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## PD-L1 Expression Is Characteristic of a Subset of Aggressive B-cell Lymphomas and Virus-Associated Malignancies

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### Abstract

**Purpose:** Programmed cell death ligand 1 (PD-L1) is an immunomodulatory molecule expressed by antigen-presenting cells and select tumors that engages receptors on T cells to inhibit T-cell immunity. Immunotherapies targeting the PD-1/PD-L1 pathway have shown durable antitumor effects in a subset of patients with solid tumors. PD-L1 can be expressed by Reed–Sternberg cells comprising classical Hodgkin lymphoma (CHL) and by malignant B cells comprising EBV-positive posttransplant lymphoproliferative disorders (PTLD). We sought to determine whether the expression of PD-L1 represents a general strategy of immune evasion among aggressive B-cell lymphomas and virus- and immunodeficiency-associated tumors.

**Experimental Design:** Using novel antibodies and formalin-fixed, paraffin-embedded (FFPE) tissue biopsies, we examined 237 primary tumors for expression of PD-L1.

**Results:** Robust PD-L1 protein expression was found in the majority of nodular sclerosis and mixed cellularity CHL, primary mediastinal large B-cell lymphoma, T-cell/histiocyte-rich B-cell lymphoma, EBV-positive and -negative PTLD, and EBV-associated diffuse large B-cell lymphoma (DLBCL), plasmablastic lymphoma, extranodal NK/T-cell lymphoma, nasopharyngeal carcinoma, and HHV8-associated primary effusion lymphoma. Within these tumors, PD-L1 was highly expressed by malignant cells and tumor-infiltrating macrophages. In contrast, neither the malignant nor the nonmalignant cells comprising nodular lymphocyte-predominant Hodgkin lymphoma, DLBCL-not otherwise specified, Burkitt lymphoma, and HHV8-associated Kaposi sarcoma expressed detectable PD-L1.

**Conclusion:** Certain aggressive B-cell lymphomas and virus- and immunodeficiency-associated malignancies associated with an ineffective T-cell immune response express PD-L1 on tumor cells and infiltrating macrophages. These results identify a group of neoplasms that should be considered for PD-1/PD-L1-directed therapies, and validate methods to detect PD-L1 in FFPE tissue biopsies. *Clin Cancer Res*; 19(13); 3462–73. ©2013 AACR.

### Introduction

Programmed cell death ligand 1 (PD-L1, also known as B7-H1) is an immunomodulatory cell-surface glycoprotein and a member of the B7 family of costimulatory molecules that is primarily expressed by antigen-presenting cells and serves to regulate the cellular immune response (1). Binding

of PD-L1 to its cognate receptor PD-1 inhibits proliferation of activated T cells in peripheral tissues leading to "T-cell exhaustion," a functional phenotype that can be reversed by PD-1 blockade (2). In clinical trials of anti-PD-1 and anti-PD-L1 antibodies, patients with solid tumors have achieved long-lasting clinical responses, thus emphasizing the importance of the PD-1/PD-L1 axis in regulating anti-tumor immunity (3, 4). Moreover, in the limited number of cases examined, clinical responsiveness to PD-1 blockade correlated with tumor-cell-specific expression of PD-L1 as detected by immunohistochemistry (IHC) using a proprietary antibody (4).

We have shown that Hodgkin lymphoma cell lines express high levels of PD-L1 transcript and protein due to multiple mechanisms including genetic amplification of *CD274* (encoding PD-L1) and constitutive AP1 signaling. Hodgkin cell lines show variable copy number gain of chromosome 9p24.1, a genomic region that includes *CD274*, *PDCD1LG2* (encoding PD-L2), and *JAK2* (5). The copy number of 9p24.1 correlates with cell surface PD-L1 protein expression due to direct amplification of *CD274*,

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org>).

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### Translational Relevance

Therapies targeting the immunomodulatory PD-1/PD-L1 signaling pathway promote marked antitumor immunity in a subset of patients with solid tumors. However, routine methods to detect PD-L1 expression in FFPE tissue biopsy sections are lacking. Here, we validate novel immunohistochemical assays amenable to FFPE tissue sections and analyze a broad group of aggressive B-cell and virus-associated malignancies for PD-L1 protein expression. We find that upregulation of PD-L1 is a general feature of both the malignant cells and the nonmalignant tumor-infiltrating macrophages of classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, T-cell/histiocyte-rich large B-cell lymphoma, and a broad group of virus- and immunodeficiency-associated malignancies, including EBV-positive diffuse large B-cell lymphoma, posttransplant lymphoproliferative disorder, plasmablastic lymphoma, primary effusion lymphoma, and extranodal NK/T-cell lymphoma. In addition, we detected PD-L1 expression on the malignant cells of nearly all EBV-associated nasopharyngeal carcinomas. These results indicate that PD-L1-positive tumor cells and tumor-infiltrating macrophages likely contribute to immune evasion in multiple lymphoid and virus-associated malignancies and suggest that these tumors may be amenable to PD-1 blockade.

and to increased JAK2 protein expression, enhanced JAK-STAT signaling, and activation of an interferon (IFN)-stimulated regulatory element/IFN-regulatory factor 1 (ISRE/IRF1) motif in the *CD274* promoter (6, 7). In a series of genetically annotated primary classical Hodgkin lymphoma (CHL) cases, we found that high copy numbers of 9p24.1 correlated with increased PD-L1 expression in Reed–Sternberg (RS) cells (5). We have further shown that: (i) PD-L1 expression is regulated by an AP1-dependent enhancer in *CD274*; (ii) AP1 signaling components JunB/cJun constitutively bind this enhancer in Hodgkin lines; and (iii) JunB and cJun are overexpressed in primary RS cells (5).

Moreover, a subset of CHL is Epstein–Barr virus (EBV) positive, and aberrant signaling through EBV-encoded gene products provides alternative mechanisms to upregulate PD-L1. In a genetically annotated series of primary CHL we found that 9p24.1 amplification and EBV-infection were mutually exclusive. Using EBV-transformed B cells, we showed that the expression of the EBV-encoded latent membrane protein (LMP)-1 promotes both AP1-signaling and JAK–STAT signaling to activate the enhancer and promoter elements of *CD274*, respectively (6). Consistent with these findings, we have detected PD-L1 in the majority of examined EBV-positive posttransplant lymphoproliferative disorders (PTLD; ref. 6). Our studies indicate that CHL and EBV-positive PTLD use several complementary mechanisms to upregulate PD-L1 in tumor cells.

CHL is also notable for its prominent inflammatory infiltrate, which includes a skewed T-cell population and abundant macrophages. In CHL, increased numbers of CD68<sup>+</sup> tumor-associated macrophages have been associated with inferior clinical outcome (8, 9). However, the biological basis for this association remains undefined.

CHL is genetically, phenotypically, and/or histologically related to additional large-cell lymphomas including primary mediastinal large B-cell lymphoma (PMLBCL), nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL), and T-cell rich, histiocyte-rich large B-cell lymphoma (TCRLBCL; refs. 10, 11). Similarly, EBV and the closely related virus human herpes virus-8 (HHV8, also known as Kaposi sarcoma (KS)-associated herpesvirus) are postulated or known drivers of additional malignancies that include HIV/immunodeficiency-related diffuse large B-cell lymphoma (DLBCL), EBV-associated DLBCL of the elderly, extranodal NK/T cell lymphoma (ENKTCL), nasopharyngeal carcinoma (NPC), plasmablastic lymphoma (PBL), endemic Burkitt lymphoma (BL), and the HHV8-associated primary effusion lymphoma (PEL) and KS (12, 13). A comprehensive analysis of PD-L1 expression in these tumor types has not been completed.

In this study, we validate 2 novel anti-PD-L1 antibodies for the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) tissue sections. We then evaluate PD-L1 expression in a range of aggressive lymphoid malignancies, including those related to CHL and those associated with EBV and/or HHV8 infection, as well as NPC and KS. We find that robust expression of PD-L1 is characteristic of the malignant cells and a large proportion of nonmalignant cells, primarily tumor-infiltrating macrophages, within the tumor microenvironment of CHL, PMLBCL, TCRLBCL, and a broad range of virus- and immunodeficiency-associated malignancies that are characterized by an ineffective immune response. Our results further provide a biological basis for the prognostic significance of tumor-infiltrating macrophages. Finally, our methods establish a template for screening FFPE tumor biopsies for PD-L1 expression by IHC using a generally available monoclonal antibody.

### Materials and Methods

#### Case selection

Cases were retrieved from the surgical pathology files of Brigham and Women's Hospital, Boston, Massachusetts; Yale School of Medicine, New Haven, Connecticut; UMass Memorial Medical Center, Worcester, Massachusetts; and from the consult files of 1 of the authors (C.D.M. Fletcher) with the approval of the institutional review boards. All tumors were diagnosed and classified according to standard World Health Organization (WHO) criteria. Representative hematoxylin and eosin (H&E)-stained slides were reviewed to confirm the presence of adequate amounts of tumor. Cases included whole tissue sections and tissue microarrays. Cases included nodular sclerosis CHL (NSCHL, 25 cases); mixed-cellularity CHL (MCCHL, 8 cases); CHL, not otherwise specified (CHL-NOS, 5 cases); nodular lymphocyte-

predominant Hodgkin lymphoma (NLPHL, 15 cases); primary mediastinal large B-cell lymphoma (PMBCL, 21 cases); T-cell/histiocyte-rich large B-cell lymphoma (TCHRBCL, 11 cases); EBV-positive DLBCL of the elderly (9 cases); EBV-positive immunodeficiency-related DLBCL (HIV-associated and iatrogenic; excluding patients with a history of solid organ transplantation, 7 cases); EBV-positive PTLD (10 cases); EBV-negative PTLD (7 cases); DLBCL, not otherwise specified (DLBCL-NOS, 66 cases); PBL (9 cases); PEL (4 cases); ENKTCL (6 cases); EBV-positive BL (7 cases); NPC (18 cases); and KS (9 cases). All cases of EBV-positive DLBCL, EBV-positive PTLD, EBV-positive BL, NPC, 4 of 6 cases of ENKTCL, and 7 of 9 cases of PBL were shown previously to be positive for EBV-encoded RNA (EBER) by *in situ* hybridization study. All EBV-negative PTLD and DLBCL-NOS cases were negative for EBER. All PEL and KS cases were shown previously to be positive for HHV8 by IHC.

### Cell culture and Western blot

Hodgkin lymphoma cell lines (L428, SUP-HD1, and HDLM2) and DLBCL cell lines (SUDHL4 and OCI-Ly1) were cultured as previously described (14). Preparation of cell lysates, SDS-PAGE, and Western blot was carried out as previously described (14) with the following antibody conditions: PD-L1 was detected with a rabbit monoclonal antibody (#10084-R015, 1:1,500, 1 µg/mL final concentration; Sino Biological) in 3% nonfat dry milk (sc-2325, Santa Cruz Biotechnologies). Blots were stripped and reprobed with anti-GAPDH antibody (FL-335, sc-25778, 1:2,000) in 3% nonfat dry milk.

### Immunohistochemistry

IHC using a rabbit anti-PD-L1 monoclonal antibody (clone 15, #10084-R015, 6.2 µg/mL final concentration; Sino Biological) was carried out using 4 µm-thick, FFPE tissue sections on a Benchmark XT autostainer (Ventana Medical System) with standard antigen retrieval methods (CC1 buffer, pH8.0, #950-124, Ventana). The UltraView Universal DAB Detection kit (#760-500, Ventana) was used according to the manufacturer's instructions. Counterstaining was done as part of the automated staining protocol using hematoxylin (#760-2021, Ventana). IHC using the mouse anti-PD-L1 monoclonal antibody (IgG1, generated in the laboratory of G. Freeman, Dana-Farber Cancer Institute, Boston, clone 339.7G11, 69 µg/mL final concentration) was carried out using the same protocol as described earlier. After staining, slides were then washed in soap water and distilled water, dehydrated in graded alcohol and xylene, mounted, and coverslipped. The protocols for double IHC staining are available in Supplementary Materials and Methods.

### Case evaluation

All IHC-stained sections were initially evaluated and scored by a hematopathologist (B.J. Chen). The same slides and the original scores were then reviewed by a second hematopathologist (S.J. Rodig) for concordance of inter-

pretation. Discrepancies in interpretation or scoring (<10% of cases) were resolved by consensus conference between the 2 pathologists. Twenty samples, representing both PD-L1-positive and -negative-staining cases, were evaluated with the rabbit and the mouse monoclonal antibodies, including EBV<sup>+</sup> PTLD, EBV<sup>-</sup> PTLD, EBV<sup>+</sup> DLBCL, EBV<sup>+</sup> Burkitt lymphoma, nasopharyngeal carcinoma, and CHL. No significant differences in staining of the malignant or nonmalignant cells were observed among the cases tested with both antibodies. Only tumors stained with the rabbit antibody were formally scored (for the graphical representations Figs. 3, and Fig. 4D–F) because: (i) this antibody is commercially available, whereas the mouse antibody is a proprietary antibody; and (ii) limited material for some cases precluded staining of the complete set of cases with the mouse antibody.

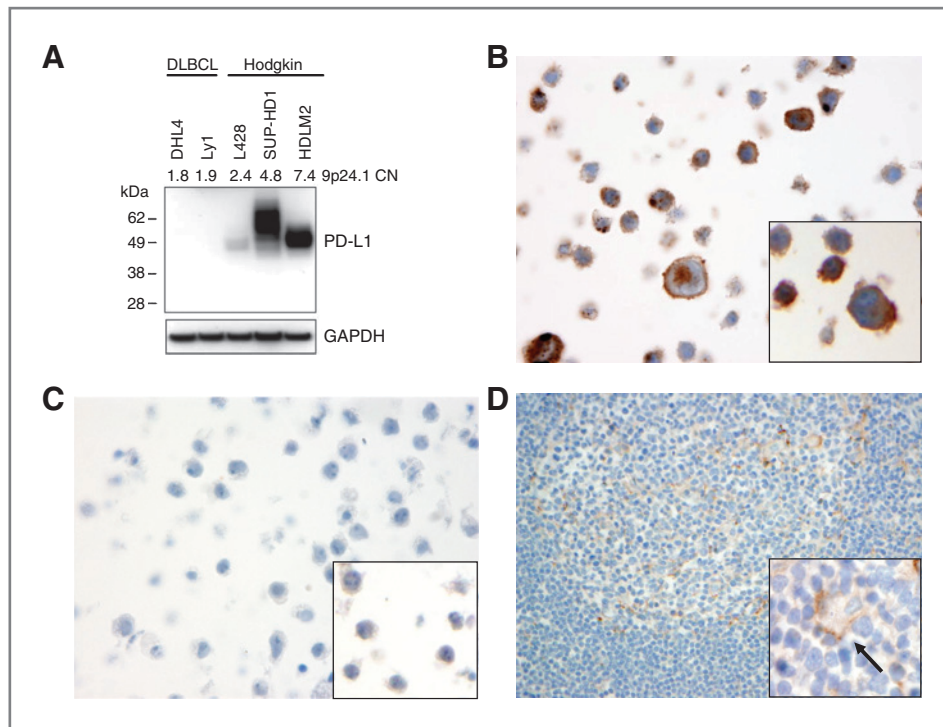
For cases stained with the rabbit anti-PD-L1 antibody, the percentage of tumor cells present in tissue sections was estimated for each case. The percentage of the total cellularity staining for rabbit anti-PD-L1, including malignant and nonmalignant cells, and the percentage of the tumor cell population staining for PD-L1 were scored independently. Staining intensity was scored as follows: 0 (no staining), 1+ (weak or equivocal staining), 2+ (moderate staining), or 3+ (strong staining). Cases with membrane staining, with or without cytoplasmic staining, and cases with only cytoplasmic staining were tabulated separately. Cytoplasmic cellular staining had to unequivocally exceed background to be considered positive. In the final tabulation (Table 1), tumor staining for PD-L1 was considered positive if 5% or more of the tumor cell population showed 2+ or 3+ membrane staining. A case was considered to have a microenvironment positive for PD-L1 if 20% or more of the total tissue cellularity showed 2+ or 3+ membrane or cytoplasmic staining in malignant and/or nonmalignant cells. The threshold for positive PD-L1 staining in malignant cells used here is comparable with that used in previous publications, using a distinct antibody (4, 15, 16). Appropriate external positive (placenta) and negative (tonsil) controls were also included with each staining run.

## Results

### Validation of PD-L1 antibodies for IHC

After testing a variety of commercially available antibodies, we identified a rabbit monoclonal antibody that showed both sensitive and specific staining for PD-L1 on a set of well-characterized cell lines and tissues (Fig. 1, Materials and Methods). In addition, we validated a novel mouse monoclonal antibody (clone 339.7G11, Materials and Methods) recognizing PD-L1 that was generated by 1 of the co-authors on this study (GF, data not shown). By Western blot analysis (Fig. 1A), the rabbit anti-PD-L1 monoclonal antibody recognized a protein of approximately 55 kDa – the size of glycosylated PD-L1 – in lysates derived from Hodgkin cell lines (L428, SUP-HD1, and HDLM2). The relative amount of PD-L1 in the Hodgkin cell lines as detected by Western blot analysis was consistent





**Figure 1.** PD-L1 antibody validation. A, Western blot analysis of cell lysates derived from genetically characterized diffuse large B-cell lymphoma (DLBCL) cell lines (DHL4, Ly1) and the Hodgkin lymphoma cell lines (L428, SUP-HD1, and HDLM2). Lysates were probed with a rabbit monoclonal antibody recognizing PD-L1 (top) showing a band at the expected size of the fully glycosylated form of PD-L1 (~55 kDa). The gene copy number (CN) for the *CD274* (*PD-L1*) locus, 9p24.1, is shown, as reported previously, for each cell line (5). Equal loading was shown by probing for GAPDH. IHC analysis of formalin-fixed, paraffin-embedded (FFPE) Hodgkin cell line HDLM2 (B) and DLBCL cell line DHL4 (C) stained with the rabbit anti-PD-L1 antibody showing membranous staining of the HDLM2 cells but no staining of DHL4 cells. Insets show similar staining patterns with the mouse monoclonal anti-PD-L1 antibody. D, FFPE human tonsil stained with rabbit anti-PD-L1 antibody showing little staining of lymphoid cells and weak membranous staining of occasional macrophages (inset, arrow).

with previously reported levels of PD-L1 expression detected by flow cytometry, and also correlated with chromosome 9p24 copy number status (5). Importantly, no band was detected in lysates derived from DLBCL cell lines (SUDHL4 and OCI-Ly1), consistent with the previously reported lack of detectable PD-L1 in these lines (Fig. 1A; ref. 5). Similarly, IHC analysis of FFPE cell pellets using the rabbit monoclonal antibody revealed robust staining of the Hodgkin cell line HDLM2 in a strong membranous pattern (Fig. 1B), but not in the DLBCL line SUDHL4 (Fig. 1C). The staining pattern was identical using the mouse monoclonal antibody (Fig. 1B and C insets). Additional IHC analysis of transfected cell lines showed specific staining by both rabbit and mouse PD-L1 antibodies of cell lines expressing human PD-L1 but not human PD-L2 (data not shown). We conclude that IHC analysis using both the rabbit and mouse monoclonal antibodies is specific for PD-L1 protein.

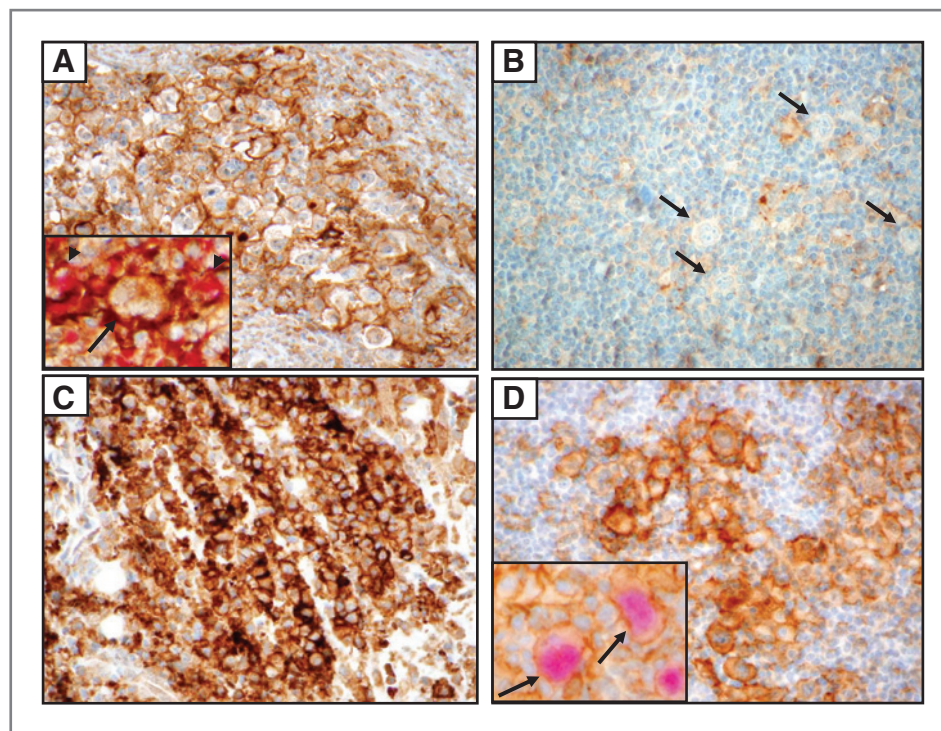
IHC analysis of human tonsil revealed little to no specific staining of the vast majority of lymphocytes within the interfollicular T-cell-rich regions and in the B-cell-rich secondary follicles using the rabbit monoclonal PD-L1 antibody (Fig. 1D). Rare lymphoid cells within reactive germinal centers did show weak membranous staining. High-power examination of human tonsil also revealed distinct membranous staining of the tonsillar epithelium

and weak, membranous staining of scattered cells morphologically consistent with macrophages (Fig. 1D inset; refs. 1, 16). IHC analysis of tonsil using the mouse monoclonal PD-L1 antibody resulted in identical findings (Supplementary Fig. S1). As an external positive control, syncytiotrophoblasts in human placental tissue showed strong membranous expression of PD-L1, as described previously (Supplementary Fig. S1; ref. 1).

We noted that, with the rabbit monoclonal antibody, a mild degree of general, nonspecific background staining was observed in tissue sections. Background staining varied according to the degree of dilution necessary to achieve the final antibody concentration optimized for IHC (Supplementary Fig. S1). Less background was associated with the more concentrated lots of antibody (6.2  $\mu\text{g}/\text{mL}$  final concentration used; lot concentrations ranged from 0.22 to 1.55  $\text{mg}/\text{mL}$ ). Very little background was noted with the mouse monoclonal antibody (Supplementary Fig. S1 and S2).

#### Expression of PD-L1 in HL and variants

Upon validation of 2 novel antibodies directed against PD-L1, we next surveyed a large cohort of Hodgkin lymphomas, including NSCHL, MCCHL, CHL-NOS, and NLPHL for PD-L1 expression by IHC. Tissue sections were comprised of a varying amount of RS cells. The median



**Figure 2.** Immunohistochemical analysis of PD-L1 in CHL, NLPHL, PMLBCL and TCRLBCL. A, representative example of nodular sclerosis classical Hodgkin lymphoma (NSCHL) stained with the rabbit anti-PD-L1 antibody showing distinct membranous staining of Reed-Sternberg (RS) cells and intratumoral macrophages. Inset shows staining with rabbit anti-PD-L1 (brown) highlighting the cell membrane of RS cells (arrow) as well as nonmalignant cells, and macrophages (arrowheads) double stained with rabbit anti-PD-L1 and the macrophage marker CD68 (red). B, representative case of nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) stained with rabbit anti-PD-L1 showing LP cells (arrows) that are negative for PD-L1. C, representative case of primary mediastinal large B-cell lymphoma (PMBCL) stained with rabbit anti-PD-L1 antibody showing predominantly membranous staining of lymphoma cells. D, representative case of T-cell/histiocyte-rich large B-cell lymphoma (TCHRBCL) stained with rabbit anti-PD-L1 antibody. Inset shows staining with mouse anti-PD-L1 (brown) highlighting macrophages adjacent to scattered malignant cells (arrow) that are double stained for PD-L1 and the B-cell lineage marker PAX5 (red).

estimated percentage of malignant cells was 5% (range 2–20%) for NSCHL, 2% (range 2–10%) for MCCHL, 50% (range 2–90%) for CHL-NOS, and 2% (range 2–5%) for NLPHL. Twenty-one of 25 (84%) cases of NSCHL, 7/8 (88%) cases of MCCHL, and 5/5 (100%) cases of CHL-NOS showed strong (2+ or 3+) membranous staining of the RS cells (Fig. 2A, Table 1, and Supplementary Table S1). Of the 33 cases of CHL showing positive staining in malignant cells, 32 cases (97%) had at least 50% of the RS cells positive for PD-L1 and 27 cases (82%) had at least 90% of the RS cells positive for PD-L1 (Fig. 3A and Supplementary Table S1). By contrast, only 2 cases of NLPHL (13%) were scored as positive for PD-L1 with only 5% of the LP cells positive in each case (Figs. 2B and 3C, and Supplementary Table S1).

Although the RS cells comprised a relatively small proportion of the overall tumor cellularity in CHL cases, nonmalignant cells – predominantly tissue macrophages – contributed to the total tumor cellularity and consistently showed strong membranous staining for PD-L1 (Supplementary Table S1). We found that 19/25 (76%) cases of NSCHL, 7/8 (88%) cases of MCCHL, and 5/5 (100%) cases of CHL-NOS showed strong (2+ or 3+) membranous and/or cytoplasmic staining for PD-L1 in at least 20% of the total

tumor cellularity, including nonmalignant cells (Fig. 3B and Supplementary Table S1). Double immunostaining for PD-L1 and CD68 on a set of cases confirmed this analysis and revealed strong membranous PD-L1 staining of CD68+ macrophages in close proximity to PD-L1-positive RS cells (Fig. 2A inset). Only 1 case of NLPHL (7%) showed PD-L1-positive staining in 20% of the total tumor cellularity. However, malignant cells in this case were estimated to comprise 5% of the cellularity and only rare LP cells (~2%) were positive for PD-L1 (Fig. 3C and D and Supplementary Table S1). Overall, the expression of PD-L1 on the malignant tumor cells was well correlated with the expression of PD-L1 on the tumor-infiltrating CD68+ macrophages in individual tumors (Supplementary Table S3).

We have previously shown that EBV-encoded LMP1 can promote PD-L1 expression in tumor cells (6). Therefore, we looked to see if the EBV status of the Hodgkin lymphoma cases correlated with PD-L1 expression. Eleven of 12 (92%) cases of Hodgkin lymphoma that were positive for EBV showed strong (2+ or 3+) membranous PD-L1 staining in malignant RS cells. One case of EBV-positive NSCHL showed weak (1+) membranous staining in 90% of RS cells (Supplementary Table S1). Twenty of 29 (69%) Hodgkin lymphoma cases that were negative for EBV had

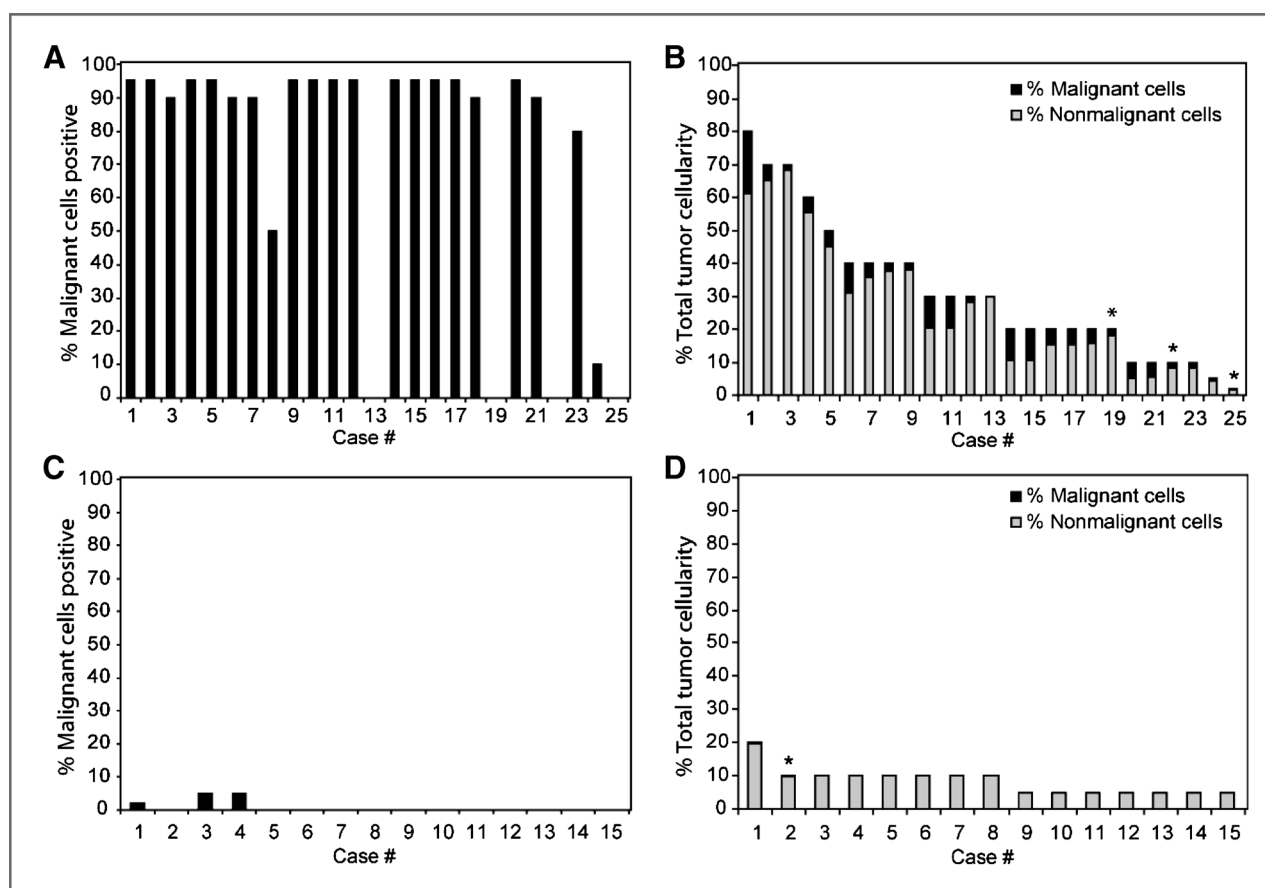


Figure 3. A and C, graphical representation of the percentage of the total malignant cells within the tumor sample staining positive for rabbit anti-PD-L1 and (B and D) graphical representation of the percentage of the total cells within the tumor sample staining positive with the rabbit anti-PD-L1 (with the contributions of malignant cells, black bars, and nonmalignant cells, gray bars, indicated) in each case of NSCHL (A, B) and NLPHL (C, D) examined. \*, cases exhibiting primarily cytoplasmic or membrane staining 1+.

malignant cells that scored positive for PD-L1 (Supplementary Table S1). Overall, EBV status did not predict a presence or absence of PD-L1 expression in Hodgkin lymphoma.

Two entities that lie on the histologic and genetic spectrum with CHL and NLPHL are PMBCL and TCHRBCL. Strong membranous PD-L1 expression was observed in 15/21 (71%) cases of PMBCL (90% median percentage of malignant cells; Fig. 2C) and 10/11 (91%) cases of TCHRBCL (10% median percentage of malignant cells; Fig. 2D). Double immunostaining for PD-L1 and PAX5 in cases of TCHRBCL showed strong membranous PD-L1 staining of the scattered, PAX5-positive malignant B cells (Fig. 2D inset). PAX5-negative cells, consistent with macrophages, adjacent to the malignant B cells also showed strong PD-L1 staining. The patterns of PD-L1 expression observed using the rabbit monoclonal antibody was identical using the mouse monoclonal antibody for IHC (Supplementary Fig. S2 and data not shown).

#### Expression of PD-L1 in EBV- and HHV8-positive malignancies

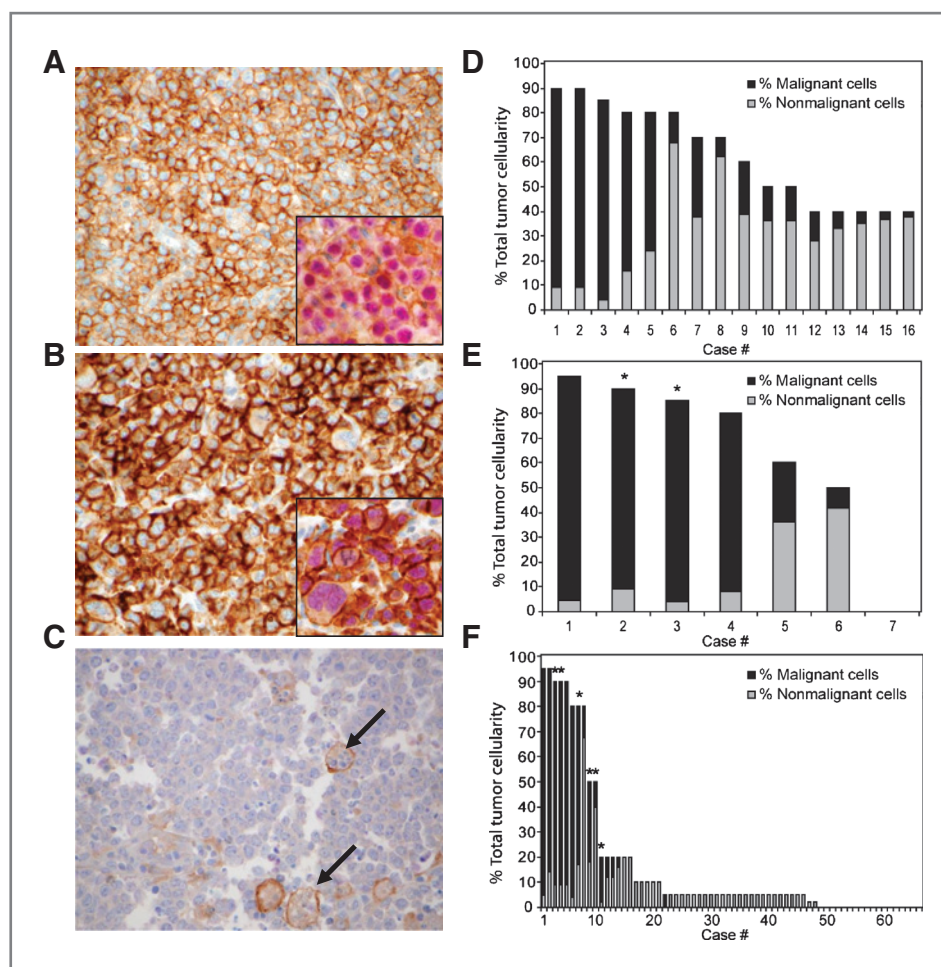
Previously, we reported a small series of EBV-positive PTLD cases that express PD-L1 (6). Using our newly char-

acterized PD-L1 antibodies, we extended our analysis to include additional aggressive EBV-positive and -negative B-cell lymphomas (Table 1). All EBV-positive DLBCLs, including 9/9 (100%) EBV-positive DLBCLs of the elderly and 7/7 (100%) EBV-positive immunodeficiency-related DLBCLs, showed strong PD-L1 membrane staining (Fig. 4A). The percentages of malignant cells staining positive for PD-L1 ranged from 5% to 90%, with 7 of 16 EBV-positive DLBCLs having at least 60% PD-L1-positive cells (Supplementary Table S2). Six of 10 (60%) EBV-positive PTLD cases showed strong PD-L1 membrane staining on at least 80% of malignant cells, consistent with our previous study (data not shown; ref. 6). Four of 7 (57%) EBV-negative PTLD cases also showed strong membranous PD-L1 tumor staining (Fig. 4B).

By contrast, only 7/66 (11%) EBV-negative DLBCL-NOS cases showed strong membranous PD-L1 tumor staining (range 10–90% of malignant cells; Fig. 4C). An additional 2 cases showed PD-L1 expression among the tumor-infiltrating macrophages (Table 1). The median tumor composition for all of the DLBCL categories ranged from 70% to 90%.

A review of the clinical, pathologic, and molecular characteristics of the cases (14%) that scored as PD-L1 positive





**Figure 4.** PD-L1 expression in EBV-positive DLBCLs, EBV-negative PTL, and DLBCL-NOS. A, representative case of an EBV-positive immunodeficiency-associated diffuse large B-cell lymphoma (DLBCL) stained with rabbit anti-PD-L1 showing distinct membranous staining of the lymphoma cells. Inset shows a case of EBV-positive DLBCL of the elderly double stained for mouse anti-PD-L1 (brown) and PAX5 (red). B, representative case of an EBV-negative posttransplant lymphoproliferative disorder (PTL) stained with rabbit anti-PD-L1 showing distinct membranous staining of the lymphoma cells. Inset shows double staining of the same case for mouse anti-PD-L1 and PAX5. C, case of DLBCL-NOS stained with rabbit anti-PD-L1 showing no staining of the lymphoma cells but scattered intratumoral macrophages filled with apoptotic debris showing weak membrane positivity for PD-L1. D–F, graphical representation of the percentage of total cells within the biopsy sample staining positive with the rabbit anti-PD-L1, and showing the contributions of malignant cells (black bars) and nonmalignant cells (gray bars) in each case of EBV+ DLBCL of the elderly/immunodeficiency-associated, EBV-negative PTL, and DLBCL-NOS, respectively. \*, cases exhibiting primarily cytoplasmic or membrane staining 1+.

revealed that, of the 8 cases with available data, 7 cases were activated B-cell (ABC) type and 1 case was germinal center B-cell (GCB) type by the cell of origin (COO) classification scheme. In comparison with all DLBCL-NOS cases for which both the PD-L1 and COO status was known (61 cases total, 27 cases ABC-type, 23 cases GCB-type; 11 cases Type 3), the association between PD-L1 expression and the ABC-subtype was statistically significant ( $P = 0.04$ , Fisher exact test).

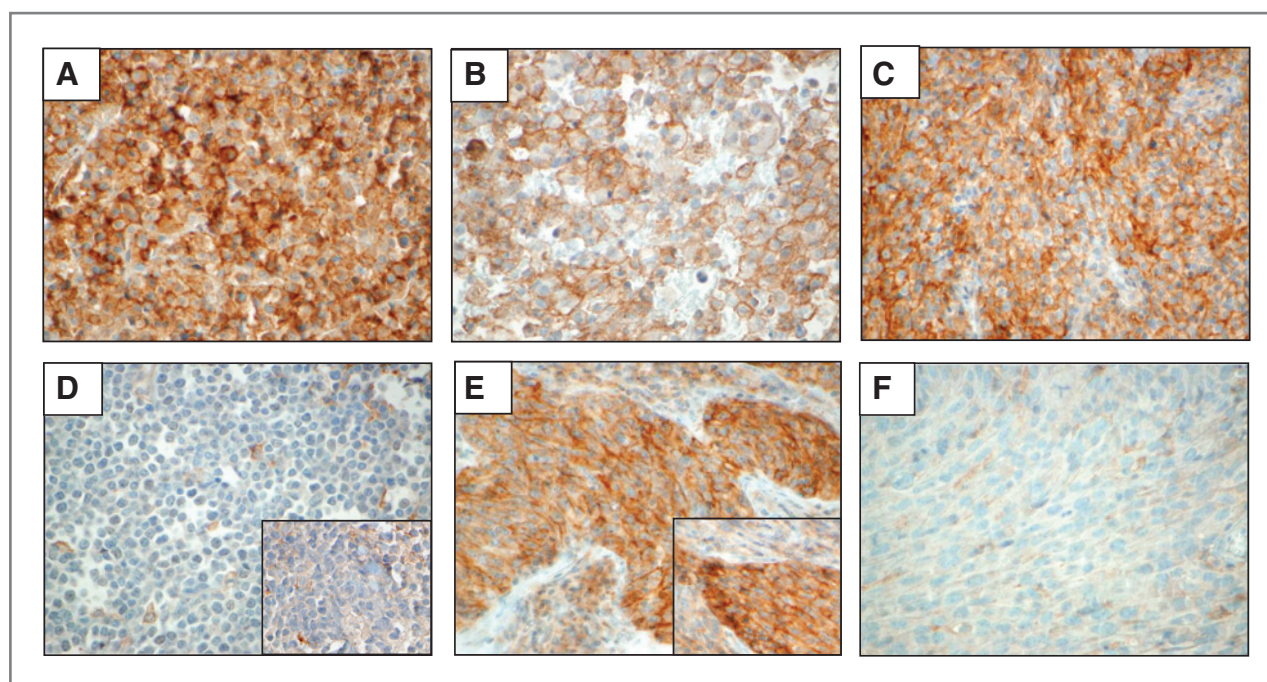
PD-L1 expression on the variable numbers of nonmalignant cells in EBV-positive DLBCL and other lymphomas consisted primarily of tumor-infiltrating macrophages (Fig. 4D–F and Supplementary Table S2). Careful morphologic review and double immunostaining for CD68 or PAX5 and PD-L1 (e.g., Fig. 4A and B insets) were used to support our histologic assessment. As for the Hodgkin lymphomas,

expression of PD-L1 by malignant cells and nonmalignant tumor-infiltrating macrophages was correlated (Supplementary Table S3).

Analysis of additional EBV- and HHV8-positive malignancies showed that 4/9 (44%) PBL, 2/4 (50%) PEL, 4/6 (67%) ENKTL, and 16/18 (89%) NPC cases showed positive PD-L1 staining in malignant cells (Fig. 5, Table 1, and Supplementary Table S2). The majority of cases had greater than 50% malignant cells positive for PD-L1, and all cases in this group had at least 10% malignant cells positive for PD-L1. By contrast, no cases of EBV-positive BL or HHV8-positive KS were positive for PD-L1 (Fig. 5 and Table 1).

For cases with available tissue, we confirmed the patterns of PD-L1 expression obtained using the rabbit monoclonal antibody with those using the mouse monoclonal





**Figure 5.** IHC analysis of PD-L1 expression in viral-associated lymphomas and additional cancers. Representative cases of (A) EBV-positive plasmablastic lymphoma (PBL), (B) HHV8-positive primary effusion lymphoma (PEL), (C) EBV-positive extranodal NK/T-cell lymphoma (ENKTCL), (D) EBV-positive Burkitt lymphoma (BL), (E) EBV-positive nasopharyngeal carcinoma (NPC), and (F) HHV8-positive KS stained with rabbit anti-PD-L1 or mouse anti-PD-L1 (insets).

antibody. We observed no differences in tumor staining between these 2 reagents (Fig. 5D and E insets and Supplementary Fig. S2).

For the majority of tumors showing positive staining, the PD-L1 expression was distinctly membranous. However, in a few cases (2 NSCHL, 1 MCCHL, 4 PMBCL, 1 EBV-positive PTLD, 2 EBV-negative PTLD, 6 DLBCL-NOS, 1 PEL, 1 ENKTCL, and 2 KS), we observed cytoplasmic staining that unequivocally exceeded background (in at least 5% tumor cells) but with little to no distinct staining of cell membranes (Fig. 4D–F, cases designated in graphs by \*, and Supplementary Table S2). Although this pattern was observed with both anti-PD-L1 antibodies, it is unclear whether such staining represents exclusively cytoplasmic PD-L1 expression, or both cytoplasmic and low-level membrane expression.

In total, we observed robust PD-L1 expression by the malignant cells and tumor-infiltrating macrophages in the majority of CHL, regardless of subtype and EBV-status, PMLBCL, TCRLBCL, EBV+ DLBCL, EBV+ and EBV- PTLD, HHV8+ PEL, and EBV+ NPC. We observed that nearly one-half of PBL and ENKTCL expressed PD-L1 as well. In contrast, only rare cases of NLPHL, DLBCL NOS, BL, and no cases of KS expressed significantly detectable PD-L1 protein.

## Discussion

EBV-associated DLBCL, PTLD, ENKTCL, PBL, NPC, and HHV8-associated PEL are rare, but often aggressive, malignancies that are largely resistant to current chemotherapeutic regimens (17–22). Given their association with

oncogenic viruses and with immunodeficiency, these tumors are attractive targets for immune-based therapies. Several novel immunomodulatory agents have shown great promise in clinical trials involving patients with advanced solid tumors, especially those using human antibodies directed against critical immune checkpoint molecules. Specifically, anti-PD-1 and anti-PD-L1-directed therapies have resulted in durable clinical responses in patients with non-small cell lung cancer, renal cell cancer, and melanoma (3, 4). Furthermore, expression of PD-L1 in a subset of tumors appeared to correlate with treatment response (4). However, the efficacy of such immune-targeted therapies in virus- and immunodeficiency-related malignancies has not been tested. Moreover, there remains a need for reliable methods to identify the critical immunoregulatory molecules expressed by individual tumors to improve patient selection.

In this study, we validated 2 novel anti-PD-L1 antibodies, 1 of which is commercially available, and showed robust PD-L1 expression in the majority of CHL/NSCHL, MCCHL, PMBCL, TCHRBL, EBV-positive DLBCL of the immunocompromised and elderly, PTLD, NPC, and ENKTCL. A subset of PBL and PEL cases revealed unequivocal PD-L1 expression, as well. These results establish PD-L1 expression as a common feature in these malignancies, which include both viral- and immunodeficiency-associated tumors, and provide a clinically applicable method for screening FFPE tumor samples on a routine basis.

In this analysis, we used a threshold of 5% positive staining of malignant cells to determine whether the tumors were scored as positive or negative for PD-L1. This threshold

**Table 1.** PD-L1 expression by select tumor types

Diagnosis	Cases	Cases with $\geq 5\%$ malignant cells positive <sup>a</sup>	Cases with $\geq 20\%$ total cellularity positive <sup>b</sup>
	<i>n</i>	<i>n</i> (%)	<i>n</i> (%)
Classical Hodgkin lymphoma (CHL)	38	33 (87)	31 (82)
Nodular sclerosis CHL	25	21 (84)	19 (76)
Mixed cellularity CHL	8	7 (88)	7 (88)
CHL-not otherwise specified (NOS)	5	5 (100)	5 (100)
Nodular lymphocyte predominant Hodgkin lymphoma	15	2 (13)	1 (7)
Primary mediastinal large B-cell lymphoma	21	15 (71)	19 (90)
T-cell/histiocyte-rich large B-cell lymphoma	11	10 (91)	11 (100)
EBV+ diffuse large B-cell lymphoma (DLBCL)	16	16 (100)	16 (100)
EBV+ DLBCL of elderly	9	9 (100)	9 (100)
EBV+ immunodeficiency associated DLBCL	7	7 (100)	7 (100)
EBV+ posttransplant lymphoproliferative disorder (PTLD)	10	6 (60)	7 (70)
EBV-negative PTLD	7	4 (57)	4 (57)
DLBCL-NOS	66	7 (11)	9 (14)
Plasmablastic lymphoma	9	4 (44)	4 (44)
Primary effusion lymphoma	4	2 (50)	3 (75)
Extranodal NK/T-cell lymphoma	6	4 (67)	5 (83)
EBV+ Burkitt lymphoma	7	0 (0)	0 (0)
EBV+ nasopharyngeal carcinoma	18	16 (89)	nd
HHV8+ Kaposi sarcoma	9	0 (0)	nd

Abbreviation: nd, not determined.

<sup>a</sup>,  $\geq 2+$  membranous staining.<sup>b</sup>,  $\geq 2+$  membranous and/or cytoplasmic staining.

was selected to conform to the cutoff-point used in previous publications (4, 15, 16). With this threshold, an association between cases scoring as positive for PD-L1 expression and clinical responsiveness to PD-1 immunotherapy in a small subset of cases has been reported (4). However, the antibody used to detect PD-L1 expression in that publication is distinct from those used here (and is not commercially available). The biological significance of a threshold of 5% positive staining remains poorly defined and deserves further investigation in future clinical trials. Moreover, given this uncertainty, we chose to present data showing the percentage of the malignant cells and the percentage of total cells within the tumor biopsy that are positive for PD-L1 expression for each case. We found that cases scored as PD-L1 positive generally expressed PD-L1 in far excess of 5% of the malignant tumor cells (Figs. 3A, C; Fig. 4D–F, and Supplementary Table S1).

PD-L1 expression is often driven by intrinsic genetic aberrations and dysregulated signaling pathways within malignant cells. We have shown that RS cells of up to 40% of NSCHLs harbor amplification of 9p24.1, a genomic region that includes *CD274*, *PDCD1LG2* (encoding PD-L2), and *JAK2* (5). In cell lines, increased 9p24 copy number is associated with increased PD-L1 expression due to both direct amplification of *CD274*, and through increased *JAK2* and enhanced *JAK-STAT* signaling (6, 7). Additional, intrinsic mechanisms driving PD-L1 expression in the RS cells of

CHL include EBV infection (32% of all CHL cases in this series) and constitutive AP1 activity. Similarly, approximately 70% of primary PMLBCLs also show gains in 9p24.1 (5) and, thus, the robust PD-L1 protein expression we observe in the majority of PMLBCLs is likely attributable, in part, to this genetic lesion. Taken together, the percentage of CHL and PMLBCL with membranous PD-L1 on the malignant cells (87% and 71%, respectively) is consistent with these earlier findings.

We have shown that EBV-transformed human B-cell lines (lymphoblastoid cell lines, LCLs) and EBV-positive PTLDS upregulate PD-L1. The results in the current study both confirm the PD-L1 protein expression by EBV-positive PTLDS using newly available, more robust antibodies and establish that the majority of additional EBV-associated malignancies express PD-L1, with the exception of BL.

In LCLs, PD-L1 induction is dependent upon constitutive signaling through the EBV-encoded protein LMP1 via its effects on both the PD-L1 enhancer and promoter (6). In both LCLs and EBV-positive PTLDS, EBV maintains a type III latency program characterized by expression of LMP1 in addition to LMP2A, the nuclear proteins EBNA-1, -2, -3 and -LP, and a series of noncoding RNAs (EBERs; refs. 7, 12, 17). Similarly, in EBV-positive CHL, EBV maintains a type II latency program that is characterized by expression of LMP1, LMP2A, EBNA1, and EBERs (23). In EBV-positive DLBCL, the virus maintains type II or type III latency

program (24), suggesting that LMP1-mediated signaling is likely to directly contribute to tumorigenesis and to the immune evasion signature for these tumors.

In contrast, the virus in endemic BL resembles that of quiescent, EBV-infected memory B cells in immunocompetent hosts and is characterized by a minimal gene expression program that includes EBNA1 and EBEBs, but not LMP1 (25). Consistent with a lack of signaling by EBV-encoded proteins in this tumor type, we found BL to be consistently negative for PD-L1. The EBV-encoded proteins, including LMP1, seem to be more heterogeneously expressed in ENKTCL and PBL than in EBV-positive PTL, CHL, and DLBCL (12, 20, 26). Therefore, the direct contribution of EBV-dependent and independent signaling pathways to the immune signature will require further analyses using cell culture models of these tumor types. Similarly, the contribution of viral-mediated signaling to expression of PD-L1 by NPC and PEL remains undefined.

Surprisingly, we observed robust PD-L1 protein expression in EBV-negative PTL. Very little is known about this tumor type, which comprises up to 30% of PTLs (27, 28). Because patients with these tumors can still respond to a reduction in immunosuppression, there is a likely role for immune surveillance in preventing or eradicating these tumors. In our cohort, EBV-negative PTL was similar to EBV-positive PTL in expressing PD-L1. Because we have shown that PD-L1 can be upregulated by mechanisms independently of viral-encoded gene products in EBV-negative CHL, we suggest that this tumor type is a good candidate for further genetic studies of the *CD274* locus.

Only rare cases of DLBCL-NOS were positive for PD-L1, either in the malignant tumor cells or the tumor-infiltrating macrophages. We have data as to the cell of origin for only a subset of the DLBCL-NOS cases (61 cases) and found that 7 of 8 cases positive for PD-L1 expression in either the malignant B cells or the tumor-infiltrating macrophages are classified as activated B-cell (ABC) type. This enrichment is statistically significant ( $P = 0.04$ , Fisher exact test), although the total number of positive-staining cases is very small. Other characteristics, including sites of disease involvement and age, were not associated with PD-L1 expression (data not shown). Given that patients with ABC-type DLBCL show inferior clinical outcome when treated with standard chemotherapy (R-CHOP), it will be of interest to determine whether PD-L1 expression, although rare in DLBCL-NOS, correlates with the cell of origin and clinical outcome using larger case series that are molecularly defined and with clinical follow-up.

Given the multiple, intrinsic mechanisms malignant cells exploit to upregulate PD-L1, an important additional result of this study is the recognition that nonmalignant cells, especially tissue macrophages, contribute to the PD-L1 expression in certain aggressive B-cell, immunodeficiency-related, and viral-related malignancies. In this regard, these tumors resemble a subset of T-cell lymphoproliferative disorders in which PD-L1 expression on dendritic cells can functionally inhibit T-cell proliferation (29). We find that the intensity of PD-L1 expression in macrophages varies

from absent to weak in normal tonsil and in tumors with little PD-L1 expression in malignant cells, such as NLP, DLBCL-NOS, and BL, to moderate and, often, very strong in PD-L1-positive tumors. For several tumor types, especially CHL and other tumors with a marked inflammatory infiltrate, a major component of the PD-L1 expression within the total tumor cellularity is derived from the tumor-infiltrating macrophages. Overall, we found a high correlation between PD-L1 expression by the malignant cells and by the nonmalignant cells within an individual tumor, regardless of tumor type, and suggests coordinate regulation of PD-L1 among malignant and tumor-infiltrating macrophages (Supplementary Table S3).

Recently, it has been recognized that the number of tumor-infiltrating macrophages in cases of CHL constitute a biomarker of inferior failure free survival and overall survival CHL (8, 9). Several studies have since confirmed this observation (30–33). However, the biological basis for this association has remained undefined. PD-L1, as a major ligand for PD-1, serves to suppress T-cell immunity – including immunity directed toward virally encoded antigens expressed in EBV-positive CHL (6). Thus, the observation that macrophages constitute a major source of PD-L1 in the tumor microenvironment of CHL suggests a critical role for the PD-1/PD-L1 signaling axis among cases of CHL with high numbers of tumor-infiltrating macrophages and an inferior clinical outcome through the suppression of antitumor immunity.

The mechanisms responsible for the recruitment of macrophages to individual tumors and the origin of PD-L1 upregulation on these cells require further definition. Cytokines, such as IFN- $\gamma$ , can upregulate PD-L1 on macrophages, potentially via the ISRE/IRF1 motif in the *CD274* promoter (5, 34). Given the cytokine-rich inflammatory microenvironment of CHL, TCRBCL, and additional viral-associated lymphomas, secreted immunomodulatory factors may be responsible for the upregulation of PD-L1 on tumor-infiltrating macrophages in these diseases. Notably, we also observed high PD-L1 expression on the tumor-infiltrating macrophages in tumors, such as EBV-positive DLBCL and EBV-positive and -negative PTL, in which the cytokine milieu is not well described. Further studies correlating the cytokine profiles of the tumor microenvironment and the relative expression of PD-L1 are needed.

Given our development and validation of novel reagents for the detection of PD-L1 protein in FFPE tissue sections, it will be of interest to more broadly survey expression of this protein in additional hematologic and non-hematologic malignancies. Others have noted that PD-L1 is expressed by a subset of T-cell lymphoproliferative disorders, especially those at cutaneous sites (29). In contrast, low-grade B-cell lymphoproliferative disorders are thought to rarely express PD-L1 (35). Given our finding that PD-L1 expression can be limited to discrete and rare tumor types, it will be important to establish a comprehensive survey of hematologic neoplasms on the basis of detailed pathologic classification. Moreover, it will be of interest to survey additional virus-associated malignancies. A very recent publication detailing



the expression of PD-L1 in the tumor-associated macrophages of human papillomavirus (HPV)-positive squamous cell carcinoma of the head and neck suggests targeting PD-1/PD-L1 signaling in HPV-associated tumors (36). Investigations of PD-L1 expression in HPV-positive cervical carcinoma, polyomavirus-positive Merkel cell carcinoma, and HCV-positive hepatocellular carcinoma are warranted.

The EBV-, HHV8-, and immunodeficiency-associated malignancies are uncommon but often aggressive, life-threatening neoplasms with limited treatment options. Our data indicate that PD-L1 expression is a common feature of these tumors. Among these tumors, EBV-positive DLBCL of the elderly is associated with a particularly dismal prognosis with a median survival of 2 years (22). As this patient population is characterized by comorbidities that complicate the use of standard chemotherapy, it is likely to be an ideal candidate for novel therapies that enhance antitumor immune responses. Although it will be important to interrogate larger tumor cohorts to confirm our findings, we suggest that clinical trials targeting PD-1 and PD-L1 may benefit patients with CHL, PMLBCL, TCHRBCL, EBV-positive DLBCL, PTL, ENKTCL, PBL, PEL, and NPC.

#### Disclosure of Potential Conflicts of Interest

G.J. Freeman has ownership interests (including patents) in the PD-1 pathway; is commercially associated with CoStim Pharmaceuticals, Bristol-Myers-Squibb, Roche, EMD-Serrono, Boehringer-Ingelheim, and Amplimmune; and is a consultant/advisory board member of CoStim Pharmaceu-

ticals. M.A. Shipp is a consultant/advisory board member of Merck and Bristol-Myers-Squibb. S.J. Rodig received a commercial research grant from Ventana Medical System. No potential conflicts of interest were disclosed by the other authors.

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