Cancer Immunology, Immunotherapy (2019) 68:1107–1120
https://doi.org/10.1007/s00262-019-02347-3

ORIGINAL ARTICLE

Metabolic remodeling contributes towards an immune-suppressive phenotype in glioblastoma

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Received: 31 October 2018 / Accepted: 17 May 2019 / Published online: 22 May 2019

Abstract

Glioblastoma (GBM) is one of the most aggressive tumors. Numerous studies in the field of immunotherapy have focused their efforts on identifying various pathways linked with tumor-induced immunosuppression. Recent research has demonstrated that metabolic reprogramming in a tumor can contribute towards immune tolerance. To begin to understand the interface between metabolic remodeling and the immune-suppressive state in GBM, we performed a focused, integrative analysis coupling metabolomics with gene-expression profiling in patient-derived GBM ($n=80$) and compared them to low-grade astrocytoma (LGA; $n=28$). Metabolic intermediates of tryptophan, arginine, prostaglandin, and adenosine emerged as immuno-metabolic nodes in GBM specific to the mesenchymal and classical molecular subtypes of GBM. Integrative analyses emphasized the importance of downstream metabolism of several of these metabolic pathways in GBM. Using CIBERSORT to analyze immune components from the transcriptional profiles of individual tumors, we demonstrated that tryptophan and adenosine metabolism resulted in an accumulation of Tregs and M2 macrophages, respectively, and was recapitulated in mouse models. Furthermore, we extended these findings to preclinical models to determine their potential utility in defining the biologic and/or immunologic consequences of the identified metabolic programs. Collectively, through integrative analysis, we uncovered multifaceted ways by which metabolic reprogramming may contribute towards immune tolerance in GBM, providing the framework for further investigations designed to determine the specific immunologic consequence of these metabolic programs and their therapeutic potential.

Keywords Glioblastoma · Immune metabolism · Tryptophan · Arginase · Prostaglandin · Adenosine

Abbreviations

1-L-MT	1-Methyl-L-tryptophan
AD-H	Adenosine pathway metabolites-high
AD-L	Adenosine pathway metabolites-low
AHR	Aryl hydrocarbon receptor
ARG-H	Arginine pathway metabolites-high
ARG-L	Arginine pathway metabolites-low
ARG2	Arginase 2
ASL	Argininosuccinate lyase
CBR	Carbonyl reductase
CIBERSORT	Cell-type identification by estimating relative subsets of RNA transcripts
CKMT1	Creatine kinase, mitochondrial 1A
COX	Cyclooxygenase
GBM	Glioblastoma
GSCs	Glioma stem cells
IDH1	Isocitrate dehydrogenase 1
IL2-Rα	Interleukin 2 receptor-subunit alpha
KEGG	Kyoto encyclopedia of genes and genomes
KMO	3-Mono-oxygenase
KYAT	Kynurenine aminotransferase
KYNU	Kynureninase
LGA	Low-grade astrocytoma
MGMT	O6-methylguanine–DNA methyltransferase
MSP	Methylation-specific PCR
NAD	Nicotinamide adenine dinucleotide
NOS1	Nitric oxide synthase 1
PG-H	Prostaglandin pathway metabolites-high

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-019-02347-3) contains supplementary material, which is available to authorized users.

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PG-L	Prostaglandin pathway metabolites-low
PGE2	Prostaglandin E2
PGF2A	Prostaglandin F2 alpha
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PLS	Partial least squares
PTGES	Prostaglandin E synthase
PTGIS	Prostaglandin I synthase
PTGS	Prostaglandin-endoperoxide synthase
QPRT	Quinolinate phosphoribosyltransferase
TCGA	The cancer genome atlas
TDO	Tryptophan 2,3-dioxygenase
TRP-H	Tryptophan pathway metaboliteshigh
TRP-L	Tryptophan pathway metaboliteslow
VIP	Variable importance in projection

Introduction

Glioblastoma (GBM) is the most common adult primary brain tumor [1]. Despite continued advances in surgery, radiation, and the identification of novel molecularly targeted agents, outcomes remain poor. Recent clinical advancements using immune checkpoint inhibitors designed to target tumor-mediated immune tolerance have revolutionized our approach to cancer therapy. Cytotoxic T-lymphocyte-associated protein 4 and programmed death-1, which negatively regulate T-cell activation, represent two specific immune checkpoints that have received recent attention, with inhibitors targeting these immune pathways demonstrating unprecedented clinical activity in multiple tumors [2–4]. Unfortunately, as with many novel therapies, these approaches have yet to translate to meaningful benefit in GBM [5]. Therefore, continued investigations designed to both understand the immune landscape of GBM and identify novel strategies to revert its immune-suppressive microenvironment are warranted.

Aberrant cellular metabolism is emerging as a novel therapeutic target, and the interplay between metabolic remodeling and immune regulation in cancer represents an active area of investigation [6, 7]. For example, enhanced glycolysis in tumors that appears to be driving its aggressive phenotype leads to a microenvironment depleted of glucose, which is a critical substrate to help support the rapid and dynamic transitions between naïve and activated states in a variety of immune cells [2]. In addition to passive consequences of metabolic reprogramming, tumors have co-opted various metabolic strategies to actively modulate the immune landscape. Tryptophan [8, 9], arginine [10], prostaglandin [11, 12], and adenosine [13] metabolism represent some of the most studied pathways actively contributing towards immune suppression. To begin to understand the relevance, these immuno-metabolic pathways may play a role in gliomagenesis, and we performed integrative, cross-platform analyses consisting of global metabolomics coupled with gene-expression profiling in patient-derived tumors. In addition to demonstrating clear metabolic reprogramming associated with these immune modulatory pathways in GBM, these comprehensive studies uncovered transcriptional programs designed to drive the observed metabolic phenotype, putative metabolic targets, and a framework for future studies designed to determine how these specific metabolic programs may actively influence the immune state.

Materials and methods

Human tumor samples and metabolomic profiling

Metabolomic profiling was performed in glioma using a combination of liquid chromatography/tandem mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) using a robust library of standards enriched with intermediates of tryptophan, arginine, prostaglandin, and adenosine metabolism, comparing patient-derived GBM ($n=80$) to low-grade astrocytoma (LGA; $n=28$). All tumors were newly diagnosed, fresh-frozen, and their integrity and histology confirmed by a staff pathologist [14]. Metabolomic studies were conducted at Metabolon (Morrisville, NC) and analyzed using methods previously described [14, 15].

Microarray and database analysis

The cancer genome atlas (TCGA) data for glioma were downloaded from http://xena.ucsc.edu. Genes associated with tryptophan, arginine, prostaglandin, and adenosine metabolism were identified using the kyoto encyclopedia of genes and genomes (KEGG) pathway database. mRNA expression data for these gene sets were compared between low-grade glioma (LGG) and GBM to generate log2 fold change (LGG at the baseline). Furthermore, Benjamini–Hochberg corrected p value < 0.05 ($-\log10$) between these two groups was used to generate volcano plots. Gene-expression profiling and molecular subtyping for O6-methylguanine–DNA methyltransferase (MGMT) promoter methylation and isocitrate dehydrogenase 1 (IDH1) mutation status were performed as previously described [16].

Cell culture and animal handling

Human GBM cell lines U251 and T98G were obtained and grown in conditions described previously [17]. The genetically engineered murine GBM TRP line and human GBM mesenchymal (MES83 and MES326) and proneural (PN19 and PN84) glioma stem cells (GSCs) were generated and

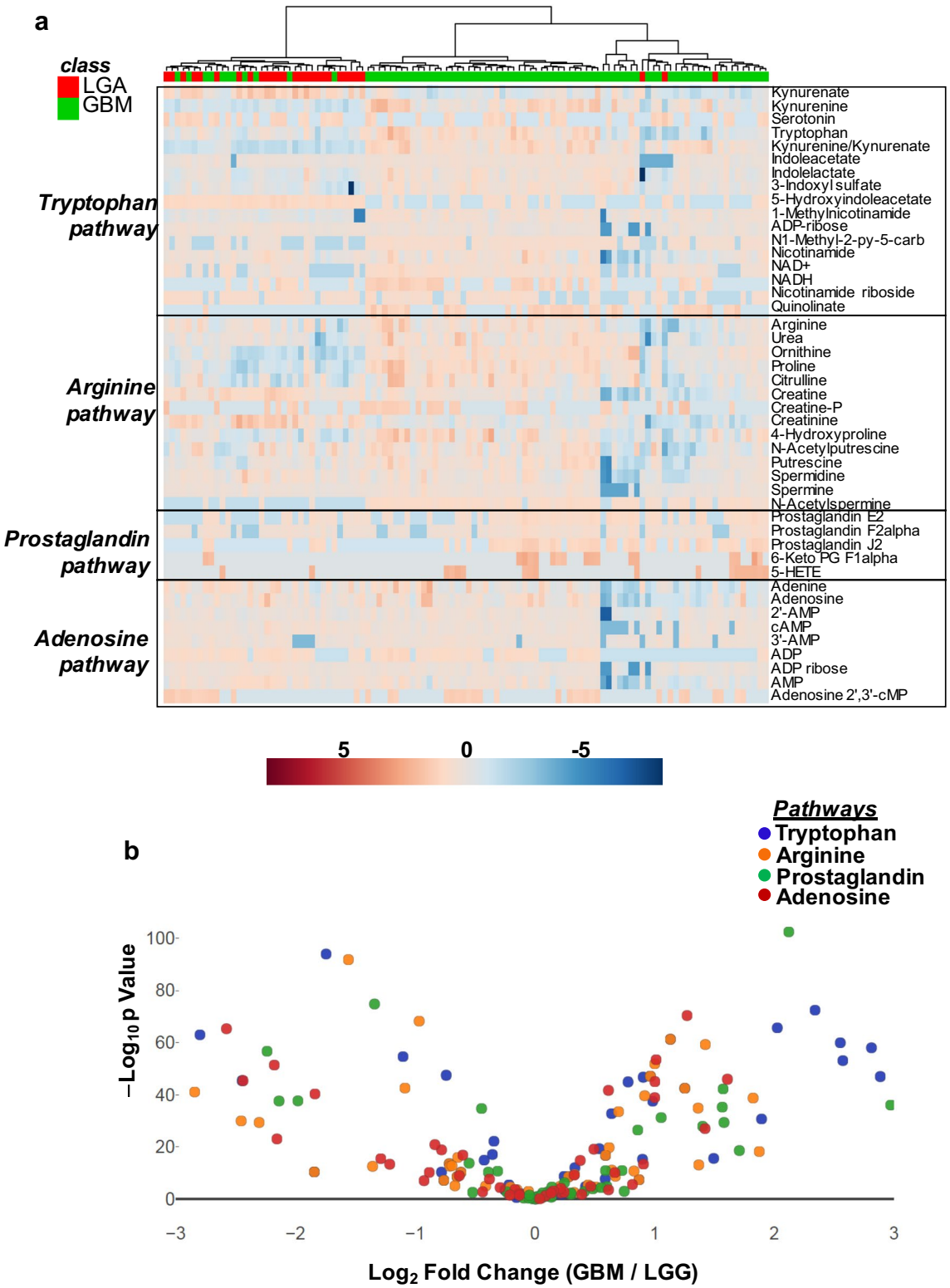


Fig. 1 Immuno-metabolic pathways are altered in GBM when compared to LGA. **a** Global metabolomic profiling was performed on patient-derived glioblastoma (GBM; $n=80$) and LGA ($n=28$) tumor samples using LG/GC–MS. Biochemicals involved in metabolic pathways with known immune consequence, including tryptophan, arginine prostaglandin, and adenosine metabolism, are presented as a heat map after normalization. **b** mRNA expression data for GBM and low-grade glioma (LGG) from the TCGA were used to analyze genes specific to tryptophan, arginine, prostaglandin, and adenosine metabolism. Differentially expressed genes ($p<0.05$) are presented as a volcano plot

grown as previously described [18, 19]. Flank and orthotopic tumor implants were performed as previously described [8].

Human PBMCs

Leukapheresis packs were obtained from healthy donors by Research Blood Components (Boston, MA) and PBMCs were isolated using Ficoll–Paque PLUS (GE Bio-Sciences, Pittsburgh, PA). PBMCs were activated for 3 days using plate-bound anti-CD3 (clone-OKT3) and anti-CD28 (clone-CD28.2) antibodies (Biolegend; San Diego, CA).

Kynurenine estimation

Mouse cells were cultured in the presence or absence of murine recombinant IFN-γ (100 ng/ml) and human cells were cultured in the presence of human recombinant IFN-γ (50 ng/ml) from Peprotech (Rocky Hill, NJ) for 3 days in the presence or absence of 1-methyl-L-tryptophan (Sigma-Aldrich, St. Louis, MO). Cell-culture supernatants were collected for kynurenine estimation using Ehrlich's reagent using methods previously described [20, 21].

Western blot

Western blot was performed using methods previously described [8]. Human indoleamine 2,3-dioxygenase (IDO1), tryptophan 2,3-dioxygenase-2 (TDO), prostaglandin I synthase (PTGIS), cyclooxygenase-1/2 (PTGS1/PTGS2), and β-actin antibodies were obtained from Cell-Signaling Technology (Boston, MA). Tryptophan 2,3-dioxygenase-2 (TDO2) antibody was obtained from Santa Cruz Biotechnology (Dallas, TX).

T-regulatory cell (Treg) polarization

$CD4^+$ T cells were isolated from C57BL/6 mouse splenocytes and purified using an untouched mouse CD4 cell-isolation kit (Invitrogen/Thermo Fisher). Cells were activated using plate-bound anti-CD3 (1 µg/ml; clone-145-2C11) and anti-CD28 (5 µg/ml; clone-37.51) antibodies (Biolegend) along with TGF-β1 (5 ng/ml) for 3 days, in ± kynurenine, and analyzed for Tregs ($CD45^+CD4^+FoxP3^+CD25^+$) using fluorochrome-conjugated antibodies (Biolegend).

M2-macrophage polarization and cell-suppression assay

Bone-marrow cells were isolated from the femur of C57BL/6 mice, polarized to an M2 macrophage phenotype in the presence of murine GM-CSF (20 ng/ml) for 6 days followed by murine IL-4 and IL-13 (20 ng/ml) (Peprotech) and analyzed for polarization with fluorochrome-conjugated antibodies, $CD45^+CD68^+CD11b^+F4/80^+$ (Biolegend). $CD8^+$ T cells were isolated from C57BL/6 mouse splenocytes and purified using an untouched mouse CD8 cell-isolation kit (Invitrogen/Thermo Fisher). $CD8^+$ T cells were stained with CellTrace (CFSE; GIBCO/Thermo) and activated with plate-bound anti-CD3 (1 µg/ml; clone-145-2C11) and anti-CD28 (5 µg/ml; clone-37.51) (Biolegend). Polarized M2 cells were added at indicated ratios ± adenosine to evaluate their capacity to suppress $CD8^+$ T-cell proliferation using CFSE dilution.

CIBERSORT

Normalized gene-expression data from GBM were used for performing cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) allowing the identification of 22 immune subtypes within a tumor through a deconvolution algorithm that uses a set of signature gene-expression values specific for these immune subsets [22].

Fig. 2 Tryptophan metabolism in GBM. **a** Schematic of tryptophan (TRP) metabolism. Red indicates metabolites upregulated in GBM when compared to LGA. Green indicates metabolites downregulated in GBM; brown indicates that the metabolite was analyzed, but was not significantly different. Metabolites in black were not detected or analyzed. Numbers in bracket demonstrate fold difference between GBM and LGA. **b** Hierarchical clustering using metabolites specific to tryptophan metabolism was performed on molecularly subtyped, patient-derived GBM ($n=56$), resulting in two distinct clusters defined as TRP-High (TRP-H) and TRP-Low (TRP-L). **c** Gene-expression profiles of GBM defined as TRP-H and TRP-L were analyzed using CIBERSORT. $*p<0.05$. **d** Described cell lines were cultured with ± 100 ng/ml human IFN-γ or 100 ng/ml human IFN-γ and 100 µM of 1-methyl-L-tryptophan (1-L-MT) for 3 days and analyzed for IDO1 and TDO2 expression. Supernatant from this cell culture was used for kynurenine estimation. **e** Described cell lines were cultured with ± 100 ng/ml human IFN-γ for 3 days and analyzed for genes involved in tryptophan metabolism. Comparisons were made to naïve and activated PBMCs

Results

To begin to understand how metabolic reprogramming may contribute towards the immune-suppressive microenvironment observed in GBM, metabolomic profiling was performed on patient-derived gliomas, comparing low-grade astrocytoma (LGA; $n=28$) with GBM ($n=80$), specifically focusing on metabolites/metabolic pathways implicated in immune tolerance, which included tryptophan, arginine, prostaglandin, and adenosine metabolism [7]. A total number of 55 biochemicals involved in these described metabolic pathways were identified in human tumors and 52% demonstrated differential accumulation between LGA and GBM. Hierarchical clustering performed on this focused panel of metabolites resulted in a clear separation between LGA and GBM (Fig. 1a). Consistent with metabolomic findings,

clear separation between LGA and GBM was observed when evaluating the expression of genes involved in these metabolic pathways using the TCGA, supporting the potential role of these metabolic pathways in gliomagenesis (Fig. 1b; Supplementary Table 1). Using integrative analyses, we went on to metabolically, molecularly, and functionally to define these metabolic pathways in further detail.

Tryptophan metabolism. Tryptophan can be metabolized to kynurenine, which is driven by the rate-limiting enzymes IDO/TDO. Kynurenine can then be exported to the microenvironment by tumors, contributing towards immune suppression at many levels, with its most notable role in contributing towards materno-fetal immune tolerance [23–25]. Although several recent studies have implicated these enzymes and/or kynurenine in gliomagenesis [8, 9, 26], its intermediary metabolism has not been studied in detail. When compared to LGA, in addition to an expected accumulation of tryptophan, kynurenine, and its biosynthetic enzymes IDO1/TDO [7], an accumulation of several indoles was observed in GBM (Fig. 2a). Similar to kynurenine, indoles appear to have the capacity of activating the aryl hydrocarbon receptor (AHR) [27] and, therefore, may have immune modulatory roles [26]. However, the enzyme-linking indoles with tryptophan metabolism are not typically present in humans [27]; therefore, the specific metabolic pathways contributing to the presence of these metabolites in the tumor microenvironment and their biologic consequence remains unclear. A particularly striking finding these integrative analyses uncovered was a clear shift towards the downstream metabolism of kynurenine in GBM. Most notable was an activation of the de novo NAD+ biosynthetic pathway, including an accumulation of the metabolites quinolinic acid and NAD+ that were coupled to several of the biosynthetic enzymes involved in this pathway.

Despite clear clustering when compared to LGA, considerable metabolic heterogeneity was still observed within GBM. Therefore, we sought to both define this metabolic heterogeneity and understand its molecular context. Of the 80 GBM specimens that were metabolomically profiled, 56 had additional tissue available to allow for cross-platform genomic/transcriptional analyses. Hierarchical clustering performed using metabolites specific to tryptophan metabolism in these tumors identified two subtypes defined as tryptophan 'high' and 'low' (Fig. 2b). Next, we evaluated gene-expression profiles of these two metabolic subtypes to provide molecular context to the observed metabolic heterogeneity. Consistent with the integrative analysis comparing GBM with LGA, IDO1 emerged as the top gene separating tryptophan 'high' and 'low' GBM on VIP analysis (Supplementary Fig. 1a). Interestingly, these studies also identified quinolinate phosphoribosyltransferase (QPRT) expression to be a central mediator driving this metabolic phenotype, further supporting the relevance of the downstream metabolism of tryptophan in GBM.

We next sought to determine if the observed metabolic heterogeneity of tryptophan metabolism in GBM could be a direct consequence of established molecular subtypes in this malignancy. To accomplish this, we performed cross-platform analyses using RNA and DNA isolated from the 56 samples, where a matched aliquot of tumor tissue was available. MGMT promoter methylation status and IDH1 mutation represent two of the strongest prognostic factors in GBM [28, 29]. We, therefore, went on to determine if these molecular subtypes differentially co-opted tryptophan metabolism to modulate the immune response. Although IDH1 and MGMT methylation status did not appear to correlate with the observed metabolic phenotype when transcriptional profiles were molecularly subtyped [30], the immuno-metabolic phenotype of tryptophan metabolism was unique to mesenchymal and classical subtypes of GBM (Fig. 2b).

To determine if tryptophan metabolism influenced the immune landscape in GBM, immune phenotypes were defined using transcriptional profiles generated from individual tumors and analyzed using CIBERSORT [22], allowing for in silico cell sorting of specific immune components. Integrative analyses coupling these metabolomic signatures with specific cellular immune subsets suggested that tryptophan metabolism contributes towards an immunosuppressive phenotype in GBM, with significantly higher levels of Tregs and M0 macrophages and lower levels of memory T cells. In addition, a trend in diminished CD8 cells was observed in tryptophan 'high' tumors (Fig. 2c). To functionally extend findings linking kynurenine metabolism with the accumulation of Tregs in GBM, we sought to determine if kynurenine contributed towards Treg polarization. As demonstrated in Supplementary Fig. 2, $CD4^+$ T cells isolated from murine splenocytes demonstrated a 44% increase in Treg polarization when cultured in the presence of kynurenine.

Finally, we sought to determine if this immune-metabolic phenotype was recapitulated in preclinical models. Although the activation of the IDO1/TDO pathway has been previously described in GBM [8, 26], a unique finding our integrative analysis uncovered was that this phenotype was specific to the mesenchymal/classical subtypes of GBM. Using subtype-specific GBM lines [18, 31], consistent with our findings involving patient-derived tumors, we were able to demonstrate that upon IFN-γ induction, mesenchymal lines demonstrated increased expression of IDO1 and kynurenine accumulation (Fig. 2d). Another novel finding from our studies was the observation of continued metabolism downstream of kynurenine in GBM. However, it remains unclear whether this occurs in tumor cells or a component of the intermediary metabolism of immune cells within the tumor microenvironment [32, 33]. We, therefore, extended our studies to evaluate its downstream metabolism. As an initial investigation, we determined the expression level of kynurenine 3-mono-oxygenase (KMO), kynureninase (KYNU), and QPRT in our preclinical models, which represent aberrantly expressed enzymes involved in the downstream metabolism of kynurenine (Fig. 2a). Consistent with clinical data, mesenchymal lines demonstrated

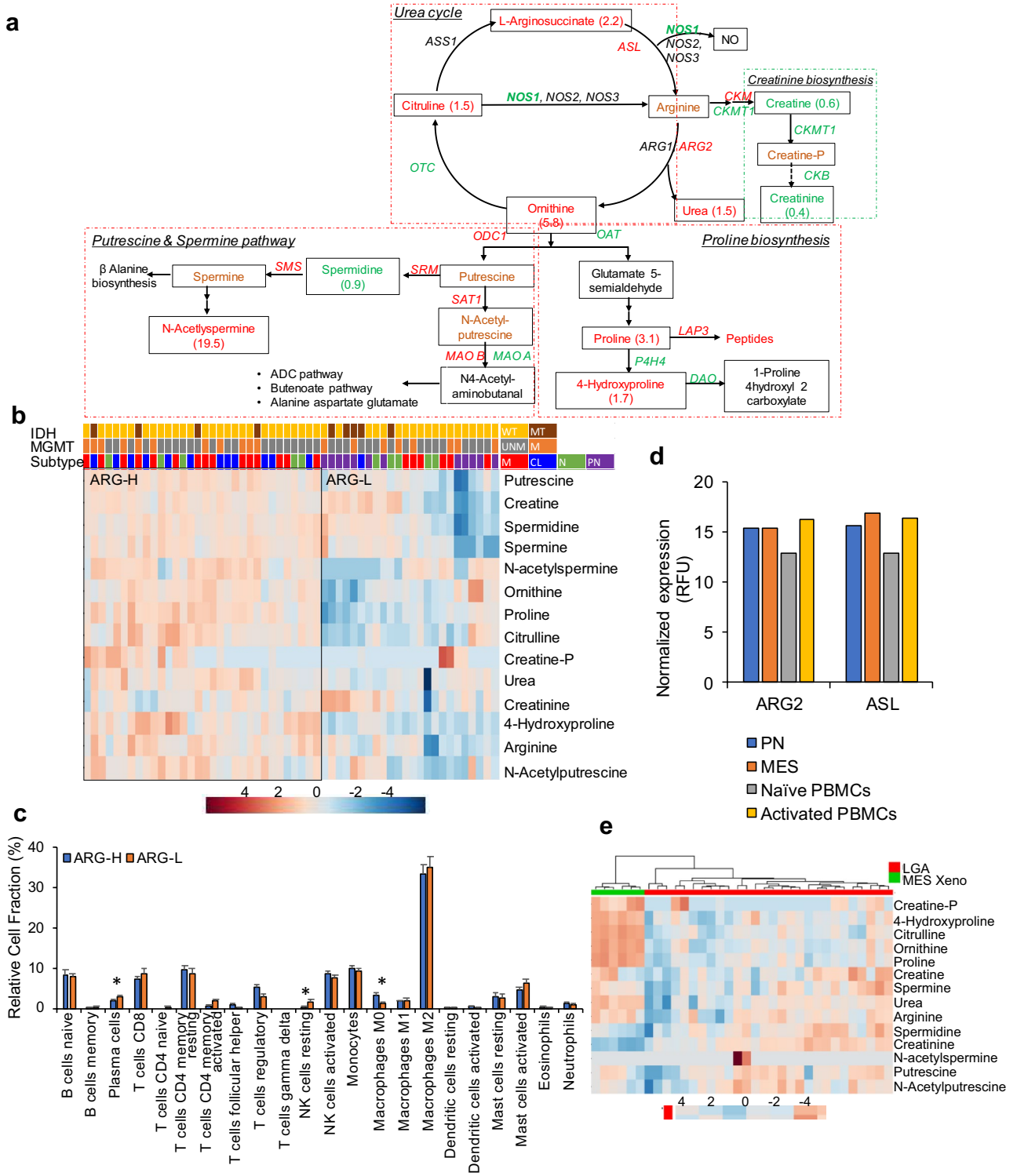


Fig. 3 Arginine metabolism in GBM. **a** Schematic of arginine metabolism. Red indicates metabolites upregulated in GBM when compared to LGA. Green indicates metabolites downregulated in GBM when compared to LGA; brown indicates that the metabolite was analyzed, but was not significantly different. Metabolites in black were not detected or analyzed. Numbers in bracket demonstrate fold difference between GBM and LGA. **b** GBM ($n=56$) with known molecular subtype, MGMT methylation status, and IDH1 mutation status was clustered using arginine pathway metabolites. Two distinct clusters were defined as ARG-High (ARG-H) and ARG-Low (ARG-L). **c** CIBERSORT analysis for 22 immune subsets was performed comparing the immune phenotype of ARG-H and ARG-L tumors. *$p<0.05$. **d** Mesenchymal (MES83 and MES326) and proneural (PN19 and PN84) GBM tumor lines were analyzed for genes involved in arginine metabolism and compared with naïve and activated PBMCs. **e** MES orthotopic tumor xenografts in nude (nu/nu) mice (green; $n=6$) were analyzed for arginine pathway metabolites using global metabolomic profiling and compared with LGA (red; $n=28$)

increased expression of all three enzymes when compared to proneural, and importantly, their relative expression was consistent with the rate-limiting enzyme IDO1 (Fig. 2e). To further explore the potential role immune cells may play a role in the downstream metabolism of kynurenine, we extended these investigations to both naïve and active human PBMCs. Increased expression of interleukin 2 receptor-subunit alpha (IL2R-α) and TNF-α in activated PBMCs served as a positive control. Although QPRT expression was diminished in PBMCs, the remainder of the enzymes displayed equivalent expression when compared to mesenchymal lines. Based on these findings, both tumor cells and immune cells appear to have a similar ability for downstream metabolism of kynurenine. Further studies designed to delineate the intermediary metabolism of kynurenine in individual cell types and the resulting immune and/or non-immune consequences are warranted.

Arginine metabolism. Arginine represents an important substrate utilized for ornithine and urea production, which contribute towards M2 macrophage-mediated immune suppression [7]. In addition to an accumulation of both ornithine and urea in GBM, integrative analyses identified additional aspects of arginine metabolism to potentially be relevant in gliomagenesis (Fig. 3a). In addition to an accumulation of metabolites central to arginine synthesis, similar to tryptophan metabolism, a particularly striking finding was an accumulation of biochemicals downstream of arginine, including spermine and proline. Accordingly, aberrant expression of several genes specific to the metabolism of these biochemicals was observed in GBM, including increased expression of enzymes associated with arginine synthesis and metabolism, argininosuccinate lyase (ASL) and arginase-2 (ARG2), respectively, and its further catabolism along the proline and spermine pathways.

Similar to our approach evaluating the kynurenine pathway, we went on to evaluate for heterogeneity of arginine metabolism in GBM, define genomic programs driving this metabolic phenotype, and determine its immune consequence. Hierarchical clustering of metabolites involved in arginine metabolism identified both arginine 'high' and 'low' subtypes (Fig. 3b). Interestingly, similar to what was observed with tryptophan metabolism, mesenchymal and classical molecular subtypes, rather than MGMT methylation or IDH1 mutation status, appeared to display aberrant arginine metabolism in GBM. In addition to upregulation of the arginine synthetic enzyme ASL, decreased expression of enzymes involved in alternate metabolic pathways of arginine, including nitric oxide synthase 1 (NOS1) and creatine kinase mitochondrial 1A (CKMT1), ranked as the most important genes defining this metabolic pathway (Supplementary Fig. 1b). The immune phenotype of arginine metabolism consisted of significant increases in M0 macrophages and a trend in Treg populations (Fig. 3c).

Fig. 4 Prostaglandin metabolism in GBM. **a** Schematic of prostaglandin metabolism. Red indicates metabolites upregulated in GBM when compared to LGA. Green indicates metabolites downregulated in GBM; brown indicates that the metabolite was analyzed, but was not significantly different between GBM and LGA. Metabolites in black were not detected or analyzed. Numbers in bracket demonstrate fold difference between GBM and LGA. **b** GBM ($n=56$) with known tumor subtype, MGMT methylation, and IDH1 status were clustered using prostaglandin pathway metabolites. Two distinct clusters were labeled as PG-High (PG-H) and PG-Low (PG-L). **c** 56 GBM tumors divided into PG-H and PG-L and used for CIBERSORT analysis for 22 immune subsets. $^*p<0.05$. **d** Described cell lines were analyzed for enzymes central to prostaglandin metabolism by western blot. **e** MES orthotopic tumors xenografts in nude (nu/nu) mice ($n=6$) were analyzed for prostaglandins using global metabolomic profiling and compared with LGA tumor metabolite data

As was noted in studies involving tryptophan metabolism, it is unclear if metabolomic profiles generated from patient-derived tumors are reflective of the tumor itself or that of intra-tumoral immune cells. We, therefore, extended our investigations to preclinical models to better define these pathways and their relevance to gliomagenesis. As an initial investigation, we evaluated the expression of enzymes involved in arginine metabolism in our preclinical models to provide insight into its intermediary metabolism, focusing specifically on the arginine synthetic and catabolic enzymes ASL and ARG2, respectively. Unlike findings involving tryptophan metabolism, differential expression was not observed between mesenchymal and proneural GBM cell lines or in PBMCs (Fig. 3d). In addition, other than an accumulation of spermidine and proline in mesenchymal cells, pathway activation did not appear to be recapitulated in preclinical models when metabolomically evaluated in vitro Supplementary Fig. 3. Conversely, accumulation of metabolites was observed in vivo when grown in an immune deficient model and compared to LGA (Fig. 3e), further supporting the relevance of this pathway in GBM and suggesting that (1) tumor cells have the capacity to independently metabolize arginine to its downstream intermediaries and (2) pathway activation may be stimulated by factors associated with the unique microenvironment in these tumors.

Prostaglandin metabolism. Prostaglandins are derived from the metabolism of arachidonic acid, which is driven by cyclooxygenase (COX or prostaglandin-endoperoxidase synthase [PTGS]). Prostaglandin-E2 (PGE2) represents one of the most well-studied downstream metabolic intermediaries of this pathway and a key mediator in immunopathology, regulating inflammation at many levels [11, 12]. PGE2 has a paradoxical role of both promoting active inflammation but also shifting from an anti-tumor to an immunosuppressive response within the tumor microenvironment. Metabolomic profiling uncovered several aspects of prostaglandin metabolism unique to GBM. Although the overexpression of PTGS2 (COX2) has been described in numerous

malignancies, including GBM, an accumulation of its resultant metabolites, PGG2 and PGH2 were not detected in our studies (Fig. 4a). However, interestingly, increased activity downstream of this metabolic node was clearly present in GBM, including the accumulation of PGE2, PGF2a, and 6-keto-PGF1a, along with increased expression of their biosynthetic enzymes (PTGES, CBR1, CBR3, and PTGIS). Beyond the generation of prostaglandins, additional aspects

of arachidonic acid metabolism were also uncovered through these integrative analyses, including metabolic programs designed to generate this biochemical through phospholipid metabolism and alternate modes of its subsequent metabolism through the eicosanoid pathway.

Unlike studies involving both tryptophan and arginine phenotypes, metabolites associated with prostaglandin metabolism were unevenly distributed, with only a small proportion of tumors (14/56) defined as 'high' and integrative analyses did not identify specific molecular subtypes or genetic programs (Fig. 4b, Supplementary Fig. 1C) that may be driving this phenotype or clear changes in the immune landscape relative to its metabolism (Fig. 4c). We went on to determine if these findings were recapitulated in preclinical models. In addition to validating increased expression of PTGIS in mesenchymal lines and established human GBM cell lines, we also uncovered an unexpected correlation with the expression levels of PTGS1 and PTGS2. Specifically, increased expression of PTGIS appeared to be consistent with increased PTGS1 expression, yet these were inversely related to PTGS2 expression (Fig. 4d). Therefore, the interplay of PTGS1 and PTGS2 and their role in the potential differential metabolism of prostaglandins in GBM is worthy of further investigation. In addition, findings were supported by metabolomic profiling of mesenchymal GBM cells grown in vivo, demonstrating accumulation of prostaglandin E2, J2, and 6-ketoprostaglandin F1-α (Fig. 4e).

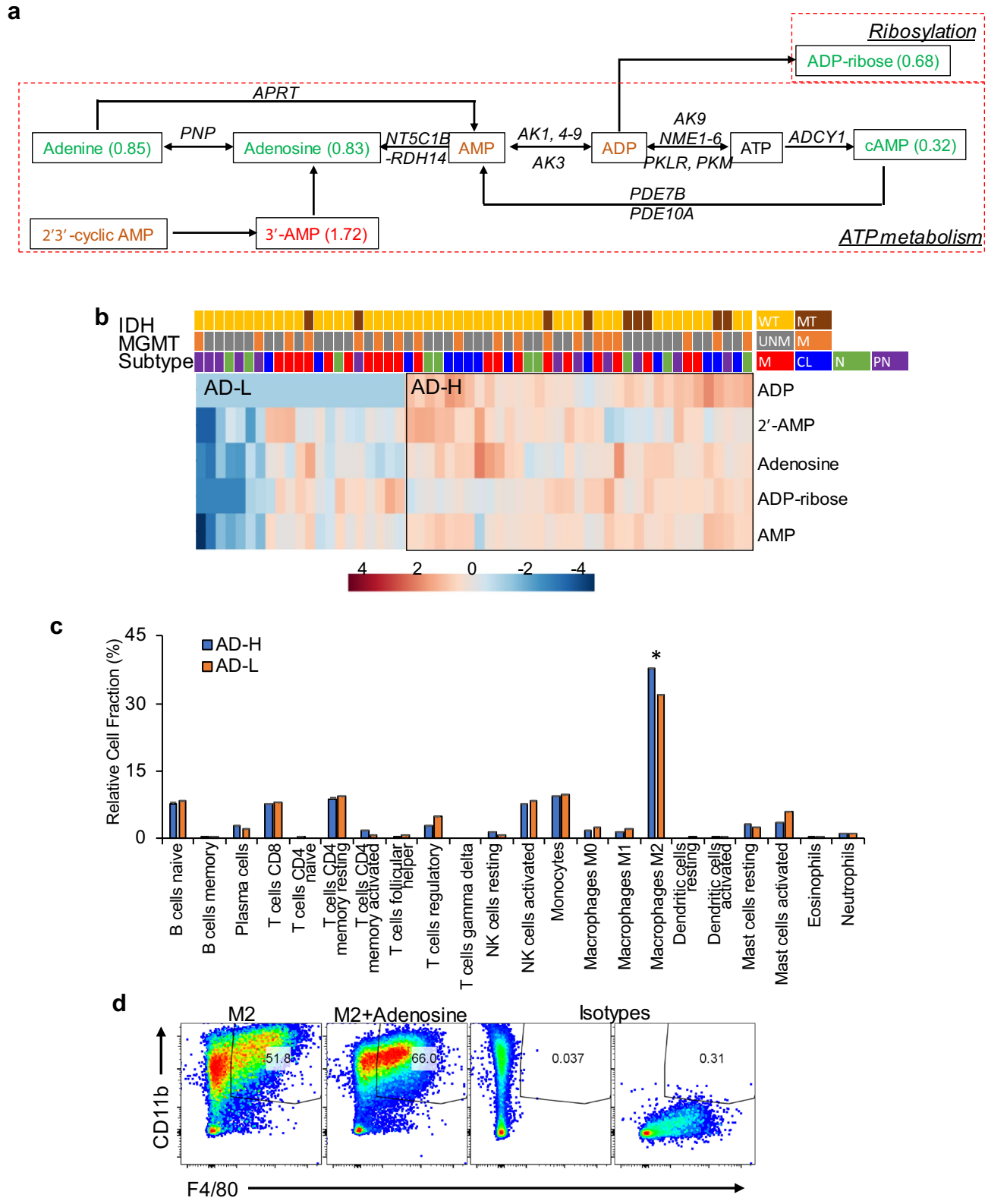
Adenosine metabolism. Although we did not demonstrate an accumulation of adenosine in GBM when compared to LGA (Fig. 5a), considerable heterogeneity was observed. We, therefore, further evaluated differential adenosine metabolism in GBM and its possible immune consequence. Approximately 2/3 of the tumors were designated as adenosine "high" and this metabolic phenotype did not appear to be specific to established molecular subtypes (Fig. 5b). However, adenosine "high" tumors did appear to confer an immune-suppressive phenotype, consisting of an accumulation of M2 macrophages when analyzed by CIBERSORT (Fig. 5c). To functionally extend findings linking adenosine metabolism with the accumulation of M2 macrophages in GBM, we sought to determine if this metabolic pathway contributed towards M2 polarization and functional suppression. Murine bone-marrow-derived macrophages demonstrated both an increase in M2 polarization (Fig. 5d) and functional suppression of CD8 cells (Supplementary Fig. 4) when cultured in the presence of adenosine. However, similar to what was observed in tumors, an accumulation of adenosine was not observed in our preclinical models in vitro or in vivo Supplementary Fig. 5A/B. Therefore, further investigation is required to better determine if this metabolic pathway plays a role in gliomagenesis.

Fig. 5 Adenosine metabolism influences the induction of M2-like macrophages in GBM. **a** Schematic of adenosine metabolism. Red indicates metabolites upregulated in GBM when compared to LGA. Green indicates metabolites downregulated in GBM; brown indicates that the metabolite was analyzed but was not significantly different between GBM and LGA. Metabolites in black were not detected. Numbers in bracket demonstrate fold difference between GBM/LGA. **b** GBM ($n=56$) with known tumor subtype, MGMT methylation, and IDH1 status were clustered using the top 5 expressed adenosine pathway metabolites. Two distinct clusters were defined as AD-High (AD-H) and AD-Low (AD-L). **c** GBM ($n=56$) tumors were defined as AD-H and AD-L and their expression profiles were analyzed by CIBERSORT to define immune subsets. **d** Murine bone-marrow-derived macrophages were polarized to the M2 phenotype and analyzed for CD11b and F4/80. This experiment has been replicated four times with similar results. $^{*}p<0.05$

Discussion

This study represents one of the first of its kind to perform integrative analyses coupling metabolomics and expression profiling to comprehensively define the interface between metabolic reprogramming and immune response in GBM. Through these studies, both tryptophan and arginine metabolism emerged as metabolic pathways particularly relevant in gliomagenesis. Numerous studies have identified adenosine as a potent anti-inflammatory molecule involved in the restoration of tissue homeostasis through the modulation of the innate and adaptive immune response [34, 35]. Extracellular adenosine levels are typically very low; however, necrotic tumors generate high levels of extracellular adenosine, which is metabolized through the dynamic and sequential actions of cell-surface enzymes (ectoenzymes), thereby contributing to an immune-suppressive state [36–38]. Surprisingly, these studies did not identify the accumulation of the potent immune-suppressing metabolite adenosine in GBM when compared to LGA. Moreover, this also appears to contradict a recent report, suggesting that this pathway is activated, including the expression of the key ectoenzymes associated with its formation, and targetable in GBM [39]. As our integrated analyses only provide a static metabolic picture, one possibility is that this metabolite is rapidly metabolized following activation. Therefore, further work designed to carefully define the intermediary metabolism of this pathway is required to better determine its potential role in gliomagenesis.

Gene-expression profiles identified numerous genes that were significantly either up- or downregulated within each metabolic pathway (Fig. 1b, Supplementary Table 1). Although the expression of many of these genes still requires validation when evaluated in the context of their metabolic profiles, these findings provide a window into potential mechanisms contributing towards individual phenotypes. For example, decreased expression of a specific enzyme



may drive metabolism towards a more 'oncogenic' upstream or parallel pathway. Furthermore, particularly relevant to prostaglandin metabolism in this study, several isoforms of an individual enzyme were identified to be differentially expressed. These findings may stimulate further investigations designed to determine the affinity of individual

isoforms to a given substrate and how this may lead to metabolic reprogramming.

One notable finding these integrative analyses offered was that although many of the observed immuno-metabolic phenotypes appeared to be independent of MGMT promoter methylation and IDH1 mutation status, two of the strongest prognostic factors in GBM, they were enriched in the mesenchymal and classical transcriptional subtypes. Interestingly, rather than representing inter-tumoral heterogeneity, we have recently demonstrated that these molecular subtypes reflect intra-tumoral heterogeneity in GBM, with mesenchymal and classical subtypes enriched in perinecrotic regions within an individual tumor [40]. We, therefore, hypothesize that the observed immuno-metabolic subtypes in GBM are a direct consequence of the diverse tumor microenvironment in this heterogeneous malignancy.

A common theme that emerged from these comprehensive metabolomic studies was an enrichment of numerous biochemicals' downstream of the established metabolic pathways in GBM. For example, although tryptophan metabolism and kynurenine accumulation have been previously described in GBM, this study represents one of the first to delineate the potential relevance of its intermediary metabolism, including an accumulation downstream metabolites involved in de novo NAD biosynthesis and salvage. Downstream of ornithine and urea, proline, and spermine metabolism was enriched in the arginine pathway. In addition, eicosanoid metabolism, along with several other biochemicals' downstream of prostaglandins, accumulated in GBM that appeared to be recapitulated in preclinical models. This suggests that targeting this metabolic pathway may be extended beyond COX inhibitors, including studies designed to test the novel targets PTGIS and PTGS1. Therefore, these findings provide the framework for a series of investigations designed to better define the intermediary metabolism of these individual pathways and their potential to be targeted.

Another novel aspect of these studies is that it allows coupling of metabolomic signatures with CIBERSORT analyses of gene-expression profiles, which defines immune-cell populations within an individual tumor, providing a previously undescribed window into the immune consequences of a given metabolic phenotype. Of the metabolic pathways evaluated, tryptophan metabolism appeared to play the most dominant role in immune tolerance in GBM, with over a twofold increase in Tregs and a strong trend in decreased $CD8^+$ T cells. These tumors also demonstrated a significant reduction in natural killer cells (NK cells), which are important for innate immune response. These results suggest that an accumulation of tryptophan-related metabolites inhibits both innate and acquired response resulting in mitigating anti-tumor immunity. Collectively, however, the immune landscape of GBM appears to be primarily influenced by M2 macrophages, which represent nearly 40% of the immune-cell population. M2 macrophages are anti-inflammatory, cytokine producing macrophages and aid in tumor angiogenesis and progression through immune suppression. This suggests that concerted efforts designed to target these immune-suppressive cells may revert the potent immune tolerance observed in this malignancy. Surprisingly, ARG-high tumors also demonstrated very similar results, with a reduction in both plasma cells and NK cells. Tumors with increased tryptophan and/or arginine metabolism appeared to have higher levels of resting M0 macrophages; however, the immune consequence of this finding remains unclear. Although an accumulation of adenosine did not appear to differentiate GBM from LGA, the subtype of increased adenosine metabolism within GBM was the only identified metabolic program associated with increased M2 macrophages. These findings support a potential immune consequence of this metabolic pathway and, again, reinforce more focused investigations to better understand its intermediary metabolism and immune consequence.

In summary, these comprehensive, integrative analyses provide insight into how metabolic remodeling contributes towards an immune-suppressive phenotype in GBM. As our understanding of how tumor-specific metabolic programs contribute to immune suppression grows, this database can be utilized to determine their role in gliomagenesis. Furthermore, by extending these investigations into preclinical models, this work provides the framework for future studies designed to define the intermediary metabolism of these pathways in further detail, evaluate their role in immune tolerance, and determine their therapeutic implications in this aggressive malignancy.

Author contributions Study design: PK and PC; experiments: PK, AP, and SK; data analysis: PK and PC; reagents: PC; and manuscript preparation: PK and PC.

Funding This work was supported by the National Institute of Health (NIH)/National Institute of Neurological Disorders and Stroke (NINDS) (R21NS090087), American Cancer Society (RSG-11-029-01), Bankhead-Coley Cancer Research Program and Cancer Research Seed Grant Awards from Beaumont Health to Prakash Chinnaiyan.

Compliance with ethical standards

Conflict of interest The authors declare that they have no potential conflict of interest.

Ethical approval GBM/glioma tissue samples were obtained from the Moffitt Cancer Center Tissue Core Facility. Institutional Review Board/Human Subjects approval (MCC16197) was obtained for this retrospective study from the ethics committee of the Moffitt Cancer Center. All animal studies were carried out under protocols approved by the IACUC (AL-16-09 and AL-18-10) at William Beaumont Research Institute.

Informed consent Patients gave written informed consent for the use of their specimens and clinical data for research and publication prior to surgical resection or following diagnosis under the Total Cancer Care Tissue Repository program at the Moffitt Cancer Center.

Animal source C57BL/6 (H-2b, CD45.2) and athymic nu/nu (NU-*Foxn1*^{nu}) mice were purchased from Charles River Laboratories (Wilmington, MA).

Cell line authentication Human GBM cell lines U87, U251, and T98G were purchased from ATCC and authenticated by STR analysis at The University of Arizona Genetics Core. MES83, MES326, PN19, and PN84 were generated, obtained, and authenticated by Dr. Ichiro Nakano's lab at Ohio State University. Murine TRP cell lines were generated, obtained, and authenticated by Dr. C. Ryan Miller's lab at The University of North Carolina School of Medicine.

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