PD-L1 expression in megakaryocytes and its clinicopathological features in primary myelofibrosis patients

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Abstract

Myeloproliferative neoplasms (MPNs) are characterized by upregulation of proinflammatory cytokines and immune dysregulation, which provide a reasonable basis for immunotherapy in patients. Megakaryocytes are crucial in the pathogenesis of primary myelofibrosis (PMF), the most clinically aggressive subtype of MPN. In this study, we aimed to explore PD-L1 (programmed death-ligand 1) expression in megakaryocytes and its clinical implications in PMF. We analyzed PD-L1 expression on megakaryocytes in PMF patients by immunohistochemistry and correlated the results with clinicopathological features and molecular aberrations. We employed a two-tier grading system considering both the proportion of cells positively stained and the intensity of staining. Among the 85 PMF patients, 41 (48%) showed positive PD-L1 expression on megakaryocytes with the immune-reactive score ranging from 1 to 12. PD-L1 expression correlated closely with higher white blood cell count (p = 0.045), overt myelofibrosis (p = 0.010), JAK2V617F mutation (p = 0.011), and high-molecular risk mutations (p = 0.045), leading to less favorable overall survival in these patients (hazard ratio 0.341, 95% CI 0.135–0.863, p = 0.023). Our study provides unique insights into the interaction between immunologic and molecular phenotypes in PMF patients. Future work to explore the translational potential of PD-L1 in the clinical setting is needed.

Keywords: PD-L1; primary myelofibrosis; megakaryocyte; JAK2; checkpoint; SP142

Introduction

Targeting the programmed death receptor 1 (PD-1) and its ligand (PD-L1) has ushered in a new era of cancer patient care. Pharmacological inhibition of the PD-1/PD-L1 axis has been associated with remarkable response in numerous solid cancers, particularly in tumors with high expression of PD-1/PD-L1, such as non-small cell lung cancer and melanoma [1–3]. Yet, patients with refractory leukemias or lymphomas usually show only modest clinical response, possibly attributed to a lower rate of checkpoint molecule overexpression or scanty T cells in the tumor region [4,5]. One notable exception is Hodgkin lymphoma, where anti-PD-1 antibodies are approved for treatment in relapsed and refractory settings due to its promising therapeutic efficacy [6]. This unique sensitivity is thought to be attributable to chromosome 9p amplification in
Reed–Sternberg cells, leading to increased transcription and overexpression of PD-L1 [7]. Interestingly, the gene encoding JAK2, the most common oncogenic driver of myeloproliferative neoplasm (MPN), shares the same locus with PD-L1 on chromosome 9p24. Prior work has shown that oncogenic JAK2 activity in MPN patients enhances PD-L1 promoter activity and PD-L1 expression in mutant cells, especially in megakaryocytes [8]. Furthermore, gene sets relevant for cell growth and energy production in T cells from healthy donors cocultured with JAK2-mutated cells are downregulated, suggesting that mutated JAK2 drives T-cell exhaustion [8]. Whole blood transcriptional profiling showed significantly higher PD-L1 expression in MPN patients compared to healthy controls [9]. Preliminary findings of another study also showed a high level of PD-1 expression in various T-cell subsets in patients with MPN-associated myelofibrosis [10]. These studies imply the presence of PD-1/PD-L1-mediated immune exhaustion in MPN patients. In addition, MPNs are susceptible to interferon treatment, suggesting that the neoplastic clones are immunogenic [11]. Taken together, these findings provide a rationale for immunotherapy in MPN patients. In fact, several trials investigating the role of immune checkpoint blockade in MPN and other myeloid malignancies are ongoing [12].

Constitutive activation of the Janus kinase/signal transducer and activator (JAK/STAT) signaling cascade is the common pathway regardless of different MPN entities and driver mutations. The activation of different downstream STAT proteins plays distinct roles in MPN phenotypes [13]. STAT5 activation is critical for cellular transformation leading to uncontrolled myeloproliferation [14] while STAT3 activation causes enhanced inflammatory cytokine production [15]. Phosphorylated STAT3 directly binds the PD-L1 gene promoter [16]; a recent report also showed the involvement of STAT3, instead of STAT5, in JAK2V617F-mediated PD-L1 expression [17]. Recruitment of different STAT proteins is determined by receptor type where EPO-R activates mainly STAT5 while MPL/TPOR activates STAT3, STAT5, and STAT1. Ultimately, the balance of STAT signaling may have an impact on the disease phenotype. For example, STAT5 is predominantly involved in polycythemia vera (PV) and STAT3 in primary myelofibrosis (PMF) [13,18].

Unlike solid cancers where the percentage of cells with PD-L1 expression and the expression intensity can be evaluated on tumor cells, there are no specific target cells in the marrow of PMF patients. In this study, we focused on megakaryocytes, which are tightly linked to the pathogenesis of PMF [19]. Clusters of morphologically abnormal megakaryocytes in the background of myeloid hyperplasia are a hallmark of PMF [19]. More recently, megakaryocytes were found to participate in various immune reactions [20] and regulate hematopoietic stem cell homeostasis [21]. Notably, small molecules targeting megakaryocytes have shown promising results in preclinical PMF models [22]. Together, these highlight the critical role of megakaryocytes in the biologic process of PMF.

This study aimed to investigate PD-L1 expression in megakaryocytes and correlated the findings with clinicopathological characteristics and gene mutations in PMF patients. Immunohistochemical (IHC) staining of marrow sections showed that PD-L1 was mainly expressed on megakaryocytes, as previously reported [8]. Using the immunoreactive scoring system, we demonstrate that PMF patients expressing PD-L1 in bone marrow (BM) megakaryocytes are more likely to have JAK2V617F mutation, advanced stage of myelofibrosis, higher white blood cell (WBC) counts, and worse survival compared to those without expression.

**Patients and methods**

**Study population and data collection**

We retrieved BM tissues from 90 PMF patients (including pre-fibrotic and overt MF) diagnosed between 2007 and 2017 at the National Taiwan University Hospital, who had complete gene mutation data and well-preserved specimens. The histopathological findings were reviewed by two hematopathologists, and diagnosis was made according to the 2016 World Health Organization classification. Clinical and genetic data were retrieved from the electronic medical records. Additionally, 48 patients with Philadelphia chromosome-negative MPNs other than PMF (15 PV, 21 essential thrombocythemia [ET], 6 post-PV MF, and 6 post-ET MF) and 12 people with normal BM were included as controls for the assessment of PD-L1 expression. The institutional review board approved this study.

**PD-L1 immunohistochemistry staining**

PD-L1 expression study was carried out using anti-PD-L1 antibody clone SP142 (Abcam, Cambridge, UK). Ten BM samples were also stained with antibody clone SP263 (Roche, Ventana, Tucson, AZ, USA) and the results were correlated with each other. In brief, BM sections of 5-μm thickness were deparaffinized with EZ prep (Ventana Medical Systems, Inc., Tucson, AZ, USA) and underwent a 64-min pretreatment using...
Cell Condition 1 solution (Ventana Medical Systems, Inc.). The slides were then incubated with anti-PD-L1 antibody for 120 min using the automated Ventana Benchmark XT. Labeling was detected with the Optiview DAB Detection Kit (Roche, Ventana, AZ, USA) as per the manufacturer’s protocol. Dilutions were 1:100 for antibody SP142 and a ready-to-use kit for antibody SP263. All sections were counterstained with hematoxylin in Ventana reagent.

Interpretation of PD-L1 expression
PD-L1 expression was reviewed and scored independently by two clinical pathologists specialized in hematology. Discrepant cases were re-reviewed until a consensus was reached. Tonsil tissue with known positivity for PD-L1 was used as positive control. We created a modified immunoreactive score (IRS), and PD-L1 staining intensity of megakaryocytes was semiquantitatively graded accordingly. IRS ranged from 0 to 12 and was the multiplication of positive cell proportion score (0–4) and staining intensity score (0–3) (supplementary material, Table S1). Positivity was defined when ≥1% of megakaryocytes show any staining intensity. Representative cases of various staining intensity are illustrated in Figure 1. Other mononuclear cells in the marrow stained variably for PD-L1 but they were not analyzed in this study due to marked heterogeneity.

Targeted next-generation sequencing analysis
The BM or peripheral blood samples, collected at the time of diagnosis, were subjected to targeted gene sequencing using TruSight myeloid panel (Illumina, San Diego, CA, USA) that targets 54 genes recurrently affected in myeloid disorders. These include full coding exons of 15 genes: BCOR, BORL1, CDK2, CEBPA, CUXI1, DNM3A, ETV6/TEL, EZH2, KDM6A, IKZF1, PHF6, RAD21, RUNX1/AML1, STA2, and ZRSR2, and hot-spots of 39 genes: ABL1, ASXL1, ATRX, BAF, CALR, CBL, CBLB, CLEC, CSF3R, FBXW7, FLT3, GATA1, GATA2, GNAS, HRAS, IDH1, IDH2, JAK2, JAK3, KIT, KRAS, KMT2A/MLL, MPL, MYD88, NOTCH1, NPM1, NRAS, PDGFRA, PENT, PTEN, PTBN1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, TET2, TP53, U2AF1, and WT1. Library preparation was performed using 50 ng of DNA according to Illumina standard protocol. Paired-end sequencing runs were performed on the Illumina HiSeq platform. Filtering was performed according to the following quality control criteria: (1) total read depth >100 and (2) variant allele frequency of at least 5%. All nonsense and frameshift variants were regarded as true mutations. The functional impact of each missense variant was estimated using the COSMIC database, ClinVar, dbSNP, Polyphen2, and SIFT. Details of sample preparation and sequencing analysis were performed as previously described [23].

Statistical analyses
Analyses were performed using SPSS Statistics v25 (IBM, Chicago, IL, USA) and GraphPad prism v9.1.2 (GraphPad, San Diego, CA, USA). Continuous variables are summarized as median and range, while categorical variables are presented as frequency. Differences between groups in continuous variables were tested with the Mann–Whitney U-test or Kruskal–Wallis test with Dunn’s post hoc multiple comparison. Categorical variables were tested using the chi-square test while sample sizes below 5 were evaluated with Fisher’s exact test. Correlation between significant parameters and PD-L1 immunoreactive score was further estimated with Spearman correlation (pre-fibrotic MF and WBC > 25 × 10⁹/l) or logistic regression (JAK2 and high-molecular risk [HMR] mutations). The proportion of variance explained by JAK2 and HMR mutations was calculated using Nagelkerke pseudo R². Survival between groups was compared using log-rank regression (Mantel–Cox). Overall survival was defined as months from diagnosis to mortality of any cause. Hazard ratio (HR) was estimated with Cox-proportional hazards regression. Leukemia-free survival (LFS) was defined as the time from the date of diagnosis to leukemic transformation. P value of <0.05 was considered statistically significant.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Representative IHC staining of cytoplasmic PD-L1 in megakaryocytes from four patients with (A) negative, (B) weak, (C) moderate, and (D) intense reaction, respectively.
Results

Study cohort and clinicopathological features

The clinical characteristics of the patients involved in the study are depicted in Table 1. Five patients were excluded as no megakaryocytes were identified from the BM specimens. PD-L1 was positively stained by IHC in 41 (48%) patients and negatively in 44 patients (52%). Positive IRS ranged from 1 (1/1) to 12 (4/3). Most of the patients were managed conservatively with oral cytoreduction agents, ruxolitinib, and/or transfusion support. Eight patients underwent hematopoietic stem cell transplantation and four had leukemia transformation during follow-up. Median clinical follow-up was 49.6 months (range 1–156 months, 95% CI 39.7–59.5 months). The average age was 59 years (range 21–88 years) and male to female ratio was 1.24. Age, gender, and conventional risk factors did not differ significantly between patients with and without PD-L1 expression except for a higher proportion of PD-L1-expressed patients having WBC > 25 × 10^9/l (Spearman’s rho −0.217, p = 0.046).

There was no difference between the two groups in the risk distribution based on the commonly used prognostic scoring system DIPSS+ (dynamic international prognostic scoring system plus) [24] or GIPSS (genetically inspired prognostic scoring system) [25]. The megakaryocytes in patients with pre-fibrotic PMF less frequently expressed PD-L1 (Spearman’s rho −0.279, p = 0.010).

Analysis of the BM from 2 patients with reactive thrombocytosis and 10 lymphoma patients without marrow involvement, which were used as the control group, showed no PD-L1 staining in megakaryocytes. BM from PV and ET patients showed modest PD-L1 expression (IRS range 1–3). The IRS in the BM from PMF patients was higher compared to that in normal marrows (p = 0.009) but did not differ significantly from other MPN entities and secondary MF (Figure 2).

Comparison of different tissue cores from the same tumor stained with clones SP142 and SP263 in 10 PMF cases showed fair agreement, although stronger staining and higher percentage of PD-L1-positive

Table 1. Comparison of clinicopathological characteristics between patients with and without PD-L1 expression.

| Characters                                | PD-L1 IRS                  | P value |
|-------------------------------------------|----------------------------|---------|
|                                           | Negative (N = 44)          |         |
|                                           | Positive (N = 41)          |         |
| Male (%)                                  | 23 (52)                    | 24 (59) |
| Age (years), median (range)               | 60 (26–88)                 | 61 (28–83) | 0.398 |
| Age >65 years, n (%)                      | 14 (32)                    | 15 (37) |
| Hemoglobin <10 g/dl, n (%)                | 16 (36)                    | 19 (49) |
| Transfusion requiring, n (%)              | 12 (27)                    | 13 (32) |
| Leukocytes, × 10^9/l, median (range)      | 14.2 (3.7–97.9)            | 14.8 (0.7–272.5) | 0.535 |
| Leukocytes, >25 × 10^9/l, n (%)           | 3 (7)                      | 9 (22) |
| Platelets, × 10^9/l, median (range)       | 364 (15–2700)              | 341 (18–1206) | 0.194 |
| Platelets, <100 × 10^9/l, n (%)           | 4 (9)                      | 3 (7) |
| Circulating blasts >1%, n (%)             | 20 (46)                    | 21 (51) |
| Pre-fibrotic myelofibrosis, n (%)         | 9 (21)                     | 1 (2.4) | 0.010 |
| DIPSS+ unfavorable karyotype, n (%)       | 6 (16)                     | 3 (8) |
| GIPSS karyotype risk distribution, n (%)  | 0.335                      |         |
| Very high                                 | 4 (10.5)                   | 1 (3) |
| Unfavorable                               | 4 (10.5)                   | 6 (16) |
| Favorable                                 | 30 (79)                    | 30 (81) |
| DIPSS+ risk distribution, n (%)           | 0.257                      |         |
| High                                      | 6 (14)                     | 6 (15) |
| Intermediate-2                            | 17 (39)                    | 19 (46) |
| Intermediate-1                            | 8 (18)                     | 11 (27) |
| Low                                       | 13 (29)                    | 5 (12) |
| Higher DIPSS+ risk (intermediate-2 and above), n (%) | 23 (52) | 25 (61) | 0.419 |
| GIPSS risk distribution, n (%)            | 0.463                      |         |
| High                                      | 6 (14)                     | 7 (17) |
| Intermediate-2                            | 9 (20.5)                   | 13 (32) |
| Intermediate-1                            | 24 (54.5)                  | 19 (46) |
| Low                                       | 5 (11)                     | 2 (5) |
| Higher GIPSS risk (intermediate-2 and above), n (%) | 15 (34) | 21 (51) | 0.110 |
cells were observed with SP263 (supplementary material, Figure S1).

Molecular features associated with PD-L1 expression in megakaryocytes

The mutational landscape of all patients is summarized in Table 2 and represented graphically in Figure 3. In line with the previous report, PD-L1 expression in our study was positively correlated with JAK2 mutation ($p = 0.011$) but negatively associated with CALR mutation, particularly the non-type I CALR mutations ($p = 0.026$) [8]. The patients with JAK2 mutation also had higher mean IRS (2.1 versus 1.1 in those with other drivers, $p = 0.037$; Figure 4). Patients with at least one HMR mutation as defined by MIPSS70+ [26], which includes mutations of ASXL1, EZH2, IDH1, IDH2, SRSF2, and U2AF1Q157, had higher PD-L1 expression ($p = 0.041$). Furthermore, when JAK2 and HMR mutations were put into a logistic regression model, both parameters were independently predictive of PD-L1 expression (JAK2 mutation $p = 0.013$, odds ratio 3.437, 95% CI 1.298–9.105; HMR mutation $p = 0.043$, odds ratio 2.540, 95% CI 1.030–6.265). The presence of JAK2 and HMR mutations collectively explain 17.9% of high PD-L1 expression ($p = 0.002$). Among the PD-L1-positive

Table 2. Molecular aberrations associated with PD-L1 expression.

| Mutations                  | PD-L1 IRS                  | $P$ value |
|----------------------------|----------------------------|-----------|
|                            | Negative ($N = 44$)     | Positive ($N = 41$) |         |
| Driver mutation, n (%)     |                            |            |
| JAK2                       | 24 (55)                   | 33 (81)    | 0.062    |
| CALR type 1/like           | 8 (18)                    | 4 (10)     | 0.265    |
| CALR non-type 1/like       | 5 (11)                    | 0          | 0.026    |
| MPL                        | 5 (11)                    | 2 (5)      | 0.277    |
| Triple negative            | 2 (5)                     | 2 (5)      | 0.942    |
| JAK2 allele burden, median (range) | 78 (21–96)               | 77 (11–98) | 0.374    |
| HMR mutations, n (%)       |                            |            |
| ASXL1                      | 11 (25)                   | 14 (34)    | 0.355    |
| EZH2                       | 6 (14)                    | 7 (18)     | 0.625    |
| SRSF2                      | 2 (5)                     | 3 (7)      | 0.587    |
| IDH1                       | 0                         | 1 (2)      | 0.297    |
| IDH2                       | 0                         | 1 (2)      | 0.297    |
| U2AF1                      | 2 (5)                     | 3 (7)      | 0.587    |

Figure 2. Comparison of PD-L1 immunoreactive score across (A) healthy BM and those of different MPN entities; Kruskal–Wallis $p = 0.0170$, Dunn’s multiple comparison test for healthy marrow versus PMF $p = 0.009$, and (B) PMF and secondary myelofibrosis.
cases, all four patients with the highest IRS (range 9–12) had JAK2V617F mutations and at least one HMR (Figure 5).

PD-L1 expression confers a worse prognosis

Patients with PD-L1 expression had shorter overall survival than those without expression (Figure 6A) but...
no difference in LFS was observed between these two groups (Figure 6B). With censoring at the time of hematopoietic stem cell transplantation, the median overall survival was not reached versus 52.7 months (95% CI 48–57 months) in PD-L1-negative and -positive groups, respectively. Additionally, a significant difference in HRs between the two groups was identified using the Wald test with Cox-proportional hazards model (HR 0.341, 95% CI 0.135–0.863, \( p = 0.023 \)). Univariable analysis identified PD-L1 expression as an unfavorable prognostic factor for OS (\( p = 0.017 \); supplementary material, Table S2). However, PD-L1 expression was not an independent prognostic factor for survival in multivariable analysis using HMR and either DIPSS+ or GIPSS as covariates (supplementary material, Table S3). Causes of mortality are summarized in supplementary material, Table S4. Eight patients died of infection, 2 died of portal vein thrombosis-related complications, 2 died of hemorrhage, and 12 died of other causes (including acute transformation and hematopoietic stem cell transplantation complication).

**Discussion**

In this study, we demonstrated that PD-L1 expression was significantly associated with overt myelofibrosis, \( JAK2V617F \), high WBC counts, and HMR mutations as defined by MIPSS70+ in PMF patients. Our findings coincide with a recent report in which PD-L1 expression was increased, along with reduced T-cell activation, during disease progression in a PMF patient, as demonstrated by single cell analysis of hematopoietic stem cells [27]. Likewise, PD-L1 expression has been shown to be an independent prognosis marker in several solid cancers [28–30]. Also, researchers found that 71% of MPN patients exhibited a significant immune response against PD-L1 by measuring spontaneous T-cell response to PD-L1-derived epitope, and the response was less frequent and weaker in those with advanced MPN [9]. This finding suggests that dysregulated antitumor immune response of T cells becomes more pronounced with disease progression. Studies also showed that megakaryocytes with \( JAK2V617F \) or \( CALR \) exon 9 mutation in MPN patients could be recognized by specific T cells, but the enhanced level of PD-L1 could induce T-cell anergy thus failing to respond to their specific antigens.

| Patient no. | IRS | JAK2V617F (allele burden) | Other mutations | DIPSS+ risk score | GIPSS risk score |
|------------|-----|--------------------------|----------------|-----------------|----------------|
| 35         | 3x3 | Positive (77%)           | ASXL1, U2AF1   | 3 (int-2)       | 3 (high)       |
| 36         | 3x3 | Positive (75%)           | SRSF2, EZH2, IDH2 | 4 (high)       | 2 (int-2)     |
| 67         | 4x3 | Positive (67%)           | ASXL1, SF3B1   | 3 (int-2)       | 2 (int-2)     |
| 85         | 3x3 | Positive (51%)           | BCORL1, KDM6A, U2AF1 | 2 (int-1)     | 2 (int-2)     |

*Figure 5.* Summary of the four cases with highest IRS in the cohort. All of them were \( JAK2V617F \) positive and carried at least one HMR mutation.

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Remarkably, immune checkpoint blockade was able to reverse the exhausted T-cell response, suggesting a potential role for immunotherapy in these patients [33]. Accordingly, several clinical trials exploring the roles of immune checkpoint blockades in PMF patients are currently ongoing [12].

It is worth noting that, in our study, all four patients with particularly high PD-L1 IRS harbored \( JAK2^{V617F} \) and at least one of the HMR mutations, including \( ASXL1, U2AF1, SRSF2, EZH2, \) and \( IDH2 \). One recent study showed that \( JAK2 \) activation enhanced PD-L1 expression via STAT3 phosphorylation, and PD-L1 expression was higher on primary cells, especially megakaryocytes, isolated from patients with \( JAK2^{V617F} \)-harboring MPN [8]. The mechanism underlying higher expression of PD-L1 in PMF patients harboring HMR mutations is not known. Yet, it is plausible that it is not the HMR \( \text{per se} \), but rather the microenvironment that induces HMR mutation and regulates PD-L1 expression. Further studies are warranted to test the hypothesis.

To our knowledge, this is the first study of PD-L1 expression status in megakaryocytes by IHC staining in PMF. Previous assessment of PD-L1 expression in immune cells and CD34+ cells from MPN patients using flow cytometry [34] and mRNA expression analysis [35] did not reveal significant correlation between PD-L1 and MPN driver gene mutations [35]. However, these studies did not focus on megakaryocytes, which are the key players in myelofibrosis.

Although only membranous, but not cytoplasmic, expression of PD-L1 is considered active expression of the protein [36], most studies of BM PD-L1 IHC staining showed cytoplasmic expression and have not observed membrane expression in megakaryocytes [8,37,38]. SP142 recognizes the epitope in the cytoplasmic tail of PD-L1 and has been shown to better delineate membranous expression of the protein [39] in numerous cell types, but most of the positively stained megakaryocytes observed in this study largely showed cytoplasmic PD-L1 staining. We believe that the heterogeneity of PD-L1 expression pattern is likely influenced by the complex mechanism behind PD-L1 expression in different cells. Additionally, in line with our findings, previous reports have shown lower reactivity of SP142 than SP263 in non-small cell lung cancer [38]. Difference in the cutoff to define positivity among cells and antibodies is important in PD-L1 analysis. Although there is no antibody approved for the assessment of PD-L1 expression in hematopoietic cancers so far, we selected antibody clone SP142 because a study has found closer association of PD-L1 expression shown by this antibody with prognosis in diffuse large B-cell lymphoma [40].
In summary, our pilot study highlighted the distribution of BM PD-L1 staining in PMF patients and its association with molecular aberrations and outcomes. Future work is required to address the application of these results in the clinical setting.

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Author contributions statement

S-HL and C-CL read and interpreted the slides, collected clinical data, performed statistical analysis, and wrote the manuscript. C-HW helped with data collection and cohort maintenance. K-PC and C-TY reviewed all the slides and confirmed the diagnosis of PMF. C-HT was responsible for next-generation sequencing data interpretation. J-HL, H-AH and J-LT jointly interpreted the data. W-CC and H-FT coordinated the study and revised the manuscript.

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**SUPPLEMENTARY MATERIAL ONLINE**

**Figure S1.** IHC staining of BM using PD-L1 antibody clone SP142 or SP263

**Table S1.** The immunoreactive score

**Table S2.** Univariable analysis (Cox regression) of the impact of different variables on overall survival

**Table S3.** Multivariable analysis for overall survival

**Table S4.** Causes of mortality and their associated PD-L1 immunoreactive scores