Increased Glioma Growth in Mice Depleted of Macrophages

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Abstract

Macrophages can promote the growth of some tumors, such as those of the breast and lung, but it is unknown whether this is true for all tumors, including those of the nervous system. On the contrary, we have previously shown that macrophages can slow the progression of malignant gliomas through a tumor necrosis factor–dependent mechanism. Here, we provide evidence suggesting that this antitumor effect could be mediated by T lymphocytes, as their number was drastically reduced in tumor necrosis factor–deficient mice and inversely correlated with glioma volume. However, this correlation was only observed in allogeneic recipients, prompting a reevaluation of the role of macrophages in a nonimmunogenic context. Using syngeneic mice expressing the herpes simplex virus thymidine kinase under the control of the CD11b promoter, we show that macrophages can exert an antitumor effect without the help of T lymphocytes. Macrophage depletion achieved by ganciclovir treatment resulted in a 33% increase in glioma volume. The antitumor effect of macrophages was not likely due to a destructive action on the tumor vasculature because phagocytosis or apoptosis of glioma cells, transduced ex vivo with a lentiviral vector expressing green fluorescent protein, was rarely observed. Their antitumor effect was also not due to a destructive action on the tumor vasculature because macrophage depletion resulted in a modest reduction in vascular density. Therefore, this study suggests that macrophages can attenuate glioma growth by an unconventional mechanism. This study also validates a new transgenic model to explore the role of macrophages in cancer.

Introduction

Tumors affecting the central nervous system are a leading cause of cancer-related death in children and young adults (1). The most frequent types are gliomas, which derive from glial cells that have acquired the ability to continuously proliferate and diffusely invade the parenchyma. In response to this aggression, immune cells, mainly macrophages, infiltrate the neoplastic tissue (2, 3). In the mouse glioma model used in the present study, for example, we have shown that macrophages account for ~8% of the cells of the tumor mass (4), which is comparable to the proportion of microglia in the normal brain. In contrast to microglia, glioma-associated macrophages originate from newly recruited monocytes, and exhibit a round, amoeboid, or elongated shape with relatively short cytoplasmic processes, rather than a highly ramified morphology (4).

The role of macrophages in glia biology is unclear, as it has not been directly tested, but rather inferred from observations made in vitro or in other tumor types (2). The traditional belief is that macrophages can potentially detect glioma cells expressing abnormal surface antigens (e.g., via the NKG2D receptor) and kill them by releasing lysosomal enzymes and reactive oxygen intermediates (5). Macrophages also have the potential to degrade such antigens into peptides that bind to MHC-I molecules for cross-presentation to CTLs (6). When activated, the latter can theoretically recognize and kill cells that display the same peptides by releasing the content of their cytotoxic granules. However, the reality is that most glioma cells escape these defense mechanisms, probably due to the lack of abnormal immunogenic antigens, down-regulation of MHC-I, induction of immunologic tolerance, and/or secretion of immunosuppressive molecules (7). In recent years, an alternative hypothesis has been proposed in which macrophages contribute to glioma progression by secreting growth factors, angiogenic molecules, extracellular matrix–degrading enzymes, and immunosuppressors (2, 3). Although this possibility is supported by several observations in the case of some cancers, such as those of the breast and lung (8–13), it has not been directly tested in gliomas. Therefore, the question of whether macrophages promote or counteract glioma progression remains unanswered and needs to be addressed in orthotopic models.

In support of a beneficial role for glioma-associated macrophages, we have recently shown that these cells stimulate their own recruitment and reduce glioma growth by expressing tumor necrosis factor (TNF; ref. 4), but the mechanism responsible for this effect is unknown. In the initial part of the present study, we found evidence suggesting that the antitumor effect of TNF could be mediated by T lymphocytes, as their number was drastically reduced in TNF-deficient mice and inversely correlated with glioma volume. However, this correlation was only seen when glioma cells, originally propagated by serial transplantation in B6 mice, were implanted into recipients with a mixed genetic background (B6 × 129S), questioning the role of macrophages in a context in which the tumor is weakly or not immunogenic. In the following sections, we confirm that glioma-associated macrophages, in a syngeneic context, exert a beneficial effect without the help of T lymphocytes, as determined using a new model of transgenic mice allowing for temporal depletion of macrophages.

Materials and Methods

Animals. TNF-deficient and wild-type mice from a B6 × 129S background were generated from breeders originally obtained from the Jackson Laboratory. Their genotypes were confirmed by PCR following the protocol provided by the supplier. Heterozygous CD11b-TK<sup>+</sup>-30 mice and wild-type littermates (B6 background) were generated as described previously (14). B6 mice were purchased from Charles River and adapted to standard laboratory conditions for 1 week before any manipulation.
All experiments were done on males aged 2 to 3 months according to procedures approved by our institution’s Animal Welfare Committee.

**Culture of glioma cells.** The glioma cell line GL261, originally propagated in B6 mice (15) and recently characterized (16), was cultured as described previously (4).

**Viral transduction of glioma cells.** GL261 cells were incubated for 24 h with vesicular stomatitis virus glycoprotein-pseudotyped lentivirus carrying the green fluorescent protein (GFP) gene at a multiplicity of infection of 10. The viral suspension was provided by Dr. Gary Kobinger (National Microbiology Laboratory, Winnipeg, Manitoba, Canada) and produced as described previously (17). Four days later, GFP+ cells were sorted using an Epic Elite ESP flow cytometer (Beckman Coulter), and then expanded in vitro before implantation.

**Intracerebral implantation of glioma cells.** A total of 5 × 10⁶ viable GL261 cells were stereotaxically injected into the right caudoputamen as described previously (4).

**Ganciclovir treatment.** Starting 7 days after tumor implantation, mice were injected i.p. twice daily for 6 days with 50 mg/kg of ganciclovir (Hoffmann-La Roche) diluted in saline. Control mice were treated identically, except that ganciclovir was substituted with saline.

**Bromodeoxyuridine labeling.** Bromodeoxyuridine (BrdUrd; Sigma-Aldrich) was dissolved in saline at a concentration of 10 μg/μL. Mice were injected twice with BrdUrd (100 μg/g) at 3h intervals, and perfused 2 h after the final injection.

**Histologic preparation.** For all histologic analyses, except for in situ hybridization, mice were transcardially perfused with 10 mL of saline, followed by ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4) over 10 min. The brains were removed, postfix for 4 h at 4°C, then cryoprotected overnight in 50 mmol/L potassium PBS supplemented with 20% sucrose. A series of sections were cut through the tumors at 40 μm using a freezing microtome, collected in cryoprotectant (30% ethylene glycol, 20% glycerol, 50 mmol/L sodium phosphate buffer; pH 7.4) and stored at −20°C until analysis. For in situ hybridization, the following modifications were applied: (a) the fixative was dissolved in borate buffer (pH 9.5) instead of phosphate buffer; (b) the brains were postfix for 48 h prior to overnight cryoprotection in the same fixative supplemented with 20% sucrose; and (c) tumor sections were cut at 30 μm.

**Immunostaining.** Immunohistochemistry and immunofluorescence were done as described previously (18) using the following primary antibodies: rat anti-BrdUrd (1:1,000; Accurate Chemicals), rabbit anti–cleaved caspase-3 (1:500; Cell Signalling Technology), rat anti-CD11b (1:1,000; BD Biosciences), rat anti-CD3ε (1:500; Serotech), rat anti-CD31 (1:1,000; BD Biosciences), rat anti-CD45 (1:1,000; BD Biosciences), rat anti-F4/80 (1:500; Serotech), and rabbit anti-Iba1 (1:2,000; Wako Chemicals). Fluorescent sections were counterstained for 1 min with 2 μg/mL of diamidino-phenylnilide (DAPI; Invitrogen). Prior to CD3ε immunostaining, sections fixed with paraformaldehyde at pH 9.5 were subjected to an antigen retrieval procedure that consisted of heating the sections in 10 mmol/L of sodium citrate buffer (pH 6.0) for 25 min in a microwave oven at 40% power.

**In situ hybridization.** Brain sections were analyzed by radioisotopic in situ hybridization according to a previously described protocol (4).

**Stereologic analyses.** Systematically sampled sections (every 10th section through the tumors) were analyzed in a blind fashion using a Stereo Investigator system (Microbrightfield) combined with a Nikon E800 microscope.

**Statistical analyses.** Unless otherwise stated, data are expressed as mean ± SE. Means were compared using the unpaired Student’s t test when the data met the assumptions of homogeneity of variance (Levene’s test). As an alternative, the Welch t test was used when the variances were unequal. Relationships between variables were assessed by Pearson correlation. All these tests used an α of 0.05 and were done with JMP software (SAS Institute).

**Results**

Decrease in glioma-infiltrating T cells in TNF-deficient mice. We have shown that macrophage-derived TNF can slow glioma growth. 

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growth through a process leading to the formation of small cavities called microcysts (4). To examine whether T cells play a role in this process, we implanted GL261 glioma cells into the brains of TNF-knockout and wild-type mice sharing the same genetic background (B6 × 129S). The animals were killed 21 days later and brain sections were immunostained for the T cell marker CD3ε. Labeled cells were found in all compartments of the tumors (Supplementary Fig. S1A and B), but not in the surrounding nonneoplastic tissue. Stereologic analysis revealed that T cell density was 4.4-fold lower in TNF-deficient mice compared with wild-type controls (Supplementary Fig. S1C). Interestingly, T cell density was directly proportional to the number of macrophages and microcysts (Supplementary Fig. S1D and E), but was inversely proportional to glioma volume (Supplementary Fig. S1F). However, such a correlation was only seen in a mixed genetic background and not in B6 mice (Supplementary Fig. S1G). A likely explanation for this discrepancy is that GL261 cells, which were originally propagated by serial transplantation in B6 mice (15), are more immunogenic and trigger a more robust adaptive immune response when implanted in a mixed genetic background compared with a pure B6 background. In support of this possibility, we found that glioma-infiltrating T cells were 3.5-fold more numerous, and that tumors were twice smaller in B6 × 129S (TNF−/−) mice compared with B6 mice (Supplementary Fig. S1C and H). These results, together with our previous findings (4), support the possibility that macrophages could reduce the growth of immunogenic glioma cells, at least in part, by promoting the recruitment of T cells with antitumor activity via the secretion of TNF. Therefore, although these results need to be confirmed by selective T cell depletion, they leave unanswered the question of whether macrophages can directly exert an antitumor effect in the absence of a significant T cell response, which prompted us to reevaluate the role of glioma-associated macrophages in a syngeneic context.

**Proliferation of glioma-associated macrophages.** Theoretically, glioma-associated macrophages could arise from three different sources: peritumoral microglia, circulating monocytes, and preexisting macrophages undergoing division. Using lethally irradiated mice reconstituted with bone marrow cells expressing GFP, we have shown that glioma-associated macrophages mainly derive from newly recruited monocytes and not from microglia (4). To determine whether these cells manifest the ability to proliferate after infiltration, we injected glioma-bearing mice (B6 background) with BrdUrd prior to sacrifice to label cells in the S phase of the cell cycle. As shown in Fig. 1A, BrdUrd+ cells were found in very large numbers in the tumors and, at a lower abundance, in the immediate vicinity. Confocal microscopic analysis showed that some BrdUrd+ cells expressed the macrophage marker Iba1 (Fig. 1B and C), which represented 1.4% (SD ± 0.3, n = 3) of the dividing cells in the tumors, but 49.9% (SD ± 10.3) of those in the adjacent normal tissue. These results indicate that macrophages and microglia located within or near gliomas have the potential to proliferate.

**Macrophage depletion increases glioma growth.** To reinvestigate the role of glioma-associated macrophages, we attempted to deplete them by exploiting the proliferative ability of these cells and their precursors. More precisely, we used transgenic mice expressing a mutated form of the herpes simplex virus 1 thymidine kinase gene (TKmut-30) under the control of the myeloid-specific CD11b promoter (14). In these mice, it is possible to deplete macrophages by the administration of ganciclovir, a produrg that is converted by viral TK to nucleotide analogues, which kill proliferating cells by inhibiting DNA synthesis. CD11b-TKmut-30 mice in a B6 background were implanted with GL261 cells, and the tumors were allowed to grow for 7 days. Thereafter, mice were injected twice daily for 6 days with ganciclovir or vehicle before being sacrificed. This treatment resulted in a 45% depletion of glioma-associated macrophages, as determined by stereologic analysis (Fig. 2A and B). In agreement with our previous observations suggesting a beneficial role for macrophages in glioma development (4), we found that tumor volume was 33% larger in ganciclovir-treated mice (Fig. 2C), and negatively correlated with macrophage density (Fig. 2D). As expected, no difference in macrophage density (33,710 ± 3,704 versus 30,238 ±...
4,141 cells/mm³; Student’s t test, \( P = 0.55 \); \( n = 5 \) per group) and glioma volume (5.1 ± 0.7 versus 4.9 ± 0.8 mm³; Student’s t test, \( P = 0.85 \); \( n = 5 \) per group) was detected in nontransgenic mice treated with ganciclovir compared with vehicle-treated controls.

To examine whether the increase in glioma growth observed after macrophage depletion was due to a reduction in the recruitment of antitumor T cells, brain sections from ganciclovir-treated mice and their controls were hybridized for TNF mRNA or immunostained for CD3. We found that the numbers of TNF-expressing macrophages and T cells were decreased in ganciclovir-treated mice (Fig. 3A–C), and positively correlated to each other (Fig. 3D). Consistently, there was also a direct relationship between the total numbers of macrophages and T cells (Fig. 3D). However, as expected from the results obtained in nontransgenic B6 mice (Supplementary Fig. S1G), we observed no correlation between T cell density and glioma volume in CD11b-TKmt⁻³⁰ mice (Fig. 3D), contrary to what we found in TNF-knockout mice and their wild-type controls (Supplementary Fig. S1F). It is unlikely that the reduction in T cells was directly due to ganciclovir because T cells collected from the spleens of CD11b-TKmt⁻³⁰ mice and cultured in the presence of IL-2 did not differ in number after a 4-day exposure to ganciclovir at a concentration as high as 10 μmol/L, as compared with untreated cells (11,444 ± 1,656 versus 10,336 ± 1,656 cells; Student’s t test, \( P = 0.65 \); \( n = 5 \) per group). As a positive control, we cultivated microglia derived from CD11b-TKmt⁻³⁰ mice, and found a 26% reduction in their number after exposure to ganciclovir.

**Figure 2.** Macrophage depletion increases glioma growth.
A, immunoperoxidase staining for Iba1 showing glioma-associated macrophages in CD11b-TKmt⁻³⁰ mice treated with saline or ganciclovir (GCV). Bar, 50 μm (main image), 10 μm (inset). B, stereologic analysis revealed a 45% reduction in the number of glioma-associated macrophages in ganciclovir-treated CD11b-TKmt⁻³⁰ mice. *, \( P < 0.0001 \) (Student’s t test). C, a 33% increase in glioma volume was found in ganciclovir-treated CD11b-TKmt⁻³⁰ mice, as determined by the Cavalieri method. *, \( P = 0.016 \) (Student’s t test). D, the number of macrophages was inversely proportional to glioma volume (Pearson correlation, \( P = 0.0054 \), \( R = -0.45 \)). Points, mice treated with ganciclovir (C) or saline (●).

**Figure 3.** Reduction in TNF-expressing cells and T lymphocytes in macrophage-depleted gliomas.
A, dark-field (main image) and bright-field (inset) photomicrographs of in situ hybridization signals for TNF mRNA in a 2-week-old glioma. Dashed line delimits the tumor area (right). Inset, in situ hybridization signals (black grains) with thionine counterstaining. Bar, 200 μm (main image), 10 μm (inset). B, stereologic analysis showed a 5-fold reduction in the number of TNF mRNA⁺ cells in gliomas from CD11b-TKmt⁻³⁰ mice treated with ganciclovir. Welch’s t test, \( P = 0.042 \). C, a 6.7-fold reduction in the number of glioma-infiltrating T cells was detected in CD11b-TKmt⁻³⁰ mice treated with ganciclovir (GCV). *, \( P = 0.0002 \) (Welch’s t test). D, Pearson correlation analyses showed that the number of glioma-infiltrating T cells correlated positively with that of TNF mRNA⁺ cells (\( P < 0.0001 \), \( R = 0.79 \)) and macrophages (\( P = 0.0002 \), \( R = 0.58 \)), but not with glioma volume (\( P = 0.066 \)).
ganciclovir exposure (213,200 ± 23,094 versus 288,833 ± 21,082 cells; Student’s t test, P = 0.039; n = 5 per group). Altogether, these results further support the idea that glioma-associated macrophages contribute to the recruitment of T cells by producing TNF, but exclude the possibility that these cells mediated the antitumor effect of macrophages observed in CD11b-TKmt-30 mice.

Phenotypic characterization of glioma-infiltrating leukocytes.
We sought to exclude the possibility that our results were due to a depletion of immune cells other than macrophages and T cells, in particular, granulocytes and natural killer cells, which are known to express CD11b (19). As we failed to unequivocally detect these cells by immunohistochemistry, we reasoned that if they were actually absent from our specimen, the proportion of macrophages versus total leukocyte population should be equal to the proportion of Iba1+ cells over the sum of Iba1+ (40,219/mm³) and CD3+ cells (24,423/mm³) found in vehicle-treated CD11b-TKmt-30 mice (Figs. 2B and 3C), i.e., 62%. In addition, almost all CD11b+ cells should express antigens that are highly specific for macrophages, including Iba1 and F4/80. To test these expectations, we immunostained glioma sections with antibodies directed against these markers or the leukocyte common antigen CD45 (Fig. 4).

Confocal microscopic analysis revealed that 64% of the CD45+ cells within the tumors expressed Iba1, which is similar to the expected 62%. Furthermore, the vast majority of the CD11b+ cells (>99%) expressed Iba1 and F4/80 (and vice versa), and none of the leukocytes examined, except one (Fig. 4D), had a multilobed nucleus typical of granulocytes. Together, these results indicate that macrophages and T cells are virtually the only immune cells that infiltrate GL261 gliomas, excluding a significant influence of other immune cells on our results.

Cytokines expressed in GL261 gliomas. As previously shown by double-labeling (4), macrophages are the main source of TNF in GL261 gliomas. TNF is a proinflammatory cytokine typically associated with the so-called type 1 inflammatory response (13), and thus, its presence suggests an important role for type 1 inflammation in the antitumor response against gliomas. This is in contrast with recent reports indicating that macrophages which populate other types of tumors have a type 2 phenotype, characterized by the expression of high levels of anti-inflammatory cytokines and low levels of proinflammatory cytokines (13). To characterize the type of inflammation involved in our model, using in situ hybridization, we examined the expression of various cytokines in glioma sections from mice killed 2 weeks after tumor implantation. We observed many strong hybridization signals for the proinflammatory molecules MCP-1 and IL-1β throughout the tumors, but very few signals for the anti-inflammatory molecules IL-4, IL-10, and transforming growth factor-β (Supplementary Fig. S2A–J). Double labeling with Iba1 revealed that macrophages were the main source of these cytokines, except transforming growth factor-β, which was predominantly expressed by Iba1-negative cells (Supplementary Fig. S2K–O). Therefore, these results confirm that glioma-associated macrophages are mainly involved in type 1 inflammatory response.
Physical interaction between macrophages and glioma cells. It is generally believed that classically activated (type 1) macrophages can kill pathogens and tumor cells by producing toxic molecules (e.g., nitric oxide, reactive oxygen intermediates). To provide evidence of the ability of macrophages to kill glioma cells in vivo, we implanted B6 mice with GL261 cells transduced with a lentiviral vector expressing GFP. Confocal imaging of brain sections immunostained for Iba1 revealed that most glioma cells were surrounded by macrophages with which many of them established physical contacts (Fig. 5A). However, we found only one clear example of macrophages phagocytosing glioma cell fragments (Fig. 5B), and very few glioma cells (approximately seven per section) completely wrapped by macrophage processes (Fig. 5C). Glioma cells showing signs of apoptosis (e.g., pyknotic nucleus, expression of cleaved caspase-3) were rarely observed (approximately three per section; Fig. 5C and D). These observations suggest that macrophages can physically interact with glioma cells, but that their antitumor activity is unlikely to be due to tumor cell killing.

Influence of macrophages on tumor vasculature. A possible alternative mechanism by which macrophages could reduce glioma growth is by promoting the destruction of the tumor vasculature and/or by inhibiting angiogenesis. To address this possibility, we immunostained brain sections from ganciclovir-treated mice and their controls with an antibody against the endothelial marker CD31 (Fig. 6A), and then estimated the density and caliber of the...
tumor vasculature using stereologic methods. We found a small (12%) reduction in vessel density after macrophage depletion (Fig. 6B), but no intergroup difference in vascular caliber (Fig. 6C), ruling out the possibility that the increase in glioma growth observed in CD11b-TK\textsuperscript{mt-30} mice treated with ganciclovir was due to a concomitant increase in vascularization.

Discussion

Accumulating evidence suggests that cancer cells that have escaped immune surveillance can exploit macrophages to their own benefit (8–13). For example, it has been proposed that tumor-associated macrophages promote angiogenesis by secreting vascular endothelial growth factor or other cytokines with direct or indirect proangiogenic activity. These cells could also facilitate tumor cell invasion and metastasis by releasing matrix-degrading enzymes such as the gelatinase matrix metalloproteinase-9. Although this mechanism seems to work in different cancers, including those of the breast and lung (8–13), the present study suggests that it does not apply to all tumors, especially gliomas. On the contrary, we found that gliomas develop faster when macrophages are compromised, suggesting that the sum of their antitumor effects are greater than that of their protumor effects, if any. This functional difference between glioma-associated macrophages and those that populate peripheral tumors may lie, at least in part, in the fact that gliomas do not metastasize outside the nervous system and do not depend on angiogenesis to grow, but rather on an alternative process called vessel cooption (20–22), in which macrophages are probably not essential.

Our results are in apparent contradiction with clinical studies indicating that a high macrophage content correlates with poor prognosis (23). However, considering the variable nature of human brain tumors, these studies do not necessarily mean that macrophages promote glioma growth, and may instead suggest that aggressive gliomas are somewhat more immunogenic than low-grade gliomas. Alternatively, it may be that aggressive gliomas cause more severe cellular and physiologic disturbances (e.g., neuronal degeneration, edema formation, and extracellular matrix remodeling), which would induce the recruitment of a higher number of macrophages to clean up the cellular debris and protect the adjacent normal tissue. Another possibility is that the density of macrophages is determined in part by that of the tumor cells, as suggested by the observation that the proportion of macrophages in GL261 gliomas (~8%) is similar to that of microglia in the normal nervous system (4). This theory would explain why macrophages were found in larger numbers in high-grade gliomas, which tend to be more homogeneous and densely packed than lower grade tumors (24, 25).

The present study leads us to conclude that macrophages use different mechanisms to slow glioma growth depending on the immunogenicity of the tumor. When it is weakly or not immunogenic, they act without the aid of lymphocytes, suggesting that they could be equipped to directly recognize and kill glioma cells. However, this idea is not supported by our observation that only rare glioma cells are phagocytosed by macrophages or show apoptotic features. The possibility that macrophages act by promoting the destruction of the tumor vasculature is also not supported by the reduction in vascular density seen in macrophage-depleted mice, suggesting, on the contrary, that macrophages reduce the vascular regression observed in this model (21). Alternatively, we propose that the antitumor effect of macrophages results from a neuroprotective activity. This hypothesis derives from the property of malignant gliomas to infiltrate the surrounding tissue by causing its destruction. Indeed, it has been reported that glioma cells produce toxic levels of glutamate that facilitate their progression, an effect that can be blocked by the administration of the glutamate receptor antagonist MK801 (26). Similarly, other potentially toxic molecules secreted by tumor cells or derived from plasma that penetrates the parenchyma through a damaged blood-brain barrier could promote glioma cell invasion. It is possible that macrophages reduce the invasive potential of glioma cells by protecting the surrounding tissue from the

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