Characterization of a Cross- Reactive, Immunodominant and HLA-Promiscuous Epitope of *Mycobacterium tuberculosis*-Specific Major Antigenic Protein PPE68

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Abstract

PPE68 (Rv3873), a major antigenic protein encoded by *Mycobacterium tuberculosis*-specific genomic region of difference (RD)1, is a strong stimulator of peripheral blood mononuclear cells (PBMCs) obtained from tuberculosis patients and *Mycobacterium bovis* bacillus Calmette Guerin (BCG)-vaccinated healthy subjects in T helper (Th)1 cell assays, i.e. antigen-induced proliferation and interferon-gamma (IFN-γ) secretion. To confirm the antigen-specific recognition of PPE68 by T cells in IFN-γ assays, antigen-induced human T-cell lines were established from PBMCs of *M. Bovis* BCG-vaccinated and HLA-heterogeneous healthy subjects and tested with peptide pools of RD1 proteins. The results showed that PPE68 was recognized by antigen-specific T-cell lines from HLA-heterogeneous subjects. To further identify the immunodominant and HLA-promiscuous Th1 cell epitopes present in PPE68, 24 synthetic peptides covering the sequence of PPE68 were individually analyzed for HLA-DR binding prediction analysis and tested with PBMCs from *M. bovis* BCG-vaccinated and HLA-heterogeneous healthy subjects in IFN-γ assays. The results identified the peptide P9, i.e. aa 121-VLTATNFFGINTIPIALTEM–145, as an immunodominant and HLA-DR promiscuous peptide of PPE68. Furthermore, by using deletion peptides, the immunodominant and HLA-DR promiscuous core sequence was mapped to aa 127-FFGINTIPIA-136. Interestingly, the core sequence is present in several PPE proteins of *M. tuberculosis*, and conserved in all sequenced strains/species of *M. tuberculosis* and *M. tuberculosis* complex, and several other pathogenic mycobacterial species, including *M. leprae* and *M. avium-intracellulare* complex. These results suggest that the peptide aa 121–145 may be exploited as a peptide-based vaccine candidate against tuberculosis and other mycobacterial diseases.

Introduction

Tuberculosis (TB) is a major infectious diseases problem of world-wide distribution and ranks among the top 10 causes of global mortality. In spite of international efforts to control TB, the most recent estimates available for global epidemiology from the World Health Organization suggest that there were 9.4 million incidence cases and 14 million prevalence cases of active disease and 1.7 million people died of TB in 2009 [1]. The impact of current efforts to reduce the global burden of TB, of means of improved diagnosis and chemotherapy, is less than expected [2]. Therefore, additional preventive efforts, which include the development of new protective vaccines against TB, are essential [2].

Previous studies have shown that interferon-gamma (IFN-γ), a cytokine secreted by T helper (Th)1 cells in large quantities, is a major player in protection against TB [3–6]. In addition, mycobacterial antigens/peptides are presented to Th1 cells mostly in association with highly polymorphic human leukocyte antigen (HLA) molecules, in particular HLA-DR [6–8]. Thus, to be effective in human populations, which are highly HLA-DR heterogeneous, the antigens/peptides selected as anti-TB vaccine candidates should be recognized by Th1 cells in HLA-DR-non-restricted (promiscuous) manner [9].

The comparative analyses of *M. tuberculosis* genome has shown the presence of several regions of difference (RD) between *M. tuberculosis* and other mycobacteria, particularly when compared with the vaccine strains of *M. bovis* BCG [10]. Among these regions, RD1 appears to be the most important region for Th1-cell stimulation because it contains genes that encode two major antigenic proteins of *M. tuberculosis* (ESAT-6 and CFP10), which were recognized by TB patients and latently infected individuals in IFN-γ assays [11–13]. However, the RD1 region has been predicted to contain genes that encode 14 *M. tuberculosis*-specific proteins [14]. By using pools of chemically synthesized peptides corresponding to each RD1 protein, it has been shown that all of these proteins were recognized by Th 1 cells from TB patients, and three of them (ESAT-6, CFP10 and PPE68) were identified as the major antigens [15]. However, PPE68, was recognized equally well by peripheral blood mononuclear cells (PBMCs) obtained from TB patients and *M. bovis* BCG vaccinated healthy subjects, and its presentation to Th1 cells was HLA-promiscuous [15]. The aim of this study was to confirm the recognition of PPE68 by Th1 cells using antigen-induced T-cell lines from *M. bovis* BCG-

Citation: Mustafa AS (2014) Characterization of a Cross-Reactive, Immunodominant and HLA-Promiscuous Epitope of Mycobacterium tuberculosis-Specific Major Antigenic Protein PPE68. PLoS ONE 9(8): e103679. doi:10.1371/journal.pone.0103679

Editor: Anil Kumar Tyagi, University of Delhi, India

Received July 2, 2013; Accepted July 6, 2014; Published August 19, 2014

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Funding: This study was supported by Kuwait University Research Sector grants MI01/10 and SRUL02/13. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The author has declared that no competing interests exist.

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vaccinated and HLA-heterogeneous healthy subjects. In addition, the HLA-promiscuous regions of PPE68 were identified by HLA-binding prediction analysis in silico, and the experimental verification was performed using overlapping synthetic peptides of PPE68 and PBMCs from M. bovis BCG-vaccinated healthy subjects in IFN-γ assays. Furthermore, the core sequence of the immunodominant peptide was identified by using deletion peptides in IFN-γ assays, and its cross-reactive nature was confirmed by demonstrating the presence in other mycobacterial species by sequence homology search.

Materials and Methods

Mycobacterial antigens and peptides
The mycobacterial antigen used in this study was irradiated whole-cell M. tuberculosis H37Ra [16]. A total of 220 peptides (25-mers, overlapping by 10 residues) corresponding to 12 proteins of RD1 (Rv3871, PE35, ORF4, PPE68, CFP10, ESAT-6, ORF8, Rv3876, Rv3877, Rv3878, ORF14 and OR15) were designed based on the amino acid sequence deduced from the nucleotide sequence of the predicted genes [17–19]. All of the peptides were synthesized by Thermo Hybaid GmbH (Ulm, Germany) using fluonemethylmethoxycarbonyl chemistry, and used as described previously [20]. In brief, the stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0.9%) by vigorous pipetting and frozen at −70°C until used. The working concentrations of each peptide were prepared by further dilution in the tissue culture medium RPMI1640. A pool of all 220 peptides (RD1pool), and pools of peptides of individual proteins were used in cell cultures to represent RD1 and single proteins, respectively.

Study subjects
The study subjects were M. bovis BCG-vaccinated healthy adults randomly selected from the group of blood donors at the Central Blood Bank, Kuwait. All of the donors were immunized with BCG vaccine following routine immunization protocol applied in Kuwait, i.e. the first immunization was given at 4 ½ years of age, followed by M. tuberculosis purified protein derivative (PPD)-skin test at 13 years of age, and a booster immunization with BCG in PPD-skin test negative subjects. At the time of inclusion in the study, all the donors were PPD-skin test positive (>10 mm, as determined with tuberculin PPD RT 23 from the Statens Serum Institute, Copenhagen, Denmark). Written informed consent was obtained from all the subjects to participate in the study, and the study protocol was approved by the Ethics Committee of the Faculty of Medicine, Kuwait University, Kuwait.

Isolation of peripheral blood mononuclear cells from M. bovis BCG-vaccinated healthy subjects and in vitro culture for IFN-γ secretion
Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats of each donor by density centrifugation according to standard procedures [20]. In brief, each buffy coat was diluted with warm tissue culture medium (RPMI 1640) at a ratio of 1:2 and gently mixed. Two volumes of the diluted buffy coat was loaded on top of 1 volume of a Lymphoprep gradient (Pharmacia Biotech., Uppsala, Sweden). After centrifugation, the white ring of the buffy coats of each donor by density centrifugation according [20]. In brief, the stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0.9%) by vigorous pipetting and frozen at −70°C until used. The working concentrations of each peptide were prepared by further dilution in the tissue culture medium RPMI1640. A pool of all 220 peptides (RD1pool), and pools of peptides of individual proteins were used in cell cultures to represent RD1 and single proteins, respectively.

Establishing antigen-reactive T-cell lines
Antigen-specific T-cell lines were established from PBMCs by stimulation with the peptide pools of RD1 and PPE68 according to standard procedures [23–25]. In brief, 2 × 10^6 cells/well were stimulated with 5 μg/ml of peptides in 96 well plates and incubated at 37°C in an atmosphere of 5% CO2 and 95% air for 6 days. Starting from day 6, IL-2 (100 U/ml) [Amersham Life Sciences, Amersham, U.K.] was added twice a week until the cell number was sufficient to be transferred to 24 well tissue culture plates (Nunc Roskilde, Denmark). The T-cell lines were maintained in 24 well plates with twice a week addition of IL-2 and tested for antigen reactivity 3–4 days after the last addition of IL-2. The T-cell lines were phenotyped for the expression of CD4 and CD8 molecules using standard procedures [26].
IFN-γ secretion by T-cell lines

The T-cell lines were tested for antigen-induced IFN-γ secretion in the wells of 96-well tissue culture plates (Nunc, Roskilde, Denmark) in the presence of autologous and allogeneic HLA-typed antigen presenting cells (APCs), as described previously [23,25]. In brief, adherent cells obtained from irradiated (24 Grays) PBMCs (seeded into the wells of 96-well plates at 1 x 10^6 cells/well) were used as APCs. The T-cell lines were added to the wells at a concentration of 5 x 10^5 cells/well. Peptides were added in triplicate at a final concentration of 5 μg/ml, and the control wells lacked the peptides. The plates were incubated at 37°C in an atmosphere of 5% CO2 and 95% air. After 3 days of incubation, the culture supernatants were collected and assayed for IFN-γ concentrations using immunoassay kits (Coulter/Immunotech, S.A., Marseille, France), as described above for PBMCs. The secretion of IFN-γ in response to a given antigen was considered positive with IFN-γ concentration ≥5 IU/ml [25].

HLA-DR binding prediction analysis of PPE68 and its peptides

HLA-DR binding prediction analysis of PPE68 and the sequence of each peptide was first performed using the ProPred server (http://www.imtech.res.in/raghava/propred/) at threshold value of 3, as described previously [27]. This server is a useful tool in locating the promiscuous binding regions that can bind to a total of 51 alleles belonging to nine serologically defined HLA-DR molecules [27–29]. These HLA-DR molecules are encoded by DRB1 and DRB5 genes including HLA-DR1 (2 alleles), DR3 (7 alleles), DR4 (9 alleles), DR7 (2 alleles), DR8 (6 alleles), DR11 (9 alleles), DR13 (11 alleles), DR15 (3 alleles) and DR51 (2 alleles). The peptides of PPE68 predicted to bind >50% HLA-DR alleles included in the ProPred were considered promiscous for binding [30].

In addition, ProPred-predicted four HLA-promiscuous and four HLA-non-binder peptides were further analyzed for HLA-DR binding predictions using two other computational prediction methods, i.e. NetMHCIIpan-2.0 [31], and Immuno Epitope Database Base (IEDB; Consensus [32], for binding to 14 alleles, including HLA DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101 and DRB1*1501 supertype alleles that are expected to cover approximately >95% of any given human population [33]. The sequences/peptides predicted to bind >50% alleles of HLA-DR molecules analyzed were considered promiscous for binding [30].

Search for sequence identity

The complete PPE68 sequence and the immunodominant and HLA-promiscuous peptide sequence (121-VLTATNFFGINTIPIALTEMDYFIR-145) were searched for identical sequences in various strains of M. tuberculosis and mycobacterial species using Protein Basic Local Alignment Search Tool (BLAST), National Center for Biotechnology Information, National Institute of Health, Bethesda, Maryland, USA, using the world wide web (WWW) server.

Results

Antigen-specific IFN-γ secretion by human T-cell lines

T-cell lines were established from HLA-heterogeneous donors by stimulating PBMCs with the RD1pool (n = 4 donors, Table 1) and PPE68 (n = 3 donors, Table 2), as the primary antigens in vitro. Phenotypic analysis showed that all of these T-cell lines belonged to the CD4+, CD8− subset of T cells. Subsequent testing for antigen-induced IFN-γ secretion demonstrated that all of the four RD1-induced T-cell lines responded to whole-cell M. tuberculosis and three of them responded to RD1pool (Table 1). When tested with the peptide pools of individual proteins of RD1, only PPE68 induced positive responses in all of the three T-cell lines responding to RD1pool (Table 1), whereas, only one T-cell line responded to 10 of the 12 ORFs of RD1 (Table 1). The IFN-γ responses of three T-cell lines established against PPE68 were also tested with whole-cell M. tuberculosis, PPE68 and peptide pools of some other RD1 proteins, and the results showed that all of these T-cell lines responded to whole-cell M. tuberculosis and PPE68, but not to other RD1 proteins (Table 2).

Identification of immunodominant and HLA-promiscuous peptide(s) of PPE68

To identify the peptides of PPE68 recognized by Th1-type cells, individual peptides of PPE68 were tested with PBMCs from 30 M. bovis BCG-vaccinated healthy subjects in IFN-γ assays. The results showed that all of the peptides induced positive responses in a proportion of donors, which ranged from 30% to 70% (Table 3). However, the best responses were observed with peptide P9 (121-VLTATNFFGINTIPIALTEMDYFIR-145), which induced positive responses in 21/30 (70%) subjects. In terms of % positives, the response induced by P9 (121–145) was comparable to the response induced by the full-length PPE68 protein (1–371) with 22/30 (73%) subjects showing positive response (Table 3). Except P9 (21–145), none of the other peptides of PPE68 qualified to be strong stimulator of Th1-type cells, because the IFN-γ responses to them were either moderate (P1, P2, P4, P8, P11, P12, P13, P14, P17, P18, P20, P21) or weak (P3, P5, P6, P7, P10, P15, P16, P19, P22, P23 and P24) (Table 3). These results suggest that, for Th1-type cell-reactivity, only P9 (121–145) was the immunodominant peptide of PPE68.

In addition to the functional assay for Th1-type cell reactivity, the sequences of PPE68 and its individual peptides were also analyzed for the presence of T-cell epitopes by predicting to bind HLA-DR molecules using the ProPred server. Because the complete PPE68 sequence (1–371) is too large, therefore, binding prediction for all of its peptides to 51 HLA-DR alleles included in ProPred, cannot be presented in a figure or a table. Instead, the summary of HLA-DR binding results are presented in table 3. However, to provide an idea of HLA-DR binding predictions, the prediction results for a small region (106 to 160 covering peptides P8, P9 and P10) to individual HLA-DR alleles, included in ProPred, are shown in fig. 1. The overall results of ProPred analysis suggest that PPE68 was a promiscuous HLA-DR binder and T-cell epitopes were scattered throughout the sequence of PPE68 (Table 3). In total, 50/51 (98%) of HLA-DR specificities included in ProPred were predicted to bind PPE68 sequences and 19 of 24 peptides were predicted to be HLA-DR binders (Table 3). However, five peptides of PPE68, i.e. P4, P7, P18, P22 and P24 were not predicted to have T-cell epitopes using ProPred, but the results of IFN-γ assays showed that all of them had Th1 cell-stimulating epitopes and induced moderate (P4) to weak responses (P7, P18, P22, P24) (Table 3). Furthermore, the peptides P5, P9, P10 and P21 were found HLA-promiscuous (Table 3, Fig. 1: data shown for P9 and P10), but only P9 qualified as a strong stimulator, whereas others were weak stimulators of IFN-γ secretion (Table 3). All other peptides were predicted to be non-promiscuous HLA-DR binders, and none of them were strong stimulators of Th1 cells in IFN-γ assays (Table 3).
Identification of immunodominant and HLA-promiscuous epitope of peptide P9 (121–145)

The immunodominant peptide P9 (121-VLTATNFFGINTIPIALTEMDYFIR-145) of PPE68 is a 25-mer and each amino acid of this sequence contributed in binding to HLA-DR molecules included in ProPred (Fig. 1). It has got six independent sequences (each a 9-mer), which were predicted to bind one (121-VLTATNFFG-129), two (122-LTATNFFGI-130, 135-IALTEM-DYF-143 and 137-LTEMDYFIR-145), 16 (128-FGINTIPIA-136) and 28 (127-FFGINTIPI-135) alleles of HLA-DR molecules included in ProPred (Fig. 1). HLA-promiscuous binding of the peptide 121–145 was also suggested by using other prediction programs for binding to HLA-DR alleles, i.e. NetMHCII 2.2, and IEDB Consensus, which predicted to bind 11/14 (79%) and 10/14 (70%) alleles of HLA-DR, respectively. Testing a series of deletion peptides of 121–145 with PBMCs of eight HLA-heterogeneous healthy subjects responding to the full-length peptide showed that the IFN-γ responses (8/8 responders) and HLA-DR binding predictions (33/51, 65%) were fully conserved for the 10-mer sequence 127-FFGINTIPIA-136 (Table 4). However, any further deletion on either side of this core peptide decreased the frequency of positive response as well as the ability to predict binding to HLA-DR alleles by ProPred (Table 4). However, variations were observed in the minimum length of peptides inducing a positive response in various donors, and even 9, 8 and 7-mer peptides, which belonged to the HLA-DR binding region but were not predicted to bind HLA-DR alleles included in the ProPred due to short length (<10 aa), could induce positive responses in PBMCs of six, five and two donors, respectively (Table 4).

A BLAST search for sequence homology with the PPE68 sequence and peptide P9 (121-VLTATNFFGINTIPIALTEMDYFIR-145) in the data base of NCBI showed that PPE68 was 100% conserved in all organisms of \textit{M. tuberculosis} complex, except BCG, and had 75% and 67% identities with PPE proteins of \textit{M. tuberculosis}.

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**Table 1.** IFN-γ secretion by RD1-induced T-cell lines from HLA-heterogeneous subjects in response to whole cell \textit{M. tuberculosis}, RD1pool and various ORFs of RD1.

| Antigen/Peptides | Concentrations of IFN-γ (IU/ml) in culture supernatants of T-celllines with HLA-type |
|------------------|-------------------------------|
|                  | DR7,10,53 | DR7,13,52,53 | DR11,13,52 | DR3,11,52 |
| \textit{M. tuberculosis} | 54 | 57 | 44 | 15 |
| RD1pool | 63 | 37 | 26 | 0.7 |
| RV3871 | <0.4 | <0.4 | 1.1 | <0.4 |
| PE35 | 22 | 1.0 | 1.4 | <0.4 |
| ORF4 | 1.0 | <0.4 | 0.5 | <0.4 |
| PPE68 | 57 | 44 | 38 | <0.4 |
| CFP10 | 40 | 0.7 | 3.0 | <0.4 |
| ESAT-6 | 71 | 2.7 | 3.0 | <0.4 |
| ORF8 | 54 | 1.0 | 2.3 | <0.4 |
| RV3876 | 69 | 1.8 | 2.6 | <0.4 |
| RV3877 | 67 | 0.4 | 1.1 | <0.4 |
| RV3878 | 41 | <0.4 | 1.0 | <0.4 |
| ORF14 | 28 | <0.4 | 0.7 | <0.4 |
| ORF15 | 29 | <0.4 | 0.4 | <0.4 |

The T-cell lines were established after stimulation of PBMCs with RD1pool and tested for antigen reactivity in IFN-γ assays, as described in the materials and methods. The positive responses (IFN-γ concentration $\geq 5$ IU/ml) are given in bold face.

doi:10.1371/journal.pone.0103679.t001

**Table 2.** IFN-γ secretion by PPE68-induced T-cell lines from HLA-heterogeneous subjects in response to whole cell \textit{M. tuberculosis} and various ORFs of RD1.

| Antigen/Peptides | Concentrations of IFN-γ (IU/ml) in culture supernatants of T-celllines with HLA-type |
|------------------|-------------------------------|
|                  | DR1,11,52 | DR2,5,51,52 | DR4,7,53 |
| \textit{M. tuberculosis} | 26 | 26 | 30 |
| PPE68 | 27 | 26 | 12 |
| ORF4 | 0.4 | 1.1 | 1.3 |
| CFP10 | 1.0 | 2.1 | 1.7 |
| ORF8 | 1.6 | 0.8 | 1.5 |
| RV3877 | 0.8 | 2.3 | 0.9 |

The T-cell lines were established after stimulation of PBMCs with the peptide pool of PPE68 and tested for antigen reactivity in IFN-γ assays, as described in the materials and methods. The positive responses (IFN-γ $\geq 5$ IU/ml) are given in bold face.

doi:10.1371/journal.pone.0103679.t002
**Table 3.** Antigen-induced IFN-γ secretion by PBMCs from 30 *M. bovis* BCG-vaccinated healthy subjects and ProPred predictions for PPE68 and its peptides (P1 to P24) to bind 51 HLA-DR alleles.

| Peptide | IFN-γ response | HLA-DR binding |
|---------|----------------|----------------|
|         | Median IU/ml | P/T | % positive | P/T | % binding |
| PPE68 (1–371) | 22 | 22/30 | 73% | 50/51 | 98 |
| P1 (1–V1TMLWHAMPPELNTARLMAGAPPA–25) | 3.6 | 16/30 | 53% | 1/51 | 2 |
| P2 (1.6–ARLMAGAPPAMLAAAGWQLLAA–40) | 4.3 | 16/30 | 53% | 6/51 | 12 |
| P3 (31–AA4NHQTLSAADQAVEVTLARNIL–55) | 1.2 | 10/30 | 33% | 22/51 | 43 |
| P4 (46–V1ELARLNSLGEANTGGSQDKALAA–70) | 3.5 | 16/30 | 53% | 0/51 | 0 |
| P5 (61–GGQSDKALAAATPMMVWQLQATSTQA–85) | 2.3 | 12/30 | 40% | 35/51 | 69 |
| P6 (76–V1WCLQATSTAQKTRAMQ7ATAQAATY–100) | 1.6 | 13/30 | 43% | 9/51 | 18 |
| P7 (91–QATAQARYTQAMATTSLPEIAA–115) | 2.6 | 14/30 | 47% | 0/51 | 0 |
| P8 (106–TPSFLPE1AAHHITQAVLTLATNFFG–130) | 3.2 | 16/30 | 53% | 3/51 | 6 |
| P9 (121–V1ELATNFINTIP1ALTEMDYFI–145) | 7.9 | 21/30 | 70% | 33/51 | 65 |
| P10 (136–ALTEMDYFI1RM/QAALAMEYQAE–160) | 2.7 | 14/30 | 47% | 38/51 | 75 |
| P11 (151–ALAMEYQAEATVNLTEKLEPMA–175) | 3.5 | 15/30 | 50% | 24/51 | 47 |
| P12 (166–LFEKLEPMASI1LDGSQSTNP1F–190) | 3.5 | 15/30 | 50% | 24/51 | 47 |
| P13 (181–AQSTNNP1F/1GMPSRSPTFVGQLP–205) | 4.6 | 17/30 | 57% | 23/51 | 45 |
| P14 (196–GSSTVPQGLLP1PAATQTLQ0LGMES–220) | 4.6 | 17/30 | 57% | 2/51 | 4 |
| P15 (211–T1QLG1EMGSPMQRQLTPQ1LPQVTS–235) | 1.9 | 13/30 | 43% | 6/51 | 12 |
| P16 (226–T1P1QLQVTS/M1FSQVGGNPAD–250) | 1.5 | 12/30 | 40% | 23/51 | 45 |
| P17 (241–T1GGN1PADEAQM1GLTQ1PSL1N–265) | 3.7 | 17/30 | 57% | 11/51 | 22 |
| P18 (256–GLLT1PSL1NH1PLAG5PSAGAGL–280) | 3.5 | 15/30 | 50% | 0/51 | 0 |
| P19 (271–G1GSP1AAGGLRAES1PL1GAGGSSL–295) | 1.8 | 14/30 | 47% | 16/51 | 37 |
| P20 (286–LPQ1AAGGLSLTTP1LMQL1KPVAP–310) | 4.2 | 16/30 | 53% | 19/51 | 37 |
| P21 (301–Q1LEKVPAPV5M1PAAAAGS1AT1GA–325) | 4.1 | 15/30 | 50% | 29/51 | 57 |
| P22 (316–AAGS1AT1GAPAAV1PGAGAM1QAG–340) | 0.8 | 10/30 | 33% | 0/51 | 0 |
| P23 (331–GAMGQGAQSGSTR1PG1MLVAPAPLAQ–355) | 0.7 | 9/30 | 30% | 18/51 | 35 |
| P24 (346–GLVAPAPLAQEREEDDE1D00WD1EE–371) | 1.4 | 11/30 | 37% | 0/51 | 0 |

*IFN-γ responses were evaluated by stimulating PBMCs with the peptides of PPE68 according to procedures described in materials and methods. The strong responses (Median concentration >5 IU/ml and %positive>70%) are given in bold face.

**Discussion**

In this study, PPE68, a major antigenic protein of *M. tuberculosis* was tested for inducing IFN-γ secretion by antigen-induced T-cell lines and identification of immunodominant peptide(s) by testing PBMCs from HLA-heterogeneous *M. bovis* BCG-vaccinated healthy humans. It has previously been shown that, PPE68, although belongs to the group of proteins encoded by *M. tuberculosis*-specific RD1 genomic segment of DNA, was recognized in Th1-cell assays (antigen-induced proliferation and IFN-γ secretion) by PBMCs from *M. tuberculosis*-infected and non-infected *M. bovis* BCG-vaccinated healthy subjects [13,34]. However, PBMCs are a mixture of various cell types present in the peripheral blood, and therefore the use of PBMCs does not conclusively rule out the recognition of PPE68 by non-T cells or the non-specific mitogenic effect of the protein. Therefore, to confirm that PPE68 was recognized by antigen-specific T cells, antigen-induced T-cell lines from HLA-heterogeneous subjects were established in this study.

Among the antigens used to establish T-cell lines were RD1pools containing peptides of 12 ORFs of RD1, and a pool consisting of the peptides of PPE68 only. Phenotypically, all of the T-cell lines were CD4+, CD8−, confirming the previous observations using

*kansasii* and *M. marinum*, respectively, whereas the sequence identities were <40% with PPE proteins of other mycobacteria, including BCG (data not shown). However, the sequence covering the immunodominant and HLA-promiscuous region of peptide 121–145, i.e. 127-FFGINTIPIA-136, was completely identical between proteins encoded by genes of PPE-family proteins present in several mycobacterial strains and species including *M. tuberculosis* complex, i.e. *M. tuberculosis* (>35 strains, including laboratory and drug-susceptible as well as all multidrug resistant clinical isolates), *M. africanum*, *M. bovis*, *M. bovis* BCG and *M. canettii* and non-tuberculous mycobacteria, e.g. *M. avium*, *M. marinum*, *M. ulcerans*, *M. kansasii* and *M. leprae* etc. (Table 5).
Figure 1. ProPred analysis of a part of PPE68 sequence (106–160) using the ProPred server (http://www.imtech.res.in/raghava/propred/) covering three overlapping peptides (P8, P9 and P10) to 51 HLA-DR alleles. The output of ProPred analysis of PPE68 sequence (aa 106–160) for binding to 51 HLA-DR alleles at the default setting (threshold value of 3) is shown in HTML II view. The sequences predicted to bind HLA-DR alleles are underlined. The obligatory anchor (starting) residues are marked in bold.
doi:10.1371/journal.pone.0103679.g001
Table 4. Analysis of peptide 121–145 and its deletions for prediction to bind HLA-DR alleles and secretion of IFN-γ by PBMCs from HLA-DR heterogeneous healthy subjects.

| Peptide sequence | HLA-DR binding | Antigen-induced IFN-γ (IU/ml) secretion by PBMCs of donors | P/T<sup>a</sup> |
|------------------|----------------|----------------------------------------------------------|---------------|
|                  |                | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
| VLTATNNFGINTIALTEMDFIR | 33/51 | 65 | 43 | 5.0 | 28 | 5.0 | 8.0 | 16 | 26 | 9.0 | 8/8 |
| ATNFGGINTIALTEMDFIR | 33/51 | 65 | 41 | 6.0 | 16 | 5.0 | 20 | 22 | 17 | 8.0 | 8/8 |
| ATNFGGINTIAL | 33/51 | 65 | 45 | 17 | 19 | 5.0 | 28 | 17 | 12 | 8.0 | 8/8 |
| ATNFGGINTIPI | 38/51 | 55 | 47 | 5.1 | 9.0 | 5.0 | 24 | 14 | 16 | 10 | 8/8 |
| FFGINTIPI | 33/51 | 65 | 51 | 5.0 | 19 | 19 | 26 | 16 | 10 | 6.0 | 8/8 |
| FFGINTIPIA | 33/51 | 65 | 50 | 5.0 | 13 | 5.0 | 8.7 | 16 | 15 | 7.0 | 8/8 |
| FGINTIPIA | 16/51 | 31 | 25 | 4.0 | 5.0 | 5.0 | 11 | 11 | 13 | 5.0 | 7/8 |
| GINTIPIA | NA<sup>b</sup> | NA | 21 | 3.0 | 1.0 | 1.5 | 26 | 17 | 9.0 | 5.0 | 6/8 |
| IINTIPIA | NA | NA | 0.5 | 3.0 | 4.0 | 3.5 | 1.0 | 3.0 | 12 | 2.0 | 1/8 |
| FFINTIPI | NA | NA | 0.5 | 2.0 | 4.0 | 1.0 | 1.0 | 3.0 | 6.0 | 4.0 | 1/8 |
| FFINTIPI | NA | NA | 57 | 2.0 | 4.0 | 5.0 | 30 | 6.0 | 15 | 5.0 | 5/8 |
| FFINTIPI | NA | NA | 28 | 4.0 | 18 | 2.5 | 10 | 12 | 17 | 5.0 | 5/8 |
| FFINTIPI | NA | NA | 28 | 2.0 | 15 | 4.0 | 30 | 8.0 | 16 | 4.0 | 4/8 |
| FFINTIPIA | NA | NA | 0.5 | 2.0 | 1.0 | 1.0 | 3.0 | 2.0 | 9.0 | 7.0 | 2/8 |
| FINTIPIA | NA | NA | 1.0 | 2.0 | 4.0 | 2.5 | 30 | 1.0 | 1.8 | 4.0 | 0/8 |

<sup>a</sup>P/T = Number of positive PBMCs donors/Number of donors tested.

<sup>b</sup>NA = Not applicable. This is because these sequences are <10 aa in length, which is the minimum requirement for ProPred to predict binding of peptide sequences to HLA-DR alleles [27].

HLA types of donors: 1(DR7,17,52,53; DQ2,6), 3(DR11,13,52; DQ7), 4(DR17,52; DQ2), 5(DR1,18,52; DQ4,5), 6(DR14,15,51,52; DQ5,6), 7(DR4,16,51,53; DQ5,8), 8(DR4,17,52,53; DQ2,8).

The regions of peptide 121–145 and its deletions predicted to bind HLA-DR alleles are shown in bold and the anchor sequences are underlined.

doi:10.1371/journal.pone.0103679.t004
similar procedures to establish T-cell lines against other antigens of 
*M. tuberculosis* [23–25]. Furthermore, the T-cell lines from all 
donors responded to whole cell *M. tuberculosis* suggesting their 
previous exposure to antigens of *M. tuberculosis* either through 
infection with *M. tuberculosis* and/or vaccination with *M. bovis* 
BCG. However, one of the four RD1-induced T-cell line did not 
respond to RD1 pool. This could have been due to the low 
frequency or absence of RD1-reactive T cells in this cell line. The 
establishment of a T-cell line from this donor could have been due 
to the antigen non-specific stimulation of *M. tuberculosis*-reactive 
T cells by IL-2, as has been shown previously with other antigens 
[35]. However, all three RD1 pool-reactive T-cell lines also 
responded to PPE68, and only one T cell line responded to nine 
other RD1 antigens, including ESAT-6 and CFP10 (Table 1). All 
of the three T-cell lines established against PPE68 responded to 
this antigen only (Table 2), which suggests that the responses to 
PPE68 were antigen-specific and not due to the activation of non-
specific T cells.

The positive responses of PBMCs from healthy subjects to 
ESAT-6/CFP10 have been considered as indication of prior 
infection of donors with *M. tuberculosis* [36–38]. Thus, the 
positive responses of T-cell lines to PPE68, but not to other RD1 
antigens, suggest that these donors were not infected with *M. 
tuberculosis*, and therefore, the positive responses to PPE68 could 
have been due to vaccination with BCG and/or exposure to 
environmental mycobacteria, as suggested previously for other 
crossreactive antigens of *M. tuberculosis*, e.g. MPT63, MPB70 and 
MPT83 etc. [29,39].

To identify immunodominant epitope(s) in PPE68, two 
approaches were used in this study. First PBMCs from HLA-
heterogeneous subjects were tested with 24 overlapping peptides 
covering the sequence of PPE68. A similar approach has 
previously been used to identify the immunodominant epitopes 
of other major antigenic proteins of *M. tuberculosis* [40–42]. The 
results showed that all of the peptides of PPE68 induced positive 
responses in a proportion of donors, but, the best responses were 
observed with peptide P9 (121–145) VLTATNFFGINTIPIALTEM-
DYFIR. Although, T-cell epitopes were present throughout 
the sequence of PPE68, the percent positive response induced by 
P9 (121–145) was comparable to the percent positive response 
induced by the peptide pool of full-length PPE68 protein (1–371) 
(P>0.05, by Z test). This feature seems to be unique to this 
peptide, because none of the single peptides of other mycobacterial 
proteins have shown similar positivity in human Th1-cell assays, as 
full-length proteins [29,39–44].

In addition to Th1-cell reactivity, the sequences of PPE68 and 
its individual peptides were analyzed for the presence of T-cell 
epitopes using the ProPred server, which predicts binding to 
molecules encoded by 51 HLA-DR alleles [27]. The ProPred 
analysis has previously been shown to identify immunodominant 
antigens and peptides of several *M. tuberculosis* proteins [28– 
30,39,40]. The overall results of ProPred analysis suggest that 
PPE68 was a promiscuous HLA-DR binder (Table 3). The 
analysis of individual peptide sequences by ProPred suggested 
that 19 of 24 peptides were predicted to be HLA-DR binders 
(Table 3). However, five peptides of PPE68, i.e. P4, P7, P18, P22 
and P24 were not predicted to have T-cell epitopes by ProPred 
analysis, but the results of IFN-γ assays showed that all of them 
had T-cell epitopes and induced moderate (P4 and P18) to weak 
responses (P7, P22, P24) (Table 3). The discrepancy between 
the HLA-DR binding and the functional assay could be due to the 
reason that ProPred, although includes the binding prediction for 
a large number of HLA-DR molecules, does not include all HLA-
DR specificities [27]. Alternatively, ProPred is not 100% accurate 
to predict the binding [28–30,39,40]. Therefore, the five non-
binding and four promiscuous peptides of PPE68 were further 
evaluated for binding predictions using two additional servers, i.e. 
NetMHCII 2.2 and IEDB Consensus, which are suggested to have

| Mycobacterial species                     | Amino acid sequence          |
|------------------------------------------|------------------------------|
| *M. tuberculosis* complex:               |                              |
| *M. tuberculosis* (>35 species)          | VLTATNFFGINTIPIALTEMDYFIR    |
| *M. bovis*                               | VLTATNFFGINTIPIALTEMDYFIR    |
| *M. bovis BCG                            | VLTATNFFGINTIPIALTEMDYFIR    |
| *M. africanum*                           | VLTATNFFGINTIPIALTEMDYFIR    |
| *M. canetti*                             | VLTATNFFGINTIPIALTEMDYFIR    |
| Non-tuberculous mycobacteria:            |                              |
| *M. kansasii*                            | VLVATNFFGINTIPIALTEADY---    |
| *M. marinum*                             | VLVATNFFGINTIPIALTEADY---    |
| *M. ulcerans*                            | VLVATNFFGINTIPIALTEADY---    |
| *M. parascrofulaceum*                    | VLVATNFFGINTIPIALTEADY---    |
| *M. abscessus*                           | VLLATNFFGINTIPIALNEADY-IR    |
| Mycobacterial species JD601              | VLVATNFFGINTIPIALTEADY---    |
| *M. avium*                               | VLVATNFFGINTIPIALTEADY---    |
| *M. smegmatis*                           | VLVATNFFGINTIPIALTEADY---    |
| *M. leprae*                              | FL1ATNFFGINTIPIALNEADYVR-    |

The 13 aa sequence of PPE68 (aa 124–136) common to all mycobacteria is given in italics and the sequence in each mycobacterial species predicted to bind HLA-DR alleles in this region is underlined. The obligatory anchor (starting) residues for HLA-DR binding are marked in bold.
doi:10.1371/journal.pone.0103679.t005
Consensus methods (data not shown). Thus, both functional as HLA-DR alleles, when analyzed by NetMHCII 2.2 and IEDB sequence also retained its promiscuous character for binding and to bind HLA-DR molecules by ProPred (Table 4). The same FFGINTIPIA-136 retained the full capacity to stimulate Th1 cells included in ProPred (Fig. 1). However, a 10 aa sequence, i.e. 127–acid of this sequence contributes in binding to HLA-DR molecules (121-VLTATNFFGINTIPIALTEMDYFIR-145) of PPE68 is a 25-mer and each amino receptors, which may be lacking in some individuals.

The immunodominant peptide P9 (121–145) of PPE68 may be useful as a peptide-based vaccine against TB and other mycobacterial diseases. FIR-145 may be useful as a peptide-based vaccine against TB and other mycobacterial diseases. Of National Centre for Biotechnology Information, USA, using Basic Local Alignment Search Tool (BLAST) for comparing protein sequences, showed that a 13 aa stretch, i.e. 124-ATNFFGINTIPIA-136, was completely identical between proteins encoded by genes of other PPE-family proteins present in various mycobacterial strains and species, e.g. M. tuberculosis, M. bovis, M. avium BCG, M. avium, M. marinum, M. ulcerans and M. leprae etc. (Table 5). These results suggest that the core region of the immunodominant peptide of PPE68, i.e. 127-FFGINTIPIA-136, is present in several pathogenic mycobacteria. Furthermore, the full length peptide 121–145 as well as peptide 127–136 were also suggested to possess CD8+ cytotoxic T cell epitopes using nHLAPred/Compred [47] and ProPred-I [48] (Table 7). Since the involvement of both CD4+ and CD8+ T cells is suggested for optimal protection against mycobacterial disease [49,50], the use of crossreactive peptide 121-VLTATNFFGINTIPIALTEMDYFIR-145 of PPE68 may be useful as a peptide-based vaccine against TB and other mycobacterial diseases.

| Table 6. Comparison of binding predictions of selected peptides of PPE68 to HLA-DR alleles using various computational methods and IFN-γ responses of PBMCs from 30 healthy subjects. |
|---------------------------------|---------------------------------|---------------------------------|----------------|
| Peptide                         | Binding to HLA-DR alleles predicted by\(^a\) | Subjects responding in IFN-γ assays\(^b\) |
| P4 (46–70)                      | 0/51 (0%)                        | 4/14 (29%)                      | 1/14 (7%)      | 16/30 (53%)    |
| P7 (91–1155)                    | 0/51 (0%)                        | 6/14 (43%)                      | 3/14 (21%)     | 14/30 (47%)    |
| P18 (256–280)                   | 0/51 (0%)                        | 4/14 (29%)                      | 0/14 (0%)      | 15/30 (50%)    |
| P22 (316–340)                   | 0/51 (0%)                        | 1/14 (7%)                       | 0/14 (0%)      | 10/30 (33%)    |
| P24 (346–371)                   | 0/51 (0%)                        | 2/14 (14%)                      | 1/14 (7%)      | 11/30 (37%)    |
| P5 (61–85)                      | 35/51 (69%)                      | 11/14 (79%)                     | 7/14 (50%)     | 12/30 (40%)    |
| P9 (121–145)                    | 33/51 (65%)                      | 11/14 (79%)                     | 10/14 (71%)    | 21/30 (70%)    |
| P10 (136–160)                   | 38/51 (75%)                      | 12/14 (86%)                     | 11/14 (79%)    | 14/30 (47%)    |
| P21 (301–325)                   | 29/51 (57%)                      | 3/14 (21%)                      | 3/14 (21%)     | 15/30(50%)     |

\(^a\)The results are shown as number of HLA-DR molecules predicted to bind/number of HLA-DR molecules tested for binding to a given peptide and the percentages are given in brackets.

\(^b\)The results are given as the number of subjects positive/the number of subjects tested with each peptide and the percentages of positive responders are given in brackets.

%positive

| Table 7. Binding predictions forPPE68peptides 121–145, 124–137 and 127–136 to HLA-class I alleles using the prediction methods nHLAPred/Compred and ProPred-I. |
|---------------------------------|---------------------------------|----------------|
| Peptide                         | Binding to HLA-class I alleles predicted by\(^a\) | |
|                                | nHLAPred/Compred | ProPred-I |
| 121-VLTATNFFGINTIPIALTEMDYFIR-145 | 25/67 (37%) | 41/47(87%) |
| 124-ATNFFGINTIPIAL-137          | 15/67(22%)     | 26/47(55%) |
| 127-FFGINTIPIA-136              | 4/67(6%)       | 15/47(32%) |

\(^a\)The results are shown as no. of HLA-class I molecules predicted to bind/number of HLA-class I molecules tested for binding to a given peptide and the percentages are given in brackets.

doi:10.1371/journal.pone.0103679.t006
doi:10.1371/journal.pone.0103679.t007
Acknowledgments

The buffy coats from healthy donors were obtained from the Central Blood Bank, Kuwait, and Fatema Shaban provided technical help.

Author Contributions

Conceived and designed the experiments: ASM. Analyzed the data: ASM. Contributed reagents/materials/analysis tools: ASM. Wrote the paper: ASM.

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