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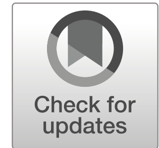

Characterisation of tumour microenvironment and immune checkpoints in primary central nervous system diffuse large B cell lymphomas

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Abstract

Primary central nervous system diffuse large B cell lymphoma (PCNS-DLBCL) is a rare and aggressive entity of diffuse large B cell lymphoma (DLBCL). Elements of the tumour microenvironment (TME) including tumour-infiltrating lymphocytes (TILs) and tumour-associated macrophages (TAMs) have been associated with survival in DLBCL but their composition and prognostic impact in PCNS-DLBCL are unknown. Programmed cell death-1 (PD1)/programmed death-ligand 1 (PD-L1) immune checkpoint may represent a therapeutic option. Here, we aimed to characterise PD1/PDL1 immune checkpoints and the composition of the TME in PCNS-DLBCL. We collected tumour tissue and clinical data from 57 PCNS-DLBCL and used immunohistochemistry to examine TAMs (CD68, CD163), TILs (CD3, CD4, CD8, PD1) and tumour B cells (PAX5/PDL1 double stains, PDL1). The *PDL1* gene was evaluated by fluorescence in situ hybridization (FISH). PAX5/PDL1 identified PDL1 expression by tumour B cells in 10/57 cases (17.5%). *PDL1* gene translocation was a recurrent cytogenetic alteration in PNCS-DLBCL (8/47.17%) and was correlated with PDL1 positive expression in tumour B cells. The TME consisted predominantly of CD163 (+) M2 TAMs and CD8 (+) TILs. Most TAMs expressed PDL1 and most TILs expressed PD1. The density of TAMs and TILs did not associate with outcome. We showed that expression of PD1 on TILs and PDL1 on TAMs, but not the expression of PDL1 on tumour B cells was correlated with better prognosis. These findings support a significant role of TME composition and PD1/PDL1 crosstalk in PCNS-DLBCL pathogenesis and bring new insights to the targeted therapy of this aggressive lymphoma.

Keywords Primary central nervous system diffuse large B cell lymphoma · Tumour microenvironment · Immune checkpoints · PDL1

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Introduction

Primary central nervous system diffuse large B cell lymphoma (PCNS-DLBCL) is listed in the WHO classification of tumours of haematopoietic and lymphoid tissues 2016 as a separate entity of diffuse large B cell lymphoma (DLBCL). It is characterised by a typical clinical presentation with tumour cells confined to the brain, the spinal cord, the leptomeninges or the eyes and remarkably worse outcome than patients with systemic DLBCL (s-DLBCL). The exclusively topographical restriction of PCNS-DLBCL to CNS raises the question of a particular microenvironment that could explain the aggressive behaviour of this disease. The immune checkpoints and the composition of the tumour microenvironment (TME) have emerged as the most important factors for both tumour survival and host defence. TME is mainly represented by include tumour-infiltrating lymphocytes (TILs) and tumour-associated macrophages (TAMs). TAMs in lymphomas have been extensively investigated. Noteworthy, several studies addressed outcome correlations by using both immunohistochemistry (with CD68 and CD163) and gene expression profiling. They especially showed adverse outcome in classical Hodgkin lymphoma (cHL) with increased TAMs [1–3]. Similar studies focusing on TILs in B cell lymphomas have demonstrated a correlation between T lymphocyte signature and a link between low amounts of TILs and a poor outcome [4]. Furthermore, high levels of CD4+ TILs have been shown to be associated with improved outcome in B cell lymphomas [5–7].

Programmed cell death-1 (PD1)/programmed death-ligand 1 (PD-L1) immune checkpoint provides an important escape mechanism from immune attack. The *PDL1* (CD274 molecule) gene encodes an immune inhibitory receptor ligand expressed by various types of tumour cells. Interaction of the encoded protein with its receptor PD1 inhibits T cell activation and cytokine production providing an immune escape for tumour cells through cytotoxic T cell inactivation. PD1 and PDL1 expression have been shown to correlate with survival and therapeutic responses in many cancers [8–10]. Thus, the PD1/PDL1 pathway has emerged as an attractive target in cancer immunotherapy in order to restore immune function. Numerous clinical trials with PD1 pathway blocking agents used alone or in combination have shown significant response in lymphoid malignancies including cHL, plasma cell myeloma or DLBCL [11–13]. PDL1 expression in tumour cells can be induced either by extrinsic signal as an adaptive response to IFN-γ released from TILs or by intrinsic signals such as genetic aberrations involving *PDL1* gene locus on chromosome 9p24.1 or latent Epstein-Barr virus (EBV) infection [14]. In cHL and primary mediastinal large B cell lymphoma (PMBL), *PDL1* gene amplification is a recurrent cytogenetic alteration and strongly correlates with PDL1 protein expression [15–17]. *PDL1* rearrangements have also been reported in lymphoid malignancies leading to aberrant expression of PDL1 favouring an immune escape and contributing to increased aggressiveness [18, 19].

The aim of this study was to evaluate the PD1/PDL1 immune checkpoints by immunohistochemistry and FISH analyses and to characterise the particular composition of the TME in PCNS-DLBCL.

Materials and methods

Patient selection

Pre-treatment biopsies from 57 immunocompetent patients with newly diagnosed PCNS-DLBCL were retrospectively retrieved from the Department of Pathology of Centre Hospitalo Universitaire (CHU) Montpellier, France. Cases with prior or concurrent low-grade B cell lymphomas and secondary CNS involvement by s-DLBCL were excluded. The study was approved by the research ethics boards of our institution (Centre des Ressources Biologiques, CRB, Montpellier) according to the Declaration of Helsinki. The written consent of patients was obtained.

Histological and immunohistochemical analysis

All cases were reviewed by five pathologists (VS, VR, VCM, LD and MP). The diagnosis of PCNS-DLBCL was made on Haematoxylin-Eosin (HE) and was based on the WHO 2016 classification of haematopoietic and lymphoid tissue. For immunohistochemical examination, 3-μm thick tissue sections from the formalin-fixed paraffin-embedded (FFPE) blocks were subjected to antigen retrieval and immunostained on a Ventana Benchmark XT autostainer (Ventana Tucson, AZ, USA). The following antibodies were used after appropriate antigen retrieval according to the manufacturer's instructions: CD20 (clone L26, Dako, Denmark A/S, 1:300), PAX5 (clone DAK-PAX5, Dako, 1:25), CD3 (clone 2GV6,Ventana, PREP Kit Ventana), CD5 (clone 4C7, Dako, Denmark A/S, 1:100), CD4 (clone SP35, Ventana, PREP Kit Ventana), CD8 (clone SP57, Ventana, PREP Kit Ventana), CD10 (clone 56C6, Menarini, California USA, 1:10), BCL6 (clone G1191E/A8, Ventana, PREP Kit Ventana), MUM1 (clone MUM1p, Dako, 1:50), P53 (clone DO7, Ventana, PREP Kit Ventana), MYC (clone EP 121, Epitomics, Burlingame, CA, USA, 1:100), CD68 (clone KP1, Dako, 1:400), CD163 (clone MRQ-26, Ventana, PREP Kit Ventana), KI67 (clone 30-9, Ventana, PREP Kit Ventana), PD1 (clone NAT105, Abcam, Paris, France, 1:100), PDL1 (clone E1L3M, Cell Signaling, Leiden, The Netherlands, 1:200). Association with EBV was examined by in situ hybridization (ISH) using EBV-encoded early nuclear RNA (EBER). For MUM1, MYC and P53, we evaluated the percentage of positive neoplastic cells as

previously described. MUM1, MYC and P53 protein expression was considered as positive if nuclear staining was observed respectively in at least 30%, 40% and 10% of the neoplastic cells [20, 21].

Evaluation of PDL1 expression by tumour cells and assessment of TME composition

For evaluation of PDL1 expression by tumour cells and for assessment of TME composition, slides were scanned at high magnification with a × 20 objective and digitised on the iScan Coreo scanner (Ventana, Roche, France) to generate an image of the whole slide. Double staining with PAX5 (nuclear B cell marker) and PDL1 was performed to evaluate PDL1 expression in the neoplastic B cells. For PDL1, both a membranous immunostaining signal on the cell surface and cytoplasmic staining within cells were recorded [22]. PD1 positive staining was evaluated in the membrane and cytoplasm of cells [23]. The percentage of positive cells was evaluated for both antibodies, and samples showing staining of any intensity in 1% or more of the respective cells were considered positive [22]. Images were obtained for PAX5/PDL1 double stains, CD3, CD4, CD8, PD1, CD68 and CD163. CD68 was used as a pan-macrophage marker staining M1 and M2 TAMs and CD163 as a M2 TAMs' marker. Images were then compared on the same screen by five pathologists (VS, VR, VCM, MP and LD) blinded to clinical data.

PDL1 expression by tumour cells The number of double-expressing cells was given in percentage as the rate of all PAX5 positive neoplastic B cells.

TILs The density of TILs was estimated with CD3 as follows: (0) absent, (1) low, (2) intermediate and (3) high. The proportion of CD4 (+), CD8 (+) and PD1 (+) TILS was manually counted. Owing to their characteristic morphology, TILs could easily be distinguished from TAMs and CD4 (+) or PD1 (+) cells with apparent morphological appearance difference from TILs were excluded from the count. The CD4 (+)/CD8 (+) TIL ratio was assessed and the number of PD1 (+) TILs was given in percentage as the rate of all CD3 (+) TILs.

TAMs The proportion of CD68 (+) cells was evaluated as follows: (0) absent, (1) low, (2) intermediate and (3) high. The CD68 (+)/CD163 (+) TAM ratio was assessed and the number of PDL1 (+) TAMs was evaluated and depicted as a proportion of all CD68 (+) cells in percentage. PAX5+/PDL1+ tumour cells were excluded from the count.

For TAM and TIL quantification, the categories low, intermediate and high were defined as follows: low, ≤ 25% of tumour area occupied by TILs or TAMs; intermediate, > 25% and ≤ 50% of tumour area occupied by TILs or TAMs; high, > 50% of tumour area occupied by TILs or TAMs.

Interphase fluorescence in situ hybridisation

Interphase FISH was performed on 3-μm thick tissue sections using split signal FISH DNA probes for *BCL2*/18q21 (probe Y5407; DAKO A/S), *BCL6*/3q27 (probe Y5408), *MYC*/8q24 (probe Y5410; DAKO A/S) and *PDL1* (PDL1, CD274 break apart probe, 9p24.1, Empire Genomics). Digital images were captured with a Metafer slide scanning platform using a Leica Axioplan fluorescence microscope (Zeiss Axio Imager M1) equipped with a charge-coupled device (CCD) camera coupled to and driven by the ISIS software (MetaSystem, FISH Imaging System, Germany). At least 100 nuclei were evaluated independently by three scorers (VS, MA, VC). Cases were considered positive when more than 15% of the cells displayed abnormalities on the FFPE tissue sections.

Statistical analyses

Fisher's exact test was used to calculate the P value with GraphPad Prism 6.0, GraphPad. Event-free survival (EFS) and progression-free survival (PFS) were estimated using the Kaplan-Meier method. Results were considered to be significant at $P < 0.05$.

Results

Patients' characteristics and clinicopathological findings

The clinicopathological characteristics of the 57 patients are depicted in Table 1. Complete remission after first line of treatment was evaluated for patients who received treatment with curative intention whereas status at last follow-up was given for the full cohort including patients with palliative treatment.

Median of follow-up was 15 months (12–108). The majority of the patients was treated with methotrexate (MTX)-based chemotherapy and received rituximab. Most patients had poor outcome showing relapse or progression and 47.2% (25/53) died of disease. As expected, most cases had non-germinal centre (GC) phenotype. No case showed *BCL2* or *MYC* gene breaks whereas MYC protein expression and MYC/BCL2 protein co-expression were respectively present in 32.1% (18/56) and 26.8% (15/56) of cases. *BCL6* and *PDL1* gene translocations were the most frequent cytogenetic aberrations occurring respectively in 22.2% (12/54) and 17% (8/47) of cases.

Table 1 Clinicopathological features of the 57 patients with PCNS-DLBCL

	Patients	Patients with PDL1+ tumour cells ($n = 10$)	Patients with PDL1− tumour cells ($n = 47$)	*P* value
Clinic				
Patients				
Mean age, years (range)	65.5 (41–89)	64 (46–89)	65.5 (41–84)	
Male, *n* (%)	32/57 (56.1)	4/10 (40)	28/47 (59.6)	0.31
Female, *n* (%)	25/57 (43.9)	6/10 (60)	19/47 (40.4)	
MSKCC prognostic class				
Class 1, *n* (%)	8/56 (14.3)	1/9 (11.1)	7/47 (14.9)	**0.06***
Class 2, *n* (%)	25/56 (44.6)	7/9 (77.8)	18/47 (38.3)	
Class 3, *n* (%)	23/56 (41.1)	1/9 (11.1)	22/47 (46.8)	
IPCG prognostic score				
Low, *n* (%)	9/36 (25)	2/6 (33.3)	7/30 (23.3)	0.31**
Intermediate, *n* (%)	15/36 (41.7)	4/6 (66.7)	21/30 (70)	
High, *n* (%)	12/36 (33.3)	0/6 (0)	12/30 (40)	
Initial management				
Palliative (radiotherapy ± corticotherapy), *n* (%)	7/51 (13.7)	1/9 (11.1)	6/42 (14.3)	1
Chemotherapy + rituximab ± radiotherapy ± corticotherapy, *n* (%)	44/51 (86.3)	8/9 (88.9)	36/42 (85.7)	
Number lines of chemotherapy				
1, *n* (%)	15/43 (34.9)	3/7 (42.9)	12/36 (33.3)	0.68
≥ 2, *n* (%)	28/43 (65.1)	4/7 (57.1)	24/36 (66.7)	
Outcome				
CR after first line of treatment, *n* (%)	19/50 (38)	4/8 (50)	15/42 (35.7)	0.43
CR at last follow up, *n* (%)	20/53 (37.7)	5/8 (62.5)	15/45 (33.3)	0.13***
AWD at last follow up, *n* (%)	8/53 (15.1)	1/8 (12.5)	7/45 (15.6)	
DOD, *n* (%)	25/53 (47.2)	2/8 (25)	23/45 (51.1)	
Relapse or progression, *n* (%)	33/50 (66)	4/8 (50)	29/42 (69)	0.42
Mean follow-up, months, range	20 (1–72)	23.4 (1–60)	19.1 (1–72)	
Tumour cells phenotype				
Non-GC phenotype positive, *n* (%)	42/53 (79.2)	7/9 (77.8)	35/44 (79.5)	1
GC phenotype positive, *n* (%)	11/53 (20.8)	2/9 (22.2)	9/44 (20.5)	
cMYC positive, *n* (%)	18/56 (32.1)	2/10 (20)	16/46 (56.5)	0.47
BCL2 positive, *n* (%)	40/57 (70.2)	6/10 (60)	34/47 (70.8)	0.46
cMYC/BCL2 positive, *n* (%)	15/56 (26.8)	1/10 (10)	14/46 (30.4)	0.26
CD5 positive, *n* (%)	2/57 (3.5)	0/10 (0)	2/47 (4.3)	1
P53 positive, *n* (%)	14/55 (25.5)	2/8 (25)	12/47 (25.5)	1
PDL1 positive, *n* (%)	10/57 (17.5)	10/10 (100)	0/47 (0)	
Cytogenetic				
BCL6 break positive, *n* (%)	12/54 (22.2)	2/9 (22.2)	10/45 (22.2)	1
PDL1 break positive, *n* (%)	8/47 (17)	8/10 (80)	0/37 (0)	**0.0001**

MSKCC, Memorial Sloan Kettering Cancer Center; *IPCG*, International Primary CNS Lymphoma Collaborative Group; *CR*, complete remission; *AWD*, alive with disease; *DOD*, dead of disease

*Comparing MSKCC class 1 + 2 versus class 3

**Comparing IPCG low + intermediate versus high

***Comparing CR versus AWD + DOD

PDL1 protein expression in tumour cells correlates with *PDL1* gene rearrangements

Using PAX5/PDL1 double stains, PDL1 protein expression by the neoplastic B cells was observed in 17.5% (10/57) of cases (Table 1, Figs. 1 and 2). When positive, at least 30% of neoplastic B cells expressed PDL1 and the mean percentage of PDL1 expressing tumour cells was 61% (30–100%).

We then investigated clinical and pathological parameters according to PDL1 protein expression in tumour cells (Table 1). We did not identify any correlation with clinical parameters although cases with PDL1 (+) tumour cells tend to present with poorer MSKCC (Memorial Sloan Kettering Cancer Center) prognostic score ($p = 0.06$). Of interest, PDL1 protein expression in tumour cells strongly correlated with the presence of *PDL1* gene rearrangements as demonstrated by the FISH analysis ($p = 0.0001$) (Figs. 1 and 2). We also identified three additional cases (3/47, 6.4%) showing more than two copies of *PDL1* suggesting polysomy for chromosome 9. However, these cases did not show PDL1 protein expression in tumour cells.

Among the ten patients with PDL1 positive expression in neoplastic B cells, two did not show *PDL1* gene translocation. Both cases had normal pattern using PDL1 break apart probe with 2 fusion signals. Both patients were in complete remission at last follow-up, after anthracycline-based chemotherapy. Concerning phenotypic features, one case had GC phenotype. This case had PD1 (+) TILs and PDL1 (+) TAMs. The other case demonstrated non-GC phenotype and showed PD1 (+) TILs without PDL1 (+) TAMs. Both cases were EBER negative.

Composition of the TME

Composition of the TME is summarised in Table 2.

TILs identified by CD3 immunostaining were observed in all cases and usually at low abundance. TILs predominantly consisted of CD8 (+) lymphocytes whereas CD4 (+) lymphocytes were less numerous in most cases (Fig. 3b–c). Moreover, a high proportion of cases (39/56, 69.6%) had TILs PD1 (+) (Fig. 3d).

Analysis of TAMs' abundance by CD68 immunostaining showed no macrophage in 10.9% (6/55) of cases, a low density of cells in 45.5% of cases (25/55), a moderate density in 34.5% (19/55) of cases and high density in 9.1% (5/55) of cases. CD68 (+) cells without CD163 co-

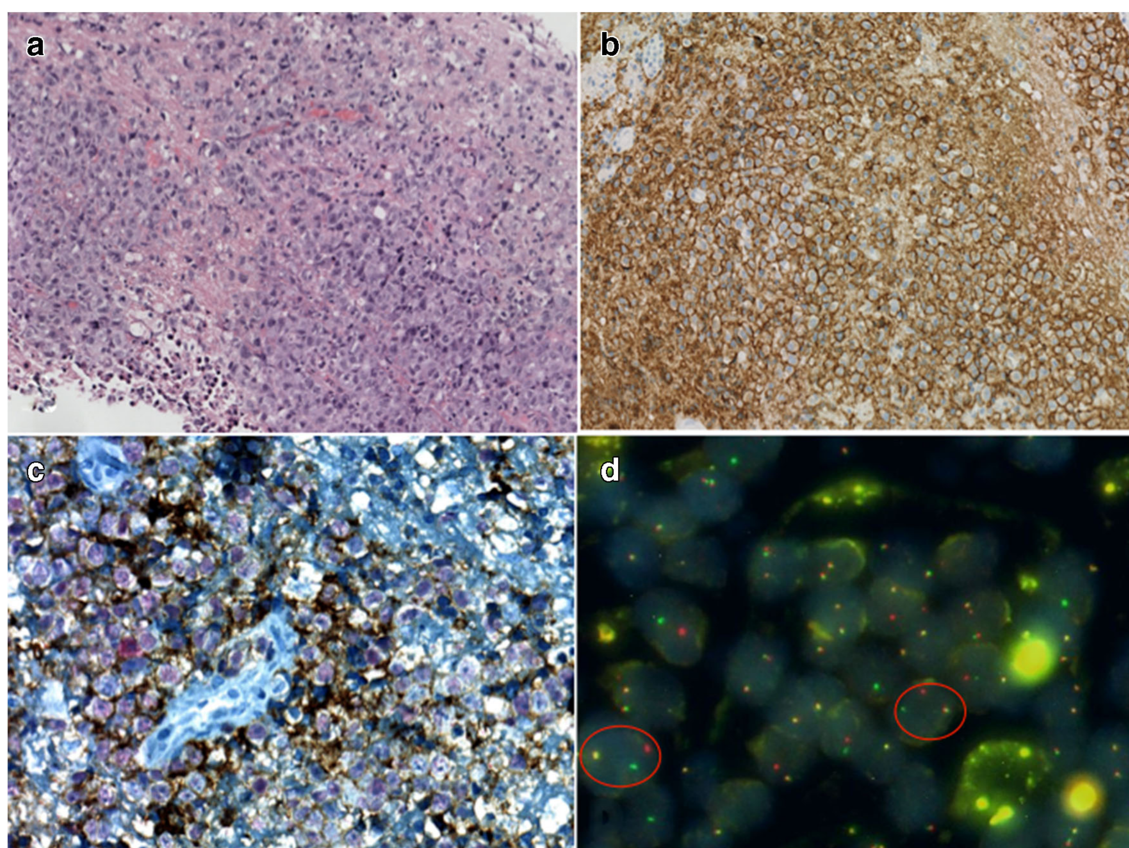

Fig. 1 PAX5 (red)/PDL1 (brown) double stains in a case of PCNS-DLBCL with *PDL1* gene translocation. **a** Haematoxylin and eosin stain (X20). **b** CD20 (X20). **c** PAX5 (red)/PDL1 (brown) X40. PDL1 (+) cells are PAX5 (+) and PAX5 (+) cells express PDL1. **d** PDL1 FISH analysis (X60). *PDL1* gene translocation with one fusion signal, one red signal and one green signal (red circles)

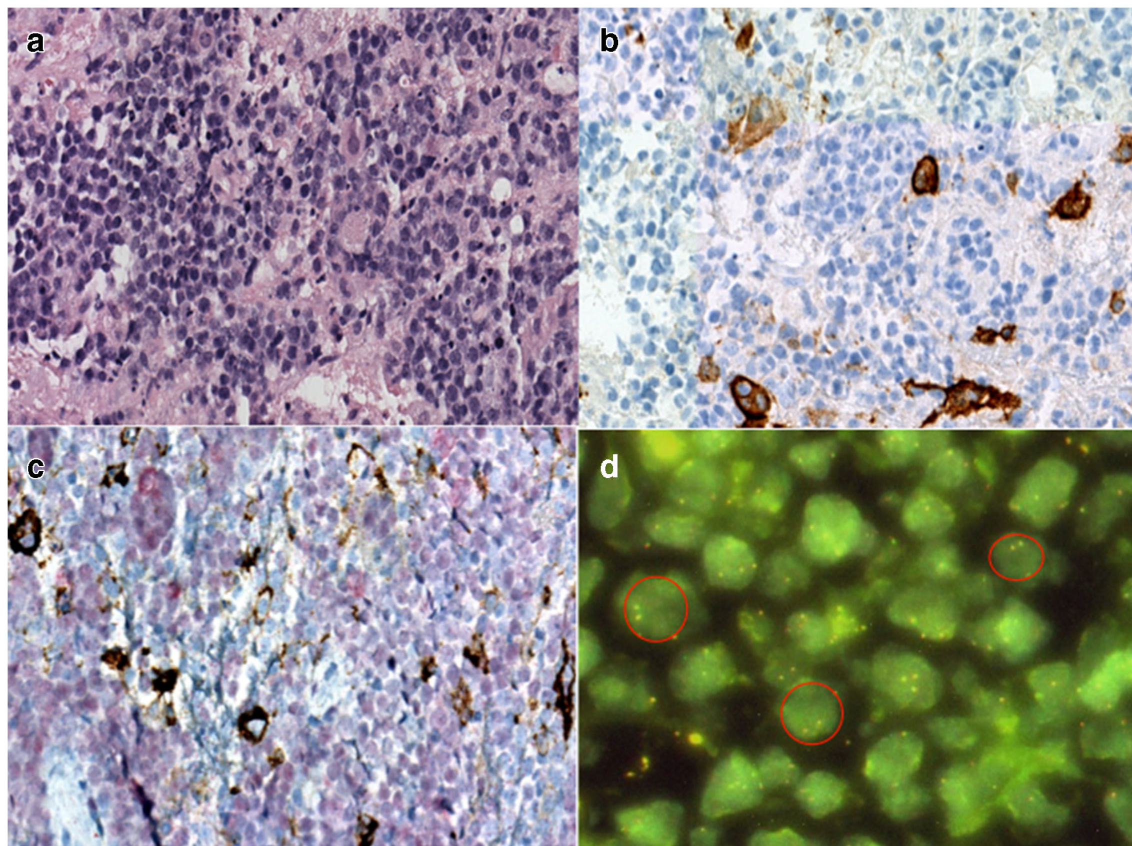

Fig. 2 PAX5 (red)/PDL1 (brown) double stains in a case of PCNS-DLBCL without *PDL1* gene translocation. **a** Haematoxylin and eosin stain (X40). **b** CD163 (X40). The microenvironment predominately consists of CD163 (+) M2 macrophages. **c** PAX5 (red)/PDL1 (brown) X40. PDL1 (+) cells are PAX5 (−), and PAX5 (+) cells do not express PDL1. **d** PDL1 FISH analysis (X60) showing normal pattern with 2 fusion signals (red circles)

expression were rare (4.1%), supporting the finding that most macrophages were M2 TAMs (Fig. 3e–f). TAMs usually expressed PDL1 (28/49, 57.1%) and rarely expressed PD1 (3/37, 6.4%) (Fig. 2b–c).

We did not identify any correlation between PD1 (+) expression in TILs and PDL1 (+) expression in tumour cells ($p = 0.25$). Nevertheless, PDL1 (+) expression in tumour cells was significantly associated with high density of TAMs ($p =$ 0,015) and tended to correlate with high density of TILs ($p =$ 0.06).

The presence of PD1 (+) TILs or PDL1 (+) TAMs is associated with better outcome

Supplementary Table 1 shows the correlation of the density of TILs or TAMs and the expression of PD1 and PDL1 in TME with clinicopathological parameters.

Density of TILs and ratio CD4 (+)/CD8 (+) did not significantly correlate with any of the clinicopathological parameters investigated although patients with ratio CD4 (+)/CD8 (+) ≥ 1 tend to present with high MSKCC prognostic score. Nevertheless, patients with TILs PD1 (+) had better outcome. PD1 positive expression in TILs was significantly correlated with complete remission at last follow-up, whereas absence of TILs PD1 (+) was significantly associated with relapse or progression and with death of disease or progressive pathology (Table 2). Furthermore, presence of TILs PD1 (+) was significantly associated with favourable impact on event-free and progression-free survival (Fig. 4a–b).

Density of TAMs did not correlate with the clinical outcome. Although patients with high density of TAMs were more frequently in complete remission after first line of treatment, we did not identify any impact on clinical presentation at diagnosis (both with MSKCC prognostic class and IPCG prognostic score) and on status at last follow-up. We observed a significant positive relationship between high density of TAMs and PDL1 positive expression in tumour cells. Moreover, the presence of PDL1 (+) TAMs significantly correlated with better survival. Patients with TAMs PDL1 (+) underwent less frequently at least 2 lines of treatment, usually achieved complete remission and showed fewer relapses/progression. Furthermore, presence of TAMs PDL1 (+) was significantly associated with favourable impact on event-free and progression-free survival (Fig. 4c–d).

Table 2 Composition of the tumour microenvironment

	Global cohort ($n = 57$)	Patients with PDL1 (+) tumour cells ($n = 10$)	Patients with PDL1 (−) tumour cells ($n = 47$)	*P* value
TILs				
Density of TILs				
0, *n* (%)	0/57 (0)	0/10 (0)	0/47 (0)	**0.06***
1, *n* (%)	38/57 (66.7)	4/10 (40)	34/47 (72.3)	
2, *n* (%)	15/57 (26.3)	4/10 (40)	11/47 (23.4)	
3, *n* (%)	4/57 (7)	2/10 (20)	2/47 (4.3)	
Ratio TILS CD4 (+)/CD8 (+)				
CD4 (+)/CD8 (+) = 1, *n* (%)	17/55 (30.9)	2/10 (20)	15/45 (33.3)	1
CD4 (+)/CD8 (+) < 1, *n* (%)	29/55 (52.7)	7/10 (70)	22/45 (48.9)	
CD4 (+)/CD8 (+) > 1, *n* (%)	9/55 (16.4)	1/10 (10)	8/45 (17.8)	
PD1 (+) TILs, *n* (%)	39/56(69.6)	9/10 (90)	30/46 (66.2)	0.25
TAMs				
Density of TAMs				
0, *n* (%)	6/55 (10.9)	2/10 (20)	4/45 (8.9)	**0.015***
1, *n* (%)	25/55 (45.5)	0/10 (0)	25/45 (55.6)	
2, *n* (%)	19/55 (34.5)	5/10 (50)	14/45 (31.1)	
3, *n* (%)	5/55 (9.1)	3/10 (30)	2/45 (4.4)	
Ratio CD68+/CD163+				
CD68 (+)/CD163 (+) = 1, *n* (%)	47/49 (95.9)	7/7 (100)	40/42 (95.2)	1
CD68 (+)/CD163 (+) > 1, *n* (%)	2/49 (4.1)	0/7 (0)	2/42 (4.8)	
PDL1 (+) TAMs, *n* (%)	28/49 (57.1)	7/10 (70)	21/39 (53.8)	0.48
PD1 (+) TAMs, *n* (%)	3/47 (6.4)	0/7 (0)	3/40 (7.5)	1

TILs, tumour-infiltrating lymphocytes; *TAMs*, tumour-associated macrophages

*Comparing 0 + 1 versus 2 + 3

**Comparing CD4 (+)/CD8 (+) ≤ 1 versus > 1

Discussion

In order to develop within immunocompetent hosts, tumour cells are able to evolve several immune escape strategies. Mechanisms leading to immune evasion that have been identified in lymphomas especially include recruitment of immunosuppressive cells such as TILs or TAMs, and dysregulation of immune checkpoints involved in the interaction of PD1 at the surface of T lymphocytes with its ligand PDL1 found at the surface of macrophages and some tumour cells [24, 25]. In this study, we aimed to characterise the TME and the immune checkpoint profiles and their association with the outcome in patients with PCNS-DLBCL.

In s-DLBCL, PDL1 may be expressed by both tumour B cells and TAMs. PDL1 expression in DLBCL has been reported around 20–30% of cases depending on the cell compartment analysed [8, 26] and on the antibody used for PDL1 identification. Of interest, using double staining of PAX5 and PDL1, some identified PDL1 positive expression in 10.5% of tumour B cells and in 15.3% of TAMs [8]. In the present work, the antibody used for PDL1 (E1L3N, Cell Signaling) detection has been previously used in several series investigating s-DLBCL and has shown a better signal-to-noise result than SP142 clone [27]. Using double staining of PAX5 and PDL1, we identified PDL1 positive expression in 17.5% (10/57) of tumour B cells. In a previous report of PCNS-DLBCL, we observed PDL1 protein expression in 37% of tumour B cells. Nevertheless, the PDL1 clone used was SP142, and we did not perform double staining to discriminate PDL1 (+) TAMs from PDL1 (+) tumour B cells.

We did not identify any correlation between PDL1 positive expression in tumour B cell and prognosis. In the largest series of s-DLBCL, patients with PDL1 (+) tumour cells had poorer prognosis [8, 28]. Of interest, as observed in our work, Pollari et al. showed, in their series of primary testicular lymphoma, that the presence of PDL1 (+) lymphoma cells was not associated with the outcome. Of interest, primary testicular and primary central nervous system lymphomas are both immune-privileged site-associated B cell lymphomas and share similar genetic and molecular features [19] rarely observed in s-DLBCL. Such distinctive biology could explain our data concerning the absence of clinical impact of PDL1 positive expression in lymphoma cells.

The structural anomalies on the chromosome 9p24.1 including gains, amplifications or translocations of the *PDL1* locus have been significantly correlated with PDL1

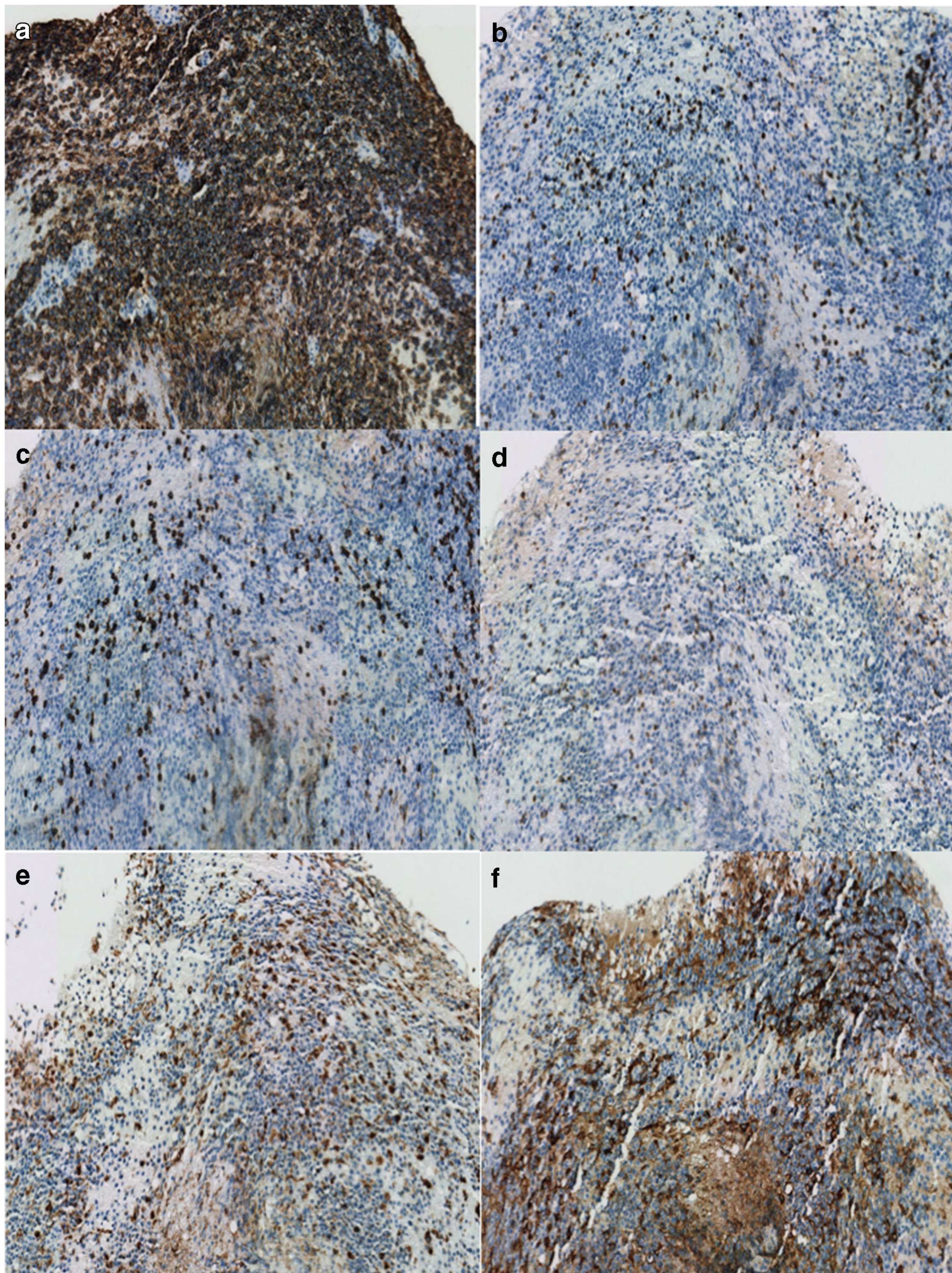

Fig. 3 Composition of the TME. **a** CD20 (X20) underlying tumour B cells. **b** CD3 (X20) showing few TILs. **c** CD8 (X20). Most TILs CD3 (+) express CD8. **d** PD1 (X20). Most TILs express PD1. **e** CD68 (× 20) showing moderate number of TAMs. **f** CD163 (× 20). TAMs CD68 (+) co-express CD163

expression in B lymphomas [19, 29]. In the present series, expression of PDL1 in tumour B cells was observed in all samples with translocations. However, in 2 cases, we identified PDL1 expression in lymphoma B cells without any cytogenetic aberrations (including polysomy for chromosome 9), indicating that there are other underlying mechanisms leading to the expression of the protein in PCNS-DLBCL. EBV infection can explain the expression of PDL1 in some cases of

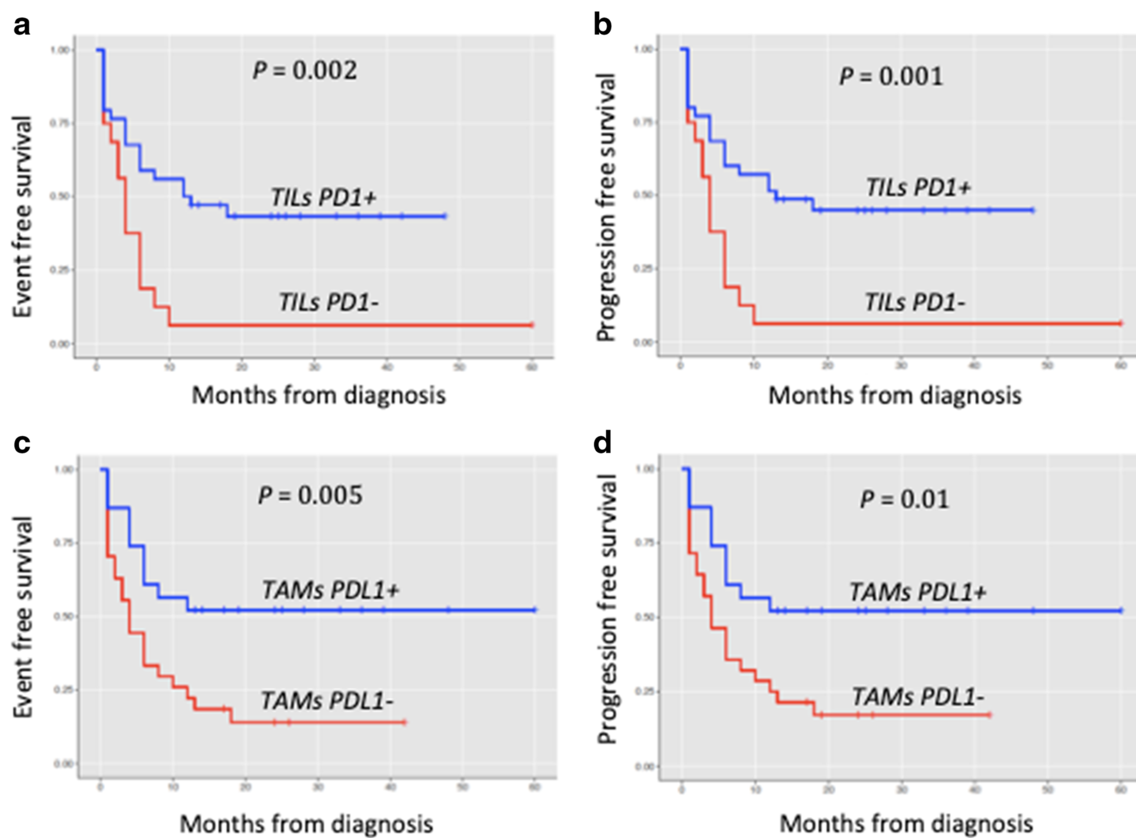

Fig. 4 Prognostic impact of PD1 protein expression in TILs and PDL1 protein expression in TAMs. **a** PD1 expression in TILs is significantly associated with a favourable impact on event-free survival. **b** PD1 expression in TILs is significantly associated with a favourable impact on progression-free survival. **c** PDL1 expression in TAMs is significantly associated with a favourable impact on event-free survival. **d** PDL1 expression in TAMs is significantly associated with a favourable impact on progression-free survival

lymphomas [30]; nevertheless, all cases in the present series were EBER negative. Active *JAK/STAT3* signalling has been associated with stimulation of PDL1 expression in lymphomas [29]. Of interest, oncogenic mutations of the *MYD88* gene lead to the activation of the JAK kinase [31] and these mutations are common in PCNS-DLBCL [32]. We thus hypothesised that *MYD88* mutations in PCNS-DLBCL are associated with the JAK kinase reactivation of STAT3 that in turn stimulates the expression PDL1.

We identified *PDL1* gene rearrangements in 17% (8/47) of cases. Translocations involving the *PDL1* locus have already been reported in PCNS-DLBCL and other immune-privileged site-associated B cell lymphomas. In their series, Chapuy et al. identified *PDL1* or *PDL2* translocations in 6% (4/66) of PCNS-DLBCL and in 4% (2/50) of primary testicular lymphomas [19]. *PDL1* gene translocations have been reported at higher frequency in PMBL and primary cutaneous diffuse large B cell lymphoma, leg type, occurring respectively in 20% (25/125) [33] and 40% (4/10) [34] of cases. Using the FISH analysis, *PDL1/PDL2* locus translocations have been reported in s-DLBCL at low frequency occurring in 3% (6/179) of cases [35]. Taken together, these results suggest that recurrent genomic rearrangements of *PDL1* underlie immune-privileged site-associated B cell lymphomas.

TILs are composed of a mixture of lymphocytes with different functions and phenotypes. The activation of both CD4 (+) and CD8 (+) TILs is needed for an efficient immune response [36]. Of interest, the ratio CD4 (+)/CD8 (+) T cells may be different for different types of cancers and DLBCL have been reported to have several fold more CD8 (+) TILs than some other cancers [37] In classical Hodgkin lymphoma, a predominance of CD4 (+) TILs is observed [38] and a high ratio CD4 (+)/CD8 (+) is associated with treatment failure [39]. On the contrary, s-DLBCL patients with ratio CD4 (+)/CD8 (+) ≥2 have better overall survival [6]. Here, we reported a predominance of CD8 (+) TILs in the background of tumour cells but we did not observe an association between prognostic and ratio CD4 (+)/CD8 (+). Presence of TILs PD1 (+) in DLBCL has been associated either with favourable, unfavourable or no prognostic effect in different studies [24]. These differences may result from methodologic and technical disparities among studies [40]. In their series of 74 primary testicular lymphoma patients, Pollari et al. showed that presence of PD1 (+) TILs predicted favourable survival [41]. In this series, we also identified a positive correlation between the outcome and the presence of PD1 (+) TILs.

Two main macrophage phenotypes have been described. They include the classically activated (or M1) macrophages

or the alternatively activated (or M2) macrophages [42]. M1 macrophages are involved in inflammatory response and antitumoral defence whereas M2 macrophages reduce inflammation and have tumour-promoting effect [43]. M2 macrophages are able to suppress the adaptive immune response through mechanisms including inhibition of T cell proliferation [42]. Part of their immunosuppressive activity is exerted by their release of chemokines (CCL17, CCL18 and CCL22) that preferentially attract T cell subsets devoid of cytotoxic function [44]. Nevertheless, in this series, although TAMs had M2 phenotype, TILs preferentially showed CD8+ cytotoxic phenotype.

Some have demonstrated that tumour cells are able to recruit and shift TAMs toward M2 function [45, 46]. In most studies, TAM density is associated with poor prognosis [47], and the presence of M2 TAMs has been shown to correlate with poor prognosis in DLBCL [48]. In the present work, we identified a polarisation to M2 macrophages in nearly all cases but we did not show any correlation between TAM density and adverse prognosis. Of interest, we identified an association between the presence of TAMs PDL1 (+) and better outcome. Such association has also been reported in primary testicular lymphoma [41], another aggressive large B cell lymphoma arising in immune-privileged site. Although PDL1 expression has been largely investigated in B cell lymphomas, its expression in TME has not been well defined and probably not separated from tumour cell expression in most studies. This could explain some discrepancies. In their series of 29 primary cutaneous DLBCL, Menguy et al. found a predominant M2 macrophages' infiltrate and demonstrated that M2 TAMs expressed PDL1 [49]. Nevertheless, they did not study any correlation with the outcome. In their series of primary intestinal DLBCL, Ishikawa et al. identified PDL1 negativity on microenvironment immune cells as a poor independent prognostic factor for overall survival. Thus, although our cases were associated with M2 macrophages in TME, usually reported as a pejorative biomarker in DLBCL, the presence of PDL1 (+) macrophage should counteract this adverse effect.

In conclusion, the TME in PNCS-DLBCL consists predominantly of M2 PDL1 (+) TAMs and CD8 (+), PD1 (+) TILs. *PDL1* gene translocation is a recurrent cytogenetic alteration in PNCS-DLBCL and correlates with PDL1 positive expression in tumour B cells. Expression of PD1 on TILs and PDL1 on TAMs, but not expression of PDL1 on tumour B cells, correlates with better prognosis. PD1 antibodies are currently approved by the US Food and Drug Administration (FDA) and tested in multiple clinical trials in B cell lymphomas with promising results. A retrospective analysis of PNCS-DLBCL and primary testicular lymphoma, which usually have *PDL1* genetic alterations, concluded that both these immune-privileged site-associated B cell lymphomas could be suitable for anti-PD1 immunotherapy [50], and a preclinical murine model concluded that anti-PD1 antibody had significant therapeutic activity against PNCS-DLBCL. Therefore, immune checkpoint inhibition are promising therapeutic options in PNCS-DLBCL and the results of underway clinical trials to test these drugs are eagerly awaited (CheckMate 647).

Author contributions Vanessa Szablewski, Melissa Alame, Valérie Costes-Martineau, Valérie Rigau and Valère Cacheux designed the research project. Vanessa Szablewski, Marion Pirel, Valérie Rigau, Luc Durand and Valérie Costes-Martineau evaluated the histological and immunohistochemical findings. Vanessa Szablewski, Melissa Alame, Laura De Oliveira, Alicia Tourneret and Valère Cacheux evaluated the cytogenetic findings. Vanessa Szablewski and Melissa Alame obtained data and wrote the main part of the manuscript. Marion Pirel, Valérie Costes-Martineau, Luc Bauchet, Michel Fabbro, Pascal Roger, Samia Gonzalez, Luc Durand, Laura De Oliveira, Alicia Tourneret Tempier, Valérie Rigau and Valère Cacheux reviewed the draft with critical comments.

Compliance with ethical standards

This study was carried out in agreements with the Declaration of Helsinki and was approved by the Centre des Ressources Biologiques (CRB) of the Centre Hospitalo Universitaire (CHU) of Montpellier, France.

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Written informed consent for the study was obtained from the patient.

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