

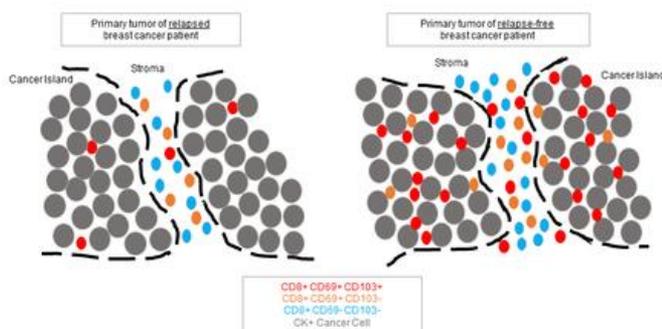
Resident memory CD8⁺ T cells within cancer islands mediate survival in breast cancer patients

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Title: Resident memory CD8⁺ T cells within cancer islands mediate survival in breast cancer patients

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Abstract:

CD8⁺ tumor-infiltrating lymphocytes (TILs) correlate with relapse-free survival (RFS) in most cancer types, including breast cancer. However, subset composition, functional status, and spatial location of CD8⁺ TILs in relation to RFS in human breast tumors remain unclear. Spatial tissue analysis via quantitative immunofluorescence showed that infiltration of CD8⁺ T cells into cancer islands is more significantly associated with RFS than CD8⁺ T cell infiltration into either tumor stroma or total tumor. Localization into cancer islands within tumors is mediated by expression of the integrin CD103, which is a marker for tissue resident memory T cells (TRMs). Analysis of fresh tumor samples revealed that CD8⁺ TRMs are functionally similar to other CD8⁺ TILs, suggesting that the basis of their protective effect is their spatial distribution rather than functional differences. Indeed, CD103⁺ TRMs, as compared to CD103⁻ CD8⁺ TILs, are enriched within cancer islands and CD8⁺ TRM proximity to cancer cells drives the association of CD8⁺ TIL densities with RFS. Together, these findings reveal the importance of cancer island localized CD8⁺ TRMs in surveillance of the breast tumor microenvironment and as a critical determinant of RFS in breast cancer patients.

Brief Summary:

Infiltration of cancer islands by resident memory CD103⁺ CD8⁺ T cells determines relapse free survival in breast cancer patients.

Introduction

The presence of tumor infiltrating lymphocytes (TILs) associates favorably with relapse-free survival (RFS) in breast cancer (1-4). CD8⁺ TILs in human breast tumors have been demonstrated to be primarily antigen experienced T cells, but little else is known about the relationship between T cell composition and spatial localization within the tumor microenvironment with RFS (5). Successful immunotherapy of breast cancer necessitates a greater understanding of the T cell infiltrate in breast tumors.

Breast tumors are segregated by receptor expression patterns into three major subtypes: estrogen receptor expressing, HER2 enriched, and basal-like/triple negative (6). These subtypes correlate with more detailed molecular expression subtypes and differ in therapeutic response and prognosis (7). Triple negative breast cancer (TNBC) is an aggressive breast cancer subset with a higher frequency of early relapse and significantly shorter time to recurrence as compared to estrogen receptor (ER⁺) breast cancer (8). Amongst breast cancer subtypes, TNBC and HER2⁺ have been clearly defined to have a positive association between RFS and presence of tumor infiltrating T cells (4).

Tumors may also be divided by their immune infiltration profiles: immune-inflamed, immune-excluded, and immune-desert (9). Immune-inflamed tumors involve infiltration of T cells into and around the tumor parenchyma (cancer islands), while immune-excluded tumors have T cell infiltration only into tumor stroma but not cancer islands. Immune-desert tumors lack T cell infiltration altogether, either in cancer islands or stroma. Beyond patient prognosis, higher levels of T cell infiltration in tumors have been shown to correlate positively with response rates to immunotherapies (10, 11). Thus, analysis of TIL infiltration patterns and characteristics is valuable for mechanistic and clinical insights into patient outcomes.

CD103, an αE integrin, key marker for tissue resident memory T cells (TRMs), with a functional role in retaining T cells within peripheral tissues via binding to E-cadherin on epithelial cells (12). In addition to CD103, another key marker for CD8⁺ TRMs is CD69, which further limits T cell tissue egress by promoting downregulation of the chemotaxis receptor G-protein-coupled sphingosine 1-phosphate receptor-1 (S1P₁) (13). Together, CD69 and CD103 are crucial determinants of CD8⁺ TRMs retention and accumulation in epithelial cell rich tissues, such as intestinal tissue, skin, lung respiratory tissue, and salivary glands (14-18).

CD8⁺ TRMs have been identified as key immune players in tumor microenvironments with survival associations in ovarian cancer, non-small-cell lung carcinoma, and melanoma (19-21). The presence of tumor infiltrating CD103⁺ CD8⁺ TRMs have been implicated to be associated with improved survival prognosis in head and neck cancer, non-small cell lung cancer, ovarian cancer, and skin cancer patients (20-23). Tumor associated TRMs from different malignancies have been demonstrated varying degrees of checkpoint molecule expression profiles and heterogeneity in functional capacities (24). A clearer understanding of the role of CD8⁺ TRMs in the breast tumor microenvironment and their relationship with prognosis is needed.

Here, phenotypic analysis of fresh and archival breast tumors unraveled mechanisms of this association. Using quantitative spatial image analysis, we demonstrate that CD103⁺ CD8⁺ TILs are enriched in the cancer islands of breast tumors. Functional profiling revealed similar cytokine production capacity of CD103⁺ CD8⁺ TRMs as compared to CD103⁻ CD8⁺ TILs, highlighting spatial localization of CD103⁺ CD8⁺ TRMs as a key phenotypic difference of this TIL subset. Finally, we demonstrate that increased densities of CD103⁺ CD8⁺ TILs in cancer islands within breast tumors is more significantly associated with RFS than CD8⁺ TILs within stroma. These

results demonstrate that CD8⁺ TRMs are a major component of immune-inflamed breast tumors and play an important role in clinical outcome.

Results

CD103⁺ CD8⁺ T cells localize to cancer islands in tumors and epithelial areas in non-cancerous breast tissue

Since CD103 engagement with E-cadherin has been shown to mediate T cell retention to epithelial tissues, we hypothesized that CD103 expression by CD8⁺ T cells resulted in unique localization within breast tissues. Human breast tumors are composed of epithelial cell derived ‘cancer islands’ and interlaced ‘stromal’ areas composed of fibroblasts, mesenchymal stem cells, and various immune cells. Using quantitative immunofluorescence (QIF) we were able to assess spatial localization of CD8⁺ T cells in tumor tissues within tissue segmented classifications of either cancer islands (CK⁺ areas) or stromal areas (CK⁻ areas) as outlined in Supplemental Figure 1. Similarly staged TNBC primary tumor formalin fixed paraffin embedded (FFPE) tissues (Supplemental Table 1) from patients with at least five years of follow-up were interrogated using QIF. These patients had no therapy prior to surgery and underwent similar chemotherapy regimens following surgery, allowing us to interrogate the relationship between the immune tumor microenvironment and relapse free survival (RFS) in treatment naïve tumors.

Tumor tissues were and non-cancerous breast tissues (NCBT) were co-stained for markers pan-cytokeratin (CK), CD8, and CD103 (Figure 1A). In breast tumors, CD8⁺ T cell density was significantly higher in stromal areas than in cancer islands (Figure 1B). However, CD103⁺ CD8⁺ T cells were highly enriched within the cancer islands of tumor tissues (Figure 1C). Similarly, in NCBT nearly all CD8⁺ T cells in the epithelial ducts expressed CD103, while the majority of

CD8⁺ T cells in stromal areas were CD103⁻ (Figure 1D, additional representative images Supplemental Figure 2). Together these data show significant localization of CD103⁺ CD8⁺ T cells to cancer islands in tumors and epithelial areas in general in human breast tissues.

CD103⁺ TRMs are a major component of CD8⁺ TILs in human breast tumors

Expression of both CD103 and CD69 has been tied to CD8⁺ TRM T cells localization and retention within peripheral tissues. To examine the phenotype CD103⁺ CD8⁺ T cells in human breast tissues, we obtained fresh surgically discarded breast tumors (both TNBC and ER⁺), NCBTs, and matched peripheral blood mononuclear cells (PBMCs) (Supplemental Tables 2 and 3). Single cell suspensions of digested tissues were analyzed by flow cytometry for canonical markers of memory T cells (Figure 2A-C; gating strategy Supplemental Figure 3). CD8⁺ T cells in both breast tumors and NCBTs were primarily composed of CD45RA⁻ CCR7⁻ effector memory cells. Further profiling of memory CD8⁺ T cells revealed that a large population co-expressed both CD69 and CD103 in breast tumors and NCBT, while CD69⁺ CD103⁺ CD8⁺ T cells were rarely found in the PBMCs of breast cancer patients. Memory composition and frequencies of CD69⁺ CD103⁺ CD8⁺ T cells were similar in ER⁺ and TNBC tumors were similar, identifying them as major cell populations in the tumor microenvironment of human breast tumors (Supplemental Figure 4A,B).

A distinct TRM gene expression signature has previously been identified for CD8⁺ T cells, including upregulation of *ITGAE*, *ITGA1*, *CD244*, and *XCL1* and downregulation of *SIPRI* (25). We examined the RNA expression levels of these genes in CD103⁺ and CD103⁻ CD8⁺ T cell populations from breast tumors and NCBT relative to circulating memory CD8⁺ T cells (Figure 2D). As expected, RNA levels of *ITGAE* were significantly higher in CD103⁺ CD8⁺ T cells relative to both circulating memory CD8⁺ T cells and CD103⁻ CD8⁺ T cells. CD103⁺ CD8⁺ T

cells also had significantly lower expression of *SIPRI* relative to both circulating memory CD8⁺ T cells and tissue CD103⁻ CD8⁺ T cells, suggesting a lack of circulation re-entry potential by these cells. Additionally, gene expression of *ITGAI*, *CD244*, and *XCLI* was significantly higher in CD103⁺ T cells compared to circulating memory CD8⁺ T cells in both breast tumor tissue and NCBT, validating them as bonafide TRMs. Interestingly, CD103⁻ CD8⁺ T cells also showed decreased levels of *SIPRI* and increased levels of *ITGAI*, *CD244*, and *XCLI* in comparison to circulating memory CD8⁺ T cells, suggesting that they may be transitioning to a TRM phenotype as well. This is also reflected by the large fraction of CD103⁻ CD8⁺ T cells in both breast tumors and NCBT expressing CD69 (Figure 2A,C), a molecule that plays an important role in inhibition of SIPRI surface expression and the resulting retention of T cells in peripheral tissue. Together this data identifies CD103⁺ CD8⁺ T cells in breast tumors and NCBT as TRMs and highlights their restricted localization to peripheral tissue sites.

CD8⁺ TIL CD103 and CD69 expression patterns differ by tumor localization

Flow cytometry data of fresh tumor tissues revealed that CD8⁺ TILs were comprised of three main populations of T cells: CD69⁺ CD103⁺, CD69⁺ CD103⁻, and CD69⁻ CD103⁻. CD69 has been shown induce T cell retention in peripheral tissues (14), but the localization of T cells within the tumor microenvironment has not been explored within the context of CD69 and CD103 expression. FFPE tumor samples were assessed by QIF for CD69, CD103, and CD8 co-staining (Figure 3A, representative images Supplemental Figure 5). As expected CD69⁺ CD103⁺ cells were highly enriched in cancer islands relative to stroma. CD69⁺ CD103⁻ cells, however, were somewhat more evenly split between stroma and cancer islands although trending toward cancer island enrichment. Intriguingly CD69⁻ CD103⁻ CD8⁺ T cells were found almost exclusively in the stroma. In agreement with our flow cytometry data, QIF rarely identified

CD69⁻ CD103⁺ CD8⁺ T cells, validating the use of CD103⁺ CD8⁺ phenotyping as a marker of CD8⁺ TRMs for larger patient cohort analysis.

CD8⁺ TRMs have a similar functional capacity compared to non-TRM tissue CD8⁺ T cells

Next we investigated the functional status of CD103⁺ CD8⁺ TRMs and CD103⁻ CD8⁺ non-TRMs from breast tumors and NCBTs. CD103⁺ CD8⁺ TRMs have previously been shown to express elevated levels of checkpoint molecules such as PD-1 (23, 26). We examined PD-1 expression in the context of CD103 and CD69 expression on CD8⁺ T cells in single cell suspensions of fresh tumor and NCBT samples by flow cytometry (Supplemental Figure 6). Higher frequencies of both CD69⁺ CD103⁺ and CD69⁺ CD103⁻ cells expressed PD-1 compared to CD69⁻ CD103⁻ cells in tumor and NCBT tissues. Thus, PD-1 expression was more associated with CD69 expression by CD8⁺ T cells rather than specifically to CD8⁺ TRMs.

Next we interrogated cytokine production capacity of CD8⁺ TIL subsets for expression of IFN γ , TNF α , and IL-2 by intracellular flow cytometry (Figure 4A,B). Memory CD45RA⁻ CD8⁺ T cells were gated on CD69⁺ CD103⁺ TRMs, CD69⁺ CD103⁻ cells, and CD69⁻ CD103⁻ cells to compare cytokine production between these subsets. CD103⁺ CD8⁺ T cells in both breast tumor and NCBTs exhibited similar cytokine production patterns as compared to CD69⁺CD103⁻ and CD69⁻ CD103⁻ CD8⁺ T cells in the same tissues in response to both phorbol myristate acetate (PMA)/ionomycin stimulation (Figure 4C) and anti-CD3 stimulation (Supplemental Figure 7).

Furthermore, similar frequencies of CD8⁺ T cell subsets from both ER⁺ and TNBC tumors produced IFN γ , TNF α , and IL-2 (Supplemental Figure 4C). To quantitatively evaluate an overall functional capacity for each CD8⁺ T cell subset in both tissue types we calculated a polyfunctionality index, which accounts for the ability of a T cell population to produce one, two, or three cytokines. No significant differences in polyfunctional capacity between CD8⁺ T

cell subsets within breast tumors or NCBTs were observed (Figure 4D). Thus, the specialized function of CD8⁺ TRMs in human breast tumors is not attributed to differential or enhanced cytokine production potential.

CD103⁺ CD8⁺ T cell infiltration of cancer islands in human breast tumors associates with relapse-free outcome

Recurrence in TNBC patients peaks three years after surgery and rapidly declines thereafter (27). We therefore divided our TNBC FFPE cohort samples into relapse and relapse-free groups. Relapse patients were defined as having recurrence within three years of primary tumor removal and relapse-free patients were defined as having no recurrence for at least five years. QIF results of CD8 and CD103 co-staining were then assessed in the context of these outcomes (Figure 5, representative images Supplemental Figures 8 and 9).

As expected, a positive trend for association between increased overall density of CD8⁺ T cells in tumor tissue and RFS ($p=0.108$) was identified (Figure 5B). However, increased density of CD8⁺ T cells localized specifically within cancer islands associated significantly with RFS ($p=0.002$). This strong association with RFS was specific to the density of CD8⁺ T cells in cancer islands, as the density of CD8⁺ T cells in stromal areas was less significantly associated with RFS ($p=0.158$).

We next examined if localization of specifically CD103⁺ CD8⁺ TRMs to cancer islands was associated with RFS in our TNBC patient cohort (Figure 5C). The density of CD103⁺ CD8⁺ TRMs in tumors from relapse-free patients was significantly higher than in tumors from relapse patients. Importantly, the density of TIL CD8⁺ CD103⁻ T cells did not associate with RFS and showed no significant differences in either the cancer islands or stroma of tumors. Higher

densities of CD8⁺ CD103⁺ TRMs were identified in relapse-free patients both in the cancer islands and stroma of tumor, suggesting that CD8⁺ TRMs are enriched in but not necessarily restricted to cancer islands allowing for migration and surveillance by CD8⁺ TRMs within the tumor microenvironment.

The measurement of CD8⁺ T cell density within cancer island tissue regions does not account for CD8⁺ T cells localized in the stroma that are very near cancer islands. To address this, we conducted spatial analysis of CD8⁺ T cells with nearest neighbor analysis in respect to CK⁺ cancer cells (Figure 6). This allowed us to examine the spatial relationship between epithelial cells and all CD8⁺ T cells within close proximity (0-25 μ m), medium proximity (25-50 μ m or 50-75 μ m), and far (>100 μ m) from CK⁺ cancer cells. As expected, in all tumors the frequency of CD8⁺ CD103⁺ T cells within 0-25 μ m of cancer cells was significantly higher than the frequency of CD8⁺ CD103⁻ T cells within 0-25 μ m of cancer cells (Figure 6B). In contrast, CD8⁺ T cells far (>100 μ m) from cancer cells were more enriched with CD8⁺ CD103⁻ T cells. Finally, a higher frequency of CD8⁺ CD103⁺ T cells localized within 0-25 μ m of cancer cells was significantly associated with RFS in the TNBC patients (Figure 6C). Strikingly, there was no significant difference in frequency of CD8⁺ CD103⁻ T cells localized within 0-25 μ m of cancer cells between the relapse-free and relapse groups. Instead, the relapse group had a significantly higher fraction of CD8⁺ CD103⁺ localized more than 100 μ m from cancer cells, demonstrating that a lack of robust cancer island infiltration by CD8⁺ TRMs associates with poor prognosis in TNBC patients.

Amongst the variables observed for total CD8⁺ T cells, CD8⁺ CD103⁺ TRMs, and CD8⁺ CD103⁻ T cells in TNBC tumors, logistic regression analysis identified the density of cancer island CD8⁺ CD103⁺ TRMs as the variable with the largest influence on predicting relapse-free outcome

(Figure 7A). Addition of other variables in a multivariate model made no further significant contribution. The density of CD8⁺ T cells in stroma correlated weakly with the density of CD8⁺ T cells in cancer islands ($\rho=0.34$, $p<0.1$) (Figure 7B). Thus, tumor stroma infiltration of CD8⁺ T cells, as described in non-inflamed tumors, does not necessarily result in cancer island infiltration of CD8⁺ T cells. However, expression of CD103 by CD8⁺ TRM TILs results in significant cancer island infiltration and retention, and most importantly, improved prognostic outcome. The density of CD8⁺ T cells in cancer islands and the density of CD8⁺ CD103⁺ TRMs in cancer islands were highly correlated ($\rho=0.97$, $p<0.0001$), while the density of total tumor CD8⁺ T cells and cancer island CD8⁺ CD103⁺ TRMs were less correlated ($\rho=0.53$, $p<0.01$). Together, this data identifies TRM CD8⁺ infiltration of cancer islands as both a strong predictor of relapse-free outcome in breast cancer patients and as a key aspect of immune-inflamed tumors.

Discussion

In this study, we show that the density of CD8⁺ T cells within cancer islands is more significantly associated with relapse-free outcome than CD8⁺ T cells in overall tumor tissue or tumor stroma. Cancer island localized CD8⁺ TILs are composed of CD103⁺ TRMs, which make up nearly half of the total CD8⁺ T cell population within breast tumors and are also the majority of CD8⁺ T cells in NCBT. While CD103⁺ CD8⁺ TRMs do not demonstrate unique functional capacity as measured by cytokine production, they do demonstrate enhanced ability to spatially localize near and amongst epithelial cells, including cancer cells. This localization of CD103⁺ CD8⁺ TRMs to cancer islands in TNBC tumors is significantly associated with RFS and furthers our current understanding of the association between CD8⁺ TILs and RFS in breast cancer.

Pathological assessment of stromal localized TILs, as opposed to cancer island localized TILs, is currently one of the primary immune evaluation parameters in breast tumors (28). Restricting analysis to tumor stromal areas rather than cancer islands is due to general less difficulty in assessment by pathologist review of H&E slides. A likely contributor to this is the significantly higher densities of CD8⁺ infiltration in stromal areas as compared to cancer islands (Figure 1). However, density of CD8⁺ TILs within cancer islands proved to be highly associated with RFS even in our relatively small cohort, while density of CD8⁺ TILs within stroma was less so (Figure 5). Strikingly stroma infiltration of CD8⁺ TILs had no correlation with cancer island infiltration of CD8⁺ TILs. Additionally, our observation that stromal CD8⁺ TILs are primarily CD69⁻ CD103⁻, which we show to have the lowest frequency of PD-1 expression leads us to question the role of stroma CD8⁺ TILs in anti-tumor immunity. Clearly there is a need for a further understanding of the dynamic infiltration of tumor tissues by CD8⁺ T cells in the context of phenotypic changes.

Our data suggest that assessment of cancer island infiltration by CD8⁺ CD103⁺ T cells may be useful in identifying CD8⁺ TRMs, predicting outcome, and assessing tumor T cell infiltration. CD103 expression in breast tumor tissues has been correlated with relapse-free survival (29). However, CD103 staining may also be attributed to expression on dendritic cells, regulatory T cells, and other lymphocytes (30-32), highlighting the need for multiplex staining for studies such as these. Quantitative immunostaining approaches offer the advantage of accurate cell subset identification, enumeration, and spatial localization within tumor microenvironments that is superior to analysis of fresh tissue digests (33). Indeed spatial localization of CD8⁺ TRMs to cancer islands and their densities within tumors would not necessarily be readily identifiable by other technologies, such as flow cytometry or RNA sequencing approaches.

A TRM gene expression signature based on single cell sequencing has recently been found to associate with good prognosis in TNBC public datasets (22). Complementary to our QIF data, Savas et. al showed that increased expression of a CD8⁺ TRM transcriptional signature was of greater prognostic value than a CD8⁺ signature alone in TNBC. Our data further demonstrate that increased density of CD8⁺ TRMs in both the cancer islands and stroma of tumor tissues comprises an immune-inflamed tumor profile. Current standard of care for primary TNBC tumors includes neoadjuvant chemotherapy prior to surgical resection, leading limited availability of fresh untreated TNBC tumor samples for research, as is evident by the predominance of ER⁺ tumor samples in our fresh tissue studies. TNBC was chosen to study the role of CD8⁺ TRMs in patient prognosis due the known positive association between CD8⁺ TILs and relapse-free outcome (4). Whether our observation of the relationship between cancer island infiltration by CD8⁺ TRMs and relapse-free outcome in our TNBC cohort is related to overall higher CD3⁺ and CD8⁺ TILs observed in TNBC relative to other breast cancer cohorts is unclear (34). A larger cohort, both validating our results and extending our findings regarding CD8⁺ CD103⁺ TRMs to other subsets of invasive breast cancer, especially hormone receptor positive disease, is needed.

Recent single cell sequencing efforts have shown breast tumor CD8⁺ TILs to be a heterogeneous population of T cells in various states of activation and differentiation (35). Our data demonstrate that CD8⁺ TRMs are a major T cell population within human breast tumors. TRMs are a subset of T cells, either CD8⁺ or CD4⁺, that are retained and long-lived in peripheral tissues (36-38). Originally identified in murine models of pathogen infection, TRMs enable critical and rapid protective immunity at peripheral tissue sites (39). TRMs facilitate this upon re-encountering antigen by secreting cytokines, such as IFN γ and TNF α , that elicit further T cell recruitment,

mature dendritic cells, activate natural killer cells, and initiate tissue-wide inflammatory pathways (40, 41). The developmental pathway of tumor TRMs and their mechanistic relationship with relapse-free outcome is not yet clear. Since tumor tissues are removed by surgical excision, the prognostic benefit of CD8⁺ TRMs likely reflects a greater systemic immunity involving circulating CD8⁺ T cells with shared antigen-specificity or at least tumor-specificity with tumor CD8⁺ TRMs. Although tumor-specificity of breast tumor TILs and TRMs has not been formally proven, T cell receptor repertoire analysis of matched patient samples has shown that certain clonotypes are enriched in the tumor and not in NCBTs (42, 43). TRMs may also develop in peripheral tissues independently of antigen presence, due to the presence of various cytokines common in tumor microenvironments, such as TGF- β , TNF- α , IL-33, and IL-15 (44, 45). Recent work has elegantly shown that TIL TRMs can be composed of both tumor-specific and tumor-nonspecific, ‘bystander’, cells (46). The presence of bystander TRMs specific for viral antigens in tumor tissues highlights the role of inflammatory cues in promoting TRM formation and cautions against assuming TRMs in tumor tissues are necessarily tumor-specific. Further studies to identify antigen specificity of breast tumor TILs will allow for a greater understanding of tumor immune composition and the role of T cells in breast cancer patients.

Vaccine induced development of CD8⁺ TRMs demonstrated effective protection against tumor induction in a murine tumor model (47). Additionally, the induction or presence of CD103⁺ CD8⁺ TRMs has been shown to enhance response to checkpoint blockade therapy in both preclinical and clinical research settings (26, 48). Although current response rates to checkpoint blockade therapies have been relatively low in breast cancer patients, focusing on patients with a significant density of intraepithelial CD8⁺ TRMs may enable more significant responses to immunotherapeutic interventions (49). Thus, a greater understanding of TRM formation,

function, and therapeutic benefit in breast tissues may enable immunotherapy strategies for breast cancer patients. Finally, evaluating the presence of CD103⁺ CD8⁺ TRMs within cancer islands may prove useful for determining patient prognosis and efficacy of various immunomodulating therapies in breast cancer patients.

Methods

Human Samples

Fresh surgically excised tumor and non-cancerous breast tissues were obtained from consented breast cancer patients undergoing standard of care treatment at City of Hope. Patient tissue characteristics are summarized in Supplementary Table 2. Scoring of tumor tissue receptor expression was performed by clinical pathologists. NCBTs were composed of tissue from prophylactic mastectomies, contralateral breast from breast cancer patients, or tumor adjacent tissue as summarized in Supplemental Table 3. Due to limited cell numbers obtained from patient tumor samples, not all analyses shown were performed on all samples. Tissue samples were provided by the City of Hope Biospecimen Repository which is funded in part by the National Cancer Institute. Other investigators may have received specimens from the same patients.

For evaluation of the relationship between outcome and the presence of CD103⁺ CD8⁺ TRMs in breast cancer patient tumors, we compiled FFPE tissues from 25 TNBC patients that had been followed for at least 5 years. Clinical characteristics are summarized in Supplemental Table 1. All patients were untreated prior to surgical removal of tumor tissue and all patients were treated with similar AC-T (doxorubicin, cyclophosphamide, paclitaxel) chemotherapy regimens following surgery. For analysis of CD103⁺ CD8⁺ TRMs in NCBT, FFPE tissues were obtained

from age-matched healthy donors with no history of breast cancer. Tissue samples were provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute.

Other investigators may have received specimens from the same subjects.

Sample Processing

Patient peripheral blood was obtained by venipuncture using heparin collection tubes, transported at room temperature from the clinic to the lab, and processed within 6 h of drawing.

PBMC were isolated via Ficoll-Paque Separation (GE Healthcare) following the manufacturer's instructions. Breast tumor and non-cancerous tissue specimens were collected by surgical resection and collected in tubes containing cold RPMI (Life Technologies, Thermo Fisher Scientific) and transported on ice to the laboratory for processing within one hour of surgery.

Tissues were minced into pieces, mechanically dissociated with a gentleMACS Dissociator (Miltenyi Biotec), and enzymatically treated with 0.2 Wunsch U/ml Liberase TM (Roche) and 10 units/ml DNase (Sigma) in RPMI for up to 1h as needed. If necessary, red blood cell (RBC) lysis was performed using RBC Lysis Buffer (Biolegend).

Flow Cytometry

Single cell suspensions were stained at RT in 2% FBS in PBS. For cytokine production assays, cells were stimulated with 50ng/ml PMA (Sigma) and 1µg/ml ionomycin (Sigma) or 1µg/ml of anti-CD3 OKT3 (Biolegend) in the presence of Golgi Plug (Biolegend) for 4 hours. Overnight fixation as needed was performed with IC Fixation Buffer (eBioscience). Fixation and permeabilization was performed with BD Cytotfix/Cytoperm buffers for intracellular cytokine staining. Antibody cocktails were diluted in Brilliant Violet Buffer (BD Biosciences) when necessary. Samples were acquired using a BD Fortessa using FACS Diva 6.1.3. Photomultiplier

tube voltages were set using BD CS&T beads. Compensation was calculated using single stained OneComp compensation beads (eBioscience). Samples were stained with fluorescently tagged antibodies detailed in Supplementary Table 4. Antibodies were titrated for optimal signal to noise ratio prior to use. Flow cytometry analysis was performed using FlowJo vX. All samples were gated on single cells, lymphocytes, and CD3⁺ CD8⁺ populations. Contour plots shown display 5% probability. The polyfunctionality index equation was applied as described by Larsen, M. *et al* (50). The polyfunctionality index was implemented in R (version 3.3.2) to take SPICE formatted csv files as inputs and output a txt file with the polyfunctionality index of each sample.

Real-Time PCR

CD8⁺ T cells were isolated from tumor tissue via culture of tissue fragments with high dose IL-2 as previously described (51). Cells were collected and sorted into CD69⁺ CD103⁺ and CD69⁺ CD103⁻ populations using an ARIA III flow cytometry sorting instrument (BD Biosciences). To serve as non TRM controls, circulating memory cells were extracted from PBMCs of age and gender matched healthy donors using a Memory CD8⁺ T cell Enrichment kit (Stem Cell Technologies). RNA from isolated cells was extracted with the RNeasy Micro Kit (Qiagen), from which cDNA was synthesized using the SuperScript VILO kit (Invitrogen). Quantitative RT-PCR reactions were run using Power SYBR Green PCR Master Mix (Applied Biosystems) for 40 cycles. RNA expression in CD69⁺CD103⁺ and CD69⁺CD103⁻ CD8 T cells was normalized relative to expression of *ACTB* and evaluated compared to gene expression in circulating CD8⁺ memory T cells from three healthy donors ($\Delta\Delta Cq$). Primers are described in Supplemental Table 4.

Immunofluorescent Staining

FFPE specimens were cut into 3-5 μ m sections and baked on glass slides. The FFPE slides were deparaffinized in xylene and then rehydrated in decreasing gradations of ethanol. Heat-induced epitope/antigen retrieval was performed in EnVision™ FLEX Target Retrieval Solution, High pH (pH 9) (K8004/5, Agilent, Santa Clara, CA, USA) or AR6 buffer (pH 6) (PerkinElmer, Hopkinton, MA, USA) using a microwave oven. Blocking was performed for ten minutes using Antibody Diluent, Background Reducing (S3022, Agilent) to minimize non-specific background staining. Primary antibodies, as shown in Supplemental Table 4, were incubated for one hour on a shaker at room temperature, detected by a horseradish peroxidase (HRP)-conjugated secondary antibody (Mach 2 Rabbit or Mouse HRP-Polymer), and then immunofluorescently labeled using the Opal™ 7- color fluorescence kit (PerkinElmer). Heat-mediated antigen retrieval using a microwave was performed in between serial stains of antibodies of interest. Cell nuclei were stained with DAPI (PerkinElmer) and the slides were mounted with ProLong Gold Antifade Reagent (P36930, ThermoFisher Scientific, Waltham, MA, USA).

Multispectral Imaging and Quantitative Immunofluorescence Image Analysis

Tissue sections were whole-slide scanned using the Vectra 3.0 System (PerkinElmer) to capture fluorescent spectra of 10x and 20x images in five channels (DAPI, FITC, Cy3, Texas Red, Cy5). Using Phenochart™ whole slide reviewer (PerkinElmer) regions of interest (ROIs) were selected and 25% of the images within the ROIs were systemically gridded and selected to unbiasedly capture tissue heterogeneity for further analysis. Images of single-stained tissues and unstained tissues were used to extract the fluorescent spectrum of each fluorophore and tissue autofluorescence in the 20x images to create a spectral library to perform multispectral unmixing using inForm® Cell Analysis (PerkinElmer). inForm® Cell Analysis or QuPath image analysis

software was used for cell segmentation, tissue segmentation, and cell phenotyping (52).

Supplemental Figure 3 summarizes the multispectral imaging and quantitative image analysis approach summarized above.

Cell Phenotype Spatial Analysis

Spatial data analysis was performed inputting cell phenotype X and Y coordinates into a K-nearest neighbor algorithm to determine nearest neighbor cells of a particular phenotype within 50 pixels (25 microns) of any cell. Images were re-coordinated so all the cells in the tissue could be represented on the same set of coordinate axes. The nearest neighbor pairs of interest were CD103⁺ CD8⁺ cell to CK⁺ cell and CD103⁻ CD8⁺ cell to CK⁺ cell. The TRM and non-TRM T-cells within 25 microns of a cancer cell were counted and then normalized by tumor area in mm².

Spatial analysis, spatial analysis related figures, and calculations were performed in R version 3.4.3. R scripts were written in house and are available at

https://github.com/TravisYTU/TRM_NN. The K-nearest neighbor algorithm was imported from the RANN package in R.

Statistics

Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from <http://exon.niaid.nih.gov> (53). Graphs and statistics were performed using Graphpad Prism 7.02. Statistics described were generated using two-tailed unpaired student T tests, one-way anovas with Holm-Sidak multiple comparison tests, or multiple comparison T tests with Holm-Sidak correction. Correlation matrices were performed using R package corrplot. Logistic regression and odds ratio analysis were performed using SAS software. Calculated p values are displayed as *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. P values <0.05 were

considered significant. For all graphs, the mean is represented by a bar and the error bars represent SEM.

Study Approval

Fresh tumor and peripheral blood were obtained from patients who gave institutional review board (IRB)-approved written informed consent prior to inclusion in the study (City of Hope IRB 05091, IRB 07047, and IRB 14346).

Author Contributions: CE and PPL designed research studies. CE, CA, TYT, AR, RW, GS, YH, ML, DLS, SS, MN, TH, and WG conducted experiments, acquired data, and analyzed data. JHY, LK, JM, SY, and YY provided clinical sample support. PF and CR assisted with statistical analysis. CE and PPL wrote the manuscript.

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Abbreviations:

cancer island, CI; central memory, CM; cytokeratin, CK; effector memory CD45RA⁺, EMRA; effector memory, EM; estrogen receptor, ER; formalin-fixed paraffin embedded, FFPE; non-cancerous breast tissue, NCBT; peripheral blood mononuclear cell, PBMC; phorbol myristate acetate, PMA; receptor tyrosine-protein kinase erbB-2, HER2; quantitative immunofluorescence, QIF; relapse free survival, RFS; sphingosine-1-phosphate receptor 1, S1P1; sphingosine-1-phosphate, S1P; tissue resident memory, TRM; triple negative breast cancer, TNBC; tumor infiltrating lymphocyte, TIL

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Figure 1. CD103⁺ CD8⁺ T cells localize to epithelial cell regions in breast tumors and non-cancerous breast tissue. Formalin fixed paraffin embedded (FFPE) tissues were assayed for expression of CD103 on CD8⁺ T cells by quantitative immunofluorescence (QIF) (A). Whole tissue sections from breast tumors or non-cancerous breast tissues (NCBTs) were stained and imaged, followed by quantitative analysis of representative fields. Pan-cytokeratin (CK; gray), CD8 (cyan), and CD103 (red) staining are depicted as composite images. Tissue segmentation algorithms based on CK staining allowed for distinct identification of cancer islands (CIs) and stroma areas or epithelial ducts and stroma areas in tumors and NCBTs respectively. T cell phenotypes (CD8⁺ CD103⁻, cyan dots; CD8⁺ CD103⁺ red dots) were created as shown for quantification and localization within segmented tissues. Scale bars represent 50µm. Total CD8⁺ T cell density in either cancer islands or stroma was assessed (B). The percentages of CD8⁺ T cells expressing CD103 within segmented areas of breast tumors (C) and NCBTs (D) were assessed. Each symbol represents data from a unique patient sample. Tumor samples n=25. NCBT samples n=8. Significance was calculated using student t-tests. ****, p<0.0001.

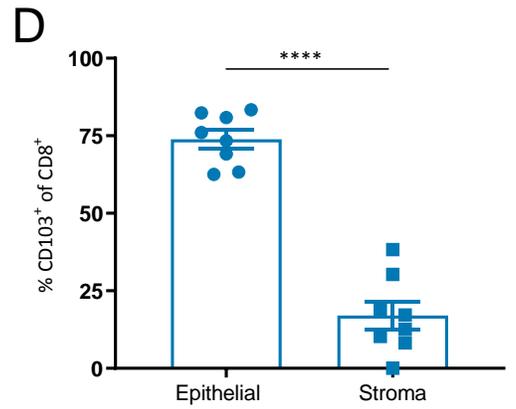
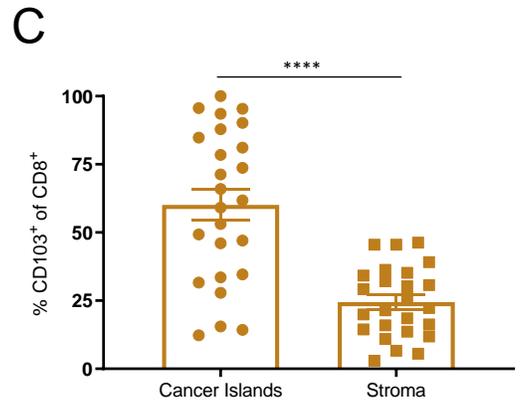
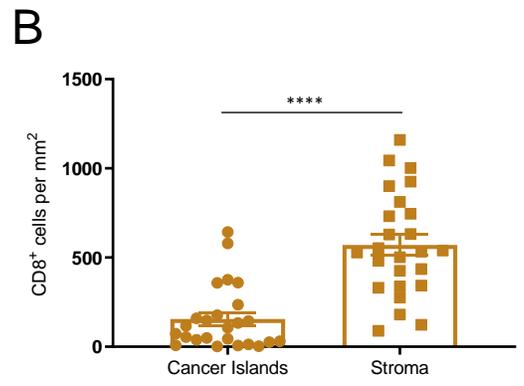
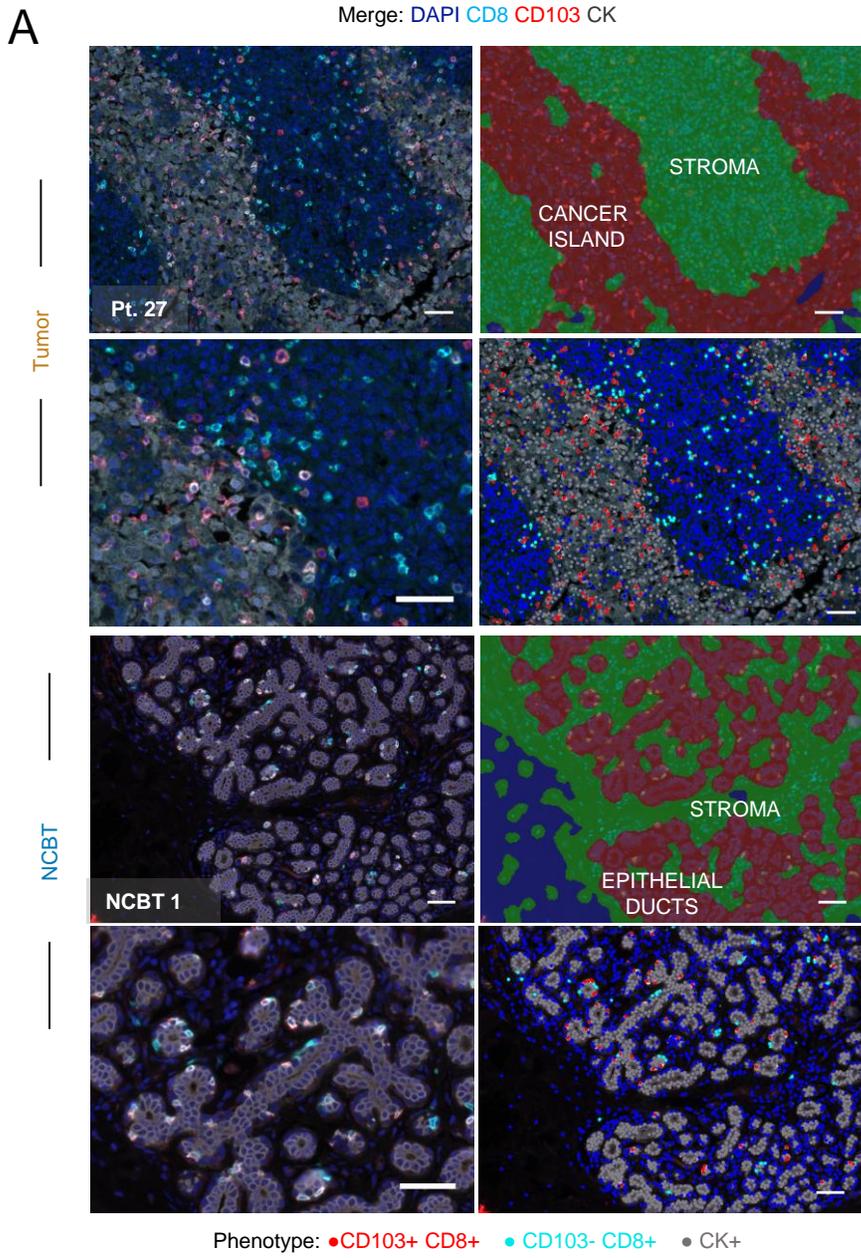


Figure 1

Figure 2. CD8⁺ tissue resident memory T cells are a major population of CD8⁺ T cells in human breast tumors and non-cancerous breast tissue. Single cell suspensions from peripheral blood mononuclear cells (PBMCs), tumors, and NCBTs were examined for expression of memory T cell and tissue resident memory T cell canonical markers CD45RA, CCR7, CD69, and CD103 by flow cytometry as shown (A). Frequencies of CD8⁺ T cells in each tissue compartment that are CD45RA⁺, CCR7⁺ (naïve), CD45RA⁻, CCR7⁺ (central memory, CM), CD45RA⁻, CCR7⁻ (effector memory, EM), or CD45RA⁺, CCR7⁻ (effector memory RA⁺, EMRA) are summarized (B). Frequencies of CD45RA⁻ CD8⁺ T cells in each tissue compartment expressing various patterns of CD69 and CD103 are summarized (C). CD103⁺ CD8⁺ T cells and CD103⁻ CD8⁺ T cells from breast tumors and NCBTs were assessed by real time PCR for gene expression. Gene expression and statistics shown are relative to control circulating memory CD8⁺ T cells (D). Each symbol represents data from a unique patient sample. Tumor samples n=36. NCBT samples n=21. PBMC samples n=24. Significance was calculated using one-way ANOVA and Holm-Sidak multiple comparison tests. *, p<0.05; **, p<0.01, ***, p<0.001, ****, p<0.0001.

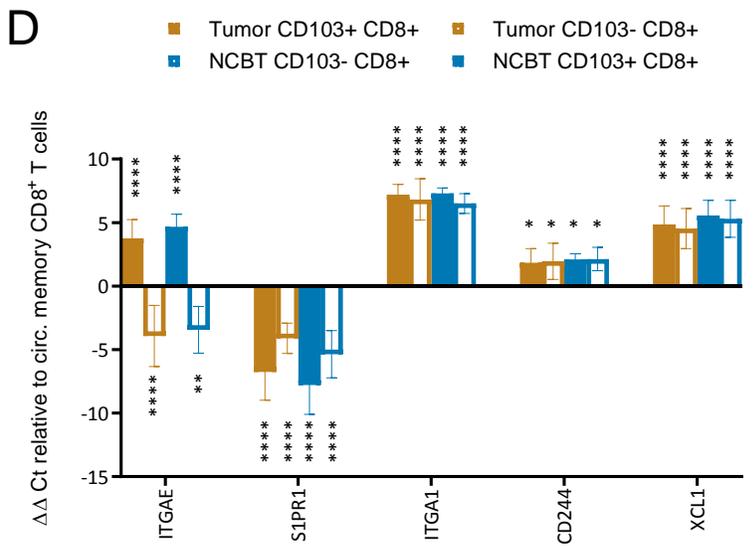
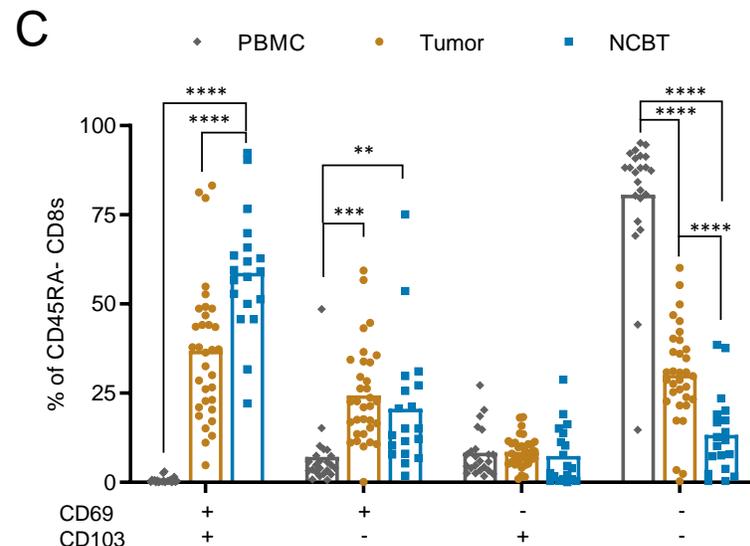
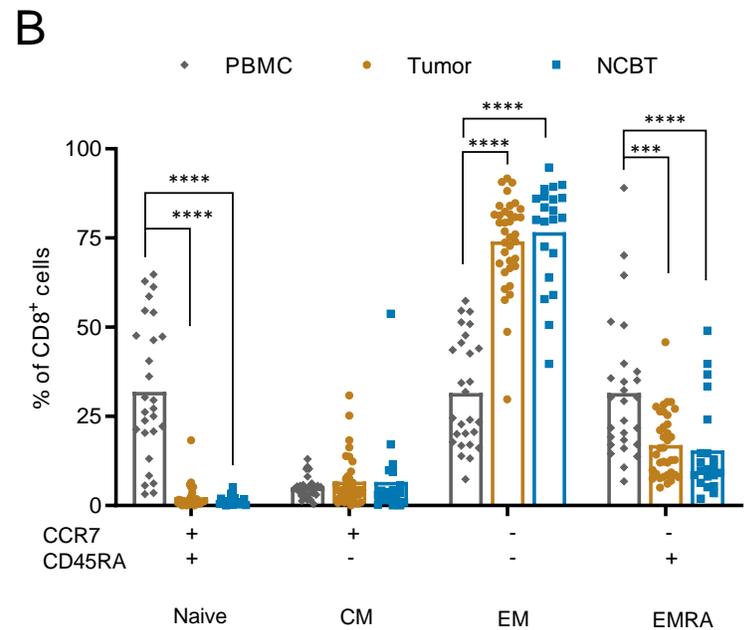
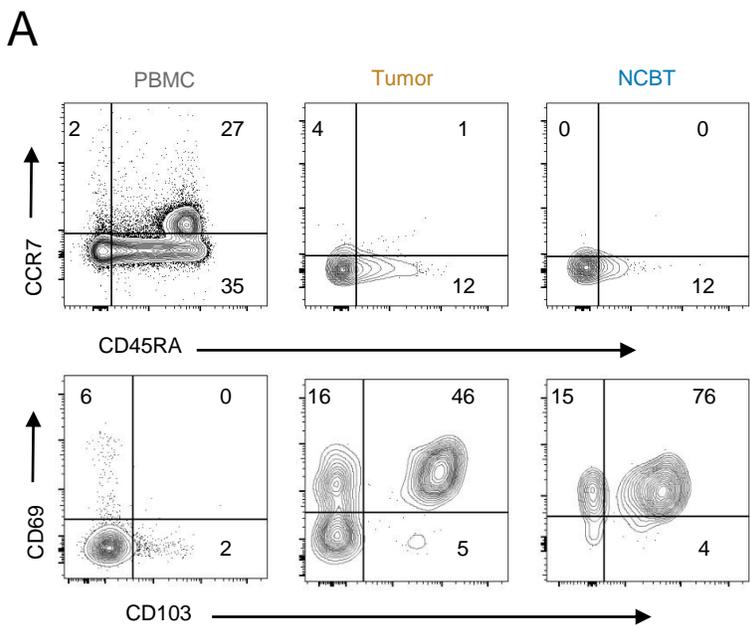
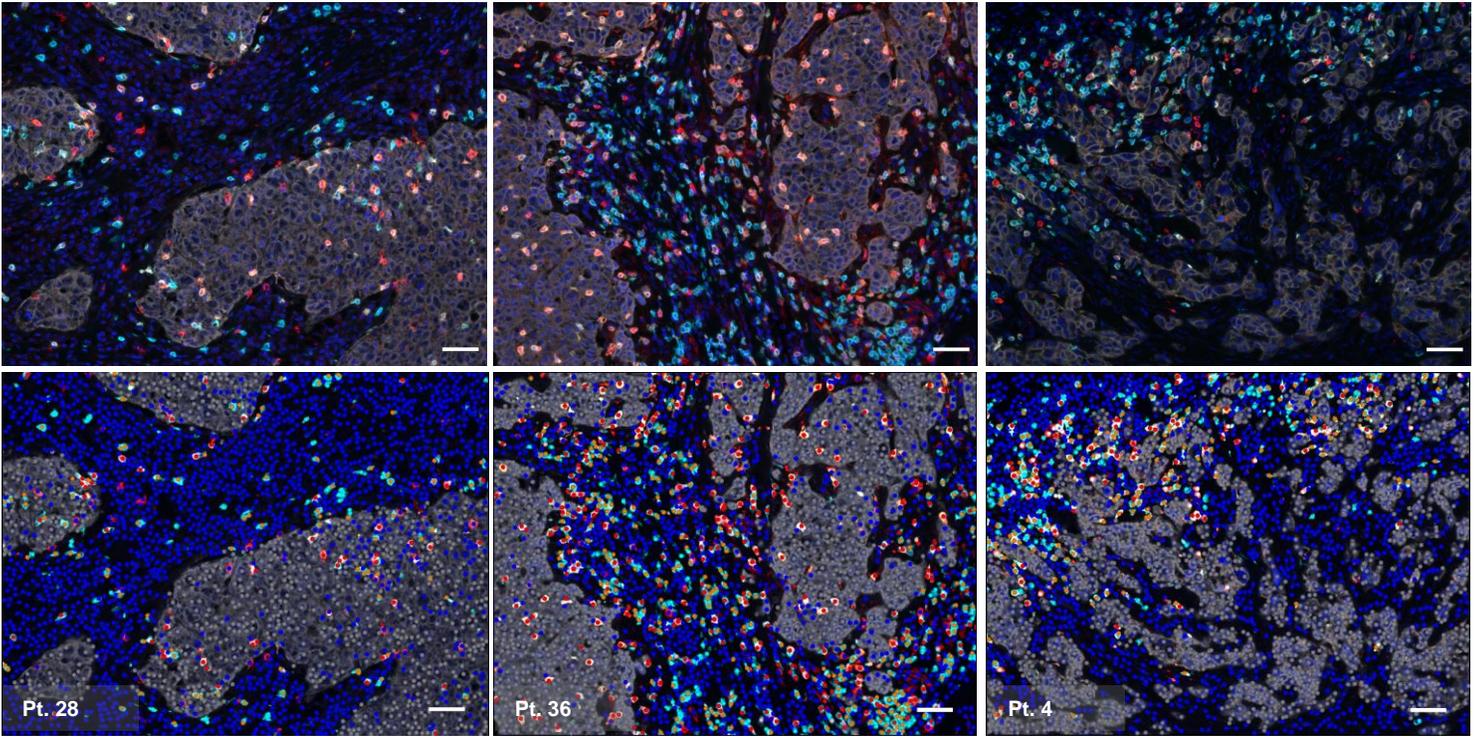


Figure 2

Figure 3. CD103 and CD69 expression by CD8⁺ TILs varies by localization within the tumor microenvironment. FFPE tumor tissues were assayed by QIF for expression of both CD69 (orange) and CD103 (red) in the context of CD8 (cyan) staining within the stroma and cancer islands (CK staining, gray). Representative merged composite images and identified T cell phenotypes (CD8⁺ CD69⁺ CD103⁺, red dots; CD8⁺ CD69⁺ CD103⁻, orange dots; CD8⁺ CD69⁻ CD103⁻, cyan dots; CK⁺, gray dots) are shown (A). Scale bars represent 50µm. Single channel representative images can be found in Supplemental Figure 4. The percentage of CD8⁺ T cells within stroma and cancer islands expressing either CD69 or CD103 was quantified as shown (B). Tumor samples n=13. Significance was calculated using one-way ANOVA and Holm-Sidak multiple comparison tests. ***, p<0.001, ****, p<0.0001.

A

Merge: DAPI CD8 CD103 CD69 PAN-CK



Phenotype: ● CD69+ CD103+ CD8+ ● CD69+ CD103- CD8+ ● CD69- CD103- CD8+ ● CK+

B

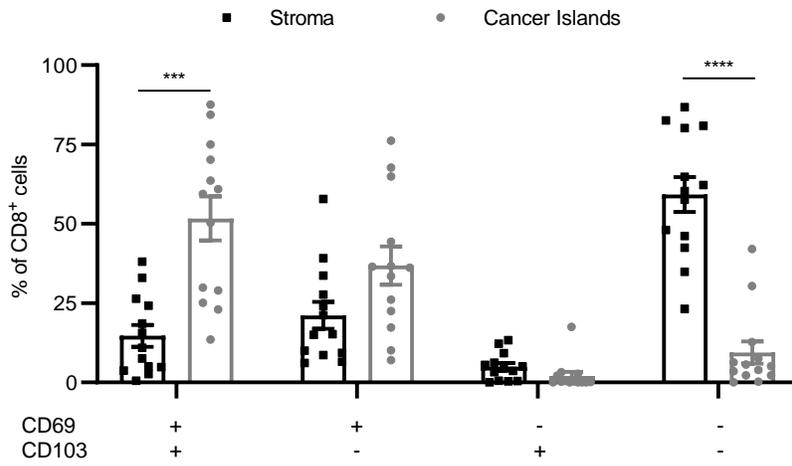


Figure 3

Figure 4. CD8⁺ TRMs have similar cytokine production capacity as other tissue infiltrating CD8⁺ T cells. CD8⁺ T cells from tumors and NCBTs were assayed by intracellular cytokine staining for IFN γ , IL-2, and TNF α following stimulation with PMA and ionomycin. Cytokine production was assayed within CD69⁺ CD103⁺, CD69⁺ CD103⁻, or CD69⁻ CD103⁻ cells as shown (A). Cytokine production profiles for different T cell populations are shown as percentage of cells within each gate producing a given cytokine (B). Cytokine production is also summarized as the number (pie slice) and type (pie arcs) of cytokines produced by each population as SPICE pie charts (C). A calculated polyfunctionality index representing the capacity of a given population to produce multiple cytokines is shown (D). Each symbol represents data from a unique patient sample. For individual cytokine data presented tumor n=19, NCBT n=9. For polyfunctional index data tumor n=18, NCBT n=9. Significance was calculated using one-way ANOVA and Holm-Sidak multiple comparison tests. No differences (p>0.05) in cytokine production capacity were identified between any groups.

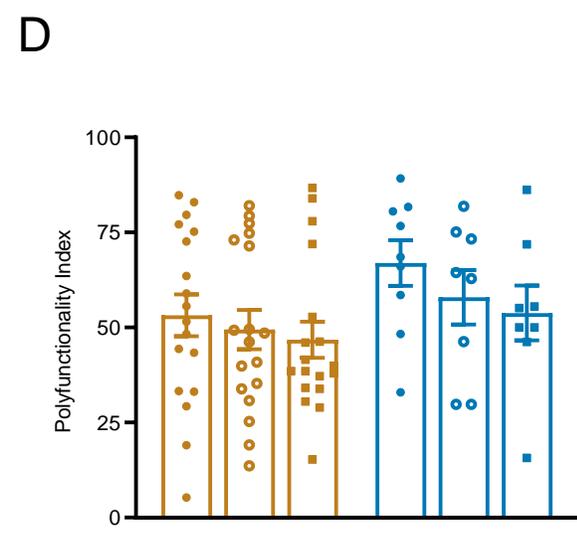
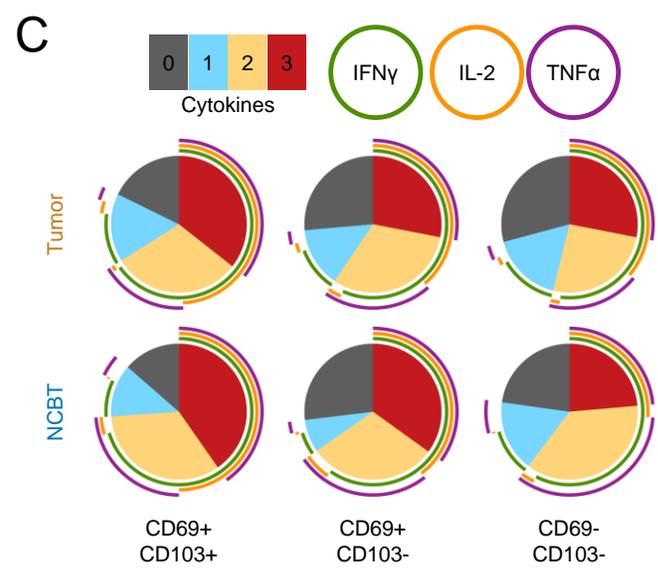
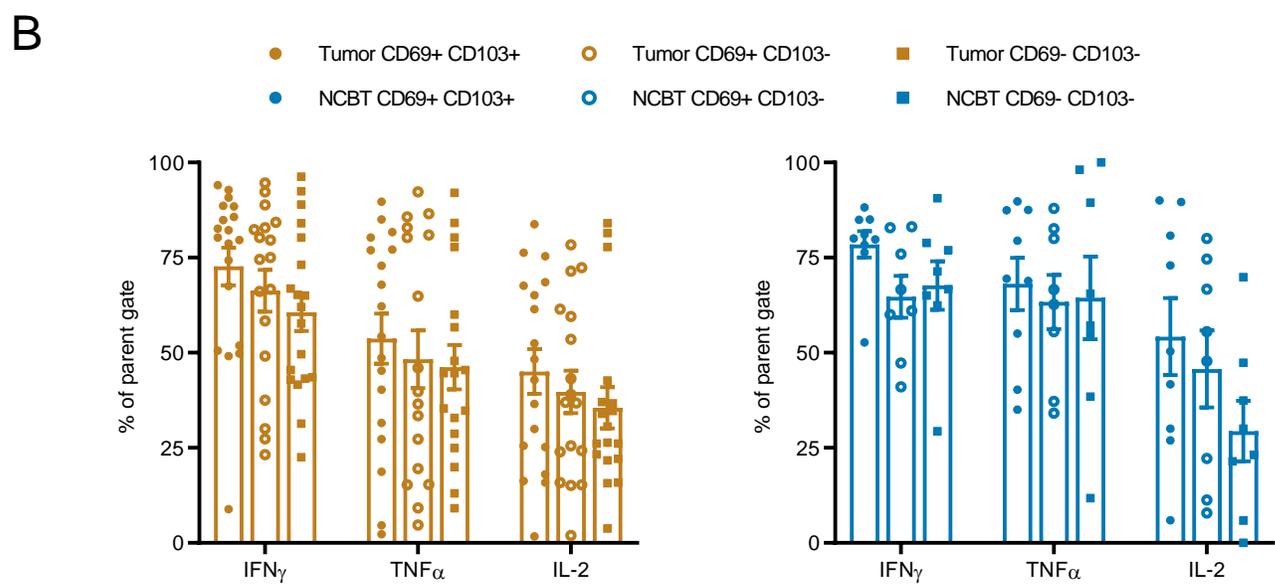
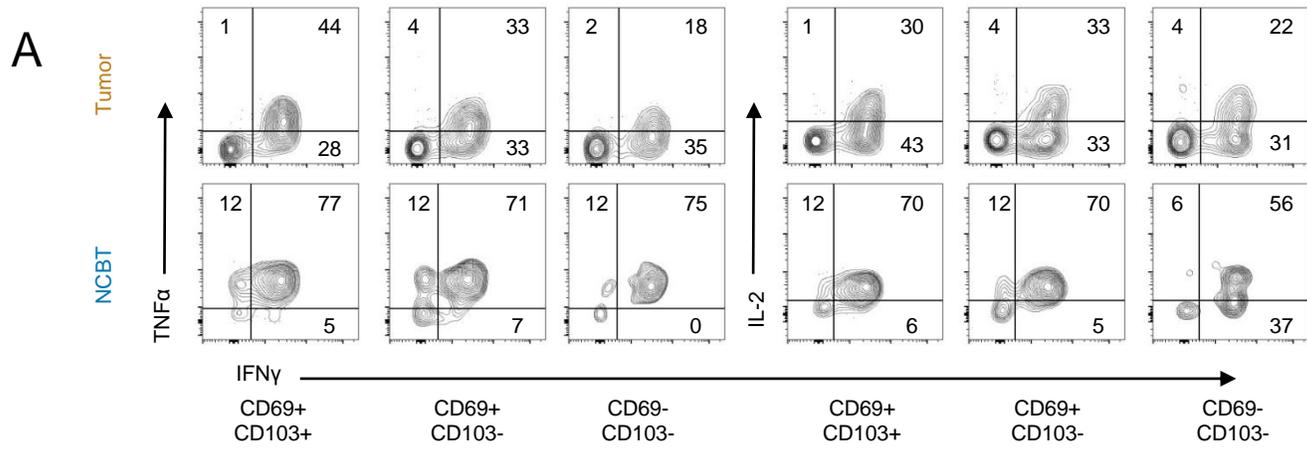


Figure 4

Figure 5. CD103⁺ CD8⁺ tissue resident memory T cell infiltration in breast tumors associates with relapse-free outcome in patients. FFPE tumors assayed for the presence of CD103⁺ CD8⁺ resident memory T cells by QIF were grouped according to relapse and relapse-free. Representative phenotype mapped images with CK (gray), DAPI (blue), and T cell type phenotypes (CD103⁻ CD8⁺, cyan dots; CD103⁺ CD8⁺ red dots) are shown (A). Scale bars represent 50µm. Summarized data of CD8⁺ T cell densities (B) or CD103⁺ CD8⁺ and CD103⁻ CD8⁺ T cell densities (C) within total tissue, CIs, and stroma are shown. Each symbol represents data from a unique patient sample. Tumor samples n=25. Significance was calculated using Holm-Sidak multiple comparison tests. ns, p>0.05, **, p<0.01, ***, p<0.001

A

Phenotype: ● CD103+ CD8+ ● CD103- CD8+ ● CK+

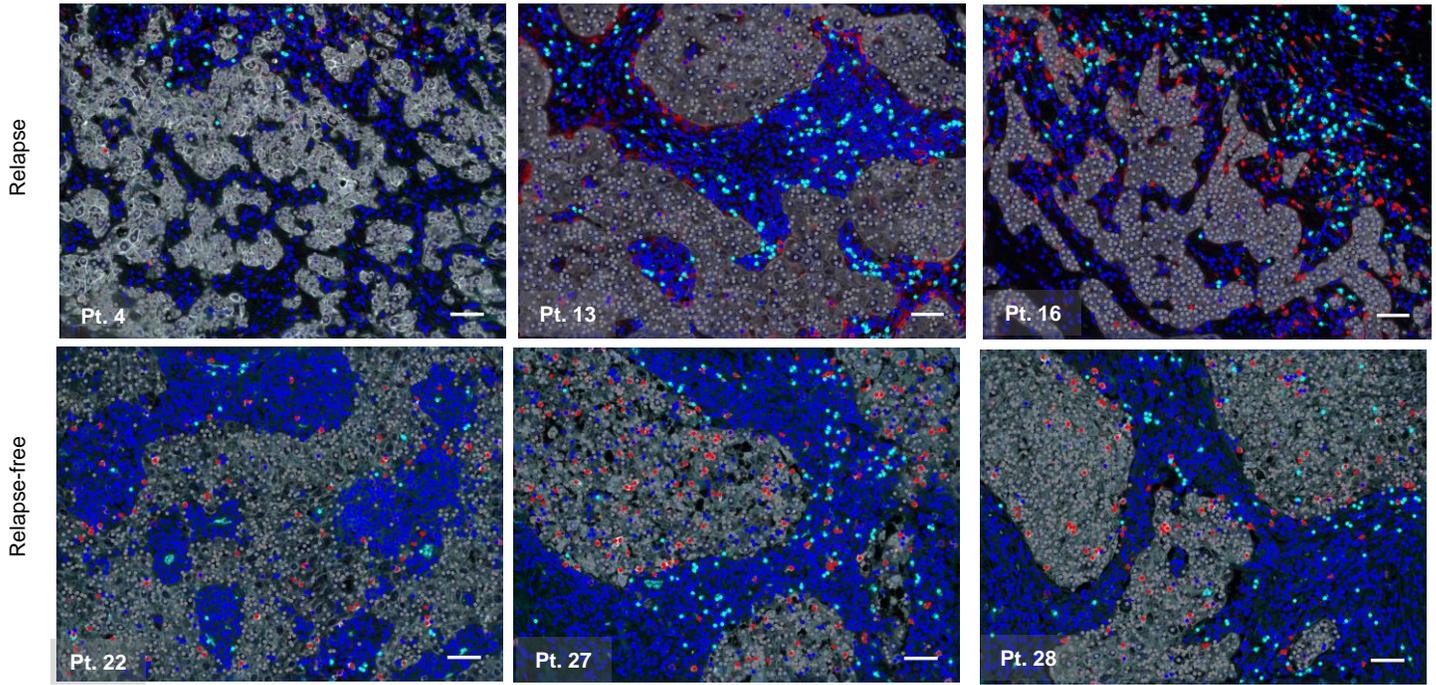
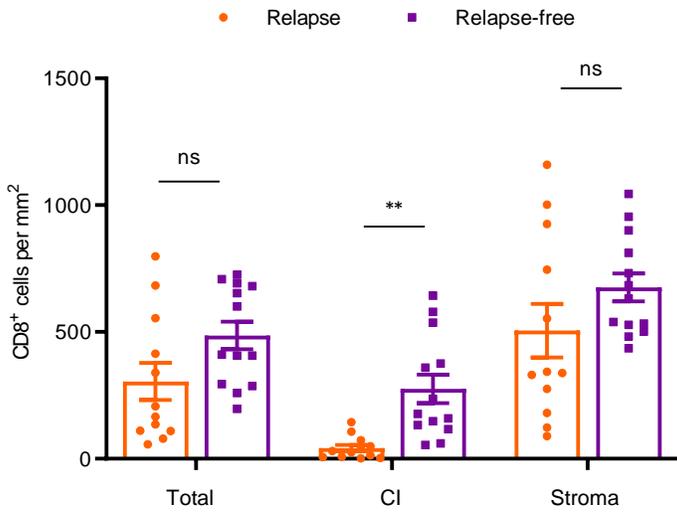
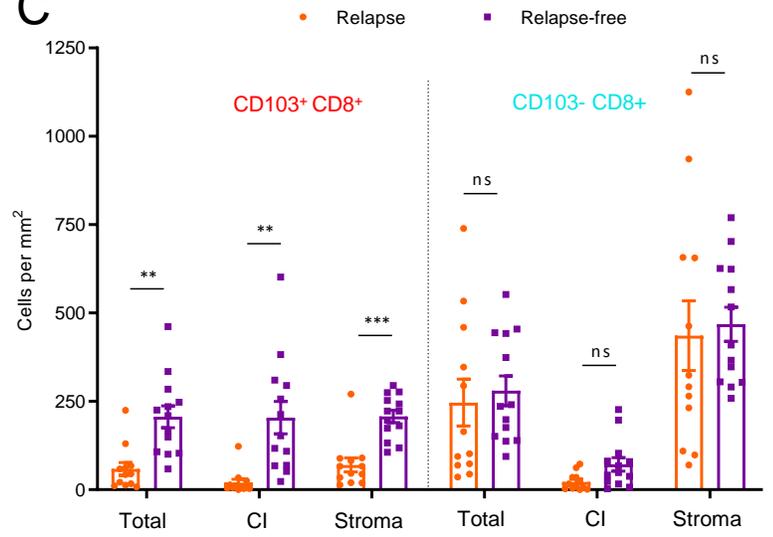
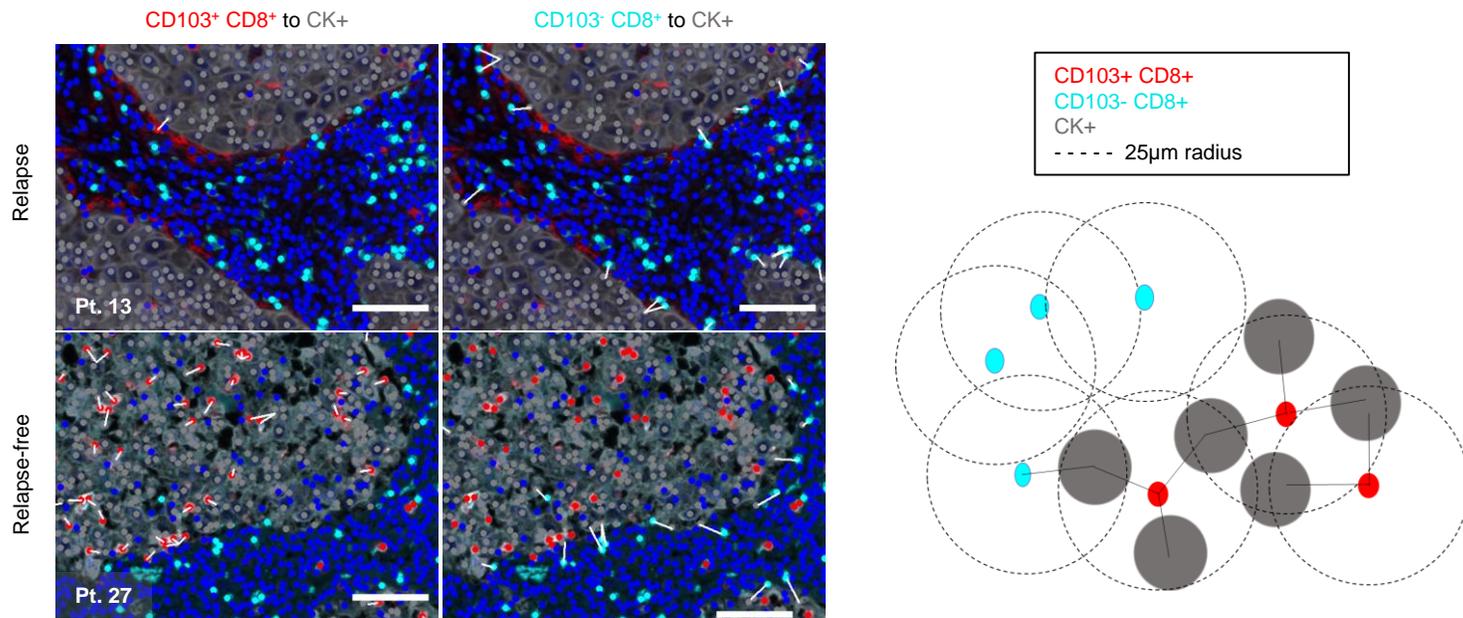
**B****C**

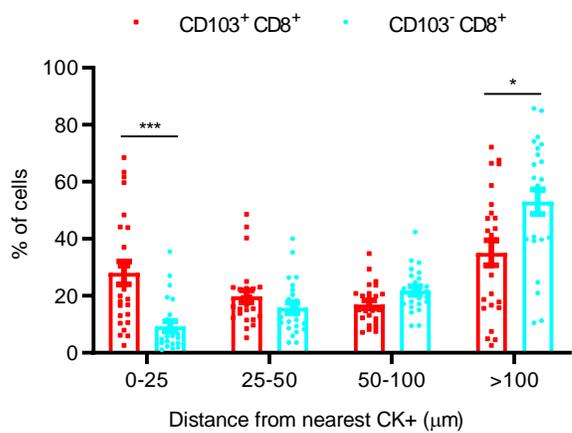
Figure 5

Figure 6. Proximity of CD103⁺ CD8⁺ TRMs and not CD103⁻ CD8⁺ T cells to cancer cells in breast tumors associates with relapse-free outcome. FFPE tissues of relapse or relapse-free (RF) patients were assessed for the spatial relationship between CD103⁺ CD8⁺ or CD103⁻ CD8⁺ and CK cancer cells. Representative phenotype mapped images with CK⁺ (grey), DAPI (blue), and T cell type phenotypes (CD103⁻ CD8⁺, cyan dots; CD103⁺ CD8⁺ red dots) are shown with white lines representing nearest neighbor analysis of T cells within 25 μ m of a CK⁺ cancer cell as depicted in a cartoon graphic(A). Spatial localization of CD103⁺ CD8⁺ and CD103⁻ CD8⁺ T cells within 0-25 μ m, 25-50 μ m, 50-100 μ m, or >100 μ m of the nearest CK⁺ cancer cell was assessed in all tumors (B) and is shown with regard to relapse and non-relapse groups as scatter plots (C). Each symbol represents data from a unique patient sample. Tumor samples n=25. Significance was calculated using Holm-Sidak multiple comparison tests. ns, p>0.05, *, p<0.05; **, p<0.01, ***, p<0.001.

A



B



C

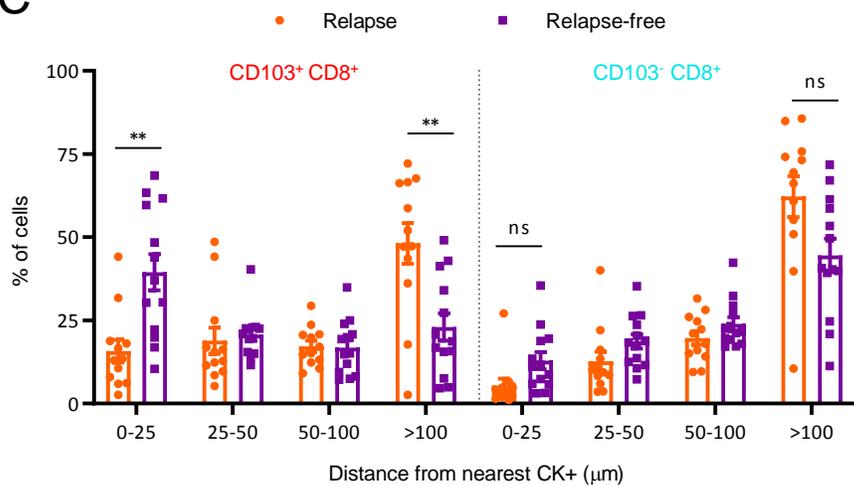


Figure 6

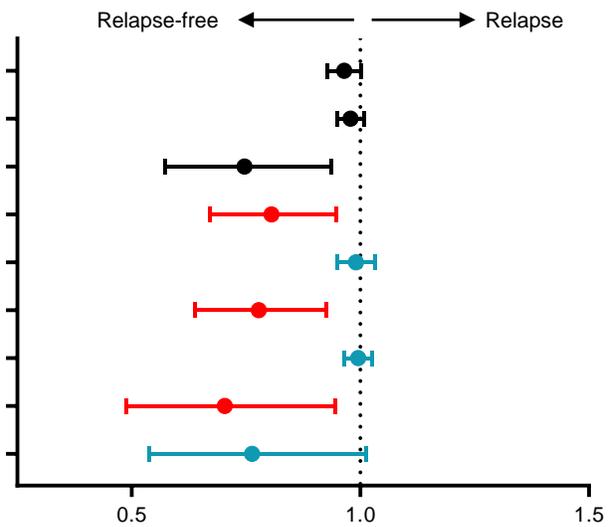
Figure 7. CD8⁺ CD103⁺ TRMs are the strongest indicator of relapse-free survival. Univariate odds ratio assessment per increment of 10 cells per mm² is shown (A), with respect to CD8⁺ T cells, CD103⁺ CD8⁺ T cells, and CD103⁻ CD8⁺ T cells in total tumor, stroma, or CIs. A correlation matrix of all variables is also presented in which r is displayed on a color scale and non-significant correlations ($p>0.05$) are marked out with an 'X' (B).

A

Univariate Odds Ratio Estimates

| Effect* | Point Estimate | 95% Wald Confidence Limits | |
|--------------------|----------------|----------------------------|-------|
| Tumor CD8+ | 0.964 | 0.928 | 1.002 |
| Stroma CD8+ | 0.979 | 0.95 | 1.008 |
| CI CD8+ | 0.732 | 0.573 | 0.936 |
| Tumor CD103+ CD8+ | 0.798 | 0.671 | 0.948 |
| Tumor CD103- CD8+ | 0.99 | 0.949 | 1.033 |
| Stroma CD103+ CD8+ | 0.769 | 0.638 | 0.927 |
| Stroma CD103- CD8+ | 0.995 | 0.965 | 1.026 |
| CI CD103+ CD8+ | 0.679 | 0.488 | 0.945 |
| CI CD103- CD8+ | 0.739 | 0.539 | 1.013 |

*Per increment 10 cells/mm²



B

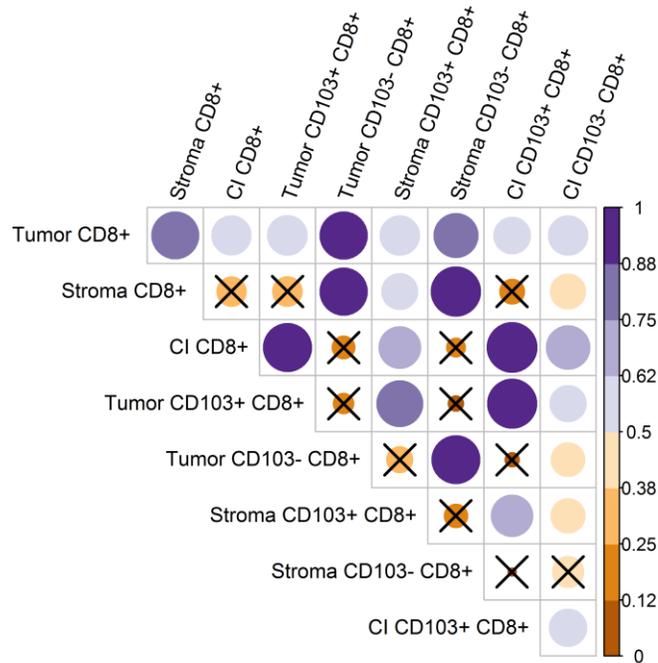


Figure 7