

PD-1 and CD103 Are Widely Coexpressed on Prognostically Favorable Intraepithelial CD8 T Cells in Human Ovarian Cancer

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Abstract

$\alpha_E(\text{CD}103)\beta_7$ is a TGF β -regulated integrin that mediates retention of lymphocytes in peripheral tissues by binding to E-cadherin expressed on epithelial cells. We recently reported that $\alpha_E(\text{CD}103)\beta_7$ specifically demarcates intraepithelial CD8⁺ tumor-infiltrating lymphocytes (CD8 TIL) in ovarian cancer and that CD103⁺ TIL have a surface profile consistent with an active effector phenotype (HLA-DR⁺, Ki67⁺, and CD127^{lo}). These findings led us to hypothesize that, over time, CD103-mediated retention of CD8 TIL within the tumor epithelium might result in chronic stimulation by tumor antigen, which in turn might lead to an exhausted phenotype. To investigate this possibility, we evaluated PD-1 expression in a large cohort of ovarian tumors ($N = 489$) with known CD103⁺ TIL content. PD-1⁺ cells were present in 38.5% of high-grade serous carcinomas (HGSC), but were less prevalent in other histologic subtypes. PD-1⁺ TIL were strongly associated with increased

disease-specific survival in HGSC (HR, 0.4864; $P = 0.0007$). Multicolor immunohistochemistry and flow cytometry revealed a high degree of PD-1 and CD103 coexpression, specifically within the CD8 TIL compartment. PD-1⁺CD103⁺ CD8 TIL were quiescent when assessed directly *ex vivo* yet were capable of robust cytokine production after pharmacologic stimulation. Moreover, they showed negligible expression of additional exhaustion-associated markers, including TIM-3, CTLA-4, and LAG-3. Thus, as hypothesized, CD103⁺ CD8 TIL express PD-1 and appear quiescent in the tumor microenvironment. However, these cells retain functional competence and demonstrate strong prognostic significance. We speculate that, after standard treatment, PD-1⁺CD103⁺ CD8 TIL might regain functional antitumor activity, an effect that potentially could be augmented by immune modulation. *Cancer Immunol Res*; 3(8); 926–35. ©2015 AACR.

Introduction

PD-1 (CD279) is an inhibitory receptor that was initially recognized as a marker of T-cell exhaustion during chronic viral infection (1). However, recent studies have revealed that PD-1 is also transiently expressed on the surface of effector T cells during earlier stages of activation (2, 3). PD-1 mediates its inhibitory effect by clustering with the T-cell receptor (TCR) in the immunologic synapse and, after contacting either of its two known ligands (PD-L1 or PD-L2), recruiting SHP-2 phosphatase to its intracellular immunoreceptor tyrosine-based switch motif (ITSM; ref. 4). SHP-2 then interferes with various aspects of T-cell function, including proliferation and cytokine production (5). In this manner, PD-1 is thought to maintain a balance between T-cell effector function and protection against excessive damage of peripheral tissues during infection (6).

Accumulating evidence suggests that the PD-1–PD-L1 axis is also frequently engaged in the cancer setting, implying that

tumors may use this pathway as a means of immune evasion (for review see ref. 7). Indeed, the presence of PD-1⁺ tumor-infiltrating lymphocytes (TIL) correlates with disease severity and poor prognosis in a number of malignancies, including breast (8, 9), renal (10), and nasopharyngeal cancer (11) as well as Hodgkin's lymphoma (12). Likewise, expression of PD-L1 is associated with poor prognosis in renal, melanoma, breast, pancreatic, stomach, bladder, lung, liver, and ovarian cancer (for review see ref. 13). Disruption of PD-1–PD-L1 interactions through the use of blocking antibodies is currently an area of intense clinical investigation, with clear efficacy seen in non–small cell lung cancer, metastatic melanoma, and renal carcinoma and promising results in bladder, head and neck, colorectal, and ovarian cancer (14).

Ovarian cancer is a particularly interesting setting to study PD-1 biology as patients are typically diagnosed at an advanced stage of disease. Moreover, TIL are often abundant in late-stage tumors yet have obviously failed to prevent tumor progression (15–19). Together, these features suggest that TIL in ovarian cancer may have experienced prolonged exposure to their cognate antigen and, as a consequence, may be functionally exhausted. In support of this notion, prior studies have shown that NY-ESO-1–specific CD8⁺ TIL from ovarian cancer patients frequently express PD-1 and are functionally impaired compared with their counterparts in peripheral blood (20). Moreover, ovarian tumor cells have been reported to express high levels of PD-L1 (21, 22).

We recently reported that intraepithelial CD8⁺ TIL in ovarian cancer express the integrin molecule $\alpha_E(\text{CD}103)\beta_7$ (23). $\alpha_E(\text{CD}103)\beta_7$ is widely expressed by intraepithelial

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Table 1. PD-1⁺ cell infiltrates in relation to clinical characteristics of the retrospective patient cohort (according to REMARK criteria)

Histologic subtype	Number of tumors	Number of tumors with PD-1 ⁺ cells present (%) ^a	Mean number of PD-1 ⁺ cells per core (when present) ^b
Serous (N = 206)		75 (36.4)	9.1
DSS (y)—median (95% CI)			
Age (y)—median (range)			
Grade			
1	11	0 (0)	—
2	56	18 (32.1)	9.4
3	139	57 (41.0)	9.0
FIGO Stage			
I	48	15 (31.3)	6.5
II	90	38 (42.2)	9.5
III	68	22 (32.4)	10.2
Mucinous (N = 30)		0 (0)	0
DSS (y)—median (95% CI)			
Age (y)—median (range)			
Grade			
1	11	0 (0)	—
2	17	0 (0)	—
3	2	0 (0)	—
FIGO stage			
I	17	0 (0)	—
II	12	0 (0)	—
III	1	0 (0)	—
Endometrioid (N = 125)		22 (17.6)	7.4
DSS (y)—median (95% CI)			
Age (y)—median (range)			
Grade			
1	82	12 (14.6)	4.6
2	35	7 (20.0)	1.8
3	8	3 (37.5)	31.8
FIGO stage			
I	69	19 (27.5)	6.4
II	50	3 (6.0)	14.1
III	6	0 (0)	—
Clear cell (N = 128)		11 (8.6)	6.1
DSS (y)—median (95% CI)			
Age (y)—median (range)			
Grade			
1	—	—	—
2	—	—	—
3	128	11 (8.6)	6.1
FIGO Stage			
I	66	5 (7.6)	4.8
II	54	6 (11.1)	7.1
III	8	0 (0)	—

^aTumors with a mean of ≥ 1 PD-1 positive cells per 0.6 mm core (2 duplicate cores, counted by two investigators).

^bMean number of PD-1 positive cells per core in those tumors scored as positive in previous column.

lymphocytes (IEL) in the skin and gut mucosa (24, 25) and is thought to mediate the retention of T cells in relevant peripheral tissues by binding to E-cadherin expressed on the surface of epithelial cells (26). $\alpha_E(\text{CD103})\beta_7$ has also been shown to enhance the cytolytic activity of T cells against E-cadherin-expressing tumor cells *in vitro* (27–29). Despite these positive aspects of $\alpha_E(\text{CD103})\beta_7$ expression by TIL, we speculated that $\alpha_E(\text{CD103})\beta_7$ -mediated retention of TIL within tumors might also have detrimental effects, by exacerbating the phenomenon of chronic antigen stimulation and immune exhaustion. Thus, in this study, we evaluated the extent of PD-1 expression by TIL in human ovarian cancer. We report that CD103 and PD-1 are highly coexpressed on intraepithelial CD8 TIL. Nonetheless, CD103⁺PD-1⁺ CD8 TIL retain functional competence and, contrary to many other tumor settings, are strongly associated with patient survival.

Materials and Methods

Tissue microarray

To assess the extent of PD-1 expression by TIL in primary ovarian tumor samples, we stained a previously described tissue microarray (TMA; ref. 5) containing 489 evaluable cores from a cohort of ovarian cancer patients treated at the BC Cancer Agency from 1984 to 2000 (OvCaRe Ovarian Tumor Bank, Vancouver, BC, Canada). All tumor tissues were obtained at the time of primary surgery, and patients in this cohort were considered optimally debulked (i.e., no macroscopic residual disease following surgery). Table 1 describes this cohort in accordance with REMARK criteria. All specimens and clinical data were obtained with either informed written consent or a formal waiver of consent under protocols approved by the Research Ethics Board of the BC Cancer Agency and the University of British Columbia.

Immunohistochemistry

To detect PD-1⁺ and CD103⁺ cells, 4- μ m sections of either whole tumor tissue or TMAs were deparaffinized and subjected to heat-induced epitope retrieval using a decloaking chamber and Diva decloaking reagent (Biocare Medical). Anti-PD-1 mouse monoclonal antibody (mAb; clone NAT105; Cell Marque) was applied at a dilution of 1/200, and rabbit anti-CD103 mAb [Epitomics clone EPR4166(2)] was applied at a dilution of 1/1,500, each for 30 minutes at room temperature. Following washing, MACH2 anti-mouse- or anti-rabbit-HRP polymer reagent was applied for 30 minutes at room temperature followed by DAB reagent. Slides were counterstained with hematoxylin, air-dried, and cover-slipped using Ecomount (Biocare).

For two-color immunohistochemical (IHC) staining, sections were stained with anti-PD-1 as described above in combination with a rabbit anti-CD103 mAb (described above) or a rabbit anti-CD3 mAb (clone SP7; Spring Bioscience). Slides were incubated with Mach 2 Double Stain #1 polymer reagent (Biocare) for 30 minutes at room temperature, and the horseradish peroxidase (HRP) and alkaline phosphatase (AP) enzyme tags were detected using Vina Green and Warp Red chromogens (Biocare), respectively. Slides were counterstained with hematoxylin.

Slides were analyzed using the Nuance Multispectral Imaging System (CRI) with a BX-53 microscope (Olympus). Single-stained slides were used to create spectral profiles for each chromogen, and these profiles were then used to discriminate the individual colors on multistained slides.

Scoring and statistical analysis

For prognostic analyses, absolute numbers of PD-1⁺ and/or CD103⁺ TIL (independent of location) in cores of the retrospective cohort TMA were enumerated by visual inspection. Slides were counted independently by two different investigators, and the average cell count per case was used for statistical analyses. Statistical analyses and HR calculations were performed using GraphPad software. Univariate survival analysis was performed using the Kaplan-Meier method, and *P* values were determined using the log-rank test. Multivariate survival analysis was performed using a Cox regression model. Differences with *P* values <0.05 were regarded as statistically significant.

Flow cytometry

Single-cell suspensions were prepared from freshly resected tumor specimens and cryopreserved, as described previously (30). After thawing, cells were stained with the following fluorescently conjugated Abs, as indicated: CD3-BV510, CD8-BV421 (Biolegend), PD-1-PerCP-eFluor710, TIM-3-APC (eBioscience), LAG-3-FITC (Enzo Life Sciences) CD103-PE, CD56-APC, CTLA-4-PECy5, and CD4-FITC (BD Biosciences). All flow cytometry was performed using a FACS Calibur that had been upgraded to 8-color capability by Cytex (DxP8). Analyses were performed using FlowJo software (Tree Star).

In vitro stimulations

Peripheral blood mononuclear cells (PBMC) obtained from normal healthy donors were stimulated for 6 days with phytohemagglutinin (PHA; 5 μ g/mL) in complete media containing 100 U/mL IL2 in the presence or absence of 2 ng/mL TGF β 1 (Peprotech). For intracellular cytokine assays, cell pellets from malignant ascites, or disaggregated tumors were stimulated in

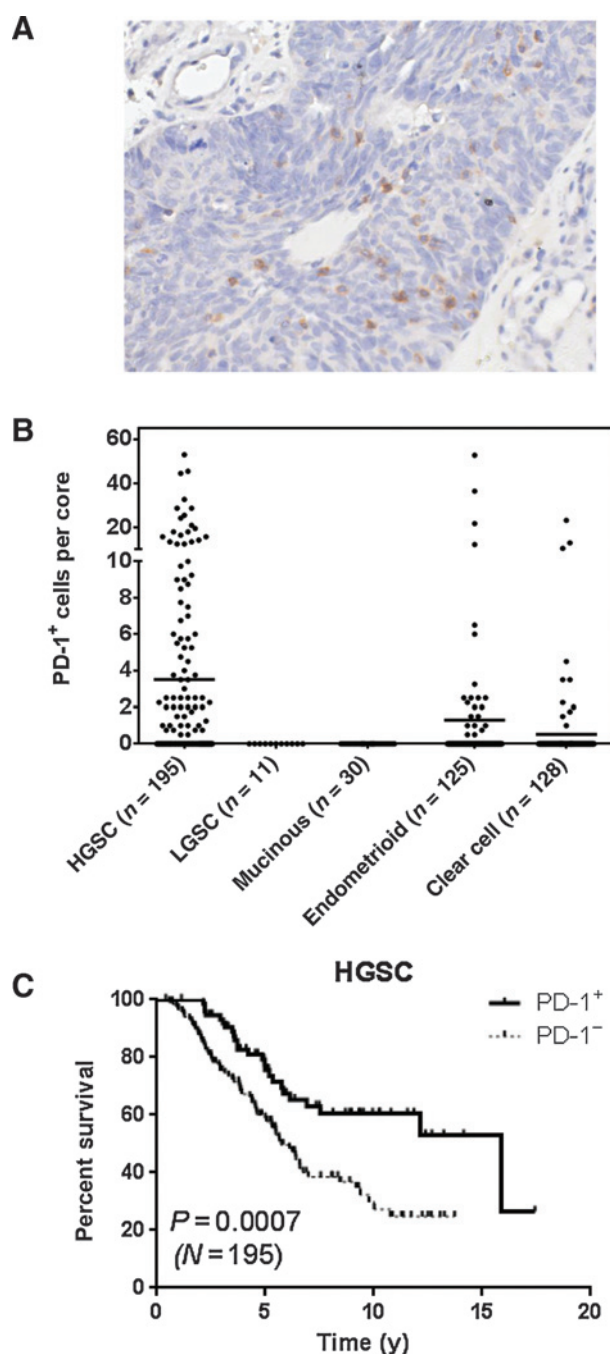


Figure 1. PD-1 expression in ovarian cancer. A retrospective TMA containing 490 ovarian cancer cases comprising the five major ovarian cancer tumor types (high-grade serous, low-grade serous, endometrioid, mucinous, and clear cell) was stained with anti-PD-1 antibody and counterstained with hematoxylin. A, representative example of an HGSC tumor containing PD-1⁺ cells (brown). B, cluster plot showing the distribution of PD-1⁺ cells within tumors of the indicated histologic subtype. Shown are the absolute numbers of PD-1⁺ cells per 0.6 mm core (average of duplicate cores). Horizontal lines indicate the mean PD-1⁺ cell density for each subtype. C, survival analysis of the HGSC cohort. Data were binarized based on the presence or absence of PD-1⁺ cells, and the log-rank test was used to compare curves.

Table 2. Disease-specific survival and HRs based on PD-1⁺ cell infiltrates

	N	PD-1 cell density ^a			Median survival (days)			P ^c
		Mean	Median	Range	PD-1 ⁻	PD-1 ⁺	HR ^b (95% CI)	
High-grade serous	195	3.3	0	0–53	2,095	5,792	0.362 (0.2293–0.5706)	0.0007
CD3 (>5)	161	4.25	0	0–53	2,704	5,792	0.5436 (0.3337–0.8857)	0.0144
CD3 (<5)	34	0	0	0–0	—	—	—	—
CD8 (>5)	151	4.53	0	0–53	3,076	5,792	0.6628 (0.3918–1.121)	0.1251
CD8 (<5)	44	0	0	0–0	—	—	—	—
CD25/FOXP3 (>5)	82	6.12	2.125	0–53	2,405	5,792	0.3009 (0.1005–0.5175)	0.0005
CD25/FOXP3 (<5)	113	1.6	0	0–44.5	2,054	2,238	0.8909 (0.4864–1.644)	0.7188
Low-grade serous	11	—	—	—	—	—	—	—
Mucinous	30	—	—	—	—	—	—	—
Endometrioid	125	1.3	0	0–53	^d	^d	0.76 (0.2490–2.320)	0.63
Clear cell	128	0.52	0	0–23	^d	2,521	1.806 (0.5647–5.773)	0.32

NOTE: P values that are statistically significant (<0.05) are shown in bold.

^aNumber of PD-1⁺ cells per 0.6-mm core (average of 2 cores), regardless of location within tumor.

^bHR calculated using the log-rank test.

^cP value using the log-rank (Mantel-Cox) test.

^d Not reached.

medium with or without PMA (50 ng/mL) plus ionomycin (250 ng/mL). Cytokine release was inhibited by adding 2 μmol/L monensin (GolgiStop; BD Biosciences) for the duration of the incubation. After 6 hours incubation, cells were recovered by centrifugation, surface stained with the indicated antibodies, and fixed and permeabilized using Cytotfix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Intracellular cytokines were detected using anti-IFNγ and anti-TNFα antibodies (BD Biosciences).

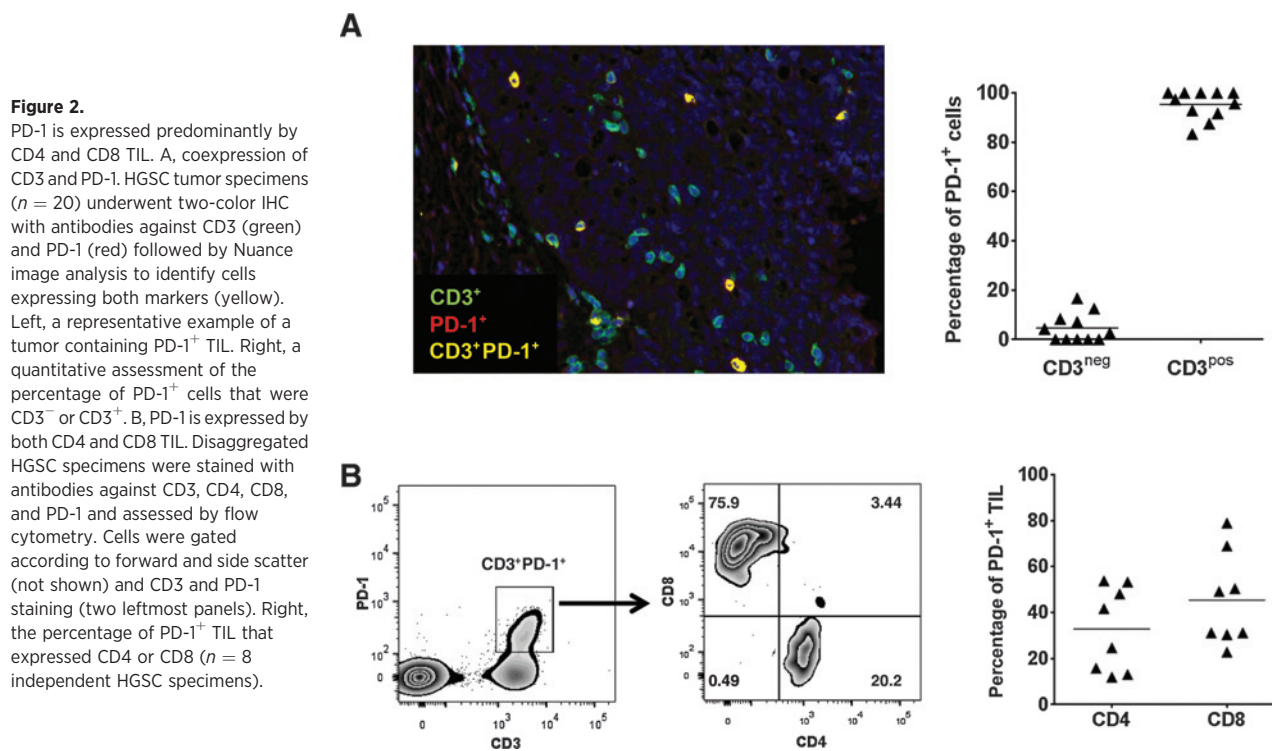
Results

Prevalence and prognostic significance of PD-1⁺ immune infiltrates in ovarian cancer

Characteristics of the patient cohort used in this study, including tumor subtypes, stages, and grades are shown in Table 1. PD-

1⁺ cellular infiltrates were observed in 108 of 489 (22.1%) of evaluable tumors, including 75 of 195 (38.5%) high-grade serous carcinomas (HGSC) and 22 of 125 (17.6%) endometrioid, 11 of 128 (8.6%) clear cell, 0 of 30 mucinous, and 0 of 11 low-grade serous (LGSC) tumors (Fig. 1 and Table 1). Among positive cases, HGSC tumors harbored a higher mean number of PD-1⁺ cells compared with endometrioid or clear cell tumors (9.1 vs. 7.4 and 6.1 per core, respectively; Table 1). In HGSC, the percentage of tumors containing PD-1⁺ cells increased with grade ($P = 0.018$), but not stage of disease ($P = 0.31$; Table 1).

In univariate analysis, the presence of PD-1⁺ cells strongly correlated with disease-specific survival in patients with HGSC [HR, 0.4864; 95% confidence interval (CI), 0.3206–0.7378; $P = 0.0007$] but not in patients with low-grade serous, mucinous, endometrioid, or clear cell cancers (Table 2 and Fig. 1). PD-1⁺



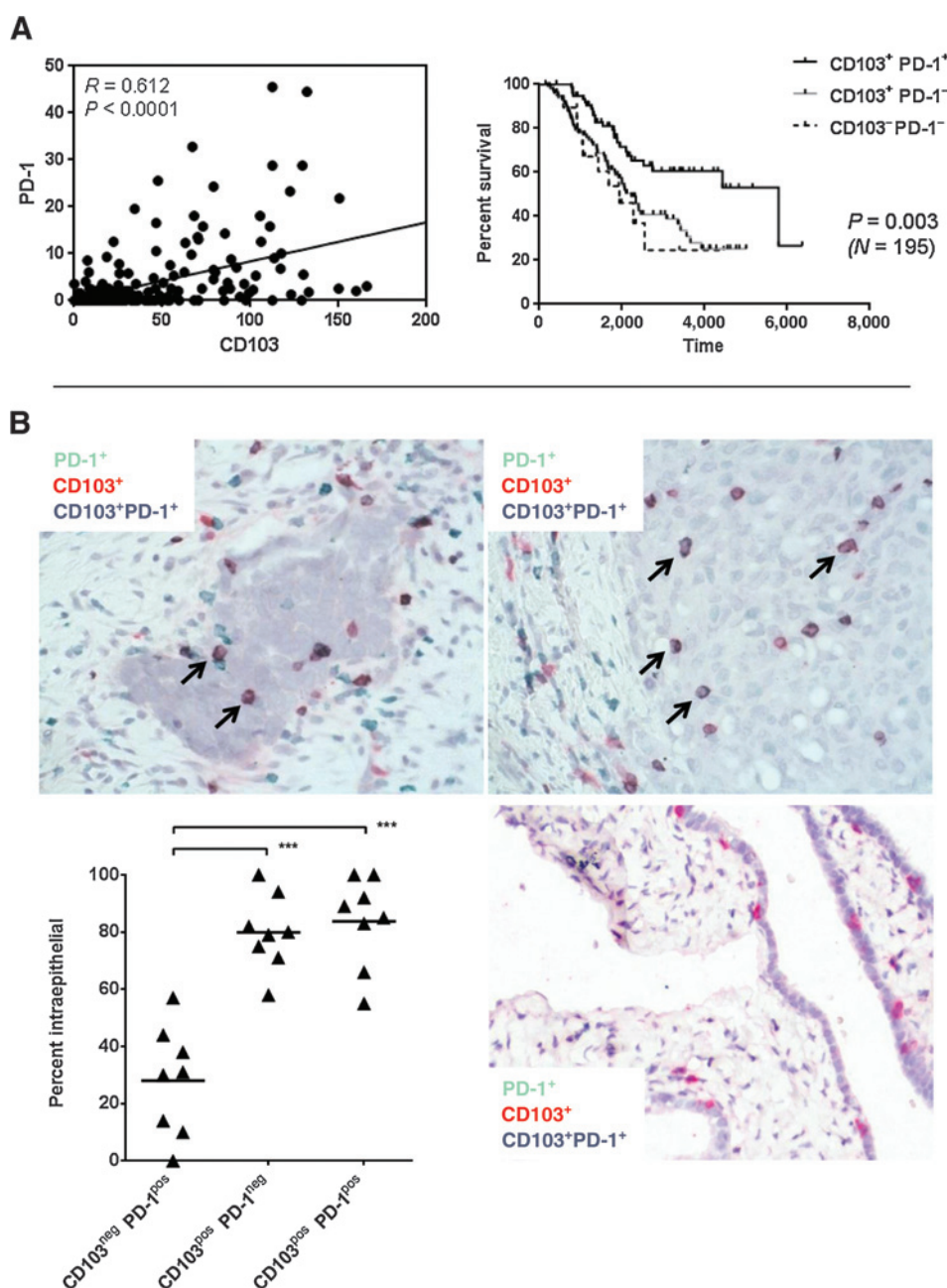


Figure 3. Coincidence and prognostic significance of PD-1⁺ and CD103⁺ cells in HGSC. Serial sections from the 490-case TMA were stained with antibodies to CD103 and PD-1. A, left, the association between the number of total PD-1⁺ and CD103⁺ cells per tumor core (based on the average of duplicate cores, regardless of location in the tumor). R value refers to the Spearman correlation for the entire cohort of 490 tumors. Right, disease-specific survival of HGSC patients based on three TIL patterns: PD-1⁺ CD103⁺, PD-1⁻ CD103⁺, or PD-1⁻ CD103⁻. The cutoff values for positivity were ≥ 1 positive cells per core for either marker. The log-rank test was used to compare curves. B, a TMA containing 20 HGSC tumors underwent two-color IHC with antibodies to PD-1 (green) and CD103 (red). Cells that were positive for both markers appear purple. Top, representative tumors containing PD-1⁺CD103⁺ TIL. Bottom left, the extent of intraepithelial localization of PD-1⁺CD103⁻, PD-1⁻CD103⁺, and PD-1⁺CD103⁺ TIL in eight HGSC tumors. Asterisks indicate a P value of <0.0001 as determined by one-way ANOVA. Bottom right, a representative sample of normal fallopian tube (from a cohort of 10) that underwent two-color IHC with antibodies to PD-1 (green) and CD103 (red) as above.

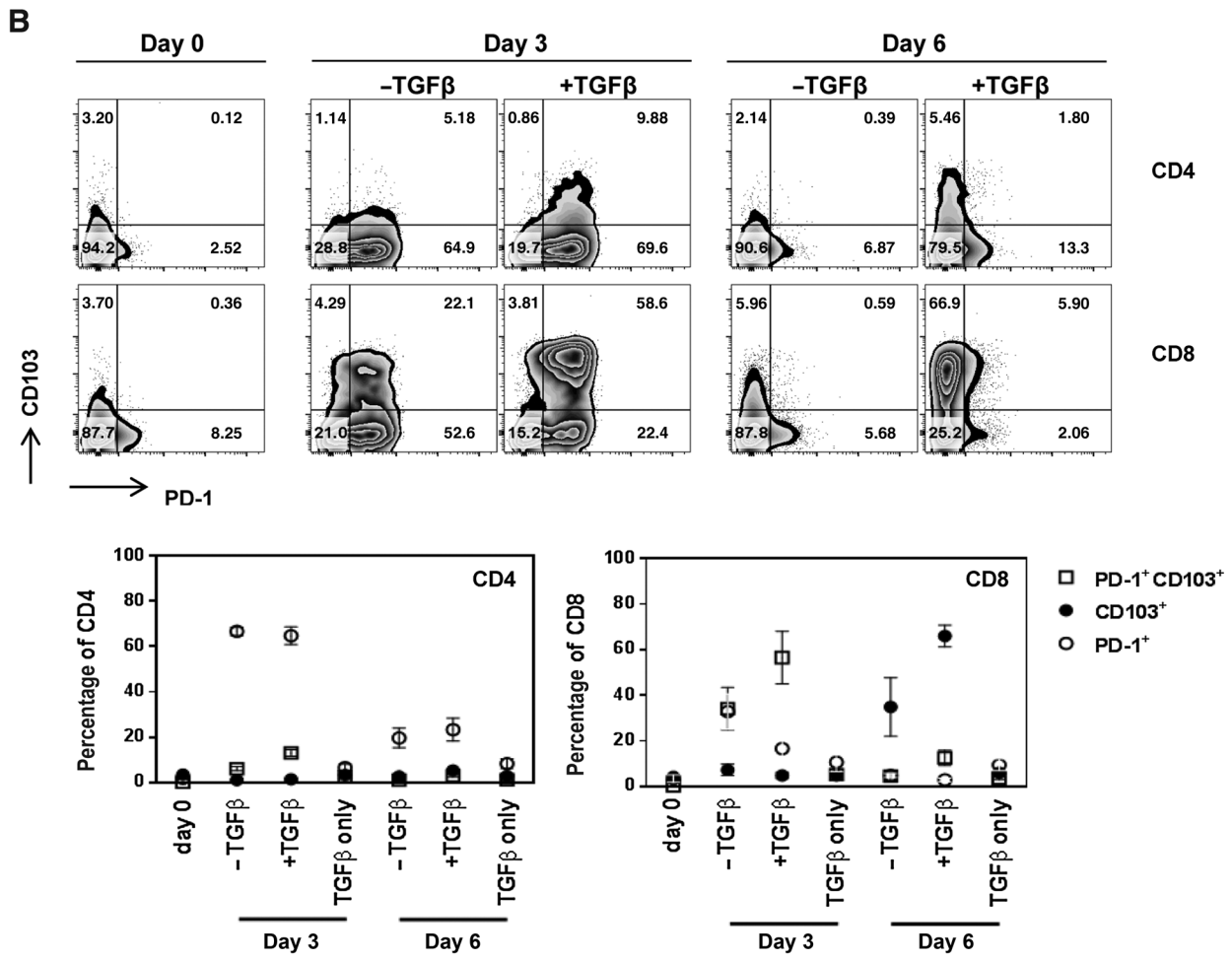
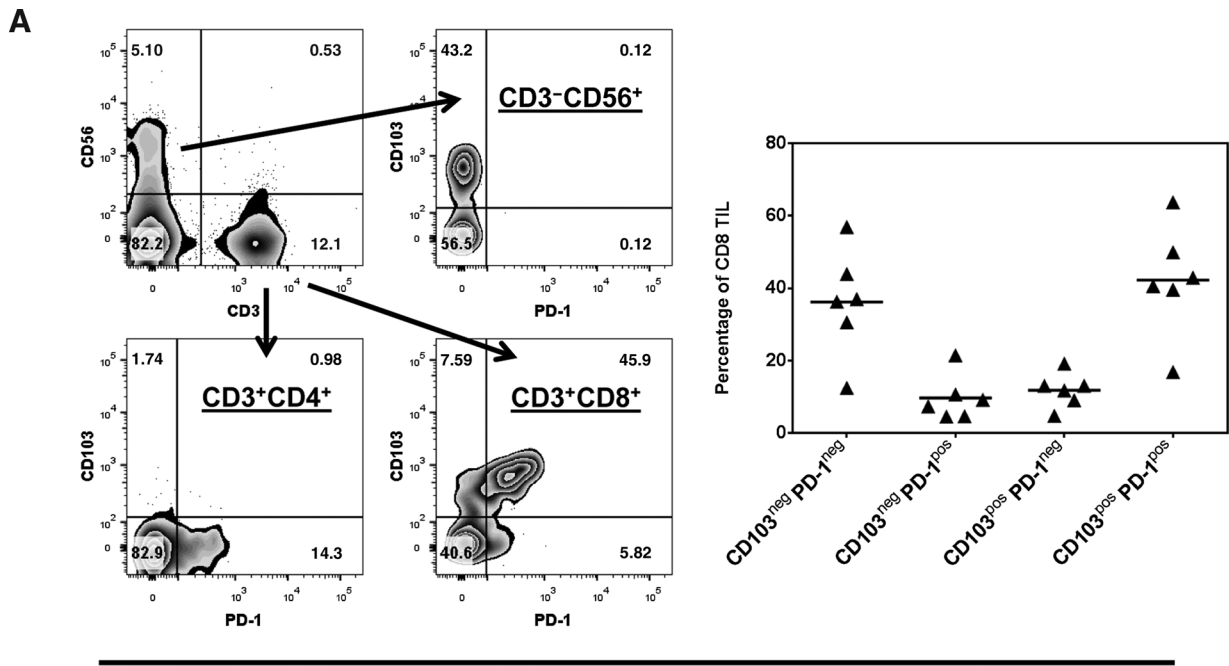
cells were positively associated with CD3⁺, CD8⁺, and CD25⁺ FOXP3⁺ TIL (Table 2), and showed independent prognostic significance relative to CD3⁺ and CD25⁺FOXP3⁺ TIL but not to CD8 TIL.

Coexpression of PD-1 and CD103 by CD8 TIL

To more precisely determine which cell type(s) express PD-1 in HGSC, we performed two-color IHC for CD3 and PD-1. PD-1 expression was largely restricted to CD3⁺ TIL (Fig. 2A). Some tumors also contained lower numbers of PD-1⁺ CD3⁻ cells (average of 4.6% of all PD-1⁺ cells); however, the identity of these cells was not further evaluated. Interestingly, PD-1⁺ CD3 TIL and PD-1⁻ CD3 TIL were often in close proximity, suggesting

that expression of PD-1 is regulated by cell-intrinsic mechanisms rather than loco-regional factors. Multicolor flow cytometry of disaggregated tumors (*n* = 8) confirmed that PD-1 expression was largely restricted to CD3⁺ TIL, including both the CD4⁺ and CD8⁺ subsets (Fig. 2B).

To initially assess the association between PD-1⁺ and CD103⁺ cellular infiltrates, serial sections of the 489 case TMA described above were stained separately with antibodies directed against PD-1 and CD103. There was a strong positive association between PD-1⁺ and CD103⁺ infiltrates in both the full cohort (i.e., all subtypes; Spearman *R* = 0.612, *P* < 0.0001) and the HGSC subtype (Spearman *R* = 0.669, *P* < 0.0001; Fig. 3A, left). Moreover, irrespective of histologic subtype, essentially all tumors that



contained PD-1⁺ cells (107/108) also contained CD103⁺ cells. This association was unidirectional, as some tumors (spanning all subtypes) contained CD103⁺ cells but lacked PD-1⁺ cells. Kaplan–Meier analysis revealed that among HGSC cases, patients with tumors containing both CD103⁺ and PD-1⁺ cells were associated with significantly increased disease-specific survival compared with those with either subset alone ($P = 0.0019$; Fig. 3A, right). Similar trends were observed for endometrioid and clear cell cancers, but the association did not reach statistical significance (data not shown).

Two-color IHC revealed that PD-1 and CD103 were frequently coexpressed on the same infiltrating cells in HGSC (Fig. 3B, top left and right). Moreover, PD-1⁺CD103⁺ cells were preferentially localized to intraepithelial regions of the tumor (Fig. 3B, lower left), as we previously reported for CD103⁺ TIL (23). In contrast, IELs from healthy fallopian tube tissue ($n = 10$) generally expressed CD103 in the absence of PD-1 (Fig. 3B, lower right). Thus, coexpression of CD103 and PD-1 appears to be a unique feature of TIL rather than IELs in general.

By flow cytometry of disaggregated HGSC samples ($n = 6$), coexpression of PD-1 and CD103 was found to be restricted to CD3⁺CD8⁺ TIL (Fig. 4A). Moreover, CD3⁺CD8⁺ TIL that were either doubly positive or doubly negative for PD-1 and CD103 were far more frequent than CD3⁺CD8⁺ TIL that were singly positive for either marker (Fig. 4A, left and right). Cells that expressed CD103 or PD-1 alone were almost always outside the CD8 compartment. Specifically, PD-1⁻CD103⁺ TIL predominantly comprised CD3⁻CD56⁺ NK cells, and PD-1⁺CD103⁻ cells predominantly comprised CD3⁺CD4⁺ T cells.

The high-degree of coexpression of PD-1 and CD103 by CD8 TIL suggested that the two molecules might be coordinately regulated. To investigate this possibility, healthy donor PBMCs were stimulated *in vitro* with PHA and TGF β , a condition that is known to induce CD103 expression on CD8 T cells (31, 32). Clear differences in the expression patterns of PD-1 and CD103 were observed. PD-1 was upregulated on both CD8 and CD4 T cells within 3 days of PHA stimulation but disappeared by day 6 (Fig. 4B). Furthermore, expression of PD-1 was not influenced by exogenous TGF β . In contrast, CD103 was expressed by CD8 T cells, but only a small proportion of CD4 T cells (Fig. 4B). Furthermore, expression of CD103 was dependent on exogenous TGF β and persisted to day 6. Negligible expression of PD-1 or CD103 was seen when cells were stimulated with TGF β alone (Fig. 4B). Thus, despite their frequent coexpression by CD8 TIL, CD103 and PD-1 appear to be regulated by distinct mechanisms.

PD-1⁺ TIL are not terminally exhausted

To investigate the functional status of PD-1⁺ TIL, six HGSC specimens were evaluated by intracellular cytokine staining. When assessed directly *ex vivo*, both CD4 and CD8 TIL were

negative for baseline cytokine production despite the presence of autologous tumor cells in the preparations (Fig. 5A). However, after stimulation with PMA and ionomycin, both the PD-1⁺ and the PD-1⁻ subsets of CD4 and CD8 TIL produced robust amounts of TNF α and/or IFN γ (Fig. 5A). The PD-1⁺CD103⁺ CD8 TIL subset produced similar levels of cytokines as the other TIL subsets. Furthermore, PD-1⁺CD103⁺ CD8 TIL showed negligible expression of the exhaustion markers TIM-3, CTLA-4, and Lag-3 (Fig. 5B). Thus, despite being quiescent directly *ex vivo*, PD-1⁺CD103⁺ CD8 TIL did not exhibit a terminally exhausted phenotype.

Discussion

The inhibitory molecule PD-1 has generated great interest as a target for immune modulation in cancer (13), yet the physiologic conditions that influence its expression are largely unknown (33). Herein, we report that in human ovarian cancer PD-1 expression by CD8 TIL is restricted to the subset expressing the integrin molecule $\alpha_E(\text{CD103})\beta_7$. We speculate that $\alpha_E(\text{CD103})\beta_7$ -mediated retention of T cells within tumor tissue might promote PD-1 upregulation by exacerbating the phenomenon of chronic antigen stimulation. Given that CD103⁺ TIL have been observed in a number of other epithelial cancers, including colon, lung, pancreatic, and bladder cancer (29, 34–37), this same phenomenon may have relevance beyond ovarian cancer. Indeed, a recent report indicates that CD103 and PD-1 are coexpressed by CD8 TIL in non-small cell lung carcinoma (38). Thus, $\alpha_E(\text{CD103})\beta_7$ may play an important role in the regulation and/or function of PD-1.

Despite the fact that PD-1 and CD103 are widely coexpressed by CD8 TIL in ovarian cancer, our results indicate these molecules are regulated by distinct mechanisms. CD103 has long been known to be upregulated on CD8 T cells after TCR stimulation in the presence of TGF β (39), a finding that was confirmed herein. However, exogenous TGF β did not influence the expression of PD-1 by either CD4 or CD8 T cells after PHA stimulation of healthy donor PBMCs. Moreover, TGF β -mediated upregulation of CD103 on CD8 T cells was maintained for at least 6 days after PHA stimulation, whereas PD-1 expression peaked on day 3 and then returned to baseline levels. Interestingly, it was reported recently that the transient upregulation of PD-1 on activated T cells can be inhibited by anti-TGF β antibodies (40), which implies that endogenous sources of TGF β may be required for PD-1 expression.

TGF β also plays a critical role in the development of tissue-resident memory CD8 T cells (Trm) during pathogen-driven immune responses. Specifically, activation of virus-specific T cells in the TGF β -rich environment of mucosal tissues leads to the upregulation of CD103, which serves as a distinguishing marker of the Trm subset (41). Adhesive interactions between

Figure 4.

PD-1 and CD103 are coexpressed on CD8⁺ T cells but demonstrate distinct mechanisms of regulation. A, coexpression of PD-1 and CD103 by CD8 TIL in HGSC. Flow cytometric analysis of cells from disaggregated HGSC samples. TIL were stained directly *ex vivo* with antibodies to CD3, CD4, CD8, CD56, PD-1, and CD103. Left, the gating strategy. Cells in the lymphocyte gate (FSC/SSC) were further gated into CD3⁺CD56⁻ T cells and CD3⁻CD56⁺ NK cells. T cells were then subdivided into CD4 and CD8 subsets, and the expression of PD-1 and CD103 was evaluated on each population. Right, a summary of the PD-1-CD103 coexpression patterns on CD3⁺CD8⁺ TIL from 6 HGSC tumors. B, TGF β induces expression of CD103 but not PD-1 on CD8 T cells. Flow cytometric analyses of healthy donor PBMCs at days 0, 3, and 6 after stimulation with PHA (5 $\mu\text{g}/\text{mL}$) in the presence or absence of TGF β (2 ng/mL), as indicated. Cells were stained with antibodies to CD3, CD4, CD8, PD-1 and CD103, and CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were assessed for expression of PD-1 and CD103. The activation status of each sample was confirmed by gating on blasts according to the FSC/SSC pattern. Top, a representative example from one donor; bottom, a summary of the results for four healthy donors.

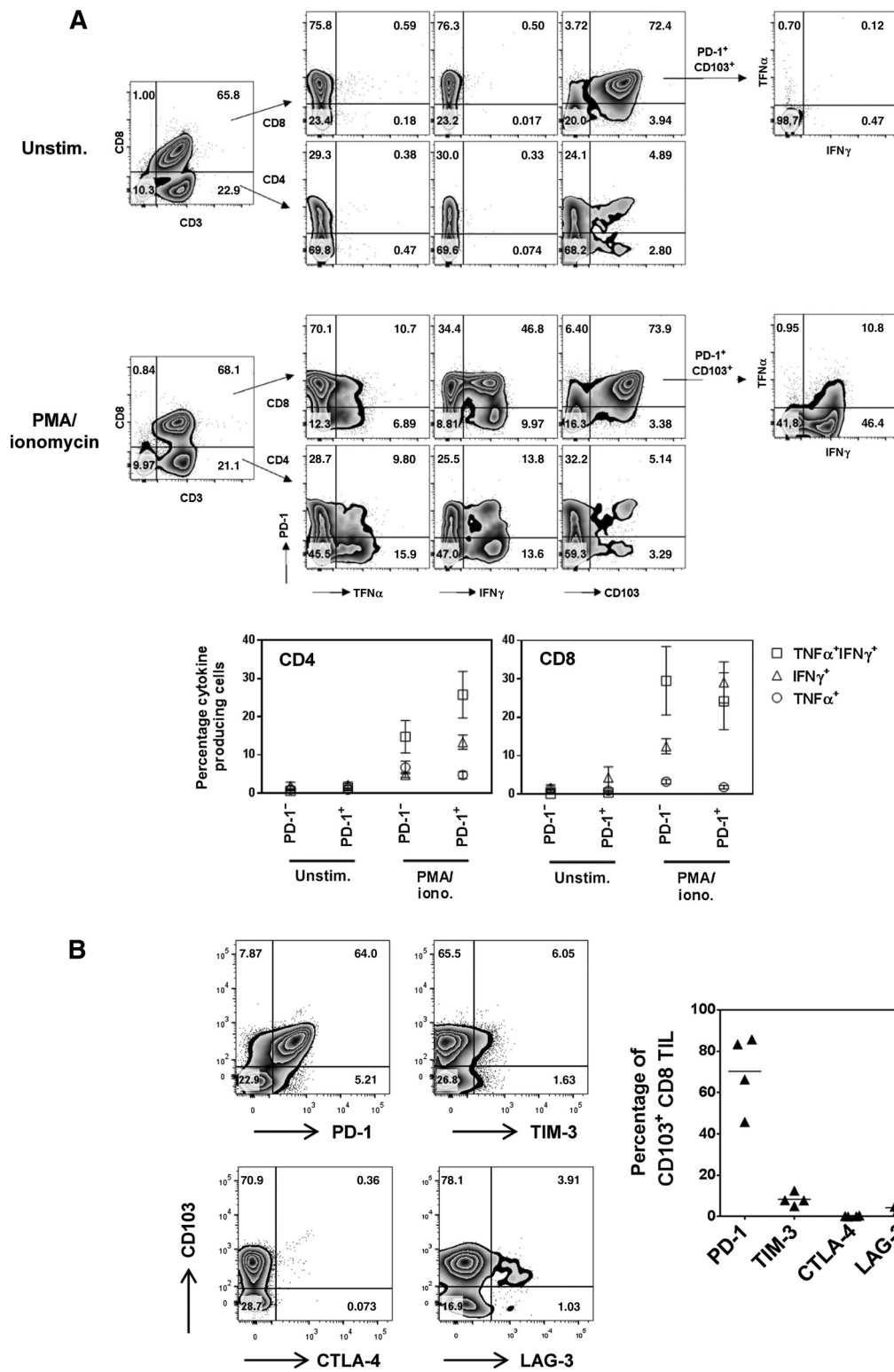


Figure 5. PD-1⁺CD103⁺ TIL are quiescent but not terminally exhausted in HGSC. **A**, flow cytometric analysis of cytokine production by TIL. HGSC tumor samples were cultured for 4 hours in the presence or absence of PMA and ionomycin (50 and 250 ng/mL, respectively). Cells were stained with antibodies to CD3, CD8, PD-1, and CD103, fixed, and then stained again to detect intracellular TNF α and IFN γ . Cells within the FSC/SSC lymphocyte gate were subdivided into CD4 and CD8 subsets for analysis. Top, representative data from one tumor; bottom, a summary of results for six samples (five ascites and one disaggregated primary tumor). **B**, flow cytometric analysis of exhaustion marker expression by CD8 TIL. Left, representative data from a tumor specimen stained directly *ex vivo* with antibodies to PD-1, TIM-3, CTLA-4, and LAG-3 (gated on CD3⁺CD8⁺ cells). Right, a summary of results for four samples (gated on CD3⁺CD8⁺CD103⁺ cells).

α_E (CD103) β_7 and E-cadherin retain Trm at sites of infection long after pathogen clearance, providing a reservoir of memory cells to protect against reexposure. However, in contrast with the CD103⁺ TIL, CD103⁺ Trm do not coexpress PD-1 (42), a finding that we corroborated by examining Trm in healthy fallopian tube tissue (Fig. 3). A possible explanation for this difference is that CD103⁺ Trm are no longer exposed to Ag once the relevant infection is cleared, whereas CD103⁺ TIL may be in continuous contact with cognate tumor Ag.

Although the vast majority of PD-1⁺ cells in HGSC were CD3⁺ T cells, some tumors also contained low numbers of CD3⁻ PD-1⁺ cells. Prior studies have shown that PD-1 can also be expressed on B cells, natural killer T (NKT) cells, NK cells, activated monocytes, and subsets of dendritic cells (DC; ref. 43). Indeed, tumor-infiltrating PD-1⁺ DCs have been shown to suppress T-cell activity in a mouse model of ovarian cancer (44); whether such cells also influence antitumor immunity in human HGSC awaits further study. Thus, although the PD-1–PD-L1 pathway is thought to be most relevant to CD4 and CD8 T cells, other immune cell types might also be influenced by this regulatory mechanism.

Our finding that PD-1 TIL are associated with favorable prognosis in HGSC was somewhat unexpected given the well-described role of PD-1 in T-cell suppression and exhaustion. This was even more surprising, given that PD-1⁺ TIL were positively associated with disease grade in both the HGSC and endometrioid subtypes. However, similar to our findings, PD-1⁺ TIL have been associated with favorable prognosis in HPV⁺ head and neck cancer (45) and follicular lymphoma (46–48). On the other hand, PD-1⁺ TIL have been associated with poor prognosis in breast and renal cancer (8, 10–12). Although speculative, these opposing prognostic effects might reflect the different histologic and molecular subtypes within each of these malignancies. In addition, levels of PD-L1 expression and the cell types expressing PD-L1 may be different between different tumor types. PD-L1 is reported to be widely expressed in ovarian cancer (21), which presumably would inhibit the antitumor activity of CD103⁺PD-1⁺ effector T cells. This may explain our observation that CD103⁺PD-1⁺ CD8 TIL are quiescent when assessed directly *ex vivo*. From this perspective, the presence of PD-1⁺CD103⁺ TIL might demarcate tumors that have thwarted the antitumor immune response through upregulation of PD-L1, a scenario referred to as "adaptive resistance" (49, 50). Subsequently, cytoreductive surgery and chemotherapy may provide an oppor-

tunity for antitumor immunity to be reset and reengaged, leading to the improved prognosis observed herein.

There is evidence in the literature suggesting that, in some settings, PD-1 is more an indicator of T-cell activation than exhaustion (2). This is consistent with our current results showing robust cytokine production by PD-1⁺ TIL, as well as our previous report showing that CD103⁺ TIL (the majority of which express PD-1) have an activated phenotype (MHC class II^{high}, Ki67^{high}, CD28^{low}, ref. 23). A similar scenario has recently been described in melanoma, where PD-1 was shown to specifically demarcate tumor-reactive CD8 T cells (51). Importantly, in this study, PD-1 expression on melanoma-derived TIL was shown to be rapidly downregulated after *in vitro* expansion, and these "reinvigorated" TIL mediated profound clinical effects after reinfusion into melanoma patients (52). If this latter finding is also applicable to ovarian cancer, then it bodes well for the possibility of using *in vitro* expanded PD-1⁺CD103⁺ TIL for the treatment of this lethal disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.R. Webb, K. Milne, B.H. Nelson

Development of methodology: J.R. Webb, K. Milne

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.R. Webb, K. Milne

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.R. Webb, B.H. Nelson

Writing, review, and/or revision of the manuscript: J.R. Webb, K. Milne, B.H. Nelson

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Milne, B.H. Nelson

Study supervision: B.H. Nelson

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