Identification of HLA-A*0201-restricted Cytotoxic T Lymphocyte Epitope from TRAG-3 Antigen

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ABSTRACT

Purpose: Identification of tumor antigen and subsequent identification of T-cell epitope from these antigens make specific immunotherapy for malignant tumor applicable. Because TRAG-3 antigen is expressed in most melanomas and 54% of non-small cell lung carcinomas and HLA-A2.1-expressing individuals cover >50% in the population of China, we aim at identifying TRAG-3-encoded peptide presented by HLA-A2.1.

Experimental Design: In our study, a HLA-A2.1-restricted CTL epitope was identified by using the following four-step procedure: (a) computer-based epitope prediction from the amino acid sequence of TRAG-3 antigen; (b) peptide-binding assay to determine the affinity of the predicted peptide with HLA-A2.1 molecule; (c) stimulation of primary T-cell response against the predicted peptide in vitro; and (d) testing of the induced CTLs toward LB373-MEL cells expressing TRAG-3 antigen and HLA-A2.1.

Results: Of the four tested peptides, effectors induced by a peptide of TRAG-3 at residue position 58–66 lysed LB373-MEL cells expressing both TRAG-3 and HLA-A2.1. Our results indicate that peptide TRAG-358–66 (ILLRDAGLV) is a new HLA-A2.1-restricted CTL epitope capable of inducing TRAG-3-specific CTLs in vitro.

Conclusions: Because TRAG-3 is a cancer/testis antigen expressed in most melanomas and half of non-small cell lung carcinomas, identification of the TRAG-3/HLA-A2.1 peptide ILLRDAGLV may facilitate peptide-based specific immunotherapy for various histological tumors.

INTRODUCTION

CT antigens family has been an attractive target for tumor immunotherapy because these antigens can be recognized by human CTLs (1–3) and expressed in a variety of malignant tumors from many kinds of histological origins but not in normal tissues, with the exception of the testis and placenta (4–6). The antigenic peptides derived from CT antigens such as MAGE-1 (7), MAGE-3 (8) and NY-ESO-1 (9) have been proven to elicit a CTL response in the context of MHC class I molecules. Moreover, immunization with these peptides-pulsed dendritic cell vaccines, as a modality of specific immunotherapy, has been applied to melanoma patients and other malignant patients and proven to have some clinical effectiveness (8, 10). However, expression of CT antigens is heterogeneous among tumors of different histological origins, different patients, and between individual lesions. These characteristics of CT antigen expression suggest that the development of specific immunotherapy based on as many antigens as possible may be clinically beneficial.

The TRAG-3 antigen, which was initially identified by Duan et al. (11) after comparing SKOV-3 to SKOV-3Tr by differential display, belongs to a growing class of CT antigens. This gene is found to be overexpressed in most carcinoma cell lines and in a high proportion of malignant melanoma and chondrosarcoma (12, 13). Recently, we found that the expression of TRAG-3 was seen in ~54% of non-small cell lung carcinoma, especially in lung adenocarcinoma (14). On the other hand, HLA-A2.1 is the most common HLA-A allele in Asian populations, especially in the Chinese, with an estimated frequency of >50% (15). In addition, it is the most common subtype of these alleles and has a ~98% expression in the HLA-A2+ Caucasians in North America. Therefore, if TRAG-3-derived HLA-A2.1-restricted CTL epitopes are identified, these peptides may be widely applied for specific immunotherapy against TRAG-3-positive tumor in clinic. In this study, we first identified four TRAG-3-derived peptides with sound affinities to the HLA-A2.1 molecule verified with MHC peptide-binding assay and epitope prediction with computer algorithms and molecular modeling. We then induced TRAG-3-specific CTLs from HLA-A2.1-positive PBMCs of four healthy donors with these candidate peptides in vitro to seek a CTL epitope from TRAG-3 antigen.
MATERIALS AND METHODS

Epitope Prediction. There are several algorithms used to predict HLA-A2.1-restricted CTL epitope. In this study, we used the automated and programmed method to identify the candidate HLA-A2.1-restricted CTL epitopes from TRAG-3 antigen. The method was established on the basis of supertype main anchor motif and quantitative motif scheme. Briefly, protein sequence from the TRAG-3 antigen encoded by smaller transcripts (11) was scanned to identify 9-mer sequences containing the HLA-A2.1 supertype main anchor motif. Position 2 and the COOH terminus of the motif is leucine (L), isoleucine (I), valine (V), methionine (M), alanine (A), or threonine (T). The 9-mer peptides were further determined with the aid of quantitative motif methods established by Parker et al. in 1993 (16).

Molecular Modeling. Models of HLA-A2.1 and binding 9-mer peptides were established from the crystal structures of the Brookhaven Protein Data Bank: 3HLA for HLA-A2.1 and 3HSA for the nonameric peptides. The HLA-A2.1 model was simplified by using only α1 and α2 domains and 18 water molecules bound on them. Molecular mechanics and dynamics calculations were performed with the Discover 3.0 package. The force field parameters used in this study were the consistent valence force field. During the molecular dynamics and minimization, a dielectric constant of 1.0 was used. A 9.0 Å cutoff distance was applied to calculate the nonbinding interaction. The peptide ligand was first relaxed by 500 steps of conjugate gradient energy minimization while maintaining fixed. It was then submitted to a 100-ps molecular dynamics calculation at 300 K. During these 100 ps, no protein atom was allowed to move. The last conformation was then solvated in a 10 Å-thick TIP3P water shell. Energy minimization of the peptide ligand, of the HLA-A2.1/ligand complex, followed by 200-ps molecular dynamics simulation of the full solvated HLA-A2.1/ligand pair was performed at 300 K.

Cell Lines. Human TAP-deficient T2-cell line and BB7.2 cell line producing mAb against HLA-A2.1 were purchased from American Type Culture Collection. LB373-MEL cell line was a generous gift from Professor Francis Brasseur (Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium). Melanoma cell line M14 was provided by Dr. Zhongmin Zou (Institute of Combined Injury, Third Military Medical University, Chongqing, China). Lung adenocarcinoma cell line A549 and breast carcinoma cell line MCF-7 were synthesized on a solid-phase simultaneous multiple peptide synthesizer based on the Fmoc strategy. Synthesis products were purified with C18 reverse-phase HPLC and yielded a purity of >93%. Lyophilized peptides were dissolved in DMSO at a concentration of 20 μg/ml and stored at −20°C.

Synthetic Peptides. Four peptides predicted were synthesized on a solid-phase simultaneous multiple peptide synthesizer based on the Fmoc strategy. Synthesis products were purified with C18 reverse-phase HPLC and yielded a purity of >93%. Lyophilized peptides were dissolved in DMSO at a concentration of 20 μg/ml and stored at −20°C.

MHC Peptide-binding Assay. The binding activity of selected peptides to HLA-A2.1 molecule was determined semi-quantitatively by measuring peptide-induced expression of HLA-A2.1 molecules on T2 cells with flow cytometry. The T2 peptide-HLA-A2.1 stabilization assay was performed according to Nijman et al. (17). Briefly, T2 cells were incubated overnight with the candidate peptides, respectively, at a concentration of 20 μg/ml in serum-free medium supplemented with β2-microglobulin (Sigma) at a concentration of 3 μg/ml. After washed with PBS-FCS, T2 cells were incubated with murine mAb against HLA-A2.1 derived from BB7.2 cells for 30 min at 4°C. T2 cells were washed twice with PBS-FCS and stained with 1:50 diluted FITC-conjugated IgG of sheep antibody to mouse immunoglobulin for 30 min. Cells were then rinsed for three times with PBS-FCS and analyzed with a FACSScan flow cytometer. The fluorescence index was calculated by using the following formula:

\[
FI = \frac{\text{MFI sample} - \text{MFI background}}{\text{MFI background}}
\]

In the formula, MFI background means the value without peptide incubation. Samples were measured in triplicate and then mean FI was calculated.

Analysis of TRAG-3 Expression with RT-PCR and Immunohistochemistry. RT-PCRs were used to analyze the expression of TRAG-3 mRNA in all tumor cell lines, including LB373-MEL, A549, M14, and MCF-7. Total RNA was isolated from tumor cell lines by using Tripure Isolation Regent kit (Progena). Synthesis of cDNA was performed with 2 μg of total RNA with the aid of a reverse transcriptase kit (Progena) and oligod(T)18 primers. Two μl of RT product were amplified with PCR by using TaqDNA polymerase (Sangon, Shanghai) in standard procedures. The following oligonucleotides were used as primers: sense 5′-GCTGGCCTGGCTCTGTC-3′; and antisense 5′-CACCCCTGTGGCTATTTACCTGGG-3′. Both primers were synthesized commercially in Sangon Company (Shanghai, China). Thirty amplification cycles were run: 1 min at 94°C; 1 min at 60°C; and 1 min at 72°C. Cycling was ceased with a final extension of 10 min at 72°C. RT-PCR products were visualized with ethidium bromide.

Moreover, the expressions of TRAG-3 protein in LB373-MEL and A549 cell lines were determined with immunohistochemistry. The first antibody to TRAG-3 antigen, generously provided by Dr. Duan Zhengfeng (Division of Hematology/Oncology, Massachusetts General Hospital, Boston, MA), is a rabbit polyclonal antibody against human TRAG-3 protein.

Induction of Peptide-specific CTL with Synthetic TRAG-3 Peptides. The CTL induction in vitro was performed in accordance with the previously described procedures.
In brief, the PBMCs of four HLA-A2.1 healthy donors were firstly obtained with centrifugation at a Ficoll-Paque density gradient and then cultured in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. These cells were pulsed by the synthetic peptides, respectively, at a final concentration of 10 μg/ml. The PBMCs were restimulated every 7 days with fresh medium containing these peptides. Recombinant interleukin 2 at a concentration of 20 units/ml was added to the culture media on day 3 and also 1 day after every stimulation. The CTL activity was then assessed on day 23.

Cytotoxicity Assay. LB373-MEL cells serving as target cells (1 × 10⁶) were labeled with Na⁵¹CrO₄ in 1 ml of RPMI 1640 supplemented with 10% FCS for 1 h at 37°C in 5% CO₂. The ⁵¹Cr-labeled cells were washed three times and then plated in triplicate at a final concentration of 1 × 10⁴ cells/well in
96-well V-bottom microtiter plates. Various concentrations of the effector cells were then added to the 51 Cr-labeled target cells. The volume in each well was adjusted to 200 μl and incubated for 4 h at 37 °C in 5% CO2. The release of the 51 Cr label was measured by collecting 100 μl of the supernatant followed by quantitation in an automated gamma counter. The percentage of specific cytotoxicity was calculated as the percentage of specific 51 Cr release using the following formula:

\[
\text{Specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100\%
\]

To explore the inhibition of cytotoxicity with anti-HLA-A2.1 mAbs, LB373-MELs (1 × 106) were incubated with anti-HLA-A2.1 mAbs and produced by BB7.2 cells for 1 h at 4°C before the cytotoxicity assay.

**RESULTS**

**Epitope Prediction and Molecular Modeling.** The sequences of TRAG-3 were initially screened for the peptides containing the supermotif for HLA-A2.1. Seven peptides consisted of nine amino residues were found containing the supermotif for HLA-A2.1. However, there are only four 9-mer peptides suitable to HLA-A2.1-restricted CTL epitope from TRAG-3 after calculated with quantitative motif method. The sequences of these peptides were HACWPAFTV, GLIQLVEGV, ILLRDAGLV, and SILLRDAGL. Molecular modeling showed that three potential CTL epitopes from TRAG-3 were suitable to the criteria of HLA-A2.1-restricted CTL epitope with the exception of HACWPAFTV. As shown in Table 1, the peptides bound to the HLA-A2.1 model structure possess a side chain of COOH-terminal anchor residues oriented into the binding groove with different distances from 17 to 19 Å. However, HACWPAFTV might not be the candidate HLA-A2.1-restricted CTL epitope because of the orientation of P9 side chain toward the solvent-accessible surface, although it has proper distance between P2 and P9.

![Fig 2](image-url)

**Fig 2** Expressions of the TRAG-3 gene in cell lines (LB373-MEL, A549, MCF-7, and M14) detected with RT-PCR. PCR amplification was performed with specific primers (sense 5'-GCTGGCCTGTCACTGTC-3'; antisense 5'-CACCCCTGTGGCATTATCTGG-3'), and PCR products were demonstrated through electrophoresis on a 1.2% agarose gel with ethidium bromide staining. PCR marker represents the DNA size marker (PCR Marker, Sagon, China). Lane 1: LB373-MEL; Lane 2: A549; Lane 3: M14; Lane 4: MCF-7; and Lane 5: PCR marker (1543, 994, 697, 515, 377, and 237 bp from top to bottom).
MHC Peptide-binding Assay. We selected four 9-mer-candidate peptides from the predicted epitope by epitope prediction and molecular modeling. These peptides were then synthesized and purified to >93% by C18 reverse-phase high-performance liquid chromatography. The binding affinity of these peptides to HLA-A2.1 was determined by using antigen processing-deficient T2 cells because their enhanced HLA-A2.1 expression when exposed to exogenous HLA-A2.1-binding peptides, HLA-A2.1-restricted MAGE-2 CTL epitope KMV-VELVHFL [amino acid position in MAGE-2; 112–120] and FLPSDFFPSV derived from hepatitis B virus (amino acid position in hepatitis B virus; 18–27), served as positive and negative controls, respectively. As shown in Table 2, all of the peptides synthesized were bound to HLA-A2.1 molecules but with different affinities. Of 4 TRAG-3 peptides selected, one (ILLRDAGLV) up-regulated the HLA-A2.1 molecular expression and showed high affinity to HLA-A2.1, whereas another (GLIQLVEGV) showed moderate affinity and the left two peptides showed low affinity.

Fig. 3 Immunohistochemistry analyses of TRAG-3 protein expressions in normal A549 lung carcinoma cell line and LB373-MEL melanoma cell line (magnification, ×200). The first antibody to TRAG-3 antigen is a polyclonal, a rabbit antibody against human TRAG-3 protein, which is expressed in *Escherichia coli* and then purified from the bacteria. A, LB373-MEL cell line; B, A549 cell line.
(HACWPAFTV and SILLRDAGL) only had low affinity to the molecule.

Expression of TRAG-3 in Tumor Cell Lines. The expressions of TRAG-3 mRNA in all cell lines in this study were analyzed with RT-PCR. Expression of positive control H9252-actin (329 bp) housekeeping gene was observed in all cell lines (Fig. 2), suggesting that mRNA isolation and RT-PCR were all successful. TRAG-3 gene was expressed in M14 cells (melanoma cell line, HLA-A2.1) and LB373-MEL cells (melanoma cell line, HLA-A2.1), although not in A549 cells (lung adenocarcinoma cell line, HLA-A2.1) and MCF-7 cells (breast carcinoma cell line, HLA-A2.1; Fig. 2). Moreover, immunohistochemistry indicated the expression of TRAG-3 protein in the cytoplasm of LB373-MEL cells (Fig. 3A) but no expression in A549 cells. (Fig. 3B)

Induction of CTL Specific to TRAG-3 Antigen by Synthetic Peptide. PBMCs from four donors were stimulated with synthetic peptides with the previously published method for CTL induction (18). Of four peptides tested, peptide TRAG-3 (58–66) (ILLRDAGLV) was able to elicit TRAG-3-specific CTLs, which could lyse LB373-MEL cells expressing both TRAG-3 and HLA-A2.1. After 21 days of stimulation with ILLRDAGLV at the concentration of 10 µg/ml once a week. On day 23, 4-h 51Cr-release assays were performed to test their cytotoxic activities against LB373-MEL cell line (HLA-A2.1+, TRAG-3+).

Inhibition of the Recognition of Effectors by Anti-HLA-A2.1 Antibody. To determine whether the peptide TRAG-3 (58–66), ILLRDAGLV-induced effectors recognized the TRAG-3-positive target tumor cells in an HLA-A2.1-restricted manner, the mAbs against HLA-A2.1 were used to block recognition by effectors. Our results showed that the anti-HLA-A2.1 antibody could significantly eliminate the cytotoxicity of the effectors against LB373-MEL melanoma cells (Fig. 6), which implied that the induced effectors lysed the LB373-MEL cells in an HLA-A2.1-restricted manner.

DISCUSSION
During the last decade, great progress has been made in the finding of CTLs recognizing melanoma-associated antigens (19,
20). These antigens mainly belong to three groups: tumor-associated tests-specific antigens (e.g., MAGE, BAGE, NY-ESO-1, and GAGE; Refs. 20–23), also called CT antigen; melanocyte differentiation antigens (e.g., tyrosinase, Melan-A/ MART-1, gp100, TRP-1, and TRP-2; Refs. 24, 25); and mutated or aberrantly expressed antigens (e.g., MUM-1, CDK4, β-catenin, and p15; Refs. 26, 27). Of these three groups, CT antigen family is predominantly regarded as targets for cancer immunotherapy because of their expressions in human different histological tumors and absent expression in normal tissues, except for the testsis, an immune-privileged organ (1–3). Therefore, the identification of T-cell epitopes from these antigens becomes a critical step in the development of peptide-based immunotherapy for cancer.

A major breakthrough in the identification of T-cell epitope was the elucidation that ligands of a certain MHC-molecule carry chemically related amino acids in certain positions, which lead to the definition of a peptide motif for every MHC allele (28). This knowledge was rapidly used to predict potential epitopes from various antigens and became the start signal for the so-called reverse immunology, which has been the most successful strategy for the identification of T-cell epitopes (29–31). This approach, including a four-step procedure of (a) computer-based epitope prediction from the amino acid sequence of a candidate antigen, (b) peptide-binding assay to determine the affinity of the predicted peptide with MHC molecule, (c) the stimulation of primary T-cell response against the predicted peptides in vitro, and (d) testing of the resulting CTLs toward target cells endogenously expressing the antigen (32). With this approach, lots of T-cell epitopes have been identified from several CT antigen such as MAGE-1 (33), MAGE-3 (34), and TRP-2 (35). Moreover, with the elucidation of crystal structures of human MHC class I molecules HLA-A2.1, molecular modeling of HLA-A2.1-restricted epitopes was also available. Lim et al. (36) found that the peptides bound to the HLA-A2.1 model structure possess a side chain of COOH-terminal anchor residue oriented into the binding groove with certain distance between the two anchor residues from 15 to 21 Å.

In this study, we first predicted four candidate epitopes from TRAG-3 antigen by using HLA-A2.1-restricted epitope prediction algorithms based on superimotif and quantitative motif methods. Secondly, we characterized four predicted candidate epitopes with molecular modeling and found that one epitope HACWPAFTV was not suitable to HLA-A2.1-restricted CTL epitope because of a side chain of COOH-terminal anchor residue oriented toward the solvent-accessible surface. Thirdly, peptide-binding assay was used to determine the affinity of every epitope with HLA-A2.1 and showed that one (ILLRDAGLV) had high affinity to HLA-A2.1, whereas another (GLIQVLQGV) had moderate affinity and the left two (HACWPAFTV and SILLRDAGL) had low affinity to HLA-A2.1 molecule. Finally, we used 51Cr-release assay to determine the induction of CTLs by every epitope. To determine whether the effector CTLs induced by TRAG-3(58–66), ILLRDAGLV peptide could recognize the TRAG-3-positive target tumor cells in an HLA-A2.1-restricted manner, we examined the inhibition of cytotoxicity with anti-HLA-A2.1 mAbs. These results showed that the cytotoxic activity of effectors against targets could be eliminated by anti-HLA-A2.1 mAbs. Thus, our present study indicates that peptide TRAG-3(58–66) (ILLRDAGLV) is a new HLA-A2.1-restricted CTL epitope capable of inducing TRAG-specific CTLs in vitro. TRAG-3 is a CT antigen containing 127 amino acids, which is expressed in most melanomas and >50% non-small cell lung carcinomas. In Chinese, the proportions of HLA-A2.1 individuals are >50%. Therefore, identification of TRAG-3(58–66) (ILLRDAGLV) peptide would contribute a lot to the design of epitope-based vaccine for melanoma patients and lung cancer patients.

In conclusion, our results suggest that TRAG-3(58–66) (ILLRDAGLV) peptide derived from TRAG-3 might be capable of inducing HLA-A2.1-restricted CD8+ CTL, which would be lethal for tumor cells expressing TRAG-3 and HLA-A2.1. Currently, we are evaluating the TRAG-3(58–66) (ILLRDAGLV) peptide-pulsed dendritic cells vaccine in HLA-A2.1+/TRAG-3+ patients with non-small cell lung carcinoma.

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