Infiltrating T cells increase IDO1 expression in glioblastoma and contribute to decreased patient survival

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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 co-cultures, 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 co-inhibit potential T cell-mediated IDO1 enhancement during therapy.
TRANSLATIONAL RELEVANCE

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, has yet to demonstrated. This study reveals that, high IDO1 mRNA levels, as assessed using the Hi-RNA-Sequencing 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 immunotherapeutically 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.
INTRODUCTION

Glioblastoma (GBM, astrocytoma, WHO grade IV) is the most common and aggressive primary central nervous system (CNS) 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) (4), as well as chromosome 1p/19q co-deletion, 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 interferon-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 (Tc), 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). Additionally, while we previously demonstrated that the upregulation of IDO1 mRNA levels inversely correlate 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- vs. 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 cytolytic T cell markers, \( CD3E \) and \( CD8A \), suggesting a T cell-associated influence on intratumoral IDO1 expression.
METHODS

The cancer genome atlas (TCGA) sample description

The TCGA data for all the cancer types analyzed in current study were accessed from the UCSC Xena browser (http://xena.ucsc.edu/). mRNA expression data represented by RNASeq (Illumina Hi-seq platform) includes RSEM normalized level 3 data present in TCGA as of April 13th, 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 13th, 2017. TCGA GBM gene expression data by AffyU133a array were also acquired from the UCSC Xena browser.

Patient samples

Peripheral blood from GBM patients were collected at the Northwestern Brain Tumor Institute (NBTI) Tissue Bank. PBMCs were isolated using Ficoll-Paque (GE Healthcare) density gradient separation and stored at liquid nitrogen tank for coculture experiments. Snap-frozen tissue and formalin-fixed, paraffin-embedded (FFPE) tissue from surgically-removed GBM were also collected at 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 are provided in Supplementary Table 1.

Animal and tissue preparation

Immunocompetent, humanized mice (NSG-SGM3-BLT) were established by implantation of human fetal liver and thymus fragments as well as haematopoietic stem
cells into immunodeficient NOD.Cg-Prkdc<sup>scid</sup> IL2rg<sup>tm1Wjl</sup> Tg (CMV-IL3, CSF2, KITLG)1Eav/MloySzJ (NSG-SGM3) mice, NOD.CB17-Prkdc<sup>scid</sup>/J (NOD-scid) mice were obtained from The Jackson Laboratory and CrTac:NCr-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 depletion experiments, 200 μg anti-human CD4 (clone OKT-4; BioXCell) and/or 200 μg anti-human CD8 (clone OKT-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 administrated 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-engrafted similar to our previous studies (22). U87 cells were acquired from ATCC and engrafted at < 10 total passages while 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, non-tumor brain tissue isolated from the contralateral hemisphere, cervical lymph node and spleen were collected, dissected and washed in ice-cold phosphate-buffered saline (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, Gene Set Enrichment Analysis

Procedures 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, while 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., La Jolla, CA). Renyi family of test statistics was computed via SAS software (version 9.4, SAS Institute Inc., Cary, NC) to determine the survival difference between two groups given the presence of crossing hazard rates. Cox proportional hazards regression analyses were performed to assess the independent contribution of the mRNA signature and clinicopathologic variables to survival prediction using MedCalc (version 16.4.3, MedCalc Software bvba, Belgium). 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 (TAM, MDSC, Treg, Neutrophil, Tc) were 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
difference between IDO1 exon expression levels. Differences were considered to be statistically significant when $P < 0.05$. Standard error of the mean (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, Supp. Fig. 1A,B), as indicated with human IDO1 mAb (Clone D5J4E, Cell Signaling Technology). IDO1 expression was not detectable in unmodified human U87 tumor intracranially-engrafted (ic.) into NOD-<i>scid</i> mice (Fig. 1E,F; Supp. Fig. 1C), although it was evident in U87 GBM cells modified to overexpress human IDO1 cDNA and engrafted into NOD-<i>scid</i> mice (Fig. 1G,H; Supp. Fig. 1, 2). In contrast to the antibody-based detection properties, <i>in situ</i> hybridization readily detected IDO1 mRNA in both primary and recurrent patient GBM tissue samples (Fig. 1I-1L; Supp. Fig. 1E, F). Unmodified intracranial U87 GBM were negative for IDO1 mRNA (Fig. 1M, N; Supp. Fig. 1G), whereas U87 modified to express IDO1 cDNA were positive (Fig. 1O, P, Supp. Fig. 1H).

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
Interestingly, while 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). Additionally, further analysis of *IDO1* mRNA in GBM revealed differential exon expression for *IDO1* (Supp. Fig. 3, Supp. Table 2), 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 Kaplan-Meier (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 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 (Supp. Table 3,4, respectively; Table 1). Karnofsky Performance status data was 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 (mIDH1/2) mutation is most common, with significantly lower *IDO1* mRNA levels (Fig. 3A; *P* < 0.05; Supp. Table 5). Decreased *IDO1* expression was also evident in mIDH1/2-associated grade III and IV glioma specimens (Fig. 3B, *P* < 0.0001, Supp. Table 5). Given the established relationship between IDH1 mutation and cytosine hypermethylation in glioma (29, 30), we also analyzed 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 1: cg10262052, 1500bp of upstream transcription start site; loci 2 and 3: cg0846577, cg24188163, gene body). Methylation was increased at 2 of the 3 CpG loci in 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, while lower glioma grade *IDO1* expression is significantly affected by mIDH 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*...
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) CD14, HLA-DR, CD312, CD115, CD163, CD204, CD301, CD206 associated with tumor-associated macrophages (TAM), (ii) CD14, CD11b, CD33, Arg1 associated with myeloid-derived suppressor cells (MDSC), and (iii) CD11b, CD16, CD66b and 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 (Supp. Fig. 4A, B; P < 0.001).

Regulation of IDO1 expression in glioblastoma

In many tissues, IDO1 expression is undetectable (20), but rapidly induced and made detectable by pro-inflammatory stimuli (31). Due to the multiple interferon-stimulated response elements (ISRE) in the promoter region of IDO1, treatment of in vitro-cultured human GBM cells with the T cell effector pro-inflammatory cytokine, IFNγ (IFNG), leads to robust IDO1 mRNA and protein expression (32, 33). To determine whether this occurs in situ, we examined TCGA data that revealed 12 IDO1-undetectable patient-resected GBM specimens, with 4 samples co-expressing detectable IFNG levels.
In contrast, of the 160 GBM specimens with detectable \textit{IDO1} expression, >50% (n=94) were absent for \textit{IFNG}. Strikingly, 43% (n=69) of GBM specimens were absent for both \textit{IFNG} and \textit{IFNB}. When the 172 total GBM samples were further stratified into \textit{IFNG}/\textit{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, \textit{CD3E}, as well as Tc-associated surface marker, \textit{CD8A}, was next assessed among the 172 patient-resected GBM samples and its correlation with \textit{IDO1}, \textit{IFNG} and patient survival. Sample stratification identified that 56% of GBM possess low \textit{CD3E} and \textit{CD8A} mRNA levels, 29% consist of low \textit{CD3E} with high \textit{CD8A} mRNA levels and 15% contain high \textit{CD3E} and high \textit{CD8A} mRNA levels (Fig. 4D). Notably, high \textit{CD3E} and \textit{CD8A} mRNA levels were correlated with higher \textit{IDO1} and \textit{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 \textit{IDO1} and \textit{IFNG} expression, as well as lower overall survival of GBM patients.

Based on the well-characterized association of activated CD8\textsuperscript{+} T cells and IFN\textgamma expression, we investigated potential associations between the expression of \textit{IFNG}, \textit{CD3E} and \textit{CD8A} mRNA. Frequency analysis indicated that GBM specimens lacking in \textit{IDO1} expression also lacked detectable \textit{CD3E} and \textit{CD8A} T cell signatures (n=12), irrespective of tumor \textit{IFNG} expression (Fig. 4E). Conversely, GBM specimens with high \textit{CD3E} and \textit{CD8A} transcript levels expressed detectable \textit{IDO1}, with a higher frequency of specimens co-expressing \textit{IFNG} (n=20). We also examined the association between
markers for CD8$^+$ T cells and Treg (Fig. 4F), as well as IDO1 mRNA and Treg (Fig. 4G), confirming a positive relationship among both correlative groups.

_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 intracranially-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 cervical lymph nodes (cLN) and spleen revealed the presence of both CD4$^+$ and CD8$^+$ human T cells that were significantly decreased when mice were co-administered T cell-depleting antibodies (Fig. 5A,B). The analysis of humanized immunocompetent mice engrafted U87 GBM revealed a predominantly human leukocyte composition in mouse spleen that was reflected in both the myeloid and lymphoid immune cell compartments (Supp. Fig. 5A). Similar to mice engrafted PDX GBM, analysis of U87 tumors confirmed the presence of human T cells in the GBM (Supp. Fig. 5B) as well as in the cLN and spleen (Supp. Fig. 5B), with a notable absence of human T cells in the contralateral brain hemisphere without tumor. Additionally, intratumoral CD4$^+$ and CD8$^+$ T cells were detectable among the CD3$^+$ GBM-infiltrating T cells (Supp. Fig. 5C), with an increased frequency of CD3$^+$CD4$^+$ T cells in association with markers indicative of immunosuppressive Treg, as compared to the cLN (Supp. Fig. 5D). CD3$^+$CD45$^+$ human myeloid cells were also enriched in brain tumors, as compared to peripheral secondary lymphoid organs. Treatment with CD4 or CD8 mAbs decreased U87 GBM-infiltrating T cell levels (Supp. Fig. 6), 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 (Supp. Fig. 7), 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 IFNγ, *ex vivo* (Fig. 5D). *In vitro* GBM patient T cell:U87 GBM cell co-cultures confirmed that *IDO1* is induced by activated T lymphocytes in an IFNγ-dependent manner (Fig. 5E; $P < 0.001$; Supp. Fig. 8B). 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 to 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 intracranially-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 IFNγ 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 IFNγ levels, in situ (Fig. 5C). These findings confirm the existence of additional mechanisms responsible for IDO1 gene expression in GBM and is 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, E, F, 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. 9 presents a model showing tumor cell - T cell interactions and supports the hypothesis that GBM-infiltrating Tc facilitate IFNγ-dependent increases in tumor cell IDO1, followed by the intratumoral accumulation of immunosuppressive Treg. This model highlights the negative repercussions of inflammatory enhancement in human GBM and is supported by the data in Fig. 3A, B, showing the inverse association between the presence of mIDH and decreased intratumoral IDO1 levels. Given that mIDH suppresses CD8+ Tc 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 to 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, E), highlighting the potential for enhancing anti-tumor 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 (Supp. Fig. 2). We further present novel data showing differential IDO1 exon expression among patient-resected GBM (Fig. 3; Supp. Table 2), 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, provide 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, 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.
Acknowledgments

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REFERENCE


Tables

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

<table>
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<td>[2.73, 7.63]</td>
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<td>2.24</td>
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Figure Legends

Figure 1. Immunohistochemical (IHC) IDO1 localization and in situ hybridization (ISH) for IDO1 in human glioblastoma (GBM). (A-H) IHC detection with a human-specific IDO1 antibody was performed in (A, B) newly diagnosed primary patient-resected GBM, (C, D) recurrent patient-resected GBM or tumors isolated from immunodeficient NOD-scid mice intracranially-engrafted (ic.) (E, F) unmodified U87 cells and (G, H) U87 overexpressing human IDO1 conjugated to an HA tag (HA-IDO1 O/E). A positive signal is indicated by red arrows in the high magnification images (B, D, F, H). (I-P) ISH for human IDO1 mRNA was performed in the same tissues described for IHC and is indicated with red arrows in the high magnification images (J, L, N, P). The proliferation marker, Ki67, was used as a positive control and appears as a red signal. All lower magnification images were obtained under 64x objective lens with a scale bar representing 400 μm.

Figure 2. mRNA expression for tryptophan catabolic enzymes in human glioma and the association of IDO1 with overall survival 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 the cancer genome atlas RNA-Hi-Seq. Illumina database. Horizontal lines in the scatter plots represents mean ± SEM. (B) Relative expression of IDO1 mRNA expression (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 3 colored groups.
based on IDO1 expression level: undetectable IDO1 mRNA (yellow; IDO1=0); IDO1 mRNA < cutoff (blue) and IDO1 mRNA > cutoff (red). Sample size (n), frequency of the representative population, and cutoff values within each grade of glioma are annotated in the IDO1 mRNA distribution dot plot. (C) Kaplan-Meier (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; ***, P < 0.001.

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 the cancer genome atlas RNA-Hi-Seq. Illumina database. The Proneural GBM subtype was further stratified into IDH1/2-wild-type (IDH\textsubscript{wt}; purple circle) and IDH1/2-mutant (IDH\textsubscript{mut}; orange square) samples. (B) IDO1 mRNA levels were compared among grade II (IDH\textsubscript{wt}; blue circle and IDH\textsubscript{mut}; red square), grade III (IDH\textsubscript{wt}; green triangle and IDH\textsubscript{mut}; purple circle), as well as grade IV (IDH\textsubscript{wt}; orange circle and IDH\textsubscript{mut}; black triangle) glioma. (C) DNA methylation analysis of cg10262052, cg08465774 and cg24188163 in grade II (IDH\textsubscript{wt}; blue circle and IDH\textsubscript{mut}; red square), grade III (IDH\textsubscript{wt}; green triangle and IDH\textsubscript{mut}; purple circle), as well as grade IV (IDH\textsubscript{wt}; orange circle and IDH\textsubscript{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. ***, $P < 0.001$, ****, $P < 0.0001$.

**Figure 4.** Gene expression correlation analysis between *IDO1, IFNG and T cells* in human glioblastoma (GBM). (A) Frequency analysis of GBM specimens with undetectable (left) or detectable (right) *IDO1* expression and stratified for the co-absence of *IFNG* and *IFNB1* (green), absence of *IFNG* and expression of *IFNB1* (purple), expression of *IFNG* and absence of *IFNB1* (blue) and co-expression for both *IFNG* and *IFNB1* (red). (B) Comparison of *IDO1* mRNA levels among GBM specimens co-absent for *IFNG* and *IFNB1* (green), absent of *IFNG* and expression of *IFNB1* (purple), expression of *IFNG* and absent of *IFNB1* (blue) and co-expression 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, B). (D) Classification of 172 GBM specimens based on the expression levels of *CD3E* and *CD8A* using the calculated cutoff values (left panel, pie chart) and *IDO1* mRNA expression (central panel, bar graph) as well as KM analysis (right panel, 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 regulatory T cells (Treg) cells, *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. *, $P < 0.05$, **, $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

**Figure 5.** Activated human T cells directly increase IDO1 expression in human glioblastoma (GBM). Humanized mice reconstituted with human immune cells (NSG-SGM3-BLT) were intracranially-engrafted patient-derived human GBM xenografts 12 or 43 (PDX12 or PDX43, respectively). (A) Representative flow plots of intracranial PDX GBM tumors, draining cervical lymph nodes (cLN) 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 monoclonal antibodies (mAb). 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. Intracranially (ic.)- or subcutaneously (sc.)-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 - 21 days post-intracranial injection. (D) *In vitro* expression analysis of human IDO1 mRNA in different GBM cells with or without addition of human interferon-gamma (IFNγ). Data represent pooled data from four independent experiments. (E) Detection of
human IDO1 mRNA in the human GBM -T cell co-culture system, in vitro. CD3+ human T cells were isolated under positive selection from GBM patient PBMCs for the co-culture experiment. Human IDO1 mRNA levels were analyzed in U87 GBM cells under different treatment conditions, or naïve 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. *: \( P < 0.05 \), **: \( P < 0.01 \), ***: \( P < 0.001 \).
FIGURE 1

<table>
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<tr>
<th>Patient-Resected Glioblastoma</th>
<th>Immunohistochemistry</th>
<th>In situ hybridization</th>
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<td>Low Mag.</td>
<td>A</td>
<td>I</td>
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<tr>
<td>High Mag.</td>
<td>B</td>
<td>J</td>
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<tr>
<td>Recurrent</td>
<td>C</td>
<td>K</td>
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<tr>
<td>Unmod. U87</td>
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<td>L</td>
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<td>NOD-scid mice i.c.</td>
<td>E</td>
<td>M</td>
</tr>
<tr>
<td>IDO1 OE U87</td>
<td>F</td>
<td>N</td>
</tr>
</tbody>
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Legend: Red arrows indicate specific markers or patterns observed in the images.
FIGURE 3

A

GBM Subtype

- Classical (n=42)
- Mesenchymal (n=55)
- Neural (n=28)
- Proneural IDH<sub>wt</sub> (n=28)
- Proneural IDH<sub>mut</sub> (n=8)

B

- Grade II, IDH<sub>wt</sub> (n=20)
- Grade II, IDH<sub>mut</sub> (n=20)
- Grade III, IDH<sub>wt</sub> (n=69)
- Grade III, IDH<sub>mut</sub> (n=180)
- Grade IV, IDH<sub>wt</sub> (n=140)
- Grade IV, IDH<sub>mut</sub> (n=9)

C

- * ****
- cg10262052
- cg08465774
- cg24188163

D

- PD-L1
- PD-L2
- PD-1
- CTLA-4
- STAT3
- CD99
- BTLA
- LAG3
- FOXP3
- FGF2

E

- IDO1 mRNA, Log2 (Gr. II and III glioma)
- IDO1 mRNA, Log2 (GBM)

Correlation coefficients and p-values:

- r=0.2993, P<0.0001
- r=0.4871, P<0.0001
- r=0.3416, P<0.0001
- r=0.3534, P<0.0001
- r=0.2366, P<0.0018
- r=0.2691, P=0.0004
- r=0.2981, P<0.0001
- r=0.2567, P=0.0007
- r=0.1865, P=0.0143
- r=0.4257, P<0.0001
- r=0.4157, P<0.0001
- r=0.5079, P<0.0001
- r=0.5644, P<0.0001
- r=0.4900, P<0.0001
- r=0.4598, P<0.0001
- r=0.1500, P=0.0005
- r=0.3577, P<0.0001
- r=0.3198, P<0.0001
- r=0.1273, P=0.0033
- r=0.4938, P<0.0001
FIGURE 5

A

Human PDX GBM

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Mouse cLN

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Mouse spleen

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B

hNSG ic. PDX GBM + IgG Ab

% CD4⁺ T cells (Gated on CD3⁺)

hNSG ic. PDX GBM + CD4/CD8 mAb

% CD8⁺ T cells (Gated on CD3⁺)

C

hNSG mice ic. U87 GBM + IgG Ab

hNSG mice ic. U87 GBM + CD4 mAb

hNSG mice ic. U87 GBM + CD8 mAb

hNSG mice ic. PDX43 + IgG Ab

hNSG mice ic. PDX43 + CD4/CD8 mAb

scid mice ic. U87

scid mice ic. IDO1-O/E U87

Nu/nu mice sc. GBM PDX

Patient-Resected Primary GBM

Patient-Resected Recurrent GBM

D

No stim. U87

U87 + IFNγ

IDO1-O/E U87

No stim. PDX39

PDX39 + IFNγ

PDX43 + IFNγ

PDX39 + IFNγ

PDX43 + IFNγ

E

IDO1 mRNA (Nor. U87-active T cell CM)

(18)

(14)

(11.25)

(10)

(7.5)

(6)

(3.75)

(2.5)

(1.25)

(0.5)

(0.25)

(0.125)

(0.0625)

(0.03125)

(0.015625)

(0.0078125)

0

IFNγ Ab:
Naïve T cell:
Act. T cell:

Direct

Trans-well

CM

U87 alone

Activated T
Infiltrating T cells increase IDO1 expression in glioblastoma and contribute to decreased patient survival

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


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