Intracerebral Interleukin 12 Induces Glioma Rejection in the Brain Predominantly by CD8\(^+\) T Cells and Independently of Interferon-\(\gamma\)

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Abstract

The prognosis of gliomas is generally poor since these tumors elude established therapeutic approaches. Immunotherapy might present an effective therapy in particular because the glioma cells are diffusely dispersed in the infiltration zone of the tumor and show a strong propensity to invade the surrounding brain along white matter tracts. Although various immune therapies for brain tumors are successful in rodents, there is currently no effective therapy in humans. In the present study, we investigated the mechanisms by which intracerebral IL-12 mediates rejection of GL261 cells in a syngenic mouse glioma model. Wild type mice revealed smaller tumors as compared to mice lacking functional T and B cells indicating that considerable immune dependent tumor rejection occurs physiologically in this model. However, glioma rejection was significantly enhanced in mice expressing IL-12 in the CNS and was predominantly dependent on the presence of CD8\(^+\) T cells while CD4\(^+\) T cells had less impact. Interestingly, the rejection of tumors was independent of IFN-\(\gamma\). Our findings contrast results obtained after in vitro or systemic stimulation with IL-12 and demonstrate that successful IL-12 induced glioma rejection critically depends on the localization, duration and time of IL-12 expression.

Key Words: Glioma, Immune therapy, Interleukin-12.

INTRODUCTION

Gliomas of the central nervous system (CNS) are graded according to their biologic behavior and are grouped into 4 distinct World Health Organization grades ranging from benign (World Health Organization Grade I) to malignant (World Health Organization Grade IV) (1). Independent of their biologic behavior, however, the most frequent gliomas have poor outcomes because of diffuse infiltration into the surrounding brain tissue that prevents complete surgical removal of neoplastic cells and can result in progressive malignancy of initially low-grade tumors. Moreover, radiation and chemotherapy have only limited effects on the survival of patients with high-grade gliomas (2). Therefore, alternative therapeutic modalities are desperately needed. Immunotherapy that prompts the host immune system to eliminate malignant cells in a specific manner has been shown to have some effect in several human malignancies. Moreover, an inverse correlation of atopic disorders and the prevalence of gliomas have been reported in a number of studies, suggesting that there is a low level of naturally occurring immunity against gliomas (3). The use of a glioma cell–specific immune response would have the advantage that all neoplastic cells could be targeted, although they are dispersed in the surrounding normal brain tissue. In recent years, a number of experimental models have demonstrated that this concept might work (4–9).

The cytokine interleukin 12 (IL-12) is crucial for the induction of antitumor T\(\text{h}1\) immune responses. It is a potent inducer of cellular immunity, an activator of T cells, and is critical for the production of interferon-\(\gamma\) (IFN-\(\gamma\)) by T cells and natural killer (NK) cells. Interleukin 12 has, therefore, been widely used in many different experimental tumor immunotherapy models; it mediates elimination of glioma cells in vivo (10), and glioma inhibition correlated with infiltration by activated CD4\(^+\) and CD8\(^+\) T cells (11). In an in vivo fibrosarcoma model as well as in vitro, several coactivators such as IFN-\(\gamma\)–induced nitrogen monoxide synthetase (NOS-2) mediate tumor rejection (12, 13). The effect of NOS-2 is, however, unclear because others have found that inhibition of NOS-2 together with IFN-\(\gamma\) treatment prolonged the survival of rats bearing gliomas (14). Moreover, IFN-\(\gamma\) was shown to inhibit angiogenesis in established brain tumors (15). Despite promising results in animal models, the transfer to human glioma therapy has not been established yet (16, 17).

The antitumorigenic potential of IL-12 is well established, but the exact pathway by which IL-12 mediates tumor rejection has not yet been identified. To elucidate the mechanisms of IL-12–induced tumor rejection in the brain, we implanted GL261 glioma cells into the cerebellum of transgenic mice that constitutively express IL-12 under
control of the glial fibrillary acidic protein (GFAP) promoter in astrocytes. The role of lymphocyte subsets and IFN-γ in tumor rejection was investigated in these mice by T-cell subset depletion and by using mice that lack functional T and B cells and mice bred on an IFN-γ-deficient background. Our results confirm previous studies that showed a beneficial effect for IL-12 in glioma rejection and demonstrate that the antitumor effects of IL-12 are mediated mainly by CD8+ T cells; surprisingly, they do not seem to depend on the presence of IFN-γ.

MATERIALS AND METHODS

Mice and Tumor Implantation

Mice of the GF-IL12 line have been previously described (18). These mice express IL-12 in the CNS under control of the GFAP promoter, resulting in a spontaneous neurological disease starting around 3 months of age. The GF-IL12 mice deficient for RAG2 (GFR) or IFN-γ (GFI), respectively, were bred on a C57BL/6 background homozygous for the disruption of either RAG2 or IFN-γ and heterozygous for the IL-12 transgene. Genotyping was performed by polymerase chain reaction (PCR) of tail DNA. The phenotype of these mice has been reported before (19); neither GFR nor GFI mice develop the spontaneous disease that occurs in GF-IL12 mice caused by lack of lymphocytes and absence of IFN-γ, respectively. If disease is triggered in GFI mice by infection with Borna disease virus, they do not develop the typical calcifications of GF-IL12 animals, which allows for histological confirmation of the disruption of the IFN-γ gene (19).

Mice were housed in the specific pathogen-free animal facility of the University Hospital of Freiburg, Freiburg, Germany. All experimental animal procedures were approved by the Regierungspräsidium Freiburg. To precede the onset of the spontaneous disease, all mice used in this study were less than 10 weeks of age at the time of tumor implantation. Because GF-IL12 mice show maximum expression of IL-12 in the cerebellum, the tumors were induced in mice of the different lines by stereotactic injection of $3 \times 10^4$ GL261 glioma cells in 3 μL PBS into the white matter of the right cerebellar hemisphere. The sulci of the cerebellum are very deep, and hence the cerebellar white matter that is suitable for tumor cell injection is very small. A reproducible tumor model was established using the following coordinates of the target point: 1 mm lateral and 2.5 mm ventral of the intersection of the lambda and the sagittal sutures and 2.5 mm below the dura mater. The injection cannula was inserted 0.3 mm beyond the target point and then retracted to the target point to establish a pouch for the injected tumor cells. Tumor cells were injected over 5 minutes followed by a 10-minute waiting period. The cannula was slowly removed during a period of 5 minutes. Shorter waiting periods or faster removal of the cannula resulted in loss of tumor cells into the cerebrospinal fluid (CSF) from which the tumor cells were eliminated by immune cells (data not shown). Marked mononuclear cell infiltration was observed in the meninges around the cerebellum, with no remaining tumor cells in these animals. None of the mice developed any neurological signs. Thus, the 20-minute period for tumor cell injection proved to be crucial for reproducible tumor development in the cerebellum.

After application of the tumor cells, the animals were monitored daily for the development of disease. In 1 series of experiments, the animals were weighed every other day. Animals were killed after 3 weeks. Mice demonstrating symptoms of raised intracranial pressure were killed earlier.

Depletion of T Cells

The GF-IL12 mice received 2 intraperitoneal injections of monoclonal anti-CD4 or anti-CD8 antibodies (clones YTS 191 and YTS 169, respectively [20]) in 200 μL sterile saline the day before and the day after implantation of tumor cells. The hybridomas were a generous gift from Dr H. Waldmann, Oxford, United Kingdom. One and 2 weeks after tumor implantation, blood samples were drawn from the tail vein of depleted and control mice, and the number of CD4+ and CD8+ T cells was determined by fluorescent activated cell sorter analysis as described (19).

Determination of the Tumor Volume

Mice were anesthetized with isoflurane (Forene, Abbott, Wiesbaden, Germany), the thoracic cavity was opened, and animals were perfusion fixed with 4% PBS buffered formaldehyde via the left ventricle. The brain was removed and fixed for 24 hours in the perfusion solution. The cerebellum was dissected from the forebrain and washed in PBS for at least 1 hour. The tissue was embedded in 4% agarose in PBS, and 60-μm sections were prepared on a vibrating blade microtome (Leica VT1000). Pilot experiments revealed that the tumor area showed only minute differences between serial sections; therefore, every fourth section was used for the determination of the tumor area (data not shown). The tumor area was measured with imaging software (Leica IM 1000) in sections stained with cresyl violet. The tumor volume was calculated as the sum of the product of tumor area, section thickness, and the factor 4. To permit both determination of the tumor volume and RNA expression in tumor-implanted cerebella in one experiment, the naive brain was snap frozen in liquid nitrogen–chilled methylbutane. From these specimens, 30-μm cryostat sections were cut, and every eighth of these was stained with hematoxylin and cosin and used for measurement of the tumor size. The tumor volume was calculated as previously described with a factor 8. The remaining sections were used for RNA extraction as described later.

RNA Isolation

Brains of tumor-bearing as well as of normal controls were removed, and one hemisphere was dissected into forebrain and cerebellum, the other hemisphere was processed for immunohistochemical examination (see later). The dissected samples were immediately snap frozen in liquid nitrogen and stored at −80°C pending RNA extraction. Total RNA was extracted with Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol. The
RNA was dissolved in TE (10 mM Tris pH 8, 1 mM EDTA) and stored at -80°C.

**RNase Protection Assays**

RNase protection assays (RPAs) for the detection of cytokine RNAs were performed as previously described (21). The RNA samples were hybridized with labeled probe sets IC5 (22), ML11, and ML26 (23). In all probe sets, a fragment of the RPL32-4A gene served as an internal loading control (24). Biomax films (Kodak) were exposed for various periods and scanned using a Microtek ScanMaker 9800 XL. The NIH Image 1.62 software was used to quantify the autoradiographs.

**Histology and Immunohistochemistry**

Mice were anesthetized with isoflurane and decapitated. The brains were removed, and the left and right hemispheres were dissected. Hemispheres were snap frozen in methylbutane chilled in liquid nitrogen and stored at -80°C until sagittal sections were prepared. Twelve-micrometer-thick sections were used for histology and immunohistochemistry. Antibodies used were against CD4, CD8, CD45R, CD31 (BD Pharmingen, Heidelberg, Germany), GFAP (Dako, Glostrup, Denmark), and MAC1 (CD11b/CD18) (a generous gift from M. Simon MPI, Freiburg, Germany). Immunohistochemistry was performed as previously described (19).

**RESULTS**

**Expression of IL-12 in the CNS Induces Rejection of GL261 Tumor Cells**

After injection of GL261 cells, the animals were monitored daily for clinical signs and were weighed every second day. No overt clinical signs were observed during the first 14 days. From Day 14 to 16, wild-type (WT) mice developed progressive ataxia and weight loss; after 4 weeks, the mean weight loss was 10.9%. Most WT mice showed severe ataxia, decreased locomotion; 30% of the WT mice developed hemiparesis. This sharply contrasted with GF-IL12 mice that showed only mild ataxia and did not develop paresis. Importantly, the GF-IL12 mice had a mean increase (7%) in body weight at 4 weeks. An inverse correlation between the change of body weight and tumor size indicated the better health of tumor-implanted GF-IL12 mice compared with WT mice.

At 4 weeks postimplantation, histological analysis of vibrating blade microtome sections of the cerebellum revealed large tumor masses in WT mice with a mean tumor volume of 44.4 µL. The tumors were confined to the cerebellum and brainstem, whereas the CSF spaces, skull, and skin overlaying the injection site were free of tumor cells. In GF-IL12 mice, the tumors were significantly smaller (mean tumor volume, 14.4 µL) and were also confined to the cerebellum. Figure 1 shows representative sections of the maximum tumor area of WT (Fig. 1A) and GF-IL12 (Fig. 1B) animals. Calculation of tumor volumes revealed significantly smaller tumors in GF-IL12 mice compared with WT mice (Fig. 1C; p ≤ 0.0013, Mann-Whitney U test). Thus, histological analysis demonstrated that IL-12 expression in the CNS mediated growth inhibition of implanted GL261 cells. In a separate experiment (see later), we determined by RPA the IL-12 messenger RNA (mRNA) expression in the tumor-implanted cerebellum (Fig. 1D). This analysis revealed an inverse correlation between IL-12 mRNA levels and tumor size, supporting the hypothesis that tumor rejection was IL-12 dependent.

**Tumor Rejection in the CNS of GF-IL12 Mice Is Accompanied by Increased Lymphocytic Infiltrates**

Immunohistochemical analysis on cryostat sections of tumor-implanted WT mice revealed scattered CD4-immunoreactive T cells in the border zone of the tumor...
and the surrounding parenchyma and to a lesser extent within the tumor (Fig. 2). There were only few CD8+ T cells in the periphery of the tumors in WT mice. No tumor cells were observed in the CSF spaces. CD45-R–immunoreactive B cells were only rarely detected, and there was prominent activation of MAC-1-immunoreactive macrophages in the border zone of the tumor and to a lesser extent within the tumor. In tumor-implanted GF-IL12 mice, the numbers of CD4+ T cells were increased compared with WT mice. Strikingly, high numbers of CD8-immunoreactive T cells were present within the tumor, tumor border, surrounding brain, and in the leptomeninges in GF-IL12 mice (Fig. 2). In both WT and GF-IL12 mice, B cells were rarely detectable; prominent infiltration by activated macrophages was observed in the tumors of GF-IL12 mice.

**FIGURE 2.** Characterization of immune cell infiltrates in GL261 tumors in the cerebellum of wild-type (WT) (A, C, E, G) and GF-IL12 (B, D, F, H) mice. Infiltration of CD4+ and CD8+ T cells is pronounced at the border zone of tumors and brain parenchyma. There are increased numbers of both T-cell types in the cerebellum of GF-IL12 mice (B, D) compared with the WT mice (A, C). Only a few CD-45R–immunoreactive B cells infiltrated the tumors of mice of both backgrounds (E, F). Strong upregulation of CD11b/CD18 (MAC-1) was seen in all animals (G, H). Original magnification: 40×.

**FIGURE 3.** RNase protection assays (RPA) for the detection of proinflammatory cytokine genes revealed induction of TNF, IL1α and IL1β in the cerebellum of wild-type (WT) mice after tumor implantation (A). In the cerebellum of GF-IL12 mice there was constitutive expression of TNF, IL1α, IL1β and IFN at high levels that was only slightly further upregulated upon tumor implantation. The calculated relative expression levels of the genes determined is depicted in (B). RPA was performed as described in the Materials and Methods section. LT, lymphotoxin; EB22/5, mouse homologue of human α₁-antichymotrypsin; ICAM-1, intercellular adhesion molecule 1; MAC-1, CD11b/CD18; NOS, nitric oxide synthase; TGF-A, transforming growth factor-A.

**Lymphocytes Are Required for Tumor Rejection in GF-IL12 Mice**

We next investigated whether lymphocytes mediate tumor rejection in GF-IL12 mice. Tumor cells were implanted into the cerebellum of GF-IL12 mice with disruption of the Rag2 gene (GFR). Mice of the GFR line do not develop the spontaneous disease phenotype of GF-IL12 mice, demonstrating that lymphocytes mediate the disease observed (19). In GFR mice, the implanted tumor cells showed an explosive growth in the cerebellum, expanding into the CSF spaces through the skull and into the skin. Because the tumors massively disseminated, it was not possible to determine the exact tumor size in these animals. This observation demonstrated...
that in GF-IL12 animals, lymphocytes are essential for the elimination of tumor cells from sites other than the CNS such as CSF spaces, skin, and bone. Moreover, this result demonstrated that in the absence of lymphocytes, intracerebral tumors became larger compared with those in WT mice, indicating that considerable intracerebral tumor rejection occurs even in WT mice. Moreover, this result suggests that NK cells (which are present in Rag2-deficient mice) are of minor relevance in tumor rejection in this model.

**RNA Expression Patterns in the CNS of WT and GF-IL12 Mice**

We next determined the expression of genes that could be involved in the lymphocyte-mediated tumor rejection. This analysis revealed that many of the genes tested were expressed at very low levels in WT mice and were strongly upregulated in GF-IL12 mice (Fig. 3; Table). In tumor-bearing WT mice, we observed strong upregulation of NOS-3 and the mouse homologue of human α-1-antichymotrypsin (EB22/5), as well as moderate upregulation of IL-1β, transforming growth factor-β, and tumor necrosis factor compared with those in control mice. Part of the upregulation of NOS-3 and EB22/5 in tumor-bearing WT mice may have come from the implanted GL261 cells because these cells express these genes in vitro (Table). Interestingly, there was marked downregulation of NOS-1 gene expression in WT mice that was most likely caused by loss of neurons in the course of tumor growth. There was significantly higher expression of NOS-2, MAC-1, intercellular adhesion molecule 1, tumor necrosis factor, IFN-γ, IL-1α, IL-1β, and transforming growth factor-β in the cerebella of GF-IL12 compared with those in WT mice. In tumor-bearing GF-IL12 mice, there were only minor changes of gene expression compared with control GF-IL12 mice (Fig. 3; Table).

**IFN-γ Is Not Involved in the Tumor Rejection in GF-IL12 Mice**

Because IFN-γ regulates several of the genes (e.g. NOS-2) that are upregulated in GF-IL12 mice (19) and might increase the immune response against the implanted tumor cells, we investigated the role of this cytokine in GF-IL12 mice with a disruption of the Ifng gene (GFI). Nontransgenic mice with a disrupted Ifng gene (GKO mice) developed brain tumors of approximately the same size as in normal WT animals (Fig. 4). Unexpectedly, GFI mice had significantly smaller tumors compared with nontransgenic mice with a disrupted Ifng gene (Fig. 4, p ≤ 0.0325, Mann-Whitney U test). Thus, IL-12 conferred the same capacity for tumor rejection in the CNS of IFN-γ-deficient animals as it did in animals with a functional Ifng gene. This suggests that IFN-γ is not a mediator of the tumor rejection seen in the cerebellum of GF-IL12 mice. One animal of the GFI line showed a very large tumor (35 μL). This outlier most likely did not express IL-12, although it had a transgenic genotype (see later).

**T Cells Mediate Tumor Rejection in GF-IL12 Mice**

To determine which lymphocyte population mediates tumor rejection, we performed 2 series of depletion experiments.
in GF-IL12 mice in which CD4⁺, CD8⁺, or both CD4⁺ and CD8⁺ T cells were depleted by intraperitoneal injection of neutralizing antibodies against the respective T-cell subset. Fluorescent activated cell sorter analysis of peripheral blood 1 and 2 weeks after antibody injection demonstrated a complete loss of the respective lymphocyte population after 1 week and very low numbers (<10% of control) at 2 weeks (Fig. 5A). The preceding experiments previously described and the first T-cell depletion experiment revealed a number of outliers with very large tumors in the transgenic animal groups. Therefore, we repeated the antibody depletion experiments and determined in the same cerebella tumor size and IL-12 mRNA expression by RPA. The RPA analysis revealed that 3 of 15 transgenic mice did not express detectable levels of IL-12 mRNA. Reverse transcription–PCR from the same RNAs with a primer specific for the transgene that contains a polyadenylation site of human growth hormone that does not occur in the mouse genome confirmed the transgenic genotype of the transgenic mice. Two (50%) of 4 transgenic animals that were depleted of both CD4⁺ and CD8⁺ T cells and 1 (25%) of 4 transgenic animals depleted of CD8⁺ T cells did not show detectable levels of IL-12 mRNA, whereas all nondepleted or CD4-depleted mice showed strong expression of IL-12 mRNA (Fig. 5B). Figure 5C shows the tumor size in the various treatment groups of the second experiment. The GF-IL12 mice (n = 2) had a mean tumor size of 0.6 μL, whereas WT mice (n = 8) had a mean tumor volume of 17.5 μL. Although depletion of CD4⁺ T cells (n = 5) led to larger tumors in GF-IL12 mice (mean tumor volume, 10.2 μL; 58% of WT), depletion of CD8⁺ T cells (n = 4: mean tumor volume, 20.9 μL; 117% of WT) or both CD4⁺ and CD8⁺ T cells (n = 4: mean tumor volume, 14.5 μL; 83% of WT) virtually abolished the tumor-rejection effect of constitutive expression of IL-12 in the cerebellum. Although we observed substantial differences between the animal groups, statistical analysis of these experiments using the Mann-Whitney U test did not show statistically significant differences (p > 0.05). This was caused by the outliers in the antibody-treated GF-IL12 mice that did not express the transgene. This phenomenon is known from previous studies using GF-IL12 mice (25), and in the present experiment, we could demonstrate the effect in 3 animals that were depleted of CD8⁺ T cells (Fig. 5B). Both experiments revealed that T cells were the tumor-rejecting cells in GF-IL12 mice, with CD8⁺ T cells exerting a more pronounced effect as compared with CD4⁺ T cells. Again, this experiment indicated that NK cells are of minor importance in the tumor immunity in GF-IL12 mice. Thus, depletion of T cells inhibited tumor rejection by eliminating the antitumor activity of CD8⁺ and/or CD4⁺ T cells, respectively, and by reducing the lymphocyte-induced reactive upregulation of transgene expression in the cerebellum of the transgenic mice (Fig. 5B). Histological examination of the tumors in hematoxylin and eosin–stained sections revealed mononuclear cells diffusely infiltrating the tumors in WT mice (Fig. 6). In GF-IL12 animals, there were very few tumor cells and highly cellular infiltrates of cells resembling lymphocytes. Depletion of CD4⁺ T cells reduced the infiltrates in the tumors of GF-IL12 mice to a small

**FIGURE 5.** Effect of T-cell depletion on tumor rejection in GF-IL12 mice. (A) A representative analysis of CD8⁺ T-cell number in peripheral blood by fluorescent activated cell sorter 1 and 2 weeks after anti-CD8 treatment (wild type [WT], n = 2; GF-IL12, n = 4; GF-IL12 CD8 depleted, n = 4). (B) Expression of IL-12 in the cerebellum of tumor-implanted mice with or without T-cell depletion. Arrowheads: 3 GF-IL12 mice depleted of CD8⁺ or CD4⁺ and CD8⁺ T cells, respectively, did not show detectable IL-12 mRNA. (C) Tumor volumes in a representative experiment. While depletion of CD4⁺ T cells had only minor effects on the capacity of GF-IL12 mice to reject implanted GL261 cells, depletion of CD8⁺ or both CD4⁺ and CD8⁺ resulted in mean tumor sizes in GF-IL12 depleted mice becoming nearly as large as in WT animals.
extent, whereas depletion of CD8\(^+\) T cells led to a marked reduction of mononuclear cells in the tumors (Fig. 6).

**DISCUSSION**

In contrast to immense advances in other areas of medicine over the last century, the prognosis for malignant gliomas has changed only little. Most patients diagnosed as having a malignant brain tumor such as high-grade astrocytoma or glioblastoma die within a very short period. Radical treatments such as partial resection or radiotherapy have only a very limited impact on the survival time and often come at the cost of loss of function. Therefore, new and less debilitating approaches for the elimination of gliomas are needed. Recently, selective activation of the immune system as a means to eliminate malignant cells from the brain has gained great attention. The main advantage of an immunologic approach is that immune cells may mediate rejection of glioma cells and leave the normal brain untouched. Previous studies have demonstrated that IL-12 may induce glioma rejection in the brain (10, 11, 13, 26–31). We show here that constitutive expression of IL-12 in the CNS induces CD8\(^+\) T-cell–mediated rejection of implanted glioma cells, and that IFN-\(\gamma\) is dispensable in this model.

In WT mice, GL261 astrocytoma cells formed large tumors in the cerebellum of syngeneic mice. The growth rate we observed is in the range of that found by other researchers (32). Subcutaneous immunization with irradiated GL261 cells 7 days before intracerebral implantation of GL261 cells improved the survival from 0% of nonvaccinated controls to 90% (32). Expression of low amounts of RNAs of major histocompatibility complex (MHC) class I and the costimulatory molecules B7-1 and B7-2 has been observed in GL261 cells, indicating that GL261 cells are moderately immunogenic (32). In preliminary experiments needed for establishing stereotactic coordinates that allowed us to establish a reproducible model, we observed that a number of brains had injection tracts that crossed or even ended in the deep cerebellar sulci. In most of these mice, no tumor was established, but there were marked meningeal infiltrates of lymphocytes and macrophages, indicating that the injected cells had leaked to the CSF spaces where they had induced an immune response. These observations suggest that although GL261 cells are recognized by the immune system in the CSF, they may evade immunologic clearance from the brain parenchyma. An earlier report showed that dendritic cells stereotactically injected into the CSF can migrate to the cervical lymph nodes, whereas dendritic cells injected into the brain stayed in the vicinity of the injection site within the brain (33). Thus, the data presented here and earlier observations on the one hand add to the concept of the “immune privilege” of the CNS and on the other hand indicate that the CSF and the brain are not “privileged equally.” Another mechanism that might play a role in the
tumors of WT mice is the active downregulation of lymphocyte functioning by the established tumor. This phenomenon is well known for a variety of tumors including gliomas (34–36).

In GF-IL12 mice, we found a high degree of tumor rejection that was significantly greater than in WT mice. Few transgenic mice had tumor volumes that were comparable to those in WT mice. This finding most likely reflects the observation that some GF-IL12 mice, although of transgenic genotype, did not produce significant amounts of IL-12 in the cerebellum (25). We have previously shown that in most GF-IL12 mice, a strong pathological stimulus such as infection with a neurotropic virus further upregulated IL-12 expression, whereas a small proportion of GF-IL12 mice failed to show detectable upregulation of IL-12 and therefore had more of a WT phenotype (25). In these experiments, we were able to confirm by reverse transcription–PCR with transgene-specific primers the very low transgene expression in the respective animals and thus could exclude insufficient genotyping of the mice. This scenario may have occurred in the current study (Figs. 1, 4), but we were not able to confirm this by reverse transcription–PCR because we did not have RNA from the animals that were used for tumor size determination. We also tried to prove the hypothesis that tumor growth was caused by the lack of IL-12 in these mice by performing in situ hybridization on vibratome sections; however, high background staining did not allow for the quantification of IL-12 mRNA. To overcome this limitation, we performed a further experiment in which we determined the tumor size in sections from fresh-frozen cerebellum and extracted RNA from the remaining tissue. This revealed an inverse correlation of tumor size and levels of IL-12 mRNA in the animals tested. As we had previously observed, a number of transgenic mice did not show detectable levels of IL-12 mRNA in the cerebellum. Interestingly, in the current experiment, all the transgenic mice that lacked detectable IL-12 expression had been depleted of CD8+ T cells. These results again confirm that not all genetically transgenic mice do express the transgene. The levels of transgene expression in the cerebellum of GF-IL12 mice are regulated in a self-limiting loop. Initial production of low levels of IL-12 recruit T cells that produce proinflammatory cytokines, namely IFN-γ, which induce astrogliosis with increased activity of the GFAP promoter and further upregulation of IL-12 production (18, 25).

We have previously shown that IFN-γ is critical for the spontaneous disease in GF-IL12 mice and also mediates the antiviral immune response in these mice after infection with a neurotropic virus (19). Moreover, a number of reports demonstrated the antitumor effect of IFN-γ in brain tumors and other neoplasms (12–15). We then examined the role of this cytokine in the rejection of GL261 cells. Surprisingly, lack of IFN-γ had no effect on tumor rejection in GF-IL12 mice, indicating a different mechanism in this specific setting. Although cytotoxic effects of IFN-γ induced NOS-2 on glioma cells have been demonstrated in vitro (13), the functions of IFN-γ in vivo seem to be much more complicated. In particular, the finding that IFN-γ can upregulate the immunosuppressive factor B7-H1 on glioma cells demonstrates that the immune response of the host might support evasion of the immune system by the glioma (36). In the GFI mice, we observed 1 animal that had a very large tumor. Most likely, this mouse did not express the transgene as previously discussed. We also tried to determine whether the effect of IL-12 might be caused by the antiangiogenic properties of this cytokine (data not shown). This analysis showed no significant differences between the vessel density of tumors in WT and GF-IL12 mice. There might be, however, differences in the initiation of neovascularization that were not investigated in the current experiments. Further studies are needed to clarify this point.

Tumor-implanted GF-IL12 mice showed increased numbers of infiltrating immune cells compared with WT mice. To determine the nature of these cells, as well as their role in tumor rejection, we injected GL261 cells into the cerebellum of GF-IL12 mice with a disruption of the Rag2 gene (GFR mice) that lack functional lymphocytes. In GFR mice, we observed extensive tumor masses in the brain and spread to the CSF spaces, the skull, and the skin. This indicates that lymphocytes are the major cell population that mediates tumor rejection in GF-IL12 mice; NK cells (which are present in GFR mice) at most play only a minor role in this model. This contrasts with several tumor models in which there are significant antitumor effects of NK cells (37–40). For optimal antitumor effectiveness of NK cells, the expression of MHC and other immunogenic molecules on the surface of the tumor cells is necessary (41). In this regard, it is noteworthy that GL261 cells express only low levels of RNA coding for MHC molecules; however, upon stimulation with IFN-γ, strong upregulation of MHC class I and class II RNAs was observed (32). In GF-IL12 mice, IFN-γ is produced mainly by infiltrating lymphocytes and NK cells. Thus, in GFR mice, the main IFN-γ-producing cells are lacking, and therefore the lymphocyte-induced upregulation of MHC genes on the tumor cells most likely does not occur. This might explain the undetectable effect of NK cells in our model.

Selective depletion of T-cell subsets demonstrated that CD8+ T cells are the predominant effector cells, whereas CD4+ T cells are less effective in this experimental paradigm. Depletion of T-cell subsets resulted in a decrease of transgene expression. Unexpectedly, depletion of CD8+ T cells led to a lack of transgene expression in 25% to 50% of transgenic mice. This finding indicates that T cells are the main inducers of transgene expression in GF-IL12 mice and that CD8+ T cells are more effective than CD4+ T cells. The injected GL261 cells on the other hand seem to influence transgene expression only marginally. Together, our experiments demonstrate that CD8+ T cells and to a lesser extent CD4+ T cells are the effector cells in GF-IL12 mice in both upregulation of transgene expression and tumor cell rejection. In line with this, most of the T cells within the tumors of GF-IL12 mice stained positively for CD8 but not CD4. Interestingly, CD4+ T cells seemed to have opposite effects on tumor growth in GF-IL12 mice, depending on the specific setting. On the one hand, GF-IL12 mice lacking CD4+ T cells had larger tumors compared with nondepleted GF-IL12 mice; on the other hand, depletion of both CD4+ and CD8+ T cells led to smaller tumors compared with CD8+ T-cell depletion.
alone. This tendency, however, was not statistically significant. A possible explanation for this is that regulatory CD4+ T cells that dampen the antitumor response are eliminated in the CD4+ T-cell–depleted mice. This is consistent with recent publications that demonstrated that depletion of regulatory CD4+ T cells induced tumor rejection in murine glioma models (4, 42). Similar mechanisms might occur in humans because high percentages of regulatory CD4+ T cells have been observed in high-grade astrocytomas and glioblastomas (43, 44).

In summary, the present study demonstrates that localized expression of IL-12 in the CNS is able to mediate rejection of malignant astrocytomas. Furthermore, this rejection is mediated via a CD8+-dependent and IFN-γ–independent pathway. The techniques that would allow for localized expression of IL-12 in the CNS of glioma patients, such as stereotactic surgery, implantation of cytokine-delivering devices, gene transfer, or minipumps, are available in many centers and have been tested in animal models. However, at the moment, we are far from a safe approach to immunologic glioma therapy. Our data indicate the feasibility of immunologic approaches to target otherwise lethal brain tumors and provide a basis for further studies.

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