Two novel squamous cell carcinoma antigen-derived HLA-A*0201-binding peptides induce *in vitro* and *in vivo* CD8$^+$ cytotoxic T lymphocyte responses

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Received December 4, 2012; Accepted January 28, 2013

DOI: 10.3892/ijo.2013.1834

Abstract. Squamous cell carcinoma antigen (SCCA) is over-expressed in many squamous cell cancers and SCCA-derived peptide-specific CD8$^+$ cytotoxic T lymphocytes can display cytotoxicity against tumor cells. In the present study, we screened the SCCA amino acid sequence for potential HLA-A*0201-binding CD8$^+$ T-cell epitopes using two predictive computational algorithms. Seven epitope candidates were selected of which $SCCA_{246-254}$(LLPNEIDGL), $SCCA_{223-231}$(SLEDVQAKV), $SCCA_{328-336}$(VLHKAFVEV) and $SCCA_{324-332}$(VLSGVLHKA) significantly stabilized HLA-A*0201 molecules on T2 cells. Both $SCCA_{328-336}$ and $SCCA_{324-332}$ induced CD8$^+$ IFN-γ^+ T-cell responses in HLA-A*0201-positive peripheral blood mononuclear cells as assessed by intracellular cytokine staining. Consistent with this, immunization with either $SCCA_{328-336}$ or $SCCA_{324-332}$ effectively elicited CD8$^+$ IFN-γ^+ T cells in HLA-A*0201 transgenic mice as visualized by IFN-γ ELISPOT assay and intracellular cytokine staining. Furthermore, CD8$^+$ T cells induced *in vitro* or *in vivo* by $SCCA_{328-336}$ or $SCCA_{324-332}$ demonstrated *in vitro* cytotoxicity against peptide-pulsed T2 cells and splenocytes, respectively. These novel SCCA-derived CD8$^+$ T-cell epitopes described, herein, may be potentially important components for diagnostic reagents and immunotherapeutic vaccines for the treatment of squamous cell carcinomas.

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Key words: squamous cell carcinoma antigen, HLA-A*0201, epitope, cytotoxic T lymphocytes

Introduction

Squamous cell carcinoma (SCC) is a malignant cancer and often occurs in squamous epithelia, including the oral cavity, esophagus, uterine cervix, anal canal and skin. In 1977, Kato and Torigoe, first discovered the squamous cell carcinoma antigen (SCCA) in a squamous cell carcinoma of the uterine cervix (1). SCCA is a new member of the serine protease inhibitors (serpins) family (2). SCCA gene locus contains SCCA1 (SerpinB3) and SCCA2 (SerpinB4) genes, which encode the neutral and acidic forms of the protein, respectively. The neutral SCCA1 is generally present inside both normal and malignant cells, whereas acidic SCCA2 is mainly secreted by malignant cells and is often elevated in patients with SCCs (3,4). Many studies have shown that elevated serum SCCA levels, including SCCA1 and SCCA2, are found in patients with SCC of cervix, head and neck, esophagus and lung (5-8). Additionally, high SCCA concentrations were found in vulvar tumors and in metastatic lymph nodes, while negative inguinal lymph nodes from the same patients presented significantly less SCCAs (9). At present, clinical data support the concept that SCCAs are SCC-specific tumor antigens and that elevated serum SCCA levels correlate well with the extent of disease in patients with SCCs. Therefore, assays for SCCAs are widely used for the diagnosis and monitoring of patients with SCC of the uterine cervix and other organs (10,11).

Many *in vitro* and *in vivo* studies have suggested an important role of SCCAs in the modulation of programmed cell death and the promotion of tumor cell survival. Suminami *et al* demonstrated that SCCA1 could significantly inhibit apoptosis induced by tumor necrosis factor (TNF)-α or interleukin (IL)-2-activated natural killer (NK) cells in tumor cells transduced with SCCA1 cDNA. Tumor cells overexpressing SCCA1 formed significantly larger tumors in nude mice than the SCCA1-negative controls (12). In addition, the suppression of SCCA1 inhibited tumor growth (13). Cancer cells naturally expressing high levels of SCCA1 were unable to survive and proliferate when SCCA1 expression was inhibited using short hairpin RNA (14). Takeda *et al*

demonstrated that SCCA2 overexpression in squamous tumor cells contributed to their survival by protecting them from TNF-α-induced apoptosis (15). The overexpression of SCCA2 in tumor cells inhibited recombinant granzyme M-induced as well as NK cell-mediated cellular apoptosis and contributed to tumor cell growth (16). Also it was confirmed that SCCAs, activated by activated STAT3, promoted the survival of squamous carcinoma cells (17). SCCA expression via STAT6 activation inhibited apoptosis of macrophages after infection with *Toxoplasma gondii* (18). Currently, it is generally accepted that overexpression of SCCAs in cancer cells can promote tumor growth and the removal of SCCA or SCCA expressing tumor cells can contribute to a better prognosis of patients with SCC.

$CD8^+$ cytotoxic T lymphocytes (CTLs) are important in combating cancer and viruses (19). Studies have suggested that $CD8^+$ T cells in metastatic lymph nodes are associated with favourable outcomes in patients with oro- and hypopharyngeal carcinoma while significantly decreased $CD8^+$ T cell numbers in metastatic lymph nodes of head and neck carcinoma facilitate cancer metastasis (20,21). In addition, the infiltration of $CD8^+$ T cells into HPV-related oropharyngeal SCC improved patient prognosis (22). A study in tonsillar squamous cell carcinoma (TSCC) patients showed a positive correlation between high numbers of infiltrating $CD8^+$ T cells and a beneficial clinical outcome (23). Moreover, memory T cells in patients may control postoperative tumor metastasis and recurrence (24) and SCCA-derived peptide-specific CTLs were cytotoxicity against tumor cells (25). Taken together, these results suggested that increased CTL numbers are involved in improved prognosis of patients with SCC. SCCA is an SCC-specific tumor antigen and the induction of $CD8^+$ T cell immunity against SCCAs may be an alternative approach for SCCs treatment. Therefore, the mapping of human $CD8^+$ T-cell epitopes from SCCAs may contribute to a better understanding of the immune correlates of protection and lead to the development of effective immunotherapeutic and immunoprophylactic vaccine strategies.

HLA-A*0201 is a prevalent MHC class I allele with a frequency of over 30% in most populations irrespective of gender or ethnicity (26). In the present study, we screened the amino acid sequences of SCCAs for potential HLA-A*0201-binding $CD8^+$ T-cell epitopes using two predictive computational algorithms. The binding affinity of peptides to HLA-A*0201 was validated using MHC-peptide complex stabilization assay. Peptides were used to stimulate HLA-A*0201-positive peripheral blood mononuclear cells (PBMCs) and immunize HLA-A*0201 transgenic mice. Peptide-specific CTLs were determined by interferon (IFN)-γ ELISPOT assay, intracellular cytokine staining (ICS) assay and *in vitro* cytotoxicity assay.

Materials and methods

Epitope prediction and peptide synthesis. Two predictive computational algorithms, SYFPEITHI (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm) and BioInformatics and Molecular Analysis Section (BIMAS) (http://www-bimas.cit.nih.gov/molbio/hla_bind/), were used to screen the complete amino acid sequences of SCCA1 and SCCA2 (GenBank accession nos.: AAB20405 and AAO92271, respectively) for potential $CD8^+$ T-cell epitopes. PAProc(http://www.paproc.de/) was used to predict the potential human proteasomal cleavage sites within the predicted peptides. Putative HLA-A*0201-binding $CD8^+$ T-cell epitopes were selected as epitope candidates. In addition, a universal Th epitope Pan-DR epitope peptide (PADRE: AKF VAA WTL KAAA) was used as a helper peptide. Peptides (>95%) were synthesized by a peptide company (ChinaPeptides Co. Ltd, Shanghai, China). High-performance liquid chromatography (HPLC) and mass spectrometry were used to analyze and verify the purity and molecular weight of these peptides. All lyophilized peptides were dissolved in PBS at a concentration of 1 mM and stored at -20˚C until assayed.

Cell line, samples and mice. The human transporter associated with antigen processing (TAP)-deficient T2 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). After written informed consent had been obtained from healthy individuals (negative for SCCs and SCCAs, recruited from Wenzhou Medical College), approximately 60 ml of an individual's blood was drawn into EDTA-treated tubes in accordance with procedures approved by the Human Research Ethics Board of Wenzhou Medical College, Wenzhou, China. PBMCs were isolated by gradient centrifugation using leukocyte separation medium and suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS). PBMCs were stained with FITC-conjugated anti-HLA-A*0201 monoclonal antibodies (mAb) and the expression of HLA-A*0201 on PBMCs was determined by flow cytometric analysis. HLA-A*0201-positive PBMCs were used for the *in vitro* $CD8^+$ T-cell induction assay. C57BL/6-Transgenic (HLA-A2.1) 1Enge/J mice (HLA-A*0201 transgenic mice) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred under specific pathogen free conditions.

MHC-peptide complex stabilization assay. The binding affinity of peptides to the HLA-A*0201 molecule was determined semiquantitatively by detecting the upregulation of peptide-inducing HLA-A*0201 molecules on T2 cells by flow cytometry. The HLA-A*0201-peptide complex stabilization assay was performed as previously described (27). Briefly, T2 cells ($1x10^5$/well) were incubated with individual peptides at different concentrations (1, 10 or 100 μM) in 24-well plates for 1 h at 37˚C, 18 h at 28˚C, followed by 3 h at 37˚C. T2 cells without peptide served as background control. After incubation, the T2 cells were incubated with FITC-conjugated anti-HLA-A*0201 mAb (BD Pharmingen, San Diego, CA, USA) for 40 min at 4˚C. Cells were washed two times with PBS and analyzed using a FACSCalibur flow cytometer (BD Biosciences, CA, USA). The mean fluorescence index (MFI) was recorded, and the fluorescence index (FI) was calculated using the following formula. FI>1 (100 μM peptide) indicated that the tested peptide had a high affinity for the HLA-A*0201 molecule.

$$FI=\frac{\text{Test MFI - background MFI}}{\text{Background MFI}}$$

Table I. SCCA-derived HLA-A*0201-restricted epitope candidates.

Peptides	Position[a]	Sequences	Proteins[b]	Scores[c]		FIs[d] Peptide concentration		
				SYFPEITHI	BIMAS	1 μM	10 μM	100 μM
$SCCA_{115-123}$	115-123	FLQEYLDAI	SCCA1,2	27	221.751	0.19	0.26	0.68
$SCCA_{246-254}$	246-254	LLPNEIDGL	SCCA1,2	27	40.928	0.09	0.19	1.10
$SCCA_{223-231}$	223-231	SLEDVQAKV	SCCA1	26	18.250	0.08	0.68	1.12
$SCCA_{328-336}$	328-336	VLHKAFVEV	SCCA1,2	25	224.653	0.11	0.32	1.11
$SCCA_{106-114}$	106-114	KLFGEKTYL	SCCA1	24	1,521.537	0.10	0.20	0.46
$SCCA_{324-332}$	324-332	VLSGVLHKA	SCCA1	24	19.425	0.14	0.23	1.35
$SCCA_{274-282}$	274-282	NMRETRVDL	SCCA1	22	4.807	0.11	0.07	0.14

[a]The location of peptides was determined according to the amino acid sequences of SCCA1 (AAB20405); [b]the peptide was conserved in SCCA1 (AAB20405) or/and SCCA2 (AAO92271); [c]the peptide binding scores were predicted using SYFPEITHI program or BIMAS program; [d]fluorescence index (FI) was calculated as described in the Materials and methods section.

In vitro generation of peptide-specific CD8$^+$ T cells. The *in vitro* CD8$^+$ T-cell induction assay was performed as previously described (25). Briefly, 8x10^6 HLA-A*0201-positive PBMCs were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μM streptomycin, given peptide (50 μM) 100 U/ml recombinant interleukin 2 (Peprotech Inc, Rocky Hill, NJ, USA). Every 3 or 4 days, half of the culture medium was removed and replaced with fresh RPMI-1640 medium. On the 28th day (1 week after the final stimulation), the peptide-stimulated PBMCs (effector cells) were tested for peptide-specific CD8$^+$ IFN-γ^+ T cells and the ability to lyse peptide-pulsed T2 cells.

*Immunization of HLA-A*0201 transgenic mice*. HLA-A*0201 transgenic mice (6-8 weeks of age) were subdivided into eight groups (5 mice/group). Peptide (250 μg) and 250 μg PADRE in 500 μl PBS were emulsified in 500 μl Complete Freund's Adjuvant (CFA). Subsequently, 1 ml of the mixture was injected subcutaneously (s.c.) into the back skin and the tail base of transgenic mice (50 μg given peptide/mouse). After 1 week, the mixture, 250 μg of the same peptide in 250 μl PBS and 250 μl Incomplete Freund's Adjuvant (IFA), were used to boost mice once a week for 4 weeks. Control mice (mock-immunized mice) received CFA and IFA but without peptide. One week after the final immunization, the spleen, lymph nodes (deltopectoral and popliteal lymph nodes), and EDTA-treated blood were harvested and prepared for single cell suspensions. The splenocytes were used for ELISPOT assay, ICS assay and *in vitro* cytotoxicity assay. Lymph node cells and PBMCs were used for ELISPOT assay. The protocols for the animal experiments in this study were approved by the Animal Ethics Committee of Wenzhou Medical College.

IFN-γ ELISPOT assay. Precoated murine IFN-γ ELISPOT 96-well plates (U-CyTech Biosciences, Utreht, The Netherlands) were used to detect peptide-specific IFN-γ-secreting cells in populations of splenocytes, lymph node cells and PBMCs. In brief, 5x10^5 splenocytes, 2x10^5 lymph node cells or 1x10^5 PBMCs in 100 μl RPMI-1640 medium were added to each well of the ELISPOT plates. The wells were divided into background control wells (without peptide) and test wells (20 μM individual peptides). The plates were then incubated for 24 h at 37˚C in a 5% CO_2 incubator. After incubation, the plates were washed with washing buffer and 100 μl fresh biotinylated anti-mouse IFN-γ antibody was added to each well. After 1 h incubation at 37˚C, the plates were washed and 100 μl fresh HRP-conjugated streptavidin was added to each well. After 1 h incubation at 37˚C, the plates were washed and 100 μl AEC solution was added to each well. After 20 min incubation at 37˚C, the reaction was stopped using tap water. An ELISPOT reader (Beijing Sage Creation Science and Technology Co. Ltd, Beijing, China) was used to count the spots. One spot indicated one peptide-specific IFN-γ-secreting cell or one spot forming cell (SFC).

ICS assay. Effector cells (peptide-stimulated PBMCs or splenocytes) were incubated with no peptide or peptide (50 μM). After 1 h, brefeldin A (BFA, 10 μM, Enzo Biochem Inc, New York, NY, USA) was added to the cells. After 5 h incubation at 37˚C, cells were washed with PBS and then fixed with 4% paraformaldehyde for 10 min at 4˚C. Cell were permealized with 1% saponin and stained with FITC-conjugated anti-human/mouse CD3 mAb, APC-conjugated anti-human/mouse CD8 mAb PE-conjugated anti-human/mouse IFN-γ mAb (eBioscience Company, San Diego, CA, USA) for 40 min at 4˚C. After washing with PBS, cells were analyzed by flow cytometry (BD Biosciences). The percentages of CD8$^+$ IFN-γ^+ T cells in total CD8$^+$ T cells were measured.

In vitro cytotoxicity assay. Based on a previously described *in vivo* cytotoxicity assay (28), an *in vitro* cytotoxicity assay was developed and used to determine the cytotoxicity of peptide-stimulated PBMCs or splenocytes against target cells. First, 2x10^6 T2 cells and 2x10^6 peptide-stimulated PBMCs were differentially labeled at 37˚C for 25 min, with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Cayman Chemical, Ann Arbor, MI, USA) to 0.5 μM (target

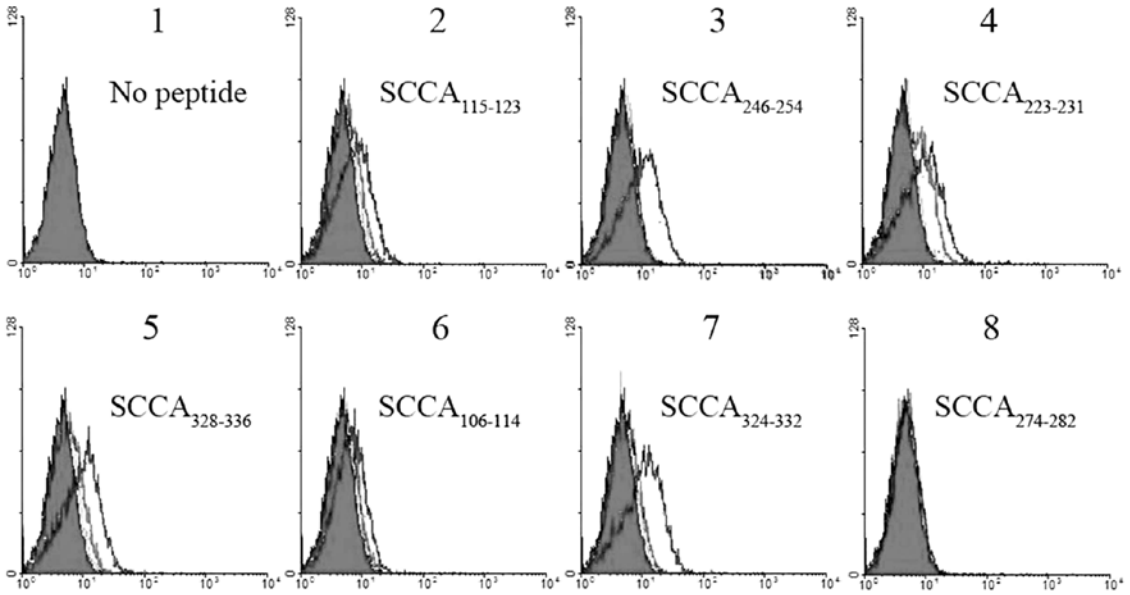

Figure 1. The binding affinity of CTL epitope candidates to HLA-A*0201. MHC-peptide complex stabilization assay was used to evaluate the binding affinity of SCCA-derived CTL epitope candidates to HLA-A*0201 molecules of T2 cells as described in Materials and methods section. (1) T2 cells without peptide; (2-8) T2 cells were incubated with individual peptides at the different concentrations [no peptide or 1 μM or 10 μM or 100 μM). The mean fluorescence intensity (MFI) of T2 cells was measured using flow cytometry and then the fluorescence index (FI) was calculated.

cells = $CFSE^{low}$) or 5 μM CFSE (effector cells = $CFSE^{high}$) concentration in PBS with 1% BSA. The labeled reaction was quenched by addition of RPMI-1640 medium containing 40% FBS. The target cells were treated with 100 μg/ml of mitomycin C (Cayman Chemical) for 40 min at 37˚C and then pulsed for 3 h with 50 μM peptide at 37˚C, and the effector cells remained unpulsed. Second, $4x10^6$ splenocytes from peptide-immunized mice were split into two equal fractions. The two populations were differentially labeled with CFSE to 0.5 μM (target = $CFSE^{low}$) or 5 μM CFSE (effector = $CFSE^{high}$) concentration in PBS with 1% BSA. The target cells were treated with 100 μg/ml of mitomycin C and pulsed for 3 h with 50 μM peptide and the effector cells remained unpulsed. Third, after being washed, $CFSE^{low}$ cells were mixed with $CFSE^{high}$ cells in a 1:1 ratio and the mix was incubated for 24 h at 37˚C. Finally, the ratio of $CFSE^{low}/CFSE^{high}$ cells was determined by flow cytometry. Specific killing percentages of peptide-pulsed $CFSE^{low}$ target cells were calculated as follows: [1 - ($CFSE^{low}/CFSE^{high}$)] x 100%.

Statistical analysis. Results were expressed as mean ± SEM. The Student's t-test was used to determine the significant differences between the mean values of the experimental groups. $p<0.05$ was considered significant.

Results

*The binding affinity of epitope candidates to HLA-A*0201*. Based on the prediction results of SYFPEITHI and BIMAS, seven high-score 9-amino acid peptides were chosen as epitope candidates for further study (Table I). Of these peptides, four peptides ($SCCA_{223-231}$, $SCCA_{106-114}$, $SCCA_{324-332}$ and $SCCA_{274-282}$) are conserved among SCCA1 while three peptides ($SCCA_{115-123}$, $SCCA_{246-254}$ and $SCCA_{328-336}$) are shared by SCCA1 and SCCA2. In addition, $SCCA_{328-336}$ and $SCCA_{324-332}$ shared a common sequence (VLHKA). Based on T2 cells, the MHC-peptide complex stabilization assay was used to evaluate the binding affinity of serial 10-fold diluted individual peptides to the HLA-A*0201 molecule. Binding of the peptide to HLA-A*0201 molecule could stabilize MHC molecules and significantly upregulate HLA-A*0201 expression on the surface of T2 cells. The fluorescence index (FI) was used to reflect the binding affinity of a peptide to the HLA-A*0201 molecule. FIs for $SCCA_{246-254}$(LLPNEIDGL), $SCCA_{223-231}$(SLEDVQAKV), $SCCA_{328-336}$(VLHKAFVEV) and $SCCA_{324-332}$(VLSGVLHKA) were dose-dependent and exceeded 1 when the peptide concentrations were 100 μM. However, the remaining three peptides ($SCCA_{115-123}$, $SCCA_{106-114}$ and $SCCA_{274-282}$) had a low affinity to HLA-A*0201 (FIs<1). Overall, these results suggest that $SCCA_{246-254}$, $SCCA_{223-231}$, $SCCA_{328-336}$ and $SCCA_{324-332}$ are high-affinity peptides, with $SCCA_{324-332}$ having the highest binding (FI=1.35). Comprehensive results are summarized in Table I, and representative results are shown in Fig. 1.

*Induction of peptide-specific CTLs from HLA-A*0201-positive PBMCs of healthy donors*. To investigate whether these peptides could induce efficiently *in vitro* $CD8^+$ T-cell responses, PBMCs from HLA-A*0201-positive, SCCA-sero-negative healthy individuals were stimulated with individual peptides. After 21 days of stimulation twice a week with peptides at a concentration of 50 μM, the induced effector cells were assessed by ICS and *in vitro* cytotoxicity assay. As shown in Fig. 2, of the seven peptides tested, both $SCCA_{328-336}$ and $SCCA_{324-332}$ were efficient at inducing peptide-specific IFN-γ-producing $CD8^+$ T cells from the PBMCs of four HLA-A*0201-positive healthy donors. The percentages of $SCCA_{328-336}$- and $SCCA_{324-332}$-specific $CD8^+$ IFN-γ^+ T cells in total $CD8^+$ T cells were 0.87±0.21 and

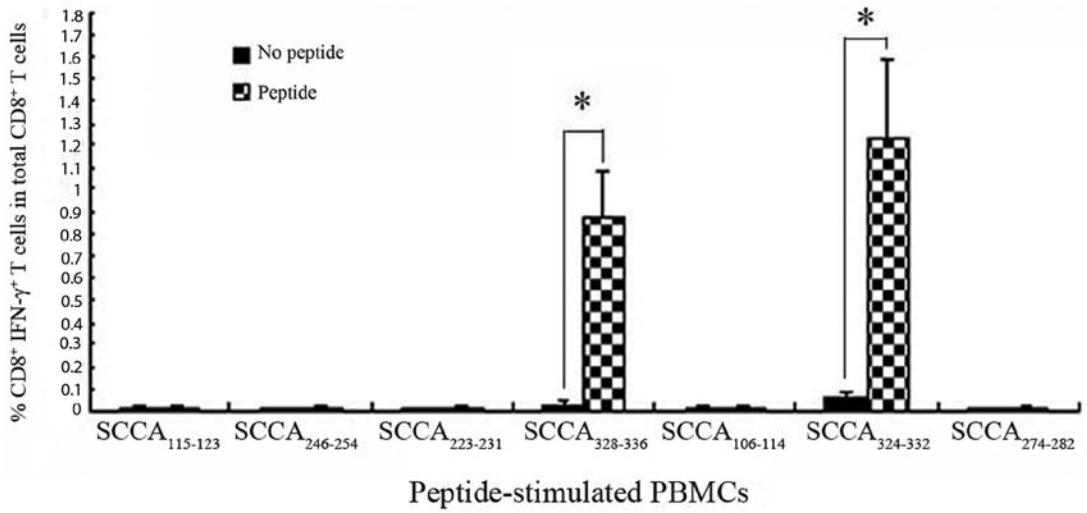

Figure 2. Percentages of peptide-specific $CD8^+$ IFN-γ^+ T cells in peptide-stimulated PBMCs from HLA-A*0201-positive healthy volunteers. HLA-A*0201-positive PBMCs were stimulated with individual peptides for three weeks. The peptide-stimulated PBMCs were restimulated *in vitro* with no peptide or the same peptide. Percentages of $CD8^+$ IFN-γ^+ T cells in total $CD8^+$ T cells were calculated using flow cytometry and presented as mean ± SD from four healthy volunteers. *Indicating the positive response to a peptide stimulation.

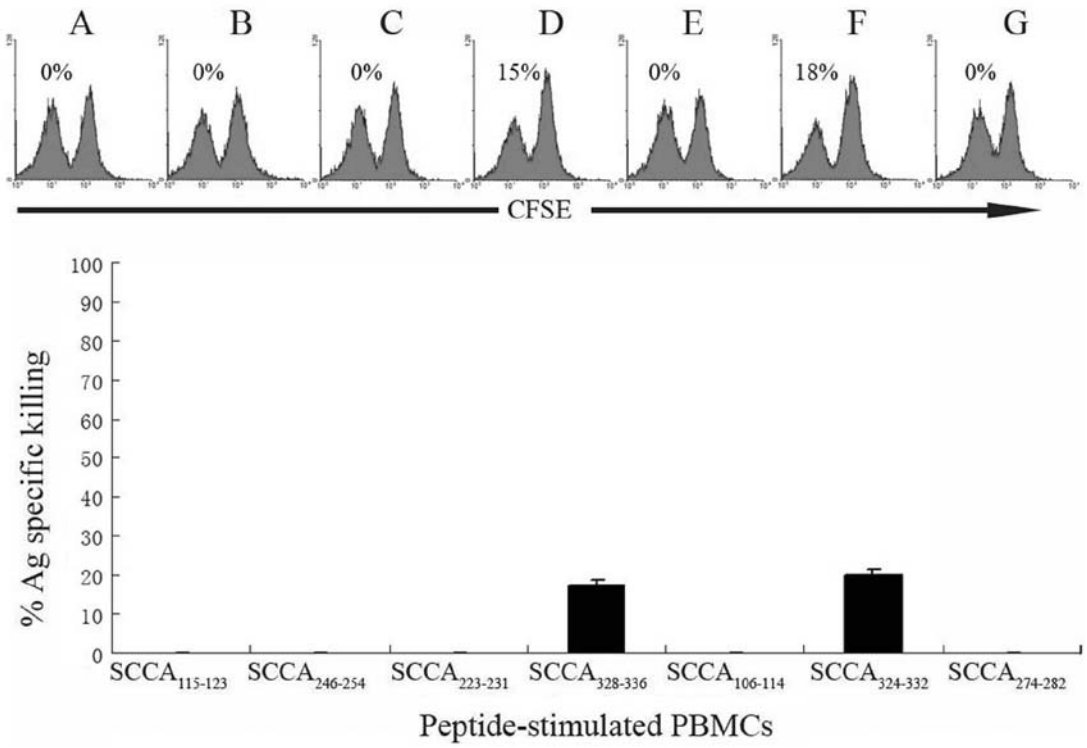

Figure 3. Cytotoxic activity of peptide-stimulated PBMCs against T2 cells pulsed with peptide. The *in vitro* cytotoxicity assay was performed to test the cytotoxic activity of peptide-stimulated PBMCs against T2 cells (effector/target =1:1) as described in Materials and methods section. Peptide-stimulated PBMCs and peptide-pulsed T2 cells were differentially labeled with CFSE (5 and 0.5 μM, respectively). The mixture of effector cells and target cells was incubated 24 h before analysis of killing percentage of targets by flow cytometry. (A-G) The figures show the representative peptide-stimulated PBMCs and the values indicate the percentage of specific lysis of target cells.

1.23±0.36%, respectively. Despite repeated attempts, the remaining five peptides did not induce $CD8^+$ T-cell response. We then investigated whether peptide-stimulated PBMCs could exhibit cytotoxicity against T2 cells. Both $SCCA_{328-336}$- and $SCCA_{324-332}$-stimulated PBMCs could lyse peptide-pulsed T2 cells (16.3-19.7 and 18.5-22.1%, respectively) (Fig. 3). However, stimulation with other peptide did not show cytotoxicity against T2 cells. Furthermore, peptide-stimulated PBMCs did not show any cytotoxicity against naked T2 cells (data not shown).

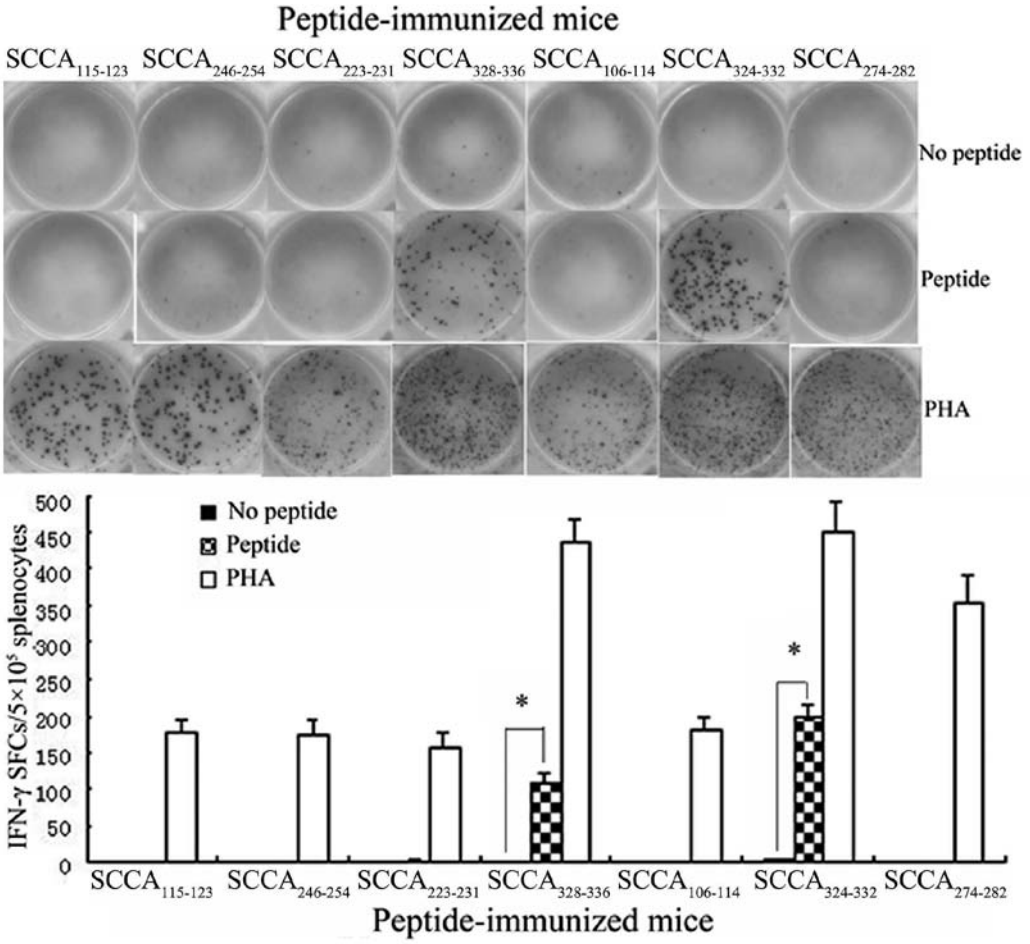

Figure 4. Peptide-specific IFN-γ-secreting cells in splenocytes of peptide-immunized HLA-A*0201 transgenic mice. Splenocytes were isolated from peptide-immunized mice and were restimulated *in vitro* with no peptide or the same peptide. The numbers of IFN-γ SFCs/5x10^5 splenocytes were detected using ELISPOT assay and presented as mean ± SD (n=5). *Indicating the positive response to a peptide.

*Immunization of HLA-A*0201 transgenic mice with SCCA$_{328-336}$ or SCCA$_{324-332}$ induces CTL responses*. To examine the CTL reactivity of these peptides *in vivo*, peptides were used to immunize HLA-A*0201 transgenic mice five times, as described in the Materials and methods section. Seven days after the final immunization, splenocytes, lymph node cells and PBMCs were prepared and peptide-specific CD8$^+$ T cells were detected by IFN-γ-secreting ELISPOT assay and ICS assay. Both SCCA$_{328-336}$ and SCCA$_{324-332}$ induced substantial IFN-γ secretion in splenocytes, lymph node cells and PBMCs detected by ELISPOT assay (Figs. 4, 5 and 6). The highest frequency of IFN-γ-secreting cells was directed against SCCA$_{324-332}$ (197±19 SFCs/5x10^5 splenocytes; 74±12 SFCs/2x10^5 lymph node cells; 45±8 SFCs/1x10^5 PBMCs) while immunization with SCCA$_{328-336}$ displayed an intermediate level of IFN-γ-secreting (107±15 SFCs/5x10^5 splenocytes, 46±9 SFCs/2x10^5 lymph node cells, 22±6 SFCs/1x10^5 PBMCs). However, the remaining peptides did not induce significant IFN-γ-secreting cells. To define the phenotype of peptide-specific IFN-γ-secreting cells, splenocytes from peptide-immunized HLA-A*0201 transgenic mice were analyzed by ICS assay. As shown in Fig. 7, splenocytes of SCCA$_{328-336}$- or SCCA$_{324-332}$-immunized mice contained high levels of peptide-specific CD8$^+$ IFN-γ$^+$ T cells. The percentage of CD8$^+$ IFN-γ$^+$ T cells in total CD8$^+$ T cells induced by SCCA$_{328-336}$ or SCCA$_{324-332}$ immunization was 0.35±0.05 and 0.65±0.07%, respectively. Additionally, mock-immunized mice showed no IFN-γ secretion in response to any peptide (data not shown). Moreover, C57BL/6 mice with a genetic background identical to the HLA-A*0201 transgenic mice but lacking the HLA-A*0201 molecule did not show peptide-specific IFN-γ-secreting CD8$^+$ T cells (data not shown). Finally, we determined whether peptide-specific IFN-γ-secreting CD8$^+$ T cells had the ability to kill target cells. As shown in Fig. 8, splenocytes from mice immunized with SCCA$_{328-336}$ displayed high levels of *in vitro* killing of peptide-pulsed splenocytes ranging from 41.2 to 51.4%. Likewise, immunization with peptide SCCA$_{324-332}$ induced efficient specific lysis in a similar range (50.1-61.2%). As expected, no lysis was observed in mock-immunized mice or C57BL/6 mice (data not shown).

Discussion

As serine protease inhibitors, squamous cell carcinoma antigen 1 (SCCA1) and squamous cell carcinoma antigen 2 (SCCA2) play important roles in host tissue homeostasis (29). Studies have reported increased SCCA expression in SCC tissues (14,25,30-32), and SCCAs are thought to contribute to the progress of SCC by protecting tumor cells from apoptosis

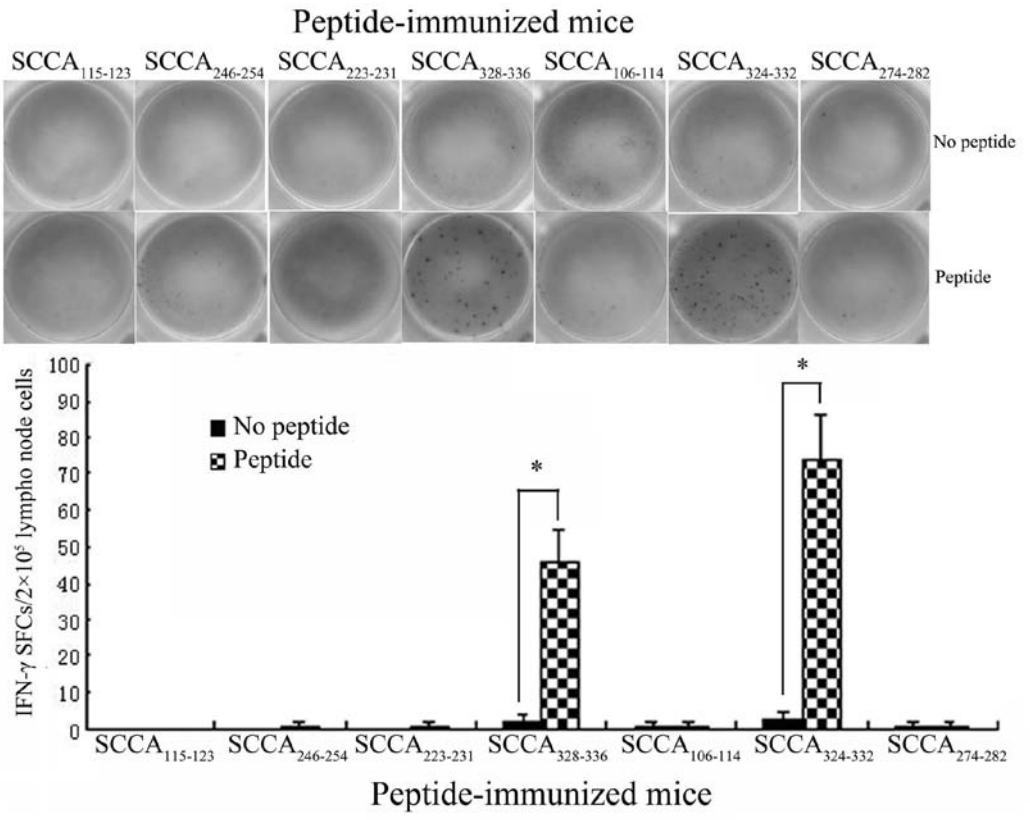

Figure 5. Peptide-specific IFN-γ-secreting cells in lymph node cells of peptide-immunized HLA-A*0201 transgenic mice. Lymh node cells were isolated from peptide-immunized mice and were restimulated *in vitro* with no peptide or the same peptide. The number of IFN-γ SFCs/2x10^5 lymph node cells was detected using ELISPOT assay and presented as mean ± SD (n=5). *Indicating the positive response to a peptide.

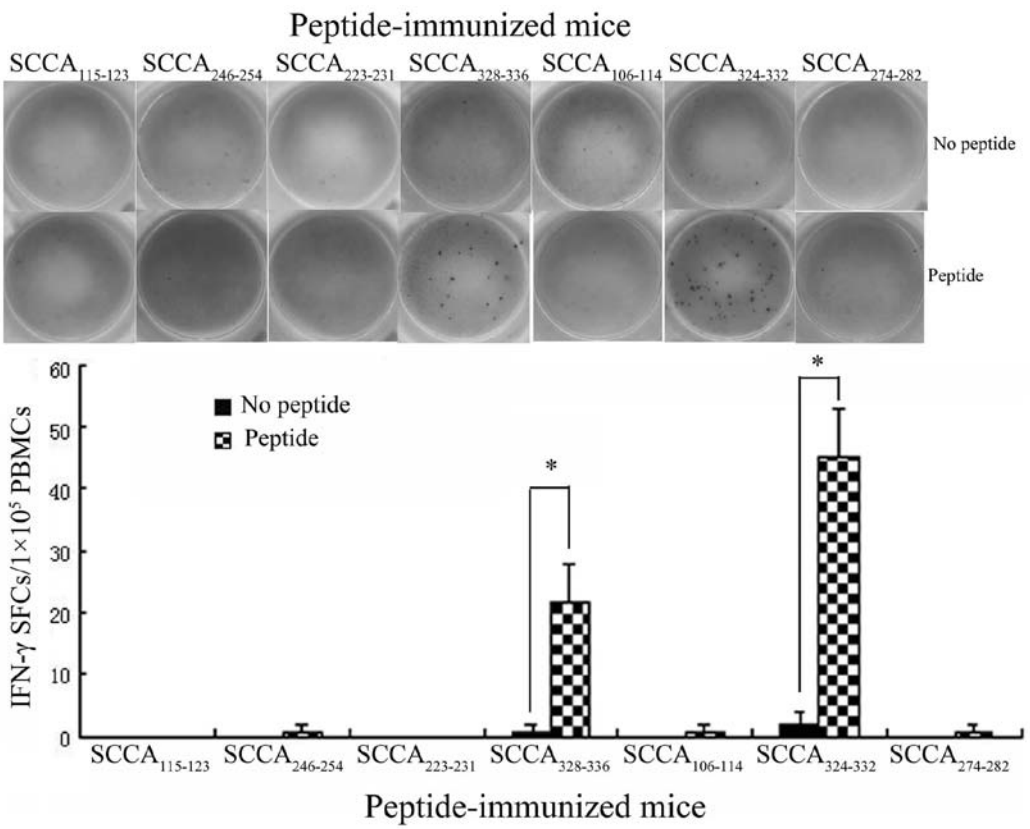

Figure 6. Peptide-specific IFN-γ-secreting cells in PBMCs of peptide-immunized HLA-A*0201 transgenic mice. PBMCs were isolated from peptide-immunized mice and were restimulated *in vitro* with no peptide or the same peptide. The number of IFN-γ SFCs/1x10^5 PBMCs was detected using ELISPOT assay and presented as mean ± SD (n=5). *Indicating the positive response to a peptide.

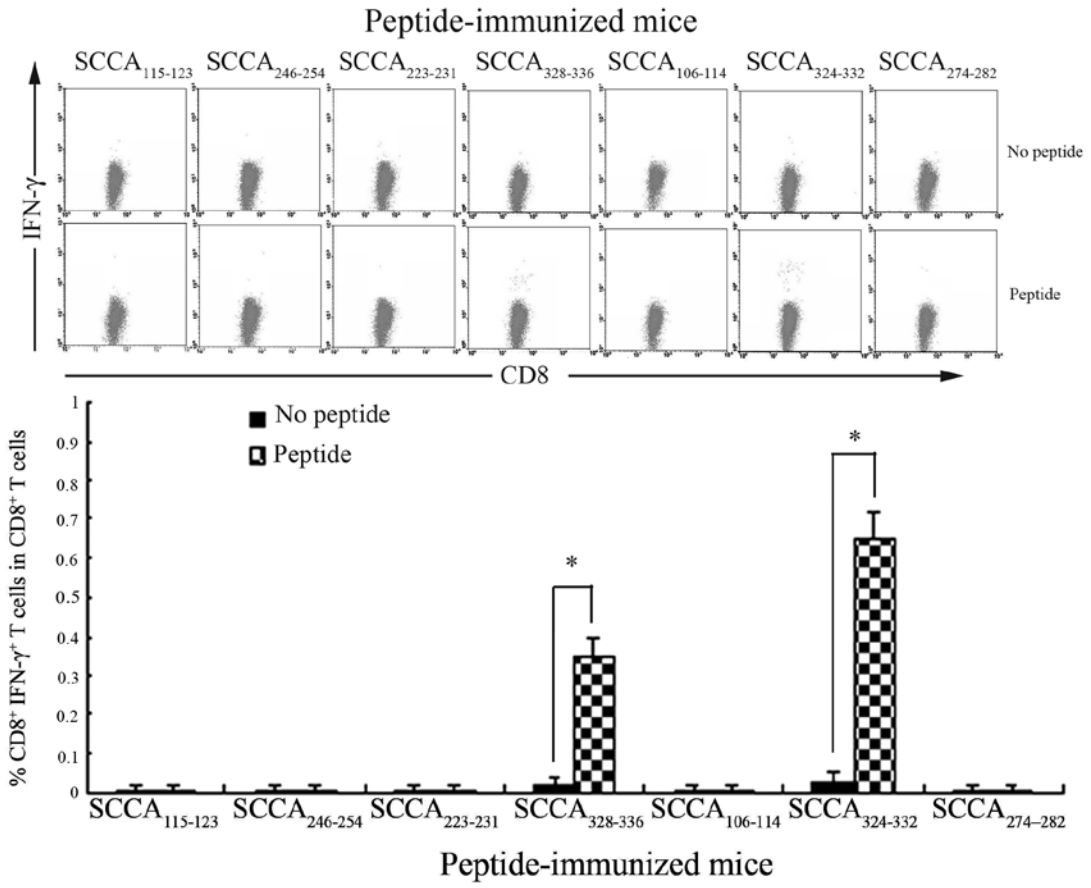

Figure 7. Peptide-specific $CD8^+$ IFN-γ^+ T cells in splenocytes of peptide-immunized HLA-A*0201 transgenic mice. Splenocytes were isolated from peptide-immunized mice and were restimulated *in vitro* with no peptide or the same peptide. The percentage of $CD8^+$ IFN-γ^+ T cells in $CD8^+$ T cells was measured using ICS assay and presented as mean ± SD (n=5). *Indicating the positive response to a peptide.

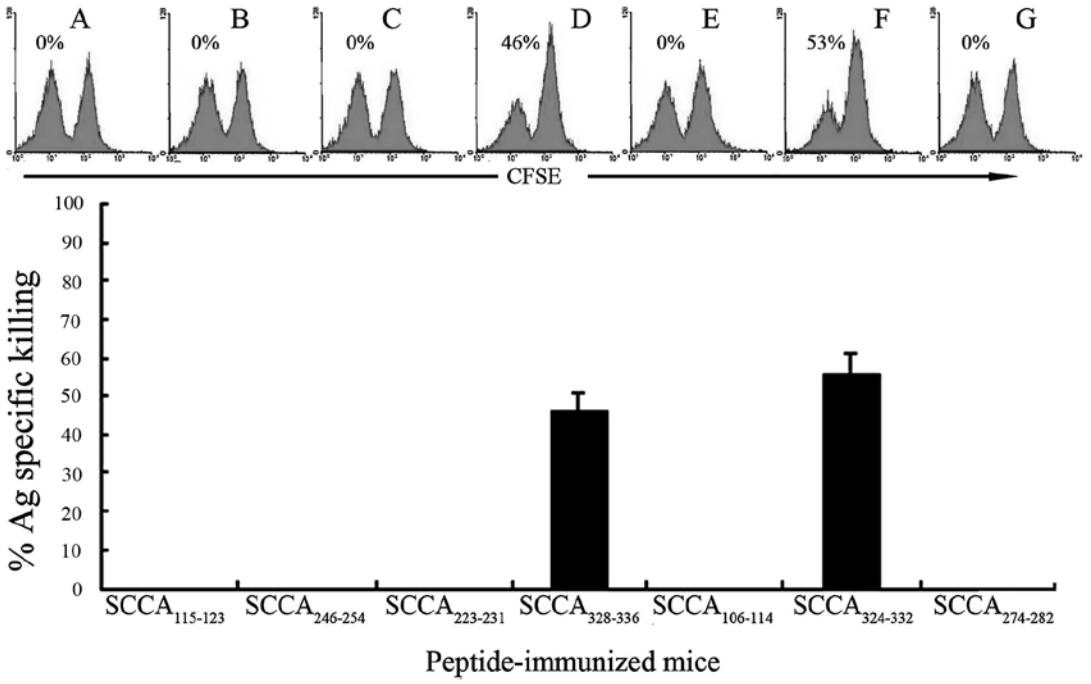

Figure 8. Cytotoxicity activity of splenocytes from peptide-immunized HLA-A*0201 transgenic mice against peptide-pulsed splenocytes. Splenocytes were isolated from peptide-immunized mice. Splenocytes and peptide-pulsed splenocytes were differentially labeled with CFSE (5 and 0.5 μM, respectively). The mixture of effector cells and target cells (effector/target =1:1) was incubated 24 h before analysis of killing percentage of targets by flow cytometry. (A-G) The figures show representative peptide-immunized mice and the values indicate the percentage of specific lysis of target cells.

and allowing cancer cells to survive (12-18). Non-SCC tissue expression of SCCAs are minimal and should not be targeted by the immune system whereas the majority of SCC tissues are positive for SCCAs and therefore are susceptible to immune killing. More importantly, a beneficial outcome of SCC is associated with increased CTL numbers (23,25). These studies clearly indicate that SCCAs are a target molecule for specific immunotherapy against SCCs (20). Therefore, it is important to define which putative peptides are dominant in the killing of target cells during the development of SCCAs-derived CTL epitope-based vaccine.

In the present study, we predicted and validated seven candidate epitopes of SCCAs. In our attempt to detect the binding affinity of peptides to the HLA-A*0201 molecule, we observed that $SCCA_{246-254}$(LLPNEIDGL), $SCCA_{223-231}$(SLEDVQAKV), $SCCA_{328-336}$(VLHKAFVEV) and $SCCA_{324-332}$(VLSGVLHKA) had high affinity for HLA-A*0201 molecules, with $SCCA_{324-332}$ exhibiting the highest binding affinity. However, although $SCCA_{115-123}$ has the highest predictive score in epitope candidates predicted by SYFPEITHI and $SCCA_{106-114}$ is the highest-score peptide in BIMAS prediction results, these two peptides had a low affinity for HLA-A*0201. Our data provide evidence that the binding affinity of candidate epitopes to HLA-A*0201 molecule does not always correlate with their predictive scores. Indeed, ours and other previous studies have demonstrated this phenomenon (27,33,34).

To determine whether these peptides could be processed by human cells and induce CTL responses, these peptides were used to stimulate HLA-A*0201-positive PBMCs *in vitro*. ICS analysis showed that both $SCCA_{328-336}$ and $SCCA_{324-332}$ induced high levels of IFN-γ-secreting $CD8^+$ T cells. HLA-A*0201/Kb transgenic mice express a chimeric MHC class I molecule composed of the α1 and α2 domain of HLA-A*0201 and the α3 domain of H-2Kb. The HLA-A*0201/Kb transgenic mouse model has been used by many investigators to evaluate the immunogenicity of HLA-A*0201-binding peptides and the ability of human HLA-A*0201 molecules to present peptides to murine T cells (35,36). In the present study, we observed that immunization of HLA-A*0201 transgenic mice with $SCCA_{328-336}$ or $SCCA_{324-332}$ induced the generation of peptide-specific $CD8^+$ IFN-γ^+ T cells. Moreover, peptide-specific IFN-γ-secreting cells were distributed in the spleen, lymph nodes and peripheral blood of peptide-immunized mice. However, as high-affinity peptides, neither $SCCA_{246-254}$ nor $SCCA_{223-231}$ induced CTL responses in HLA-A*0201-positive PBMCs or HLA-A*0201 transgenic mice. We speculate that possible explanations for the present findings are: i) neither $SCCA_{246-254}$ nor $SCCA_{223-231}$ are cross-presented to $CD8^+$ T cells as previously described (37); ii) both $SCCA_{246-254}$ and $SCCA_{223-231}$ have higher dissociation rates from HLA-A*0201 molecules than $SCCA_{328-336}$ and $SCCA_{324-332}$. It has been shown that the binding affinity of TCR for MHC/peptide complex is not a single critical determinant for the activation of $CD8^+$ T cells. Previous studies indicated that the immunogenicity of peptides correlates with the stability of MHC/peptide complex formation, but there is no strict correlation between functional activity of the various peptides and their MHC binding efficiency (38,39). Furthermore, as expected, significant $CD8^+$ T cell responses were not observed in mock-immunized mice or peptide-immunized control mice (data not shown). This indicated that the response to these two peptides is restricted by HLA-A*0201 and not by murine MHC allele.

Besides producing IFN-γ, $CD8^+$ T cells also contribute to host protection by exerting cytolytic functions. *In vitro* and *in vivo* techniques for assessing lysis, such as CFSE-based assay, have been developed and use effector and target cell populations labeled with different concentrations of CFSE, so that cytotoxicity activity can be assessed by detecting fluorescence using flow cytometry (40). Many studies have confirmed that peptide-pulsed splenocytes can be used as target cells for evaluating the cytotoxicity of splenocytes from peptide-immunized mice (41-43) while peptide-pulsed T2 cells can serve as target cells for evaluating the cytotoxic function of peptide-stimulated HLA-A*0201-positive PBMCs (44-46). In the present study, we used an *in vitro* CFSE-based cytotoxicity assay to determine whether peptide-induced $CD8^+$ T cells could display *in vitro* cytotoxicity against target cells. Both $SCCA_{328-336}$- and $SCCA_{324-332}$-stimulated PBMCs exhibited the capacity to lyse peptide-pulsed target cells. The percentages of peptide-pulsed T2 cells lysis in SCCA-derived peptide-stimulated PBMCs (16.3-22.1%, effector:target =1:1) are in line with percentages detected in papillomavirus binding factor-derived peptide-stimulated PBMCs (30%, effector: target =2:1) (45), and in HCV-derived peptide-stimulated PBMCs (26.5-41.6%, effector: target =5:1) (46). Furthermore, splenocytes from $SCCA_{328-336}$- or $SCCA_{324-332}$-immunized HLA-A*0201 transgenic mice showed specific *in vitro* killing of peptide-pulsed splenocytes (41.2-61.2%), whereas no lysis was observed in mock-immunized mice or background mice. Our results appear to correspond with those seen in other antigen-derived peptide-immunized mice using *in vivo* cytotoxicity assays (41-43).

Based on PBMCs from HLA-$A24^+$ patients with SCC, Shigenori *et al* identified two immunodominant CTL epitopes from SCCA1. These two peptides, [$SCCA_{112-120}$(TYLFLQEYL) and $SCCA_{215-224}$(QYTSFHFASL)], were found to effectively induce peptide-specific CTLs, displaying cytotoxicity against HLA-A24-positive $SCCA^+$ cancer cells (25). Except these epitopes, no other SCCA-derived epitopes have been identified. To our knowledge, this is the first report identifying SCCA-derived HLA-A*0201-restricted CTL epitopes. It is noteworthy that the present identified epitopes, $SCCA_{328-336}$ and $SCCA_{324-332}$, have the following features: i) they are recognized by murine T cells from HLA-A*0201 transgenic mice and by HLA-A*0201-positive $CD8^+$ T cells, and can induce CTL responses; ii) $SCCA_{328-336}$ is shared by SCCA1 and SCCA2 while $SCCA_{324-332}$ is conserved among SCCA1; iii) these two peptides share five amino acids (VLHKA), which may expain why both $SCCA_{328-336}$ and $SCCA_{324-332}$ were recognized by HLA-A*0201 molecules and induced CTL response.

In conclusion, we demonstrated that both $SCCA_{328-336}$ and $SCCA_{324-332}$ have high affinity for HLA-A*0201 molecules and are more efficient at inducing *in vitro* and *in vivo* $CD8^+$ T-cell response. We further showed that both $SCCA_{328-336}$- and $SCCA_{324-332}$-specific $CD8^+$ T cells could kill peptide-pulsed target cells. Therefore, these two novel HLA-A*0201-restricted epitopes may potentially contribute to the development of diagnostic tools as well as therapeautic CTL epitope-based vaccines for SCCs. Further studies to demonstrate the cytotoxicity of peptide-specific $CD8^+$ T cells against SCC-derived tumor cells and the long-term protection against SCCs are needed.

Acknowledgements

This study was supported by the National Science Foundation of China (30800050 and 31070143), the Natural Science Foundation of Zhejiang province (Y2090744), and the Planned Science and Technology Project of Wenzhou (H20100066 and Y20090338).

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