Programmed death ligand 1 (PD-L1) is expressed by non-Hodgkin lymphomas
and inhibits the activity of tumor-associated T cells

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None to declare
Translational statement

Impaired host immunity is thought to play a role in the pathogenesis and progression of lymphoma. Expression of the negative T cell regulator PD-L1 appears to facilitate immune tolerance of various carcinomas. Here, we describe the spectrum of expression of PD-L1 among non-Hodgkin lymphomas and evaluate its functional activity in suppressing T cell responses. *In vitro* experiments using established cell lines and primary lymphoma specimens demonstrate that both T cell and B cell lymphomas express biologically active PD-L1, and that suppression of tumor-associated T cells can be reversed by PD-L1 blockade. Among diffuse large B cell lymphomas, the most common non-Hodgkin lymphoma in adults, we found that PD-L1 is expressed only in the non-germinal center subtype, which carries a poorer prognosis and frequently recurs after conventional chemoimmunotherapy. Our results suggest that targeting PD-L1 may be an effective anti-lymphoma immunotherapy for certain histologic subtypes.
Abstract

Purpose: Programmed death ligand 1 (PD-L1) is expressed on antigen presenting cells and inhibits activation of T cells through its receptor, PD-1. PD-L1 is aberrantly expressed on some epithelial malignancies and Hodgkin lymphomas, and may prevent effective host anti-tumor immunity. The role of PD-L1 in non-Hodgkin lymphomas (NHL) is not well characterized.

Experimental Design: PD-L1 expression was analyzed in cell lines and lymphoma specimens using flow cytometry and immunohistochemistry. Functional activity of PD-L1 was studied by incubating irradiated lymphoma cells with allogeneic T cells with or without anti-PD-L1 blocking antibody; T cell proliferation and IFN-γ secretion served as measures of T cell activation. Similar experiments were performed using cultures of primary lymphoma specimens containing host T cells.

Results: PD-L1 was expressed uniformly by anaplastic large cell lymphoma (ALCL) cell lines, but rarely in B cell NHL, confined to a subset of diffuse large B cell lymphomas (DLBCL) with activated B cell features (3 of 28 cell lines and 24% of primary DLBCL). Anti-PD-L1 blocking antibody boosted proliferation and IFN-γ secretion by allogeneic T cells responding to ALCL and DLBCL cells. In autologous cultures of primary ALCL and DLBCL, PD-L1 blockade enhanced secretion of inflammatory cytokines IFN-γ, GM-CSF, IL-1, IL-6, IL-8, IL-13, TNF-α, and MIP-1α. In establishing cell lines from an aggressive PD-L1+ mature B cell lymphoma, we also noted that PD-L1 expression could be lost under certain in vitro culture conditions.

Conclusions: PD-L1 may thwart effective anti-tumor immune responses and represents an attractive target for lymphoma immunotherapy.
Introduction

PD-1, a member of the CD28 family, is an inhibitory receptor expressed on the surface of T cells which functions to physiologically limit T cell activation and proliferation. Its ligand, PD-L1 (B7-H1/CD274), is expressed on antigen presenting cells. Binding of PD-L1 to its receptor inhibits T cell activation and counterbalances T cell stimulatory signals, such as the binding of B7 to CD28.

Dysregulation of the PD-1/PD-L1 pathway has been implicated in a wide variety of diseases. Impairment of PD-1/PD-L1 signaling can lead to autoimmune disease in murine systems. In humans, several single nucleotide polymorphisms in PD-1 have been associated with an increased risk of rheumatological disease. Conversely, upregulation of PD-1 signaling is associated with the persistence of chronic infections, including HIV, Helicobacter pylori, and schistosomiasis.

PD-L1 is not expressed by normal epithelial tissues, but it is aberrantly expressed on a wide array of human cancers. In this context, PD-L1 may promote cancer progression by disabling the host anti-tumor response. Its expression on tumor cells has been associated with poorer prognosis in renal cell carcinoma, breast cancer, Wilms tumor, pancreatic cancer, ovarian cancer, urothelial cancer, gastric cancer, esophageal cancer, and hepatocellular carcinoma. In murine systems, melanoma cells engineered to express PD-L1 are resistant to cytotoxic T lymphocyte (CTL)-mediated lysis and exhibit more aggressive tumor growth than wild-type melanoma. Moreover, melanoma cells expressing PD-L1 can induce apoptosis in tumor-specific CTLs.

Compared to solid tumors, the spectrum of expression and biological activity of PD-L1 in lymphomas is incompletely characterized. Using immunohistochemistry, Brown et al reported PD-L1 expression on 7 of 11 peripheral T cell lymphomas and 0 of 16 B cell non-Hodgkin
lymphomas (NHL). PD-L1 was detected by RT-PCR in 5 ALK+ anaplastic large cell lymphoma (ALCL) cell lines and by immunohistochemistry in 18 primary ALK+ ALCL specimens. Another series reported that PD-L1 was expressed in 4 of 14 diffuse large B cell lymphomas (DLBCL), 0 of 9 T cell lymphomas, and 8 of 13 classic Hodgkin lymphoma (HL) cases.

In this report, we describe the pattern of expression of PD-L1 in a large series of primary human lymphoma specimens (n=110) and NHL cell lines (n=34). Using both cell lines and primary tumor specimens, we demonstrate that PD-L1 expressed on tumor cells is immunologically active in suppressing the activation of tumor-associated T cells. These results suggest PD-L1 blockade as a potentially useful strategy for lymphoma immunotherapy.
Materials and Methods

Cell lines and clinical sample preparation

Raji, Ramos, and Daudi human Burkitt lymphoma, and Jurkat T cell lymphoblastic leukemia cell lines were obtained from the American Type Culture Collection (Manassas, VA). SU-DHL-1, SU-DHL-4, SU-DHL-6, SU-DHL-8, SU-DHL-9, SU-DHL-16, BCBL-1, Karpas 299, DEL, Hut78, and SUP-M2 were gifts from Dr. Linda Baum (UCLA, Los Angeles, CA). Granta-519, JeKo-1, and REC-1 were gifts from Dr. William Matsui (Johns Hopkins University, Baltimore, MD). OCI-Ly-2, -3, -7, -10, -19, HBL-1, SUDHL-2, and U2932 were gifts from Dr. Louis Staudt (National Cancer Institute, Bethesda, MD). SU-DHL-5, SU-DHL-7, SU-DHL-10, NU-DHL-1, and USC-DHL-1 were gifts from Dr. Alan Epstein (University of Southern California, Los Angeles, CA). RC-K8 and MC116 cells were gifts from Dr. Izidore Lossos (University of Miami, Miami, FL). BJA-B was a gift from Dr. Elliott Kieff (Harvard, Boston, MA). Unless otherwise specified, tumor cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) plus 10% heat-inactivated fetal calf serum (FCS)(Omega Scientific, Tarzana, CA), 100 Units/ml penicillin/streptomycin, 2 mM L-glutamine, and 50 μM β-mercaptoethanol (“RPMI complete medium”; all supplements from Invitrogen), at 37°C in 5% CO₂. SU-DHL-6 and SU-DHL-8 cells were cultured in RPMI complete medium plus 20% FCS. Hut78 cells were cultured in Isocove’s Modified Dulbecco’s Medium (IMDM; Invitrogen) plus 20% FCS. The OCI-Ly series, SU-DHL-2, U2932, and HBL-1 were cultured in IMDM complete medium plus 20% fresh human plasma (heparinized) instead of FCS.

Primary lymphoma specimens were obtained from lymph node biopsies or involved peripheral blood after written informed consent approved by the UCLA Institutional Review Board, enriched by Ficoll-Hypaque sedimentation (GE Healthcare, Piscataway, NJ), and
cryopreserved in liquid nitrogen. For analysis, specimens were thawed quickly in a 37°C water bath and washed twice with warm RPMI complete medium before use.

**Cytokine stimulation of B cell lines**

Ramos, Daudi, SU-DHL-4, and SU-DHL-6 cells were cultured in RPMI complete medium containing IFN-γ 2000 RU/ml (R&D Systems, Minneapolis, MN), or CpG oligodeoxynucleotide (ODN) 10103 10 µg/ml (sequence 5’-TCGTCGTTTTCGGTCGTTTTT-3’) (Coley Pharmaceuticals Group, Wellesley, MA) plus IL-4 at 2 ng/ml (R&D Systems) for 24-48 hours. Daudi, Ramos, SU-DHL-4, SU-DHL-6, OCI-Ly-3, and HBL-1 cells were cultured in complete medium containing IL-6, IL-10, or both at 50 ng/ml (R&D Systems) for 24 to 72 hours. PD-L1 expression was analyzed by flow cytometry.

**Flow cytometry**

Monoclonal antibodies (mAbs) used to measure expression of cell surface markers by flow cytometry included: Phycoerythrin (PE)-conjugated anti-human PD-L1/B7-H1 (clone M1H1), PD-L2/B7-DC PE (clone M1H18), and PD-1 PE (clone M1H4) from eBioscience (San Diego, CA); and CD3 fluorescein isothiocyanate (FITC)(clone HIT3a), CD3 PE (clone UCHT1), CD4 PE (clone RPA-T4), CD8 PE (clone RPA-T8), CD20 FITC (clone L27), CD30 PE or CD30 FITC (clone BerH8), EMA/CD227/MUC1 FITC (clone HMPV), and appropriate isotype controls, all from BD Biosciences (Carlsbad, CA). Stained tumor cells were analyzed using a BD FACSCaliber flow cytometer (BD Biosciences) with FCS Express software (De Novo Software, Los Angeles, CA).
**Immunohistochemistry**

Frozen sections were cut at 2-4 microns and immediately fixed in cold acetone for 20 minutes at 4°C. After air drying for 10 minutes, slides were incubated overnight with mouse anti-PD-L1 Ab (clone MIH1, eBioscience). Slides were then incubated with DakoCytomation Envision+ System labeled polymer horseradish peroxidase (HRP)-anti-mouse for 30 minutes (DAKO, Carpinteria, CA), followed by the diaminobenzidine (DAB) reaction. The sections were counterstained with hematoxylin.

For formalin-fixed specimens, histologic sections from paraffin-embedded tissue blocks were subjected to heat-induced epitope retrieval using a steamer at 95°C for 25 minutes in 0.01 M citrate buffer, pH 6.0 (for PD-L1), or at 115°C for 3 minutes in 0.1 mM EDTA pH 8.0 for CD10, BCL6 and MUM1. Sections were incubated with mouse mAbs to CD10 (Vector laboratories, Burlingame, CA), BCL6 (DAKO), and MUM1 (DAKO), followed by antibody localization using the DakoCytomation Envision+ System-HRP-labeled polymer (DAKO). After a 10 minute incubation with DAB, sections were counterstained with hematoxylin. Staining for PD-L1 in paraffin sections was performed using a mAb (clone 5H1, provided by Dr. Lieping Chen, Johns Hopkins University) at BioPillar Laboratories (Monmouth Junction, New Jersey) using previously described methods,¹¹ or using a polyclonal rabbit antiserum (Lifespan Biosciences, Seattle, WA), followed by DAKO DakoCytomation Envision+ labeled polymer HRP-anti-rabbit detection.

**Allogeneic T cell proliferation assays**

T cells were enriched from whole blood obtained from healthy donors who gave informed consent using the RosetteSep® T cell enrichment cocktail (StemCell Technologies, Vancouver, BC), following the manufacturer’s protocol. 2 x 10⁵ enriched T cells were cultured...
in RPMI complete medium at a 10:1 ratio with irradiated (3000R) Karpas 299 cells in 96-well U-bottom plates (Nunc, Rochester, NY). Cells were fed every 2 days with fresh medium containing 10 IU/ml IL-2 (Chiron, Emeryville, CA). After 1 week, T cells were harvested, counted and re-plated in quadruplicate with fresh Karpas 299 cells (irradiated 3000R) at effector:target (E:T) ratios of 2:1 and 1:1 with 2 x 10^4 tumor cells per well in 96-well U-bottom plates with or without 10 μg/ml anti-PD-L1 (clone M1H1) or mouse IgG1 isotype control mAbs (eBioscience). After 4 days, anti-PD-L1 and control mAbs were replenished before cells were pulsed with 1 μCi/well ^3[H]-thymidine (MP Biomedicals, Solon, OH); cells were harvested 16 hours later. Incorporated radioactivity (counts per minute, cpm) was measured using a β-liquid scintillation analyzer (PerkinElmer, Waltham, MA), and results from quadruplicate cultures reported as arithmetic means ± standard deviation.

**Derivation of PD-L1-expressing lymphoma cell lines LC-96 and RS-27**

An 18-year old female (LC-96) presented with rapidly-progressive cervical and abdominal lymphadenopathy, ascites, and pleural effusions. Cervical lymph node biopsy confirmed ALK^+ ALCL. Malignant ascites fluid was collected at therapeutic paracentesis, then cells were isolated by centrifugation and cryopreserved. Cell-free ascites fluid was obtained by centrifugation and 0.45 μm filtration. A sample of unmanipulated ascites fluid was placed into immediate culture supplemented 1:1 with Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FCS, 100 Units/ml penicillin/streptomycin, 2 mM L-glutamine, and 50 μM β-mercaptoethanol (Invitrogen) at 37°C in 5% CO₂. As tumor cells slowly grew over a period of one month, supplementation of the culture with cell-free ascites fluid was gradually decreased (from 40% to 5%), until the resulting LC-96 cell line was able to grow in DMEM containing
15% FCS. The surface immunophenotypes of the primary ascites cells and the resulting LC-96 cell line were determined by flow cytometry as described above.

Peripheral blood mononuclear cells (PBMC) were obtained from a patient (RS-27) with peripheral blood involvement with an aggressive DLBCL. Flow cytometry demonstrated a monomorphic B cell population expressing CD19, CD20, CD22, and FMC7, with surface κ light chain restriction. The cells did not express BCL1, CD5, CD10, or CD38, and FISH was negative for t(11;14) and c-myc translocations (data not shown). After Ficoll-Hypaque isolation, PBMC were cryopreserved in liquid nitrogen. Thawed cells were initially cultured in DMEM complete medium containing 20% FCS plus 10% fresh human serum and 10% 0.45 μm-filtered LC-96 ascites fluid, as described in Results.

**Cytokine analyses**

For allogeneic experiments, supernatants from co-cultures of Karpas 299 cells and healthy donor T cells, as described above under allogeneic T cell proliferation assays, were collected after 4 days incubation and analyzed for IFN-γ by enzyme-linked immunosorbent assay (ELISA; R&D Systems). 96-well Maxisorp plates (Nunc) were coated with mouse anti-human IFN-γ antibody, then washed and blocked with 1% BSA in PBS for 1 hour. Supernatants were added and incubated for 2 hours, followed by biotinylated goat anti-human IFN-γ antibody (50 ng/ml). Detection was performed using streptavidin-conjugated HRP and hydrogen peroxide-tetramethylbenzidine substrate, and absorbance determined at 450 nm/570 nm with a SPECTRAmax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Recombinant human IFN-γ was used to generate a standard curve.

For autologous tumor cell-T cell co-cultures, cryopreserved LC-96 and RS-27 primary tumor specimens were thawed at 37˚C, washed twice with warm RPMI complete medium, and 2-
2.5 x 10^5 cells per well were plated in six replicates in a 96-well U-bottom plate in RPMI complete medium. Phytohemaglutinin (PHA; Sigma, St. Louis, MO) was added at 0, 0.5, or 1 μg/ml with or without anti-PD-L1 or mouse IgG1 isotype control mAbs at 10 μg/ml. Cells were incubated for 5 days at 37°C in a 5% CO₂ humidified incubator. Supernatants were collected and analyzed for IFN-γ by ELISA.

Cytokine multiplex analysis was performed on cell-free malignant LC-96 ascites fluid, spent media from the LC-96 cell line, and primary LC-96 cells treated as above with PHA and anti-PD-L1 or isotype control antibody. Supernatants were analyzed for levels of 16 cytokines (GM-CSF, IFN-γ, IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, IL-13, TARC, IFN-α, SDF-1β, MIP-1α, and MIG) using SearchLight® protein array multiplex sandwich-ELISA (Pierce Biotechnology, Woburn, MA).
Results

PD-L1 is widely expressed by ALCL but uncommonly among B cell non-Hodgkin lymphoma cell lines

We screened a large panel of human lymphoma cell lines for PD-L1 expression using flow cytometry (Table 1). Representative histograms are shown in Figure 1A. Three of the 28 B cell lymphoma cell lines tested expressed PD-L1. Two of the positive lines (OCI-Ly-10 and HBL-1) have been classified as DLBCL of the activated B cell (ABC) subtype based on gene expression profiling, while the third (RC-K8) is known to have constitutive upregulation of the NF-κB pathway, a hallmark of the ABC phenotype. In contrast, 5 of 6 T cell lymphoma cell lines exhibited expression of PD-L1, including all 4 ALCL lines tested. PD-L1 expression was strongest amongst the ALCL lines, with 3 of 4 lines showing 2-log increases over isotype controls, whereas Jurkat cells (T cell acute lymphoblastic leukemia) had low-level expression of PD-L1, and Hut78 (Sezary syndrome) was negative for PD-L1. PD-L1 mRNA expression correlated with protein expression, with the highest levels found in ALCL and a subset of ABC DLBCL lines (Supplemental Table 1). In addition, all cell lines were screened for PD-1 and PD-L2 expression. Only Jurkat cells expressed PD-1 (data not shown), and no cell line expressed PD-L2.

We then attempted to induce PD-L1 expression in B cell lymphoma lines that did not constitutively express it, as stimulation of some tumor cells with IFN-γ can induce PD-L1 expression. Ramos, Daudi, SU-DHL-4, and SU-DHL-6 were incubated with IFN-γ or CpG plus IL-4 for 24-48 hours, and PD-L1 expression monitored by flow cytometry. No cell line responded to IFN-γ, and only the Ramos cell line showed modestly increased PD-L1 expression 48 hours after stimulation with CpG plus IL-4 (Figure 1B). In ALCL, PD-L1 expression is induced by STAT3 signaling, and IL-6 and IL-10 are both potent inducers of STAT3.
Therefore, we asked whether IL-6 and IL-10 stimulation of NHL lines could upregulate PD-L1. Daudi, Ramos, SU-DHL-4, SU-DHL-6, OCI-Ly-3, and HBL-1 cells were cultured with IL-6, IL-10, or both at 50 ng/ml for 24 to 72 hours, but none showed significant increase in PD-L1 expression (data not shown). Thus, PD-L1 expression is not readily altered in cultured lymphoma cells by exogenous cytokines.

**PD-L1 is expressed by a subset of primary human diffuse large B cell lymphomas**

We next tested 68 lymphoma tissue specimens for expression of PD-L1 (Table 2A-B). Thirty-three DLBCL, 3 primary mediastinal B cell lymphomas (PMBCL) and 9 HL were analyzed by immunohistochemistry in frozen specimens. Single cell suspensions of 23 additional B cell NHL specimens, including 16 follicular lymphomas (FL), were analyzed by flow cytometry. Expression of PD-L1 among B cell NHL specimens was heterogeneous. Twenty-seven percent of DLBCL specimens demonstrated expression of PD-L1. In contrast, all 3 PMBCL specimens were PD-L1+. PD-L1 was not expressed in any cases of FL (n=16), small lymphocytic lymphoma (n=2), marginal zone lymphoma (n=3), or single cases of Burkitt or mantle cell lymphoma. Eight of 9 HL expressed PD-L1 in Reed-Sternberg cells, in concordance with previous observations.28

DLBCL were classified into germinal-center B (GCB) or non-GCB subtype based on the immunohistochemical markers CD10, BCL6, and MUM1, which correlate with cell of origin subtype as determined by gene expression profiling.29 Of 33 evaluable frozen cases, 19 were GCB and 14 were non-GCB. Only 1 of the GCB tumors expressed PD-L1. In contrast, 8 of 14 (57%) of non-GCB tumors expressed PD-L1 (P = 0.0004 by Fisher’s exact test). This pattern of expression parallels that seen in our cell lines, where PD-L1 expression was found in 3 of 6 DLBCL lines with ABC features, but in 0 of 7 GCB DLBCL cell lines (Table 1).
We next performed immunohistochemistry for PD-L1 expression in a separate set of 42 formalin-fixed, paraffin-embedded lymphoma specimens (Table 2C), which required a different mAb (5H1), previously used to stain PD-L1 in paraffin sections. Cases were considered positive when the majority of tumor cells stained for PD-L1. All 7 FL were negative, while 4 of 5 ALCL and 6 of 30 DLBCL were positive. Representative images are shown in Supplemental Figure 1. Of note, tumor-associated histiocytes stained positive for PD-L1 in 9 of 24 (38%) DLBCL in which tumor cells were negative. Similarly, PD-L1+ histiocytes were also found in FL surrounding tumor cell follicles. Of DLBCL specimens, 11 were GCB and 19 were non-GCB. None of the GCB DLBCL stained for PD-L1, whereas 6 (32%) of the non-GCB DLBCL were positive for PD-L1 (P = 0.061), consistent with results we obtained in frozen sections. Comparative results of the frozen and paraffin DLBCL series are shown in Table 2D.

Of note, we also stained 91 formalin-fixed, paraffin-embedded lymphoma specimens using a polyclonal rabbit anti-PD-L1 antiserum (Lifespan Biosciences). Using this methodology, 18 of 22 (82%) DLBCLs expressed PD-L1 (data not shown). Because of discordance between paraffin and frozen section results, we tested the polyclonal anti-PD-L1 antibody on cell pellets of several PD-L1-negative B cell lines and a Daudi lymphoma xenograft. As several of these negative controls stained positive, we concluded that this antibody was unreliable for detecting PD-L1 expression in lymphomas.

**PD-L1 expressed by ALCL inhibits the proliferation and cytokine secretion of allogeneic T cells**

We next asked whether PD-L1 expressed by lymphoma cells was biologically active in attenuating host immune responses. Because PD-L1 was strongly expressed in both ALCL cell lines and tumor specimens, we chose this as our initial in vitro model. We hypothesized that
antibody blockade of PD-L1 would result in greater T cell activity, demonstrating that the presence of PD-L1 on target tumor cells serves to inhibit T cell responses. In cultures of donor allogeneic T cells primed for 7 days with irradiated ALCL cells, both T cell proliferation and IFN-γ secretion were markedly increased in the presence of a blocking anti-PD-L1 antibody (Figure 2A). In contrast, anti-PD-L1 did not alter proliferation or IFN-γ secretion of T cells incubated in the absence of tumor targets. As a control, irradiated SU-DHL-4 cells, which do not express PD-L1, were used as targets. In this case, PD-L1 blockade did not alter the degree of T cell proliferation or IFN-γ secretion (Figure 2B). Even in 5-day cultures of unprimed normal donor T cells plus irradiated ALCL target cells, IFN-γ secretion was uniformly increased in the presence of anti-PD-L1 (Figure 2C). The differences seen in T cell proliferation were smaller and not consistently statistically significant. Nonetheless, these results demonstrate functional expression of immunosuppressive PD-L1 by ALCL cells.

**PD-L1 expression by primary ALCL attenuates the activity of tumor-associated T cells**

To further study tumor-T cell interactions, cryopreserved malignant ascites from a patient with newly diagnosed ALK+ ALCL (LC-96; see Methods), containing approximately equivalent proportions of PD-L1-expressing tumor cells and tumor-associated T cells, was used as an autologous system (Figure 3A). The primary tumor cells within the ascites (PD-L1+, EMA+, and CD30+) were associated with a mixture of CD4+ and CD8+ T cells.

Cells from the ascites were incubated for 5 days with anti-PD-L1, isotype control antibody, or media alone, plus different concentrations of PHA to serve as a polyclonal T cell activator, and supernatants assayed for IFN-γ secretion as an indicator of T cell stimulation (Figure 3B). Without the addition of PHA (media alone), even with addition of IL-2 (10 μg/ml),
no IFN-γ secretion was seen. Yet in the presence of PHA (0.5 μg/ml or 1.0 μg/ml), anti-PD-L1 provoked a marked increase in IFN-γ secretion ($P < 0.0001$ by one-way ANOVA compared to isotype control antibody or media alone). Thus, PD-L1 expressed by fresh, primary ALCL cells can suppress the function of tumor-associated autologous T cells.

Ascites cells were serially passaged in cell culture to derive a new ALCL cell line, designated LC-96 (see Methods). The immunophenotype of the LC-96 cell line mirrored that of the primary ascites tumor cells (Figure 4A, lower panel), with strong expression of PD-L1, EMA, and CD30, but without expression of PD-1, CD3, CD4, or CD8. FISH analysis revealed a t(2;5)(p23;q35) NPM-ALK translocation, which was also observed in the primary clinical specimen (data not shown). When LC-96 cells were incubated with PHA, with or without anti-PD-L1 antibody, there was no secretion of IFN-γ (data not shown), demonstrating that T cells, not tumor cells are the source of IFN-γ in the primary ascites cultures after PD-L1 blockade.

To further characterize the T cell response induced by PD-L1 blockade, we quantitated the secretion of 16 cytokines using multiplex ELISA. First, we measured the cytokines present in cell-free ascites fluid, which would reflect the tumor environment in situ (Supplemental Figure 2A). Interestingly, the fluid contained high levels of IL-6, as well as IL-10, and SDF-1β. We next surveyed cytokines in spent culture media from the established LC-96 cell line (Supplemental Figure 2B), to discern which might be products of tumor cells themselves. High levels of IL-8, IL-10, and SDF-1β were observed, indicating these as likely products of primary tumor cells in vivo.

Next, primary ascites cells were incubated with or without PHA and anti-PD-L1 antibody, and the cytokine profile determined (Supplemental Figure 2C). Without PHA, most cytokines were secreted at low levels. However, the addition of anti-PD-L1 resulted in increased secretion of IL-6, IL-8, TNF-α, and MIP-1α compared to control antibody or media alone. The
addition of PHA resulted in further enhancement of cytokine secretion, including GM-CSF, IFN-γ, IL-1, IL-6, IL-8, TNF-α, IL-13, and MIP-1α. Levels of IL-2, IL-4, IL-5, IL-10, IFN-α, TARC, SDF-1β, and MIG were not altered by PD-L1 blockade (data not shown).

**Functional PD-L1 expression by an aggressive primary B cell lymphoma**

To demonstrate that PD-L1 can also be immunologically active when expressed by B cell lymphomas, analogous experiments were performed using a tumor sample from a patient with an aggressive DLBCL (RS-27), which lacked expression of CD10, consistent with non-GCB phenotype. Circulating tumor cells strongly co-expressed PD-L1 and CD20, as measured by flow cytometry (Figure 4A). Unmanipulated RS-27 PBMCs containing approximately 75% tumor cells and 20% CD3+ T cells (Figure 4A), were cultured with PHA in the presence or absence of anti-PD-L1. After 5 days, supernatants were assayed for IFN-γ secretion (Figure 4B). As described above with the LC-96 ALCL tumor cell-T cell mixture, PD-L1 blockade resulted in increased IFN-γ secretion by tumor-associated T cells (P = 0.009 by one-way ANOVA), indicating functional inhibition of T cells by PD-L1 expressed by the B cell lymphoma.

**PD-L1 expression may be lost or attenuated during serial in vitro passage of lymphoma cells**

In deriving the new RS-27 B cell lymphoma line, we discovered that expression of PD-L1 can be lost during in vitro culture (Figure 4C). After thawing, primary tumor cells from patient RS-27 were initially cultured in medium containing 20% FCS plus 10% fresh human serum and 10% 0.45 μm-filtered LC-96 ascites fluid (as a source of lymphoma-derived growth factors). Tumor cells slowly expanded under these conditions, and when weaned slowly from
LC-96 ascites and fresh human serum, continued to express high levels of PD-L1 and CD20. However, if cells were weaned rapidly (over 2 weeks) into media containing 20% FCS and 5% pooled human AB serum, PD-L1 expression was almost entirely lost. Accordingly, T cell proliferation and IFN-γ production were only increased by PD-L1 blockade when allogeneic T cells were incubated with RS-27 PD-L1-positive cells (Supplemental Figure 3). Culture of PD-L1-negative RS-27 cells for 48 hours in media containing 10% fresh human serum, or CpG plus IL-4, could not restore PD-L1 expression (data not shown). Thus, PD-L1 expression by B cell lymphomas can easily be lost upon tumor cell establishment and serial passage in vitro.

We also observed attenuation of PD-L1 expression under different culture conditions in the established OCI-Ly-10 ABC DLBCL cell line (Figure 4D). When grown in media supplemented with 20% human plasma, the cells displayed bright expression of PD-L1 (MFI 465). However, when the same cells were transferred to media containing 20% FCS, the cells appeared less healthy, as evidenced by increased numbers of dead cells with lower forward scatter, and PD-L1 expression diminished in the viable cells (MFI 72). When the cells were transferred back to 20% human plasma, PD-L1 expression returned to its previous level (MFI 664, data not shown). Therefore, culture conditions can alter the expression of PD-L1 even among well-established lymphoma cell lines.
Discussion

Cancers use multiple mechanisms to thwart endogenous host anti-tumor immunity. While accumulating data indicate that expression of the negative T cell regulatory molecule PD-L1 by tumor cells or tumor-associated antigen presenting cells represents an important pathway whereby cancers evade host immunity, only limited data has been available regarding the expression of PD-L1 among common NHL subtypes and its ability to suppress autologous T cell functions.

We studied the spectrum of PD-L1 expression among human lymphomas, and demonstrated its capacity to impair the function of tumor-associated T cells in both T and B cell lymphomas. This is the largest reported series of PD-L1 expression in human lymphoma cell lines and primary tumors, and encompasses the most common B cell NHL subtypes. We observed near uniform expression of PD-L1 in ALCL cell lines and primary tumors. In contrast, we found that among B cell lymphomas, PD-L1 expression is essentially confined to a subset of the clinically-important ABC/non-GCB subtype of DLBCL. We further demonstrated that PD-L1 expressed by lymphoma cells is biologically active, with antagonist antibody blockade resulting in increased activation of adjacent T cells. This was true in allogeneic models using ALCL or DLBCL tumor cells as targets, as well as in primary tumor specimens of ALCL and DLBCL containing mixtures of lymphoma cells and autologous lymphocytes. Further studies will be required to confirm whether PD-L1 blockade results in similar effects in primary NHL specimens from lymph nodes or other extranodal sites of tumor.

Our observations regarding the pattern of expression of PD-L1 in human lymphomas is consistent with other reports in smaller series. Brown and colleagues reported PD-L1 expression in 7 of 11 peripheral T cell lymphomas, including ALCL, and in 0 of 16 B cell NHL. Marzec and colleagues reported PD-L1 staining in 100% of 18 ALCLs, although the polyclonal anti-
PD-L1 antibody used in this study possibly yielded false-positive cases (see below). Wilcox and colleagues reported PD-L1 expression in 15% of 131 T cell lymphomas, including 3 of 9 ALCL. Xerri and colleagues reported PD-L1 expression in 4 of 14 DLBCL (including 2 PMBCL), but not in follicular (n=8), mantle cell (n=4), marginal zone (n=4), or Burkitt lymphomas (n=3), similar to our own results. PD-L1 is frequently expressed in HL within Hodgkin and Reed-Sternberg cells, as reported in a series of 4 cases by Yamamoto and colleagues, consistent with our findings. PD-L1 expression by PMBCL is not surprising, since gene expression profiling has revealed increased PD-L1 and PD-L2 mRNAs in this lymphoma, and given the close biologic relationship of this disease to HL.

It appears that PD-L1 expression in B cell lymphomas is uncommon. Interestingly, we found that among DLBCL cell lines and primary tumor specimens, PD-L1 expression was almost entirely confined to the ABC/non-GCB subtype (Tables 1-2). The ABC DLBCL subtype identified by gene expression profiling is associated with inferior survival compared to the GCB subtype, even in cohorts of patients treated with rituximab. ABC DLBCL is characterized biologically by upregulation of NF-κB, but numerous other differences from GCB DLBCL exist. It is possible that PD-L1 expression is one of several “virulence factors” that lead to the inferior prognosis among ABC DLBCL, and we suggest this hypothesis be tested in a large series of molecularly-classified DLBCL with associated clinical outcome data. The aggressive B cell lymphoma from which we derived the PD-L1+ RS-27 cell line (Figure 4) was indeed virulent; the patient died of CNS disease despite high-dose chemotherapy and allogeneic stem cell transplantation. Finally, the low frequency of PD-L1 expression we observed in DLBCL cell lines (50% among ABC-type, Table 1) may underestimate the true prevalence in primary DLBCL. In establishing the RS-27 cell line, we observed that PD-L1 expression was easily lost during serial passage of the cells. Thus, loss of PD-L1 expression may have occurred during the
establishment of other human lymphoma cell lines, as appears to be the case in melanoma. While virtually all primary melanomas express PD-L1, most melanoma cell lines do not.9

PD-L1 expressed by leukocytes within the tumor microenvironment may play a role in host immune suppression even when not expressed by the tumor cells themselves. We observed that 38% of PD-L1 negative DLBCLs were infiltrated by PD-L1+ histiocytes. Increased numbers of lymphoma-associated macrophages has been associated with worse prognosis in FL38 and HL,39 although the same association is not seen in DLBCL.40,41 Thus, further studies examining the relationships between lymphoma-associated macrophages, PD-L1 expression, and clinical outcome are warranted. For such studies it will be important to utilize the monoclonal antibodies M1H1 or 5H1. In our experience, the polyclonal anti-PD-L1 antiserum used by Marzec et al23 appears to be less specific for PD-L1 than reported (see Results).

Several investigators have also shown that the PD-1/PD-L1 axis can also influence the function of lymphoma-infiltrating T cells in cases of PD-L1 negative human lymphomas. Yang and colleagues found that B cell lymphoma-associated Treg cells could express PD-L1 and suppress the function of PD-1+ tumor-associated T cells, an effect partially reversible by PD-L1 blockade.42 Similarly, Neelapu et al found that PD-1 was markedly upregulated on tumor-derived and peripheral blood T cells in FL in association with impaired Th1 cytokine secretion.43 Antibody blockade of PD-1 improved proliferation of tumor-derived T cells and promoted the activation of NK cells.44

As in other series, we found near uniform expression of PD-L1 in ALCL, a rare but clinically distinct T cell neoplasm. Nonetheless, PD-L1 is not a feature of all peripheral T cell lymphomas. Wilcox and colleagues30 found that PD-L1 was expressed by T cell lymphoma tumor cells in only a minority of cases, yet often expressed by tumor-associated stromal histiocytes, just as we observed in DLBCL. Our report adds to these findings in demonstrating
the ability of PD-L1 on ALCL and DLBCL cells to suppress the responses of both allogeneic and autologous tumor-associated T cells (Figures 2-4 and Supplemental Figures 2-3).

In our cultures of primary ALCL ascites cells containing autologous T cells, PD-L1 blockade enhanced the production of IFN-γ, as well as GM-CSF, IL-1, IL-6, IL-8, TNF-α, IL-13, and MIP-1α. We also noted that the ascites fluid representing the tumor microenvironment of this case contained high levels of IL-6, IL-10, and SDF-1β, while the established LC-96 cell line secreted these same cytokines, plus IL-8. These cytokines likely play roles in the pathogenesis and clinical manifestations of ALCL, having been detected in ALCL and other lymphomas.45-48 Intriguingly, SDF-1 (CXCL12) is associated with cancer metastasis49 and angiogenesis,41 and since most ALCL express the SDF-1 receptor CXCR4,50 this suggests a possible autocrine feedback loop in this lymphoma subtype.

In conclusion, the current work adds to a growing body of literature documenting the important role of PD-1/PD-L1 signaling in the pathogenesis of NHL. PD-L1 is highly expressed in ALCL, HL, and some poor-prognosis DLBCL of the ABC/non-GCB subtype, where it acts to negatively regulate adjacent T cells. Targeting the PD-1/PD-L1 pathway using antagonistic monoclonal antibodies may thus be an attractive approach to lymphoma immunotherapy.
References


Table 1. Expression of PD-L1 among 34 human lymphoma cell lines
Expression of PD-L1 and PD-L2 was measured by flow cytometry. “++” indicates ≥ 2 logs mean fluorescence intensity (MFI) above isotype control; “+” indicates < 2 logs MFI above control. Abbreviations: ALCL, anaplastic large cell lymphoma; T-ALL, precursor T acute lymphoblastic leukemia; DLBCL, diffuse large B cell lymphoma; GCB, germinal center B subtype; ABC, activated B cell subtype.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lymphoma subtype</th>
<th>PD-L1</th>
<th>PD-L2</th>
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<tr>
<td>B cell lines</td>
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</tr>
<tr>
<td>Daudi</td>
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</tr>
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<td>Ramos</td>
<td>Burkitt</td>
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<td>BJA-B</td>
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<td>NU-DHL-1</td>
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<td>ALCL</td>
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<td>Jurkat</td>
<td>T-ALL</td>
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<tr>
<td>Hut78</td>
<td>Sezary syndrome</td>
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Table 2. Expression of PD-L1 in primary lymphoma tissue specimens

Frozen sections (A) and flow cytometry (B) on cryopreserved specimens were performed with the anti-PD-L1 antibody clone M1H1. Paraffin sections (C) were stained with clone 5H1. Frozen and paraffin sections of diffuse large B cell lymphoma were classified as germinal center origin or non-germinal center origin (D). PD-L1 expression in DLBCL occurs almost exclusively in tumors of non-germinal center origin.

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<thead>
<tr>
<th>Lymphoma subtype</th>
<th># Cases</th>
<th># PD-L1+</th>
<th>% PD-L1+</th>
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<tr>
<td><strong>Frozen</strong></td>
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<td>Hodgkin lymphoma*</td>
<td>9</td>
<td>8</td>
<td>89%</td>
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<td>Diffuse large B cell lymphoma</td>
<td>33</td>
<td>9</td>
<td>27%</td>
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<tr>
<td>Primary mediastinal B cell</td>
<td>3</td>
<td>3</td>
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<th># PD-L1+</th>
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<td>16</td>
<td>0</td>
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<td>Small lymphocytic lymphoma / chronic lymphocytic leukemia</td>
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<tr>
<td><strong>Paraffin</strong></td>
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<td>Anaplastic large cell lymphoma</td>
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<td>30</td>
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<th>non-GCB</th>
<th>P-value</th>
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<tr>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td></td>
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<tr>
<td>Frozen (M1H1 antibody)</td>
<td>33</td>
<td>1 (5%)</td>
<td>18 (95%)</td>
<td>0.0004</td>
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<td></td>
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<td>8 (57%)</td>
<td>6 (42%)</td>
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<tr>
<td>Paraffin (5H1 antibody)</td>
<td>30</td>
<td>0 (0%)</td>
<td>11 (100%)</td>
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<td></td>
<td></td>
<td>6 (32%)</td>
<td>13 (68%)</td>
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*Staining in Hodgkin and Reed-Sternberg cells.*
Figure legends

Figure 1. High-level PD-L1 expression among ALCL but not most B cell NHL cell lines.  (A) Flow cytometric analysis of PD-L1 expression is shown for 8 representative NHL cell lines (among 34 described in Table 1). Consistent high-level PD-L1 expression is a feature of ALCL cell lines, but not B cell lines.  (B) Low-level expression of PD-L1 and PD-L2 is induced in Ramos B cell lymphoma cells after incubation with CpG plus IL-4 for 48 hours.

Figure 2. PD-L1 blockade enhances the activation of T cells co-cultured with allogeneic ALCL cells.  (A) Irradiated Karpas 299 ALCL cells (PD-L1+), were stimulated for 1 week with T cells from 2 healthy donors, then incubated with freshly irradiated Karpas 299 target cells at 2:1 and 1:1 E:T ratios in the presence of media alone, anti-PD-L1 antibody, or control antibody. After 4 days, supernatants were collected for IFN-γ measurement (lower panels), or antibody was replenished and cells pulsed with $[^3]$H-thymidine overnight to measure T cell proliferation (upper panels). Data are represented as mean ± SD of quadruplicate cultures. $P$ values shown are for anti-PD-L1 antibody versus isotype control antibody by one-way ANOVA.  (B) PD-L1 blockade does not affect T cells incubated with PD-L1-negative tumor cells. Incubation of healthy donor T cells with a B cell lymphoma line that does not express PD-L1 (SU-DHL-4) show that anti-PD-L1 antibody does not significantly alter allo-specific proliferation (upper panel) or IFN-γ secretion (lower panel).  (C) PD-L1 blockade augments activation of allogeneic T cells directly stimulated with ALCL cells. Irradiated Karpas 299 cells were incubated for 5 days with T cells from 3 healthy donors at 4:1 and 2:1 E:T ratios, in the presence of anti-PD-L1 antibody, control antibody, or media alone. Supernatants were collected for IFN-γ measurement by ELISA (bottom panels), or cells were pulsed with $[^3]$H-thymidine overnight to measure T cell proliferation (upper panels). Proliferation data are represented as mean ± SD of quadruplicate cultures. $P$ values shown are for anti-PD-L1 antibody versus isotype control antibody.

Figure 3. PD-L1 blockade enhances the activation of T cells in the presence of autologous ALCL cells that express PD-L1.  (A) Immunophenotyping of malignant ascites from a patient with newly-diagnosed ALK+ ALCL and cultured cell line (LC-96) from the same patient. The top two rows show results for the primary tumor (ascites), with far left panel displaying forward
and side scatter plot for analysis of tumor (large cell) and lymphocyte (small cell) gates. The red curve in each histogram represents the surface marker, and unstained cells are shown in grey. Tumor cells (top row) were mostly negative for CD3, CD4, CD8, and PD-1, but expressed PD-L1, EMA, and CD30. The lymphocyte gate (middle row) contains predominantly CD3+ T cells, with a mixture of CD4+ and CD8+ T cells having low-level expression of PD-1, PD-L1, and CD30. The immunophenotype of the derived LC-96 cell line is shown in the bottom row. Tumor cells are strongly positive for PD-L1, EMA, and CD30, without expression of CD3, CD4, CD8, or PD-1. (B) LC-96 primary ascites cells, containing approximately equivalent proportions of PD-L1-expressing tumor cells and tumor-associated T cells, were incubated for 5 days with PHA to activate T cells in the presence of media alone, anti-PD-L1 antibody, or isotype control antibody. In the presence of PHA 0.5 or 1.0 μg/ml, cells incubated with anti-PD-L1 antibody secreted more IFN-γ than controls (P < 0.0001 by one-way ANOVA). Results shown are representative of 3 independent experiments.

**Figure 4.** PD-L1 expressed in primary B cell lymphoma is biologically active but readily lost during in vitro culture. (A) PBMC from a patient with aggressive mature B cell lymphoma were analyzed by flow cytometry. Malignant cells co-express CD20 and PD-L1 and comprise 75% of the total. The specimen also contains 11% CD4+ T cells and 9% CD8+ T cells. (B) PD-L1 blockade enhances the activation of T cells in the presence of autologous B cell lymphoma expressing PD-L1. Patient PBMC were incubated in triplicate for 5 days with PHA 0.5 μg/ml in the presence of media alone, anti-PD-L1 antibody, or isotype control antibody, and IFN-γ measured in supernatants by ELISA. Data are represented as mean ± SD of triplicate cultures. Results are representative of 2 independent experiments. (C) PD-L1 expression by primary B cell lymphoma can be lost during serial in vitro passage. Primary tumor cells obtained directly from peripheral blood co-express PD-L1 and CD20. Cells were initially cultured in vitro with human serum, and weaned either rapidly or gradually to medium containing FCS, while monitoring PD-L1 and CD20 expression by flow cytometry. (D) PD-L1 expression may be attenuated based on cell culture conditions. OCI-Ly-10 cells, which express PD-L1, were cultured in 20% human plasma or 20% FCS. PD-L1 expression was substantially lower when the cells were grown in 20% FCS (MFI = 72) versus 20% human plasma (MFI = 465).
Figure 1

A

Karpas 299 (ALCL)  
SU-DHL-1 (ALCL)  
SUP-M2 (ALCL)  
Jurkat (T-ALL)  

Ramos (Burkitt)  
SU-DHL-6 (DLBCL)  
HBL-1 (DLBCL)  
OCI-Ly-10 (DLBCL)  

B

Ramos: No stimulation  
Ramos: CpG + IL-4 stimulation  

PD-L1  
PD-L2  
PD-L1  
PD-L2
Figure 2

A

T cell donor 1

T cell donor 2

B

PD-L1 negative targets

C

T cell donor 1

T cell donor 2

T cell donor 3

*P* values are indicated for each condition.
Figure 3

A

Primary tumor (ascites)

CD3

CD4

CD8

PD-1

PD-L1

EMA

CD30

Cell Line

CD30 FITC

PD-L1 PE

FSC-H

SC-H

B

IFN-γ (pg/ml)

None

PD-L1 antibody

Control antibody

PHA 0

PHA 0.5

PHA 1.0

P < 0.001

P < 0.001

P < 0.001

Cell conditions
**Figure 4**

### A

**Primary RS-27 tumor**

- PD-L1 PE vs. CD20 FITC
- PD-L1 PE vs. CD3 FITC
- PD-L1 PE vs. CD8 FITC

### B

**Cell conditions**

- IFN-γ (pg/ml)
- PHA 0 vs. PHA 0.5

- None
- PD-L1 antibody
- Control antibody

**P = 0.009**

### C

**Primary tumor**

- RS-27 cell line
  - Weaned rapidly from human serum
  - Weaned gradually from human serum

### D

**OCI-Ly-10, 20% human plasma**

- MFI = 465

**OCI-Ly-10, 20% fetal calf serum**

- MFI = 72
Programmed death ligand 1 (PD-L1) is expressed by non-Hodgkin lymphomas and inhibits the activity of tumor-associated T cells


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