

Identification of *Mycobacterium tuberculosis* PPE68-Specific HLA-A*0201-Restricted Epitopes for Tuberculosis Diagnosis

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Abstract PPE68 is a *Mycobacterium tuberculosis*-specific protein which is absent from the vaccine strains of BCG. A panel of 14 PPE68-derived peptides predicted to bind to HLA-A*0201 was synthesized. The HLA-A*0201 restriction of these peptides was determined in T2 cell line and HLA-A*0201 transgenic mice. The specificity of peptides was assessed in pulmonary tuberculosis (TB) patients using IFN- γ enzyme-linked immunospot (ELISPOT) assay, and immunodominant peptides were further used to evaluate their diagnostic potential in HLA-A*0201-positive pulmonary TB patients. 13 out of 14 peptides were identified as high-affinity binders. Of these peptides, 12 peptides induced significant IFN- γ -secreting T cell response in transgenic mice and 9 peptides were efficiently recognized by peripheral blood mononuclear cells of 10 HLA-A*0201-

positive TB patients. Four immunodominant HLA-A*0201-restricted epitopes (PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–148}, and PPE68_{148–156}) were recognized by the most of 80 HLA-A*0201-positive TB patients (81, 86, 74, and 84 %, respectively). These epitopes may be used for a potential diagnosis of *M. tuberculosis* infection.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), is still a major public health problem [25]. At present, one-third of the world's population has been infected with *M. tuberculosis*. In 2013, there were an estimated 9 million new cases of TB and 1.5 million people died from TB, including more than one million deaths among HIV-negative individuals and 360,000 among HIV-positive people [31]. In China, about one million new cases of active TB are reported each year according to the national wide TB survey in 2013 [31]. Although Bacille

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Calmette Guerin (BCG) has been used for almost a century, the protective efficacy of BCG is highly variable and limited. Currently, the main strategies pursued to improve TB control are accurate diagnosis, efficient drug treatment, and the development of a new effective TB vaccine. The traditional diagnostic methods for TB include sputum culture, polymerase chain reaction (PCR), tuberculin skin test (TST), etc. However, *M. tuberculosis* culture and PCR depend on a certain bacterial load. The most widely used TST is purified protein derivative (PPD) assay, which has been an important tool for detecting TB infection (both latent and active TB). However, because of cross reaction to proteins from BCG and nontuberculous mycobacteria, PPD assay has several drawbacks such as poor specificity and limited sensitivity in TB diagnosis [10, 12]. False-positive results are common because of environmental mycobacterial exposure and previous BCG vaccination. Therefore, the limitations of the TST indicate the need for an improved approach to the diagnosis of TB infection. Therefore, *M. tuberculosis*-specific antigens could be an alternative approach for the immunological diagnosis of TB.

Previous studies indicated that all clinical isolates of *M. tuberculosis* and *M. bovis* have a unique Region of difference 1 (RD-1) which is absent in all vaccine strains of BCG [3]. RD1 region contains genes encoding a specific number of proteins, including early secretory antigen target 6 (ESAT-6), culture-filtered protein 10 (CFP-10), PPE68, etc. [3, 18]. EAST-6 and CFP-10 have been intensively investigated and identified as strong targets of the cellular immune response in TB patients and outstanding diagnostic targets for TB diagnosis [10, 19]. In addition, ESAT-6- and CFP-10-based diagnostic reagent such as T-SPOT.TB assay has been released to the market for clinical diagnosis of TB [17, 26]. However, the sensitivity obtained with these proteins is insufficient [17, 26]. Thus, it is necessary to find other antigens with diagnostic potential.

PPE68 (368 aa, encoded by Rv3873) is a membrane protein expressed on the surface of *M. tuberculosis* and *M. tuberculosis*-infected cells [8]. The ability of PPE68 inducing high levels of IFN- γ secretion by T cells of TB patients supports the usefulness of PPE68 as an antigen with diagnostic potential [15, 18, 19]. However, the cross reactivity between PPE68 and proteins from BCG and environmental mycobacterial strains may restrict its application in TB diagnosis [4, 15, 19]. The cross reaction would lead to false-positive reactions and may be resolved using highly specific PPE68-specific T cell epitopes.

HLA-A*0201 is a common HLA-A allele with a frequency of over 30 % in most populations irrespective of gender or ethnicity [5]. The study presented herein was designed to identify PPE68-derived HLA-A*0201-binding

peptides and evaluate IFN- γ -secreting T cell responses to these peptides in HLA-A*0201 transgenic mice and TB patients. Fourteen putative PPE68-derived HLA-A*0201-binding peptides were selected and tested by ex vivo MHC-peptide complex stabilization assay and IFN- γ enzyme-linked immunospot (ELISPOT) assay. Four highly sensitive PPE68-specific HLA-A*0201-restricted epitopes were identified as a diagnostic candidates for human TB infection.

Materials and Methods

Prediction of T cell Epitopes in PPE68 Protein and Synthesis of Peptides

SYFPEITHI (<http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm>) [23], a T cell epitope prediction algorithm, was used to predict HLA-A*0201-binding peptides within PPE68 protein of *M. tuberculosis* H37Rv strain (Genbank accession number: NP_857540). In addition, PAPROC (<http://www.paproc.de/>) [14] was used to predict the potential human proteasomal cleavage sites within the predicted peptides. Epitope candidates were synthesized at ChinaPeptides Co., Ltd. (Shanghai, China) and purified to >95 % as confirmed by high-performance liquid chromatography and mass spectrometry. A universal Pan-DR Th epitope peptide (PADRE: AKFVAAWTLK-AAA) was synthesized and used as a helper peptide. A dengue virus-derived HLA-A*0201-restricted CD8⁺ T cell epitope NS4b₄₀₋₄₈(TLYAVATTI) was used as a positive control [30]. All peptides were dissolved in PBS to a concentration of 1 mM and stored at -20 °C until use.

Cell Line, Mice, and Study Subjects

The human transporter associated with antigen processing (TAP)-deficient T2 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). 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 in specific pathogen-free conditions. One hundred sputum culture positive with PPD skin test and chest X-ray confirmed pulmonary TB patients (90 HLA-A*0201 positive, and 10 HLA-A*0201 negative, HLA-A*0201 was confirmed using the PCR sequence-specific primers [27]) (median age 48 \pm 26 (22–76)) were recruited from the department of clinical laboratory, the third affiliated hospital of Wenzhou Medical University, China. Twenty healthy participants, BCG-vaccinated donors (median age 23 \pm 2 (20–25)) with no known exposure to *M. tuberculosis*, were recruited from Wenzhou Medical University. All donors gave written

informed consent before peripheral blood donation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using human lymphocyte density gradient centrifugation [11] and resuspended in RPMI-1640 medium supplemented with 2 Mm L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 % fetal bovine serum (FBS). The study was approved by the Human Research Ethics Board of Wenzhou Medical University.

MHC-Peptide Complex Stabilization Assay

To determine the affinity of peptides to HLA-A*0201 molecules, peptide-induced HLA-A*0201 up-regulation on T2 cells was measured as described previously [30]. Briefly, T2 cells (2×10^5 cells) were incubated in serum-free RPMI-1640 medium in the absence or presence of peptide (1, 10, 100 µM) for 1 h at 37 °C/5 % CO₂ incubator. Cells were then incubated for 12 h at 26 °C, and returned to 37 °C for a 3 h incubation. Cells were stained with FITC-conjugated anti-HLA-A*0201 mAb (BD Pharmingen, CA, USA) for 40 min at 4 °C, and mean fluorescence intensity (MFI) was recorded with a FACS Calibur flow cytometer (BD bioscience, CA, USA). The fluorescence index (FI) was calculated as follows. Peptide with FI ≥ 1 (peptide concentration = 100 µM) was considered as high-affinity peptide [32], which means that this peptide could increase the expression of HLA-A*0201 molecules on T2 cells by at least 1-fold.

$$FI = \frac{\text{MFI with peptide} - \text{MFI without peptide}}{\text{MFI without peptide}}$$

Immunization of HLA-A*0201 Transgenic Mice

Female HLA-A*0201 transgenic mice (6–8 weeks of age) were subdivided into 14 groups (4 mice/group). Mice were immunized subcutaneously with peptide (50 µg/mouse) and PADRE (50 µg/mouse) emulsified in complete Freund's adjuvant (CFA). One week later, mice were boosted with the same peptide emulsified in Incomplete Freund's adjuvant (IFA) for three additional times at weekly intervals. In addition, control mice (mock-immunized mice) were immunized with CFA and IFA without peptide according to the same immunization protocol. One week after the fourth immunization, splenocytes and lymph node cells (from deltopectoral and popliteal lymph nodes) were prepared and tested using IFN- γ enzyme-linked immunospot (ELISPOT) assay. All animal experiments were performed following the Institutional Animal Care and Use Committee-approved protocols of Wenzhou Medical University.

Murine IFN- γ ELISPOT Assay

Murine IFN- γ ELISPOT assays were performed in pre-coated 96-well plates (U-CyTech Company, Utrecht, The Netherlands) according to the manufacture's instructions. Firstly, splenocytes and lymph node cells were resuspended in RPMI-1640 medium, and adjusted to 5×10^6 cells/ml and 2×10^6 cells/ml, respectively. Splenocytes (5×10^5 cells/well) and lymph node cells (2×10^5 cells/well) were added to each well of the ELISPOT plates. Background control wells (without peptide) and test wells (with 10 µg/ml peptide) were set in duplicate wells. Subsequently, the plates were incubated for 24 h at 37 °C/5 % CO₂ incubator and then incubated with biotinylated anti-mouse IFN- γ mAb for 1 h at 37 °C. Finally, plates were incubated with streptavidin-horseradish peroxidase, and developed using fresh ACE solution as a substrate. IFN- γ spots (Spot-forming cells, SFCs) were counted using an ELISPOT reader (Beijing SageCreation Science Co., Ltd. Beijing, China). Peptide-specific T cell frequency was expressed as IFN- γ SFCs/ 5×10^5 splenocytes or 2×10^5 lymph node cells.

Human IFN- γ ELISPOT Assay

Human IFN- γ ELISPOT kit (pre-coated 96-wells plates) was purchased from U-CyTech Company and the assay was performed as described above. In brief, PBMCs from 10 HLA-A*0201-positive TB patients, 10 HLA-A*0201-negative TB patients, and 20 healthy BCG-vaccinated donors were resuspended in RPMI-1640 medium and adjusted to 5×10^5 cells/ml. PBMCs (5×10^4 cells/well) were seeded into each well of the ELISPOT plates, and background control wells (without peptide) and test wells (with 10 µg/ml individual peptide) were set. After 24 h incubation at 37 °C, the plates were washed and processed as described above. IFN- γ SFCs were counted, and the frequency of peptide-specific T cells was expressed as IFN- γ SFCs/ 5×10^4 PBMCs. Responses to tested peptides were scored as positive if the number of IFN- γ spots was greater than 5 SFCs/ 5×10^4 PBMCs after subtraction of the background and this number also had to be at least twice the frequency found in the background wells [15].

Evaluation of the Sensitivity of Immunodominant PPE68-Derived Peptides

The sensitivity of 4 immunodominant PPE68-derived peptides (PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–148}, and PPE68_{148–156}) was further evaluated in PBMCs of 80

additional HLA-A*0201-positive TB patients using human IFN- γ ELISPOT assay as described above (1×10^5 PBMCs/well). Meanwhile, the commercial ELISPOT-based T-SPOT.TB kit (containing ESAT-6 and CFP-10) was purchased from Oxford Immunotec Ltd. (Abingdon, UK) and used to detect the frequencies of ESAT-6- and CFP-10-specific IFN- γ -secreting T cells in PBMCs of 80 TB patients as described previously [17]. In brief, PBMCs (1×10^5 cells/well) were seeded into each well of the ELISPOT plates, background control wells (without proteins) and test wells (with ESAT-6 or CFP-10) were set in duplicate wells, and plates were incubated for 24 h at 37 °C/5 % CO₂ incubator. The plates were washed and then incubated with alkaline phosphatase-conjugated anti-human IFN- γ mAb for 1 h at 37 °C. After a subsequent wash, the plates were developed using BCIP/NBT solution as a substrate. IFN- γ SFCs were counted using an ELISPOT reader and expressed as IFN- γ SFCs/ 1×10^5 PBMCs. Responses to tested peptides were scored as positive if the number of IFN- γ spots was greater than 5 SFCs/ 1×10^5 PBMCs after subtraction of the background and this number also had to be at least twice the frequency found in the background wells [15].

Statistical Analysis

Results were expressed as mean \pm SEM. 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

Affinity of Peptides to HLA-A*0201 Molecules

Epitope candidates were selected on the basis of the high predictive scores and proteasomal cleavage sites (C terminus). According to the prediction results, a panel of 14 peptides (each represents an epitope candidate) was synthesized (as shown in Table 1). BLAST results showed that PPE68_{126–134} is conserved in some PPE family proteins (PPE2, PPE3, PPE46, PPE47, and PPE66), while others have no sequence homology with any known mycobacterial proteins. Of 14 peptides, 8 peptides are nonapeptides, while other 6 peptides belong to decapeptides. Four overlapping peptides (PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–149}, and PPE68_{148–156}) span a sequence (residues 126 to 156). MHC-peptide complex stabilization assay was used to measure the expression of HLA-A*0201 molecules on T2 cells induced by peptide exposure, with high-affinity peptides inducing HLA-A*0201 up-regulation more strongly than

low-affinity peptides. FI ≥ 1 (peptide concentration = 100 μ M) is a normally accepted threshold for high affinity [32]. As shown in Fig. 1 and Table 1, when peptide concentration reached 100 μ M, of the 14 peptides, only PPE68_{26–34} was low-affinity peptide (FI = 0.65); other peptides and positive control peptide bound to HLA-A*0201 molecules with a FI ≥ 1 .

Immunogenicity of Peptides in HLA-A*0201 Transgenic Mice

To test the potential immunogenicity of epitope candidates, HLA-A*0201 transgenic mice were immunized subcutaneously with individual peptides. Splenocytes and lymph node cells from immunized mice were restimulated in vitro with the corresponding peptide and the frequencies of peptide-specific IFN- γ -secreting cells were detected by IFN- γ ELISPOT assay. Positive control peptide elicited significant IFN- γ response. Of 13 high-affinity peptides, PPE68_{37–45} and PPE68_{44–52} did not elicit significant IFN- γ -secreting cells, while the other peptides induced high levels of IFN- γ -secreting cells in both spleen (Fig. 2a) and lymph nodes (Fig. 2b). Among 14 epitope candidates, PPE68_{299–308} immunization induced the highest frequency of IFN- γ -secreting cells in both spleen and lymph nodes (307 ± 23 SFCs/ 5×10^5 splenocytes; 174 ± 21 SFCs/ 2×10^5 lymph node cells) (Fig. 2a and b). In addition, there was no significant peptide-specific IFN- γ -secreting T cell response in mock-immunized transgenic mice (data not shown). Because HLA-A*0201 transgenic mice are produced on the basis of C57BL/6 mice, in order to determine if these fourteen epitope candidates could induce IFN- γ -secreting T cell response in C57BL/6 mice, the immunogenicity of fourteen peptides was evaluated in C57BL/6 mice and no significant IFN- γ responses were observed (data not shown).

Identification of the Immunodominant PPE68-Specific HLA-A*0201-restricted Epitopes

To further determine whether these peptides are naturally processed and presented in human cells, PBMCs from 10 HLA-A*0201-positive TB patients were tested for their capacity to recognize peptide and secrete IFN- γ . PBMCs from each TB patients were stimulated with individual peptides, and the frequencies of peptide-specific IFN- γ -secreting cells were then detected by ELISPOT assay. As shown in Fig. 3b, the percentages of responding patients varied between 10 and 100 % for different peptides. Of 14 peptides, 9 peptides (PPE68_{148–156}, PPE68_{133–141}, PPE68_{166–174}, PPE68_{126–134}, PPE68_{64–73}, PPE68_{200–209}, PPE68_{105–114}, PPE68_{140–149}, and PPE68_{277–284}) were recognized for at least 50 % of the TB patients. Notably, PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–149},

Table 1 *Mycobacterium tuberculosis* PPE68-derived epitope candidates and their affinity for HLA-A*0201 molecule

Peptides	Position	Sequences	Proteins	Predictive scores	FI ^a		
					Peptide concentration		
				SYFPEITHI	1 μ M	10 μ M	100 μ M
PPE68 ₃₇₋₄₅	37-45	ALDAQAVEL	PPE68	26	0.07	0.63	3.5
PPE68 ₅₋₁₃	5-13	AMPPELNTA	PPE68	23	0	0.2	2.67
PPE68 ₄₄₋₅₂	44-52	ELTARLNSL	PPE68	23	0	0.14	1.42
PPE68 ₁₄₈₋₁₅₆	148-156	ALAMEVYQA	PPE68	23	1.27	1.59	3.08
PPE68 ₂₆₋₃₄	26-34	AAAAGWQTL	PPE68	22	0	0.24	0.65
PPE68 ₁₃₃₋₁₄₁	133-141	ALTEM DYFI	PPE68	22	0.2	1.07	3.22
PPE68 ₁₆₆₋₁₇₄	166-174	KLEPMASIL	PPE68	22	0.05	0.29	1.79
PPE68 ₁₂₆₋₁₃₄	126-134	GINTIPIAL	PPE2, PPE3, PPE46, PPE47, PPE66, PPE68	21	0.02	0.21	2.24
PPE68 ₆₄₋₇₃	64-73	ALAAATPMVV	PPE68	24	0.22	0.84	3.37
PPE68 ₂₀₀₋₂₀₉	200-209	QLPPAATQTL	PPE68	24	0.03	0.22	2.68
PPE68 ₁₀₅₋₁₁₄	105-114	SLPEIAANHI	PPE68	23	0.01	0.26	2.23
PPE68 ₁₄₀₋₁₄₉	140-149	FIRMWNQAAL	PPE68	22	0.1	0.29	1.16
PPE68 ₂₇₇₋₂₈₆	277-286	LLRAESLPGA	PPE68	22	0.07	0.22	2.12
PPE68 ₂₉₉₋₃₀₈	299-308	LIEKPVAPSV	PPE68	22	0.01	0.28	1.13

^a FI = [Mean fluorescence intensity(MFI) sample – MFI background]/MFI background

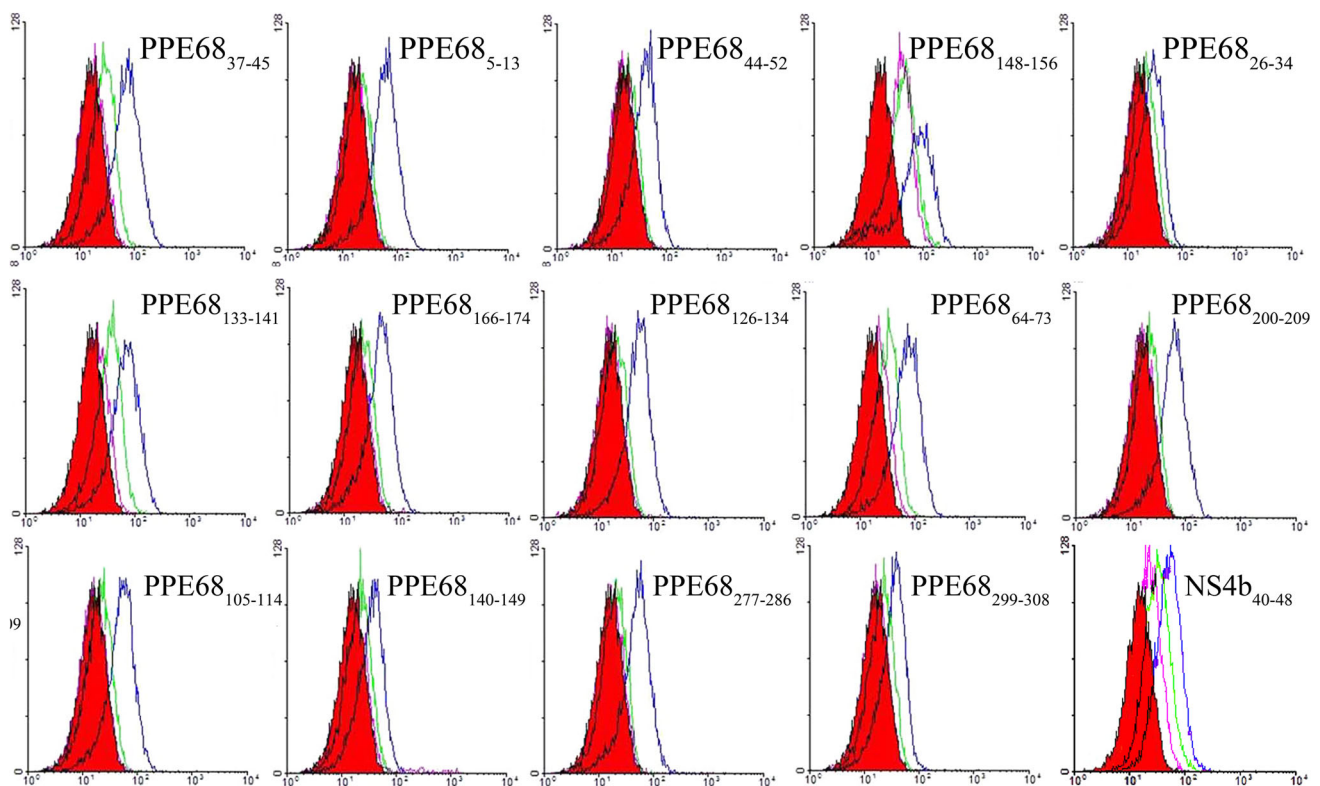


Fig. 1 The binding affinity of PPE68-derived peptides to HLA-A*0201 molecule. Based on T2 cells, MHC-peptide complex stabilization assay was used to evaluate the binding affinity of PPE68-derived CD8⁺ T cell epitope candidates and positive control (NS4b₄₀₋₄₈) to HLA-A*0201 molecules as described in “Materials

and Methods” section. T2 cells were incubated with individual peptides at the different concentrations [no peptide (*red*) or 1 μ M (*purple*) or 10 μ M (*green*) or 100 μ M (*blue*)]. The mean fluorescence intensity of T2 cells was measured using flow cytometry and then the fluorescence index (FI) was calculated

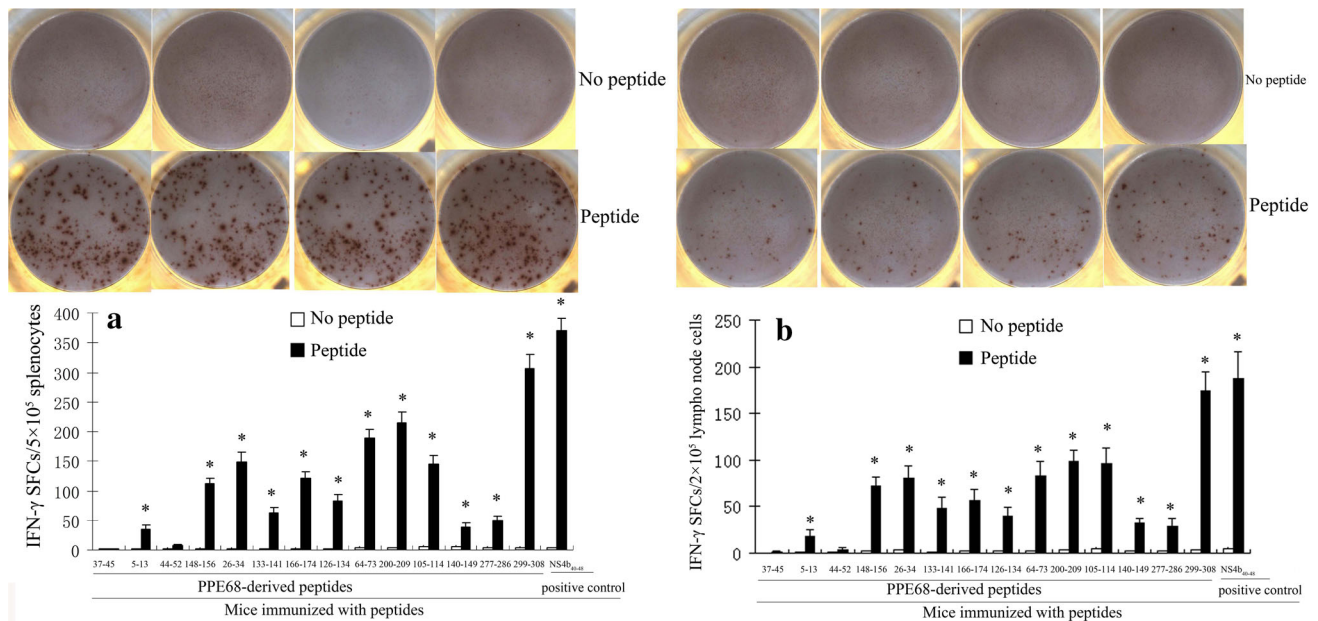


Fig. 2 Peptide-specific IFN- γ -secreting cells in splenocytes and lymph node cells of peptide-immunized HLA-A*0201 transgenic mice. Splenocytes and lymph node cells were isolated from peptide-immunized mice and were restimulated in vitro with no peptide or the

same peptide. The frequencies of IFN- γ SFCs/ 5×10^5 splenocytes (a) or IFN- γ SFCs/ 2×10^5 lymph node cells (b) were detected using ELISPOT assay and presented as mean \pm SD ($n = 4$). Asterisk indicating the positive response to a peptide

and PPE68_{148–156} were recognized by 100, 100, 80, and 90 % of the TB patients, respectively. The frequencies of peptide-specific IFN- γ -secreting cells specific for PPE68_{126–134}, PPE68_{133–141}, and PPE68_{148–156} were (median response and interquartile range) 18 (6–49), 18 (7–38), and 15 (1–37) per 5×10^4 PBMCs, respectively. Patients responded weakly to PPE68_{140–149} yielding relatively few spots (3–8 SFCs/ 5×10^4 PBMCs) (Fig. 3a). We next assessed the HLA-A*0201 restriction and specificity of PPE68-derived peptides by ELISPOT assay in 10 HLA-A*0201-negative TB patients and 20 HLA-A*0201-positive BCG-vaccinated donors. As shown in Fig. S1a, for HLA-A*0201-negative TB patients, the responders to PPE68-derived peptides, as well as the numbers of IFN- γ SFCs, were significantly lower than HLA-A*0201-positive TB patients. As expected, in the BCG-vaccinated individuals, the responses to peptides were absent (Fig. S1b). All of these results suggested that these four peptides (PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–149}, and PPE68_{148–156}) represent the immunodominant PPE68-specific HLA-A*0201-restricted epitopes. These four peptides were then chosen as diagnostic peptides for subsequent experiments.

Diagnostic Potential of Immunodominant PPE68-Specific HLA-A*0201-restricted Epitopes

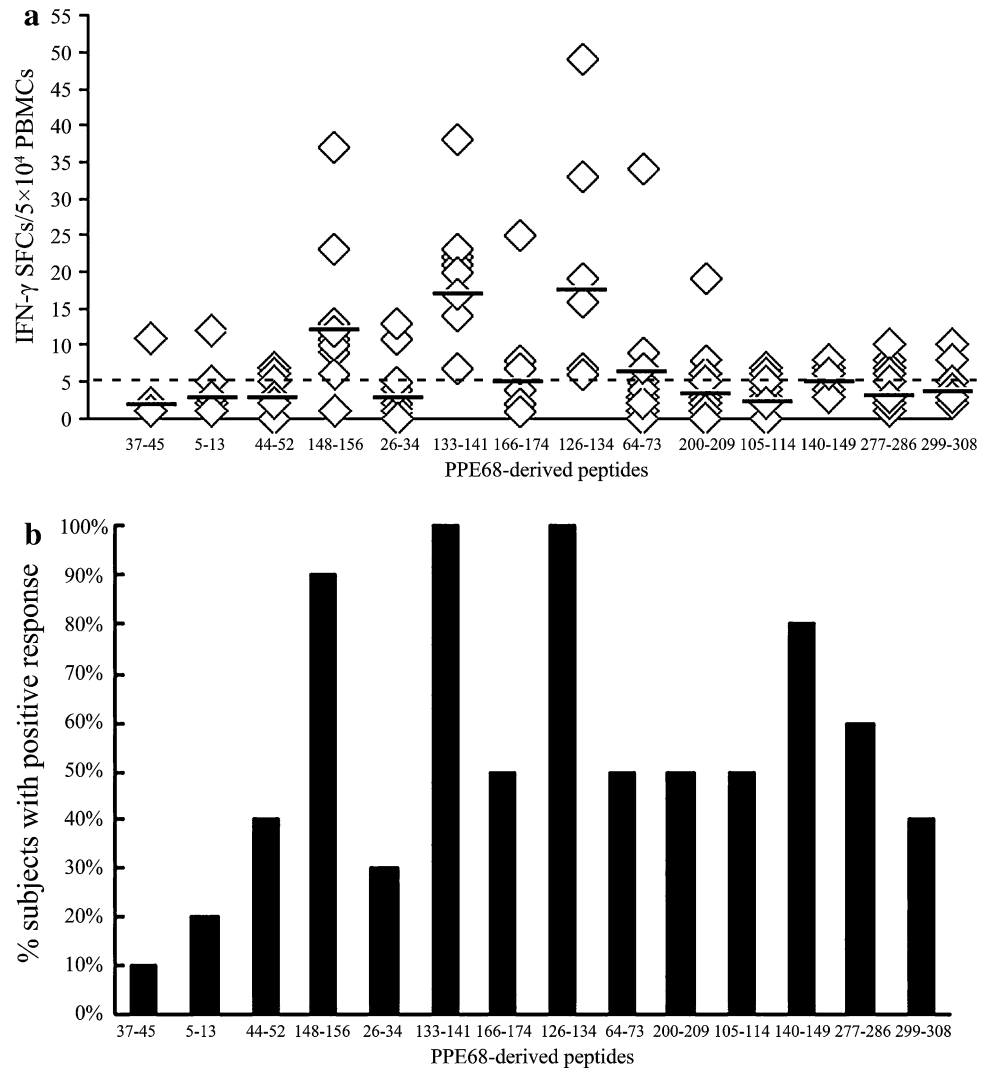
To test the diagnostic potential of the PPE68-specific HLA-A*0201-restricted epitopes, the immunodominant peptides including PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–149}, and PPE68_{148–156} were selected for diagnostic test in 80 HLA-

A*0201-positive TB patients. To compare the relative sensitivity of above peptides in TB diagnosis, ESAT-6 and CFP-10 (components of T-SPOT.TB kit) were used as positive controls. The IFN- γ ELISPOT assay responses to 4 PPE68-derived peptides, ESAT-6 and CFP-10 are summarized in Fig. 4a. The percentages of responding patients for PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–149}, PPE68_{148–156}, ESAT-6, and CFP-10 were 81, 86, 74, 84, 88, and 84 %, respectively (Fig. 4b). The frequencies of peptide-specific IFN- γ -secreting cells for all responding patients were (median response and interquartile range) 39 (7–132), 34 (7–111), 26 (5–88), 44 (6–142), 47 (5–128), and 51 (7–183) per 1×10^5 PBMCs, respectively (Fig. 4a).

Discussions

Mycobacterium tuberculosis infection is routinely being detected by TST against PPD, a crude extract from *M. tuberculosis*. This test has poor specificity, due to cross reaction with environmental mycobacteria and vaccination with BCG. New in vitro T cell-based interferon- γ release assays (IGRAs), which utilize *M. tuberculosis*-specific antigens, have been introduced into clinical diagnosis in recent years [22, 24]. At present, there are two available IGRAs: the QuantiFERON-TB Gold In-Tube test (QFT-GIT) and T-SPOT.TB test. T-SPOT.TB assay (ELISPOT-based IGRAs) contains a mixture of couples of epitopes derived from ESAT-6 and CFP-10, and determines the

Fig. 3 IFN- γ ELISPOT assay responses to peptides in PBMCs of HLA-A*0201-positive TB patients. **a** Frequencies of peptide-specific IFN- γ -secreting SFCs were enumerated by ELISPOT assay in 10 HLA-A*0201-positive TB patients. Each *horizontal bar* represents the median response for each peptide. The *broken horizontal line* represents the predefined cutoff point (5 SFCs/5 \times 10⁴ PBMCs). Points above the *broken horizontal line* represent individuals with a positive response to a give peptide, **b** Percentages of HLA-A*0201-positive TB patients who responded to each peptide



number of INF- γ -secreting cells with the use of an ELISPOT assay [16]. Although T-SPOT.TB improved the specificity for the diagnosis of TB, its sensitivity is insufficient [6, 17, 26]. Recent study indicated potentials for further improvement of this test [2]. Therefore, it has been of interest to search fine epitopes from other antigens and improve the diagnosis of TB.

In the present study, we sought out to identify a few immunodominant PPE68-derived HLA-A*0201-restricted epitopes that have both high specificity and sensitivity in TB diagnosis. 13 of 14 PPE68-derived epitope candidates bound to HLA-A*0201 molecules with high affinity, and 12 peptides induced IFN- γ -secreting cells in HLA-A*0201 transgenic mice. However, two high-affinity peptides, PPE68₃₇₋₄₅ and PPE68₄₄₋₅₂, did not induce significant IFN- γ -secreting cell response in transgenic mice. Conversely, one low-affinity peptide, PPE68₂₆₋₃₄, elicited significant IFN- γ release. The possible explanations may be

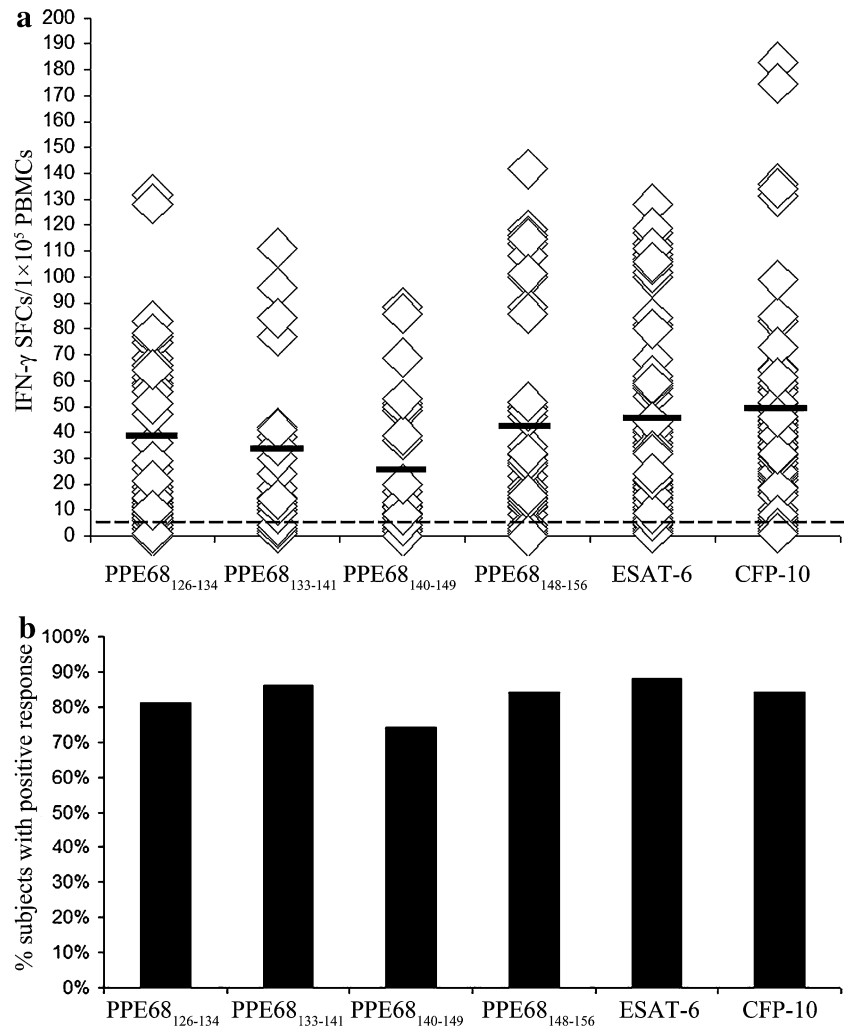
(1) neither PPE68₃₇₋₄₅ nor PPE68₄₄₋₅₂ is cross-presented to CD8⁺ T cells as for other antigens [21], so they could not induce IFN- γ response; (2) both PPE68₃₇₋₄₅ and PPE68₄₄₋₅₂ have higher dissociation rates from HLA-A*0201 molecules than other high-affinity peptides. In contrast, PPE68₂₆₋₃₄ might has low dissociation rates from HLA-A*0201 molecules. Indeed, many studies have indicated that there is no strict correlation between functional activity of peptides and their HLA binding efficiency [1, 28]. High-affinity peptides did not always induce immune response [9, 13], whereas low-affinity peptides triggered CD8⁺ T cell response [29].

To know if these peptides could be processed and presented in human cells, 14 peptides were further tested in ex vivo IFN- γ ELISPOT assay in 10 HLA-A*0201-positive patients with active pulmonary TB. Nine PPE68-derived peptides were recognized by PBMCs from >50 % of the patients tested. 100, 100, 80, and 90 % of the TB

Fig. 4 IFN- γ ELISPOT assay responses to four peptides and two proteins in PBMCs of HLA-A*0201-positive TB patients.

a The frequencies of four peptides (PPE68₁₂₆₋₁₃₄, PPE68₁₃₃₋₁₄₁, PPE68₁₄₀₋₁₄₉, and PPE68₁₄₈₋₁₅₆) and two proteins (ESAT-6 and CFP-10)-specific IFN- γ -secreting SFCs in 80 additional HLA-A*0201-positive TB patients were detected using ELISPOT assay and T-SPOT.TB assay, respectively. Each *horizontal bar* represents the median response for each peptide. The *broken horizontal line* represents the predefined cutoff point (5 SFCs/l $\times 10^5$ PBMCs). Points above the *broken horizontal line* represent individuals with a positive response to a give peptide.

b Percentages of HLA-A*0201-positive TB patients who responded to each peptide



patients responded to PPE68₁₂₆₋₁₃₄, PPE68₁₃₃₋₁₄₁, PPE68₁₄₀₋₁₄₉, and PPE68₁₄₈₋₁₅₆, respectively, identifying these overlapping peptides as immunodominant epitopes in humans. Although PPE68₁₂₆₋₁₃₄ was recognized by one HLA-A*0201-negative individual, no significant IFN- γ T cell response to peptides could be detected in all BCG-vaccinated donors tested. Therefore, these peptides showed an excellent specificity for discrimination between HLA-A*0201-positive active TB patients and BCG-vaccinated donors. Subsequently, by the same approaches, we investigated the diagnostic performance of epitope-based ELISPOT assay for TB diagnosis and our study has highlighted the immunodominance of these four overlapping peptides in 80 HLA-A*0201-positive pulmonary TB patients. The sensitivities of 4 peptides each in TB diagnosis are 81, 86, 74, and 84 %, respectively. Given ESAT-6- and CFP-10-based diagnostic reagent (T-SPOT.TB assay) has been released to the market for clinical diagnosis of TB [17], we compared these four peptides with ESAT-6 and CFP-10 for the sensitivity in TB diagnosis. The sensitivity of epitope-

based ELISPOT assay was similar to that of ESAT-6- and CFP-10-based T-SPOT.TB assay (88 and 84 %, respectively) in the present study. In total, the differences between these four peptides and ESAT-6 and CFP-10 are not so significant either in terms of proportion of responders or the frequencies of IFN- γ -secreting cells.

In the last decade, although some researchers have described some PPE68-derived peptides which could elicit IFN- γ -secreting cells and explored their potential diagnostic role in TB, no CD8⁺ T cell epitope has ever been reported. For example, PPE68₁₁₈₋₁₃₅ (VLTATNFFGINTIPIALT) was identified as an immunodominant epitope which is recognized by both TB patients and BCG-vaccinated individuals [20]. Cockle et al. reported four immunodominant peptides, PPE68₉₇₋₁₁₆(TQAMATTPSLPEIAANHITQ), PPE68₁₀₅₋₁₂₄(SLPEIAANHITQAVLTA TNF), PPE68₁₂₁₋₁₄₀(ATNFFGINTIPIALTEMDYF), and PPE68₁₂₉₋₁₄₈(TIPIALTEMDYFIRMWNQAA), in *M. bovis*-infected cattle [7]. Based on TB patients, Mustafa et al. identified an immunodominant peptide PPE68₁₂₁₋₁₄₅

(VLATNFFGINTIPIALTEMDYFIR) and limited the promiscuous Th1 cell epitope region to the sequence 127-FFGINTIPIA-136 [19]. Although these studies demonstrated high levels of IFN- γ release and median percentages of responding patients specific for these peptides, they neither determined the minimal immunodominant peptides nor defined the HLA restriction of these peptides. Compared with the present identified epitopes, we can find that PPE68_{97–116}, PPE68_{105–124}, PPE68_{121–140}, and PPE68_{129–148} contain the complete or partial sequences of PPE68_{105–114}, PPE68_{126–134}, PPE68_{133–141}, and PPE68_{140–149}, respectively. PPE68_{118–135} covers the sequence of PPE68_{126–134}, while PPE68_{121–145} covers two epitopes (PPE68_{126–134} and PPE68_{133–141}).

At present, the epitope-based diagnostic test is a convenient method for the diagnosis of active TB. Compared to TST, the main advantage of *M. tuberculosis*-specific T cell epitope as a TB diagnostic reagent is its sensitivity and specificity. Considering the high specificity and moderate sensitivity for the diagnosis of active TB, these four epitopes would provide a supplementary tool for screening this disease. It is possible to combine these epitopes as ingredients in a highly sensitive and specific ELISPOT-based or tetrameric HLA-A*0201-peptide complex (tetramer)-based diagnostic reagent. In addition, these four peptides span a peptide sequence (residues 126–156, GINTIPIALTEMDYFIRMWNQAALAMEVYQA), it is very likely that this long peptide could be used as a diagnostic reagent for TB.

Taken together, the present study described 13 PPE68-derived HLA-A*0201-binding peptides and 4 immunodominant epitopes (PPE68_{126–134}, PPE68_{133–141}, PPE68_{140–149}, and PPE68_{148–156}). To our knowledge, this is the first study reporting PPE68-specific HLA-A*0201-restricted epitopes, and using PPE68-derived epitope-based ELISPOT assay for TB diagnosis. These immunodominant PPE68-derived HLA-A*0201-restricted epitopes may represent a new diagnostic tool for TB. The population coverage rate of HLA-A*0201 (about 30 % in most populations) limits the broad application of above-mentioned immunodominant epitopes in TB diagnosis. Since the total population coverage rate of three HLA-A alleles (A*0201, A*1101 and A*2402) exceeds 80 % [5, 33], we next would identify HLA-A*1101-, A*2402-restricted epitopes in PPE68. A number of such defined T cell epitopes would form the basis of universal T cell epitope-based in vitro diagnostic reagent for TB.

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