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 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/MDSC subset marker CD15, which is reduced, provide the best index of glioma grade.

Conclusions: Grade II and IV astrocytomas can be clearly differentiated based on the expression of certain M2 markers in tumor tissues while 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.

Translational Relevance: Glioma grading by conventional histopathology can be problematic due to sample location and quality. As revealed by PCA and 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 grades 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.
Introduction

Gliomas account for approximately 50% of all primary brain neoplasms (1,2), the most common being the highly malignant Grade IV glioblastoma multiforme (GBM; ref. 3). GBMs are aggressive, rapidly progressing, infiltrative, parenchymal neoplasms, with a poor prognosis (4). Histologically, GBMs 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 GBMs, 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 GBMs 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 anti-tumor activity (13,14) and, based on 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 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 a mouse skin and breast tumor models as well as human CD8+ T cells in vitro (20). Moreover, IL-10-producing CD4 T cells have been implicated in the anti-tumor 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). Since 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 demonstrated 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 GBM 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, III and IV (GBM) 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 (29). 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 obtained under a Thomas Jefferson University Institutional Review Board approved protocol. Patient demographics, disease characteristics and treatment are summarized in Supplemental Table 1. Pathological analysis of tumor specimens was performed by two board certified neuropathologists (MTC and LCK) and graded according to 2007 WHO criteria as oligodendrogliomas, Grade II astrocytomias, Grade III anaplastic astrocytomias, and Grade IV glioblastoma (GBM). 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 polymerase chain reaction (QRT-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 (Supplemental Table 2), 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% 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), GFAP (EP672Y, Abcam; 1:200). Polyclonal rabbit anti-human CD204 (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 DAPI (Vector Laboratories). Slides were imaged with an Olympus Provis AX-70 microscope equipped with a CCD camera and Spot image software (Diagnostic Instruments). Sections were re-stained 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) then with the following prediluted biotinylated antibodies specific for human: CD14 (Ventana); CD68, (clone KP1, Dako Corporation); 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 20X 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), CD163 (215927, R&D Systems) and analyzed by the BD FACSCalibur system (BD Bioscience). Data analysis was performed 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 analysis of variance and Dunn’s multiple comparison post-test using Prism 5
software (GraphPad Software, Inc.), and the Log-Rank test using JMP (SAS Institute). Spearmen 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 $\sqrt{2} \times \text{lower 95\% C.I.}$ of the population mean (mean 6.32, lower 95\% C.I. 2.63).

Principal component analysis (PCA) was performed to assess patterns in gene expression and Mixture Discriminant Analysis (MDA; ref. 33,34) to determine the combination of genes which best characterize astrocytomas of different grades. For MDA, data from the second class (AIII specimens) was modeled as a mixture of three 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 performed 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 GBM and there is a trend towards higher IL-10, TGF-β1 and TGF-β2 mRNA levels in grade III astrocytoma (Fig. 1A). The Thelper/Treg marker CD4 was found at relatively high but comparable levels in the samples tested while 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 two 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 while elevation of the more weakly expressed M2 marker CD206 was limited to GBM specimens. Levels of mRNAs specific for the two variants of Foxp3 found in humans, i1 and i2 (35), were very low, showing a non-significant trend towards higher levels in high grade gliomas (Supplemental Fig. 1A). In GBM 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 (Supplemental Table 3). Conventional PCR analysis of representative samples from the tissues assessed in Figure 1A confirms that human rather than CMV IL-10 mRNA is being detected (Supplemental Fig. 1B). Consistent with the mRNA results, immunohistochemical analysis of sections from representative All, Alll and GBM specimens showed that numbers of CD14, CD68, and CD163–positive cells increase...
with tumor grade (Fig. 1B). Flow cytometric analysis of cells recovered from GBM 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 GBM**

We next stained GBM tumor tissue sections for IL-10 and counter-stained 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±9 percent 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 (Supplemental Fig. 2). 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 (Supplemental Fig. 3).

**Cells of the monocyte lineage isolated from GBM tissues elaborate IL-10 ex vivo**

To more conclusively establish whether cells of the monocyte lineage in GBM 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,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,D). In addition, the CD14+ cells stained for IL-10, CD14, CD68, and CD163 in cytospins (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 towards higher expression in GBM. This includes the markers of M2 monocyte phenotype and function CD163, CD204, TGFβ1, and IL-10. However other genes associated with M2 monocytes, such as indoleamine 2,3-dioxygenase (IDO)-1, PPRγ, IRF4, arginase-1 and genes expressed by MDSCs and M1 monocytes, including CD15, IL-12, IRF5, and TNFα, are found in very low copy numbers with no difference between glioma grades (Fig. 4A and Supplemental Fig. 4). 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 GBM, 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 Supplemental Fig. 5.

**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 percent of total variation explained by 2, 3 and 4 principal components respectively, was performed 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 Figure 5A. The expression of genes that are near each other in the vector plots, for example CD14, CD68, CD163, CD204, and IL-10, are 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 GBM specimens and fair separation between those of AII and AIII, as well as between AIII and GBM. In addition,
there is a geometric link between the arrows and symbols indicating that higher levels of CD14, CD68, CD204, IL10, and CD163 are significant in characterizing GBM samples while 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 performed 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, CD163 and CD204 mRNA levels. The overall cross-validated misclassification rate is 9% (4/44). Specimens assessed by histopathology as AII and GBM are perfectly separated in model fit. All of the misclassifications come from imperfect discrimination between AIII and other grades in spite of the fact that AIII samples are allowed to be heterogeneous and non-normal. Specifically these follow a mixture of three distributions. Consequently, 14% (2/14) and 7% (1/14) of histopathologically-defined AIII specimens show gene expression profiles that are more consistent with AII and GBM samples respectively. Only a single out of 22 GBM specimens exhibited molecular characteristics better resembling those of most of the samples in one of the AIII subclasses.

**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 that had undergone surgery for astrocytomas classified as Grade III by
WHO histopathological 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 weeks compared to 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-β1 and TGF-β2 (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). Based on 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-β1 and monocyte and M2 phenotypic markers were found to concordantly increase with WHO glioma grade. Cells staining positively for IL-10 throughout the GBM 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 GBM tissues on the basis of CD14 expression, elaborated IL-10 in vitro. We therefore conclude that the majority of cells in GBMs 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 GBMs, 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 AIII tumors, albeit at a lower level than is evident for GBM 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), AIII tumors also segregate into three groups by IL-10 and M2 marker expression: 1/ neither IL-10 nor M2 markers; 2/ M2 phenotypic markers but low levels of IL-10; 3/ high levels of both M2 markers and IL-10. Like the first AIII subset, cells with an M2 phenotype are rare in Grade II astrocytomas, 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 monocyte, 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 biological 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-\(\beta_1\), provide a reliable correlate of WHO tumor grade as determined by histopathology. For the most part, there is good concordance between histopathology and gene expression grading of the tumors with AII and GBM 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 AIII 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 two 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 GBM. 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 anti-tumor 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.

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**Figure Legends**

Figure 1. Genes encoding immunomodulatory factors and monocyte phenotypic markers are expressed at higher levels in gliomas of increasing grade with GBM containing cells coordinately expressing M2 markers. A, levels of mRNAs specific for the indicated genes in 10 epilepsy (Ep), 10 oligodendroglioma (Oligo), 8 All, 15 AllIII and 43 GBM specimens presented as the mean ± SD of the normalized mRNA copy number. Significant differences between groups determined by Kruskal-Wallis with Dunn’s post-test are indicated by * (p < 0.05), ** (p <0.01) and *** (p < 0.001). B, representative images of paraffin sections from All, AllIII, and GBM stained with CD14, CD68, CD163 antibodies (brown). Hematoxylin was used for nuclear staining (blue) (bars = 50 µm). C, four-color flow cytometric analysis of cells from a representative GBM surgical specimen for expression of CD11b, CD14, CD68, and CD163. Values in the contour plots indicate the percentage of total cells in each quadrant.

Figure 2. Monocyte/microglia and M2-monocyte markers co-localize with IL-10 in the parenchyma and necrotic areas of GBM. A, representative images of GBM parenchyma stained for CD14, CD11b, CD68, CD163, CD204 (green), IL-10 (red) and DAPI (blue), both singly and merged. B (left), a necrotic area from a representative GBM specimen double-immunostained for CD163 (green) and IL-10 (red); (right) the same section re-stained with H&E (scale bars = 20 µm).

Figure 3. CD14-positive cells enriched from GBM specimens express the phenotype and cytokines characteristic of M2 monocytes. A, representative flow cytometric plots
for CD11b and CD14 of cells separated by CD14-specific magnetic beads. Numbers indicate the percent of total cells in each quadrant. B, *ex vivo* IL-10 secretion by the two cell subsets in overnight culture, determined by ELISA, presented as the mean plus SD of quadruplicate wells. None detected denotes cytokine levels below the limit of detection (<10 pg). C, the normalized mean ± SD copies of mRNAs specific for monocyte and M2 phenotypic markers and immunomodulatory factors in CD14+ and, D, CD14- cell subsets isolated from GBM tissues. E, cytopsins of CD14+ cells, isolated from a representative GBM specimen, stained with antibodies to CD14, CD68, CD163 (green), IL-10 (red) and Dapi (blue).

Figure 4. Genes encoding phenotypic and functional markers of various immunomodulatory cell subsets are differentially expressed and correlated in diverse glioma grades. A, heat map depicting expression of the indicated genes (columns) in 8 AII, 15 AIII and 23 GBM specimens (rows) with colors corresponding to the expression level (scale bar). B, heat map 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 shown as colors corresponding to the scale bar). Gray represents gene combinations without significant correlation.

Figure 5. The expression of genes encoding monocyte phenotype markers and IL-10 differentiates glioma grades. Levels of mRNAs from individual specimens of different WHO grade astrocytomas (8, AII; 14, AIII; 22, GBM) specific for the genes indicated in the figures were used for PCA and MDA analysis which are represented by two-
dimensional visualizations. In both, the symbols represent independent patient data (blue - AII, green – AIII, red – GBM). A, PCA projections of the first two principal components, the arrows representing individual genes with the points directed at their loading coordinates. B, MDA canonical coordinates presented with the centers for each grade and, in the case of AIII, the three subclass centers, indicated with crosses of the corresponding color.

Figure 6. The CD163-positive cell content of Grade III astrocytomas has prognostic value. A, representative paraffin-embedded sections of specimens with low (left) and high (right) levels of CD163+ cells (bar = 50 µm). B, percentage of CD163 positive cells in low (n=23) and high (n=13) groups. The upper and lower boundaries of the boxes indicate the 75th and 25th percentile of the data set, respectively, while the line within the box indicates the median value. Maximum and minimum data values are represented by the upper and lower whiskers. *** denotes a statistically significant difference between the groups determined by the Mann Whitney test (p<0.001). C, Kaplan-Meier survival curves of patients with high versus low frequencies of CD163+ in their tumor specimens, the arrow indicating the 5 year point at which survival is significantly different by the Log-rank test (p < 0.003).
Figure 1

A

IL-10 & TGF-β1 gene expression

CD4 gene expression

CD8b gene expression

B

All

AllIII

GBM

CD14

CD68

CD163

C

CD14

CD11b

CD68

CD163

CD14

CD11b

CD68

CD163
Figure 2

A

B
Figure 3

A

CD14- population

1.3

0.7

CD14+ population

1.3

79

CD11b

CD14

B

IL-10 secretion (ng/35,000 cells)

none-detected

C

Gene expression

2.0

1.5

1.0

0.5

0.0

CD11b

CD68

CD163

CD204

CD206

D

E

CD14

IL-10

CD68

IL-10

CD163

IL-10
Figure 4
Figure 6

A

B

C

% CD163 Positive cells

***

CD163 expression level

Low

Hi

p<0.003

Weeks of follow-up

Percent survival

Low

Hi

p<0.003
Clinical Cancer Research

Glioma grade is associated with the accumulation and activity of cells bearing M2 monocyte markers


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