Table 2 Summary of clinical courses of patients administered survivin-2B80-88 peptide vaccine

	Peptide dose	Adverse events (Grade)[a]	DTH skin test	Tetramer staining assay % of HLA-A24/survivin-2B80-88 peptide tetramer-positiv e CTL (post-/pre-vaccination)	Reduction rate (judgment)
1	0.1 mg × 6	Induration at the injection site (Grade 1)	(–)	Increased (1.29/0.04)	19.4% (SD)
2	0.1 mg × 6	Induration at the injection site (Grade 1)	(–)	Increased (1.34/0.07)	17.6% (SD)
3	0.1 mg × 2	None	(–)	NA	NA
4	0.1 mg × 6	Induration at the injection site (Grade 1)	(–)	No change (0.28/0.06)	−52.5% (PD)
5	1.0 mg × 3	None	(–)	NA	NA
6	1.0 mg × 6	Induration at the injection site (Grade 1)	(–)	No change (0.06/0.10)	−68.0% (PD)
7	1.0 mg × 5	Induration at the injection site (Grade 1)	(–)	Increased (0.60/0.15)[b]	NA
8	1.0 mg × 6	Induration at the injection site (Grade 1)	(–)	Increased (0.78/0.07)[b]	−87.6% (PD)
9	1.0 mg × 6	None	(–)	Increased (1.42/0.13)[b]	−81.4% (PD)

NA not available

[a] Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC)

[b] Evaluation after third vaccination

through the protocol because of disease progression. Six patients (cases 1, 2, 4, 6, 8 and 9) received the complete regimen including six vaccinations and were evaluated. Table 2 summarizes the immunological and clinical outcomes of the nine patients.

Safety

Peptide vaccination was well tolerated in all nine patients. As shown in Table 2, no hematologic, cardiovascular, hepatic, or renal toxicity was observed. No other severe adverse events such as fever and fatigue were observed during or after vaccination in any patient. As minor side effect, six patients (cases 1, 2, 4, 6, 7 and 8) developed grade 1 local skin reactions with redness and swelling at the injection sites.

DTH skin test

A DTH skin test was performed at each vaccination and assessed 48 h later. No positive DTH reaction was observed in any patient.

Tetramer staining assay

Peripheral blood lymphocytes of patients using HLA-A24/survivin-2B80-88 peptide tetramers were available from seven patients (cases 1, 2, 4, 6, 7, 8 and 9). HLA-A24/survivin-2B80-88 peptide tetramer analysis revealed a significant increase in the peptide-specific CTL frequency of CD8-positive T cells after the vaccination in five patients (cases 1, 2, 7, 8 and 9) (Table 2). In cases 1 and 2, the frequency of HLA-A24/survivin-2B80-88 peptide tetramer-positive CTLs was increased from 0.04 to 1.29% and 0.07 to 1.34%, respectively, after the sixth vaccination (Fig. 2a, b). In case 7 who could not receive the complete regimen due to disease progression, HLA-A24/survivin-2B80-88 peptide tetramer analysis after the third vaccination revealed an increase in the peptide-specific CTL frequency from 0.15 to 0.60% (Fig. 2c). In cases 8 and 9, peptide-specific CTL frequency increased from 0.07 to 0.78% and 0.13 to 1.42%, respectively, after the third vaccination (Fig. 2d, e). In these five patients, these CTLs did not show any increases of frequency with the HLA-A24/HIV peptide tetramer.

Clinical responses

As indicated in Table 2, the two patients (cases 1 and 2) who showed increased frequencies of tetramer-positive CTLs showed slight reduction of the tumor volume after six-vaccination therapy. One responder (case 1) with right ureteral cancer who developed chemotherapy-refractory obturator lymph node metastasis chose to continue vaccination after finishing this phase I study because there were no severe adverse events. Before this study, as shown in Fig. 3a, pelvic CT showed that the recurrent right obturator node metastasis was 60 × 25 mm in size. The metastatic nodal disease was decreased to 46 × 15 mm after the sixth vaccination (arrow, Fig. 3b) and to 45 × 14 mm 21 months after first vaccination (arrow, Fig. 3c). In this patient with advanced urothelial cancer, slight reduction of the tumor volume was observed for 2 years, which was considered as minor response.

Discussion

High-throughput gene expression profiling of cancer has led to the discovery of many novel genes associated with it.

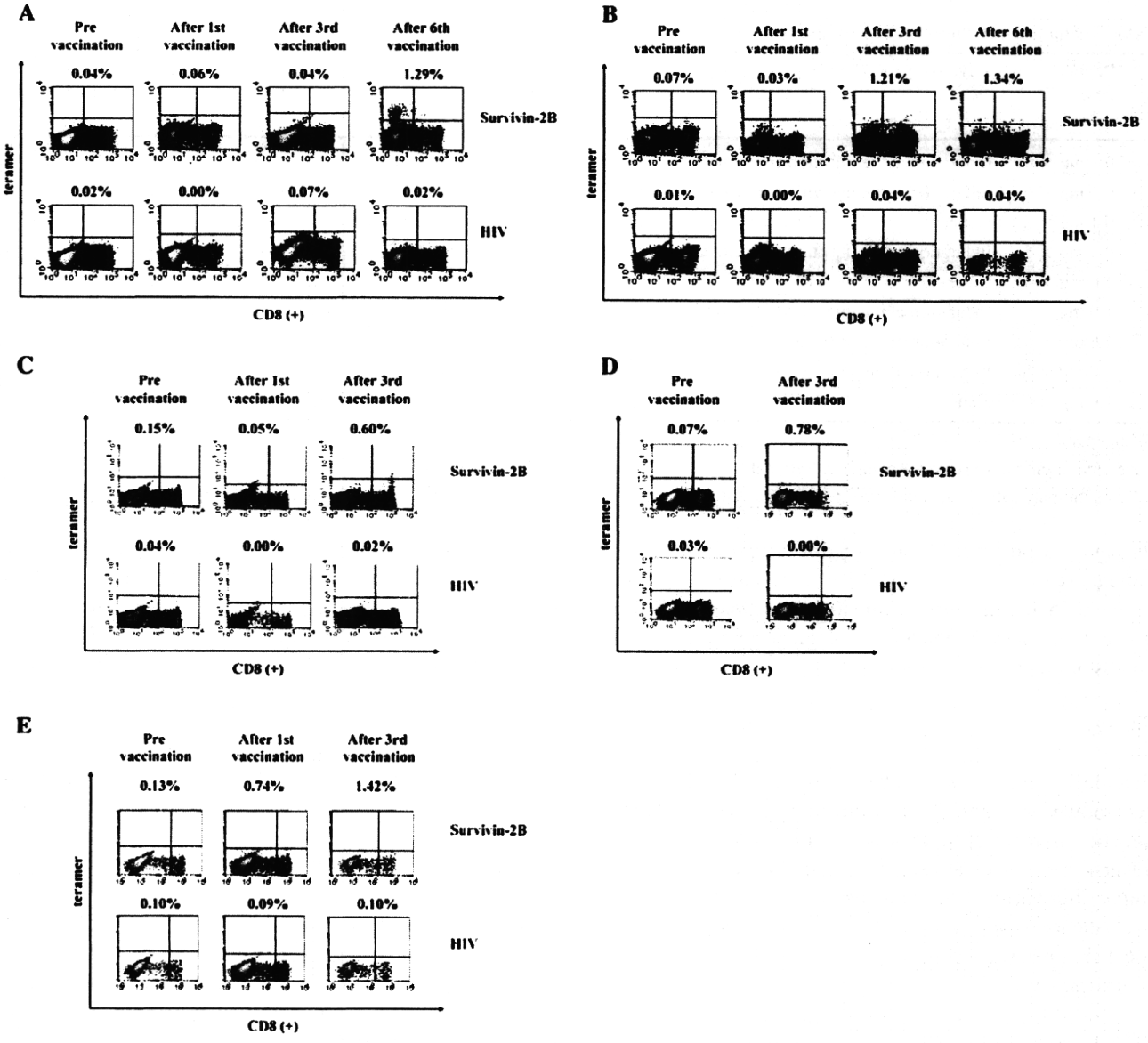

Fig. 2 Frequency of peptide-specific CTLs analyzed by HLA-A24/survivin-2B80-88 peptide tetramer analysis (**a** case 1, **b** case 2, **c** case 7, **d** case 8 and **e** case 9). Lymphocytes were collected from peripheral blood of the patients before and after the first, third and sixth vaccinations, stained with an FITC-labeled anti-CD8 mAb and PE-labeled HLA-A24/survivin-2B80-88 peptide tetramer, and analyzed by flow cytometry. As a negative control, a tetramer with an HIV peptide was used. The frequency of CTL precursors was calculated as the number of tetramer-positive cells over the number of CD8-positive cells. The frequencies of HLA-A24/survivin-2B80-88 peptide tetramer -positive CTLs were increased from 0.04 to 1.29%, and 0.07 to 1.34% after the sixth vaccination in cases 1 and 2, respectively (**a** and **b**). In case 7, 8 and 9 peptide-specific CTL frequency increased from 0.15 to 0.60%, 0.07 to 0.78% and 0.13 to 1.42%, respectively, after the third vaccination (**c**–**e**)

An increasing number of T-cell epitopes derived from these various tumor-associated antigens have been reported and proved to play significant roles in CTL-based immunotherapy. Survivin was originally identified as a member of the inhibitor of apoptosis protein family. It has the capability to inhibit caspase-3, -7, and -9 in cells receiving an apoptotic stimulus and may lead to tumor initiation, progression, and therapeutic resistance [4]. It is expressed in colorectal, breast, and urothelial cancers but is hardly detectable in normal, differentiated adult tissues [2–5]. Moreover, overexpression of survivin in cancer cells is associated with unfavorable clinicopathologic variables such as poor prognosis and shorter patient survival rates.

Because of its cancer-specific expression and function of protecting cancer cells from apoptotic stimuli, survivin might be an ideal target for CTL-based immunotherapy. We focused on a survivin-derived peptide carrying the HLA-A24 binding motif. This HLA-A24 genotype is

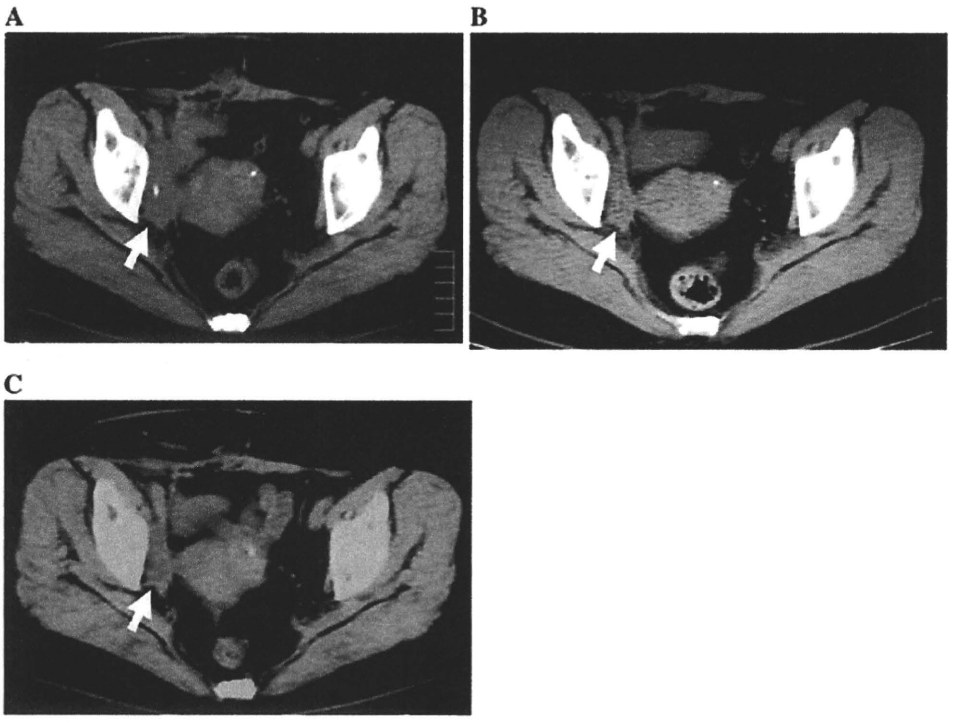

Fig. 3 Pelvic CT findings of case 1 patient. Pelvic CT shows slight reduction of recurrent right obturator node tumor size after the sixth vaccination. The vaccinations were continued and, after 21 months, the size of the recurrent right obturator node tumor was almost unchanged. Arrows, recurrent right obturator node masses and changes of the tumor size (mm) are shown as follows: pre (**a** 60 × 25), after sixth vaccination (**b** 46 × 15), and after 21 months (**c** 45 × 14)

predominant in Japanese (about 60%) and less abundant in Caucasians (17%), Blacks (9%), and Hispanics (27%) [14]. Previously we reported that survivin-2B80-88, a survivin-derived peptide carrying the HLA-A24 binding motif, was established and CD8-positive CTLs were successfully induced by stimulation with this peptide in vitro [5]. In addition, we demonstrated that survivin was expressed in a large proportion of bladder cancer specimens, and this survivin-2B-derived peptide could induce CTL responses in the context of HLA-A24 [9]. On the basis of these observations, we started a phase I clinical study of survivin-2B80-88 peptide vaccination for patients with advanced or recurrent urothelial cancer.

The primary end point in the current clinical trial was to evaluate the safety and toxicity of survivin-2B80-88 peptide vaccination. In this study, there was no severe adverse event during or after vaccination in either the 0.1 or 1.0 mg group. Thus, we concluded that the survivin-2B80-88 peptide vaccine was safe and could be repeatedly injected into patients without serious side effects.

The secondary aims of our study were to evaluate vaccine-induced specific immune reactions and clinical responses. With respect to the immunological response, by objective scientific HLA-A24/peptide tetramer analysis, we could confirm that the peptide-based vaccines activated peptide-specific CTLs in some patients. Under conditions in which HIV peptide-specific CTL frequencies in PBMCs remained at background levels (less than 0.1%), the frequencies of tetramer-positive CTLs were increased after the vaccination in five cases. However, no positive DTH reaction was observed in any vaccination of the nine patients. A previous study reported that there was a positive correlation between DTH and clinical responses [15]. The CTL response induced by the survivin-2B peptide vaccine might not be sufficient to induce cytotoxic activity against bulky recurrent masses and induce dramatic clinical regression in patients with an advanced urothelial cancer.

HLA-A24/survivin-2B80-88 peptide tetramer analysis revealed tetramer-positive cells were detected in CD8-positive population (Fig. 2a–e), however, they were considered to be non-specific binding since they could be eliminated after titration of the tetramer. In order to confirm if CD8±/tetramer± cells represent the survivin2B80-88-specific CTL, CD8±/tetramer± T-cells were cloned from PBMCs of vaccinated patients by single cell sorting and analyzed for tetramer reactivity and specific killing activity. The CTL clones that were approximately 98% positive for the survivin2B80-88 tetramer showed the peptide-specific killing activity, indicating that CD8±/tetramer± cells represent the survivin2B80-88-specific CTLs (data not shown).

This survivin-2B80-88 peptide vaccination therapy may have limitations to induce clinically relevant results because of using only single HLA class I restricted peptides. Recently, a number of survivin epitopes restricted to several additional HLA molecules have been identified [16], and several clinical trials of immunotherapy based on survivin-derived peptides have been initiated. It would be interesting to identify and use other HLA class I or HLA class II survivin epitopes to induce more

survivin-specific CTLs. Furthermore, it is possible that vaccination with the peptide in combination with some cytokines may be able to lead to stronger immune responses both in the induction and effector phases [17, 18]. On the basis of the information obtained from this study, further studies are required to evaluate the efficacy of the survivin-2B peptide vaccine in combination with various adjuvant drugs such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2 and interferon (IFN). Our preliminary clinical study suggested that survivin-2B80-88 peptide vaccination in conjunction with IFN-α was more effective than the peptide alone in colon and pancreas cancer patients. Moreover, heat shock protein (HSP)-peptide complexes elicited antitumor responses in studies on immunization protocols. In our laboratories, we have found that HSPs such as Hsp70 and Hsp90 could be subjected to receptor-mediated uptake by antigen-presenting cells with subsequent representation of the HSP-associated peptides to HLA class I molecules on antigen presenting cells, facilitating efficient cross-presentation [19]. Toll-like receptors (TLR) have an essential role in the innate immune recognition of antigens [20]. Thus, it should be effective to use TLR-mediated signaling pathways to induce more survivin-specific CTLs.

Although our study consisted of only a limited number of patients, these preliminary data seem to suggest that survivin-2B peptide vaccination is safe without serious adverse events. As the first step, this study revealed that survivin-2B peptide-based vaccines activated peptide-specific CTLs and may be considered for potential immune and clinical efficacy in HLA-A24 positive/survivin-expressing patients with urothelial cancer. In the future, if the efficacy and safety of this vaccination therapy are established, we might be able to use this vaccine as an adjuvant therapy for high-risk non-immune-suppressed patients before systemic chemotherapy.

Conclusion

This phase I clinical study indicates that survivin-2B80-88 peptide-based vaccination is safe and should be further considered for potential immune and clinical efficacy in HLA-A24+ survivin-expressing patients with urothelial cancer.

Acknowledgments Kumiko Shimozawa and Emiri Nakazawa provided technical assistance. This study was supported in part by a grant-in-aid for Clinical Cancer Research from the Ministry of Health, Labor and Welfare and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 17390441 to T. Tsukamoto), and research grants from the Stiftelsen Japanese-Swedish Research Foundation, and Gotaro Sugawara Research Fund for Urological Diseases.

References

1. Rosenberg SA (1999) A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 10:281–287
2. Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 3:917–921
3. Kawasaki H, Altieri DC, Lu CD et al (1998) Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 58:5071–5074
4. Tamm I, Wang Y, Sausville E et al (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58:5315–5320
5. Hirohashi Y, Torigoe T, Maeda A et al (2002) An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. Clin Cancer Res 8:1731–1739
6. Idenoue S, Hirohashi Y, Torigoe T et al (2005) A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins. Clin Cancer Res 11:1474–1482
7. Tsuruma T, Hata F, Torigoe T et al (2004) Phase I clinical study of anti-apoptosis protein, survivinderived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer. J Transl Med 2:19–29
8. Tsuruma T, Iwayama Y, Ohmura T et al (2008) Clinical and immunological evaluation of anti-apoptosis protein, survivin-derived peptide vaccine in phase I clinical study for patients with advanced or recurrent breast cancer. J Transl Med 6:24
9. Kitamura H, Torigoe T, Honma I et al (2006) Expression and antigenicity of survivin, an inhibitor of apoptosis family member, in bladder cancer: implications for specific immunotherapy. Urology 67:955–959
10. Koh YT, Higgins SA, Weber JS et al (2006) Immunological consequences of using three different clinical/laboratory techniques of emulsifying peptide-based vaccines in incomplete Freund's adjuvant. J Trans Med 4:42
11. Altman JD, Moss PA, Goulder PJ et al (1996) Phenotypic analysis of antigen-specific T lymphocytes. Science 274:94
12. Sato Y, Sahara H, Tsukahara T et al (2002) Improved generation of HLA class/peptide tetramers. J Immunol Methods 20:177–184
13. Sato Y, Nabeta Y, Tsukahara T et al (2002) Detection and induction of CTLs specific for SYT-SSX-derived peptides in HLA-A24(+) patients with synovial sarcoma. J Immunol 169:1611–1618
14. Date Y, Kimura A, Kato H et al (1998) DNA typing of the HLA-A gene: population study and identification of four new alleles in Japanese. Tissue Antigens 47:93–101
15. Nestle FO, Alijagic S, Gilliet M et al (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4:328–332
16. Andersen MH, Pedersen LO, Capeller B et al (2001) Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. Cancer Res 61:5964–5968
17. Ojima T, Iwahashi M, Nakamura M et al (2006) The boosting effect of co-transduction with cytokine genes on cancer vaccine therapy using genetically modified dendritic cells expressing tumor-associated antigen. Int J Oncol 28:947–953
18. Svane IM, Nikolajsen K, Walter MR et al (2006) Characterization of monocyte-derived dendritic cells maturated with IFN-alpha. Scand J Immunol 63:217–222
19. Kurotaki T, Tamura Y, Ueda G et al (2007) Efficient cross-presentation by heat shock protein 90-peptide complex-loaded dendritic cells via an endosomal pathway. J Immunol 179:1803–1813
20. Kumagai Y, Takeuchi O, Akira S (2008) TLR9 as a key receptor for the recognition of DNA. Adv Drug Deliv Rev 60:795–804

Biochemical and Biophysical Research Communications 379 (2009) 765–770

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Wild-type AIRE cooperates with p63 in HLA class II expression of medullary thymic stromal cells ☆

Akiko Tonooka [a], Terufumi Kubo [a], Shingo Ichimiya [a,*], Yutaka Tamura [b], Tanja Ilmarinen [c], Ismo Ulmanen [c], Sachiko Kimura [d], Shigeaki Yokoyama [d], Yoshihide Takano [a], Tomoki Kikuchi [a], Noriyuki Sato [a]

[a] Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan
[b] Department of Bioinformatics, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba 260-8670, Japan
[c] Department of Molecular Medicine, National Public Health Institute, Biomedicum Helsinki, Haartmaninkatu 8, 00290 Helsinki, Finland
[d] Division of Pathology and Laboratory Medicine, Hokkaido Medical Center for Child Health and Rehabilitation, Kanayama 1-1, Teine-ku, Sapporo 060-0041, Japan

ARTICLE INFO

Article history:
Received 17 December 2008
Available online 3 January 2009

Keywords:
AIRE
p63
Thymic selection
Autoimmunity

ABSTRACT

During T cell development in the thymus, autoreactive T cells are deleted through a mechanism that is actively supported by medullary epithelial cells. These epithelial cells possess particular transcription factors including autoimmune regulator (AIRE), which is responsible for regulating expression of self-antigens, as well as p63, a p53-like molecule. Here we present evidence suggesting interaction of AIRE with p63 through a SAND domain and a transactivation domain, respectively. Interestingly an AIRE molecule with a mutated SAND domain of G228W, whose genetic alteration is inherited in an autosomal dominant manner, could not establish a complex with p63 as indicated by immunoprecipitation and molecular modeling analyses. Further in vitro study indicated that the G228W mutation led to downregulation of the transcription levels of CIITA and, accordingly, the cell surface expression of HLA class II molecules in thymic epithelial cells with p63. This indicates novel involvement of AIRE and p63 in the regulation of HLA class II, and suggests that defects in the AIRE–p63 interaction may lead to malfunction of HLA-based selection of self-reactive helper $CD4^+$ T cells in the thymus.

To establish immunosurveillance, which is largely conducted by effector T cells, the thymus generates fully functional T cell repertoires equipped with immunological self-tolerance [1,2]. This central process of the thymus is mainly achieved by major histocompatibility complex (MHC)-based positive and negative selection of developing T cells in the cortical and medullary regions, respectively. Since helper $CD4^+$ T cells constituting major peripheral T cells control most immune cells, including cytotoxic $CD8^+$ T cells, B cells, NK cells, macrophages and dendritic cells, dysregulation of the selection event of helper T cells would cause an unfavorable shift into systemic autoimmune-prone conditions [3,4]. Medullary thymic epithelial cells mainly take part in negative selection of immature T cells after rearrangement of T cell receptor genes and, as a result, are believed to prevent the escape of autoreactive T cells from the thymus [5].

The gene encoding human AIRE (autoimmune regulator), a 545 amino acid molecule, was reported to be behind the non-HLA-linked disorder autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) [6,7]. APECED exhibits a broad spectrum of autoimmune phenotypes affecting many organs such as the parathyroid and adrenal glands, pancreas, epidermis, liver and bone marrow. This phenotypic feature has led to a hypothesis of the functional properties of AIRE in terms of thymic selection. Consequently, analyses of AIRE-deficient mice and other studies have revealed that medullary thymic epithelial cells express AIRE, which helps to purge immature self-reactive T cells at the stage of negative selection during T cell development [8–11]. Accordingly, the discovery of AIRE has had a decisive impact on the understanding of the regulatory mechanism of medullary thymic epithelial cells in terms of intrathymic T cell development and autoimmune-related disorders [12]. Thus far, the functional significance of AIRE in medullary thymic epithelial cells has been highlighted with regard to T cell selection and antigen presentation.

We have previously reported the expression of a p53-related transcription factor, p63, in human medullary thymic epithelial cells [13,14], which evidence prompted us to examine whether the function of AIRE might be controlled by p63 because transcriptional regulators occasionally constitute an oligo-molecular

☆ This work was supported by the Japan Society for the Promotion of Science to S. Ichimiya and in part by the EU FP6 Program project EURAPS, the Academy of Finland, Sigrid Juselius Foundation and the Paulo Foundation to I. Ulmanen and T. Ilmarinen.

* Corresponding author. Fax: +81 11 643 2310.
E-mail address: ichimiya@sapmed.ac.jp (S. Ichimiya).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.123

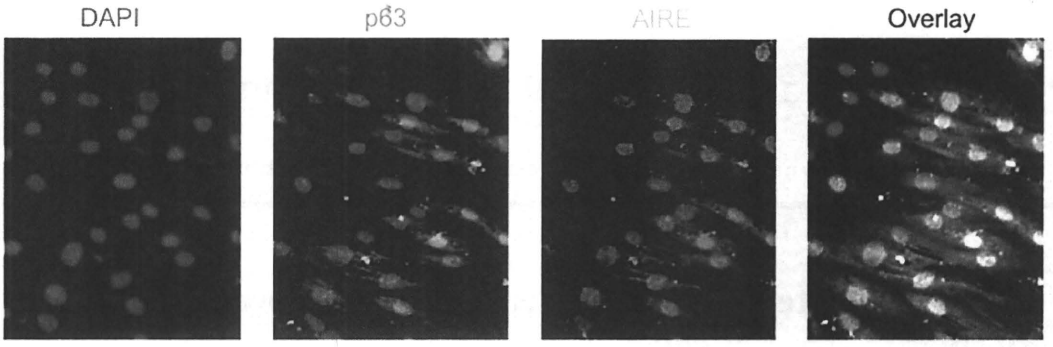

Fig. 1. Colocalization of AIRE and p63 of a p53-related transcription factor in medullary thymic epithelial cells of primary culture. Images were obtained from immunofluorescence microscopy after double-staining with p63 (green) and AIRE (red) and subsequent nuclear staining with DAPI (blue). Original magnification: x400.

complex. Our experimental evidence suggested that, when AIRE was introduced into cells together with p63 mimicking the native status of medullary thymic epithelial cells, the two molecules could interact through the association of a SAND domain of AIRE and a transactivation (TA) domain of p63. Interestingly, a naturally occurring mutation with autosomal dominant inheritance in the SAND domain of AIRE (G228W-AIRE) could not bind to the TA domain of p63, probably due to protrusion of the tryptophan residue from the original structure of the SAND domain. Furthermore, the surface expression of HLA-DR was affected by combinatory expression of p63 and G228W-AIRE in thymic epithelial cells due to the downregulation of CIITA in that state. These findings illustrate a cooperative role of p63 with AIRE in the regulation of HLA class II of medullary thymic epithelial cells and will help elucidate the mechanism of the genetic defect causing G228W-AIRE mutation.

Experimental procedures

Tissues and cell culture. Normal human thymuses were obtained from patients <3 years of age undergoing cardiovascular surgery for congenital cardiac disease in the Hokkaido Medical Center for Child Health and Rehabilitation in Japan. The procedures used for primary thymic epithelial cell culture were previously [14]. Human P1.4 thymic epithelial cells and HEK293 cells were maintained in MEM with 10 mM HEPES, and DMEM, respectively, both of which were supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 50 μg/ml streptomycin. All cells were cultured at 37 °C in a humidified atmosphere in 5% CO_2.

Antibodies and immunohistochemistry. A 4A4 mouse anti-p63 mAb and H137 rabbit anti-p63 pAb were purchased from Lab Vision and Santa Cruz Biotechnology, respectively. Mouse anti-HLA class II (L243), anti-HA (HA-7), anti-GFP (JL-8), and anti-myc (9E10) mAbs were also used in this study. A rabbit anti-AIRE pAb was produced by immunization of polypeptides of the SAND domain. The procedures conducted for single- or double-staining analysis were as previously described [13].

Plasmid DNAs. Expression plasmids of pEGFPC2 carrying wild-type AIRE (EGFP-AIRE) or G228W-AIRE (EGFP-G228W) were used in this study [15]. We newly constructed six EGFP-tagged deleted forms based on EGFP-AIRE by PCR techniques. Expression plasmids of pcDNA3.1 carrying HA-tagged p63 and ΔNp63 cDNA were also employed. To establish stable transformants, expression plasmids of pIREShyg2 carrying p63 (pIREShyg2-p63) and pIRESpuro-myc carrying AIRE (pIRESpuro-myc-AIRE) and G228W-AIRE (pIRESpuro-myc-G228W-AIRE) were constructed.

Flow cytometry. For detecting surface HLA-DR molecules, cells were detached from the tissue culture bottles using PBS containing EDTA. Then the cells were stained with L243 as the primary antibody. After washing with PBS three times, the cells were subsequently stained with a rabbit anti-mouse pAb conjugated with FITC. After washing, these cells were subjected to analysis with FACS Calibur (BD Biosciences). All data shown were obtained with gates set on living cells and analyzed with CellQuest software.

Calculation of interaction energy and electrostatic complementarity. The three-dimensional structures of the SAND domain of AIRE (aa 194–279) and the TA domain of p63 (aa 1–55) were constructed with MOE (CCG, Montreal, Canada) based on the Brookhaven Protein Databank 1OLZ and 2OJQ, respectively. The structure and molecular mechanics of these complexes were analyzed by using the Amber99 force field in MOE as well as ZDOCK (Boston University, MA). To calculate interaction energy and electrostatic complementarities, we employed MOE and MolFeat-EC (FiatLux, Tokyo, Japan), as previously described with some modification [16,17].

Results

Colocalization of AIRE and p63 in human thymic epithelial cells

First we studied the expression of AIRE and p63 in medullary thymic epithelial cells of primary culture to reconcile the evidence observed in thymic tissue sections [13,14]. Medullary thymic epithelial cells of primary culture showed typical flat shapes with round nuclei forming small cell clusters (Fig. 1). Indeed, we could find a cell population expressing AIRE and p63 in their nuclei of medullary thymic epithelial cells of primary culture. Thus, AIRE was certainly colocalized with p63 in the nuclei of these human thymic epithelial cells.

Association of AIRE with p63

To examine whether AIRE had the capacity to associate with p63, we next performed immunoprecipitation experiments. The AIRE protein is constituted of multiple structural domains, including a homogeneously staining region (HSR) domain at the N-terminus, a nuclear localization signal (NLS), an Sp100, AIRE, NucP41/75 and DEAF-1 (SAND) domain and two plant homeodomain-type zinc-finger (PHD) domains at the C-terminus (Fig. 2A). By using EGFP-tagged deletion constructs covering the whole domain structures of such AIRE proteins, results from immunoprecipitation experiments interestingly indicated that p63 could bind to the NLS/SAND domain of AIRE, whereas ΔNp63, an alternative spliced form of p63 lacking its TA domain, did not associate with any type of domain of AIRE (Fig. 2B and C). These results implied that the TA domain of p63 was responsible for the interaction with the NLS/SAND domain of AIRE. Further analysis suggested that an EGFP-tagged construct containing only the SAND domain would bind to p63 (Fig. 2C). Thus, it was suggested that the SAND domain of

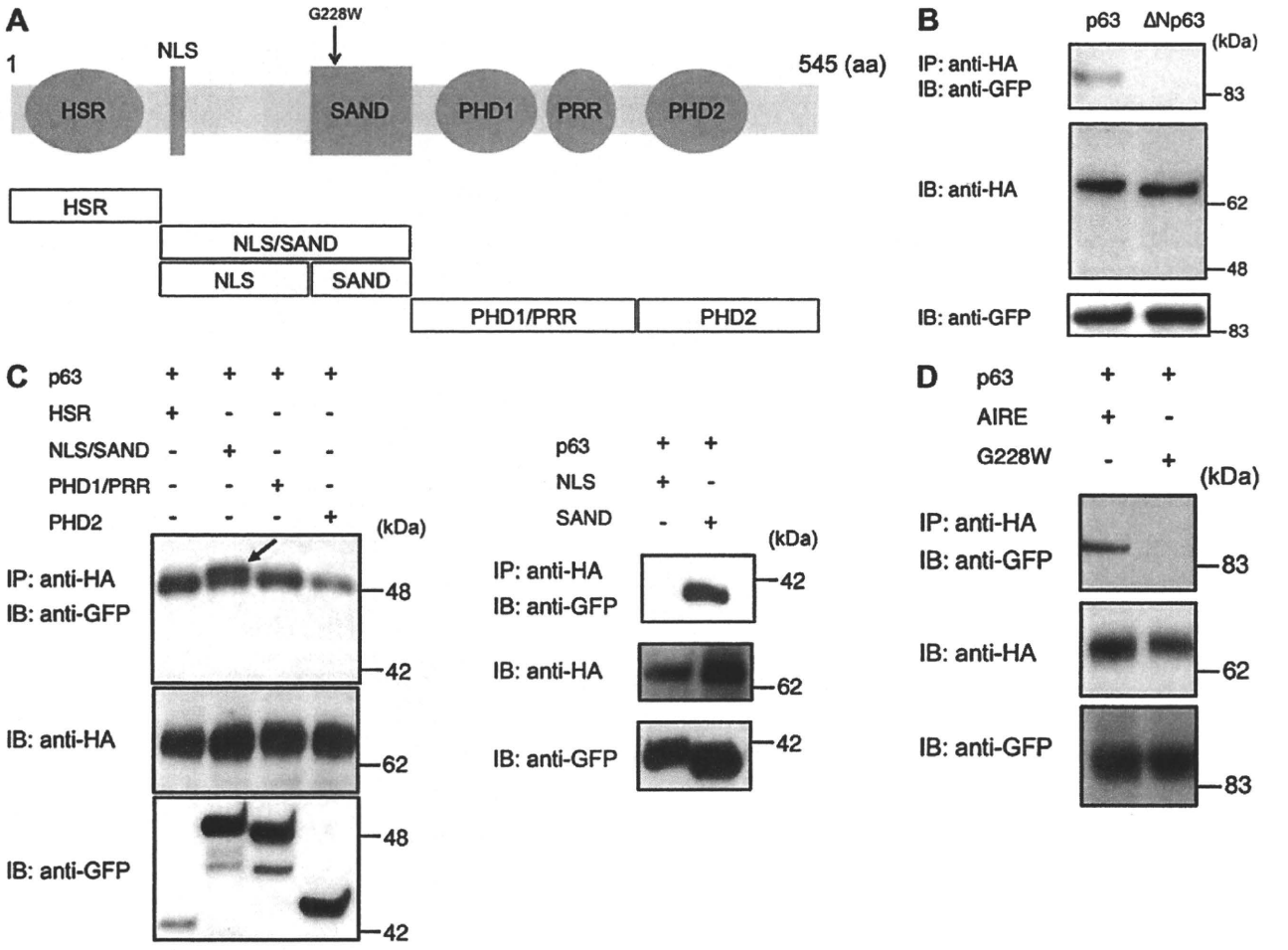

Fig. 2. Interaction of p63 with AIRE. (A) Functional domains of an AIRE protein and the respective fragments employed to transfect cells. To cover the whole domain structure of AIRE, six different constructs with an EGFP tag, EGFP-HSR, EGFP-NLS/SAND, EGFP-NLS, EGFP-SAND, EGFP-PHD1/PRR and EGFP-PHD2, were made. (B) Immunoprecipitation analysis of HEK293 cells transiently transfected with HA-p63 or HA-ΔNp63 expression plasmids and EGFP-AIRE. (C) Interaction of p63 with the NLS/SAND domain of AIRE was suggested by immunoprecipitation in a left panel (the arrow indicating EGFP-NLS/SAND), whereas no association with p63 was indicated for the other constructs. Bands due to immunoglobulin heavy chains are detected in each lane. In a right panel, the p63 protein was indicated to interact with the SAND domain, but not the NLS domain of AIRE. (D) The p63 protein was indicated to interact with wild-type AIRE, whereas p63 did not associate with AIRE carrying G228W-mutation in the SAND domain.

wild-type AIRE (wt-AIRE) could have a capacity to directly bind to the TA domain of p63.

This evidence prompted us to perform further experiments to determine whether naturally occurring mutant AIRE carrying the missense mutation G228W in its SAND domain (G228W-AIRE) could bind to p63. This type of missense mutation of AIRE is inherited in an autosomal dominant manner, whereas most mutant types of AIRE are recessively transmitted [18,19]. Compared to the binding capacity of wt-AIRE, G228W-AIRE seemed to abolish the binding of AIRE to p63 (Fig. 2D).

Effects of a G228W moiety of mutant AIRE on AIRE-p63 interaction

Given that the physical association of wt-AIRE with p63 was inhibited by G228W mutation of AIRE, we employed molecular modeling to determine how the G228W portion of AIRE abrogated the interaction of AIRE with p63. The interaction energies of the SAND domains of wt-AIRE and G228W-AIRE with the TA domain of p63 were calculated to be −340.7 kcal/mol and −254.1 kcal/mol, respectively, implying that the wt-AIRE/p63 complex constituted a more stable form than the G228W-AIRE/p63 complex (Table 1). Following this analysis, we further modeled how G228W-AIRE inhibited the robust interaction with the TA domain of p63 (Fig. 3A). The manner of binding of the SAND domain of wt-AIRE to the TA domain of p63 was proposed to be the sole feasible binding pattern. In contrast, due to the hydrophobic contact repulsion of tryptophan at position 228, the SAND domain of G228W-AIRE prevented active interaction of wt-AIRE with the TA domain of p63. In addition to such a docking simulation, we investigated the electrostatic complementarity (EC) for quantitative analysis of the protein–protein interaction between the SAND domain of wt-AIRE and the TA domain of p63. This was in accord with our observation that the value of EC of wt-AIRE/p63 (0.34) was much higher than that of G228W-AIRE/p63 (EC = −0.05) (Table 1). Indeed, the protein–protein interfaces of the SAND domain of wt-AIRE and the

Table 1
Interaction energy (IE) and electrostatic complementarity (EC) between two portions of the SAND domains of wt-AIRE and G228W-AIRE and the TA domain of p63 as depicted in wt-AIRE/p63 and G228W-AIRE/p63, respectively. IE and EC were calculated by parse and Amber analyses, respectively.

	p63/wt-AIRE	p63/G228W-AIRE
IE (kcal/mol)	−340.7	−254.1
EC (Person's)	0.34	−0.05

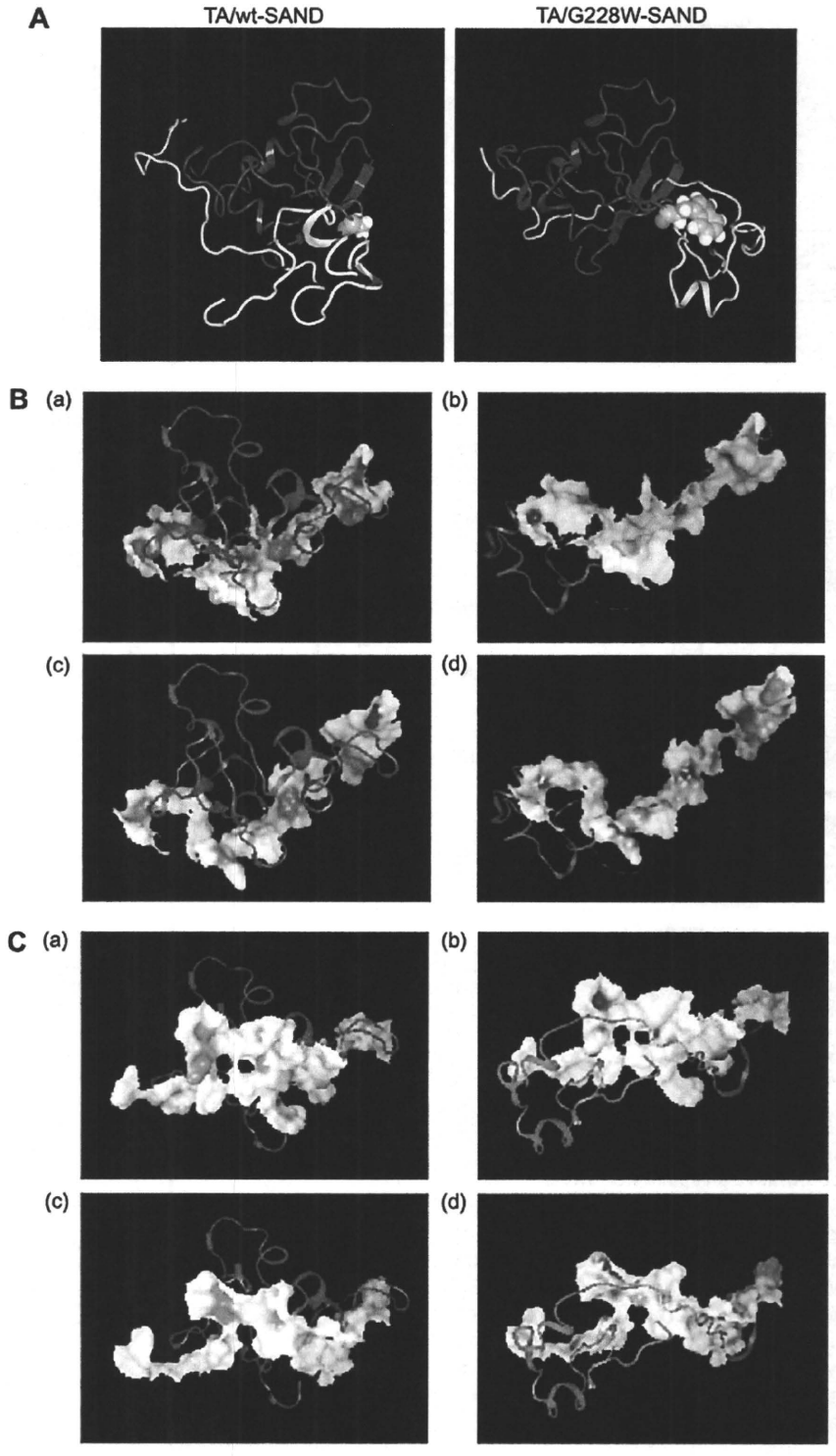

Fig. 3. Molecular interaction models of the SAND domains of wild-type AIRE and G228W-AIRE with the TA domain of p63. (A) The atomic structures of glycine^{228} of the wild-type AIRE and tryptophan^{228} of G228W-AIRE within the predicted SAND domain are shown in the left and right panels, respectively. Due to the bulging of tryptophan^{228}, the surface interactions of the G228W-SAND domain and the TA domain seem to be interrupted. The putative domain structure of the SAND domain is demonstrated in blue and that of the TA domain is in yellow. (B) The electrostatic potential on the molecular surfaces buried in the interface between the wild-type SAND domain (green) and the TA domain (blue). (a) EC on the buried molecular surface of the TA domain generated by its own charges. (b) EC of the TA domain generated by charges on the wild-type SAND domain. (c) EC of the wild-type SAND domain generated by charges on the TA domain. (d) EC of the wild-type SAND domain generated by its own charges. (C) Electrostatic potential on the molecular surfaces buried in the interface between the G228W-SAND domain (green) and the TA domain of p63 (blue). (a) EC on the buried molecular surface of the TA domain generated by its own charges. (b) EC of the TA domain generated by charges on the G228W-SAND domain. (c) EC of the G228W-SAND domain generated by charges on the TA domain. (d) EC of the G228W-SAND domain generated by its own charges.

TA domain of p63 had anticorrelated and complementary surface electrostatic potentials when compared to those of the SAND domain of G228W-AIRE and TA domain of p63 (Fig. 3B and C). These results further supported our finding that the G228W residue might be unfavorable when the SAND domain of AIRE accesses the TA domain of p63.

Involvement of AIRE/p63 complex in the regulation of HLA class II expression

To further address the question of functional consequences of the G228W mutation of AIRE, we established P1.4 cells stably expressing wt-AIRE or the G228W-AIRE mutant together with p63 as well as p63 alone (Fig. 4A). Especially focusing on $CD4^+$ T cell selection, flow cytometry analysis of the cell surface expression of HLA class II was conducted to these cells. Results revealed that the levels of HLA class II were slightly downregulated in P1.4 cells with p63 alone (mean fluorescence intensity, MFI = 68), when compared to mock transfectants (MFI = 77) as shown in Fig. 4B. More interestingly the levels of HLA class II of P1.4 cells with p63 and wt-AIRE were relatively increased (MFI = 125), while the levels of HLA class II of P1.4 cells expressing p63 and G228W-AIRE (MFI = 70) were found to be similar to those of P1.4 cells of mock controls. These suggest that, in the presence of p63, wt-AIRE but not G228W-AIRE could have a capacity to maintain the levels of HLA class II. It is well investigated that the expression of HLA class II is chiefly regulated by a class II master regulator, CIITA, in antigen-presenting cells [20]. Quantitative PCR analysis of these P1.4 cells expressing p63 suggested that G228W-AIRE suppressed

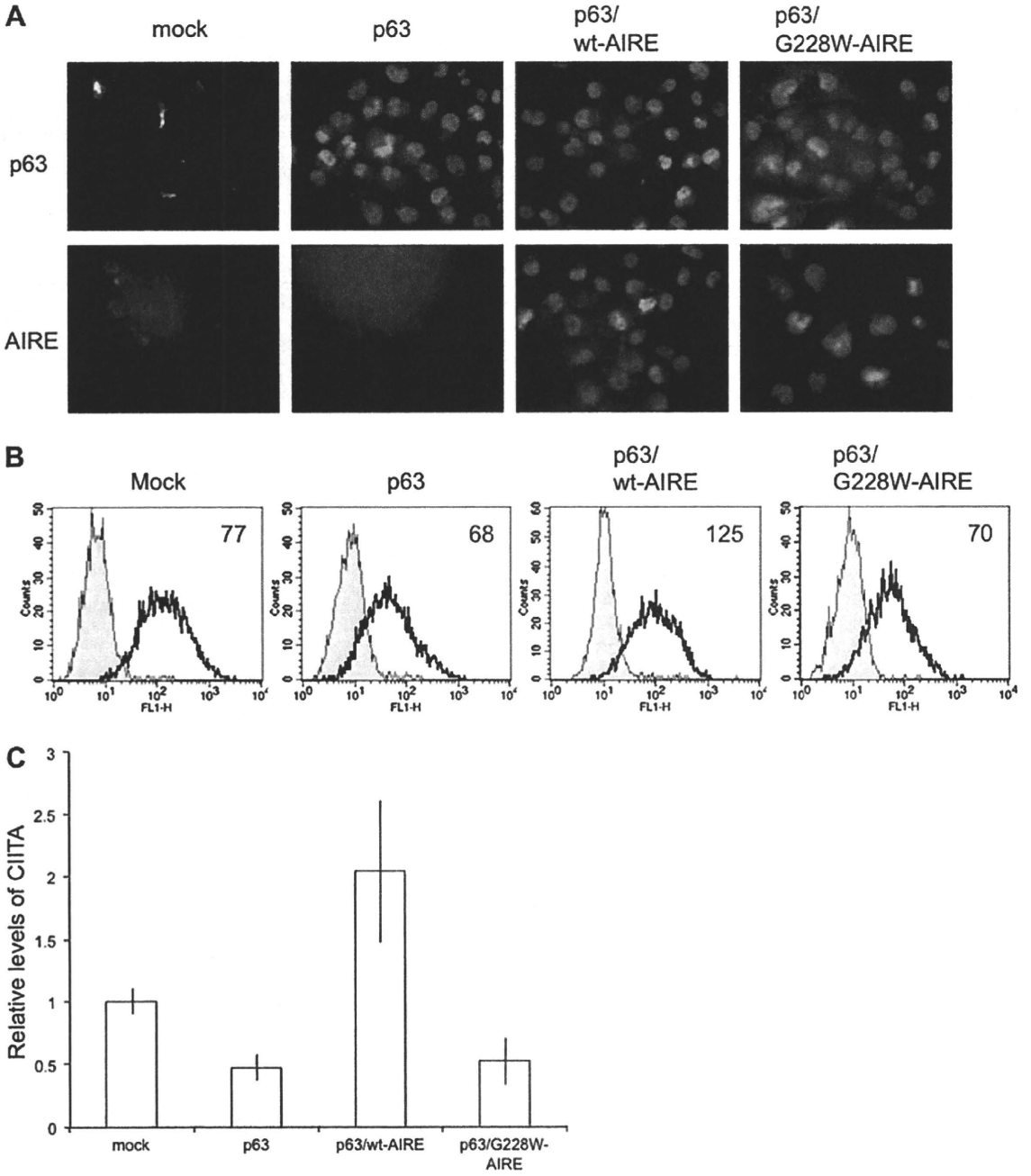

Fig. 4. The roles of AIRE and p63 in the regulation of HLA class II expression. (A) Immunohistochemical analysis was performed using anti-p63 and anti-myc antibodies to confirm expression phenotypes of P1.4 stable transformants as indicated in mock, p63 (alone), p63/wild-type-AIRE and p63/G228W-AIRE. (B) FACS analysis of cell surface expression of HLA class II on P1.4 transformants. The expression of HLA class II is depicted by the solid line with isotype control as indicated by shaded areas. The mean fluorescence intensity (MFI) is also noted in the upper right corner of each figure. *x*-axis: signal intensity in arbitrary units, *y*-axis: number of cells. A representative data is shown from three independent experiments. (C) Quantitative PCR analysis of CIITA transcripts in P1.4 transformants. To evaluate the levels of CIITA in cells, the ΔΔCT method was employed to analyze triplicate specimens. Values are expressed at the mean ± SD for each group.

the levels of transcripts of CIITA (Fig. 4C). When compared to the endogenous levels of CIITA transcripts of P1.4 cells, the levels were 2.0-fold in P1.4 cells expressing wt-AIRE and p63, whereas P1.4 cells expressing G228W-AIRE and p63 showed significantly decreased amounts of CIITA transcripts (0.5-fold).

Discussion

Comparative studies of the molecular structure of the SAND domain with those of Sp100b and GMEB1/2 suggest that, in addition to the structural similarity, AIRE also shares their functions and that the SAND domain of AIRE might contribute to protein–protein interaction as well as DNA binding [21,22]. Based on this feature, the mutated SAND domain of G228W-AIRE would be predicted to disturb intermolecular interactions with other molecules. Furthermore, the G228W mutant has a putative dominant negative effect by interfering with the transactivation capacity of the AIRE protein oligomer [15,19]. Since a broad range of tissue-restricted antigens encompassing a large set of tissue-specific molecules in medullary thymic epithelial cells is presumably dependent on promiscuous gene expression by AIRE, the G228W mutant is expected to cause alteration of expression profiles of tissue-restricted antigens in these cells [23]. This might support the abnormal selection of immature T cells in patients with the G228W mutation. In addition to this putative mechanism, our hypothesis focusing on altered expression of HLA class II molecules of medullary thymic epithelial cells in concert with p63 would also account for the reason why the G228W mutation promotes the emergence of self-reactive T cells. Thus, it might be possible that, in addition to AIRE-related functions, the G228W mutant could affect AIRE-independent functions via HLA class II modulation. This may reflect the phenotype of patients with the G228W mutation, which is not typical for APECED.

The physiological relevance of p53-related factors of the human thymus has been suggested by previous reports. For example, mutations of the *p63* gene cause ectrodactyly-ectodermaldysplasia cleft lip/palate (EEC) and EEC-related syndromes [24]. Some of those syndromes exhibit histological abnormalities of the thymus and prominent depletion of cells in T-zones of lymph nodes [25]. Since the nuclear factor or factors responsible for the function of medullary thymic epithelial cells have been incompletely studied, the discovery of AIRE as well as a p53 family member localized in these cells allows us to investigate the regulatory networks in them, which may contribute to thymic selection. The fact that AIRE and p63 might mutually regulate each other's functions would suggest a complex mechanism in the modulation of medullary thymic epithelial cell function and provides an opportunity to further understand how promiscuous gene transcription is achieved within the cells. From a broader point of view, p63 is thought to be a potential determinant of the stem cell properties of epithelia by regulating a wide range of target genes [26]. With regard to gene expression of medullary thymic epithelial cells, it poses the question of whether p63 is associated with the fundamental role of AIRE. In another study, we found that p63 might take part in the regulation of claudin-1, which widely join medullary cells, including epithelial cells and dendritic cells [14]. Besides cell surface expression of HLA class II, AIRE in conjunction with p63 might also have a role in the instruction of cell surface molecules required for establishing the medullary cell network.

In summary, we described a potential complex formed of AIRE and p63 and the regulatory role of the complex in CIITA expression of medullary thymic epithelial cells. It remains to be seen whether the putative role of AIRE/p63 in the regulation of HLA class II is relevant for explaining the molecular phenotypes of other mutations of AIRE. However, AIRE function seems, at least in part, to be more understandable by adding p63 in the context of HLA class II expression.

References

[1] C.M. Witt, E.A. Robey, Thymopoiesis in 4 dimensions, Semin. Immunol. 17 (2005) 95–102.
[2] M. Gatzka, C.M. Walsh, Apoptotic signal transduction and T cell tolerance, Autoimmunity 40 (2007) 442–452.
[3] C. Viret, C.A. Janeway Jr., MHC and T cell development, Rev. Immunogenet. 1 (1999) 91–104.
[4] M. Carneiro-Sampaio, A. Coutinho, Tolerance and autoimmunity: lessons at the bedside of primary immunodeficiencies, Adv. Immunol. 95 (2007) 51–82.
[5] G. Anderson, P.J. Lane, E.J. Jenkinson, Generating intrathymic microenvironments to establish T-cell tolerance, Nat. Rev. Immunol. 7 (2007) 954–963.
[6] K. Nagamine, P. Peterson, H.S. Scott, J. Kudoh, S. Minoshima, M. Heino, K.J. Krohn, M.D. Lalioti, P.E. Mullis, S.E. Antonarakis, K. Kawasaki, S. Asakawa, F. Ito, N. Shimizu, Positional cloning of the APECED gene, Nat. Genet. 17 (1997) 393–398.
[7] The Finnish-German APECED Consortium, An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains, Nat. Genet. 17 (1997) 399–403.
[8] M.S. Anderson, E.S. Venanzi, L. Klein, Z. Chen, S.P. Berzins, S.J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, D. Mathis, Projection of an immunological self shadow within the thymus by the aire protein, Science 298 (2002) 1395–1401.
[9] A. Liston, S. Lesage, J. Wilson, L. Peltonen, C.C. Goodnow, Aire regulates negative selection of organ-specific T cells, Nat. Immunol. 4 (2003) 350–354.
[10] M.S. Anderson, E.S. Venanzi, Z. Chen, S.P. Berzins, C. Benoist, D. Mathis, The cellular mechanism of Aire control of T cell tolerance, Immunity 23 (2005) 227–239.
[11] J.B. Johnnidis, E.S. Venanzi, D.J. Taxman, J.P. Ting, C.O. Benoist, D.J. Mathis, Chromosomal clustering of genes controlled by the aire transcription factor, Proc. Natl. Acad. Sci. USA 102 (2005) 7233–7238.
[12] M.H. Cheng, A.K. Shum, M.S. Anderson, What's new in the Aire?, Trends Immunol 28 (2007) 321–327.
[13] T. Kikuchi, S. Ichimiya, T. Kojima, L. Crisa, S. Koshiba, A. Tonooka, N. Kondo, P.T. van DerSaag, S. Yokoyama, N. Sato, Expression profiles and functional implications of p53-like transcription factors in thymic epithelial cell subtypes, Int. Immunol. 16 (2004) 831–841.
[14] S. Ichimiya, T. Kojima, Cellular networks of human thymicmedullarystromas coordinated by p53-related transcription factors, J. Histochem. Cytochem. 54 (2006) 1277–1289.
[15] M. Halonen, H. Kangas, T. Rüppell, T. Ilmarinen, J. Ollila, M. Kolmer, M. Vihinen, J. Palvimo, J. Saarela, I. Ulmanen, P. Eskelin, APECED-causing mutations in AIRE reveal the functional domains of the protein, Hum. Mutat. 23 (2004) 245–257.
[16] A.J. McCoy, V. Chandana Epa, P.M. Colman, Electrostatic complementarity at protein/protein interfaces, J. Mol. Biol. 268 (1997) 570–584.
[17] M. Shimizu, H. Nakamura, T. Hirabayashi, A. Suganami, Y. Tamura, T. Maruyama, Ser515 phosphorylation-independent regulation of cytosolicphospholipase A2a (cPLA2a) by calmodulin-dependent protein kinase, Cell Signal 20 (2008) 815–824.
[18] F. Cetani, G. Barbesino, S. Borsari, E. Pardi, L. Cianferotti, A. Pinchera, C. Marcocci, A novel mutation of the autoimmune regulator gene in an Italian kindred with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, acting in a dominant fashion and strongly cosegregating with hypothyroid autoimmune thyroiditis, J. Clin. Endocrinol. Metab. 86 (2001) 4747–4752.
[19] T. Ilmarinen, P. Eskelin, M. Halonen, T. Rüppell, R. Kilpikari, G.D. Torres, H. Kangas, I. Ulmanen, Functional analysis of SAND mutations in AIRE supports dominant inheritance of the G228W mutation, Hum. Mutat. 26 (2005) 322–331.
[20] G. Drozina, J. Kohoutek, N. Jabrane-Ferrat, B.M. Peterlin, Expression of MHC II genes, Curr. Top. Microbiol. Immunol. 290 (2005) 147–170.
[21] M.J. Bottomley, M.W. Collard, J.I. Huggenvik, Z. Liu, T.J. Gibson, M. Sattler, The SAND domain structure defines a novel DNA-binding fold in transcriptional regulation, Nat. Struct. Biol. 8 (2001) 626–633.
[22] P.L. Surdo, M.J. Bottomley, M. Sattler, K. Scheffzek, Crystal structure and nuclear magnetic resonance analyses of the SAND domain from glucocorticoidmodulatory element binding protein-1 reveal deoxyribonucleic acid and zinc binding regions, Mol. Endocrinol. 17 (2003) 1283–1295.
[23] J. Gotter, B. Brors, M. Hergenhahn, B. Kyewski, Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters, J. Exp. Med. 199 (2004) 155–166.
[24] T. Rinne, H.G. Brunner, H. van Bokhoven, p63-associated disorders, Cell Cycle 6 (2007) 262–268.
[25] H. Frick, D.M. Munger, J.C. Fauchere, T. Stallmach, Hypoplastic thymus and T-cell reduction in EECUT syndrome, Am. J. Med. Genet. 69 (1997) 65–68.
[26] M. Senoo, F. Pinto, C.P. Crum, F. McKeon, p63 is essential for the proliferative potential of stem cells in stratified epithelia, Cell 129 (2007) 523–536.

Human Immunology 70 (2009) 89-95

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Clonal diversity of cytotoxic T lymphocytes that recognize autologous oral squamous cell carcinoma

Jun-Ichi Kobayashi [a,b], Yoshihiko Hirohashi [a,*], Toshihiko Torigoe [a], Yoshitaka Michifuri [a,b], Takashi Yamamoto [a,b], Yasuaki Tamura [a], Kenjiro Kamiguchi [a], Akihiro Miyazaki [b], Akira Yamaguchi [b], Hiroyuki Hariu [a], Hiroyoshi Hiratsuka [b], Noriyuki Sato [a]

[a] Department of Pathology, Sapporo Medical University, School of Medicine, Sapporo, Japan
[b] Department of Oral Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan

ARTICLE INFO

Article history:
Received 17 March 2008
Accepted 13 November 2008
Available online 3 January 2009

Keywords:
Oral cancer
Squamous cell carcinoma
HLA-A24
CTL, tumor antigen

ABSTRACT

Cytotoxic T lymphocytes (CTLs) play an essential role in immunologic responses for tumor rejection. In the past decade, various melanoma tumor–associated antigens (TAAs) have been identified, and several clinical trials of vaccination immunotherapy and adoptive immunotherapy using such antigens with or without adjuvants have had fascinating results. However, this has not been the case with oral squamous cell carcinoma (OSCC) because of the difficulty of establishing oral cancer cell lines and CTLs against autologous oral cancer cells. Therefore, few oral cancer antigens have been identified with such CTLs.

We herein present the successful establishment of an oral squamous cell carcinoma cell line, POT-1, and an HLA-A24–restricted CTL line (TcPOT-1) from a patient's autologous peripheral blood lymphocytes. TcPOT-1 recognized autologous POT-1 cells in an HLA-A24–restricted manner, and also allogeneic HLA-A24 ( ) OSCC cell lines OSC-70 and HSC-2. We also succeeded in isolating two distinct CTL clones from TcPOT-1, HLA-A24–restricted CTL clone 4F11 and HLA-A33–restricted clone 4A11. Both of these clones recognized autologous POT-1 but not allogeneic OSSC cell lines. These data imply that the TcPOT-1 CTL line may include several CTL subpopulations with distinct antigen specificities, such as an HLA-A24–restricted POT-1–specific clone, HLA-A33–restricted POT-1–specific clone, and HLA-A24–restricted allogeneic OSCC-recognizing clone. Therefore, precise analysis of TcPOT-1–recognizing antigens may provide us with important information on as-yet-unknown tumor rejection antigens in OSCC.

1. Introduction

Recently, huge numbers of tumor–associated antigens (TAAs) have been identified and characterized. For most of them, this has been done through autologous cytotoxic T lymphotye (CTL) clones isolated from peripheral blood mononuclear cells (PBMCs) of melanoma patients [1,2]. Even now, however, little is known about TAAs recognized by autologous CTLs in oral squamous cell carcinoma (OSCC) because of the difficulties in the generation of autologous pairs of CTL clones and OSCC cell lines. Recent advances in reverse immunogenetic strategies have facilitated the identification of antigenic peptides, but such analysis harbors the risk that *in vitro* CTL reactions to allogeneic tumor cells do not always reflect *in vivo* immune reactions against autologous tumor cells. Hence, it is very important to analyze CTLs that recognize autologous OSCC cells for precise identification of tumor-rejection antigens. Thus far, great efforts have been made to establish such autologous pairs of CTLs and tumor cell lines, but notable achievements still remain very rare for OSCC.

Previously, we reported the establishment of OSCC cell line OSC-20 and HLA-DR–restricted autologous CD4 T cells [3,4]. In a subsequent report, we also showed that a human enolase- –derived antigenic peptide could be presented by HLA-DR molecules and might be a target antigen of CD4 T cells [5]. This was the first report concerning the characterization of an OSCC antigenic peptide. However, because enolase- is a ubiquitous self-antigen, further analysis is required to explain the tumor specificity of the CD4 T cells. Taking into consideration that CD8 T cells are an essential component for tumor rejection, identification of HLA class I–restricted antigenic peptides has great importance in the development of cancer immunotherapy. In this context, we have attempted to establish more pairs of OSCC cell lines and CTLs. Herein, we report the establishment of a novel autologous pair of OSCC cell lines, POT-1 and CTL line TcPOT-1, which recognized POT-1 cells in an HLA-A24–restricted manner. We isolated two CTL clones, 4F11 and 4A11, from the TcPOT-1 CTL line. Clone 4F11 recognized POT-1 cells in an HLA-A24–restricted manner, whereas clone 4A11 recog-

* Corresponding author.
E-mail address: hirohash@sapmed.ac.jp (Y. Hirohashi).

0198-8859/09/$32.00 - see front matter
doi:10.1016/j.humimm.2008.11.004

nized POT-1 cells in an HLA-A33–restricted manner. Although parental TcPOT-1 recognized allogeneic HLA-A24 ( ) OSCC cell lines as well as POT-1, CTL clones 4F11 and 4A11 recognized only autologous POT-1 cells. These data indicate that the TcPOT-1 CTL line is composed of several CTL clones with distinct antigen specificities, which may recognize shared OSCC antigens and POT-1–specific antigens.

2. Subjects and methods

2.1. Cell lines

The oral squamous cell carcinoma cell line POT-1 was established from a surgically resected specimen of a 60-years-old female patient with moderately differentiated oral squamous cell carcinoma, and was cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Palo Alto, CA). The HLA genotype of POT-1 was HLA-A*2402/3303, B*4403/5201, Cw*1202/1403, DRB1*1302/1502, DQA1*0102/0103, DQB1*0601/0604, DPB1*0401/ 0901. An Epstein-Barr (EB) virus–transformed B-cell line (POT-1 EBV) was established from autologous PBMCs using B95-8 culture supernatant. OSC-19 (HLA-A2), OSC-20 (HLA-A2/A11, B*4601, Cw*0102), and OSC-70 (HLA-A24) oral squamous cell carcinoma cell lines were also established in our laboratory. Erythroleukemia cell line K562 was purchased from ATCC. HSC-2 (HLA-A*2402/3302, B*44031/5401, Cw*0102/1403), and HSC-3 (HLA-A*0201/2402, B*4801/5201, Cw*0803/1202) oral cell carcinoma cell lines were purchased from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). All of these cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS. Autologous PBMCs from the patient were density-gradient centrifuged with Lymphoprep (Axis Shied, Oslo, Norway) and frozen at 80 C for storage.

2.2. FACScan analysis and monoclonal antibodies

Cells were incubated with murine monoclonal antibodies (mAbs) at saturating concentration for 30 minutes on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with FITC for 30 minutes. Samples were analyzed using a FACScan analyzer (Becton Dickinson, Mountain View, CA). Anti–HLA class I (W6/32), anti–HLA-DR (L243), and anti–HLA-A24 (C7709A2.6) mAbs, as well as anti-HLA-B,C (B1.23.2), were prepared from hybridomas. An anti-A33 monoclonal antibody (Cat. no. 0612HA) was purchased from One Lambda (Canoga Park, CA). Anti-A24 and anti–HLA-B,C hybridomas were kind gifts from Dr. P. G. Coulie (Ludwig Institute for Cancer Research, Brussels, Belgium). To increase the protein expression of HLA molecules, cell lines were treated with 100 U/ml interferon (IFN)– for 48–72 hours.

2.3. CTL induction and establishment of CTL clones

Mixed lymphocyte tumor cell culture was performed as described previously [6]. Briefly, CD8 T cells were positively separated from an OSCC patient's PBMCs using immunomagnetic beads coupled with an anti-CD8 mAb (Miltenyli Biotech GmbH, Germany) following the manufacturer's protocol. Then 2 10^6 CD8 T cells were cocultured in a 24-well plate with 2 10^5 irradiated POT-1 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; a kind gift from Takeda Pharmaceutical Co., Osaka, Japan) was added at a final concentration 20 IU/ml. The same mixed lymphocyte tumor cell culture procedure was performed every 7 days. After stimulation four times, cytotoxic activity and IFN- secretion were measured with the standard ^{51}Cr release assay and enzyme-linked immunoabsorbent assay (ELISA), respectively.

To obtain the CTL clones, standard limiting dilution was performed as described previously [7]. Briefly, series of diluted CTLs were incubated with 1 10^4 100 Gy-irradiated LG2-EBV (a kind gift from Dr. B. J. Eynde, Ludwig Institute for Cancer Research, Brussels, Belgium) and 5 10^4 allogeneic PBMCs as feeder cells in 200 l of AIM-V medium supplemented with 10% HS, the anti-CD3 Ab (40 ng/ml) (BD), and 100U/mL rIL-2 in 96-well round plates. On day 7, the culture medium was half exchanged for AIM-V supplemented with 10% HS and 100 U/mL rIL-2. On day 14, growing wells were transferred to 24-well culture plates and the specific cytotoxic activity was examined by ^{51}Cr release assay.

2.4. Cytotoxicity assay and ELISA

The lytic activity of CTLs was tested by ^{51}Cr release assay as described previously [6]. Briefly, 2000 target cells labeled with 100 Ci of chromium were incubated in 96-well microtiter plates with CTLs at different E/T ratios in 200 l of AIM-V medium. ^{51}Cr release was measured after 6 hours' incubation in a 37 incubator. For the inhibition assay with monoclonal antibodies (mAbs), ^{51}Cr-labeled target cells were incubated with appropriate concentrations of mAbs at room temperature for 60 min, before ^{51}Cr release assay. Percent cytotoxicity was calculated as: % Cytotoxicity (experimental release spontaneous release)/(maximum release spontaneous release) 100. All target cells were treated with 100 U/ml IFN- for 48–72 hours before assay.

For the detection of IFN- in the culture supernatant, standard IFN- ELISA (R&D Systems, Minneapolis, MN) was performed following the manufacturer's instructions.

For the specific inhibition with antibodies, an anti–HLA-class I mAb (W6/32), anti–HLA-B,C mAb (B1.23.2), anti–HLA-A24 mAb (C7709A2.6), and anti–HLA-DR mAb (L243) were added at half the volume of the hybridoma culture supernatant.

2.5. Immunoselection

For the immune selection of cancer cells, 1 10^6 POT-1 cells were cocultured with 1 10^8 TcPOT-1 clones for 6 hours, and then the CTLs were removed. The same coculture procedure was performed weekly. After immunoselection four times, POT-1 variant cells were established.

3. Results

3.1. Establishment of the oral squamous cell carcinoma cell line POT-1

For successful identification of TAAs, it is essential to analyze an autologous tumor cell line and CTL pair. Therefore, we attempted to establish an oral squamous cell carcinoma cell line. A surgically resected specimen of oral squamous cell carcinoma from a 60-year-old female patient was cultured in RPMI-1640 supplemented with 10% FBS as described in Subjects and methods, and we successfully established oral squamous cell carcinoma cell line POT-1. The primary lesion of the carcinoma specimen was well-differentiated squamous cell carcinoma (Fig. 1A). This cell line had a standard doubling time of 48–72 hours, and grew stably through more than 50 passages. Moreover, this cell line exhibited tumor-forming ability in immune-compromised host mice, and the tumors were typical squamous cell carcinoma resembling the primary lesion (Fig. 1B).

For immunologic evaluation of the POT-1 cell line, the surface MHC molecules were then examined by FACScan analysis. As shown in Fig. 2, POT-1 expressed a pan-HLA class I molecule (W6/32), HLA-B,C (B1.23.2), HLA-A24 (C7709A2.6), HLA-A33, and a relatively low level of HLA-DR (L243). The expression levels of HLA-DR molecules were enhanced by IFN- treatment for 48 hours, but those of HLA-class I molecules were not. These HLA expression profiles might indicate that immune cells can interact with POT-1 through HLA-class I molecules.

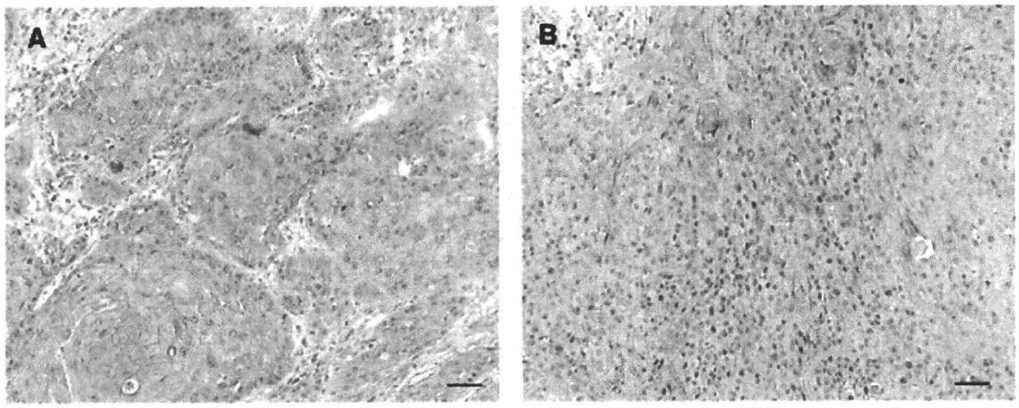

Fig. 1. Pathologic findings of the primary oral squamous cell carcinoma lesion of the POT-1 patient (A), and the tumor tissue in immune-deficient mice (B). Hematoxylin and eosin stain; magnification 100 (scale bar, 100 m).

3.2. Induction and analysis of CTLs

To establish CTLs that recognized the POT-1 cell line, we stimulated PBMCs with autologous POT-1 several times. After stimulation four times, the specific reactivity was evaluated by ^{51}Cr release assay (Fig. 3A). The CTLs (TcPOT-1) recognized autologous POT-1 specifically, but not the autologous EB-virus–transformed B cells (POT-1 EBV) or erythroleukemia cell line K562 used as negative controls. For further analysis of TcPOT-1, cytotoxic activity was measured against the allogeneic OSCC cell line by ^{51}Cr release assay (Fig. 3A). TcPOT-1 line recognized allogeneic OSCC cell line HSC-2 (HLA-A*2402/3302, B*44031/5401, Cw*0102/1403) cell line, but did not OSC-19 and OSC-70 cell lines. To confirm the HLA restriction of TcPOT-1, blocking assays with HLA-specific antibodies were performed. As shown in Fig. 3B, the cytotoxic activity was blocked by an anti–HLA-class I antibody (W6/32). The cytotoxic reactivity was also likely to be inhibited with anti–HLA-A24 antibody (C7709A2.6) but not inhibited by an anti-HLA-B,C antibody (B1.23.2) or anti-HLA-DR antibody (L243). Inasmuch as we could observe independent experiments, some populations of TcPOT-1 line might be restricted HLA-A24 molecule, and this suggests the possibility that TcPOT-1 recognized HSC-2 on the context of HLA-A24 molecule.

3.3. Establishment of TcPOT-1 clones

For detailed analysis of TcPOT-1, CTL TcPOT-1 clone lines were generated by the standard limiting dilution method as described in Subjects and methods. We successfully established two CTL clones, designated 4F11 and 4A11. As shown in Fig. 4A, clones 4F11 and 4A11 both recognized the autologous POT-1 cell line specifically compared with POT-1 EBV cells used as a negative control. Next, blocking assays using mAbs were performed to identify the HLA-restriction elements. As shown in Fig. 4B, the IFN- –secreting ability of clone 4F11 was blocked by anti–HLA-class I and anti–HLA-A24 antibodies, indicating that 4F11 was an HLA-A24–restricted CTL. On the other hand, the IFN- secretion of clone 4A11 was inhibited by an anti–HLA-class I antibody, but not other antibodies, including anti–HLA-A24 and HLA-B,C (Fig. 4C). This highly suggested that clone 4A11 is restricted to another allele of the HLA-A locus, HLA-A33. To analyze clone 4F11 and 4A11 in detail, the

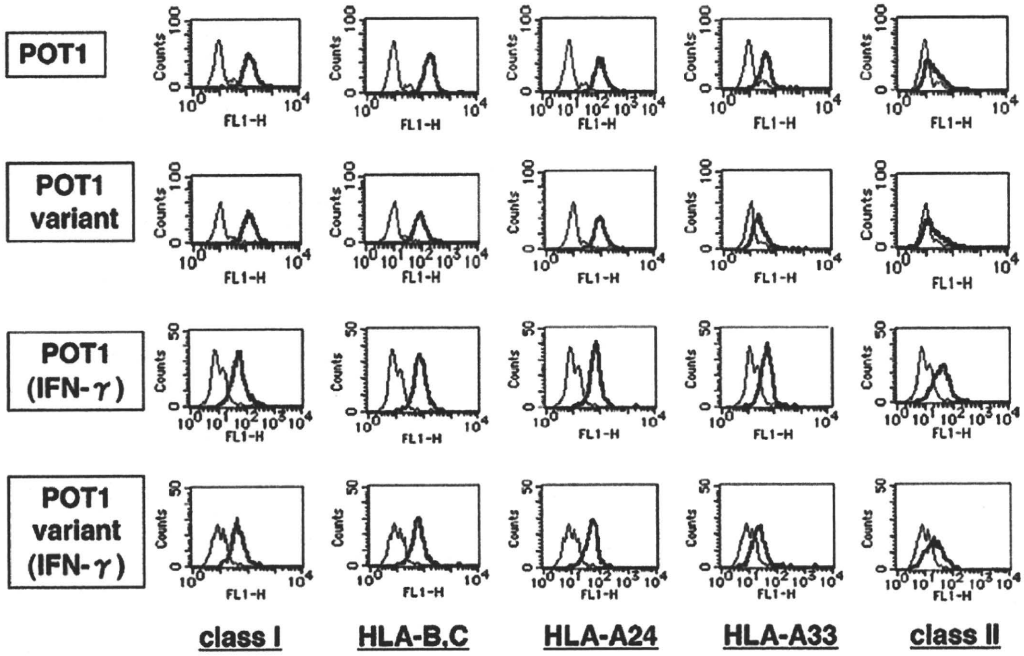

Fig. 2. FACScan analysis of the surface HLA molecules. Several cell lines were evaluated with FACScan analysis. Anti–pan-HLA-class I (W6/32), anti-HLA-B,C (B1.23.2), anti-HLA-A24 (C7709A2.6), anti–HLA-A33 (0612HA) and anti–HLA-DR (L243) monoclonal antibodies were used for specific detection.

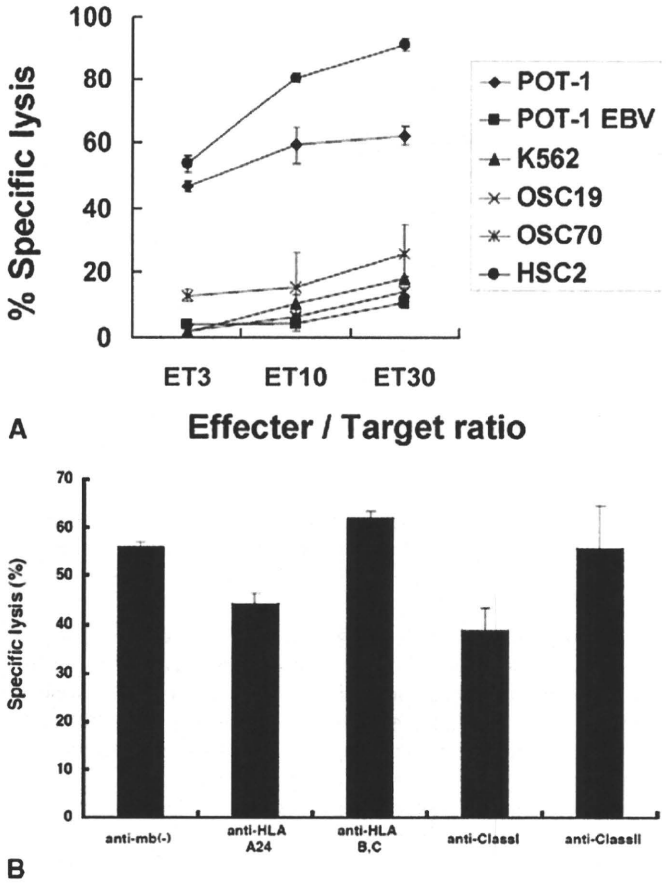

Fig. 3. Cytotoxic activity of TcPOT-1 CTL line. (A) TcPOT-1 CTL cells were examined for cytotoxic activity against autologous oral squamous cell carcinoma cell line POT-1 by ^{51}Cr release assay as described in Subjects and methods. POT-1 EBV is the autologous EBV-B cell line. (B) Cytotoxic activity against allogeneic oral squamous cell carcinoma was examined. Allogeneic oral squamous cell lines OSC-19 (HLA-A2), OSC-70 (HLA-A24) and HSC-2 (HLA-A*2402/3302, B*44031/5401, Cw*0102/1403) were used as target cells. (C) Blocking assays with HLA-specific mAbs was performed as described in Subjects and methods. Anti-pan-HLA-class I (W6/32), anti-HLA-B,C (B1.23.2), anti-HLA-A24 (C7709A2.6), and anti-HLA-DR (L243) monoclonal antibodies were used. Similar results could be obtained with independent experiments three times.

reactivity against allogeneic OSCC cell lines was examined (Fig. 4B and 4C). Clones 4F11 and 4A11 recognized the autologous POT-1 cell line but not allogeneic OSCC cell lines including OSC-19, OSC-20, HSC-2, and HSC-3. These data indicated that 4F11 and 4A11 recognized the POT-1 specific antigen in an HLA-A24- and HLA-A33-restricted manner, respectively.

3.4. *Immune selection of POT-1 and establishment of immune-tolerant subline POT-1 variant*

For further analysis of TAAs of POT-1, we established a POT-1 subline that was immunoselected by coculture with both CTL clones 4F11 and 4A11. The established subline was termed as a POT-1 variant. As shown in Fig. 5, CTL clones 4F11 and 4A11 recognized POT-1, but not POT-1 variant or the negative controls POT-1 EBV and K562. However, the TcPOT-1 line did recognize POT-1 variant as well as POT-1, suggesting TcPOT-1 line mainly recognized different antigens as CTL clones 4F11 and 4A11. For immunologic evaluation of POT-1 variant, cell surface HLA expression was also examined by FACScan analysis (Fig. 2). The expression levels of HLA-class I, HLA-B,C, and HLA-A24 showed no significant difference compared with POT-1. The expression of HLA-A33 was relatively deceased compared with POT-1, but the expression of HLA-A33 was restored with IFN- treatment. Thus, the expression levels of HLA molecules might not be critical for the immunoescape of POT-1 variant cells, and POT-1 variant might lose some antigen gene expression or antigen-presenting machinery. Therefore, OSCC cell line POT-1 seems to express at least three different TAAs, and TcPOT-1, 4F11, and 4A11 recognize independent antigens.

4. Discussion

Oral squamous cell carcinoma is a major malignancy all over the world, with an annual mortality rate 5–10 per 100,000 in industri-

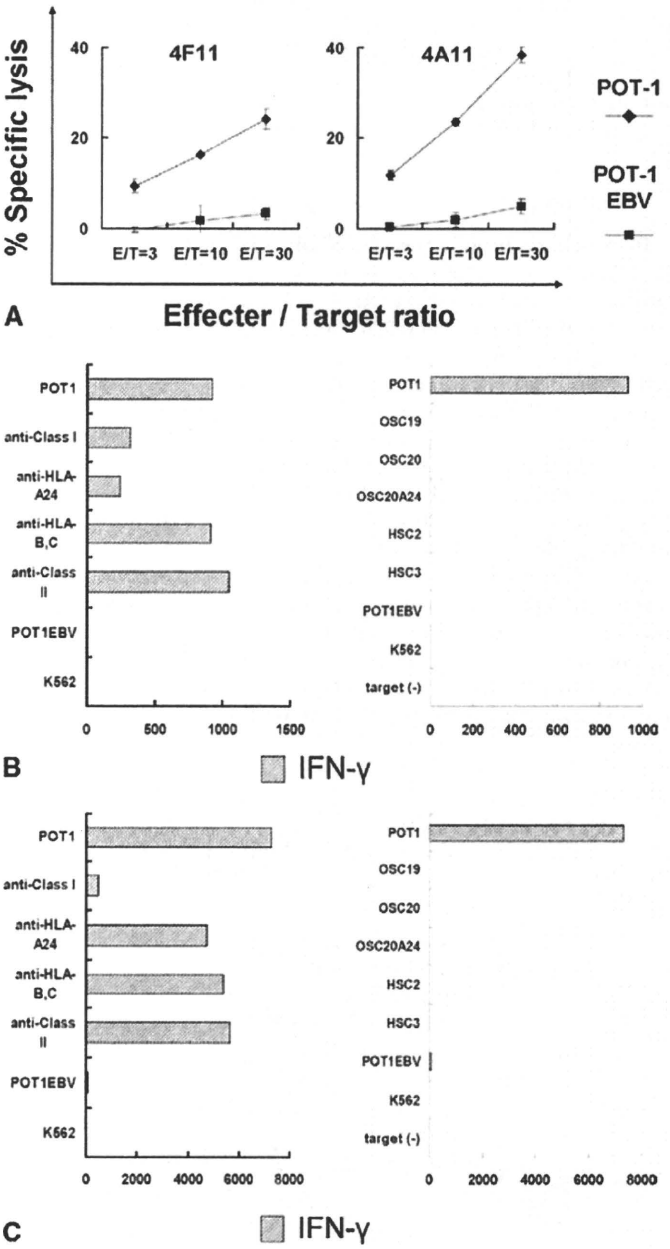

Fig. 4. Establishment and characterization of CTL clones 4-F-11 and 4-A-11. (A) Cytotoxic activities of CTL clones 4-F-11 and 4-A-11 for autologous OSCC cell line POT-1 and autologous EBV-B cell line POT-1 EBV were examined by ^{51}Cr release assay. (B) Reactivities of CTL clone 4F11. Blocking assays with mAbs were performed with CTL clones using IFN- ELISA. Anti-pan-HLA-class I (W6/32), anti-HLA-B,C (B1.23.2), anti-HLA-A24 (C7709A2.6), and anti-HLA-DR (L243) monoclonal antibodies were used. 4F11 reactivity for allogeneic OSCC cell lines were evaluated. Allogeneic oral squamous cell lines OSC-19 (HLA-A2), OSC-20 (HLA-A2/A11, B*4601, Cw*0102), OSC-20-A24 HLA-A24 transfected OSC-20 cell line, OSC-70 (HLA-A24) and HSC-2 (HLA-A*2402/3302, B*44031/5401, Cw*0102/1403) were used as target cells. (C) Reactivities of CTL clone 4A11.

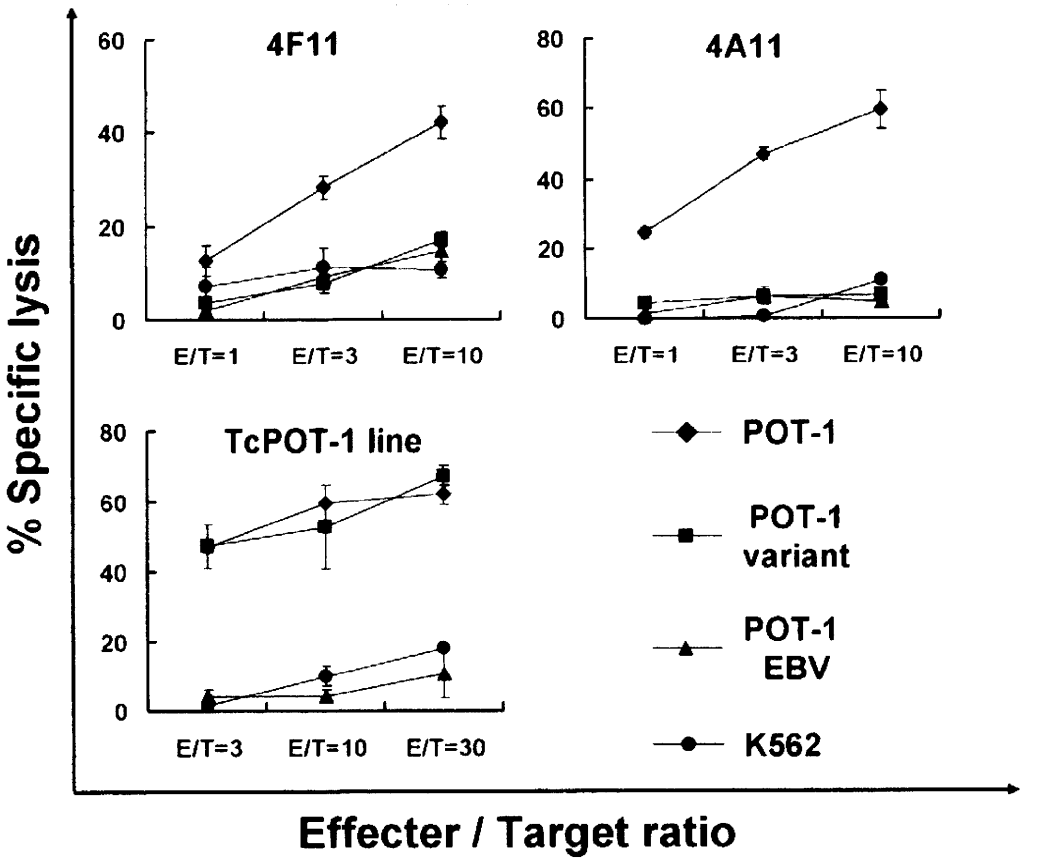

Fig. 5. Immunologic escape of POT-1 variant. Cytotoxic activity of CTL clones 4F11 and 4A11 and TcPOT-1 CTL line were assessed the reactivity for POT-1 and POT-1 variant cell line.

alized countries. Surgical resection, chemotherapy and radiation therapy are the major treatments for OSCC. With recent progress, the outcome of the disease has greatly improved. However, even now, the prognosis for advanced cases (i.e. with neck lymph node metastasis or distant metastasis), is still not favorable. Therefore, another breakthrough in OSCC therapy is desired.

With recent innovative advances in cancer immunity initiated from the identification of the MAGE gene family and other melanoma antigens [8,9], immune therapy can be one of the representative therapies for malignancies. Using such melanoma antigens, several groups reported the efficacy of cancer immunotherapy for advanced melanoma patients [10,11]. There are three major approaches to identifying TAAs. These reports prompted the further analysis of other epithelial malignancies. Previously, we reported autologous cancer cell line and CTL pairs from several epithelial malignancies [3,12–15]. By analysis of those CTLs, we succeeded in isolating novel cancer-associated antigens [5,16], as well as antigenic peptides by reverse immunogenetic analysis [7,17–19]. With this progress, we started a clinical trial for cancer vaccine therapy with an antigenic peptide derived from one of the cancer-associated antigens, survivin [20]. Some patients showed decreases in tumor markers and remarkable tumor regression. These findings suggested that cancer vaccine therapy might also be effective for OSCC patients.

In this study, we successfully established OSCC cell line POT-1 and autologous CTL line Tc-POT-1. TcPOT-1 did recognized allogeneic OSCC cell line HSC-2. This suggests TcPOT-1 line recognized POT-1 and HSC-2 shared TAA (antigen C in Fig. 6). Inasmuch as POT-1 and HSC-2 shared several HLA-class I molecules, we cannot definitely conclude which HLA is the restriction element of TcPOT-1. However, the cytotoxic activity of TcPOT-1 tended to be inhibited with anti-HLA-A24 mAb, HLA-A24 might be one candidate. Because antigen C was shared with allogeneic cell line, this is presumed to be overexpressed antigen. As previously reported, cancer-testis antigens and Wilms' tumor 1 (WT1) antigens express in OSCC [21,22]. There is possibility that antigen C is one of the cancer-testis antigen or WT1. Both 4F11 and 4A11 recognized only autologous cell line, highly suggesting these CTL clones recognized POT-1–specific antigens (antigens A and B in Fig. 6). Autologous cell–specific antigens are usually coded by mutated genes [23]. Thus, antigens A and B seem to be coded with some mutated genes. However, at this moment, we cannot conclude that antigen A and antigen B are coded in same genes or different genes. The gene cloning will resolve all of these questions.

Some analyses of OSCC cancer immunity have been reported, including *in vivo* analysis of immunologic parameters. Cruz *et al.* showed that $_2$ microglobulin expression in cancer tissues was down-regulated in 17 of 47 OSSC cases [24]. The decreased level of $_2$ microglobulin correlated well with decreased expression of HLA class I and decreased infiltration of immune cells such as CD8 T cells. Matsui *et al.* reported that antigen-presenting machinery such as TAP1/2, LMP7/tapasin, and PA28 / were downregulated in OSCC cell lines [25]. We also examined the HLA-class I expression level of OSCC in surgically resected specimens using the novel anti-HLA class I antibody EMR8-5. A decrease in HLA-class I was observed in 22 of 81 cases at several levels (data not shown). These findings indicated that the immunologic surveillance *in vivo* was potent enough to reject cancer cells. Therefore, vaccination therapy with antigenic peptides of OSCC patients might have enough potential to remove cancer cells. Recently we reported that HLA-class I downregulation was one of the factors for poor prognoses in bladder cancer and osteosarcoma [26,27]. These findings also sup-

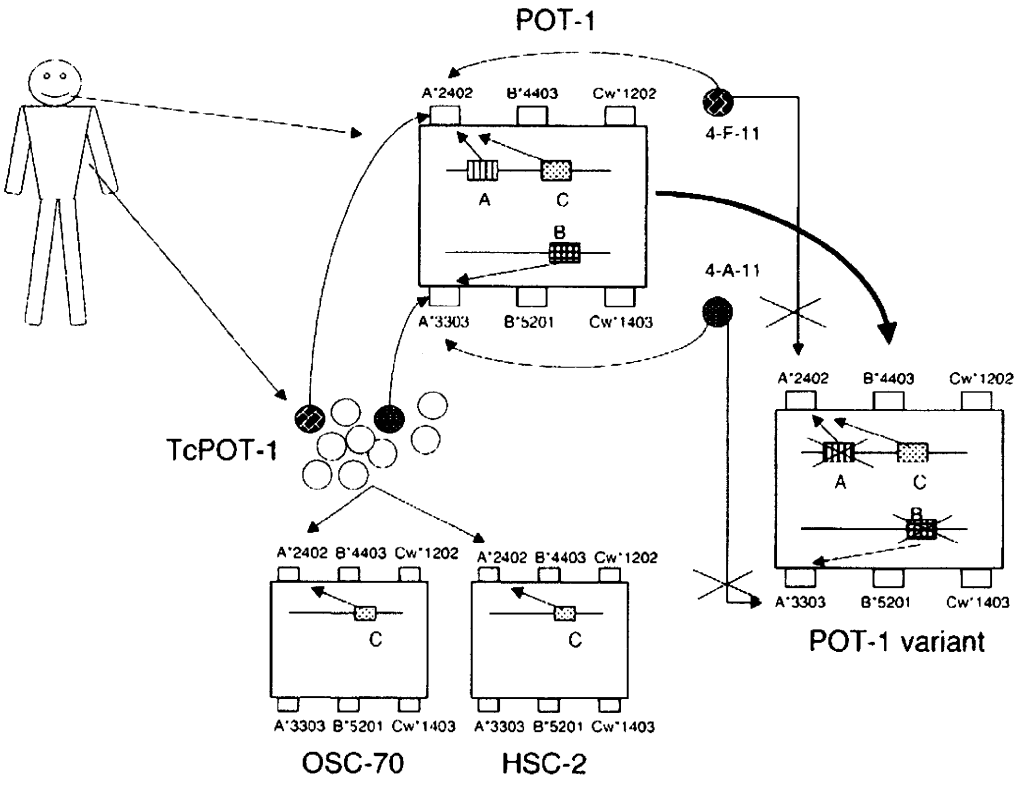

Fig. 6. POT-1 was established from a 60-year-old female OSCC patient. TcPOT-1 contains several clone populations of CTLs specific for more than 3 TAAs. One was the highly likely HLA-A24 restricted clone specific for OSCC shared TAA (C). The others were an HLA-A24 restricted clone specific for POT-1-specific antigen (A) and an HLA-A33 restricted clone specific for POT-1-specific antigen (B).

port the idea that immunoreaction against cancer cells has sufficient competency for cancer rejection.

Recently, Morgan *et al.* reported remarkable results for adoptive cancer immune therapy [28]. After transduction of anti–MART-1, gp100, NY-ESO-1, and p53 TCRs into autologous T lymphocytes and expansion of CTLs *in vitro*, more than 1 billion CTLs were transferred into advanced melanoma patients with 720,000 IU/kg IL-2 every 8 hours. One month later, two of 17 patients showed partial responses (PR) of solid tumors, and the tumor regression lasted more than 10 months. This report showed the CTLs were powerful for tumor regression in advanced melanoma patients. This was also the case with the POT-1 patient. The CTL cytotoxicity of TcPOT-1 was 60% at an E/T ratio of 30 and was quite high compared with the autologous CTLs we previously reported. After surgical resection of the oral squamous cell carcinoma, the patient is still disease free and has enjoyed good quality of life for more than 4 years. One of the reasons for the good prognosis was the high CTL reactivity. Furthermore, the CTL precursor frequency seemed to be quite high because we succeeded in inducing CTLs from the POT-1 patient's PBMCs three times over 3 years. These findings also support the idea that CTL is quite potent and plays an essential role in cancer immunosurveillance.

As shown in Fig. 6, the TcPOT-1 CTL line and CTL clones 4F11 and 4A11 recognized more than 3 TAAs. Because HLA-A24 and A33 loci are common throughout the world, further analysis of TcPOT-1 and clones 4-F-11 and 4-A-11 should be informative for the analysis of OSCC cancer immunity.

Acknowledgments

We thank Drs. P. G. Coulie and B. J. Van den Eynde for kindly providing cell lines.

References

[1] Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol 2006;24:175–208.
[2] Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 1999;10:281–7.
[3] Miyazaki A, Sato N, Takahashi S, Sasaki A, Kohama G, Yamaguchi A, et al. Cytotoxicity of histocompatibility leukocyte antigen-DR8-restricted CD4 killer T cells against human autologous squamous cell carcinoma. Jpn J Cancer Res 1997;88:191–7.
[4] Sato N, Nabeta Y, Kondo H, Sahara H, Hirohashi Y, Kashiwagi K, et al. Human CD8 and CD4 T cell epitopes of epithelial cancer antigens. Cancer Chemother Pharmacol 2000;46 (Suppl):S86–90.
[5] Kondo H, Sahara H, Miyazaki A, Nabeta Y, Hirohashi Y, Kanaseki T, et al. Natural antigenic peptides from squamous cell carcinoma recognized by autologous HLA-DR8-restricted CD4 T cells. Jpn J Cancer Res 2002;93:917–24.
[6] Sato T, Sato N, Takahashi S, Koshiba H, Kikuchi K. Specific cytotoxicity of a long-term cultured T-cell clone on human autologous mammary cancer cells. Cancer Res 1986;46:4384–9.
[7] Maeda A, Ohguro H, Nabeta Y, Hirohashi Y, Sahara H, Maeda T, et al. Identification of human antitumor cytotoxic T lymphocytes epitopes of recoverin, a cancer-associated retinopathy antigen, possibly related with a better prognosis in a paraneoplastic syndrome. Eur J Immunol 2001;31:563–72.
[8] van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643–7.
[9] Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannelli JR, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-nized by theumor infiltrating lymphocytes. J Exp Med 1994;180:347–52.
[10] Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321–7.
[11] Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 1999;80:219–30.
[12] Kashiwagi K, Ikeda H, Hirohashi Y, Yamamoto M, Idenoue S, Hirai I, et al. Analysis of a shared pancreatic cancer antigen recognized by an HLA-A*2601-restricted cytotoxic T-lymphocyte clone. Pancreas 2003;26:e81–8.

[13] Ueda D, Sato N, Matsuura A, Sasaki A, Takahashi S, Ikeda H, et al. T-cell receptor gene structures of HLA-A26-restricted cytotoxic T lymphocyte lines against human autologous pancreatic adenocarcinoma. Jpn J Cancer Res 1995;86:691–7.
[14] Yasoshima T, Sato N, Hirata K, Kikuchi K. The mechanism of human autologous gastric signet ring cell tumor rejection by cytotoxic T lymphocytes in the possible context of HLA-A31 molecule. Cancer 1995;75:1484–9.
[15] Nabeta Y, Kawaguchi S, Sahara H, Ikeda H, Hirohashi Y, Goroku T, et al. Recognition by cellular and humoral autologous immunity in a human osteosarcoma cell line. J Orthop Sci 2003;8:554–9.
[16] Tsukahara T, Nabeta Y, Kawaguchi S, Ikeda H, Sato Y, Shimozawa K, et al. Identification of human autologous cytotoxic T-lymphocyte-defined osteosarcoma gene that encodes a transcriptional regulator, papillomavirus binding factor. Cancer Res 2004;64:5442–8.
[17] Hirohashi Y, Torigoe T, Maeda A, Nabeta Y, Kamiguchi K, Sato T, et al. An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. Clin Cancer Res 2002;8:1731–9.
[18] Idenoue S, Hirohashi Y, Torigoe T, Sato Y, Tamura Y, Hariu H, et al. A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins. Clin Cancer Res 2005;11:1474–82.
[19] Hariu H, Hirohashi Y, Torigoe T, Asanuma H, Hariu M, Tamura Y, et al. Aberrant expression and potency as a cancer immunotherapy target of inhibitor of apoptosis protein family, Livin/ML-IAP in lung cancer. Clin Cancer Res 2005;11:1000–9.
[20] Tsuruma T, Hata F, Torigoe T, Furuhata T, Idenoue S, Kurotaki T, et al. Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer. J Transl Med 2004;2:19.
[21] Eura M, Ogi K, Chikamatsu K, Lee KD, Nakano K, Masuyama K, et al. Expression of the MAGE gene family in human head-and-neck squamous-cell carcinomas. Int J Cancer 1995;64:304–8.
[22] Oji Y, Inohara H, Nakazawa M, Nakano Y, Akahani S, Nakatsuka S, et al. Overexpression of the Wilms' tumor gene WT1 in head and neck squamous cell carcinoma. Cancer Sci 2003;94:523–9.
[23] Mandruzzato S, Brasseur F, Andry G, Boon T, van der Bruggen P. A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma. J Exp Med 1997;186:785–93.
[24] Cruz I, Meijer CJ, Walboomers JM, Snijders PJ, Van der Waal I. Lack of MHC class I surface expression on neoplastic cells and poor activation of the secretory pathway of cytotoxic cells in oral squamous cell carcinomas. Br J Cancer 1999;81:881–9.
[25] Matsui M, Ikeda M, Akatsuka T. High expression of HLA-A2 on an oral squamous cell carcinoma with down-regulated transporter for antigen presentation. Biochem Biophys Res Commun 2001;280:1008–14.
[26] Kitamura H, Torigoe T, Honma I, Sato E, Asanuma H, Hirohashi Y, et al. Effect of human leukocyte antigen class I expression of tumor cells on outcome of intravesical instillation of bacillus Calmette-Guerin immunotherapy for bladder cancer. Clin Cancer Res 2006;12:4641–4.
[27] Tsukahara T, Kawaguchi S, Torigoe T, Asanuma H, Nakazawa E, Shimozawa K, et al. Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. Cancer Sci 2006;97:1374–80.
[28] Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 2006;314:126–9.

Journal of Translational Medicine BioMed Central

Research

Open Access

Comparative study on the immunogenicity between an HLA-A24-restricted cytotoxic T-cell epitope derived from survivin and that from its splice variant survivin-2B in oral cancer patients

Jun-ichi Kobayashi[1,2], Toshihiko Torigoe*[1], Yoshihiko Hirohashi[1], Satomi Idenoue[3], Akihiro Miyazaki[2], Akira Yamaguchi[2], Hiroyoshi Hiratsuka[2] and Noriyuki Sato[1]

Address: [1]Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan, [2]Department of Oral Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan and [3]Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan

Email: Jun-ichi Kobayashi - jkoba@sapmed.ac.jp; Toshihiko Torigoe* - torigoe@sapmed.ac.jp; Yoshihiko Hirohashi - yhirohashi@yahoo.co.jp; Satomi Idenoue - idenoue@sapmed.ac.jp; Akihiro Miyazaki - amiyazak@sapmed.ac.jp; Akira Yamaguchi - yamaguch@sapmed.ac.jp; Hiroyoshi Hiratsuka - hiratuka@sapmed.ac.jp; Noriyuki Sato - nsatou@sapmed.ac.jp

* Corresponding author

Published: 6 January 2009

Journal of Translational Medicine 2009, **7**:1 doi:10.1186/1479-5876-7-1

Received: 30 July 2008
Accepted: 6 January 2009

This article is available from: http://www.translational-medicine.com/content/7/1/1

Abstract

Background: We previously reported an HLA-A24-restricted cytotoxic T-cell epitope, Survivin-2B80-88, derived from a splice variant of survivin, survivin-2B. In this report, we show a novel HLA-A24-restricted T-cell epitope, Survivin-C58, derived from a wild type survivin, and compared their immunogenicity in oral cancer patients.

Methods: By stimulating peripheral blood lymphocytes of HLA-A24-positive cancer patients with Survivin-C58 peptide *in vitro*, the peptide-specific CTLs were induced. In order to compare the immunogenic potential between C58 peptide and 2B80-88 peptide, peripheral blood T-cells from thirteen HLA-A24-positive oral cancer patients were stimulated with either or both of these two peptides.

Results: Survivin-2B80-88 peptide-specific CTLs were induced from four patients, and C58 peptide-specific CTLs were induced from three out of eight patients with over stage II progression. The CTLs exerted cytotoxicity against HLA-A24-positive tumor cells. In contrast, CTL induction failed from a healthy volunteer and all four patients with cancer stage I.

Conclusion: It was indicated that a splicing variant-derived peptide and wild type survivin-derived peptide might have a comparable potency of CTL induction, and survivin targeting immunotherapy using survivin-2B80-88 and C58 peptide cocktail should be suitable for HLA-A24+ oral cancer patients.

Background

Survivin, an inhibitor of apoptosis protein, is highly expressed in the vast majority of cancers [1,2]. Survivin has been shown to increase tumor resistance to apoptotic stimuli, such as radiation and chemotherapy [3,4]. In agreement with these findings, a number of reports demonstrate that survivin expression in cancer cells has a prognostic value and is associated with increased tumor recurrence and shorter patient survival [5-10], although the opposite correlation is reported in certain cancers [11]. So far, four different splicing variants of human survivin have been described, including survivin-2!, survivin-2B, survivin-ΔEx3, and survivin-3B [12-15]. While survivin-2! and survivin-3B are truncated forms, survivin-2B results from alternating splicing at the interface between exon 2 and exon 3, leading to insertion of an additional exon, termed exon 2B, in BIR domain. Since BIR domain is a functional domain that is important for the anti-apoptotic activity of survivin, survivin-2B is predicted to be non-anti-apoptotic [16,17].

Survivin was originally detected only in normal thymus, testis and placenta; however, low levels of wild type survivin was detected in other normal tissues, such as activated T-cells, vascular endothelial cells, and hematopoietic cells by more sensitive methods [3,18,19]. Wild type survivin is known to have an essential role in the mitosis [3,18,20]. It forms a complex with the chromosomal passenger proteins during mitosis and regulates mitotic progression. In contrast, the protein levels as well as the mRNA levels of survivin-2B and other survivin variants are far less than that of wild type survivin, and they are dispensable in such a mitotic checkpoint [17,21].

Since survivin expression is very low in normal differentiated adult tissues as compared with that in cancer tissues, survivin is considered to be an ideal molecular target for cancer immunotherapy. With this mind, we attempted to identify a HLA-A24-restricted cytotoxic T-lymphocyte (CTL) epitopes of survivin that were suitable for cancer vaccine, since HLA-A24 was the most frequent allele in Japanese. In our previous report, three peptides derived from survivin and its splicing variant survivin-2B were examined for HLA-A24-binding affinity and immunogenicity [22]. It was shown that Survivin-2B80-88 peptide (amino acid sequence AYACNTSTL), which was derived from a splicing variant survivin-2B-specific exon2B, was capable of inducing CTLs that had killing activity to HLA-A24+ cancer cells. Following this report, we provided further evidence that Survivin-2B80-88 was highly immunogenic in various cancer patients, including those with gastric cancer, breast cancer, and colorectal cancer [23]. Based on these results ex vivo, we have conducted phase I clinical trials assessing the adverse event and efficacy of Survivin-2B80-88 peptide vaccination in patients with advanced colorectal cancer, breast cancer, lung cancer, bladder cancer, and oral cancer [24-26].

Though we failed to identify an HLA-A24-restricted CTL epitope derived from wild type survivin in the initial study, a number of epitopes have been identified from wild type survivin that are restricted to other HLA class I alleles, such as A1, A2, A11, and B35 [27-29], some of which have been applied for clinical trials [30,31]. More recently, Andersen, et al. demonstrated that wild type survivin-derived Sur20-28 peptide (amino acid sequence STFKNWPFL) was capable of inducing the peptide-specific CD8-positive T-cells from PBMCs of HLA-A24+ cancer patients, although HLA-A24-restricted killing activity of the peptide-specific T-cells against survivin-positive cancer cells has not been assessed [32]. In this study, we present a novel CTL epitope Survivin-C58 peptide derived from wild type survivin. The peptide-specific CTLs induced from peripheral blood mononuclear cells (PBMCs) of oral cancer patient exerted HLA-A24-restricted cytotoxicity against the tumor cells. Then, we stimulated PBMCs of oral cancer patients with either or both Survivin-C58 and Survivin-2B80-88 peptides, and the consequent CTLs were examined for the peptide-specificity and cytotoxicity against HLA-A24+ tumor cells. We demonstrate here for the first time a comparative study on the potency of inducing CTLs in vitro between wild type survivin-derived peptide and survivin-2B-derived peptide, which indicates the comparable potency of CTL induction in oral cancer patients.

Materials and methods

Patients and samples

Surgically-resected cancer specimens and PBMCs used in this study were obtained from HLA-A*2402+ patients with breast cancer or oral cancer who were hospitalized at Sapporo Medical University Hospital after obtaining their informed consent. The patients' clinicopathological profiles were listed on the table.

Cell lines and culture media

Human breast cancer cell lines, HMC-1 and HMC-2, human oral squamous cell carcinoma (OSCC) cell lines, OSC19, OSC20, OSC30, OSC40, OSC70, and POT1 were established in our laboratory. OSCC cell lines HO-1-NH, KOSC-3, HSC-2, HSC-3, and HSC-4 were purchased from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). OSCC cell line SAS was obtained from the Institute of Development, Aging and Cancer Tohoku University (Tohoku, Japan). Human embryonic kidney cell line 293T, breast cancer cell line MCF7, lymphoma cell line Daudi and leukemia cell line K562 were purchased from American Type Culture Collection (Manassas, VA). C1R-A24 and C1R-A31, lymphoblastoid cell line C1R transfectants with HLA-A*2402 and HLA-A*31012 cDNA

respectively, were kind gifts from Dr. M. Takiguchi (Kumamoto University School of Medicine, Kumamoto, Japan). T2-A24, a stable transfectant of T2 cells with HLA-A*2402 cDNA was a kind gift from Dr. K. Kuzushima (Aichi Cancer Research Institute, Nagoya, Japan).

293T cells and breast cancer lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 mg/ml streptomycin at 37°C in humidified 5% CO_2 atmosphere. All the OSCC cell lines were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin G, and 100 #g/ml streptomycin at 37°C in a humidified 5% CO2 atmosphere. OSC20-A24, a stable transfectant of OSC20 with HLA-A*2402 cDNA was cultured in a medium supplemented with 800 ng/ml of puromycin (Sigma-Aldrich, St. Louis, MO). Hygromycin B (0.5 mg/ml, WAKO chemicals, Osaka, Japan) or G418 (800 #g/ml, GIBCO/Invitrogen Corp., Carlsbad, CA) was continuously added to the culture medium for C1R transfectants and T2 transfectant, respectively.

RT-PCR Analysis

A set of total RNA from normal human adult tissues was purchased from Clontech (human total RNA master panel). Total RNA was isolated from cultured cells by using ISOGEN reagent (Nippon Gene, Tokyo, Japan). The cDNA mixture was synthesized from 1 mg of total RNA by reverse transcription using Superscript II and oligo(dT) primer (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol. PCR amplification was performed in 50 ml of PCR mixture containing 1 mL of the cDNA mixture, KOD Plus DNA polymerase (Toyobo, Osaka, Japan), and 50 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 57°C for 30 s, and extension at 68°C for 1 min. Primer pairs used for RT-PCR analysis were 5'-TCAAGGACCACCGCATCTCTAC-3' and 5'-GCACTTTCT-TCGCAGTTTCCTC-3' as a forward and a reverse primer, respectively. Expected sizes of PCR products for wild type survivin, survivin-2B, and survivin-DEx3 were 355 bp, 424 bp, and 236 bp, respectively. As an internal control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was detected by using a forward primer 5'-ACCACAGTC-CATGCCATCAC-3' and a reverse primer 5'-TCCACCAC-CCTGTTGCTGTA-3' with an expected PCR product of 452 bp. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.0% agarose gel. Nucleotide sequence of the PCR products was confirmed by direct sequencing using ABI Genetic analyzer PRIM 310 and an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

Western blotting

Cultured cells were washed in ice-cold PBS, lysed by incubation on ice in a lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, protease inhibitor cocktail; Complete, Roche Diagnostics, Inc., Basel, Switzerland], and clarified by centrifugation at 15,000 rpm for 20 minutes at 4°C. The whole-cell lysates were boiled for 5 minutes in the presence of SDS sample buffer, resolved by 10% SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA). The membranes were then incubated with blocking buffer (5% nonfat dry milk in PBS) for 1 hour at room temperature and then incubated for 40 minutes with mouse anti-human survivin monoclonal antibody (Santa Cruz Biotechnology) or mouse anti-ß-actin monoclonal antibody AC-15 (Sigma-Aldrich). After washing three times with wash buffer (0.1% Tween-20 in PBS), the membrane was reacted with peroxidase-labeled goat anti-mouse IgG antibody (KPL, Gaithersburg, MD) for two hours. Finally, the signal was visualized by using an enhanced chemiluminescence (ECL) detection system (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's protocol.

Peptides and Cytokines

Wild type survivin-derived peptides carrying HLA-A24 binding motif Survivin-C58 (amino acid sequence FFCFKELEGW), a splicing variant survivin-2B-derived peptide Survivin-2B80-88 (AYACNTSTL) [22], EBV LMP2-derived HLA-A24 binding peptide (TYGPVFMSL) [33], HIV env-derived HLA-A24 binding peptide (RYL-RDQQLLGI) [34], CMV pp65-derived HLA-A24 binding peptide (QVDPVAALF), mouse VSV-derived peptide VSV8 (RGYVYQGL), and synovial sarcoma chromosomal translocation product SYT-SSX-derived SS393 peptide and K9I peptide (GYDQIMPKK and GYDQIMPKI respectively) [35,36] were purchased from SIGMA Genosys (Ishikari, Japan). They were resolved in DMSO at the concentration of 5 mg/ml and stored at -80°C. Human recombinant interleukin (IL)-2 was a kind gift from Takeda Pharmaceutical Co. (Osaka, Japan). Human recombinant GM-CSF was a kind gift from Kirin (Tokyo, Japan) and Novartis Pharmaceutical (Basel, Switzerland). Human recombinant IL-4 and IL-7 were purchased from Invitrogen (San Diego, CA).

Peptide Binding Assay

Peptide binding affinity to HLA-A24 was assessed by HLA-A24 stabilization assay as described previously [22]. Briefly, after incubation of T2-A24 cells in culture medium at 26°C for 18 h, cells (2×10^5) were washed with PBS and suspended with 1 ml of Opti-MEM (Life Technologies,