Human Cancer Biology

Glioma Grade Is Associated with the Accumulation and Activity of Cells Bearing M2 Monocyte Markers

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Abstract

Purpose: This study is directed at identifying the cell source(s) of immunomodulatory cytokines in high-grade gliomas and establishing whether the analysis of associated markers has implications for tumor grading.

Experimental Design: Glioma specimens classified as WHO grade II–IV by histopathology were assessed by gene expression analysis and immunohistochemistry to identify the cells producing interleukin (IL)-10, which was confirmed by flow cytometry and factor secretion in culture. Finally, principal component analysis (PCA) and mixture discriminant analysis (MDA) were used to investigate associations between expressed genes and glioma grade.

Results: The principle source of glioma-associated IL-10 is a cell type that bears phenotype markers consistent with M2 monocytes but does not express all M2-associated genes. Measures of expression of the M2 cell markers CD14, CD68, CD163, and CD204, which are elevated in high-grade gliomas, and the neutrophil/myeloid-derived suppressor cell (MDSC) subset marker CD15, which is reduced, provide the best index of glioma grade.

Conclusions: Grade II and IV astrocytomas can be clearly differentiated on the basis of the expression of certain M2 markers in tumor tissues, whereas grade III astrocytomas exhibit a range of expression between the lower and higher grade specimens. The content of CD163+ cells distinguishes grade III astrocytoma subsets with different prognosis. Clin Cancer Res; 19(14): 3776–86. ©2013 AACR.

Introduction

Gliomas account for approximately 50% of all primary brain neoplasms (1, 2), the most common being the highly malignant grade IV glioblastoma multiforme (3). Glioblastoma multiformes are aggressive, rapidly progressing, infiltrative, parenchymal neoplasms, with a poor prognosis (4). Histologically, glioblastoma multiformes are characterized by hypercellularity, nuclear pleomorphism, microvascular proliferation, and pseudopalisading necrosis (5) and are associated with reactive gliosis, microglial activation, and disrupted vasculature (6). Dissemination of tumor cells into surrounding brain tissue makes complete surgical removal of neoplastic tissue impossible and the resistance of residual cells to conventional radiotherapy and chemotherapy inevitably results in tumor recurrence (7, 8). Given these clinical challenges and the refractory nature of glioblastoma multiformes, there is considerable interest in whether immune mechanisms may have therapeutic value (9, 10).

While CNS tissues are normally immunologically privileged, the diminished barrier function of the tumor vasculature in glioblastoma multiformes may be expected to facilitate immune cell entry into the tumor parenchyma, and a number of studies have documented the presence of immune cells, both lymphocytes and macrophages, in tumor tissues (11, 12). However, many of these cells do not appear to have antitumor activity (13, 14) and, on the basis of the factors they produce, may be more likely to contribute to tumor progression. Tumor and tumor-infiltrating cells are known to produce a variety of factors thought to be tumor-promoting factors including angiogenic VEGF and interleukin (IL)-8, immunomodulatory IL-10, and TGF-β1, which has both angiogenic and immunomodulatory properties (15). IL-10 is of particular interest to the development of tumor immunotherapy (16). This cytokine inhibits the expression of a wide range of inflammatory cytokines in vitro as well as in vivo (17), suppressing the activation and expansion of T cells that are important contributors to cellular immunity (18, 19). On the other hand, exogenously administered IL-10 has recently been shown to activate tumor-specific CD8+ cells in mouse skin and breast tumor models as well as human CD8+ T cells in vitro (20). Moreover, IL-10–producing CD4 T cells have...
Translational Relevance

Glioma grading by conventional histopathology can be problematic due to sample location and quality. As revealed by principal component analysis (PCA) and mixture discriminant analysis (MDA), analysis of the expression of mRNAs encoding CD14, CD68, CD163, CD204, and CD15, markers associated with immunomodulatory cell subsets, and TGF-β1 proves to be highly accurate in discriminating between grade II and IV astrocytomas. Tumors classified as grade III astrocytomas appear heterogeneous using this approach and can be segregated into subsets with different prognosis by immunohistochemical quantification of their content of CD163-positive cells. These findings raise the possibility that strategies targeting the immunomodulatory cells infiltrating high-grade gliomas may have therapeutic value.

been implicated in the antitumor response in glioma-vaccinated mice (21). Hence, the source of IL-10, the activity of concomitantly produced factors, as well as the nature of the tumor, may dictate whether it enhances or inhibits tumor immunity. IL-10 can be produced by a wide variety of cells including M2 monocytes, type 2 CD4+ T-helper cells, myeloid-derived suppressor cells (MDSC), a subset of CD8 T cells, mast cells, and CD4+ CD25+Foxp3+ Treg cells. While IL-10 is known to be expressed at elevated levels in malignant gliomas, the identity of its cell source is controversial (22). As TGF-β is produced by glioma cells and known to induce Treg (23), there is some speculation that IL-10 is produced by Treg infiltrating high-grade gliomas (24). However, Tregs have not consistently been shown in glioma tissues (25).

Tumor infiltration by MDSCs has also been proposed as the cause of IL-10 production in gliomas (26), and patients with glioblastoma multiformes have elevated circulating levels of diverse CD33+ HLA-DR– MDSC subsets in their blood (27). Nevertheless, the cells directly implicated in IL-10 production in glioma tissues express the monocyte/activated microglia marker CD68 (28) and astrocytoma grades II–IV (glioblastoma multiforme) tissues contain increasing numbers of cells bearing CD68 and the M2 phenotype markers CD163 and CD204 (29). However, it has yet to be established whether IL-10 production in gliomas is associated with M2 phenotype cells. Moreover, these observations could be explained by the infection of glioma cells with cytomegalovirus (CMV), which expresses a gene product that is structurally distinct but has some of the functional characteristics of IL-10 (30) and causes M2 polarization of monocytes in vitro (31). To more clearly define the source of IL-10 in malignant gliomas and, more importantly, to elucidate the relationship between tumor malignancy and the content and activity of cells elaborating immunomodulatory cytokines, we have examined a broad panel of gliomas of different subtypes and histologic grades for cell infiltration and gene expression. We show here that IL-10 is produced in high-grade gliomas by cells with certain M2 characteristics and that these cells are present in decreasing numbers and activity in lower grade gliomas. We propose that analysis of the expression of a subset of M2 markers may provide an accurate, adjunct approach to classifying glioma malignancy.

Materials and Methods

Tissue and cell culture specimens

Primary brain tumor and epilepsy samples, used as a surgical, non-tumor CNS tissue control, were discarded tissues from patients undergoing surgery at Thomas Jefferson University Hospital (Philadelphia, PA) obtained under a Thomas Jefferson University Institutional Review Board–approved protocol. Patient demographics, disease characteristics, and treatment are summarized in Supplementary Table S1. Pathologic analysis of tumor specimens was conducted by 2 board-certified neuropathologists (M.T. Curtis and L.C. Kenyon) and graded according to 2007 WHO criteria as oligodendrogliomas, grade II astrocytomas, grade III anaplastic astrocytomas, and grade IV glioblastoma. Tissues were harvested in bulk or by the Myriad tissue removal device (NICo Corporation), snap-frozen, and stored at −130°C or processed for cell culture as previously described (32).

Quantitative real-time reverse transcription PCR

Total RNA was extracted from tissues using the Absolutely RNA Miniprep Kit (Agilent Technologies), reverse transcribed into cDNA with MMLV reverse transcriptase (Promega), and then quantified by real-time PCR with gene-specific primers and probes (Supplementary Table S2), Brilliant QPCR Master Mix (Agilent Technologies), and a Bio-Rad iCycler (Bio-Rad Laboratories). Samples were run in duplicate with cDNA gene standards used to determine copy numbers which were normalized to the number of copies of the L13a housekeeping gene in each sample.

Microscopy

For immunofluorescent staining, frozen sections and cells centrifuged onto slides were fixed with methanol, blocked with 2% bovine serum albumin (BSA; Sigma) and Fc receptor blocker (Innovex Biosciences), then incubated with primary monoclonal antibodies specific for human: IL-10, (JES3-12G8, AbD Serotec; 1:50); CD163 (EDHu-1 AbD Serotec; 1:200); CD11b (ICRF44, AbD Serotec; 1:100); CD14 (UCHM1, AbD Serotec; 1:100); CD68 (KP1, Dako; 1:100); and glial fibrillary acidic protein (GFAP; EP672Y, Abcam; 1:200). Polyclonal rabbit anti-human CD204 antibody (Prestige Anti-MSR, Sigma; 1:50) was also used. Incubation with Alexa Fluor–conjugated secondary antibodies (Invitrogen, 1:400) followed and, finally, Vectashield-mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Slides were imaged with an Olympus Provis AX-70 microscope equipped with a charge-coupled device (CCD) camera and Spot image software (Diagnostic Instruments). Sections were retained...
with hematoxylin and eosin (H&E) and photographed using light microscopy, where noted.

For immunohistochemistry, formalin-fixed, paraffin-embedded tissues were mounted onto slides, deparaffinized, rehydrated, and subjected to epitope retrieval using Target Retrieval Solution (# S1699, Dako Corporation) or heat. Immunostaining was carried out using a Dako Autostainer (model LV-1, Dako Corporation) with incubation in avidin and biotin blocking solution (Biocare Medical) and then with the following prediluted biotinylated antibodies specific for human: CD14 (Ventana); CD68, (clone KP1, Dako Corporation); and CD163 (Ventana). Slides were then treated with 3% H2O2 and bound antibody detected using the SuperPicture 3rd Generation IHC Detection Kit HRP polymer conjugate (Invitrogen). Cells in paraffin sections stained with CD163 antibody (clone MRQ-29, Cell Marque) using the ultraView Universal DAB Method (Ventana) with hematoxylin counter stain were scanned at ×20 on the Aperio Scan Scope XT (Aperio) and quantified with a modified membrane detection algorithm to detect the number of positively stained cells and a nuclear algorithm to determine the total number of cells.

**Cell separation, flow cytometry, and in situ ELISA**

CD14+ cells were magnetically isolated from single-cell suspensions using CD14 MicroBeads (Miltenyi Biotec Inc.). For flow cytometry, the cells were stained with fluorescently labeled monoclonal antibodies specific for human CD11b (M1/70.15.11.5, Miltenyi Biotech), CD14 (M5E2, BD Bioscience), CD68 (eBio Y1/82A, eBioscience), and CD163 (215927, R&D Systems), and analyzed by the BD FACSCalibur system (BD Bioscience). Data analysis was conducted with FlowJo software (Tree Star Inc.). For in situ ELISA, cells (35,000) were incubated in the wells of Immulon 4HBX 96-well plates (Dynex Technologies) coated with anti-IL-10 monoclonal antibodies (Mabtech) and blocked with 1% BSA. Plate-bound cytokine was labeled with biotinylated IL-10–specific monoclonal antibodies and developed using the Vectastain ABC Elite Kit (Vector Laboratories) and SureBlue TMB (KPL). Enzyme–substrate reactions were terminated by adding TMB stop solution (KPL) and absorbance measured on a microplate reader (Molecular Devices).

**Statistical analysis**

Statistical significance was assessed with the Mann–Whitney test, Kruskal–Wallis one-way ANOVA, and Dunn multiple comparison posttest using Prism 5 software (GraphPad Software, Inc.), and the log-rank test using JMP (SAS Institute). Spearman correlation was used to assess relationships between pairs of genes and the results expressed using MultiExperiment Viewer software (Dana Farber Cancer Institute). The threshold for determining low versus high CD163 cell content was calculated by JMP as $\sqrt{2} \times$ lower 95% confidence interval (CI) of the population mean (mean, 6.32; lower 95% CI, 2.63).

Principal component analysis (PCA) was conducted to assess patterns in gene expression, and mixture discriminant analysis (MDA; refs. 33, 34) to determine the combination of gene activities which best characterize astrocytomas of different grades. For MDA, data from the second class (AII specimens) were modeled as a mixture of 3 Gaussian densities and the set of most discriminant genes determined via leave-one-out cross-validation, repeated such that each observation in the sample was used once as the validation data. PCA and MDA were conducted with the R programming language (R Development Core Team, version 2.12.2).

**Results**

**Higher grade astrocytomas express elevated levels of IL-10 and TGF-β and genes associated with the M2 monocyte phenotype**

By comparison with epilepsy specimens, IL-10, TGF-β1, and TGF-β2 mRNA levels are significantly elevated in glioblastoma multiformes and there is a trend toward higher IL-10, TGF-β1, and TGF-β2 mRNA levels in grade III astrocytoma (Fig. 1A). The T-helper/Treg marker CD4 was found at relatively high but comparable levels in the samples tested, whereas the less abundant T-cell marker CD8 was lowest in high-grade gliomas. In contrast, mRNAs specific for the monocyte markers CD11b, CD14, and CD68, the latter 2 generally being more abundant, were found at levels which increased as tumor grade progressed. The M2 markers CD163 and CD204 showed a similar pattern of increasing expression, whereas elevation of the more weakly expressed M2 marker CD206 was limited to glioblastoma multiforme specimens. Levels of mRNAs specific for the 2 variants of Foxp3 found in humans, i1 and i2 (35), were very low, showing a nonsignificant trend toward higher levels in high-grade gliomas (Supplementary Fig. S1A). In glioblastoma multiforme tissues, the levels of mRNAs specific for IL-10 are more closely correlated with those of the M2 cell phenotype than with the levels of mRNAs associated with other regulatory cell types (Supplementary Table S3). Conventional PCR analysis of representative samples from the tissues assessed in Fig. 1A confirms that human rather than CMV IL-10 mRNA is being detected (Supplementary Fig. S1B). Consistent with the mRNA results, immunohistochemical analysis of sections from representative AII, AIII, and glioblastoma multiforme specimens showed that numbers of CD14+, CD68-, and CD163-positive cells increase with tumor grade (Fig. 1B). Flow cytometric analysis of cells recovered from glioblastoma multiforme tissues indicates that a single-cell subset coordinately expresses CD14, CD11b, CD68, and CD163 (Fig. 1C).

**Cells bearing M2 monocyte markers produce IL-10 in glioblastoma multiforme**

We next stained glioblastoma multiforme tumor tissue sections for IL-10 and counterstained for monocyte lineage and M2 markers. Cells expressing monocyte and M2 markers were found dispersed throughout the tumor parenchyma (Fig. 2A). While the number varied between different tumor specimens, on average 15% ± 5% of the total cell number in areas of non-necrotic tumor were identified as...
bearing M2 markers. Approximately 50% of these cells are positive for IL-10. Less than 1% of the IL-10+ cells lacked a monocyte or M2 marker. Elevated numbers of cells expressing the M2 marker CD163 and IL-10 were seen in areas surrounding necrosis (Fig. 2B). No overlap was seen between GFAP and IL-10 staining (Supplementary Fig. S2). In addition, we noted a coincidental reduction in the expression of mRNAs specific for IL-10, CD163, and CD204 but not TGF-β1 and TGF-β2, during serial passage of tumor cells in vitro (Supplementary Fig. S3).
Cells of the monocyte lineage isolated from glioblastoma multiforme tissues elaborate IL-10 ex vivo

To more conclusively establish whether cells of the monocyte lineage in glioblastoma multiforme tissues are actively producing IL-10, we separated cells recovered from the tissues into CD14-positive and -negative subsets, the former also being positive for CD11b (Fig. 3A). CD14+ but not CD14− cells elaborated IL-10 in culture (Fig. 3B). At culture, the CD14+ exhibited relatively high levels of mRNAs specific for monocyte and M2 markers including CD11b, CD68, CD163, CD204, CD206, as well as for IL-8, IL-10, TGF-β1, and VEGF (Fig. 3C and D). In contrast, the CD14− cells expressed higher levels of mRNAs specific for TGF-β2 and VEGF, lower while still substantial levels of CD68, but little of the other mRNAs (Fig. 3C and D). In addition, the CD14+ cells stained for IL-10, CD14, CD68, and CD163 in cytopsins (Fig. 3E).
Expression levels and correlation patterns of myeloid and immunomodulatory genes differ between different grade gliomas

Using a more comprehensive panel of genes, we probed for associations between cell phenotype and immunomodulatory functions in different grade gliomas. The results depicted in the heat map of Fig. 4A show that a subset of genes is expressed at moderate-to-high levels in gliomas with a trend toward higher expression in glioblastoma multiformes. This includes the markers of M2 monocyte phenotype and function CD163, CD204, TGF-β1, and IL-10. However, other genes associated with M2 monocytes, such as indoleamine 2,3-dioxygenase (IDO)-1, PPARγ, IRF4, arginase-1 (ARG1), and genes expressed by MDSCs and M1 monocytes, including CD15, IL-12, IRF5, and TNF-α, are found in very low copy numbers with no difference between glioma grades (Fig. 4A and Supplementary Fig. S4). Figure 4B shows a heat map representing the extent of Spearman correlation between the expression levels of the different genes where correlations are statistically significant. While there is a strong association between the expression of monocyte marker and immunomodulatory genes throughout, correlations between IL-10, TGF-β1, and M2 phenotype markers increase in conjunction with tumor grade. Other genes overexpressed in glioblastoma multiformes, such as the stem cell marker CD133, show no correlation with genes encoding either M2 markers or immunomodulatory cytokines. Interestingly, despite low levels of expression, there are strong correlations between the activities of a variety of different genes. While the significance is not yet known,
certain of these patterns, for example, those including CD15, differ between tumor grades. A more detailed scatter plot visualization of the relationships between IL-10 mRNA levels and those of CD11b, CD14, CD15, CD163, CD204, and TGF-β1 in the different tumor specimens is shown in Supplementary Fig. S5.

Analysis of the expression of genes encoding immunomodulatory cell phenotypic and functional markers can be used for tumor grading

PCA, based on a correlation matrix with 82%, 89%, and 94% of total variation explained by 2, 3, and 4 principal components, respectively, was conducted to determine whether or not there may be latent associations common to the genes encoding monocyte (CD11b, CD14, CD68), M2 (CD163, CD204), and MDSC (CD15) phenotypic markers as well as IL-10 and TGF-β1. The scores for individual samples (symbols) and the amount by which the expression of each gene “loads” on, or correlates with, the components (represented by the direction and length of the loading plot vectors) are shown in Fig. 5A. The expression of genes that are near each other in the vector plots, for example, CD14, CD68, CD163, CD204, and IL-10, is expected to be associated, as would be the case if M2 cells are the source of IL-10. The PCA plot shows a very good separation between the scores of AII and glioblastoma multiforme specimens and fair separation between those of AII and AIII as well as between AIII and glioblastoma multiforme. In addition, there is a geometric link between the arrows and symbols indicating that higher levels of CD14, CD68, CD204, IL-10, and CD163 are significant in characterizing glioblastoma multiforme samples, whereas a higher level of CD15 may be characteristic of AII.

To establish which combination of markers provides the best discrimination between histologically diagnosed tumor types, we conducted MDA using mRNA levels for the genes assessed by PCA and the additional MDSC marker ARG1. The feature set obtained (Fig. 5B) is composed of CD14, CD15, CD68, CD204, IL-10, and CD163 and is significant in characterizing glioblastoma multiforme samples, whereas a higher level of CD15 may be characteristic of AII. To establish which combination of markers provides the best discrimination between histologically diagnosed tumor types, we conducted MDA using mRNA levels for the genes assessed by PCA and the additional MDSC marker ARG1. The feature set obtained (Fig. 5B) is composed of CD14, CD15, CD68, CD204, IL-10, and CD163 and is significant in characterizing glioblastoma multiforme samples, whereas a higher level of CD15 may be characteristic of AII. To establish which combination of markers provides the best discrimination between histologically diagnosed tumor types, we conducted MDA using mRNA levels for the genes assessed by PCA and the additional MDSC marker ARG1. The feature set obtained (Fig. 5B) is composed of CD14, CD15, CD68, CD204, IL-10, and CD163 and is significant in characterizing glioblastoma multiforme samples, whereas a higher level of CD15 may be characteristic of AII.

Figure 4. Genes encoding phenotypic and functional markers of various immunomodulatory cell subsets are differentially expressed and correlated in diverse glioma grades. A, heatmap depicting expression of the indicated genes (columns) in 8 AII, 15 AIII, and 23 glioblastoma multiforme specimens (rows) with colors corresponding to the expression level (scale bar). B, heatmap representation of the extent of statistically significant (P < 0.05). Spearman correlation between the expression levels of the indicated gene pairs in different tumor grades (Spearman coefficients are shown as colors corresponding to the scale bar). Gray represents gene combinations without significant correlation.
gliomas and, likely, different subsets of grade III astrocytoma gene expression as discriminating between different grade numbers of CD163

Subjects with grade III astrocytomas containing low numbers of CD163\(^+\) cells have a survival advantage

MDA analysis identifies CD14, CD68, CD163, and CD204 gene expression as discriminating between different grade gliomas and, likely, different subsets of grade III astrocytomas. As a first test of this latter hypothesis, we obtained tumor specimens from 36 individuals who had undergone surgery for astrocytomas classified as grade III by WHO histopathologic criteria and assessed CD163\(^+\) cell content by immunohistochemistry and an automated cell counting system (Fig. 6A). The percentage of CD163\(^+\) cells in each specimen segregated into statistically disparate groups of low versus high CD163 cell content (Fig. 6B). Individuals whose tumors contained low levels of CD163\(^+\) cells have significantly greater 5-year survival, with a median survival of 239 compared with 57 weeks for individuals with high numbers of the cells in their tumors (Fig. 6C).

Discussion

Higher grade gliomas express elevated levels of factors with known immunomodulatory properties including IL-10, TGF-\(\beta\)1, and TGF-\(\beta\)2 (36–38). These factors could be produced by tumor cells, incorporated CNS resident cells such as reactive astrocytes, or by tumor-infiltrating cells such as CD4\(^+\)CD25\(^+\) FOXP3\(^+\) Treg cells (39), MDSCs (26), and M2 monocyte lineage cells (29). On the basis of their production of IL-10 in culture, a recent study has concluded that IL-10 is largely produced by glioma stem cells (40). However, in our analyses of different grade gliomas, no correlations were detected between the expression of genes associated with Tregs, MDSCs, glioma stem cells (CD133), and IL-10. On the other hand, levels of mRNAs specific for IL-10, TGF-\(\beta\)1, and monocyte and M2 phenotypic markers were found to concordantly increase with WHO glioma grade. Cells staining positively for IL-10 throughout the glioblastoma multiforme parenchyma as well as in accumulations around areas of necrosis generally express the M2 markers CD163 and CD204. Moreover, only cells positive for CD14, CD11b, CD68, and CD163, isolated from glioblastoma multiforme tissues on the basis of CD14 expression, elaborated IL-10 \textit{in vitro}. We therefore conclude that the majority of cells in glioblastoma multiformes that produce IL-10 phenotypically resemble M2 monocytes rather than MDSCs, Treg, or glioma cells. However, gene expression analysis reveals that these cells are unlikely to be conventional M2 monocytes as genes associated with human M2 function, such as IDO-1, are not expressed.

In our view, the genesis of the IL-10–producing cells expressing select M2 markers in high-grade gliomas is not due to CMV infection and remains to be established. While we readily identified CMV IL-10 mRNA in epilepsy specimens, CMV IL-10 mRNA was not detected in any of our high-grade astrocytoma specimens.

Cells bearing M2 phenotype markers, while common and the principle cell source of IL-10 in glioblastoma multiformes, do not all express this cytokine. We speculate that this may reflect the involvement of different mechanisms in cell recruitment and activation to secrete IL-10. Cells with M2 phenotype markers are also generally detectable in AII tumors, albeit at a lower level than is evident for glioblastoma multiforme and with greater variability in IL-10 expression. Consistent with our previous observation of distinct radiographic phenotypes within grade III astrocytomas that differ in prognosis (41), AII tumors also segregate into three classes.
groups by IL-10 and M2 marker expression: (i) neither IL-10 nor M2 markers, (ii) M2 phenotypic markers but low levels of IL-10, and (iii) high levels of both M2 markers and IL-10. Like the first AII subset, cells with an M2 phenotype are rare in grade II astrocyomas, which rarely express significant levels of IL-10. In fact, our single AII specimen with a substantial IL-10 mRNA signal expressed low levels of M2 phenotype markers, suggesting that IL-10 was being produced by a different cell type. This is consistent with the prior observation of IL-10 production by a GFAP-positive cell in a glioma (22).

Comparative analysis of the expression of a variety of genes encoding macrophage, M2, and MDSC phenotypic markers as well as IL-10 and TGF-β1 confirmed associations between M2 phenotype markers and IL-10, primarily in high-grade gliomas. PCA of the patterns of expression of CD14, CD68, CD163, CD204, and IL-10 shows that these are all likely to share a biologic commonality. However, while there is a general correlation at the gene expression level between the presence of M2 phenotypic markers and IL-10 production in tumors of increasing malignancy, this relationship is not absolute. In addition to the disconnect between the presence of cells with M2 markers and IL-10 production in certain grade III astrocytomas, specimens from all glioma grades were identified with relatively low levels of M2 phenotype mRNA but moderate IL-10 mRNA. In fact, MDA shows that measures of the combined gene expression of CD14, CD68, CD163, CD204, and CD15, all elevated in high-grade gliomas with the exception of the latter, but not IL-10 or TGF-β1, provide a reliable correlate of WHO tumor grade as determined by histopathology. For the most part, there is a good concordance between histopathology and gene expression grading of the tumors with AII and glioblastoma multiformes clearly separated by both approaches. Misclassifications between the MDA model and conventional histopathology more commonly involve tumors classified by histopathology as AIII, where the distributions of gene expression are more diverse. Can analysis of the expression of M2 markers identify subsets of AII not evident by histopathology? As an initial test of this hypothesis, we used the content of CD163+ cells in tumor specimens to segregate 36 patients with grade III astrocytomas into 2 groups, finding that low numbers of these cells are associated with longer survival. We also examined survival and CD163 cell content in tumors from the subset of patients diagnosed with a grade III astrocytoma that underwent a subtotal resection (to remove bias from the potentially more curative gross total resections) and were subsequently treated with radiation and chemotherapy. Mean survival of the CD163 high and low cohorts was 39 ± 10 and 286 ± 95 weeks, respectively (P = 0.01 by the log-rank test).

IL-10 has well-established immunomodulatory properties and is elaborated by a number of different cell types in a variety of cancers including melanoma (42), non–small cell lung cancer (43), and B-cell lymphoma (44) as well as glioblastoma multiformes. This has led to speculation that IL-10 contributes to an immunosuppressive tumor microenvironment, a concept that is challenged by the observation...
in mice that IL-10 can activate tumor-specific CD8 T cells (20). This does not appear to be the case for high-grade gliomas. While studies in animal models and human clinical trials suggest that glioma cells are highly immunogenic (45–47), there is little evidence that immune mechanisms naturally control brain tumor growth. Cells infiltrating gliomas have generally been found to have immunomodulatory (48) as opposed to antitumor functions. The role of IL-10—producing cells in glioma progression can only be understood in the context of other factors produced in concert with IL-10. The M2 phenotype cells identified as producing IL-10 in this study also produce immunomodulatory and angiogenic factors, such as TGF-β1, IL-8, and VEGF, that are likely to impact glioma progression. Thus, intervention to selectively prevent their activity in gliomas may have greater therapeutic impact than targeting particular immunomodulatory or angiogenic pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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