Tumor-infiltrating lymphocytes expressing the tissue resident memory marker CD103 are associated with increased survival in high-grade serous ovarian cancer.

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The immune system has a strong influence on clinical outcomes in cancer, as evidenced by the association between tumor-infiltrating lymphocytes (TIL) and patient survival. TIL actively enter the malignant epithelium of tumors, where they are thought to mount cytolytic responses against tumor cells. The mechanisms that promote the intraepithelial localization of TIL remain poorly understood. We addressed this issue in ovarian cancer, an aggressive malignancy in which CD8$^+$ TIL correlate with survival. We show that the subset of CD8$^+$ TIL localized to tumor epithelium expresses CD103 ($\alpha_E/\beta_7$ integrin), a molecule commonly found on normal mucosal intraepithelial lymphocytes and tissue-resident memory T cells. CD8$^+$ TIL that express CD103 have a highly activated and cytolytic phenotype and are strongly associated with patient survival. Thus, CD103 demarcates the subset of CD8$^+$ TIL that mediates protective tumor immunity. CD103 may serve as a useful marker to enrich these cells for cancer immunotherapy.
Abstract

Background The presence of CD8\(^+\) tumor-infiltrating lymphocytes (TIL) is associated with prolonged survival in high-grade serous ovarian cancer (HGSC) and other epithelial cancers. Survival is most strongly associated with intraepithelial versus intrastromal CD8\(^+\) TIL, however the mechanisms that promote the intraepithelial localization of TIL remain poorly understood. We hypothesized that intraepithelial CD8\(^+\) TIL, like normal mucosal intraepithelial lymphocytes, might express CD103, a subunit of \(\alpha_E/\beta_7\) integrin which binds E-cadherin on epithelial cells.

Methods A large collection of primary ovarian tumors (HGSC, endometrioid, mucinous and clear cell) was analyzed by immunohistochemistry for the presence of TIL expressing CD103. The activation and differentiation status of CD103\(^+\) TIL were assessed by flow cytometry. The prognostic significance of TIL subsets was evaluated by Kaplan-Meier analysis.

Results CD103\(^+\) TIL were present in all major ovarian cancer subtypes and were most abundant in HGSC. CD103\(^+\) TIL were preferentially localized to epithelial regions of tumors and were comprised predominantly of CD8\(^+\) T cells expressing activation (HLA-DR, Ki-67, PD-1) and cytolytic (TIA-1) markers, as well as CD56\(^+\) NK cells. Tumor infiltration by CD103\(^+\) TIL was strongly associated with patient survival in HGSC. Tumors containing CD8\(^+\) TIL that were CD103\(^-\) showed poor prognosis equivalent to tumors lacking CD8\(^+\) TIL altogether.

Conclusions CD103\(^+\) TIL comprise intraepithelial, activated CD8\(^+\) T cells and NK cells and are strongly associated with patient survival in HGSC. CD103 may serve as a useful marker for enriching the most beneficial subsets of TIL for immunotherapy.
Introduction

Epithelial ovarian cancer (EOC) is the fifth most frequent cause of death due to cancer in women worldwide (1). The overall five-year survival rate is 44% (2) and depends on stage at diagnosis, histological subtype and extent of surgical de-bulking. In addition, a number of studies have demonstrated that the presence of tumor-infiltrating lymphocytes (TIL) in surgically resected tumor tissue correlates with a favorable prognosis, particularly in patients with high-grade serous ovarian cancer (HGSC). For example, in a landmark study by the Coukos group, retrospective analysis of 186 stage III-IV EOC patients revealed that the five-year overall survival rate was 38% for patients with CD3+ tumor-infiltrating lymphocyte (TIL) positive tumors, compared to only 4.5% for CD3+ TIL negative tumors (3). Follow up studies revealed that the CD8+ subset of TIL correlates most strongly with survival (4). Moreover, although TIL are found in all EOC subtypes, their beneficial effects are most pronounced in HGSC (5). Importantly, the prognostic effect is strongest for CD8+ TIL that are localized within the epithelial component of tumors rather than the associated stroma (5). Despite this association, the mechanisms that promote the localization of TIL to tumor epithelium remain poorly understood.

Retention of T cells within epithelial tissues under a variety of normal and pathological conditions is known to be mediated, at least in part, through expression of the αE(CD103)/β7 integrin molecule (referred to hereafter as CD103) (6). In healthy individuals, CD103 is expressed on <2% of circulating peripheral blood lymphocytes but widely expressed on intraepithelial lymphocytes (IEL) of the gut mucosa and skin (7, 8). The only known ligand for CD103 is the epithelial cell surface molecule E-cadherin, and adhesive interactions between CD103 and E-cadherin are thought to be responsible for retention of antigen-specific lymphocytes within epithelial tissues (6). Interestingly, CD103 is also expressed by tissue-infiltrating, allo-reactive CD8+ T cells during transplant rejection (9) and graft-versus-host disease (10). In the infectious disease setting, CD103 is increasingly being recognized as a definitive marker of ‘tissue resident memory’ CD8+ T cells (CD8+ TRM) (11). Virus-specific CD8+ T RM have been found in tissues such as skin (12), sensory ganglia (13), brain (14), lung (15) and tonsils (16).
CD8+ TRM are stably retained at the site of primary infection for long periods of time after disease resolution, even in the complete absence of persisting antigen (17) and are thought to play a pivotal role in conferring immunity against re-exposure to the same pathogen (13, 18).

Recent evidence suggests that CD103 also plays an important role in immunity against cancer, and in particular, cancers of epithelial origin (19-23). For example, CD103:E-cadherin interactions have been implicated in the recognition of tumor cells by cytotoxic T cells (CTL) in colon (19), lung (20) and pancreatic cancer (21). We recently reported that the ascites compartment of some HGSC patients contains large numbers of CD8+CD103+ T cells with an effector memory phenotype (24). CD103 is reported to be recruited to the immunological synapse during tumor cell recognition, where it mediates adhesive interactions with E-cadherin on the target tumor cell, resulting in polarization of lytic granules and potentiated cytotoxic activity (20). In a lung cancer xenograft model, CD103 surface expression was upregulated on TIL after migration to the tumor and co-engagement of TCR and TGF-β receptors at that site (25). Together, these findings lead us to hypothesize that CD103 expression might demarcate intraepithelial, tumor-reactive TIL that confer favorable prognosis in epithelial cancers.

Using a new anti-CD103 antibody that works with formalin-fixed, paraffin embedded tissues (26), we have performed the first analysis of the intratumoral location and prognostic influence of CD103+ TIL in human cancer. We show herein that CD103 is widely expressed by TIL in human ovarian tumors and that it is preferentially expressed by intraepithelial versus stromal CD8+ TIL in both ovarian and breast tumors. In some patients, CD103 is also expressed by tumor-infiltrating NK cells. Importantly, CD103+ TIL are strongly associated with survival in patients with HGSC, indicating that CD103 demarcates a clinically beneficial TIL subset that warrants further analysis as a potential effector cell population for immunotherapy.

Materials and Methods

Patients
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. The prospective cohort was comprised of HGSC patients admitted to the BCCA between August 2007 and August 2011. Patients were treated with primary cytoreductive surgery followed by chemotherapy with carboplatin with or without paclitaxel. Tumor tissue obtained during primary cytoreductive surgery was fixed in formalin and embedded in paraffin using standard methodologies. A tissue microarray was constructed with duplicate 1.0 mm cores from representative tumor areas using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). Immunohistochemical analyses were performed on either whole tumor sections or on tissue microarrays, as indicated.

The retrospective cohort used for survival analysis has been previously described (5). In brief, tumor samples were obtained from EOC cases of various histological subtypes seen 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 had no macroscopic residual disease following surgical cytoreduction. Table 1 describes this cohort in accordance with REMARK criteria. The breast tumor microarray utilized has also been previously described (27) and contained 24 primary breast tumors with no specific selection criteria.

**Immunohistochemistry**

To detect CD103+ cells, 4 μM 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-CD103 rabbit monoclonal Ab (Epitomics clone EPR4166(2), Burlingame, CA) was applied at a dilution of 1/1000 for 30 minutes at room temperature. Following washing, anti-rabbit-HRP polymer reagent was applied for 30 minutes at room temperature followed by DAB reagent. The slides were counterstained with hematoxylin, dehydrated and cover-slipped using Cytoseal 60.
For two-color immunohistochemical staining, sections were stained with rabbit anti-CD103 as described above in combination with a murine primary antibody against either CD8 (C8/144B) or CD56 (123C3.D5). 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.

For the CD103/CD8/cytokeratin triple stain, rabbit anti-CD103 and murine anti-CD8 antibodies were added for 30 minutes at room temperature followed by Mach 2 Double Stain 2 polymer for 30 minutes. CD103 was detected with DAB chromogen and CD8 was detected with Warp Red. Slides were subjected to a denaturation protocol followed by a second round of staining with pan-Cytokeratin antibody (clone PCK-26, Sigma-Aldrich St. Louis, MO). Slides were incubated with Mach 2 Mouse-AP polymer followed by Perma-Green Plus AP chromogen (Diagnostic Biosystems, Pleasanton, CA).

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 colours on multi-stained slides. False color images were generated using Metamorph software (Molecular Devices, Sunnyvale, CA).

Flow cytometry

Single-cell suspensions were prepared from freshly excised tumor specimens and cryopreserved, as described (28). After thawing, cells were stained with the following fluorescently conjugated Abs: CD3-BV510, PD-1-PECy7 (Biolegend, San Diego, CA), CD103-PECy7 (Ebioscience, San Diego, CA), CD8-BV421, CD103-PE, HLA-DR-PE, CD127-AF647, CD56-PE/APC, CD4 FITC, and Ki67-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).
Scoring and statistical analysis

Scoring for epithelial localization of CD8 and CD103 markers was undertaken by an experienced histopathologist (PHW) blinded to results of other markers or case identity. Positively stained cells that had morphological features compatible with lymphocytes were counted according to two localization patterns: (1) intraepithelial infiltrates, defined as TIL within cancer cell nests or in direct contact with cancer cells; and (2) stromal infiltrates, defined as TIL within stromal areas that lack direct contact with epithelial cells. For prognostic analyses, absolute numbers of CD103+ TIL (independent of location) in cores of the retrospective cohort TMA were enumerated by visual inspection. Duplicate cores were counted independently by two different investigators and the average cell count per core 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. P-values <0.05 were regarded as significant.

Results

CD103 is preferentially expressed by intraepithelial TIL

To assess the frequency and distribution of CD103+ TIL in primary ovarian tumor samples, we used a recently developed anti-CD103 antibody (26) to stain a previously described tissue microarray (5) containing cores from a cohort of 497 EOC solid tumor specimens. CD103+ TIL were detectable in tumors of all stages and grades and in all four major EOC subtypes (Fig. 1 and Table 1). In all tumor subtypes, CD103+ TIL appeared to be preferentially localized to the epithelial regions of tumors (Fig. 1A). The density of CD103+ TIL within each subtype ranged from negligible to heavy infiltration (Fig. 1B) and the highest mean frequency of CD103+ TIL was seen in serous, followed by the endometrioid, clear cell and mucinous subtypes, respectively (Fig. 1B and Table 2).
Focusing on the high grade serous subtype (HGSC), we then quantified the intratumoral location of CD103+ TIL as epithelial or stromal. Serial sections of a prospective HGSC TMA were stained with H&E, anti-CD8 or anti-CD103 and localization was evaluated by an experienced pathologist. There was a statistically significant bias in the localization of CD103+ versus CD8+ TIL to tumor epithelium with an average epithelial:stromal density ratio of 2.29 for CD103+ TIL versus 1.36 for CD8+ TIL (p=0.008 by the Wilcoxin test, n=34) (Fig. 2A). The biased intraepithelial localization was particularly evident in those tumors with denser CD103+ TIL infiltration.

As a complementary approach, we performed triple staining of HGSC tumors to simultaneously detect CD8, CD103 and cytokeratin, the latter being a well-established marker of tumor epithelium. Consistent with the above results, CD8+CD103+ TIL were preferentially localized to cytokeratin-positive regions of the tumor (Fig. 2B). In addition, we evaluated the localization of CD103+ TIL in breast cancer, where epithelial and stromal regions are typically more distinct than in HGSC. CD103+ TIL were present in 5 of 20 invasive breast cancer specimens analyzed, and they again showed preferential localization to tumor epithelium (Fig. 2C). Thus, as seen with normal mucosal IEL, CD103 is preferentially expressed by ‘intraepithelial’ TIL in ovarian and breast cancer.

The only known ligand for CD103 is the epithelial surface molecule E-cadherin (6). Consistent with prior reports (29), E-cadherin expression was variable across HGSC tumors, ranging from negative to strong expression (Fig. 3). However, there was no obvious correlation between E-cadherin staining intensity and the presence of CD103+ TIL (p=0.667, Pearson test, n=60). Thus, infiltration by CD103+ TIL is determined by factors other than E-cadherin expression.

CD103+ TIL are comprised of activated CD8+ T cells and CD56+ NK cells

To determine the lineage and activation status of CD103+ TIL, multi-parameter flow cytometry was performed on disaggregated tumor preparations from 6 HGSC solid tumor specimens (Fig. 4A). In most patients, the majority of CD103+ TIL were CD3+CD8+ T lymphocytes. The second most common subset of CD103+ TIL was comprised of CD3+CD56+ NK cells. In some tumors, we also observed small numbers of
CD3⁺CD4⁺CD8⁻ and CD3⁺CD8⁻CD4⁻ T cells among CD103⁺ TIL (Fig. 4A). CD103 staining was not detected on CD20⁺ B cells, FOXP3⁺ lymphocytes or CD14⁺ monocytes; CD11c⁺ DC were too rare to assess (data not shown). The presence of tumor-infiltrating CD103⁺CD8⁺ and CD103⁺CD56⁺ lymphocytes was confirmed by multi-parameter IHC using antibodies against CD103, CD8 and CD56 (Fig. 4B). Consistent with the flow cytometry results, both CD8⁺ and CD56⁺ cells expressing CD103 were readily detectable by IHC and were localized primarily to tumor epithelium.

We also compared the activation and differentiation status of CD103⁻ and CD103⁺ TIL by flow cytometry. Fig 5 shows representative plots from tumors in which the CD103⁺ TIL population was predominantly CD8⁺ T cells (Case #014) or a mixture of CD8⁺ T cells and NK cells (Case #042). Consistent with the above observation that most CD103⁺ TIL were either CD8⁺ T cells or CD56⁺ NK cells, CD103⁺ TIL cells universally expressed TIA-1, a marker of cytolytic potential. Compared to their CD103⁻ counterparts, CD103⁺CD8⁺ TIL had markedly increased expression of the activation markers HLA-DR, Ki-67 and PD-1 and lower levels of IL-7 receptor α chain (CD127).

**CD103⁺ TIL are associated with increased survival in HGSC**

We next assessed the relationship between CD103⁺ TIL and patient survival, using two distinct statistical approaches, both of which have previously been used to demonstrate the prognostic significance of TIL in EOC. The first approach used an absolute value (> 5 CD103⁺ TIL per 0.6 mm core) to stratify the cohort into infiltrated versus non-infiltrated subsets, similar to as has been described previously (3, 4). Using this strategy, the presence of CD103⁺ TIL correlated with increased disease-specific survival in HGSC (P=0.0003) and mucinous EOC (P=0.0481), but not endometrioid or clear cell cancers (Table 2 and Fig. 6A). The second approach relied upon the quantitative characteristics of the cohort to determine the cutoff value. As shown in Table 2, the distribution of CD103⁺ TIL infiltration was positively skewed (ie. the mean was significantly higher than the median) for all 4 histologic subtypes. Similar patterns of positive skewing have been observed previously for other TIL subsets and has led to the use of the lowest tertile as an approach for stratifying cohorts into infiltrated versus non-infiltrated subsets (4). When the lowest tertile approach was used, the presence of
CD103+ TIL again correlated with increased disease-specific survival in HGSC ($P<0.0001$). However, using this strategy, CD103+ TIL infiltration did not reach statistical significance in mucinous, endometrioid or clear cell EOC, likely due to the disproportionately low tertile values obtained in each of these subtypes (Table 2). CD103+ TIL infiltration remained a significant factor in multivariate survival analysis when age, stage, and grade were used as co-variants (Hazard ratio = 0.509; 95% Confidence Interval = 0.331-0.783; $P = 0.002$). Importantly, whereas previous studies have shown that only ‘intraepithelial’ CD8+ TIL are associated with survival in HGSC, herein, TIL were scored solely on the basis of CD103 expression, which obviated the need to precisely account for their localization within the tumor.

Finally, we attempted to determine whether CD103+ CD8 cells or CD103+ NK cells were primarily responsible for the observed prognostic effect in HGSC. Although it is technically feasible to perform three-color IHC with antibodies to CD8, CD56 and CD103, the resulting images are difficult to score reliably on the scale of a large TMA. Therefore, as an alternate approach, we stained serial sections of the TMA with antibodies to CD8 and CD103 and then scored tumors as positive for CD8+ and/or CD103+ TIL using a threshold of >5 cells per core. The majority of tumors were positive for both CD103+ and CD8+ TIL and, accordingly, were associated with favorable prognosis (Fig. 6B). In contrast, tumors that contained CD8+ TIL which lacked CD103 expression were associated with inferior survival, similar to those tumors lacking CD8+ TIL altogether, implying that it is the CD103+CD8+ TIL cells that are primarily responsible for improved prognosis. Moreover, very few tumors contained CD103+ TIL without CD8+ TIL, therefore we were unable to precisely infer the contribution of CD103+ NK cells to prognosis.
CD8⁺ TIL are strongly associated with patient survival in a broad range of epithelial cancers (30), however the mechanisms that promote their localization to tumor epithelium remain poorly understood. We examined the hypothesis that intraepithelial CD8⁺ TIL, like normal mucosal IEL and T RM, might express the αE/β7 integrin subunit CD103. Indeed, we found that CD103 expression is a common feature of intraepithelial CD8⁺ TIL in the four major subtypes of EOC. CD103⁺ TIL were found to have a highly activated, cytolytic phenotype, and included both CD8⁺ T cells and CD56⁺ NK cells. Consistent with their cytolytic phenotype, CD103⁺ TIL were strongly associated with survival in HGSC as well as mucinous EOC. Although CD103⁺CD8⁺ TIL have been reported in other epithelial cancers (19-23), to the best of our knowledge this is the first demonstration of their prognostic significance in any cancer. Our data indicates that CD103 demarcates a subset of TIL that appear to mediate protective anti-tumor immunity in HGSC and warrant further investigation as effector cells for immunotherapy.

In our analysis of 497 EOC tumors, the density of CD103⁺ TIL ranged widely, from 0 to >300 CD103⁺ TIL per tumor core. Although the underlying cause of this variability is unknown, earlier in vitro studies have demonstrated that upregulation of CD103 on the surface of CD8⁺ T cells is dependent upon the concurrent stimulation of the TCR and TGF-β receptors (19, 31). Based upon this dual signal model, variability in CD103⁺ TIL density could therefore reflect inter-patient differences in the levels of TGF-β, cognate antigens, or both. Although TGF-β is widely considered an immunosuppressive factor, it can facilitate immune function in some physiological contexts. For example, gut mucosal tissue contains resident IELs that upregulate CD103 expression in response to stimulation with cognate microbial Ag and locally expressed TGF-β (32). This culminates in the retention of IEL through adhesive interactions between αE(CD103)/β7 integrin and E-cadherin on gut epithelial cells (11). Accordingly, CD103 knockout mice show substantially reduced numbers of IEL in gut mucosa (33). Furthermore, allograft rejection involves infiltration of grafted tissues by CD8⁺ T cells, which express CD103 upon exposure to TGF-β in the local microenvironment (9). Importantly, allogeneic islet cell
transplants show increased *in vivo* persistence in CD103-deficient mice due to impaired infiltration of T cells into graft tissue (34). Ovarian cancers have long been known to express TGF-β (35), and indeed TGF-β expression has been linked to metastatic potential (36) and to the process of epithelial to mesenchymal transition (EMT) (37). Moreover, we previously reported that the malignant ascites of some HGSC patients contains CD103-expressing CD8+ T cells which correlate with the amount of TGF-β in the ascites fluid (24). Thus, the density of CD103+ TIL in solid tumors might reflect local TGF-β levels.

The fact that T cells also require antigen stimulation to upregulate expression of CD103 implies that CD103+ TIL may be responding to antigens in the tumor microenvironment. Although there is currently a paucity of well-characterized tumor antigens to test this hypothesis comprehensively in EOC, we previously showed that ascites-derived CD8+ T cells specific for the cancer-testis antigen NY-ESO-1 were predominantly CD103+ (24). The notion that CD103 is a marker of antigen-specific T cells is further supported by recent studies demonstrating CD103 expression by influenza-specific CD8+ T cells in human lung (15) and EBV-specific cells in human tonsil (16). CD103 is also expressed by virus-specific CD8+ cells in mouse brain after experimental infection with vesicular stomatitis virus (VSV) (14, 18). In the above examples, CD103 demarcates tissue resident memory CD8+ T cells (CD8+ T<sub>RM</sub>) which persist at sites of antigen encounter, even long after pathogen clearance (for review see (11, 38, 39)). Finally, in the present study it is noteworthy that CD103+ TIL express high levels of PD-1 (Fig. 5), which has been shown to be marker of tumor-reactive TIL in melanoma (40).

At present, the antigens recognized by the vast majority of TIL in ovarian cancer remain poorly defined. Based upon the evidence described above, we speculate that CD103 may specifically demarcate TIL that are tumor-specific and, as such, may serve as a useful marker for enriching tumor-reactive TIL for antigen discovery efforts. On the other hand, given that CD103 is also a marker by T<sub>RM</sub>, it is also conceivable that CD103+CD8+ TIL are specific for pathogen antigens rather than tumor antigens. This possibility is consistent with the recent hypothesis that many “ovarian” cancers actually
originate in the fallopian tubes (41) which constitute a barrier tissue that is continuously exposed to various microbial agents. Indeed, similar to other mucosal tissues, the mucosal surface of the female genital tract is known to harbor intraepithelial T cells that express CD103 (42). Thus, it is plausible that at least some of the CD103+ TIL in EOC may represent pathogen-specific T_{RM} that are remnants of prior immune response(s) in the fallopian tubes or neighboring mucosal tissues. However, this alternative hypothesis needs to be reconciled with the strong prognostic effect of CD103+ TIL observed herein, which is indicative of tumor-specificity rather than pathogen specificity.

In addition to its potential utility as both a prognostic marker and a tool for antigen discovery, CD103 provides a convenient cell surface marker for investigating the functional phenotype of intraepithelial TIL. Previously, CD103+CD8+ T cells have been shown to have regulatory/immunosuppressive properties, at least in some settings (43). Moreover, CD103 is often expressed on conventional regulatory CD4 T cells (Tregs) (44). Although Tregs are known to influence outcomes in ovarian cancer (4), we did not detect significant numbers of CD103-expressing Tregs during our analyses. In contrast, we demonstrate herein that, compared to their CD103− counterparts, CD103−CD8+ TIL express higher levels of the activation markers HLA-DR, PD-1 and Ki-67 and lower levels of the naïve T cell marker CD127. Most strikingly, virtually all CD103+ TIL expressed TIA-1, a marker of cytolytic potential that is strongly associated with survival in EOC (45) and breast cancer (46). The strong association between CD103+ TIL and patient survival, together with their universal expression of the cytolytic marker TIA-1, suggests that CD103 demarcates a subset of highly activated, cytolytic CD8+ TIL. However, the expression of PD-1 by CD103+CD8+ TIL could also signify functional exhaustion (47). Indeed, one can imagine that CD103+ TIL might be trapped within the tumor bed through interactions with E-cadherin expressed on tumor cells, and that they subsequently become exhausted due to chronic antigen stimulation. Indeed, the fact that these cells express high levels of PD-1 suggests their anti-tumor activity might be further enhanced through blockade of PD-1 or other inhibitory pathways.

Our finding that CD103 is preferentially expressed by intraepithelial TIL also has practical implications for TIL scoring. In particular, a worldwide task force is being assembled to validate the prognostic significance of TIL as defined by the so-called
Immunoscore (48). The ultimate goal of this effort is to bring the Immunoscore into standard clinical practice as a prognostic biomarker. Colorectal cancer is likely to be the first indication, but other tumor sites are being considered. It is often difficult to distinguish between epithelial versus stromal compartments of tumors, especially for non-experts. Our results suggest that immunohistochemical detection of CD103+ TIL may provide an objective surrogate means to score intraepithelial TIL. Indeed, we showed a strong correlation between CD103+ TIL and survival in HGSC without the need to discriminate their epithelial versus stromal location (Fig. 6A).

Although the majority of CD103+ TIL observed herein were CD8+ T cells, the next largest subset was comprised of CD56+ NK cells. Like CD8+ T cells, NK cells express CD103 when exposed to TGF-β (49), however it is unknown whether they require a second signal equivalent to antigen stimulation. In healthy individuals, CD103+CD56+ NK cells are found in microenvironments rich in TGF-β, including uterine decidua (50) and tonsil. Very little is known about the functional profile or prognostic significance of CD3+CD56+ NK cells in HGSC. To date, greater attention has been focused on CD56+CD3+ NKT-like cells, which correlate with poor outcome as defined by platinum resistance (51). Because CD103+ NK cells were almost always found together with CD103+CD8+ TIL, we were unable to precisely define their independent prognostic significance. However, given the strong association between CD103+ TIL and survival, further study regarding the role of CD103+CD56+ TIL in tumor immunity is clearly warranted.

In closing, the strong association between intraepithelial CD8+ TIL and patient survival has spawned great interest in the use of TIL as predictive and prognostic biomarkers and as effector cells for immunotherapy. We propose that CD103, by serving as a definitive marker of these cells, has the potential to dramatically increase our understanding of TIL function, antigen specificity and immunotherapeutic potential, opening new avenues in our understanding of tumor immunity in EOC and other cancers.
References

Running title: Prognostic significance of CD103+ TIL in ovarian carcinoma


Running title: Prognostic significance of CD103+ TIL in ovarian carcinoma


Figure Legends

Figure 1

CD103 is expressed by TIL in the major subtypes of ovarian cancer. A A retrospective TMA containing 497 ovarian cancer cases comprising all four major ovarian cancer tumor types (HGSC, endometrioid, mucinous and clear cell) was stained with anti-CD103 antibody and counterstained with hematoxylin. A representative image of each tumor subtype is shown. B Cluster plot showing the distribution of CD103+ TIL within EOC tumors of the indicated histologic subtype. The mean CD103+ TIL density for each subtype is indicated by a horizontal line.

Figure 2

CD103+ TIL are preferentially localized within the intraepithelial regions of HGSC tumors. A Serial sections of a HGSC prospective TMA were stained with 1) hematoxylin and eosin, 2) anti-CD103 or 3) anti-CD8 Abs and the density of CD103+ and CD8+ cells within epithelial versus stromal regions was scored by a pathologist. The graph depicts a pairwise comparison of the density of CD103+ and CD8+ TIL in epithelial versus stromal regions of each tumor and statistical comparison using the Wilcoxon test. B A representative HGSC sample was triple-stained with antibodies against CD8 (red), CD103 (green) and cytokeratin (blue). The image was false-colored to highlight cells that were double positive for CD8 and CD103 (yellow). The majority of CD103+ TIL were found in epithelial (cytokeratin-positive) regions.

Figure 3

CD103+ TIL do not correlate with E-cadherin expression by tumors. Serial sections of a HGSC prospective TMA were stained antibodies to E-cadherin (left panels) or CD103 (right panels). Representative examples are shown of tumors with low (top panels), intermediate (middle panels), and high (lower panels) densities of CD103+ TIL.

Figure 4
CD103 is expressed predominantly by intraepithelial CD8+ T cells and CD56+ NK cells in HGSC. A FACS analysis of CD103+ TIL in primary ovarian tumors. Tumor tissue obtained from six HGSC patients at the time of initial de-bulking surgery was enzymatically dissociated into single-cell suspension and stained with antibodies to CD103, CD3, CD4, CD8 and CD56, followed by flow cytometry. Events were first gated on CD103+ TIL populations and then sequentially into the indicated subpopulations. B Immunohistochemical analysis of CD103+ TIL in primary ovarian tumors. Sections of two representative HGSC tumors were dual-stained with antibodies to CD8 (red) and CD103 (green) (upper panel), or CD103 (red) and CD56 (green) (lower panel). Images were false colored to highlight cells that are positive for both markers (yellow). Results are representative examples from over 50 HGSC tumors.

Figure 5
Cell surface phenotype of CD103+ TIL in HGSC tumors. Tumor tissue obtained from two HGSC patients at the time of initial de-bulking surgery was enzymatically dissociated into single-cell suspension and stained with antibodies to CD103 plus the indicated markers, followed by flow cytometry. Upper panels are gated on the FSC/SSC lymphocyte gate. Lower panels are gated on CD8+ lymphocytes.

Figure 6
Prognostic value of CD103+ TIL in the four major subtypes of ovarian cancer. A A retrospective TMA containing samples from 497 cases comprising the four major histological subtypes of EOC was stained with anti-CD103 antibody. Total numbers of CD103+ TIL were determined without regard to their epithelial or stromal location. Data were binarized based on a threshold of ≤5 or >5 CD103+ cells per core (average of 2 cores/case) followed by Kaplan-Meier analysis. The log-rank test was used to compare curves and P-values less than 0.05 were considered significant. B Sequential sections from the TMA described in A were stained separately with antibodies to CD8 and CD103. Total numbers of CD8+ and CD103+ TIL (irrespective of location) were determined using a cutoff of ≤5 or >5 cells per core (average of 2 cores/case).
Meier analysis was used to compare outcomes for patients with tumors that were positive for one, both or neither marker.
Webb et. al. Fig. 2

A

![Graph showing localization of CD103 to CD8 with p=0.008](image)

B

![Immunofluorescence image showing CD8, CD103, and cytokeratin](image)

C

![Histological image](image)
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A

- > 5 CD103⁺ TIL per core
- ≤ 5 CD103⁺ TIL per core

HGSC

Mucinous

Endometrioid

Clear Cell

% Disease-specific survival vs. Days

p=0.0003 (n=198)
p=0.0481 (n=31)
p=0.1357 (n=125)
p=0.5221 (n=131)

B

CD8⁺ CD103⁺
CD8⁺ CD103⁻
CD8⁻ CD103⁻

% Disease-specific survival vs. Days

p=0.0039

Days
Table 1
CD103+ TIL in relation to clinical characteristics of the retrospective patient cohort (according to REMARK criteria)

<table>
<thead>
<tr>
<th>Histologic subtype</th>
<th>CD103 TIL Present (%)</th>
<th>Mean Number of CD103+ TIL per core (when present)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous (N= 210)</td>
<td>6.9 (4.6-9.2)</td>
<td>172 (81.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46.4</td>
</tr>
<tr>
<td></td>
<td>DSS (yrs) – median (95% C.I.)</td>
<td>60 (37.6-86)</td>
</tr>
<tr>
<td></td>
<td>Age (yrs) – median (range)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>142</td>
</tr>
<tr>
<td>FIGO Stage</td>
<td>I</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>69</td>
</tr>
<tr>
<td>Mucinous (N=31)</td>
<td>19 (61.3)</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>DSS (yrs) – median (95% C.I.)</td>
<td>not reached</td>
</tr>
<tr>
<td></td>
<td>Age (yrs) – median (range)</td>
<td>55.0 (25.4-82.5)</td>
</tr>
<tr>
<td>Grade</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>FIGO Stage</td>
<td>I</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1</td>
</tr>
<tr>
<td>Endometrioid (N=125)</td>
<td>87 (69.6)</td>
<td>21.6</td>
</tr>
<tr>
<td></td>
<td>DSS (yrs) – median (95% C.I.)</td>
<td>not reached</td>
</tr>
<tr>
<td></td>
<td>Age (yrs) – median (range)</td>
<td>53.9 (29.4-88.1)</td>
</tr>
<tr>
<td>Grade</td>
<td>1</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>FIGO Stage</td>
<td>I</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>6</td>
</tr>
<tr>
<td>Clear cell (N=131)</td>
<td>61 (46.6)</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>DSS (yrs) – median (95% C.I.)</td>
<td>not reached</td>
</tr>
<tr>
<td></td>
<td>Age (yrs) – median (range)</td>
<td>54.8 (28.1-89.0)</td>
</tr>
<tr>
<td>Grade</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>131</td>
</tr>
<tr>
<td>FIGO Stage</td>
<td>I</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>8</td>
</tr>
</tbody>
</table>

* tumors with a mean of $\geq 1$ CD103 positive cells per 0.6 mm core (2 duplicate cores, each counted once by two independent investigators)

** mean number of CD103 positive cells per 0.6 mm core in those tumors scored as positive in previous column
Table 2
Disease specific survival and hazards ratio based upon the presence of CD103+ TIL.

<table>
<thead>
<tr>
<th>CD103 density a</th>
<th>Median survival (days) (absolute cutoff approach)</th>
<th>Median survival (days) (lowest tertile approach)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95% C.I.</td>
<td>Hazard ratio b</td>
</tr>
<tr>
<td>N</td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>HGSC</td>
<td>40.19</td>
<td>20.8</td>
</tr>
<tr>
<td>mucinous</td>
<td>5.34</td>
<td>2.0</td>
</tr>
<tr>
<td>endometrioid</td>
<td>15.07</td>
<td>3.8</td>
</tr>
<tr>
<td>clear cell</td>
<td>7.61</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a based on mean number of CD103+ TIL per 0.6 mm core (average of 2 cores), regardless of location within tumor
b Hazard ratio calculated using logrank
c P-value using Log-rank (Mantel-Cox) test
** - not reached
Tumor-infiltrating lymphocytes expressing the tissue resident memory marker CD103 are associated with increased survival in high-grade serous ovarian cancer


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