Immunological evaluation of a novel HLA-A2 restricted phosphopeptide of tumor associated Antigen, TRAP1, on cancer therapy

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1. Introduction

TRAP1 is a homolog of the heat shock protein 90 (Hsp90) chaperon that was majorly found in mitochondria [1,2]. Accumulated evidence has indicated TRAP1 is highly expressed in a wide range of carcinomas, including colorectal [3], non-small cell lung cancer [4] and breast cancer [5]. These highly expressed TRAP1 can present multiple functions via the interaction with cyclophilin D [6] and succinate dehydrogenase (SDH) tumor suppression factors, respectively, to suppress the cell apoptosis and promote the cell transformation [7–9]. On the other hand, downregulation of TRAP1 level was shown to accelerate cancer cell apoptosis and to inhibit the neoplastic transformation [4], which indicated that targeting TRAP1 is a potential strategy for cancer therapy. Previously, Hsp90 inhibitor and its derivatives were hypothesized as potential drug candidates to inactivate TRAP1 [10,11] due to the sequence similarity with Hsp90 protein. However, their anti-tumor potency remained controversial since these molecules may lack the specificity to distinguish TRAP1 from other chaperone homologs, raising a concern of interfering the client protein expression and homeostasis in normal cells [12,13]. Thus, a novel TRAP1-targeting strategy is required.

Major histocompatibility complex class I (MHC I) are expressed on the surface of nucleated cell to display a broad repertoire of peptides for recognition by CD8+ cytotoxic T lymphocytes (CTL) via the T cell receptors (TCRs). In the human immune system, MHC I molecules are referred to as human leukocyte antigens class I molecules (HLAs). Typically, these peptides are predominantly 9–12 amino acids in length, which are generated from the proteasome degradation of intracellular proteins and are then transported into the endoplasmic reticulum via the transporter associated with antigen processing (TAP) to form the complex with MHC I proteins (pMHC I) [14]. Basically, thousands of different peptides can be loaded on individual MHC I allele, and it was estimated that one peptide can be presented 1 to 4000 copies per cell [15], leading the exposed peptide-MHC I complex (pMHC I) as “nature’s gene chip” to display internal proteome of cell for scanning by the circulating CD8+ T cell.

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a process called the immune surveillance [16]. Mutation in gene product or changes in protein expression level associated with cell transformation or viral infection would alter the peptide frame displayed on the HLA I proteins [17], which serves as a flag to probe the transformed cell for CTL recognition. Sykulev and colleagues have demonstrated that immune surveillance is highly sensitive, such that a single antigenic peptide-MHC complex can be readily sensed and discriminated by CD8+ T cell and trigger degradation [18]. Previously, study reports demonstrated that high abundant TRAP1 was phosphorylated via the ERK pathway to regulate the metabolic mechanism and to promote the tumor proliferation [19,20]. In general, the highly expressed phosphoproteins would be ubiquitinated by E3 ligase for rapid proteasome degradation to regulate their activities for maintaining the cellular homeostasis [20]. Interestingly, such mechanism was found to elevate the extent of phosphopeptide presented on MHC I molecules [21–23]. By mass spectrometry analysis, results revealed that pHMCI complex presents the peptide with phosphorylation, and indicated that this post translational modification can enhance the affinity with HLA protein and lead to CTL priming [23]. Therefore, the MHC I-bound peptides derived from the tumor-associated TRAP1 in cancer cells could be tumor specific, and may be a potential vaccine candidates for development of cancer immunotherapy [24].

TC1/AAD is an HLA-A2 gene-transfected mouse lung epithelial cancer cell line, which can express a chimeric class I molecule, AAD, consisting of α1 and α2 domains of the HLA-A*0201 for peptide binding specificity and α3 transmembrane domain of the mouse H2-Db to stabilize the molecule on mouse cell membrane [25,26]. Such hybrid HLA-AAD molecule was designed to present the human epitope for murine T cell recognition. It should be noted that the TRAP1 from human and mouse are highly similar based on the sequence alignment from Uniprot database with the respective access numbers of Q12931 and Q9CQ1 (supplemental Fig. S1). Therefore, it is reasonable to expect TC1/AAD cells to present the identical HLA-A2 restricted peptides of TRAP1 as those found in human cell line. In the study, MS coupling liquid chromatography (LCMS) was carried out to analyze the peptide pools immunoprecipitated from TC1/AAD cells [27,28]. The mono HLA allele lead the determination of HLA-type of MS-identified sequence becomes straightforward. Through phosphoproteomic analysis, a novel HLA-A2-restricted phosphopeptide of TRAP1 was identified in TC1/AAD cells. The peptide contains a novel phosphorylation site that was first observed in the TRAP1 protein. Via the immunological characterization and tumor challenge assays, the TRAP1-derived HLA-A2 restricted phosphopeptide was examined as a potential agent for the development of cancer immunotherapeutic treatment.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jvacx.2019.100017.

2. Material and methods

2.1. Animals and cell lines

C57BL/6 wild-type mice were obtained from the National Animal Center. AAD (containing the alpha1 + alpha2 domains of HLA-A2 and the alpha3 domain of H-2Db) transgenic mice were purchased from The Jackson Laboratory (Sacramento, CA). All animal studies were approved by the Institutional Animal Care and Use Committee of the NHRI (NHRI-IACUC-105156). The mouse lung cancer epithelial TC-1 cells were kindly provided by Dr. T. C. Wu (Johns. Hopkins University, USA). The TC-1/AAD was derived from parental TC-1 cells expressing AAD from AAD transgenic mice. In brief, the DNA fragment encoding the AAD chimeric protein was amplified with a pair of primers (5’-CCCAAGCT TATGCGGCTATGGGCCTCCCGA-3’ and 5’-GGCTCTAGATCATACACTT ATTACCTGGGAC-3’) from splenocytes of HLA- AAD transgenic mice. The amplified DNA fragment was further cloned into pcDNA4/TO/myc-His plasmid (Thermo Fisher Scientific Inc., USA) to generate pcDNA4/TO/myc-His/AIDS. The TC-1 cells were transfected with pcDNA4/TO/myc-His/AIDS to generate the TC-1/AAD stable cell line. The human lung cancer H2981 cell line (HLA-A2 positive) was a gift from Dr. Y-P, Sher [29]. The cell lines were cultivated in complete RPMI medium (RPMI 1640, Gibco) containing 10% heat-inactivated fetal bovine serum (HyClone), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Gibco), 1 mM sodium pyruvate, and 5 mM HEPES in the incubator at 37 °C with 5% CO2.

2.2. Western blot analysis

The harvested cells were washed with chilled PBS buffer and then lysed with RIPA buffer (0.1% in 100 mM PBS buffer, pH 7.8) containing a protease inhibitor tablet (complete, Roche). AAD transgenic mouse lung tissue served as a control in the SDS page electrophoresis. The lung tissue was pulverized with ceramic beads (100 μm, EE-TEC Ltd., Taiwan) in an ice bath and then lysed with 10 mL RIPA buffer at 4 °C. The protein concentrations of the cell lysate and tissue control were quantified with a BCA kit (BCA Protein Assay Kit, Pierce). The BLUeye prestained protein ladder (GenDireX, Canada) was used for SDS-PAGE. A total of 50 μg protein was separated by 4–12% SD-S-PAGE (NuPAGE, ThermoFisher Scientific) and then transferred to a PVDF membrane. To reduce nonspecific binding, washing with PBS buffer containing 0.05% Tween 20 was after every wash step. The blotting membrane was blocked with 5% non-fat milk in PBST at 4 °C overnight. Subsequently, the membrane was cut and separately incubated with diluted anti-TRAP1 (1:1000 in PBST, rabbit polyclonal, ab8227, Abcam) and anti-actin (1:10,000 in PBST, rabbit polyclonal, ab151239, Abcam). The membranes were then stained with horseradish peroxidase (HRP)-conjugated detection antibodies (1:10,000 in PBST, goat polyclonal, ab6721, Abcam) in enhanced chemiluminescence solution (Millipore).

2.3. Immunohistochemical staining

The lung non-small cell cancer tissue array was purchased from US Biomax (LC10012). The TRAP1 expression level in clinical specimens from patients with lung cancer was evaluated by immunohistochemical staining as previously described [30]. The specimens were obtained from the files of the Department of Pathology of China Medical University Hospital in compliance with protocols approved by the CMUH IRB (DM999-IRB-131). A pair of tumor tissues and adjacent tissue were used to compare the staining intensity, and 44 paired specimens from a total of 45 cases were included in the analysis due to the loss of one core from the tissue array. The tissue sections were incubated with rabbit anti-human TRAP1 protein (LS-C31383; LifeSpan Biosciences) and horseradish peroxidase-conjugated Avidin-biotin complex (ABC) from the Vectastain Elite ABC Kit (Vector Laboratories) and AEC chromogen (Vector Laboratories). The TRAP1 staining intensity in all samples was scored by a pathologist.

2.4. Isolation of HLA-AAD peptides

HLA-AAD-bound peptides were obtained by the immunoprecipitation as previously described [31] with slight modifications. The flow chart of CTL peptide identification is shown in supplemental Fig. S2. TC1/AAD cells were harvested from two spinner flasks (1 × 108 cells/liter), lysed with CHAPS solution (1% in chilled PBS buffer, pH 7.8) and then centrifuged at 2000 rpm for 10 min to remove the cell debris. The HLA-AAD-peptide complex was

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immunoprecipitated using an anti-HLA A2 antibody (BB7.2-)immobilized column. After heat denaturation (95 °C, 10 min) and a cooling step, the associated peptides were extracted sequentially using an ultracentrifuge column (10 kDa cutoff membrane, Milli-pore, Billerica, MA) and reverse-phase spin column (C18, Invitrogen) [31,32]. The desalted analyte was lyophilized and then resuspended in 5% formic acid solution for LC-MS/MS analysis.

2.5. Identification of HLA-AAD-bound peptides by LC-MS/MS

A Q Exactive mass spectrometer (Thermo Scientific, USA) coupled to an Ultimate 3000 RSLC system (Dionex) was utilized for HLA-peptide sequencing. The complex peptide mixture was separated using a reverse-phase column (C18, Acclaim pepmap RSLC, 75 μm × 150 mm, 2 μm pore size, Dionex). The mobile phases were prepared as follows: A: 0.1% FA in 5% acetonitrile (ACN) (V/V), and B: 0.1% FA in 95% ACN (V/V). A linear gradient was initiated from 1% B to 25% B in 40 min, and then increased to 80% B in 18 min to elute all the analytes in the column. Full MS was performed within the range of m/z 300–2000, and the ten most intense ions in the MS scan were subjected to fragmentation to acquire the MS/MS (tandem MS) spectra. The MS raw data was converted into peak list (PKL) using Proteome Discoverer 1.3. Sequence analysis was carried out using the MASCOT searching engine with self-constructed databases (mouse species, UniProt) with parameters including the following: no enzyme, variable modification of oxidation (M) and phosphorylation (ST, Y), intensity ratio cutoff of 10%, tolerance of ±0.002 Da, mass tolerance of ±0.0 ppm. The MS spectra of the identified sequences were confirmed by further manual examination.

2.6. Prediction of binding motif of peptide

The MHC class I binding predictions were performed using IEDB analysis resource NetMHC (ver. 4.0) tool [33–35] with parameters including the following: human allele of HLA-A*02:01, mouse allele of H2-D^b and peptide length of 8–12 amino acids.

2.7. T2 cell-based stabilization assay

The binding affinity between the TAP-deficient T2-cell stabilization assay as described previously [36]. Briefly, 2 × 10^5 T2 cells (ATCC, HLA-A2-positive) were incubated with 10 μM synthetic peptide (purity greater than 95%, UV 210 nm) in DMEM containing 0.1% FBS, 5 × 10^{-5} M β-mercaptoethanol, and 5 × 10^{-7} M β2-microglobulin at 25 °C in a 5% CO2 filling incubator overnight. Subsequently, the temperature was adjusted to 37 °C for 3 h incubation, stained with PE-conjugated anti-HLA-A2 antibody (BB7.2, BD Bioscience, USA), and then detected the mean fluorescence intensity (MFI) by flow cytometry (FACScalibur, BD Bioscience, USA). Cell marked with isotype Ab (27–35, monoclonal anti-CD8, BD) served as control in the T2 assay. The affinity was evaluated using FCS Express software (De Novo Software).

2.9. Intracellular staining

Splenocytes (5 × 10^6) were harvested from peptide-immunized AAD transgenic mice and incubated with the corresponding immunogen (final concentration 10 μg/ml) overnight at 37 °C with 5% CO2. After the incubation period, the cells were washed once with chilled PBS and stained with FITC-conjugated mononclonal anti-mouse CD8 antibody (53–6.7, BD). The FITC-labeled cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin/PBS for 5 mins, and then the supernatant was decanted. Intracellular cytokine staining was detected with PECy7-conjugated anti-mouse IFN-γ (R3-34, BD), and the number of stained cells was then determined by flow cytometry (BD Bioscience). The data analysis was performed using FCS Express software (De Novo Software).

2.10. In vivo killing assay

Splenocytes (5 × 10^7) were harvested from AAD transgenic mice and then incubated with either peptide (5 μg/ml) or PBS (untreated control) for 30 min at 37 °C. After incubation, carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) was used to label the peptide-pulsed spleen cells and untreated control at a concentration of 10 μM and 1 μM, respectively, for 15 min at 37 °C. Ice-cold complete RPMI medium was then added to stop the CFSE-labeling reaction. The non-peptide pulsed cells and the peptide-pulsed splenocytes were mixed at a ratio of 1:1, and a total of 2 × 10^7 CFSE-labeled cells were injected into the peptide-immunized AAD transgenic mice via the tail vein. At 18 h post-adoptive transfer, the spleen cells were harvested and analyzed by flow cytometry (BD Bioscience). The data analysis was performed using FCS Express software (De Novo Software).

2.11. 51Cr release assay

The CD8+ T cells were isolated from spleen of KLIpS-immunized mice using the CD8+ T Cell Isolation Kit (Miltenyl Biotech). H2981 cells (5 × 10^5/ml) were labeled with 100 μCi of 51Cr (Na_2CrO_4, PerkinElmer, MA) at 37 °C for 1 h as the target cells. The CD8+ T cells (5 × 10^5) was then mixed with 51Cr-labeled target cells (5 × 10^5) at a ratio of 100:1 (Effector : Target, E : T), and incubated at 37 °C for 18 h. After the incubation period, the supernatants were harvested to measure the radioactivity using a gamma counter. The percentage of specific lysis was calculated using the following formula: 100 × [(experimental release – spontaneous release)/(maximal release – spontaneous release)].
2.12. Tumor challenge assay

AAD transgenic mice aged 6–8 weeks were inoculated with 2 × 10^5 TC1/AAD cells for tumor development. One week after the tumor injection, the tumor-bearing mice (n = 5) were immunized twice with 50 μg peptide in IFA with PADRE on Day 7 and 14 by footpad injection. Tumor-bearing mice (n = 5) immunized with PBS buffer served as the untreated control group in the assay. Tumor size was monitored twice a week by palpation with calipers, and the tumor volume was calculated using the following formula: length × width × width/2. The tumor-bearing mice were sacrificed when the tumor size exceeded 2000 mm^3.

2.13. Statistical analysis

Statistical significance was evaluated by the Student’s t-test (two-tailed) and ANOVA at the 5% level.

3. Results

3.1. Expression levels of TRAP1 in lung cancer cells and tumors

To assess the TRAP1 expression level in mouse and human lung cancer cell lines, we quantified this protein target using a western blot assay and compared its abundance to that in normal mouse lung cells. Consistent with another study [40], the expression level of TRAP1 in cancer cell lines was significantly higher in the tested lung cancer cell lines, but it was nearly undetectable in normal lung tissue, as shown in Fig. 1A. In order to validate the expression of TRAP1, immunohistochemical staining was carried out to assess the abundance of TRAP1 in human lung tumor tissues and their adjacent normal tissues. IHC results showed that positive TRAP1 expression was located in the cytoplasm as red color staining, as shown in Fig. 1B. The strong staining of TRAP1 in tumor tissue and normal tissue were 38.6% (17/44) and 15.9% (7/44), respectively, and the difference was statistically significant. Among these 7 normal tissue samples with strong staining of TRAP1, 5 of them were sampled from the adjacent tumor tissues with highly abundant of TRAP1, suggesting the high expression rate of TRAP1 could be found in normal tissues adjacent to the tumor tissue.

3.2. Identification of HLA-A2-bound peptides by mass spectrometry

In the study, LCMS was conducted to analyze the HLA-AAD-bound peptides that immunoprecipitated from TC1/AAD cells. The MASCOT search resulted in the identification of a total of 12 significant HLA-A2-restricted peptides in 1 × 10^5 TC1/AAD cells, and their sequences were further confirmed by manual inspection of their corresponding tandem MS spectra (Table S1). By importing the sequences into the IEDB (www.IEDB.org) database, all the MS-identified peptides fit the binding motif of HLA-A2 (A'02:01) molecule but not the mouse MHC molecule (H2-D^d). Among these MS-identified peptides, KLPsSVETDLI (abbreviate as KLPs, lowercase letter denotes phosphate group in the article) and RQLPSPSSGVEL (ROLPs) were phosphopeptides derived from the cancer-associated protein of TRAP1_mouse (Q9CQ1, UniProt) and HSP27_mouse (P14602, UniProt), respectively. Additionally, synthetic references were prepared and their tandem MS spectra presented nearly similar b/y fragment patterns as those acquired from the immunoprecipitated phosphopeptides, which confirmed the sequence identities of the phosphopeptides (Figs. S3 and S4). It is noteworthy that ROLPs is a known HLA-A2-restricted CTL epitope that has been detected in human ovarian carcinoma (cov413) [41] and melanoma cells (DM331) [27,42]. Such Hsp27-derived HLA-A2 restricted phosphopeptide was first observed in a mouse lung cancer cell line, further supporting the MS sequencing results of the study. As the structure features indicated phosphate group of HLA-phosphopeptide is critical for T cell receptor (TCR) recognition, [43], the immunogenicity of TRAP1-derived phosphopeptide on CTL induction was analyzed in the following study.

3.3. HLA-peptide stabilization assay

A stabilized peptide-HLA (pHLA) complex is essential for TCR recognition. The T2 cell stabilization assay was applied to assess the binding affinity of the phosphopeptide to HLA-A2 molecule. The T2 cell line is TAP-deficient but expresses peptide-unloaded HLA-A2 molecules on the cell membrane, which can be stabilized by docking with allele-restricted peptides. As shown in Fig. 2, the KLPs was found to form a stabilized complex with the HLA-A2 molecule in the T2 cell as well as the positive control of YMLDLQ-PET peptide (HLA-A2 binder). In contrast, only a few or a less stabilized HLA-peptide complex was detected when T2 cell was cocultured with non-phosphorylated peptide (KLIS). The observed results indicated that such site-specific phosphate moiety of HLA-A2 phosphopeptide is critical for the peptide binding affinity with HLA-A2 molecule, and such result is consistent with the finding of other investigations [21].

3.4. Immunization with KLPs induces cytokine responses in transgenic mice

Based on the T2 assays result, we further evaluated the T cell-inducing ability of KLPs in the animals of C57BL/6 wild type (WT) and AAD transgenic mice. ELISPOT result revealed that a high frequency of interferon gamma (IFN-γ)-secreting cells was detected only in the lymph nodes of peptide-immunized AAD...
transgenic mice, but not in the wild-type control, as shown in Fig. 3A. The mice-immunization results not only validated the binding motif of KLipS, but also indicated that the cytokine response was mediated by the AAD molecule rather than other mouse MHC molecules. Subsequently, the AAD transgenic mice were immunized with peptides of KLIpS, KLIS (non-phosphorylated counterpart), YML and VYC to compare their efficacy for cytotoxic T cell induction. Upon peptide restimulation, ELISPOT assay results demonstrated that a significantly larger number of IFN-γ-secreting cells in the lymph nodes of AAD transgenic mice immunized with KLIPs (spots: 230 ± 45) or YML peptide (spots: 161 ± 81, positive control), but such cytokine response was not observed in the mice immunized with either KLIS or VYC peptide (Fig. S5). Moreover, a cross-restimulation assay was performed, and the robust cytokine response was restricted to KLIPs but not to its non-phosphorylated counterpart, as shown in Fig. 3B. Additionally, an intracellular staining assay was introduced to evaluate cytokine expression within a heterogeneous population of cells. After fixing the cell membrane and Golgi apparatus, the accumulated IFN-γ was detected in the CD8+ T cell population (3.7%) of splenocytes from KLIPs-immunized AAD transgenic mice, which made up a relatively smaller population in AAD transgenic mice immunized with KLIS (0.5%) or VYC (0.7%), as shown in Fig. 3C and 3D. The cytokine secretion assay confirmed that KLIPs is immunogenic and capable of inducing effector CD8+ T cells through the HLA-AAD molecule in transgenic mice.

3.5. Cytolytic activity of KLIPs-specific CTLs

In addition to the cytokine response of peptide restimulation, we further evaluated the cytolytic activity of KLIPs-specific CTLs on different cell targets in vivo and in vitro. In the in vivo killing assay, the naïve splenocytes of transgenic mice was pulsed with phosphopeptide to mimic the cancer cell in native-like condition. A mixture of equal amounts of epitope-pulsed (CFSE<sup>high</sup>) and untreated (CFSE<sup>low</sup>) splenocytes was injected into the KLIPs-immunized AAD transgenic mice through the tail vein. Then the splenocytes of recipient were then harvested to determine the percentage of CFSE<sup>high</sup> and CFSE<sup>low</sup> among the total CFSE<sup>+</sup> cells. As shown in Fig. 4A, nearly half of the CFSE<sup>high</sup> were diminished in mice immunized with either KLIPs (48.11 ± 18.36%) or the CTL peptide (61.7 ± 41.34%), HLA is the positive control. As such, the results evidenced the stimulated antigen-specific T cell can recognize and induce cytolsis of target cells with the HLA-AAD-KLIPs complex in vivo.

Apart from phosphopeptide-pulsed splenocytes, the CTL recognition on human cancer cell line was examined in the study. H2981 is an HLA-A2 positive human lung cancer cell line that expresses high abundant TRAP1 proteins as those found in TC1/AAD cell, which suggested that KLIPs could be expressed in H2981 cells as TC1/AAD cell line. We, therefore, extracted the CD8+ T cells from KLIPs immunized mice to evaluate the cytolytic activity on H2981 using an in vitro chromium (51Cr) release assay. In Fig. 4B, the results demonstrated that up to 20% of 51Cr-labeled H2981 (target, T) was lysed when incubating with mouse effector (effecter, E) at the ratio of 100:1 (E:T), while this cytolytic activity was markedly inhibited when either TCR or HLA was blocked with anti-CD8 or anti-HLA antibody, respectively. As such, the results revealed that the recognition of effector CD8+ T cell was mediated through the interaction of HLA and T cell receptor (TCR) molecules, but also demonstrated the capability of KLIPs-stimulated T cell to cytolsis the target cell with HLA-A2 restricted KLIPs peptide.

3.6. Anti-tumor effect of KLIPs-specific CTLs

Based on the killing effect on cancer cells, a tumor challenge model was developed to examine the cytolytic effect of KLIPs-specific CTLs in tumor tissue. Fig. 5 profiles the tumor size in mice immunized with peptide antigen and PBS (control), respectively. In the tumor challenge assay, tumor-bearing mice (n = 5) immunized with KLIS or PBS buffer were all sacrificed by 7 weeks. Immunization of KLIPs peptide caused a significant reduction of tumor growth and prolonged the survival period of tumor-bearing mice for more than 7 weeks, demonstrating the potency of KLIPs vaccination as a therapeutic strategy for cancer treatment.

4. Discussion

Lung cancer is one of the most prevalent cancers worldwide and also a leading cause of malignant-related death because of its highly metastatic potential. Systemic chemotherapy is commonly used for lung cancer treatment, which could improve the survival rate but might provoke serious side effect. Currently, immunotherapy is considered as a candidate strategy since increasing ev-
This phosphopeptide in HLA-A2 transgenic mice could induce complex assembly, we also demonstrated that immunization of compared to the non-phosphorylated counterpart [43]. Apart from residues of the HLA-A2 molecule [43]. Our study is consistent with terminal residue could form an electrostatic interaction with polar has indicated that the phosphate conjugated on the forth N-terminal residue of KLIpS contributes unique features for peptide-specific T cell induction. First, the phosphate moiety on the fourth N-terminal residue of KLIpS enhances the binding affinity of KLIpS with the HLA-A2 molecule. A structural investigation on HLA-peptide binding was performed to assay the specificity of peptide-induced IFN-γ-secreting cells from peptide-immunized AAD transgenic mice. (Student’s t-test for KLIpS restimulation: KLIS vs KLIpS immunization; for KLIpS immunization: KLIS vs KLIpS restimulation, **, p < 0.01). The error bar represented the standard deviation (SD) of triplicate experiments. (C) Six AAD transgenic mice were vaccinated with KLIpS, as described in the legend of Fig. 2A. Detection of the effector T cell population in 1 × 10⁶ splenocytes of peptide-immunized AAD-transgenic mouse using flow cytometry. The upper-right corners revealed a significant population of stimulated IFN-γ-secreting CD8⁺ T cells among the lymphocytes of KLIpS-immunized AAD transgenic mice. (D) The bar graph shows the percentage of IFN-γ-secreting cells in CD8⁺ T cells (Student’s t-test, KLIS vs KLIpS, **, p < 0.01). The error bar represented the standard deviation (SD) of triplicate experiments.

dences showed that host immune system can confer significant survival advantages. To meet the success of immunotherapeutic treatment, ideal targets are essential and critical. In the study, we demonstrated that TRAP1 was highly expressed in variety of lung cancer cell lines and patient tissues, but nearly silenced in normal cell. With the aid of MS analysis, one novel TRAP1-derived HLA-A2-restricted phosphopeptide, KLIpS, was identified from the AAD gene-transfected mouse lung epithelial cancer cell. The phosphorylation site of KLIpS fits the consensus motif of kinases, R/KxxS/T. To our knowledge, it was the first HLA-A2 restricted phosphopeptide, KLIpSVETDI, identified from the tumor-associated antigen of TRAP1. The correlation between the new phosphorylation site and TRAP1 functionality was not clear, but increasing phosphorylation of the protein suggested an altered kinase activity in mitochondria [44].

Other than the phosphate dynamics of the protein, the pharmaceutic moity on the fourth N-terminal residue of KLIpS contributes unique features for peptide-specific T cell induction. First, the phosphate enhanced the binding affinity of KLIpS with the HLA-A2 molecule. A structural investigation on HLA-peptide binding has indicated that the phosphate conjugated on the forth N-terminal residue could form an electrostatic interaction with polar residues of the HLA-A2 molecule [43]. Our study is consistent with previous observations that assay did present that KLIpS could form a stabilized complex with HLA-A2 molecule in the TAP-deficient as compared to the non-phosphorylated counterpart [43]. Apart from complex assembly, we also demonstrated that immunization of this phosphopeptide in HLA-A2 transgenic mice could induce IFN-γ-secreting CD8⁺ T cell in splenocytes during antigen stimula-
potency of CTL on cell targets. Based on the peptide density information and procedure optimization, we expect to develop a novel peptide-based vaccination approach for improving the outcome of CTL responses on the treatment of cancer therapy.

Author contributions

Concept and design: SJ Liu, WC Sung.
Development of methodology: Y-P Sher, S-J Liu, W-C Sung.
Acquisition of data (provided animals, acquired and managed patients, provided facilities): I-H Chen, S-J Liu.
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): M-H Lin, K-Y Shen, BS Liu, Y-P Sher, G-C Tseng.

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Conflict of interest

No potential conflicts of interest were disclosed.

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