

Expression of Programmed Cell Death 1 Ligand 2 (PD-L2) Is a Distinguishing Feature of Primary Mediastinal (Thymic) Large B-cell Lymphoma and Associated With *PDCD1LG2* Copy Gain

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Abstract: Primary mediastinal (thymic) large B-cell lymphoma (PMBL) and diffuse large B-cell lymphoma (DLBCL) are tumors with distinct clinical and molecular characteristics that are difficult to distinguish by histopathologic and phenotypic analyses alone. Programmed cell death 1 ligand 2 (PD-L2) is a cell surface protein expressed by activated macrophages and dendritic cells that binds PD-1 on T cells to inhibit immune responses. Amplification and/or translocations involving chromosome 9p24.1, a region that includes *PDCD1LG2*-encoding PD-L2, is a common event in PMBL but not DLBCL and suggests that PD-L2 expression might be a distinguishing feature of PMBL. We developed an assay for the immunohistochemical detection of PD-L2 protein in fixed biopsy specimens (PD-L2 IHC), which we applied to a cohort of PMBLs and DLBCLs. For a subset of cases, we correlated the results of PD-L2 IHC with *PDCD1LG2* copy number (CN) as determined by quantitative polymerase chain reaction. Twenty-three of 32 (72%) PMBLs but only 1 of 37 (3%) DLBCLs were positive by PD-L2 IHC. Among PMBLs with *PDCD1LG2* CN gain, all were positive by PD-L2 IHC. One PMBL without CN gain was positive by PD-L2 IHC. When expressed in PMBL, PD-L2 was restricted to tumor cells and not detected on intratumoral macrophages. We conclude that PD-L2 protein is robustly expressed by the majority of PMBLs but only rare DLBCLs and often associated with *PDCD1LG2* copy gain. PD-L2 IHC may serve as a useful ancillary test for distinguishing

PMBL from DLBCL and for the rational selection of patients for therapeutic antibodies that inhibit PD-1 signaling.

Key Words: PD-1, PD-L2, primary mediastinal large B-cell lymphoma, diffuse large B-cell lymphoma, *PDCD1LG2*

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Primary mediastinal (thymic) large B-cell lymphoma (PMBL) and diffuse large B-cell lymphoma, not otherwise specified (DLBCL) are aggressive tumors of mature B cells with distinct clinical and genetic characteristics that can be difficult to distinguish in routine surgical pathology practice.^{1–5} PMBL and DLBCL have extensive histomorphologic and phenotypic similarities including a sheet-like growth pattern of large lymphoid cells and the expression of pan-B lineage markers such as CD19, CD20, CD79a, and PAX5.^{6,7} Other phenotypic markers useful for the subclassification of B-cell lymphomas, including BCL6, IRF4/MUM1, and CD30, can be expressed by PMBL and DLBCL. MAL, TRAF1, nuclear cRel, CD23, and CD200 are preferentially expressed by PMBL, and immunohistochemical (IHC) analysis for 1 or more can facilitate final tumor classification, but no single marker is entirely sensitive and specific.^{8–12}

Programmed cell death 1 (PD-1) and the programmed death ligands (PDLs), PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC), form a signaling network that limits T-cell immunity during chronic viral infection and during pregnancy.^{13,14} A subset of malignant tumors co-opts the PD-1 signaling axis to evade immune surveillance.¹⁵ Targeting PD-1 signaling with inhibitory antibodies can relieve the block in antitumor immunity and achieve durable clinical responses in a subset of patients with solid tumors.^{16,17} We, and others, have found that 70% of PMBLs but <5% of DLBCLs, have amplification of chromosome 9p24.1—a region that includes *CD274* (encoding PD-L1) and *PDCD1LG2* (encoding PD-L2).^{5,18–20} Consistent with these results, we have found that PMBL cell lines express PDLs, whereas

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DLBCL cell lines do not.¹⁸ These results indicate that *CD274* and *PDCD1LG2* amplification can result in increased PD-L1 and PD-L2 protein production.

Recently, we surveyed the protein expression of PD-L1 on aggressive lymphomas and found tumor-specific expression of PD-L1 to be common in PMBL but not DLBCL.²¹ We also found that PD-L1 is highly expressed by tumor-associated macrophages (TAMs) in most PMBLs and in a subset of DLBCLs—a result that complicates the interpretation of PD-L1 IHC.²¹ A detailed examination of PD-L2 protein expression in PMBL and DLBCL has not been performed because of the lack of quality antibodies that are amenable to IHC on formalin-fixed paraffin-embedded (FFPE) tissue sections. Herein, we establish an IHC staining protocol for the detection of PD-L2 protein in FFPE samples that we apply to a series of fixed, primary PMBL and DLBCL tissue biopsies. For a subset of cases with matched frozen tissue, we compared the results of PD-L2 IHC with the results of *PDCD1LG2* copy number (CN) analysis.

MATERIALS AND METHODS

Cell lines

Cell lines (300.19, SU-DHL-4, Karpas 1106 P, HDLM-2) were maintained as previously described.^{18,22} FFPE cell pellets were generated by harvesting 10^7 cells, washing, and fixing in 2 mL 10% formalin at room temperature for 20 minutes. Cells were then washed, suspended in matrigel, processed, embedded in paraffin as a cell pellet tissue microarray, and cut onto glass slides as per standard histologic procedures.

Case Selection

All FFPE biopsy samples (PMBL, $n = 32$; DLBCL, $n = 37$) were obtained from the files of the Department of Pathology at Brigham and Women's Hospital, Boston, MA, with institutional internal review board approval. Diagnoses were established according to the criteria of the World Health Organization classification.¹ Cases classified as PMBL were confirmed, as part of this study, to satisfy the combination of clinical, radiologic, morphologic, and phenotypic criteria for this entity. These include radiologic identification of an isolated mediastinal mass in a young or middle aged individual with or without local extension into adjacent tissues and organs, a morphologic pattern showing sheets of large atypical lymphoid cells with or without a sclerotic background and scattered thymic remnants, and positive staining of tumor cells for mature B lymphoid markers, TRAF1 and/or cRel, and/or CD23 and/or CD30, and an absence of CD15 expression as previously described.²³ For a subset of cases (PMBL, $n = 12$; DLBCL, $n = 9$), matched frozen tumor samples were obtained at the time of biopsy.

Immunohistochemistry

IHC analysis was performed using 4- μ m-thick, formalin or B+ -fixed, FFPE tissue sections. Slides were

baked, deparaffinized in xylene, and passed through graded alcohols; antigen was then retrieved with 10 mM citrate buffer, pH 6.0 (Invitrogen/Life Technologies, Grand Island, NY), in a steamer for 30 minutes. All further steps were carried out at room temperature in a hydrated chamber. Slides were pretreated with Peroxidase Block (Dako, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity and then washed in 50 mM Tris-Cl, pH 7.4. Slides were blocked using Protein Block (Dako) as per manufacturer's instruction and subsequently incubated with anti-PD-L2 (clone 366C.9E5, 0.14 μ g/mL final concentration, generated in the laboratory of G. Freeman, gordon_freeman@dfci.harvard.edu) in Da Vinci green diluent (BioCare Medical, Concord, CA) for 1 hour or anti-CD68 (clone PG-M1, Dako, 1:150 dilution) in antibody diluent (Dako) for 1 hour. Slides were then washed in Tris buffer and, for anti-PD-L2, treated with anti-mouse horseradish peroxidase-conjugated antibody (Power-Vision; Leica Biosystems, Buffalo Grove, IL) for 30 minutes or, for anti-CD68, treated with mouse envision secondary antibody (Dako) for 30 minutes. After further washing, immunoperoxidase staining was developed using a 3,3'-diaminobenzidine chromogen (Dako) for 5 minutes. Slides were counterstained with hematoxylin, dehydrated in graded alcohol and xylene, and mounted and coverslipped.

Double staining for PD-L2 and CD68 was performed using an automated staining system (Bond III; Leica Biosystems). Heat-induced antigen retrieval was performed using ER1 solution (pH 6) (Leica Biosystems) for 30 minutes. Anti-PD-L2 antibody was incubated for a total of 2 hours with 2 separate applications. Postprimary AP blocking reagents were incubated for 20 minutes, followed by 15 minutes of AP-labeled polymer. Red substrate was incubated for 15 minutes. All reagents were components of the Bond Polymer AP Red detection system (Leica Biosystems). Anti-CD68 (PG-M1) immunostaining was performed subsequently with a 1:150 dilution using Bond Primary Antibody Diluent. CD68 primary antibody was incubated for 1 hour, followed by 8 minutes of postprimary blocking reagent, 12 minutes of horseradish peroxidase-labeled polymer, and 5 minutes of peroxidase block. All reagents were components of the Bond Polymer Refine detection system (Leica Biosystems). Slides were then taken off the autostainer, and HighDef Blue IHC chromogen (HRP) (Enzo Life Sciences, Farmingdale, NY) was applied manually and incubated for 10 minutes. Slides were coverslipped with Dako Faramount Aqueous Mounting medium (Dako).

Evaluation and Scoring of IHC Staining

Reactivity for PD-L2 was determined and scored by 2 hematopathologists (M.S. and S.J.R.). Discrepant results in staining interpretation ($< 5\%$ of cases) were resolved in a consensus conference. For each stained slide, the percentage of tumor cells showing positive staining for PD-L2 was recorded in 10% increments (0% to 100%). In addition, the intensity of positive staining was recorded: (–) = no staining detected, (1+) = weak

staining, (2+) = moderate staining, (3+) = strong staining. A case was scored as positive if at least 20% of the tumor cells stained positive for PD-L2 with an intensity of 1+, 2+, or 3+.

DNA Isolation and *PDCD1LG2* CN Analysis

Genomic DNA was extracted from cryostat sections of frozen biopsy tissues as previously described.^{18,24} CN of *PDCD1LG2* (locus of PD-L2) at chromosome 9p24.1 was assessed with the Taqman CN assay Hs00797077_cn. RNase P was used as reference control (#4403326). Quantitative polymerase chain reaction (PCR) was performed with Taqman Universal Genotyping Master Mix (Applied Biosystems/Life Technologies) on an ABI 7300/7500 real-time PCR machine according to the manufacturer's protocol. The CN of the loci of PD-L2 was inferred by the $2^{-\Delta\Delta CT}$ method,²⁵ normalized to the mean of 9 normal samples (2 reactive tonsils and 7 reactive lymph nodes). A CN above the threshold of 2.2 was assigned as CN gain, consistent with previous analyses.¹⁸ Hodgkin lymphoma cell lines with well-defined CN status of this region were used as positive controls.¹⁸

Imaging and Statistics

Cases were photographed with an Olympus BX41 microscope with $\times 100/0.75$ Olympus UPlanFL (Olympus, Melville, NY) objective at $\times 1000$ final magnification. All pictures were taken using Olympus QColor3 camera and analyzed with acquisition software QCapture v2.60 (QImaging, Burnaby, BC, Canada) and Adobe Photoshop 6.0 (Adobe, San Jose, CA).

RESULTS

Validation of Anti-PD-L2 Antibody for IHC

We optimized IHC staining using a mouse monoclonal antibody targeting human PD-L2 (PD-L2 IHC) on a series of genetically defined, FFPE cell lines. We observed no staining of an untransfected, mouse pre-B-cell line 300.19 (Fig. 1A) and no staining of line 300.19 stably transfected with full-length, human PD-L1 (Fig. 1B). In contrast, we observed intense staining of line 300.19 stably transfected with full-length, human PD-L2 in a cytoplasmic and cell membrane pattern (Fig. 1C). The DLBCL cell line SU-DHL-4, which has the normal 2 copies of 9p24.1 and no detectable PD-L2 protein by flow immunophenotyping or western blot analysis,^{18,21} showed no staining for PD-L2 protein by IHC (Fig. 1D). In contrast, the PMBL cell line Karpas 1106 P, which has intermediate-level amplification of *PDCD1LG2* at 9p24.1 (average 3.8 copies) and expresses PD-L2 protein,¹⁸ showed weak but positive staining for PD-L2 by IHC (Fig. 1E). The Hodgkin lymphoma cell line, HDLM-2, which has high-level amplification of *PDCD1LG2* at 9p24.1 (> 7 copies) and expresses PD-L2 protein,^{18,21} showed intense cytoplasmic and membrane staining for PD-L2 by IHC (Fig. 1F). These results verify that PD-L2 IHC is sensitive and specific and demonstrate a correlation between the intensity of staining and *PDCD1LG2* CN.

Staining of human lymphoid tissues revealed staining of expected structures. Specifically, reactive lymph nodes showed weak staining in a pattern consistent with follicular dendritic cells within germinal centers (Figs. 1G, H). Lymphocytes within the B-cell-rich follicles and T-cell-rich interfollicular regions were negative for staining. Similarly macrophages within germinal centers, interfollicular regions, and subcapsular sinuses were negative for PD-L2 (Fig. 1H, data not shown). Upon staining additional tissue specimens, we observed positive staining of activated macrophages and multinucleated giant cells associated with marked, acute inflammation in a distinct, membranous pattern (Fig. 1I). These data suggest that PD-L2 is minimally expressed by immune cells within reactive lymphoid tissue but can be induced to high levels on tissue macrophages and giant cells associated with acute inflammation.

Expression of PD-L2 in PMBL and DLBCL

Next, we investigated PD-L2 expression by IHC in a collection of 32 PMBLs and 37 DLBCLs. There was a spectrum of staining patterns across tumor samples. Cases scored as positive for PD-L2 showed distinct membranous staining in $\geq 20\%$ tumor cells at 3+ (Fig. 2A), 2+ (Fig. 2B), or 1+ (Fig. 2C) intensity. Cases not fulfilling these criteria were scored as negative for PD-L2 (Fig. 2D). Twenty-three of 32 (72%) PMBLs were scored as positive for PD-L2 by this method (Table 1). In contrast, only 1 of 37 (3%) DLBCLs were scored as positive ($P < 0.001$, Fisher exact test). Among the positive PMBLs, 19 cases (83%) had membranous PD-L2 expression in $\geq 60\%$ of the malignant cells, and 4 cases (17%) had similar staining in 20% to 59% of the malignant cells (Table 2). The majority of positive PMBLs expressed PD-L2 in a membranous pattern and at 3+ intensity (18/23, 78%) (Table 2). Four PMBLs (17%) showed positive staining at 2+ intensity, and 1 PMBL (4%) showed positive staining 1+ intensity (Table 2). The single DLBCL case scored as positive for PD-L2 showed positive staining of 20% of the malignant cells at 2+ intensity. Rereview of the clinical, histomorphologic, and phenotypic features of this case did not suggest misclassification of a PMBL (data not shown). Taken together these findings indicate that PD-L2 IHC is a highly specific (97%) and moderately sensitive (72%) method for distinguishing PMBL from DLBCL.

9p24.1/*PDCD1LG2* CN and PD-L2 Expression in PMBL and DLBCL

Up to 70% of PMBLs have amplification of chromosome 9p24.1, a region that includes the genes encoding both PD-1 ligands, *PDCD1LG2* and *CD274*.^{18,26} In primary PMBL, there is a close association between *PDCD1LG2* copy gain and increased PD-L2 transcript abundance.¹⁸ We therefore evaluated the relationship between *PDCD1LG2* CN and PD-L2 protein expression in 12 PMBLs and 9 DLBCLs for which we had matched frozen and paraffin tissue samples using a quantitative PCR assay for *PDCD1LG2* CN and PD-L2 IHC (for

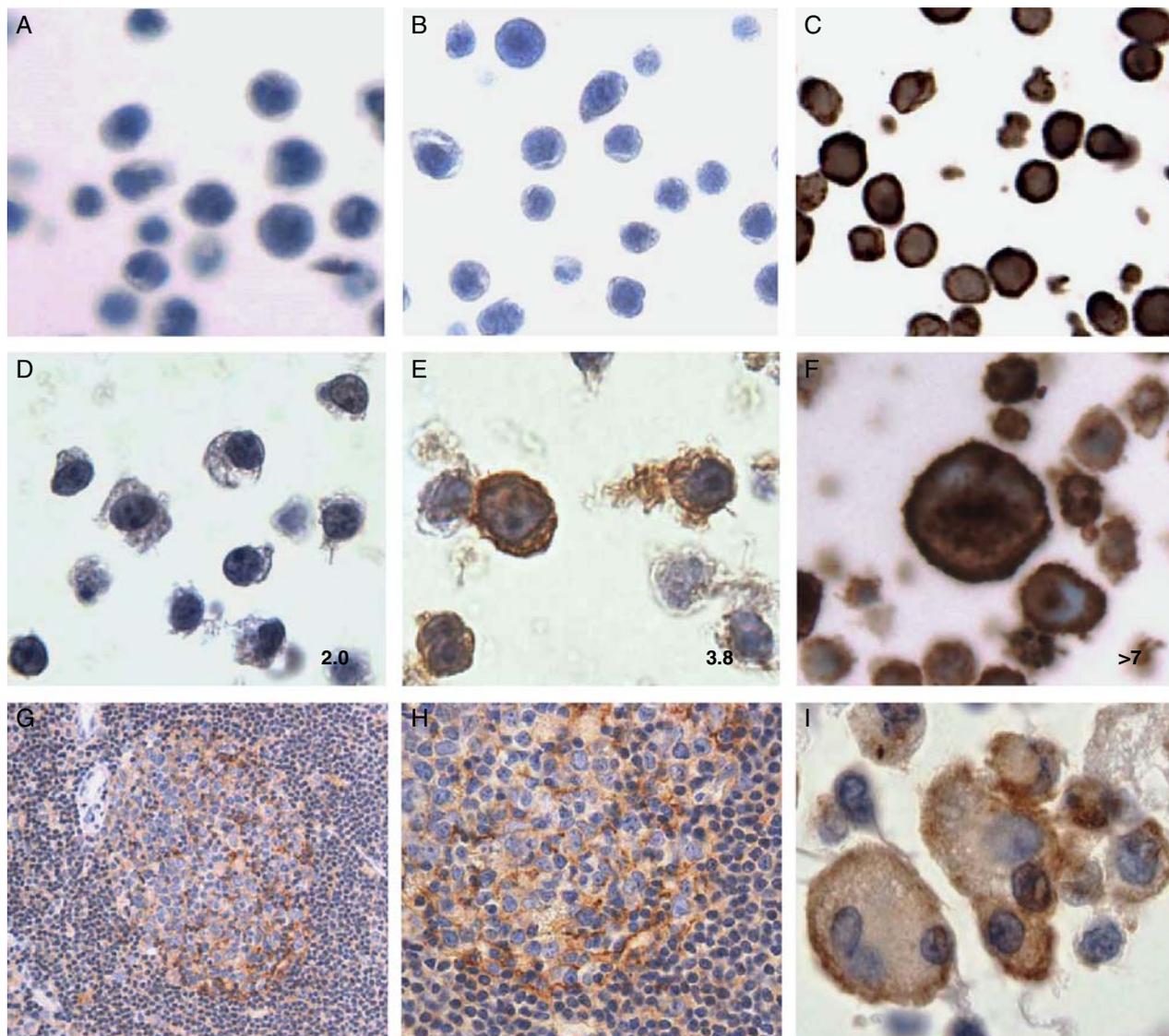


FIGURE 1. IHC staining of FFPE cell lines and select tissues with anti-PD-L2 antibody. Staining of (A) untransfected murine pre-B-cell line, 300.19, (B) pre-B line stably expressing human PD-L1, (C) pre-B line stably expressing human PD-L2, (D) DLBCL cell line SU-DHL-4, (E) mediastinal large B-cell lymphoma cell line Karpas 1106 P, (F) Hodgkin lymphoma line HDLM-2, (G) lymph node showing a reactive germinal center, (H) high-power image of a reactive germinal center, (I) macrophages, including a multinucleated giant cell, associated with necrotic tissue. The average *PDCD1LG2* CN for each human cell line is indicated in the bottom right corner of each image.

details see the Materials and methods section). Previously characterized Hodgkin lymphoma cell lines with known low (L428, 2.6 copies), intermediate (SUP-HD-1, 3.3 copies), and high (HDLM-2, > 7 copies) *PDCD1LG2* CN served as positive controls (Fig. 3).¹⁸ None of the DLBCLs showed amplification of *PDCD1LG2* consistent with the lack of staining for PD-L2 by IHC (Fig. 3). In contrast, 9 of 12 (75%) PMBLs had *PDCD1LG2* amplification, and all 9 were positive for PD-L2 by IHC (Figs. 3A, B). Interestingly, 1 PMBL without *PDCD1LG2* copy gain (PMBL case #2) was positive for PD-L2 by IHC. Whereas 2 of 3 PMBLs lacking *PDCD1LG2* CN

gain (PMBL cases #1 and #3) showed no PD-L2 staining, cases with even low-level *PDCD1LG2* CN gain (ie, PMBL case #5, 2.3 average copies) exhibited distinctly positive, membranous PD-L2 staining (Fig. 3B). As expected, cases with high CN gain (ie, PMBL case #12, 10.9 average copies) were associated with very intense, membranous staining for PD-L2 (Fig. 3B). These data indicate that PD-L2 IHC effectively captures PD-L2 protein expression in all cases of PMBL with *PDCD1LG2* CN gain, including those with low-level gain, and identifies additional cases in which PD-L2 may be dysregulated by alternative mechanisms.

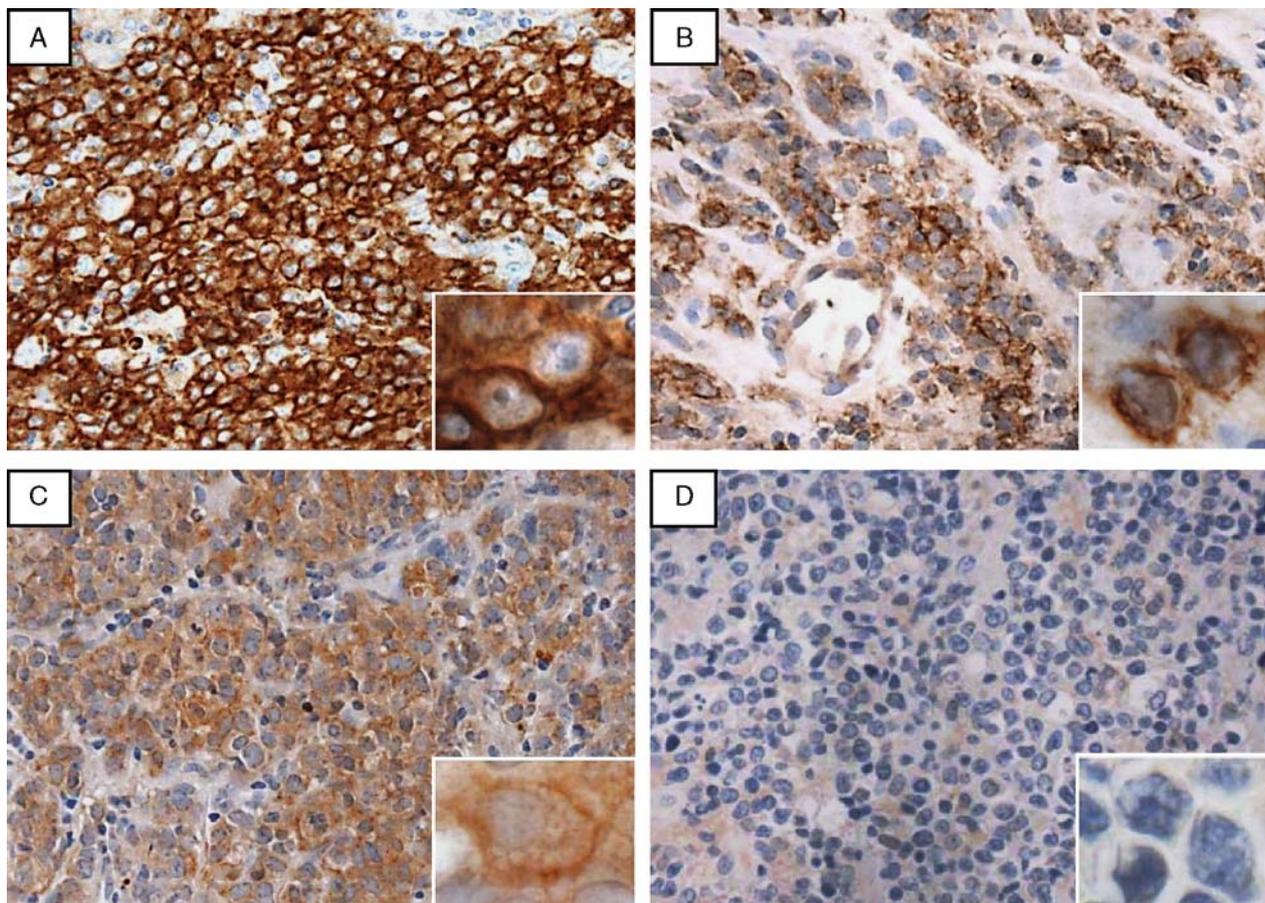


FIGURE 2. PD-L2 IHC staining patterns in PMBL. Cases showing (A) intense membranous staining scored as 3+, (B) moderate membranous staining scored as 2+, (C) light membranous staining scored as 1+, (D) no membranous staining scored as 0. High magnification images to show cytoplasmic detail are given (insets).

Tumor-associated Macrophages Do Not Express Detectable PD-L2 in PMBL and DLBCL

Our data revealed low to undetectable PD-L2 expression on the resident macrophage populations colonizing reactive lymphoid tissue but high expression on tissue macrophages at sites of acute inflammation. We therefore wished to determine whether PD-L2 protein is detected on TAMs in PMBL and DLBCL. Review of PD-L2 IHC revealed that distinct, membranous staining for PD-L2 was restricted to the large, neoplastic tumor cells despite the presence of small lymphocytes and macrophages within the tumor microenvironment (Fig. 4A). A case of PMBL that was double-stained for PD-L2 and the macrophage marker CD68 revealed distinct cellular populations expressed the 2 proteins (Fig. 4B). In addition,

cases of PMBL and DLBCL without any positive staining for PD-L2 contained frequent CD68⁺ TAMs (Figs. 4C–F, respectively).

DISCUSSION

PMBL is an aggressive large B-cell lymphoma with a distinct clinical presentation and course.^{3,7} The major differential diagnosis for PMBL is classical Hodgkin lymphoma (cHL) and DLBCL.¹ For the practicing pathologist, the distinction between PMBL and cHL is usually straightforward, although occasional cases can exhibit features intermediate between PMBL and cHL.²⁷ In contrast, the distinction between PMBL and DLBCL on histopathologic and phenotypic findings alone is often very difficult in the absence of ancillary clinical and

TABLE 1. PD-L2 Expression in PMBL and DLBCL

Diagnosis	No. Cases	Positive* (n [%])	Negative (n [%])
PMBL	32	23 (72)	9 (28)
DLBCL	37	1 (3)	36 (97)

*Positive: membranous PD-L2 staining ≥ 20% tumor cells with 1+, 2+, or 3+ intensity.

TABLE 2. Percentage and Intensity of PD-L2 Expression in PMBL and DLBCL

Diagnosis	PD-L2 +	Percentage		Intensity		
		≥ 60%	20%-59%	+++	++	+
PMBL (n [%])	23	19 (83)	4 (17)	18 (78)	4 (17)	1 (4)
DLBCL (n [%])	1	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)

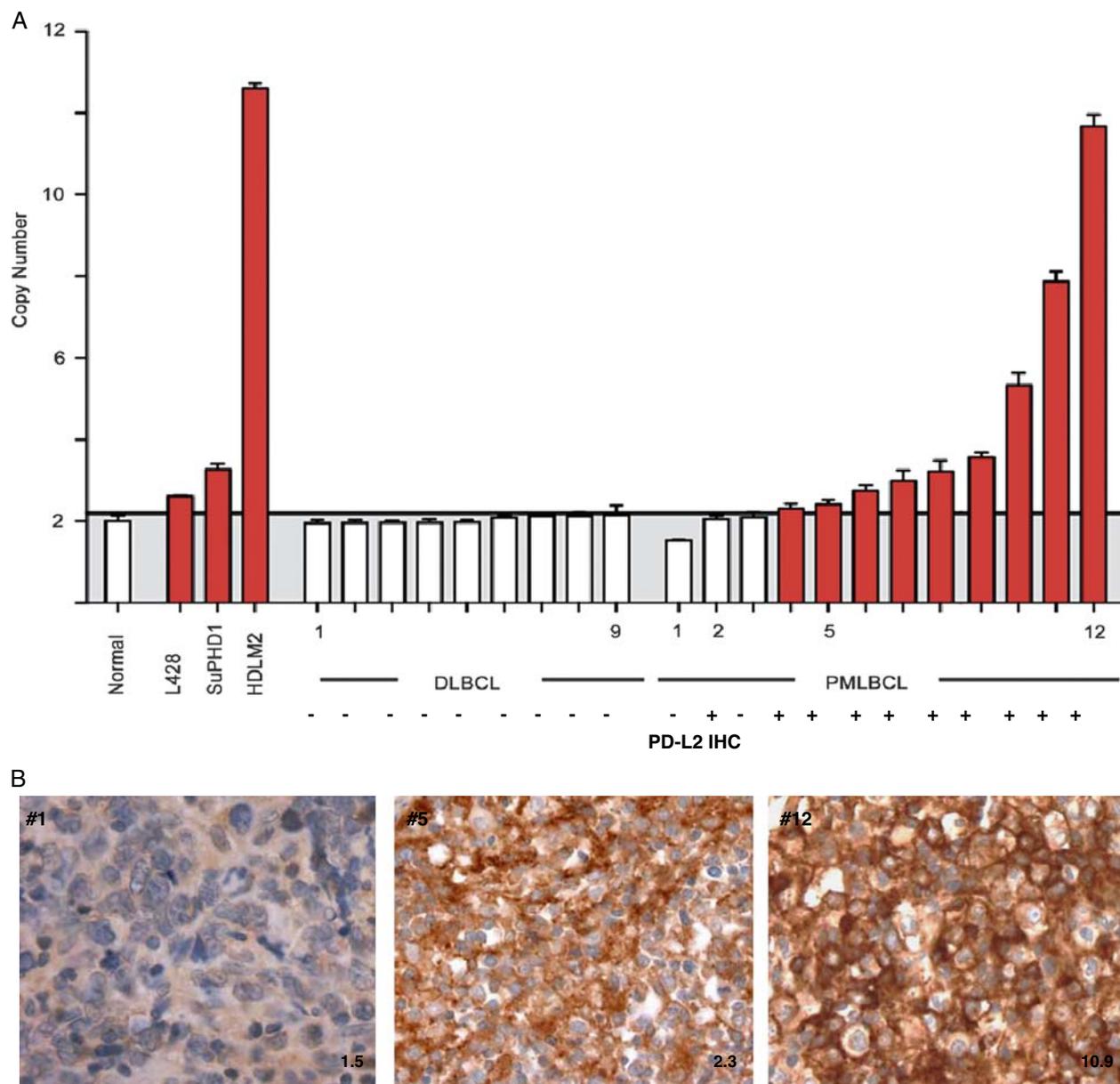


FIGURE 3. Correlation between *PDCD1LG2* CN and PD-L2 IHC for select DLBCLs and PMBLs. A, *PDCD1LG2* CN as determined by quantitative PCR for 9 normal controls, Hodgkin lymphoma cell lines L428, SUP-HD-1, and HDLM-2, 9 DLBCLs, and 12 PMBLs. The corresponding PD-L2 IHC result is indicated below the *PDCD1LG2* CN for each case. Cases without CN gain (white bars) and with CN gain (red bars) are indicated. B, PD-L2 IHC corresponding to PMBL cases #1, #5, and #12, respectively, in (A) with the *PDCD1LG2* CN indicated in the bottom right corner of each image.

radiographic data.⁶ This distinction is important, as treatment regimens are increasingly tailored to patients with PMBL,^{28,29} and, in an era of molecularly targeted therapies, it is critical to ensure that the appropriate patient population is selected for clinical trials.³⁰

Several proteins, detectable by IHC in FFPE biopsy specimens, have been proposed to distinguish PMBL from DLBCL. These include MAL, activated (phosphorylated) STAT6 (p-STAT6), p63, TRAF1, activated (nuclear) cRel, TNFAIP2, CD23, and CD200.^{8,10-12,31,32} The most robust of these markers show sensitivities

ranging from 69% (CD23) to 94% (CD200) and specificities ranging from 77% (TRAF1) to 97% (MAL).¹² In this study we found that the expression of PD-L2 is 72% sensitive and 97% specific for the diagnosis of PMBL relative to DLBCL and therefore comparable in sensitivity to most and superior in specificity to almost all IHC markers described to date.

PD-L2 and PD-L1 are unique among the diagnostic markers of PMBL, because these proteins are often overexpressed as a consequence of specific, genetic lesions. Abnormalities involving chromosome 9p24.1 are

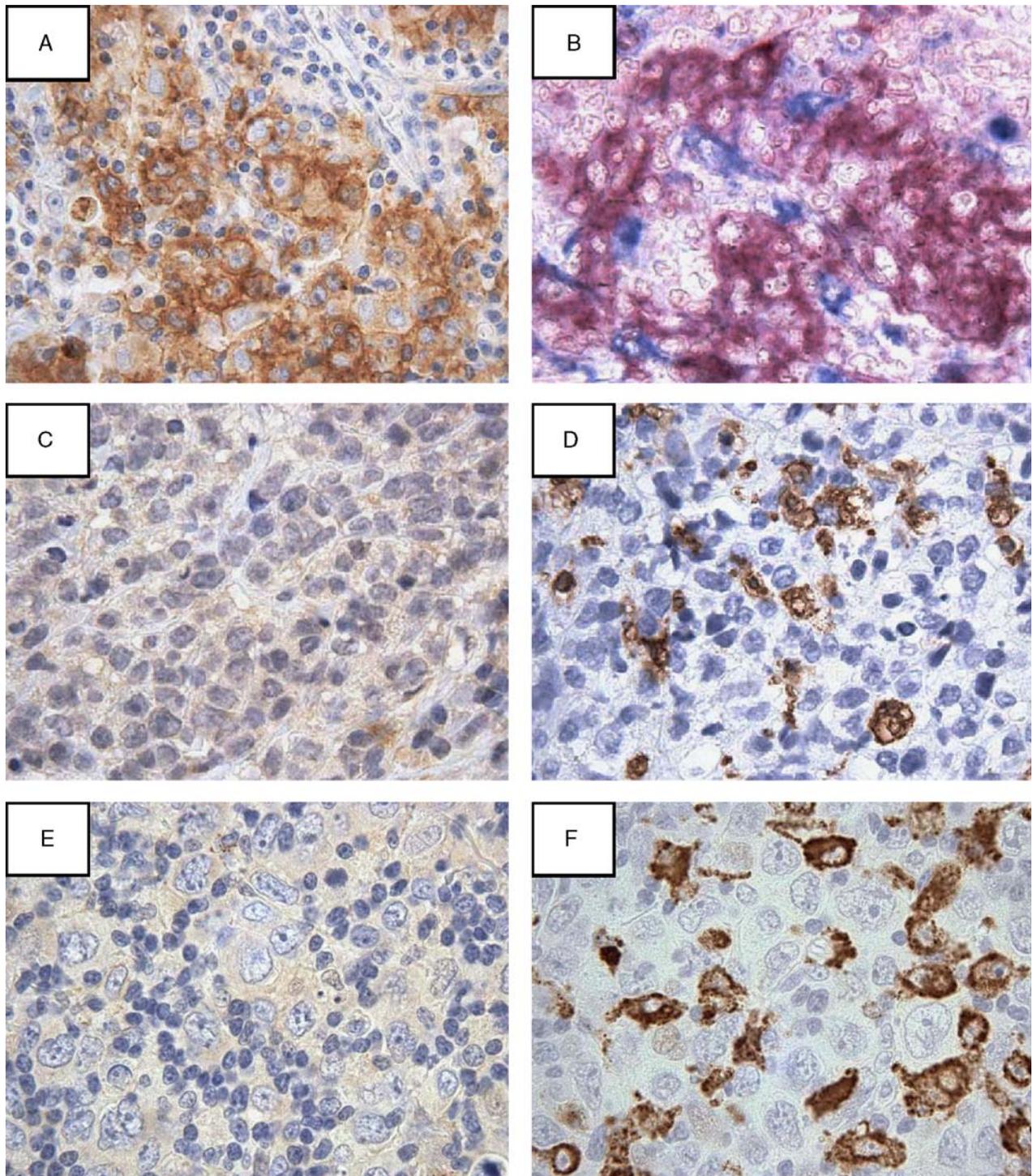


FIGURE 4. Expression of PD-L2 within the tumor microenvironment of PMBL and DLBCL. A, Case of PMBL stained with anti-PD-L2 antibody and showing positive staining of tumor cells (positive staining = brown). B, The case of PMBL shown in (A) double-stained with anti-PD-L2 and anti-CD68 antibodies and showing positive staining in distinct cellular populations (positive staining for anti-PD-L2 = red, positive staining for anti-CD68 = blue). C, A second case of PMBL stained with anti-PD-L2 antibody and showing no positive staining of tumor cells (positive staining = brown). D, The case of PMBL shown in (C) stained with anti-CD68 antibody and showing positive staining intratumoral macrophages (positive staining = brown). E, Case of DLBCL stained with anti-PD-L2 antibody and showing no positive staining of tumor cells (positive staining = brown). F, The case of DLBCL shown in (E) stained with anti-CD68 antibody and showing positive staining intratumoral macrophages (positive staining = brown).

common in PMBL and in cHL, occurring in approximately 70% and 30% of cases, respectively; in contrast, these lesions are rare in DLBCL.^{18,24,33} The frequency of 9p24.1 abnormalities in PMBL suggests critical biological roles for 4 major genes that reside at this locus, *PDCD1LG2*, *CD274*, *JAK2*, and *JMJ2DC*, and amplification of 9p24.1 is often associated with increased transcription of all 4 genes.^{18,20,26,30,33,34} However, gene expression profiling studies indicate that the relative overexpression of PD-L2 typically exceeds that of PD-L1 in PMBL.^{18,20} Indeed, PD-L2 was the best overall gene transcript detected on a tissue microarray for distinguishing PMBL from DLBCL with 5.6-fold more expression in PMBL compared with DLBCL.²⁰ This may explain the exceptionally high specificity for PMBL that we observe with PD-L2 IHC.

Gene CN gain is not the only mechanism for deregulation of PD-1 ligands but is the most common one in B-cell malignancies.^{18,33} Translocations involving *PDCD1LG2* or *CD274* can occur and result in marked upregulation of the respective target genes.^{33,34} On the basis of integrative genetic and expression data, other mechanisms of deregulation also likely exist.³³ In this study, we focused on the detection of *PDCD1LG2* amplification, which showed excellent correlation with PD-L2 IHC. However, we also found a case of PMBL that was positive for PD-L2 by IHC but lacked *PDCD1LG2* amplification. The origin of PD-L2 deregulation in this case is unknown but, on the basis of prior published analyses of PMBL, a translocation involving *PDCD1LG2* is possible.

The interpretation of PD-L2 IHC is facilitated by the specificity of the stain for malignant cells. We have found that TAMs express little PD-L2 in PMBL and DLBCL. In contrast, PD-L1 is highly expressed by TAMs in a wide variety of aggressive lymphomas, including PMBL and a subset of DLBCL.²¹ TAMs expressing high PD-L1 protein are also characteristic of other tumor types and thought to have clinical significance.^{35,36} The differential expression of PD-L2 and PD-L1 on TAMs in PMBL may be attributable to the differential sensitivity of the *PDCD1LG2* and *CD274* promoters to cytokine-mediated JAK-STAT signaling. The human *PDCD1LG2* promoter contains a partially conserved interferon-stimulated regulatory element/interferon-regulatory factor 1 (ISRE/IRF1) motif that is weakly responsive to JAK-STAT signaling.¹⁸ In contrast, human *CD274* promoter contains a highly conserved ISRE/IRF1 motif that is highly responsive to JAK-STAT signaling.^{18,37} Thus the multitude of cytokines within the tumor microenvironment of PMBL and DLBCL may be less effective in inducing PD-L2 than PD-L1.

Our results have clinical implications for patients with PMBL. Recent trials of anti-PD-1 and anti-PD-L1 antibodies have shown dramatic and long-lasting clinical responses in a subset of patients with solid tumors including melanoma, non-small cell lung cancer, and renal cell carcinoma.^{16,17} These findings emphasize the importance of the PD-1/PD-L1 axis in regulating antitumor

immunity and the benefits of targeting this signaling pathway in clinical practice. In addition, clinical responsiveness to PD-1 blockade correlated with tumor-specific expression of PD-L1 as detected by IHC in a small series of cases for which biopsy tissues were available for analysis.¹⁶ Our data indicate that up to 70% of patients with PMBL are rational candidates for immunotherapy that targets PD-1 signaling. Given that PMBLs typically express both PD-L2 and PD-L1,^{18,21} PD-1 receptor blockade should be considered a better option than selective PD-L1 blockade.

In summary, we have validated a protocol for the detection of PD-L2 in FFPE tissue sections by standard IHC. We find that PD-L2 IHC is sensitive and specific, with robust positive staining of cell lines that harbor even low-level *PDCD1LG2* CN gain. The majority of PMBLs (72%) exhibit distinct, membranous PD-L2 expression, whereas the vast majority of DLBCLs (97%) do not. In most, but not all, cases of PMBL, positive staining for PD-L2 is associated with *PDCD1LG2* CN gain. Finally, we find that PD-L2 expression is restricted to neoplastic cells within the tumor microenvironment. PD-1 blockade, which is currently being evaluated in clinical trials, may be a possible new treatment option for patients with PMBL.

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