

Clinicopathological Features and Implications of PD-L1 Expression in Colorectal Carcinoma and the Associated CD68-Positive Macrophages: An Analytical Cross-sectional Study

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ABSTRACT

Introduction: Colorectal carcinoma exhibits marked heterogeneity in its tumour immune microenvironment, and immune escape through PD-L1 expression may interact with macrophage infiltration. However, the relationship between PD-L1 expression and CD68-positive tumour-associated macrophage density in colorectal carcinoma remains incompletely defined, particularly in relation to routine clinicopathological features. Therefore, evaluating this association may help improve the biological interpretation of the tumour microenvironment in colorectal carcinoma.

Aim: To evaluate the association between PD-L1 expression with macrophage infiltration in colorectal carcinoma and their clinicopathological correlation.

Materials and Methods: This single-centre, hospital-based analytical cross-sectional study conducted in the Department of Pathology, Chettinad Hospital and Research Institute, Chennai, Tamil Nadu, India included 30 cases of Stage-I-IV colorectal carcinoma evaluated using archival blocks and prospective specimens reported between February 2024 and January 2026 (24 months). Clinicopathological parameters (age, sex, tumour site, histological type, histological grade, depth of tumour invasion, lymph node status, overall TNM stage, lymph node metastasis and tumour-infiltrating lymphocyte status) were recorded, and immunohistochemistry for PD-L1 and CD68 was performed on formalin-fixed paraffin-embedded tissue sections,

with PD-L1 assessed by Tumour Proportion Score (TPS) and Combined Positive Score (CPS). Associations between PD-L1 expression, CD68-positive tumour-associated macrophage density and clinicopathological variables were analysed using the chi-square test or Fisher's-exact test, as appropriate, with $p < 0.05$ considered statistically significant.

Results: The mean age was 56.5 ± 9.9 years, and 13/30 patients (43.3%) were aged ≥ 60 years. Males constituted 17/30 cases (56.7%). Conventional adenocarcinoma was the commonest histological type, seen in 24/30 cases (80.0%), while 16/30 tumours (53.3%) were moderately differentiated, 15/30 (50.0%) were T3, and 12/30 (40.0%) were Stage-III. PD-L1 positivity by TPS was observed in 13/30 cases (43.3%), while high CD68-positive TAM density was noted in 10/30 cases (33.3%). Among PD-L1-positive tumours, 8/13 cases (61.5%) showed high CD68-positive TAM density, compared with 2/17 PD-L1-negative cases (11.8%) ($p = 0.009$). PD-L1 positivity was significantly associated with right-sided tumour location {8/13 (61.5%); $p = 0.011$ }, poor differentiation {6/13 (46.2%); $p = 0.004$ }, brisk TILs {7/13 (53.8%); $p = 0.011$ }, T4 stage {9/13 (69.2%); $p = 0.002$ }, N2 stage {5/13 (38.5%); $p = 0.004$ }, Stage-IV disease {6/13 (46.2%); $p = 0.013$ }, and lymph node metastasis {%; $p = 0.009$ }.

Conclusion: PD-L1 expression was significantly associated with CD68-positive macrophage infiltration and adverse clinicopathological features in colorectal carcinoma, supporting its potential role as a prognostic biomarker and therapeutic target.

Keywords: CD68, Colorectal carcinoma, Immunohistochemistry, PD-L1, Tumour-associated macrophages, Tumour microenvironment

INTRODUCTION

Colorectal carcinoma is a major global health problem and remains one of the most frequently diagnosed and lethal malignancies worldwide. According to the 2024 GLOBOCAN update based on 2022 estimates, colorectal cancer accounted for 9.6% of all newly diagnosed cancers globally and 9.3% of all cancer deaths, making it the third most commonly diagnosed cancer and the second leading cause of cancer-related mortality worldwide [1]. Bray F et al., further noted that the continuing rise in absolute case numbers is being driven by population growth, ageing, and epidemiological transition, underscoring the need for improved biological stratification of colorectal carcinoma beyond conventional stage-based assessment [1]. Although TNM staging, histological subtype, grade, and lymph node status remain the backbone of prognostic evaluation, increasing evidence indicates that colorectal carcinoma behaviour is also strongly shaped by the tumour immune microenvironment.

In particular, immune escape through the programmed cell death protein-1/Programmed Death Ligand-1 (PD-1/PD-L1) axis has emerged as an important mechanism by which tumour cells evade cytotoxic antitumour responses. Siraj AK et al., in a study of 1,428 middle eastern colorectal cancers, demonstrated PD-L1 expression in 37.3% of tumour cells and showed significant associations with deficient mismatch repair, poor differentiation, larger tumour size, mucinous histology, and an inflamed immune phenotype, supporting the biological relevance of PD-L1 in colorectal cancer progression [2]. Shan T et al., also reported that PD-L1 expression in colon cancer was significantly associated with lymph node metastasis and poorer survival, indicating that PD-L1 may have both prognostic and therapeutic importance [3].

The tumour microenvironment in colorectal carcinoma is not determined by lymphocytes alone; tumour-associated macrophages also play a critical role in tumour growth, invasion, angiogenesis,

immunomodulation, and metastatic spread. Hou S et al., described tumour-associated macrophages as key regulators of colorectal cancer metastasis through secretion of cytokines, chemokines, proteases, and growth factors that remodel the local immune niche and facilitate tumour dissemination [4]. In addition, Yang C et al., demonstrated that macrophage polarisation patterns, particularly a higher CD163+/CD68+ ratio at the invasive front, were associated with epithelial-mesenchymal transition and worse prognosis in colorectal cancer, indicating that macrophage-rich microenvironments may contribute directly to aggressive tumour biology [5]. These observations are clinically relevant because immune-checkpoint blockade has transformed the treatment landscape of a biologically defined subset of colorectal cancers. Saberzadeh-Ardestani B et al., noted that nearly 15% of colorectal carcinomas are mismatch repair-deficient or microsatellite instability-high and that these tumours are characterised by abundant neoantigens, dense immune infiltration, and sensitivity to PD-1/PD-L1-axis inhibition [6]. However, PD-L1 expression in colorectal carcinoma remains heterogeneous, and its relationship with macrophage infiltration is incompletely characterised [7]. Against this background, the objectives of the present study were to evaluate the association between PD-L1 expression and macrophage infiltration in colorectal carcinoma.

MATERIALS AND METHODS

This was a single-centre, hospital-based, analytical cross-sectional study conducted in the Department of Pathology, Chettinad Hospital and Research Institute, Chennai, Tamil Nadu, India with archival blocks and prospective cases of colorectal carcinoma reported between February 2024 and January 2026. The study was approved by the Institutional Human Ethics Committee (IHEC; IHEC-II/0966/25).Patients diagnosed with Stage-I to Stage-IV colorectal carcinoma who had undergone surgical resection, as well as colonoscopic biopsy specimens diagnosed as infiltrating carcinoma, were included in the study. Patients who had received neoadjuvant chemoradiotherapy were excluded because treatment could alter PD-L1 expression. Cases showing aberrant immunohistochemical staining with non-contributory results and colonoscopic biopsy specimens demonstrating only dysplasia were also excluded.

The sample size was calculated using the single-proportion formula, $n=Z^2pq/e^2$, taking the expected proportion of PD-L1 positivity in colorectal carcinoma from Gao H et al., who reported PD-L1 positive expression in 22% (39/174) of colorectal carcinoma cases [8]. For the calculation, the prevalence (p) was assumed to be 0.22, $q=1-p=0.78$, the standard normal deviate (Z) at 95% confidence level was taken as 1.96, and the absolute precision (e) was set at 0.165. Using these assumptions, the estimated minimum sample size was rounded off to 30 cases; enrolled using non-probability sampling technique - convenience sampling.

Demographic variables including age, sex, and relevant family history were recorded. Tumour-related details, including laterality, exact anatomical site, histological type, histological grade, lymph node status, and pathological stage, were documented from the resection specimens, and staging was interpreted.

Tumour-infiltrating lymphocytes were assessed on routine haematoxylin and eosin-stained sections at the invasive tumour front and within the tumour stroma, avoiding areas of necrosis, ulceration and pre-existing lymphoid aggregates. TILs were graded semi-quantitatively based on the density and distribution of lymphocytic infiltration. Absent/scant TILs were defined as no or only occasional lymphocytes within the tumour stroma; non-brisk TILs were defined as focal or patchy lymphocytic infiltration without a continuous band-like inflammatory response; and brisk TILs were defined as dense, diffuse or band-like lymphocytic infiltration at the invasive margin and/or tumour stroma, involving a substantial proportion of the tumour-stromal interface. All available haematoxylin and eosin-stained slides

were reviewed first to confirm the diagnosis, assess adequacy, and identify a representative paraffin block containing viable invasive tumour for immunohistochemical analysis. Formalin-fixed, paraffin-embedded tissue blocks from the selected cases were then used for immunohistochemistry. Sections of routine thickness were cut from the representative blocks and mounted on charged slides for staining. Primary antibodies against PD-L1 (rabbit monoclonal antibody (clone: RM320)) and CD68 (rabbit monoclonal antibody (clone: KP1)) were applied. CD68 was used as a pan-macrophage marker for assessment of tumour-associated macrophage infiltration. Normal colonic epithelium was included as an external control with each batch. Cases showing aberrant cytoplasmic staining or technically unsatisfactory staining were repeated. The immunohistochemical interpretation was performed independently by two pathologists. PD-L1 expression was assessed on tumour cells and immune cells, and the results were documented using both the TPS and the CPS [9,10]. TPS represents the percentage of viable tumour cells showing PD-L1 staining, whereas CPS is calculated as the number of PD-L1-positive tumour cells and immune cells divided by the total number of viable tumour cells, multiplied by 100; a minimum of 100 viable tumour cells is generally recommended for reliable PD-L1 assessment. PD-L1 was considered immunohistochemically positive when membranous or cytoplasmic staining of any intensity was present in at least 1% of the TPS, while complete absence of cytoplasmic staining in the presence of an adequate internal control was considered negative [11].

STATISTICAL ANALYSIS

Data were entered in Microsoft Excel and analysed using Statistical Package for the Social Sciences (SPSS) version 27.0 (IBM Corp., Armonk, NY, USA). Categorical variables were summarised as frequencies and percentages, while continuous variables were expressed as mean and standard deviation after assessment of distribution. The association between PD-L1 expression and clinicopathological variables, as well as the association between CD68-positive TAM density and relevant clinicopathological parameters, was analysed using the Chi-square test or Fisher's-exact test wherever appropriate. For all analyses, a two-sided p-value of less than 0.05 was considered statistically significant.

RESULTS

Among the 30 colorectal carcinoma cases, the mean age was 56.5±9.9 years, and the largest proportion of patients were aged ≥60 years {13/30 (43.3%)}. Males were slightly more common than females {17/30 (56.7%) vs 13/30 (43.3%)}, and a family history of colorectal or gastrointestinal malignancy was present in 5/30 cases (16.7%) [Table/Fig-1]. Left-sided tumours were the most frequent {12/30 (40.0%)}, followed by right colon lesions {10/30 (33.3%)} and rectosigmoid/rectal tumours {8/30 (26.7%)}. Conventional adenocarcinoma, NOS was the predominant histological type {24/30 (80.0%)}. Most tumours were moderately differentiated {16/30 (53.3%)}, pathologically T3 {15/30 (50.0%)}, node-negative {16/30 (53.3%)}, and Stage-III overall {12/30 (40.0%)}, while lymph node metastasis was seen in 14/30 cases (46.7%). Tumour-infiltrating lymphocytes were most often non-brisk {12/30 (40.0%)}.

		Overall (N=30)
Demographic characteristics		
Age (years), Mean (SD)		56.5 (9.9)
Age group, n (%)	<50 years	8 (26.7)
	50-59 years	9 (30.0)
	≥60 years	13 (43.3)
Sex, n (%)	Male	17 (56.7)
	Female	13 (43.3)
Family history of colorectal/ gastrointestinal malignancy, n (%)	Present	5 (16.7)
	Absent	25 (83.3)

Clinicopathological characteristics		
Tumour site/location, n (%)	Right colon	10 (33.3)
	Left colon	12 (40.0)
	Rectosigmoid/rectum	8 (26.7)
Histological type, n (%)	Conventional adenocarcinoma, NOS	24 (80.0)
	Mucinous adenocarcinoma	4 (13.3)
	Signet-ring cell carcinoma	2 (6.7)
Histological grade, n (%)	Well-differentiated	8 (26.7)
	Moderately differentiated	16 (53.3)
	Poorly differentiated	6 (20.0)
Pathological T stage, n (%)	T1	1 (3.3)
	T2	4 (13.3)
	T3	15 (50.0)
	T4	10 (33.3)
Pathological N stage, n (%)	N0	16 (53.3)
	N1	9 (30.0)
	N2	5 (16.7)
Overall TNM stage, n (%)	Stage-I	3 (10.0)
	Stage-II	8 (26.7)
	Stage-III	12 (40.0)
	Stage-IV	7 (23.3)
Lymph node metastasis, n (%)	Present	14 (46.7)
	Absent	16 (53.3)
Tumour-infiltrating lymphocytes, n (%)	Brisk	8 (26.7)
	Non-brisk	12 (40.0)
	Absent/scant	10 (33.3)
PD-L1 immunohistochemical expression		
PD-L1 expression status by TPS (cut-off $\geq 1\%$), n (%)	Positive	13 (43.3)
	Negative	17 (56.7)
TPS category, n (%)	<1%	17 (56.7)
	1-9%	5 (16.7)
	10-49%	5 (16.7)
	$\geq 50\%$	3 (10.0)
CPS category, n (%)	<1	1 (3.3)
	1-9	8 (26.7)
	10-19	9 (30.0)
	≥ 20	12 (40.0)
Tumour cell staining pattern, n (%)	Negative	17 (56.7)
	Membranous only	4 (13.3)
	Membranous with cytoplasmic staining	9 (30.0)
Staining intensity among PD-L1-positive cases (n=13), n (%)	Weak	5 (38.5)
	Moderate	5 (38.5)
	Strong	3 (23.1)
CD68-positive tumour-associated macrophage infiltration		
CD68-positive TAM density, n (%)	Low	9 (30.0)
	Moderate	11 (36.7)
	High	10 (33.3)
Predominant compartment/distribution, n (%)	Stromal predominant	15 (50.0)
	Intratumoural predominant	8 (26.7)
	Mixed stromal and intratumoural	7 (23.3)

[Table/Fig-1]: Baseline demographic, clinicopathological, PD-L1 expression, and CD68-positive tumour-associated macrophage characteristics of colorectal carcinoma cases.

PD-L1 positivity by TPS using a cut-off of $\geq 1\%$ was observed in 13/30 cases (43.3%), with TPS $\geq 50\%$ in 3/30 cases (10.0%), while CPS ≥ 20 was seen in 12/30 cases (40.0%). CD68-positive tumour-

associated macrophage infiltration was low in 9/30 cases (30.0%), moderate in 11/30 cases (36.7%), and high in 10/30 cases (33.3%), with stromal predominance seen in 15/30 tumours (50.0%).

There was a statistically significant association between PD-L1 expression and CD68-positive tumour-associated macrophage density in colorectal carcinoma ($p=0.009$) [Table/Fig-2]. Among PD-L1-negative cases, low and moderate CD68-positive macrophage density predominated, seen in 8/17 cases (47.1%) and 7/17 cases (41.2%), respectively, whereas only 2/17 cases (11.8%) showed high CD68-positive macrophage density. In contrast, among PD-L1-positive cases, high CD68-positive macrophage density was the most frequent pattern, observed in 8/13 cases (61.5%), followed by moderate density in 4/13 cases (30.8%), while low density was seen in only 1/13 case (7.7%). PD-L1 positivity was significantly more frequent in right-sided tumours {8/13 (61.5%)} than in left-sided tumours {2/13 (15.4%)} or rectosigmoid/rectal tumours {3/13 (23.1%)} ($p=0.011$) [Table/Fig-3]. A significant association was also observed with histological type, as all mucinous adenocarcinomas {4/4 (100.0%)} and signet-ring cell carcinomas {2/2 (100.0%)} were PD-L1 positive, whereas all PD-L1-negative cases were conventional adenocarcinoma, NOS {17/17 (100.0%)} ($p=0.007$). Poorly differentiated tumours showed a strong association with PD-L1 positivity, accounting for 6/13 PD-L1-positive cases (46.2%), while no poorly differentiated tumour was seen among PD-L1-negative cases {0/17 (0.0%)} ($p=0.004$). Brisk tumour-infiltrating lymphocytes were more common in PD-L1-positive tumours {7/13 (53.8%)} than in PD-L1-negative tumours {1/17 (5.9%)} ($p=0.011$). Advanced disease was also associated with PD-L1 positivity, including T4 stage {9/13 (69.2%); $p=0.002$ }, N2 stage {5/13 (38.5%); $p=0.004$ }, Stage-IV disease {6/13 (46.2%); $p=0.013$ }, and lymph node metastasis {10/13 (76.9%); $p=0.009$ }.

CD68-positive TAM density	PD-L1 negative	PD-L1 positive	Total
Low	8 (47.1)	1 (7.7)	9 (30.0)
Moderate	7 (41.2)	4 (30.8)	11 (36.7)
High	2 (11.8)	8 (61.5)	10 (33.3)

[Table/Fig-2]: Association between PD-L1 expression and CD68-positive tumour-associated macrophage density in colorectal carcinoma. p -value=0.009 (Chi-square test), indicating a significant positive association between increasing CD68-positive macrophage density and PD-L1 expression

		PD-L1 negative	PD-L1 positive	p-value
Age group	<50 years	7 (41.2)	1 (7.7)	0.088
	50-59 years	5 (29.4)	4 (30.8)	
	≥ 60 years	5 (29.4)	8 (61.5)	
Sex	Male	8 (47.1)	9 (69.2)	0.399
	Female	9 (52.9)	4 (30.8)	
Tumour site/location	Right colon	2 (11.8)	8 (61.5)	0.011*
	Left colon	10 (58.8)	2 (15.4)	
	Rectosigmoid/rectum	5 (29.4)	3 (23.1)	
Histological type	Conventional adenocarcinoma, NOS	17 (100.0)	7 (53.8)	0.007*
	Mucinous adenocarcinoma	0 (0.0)	4 (30.8)	
	Signet-ring cell carcinoma	0 (0.0)	2 (15.4)	
Histological grade	Well differentiated	7 (41.2)	1 (7.7)	0.004*
	Moderately differentiated	10 (58.8)	6 (46.2)	
	Poorly differentiated	0 (0.0)	6 (46.2)	
Tumour-infiltrating lymphocytes	Brisk	1 (5.9)	7 (53.8)	0.011*
	Non-brisk	8 (47.1)	4 (30.8)	
	Absent/scant	8 (47.1)	2 (15.4)	
Pathological T stage	T1	1 (5.9)	0 (0.0)	0.002*
	T2	4 (23.5)	0 (0.0)	
	T3	11 (64.7)	4 (30.8)	
	T4	1 (5.9)	9 (69.2)	

Pathological N stage	N0	13 (76.5)	3 (23.1)	0.004*
	N1	4 (23.5)	5 (38.5)	
	N2	0 (0.0)	5 (38.5)	
Overall TNM stage	Stage-I	3 (17.6)	0 (0.0)	0.013*
	Stage-II	7 (41.2)	1 (7.7)	
	Stage-III	6 (35.3)	6 (46.2)	
	Stage-IV	1 (5.9)	6 (46.2)	
Lymph node metastasis	Present	4 (23.5)	10 (76.9)	0.009*
	Absent	13 (76.5)	3 (23.1)	

[Table/Fig-3]: Association of PD-L1 expression with demographic and clinicopathological characteristics in colorectal carcinoma.

*Statistically significant at $p < 0.05$

High CD68-positive tumour-associated macrophage density was frequent in right-sided tumours, with 8/10 high-CD68 cases (80.0%) located in the right colon, while no right-sided tumour was present in the low-CD68 group {0/9 (0.0%)} ($p=0.003$). Poorly differentiated tumours were also enriched in the high-CD68 group {5/10 (50.0%)}, whereas well-differentiated tumours were predominantly seen in the low-CD68 group {5/9 (55.6%)} ($p=0.028$). Advanced disease demonstrated a similar pattern, with Stage-IV tumours forming 6/10 cases (60.0%) in the high-CD68 group, while Stage-I and Stage-II tumours were absent from this category {0/10 (0.0%)} ($p=0.016$). Lymph node metastasis was present in 8/10 high-CD68 cases (80.0%) compared with 1/9 low-CD68 cases (11.1%) ($p=0.006$). Brisk tumour-infiltrating lymphocytes were also more common in the high-CD68 group {6/10 (60.0%)}, whereas absent or scant lymphocytes predominated in the low-CD68 group {6/9 (66.7%)} ($p=0.014$) [Table/Fig-4].

		Low CD68	Moderate CD68	High CD68	p-value
Tumour site/ location	Right colon	0 (0.0)	2 (18.2)	8 (80.0)	0.003*
	Left colon	5 (55.6)	5 (45.5)	2 (20.0)	
	Rectosigmoid/rectum	4 (44.4)	4 (36.4)	0 (0.0)	
Histological grade	Well-differentiated	5 (55.6)	2 (18.2)	1 (10.0)	0.028*
	Moderately differentiated	4 (44.4)	7 (63.6)	5 (50.0)	
	Poorly differentiated	0 (0.0)	1 (9.1)	5 (50.0)	
Overall TNM stage	Stage-I	2 (22.2)	1 (9.1)	0 (0.0)	0.016*
	Stage-II	4 (44.4)	4 (36.4)	0 (0.0)	
	Stage-III	2 (22.2)	5 (45.5)	5 (50.0)	
	Stage-IV	1 (11.1)	0 (0.0)	6 (60.0)	
Lymph node metastasis	Present	1 (11.1)	5 (45.5)	8 (80.0)	0.006*
	Absent	8 (88.9)	6 (54.5)	2 (20.0)	
Tumour-infiltrating lymphocytes	Brisk	0 (0.0)	2 (18.2)	6 (60.0)	0.014*
	Non-brisk	3 (33.3)	6 (54.5)	3 (30.0)	
	Absent/scant	6 (66.7)	3 (27.3)	1 (10.0)	

[Table/Fig-4]: Association of CD68-positive tumour-associated macrophage density with clinicopathological characteristics in colorectal carcinoma.

*Statistically significant at $p < 0.05$

PD-L1 expression was significantly associated with several adverse clinicopathological features in colorectal carcinoma [Table/Fig-5]. All poorly differentiated tumours were PD-L1 positive {6/6 (100.0%)} compared with 7/24 tumours without poor differentiation (29.2%) ($p=0.003$). PD-L1 positivity was also markedly higher in right-sided tumours {8/10 (80.0%)} than in non-right-sided tumours {5/20 (25.0%)} ($p=0.007$), and in T4 disease, where 9/10 cases (90.0%) were positive compared with 4/20 non-T4 tumours (20.0%) ($p < 0.001$). Advanced-stage disease showed a strong relationship with PD-L1 expression, with 12/19 Stage-III/IV tumours (63.2%) being positive compared with 1/11 Stage-I/II tumours (9.1%) ($p=0.007$). Similarly, node-positive cases demonstrated greater PD-L1 positivity than node-negative cases {10/14 (71.4%) vs 3/16 (18.8%); $p=0.009$ }. PD-L1 expression was also more common

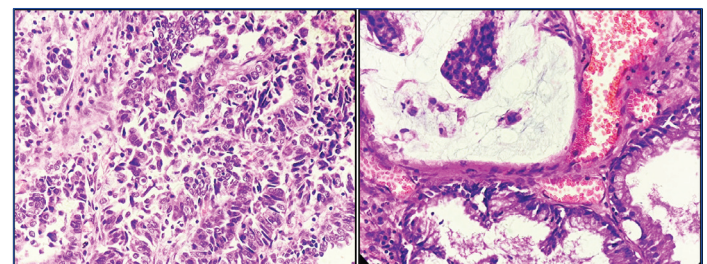
in tumours with high CD68-positive macrophage density {8/10 (80.0%)} than in tumours without high CD68 density {5/20 (25.0%)} ($p=0.007$), and in tumours with brisk tumour-infiltrating lymphocytes {7/8 (87.5%)} than in those without brisk tumour-infiltrating lymphocytes {6/22 (27.3%)} ($p=0.009$). These findings indicate that PD-L1 expression was associated with macrophage-rich and immune-active tumour microenvironments, as well as with adverse clinicopathological features.

Adverse/ prognostically relevant feature	PD-L1 positive when feature present	PD-L1 positive when feature absent	p-value	Interpretation
Poor differentiation	6/6 (100.0)	7/24 (29.2)	0.003*	Marked enrichment of PD-L1 positivity in poorly differentiated tumours
Right-sided location	8/10 (80.0)	5/20 (25.0)	0.007*	PD-L1 positivity was more frequent in right-sided tumours
T4 disease	9/10 (90.0)	4/20 (20.0)	<0.001*	PD-L1 positivity clustered with locally advanced tumours
Stage-III/IV disease	12/19 (63.2)	1/11 (9.1)	0.007*	Advanced-stage disease showed substantially higher PD-L1 positivity
Lymph node metastasis	10/14 (71.4)	3/16 (18.8)	0.009*	Node-positive cases showed greater PD-L1 expression
High CD68-positive TAM density	8/10 (80.0)	5/20 (25.0)	0.007*	Macrophage-rich tumours were more likely to express PD-L1
Brisk TILs	7/8 (87.5)	6/22 (27.3)	0.009*	Immune-rich tumours showed higher PD-L1 expression

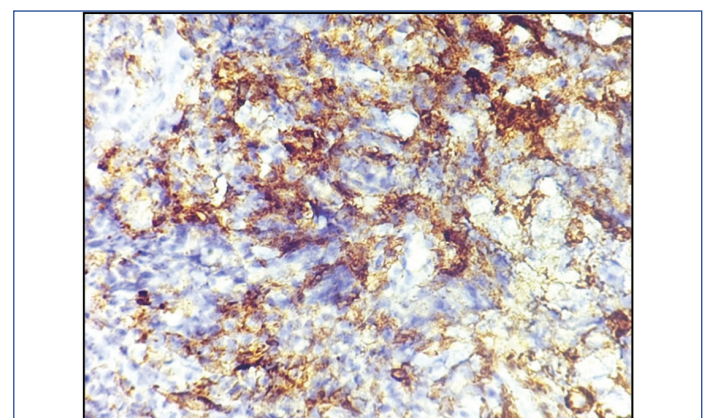
[Table/Fig-5]: Summary of prognostically relevant PD-L1 expression patterns in colorectal carcinoma.

*Statistically significant at $p < 0.05$

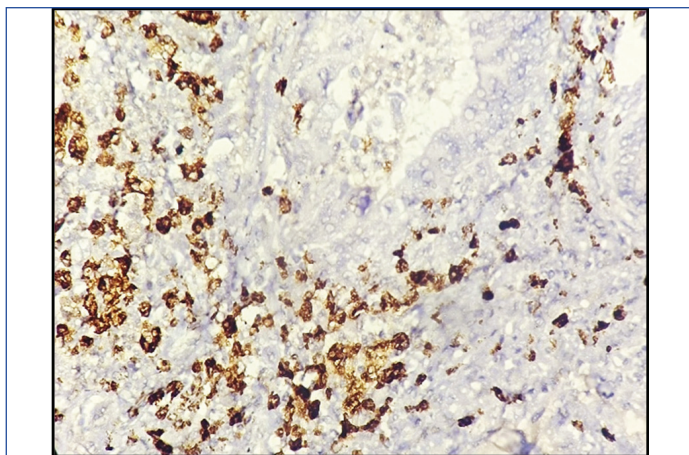
The H&E section of conventional adenocarcinoma and mucinous adenocarcinoma, PD-L1-positive tumour and immune cells showing moderate membranous staining and CD 68-positive immune cells are shown in [Table/Fig-6-8].



[Table/Fig-6]: H&E section of conventional adenocarcinoma and mucinous adenocarcinoma 40x, respectively.



[Table/Fig-7]: PD-L1-positive tumour and immune cells in 40x showing moderate membranous staining.



[Table/Fig-8]: CD 68-positive immune cells in 40x.

DISCUSSION

The demographic and baseline clinicopathological profile in the present study was broadly in keeping with the usual epidemiology of colorectal carcinoma, while also reflecting the selection of resected and biopsy-confirmed cases. The mean age of 56.5 ± 9.9 years and the slight male predominance (56.7%) are consistent with Luo C et al., (2019), which is typically more common after the fifth decade and shows a modest male excess [12]. The predominance of conventional adenocarcinoma, NOS (80.0%) also mirrors Stachler MD (2019), where conventional adenocarcinoma remains the major histological subtype, while mucinous and signet-ring variants form smaller but biologically distinct subsets [13]. The distribution of T3/T4 lesions and Stage-III/IV cases in this cohort suggests that a substantial proportion of tumours had already acquired invasive and metastatic capability at diagnosis, which is relevant because both tumour progression and microenvironmental remodelling are known to influence immune-checkpoint expression and macrophage recruitment, as noted by Chen K et al., (2021) [14].

A central finding of the study was the PD-L1 positivity rate of 43.3% by TPS using a $\geq 1\%$ threshold, with 10.0% of cases showing TPS $\geq 50\%$ and 40.0% showing CPS ≥ 20 . This frequency is plausible within the wide range reported in colorectal carcinoma, where PD-L1 prevalence varies substantially depending on antibody clone, tissue sampling, scoring method, and threshold for positivity. Frančina M et al., (2024) highlighted that lack of uniform scoring systems and cut-offs is a major reason for variability across colorectal cancer studies, and specifically discussed TPS and CPS as two currently used approaches for PD-L1 assessment [15]. In Valentini AM et al., (2018), PD-L1 positivity in neoplastic cells was reported in 25% of colorectal cancers using a 5% cut-off, while positivity in infiltrating immune cells was much higher at 78%, supporting the idea that colorectal cancer PD-L1 biology is highly dependent on whether one evaluates tumour cells alone or the broader immune microenvironment [11]. Our higher TPS positivity than the Valentini tumour-cell rate is therefore explainable by the lower positivity threshold of 1% and by the known focality of PD-L1 expression in colorectal tumours.

The significant association between PD-L1 positivity and increasing CD68-positive tumour-associated macrophage density was one of the most biologically meaningful findings in this study. High TAM density was present in 61.5% of PD-L1-positive tumours but in only 11.8% of PD-L1-negative tumours, whereas low TAM density predominated among PD-L1-negative cases (47.1% vs 7.7%; $p=0.009$). This pattern supports a model in which macrophage-rich tumour microenvironments participate in adaptive immune resistance, either by producing cytokines that induce PD-L1 or by being recruited into tumours already characterised by chronic inflammatory signalling, in corroboration with Zhang H et al., (2023) [16]. Valentini AM et al., (2018) emphasised that PD-L1 should be interpreted together with infiltrating immune cells rather than as a

purely tumour-cell phenomenon, because colorectal carcinomas can be separated into distinct immune microenvironment subsets based on the combined presence or absence of PD-L1 on neoplastic and immune cells [11]. The current findings extend that concept by showing that pan-macrophage enrichment, as captured by CD68, tracks closely with PD-L1 expression in this cohort [17].

Another important observation was the enrichment of PD-L1 expression in adverse pathological subsets. PD-L1 positivity was significantly more frequent in right-sided tumours, poorly differentiated tumours, tumours with brisk TILs, T4 lesions, N2 disease, Stage-IV disease, and cases with lymph node metastasis. This overall pattern is partly concordant with the published literature. Li Y et al., in a systematic review and meta-analysis, found that PD-L1 expression in colorectal cancer was significantly associated with poor differentiation (OR 3.47, 95% CI 1.37–8.77, $p=0.008$) and right-sided colon cancer (OR 2.38, 95% CI 1.57–3.60, $p<0.0001$), even though pooled associations with lymph node metastasis and TNM stage were not statistically significant in that analysis [18]. In Valentini AM et al., tumour-cell PD-L1 expression was likewise associated with right-sided location ($p=0.004$) and G3 tumour grade ($p<0.001$) [11]. The association of PD-L1 with right-sided location deserves particular emphasis. In the present study, 80.0% of right-sided tumours were PD-L1 positive compared with only 25.0% of non-right-sided tumours. Right-sided colorectal cancers are biologically distinct from left-sided tumours; they are more often associated with mismatch-repair deficiency, microsatellite instability, BRAF mutation, serrated-pathway carcinogenesis, mucinous or medullary morphology, and a more inflamed immune milieu. Chen K et al., note that the evidence for right-sided colorectal cancer as a poor prognostic factor is strongest in metastatic disease [14], while KEYNOTE-164 also described MSI-H/dMMR metastatic colorectal cancers as often arising on the right side and being more poorly differentiated [19]. Since MSI-H/dMMR tumours also tend to show higher immune infiltration and greater checkpoint upregulation, the right-sided enrichment of PD-L1 in this cohort is biologically plausible and potentially therapeutically relevant [18].

The histological findings also fit known subtype biology. All mucinous adenocarcinomas and signet-ring cell carcinomas in this study were PD-L1 positive, while all PD-L1-negative tumours belonged to the conventional adenocarcinoma. Luo C et al., summarised that mucinous colorectal adenocarcinoma accounts for roughly 10%–20% of colorectal cancers, occurs more frequently in the proximal colon, is often diagnosed at a more advanced stage, and shows higher rates of lymph node infiltration and peritoneal spread than non-mucinous tumours [12]. Inamura K et al., (2015) and Yalcin S and Onguru O (2017) noted shared molecular features between mucinous and signet-ring carcinomas, including MSI-H and frequent BRAF alterations [20,21]. These biological links help explain why the non-conventional histologies in the present study clustered with PD-L1 positivity, advanced stage, and aggressive features. The finding that all poorly differentiated tumours were PD-L1 positive further supports the view that dedifferentiation in colorectal carcinoma is frequently accompanied by a more immunologically active but immune-evasive phenotype [18].

The TIL and CD68 results together suggest that PD-L1 expression in colorectal carcinoma is not simply a marker of immune escape, but also a readout of an inflamed tumour microenvironment. Brisk TILs were significantly more common in PD-L1-positive tumours (53.8%) and in the high-CD68 group (60.0%), whereas absent/scant TILs were concentrated in low-CD68 tumours (66.7%). This is consistent with the concept of adaptive immune resistance, in which an ongoing antitumour lymphocytic response is accompanied by compensatory checkpoint upregulation. Pagès F et al., (2018) demonstrated in the Immunoscore validation study that the density and location of immune infiltrates in colon cancer carry major prognostic value [22], and Ros J et al., (2023) reinforced the importance of TIL quantification in

colorectal carcinoma biology [23]. Valentini AM et al., also proposed that PD-L1-positive colorectal cancers with concomitant immune-cell infiltration represent a distinct immunological subset rather than a uniformly “cold” tumour [11]. The co-existence of brisk TILs, high TAM density, and PD-L1 positivity in our cohort likely reflects this inflamed-but-suppressed microenvironment.

The CD68 findings merit careful interpretation because Koelzer VH et al., (2016) have shown compartment-specific and phenotype-specific heterogeneity [10]. In the present study, higher CD68 density was associated with right-sided location, poor differentiation, Stage-IV disease, lymph node metastasis, and brisk TILs. This pattern suggests that, in this cohort, overall macrophage accumulation tracked with aggressive and immune-reactive disease. However, not all published data show the same direction for pan-macrophage markers. Koelzer VH et al., reported that strong stromal CD68 infiltration correlated with less lymph node metastasis and less tumour budding at the invasive front, underscoring that macrophage function depends on microlocalisation and not simply total numbers [10]. Kou Y et al., (2022) has similarly emphasised that pan-macrophage CD68 is less biologically specific than polarisation markers such as CD163 or CD86 [17]. Even so, the present results remain meaningful because they indicate that macrophage-rich tumours were significantly more likely to be PD-L1 positive, node-positive, and advanced-stage, pointing toward clinically relevant crosstalk between myeloid infiltration and checkpoint biology.

From a therapeutic standpoint, the observed convergence of PD-L1 positivity, brisk TILs, high TAM density, right-sided location, poor differentiation, and advanced stage supports the idea that PD-L1 in colorectal carcinoma is best interpreted as part of a broader immune-contexture rather than as a stand-alone marker. In metastatic colorectal cancer, the clearest benefit from PD-1/PD-L1 axis blockade is currently seen in MSI-H/dMMR tumours. KEYNOTE-177 established pembrolizumab as effective first-line therapy in MSI-H/dMMR metastatic colorectal cancer, and the final analysis confirmed durable anti-tumour benefit with longer progression-free survival and fewer treatment-related events than chemotherapy [24]. In KEYNOTE-164, pembrolizumab produced an objective response rate of 33% in previously treated MSI-H/dMMR colorectal cancer, with median duration of response not reached. Because MSI-H/dMMR tumours are often right-sided, poorly differentiated, and immune-infiltrated, the present findings are directionally consistent with the phenotype of colorectal cancers most likely to respond to checkpoint inhibition [19]. At the same time, PD-L1 expression alone is not sufficient for treatment selection, and its greatest current value may lie in refining the biological interpretation of the tumour microenvironment and identifying subsets that warrant integrated assessment with MMR/MSI status and other immune biomarkers [23].

Limitation(s)

The present study had certain limitations. It was a single-centre, hospital-based study which may have limited the generalisability of the results. Because of the cross-sectional design, only associations could be assessed, and causal or temporal relationships could not be established. The relatively small sample size limits the generalisability of the findings. The small cohort also increases the possibility of both type I and type II errors. The use of convenience sampling may also have introduced selection bias. In addition, immunohistochemical assessment of PD-L1 is influenced by antibody clone, staining protocol, scoring method, and cut-off values, and despite independent interpretation by two pathologists, some degree of interobserver variation remains possible. CD68 was used as a pan-macrophage marker and did not distinguish between different macrophage phenotypes such as M1 and M2 subsets. Molecular parameters such as mismatch repair status, microsatellite instability, and KRAS/BRAF mutation status were not evaluated, and survival or follow-up data were not available to directly validate the prognostic implications of PD-L1 expression. No

correction for multiple comparisons, such as Bonferroni correction or false discovery rate adjustment, was applied.

CONCLUSION(S)

In conclusion, the present study demonstrated a significant association between PD-L1 expression and CD68-positive tumour-associated macrophage infiltration in colorectal carcinoma, indicating an important interaction between immune checkpoint expression and the tumour microenvironment. PD-L1 positivity was significantly associated with right-sided tumour location, non-conventional histological subtypes, poor differentiation, brisk tumour-infiltrating lymphocytes, advanced T and N stage, higher overall TNM stage, and lymph node metastasis, thereby supporting its association with aggressive tumour behaviour. Increased CD68-positive macrophage density was also linked to adverse pathological features and immune-rich tumours. These findings suggest that PD-L1 may serve as a useful prognostic biomarker and a potential therapeutic target in colorectal carcinoma, while also highlighting the relevance of macrophage infiltration in tumour progression and immune modulation.

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