International Journal of Medical, Medicine and Health Sciences
ISSN: 2517-9969
Vol:12, No:4, 2018

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 [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-g), 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

The study was supported by Kuwait University grant no. MI02/12 and Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait.

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: abusalim@hsc.edu.kw).

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% CO_2 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-g, 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 $\geq$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], NetMHCII2.2 [62] and MHC-2Pred [63] for HLA-DR alleles, NetMHCII2.2 for HLA-DP alleles [62], and NetMHCII2.2 [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-g, 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 over-lapping 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-g. 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-g, Rv3873, Rv3874 and Rv3875 induced significant concentration (>5 pg/ml) of IFN-g from PBMC of TB patients but only Rv3873 induced significant concentration of IFN-g 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%)

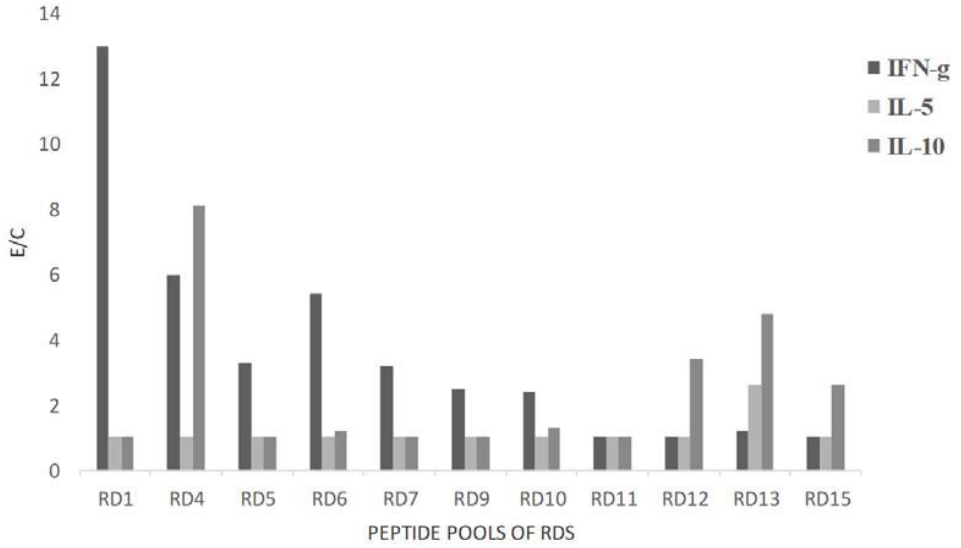

Fig. 1 Secretion (E/C) of Th1, Th2 and Treg cytokines by PBMC of TB patients in response to Peptide pools of RDs

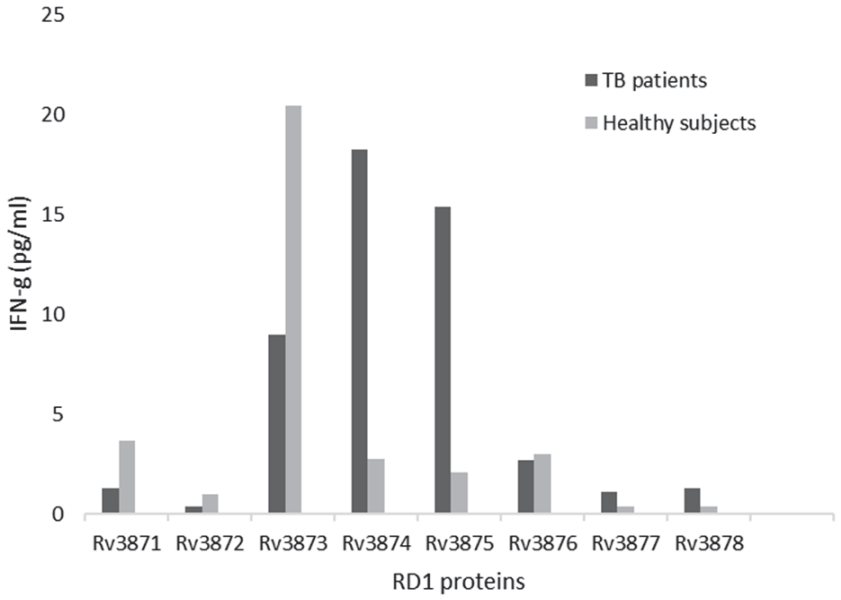

Fig. 2 Antigen-induced IFN-g secretion (pg/ml) by PBMC from TB patients and BCG-vaccinated healthy subjects in response to peptide pools of RD1 proteins

VITMLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLSAALDAQAVELTARLNSLGEAWTGGGSDKALAAATPM
VVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLTATNFFGINTIPIALTEMDYFIRMWNQAA
LAMEVYQAETAVNTLFEKLEPMASILDPGASQSTTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQQLTQP
LQQVTSLFSQVGGTGGGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLIEKP
VAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDEDDWDEEDDW

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 ≥50% HLA-DR alleles) but only P9 was found to be immunodominant for Th1 cell reactivity, i.e. with positive responses in ≥50% tested subjects (as shown in Table II).

HLA-DR binding prediction analysis using two other servers, i.e. NetMHCII2.2 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 HLADP by NetMHCII2.2 server and HLA-DQ by NetMHCII2.2 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
NetMHCII2.2	HLA-DR	9
NetMHCII2.2	HLA-DP	12
NetMHCII2.2	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.

ACKNOWLEDGMENT

The technical support by Fatima Shaban for conducting cytokine assays is gratefully acknowledged.

REFERENCES

[1] Daniel TM. 2006. The history of tuberculosis. Resp Med 100: 1862-1870
[2] WHO Report. 2017. Global tuberculosis report. http://www.who.int/tb/publications/global_report/en/
[3] Mustafa AS, Al-Attiyah R. 2003. Tuberculosis: Looking beyond BCG vaccines. *J Postgrad Med* 49:129-140.
[4] Mustafa AS. 2005. Progress towards the development of new anti-tuberculosis vaccines. In: Focus on Tuberculosis Research, Editor: Lucy T. Smithe, Nova Science Publishers, Inc., New York, pp. 47-76.
[5] Mustafa AS. 2012. What's new in the development of tuberculosis vaccines. *Med Princ Pract* 21:195-196.
[6] Mustafa AS. 2018. Antigens for DNA vaccines against tuberculosis. *Mycobact Dis* 8:255. doi:0.4172/2161-1068.1000255.
[7] Mustafa AS. 2016. BCG pros and cons and new/improved vaccines for tuberculosis. In: Text Book of Biochemistry, Biotechnology, Allied and Molecular Medicine, Fourth Edition. Editors: Talwar GP, Hasnain SE, Sarin SK & Sayed Hasnain, Publisher: PHI Learning Private Limited, Delhi, India. Chapter 117, pp. 1347-1353.
[8] Nuttall JJ, Eley BS. 2011. BCG Vaccination in HIV-Infected Children. *Tuberc Res Treat* 2011:712736.
[9] Mustafa AS. 2009. Vaccine potential of *Mycobacterium tuberculosis*-specific genomic regions: in vitro studies in humans. *Expert Rev Vaccines* 8:1309-1312.
[10] Mustafa AS, Amoudy HA, Oftung F, Wiker HG, Abal AT, Ravn P, Andersen P. 1998. Comparison of antigen-specific T-cell responses of tuberculosis patients using complex or single antigens of *Mycobacterium tuberculosis*. *Scand J Immunol* 48:535-543.
[11] Al-Attiyah R, Mustafa AS, Abal AT, Madi NM, Andersen P. 2003. Restoration of mycobacterial antigen-induced proliferation and interferon-γ responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy *FEMS Immunol Med Microbiol* 38:249-256.
[12] Al-Attiyah R, Mustafa AS, Abal AT, El-Shamy AM, Dalemans W, Skeiky YAW. 2004. *In vitro* cellular immune responses to complex and newly defined recombinant antigens of *Mycobacterium tuberculosis*. *Clin Exp Immunol* 138:139-144.
[13] Al-Attiyah R, N Madi, AS El-Shamy, W Harald, P Andersen, Mustafa AS. 2006. Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of *Mycobacterium tuberculosis*. *FEMS Immunol Med Microbiol* 47:254-461.
[14] Al-Attiyah R, Mustafa AS. 2008. Characterization of human cellular immune responses to novel *Mycobacterium tuberculosis* antigens encoded by genomic regions absent in *Mycobacterium bovis* BCG. *Infect Immun* 76:4190-4198.
[15] Mustafa AS, El-Shamy AM, Madi NM, Amoudy HA, Al-Attiyah R. 2008. Cell mediated immune responses to complex and single mycobacterial antigens in tuberculosis patients with diabetes. *Med Princ Pract* 17:325-330
[16] Mustafa AS, Al-Attiyah R. 2009. Identification of *Mycobacterium tuberculosis*-specific genomic regions encoding antigens those induce qualitatively opposing cellular immune responses. *Ind J Exp Biol* 47:498-504.
[17] Al-Attiyah R, Mustafa AS. 2010. Characterization of human cellular immune responses to *Mycobacterium tuberculosis* proteins encoded by genes predicted in RD15 genomic region that is absent in

Mycobacterium bovis BCG. *FEMS Immunol Med Microbiol* 59:177-187.
[18] Mustafa AS, Al-Saidi F, El-Shamy ASM and Al-Attiyah R. 2011. Cytokines in response to proteins predicted in genomic regions of difference of *Mycobacterium tuberculosis*. *Microbiol Immunol* 55:267-278.
[19] Dheda K, Schwander SK, Zhu B, van Zyl-Smit RN, Zhang Y. 2010. The immunology of tuberculosis: From bench to bedside. *Respirology* 15:433–450.
[20] Mustafa AS, Qvigstad E. 1989. HLA-DR restricted antigen induced proliferation and cytotoxicity mediated by CD4+ T cell clones from subjects vaccinated with killed *M. leprae*. *Int J Lepr Other Mycobact* Dis 57: 1 -11.
[21] Mustafa, AS, Lundin KEA, Oftung F. 1993. Human T cells recognize mycobacterial heat shock proteins in the context of multiple HLA-DR molecules. Studies in healthy subjects vaccinated with *Mycobacterium bovis* BCG and *Mycobacterium leprae*. *Infect Immun* 61: 5294-5301.
[22] Oftung F, Geluk A, Lundin KEA, Meloen RH, Thole JER, Mustafa AS, Ottenhoff THM. 1994. Mapping of multiple HLA Class II restricted T cell epitopes of the mycobacterial 70 kDa heat shock protein. *Infect Immun* 62: 5411-5418.
[23] Mustafa AS, Deggerdal A, Lundin KEA, Meloen RM, Shinnick TM, Oftung F. 1994. An HLA-DRw53-restricted T-cell epitope from a novel *Mycobacterium leprae* protein antigen important to the human memory T-cell repertoire against *M. leprae*. *Infect Immun* 62: 5595-5602.
[24] Mustafa AS. 1995. Identification of mycobacterial peptide epitopes recognized by CD4+ T-cells in association with multiple MHC class II molecules. *Nutrition* 11: 657-660.
[25] Mustafa AS, KEA Lundin, RM Meloen, TM Shinnick, AFW Coulson, F Oftung. 1996. HLA-DR4 restricted T cell epitope from the mycobacterial 60 kDa heat shock protein (HSP60) do not map to the sequence homology regions with human HSP60. *Immunology* 87:421-427.
[26] Mustafa AS, Oftung F. 1996. Amino acid sequence and HLA-DR restriction of peptide defined T cell epitopes from somatic antigens of mycobacteria. *Kuwait Med J* (Supplementary Issue) 244-247.
[27] Mustafa AS, Nadeem S, Amoudy HA. 1996. Identification of HLADR molecules frequently expressed by peripheral blood mononuclear cells of healthy subjects responding to mycobacterial antigens. *Kuwait Med J* (Supplementary Issue) 323-327.
[28] Oftung F, Lundin KEA, Geluk G, Shinnick TM, Meloen R, Mustafa AS. 1997. Primary structure and MHC restriction of peptide defined T cell epitopes from recombinantly expressed mycobacterial protein antigens. *Med Princ Pract* 6: 66-73.
[29] Mustafa AS, Shaban FA, Abal AT, Al-Attiyah R, Wiker HG, Lundin KEA, Oftung F, Huygen K. 2000. Identification and HLA-restriction of naturally derived Thl-cell epitopes from the secreted *Mycobacterium tuberculosis* antigen 85B recognized by antigen-specific human CD4+ T-cell lines. *Infect Immun* 68:3933-3940.
[30] Mustafa AS, Lundin KEA, Meloen RH, Oftung F. 2000. Crossreactive epitopes and HLA-restriction elements in human T cell recognition of the *Mycobacterium leprae* 18-kD heat shock protein. *Clin Exp Immunol* 120:85-92.
[31] Mustafa AS, Shaban FA, Al-Attiyah RJ, Abal AT, El-Shamy AM, Andersen P, Oftung F. 2003. Human Th1 cell lines recognize the Mycobacterium tuberculosis ESAT-6 antigen and its peptides in association with frequently expressed HLA class II molecules. *Scand J Immunol* 57:125-134.
[32] Al-Attiyah R, Mustafa AS. 2004. Computer-assisted prediction of HLA-DR binding and experimental analysis for human promiscuous Th1 cell peptides in a novel 24-kDa secreted lipoprotein (LppX) of *Mycobacterium tuberculosis*. *Scand J Immunol* 59:16-24.
[33] Mustafa AS, Abal AT, Shaban F, El-Shamy AM, Amoudy HA. 2005. HLA-DR binding prediction and experimental evaluation of mycolyltransferase (Ag85B), a major secreted antigen of *Mycobacterium tuberculosis. Med Princ Pract* 14:140-146.
[34] Mustafa AS. 2000. HLA-restricted immune response to mycobacterial antigens: relevance to vaccine design. *Hum Immunol* 61:166-171.
[35] Mustafa AS, Al-Attiyah R. 2004. *Mycobacterium tuberculosis* antigens and peptides as new vaccine candidates and immunodiagnostic reagents against tuberculosis. *Kuwait Med J* 36: 171-176.
[36] Mustafa AS. 2005. Mycobacterial gene cloning and expression, comparative genomics, bioinformatics and proteomics in relation to the development of new vaccines and diagnostic reagents. *Med Princ Pract* 14 Suppl 1:27-34.
[37] Mustafa AS. 2010. Cell mediated immunity assays identify proteins of diagnostic and vaccine potential from genomic regions of difference of *Mycobacterium tuberculosis*. *Kuwait Med J* 42:98-105.
[38] Mustafa AS. 1997. Development of improved vaccines against tuberculosis. *Med Princ Pract* 6: 55.
[39] Mustafa AS. 2001. Biotechnology in the development of new vaccines and diagnostic reagents against tuberculosis. *Curr Pharm Biotechnol* 2: 157-173.
[40] Mustafa AS. 2012. What's New in the Development of Tuberculosis Vaccines. *Med Princ Pract* 21:195-196.
[41] Mustafa AS, Godal T. 1983. In vitro induction of human suppressor T cells by mycobacterial antigens. BCG activated OKT4 + T cells mediate suppression of antigen induced proliferation. *Clin Exp Immunol* 52: 29-37.
[42] Mustafa AS, Godal T. 1985. BCG induced suppressor T cells. Optimal conditions for *in vitro* induction and mode of action. *Clin Exp Immunol* 62: 474-481.
[43] Mustafa AS, Kvalheim G, Degre M, Godal T. 1986. *Mycobacterium bovis* BCG induced human T cell clones from BCG vaccinated healthy subjects: antigen specificity and lymphokine production. *Infect Immun* 53: 491-497.
[44] Mustafa AS. 2005. Recombinant and synthetic peptides to identify *Mycobacterium tuberculosis* antigens and epitopes of diagnostic and vaccine relevance. *Tuberculosis* (Edinb) 85:367-76.
[45] Mustafa AS, Shaban FA. 2006. ProPred analysis and experimental evaluation of promiscuous Th1 cell epitopes of three major secreted antigens of *Mycobacterium tuberculosis*. *Tuberculosis* (Edinb) 86:115-24.
[46] Hanif SNM, El-Shammy AM, Al-Attiyah R, Mustafa AS. 2008. Whole blood assays to identify Th1 cell antigens and peptides encoded by *Mycobacterium tuberculosis*-specific RD1 genes. *Med Princ Pract* 17:244-249.
[47] Mustafa AS. 2009. HLA-promiscuous Th1-cell reactivity of MPT64 (Rv1980c), a major secreted antigen of *Mycobacterium tuberculosis*, in healthy subjects. *Med Princ Pract* 18:385-392.
[48] Mustafa AS, Al-Attiyah R. 2009. Identification of *Mycobacterium tuberculosis*-specific genomic regions encoding antigens those induce qualitatively opposing cellular immune responses. *Ind J Exp Biol* 47:498-504.
[49] Mustafa AS. 2009. Th1-cell reactivity and HLA-DR binding prediction for promiscuous recognition of MPT63 (Rv1926c), a major secreted protein of *Mycobacterium tuberculosis*. *Scand J Immunol* 69:213-222.
[50] Al-Attiyah R, Mustafa AS. 2010. Characterization of human cellular immune responses to *Mycobacterium tuberculosis* proteins encoded by genes predicted in RD15 genomic region that is absent in *Mycobacterium bovis* BCG. *FEMS Immunol Med Microbiol* 59:177-187.
[51] Mustafa AS. 2011. Comparative evaluation of MPT83 (Rv2873) for T helper-1 cell reactivity and identification of HLA-promiscuous peptides: studies in *M. bovis* BCG-vaccinated healthy subjects. *Clin* Vaccine *Immunol* 18: 1752-1759.
[52] Mustafa AS. 2010. In silico binding predictions for identification of HLA-DR-promiscuous regions and epitopes of Mycobacterium tuberculosis protein MPT64 (Rv1980c), and their recognition by human Th1 cells. *Med Princ Pract* 19:367-372.
[53] Shaban K, Amoudy HA, Mustafa AS. 2013. Cellular immune responses to recombinant *Mycobacterium bovis* BCG constructs expressing major antigens of region of difference 1 of *Mycobacterium tuberculosis*. *Clin Vaccine Immunol* 20:1230-1237.
[54] Amoudy HA, Ebrahimi BH, Mustafa AS. 2014. Immune responses against *Mycobacterium tuberculosis*-specific proteins PE35 and CFP10 in mice immunized with recombinant *Mycobacterium vaccae*. *Saudi Med J* 35:350-359.
[55] Hanif SNM, Al-Attiyah R and Mustafa AS. 2010. Molecular cloning, expression, purification and immunological characterization of three low molecular weight proteins encoded by genes in genomic regions of difference of *Mycobacterium tuberculosis*. *Scand J Immunol* 71:353-361.
[56] Hanif SN, Al-Attiyah R, Mustafa AS. 2011. Cellular immune responses in mice induced by *M. tuberculosis* PE35-DNA vaccine construct. *Scand J Immunol* 74:554-60.
[57] Al-Attiyah R, El-Shazly A, Mustafa AS. 2012. Comparative analysis of spontaneous and mycobacterial antigen-induced secretion of Th1, Th2 and pro-inflammatory cytokines by peripheral blood mononuclear cells of tuberculosis patients. *Scand J Immunol* 75:623-632.
[58] Mustafa AS. 2014. T-helper 1, T-helper 2, pro-inflammatory and anti-inflammatory cytokines in tuberculosis. *IJMPBS* 3: 1-14.

[59] Mustafa AS. 2016. Immune responses to candidate vaccine antigens delivered through naked plasmid and mycobacterial vectors. *Open Conf Proc J* 7:153-199.
[60] Mustafa AS, Al-Attiyah R, Hanif SNM, Shaban FA. 2008. Efficient testing of pools of large numbers of peptides covering 12 open reading frames of *M. tuberculosis* RD1 and identification of major antigens and immunodominant peptides recognized by humanTh1 cells. *Clin Vaccine Immunol* 15:916-924.
[61] Mustafa AS. 2014. Characterization of a cross-reactive, immunodominant and HLA-promiscuous epitope of *Mycobacterium tuberculosis*-specific major antigenic protein PPE68. PLoS One 9:e103679.
[62] Singh H, Raghava GPS. 2001. ProPred: Prediction of HLA-DR binding sites. Bioinformatics 17: 1236-1237.
[63] Nielsen M, Lund O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. *BMC Bioinformatics* 2009; 10:296.
[64] [http://www.imtech.res.in/raghava/mhc2pred.
[65] Jurtz V, Paul S, Andreatta M, Marcatili P, Peters B, Nielsen M. 2017. NetMHCpan-4.0: Improved peptide–MHC class I interaction predictions integrating eluted ligand and peptide binding affinity. *J Immunol* 199:3360-3368.
[66] Calis JJ, Maybeno M, Greenbaum JA, Weiskopf D, De Silva AD, Sette A, Kesmir C, Peters B. 2013. Properties of MHC class I presented peptides that enhance immunogenicity. *PloS Comp. Biol.* 8:361.