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이학석사 학위논문

# 고형암에서의 조직 상주 기억 T 세포의 표현형 차이

# Phenotypic difference of tissue-resident memory T cells in solid tumor

울 산 대 학 교 대 학 원

의 과 학 과

박 혜 선

# 고형암에서의 조직 상주 기억 T 세포의 표현형 차이

지 도 교 수  공 경 엽

이 논문을 이학석사학위 논문으로 제출함

2021 년 2 월

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2021 년 2 월

**ABSTRACT**

The presence and clinical importance of tissue-resident memory T ($T_{RM}$) cells have been recently described in association with various cancer types. However, the frequency and phenotypic characteristics of $T_{RM}$ cells are largely unknown. We analyzed single-cell populations of colorectal cancer (CC, n=18), stomach cancer (SC, n=13), renal cell carcinoma (RCC, n=19), and breast cancer (BC, n=16) by dissociation of tumor tissue with collagenase/hyaluronidase. We investigated populations of naïve, effector, and memory T and $T_{RM}$ cells by flow cytometry. Compared with the other tumor types, CC was associated with a significantly lower percentage of $CD8^+$ T cells ($P < 0.001$) among $CD3^+$ T cells. Memory T cells were generally the dominant population. Among $CD4^+$ cells, CC was associated with a significantly higher proportion of $CD103^+$ T cells than other tumor types ($P < 0.001$). Among $CD8^+$ cells, CC and SC were associated with higher $CD103^+$ T cell proportions than RCC and BC ($P < 0.001$). Significantly more $CD8^+$ than CD4+ cells expressed CD103 ($P < 0.001$). In association with SC, RCC, and BC, $CD8^+$ T cells had a similar T cell phenotype composition pattern: fewer effector T cells and more memory-type T cells among $CD103^+$ cells compared with $CD103^-$ cells ($P < 0.05$). Immune cell population composition was not significantly associated with pT stage or tumor-infiltrating lymphocyte levels. $T_{RM}$ cell abundance and phenotypes varied among CC, SC, RCC, and BC. Further studies regarding the clinical significance and functional differences of $T_{RM}$ associated with various tumors are warranted.

**Keywords**: human cancer, single cell, CD103, tissue-resident memory T cell, $T_{RM}$

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## INTRODUCTION

### Introduction

The tumor microenvironment contains various types of immune cells. Among them, T cells are the main source of antitumor immunity. Naïve T cells can be differentiated into effector and memory cell types after exposure to cognate antigens of each T cell. Traditionally, T cells are divided into different cell types according to the expression of CD45RO, CCR7/CD62L, and CD95: naïve cells (Tn, defined as $CD45RO^{-}CCR7^{+}CD62L^{+}CD95^{-}$), memory stem cells (TSCM, $CD45RO^{-}CCR7^{+}CD62L^{+}CD95^{+}$), central memory cells (TCM, $CD45RO^{+}CCR7^{+}CD95^{+}$), effector memory cells (TEM, $CD45RO^{+}CCR7^{-}CD95^{+}$), and effector cells (Teff, $CD45RO^{-}CCR7^{-}CD95^{+}$).(1) We previously identified the presence of effector cells and various memory T cells in breast cancer tissue.(2) About 80% of T cells in breast cancer tumor-infiltrating lymphocytes (TILs) expressed the memory cell phenotype.

Tissue-resident memory T (TRM) cells are a subset of specialized T cells that resides in tissues and are responsible for enhanced regional immunity.(3) Among various markers expressed on TRM cells, CD103 is a representative TRM cell marker. CD103 is an integrin alpha protein encoded by the ITGAE gene. CD103 is a part of the heterodimeric integrin molecule αEβ7 with integrin beta 7. E-cadherin, which is an adhesion molecule in epithelial cells, is the ligand for αEβ7. In addition to the expression of CD103, which can interact with epithelial cells, TRM cells have lower expression levels of receptors for sphingosine 1 phosphate (S1P1).(4) S1P1 is highly present in blood, and the gradient of S1P1 between blood and tissue drives immune cells with high levels of S1P1 receptors into the circulation. Therefore, reduced expression of S1P1 receptors is associated with increased tissue retention of TRM cells. CD69, which is associated with recent T cell activation, is another well-known TRM cell marker. CCR7 is also known to be decreased in TRM cells, but the relationship between CD103 expression and the traditional naïve–effector–memory phenotype of T cells has not been fully explored.

The presence of $CD103^{+}$ T cells has been described in association with several tumor types.(3, 5, 6) A high abundance of $CD103^{+}$ T cells in the tumor microenvironment was associated with a better prognosis among patients with melanoma, renal cell carcinoma (RCC), and head and neck squamous cell carcinoma, as well as non-small cell lung, ovarian, endometrial, cervical, pancreatic, and breast cancer (BC). Remarkably, $CD103^{+}$ T cells

showed tumor-reactive cytolytic activity upon stimulation with autologous tumor cells in association with non-small cell lung cancer, melanoma, and head and neck squamous cell carcinoma.(6, 7) Therefore, investigations of the associations of $CD103^+$ T cells with various tumor types might inform improvements of antitumor immunotherapy.

This study aimed to characterize the abundance and phenotypic characteristics of CD103+ T cells in different cancer tissues. We analyzed naïve, effector, and memory T cell subtypes and CD103 expression in four different cancer types: colorectal cancer (CC), stomach cancer (SC), RCC, and BC.

## MATERIAL AND METHODS

### Patients and Clinicopathologic Characteristics

Primary cancer tissues (CC, n=18; SC, n=13; RCC, n=19; and BC, n=16) without any previous treatment were obtained from operations performed at the Asan Medical Center (Supplementary Table 1). This study was approved by the Institutional Review Board of Asan Medical Center (IRB 2016-0935). Whole sections of the hematoxylin and eosin (H&E)–stained slides were reviewed for analysis of histologic grade, pT stage, and pN stage. We also evaluated the levels of stromal TILs using full sections in 10% increments (defined as the mean percentage of plasma cells and lymphocytes in the stroma of invasive carcinoma; if there was <10% of the area involved, 0%, 1%, 2%, or 5% level criteria were used).(8)

### Single-Cell Dissociation

Cancer tissues were placed in RPMI1640 medium for 2 h postoperatively. Tissues were into 2-3 mm pieces and washed with phosphate-buffered saline (PBS; pH 7.4) with 1 × ZellShield anti-contaminant agent (Minerva Biolabs, Berlin, Germany). Fragmented tissues were placed in digestion medium (Dulbecco's Modified Eagle Medium [DMEM-12], 2% fetal bovine serum [FBS], 1% penicillin/streptomycin, 10 μg/mL insulin, and 10 ng/mL epi-

dermal growth factor) with 1 × collagenase/hyaluronidase (GenDEPOT, TX, USA) and DNase I (CA095-005; GenDEPOT) and incubated at 37°C for 1-2 h using a rotator. The sample was placed in a 70 μm pore nylon mesh strainer and incubated for 3-5 min using 0.25% EDTA to separate single cells. After washing with Hank's balanced salt solution containing 2% FBS, cells were centrifuged at 400 g for 5 min. Single cells were counted and stored frozen in a deep freezer.

**Fluorescence-Activated Cell Sorting (FACS) Analysis**

To analyze the composition of immune cells, we performed FACS analysis using anti-CD3, CD8, CD45RO, CCR7, and CD95. Also, for $T_{RM}$ cell analysis, we used anti-CD69 and anti-CD103 antibodies. Briefly, the prepared single cells were washed with FACS buffer (PBS containing 2% FBS) and then surface-stained at room temperature in the dark for 30 min with the following antibodies: IgG (BioLegend, CA, USA), Pacific blue-labeled anti-DAPI (Invitrogen, USA), APC-Cy7-labeled anti-CD3 (SK7; BioLegend), PercP-Cy5.5-labeled anti-CD8 (RPA-T8; BioLegend), PE-Cy7-labeled anti-CCR7 (G043H7; BD Biosciences, USA), PE-labeled anti-CD45RO (UCHL1; BioLegend), APC-labeled anti-CD95 (DX2; BioLegend), FITC- and PE-labeled anti-CD103 (Ber-ACT8; BioLegend), BV510-labeled anti-CD69 (FN50; BioLegend), and APC-Cy7-labeled anti-CD3 (SK7; BD Biosciences). After being washed with FACS buffer, the stained cells were assessed using a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, OR, USA).

**Statistical Analysis**

All statistical analyses were performed using SPSS statistical software (version 18; SPSS, Chicago, IL, USA). The Mann–Whitney test and Spearman correlation analysis were used, as appropriate. All tests were two-sided and statistical significance was set at 5%.

**Results-**

**Single-Cell Dissociation Yield**

To provide basic knowledge about dissociated single cells in CC, SC, RCC, and BC, we analyzed the number of individual cells obtained from tumor tissues (25-40 mg). We could retrieve 1.5–26 × $10^5$ cells/mg for CC, 1.4–9.6 × $10^5$ cells/mg for SC, 20–450 × $10^5$ cells for RCC, and 0.8–11 × $10^5$ cells/mg for BC.

**T Cell Differentiation**

The presence of memory and effector cells in the tumor microenvironment might suggest the existence of tumor-reactive T cells. We analyzed T cell differentiation status with anti-CD3, CD8, CD45RO, CCR7, and CD95 antibodies (Fig. 1A). Compared with the other tumor types (SC median, 35.8% [range, 25.4–59.2%]; RCC, 45.5% [23.2–77.3%]; BC, 42.9% [31.1–87.6%], CC (24.2%; 2.7–40.4%) was associated with a significantly lower percentage of $CD8^+$ T cells ($P < 0.001$). Except for $CD4^+$ T cells associated with CC, memory T cells were the dominant population (Fig. 2A-D). Among both $CD4^+$ and $CD8^+$ T cells, there was a significantly smaller proportion of $T_n$ cells than $T_{eff}$ and T memory cells in association with RCC and BC ($P < 0.05$). In all tumors, among both $CD4^+$ and $CD8^+$ T cells, there was a significantly smaller proportion of $T_{eff}$ cells than T memory cells ($P < 0.05$). $CD8^+$ T cells had significantly more $T_{eff}$ cells than $CD4^+$ T cells in all tumor types except SC ($P < 0.01$). $CD4^+$ T cells had significantly more memory type T cells than $CD8^+$ T cells in RCC and BC ($P < 0.01$).

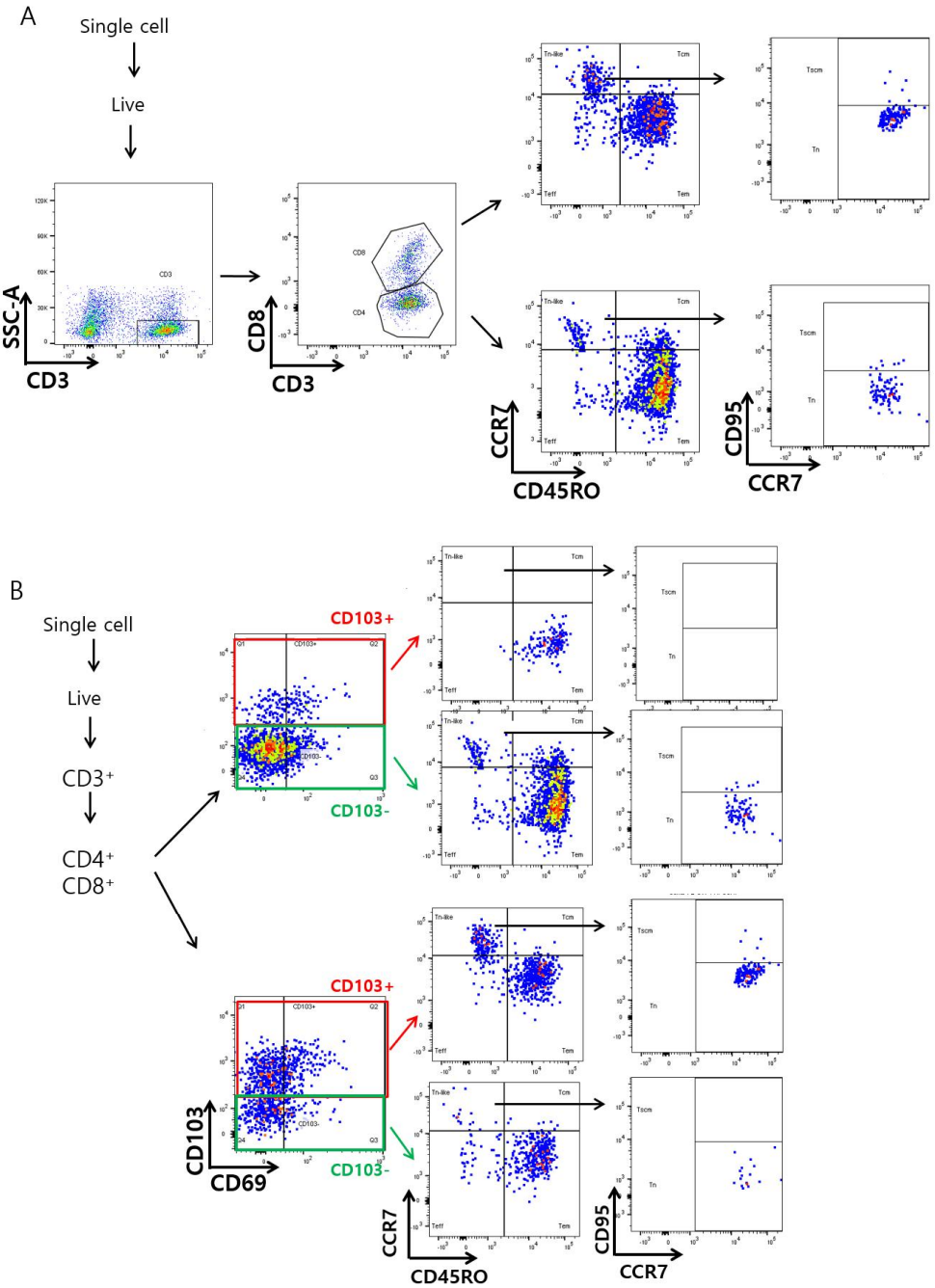


Figure 1. FACS analysis scheme. (A) T cell differentiation. (B) T cell differentiation according to CD103/CD69 positivity.

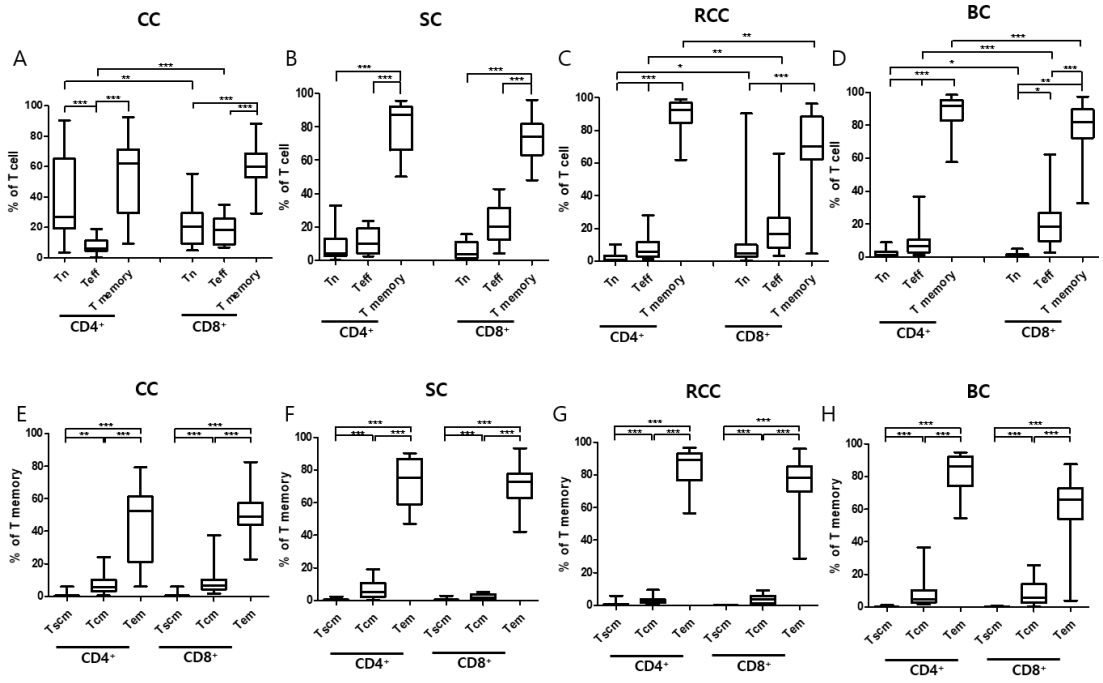


Figure 2. Subtypes of T cells. (A-D) T cell phenotype composition associated with colorectal cancer (CC, A), stomach cancer (SC, B), renal cell carcinoma (RCC, C), and breast cancer (BC, D). (E-H) Memory T cell subtypes in CC (E), SC (F), RCC (G), and BC (H).

Memory T cell subtypes were further analyzed. Most memory-type T cells were $T_{em}$ cells (Fig 2E-H).

**Expression of CD69 and CD103**

To analyze $T_{RM}$ cells, we performed FACS analysis with anti-CD69 and anti-CD103 antibodies. Among $CD4^+$ cells, the proportion of $CD103^+$ T cells associated with CC was significantly higher than that associated with tumors affecting organs (CC, 49.6% [17.8–82.6%]; SC, 7.9% [1.5–27.9%]; RCC, 3.7% [0.7–15.0%]; BC, 15.0% [4.7–27.9%]) ($P < 0.001$, Fig 3A). Among $CD8^+$ cells, CC and SC were associated with a higher $CD103^+$ T cell proportion than RCC and BC (CC, 60.0% [21.4–92.2%]; SC, 64.4% [33.5–92.0%]; RCC, 15.8% [3.4–54.8%]; BC, 45.6% [9.0–87.2%]) ($P < 0.001$, Fig 3A).

Since previous studies reported that $CD103^+$ cells are more abundant among $CD8^+$ T cells than among $CD4^+$ T cells,(9) we compared the $CD103^+$ cell proportion between $CD4^+$ and $CD8^+$ T cells and confirmed that $CD103^+$ cells were significantly more abundant among $CD8^+$ than among $CD4^+$ T cells ($P < 0.001$, Fig 3B).

The proportion of $CD103^+$ and $CD69^+$ double-positive cells was significantly lower in association with RCC than other cancer types among both $CD4^+$ and $CD8^+$ cells ($P < 0.01$, Fig 3C and 3D, Table 1). $CD4^+$ T cells expressing either CD103 or CD69 comprised 53.0% of CC cells, 28.8% of SC cells, 20.0% of RCC cells, and 28.9% of BC cells. CC was associated with a higher level of $CD4^+$ T cells expressing either CD103 or CD69. Among $CD8^+$ T cells, the following proportions were positive for CD103 or CD69 according to cancer type: 69.2% for CC, 70.3% for SC, 36.8% for RCC, and 61.8% for BC. RCC was associated with a lower level of $CD8^+$ T cells expressing either CD103 or CD69.

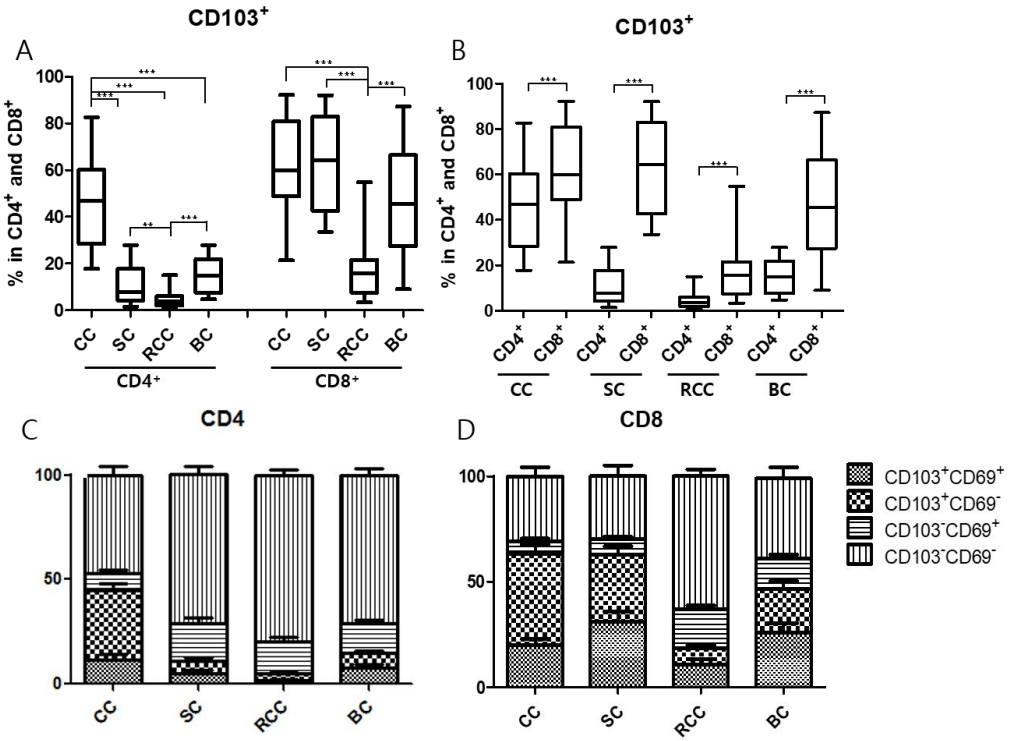


Figure 3. CD69 and CD103 expression in T cells. (A) $CD103^+$ cells among $CD4^+$ and $CD8^+$ cells. (B) $CD103^+$ cells among $CD4^+$ and $CD8^+$ cells. (C) Proportion of CD69 and/or CD103 expressing cells in $CD4^+$ cells. (D) Proportion of CD69 and/or CD103 expressing cells in $CD8^+$ cells.

Table 1. Proportion of cells expressing CD103 or CD69 among $CD4^+$ and $CD8^+$ T cells

| | CC (%) | SC (%) | RCC (%) | BC (%) |
|---|---|---|---|---|
| **$CD4^+$ cells** | | | | |
| $CD103^+CD69^+$ | 11.6 (1.3-28.8) | 5.2 (0.3-17.2) | 1.9 (0.1-7.6) | 7.9 (1.2-19.0) |
| $CD103^+CD69^-$ | 33.4 (12.5-55.7) | 5.9 (1.0-16.7) | 3.1 (0.6-9.2) | 7.1 (1.6-11.5) |
| $CD103^-CD69^+$ | 8.0 (1.6-24.0) | 17.7 (6.9-44.1) | 15.0 (2.8-35.9) | 13.9 (3.4-29.8) |
| $CD103^-CD69^-$ | 47.0 (14.1-80.0) | 71.2 (39.4-91.6) | 80.0 (51.1-95.4) | 71.1 (54.6-89.5) |
| **$CD8^+$ cells** | | | | |
| $CD103^+CD69^+$ | 19.9 (3.1-49.0) | 31.1 (12.1-68.2) | 10.7 (0.9-44.6) | 25.9 (1.4-55.9) |
| $CD103^+CD69^-$ | 43.3 (14.3-85.2) | 31.7 (14.7-64.2) | 7.9 (1.7-23.4) | 20.5 (4.9-69.6) |
| $CD103^-CD69^+$ | 5.9 (0.0-19.1) | 7.5 (1.4-16.1) | 18.2 (3.7-33.3) | 14.4 (0.2-34.2) |
| $CD103^-CD69^-$ | 30.8 (7.38-64.3) | 29.7 (5.2-59.6) | 63.2 (29.2-92.1) | 38.2 (12.5-80.5) |

BC, breast cancer; CC, colorectal cancer; SC, stomach cancer; RCC, renal cell carcinoma

**T Cell Differentiation Status of Cells According to CD103 Expression**

To explore the relationship between CD103 expression and the traditional naïve–effector–memory phenotype classification of T cells, we investigated T cell differentiation status according to CD103 expression (Supplementary Table 2). We compared the $CD103^+$ and $CD103^-$ cell proportions associated with each T cell phenotype. Among $CD4^+$ T cells associated with CC, a significantly higher proportion of $CD103^+$ cells than $CD103^-$ T cells were of the $T_n$ and $T_{eff}$ phenotypes ($P < 0.05$, Fig 4A). In contrast, in association with SC, the $T_n$ and $T_{eff}$ phenotypes were more commonly associated with $CD103^-CD4^+$ T cells than $CD103^+CD4^+$ T cells ($P < 0.05$, Fig 4B). $CD8^+$ T cells associated with SC, RCC, and BC had a similar T cell phenotype composition pattern: fewer $T_{eff}$ cells and more memory type T cells among $CD103^+$ cells than $CD103^-$ cells ($P < 0.05$, Fig 4B-D).

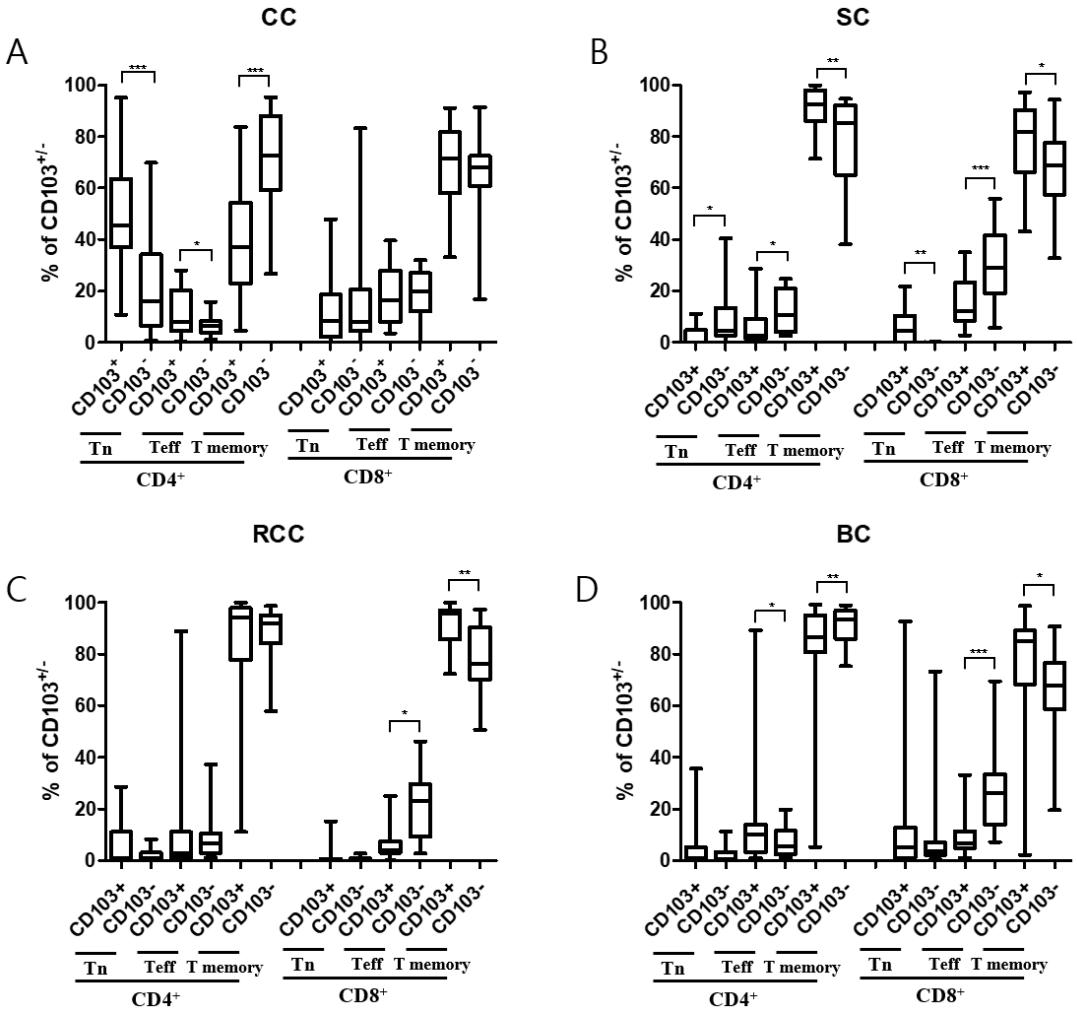


Figure 4. Comparison of $CD103^+$ and $CD103^-$ T cells for each T cell phenotype in association with colorectal cancer (CC) (A), stomach cancer (SC) (B), renal cell carcinoma (RCC) (C), and breast cancer (BC) (D).

**Analysis of CCR7 Positivity in $CD103^+$ Cells**

Since $T_{RM}$ cells are also known to have a lower CCR7 expression, we analyzed CCR7 expression according to CD103 positivity. In contrast to previous findings, we observed higher CCR7 positivity among $CD103^+$ cells of all tumor types except for $CD4^+$ SC cells (Fig 5).

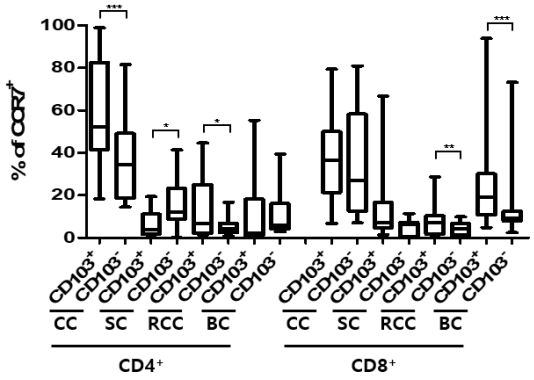


Figure 5. Comparison of composition of $CCR7^+$ cells between $CD103^+$ and $CD103^-$ T cells.

**Characteristics of Tumors with a High Proportion of CD103$^+$ T Cells**

To characterize tumors with more CD103$^+$ T cells, we divided cases into two groups according to the median CD103$^+$ cell count in each organ. The cut-off points for CD4$^+$ cells were 45.2% for CC, 10.9% for SC, 4.9% for BC, and 14.6% for RCC. The cut-off points for CD8$^+$ cells were 62.9% for CC, 62.3% for SC, 18.5% for BC, and 47.5% for RCC. We compared immune cell compositions between the low-CD103$^+$ and high-CD103$^+$ groups. Tumors with high proportions of CD103$^+$ cells in CD4$^+$ T cells had higher levels of CD103 positivity in CD8$^+$ cells in association with CC and SC, while tumors with high proportions of CD103$^+$ cells in CD8$^+$ T cells had higher levels of CD103 positivity in CD4$^+$ cells in CC, SC, and RCC. For the other immune cell compositions, there were no associations observed according to the proportion of CD103$^+$ cells.

**Comparison of Immune Cell Composition According to pT Stage and TIL Levels**

We compared immune cell compositions according to pT stage. pT stage was classified as low (pT1 & 2 for CC and SC; pT1 for RCC and BC) and high (pT3 & 4 for CC and SC; pT2 & 3 for RCC and BC). The immune cell population composition was not associated with pT stage (Supplementary Table 3).

To find associations between the TIL levels and immune cell composition, we analyzed the level of TILs in H&E slides. Among CD8$^+$ T cells, the proportion of memory phenotype cells positively correlated with the abundance of TILs for all tumor types except BC (Supplementary Table 4).

**Discussion**

$T_{RM}$ cells have been characterized by elevated CD103 and CD69 expression and reduced S1P1 expression. CD69 is also known as an inhibitor of S1P1, blocking lymphocyte egress into the circulation.(10) We demonstrated the presence of CD103 and CD69 double-positive and single-positive cell populations in association with four cancer types. The compositions of cells expressing CD103 and CD69 varied among the tumor types. $CD8^+$ T cells expressing either CD103 or CD69 constituted about 70% of CC, SC, and BC cells, while RCC was associated with larger CD103 and CD69 double-negative populations. The presence of CD103 expressing $CD8^+$ T cells has been studied in various cancer types with varying methods. In association with malignant glioma, it has been shown by flow cytometry that 20-57% of $CD8^+CD3^+$-infiltrating T cells expressed CD103.(11) Additionally, in association with metastatic melanoma, researchers used flow cytometry to demonstrate that 30% of $CD8^+$ TIL cells were $CD69^+CD103^+$ tumor-resident T cells.(12) Other flow cytometry experiments demonstrated that, in untreated early-stage non-small cell lung carcinoma TILs, the proportion of $CD103^+$ cells was 32% that of $CD8^+$ cells.(13) Another group used two-color immunofluorescence staining to show that, among bladder urothelial cell carcinoma $CD8^+$ T cells, 76% were $CD103^+$ cells.(14) Another study used immunohistochemical staining to show that 40% of high grade serous ovarian cancer TILs were $CD103^+$ cells.[16]

In most related previous studies, $T_{RM}$ cells have been studied among $CD8^+$ TILs. CD103 positivity in $CD4^+$ T cells has not been comprehensively analyzed. It is known that $CD103^+$ T cells are more frequently present in $CD8^+$ T cells than $CD4^+$ T cells.(7) Also, in this study, the $CD8^+CD103^+$ T cell abundance exceeded that of $CD4^+CD103^+$ T cells in association with four tumor types (CC, SC, BC, and RCC). Flow cytometry has been used to show that most $CD103^+$ TILs associated with high-grade ovarian cancer were $CD8^+$ T cells ($P < 0.001$) by flow cytometry.[18] In intratumor areas and adjacent non-tumor areas of

esophageal squamous cell carcinoma, among $CD103^+$ cells, $CD8^+$ cells have been shown to be more highly expressed than $CD4^+$ cells by co-localization immunofluorescence. This means that primary CD103-expressing cells could be $CD8^+$ cells.(15) However, in a study of TILs in non-small cell lung cancer, $CD103^+CD4^+$ TILs appeared to produce the most TNF-α and IFN-γ compared with all other $CD8^+$ TILs regardless of CD103 or CD69 expression.(16) In another study of lung fibrosis associated with fungal antigen, lung tissue-resident $CD44^{hi}CD69^{hi}CD4^+$ T cells consisted of $CD103^{lo}CD4^+$ $T_{RM}$ cells that expressed profibrotic cytokine genes and $CD103^{hi}$ $T_{reg}$ cells that expressed inhibitory genes against fibrosis.(17) Based on those results, it is necessary to investigate further regarding the functional role of CD103 positivity in $CD4^+$ and $CD8^+$ T cells.

CCR7 expression in $T_{RM}$ cells has been reported to be low,(18) but the relationship between CD103 expression and the traditional naïve–effector–memory phenotype classification of T cells has not been fully explored. In early lung cancer cytotoxic $CD8^+$ T cells, both $CD103^+$ and $CD103^-$ cells mostly express a late-stage effector memory phenotype ($CD28^-$ $CD27^-CD45RO^+CD45RA^-CCR7^-$). However, a comparison of compositions of a late-stage effector memory phenotype between $CD103^+$ and $CD103^-$ cells was not conducted in that study.(19) In head and neck squamous cell carcinoma and ovarian cancer, $CD103^+CD39^+CD8^+$ TILs have showed lower CD62L and CCR7 expression than $CD103^+CD39^-$ and $CD103^-CD39^-$ TILs.(6) However, in our study, expression of CCR7 was not significantly lower in $CD103^+$ cells than $CD103^-$ cells, in either the $CD8^+$ or $CD4^+$ cells of four cancer types (CC, SC, BC, and RCC). This difference may have occurred because of the differing sites of tumor origin.

We did not find significant differences in immune cell population compositions, including $CD103^+$/$CD103^-$ cells, between the low and high T stages for each tumor type. Similar to our study, Workel et al. found that, in endometrial adenocarcinoma (n=305), $CD103^+$ density showed no difference depending on the FIGO grade and stage.(20) Also, Webb et al.

found that, in ovarian epithelial tumors (n=366), the number of $CD103^+$ TILs was not proportional or inversely proportional to the FIGO stage.(21) Koh et al. showed that, in lung squamous cell carcinoma (n=378), intratumoral $CD103^+$ TILs were less abundant in association with a higher stage, but statistical significance was not observed ($P = 0.384$).(22) On the other hand, Komdeur et al. found that, in cervical cancer (n=460), there were fewer $CD103^+$ cells in association with high-stage disease. The authors suggested that this might have been because equilibrium is induced early in cancer during the competition between T cells and tumor cells, and later, the tumor cells that avoided T cell attacks continued to proliferate.(23) All of these aforementioned studies used immunohistochemical staining of immune cells, unlike our study which used flow cytometry. Those studies might have been affected by false-positive staining, since it is not only T cells that express CD103 molecules.

Functional studies have been executed to identify the physiologic function of $T_{RM}$ cells, but little is understood. Some studies have demonstrated the expression of immune checkpoint molecules in $CD103^+CD8^+$ cells. Edwards et al. showed that the $CD103^+CD69^+$ subset expressed more PD-1 and 2B4 than the $CD103^+CD69^-$ and $CD103^-CD69^-$ subsets in malignant melanoma.(12) Ganesan et al. demonstrated $CD103^+$ cells expressed PD-1, TIM-3, and 4-1BB in non-small cell lung cancer and head and neck squamous cell carcinoma.(13) In $CD8^+$ T cells of high-grade serous carcinoma, co-expression of CD103 and PD-1 accounted for 38.5% of the cells. PD-1 positivity was associated with a favorable prognosis.(24) A study investigating esophageal squamous cell carcinoma showed that $CD103^+$ T cells were associated with a favorable prognosis, but $CD103^+$ T cells co-expressed CTLA-4 rather than PD-1.(15)

The cytotoxic function of $CD103^+CD8^+$ T cells has been reported at the RNA level (*IFNG, GZMA, GZMB, SEMA7A, KLRB1, CCL3, STAT1, RAB27A, IL21R, and FKBP1A*) (13) as well as the protein level (granzyme A, granzyme B, perforin, and CD107a).(7) (15) (25) (26) Some functional studies demonstrated that $CD8^+CD103^+$ T cells expressed cyto-

kines, such as IFN-γ and TNF-α.(13) (27) In ascites of high grade serous ovarian carcinoma, the level of TGF-β was correlated with CD103 expression in ascites. Franciszkiewicz et al. showed that the interaction between CD103 and E-cadherin of tumor cells was associated with cytokine release.(28)

Adoptive TIL therapy has been successful for treating malignant melanoma. To acquire sufficient TILs *in vitro*, Rohaan et al. conducted a rapid expansion protocol (REP) by triggering T cell receptor complexes using T cell growth factor and feeder cells.(29) We previously demonstrated the feasibility of TIL culture from breast cancer.(2) We studied $CD3^+$ and $CD103^+$ expression in REP TILs of CC (1 cases), SC (5 cases), and BC (16 case), although they were performed on samples from different patients than those analyzed in this study (Fig 6). Among $CD3^+$ cells, all tumor types had more $CD103^+$ cells in $CD8^+$ cells than in $CD4^+$ cells, which was the same pattern observed in the present study. However, the proportion of $CD103^+$ cells was significantly lower in association with REP TILs than in TILs dissociated from tumor tissue in this study. Induction of $T_{RM}$ cell differentiation during TIL culture might potentiate the trafficking of injected TILs to tissue sites and lead to tissue retention of TILs to improve the efficacy of adoptive TIL therapy.(30) Cytokines, such as TGF-β, IL-15, TNF, and IL-33, have been reported to be associated with $T_{RM}$ cells.(31) Therefore, further studies targeting improvements in the proportions of $T_{RM}$ cell subsets in cultured TILs or other T cell immunotherapeutics are necessary.

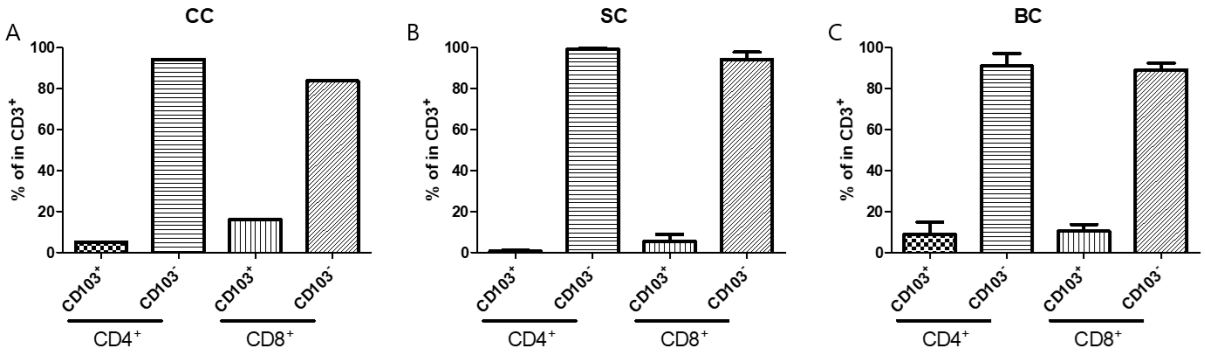


Figure 6. Composition of $CD103^+$ in $CD4^+$ and $CD8^+$ in *in vitro* rapidly expanded tumor-infiltrating lymphocytes. A) colorectal cancer (CC), B) stomach cancer (SC), C) breast cancer (BC).

The limitations of this study include the different stages of the four cancer types. Since we focused on mass-forming tumors from operation specimens with primary cancer without previous treatment, available tumors for CC and GC had higher pT stages (pT3 or pT4). For BC, because neoadjuvant systemic treatment is usually applied to higher pT-stage primary tumors, available tumors were those with relatively lower pT stages (pT1 or pT2). Short follow-up periods also precluded analysis of the prognostic significance of $CD103^+$ cells with different phenotypes. Further studies regarding the clinical significance and functional differences of $T_{RM}$ cells associated with various tumor types are warranted.

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**SUPPLEMENTARY TABLES**

Supplementary Table 1. Clinicopathologic characteristics

| Characteristic | | CC (%) | SC (%) | RCC (%) | BC (%) |
|---|---|---|---|---|---|
| Age (years) | ≤50 | 7 (39) | 2 (15) | 5 (26) | 11 (69) |
| | >50 | 11 (61) | 11 (85) | 14 (74) | 5 (31) |
| Sex | Male | 9 (50) | 8 (62) | 17 (89) | 0 (0) |
| | Female | 9 (50) | 5 (38) | 2 (11) | 16 (100) |
| pT | 1 | | | 15 (79) | 5 (31) |
| | 2 | 4 (22) | 4 (31) | 1 (5) | 11 (69) |
| | 3 | 12 (67) | 3 (23) | 3 (16) | |
| | 4 | 2 (11) | 6 (46) | | |
| pN | 0 | 9 (50) | 3 (23) | | 12 (75) |
| | 1 | 4 (22) | 1 (8) | | 4 (25) |
| | 2 | 5 (28) | 5 (38) | | |
| | 3 | | 4 (31) | | |
| | X | | | 19 (100) | |
| MSI status | MSI-low | 0 (0) | | | |
| | MSI-high | 4 (22) | | | |
| | MSS | 13 (72) | | | |
| EBV in situ hybridization | Positive | | 3 (23) | | |
| | Negative | | 10 (77) | | |
| Subtype | Clear cell RCC | | | 18 (95) | |
| | Chromophobe RCC | | | 1 (5) | |
| | HR+ | | | | 10 (63) |
| | HER2+ | | | | 3 (19) |
| | HE+/HER2+ | | | | 1 (6) |
| | TNBC | | | | 2 (13) |
| TIL (%) | median (range) | 7.5 (1-40) | 30 (2-80) | 5 (0-60) | 10 (0-80) |

BC, breast cancer; CC, colorectal cancer; SC, stomach cancer; RCC, renal cell carcinoma; TIL, tumor-infiltrating lymphocyte

Supplementary Table 2. Naïve–effector–memory phenotype of $CD103^+$ and $CD103^-$ cells in $CD4^+$ and $CD8^+$ T cells.

| Phenotype | | CC (%) | SC (%) | RCC (%) | BC (%) |
|---|---|---|---|---|---|
| In $CD4^+$ | | | | | |
| $T_n$ | $CD103^+$ | 45.6 (10.8–95.1) | 0.0 (0.0–11.1) | 1.0 (0.0–28.6) | 1.0 (0.0–35.7) |
| | $CD103^-$ | 15.9 (0.7–69.8) | 4.4 (0.0–40.5) | 1.1 (0.1–8.3) | 0.6 (0.0–11.3) |
| $T_{eff}$ | $CD103^+$ | 7.8 (0.3–28.0) | 2.8 (0.0–28.6) | 3.0 (0.0–88.9) | 10.2 (1.0–89.2) |
| | $CD103^-$ | 6.4 (1.0–15.8) | 10.6 (2.6–24.7) | 6.9 (1.2–37.4) | 5.5 (1.1–19.8) |
| $T_{memory}$ | $CD103^+$ | 37.0 (4.6–83.8) | 92.5 (71.4–100.0) | 94.1 (11.1–100.0) | 86.4 (5.4–99.1) |
| | $CD103^-$ | 72.5 (26.7–95.3) | 85.1 (38.1–94.7) | 91.9 (57.9–98.6) | 93.4 (75.3–98.9) |
| In $CD8^+$ | | | | | |
| $T_n$ | $CD103^+$ | 8.4 (0.0–47.9) | 4.5 (0.0–21.7) | 0.0 (0.0–15.2) | 5.4 (0.0–92.7) |
| | $CD103^-$ | 8.0 (0.0–83.3) | 0.0 (0.0–0.1) | 0.4 (0.0–2.8) | 3.7 (0.5–73.2) |
| $T_{eff}$ | $CD103^+$ | 16.6 (3.5–39.6) | 12.3 (2.8–35.1) | 4.1 (0.0–25.0) | 6.9 (1.1–33.3) |
| | $CD103^-$ | 19.8 (0.0–32.0) | 29.2 (5.6–55.9) | 23.1 (2.8–46.3) | 26.2 (7.3–69.5) |
| T memory | $CD103^+$ | 71.6 (33.1–91.2) | 81.8 (43.2–97.2) | 95.9 (72.3–100.0) | 84.9 (2.3–98.7) |
| | $CD103^-$ | 68.2 (16.7–91.4) | 68.7 (32.8–94.4) | 76.3 (50.8–97.3) | 67.9 (19.5–90.7) |

BC, breast cancer; CC, colorectal cancer; SC, stomach cancer; RCC, renal cell carcinoma; $T_{eff}$, effector T cell; $T_{memory}$, memory T cell; $T_n$, naïve T cell

Supplementary Table 3. Comparison of immune cell population according to pT stage (low versus high, Mann–Whitney U-test)

| Population composition | *P*-value | | | |
|---|---|---|---|---|
| | CC | SC | RCC | BC |
| $CD45^+$ | 0.10 | 0.20 | 0.31 | 1.00 |
| $CD3^+$ | 0.08 | 0.71 | 0.36 | 1.00 |
| B cell | 0.28 | 0.83 | 0.96 | 0.66 |
| NK | 0.57 | 0.83 | 0.53 | 0.38 |
| NKT | 0.65 | 0.94 | 0.31 | 0.18 |
| $CD4^+$ | 0.28 | 0.10 | 0.96 | 0.57 |
| $CD4^+T_n$ | 0.51 | 0.20 | 0.41 | 0.11 |
| $CD4^+T_{eff}$ | 0.88 | 0.33 | 0.12 | 0.91 |
| $CD4^+T_{memory}$ | 0.10 | 0.26 | 0.10 | 0.58 |
| $CD4^+CD103^+$ | 0.51 | 0.60 | 0.74 | 0.58 |
| $CD4^+CD103^+T_n$ | 0.38 | 0.22 | 0.30 | 0.08 |
| $CD4^+CD103^+T_{eff}$ | 0.94 | 0.91 | 0.12 | 0.58 |
| $CD4^+CD103^+T_{memory}$ | 0.06 | 0.94 | 0.25 | 0.39 |
| $CD4^+CD103^-$ | 0.51 | 0.58 | 0.65 | 0.57 |
| $CD4^+CD103^-T_n$ | 0.23 | 0.20 | 0.52 | 0.06 |
| $CD4^+CD103^-T_{eff}$ | 0.57 | 0.15 | 0.12 | 0.74 |
| $CD4^+CD103^-T_{memory}$ | 0.23 | 0.26 | 0.10 | 0.83 |
| $CD8^+$ | 0.28 | 0.11 | 0.53 | 0.58 |
| $CD8^+T_n$ | 0.81 | 0.11 | 0.53 | 0.91 |
| $CD8^+T_{eff}$ | 0.96 | 0.85 | 0.06 | 0.44 |
| $CD8^+T_{memory}$ | 0.57 | 0.71 | 0.06 | 0.74 |
| $CD8^+CD103^+$ | 0.80 | 0.50 | 0.80 | 0.22 |
| $CD8^+CD103^+T_n$ | 0.72 | 0.14 | 0.57 | 0.74 |
| $CD8^+CD103^+T_{eff}$ | 0.80 | 1.00 | 0.48 | 0.89 |
| $CD8^+CD103^+T_{memory}$ | 0.80 | 0.71 | 0.46 | 0.83 |
| $CD8^+CD103^-$ | 0.80 | 0.50 | 0.65 | 0.22 |
| $CD8^+CD103^-T_n$ | 0.44 | 1.00 | 0.22 | 0.98 |
| $CD8^+CD103^-T_{eff}$ | 0.74 | 0.41 | 0.08 | 0.51 |
| $CD8^+CD103^-T_{memory}$ | 0.28 | 0.33 | 0.08 | 0.32 |

BC, breast cancer; CC, colorectal carcinoma; SC, stomach cancer; RCC, renal cell carcinoma; $T_{eff}$, effector T cell; $T_{memory}$, memory T cell; $T_n$, naïve T cell

Supplementary Table 4. Spearman correlation test for the level of TILs and various immune cell composition (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

| Composition | rho | | | |
|---|---|---|---|---|
| | CC | SC | RCC | BC |
| $CD4^+$ | -0.14 | -0.67* | -0.16 | 0.30 |
| $CD4^+T_n$ | -0.28 | -0.27 | -0.71** | -0.54* |
| $CD4^+T_{eff}$ | 0.25 | 0.07 | -0.54* | -0.36 |
| $CD4^+T_{memory}$ | 0.26 | -0.01 | 0.57* | 0.46 |
| $CD4^+CD103^+$ | -0.31 | 0.31 | -0.09 | 0.15 |
| $CD4^+CD103^+T_n$ | -0.11 | 0.16 | -0.26 | -0.41 |
| $CD4^+CD103^+T_{eff}$ | 0.33 | -0.12 | -0.44 | -0.42 |
| $CD4^+CD103^+T_{memory}$ | 0.06 | 0.01 | 0.5* | 0.47 |
| $CD4^+CD103^-$ | 0.30 | -0.34 | -0.15 | -0.16 |
| $CD4^+CD103^-T_n$ | -0.29 | -0.32 | -0.14 | -0.43 |
| $CD4^+CD103^-T_{eff}$ | 0.31 | 0.18 | -0.05 | -0.20 |
| $CD4^+CD103^-T_{memory}$ | 0.25 | -0.06 | 0.03 | 0.43 |
| $CD8^+$ | 0.15 | 0.62* | 0.22 | -0.31 |
| $CD8^+T_n$ | -0.34 | -0.55 | -0.36 | -0.46 |
| $CD8^+T_{eff}$ | 0.34 | -0.24 | -0.46* | -0.14 |
| $CD8^+T_{memory}$ | 0.32 | 0.34 | 0.47* | 0.54* |
| $CD8^+CD103^+$ | -0.24 | 0.36 | -0.32 | 0.00 |
| $CD8^+CD103^+T_n$ | -0.28 | -0.51 | -0.26 | -0.17 |
| $CD8^+CD103^+T_{eff}$ | 0.16 | -0.36 | -0.55* | -0.39 |
| $CD8^+CD103^+T_{memory}$ | 0.18 | 0.47 | 0.5* | 0.39 |
| $CD8^+CD103^-$ | 0.21 | -0.36 | 0.22 | 0.00 |
| $CD8^+CD103^-T_n$ | -0.18 | -0.19 | -0.35 | -0.51* |
| $CD8^+CD103^-T_{eff}$ | 0.41 | -0.09 | -0.17 | -0.05 |
| $CD8^+CD103^-T_{memory}$ | 0.10 | -0.04 | 0.18 | 0.43 |

BC, breast cancer; CC, colorectal cancer; SC, stomach cancer; RCC, renal cell carcinoma; $T_{eff}$, effector T cell; $T_{memory}$, memory T cell; $T_n$, naïve T cell
*$P < 0.05$, **$P < 0.01$

## 국문요약

조직 상주 기억 T (TRM) 세포의 존재 및 임상적 중요성은 최근 다양한 암 유형과 관련하여 설명되었습니다. 그러나 TRM 세포의 빈도와 표현형 특성은 거의 알려지지 않았습니다. 본 연구는 결장 직장암 (CC, n = 18), 위암 (SC, n = 13), 신세포 암종 (RCC, n = 19) 및 유방암 (BC, n = 16)의 수술 검체를 효소를 이용하여 단세포로 분리한 후 CD103 과 CD69 이중양성 및 단일양성 세포 집단 및 메모리 T 세포 표현형을 유세포분석기를 이용하여 분석했습니다. CD103 과 CD69 를 표현하는 세포의 구성은 종양 종류에 따라 다양했습니다. CD103 또는 CD69 중 하나를 발현하는 $CD8^+$ T 세포는 CC, SC, BC 의 약 70%를 차지했고, RCC 는 더 큰 CD103 및 CD69 이중 음성 모집단과 연관되었습니다. 다른 종양 유형과 비교하여 CC 는 $CD3^+$ T 세포 중에서 $CD8^+$ T 세포의 비율이 상당히 낮았으며, $CD4^+$ 세포 중에서 CC 는 다른 종양 유형에 비해 $CD103^+$ T 세포의 비율이 상당히 높았습니다 이전 연구에서는 $CD103^+$ 세포가 $CD4^+$ T 세포보다 $CD8^+$ T 세포에 더 풍부하다고 보고되었기 때문에, $CD4^+$와 $CD8^+$ T 세포 사이의 $CD103^+$ 세포 비율을 비교한 결과 $CD4^+$ T 세포보다 $CD8^+$ T 세포에 $CD103^+$ 세포가 훨씬 더 풍부하다는 것을 확인했습니다. TRM 세포는 CCR7 발현이 낮은 것으로 알려져 있기 때문에 CD103 발현에 따라 CCR7 발현을 분석했습니다. 이전의 연구 결과와 대조적으로, $CD4^+$ SC 세포를 제외한 모든 종양 유형의 $CD103^+$ 세포에서 CCR7 발현이 더 높다는 것을 관찰했습니다. 면역 세포 집단 구성은 pT 단계 또는 종양 침윤 림프구 수준과 유의하게 연관되지 않았습니다. TRM 세포양과 표현형은 CC, SC, RCC 및 BC 간에 차이가 있었으며, 그에 대한 임상적 중요성 및 기능적 차이에 대한 추가 연구가 필요합니다.



**핵심어** : 사람암, 단세포, CD103, 조직 상주 기억 T 세포