Clinical Cancer Research

Expression of PD-1 by T Cells in Malignant Glioma Patients Reflects Exhaustion and Activation

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Abstract

Purpose: Glioblastoma (GBM) is the most common primary malignant tumor in the central nervous system. Our recent preclinical work has suggested that PD-1/PD-L1 plays an important immunoregulatory role to limit effective antitumor T-cell responses induced by active immunotherapy. However, little is known about the functional role that PD-1 plays on human T lymphocytes in patients with malignant glioma.

Experimental Design: In this study, we examined the immune landscape and function of PD-1 expression by T cells from tumor and peripheral blood in patients with malignant glioma.

Results: We found several differences between PD-1$^+$ tumor-infiltrating lymphocytes (TIL) and patient-matched PD-1$^+$ peripheral blood T lymphocytes. Phenotypically, PD-1$^+$ TILs exhibited higher expression of markers of activation and exhaustion than peripheral blood PD-1$^+$ T cells, which instead had increased markers of memory. A comparison of the T-cell receptor variable chain populations revealed decreased diversity in T cells that expressed PD-1, regardless of the location obtained. Functionally, peripheral blood PD-1$^+$ T cells had a significantly increased proliferative capacity upon activation compared with PD-1$^-$ T cells.

Conclusions: Our evidence suggests that PD-1 expression in patients with glioma reflects chronically activated effector T cells that display hallmarks of memory and exhaustion depending on its anatomic location. The decreased diversity in PD-1$^+$ T cells suggests that the PD-1–expressing population has a narrower range of cognate antigen targets compared with the PD-1 nonexpression population. This information can be used to inform how we interpret immune responses to PD-1–blocking therapies or other immunotherapies.

Introduction

Glioblastoma (GBM) is the most common primary brain tumor in adults and remains one of the most lethal of all human cancers (1). Current standard of care includes surgical resection, chemotherapy, and radiotherapy and is associated with a median survival of 12 to 18 months (2–4). The dismal outcome of this disease clearly highlights the need for new approaches in therapy. Novel treatment strategies, such as GBM-targeted immunotherapy and vaccination therapy, have shown promise in both preclinical and clinical trials (5–9). However, the survival benefit has been indeterminate. Antitumor immune responses induced by immunotherapy and, more specifically active vaccination in GBM, may in turn induce an adaptive immune resistance mechanism mediated by immune checkpoints such as signaling of programmed death 1 and its ligand (PD-1/PD-L1; refs. 10–12).

PD-1 is a cell surface coinhibitory receptor that is expressed on T cells after activation and limits the immune response, resulting in what are traditionally considered functionally

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Translational Relevance

Inhibition of the PD-1/PD-L1 axis is currently being explored as a potential immunotherapy for patients with malignant gliomas. Typically, PD-1 expression on T cells in the context of cancer has been exclusively thought of as a marker of exhaustion, and current PD-1 checkpoint therapies are thought to be effective due to their ability to reactivate exhausted T-cell populations. In our study, we sought out to define and characterize the PD-1 expression on T cells in the context of malignant gliomas. We found that PD-1 expression on T cells in the tumor reflects both exhaustion and chronic activation. However, in peripheral blood T cells, PD-1 expression reflects antigen experience and chronic activation more than exhaustion. The results from this study may help inform our use of PD-1 as a biomarker and also be used to assess the clinical efficacy of using PD-1 checkpoint blockade in this malignancy.

"exhausted" T cells (13, 14). In normal physiologic states, the function of this immune checkpoint protein is to inhibit or reduce autoimmunity and to maintain homeostasis during chronic infections (15, 16). In the setting of cancer, the PD-1/PD-L1 pathway may be an adaptive mechanism by the tumor to evade antitumor immune attack. Although PD-1 has previously been considered to be a marker of terminally differentiated exhausted T cells, PD-1 expression may in fact be a marker of continued effector or memory function needed to maintain pathogen or tumor control while minimizing tissue damage in a chronic state (17). In fact, a number of recent studies have shown that PD-1 expression of $CD8^+$ T cells early after activation may temporally coincide with rapid proliferation and production of signature effector cytokines and be an important signaling pathway in programming of memory $CD8^+$ T cells (18–20).

In this study, we set out to define and characterize PD-1 expression in tumor-infiltrating lymphocytes (TIL) and peripheral blood from patients diagnosed with malignant glioma. Numerous studies have shown PD-L1 expression in GBM as well as PD-1 expression in a subset of GBM TILs (10, 12, 13). PD-1 expression on malignant glioma TILs in comparison with peripheral blood has not been investigated, nor has the function of these malignant glioma PD-1^+ TILs been examined in this detail. We found that the proportion of TILs expressing PD-1 was significantly elevated when compared with T cells in patient peripheral blood and T cells from healthy controls. Furthermore, we found increased levels of activation and exhaustion markers on PD-1^+ TILs when compared with PD-1^+ peripheral blood T cells, while we found increased markers of memory/antigen experience on the peripheral blood PD-1^+ T cells. Our results highlight the complex relationship between PD-1 expression on T cells and its relation to exhaustion and chronic activation within human malignant brain tumors.

Materials and Methods

Patients

We collected 52 patient tumor and blood specimen samples and an additional 14 peripheral blood samples from healthy donors at University of California, Los Angeles (Los Angeles, CA). In parallel, matched TILs and peripheral blood were obtained from 6 consenting patients with glioblastoma who presented to the University of Heidelberg (Heidelberg, Germany) neurosurgery service for surgical removal of their tumors. All patients provided written informed consent, and this study was conducted in accordance with recognized ethical guidelines and under Institutional Review Board–approved protocols from both institutions.

Collection of PBMCs for immune monitoring

Peripheral blood was drawn from patients prior to surgical resection of their gliomas. Once drawn, we diluted peripheral blood at 1:1 dilution of RPMI media (Thermo Fisher Scientific catalog no. MT10041CV); peripheral blood mononuclear cells (PBMC) were harvested through subsequent extraction with Ficoll (Thermo Fisher Scientific catalog no. 45-001-750) gradient. After a series of two washes in RPMI media, we placed cells in freezing media made of 90% Human AB serum (Thermo Fisher Scientific catalog no. MT35060CI) and 10% DMSO (Sigma catalog no. C6295-50ML) and stored in liquid nitrogen.

Collection of TILs

TILs were collected in two ways. In the first method, we collected tumor and digested the tissue via incubation with collagenase (1 mg/mL; Advance Biofactures Corporation) and DNAse (0.02 mg/mL; Sigma catalog no. D4263-5VL) in RPMI media overnight on the day of resection. TILs were isolated using Percoll (Sigma catalog no. P4937) gradient and washed in RPMI twice. In the second method, patient-resected tumors were first digested using Miltenyi's Brain Tumor Dissociation Kit (Miltenyi Biotec catalog no. 130-095-42) and gentleMACS Dissociator (catalog no. 130-093-235) and labeled with $CD45^+$ microbeads (catalog no. 130-045-801). $CD45^+$ cells were positively selected for using Miltenyi LS columns (catalog no. 130-042-401) and MidiMACS Separator. TILs were frozen in the same freezing media as the PBMCs.

Flow cytometric analysis of PBMCs and TILs

We thawed donor PBMCs and patient PBMCs and TILs at 37°C for 3 minutes in a water bath and immediately washed with RPMI media to remove DMSO. We resuspended cells at 1×10^6 cells/100 μL PBS.

In the characterization of PD-1 expression of TILs and PBMCs, we first stained cells with live-dead Zombie Yellow stain (BioLegend, catalog no. 423104). In two separate experimental reactions, we stained cells with a 7-antibody and an 8-antibody panel. Two antibody panels were necessary to investigate all of the surface markers of interest due to the spectral limitations of the flow cytometer. The first antibody cocktail included seven antibodies: CD3 Pe-Cy7 (eBioscience, catalog no. 25-0038-42), CD4 Alexa Fluor 700 (BD Biosciences, catalog no. 557922), CD8 Pacific Blue (BD Biosystems, catalog no. 558207), CD16 FITC (BD Biosystems, catalog no. 555406), CTLA-4 PE (BD Biosystems, catalog no. 555853), PD-1 AF647 (eBioscience, catalog no. 51-9969-73), and CD69 PerCP-CY5.5 (BD Biosystems, catalog no. 560738) antibodies where each antibody was diluted to a 1:40 antibody to buffer ratio. The second 8-antibody cocktail contained CD3 Pe-Cy7 (eBioscience, catalog no. 25-0038-42), CD4 Alexa Fluor 700 (BD Biosystems, catalog no. 557922), CD8 Pacific Blue (BD Biosystems, catalog no. 558207), CD16 FITC (BD Biosystems, catalog no. 555406), CD19 PE (BD Biosystems, catalog no. 555413), CD25 APC-Cy7 (BD Biosystems, catalog

no. 557753), CD127 AF647 (BD Biosystems, catalog no. 558598), and CD69 PE-Cy5 (BD Biosystems, catalog no. 555532) antibodies at the same dilution as above.

We used a BD LSRII flow cytometer and the BD FACS diva software to analyze all cell samples. Cytometer settings were restricted to the nine colors used. We used unstained controls to adjust FSC and SSC voltages such that the lymphocyte population became visible and then acquired single-color compensation controls, adjusting voltages such that the stained cells did not exceed 10^5. We set gates on stained controls in comparison with the negative control and recorded data from all samples, while subsequently applying compensation controls. We recorded staining expression at 80,000 to 100,000 events per sample, as the quantity of T cells was variable from specimen to specimen. Thus, we analyzed the data acquired from the flow cytometer by using frequency measurements rather than absolute numbers to compare sample expression profiles using FlowJo.

Mass cytometry

Patient PBMCs and TILs were collected as described above. Between 1 to 2 million patient PBMCs and TILs were labeled with Cell-ID Cisplatin (Fluidigm, catalog no. 201064) to assess for live/dead and then labeled with a variety of metal-conjugated antibodies. From the patient PBMCs, 1×10^6 cells were set aside and labeled with a smaller panel containing only cell lineage markers to aid in setting the proper gates of other markers. After labeling, cells were fixed using MaxPar Fix and Perm buffer (Fluidigm, catalog no. 201067) and labeled for single-cell discrimination with Cell-ID Intercalator-Ir (Fluidigm, CA 201192A) and run on the Helios CyTOF system.

The collected mass cytometry data were analyzed as described previously (21). Briefly, samples were normalized utilizing a bead standard, then manually gated to remove debris and dead cells. Raw marker intensities were transformed using hyperbolic inverse sine with cofactor of 5. Cell population identification was carried out utilizing the FlowSOM (22) and ConsensusClusterPlus packages in R using 11 lineage markers, with the number of clusters determined by elbow criterion. For differential analysis of cell population abundance and functional marker expression, we fit fixed and mixed linear models to our data and performed testing using the general linear hypothesis function glht in R. To account for multiple testing, we applied the Benjamini–Hochberg adjustment with a false discovery rate of 5%.

FACS of $CD3^+$ $PD\text{-}1^+$ and $CD3^+$ $PD\text{-}1^-$ T-cell populations

As described above, we thawed frozen donor PBMCs and patient PBMCs and TILs, stained samples with CD3 Pe-Cy7 (eBioscience, catalog no. 25-0038-42) and PD-1 AF647 (BD Biosystems, catalog no. 558598) and sorted samples with the Aria II cell sorter (Becton Dickinson) into $PD\text{-}1^+$ and $PD\text{-}1^-$ T-cell populations. We defined $PD\text{-}1^+$ T cells as $CD3^+$ and $PD1^+$ and $PD\text{-}1^-$ T cells as $CD3^+$ and $PD1^-$.

RNA gene expression

We used the NanoString nCounter Dx Analysis System, which measures the expression of 511 human immune function genes through digital readouts of the abundance of mRNA transcripts to analyze cell lysates of PD-1–expressing and PD-1 void cells (23). The nCounter Dx Analysis System uses gene-specific probe pairs that hybridize directly to mRNA samples. A Reporter Probe carries the fluorescent signal; a Capture Probe allows the complex to be immobilized for data collection. We included, in each assay, six positive quality controls comprised of a linear titration of *in vitro* transcribed RNA transcripts and corresponding probes, and eight negative quality controls consisting of probes with no sequence homology to human RNA sequences. Each Human Immunology assay run includes a reference sample consisting of *in vitro* transcribed RNAs of the 13 targets that are used for normalization purposes.

TCR V-β sequencing

We extracted genomic DNA from sorted $CD3^+$ $PD\text{-}1^+$ and $CD3^+$ $PD\text{-}1^-$ T cells from malignant glioma patient TILs and PBMCs and from healthy donor PBMCs using DNeasy Blood & Tissue Kit (Qiagen catalog no. 69504) and sequenced DNA at the T-cell receptor (TCR) V-β CDR3 region using high-throughput sequencing in collaboration with Adaptive Biotechnologies, as we have recently published (24).

IFNγ activation assay

$CD3^+$ $PD\text{-}1^-$ and $CD3^+$ $PD\text{-}1^+$ cells were sorted in a similar manner described above. In a 96-well plate, we added 2×10^5 cells into each well at a concentration of 1×10^6 cells/mL. For the cells that were to be activated, we added 50 ng/mL anti-CD3 Ab (BioLegend, catalog no. 317304) and 1 μg/mL anti-CD28 Ab (BD Biosciences, catalog no. 555725). In all wells, we added 50 IU/mL IL2. After 24 hours, the supernatant was collected and fresh media with 50 IU/mL IL2 were added in each well, and the supernatant was collected 24 hours later (total of 48 hours after activation). To measure IFNγ levels, we used the Human IFNγ Uncoated ELISA Kit (Thermo Fisher Scientific, catalog no. 88-7613-86).

Statistical analysis

The percentage of T lymphocytes expressing the various T-cell markers studied were compared between patient PBMCs, TILs, and donor PBMCs. Unpaired Welch *t* test was applied to compare the means of the frequencies of expression with two-sided *P* values (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

Results

Glioma-infiltrating T cells have elevated expression of PD-1 and other markers of T-cell exhaustion and activation compared with peripheral T cells

We recently demonstrated a critical role for PD-L1 on T cells infiltrating murine gliomas, as well as analogous human tumors (10, 12). To examine the role of PD-1 in human glioma, we first analyzed the PD-1 expression profile using traditional flow cytometric approaches in TILs and matched peripheral blood from 14 patients with malignant glioma undergoing surgical resection at UCLA Medical Center (Los Angeles, CA; Fig. 1A). The clinical and pathologic characteristics of our patient population are outlined in Table 1. Similarly, we simultaneously analyzed the expression of PD-1 in the peripheral blood T cells of 14 healthy donors (Supplementary Fig. S1A).

PD-1 was significantly elevated in the $CD3^+$ TIL population (mean ± SE: 51.7% ± 7.2%) compared with the $CD3^+$ glioma patient PBMC population (19.7% ± 1.8%, $P = 0.0005$) and healthy donor PBMCs (17.0% ± 2.4%, $P = 0.02$; Fig. 1A). The proportion of TILs expressing PD-1 was significantly elevated in

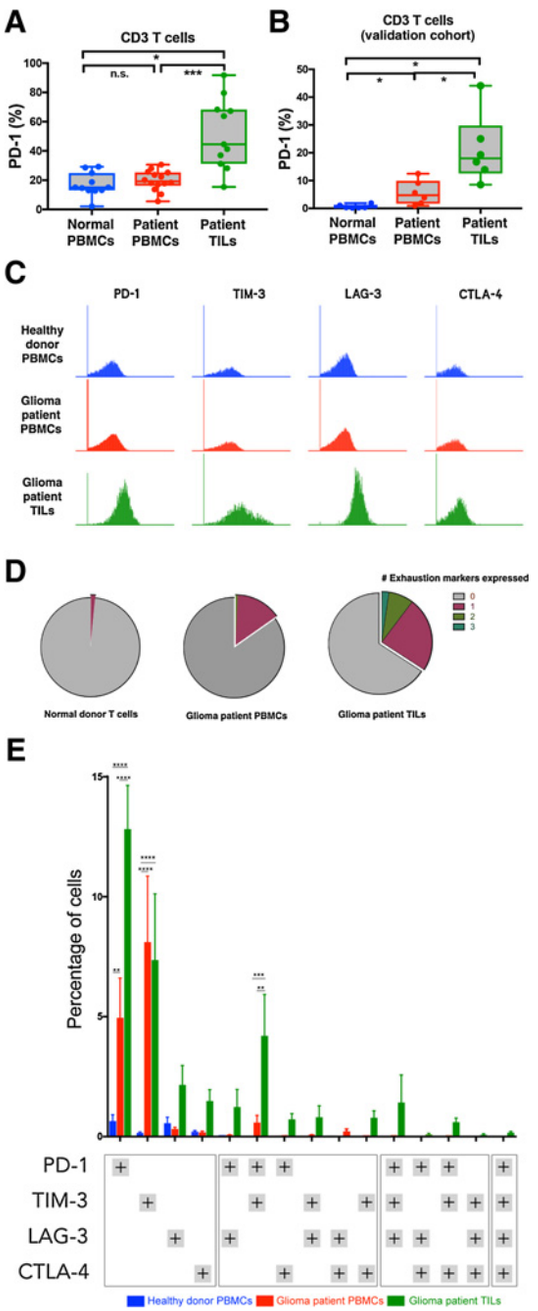

Figure 1.

CD3$^+$ T cells from patients with malignant glioma demonstrate significantly elevated expression of PD-1 compared with healthy donor PBMCs and glioma patient PBMCs. **A,** Percent of PD-1 expression on CD3$^+$ T cells from all the patient samples as measured by flow cytometry (*, $P < 0.05$; **, $P \leq 0.005$; *** $P \leq 0.0005$). **B,** The percentage of PD-1–expressing CD3$^+$ T cells from a validation cohort of patients with malignant glioma from the University of Heidelberg (*, $P \leq 0.05$). **C,** Histogram of the expression levels of the different exhaustion markers (PD-1, TIM3, LAG3, CTLA-4). **D,** Percentage of CD3$^+$ T cells that express one or more markers of exhaustion (PD-1, TIM3, LAG3, CTLA-4). **E,** The percentage of cells expressing different combinations of the various exhaustion markers (**, $P \leq 0.005$; ***, $P \leq 0.0005$). Blue bars, healthy donor PBMC samples; red bars, glioma patient PBMC samples; and green bars, glioma patient TIL samples.

the CD3$^+$CD4$^+$ T-cell population (mean ± SE: 53.2% ± 6.8%) as compared with patient peripheral blood (17.6% ± 2.6%, $P < 0.0004$) or donor peripheral blood (17.3% ± 1.9%, $P < 0.0001$; Supplementary Fig. S1B). Similarly, the percentage of CD3$^+$CD8$^+$ cells expressing PD-1 was also significantly higher in TILs (51.9% ± 7.39%) than on patient peripheral blood T cells (22.5% ± 3.13%; $P < 0.0023$) or donor peripheral blood T cells (15.9% ± 2.24%; $P < 0.0005$; Supplementary Fig. S1C). To validate these findings independently, PD-1 expression was analyzed on GBM TILs, matched patient peripheral blood T cells, and normal controls from a separate cohort of 6 patients with GBM and 6 healthy controls at the University of Heidelberg (Heidelberg, Germany). Similarly, PD-1 expression was significantly increased in the GBM TIL population when compared with matched patient peripheral blood T cells and healthy patients' T cells (Fig. 1B).

Because PD-1 has typically been associated with an exhausted phenotype, in addition to PD-1, the expression of three other exhaustion markers (CTLA-4, TIM-3, and LAG-3) was measured in the patients from our German validation cohort (Fig. 1C). The GBM TIL population showed greater expression of TIM-3 and LAG-3 and marginal increase in expression of CTLA-4 compared with both healthy donor PBMCs and patient PBMCs. To examine this further, we noted the percentage of cells in each population that showed one more of the exhaustion markers (Fig. 1D). The GBM TIL population showed the largest subpopulation of cells that expressed at least one of the exhaustion markers and the only population that contained cells that expressed more than two of the exhaustion markers. In contrast, peripheral blood T cells from matched patients rarely had up to two markers of exhaustion and T cells from healthy controls almost uniformly had no markers of exhaustion except for a small percentage with only one marker. In addition, we examined the proportion of CD3$^+$ cells within each combination of exhaustion markers. There was a greater percentage of CD3$^+$ TILs expressing two or more exhaustion markers across all the different combinations of exhaustion markers than PBMCs from the patients or controls (Fig. 1E). Our results suggest that malignant glioma patients possess elevated frequencies of PD-1 and other exhaustion markers on T cells from the tumor and in the peripheral blood compared with normal healthy volunteers.

To explore further the findings from our standard fluorescent antibody–based flow cytometry, we conducted mass cytometry (21, 25, 26) on an additional 28 matching high-grade glioma patient TILs and PBMCs. A panel of immune lineage, memory, trafficking, activation, and exhaustion markers were used to stain these paired immune cell populations simultaneously (Supplementary Table S1). To analyze the CD3$^+$ PD-1$^+$ population from this set of patients, we employed FlowSom (22), an R package that uses machine learning algorithms to analyze mass cytometry data in an unbiased manner. We ran a principal component analysis using the median expression levels of all the T-cell markers from each individual patient as variables. An unsupervised clustering algorithm was used to generate a tSNE map of phenotypically defined population clusters. The principal component analysis showed a clear separation between the CD3$^+$ PD-1$^+$ PBMCs and the CD3$^+$ PD-1$^+$ TILs (Fig. 2A). The unsupervised clustering algorithm grouped a majority of the CD3$^+$ PD-1$^+$ TILs in distinct clusters from the CD3$^+$ PD-1$^+$ PBMCs (Fig. 2B; Supplementary Fig. S2A).

We next looked at each individual marker and its median expression levels in all the PBMCs and TILs. For each individual

Table 1. The clinical and pathological characterization of our patient population

	Flow cytometry/gene expression/TCR sequencing	Mass cytometry	Activation IFNγ studies	All studies
Total number	20	28	4	52
Mean age at diagnosis	51.3 years	51.4 years	43.1 years	50.7 years
Diagnosis	Number (percent)	Number (percent)	Number (percent)	Number (percent)
Glioblastoma	18 (90.0%)	20 (71.4%)	3 (75.0%)	41 (78.8%)
Gliosarcoma	0 (0%)	1 (3.6%)	0 (0%)	1 (1.9%)
Anaplastic oligodendroglioma	0 (0%)	3 (10.7%)	0 (0%)	3 (5.8%)
Anaplastic astrocytoma	2 (10.0%)	4 (14.3%)	1 (25.0%)	7 (13.5%)
Sex				
Male	13 (65.0%)	17 (60.7%)	4 (100%)	34 (65.4%)
Female	7 (35.0%)	11 (39.3%)	9 (0%)	27 (51.9%)
IDH				
Mutated	4 (20.0%)	7 (25.0%)	2 (50.0%)	13 (25.0%)
Wild type	15 (75.0%)	21 (75.0%)	2 (50.0%)	38 (73.1%)
Unknown	1 (5.0%)			1 (1.9%)
MGMT				
Methylated	10 (50.0%)	11 (39.3%)	0 (0%)	21 (40.4%)
Nonmethylated	10 (50.0%)	16 (57.1%)	3 (75.0%)	29 (55.8%)
Unknown		1 (3.6%)	1 (25.0%)	2 (3.8%)
Presentation				
New diagnosis	14 (70.0%)	16 (57.1%)	0 (0%)	30 (57.7%)
Recurrent	6 (40.0%)	12 (42.9%)	4 (100%)	22 (42.3%)

marker, we highlighted the regions on the generated tSNE map where the marker was most highly expressed. For the T-cell exhaustion markers, there was significant increase in TIM-3, PD-L1, and CTLA-4 in the TIL population compared with the PBMC population (Fig. 2C). Among T-cell activation markers, there was a significant increase in expression of CD38 and HLA-DR in the TIL population compared with the PBMC population (Fig. 2D). Meanwhile, among the antigen experience and T-cell memory markers: CD45RA, CD27, and CD127, there was a significant decrease in the TIL population compared with the PBMC population (Fig. 2E). In 4 recent patients, two additional T-cell exhaustion markers CD39 and TIGIT were incorporated into our panel, and the expression levels were measured between the PBMC and TIL groups. We found a significant increase in CD39 in the TIL population (Supplementary Fig. S2C) but a nonsignificant difference in TIGIT expression between the two groups (Supplementary Fig. S2d). A heatmap of each analyzed patient's marker expression level was also generated. There was increased expression of various exhaustion and activation markers in the TIL population and increased expression of antigen experience markers in the PBMC population (Fig. 2F). The results generated from the mass cytometry data strongly suggest phenotypic differences exist between $CD3^+$ $PD\text{-}1^+$ cells from malignant glioma patient TILs and PBMCs.

To further investigate the possible relationship of PD-1 expression with T-cell exhaustion, activation, and memory phenotypes, we sought to characterize the gene expression of the $PD\text{-}1^-$ and $PD\text{-}1^+$ cells by performing a tailored comparison panel on the PBMCs and TILs from three randomly selected patients and PBMCs from one healthy individual using RNA hybridization probe technology (23). When the normalized RNA counts of $PD\text{-}1^+$ T cells and $PD\text{-}1^-$ T cells were divided to create a ratio, we found that the ratio of $PD\text{-}1^+/PD\text{-}1^-$ gene expression in malignant glioma TILs had an elevated fold change for markers of exhaustion (TIM-3, CTLA-4, TIGIT) when compared with the peripheral blood T cells (Supplementary Fig. S3A). Gene expression analysis of the same samples uncovered an increase in several activation markers (OX-40, 4-1BB, CD25, BIM, ICOS) of TILs when compared with patient peripheral blood T cells (Supplementary Fig. S3B). We saw a reversal of this $PD1^+/PD1^-$ fold change for markers of memory (Supplementary Fig. S3C). These data suggest that PD-1 expression reflects activation and exhaustion in TILs and may be a marker of antigen experience in peripheral blood T cells of malignant glioma patients, similar to our findings from our phenotypic analyses.

$PD\text{-}1^+$ T cells demonstrate decreased TCR diversity

We used high-throughput DNA sequencing of the TCR V-β region to determine the TCR diversity of $CD3^+$ $PD\text{-}1^+$ and $CD3^+$ $PD\text{-}1^-$ T cells FACS-sorted from one matched patient tumor and PBMC samples, two additional distinct patient PBMC samples, and normal donor PBMCs. Because our data suggested that PD-1 reflects antigen experience, activation, and exhaustion in our malignant glioma patient samples, it was unclear what the TCR repertoire would reflect. In fact, we saw an increase in the diversity of $PD\text{-}1^-$ T cells from all populations of sorted T cells (Fig. 3A). As expected, we saw a trend toward the least amount of TCR diversity in the TIL population, but our limited number of sorted TIL precludes a clear assessment. Among the predicted productive TCR rearrangements, we also saw an expansion of clonal T-cell populations in the PBMC and TIL populations in patients with malignant glioma, possibly signifying expansion of tumor antigen-specific T cells not seen in healthy donors (Fig. 3B). These findings suggest that $PD\text{-}1^+$ T cells are enriched for a restricted TCR population, regardless of where the cells were obtained.

$PD\text{-}1^+$ peripheral T cells of patients with malignant gliomas exhibit an increased rate of activation compared with $PD\text{-}1^-$ T cells

To examine the functional differences between these T-cell populations, we sorted $PD\text{-}1^+$ and $PD\text{-}1^-$ $CD3^+$ peripheral blood T cells from patients with malignant glioma. Because of limited numbers of $PD\text{-}1^+$ T cells in nonexpanded PBMCs, we pooled together PBMCs from multiple patients. The sorted T cells were

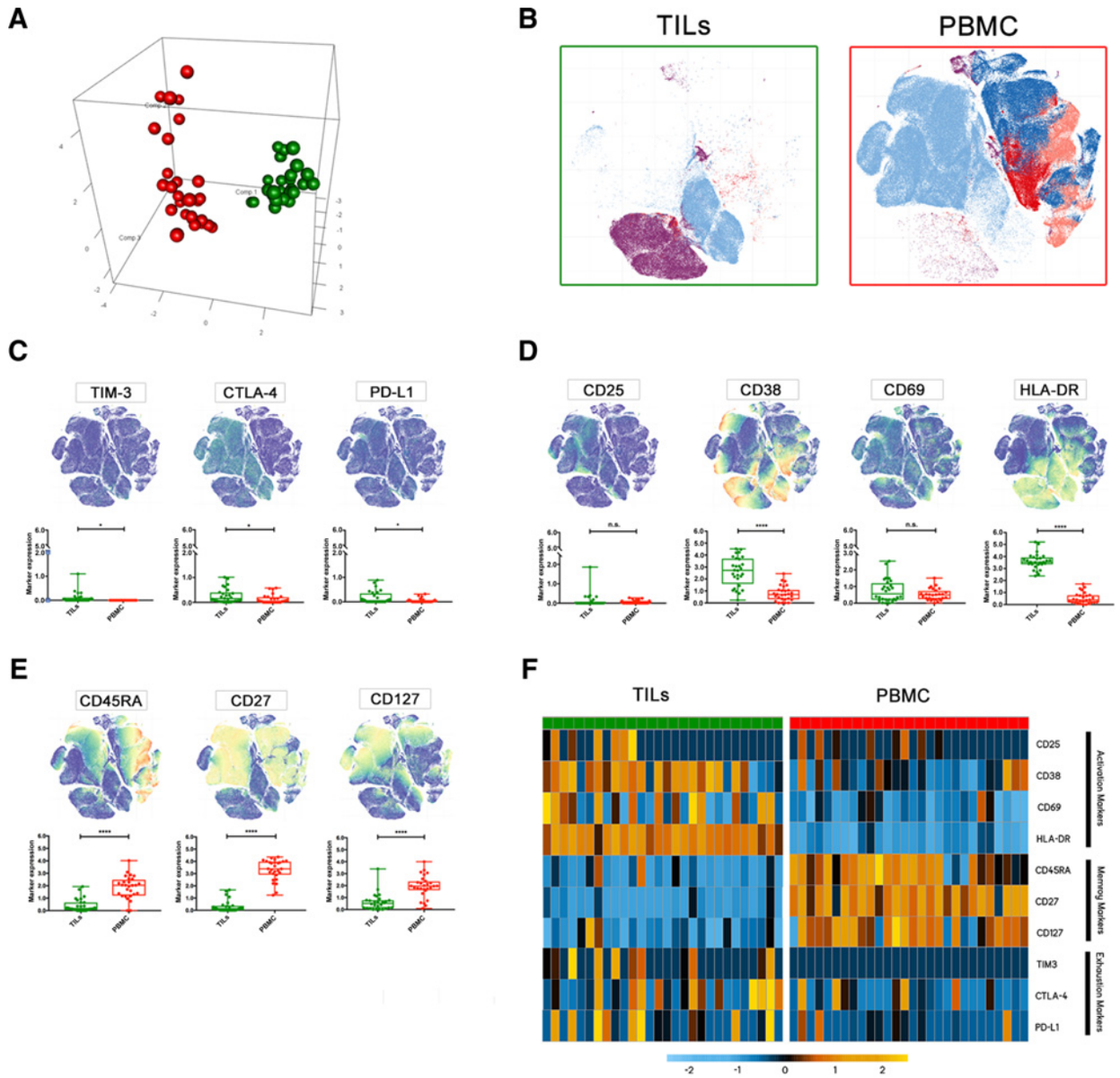

Figure 2.

High dimensional analysis of mass cytometry data shows elevated expression of markers of activation and exhaustion on $CD3^+$ $PD\text{-}1^+$ TILs and markers of activation and antigen experience on $CD3^+$ $PD\text{-}1^+$ PBMCs from patients with malignant glioma. **A,** A PCA plot showing $CD3^+$ $PD\text{-}1^+$ TILs (green dots) and PBMCs (red dots). Each dot represents an individual patient. **B,** tSNE plots showing which clusters the $CD3^+$ $PD\text{-}1^+$ TILs were grouped into (green box) and which clusters the $CD3^+$ $PD\text{-}1^+$ PBMCs were grouped into (red box) by the clustering algorithm. **C,** The median expression levels of various activation markers in TILs (green dots) and PBMCs (red dots). The same tSNE plot as **B** was colored to show which cells expressed the particular marker (*, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$). **D,** Antigen experience/memory markers. **E,** Exhaustion markers. **F,** A heatmap of the median expression pattern for each of the exhaustion, activation, and memory/antigen experience markers for each individual patient.

placed in culture and activated using agonistic CD3/28 antibodies. At 24 and 48 hours postactivation, supernatants were collected and the IFNγ levels measured. At 24 hours, the amount of IFNγ produced by the $CD3^+$ $PD\text{-}1^+$ cells was 2.4× higher than the IFNγ produced by the $CD3^+$ $PD\text{-}1^-$ cells (Fig. 4a). By 48 hours poststimulation, the amount of IFNγ produced by the $CD3^+$ $PD\text{-}1^+$ cells was 8.8× higher than the IFNγ produced by the $CD3^+$ $PD\text{-}1^-$ cells (Fig. 4b).

Discussion

We previously demonstrated that the PD-1/PD-L1 axis plays an important immunoregulatory role in antitumor T-cell function induced by DC vaccination (10, 12). Several other studies have described T-cell exhaustion and dysfunction within the tumor microenvironment and in relation to PD-1 expression. Thommen and colleagues showed in non–small cell lung cancer patients that PD-1 expressing T cells from the tumor had higher expression of

various exhaustion markers, had higher upregulation of genes related to proliferation and activation, and were more clonal (27). Analyzing TILs from both human GBM and murine tumor models, Woroniecka and colleagues showed that TILs had a more prominent exhaustion signature compared with the peripheral blood from patients with GBM and from healthy donor PBMCs (28). However, a comprehensive, high-dimensional analysis of PD-1 expression by human T lymphocytes in patients with malignant glioma has not been described. In our study, we found several differences in both phenotypic characteristics and function between PD-1$^+$ TILs and matched PD-1$^+$ peripheral blood T lymphocytes extracted from patients with malignant glioma. Notably, we found that PD-1 expression in the glioma TIL context may reflect T-cell activation in addition to T-cell exhaustion, but in the periphery, PD-1 expression on T cells may indicate activation and antigen experience (Fig. 5).

Expression of PD-1 on peripheral blood T cells of patients with malignant tumors, including malignant glioma, has previously been reported (29–34). In most of these previous studies, PD-1 was described as a marker of exhaustion by terminally

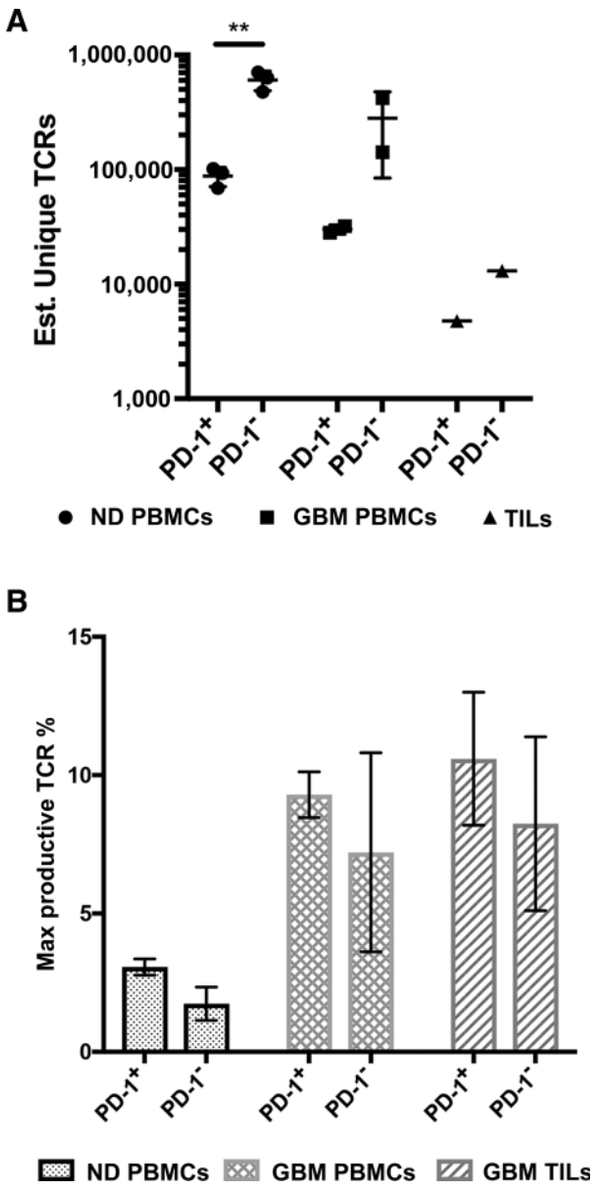

Figure 3.

PD-1$^+$ T cells have decreased TCR diversity compared with PD-1$^-$ cells. **A,** The number of estimated unique TCR sequences in PD-1$^-$ and PD-1$^+$ populations in healthy donor PBMCs, glioma patient PBMCs, and glioma patient TILs. Each point represents a single patient. Circle points, healthy donor PBMCs; square points, glioma patient PBMCs; triangle points, glioma patient TILs (**, $P \leq 0.005$). **B,** The frequency of a specific TCR rearrangement in the populations. The populations shown are the CD3$^+$ PD-1$^-$ or CD3$^+$ PD-1$^+$ populations from healthy donor PBMCs (dotted bar), glioma patient PBMCs (the checkerboard bar), and glioma patient TILs (slanted stripes bar).

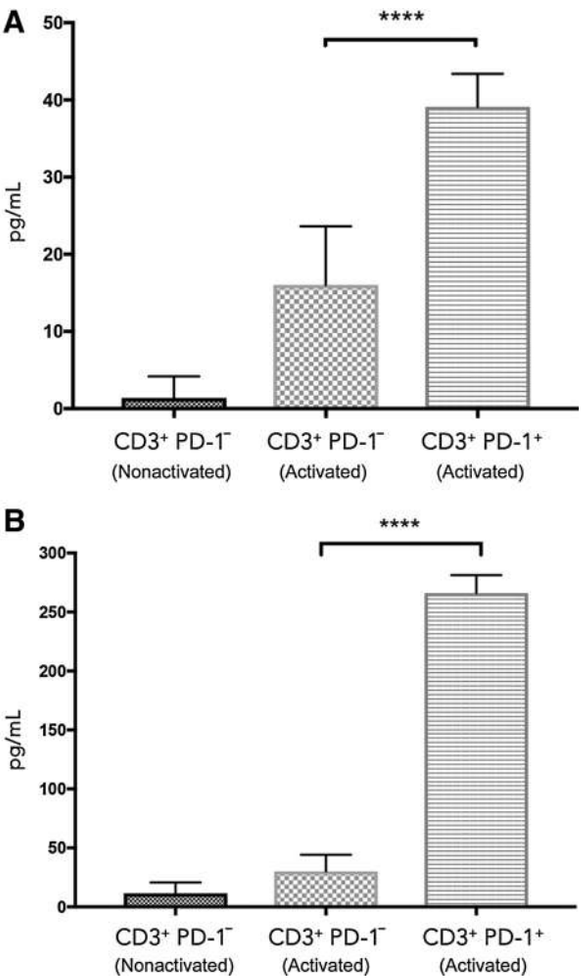

Figure 4.

CD3$^+$ PD-1$^+$ T cells from pooled glioma patient PBMCs produce higher levels of IFNγ compared to the CD3$^+$ PD-1$^-$ T cell population after T cell activation. **A,** The amount of IFNγ produced in CD3$^+$ PD-1$^-$, and CD3$^+$ PD-1$^+$ T cells from four glioma patient PBMCs 24 hours after CD3/CD28 activation. The dotted bar represents CD3$^+$ PD-1$^-$ T cells that were not activated. The checkerboard bar represents CD3$^+$ PD-1$^-$ T cells that were activated. The horizontal stripes bar represents CD3$^+$ PD-1$^+$ T cells that were activated. The results presented are from a representative experiment that has been repeated multiple times with similar findings. **B,** IFNγ produced at 48 hours from the same set of cells as in **A**.

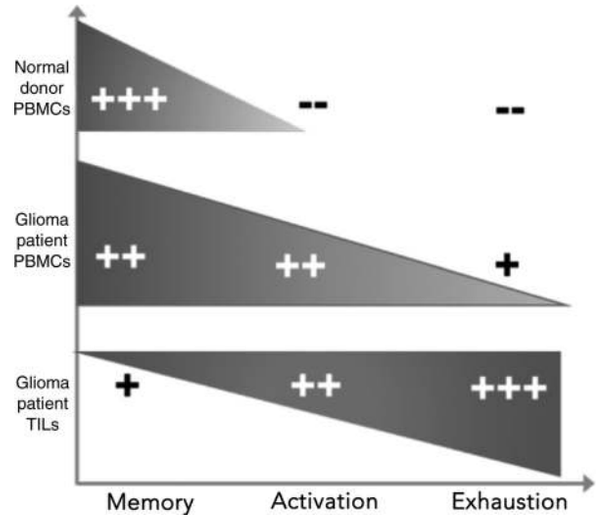

Figure 5.
A schematic indicating what PD-1 expression means in the context of malignant glioma.

differentiated effector T cells. But these reports failed to describe the phenotypic and functional differences between PD-1$^+$ T cells in the peripheral blood and PD-1$^+$ TILs. In our study, we performed a comprehensive analysis of PD-1 expression on T lymphocytes isolated from the tumors and peripheral blood of patients with malignant glioma. We found that PD-1 was significantly elevated in glioma patient TILs when compared with T cells in glioma patient peripheral blood and T cells from healthy controls. PD-1$^+$ TILs are enriched for markers of activation and exhaustion (CTLA-4, TIM-3, HLA-DR, and CD38) when compared with PD-1$^+$ peripheral blood T cells. In contrast, peripheral blood PD-1$^+$ T cells express a complete array of central memory, effector memory, and even the CD45RA$^+$ effector memory T-cell subsets (CD45RO, CD45RA, CD27, and CD127), suggesting diverging functionality between PD-1$^+$ T cells obtained from distinct anatomic locale.

In the setting of chronic viral infections, similar trends have been observed. PD-1 expression has been associated with T-cell exhaustion in both mouse LCMV models and humans infected with HIV (35, 36). In studies examining the transcriptional profile of the exhausted T-cell population from a chronic LCMV mouse model, the exhausted population remained distinct from the memory T-cell population and had increased transcription of various exhaustion markers such as PD-1, TIM-3, and CTLA-4 and activation markers such as ICOS and OX40 (37, 38). In contrast, previous reports have shown that in healthy controls, PD-1$^+$ peripheral CD8$^+$ T cells do not show exhausted characteristics, but instead exhibit hallmarks of effector memory T cells (39). In a subset of the patients, CD45RO was included in the mass cytometry analysis, which allowed a more thorough exploration of the T-cell memory subsets. We found that the expression CD45RO is uniformly expressed on all CD3$^+$ PD1$^+$ T cells from the tumor but variably expressed by the peripheral blood population. In the peripheral blood, there are multiple populations of PD-1$^+$ T cells that do not express CD45RO and are likely long-lived stem memory T_{SCM} or effector memory T_{EMRA} cells. Thus, our data support the conclusion that the phenotype of PD-1$^+$ T cells obtained from tumor tissue represents a chronically activated, exhausted effector T-cell population (CD45RO$^+$ CD27$^-$ IL-7R$^-$ HLA-DR$^+$ CD38$^+$ TIM-3$^+$ LAG-3$^+$ CTLA-4$^+$), while the PD-1$^+$ T-cell population obtained from peripheral blood represents the full complement of memory T-cell populations (T_{CM}, CD45RO$^+$ CD27$^+$ CD45RA$^-$ IL7R$^+$; T_{EM}, CD45RO$^+$ CD45RA$^-$ CD27$^-$ IL7R$^-$; and T_{EMRA}, CD45RO$^-$ CD27$^-$ CD45RA$^+$ IL7R$^-$). Therefore, our data would suggest that PD-1 expression by peripheral blood T cells may not be a good surrogate or indicator for exhaustion or tumor immune evasion. Such information must be considered when applied as a biomarker in clinical immunotherapy trials.

Interestingly, we found that PD-1 expression was associated with increased TCR clonality and decreased diversity in both the TIL population and peripheral blood T cells of patients with malignant glioma. As such, increased clonal expansion of the PD-1$^+$ T-cell population may reflect the activation and expansion of specific antitumor antigen T cells when compared with PD-1$^-$ T cells, although future studies are needed to confirm this. In addition, our mass cytometry data revealed a significant increase in expression of CD39 in the CD3$^+$ PD-1$^+$ TIL population compared with the CD3$^+$ PD-1$^+$ PBMC population. CD39, an extracellular ATPase commonly associated with regulatory T cells, has been shown to be coexpressed with PD-1 on antigen-specific but exhausted CD8$^+$ T cells in a chronic viral setting (40). More recently, CD39 has been proposed to be expressed by tumor-specific T cells (41, 42). The increase of clonality and CD39 expression in the PD-1$^+$ TILs further supports their dual function phenotype of antitumor specific activation and exhaustion. Increased TCR clonality has been correlated with better outcomes after anti–PD-1 therapy in melanoma and has been associated with higher PD-1 expression in soft tissue sarcomas (11, 43). Further investigation is needed to study the association between TCR clonality and PD-1 expression in the context of tumor antigen-specific T lymphocytes.

Finally, to characterize PD-1$^+$ peripheral blood T cells in patients with malignant glioma, we investigated the functional activation differences between PD-1$^+$ and PD-1$^-$ peripheral blood T cells from patients with GBM. Traditionally, it has been held that effector, memory, and naïve T cells have different response rates, as measured by amount of IFNγ produced, after activation. We found that PD-1$^+$ malignant glioma patient PBMC T cells exhibited an increased rate of IFNγ production when compared with PD-1$^-$ PBMC T cells from the same patients. Increased IFNγ is consistent with an activated memory T-cell phenotype, further highlighting that PD-1 expression in peripheral T cells is not a marker of exhaustion as we would expect in the PD-1$^+$ population in the tumor microenvironment.

In conclusion, we demonstrate that PD-1 expression in patients with malignant glioma reflects chronically activated effector T cells that display both hallmarks of memory and exhaustion depending on its location. The use of anti–PD-1/PD-L1 antibody blockade is currently being studied in malignant glioma clinical trials. Further exploration of the effect of these checkpoint inhibitors on PD-1–expressing T cells in different anatomical environments is warranted both to understand the use of PD-1 expression as a therapeutic biomarker as well as its importance for clinical efficacy.

Disclosure of Potential Conflicts of Interest

N. Kotecha holds ownership interest (including patents) in Cytobank Inc. W.H. Yong reports receiving commercial research grants from Amgen. No potential conflicts of interest were disclosed by the other authors.

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