Expression of Programmed Death 1 Ligand in Different Compartments of Chronic Lymphocytic Leukemia

Maciej Grzywnowicz a Agnieszka Karczmarczyk a Katarzyna Skorka a
Małgorzata Zajac a Joanna Zaleska a Sylwia Chocholska b Waldemar Tomczak b
Krzysztof Giannopoulos a, b

a Department of Experimental Hematooncology and b Hematooncology and BMT Unit, Medical University of Lublin, Lublin, Poland

Key Words
Chronic lymphocytic leukemia · Programmed death 1 ligand · Microenvironment · Thalidomide

Abstract
Background: The programmed death 1 (PD-1) receptor pathway is responsible for the negative regulation of both T and B lymphocytes upon activation of these cells. There is growing evidence that chronic lymphocytic leukemia (CLL) cells exploit the PD-1 ligand (PD-L1) to resist antitumor immune reactions and maintain their survival by shaping their own microenvironment. Methods: We used a quantitative RT-PCR method to analyze PD-L1 gene expression in bone marrow and peripheral blood mononuclear cells, representing the proliferation and accumulation compartments of CLL. Results: PD-L1 expression was found to be significantly higher in 112 CLL patients than in controls. Levels of PD-L1 expression in bone marrow and peripheral blood were comparable and showed a positive correlation. Furthermore, expression of PD-L1 strongly correlated with expression of PD-1 receptor in mononuclear cells from the same compartment, and was not affected by incubation with immunomodulatory drug thalidomide. Conclusion: PD-L1 expression is shared between CLL cells localized in distinct disease compartments, demonstrating that PD-1/PD-L1 a universal target for therapy.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of adult B cell malignancy in both the USA and the European Union [1, 2]. CLL is characterized by the proliferation and accumulation of monoclonal CD5+ B cells in the peripheral lymphoid organs and bone marrow, accompanied by overrepresentation of CLL cells in peripheral blood [3]. Leukemic cells interact with bystander cells in certain tissues, creating a microenvironment which supports the proliferation of malignant cells and promotes their survival by inhibiting apoptosis and protecting from immune elimination [4]. One of the potential mechanisms responsible for evading cytotoxic T lymphocytes by leukemic cells in CLL might be the pathway of programmed death 1 (PD-1, CD279). PD-1 is a costimulatory receptor expressed on
T and B lymphocytes upon activation of these cells. PD-1, via interactions with PD-1 ligand (PD-L1, CD274, B7-H1) limits the effector functions of T cells and prevents autoimmune events [5]. The PD-L1/PD-1 pathway has been found to cause ‘exhaustion’ of tumor-infiltrating PD-1\(^\text{high}\) T cells through upregulation of PD-L1 on tumor cells [6]. PD-1-mediated ‘exhaustion’ of T cells could be reversed with PD-1 blockade [7]. In CLL, CD8+ T cells have been found to express many immunosuppressive ‘exhaustion’ features including PD-1 [8–10]. Expansion of T cells expressing PD-1 has been shown to correlate with an inferior outcome in CLL patients [8, 9]. Regardless of the ‘exhausted’ phenotype, T lymphocytes in CLL are functionally able to retain cytokine production [10]. Overexpression of PD-L1 on tumor cells could therefore play an important role in maintaining the immunosuppressive microenvironment [11]. Expression of PD-L1 on tumor cells has been identified in several types of cancers and in hematological malignancies; however, in non-Hodgkin lymphomas, the expression of PD-L1 is sparsely characterized [11, 12]. Previously, we showed the simultaneous expression of PD-L1 and PD-1 on CLL cells [13]. Here, we present the characterization of expression of PD-L1 in CLL, including the comparison of cells isolated from two different microenvironmental compartments: the bone marrow and peripheral blood. Moreover, we assess the influence of the immunomodulatory drug thalidomide on the expression of PD-L1 in leukemic cells in CLL, as lenalidomide, an analog of thalidomide, has been found to modulate PD-1/PD-L1 expression in hematological malignancies [14, 15].

**Materials and Methods**

**Ethics Statement**

This study was approved by the Ethics Committee of the Medical University of Lublin (No. KE-0254/150/2008). Written informed consent was obtained from all patients with respect to the use of their blood for scientific purposes.

**Patients**

For the experiments, peripheral blood samples and bone marrow aspirates were collected from 112 CLL patients treated in the Department of Hematooncology, Medical University of Lublin, Poland. All samples were collected at the point of diagnosis. To avoid contamination risk, during bone marrow aspiration, we used the first collected aspirate. The clinical characteristics of the patients are summarized in table 1. Peripheral blood samples were collected from 30 age- and sex-matched (15 men and 15 women with a median age of 63 years) healthy volunteers (HV) were used as a control.

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics of patients</th>
<th>Total (n = 112)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>40</td>
</tr>
<tr>
<td>Male</td>
<td>72</td>
</tr>
<tr>
<td><strong>Age (median, range)</strong></td>
<td>64 (31–85)</td>
</tr>
<tr>
<td><strong>Binet Stage</strong></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>37</td>
</tr>
<tr>
<td>B</td>
<td>47</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
</tr>
<tr>
<td><strong>Cytogenetics</strong></td>
<td></td>
</tr>
<tr>
<td>t(14q deletion)</td>
<td>5</td>
</tr>
<tr>
<td>t(11q deletion)</td>
<td>14</td>
</tr>
<tr>
<td>t(12q trisomy)</td>
<td>4</td>
</tr>
<tr>
<td>Normal karyotype</td>
<td>14</td>
</tr>
<tr>
<td>t(13q deletion as sole abnormality)</td>
<td>25</td>
</tr>
<tr>
<td>Various abnormalities</td>
<td>2</td>
</tr>
<tr>
<td><strong>ZAP-70 (cut-off 20%)</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
</tr>
<tr>
<td>Negative</td>
<td>69</td>
</tr>
<tr>
<td><strong>CD38 (cut-off 30%)</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>25</td>
</tr>
<tr>
<td>Negative</td>
<td>82</td>
</tr>
<tr>
<td><strong>Mutational status of IgVH</strong></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>33</td>
</tr>
<tr>
<td>Unmutated</td>
<td>47</td>
</tr>
<tr>
<td>n/a</td>
<td>32</td>
</tr>
</tbody>
</table>

* Patients with ZAP-70 expression lower or higher than 20% were classified as ZAP-70 negative or positive respectively.

** Cell Isolation, RNA Preparation and mRNA Reverse Transcription **

Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) from aspirates were isolated with Ficoll density gradient centrifugation (Biochrom AG, Berlin, Germany). The viability of the cells obtained was always >95% as determined by Trypan blue exclusion. Viable cells were quantified in a Neubauer chamber (Zeiss, Oberkochen, Germany). For total RNA isolation, 10 million cells were pelleted and stored at –80°C. The QIAamp RNA blood mini kit (Qiagen, Venlo, the Netherlands) for RNA isolation from PBMC was used according to the manufacturer’s instructions. From each sample, 1 μg of total RNA was reverse-transcribed to 20 μl of cDNA using a QuantiTect reverse transcription kit (Qiagen). For quantitative (q)RT-PCR reactions, 1 μl of cDNA of each sample was used.

**Quantitative RT-PCR**

For quantitative measurements of the mRNA expression of PD-L1, qRT-PCR was performed with Fast Start Universal SYBR Green Master methodology according to the manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany). Primer design was based on the NM-014143 sequence of the PD-L1 transcript (NCBI reference sequence database) with the forward primer:
5′-TATGGTGGTGCCGACTACAA-3′ and reverse primer: 5′-TGCTTGCTCAGATGACTTCG-3′. Primers for PD-1 were based on the NM-005018 sequence of the PDCD1 gene with the forward primer: 5′-CTCAGGGTGACAGAGAGAAG-3′ and reverse primer: 5′-GACACCAACCACCAAGGT-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a constitutively expressed housekeeping gene. A thermocycling program was set for 40 cycles of 15 s at 95 °C, and 1 min at 60 °C with an initial de-naturation step at 95 °C for 10 min on the ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, Calif., USA). Particular expression levels were presented as a two raised to the Δ of cycle threshold (2ΔCt) power, where ΔCt is the Ct value for GAPDH minus the Ct value for PD-L1.

Leukemic B Cell Incubation with Thalidomide
For functional studies, CD19+ cells from PBMC samples of 14 CLL patients (3 females and 11 males with a median age of 65 years) were separated in a magnetic field according to the manufacturer’s protocol (MACS Milltenyi Biotec). Cells were cultured for 24 h in a standard medium consisting of RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% (v/v) FCS, 50 units/ml of penicillin, 50 mg/ml streptomycin and 100 mg/ml neomycin with and without the addition of 10 µg/ml of thalidomide. After culture, cells were harvested and analyzed for PD-L1 expression using qRT-PCR.

Statistical Analysis
PD-L1 expression was presented as median values. The Mann-Whitney U test was used to evaluate the differences between subgroups of patients. In functional studies, the Wilcoxon signed-rank test was used to calculate the difference in PD-L1 expression between patients’ cells cultured with and without thalidomide. Correlations of variables were computed with the Spearman rank correlation coefficient. The Kaplan-Meier method and the log-rank test were used to assess time for the first treatment in different groups of patients.

Results
PD-L1 Is Overexpressed in PBMC in CLL and Shows a Strong Correlation between Compartments
Levels of expression of the PD-L1 transcript in the qRT-PCR analyses were higher in PBMC from CLL patients than in those from the HV group (0.144 vs. 0.022, p < 0.0001). Considering leukemic cell compartmentalization, the expression of PD-L1 in the mononuclear cells was comparable in the peripheral blood and bone marrow compartments (0.144 vs. 0.116; fig. 1a). There was a positive correlation between the expression of PD-L1 in the peripheral blood and bone marrow (r = 1, p < 0.0001; fig. 1b).

PD-L1 Expression Correlates with Expression of PD-1 in Mononuclear Cells
Earlier, we found coexpression of PD-1 and PD-L1 on the same CLL cell on a protein level. In this study, we analyzed the correlation between the expression of the PD-1 receptor and its ligand in CLL cells. The expression of PD-L1 correlated with PDCD1 expression in the peripheral blood (r = 0.34, p = 0.0002; fig. 1c) and bone marrow samples (r = 0.5, p < 0.0001; fig. 1d).

PD-L1 Expression Is Independent of Clinical Parameters and Prognostic Markers in CLL
The expression of PD-L1 was correlated with the clinical parameters of CLL patients. There were no correlations with age, LDH levels and white blood cell count. No differences of PD-L1 expression in PBMC or BMMC were found in males and females or in groups of patients classified according to different stages of disease (Binet staging system). Considering the prognostic factors, PD-L1 expression was found to be independent of the expression of ZAP-70, CD38 and the mutational status of IGHV (fig. 2a–c). Analysis of cytogenetic abnormalities showed that the lowest PD-L1 expression was in patients with the 11q deletion and highest in those with the 13q deletion in both the bone marrow and peripheral blood (fig. 2d).

The Immunomodulatory Drug Thalidomide Does Not Regulate PD-L1 Expression in CLL Cells
Cell culture of CD19+ cells from 14 CLL patients with the addition of the immunomodulatory drug thalidomide showed that samples treated with thalidomide tended to have greater PD-L1 expression than nontreated controls (1.22 vs. 0.37, p = 0.067; fig. 3).

Discussion
It has been found that cancer cells develop a large variety of mechanisms in order to maintain their survival [16]. Modulation of the cancer cell phenotype along with expressing negative costimulatory molecules is one of the effective ways to evade the antitumor action of immune system. The PD-1/PD-L1 pathway has been shown to function as such an escape mechanism. High expression of PD-L1 can be utilized by cancer cells to sustain immune tolerance by promoting PD-1+ T lymphocytes [17]. The precise result of PD-1 receptor activation is unknown, although it has been found to affect signal transduction from both T and B lymphocyte receptors [18, 19]. In T cells, PD-L1, via PD-1 binding, was shown to induce the formation of PD-1/T lymphocyte receptor microclusters, which are crucial for the regulation of T lymphocyte receptor signaling [18]. It has been described that block-
ade of PD-L1 restores and improves the antitumor function of PD-1+ T lymphocytes [20, 21], making the PD-1/PD-L1 pathway a target in specific-antibody anticancer therapy [22, 23]. Since both elements of the pathway are overexpressed in CLL, they could be used as potential targets for the immunotherapy of this disease.

Malignant cells can use immune regulatory proteins to evade T cell recognition through constitutive oncogenic signaling, which is defined as innate immune resistance. In this report, we found that the level of the PD-L1 transcript is upregulated in CLL cells regardless of the microenvironment. The cause of the high level of PD-L1 expression in CLL compartments is undetermined; however, here we provide the evidence that this phenomenon might possibly be independent of the microenvironment. The PD-L1 gene is localized in the short arm of the chromosome 9p24 region. It is therefore a target for recurrent amplification of the 9p24.1 fragment in lymphoid malignancies [24], which leads to overexpression of PD-L1. Extended amplification of the 9p24 region also induces expression of the Janus kinase 2 (JAK2) locus, which augments signaling between JAK and the signal transducer and activator of transcription (STAT). STAT3 was shown to drive PD-L1 expression through binding to the promoter region [25], and to regulate microRNAs in CLL [26]. MicroRNA has been described as a negative regulator of PD-L1 expression; therefore, the activation of STAT3 might lead to the deregulation of microRNAs.

**Fig. 1.** Expression of PD-L1 in peripheral blood and bone marrow. 

| a | Expression of the PD-L1 transcript normalized to GAPDH expression. Median expression of PD-L1 was higher in CLL patients than in healthy controls (0.144 vs. 0.022). b PD-L1 expression in PBMC and BMMC was found to be comparable, and it showed a strong correlation. Expression of PD-L1 correlated with the expression of the PDCD1 receptor transcript in both peripheral blood (c) and bone marrow (d).
possibly resulting in the regulation of the PD-L1 level [27]. Moreover, STAT3 is activated via the CD40/CD40L pathway [28], and this activation mediates the resistance of CLL cells to apoptosis [29]. Another mechanism possibly related to the upregulation of PD-L1 is the phosphatidylinositide 3-kinase/protein kinase B (PI3K-AKT) pathway, which is persistently activated due to B lymphocyte receptor signaling in CLL [30]. It has been found that this pathway and the loss of the phosphatase and tensin homolog (PTEN) induce PD-L1 expression [31]. In Hodgkin lymphoma cells, activating protein 1 (AP-1) is also related to PD-L1 expression [24] and this mechanism has recently been shown to be involved in CLL biology [32].

Expression of PD-L1 in CLL could represent the adaptation of leukemic cells to the antitumor immune response. Interferons (IFN), mainly IFNγ, produced by T cells, natural killer cells, epithelial cells and stromal cells in normal tissue, posttranscriptionally increase PD-L1 expression [33–35]. It has been shown that surface PD-L1 expression strongly correlates with lymphocytic infiltration and intratumoral IFNγ production [36]. Additionally, IFNγ is elevated in the serum of CLL patients [37]. Moreover, lenalidomide, an immunomodulatory
drug used in CLL therapy, has been shown to induce the production of proinflammatory cytokines, including IFNγ [14, 38]. In multiple myeloma, lenalidomide downregulates PD-L1 expression [14, 15]. There is also a negative feedback between PD-L1 and IFNγ since the PD-1/PD-L1 interaction impairs IFNγ production in CD8+ T cells, and PD-L1 blockade in non-Hodgkin lymphomas leads to increased cytokine production by T cells [39, 40]. Immumodulatory drugs, like thalidomide and lenalidomide, reduce numbers of regulatory T cells in CLL patients [41]; this is the opposite mechanism to the PD-1/PD-L1 pathway which is able to promote the development of regulatory T cells [42, 43]. In functional studies, we assessed the influence of thalidomide, an analog of lenalidomide [44], on the expression of PD-L1 in leukemic cells in CLL. We observed no significant change in PD-L1 expression by CLL cells upon thalidomide incubation, suggesting that thalidomide might not directly affect the expression of PD-L1 in CLL.

The detailed mechanism of action and the regulation of the PD-1/PD-L1 axis in CLL are not known; however, there is growing evidence about their significance in this disease. Expression of the PD-1 receptor is elevated on CD8+ T lymphocytes in CLL and the PD-1/PD-L1 pathway contributes to immunological synapse dysfunction between leukemic and antitumor T cells and IFNγ production [8, 40]. Brusa et al. [40] reported that the expression of PD-L1 is elevated on circulating CLL cells, proliferating particularly under stimulation when compared to nonmalignant B cells from healthy donors. This work points to the overexpression of the PD-L1 transcript; this is coherent with the flow cytometric analysis of Brusa et al. [40]. PD-L1+ leukemic cells and PD-1+ T cells were found to be in close contact and then localized in proliferation centers in the lymph nodes of CLL patients, demonstrating that there is a close functional interaction between PD-L1+ leukemic cells and infiltrating T cells [40]. Our study shows that the high level of expression of the PD-L1 transcript appears to be comparable between circulating malignant B cells and those in the bone marrow compartment. The expression of the ligand correlates with PD-1 receptor expression in both compartments, and is also upregulated in the peripheral blood and bone marrow [45]. Ramsay et al. [8] described the clinical link of PD-L1 expression on CLL cells to a poor prognosis. In their study, patients with higher PD-L1 expression were classified as poor-prognosis patients (median survival: 38 months) or good-prognosis patients (median survival: >10 years). In this study, we analyzed influence of PD-L1 expression on time to first treatment of the CLL patients and we found no association (data not shown). The elevated expression of PD-L1 did not correlate with unfavorable genomic aberrations or with other prognostic factors in CLL.

In conclusion, key events for CLL development occur mainly in the lymphoid tissues and bone marrow; however, our results prove that PD-L1 expression is shared between CLL cells localized in distinct disease compartments with different microenvironments. This fact should be considered when designing future immunotherapies targeting PD-1/PD-L1 in CLL.

Acknowledgement

This work was supported by grants from the Foundation for Polish Science FNP FOCUS 01/08, Ministry of Science and Higher Education IUVENTUS PLUS MNISW IP2011014171 and Medical University of Lublin DS462.

Disclosure Statement

The authors declare no competing financial interests.
PD-L1 in Chronic Lymphocytic Leukemia

References


