Specific Expression of PD-L1 in RELA-Fusion Supratentorial Ependymoma: Implications for PD-1 Targeted Therapy

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Abstract

Background—A desperate need for novel therapies in pediatric ependymoma (EPN) exists, as chemotherapy remains ineffective and radiotherapy often fails. EPN have significant infiltration of immune cells, which correlates with outcome. Immune checkpoint inhibitors provide an avenue for new treatments. This study characterizes tumor infiltrating immune cells in EPN and aims at predicting candidates for clinical trials using checkpoint inhibitors targeting PD-L1/PD-1.

Methods—The transcriptomic profiles of the primary study cohort of EPN and other pediatric brain tumors were interrogated to identify *PD-L1* expression levels. Transcriptomic findings were validated using western blotting, immunohistochemistry and flow cytometry.

Results—We evaluated *PD-L1* mRNA expression across four intracranial subtypes of EPN in two independent cohorts and found supratentorial RELA fusion (RELA) tumors to have significantly higher levels. There was a correlation between high gene expression and protein PD-L1 levels in RELA tumors by both Western blot and immunohistochemisty. Investigation of EPN cell populations revealed PD-L1 was expressed on both tumor and myeloid cells in RELA. Other subtypes had little PD-L1 in either tumor or myeloid cell compartments. Lastly, we measured PD-1 levels on tumor infiltrating T-cells and found RELA tumors express PD-1 in both CD4 and CD8 T-cells. A functional T-cell exhaustion assay found RELA T-cells to be exhausted and unable to secrete IFNγ on stimulation.

Conclusions—These findings in RELA suggest tumor evasion and immunsuppression due to PD-L1/PD-1 mediated T-cell exhaustion. Trials of checkpoint inhibitors in EPN should be enriched for RELA tumors.

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Conflict of Interest:
There are no conflicts of interest for this study.

Keywords

C11orf95-RELA; Ependymoma; PD-L1; PD-1; Immunsuppression

Introduction

Pediatric ependymoma (EPN) is the third most common central nervous system (CNS) tumor and these tumors usually arise from the lining of the ventricles of the CNS[1]. EPN present intracranially as four main types, two in the supratentorial space and two in the posterior fossa. Posterior fossa EPN arise in the 4th ventricle and are divided on the basis of expression and methylation into Group A and Group B. Each sub-type of EPN possesses unique clinical characteristics, prognosis, microarray gene expression profiles, and mutations[2]. Supratentorial EPN are divided based on mutational drivers namely, *C11orf95-RELA* (RELA) fusions and *YAP1* (YAP) fusions. These two supratentorial subtypes are genetically and clinically distinct with data suggesting RELA patients have a poorer outcome than those with YAP[3]. As is the case in all intracranial EPN, there is little evidence that chemotherapy benefits RELA patients and treatment for these patients has remained stagnant with gross total resection and radiation as the standard of care. It is critical to develop new treatment strategies for these patients.

Immune checkpoint inhibitors, which overcome tumor associated immunosuppression, have proven effective in several adult tumors[4] and has become a particular interest in brain tumors[5]. One class of these inhibitors target the interaction of Programmed Cell Death Ligand 1(PD-L1) and its receptor Programmed death 1(PD-1). PD-1 is expressed on antigen-activated T-cells, B-cells, natural killer cells, and often on tumor infiltrating leukocytes, while its ligand, PD-L1 is normally expressed on dendritic cells, myeloid cells, and epithelial cells. The PD-1/PD-L1 axis in physiologic conditions helps to mitigate and dampen the immune response to a stimulus[6]. In the context of tumors, PD-L1 is found on tumor cells and on infiltrating myeloid cells. As PD-1 and PD-L1 interact, T-cells activity is diminished and they take on an exhausted phenotype[7]. Preventing this PD-L1/PD-1 interaction by a PD-1 inhibitor, such as pembroluzimab and nivolumab, has shown antitumor effects by reversing T-cell exhaustion. These agents are now approved in certain adult tumors, such as melanoma[8]. With respect to CNS tumors, there is evidence in adult glioblastoma that PD-1's interaction with PD-L1 contributes to the profound immunosuppression seen in this tumor[5]. Our lab has shown that EPN have significant infiltration of immune cells[9]. We have also shown that the phenotype of these immune cells correlate with outcome and molecular subtypes in the tumor immune microenvironment of posterior fossa EPN[10] and therefore investigation of immune therapies in this tumor is particularly important.

Given that PD-L1 expression on patient tumors is a current predictive marker of response to PD-1 checkpoint blockade[11], the aim of our current study was to evaluate supratentorial and posterior fossa EPN for PD-L1/PD-1. We examined *PD-L1* transcript and protein expression levels across EPN supratentorial subtypes and posterior fossa subtypes. Flow cytometry was also utilized to identify which EPN microenvironment cell type expressed PD-L1 protein

and to characterize the tumor infiltrating immune cells. These studies may predict which EPN patients will have a positive response to future clinical trials with PD-L1/PD-1 checkpoint inhibitors in pediatrics.

Materials and Methods

Study Approval

Primary patient tumor and normal brain samples were obtained from Children's Hospital Colorado and collected in accordance with local and federal human research protection guidelines and institutional review board regulations (COMIRB 95-500 and 09-0906). Informed consent was obtained for all specimens collected.

Transcriptomic analysis

The primary study cohort of EPN samples consists of: 20 primary Group A EPNs, 21 primary Group B EPNs, 9 recurrent Group A EPNs, 8 recurrent Group B EPNs, 11 RELA EPNs, 2 YAP EPNs, and 5 myxopapillary (Myxo) EPNs. Transcriptomic profiles from each sample were interrogated using the Human Genome U133plus2 Array (Affymetrix) platform as described previously[10]. Posterior fossa EPN subgrouping was determined using nonnegative matrix factorization clustering analysis as previously described[12]. Supratentorial EPN subgrouping was determined using signature transcript genes, ARL4D (Probeset ID#203586), CLDN1 (222549), RELA (201783), L1CAM (204584) as described by Patjler et al[3]. We then validated these findings by examining RELA fusion protein in a subset of the characterized samples by western blot analysis. From this transcriptomic dataset we extracted *PD-L1* (CD274, Geneprobe #227458) to identify association of PD-L1 expression with the respective EPN subtypes. These results were validated in part using an independent EPN patient cohort[3].

We then extrapolated gene expression of *PD-L1* from all pediatric and adult tumors in our expanded cohort, which includes 9 Diffuse Intrinsic Pontine Gliomas, 15 Supratentorial Glioblastomas, 6 Thalamic Glioblastomas, 4 Posterior Fossa/Spinal Glioblastomas, 4 infant Glioblastomas, 11 Radiation Induced Gliomas, 14 adult Glioblastomas, 2 WNT Medullobastomas, 12Sonic Hedgehog Medulloblastomas, 14 Group 3 Medulloblastomas, 17 Group 4 Medulloblastomas, 27 Atypical Teratoid Rhabdoid Tumor, 6 Pleomorphic Xanthoastrocytoma, 12 posterior fossa Pilocytic Astrocytoma, 7 Supratentorial Pilocytic Astrocytoma, 4 Supratentorial Ganglioglioma, 8 Posterior Fossa Ganglioglioma, 27 Adamantinomatous Craniopharyngioma, 10 Meningiomas, 17 Embryonal Tumors, and 5 Choroid Plexus Papillomas. Twenty seven normal brain samples obtained from autopsy or epilepsy surgery were included as controls.

Western Immunoblots

Lysates were extracted from 9 RELA, 2 YAP, 13 Group A, 9 Group B, and 6 Normal brain samples. Primary antibodies used were PD-L1 (Cell Signaling; 13684, cloneE1L3N), RELA (Cell Signaling; 8242, clone D14E12), and β-actin (Cell Signaling; 12262, clone 8H10D10) was used as protein loading control. Bands were visualized with Immobilon Western

Chemiluminescent HRP substrate (Millipore) on a Syngene G Box imager. Densitometry measurements were made using ImageJ software.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were stained using standard immunohistochemical techniques for CD4 and CD8 and cell numbers per high-power field were scored as previously described[10]. PD-L1 was performed using rabbit monoclonal PD-L1/CD274 *(SP263)* antibody (Ventana/Spring Bioscience). The antibody was used according to the manufacturer's protocol. For PD-L1, a subjective scoring scale was used. Scoring incorporated images in multiple microscopic fields. IHC was scored as zero (0) if no cells were positive. Very rare (<1% overall) IHC-positive cells confined to the extreme edge/perimeter of tumor were not counted, especially if adjacent to normal nontumorous CNS tissue. Tumors were scored as 1 if weak IHC positivity was seen, with scattered positive tumor and myeloid staining. Tumors with medium IHC positivity were scored as 2. Those with high positivity were scored as 3 and additionally had to show strong IHC signal and diffuse distribution.

Flow Cytometry

EPN patient samples were mechanically dissociated into single cell suspensions and viably frozen as previously described[9]. Cells were gently thawed and suspended in PBS supplemented with 10% FBS. Myeloid cells were distinguished from tumor cells by co-expression of CD45 and CD11b as described previously[9]. PD-L1 (eBiosciences, catalog number: 25-5983, Clone: MIH1) staining was measured on both myeloid cells and tumor. Myeloid and T-cell characterization was performed as previously described[9]. Flow cytometry was performed using a BD Gallios equipped with 561 laser and flow data was analyzed using FloJo v10.7.

T-Cell Exhaustion Assay

Disaggregated tumor samples were stained with fluorophore conjugated antibodies CD45, CD3, and CD4 or CD8. T-cells were isolated using Beckman-Coulter Mo Flo Astrios EQ flow sorter and stimulated for 48 hours with PMA/ionomycin in RPMI media with 10% FBS and 1% penicillin streptomycin, after which supernatant was collected. A high sensitivity Milliplex Map kit (Millipore) was used to measure the concentration of 21 common cytokines (ITAC, GM-CSF, fractalkine, IFNγ, IL-10, MIP-3α, IL-12 (p70), IL-13, IL-17A, IL-1β, IL-2, IL-21, IL-4, IL-23, IL-5, IL-6, IL-7, IL-8, MIP-1α, MIP-1β, TNFα).

850K Methylation Array

DNA was isolated from snap frozen tumor specimen using All Prep DNA/RNA isolation kit (Qiagen). DNA methylation was assessed using the Infinium® MethylationEPIC BeadChip (Illumina). 750 nanograms of DNA per sample were submitted for bisulfite conversion using Zymo EZ DNA Methylation kits (Zymo Research Corp). Following the manufacturer's protocol, bsDNA was amplified, fragmented and hybridized to BeadChips. The chips were stained and then read with an iScan (Illumina), generating IDAT files to be used in subsequent statistical analyses. IDAT files were batch normalized and background corrected

using default settings of the R package ChAMP. The absolute value was calculated across probes located within and near the promoter and gene body of *PD-L1*.

Statistical Analysis

Statistical analyses were performed using R bioinformatics, Prism (GraphPad v6), and Excel (Microsoft) software. For all tests, statistical significance was defined as $p < 0.05$ using Student t-test.

Results

Characterization of Supratentorial EPN subtypes

In order to identify EPN subtype specific PD-L1 expression, EPN samples were divided into the three molecular subtypes using negative matrix factorization clustering of gene expression profiles: Supratentorial, Group A and Group B EPN, as previously described[13]. We further characterized the 12 supratentorial EPN patients in order to identify the common *C11orf95-RELA* translocation or *YAP1 fusion.*

We utilized a subtype associated signature geneset to distinguish RELA translocation samples from YAP fusion samples[3]. We analyzed the differential gene expression of *ARL4D, CLDN1, RELA*, and *L1CAM* and were able to identify the two unique subtypes within the ST location: 10 RELA and 2 YAP (Supplemental Figure S1A). We further validated these findings by analyzing the RELA gene expression across our EPN patient samples. We found the patients identified as supratentorial RELA subtype, had significantly higher levels of RELA gene expression compared to the YAP, Group A and Group B tumors (Supplemental Figure S1B). Finally, we measured RELA protein levels across the 12 supratentorial and posterior fossa tumors in order to confirm presence of the RELA fusion gene. In the patients with RELA subtype geneset expression, we were able to validate the fusion protein at 100kDa by Western blot (Supplemental Figure S1C). With the proper characterization of our EPN samples, we were then able to perform the subsequent PD-L1/PD-1 analyses.

PD-L1 transcript levels are highest in RELA Tumors

PD-L1 expression can be used to identify patients with lung cancer and melanoma that are more likely to benefit from checkpoint inhibitors such as pembroluzimab or nivolumab[14]. We therefore wanted to evaluate the gene expression of *PD-L1* across four of the intracranial subtypes of EPN. We interrogated transcriptomic data from EPN subtypes to evaluate gene expression of *PD-L1* in 12 RELA, 2 YAP, 29 Group A (20 primary Group A, 9 Group A recurrent), 29 Group B (21 primary Group B and 8 Group B recurrent) patients (Figure 1A). RELA patients have significantly higher levels of *PD-L1* than Group A and Group B patients, and trended towards significance in comparison to our limited cohort of YAP patients. We validated our cohort findings with an independent *PD-L1* transcription analysis in a larger EPN transcriptomic cohort (Figure 1B)[3]. In this cohort, RELA expression levels were significantly higher than in YAP and Group A patients. The discrepancy in PFB_1 mRNA expression between our cohort and the validation cohort may come down to a difference in the utilization of subtyping technologies between institutions (transcriptome vs.

methylome) and in the difference of patient age between the cohorts. In our cohort, Group A patients had a median age of 3 years (range 0.5 to 17 years), Group B patients were a median age of 2 years (range 1 to 13 years), RELA patients were a median age of 5.5 years (range 1.9 to 20 years), and lastly, YAP had a mean age of 0.5 years, maximum of 0.8 and minimum of 0.2 years old. The validation cohort Group A patients had a median age of 3.5 years, (range 0.8 to 12 years), Group B patients were a median age of 12 (range 11 to 15 years), RELA patients were a median age of 7 years (range 0.75 to 17 years), and lastly, YAP had a median age of 1 year (range of 0.8 to 9 years). Also, in our cohort, at recurrence, Group B tumors significantly upregulated *PD-L1* expression compared to primary tumors. We did not see a change in *PD-L1* expression at recurrence in Group A patients.

It has been shown that differential DNA methylation across the EPN molecular subtypes' results in unique gene expression profiles. We evaluated whether DNA methylation affects the *PD-L1* transcription in RELA using Illumina 850K methylation array. We found there was no differential methylation in the 12 probes that span the promoter region of *PD-L1* across all of our EPN patient samples (Supplemental Figure S2). This suggests that *PD-L1* expression in EPN is regulated by a mechanism other than methylation of the specific promoter region of the gene.

PD-L1 Protein Expression is highest in RELA

In previous studies, we have shown that microarray expression can be used to predict protein expression[15,16]. To ensure our *PD-L1* gene expression correlated with protein levels, we used lysates from snap frozen patient samples from available RELA, YAP, Group A, Group B, and normal brain. We found that PD-L1 protein levels were higher in RELA tumors compared to patients from other EPN subtypes (Figure 2A and B). Protein levels, as measured by densitometry, correlated very strongly with gene expression levels across EPN subtypes.

Involvement in clinical trials for PD-1/PD-L1 blockade requires immunohistochemistry-positive staining for PD-L1, thus, we tested and scored 8 pediatric EPN paraffin fixed tissues (4 RELA, 2 Group A, and 2 Group B). All RELA tumors tested positive for PD-L1 (Figure 3). Two RELA tumors tested had diffuse strong staining (score 3) (Figure 3A), or high positivity, 1 had medium positivity (score 2) (Figure 3B) and one had low positivity (score 1) (Figure 3C), with patchy staining. All four posterior fossa EPN scored 0, with no staining present (Figure 3D). No apparent restriction to discrete cells that would be indicative of immune cell staining was found, but rather extensive staining of tumor cells.

Based on our observation that *PD-L1* gene expression correlated strongly with protein expression (Supplemental Table S1), *PD-L1* transcript may therefore provide a valuable surrogate for determination of PD-L1 protein levels for clinical trial selection. We therefore examined *PD-L1* gene expression in our transcriptomic dataset containing the majority of pediatric and some adult brain tumor types. We established a minimum expression level of 3 as our cutoff, as this represents a value double the background level in our microarray data (Supplemental Figure S3). We were able to see bands on our western blots at expression levels as low as 2.49, but chose 3 as our cut off in order to be more stringent. From this cutoff we determined the percent of *PD-L1* positive patient tumor samples for each tumor

type that was further subdivided by either molecular subtype or anatomic location within the CNS (Figure 4). This analysis showed that Adamantinomatous Craniopharyngioma, low grade Astrocytomas and some high grade Astrocytomas expressed *PD-L1* at levels equal to or higher than RELA, whereas some other common tumor types, in particular Medulloblastomas and Diffuse Intrinsic Pontine Gliomas, showed no *PD-L1* expression. These data suggests that *PD-L1* transcript can be helpful in determining which patients would be candidates for future clinical trials, although this would require some protein expression validation.

PD-L1 found on both tumor and myeloid cells in RELA

It is known that PD-L1 can be upregulated by either tumor cells or myeloid cells. Given that EPN have significant infiltration of immune cells, particularly myeloid cells, we investigated which cellular population was expressing PD-L1[9,16]. Using flow cytometry on viable frozen disaggregated EPN from different subtypes, we identified PD-L1 to be expressed on both tumor and myeloid cell populations for the RELA samples with high *PD-L1* transcriptomic expression (Supplemental Figure S4). The patient with low *PD-L1* gene expression had little expression on either tumor or myeloid cells (RELA_790) (Figure 5A and 5B). As was seen by both gene expression and Western blot protein analysis, YAP, Group A and Group B tumors had very little PD-L1 on either tumor or myeloid cell compartments. These data shows that both tumor and myeloid cells upregulate PD-L1 in RELA patients.

T-cells in RELA patients express high levels of PD-1 and are exhausted

We have previously shown that T-cell infiltration, specifically CD4 infiltration, correlated with outcome in EPN patients but its association with EPN molecular subtypes was not evaluated[16]. We therefore measured T-cell infiltration in RELA, Group A and Group B EPN patients by immunohistochemistry. We found that RELA patients had significantly more CD4 infiltration than either posterior fossa subtype (Figure 6A). CD8 infiltration was also significantly higher in RELA patients compared to Group B, but not when compared to Group A (Figure 6A).

T-cell PD-1 interaction with tumor or myeloid PD-L1 results in inhibition of T-cell proliferation and subsequent T-cell exhaustion. T-cell exhaustion is one of the mechanisms by which tumors evade immune mediated clearance[4]. In order to identify if this immune suppressive mechanism was present in pediatric EPN subtypes, we measured PD-1 levels on both CD4 and CD8 EPN infiltrating T-cells by flow cytometry (Supplemental Figure S5). We found that RELA do in fact express PD-1 on both of their infiltrating T-cell populations (Figure 6B). We also found the same to be true for Group A and Group B patient samples.

Using a functional T-cell exhaustion assay, earlier studies by our group had showed that Group A infiltrating T-cells were unable to induce cytokine secretion in response to a non-specific stimulus in comparison to Group B[10]. In order to evaluate whether RELA infiltrating T-cells were equally exhausted, we repeated this assay using RELA tumors. Following PMA/ionomycin stimulation, T-cells from RELA were unable to secrete IFN-γ (Figure 6C). These findings in RELA suggest that tumor evasion and immunosuppression is due to PD-L1/PD-1 mediated T-cell exhaustion. In Group A patients, we found high levels

of PD-1 on both subsets of T-cells (Figure 6B). Similar to the RELA patients, the T-cells in Group A patients were unable to produce a significant level of IFN-γ in response to PMA/ionomycin suggesting the T-cells are also exhausted (Figure 6C). However, given we found very little PD-L1 in Group A patients, it is likely the mechanism of T-cell exhaustion is due to the previously described Group A inflammatory microenvironment driven by tumor secreted IL-6[12]. Upon investigation of IL-6 secretion across EPN subtypes, we found that Group A secrete significantly higher amounts of IL-6 than any other subtype and RELA patients do not secrete IL-6 at all (Supplemental Figure S6). Group B patients also expressed high levels of PD-1, however both CD4 and CD8 T-cells were able to secrete IFN-γ after PMA/Ionomycin stimulation which indicates that PD-1 levels are due to normal T-cell antigen-activation, as has been a documented function of PD-1[6].

Discussion

EPN is an aggressive childhood brain tumor with poor survival and significant long-term morbidity. Standard therapy has not changed in two decades and consists of surgery and radiation. Chemotherapy and small molecule inhibitors have failed and up to half the children with EPN will relapse within a decade and almost all of these relapsed patients will die[17]. There is a vital need for novel therapeutic approaches for these very high risk patients. Given the recent successes of immunotherapy, such as the PD-1 checkpoint inhibitor studies in adult cancers, this approach should be explored in resistant brain tumors[18]. Careful selection of pediatric populations to test these agents is paramount if the agents are to be successfully introduced in the resistant tumors of childhood. Pediatric tumors are rarer and generally have less mutational load than those of adults[19]. It is also thought, that due to this generally decreased mutational load, immune checkpoint inhibitors would be less effective[20]. It is apparent from our data there may be significant differences in *PD-L1* expression status even between subtypes of a tumor. Chromothrypsis, and thus mutational load, identified in RELA tumors may result in increased neoantigen expression[21]. The relatively high level of neoantigens in RELA may underlie the exhausted T-cell phenotype inferred by *PD-L1* expression in these tumors. Lastly, NF-kB activity has been implicated as a driver of *PD-L1* expression in melanoma[22]. Collectively, these data provide optimism that RELA might be responsive to immune checkpoint inhibitors[23].

In our study, we showed that expression of PD-1 on the T-cells, and PD-L1 on the myeloid and tumor cells was common amongst the RELA fusion subtype but uncommon in the other 3 types of EPN. We have also shown that T-cells isolated from RELA tumors are nonresponsive to stimulus *ex vivo* and do not produce inflammatory cytokines. It is reasonable to assume that this T-cell phenotype in RELA tumors is secondary to the PD-1/PD-L1 interaction. This would support selectively recruiting RELA fusion positive EPN to a PD-1 inhibitor trial given the data about the predictive value of PD-L1[8]. Questions remain how reliable PD-L1 expression is in predicting response to a PD-1 inhibitor and whether the level of PD-L1 expression or protein is important, although currently PD-L1 expression remains the best predictor of response to PD-1 inhibitor therapy[14,24].

Our data also shows that T-cells isolated from YAP fusion tumors and Group A tumors are also non-responsive *ex vivo* and do not produce inflammatory cytokines. Yet these tumors

lack PD-L1 and a PD-1 interaction with PD-L1 does not explain why the T-cells are functionally exhausted in these tumors. This is in comparison to the Group B tumors with functional T-cells and excellent clinical outcomes[10]. This would suggest that while PD-1 inhibitors would be reasonable to try in RELA, an alternative immunotherapy approach may be necessary in YAP and Group A tumors. Indeed, our previous work would suggest that an immunotherapy approach to Group A may involve inhibiting an IL-6/myeloid interaction[12]. We know from previous work that the variation in immunobiology amongst different childhood brain tumors is profound and this paper and others from our group documents that this extends into tumor subtypes. Extending our understanding of differing immunobiology among tumors and their subtypes is necessary in order to provide better clinical care, maximizing personalized medicine and rational treatment for these patients.

A technical point from our observations in this study may help enrich patients likely to respond to a particular immunotherapy. We observed that transciptome data corresponded well to protein by both Western blot and IHC staining for PD-L1. With the expansion of genetic and transcriptome evaluation of tumors at diagnosis, this observation should be extended to other tumors and the relationship of expression to protein for other immunomodulating factors should be explored. Given the existence of large expression data banks this may improve our ability to choose appropriate immunotherapy agents to explore for different tumors and their subtypes.

This study investigated the presence of PD-L1 in EPN by immunohistochemistry. There is presently some difficulty surrounding the use of this technique to interpret PD-L1 in EPN, as identified by a recent study[25]. Despite these problems, their study concluded that PD-L1 was absent in all pediatric EPN. In contrast, our study clearly identified the presence of PD-L1 in supratentorial RELA EPN. Importantly, the antibody used was recently described as one of high reliability[26]. These results enhance the rigor and reliability of this study. In conclusion, any PD-1 inhibitor therapy trial, if conducted in EPN should be enriched for relapsed RELA patients. Alternative immunotherapies should be developed for the other subtypes of EPN with functionally exhausted T-cells but without evidence of PD-L1 expression on myeloid or tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations Key

EPN   Ependymoma

Group A	Posterior Fossa Ependymoma Group A
Group B	Posterior Fossa Ependymoma Group B
RELA	Supratentorial C11orf95-RELA Fusion
YAP	Supratentorial YAP1 fusion
PD-L1	Programmed Death Ligand 1
PD-1	Programmed Death 1
Myxo	Myxopapillary Ependymoma
ITAC	Interferon-inducible T-cell alpha chemoattractant
MIP-1α	Macrophage Inflammatory Protein-1 alpha
MIP-1β	Macrophage Inflammatory Protein-1 beta
bsDNA	bisulfide Deoxyribonucleic acid
ARL4D	ADP Ribosylation Factor Like GTPase 4D
CLDN1	Claudin 1
RELA	RELA Proto-Oncogene, NF-κB Subunit
L1CAM	L1 Cell Adhesion Molecule
PMA	Phorbol 12-myristate 13-acetate

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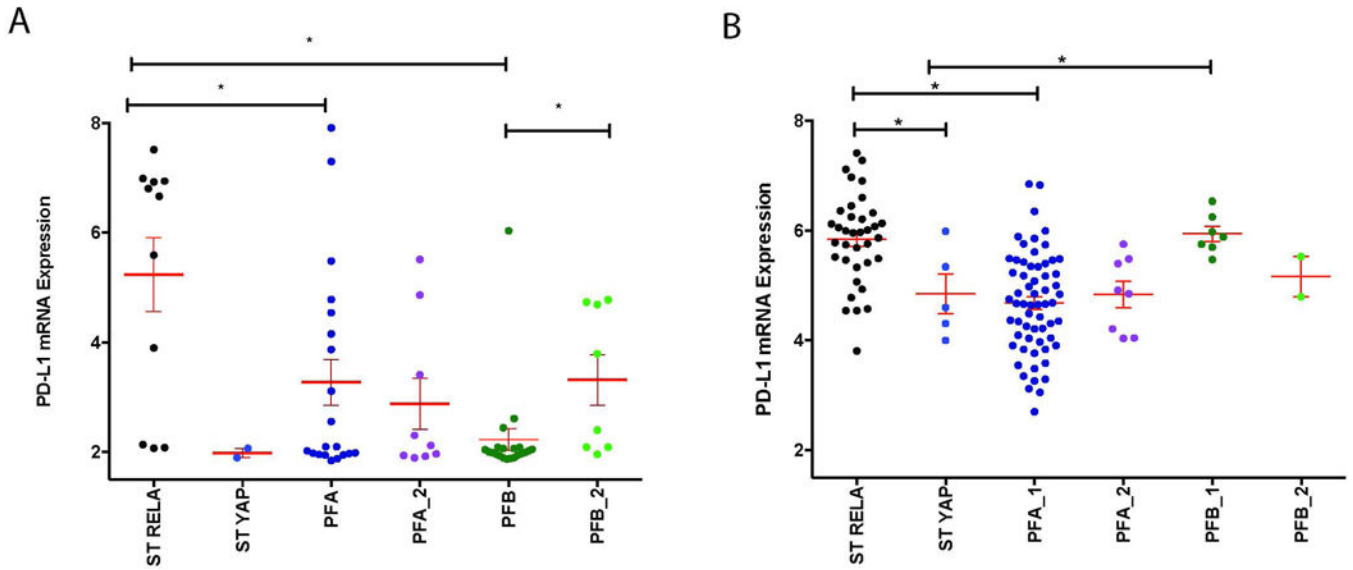
Fig. 1.

PD-L1 gene expression in two independent EPN patient cohorts. (A) mRNA expression of *PD-L1* in our primary cohort of supratentorial RELA fusion (ST RELA), supratentorial YAP fusion (ST YAP), posterior fossa group A (PFA_1) (primary tumor), posterior fossa group A (PFA_2) (recurrent tumor), posterior fossa group B (PFB_1) (primary tumor), and posterior fossa group B (PFB_2) (recurrent tumor) samples. *p<0.05 (B) mRNA expression of *PD-L1* in validation cohort of ST RELA, ST YAP, PFA_1, PFA_2, PFB_1, and PFB_2 samples. *p<0.05
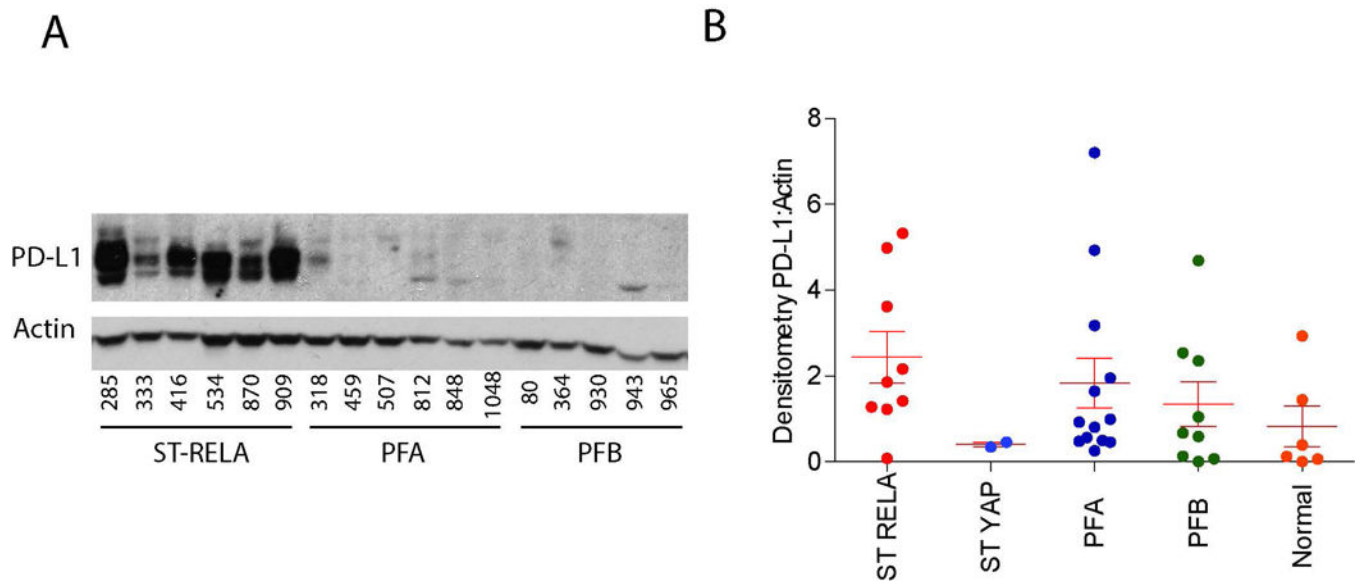
Fig. 2.

PD-L1 protein expression in our EPN patient cohort. *PD-L1* protein expression in supratentorial YAP fusion (ST YAP) (2), supratentorial RELA fusion (ST RELA) (9), posterior fossa Group A (PFA) (13), posterior fossa Group B (PFB) (9) EPN, and Normal brain samples (6). Densitometry ratio of *PD-L1* to Actin.
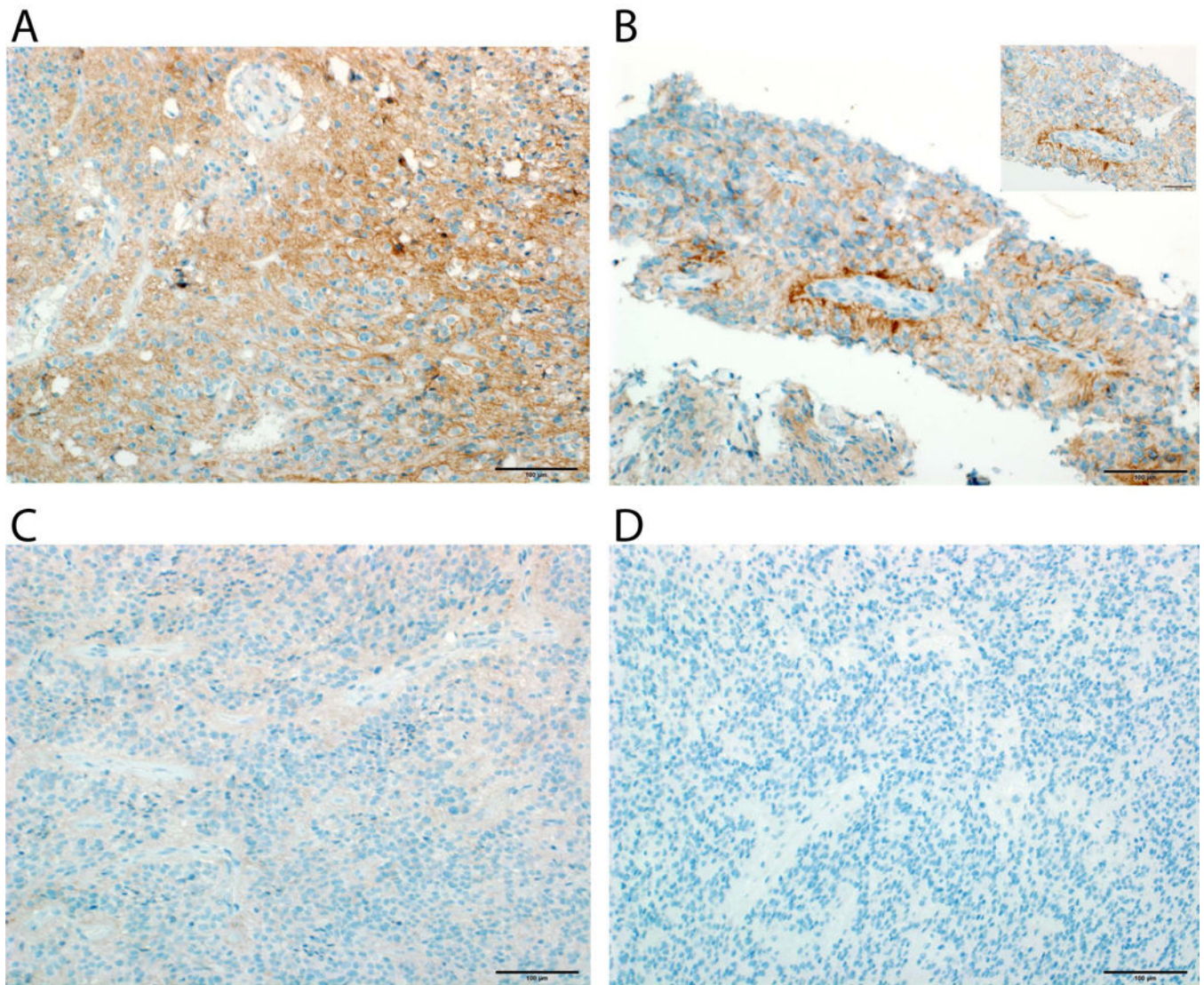
Fig. 3.
Representative examples of PD-L1 staining patterns in pediatric EPN. (A) Strong IHC signal and diffuse distribution seen (score 3) (RELA tumor, UPN 909) (20× magnification). (B) Medium staining intensity (score 2) (RELA tumor, UPN 1158) (20× and 40× magnification). (C) Low staining intensity (score 1) (RELA tumor, UPN 870) (20× magnification). (D) Posterior Fossa EPN with no *PD-L1* staining present (score 0) (Group B tumor, UPN 930).
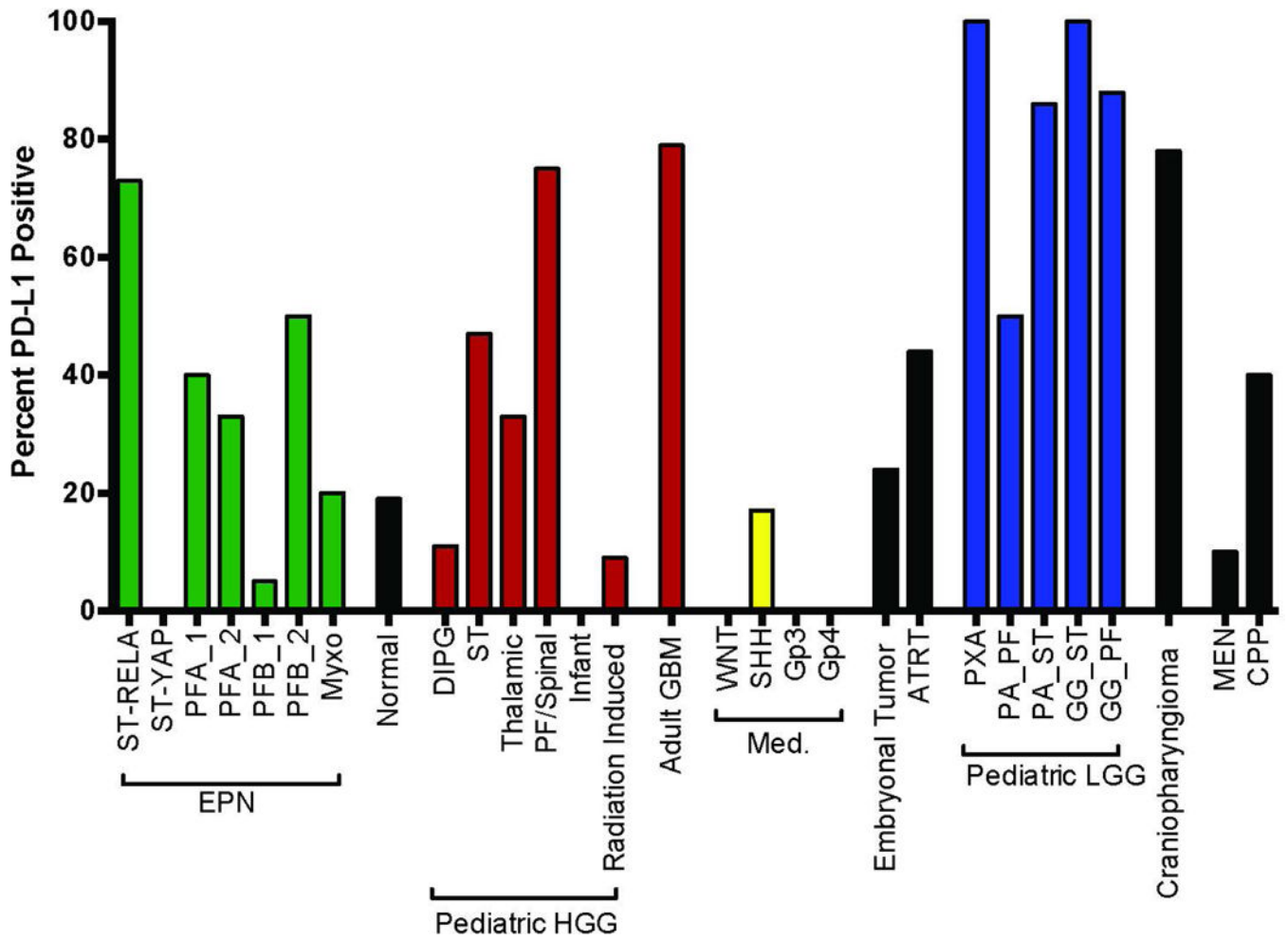
Fig. 4.
Percent *PD-L1* positive patients per diagnosis across all pediatric brain tumors in our cohort, determined by gene expression.
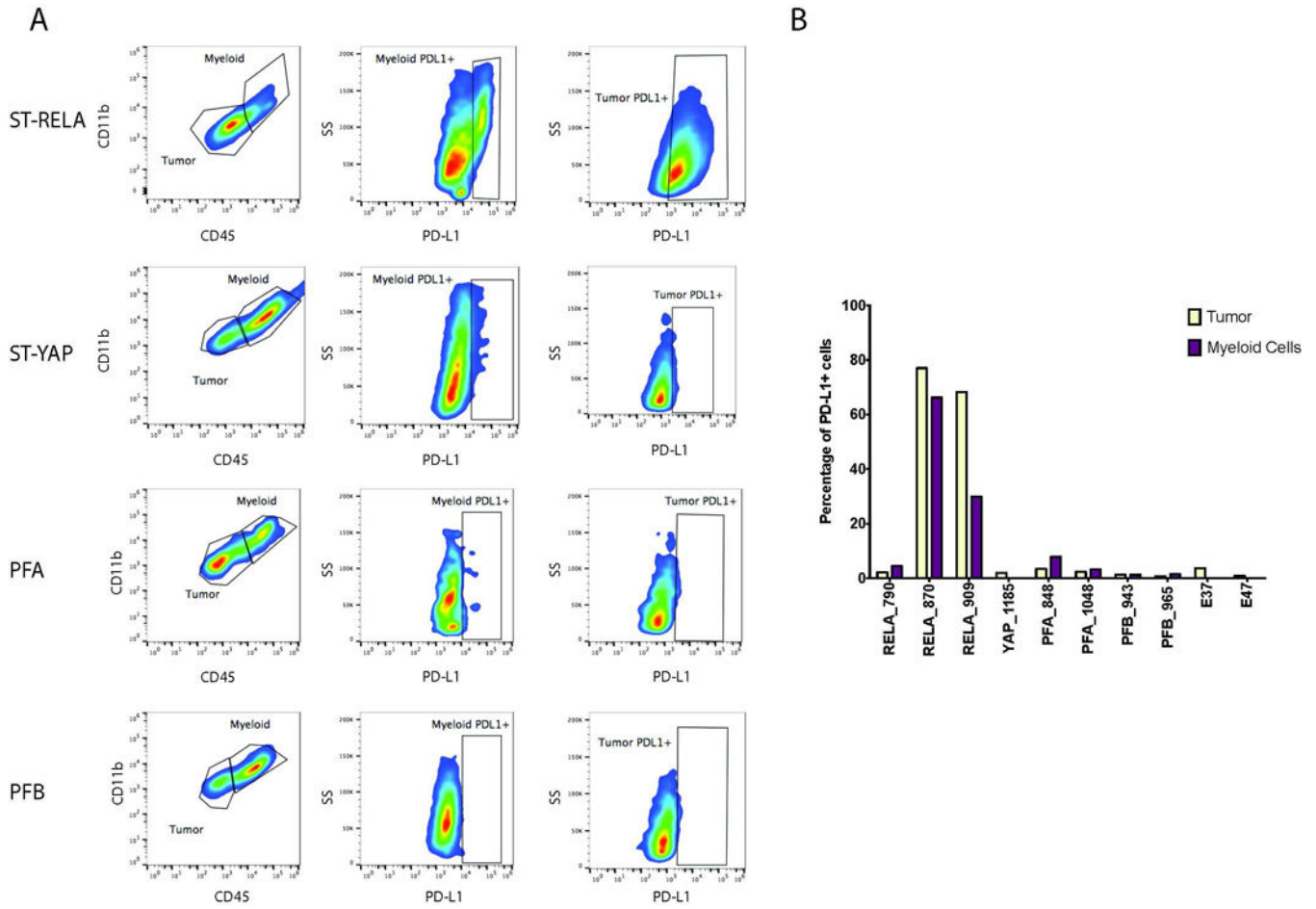
Fig. 5.
Supratentorial RELA fusion (ST RELA) EPN express *PD-L1* on both tumor and myeloid cells. (A) Flow Cytometry reveals only ST RELA tumor samples expressed *PD-L1* on both tumor and myeloid cells. (B) Percentage of myeloid and tumor cells that were positive for *PD-L1* in ST RELA, supratentorial YAP fusion (ST YAP), posterior fossa Group A (PFA), posterior fossa Group B (PFB) and normal patient samples.
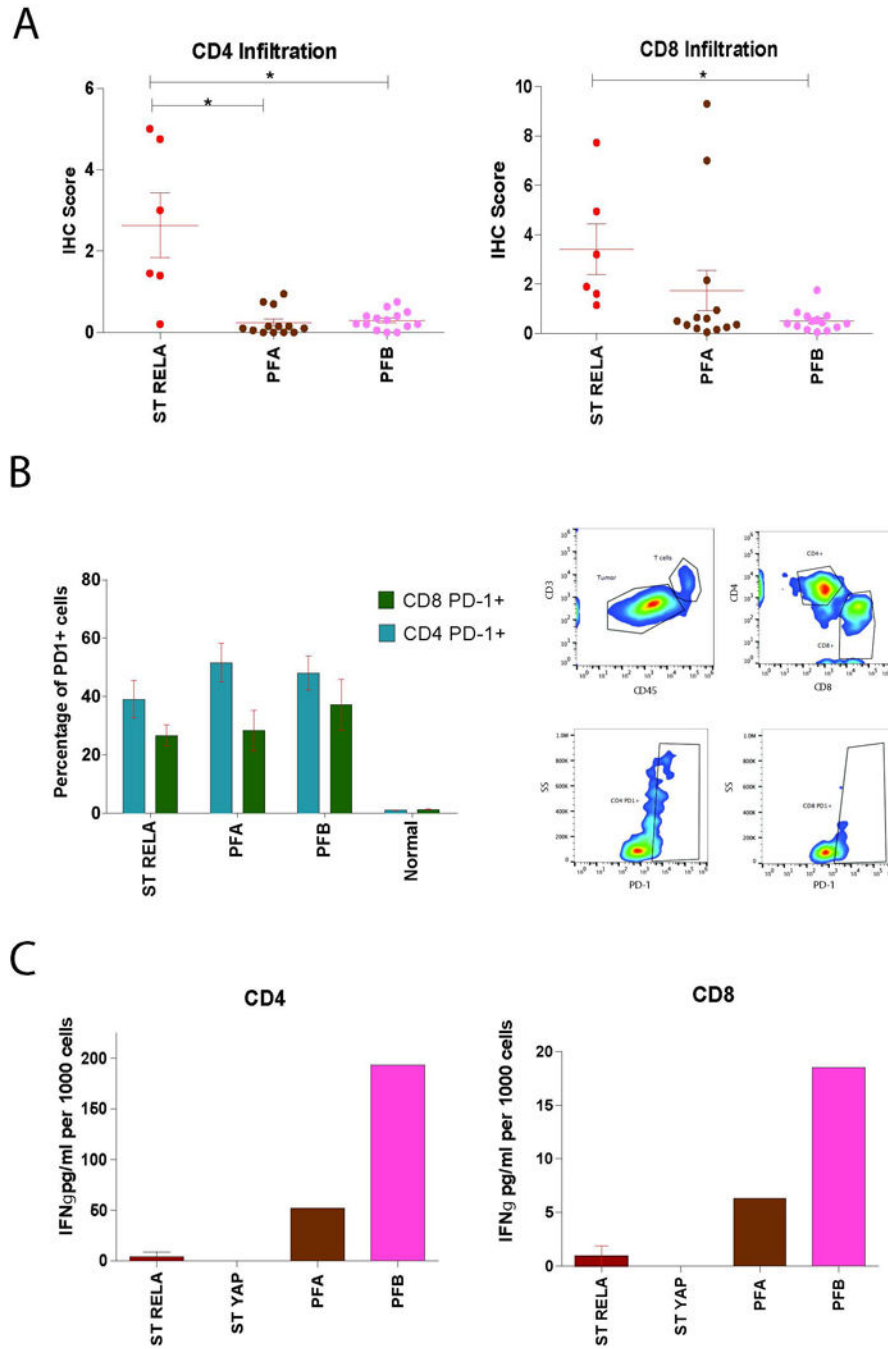
Fig. 6.

Infiltrating T-cells in supratentorial RELA fusion (ST RELA) tumors express high levels of *PD-1* and are functionally exhausted. (A) IHC scoring performed identifying infiltrating CD4 and CD8 T-cells in ST RELA, posterior fossa Group A (PFA) and posterior fossa Group B (PFB) patient samples. *p<0.05 (B) Flow cytometry characterization of *PD-1* expression on ST RELA, PFA, PFB, and normal brain samples in CD4 and CD8 T-cells. (C)

Secretion of IFNγ in CD4 and CD8 T-cells from ST RELA, supratentorial YAP fusion (ST YAP), PFA, and PFB patient samples. Results normalized per 1000 cells.