

Identification of Promiscuous Epitopes for Cellular Immune Responses in the Major Antigenic Protein Rv3873 Encoded by Region of Difference 1 of *Mycobacterium tuberculosis*

Abu Salim Mustafa

Abstract—Rv3873 is a relatively large size protein (371 amino acids in length) and its gene is located in the immunodominant genomic region of difference (RD)1 that is present in the genome of *Mycobacterium tuberculosis* but deleted from the genomes of all the vaccine strains of *Bacillus Calmette Guerin* (BCG) and most other mycobacteria. However, when tested for cellular immune responses using peripheral blood mononuclear cells from tuberculosis patients and BCG-vaccinated healthy subjects, this protein was found to be a major stimulator of cell mediated immune responses in both groups of subjects. In order to further identify the sequence of immunodominant epitopes and explore their Human Leukocyte Antigen (HLA)-restriction for epitope recognition, 24 peptides (25-mers overlapping with the neighboring peptides by 10 residues) covering the sequence of Rv3873 were synthesized chemically using fluorenylmethyloxycarbonyl chemistry and tested in cell mediated immune responses. The results of these experiments helped in the identification of an immunodominant peptide P9 that was recognized by people expressing varying HLA-DR types. Furthermore, it was also predicted to be a promiscuous binder with multiple epitopes for binding to HLA-DR, HLA-DP and HLA-DQ alleles of HLA-class II molecules that present antigens to T helper cells, and to HLA-class I molecules that present antigens to T cytotoxic cells. In addition, the evaluation of peptide P9 using an immunogenicity predictor server yielded a high score (0.94), which indicated a greater probability of this peptide to elicit a protective cellular immune response. In conclusion, P9, a peptide with multiple epitopes and ability to bind several HLA class I and class II molecules for presentation to cells of the cellular immune response, may be useful as a peptide-based vaccine against tuberculosis.

Keywords—*Mycobacterium tuberculosis*, Rv3873, peptides, vaccine

I. INTRODUCTION

TUBERCULOSIS (TB) is an infectious diseases caused by the acid-fast bacterium known as *Mycobacterium tuberculosis*. TB can affect any organ of the human body but lungs are the primary target. TB is known to humans since antiquity, and it may have killed the highest number of people in the world, as compared to any other microbial pathogen

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Abu Salim Mustafa is Professor of Molecular Microbiology and Immunology at the Department of Microbiology, Faculty of Medicine, Health Sciences Centre, Kuwait University, Kuwait (phone: +965-24636505, e-mail: abusolim@hsc.edu.kw).

[1]. According to the most recent worldwide estimates from the World Health Organization (WHO), TB was the ninth leading cause among all causes of human death in 2016 [2]. Furthermore, it was the leading cause of death from a single infectious agent, ranking above HIV/AIDS [2]. According to the estimates by WHO, 10.4 million people became sick with TB, and 1.3 million and 0.38 million TB deaths occurred among HIV-negative and HIV-positive people, respectively [2]. Most deaths from TB could be prevented with early diagnosis, followed by effective chemotherapy using the available anti-TB drugs. However, drug resistance is a problem of major concern in controlling TB and a continuing global threat. In 2016, there were 0.6 million new TB cases with resistance to rifampicin, the most effective first-line anti-TB drug, of which 0.49 million had multidrug-resistant TB [2]. The problem of drug-resistance is having a negative effect in controlling the global burden of TB [2]. Therefore, it is essential that effective preventive tools, including vaccines against TB, are made available to fulfill the WHO's aim of ending the global TB epidemic by 2035 [2].

The currently available vaccine against TB is the *Bacillus Calmette-Guerin* (BCG), which is an attenuated strain of *Mycobacterium bovis* [3]. Although, vaccination with *M. bovis* BCG provides some degree of protection against childhood TB and severe forms of TB in adults, it has failed to provide consistent protection, especially in poor and developing countries of Asia and Africa, against pulmonary TB in adults [4]-[6]. Furthermore, vaccination with *M. bovis* BCG is not recommended in HIV-infected subjects, because due to the immunocompromised state of these individuals, the live organisms may grow unrestricted and cause TB-like disease by themselves [7], [8].

In immunocompetent people, the infection with *M. tuberculosis* activates the immune response to its antigens [9]-[17]. It has previously been shown that protective cellular immune responses are mediated by T helper (Th)1 and T cytotoxic (Tc) cells, whereas Th2 and Treg cells mediate pathogenesis in TB [18]. The indicator cytokines for Th1, Th2 and Treg responses are interferon gamma (IFN- γ), interleukin (IL)-5 and IL-10, respectively [18]. In general, the mycobacterial antigens/peptides are presented to Th and Tc cells in association with highly polymorphic human leukocyte antigen (HLA) class II and class I molecules, respectively [19]-[32]. Since human populations are highly heterogeneous

with respect to the expression of HLA molecules [33], it is essential that the antigens/peptides selected as candidates for TB vaccine development should bind several HLA molecules [34]-[36].

The studies involving comparative genome analyses of *M. tuberculosis* with other mycobacteria have revealed the existence of many regions of differences (RDs) that are specific for *M. tuberculosis* and deleted in all strains of BCG vaccines, i.e. RD1, RD4 to RD7, RD9 to RD13, and RD15 [37]-[39]. In this paper, the results of our work are presented in relation to cellular immune responses to RDs in humans, and the identification of immunodominant proteins and peptides. Furthermore, the immunodominant epitopes were identified and their HLA-restriction was determined by prediction analysis and wet-lab experiments.

II. MATERIALS AND METHODS

A. Study Participants and Isolation of Peripheral Blood Mononuclear Cells

The study participants were pulmonary TB patients (n = 88) from the Chest Diseases Hospital, and healthy blood donors (n = 75) from the Central Blood Bank, Kuwait. Informed written consent was obtained from all participants. The study received approval from the Joint Ethical Committee of the Health Sciences Centre and Ministry of Health, Kuwait. Peripheral blood mononuclear cells PBMCs were isolated from the blood of study participants by flotation on Lymphoprep gradients, as described previously [40]-[42].

B. Chemically Synthesized Peptides

Overlapping synthetic peptides (25-mers, overlapped with the neighboring peptides by 10 residues) spanning the sequence of the proteins encoded by genes *M. tuberculosis*-specific RDs, i.e. RD1, RD4 to RD7, RD9 to RD13, and RD15 of *M. tuberculosis*, were synthesized using fluorenylmethyloxycarbonyl chemistry, as described previously [43]-[48].

C. Antigen Induced Secretion of Th1, Th2 and Treg Cytokines from PBMC

PBMCs were seeded in 96-well microtitre plates and stimulated with peptide pools of RDs, peptide pools of single proteins and individual peptides using standard procedures [49]-[52]. The culture plates were incubated at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air [53]-[55]. On day 6, the culture supernatants (100 µl) were collected from each well and used for cytokine estimations.

D. Cytokine Assays

The supernatants collected from the cultures of PBMCs were used to determine the concentrations of secreted IFN-γ, IL-5 and IL-10 using standard procedures [54]-[58]. The results are presented in terms of cytokine concentrations (pg/ml) and E/C (cytokine concentration in antigen/peptide-stimulated cultures/cytokine concentration in cultures lacking antigen/peptide). The cytokine responses to a given antigen were considered significant when % responders were ≥40%

[59], [60].

E. HLA-Binding Prediction Analysis

The binding predictions for Rv3873 peptides to highly polymorphic HLA molecules were performed using servers available online, which included ProPred [61], NetMHCII.2 [62] and MHC-2Pred [63] for HLA-DR alleles, NetMHCII.2 for HLA-DP alleles [62], and NetMHCII.2 [62] and MHC-2Pred [63] for HLA-DQ alleles of HLA class II molecules. In addition, binding predictions to HLA class I molecules was performed using NetMHCpan-4.0 [64]. Furthermore, the prediction for the presence of Tc epitopes in P9 was performed using IEDB class I immunogenicity predictor [65], [66].

III. RESULTS AND DISCUSSION

In order to determine the cellular immune responses of *M. tuberculosis*-infected humans in terms of Th1, Th2 and Treg responses, PBMCs of TB patients were tested with chemically synthesized peptide pools of RDs for secretion of IFN-γ, IL-5 and IL-10, respectively. The results showed that RD1 was the most important region for the stimulation of Th1-cells (as shown in Fig. 1).

To further identify the reactivity of individual RD1 proteins in cellular immune response, chemically synthesized overlapping peptides covering the complete sequences of eight RD1 proteins (Rv3871, Rv3872, Rv3873, Rv3874, Rv3875, Rv3876, Rv3877 and Rv3878) were tested in Th1 cell assays, i.e. secretion of IFN-γ. The results showed that all of these proteins were recognized by Th-1 cells from some TB patients, but three of them (Rv3873, Rv3874 and Rv3875) were recognized by Th1-cells from more than 50% patients and thus qualified as immunodominant antigens encoded by RD1 (as shown in Table I). However, Rv3873 was the only protein that qualified to be the immunodominant Th1 cell antigen for healthy subjects (as shown in Table I).

In terms of the concentration of secreted IFN-γ, Rv3873, Rv3874 and Rv3875 induced significant concentration (>5 pg/ml) of IFN-γ from PBMC of TB patients but only Rv3873 induced significant concentration of IFN-γ from the PBMCs of healthy subjects (as shown in Fig 2). These findings suggested that Rv3873 is not a *M. tuberculosis*-specific protein and has cross-reactive Th1-cell epitopes. The recognition of Rv3873 by T cells from healthy subjects has also been confirmed by testing antigen-induced T cell lines [61]. Further studies were conducted to identify the immunodominant, HLA-promiscuous and cross-reactive Th1-cell epitope(s) of Rv3873 by using its individual peptide sequences in HLA-binding prediction and Th1-cell assays.

Rv3873 is a relatively large size protein and contains 371 amino acids (as shown in Fig. 3). To cover the entire sequence of Rv3873, a total of 24 peptides (P1 to P24, 25-mers overlapping by 10 residues with the neighboring peptides). All the peptides were analyzed for HLA-DR binding using a web-based server (ProPred to predict binding 51 HLA-DR alleles) and tested with PBMCs of healthy subjects for Th1 responses.

TABLE I

TH1 CELL REACTIVITY OF RD1 PROTEINS USING PERIPHERAL BLOOD MONONUCLEAR CELLS FROM TB PATIENTS AND HEALTHY SUBJECTS

RD1 protein	% positivity in Th1 cell assays with PBMCs from TB patients	% positivity in Th1 cell assays with PBMCs from healthy subjects
Rv3871	30	53
Rv3872	28	33
Rv3873	68	73
Rv3874	80	47
Rv3875	80	37
Rv3876	48	50
Rv3877	33	20
Rv3878	40	17

TABLE II

HLA-PROMISCUOUS PEPTIDES OF PPE68 AND THEIR TH1 CELL REACTIVITY WITH PBMCs FROM HEALTHY SUBJECTS

Peptide	ProPred analysis for binding to HLA-DR No. of alleles binding/tested (%binding)	Th1 cell reactivity No. of subjects positive/tested (%positive)
P5 (aa 61-85)	35/51 (69%)	8/30 (27%)
P9 (aa 121-145)	33/51 (65%)	15/30 (50%)
P10 (aa 136-160)	38/51 (75%)	10/30 (33%)
P21 (aa 301-325)	29/51 (57%)	9/30 (30%)

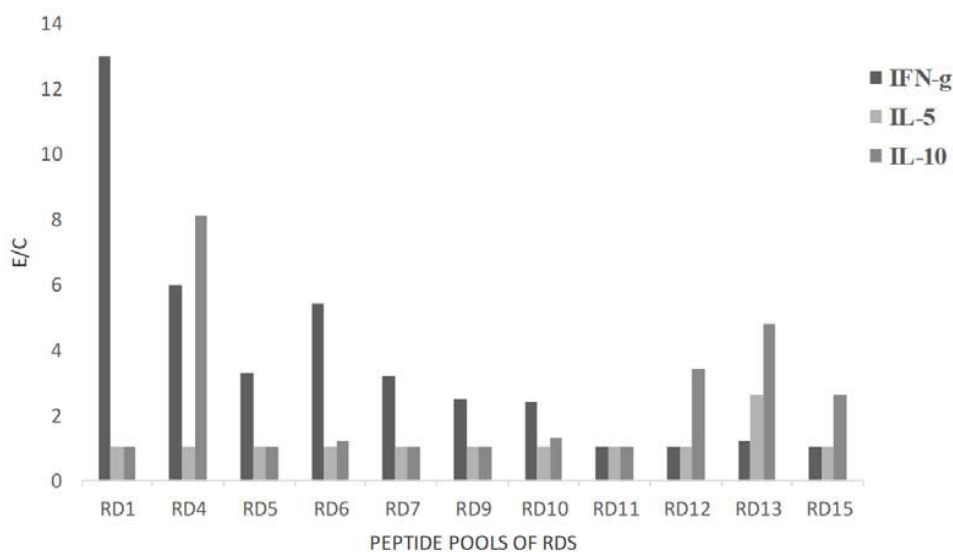


Fig. 1 Secretion (E/C) of Th1, Th2 and Treg cytokines by PBMC of TB patients in response to Peptide pools of RDs

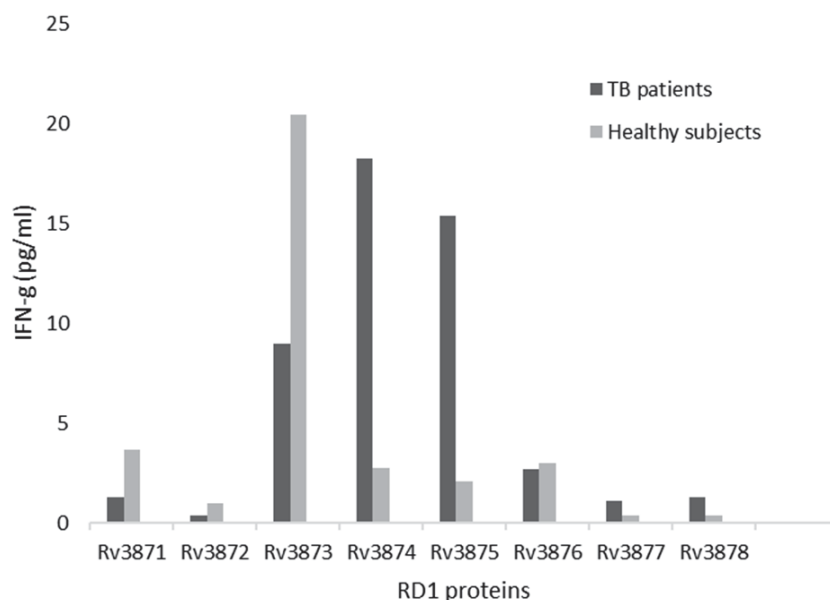


Fig. 2 Antigen-induced IFN-g secretion (pg/ml) by PBMC from TB patients and BCG-vaccinated healthy subjects in response to peptide pools of RD1 proteins

VITMLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQVELTARLNSLGEAWTGGGSDKALAAATPM
 VVWLQASTAQKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLTATNFFGINTIPIALTEMDYFIRMWNQAA
 LAMEVYQAETAVNTLFEKLEPMASILDPGASQSTTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQQLTQP
 LQQVTSLSFSQVGGTGGGNPADEEAAQMGLLGTSPLSNHLPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPMSQLIEKP
 VAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREDEDDWDEEDDW

Fig. 3 Amino acid (1 to 371) sequence of Rv3873

The results showed that four peptides (P5, P9, P10 and P21) were predicted to be promiscuous for binding to HLA-DR alleles (binding to $\geq 50\%$ HLA-DR alleles) but only P9 was found to be immunodominant for Th1 cell reactivity, i.e. with positive responses in $\geq 50\%$ tested subjects (as shown in Table II).

HLA-DR binding prediction analysis using two other servers, i.e. NetMHCII.2 and MHC-2Pred showed that P9 was capable of binding promiscuously to various HLA-DR alleles (as shown in Table III). Similarly, several alleles were predicted to bind P9 when analyzed for binding to HLA-DP by NetMHCII.2 server and HLA-DQ by NetMHCII.2 and MHC2Pred servers (as shown in Table IV). In addition, analysis with NetMHC4.0, a server predicting binding of peptides to HLA class I molecules, suggested the binding of P9 to 6/12 (50%) alleles. The HLA-binding prediction analysis to HLA class I and class II molecules further suggested that several epitopes (ranging between 5 to 13) were present in P9 for binding to HLA-DR, HLA-DP, HLA-DQ and HLA class I alleles (Table IV). Furthermore, the use of IEDB class I immunogenicity predictor server yielded a high score (0.94), which indicated a greater probability of P9 to elicit a protective cellular immune response.

TABLE III
 HLA-DR BINDING PREDICTION ANALYSIS OF P9 OF Rv3873 TO
 POLYMORPHIC HLA CLASS II MOLECULES

Prediction server	Binding predictions to HLA class II molecules		
	HLA-DR	HLA-DP	HLA-DQ
	No. of alleles binding/tested (%binding)		
NetMHCII 2.2	11/14 (79%)	16/20 (80%)	6/6 (100%)
MHC2Pred	14/14 (100%)	NA	5/5 (100%)

TABLE IV
 HLA BINDING PREDICTION ANALYSIS OF P9 FOR NUMBER OF EPITOPES

Prediction server	HLA molecule	No. of epitopes
ProPred	HLA-DR	6
MHC2Pred	HLA-DR	13
MHC2Pred	HLA-DQ	5
NetMHCII.2	HLA-DR	9
NetMHCII.2	HLA-DP	12
NetMHCII.2	HLA-DQ	8
NetMHC4.0	HLA class I	10

IV. CONCLUSION

A single peptide P9 of Rv3873 was found immunodominant and promiscuous with multiple epitopes for binding to both HLA class I and class II molecules for presentation to TC and Th1 cells, respectively. These results suggest that the peptide P9 may be useful in exploring the possibility of developing a

peptide-based vaccine against TB.

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