Kupfer-type immunological synapse characteristics do not predict anti-brain tumor cytolytic T-cell function in vivo

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To analyze the in vivo structure of antigen-specific immunological synapses during an effective immune response, we established brain tumors expressing the surrogate tumor antigen ovalbumin and labeled antigen-specific anti-glioma T cells using specific tetramers. Using these techniques, we determined that a significant number of antigen-specific T cells were localized to the brain tumor and surrounding brain tissue and a large percentage could be induced to express IFNγ when exposed to the specific ovalbumin-derived peptide epitope SIINFEKL. Detailed morphological analysis of T cells immunoreactive for tetramers in direct physical contact with tumor cells expressing ovalbumin indicated that the interface between T cells and target tumor cells displayed various morphologies, including Kupfer-type immunological synapses. Quantitative analysis of adjacent confocal optical sections was performed to determine if the higher frequency of antigen-specific antiangioma T cells present in animals that developed an effective antitumor immune response could be correlated with a specific immunological synaptic morphology. Detailed in vivo quantitative analysis failed to detect an increased proportion of immunological synapses displaying the characteristic Kupfer-type morphology in animals mounting a strong and effective antitumor immune response as compared with those experiencing a clinically ineffective response. We conclude that an effective cytolytic immune response is not dependent on an increased frequency of Kupfer-type immunological synapses between T cells and tumor cells.

anti-tumor immunity | brain tumors | immunotherapy

Interactions between effector T cells and target antigen-presenting cells (APCs) reveal a complex, spatiotemporally dynamic machinery at the intercellular interface. Immunological synapses (IS) were first characterized in the context of CD4+ T-cell recognition of antigen presented by professional APCs. According to Monks et al. (1) and subsequently supported by many studies (2–9), upon ligation of the MHC/peptide complex by the T-cell receptor (TCR), several membrane-associated proteins, including the TCR complex and downstream kinases, become concentrated at the center of the contact interface, the central supramolecular activation complex (cSMAC), whereas others [e.g., lymphocyte function-associated antigen 1 (LFA-1), talin, and CD45] are excluded to form an outer concentric ring, the peripheral supramolecular activation complex (pSMAC) (1, 5, 10, 11). The structures displayed by CD8+ cytotoxic T lymphocytes (CTL) as they interact with target cells share several important morphological and functional characteristics with the CD4/APC interaction (5, 12–18). Stinchcombe et al. (12) examined the synapses between primary mouse CD8+ CTL and mouse mastocytoma targets, describing in detail the formation of cSMAC and pSMAC at the cytotoxic interface and how cytolytic granules are brought to a secretory subdomain within the cSMAC to focus cytotoxicity onto the targets (5, 12, 13). On the other hand, the need for stable IS for target-cell killing has been questioned by the observation that low levels of antigen on APCs stimulate Fas-mediated CTL killing without formation of stable Kupfer-type IS (19–21). Thus, the function of stable IS induced by higher antigen levels may be related more closely to perforin-mediated cytotoxicity (20) and/or cytokine secretion (15, 22). In addition to these in vitro studies, we and others have described SMACs in T cells in vivo, and the resulting cellular reorganization in “postsynaptic” target cells during the effector phase of an antiviral immune response (14–16, 25, 24) and in human brain tumors (17). Thus, although IS appear relevant to viral clearing and cytotoxicity in vitro and in vivo (14–17, 23, 24), their absolute requirement has been questioned (19–21, 25).

To study the role of Kupfer-type IS during an antigen-directed brain tumor immune response in vivo, we examined contacts between CTLs and targets in a well-characterized brain tumor model (26, 27), comparing 2 situations that differ in the effectiveness of the immune response. GL26 glioma tumors in the mouse brain do not elicit effective immunity and kill the hosts. However, they can be treated successfully with an immunostimulatory regimen of adenoviral (Ad) vectors encoding the cytokine Fms-like tyrosine kinase 3 ligand (Flt3L) and the conditionally cytotoxic transgene thymidine kinase (TK), inducing strong anti-tumor CTL response (26). To assess antigen specificity using MHC tetramers and cognate epitope-induced IFNγ production, GL26 glioma cells were engineered to express the antigen chicken ovalbumin.

We predicted that in treated animals, a higher proportion of IS would display typical Kupfer-type cSMAC/pSMAC IS morphology. Although antigen-specific antiangioma T cells were indeed enriched in tumors and brains of treated animals, these T cells displayed various synaptic morphologies, and there was no increased proportion of Kupfer-type IS in the animals mounting a strong immune response.

Results

Flt3L/TK Treatment Induces Effective Antitumor Immune Responses.

The stable cell line GL26-cOVA expressed ovalbumin as assessed by immunoblotting (Fig. S1 A and B) or immunocytochemistry (Fig. S1 C and D), and brain tumors derived from these cells expressed ovalbumin as assessed by immunohistochemistry (Fig. S1 E and F). As has been described before using wild-type GL26 glioma cells (28) 20,000 GL26-cOVA cells implanted straitly...
developed into a large tumor (Fig. 1A and Fig S1 E and F) that killed untreated hosts. Treatment with intratumoral injection of Ad.Flt3L and (Ad.TK) and systemic ganciclovir enabled ∼50% of the animals to clear the tumor and survive, whereas animals injected with saline died within 30 days of tumor implantation (Fig. 1B). Saline-injected animals killed at 27 days had large tumors occupying most of the striatum; treated animals had much smaller tumors (Fig. 1A).

Flow cytometry of lymphocytes labeled with anti-CD3ε or anti-CD8 antibodies and ovalbumin-derived peptide epitope SIINFEKL-H2Kβ tetramers revealed more ovalbumin-specific T cells among the tumor-infiltrating cells in the treated animals than in controls (Fig. 1C), a difference that was not seen in the cervical lymph nodes or spleen (Fig. S2). Similarly, tumor-infiltrating CD3+ CD8+ cells from treated animals produced IFNγ in response to SIINFEKL at a higher frequency than did those from controls (Fig. 1D), and this difference was not observed in cells from the lymph nodes or spleen (Fig. S3). Total numbers of CD3ε immureactive cells were similar between groups (Fig. S4 A and B), although the tumors in treated animals displayed areas of very high T-cell density that were not observed in the controls (Fig. S4). Likewise, total numbers of brain-infiltrating CD4+, CD8+, and Foxp3+ cells did not differ between treated and control animals, although in treated animals all these cell types accumulated most densely in a smaller area of the brain corresponding to the remnants of the treated tumor and the immediately surrounding brain (Fig. S5).

Figure 1. Flt3L plus TK and ganciclovir treatment eliminates GL26 tumors and induces tumor infiltration of antigen-specific CD8+ T cells. (A) Comparison of GL26-cOVA tumors injected with saline (Top, or Flt3L/TK plus ganciclovir (Bottom). Brains were labeled using antibodies to CD8 to detect tumor-infiltrating T cells. Yellow arrows show extent of tumor by day 27. (Scale bar, 1 mm.) (B) Kaplan–Meier diagram comparing survival of Flt3L/TK-treated mice (solid red line) with saline-injected animals (dashed blue line). (C) Flow cytometry comparing frequencies of SIINFEKL-H2Kβ tetramer-labeled CD3ε/CD8+ tumor-infiltrating lymphocytes from saline-treated mice (Upper plots), and Flt3L/TK-treated mice (Lower plots). Cells were gated on CD8 and CD3 expression (Left plots), and tetramer binding was assessed within that population (Right plots; Y axis is side scatter). Percentages on each density plot are the proportion of cells falling within the zone defined by the small black rectangle. Each pair of density plots represents one animal. (D) The column scatter graph shows the percentage of CD8ε/CD3ε+ cells that bind tetramer for 7 saline mice (black triangles) and 13 Flt3L/TK mice (brown triangles). Horizontal bars are group means; *, significantly greater than saline group (P < 0.05). (E) Density plots comparing the frequency of cells producing IFNγ in response to SIINFEKL stimulation in tumor-infiltrating lymphocytes from saline-treated mice (Upper) and from Flt3L/TK-treated mice (Lower). (F) The column scatter graph shows the percentage of CD8ε/CD3ε+ cells that produce IFNγ in response to SIINFEKL stimulation for 7 saline-treated mice (black triangles) and 13 Flt3L/TK-treated mice (brown triangles). Horizontal bars are group means; *, greater than saline (P < 0.05).
an alternative pattern (consisting of a co-concentration of LFA-1 and tetramer at the contact interface) was observed also. Fig. 3C shows a single ovalbumin-expressing tumor cell being attacked simultaneously by a CTL with Kupfer-type morphology (K, Left), and by a second CTL with both markers concentrated at the contact (N, Right). The degree of colocalization or mutual exclusion is shown in the intensity plots in Fig. 3, which show the relative fluorescent intensity associated with LFA-1 or tetramer, measured along a path drawn following the T-cell membrane, from one side of the contact to the other, for each contact. Both kinds of morphology and various intermediate morphologies were observed in both treated and control animals.

**Distribution of Synapse Phenotype Is Not Correlated with Effectiveness of Immune Response.** Following the observation of T-cell/target interfaces displaying Kupfer-type SMAC organization in both treated and control animals, we undertook a larger-scale, quantitative analysis of the morphologies of contacts. Tissue from 2 groups was immunolabeled for ovalbumin (to identify target glioma cells), LFA-1 (to identify the pSMAC), and CD3ε (to identify cSMAC). We then captured confocal stacks including >50 contacts from each group. Contacts were defined by polarized CD3ε at a T-cell/target interface and quantified. For each contact we identified the optical section that most centrally bisected T-cell/target cell contact; on this and adjacent sections (total, 3 sections per contact), we delineated the area at the center of the contact (representing the cSMAC) and the area to each side (the pSMAC) and recorded the mean fluorescent energy of the LFA-1 channel and the CD3ε channel. This procedure yielded a measure of the extent to which each contact was organized into Kupfer-type SMAC morphology, corresponding to high central and low peripheral CD3ε and low central and high peripheral LFA-1. Fig. 4 shows confocal images of a typical Kupfer-type synapse from an animal treated with Flt3L/TK and the quantitative data extracted from this synapse. Fig. 4B shows a synapse with the alternative distribution, i.e., co-concentration of the 2 markers at the contact interface. When we examined the contacts in tissue from saline-treated mice, we observed the same diversity of interfaces found in animals treated with Flt3L/TK. Fig. 5 shows confocal micrographs and quantitative data from a Kupfer-type synapse found in a saline-treated animal, and Fig. 5B shows a synapse with both CD3ε and LFA-1 concentrated at the interface.

We predicted that the effective antitumor immune response in animals treated with Flt3L/TK would be reflected in a higher frequency of IS displaying canonical Kupfer-type morphology. Averages from three sections per contact were plotted as a ratio of LFA-1 intensity in the cSMAC to LFA-1 intensity in the pSMAC to yield distributions of the preponderance of Kupfer-type morphology (Fig. 6A). These data also were evaluated with respect to CD3ε concentration in the IS, with LFA-1−/−depleted cSMAC in 1 group (Kupfer-type IS; Fig. 6B) and LFA-1−/−enriched cSMAC in a second group (non–Kupfer-type IS; Fig. 6B) to determine whether polarization of CD3ε correlated with LFA-1 distribution. Distributions were indistinguishable between groups (Fig. 6). To determine whether antigen-specific T cells form comparable IS with target tumor cells, we implanted GL26-cOVA or GL26 cells into RAG1−/− mice and treated the tumors with Flt3L/TK. Two days later, we transplanted 10^7 OT-I splenocytes into treated mice. Nine days later, animals were perfused, and tumors were analyzed for the presence of OT-I cells. OT-I cells infiltrated only tumors expressing ovalbumin (Fig. S6). Polarization of CD3ε (Fig. S7A and B), and IFNγ at contacts (Fig. S7C) suggested the formation of IS; unexpectedly low levels of LFA-1 precluded complete characterization.
Survival, increased tumor infiltration of glioma antigen-specific T cells, and an increase in their IFN-γ production in response to antigen challenge. A higher proportion of T-cell/glioma contacts displaying the characteristic cSMAC/pSMAC morphology of Kupfer-type IS was expected. However, treatment efficacy failed to correlate with an increase in the proportion of Kupfer-type IS between T cells and glioma cells detected in treated animals. In vitro studies suggest that IS underpin intercellular immune interactions. We tested the relevance of IS to in vivo brain tumor immune responses. In culture, CD8⁺ CTL form SMACs only during contact with targets displaying the appropriate antigenic epitopes (3, 5, 19), although the level of antigen displayed by MHC, the concentration of adhesion molecules, and the nature of the epitopes and APC influence the type of junctions formed (6, 15, 30–33). Because lysis by CTLs depends on antigen recognition and cell-to-cell contact, it has been proposed that SMACs are necessary for effective and selective lysis of targets to occur (i.e., to restrict cytotoxic consequences to the appropriate target cells) (4, 12, 13, 15). The amount of antigen required to activate CTL cytolysis in vitro, however, is less than that required to form stable IS, suggesting that at least some forms of CD8⁺ T-cell-mediated cytotoxicity do not require stable IS (20, 37, 38). It is possible that Fas-mediated cytotoxicity, lytic-granule-mediated cytotoxicity, and cytokine secretion may each be accompanied by differential morphological synaptic specializations (4, 6, 12, 15, 19–22, 25, 34–36).

To demonstrate the necessity of IS for immune-mediated tumor cytotoxicity in vivo is more challenging; within the tumor microenvironment T cells simultaneously may contact several cells, which may display appropriate antigenic epitopes. Nevertheless, Kupfer-

### Discussion

The morphology of IS would be predicted to reflect the therapeutic efficiency of the antitumoral immune response. In our experiments, treatment-induced therapeutic efficiency was reflected in increased survival, increased tumor infiltration of glioma antigen-specific T cells, and an increase in their IFNγ production in response to antigen challenge. A higher proportion of T-cell/glioma contacts displaying the characteristic cSMAC/pSMAC morphology of Kupfer-type IS was expected. However, treatment efficacy failed to correlate with an increase in the proportion of Kupfer-type IS between T cells and glioma cells detected in treated animals. In vitro studies suggest that IS underpin intercellular immune interactions. We tested the relevance of IS to in vivo brain tumor immune responses. In culture, CD8⁺ CTL form SMACs only during contact with targets displaying the appropriate antigenic epitopes (3, 5, 19), although the level of antigen displayed by MHC, the concentration of adhesion molecules, and the nature of the epitopes and APC influence the type of junctions formed (6, 15, 30–33). Because lysis by CTLs depends on antigen recognition and cell-to-cell contact, it has been proposed that SMACs are necessary for effective and selective lysis of targets to occur (i.e., to restrict cytotoxic consequences to the appropriate target cells) (4, 12, 13, 15). The amount of antigen required to activate CTL cytolysis in vitro, however, is less than that required to form stable IS, suggesting that at least some forms of CD8⁺ T-cell-mediated cytotoxicity do not require stable IS (20, 37, 38). It is possible that Fas-mediated cytotoxicity, lytic-granule-mediated cytotoxicity, and cytokine secretion may each be accompanied by differential morphological synaptic specializations (4, 6, 12, 15, 19–22, 25, 34–36).

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### Fig. 4
Confocal micrographs of T cells and targets from GL26-cOVA tumors in mice injected with Flt3L/TK. (A) A synapse that exhibits the Kupfer-type SMAC organization. (B) An interface with an alternative distribution (i.e., co-concentration of LFA-1 and CD3ε). The left and middle images in the top rows show the distribution of CD3ε (magenta) and LFA-1 (red), respectively. In the LFA-1 image, a yellow arrow shows the point at which the T cell contacts the target. The right image in the top row shows the combination of these channels together with immunolabeling for ovalbumin (green) and DAPI (blue). Here the T cell is marked “T,” and the ovalbumin-expressing target is marked “O.” On the bottom right is another merged image in which the areas corresponding to the cSMACs are marked with solid yellow lines and the pSMACs with broken white lines. In these regions of interest, mean fluorescent density was recorded for LFA-1 and CD3ε on three adjacent sections through the T-cell/target cell contact; these data are shown in the bar charts (Bottom rows left and middle). The light gray bar is the measurement from the section centrally bisecting the contact, and the white and dark gray bars are from the adjacent sections. The bar chart at the left of the bottom row shows measurements of CD3ε, and the bar chart in the middle of the bottom row shows LFA-1. The quantification and statistical analysis of all contacts studied are shown in Fig. 6.

### Fig. 5
Confocal micrographs of T cells and targets from GL26-cOVA tumors in mice injected with saline. (A) A synapse that exhibits the Kupfer-type SMAC organization. (B) An interface with an alternative distribution (i.e., co-concentration of LFA-1 and CD3ε at the center of the interface). The left and middle images in the top rows show CD3ε (magenta) and LFA-1 (red). In the LFA-1 image, a yellow arrow indicates the point at which the T cell contacts the target. The right images in the top rows show the combination of these channels together with immunolabeling for ovalbumin (green) and DAPI (blue). In these images, the T cell is marked “T,” and the ovalbumin-expressing target is marked “O.” The bottom right panels show another merged image in which the areas corresponding to the cSMACs are marked with solid yellow lines and the pSMACs with broken white lines. In these regions of interest, mean fluorescent density was recorded for LFA-1 and CD3ε on three adjacent sections through the T-cell/target cell contact, and these data are shown in the bar charts in left and middle panels of the bottom rows. The light gray bar is the measurement from the section centrally bisecting the contact, and the white and dark gray bars are from the adjacent sections. The bar charts at the left of the bottom rows show measurements of CD3ε, and the bar charts in the middle of the bottom rows show LFA-1. The quantification and statistical analysis of all contacts studied are shown in Fig. 6.
that do not (saline). (\textsuperscript{14, 17, 23, 24, 37–39}).

The causal relevance for IS was studied to assess (i) the antigen specificity of IS during an antitumor immune response and (ii) whether the morphology of IS would reflect the therapeutic efficiency of the in vivo immune response. A tumor model was developed in which tumor cells express the model antigen ovalbumin to facilitate the assessment of the antigen specificity of the interactions in vivo. We found that tetramer-positive CD8\textsuperscript{+} T cells indeed participated in the formation of Kupfer-type IS with target tumor cells expressing the cognate antigen. We examined CTLs infiltrating syngeneic glioma tumors treated with Ad.Fl3L and Ad.TK followed by ganciclovir and in untreated tumors. The advantage of this comparison is that a large number of previous studies have described the induction of an effective immune response in the former and its absence from the latter (26–28, 40). This CD8\textsuperscript{+} T-cell–dependent immune response is thought to be initiated by dendritic cells activated by the combination of Flt3L and Toll-like receptor 2 ligands released from dying tumor cells (28).

In view of the quantitative difference between treated mice and controls, we gathered detailed CD3ε and LFA-1 distribution data from a large number of contacts between T cells and targets from both groups. Our expectation was that, even if SMACs were present in both groups, examination of a large body of data would reveal an increased occurrence of Kupfer-type IS commensurate with other measures of immune response strength, such as the frequency of SIINFEKL–specific IFN-γ-secreting T cells. This expectation was not borne out. Although the immune response was substantially stronger in treated animals, there was no increase in the frequency of Kupfer-type IS.

A number of limitations of the strategy employed here must be considered in interpreting the result that the frequency and characteristics of Kupfer-type IS did not reflect the strength of the immune response. First, we wished to compare IS between animals that differed in the clinical effectiveness of their antitumor immune response rather than performing our comparison of IS in an all-or-none paradigm. However, we believe an all-or-none paradigm (see Figs. S6 and S7) is less informative, because with no T cells in the control animals, it becomes impossible to correlate and quantify differences of IS with strength of immune responses. Therefore, we believe the paradigm used represents a reasonable tradeoff to explore the physiological role of IS in a relevant model of immune-mediated tumor rejection.

A second caveat is that we selected contacts with polarized CD3ε between T cells and OVA-expressing tumor cells in both groups; this selection was necessary as an indication of contact formation between T cells and targets. The selection of cells with polarized CD3ε might have biased our study toward T cells already forming IS in both groups. Because there were similar numbers of T cells in both groups, we expected that the higher proportion of activated antigen-specific T cells in the treated group would be reflected in a higher proportion of effective cytolytic interactions and a higher proportion of Kupfer-type IS, if these structures correlate with the strength of the in vivo immune response. A third caveat is that the quantitative morphological analysis described here was limited to CD3ε and LFA-1 distribution. These molecules were chosen because of the wealth of studies describing and characterizing their involvement in the cSMAC and pSMAC of typical Kupfer-type synapse. Future studies could focus attention on whether the distribution of other synapse-associated molecules provides a better indication of the strength of the anti-brain tumor immune response in vivo. Equally, it is likely that dynamic imaging studies will be necessary to address the function of IS in vivo.

In summary, the results of our studies demonstrate the existence of Kupfer-type antigen-specific IS, accompanied by a wide range of other synapic morphologies in vivo, during immune-mediated elimination of brain tumors. This same continuum of synaptic morphologies was detected both when antigen-specific T cells engaging tumor cells were labeled with specific tetramers and when we carefully characterized the structure of IS through a serially quantitative assessment of the distribution of CD3ε and LFA-1. Elucidating the role of Kupfer-type IS during ongoing in vivo immune responses and the precise morphological correlates of CTL cytotoxicity may require the development of novel imaging techniques to detect simultaneously the morphology and function of T cells by optical means in live animals. Nevertheless, the weight of available data supports the hypothesis that an effective T-cell immune response is mediated by a range of morphological synaptic specializations rather than relying solely on the formation of Kupfer-type IS.

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**Fig. 6.** Quantitative analysis of the frequency of T-cell/glioma cell contacts displaying various distributions of LFA-1 and CD3ε in Flt3L/TK-treated mice (pink triangles), or in control mice injected with saline (black triangles). This figure illustrates detailed quantitative analysis of 111 IS interfaces in which the distribution of CD3ε and LFA-1 at each interface was quantitated in at least three adjacent optical sections in the three regions of interest (as in Figs. 4 and 5). All interfaces were identified by CD3ε strongly polarized toward ovalbumin-expressing glioma cells and were colabeled with LFA-1. (A) Each triangle represents one immunological synapse, with the degree of LFA-1 focusing (i.e., the ratio of LFA-1 in the cSMAC to LFA-1 in the pSMAC) displayed on the vertical axis. IS conforming to the Kupfer-type cSMAC pattern should fall at the bottom of this distribution (indicated by the blue bracket at the right of the figure). IS at the top of the distribution show LFA-1 which tends to colocalize with CD3ε at the interface (green bracket). IS from saline-injected mice are in the left column, and those from Flt3L/TK-treated mice are in the right column. This graph indicates that there is a wide range of LFA-1 distribution at IS interfaces, but there is no difference between animals mounting a therapeutically effective immune response (Flt3L/TK), and those that do not (saline). (B) The total population of IS was divided into two subsets: On the left are the IS in which LFA-1 was depleted at the contact site (i.e., the ratio of LFA-1 in the cSMAC to LFA-1 in the pSMAC is < unity, displaying the pattern of Kupfer-type IS). On the right are those in which LFA-1 was enriched at the contact. The vertical axes represent the degree to which the CD3ε was concentrated in the cSMAC. These two figures indicate that the degree of CD3ε polarization toward the target tumor cells did not vary with the segregation pattern of LFA-1. The entire experiment was repeated twice, with similar results. Differences between groups were analyzed by repeated-measures ANOVA, and resulting P values are shown. Differences between groups were not statistically significant.
Materials and Methods

Animals, Tumor Implantation, Survival Studies, and Immunohistochemistry. Animal experiments complied with Cedars-Sinai Medical Center's Institutional Animal Care and Use Committee. C57BL/6 mice were implanted with GL26 cells and treated with Ad.FIL3/LTK or saline as described in more detail in SI Materials and Methods and in ref.30. For the survival study, 3 mice were injected intratumorally with saline, and 17 animals were injected with Ad.FIL3/LTK and given ganciclovir. Immunohistochemical labeling of ovalbumin, CD3ε, and LFA-1 for quantitative studies was repeated in 2 independent experiments which yielded similar results. C57BL/6 mice were implanted with GL26-OVA and treated 17 days later with Ad.FIL3/LTK (n = 7) or saline (n = 5); 29 days after tumor implantation, animals were perfused with oxygenated Tyrode's solution, followed by 4% paraformaldehyde, and brains postfixed for 24–48 h. 50 μm coronal sections were vibratome cut, immunolabeled, and visualized using immunofluorescence or peroxidase histochemistry using rat anti-mouse CBB, Syrian hamster anti-mouse CD3ε (1:500) (BD Biosciences), rat anti-mouse LFA-1, and rabbit anti-ovalbumin, as described in detail in SI Materials and Methods and refs. 15, 28, and 40.

In Situ Tetramer Labeling. At 29 days after tumor implantation, mice were anesthetized and perfused transcardially with oxygenated Tyrode's solution. Brains were removed, fixed in 2% paraformaldehyde in PBS at room temperature for 30 min, stored overnight in PBS at 4 °C, and embedded in 4% low-melt agarose in PBS. 200 μm sections were cut in ice-cold PBS and stained free-floating for 24 h at 4 °C. Sections were rinsed in PBS, heated to 95 °C in a specific T cell staining buffer, and treated with unconjugated SIINFEKL-H2-KK MHC tetramers (1:200) (Beckman Inc.), because of the rapid bleaching of aliphophocyanin, we used rabbit anti-aliphophocyanin antibodies (Novus Biologicals) 1:500 and goat anti-rabbit conjugated to Alexa 488 (1:500) to visualize tetramers. Multiple labeling of tetramer-stained sections proceeded as follows: rat anti-CD8 (1:5000) (Serotec), or rat anti-LFA-1 (1:500) (BD Biosciences) were detected with Alexa 594-conjugated goat anti-rat (1:500) (Invitrogen) and Alexa 488 (1:500) to visualize tetramers. Multiple labeling of tetramer-stained sections proceeded as follows: rat anti-CD8 (1:5000) (Serotec), or rat anti-LFA-1 (1:500) (BD Biosciences) were detected with Alexa 594-conjugated goat anti-rat (1:500) (Invitrogen) and Alexa 488 (1:500) (Invitrogen). In experiments labeling both tetramers and ovalbumin simultaneously, tetramers were visualized with Dylight 488 goat anti-rabbit Fab fragments (1:50) (Jackson). Ovalbumin was detected with specific rabbit antibodies (1:500) (Abcam), following blocking with unconjugated goat anti-rabbit Fab (1:100) (Jackson) and was visualized with goat anti-rabbit Alexa 594 (1:500) (Invitrogen). The use of unconjugated goat anti-rabbit Fab was optimized to avoid cross-labeling, and controls were included in each experiment.

Confocal Microscopy, Analysis, and Statistics. Subcellular distribution of LFA-1 and CD3ε was analyzed at 136 controls between T cells and ovalbumin-expressing glioma cells in brain sections from 12 mice (7 in the first experiment and 5 in the second). Sections were captured with Leica TCS-SP2 confocal microscope with the PlanAPO 63× 1.4 NA oil objective and LCS ConfoSoft Software (Leica Microsystems) (14–16). A potential contact interface was defined as the junction between a T cell and an ovalbumin-positive glioma cell in which CD3ε was strongly polarized toward the interface and CD3ε and LFA-1 were both present at the interface in at least 3 sequential optical sections. To assess the extent to which the 2 proteins were distributed in the pattern characteristic of the Kappa–ypsilon synapse, mean fluorescence density was measured in 3 adjacent regions in the T-cell side of the contact, the 1 in the center and 1 on each side, in each of 3 successive sections. The overall difference between Ad.FIL3/LTK-treated and saline groups in the degree of LFA-1 exclusion from the cSMAC (Fig. 6A) or the distribution of CD3 towards the cSMAC (Fig. 6B) was compared by repeated-measures ANOVA, with treatment group as the between-subjects variable, using NCSS software. For quantitative analysis of the distribution of tetramers, the LFA-1 staining using anti-tetramer antibody, the relative fluorescent intensity was measured along a path as illustrated in Fig 3.

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Supporting Information

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SI Materials and Methods

Animals, Surgical Procedures, Plasmids, Viral Vectors, and Cell Lines. Female C57BL/6J and Rag1−/− (B6.129S7-Rag1tm1Mom/J; stock number 002216) were obtained from Jackson Laboratories. OT-1 Rag1−/− mice were kindly provided by Toni Ribas (UCLA). All mice were treated with ganciclovir for only 2 days. M. carriage was recorded and analyzed. The brain sections were examined for 5 min on ice, then added OVA H-2Kb tetramer (1:200) (Beckman Inc.) were used. Sections were washed 10 times with PBS-T and then were incubated in rabbit anti-ovalbumin polyclonal antibody (Abcam) and mouse anti-β actin (Sigma).

Flow Cytometry. Splenocytes, lymphocytes from cervical lymph nodes, and tumor-infiltrating immune cells were isolated and analyzed as described in ref. 3. Briefly, immune cells were labeled with antibodies in cell surface-staining buffer (0.1 M PBS, w/o Ca2+, Mg2+, with 1% FBS, 0.1% sodium azide) for analysis by flow cytometry using a FACScan flow cytometer (Beckton Dickinson). To analyze OVA H-2Kb tetramer-specific CD8+ T cells, we first stained cells with CD3-PerCP and CD8a-FTTC (BD Biosciences) for 5 min on ice, then added OVA H-2Kb tetramer-PE (Beckman Coulter), and incubated cells at room temperature for another 25 min. In addition to CD3-PerCP and CD8a-FTTC, intra-cellular IFNγ was labeled for flow cytometry with IFNγ-PE (BD Biosciences) using the Cytofix/Cytoperm Fixation/Permabilization Solution Kit with BD GolgiPlug (BD Biosciences) according to the manufacturer’s instructions.

In Situ Tetramer Labeling of SIINFEKL-Specific CD8+ T Cells in Brain. Wild-type C57BL/6 mice implanted with GL26-cOVA or GL26 cells were used in these experiments. Tumor implantation and Ad.FlI3L/TK treatment or saline injections were performed as described above. At 29 days after tumor implantation, mice were anesthetized with ketamine and xylazine and were perfused transcardially with oxygenated Tyrode’s solution. Brains were removed and fixed in 2% paraformaldehyde in PBS at room temperature for 30 min, stored overnight in PBS at 4°C, embedded in 4% low-melt agarose in PBS, patted dry, and secured to a vibratome block with glue. After the glue had set for at least 1 min, the block was placed in a vibratome bath containing ice-cold PBS. Vibratome sections, 200-μm thick, were generated at minimum speed and maximum amplitude using a standard double-edged razor blade. Sections were stained fixed-floating in 0.5 mL staining buffer containing primary antibodies, 2% normal goat serum in 0.1 M PBS, and 0.01% sodium azide in 24-well tissue-culture plates at 4°C on a rocking platform overnight. Primary antibodies recognizing CD8 (1:5,000) (Serotec) and lymphocyte function-associated antigen 1 (LFA-1; 1:500) (BD Bioscience) and an APC-conjugated SIINFEKL-H-2Kb MHC tetramer (1:200) (Beckman Inc.) were used. Sections were washed five times with PBS at 4°C and then were fixed with 2% paraformaldehyde for 1 h at room temperature. Sections were washed again in PBS and then were incubated overnight at room temperature with rabbit anti-ovalbumin (Novus Biologicals) diluted 1:500 in staining buffer. Sections were washed five times with PBS containing Triton-X (PBS-T) for at least 30 min and then were incubated with appropriate secondary antibodies in staining buffer (Alexa 488-conjugated goat anti-rabbit at 1:500 and Alexa 594-conjugated goat anti-rat at 1:500, both from Invitrogen). Finally, sections were washed five times for at least 30 min, incubated with 5 μg/mL DAPI (Invitrogen), washed, and mounted. For labeling with tetramer, LFA-1, and ovalbumin, tetramers were detected with Dylight 488 goat anti-rabbit Fab (1:50) (Jackson). Sections were washed five times with PBS-T for at least 30 min and blocked with unconjugated goat anti-rabbit Fab (1:100) (Jackson) for 1 h at room temperature. Sections were washed five times in PBS-T at room temperature for 1 h and incubated in rabbit anti-ovalbumin (Abcam) at 1:500 for 48 h at room temperature in staining buffer. Sections were washed five times in PBS-T at room temperature for 30 min and then were incubated overnight with goat anti-rabbit Alexa 594 (1:500) (Invitrogen) and Alexa 546-conjugated goat anti-rat (1:500) (Invitrogen). Sections were washed and then were incubated with DAPI for 10 min, washed, and mounted with Prolong (Invitrogen).

Immunohistochemistry. For immunohistochemistry not involving MHC tetramers, animals were anesthetized and transcardially perfused with 200–500 μL of oxygenated Tyrode’s solution, then with 4% paraformaldehyde, and were postfixed for 24–48 h in the same fixative. Fifty-micrometer coronal sections were cut serially through the striatum on a Leica vibratome, immunolabeled, and visualized using immunofluorescence or peroxidase histochemistry using the following primary antibodies: rat anti-mouse CD8 (1:5,000) (Serotec), Syrian hamster anti-mouse CD3ε (1:500) (BD Bioscience), rat anti-mouse LFA-1 (1:500) (BD Bioscience), rat anti-mouse CD4 (1:2,000) (Serotec), rat anti-mouse Fsp3 (1:500) (eBioscience), and rabbit anti-ovalbumin (1:500) (Abcam), as described in detail in refs. 1 and 4–6.

Confocal Microscopy and Analysis. Subcellular distribution of LFA-1 and CD3ε was analyzed in detail at contacts between T cells and ovalbumin-expressing target cells in striatal sections from 12 mice. In total more than 100 immunological synapses (IS) were recorded and analyzed. The brain sections were examined using a Leica TCS-S2 confocal microscope with the PlanAPO
63× 1.4 NA oil objective and LCS Confocal Software (Leica Microsystems), as described in much detail elsewhere (4, 7, 8). Briefly, a stack of confocal sections was captured to include the whole of each contact with a z-section interval of 244 nm. A contact was defined as an interface between a CD3ε+/LFA-1+ cell and an ovalbumin-positive cell, in which the two T-cell markers were both visible at the interface in at least three sequential sections. Fluorescence channels were scanned sequentially to reduce interchannel contamination, using 488-nm argon and 542- and 590-nm helium neon laser lines for Alexa dyes 488, 546, and 594, respectively. To assess the extent to which the two proteins were distributed in the pattern characteristic of the Kupfer-type synapse (i.e., CD3ε concentrated at the center of the contact and LFA-1 excluded to a peripheral zone), mean fluorescence density was measured in 3 adjacent regions on the T-cell side of the contact: one measurement in the center and one on each side, in each of three successive sections. A similar analysis was performed for a smaller number of sections labeled with tetramers and various combinations of immunofluorescent labels. Where both labels were restricted to the same part of the membrane, the region analysis was replaced by measurement of the relative fluorescent intensity along a path, as illustrated in Fig. 3.

Quantification of CD3ε, CD4, CD8, and Foxp3 Immunoreactive Staining. Distributions and numbers CD3ε, CD4, CD8, and Foxp3 immunoreactive cell populations were estimated in the striatum and cortex using the Optical Fractionator routine of the StereoInvestigator program (Microbrightfield) and a computer-assisted image analysis system with a Zeiss microscope connected to a digital camera through a zoom set (Carl Zeiss MicroImaging, Inc.). Results were expressed as the estimated absolute number of positive cells in the anatomical regions analyzed and, in the case of CD3ε, counts per counting site. Quantification was performed as in earlier publications from our laboratory (4, 7, 8).

Characterizing Antigen-Specific Immunological Synapses in Rag1−/− Mice. Rag1−/− mice (B6.129S7-Rag1tm1Mom/J stock number 002216) on a C57BL/6 background were purchased from Jackson Laboratories. For adoptive transfer of antigen-specific T cells into mice with established tumors, recipient Rag1−/− mice were implanted with tumors, treated with Ad.Flt3L and Ad.TK at day 16 as described above, and injected with ganciclovir twice daily for 2 days. Eighteen days after implantation, tumor-bearing mice were injected via the tail vein with 1×10⁷ splenocytes from OT-1 Rag1−/− mice in 500 μL PBS. Nine days after adoptive transfer, mice were killed and perfused with oxygenated Tyrode’s solution and 4% paraformaldehyde. Brains then were sectioned at 50 μm and processed for immunohistochemical labeling of LFA-1, CD3ε, and ovalbumin. This experimental paradigm is shown schematically in Fig. S6A.

Fig. S1. Development of the ovalbumin-expressing glioma cell line GL26-cOVA. (A) A restriction map of the mammalian expression plasmid pCI-neo-OVA. (B) An image of light-sensitive film exposed to chemiluminescent label on an immunoblot prepared from wild-type GL26 cells (Left lane) or GL26-cOVA cells (Right lane) and probed with polyclonal rabbit anti-ovalbumin antibody (Upper band), and mouse anti-β actin (Lower band). (C and D) Immunofluorescent labeling of ovalbumin (red) in GL26-WT and GL26-cOVA cells, respectively. Cells were grown on glass coverslips for 24 h and then immunolabeled for ovalbumin (red), and with DAPI (blue). (Scale bar, 70 μm.) (E and F) Expression of ovalbumin in GL26-cOVA–derived tumor in an entire coronal brain section taken 22 days after intracranial implantation. (E) Ovalbumin expression was visualized by immunohistochemistry with polyclonal rabbit anti-ovalbumin (green). (F). The green channel from (E) is superimposed on an image of nuclei labeled with DAPI (blue) to show position of tumor in the brain; on the contralateral hemisphere, the cerebral cortex (Cx) and the striatum (St) are outlined by broken white lines. (Scale bar, 1 mm.)
Fig. S2. Flow cytometry study comparing OVA-specific T cells in spleen and cervical lymph nodes in Flt3L/TK-treated and saline-injected animals using antigen-specific tetramers. (A) Data from spleen. Upper two density plots are from a representative saline-treated mouse, and lower two density plots are from an Flt3L/TK-treated mouse. For each sample, cells were gated on CD8 and CD3 expression (Left plot), and tetramer binding was assessed within that population (Right plot). Percentages on each density plot are the proportions of cells falling within the zone defined by the small black rectangle. Each pair of density plots represents one animal. The column scatter graph to the right of the density plots shows the percentage of CD8⁺/CD3⁺ cells that bind tetramer for 7 saline-treated mice (black squares) and 13 Flt3L/TK-treated mice (brown squares). Horizontal bars are group means. (B) Data from cells isolated from cervical lymph nodes, analyzed as in A.
Fig. S3. Flow cytometry analysis comparing SIINFEKL-induced IFNγ production by cells in spleen and cervical lymph nodes in Flt3L/TK-treated and saline-injected animals. (A) Data from spleen. The upper two density plots are from a representative saline-treated mouse, and the lower two density plots are from an Flt3L/TK-treated mouse. For each sample, cells were gated on CD8 and CD3 expression (Left density plots), and intracellular IFNγ immunolabeling was assessed within that population (Right density plots). Percentages on each density plot are the proportions of cells falling within the zone defined by the small black rectangle. Each pair of density plots represents one animal. The column scatter graph to the right of the density plots shows the percentage of CD8+/CD3+.
**Fig. S4.** Quantitative distribution of CD3ε-immunoreactive cells in Flt3L/TK- or in saline-treated tumors. (A) Coronal sections stained with immunoperoxidase for CD3ε. (Scale bar, 1 mm.) Micrographs on right are higher-power images (Scale bar, 150 μm) of the areas of the full-brain sections marked by small black rectangles. (B) A column scatter graph comparing the total number of CD3ε-immunoreactive cells estimated by counting cells in a large number of counting frames, using a computerized stereological method described in SI Materials and Methods. There was no statistically significant difference between these measurements. (C) A column scatter graph showing the number of cells counted in each counting frame reveals that counting frames with the highest density of CD3ε-immunoreactive cells were found more often in the Flt3L/TK-treated tumors. Such areas of high lymphocyte density also can be seen in the micrographs in A.
Fig. S5. Quantitative distribution of CD4, CD8, and Foxp3 immunoreactive cells in Flt3L/TK- or saline-treated tumors. (A) Coronal sections stained with immunoperoxidase for the three antigens. (Scale bar, 1 mm.) Micrographs on right are higher-power images (Scale bar, 150 μm) of the areas of the full-brain sections delineated by small black rectangles. (B) Column scatter graphs comparing the total number of immunoreactive cells for each cell type, estimated stereologically; there was no statistically significant difference between these measurements (two-tailed, unpaired t-test: CD4, $P = 0.87$; CD8, $P = 0.66$; Foxp3, $P = 0.99$).
Infiltration of tumors by adoptively transferred, ovalbumin-specific, OT-I T cells. (A) A schematic representation of the experimental design. On day 0, Rag1−/− mice were implanted with wild-type GL26 tumors or GL26-cOVA tumors. On day 16, they were treated with intratumoral injection of Ad.FL3L and Ad.TK and were treated with ganciclovir; 2 days later they were injected via the tail vein with 10 million OT-I splenocytes. At day 27, they were perfused, and coronal brain sections were immunolabeled for ovalbumin and CD3ε. (Scale bar, 150 μm.) (B) Fluorescent photomicrographs of such brain sections. In GL26-cOVA tumors (Left), the majority of the tumor cells (large DAPI-stained nuclei) are ovalbumin immunoreactive, and a large number of infiltrating CD3ε+ OT-I cells (white arrows) are observed. Both are absent from the wild-type tumors (Right). Borders between tumors and adjacent brain are marked with broken white lines on the 20× images. (Scale bar, 50 μm.)
Fig. S7. Confocal micrographs of T cells and targets from GL26-cOVA tumors in Rag1−/− mice adoptively receiving OT-I splenocytes. In (A) and (B), the left and middle images of the top rows show immunofluorescence corresponding to ovalbumin (green) and CD3ε (magenta), respectively. The next images in the top rows show the DAPI (blue), and the images on the right are the overlays of these channels. The panels on the bottom left rows also are merged images in which the areas putatively corresponding to the central supramolecular activation complexes (cSMACs) are marked with solid yellow lines and the peripheral supramolecular activation complexes (pSMACs) with broken white lines. In these regions of interest, mean fluorescent density was recorded for CD3ε on three adjacent sections through the T-cell/target cell contact, and these data are shown in the bar charts on the bottom rows, right. In each case, the black bar is the measurement from the section most centrally bisecting the contact, and the white and light gray bars are measured from the adjacent sections above and below. (C) The polarization of IFNγ (green) at a synaptic interface between the immune cell and the ovalbumin-expressing (red) tumor cell. The third image from the left shows DAPI, and right panel is a merged image. (Scale bars, 5 μm.)