Background. Therapeutic targeting of the immune checkpoints cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4) and PD-1/PD-L1 has demonstrated tumor regression in clinical trials, and phase 2 trials are ongoing in glioblastoma (GBM). Previous reports have suggested that responses are more frequent in patients with tumors that express PD-L1; however, this has been disputed. At issue is the validation of PD-L1 biomarker assays and prognostic impact.

Methods. Using immunohistochemical analysis, we measured the incidence of PD-L1 expression in 94 patients with GBM. We categorized our results according to the total number of PD-L1-expressing cells within the GBMs and then validated this finding in ex vivo GBM flow cytometry with further analysis of the T cell populations. We then evaluated the association between PD-L1 expression and median survival time using the protein expression datasets and mRNA from The Cancer Genome Atlas.

Results. The median percentage of PD-L1-expressing cells in GBM by cell surface staining is 2.77% (range: 0%–86.6%; n = 92), which is similar to the percentage found by ex vivo flow cytometry. The majority of GBM patients (61%) had tumors with at least 1% or more PD-L1-positive cells, and 38% had at least 5% or greater PD-L1 expression. PD-L1 is commonly expressed on the GBM-infiltrating T cells. Expression of both PD-L1 and PD-1 are negative prognosticators for GBM outcome.

Conclusions. The incidence of PD-L1 expression in GBM patients is frequent but is confined to a minority subpopulation, similar to other malignancies that have been profiled for PD-L1 expression. Higher expression of PD-L1 is correlated with worse outcome.

Keywords: cancer stem cells, glioblastoma, immune suppression, PD-1, PD-L1.
pembrolizumab) have also been clinically tested in melanoma patients, with FDA approval of nivolumab and pembrolizumab in 2014. Ipilimumab, co-administered with nivolumab in melanoma patients, has demonstrated an objective response rate of 47%, indicating that targeting both of these immune checkpoints can result in potent synergy. Antibody targeting of the PD-1/PD-L1 axis has demonstrated robust preclinical efficacy in an established murine model of glioma, including use in synergy with ipilimumab.15

Currently, Bristol-Meyers Squibb is sponsoring a phase 3 clinical trial of nivolumab and ipilimumab in GBM patients. As such, we wanted to further delineate the prognostic impact of the PD-1/PD-L1 axis in GBM patients, clarify whether the glioblastoma stem cells (GSCs) and/or the infiltrating immune cells are contributing to this mechanism of immune suppression within the GBM microenvironment, and verify if GBMs possess such extraordinarily high levels of PD-L1 expression as has been previously reported.

Methods

Cell Lines
The murine glioma GL261 cell line was obtained from the National Cancer Institute–Friederich Cancer Research Tumor Repository, and the U87 cell line was obtained from ATCC. Both were maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin/streptomycin (Life Technologies), and 1% L-glutamine (Life Technologies). The cell cultures were split (1:2) every 3 days to ensure logarithmic growth. For flow cytometry, the cells were harvested, washed, and then stained for 30 minutes at 4°C (U87, M1H1; GL261, M1H5; BD Biosciences). Cell lines WM35 and HEK293 were obtained from ATCC and maintained in Roswell Park Memorial Institute medium (RPMI) 1640 GlutaMAX supplemented with 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, and penicillin/streptomycin (Life Technologies). GSCs were cultured in Dulbecco’s modified Eagle's medium F-12 containing 20 ng/mL of epidermal growth factor, basic fibroblast growth factor (Sigma), and B27 (1:50; Invitrogen) as a neural stem cell-permissive medium (neurosphere medium) and passaged every 5–7 days. The characteristics of these cells (including the cytogenetics, limiting dilution assays, tumorigenicity, CD133 expression, and immune-suppressive properties) have been previously published.16,17 GSCs were washed, dissociated with Accutase, and examined for PD-L1 expression by flow cytometry (M1H1; BD Biosciences).

Glioma Tumor Microarrays
A GBM-specific tissue microarray (TMA) was constructed under protocol PA12-0136 and contained 99 GBMs that have been previously described.1,18 For TMA construction, two 1 mm cores were obtained per tumor sample. The rationale for using a TMA was to facilitate analysis of the largest number of tumor samples possible. The study neuropathologist (G.N.F.) gathered the tissue sections from the archived paraffin blocks and confirmed the tumor pathologic type. The time from resection to fixation was <20 minutes in all cases, in accordance with the Clinical Laboratory Improvement Amendments standard.

PD-L1 Immunohistochemistry
The antibodies used in this study included Abcam ab 58810, rabbit anti-PD-L1 polyclonal antibody, 1:200 (Abcam); Abcam clone EPR1161(2), rabbit anti-PD-L1 monoclonal antibody (mAb), 1:100, (Abcam); clone 7G11 mouse anti-PD-L1 mAb, 1:40, (kindly supplied by Dr. Gordon Freeman, Harvard Medical School, Boston, Massachusetts); and clone SH1 anti-PD-L1 mAb, 1:100, (kindly supplied by The Mayo Clinic). The 4 μm paraffin-fixed TMA sections were deparaffinized in xylene and rehydrated through graded alcohols (100%, 95%–80%). Antigen retrieval was carried out using citric acid buffer (pH 6.0) for 30 minutes in a steam bath (ab 55810) or using PT Module (EPR1161[2], 7G11, SH1; Lab Vision Corporation). Endogenous peroxidase activity was quenched by immersion in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature (ab 58810) or by 3% hydrogen peroxide for 10 minutes at room temperature (EPR1161[2], 7G11, SH1). Nonspecific binding of the primary antibody was blocked by incubating the sections with 1:100 normal goat serum for 20 minutes at room temperature (ab 58810) or 10% normal horse serum for 30 minutes at room temperature (EPR1161[2], 7G11, SH1). Sections were then incubated with the respective primary antibody at 4°C overnight. The next day, the slides were incubated with 1:200 secondary biotinylated horse anti-rabbit Ig antibody (ab58810, 7G11, SH1; Vectastain ABC Kit; Vector Laboratories) at room temperature for 1 hour or with secondary biotinylated horse anti-mouse Ig/rabbit Ig antibody (EPR1161[2], RTU, Vectastain Universal Elite ABC Kit; Vector Laboratories) at room temperature for 30 minutes. After another 1 hour incubation with the avidin-biotin peroxidase complex (ab58810, 7G11, SH1; Vectastain ABC Kit, Vector laboratories) or 30 minutes incubation with the avidin-biotin peroxidase complex (EPR1161[2] RTU, Vectastain Universal Elite ABC Kit; Vector Laboratories), visualization was performed with the chromagen 3, 3′-diaminobenzidine (ab58810, DAB, Sigma-Aldrich Corp.; EPR1161[2], 7G11, SH1; DAB, Dako). The slides were counterstained with hematoxylin and cover slipped with PerMount.

Ultimately after review with the study neuropathologist (G.N.F.), we found the staining provided by ab58810, 7G11, and SH1 to be unsatisfactory despite optimization. We proceeded with the staining provided by EPR1161(2) for our ultimate quantification and analysis. For positive controls, sections of human placenta, tonsil tissues, and HEK 293 FFPE cell blocks transfected with PD-L1 (Supplementary material, Figure S1 and Supplementary Materials and Methods) were used. Omission of the primary antibody for placenta, tonsil tissue, and HEK293 FFPE cell blocks transfected with PD-L1 was used as a negative control for staining, as well as WT HEK293 FFPE cell blocks with or without primary antibody. Positive cells showed brownish, intense membrane staining, while negative controls and unstained cells were blue.

Imaging was performed using the Perkin Elmer Vectra 2 microscope. To prevent the influence of human error and bias in tabulating our PD-L1 positivity for the survival analysis, quantitation was performed using Perkin Elmer InForm 2.1 software. In brief, an educated user trained the software to identify the
various cell types of interest based on several characteristics including nuclear and cell size, shape, and intensity. Concurrently, the software, using a user-specified threshold, identified DAB-positive cells. Once the system was able to appropriately identify and count the lymphocytes and the threshold was set, a batch analysis was performed on all TMA images. After the analysis was complete, an additional review was performed to confirm that the software had properly identified the desired populations of cells. Cells identified as lymphocytes that were DAB positive were counted and compared with the total cell population.

Glioblastoma Flow Cytometry
To secondarily validate the PD-L1 findings, GBM surgery specimens were processed within 4 hours after resection. Briefly, the tumors were washed in RPMI medium and dissected to remove blood products and surrounding nontumor tissue. The tumor was then mechanically dissociated, enzymatically digested for 1 hour in Liberase at 37°C, passed sequentially through 100 and 70 micron filters, incubated in red cell lysis buffer for 15 minutes, and then spun, blocked, and stained with the PD-L1 antibody (329713; Biolegend) and CD11b antibody (555388; BD Biosciences) or isotype control for 20 minutes at 4°C. For GBM-infiltrating T cell flow cytometry, the filtrate was pelleted by centrifugation and then resuspended in Percoll at a density of 1.03 followed by a 1.095 density underlay. Fluorescence-activated cell sorting (FACS) buffer (PBS + 2% BSA) was gently overlayed on the cell suspension, and the completed gradient was centrifuged for 15 minutes at 1200 g with no brake. Cells were harvested from between the gradients and washed in FACS buffer (PBS + 2% BSA). Cells were refiltered and washed as necessary. T cells were then isolated using the BD Biosciences Human T Lymphocyte Enrichment Set. These cells were then blocked and stained with both CD4 and CD8 antibodies (eBiosciences, 17-0048-41 and 11-0086-41) and either PD-1 or PD-L1 antibodies (Biolegend, 329 905 and 329 705) or the respective isotype controls. Data acquisition was performed using the Beckman Coulter Gallios flow cytometer (Beckman Coulter). Analysis was performed using FlowJo software.

The Cancer Genome Atlas Analysis
Please see the Supplementary Materials and Methods for details on our analysis of The Cancer Genome Atlas (TCGA).

Statistics
For TCGA data analysis, statistical analyses were performed in R (version 3.0.1) (http://www.r-project.org/), and the statistical significance was defined as a P value <.05. For immunohistochemical survival analyses, Kaplan-Meier curves were generated via GraphPad Prism 6 (Graphpad) software, and significance was evaluated using the log-rank (Mantel-Cox) test and Cox proportional hazards models via R-3.1.2 with package survival (v2.38-1). The ANOVA, 2-sided 2-sample test or paired t test, as appropriate, was used for all other data comparisons using GraphPad Prism 6 software. A threshold of P < .05 was used to determine significance in each experiment.

Results
PD-L1 Expression in Glioblastoma Is Not Ubiquitous
In order to elucidate the expression frequency and incidence in GBM of PD-L1 expression, we stained the GBM TMA with 7G11, 5H1, and ab58810 (Supplementary material, Fig. S2). This revealed heterogenous staining, with the vast majority of the cells demonstrating both membrane and cytoplasmic staining. In the case of the ab58810 antibody, >95% of the GBMs on the TMA had robust and ubiquitous expression of PD-L1. Since these results were outside the levels previously reported for other solid malignancies and since we could not secondarily validate these findings using ex vivo flow cytometry, we therefore developed an immunohistochemical approach using clone EPR1161(2). As a control, HEK293 cells were transfected with PD-L1 with an efficiency of 32.7% as examined by flow cytometry (Supplementary material, Fig. S1). On positive controls of placenta and PD-L1 transfected HEK293, we found specific membrane staining (Fig. 1A-C). EPR1161(2) staining was then conducted on the GBM TMA, which demonstrated membrane-specific staining of PD-L1 (Fig. 1D). Frequently, the PD-L1 expression was found on small round cells, which resembled lymphocytes, with lymphocytes representing up to 28.6% of the positive cells counted (mean: 6.28%) (Fig. 1D). PD-L1 has previously been shown to be expressed on T cells and to play a role in dendritic cell maturation.19 We also found cell-surface staining on other, more pleiomorphic cells within the glioblastoma (Fig. 1E). The median percentage of PD-L1-expressing cells within the glioblastoma was 2.77% (range: 0%–86.6%; n = 94). Notably, the median percentage expression of PD-L1 on lymphocytes was enriched to 69.8%. The majority of GBM patients (60.6%) had tumors with at least 1% or more PD-L1-positive cells; 38.3% had at least 5% or greater PD-L1 expression, 17% had 25% or greater expression, and 5.32% had 50% or greater PD-L1 positive cells.

Validation of PD-L1 Expression in Glioblastoma
To determine if the PD-L1 expression was an accurate assessment, we next used a second methodology to confirm the frequency of PD-L1 positive cells in GBM. Direct ex vivo staining of GBMs by flow cytometry demonstrated a median level of 3.5% PD-L1 surface-expressing cells (range: 0.64–6.7%; n = 5) (Fig. 1F and Supplementary material, Fig. S3). We next expanded this analysis to ascertain if the infiltrating lymphocytes were accounting for the PD-L1 expression, given the morphology observed on the immunohistochemical staining. Using ex vivo flow cytometry of the GBM, the CD4 and CD8 T cell populations were sorted, and both populations were found to express PD-L1 and PD-1 (Fig. 2). To ascertain if the GSCs are a contributing population for PD-L1 expression, a panel was tested and found to have minimal expression; however, PD-L1 expression is observed in laboratory glioma cell lines such as GL261 and U87 (Fig. 3).

PD-1/PD-L1 Axis Expression Is a Negative Prognosticator for Survival in Glioblastoma
We grouped patients into percentiles based on the relative expression of PD-1 and PD-L1 mRNA and then determined cutoff
points to significantly split (log-rank test $P$ value <.05) the samples into low/high mRNA groups. For PD-L1 and PD-1, we found that high expression is associated with significantly shorter survival ($P = .023$ and $P = .028$, respectively) (Fig. 4A). The relationship between overall survival (OS) and PD-L1 expression levels (dichotomized at cutoff point 0.37 into low and high) and age (dichotomized at 65 years into younger and older) was examined using a Cox proportional hazards model. Both factors were statistically significant via univariate regression analyses and were included in the multivariable regression model. Patients having high PD-L1 expression levels had an increased estimated risk of death when compared with those having a low relative expression (hazard ratio [HR] = 1.54; 95% CI = 1.05–2.28; $P = .0231$). Older patients had an increased estimated risk of death when compared with younger patients (HR = 1.7; 95% CI = 1.16–2.49; $P = .006$). Multivariate analysis demonstrated that both PD-L1 and age are independent factors negatively impacting survival (PD-L1 high versus low: HR = 1.52; 95% CI = 1.03–2.25; $P = .0343$); older versus younger: HR = 1.68; 95% CI = 1.14–2.47; $P = .0084$). The MGMT promoter methylation status was excluded as a covariate because it was not complete for the 152 patients.

To further evaluate the prognostic influence of PD-L1 at the protein level, we analyzed the immunohistochemical expression and correlated this with survival. Using the median as a cutoff point, we found that patients with >2.77% of PD-L1...
Markers of Cytolytic Activity are Associated With Expression of Immunosuppressive Factors

Recent findings have suggested that cytolytic activity is tied with the expression of immunosuppressive markers. As such, we evaluated surrogate markers of T cell cytolytic activity such as granzyme A and perforin 1 on survival and specifically interacting with PD-L1 expression (Fig. 5). We found that high expression of granzyme A (Fig. 5A), perforin 1 (Fig. 5B), and PD-L1 (Fig. 4B) were indicators of poor survival relative to patients with low expression, including in combination (Fig. 5C and D). These results are likely because of the strong co-associated expression as demonstrated with perforin 1 (PRF1) to PD-L1 (CD274) (r = 0.34; P < .0001; Fig. 6A); perforin 1 with PD-1 (PDCD1) (r = 0.37; P < .0001; Fig. 6B); granzyme A (GZMA) and PD-1 (r = 0.54; P > .0001; Fig. 6C) and granzyme A with perforin (r = 0.55; P < .001; Fig. 6D). We also examined the association between PD-1 and PD-L1 with T cell markers such as CD3, CD4, and CD8. As expected, PD-1 was associated with all markers of T cell subsets including CD3, CD4, and CD8. Interestingly—and confirmatory of the immunohistochemistry and ex vivo flow cytometry—was an association of PD-L1 with CD3 (r = 0.21; P = .0099) and CD4 (r = 0.33; P < .001) (Supplementary material, Fig. S4) indicating the association with suppressed/exhausted T cells and/or Tregs.

Discussion

Precision medicine, in which a therapeutic is matched to the genetic features of a malignancy, will become increasingly more common, even in the context of immune therapeutics. The precedent for this type of strategy in GBM was set by the selection of patients with GBM tumors that express the epidermal growth factor receptor variant III to receive rindopepimut. Currently, there is an active phase 2 clinical trial of a PD-1 inhibitor in GBM patients that does not require a specified expression level of PD-L1 (NCT02017717). An analysis of PD-L1 expression in GBM tumors has recently been published, in which 88% of newly diagnosed GBMs demonstrated robust, diffuse, and near-ubiquitous staining. By the investigators’ own admission, this was acknowledged as being highly relative to other types of tumors such as melanoma and non–small cell lung carcinoma, in which only about 30% have PD-L1 expression. In the previous GBM PD-L1 expression study, the investigators used the noncommercial 5H1 anti-PD-L1 antibody, which was used to correlate the clinical response in (P = .0031) (Fig. 4C). The multivariate Cox proportional hazards regression model was also used to investigate the conditional effects of PD-L1 expression to OS, given other demographic and clinical factors (Supplementary material, Table S1). In the multivariate analysis, low PD-L1 expression was still predictive of long OS (HR = 0.554; P = .018). Such an association was unchanged when the dichotomized PD-L1 expressed was replaced with continuous measure (HR = 1.016; P = .0089). None of the interactions between PD-L1 expressions with other covariates such as age, sex, KPS, and extent of resection were significant, indicating that PD-L1 is a prognostic factor for OS in the multivariate setting.
the melanoma clinical trial. However, this correlation has recently been called into question. Notably, Bristol-Meyers Squibb is using the 28-8 antibody for the Phase 3 GBM clinical trial, for which the commercial kit should be available soon from AbCam. The challenges of companion biomarkers, including their sensitivity and specificity, staining methods, and antibodies used (including their intrinsic abilities to detect various levels of PD-L1 expression, their cutpoints for being declared positive, and variability, among other limitations) have been previously documented and provide significant logistical challenges for the implementation of precision medicine.

Because the incidence of PD-L1 expression on GBM was unclear using different immunohistochemical analysis, 2 methods were used here to define PD-L1 expression in GBM. The ex vivo flow cytometry and immunohistochemical analysis both yielded modest but consistently positive PD-L1 expression in most GBM samples, further supporting the validity of these methods. While the total number of PD-L1 positive-staining cells was not routinely high, we used a stringent immunohistochemical testing protocol validated with a PD-L1-expressing U293 cell line. Our reported PD-L1 expression frequency is consistent with the descriptions of PD-L1 expression by other groups.

Fig. 3. PD-L1 expression on glioma cells. (A) Histogram plots showing expression levels of PD-L1 on glioma stem cells. (B) Histogram plots showing expression levels of PD-L1 on representative glioma cell lines. The dotted line represents the isotype control, and the black line denotes PD-L1 expression.
Given the discrepancy between the findings of Berghoff et al and our group reporting a median percentage of PD-L1 expressing cells of 2.7%, there may be a significant difference of anticipated benefit to checkpoint blockade therapeutics and need for stratification. Multiple steps are required for an optimized antitumor immune response including the presence of an antigenic target, immune cell activation, trafficking of lymphocytes to the tumor microenvironment, and maintenance of the effector function. It is possible that a small amount of PD-L1 expression could be sufficient to restrain the immune system and that the clinical responders will be the subset of patients with antigen-enriched tumors in which there are preexisting T cells within the GBM microenvironment, and maintenance of the effector function. However, if we accept the adaptive resistance model proposed in melanoma, where brisk T cell infiltrates induce PD-L1 expression through mediators such as IFN-γ,33,34 it is possible that the relatively low PD-L1 expression found here could be indicative of relatively low T cell infiltration, specifically of CD8+ cells and their associated pro-inflammatory cytokines in the tumor microenvironment, which would mean that most GBMs would not respond to PD-1 blockade therapy. This is also akin to recent data emerging on EGFR- and ALK-driven non–small cell lung cancers, in which relatively low levels of T cell infiltrates and PD-L1 expression on tumor cells were found compared with more mutated smoking-related lung cancers.35 In fact, the possibility of PD-1 blockade being contraindicated in these lung cancers versus more highly mutated smoking-related lung cancer is now being debated, and this may also need to be considered in GBM.

Preclinical testing of immune checkpoint efficacy has been conducted in clonotypic model systems.2,15 Given the heterogeneity and lower levels of PD-L1 in human GBM relative to the clonotypic cell lines, the therapeutic benefit in patients may be lower than that observed within these models. We found that commonly used glioma cell lines demonstrated robust expression of PD-L1. However, GSCs do not express appreciable levels of PD-L1. Testing the therapeutic effects of checkpoint inhibitors would be challenging in humanized GSC xenograft murine models. However, PD-L1 knockout glioma cell lines could be created and mixed with the PD-L1-expressing parental lines to determine the threshold level of expression that correlates with a therapeutic effect. This expression level...
could then be validated in a prospective manner in the clinical trial. Alternatively, the threshold level of PD-L1 expression and clinical response may be identified in a retrospective manner in ongoing clinical trials. On a final note, our study used an automated system for non-biased quantification of PD-L1 positive status. Once the computer is properly trained, multiple slides can be quantified quickly, easily, and reproducibly, allowing for reliable stratification of patients in clinical trials.

In contrast to the previously reported modest improvement in survival across various tumor types with high expression of cytolytic factors, we found the opposite result in GBM patients. High expression of GZMA and PRF1 were significantly associated with lower survival in GBM patients. This discrepancy can be reconciled if negative prognostic, immune suppression predominates and overrides the effector responses. By both immunohistochemistry and flow cytometry, tumor-infiltrating lymphocytes expressed PD-L1. PD-L1 expression has been previously documented on suppressed T cells, and our findings would support the notion that PD-L1 expression on T cells within the GBM microenvironment may represent a highly suppression-prone phenotype, especially since the simultaneous expression of GZMA/PRF1 with PD-1 may be a marker for these cells. Finally, the operational mechanisms of immune suppression may be different in various GBM subsets since PD-L1 expression has been shown to be more pronounced in the mesenchymal GBM type, which has also been shown to have a higher inflammatory effector signature relative to other subtypes.

In the survival analysis using both the TCGA and TMA data sets, our study found that high PD-L1 expression is a statistically significant negative prognosticator. When the TCGA dataset was analyzed using a multivariate assessment of PD-1 and PD-L1 expression, significant impact was found on outcome. The Bergoff et al study found no prognostic impact of PD-L1 expression in GBM patients. However, they used level 2 Agilent microarray gene expression data for their analysis. Indeed, when
we reviewed that same data, we did not find any survival differ-
ence between high- and low-expressing groups, but we did
find differences when using the RNASeq data, which were con-
sistent with the outcomes obtained from the GBM TMA. Previ-
ous studies have shown that the 2 Agilent and the RNASeq data
are often discordant.43

**Conclusion**

PD-L1 expression in GBM patients is frequent but is largely con-
ained to a minority subpopulation—similar to other malignan-
cies that have been profiled for PD-L1 expression. Higher
expression of PD-L1 is correlated with worse outcome, and
proper stratification of PD-L1-positive and -negative patients
may become an important criterion for high-quality immuno-
therapeutic trials in GBM. Expression of PD-L1 on infiltrating
lymphocytes also suggests that a unique immune-suppressive
pathway may operate in GBM.

**Supplementary Material**

Supplementary material is available at Neuro-Oncology Journal
online (http://neuro-oncology.oxfordjournals.org/).

**Fig. 6.** Correlation of markers of cytotoxicity and immunsuppression in glioblastoma. (A) High expression of PRF1 is correlated positively with
PD-L1. (B) High expression of PD-1 is positively correlated with PRF1 and (C) GZMA. (D) GZMA and PRF1 expression are positively correlated with
each other.

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**References**

1. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, et al. Incidence and
prognostic impact of Foxp3+ regulatory T cells in human gliomas.


