Infiltrating T Cells Increase IDO1 Expression in Glioblastoma and Contribute to Decreased Patient Survival

Lijie Zhai\textsuperscript{1}, Erik Ladomersky\textsuperscript{1}, Kristen L. Lauing\textsuperscript{1}, Meijing Wu\textsuperscript{1}, Matthew Genet\textsuperscript{1}, Galina Gritsina\textsuperscript{1}, Balazs Györfy\textsuperscript{2,3}, Priscilla K. Brastianos\textsuperscript{4,5}, David C. Binder\textsuperscript{6}, Jeffrey A. Soxman\textsuperscript{7,8}, Francis J. Giles\textsuperscript{7,8}, Charles D. James\textsuperscript{1,6,9}, Craig Horbinski\textsuperscript{1,8,10}, Roger Stupp\textsuperscript{1,7,8}, and Derek A. Wainwright\textsuperscript{1,7,8,11}

Abstract

**Purpose:** Indoleamine 2,3 dioxygenase 1 (IDO1) mediates potent immunosuppression in multiple preclinical models of cancer. However, the basis for elevated IDO1 expression in human cancer, including the most common primary malignant brain tumor in adults, glioblastoma (GBM), is poorly understood. The major objective of this study is to address this gap in our understanding of how IDO1 expression contributes to the biology of GBM, and whether its level of expression is a determinant of GBM patient outcome.

**Experimental Design:** Patient-resected GBM, The Cancer Genome Atlas, human T-cell-GBM cocultures, as well as nu/nu, NOD-scid, and humanized (NSG-SGM3-BLT) mice-engrafted human GBM form the basis of our investigation.

**Results:** In situ hybridization for IDO1 revealed transcript expression throughout patient-resected GBM, whereas immunohistochemical IDO1 positivity was highly variable. Multivariate statistical analysis revealed that higher levels of IDO1 transcript predict a poor patient prognosis (P = 0.0076). GBM IDO1 mRNA levels positively correlated with increased gene expression for markers of cytolytic and regulatory T cells, in addition to decreased patient survival. Humanized mice intracranially engrafted human GBM revealed an IFN-\gamma-associated T-cell-mediated increase of intratumoral IDO1.

**Conclusions:** Our data demonstrate that high intratumoral IDO1 mRNA levels correlate with a poor GBM patient prognosis. It also confirms the positive correlation between increased GBM IDO1 levels and human-infiltrating T cells. Collectively, this study suggests that future efforts aimed at increasing T-cell–mediated effects against GBM should consider combinatorial approaches that coinhibit potential T-cell–mediated IDO1 enhancement during therapy. *Clin Cancer Res;* 1–11. ©2017 AACR.

Introduction

Glioblastoma (GBM, astrocytoma, WHO grade IV) is the most common and aggressive primary central nervous system cancer in adults (1). Numerous efforts have been made to identify prognostic biomarkers for GBM patients, resulting in the determination of O-6-methylguanine-DNA methyltransferase (MGMT) gene promoter methylation (2, 3), mutant isocitrate dehydrogenase 1 and 2 (mIDH1/2; ref. 4), as well as chromosome 1p/19q codeletion, as important indicators of tumor malignancy and/or response to specific therapies (5, 6). Novel immunotherapies, which have caused the reconsideration of patient management among multiple malignancies including melanoma (7, 8) and lung cancer (9, 10), have been largely unsuccessful in treating GBM. A caveat associated with this lack of success is the relative dearth of knowledge regarding biomarkers that could be used to guide patient selection for specific immunotherapies.

Indoleamine 2,3-dioxygenase 1 (IDO1) is an IFN-inducible tryptophan (Trp) catabolic enzyme (11) that facilitates immunosuppression in cancer (12) through regulatory T cell (Treg CD3\(^+\)CD4\(^+\)CD25\(^+\)FoxP3\(^+\))–mediated suppression of cytolytic CD8\(^+\) effector T cells (Te), as demonstrated in preclinical GBM models (13). The protein level and/or activity of IDO1 has been correlated with prognosis in several tumor types (14–19). In GBM, however, interpretations of the prognostic significance of
IDO1 expression have been inconsistent, with immunohistochemical analysis revealing cellular positivity ranging from 8% to 96%, respectively (20, 21). In addition, although we previously demonstrated that the upregulation of IDO1 mRNA levels inversely correlated with glioma patient survival (22), microarray-based expression analysis did not prognostically stratify GBM patient survival, possibly due to an inability to quantify full-length IDO1 mRNA transcript as a result of oligo-based hybridization technology.

Here, we have compared mRNA- versus antibody-based IDO1 detection in GBM surgical specimens and determined a correlation between high IDO1 mRNA levels and decreased patient survival for grade II, III, or IV (GBM) gliomas. Increased IDO1 mRNA was associated with a commensurate increase in the expression for cytotoxic T-cell markers, CD3E and CD8A, suggesting a T-cell–associated influence on intratumoral IDO1 expression.

**Materials and Methods**

**The Cancer Genome Atlas sample description**

The Cancer Genome Atlas (TCGA) data for all cancer types analyzed in the current study were accessed from the UCSC Xena browser (http://xena.ucsc.edu/). mRNA expression data represented by RNASeq (Illumina Hi-seq platform) include RSEM normalized level 3 data present in TCGA as of April 13, 2017. DNA methylation data and exon expression RNASeq data were extracted from the same TCGA dataset. Glioma patient data were also acquired from the Molecular Brain Neoplasia Data (REMBRANDT) database (https://wiki.cancerimagingarchive.net/display/Public/REMBRANDT) as of April 13, 2017. TCGA GBM gene expression data by AffyU133a array were also acquired from the UCSC Xena browser.

**Patient samples**

Peripheral blood from GBM patients was collected at the Northwestern Brain Tumor Institute (NBTI) Tissue Bank. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (GE Healthcare) density gradient separation and stored in liquid nitrogen for coculture experiments. Snap-frozen tissue and formalin-fixed, paraffin-embedded tissue from surgically removed GBM was also collected through the NBTI Tissue Bank. All tumors were diagnosed according to WHO diagnostic criteria by Dr. Craig Horbinski. Detailed information for patient tissue samples used in gene expression analysis and in situ RNA hybridization/immunohistochemistry is provided in Supplementary Table S1.

**Glioblastoma (GBM) is the most common primary malignant brain tumor in adults with a dismal prognosis. Increased IDO1 expression is associated with decreased survival among glioma patients, but the significance of IDO1 expression exclusively in GBM patients is yet to be demonstrated. This study reveals that high IDO1 mRNA levels, as assessed using the Hi-RNA-Seq, Illumina platform, are consistently associated with decreased GBM patient survival. It also shows that infiltrating human T cells directly increase IDO1 expression in GBM. Given the growing number of clinical trials aimed at immuno therapeutically enhancing T cell functions in GBM, these data suggest that the further inhibition with an IDO1 inhibitor may increase the number of individuals that respond favorably in the clinic.**

**Translational Relevance**

**Animal and tissue preparation**

Immunocompetent, humanized mice (NSG-SGM3-BLT) established by implantation of human fetal liver and thymus fragments as well as hematopoietic stem cells into immunodeficient NOD. Cg-Prkdc<sup>scid</sup> II2rg<sup>tm1Wjl</sup>Tg (CMV-IL3, CSF2, KITLG)1Eav/MloySzl (NSG-SGM3) and NOD.CB17-Prkdc<sup>scid</sup>/1 (NOD-scid) mice were obtained from The Jackson Laboratory, and C57BL/6N-Foxn1<sup>nu</sup> mice were obtained from Taconic. Mice were maintained under specific pathogen-free conditions in the Northwestern University Center for Comparative Medicine. For T-cell depletions experiments, 200 μg anti-human CD4 (clone OX-T; BioXCell) and/or 200 μg anti-human CD8 (clone OX-8; BioXCell) was delivered/mouse by intraperitoneal (i.p.) injection 3 days prior to tumor cell engraftment and maintained every 3 days until experimental endpoints. Mouse IgG2b (clone MPC-11; BioXCell) and mouse IgG2a (clone C1.18.4, BioXcell) were administered in the same dose and approach as isotype control. For orthotopic brain tumor mouse modeling, 3 × 10<sup>5</sup> human U87, PDX12, or PDX43 GBM cells were intracranially (i.c.)-engrafted similar to our previous studies (22). U87 cells were acquired from the ATCC and engrafted at ≤10 total passages, whereas PDX tumor cells were provided by the laboratory of Dr. C. David James, PhD, from continuously propagated GBM subcutaneously engrafted in nude mice. Human GBM was not tested for mycoplasma prior to analysis. Mice were euthanized at the indicated time point(s). Brain tumor, nontumor brain tissue isolated from the contralateral hemisphere, cervical lymph node (cLN), and spleen were collected, dissected, and washed in ice-cold PBS, frozen in liquid nitrogen and stored at −80°C until analysis or processed for other techniques. Patient-derived GBM xenografts (PDX) were prepared as previously reported (23, 24). Procedures for all mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Northwestern University and are in compliance with national and institutional guidelines.

**Glioma cell lines, coculture assays, immunohistochemistry, in situ mRNA hybridization, flow cytometry, Western blotting, real-time RT-PCR, HPLC, and Gene Set Enrichment Analysis**

These procedures are described in the Supplementary Materials and Methods section.

**Statistical analysis**

The cutoff value for each gene expression level was determined with Cutoff Finder software (http://molpath.charite.de/cutoff/) using significance as the cutoff optimization method (25). Kaplan–Meier (KM) survival analysis was performed to estimate the survival distribution, whereas the log-rank test was used to assess the statistical significance of differences between the stratified survival groups using GraphPad Prism (version 6, GraphPad Software, Inc.). Renyi family of test statistics was computed via FDR<sup>adj</sup> < 0.05 was considered significant. Cox proportional hazards regression analysis was performed to assess the independent contribution of the mRNA signature and clinicopathologic variables to survival

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prediction using MedCalc (version 16.4.3, MedCalc Software bvba). Pearson’s correlation was used to analyze the relationship between each two genes’ mRNA expression level. Comparisons between multiple groups were analyzed by one-way ANOVA using GraphPad Prism software. The correlation between IDO1 mRNA levels and different cell types [tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), Treg, Neutrophil, and Tc] was examined by Canonical Correlation analysis, where each cell type was defined by a linear combination of the corresponding signature marker genes. One-way repeated measurement analysis of variance, followed by Tukey multiple comparison, was conducted to determine the difference between IDO1 exon expression levels. Differences were considered to be statistically significant when \( P < 0.05 \). SEM is presented as the error bar in all bar graphs, and mean ± SEM was utilized to describe the data throughout the text unless specifically noted.

**Results**

**Comparison of IDO1 expression by mRNA and protein detection methods in GBM**

Previous studies have demonstrated highly variable IDO1 levels in GBM using immunohistochemical detection. In accordance with a recent independent report (26), minimal IDO1 protein expression was detected in both newly diagnosed and recurrent surgical GBM specimens (Fig. 1A–D; Supplementary Fig. S1A and S1B), as indicated with human IDO1 mAb (Clone D5J4E; Cell Signaling Technology). IDO1 expression was not detectable in unmodified human U87 tumor i.c.-engrafted into NOD-scid mice (Fig. 1E and F; Supplementary Fig. S1C), although it was evident in U87 GBM cells modified to overexpress human IDO1 cDNA and engrafted into NOD-scid mice (Fig. 1G and H; Supplementary Fig. S1A and S1B), as indicated with human IDO1 mAb (Clone OF3; Cell Signaling Technology). IDO1 expression was not detectable in cranial U87 GBM were negative for IDO1 mRNA (Fig. 1I and J; Supplementary Fig. S1G), whereas U87 modified to express IDO1 cDNA were positive (Fig. 1O and P; Supplementary Fig. S1H).

**Patient survival and IDO1 expression in different grades of gliomas**

To follow-up our previous work, we evaluated the surgical specimen cohort using Hi-RNA sequencing technology for the mammalian tryptophan catabolic genes, IDO1 and TDO2, as well as the pseudogene, IDO2, for WHO grade II, grade III, and grade IV (GBM) glioma, as accessed through the TCGA database. IDO1, IDO2, and TDO2 (tryptophan 2,3-dioxygenase) mRNA expression levels were quantified and correlated with overall survival (OS). IDO1 expression progressively increased with tumor grade (Fig. 2A; \( P < 0.0001 \)). Interestingly, although a large subset of grade II and III glioma specimens were undetectable for IDO1 mRNA, 95% of GBM specimens possessed detectable IDO1 mRNA levels (Fig. 2B). In contrast, IDO2 expression did not change with tumor grade, and the majority of samples analyzed were undetectable for gene transcript (Fig. 2A). In accordance with a previous report, TDO2 mRNA levels were significantly increased in grade IV glioma (27). In addition, further analysis of IDO1 mRNA in GBM revealed differential exon expression for IDO1 (Supplementary Fig. S3; Supplementary Table S2), indicating the possibility of multiple IDO1 mRNA isoforms being expressed in GBM.

Based on the correlation between increasing glioma grade and increasing IDO1 mRNA, we examined whether high IDO1 expression correlates with patient survival using KM analysis. Cutoff Finder was utilized to generate individual cutoff values within each glioma grade. IDO1 mRNA levels were stratified into IDO1-low and -high expressing groups based on the determined cutoff
Figure 2.
mRNA expression for tryptophan catabolic enzymes in human glioma and the association of IDO1 with OS in glioma patients. 

A, The mRNA expression levels for IDO1, IDO2, and TDO2 in grade II (green; n = 226) and grade III (blue; n = 249) and grade IV (GBM; red; n = 172) analyzed from TCGA RNA-Hi-Seq Illumina database. Horizontal lines in the scatter plots represent mean ± SEM.

B, Relative expression of IDO1 mRNA levels (left column) in grade II (top), grade III (middle) and grade IV (bottom) glioma and the corresponding survival (right column). Each dot represents one patient sample that is displayed in three colored groups based on IDO1 expression level: undetectable IDO1 mRNA (yellow; IDO1 = 0); IDO1 mRNA < cutoff (blue); and IDO1 mRNA > cutoff (red).

C, KM survival analysis of grade II (left), grade III (center), and grade IV (right) glioma patients stratified by IDO1-low (green; below the cutoff) and IDO1-high (red; equal or above the cutoff) expression levels. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
values. High IDO1 mRNA levels were significantly correlated with decreased patient survival across all 3 glioma patient tumor grades (Fig. 2C; \( P < 0.05 \)). Because clinicopathologic parameters including age, tumor grade, and mode of therapy can also contribute to the prognosis of glioma patients, multivariate COX regression analysis was performed to evaluate whether IDO1 mRNA levels can be utilized as an independent prognostic factor. The results indicate that IDO1 mRNA expression functions as an independent prognostic factor in grade II and III glioma, as well as for GBM patients (Supplementary Tables S3 and S4, respectively; Table 1). 

Karnofsky Performance status data were missing in up to 50% of the analyzed specimens and were therefore not included in the multivariate analysis.

**IDO1 expression with respect to GBM subtype and IDH1 mutation status**

Given the commonly used transcriptome-based classification of GBM (28), we examined IDO1 mRNA expression with respect to GBM transcriptional subclasses. One-way ANOVA identified the proneural GBM subtype, among which isocitrate dehydrogenase 1 and 2 (\( \text{IDH1/2} \)) mutation is most common, with significantly lower IDO1 mRNA levels (Fig. 3A; \( P < 0.05 \); Supplementary Table S5). Decreased IDO1 expression was also evident in \( \text{mIDH1/2} \)-associated grade III and IV glioma specimens (Fig. 3B, \( P < 0.0001 \); Supplementary Table S5). Given the established relationship between IDH1 mutation and cytotoxic hypermutation in glioma (29, 30), we also examined genomic CpG motifs for DNA methylation throughout the human IDO1 gene locus. TCGA analysis of the DNA methylation status identified 3 CpG loci within the human IDO1 gene locus (locus 1: cg10262052, 1,500 bp of upstream transcription start site; loci 2 and 3: cg0846577, cg24188163, gene body). Methylation was increased at 2 of the 3 CpG loci in \( \text{mIDH1/2} \) grade II and III glioma (Fig. 3C). In contrast, methylation patterns did not significantly change at any CpG locus among GBM specimens, confirming that although lower glioma grade IDO1 expression is significantly affected by \( \text{mIDH1} \) status, independent mechanisms appear to regulate IDO1 mRNA levels in GBM.

Due to IDO1 activity acting as an immunosuppressant, we examined IDO1 expression with respect to the expression of genes that influence immune response. Using Pearson’s correlation analysis, we found significant relationships between mRNA expression for IDO1 and \( PD-L1 \) (\( r = 0.2993 \) and \( r = 0.4157 \)), \( PD-L2 \) (\( r = 0.4871 \) and \( r = 0.5079 \)), \( PD-1 \) (\( r = 0.3416 \) and \( r = 0.5644 \)), \( CTLA-4 \) (\( r = 0.3534 \) and \( r = 0.4900 \)), \( STAT3 \) (\( r = 0.2366 \) and \( r = 0.4598 \)), \( CD39 \) (\( r = 0.2691 \) and \( r = 0.1500 \)), \( BTLA \) (\( r = 0.2981 \) and \( r = 0.3577 \)), \( LAG3 \) (\( r = 0.2567 \) and \( r = 0.3196 \)), \( FOXP3 \) (\( r = 0.1865 \) and \( r = 0.1273 \)), and \( FGL2 \) (\( r = 0.2467 \) and \( r = 0.4983 \)) both in GBM and grade II/III glioma, respectively, (\( P < 0.01 \) and \( P < 0.001 \), respectively; Fig. 3D and E). These results suggest that increased IDO1 expression is most evident in tumors expressing additional immunosuppressive factors.

We also examined IDO1 expression with respect to immune cell infiltrates in tumor, including the markers: (i) \( \text{CD14} \), \( \text{HLA-DR} \), \( \text{CD32} \), \( \text{CD115} \), \( \text{CD163} \), \( \text{CD204} \), \( \text{CD301} \), and \( \text{CD206} \) associated with TAMs, (ii) \( \text{CD14} \), \( \text{CD11b} \), \( \text{CD33} \), and Arg1 associated with MDCs, and (iii) \( \text{CD11b} \), \( \text{CD16} \), \( \text{CD68} \), and \( \text{ELANE} \) associated with neutrophils. Correlation analyses showed that GBM-derived IDO1 mRNA levels positively correlate with the expression of all of the cell-type-specific marker genes (Supplementary Fig. S4A and S4B; \( P < 0.001 \)).

**Regulation of IDO1 expression in GBM**

In many tissues, IDO1 expression is undetectable (20), but rapidly induced and made detectable by proinflammatory stimuli (31). Due to the multiple interferon-stimulated response elements in the promoter region of IDO1, treatment of \( \text{in vitro} \)-cultured human GBM cells with the T-cell effector proinflammatory cytokine, IFN\( \gamma \) (IFNG), leads to robust IDO1 mRNA and protein expression (32, 33). To determine whether this occurs \( \text{in situ} \), we examined TCGA data that revealed 12 IDO1-undetectable patient-resected GBM specimens, with 4 samples coexpressing detectable IFNG levels (Fig. 4A). In contrast, of the 160 GBM specimens with detectable IDO1 expression, \( >50\% \) (\( n = 94 \)) were absent for IFNG. Strikingly, 43% (\( n = 69 \)) of GBM specimens were absent for both IFNG and IFNB. When the 172 total GBM samples

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**Table 1. Multivariate analysis of IDO1 mRNA levels as an independent prognostic marker in GBM patients (n = 148)**

<table>
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<th>Variables</th>
<th>Total number of event patients</th>
<th>Death N (%)</th>
<th>Survival (months) KM analysis</th>
<th>P</th>
<th>Multivariate Cox regression HR (95% CI)</th>
<th>P</th>
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<tr>
<td>&lt;50</td>
<td>33</td>
<td>21 (63.6)</td>
<td>14.5 (12.7–21.9)</td>
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<td>( \geq 50 )</td>
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<td>81 (70.4)</td>
<td>13.0 (10.4–15.4)</td>
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<td>35 (68.6)</td>
<td>11.3 (10.5–15.9)</td>
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<td>Low</td>
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<td>71 (67.0)</td>
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T Cells Enhance IDO1 Expression in Human Glioblastoma

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Figure 3.
Correlation of GBM IDO1 mRNA levels with IDH mutation, DNA methylation, and immunosuppressive gene expression. A, IDO1 mRNA levels were compared among classical (blue circle), mesenchymal (red square), and neural (green triangle) GBM subtypes analyzed from TCGA RNA-Hi-Seq. Illumina database. The proneural GBM subtype was further stratified into IDH1/2-wild-type (IDH wt; purple circle) and IDH1/2-mutant (IDH mut; orange square) samples. B, IDO1 mRNA levels were compared among grade II (IDH wt, blue circle and IDH mut, red square), grade III (IDH wt, green triangle and IDH mut, purple circle), as well as grade IV (IDH wt, orange square and IDH mut, black triangle) glioma. Pearson's correlation analysis for IDO1 mRNA with PD-L1, PD-L2, PD-1, CTLA-4, STAT3, CD39, BTLA, LAG3, FOXP3, and FGL2 in (D) GBM and (E) pooled grade II and III glioma. Each small circle in the plot represents a single patient data point. For all the scatter plots, horizontal lines represent mean ± SEM. ***, P < 0.001 and ****, P < 0.0001.
were further stratified into IFNG/IFNB-expressing, versus non-expressing groups, no difference was found among IDO1 mRNA levels (Fig. 4B), nor did this stratification yield any correlation with GBM patient survival (Fig. 4C).

TCGA analysis for human T cell-specific surface marker, CD3E, as well as Tc-associated surface marker, CD8A, was next assessed among the 172 patient-resected GBM samples and its correlation with IDO1, IFNG, and patient survival. Sample stratification

Figure 4.
Gene expression correlation analysis between IDO1, IFNG, and T cells in human GBM. A, Frequency analysis of GBM specimens with undetectable (left) or detectable (right) IDO1 expression and stratified for the coabsence of IFNG and IFNB1 (green), absence of IFNG and expression of IFNB1 (purple), expression of IFNG and absence of IFNB1 (blue), and coexpression for both IFNG and IFNB1 (red). B, Comparison of IDO1 mRNA levels among GBM specimens coabsent for IFNG and IFNB1 (green), absent of IFNG and expression of IFNB1 (purple), expression of IFNG and absent of IFNB1 (blue), and coexpression for both IFNG and IFNB1 (red). Sample size (n) of each classification group is same as in A. C, KM analysis of GBM patients based on the stratification of IFNG and IFNB1 as performed in A and B. D, Classification of 172 GBM specimens based on the expression levels of CD3E and CD8A using the calculated cutoff values (left, pie chart) and IDO1 mRNA expression (middle, bar graph) as well as KM analysis (right, survival curves) based on this classification. E, 172 GBM samples were first classified into 4 groups based on IDO1 and IFNG expression status (undetectable = 0 or detectable > 0), then a frequency analysis was further performed using the same CD3E and CD8A-based stratification as in A within each of the four groups. Sample size (n) is the same as in D. Canonical correlation analysis of (F) CD8+ T-cell marker genes, CD3E and CD8A, with those of Tregs, CD3E, CD4, CD25, and FOXP3, as well as (G) Treg marker genes with IDO1 mRNA expression. Each blue spot represents a GBM patient data point. A regression line was fitted to the dot plot. **, \( P < 0.01; \) ****, \( P < 0.001; \) and ******, \( P < 0.0001. \)
identified that 56% of GBM possess low CD3E and CD8A mRNA levels, 29% consist of low CD3E with high CD8A mRNA levels, and 15% contain high CD3E and high CD8A mRNA levels (Fig. 4D). Notably, high CD3E and CD8A mRNA levels were correlated with higher IDO1 and IFNG mRNA levels, as well as decreased patient survival (P < 0.01). These results suggest that greater T-cell infiltration of tumor is associated with higher IDO1 and IFNG expression, as well as lower OS of GBM patients.

Based on the well-characterized association of activated CD8⁺ T cells and IFNG expression, we investigated potential associations between the expression of IFNG, CD3E, and CD8A mRNA. Frequency analysis indicated that GBM specimens lacking in IDO1 expression also lacked detectable CD3E and CD8A T-cell signatures (n = 12), irrespective of tumor IFNG expression (Fig. 4E). Conversely, GBM specimens with high CD3E and CD8A transcript levels expressed detectable IDO1, with a higher frequency of specimens coexpression IFNG (n = 20). We also examined the association between markers for T-cell infiltration of tumor is associated with higher IDO1 and IFNG expression, as well as lower OS of GBM patients.

T cells directly regulate IDO1 expression in human GBM

To further explore associations between T cells and IDO1 expression in GBM, humanized immunocompetent mice (hNSG; NSG-SGM3-BLT) were i.c.-engrafted with human patient-derived GBM xenografts (PDX) 12 or 43 and treated with or without T-cell–depleting antibodies. Analysis of isolated GBM tumors, draining cLNs, and spleen revealed the presence of both CD4⁺ and CD8⁺ human T cells that were significantly decreased when mice were coadministered T-cell–depleting antibodies (Fig. 5A and B). The analysis of humanized immunocompetent mouse-engrafted U87 GBM revealed a predominantly human leukocyte composition in mouse spleen that was reflected in both the myeloid and lymphoid cell compartments (Supplementary Fig. S5A). Similar to mice engrafted PDX GBM, analysis of U87 tumors confirmed the presence of human T cells in the GBM (Supplementary Fig. S5B) as well as in the cLN and spleen (Supplementary Fig. S5B), with a notable absence of human T cells in the contralateral brain hemisphere without tumor. In addition, intratumoral CD4⁺ and CD8⁺ T cells were detectable among the CD3⁺ GBM-infiltrating T cells (Supplementary Fig. S5C), with an increased frequency of CD3⁺CD4⁺ T cells in association with markers indicative of immunosuppressive Treg, as compared with the cLN (Supplementary Fig. S5D). CD3⁺CD45⁺ human myeloid cells were also enriched in brain tumors, as compared with peripheral secondary lymphoid organs. Treatment with CD4 or CD8 mAbs decreased U87 GBM infiltrating T-cell levels (Supplementary Fig. S6), confirming the availability of multiple GBM models for investigating the interactions between human brain tumor and human immune cells, in situ.

Having validated the presence of human T cells in humanized mice with intracranial human PDX or U87 GBM, we further explored the hypothesis that T cells directly increase IDO1 expression in human glioma. Results from mice depleted for CD4⁺ and CD8⁺ T cells confirm that immune cell neutralization does not affect tumor growth (Supplementary Fig. S7) and that T-cell depletion decreases tumor IDO1 mRNA levels (P < 0.001, respectively; Fig. 5C). Similarly, human IDO1 mRNA is undetectable in unmodified GBM isolated from T-cell–deficient mice, but present in GBM tumors engineered to express human IDO1 cDNA (IDO1 O/E U87) and engrafted into mice without human T cells, as well as in surgically resected GBM patient tumor. Expression for the T-cell–specific marker, CD3E, as well as the T-cell effector cytokine, IFNG, is readily detectable in normal hNSG mice, but is absent or decreased in the majority of GBM engrafted into mice depleted or deficient for human T cells. The analysis of subcutaneously propagated human PDX GBM further confirmed the lack of IDO1 expression in T-cell–deficient nude mice, although the transcript is potently induced after stimulation of PDX GBM cells with human IFNg, ex vivo (Fig. 5D). In vitro GBM patient T-cell: U87 GBM cell cocultures confirmed that IDO1 is induced by activated T lymphocytes in an IFNg-dependent manner (Fig. 5E; P < 0.001; Supplementary Fig. S8B). Taken together, these data support the hypothesis that GBM-infiltrating T cells directly increase immunosuppressive IDO1 expression in human GBM.

Discussion

IDO1 is recognized as an important mediator of immunosuppression in cancer (12, 22, 34, 35). To address the potential usefulness of IDO1 expression as a prognostic tool for glioma patients, we compared the sensitivity of protein- and mRNA-based detection methods for IDO1 in human GBM. Our data indicate that the quantification of tumor IDO1 mRNA expression yields high prognostic value for GBM patient outcome. This also proved to be the case for grade II and grade III glioma. mRNA results also showed that IDO1 expression (i) increases with glioma grade; (ii) is distinct among GBM molecular subtypes; (iii) is decreased in IDH mutant when compared with IDH wild-type tumors; (iv) is correlated with the expression of other immunosuppressive mediators; and (vii) intratumorally increases in association with increased expression for Tc and Treg markers.

To evaluate relationships between IDO1 expression and tumor-infiltrating T lymphocytes, our use of humanized immunocompetent (NSG-SGM3-BLT) mice i.c.-engrafted with human GBM showed that tumor IDO1 expression is influenced by infiltrating CD4⁺ and CD8⁺ T cells. In contrast to the general assumption that IFNy is the primary regulator of IDO1 expression, our analysis of 172 patient-resected GBM revealed that 59% of tumor specimens are undetectable for IFNG (Fig. 4A). Coincidently, PDX12 tumor IDO1 expression increased in a T-cell–dependent manner that was not associated with a commensurate increase in IFNg levels, in situ (Fig. 5C). These findings confirm the existence of additional mechanisms responsible for IDO1 gene expression in GBM and are the subject of an ongoing investigation by our group. Therefore, we could conclude that IFNg is sufficient, but not required, for increasing IDO1 levels in human GBM.

We found significant correlations between GBM IDO1 levels, decreased patient survival, and increased marker expression for Tc and Treg (Fig. 4D–G). This finding aligns with our previous observations in syngeneic, immunocompetent, intracranial mouse GBM models which showed that: (i) tumor cell IDO1 facilitates local Treg accumulation; (ii) Tc and Treg coincidently infiltrate IDO1-expressing tumors; and (iii) tumor cell IDO1 expression decreases animal subject survival (13, 36). Supplementary Fig. S9 presents a model showing tumor cell–T-cell interactions and supports the hypothesis that GBM-infiltrating Tc facilitate IFNg-dependent increases in tumor cell IDO1, followed by the intratumoral accumulation of immunosuppressive Treg. This model highlights the negative repercussions of...
Figure 5.
Activated (Act.) human T cells directly increase IDO1 expression in human GBM. Humanized mice reconstituted with human immune cells (NSG-SGM3-BLT) were i.c.-engrafted patient-derived human GBM xenografts 12 or 43 (PDX12 or PDX43, respectively). A, Representative flow plots of intracranial PDX GBM tumors, draining cLNs, or spleen isolated at the time of symptomatic onset are shown for mice treated with IgG antibodies (Ab) or CD4 and CD8 T-cell–depleting mAbs. Data are representative of one mouse from each treatment group and tissue type. B, Quantification of flow cytometric data of the frequency and absolute number for CD4+ and CD8+ T cells in the GBM, draining cLN, and spleen of IgG Ab control– or CD4/CD8 mAb–treated humanized mice engrafted intracranial PDX12 or PDX43 GBM (n = 5/group). C, mRNA expression levels for human IDO1, CD3E, or IFNG was quantified and compared among isolated intracranial human GBM (n = 4–9/group). Unmodified U87, U87 modified to constitutively express human IDO1 cDNA (IDO1-O/E U87), PDX12, PDX39, PDX43, or patient-resected primary and recurrent GBM were included in the comparison. I.c.- or s.c.-engrafted tumors were resected from T-cell–deficient mice (Nu/nu or scid) or patient-resected primary and recurrent GBM were included in the comparison. I.c.- or s.c.-engrafted tumors were resected from T-cell–deficient mice (Nu/nu or scid) or humanized mice with or without treatment of T-cell–depleting antibodies between 14 and 21 days postintracranial injection. D, In vitro expression analysis of human IDO1 mRNA in different GBM cells with or without addition of human IFNγ. Data represent pooled data from four independent experiments. E, Detection of human IDO1 mRNA in the human GBM–T-cell coculture system, in vitro. CD3+ human T cells were isolated under positive selection from GBM patient PBMCs for the coculture experiment. Human IDO1 mRNA levels were analyzed in U87 GBM cells under different treatment conditions, or naive and activated T cells were analyzed in isolation and measured by real-time RT-PCR. Data were compiled from three independent experiments.

hNSG, immunocompetent humanized mice (NSG-SGM3-BLT); SCID, NOD-scid mice; Nu/nu, nude mice; ND, not detectable. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
inflammatory enhancement in human GBM and is supported by the data in Fig. 3A and B, showing the inverse association between the presence of mIDH and decreased intratumoral IDO1 levels. Given that mIDH suppresses CD8+ T cell accumulation in glioma (37), in addition to the favorable prognosis it carries for GBM patient survival (38), it is tempting to speculate that the mIDH suppression of GBM-infiltrating Tc increases patient survival by virtue of the additional suppression of IDO1 and Treg accumulation in those patient tumors.

Our observations suggest that future immunotherapeutic strategies incorporating IDO1 inhibition into a GBM patient treatment regimen may be more effective in treating classical, mesenchymal, and neural GBM subtypes, when compared with the lower IDO1-expressing proneural GBM and glioma grades II and III. These data further infer that GBM patients with high baseline tumor-infiltrating T cell levels, or those who enroll on T-cell–enhancing therapeutic treatments, may maximally benefit from the addition of an IDO1 inhibitor. Our data also indicate a gene expression correlation between IDO1 and other mediators of immunosuppression (Fig. 3D and E), highlighting the potential for enhancing antitumor effects when combining IDO1 inhibition with a blockade of multiple immune checkpoint targets.

In conclusion, we show for the first time that IDO1 mRNA levels are a useful, sensitive, and prognostic predictor of grade II–IV glioma patient survival. We also introduce a new, enzymatically active modified U87 human GBM cell line that expresses an C-terminus HA tag conjugated to human IDO1 (Supplementary Fig. S2). We further present novel data showing differential IDO1 exon expression among patient-resected GBM (Fig. 3; Supplementary Table S2), highlighting complex transcriptional mechanisms that are likely associated with the expression of multiple isoforms. Analysis of IDO1 expression in GBM isolated from humanized mice-engrafted PDX43 and U87, versus PDX12 GBM, provides new comparative platforms for investigating IFNγ-independent mechanisms of IDO1 regulation by tumor-infiltrating T cells. Given the past preclinical strategies demonstrating the survival benefit of pharmacologic IDO1 blockade in preclinical brain tumor models (39), in addition to clinical trials evaluating IDO1-targeting strategies in glioma patients (NCT02052648 and NCT02764151), the data collectively suggest that future treatment approaches designed to enhance T-cell–mediated antitumor immunity may maximally benefit from the further addition of an IDO1 inhibitor.

Disclosure of Potential Conflicts of Interest
P.K. Bastiananos reports receiving speakers bureau honoraria from Genentech and Merck and is a consultant/advisory board member for Angiogenics and Roche. C. Horbinski is a consultant/advisory board member for AbbVie. R. Stupp is a consultant/advisory board member for AbbVie, Celgene, EMD Serono, Merck Sharp & Dohme Corp., Novartis, Novocure, and Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: L. Zhai, F.J. Giles, C.D. James, C. Horbinski, D.A. Wainwright
Development of methodology: L. Zhai, F.J. Giles, C. Horbinski, D.A. Wainwright
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Zhai, E. Ladomersky, K. Lauing, C. Gritsina, C. Horbinski, D.A. Wainwright
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Zhai, K. Lauing, M. Wu, M. Genet, B. György, F.J. Giles, C. Horbinski, R. Stupp, D.A. Wainwright
Writing, review, and/or revision of the manuscript: L. Zhai, E. Ladomersky, M. Genet, P.K. Bastiananos, D.C. Binder, J.A. Sosman, F.J. Giles, C. Horbinski, R. Stupp, D.A. Wainwright
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Zhai, G. Grisina, B. György, D.A. Wainwright
Study supervision: D.A. Wainwright

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Infiltrating T Cells Increase IDO1 Expression in Glioblastoma and Contribute to Decreased Patient Survival

Lijie Zhai, Erik Lademersky, Kristen L. Lauing, et al.

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