PD-1 and CD103 are widely co-expressed on prognostically favorable intraepithelial CD8 T cells in human ovarian cancer

John R. Webb\textsuperscript{a,b}, Katy Milne\textsuperscript{a} and Brad H. Nelson\textsuperscript{a,b,c}

\textsuperscript{a}Trev and Joyce Deeley Research Centre, British Columbia Cancer Agency, Victoria, Canada, \textsuperscript{b}Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada, \textsuperscript{c}Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada.

* Address correspondence to Dr. John R. Webb, Trev and Joyce Deeley Research Centre, British Columbia Cancer Agency, Victoria, BC, Canada, V8R 6V5. (Tel) 250-519-5706, (FAX) 250-519-2040, (email) jwebb@bccrc.ca

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Abstract

αE(CD103)β7 is a TGFβ-regulated integrin that mediates retention of lymphocytes in peripheral tissues by binding to E-cadherin expressed on epithelial cells. We recently reported that αE(CD103)β7 specifically demarcates intraepithelial CD8+ tumor-infiltrating lymphocytes (CD8 TIL) in ovarian cancer and that CD103+ TILs have a surface profile consistent with an active effector phenotype (HLA-DR+, Ki67+, CD127lo). These findings led us to hypothesize that, over time CD103-mediated retention of CD8 TILs 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+ TILs were strongly associated with increased disease-specific survival in HGSC (hazard ratio=0.4864; P=0.0007). Multi-color immunohistochemistry and flow cytometry revealed a high degree of PD-1 and CD103 co-expression, specifically within the CD8 TIL compartment. PD-1+CD103+ CD8 TILs 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 TILs 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 TILs might regain functional antitumor activity, an effect that potentially could be augmented by immune modulation.
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 immunological 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) [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 [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 [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, TILs are often abundant in late-stage tumors yet have obviously failed to prevent tumor progression [15-19]. Together, these features suggest that TILs 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+ TILs from ovarian cancer patients frequently express PD-1 and are functionally impaired compared to 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+ TILs in ovarian cancer express the integrin molecule αE(CD103)β7 [23]. αE(CD103)β7 is widely expressed by intraepithelial 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]. αE(CD103)β7 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 αE(CD103)β7 expression by TILs, we speculated that αE(CD103)β7-mediated retention of TILs within tumors might also have detrimental effects, by exacerbating the phenomenon of chronic antigen stimulation and immune exhaustion. Thus, in the current study we evaluated the extent of PD-1 expression by TILs in human ovarian cancer. We report that CD103 and PD-1 are highly co-expressed on intraepithelial CD8 TILs. Nonetheless, CD103+PD-1+ CD8 TILs 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 TILs in primary ovarian tumor samples, we stained a previously described tissue microarray (5) containing 489 evaluable cores from a cohort of ovarian cancer patients treated at the BC Cancer Agency from 1984 to 2000 (OvCaRe Ovarian Tumour 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 uM sections of either whole tumor tissue or tissue microarrays were deparaffinized and subjected to heat-induced epitope retrieval using a decloaking chamber and Diva decloaking reagent (Biocare Medical, Concord, CA). Anti-PD-1 mouse monoclonal antibody (mAb; clone NAT105, Cell Marque, Rocklin, CA) was applied at a dilution of 1/200, and rabbit anti-CD103 mAb (Epitomics clone EPR4166(2), Burlingame, CA) was applied at a dilution of 1/1500, each for 30 min 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, Pleasanton, CA). 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, Woburn MA) 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 multi-stained slides.

**Scoring and statistical analysis**

For prognostic analyses, absolute numbers of PD-1$^+$ and/or CD103$^+$ TILs (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 hazard ratio calculations were performed using Graphpad software. Univariate survival analysis was performed using the Kaplan-Meier method, and $P$ values were determined using 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 [30]. After thawing, cells were stained with the following fluorescently conjugated Abs, as indicated: CD3-BV510, CD8-BV421 (Biolegend, San Diego, CA), PD-1-PerCP-eFluor710, TIM-3-APC (eBioscience, San Diego, CA), LAG-3-FITC (Enzo Life Sciences, Farmingdale, NY) CD103-PE, CD56-APC, CTLA-4-PECy5 and CD4-FITC (BD Biosciences, San Jose, CA). All flow cytometry was performed using a FACS Calibur that had been upgraded to 8-color capability by Cytek (DxP8). Analyses were performed using FlowJo software (Tree Star, Ashland, OR).
**In vitro stimulations**

Peripheral blood mononuclear cells (PBMC) obtained from normal healthy donors were stimulated for 6 days with phytohemagglutinin (PHA) (5 ug/ml) in complete media containing 100 U/ml IL2 in the presence or absence of 2 ng/ml TGFβ1 (Peprotech, RockyHill, NJ). For intracellular cytokine assays, cell pellets from malignant ascites or disaggregated tumors were stimulated in medium with or without PMA (50 ng/ml) plus ionomycin (250 ng/ml). Cytokine release was inhibited by adding 2 uM monensin (GolgiStop, BD Biosciences) for the duration of the incubation. After 6 h incubation, cells were recovered by centrifugation, surface stained with the indicated antibodies, and fixed and permeabilized using Cytofix/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 75/195 (38.5%) high-grade serous carcinomas (HGSC) and 22/125 (17.6%) endometrioid, 11/128 (8.6%) clear cell, 0/30 mucinous and 0/11 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 to endometrioid or clear cell tumors (9.1 versus 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 (hazard ratio=0.4864; 95% confidence interval=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\(^+\) cells were positively associated with CD3\(^+\), CD8\(^+\) and CD25\(^+\)FOXP3\(^+\) TILs (Table 2), and showed independent prognostic significance relative to CD3\(^+\) and CD25\(^+\)FOXP3\(^+\) TILs but not to CD8 TILs.

**Co-expression of PD-1 and CD103 by CD8 TILs**

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\(^+\) TILs (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 TILs and PD-1\(^-\) CD3 TILs 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\(^+\) TILs, 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 to 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 co-expressed on the same infiltrating cells in HGSC (Fig 3B, upper 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+ TILs [23]. In contrast, intraepithelial lymphocytes from healthy fallopian tube tissue (n = 10) generally expressed CD103 in the absence of PD-1 (Fig 3B, lower right). Thus, co-expression of CD103 and PD-1 appears to be a unique feature of TILs rather than intraepithelial lymphocytes in general.

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

The high-degree of co-expression of PD-1 and CD103 by CD8 TILs suggested that the two molecules might be coordinately regulated. To investigate this possibility, healthy donor PBMCs were stimulated \textit{in vitro} with PHA and TGFβ, 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 TGFβ. 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 TGFβ and persisted to day 6. Negligible expression of PD-1 or CD103 was seen when cells were stimulated with TGFβ alone (Fig 4B). Thus, despite their frequent co-expression by CD8 TILs, CD103 and PD-1 appear to be regulated by distinct mechanisms.

**PD-1+ TILs are not terminally exhausted**

To investigate the functional status of PD-1+ TILs, 6 HGSC specimens were evaluated by intracellular cytokine staining. When assessed directly ex vivo, both CD4 and CD8 TILs 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 TILs produced robust amounts of TNFα and/or IFNγ (Fig 5A). The PD-1+CD103+ CD8 TIL subset produced similar levels of cytokines as the other TIL subsets. Furthermore, PD-1+CD103+ CD8 TILs 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 TILs 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 TILs is restricted to the subset expressing the integrin molecule αE(CD103)β7. We speculate that αE(CD103)β7-mediated retention of T cells within tumor tissue might promote PD-1 upregulation by exacerbating the phenomenon of chronic antigen stimulation. Given that CD103+ TILs 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 co-expressed by CD8 TILs in non-small cell lung carcinoma [38]. Thus αe(CD103)β7 may play an important role in the regulation and/or function of PD-1.

Despite the fact that PD-1 and CD103 are widely co-expressed by CD8 TILs 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 TGFβ [39], a finding that was confirmed herein. However, exogenous TGFβ did not influence the expression of PD-1 by either CD4 or CD8 T cells after PHA stimulation of healthy donor PBMCs. Moreover, TGFβ-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-TGFβ antibodies [40], which implies that endogenous sources of TGFβ may be required for PD-1 expression.

TGFβ 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 TGFβ-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 αE(CD103)β7 and E-cadherin retain Trm at sites of infection long after pathogen clearance, providing a reservoir of memory cells to protect against re-exposure. However, in contrast to the CD103+ TILs, CD103+ Trm do not co-express PD-1 [42], a finding that we corroborated by
Running title: Co-expression of PD-1 and CD103 by CD8 TIL in ovarian cancer

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+ TILs 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) [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 TILs 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+ TILs were positively associated with disease grade in both the HGSC and endometrioid subtypes. However, similar to our findings, PD-1+ TILs have been associated with favorable prognosis in HPV+ head and neck cancer [45] and follicular lymphoma [46-48]. On the other hand, PD-1+ TILs 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 TILs are quiescent when assessed directly ex vivo. From this perspective, the presence of PD-1+CD103+ TILs 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 opportunity for antitumor immunity to be reset and re-engaged, 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⁺ TILs, as well as our previous report showing that CD103⁺ TILs (the majority of which express PD-1) have an activated phenotype (MHC class II⁺, Ki67⁺, CD28⁻) [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 TILs was shown to be rapidly down-regulated after in vitro expansion, and these ‘reinvigorated’ TILs mediated profound clinical effects after re-infusion 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⁺ TILs for the treatment of this lethal disease.
Running title: Co-expression of PD-1 and CD103 by CD8 TIL in ovarian cancer

References


Running title: Co-expression of PD-1 and CD103 by CD8 TIL in ovarian cancer


Figure Legends

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 a 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.

Figure 2

**PD-1 is expressed predominantly by CD4 and CD8 TILs.** **A.** Co-expression of CD3 and PD-1. HGSC tumor specimens (n=20) underwent two-color IHC with antibodies against CD3 (green) and PD-1 (red) followed by Nuance image analysis to identify cells expressing both markers (yellow). The panel on the left shows a representative example of a tumor containing PD-1+ TILs. The panel on the right shows a quantitative assessment of the percentage of PD-1+ cells that were CD3− or CD3+. **B.** PD-1 is expressed by both CD4 and CD8 TILs. Disaggregated HGSC specimens were stained with antibodies against CD3, CD4, CD8 and PD-1 and assessed by flow cytometry. Cells were gated according to forward and side scatter (not shown) and CD3 and PD-1 staining (two leftmost panels). The right panel shows the percentage of PD-1+ TILs that expressed CD4 or CD8 (n= 8 independent HGSC specimens).
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Figure 3

Co-incidence 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. The left panel shows 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. The right panel shows disease-specific survival of HGSC patients based on three TIL patterns: PD-1$^+$ CD103$^+$, PD-1$^-$ CD103$^+$ or PD-1$^-$ CD103$^-$. The cut-off values for positivity were $\geq 1$ positive cells/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. The upper panels show representative tumors containing PD-1$^+$CD103$^+$ TILs. The lower left panel shows the extent of intraepithelial localization of PD-1$^+$CD103$^-$, PD-1$^-$CD103$^+$, and PD-1$^-CD103^+$ TIL in 8 HGSC tumors. Asterisks indicate a $P$-value of $<0.0001$ as determined by one-way ANOVA. The lower right panel shows 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.

Figure 4

PD-1 and CD103 are co-expressed on CD8$^+$ T cells but demonstrate distinct mechanisms of regulation. A. Co-expression of PD-1 and CD103 by CD8 TILs in HGSC. Flow cytometric analysis of cells from disaggregated HGSC samples. TILs were stained directly ex vivo with antibodies to CD3, CD4, CD8, CD56, PD-1 and CD103. The left panels show 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. The right panel shows a summary of the PD-1/CD103 co-expression patterns on CD3$^+$CD8$^+$ TILs from 6 HGSC tumors. B. TGFβ 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 ug/ml) in the presence or absence of TGFβ (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. The upper panel shows a representative example from one donor, and the bottom panels show a summary of the results for 4 healthy donors.

**Figure 5**

**PD-1+CD103+ TILs are quiescent but not terminally exhausted in HGSC.**  
*Panel A.* Flow cytometric analysis of cytokine production by TILs. HGSC tumor samples were cultured for 4 hours in the presence or absence of PMA and ionomycin (50 ng/ml 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 TNFα and IFNγ. Cells within the FSC/SSC lymphocyte gate were subdivided into CD4 and CD8 subsets for analysis. The upper panels show representative data from one tumor, and the bottom panels show a summary of results for 6 samples (5 ascites and 1 disaggregated primary tumor).  
*Panel B.* Flow cytometric analysis of exhaustion marker expression by CD8 TILs. The left panel shows 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). The right panel shows a summary of results for four samples (gated on CD3+CD8+CD103+ cells).
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A

B

C

HGSC

PD-1+

PD-1-

Percent survival

Time (yrs)

P=0.0007

(N=195)
A

\[ R = 0.612 \quad P < 0.0001 \]

B

PD-1^+ 
CD103^+ 
CD103^+PD-1^+

\[ P = 0.003 \] 
\( N = 195 \)
**Table 1.** PD-1⁺ cell infiltrates in relation to clinical characteristics of the retrospective patient cohort (according to REMARK criteria)

<table>
<thead>
<tr>
<th>Histologic subtype</th>
<th>Number of tumors</th>
<th>Number of tumors with PD-1⁺ cells present (%)*</th>
<th>Mean Number of PD-1⁺ cells per core (when present)**</th>
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<td>75 (36.4)</td>
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<td>DSS (yrs) – median (95% C.I.)</td>
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<td>Age (yrs) – median (range)</td>
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<td>DSS (yrs) – median (95% C.I.)</td>
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<tr>
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</table>

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

** mean number of PD-1 positive cells per core in those tumors scored as positive in previous column
Table 2. Disease-specific survival and hazard ratios based on PD-1$^+$ cell infiltrates.

<table>
<thead>
<tr>
<th>PD-1 cell density $^a$</th>
<th>Median survival (days)</th>
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<td>$N$</td>
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<td>Median</td>
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<tr>
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<tr>
<td>CD3 (&gt;5)</td>
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<td>CD3 (&lt;5)</td>
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<tr>
<td>CD8 (&gt;5)</td>
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<td>4.53</td>
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<tr>
<td>CD8 (&lt;5)</td>
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</tr>
<tr>
<td>CD25/FOXP3 (&gt;5)</td>
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<td>6.12</td>
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<tr>
<td>CD25/FOXP3 (&lt;5)</td>
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<tr>
<td>clear cell</td>
<td>128</td>
<td>0.52</td>
</tr>
</tbody>
</table>

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

$^b$ Hazard ratio calculated using Log-rank

$^c$ $P$-value using Log-rank (Mantel-Cox) test

** not reached
PD-1 and CD103 are widely co-expressed on prognostically favorable intraepithelial CD8 T cells in human ovarian cancer

John R Webb, Katy Milne and Brad H. Nelson

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