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Research Article

Cancer Immunology Research

Tumor PDCD1LG2 (PD-L2) Expression and the Lymphocytic Reaction to Colorectal Cancer

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Abstract

Expression of the immune checkpoint ligand CD274 (programmed cell death 1 ligand 1, PD-L1, from gene *CD274*) contributes to suppression of antitumor T cell–mediated immune response in various tumor types. However, the role of PDCD1LG2 (PD-L2, CD273, from gene *PDCD1LG2*) in the tumor microenvironment remains unclear. We hypothesized that tumor PDCD1LG2 expression might be inversely associated with lymphocytic reactions to colorectal cancer. We examined tumor PDCD1LG2 expression by IHC in 823 colon and rectal carcinoma cases within two U.S.-nationwide cohort studies and categorized tumors into quartiles according to the percentage of PDCD1LG2–expressing carcinoma cells. We conducted multivariable ordinal logistic regression analysis to assess the associations of tumor PDCD1LG2 expression with Crohn-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, or tumor-infiltrating lymphocytes, controlling for potential confounders, including microsatellite instability, CpG island methylator phenotype, long-interspersed nucleotide element-1 methylation, and *KRAS*, *BRAF*, and *PIK3CA* mutations. Tumor PDCD1LG2 expression was inversely associated with Crohn-like lymphoid reaction ($P_{trend} = 0.0003$). For a unit increase in the three-tiered ordinal categories of Crohn-like lymphoid reaction, a multivariable OR in the highest (vs. lowest) quartile of the percentage of PDCD1LG2–expressing tumor cells was 0.38 (95% confidence interval, 0.22–0.67). Tumor PDCD1LG2 expression was not associated with peritumoral lymphocytic reaction, intratumoral periglandular reaction, tumor-infiltrating lymphocytes, or patient survival ($P_{trend} > 0.13$). Thus, tumor PDCD1LG2 expression is inversely associated with Crohn-like lymphoid reaction to colorectal cancer, suggesting a possible role of PDCD1LG2-expressing tumor cells in inhibiting the development of tertiary lymphoid tissues during colorectal carcinogenesis. *Cancer Immunol Res; 5(11); 1–10. ©2017 AACR.*

Introduction

Immunotherapy has emerged as a promising strategy for cancer treatment (1). Studies suggest that tumor expression of immune checkpoint ligands triggers the transient downregulation of T-cell activity at the tumor–immune interface, leading to immune evasion (1). Therapeutic antibodies targeting the immune checkpoint receptor PDCD1 (programmed cell death 1, PD-1, from gene *PDCD1*) and its ligand, CD274 (PDCD1 ligand 1, PD-L1,

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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from gene *CD274*), are effective for treatment of various types of tumors (1, 2). However, the clinical efficacy of these checkpoint blockade immunotherapies varies by tumor type, tumor molecular characteristics, and immune-cell infiltrates, in addition to aberrant expression of immune checkpoint ligands in the tumor microenvironment (2–6).

Colorectal carcinogenesis involves a heterogeneous oncogenic process influenced by differing sets of genetic and epigenetic alterations, diet, microbiota, and host immunity (7–10). Potent lymphocytic reactions and higher density of T cells in colorectal carcinoma tissue have been associated with better patient survival, supporting a major role of antitumor immunity in inhibiting tumor progression (11–16). Previous studies have linked intense lymphocytic reaction to colorectal cancer to high tumor neoantigen load and certain tumor molecular characteristics, including high-level microsatellite instability (MSI-high; refs. 15–18). Evidence attests to the important role of CD274 (PD-L1) in suppressing antitumor immunity in a number of tumor types including colorectal cancer (1–3). PDCD1LG2 (PDCD1 ligand 2, PD-L2, from gene *PDCD1LG2*) is implicated in inducing immune tolerance under physiologic and pathologic conditions (1, 19–21); however, the potential role of PDCD1LG2 in the tumor microenvironment remains poorly understood. We hypothesized that tumor PDCD1LG2 expression might be inversely associated with lymphocytic reaction to colorectal cancer.

To test this hypothesis, we utilized colorectal carcinoma cases identified in two U.S.-nationwide prospective cohort studies and examined tumor PDCD1LG2 expression in relation to four histologic patterns of lymphocytic reactions: Crohn-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, and tumor-infiltrating lymphocytes (TILs). Our comprehensive database integrating tumor immunity data with relevant clinical, pathologic, and tumor molecular information enabled us to investigate the independent association of tumor PDCD1LG2 expression with lymphocytic reactions to colorectal cancer, controlling for potential confounders. In addition, as a secondary exploratory analysis, we assessed the prognostic value of PDCD1LG2 expression in colorectal carcinoma.

Materials and Methods

Study populations and histopathologic features

We utilized two prospective cohort studies: the Nurses' Health Study (121,701 women followed since 1976) and the Health Professionals Follow-Up Study (51,529 men followed since 1986; ref. 22). Participants have been sent follow-up questionnaires biennially to collect updated information on lifestyle factors and to identify newly diagnosed diseases, including colorectal cancers. The National Death Index was used to ascertain deaths of study participants and to identify unreported lethal colorectal cancer cases. Study physicians reviewed medical records of colorectal cancer patients to extract clinical information, including the American Joint Committee on Cancer stage, tumor location, and recorded cause of death in deceased individuals. Patients were followed until death or January 2012, whichever came first. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from hospitals across the United States, where participants with colorectal cancer had undergone tumor resection. The study pathologist (S. Ogino), who was blinded to other data, performed centralized pathology review and recorded histopathologic features. Tumor differentiation was defined as well to moderate (>50% glandular area) or poor (≤50% glandular area). Four histologic patterns of lymphocytic reactions, Crohn-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, and TILs, were scored as 0, 1, 2, or 3 by a pathologist (S. Ogino), as described previously (16). Because of the limited numbers of cases with scores of 2 and 3 in each lymphocytic reaction pattern, we categorized lymphocytic reaction patterns as negative/low (score 0), intermediate (score 1), or high (scores 2-3), as described previously (16, 23). Crohn-like lymphoid reactions were defined as a transmural lymphoid reaction across the full thickness of the bowel wall (16). A peritumoral lymphocytic reaction was defined as discrete lymphoid reactions surrounding tumors (16). An intratumoral periglandular reaction was defined as lymphocytic reactions in tumor stroma within the tumor mass (16). TILs were defined as lymphocytes on top of tumor cells in tissue sections (16). An agreement study on the four lymphocytic reaction patterns that encompassed more than 400 tumors was performed, and we observed a good concordance between independent reviews by two pathologists (S. Ogino and J. Glickman; ref. 16). The overall lymphocytic reaction score (ranging from 0 to 12) was calculated as the sum of scores for the four patterns of histologic lymphocytic reaction, as described previously (16). Of the 1,546 colon and rectal carcinomas diagnosed by 2008 that had available tumor FFPE blocks, we constructed tissue microarrays (TMA) from 1,014 cases (24). When constructing TMA blocks, we punched tumor areas, including tumor center and tumor margin, to select up to four 0.6-mm tissue cores from each tumor, considering spatial heterogeneity. We excluded 191 cases, in which PDCD1LG2 expression was unevaluable due to core exhaustion, detachment of cores during staining procedure, or insufficient staining quality. Consequently, we analyzed a total of 823 colorectal carcinoma cases with available PDCD1LG2 protein expression data (Supplementary Fig. S1). Tissue collection and analyses were approved by the human subjects committee at the Harvard T.H. Chan School of Public Health and the Brigham and Women's Hospital (Boston, MA).

IHC

For PDCD1LG2 (PD-L2) IHC, we utilized an automated staining system (Bond III, Leica Biosystems) following the manufacturer's protocols, with the use of an mAb to PDCD1LG2 that was generated in the laboratory of G.J. Freeman (clone 366C.9E5; mouse IgG1 kappa; dilution, 1:6,000; refs. 25–28). Prebaked sections were dewaxed, rehydrated, and incubated in ER2 solution (pH8; Leica Biosystems) for 30 minutes. Slides were incubated with anti-PDCD1LG2 for a total of 2 hours with two separate applications, followed by 8 minutes of post-primary blocking reagent, 12 minutes of horseradish peroxidase–labeled polymer, 5 minutes of peroxidase block, and 15 minutes of DAB developing. All reagents were components of the Bond Polymer Refine Detection system (Leica Biosystems; catalog number, DS9800). Slides were then counterstained by hematoxylin for 5 minutes. The positive control for PDCD1LG2 IHC was human tonsil and colorectum, in which we observed positive staining on immune cells, including dendritic cells in germinal centers within mucosa-associated lymphoid tissues and Crohn-like lymphoid reactions (Fig. 1A). Smooth muscle cells in these human specimens served as internal negative controls. To control for potential nonspecific binding of anti-PDCD1LG2, we used an isotype-identical antibody (clone DAK-GO1; mouse

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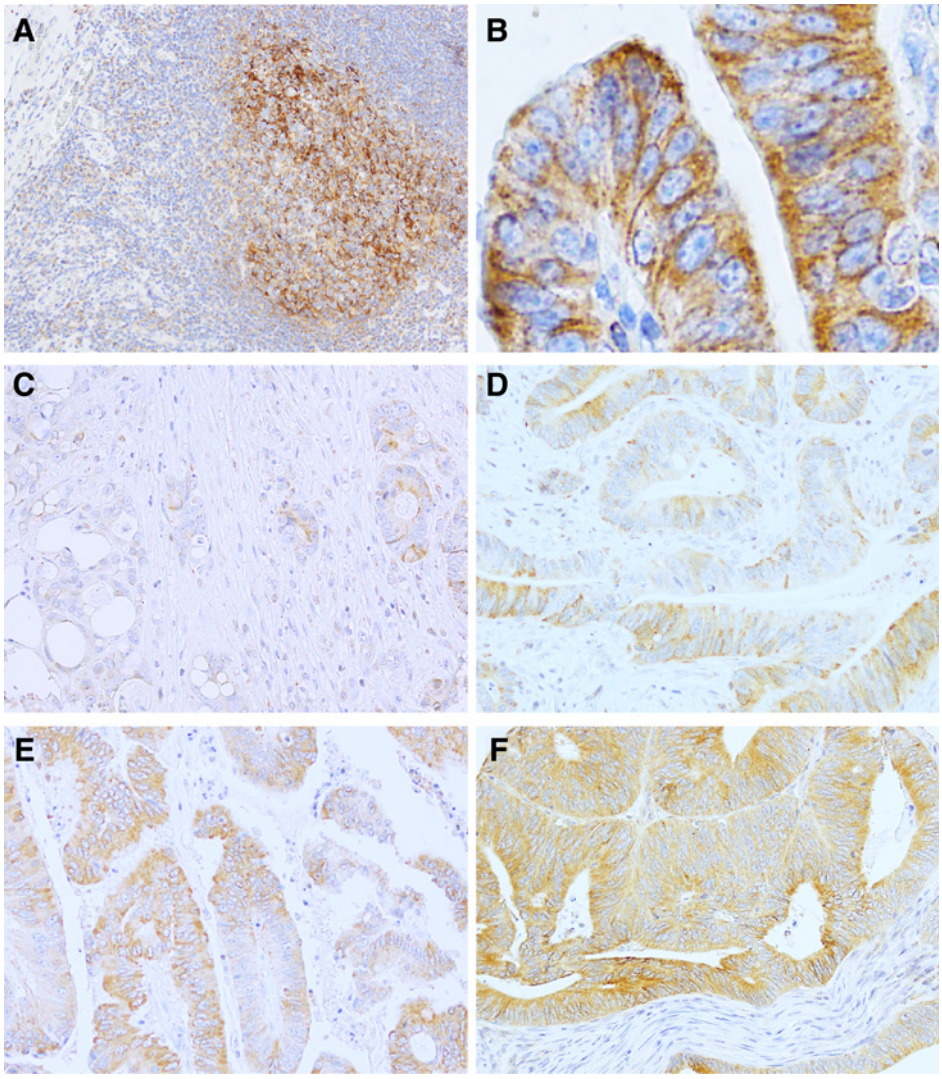

Figure 1.
IHC for PDCD1LG2 (PD-L2) in human tonsil (**A**) and colorectal carcinoma tissue (**B–F**). A strong expression of PDCD1LG2 is observed on immune cells, including dendritic cells in a germinal center, which served as positive controls (**A**). PDCD1LG2 expression is observed in the cytoplasm and on the membrane of colorectal carcinoma cells (**B**). Examples of PDCD1LG2 immunostaining in each quartile category of fraction of PDCD1LG2-expressing tumor cells are shown [quartile 1 (**C**), quartile 2 (**D**), quartile 3 (**E**), and quartile 4 (**F**)].

IgG1 kappa; dilution, 1:6000; Agilent, catalog number, X0931) with replacement of primary anti-PDCD1LG2 in the same IHC assay, which yielded negative staining (Supplementary Fig. S2). IHC expression of PDCD1LG2 was interpreted by a pathologist (Y. Masugi) unaware of other data. PDCD1LG2 expression was observed in the cytoplasm and on the membrane of colorectal carcinoma cells (Fig. 1B). We recorded the percentage of PDCD1LG2^{+} carcinoma cells in the cytoplasm and/or membrane (range, 0–100; mean, 55; median, 60; and interquartile range, 20–80) and categorized tumors into quartiles according to the percentage of PDCD1LG2-expressing tumor cells either in the cytoplasm or on the membrane (Fig. 1C–F). A subset of colorectal cancer cases ($n = 142$) was examined independently by a second pathologist (A. da Silva), and the concordance between the two observers was reasonable with a Spearman correlation coefficient of 0.74 ($P < 0.0001$; for the percentage of positive-staining tumor cells) and a weighted κ of 0.62 [95% confidence interval (CI), 0.53–0.72; for quartile categories of the percentage of positive-staining tumor cells]. PDCD1LG2 expression on stromal cells, including immune cells, was evaluated on the basis of the presence or absence of cytoplasmic and/or membrane staining in stromal cells within tumor tissues. We examined CD274 (PD-L1) expression by IHC and categorized tumor CD274 expression as low or high, as described previously (29, 30). Immunostaining for CD3, CD8, CD45RO (from gene *PTPRC*), and FOXP3 was conducted and the density of T cells in tumor tissue measured with the Ariol image analysis system (Genetix), as described previously (14, 31).

DNA analyses

DNA was extracted from archival colorectal cancer tissue blocks. MSI status was analyzed with the use of 10 microsatellite markers (*BAT25*, *BAT26*, *BAT40*, *D2S123*, *D5S346*, *D17S250*, *D18S55*, *D18S56*, *D18S67*, and *D18S487*), as described previously (22). We defined MSI-high as the presence of instability in ≥30% of the markers, and non–MSI-high as instability in <30% of the markers (22). Methylation analyses for eight promoters specific for the CpG island methylator phenotype (CIMP; *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) were conducted using bisulfite-treated DNA and real-time PCR, as described previously (22). CIMP-high, CIMP-low,

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and CIMP-negative were defined as ≥6/8, 1/8–5/8, and 0/8 methylated promoters, respectively, according to the previously established criteria (22). Long-interspersed nucleotide element-1 (LINE-1) methylation was determined using bisulfite modification of DNA followed by PCR and pyrosequencing (22). PCR and pyrosequencing were performed for *BRAF* (codon 600), *KRAS* (codons 12, 13, 61, and 146), and *PIK3CA* (exons 9 and 20; ref. 22). We performed quantitative PCR to measure the amount of *Fusobacterium nucleatum* (*F. nucleatum*) DNA in tumor tissue, as described previously (32). We categorized colorectal carcinoma cases with detectable *F. nucleatum* DNA as low or high, using the median as the cut-off point amount among *F. nucleatum*-positive carcinomas (32).

Statistical analysis

Statistical analyses were conducted using SAS (version 9.4, SAS Institute) and all *P* values were two sided. Our primary hypothesis testing was an assessment of the associations of tumor PDCD1LG2 expression (as a quartile predictor variable) with the four histologic patterns of lymphocytic reaction (Crohn-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, and TILs) as outcome variables. Because we examined the four outcome lymphocytic reaction variables, we adjusted the two-sided α level to 0.01 (≈0.05/4) by Bonferroni correction for multiple hypothesis testing.

We performed multivariable logistic regression analysis to control for potential confounders. The multivariable model initially included age at diagnosis (continuous), sex (female vs. male), year of diagnosis (continuous), family history of colorectal cancer in a first-degree relative [present vs. absent vs. missing (1.3% missing)], tumor location [proximal colon vs. distal colon vs. rectum vs. missing (0.5% missing)], MSI status [MSI-high vs. non–MSI-high vs. missing (2.9% missing)], CIMP status [high vs. low/negative vs. missing (7.9% missing)], *BRAF* mutation [mutant vs. wild type vs. missing (2.2% missing)], *KRAS* mutation [mutant vs. wild type vs. missing (2.7% missing)], *PIK3CA* mutation [mutant vs. wild type vs. missing (8.6% missing)], and LINE-1 methylation level (continuous). For the cases with missing data on LINE-1 methylation (2.7% missing), we assigned a separate indicator variable. A backward elimination with a threshold of $P = 0.05$ was used to select covariates for the final model. For cases with missing information in any of the categorical variables, we included those cases in the majority category of a given covariate to limit the degrees of freedom of the final models. We confirmed that excluding the cases with missing information in any of the covariates did not substantially alter results through sensitivity analyses (data not shown). We assessed the proportional odds assumption in an ordinal logistic regression model, which was generally satisfied ($P > 0.05$).

To assess associations between tumor PDCD1LG2 expression and other categorical variables, the χ^2 test was performed. To compare mean age and mean LINE-1 methylation levels, an ANOVA assuming equal variances was performed. All of those cross-sectional analyses for clinical, pathologic, and molecular associations were secondary exploratory analyses, with adjusted two-sided α level of 0.003 (≈0.05/15) for multiple hypothesis testing.

We conducted Kaplan–Meier analysis to compare survival between patient groups. Deaths from causes other than colorectal cancer were censored in colorectal cancer–specific mortality analyses. To adjust for confounding, we performed Cox proportional hazards regression analysis and calculated HR for mortality. The multivariable Cox proportional hazards regression models initially included disease stage (I/II vs. III/IV/unknown) in addition to the same set of variables as in the aforementioned initial multivariable logistic regression analysis. We performed a backward elimination with a threshold of $P = 0.05$ to select variables for the final multivariable model. To limit the degrees of freedom of the models, we included cases with missing information in a categorical covariate in the majority category. For the cases with missing data on LINE-1 methylation, we assigned a separate indicator variable. We confirmed that excluding the cases with missing data in any of the covariates did not materially alter results (data not shown). The proportionality of hazards assumption was satisfied ($P > 0.05$) by evaluating time-dependent variables, which were the cross-product of the PDCD1LG2 expression variable and survival time.

Results

We examined tumor expression of PDCD1LG2 (PD-LG2) by IHC in 823 colorectal carcinoma cases within the two U.S.-nationwide cohort studies (Fig. 1). Tumors were categorized into quartiles according to the percentage of PDCD1LG2-expressing carcinoma cells (median, 60%; interquartile range, 20%–80%). Clinical, pathologic, and molecular characteristics according to tumor PDCD1LG2 expression in colorectal cancer are summarized in Table 1. Higher fractions of PDCD1LG2-expressing tumor cells were associated with female ($P = 0.0001$), well-to-moderate tumor differentiation ($P = 0.001$), and a high level of tumor CD274 (PD-L1) expression ($P = 0.001$). We performed multivariable ordinal logistic regression analysis to assess relationships of sex (as a predictor variable) and tumor PDCD1LG2 expression (as an outcome variable) and found that female gender remained significantly associated with PDCD1LG2 expression (for a unit increase of quartile categories of PDCD1LG2 expression; OR, 1.68; 95% CI, 1.29–2.18; $P = 0.0001$; Supplementary Table S1). There was a trend toward higher tumor PDCD1LG2 expression associated with LINE-1 hypomethylation (OR for 30% decrease in methylation level, 1.65; 95% CI, 1.11–2.44; $P = 0.013$ with the adjusted α level of 0.004; Supplementary Table S1). Tumor PDCD1LG2 expression was not significantly associated with the other characteristics examined ($P > 0.03$; with the adjusted α level of 0.003; Table 1). PDCD1LG2-expressing stromal cells, including immune cells, were found in 630 (78%) of 810 cases. The presence of PDCD1LG2-expressing stromal cells was not significantly associated with any clinical, pathologic, or molecular features ($P > 0.007$; with the adjusted α level of 0.003; Supplementary Table S2).

Table 2 shows the distribution of colorectal cancer cases according to tumor PDCD1LG2 expression and the four histologic patterns of lymphocytic reaction. Tumor PDCD1LG2 expression correlated inversely with Crohn-like lymphoid reaction ($P = 0.0002$, by Spearman correlation test). In our primary hypothesis testing, we used ordinal logistic regression analysis to assess the association of tumor PDCD1LG2 expression (an ordinal quartile predictor variable (1–4)] with Crohn-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, or TILs as an outcome variable (ordinal 3-tiered; negative/low, intermediate, and high; Table 3; Supplementary Table S3). Tumor PDCD1LG2 expression was inversely associated with Crohn-like lymphoid reaction in the

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Table 1. Clinical, pathologic, and molecular features according to tumor PDCD1LG2 (PD-L2) expression in 823 colorectal cancer cases

		Fraction of PDCD1LG2-expressing tumor cells (quartile)[a]				
Characteristic[b]	**Total ($n = 823$)**	**0%–20%, quartile 1 ($n = 210$)**	**21%–50%, quartile 2 ($n = 187$)**	**51%–80%, quartile 3 ($n = 247$)**	**81%–100%, quartile 4 ($n = 179$)**	**P^c**
Mean age ± SD (y)	68.7 ± 9.0	68.1 ± 9.1	68.8 ± 10.1	69.3 ± 8.7	68.5 ± 8.1	0.60
Sex						0.0001
Men	364 (44%)	98 (47%)	96 (51%)	117 (47%)	53 (30%)	
Women	459 (56%)	112 (53%)	91 (49%)	130 (53%)	126 (70%)	
Year of diagnosis						0.66
Prior to 1996	272 (33%)	76 (36%)	59 (32%)	77 (31%)	60 (34%)	
1996–2000	284 (35%)	62 (30%)	72 (39%)	88 (36%)	62 (35%)	
2001–2008	267 (32%)	72 (34%)	56 (30%)	82 (33%)	57 (32%)	
Family history of colorectal cancer in a first-degree relative						0.58
Absent	642 (79%)	164 (80%)	138 (75%)	196 (80%)	144 (80%)	
Present	170 (21%)	42 (20%)	45 (25%)	48 (20%)	35 (20%)	
Tumor location						0.040
Cecum	146 (18%)	45 (22%)	40 (22%)	39 (16%)	22 (12%)	
Ascending to transverse colon	260 (32%)	68 (33%)	58 (31%)	67 (27%)	67 (38%)	
Splenic flexure to sigmoid	249 (30%)	56 (27%)	46 (25%)	90 (36%)	57 (32%)	
Rectosigmoid and rectum	164 (20%)	40 (19%)	42 (23%)	51 (21%)	31 (18%)	
Disease stage						0.39
I	171 (23%)	39 (21%)	42 (24%)	50 (22%)	40 (24%)	
II	250 (33%)	61 (33%)	63 (37%)	78 (34%)	48 (29%)	
III	216 (29%)	54 (29%)	44 (26%)	74 (32%)	44 (26%)	
IV	120 (16%)	33 (18%)	23 (13%)	29 (13%)	35 (21%)	
Tumor differentiation						0.001
Well to moderate	746 (91%)	177 (84%)	171 (92%)	234 (95%)	164 (92%)	
Poor	75 (9%)	33 (16%)	14 (8%)	13 (5%)	15 (8%)	
MSI status						0.86
Non–MSI-high	663 (83%)	167 (82%)	149 (82%)	203 (84%)	144 (85%)	
MSI-high	136 (17%)	37 (18%)	33 (18%)	40 (16%)	26 (15%)	
CIMP status						0.047
Low/negative	627 (83%)	154 (79%)	141 (82%)	183 (81%)	149 (90%)	
High	131 (17%)	40 (21%)	30 (18%)	44 (19%)	17 (10%)	
BRAF mutation						0.52
Wild type	686 (85%)	171 (83%)	161 (88%)	208 (84%)	146 (85%)	
Mutant	119 (15%)	34 (17%)	21 (12%)	39 (16%)	25 (15%)	
KRAS mutation						0.27
Wild type	474 (59%)	111 (55%)	117 (65%)	145 (59%)	101 (59%)	
Mutant	327 (41%)	92 (45%)	64 (35%)	100 (41%)	71 (41%)	
PIK3CA mutation						0.43
Wild type	639 (85%)	150 (83%)	154 (88%)	204 (86%)	131 (82%)	
Mutant	113 (15%)	31 (17%)	22 (13%)	32 (14%)	28 (18%)	
Mean LINE-1 methylation level ± SD (%)	62.2 ± 9.7	63.5 ± 9.9	61.9 ± 9.7	61.7 ± 9.9	61.6 ± 8.9	0.15
Fusobacterium nucleatum DNA						0.44
Negative	574 (87%)	139 (85%)	129 (85%)	183 (89%)	123 (89%)	
Low	42 (6%)	15 (9%)	8 (5%)	12 (6%)	7 (5%)	
High	42 (6%)	10 (6%)	14 (9%)	10 (5%)	8 (6%)	
Tumor CD274 (PD-L1) expression level						0.001
Low	296 (38%)	84 (44%)	80 (45%)	86 (36%)	46 (27%)	
High	478 (62%)	106 (56%)	98 (55%)	150 (64%)	124 (73%)	

Abbreviation: CIMP, CpG island methylator phenotype.

[a]Tumors were categorized into quartiles according to the percentage of PDCD1LG2-expressing carcinoma cells (quartile 1, 0%–20%; quartile 2, 21%–50%; quartile 3, 51%–80%; quartile 4, 81%–100%).

[b]Percentage indicates the proportion of cases with a specific clinical, pathologic, or molecular feature in colorectal cancer cases with each quartile category of tumor PDCD1LG2 expression.

[c]To assess associations between the quartile categories of tumor PDCD1LG2 expression and categorical data, the χ^2 test was performed. To compare mean age and mean LINE-1 methylation, an ANOVA was performed. We adjusted two-sided α level to 0.003 ($\approx$0.05/15) by simple Bonferroni correction for multiple hypothesis testing.

univariable and multivariable ordinal logistic regression analyses ($P_{trend} < 0.0004$). For a unit increase in the ordinal categories of Crohn-like lymphoid reaction, a multivariable OR in the highest (vs. lowest) quartile of the percentage of PDCD1LG2-expressing tumor cells was 0.38 (95% CI, 0.22–0.67). We did not observe any statistically significant association of tumor PDCD1LG2 expression with peritumoral lymphocytic reaction, intratumoral periglandular reaction, or TILs ($P_{trend} > 0.06$; with the adjusted α level of 0.01). In addition, we found an inverse correlation between tumor PDCD1LG2 expression and the overall lymphocytic reaction score (Spearman correlation coefficient $r = -0.11$, $P = 0.006$; Supplementary Table S4). In a multivariable ordinal logistic regression analysis, tumor PDCD1LG2 expression was not significantly associated with the overall lymphocytic reaction score ($P_{trend} = 0.046$, with the adjusted α level of 0.01; Supplementary Table S5). As an exploratory analysis, we conducted Spearman

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Table 2. Distribution of colorectal cancer cases according to tumor PDCD1LG2 (PD-L2) expression and histologic lymphocytic reaction patterns

		Fraction of PDCD1LG2-expressing tumor cells (quartile)[a]				
	Total no.	0%–20%, quartile 1	21%–50%, quartile 2	51%–80%, quartile 3	81%–100%, quartile 4	P^b
Crohn-like lymphoid reaction ($n = 680$)						0.0002
Negative/low	505 (74%)	121 (66%)	105 (71%)	151 (76%)	128 (84%)	
Intermediate	127 (19%)	42 (23%)	31 (21%)	39 (20%)	15 (10%)	
High	48 (7%)	19 (10%)	11 (7%)	9 (5%)	9 (6%)	
Peritumoral lymphocytic reaction ($n = 811$)						0.80
Negative/low	118 (15%)	32 (15%)	24 (13%)	31 (13%)	31 (18%)	
Intermediate	573 (71%)	145 (70%)	132 (71%)	178 (74%)	118 (67%)	
High	120 (15%)	30 (14%)	30 (16%)	32 (13%)	28 (16%)	
Intratumoral periglandular reaction ($n = 813$)						0.10
Negative/low	102 (13%)	24 (12%)	16 (9%)	30 (12%)	32 (18%)	
Intermediate	618 (76%)	160 (77%)	146 (78%)	186 (77%)	126 (71%)	
High	93 (11%)	23 (11%)	25 (13%)	26 (11%)	19 (11%)	
TILs ($n = 812$)						0.07
Negative/low	594 (73%)	149 (72%)	128 (69%)	177 (73%)	140 (79%)	
Intermediate	128 (16%)	33 (16%)	30 (16%)	42 (17%)	23 (13%)	
High	90 (11%)	25 (12%)	28 (15%)	23 (10%)	14 (8%)	

[a]Tumors were categorized into quartiles according to the percentage of PDCD1LG2–expressing carcinoma cells (quartile 1, 0%–20%; quartile 2, 21%–50%; quartile 3, 51%–80%; quartile 4, 81%–100%).

[b]*P* value was calculated by Spearman correlation test between tumor PDCD1LG2 expression (ordinal quartile categories; 1–4) and each histologic pattern of lymphocytic reaction (ordinal categories; negative/low, intermediate, and high). Because we assessed four primary outcome variables, we adjusted two-sided α level to 0.01 ($\approx$0.05/4) by simple Bonferroni correction.

correlation tests to assess the associations of tumor PDCD1LG2 expression [ordinal quartile categories (1–4)] with $CD3^+$ cell, $CD8^+$ cell, PTPRC $(CD45RO)^+$ cell, and $FOXP3^+$ cell densities (cells/mm^2; continuous variables) in tumor tissue and found only weak correlations [Spearman correlation coefficient $r < 0.25$ (0.24 for $CD3^+$ cells, 0.13 for $CD8^+$ cells, 0.12 for $PTPRC^+$ cells, and 0.06 for $FOXP3^+$ cells)].

As a secondary analysis, we conducted Kaplan–Meier analysis and Cox proportional hazards regression analysis to examine a prognostic role of tumor PDCD1LG2 expression in colorectal

Table 3. Ordinal logistic regression analysis to assess the association of tumor PDCD1LG2 (PD-L2) expression (predictor) with histologic lymphocytic reaction (outcome)

		Univariable OR (95% CI)	Multivariable OR (95% CI)[a]
Model for Crohn-like lymphoid reaction as an ordinal outcome variable ($n = 680$)			
Fraction of PDCD1LG2-expressing tumor cells (quartile)[b]	Quartile 1	1 (reference)	1 (reference)
	Quartile 2	0.78 (0.49–1.24)	0.79 (0.48–1.30)
	Quartile 3	0.61 (0.39–0.95)	0.58 (0.36–0.93)
	Quartile 4	0.38 (0.22–0.64)	0.38 (0.22–0.67)
	P_{trend}[c]	0.0002	0.0003
Model for peritumoral lymphocytic reaction as an ordinal outcome variable ($n = 811$)			
Fraction of PDCD1LG2-expressing tumor cells (quartile)[b]	Quartile 1	1 (reference)	1 (reference)
	Quartile 2	1.18 (0.77–1.82)	1.20 (0.78–1.85)
	Quartile 3	1.06 (0.71–1.58)	1.09 (0.73–1.64)
	Quartile 4	0.97 (0.63–1.50)	1.01 (0.65–1.57)
	P_{trend}[c]	0.81	0.98
Model for intratumoral periglandular reaction as an ordinal outcome variable ($n = 813$)			
Fraction of PDCD1LG2-expressing tumor cells (quartile)[b]	Quartile 1	1 (reference)	1 (reference)
	Quartile 2	1.28 (0.81–2.04)	1.32 (0.83–2.11)
	Quartile 3	0.95 (0.61–1.46)	0.99 (0.64–1.53)
	Quartile 4	0.72 (0.45–1.14)	0.75 (0.47–1.20)
	P_{trend}[c]	0.10	0.16
Model for TILs as an ordinal outcome variable ($n = 812$)			
Fraction of PDCD1LG2-expressing tumor cells (quartile)[b]	Quartile 1	1 (reference)	1 (reference)
	Quartile 2	1.19 (1.78–1.82)	1.34 (0.84–2.13)
	Quartile 3	0.92 (0.61–1.39)	0.92 (0.58–1.44)
	Quartile 4	0.67 (0.42–1.07)	0.81 (0.49–1.35)
	P_{trend}[c]	0.07	0.23

[a]The multivariable ordinal logistic regression analysis model initially included age, sex, year of diagnosis, family history of colorectal carcinoma in any parent or sibling, tumor location, MSI, CpG island methylator phenotype, *KRAS, BRAF*, and *PIK3CA* mutations, and LINE-1 methylation level. A backward elimination with a threshold of $P = 0.05$ was used to select variables in the final models. Variables remaining in the final multivariable model for Crohn-like lymphoid reaction were shown in Supplementary Table S3.

[b]Tumors were categorized into quartiles according to the percentage of PDCD1LG2–expressing carcinoma cells (quartile 1, 0%–20%; quartile 2, 21%–50%; quartile 3, 51%–80%; quartile 4, 81%–100%).

[c]P_{trend} value was calculated by the linear trend across the ordinal quartile categories of tumor PDCD1LG2 expression (1–4; as a predictor variable) in the ordinal logistic regression model for each histologic pattern of lymphocytic reaction (negative/low, intermediate, and high; as an ordinal outcome variable). Because we assessed four primary outcome variables, we adjusted two-sided α level to 0.01 ($\approx$0.05/4) by simple Bonferroni correction.

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Table 4. Tumor PDCD1LG2 (PD-L2) expression and colorectal cancer mortality

		Colorectal cancer–specific mortality			Overall mortality		
Fraction of PDCD1LG2-expressing tumor cells[a]	Total no.	No. of events	Univariable HR (95% CI)	Multivariable HR (95% CI)[b]	No. of events	Univariable HR (95% CI)	Multivariable HR (95% CI)[b]
Quartile 1	207	71	1 (reference)	1 (reference)	122	1 (reference)	1 (reference)
Quartile 2	185	58	0.87 (0.61–1.23)	0.99 (0.70–1.41)	106	0.93 (0.71–1.20)	1.03 (0.79–1.34)
Quartile 3	246	63	0.70 (0.50–0.98)	0.68 (0.48–0.95)	130	0.85 (0.67–1.09)	0.84 (0.65–1.07)
Quartile 4	179	59	0.92 (0.65–1.31)	0.95 (0.67–1.34)	95	0.85 (0.65–1.11)	0.88 (0.67–1.15)
P_{trend}[c]			0.35	0.26		0.17	0.14

[a]Tumors were categorized into quartiles according to the percentage of PDCD1LG2–expressing carcinoma cells (quartile 1, 0%–20%; quartile 2, 21%–50%; quartile 3, 51%–80%; quartile 4, 81%–100%).

[b]The multivariable Cox regression model initially included age, sex, year of diagnosis, family history of colorectal carcinoma in any parent or sibling, tumor location, disease stage, MSI, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and LINE-1 methylation level. A backward elimination with a threshold of $P = 0.05$ was used to select variables in the final models.

[c]P_{trend} value was calculated across the ordinal quartile categories of tumor PDCD1LG2 expression (1–4) as a continuous variable in the Cox regression models.

cancer. No statistically significant association between tumor PDCD1LG2 expression and colorectal cancer–specific or overall mortality (Table 4; Supplementary Fig. S3) was observed. In addition, we conducted multivariable Cox proportional hazards regression analyses and found that stronger Crohn-like lymphoid reaction was associated with favorable patient outcome in our current dataset ($P_{trend} = 0.0005$ for colorectal cancer-specific mortality, $P_{trend} < 0.0001$ for overall mortality; Supplementary Table S6).

Discussion

In the current study, we tested the hypothesis that tumor PDCD1LG2 (PD-L2) expression might be inversely associated with lymphocytic reactions to colorectal cancer. We found an inverse association between tumor PDCD1LG2 expression and Crohn-like lymphoid reaction, independent of potential confounders, including MSI status, CIMP status, and LINE-1 methylation; these specific tumor molecular characteristics have been associated with the abundance of lymphocytic infiltration in colorectal carcinoma tissue (16). Our results suggest a possible role of PDCD1LG2-expressing tumor cells in suppression of antitumor immune responses to colorectal cancer.

Colorectal cancer represents a heterogeneous group of tumors that results from not only progressive accumulations of differing sets of somatic molecular alterations, but also various host–tumor interactions, including antitumor immunity (33–35). The assessment of host immunity against colorectal cancer in the tumor microenvironment is increasingly important in translational research and routine clinical practice for tumor classification (36, 37). A strong histologic lymphocytic reaction to colorectal carcinoma has been associated with better patient survival (15, 16). These data indicate that histopathologic characterization of lymphocytic infiltrates in cancer tissue is of value when assessing the magnitude of antitumor immune response to colorectal malignancies.

We found that tumor PDCD1LG2 expression was inversely associated with Crohn-like lymphoid reaction, but not with peritumoral lymphocytic reaction, intratumoral periglandular reaction, or TILs, suggesting the unique immunologic nature of Crohn-like lymphoid reaction. Crohn-like lymphoid reaction is characterized by the transmural presence of discrete lymphocytic aggregates with tertiary lymphoid structures, including germinal centers, T cell–rich area, and high endothelial venules (38–40). Previous studies have linked strong Crohn-like lymphoid reaction to favorable outcomes in patients with colorectal carcinoma (15, 16, 38, 39). Tertiary lymphoid tissues, such as Crohn-like lymphoid reaction, have a potential role in maintaining adaptive immune responses to colorectal cancer, as a local source of antigen-specific effector B and T cells (39, 40). Although the precise role of PDCD1LG2 in the tumor microenvironment remains to be investigated, PDCD1LG2 appears to be important in modulation of type II Th2 cell immune responses under physiologic and pathologic conditions (19). It is possible that PDCD1LG2 may inhibit functional activities of Th2 cells, such as precursors of follicular Th cells that are responsible for the formation of germinal centers (41), in the tumor microenvironment, leading to downregulation of Crohn-like lymphoid reaction against colorectal carcinoma.

The colocalization between PDCD1 (PD-1)-expressing cells and T-cell infiltrates has been reported in various solid tumors (2, 42). A study on several tumor types, including colorectal cancer, has demonstrated strong positive correlations of *PDCD1LG2* and *CD274* with the IFNG (IFN-γ) and CD8 signaling pathways (21). In the tumor microenvironment, PDCD1LG2 can be induced by several proinflammatory cytokines, such as IFNG, IL4, and CSF2 (colony-stimulating factor 2), that can be produced by $CD8^+$ cytotoxic T cells, $CD4^+$ type I helper T (Th1) cells, $CD4^+$ Th2 cells, and macrophages (1, 2). Although we observed a positive association of PDCD1LG2 expression with CD274 (PD-L1) expression or $CD3^+$ cell density in tumor tissues, we recognize the limitations of TMA-based singleplex IHC in the assessment of spatial relationships of PDCD1LG2-expressing cells or CD274-expressing cells with various subpopulations of immune cells. Future studies utilizing multiplex IHC of whole tissue sections assisted with digital image analysis system would likely unveil the possible spatial associations of the two PDCD1 ligands with specific immune-cell fractions.

PDCD1LG2 expression is commonly observed in dendritic cells and macrophages within secondary and tertiary lymphoid organs throughout the body, whereas CD274 is more broadly expressed on antigen-presenting cells and other hematopoietic cell types (1, 19, 43), suggesting differing functional roles of the two PDCD1 ligands in the immunomodulation of host immunity. PDCD1LG2 and CD274 can be expressed in stromal cells, including immune cells, within tumor tissues of various tumor types (18, 42, 43), which is consistent with our previous (29) and current findings on colorectal cancer. In the tumor microenvironment, PDCD1LG2 and CD274 may be induced in a variety of immune and nonimmune cells by the adaptive mechanism, depending on the milieu of inflammatory cytokines (1, 2). These lines of evidence, together with our findings, underscore the

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complex interactions between neoplastic and immune cells in the tumor microenvironment.

Our data suggesting an association of tumor PDCD1LG2 expression with female gender are intriguing. Female gender was associated with increased PDCD1LG2 expression in a study of hematopoietic neoplasms (44), similar to our findings in colorectal carcinoma. Sex hormones, such as estrogens, may upregulate cellular expression of immune checkpoint molecules, including the PDCD1 and its ligand CD274, in a variety of cell types (45, 46). Taken together, our population-based data will likely inform further mechanistic studies to clarify potential roles of female sex hormones in the regulation of the immune checkpoint pathway in the tumor microenvironment.

In our population-based data, we found a trend toward higher tumor PDCD1LG2 expression associated with LINE-1 hypomethylation, although not statistically significant at the α level after adjusting for multiple hypothesis testing. $FOXP3^+$ cell density in colorectal cancer tissue, which has been inversely associated with tumor CD274 expression (29), was not correlated with PDCD1LG2 expression in the current analysis, suggesting a differential influence of the two PDCD1 ligands on regulatory T cells in colorectal cancer microenvironments.

In our exploratory analysis, tumor PDCD1LG2 expression was not significantly associated with colorectal cancer mortality, consistent with a study using gene expression data on *PDCD1LG2* from a colonic adenocarcinoma cohort in The Cancer Genome Atlas dataset (21). Whereas one study showed that PDCD1LG2 expression in esophageal cancer was associated with poor prognosis, studies on other tumor types, such as pancreatic carcinoma, hepatocellular carcinoma, breast carcinoma, and ovarian carcinoma, did not show a prognostic role of tumor PDCD1LG2 expression (19, 47). Further studies are needed to determine the prognostic role of PDCD1LG2 expression in different types of malignant tumors.

One study reported that not only CD274, but also PDCD1LG2 status was useful for predicting clinical response to pembrolizumab (mAb to PDCD1) in patients with head and neck carcinoma (43). Although tumor PDCD1LG2 expression was not significantly associated with colorectal cancer survival in our current study, tumor PDCD1LG2 expression was inversely associated with Crohn-like lymphoid reaction, the latter of which has been an important arm of the adaptive immune response in colorectal carcinogenesis. Therefore, immunotherapy strategies that block the PDCD1LG2/PDCD1 axis may prolong patient survival through reactivation of antitumor immunity, and our current findings may have clinical implications.

We recognize limitations of the current study. First, the cross-sectional nature of the study makes it challenging to determine the causal relationship between PDCD1LG2 expression and Crohn-like lymphoid reaction. Nonetheless, our specific hypothesis is based on several lines of evidence indicating that PDCD1LG2 checkpoint ligand expression contributes to suppression of T cell–mediated immune reaction (1, 2, 19, 20, 28, 43). Another limitation is the lack of standardized antibody and evaluation methods for PDCD1LG2 IHC in FFPE tissues. We used the automated immunostaining system as well as the established mAb to PDCD1LG2 that has been validated in previous studies (25–28). Considering the binding activity of the PDCD1LG2 ligand to its cognate cell surface receptors on immune cells, a fraction of tumor cells with PDCD1LG2 expression on the cell surface membrane is likely important. However, membranous PDCD1LG2 staining was considerably masked by cytoplasmic staining, which likely caused an underestimation of membrane PDCD1LG2 expression and limited its reproducibility when assayed. Hence, we assessed fractions of tumor cells that expressed PDCD1LG2 in the cytoplasm and/or membrane, which is a method similar to that in the previous studies on solid tumors (27, 28). A blinded and independent assessment of tumor PDCD1LG2 expression was performed, and we confirmed substantial interobserver agreement between the two pathologists. Despite limitations of IHC evaluation for PDCD1LG2 expression, we could demonstrate a significant and strong inverse association between tumor PDCD1LG2 expression and Crohn-like lymphoid reaction.

Strengths of our current study include the use of our molecular pathologic epidemiology (48) database of a large number of colorectal carcinoma cases in the two independent prospective cohort studies, which integrates clinicopathologic features, major tumor molecular features, and tumor immunity status in colorectal cancer tissue. Our study design (49, 50) and population-based database allowed us to rigorously investigate the association of tumor PDCD1LG2 expression with lymphocytic reaction to colorectal cancer, controlling for multiple potential confounders. In addition, our colorectal cancer specimens were collected from a large number of hospitals in diverse settings across the United States, which increases the generalizability of our findings.

In conclusion, we have shown that tumor PDCD1LG2 expression is inversely associated with Crohn-like lymphoid reactions to colorectal carcinoma, suggesting a possible influence of PDCD1LG2-expressing tumor cells on adaptive antitumor immunity. Upon validation, our human population data can likely inform translational research on the development of immunotherapy strategies targeting immune checkpoint mechanisms.

Disclosure of Potential Conflicts of Interest

F.S. Hodi reports receiving a commercial research grant from Bristol-Myers Squibb (to institution), other commercial research support from Genentech (to institution for clinical trial support), and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, Celldex, EMD Serono, Genentech, and Merck. G.J. Freeman has received speakers bureau honoraria from Expert Connect, MI Bioresearch, and MPM Capital and has ownership interest (including patents) in AstraZeneca, Bristol-Myers Squibb, Dako, EMD Serono, Merck, Novartis (all are related to licensed patents in the PD-1/PD-L1 field), and Roche. S.J. Rodig reports receiving a commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member for PerkinElmer Inc. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The authors assume full responsibility for analyses and interpretation of these data.

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