




BRIEF REPORT

Preclinical efficacy of immune-checkpoint monotherapy does not recapitulate corresponding biomarkers-based clinical predictions in glioblastoma

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ABSTRACT

Glioblastoma (GBM) is resistant to most multimodal therapies. Clinical success of immune-checkpoint inhibitors (ICIs) has spurred interest in applying ICIs targeting CTLA4, PD1 or IDO1 against GBM. This amplifies the need to ascertain GBM's intrinsic susceptibility (or resistance) toward these ICIs, through clinical biomarkers that may also “guide and prioritize” preclinical testing. Here, we interrogated the TCGA and/or REMBRANDT human patient-cohorts to predict GBM's predisposition toward ICIs. We exploited various broad clinical biomarkers, including mutational or predicted-neoantigen burden, pre-existing or basal levels of tumor-infiltrating T lymphocytes (TILs), differential expression of immune-checkpoints within the tumor and their correlation with particular TILs/Treg-associated functional signature and prognostic impact of differential immune-checkpoint expression. Based on these analyses, we found that predictive biomarkers of ICI responsiveness exhibited inconsistent patterns in GBM patients, i.e., they either predicted ICI resistance (as compared with typical ICI-responsive cancer-types like melanoma, lung cancer or bladder cancer) or susceptibility to therapeutic targeting of CTLA4 or IDO1. On the other hand, our comprehensive literature meta-analysis and preclinical testing of ICIs using an orthotopic GL261-glioma mice model, indicated significant antitumor properties of anti-PD1 antibody, whereas blockade of IDO1 or CTLA4 either failed or provided very marginal advantage. These trends raise the need to better assess the applicability of ICIs and associated biomarkers for GBM.

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Introduction


Glioblastoma (GBM), a sub-type of high-grade glioma, is an aggressive type of cancer afflicting the central nervous system (CNS) (associated with very low median survival of 12–15 mo).^{1,2} GBM is resistant to most existing multimodal therapies.^{3–5} Nearly 160 FDA-approved anti-GBM drugs have been applied, yet only a few are implemented as standard-of-care (e.g., temozolomide, since 2005).² Current GBM treatment paradigm consists of maximal surgical resection followed by radiotherapy *plus* temozolomide.² However, even this only marginally improves the prognosis of GBM patients.^{3–5} This disturbingly negative situation advocates application of novel anti-GBM therapies.

Anticancer immunotherapy especially immune-checkpoint inhibitors (ICIs), have shown great promise against aggressive cancers like melanoma and lung cancer that had otherwise failed to sufficiently respond to conventional therapies.^{2,6} Immune-checkpoints like Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4), Programmed Cell Death 1 (PD1) and Indoleamine 2,3-Dioxygenase 1 (IDO1) primarily aim to avoid autoimmune

reactions and hence typically function to inhibit T cell effector responses (including anticancer T cell immunity).^{7,8} Thus, ICIs help “revive” anticancer immunity by blocking these checkpoints.^{2,6} Of note, while CTLA4 and PD1 are mainly expressed on T cells, yet IDO1 can be derived from multiple sources, including cancer cells, innate immune cells and stromal cells.⁷

Past research has revealed GBM's relative susceptibility to highly efficacious immunotherapies like oncolytic viruses or dendritic cell (DC)-based vaccines^{5,9,10} – a major motivation behind using anti-CTLA4 antibodies (Abs), anti-PD1 Abs or IDO1 inhibitors against GBM.^{2,11} It has been reported that IDO1 can be upregulated during gliomagenesis thereby making it an attractive target for GBM immunotherapy.¹² On the other hand, studies on neuronal autoimmune disorders have shown that the CNS particularly exploits the PD1-axis for maintaining immune-tolerance.² Hence, anti-PD1 Ab is currently being prioritized for GBM immunotherapy.² In fact the anti-PD1 Abs (e.g., NCT02085070, NCT02337491, NCT01952769) and anti-CTLA4 Abs (e.g., NCT01950195, NCT01703507, NCT02107755, NCT02097732,

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NCT02115139) are currently being tested, also as monotherapies, against GBM in various phase I/II/III clinical trials.²

Therapeutically challenging cancer-types like melanoma have responded to ICI monotherapy in a remarkable fashion, both in preclinical and clinical settings.^{13,14} To this end, it is necessary to identify whether (and to what extent) GBM responds to ICI monotherapy; and whether such responsiveness can be predicted by broad clinical biomarkers. This can help understand whether GBM exhibits pre-existing (intrinsic) susceptibility to ICIs like melanoma or lung cancer. These trends would not only help to delineate the most suitable subset of patients to be treated with ICIs, but also those that should be avoided (owing to the severity of possible autoimmune toxicities) or treated with additional therapies to augment ICI's impact. But, while the clinical results of ICIs against GBM are awaited yet some preclinical studies have presented contradictory results, reporting both success¹⁵ and complete failure¹⁶ of anti-CTLA4 or anti-PD1 mono-immunotherapies.

Recently, various predictive biomarkers of ICI responsiveness have been delineated that can help in broadly predicting whether GBM could be susceptible to ICI monotherapy.⁶ Such broad predictive biomarkers include (but are not limited to) the following:^{6,17,18} (1) overall mutational burden, which is a surrogate marker for neoantigen burden (neoantigen-specific T cells are particularly active in tumors responding to ICIs thereby making high mutational/neoantigen burden crucial for ICI responsiveness), (2) differential expression of immune checkpoints, (3) pre-existing or basal levels of tumor-infiltrating T lymphocytes (TILs), (4) correlation of immune-checkpoint expression with particular TILs/Treg-associated polarization or effector function markers and (5) prognostic impact of differential immune-checkpoint expression.^{2,6,17,18} In terms of consistency, current clinical data shows that high pre-existing/basal density of TILs and high mutational/neoantigen burden together predict positive responsiveness to ICIs.^{2,6} These biomarkers are considered to be resulting from prolonged carcinogenic insults and mutagenic clonal evolution.^{6,17,18} Carcinogenic insults, in particular, are considered to be the predominant source of high mutational burden (i.e., non-synonymous somatic single-nucleotide variations).^{19,20} In fact, some recent genetic analyses have shown that specific mutagens or carcinogens induce distinct mutational lesions in particular cancer types²¹ like mutational signatures 4 and 29 (e.g., C>A or CC>AA mutations induced by tobacco mutagens, hence showing high presence in smokers and/or those who chew tobacco), signature 7 (e.g., CC>TT mutations induced by ultraviolet radiation), signature 22 (e.g., T>A mutations induced by aristolochic acid) and signature 24 (e.g., C>A mutations induced by aflatoxin).²¹ In line with this, melanoma (SKCM), lung cancer (LUAD/LUSC) and bladder cancer (BLCA) represent one of the most ICI-responsive cancers; since a sizeable subset of patients of these cancer types possesses high mutational/neoantigen burdens and high pre-existing TILs.^{2,6}

Of note, anti-PD1 Abs have shown promising results in patients with “hypermutant-GBM” (i.e., a pediatric-GBM

“sub-type” with high mutational burden resulting from biallelic mismatch repair deficiency or bMMRD).²² However, it is necessary to consider that hypermutant GBM has the highest known mutational/neoantigen burden of all human cancers (even higher than melanoma and lung cancer). Hence, as such it represents an exception, because typical (adult) GBM exhibits much lower mutational burden.¹ Also, a very limited number of hypermutant GBM patients were tested for the efficacy of anti-PD1 Abs.

Thus, for adult-GBM, it is necessary to ascertain whether the above-mentioned broad predictive biomarkers can estimate the responsiveness of GBM to single-agent ICIs. To this end, our primary aim was 2-fold, i.e., first to exploit these broadly applicable biomarkers to predict whether GBM has a predisposition for positive responses toward ICIs targeting CTLA4, PD1 or IDO1 in patients. This was tested by using two-independent (publicly-accessible) cohorts of GBM patients as applicable, i.e., The Cancer Genome Atlas (TCGA)²³⁻²⁵ and REpository of Molecular BRAin Neoplasia DaTa (REMBRANDT).²⁶ And second, to use these biomarkers-based outcomes for “guiding and prioritizing” preclinical testing of corresponding ICI monotherapy in an orthotopic-murine glioma model.

Results

Glioblastoma exhibits relatively low mutational and predicted neoantigen burden

Considering the broad utility of high number of non-synonymous SNVs, further referred to as “mutational-burden,” as a positive predictive biomarker of ICI responsiveness,^{19,20} we first decided to ascertain the positioning of GBM, in this regard, relative to other cancer types. TCGA GBM data set was primarily used for these analyses since it provides systematic information on somatic mutational counts in different cancer types.

In line with various previous analyses,^{2,6} we observed that cancer types harboring carcinogen-induced mutational signatures²⁵ had significantly higher mutational burden (Fig. 1A), e.g., melanoma (SKCM), lung cancer (LUAD/LUSC) or bladder cancer (BLCA). Instead, GBM, which does not usually possess a (non-therapeutic) carcinogen-induced mutational signature, clustered with other similar cancer types and exhibited relatively low mutational burden (Fig. 1A).^{2,6}

Moreover, we observed that GBM not only exhibits low mutational burden on the level of the tumor, but also on the level of human GBM-derived cancer cell lines (Fig. 1B). In contrast, the representative cell lines from ICI-responsive cancer types like SKCM, LUAD or BLCA, also exhibited higher mutational burdens. Of note, we observed a significant positive correlation between mutational burdens in various tumor types and the mutational burdens in the corresponding human cancer cell lines (Fig. 1B). However, quantitatively speaking, the cancer cell lines had approximately five times less median mutational burden than the corresponding tumor types (Fig. 1B), possibly because some tumor cells with high mutational counts might be too genetically unstable for long-term persistence in *in vitro* culturing conditions.

Next, in an analysis depicting significant positive correlation between overall mutational burden and burden of predicted

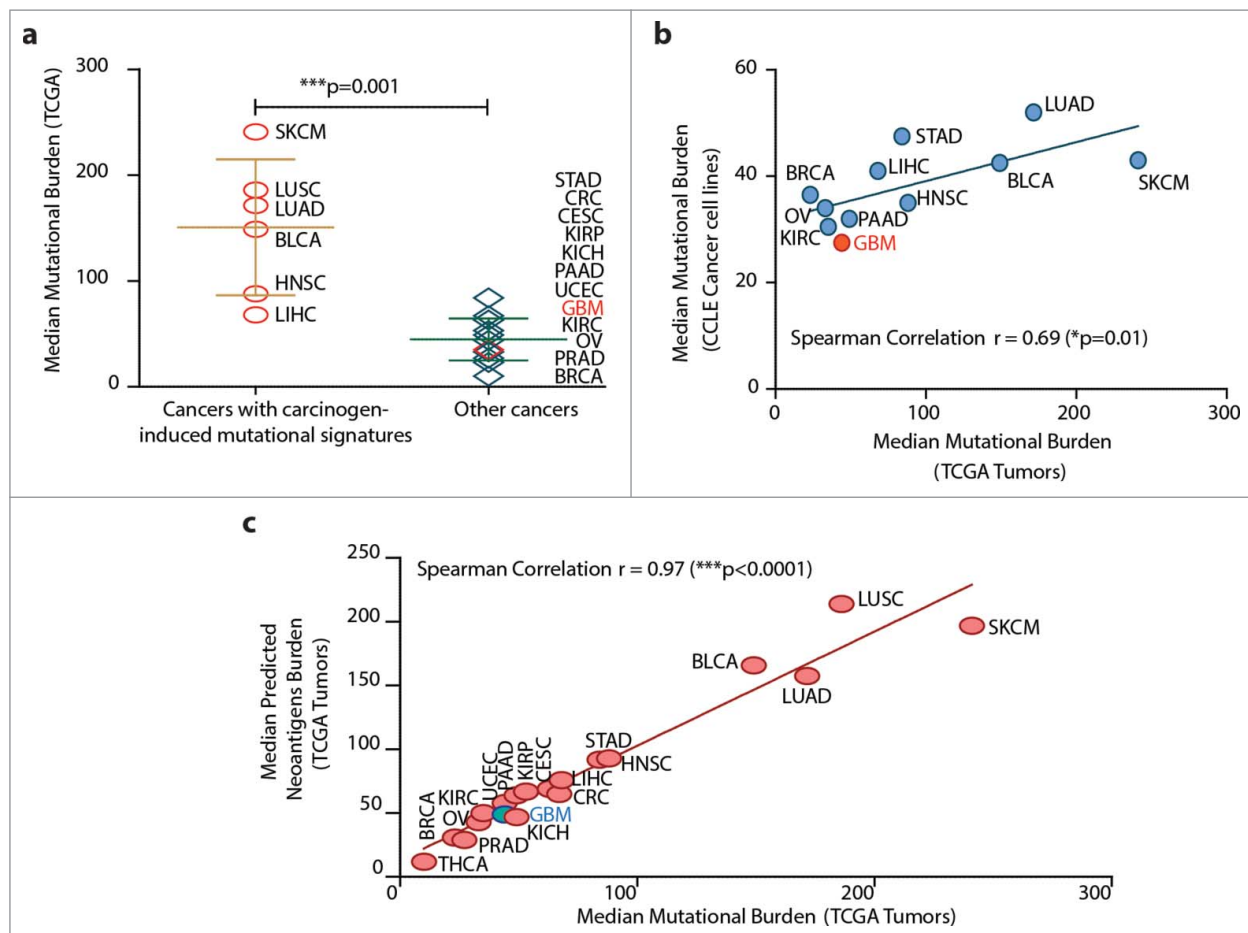


Figure 1. Glioblastoma (GBM) has one of the lowest overall mutational and predicted neoantigen burdens. (A) Presence or absence of carcinogen-induced mutational signatures (derived from COSMIC-database: <http://cancer.sanger.ac.uk/cosmic/signatures>) was used as a means to “classify” median (somatic) mutational burdens of 18 TCGA cancer-data sets (mean \pm s.d., Mann–Whitney test; p -value as indicated); (B) Correlation between median mutational burdens of different TCGA cancer-data sets and median mutational counts of corresponding cell lines belonging to these cancer types from the CCLE-data sets. (C) Correlation of median mutational burdens and median-predicted neoantigen burden from the respective TCGA data sets derived from The Cancer Immunome Atlas at <http://tcia.at>. Abbreviations: BLCA, bladder urothelial carcinoma; BRCA, breast cancer; CESC, cervical squamous cell carcinoma; CRC, colorectal carcinoma; GBM, glioblastoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe cancer; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian cancer; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; THCA, thyroid cancer; UCEC, uterine corpus endometrial carcinoma; Of note, mutational burden refers to nonsynonymous somatic single nucleotide variations.

neoantigens (a phenomenon well established in the literature),^{19,20} GBM also displayed one of the lowest predicted-neoantigen burdens, when compared with the typical ICI-responsive cancer types like SKCM, LUAD/LUSC and BLCA (Fig. 1C).

In conclusion, our analysis shows that most GBM tumors are at the lower end of the mutational/neoantigen-burden’s scale, compared with typical ICI-responsive cancer types.

Glioblastoma exhibits lower expression of *CTLA4*, *PDCD1* and *IDO1* compared with melanoma, lung cancer and bladder cancer

The observation that, relative to typical ICI-responsive cancer types, GBM has much lower mutational and/or neoantigen’s burden made us curious about the overall expression of major immune-checkpoint coding genes, i.e., *CTLA4*, *PDCD1* (codes for PD1) and *IDO1*, in GBM. To this end, we first decided to exploit the TCGA data set to analyze the differential expression of these immune checkpoints in GBM, as compared with

typical ICI-responsive cancer types like SKCM, LUAD/LUSC and BLCA. Interestingly, SKCM, LUAD/LUSC and BLCA expressed *CTLA4* (Fig. 2A), *PDCD1* (Fig. 2B) and *IDO1* (Fig. 2C) immune checkpoints at significantly higher levels than GBM. LUAD in general exhibited relatively high expression of all three immune checkpoints.

Genomic DNA copy-number alterations (CNAs) are key genetic events in human cancer progression such that CNAs of particular genes may influence their subsequent expression,^{27,28} e.g., amplification-type CNAs of a gene have higher (but not absolute) probability of associating with upregulation of corresponding mRNA(s).^{27,29} Moreover, occurrence of CNAs, along with nucleotide mutations, is a good indication of genomic instability/variability associated with particular genes.³⁰ Interestingly, in the TCGA cohorts, higher percentage of LUAD/LUSC, SKCM and BLCA patients exhibited CNAs (like deletions or amplifications) of *CTLA4*, *PDCD1* and *IDO1* genes, as compared with GBM (Fig. 2D). Importantly, no GBM patient in this cohort had amplification-type CNAs of *CTLA4*, *PDCD1* and *IDO1* as

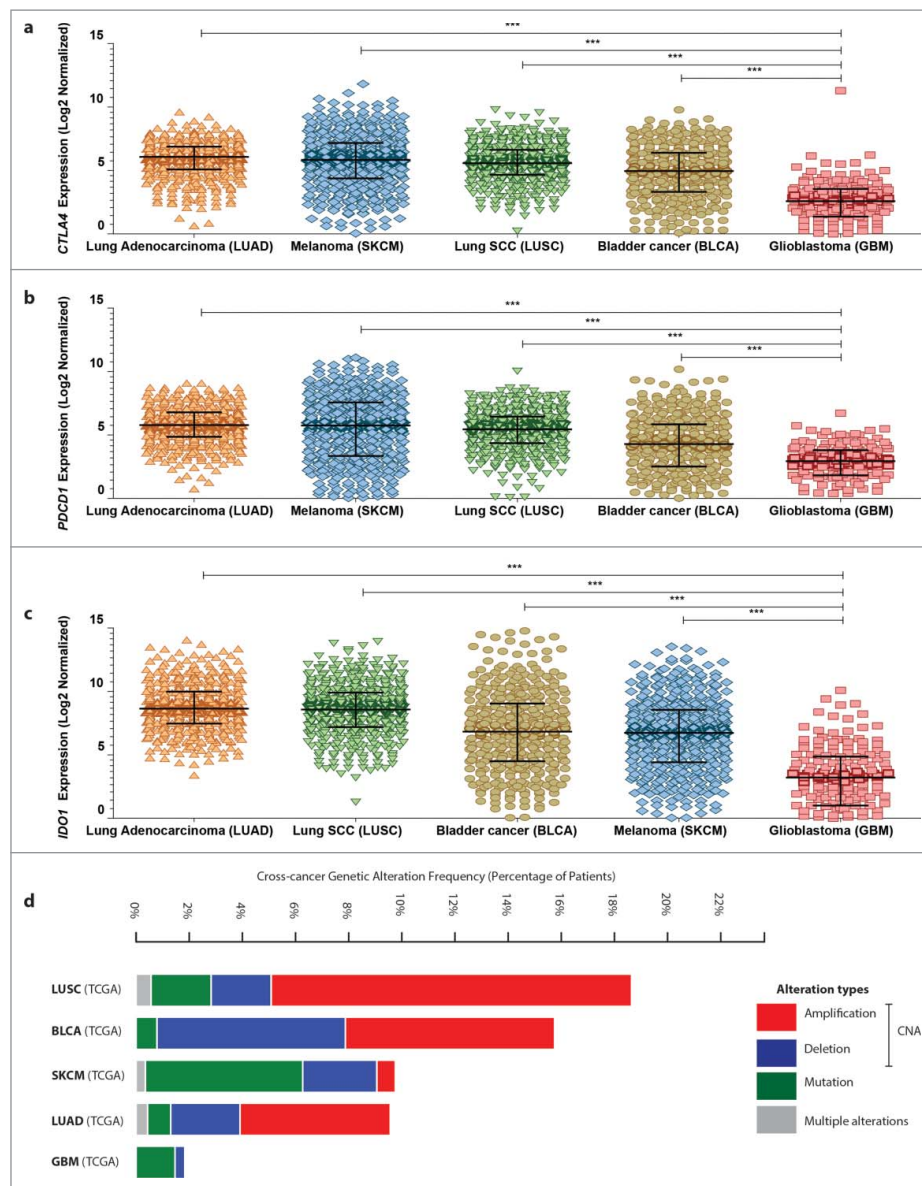


Figure 2. Melanoma, lung cancer and bladder cancer exhibit significantly higher expression of *CTLA4*, *PDCD1* or *IDO1* than glioblastoma in human patients. Analysis of differential expression of *CTLA4* (A), *PDCD1* (B) or *IDO1* (C) between lung adenocarcinoma (LUAD; n = 517), melanoma (SKCM; n = 472), lung squamous cell carcinoma (SCC) (LUSC; n = 501), bladder cancer (BLCA; n = 408) and glioblastoma (GBM; n = 166) (from TCGA cohorts) (data are log2 normalized and presented as median with inter-quartile range; One-way ANOVA; significance set at $p < 0.05$; *** $p < 0.001$). Of note RNASeq, and not microarray, expression data was used for this analysis since comprehensive microarray data are not available for melanoma and bladder cancer within TCGA data set. (D) A combined cross-cancer genetic alteration frequency analysis (expressed as % of patients) for *CTLA4*, *PDCD1* and *IDO1* genes was performed using the TCGA cohorts of LUAD, LUSC, SKCM, BLCA and GBM. The graph indicates presence of either genetic mutations or specific copy-number alterations (CNA; like genetic deletion or amplification) or presence of multiple such alterations simultaneously (as indicated by the color-code in the legend within the figure).

opposed to other cancer types analyzed here (Fig. 2D). This indicates that *CTLA4*, *PDCD1* and *IDO1* have higher chance of experiencing CNAs in ICI-responsive cancer types as compared with GBM.²⁹ On the other hand, overall percentage of patients with mutations in these immune-checkpoint coding genes in GBM were to a certain extent comparable to BLCA, LUAD/LUSC (Fig. 2D).

In conclusion, GBM patients exhibit significantly lower mRNA expression of *CTLA4*, *PDCD1* and *IDO1* as well as lower tendency of displaying amplification-type CNAs associated with these genes, when compared with typical ICI-responsive cancer types like melanoma, lung cancer and bladder cancer.

Overall expression of *CTLA4*, *PDCD1* and *IDO1* is not drastically altered by gliomagenesis

Tumors tend to exploit immune checkpoints for immune tolerance and escape from anticancer T cells and hence frequently, tumor tissue has been reported to exhibit higher expression of immune checkpoints than corresponding normal tissue.^{7,18} To this end, we analyzed this in the GBM/normal brain samples from the TCGA and REMBRANDT data sets (Fig. 3). Interestingly, only in TCGA GBM cohort, *CTLA4* showed significantly higher expression than corresponding normal brain samples (Fig. 3A). Although other analyses did not show significant upregulation of various checkpoints (Figs. 3B–F), it seemed

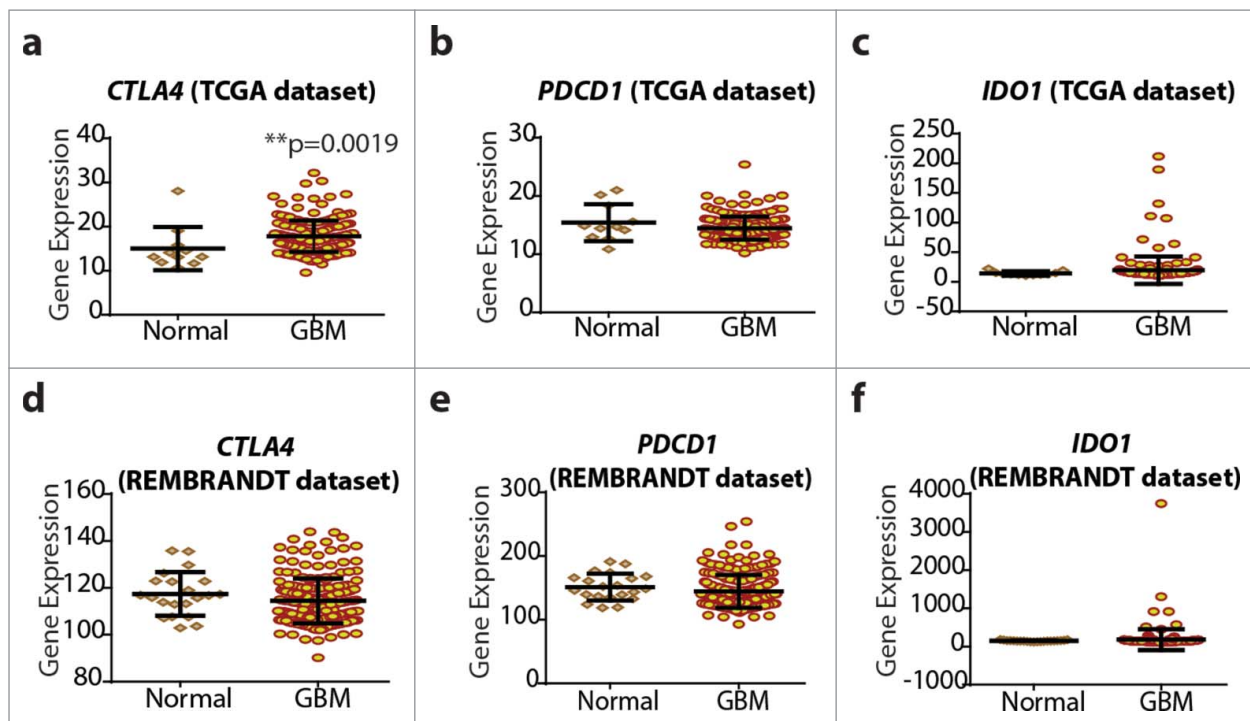


Figure 3. Glioblastoma (GBM) tumor tissue does not show strong upregulation of *CTLA4*, *PDCD1* and *IDO1* (B, E) and *IDO1* (C, F) between normal brain tissue sample (for REMBRANDT, $n = 21$; for TCGA, $n = 11$) and GBM tissue sample (for REMBRANDT, $n = 214$; for TCGA, $n = 202$) (mean \pm s.d., Mann–Whitney test; p -value as indicated). Of note, TCGA and REMBRANDT data sets were analyzed by different expression-analysis platforms and standardized by different post-processing and normalization analyses and hence their overall gene-expression counts have different (but proportional) scales.

that a small subset of patients with GBM tumors (5–10%) tend to show higher *IDO1* expression than their normal counterparts (Figs. 3C and F). Although these trends were not significant, these were consistently observed in both TCGA and REMBRANDT data sets.

Overall, it seems that the oncogenic process of gliomagenesis by itself does not cause significant upregulation of at least *PDCD1* and *IDO1* gene expression, with the exception of *CTLA4* in the TCGA patient cohort.

Glioblastoma exhibits relatively low pre-existing presence of tumoral T cell biomarkers

Higher pre-existing or basal TILs positively predict responses to ICIs, e.g., melanoma tumors with high basal/pre-existing TILs tend to respond better to ICIs rather than tumors with low to negligible TILs.^{6,7,18} Thus, the amount of TILs is a suitable broad biomarker of ICI responsiveness.

Estimation of TILs can be achieved either via the “classical” immunophenotyping technique (immunohistochemistry or immunostaining-driven flow cytometry) or through the more recent technique of using (pre-established) T-cell-specific gene signatures.^{31–33} Since we did not have access to substantial number of GBM tumor tissues for the classical analyses, we decided to utilize the latter technique with the TCGA GBM cohort (as reported by us previously).^{5,33} TCGA GBM cohort was preferred since it is completely composed of primary-resected GBM tissues, thereby allowing for the best estimation of pre-existing/basal TILs.

Pan-cancer T cell subtype-specific gene signatures available from The Cancer Immunome Atlas³⁴ were used to estimate the

total TIL-fractions within 19 different TCGA tumor cohorts (Fig. 4A). Separate genetic signatures associated with different T cell sub-populations were layered together to estimate the overall TIL fractions for each cancer type. Interestingly, among these 19 different solid tumor types, GBM exhibited the least amount of TIL fractions (Fig. 4A). This was especially evident when compared with the higher amounts of TIL fractions in typically ICI responsive cancer types like SKCM, LUSC/LUAD and BLCA (Fig. 4A).

In conclusion, GBM exhibits one of the lowest basal/pre-existing TIL-associated genetic signatures among various solid tumor types.

CTLA4 and IDO1 show association with Treg and effector T-cell-associated biomarkers

Low occurrence of basal TIL-associated genetic signatures in GBM raised the need to ascertain the exact association of above immune checkpoints with T cell polarization or activity-associated genetic signatures.^{2,6} We gave due consideration to two currently described scenarios in this regard, i.e., (1) the “canonical” association between immunosuppressive Treg cells and immune checkpoints, considering that the function of Tregs and *CTLA4*, *PDI* and *IDO1* in enforcing immunosuppression is overlapping^{2,6,17,34,35}; and (2) the “paradoxical” association between immune checkpoints and T cell-effector markers (like $\text{IFN}\gamma$, Granzyme B-perforin), since activation of T cells eventually causes upregulation of immune checkpoints as an auto-regulatory loop in later stages of effector function (to avoid autoimmunity and resolve inflammation).^{2,6}

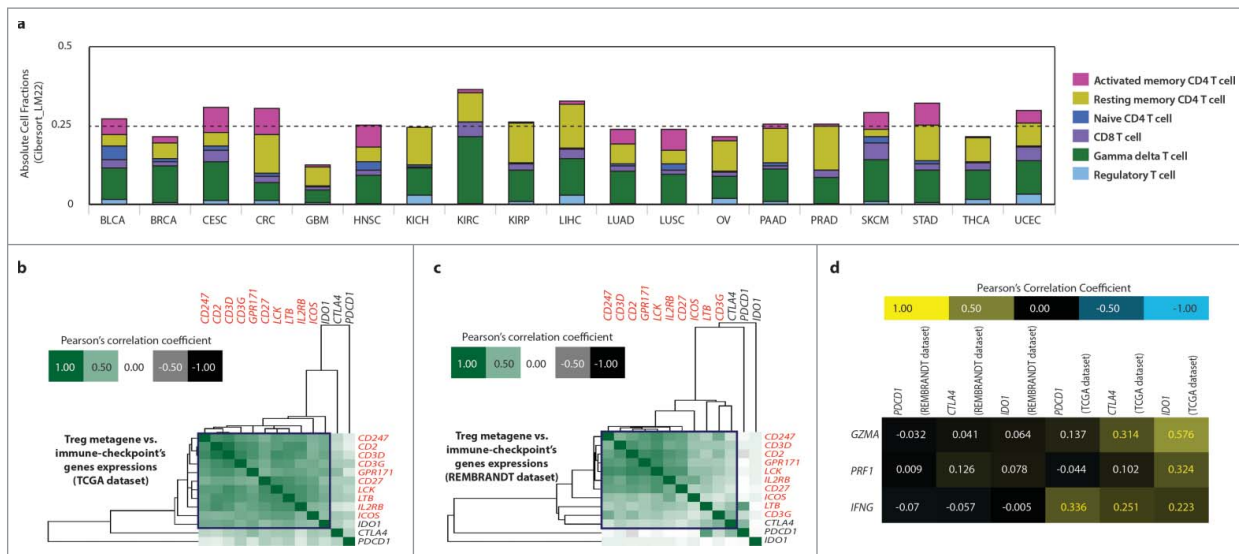


Figure 4. Glioblastoma (GBM) exhibits sparse basal T cell-infiltrates and correlation of *CTLA4* and *IDO1* with T-cell-associated polarization biomarkers. (A) The overall (absolute) presence of different T-cells-associated genetic signatures or metagenes (indicated as color code) was estimated across 19 TCGA cancer-data sets using The Cancer Immunome Atlas. (B, C, D) Correlation of *CTLA4*, *PDCD1*, *IDO1* expression with, GBM-specific Treg-metagene⁵ across TCGA (B) and REMBRANDT data sets (C) (blue-box indicates co-clustering of immune checkpoint with Treg-metagene); or with, *GZMA*, *PRF1* and *IFNG* across the same data sets (D). BLCA, bladder urothelial carcinoma; BRCA, breast cancer; CESC, cervical squamous cell carcinoma; CRC, colorectal carcinoma; GBM, glioblastoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe cancer; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian cancer; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; THCA, thyroid cancer; UCEC, uterine corpus endometrial carcinoma.

To this end, we first analyzed the correlation between *CTLA4*, *PDCD1* or *IDO1* expression levels and a previously established Treg-specific genetic signature or metagene.⁵ Here, a Treg-specific metagene refers to a “multi-gene expression pattern”³³ where aggregated patterns of various Treg-associated genes (experimentally validated by independent studies)^{31,32} are clustered to represent a correlated expression or co-expression matrix. This is because the tendency of these genes (i.e., *CD247*, *CD2*, *CD3D*, *CD3G*, *GPR171*, *CD27*, *LCK*, *LTB*, *IL2RB* and *ICOS*) to show co-expression has statistically higher (but not absolute) chance of indicating the presence of Tregs.^{31,33,34} The principle behind such immune cell-specific metagenes is discussed in details, elsewhere.^{31,33,34} In this study, the rationale was, if an immune-checkpoint coding gene co-clusters with the Treg-metagene within human GBM tissue than the probability of that immune-checkpoint being expressed on Tregs is higher (but not absolute).

Interestingly, in a cohort-dependent fashion, either *IDO1* (TCGA, Fig. 4B) or *CTLA4* (REMBRANDT, Fig. 4C), but not *PDCD1*, showed considerable co-clustering with Treg metagene (as indicated by the tendency of *IDO1* or *CTLA4* to show nodal association with the “core” Treg-metagene). Of note, the “core” Treg metagene is indicated by red font coloring of the respective genes on the heatmaps (Figs. 4B and C). Noteworthy, this meta-analysis showed that the correlation between *CTLA4* or *IDO1* and Treg metagene was rather weak, as indicated by the lack of overlap between these immune checkpoints and the “core” of the metagene (Figs. 4B and C).

Next, we correlated *CTLA4*, *PDCD1* and *IDO1* expression levels with markers of T cell effector function, i.e., *GZMA* (coding granzyme B), *PRF1* (coding perforin) and *IFNG* (coding IFN γ) which is displayed in a correlation heatmap. Interestingly, we observed a, cohort-specific, consistent positive

correlation between these effector function-related factors (*GZMA*, *PRF1*, *IFNG*) and *IDO1* (TCGA-cohort, Fig. 4D). On the other hand, in this cohort, *CTLA4* achieved the next best correlation (with *GZMA*, *IFNG*), followed by *PDCD1* (only with *IFNG*) (Fig. 4D).

In conclusion, mainly *CTLA4* and *IDO1* exhibited correlation with T cell polarization/function-related genetic markers, in a cohort-specific manner.

Differential *CTLA4*, *PDCD1* and *IDO1* expression fail to exhibit definitive prognostic impact in glioblastoma patients

In various publications it has been shown that high expression of immune checkpoints may associate with negative patient prognosis.^{17,34,35} To this end, we decided to ascertain the prognostic impact of immune-checkpoint gene expression in TCGA and REMBRANDT GBM cohorts. The differential expression of *CTLA4*, *PDCD1* and *IDO1* did not show strong association with poor or prolonged overall survival (OS) in GBM patients, neither in TCGA (Figs. 5A–C) nor in REMBRANDT (Figs. 5D–F) data sets. The hazard ratios (HRs) in all cases were neither less than 0.90 nor more than 1.10 thereby further substantiating the seemingly null prognostic impact of these immune-checkpoints’ expression levels in GBM patients.

Anti-PD1 therapy shows stronger preclinical efficacy than anti-*CTLA4* or *IDO1*-targeted therapies

The clinical predictions on GBM responsiveness to ICIs on the basis of broad biomarkers were mixed. The predictive biomarkers of ICI responsiveness exhibited contradictory patterns in GBM patient cohorts, such that (i) four sets of biomarkers

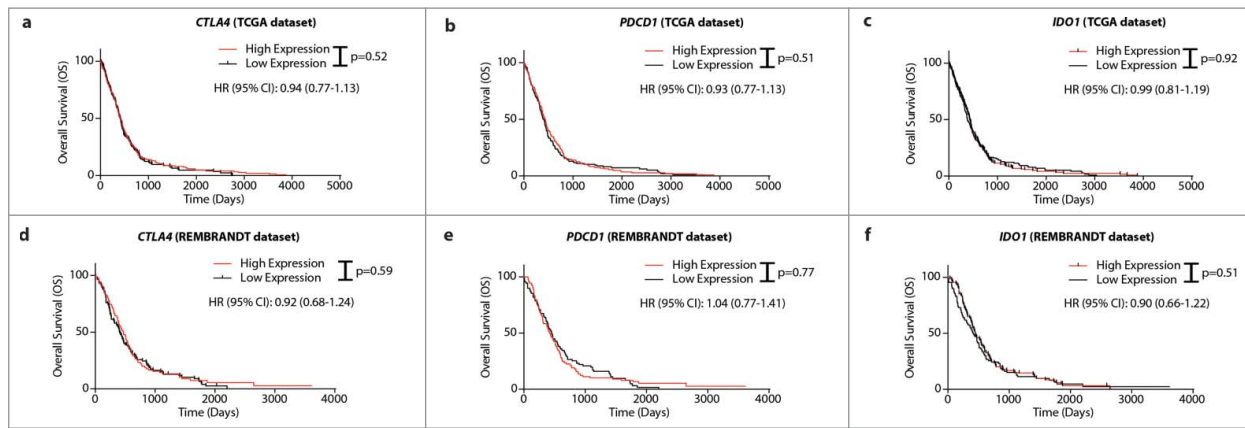


Figure 5. *CTLA4*, *PDCD1* and *IDO1* differential expression does not exhibit significant prognostic impact in GBM patients. TCGA GBM-cohort (n = 540, A–C) and REMBRANDT GBM-cohort (n = 178, D–F) stratified (median) into “high-expression” (red; TCGA, n = 271; REMBRANDT, n = 89) or “low-expression” (black; TCGA, n = 269; REMBRANDT, n = 89) and represented as Kaplan–Meier plots (log-rank (Mantel–Cox) test; hazard ratios (HR) +95% confidence interval or CI).

predicted ICI-resistance (i.e., low mutational/predicted-neoantigen burden, relatively low expression of immune-checkpoint coding genes, low basal/pre-existing TIL-associated signatures and no discernible prognostic impact of differential expression of immune-checkpoints), while (ii) two sets of biomarkers predicted susceptibility to at least anti-CTLA4 Abs and/or IDO1 inhibitors (i.e., high *CTLA4* expression in GBM tissue compared with normal tissue and correlation of *CTLA4/IDO1* with T cell polarization/function-related signatures).

These contradictory patterns on biomarker level made it challenging to prioritize, in an objective manner, the preclinical application of specific ICIs in murine settings. To this end, we decided to experimentally test the efficacy of ICIs targeting all three immune checkpoints, i.e., CTLA4, PD1 or IDO1 in orthotopic GL261 glioma-bearing mice. In one case, we administered pharmacological IDO1 inhibitors, i.e., 1-Methyl-DL-tryptophan (1-DL-MT, a mixture of levorotary/L and dextrorotary/D stereoisomers of 1-MT) or 1-Methyl-D-tryptophan (i.e., 1-D-MT)¹² (Fig. 6A). In another case, we administered anti-CTLA4 or anti-PD1 Abs (Fig. 6B). Interestingly, both 1-DL-MT and 1-D-MT failed to prolong survival of GL261 glioma-bearing mice (Fig. 6C). This failure of IDO1 targeting was “phenocopied” on the level of *Ido1*^{-/-} mice (Fig. 6D). Anti-CTLA4 therapy was able to only marginally ($p = 0.05$) extend the median survival of GL261 glioma-bearing mice (Fig. 6E). However, interestingly only anti-PD1 therapy significantly prolonged the median survival of GL261 glioma-bearing mice allowing some long-term survival (Fig. 6F).

ICIs like anti-CTLA4 and anti-PD1 antibodies, exert their anticancer effects by effectively reducing the intratumoral persistence of CTLA4⁺ and PD1⁺ T cells (especially CTLA4⁺/PD1⁺ Treg cells), respectively.^{7,17,36} To this end, we interrogated whether brains inoculated with GL261-gliomas possessed CTLA4⁺/PD1⁺ T cell infiltrates; and whether ICIs targeting CTLA4/PD1 affected their persistence. GL261-glioma inoculated brains were indeed infiltrated by CTLA4⁺ or PD1⁺, CD4⁺ T cells, CD8⁺ T cells and Tregs (PD1⁺ T cells > CTLA4⁺ T cells) (Figs. 6G–L). Notably, more than half of Tregs infiltrating the GL261-glioma inoculated brains did not express CTLA4 or PD1 (Figs. 6I and L). However, in line with expectations, anti-

CTLA4 or anti-PD1 antibodies were indeed able to effectively reduce the amount of CTLA4⁺ or PD1⁺ T cells infiltrating the GL261-glioma inoculated brains, respectively (Figs. 6G–L). Last but not least, we also confirmed that GL261 glioma cells tend to express surface PD-L1, one of the major ligands required for PD1 activation (Fig. S1).

Next, we decided to position our experimental results relative to the existing *status quo* in the field of ICI-based preclinical treatment of glioma, through a systematic literature meta-analysis. A survey of 15 relevant preclinical studies (Box S1) exploring the impact of ICIs targeting CTLA4, PD1 or IDO1 in preclinical (orthotopic) glioma murine models (most studies used either GL261 or SMA560 glioma), showed an interesting result (Fig. S2). ICIs targeting CTLA4 or IDO1 were reported, by most studies, to fail in significantly prolonging median survival of glioma-bearing mice (Fig. S2). In contrast, relatively more studies reported success of anti-PD1 therapy in prolonging median survival of glioma-bearing mice (Fig. S2).

Overall this shows that the propensity of anti-PD1 therapy to succeed in glioma-bearing mice is higher than IDO1-inhibitors or anti-CTLA4 therapy.

Discussion

Our observations reveal a discrepancy between broad predictive biomarkers of ICI responsiveness and preclinical efficacy of respective ICI monotherapy in GBM. The majority of broad biomarkers predicted ICI resistance in GBM. Few biomarkers, however, predicted some efficacy for ICIs targeting IDO1 or CTLA4 but not PD1. On the other hand, opposite to the biomarker-based trends, the preclinical efficacy of these ICIs in orthotopic GL261-glioma model revealed a significant susceptibility to anti-PD1 Abs, whereas the tumor-rejecting ability of IDO1 or CTLA4 targeting ICIs was null or poor, respectively.

The clinical biomarker analysis in GBM patients delineated low mutational/neoantigen burden, relatively lower tumoral expression of immune checkpoints and sparse pre-existing levels of TILs, features that do suggest that unlike melanoma or lung cancer, adult-GBM probably does not have intrinsic predisposition toward therapies targeting immune checkpoints.^{2,6,16} This does not mean that a subset-of-patients of

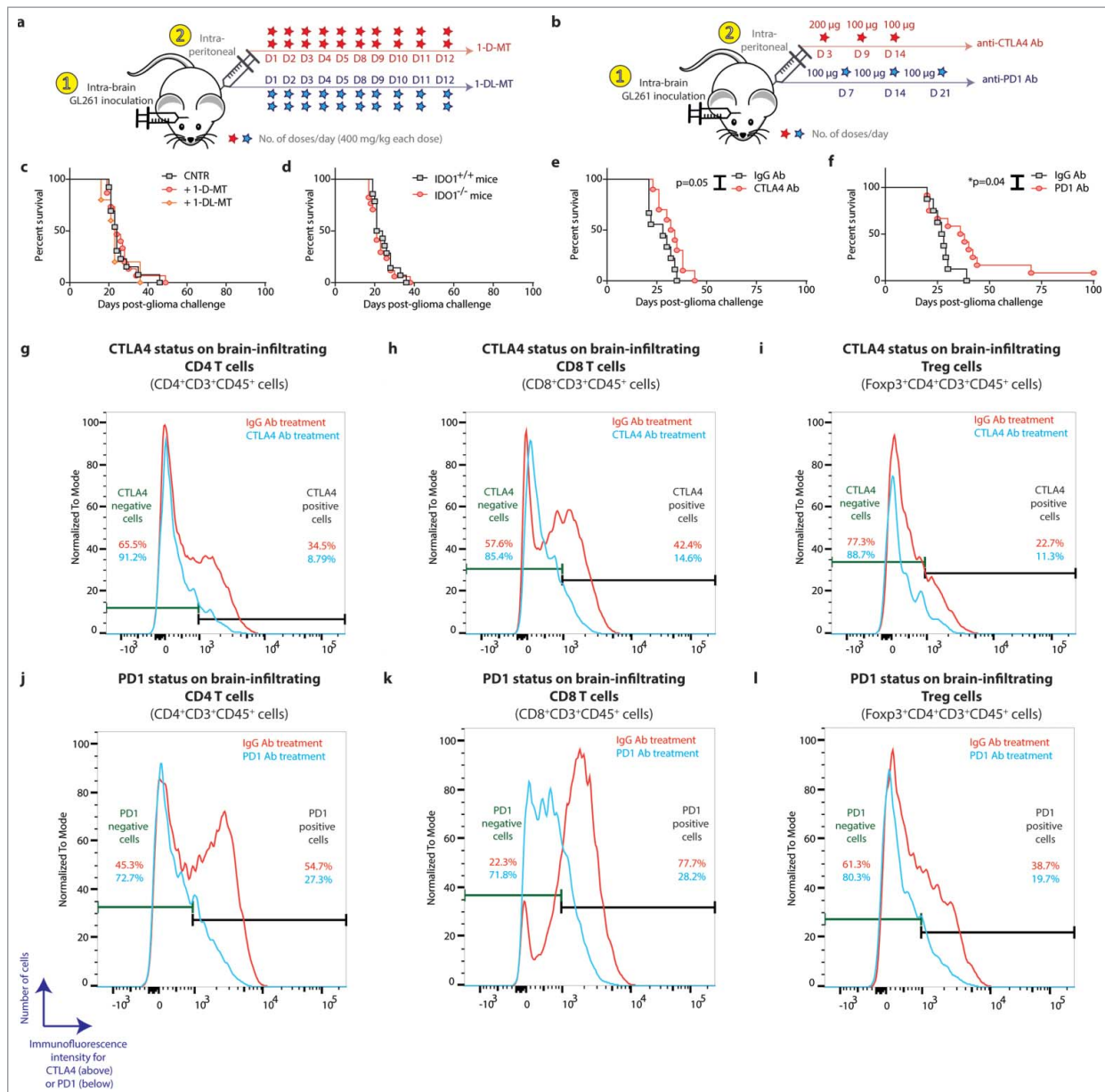


Figure 6. Anti-PD1 mono-immunotherapy exhibits the highest therapeutic efficacy against preclinical glioma. (A, B) Mice inoculated with GL261 cells intra-axially,⁵ were treated with IDO1-inhibitors (A) or anti-CTLA4, anti-PD1 antibodies (B). Kaplan–Meier survival curves for glioma-bearing mice treated with IDO1 inhibitors (CNTR, n = 13; +1-D-MT, n = 15; +1-DL-MT, n = 5) (C), *Ido1*^{+/+} (n = 14) vs. *Ido1*^{-/-} (n = 17) mice (D) or treated with anti-CTLA4 (IgG Ab, n = 9; CTLA4 Ab, n = 10) (E) or anti-PD1 (IgG Ab, n = 8; PD1 Ab, n = 12) (F) antibodies (Log-rank-(Mantel-Cox)-test). (G–L) C57BL/6 mice were inoculated with live GL261 cells (Day 0), intra-axially and either injected with respective IgG antibodies (Ab), i.e., control mice or treated with anti-CTLA4 Ab (G–I) or anti-PD1 Ab (J–L). Thereafter the mice were killed at day 18–22 post-GL261 inoculation and the brains were isolated. Initially, total mononuclear immune cells were counted. Thereafter these were processed for FACS-based immunophenotyping for (G) CTLA4⁺CD4⁺T cells, (H) CTLA4⁺CD8⁺T cells, (I) CTLA4⁺Treg cells, (J) PD1⁺CD4⁺T cells, (K) PD1⁺CD8⁺T cells and (L) PD1⁺Treg cells. The histograms are representative of n = 3–4 mice/group (the percentage of CTLA4 or PD1 negative and positive T cells are indicated through the agency of histogram).

GBM will not respond to ICI monotherapy (at least partially), however, these patients will have to be either delineated more stringently or alternative GBM-specific immune checkpoints will have to be characterized. Moreover, the biomarkers used in this study to estimate the ICI responsiveness of GBM are predictive of broad “resistant-phenotype,” and hence they may ignore particular GBM-specific features. Last but not least, our results are not representative of the ICI responsive, hypermutant pediatric-GBM since on one hand, hypermutant GBM patients are not well represented in TCGA/REMBRANDT cohorts and on the other hand, orthotopic GL261-glioma is not *per se* a good model of pediatric-GBM.

Interestingly, a recent study has shown that preclinical glioma models like GL261 do possess some neoantigens and may possess higher mutational burden than a typical primary human GBM tissue.¹ Moreover, another recent study showed that murine cancer cell lines with higher mutational burden (e.g., cell lines derived from carcinogen-induced tumor) respond better to immune-checkpoint therapy than those with lower mutational burdens (e.g., cell lines derived from spontaneous or GEMM-derived tumors).³⁷ Thus, it is tempting to speculate that GL261-based gliomas are more representative of medium-to-hyper mutant GBM rather than typical low mutational burden (adult) GBM, and hence are more responsive to

ICIs as shown here and in previous studies.¹⁵ This is an interesting hypothesis that needs further validation. More research is required to understand the determinants of anti-glioma immunity and immunosurveillance in preclinical model and compare these with clinical determinants to reach better consensus on suitable biomarkers.^{38,39} Last but not least, beyond these glioma cell line-related differences there is also a distinct possibility that mice-to-human differences in CNS-associated immune responses and general biology of immune checkpoints may also be a source of the discrepancy between clinical biomarker-based predictions and preclinical therapeutic efficacies.^{39,40} For instance, we observed that more than half of the brain-infiltrating Tregs in GL261 glioma-bearing mice did not express considerable amounts of CTLA4 or PD1. This might indicate that alternative immune checkpoints (possibly specific to brain or CNS milieu) might be operating in a GBM micro-environment. Thus, in GBM contexts, CTLA4⁺/PD1⁺ Tregs could be either minor enforcers of immunosuppression⁴¹ or susceptible to being replaced by T cells expressing alternative immune checkpoints following ICIs treatment. For instance, a recent preclinical study found T cells exploiting T-cell immunoglobulin mucin-3 (TIM3) to drive lung cancer growth despite anti-PD1 therapy.⁴² Thus, future studies will have to concentrate on delineating such alternative GBM-associated immune checkpoints to design more tumor-specific ICIs.

Considering the relatively low intrinsic susceptibility of GBM to immunotherapy, ICIs may have a better chance of showing therapeutic efficacy if they are combined with other immunotherapies that can correct the low pre-existing TILs density within GBM tissue. Highly efficacious immunotherapies like next-generation DC vaccines (e.g., DC vaccines based on glioma cells that underwent immunogenic cell death or ICD),^{5,43,44} adoptive or chimeric antigen receptor (CAR) T cell immunotherapy (e.g., CAR T cells targeted against the glioma antigen IL13R α 2)⁴⁵ or oncolytic viruses (e.g., Newcastle disease virus)⁹ can help in increasing intra-GBM TILs density thereby creating a conducive scenario for ICIs response. Such “smart” combinations will have to be tried pre-clinically and if found successful their translation toward the clinic should be expedited.

It is worth mentioning that a recent report estimating safety of anti-PD1 therapy (pembrolizumab) in combination with anti-angiogenic therapy (bevacizumab) in recurrent GBM, found this combination to be safe for patients, however, all enrolled patients experienced progressive disease despite therapy.⁴⁶ As such it is too early to draw strong conclusions based on the above report, considering the very small number of patients, absence of proper control arms and follow-up criteria not tailored for long-term ICI response analysis. As the results of ongoing phase II/III ICI-clinical trials in GBM come out, the scenario would clear up, also with respect to the discrepancies we observe.²

Materials and methods

Cell culture, orthotopic GBM mice model and ICIs administration

GL261 murine glioma cells (received as a gift from Dr Eyupoglu, University of Erlangen, Germany) were cultured at 37°C

under 5% CO₂ in DMEM containing 4.5 g/L glucose and 0.11 g/L sodium pyruvate, supplemented with 2 mM glutamine, 100 units/mL penicillin, 100 μ g/L streptomycin and 10% fetal calf serum. For orthotopic glioma murine model, female C57BL/6J mice (8–10 weeks old) were purchased from Harlan (Horst) or KU Leuven internal stock *ad libitum*. Animals were handled in accordance with the KU Leuven bioethics regulations. To generate intra-brain GBM, the mice were intra-axially injected with 5 \times 10⁵ GL261 cells as described previously.⁴⁷ In brief, mice were anesthetized, set in a stereotactic frame (Kopf Instruments) and injected (in sterile circumstances) with the GL261 cells at 2 mm lateral and 2 mm posterior from the bregma, and at 3 mm underneath the dura mater. After intra-brain inoculation, the mice were monitored 2–3 times per week and clinical symptoms were noted with a neurologic-deficit grading scale modified from an experimental autoimmune encephalomyelitis model, described in the past.⁴⁷ Mice were classified as long-term survivors if their survival reached beyond three times the median survival of the untreated control or CNTR mice. Also, as applicable, some animals received intra-peritoneal injections of anti-CTLA4 Abs (4F10) or anti-PD1 Abs (RMP1–14) (these antibodies were received from Louis Boon, Bioceros, Netherlands). IDO1 inhibitors (1-D-MT or 1-DL-MT, purchased from Sigma-Aldrich) were administered via oral gavage. The administration schedules and doses of these ICIs are described either in the figures as schemas or within the figure legends. As applicable, either representatives of two independent mice experiments (PD1, CTLA4 experiments) were shown or power analysis based on previous publications^{47–49} was used to reach necessary sample sizes in individual experiments (IDO1 experiments).

Literature meta-analysis for preclinical efficacy of ICIs in murine glioma model

PubMed was searched for applicable studies conducted in mouse, until 9th January, 2017. The following search keywords were used: (ido OR ctla-4 OR pd-1) AND (glioblastoma OR glioma) AND (murine OR mice OR mouse OR *in vivo*). To distinguish potentially relevant studies, the catalog of articles identified in the earlier search, were also scanned manually. Studies within the catalog were considered qualified if they met all of the subsequent criteria: (1) presented Kaplan–Meier plot based overall survival data and (2) data generated in syngeneic immunocompetent mice model. Studies were excluded because of following reasons: (1) not sufficient survival data reported, (2) letters, reviews, commentary, perspectives, case reports, conference abstracts, editorials or expert opinion, (3) studies reporting xenograft results in immunodeficient or humanized mice. Overall these search criteria helped short-list 15 research articles^{12,15,16,50–61} mentioned in Box S1.

Analysis of T cell biomarker-associated genetic signature and immune checkpoints

The metagene associated with Treg cells^{31,33} was derived from our previously published analysis, where GBM-tailored T cell-metagenes were established.⁵ The co-expression of *CTLA4*, *PDCD1* and *IDO1* was analyzed with respect to this Treg-

metagene in TCGA and REMBRANDT GBM patient data sets to generate a (Pearson's) correlation submatrix. Data retrieved from cBioPortal⁶² or Project *β*stasis web-portals were used for the above matrix generation, as applicable. In another case, *CTLA4*, *PDCD1* and *IDO1* were correlated with expression of *IFNG*, *GZMA*, *PRF1* in TCGA and REMBRANDT. These coefficient values were used for hierarchical clustering³³ through the Cluster 3.0 software⁶³ and the gene co-expression matrices were visualized as heatmaps through TreeView.⁶⁴ Last but not least, to estimate the basal or pre-existing (total) T cell infiltrates-associated genetic signatures in different cancer types, we used the Cancer Immunome Atlas (<https://tcia.at/home>),³⁴ to analyze the absolute amounts of tumor-associated T cell genetic signatures in 19 different TCGA tumor types (mentioned in the figure or figure legends). The "cell type fractions plot" was used to generate absolute fraction values with Cibersoft_LM22 deconvolution methodology.³⁴

Analysis of CTLA4/PD1 expression on brain-infiltrating T cells

Brain-infiltrating mononuclear immune cells were isolated from GL261-inoculated mice (treated with IgG Ab or anti-CTLA4/PD1 Ab) as detailed previously.^{5,9,10} Surface staining of these mononuclear immune cells was performed with anti-CD4 PerCP-Cyanine5.5/APC-eF780, anti-CD3 FITC/PE/eFluor[®] 450, anti-CD8 BV421/eFluor[®] 605NC mAbs, anti-CTLA4 PE or anti-PD1 PE eBioscience or BD). Intracellular FoxP3 was detected using a FoxP3-PE staining kit (eBioscience) according to the manufacturer's instructions. Data acquisition was performed on LSRFortessa flow cytometer (BD Biosciences) and the FlowJo software was used for histogram analysis.

Prognostic impact and differential expression analysis of immune checkpoints

The differential expression levels of *CTLA4*, *PDCD1* or *IDO1* and associated clinical survival information (overall survival or OS) was retrieved and analyzed for the TCGA GBM patient data set (n = 540)^{23,24} and REMBRANDT GBM patients (n = 178)²⁶ using the PROGgeneV2 web-platform⁶⁵ and Project *β*stasis web-platform, respectively. These platforms stratified the respective patients on the basis of the median gene expression profile into two risk-groups, i.e., high risk or low risk.³³ The respective patient risk groups were plotted with respect to OS to generate Kaplan–Meier curves using the Graphpad Prism software. HR (and its 95% confidence interval) and log-rank (Mantel-Cox) *p* values were calculated (statistical significance threshold set at *p* < 0.05).³³ Patients surviving beyond the follow-up thresholds were censored. Last but not least, differences in expression of *CTLA4*, *PDCD1* and *IDO1* between normal brain tissue sample (for REMBRANDT, n = 21; for TCGA, n = 11) and GBM tissue sample (for REMBRANDT, n = 214; for TCGA, n = 202) were analyzed through the Project *β*stasis web-platform. Of note, only those GBM patients' data were analyzed within the TCGA data set, whose GBMs could be pathologically subdivided into classical, mesenchymal, neural or pro-neural phenotypes.

Cross-cancer analysis for differential expression and genetic alterations of immune checkpoints

Data for differential expression (RNASeq-based) analysis and genetic alterations (mutations or CNAs in respective genes) of *CTLA4*, *PDCD1* or *IDO1* between lung adenocarcinoma (LUAD; n = 517), melanoma (SKCM; n = 472), lung squamous cell carcinoma (SCC) (LUSC; n = 501), bladder cancer (BLCA; n = 408) and glioblastoma (GBM; n = 166) were retrieved from respective TCGA data sets available from the cBioPortal⁶² and further analyzed as detailed in the figure legends.

Analysis of mutational burden and predicted-neoantigens burden

The median somatic mutational burdens (i.e., non-synonymous somatic single nucleotide variations) for different TCGA cancer types were derived from Cancer Immunome Atlas (<https://tcia.at/home>),³⁴ Major TCGA cancer types were classified to carry (validated) carcinogen-associated mutational signatures²¹ on the basis of the Catalog of Somatic Mutations in Cancer (COSMIC) database (<http://cancer.sanger.ac.uk/cosmic/signatures>) (see Results text for details). The mutational burden graph of these two groups was then plotted. In another case, the somatic mutational counts of various human cancer cell lines available in the Cancer Cell Line Encyclopedia (CCLE) database (<https://portals.broadinstitute.org/ccle/>), were retrieved from the cBioPortal⁶² database and correlated (Spearman's) with the (above) corresponding TCGA median mutational burdens. Last but not least, the overall median mutational burdens and predicted neoantigens burdens of various TCGA cancer types were derived from the Cancer Immunome Atlas (<https://tcia.at/home>),³⁴ and plotted to derive overall correlation (Spearman's).

Statistical analysis

All statistical analyses were performed using either Prism software (GraphPad Software) or GraphPad QuickCalcs online software (<http://www.graphpad.com/quickcalcs/index.cfm>). Log-rank (Mantel-Cox) test or Mann–Whitney statistical test were used for statistical analysis, as applicable and unless otherwise mentioned (indicated in figure legends). The significance level was set at *p* < 0.05 (**p* < 0.05, ***p* < 0.01, ****p* < 0.001; values indicated in the figures).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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