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Recurrence of Intracranial Tumors following Adoptive T Cell Therapy Can Be Prevented by Direct and Indirect Killing Aided by High Levels of Tumor Antigen Cross-Presented on Stromal Cells

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Elimination of peripheral tumors by adoptively transferred tumor-specific T cells may require killing of cancer cells and tumor stromal cells. Tumor Ags are cross-presented on stromal cells, resulting in direct cytotoxic T cell (CTL) killing of both Ag-expressing cancer cells and stromal cells. Indirect killing of Ag loss variant cells also occurs. We show here that similar processes occur in a brain tumor stromal environment. We used murine cancer cell lines that express high or low levels of a peptide Ag, SIYRYYGL (SIY), recognized by transgenic 2C CD8+ T cells. The two cell lines are killed with equivalent efficiency by 2C T cells in vitro. Following adoptive transfer of 2C T cells into mice with established SIY-Hi or SIY-Lo brain tumors, tumors of both types infused with H-2k tumor cells expressing high levels of SIY peptide. Adoptively transferred 2C T cells are able to kill cross-presenting H-2b stromal cells but not H-2k tumor cells. In peripheral models, this paradigm led to a small static tumor. In the brain, activated 2C T cells were able to kill cross-presenting CD11b+ cells and completely eliminate the H-2b tumors in most mice. Targeting brain tumor stroma or increasing Ag shedding from tumor cells to enhance cross-presentation may improve the clinical success of T cell adoptive therapies. The Journal of Immunology, 2009, 183: 1828–1837.

T cell-mediated immunotherapies for cancer currently in development include vaccination strategies and adoptive transfer of tumor-specific T cells (reviewed in Refs. 1–3). The results of clinical trials evaluating adoptive T cell therapies have been mixed, with particular types of cancer showing some beneficial response. Treatment of melanoma in particular shows promise (4–6), but Ag loss variant (ALV)3 cancer cells remain a challenge for Ag-specific T cell therapies (5, 7).

Adoptive T cell therapies for brain tumors have also been investigated for >25 years in animal models (8–15) and in clinical trials (16–26). Early trials used activated T cell populations that were not specific for tumor Ags, but more recent preclinical studies have outlined approaches to improve the effectiveness of adoptively transferred Ag-specific T cells (27–37). Clinical trials evaluating autologous, ex vivo-activated T cells from peripheral blood or tumor- or vaccine-draining lymph nodes have demonstrated partial responses against gliomas, even in patients that previously failed to respond to multiple forms of therapy (20, 21, 26, 38).

Tumors outside the CNS are easily accessed by primed CTL, but even in cases of strong antitumor responses, it is clear that new approaches for targeting ALV cancer cells that lead to tumor recurrence are needed to improve the success of T cell-based therapies (5, 7, 39, 40). Similarly, T cell responses can also be induced in the brain following peptide vaccination targeting EGFRvIII on malignant gliomas, but EGFRvIII-negative tumors recur in patients (41).

Successful clearance of solid peripheral tumors and prevention of relapse from ALV outgrowth depend on elimination of malignant cancer cells as well as bone marrow and non-bone marrow-derived stromal cells that support the growing tumor (42–47). Adoptively transferred CTL can target both cancer cells and stromal cells that cross-present tumor Ag shed by cancer cells or internalized via phagocytosis. ALV cancer cells sheltered within the tumor stroma can be eliminated via a bystander effect by killing cross-presenting CD11b+ stromal cells that are sensitized with sufficient tumor Ag released by dying cancer cells, irradiation, or chemotherapy (46).

The importance of targeting stroma has been established previously for a variety of peripheral tumors (42–47), but brain tumors differ from peripheral tumors in a number of ways. For example, gliomas do not induce lymphangiogenesis (48), and microglia associated with gliomas are particularly active at secreting TGF-β that contributes to invasiveness (49). Gliomas are also comprised of different stromal elements than peripheral tumors. Fibroblasts contribute to T cell-mediated peripheral tumor rejection (44). However, fibroblasts are absent from gliomas and orthotopic murine brain tumors (50).

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2 Abbreviations used in this paper: ALV, Ag loss variant; eGFP, enhanced GFP; MHC I, MHC class I; PI, propidium iodide.

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Differences in brain and peripheral immune responses to tumors, and in the stroma of brain and peripheral tissues, led us to investigate whether cross-presentation of brain tumor Ags by stroma is also important for brain tumor rejection by adoptively transferred CTL. Mice were intracranially infused with cancer cells expressing high or low levels of a defined model peptide Ag, SIYRYYGL (SIY). High- and low-Ag-expressing cell lines have been shown to have differences in ALV outgrowth in s.c. models (46). Tumors were allowed to establish for several days before treatment to allow tumor stromal cells to organize. Mice received an adoptive transfer of 2C T cells that recognize SIY peptide presented on MHC class I (MHC I) haplotype K\(^b\), and T cell responses to both tumors were characterized. Cross-presentation of tumor Ags by CD11b\(^+\) brain tumor stromal cells was directly assessed using high-affinity single-chain TCR monomers that bind tumor peptide/MHC complexes. We also observed antitumor effects of adoptively transferred 2C T cells when stromal cross-presentation was the only mechanism of peptide/MHC presentation in the tumors.

Materials and Methods

**Mice, cell lines, and reagents**

C57BL/6 RAG1\(^{-/-}\) mice from The Jackson Laboratory are maintained as a colony at the University of Illinois. 2C TCR transgenic mice are maintained as a heterozygous colony by crossing with C57BL/6 mice and screened for expression of the 2C TCR on PBL by flow analysis with 1B2 clone. Mice were 2–6 mo of age at the time of experiments. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois (Urbana-Champaign, IL). MC57-SIY-Lo and MC57-SIY-Hi cells (derived from C57BL/6 mouse fibrosarcoma MC57), and PRO4L-SIY and PRO4L-gg33 cells (derived from C3H/HeN mouse undifferentiated spindle cell cancer 1591-PRO4L) were generated at the University of Chicago and have been described previously (45, 46). Cancer cell lines were grown in complete RPMI 1640 medium containing 5 mM HEPES, 10% FCS, 1.3 mM Na-glutamine, 50 mM P-2-ME, penicillin, and streptomycin. Anti-K\(^b\) and isotype control Abs were purchased from BD Pharmingen. 1B2 mAb specific for the 2C TCR was purified from the culture supernatant of 2C TCR transgenic 2C T cells from spleens of 2C TCR transgenic mice, and staining with 1B2 Ab was allowed to establish for several days before treatment to allow tumor stromal cells to organize. Mice received an adoptive transfer of 2C T cells that recognize SIY peptide presented on MHC class I (MHC I) haplotype K\(^b\), and T cell responses to both tumors were characterized. Cross-presentation of tumor Ags by CD11b\(^+\) brain tumor stromal cells was directly assessed using high-affinity single-chain TCR monomers that bind tumor peptide/MHC complexes. We also observed antitumor effects of adoptively transferred 2C T cells when stromal cross-presentation was the only mechanism of peptide/MHC presentation in the tumors.

**Cytotoxic assays**

2C T cells from spleens of 2C TCR transgenic mice were prepared by mechanical dissociation and ammonium chloride buffer lysis of erythrocytes. Splenocytes were incubated for 1 h at 37°C and washed three times before incubating with 2C T cells at different E:T ratios for 4 h. Chromium release was measured using a Beckman gamma counter, and specific release was calculated using the standard formula: ([sample counts – spontaneous counts]/[maximum counts – spontaneous counts]) \times 100.

**Cancer cell infusions and T cell adoptive transfers**

Cancer cells were trypsinized and collected, washed twice with HBSS, and stereotaxically infused into the brain. Each mouse received an infusion of 5 \times 10^6 cells in 300 nL in ventral stratum (antero-posterior, 0.5 mm; lateral, 2.5 mm; dorsal-ventral, –4 mm). In all experiments unless specified otherwise, a small tumor challenge of ~1–2 \times 10^5 cells was prepared the same way and injected s.c. into the flank to ensure peripheral afferent immune responses leading to activation of adoptively transferred 2C T cells. 2C T cells were adoptively transferred by preparing a single-cell suspension of lymph node and spleen cells from 2C TCR transgenic mice lysing erythrocytes with ammonium chloride buffer, and washing twice with HBSS. Mice were injected with 5 \times 10^7 naive 2C lymphocytes into the tail vein 1, 5, 6, or 10 days after tumor implantation.

**Histological analysis of T cell response**

Mice were sacrificed at 1, 3, or 5 days following 2C T cell transfer or followed for survival. Criteria for euthanasia were 75% of baseline body weight or signs of neurological impairment, in accordance with Institutional Animal Care and Use Committee guidelines. Unfixed, frozen 10-\mu m-thick tissue sections were stained with 1B2 Ab amplified with HRP/Pyramid-biotin (PerkinElmer) and streptavidin-Alexa 594 (Invitrogen) and counterstained with 4',6-diamidino-2-phenylindole (Invitrogen). Other sections were stained with anti-CD31 (PECAM), followed by biotinylated rabbit anti-ram IgG (Vector Laboratories) and streptavidin-Alexa 594. Tumor volume was systematically reconstructed by measuring tumor area on hematoxylin-stained sections taken at regular intervals through the brain.

**Analysis of cross-presentation by CD11b\(^+\) stromal cells**

CD11b\(^+\) cells were purified from MC57, MC57-SIY-Lo, and MC57-SIY-Hi brain tumors (established for 10 days before adoptive transfer of 2C T cells) and PRO4L-SIY brain tumors (established for 7 days before adoptive transfer of 2C T cells) 2 or 3 days following 2C T cell adoptive transfer using anti-CD11b-labeled magnetic beads (Miltenyi Biotec). Brains were dissected out, minced, and incubated with Dispsue (5 U/ml; Life Technologies) at 33°C for 30 min in HIBA medium (Brainbits). Cells were then washed with RPMI 1640 medium containing 10% FCS to inactivate Dispsue and passed through 100- and 40-\mu m filters sequentially. Erythrocytes were lysed with ammonium chloride buffer, and cells were washed with HIBA A before following the manufacturer’s protocol for separation of CD11b\(^+\) cells using a magnetic column. Purified cells were then stained with anti-CD11b-Pacific Blue (Caltag Laboratories) and anti-sIYRYYGL (Fig. 1A). Cancer cell lines expressing different surface levels of SIY peptide (MC57-SIY-Lo, MC57-SIY-Hi, PRO4L-SIY) were grown in complete RPMI 1640 medium containing 5 mM HEPES, 10% FCS, 1.3 mM Na-glutamine, 50 mM P-2-ME, penicillin, and streptomycin. Anti-K\(^b\) and isotype control Abs were purchased from eBioscience, and anti-CD31 (PECAM-1) was obtained from BD Pharmingen. 1B2 mAb specific for the 2C TCR was purified from the hybridoma and biotinylated in our laboratory. Expression and purification of soluble, single-chain 2C-m67 TCR monomers that bind SIYR\(^b\)(46) at low nanomolar affinity were described previously (46). Monomers were chemically biotinylated at multiple sites via free amine groups using EZ-Link NHS-PEO4 Biotinylation Kit (Pierce). Streptavidin-allophycocyanin (Invitrogen) was used as a secondary. For flow cytometry experiments, cancer cells were stained and washed twice with PBS/BSA before analysis on a BD FACSCanto or BD LSR II.

**Statistical analyses**

GraphPad Prism software was used to analyze survival curves by log-rank test and area/volume data by unpaired \(t\) test. Significance was considered \(p < 0.05\).

**Results**

**Cancer cell lines expressing different surface levels of SIY peptide are killed equally well by 2C T cells in vitro**

MC57 cancer cell lines were evaluated for MHC I haplotype K\(^b\) epitope expression to rule out the possibility that differences in surface levels of MHC I result in differences in killing by CD8 plus 2C T cells. All three MC57 lines (parental, SIY-Lo, and SIY-Hi) express similar levels of K\(^b\) (Fig. 1A). We also confirmed that PRO4L-SIY cancer cells (H-2\(^d\)) do not express K\(^b\). Next, we measured the relative surface levels of SIY peptide complexed with K\(^b\) on the cancer cells using biotinylated single-chain high-affinity 2C TCR monomers (2C-m67 TCR). 2C-m67 TCR binding was detected with fluorescent streptavidin-allophycocyanin. The mean fluorescence intensity of MC57-SIY-Lo cells was increased ~2.6-fold over untransduced MC57 parental cells. MC57-SIY-Hi cell levels were increased ~16-fold over MC57-SIY-Lo cells (Fig. 1B). The genes for enhanced GFP (eGFP) and SIY were fused in the expression construct used to generate these cell lines; therefore, eGFP levels can also serve as an index of SIY Ag expression. The mean eGFP fluorescence of MC57-SIY-Lo and MC57-SIY-Hi cancer cells were 1.6- and 17-fold over untransduced parental MC57, respectively (Fig. 1C). PRO4L-SIY cells express the highest level of eGFP, but SIY cannot be presented on H-2\(^d\) and thus is not recognized by the wild-type 2C TCR or single-chain 2C-m67 TCR monomers. eGFP expression serves as the only readout of SIY peptide present in PRO4L-SIY cancer cells.

Next, we evaluated the cytotoxic effector function of 2C T cells against the MC57 cancer cell lines in vitro. Equivalent \(^{51}\)Cr release was measured from MC57-SIY-Hi and MC57-SIY-Lo cells incubated with preactivated 2C T cells at different effector to target cell ratios (Fig. 1D). The parental MC57 line was poorly killed by 2C T cells, demonstrating specificity for the SIY Ag. In a separate assay, PRO4L-SIY showed low levels of chromium release similar
to parental MC57 when incubated with activated 2C T cells, confirming the MHC restriction of the 2C TCR to K\(^{b}\) (data not shown).

**Adoptively transferred 2C CTL initially cleared established brain tumors expressing high or low levels of Ag**

To establish an in vivo brain tumor model to assess the therapeutic effect of adoptively transferred tumor-specific 2C T cells, we infused MC57, MC57-SIY-Lo, and MC57-SIY-Hi cancer cells into the brains of syngeneic C57BL/6 RAG\(^{-/-}\) mice and waited several days for the tumor and stroma to establish. Unactivated, naive 2C T cells were adoptively transferred into the tumor-bearing mice on day 5. On the same day, a small number of the same cancer cells were injected s.c. to ensure a robust afferent immune response and activation of the transferred 2C CTL. One day following treatment, there were few 2C T cells infiltrating either SIY-Hi or SIY-Lo brain tumors (Fig. 2A), and both tumors were well-established and growing (Fig. 3A). Three days following transfer of 2C T cells, both MC57-SIY-Hi and MC57-SIY-Lo tumors were infiltrated by massive numbers of 2C T cells (Fig. 2B). Five days following T cell transfer, both tumor types were almost completely eliminated based on histological examination (Fig. 3B). 2C T cell accumulation was still observed at the sites of both SIY-Hi and SIY-Lo tumors (Fig. 2C), but there was little evidence of residual tumor. In these brains, T cells were mostly restricted to the tumor hemisphere but not restricted to the tumor, raising some concern about autoimmunity. However, no changes in brain morphology were observed, and long-term survivors showed no evidence of behavioral abnormalities at any point. Impressively, MC57-SIY-Hi tumors were almost completely eliminated within 2 days of the surge of T cell infiltration despite a faster growth rate than MC57-SIY-Lo (Fig. 3, C and D; note difference in y-axis scale). This faster growth rate was also observed in cell culture (data not shown).

If the s.c. injection of cancer cells was omitted, some 2C T cells infiltrated both SIY-Hi and SIY-Lo brain tumors by day 5, but the response was greatly diminished (Fig. 2D). Likewise, 2C T cells activated by a s.c. injection of MC57-SIY-Hi cells were able to infiltrate parental (SIY-negative) MC57 tumors in the brain (Fig. 2E), suggesting local inflammatory cues existed that recruited the activated 2C T cells. However, the possibility that 2C T cells expressing dual TCR may have recognized additional tumor Ags cannot be ruled out. Compared with MC57-SIY-Hi or SIY-Lo brain tumors, substantially fewer 2C T cells were retained in parental MC57 brain tumors at 5 days posttransfer (Fig. 2E compared with Fig. 2C).

In a unilateral brain tumor model, adoptively transferred 2C CTL completely eliminated SIY-Hi brain tumors and cured mice while mice with SIY-Lo tumors usually relapsed

Survival experiments were done to determine whether tumor-infiltrating 2C CTL were able to completely cure mice with brain tumors expressing high or low levels of SIY peptide. Mice with 5-day-old unilateral MC57-SIY-Hi, MC57-SIY-Lo, or MC57 parental brain tumors received unactivated 2C T cell transfers and a small s.c. injection of the same cancer cells to ensure activation of the transferred cells. 2C T cell treatment cured 100% of mice with MC57-SIY-Hi tumors and significantly extended survival of mice with MC57-SIY-Lo tumors (median survival, 46 vs 17 days with no 2C transfer; Fig. 4A). Mice with MC57-SIY-Lo brain tumors that reached criteria for euthanasia were confirmed to have recurring brain tumors postmortem. Mice with either MC57-SIY-Hi or MC57-SIY-Lo brain tumors that did not receive 2C T cells survived similarly as mice with parental MC57 brain tumors treated with 2C T cells (median survival times of 16, 17, and 18 days, respectively; Fig. 4A).

In a bilateral brain tumor model, adoptively transferred 2C CTL eliminated SIY-Hi tumors but failed to eliminate SIY-Lo tumors

To evaluate whether ineffective priming or cytotoxic effector function of adoptively transferred 2C T cells were responsible for the observed relapses of mice with MC57-SIY-Lo brain tumors, survival experiments were done to determine whether 2C CTL could clear both SIY-Hi and SIY-Lo brain tumors present in the same
mouse. MC57-SIY-Hi and MC57-SIY-Lo cells were infused bilaterally into the brains of mice. Mice received 2C T cell transfers and s.c. injections of both SIY-Hi and SIY-Lo cancer cells bilaterally to activate the CTL 5, 6, or 10 days later to determine any effect of timing. In Fig. 4, B–D, each mouse that received 2C T cells is represented by two curves, showing survival time dependent on which brain tumor recurred. Brains of mice that reached criteria for euthanasia were histologically examined postmortem to determine which tumor had recurred. When 2C T cell adoptive transfers were given on day 5, MC57-SIY-Hi tumors were eliminated in all mice, indicating that the 2C CTL had been sufficiently activated and were able to kill (Fig. 4A). However, two of five mice died from recurring MC57-SIY-Lo tumors, even though MC57-SIY-Hi tumors were rejected in the same mice.

The timing of adoptive transfer with respect to tumor growth was critical. Control mice with bilateral tumors that did not receive 2C T cell treatment survived only 14 days (Fig. 4A). Mice receiving 2C T cells on day 5 following cancer cell infusion included three long-term survivors (out of five), and all mice rejected MC57-SIY-Hi tumors (Fig. 4B). Mice receiving 2C T cells on day 6 did not survive, but death was significantly delayed by 2C T cell treatment (Fig. 4C). In four of five cases, recurring MC57-SIY-Lo tumors were responsible for the relapse, and MC57-SIY-Hi tumors were eliminated. If 2C T cell treatment was given on day 10 (i.e., only a few days before mice normally succumb to the tumors), both MC57-SIY-Hi and MC57-SIY-Lo tumors continued to grow and were rapidly fatal (Fig. 4D). Presumably, CTL killing cannot keep up with the rapidly dividing cancer cells once the tumor reaches a critical mass. In this regard, it is important to note that by day 12 following infusion, the tumor masses occupied >50% of the brain in some coronal planes assessed by magnetic resonance imaging (data not shown).

Adoptively transferred 2C T cells persist in vivo but no longer recognize MC57-SIY-Lo brain tumors that recur due to Ag loss

To address the possibility that adoptively transferred 2C T cells did not persist in mice with MC57-SIY-Lo brain tumors that recurred, we examined tumors, draining cervical lymph nodes, and spleen tissue collected from mice when they reached criteria for euthanasia. 1B2 mAb staining revealed substantial numbers of 2C T cells in recurring brain tumors and lymphoid tissue of all mice evaluated 34 or 35 days following adoptive transfer.
transfer of 2C T cells (n = 3; supplemental Fig. 1). To determine whether the recurring MC57-SIY-Lo brain tumors were SIY-ALV cells no longer recognized by 2C T cells, tumors were explanted from mice (n/H11005 = 5) when they reached criteria for euthanasia following relapse 31–51 days following 2C T cell transfer. All mice had received 2C T cell transfers and s.c. cancer cell injections on day 5. Tumors were dissociated and then cultured to enrich for live tumor cells. Explanted cancer cells were then stained with anti-Kb mAb and analyzed by flow cytometry. All recurring tumors expressed normal levels of MHC I-Kb (Fig. 5A). To detect surface levels of SIY peptide bound to Kb, explanted cancer cells were also stained with biotinylated single-chain 2C-m67 TCR monomer (Fig. 5B). Flow analysis revealed that the level of 2C-m67 TCR binding to explanted tumor samples was the same as SIY-negative parental MC57 tumor cells, indicating that recurring MC57-SIY-Lo tumors developed from SIY-loss variants not recognized by 2C T cells.

CD11b+ stromal cells in brain tumors expressing high levels of Ag cross-present tumor peptide on MHC I

We hypothesized that one possible mechanism by which adoptively transferred 2C T cells can eliminate SIY-Hi but not SIY-Lo brain tumors may be related to stromal cell cross-presentation of available tumor Ag to CTL in the tumors. To evaluate the role of CD11b+ stromal cells, MC57-SIY-Hi, SIY-Lo, or parental cells were infused into the brains of mice. Ten days later, all mice received an adoptive transfer of naive 2C T cells and a s.c. injection of MC57-SIY-Hi cells to activate the transferred cells. Two days following 2C T cell treatment, when T cells are extravasating into the brain tumors in large numbers and presumably interacting with stromal cells, mice were sacrificed and brains were excised. CD11b+ cells purified from dissociated brain tumors were incubated with biotinylated 2C-m67 TCR monomers. Flow cytometry analysis showed that CD11b+ cells purified from MC57-SIY-Hi brain tumors presented SIY peptide bound to Kb on their surface (Fig. 6A, right panel). However, no increase in surface levels of SIY/Kb was detectable on CD11b+ cells purified from SIY-Lo brain tumors (Fig. 6A, middle panel). CD11b+ cells from parental (SIY-negative) brain tumors served as a negative staining control (Fig. 6A, left panel). Tumor peptide was therefore cross-presented by CD11b+ tumor stromal cells to 2C T cells infiltrating MC57-SIY-Hi brain tumors 2 days posttransfer but not on CD11b+ cells from MC57-SIY-Lo brain tumors. Fig. 6B shows 2C-m67 TCR staining of two independent CD11b+ cell isolations from MC57-SIY-Lo brain tumors that stain similarly to CD11b+ cells from MC57 parental tumors (12.7, 15.6, and 12.1 mean fluorescent units, respectively), whereas two independent CD11b+ cell isolations from MC57-SIY-Hi brain tumors express significantly higher levels of SIY/Kb on the cell surface (64.9 and 84.3 MFU). The percentage of dead CD11b+ stromal cells in SIY-Hi and SIY-Lo tumors 2 days following injection of 2C T cells were also compared, and no differences were observed (data not shown). Whether bystander elimination of ALV cells in MC57-SIY-Hi tumors occurred as a

4 The online version of this article contains supplemental material.

**FIGURE 3.** Regression of both MC57-SIY-Hi and MC57-SIY-Lo brain tumors after 2C T cell transfer. A. One day after 2C T cell transfer (6 days after bilateral infusion of SIY-Hi and SIY-Lo cancer cells into striatum), tumors are similarly well-established. B. Five days after 2C T cell transfer (10 days after bilateral infusion of cancer cells into striatum), both tumors have nearly completely regressed. Arrows indicate location of tumors. C and D. Quantitation of regression of both MC57-SIY-Hi and MC57-SIY-Lo brain tumors after 2C T cell transfer. Mice with bilateral tumors that received 2C T cells and a small s.c. infusion of MC57-SIY-Hi cells on day 5 were euthanized on day 6 (1 day after 2C transfer), day 8 (3 days after 2C transfer), and day 10 (5 days after 2C transfer). Both MC57-SIY-Hi and MC57-SIY-Lo tumors regressed within 5 days of receiving 2C T cells. n = 4–8 mice/group at each time point. Two-way ANOVA of treatment and time, significant interaction, p < 0.001; Bonferroni comparison of 2C vs control on day 5, p < 0.001.
result of increased 2C T cell killing of stromal cells cross-presenting SIY peptide cannot be confirmed, perhaps because stromal cell killing occurs later than 2 days after T cell transfer. 2C T cells only begin to appear in the brain between 1 and 3 days following adoptive transfer. However, direct assessment of the ability of brain tumor APC to cross-present tumor-derived peptides demonstrated that cross-presentation can and does occur in the brain when sufficient tumor Ag is available.

Activated 2C CTL can also suppress the growth of SIY-expressing tumors without direct cancer cell recognition, possibly by targeting CD11b^+ stromal cells cross-presenting SIY peptide

To further examine whether stromal cell cross-presentation of tumor Ag enhances tumor elimination, we characterized the 2C T cell response to allogeneic H-2^k PRO4L-SIY tumors in the brain. Despite the inability of 2C T cells to directly recognize SIY peptide on the cell surface due to MHC mismatch, activated 2C T cells had potent antitumor effector function in these mice with high-Ag-expressing tumors. PRO4L-SIY cells were infused into the brains of H-2^k C57BL/6 Rag1^-/- mice. Five days later, naive 2C T cells were adoptively transferred into the mice. Half of the mice were also received a small inoculation of MC57-SIY-Hi cells s.c. to activate the T cells. All s.c. MC57-SIY-Hi cells were cleared, and no s.c. tumors grew out in any of the mice. In the absence of the s.c. injection of MC57-SIY-Hi cells, no survival benefit was conferred by the adoptively transferred 2C T cells (Fig. 7A). However, with the s.c. challenge of MC57-SIY-Hi tumor, whereas four of five mice succumbed to MC57-SIY-Lo recurring tumors. Value of p < 0.01 for MC57-SIY-Hi vs MC57-SIY-Lo. D, When 2C T cells were injected on day 10, no therapeutic effect was achieved for either tumor.

FIGURE 4. Effect of adoptively transferred 2C T cells on survival of mice bearing unilateral or bilateral SIY-Hi and SIY-Lo brain tumors. A, Median survival of untreated mice with unilateral brain tumors was 16 days for MC57-SIY-Hi and 17 days for MC57-SIY-Lo. Median survival was 18 days for parental MC57 with 2C T cell transfer. Mice with tumors expressing either high or low levels of SIY peptide lived significantly longer when treated with 2C T cells 5 days following infusion of cancer cells, compared with no treatment (p < 0.01). Approximately 80% of mice with MC57-SIY-Lo brain tumors relapsed and died; median survival was 46 days. One hundred percent of MC57-SIY-Hi tumor mice were cured by 2C T cell treatment. Survival of MC57-SIY-Hi mice that received 2C T cells differed significantly from MC57-SIY-Lo mice that received 2C T cells (p < 0.05). B–D, Effect of adoptively transferred 2C T cells on survival of mice bearing bilateral brain tumors. 2C T cells can eliminate MC57-SIY-Hi tumors, but some MC57-SIY-Lo tumors recur in the same mice. Each mouse treated with 2C T cells is represented by two curves. When mice reached criteria for euthanasia, brains were examined histologically to determine which tumor proved lethal. The therapeutic effect of 2C T cells decreased with a delay in treatment as tumors became larger. In mice with bilateral tumors that did not receive 2C T cells, both tumors progressed rapidly, and mice had a median survival of 14 days. The “no 2C” mice (n = 2) in B–D are the same. B, When 2C T cells were given on day 5, MC57-SIY-Hi tumors were eliminated in all mice, but two mice eventually succumbed to MC57-SIY-Lo tumor recurrences. No tumor was evident histologically on the MC57-SIY-Hi side of the brain. Value of p < 0.01 for MC57-SIY-Hi vs MC57-SIY-Lo. C, When 2C T cells were given on day 6 following cancer cell infusion, one mouse succumbed to a recurring MC57-SIY-Hi tumor, whereas four of five mice succumbed to MC57-SIY-Lo recurring tumors. Value of p < 0.01 for MC57-SIY-Hi vs MC57-SIY-Lo. D, When 2C T cells were injected on day 10, no therapeutic effect was achieved for either tumor.

Activated 2C CTL can also suppress the growth of SIY-expressing tumors without direct cancer cell recognition, possibly by targeting CD11b^+ stromal cells cross-presenting SIY peptide.
were immunostained using 4D11 Ab (anti-Ly-49G). Similar numbers of NK cells were observed in tumors of untreated mice and mice that received 2C T cells 1–3 days following T cell transfer, and the number of NK cells was proportional to the size of the tumor, suggesting that NK cells may also participate in the clearance of PRO4L-SIY brain tumors following 2C T cell transfer. However, the recruitment of NK cells to the brain was independent of whether tumors arose from variant cells that do not express SIY peptide. Explanted tumor cells from mice (n = 5) that relapsed from MC57-SIY-Lo brain tumors following adoptive transfer of 2C T cells were evaluated for MHC I expression with anti-K\(^b\) Ab and for SIY/K\(^b\) expression with 2C-m67 TCR monomer staining. A. Tumors that recurred expressed normal levels of K\(^b\). B. Tumor cells no longer expressed measurable surface levels of SIY/K\(^b\) recognized by 2C T cells. Cultured parental MC57 cells and MC57-SIY-Lo cells were used as negative and positive controls, respectively.

**FIGURE 6.** CD11b\(^+\) stromal cells in MC57-SIY-Hi brain tumors but not SIY-Lo brain tumors cross-present tumor Ag on MHC I. CD11b\(^+\) cells were purified from established MC57, SIY-Lo, and SIY-Hi brain tumors 2 days after adoptive transfer of 2C T cells and stained with 2C-m67 TCR monomers. A. Representative plots of dual-stained (anti-CD11b and biotinylated 2C-m67 TCR monomers) purified brain tumor APC from MC57, SIY-Lo, and SIY-Hi brain tumors. B. 2C-m67 TCR staining of independent samples of purified live CD11b\(^+\) cells collected from different mice demonstrated that detectable levels of cross-presentation of SIY peptide on CD11b\(^+\) stromal cells occurs only in tumors with high levels of available tumor Ag (MC57-SIY-Hi). Staining levels of CD11b\(^+\) cells from MC57-SIY-Lo brain tumors were indistinguishable from those of CD11b\(^+\) cells from parental MC57 tumors. Two brain tumors were pooled per sample, and two samples per group were evaluated.

**Discussion**

Our finding of a differential effect of adoptive T cell therapy on brain tumors expressing high or low levels of the target Ag supports the idea that stroma plays a role in T cell-mediated rejection of brain tumors. In the present experiments, activated CD8\(^+\) T cells were able to kill cancer cells expressing high or low levels of Ag efficiently, yet brain tumors expressing low levels of Ag recurred as a result of ALV cancer cells.

In these studies, the tumor stroma was allowed to establish before initation of treatment to best model the stromal environment T cells would encounter in an endogenously arising brain tumor. Stromal cells with macrophage/microglia surface markers represent approximately one-third of all cells in glioma biopsies (52, 53) and between 5 and 35% of the total tumor burden in rodent glioma models (54, 55). We were able to purify and characterize a population of CD11b\(^+\) macrophage/microglia stromal cells representing ~10% of total tumor burden in the present experiments.

From studies evaluating adoptive T cell therapy in peripheral tumor models, we know that cross-presentation of tumor Ags by stroma allows activated T cells to kill stromal cells (46, 47). Using single-chain, high-affinity TCR monomers, we have directly demonstrated that brain tumor CD11b\(^+\) stromal cells can cross-present...
tumor Ags at detectable levels in tumors expressing high levels of tumor Ag but not in tumors with low levels of Ag and that these cross-presenting stromal cells are likely targeted and eliminated by Ag-specific, adoptively transferred T cells.

Stromal cell cytolysis may enhance the therapeutic effect conferred by adoptively transferred T cells in three ways. First, trophic support provided by stromal cells is eliminated. Stromal cells can enhance tumor growth by providing growth factors and stimulating angiogenesis. Destruction of tumor stroma results in a less hospitable environment for residual cancer cells, including ALV, that escape direct T cell killing and continue to proliferate. Thus, presentation of Ag by stromal cells could play an important role in preventing recurrences. Second, elimination of stromal cells may remove a source of T cell suppression. Stromal cells can serve as a physical barrier for tumor-infiltrating T cells and have multiple mechanisms for suppressing CTL. We did not examine the phenotype of the CD11b+ stromal cells in the present experiments, but in other tumor models, we have found that cancer cells infused into the brain resulted in an influx of Gr1+CD11b+ suppressor phenotype cells (56). Elimination of these myeloid-derived suppressor cells removes one form of suppression on tumor-infiltrating CTL.

Third, brain tumor stroma may provide a niche that supports chemoresistant and radiation-resistant cancer stem cells that express low or no MHC I- or NK cell-activating ligands on their surface (57); therefore, targeting tumor stroma may be an ideal way to eliminate these cells.

In the present experiments, the extent to which the tumors had progressed before T cell therapy was initiated determined whether a differential effect of T cells on the growth of high- and low-Ag-expressing tumors was observed. After adoptive T cell treatment on day 5 after tumor cell infusion, both MC57-SIY-Hi and MC57-SIY-Lo tumors were established but treatable, and they initially regressed within 5 days. If mice had been monitored for only 35 days, it would have appeared that all mice were long-term survivors. Instead, two mice relapsed from MC57-SIY-Lo tumors and were euthanized at 42 and 54 days. When the transfer of 2C T cells occurred on day 6 after tumor infusion, all of the MC57-SIY-Lo tumors recurred, with a median survival time of 65 days. However, the MC57-SIY-Hi tumors were completely cleared in 9 of 10 mice when the adoptive transfer occurred on day 5 or 6 after tumor infusion. Finally, if T cell adoptive therapy was delayed until day 10 following cancer cell infusion, it was too late for a therapeutic effect for either tumor, and mice died within the next 4 days with no prolongation of survival compared with untreated controls. Thus, at intermediate time points when the tumors were well-established but treatable, the abundance of Ag available for cross-presentation may have prevented recurrences of the MC57-SIY-Hi tumors (9 of 10 long-term survivors), and the scarcity of Ag made it more likely that recurrences would occur in the MC57-SIY-Lo tumors (only 3 of 10 long-term survivors).

Two lines of evidence, histological evaluation and calculations of tumor growth patterns, suggest that T cells initially cleared nearly all of the cancer cells from the brain. Following T cell therapy at the most effective time point, there was no clear histological evidence of tumor on day 10, 5 days after T cell transfer. Calculated estimations of tumor growth are consistent with the idea that very few cancer cells remained at that time point. The median survival time for untreated bilateral tumor mice was 14 days, and 17 days for unilateral tumors, in the absence of T cell therapy. If there were only a single surviving cancer cell after the T cell treatment, with a doubling time of 24 h, it would take 2 wk to re-establish the original tumor inoculation and another 2–3 wk

FIGURE 7. Activated 2C CTL are unable to directly recognize H-2^K^ expressing PRO4L-SIY brain tumors but are able to significantly prolong survival and achieve cures in tumor-bearing mice. A. In the absence of a s.c. infusion of MC57-SIY-Hi cancer cells to activate the T cells, 2C T cell transfer had no effect on survival of mice bearing PRO4L-SIY tumors in the brain (n = 5). B. With a concurrent s.c. infusion of MC57-SIY-Hi cancer cells, 2C T cell transfer significantly prolonged survival of mice with PRO4L-SIY brain tumors (n = 5; p < 0.01). Brains were examined for residual tumor, but none was found in the long-term survivors. C and D. Growth inhibition by 2C T cell adoptive transfer was specific to PRO4L tumors expressing SIY peptide. Mice received bilateral cancer cell infusions of PRO4L-SIY and PRO4L-gp33 in the brain, followed by 2C T cell transfer on day 5. Mice were euthanized on day 10 (5 days after 2C transfer). Brain tumor volumes were reconstructed by quantifying tumor areas of sections through the extent of the brains: PRO4L-SIY in C and PRO4L-gp33 in D. n = 8 for 2C and n = 5 for control, p < 0.05 for PRO4L-SIY.

FIGURE 8. CD11b+ stromal cells from PRO4L-SIY brain tumors cross-present tumor peptide following 2C T cell adoptive transfer. Mice with PRO4L-SIY brain tumors established for 1 wk were adoptively transferred unactivated 2C T cells or control wild-type lymphocytes and given a s.c. infusion of MC57-SIY-Hi cells to activate T cells. A. 2C-m67 TCR monomer staining detected higher levels of cross-presentation of SIY peptide by CD11b+ cells in tumors of mice treated with 2C T cells than in mice that received control lymphocyte transfers. B. Proportionally more CD11b+ stromal cells were dead, possibly due to 2C T cell killing, in PRO4L-SIY brain tumors of mice that received 2C T cell transfers compared with controls (40 vs 25%, respectively, % PI+CD11b+/all CD11b+).
to grow to a size of ~1 cm³, the point of producing neurological symptoms. In cases of recurring MC57-SIY-Lo tumors, we were able to demonstrate that the cancer cells were ALV. It may be that the residual tumor stroma nurtured a few ALV cells and allowed them to repopulate the tumor.

Our experiments with allogeneic PRO4L-SIY tumors suggest that activated T cells can clear an established brain tumor by targeting the tumor stroma, without any direct recognition of the cancer cells. PRO4L-SIY tumors cannot be directly killed by 2C T cells but do provide SIY peptide for cross-presentation on stromal cells. Cross-presentation of brain tumor peptides to CD4 T cells (58) and CD8 T cells (59) has been reported previously, inferred from experiments of MHC mismatch between T cells and tumor cells. In the present experiments, we were able to directly detect cross-presentation of tumor Ag on CD11b⁺ stromal cells using single-chain, high-affinity TCR, as well as demonstrate the apparent targeting and killing of these cross-presenting stromal cells.

PRO4L-SIY brain tumor growth was controlled following an adoptive transfer of 2C T cells, and the response was tumor specific because the growth of PRO4L-gp33 tumors was not affected by 2C T cells. Indeed, activated 2C T cells were able to completely eliminate PRO4L-SIY tumors from the brain. This is in contrast to peripheral tumor models where an equilibrium was established between the transferred T cells and residual PRO4L-SIY cells (47), and tumors grew out in mice that were subsequently depleted of CD8⁺ T cells. Plautz et al. (58) observed a similar phenomenon with CD4 T cells adoptively transferred into mice bearing MCA tumors in the brain, which do not express MHC II, but were nevertheless eliminated by T cell treatment. In addition to killing stromal cells, it is unknown whether other effector mechanisms are involved in 2C T cell rejection of PRO4L-SIY brain tumors. We did not examine the possibility that the degree of NK cell cytotoxicity may be changed in the presence of activated CD8⁺ T cells, but we did observe similar numbers of NK cells in PRO4L-SIY tumors with or without adoptive T cell therapy. However, it is possible that NK cells may have been more cytotoxic in the presence of 2C T cells interacting with cross-presenting stromal cells. Shanker et al. (60) observed that CD8⁺ T cells allow rejection of Ag-negative cancer cells variants by a mechanism dependent on NK cells, and Wu et al. (57) demonstrated that the production of IFN-γ causes glioma stem cells to be susceptible to killing by NK cells. It is thus possible that the interaction of 2C T cells with cross-presenting stromal cells also enhances NK cell function, allowing rejection of allogeneic PRO4L-SIY cells but not PRO4L-gp33 cells. This possibility warrants further investigation.

In addition to stromal microglia/macrophages, other elements of tumor stroma may be targets of T cells. Liver endothelial cells are able to cross-present Ags from apoptotic tumor cells (61, 62). We observed a reduction of in the number endothelial cells in PRO4L-SIY brain tumors of mice treated with 2C T cells. This could be a consequence of the reduced tumor size but requires further study.

With respect to therapeutic implications of these results, targeting tumor stroma in addition to malignant cancer cells may help prevent tumor recurrence. In peripheral tumor models, low-Ag-expressing tumors can be damaged by radiation or chemotherapy to produce a surge of Ag available for cross-presentation, and an appropriately timed adoptive T cell treatment can become more effective (46). The present results suggest such a strategy may be effective for brain tumors expressing endogenous levels of Ag as well. Other strategies for delivering tumor Ag, e.g., via viral vectors, could also enhance T cell targeting of stroma and help prevent tumor recurrence.

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Disclosures
The authors have no financial conflict of interest.

References


Supplemental Figure 1. Adoptively transferred 2C T cells persist in mice that relapse from recurring MC57-SIY-Lo brain tumors. Mice were infused in the brain with MC57-SIY-Lo cancer cells and received a 2C T cell transfer and a s.c. MC57-SIY-Lo cell infusion to activate 2C T cells on day 5. Tissues were collected when mice (n=3) reached criteria for euthanasia following relapse 34 to 35 days later. Immunostaining with 1B2 antibody demonstrated persistence of 2C T cells in all mice. Representative images are shown. Red = 2C T cells stained with 1B2 clonotypic antibody. Blue = DAPI counterstain. A. Brain tumor, scale bar = 1mm. B. Spleen, scale bar = 200 μm. C. Draining cervical lymph node, scale bar = 200 μm.

Supplemental Figure 2. Endothelial cell (CD31/PECAM) staining is reduced in PRO4L-SIY brain tumors of mice treated with 2C T cells. Mice bearing PRO4L-SIY tumors were treated with 2C T cells, injected s.c. with a small burden of PRO4L-SIY cells on day five to activate 2C T cells, and euthanized on day ten. Brain sections were stained with an antibody against CD31/PECAM, and visualized with SA-594. The area of endothelial cell staining was reduced in the 2C T cell treated mouse (A) compared to control mice that did not receive 2C T cell adoptive transfers (B). Scale bars= 200μm. C. The mean area of stained vessels was quantified. n’s = 8 for 2C T cell treatment, 5 for controls. One to two random brain tumor sections per mouse were analyzed. p < 0.01 for 2C T cell treatment.