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学 位 申 請 論 文

Induction and analysis of cytotoxic T lymphocytes that recognize autologous oral squamous cell carcinoma

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学位申請論文

Induction and analysis of cytotoxic T lymphocytes that recognize autologous oral squamous cell carcinoma

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Abbreviations: APCs, antigen-presenting cells; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; IFN, interferon; mAb, monoclonal antibody; MLTC, mixed lymphocyte tumor cell; NAP, natural antigen peptide; OSCC, oral squamous cell carcinoma; PBMC, peripheral blood mononuclear cell; SCCHN, squamous cell carcinoma of the head and neck; SCID, severe combined immunodeficiency; TAA, tumor-associated antigen

ABSTRACT

For tumor immunity, it is necessary that specific cytotoxic T lymphocytes (CTLs) recognize tumor-specific antigens and exhibit antitumor effects. The purpose of this research was to establish and analyze a novel autologous tumor-CTL pair. Primary culture of biopsy tissue and peripheral blood mononuclear cells from the same patient was performed, and we were successful in establishing an autologous tumor-CTL pair. Co-culture of the tumor cells and the CTLs (TcOTM) was performed to determine the cytotoxic activity of the CTL. Further, we also verified that the CTL clone recognized the HLA-A24-restricted peptide to exert its cytotoxicity. The CTL clone showed an anti-tumor effect against autologous cancers by recognizing the HLA-A24-restricted peptide and exerted cytotoxicity against the allogenic HLA-A24 cell line. Since the natural antigen peptide (NAP) obtained from the analysis of the autologous tumor-CTL pair was actually expressed on the cancer cell surface, the NAP that is recognized by TcOTM clone may be recommended for immediate clinical application. The

results from this study indicated that the TcOTM clone recognized the tumor antigenic peptide presented by HLA-A24 cells. The findings from the present study can help identify unknown NAPs for oral cancer in the future.

Keywords: oral cancer, CTL, tumor antigen, autologous tumor-CTL pair

1. Introduction

According to the database of the WHO, oral cancer is the 8th most common cancer in men and the 10th most common one in women worldwide. In the overall population, oral cancer ranks 10th among the most common cancers, and its incidence is continuously increasing [1]. Currently, it is possible to achieve high rates of positive outcome by surgery or radiotherapy without functional complications and esthetic problems in early stage oral cancers [2]. However, in locally advanced or recurrent oral cancers, although the combination of surgery, radiotherapy, and chemotherapy has been found to be more effective than single modality therapy, this combination treatment has failed to produce remarkable

increases in overall survival or recurrence-free survival rates because of loco-regional recurrence and distant metastasis [2]. Therefore, advances in new therapeutic modalities for patients with locally advanced or recurrent oral cancers are urgently needed. As a new treatment method, cetuximab is currently the only monoclonal antibody approved for management of squamous cell carcinoma of the head and neck (SCCHN), but other strategies such as immune checkpoint openers are attracting a great deal of attention [3, 4]. In particular, immune checkpoint openers offer promising outcomes for the treatment of SCCHN, although their clinical responses may be restrictive [5].

Indeed, since the discovery of the first human tumor-associated antigen (TAA) in 1991, many clinical trials for cancer vaccines have shown promise in pilot clinical studies [6-10]. In SCCHN, single or multi-peptide vaccines have also been shown to improve the prognosis of patients with advanced stages of disease [11, 12]. Previously, our group identified an HLA-A24–restricted antigenic peptide, survivin-2B80-88 (AYACNTSTL), which is recognized by $CD8^+$ cytotoxic T

lymphocytes (CTLs) and functions as an immunogenic molecule in patients with cancers of various histological origins and immunological responses [11, 13-16]. In another previous study, we also established a novel autologous tumor-CTL pair of oral squamous cell carcinoma (OSCC), which recognized the tumor cells in an HLA-A24–restricted manner [17]. However, little is known so far about TAAs recognized by autologous CTLs in OSCC, and very few attempts have been made at identifying these yet-unknown TAAs because the establishment of such autologous pairs of tumor cell lines and CTLs requires great effort. Therefore, in this study, we aimed to establish and analyze an autologous tumor-CTL pair of OSCC that was capable of identifying a novel HLA-A24–restricted TAA in oral cancer.

Recently, cancer stem-like cells (CSCs)/ tumor-initiating cells (TICs) that are essential for tumor maintenance, recurrence, and distant metastasis has been extensively studied [18-21]. The results of the establishment of an autologous tumor-CTL pair, which is the aim of our present study, will contribute to

improvements in CSC/TIC-targeting immunotherapy due to the identification of a natural antigen peptide (NAP) from the tumor cell lysate that can be recognized by autologous CTLs. Such improvements in CSC/TIC-targeting immunotherapy for oral cancer will lead to the development of more effective peptide vaccine therapies.

2. Materials and methods

2.1. Cell lines and primary culture

The OSCC cell line, OTM, was established from biopsy specimens resected from a 64-year-old female patient with moderately differentiated OSCC and was cultured in Dulbecco's Modified Eagle's Medium / Ham's F12 (DMEM/F12) medium (Life Technologies, Carlsbad, CA). The HLA genotype of the OTM cells was HLA-A*2402/2603, B*4001/5101, C*1402/1502. OSCC cell lines OSC19 (HLA-A2) and OSC70 (HLA-A24) [22] were also established in our laboratory. HSC-2 (HLA-A24) OSCC cell lines were purchased from the Human Science

Research Resources Bank (HSRRB, Osaka, Japan). Erythroleukemia cell line K562 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HSC-2, OSC19, OSC70, and K562 cell lines were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA). All the media were supplemented with 10% fetal bovine serum (FBS; Invitrogen, Palo Alto, CA) and 1% penicillin, and all the cultures were carried out in a 5% CO_2 incubator at 37°C.

2.2. Flow cytometric analysis and monoclonal antibodies

The cells were incubated with murine monoclonal antibodies (mAbs) at saturating concentration for 30 min on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with FITC for 30 min. The samples were then analyzed using a BD LSR II flow cytometer with FACS Calibur (Becton Dickinson, Mountain View, Biosciences). Anti-HLA-ABC (W6/32; AbD serotec), anti-HLA-DR (L243; ATCC, Manassas, VA, USA), and anti-HLA-A24 (C7709A2; a gift from Dr. P. G. Coulie; Ludwig Institute for Cancer Research, Brussels,

Belgium) mAbs were used. To increase the protein expression of HLA, the cell lines were treated with 100 U/ml interferon (IFN)–γ for 48–72 h.

2.3. CTL induction and establishment of CTL clones

Autologous PBMCs from the OTM donor patient were subjected to density gradient centrifugation with Lymphoprep (Axis Shied, Oslo, Norway) and frozen at -80°C for storage.

Mixed lymphocyte tumor cell (MLTC) culture was performed as described previously [23]. Briefly, CD8+ T cells were positively separated from the OSCC patient's PBMCs using immunomagnetic beads coupled with an anti-CD8 mAb (Miltenyi Biotech GmbH, Germany) following the manufacturer's protocol. Then, 2×10^6 CD8+ T cells were co-cultured in a 24-well plate with 2×10^5 irradiated OTM cells as antigen-presenting cells (APCs) in 2 ml of AIM-V medium supplemented with 10% human serum. Three days later, human recombinant interleukin-2 (IL-2; Takeda Pharmaceutical Co., Osaka, Japan) was added at a final concentration of 20 IU/ml. The same MLTC culture procedure was

performed every 7 days. After stimulation four times, the cytotoxic activity and IFN-γ secretion were measured using the LDH release cytotoxicity assay and ELISpot assay, respectively.

To obtain the CTL clones, standard limiting dilution was performed as described previously [24, 25]. The series of diluted CTLs (1×10^4) were then incubated with 100 Gy-irradiated 5×10^4 allogeneic PBMCs as feeder cells in 200 μl of AIM-V medium supplemented with 10% human serum and 100 U/ml rIL-2 in 96-well round plates. The allogenic PBMCs as feeder cells were isolated from three healthy volunteer donors using Lymphoprep Isolation of $CD8^+$ cells, and establishment of phytohemagglutinin (PHA; 5 l g/ml; Wako Chemicals, Osaka, Japan) blast was performed as described. $CD8^+$ T cells were stimulated with a peptide-pulsed PHA blast once in 2 weeks. On day 7, half of the culture medium was replaced with AIM-V medium supplemented with 10% human serum and 100 U/ml rIL-2. Growing wells could be observed on days 14–20. The cells were

then transferred to 24-well culture plates, and the specific cytotoxic activity was examined using the LDH release cytotoxicity assay.

For the first limiting dilution, CTLs were distributed among 45 wells in 24-well plates, and for the second dilution, CTLs were distributed among 31 wells. Theoretically, co-culturing CTLs and OTM cells that have become a single clone should help verify the cytotoxic activity of the CTLs using the ELISpot assay. Accordingly, on day 14, we assessed the reactivity of the $CD8^+$ T cells using interferon (IFN)-γ enzyme linked ELISpot assay. The cytotoxic activity of the T cells was assessed using the LDH release cytotoxicity assay.

2.4. Interferon-γ ELISpot Assay

CTL induction was performed as described previously. All the target cells were treated with 100 U/ml IFN-γ for 48–72 h before performing the ELISpot assay as described. For the detection of IFN-γ in the culture supernatant, standard IFN-γ ELISA (R&D Systems, Minneapolis, MN) was performed following the manufacturer's instructions. For specific inhibition with antibodies,

anti-HLA-class I mAb (W6/32), anti-HLA-A24 mAb (C7709A2), and anti-HLA-DR mAb (L243) were added at half the volume of the hybridoma culture supernatant.

2.5. LDH release cytotoxicity assay

The antigen-specific lytic activity of the CTL clones was evaluated using an LDH Cytotoxicity Detection Kit (Takara Bio, Otsu, Japan) following the manufacturer's protocol. Target cells (10×10^3 cells/well) were incubated with various numbers of effector cells for 7 h at 37°C in 96-well V-bottomed plates, and the cytotoxicity was calculated using the culture supernatants. The percentage of specific lysis was calculated as [(experimental release – spontaneous release) / (maximum release – spontaneous release)] × 100. Cytotoxicity blocking assays were performed using mAbs. Tumor targets were incubated with the hybridoma culture supernatants of anti-HLA-class I mAb (W6/32), anti-HLA-A24 mAb (C7709A2), anti-HLA-A1, A11, A26 mAbs (8.L.101), and anti-HLA-DR mAb (L243) for 1 h at room temperature.

The experiments conducted in the present study were approved under the

institutional guidelines for the use of human subjects in research. The patients and their families, as well as the healthy donors, provided informed consent for the use of blood samples and tissue specimens in our research.

3. Results

3.1. Establishment of the OSCC cell line, OTM

A new OSCC cell line was established in order to identify TAAs. As shown in Fig. 1A, a biopsy specimen of carcinoma of the right buccal mucosa from a 64-year-old woman patient was cultured in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin. We then successfully established the OSCC cell line, OTM. The primary lesion of the carcinoma specimen was a moderately differentiated squamous cell carcinoma (Fig. 1B, C) and revealed a 4C mode of invasion, which was diffuse invasion of cord-like type, according to the Y-K classification. [26]. Papanicolaou staining showed an increase in the nuclear/cytoplasmic ratio and aggressive nuclear fission (Fig. 2A). Additional

immunohistochemical studies revealed atypical cells as positive for AE1/AE3 (pancytokeratin) (Fig. 2B). The cell line had a standard doubling time of 48 h and grew stably through more than 20 passages (Fig. 3). Moreover, this cell line exhibited tumor-forming ability in severe combined immunodeficiency (SCID) mice, and the tumors were typical squamous cell carcinomas resembling the primary lesion (Fig. 4). For immunological evaluation of the OTM cell line, the expression of surface MHC molecules was then examined by flow cytometric analysis. As shown in Fig. 5, the OTM cells expressed a pan-HLA class I molecule (W6/ 32) and HLA-A24 (C7709A2), but a negative level of HLA-A2 (BB7.2) and HLA-DR (L243) was observed. These HLA expression profiles suggested that immune cells could interact with OTM cells through HLA-class I molecules.

3.2. Induction and analysis of CTLs and establishment of TcOTM clones

To establish CTLs that recognized the OTM cells, we stimulated PBMCs with autologous OTM cells several times. The specific reactivity was then evaluated

using the Interferon-γ ELISpot assay (Fig. 6). The CTL line (TcOTM) recognized autologous OTM cells specifically, but not cells of the erythroleukemia cell line K562, which were used as a negative control. For detailed analysis of the TcOTM clones, they were generated using the standard limiting dilution method, and we successfully established a CTL clone, designated as CTL3. As shown in Fig. 7, CTL3 recognized the autologous OTM cells specifically, but did not recognize K562 cells, which were used as a negative control. Furthermore, the specific reactivity was evaluated using the LDH release cytotoxicity assay (Fig. 8). CTL3 exerted a cytotoxic potential on OTM cells in a dose-dependent manner. Next, we performed limiting dilution again for the establishment of a single TcOTM clone and identified the most positive spot in the group of 3–1 for CTL3 (Fig. 9).

Next, to identify the HLA restriction of elements, blocking assays using several mAbs were performed against CTL3-1. As shown in Fig. 10, the cytotoxic activity of clone CTL3-1 was blocked by the use of anti-HLA-class I mAb (W6/32)

and HLA-A24 mAb (C7709A2), but not by anti-HLA-A26 mAb (ab33883) or anti HLA-DR mAb (L243), which indicated that clone CTL3-1 was an HLA-A24-restricted CTL. For further analysis of CTL3-1, we measured its cytotoxic activity against allogenic OSCC cell lines using the LDH release cytotoxicity assay (Fig. 11). CTL3-1 recognized allogeneic HLA-A24–positive OSCC cells such as HSC-2 and OSC-70 as well as the autologous OTM cells, but did not recognize OSC-19 (HLA-A2) cells. These data indicate that CTL3-1 recognized a shared antigen in an HLA-A24–restricted manner.

4. Discussion

OSCC is one of the most common human cancers worldwide. The establishment of cell lines from human cancers is important for investigating the biology of cancer cells at the cellular level. However, human OSCCs have been generally difficult to establish in culture as cell lines [22]. Three major factors have been suggested to be responsible for the failure of establishment of such cell

lines: fungal or bacterial contamination, overgrowth of fibroblasts, and inadequate nutrients in the culture medium [27]. Previously, we have also made many efforts to establish cell lines from human OSCCs [15, 22, 28]. In this study, as well, using approximately 20 biopsy or surgically resected specimens of different OSCCs, we succeeded in establishing only one cell line (OTM). Furthermore, we successfully established the CTL line TcOTM, which reacts with autologous OTM cells established from the PBMCs of one patient by using MLTC. We also found that the TcOTM clone recognized the allogenic OSCC cell lines OSC-70 and HSC-2 as well as the autologous OTM cell line. We also found that the OTM, OSC-70, and HSC-2 cells shared a TAA in a HLA-A24–restricted manner, as the cytotoxic activity of the TcOTM clone was clearly inhibited with the use of the anti-HLA-A24 mAb. Although the HLA-A24–restricted TAA that was recognized by the TcOTM clone could not be identified, it showed potential for further development toward clinical applications. With the recent innovative advances in cancer immunotherapy that started since the identification of the

MAGE gene family and other melanoma antigens [7, 10], immunotherapies have been considered one of the promising therapies for many malignancies. Using melanoma antigens, several groups have reported the efficacy of cancer immunotherapy for advanced melanoma patients [8, 29].

There are several approaches for identifying TAAs. Previously, we established an autologous cancer cell line and CTL pairs from several epithelial malignancies [30-32] and succeeded in isolating novel cancer-associated antigens [33, 34] as well as antigenic peptides by reverse immunogenetic analysis [13, 25, 35, 36]. With this progress, we started a clinical trial for cancer vaccine therapy using an antigenic peptide derived from one of the cancer-associated antigens, survivin [11, 37]. Some patients showed decreases in tumor marker expression and remarkable tumor regression. These findings suggested that cancer vaccine therapy might also be effective for OSCC patients.

We have found novel tumor antigens using various methods such as gene expression cloning, bioinformatics, reverse immunology, transcriptome analysis,

and peptidome analysis. Peptidome analysis enabled the biochemical analysis of NAP in living cells using the autologous tumor-CTL pair [33, 38, 39]. In the field of tumor immunology, the investigations for OSCC therapy are few as compared to those on other origin malignancies. Our data from the present study revealed that a clone of CTLs, TcOTM, that specifically lysed OTM cells in an HLA-A24–restricted fashion in oral cancer.

Recent clinical trials of multiple peptide vaccinations for HLA-A24–positive advanced head and neck cancer patients have revealed induction of immune responses and improved overall survival [12]. Therefore, it is necessary to accumulate a large number of available peptides with regard to HLA typing. HLA-A24 is the most common HLA class I allele in the Japanese population, and 60% of Japanese individuals (95% of whom have an A*24:02 genotype), 20% of Caucasians, and 12% of Africans have been found to be positive for HLA-A24 [40]. Therefore, in the future, we need to identify new TAAs that are mainly HLA-A24–restricted or other HLA class I allele–restricted. Our autologous tumor-CTL pair

model established in the present study would be very useful in the identification of a NAP and may contribute to the development of more effective cancer immunotherapies in the future.

5. Conclusion

We successfully established a novel OSCC cell line and induced a specific CTL clone from autologous PBMCs of an OSCC patient. The CTL clone exhibited tumor-specific cytotoxic activity against allogenic HLA-A24–positive oral cancer cell lines as well as the autologous OSCC cell line and recognized shared OSCC antigens. Further, precise analysis of TcOTM–recognized antigens will provide important baseline information on an as-yet-unknown NAP in OSCC.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.

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Figure Legends

Fig. 1. A: A 64-year-old woman's right buccal mucosa cancer, T2N1M0 (Stage Ⅲ) Tumor size: 24 × 22 mm. B, C: Result of the biopsy and histopathological images revealed squamous cell carcinoma. Moderately differentiated squamous cell carcinoma, Mode of invasion was YK-4C.

Fig. 2. A: Papanicolaou staining showed an increase in the nuclear/cytoplasmic ratio and aggressive nuclear fission. B: Additional immunohistochemical studies revealed atypical cells as positive for AE1/AE3 (pancytokeratin).

Fig. 3. Image of the culture of a portion of the biopsy tissue, as observed under a phase contrast microscope. A: After culture for 3, 5, and 7 days. B: Repeated passages, taking stock of 20 generations each day; the cells showed stable proliferation.

Fig. 4. The novel OSCC cell line, OTM, exhibited tumor-forming ability in severe combined immunodeficiency mice, and the tumors were typical squamous cell carcinomas resembling the primary lesion. A: Subcutaneous injection of cultured cancer cells on the back of an SCID mouse (severe combined immunodeficiency mouse) strain (1.0×10^4)/ml to 1 ml. After 30 days of culture, tumor formation was observed on the back of the mouse. B: Grown tumor tissue ablation, and the image of histopathologically squamous cell carcinoma.

Fig. 5. FACScan analysis of the surface HLA molecules. OTM cell line was evaluated by FACscan analysis. HLA-class I mAb (W6/32), HLA-A24 mAb (C7709A2), HLA-A2 mAb (BB7.2), and HLA-DR mAb (L243) were used for specific detection. The OTM cell surface was observed for expression of HLA Class I and HLA-A24 molecules.

Fig. 6. Cytotoxic activity of the TcOTM CTL line. Blood collected from the same

patient was used to establish OTM cells. Centrifugation was used to separate the $CD8^+$ lymphocytes using microbeads. To co-culture, TcOTM was separated from the OTM $CD8^+$ lymphocytes, and the cytotoxic activity was measured using the ELISpot Assay. High specific cytotoxic activity towards OTM cells can be clearly observed.

Fig. 7. Limiting dilution of bulk TcOTM. Limiting dilution was used, but there was a different reaction in each well. The most positive spot was found for CTL3.

Fig. 8. Cytotoxic activities of the CTL clone3. In ET ratio 10 and ET ratio of 30, tumor-specific lysis against OTM cells was significantly higher than that against the K562 cells.

Fig. 9. Limiting dilution of CTL clone3. CTL3 was further subjected to limiting dilution to isolate a single clone. CTL3-1 showed the highest reaction and was

therefore selected for use in the subsequent experiments.

Fig. 10. Blocking assays with mAbs were performed with CTL3-1 to identify the HLA restriction of elements by using the LDH release cytotoxicity assay. The use of W6/32 and C7709A2 antibodies decreased the cytotoxic activity of CTL3-1. The data indicated that CTL3-1 exerted its cytotoxicity by recognizing peptides bound to HLA-A24 of the OTM cells.

Fig. 11. Cytotoxicity of CTL3-1 against autologous and allogenic OSCC cell lines with various E/T ratios. TcOTM CTL cells were examined for their cytotoxic activity against the OTM cells using the LDH release cytotoxicity assay. Cytotoxic activity against the allogeneic OSCC OTM cells was examined. Allogeneic OSCC cell lines HSC-2 (HLA-24), OSC-70 (HLA-A24), and OSC-19 (HLA-A2) were used as target cells. CTL3-1 recognized allogeneic HLA-A24–positive OSCC cells. These data indicate that CTL3-1 recognized a shared antigen in an HLA-A24–

restricted manner.

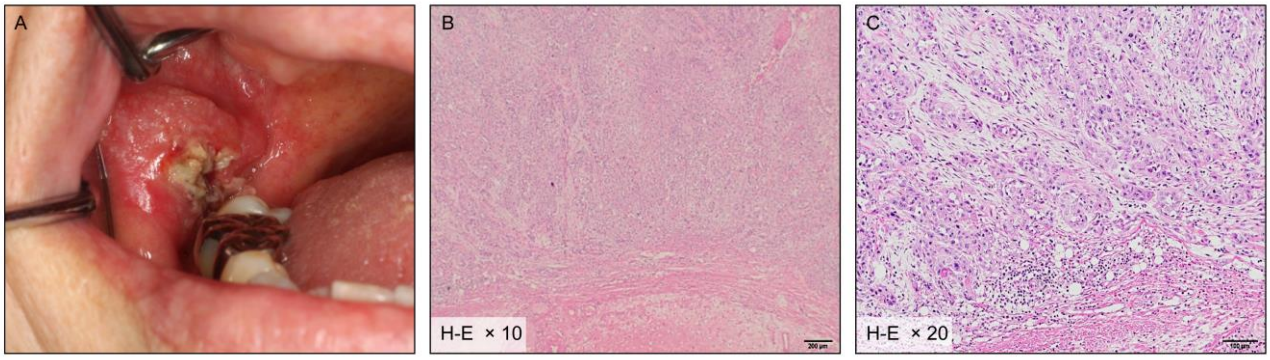

Figure.1.

A: Photo of the buccal mucosa tumor.

B.C: Result of the biopsy and histopathological images.

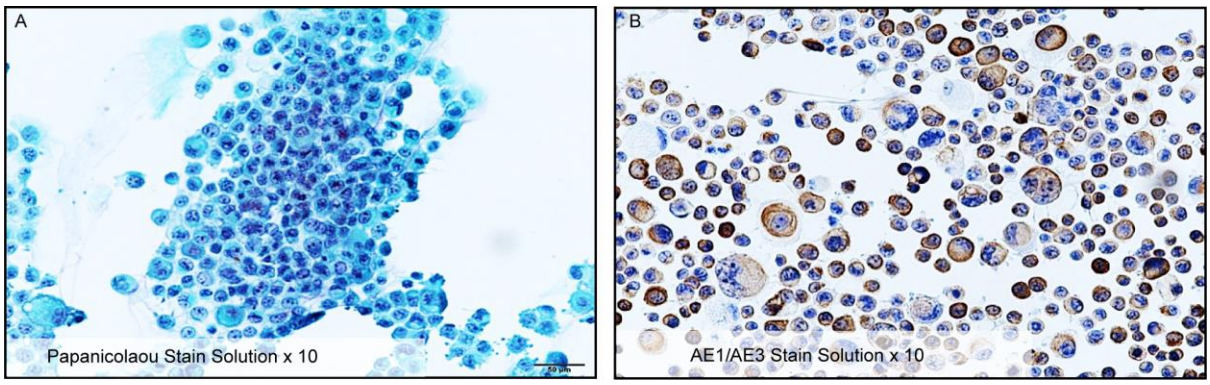

Figure.2.

A: Papanicolaou staining

B: AE1/AE3 (pancytokeratin) staining.

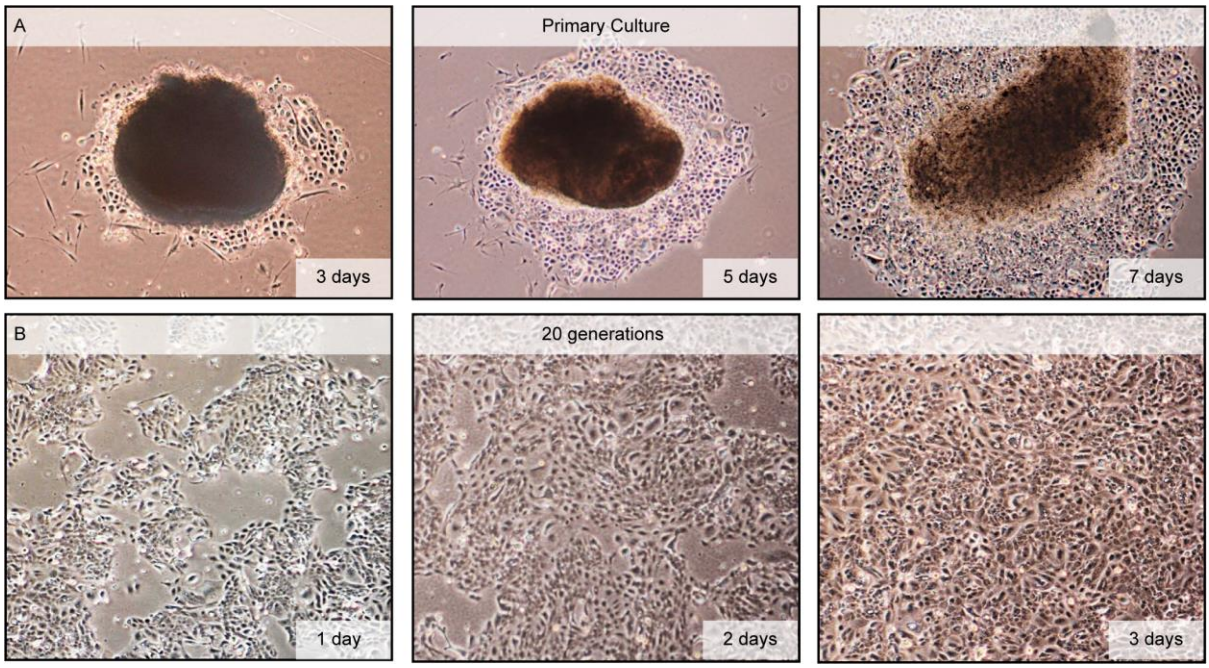

Figure.3.

Image of the culture of a portion of the biopsy tissue.

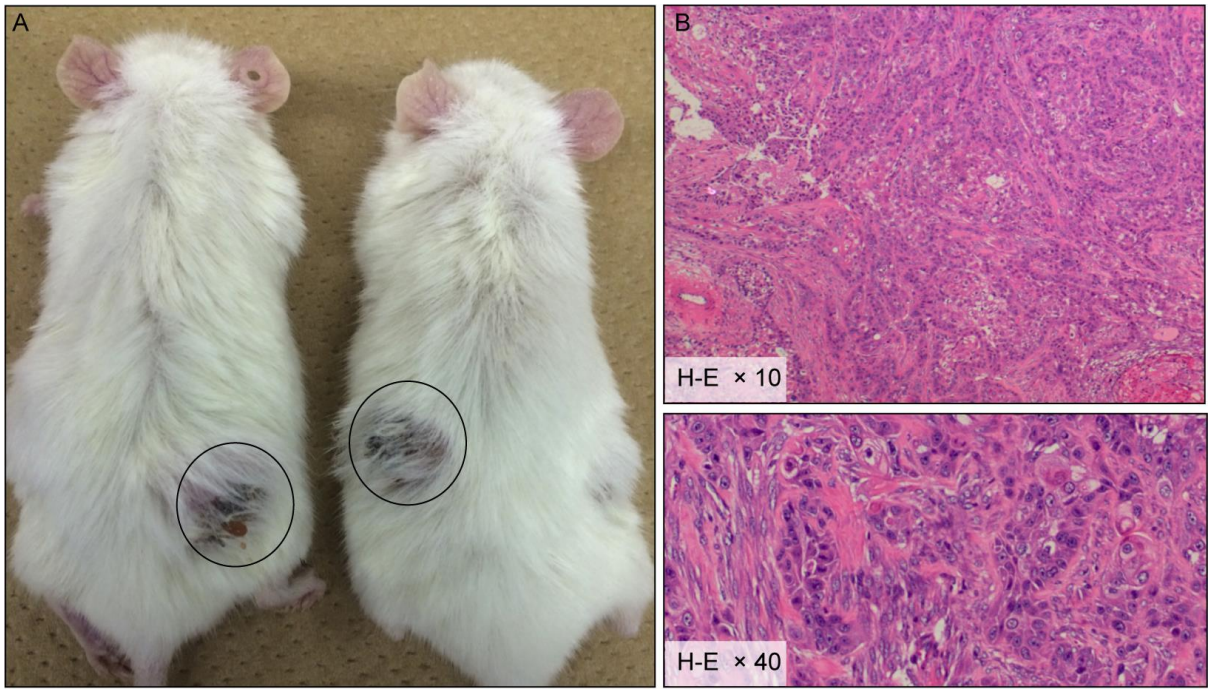

Figure.4.

The novel OSCC cell line, OTM, exhibited tumor-forming ability in severe combined immunodeficiency mice.

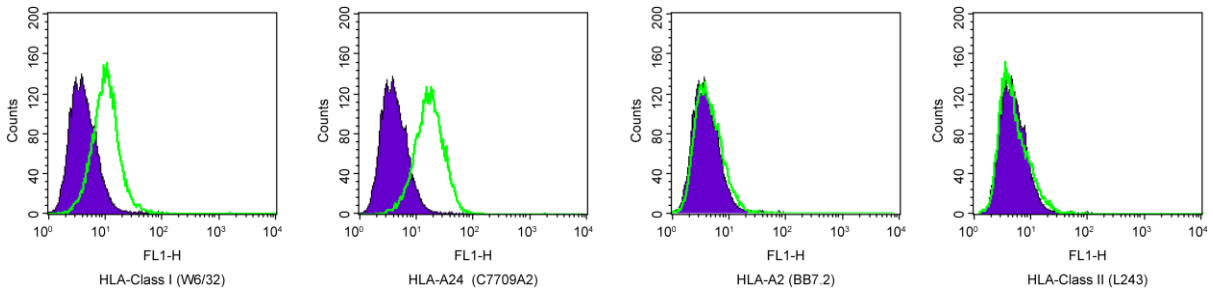

Figure.5.

FACScan analysis of the surface HLA molecules.

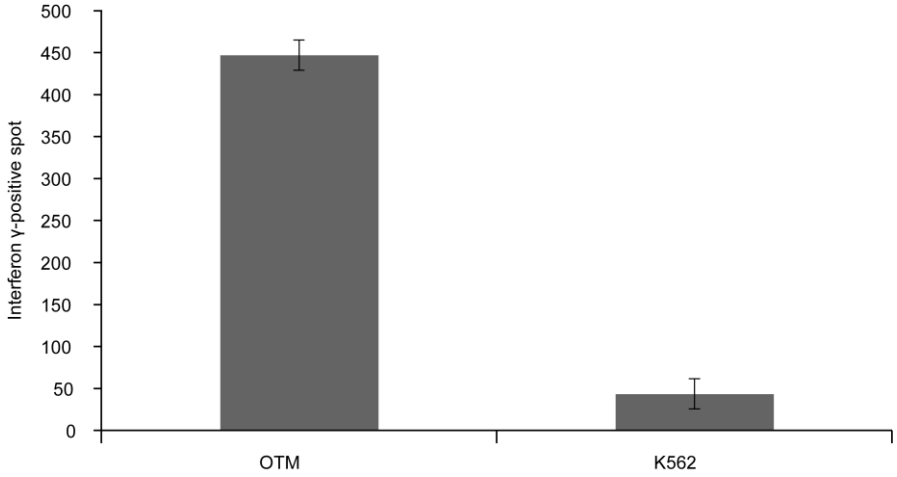

Figure.6.

Cytotoxic activity of the TcOTM CTL line.

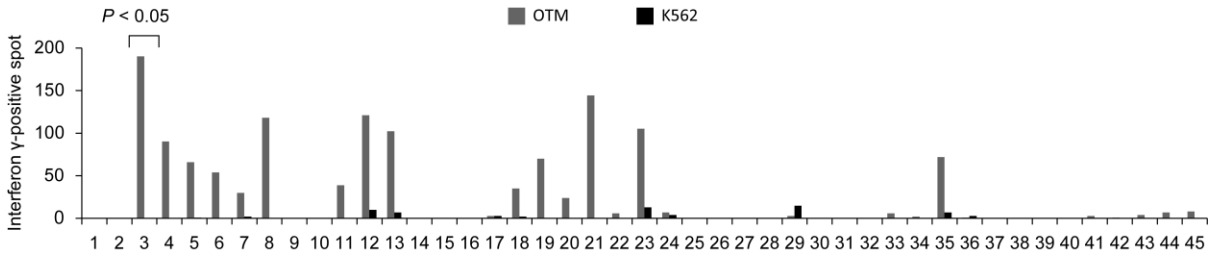

Figure.7.

Limiting dilution of bulk TcOTM.

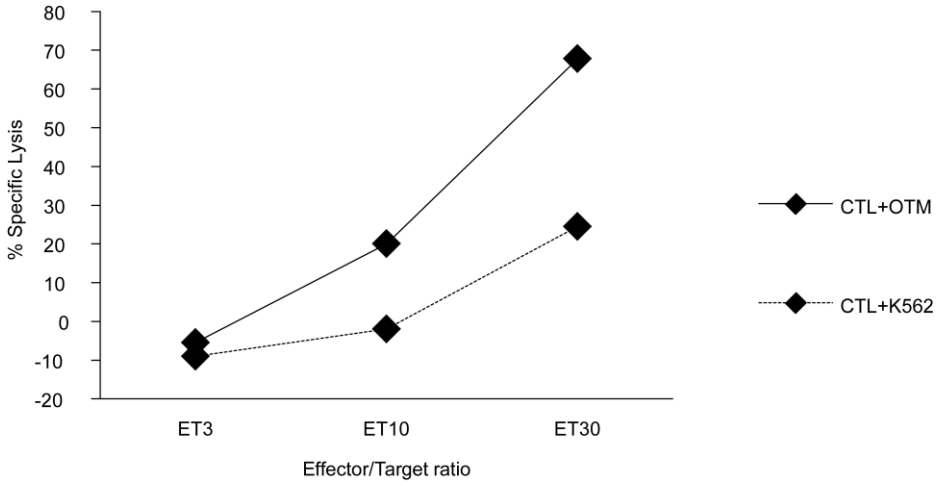

Figure.8.

Cytotoxic activities of the CTL clone3.

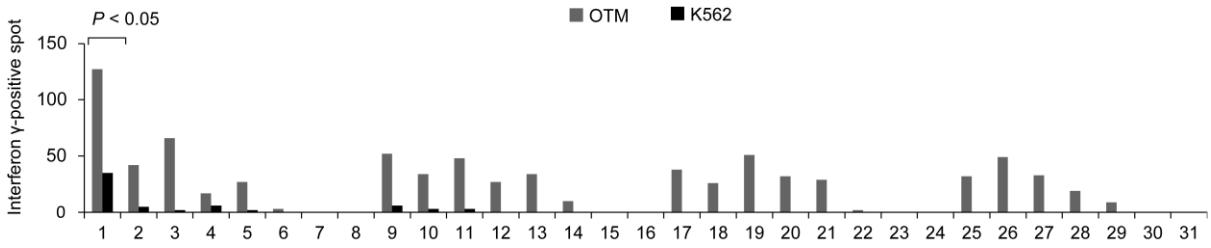

Figure.9.

Limiting dilution of CTL clone3.

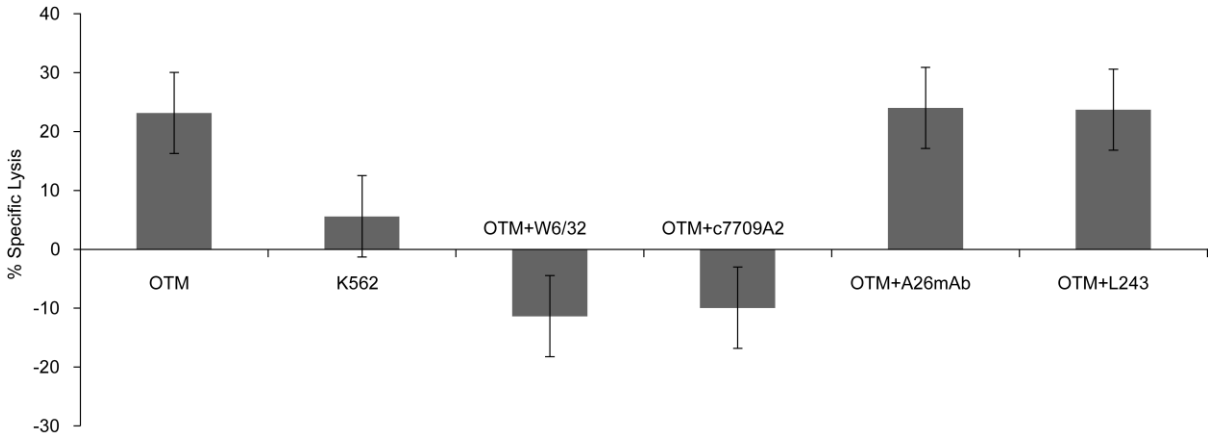

Figure.10.

Blocking assays with mAbs were performed with CTL3-1 to identify the HLA restriction of elements by using the LDH release cytotoxicity assay.

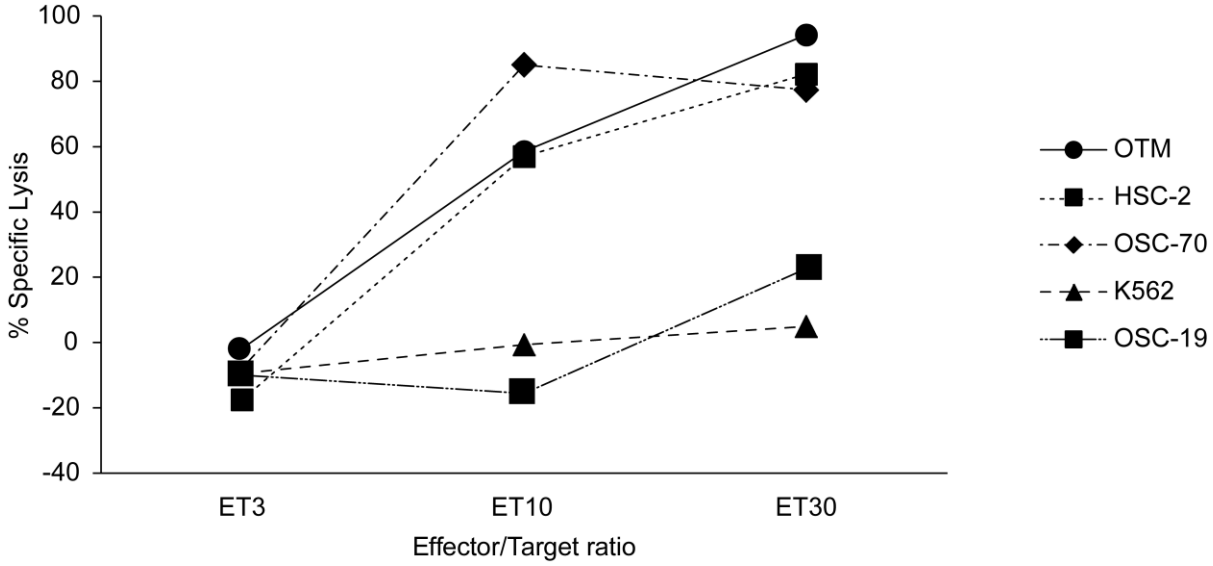

Figure.11.

Cytotoxicity of CTL3-1 against autologous and allogenic OSCC cell lines with various E/T ratios.