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ORIGINAL ARTICLE

PD-L1 and IDO1 are potential targets for treatment in patients with primary diffuse large B-cell lymphoma of the CNS

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ABSTRACT

Background: Programmed cell death 1 (PD-1) and its ligands PD-L1 and PD-L2, as well as Indoleamine 2,3-deoxygenase (IDO1) can be expressed both by tumor and microenvironmental cells and are crucial for tumor immune escape. We aimed to evaluate the role of PD-1, its ligands and IDO1 in a cohort of patients with primary diffuse large B-cell lymphoma of the CNS (PCNSL).

Material and methods: Tissue microarrays (TMAs) were constructed in 45 PCNSL cases. RNA extraction from whole tissue sections and RNA sequencing were successfully performed in 33 cases. Immunohistochemical stainings for PD-1, PD-L1/paired box protein 5 (PAX-5), PD-L2/PAX-5 and IDO1, and Epstein-Barr virus encoding RNA (EBER) *in situ* hybridization were analyzed.

Results: High proportions of PD-L1 and PD-L2 positive tumor cells were observed in 11% and 9% of cases, respectively. High proportions of PD-L1 and PD-L2 positive leukocytes were observed in 55% and 51% of cases, respectively. RNA sequencing revealed that gene expression of *IDO1* was high in patients with high proportion of PD-L1 positive leukocytes ($p = .01$). Protein expression of IDO1 in leukocytes was detected in 14/45 cases, in 79% of these cases a high proportion of PD-L1 positive leukocytes was observed. Gene expression of *IDO1* was high in EBER-positive cases ($p = .0009$) and protein expression of IDO1 was detected in five of six EBER-positive cases.

Conclusion: Our study shows a significant association between gene and protein expression of IDO1 and protein expression of PD-L1 in the tumor microenvironment of PCNSL, possibly of importance for prediction of response to immunotherapies.

Introduction

Primary diffuse large B-cell lymphoma (DLBCL) of the central nervous system (PCNSL) is a distinct entity of DLBCL that affects predominantly elderly patients [1]. It represents about 2–3% of all lymphomas, with an increasing incidence in the last three decades [2]. Despite improved treatment results for systemic DLBCL (sDLBCL), PCNSL still has a dismal outcome with almost no improvement in survival [1].

The role of the immune system in cancer initiation, progression and invasion has been established [3], where tumor cells evade the immune system mainly by disabling the immune cells in what is called 'tumor immune escape'.

Programmed death receptor 1 (PD-1) and its ligands, PD-L1 and PD-L2, are part of an immune checkpoint pathway responsible for the maintenance of self-tolerance and control of excessive immune responses in order to protect tissues from immune-mediated damage in a healthy host [4]. In

cancer, the binding of PD-L1 or PD-L2 to PD-1 inhibits the proliferation of activated T-lymphocytes and allows tumor cells to escape the antitumor adaptive immune response [5]. In recent years, the introduction of inhibitors that block the PD-1-PD-L1/PD-L2 interaction has shown promising responses in several solid tumors [6]. In lymphomas, studies on classical Hodgkin lymphoma (cHL) have resulted in high response rates to PD-1 blockade in patients with refractory disease [7], and another study demonstrated that PD-1 blockade restores T-cell function *in vitro* in Epstein-Barr Virus (EBV)-positive DLBCL patients [8]. In a previous study of five patients with refractory PCNSL, all patients showed response when treated with PD-1 blockade [9].

Indoleamine 2,3 dioxygenase (IDO1) is a rate-limiting metabolic enzyme involved in the kynurenine pathway and is responsible for degrading the essential amino acid tryptophan into L-kynurenine, which blocks T-cell proliferation and

induces T-cell death, facilitating immune tolerance in healthy hosts [10]. Several preclinical and clinical studies have investigated different IDO1 inhibitors and shown beneficial therapeutic effects of IDO1 inhibitors when combined with cytotoxic chemotherapies and/or other immunotherapies in different malignancies [11–14].

In the current study, we aimed to evaluate the protein expression of PD-1 and its ligands and other markers related to PD-1/PD-L1, identified by RNA sequencing in relation to the clinical characterization and immunophenotypic features in formalin-fixed, paraffin-embedded (FFPE) tumor biopsies of PCNSL.

Materials and methods

Patients

In this cohort study, 132 patients diagnosed with PCNSL between 1996 and 2015 at the Department of Pathology, Uppsala University Hospital were identified. Patients infected with human immunodeficiency virus (HIV) were excluded ($n = 2$). Patients were classified into three prognostic groups according to the Memorial Sloan-Kettering Cancer Center (MSKCC) based on age and Karnofsky performance status (KPS): class 1 included patients <50 years; class 2 included patients ≥50 years and KPS ≥ 70; and class 3 included patients ≥50 years KPS < 70 [15]. Data on clinical presentation and outcome for 96 of these patients has previously been published [2].

The study was approved by the Regional Ethical Review Board in Uppsala, Sweden (EPN 2008/246 and 2014/233).

Tissue samples

FFPE diagnostic biopsies were reviewed by two hematopathologists (CS, MA). The diagnosis was confirmed as PCNSL according to the 2008 WHO classification of Tumors of Hematopoietic and Lymphoid Tissues [1]. Tissue microarrays (TMAs) were constructed in a total of 45 cases with sufficient tumor tissue using standard techniques, containing two cores of one mm in diameter per case.

Immunohistochemistry

Immunohistochemical (IHC) stainings for CD10, BCL6, MUM1, BCL2 and MYC were performed on whole tissue sections using fully automated protocols (DAKO Autostainer Link48). IHC stainings for PD-1, double stains for PD-L1/paired box protein 5 (PAX-5), PD-L2/PAX-5, CD68 and IDO1 were performed on TMA (Supplementary methods).

Evaluation of immunohistochemical stainings

All IHC stainings were evaluated manually. Assessment of positive tumor cells and tumor microenvironment cells was based on morphology and comparison with IHC stainings for B-cell marker (CD20), T-cell marker (CD3) and macrophage marker (CD68). Double stainings for PAX-5/PD-L1 and PAX-5/

PD-L2 were performed for identification of positive tumor cells

Evaluation of tumor cells

The estimation of positive tumor cells for CD10, BCL6 and MUM-1 was based on Hans algorithm to classify tumors as germinal center-derived subtype (GCB) and non-germinal center-derived subtype (non-GCB) [16]. Estimation of positive tumor cells for MYC and BCL2 was based on cut off values of 70% and 40% respectively, as previously described [17].

Tumor cells with brown membranous PD-L1 or PD-L2 staining and red nuclear PAX-5 staining were designated as positive. Proportions of positive PD-L1 and PD-L2 tumor cells were calculated by dividing the number of positive tumor cells by all the tumor cells.

Evaluation of microenvironment cells

All immune cells such as lymphocytes, plasma cells, macrophages, mast cells, and dendritic cells were defined as leukocytes. The small round lymphocytes were further identified as tumor infiltrating lymphocytes (TIL) which mainly consisted of CD3-positive T-lymphocytes identified by CD3 stainings on consecutive sections.

Leukocytes with brown membranous PD-L1 or PD-L2 staining but without red nuclear PAX-5 staining were designated as positive leukocytes. Proportions of positive PD-L1 and PD-L2 leukocytes were calculated by dividing the number of positive leukocytes by the total number of leukocytes. Optimal cut off values for high and low proportions of PD-L1 and PD-L2 positive cells were determined by receiver operating characteristic (ROC) curves with Youden's index calculated for each marker. The following cut off values for high proportion were used: >85% PD-L1 and >6% PD-L2 for leukocytes, and >80% PD-L1 and >60% PD-L2 for tumor cells. PD-1 expression on leukocytes was manually evaluated semiquantitatively in 40x magnification in 10 high-power fields (HPF) and grouped as: 0: no positive cells/HPF; 1: <10 positive cells/HPF; 2: 10–30 positive cells/HPF; 3: >30 positive cells/HPF. IDO1 expression was assessed as: negative; focal positive; and diffuse positive cytoplasmic expression in leukocytes.

In situ hybridization for Epstein-Barr virus-encoded RNA

Epstein-Barr virus (EBV) was detected by in situ hybridization with a fluorescein-labeled oligonucleotide probe intended to identify cells expressing Epstein-Barr encoded RNA (EBER) in the TMA cohort. In situ hybridization was performed on a Ventana Benchmark Autostainer using ISH iVIEW blue plus detection kit according to manufacturer's instructions. Light microscopy was used to assess the staining where cell nuclei with dark blue labeling were considered positive for EBER.

FISH for MYC, BCL2 and BCL6

Fluorescence *in situ* hybridization (FISH) was performed on the TMA cohort (Supplementary methods).

RNA extraction and quality assessment

Extraction of the total RNA from the 45 diagnostic FFPE samples was performed from $\geq$ three 10-μm tissue sections using the AllPrep DNA/RNA FFPE Kit (Qiagen, Valencia, CA) and 33 samples were found adequate for evaluation. Total RNA was eluted in 30 μl RNase-free water. RNA quantity was measured by fluorometric quantitation using the Qubit RNA HS assay kit (ThermoFisher Scientific, Waltham, MA) while RNA integrity was assessed with the 4200 TapeStation System (Agilent Technologies, Waldbronn, Germany) using the DV_{200} metric. Samples were stored in $-80\,^{\circ}$C directly after extraction.

Library construction and RNA sequencing

The Illumina TruSeq RNA Exome protocol (Illumina, San Diego, CA) was used to prepare RNAseq libraries according to the manufacturer's protocol. For samples with low RNA quality (DV_{200} 30–50%), 100 ng input RNA was used for library construction while for medium (DV_{200} 50–70%) and high (DV_{200} >70%) quality samples, 40 ng and 20 ng input RNA were used respectively. Libraries were sequenced on a HiSeq2500 (Illumina, San Diego, CA) with a 2*126 setup at the National Genomics Infrastructure (NGI), Science for Life Laboratory, Uppsala, Sweden. Demultiplexing was performed using bcl2fastq_v2.19.1.403 and data was processed using the NGI-RNAseq pipeline (https://github.com/SciLifeLab/NGI-RNAseq). In brief, the NGI-RNAseq pipeline is a best-practice analysis pipeline used for RNA sequencing data at NGI. The pipeline uses Nextflow (www.nextflow.io), a bioinformatics workflow tool. It preprocesses raw data from FastQ inputs, aligns the reads and performs extensive quality-control on the results.

Statistical analysis

Overall survival (OS) was defined as time from diagnosis to time at last follow up or death from any cause. All deceased patients either died due to lymphoma or with lymphoma. Patients who were alive at last follow up were censored in the analyses. Survival curves and univariate analyses were performed using the Kaplan–Meier method and the log-rank test to compare differences between groups. Tabulated values were compared using the Chi-square or Fisher's exact test. Wilcoxon rank-sum test was used to compare mean differences between groups. *p*-Values <.05 were considered significant. Statistical analyses were performed using Statistica 13 software (StatSoft Scandinavia AB) and R Studio 1.1.383 (www.r-project.org). Differential gene expression analysis was performed with DESeq2 [18] using the negative binomial generalized linear model (GLM) fitting and Wald statistics for testing significance of coefficients. Bonferroni-corrected *p*-values of <.05 were considered significant. Results from the differential gene expression analyses of dichotomized EBER, as well as high proportions of PD-L1 and PD-L2 positive leukocytes were combined and genes with significant differential expression across these parameters were identified.

Results

Clinical characterization, immunophenotyping and rearrangement status

Clinical features of PCNSL patients in the TMA cohort ($n = 45$) compared to the whole cohort ($n = 132$) are summarized in Table 1. In brief, there were no major clinical differences between these groups (Table 1). Median age at diagnosis was 65.5 years (range 31–82 years).

IHC features in the TMA cohort and the whole cohort are summarized in Supplementary Table 1. According to the Hans algorithm, most cases belonged to the non-GCB subtype, 39/45 (87%) cases, while GCB subtype was detected in 6/45 (13%) cases. MYC, BCL2, and BCL6 were expressed in tumor cells in 23/45 (51%), 40/45 (89%), and 24/45 (53%) cases, respectively. MYC and BCL2 were concurrently expressed in 22/45 (49%) cases.

Analyses performed on the TMA cohort are summarized in Table 2. There was no protein expression of PD-1 on tumor cells. PD-1 protein expression was detected on TIL in 30/45 (67%) cases. In only one of the cases was there also protein expression of PD-1 on other leukocytes.

High proportions of PD-L1 and PD-L2 positive tumor cells were observed in 11% and 9% of cases respectively (Figure 1(B,D)) and high proportions of PD-L1 and PD-L2 positive leukocytes were observed in 55% and 51% of cases respectively (Figure 1(A,C)).

IDO1 cytoplasmic protein expression was detected in leukocytes in 14/45 (31%) cases, focal positive expression in 12 cases and diffuse positive expression in 2 cases (Figure 2). IDO1 was expressed on tumor cells in one of these 14 cases.

In situ hybridization for EBER was positive in tumor cells in 6/45 (13%) cases. In EBER positive cases, 3 had a previous history of renal transplantation.

FISH analysis revealed no translocation of *MYC* (0/45), one case with translocation of *BCL2* (1/45), and translocation of *BCL6* was detected in 10/44 cases, one case failed due to technical error.

RNA sequencing results

Statistically significant associations of gene expression levels by phenotype were identified (data not shown). We identified genes with differential expression across EBER and proportion of PD-L1, and PD-L2 positive leukocytes.

IDO1 gene expression was found to be differentially expressed against EBER on tumor cells and high proportions of PD-L1 positive leukocytes, while Solute Carrier Family 2 Member 3 (SLC2A3) gene expression was found to be differentially expressed against high proportion of PD-L2 positive leukocytes.

Table 1. Clinical features of patients with PCNSL in the TMA cohort ($n = 45$) and the whole cohort ($n = 132$).

Parameters	TMA cohort ($n = 45$)	Whole cohort ($n = 132$)	P-Value
Age in years median, range	65.5 (31–82)	66 (17–95)	.97*
0–59	10 (22%)	30 (23%)	
60–69	18 (40%)	55 (42%)	
≥ 70	17 (38%)	47 (35%)	
Gender			.11*
Female	27 (60%)	59 (45%)	
Male	18 (40%)	73 (55%)	
MSKCC			.96**
Class 1	4 (9%)	14 (10%)	
Class 2	12 (27%)	37 (28%)	
Class 3	15 (33%)	41 (31%)	
Missing information	14 (31%)	40 (31%)	
LDH			.46*
Normal	7 (15%)	31 (23%)	
High	12 (27%)	31 (23%)	
Missing information	26 (58%)	70 (54%)	
Treatment			.46*
Curative (intent)	30 (67%)	78 (59%)	
Palliative	10 (22%)	36 (27%)	
Missing information	5 (11%)	18 (14%)	
Follow-up, median (range), months			
Whole cohort	3.6 (0.02–134)	3.3 (0.02–172)	.38***
Curative group	10 (0.3–134)	12 (0.3–172)	.71***
2-yr OS			
Whole cohort	26%	22%	
Curative group	40%	35%	
5-yr OS			
Whole cohort	22%	17%	
Curative group	33%	27%	

TMA: tissue microarray; MSKCC: Memorial Sloan-Kettering Cancer Center; LDH: lactate dehydrogenase; OS: Overall survival.
*p-Value: comparison was assessed by using Chi-square test.
**p-Value: comparison was assessed by using Fisher exact test.
***p-value: comparison was assessed by using Wilcoxon test.

Association between gene and protein expression of IDO1 with protein expression of PD-1 ligands in the tumor microenvironment and with EBER

Gene expression level of *IDO1* was associated with high proportion of PD-L1 positive leukocytes ($p = .01$) (Figure 3). There was no significant association between *IDO1* gene expression and high proportion of PD-L2 positive leukocytes.

In all cases with positive protein expression of IDO1 ($n = 14$), positive protein expression of PD-L1 on leukocytes was detected with at least one positive cell. In 11/14 (79%) cases there was a high proportion of PD-L1 positive leukocytes.

Gene expression of *IDO1* was associated with EBER ($p = .0009$) (Figure 3). In addition, an association was detected between EBER and positive protein expression of IDO1 (five of six positive EBER cases had positive protein expression of IDO1).

A significant association was found between high proportion of PD-L1 and PD-L2 positive tumor cells and EBER (all cases with high proportions of PD-L1 ($n = 5$) and PD-L2 ($n = 4$) positive tumor cells were positive for EBER).

Slc2a3

Gene expression of *SLC2A3* was associated with a high proportion of PD-L2 positive leukocytes ($p = .002$). We failed to detect any associations between gene expression of *SLC2A3* and high proportion of PD-L1 positive leukocytes or EBER (data not shown).

Table 2. Proportions of PD-1, PD-L1, PD-L2 and IDO1 positive cells, and EBER status in patients with PCNSL in the TMA cohort ($n = 45$).

Parameters	Number of patients (%)
PD-1 on TC	0 (0%)
[a]PD-1 on TIL	
0: no positive cell/HPF	15 (33%)
1: <10 positive cells/HPF	17 (38%)
2: 10-30 positive cells/HPF	9 (20%)
3: >30 positive cells/HPF	4 (9%)
PD-L1 expression on TC	
Expressed by at least one TC	16 (35.5%)
Expressed with cut off 80%	5 (11%)
PD-L1 expression on leukocytes	
Expressed by at least one leukocyte	43 (95.5%)
Expressed with cut off 85%	25 (55%)
PD-L2 expression on TC	
Expressed by at least one TC	5 (11%)
Expressed with cut off 60%	4 (9%)
PD-L2 expression on leukocytes	
Expressed by at least one leukocyte	41 (91%)
Expressed with cut off 6%	23 (51%)
IDO1 protein expression in leukocytes	
Negative	31 (69%)
Focal positive	12 (27%)
Diffuse positive	2 (4%)
IDO1 protein expression on TC	1 (2%)
Positive *in situ* hybridization for EBER	6 (13%)

TIL: Tumor infiltrating lymphocytes; HPF: High power field; TC: tumor cells; EBER: Epstein-Barr virus encoded RNA.
[a]PD-1 protein expression was detected mainly on TIL, while there was one case with PD-1 expression on other leukocytes.

Survival analysis

Younger age and lower MSKCC score were associated with better outcome in our cohort ($p < .0001$ and $p = .004$, respectively). In the whole TMA cohort, there was no
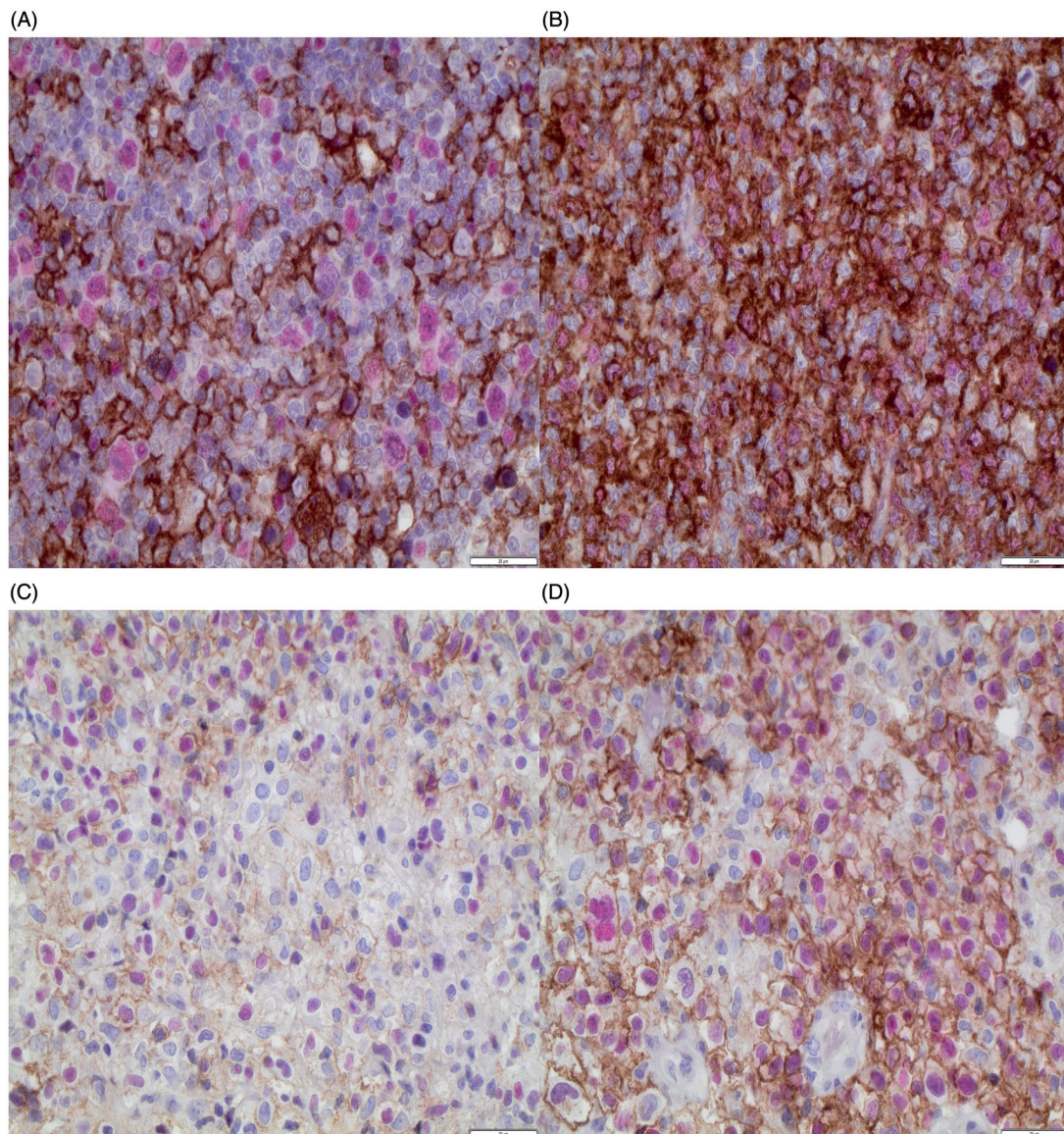
(A) (B)

(C) (D)

Figure 1. Brown membranous protein expression of PD-L1 and PD-L2 on leukocytes without red nuclear PAX-5 staining (A and C). Brown membranous protein expression of PD-L1 and PD-L2 on tumor cells with red nuclear PAX-5 staining (B and D).
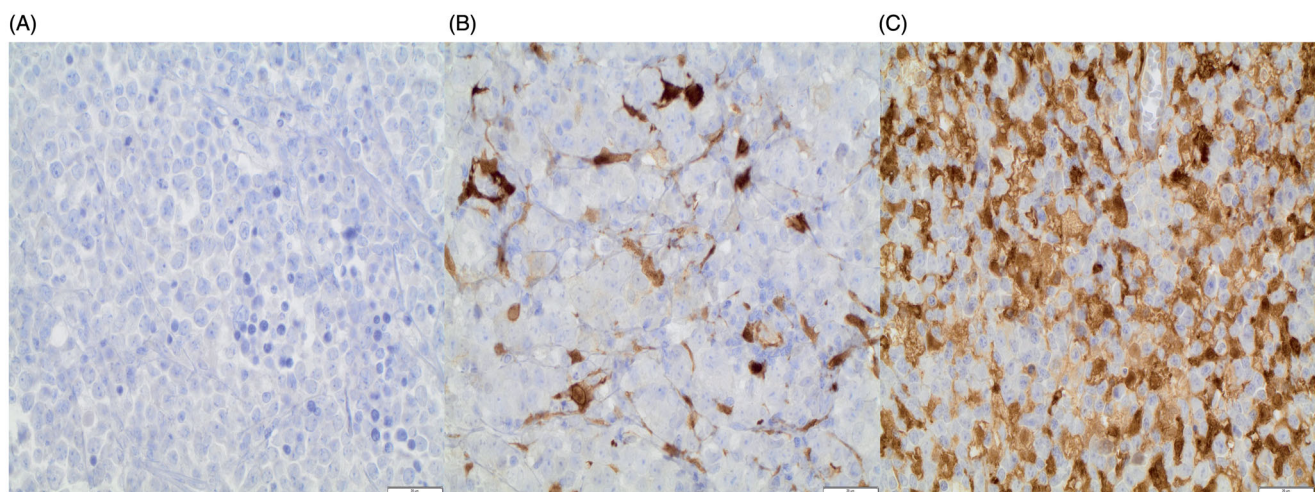
(A) (B) (C)

Figure 2. Cytoplasmic IDO1 protein expression in leukocytes: Negative (A), positive focal expression (B), positive diffuse expression (C).
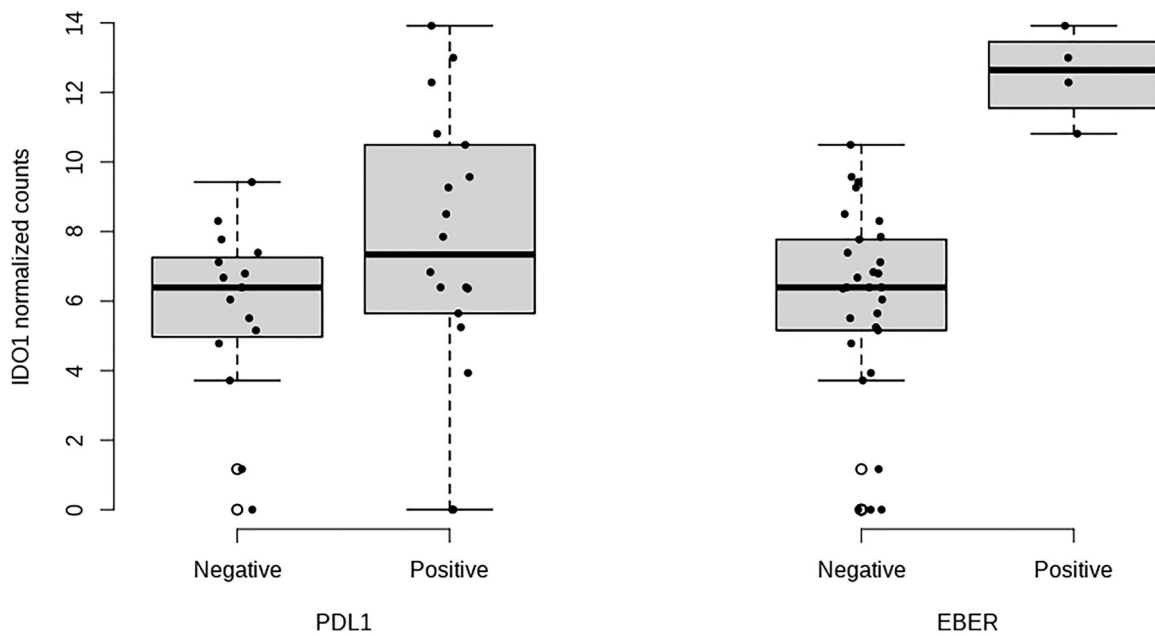
Figure 3. Box plot showing IDO1 gene expression values by PD-L1 protein expression group on leukocytes (DESeq2 adjusted $p = .013$) and by EBER status on tumor cells (DESeq2 adjusted $p = .0010$).

significant difference in survival regarding protein expression of PD-1 on TIL, PD-L1, PD-L2 on leukocytes or tumor cells, or of IDO1 on leukocytes (data not shown). When limiting the analysis to patients undergoing curative-intended treatment ($n = 30$), the protein expression of PD-L1, PD-L2 and IDO1 on leukocytes showed a tendency to better outcome (Supplementary Figures 1–3). Survival analyses regarding protein expression of IDO1 on tumor cells was not possible to perform since only one case had positive protein expression of IDO1 on tumor cells.

There was no difference in survival between patients with GCB and non-GCB subtypes and there was no negative prognostic impact of double expression of MYC and BCL2 (data not shown). BCL6 translocation did not affect survival.

Discussion

In the present study, RNA sequencing of FFPE tissue revealed that increased gene expression of IDO1 was associated with a high proportion of PD-L1 positive leukocytes in PCNSL patients. This finding was confirmed by IHC staining for IDO1, 79% of cases with positive protein expression of IDO1 also had a high proportion of PD-L1 positive leukocytes in the tumor microenvironment in our cohort of PCNSL patients. PD-L1-positive leukocytes are mainly tumor-associated macrophages (TAMs). TAMs also express PD-L2, and PD-L1/2 expression is mediated by Stat3 activation [19] and the significance of PD-L1 and PD-L2 on TAMs in immune suppression has been tested using mouse models [20].

High expression of PD-L1 and IDO1 on macrophages/microglia was observed in an earlier study on five PCNSL patients, which is in concordance with our findings and may explain the immune escape of lymphoma cells in PCNSL [21].

In this cohort, PD-1 was expressed on TIL in 67% of cases while there was no protein expression of PD-1 on tumor cells. High proportions of PD-L1 and PD-L2 positive leukocytes were observed in about half of the cases, while high proportions of PD-L1 and PD-L2 positive tumor cells were observed in about 10% of the cases. In our cohort, there was no prognostic impact of protein expression of PD-1 on TIL, or of PD-L1 or PD-L2 on leukocytes or tumor cells. A few previous studies have investigated PD-1 and its ligands in PCNSL with varying results regarding expression of these proteins on tumor cells and tumor microenvironment cells and their prognostic role. In one study, high protein expression of PD-1 was found to be associated with inferior outcome in PCNSL. Also, high protein expression of PD-L1 and PD-L2 in tumor microenvironment was detected in about 13% and 42% respectively, and did not show significant association with survival [22], whereas others found high expression of PD-1 on TILs to be associated with superior outcome [23]. Yet, another study detected PD-L1 expression on tumor cells in about 4% and on tumor microenvironment cells in 52% of patients. Furthermore, PD-L1 expression on tumor cells seemed to be associated with better prognosis while there was no significant difference in survival regarding expression of PD-L1 in the tumor microenvironment [24].

We demonstrated a strong association between IDO1 gene and protein expression with EBER which is in agreement with findings in one previous study that showed that EBV infection induces mRNA, protein and enzymatic activity of IDO1 in the tumor microenvironment [25]. We identified a strong association between high proportions of PD-L1 and PD-L2 positive tumor cells with EBER. This relationship has previously been identified in sDLBCL as well as in PCNSL [26, 27] where EBV-positive PCNSL exhibits another mechanism for the upregulation of PD-L1. EBV-positivity in PNCSL without association to HIV or other immune suppression has also been observed in other studies [28]. LMP-1 gene deletion and EBV-nuclear antigen 2 expression may drive lymphomagenesis in these cases [29].

We reported positive protein expression of IDO1 in leuko-cytes in 31% of all patients with no significant prognostic impact. Thus, the prognostic role of IDO1 in PCNSL is still unclear and not well investigated. In sDLBCL, however, one previous study found high protein expression of IDO1 in the tumor microenvironment to be associated with superior out-come [30] and another study demonstrated an inferior out-come for patients with high IDO1 expression on tumor cells [31]. We detected protein expression of IDO1 on tumor cells in only one patient, which is in accordance with another study on sDLBCL in which IDO1 expression on tumor cells was detected in few patients [30]. Furthermore, IDO1 expres-sion was associated with a less favorable prognosis in differ-ent human cancers when IDO1 was expressed by tumor cells [32,33], whereas IDO1 expression in the tumor stroma appears to be associated with better prognosis in cancer [34–36]. This suggests that IDO1 expression on tumor cells and tumor microenvironment cells may have differ-ent functions.

Immune checkpoint inhibitors have revolutionized the treatment of several malignancies. However, resistance to immunotherapy is reported as a common limitation [37,38]. IDO1 is involved in developing resistance to immune check-point inhibitors [39,40], thus IDO1 inhibitors may represent an alternative strategy in cancer immunotherapy in combin-ation with immune checkpoint inhibitors [41]. Indeed, in two clinical trials, combination therapy using IDO1 inhibitors and PD-1 inhibitors has shown encouraging results in patients with squamous cell carcinoma of the head and neck [42].

The lack of prognostic impact of double expression of MYC and BCL2 in 49% of the cases is in line with a previous, recently published study on PCNSL [43]. The frequency of translocations of MYC, BCL2 and BCL6 in our cohort is in accordance with others [44]. The foremost strength of our study is the comprehensive investigation of tumor and tumor microenvironment characteristics in PCNSL patients using numerous robust methods, including IHC stainings, FISH, in situ hybridization for EBER and RNA sequencing. Limitations include the rather low number of cases studied, which limits the statistical analyses. However, most studies on PCNSL consist of a limited number of patients due to the rarity of the disease and sparse diagnostic tumor samples. There were no major clinical differences in our study cohort compared to the population-based cohort previously pub-lished [2], consequently we believe that even though the number of cases included was limited, selection bias is prob-ably of minor importance.

Conclusion

Our study is the first to demonstrate a significant association between both gene and protein expression of IDO1 and pro-tein expression of PD-L1 in the tumor microenvironment in a well-characterized cohort of PCNSL patients. Both IDO1 and PD-L1 appear to be crucial immunosuppressive molecules, and further studies including treatment with IDO1 inhibitors in combination with other immunotherapeutic strategies may be warranted.

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