

PD-L1 (B7-H1) Expression by Urothelial Carcinoma of the Bladder and BCG-Induced Granulomata

Associations With Localized Stage Progression

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Dr. Eugene Kwon has filed a patent application pertaining to B7-H1 as a prognostic marker in cancer.

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BACKGROUND. PD-L1 (programmed death ligand 1, B7-H1) is a cell surface glycoprotein that can impair T-cell function. PD-L1 is aberrantly expressed by multiple human malignancies and has been shown to carry a highly unfavorable prognosis in patients with kidney cancer. The role of PD-L1 was evaluated as a mechanism for local stage progression in urothelial carcinoma (UC) of the bladder.

METHODS. Using immunohistochemistry, PD-L1 expression was evaluated in a cohort of 280 high-risk UCs of the bladder. PD-L1 was modeled as a predictor of bladder cancer stage using ordinal logistic regression. Other covariates evaluated as potential confounders included age, gender, tumor grade, and lymphocytic infiltration. Further, PD-L1 was evaluated as a potential mechanism of bacillus Calmette-Guerin (BCG) failure in the subset of high-risk nonmuscle-invasive tumors that received this treatment.

RESULTS. PD-L1 expression was observed in 7% of pTa, 16% of pT1, 23% of pT2, 30% of pT3/4, and 45% of carcinoma in situ (CIS) tumors. PD-L1 expression was associated with high-grade tumors (odds ratio [OR] = 2.4, $P = .009$) and tumor infiltration by mononuclear cells (OR = 5.5, $P = .004$). We observed that the key determinants of stage progression in this cohort were World Health Organization/International Society of Urologic Pathology (WHO/ISUP) high-grade tumor pathology (OR = 4.77, 95% confidence interval [CI]: 2.73–8.34; $P < .001$) and PD-L1 expression (OR = 2.20, $P = .012$). PD-L1 expression was found to be extremely abundant in the BCG-induced bladder granulomata in 11 of 12 patients failing BCG treatment.

CONCLUSIONS. Collectively, these data indicate that tumor PD-L1 may facilitate localized stage-advancement of UC and attenuate responses to BCG immunotherapy by neutralizing T cells that normally guard against cancer invasion from the epithelium into the bladder musculature. *Cancer* 2007;109:1499–505.

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There are several predictors of cancer-specific survival for patients with urothelial carcinoma (UC) of the bladder, the most important of which is probably tumor stage.¹ As UCs progress in stage from involving only the bladder epithelium (pTa and pTis), into the lamina propria (pT1), and then deeper into the detrusor muscle and beyond (pT2–pT4), the risk of pelvic lymph node involvement and systemic metastases increases dramatically.¹ Given that it is metastatic dissemination that ultimately leads to the death of patients who fail surgical and/or chemotherapeutic intervention, local progression of UC from the epithelium into the bladder musculature

encompasses a seminal event that is associated with fatal consequences.

The question therefore arises as to what factors are responsible for localized stage progression of UC and, perhaps more important, whether any of these factors can be therapeutically targeted. The most widely recognized predictors of stage progression in nonmuscle-invasive tumors are, in estimated order of importance: the presence of carcinoma in situ (CIS), tumor grade, tumor size, the number of bladder tumors, and the previous bladder tumor recurrence rate.² Although these factors certainly warrant consideration when counseling bladder cancer patients, they are only indirectly modifiable. That is to say, such factors cannot be directly targeted with therapeutic intent.

Hence, the goal of our present study was to test whether an immune inhibitory molecule, PD-L1 (programmed death ligand-1, B7-H1), might act a potential mediator of stage progression in UC of the bladder. PD-L1 is a coregulatory ligand that can inhibit immune responses by either binding to PD-1 (programmed death-1) or a putative non-PD-1 receptor on the surface of T lymphocytes to induce antigen-specific T-cell apoptosis or anergy. Because T lymphocytes play a central role in mediating acquired antitumoral immunity, it has been proposed that expression of PD-L1, and other T-cell-inhibitory coregulatory ligands, may endow tumors with a mechanism to evade host immune destruction.³ Moreover, PD-L1 can be therapeutically targeted and recent *in vivo* studies have demonstrated that antibody-mediated blockade of PD-L1 is capable of facilitating the rejection of certain PD-L1-expressing tumors in several murine models.^{4,5} Prompted by our previous finding that PD-L1 expression strongly correlates with aggressive renal cell carcinoma progression and adverse outcome,^{6,7} we hypothesized that increased expression of PD-L1 might similarly facilitate UC progression by rendering bladder tumors more able to evade local immune mechanisms and, thus, invade beyond the bladder mucosa.

Herein we report that increasing tumor cell PD-L1 expression predicts localized UC stage progression, independent of tumor grade. Also, PD-L1 levels are highest in CIS and within granulomata of bladder tissues of patients failing bacillus Calmette-Guerin (BCG) therapy. To our knowledge, expression of PD-L1 by pathogen-induced granulomata has not previously been described. Collectively, these observations indicate that PD-L1 may act to abrogate host T-cell-mediated immunity to foster UC progression into deeper structures of the bladder and, perhaps, even undermine host immune responses against pathogen as well.

MATERIALS AND METHODS

Study Cohort

Two clinically distinct patient groups were examined in the current study to determine the role of PD-L1 expression in the switch from a nonmuscle-invasive high-risk phenotype to a muscle-invasive high-risk phenotype. The first group consisted of 44 patients with nonmuscle-invasive UCs that were prospectively identified from 1997 to 2000 and considered to be at high risk of stage progression. These cases were defined as high risk because of the presence of CIS ($n = 14$), pathologic grade 3/3 ($n = 22$), tumor size ≥ 3 cm ($n = 15$), multiple (≥ 3) tumors ($n = 13$), and/or microscopic invasion of the lamina propria ($n = 20$). All of these patients were treated with intravesicle BCG after initial transurethral resection of their bladder tumors (TURBT) and followed for recurrences thereafter. The median number of BCG doses administered was 12, with 29 patients receiving more than the 6 doses of the induction regimen. The second group consisted of 236 radical cystectomy cases treated from 1983 to 2002 for muscle-invasive UC of the bladder. Study exclusion criteria were: neoadjuvant systemic chemotherapy, previous pelvic irradiation, inadequate tissue for immunohistochemical evaluation, and lack of informed consent. Institutional Review Board (IRB) approval was obtained from the participating institutions.

Immunohistochemistry

The method of immunohistochemistry used in the current study and its validation has been previously described elsewhere.⁷ Briefly, paraffin-embedded tumor specimens were deparaffinized in xylene and rehydrated in a graded series of alcohols. Slides were unmasked in Target Retrieval Solution (DakoCytomation, Glostrup, Denmark) using a Decloaking Chamber (Biocare Medical, Walnut Creek, Calif) and then blocked for endogenous peroxidase for 5 minutes with a peroxidase blocking solution. Slides were then rinsed in TRIS-buffered saline with 0.1% Tween-20 (TBST), incubated for 30 minutes with 1.5% normal horse serum in TBST, rinsed in TBST, and blocked for endogenous avidin and biotin. Slides were then incubated overnight at 4°C with an anti-PD-L1 antibody (clone 5H1) that was created and is produced at our institution.^{3,8} Tissue slides were incubated with anti-PD-L1 antibody at a concentration of 1:100. This step was followed by 30 minutes of incubation with biotinylated horse antimouse immunoglobulin G and avidin/biotin complex reagent. Slides were amplified using a Tyramide Signal Amplification Biotin System (Perkin-Elmer, Boston, Mass) and incubated in 3-amino-9-ethylcarbazole chromogen.

Isotype-matched antibodies were used to control for nonspecific staining.

Pathologic Evaluation

Hematoxylin/eosin and PD-L1-stained sections were reviewed by a urologic pathologist (T.J.S.) blinded to clinical outcome. Criteria evaluated included: World Health Organization/International Society of Urologic Pathology (WHO/ISUP) histologic subtype and grade,⁹ TNM 2002 pathologic tumor stage,¹⁰ the presence and type of intratumoral lymphocytic infiltration, and the quantity and location of PD-L1 staining. The tumor was considered positive for PD-L1 if $\geq 1\%$ of tumor cells had histologic evidence of plasma membrane staining.

Statistical Methods

All statistical analyses were conducted with v. 2.3.1 of the *R* statistical software for Windows. For statistical analyses, pT3 and pT4 tumors were grouped into a single category. Using the Wilson score method of calculating confidence intervals for proportions,¹¹ univariate bar plots with 95% confidence intervals (95% CI) were constructed to visually explore the relations between tumor PD-L1 expression and gender, patient age, tumor grade, pathologic stage, and intratumoral lymphocytic inflammation. All of these variables were included in the multivariate regression models described below.

To model the ability of PD-L1 staining to predict tumor stage, proportional odds (a.k.a. cumulative logit) ordinal logistic regression models were fitted using the *Hmisc* and *Design* packages for *R*. Based on its unique biologic characteristics, UC in situ was considered to comprise the highest ordinal stage category. The assumptions of stage ordinality and multivariate proportional odds were confirmed graphically using methods described by Harrell.¹² To assess for potential confounding with PD-L1, covariates were tested for their association with PD-L1 using the binary logistic regression. In addition, all variables included in the multivariate model were individually assessed in the full multivariate model for statistical interaction with PD-L1. All reported *P*-values are 2-sided, rounded to 3 significant digits, and considered significant if $\leq .050$.

RESULTS

The clinical characteristics of the study cohort are shown in Table 1. As expected, the large majority of tumors were high-grade, muscle-invasive, and occurred in males. Overall, PD-L1 immunostaining was noted in 28% of specimens (Fig. 1A,B). However,

TABLE 1
Baseline Characteristics of the Patient Cohort

	Frequency no. (%)
Age, mean [IQR*]	66.4 [60.3–67.0]
Sex	
Women	53 (19)
Men	227 (81)
Pathologic stage	
pTa	11 (4)
pT1	19 (7)
pT2	52 (19)
pT3/4	184 (66)
Cis	14 (5)
Tumor grade	
Low	74 (26)
High	206 (74)
Lymphocytic infiltration	
None	28 (10)
Focal	90 (32)
Diffuse	162 (58)
PD-L1 IHC staining	
Negative	203 (73)
Positive	77 (28)

* Interquartile range.

when examined by stage category, the proportion of PD-L1 expression was 7% for pTa, 16% for pT1, 23% for pT2, 30% for pT3/4, and 45% for CIS (Fig. 2A). No plasma membrane expression of PD-L1 was found in the normal urothelial adjacent to malignant urothelium. PD-L1 expression was univariately associated with high-grade tumors (OR = 2.4, 95% CI: 1.20–4.72; *P* = .009) and the presence of intratumoral lymphocytic infiltration (OR = 5.5, 95% CI: 1.27–23.80; *P* = .004) but not with age (OR = 1.0, 95% CI: 0.98–1.03; *P* = .727) or gender (OR = 2.4, 95% CI: 0.54–2.04; *P* = .885). The proportions of PD-L1 positivity found within each category of tumor grade and intratumoral lymphocytic infiltration are shown in Figure 2B,C.

The results of the proportional odds ordinal logistic regression modeling to predict tumor stage are shown in Table 2. Univariately, only tumor grade and the presence of PD-L1 staining predicted increasing tumor stage. These same 2 variables remained significant predictors of tumor stage in the multivariate regression model adjusting for all of the covariates. None of the interaction terms between PD-L1 staining and the other variables included in the multivariate model were significant, and therefore these were not included in the final model.

Lastly, 16 of the 44 BCG-treated patients with nonmuscle-invasive tumors developed recurrent superficial bladder cancer. Only 3 of these patients

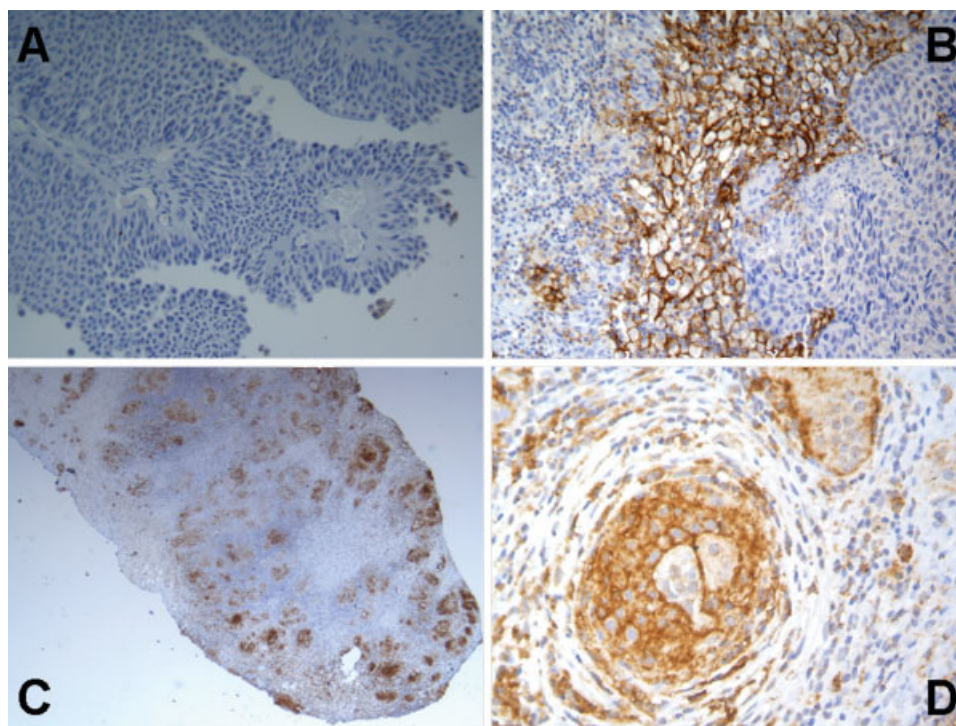


FIGURE 1. PD-L1 immunohistochemistry in bladder cancer. (A) Urothelial carcinoma demonstrating absence of PD-L1 staining (20×). (B) Urothelial carcinoma demonstrating marked membranous PD-L1 staining (20×). (C) Low-power (2.5×). (D) High-power (40×) images of PD-L1 positive BCG granulomas from patients with recurrent bladder cancer.

were PD-L1-positive in their original tumor specimen and the initial TNM stages of these patients were pTa in 10 cases, pT1 in 3 cases, and CIS in 3 cases. Histologically, 12 of the 16 recurrences had BCG granulomata and 11 of these granulomata showed a pattern of diffuse and intense (>90% of cells) PD-L1 staining (Fig. 1C,D). None of the tumor cells evaluated in the 4 specimens without granulomatous inflammation expressed PD-L1 and only 2 of the 12 granulomatous cases had weak (<5% of cells) PD-L1-positive tumor cells.

DISCUSSION

Several large series have established that the 5-year cancer-specific survival of patients undergoing radical cystectomy for UC is highly dependant on localized pathologic tumor stage (pT).^{1,13} These studies also support that infiltration of UC into the muscular layer of the bladder represents a threshold event that is associated with grave clinical outcomes. With this in mind, we postulated that one mechanism whereby UC cells might invade progressively into the bladder wall is by subverting host immunity that might normally militate against tumor progression. We further surmised that expression of PD-L1 by UC cells might

contribute to this process. These hypotheses emanate from both historical as well as relatively recent observations reported in the literature. First, bladder cancer patients can manifest acquired immune dysfunction that appears to affect lymphocytes and that is related to tumor stage.^{14–16} Second, nonmuscle-invasive UC of the bladder is one of the most responsive human malignancies to immunotherapy.¹⁷ As such, host immunity appears to modulate bladder cancer pathogenesis. Third, it has recently become clear that T-cell-inhibitory PD-L1 is aberrantly expressed by many forms of cancer and, in this context, has been implicated as a mechanism for tumors acquiring the ability to evade the host immune system.^{3,8} Consistent with this, increased tumor PD-L1 expression has recently been reported to predict poor outcomes for patients with carcinomas of the esophagus and kidney.^{6,7,18}

Herein we describe several novel observations that generally support our overall hypothesis that PD-L1 contributes to the pathogenesis of bladder cancer. Related to this, we focused our study on relatively high-risk forms of bladder cancer to ascertain the extent to which PD-L1 contributes to host immune evasion in order to promote localized stage progression of urothelial malignancies toward in-

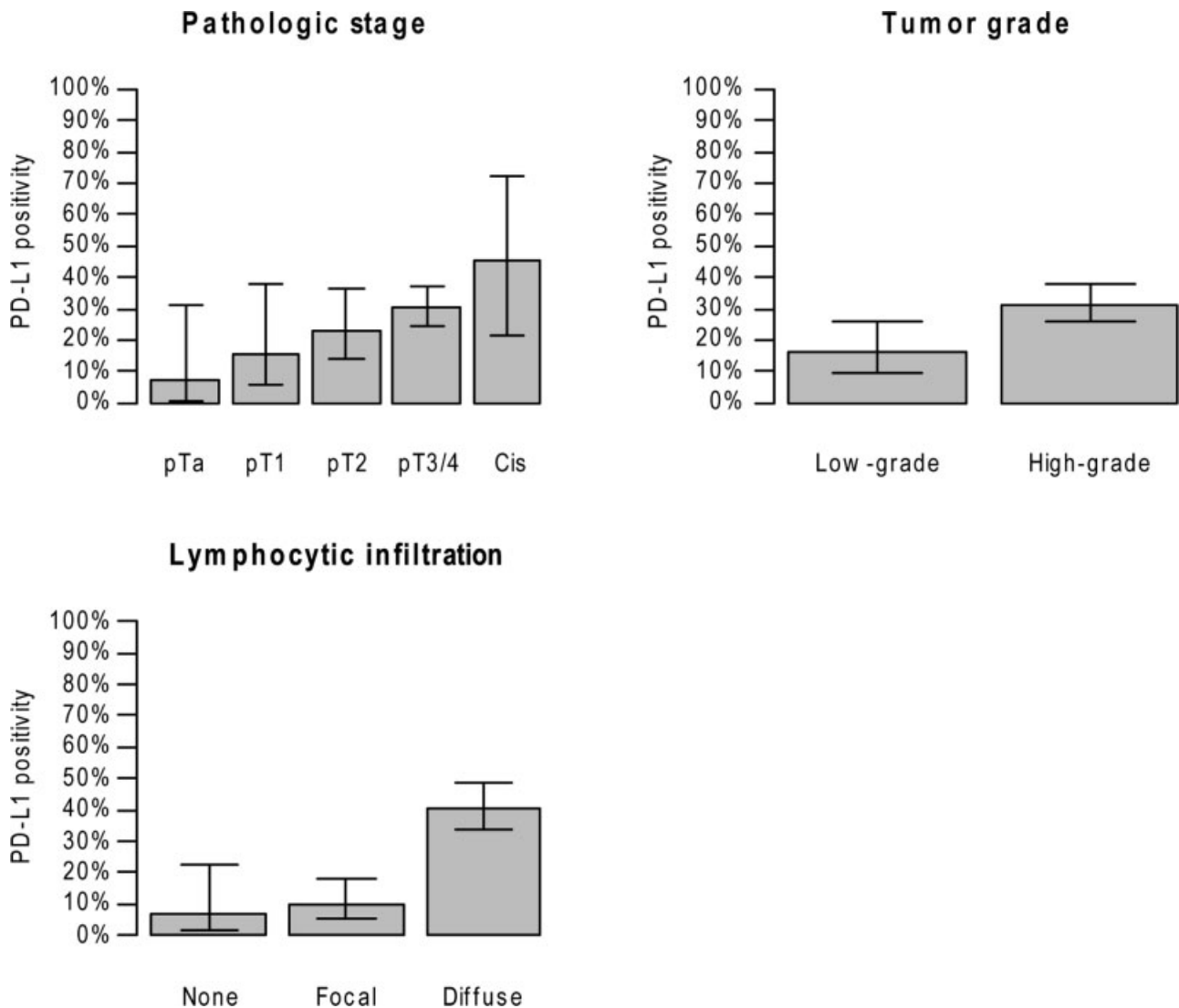


FIGURE 2. PD-L1 expression in bladder cancer stratified by: (A) TNM 2002 pathologic tumor stage, (B) World Health Organization/International Society of Urologic Pathology (WHO/ISUP) tumor grade, and (C) amount of intratumoral lymphocytic infiltration. Boxes represent the proportion of positive cases and the error bars represent the 95% confidence interval for the proportion.

creasingly deadly tumors. One primary observation that we report in our present study is that the amounts of PD-L1 expressed by urothelial tumor cells increase steadily with advancing local tumor stage. Specifically, when PD-L1 is modeled as a predictor of tumor stage, increasing levels of PD-L1 expression by urothelial tumor cells more than double the risk of advancing from one stage category to the next, independent of tumor grade, age, gender, and lymphocytic infiltration. The independence from tumor grade suggests that the presence of the PD-L1 glycoprotein on the surface of UC cells may affect malignant stage progression via processes that are distinct from the tumor's intrinsic intracellular machinery and implies that the host's immune response

is a factor as well. Specifically, although PD-L1 is a T-cell coregulatory molecule whose expression is normally restricted to macrophage-lineage cells as well as subsets of activated T cells, our study suggests some bladder tumor cells acquire the ability to express PD-L1, which may then function to paralyze responses by local mediators of antitumoral immunity.

Another interesting observation from our study is that UC in situ exhibited the highest degree of PD-L1 expression, with PD-L1 being apparent in roughly 40% of all CIS-containing specimens examined. Moreover, the majority of patients that failed BCG immunotherapy exhibited extremely intense PD-L1 expression within the BCG granulomas found in

TABLE 2
Proportional Odds Ordinal Logistic Regression Models Predicting Tumor Stage

	Univariate			Multivariate		
	OR*	[95%CI] [†]	P	OR	[95%CI]	P
Age	1.00	[0.97–1.02]	.688	1.00	[0.97–1.02]	.705
Sex			.728			.529
Men	1			1		
Women	0.90	[0.49–1.64]		0.81	[0.43–1.54]	
Tumor grade			<.001			<.001
Low	1			1		
High	5.03	[2.90–8.71]		4.77	[2.73–8.34]	
Lymphocytic infiltration			.679			.118
None	1			1		
Focal	1.07	[0.44–2.59]		1.23	[0.49–3.08]	
Diffuse	0.85	[0.37–1.94]		0.67	[0.28–1.60]	
PD-L1 staining			.010			.012
Negative	1			1		
Positive	2.09	[1.18–3.73]		2.20	[1.17–4.13]	

* Odds ratio.

[†] 95% confidence interval.

proximity to their recurrent tumors. The potential significance of these findings is described in what follows. In a large series of 2596 pTa and pT1 bladder tumors (treated in 7 separate EORTC clinical trials), it has been reported that the presence of urothelial CIS represents the single most important clinical predictor of bladder cancer stage progression.² In fact, nearly 50% of bladder cancer patients with concomitant CIS are expected to experience stage progression of their disease.¹⁹ Consequently, CIS is most often treated with aggressive endoscopic resection, followed by intravesical instillation of BCG, one of the earliest and most effective forms of tumor immunotherapy. That CIS is highly amenable to immunotherapy is supported by rates of BCG treatment response ranging from 75% to 80%.¹⁹ At first glance, it may seem paradoxical that CIS—a bladder cancer subtype that we show expresses high level of immunosuppressive protein PD-L1—responds so well to intravesical BCG immunotherapy. However, patients experiencing responses to BCG therapy are not typically afforded lifelong antitumor protection, and they fail BCG therapy at a median of roughly 5 to 6 years with a 5-year cancer-free survival rate of only about 60%.¹⁹ Thus, it appears that BCG initially induces a robust immune response against CIS that overrides local mechanisms of immunosuppression imparted by the tumor. Over time, enhanced levels of intratumoral PD-L1 (expressed by the tumor cells, infiltrating lymphocytes, and/or organized BCG-induced granulomata) may cause the antitumoral efficacy of

BCG immunotherapy to wane, thereby permitting CIS to progress to more invasive forms of cancer. In support of this, we demonstrate that, although PD-L1 is present in roughly 40% of CIS tumors before BCG treatment, the amount of expression by these tumor cells is relatively low (median expression level was about 5% of tumor cells). In stark contrast, CIS cases that ultimately failed BCG therapy exhibited approximately 15–20-fold higher levels of PD-L1 expression, predominantly within BCG granulomas. As such, PD-L1 expression is not a static feature within bladder cancer but, rather, is a dynamic feature that changes over time and in response to treatment. And as we ultimately show, increasing levels of PD-L1 expression within the bladder tumor microenvironment correlates with increased local aggressiveness of this cancer.

Our data suggest several potential roles for PD-L1 that pertain to bladder cancer. Specifically, the PD-L1 expressed by bladder tumors (including CIS) may equip urothelial tumor cells with a molecular weapon to impair host antitumoral immune cells and facilitate to aggressive cancer progression. Moreover, increased expression of T-cell-inhibitory PD-L1 by mononuclear cells that are recruited into bladder tissues in response to BCG therapy (ultimately to form multiple foci of chronic granulomatous inflammation) may contribute to a decline in the effectiveness of BCG therapy over time. In addition, the intense PD-L1 expression circumscribing BCG-induced granulomata might further act to inhibit or sequester away from the host immune system the antigen-presenting cells (APCs) that are attempting to process either BCG and/or tumor-related antigens that are critical for the propagation of antitumoral immunity. Such a mechanism could conceivably limit the effectiveness of BCG immunotherapy and, perhaps more important, even favor the establishment of chronic infection through impairment of host cell-mediated immunity. This proposal is somewhat analogous to the role of PD-L1 in promoting chronic viral infections (such as HIV) that has recently been reported.^{20–22} In summary, the longitudinal accumulation of PD-L1-expressing cells within and around BCG-induced granulomata may inhibit T-cell interactions with relevant APCs, or responses directed against tumor or pathogenic antigens, to ultimately abrogate the effectiveness of BCG immunotherapy.

We acknowledge that any functionality of PD-L1 in promoting bladder cancer progression and treatment failure can only be inferred in a retrospective human study of the nature we have conducted and that numerous overlapping mechanisms for BCG failure and tumor progression likely exist. Clearly, further in vitro and in vivo mechanistic studies are

warranted to fully elucidate the role of PD-L1 in bladder cancer and BCG response. Nevertheless, our data demonstrate strong associations between increasing tumor cell PD-L1 expression and UC progression, suggesting that PD-L1 may be a factor promoting localized stage progression and perhaps facilitating the eventual loss of BCG effectiveness over time. More generally, our study indicates that T lymphocytes may represent a critical immune barrier against tumor progression in bladder cancer. Given that in vivo antibody-mediated blockade has recently been shown to potentiate antitumoral immunity in murine studies,^{4,20,23} it appears that PD-L1 may serve as a viable prognostic marker to predict UC risk as well as a rational immunotherapeutic target for the treatment of urothelial bladder carcinoma.

CONCLUSION

We describe a potentially novel mechanism for local stage progression of UCs arising in the bladder. We demonstrate that PD-L1, a negative coregulatory molecule that inhibits antigen-specific T-cell activity, acts as a predictive marker for local tumor stage progression, independent of tumor grade. PD-L1 levels are highest in CIS and in granulomata developing within the bladder tissues of patients failing BCG therapy. Collectively, these observations suggest that PD-L1 may act to abrogate host T-cell-mediated immunity to foster UC progression into deeper structures of the bladder. Moreover, our studies suggest that BCG induces strong PD-L1 expression in host tissues that might have important implications for antitumoral tumor immunotherapy as well as, perhaps, mechanisms of host immune response impairment against various pathogens.

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