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RESEARCH ARTICLE

Polymorphisms and overexpression of immune checkpoints PD-1, PD-L1, and CTLA-4 in DLBCL: biomarker insights from a case–control study

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Abstract

Background Diffuse large B-cell lymphoma (DLBCL) is a biologically heterogeneous lymphoma with variable treatment outcomes. Immune checkpoints are key regulators of tumor-immune interactions and important targets in cancer therapy. This study investigates the roles of *PD-1*, *PD-L1*, and *CTLA-4* gene polymorphisms, along with their mRNA and protein expression, in DLBCL pathogenesis.

Methods This case–control study included 20 patients with newly diagnosed DLBCL and 20 age- and sex-matched healthy controls. SNPs in *PD-1* (rs2227981), *PD-L1* (rs4143815), and *CTLA-4* (rs231775) were genotyped. Gene expression was assessed via RT-qPCR, and protein levels on immune cells were analyzed by flow cytometry.

Results *PD-1*, *PD-L1*, and *CTLA-4* mRNA levels were significantly elevated in DLBCL patients ($p=0.018$, $p=0.006$, $p=0.031$, respectively). These SNPs were associated with expression changes (PD-1: $p=0.009$; PD-L1: $p=0.007$; CTLA-4: $p=0.018$). Flow cytometry showed elevated percentages of $CD4^+PD\text{-}1^+$ ($p=0.010$) and $CD4^+PD\text{-}L1^+$ ($p=0.002$) cells in patients. No significant clinical differences were found among DLBCL subtypes.

Conclusion These results suggest that overexpression of these checkpoints and related genetic variants may contribute to immune escape and disease progression in DLBCL. PD-1, PD-L1, and CTLA-4 may serve as potential biomarkers for prognosis and personalized therapy. Further large-scale studies are needed to confirm these findings.

Keywords Diffuse large B-Cell lymphoma · PD-1 · PD-L1 · CTLA-4 · Gene expression

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive subtype of non-Hodgkin lymphoma (NHL) in adults, accounting for approximately 30–40% of newly diagnosed NHL cases [1, 2]. Although the exact etiology of DLBCL remains unclear, it has been associated with infectious, environmental, and genetic/epigenetic factors. Based on gene expression profiles, DLBCL is classified into molecular subtypes, primarily germinal center B-cell-like

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(GCB) and activated B-cell-like (ABC) [1]. Prognosis is most commonly assessed using the International Prognostic Index (IPI) and its modified versions. The standard treatment involves a CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) in combination with rituximab, and disease staging is performed using the Lugano modification of the Ann Arbor system [1]. DLBCL pathogenesis is driven by the accumulation of genetic and epigenetic alterations affecting proto-oncogenes, tumor suppressor genes, and immune evasion pathways. Loss of major histocompatibility complex (MHC) class I molecules has been reported in approximately 60% of cases [3].

Programmed cell death protein 1 (PD-1, encoded by *PDCD1*) is an immune checkpoint receptor encoded on chromosome 2q37.3 and is expressed on activated T cells, B cells, natural killer (NK) cells, and monocytes [4]. PD-1 exerts its function through binding to its ligands, PD-L1 (Programmed Death-Ligand 1, CD274) and PD-L2 (Programmed Death-Ligand 2, CD273), which are encoded on chromosome 9p24.1. These ligands, expressed on antigen-presenting and tumor cells, interact with PD-1 to suppress effector T-cell activation and enhance regulatory T-cell (Treg) function, thereby attenuating immune responses. While the PD-1/PD-L1 axis plays a physiological role in maintaining immune homeostasis, tumor cells exploit this pathway as a mechanism of immune evasion. Several studies have demonstrated that PD-1 and PD-L1 expression levels vary significantly across cancer types and tumor subtypes [5, 6]. Moreover, the prognostic significance of PD-1 and PD-L1 expression in DLBCL has been supported by multiple investigations [7, 8].

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is another key immune checkpoint molecule, encoded by a gene located on chromosome 2q33.2. Unlike PD-1, CTLA-4 is primarily expressed on activated T and B lymphocytes, where it modulates immune responses through interactions with its ligands CD80 and CD86. The expression levels of CTLA-4 and CD80 play a critical role in regulating immune activity and have been shown to contribute to immune evasion mechanisms in various tumor types. These molecules have been associated with multiple cancers as well as autoimmune diseases, and are reported to play dual roles in both immune regulation and cancer development [9–11].

Polymorphisms in immune checkpoint genes such as *PD-1*, *PD-L1*, and *CTLA-4* may influence susceptibility to various cancers, including diffuse large B-cell lymphoma (DLBCL). For instance, the *PD-1.5* polymorphism (rs2227981, +7785C > T) has been associated with multiple malignancies, including non-small cell lung cancer, cervical cancer, and breast cancer [12, 13]. Similarly, the PD-L1 polymorphism (rs4143815, −14C > G), located in the 3′ untranslated region (UTR) of the gene, has been shown to enhance *PD-L1* expression by altering miR-570 binding affinity, thereby suppressing anti-tumor-immune responses and increasing cancer risk across different tumor types [12, 14]. The *CTLA-4* polymorphism (rs231775, +49A > G) has been linked to both cancer and autoimmune diseases by altering the protein's functional properties [15, 16]. In contrast, the *CD80* polymorphism (rs2228017, +452G > A), which represents a synonymous variant in exon 3 affecting a valine codon, has not been consistently associated with cancer in previous studies [17]. Collectively, these genetic variations may influence the regulation and expression of their respective genes, particularly in the context of *PD-1*, *PD-L1*, and *CTLA-4*, and could play critical roles in modulating immune evasion mechanisms and disease progression.

To date, no study in Turkey has comprehensively evaluated the genetic polymorphisms, mRNA expression, and surface protein levels of *PD-1*, *PD-L1*, *CTLA-4*, and *CD80* in newly diagnosed DLBCL patients within the same cohort. This study aims to investigate the associations between these immune checkpoint molecules and DLBCL pathogenesis and prognosis. By linking genetic variants with expression profiles, we seek to identify potential biomarkers that may improve patient stratification and guide future targeted therapies.

Materials and methods

Study population

Between January 2019 and December 2020, a total of 20 patients (10 males and 10 females) with histologically or cytologically confirmed diffuse large B-cell lymphoma (DLBCL) were prospectively enrolled at [Akdeniz University Hospital]. An age- and sex-matched control group comprising 20 healthy volunteers with no history of malignancy or severe systemic illness was also included. Healthy status was confirmed via routine clinical evaluations and laboratory screening. Ethical approval was obtained from the Ethics Committee, Medical Faculty of Akdeniz University (Protocol No: 70904504/11), and all procedures were conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent prior to enrollment. Peripheral blood samples (≥5 mL) were collected from each individual using both EDTA and heparin-coated Vacutainer tubes; EDTA tubes were used for DNA extraction and genotyping, while heparin tubes were used for flow cytometry.

Staging was performed according to the Ann Arbor classification system using 18F-fluorodeoxyglucose positron emission tomography-computed tomography (FDG-PET-CT). Prognostic assessment was conducted based on the Rituximab-International Prognostic Index (R-IPI). Clinical data, including treatment response and survival status, were obtained through electronic medical records and

direct patient interviews. Survival time was defined as the interval between initial diagnosis and either death or the last follow-up date. Inclusion criteria comprised patients aged > 18 years with newly diagnosed, untreated DLBCL confirmed by histopathology. Exclusion criteria included any prior history of malignancy, organ transplantation, autoimmune or chronic immunosuppressive conditions.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the EZ1 Advanced XL system (Qiagen, Hilden, Germany). DNA purity and concentration were measured with a DS-11 spectrophotometer (DeNovix), and samples were stored at −20 °C until use. Genotyping of *PD-1* (rs2227981), *PD-L1* (rs4143815), *CTLA-4* (rs231775), and *CD80* (rs2228017) polymorphisms was performed via PCR, followed by capillary electrophoresis using a 3500 DNA Analyzer (Applied Biosystems). SNP identification was based on reference sequences from NCBI dbSNP and Ensembl databases.

qRT-PCR

Expression levels of *PD-1*, *PD-L1*, *CTLA-4*, and *CD80* were quantified using real-time reverse transcription PCR (RT-qPCR). Total RNA was isolated from peripheral blood using the NucleoSpin RNA Blood Midi kit (Macherey–Nagel, Germany), and reverse transcription was performed with the AccuPower RT PreMix kit (Bioneer). Complementary DNA (cDNA) was amplified using gene-specific primers, and reactions were run in triplicate.

Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as the endogenous control.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation. Flow cytometry was performed to assess *PD-1*, *PD-L1*, and *CTLA-4* expression in T lymphocytes. Cells were stained with fluorescently labeled monoclonal antibodies specific for C*D3*, *CD4*, *CD8*, *PD-1*, *PD-L1*, and *CTLA-4*. Data were acquired on an 8-color FACSCanto II flow cytometer and analyzed with FACSDiva software (BD Biosciences, USA). Sample preparation, staining procedures, data acquisition, and T-cell identification were conducted as previously described [18]. Helper T lymphocytes ($CD3^+CD4^+$) and cytotoxic T lymphocytes ($CD3^+CD8^+$) were assessed for *PD-1*, *PD-L1*, and *CTLA-4* expression. Marker-positive cell frequencies were calculated relative to isotype control staining. A detailed stepwise gating strategy was implemented to ensure accurate identification of lymphocyte subsets. Sequential gating was performed to exclude debris and doublets, define $CD3^+$ T cells, and subsequently separate $CD4^+$ helper and $CD8^+$ cytotoxic T cells before assessing PD-1, PD-L1, and CTLA-4 expression. Percentages for each gated population were indicated on the respective plots to facilitate data interpretation and reproducibility. Further technical details, including antibody clones, fluorochromes, and primer sequences, are provided in the Supplementary Material (Supplementary Tables S.1–S.2).

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 22.0 and R version 3.3.2 (IBM Corp., Armonk, NY, USA). The Shapiro–Wilk test assessed normality. Non-normally distributed variables were analyzed using the Mann–Whitney U test or Kruskal–Wallis H test, with Dunn's post hoc test for pairwise comparisons. Normally distributed variables were evaluated using independent samples t-tests or one-way ANOVA, as appropriate. The assumption of homogeneity of variances was verified using Levene's test. All tests were two-tailed. Categorical variables were compared using the Chi-square or Fisher–Freeman–Halton exact test. Results are presented as mean ± standard deviation (SD), median (interquartile range, Q1–Q3), or counts and percentages (*n*, %) where applicable. A p-value < 0.05 was considered statistically significant. Given the exploratory nature of the study and the limited sample size, no formal correction for multiple comparisons (such as Bonferroni or false discovery rate adjustment) was applied. However, effect sizes and 95% confidence intervals were reported to support the interpretation of p-value trends and to provide context for the magnitude of observed differences.

Results

Patient demographics and clinical data

The study consisted of 9 patients with the germinal center B-cell-like (GCB) subtype and 11 with the non-GCB subtype of DLBCL. There were no statistically significant differences between the two subtypes in terms of clinical or demographic characteristics, baseline laboratory values at diagnosis, or prognostic indicators ($p > 0.05$; Supplementary Tables S.3–S.5).

Polymorphism analysis

The distribution of *PD-1* (rs2227981), *PD-L1* (rs4143815), *CTLA-4* (rs231775), and *CD80* (rs2228017) genotypes did not significantly differ between the patient and control

groups or between DLBCL subtypes ($p > 0.05$; Table 1, Supplementary Table S.6). Odds ratio (OR) analyses under the dominant genetic model (combined heterozygote and homozygote variant genotypes vs. wild-type) similarly revealed no significant associations between these polymorphisms and DLBCL susceptibility. Representative Sanger sequencing chromatograms for these polymorphisms are presented in Supplementary Fig. 1.

Gene expression

Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ (Livak) method, with GAPDH as the endogenous control. Compared with healthy controls, DLBCL patients showed significantly higher expression of *PD-1* (1.62-fold, $p = 0.001$), *PD-L1* (3.67-fold, $p = 0.001$), and *CTLA-4* (1.48-fold, $p = 0.001$), whereas *CD80* expression was slightly lower but not statistically significant ($p > 0.05$) (Table 2). Summary statistics of cycle threshold (Ct) values (n, mean ± SD) for *PD-1*, *PD-L1*, *CTLA-4*, and *CD80* are provided in Supplementary Table S8.

These results are summarized in Table 2 and graphically presented in Fig. 1. Figure 1 illustrates the mean ± SD relative expression values, highlighting the increased *PD-1*, *PD-L1*, and *CTLA-4* expression in patients, whereas *CD80* shows no significant difference.

Table 2 Relative mRNA expression of immune checkpoint genes in DLBCL patients and controls

Gene	Relative expression ($2^{-\Delta\Delta Ct}$, mean ± SD)	95% CI	p-value
PD-1	1.62 ± 0.25	1.35–1.94	0.001
PD-L1	3.67 ± 0.41	3.10–4.25	0.001
CTLA-4	1.48 ± 0.20	1.25–1.76	0.001
CD80	0.92 ± 0.18	0.70–1.21	0.110

Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the reference gene. Data are shown as mean ± SD and 95% CI. Comparisons between patients and controls were performed using the Mann–Whitney U test ($\alpha = 0.05$)

Genetic polymorphisms and their impact on gene expression

In DLBCL patients, the *PD-1* (rs2227981) C/T genotype was significantly associated with increased *PD-1* expression compared to controls ($p = 0.012$). Similarly, the *PD-L1* (rs4143815) C/G and G/G genotypes were associated with significantly elevated *PD-L1* expression levels ($p = 0.007$ and $p = 0.003$, respectively). For *CTLA-4* (rs231775), the G/A genotype was linked to significantly higher expression in the patient group ($p = 0.006$). In contrast, no significant differences in *CD80* expression were observed between genotypes in patients and controls ($p > 0.05$).

Table 1 Distribution of PD-1, PD-L1, CTLA-4, and CD80 genotypes in patient and control groups

Gene/SNP	Genotype	Patients n (%)	Controls n (%)	OR (95% CI)	p value	HWE p (controls)
PD-1 rs2227981	TT	5 (25.0)	6 (30.0)	Ref	–	0.016
	CT	15 (75.0)	14 (70.0)	–	–	–
	CT + TT vs TT	–	–	1.26 (0.33–4.84)	0.723	–
PD-L1 rs4143815	GG	7 (35.0)	8 (40.0)	Ref	–	0.007
	CG	10 (50.0)	4 (20.0)	–	–	–
	CC	3 (15.0)	8 (40.0)	–	–	–
	CG + GG vs CC	–	–	3.40 (0.80–14.36)	0.086	–
CTLA-4 rs231775	GG	2 (10.0)	4 (20.0)	Ref	–	0.964
	GA	13 (65.0)	10 (50.0)	–	–	–
	AA	5 (25.0)	6 (30.0)	–	–	–
	GA + AA vs GG	–	–	2.02 (0.38–10.86)	0.563	–
CD80 rs2228017	GG	16 (80.0)	16 (80.0)	Ref	–	0.619
	GA	4 (20.0)	4 (20.0)	–	–	–
	GA vs GG	–	–	1.00 (0.23–4.37)	1.000	–

HWE Hardy–Weinberg equilibrium; tested in controls using the χ^2 goodness-of-fit test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated under the dominant genetic model (combined heterozygote and homozygote variant genotypes vs. wild-type). Genotype and allele frequencies were compared between patients and controls using the χ^2 or Fisher's exact test, as appropriate. Deviations from HWE were considered nonsignificant at $p > 0.05$

Ref reference genotype, n number of subjects

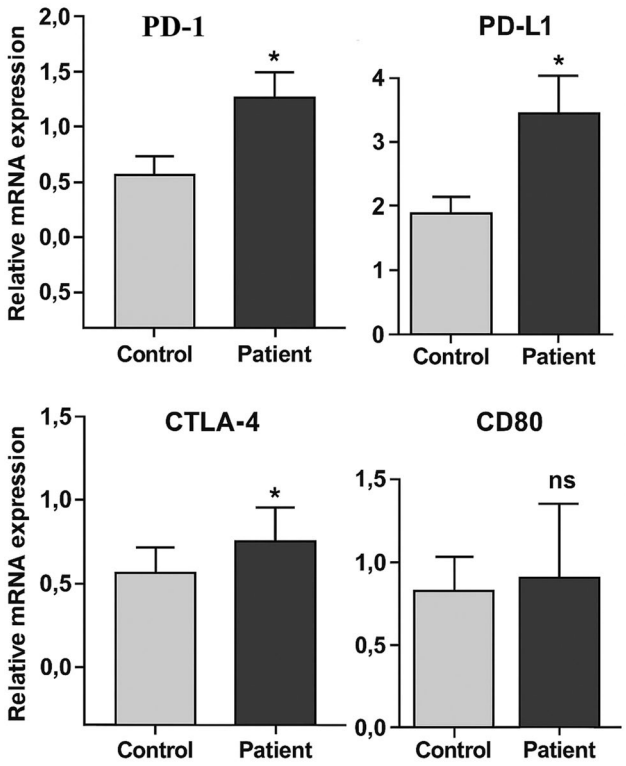

Fig. 1 Relative mRNA expression ($2^{\wedge-\Delta\Delta Ct}$) levels of *PD-1*, *PD-L1*, *CTLA-4*, and *CD80* in DLBCL patients and healthy controls. Bars represent mean ± SD. Statistical comparisons were performed using the Mann–Whitney *U* test ($\alpha = 0.05$). *$p < 0.05$; ns, not significant

Flow cytometry findings

Flow cytometry analysis revealed that the proportions of $CD4^+PD\text{-}1^+$ and $CD4^+PD\text{-}L1^+$ T cells were significantly higher in DLBCL patients compared to controls ($p = 0.010$ and $p = 0.002$, respectively). Although other immune cell subsets—such as $CD4^+CTLA\text{-}4^+$, $CD8^+PD\text{-}1^+$, and $CD8^+PD\text{-}L1^+$ cells—also exhibited elevated levels in patients, these differences did not reach statistical significance ($p > 0.05$).

Subtype-specific analysis showed that GCB patients had higher frequencies of $CD4^+PD\text{-}L1^+$, total $CD8^+$ T cells, and $CD3^+CD4^+PD\text{-}1^+PD\text{-}L1^+$ T cells. In contrast, non-GCB patients demonstrated increased proportions of $CD4^+PD\text{-}1^+$, $CD8^+PD\text{-}L1^+$, and $CD8^+CTLA\text{-}4^+$ cells (Tables 3 and 4). Consistent with the exploratory design of the study, effect sizes and 95% confidence intervals were also evaluated to support the observed p-value trends and assess the magnitude of differences.

Quantitative comparisons of T-cell subsets across GCB and non-GCB subtypes are summarized in Table 4.

As described in the Materials and Methods section, flow cytometric analyses were performed using a stepwise gating strategy to define lymphocyte populations and to distinguish $CD4^+$ helper and $CD8^+$ cytotoxic T-cell subsets. Representative sequential gating plots from a DLBCL patient and a healthy control are presented in Fig. 2, illustrating the gating hierarchy and the corresponding percentages for each population. In addition, complementary flow cytometry plots for $CD4^+$ and $CD8^+$ T-lymphocyte analyses in the

Table 3 Levels of flow cytometry results (%) in patients with DLBCL and control groups

		Patient group (*n*:20)	Control group (n:20)	*p* value
Lymphocyte[a]	Mean ± SD	65.10 ± 19.19	65.04 ± 19.62	0.992
$(CD4^+)$[b]	Median (Q1-Q3)	36.85 (14.85–43..65)	36.05 (16.65–40.50)	0.602
$(CD4^+PD\text{-}1^+)$[b]	Median (Q1-Q3)	30.20 (16.10–40.05)	13.60 (6.80–25.65)	**0.010***
$(CD4^+ PD\text{-}L1^+)$[b]	Median (Q1-Q3)	32.00 (23.40–34.75)	12.25 (4.30–19.10)	**0.002***
$(CD4^+CTLA\text{-}4^+)$[b]	Median (Q1-Q3)	21.65 (8.60–41.55)	14.95 (5.90–23.60)	0.121
$(CD8^+)$[a]	Mean ± SD	25.60 ± 9.80	25.25 ± 12.37	0.920
$(CD8^+PD\text{-}1^+)$[a]	Mean ± SD	34.6 ± 17.01	32.62 ± 20.18	0.738
$(CD8^+ PD\text{-}L1^+)$[b]	Median (Q1-Q3)	30.80 (27.30–39.85)	30.55 (22.20–34.70)	0.602
$(CD8^+CTLA\text{-}4^+)$[b]	Median (Q1-Q3)	33.45 (21.65–53.90)	22.35 (8.00–44.00)	0.201
$(CD3^+CD4^+PD\text{-}1^+ PD\text{-}L1^+)$[b]	Median (Q1-Q3)	3.25 (0.60–5.95)	2.10 (0.35–4.40)	0.461
$(CD3^+CD8^+PD\text{-}1^+ PD\text{-}L1^+)$[b]	Median (Q1-Q3)	1.55 (0.30–4.65)	4.50 (0.40–8.85)	0.341

Difference between groups is statistically significant. Effect sizes (absolute differences with 95% confidence intervals) were calculated to illustrate the magnitude of group differences. No formal correction for multiple comparisons was applied due to the exploratory nature and limited sample size of this studyDifference between groups is statistically significant. Effect sizes (absolute differences with 95% confidence intervals) were calculated to illustrate the magnitude of group differences. No formal correction for multiple comparisons was applied due to the exploratory nature and limited sample size of this study

$\alpha = 0.05$; *n* = number of participants; *SD* standard deviation, *Q1–Q3* quartile 25% (Q1)–quartile 75% (Q3)

[a]Independent samples *t*-test

[b]Mann–Whitney *U* test

Table 4 Levels of flow cytometry results (%) in patients with DLBCL and control groups

		GCB (*n*: 9)	Non-GCB (*n*: 11)	Control group (*n*:20)	*p* value
(Lymphocyte)**	Mean ± SD	62.38 ± 21.69	67.33 ± 17.64	65.04 ± 19.62	0.854
$CD4^+$	Median (Q1–Q3)	39.20 (12.50–41.50)	34.50 (16.00–48.70)	36.05 (16.65–40.50)	0.740
$CD4^+PD\text{-}1^+$	Median (Q1–Q3)	24.70 (16.70–38.00)	34.60 (15.50–56.00)[c]	13.60 (6.80–25.65)[b]	**0.034***
$CD4^+$ $PD\text{-}L1^+$	Median (Q1–Q3)	33.80 (33.00–51.70)[c]	30.10 (13.40–32.00)	12..25 (4.30–19.10)[a]	**0.005***
$CD4^+CTLA\text{-}4^+$	Median (Q1–Q3)	23.50 (15.20–31.50)	20.10 (8.60–41.70)	14.95 (5.90–23.60)	0.297
$(CD8^+)$**	Mean ± SD	29.31 ± 9.96	22.56 ± 8.99	25.25 ± 12.37	0.404
$(CD8^+$ $PD\text{-}L1^+)$**	Mean ± SD	43.40 ± 18.25	27.40 ± 12.50	32.62 ± 20.18	0.147
$CD8^+$ $PD\text{-}L1^+$	Median (Q1–Q3)	29.90 (25.00–38.70)	31.10 (29.60–41.00)	30.55 (22.20–34.70)	0.777
$CD8^+CTLA\text{-}4^+$	Median (Q1–Q3)	33.20 (19.80–41.00)	33.70 (23.50–83.00)	22.35 (8.00–44.00)	0.188
$CD3^+CD4^+PD\text{-}1^+$ $PD\text{-}L1^+$	Median (Q1–Q3)	4.50 (2.50–13.00)	2.50 (0.30–4.50)	2.10 (0.35–4.40)	0.404
$CD3^+CD8^+PD\text{-}1^+$ $PD\text{-}L1^+$	Median (Q1–Q3)	4.20 (0.60–9.60)	1.20 (0.10–2.50)	4.50 (0.40–8.85)	0.132

Kruskal–Wallis H test; post hoc: Dunn's test; ANOVA; $\alpha = 0.05$; n = number of participants; SD = standard deviation; Q1–Q3: 25th percentile (Q1)–75th percentile (Q3). GCB and non-GCB refer to the germinal center B-cell–like and non-germinal center B-cell–like molecular subtypes of diffuse large B-cell lymphoma (DLBCL); therefore, the sample sizes shown for these groups represent DLBCL patient subgroups (e.g., $n = 9$ and $n = 11$) rather than the total cohort. Difference between groups is statistically significant

[a]Significant difference versus the GCB subgroup

[b]Significant difference versus the non-GCB subgroup

[c]Significant difference versus the control group. Effect sizes (absolute differences with 95% confidence intervals) were calculated to illustrate the magnitude of group differences. No formal correction for multiple comparisons was applied due to the exploratory nature and limited sample size of this study

patient and control groups are provided in Supplementary Figures S2 and S3. A visual summary of subgroup comparisons of $CD4^+PD\text{-}1$ and $CD4^+PD\text{-}L1$ expression across DLBCL molecular subtypes (GCB and non-GCB) and the control group is shown in Supplementary Figure S4. Supplementary Figure S4 was derived from the median (IQR) summary statistics reported in Tables 3 and 4 and visually summarizes the group differences presented in those tables.

Comparison of GCB and non-GCB subtypes

Flow cytometry analysis revealed significant differences between GCB and non-GCB subtypes, particularly in $CD4^+PD\text{-}1^+$ and $CD4^+PD\text{-}L1^+$ T-cell populations when compared to controls ($p = 0.034$ and $p = 0.005$, respectively). Distinct gene expression profiles and immune phenotypes were identified among DLBCL patients, with elevated expression of *PD-1*, *PD-L1*, and *CTLA-4* genes correlating with specific polymorphisms. These findings suggest potential utility of these immune checkpoints as biomarkers for disease stratification and therapeutic decision-making.

In addition, flow cytometry data highlighted immunophenotypic differences between molecular subtypes, providing insights into potential immune evasion mechanisms in DLBCL. Finally, molecular cytogenetic review of archived patient samples revealed no evidence of common chromosomal translocations, including t(8;14)(q24;q32) (MYC–IGH), t(14;18)(q32;q21) (IGH–MALT1), t(14;18)(q32;q21) (IGH–BCL2), or t(11;14)(q13;q32) (IGH–CCND1).

Discussion

This study investigated the relationship between genetic polymorphisms and expression levels of the immune checkpoint molecules PD-1, PD-L1, CTLA-4, and CD80 in patients with diffuse large B-cell lymphoma (DLBCL). Our findings support a significant role for these molecules in DLBCL pathogenesis. Overall, we observed significantly increased PD-1, PD-L1, and CTLA-4 mRNA expression in patients compared with healthy controls (Table 2), together with higher proportions of $CD4^+PD\text{-}1^+$ and $CD4^+PD\text{-}L1^+$ T cells by flow cytometry (Tables 3, 4). Our findings showing increased *PD-1*/*PD-L1*/*CTLA-4* expression but specific polymorphic variants suggest that immune exhaustion may not be reversed effectively by current inhibitors, possibly due to intrinsic genetic regulation of these pathways [12, 16]. Despite reports of PD-L1 expression and an immune-infiltrated microenvironment in DLBCL, clinical responses to immune checkpoint inhibitors have generally been limited and heterogeneous in most DLBCL settings. In this context, our concurrent findings of increased PD-1, PD-L1, and CTLA-4 expression together with higher circulating $CD4^+PD\text{-}1^+$ and $CD4^+PD\text{-}L1^+$ T-cell fractions support the possibility that DLBCL can appear quantitatively "inflamed" yet remain functionally suppressed through

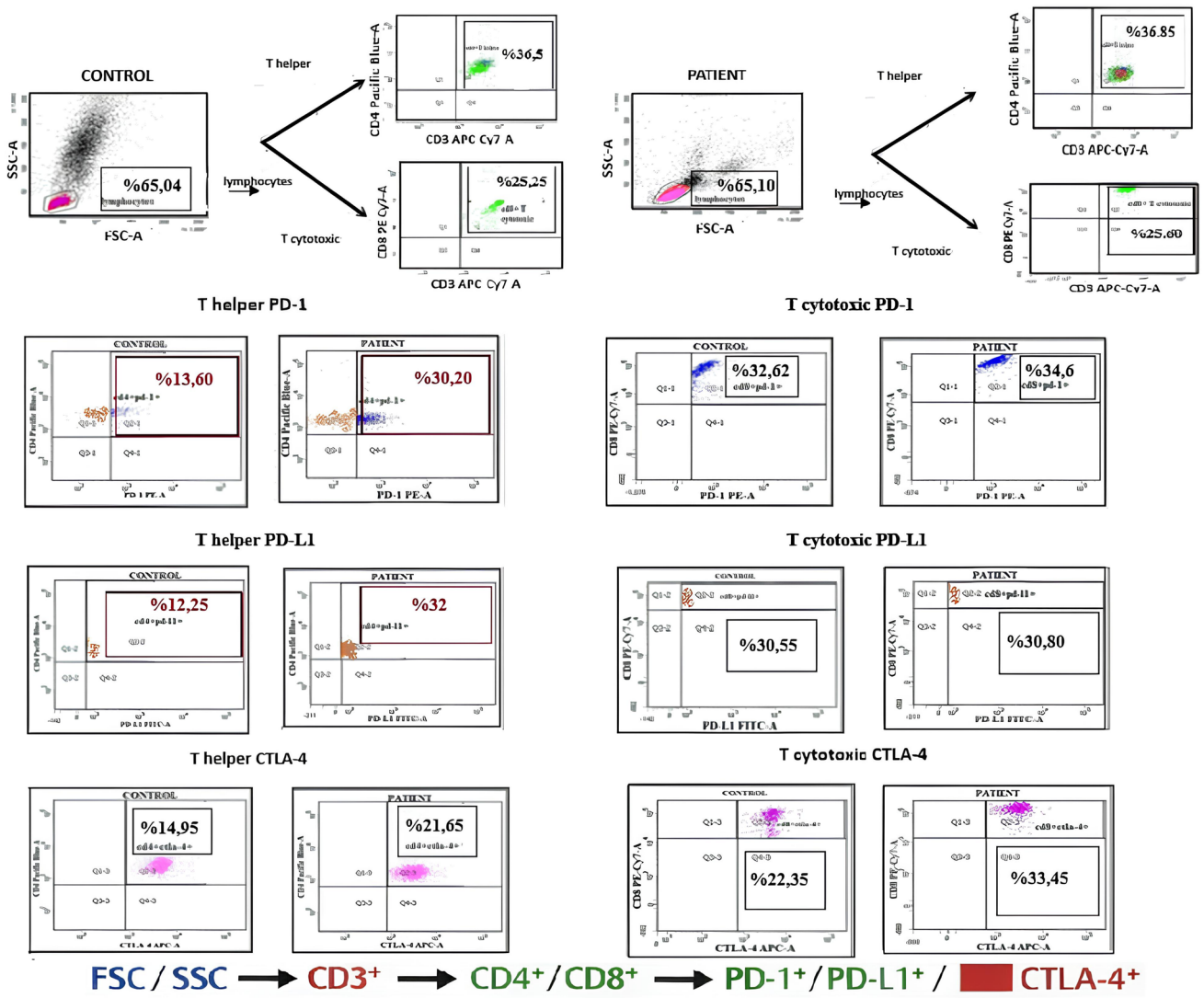

Fig. 2 Stepwise flow cytometric gating strategy illustrating $CD4^+$ and $CD8^+$ T-cell subsets expressing PD-1, PD-L1, and CTLA-4 in DLBCL patients and controls

layered checkpoint signaling, which may contribute to suboptimal reversibility with single-agent blockade.

Demographic characteristics of the study were consistent with global and regional DLBCL epidemiological data. The patient group included 20 individuals (10 females, 10 males) with a mean age of 59.95 ± 15.78 years, which aligns with previously reported population-level trends (Supplementary Table S.3) [2].

Earlier classifications, such as the 2016 World Health Organization (WHO) system, divided diffuse large B-cell lymphoma into two main molecular subtypes: germinal center B-cell-like (GCB) and activated B-cell-like (ABC). GCB subtypes were often linked to *BCL2* translocations and *PTEN* deletions, while ABC subtypes were characterized by chronic BCR signaling and NF-κB pathway activation [1]. Genetic alterations in oncogenes including *BCL2*, *BCL-xL*, *BCL6*, and *MYC* were recognized as key drivers of DLBCL pathogenesis [19]. However, the 2022 International Consensus Classification (ICC) and 5th edition WHO classification have moved beyond this dichotomous model, introducing a more refined taxonomy that includes genetically defined entities such as MYC/BCL2 and/or BCL6 rearranged high-grade B-cell lymphoma (so-called "double-hit" or "triple-hit" lymphomas), EBV-positive DLBCL, and other molecularly distinct subsets. This reflects the increasing appreciation of the biological heterogeneity within DLBCL [19]. In our study, molecular analyses did not detect hallmark translocations such as t(8;14)(q24;q32) (MYC–IGH), t(14;18)(q32;q21) (IGH–BCL2), or t(11;14)(q13;q32) (IGH–CCND1). Additionally, no statistically significant differences were observed between GCB and non-GCB subtypes in terms of clinical and prognostic features

including B symptoms, ECOG performance status, Ann Arbor staging, or laboratory parameters (Supplementary Tables S.4 and S.5). These findings may be influenced by both the limited sample size and the evolving criteria used to define molecular subtypes.

High *PD-1*/*PD-L1* expression in DLBCL tumor cells and within the tumor microenvironment is associated with poor prognosis, particularly in non-GCB subtypes [20]. *PD-L1*, expressed by both malignant B cells and tumor-associated macrophages, has been identified in approximately 20–30% of DLBCL cases. However, *PD-L2* expression shows no significant subtype-specific correlation [21, 22]. Blockade of PD-L1 has been shown to enhance cytokine secretion and restore antitumor immune responses, highlighting its therapeutic potential [21].

In our study, *PD-1* and *PD-L1* mRNA expression levels were significantly elevated in patients compared to controls ($p = 0.001$), consistent with prior observations [8]. No significant differences were found in expression between GCB and non-GCB subtypes ($p > 0.05$), aligning with previous findings [22]. Flow cytometry analysis further revealed increased percentages of CD4 + PD-1 + ($p = 0.010$) and CD4 + PD-L1 + ($p = 0.002$) T cells in patients compared to controls. Additionally, there were statistically significant differences between control and both GCB and non-GCB groups ($p = 0.034$ and $p = 0.005$, respectively). These findings collectively underscore the role of PD-1/PD-L1 axis in the pathogenesis and prognosis of DLBCL and support ongoing interest in targeting this pathway therapeutically [20, 21]. Taken together, our results indicate that DLBCL in our cohort is not "immune silent"; rather, it appears immune-infiltrated but dominated by inhibitory checkpoint signaling—an arrangement that can plausibly contribute to the limited and heterogeneous clinical activity reported for PD-1/PD-L1 blockade in DLBCL.

Genetic polymorphisms in the *PD-1* and *PD-L1* genes have been increasingly studied for their roles in cancer susceptibility. The PD-1 rs2227981 (C/T) polymorphism, also known as PD-1.5, has demonstrated inconsistent associations across cancer types. While some studies have suggested a possible link with colorectal and non-small cell lung cancer risk, others, including recent meta-analyses, report no significant association or even protective effects depending on the cancer type and population examined [23, 24]. Specifically, no association was found between *PD-1.5* and endometrial cancer risk in the Iranian population, and similarly, no significant relationship was observed between this SNP and NHL susceptibility [24, 25]. In our study, however, *PD-1* expression was significantly elevated in individuals with the C/T genotype compared to controls ($p = 0.012$), suggesting a potential functional impact. Previous reports have demonstrated that the PD-1 rs2227981 (C/T) polymorphism, located in the promoter region of the PDCD1 gene, can influence transcriptional regulation by altering transcription factor binding motifs (e.g., NFAT and FOXO1) and enhancing promoter activity, thereby leading to increased PD-1 expression on T cells [23–25]. This upregulation amplifies inhibitory signaling through SHP2-mediated dephosphorylation of downstream kinases, resulting in reduced T-cell proliferation and cytotoxic activity. This functional shift likely contributes to immune exhaustion and may help explain the elevated PD-1 levels observed in our DLBCL cohort, consistent with the limited responsiveness to PD-1/PD-L1 inhibitors reported in previous DLBCL studies [27, 28].

On the other hand, the *PD-L1* rs4143815 polymorphism, located in the 3′ untranslated region, has been more consistently linked to altered cancer risk and prognosis. Studies have shown its association with multiple cancers, including non-small cell lung cancer, ovarian cancer, and hepatocellular carcinoma, often through modulation of miRNA binding and gene expression [23]. However, like *PD-1.5*, no significant relationship between rs4143815 and NHL risk was found in prior research (Hoseini et al., 2020). In our study, we observed significantly increased *PD-L1* expression in patients carrying the C/G and G/G genotypes compared to controls ($p = 0.007$ and $p = 0.003$, respectively), underscoring a potential regulatory role in DLBCL pathogenesis. This variant lies within the 3′UTR of the *PD-L1* gene and can disrupt microRNA (miR-570) binding sites, enhancing *PD-L1* mRNA stability and translation efficiency [23–25]. The consequent overexpression of *PD-L1* on tumor and immune cells promotes persistent T-cell inhibition within the tumor microenvironment, contributing to immune evasion and potentially reduced responsiveness to PD-1/PD-L1 blockade therapies. Clinically, this mechanism may partially explain the limited efficacy of PD-1/PD-L1 inhibitors in DLBCL despite high *PD-L1* expression levels.

CTLA-4 is a critical immune checkpoint receptor that regulates T-cell activation and is closely associated with the progression of hematologic malignancies. In particular, the *CTLA-4* rs231775 (49A/G) polymorphism has been associated with elevated genetic susceptibility to several hematologic malignancies, such as NHL, multiple myeloma, and leukemia. Subtype-specific analyses in NHL have shown that the A/A genotype is more frequently observed in patient populations and represents a significant risk factor [16]. Elevated *CTLA-4* expression has been implicated in supporting the development of lymphoma stem cells and facilitating cellular proliferation and invasion through TGF-β signaling pathways [26]. In our study, *CTLA-4* expression was also found to be significantly higher in the patient group ($p = 0.001$; Table 2; Fig. 1), further supporting the immunosuppressive role of this molecule.

Importantly, PD-1/PD-L1 and CTLA-4 mediate immune suppression at different stages of the anti-tumor

response: PD-1 primarily dampens effector T-cell function in peripheral tissues and within the tumor microenvironment, whereas CTLA-4 predominantly constrains early T-cell priming and the CD80/CD86 costimulatory balance. Therefore, the concurrent upregulation of PD-1/PD-L1 and CTLA-4 observed in our cohort—together with genotype–expression associations for PD-L1 rs4143815 and CTLA-4 rs231775—supports the concept of a broader inhibitory checkpoint program rather than a single dominant node.

Despite the frequent presence of elevated *PD-1* and *PD-L1* expression and a highly immune-infiltrated tumor microenvironment, DLBCL generally exhibits only modest clinical responses to single-agent checkpoint inhibitors targeting the PD-1/PD-L1 axis. In an early phase I trial, nivolumab achieved an overall response rate of 36% and a complete response rate (CR) of 18% in relapsed or refractory DLBCL; however, subsequent studies with PD-1/PD-L1 inhibitors, including pembrolizumab, have reported considerably lower response rates, indicating limited clinical benefit from monotherapy [27, 28]. These findings suggest that elevated *PD-1 and PD-L1* expression in DLBCL may primarily reflect T-cell exhaustion and the presence of multiple overlapping immunosuppressive pathways, rather than effective immune activation. Mechanistically, while PD-1/PD-L1 blockade targets inhibitory signaling in effector T cells, CTLA-4 modulates T-cell priming and co-stimulation at an earlier stage. Therefore, the concurrent overexpression of PD-1, PD-L1, and CTLA-4 observed in our study may indicate a multilayered inhibitory network that reduces the reversibility of immune suppression with single-agent checkpoint inhibition. Collectively, these insights help explain the limited and inconsistent therapeutic responses observed in DLBCL and highlight the rationale for combined or sequential immune-targeted approaches in future studies.

Although *CD80* expression was higher in the control group, the difference was not statistically significant ($p = 0.110$; $p > 0.05$; Table 2; Fig. 1). These results may indicate that impaired immune-regulatory function of CD80 contributes to tumor progression. Genotypic analysis revealed that individuals carrying the CTLA-4 rs231775 G/A genotype exhibited significantly elevated CTLA-4 expression levels ($p = 0.006$), while no significant differences were observed among other genotypes ($p > 0.05$). This observation is consistent with the hypothesis that the G allele may lead to increased expression of less suppressive *CTLA-4* isoforms [16]. In contrast, the *CD80* rs2228017 polymorphism did not appear to have a significant effect on *CD80* expression in either patient or control groups ($p > 0.05$), suggesting that this variant may have limited functional impact.

Conclusions

This study offers a comprehensive evaluation of immune checkpoint dynamics in DLBCL by integrating gene expression profiling and polymorphism analysis of *PD-1*, *PD-L1*, *CTLA-4*, and *CD80*. Our findings revealed significantly increased expression levels of *PD-1*, *PD-L1*, and *CTLA-4* in DLBCL patients compared to healthy controls, consistent with their known roles in promoting immune escape in the tumor microenvironment. Although *CD80* expression was higher in controls, the lack of statistical significance points to the need for deeper functional analysis. Notably, specific genetic variants, particularly *CTLA-4* rs231775 and *PD-L1* rs4143815, were associated with altered expression levels, suggesting a genetic basis for immune regulation in DLBCL. These associations reinforce recent findings on the role of immune checkpoint gene polymorphisms in modulating cancer susceptibility and immune responses. The dual function of these molecules—as both immune regulators and facilitators of tumor-immune evasion—highlights the complexity of immune modulation in malignancies.

A key strength of this study is its originality; to our knowledge, this is the first investigation to simultaneously assess both gene-level expression and key immune-related SNPs across multiple checkpoint molecules in DLBCL patients. This dual approach provides a more nuanced view of immune dysregulation in this heterogeneous disease and offers potential biomarkers for risk stratification and therapeutic targeting. However, several limitations should be acknowledged. The relatively small sample size may limit the statistical power and generalizability of our findings. Furthermore, functional validation of SNP effects and longitudinal clinical correlation were beyond the scope of this study. Despite these limitations, the current work lays essential groundwork for future large-scale, multicenter studies.

In conclusion, our findings underscore the relevance of immune checkpoint pathways in the pathogenesis of DLBCL and support the integration of immunogenetic biomarkers into personalized therapeutic strategies. Further studies are warranted to validate these results and explore the potential of combination immunotherapies in improving clinical outcomes for DLBCL patients.

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experiments, contributed to data acquisition and analysis, and drafted the manuscript. S.D.A. and N.S.E. contributed to laboratory processes/technical procedures and supported the preparation and organization of figures and tables, while Y.K. contributed to the statistical interpretation of the data; all of these authors critically revised the manuscript for important intellectual content. All authors contributed to the study design and/or interpretation of the data, reviewed the manuscript, approved the final version for publication, and agree to be accountable for all aspects of the work.

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Declarations

Conflict of interest All authors made substantial contributions to the design of this work andwere involved in well drafting the work and critically reviewing the workfor intellectual content. All authors gave final approval of the version to bepublished and agree to be accountable for all aspects of the work inensuring that questions related to the accuracy or integrity of any part ofthe work are appropriately investigated and resolved.

Ethics approval statement All participants were informed of the study procedures, as stated in the study (Protocol No:70904504/11) approved by the Ethics Committee, Medical Faculty of Akdeniz University and they were provided written consent prior to initiation of study procedures. The study was performed in accordance with the Declaration of Helsinki.

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