# Increase of Peripheral Blood CD57+ T-Cells in Patients with Oral Squamous Cell Carcinoma

MASAKI IIDA[1*], EIJI TAKAYAMA[2], KOSUKE NAGANAWA[2,3*], KENJI MITSUDO[1], MAKOTO ADACHI[3], JUN-ICHI BABA[1], MASAKO FUJIMOTO-MUTO[3], MASAYUKI MOTOHASHI[3], MASAKO MIZUNO-KAMIYA[2], HARUMI KAWAKI[2], MITOMU KIOI[1], MASAO ICHINOSE[4], SHIN-ICHIRO SUMITOMO[3], YASUNORI MURAMATSU[3], MICHIO SHIKIMORI[3], IWAI TOHNAI[1] and NOBUO KONDOH[2]

[1]*Department of Oral and Maxillofacial Surgery, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa, Japan;*
[2]*Departments of Oral Biochemistry, and* [3]*Oral and Maxillofacial Surgery, School of Dentistry, Asahi University, Mizuho, Gifu, Japan;*
[4]*Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan*

**Abstract.** *Background/Aim: The subset of T-cells positive for expression of cluster of differentiation (CD) 57 has been associated with various cancer phenotypes. However, the presence of CD57+ T-cells in patients with oral squamous cell carcinoma (OSCC) has yet to be confirmed. In the present study, we examined the diagnostic significance of the presence of CD57+ T-cells in peripheral blood (PB) from patients with OSCC. Materials and Methods: The subset of CD57+ T-cells in PB was analyzed in 43 patients with OSCC by fluorescence-activated cell sorting (FACS) analysis. Results: The proportion of CD57+ T-cells, including both CD8+ and CD4+ subsets, significantly increased with clinical stage, especially in parallel with tumor size. Conclusion: Our results suggest that an increase in the population of CD57+ T-cells is a potent prognostic marker and may also influence the systemic immunity of patients with OSCC.*

Oral squamous cell carcinoma (OSCC) is the most frequent malignant tumor of the oral cavity, covering more than 80% of all oral malignancies (1). For patients with locally advanced OSCC, surgery with or without radiotherapy has been widely accepted as the standard treatment and thought to be the most curative therapy. However, the disease is still associated with a low overall survival rate of 60% (2), and for many patients, it remains difficult to predict the risk of metastasis and their prognosis.

Small but substantial subsets of peripheral blood (PB) lymphocytes positive for cluster of differentiation (CD) 57 express T-cell receptors (TCR) with both α and β chains ($CD57^+$ T-cells). The proportion of these cells in PB increases with age in healthy individuals (3). $CD57^+$ T-cells, expressing CD8, produce a large amount of interferon-gamma (IFN-γ), and have strong cytotoxic activity (3). The proportion of the $CD57^+$ T-cells in PB is increased in patients who have received a bone marrow transplant (4, 5), as well as in patients suffering from any of the following: rheumatoid arthritis (6), human immunodeficiency virus (HIV) infection (7), and malaria infection (8). Recently, $CD57^+$ T-cells expressing CD8 in PB from healthy individuals was characterized as a terminally-differentiated (Td) phenotype, by being negative for both CD27 and CD28 (9). Many previous studies have also demonstrated an expansion of Td-$CD8^+CD57^+$ T-cells in the PB of individuals infected with the cytomegalo virus (CMV) (10-12). On the other hand, CMV seropositivity is associated with a dramatic increase in the expression of so-called non-classical $CD57^+$ T-cells, expressing CD4 (13). Furthermore, CMV-specific $CD4^+$ T-cells are characterized by a $CD45RO^+$ $CD27^+$ ($CD28^-$) mature effector memory phenotype (14). Thus, phenotypically mature non-classical $CD4^+CD57^+$ T-cells exhibit functional properties that are different from those of Td-$CD8^+CD57^+$ T-cells and conventional $CD4^+$ ($CD57^-$) T-cells.

The clinical course of various types of cancer is influenced by host immune responses in which tumor-infiltrating immunocytes and PB cells play important roles. These

*These Authors contributed equally to the study.

*Correspondence to:* Professor Nobuo Kondoh, Department of Oral Biochemistry, School of Dentistry, Asahi University, 1851 Hozumi, Mizuho-shi, Gifu 501-0296, Japan. Tel: +81 583291416, Fax: +81 583291417, e-mail: nkondoh@dent.asahi-u.ac.jp

*Key Words:* Oral squamous cell carcinoma, CD57, T-cells, peripheral blood.

Table I. *Clinical parameters of the enrolled patients with oral squamous cell carcinoma.*

| | Stage | | | | | |
|---|---|---|---|---|---|---|
| | Total | I | II | III | IV | *p*-Value |
| No. of patients | 43 | 4 | 7 | 13 | 19 | |
| Gender | | | | | | |
| Male | 32 | 3 | 4 | 11 | 14 | 0.6120 |
| Female | 11 | 1 | 3 | 2 | 5 | |
| Age (years) | 65.9±8.13 | 69.2±2.87 | 63.8±10.2 | 68.9±7.53 | 63.9±8.08 | 0.2418 |
| Location | | | | | | |
| Tongue | 22 | 4 | 5 | 8 | 5 | 0.5566 |
| Lower gingiva | 9 | 0 | 1 | 2 | 6 | |
| Upper gingiva | 5 | 0 | 1 | 2 | 2 | |
| Oral floor | 4 | 0 | 0 | 1 | 3 | |
| Buccal mucosa | 3 | 0 | 0 | 0 | 3 | |
| Palate | 0 | 0 | 0 | 0 | 0 | |
| Tumor size | | | | | | |
| T1 | 4 | 4 | 0 | 0 | 0 | 0.0001 |
| T2 | 9 | 0 | 7 | 1 | 1 | |
| T3 | 14 | 0 | 0 | 12 | 2 | |
| T4 | 16 | 0 | 0 | 0 | 16 | |
| Distant metastasis | | | | | | |
| Negative | 22 | 4 | 5 | 8 | 5 | 1.0000 |
| Positive | 0 | 0 | 0 | 0 | 0 | |
| Lymph node metastases | | | | | | |
| N0 | 29 | 4 | 7 | 10 | 8 | 0.0023 |
| N1 | 4 | 0 | 0 | 3 | 1 | |
| N2 | 10 | 0 | 0 | 0 | 10 | |

The clinical staging and classification were based on the Union Internationale Contra le Cancer staging system as described in (22).

immune cells also correlate with tumor progression and prognosis in various types of cancer. Histochemical and multicolor fluorescence-activated cell sorting (FACS) analyses of local tumor infiltration and circulation of $CD57^+$ lymphocytes in patients with different types of cancers have indicated that the number of $CD57^+$ lymphocytes is closely correlated with both tumor progression and prognosis of these patients (15-21). However, it is still unknown whether locally tumor-infiltrating and systemically-circulating $CD57^+$ lymphocytes are correlated with the progression and prognosis of OSCC.

In the present study, we examined the diagnostic significance of $CD57^+$ T-cells in PB from patients with OSCC using FACS analysis and compared the data with clinical states.

## Materials and Methods

*Patients with OSCC*. Forty-three patients with OSCC treated at Yokohama City University Hospital between April 2010 and March 2013 were enrolled in the present study [32 men and, 11 women; mean standard deviation (SD) age=65.9 (±8.13) years, range=51-80 years] (Table I). The primary lesion was confirmed by biopsy specimen, and cervical lymph nodes and distant metastases were assessed by positron emission tomography-CT, contrast enhanced CT, magnetic resonance imaging and ultrasonography. Clinical staging was based on the 2009 Union Internationale Contra le Cancer staging system (22). The Ethics Committee of Yokohama City University Hospital approved the study protocols (B10010728), and informed consent was obtained from all patients prior to initiation of the study. The Ethics Committee of Asahi University School of Dentistry approved the study protocols (20071).

*Flow cytometric analysis of PB*. We previously described the method for FACS analysis using PB (19). A 0.05-ml aliquot of PB was incubated with the following monoclonal antibodies (mAbs) for 30 min on ice: fluorescein isothiocyanate (FITC)-conjugated mAb specific for CD57 (NC1), phycoerythrin (PE)-conjugated mAb specific for the β chain of TCR (TCRβ) (BMA031), and allophycocyanin (APC)-conjugated mAb specific for CD4 (13B8.2) (all Beckman Coulter Immunotech, Marseille, Bouches-du-Rhône, France); peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated mAbs specific for CD8 (SK1), CD4 (SK3), and CD25 (M-A251) (BD Biosciences, San Jose, California, USA); APC-conjugated mAb specific for FOXP3 (236A/E7) (eBioscience, San Diego, California, USA). Red blood cells were lysed using a FACS Lysing Solution (BD Biosciences), and the peripheral blood mononuclear cells (PBMCs) were then fixed and permeabilized by using FACS Permeabillizing Solution

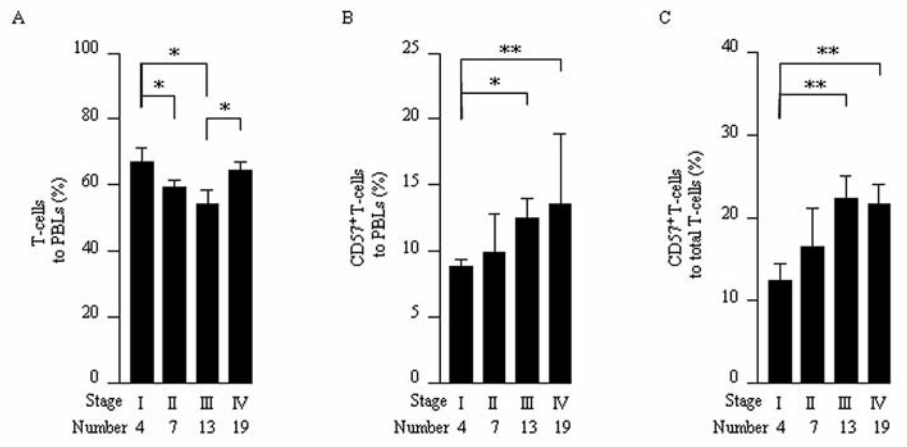


Figure 1. *Analysis of cluster of differentiation (CD) 57+ T-cells in peripheral blood (PB) from patients with oral squamous cell carcinoma by tumor stage. The proportion of total T-cells to PB lymphocytes (PBLs) (A), CD57+ T-cells to PBLs (B), and of CD57+ T-cells to total T-cells (C) are indicated. Data are mean±SD.*p<0.05, **p<0.01.*

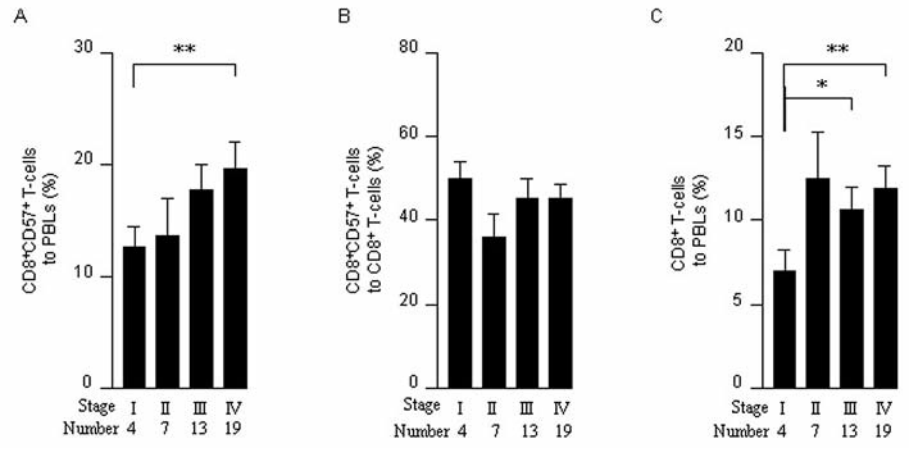


Figure 2. *Analysis of CD57+ T-cell subsets of CD8+ T-cells from patients by tumor stage. The proportion of CD8+CD57+ T-cells to PBLs (A), to CD8+ T-cells (B), and of CD8+ T-cells to total PBLs (C) are indicated. Data are mean±SD.*p<0.05, **p<0.01.*

2 (BD Biosciences). The PBMCs were resuspended in phosphate-buffered saline containing 2% fetal calf serum, 1 mM disodium ethylenediaminetetra-acetic acid and 0.1% sodium azide and then analyzed by FACSCalibur (BD Bioscience) with Cell Quest software (BD Biosciences). The proportions of different subsets of T-cells were analyzed with the Flowjo software (Tree Star, Ashland, Oregon, USA).

*Statistics*. All analysis was performed using Stat Mate version 4.0 software (Atms, Bunkyo, Tokyo, Japan). Data are expressed as means with the SD and standard error (SE). Welch's *t*-test was applied to determine the significance of differences between two groups. One-way ANOVA was applied to determine the significance of differences among groups overall. *p*-Values less than 0.05 were considered to be statistically significant.

## Results

*Clinicopathological characteristics of patients with OSCC*. Clinical parameters of the 43 enrolled patients with OSCC classified by clinical stage are shown in Table I. The sampling data showed that no significant differences in the gender, age and the primary tumor sites were found; however, the tumor sizes and the presence of lymph node metastases differed significantly among the clinical stages.

*CD57+ T-cells in PB from patients with OSCC*. Whole PB lymphocytes (PBLs) from patients with OSCC were stained with mAbs against TCRβ, CD57, CD8 and CD4. Subsets of

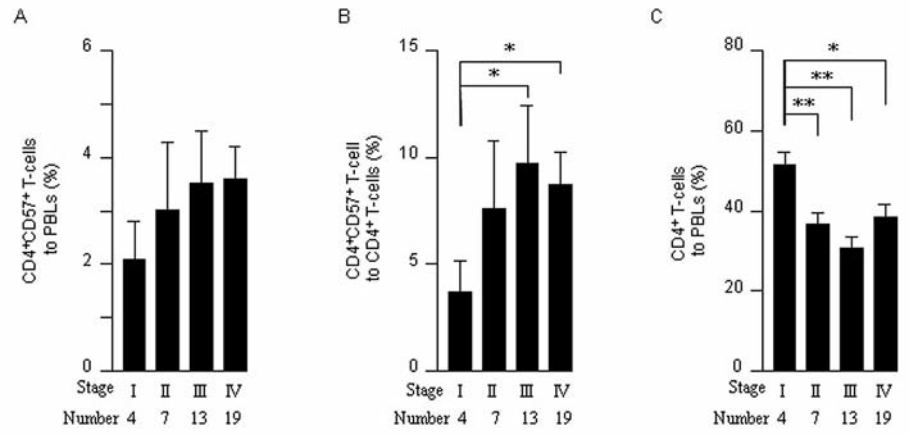


Figure 3. *Analysis of CD57+ T-cell subsets of CD4+ T-cells from patients by tumor stage. The proportion of CD4+CD57+ T-cells to PBLs (A), to CD4+ T-cells (B), and of CD4+ T-cells to total PBLs (C) are indicated. Data are mean±SD.*p<0.05, **p<0.01.*

T-cells expressing TCRβ were determined. Although the proportion of T-cells to total PBLs from patients with OSCC fluctuated with clinical stage (Figure 1A), the ratios of CD57+ T-cells to total T-cells and to total PBLs significantly increased with increasing clinical stage of OSCC (Figure 1B and C).

*CD8+CD57+ and CD4+CD57+ T-cells in PB from patients with OSCC*. The CD8+CD57+ T-cell subset as a proportion of total PBLs was significantly increased in higher clinical stages (stages III and IV) of OSCC than in lower stages (stages I and II) (Figure 2A and C), in accordance with the expansion of the total CD8+ T-cell subset. The ratio of CD8+CD57+ T-cells to total CD8+ T-cells was unchanged among stages (Figure 2B).

On the other hand, the ratio of CD4+CD57+: CD4+ T-cells was significantly increased in clinical stages III and IV (Figure 3B). However, the ratio of CD4+CD57+ T-cells to total PBLs showed no significant change among stages (Figure 3A). This may be attributable to the decrement of CD4+ T-cells as a proportion of total PBLs from stages II-IV (Figure 3C).

*Correlation of the CD57+ T-cell subsets and tumor size*. Clinical stages for OSCC are generally defined by the tumor size and status regarding the presence of lymph node metastases. Therefore, we classified the size of the tumors according to the TNM classification (Table II), and evaluated the population of CD57+ cells among them. As shown in Figure 4, the proportions of CD57+ and CD8+CD57+ T-cell subsets/total PBLs were significantly increased in the patients harboring larger tumors classified as T3 and T4 compared to T1. Furthermore, the CD4+CD57+ T-cell subset of CD4+ T-cells was somewhat increased in patients with T4 tumors (Figure 4C). We also evaluated the populations of CD57+ T-cells among patients with different lymph node status, however, no particular correlation was observed (data not shown).

Table II. *Clinical parameters of enrolled patients with oral squamous cell carcinoma.*

| | Tumor size | | | | |
|---|---|---|---|---|---|
| | T1 | T2 | T3 | T4 | *p*-Value |
| No. of patients | 4 | 9 | 14 | 16 | |
| Gender | | | | | |
| Male | 3 | 6 | 12 | 11 | 0.6838 |
| Female | 1 | 3 | 2 | 5 | |
| Age (years) | 69.2±2.87 | 65.6±9.47 | 66.9±7.83 | 64.4±8.52 | 0.2597 |
| Location | | | | | |
| Tongue | 4 | 7 | 7 | 4 | 0.6556 |
| Lower gingiva | 0 | 1 | 3 | 5 | |
| Upper gingiva | 0 | 1 | 2 | 2 | |
| Oral floor | 0 | 0 | 1 | 3 | |
| Buccal mucosa | 0 | 0 | 1 | 2 | |
| Palate | 0 | 0 | 0 | 0 | |
| Stage | | | | | |
| I | 4 | 0 | 0 | 0 | 0.0001 |
| II | 0 | 7 | 0 | 0 | |
| III | 0 | 1 | 12 | 0 | |
| IV | 0 | 1 | 2 | 16 | |
| Lymph node metastases | | | | | |
| N0 | 4 | 7 | 10 | 8 | 0.2906 |
| N1 | 0 | 1 | 2 | 1 | |
| N2 | 0 | 1 | 2 | 7 | |

## Discussion

In the present study, we demonstrated that increases in the proportions of CD57+ T-cells, including both CD8+ and CD4+ T-cell subsets, in PB of 43 patients with OSCC were significantly correlated with clinical stage, especially in parallel with tumor size. The extent of tumor-infiltrating CD57+ cells

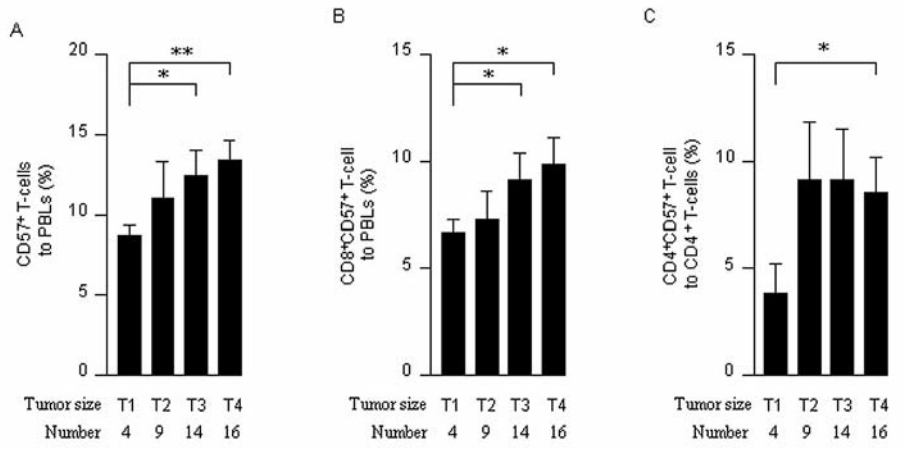


Figure 4. *Analysis of CD57+ T-cell subsets in patients by tumor sizes according to TNM classification. The proportion of CD57+ T-cells (A), CD8+CD57+ T-cells (B) to PBLs, and CD4+CD57+ T-cells to CD4+ T-cells (C) are indicated. Data are mean±SD.*p<0.05, **p<0.01.*

has been reported in various tumor types, including, lung cancer (16), melanoma (9), and gastric cancer (15, 17, 18).

The ratio of $CD8^+CD57^+$ T-cells to total PBLs from patients with OSCC significantly increased with the progression of clinical stage. The increase of $CD57^+$ T-cells has been correlated with the production of antitumor cytokine, IFN-γ from PB (3). A previous study has indicated CD8+CD57+ T-cells are also increased in PB from patients with early gastric cancer, expressing higher levels of perforin and granzyme B which exert cytotoxicity (17). Likewise, $CD8^+CD57^+$ T-cells infiltrating into metastatic melanoma tissues also expressed granzyme B and a high level of IFN-γ (9). It has been considered that the $CD8^+CD57^+$ T-cells are terminally-differentiated cytotoxic T-lymphocytes, and are increased in PB from patients with tumor (9). Therefore, increased numbers of $CD8^+CD57^+$ T-cells in patients with OSCC include the Td-cytotoxic T-lymphocytes that could affect the systemic immunity of patients. These reports, together with our results, demonstrate that the evaluation of the $CD57^+$ T-cell subsets could provide effective indices for the immunological status and prognosis of malignancies.

Although the population of $CD4^+$ T-cells was reduced, the ratio of the $CD4^+CD57^+$ T-cells to total $CD4^+$ T-cells was increased in patients with OSCC, in parallel with the progression of clinical stages. A number of reports have indicated expansions in the $CD4^+CD57^+$ T-cell subset in PB from patients suffering from colorectal cancer (23), gastric cancer (17, 18) and hepatitis C virus-related hepatocellular carcinoma (19). Based on our results, the $CD4^+CD57^+$ T-cells may act as effector cells for OSCC progression. In addition, there have been reports of an increase in regulatory T-cells in some malignancies (24, 25), suggesting the possibility that the $CD4^+CD57^+$ T-cells share a function as regulators in OSCC. However, the $CD4^+CD57^+$ T-cells in PB did not express either of the regulatory T-cell markers (data not shown) CD25 (19) or cytoplasmic FOXP3 (26).

In order to elucidate the mechanisms of generation and functional significance of the $CD57^+$ T-cell subsets in patients with OSCC, further intensive studies will be required. However, our results not only suggest that the increase in the population of $CD57^+$ T-cells has prognostic value, but may also have an influence on the systemic immunity of patients with OSCC.

## Conflicts of Interest

The Authors declare that there are no conflicts of interest.

## Acknowledgements

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