binding of anti-CD28 Fab/folate preparations to CD28, a different competitive inhibition assay was performed. Primary C57BL/6 splenocytes were incubated for 20 min at 37 °C with indicated concentrations of anti-CD28 Fab/folate. Biotinylated anti-CD28 IgG was added to the cells and incubated at 37 °C for an additional 20 min. Cells were incubated with SA:PE in 0.01% PBS-BSA for 20 min at 4 °C. Cells were washed 3 \times in 0.01% PBS-BSA before analysis using a Coulter EPICS XL instrument.

³H-Thymidine Incorporation Assay. KB cells were treated with mitomycin C at 1 mg/mL for 2 h at 37 °C. 10⁴ KB cells were incubated with 10⁵ TCR/RAG splenocytes in folate-free RPMI and various concentrations of a conjugate of an anti-TCR antibody (KJ16 Fab) with folate (to stimulate the TCR) and various concentrations of anti-CD28 Fab/folate. Cells were cultured in 96-well flat bottom plates for 48 h at 37 °C and 5% CO₂. Cells were pulsed with ³H-thymidine (ICM Pharmaceuticals) for 16 h and harvested using a PHD cell harvester. Thymidine incorporation was assessed using a Beckman LS1801 beta-scintillation counter.

MRI Analysis. Mice were anesthetized using 100 mg/kg ketamine and 15 mg/kg xylazine. MRI data acquisition used a single loop small coil with 14 mm diameter placed around the mouse head and proton MR signals were detected using 4.7 T Varian MR scanner at 200 MHz. Coronal MR images of mouse brain were obtained using SEMS pulse sequence with interleaved slice acquisition. Twenty slices were acquired without gap during each

scan using slice thickness of 0.5 mm. Repetition time TR was 750 ms and echo time TE was 25 ms. Data matrix had 256×128 complex points and FOV was 30×30 mm. Duration of each MRI experiment was 6.4 min using NEX = 4. Data processing of MR raw data and images was performed using V and TkViewit software packages (BMRL, University of Illinois) and ImageJ (version 1.28). For volume measurements following the sc injection of anti-CD28 Fab/folate, the number of animals (n) equaled 9 (IL-12), 12 (IL-12 + anti-CD28 Fab/folate), and 6 (PBS). For volume measurements of intracerebroventricular (ICV) treated animals, $n = 5$ (IL-12), 4 (IL-12 + anti-CD28 Fab/Fol), and 4 (PBS). Data from mice that received IL-12 alone and IL-12 + ICV PBS were combined, as no significant differences were found between these groups. Statistical analysis was done using SAS JMP software.

IL-12 and Anti-CD28 Fab/Folate Treatments and Survival Analysis. SV11⁺ tumor mice were treated for 10 days starting at day 85 of age with intraperitoneal (ip) injections of either 100 μ L of 1% normal mouse serum (NMS) in PBS or 0.1 μ g (5 μ g/kg) murine IL-12 in 100 μ L of 1% NMS in PBS. Anti-CD28 Fab/folate was delivered by one of four routes: iv (2 μ g (0.1 mg/kg) on days 4, 7, 9 of IL-12 treatment); sc (2 μ g on days 4, 7, 9); ICV as a single injection (5 μ g (0.25 mg/kg) on day 6); or ICV as a continuous infusion (a total of 25 μ g (1.5 mg/kg) infused from an osmotic minipump on days 6–21). Control mice were injected with PBS for each route of anti-CD28 Fab/folate delivery. Mice were checked daily for health status and weight. If mice were lethargic or weighed less than 75% of their baseline body weight they were euthanized. Survival statistics were analyzed by Kaplan–Meier estimates and log-rank test using SAS JMP software.

Intracerebroventricular (ICV) Injections and Cannula Implantations with Minipumps. For ICV injections and infusions, the tip of the needle or cannula was stereotaxically placed into the lateral ventricle; from bregma: 0.8 mm lateral, 0.5 mm posterior, and 3.1–3.3 mm ventral. Animals were anesthetized using 100 mg/kg ketamine and 15 mg/kg xylazine. The exposed skull was trephined with a 26G needle. For ICV injections, a 27G Hamilton syringe was used to inject 5 μ g/5 μ L anti-CD28 Fab/folate or PBS. Cannulas (Plastics One) were held in place with Lock-tite and attached with vinyl tubing to an osmotic minipump (Alzet). The pump was implanted subcutaneously at the nape of the neck and filled with 25 μ g anti-CD28 Fab/folate in PBS or PBS alone. MRI imaging was used to test whether the osmotic minipump was able to deliver fluid to the indwelling cannula implanted into the lateral ventricle. A Teflon cannula was implanted into the lateral ventricle of a nontumor mouse using the same coordinates as the ICV injections and connected to an osmotic minipump filled with gadolinium. As visualized by MRI, the gadolinium in the pump was delivered to the ventricles and infused the surrounding brain tissue (data not shown).

Immunohistochemistry (IHC). Animals were injected with 50 μ g BrdU/g 2 h before sacrifice. Brains were dissected, embedded in Cryomatrix, and frozen at -80 °C. Ten micrometer sections were cut on a cryostat and fixed using 95% ethanol at -20 °C. Slides were rehydrated and washed between incubations in PBS-0.1%Tween20 (PBST). Sections were blocked with Superblock (Pierce) for 1.5 h. Primary anti-CD8 antibody was incubated at room temperature for 30 min. Slides were incubated with secondary biotinylated goat anti-rat antibody at room temperature for 20 min and incubated with SA-488 for

15 min. Sections were coverslipped or incubated in cold 2.7% formaldehyde-0.01% Tween20 at 4 °C for 45 min. Slides washed $2 \times$ in PBST, $1 \times$ in NaCl/MgCl₂ buffer, and incubated 45 min in DNase I, 50 U/150 μ L in NaCl/MgCl₂ buffer. Slides were blocked in MOM blocker (Vector), 5 min, and incubated in primary anti-BrdU antibody at 4 °C overnight. Secondary biotinylated anti-mouse antibody was incubated for 10 min at room temperature and visualized using SA-594 stained 15 min.

RESULTS

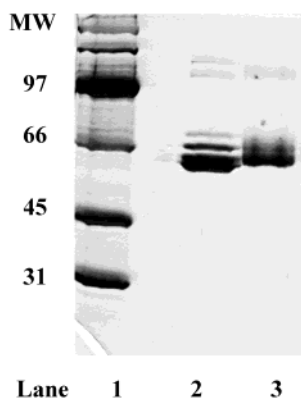
Construction and Characterization of Anti-CD28 Fab/Folate. To eliminate potential complications of tumor-independent, Fc-receptor-mediated activation of T cells, Fab fragments of the anti-CD28 antibody PV-1 were used in these studies. Fab fragments, prepared by digestion with papain coated beads, were run over a protein A column to remove undigested IgG and Fc regions. Fab fragments were labeled with folic acid using the EDC reaction, which links carboxyl groups of folic acid to primary amine groups on the protein. Fab fragments and folate-labeled Fab fragments were examined by SDS–PAGE (Figure 1A). The Fab/folate preparation (lane 3) migrated slightly slower than the Fab fragment (lane 2) suggesting that the protein was successfully coupled to folic acid molecules, given that the molecular weight of folate is 441.

The bispecific conjugate was tested for binding capability to its respective ligands using competition assays and flow cytometry. FR binding of anti-CD28 Fab/folate was tested using *N*- α -pteroyl-*N*- ϵ -(4'-fluoresceinthiocarbonyl)-*L*-lysine (PLF), a fluorescein derivative of the lysine analogue of folic acid (25). Anti-CD28 Fab/folate was able to inhibit the binding of PLF in a dose dependent manner, retaining its ability to bind FR after EDC coupling (Figure 1B and data not shown). Conjugation of the folate to the larger antibody substantially reduced its affinity, by approximately 2 orders of magnitude on a molar basis. Flow cytometry was used to test CD28 binding of anti-CD28 Fab/folate conjugate. Anti-CD28 Fab/folate conjugate blocked the binding of biotinylated anti-CD28 IgG to C57Bl/6 mouse splenocytes, as detected with PE-labeled streptavidin (Figure 1C). Together, these results indicate that anti-CD28 Fab/folate is functional and can bind both FR and CD28.

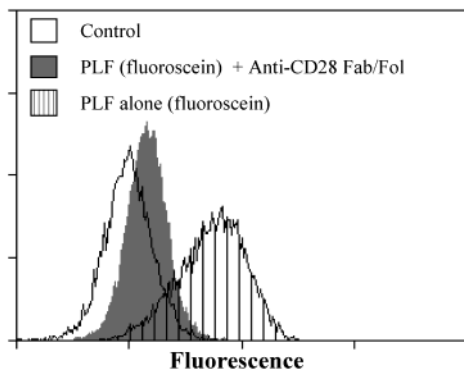
Cross-linking of the tumor cell with CD28 on a T cell by the bispecific conjugate is required for CD28-mediated activation and signaling. The ability of the Fab/folate conjugate to costimulate T cells was assessed using ³H-thymidine incorporation into primary splenocytes, upon activation with an anti-TCR Fab/folate conjugate. Primary splenocytes from a V β 8+ TCR/RAG mouse were cultured with the tumor cell line KB (mitomycin C treated) that expresses the high affinity folate receptor. In the presence of an anti-V β 8 Fab fragments conjugated to folate (KJ16 Fab/folate) (26), T cells proliferated in a dose dependent manner by cross-linking of the TCR to the FR (Figure 2A). Cultures were treated with a concentration of KJ16 Fab/folate (10 ng/mL) below maximal stimulation and various concentrations of anti-CD28 Fab/folate. As predicted from many other studies with anti-CD28 antibodies, anti-CD28 Fab/folate synergized with the KJ16 Fab/folate (Figure 2B). Anti-CD28 Fab/Fol by itself had no effect on proliferation, as expected (data not shown). Taken together, these results indicate that the binding and cross-linking of the anti-CD28 Fab/folate is functional and the conjugate is biologically active.

Systemic Administration Routes of Anti-CD28 Fab/Folate. In previous studies, we found that a 10 day

A. Anti-CD28 Fab/Folate gel electrophoresis



B. Anti-CD28 Fab/Folate binds folate receptor



C. Anti-CD28 Fab/Folate binds CD28

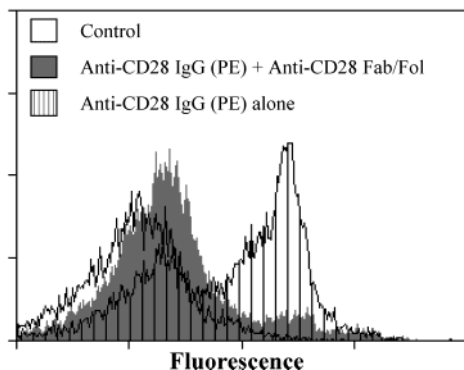


Figure 1. Anti-CD28 Fab/Folate preparation. (A) SDS-PAGE gel of unreduced anti-CD28 Fab preparation. Lane 1, MW standards. Lane 2, anti-CD28 Fab. Lane 3, conjugated anti-CD28 Fab/folate. (B) Flow cytometric analysis demonstrating that anti-CD28 Fab/folate (2×10^{-6} M) inhibits binding of the folic acid analogue PLF (*N*- α -pteroyl-*N*- ϵ -(4'-fluoresceinthiocarbamoyl)-L-lysine, 10^{-8} M). (C) Flow cytometric analysis showing folate conjugated anti-CD28 Fab (2×10^{-6} M) can inhibit binding of divalent anti-CD28 IgG (7×10^{-8} M) to CD28.

course of IL-12 leads to a modest, but significant, increase in survival of SV11 mice (28). After IL-12 treatment, T cells were found to migrate into the brain tumor. We reasoned that by providing daily IL-12 intraperitoneal (ip) injections, followed by anti-CD28 Fab/folate treatments beginning on day 4, enhanced costimulatory signals could be delivered to infiltrating lymphocytes in the choroid plexus tumor. SV11 mice were treated with IL-12 and various systemic routes of delivery (intraperitoneal, intravenous, and subcutaneous) of anti-CD28 Fab/folate. SV11 animals received anti-CD28 Fab/folate on days 4, 7, and 10 after onset of IL-12 treatment.

A regimen of three sc injections of anti-CD28 Fab/folate resulted in enhancement of survival and was further

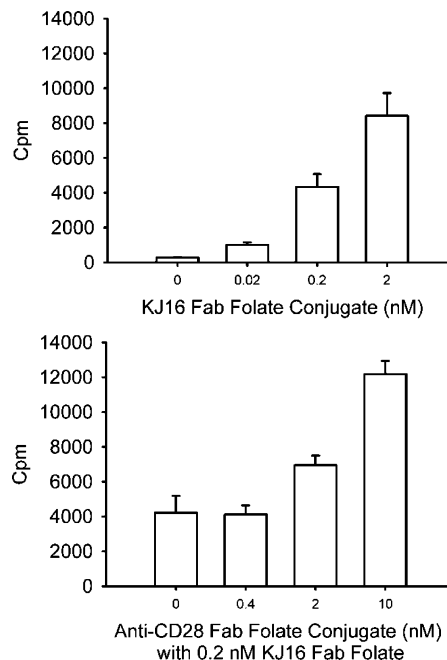


Figure 2. Functional activity of conjugated anti-CD28 Fab/Folate. 3 H-Thymidine incorporation into splenocytes after incubation with the folate receptor expressing cell line KB, with varying concentrations of anti-TCR antibody-ligand conjugate (KJ16 Fab/folate) and anti-CD28 Fab/folate. A. Increasing stimulation of the TCR increases proliferation. B. At a given concentration of the TCR increases proliferation.

studied with MRI-based volumetric analysis as the endpoint. Subcutaneous anti-CD28 Fab/folate administration ($2 \mu\text{g} \times 3$ days) in combination with daily IL-12 ($0.1 \mu\text{g}$ ip for 10 days) increased mouse survival from an average of 106 days with PBS alone ($n = 37$), to 115 days with IL-12 alone ($n = 55$, $p < 0.001$ versus PBS), to 121 days with the combination of IL-12 and anti-CD28 Fab/folate ($n = 44$, $p < 0.05$ versus IL-12 alone, Figure 3A).

The 10 day tumor progression from day 85 to day 95 was followed using MRI (Figure 3B and 3C). Volume reconstruction of images was used to assess tumor growth. Tumors of PBS treated animals ($n = 6$) increased by 263% while tumors of IL-12 treated animals ($n = 9$) increased only 81% ($p < 0.05$, Figure 3B). Animals treated with IL-12 in conjunction with anti-CD28 Fab/folate ($n = 9$) decreased tumor growth even further, growing only 8% over 10 days ($p < 0.05$ versus IL-12 alone, Figure 3B).

Intracerebroventricular (ICV) Injection of Anti-CD28 Fab/Folate. It is possible that relatively short circulating lifetime of Fab fragments minimizes their benefits because most of the conjugate does not reach the brain tumor. Thus, the effects of direct administration of anti-CD28 Fab/folate into the brain were investigated. Animals were treated daily for 10 days ip with PBS or IL-12. To allow for T cell infiltrate into choroid plexus tumors mediated by IL-12, ICV anti-CD28 Fab/folate injection was administered on day 6. Figure 4A shows that animals treated with a single administration of $5 \mu\text{g}$ of anti-CD28 Fab/folate ICV in combination with daily IL-12 ip survived an average of 122 days ($n = 17$), while animals treated with IL-12 alone or IL-12 plus ICV PBS ($n = 14$) survived 114 days ($p < 0.02$). Mice treated with anti-CD28 Fab/folate but without IL-12 pretreatment ($n = 3$) did not survive longer than PBS controls ($n = 7$). The increase in survival in animals treated with the combination of IL-12 and anti-CD28 Fab/folate is com-

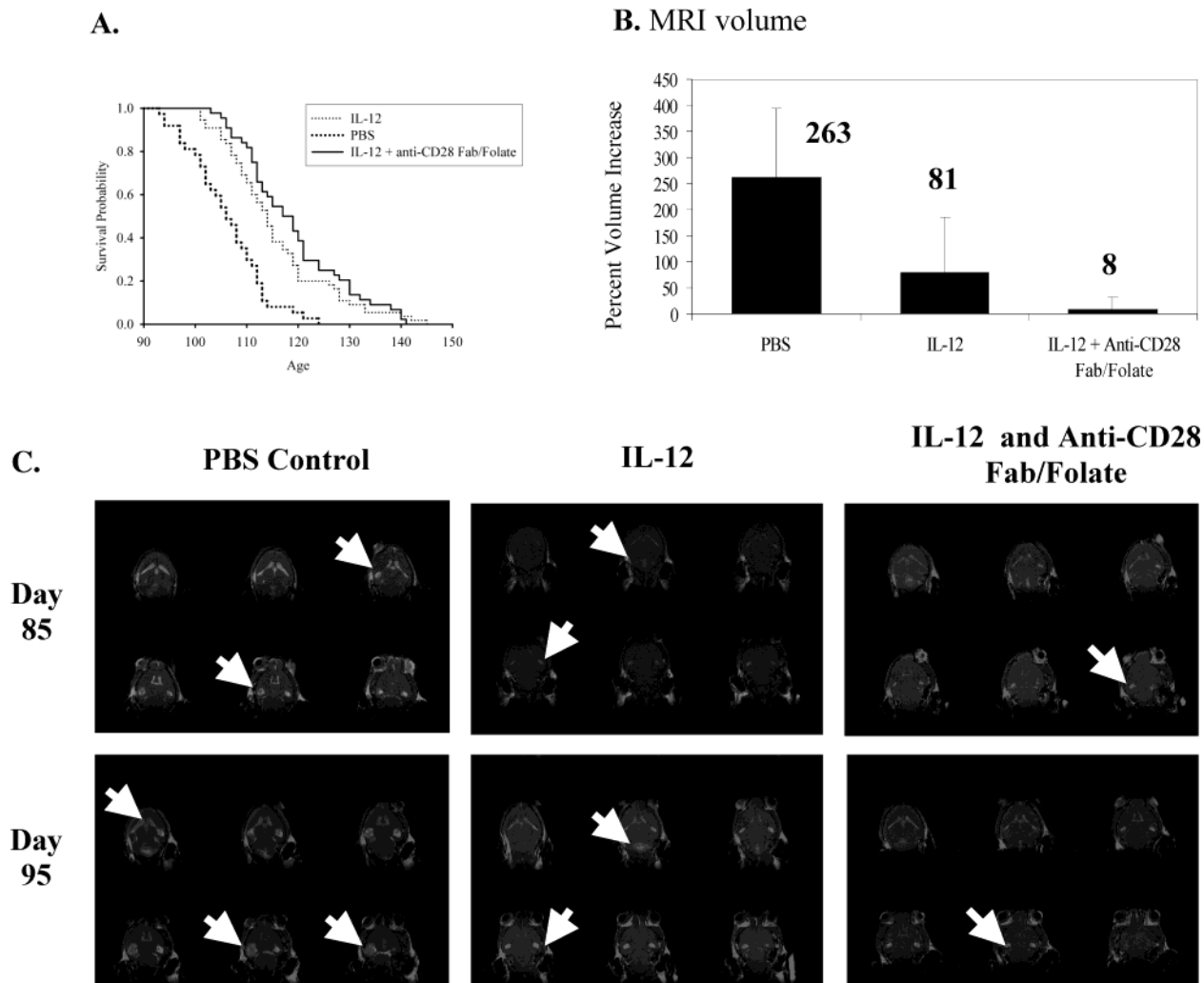


Figure 3. Response to subcutaneous administration of anti-CD28 Fab/folate. (A) Kaplan–Meier survival curves of three treatment groups showing longest survival time for animals treated with IL-12 in combination with Anti-CD28 Fab/folate ($p < 0.02$ versus IL-12 alone). (B) MRI analysis showing percent volume increase in choroid plexus tumor size over 10 day treatment. Mice treated with IL-12 + anti-CD28 Fab/folate had smaller increases than mice treated with IL-12 alone ($p < 0.05$). PBS ($n = 6$), IL-12 ($n = 9$), IL-12 + anti-CD28 Fab/folate ($n = 9$). (C) Representative MRI images showing 10 day tumor progression of tumors in mice treated with PBS, IL-12, and IL-12 plus 3 sc injections of Anti-CD28 Fab/folate conjugate. Arrows point to choroid plexus tumors.

parable to experiments described above using three sc injections of $2 \mu\text{g}$ anti-CD28 Fab/folate (Figure 3A), indicating that a single direct administration of bispecific conjugate to the site of the tumor can produce similar therapeutic effects. The average survival of untreated animals was 106 days, 21 days past start of treatment.

Immunohistochemistry of brain sections from ICV injected animals revealed a proliferating T cell infiltrate. To evaluate the proliferative state of both the tumor and T cells, animals in treatment groups were injected with BrdU 2 h before sacrifice and brain sections stained for BrdU. To examine whether cytotoxic T cells are present in the infiltrate, double staining was also performed with an anti-CD8 antibody. Consistent with the rapid growth observed by MRI, tumor cells were highly proliferative. Within the 2 h window of BrdU exposure, 15–20% of tumor cells were found to be proliferating. In addition, a double positive CD8 and BrdU population was found in anti-CD28 Fab/folate treated animals (Figure 4B), indicative of a tumor-reactive CTL infiltrate into choroid plexus tumors.

Continuous Delivery of Anti-CD28 Fab/Folate. The effect of a 14 day continuous ICV infusion of anti-CD28 Fab/folate through an osmotic minipump was tested in combination with daily IL-12 ip injections. To con-

trol for tumor variability inherent in the SV11 model, mice were imaged by MRI for tumors and matched for tumor size among treatment groups at the start of the experiment. Animals received anti-CD28 Fab/folate into the right lateral ventricle, through an indwelling cannula connected to a surgically implanted osmotic minipump. The animals received $0.25 \mu\text{L/h}$ for 14 days of either PBS or a total dose of $25 \mu\text{g}$ anti-CD28 Fab/folate. Mice were analyzed by MRI (Figure 5A) on day 1 of treatment and 21 days after treatment (in contrast to MRI analyses after 10 days of treatment described earlier). The PBS treated animals ($n = 4$) had a significant increase in tumor size, 73 mm^3 within the 21 day period (Figure 5B). The IL-12 treated tumors ($n = 5$) grew 30 mm^3 , while IL-12 and anti-CD28 Fab/folate treated tumors ($n = 4$) had little growth, 2 mm^3 ($p < 0.05$ versus IL-12, Figure 5B). After anti-CD28 Fab/folate and IL-12 treatments were stopped, tumors rapidly progressed. Both treated groups survived significantly longer than PBS controls ($p < 0.05$, data not shown), but the slower growth of the tumors in mice treated with anti-CD28 Fab/folate did not translate into survival benefit, compared to IL-12 alone. The smaller sample sizes in this experiment (n 's = 4 and 5) may have contributed to the lack of effect on survival.

B. Proliferating CTLs in tumor

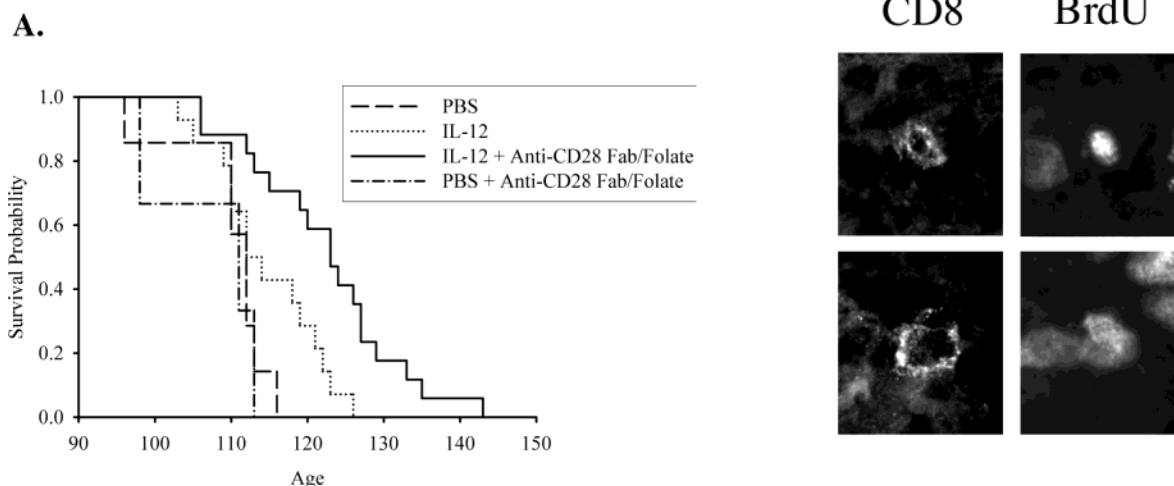


Figure 4. Response to intracerebroventricular (ICV) administration of anti-CD28 Fab/folate. (A) Kaplan–Meier survival curves of four treatment groups showing longest survival for mice treated with IL-12 in combination with anti-CD28 Fab/folate ($p < 0.02$ versus IL-12 alone). (B) Immunohistochemistry of tumor sections from ICV injected animals. CD8⁺/BrdU double positive cells can be seen within the choroid plexus tumors (60 \times magnification, IL-12 and anti-CD28 Fab/folate treated animal).

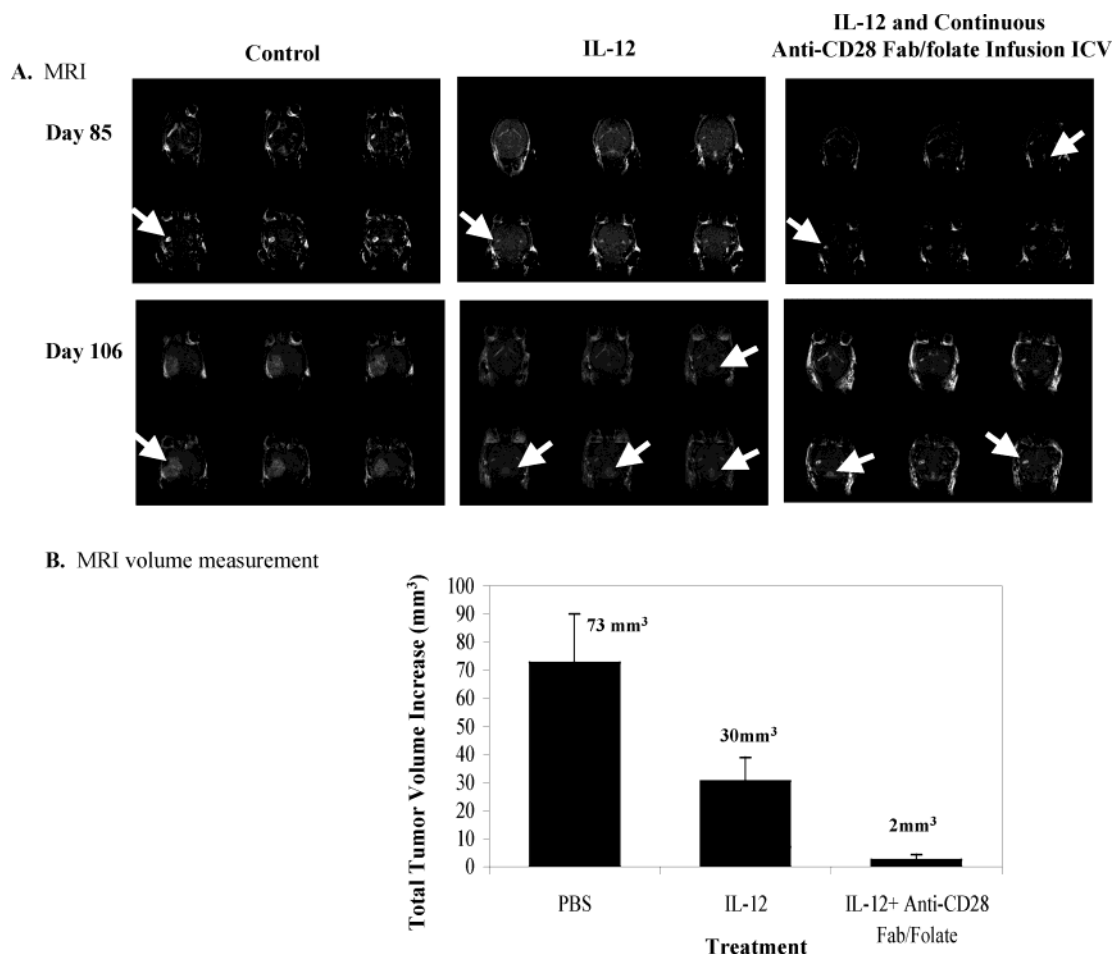


Figure 5. Response to continuous ICV infusion with anti-CD28 Fab/folate. (A) Representative MRI images showing 21 day treatment and progression of tumors after treatment with PBS, IL-12 ip, IL-12 ip + ICV PBS infusion, or IL-12 ip + ICV anti-CD28 Fab/folate infusion. Arrows point to choroid plexus tumors. (B) MRI analysis of tumor volume growth over 21 day treatment. Volumes expressed as mm³ of tumor growth. PBS ($n = 4$), IL-12 ip ($n = 5$), IL-12 ip + anti-CD28 Fab/folate ICV infusion ($n = 4$).

DISCUSSION

Although immune responses can be readily elicited within transplanted tumor models, attempts at eliciting native host immune responses against endogenously

arising tumors have been less successful (29, 30). Among the challenges posed by endogenously arising tumors are that host T cells are tolerant to the tumor. During thymic education, self-reactive T cells undergo apoptosis as the

normal developmental pathway which protects against autoimmunity. However, the process of central tolerance is not complete and allows the escape of low-affinity self-reactive T cells. The T cell repertoire in tumor bearing SV40 transgenic mice is shaped by clonal deletion, as a consequence of the fact that tumor antigens are expressed at low levels in the thymus (31). Even though SV40 transgenic mice undergo central tolerance through the expression of Tag during thymic selection, a population of low-avidity autoreactive immune CTLs can escape thymic deletion (23, 32). The polyclonal population of self-reactive T cells which escape deletion during negative selection has been related to T cell avidity (33). Highest affinity T cells are deleted in thymus while lower affinity, immunorecessive epitopes persist and offer opportunity for immune-based therapy (34).

The effectiveness of a CTL response in the control of SV40 tumors is dependent on the degree of central tolerance and the degree of SV40 antigen expression. Positively selected T cells can also be anergized peripherally as a result of weak stimulating signals received by immature dendritic cells expressing low amounts of pMHC or presentation of pMHC to a low affinity TCR in the absence of costimulatory signals such as B7. T cells with low avidity TCRs are not necessarily deficient in proliferative capacity and can be effectively stimulated by altered peptide ligands with higher affinity for the TCR (35). A higher affinity ligand promoted the expansion of these low-avidity self-reactive T cells with increased magnitude of response and maintenance of effector function upon immunization with the peptide. This suggests that the defect in tolerized or anergic CTL responses is not an intrinsic problem but rather a defect in T cell activation (35). T cell survival is based upon total strength of stimulation consisting of quantity and affinity for antigen, duration of stimulation, degree of costimulation, and T cell cytokine responsiveness (36), and thus additive avenues of activation are most likely needed for a long-term response.

We have previously found that IL-12 stimulates a T cell response against endogenous SV11 tumors (28). The present study demonstrates that the antitumor effect of IL-12 is enhanced when combined with the bispecific ligand-antibody conjugate, anti-CD28 Fab/folate. Anti-CD28 Fab/folate targets CD28 on T cells and the FR on tumor cells to enhance a specific antitumor CTL response. To promote tumor specificity and infiltration, the bispecific antibody conjugate was constructed from an anti-CD28 Fab fragment and directly conjugated to folic acid. After chemical conjugation, both CD28 and FR binding were retained and anti-CD28 Fab/folate was able to induce T cell proliferation *in vitro* in combination with an anti-TCR/folate antibody conjugate.

The dosing schedule and route of administration of anti-CD28 Fab/folate influenced the effectiveness of the treatment *in vivo*. Although intravenous administration of anti-CD28 Fab/folate in combination with daily intraperitoneal injections of IL-12 did not significantly prolong survival, an increase in infiltrating activated T cells in the tumors suggested that peripheral administration of anti-CD28 Fab/folate was effective in activating T cells focally within the area of the tumor (data not shown). Subcutaneous administration would be expected to provide a more sustained delivery of the agent to the brain, compared to intravenous administration. Consistent with this idea, subcutaneous administration of anti-CD28 Fab/folate in combination with daily intraperitoneal injections of IL-12 over a 10 day period resulted in increased survival and a dramatic decrease in tumor growth as

assessed by MRI. Given the fact that choroid plexus epithelium is on the blood side of the blood-CSF barrier and the finding that the tumors enhanced with gadolinium, peripheral injections would be expected to have access to the tumors. However, the relatively short circulating lifetime of Fab fragments, and the possibility that FR+ macrophages are in the periphery, may reduce the effective concentration Fab/folate conjugates that make it to the brain.

The possibility that the bispecific agent acts in the local tumor environment to enhance costimulation of infiltrating T cells was supported by the observations that local administration of anti-CD28 Fab/folate at the site of the choroid plexus tumors was also effective. The survival of mice treated with a single ICV injection of anti-CD28 Fab/folate was comparable to results seen with three subcutaneous injections of the bispecific antibody conjugate. This is significant considering that at the start of treatment, tumors in these animals have rapid tumor growth with high levels of large-T antigen and highly neoplastic pathology (21, 37). The strongest effect on tumor growth assessed by MRI was achieved through the 2 week continuous local ICV administration of anti-CD28 Fab/folate. Choroid plexus tumors in animals treated with anti-CD28 Fab/folate exhibited little or no growth 21 days after initiation of treatment.

Proliferating CD8⁺ T cells were seen in brain sections of animals treated with IL-12 in combination with ICV injections of anti-CD28 Fab/folate. In efforts to examine whether the specificity of the CD8⁺ tumor infiltrating cells are specific for the most dominant SV40 Tag epitope (epitope IV bound to K^b), we examined splenocytes with tetramers of epitope IV/K^b. However, we were unable to detect significant binding (data not shown), consistent with findings of Schell that the T antigen specific T cells stimulated in SV11 mice by a T antigen vaccine are specific for epitope V (23). Thus, if the T cells stimulated by IL-12 and anti-CD28 Fab/folate are specific for large T antigen, it is possible that they are specific for the immunorecessive epitope V.

Two other SV40 transgenic tumor models have shown recently that strong activating signals are required to overcome T cell tolerance. SV40 under an α -amylase promoter produces endogenously arising osteosarcomas; naive SV40 epitope I specific T cells adoptively transferred into these SV40 tumor-expressing mice lacked effector function due to peripheral T cell anergy (38). However, treatment with an agonist antibody against CD40 in addition to the adoptively transferred CTLs prevented the development of T cell tolerance and promoted the development of CTL effector function, clonal expansion, and memory cell development (38). Upon CD40 ligation, dendritic cells upregulate B7 expression as well as secrete IL-12. In contrast, in an SV40 prostate mouse model, CD40 ligation in combination CpG injections failed to reverse SV40 T cell tolerance, but CTLs against the subdominant Tag epitope V could be elicited (39).

Overcoming self-tolerance for activation of naive low-affinity antitumor CTLs requires a considerable synergistic engagement of a variety of receptors on naive T cells. Increasing evidence points to a three signal model for T cell activation. While the TCR/pMHC interaction confers antigenic specificity, CD28 and IL-12 are needed to amplify the low-affinity activation. While both CD28 and IL-12 can stimulate T cell proliferation and cytokine secretion, growing evidence indicates that CD28 is needed for the development of CTL effector function and IL-12 is needed for T cell memory formation. In the develop-

ment of antitumor immunotherapies, a specific and strong immune response must be elicited to activate tumor-tolerized T cells. The most successful treatment will most likely comprise a combination of therapies that stimulate signaling pathways needed to activate and sustain a specific antitumor T cell response.

ACKNOWLEDGMENT

We thank Laurie Rund and Lori Carr for technical assistance, and Dr. Todd Schell for comments on the manuscript. Supported by NIH grants CA77499 (E.J.R.) and AI3599 (D.M.K.).

LITERATURE CITED

- (1) Harding, F. A., McArthur, J. G., Gross, J. A., Raulet, D. H., and Allison, J. P. (1992) CD28-mediated signaling costimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356**, 607–609.
- (2) Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B., and Mak, T. W. (1993) Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**, 609–612.
- (3) Bachmann, M. F., McKall-Faienza, K., Schmits, R., Bouchard, D., Beach, J., Speiser, D. E., Mak, T. W., and Ohashi, P. S. (1997) Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* **7**, 549–557.
- (4) Salomon, B., and Bluestone, J. A. (2001) Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu. Rev. Immunol.* **19**, 225–252.
- (5) Kundig, T. M., Shahinian, A., Kawai, K., Mittrucker, H. W., Sebзда, E., Bachmann, M. F., Mak, T. W., and Ohashi, P. S. (1996) Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity* **5**, 41–52.
- (6) Morgan, D. J., Kreuwel, H. T., Fleck, S., Levitsky, H. I., Pardoll, D. M., and Sherman, L. A. (1998) Activation of low avidity CTL specific for a self-epitope results in tumor rejection but not autoimmunity. *J. Immunol.* **160**, 643–651.
- (7) Manning, T. C., Rund, L. A., Gruber, M. M., Fallarino, F., Gajewski, T. F., and Kranz, D. M. (1997) Antigen recognition and allogeneic tumor rejection in CD8⁺ TCR transgenic/RAG^{-/-} mice. *J. Immunol.* **159**, 4665–4675.
- (8) Townsend, S. E., and Allison, J. P. (1993) Tumor rejection after direct costimulation of CD8⁺ T cells by B7-transfected melanoma cells. *Science* **259**, 368–370.
- (9) Curtsinger, J. M., Lins, D. C., and Mescher, M. F. (2003) Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J. Exp. Med.* **197**, 1141–1151.
- (10) Ushio, H., Tsuji, R. F., Szczepanik, M., Kawamoto, K., Matsuda, H., and Askenase, P. W. (1998) IL-12 reverses established antigen-specific tolerance of contact sensitivity by affecting costimulatory molecules B7-1 (CD80) and B7-2 (CD86). *J. Immunol.* **160**, 2080–2088.
- (11) Fallarino, F., Uyttenhove, C., Boon, T., and Gajewski, T. F. (1996) Endogenous IL-12 is necessary for rejection of P815 tumor variants in vivo. *J. Immunol.* **156**, 1095–1100.
- (12) Fallarino, F., Uyttenhove, C., Boon, T., and Gajewski, T. F. (1999) Improved efficacy of dendritic cell vaccines and successful immunization with tumor antigen peptide-pulsed peripheral blood mononuclear cells by coadministration of recombinant murine interleukin-12. *Int. J. Cancer* **80**, 324–333.
- (13) Hallez, S., Detremmerie, O., Giannouli, C., Thielemans, K., Gajewski, T. F., Burny, A., and Leo, O. (1999) Interleukin-12-secreting human papillomavirus type 16-transformed cells provide a potent cancer vaccine that generates E7-directed immunity. *Int. J. Cancer* **81**, 428–437.
- (14) Ross, J. F., Chaudhuri, P. K., and Ratnam, M. (1994) Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* **73**, 2432–2443.
- (15) Garin-Chesa, P., Campbell, I., Saigo, P. E., Lewis, J. L., Jr., Old, L. J., and Rettig, W. J. (1993) Trophoblast and ovarian cancer antigen LK26. Sensitivity and specificity in immunopathology and molecular identification as a folate-binding protein. *Am. J. Pathol.* **142**, 557–567.
- (16) Brinster, R. L., Chen, H. Y., Messing, A., van Dyke, T., Levine, A. J., and Palmiter, R. D. (1984) Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. *Cell* **37**, 367–379.
- (17) Van Dyke, T., Finlay, C., and Levine, A. J. (1985) A comparison of several lines of transgenic mice containing the SV40 early genes. *Cold Spring Harb. Symp. Quant. Biol.* **50**, 671–678.
- (18) Palmiter, R. D., Chen, H. Y., Messing, A., and Brinster, R. L. (1985) SV40 enhancer and large-T antigen are instrumental in development of choroid plexus tumours in transgenic mice. *Nature* **316**, 457–460.
- (19) Schirmbeck, R., Zerrahn, J., Kuhrober, A., Deppert, W., and Reimann, J. (1993) Immunization of mice with the N-terminal (1–272) fragment of simian virus 40 large T antigen (without adjuvants) specifically primes cytotoxic T lymphocytes. *Eur. J. Immunol.* **23**, 1528–1534.
- (20) Mylin, L. M., Schell, T. D., Roberts, D., Epler, M., Boesteanu, A., Collins, E. J., Frelinger, J. A., Joyce, S., and Tevethia, S. S. (2000) Quantitation of CD8⁺ T-lymphocyte responses to multiple epitopes from simian virus 40 (SV40) large T antigen in C57BL/6 mice immunized with SV40, SV40 T-antigen-transformed cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes. *J. Virol.* **74**, 6922–6934.
- (21) Schell, T. D., Mylin, L. M., Georgoff, I., Teresky, A. K., Levine, A. J., and Tevethia, S. S. (1999) Cytotoxic T-lymphocyte epitope immunodominance in the control of choroid plexus tumors in simian virus 40 large T antigen transgenic mice. *J. Virol.* **73**, 5981–5993.
- (22) Webb, S., Morris, C., and Sprent, J. (1990) Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell* **63**, 1249–1256.
- (23) Schell, T. D. (2004) In vivo expansion of the residual tumor antigen-specific CD8⁺ T lymphocytes that survive negative selection in simian virus 40 T-antigen-transgenic mice. *J. Virol.* **78**, 1751–1762.
- (24) Patrick, T. A., Kranz, D. M., Zachary, J. F., and Roy, E. J. (1998) Intracerebral bispecific ligand-antibody conjugate increases survival of animals bearing endogenously arising brain tumors. *Int. J. Cancer* **78**, 470–479.
- (25) McAlinden, T. P., Hynes, J. B., Patil, S. A., Westerhof, G. R., Jansen, G., Schornagel, J. H., Kerwar, S. S., and Freisheim, J. H. (1991) Synthesis and biological evaluation of a fluorescent analogue of folic acid. *Biochemistry* **30**, 5674–5681.
- (26) Cho, B. K., Roy, E. J., Patrick, T. A., and Kranz, D. M. (1997) Single-chain Fv/folate conjugates mediate efficient lysis of folate-receptor-positive tumor cells. *Bioconjugate Chem.* **8**, 338–346.
- (27) Abe, R., Vandenberghe, P., Craighead, N., Smoot, D. S., Lee, K. P., and June, C. H. (1995) Distinct signal transduction in mouse CD4⁺ and CD8⁺ splenic T cells after CD28 receptor ligation. *J. Immunol.* **154**, 985–997.
- (28) Roy, E. J., Gawlick, U., Orr, B. A., Rund, L. A., Webb, A. G., and Kranz, D. M. (2000) IL-12 treatment of endogenously arising murine brain tumors. *J. Immunol.* **165**, 7293–7299.
- (29) Schell, T. D., and Tevethia, S. S. (2001) Control of advanced choroid plexus tumors in SV40 T antigen transgenic mice following priming of donor CD8⁺ T lymphocytes by the endogenous tumor antigen. *J. Immunol.* **167**, 6947–6956.
- (30) Cordaro, T. A., de Visser, K. E., Tirion, F. H., Schumacher, T. N., and Kruisbeek, A. M. (2002) Can the low-avidity self-specific T cell repertoire be exploited for tumor rejection? *J. Immunol.* **168**, 651–660.
- (31) Zheng, X., Gao, J. X., Zhang, H., Geiger, T. L., Liu, Y., and Zheng, P. (2002) Clonal deletion of simian virus 40 large T antigen-specific T cells in the transgenic adenocarcinoma of mouse prostate mice: an important role for clonal deletion in shaping the repertoire of T cells specific for antigens overexpressed in solid tumors. *J. Immunol.* **169**, 4761–4769.

- (32) Oehen, S. U., Ohashi, P. S., Burki, K., Hengartner, H., Zinkernagel, R. M., and Aichele, P. (1994) Escape of thymocytes and mature T cells from clonal deletion due to limiting tolerogen expression levels. *Cell Immunol.* *158*, 342–352.
- (33) Bouneaud, C., Kourilsky, P., and Bousso, P. (2000) Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity* *13*, 829–840.
- (34) Ohlen, C., Kalos, M., Hong, D. J., Shur, A. C., and Greenberg, P. D. (2001) Expression of a tolerizing tumor antigen in peripheral tissue does not preclude recovery of high-affinity CD8⁺ T cells or CTL immunotherapy of tumors expressing the antigen. *J. Immunol.* *166*, 2863–2870.
- (35) de Visser, K. E., Cordaro, T. A., Kessels, H. W., Tirion, F. H., Schumacher, T. N., and Kruisbeek, A. M. (2001) Low-avidity self-specific T cells display a pronounced expansion defect that can be overcome by altered peptide ligands. *J. Immunol.* *167*, 3818–3828.
- (36) Gett, A. V., Sallusto, F., Lanzavecchia, A., and Geginat, J. (2003) T cell fitness determined by signal strength. *Nat. Immunol.* *4*, 355–360.
- (37) Van Dyke, T. A., Finlay, C., Miller, D., Marks, J., Lozano, G., and Levine, A. J. (1987) Relationship between simian virus 40 large tumor antigen expression and tumor formation in transgenic mice. *J. Virol.* *61*, 2029–2032.
- (38) Staveley-O'Carroll, K., Schell, T. D., Jimenez, M., Mylin, L. M., Tevethia, M. J., Schoenberger, S. P., and Tevethia, S. S. (2003) In vivo ligation of CD40 enhances priming against the endogenous tumor antigen and promotes CD8⁺ T cell effector function in SV40 T antigen transgenic mice. *J. Immunol.* *171*, 697–707.
- (39) Grossmann, M. E., Davila, E., and Celis, E. (2001) Avoiding Tolerance Against Prostatic Antigens With Subdominant Peptide Epitopes. *J. Immunother.* *24*, 237–241.

BC049911E