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ORIGINAL ARTICLE

In vitro and *in vivo* identification of a novel cytotoxic T lymphocyte epitope from Rv3425 of *Mycobacterium tuberculosis*

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ABSTRACT

The identification of novel cytotoxic T lymphocyte (CTL) epitopes is important to analysis of the involvement of $CD8^+$ T cells in *Mycobacterium tuberculosis* infection as well as to the development of peptide vaccines. In this study, a novel CTL epitope from region of difference 11 encoded antigen Rv3425 was identified. Epitopes were predicted by the reversal immunology approach. Rv3425-p118 (LIASNVAGV) was identified as having relatively strong binding affinity and stability towards the HLA-A*0201 molecule. Peripheral blood mononuclear cells pulsed by this peptide were able to release interferon-γ in healthy donors (HLA-A*02^+ purified protein derivative$^+$). In cytotoxicity assays *in vitro* and *in vivo*, Rv3425-p118 induced CTLs to specifically lyse the target cells. Therefore, this epitope could provide a subunit component for designing vaccines against *Mycobacterium tuberculosis*.

Key words Cytotoxic T lymphocyte, epitope, *Mycobacterium tuberculosis*, Rv3425.

Tuberculosis continues to be a major global health problem. Although there has been considerable success in addressing this problem, the number of new cases continues to grow, approaching 10 million in 2010; in developing countries, the problem is particularly serious (1). The attenuated *Mycobacterium bovis* strain (BCG), which has been the only TB vaccine used in almost a century, confers highly variable protection (2). The increasing emergence of multi-drug resistant and extensively drug resistant strains of *M. tuberculosis* and the lack of an effective vaccine against the infectious form of TB have made TB control even more problematic (3, 4).

Cellular immunity is essential for fighting infections caused by intracellular pathogens, including *M. tuberculosis* (5). Epitope-specific *M. tuberculosis* reactive $CD8^+$ T cells are reportedly present in very large numbers in the circulation of PPD (purified protein derivative) positive individuals and patients with active TB, which indicates the importance of $CD8^+$ T cells in immunity to *M. tuberculosis* (6, 7). It also emphasizes that vaccines that activate $CD8^+$ T cell subsets could be effective tools for controlling *M. tuberculosis*. The bottleneck for the development of these peptide vaccines is identification of CTL epitopes. Many major histocompatibility complex class I-restricted

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List of Abbreviations: BCG, bacillus Calmette-Guérin; β2-M, β2-microglobulin; CD, cluster of differentiation; CTL, cytotoxic T lymphocyte; DC_{50}, half-life of dissociation; ELISPOT, enzyme-linked immunospot; ESI-MS, electrospray ionization-mass spectrometry; E:T, effector cell: target cell; FI, fluorescence index; HBV, hepatitis B virus; HLA, human leukocyte antigen; IFA, incomplete Freund's adjuvant; IFN-γ, interferon-γ; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; LDH, lactate dehydrogenase; MFI, mean fluorescence intensity; *M. tuberculosis, Mycobacterium tuberculosis*; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPD, purified protein derivative; RD, region of difference; RP-HPLC, reverse phase-high performance liquid chromatography; TAP, transporter associated with antigen processing; TB, tuberculosis; Th, T helper; TNF, tumor necrosis factor.

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CTL epitopes continue to be identified from antigens of *M. tuberculosis* (5, 8–10).

Genome comparative analysis has revealed that several genomic regions of *M. tuberculosis* are deleted in BCG and other mycobacteria. These deleted regions (RDs) have been predicted to encode over 100 proteins. To overcome cross-reaction between BCG vaccine and *M. tuberculosis*, great efforts have been made to identify antigens that are missing in BCG but present in *M. tuberculosis*, such as those encoded by the genes in the RD region (11). Rv3425, an RD-11 region encoded protein, is a novel immunodominant antigen in *M. tuberculosis* that induces humoral and cellular immune responses in mice. In the BCG genome, the nucleotide sequence for the C-terminal fragment of Rv3425 is absent; however, its N-terminal fragment is conserved as a fusion protein with the N-terminal fragment of Rv3429. This fusion protein is annotated as BCG3495 in the BCG Pasteur genome. Rv3425 is expressed in *M. tuberculosis* H37Rv during exponential growth *in vitro*, and recognized by patients with both pulmonary and extra-pulmonary TB (12, 13). Therefore, we deduced that Rv3425 might contain $CD8^+$ T cell epitopes.

Because of the high prevalence of the HLA-A2 supertype in Chinese subjects, we performed reversal immunology approach to search for potential HLA-A*02-restricted epitopes in Rv3425. We identified novel cytotoxic T lymphocyte epitopes by *in vitro* and *in vivo* assays.

MATERIALS AND METHODS

Human subjects

Peripheral heparinized venous blood samples were obtained from seven healthy donors (HLA-A*02^+ PPD^+). The ethics committee of Zhengzhou University approved this sample collection.

Cell line

A human TAP-deficient T2 cell line (14) transfected with HLA-A*0201 was cultured in IMDM supplemented with 10% FBS in an incubator with a humidified atmosphere containing 5% CO_2. The T2 cell line was provided by Professor Yu-Zhang Wu (Third Military Medical University, China).

Peptide prediction and synthesis

The internet services of BIMAS, SYFPEITHI and NetCTL (15–17) were all used for epitope prediction. Native peptides with prediction scores ranked in the top three by at least two prediction tools were selected. A standard solid phase Fmoc strategy was used for 9-mer predicted peptides synthesis. The products were purified to more than 95% purity by RP-HPLC and their sequences confirmed by ESI-MS. As a positive control for the HLA-A*0201 binding assay, COX-2_{321} (ILIGETIKI) was used (18). The IA^b-restricted HBV core antigen-derived Th epitope (sequence 128–140: TPPAYRPPNAPIL) was used as a T helper epitope in the *in vivo* assay (19, 20).

Transgenic mice

HLA-A2.1/K^b transgenic mice were kindly supplied by Professor Xue-Tao Cao (Second Military Medical University, China) (21). The mice were bred and maintained in specific pathogen-free facilities. In the present experiment, mice at 8 to 12 weeks of age were used.

T2 binding affinity

To determine the binding properties of the candidate peptides toward the HLA-A*0201 molecule, up-regulation of peptide-induced HLA-A*0201 molecule on T2 cells was examined according to a previously described protocol (22). Briefly, T2 cells were incubated with peptides (50 μg/mL) in serum-free IMDM containing 3 μg/mL human β2-M (Sigma, St Louis, MO, USA) at 37°C for 18 hr. The cells were then washed twice and incubated with anti-HLA-A2 monoclonal antibody (BB7.2). After being washed three times, the cells were then treated with fluorescein isothiocyanate-labeled goat IgG anti-mouse immunoglobulin (Sigma). The cells were then harvested and analyzed by a FACSCalibur flow cytometer (Becton Dickinson, Sparks, MD, USA). The FI was calculated by the following formula: FI = (MFI sample - MFI background)/MFI background, where MFI background represents the value without the peptide.

Peptide/HLA-A*0201 complex stability assay

T2 cells were incubated with peptides (50 μg/mL) in serum-free IMDM medium containing 3 μg/mL β2-M at 37°C for 18 hr. The cells were then washed to remove free peptides and incubated with 10 μg/mL brefeldin-A for 2, 4 and 6 hr. The cells were then washed twice, stained and analyzed by flow cytometer. The DC_{50} was defined as an estimate of the time required for 50% reduction of the MFI value recorded at time 0.

In vitro expansion of peptide-specific $CD8^+$ T cells

Induction of CTLs i*n vitro* was performed in accordance with the procedures previously described (23). PBMCs were separated from the peripheral blood of HLA-A*02^+ PPD^+ healthy donors by Ficoll-Hypaque density gradient centrifugation. These cells were then stimulated every 7 days with 9-mer peptides (10 μg/mL) and β_2-M

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($3\ \mu$g/mL) in IMDM medium supplemented with 10% FBS. On the third day of the first stimulation and after each subsequent stimulation, human recombinant IL-2 (50 U/mL) was added. The cells were employed for further experiments on day 21.

In vivo expansion of peptide-specific CD8$^+$ T cells

Three groups of HLA-A2.1/K^b transgenic mice (four mice in each group) were immunized at the base of the tail with 100 μg of various peptides and 140 μg of the Th epitope prepared in IFA on days 0, 5 and 10 (24, 25). One group of mice receiving IFA containing PBS and another Th peptide were used as negative controls. On day 11, spleen lymphocytes (5×10^7 cells in 10 mL) were separated and re-stimulated with peptide (10 μg/mL) *in vitro*. On day 7 after re-stimulation, the specific cytotoxicity assay was employed.

Enzyme-linked immunospot assay for interferon-γ

A commercial kit (human IFN-γ precoated ELISPOT kit, Dakewe Biotech, Shenzhen , China) was used for ELISPOT assay. Effector cells (1×10^5) and stimulator cells (peptide-pulsed T2 cells, 1×10^5) were co-cultured in 96-well microplates coated with antibody specific for human IFN-γ (26). After incubation at 37°C for 16 hr, the cells were removed and the plates processed. The number of spots was determined automatically using a computer-assisted spot analyzer (Dakewe Biotech).

Cytotoxic activity of peptide-specific T cells

A non-radioactive method for assaying cytotoxic T lymphocytes was employed. It was based on measurement of the LDH released from target cells at graded E:T ratios (12.5:1, 25:1 and 50:1, CTLs from the PBMCs of healthy donors; 20:1, 40:1 and 80:1, CTLs from the spleen lymphocytes of transgenic mice) (26). Briefly, T2 cells loaded with 10 μg/mL peptide at 37°C for 4 hr were used as target cells. The target cells (1×10^4/well) were then co-cultured with various numbers of effector cells at 37°C for 5 hr. The percentage of specific lysis of the target cells was determined according to the following equation: percentage of specific lysis = ([experimental release - effector spontaneous release - target spontaneous release]/[target maximum release - target spontaneous release]) $\times$ 100.

Statistical analysis

All data are expressed as means $\pm$ S.D. Significance was analyzed by one way ananlysis of variance. $P < 0.05$ was considered significant. All statistical analyses were performed with SPSS 10.0.

RESULTS

Prediction and synthesis of candidate peptides

First, we selected three candidate peptides (p118, p43, and p29) derived from Rv3425 protein with optimal predicted scores (prediction score ranked in the top three by at least two prediction tools) towards the HLA-A*0201 molecule for further study (Table 1). Rv3425-p118 peptide is involved in the N-terminal fragment of Rv3425 and conserved in the virulent strain of *M. bovis* and BCG. We confirmed the molecular weights of the peptides by ESI-MS (Table 2).

Relative affinities and stabilization capacities of the synthetic peptides

To evaluate the binding affinities of these peptides to the HLA-A*0201 molecule and the stability of the peptide/HLA-A*0201 complexes *in vitro*, we used binding affinity and stability assays. We labeled the affinities and stabilization capacities of the nonamer peptides as FI and DC_{50}, respectively (Table 2). Of the three candidates, Rv3425-p118 (LIASNVAGV) showed the highest binding affinity and stability ($DC_{50} > 2$ hr) towards HLA-A*0201. We therefore chose this peptide for further study.

Rv3425-p118 elicits interferon-γ production by T cells from purified protein derivative positive healthy donors

We tested by an ELISPOT assay whether peptide-specific CTLs can cause release of IFN-γ from expanded PBMCs from HLA-A*02$^+$ PPD$^+$ individuals when they have been challenged with the peptides *in vitro*. We stimulated PBMCs from seven donors with Rv3425-p118, Rv3425-p43 and Rv3425-p29. We used PBS and PHA as negative and positive controls, respectively. Rv3425-p118 induced the PBMCs of two donors (D1 and D5) to produce IFN-γ (Fig. 1). PHA, a positive control, elicited IFN-γ production by PBMCs from all seven donors; PBS did not elicit IFN-γ production by any of them (partial data not shown).

In vitro cytotoxic activity of peptide-specific T cells

In addition to performing the IFN-γ release assay, we carried out an LDH release assay to test the cytotoxic activities of the CTLs induced by Rv3425-p118. We investigated whether Rv3425-p118 triggers specific and functional CTL

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Table 1. Prediction of HLA-A*0201 restricted epitopes from Rv3425 by NetCTL, SYFPEITHI, and BIMAS

		Scores (Rank)		
Peptide	Sequence	NetCTL	SYFPEITHI	BIMAS
p118	LIASNVAGV	1.0900 (1)	29 (1)	37.393 (4)
p43	SLEDELDEL	1.0680 (2)	28 (2)	6.317 (10)
p29	QLRELAYSV	0.9259 (3)	26 (3)	21.672 (6)

Table 2. ESI-MS data and the HLA-A*0201 binding affinity and stability of the candidate peptides

		ESI-MS $[M + H]^+$			
Protein	Peptide	Calculated	Observed	FI	DC_{50}
Rv3425	p118	843.1	843.2	0.79	>2 hr
	p43	1062.1	1062.2	0.64	<2 hr
	p29	1078.2	1078.3	0.32	<2 hr
COX-2	p321[a]	999.6	1000.3	1.16	>4 hr

DC_{50}, half-life of disassociation of peptide/HLA-A*0201 complexes; FI, (MFI of the peptide - MFI background)/(MFI background).
[a]Positive peptide.

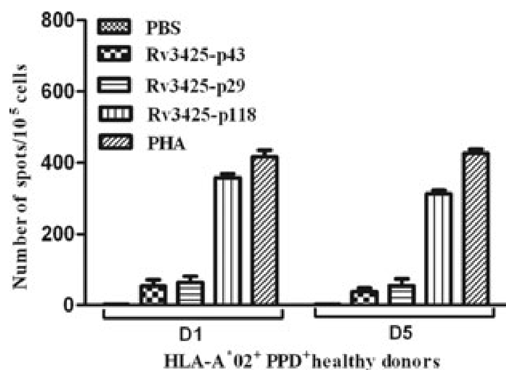

Fig. 1. IFN-γ release ELISPOT assay to detect IFN-γ release by CTLs induced by Rv3425-p118, Rv3425-p43 and Rv3425-p29 from PBMCs of healthy donors. These peptides were used to induce CTLs from PBMCs of two HLA-A*02+ PPD+ healthy donors (D1 and D5) *in vitro*. T2 cells loaded with peptide for 4 hr were used as stimulators. PBS served as negative control and PHA as positive control.

responses that lyse target cells. PBMCs from donors 1 and 5 were isolated and stimulated with Rv3425-p118 *in vitro*. We measured the cytotoxic activity of the responding cells by an LDH cytotoxicity assay. Peptide-loaded T2 cells served as target cells. As shown in Fig. 2, we generated peptide-specific $CD8^+$ T cells that specifically lysed peptide-pulsed T2 cells. The percentage of specific lysis of Rv3425-p118 was 16.4% at an E:T ratio of 50:1.

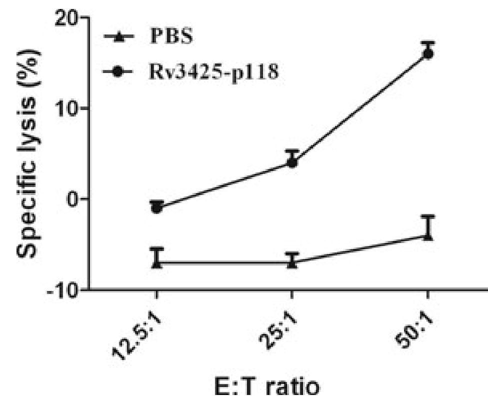

Fig. 2. LDH release cytotoxic assay to measure specific lysis of target cells by CTLs induced by Rv3425-p118. The E:T ratios were 12.5:1, 25:1 and 50:1. CTLs induced by PBS were used as negative controls. Specific lysis of T2 cells incubated with Rv3425-p118 peptide by CTLs generated from PBMCs of healthy donor D5 is shown. The CTLs were induced by Rv3425-p118.

In vivo induction of peptide-specific cytotoxic T lymphocytes in HLA-A2.1/K^b transgenic mice

We proved that CTLs induced by Rv3425-p118 do lyse target cells *in vitro*. However, the *in vivo* environment is more complex. It is more important to determine whether the peptide can be naturally processed, presented, and then induce peptide-specific CTLs *in vivo*. Therefore, we used HLA-A2.1/K^b transgenic mice as *in vivo* animal model. As shown in Fig. 3, the percentage of specific lysis of Rv3425-p118 increased to 13.7% at an E:T ratio of 80:1.

Although the mice were immunized with the epitope, their body weights were not significantly different from those of the control group mice, which indicates that Rv3425-p118 might have low toxicity (data not shown). All these *in vivo* results suggest that Rv3425-p118 can be naturally processed and presented and induce potent peptide-specific CTL responses *in vivo*.

DISCUSSION

Traditionally, identification of T cell epitopes has required synthesis of overlapping peptides that span the entire length of a protein, followed by experimental assays such as *in vitro* intracellular cytokine staining for each peptide to assess T cell activation. This method is economically viable only for single proteins or pathogens that consist of several proteins. Therefore, researchers have developed alternative computational approaches for predicting T cell epitopes that have significantly decreased the experimental burden previously associated with epitope identification. In practice, using only one computational algorithm to

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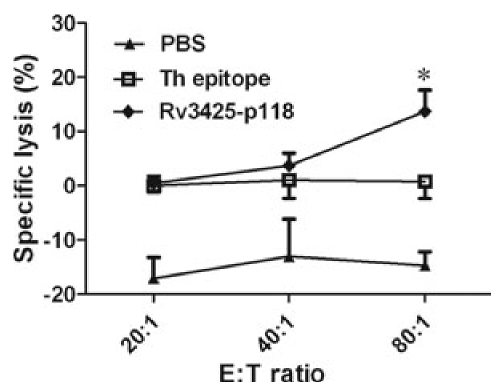

Fig. 3. Specific lysis of T2 cells incubated with synthetic peptide by CTLs generated from immunized HLA-A2.1/K^b transgenic mice. Mice were injected subcutaneously with 100 μg of each candidate peptide emulsified in IFA in the presence of 140 μg of IA^b-restricted HBV-core$_{128}$ Th epitope on days 0, 5, and 10. Mice injected with IFA containing PBS or T helper epitope were used as negative controls. On day 11, the animals were killed and their spleen lymphocytes re-stimulated *in vitro* by the same peptide for an additional 6 days. Amount of LDH release was measured. Data are represented as means ± S.D. (*) represents $P < 0.05$ *vs* Th epitope group ($n = 4$).

predict CTL epitopes may lead to large numbers of false positives and false negatives. More recently, comprehensive validation using data from several different types of prediction software has proved accurate and has considerably reduced the cost of epitope discovery. However, because the different prediction tools are based on different computational algorithms, their predictions can be different. If we do not choose with care, we can miss the immunodominant epitopes. Therefore, in this study we chose three prediction tools based on different computational algorithms to achieve results that are more accurate.

One of the approaches to finding a new generation of effective and safe vaccines is epitope-based DNA vaccination, which enables focusing of the immune response on important and highly conserved epitopes (27). This approach provides the opportunity to use specific epitopes to shift the immune system toward a Th1- or Th2-mediated immune response and eliminate unwanted responses. In addition, CTL epitope-based immunization has the advantage of eliciting an immune response only against the protective epitope and avoidance of epitope drift in *M. tuberculosis* infections (28). Studies of *M. tuberculosis* in humans have shown that induction of broad T-cell mediated immunity to *M. tuberculosis* and type 1 cytokines, including IL-2, IFN-γ and TNF-α, could be essential to TB vaccine design (29). Vaccines based on CTL epitopes represent a logical approach to generating effective cellular immunity in both the prophylactic and therapeutic settings because multiple epitopes can be incorporated into the vaccine design with the goal of inducing broadly reactive responses by multiple CTL clones directed against different epitopes. Multi-CTL epitope DNA vaccines have been reported to induce broad CTL responses against HIV, HBV, severe acute respiratory syndrome coronavirus and others (30–32). Whereas epitope-based vaccines are limited with respect to HLA polymorphism and population coverage, the use of supertype-restricted epitopes provides a means to address this problem (33).

In conclusion, we have identified a novel HLA-A2-restricted T cell epitope derived from Rv3425. Our results show that Rv3425-p118 (LIASNVAGV) could serve as a candidate for a peptide vaccine against *M. tuberculosis*.

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DISCLOSURE

There are no conflicts of interest.

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