

Immune Infiltration of Spontaneous Mouse Astrocytomas Is Dominated by Immunosuppressive Cells from Early Stages of Tumor Development

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Abstract

Immune infiltration of advanced human gliomas has been shown, but it is doubtful whether these immune cells affect tumor progression. It could be hypothesized that this infiltrate reflects recently recruited immune cells that are immediately overwhelmed by a high tumor burden. Alternatively, if there is earlier immune detection and infiltration of the tumor, the question arises as to when antitumor competency is lost. To address these issues, we analyzed a transgenic mouse model of spontaneous astrocytoma (*GFAP-V¹²HA-ras* mice), which allows the study of immune interactions with developing glioma, even at early asymptomatic stages. T cells, including a significant proportion of Tregs, are already present in the brain before symptoms develop, followed later by macrophages, natural killer cells, and dendritic cells. The effector potential of CD8 T-cells is defective, with the absence of granzyme B expression and low expression of IFN- γ , tumor necrosis factor, and interleukin 2. Overall, our results show an early defective endogenous immune response to gliomas, and local accumulation of immunosuppressive cells at the tumor site. Thus, the antiglioma response is not simply overwhelmed at advanced stages of tumor growth, but is counterbalanced by an inhibitory microenvironment from the outset. Nevertheless, we determined that effector molecule expression (granzyme B, IFN- γ) by brain-infiltrating CD8 T-cells could be enhanced, despite this unfavorable milieu, by strong immune stimuli. This potential to modulate the strong imbalance in local antiglioma immunity is encouraging for the development and optimization of future glioma immunotherapies. *Cancer Res*; 70(12): 4829–39. ©2010 AACR.

Introduction

Interactions between cancer and the immune system have been proposed to rely on three processes: elimination (immunosurveillance), equilibrium, and escape, leading to immunologic sculpting of the tumor (immunoeediting). Evidence for these mechanisms comes from mouse models, observation of large cohorts of immunosuppressed patients showing increased cancer incidence, and in certain tumors, correlation between immune infiltration and clinical outcome (1–3). In mice with defined immunodeficiencies or

after antibody blockade/depletion of specific immunologic components, both innate and adaptive immune responses affected tumor growth (3). However, whether the theory of immunoeediting is applicable to primary brain tumors has not yet been addressed.

Gliomas and, in particular, malignant astrocytomas have a poor prognosis and are to date incurable. These tumors are infiltrated by macrophages, CD4 and CD8 T lymphocytes, and natural killer (NK) cells (4). However, the correlation between this immune infiltrate and clinical outcome is controversial, partly due to the lack of multivariate analyses taking into account tumor grade, patient treatment before and after surgery, and tumor-infiltrating immune cell phenotype. Finally, analyses of the glioma immune infiltrate are performed only at advanced tumor stages in patients. Thus, at least at these late stages, gliomas are not ignored by the immune system, but earlier tumor-immune interactions remain to be elucidated. Indeed, deficient immunosurveillance of gliomas may be explained by defective afferent immunity delaying the induction of immune effector mechanisms, which are then overwhelmed by the tumor mass (5). Alternatively, an antiglioma immune infiltrate appearing earlier might shape the tumor to develop less immunogenic variants or immune escape mechanisms. These issues must be studied in the context of the brain, as this site

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imposes such a specialized interface between cancer and the immune system.

Immune privilege of the brain, originally defined by prolonged allograft survival (6), has been reconsidered as a process concerning the brain parenchyma only (7). Moreover, this status is perturbed during brain inflammation. It relies on unique features such as tight regulation of trafficking through the blood-brain barrier, the absence of conventional lymphatic drainage, enrichment in local potent immunosuppressive factors such as transforming growth factor- β (8), and the absence of resident parenchymal dendritic cells (DC). Endogenous brain cells such as microglial cells and astrocytes could participate in local immune responses and promote the recruitment of other cell types (9, 10). However, afferent immunity and cell-transported antigen egress from the brain might still be impaired (11). Nevertheless, in inflammatory or tumoral situations, T cells are eventually activated, induced to proliferate, and imprinted with the necessary adhesion molecules that facilitate their migration to the brain (12–16).

Glioma cells might escape T-cell recognition by downregulation of MHC molecules, and local hypoxia might impede T-cell function (17). Active immunosuppressive mechanisms in human gliomas include soluble factors such as transforming growth factor- β (18, 19) and cell surface molecules (20, 21). Several recent studies have focused on glioma-infiltrating immunosuppressive cells, of which Tregs are the best defined (22, 23). However, the most abundant glioma-infiltrating cells are macrophages/activated microglia. They represent either an influx of peripheral monocytes that differentiate into macrophages, or the *in situ* proliferation and activation of resident microglia. Whether they promote or antagonize tumor growth is unclear (24).

To study tumor-immune system interactions, murine models of intracranially implanted tumors have been invaluable, facilitating analysis of effector T-cell entry and functionality in the brain (14, 16, 25, 26), and of how tumors can escape immune effector mechanisms (17). However, in these models, the blood-brain barrier is inevitably breached by tumor implantation, and transformed cells rapidly establish a tumor mass, leading to an acute response, with early influx of immune cells into the brain. To better model slowly progressive glioma growth, genetic models of spontaneous cancer are now emerging (27).

Here, we used a transgenic model of spontaneous astrocytoma (*GFAP-V¹²HA-ras* B8, referred to hereafter as RasB8 mice) to study immune responses at occult stages of glioma development. In this model, activated ras is selectively expressed in astrocytes under the control of the glial fibrillary acidic protein (*GFAP*) promoter. Hemizygous RasB8 mice gradually develop astrocytic hyperplasia and more aggressive solitary or multifocal grade II/III astrocytoma-like lesions by 12 weeks. Additional mutations occur, such as loss of PTEN and p16 expression, and most mice develop symptoms and die between 2 and 6 months of age (28, 29). At early asymptomatic stages, we already detected immune infiltration of RasB8 brains, including infiltrating Tregs and granzyme B–negative CD8 T-cells, thereby unfavorable for antitumor immunity. However, we showed that partial local immune

competency could be achieved with peripheral vaccination with virus, or an intracranial immunogenic stimulus.

Materials and Methods

Mice and vaccinations

Hemizygous transgenic RasB8 mice, endogenously expressing a single allele of oncogenic V¹²HA-ras, have been previously described (28). RasB8 mice were maintained on a CD1 background, or backcrossed at least seven generations to the 129SvPas (H-2^b) background (RasB8 \times 129SvPas) where indicated, and were genotyped as described. Mice exhibiting one/several of the following signs were considered symptomatic: weight loss, ruffled fur, hunched back, decreased activity/prostration, paresis/paralysis, convulsions, or irregular breathing. Mice were sacrificed by CO₂ asphyxia and all procedures were approved by the Institutional Ethical Committee and the Cantonal Veterinary Office. Vaccination of RasB8 \times 129SvPas mice was performed using a recombinant vaccinia virus encoding β -galactosidase (VV-LacZ), which was kindly provided by D. Pinschewer (University of Geneva, Geneva, Switzerland). Brains were analyzed 7 days after intraperitoneal infection with 2×10^6 plaque-forming units of VV-LacZ. The RasB8 \times 129SvPas background was chosen to facilitate the detection of CD8 T-cell responses to defined H-2^b-restricted epitopes of VV and β -galactosidase (30).

Cell preparations

After transcardial perfusion with isotonic Ringer's solution, brains were dissociated by mechanoenzymatic digestion as described (14), and brain-infiltrating leukocytes (BIL) were partially purified by ultracentrifugation (30,000 \times g) on a continuous 30% Percoll gradient (Fluka).

Immunohistochemistry

Ringer's-perfused brains were frozen in 2-methylbutane. Cryosections (7 μ m) were fixed in 3.7% formaldehyde. Cryosections were microwaved before staining for Ki-67 or isotype control (BD Biosciences). Alternatively, cryosections were directly stained for GFAP or with isotype control (Dako), and CD45 or with isotype control (BD Biosciences). Secondary antibodies included Envision goat anti-mouse horseradish peroxidase (Dako), donkey anti-rabbit horseradish peroxidase (Immunoresearch), revealed with DAB chromogen (Dako), and goat anti-rat biotin (Molecular Probes) used with Vectastain A+B kit (Vector Laboratories) and revealed with AEC (Sigma). Cryosections were counterstained with hematoxylin. Images were recorded on an Axio-cam CCD camera–equipped Axiophot microscope, and analyzed with Axiovision software (Zeiss).

Flow cytometry

After FcR blocking, the following antibodies were used: CD3 (145-2C11), CD4 (GK1.5, H129.19, RM4-5), CD8 (53-6.7), CD11b (M1-70), CD11c (HL3), CD25 (7D4, PC61), CD44 (IM7), CD45 (30-F11), CD45R/B220 (RA3-6B2), CD49b (DX5), CD49d/anti- α_4 integrin (9C10), CD69 (H1.2F3), CD103 (M290), anti- $\alpha_4\beta_7$ integrin/LPAM (DATK32), GR1/Ly6G

(RB6-8C5), IFN- γ (XMG1.2), interleukin 2 (JES6-5H4), tumor necrosis factor (MP6-XT22), and appropriate control isotypes from BD Biosciences or BioLegend; anti-FoxP3 (FJK-16S) and control isotype from eBiosciences; anti-granzyme B (GB11) and control isotype from Caltag; and CD62L from Immuno-Kontakt. eBiosciences (FoxP3 staining) or BD Biosciences (IFN- γ , tumor necrosis factor, interleukin 2, granzyme B) permeabilization and fixation kits were used according to the instructions of the manufacturer. Where indicated, cells were restimulated with phorbol 12-myristate 13-acetate and ionomycin in a monensin solution for 6 hours or with a VV peptide (B8R₂₀₋₂₇, TSYKFESV). Live leukocytes were gated on scatter and analyzed using BD Bioscience or Beckman Coulter cytometers, and results processed with FlowJo software (Tree Star, Inc.).

Statistical analysis

Statistical analyses were performed using Prism 4.0 software (GraphPad), and considered statistically significant if $P < 0.05$.

Results

Early and late brain infiltration by leukocytes in RasB8 mice

As previously shown in the initial characterization of this model, widespread astrocytic hyperplasia leads to a gradual increase in the number of GFAP⁺ astrocytes between 3 and 12 weeks. Moreover, astrocytes in the neoplastic lesions exhibit nuclear atypia and higher bromodeoxyuridine labeling (29).

We confirmed higher GFAP expression in the brains of symptomatic RasB8 mice by immunohistochemistry. Hematoxylin counterstain revealed larger pleiomorphic nuclei in astrocytoma lesions, also expressing the Ki-67 proliferation marker (Fig. 1; Supplementary Fig. S1). We immunostained sections adjacent to those showing Ki-67⁺GFAP⁺ regions and found a CD45⁺ leukocyte infiltration in neoplastic lesions, but not in surrounding tissue. CD45 expression was lower in resident resting microglia than in other BILs and was not detected by immunohistochemistry. Thus, CD45 immunohistochemistry shows only infiltration by CD45^{high} leukocytes recruited from the periphery or activated resident microglial cells. This is consistent with the absence of CD45⁺ staining in age-matched nontransgenic controls (Fig. 1).

RasB8 mice were described to develop progressive glial hyperplasia and low-grade astrocytomas from the age of 4 weeks, and high-grade lesions from the age of 12 weeks (29). To analyze the involvement of different leukocyte subsets during gliomagenesis, BILs were isolated from asymptomatic RasB8 mice (at 4, 8, and 12 weeks), and from symptomatic mice (mean, 13; range, 10–14 weeks), and were compared with BILs from nontransgenic littermates. Individual variability in leukocyte infiltration, tumor progression, and development of symptoms was expected due to the outbred background and stochastic accumulation of genetic lesions, and parallels variability in human gliomagenesis. We therefore analyzed groups of individual mice and not pooled BILs, using multicolor flow cytometry to characterize BILs and to evaluate the dynamics of leukocyte infiltration during astrocytoma progression. As

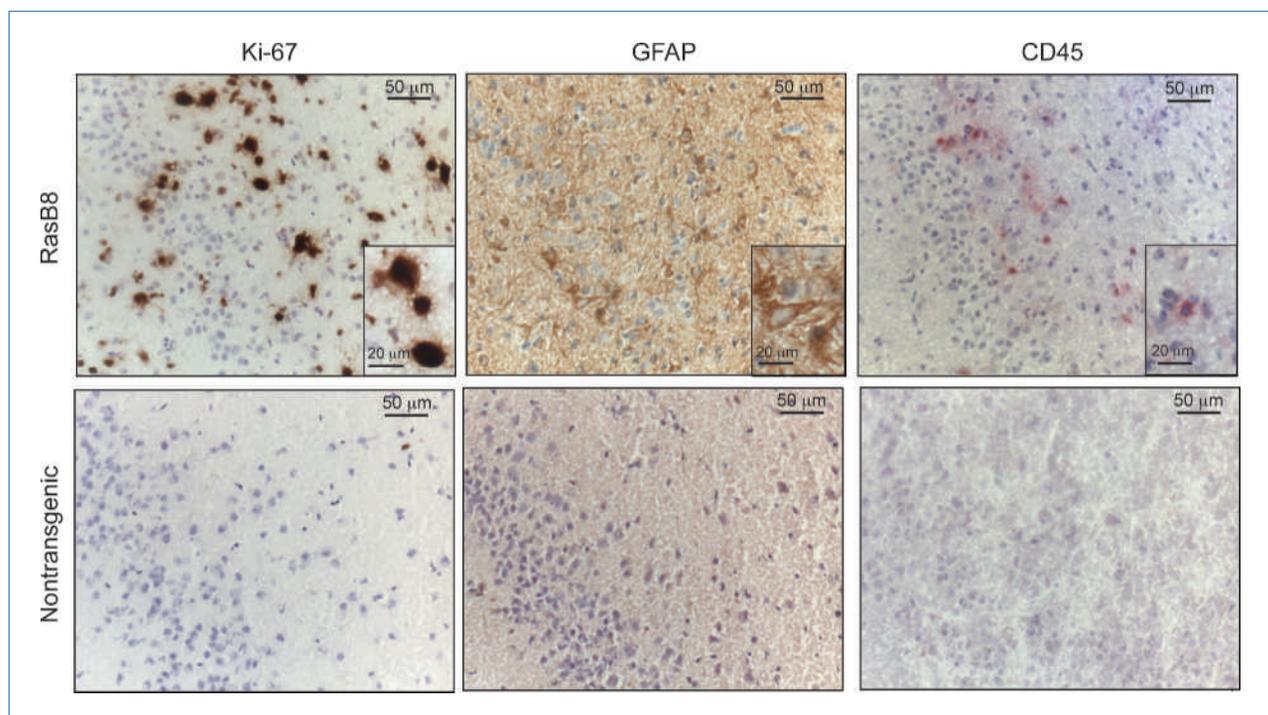


Figure 1. Leukocytes colocalize with pathologic lesions in ill RasB8 mice. Representative Ki-67, GFAP, and CD45 staining of corresponding neoplastic zones in serial brain sections from an ill RasB8 mouse, with control staining in a nontransgenic age-matched mouse.

expected, statistical analysis of each group of mice was not always representative of immune infiltration of individual mice, but is provided as an aid to understand the overall kinetics. The contribution of each leukocyte subset was expressed as a proportion of the total CD45⁺ leukocyte population, i.e., resident microglia as well as infiltrating leukocytes (Figs. 2 and 3). In contrast to immunohistochemistry, resting CD45^{mid} microglial cells in addition to CD45^{high} cells are detected by flow cytometry, and constitute the major leukocyte population in BILs of nontransgenic and transgenic asymptomatic mice. However, in ill RasB8 mice, the balance switches towards predominantly CD45^{high} cells (Fig. 2A and B). At the early ages of 4 and 8 weeks, there was no significant immune infiltration of RasB8 brains. However, CD4 and CD8 T-cells (Fig. 3), but no other leukocyte subset, infiltrated the brains of still asymptomatic RasB8 mice by 12 weeks. A significant proportion of CD4 T cells was CD4⁺CD25⁺, which includes Tregs. In terminally ill mice, infiltration by macrophages/activated microglia (Fig. 2B), CD4 and CD8 T-cells, NK cells, and DCs (Fig. 3), was statistically significant. Moreover, we found an even higher proportion of CD4⁺CD25⁺ Tregs. CD45^{high}CD11c⁺ DCs were predominantly CD11b⁺CD8⁻, with a low proportion (mean, 1.8%) of CD11b⁺Gri1⁺ inflammatory DCs; they expressed high levels of MHC class II and were CD86⁺ (Supplementary Fig. S2). Despite a trend for infiltration by B cells, NK T-cells, and myeloid-derived suppressor cells (Fig. 3), their contribution to the infiltrate was variable and did not differ significantly from nontransgenic littermates. Thus, at 12 weeks, when pathologic lesions progress to high-grade astrocytomas but

mice are still asymptomatic, influx of leukocytes begins with a T-cell infiltrate, including Tregs. Infiltration by macrophages, NK cells, and DCs becomes detectable only in terminally ill mice. To understand the role of CD4 and CD8 T-cells in early immune responses to astrocytomas, we consequently analyzed them in 12-week-old, asymptomatic RasB8 mice.

Expression of $\alpha_4\beta_1$ and $\alpha_E\beta_7$ integrins by brain-infiltrating CD8 and CD4 T-cells at early stages of astrocytoma progression

In autoimmune diseases and in mice implanted intracranially with fibrosarcomas, $\alpha_4\beta_1$ integrin plays a key role in facilitating T-cell entry into the brain (14, 31). However, its importance was never addressed in human gliomas or murine spontaneous astrocytomas. Because α_4 integrin associates either with β_1 or β_7 integrins as a heterodimer at the cell surface, upregulation of α_4 and β_1 in the absence of β_7 corresponds to preferential $\alpha_4\beta_1$ upregulation. In brain-infiltrating CD8 and CD4 T-cells of 12-week-old RasB8 mice, we indeed found such integrin expression (Fig. 4A) that was at a higher level than in T cells isolated from cervical or inguinal lymph nodes. Upregulation of β_1 integrin in brain-infiltrating CD4 and CD8 T-cells confirmed its availability for pairing with α_4 integrin (data not shown).

In intracranially implanted tumors, the induction of $\alpha_E\beta_7$ integrin expression in CD8 T-cells correlated with their retention in the tumor site and with an activated effector phenotype (25). We analyzed $\alpha_E\beta_7$ integrin expression in BILs of 12-week-old RasB8 mice (Fig. 4B; mean, 42.1%; range

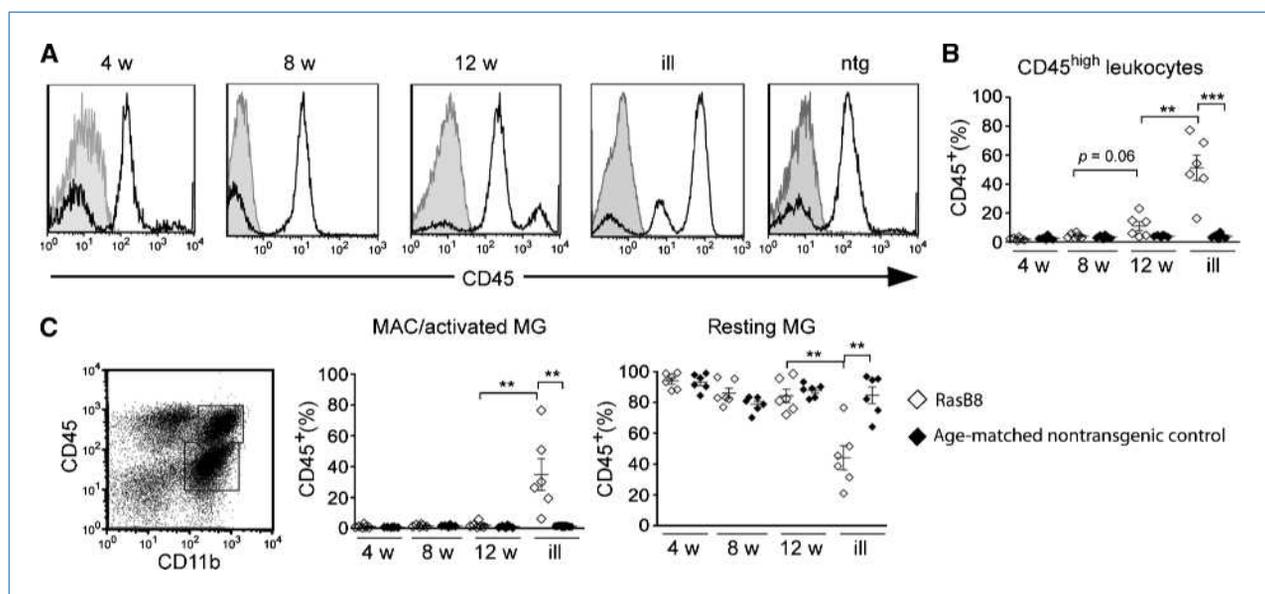


Figure 2. Brain infiltration is composed mainly of CD45^{mid} resting microglia in asymptomatic RasB8 and nontransgenic mice, but of CD45^{high} cells in ill mice. BILs from individual RasB8 mice or nontransgenic littermates were characterized by flow cytometry at 4, 8, and 12 wk (w), and in terminally ill mice, as well as in age-matched nontransgenic (ntg) controls ($n = 6$ for each group; \blacklozenge , RasB8; \diamond , ntg). A, representative CD45 histograms in RasB8 mice and in a nontransgenic control, age-matched to the ill mouse (solid lines, CD45 staining; gray histograms, isotype control). B, scatterplot summarizes the CD45^{high} population at indicated times. C, CD11b versus CD45 dot plot in an ill RasB8 mouse, showing CD11b⁺CD45^{high} macrophages (MAC)/activated microglia (MG), and CD11b⁺CD45^{mid} resting microglia. Scatterplots (with means \pm SEM) summarize the MAC/MG populations at indicated times. **, $P < 0.01$; ***, $P < 0.001$, significant P values determined by Student's t test.

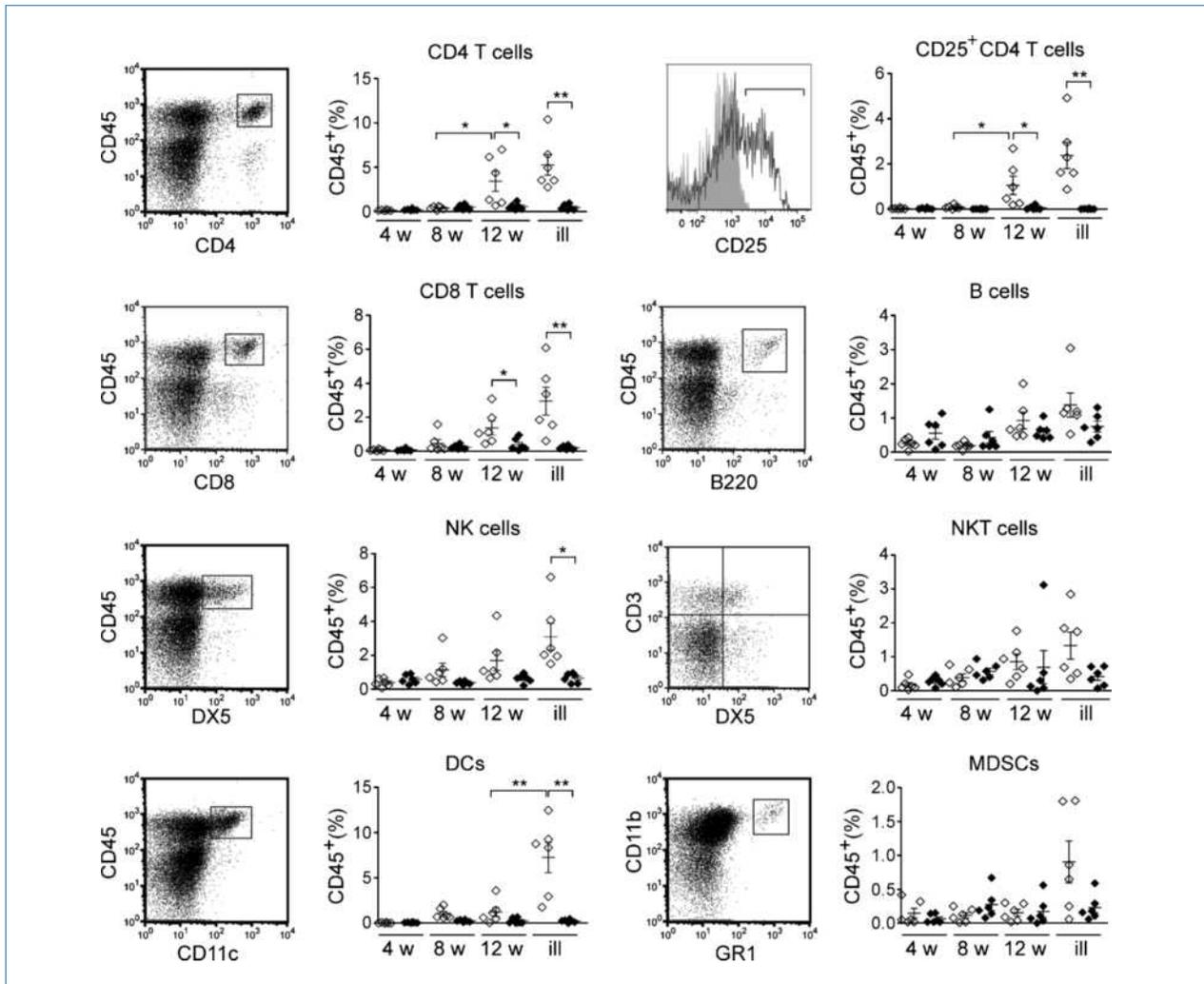


Figure 3. Early RasB8 brain infiltration by T cells occurs at an asymptomatic stage before accumulation of other leukocyte subsets in ill mice. BILs from RasB8 mice or nontransgenic littermates were characterized by flow cytometry at different stages of tumor development. Representative BIL stainings of ill RasB8 mice (dot plots and histogram plot) and kinetic analyses, with means \pm SEM ($n = 6$ for each group; \blacklozenge , transgenic; \diamond , nontransgenic age-matched control). The indicated regions were used to define each population. CD4 T cells ($CD45^{high}CD4^{+}$) and $CD45^{high}CD4^{+}CD25^{+}$ T cells (histogram gated on the $CD45^{high}CD4^{+}$ population); gray histogram, isotype control; solid line, CD25; CD8 T cells ($CD45^{high}CD8^{+}$); B cells ($CD45^{high}B220^{+}$); NK cells (B1, $CD45^{high}DX5^{+}CD3^{-}$); NK T-cells ($CD45^{high}DX5^{+}CD3^{+}$, DX5 versus CD3 plot gated on $CD45^{+}$ cells); DCs ($CD45^{high}CD11c^{+}$) and myeloid-derived suppressor cells (MDSCs, $CD45^{high}GR1^{+}CD11b^{+}$, GR1 versus CD11b plot gated on $CD45^{+}$ cells). w, weeks. *, $P < 0.05$; **, $P < 0.01$, significant P values determined by Student's t test.

24.9–62.9%) and found similar proportions of $\alpha_E\beta_7^{+}$ brain-infiltrating CD8 T-cells as in human malignant gliomas (25). Some CD4 T-cells also expressed $\alpha_E\beta_7$ integrin (mean, 20.7%; range 13.6–40.5%). Given the important proportion of FoxP3⁺ Tregs among brain-infiltrating CD4 T-cells at asymptomatic stages as well as in ill RasB8 mice (Fig. 5A; mean, 20.6% and 35.3%, respectively; $P = 0.05$), we addressed whether $\alpha_E\beta_7$ integrin was preferentially expressed in Tregs. Among brain-infiltrating CD4 T-cells of 12-week-old RasB8 mice, $\alpha_E\beta_7$ integrin was expressed by a mean of 71% of Tregs, whereas it was poorly expressed by FoxP3⁻ CD4 T-cells (9%), or by Tregs in draining cervical lymph nodes (16%) or non-draining inguinal lymph nodes (15%; Fig. 5B).

Defective effector phenotype of brain-infiltrating CD8 T-cells in RasB8 mice

To determine whether CD8 T-cells express cytotoxic molecules and cytokines at early stages of RasB8 brain infiltration, we compared their effector molecule expression after *ex vivo* restimulation with expression in CD8 T-cells isolated from draining cervical lymph nodes and spleens (Fig. 6A1). Tumor necrosis factor was expressed by brain-infiltrating CD8 T-cells (mean, 24.4%) but in lower proportions than in peripheral sites (cervical lymph nodes, 41.7%; spleens, 39.6%). Expression of IFN- γ was low (2.2%), but with slightly higher percentages of CD8 T-cells expressing IFN- γ in cervical lymph nodes (6.9%) and spleens (9.5%). Few brain-infiltrating

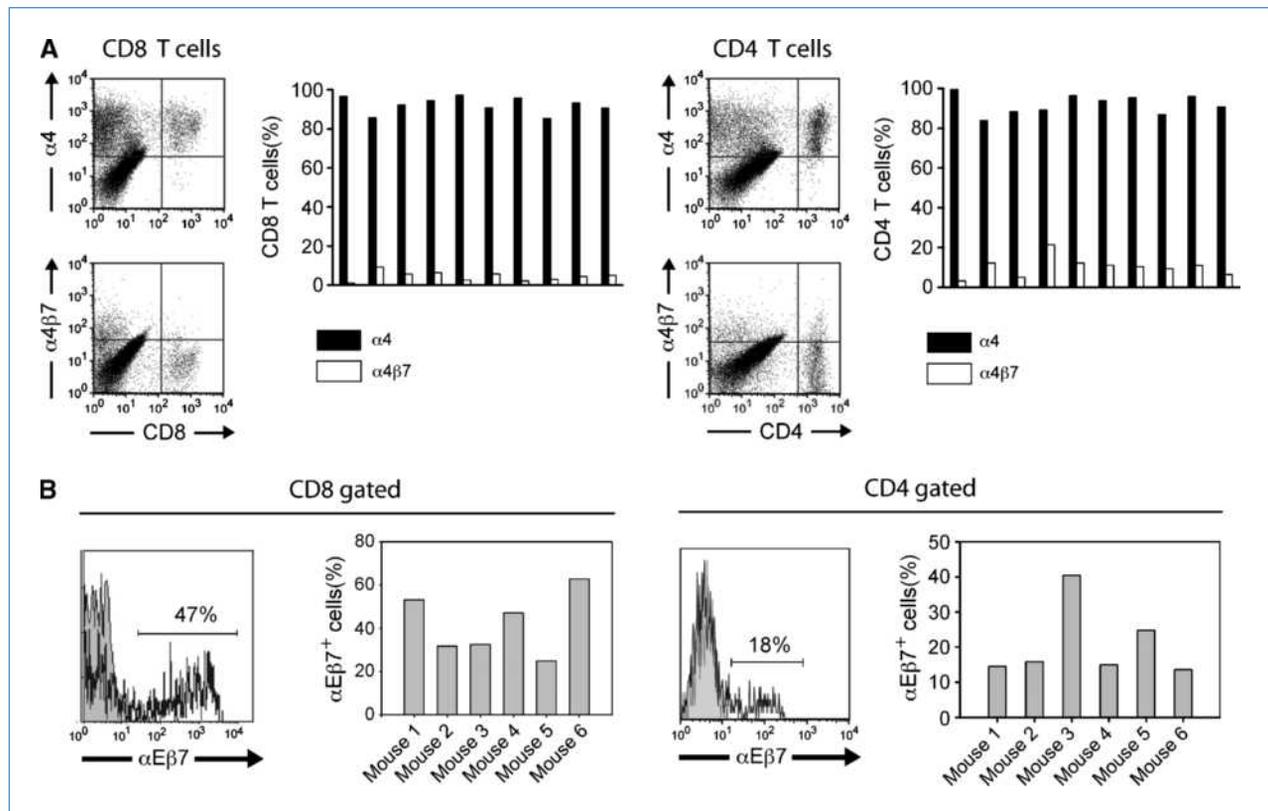


Figure 4. Brain-infiltrating CD4 and CD8 T cells in 12-wk-old RasB8 mice express integrins involved in tropism to and retention in the brain. Representative dot plots and histogram plots are shown, and results are summarized in graphs in which columns represent expression of integrins in individual mice. A, given expression of $\alpha 4$ integrin either as $\alpha 4\beta 7$ or $\alpha 4\beta 1$ heterodimer at the cell surface, expression of $\alpha 4\beta 1$ integrin in RasB8 brain-infiltrating CD4 and CD8 T cells is inferred by positive $\alpha 4$ and negative $\alpha 4\beta 7$ stainings. B, expression of $\alpha E\beta 7$ integrin by CD4 and CD8 T cells in BILs of 12-wk-old RasB8 mice. Gray histogram, isotype control; solid line, $\alpha E\beta 7$. The percentages noted in the histograms are from individual representative mice.

CD8 T-cells expressed interleukin 2 (0.2%) and granzyme B (1%). Overall brain-infiltrating CD8 T-cells in asymptomatic RasB8 mice exhibited a phenotype consistent with partially defective effector function, although they had a non-naïve CD62L^{CD44}^{high} phenotype (data not shown). Granzyme B expression was absent, whether or not they were restimulated with phorbol 12-myristate 13-acetate/ionomycin *ex vivo* (1.0% and 1.3%, respectively; data not shown), and did not increase at later stages in ill RasB8 mice (Fig. 6A3).

Induction of enhanced effector functions in T cells infiltrating RasB8 brains

To determine whether the RasB8 brain microenvironment inevitably overwhelms the effector function of CD8 T cells, we induced a more substantial immune infiltrate in response to peripheral vaccination with VV-LacZ recombinant virus. This approach was chosen to stimulate T cells specific for the β -galactosidase antigen encoded in the transgene (thus expressed as an astrocytoma-associated antigen). Although β -galactosidase-specific T cells were at/below the limit of *ex vivo* detection in VV-LacZ vaccinated mice, vaccination did induce T cells that infiltrated the brains of transgenic mice, and which had enhanced capacity to express IFN- γ . These T cells included viral antigen-specific CD8 T cells

(Fig. 6B1), as well as CD4 T cells (Fig. 6B3). Moreover, the CD4/CD8 ratio in the BILs was significantly and consistently decreased after viral vaccination (Fig. 6B2). Viral vaccination induced no detectable T-cell infiltration of brains of non-transgenic littermate control mice (data not shown). We also tested brain-infiltrating CD8 T-cells for granzyme B expression after viral vaccination, but this remained negative (data not shown). Indeed, the only circumstances in which we could detect an infiltrate of granzyme B⁺ CD8 T-cells, was after intracranial implantation of immunogenic P815 mastocytoma cells directly into the brain of RasB8 \times BALB/c transgenic mice (Supplementary Fig. S3). Treg proportions in BILs were unchanged by the implantation (Supplementary Fig. S3C), indicating that the local immunosuppressive environment was permissive for CD8 T-cell expression of granzyme B when a sufficiently strong immune stimulus was applied locally.

Discussion

Analyses of human malignant astrocytoma and intracranially implanted mouse models highlight the complexity of interactions between the immune system and gliomas.

Despite compelling data showing glioma-infiltrating immune cells and several mechanisms of immune escape (3, 32, 33), the dynamics of these local interactions were not directly explored in spontaneous glioma. Indeed, it is unknown whether the immune infiltrate in advanced spontaneous glioma reflects the recent arrival of immune cells following rupture of brain integrity, with an immune response immediately overwhelmed by high tumor burden. Otherwise, earlier during gliomagenesis, immune effector mechanisms could initially restrain glioma growth and lead to the selection of less immunogenic tumor cells, or immunosurveillance might be inefficient from the outset. To better understand the dynamics of these interactions, we used the RasB8 spontaneous glioma model, in which astrocytomas gradually develop and recapitulate histopathologic features of human gliomas.

Analysis of RasB8 mice revealed immune infiltration of the brain parenchyma before mice exhibited malignancy-associated symptoms. Thus, the immune system is alerted at a relatively early stage of gliomagenesis. CD8 and CD4 T cells, including a significant proportion of Tregs, infiltrated the brains of asymptomatic 12-week-old mice. In terminally ill mice, we observed a more intense infiltration, including macrophages, DCs, and NK cells in addition to T cells. This does not exclude the involvement of innate immune cells at earlier stages, but can reflect their more gradual mobilization by a progressively developing tumor, in contrast to the acute immune infiltrate seen in implanted tumors.

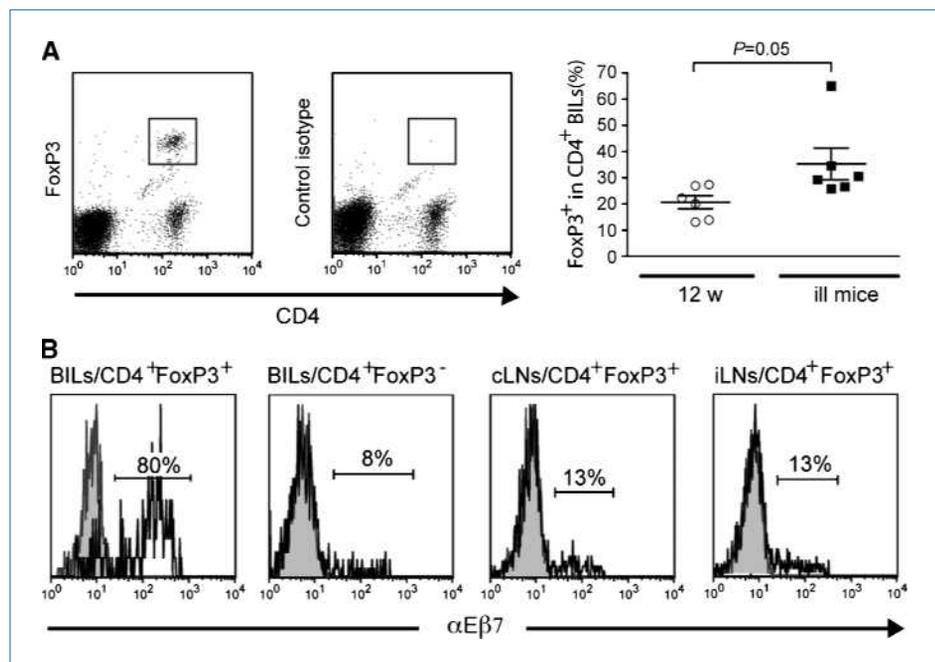
The phenotype of innate immune cells infiltrating RasB8 brains may not promote efficient anti-glioma immunity. Although the nature of brain antigen-presenting cells is still debated, recent evidence points to CD11c⁺ DCs as antigen-presenting cells playing a critical role at least for naïve T-cell priming and generation of memory T cells (34). The DCs we identified late

in the disease were mainly CD11c⁺CD11b⁺CD8⁻ DCs, which might be inefficient for antigen cross-presentation and priming of CD8 T-cells, despite higher levels of MHC class II and CD86 than CD11c⁻CD11b⁺ cells (Supplementary Fig. S2). This is also in line with the study of Biollaz and colleagues, in which CD11c⁺CD11b⁺CD8⁻ DCs infiltrating intracranially implanted GL261 tumors exhibited poor T-cell activation capacities, but induced Tregs (35).

Concerning other innate immune cells, CD45^{high}CD11b⁺ cells were detected only in ill mice. Estimated absolute cell numbers from populations shown in Fig. 2B suggest an increase in CD45^{mid}CD11b⁺ microglial cells (from <35,000 to >60,000 cells per brain at late stages) and show massive recruitment of CD45^{high}CD11b⁺ cells (from <1,000 to >56,000 cells per brain). The significance of tumor-associated macrophages/activated microglia in glioma is debated, as in other tumors, where hypoxia-induced tumor-associated macrophages were associated with poor prognosis, angiogenesis, and tumor invasiveness (36). Glioma tumor-associated macrophages/activated microglia were reported to have poor tumoricidal activity and capacity to activate antitumor effector T cells (23). Moreover, M2 macrophages, associated with tolerance and anti-inflammatory properties, were detected in gliomas and correlated with tumors of higher grade (37).

Two prerequisites for efficient T-cell antitumor immunity are the ability to traffic to the tumor site and the expression of effector molecules. RasB8 brain-infiltrating CD8 T-cells show a partially defective effector phenotype, despite the up-regulation of integrins associated with preferential homing to and retention in the brain. Entry of immune cells into the noninflamed brain is usually low and subjected to tight regulation at the blood-brain barrier. In contrast, in multiple sclerosis and experimental autoimmune encephalomyelitis,

Figure 5. Activated $\alpha\text{E}\beta_7^+$ Tregs accumulate in the brains of RasB8 mice. **A**, infiltration of RasB8 brains of 12-wk-old and ill mice by FoxP3⁺CD4⁺ Tregs (mean, 20.6% and 35.3%, respectively; $P = 0.05$). Representative dot plot shows gating of the FoxP3⁺CD4⁺ population compared with isotype control staining in a 12-wk-old mouse (w). The indicated region was used to calculate the values shown in the scatter plot. P value calculated using a t test. **B**, expression of $\alpha\text{E}\beta_7$ integrin by FoxP3⁺ or FoxP3⁻ CD4⁺ T cells in BILs of 12-wk-old RasB8 mice ($n = 4$), and by FoxP3⁺ CD4⁺ T cells in draining cervical lymph nodes (cLNs) or nondraining inguinal lymph nodes (iLNs). Gray histogram, isotype control; solid line, $\alpha\text{E}\beta_7$. Representative of one of two experiments with similar results.



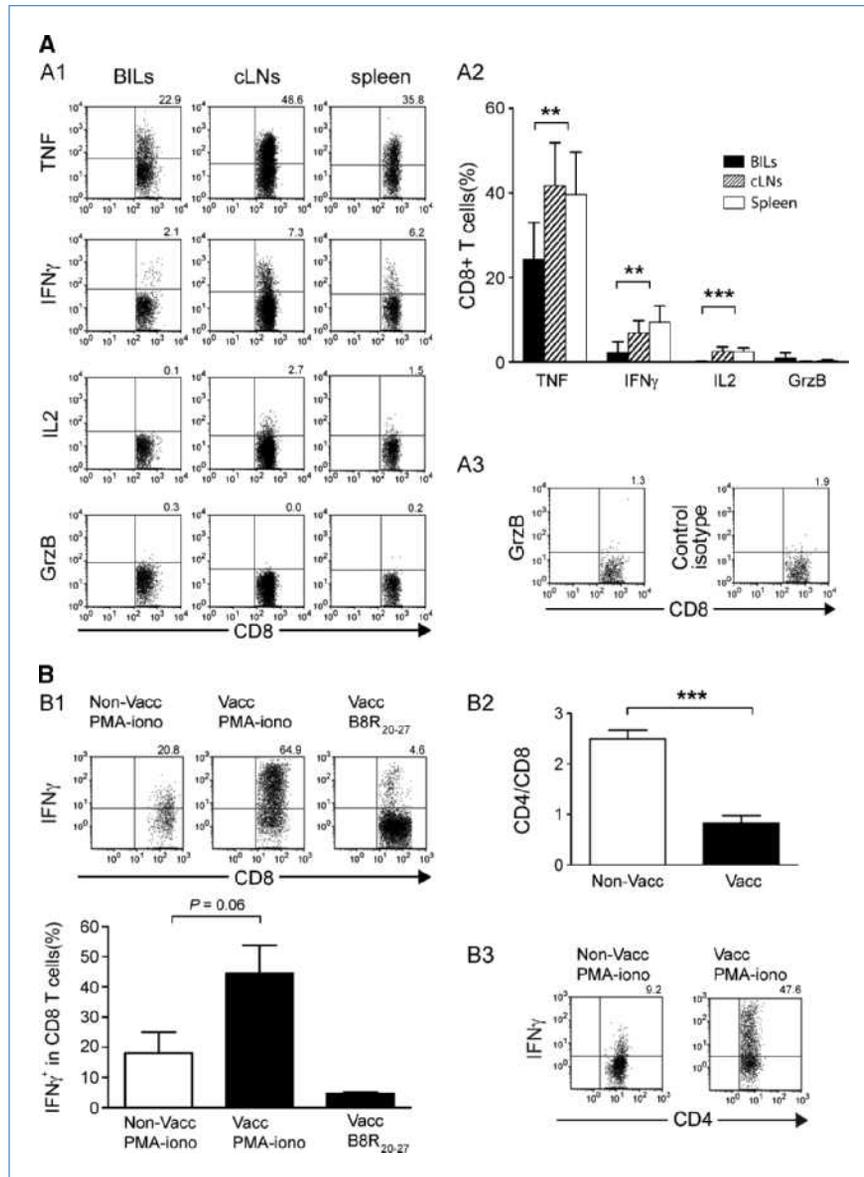


Figure 6. CD8 T cells infiltrating brains of RasB8 mice exhibit a partially defective effector phenotype but expression of IFN- γ by brain-infiltrating CD4 and CD8 T cells could be stimulated by vaccination. **A**, defective effector phenotype of brain-infiltrating CD8 T cells. Representative stainings of tumor necrosis factor (TNF), IFN- γ , interleukin 2 (IL2), and granzyme B (GrzB) expression gated on CD8 T cells from BILs, draining cervical lymph nodes, or spleens of 12-wk-old RasB8 mice (A1). Quadrants were defined according to isotype controls and used to calculate the values shown in the histogram with means and SEM (A2, $n = 6$). A representative dot plot for granzyme B expression by brain-infiltrating CD8 T cells in an ill RasB8 mouse is shown and compared with isotype control (A3, $n = 4$, mean expression granzyme B⁺ CD8 T cells, $1.96 \pm 0.79\%$). Numbers above the quadrants correspond to percentages of CD8 T cells. **B**, peripheral vaccination with β -galactosidase recombinant vaccinia virus (VV-LacZ). BILs isolated from infected (Vacc) and noninfected (Non-Vacc) RasB8 \times 129SvPas mice were analyzed 7 days after infection. IFN- γ expression gated on CD8 T cells is shown after *ex vivo* restimulation with phorbol 12-myristate 13-acetate/ionomycin (PMA-iono) or with a VV peptide (B8R₂₀₋₂₇, TSYKFESV; B1). Quadrants in representative dot plots were defined according to isotype control staining. Values in the histogram represent means from all mice, \pm SEM ($n = 4$). Ratio of CD4 to CD8 T cells in BILs from VV-LacZ noninfected and infected mice is also represented with means and SEM (B2). Dot plots of IFN- γ expression gated on CD4 T cells from BILs of VV-LacZ noninfected and infected mice are shown ($n = 2$), with quadrants defined according to isotype control staining (B3). P value was determined with ANOVA (A2) or Student's t test (B1 and B2; **, $P < 0.01$; ***, $P < 0.001$). The numbers above the quadrants correspond to percentages of CD8 or CD4 T cells as indicated.

in vitro and *in vivo* experiments with anti- α_4 blocking antibodies showed a key functional role of $\alpha_4\beta_1$ in controlling the entry of T cells into the brain (31, 38, 39). Less is known in tumoral situations, although $\alpha_4\beta_1$ integrin facilitates T-cell

access to the brain in acute responses to intracranially implanted fibrosarcomas and melanomas (14, 16). We show for the first time that this integrin is also upregulated in T cells infiltrating spontaneous gliomas. Furthermore, we

noted the expression of $\alpha_E\beta_7$ integrin by CD8 T-cells, which was associated with their retention in the brain (25), and with granule exocytosis and antitumor activity in E-cadherin-expressing lung cancers (40). E-cadherin is not detected in gliomas, but it is assumed that other $\alpha_E\beta_7$ ligands exist because E-cadherin-independent adhesion of $\alpha_E\beta_7^+$ lymphocytes to endothelial and epithelial cells was described (41). It is likely that the transforming growth factor- β -rich glioma microenvironment plays a role in the induction of $\alpha_E\beta_7$ integrin (42). Indeed, in RasB8 brains, we confirmed transforming growth factor- β expression by quantitative reverse transcription-PCR (Supplementary Fig. S4).

However, CD8 T-cells infiltrating RasB8 brains strikingly differed from those observed in implanted models. In a model using intracranial implantation of fibrosarcomas, antigen-experienced CD8⁺ T cells proliferated in the brain and upregulated their IFN- γ and granzyme B expression (25). CD8 T-cells infiltrating intracranially implanted GL261 glioma similarly expressed effector molecules (43),⁴ although these cells did not enable GL261 tumor rejection without TLR stimulation (44) or depletion of Tregs (43, 45). In RasB8 mice, inefficient priming is one possible explanation for poor effector molecule expression by brain-infiltrating CD8 T-cells. Indeed, Thomas and colleagues showed that brain tumor antigens draining to cervical lymph nodes generated a low-magnitude local CD8 T-cell response (46). Although in RasB8 mice, elevated numbers of activated T cells were not detected in cervical lymph nodes (data not shown), this is likely due to the gradual kinetics of tumorigenesis. Moreover, central tolerance to the β -galactosidase component of the transgene was absent because CD4 and CD8 T-cells proliferated in RasB8 mice immunized with β -galactosidase protein (data not shown). Another hypothesis for the lack of effector molecule expression implicates local immunosuppressive factors and cells. These could progressively inhibit proliferation and full activation of CD8 T-cells early after infiltration. The importance of Tregs was addressed in acute immune responses to intracranially implanted gliomas (33, 45); Treg depletion alone or in combination with vaccination had an effect on survival and antitumor immunity (43, 45, 47, 48). Tregs accumulate simultaneously with CD8 T-cells in the brains of asymptomatic RasB8 mice and express $\alpha_E\beta_7^+$ integrin, which correlates with an effector/memory phenotype, and their redirection to inflammatory sites (49). Moreover $\alpha_E\beta_7^+$ Tregs were recently shown to accumulate in tumors and to potentially suppress antitumor CD8 T-cells (50). Tumor-associated macrophages/activated microglia observed in ill mice may also further perturb the local response and favor tumor progression. These late effects are consistent with observations in human glioma (23, 37). However, importantly, the possibility to study early phases of the immune response in the RasB8 model has revealed Tregs as the main early immunosuppressive cells, suggesting that they tilt the balance towards tumor escape.

⁴ Our unpublished data.

Our peripheral vaccination experiments (Fig. 6B) indicated that a potentially clinically relevant approach could tilt the intracranial immune balance towards Tc1/Th1 (IFN- γ secreting) effector functions. Moreover, even symptomatic RasB8 mice show unimpaired *in vitro* T-cell mitogen responses, and only minor changes in CD4/CD8 T-cell ratios in lymph nodes (Supplementary Fig. S5), suggesting that peripheral stimulation of antitumor immunity might be feasible. However, the absence of granzyme B expression by brain-infiltrating T cells after vaccination could indicate that a stronger local immune stimulus (as illustrated in Supplementary Fig. S3), or inhibition of Tregs or factors such as STAT-3, will be necessary for the induction of polyfunctional antitumor T cells expressing a range of effector molecules.

These encouraging results suggest that future immunotherapies might function in the context of local Tregs, provided the immune stimulation and brain tumor infiltration by effector T cells are sufficient. Moreover, observations made in RasB8 mice at early stages might be relevant for the design of complementary immunotherapies for unresectable lower grade gliomas. Other approaches to generate mouse models recapitulating progressive genetic alterations and histopathologic features of human glioma have been or are being developed. These include models allowing retroviral gene transfer to selected precursor cells, or models using transgenesis to target individual or multiple genetic pathways (27). However, to our knowledge, these models have not been used to examine the issue of host immunity or immunosuppression.

Our study has taken a first step in addressing early phases and dynamics of the immune response in spontaneous glioma. Elimination or equilibrium phases are unlikely to take place in these tumors in view of the early unbalanced response towards immunosuppression. The lack of an effective pre-existing CD8 T-cell response, the abundant Treg infiltrate, and the potential role of tumor-associated macrophages at late stages of disease provide specific targets for future multimodal glioma therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Immune Infiltration of Spontaneous Mouse Astrocytomas Is Dominated by Immunosuppressive Cells from Early Stages of Tumor Development

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