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Original Article

ORIGINAL ARTICLE

A study of the immune infiltrate and patient outcomes in esophageal cancer

Melissa J.Conroy[1,*,†], Susan A.Kennedy[1,†], Suzanne L.Doyle[2], Brian Hayes[3], Maria Kavanagh[1], Eric P.van der Stok[4], Katie O'Sullivan[1], Mary-Clare Cathcart[1], John V.Reynolds[5] and Joanne Lysaght[1]

[1]Department of Surgery, Cancer Immunology and Immunotherapy Group, Trinity Translational Medicine Institute, Trinity College Dublin, St James's Hospital, Dublin 8, Ireland, [2]School of Biological Sciences, Dublin Institute of Technology, Ireland, [3]Department of Histopathology, Cork University Hospital and Department of Pathology, University College Cork, Ireland, [4]Department of Surgical Oncology, Erasmus MC Cancer Institute, The Netherlands and [5]National Esophageal and Gastric Centre, St. James's Hospital, Dublin, Ireland.

*To whom correspondence should be addressed. Tel: +353-1-8963620; Email: meconroy@tcd.ie
†These authors contributed equally to this work.

Abstract

Objectives: Cancer patient outcomes and selection for novel therapies are heavily influenced by the immune contexture of the tumor microenvironment. Esophageal cancer is associated with poor outcomes. In contrast to colorectal cancer, where the immunoscore is increasingly used in prognostic staging, little is known about the immune cell populations in esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (SCC), and their clinical significance.
Methods: Tissue microarrays were constructed from resected tumor tissue of 72 EAC patients and 23 SCC patients. Immunohistochemical staining of CD3, CD8, CD56, CD68, CD45RO, CD69, IFN-γ, IL-10, IL-4, IL-17, TGF-β, FOXP3 and CD107a was performed. Positivity was examined in both the stromal and epithelial compartments. Statistical analysis was performed to identify differences in immune cell infiltration and functional phenotypes between cancer subtypes and tissue compartments.
Results: This study identified that esophageal tumors are enriched with $CD45RO^+$ and $CD8^+$ cells and such positivity is significantly higher in SCC compared with EAC. Furthermore, the expression of CD45RO positively correlates with that of CD8 within the tumors of both patient cohorts, suggesting a dominance of memory cytotoxic T cells. This is supported by strong positivity of degranulation marker CD107a in the stromal compartment of EAC and SCC tumors. Cytokine staining revealed a mixed pro- and anti-inflammatory profile within EAC tumors.
Conclusions: Esophageal tumors are enriched with memory cytotoxic T cells. Applying these measurements to a larger cohort will ascertain the clinical utility of assessing specific lymphocyte infiltrates in EAC and SCC tumors with regards to future immunotherapy use, patient prognosis and outcomes.

Keywords: CD8+ T cells, esophageal cancer, tumor microenvironment, immune contexture

Introduction

Globally, gastrointestinal cancers are a leading cause of cancer death, with WHO estimating that >1.6 million deaths in 2018 were attributable (www.who.int). Of these, the incidence of esophageal cancer has increased markedly over the last 40 years with overall survival rates of <20% and ~50% for patients who can be treated with curative intent (1). Therefore, the need for research to develop therapeutics and innovative tests to improve survival, and to predict and enhance response to therapies such

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Abbreviations

CRT	chemoradiotherapy
EAC	esophageal adenocarcinoma
EBV	Epstein Barr virus
IFN	interferon
IL	interleukin
MSI	microsatellite instability
PD-1	programmed cell death 1
PD-L1	programmed cell death 1 ligand 1;
SCC	squamous cell carcinoma
TGF	transforming growth factor
TME	tumor microenvironment
Treg	regulatory T

as chemotherapy, immunotherapy and radiation therapy is urgently required.

In the modern era, immunotherapy is revolutionizing cancer treatment for many tumor types, in particular malignant melanoma and non-small cell lung cancer, where anti-PD1, anti-PDL-1 and anti-CTLA 4 therapies have had major clinical impact (2,3). In gastrointestinal cancer, the International Immunoscore Project has attempted to standardize immune measurements in lower gastrointestinal cancer (4). The Immunoscore for colorectal cancer is based on the T cell markers CD3 and CD8 and has demonstrated significant potential as an adjunct or alternative to the TNM cancer staging system (4). The score is based on the enumeration of lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO) in the core and invasive margin, with a high immunoscore being a positive prognostic feature, offering promise in personalized selection of adjuvant therapies. Notwithstanding encouraging evaluation of its efficacy, the Immunoscore has been challenged for not considering other immune cell infiltrates, such as macrophages and NK cells, and the heterogeneity of the T cell infiltrate (5).

The Immunoscore has not been studied for esophageal cancer. In gastric cancer, a new 4-score system incorporating CD8, PD-L1 and PD-1 on immune cells and PD-L1 on tumor cells might have superior prognostic application (6). The 11-score system incorporating four immune cell types ($CD8^+$, $CD4^+$, $FoxP3^+$ and $CD33^+$ cells), four inhibitory receptors (PD-1/PD-L1/Tim-3/LAG-3), two stimulatory checkpoints (OX-40/ICOS) and IDO1 could predict overall survival in squamous cell carcinoma (SCC) (7). This may suggest that a more extensive immunoscore may be required for esophageal cancer compared with what is currently proving successful for colorectal cancer. From our own group, we have reported alterations in intratumoral T cell phenotype in esophageal adenocarcinoma (EAC), and impaired T cell migratory capacity (8,9). These studies were performed using fresh tumor tissue samples, which is not a feasible or cost-effective option for standardized clinical prognostic immune-response measurements for solid tumors. Therefore, the study reported here pilots a broader immunoscoring system using formalin fixed and paraffin-embedded tumor samples. Moreover, this study has expanded immune infiltration analysis to 13 markers indicative of Th1, Th2, Th17, Treg, cytotoxic immune responses and macrophages, and studied both EAC and esophageal SCC patients.

We report herein differential expression of cytotoxic T cell marker CD8 and memory marker CD45RO between SCC and EAC, with greater expression in SCC. In addition, CD45RO positively correlates with that of CD8, suggesting a dominance of memory cytotoxic T cells. In addition, cytokine staining revealed a mixed pro- and anti-inflammatory profile within EAC and SCC tumors, which aligns with our previous findings and suggests that multiple subtypes of T cells and immune cells are present within esophageal tumors.

Patients and Methods

Subjects

A total of 95 consecutive consenting patients, attending the National Esophageal and Gastric Centre at St. James's Hospital, Dublin were enrolled in this study. The patient group included 72 patients with EAC and 23 patients with SCC. The study comprised of 59 males and 36 females with an average age of 64 years (Table 1).

Informed consent

Informed consent was obtained from all individual participants included in the study.

Compliance with ethical standards

The study received ethical approval from the St James's Hospital Ethics Review Board and it conforms to the provisions of the Declaration of Helsinki.

Tissue microarray generation

Tissue microarrays were created from paraffin-embedded EAC and SCC resected tumor specimens that were collected between July 1998 and December 2009. A pathologist marked the areas containing viable tumor. Cores were taken from tumor-containing areas and arrayed in a paraffin block. The invasive edge of the tumor was marked and included wherever possible. Core specimens were sampled for a total of 72 EAC patients and 23 SCC patients.

Immunohistochemistry

Immunohistochemical staining was optimized using full-face sections of esophageal cancer tissue to ensure staining specificity and optimal antibody concentrations. Two microarrays, sections

Table 1. Demographic data

Esophageal adenocarcinoma	72 (75.8%)
Esophageal squamous cell carcinoma	23 (24.2%)
Mean age (range years)	EAC: 64.15 (37–81), SCC: 64.74 (47–79)
Sex ratio (M:F)	EAC (57:15), SCC (2:21)
Tumor pathologic stage N (% of cancer type)	
T0	EAC: 0 (0%), SCC: 0 (0%)
T1	EAC: 9 (12.5%), SCC: 2 (8.7%)
T2	EAC: 14 (19.4%), SCC: 5 (21.7%)
T3	EAC: 45 (62.5%), SCC: 16 (69.6%)
T4	EAC: 4 (5.6%), SCC: 0 (0%)
Nodal status	
Positive	EAC: 50 (69.4%), SCC: 14 (60.9%)
Negative	EAC: 22 (30.6%), SCC: 9 (39.1%)
Mean body mass index (kg/m^2) (range)[a]	EAC: 26.27 (17.43–43.26), SCC: 21.81 (16.67–26.84)

Demographic table showing age, sex, cancer type, tumor pathologic stage, nodal status and BMI for the patient cohort. BMI, body mass index.
[a]BMI was not available for 19 EAC and 6 SCC patients.

of 4 μm were stained for 2 h at RT with anti-CD3 (clone F7.2.38) 1:100 dilution, anti-CD8 (clone C8/144B) pre-diluted, anti-CD45RO (clone UCHL1) 1:150 dilution and anti-CD68 (Clone KP1) pre-diluted from (Dako Agilent, Carpinteria, CA). Anti-IL-17a (clone eBio64Dec17) 1:500, anti-CD107a (clone eBioH4A3) 1:500 and anti-TGF-β (clone eBioTB2F) 1:1500 from (eBioscience, Thermo Fisher Scientific, Waltham, MA). Anti-Foxp3 (1:50) and anti-IL-4 (1:500) from Abcam, Cambridge, UK. Anti-CD56 (clone MEM-188) 1:100 and anti-IFN-γ (clone G-23) 1:100 from Immunotools, Friesoythe, Germany. Anti-IL-10 (1:100) and anti-CD69 (1:50) from R&D systems, UK. The grading was performed by three independent scorers (one of whom was a histopathologist) and averaged. Scoring for IL-10, IL-4, IL-17 IFN-γ and TGF-β was on the basis of intensity (0–3), where 0 was negative, 1 was weak, 2 was moderate and 3 represented strong staining. The quantity of positive cell staining for CD3, CD8, CD69, CD56, CD68, Foxp3, CD45RO and CD107a was scored as 0, 1, 10, 25, 50, 75, 90, or 100%. The data for tumor epithelium and stroma were analysed separately.

Within the field of view, which was the same magnification of 20× used by each of the three graders for each core, an estimated percentage of positively stained cells ranging from 0, 1, 10, 25, 50, 75, 90 or 100% amongst unstained cells was estimated. For example, 25% positive would suggest that for every 100 cells, 25 are positive. This scoring system was applied for markers determining subpopulations of infiltrating immune cells such as CD8 and CD56, where individual cells could be counted. However, this system was not applied to the grading system for cytokines as these stains were diffuse. Instead cytokine positivity was quantified as follows; 0-negative, 1-weak, 2-moderate and 3-strong staining.

Tumor tissue preparation

Tumor biopsies were collected during surgical resection. Tumor tissue was digested in 125 U/ml collagenase type IV in HBSS containing 4% FBS for 30 min on a shaking incubator at 37°C at 180 RPM before being passed through a 70 μm polypropylene filter (Filcon; BD Bioscience, San Jose, CA) to discard debris. Cells were washed twice with HBSS and centrifuged at 1300 RPM for 3 min.

Flow cytometric analysis

Quantification of cytotoxic memory T cells in EAC tumor

Intratumoral immune cells were stained with anti-CD3-conjugated with PEeflour610, anti-CD8 conjugated with Brilliant Violet 421 and anti-CD45RO conjugated with FITC (BD Biosciences, Oxford UK). $CD45RO^+$ $CD3^+$ T cells were quantified as a proportion of $CD3^+$ lymphocytes. $CD45RO^+$ $CD8^+$ T cells were quantified as a proportion of $CD8^+$ $CD3^+$ lymphocytes. Cells were acquired using the CyAn ADP (Beckman Coulter, Brea, CA) flow cytometer and analysed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (Version 5). The paired non-parametric *t*-test was applied to determine significant differences in the immune markers between tumor and stroma. The R project for statistical computing (version 3.5.1) was used to generate Figure 3 (10). The 'corrplot' package was used to generate visualizations of Spearman correlations (11). Kaplan–Meier survival curves and log-rank (Mantel–Cox) tests were used to assess any associations between immune marker expression and overall survival. Survival was calculated as the time, in months, from the day of diagnosis until death of the patient. The last follow-up date was used as a cut-off time point for survival calculations. Low expression of each immune marker was classified as ≤ median value and high expression was classified as ≥ median value.

Results

Significantly higher percentage of cells expressing CD45RO, CD8 and CD3 within the stromal compartments of EAC and SCC tumors

Staining of immune cell surface markers was performed on 72 EAC patients and 23 SCC patients; T cell markers CD3 and CD8, natural killer cell marker CD56, macrophage marker CD68, memory T cell marker CD45RO and activation marker CD69. Significantly stronger staining of CD3, CD8 and CD45RO was observed in the stroma compared with the tumor islets of EAC and SCC tissue sections, based on average % positive cells per patient (mean ± SEM CD68: EAC Tumor versus EAC Stroma: 5.83 ± 0.93% versus 9.05 ± 0.65%, $P < 0.001$, CD3: EAC Tumor versus EAC Stroma: 5.82 ± 0.86% versus 13.66 ± 1.08%, $P \leq 0.001$, SCC Tumor versus SCC Stroma: 7.54 ± 1.27% versus 19.49 ± 1.73%, $P \leq 0.001$, CD8: EAC Tumor versus EAC Stroma: 4.8 ± 0.69% versus 18.5 ± 1.4%, $P \leq 0.001$, SCC Tumor versus SCC Stroma: 9.7 ± 1.9% versus 24.7 ± 2.2%, $P \leq 0.001$, CD45RO: EAC Tumor versus EAC Stroma: 9.1 ± 0.66% versus 13.1 ± 0.94%, $P \leq 0.01$, SCC Tumor versus SCC Stroma: 11.9 ± 1.5% versus 24.4 ± 1.96, $P \leq 0.001$, Figure 1). Interestingly, there was significantly stronger staining within the SCC stroma compared with EAC stroma for CD3, CD8 and CD45RO (EAC versus SCC: CD3: $P < 0.05$, CD8: $P < 0.05$, CD45RO: $P \leq 0.001$, Figure 1). To confirm that esophageal tumors are enriched with memory $CD8^+$ T cells, CD8 and CD45RO co-staining was performed on immune cells isolated from fresh tumor explants from 3 EAC patients and flow cytometric analysis was performed. Our data revealed that the high percentage of CD45RO positivity was specific to the $CD8^+$ subset of T cells (mean ± SEM of $CD45RO^+CD8^+$ T cells versus $CD45RO^+CD3^+$ T cells; 79.73 ± 8.5% versus 15.10 ± 8.6%, $P = 0.1$, Figure 1E).

Significantly higher expression of IL-17 and TGF-β within the stromal compartments of EAC and SCC tumors while IL-10 is significantly enriched within the EAC tumor islets

Staining of the inflammatory Th1 cytokine IFN-γ, the Th17 cytokine IL-17, the Th2/ regulatory T cell cytokines IL-4, IL-10 and TGF-β and the regulatory T cell transcription factor FoxP3, was performed on 72 EAC patients and 23 SCC patients. IL-17 and TGF-β staining is significantly stronger in the stroma of EAC and SCC tumor tissue sections, based on average % positive cells per patient (mean ± SEM IL-17: EAC Tumor versus EAC Stroma: 0.82 ± 0.07% versus 1.1 ± 0.05%, $P \leq 0.001$, SCC Tumor versus SCC Stroma: 0.73 ± 0.07% versus 1.1 ± 0.07%, $P < 0.01$, TGF-β: EAC Tumor versus EAC Stroma: 1.4 ± 0.09% versus 1.8 ± 0.1%, $P \leq 0.01$, SCC Tumor versus SCC Stroma: 1.4 ± 0.1% versus 2.2 ± 0.1%, $P \leq 0.01$, Figure 2A). IL-10 is significantly more abundant within the tumor than the stroma, which indicates an immunoregulatory role in EAC (mean ± SEM in EAC Tumor versus EAC Stroma: 1.9 ± 0.09% versus 1.5 ± 0.09%, $P \leq 0.01$, Figure 2A), while Th1 cytokine IFN-γ is stronger in the stroma of SCC tumors only (mean ± SEM in SCC Tumor versus SCC Stroma: 0.6 ± 0.1% versus 1.2 ± 0.07%, $P < 0.001$). Interestingly, the Treg marker FOXP3 staining is significantly stronger in the stroma of SCC tumor tissue sections based on average % positive cells per patient (SCC Tumor versus SCC Stroma: 3.2 ± 0.79% versus 10.2 ± 1.05%, $P \leq 0.001$, Figure 2A). Overall the intensity of the inflammatory cytokines IFN-γ and IL-17 were lower in both EAC and SCC compared with the Th2/ regulatory T cell cytokines.

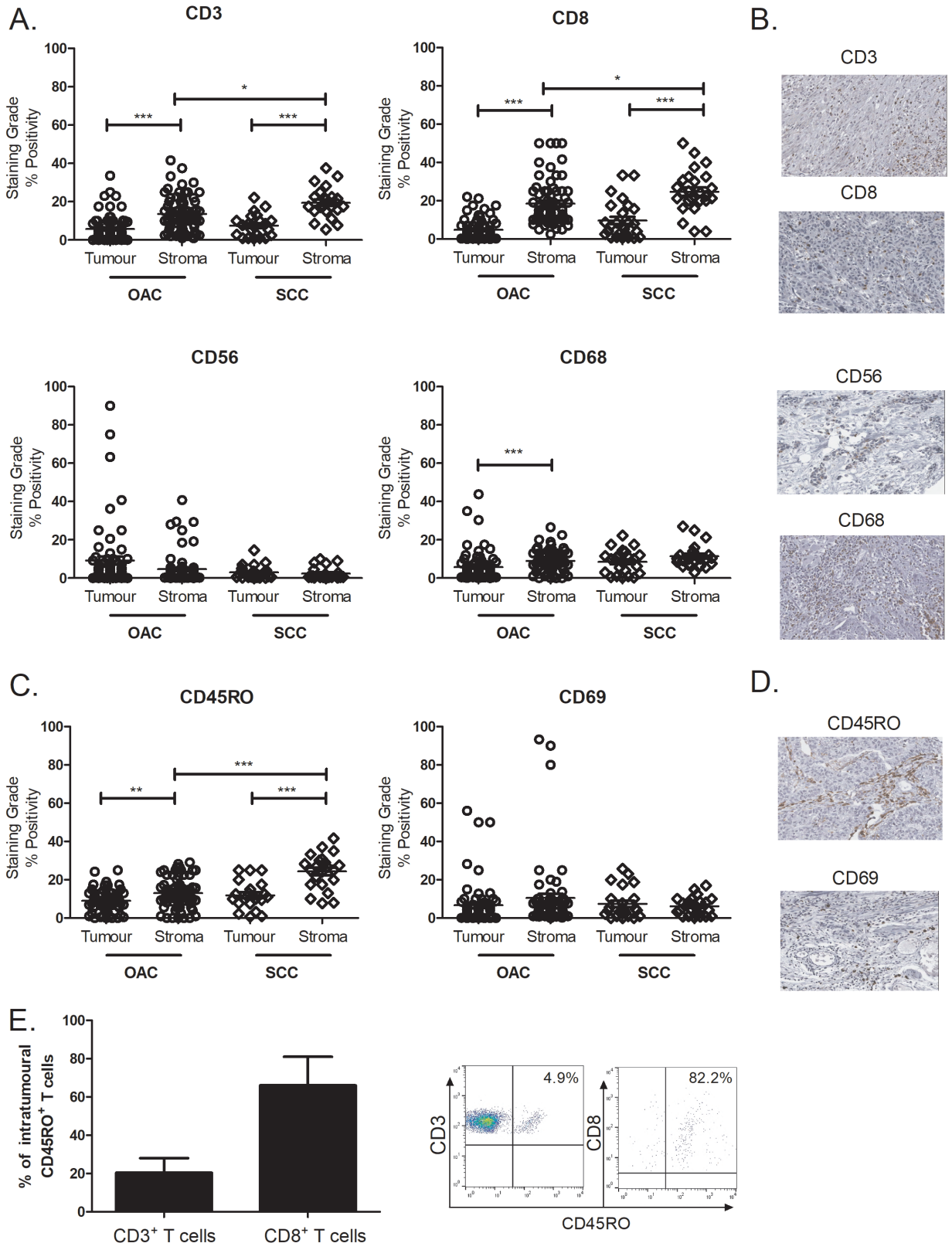

Figure 1. Significantly higher expression of CD45RO, CD8 and CD3 within the stroma of EAC and SCC tumors. Staining of CD3, CD8, CD56, CD68, CD45RO and CD69 was performed on 72 EAC patients and 23 SCC patients. (**A**) Scatterplots showing CD3 and CD8 staining is significantly stronger in the stroma of EAC and SCC tissue sections and CD68 staining is significantly stronger in the stroma of EAC tissue (based on average % positivity per patient). (**B**) Representative images of immunohistochemical staining of CD3, CD8, CD56 and CD68 in esophageal cancer tissue. (**C**) Scatterplots showing CD45RO staining is significantly stronger in the stroma of EAC and SCC tissue sections (based on average % positive cells per patient). (**D**) Representative images of immunohistochemical staining of CD45RO and CD69 in esophageal cancer tissue. (**E, left**) Bar chart showing % frequencies of $CD3^+CD45RO^+$ T cells and $CD8^+CD45RO^+$ T cells in tumor explants from 3 EAC patients. (**E, right**) Representative dot plots of gating on $CD3^+CD45RO^+$ T cells as a percentage of the total T cell population and $CD8^+CD45RO^+$ T cells as a percentage of the $CD8^+$ T cell population. $^*P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ paired non-parametric *t*-test.

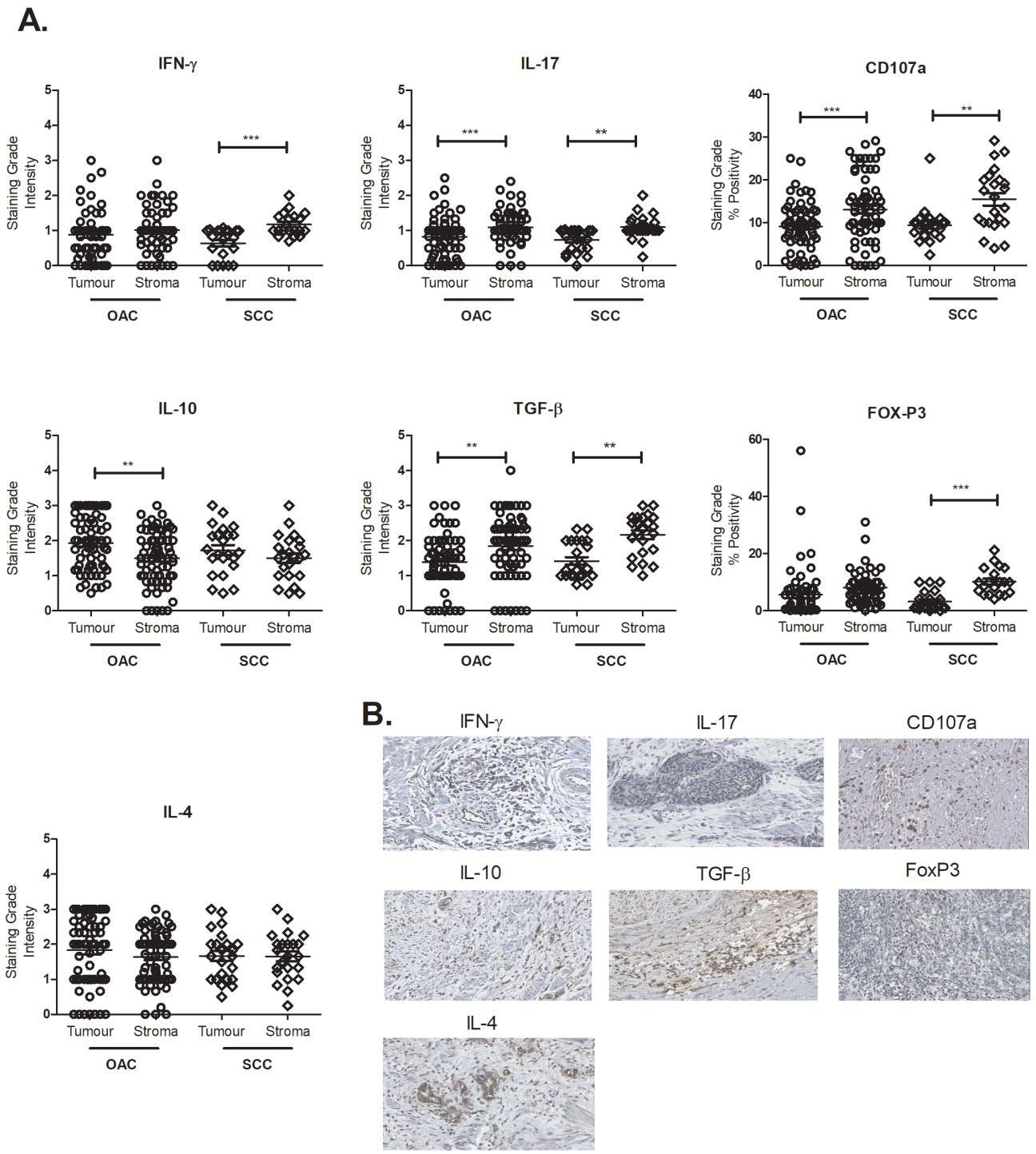

Figure 2. Significantly different levels of cytokines, degranulation marker CD107a and FoxP3 within the tumor and stromal compartments of EAC and SCC tumors. Staining of IFN-γ, IL-10, IL-4, IL-17, TGF-β, FoxP3 and CD107a was performed on 72 EAC patients and 23 SCC patients. (**A**) Scatterplots showing IL-17 and TGF-β staining is significantly stronger in the stroma of EAC and SCC tumor tissue sections (based on average staining intensity), while IFN-γ is stronger in SCC stroma only and IL-10 is significantly lower in the EAC stromal compartment compared with the tumor. FoxP3 staining is significantly stronger in the stroma of SCC tumor tissue sections and CD107a staining is significantly stronger in the stroma of EAC and SCC tumor tissue sections (based on average % positivity per patient) (**B**) Representative images of immunohistochemical staining of IFN-γ, IL-10, IL-4, IL-17, TGF-β, FoxP3 and CD107a in esophageal cancer tissue. $^{*}P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ paired non-parametric *t*-test.

Significantly higher expression of the degranulation marker CD107a within the stromal compartments of EAC and SCC tumors

In light of surface marker analysis data indicating $CD8^{+}$ T cell enrichment within the stromal compartments of EAC and SCC tumors, staining of degranulation marker CD107a was performed on 72 EAC patients and 23 SCC patients as an indicator of cytotoxicity. CD107a expression is significantly stronger in the

stroma of EAC and SCC tumor tissue sections compared with the tumor islets (mean ± SEM: EAC Tumor versus EAC Stroma: 9.1 ± 0.7% versus 13.1 ± 0.9%, $P \leq 0.001$, SCC Tumor versus SCC Stroma: 9.5 ± 0.8% versus 15.5 ± 1.5%, $P < 0.01$, Figure 2).

Significant correlations between CD8 and CD45RO in both EAC and SCC tumor issue

Corrplots were generated to visualize Spearman correlation values between CD8, FOXP3, CD3, CD45RO, and CD107a in both the tumor and stroma from 19 SCC and 48 EAC patients. Correlation r values of >0.6 were defined as strong correlations. Tumor and stromal expression of CD8, CD3 and CD45RO were all found to significantly positively correlate ($r > 0.6$, $P \leq 0.001$) with each other in the EAC cohort (Figure 3). In the SCC patients, a significant positive correlation ($r > 0.6$, $P \leq 0.01$) was also identified between CD45RO and CD8 expression (Figure 3). These data indicate a prevalence of memory CD8+ T cells within both EAC and SCC tumors.

No significant associations were observed between CD3, CD8 or CD45RO expression and EAC or SCC patient survival

The Kaplan–Meier survival curves were used to elucidate any associations between CD3, CD8 and CD45RO expression and overall survival (Figure 4). Survival was calculated as the time, in months, from the day of diagnosis until death of the patient. The last follow-up date was used as a cut-off time point for survival calculations. Low expression of each immune marker was classified as ≤ median value and high expression was classified as ≥ median value (Table 2). The log-rank (Mantel–Cox) test revealed that there were no significant associations between CD3, CD8 and CD45RO expression in EAC or SCC tumor or stroma and overall patient survival (Table 2). (EAC $n = 59$, SCC $n = 21$). No correlations were observed with TMN staging (data not shown).

Discussion

Esophageal cancer can be considered an aggressive malignancy, often presenting late, and with a poor prognosis and limited effective therapeutic options (1,12). In the wake of an immunotherapy revolution and given the prognostic promise of the Immunoscore classification system in colorectal cancer, a similar development in esophageal cancer has appeal, for prognostication, selection of adjuvant therapy and for selection of specific immune therapies (4). Recent studies have identified associations between intratumoral expression of CD3, CD8, FoxP3 and immune checkpoint molecules and survival, recurrence, and perioperative characteristics in esophageal cancer patients (7,13). Therefore, there is some basis to suggest that assessment of immune function within such tumors may have clinical relevance and value (7).

This study addressed some shortfalls of the immunoscore system and expanded upon our previous findings on T cells in esophageal cancer, by assessing the intratumoral expression of multiple T cell markers and considering the profiles and phenotypes of T cells within esophageal tumor and stroma. In light of recent publications identifying associations between intratumoral NK cells, macrophages and the antigen presenting molecule HLA-DR and clinical parameters such as tumor stage, survival and post-operative prognosis, it is evident that the incorporation of multiple immune cell subsets, phenotypes and profiles may be required to truly reflect the immune landscape of esophageal tumors (14,15).

In this study, a marked expression of CD3, CD8 and CD45RO within the stroma of esophageal tumors was evident, and positive correlations between CD45RO and CD8 were confirmed, indicating that memory cytotoxic T cells are key players in the immune component of the tumor microenvironment (TME). A strong infiltration of memory CD8+ T cells was confirmed in fresh esophageal tumor explants by co-staining for CD45RO and CD8. However, there was no evidence that these markers alone were significantly associated with survival. Previous studies have demonstrated that CD45RO predicted a favorable outcome in esophageal cancer and while not significant, our findings support this with substantially longer survival observed in SCC patients with high CD45RO tumors (16). In contrast, however a marked difference was observed between SCC patients with high and low CD3, with lowest survival in those patients with high intratumoral CD3. The Treg cell infiltration has been associated previously with poorer prognosis in SCC and we propose that such Treg infiltrations might contribute to lower survival in our SCC cohort (17).

Interestingly, our data also revealed significantly stronger CD45RO, CD8 and CD3 within SCC stroma, compared with OAC. Such findings may reflect differences in the cytotoxic T cell response between the esophageal tumor types. For instance, differences in the expression of cancer testis antigens and neoantigens have been reported between the two tumor types with melanoma-associated antigen-A expression detected in over 50% of SCCs and in only a modest percentage (15%) of EACs (18,19). Furthermore, esophageal cells have been shown to invoke a cytotoxic T cell response via the expression of specific neoantigens (20). Therefore, the differences in CD45RO, CD8 and CD3 staining between EAC and SCC stroma are most probably a representation of their immunogenicity and immune infiltration.

The present study supports our previous reports of the mixed pro- and anti-inflammatory profile within the esophageal TME, with strong expression of IL-17 and TGF-β in stroma of both EAC and SCC tumors, while IFN-γ and FoxP3 expression is strongest in SCC stroma and IL-10 expression is highest in EAC tumor (8). While this study indicates that memory cytotoxic CD8+ T cells are enriched within esophageal tumors, it does not identify any associations between the strongly expressed immune markers and clinical parameters such as survival or TNM stage. Since the majority of this cohort has advanced stage cancer and nodal positivity, therefore, it is probably that the memory cytotoxic CD8 T cells are not sufficient to elicit an effective antitumor immune response or may have been hindered by neo-adjuvant therapy. Future work to identify immune cell infiltrate prior to any treatment and to elucidate expression of immunosuppressive elements within the TME might reveal the prognostic potential of immunophenotyping and why the presence of these tumor killing cells is not conferring improved survival. For instance, we have previously reported PD-1 positivity on T cells from esophageal tumors and it is probable that the PD-1/PD-L1 pathways among others may play a role in inhibiting an effective antitumor immune response (21). We suggest that future studies should not only bring our current panel of immune markers of interest forward into larger cohorts to validate our insights into the immune landscape of esophageal tumors but should also incorporate inhibitory and stimulatory markers such as PD-1, LAG-3, TIM-3, PD-L1, PD-L2, TIGIT, NKG2A, OX40 and ICOS1. Duan *et al.* have demonstrated the usefulness of similar panels in SCC and it is probable that larger panels will yield more clinical utility than the more modest immunoscore (7). In addition to PD-1 and PD-L1 expression in EAC tumors, PD-L2 positivity

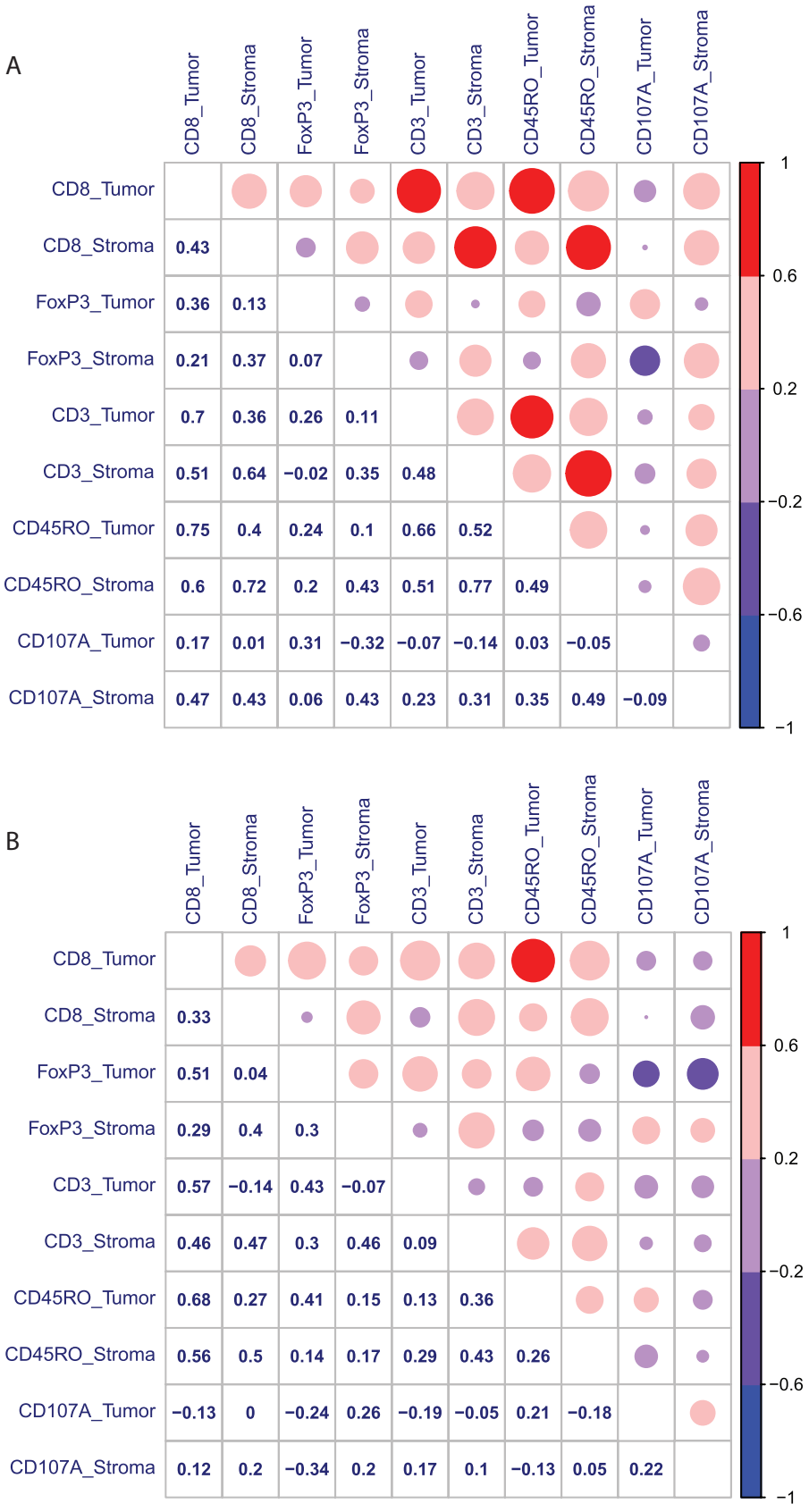

Figure 3. Significant correlations between CD8 and CD45RO in both EAC and SCC tumor tissue. Corrplots were generated to visualize spearman correlation values between CD8, FoxP3, CD3, CD45RO, and CD107a in both the tumor and stroma from SCC (n = 19) and EAC (n = 48) patient cohorts. The scale on the right ranges from R values of −1 to +1 in increments of 0.4. R values between −0.6 and −1 (blue) or between 0.6 and 1 (red) were deemed as strong correlations. All R values are displayed numerically on the left-hand side of the corrplot and all R values are displayed in the form of colored circles on the right-hand side as per the scale. Red circles denote positive correlation values between 0.6 and 1. Blue circles denote negative correlation values between −0.6 and −1. (**A**) Tumor and stromal expression of CD8, CD3 and CD45RO were all found to be strongly positively correlating (r > 0.6) with each other in the EAC cohort. (**B**) A strong positive correlation (r > 0.6) was identified between CD45RO expression in the tumor and CD8 tumor expression in the SCC cohort.

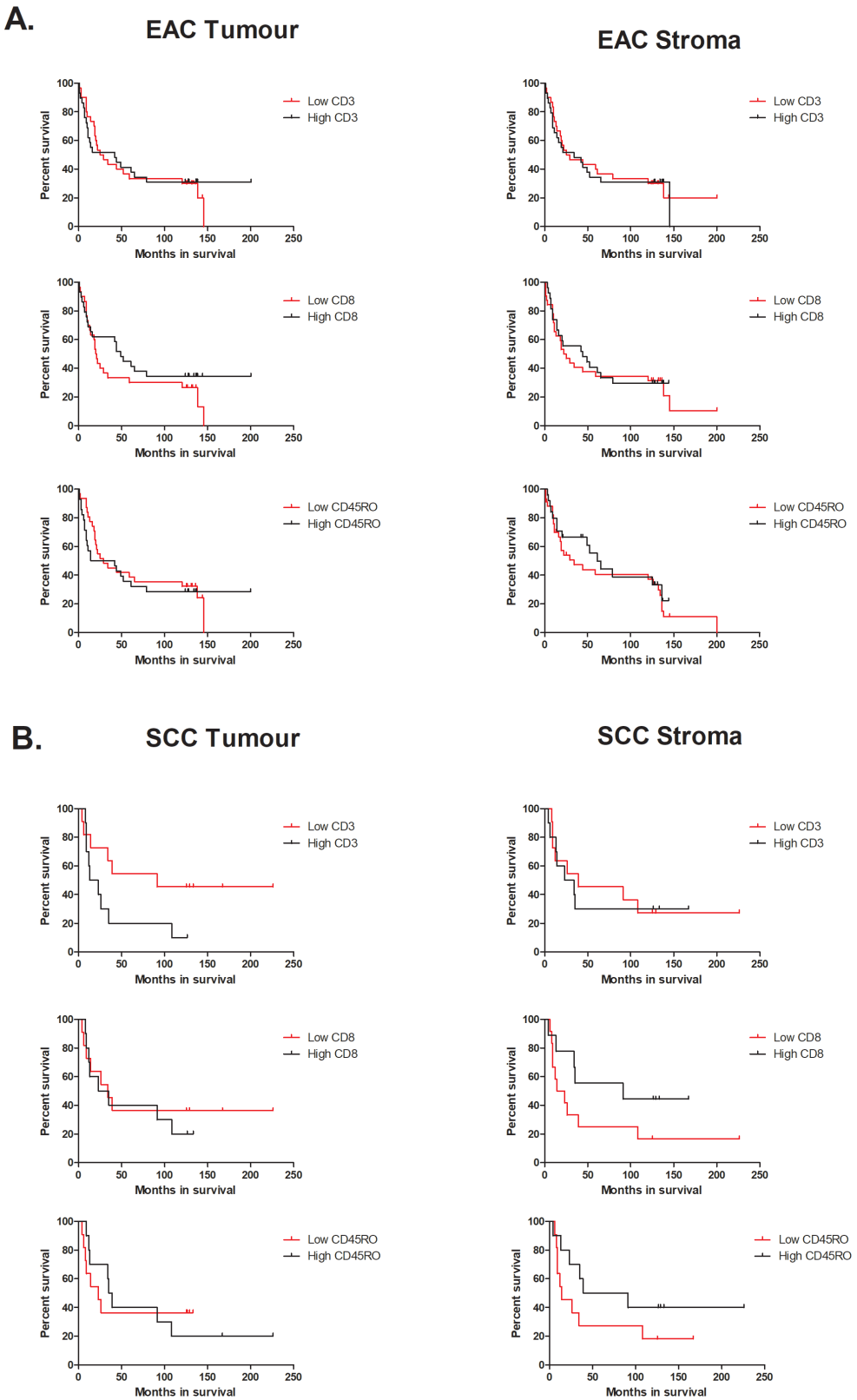

Figure 4. No significant association between CD45RO, CD8 or CD3 expression in EAC or SCC tumor tissue and overall survival. (**A**) Patients with low (≤ median value) CD3, CD8 or CD45RO expression in the EAC tumor (left) or stroma (right) (red line) had similar survival to those patients with high (≥ median value) CD3, CD8 or CD45RO expression (black line), as represented by a Kaplan–Meier survival curves, $n = 59$. (**B**) Patients with low (≤ median value) CD3, CD8 or CD45RO expression in the SCC tumor (left) or stroma (right) (red line) had similar survival to those patients with high (≥ median value) CD3, CD8 or CD45RO expression (black line), as represented by a Kaplan–Meier survival curves, $n = 21$. Associations were tested using a log-rank (Mantel–Cox) test.

Table 2. Overall survival statistical data

Survival statistics	Median %	*P*-value[a]	HR	95% CI of ratio
CD3 EAC tumor	3.6	0.9	1.034	0.5643 to 1.893
CD8 EAC tumor	2.8	0.2669	1.410	0.7686 to 2.588
CD45RO EAC tumor	5	0.6383	0.8635	0.4683 to 1.592
CD3 EAC stroma	13.75	0.8423	0.9405	0.5140 to 1.721
CD8 EAC stroma	15	0.7543	1.103	0.5981 to 2.033
CD45RO EAC stroma	17.5	0.5003	1.244	0.6597 to 2.344
CD3 SCC tumor	7.75	0.0761	0.3804	0.1307 to 1.107
CD8 SCC tumor	8	0.6409	0.7845	0.2830 to 2.175
CD45RO SCC tumor	11.2	0.9784	1.014	0.3642 to 2.824
CD3 SCC stroma	17.6	0.8499	0.9055	0.3239 to 2.531
CD8 SCC stroma	24.1	0.1443	2.151	0.7692 to 6.017
CD45RO SCC stroma	26.6	0.1803	2.032	0.7203 to 5.734

Table showing the survival statistics (median, *P*-value, HR and 95% CI of ratio) for each of the immune markers. CI, confidence interval; HR, Hazards ration.
[a]Associations were tested using a log-rank (Mantel–Cox) test.

has been detected in more than half of EAC tumors and its role and therapeutic potential in this milieu certainly warrants further investigation (22).

At a time when numerous clinical trials are actively assessing the therapeutic potential of the PD-1/PD-L1 pathway via the use of Nivolumab, Pembrolizumab, Avelumab and Durvalumab in esophageal and gastric cancer, it appears imperative to assess their expression in the tumor and relate this and other immune parameters in response to such therapies (23). Sophisticated stratification tools are required, as efficacy is only demonstrated in ~22–27% of patients with PD-L1^{+} tumors. Alteration in expression of the therapeutic target caused by first-line chemotherapy and radiotherapy must also be considered to identify the optimal timing of such therapies (23). We have previously reported preliminary evidence demonstrating lower PD-1 expression on intratumoral T cells from EAC patients following chemoradiotherapy (CRT), compared with those treatment naive EAC patients (21). However, results from the phase III ONO-4538-12 (ATTRACTION 2) trial demonstrated that Nivolumab improves overall survival for heavily pre-treated gastro-esophageal cancer patients, suggesting that CRT does not impact subsequent efficacy of targeting PD-1 in the absence of concurrent CRT (24). Further work to dissect the impact of different regimens of chemotherapy and radiotherapy would be very worthwhile and it is still important to consider that the timing of immune profiling relative to first line CRT may inform the timing of the associated immunotherapy.

Our data suggest that the strong expression of CD45RO, CD8, CD3 and CD107a within esophageal tumors represents the presence of cytotoxic memory CD8^{+} T cells within the stroma of such tumors. Since the majority of PD-L1 expression is detected in myeloid cells at the invasive margin of such tumors, it is probable that cytotoxic T cells are being functionally constrained within the stroma and that they are being regulated via checkpoint molecules (25). Thompson *et al.* reported that a higher CD8 infiltration correlated with lower survival, and patients with higher CD8^{+} T cell densities also have higher PD-L1 expression (25). This study provides further evidence to at least suggest that the existing immunoscore applied to colorectal cancer may not be sufficient for prognostication in esophageal cancer, nor would it accurately stratify patients for targeted immunotherapy.

Approximately 40% of gastro-esophageal tumors are PD-L1^{+}, but in distinct genomic subsets this may be much greater, for example it is between 50% and 94% in the EBV subtype, and between 33% and 45% in MSI high tumors (26). Recent integrated genomic characterizations have revealed that there were no EAC tumors among the MSI^{HIGH} or EBV^{+} subtypes but they did identify MSI^{HIGH} or EBV^{+} subtypes among gastro-esophageal junctional adenocarcinomas that were not clearly of esophageal origin (27). In light of these data, these biological factors are being incorporated in the design of ongoing clinical trials in the gastro-esophageal adenocarcinoma cohorts (23). Defective mismatch repair genes have been identified as being predictive of response to PD-1 inhibition with mismatch repair-deficient tumors constituting 17–21% of gastric cancers and exhibiting strong expression of several immune checkpoints including PD-1 and PD- L1 (28,29). In fact, data indicate a higher treatment response rate among gastric cancer patients with mismatch repair-deficient tumors (28). Based on these observations, genetic or exogenous influences such as viral infection impact on the immune cell activity within the esophageal TME, and may correlate with immunotherapy treatment response. Therefore, they should be the focus of future studies and trials.

In conclusion, the current immunoscoring approaches in colorectal cancer may be an insufficient representation of the immune profile of the TME in esophageal cancer, and further studies on larger cohorts are needed to identify its prognostic or therapeutic significance, and refine and adapt an appropriate scoring panel for EAC and SCC. Incorporation of the immunosuppressive elements of the TME into such scores may be required, as is showing promise in SCC and gastric cancer (6,7). In this study, an extended 13-factor panel in EAC and SCC indicated a strong infiltration of cytotoxic memory CD8^{+} T cells within the stroma of such tumors, but in this cohort it did not correlate with survival. It also seems obvious that the inclusion of immune checkpoint markers should be routine in future assessments. At a time when a plethora of immunotherapy clinical trials are ongoing for patients with EAC, gastro-esophageal adenocarcinoma, gastric and gastro-esophageal junctional adenocarcinoma, more sophisticated approaches are required to stratify patients for treatment and provide better prognostic utility.

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Author contributions

All authors have contributed significantly to this manuscript. M.J.C prepared the manuscript, figures, tables and analysed data. S.A.K performed the statistical analysis and prepared figures. M.C.C, S.L.D and E.P.S generated the TMAs and performed the experiments. B.H, M.K and K.O'S graded the sections. J.V.R was involved in study design and manuscript preparation. J.L made substantial contribution to the conception and design of the project, interpretation of the data and overall supervision of the project. All authors were involved in the drafting and critical appraisal of the manuscript and have given approval of the final version for publication.

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