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Immunology Letters 84 (2002) 57–62

Immunology Letters

www.elsevier.com/locate/

Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues

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Received 7 May 2002; received in revised form 3 June 2002; accepted 5 June 2002

Abstract

PD-1 is a member of the immunoglobulin superfamily expressed on immune cells, including T and B cells, and is involved in the delivery of inhibitory signal upon engagement of its ligands, PD-L1 and PD-L2. While the expression profile of PD-1 has been well documented, the analysis of PD-L1 and PD-L2 distributions on a protein basis has not been carried out because of the lack of available monoclonal antibodies specific for the molecules. In this study, we established two monoclonal antibodies, 1-111A and 122, specific for murine PD-L1 and PD-L2, respectively, and examined their expression profiles. Based on flow cytometric analyses, the expression of PD-L1 was detected in a variety of lymphohematopoietic cell types, including a minor proportion of T and B cells in the spleen, majority of pre-B cells and myeloid cells in bone marrow and subsets of thymocytes, while the expression of PD-L2 was not observed in the lymphohematopoietic cells at all. Notably, a significant proportion of the most immature lineage-marker-negative and c-Kit-positive bone marrow cells containing stem cells did express PD-L1. Following mitogenic stimulation, essentially all lymphocytes expressed PD-L1. Furthermore, a variety of leukemic lines also expressed PD-L1, while none of them did PD-L2. Thus, present results demonstrate the distinct expression patterns of PD-L1 and PD-L2 with the cells of lymphohematopoietic tissues exclusively expressing the former.

Keywords: PD-1; PD-L1; PD-L2; T-cell; Inhibitory receptor; Negative signal

1. Introduction

PD-1 has been shown to be one of the inhibitory receptors expressed on immune cells like T and B cells [1]. Gene disruption studies suggest that the PD-1 molecule is involved in the control of auto-immune responses, since PD-$1^{-/-}$ B6 mice develop lupus-like auto-immune diseases and PD-$1^{-/-}$ BALB/c mice suffer from autoimmune cardiomyopathy [2,3]. These phenotypes, however, become explicit late in life. On the contrary, mice lacking CTLA-4, another inhibitory receptor expressed on T-cells, develop a massive polyclonal lymphoproliferation as early as 5–6 days after birth [4,5]. This strongly suggests that PD-1 has a physiological role distinct from CTLA-4, even though both receptors deliver similar inhibitory signals in immune cells.

CTLA-4 exerts an inhibitory signal in CD4 positive T-cells upon engagement of its ligands, B7-1 and B7-2 [6,7]. Since PD-1 is functionally and structurally related to CTLA-4, it was postulated that ligands for PD-1 might be members of the B7 gene family. In a search for candidates in the B7 family proteins, two membrane proteins related to B7 molecules containing two immunoglobulin-fold domains, PD-L1 and PD-L2, were demonstrated to interact directly with the soluble PD-1/

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0165-2478/02/$ - see front matter
PII: S0165-2478(02)00142-6

Fcγ chimera protein in an in vitro binding assay [8,9]. Engagement of PD-1 by PD-L1 or PD-L2/Fcγ fusion protein was shown to inhibit proliferation and cytokine production in T-cells [8,9]. CTLA-4 contains an amino acid stretch like an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail, which recruits src homology 2-domain-containing tyrosine phosphatase, SHP-2 [10–13]. Although, PD-1 also contains an amino acid stretch like an ITIM, the N-terminal tyrosine residue in the putative ITIM motif is not involved in the delivery of negative signal and rather another C-terminal tyrosine residue plays a pivotal role in the execution of an inhibitory signal cascade through recruitment of SHP-2 [14].

In order to determine the physiological roles of PD-1-PD-L1/PD-L2 interaction, it is necessary to examine the distribution and expression profiles of the PD-L1 and PD-L2 proteins. In the present study, we established monoclonal antibodies specific for mouse PD-L1 and PD-L2 molecules, and examined the distribution and expression patterns of the ligands in the cells of lymphohematopoietic tissues.

2. Materials and methods

2.1. Mice

BALB/c mice (6–9-week-old, female) were purchased from Japan Clea and maintained in our animal facility.

2.2. Preparation of PD-L1 molecule

A cDNA encoding His-tagged murine PD-L1 was digested with EcoRI and NotI and incorporated into an expression plasmid, pVL1393 (Clontech). The resultant vector together with Bsu36I-digested BacPAK6^{TM} DNA (Clontech) was introduced into SF9 insect cells (Invitrogen) for packaging according to the manufacturer's protocol. HiFive insect cells (Invitrogen) were infected with the viral particles at 27 °C for 2 days, and lyzed with 50 mM Tris–HCl, pH 7.5 containing 1% Triton X-100, 10 mM EDTA, 150 mM NaCl, and a mixture of protein inhibitors (Amersham). PD-L1 protein in the cell lysate was purified using Ni Sepharose column chromatography (Qiagen).

2.3. Expression and purification of PD-L2/human Ig chimera

A human renal cell carcinoma line, 293T, was transfected with a plasmid, pEF-BOS NeoSE, containing a cDNA encoding mouse PD-L2/human Ig chimera protein at a SmaI cloning site. The culture supernatant of the transfectant resistant to 1 mg/ml of G418 (Nacalai tesque, Osaka, Japan) was collected and PD-L2/human Ig chimera was purified on Protein A Sepharose column chromatography (Amersham).

2.4. cDNA transfection

P815 mastocytoma line and A20.J B lymphoma line were transfected with an expression vector, pApuroXS, containing cDNA for murine PD-L1 and pEF-BOS NeoSE containing cDNA for murine PD-L2 by electroporation followed by selection with puromycin and G418, respectively.

2.5. Preparation of monoclonal antibodies specific for PD-L1 and PD-L2

An emulsion of PD-L1 or PD-L2 protein plus complete Freund's adjuvant (DIFCO) was injected into two 8-week-old female Whister rats (SLC Japan, Shizuoka, Japan). After the immunization, the regional lymph node cells, 2×10^8, were fused with SP2/0, 2×10^8, using PEG1500 (Amersham). After selection with RPMI1640 containing HAT (Sigma), 10% Origen (Igen), 10% fetal calf serum, 10^{-5} M 2-mercaptoethanol, penicillin and streptomycin, the culture supernatants were examined on flow cytometry for their reactivity to PD-L1 or PD-L2 using P815/PD-L1 or A20.J/PD-L2. Established hybridomas were injected into BALB/c nu/nu mice and ascites were obtained, from which monoclonal antibodies, 1-111A and 122, specific for PD-L1 and PD-L2, respectively, were purified on Protein G Sepharose column chromatography. For flow cytometric analyses, the monoclonal antibodies were conjugated with biotin using sulfo–NHS–LC–biotin (EZ-Link, Pierce) according to the manufacturer's protocol.

2.6. Western blot analysis

Both P815 cells transfected with a vector containing a cDNA encoding murine PD-L1 in a normal direction and in a reversed direction were lyzed with 50 mM Tris–HCl, pH 7.5, containing 1% Triton X-100, 10 mM EDTA, 150 mM NaCl, and a mixture of protein inhibitors on ice for 30 min. The samples were subjected to a 10% SDS-PAGE and the separated protein bands were transferred onto a PVDF membrane, which was blocked with PBS containing 5% skimmed milk and 0.05% Tween-20 at room temperature for 2 h. After treating with rat *anti*-mouse PD-L1 monoclonal antibody, 1-111A, the membrane was soaked in a solution containing horseradish peroxidase-conjugated *anti*-rat IgG (H+L) (KPL) followed by substrate. Finally, protein band was visualized using ECL chemiluminescence reagent (Pierce).

2.7. Antibodies

Anti-mouse B220, IgM, CD19, CD3, CD4, CD8, Thy1.2, CD11b, CD11c, c-Kit, TER19 and PE-conjugated streptavidin were purchased from Pharmingen, and anti-pan NK cell marker, DX-5, and Gr-1 from eBioscience, and streptavidin-Red670 from GibcoBRL, respectively.

2.8. Cell culture and flow cytometric analysis

Spleen cells were treated with hypotonic buffer at room temperature for 2 min to remove erythrocytes and washed three times with the complete RPMI1640 medium. For stimulation, the resultant cells were incubated with 2 μg/ml of concanavalin A (ConA, Sigma) or 50 μg/ml of lipopolysaccharide (LPS, Sigma) in a six-well culture plate at 2×10^6 cells/ml at 37 °C with 5% CO_2 for 2 days. The blast cells were pretreated with 2.4G2 specific for FcIIγR and stained with biotin-conjugated rat *anti*-mouse PD-L1 or PD-L2 mAbs, followed by streptavidin-PE. Freshly isolated splenocytes, thymocytes, and bone marrow cells were similarly examined for PD-L1/PD-L2 expression on two-, or, three-color flow cytometry using LSR and FACS Vantage (Becton Dickinson).

3. Results

3.1. Monoclonal antibodies specific for PD-L1 and PD-L2

Northern blot analysis indicated that mouse P815 and A20 cell lines exhibited no detectable PD-L1 and PD-L2 transcripts, respectively (data not shown). We, therefore, established a PD-L1-expressing clone of P815 (P815-PD-L1) and a PD-L2-expressing clone of A20 (A20-PD-L2) by cDNA transfection, the expression of each transcript being confirmed by Northern blotting (data not shown). Rats were immunized with purified His-tagged PD-L1 protein and PD-L2/human Ig chimera protein as described in Section 2. Monoclonal antibodies, which specifically recognize these transfectants were screened, and clones, 1-111A and 122, were established (Fig. 1A). 1-111A mAb reacted to P815-PD-L1, but not to parental P815, evidenced by flow cytometric staining, and specifically immunoblotted a ~65 kDa protein from the cell lysate of P815/PD-L1

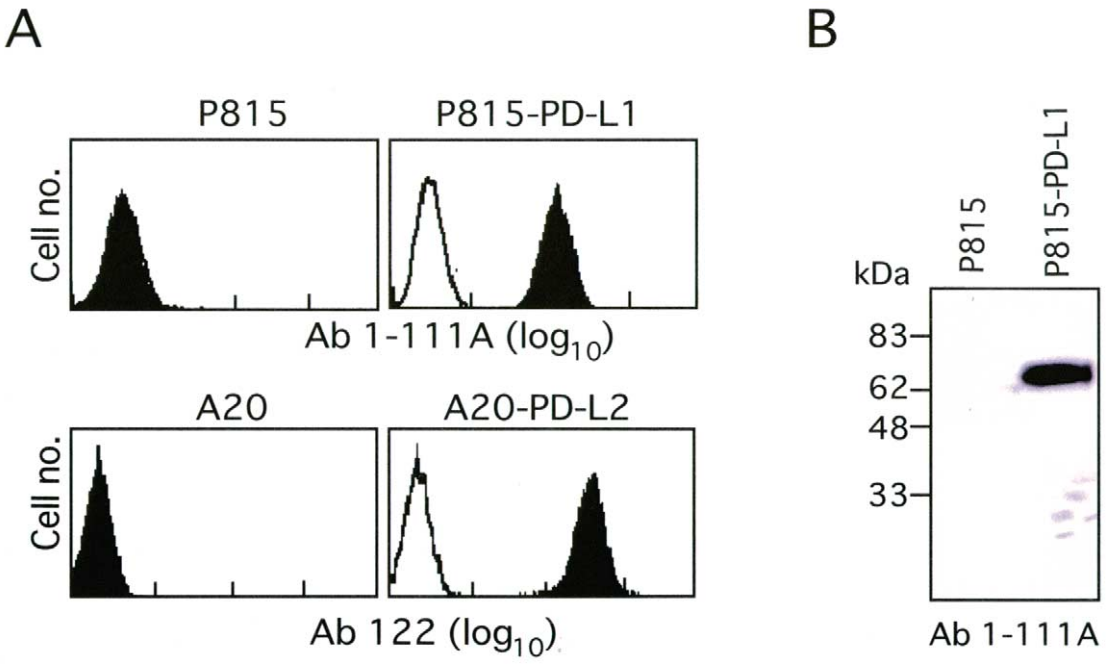

Fig. 1. Establishment of monoclonal antibodies specific for PD-L1 and PD-L2. (A) Flow cytometric analyses of the specificity of newly established rat monoclonal antibodies, 1-111A and 122, specific for murine PD-L1 and PD-L2, respectively. P815 cells transfected with expression plasmids containing either cDNA encoding mouse PD-L1 in a normal direction or that in a reversed direction were stained with biotin-conjugated 1-111A, followed by PE-conjugated streptavidin (upper panels), and similarly A20 cells with cDNA encoding mouse PD-L2 in a normal direction and reversed direction were treated with biotin-conjugated 122, followed by PE-conjugated streptavidin (lower panels). (B) Specificity of 1-111A on Western blot analysis. Both P815 cells lacking and expressing PD-L1 molecule were lyzed and subjected to the standard Western blot analysis using 1-111A.

(Fig. 1B), indicating that the mAb recognized intact as well as denatured PD-L1 molecules. Although, not shown, 1-111A mAb also immunoprecipitated the same ~65 kDa protein. On the other hand, 122 mAb specifically recognized A20/PD-L2, but not parental A20. This mAb, however, could not be used for immunoprecipitation or immunoblotting. Survey of murine cell lines indicated that the reactivity of 1-111A and 122 was well correlated with the expression of PD-L1 and PD-L2 transcripts (data not shown). We thus concluded that 1-111A was specific for PD-L1 and 122 for PD-L2.

3.2. PD-L1, but not PD-L2, is expressed on the vast majority of activated lymphocytes

A minor proportion of freshly isolated normal spleen cells expressed PD-L1 (Fig. 2A). Two-color analysis revealed that about half of normal B cells, dendritic cells, macrophages, and NK cells significantly expressed PD-L1 molecules on their cell surface, while only a marginal proportion of resting T-cells expressed PD-L1 (Fig. 2B). Upon stimulation with ConA or LPS for 2 days, however, the vast majority of T and B cells were induced to express PD-L1 strongly (Fig. 2A), indicating that PD-L1 is one of the lymphocyte activation antigens. Consistent with a previous report on Northern blot analysis [8], PD-L1 was also induced by interferon-γ (data not shown). It is, thus, suggested that a minor PD-L1$^+$ lymphocyte population in the fresh spleen may represent those that have been activated in vivo. On the other hand, no significant expression of PD-L2 was ever detected in the normal lymphoid cells even after activation in vitro. Based on these results, PD-L1 and PD-L2 exhibit distinct tissue expression profiles.

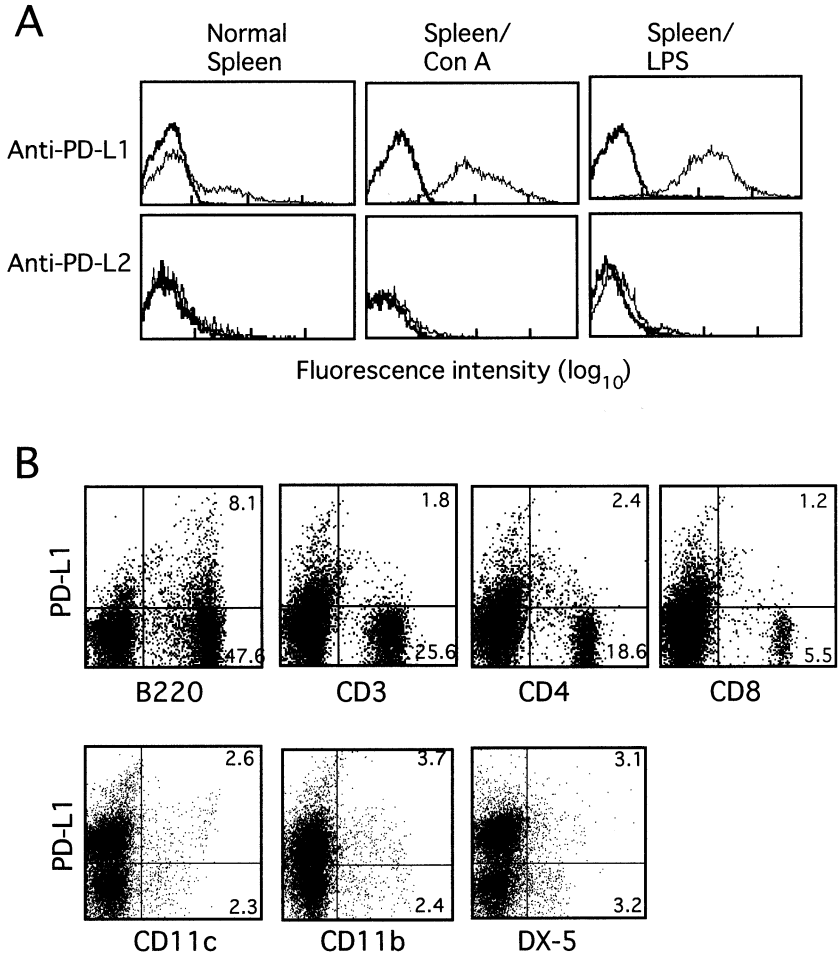

Fig. 2. Analyses of PD-L1 and PD-L2 expression on splenocytes. (A) Flow cytometric analysis of the expression of PD-L1 and PD-L2 on the surface of untreated, ConA-treated, and LPS-treated spleen cells. Freshly isolated splenocytes were treated with either biotin-conjugated 1-111A or 122, followed by PE-conjugated streptavidin (left panels). Similarly, splenocytes cultured with either ConA (middle panels) or LPS (right panels) for 2 days were stained and analyzed on flow cytometry. (B) Two-color flow cytometric analysis of freshly isolated splenocytes. Splenocytes were prepared from BALB/c mice and stained with biotin-conjugated 1-111A monoclonal antibody in combination with FITC-conjugated CD3, CD4, CD8, B220, CD11b, CD11c or DX-5 monoclonal antibody, followed by PE-conjugated streptavidin.

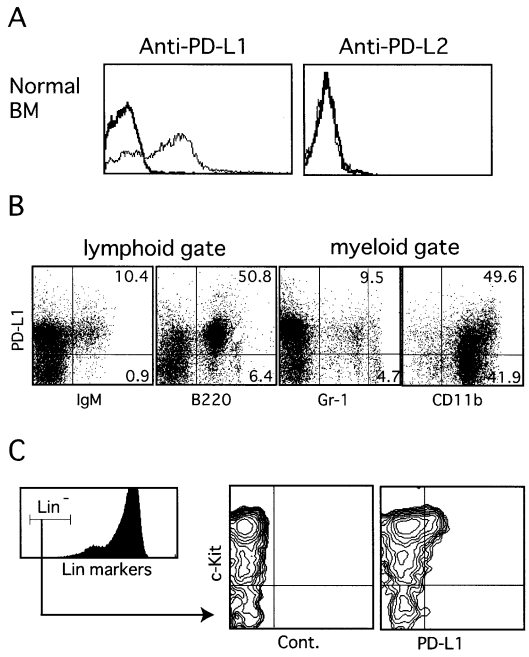

Fig. 3. Examination of the expression of PD-L1 and PD-L2 on normal bone marrow cells. (A) Flow cytometric analysis of PD-L1 and PD-L2 expression on freshly isolated bone marrow cells. Normal bone marrow cells prepared from BALB/c mice were stained with biotin-conjugated 1-111A, followed by PE-conjugated streptavidin. (B) Two-color flow cytometric analysis of normal bone marrow cells. Bone marrow cells were isolated from BALB/c mice and stained with biotin-conjugated 1-111A monoclonal antibody in combination with FITC-conjugated IgM, B220, Gr-1, or CD11b monoclonal antibody, followed by PE-conjugated streptavidin. For detailed analyses, lymphoid and myeloid regions were determined based on the FACS profile of forward scattering versus side scatterimg plot and separately depicted, respectively. (C) Three-color flow cytometric analysis of bone marrow cells. Freshly isolated bone marrow cells were stained with FITC-conjugated monoclonal antibodies specific for lineage markers including Thy 1.2, B220, TER-119, Gr-1, MAc-1, CD19, and DX-5, APC-conjugated anti-c-kit mAb, and biotin-conjugated anti-PD-L1 mAb, followed by PE-conjugated streptavidin. After lineage negative region was confined, the expression profile of c-kit versus PD-L1 was determined.

3.3. PD-L1 is expressed on the majority of pre-B cells and myeloid cells in the normal bone marrow

As shown in Fig. 3A, PD-L1 was expressed on the majority of normal BM cells. Two-color analysis indicated that the vast majority of $B220^+$ pre-B cells and IgM^+ B cells and more than half of myeloid cells (granulocytes and macrophages) in the BM expressed PD-L1 on their cell surface (Fig. 3B). In $Gr\text{-}1^+$ population, a proportion of $PD\text{-}L1^+$ cells tended to be higher in $Gr\text{-}1^{low}$ immature granulocytes than in $Gr\text{-}1^{high}$ maturated granulocytes. Furthermore, among lineage-marker negative (lin-) population, a minor yet significant proportion of $c\text{-}Kit^+$ population representing the most immature progenitors also exhibited a significant level of PD-L1 (Fig. 3C). Again, no detectable expression of PD-L2 was detected either in the normal BM cells.

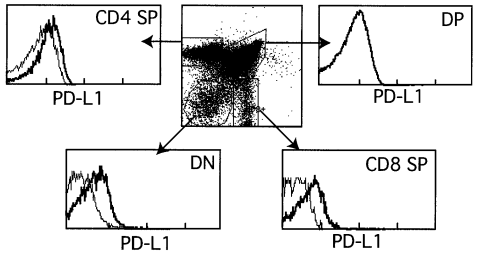

Fig. 4. Determination of PD-L1 expression on thymocytes. Freshly isolated thymocytes were stained with PE-conjugated anti-CD4, FITC-conjugated anti-CD8, and biotin-labeled anti-PD-L1 monoclonal antibodies, followed by Red670-conjugated streptavidin. Cell regions were determined based on the FACS profile of CD4 versus CD8 plot to confine $CD4^-CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^-$, $CD4^+CD8^+$ populations. Each regions were analyzed for the expression of PD-L1 molecules on their cell surface, and depicted as histograms, respectively.

3.4. Immature DN, but not DP, thymocytes express significant PD-L1

In the thymus, PD-L1 was expressed marginally yet explicitly. Expression profiles of PD-L1 were analyzed in distinct thymocyte subsets by using three-color analysis. As shown in Fig. 4, the most immature CD4/CD8-double negative (DN) population significantly expressed PD-L1, and the mature CD4 or CD8-single positive (SP) population also did albeit rather marginally. In contrast, CD4/CD8-double positive (DP) population representing the vast majority of normal thymocytes did not express PD-L1 at all. Although not shown, PD-L2 was not expressed on thymocytes at all.

Table 1
Expression of PD-1 ligands on leukemic cell lines

Lineages	Lines	PD-L1	PD-L2
B-cell lineage	A20.2J	+	–
	BAL.17	+	–
Myeloma	P3U	+	–
	SP2/0	+	–
	PAI	+	–
	X63	+	–
	J558L	+	–
T-cell lineage	DL3	+	–
	EL4	–	–
	YAC-1	–	–
	BW5147	+	–
Myeloid lineage	WEHI-3	+	–
	FBL-3	+	–
	P815	–	–

3.5. Expression of PD-L1 on various tumor cell lines of lymphohematopoietic origin

We finally examined the expression of PD-L1 and PD-L2 on tumor cell lines of lymphohematopoietic origin. As summarized in Table 1, the majority of leukemia and lymphoma lines of T-, B-, and myeloid lineages exhibited PD-L1 but not PD-L2.

4. Discussion

In the present study, we have developed monoclonal antibodies specific to ligands for the PD-1 receptor, PD-L1 and PD-L2, and systematically examined their expression profiles in normal lymphohematopoietic cells. The results indicated the following findings. First, PD-L1 was expressed on almost all the types of lymphohematopoietic cells at varying levels, while PD-L2 was not at all on any cell types examined. Previous studies indicated that PD-L1 transcripts were detected not only in the lymphoid organs but also other tissues such as heart, lungs and kidney but not in liver, whereas PD-L2 transcript was expressed rather selectively in the liver [8,9]. Thus, expression profiles of PD-L1 and PD-L2 were quite distinct and largely un-overlapped; however, the possible biological significance of this remains to be seen. Second, in T and B cells, PD-L1 expression was strongly induced by mitogenic stimulation as well as by interferon-γ. This is reminiscent of PD-1 receptor expression [15], and thus both PD-1 and PD-L1 were concomitantly up-regulated following lymphocyte activation. Third, PD-L1 was strongly expressed on the developing lymphohematopoietic cells in the normal BM, including pre-B cells and myeloid cells. Notably, a significant proportion of the most immature c-Kit$^+$ lin$^-$ progenitor population expressed PD-L1. Finally, the most immature DN thymocytes exhibited PD-L1 expression, while DP thymocytes did not at all, suggesting regulated expressions during the thymocyte development. The PD-1 receptor was also expressed selectively in a minor population of DN thymocytes, and suggested to affect β-selection process [16,17].

PD-1 deficient mice develop characteristic autoimmune diseases, and it is suspected that PD-1 ligands expressed on normal somatic cells play an important role in protecting them from potentially autoreactive T-cells expressing PD-1 molecules [1]. Indeed, we have observed that expression of PD-L1 on the target cells provided a significant protective effect from direct cytotoxicity by the specific cytotoxic T-cells expressing PD-1 (CTL) (submitted for publication). It is therefore quite possible that PD-L1 expressed on the normal lymphohematopoietic cells may in part function to veto the effects of potentially autoreactive T-cells at the effector phase. Importantly, a number of tumor lines of lymphohematopoietic origin expressed PD-L1, suggesting that PD-L1 may provide a mechanism for such tumor cells to escape from immune survaillance in the hosts, and thus PD-L1 may be one of the effective molecular targets for cancer immunotherapy.

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