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Brain Microenvironment Promotes the Final Functional Maturation of Tumor-Specific Effector CD8+ T Cells

Frédéric Masson,* Thomas Calzascia,* Wilma Di Berardino-Besson,* Nicolas de Tribolet,† Pierre-Yves Dietrich,* and Paul R. Walker3*

During the priming phase of an antitumor immune response, CD8+ T cells undergo a program of differentiation driven by professional APCs in secondary lymphoid organs. This leads to clonal expansion and acquisition both of effector functions and a specific adhesion molecule pattern. Whether this program can be reshaped during the effector phase to adapt to the effector site microenvironment is unknown. We investigated this in murine brain tumor models using adoptive transfer of tumor-specific CD8+ T cells, and in spontaneous immune responses of patients with malignant glioma. Our data show proliferation of Ag-experienced tumor-specific T cells within the brain parenchyma. Moreover, CD8+ T cells further differentiated in the brain, exhibiting enhanced IFN-γ and granzyme B expression and induction of αEβ7 integrin. This unexpected integrin expression identified a subpopulation of CD8+ T cells conditioned by the brain microenvironment and also had functional consequences: αEβ7-expressing CD8+ T cells had enhanced retention in the brain. These findings were further investigated for CD8+ T cells infiltrating human malignant glioma; CD8+ T cells expressed αEβ7 integrin and granzyme B as in the murine models. Overall, our data indicate that the effector site plays an active role in shaping the effector phase of tumor immunity. The potential for local expansion and functional reprogramming should be considered when optimizing future immunotherapies for regional tumor control. The Journal of Immunology, 2007, 179: 845–853.

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ntitumor immune responses can be separated in two phases: the priming phase that occurs in secondary lymphoid organs and the effector phase that occurs at the site of tumor growth. It is generally accepted that during the priming phase, cross-presentation of tumor Ag to naive tumor-specific CD8+ T cells leads to their clonal expansion in secondary lymphoid organs (1, 2). CD8+ T cells fully differentiate into effector T cells and memory cells in function of the costimulation signals, the duration of antigenic stimulation, and the help provided by CD4+ T cells. Additionally, it was reported in different models that professional APCs imprint specific adhesion molecule patterns on CD8+ T cells, determining their tissue tropism (2–5). Thus, during the priming phase, tumor-specific CD8+ T cells undergo an extensive program of differentiation. By contrast, the effector phase of CD8+ T cell-mediated tumor immunity has often been studied only in terms of tumor rejection and survival. Considering recent advances and interest in treating cancer by adoptive transfer of activated T cells (6, 7), a comprehensive understanding of immune function at the effector stage becomes essential. Nonlymphoid tissues and tumor-invaded tissues differ profoundly from secondary lymphoid organs, both in cellular composition and in soluble factors secreted by stromal cells. Such considerations can impose a significant bias on T cell immunity. Many mechanisms of tumor-associated immunosuppression have also been reported (8). Furthermore, even the local microenvironment in the absence of tumor is important. For example, the lung microenvironment limits proliferation of effector memory T cells (9, 10). For the brain, low immune reactivity (e.g., extended allograft survival) is well documented (11), but paradoxically, excessive immune reactions feature in many CNS pathologies. Thus, for cerebral malignancies, both the tumor and the brain microenvironments could influence the phenotypic program that effector T cells have acquired during the priming phase. To understand deleterious or beneficial effects of the effector site is a major issue for elaborating new immunotherapeutic approaches for brain tumors.

Diverse mechanisms appear to regulate entry and retention of memory T cells in different tissues. Klonsowski et al. (12) suggested that in many peripheral tissues, the turnover of effector memory cells would be mostly assured by the recruitment of circulating memory cells. By contrast, the dynamics were proposed to be different for effector memory cells residing in the brain and the intestinal lamina propria. The major mechanism proposed was local self renewal of effector cells that stably seeded these sites during primary responses. However, T cell proliferation in extralymphoid sites remains a controversial issue (13), and mechanisms responsible for retaining T cells in the brain have not been elucidated.

The retention of T cells in tissue following their extravasation depends on interactions of adhesion molecules with extracellular matrix or with counterreceptors expressed by stromal cells. Adhesion molecules induced during the priming phase promote the selective interaction of T cells with inflamed endothelium, which leads to their extravasation to the underlying tissue. It is not known whether such adhesion molecule patterns induced during the priming phase can be reprogrammed during the effector phase within the tissue. Several studies highlighted the role of αEβ7 integrin for
T cell retention in the intestinal epithelia or the skin in certain pathologies (14–16). It was also reported that this integrin can act as a signaling molecule to provide costimulation signals (17). We previously reported that αEβ7 integrin expression was completely down-regulated on tumor-specific CD8+ T cells during the priming phase, whereas a significant proportion of cells expressed this integrin in the brain (2). However, it was not determined whether Ag-experienced CD8+ T cells were reprogrammed within the brain to reinduce αEβ7 expression.

In this study, we analyzed the final functional maturation of tumor-specific effector CD8+ T cells occurring during the effector phase of brain tumor immunity in mouse brain tumor models and in spontaneous immune responses of patients with malignant glioma. In brain tumor models, Ag presentation by tumor cells induced intracerebral (i.c.) proliferation of Ag-experienced CD8+ T cells. During this process, a further differentiation of Ag-experienced CD8+ T cells occurred, characterized by enhanced IFN-γ and granzyme B expression and induction of αEβ7 integrin expression that facilitated T cell retention in the brain. Furthermore, a proportion of CD8+ T cells infiltrating human gliomas expressed granzyme B and showed expression of αEβ7 integrin as in murine models.

Materials and Methods

Mice

P14 TCR transgenic (Tg) mice bearing a Vα2Vβ8.1 TCR specific for the H-2D^d/ gp33–41 complex were from H. Pircher (University of Freiburg, Freiburg, Germany). H-2K^d knockout animals were from F. Lemonnier (Institut Pasteur, Paris, France). αE (CD103)-deficient mice were from C. Parker (University of Harvard, Boston, MA) and H. MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland). P14 × enhanced GFP Tg mice were obtained by breeding P14 TCR Tg mice with enhanced GFP Tg mice from M. Okabe (Osaka University, Suita Osaka, Japan). VM mice were from Institute of Animal Health. C57BL/6, BALB/c, and OTI mice were from Charles River Laboratories.

Cell preparations

PBMCs were purified on Ficoll (Pharmacia). Brain-infiltrating leukocytes (BILs) were isolated from Ringer’s perfused brains, as previously described (2). Bone marrow (BM)-derived dendritic cells (BMDC) were generated by BM cell culture in DMEM containing 6% FCS and rGM-CSF (PeproTech) at 20 ng/ml. BMDC were harvested on day 6, and then maturation was achieved with LPS (Sigma-Aldrich) at 50 ng/ml for a further 2 days.

Tumor cell implantation

The MC57-glycoprotein (GP) and nontransfected MC57 fibrosarcoma cell lines were from R. Zinkernagel (Institute of Experimental Immunology, Zurich, Switzerland). The MT539MG glioma (MT) was from G. Gillespie (University of Birmingham, Birmingham, AL) and transfected with cDNA for OVA257–264 peptide. Kit264/56 (Miltenyi Biotec), and CD103 (Ber-ACT8; BD Biosciences). The MT539MG glioma (MT) was from G. Gillespie (University of Birmingham, Birmingham, AL) and transfected with cDNA for OVA257–264 peptide (SHINEKLM). Cells were cultured in DMEM/6% FCS/20 μM human rIL-2 (Chiron). In some experiments, P14 T cells were first activated in vivo before expansion ex vivo. Briefly, cells were isolated from inguinal lymph nodes (LN) after s.c. MC57-GP implantation into C57BL/6 mice previously transferred with naive P14 T cells. Cells were then cultured for 72 h in human rIL-2-containing medium, and CD8+ T cells were positively isolated by magnetic separation (Miltenyi Biotec).

Adaptive transfer of TCR Tg cells

Naïve P14 or P14-GFP CD8+ T cells were positively isolated from spleen and LN by magnetic separation (Miltenyi Biotec). In some experiments, CD62L+ cells were eliminated from the activated cell mix using biotin-conjugated anti-CD62L Ab (MEL14; BD Biosciences) and anti-biotin-coated magnetic beads (Miltenyi Biotec). Activated TCR Tg cells were labeled with CFSE (Molecular Probes), as described (18), before adoptive transfer. For adoptive transfer experiments, 5–10 x 10^6 naive or activated TCR Tg cells were injected i.v. into recipient mice.

In vivo i.c. cytotoxic assay

Splenocytes from C57BL/6 mice were pulsed with 10 μM gp33–41 peptide or OVA257–264 peptide. Then, gp33–41–peptide-pulsed target cells were labeled with 10 μM CFSE (Molecular Probes) and OVA257–264 peptide pulsed control target cells with 1 μM CFSE to generate CFSEhi and CFSElo target cells. Equal numbers of target cells were then injected i.c. (1 x 10^6 cells in 5 μl of methylcellulose). Differentially CFSE-labeled target cells were detected in BILs by flow cytometry allowing the evaluation of specific cytotoxicity.

Survival analysis

Mice implanted i.c. with tumor cells were sacrificed according to institutional and cantonal animal welfare rules (20% weight loss and/or presence of adverse symptoms). Survival was plotted using Kaplan-Meier analysis.

Generation of BM chimeras

Recipient (C57BL/6 x DBA/2)F1 (B6D2) mice were irradiated, as described (2), and then after 16 h they were injected i.v. with 10^6 BM cells (B6D2 or DBA/2). Chimeras were allowed to reconstitute for at least 7 wk before use.

Intracellular staining

Permeabilization for intracellular IFN-γ and granzyme B was performed using the Cytofix/Cytoperm kit (BD Biosciences). Intracellular granzyme B expression was assessed on BILs directly ex vivo, whereas intracellular IFN-γ was assessed after BIL restimulation with 1 μM gp33–41 peptide or OVA257–264 peptide for 6 h in the presence of 2 μM monensin (BD Biosciences).

In vivo retention assay

CD8+ T cells were positively isolated from spleen of BALB/c wild-type (WT) or αE−/− mice by magnetic separation (Miltenyi Biotec), then activated by coculture with irradiated splenocytes in DMEM containing 6% FCS, 20 μM 2-ME, and 2.5 μg/ml Con A (Sigma-Aldrich). After 3 days, cultures were split, and 30 U/ml human rIL-2 (Chiron) and 10 ng/ml human rTNF-β (PeproTech) were added for 3 additional days to induce expression of αEβ7 on at least 95% of activated CD8+ T cells from BALB/c WT mice. Equal numbers of BALB/c WT and αE−/− cells were mixed together, labeled with 5 μM CFSE (Molecular Probes), then injected into mice either i.c. (1 x 10^6 cells in 5 μl of methylcellulose) or i.v. (7–8 x 10^6 cells in 250 μl of PBS). Four days later, CFSE-labeled CD8+ T cells differentially expressing αEβ7 were detected in BILs, PBMCs, and splenocytes by flow cytometry. Retention index was calculated by the ratio of αEβ7+ to αEβ7− cells among CFSE−/CD8− gated T cells (corrected for the input ratio).

Abbreviations used in this paper: i.c., intracerebral; BIL, brain-infiltrating leukocyte; BM, bone marrow; BMDC, BM-derived dendritic cell; cLN, cervical lymph node; LN, lymph node; Tg, transgenic; WT, wild type; GP, glycoprotein.
**Fluorescence microscopy**

Brains were perfused with 3% paraformaldehyde in PBS, postfixed overnight in PBS/3% paraformaldehyde/20% sucrose at 21°C, then frozen on dry ice. For the detection of α5 integrin-expressing cells, 7-μm brain cryosections were blocked with PBS/5% BSA/2% mouse serum/0.1% Tween 20, and then stained with anti-α5 Ab (M293; BD Biosciences) or control Ab. Primary Ab were revealed with an Alexa 546-conjugated anti-rat Ab (Molecular Probes), and analysis was by fluorescence microscopy (Zeiss).

**Immunohistochemistry**

Brain cryosections (7 μm) were stained for CD8 (H35-17.2; hybridoma supernatant prepared in-house) or with isotype control Ab, as previously described (19).

**Human tumor biopsies**

Biopsies from patients with malignant glioma were collected during surgery, after informed consent and with Institutional Ethical Committee approval. Single-cell suspensions were obtained after either mild or more aggressive enzymatic digestion, according to the characteristics of the biopsy. Mild conditions (0.1% collagenase D, Roche Diagnostics; 0.27 μM Nα-tosyl-l-lysine chloromethyl ketone hydrochloride and 0.002% DNase I, both from Sigma-Aldrich) allowed ex vivo analysis for Ge 460, Ge 462, and Ge 465. Harsher conditions (0.1% collagenase IA/0.05% protease I/0.002% DNase II, all from Sigma-Aldrich) were used for Ge 293, Ge 319, Ge 397, and Ge 421. This latter treatment degraded CD3 and CD8, and cells required culture overnight for re-expression of these markers.

**Results**

**Ag-experienced P14 CD8+ T cells infiltrating the brain of tumor-implanted mice are potent cytotoxic cells**

We analyzed the kinetics of infiltration of tumor-specific CD8+ T cells in the brain of mice implanted i.c. with tumor cells. We chose an adoptive transfer strategy of naïve P14-GFP TCR Tg CD8+ T cells specific for the H-2Dp-restricted gp33-41 epitope of the lymphocytic choriomeningitis virus glycoprotein combined with i.c. injection of MC57-GP tumor cells expressing lymphocytic choriomeningitis virus glycoprotein. There was a dramatic increase in absolute numbers of brain-infiltrating P14-GFP CD8+ T cells between days 8 and 12 after tumor implantation (Fig. 1A) that were localized in the ipsilateral hemisphere (Fig. 1B). Tumor was detected at day 5, but had regressed by day 12 after implantation, with CD8+ T cells infiltrating the implantation site (Fig. 1C). We then studied whether brain-infiltrating CD8+ T cells could exert their cytotoxicity in vivo within the brain. We developed an in vivo cytotoxicity test in which gp33-41 or OVA257-264 peptide-pulsed target cells differentially labeled with CFSE were injected i.c. into the brain of mice preimplanted with the tumor. The gp33-41 peptide-pulsed target cells were specifically killed in the context of the brain microenvironment (Fig. 1D). Survival analysis confirmed that P14 CD8+ T cells were involved in the rejection of i.c. implanted MC57-GP (Fig. 1E).

**Tumor cells induce i.c. proliferation of Ag-experienced P14 CD8+ T cells infiltrating the brain**

The kinetics of CD8+ T cell accumulation in the brain of tumor-implanted mice suggested that these cells had proliferated locally within the brain tumor during the effector phase. We addressed this issue by the adoptive transfer of preactivated P14 CD8+ T cells (gp33-41 specific) and OTI CD8+ T cells (OVA257-264 specific) labeled with CFSE into mice preimplanted i.c. with MC57-GP. Proliferation of adoptively transferred CD8+ T cells was then analyzed in the brain and cervical LNs (cLNs), the latter having been previously defined as the site of priming of naive CD8+ T cells (2). Activated P14 CD8+ T cells proliferated only in the brain and not in the cLNs of tumor-implanted mice, the divisions continuing at least until 7 days after transfer (Fig. 2A). We confirmed that proliferation of activated P14 CD8+ T cells was strictly restricted to the brain because no divided P14 CD8+ T cells were found in the spleen or in the blood (Fig. 2B). By contrast, OTI CD8+ T cells proliferated neither in the brain, nor in the cLNs (Fig. 2A), suggesting that i.c. proliferation of activated P14 CD8+ T cells was Ag specific. This was further confirmed by experiments in which activated P14 CD8+ T cells were implanted into mice preimplanted with either MC57-GP or nontransfected MC57 (Fig. 2C).

We previously demonstrated that priming of naïve P14 CD8+ T cells requires cross-presentation of tumor Ag (2). Because the identity of the APC can strongly influence the function and the fate of CD8+ T cells, it was crucial to determine whether the i.c. proliferation of activated P14 CD8+ T cells during the effector phase was induced by cross-presentation of tumor Ag by a professional APC, or by direct Ag presentation by tumor cells. We therefore assessed whether activated P14 CD8+ T cells could proliferate in the brain of mice lacking the MHC class I allele presenting the immunodominant gp33-41 epitope. Activated P14 CD62L− CD8+ T cells divided similarly in the brain of H-2KbD− knockout mice and in WT mice, indicating that tumor cells directly induced the proliferation of activated P14 CD8+ T cells (Fig. 2D) in contrast...
to the requirement for cross-presentation for naïve P14 CD8+ T cells (data not shown) (2). Tumor cells can also directly induce the proliferation of activated P14 CD8+ T cells in a noncerebral site, as demonstrated by CFSE dilution of P14 CD62Llow CD8+ T cells infiltrating the same tumor-implanted s.c. in chimeric mice reconstituted with either F1 B6D2 BM (H-2b) or DBA/2 BM (H-2d) (Fig. 2E). Because malignant astrocytomas are aggressive and potentially immunosuppressive tumors, we determined whether

**FIGURE 2.** Tumor cells induce i.c. proliferation of Ag-experienced P14 CD8+ T cells. A, TCR Tg CD8+ T cells (P14 or OTI) activated in vitro with irradiated, peptide (gp33–41 or OVA257–264)-pulsed splenocytes were harvested after 7–8 days, labeled with CFSE, and infused i.v. into mice implanted i.c. with MC57-GP 4 days previously. CFSE dilution of H-2Dd/gp33−41 tetramer-positive (P14 T cells) or negative cells (OTI T cells) from BILs or cLNs was assessed by flow cytometry at the indicated times after tumor implantation. Dot plots are gated on CD8+ cells and are representative of three experiments. B, Activated P14 CD8+ T cells were enriched for CD62Llow cells (see Materials and Methods), CFSE labeled, then infused i.v. into WT mice implanted i.c. 4 days previously with MC57-GP. After 4 days, CFSE dilution of Vα2+ cells isolated from the indicated sites was determined by flow cytometry. Dot plots are gated on CD8+ cells, except for the brain, and are representative of six experiments. C, Activated P14 CD8+ T cells were labeled with CFSE and infused i.v. into mice implanted i.c. with MC57-GP or nontransfected MC57 cells 4 days previously. After 4 days, CFSE dilution of H-2Db/gp33−41 tetramer+ cells was assessed by flow cytometry of BILs. Dot plots are gated on CD8+ cells and are representative of three experiments. D, CFSE-labeled activated CD62L− P14 T cells were infused i.v. into H-2KdDb-deficient or WT mice implanted i.c. 4 days previously with MC57-GP. After 4 days, CFSE dilution of H-2Dd/gp33−41 tetramer− BILs was determined by flow cytometry. Dot plots are gated on CD8+ cells and are representative of three experiments. E, CFSE-labeled activated CD62L− P14 T cells were infused i.v. into B6D2→B6D2 or DBA/2→B6D2 BM chimeras implanted s.c. with MC57-GP 4 days previously. After 4 days, CFSE dilution of Vα2+ cells isolated from the s.c. tumor was determined. Dot plots are gated on CD8+ cells and represent three experiments. F, CFSE-labeled activated CD62L− P14 T cells were infused i.v. into mice implanted i.c. with GL261 glioma cells 14 days previously. On the same day, 5 × 10^5 gp33−41 or OVA257–264 peptide-pulsed BMDC were injected i.c. in the same mice. After 4 days, CFSE dilution of Vα2+ cells isolated from the indicated sites was determined. Dot plots represent one of four experiments for OVA257–264 peptide-pulsed BMDC, or nine experiments for gp33−41 peptide-pulsed BMDC.
FIGURE 3. Ag-experienced P14 CD8+ T cells proliferating in the brain further differentiated into fully functional effector T cells. Naïve P14 CD8+ T cells were activated for 8 days in vitro with irradiated gp33-41 peptide-pulsed splenocytes, CFSE labeled, then infused i.v. into recipient mice implanted i.c. with MC57-GP 4 days previously. A. After an additional 4 days, BILs were isolated, restimulated in vitro for 6 h with gp33-41 or OVA257–264 peptides, and assessed for expression of intracellular IFN-γ by flow cytometry. Dot plots are gated on Vα2+ cells. Graph shows IFN-γ expression quantified as the geometric mean fluorescence index (GMFI ± SEM, n = 4) as a function of CFSE content (lanes 1–6 on the dot plots). B. Isolated BILs were directly assessed for intracellular granzyme B expression. Dot plots gated on Vα2+ cells show staining for granzyme B. Graph shows granzyme B expression quantified as the geometric mean fluorescence index (GMFI ± SEM, n = 6) as a function of CFSE content (lanes 1–6 on the dot plots).

activated P14 CD8+ T cells retained the ability to proliferate in a glioma microenvironment. Following i.c. boosting with gp33-41 peptide-pulsed BMDC, activated P14 CD62L-CD8+ T cells strongly proliferated in the brains of mice with established GL261 glioma (Fig. 2F). Overall, these data provide evidence that proliferation of Ag-experienced P14 CD8+ T cells driven by tumor cells (and potentially by other APCs) can occur within the brain tumor microenvironment.

Ag-experienced P14 CD8+ T cells proliferating i.e., further differentiated into fully competent effector cells

Effectors are acquired by CD8+ T cells during the priming phase after Ag presentation by professional APCs to naïve CD8+ T cells in secondary lymphoid organs. We asked whether Ag-specific interaction of activated P14 CD8+ T cells with tumor cells in the brain could modify their effector functions. Although nondividing P14 CD8+ T cells (i.e., activated in vitro before adoptive transfer) expressed intracellular granzyme B and IFN-γ, the expression level of these effector molecules dramatically increased with the number of divisions (Fig. 3). In addition, IL-7R expression decreased on divided cells (data not shown). These functional changes also occurred in a noncerebral site, because activated P14 CD8+ T cells proliferating within s.c. MC57-GP tumor up-regulated intracellular granzyme B and IFN-γ (data not shown). Thus, Ag-experienced P14 CD8+ T cells proliferating in the tumor site further differentiated into fully competent effector cells.

Induction of αβ integrin expression on brain tumor-infiltrating CD8+ T cells

We previously reported that during the priming phase, a specific pattern of adhesion molecule expression is imprinted on brain tumor-specific CD8+ T cells by APCs from the brain able to cross-present tumor Ag (2). For most of the adhesion molecules analyzed, the hierarchy of expression was recapitulated in the effector site, except for αβ integrin. Indeed, its expression was completely down-regulated on P14 CD8+ T cells during priming, whereas a significant proportion of cells expressed this integrin in the brain. We asked whether Ag-experienced CD8+ T cells were reprogrammed within the brain to modify αβ integrin expression. We analyzed αβ integrin expression on P14-GFP CD8+ T cells infiltrating the brain of tumor-implanted mice at different time points. αβ expression was induced on brain-infiltrating P14-GFP CD8+ T cells between days 8 and 12 after tumor implantation (~70% of P14-GFP CD8+ T cells expressed αβ integrin at day 12) (Fig. 4, A and B) and remained stable until day 18, whereas there was no significant expression in the LNs (Fig. 4B) or in the blood (data not shown). This strongly suggests that this integrin was induced locally in the brain tumor microenvironment. Moreover, this up-regulation was related to the brain rather than to the tumor microenvironment because P14-GFP CD8+ T cells infiltrating the same tumor implanted s.c. failed to express αβ integrin (Fig. 4A). The kinetics of αβ integrin induction correlated with the rate of P14-GFP CD8+ T cell accumulation in the brain, suggesting either that αβ integrin was induced on tumor-specific T cells proliferating in the brain, or that some αβ+ cells migrated to the brain at this time point. To discriminate between these two hypotheses, we analyzed whether αβ integrin expression was induced on adoptively transferred activated P14 CD8+ T cells proliferating in the brain of tumor-implanted mice. To generate activated P14 CD8+ T cells negative for αβ integrin expression, it was necessary to prime cells in vivo, because in vitro priming with peptide-pulsed splenocytes induced this integrin. αβ integrin expression was induced on a proportion of P14 CD62L-CD8+ T cells dividing in the brain, which increased with each division to reach ~60% of cells expressing this integrin at CFSE dilution 6 (Fig. 4C). In addition, we analyzed the localization of P14-GFP CD8+ T cells expressing β integrin (reflecting αβ integrin expression in the absence of αβ integrin expression (2)): these cells were widely distributed in the parenchyma of the ipsilateral hemisphere of the brain (Fig. 4D). Finally, we found that αβ integrin expression was expressed by a proportion of CD8+ T cells infiltrating i.c. implanted glioma (MT-CW3) and mastocytoma (P815-CW3) (Fig. 4E). These data demonstrate that αβ integrin expression by a proportion of brain tumor-infiltrating CD8+ T cells can be generalized to other murine neoplasms. We then assessed whether the acquisition of αβ integrin expression by brain-infiltrating tumorspecific CD8+ T cells had functional consequences for their retention in the brain. After activation, CD8+ T cells derived from WT BALB/c mice (strongly expressing αβ integrin) or from αβ+ mice were labeled with CFSE and injected i.c. into WT mice (Fig. 4F). Before i.c. injection, the relative ratio of the two populations isolated from the brain was calculated. CD8+ T cells expressing αβ integrin were retained ~3 times more efficiently than CD8+ T cells derived from
pressed by 20–57% of CD8 *+* gliomas (Fig. 5, for the input ratio). Means proliferation of panel SEM, and s.c. tumor histogram is representative of four experiments. cLNs yielded insufficient cells for analysis at day 18.

We next asked whether our results in murine models showing infiltration of human gliomas injected i.v. Histograms show the either i.c. or i.v. into recipient mice. Four days after injections, cells were isolated from the brain of mice injected i.c. or from blood and spleen of mice indicated tissues (lower).

These differences are not due to an in vivo infiltration of CD8 *+* cells (Fig. 4G). These results are consistent with the data shown. These results suggest that CD8 *+* expression promotes T cell retention in the brain by increasing either T cell adhesion or T cell survival.

**α*ε*β*7* integrin and granzyme B are expressed by CD8 *+* T cells infiltrating human gliomas**

We next asked whether our results in murine models showing CD8 *+* T cells could be also extended to human brain tumors. **α*ε*β*7* integrin was expressed by 20–57% of CD8 *+* CD3 *+* T cells infiltrating human malignant gliomas (Fig. 5, A and B). By contrast, **α*ε*β*7* integrin was poorly expressed by CD8 *+* CD3 *+* T cells isolated from the blood (range of 0.5–5%) (Fig. 5, A and B), demonstrating that **α*ε*β*7* integrin is specifically expressed by CD8 *+* T cells infiltrating brain tumors, suggesting a local induction of this integrin as in murine models. **α*ε*β*7* integrin is differentially regulated on CD4 *+* T cells, as only 2–7% of tumor-infiltrating CD8 *+* CD3 *+* T cells (reflecting CD4 *+* T cells) expressed the integrin (Fig. 5B). We also showed that between 32 and 70% of CD8 *+* T cells were maintained within the glioma microenvironment (Fig. 5C). Finally, we showed that a proportion of **α*ε*β*7* T cells expressed granzyme B (62% ± 6, mean ± SEM, n = 6). Interestingly, the percentage of granzyme *+*
cells among $\alpha_\beta_7^+$ CD8$^+$ T cells was significantly higher than among $\alpha_\beta_7^-$ CD8$^+$ T cells (48% ± 5, mean ± SEM, n = 6) ($p = 0.012$; paired Student’s $t$ test). Overall, these results suggest that $\alpha_\beta_7$ expression by brain tumor-infiltrating T cells may identify a subset of CD8$^+$ T cells that were further differentiated within the effector site.

Discussion

Conceptual and methodological advances in tumor immunology now offer many promising approaches to induce tumor-specific immunity (7, 20). However, clinical outcome of tumor immunotherapy may well differ according to the tissue affected by malignancy. Our study has explored this issue of regional tumor immunity for the brain, studying murine brain tumor models and human gliomas. We found that tumor-specific CD8$^+$ T cells strongly accumulated in the brain of mice bearing an i.c. tumor. Furthermore, effector CD8$^+$ T cells proliferated i.c. after encounter with tumor cells, demonstrating a surprising aptitude of the brain to accommodate proliferation of tumor-specific Ag-experienced CD8$^+$ T cells. In the models used in our study, priming of naive P14 CD8$^+$ T cells was dependent on cross-presentation of tumor Ag, but direct Ag presentation by tumor cells was sufficient to induce the i.c. proliferation of Ag-experienced P14 CD8$^+$ T cells. These differences in the mode of activation may be due to different requirements for costimulation signals between naive and Ag-experienced T cells (21–23). However, it is noteworthy that even in the absence of direct tumor Ag presentation, local proliferation can still be induced with the aid of i.c. implanted dendritic cells (Fig. 2F). Our data extend the observations made by Klonowski et al. (12) in an elegant study of memory T cell migration using a parabiotic mouse model. In this earlier work, it was predicted that special mechanisms may regulate pools of memory T cells in the brain and intestinal lamina propria, because of restricted accessibility of these sites to circulating memory cells. Our data indicated that local Ag-driven proliferation could compensate for the restricted accessibility of the CNS for circulating memory cells.

In addition, we showed that tumor-specific CD8$^+$ T cells infiltrating the brain of tumor-bearing mice are capable of full effector function within this specialized site. Interestingly, i.e. proliferation is associated with enhanced granzyme B and IFN-γ expression by CD8$^+$ T cells. This demonstrates that Ag-experienced T cells can further differentiate into fully competent effector cells during the effector phase of brain tumor immunity. Granzyme B expression by a significant proportion of CD8$^+$ T cells infiltrating malignant glioma from patients with progressive disease gives a valuable insight into the function of glioma-infiltrating T cells. Indeed, there are currently few tests that can be performed on T cells isolated from brain tumors in patients without in vitro manipulation or prior knowledge of Ag specificity. Although tumor reactivity of these human T cells is not defined in this study, our previous data based on TCR spectratyping suggested that the repertoire of CD8$^+$ T cells infiltrating human glioma reflects Ag-driven, oligoclonally expanded T cells (24). Our demonstration that a major component of the cytotoxic machinery is intact on many malignant glioma-infiltrating CD8$^+$ T cells clearly raises the issue of why there is little evidence for T cell-mediated immune control in these generally lethal tumors. One explanation may be that even if functional brain tumor-specific CD8$^+$ T cells infiltrate the tumor, they are quantitatively insufficient to impact on the tumor and are ultimately overwhelmed by the tumor mass (25) (data not shown). There is also a rich literature detailing a plethora of potential active and passive glioma immune escape mechanisms that may provide further clues (24). Indeed, certain studies have suggested that brain tumor immune escape may be due to deficits at the effector phase of the response, which can be corrected by local immunotherapy (26). However, the importance of glioma immune escape in vivo, at the tumor site in the brain, has rarely been established, and so to understand the apparent ineffectiveness of tumor-infiltrating
CD8\(^+\) T cells will require approaches that better analyze the local tumor site.

The expression of \(\alpha_\beta_2\) by tumor-specific CD8\(^+\) T cells infiltrating the brain represents an original and intriguing finding that raises important issues. Similar kinetics of \(\alpha_\beta_2\) integrin induction were described for CD8\(^+\) T cells infiltrating renal allografts as well as for effector CD8\(^+\) T cells infiltrating intestinal epithelium during graft-vs-host disease (15, 27). These kinetics suggested a local induction of the integrin on CD8\(^+\) T cells. However, the authors did not exclude the possibility that \(\alpha_\beta_2\) integrin could be induced on CD8\(^+\) T cells in other sites, an event preceding their migration in the site of inflammation. In this study, we show definitive evidence that \(\alpha_\beta_2\) integrin is induced locally on brain-infiltrating CD8\(^+\) T cells. Importantly, this process depends on their local proliferation, because undivided CD8\(^+\) T cells did not acquire \(\alpha_\beta_2\) integrin. Because TGF-\(\beta\) is a key factor in \(\alpha_\beta_2\) induction in vitro and in vivo in other sites (15, 27, 28), it is likely that the TGF-\(\beta\) locally present in the brain (29) plays a role in inducing \(\alpha_\beta_2\) expression on tumor-specific CD8\(^+\) T cells in our murine models, as well as on CD8\(^+\) T cells infiltrating human gliomas. Overall, our data suggest that sequestration of T cells in the brain, rather than the particularities of a given tumor, is important for this phenotypic reprogramming. However, to our knowledge, \(\alpha_\beta_2\) expression was never reported for CD8\(^+\) T cells in other brain pathologies. For CD4\(^+\) T cells, our data from T cells infiltrating human glioma indicate that CD3\(^+\)CD8\(^+\) T cells lacked \(\alpha_\beta_2\) expression, as did CD4\(^+\) T cells infiltrating the brain following i.c. injection of bacteria (30), whereas \(\alpha_\beta_2\) expression was detected on CD4\(^+\) regulatory T cells in an experimental allergic encephalomyelitis model (31). Thus, effector cell phenotype is a consequence of both immune stimulus and tissue and cannot be generalized for any T cell subset or brain pathology. Because \(\alpha_\beta_2\) expression is uniformly down-regulated on tumor-specific CD8\(^+\) T cells during priming in vivo (2), its reinduction described in this work represents important new evidence for tumor immunity that adhesion molecule patterns initially dictated by cross-presentation during the priming phase can be reprogrammed during the effector phase. Our in vivo experiments are in accordance with the in vitro work of Mora et al. (3), showing that dendritic cells from different lymphoid organs could reorient specific adhesion molecule patterns acquired by effector or effector memory T cells.

The integrin \(\alpha_\beta_2\) mediates adhesion of intraepithelial T lymphocytes to epithelial or endothelial cells, either by interaction with its major counterreceptor E-cadherin (32), or via E-cadherin-independent interactions (33, 34). E-cadherin is not widely expressed in the CNS (35) and is not expressed in gliomas, but it was reported in brain tumor metastasis of different origins (36, 37). Interaction of \(\alpha_\beta_2\), CD8\(^+\) T cells with E-cadherin-expressing tumor cells could have functional consequences, by increasing T cell adhesion to tumor cells and triggering directional egress of lytic granules augmenting specific cytotoxicity, as recently reported for \(\alpha_\beta_2\), CD8\(^+\) T cells interacting with epithelial tumor cells (38, 39). We demonstrated that \(\alpha_\beta_2\)-expressing CD8\(^+\) T cells have an enhanced retention in the normal brain. We consider the most likely explanation is that \(\alpha_\beta_2\) expressed by activated CD8\(^+\) T cells increased their adhesion to the brain stroma. However, we cannot exclude that \(\alpha_\beta_2\), CD8\(^+\) T cells had an enhanced survival in the brain microenvironment.

Overall, our results indicate that the tumor site should be considered not only for its negative aspects, but also for its potential beneficial effects in reshaping brain tumor immunity. Indeed, the brain tumor microenvironment is permissive for local T cell proliferation and the maintenance of critical T cell effector functions, and can induce T cell adhesion molecule reprogramming. Notably, we have now taken the first steps in defining this for human glioma, because a proportion of tumor-infiltrating CD8\(^+\) T cells expressed granzyme B and \(\alpha_\beta_2\) integrin. One promising approach in tumor immunotherapy is the adoptive transfer of tumor-specific CD8\(^+\) T cells amplified in vitro (6, 40–42). Thus, whereas certain cancer immunotherapy protocols may privilege in vivo expansion of adoptively transferred T cells within secondary lymphoid organs (43, 44), the consequences of efficient proliferation at the tumor site must also be considered. Considering our results, certain functionally significant changes, including the induction of \(\alpha_\beta_2\) integrin, only occurred within the brain and not in lymphoid organs. Identifying the positive effects of the tumor site for T cell function provides opportunities for optimizing immunotherapies for regional tumor control. Overall, our detailed analysis of tumor immunity in the brain demonstrates the importance of the CNS microenvironment in shaping immune responses from the priming stage (2) through to the effector phase of the response.

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Disclosures

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