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BRIEF CONCLUSIVE REPORT

PD-L1 expression in peripheral blood granulocytes at diagnosis as prognostic factor in classical Hodgkin lymphoma

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Abstract

Hodgkin lymphoma (HL) is a neoplastic disease in which the inflammatory microenvironment plays a pivotal role in the tumorigenesis. Neutrophilia is a typical finding in HL at diagnosis and, in particular, in association with lymphocytopenia, is a negative prognostic factor. As the immune checkpoint Programmed Death (PD)-L1/PD-1 has become an important therapeutic target, we were interested in the expression of PD-L1 in peripheral blood (PB) leukocytes using flow cytometry and RT-PCR in patients with HL and healthy controls. Granulocytes were the major PB cell fraction expressing PD-L1. PD-L1 expression on granulocytes was higher in patients with HL than in controls and correlated with lower T-cell numbers in PB. We analyzed for associations between PD-L1 expression in PB granulocytes at the time of diagnosis with patient characteristics and outcome in 126 patients with HL treated with standard chemotherapy adriamycin, bleomycin, vinblastine, and dacarbazine. Increased PD-L1 expression in PB associated with advanced disease, systemic symptoms, positive interim positron emission tomography, and inferior progression-free survival (PFS). PFS at 4 years was 81% (95% C.I., 71–87%) in patients with normal PD-L1 expression and 56% (95% C.I., 35–72%) in patients with higher-than-normal PD-L1 expression ($p = 0.002$). In

conclusion, PD-L1 expression in PB could become a potentially actionable prognostic factor in HL.

KEYWORDS

Hodgkin lymphoma, immune checkpoint, PD-L1, prognosis

Summary Sentence

Expression of PD-L1 in peripheral blood reflects disease burden and predicts interim PET result and prognosis in classical HL.

1 | INTRODUCTION

An abundant inflammatory microenvironment surrounding the neoplastic Hodgkin and Reed/Sternberg (HRS) cells is one of the hallmarks of Hodgkin lymphoma (HL).[1] Disease activity is often accompanied by systemic inflammatory reactions, mediated by cytokines, chemokines, and direct cellular cross-talk. These interactions result in typical alterations in the WBCs in HL, including leukocytosis due to neutrophilia, and lymphocytopenia.[2] These alterations have been included in the international prognostic score (IPS) defined 20 years ago.[3] In more recent years, the ratio between myeloid and lymphoid cells in peripheral blood (PB), in particular the monocyte/lymphocyte and the neutrophil/lymphocyte (N/L) ratio, has been described as prognostic factors in HL.[4–9] Various cut levels have been reported varying from 4.3 to 6.4,[4–7] with the largest study identifying 6 as optimal cut-point.[5]

The alteration of the myeloid/lymphoid cell ratio is an indicator of an immunosuppressive milieu in HL. HRS cells evade antitumor immunity by several mechanisms including activation of the immune checkpoint, in particular the axis between Programmed Death (PD)−1 and its ligand PD-L1.[8,9] PD-L1 is overexpressed on HRS cells of most cases of HL due to the amplification of the genomic region on chromosome 9p24 encoding PD-L1/PD-L2 genes.[10,11] PD-L1 overexpression in HL is not limited to the malignant HRS cells, but is also present on leukocytes in the tumor microenvironment.[12,13] Tumor-associated macrophages can colocalize with the HRS cells, forming "PD-L1 niche"s.[12] Both the intensity of PD-L1 expression on HRS cells and high PD-L1 expression in the microenvironment have been linked to unfavorable outcome following standard therapy in some, but not all studies.[11,13,14]

Neutrophils can express PD-L1 following exposure to proinflammatory cytokines.[15] PD-L1-expressing neutrophils show defective neutrophil function.[16] This expression has been linked to the acquisition of immunosuppressive function suggesting a role for neutrophils as players in the orchestration of the adaptive immune response, in particular inhibition of T cell responses.[15] We hypothesized that granulocytic PD-L1 expression would be associated with signs of a systemic immunosuppressive milieu, as reduced T cell counts in PB. In addition, we were interested in potential associations of PD-L1 expression with patient characteristics and outcome.

2 | MATERIAL AND METHODS

2.1 | Patients and healthy controls

We included 126 patients diagnosed with Hodgkin lymphoma. All patients were treated with the standard chemotherapy protocol adriamycin, bleomycin, vinblastine, and dacarbazine (ABVD) for 3–6 cycles according to stage and risk factors, between June 2010 and February 2019. Treatment response was evaluated using 18-fluorodeoxyglucose (FDG) positron emission tomography (PET) after 2 cycles ABVD. Metabolic activity in the tumor tissue was evaluated according to the 5-point Deauville scoring (DS) system. Interim PET (iPET) was considered positive when FDG uptake was found moderately (DS 4) or markedly (DS 5) increased when compared to the liver. Therapy was intensified with dose-escalated bleomycin, etoposide, adriamycin, cyclophosphamide, oncovin, procarbazine, and prednisone (BEACOPP) regimen in 14 patients for PET positivity.

Controls consisted of 37 healthy volunteers. The study protocol was approved by the institutional review board and conducted in accordance with the principles of the Declaration of Helsinki. All patients provided written informed consent.

2.2 | Identification and isolation of PB leucocyte populations

All blood samples were collected at the time of diagnosis and analyzed in the Laboratory of Hematology at our institution with automatic blood cell analyzers for routine CBC. Leukocyte populations were prepared from EDTA-anticoagulated fresh blood samples. Buffy-coats were used to extract RNA from total leukocyte populations. Leukocyte population were separated by Ficoll density centrifugation. The high-density fraction containing >95% neutrophils was treated with a hypotonic erythrocyte lysis buffer. T-cells were prepared from the low-density fraction using the Pan T Cell Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions obtaining a purity of >95%.

2.3 | Flow cytometry

Fresh whole blood samples were incubated with fluorescence-labeled antibodies for 30 min at room temperature and washed after red cell lysis. Stained cells were acquired on a BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed using the BD FACSDiva 8.0 software. Seven-color antibody panels were used to assess expression of PD-1 and PD-L1 on leukocyte populations including BV421-conjugated PD-1 (CD279, clone EH12.1; Becton Dickinson) and PC7-conjugated PD-L1 (clone PD-L1; Beckman Coulter). A minimum of 100,000 events per tube were recorded. Cytometer setup was daily optimized by BD FACSDiva CST (Becton Dickinson).

2.4 | RT-PCR

RNA was extracted using Trizol reagent (EuroGOLD TriFast; Euro-Clone, Pero, Italy). Following reverse transcription, expression levels PD-L1 mRNA were analyzed using a semi-quantitative PCR assay (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA), using β-actin as reference gene. Primers for PD-L1 were designed using primer designing tool-NCBI-NIH: forward primer, 5′-AAT GTG ACC AGC ACA CTG AGA A-3′; reverse primer 5′-CCT TTC ATT TGG AGG ATG TGC C-3′. A melting curve (62–95°C) was generated at the end of each run to verify specificity of the reactions. Expression was defined as 2–ΔCt, where ΔCt is Ct (test gene) − Ct (reference gene).

2.5 | Statistical analysis

The Wilcoxon signed rank test was used for 2-sample comparisons according to dichotomized patient characteristics, whereas the Kruskal–Wallis test was used for more than 2 group comparisons. The Student's t-test was used for comparisons of expression levels between cell fractions.

Multivariate logistic regression analysis was used to identify patient characteristics independently associated with iPET results after 2 cycles.

The primary end-point was progression-free survival (PFS), with progression during treatment, lack of complete remission at the end of first-line treatment, relapse and death from any cause counted as events. Planned change of therapy due to interim PET-positivity in the absence of progression in 14 patients was not counted as event. Subsequent change due to persisting disease activity to salvage therapy and consolidation with autologous stem cell transplantation was counted as event (8 of 14 patients). Survival curves were estimated using the Kaplan–Meier product limit method. Log-rank tests were used to analyze for differences in PFS. Cox regression was used for multivariate analysis of PFS. Backward and forward selection of variables was performed to obtain the best model. We set the p value at 0.1 to maintain the parameter in the model. A p value < 0.05 was considered statistically significant. Computations were performed using the Stata 10.0 software (Stata Corp., College Station, TX).
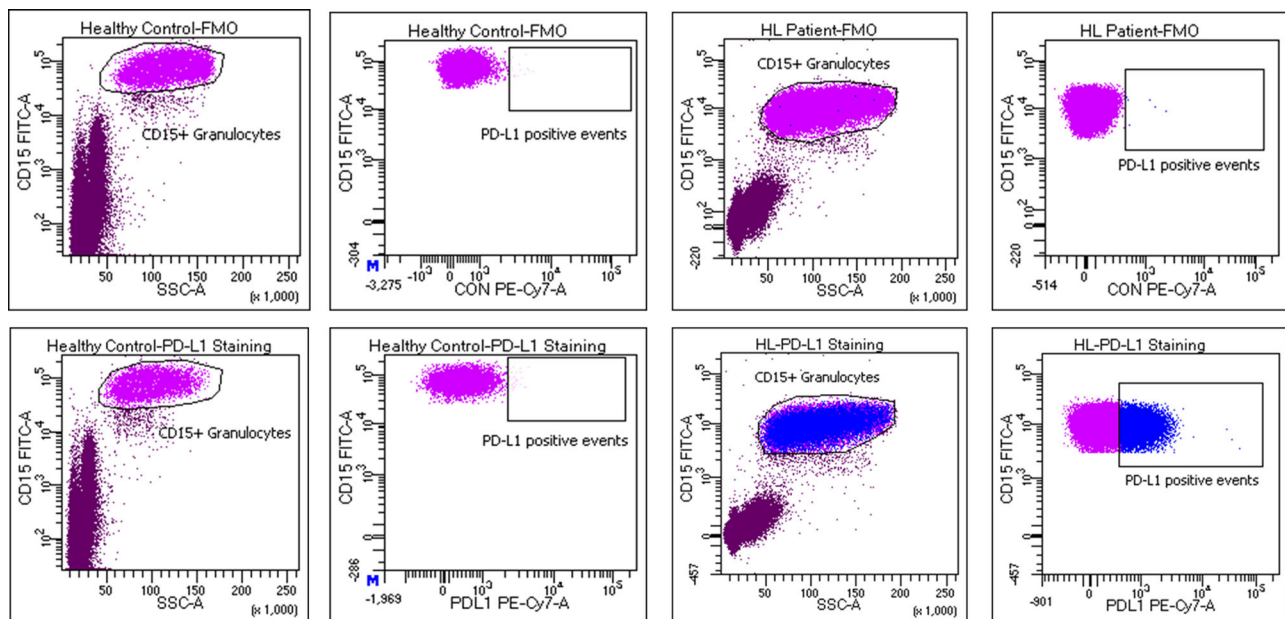

FIGURE 1 **PD-L1 expression in peripheral blood CD15+ granulocytes assessed by flow cytometry**. Example of PD-L1 expression on CD15+ granulocytes from PB of a patient with HL (right panels) and in a control subject (left panels). Granulocytes in whole blood samples were identified by staining with CD15 antibodies and side scatter (SSC) characteristics. PD-L1 expression was assessed using PC7-conjugated PD-L1 (clone PD-L1; Beckman Coulter). The upper panels show the FMO (fluorescence minus one) control tube for a control subject (upper left panels) and a patient with HL (upper right patient). A minimum of 100,000 events per tube were recorded. The proportion of PD-L1+/CD15+ granulocytes was 0.2% in the healthy control, and 34% in the HL patient
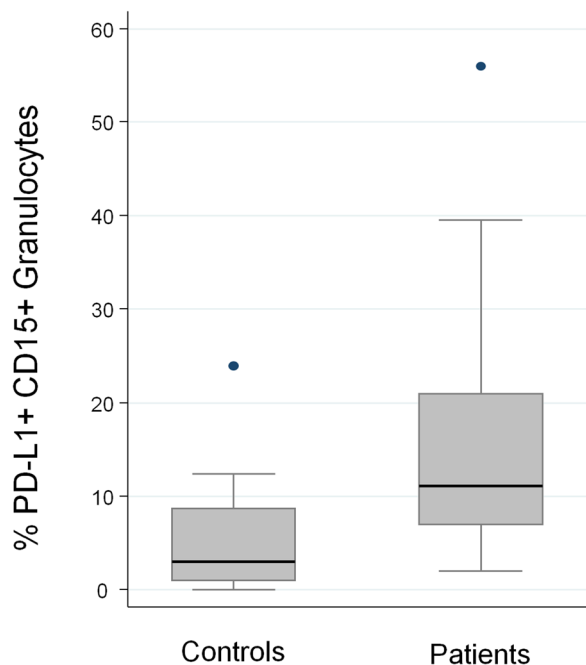
FIGURE 2 PD-L1 expression in peripheral blood CD15+ granulocytes assessed by flow cytometry compared with healthy individuals. PD-L1 expression on PB neutrophils in 9 control subjects and 25 patients with HL. Results of flow cytometry are summarized and shown as percentage of positive CD15+ granulocytes. The upper border of the box indicates the upper quartile (75th percentile), whereas the lower border indicates the lower quartile (25th percentile), and the horizontal line in the box the median. The vertical lines are the whiskers indicating the upper and lower adjacent values. The difference in the proportion of PD-L1-positive granulocytes was significant ($p = 0.01$)

3 | RESULTS AND DISCUSSION

We first examined cell surface PD-L1 expression using flow cytometry on granulocytes from PB of 9 healthy subjects and 25 patients with HL at diagnosis. An exemplary flow cytometry plot is shown in Figure 1. PD-L1 was expressed on 11.1% (median, range 2.0–59%) of granulocytes from HL, whereas it was expressed on 3.0% of control granulocytes (median, range 0–24%) ($p = 0.01$) (Figure 2). The significant increase in the proportion of PD-L1-positive neutrophils in patients became even more evident when analyzed in absolute numbers. Patients had a median number of circulating PD-L1+ neutrophils of 873×10^6/L versus 53×10^6/L in controls.

We extended the study of PD-L1 expression to various PB cell fractions in 20 patients. The median number of circulating PD-L1+ monocytes was 63×10^6/L in patients with HL versus 12×10^6/L in controls (Figure 3). A slight increase in the relative proportion of PD-L1-expressing cells in the lymphocyte fraction did not translate into a difference in the absolute number of PD-L1+ T cells and B cells in PB (Figure 3). This analysis shows that the major fraction of PD-L1-expressing cells in PB of patients with HL are CD15+ granulocytes.

As we were interested in a potential axis between PD-L1 expression on neutrophils and T cells, we next examined the ratio between these

TABLE 1 Association between PD-L1 expression and patient characteristics in 126 patients with HL treated with ABVD

Parameter	Variable	Cases (n = 126)	PD-L1 low (n, %)	PD-L1 high (n, %)	p
Age	≤45 aa	80	67 (84%)	13 (16%)	0.05
	>45 aa	46	31 (67%)	15 (33%)	
Gender	Female	67	52 (78%)	15 (22%)	1.0
	Male	59	46 (78%)	13 (22%)	
Stage	Early	50	45 (90%)	5 (10%)	**0.008**
	Advanced	76	53 (70%)	23 (30%)	
B Symptoms	No	73	63 (86%)	10 (14%)	**0.009**
	Yes	53	35 (66%)	18 (34%)	
IPS	IPS 0–2	90	75 (83%)	15 (17%)	**0.03**
	IPS 3–7	36	23 (64%)	13 (36%)	
N/L ratio	<6	86	70 (81%)	16 (19%)	0.17
	>6	40	28 (70%)	12 (30%)	
iPET	5pDS 1–3	90	77 (86%)	13 (14%)	**0.001**
	5pDS 4–5	29	15 (52%)	14 (48%)	

IPS, international prognostic score; iPET, interim positron emission tomography; N/L ratio, neutrophil/lymphocyte ratio. 5pDS, 5-point Deauville score. Significant p values are shown in bold.

parameters. We observed a negative correlation between the proportion of PD-L1 expression on granulocytes and the number of T cells in PB (rho of Spearman's rank correlation = -0.51, $p = 0.01$) (Figure 4).

We then analyzed for associations between PD-L1 expression in PB and clinical characteristics and prognosis in a retrospective study on a cohort of 126 patients with HL treated with ABVD. For this part of our study, we used RT-PCR analysis of stored buffy coat PB samples. We therefore first validated PD-L1 expression using RT-PCR with flow cytometry and observed a strong correlation between these 2 methods in 25 samples (rho of Spearman's rank correlation = 0.73, $p < 0.001$) (Figure S1). Using RT-PCR on different PB sample fractions, we confirmed that PD-L1 was mainly expressed in the granulocyte fraction (Figure 5).

Using the 95% percentile of PD-L1 expression in the fraction of 37 controls, we defined the cut-off between low and high expression and analyzed for associations between PD-L1 expression levels and patient characteristics in a cohort of 126 patients (Table 1). Twenty-eight patients (22%) were defined as *high expressors*. There was a significant association between high PD-L1 expression levels and advanced stage, presence of B symptoms, high IPS, and the interim PET response, with a particular high fraction of PD-L1-positive patients being PET positive after 2 cycles of ABVD (Table 1).

The association of high PD-L1 expression with poor clinical prognostic factors translated into an association between high PD-L1 levels and inferior PFS. PFS was defined as the time from diagnosis to disease progression during treatment, lack of complete remission at the end of first-line treatment, relapse, and death from any cause. Planned change of therapy from ABVD to BEACOPP due to interim PET-positivity in the absence of progression in 14 patients was not counted as event. Subsequent change due to persisting disease activity to salvage therapy and consolidation with autologous stem cell transplantation
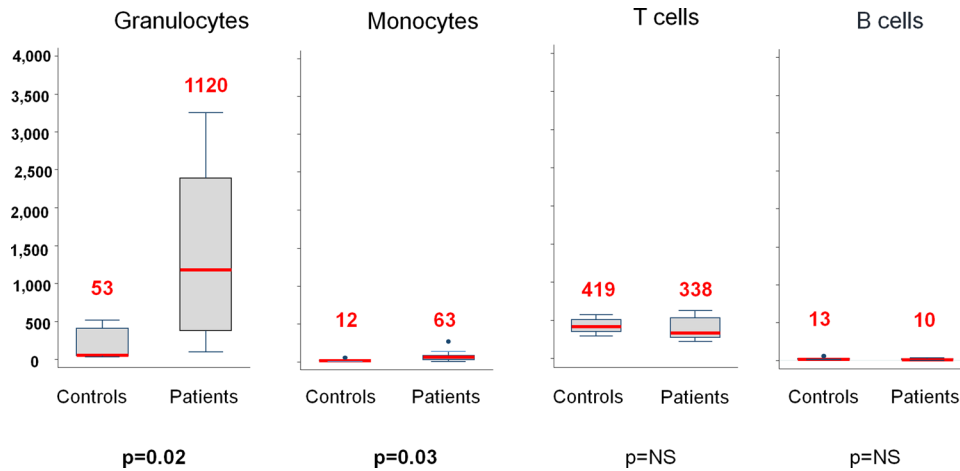
FIGURE 3 PD-L1 expression on various peripheral blood fractions assessed by flow cytometry. Results of expression analysis in 5 control subjects and 20 patients with HL are summarized and shown as numbers of positive cells ($\times10^6$/L), with the median number of positive cells indicated by the numbers in red. The difference in the number of PD-L1-positive granulocytes and monocytes was significant

was counted as event (8 of 14 patients). At a median observation of 48 months PFS was 81% (95% C.I., 71−87%) in patients with PD-L1 expression in the normal range and 56% (95% C.I., 35−72%) in patients with higher than normal PD-L1 expression ($p = 0.002$) (Figure 6).

In addition to PD-L1 expression, several other patient characteristics that are available at diagnosis proved to be of prognostic significance in univariate analysis in our cohort of ABVD-treated patients, such as N/L ratio greater than 6, advanced stage (IIB-IV), and presence of B-symptoms (Table 2). Including these significant parameters into a multivariate analysis, only high PD-L1 expression maintained its prognostic significance (HR, 2.2; 95% C.I., 1.04−4.5; $p = 0.004$) (Table 2).

Interim PET performed after 2 cycles ABVD and evaluated according to the 5-point DS is the strongest outcome predictor in HL.[17] Data on interim PET were available for 119 patients and confirmed its role as strong outcome predictor ($p < 0.0001$, data not shown). Inclusion of interim PET into the multivariable analysis abrogated the role of all other prognostic factors available at diagnosis (data not shown).

However, using a multivariate logistic regression analysis to identify factors available at diagnosis predictive for the interim PET result, we found that high PD-L1 expression levels were the only independent risk factor for PET positivity after 2 cycles ABVD (HR 4.3; 95% C.I. 1.6−11.57, $p = 0.004$) (Table 2).

To our knowledge, this is the first report on PD-L1 expression in PB at HL diagnosis. We identified granulocytes as the major PD-L1-expressing cell population in PB. This could further support a potential important functional role for these myeloid cells in the peculiar inflammatory state of HL. High PD-L1 expression on granulocytes may be an indicator of the immunosuppressive capacity of these cells and an indicator of a general immunosuppressed state in advanced HL at diagnosis. Neutrophils are a functionally heterogeneous population. In cancer and other chronic conditions, stimulation of neutrophils with weak signals induces a pathologic state of activation with immature phenotype and morphology, a relatively weak phagocytic activity and production of reactive oxygen species, NO, and arginase.[18] A
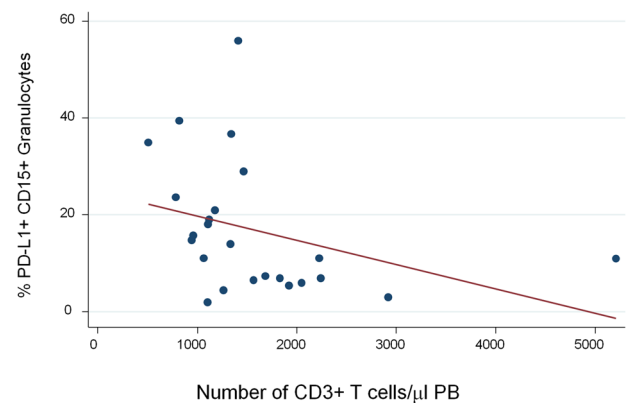

FIGURE 4 Correlation between proportion of PD-L1-expressing CD15+ granulocytes and number of circulating CD3+ T cells in 24 patients with HL. Rho of Spearman rank correlation for PD-L1 on CD15+ granulocytes and T cells is −0.51 ($p = 0.01$)

distinctive feature of the latter cell population is their capacity to suppress the adaptive immune response and to contribute to cancer progression. These cells are now collectively called myeloid-derived suppressor cells (MDSC). Our finding of increased PD-L1 expression on granulocytes at HL diagnosis raises the possibility that these cells have features of MDSC. An expansion of mature neutrophils with immunosuppressive characteristics has been described in patients with lymphoma.[19] Romano et al.[20,21] found increased levels of MDSC that was accompanied by increased levels of arginase in patients with HL.

The association between PD-L1 expression on granulocytes and a reduced number of circulating T cells supports the hypothesis that these cells may indeed have immunosuppressive functions. In this line, Wang et al.[22] showed that tumor-infiltrating neutrophils in gastric cancer express PD-L1 and effectively suppress T cells in vitro. The multiple interactions of tumor-associated neutrophils in the tumor microenvironment have become a subject of increasing interest in the last decade; however, data on the role of neutrophils in the HL tissue are scarce.[23,24]
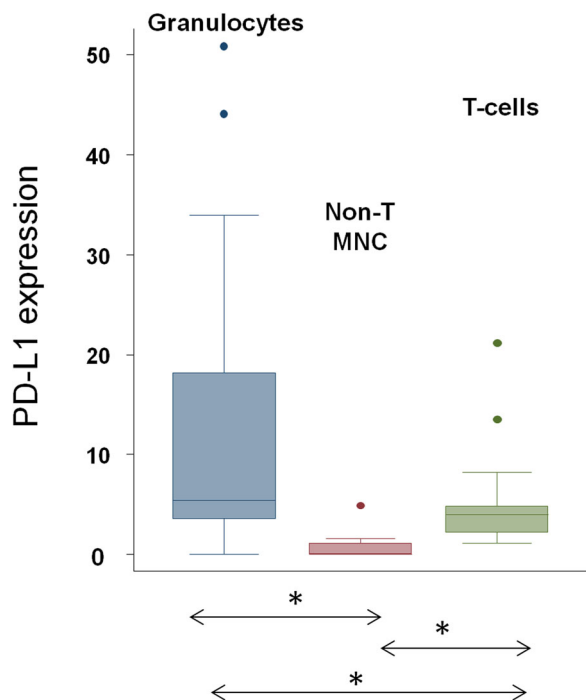
FIGURE 5 **PD-L1 expression in peripheral blood neutrophils assessed by RT-PCR**. For PD-L1 expression in peripheral blood neutrophils, T cells, and non-T cell mononuclear cells (MNC), 17 patients with HL were assessed by RT-PCR. The relative expression was 13.4 ± 3.8 in neutrophils, 0.7 ± 0.3 in mononuclear non-T cells, and 5.3 ± 1.2 in T cells (mean ± SEM). * indicates significant *p* values of paired *t*-test for comparison between cell fractions

FIGURE 6 **PD-L1 expression and its association with PFS**. Progression-free survival according to PD-L1 expression in peripheral blood in 126 patients with HL treated with ABVD. The estimated 4-year PFS was 82% (95% C.I. 71–87%) in 98 patients with PD-L1 expression in the normal range (19 events) and 56% (95% C.I., 35−72%) in 28 patients with high PD-L1 expression (13 events). *p* indicates significance level of log-rank test

PD-L1 expression was not independent from iPET to predict prognosis. This is similar to recent data on the prognostic value of NLR that was lost when iPET response was included as an outcome parameter.[25]

PD-L1 was the only parameter available at diagnosis to predict the iPET result. This could assume particular interest in identifying patients at diagnosis in need for treatment strategies alternative to standard ABVD. Antibodies targeting the PD-1 pathway showed encouraging results in clinical studies including heavily pretreated patients.[26]
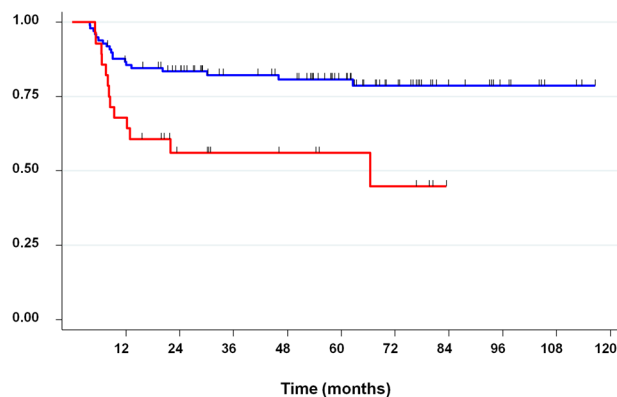
Addition of checkpoint inhibitors to first-line therapy is already subject of clinical trials. PD-L1 expression could be explored as a useful PB biomarker for therapeutic interventions targeting the immune checkpoint.

There are some limitations to our study. Due to the retrospective nature of the study, we used RT-PCR for expression analysis. Flow cytometry analysis could be a simpler and direct method for expression analysis in prospective studies. We used CD15 as marker to identify neutrophilic granulocytes. CD15 is expressed also on other granulocytic cells, as eosinophils.[27] However, PD-L1-expressing CD15+ granulocyte counts were 8 times higher than the patient's respective eosinophil counts, indicating that neutrophils were the major fraction of PD-L1-expressing granulocytes. PD-L1 expression could be altered by concomitant infections or systemic inflammations due to other causes that are known to up-regulate PD-L1 expression.[28] Patients in our cohort appeared not to have concomitant active infections or inflammatory diseases in addition to HL at diagnosis.

TABLE 2 Patient characteristics available at diagnosis as predictors of progression-free survival and interim PET in 126 patients treated with ABVD

Prediction of progression free survival		Prediction of interim PET								
Parameter		Univariate analysis				Multivariate analysis			Logistic regression	
		HR	95% C.I.	*p*		HR	95% C.I.	*p*	HR	95%C.I.	*p*
PD-L1	High vs Low	2.9	1.4–5.9	**0.003**		2.2	1.04–4.5	**0.004**	4.3	1.6–11.5	**0.004**
N/L ratio	>6 vs. ≤6	2.3	1.2–4.7	**0.02**		2.0	0.98–4.0	0.06	1.1	0.4–2.8	0.9
Stage	Adv. vs. early	2.7	1.2–6.3	**0.02**		1.7	0.55–4.4	0.2	2.8	0.9–8.9	0.08
B Symp.	Yes vs. no	2.9	1.4–5.9	**0.004**		1.8	0.79–4.0	0.1	1.3	0.5–3.6	0.6
IPS	3–7 vs. 0–2	1.9	0.9–3.9	0.07							

IPS, international prognostic score; N/L ratio, neutrophil/lymphocyte ratio. HR, hazard ratio; C.I. confidence interval; Adv., advanced; Symp., symptoms. Significant *p* values are shown in bold.

In conclusion, increased PD-L1 expression may indicate an activation state of neutrophils with potentially immunosuppressive function, that has to be confirmed by functional studies, in HL. The identification of this actionable prognostic marker at diagnosis might help to develop personalized therapeutic strategies.

AUTHORSHIP

S. H. and A. C. designed the research and wrote the paper. I. Z., E. C., F. F, M. V., F. C., and S. B. performed the experiments; L. M. L. and M. M. analyzed the histology specimens; S. A., V. R., and A. G. analyzed the PET data; E. G. and F. D. collected and analyzed the clinical data; V. D. S. contributed to writing. All authors approved the manuscript.

DISCLOSURE

The authors declare no competing financial interests.

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