International Journal of Pharma Medicine and Biological Sciences

ISSN 2278 – 5221 www.ijpmbs.com
Vol. 3, No. 4, October 2014

Research Paper

T-HELPER 1, T-HELPER 2, PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINES IN TUBERCULOSIS

Abu Salim Mustafa[1]*

*Corresponding Author: ***Abu Salim Mustafa*** ✉ *abusalim@hsc.edu.kw*

Cytokines are critical for protection and pathogenesis in tuberculosis. In general, the T-helper (Th)1 and pro-inflammatory cytokines are considered to have a role in protection, and the anti-inflammatory and Th2 cytokines in susceptibility/pathogenesis of tuberculosis. To understand better the role of cytokines in tuberculosis, we have studied in vitro secretion of the above cytokines from the Peripheral Blood Mononuclear Cells (PBMCs) of tuberculosis patients (diabetic and non-diabetic) and healthy subjects. PBMCs were incubated in vitro with complex mycobacterial antigens and pools of peptides corresponding to *M. tuberculosis*-specific genomic Regions of Differences (RDs). The culture supernatants were assayed for the amount of cytokines released after 6 days of incubation. In general, the concentrations of antigen-induced Th2 cytokines were low/undetected and the pro-inflammatory cytokines were non-discriminatory. With respect to Th1 and anti-inflammatory cytokines, the antigens could be divided into three groups; the first with Th1-bias (culture filtrate of *M. tuberculosis*, RD1, RD5, RD7 and RD9), the second with anti-inflammatory-bias (whole bacilli and cell walls of *M. tuberculosis*, RD12 and RD13), and the third without any bias (*M. bovis* BCG, RD4, RD10, RD6, RD11 and RD15). However, among the peptide pools, RD1 peptides induced strongest Th1-bias, and the addition of RD12 and RD13 peptides to PBMCs cultures inhibited the RD1-induced Th1-cell reactivity. The analyses of data for cytokines in diabetic and non-diabetic TB patients and healthy subjects showed a lower Th1:Th2/anti-inflammatory cytokines in diabetic TB patients, which may explain, at least in part, a faster deterioration in their clinical conditions.

Keywords: *M. tuberculosis*, Cytokines, RDs, Proteins, Peptides

INTRODUCTION

Tuberculosis (TB) in *Homo sapiens* is primarily caused by *Mycobacterium tuberculosis*, which is among the most successful human pathogens. Because of its adverse effects on human health, the World Health Organization (WHO) declared TB a global public health emergency in 1993 (World Health Organization, 2012). According to WHO, about one-third of the world's population is latently infected with *M. tuberculosis* and 10%

[1] Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait.

of the infected individuals will develop active disease in their life time (Tuberculosis Fact sheet N°104, 2014). Furthermore, the most recent estimates by WHO suggest that 8.3 to 9 million people developed active disease, and 1.3 to 1.6 million people died from TB in 2011 (World Health Organization, 2012). A large proportion of TB patients reside in the poor developing countries of Asia (59%) and Africa (26%) (World Health Organization, 2012), which suffer from poverty, unhygienic living conditions, and poor healthcare infrastructure. Diabetes is another major international health problem affecting 285 million adults and causing 3.96 million deaths in 2010 (Roglic and Unwin, 2010). The association between diabetes and tuberculosis, and their synergistic role to aggravate each other is well established (Dooley and Chaisson, 2009). However, the immunological basis of this association is not very clear.

The primary natural reservoirs of *M. tuberculosis* are humans. Upon entering into the human body, mostly through aerosol infections, and reaching the lungs, *M. tuberculosis* is taken up by phagocytic cells, e.g., macrophages, monocytes and dendritic cells, etc. (Henderson *et al*., 1997), which act as antigen presenting cells and present the antigens of *M. tuberculosis* to T cells (van Crevel *et al*., 2002). The interaction of infected phagocytes with T cells causes the release of various types of soluble molecules known as cytokines (Henderson *et al*., 1997). The functional diversity of T cells, and the type of cytokines they produce, is important for protection and pathogenesis in TB (Mustafa, 2001; 2009; 2012). The functional division of T cells into T helper 1 (Th1) and Th2 was first document in 1986 in mice (Mosmann *et al*., 1986). In general, Th1 cells and their cytokines are considered protective against TB (Mustafa, 2001; 2009; 2012). In particular, interferon-gamma (IFN-γ), the signature cytokine produced by Th1 cells, is considered to mediate protective immunity against *M. tuberculosis* by activating infected phagocytes to kill the intracellular bacilli, whereas Th2 cells release IL-4 and IL-5 associated with the lack of protection against TB (Al-Attiyah *et al*., 1996; 2003; Flynn, 2004; Bai *et al*., 2004; Mustafa, 2013; 2010; O'Garra *et al*., 2013). In addition, the anti-inflammatory cytokine IL-10, produced by many hematopoietic cells, including phagocytes and lymphocytes, helps in pathology, reduces resistance and causes chronic progressive TB (O'Garra *et al*., 2013). Furthermore, IL-10 deactivates macrophages and down regulates the secretion of the protective cytokine IFN-γ by Th1 cells (Turner *et al*., 2002). IL-10 has a major role in suppressing macrophage and dendritic cell functions, which are required for the capture, control, and initiation of immune responses to *M. tuberculosis* (Redford *et al*., 2011). It has been proposed that IL-10 is linked with the ability of *M tuberculosis* to evade immune responses and mediates long-term infections in the lung. Furthermore, the infected phagocytes release innate-immune-response-related pro-inflammatory cytokines IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α), which help to slow mycobacterial growth by various mechanisms, including the recruitment of fresh monocytes into the lesions and activation to kill the phagocytized mycobacteria (Zuñiga *et al*., 2012).

In this study, to investigate the role of various mycobacterial antigens in protection and susceptibility to pulmonary TB in diabetic and non-diabetic TB patients, a battery of complex mycobacterial and region of difference (RD) antigens of *M. tuberculosis* were studied for the

secretion of Th1 (IFN-γ, TNF-β), Th2 (IL-4, IL-5), proinflammatory (IL1-β, IL-6 and TNF-α) and antiinflammatory (IL-10) cytokines from peripheral blood monocular cells of TB patients (with and without diabetes) and BCG-vaccinated healthy subjects.

MATERIALS AND METHODS

TB Patients and Healthy Subjects

The patients included in the study were newly diagnosed and culture-confirmed cases of pulmonary TB (with and without diabetes) and admitted in the Chest Diseases Hospital, Kuwait. *M. bovis* BCG-vaccinated healthy subjects were the blood donors at the Central Blood Bank, Kuwait. Informed written consent was obtained from all patients and healthy subjects, and the study received approval from the Ethical Committee, Faculty of Medicine, Kuwait University, Kuwait.

Complex Mycobacterial Antigens and Synthetic Peptides of *M. tuberculosis*

The complex mycobacterial antigens used in this study were killed *M. tuberculosis* H37Rv (MTB), killed *M. bovis* BCG, *M. tuberculosis* culture filtrate (MT-CF) and *M. tuberculosis* cell walls (MT-CW) (Mustafa *et al*., 2003; Al-Attiyah and Mustafa, 2004; Al-Attiyah *et al.,* 2004; Mustafa *et al.,* 2005; Mustafa and Shaban, 2006). All of these antigens were provided by Brennan (Colorado State University, Fort Collins) through the repository of TB research materials at the National Institute of Allergy and Infectious Diseases (NIH contract no. AI-25147). Overlapping synthetic peptides (25-mers, overlapped with the neighboring peptides by 10 residues) spanning the sequence of putative proteins encoded by genes in the regions of difference (RD)1, RD4 to RD7, RD9 to RD13, and RD15 of *M. tuberculosis* were designed, synthesized and used in the form of peptide pools, as described previously (Mustafa *et al.,* 2002; Al-Attiyah and Mustafa, 2010; Mustafa and Shaban, 2010; Al-Khodari *et al.,* 2011).

Antigen Stimulation of Peripheral Blood Mononuclear Cells for Proliferation and Cytokine Secretion

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood of TB patients (n=47) and *M. bovis* BCG-vaccinated healthy subjects (n=38) by flotation on Lymphoprep gradients and used in 96-well plates for stimulation with various antigens, as described previously (Mustafa *et al*., 2011; Hanif *et al*., 2008; Mustafa *et al*., 2008; 2011; 2010). The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. On day 6, the culture supernatants (100 μL) were collected from each well and used for cytokine estimations, as given below. The remaining cultures were pulsed with 1 μCi [^{3}H]thymidine (Amersham Life Science, Amersham, United Kingdom) and harvested (Skatron Instruments As, Norway) using standard procedures (Mustafa, 2009; 2014; Al-Attiyah *et al*., 2003).

The radioactivity incorporated was obtained as counts per minute (cpm). The average cpm was calculated from triplicate cultures stimulated with each antigen or peptide pool, as well as from triplicate wells of negative control cultures lacking antigen. The cell proliferation results were calculated as the Stimulation Index (SI), which is defined as follows: SI = cpm in antigen or peptide-stimulated cultures/cpm in cultures lacking antigen or peptide. PBMC cultures from a subject were considered responder to a given antigen when SI value was ≥3 (Al-Attiyah and Mustafa, 2008; 2009; Mustafa and Al-Attiyah, 2009).

Immunoassays for the Quantitation of Cytokines

The supernatants collected from the cultures of PBMC of TB patients before the [^{3}H]thymidine pulse, were assayed to determine the concentrations of secreted cytokines by using FlowCytomix kits (Bender Medsystems Inc., Austria), as described previously (Al-Attiyah and Mustafa, 2008). These kits permit simultaneous quantification of pro-inflammatory (TNF-α, IL-6 and IL-1β), Th1 (IFN-γ, TNF-β), Th2 (IL-4 and IL-5) and antiinflammatory (IL-10) cytokines. The samples were analyzed by flow cytometry using Coulter Epics FC500 (Beckman Coulter Inc.). The mean concentration of each cytokine was expressed as pg/mL. The minimum detectable concentrations of IL-1β, IL-4, IL-5, IL-6, IL-10, TNF-α, TNF-β, and IFN-γ were 4.5 pg/mL, 6.4 pg/mL, 5.3 pg/mL, 4.7 pg/mL, 6.9 pg/mL, 7.9 pg/mL, 3.2 pg/mL, and 7.0 pg/mL, respectively. In response to antigenic stimuli, the values of E/C that were $\geq$2 were considered positive responses (Al-Attiyah *et al.*, 2008). E/C is defined as follows: E/C = cytokine concentration in antigen-stimulated cultures/cytokine concentration in cultures lacking antigen. In the experiments where the concentrations of cytokines in control cultures lacking antigens were not detectable, the E/C values were determined by dividing the concentration of a given cytokine in antigen-stimulated cultures with the minimum detectable concentration of the same cytokine. The cytokine responses to a given antigen were considered significant when % responders were $\geq$40%. Furthermore, based on the IFN-γ:IL10 ratios, the antigens were grouped to have Th1-bias (IFN-γ:IL10 >2), anti-inflammatory-bias (IFN-γ:IL10 <0.3) and no bias (IFN-γ:IL10 >0.3 to <2).

STATISTICAL ANALYSIS

The antigen-induced proliferation and IFN-γ secretion results with antigens and peptide pools were statistically analyzed for significant differences between TB patients and healthy subjects using a nonparametric Mann-Whitney U test for two independent samples. *P* values of <0.05 were considered significant.

RESULTS

PBMCs from TB patients secreted the proinflmmatory cytokine IL-6 in response to all complex mycobacterial antigens and RD peptides, whereas IL1-β and TNF-α were secreted in response to complex mycobacterial antigens only (Table 1). Among the Th1 cytokines, TNF-β and IFN-γ were secreted in response to all complex mycobacterial antigens (Table 1). TNF-β was also secreted in response to RD1, RD6 and RD13 and IFN-γ was secreted in response to RD1, RD4, RD5, RD6, RD7, RD9 and RD 10 (Table 1). The secretion of Th2 cytokines IL-4 and IL-5 could not be detected in response to any antigen, except IL-5 in response to RD13 (Table 1). The anti-inflammatory cytokine IL-10 was detected in response to all complex mycobacterial antigens and RD4, RD6, RD12, RD13 and RD15 (Table 1). The calculation of IFN-γ:IL-10 ratios suggested Th1 bias for MTCF, RD1, RD5, RD7 and RD9 (Table 1), and antiinflammatory bias for MTB, MTCW, RD12 and RD13, and no bias for *M. bovis* BCG, RD4, RD6, RD10, RD11 and RD15 (Table 1).

A comparative analysis, in terms of % responders, was performed for antigen-induced proliferation and secretion of IFN-g and IL-10 by PBMCs of TB patients and healthy subjects. The results showed that best responses in both groups were obtained with complex mycobacterial antigen and RD1, RD7 and RD9 in proliferation and IFN-γ assays (Table 2);

Table 1: Secretion of Cytokines (E/C) by PBMCs of Tuberculosis Patients in Response to the Complex mycobacterial antigens and peptide pools of *M. tuberculosis*-specific RDs

Antigen/Peptide	E/C of Cytokines								
	IL1-β	IL-6	TNF-α	TNF-β	IFN-γ	IL-4	IL-5	IL-10	IFN-γ:IL-10
BCG	**21**	**48**	**10**	1.9	**62**	<1	<1	**150**	0.4
MTB	**28**	**58**	**9.4**	**4.4**	**68**	1.2	1.0	**251**	0.3
MTCF	0.9	**65**	**3.9**	**5.2**	**81**	<1	<1	**15**	**5.4**
MTCW	**23**	**73**	**6.7**	**3.3**	**44**	<1	<1	**239**	0.2
RD1	1.5	**24**	1.4	**2.0**	**13**	<1	<1	1.0	**13**
RD4	1.4	**20**	<1	<1	**6.0**	<1	<1	**8.1**	0.7
RD5	1.1	**13**	<1	<1	**3.3**	<1	<1	1.0	**3.3**
RD6	1.6	**21**	1.0	**2.1**	**5.4**	1.4	1.1	**12**	0.5
RD7	<1	**2.0**	<1	1.3	**3.2**	<1	<1	1.0	**3.2**
RD9	<1	**2.5**	<1	<1	**2.5**	<1	<1	1.0	**2.5**
RD10	1.4	**25**	<1	<1	**2.4**	<1	<1	1.3	**1.8**
RD11	<1	**4.5**	1.1	1.6	<1	<1	1.0	1.0	1.0
RD12	<1	**5.0**	<1	1.6	<1	<1	<1	**3.4**	0.3
RD13	1.5	**14**	1.7	**5.1**	1.2	1.4	**2.6**	**4.8**	0.25
RD15	1.4	**36**	<1	<1	<1	<1	<1	**2.6**	0.38

Note: E/C = Concentration of the cytokine secreted by PBMCs in the presence of antigen/ Concentration of the cytokine secreted by PBMCs in the absence of antigen. The positive values (E/C $\geq$ 2.0) are given in bold.

Table 2: Responders in proliferation with PBMCs obtained from pulmonary tuberculosis patients (n=18) and healthy blood donors (n=18) in response to the complex mycobacterial antigens and peptide pools of *M. tuberculosis* RDs

Antigen peptide	% responders for pulmonary TB patients in			% respondersfor healthy blood donors in		
	Proliferation	IFN-γ	IL-10	Proliferation	IFN-γ	IL-10
BCG	**77**	**73**	**90**	**96**	**98**	**100**
MTB	**81**	**76**	**100**	**96**	**100**	**94**
MTCF	**87**	**89**	**50**	**98**	**100**	**78**
MTCW	**90**	**73**	**100**	**96**	**98**	**94**
RD1	**74**	**48**	22	**62**	**56**	17
RD4	**53**	21	33	27	7	6
RD5	32	11	33	18	2	17

Table 2 (Cont.)

Antigen peptide	% responders for pulmonary TB patients in			% respondersfor healthy blood donors in		
	Proliferation	IFN-γ	IL-10	Proliferation	IFN-γ	IL-10
RD6	**45**	21	28	29	7	6
RD7	**51**	21	28	**51**	**40**	22
RD9	**48**	28	**44**	**42**	**42**	17
RD10	38	23	28	36	22	11
RD11	29	12	28	29	13	28
RD12	38	12	**83**	**44**	24	**44**
RD13	23	11	**50**	**44**	22	**44**
RD15	27	9	22	18	13	11

Note:% responders >40 were considered significant and such values in the table are marked in bold.

Table 3: Inhibitory effect of RD12 and RD13 peptides on RD1-induced proliferation and IFN-γ Secretion by PBMCs of healthy subjects

Assay	PBMCs stimulated with peptides		
	RD1 alone	RD1+RD12	RD1+RD13
Proliferation SI (% inhibition)	10 (0%)	2.2 (78%)	4 (60%)
IFN-γ U/ml (% inhibition)	4 (0%)	1.6 (53%)	2.8 (21%)

Note: SI = stimulation index, as defined in the materials and methods

Table 4: Secretion of cytokines (E/C) by PBMCs of non-diabetic (18) and diabetic (11) tuberculosis patients and healthy subjects (n=20) in response to complex mycobacterial antigens and peptide pools of *M. tuberculosis*-specific RDs

Antigen/peptide	TNF-β			IFN-γ			IL-10			IFN-γ:IL-10		
	TB		Healthy	TB		Healthy	TB		Healthy	TB		Healthy
	Diabetic	Non Diabetic		Diabetic	Non Diabetic		Diabetic	Non Diabetic		Diabetic	Non Diabetic	
BCG	3.5	9.4	6.6	46	308	66	20	24	5.5	3.7	13	21
MTB	1.5	10.4	4.6	41	211	81	33	32	48	2.0	6.6	3.0
MTCF	6.4	20	21	47	384	332	5.3	12	1.1	15	33	399
MTCW	4.0	18	9.7	51	325	156	25	15	3.6	3.3	22	74
RD1	3.4	4.4	1.0	37	58	14	7.0	1.0	1.0	8.6	58	23
RD4	<1	1.0	1.0	7.5	<1	1.0	4.0	1.0	1.0	NA	NA	NA
RD6	1.0	1.0	1.0	4.7	1.0	5.0	2.2	1.0	1.0	NA	NA	NA
RD10	<1	1.0	1.0	<1	1.0	1.1	5.6	1.0	1.0	NA	NA	NA

Note: E/C = Concentration of the cytokine secreted by PBMCs in the presence of antigen/ Concentration of the cytokine secreted by PBMCs in the absence of antigen. The positive values (E/C > 2.0) are given in bold. NA = not applicable

whereas in IL-10 assays, the highest responses were observed with complex mycobacterial antigens, RD12 and RD13 (Table 2). Furthermore, peptide mixing experiments showed that RD12 and RD13 peptides inhibited RD1-induced Th1-cell reactivity as indicated by reduced proliferation and IFN-g secretion by PBMCs of healthy subjects (Table 3).

The secretion of Th1 cytokines, TNF-β and IFN-γ, and antiinflammatory cytokine IL-10 was further studied in type II diabetic and non-diabetic pulmonary TB patients and *M. bovis* BCG-vaccinated healthy subjects in response to complex mycobacterial antigens and peptide pools of RD1, RD4, RD6 and RD10. In general, diabetic TB patients secreted lower concentrations of Th1 cytokines and higher concentrations of antiinflammatory cytokine IL-10, as compared to non-diabetic TB patients and healthy subjects (Table 4). Furthermore, the analysis of cytokine ratios demonstrated that IFN-γ:IL-10 ratios were significantly ($P < 0.05$) and consistently lower in diabetic TB patients, as compared to non-diabetic TB patients and healthy subjects in response to all antigens that induced detectable concentrations of these cytokines in the tested subjects (Table 4).

DISCUSSION

In this study, antigen-induced proliferation and cytokine secretion were studied using PBMCs from diabetic and non-diabetic TB patients and BCG-vaccinated healthy subjects. The antigens used were complex mycobacterial antigens and peptide pools corresponding to the *M. tuberculosis* genomic regions RD1, RD4 to RD7, RD9 to RD13, and RD15, which are absent in all strains of *M. bovis* BCG vaccines (Behr *et al*., 1999; Mustafa and Al-Attiyah, 2003; Mustafa, 2005; 2012; 2013). The study of cellular proliferation and cytokine responses with respect to antigens recognized, particularly the RD antigens, is important for the understanding of protective and pathological immune mechanisms in TB and identification of antigens suitable for the diagnosis and development of new vaccines (Mustafa and Al-Attiyah, 2004; Mustafa, 2005; Hanif *et al*., 2010; Hanif *et al*., 2010; Hanif *et al*., 2011; Shaban *et al*., 2013; Mustafa, 2002). Although, some of the cytokines could be detected in PBMCs cultures within 24 to 48 h of incubation (Al-Attiyah *et al*., 2012), both proliferation and cytokine secretions were studied on day 6 in this work. This is because day 6 is optimal for proliferation and secretion of cytokines IFN-γ and TNF-β (Al-Attiyah *et al*., 2012; Mustafa and Godal, 1983; 1985), and practically convenient for all other cytokines reported in this study (Al-Attiyah *et al*., 2012). Cellular proliferation was studied using the standard assay of [^{3}H] thymidine incorporation into DNA of dividing cells (Mustafa *et al*., 1986; Mustafa, 1988; Mustafa and Qvigstad, 1989), whereas the cytokine concentrations in culture supernatants were estimated by the flow cytomix assay (Al-Attiyah and Mustafa, 2008; 2009; Mustafa and Al-Attiyah, 2009). As compared to regular 96-well plate based uniplex enzyme-linked immunosorbent assays, which detect only one cytokine at a time (Agarwal *et al*., 1999; Pacsa *et al*., 2000; Mustafa *et al*., 2006), the flowcytomix assays are multiplex and allow quantification of multiple cytokines using very low sample volume, i.e., 25 μL (Al-Attiyah and Mustafa, 2008; 2009; Mustafa and Al-Attiyah, 2009).

The overall results suggest that the complex mycobacterial antigens induced large concentrations of both protective (IFN-γ) and

pathologic (IL-10) cytokines from PBMCs of TB patients and healthy subjects (Tables 1 and 2). IL -10 is linked with the ability of *M. tuberculosis* to evade immune responses, and mediates long-term infections in the lung (Turner *et al.*, 2002; Redford *et al.*, 2011). Therefore, the complex mycobacterial antigens inducing large concentrations of IL-10 will not be appropriate as vaccines against TB. On the other hand, some of the RDs preferentially activated protective cytokine IFN-γ in the absence of detectable IL-10, i.e., RD1, RD7 and RD9 and others induced the secretion of large quantities of pathologic cytokine IL-10, without inducing detectable IFN-γ secretion, i.e., RD12 and RD13. Furthermore, addition of RD12 and RD13 antigens to PBMCs cultures stimulated with RD1 was inhibitory for RD1-induced proliferations and IFN-γ secretion (Table 3). These results suggest that antigens of RD1, RD7 and RD9 would be suited as new vaccines; whereas, the use of antigens of RD12 and RD13 may be avoided in TB vaccine preparations. In line with this suggestion, it has been shown that the antigens of RD1, RD7 and RD 9 are expressed in *M. tuberculosis* (Amoudy et al., 2006; Amoudy and Mustafa, 2008; Hanif *et al*., 2011) and have vaccine potentials in animal models of TB (Zhang *et al*., 2006; Bai *et al*., 2008; Maue *et al*., 2007; Ansari *et al*., 2011; Baldwin *et al*., 2009). Important among them are ESAT-6, CFP10, PE35 and PPE86 of RD1, RV2346 of RD7 and RV3619 of RD9 (Hanif and Mustafa, 2013).

When the data were compared for protective (Th1 cytokines) and the pathologic (antiinflammatory cytokine IL-10) between diabetic and non-diabetic TB patients and healthy subjects, the diabetic TB patients showed the lowest concentrations of Th1 cytokines. These results confirm previous reports demonstrating lower IFN-γ secretion by diabetic TB patients, when compared with non-diabetic TB patients and healthy subjects (Tsukaguchi *et al*., 1997; Sun *et al.*, 2012). Although, absolute IL-10 concentrations were similar in the three groups of patients, lower IFN-γ shifted the balance towards the antiinflammatory cytokine in diabetic patients. These results suggest that the cytokine balance between protective and pathologic cytokines will be more relevant for resistance and susceptibility to TB disease, as compared to the absolute quantities of these cytokines.

CONCLUSION

The results reported in this study suggest that antigens of *M. tuberculosis*-specific genomic region RD1, RD7 and RD9 will be relevant for new vaccine development against TB, because these RDs induce secretion of protective Th1 cytokines. Furthermore, the shift towards antiinflammatory and Th2 cytokines may explain the faster deterioration of clinical conditions in diabetic TB patients.

ACKNOWLEDGMENT

The study was supported by Kuwait University Research Sector grants MI02/12, MI01/10 and SRUL02/13.

REFERENCES

1. Agarwal R, Elbishbishi E A, Chaturvedi U C, Nagar R and Mustafa A S (1999), “Profile of transforming growth factor-beta 1 in patients with dengue haemorrhagic fever”, *Int J Exp Pathol.,* Vol. 80, pp. 143-149.
2. Al-Attiyah R, El-Shazly A and Mustafa A S (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.,* Vol. 75, pp. 623-632.

3. Al-Attiyah R, Madi N, El-Shamy A S, Wiker H, Andersen P and Mustafa A S (1996), "Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of *Mycobacterium tuberculosis*", *FEMS Immunol Med Microbiol.,* Vol. 47, pp. 254-261.

4. Al-Attiyah R and Mustafa AS (2004), "Computer-assisted prediction of HLA-DR binding and experimental analysis for human promiscuous Th1-cell peptides in the 24 kDa secreted lipoprotein (LppX) of *Mycobacterium tuberculosis"*, *Scand J Immunol.*, Vol. 59, pp. 16-24.

5. Al-Attiyah R and Mustafa A S (2008), "Characterization of human cellular immune responses to novel *Mycobacterium tuberculosis* antigens encoded by genomic regions absent in *Mycobacterium bovis* BCG", *Infect Immun,* Vol. 76, pp. 4190-4198.

6. Al-Attiyah R and Mustafa A S (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, Vol.* 59, pp. 177-187.

7. Al-Attiyah R, Mustafa A S, Abal AT, El-Shamy A S, Dalemans W *et al.* (2004), "In vitro cellular immune responses to complex and newly defined recombinant antigens of *Mycobacterium tuberculosis*", *Clin Exp Immunol,* Vol. 138, pp. 139-144.

8. Al-Attiyah R, Mustafa A S, Abal A T, Madi N M and Andersen P (2003), "Restoration of mycobacterial antigen-induced proliferation and interferon-gamma responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy", FEMS *Immunol Med Microbiol.,* Vol. 38, pp. 249-256.

9. Al-Attiyah R, Shaban F A, Wiker H G, Oftung F and Mustafa A S (2003), "Synthetic peptides identify promiscuous human Th1 cell epitopes of the secreted mycobacterial antigen MPB70", *Infect Immun,* Vol. 71, pp. 1953-1960.

10. Al-Attiyah R J and Mustafa A S (2009), "Mycobacterial antigen-induced cell proliferation and Th1 and Th2 cytokine secretion by peripheral blood mononuclear cells of diabetic and non-diabetic tuberculosis patients and *Mycobacterium bovis* BCG vaccinated healthy subjects", *Clin Exp Immunol,* Vol. 158, pp. 64-73.

11. Al-Khodari NY, Al-Attiyah R and Mustafa A S (2011), "Identification, diagnostic potential, and natural expression of immunodominant seroreactive peptides encoded by five Mycobacterium tuberculosis-specific genomic regions", *Clin Vaccine Immunol,* Vol. 18, pp. 477-82.

12. Amoudy HA, MB Al-Turab and A S Mustafa (2006), "Identification of transcriptionally active open reading frames within the RD1 genomic segment of *Mycobacterium tuberculosis*", *Medical Principles and Practice,* Vol. 15, pp. 137-144.

13. Amoudy H A and Mustafa A S (2008), "Amplification of Six Putative RD1 Genes of *Mycobacterium tuberculosis* for Cloning

and Expression in *Escherichia coli* and Purification of Expressed Proteins", *Med Princ Pract,* Vol. 17, pp. 378-384.

14. Ansari M A, Zubair S, Mahmood A, Gupta P, Khan A A, *et al.* (2011), "RD antigen based nanovaccine imparts long term protection by inducing memory response against experimental murine tuberculosis", PLoS One 6, pp. e22889.

15. Bai X, Wilson S E, Chmura K, Feldman N E and Chan E D (2004), "Morphometric analysis of Th1 and Th2 cytokine expression in human pulmonary tuberculosis", *Tuberculosis,* Vol. 84, pp. 375–385.

16. Bai Y, Xue Y, Gao H, Wang L, Ding T, *et al.* (2008), "Expression and purification of *Mycobacterium tuberculosis* ESAT-6 and MPT64 fusion protein and its immunoprophylactic potential in mouse model", *Protein Expr Purif ,* Vol. 59, pp. 189-196.

17. Baldwin S L, Bertholet S, Kahn M, Zharkikh I, Ireton G C, *et al.* (2009), "Intradermal immunization improves protective efficacy of a novel TB vaccine candidate", *Vaccine,* Vol. 27, pp. 3063-3071.

18. Behr M A, Wilson M A, Gill W P, Salamon H, Schoolnik G K*, et al*. (1999), "Comparative genomics of BCG vaccines by whole-genome DNA microarray*", Science,* Vol. 284, pp. 1520-1523.

19. Dooley K E and Chaisson R E (2009), "Tuberculosis and diabetes mellitus: convergence of two epidemics", *Lancet Infect Dis,* Vol. 9, pp. 737-746.

20. Flynn J (2004), "Immunology of tuberculosis and implications in vaccine development*", Tuberculosis,* Vol. 4, pp. 93-101.

21. Hanif S N, Al-Attiyah R and Mustafa A S (2011), "Cellular immune responses in mice induced by *M. tuberculosis* PE35-DNA vaccine construct", *Scand J Immunol,* Vol. 74, pp. 554-560.

22. Hanif S N M, Al-Attiyah R and Mustafa A S (2010), "DNA vaccine constructs expressing *Mycobacterium tuberculosis*-specific genes induce immune responses", *Scand J Immunol,* Vol. 72, pp. 408-415.

23. Hanif S N M, Al-Attiyah R and Mustafa A S (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,* Vol. 71, pp. 353-361.

24. Hanif S N M, Al-Attiyah R and Mustafa A S (2011), "The natural expression of genes encoding major antigens of Rd1 and Rd9 in *M. tuberculosis* and other mycobacteria", *Mycobact Dis,* Vol. 1, pp. 105.

25. Hanif S N M, El-Shammy A M, Al-Attiyah R and Mustafa A S (2008), "Whole blood assays to identify Th1 cell antigens and peptides encoded by *Mycobacterium tuberculosis*-specific RD1 genes", *Med Princ and Pract,* Vol. 17, pp. 244-249.

26. Hanif S N M and Mustafa A S (2013), "Advances on TB vaccine*",* In *Vaccines and Vaccine Technologies,* edited by JR Vasconcelos and published by OMICS Group, pp. 002-007.

27. Henderson R A, Watkins S C and Flynn J L (1997), "Activation of human dendritic cells following infection with *Mycobacterium*

tuberculosis", *J Immunol,* Vol. 159, pp. 635-643.

28. Maue A C, Waters W R, Palmer M V, Nonnecke B J, Minion F C, *et al.* (2007), "An ESAT-6:CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. bovis*", *Vaccine,* Vol. 25, pp. 4735-4746.

29. Mosmann T R, Cherwinski H, Bond M W, Giedlin M A and Coffman R L (1986), "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins", *J Immunol,* Vol. 136, pp. 2348-2357.

30. Mustafa A S (1988), "Identification of recombinant antigens shared between three candidate anti leprosy vaccines, killed *M. leprae*, BCG and *Mycobacterium w.", Int J Lepr Other Mycobact Dis,* Vol. 56, pp. 265 273.

31. Mustafa A S (2001), "Biotechnology in the development of new vaccines and diagnostic reagents against tuberculosis", *Curr Pharm Biotechnol,* Vol. 2, pp. 157-173.

32. Mustafa A S (2002), "Development of new vaccines and diagnostic reagents against tuberculosis", *Mol Immunol, Vol.* 39, pp. 113-119.

33. Mustafa A S (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,* Vol. 14, pp. 27-34.

34. Mustafa A S (2005), "Progress towards the development of new anti-tuberculosis vaccines", In: *Focus on Tuberculosis Research*, Editor: Lucy T. Smithe, pp. 47-76, Nova Science Publishers, Inc., New York.

35. Mustafa A S (2005), "Recombinant and synthetic peptides to identify *Mycobacterium tuberculosis* antigens and epitopes of diagnostic and vaccine relevance", *Tuberculosis (Edinb),* Vol. 85, pp. 367-376.

36. Mustafa A S (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,* Vol. 69, pp. 213-222.

37. Mustafa AS (2009) HLA-promiscuous Th1-cell reactivity of MPT64 (Rv1980c), a major secreted antigen of *Mycobacterium tuberculosis*, in healthy subjects. *Med Princ Pract ,* Vol. 18, pp. 385-392.

38. Mustafa A S (2009), "Vaccine potential of *Mycobacterium tuberculosis*-specific genomic regions: in vitro studies in humans", *Expert Rev Vaccines,* Vol. 8, pp. 1309-1312.

39. Mustafa A S (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,* Vol. 19, pp. 367-372.

40. Mustafa A S (2010), "Cell mediated immunity assays identify proteins of diagnostic and vaccine potential from genomic regions of difference of *Mycobacterium tuberculosis*", *Kuwait Med J,* Vol. 42, pp. 98-105.

41. Mustafa A S (2011), "Comparative evaluation of MPT83 (Rv2873) for T helper-1 cell reactivity and identification of HLA-promiscuous peptides in *Mycobacterium bovis* BCG-vaccinated healthy subjects", *Clin Vaccine Immunol,* Vol. 18, pp. 1752-1759.

42. Mustafa A S (2012), "Proteins and peptides encoded by *M. tuberculosis*-specific genomic regions for immunological diagnosis of tuberculosis*", J Mycobac Dis,* Vol. 2, pp. e114.

43. Mustafa A S (2012), "What's New in the Development of Tuberculosis Vaccines", *Med Princ Pract,* Vol. 21, pp. 195-196.

44. Mustafa A S (2013), "In silico analysis and experimental validation of *Mycobacterium tuberculosis*-specific proteins and peptides of *Mycobacterium tuberculosis* for immunological diagnosis and vaccine development", *Med Princ Pract,* Vol. 22, Suppl 1, pp. 43-51.

45. Mustafa A S (2013), " Diagnostic and Vaccine Potentials of ESAT-6 Family Proteins encoded by *M. tuberculosis* genomic regions absent in *M. bovis* BCG", Mycobact Dis, *J Mycobact Dis*, Vol. 3, p. 129.

46. Mustafa A S (2014), "Characterization of a cross-reactive, immunodominant and HLA-promiscuous epitope of *Mycobacterium tuberculosis*-specific major antigenic protein PPE68", *PLoS One*, Vol. 9, No. 8, pp. e103679

47. Mustafa A S, Abal A T, Shaban F, El-Shamy A M and Amoudy H A (2005), "HLA-DR binding prediction and experimental evaluation of T-cell epitopes of mycolyl transferase 85B (Ag85B), a major secreted antigen of *Mycobacterium tuberculosis*", *Med Princ Pract,* Vol. 14, pp. 140-146.

48. Mustafa A S and Al-Attiyah R (2003), "Tuberculosis: Looking beyond BCG vaccines", *J Postgrad Med,* Vol. 49, pp. 129-140

49. Mustafa A S and Al-Attiyah R (2009), "Identification of *Mycobacterium tuberculosis*-specific genomic regions encoding antigens inducing protective cellular immune responses", *Indian J Exp Biol ,* Vol. 47, pp. 498-504.

50. Mustafa A S, Al-Saidi F, El-Shamy A S M, *et al.* (2011), "Cytokines in response to proteins predicted in genomic regions of difference of *Mycobacterium tuberculosis*", *Microbiol Immunol,* Vol. 55, pp. 267-278.

51. Mustafa A S, Cockle P J, Shaban F, Hewinson R G and Vordermeier H M (2002), "Immunogenicity of *Mycobacterium tuberculosis* RD1 region gene products in infected cattle", *Clin Exp Immunol.,* Vol. 130, pp. 37-42.

52. Mustafa A S and 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,* Vol. 57, pp. 1 11.

53. Mustafa A S, El-Shamy A M, Madi N M, Amoudy H A and Al-Attiyah R (2008), "Cell mediated immune responses to complex and single mycobacterial antigens in tuberculosis patients with diabetes", *Med Princ and Pract,* Vol. 17, pp. 325-330.

54. Mustafa A S, G Kvalheim, M Degre and Godal (1986), *"Mycobacterium bovis* BCG induced human T cell clones from BCG vaccinated healthy subjects: antigen specificity and lymphokine production", *Infect Immun,* Vol. 53, pp. 491 497.

55. Mustafa A S and R Al-Attiyah (2004), *"Mycobacterium tuberculosis* antigens and peptides as new vaccine candidates and immunodiagnostic reagents against tuberculosis", *Kuwait Med J,* Vol. 36, pp. 171-176.

56. Mustafa A S, R Al-Attiyah S N M, Hanif F A and Shaban (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 human Th1 cells", *Clin Vaccine Immunol,* Vol. 15, pp. 916-924.

57. Mustafa A S and Shaban F (2010), "Mapping of Th1-cell epitope regions of *Mycobacterium tuberculosis* protein MPT64 (Rv1980c) using synthetic peptides and T-cell lines from *M. tuberculosis*-infected healthy humans", *Med Princ Pract ,* Vol. 19, pp. 122-128.

58. Mustafa A S and Shaban F A (2006), "ProPred analysis and experimental evaluation of promiscuous T-cell epitopes of three major secreted antigens of *Mycobacterium tuberculosis*", *Tuberculosis (Edinb),* Vol. 86, pp. 115-124.

59. Mustafa A S, Shaban F A, Al-Attiyah R, Abal A T, El-Shamy A M, *et al.* (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,* Vol. 57, pp. 125-134.

60. Mustafa A S and 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,* Vol. 52, pp. 29 37.

61. Mustafa A S and Godal T (1985), "BCG induced suppressor T cells. Optimal conditions for *in vitro* induction and mode of action", *Clin Exp Immunol,* Vol. 62, pp. 474 481.

62. Mustafa A S, Y A Skeiky, R Al-Attiyah, M R Alderson, R G Hewinson *et al.* (2006), "Immunogenicity of *M. tuberculosis* antigens in *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle", *Infect Immun,* Vol. 74, pp. 4566-4572.

63. O'Garra A, Redford P S, McNab F W, Bloom C I, Wilkinson R J, *et al.* (2013), "The immune response in tuberculosis", *Annu Rev Immunol ,* Vol. 31, pp. 475-527.

64. Pacsa A S, R Agarwal, E A Elbishbishi, U C Chaturvedi, R Nagar and A S Mustafa (2000), "Role of interleukin 12 in patients with dengue haemorrhagic fever", *FEMS Immunol Med Microbiol,* Vol. 20, pp. 151-155.

65. Redford P S, Murray P J and O'Garra A (2011), "The role of IL-10 in immune regulation during *M. tuberculosis* infection", *Mucosal Immunol,* Vol. 4, pp. 261-70.

66. Roglic G and Unwin N (2010), "Mortality attributable to diabetes: estimates for the year 2010", *Diabetes Res Clin Pract,* Vol. 87, pp. 15-19.

67. Shaban K, Amoudy H A and Mustafa A S (2013), "Cellular immune responses to recombinant *Mycobacterium bovis* BCG constructs expressing major antigens of region of difference 1 of *Mycobacterium tuberculosis*", *Clin Vaccine Immunol,* Vol. 20, pp. 1230-1237.

68. Sun Q, Zhang Q, Xiao H, Cui H and Su B (2012), "Significance of the frequency of CD4+CD25+CD127- T-cells in patients with pulmonary tuberculosis and diabetes mellitus", *Respirology,* Vol. 17, pp. 876-82.

69. Tsukaguchi K, Okamura H, Ikuno M, Kobayashi A, Fukuoka A, *et al.* (1997), "The relation between diabetes mellitus and IFN-gamma, IL-12 and IL-10 productions by CD4+ alpha beta T cells and monocytes in patients with pulmonary tuberculosis", *Kekkaku,* Vol. 72, pp. 617-622.

70. Tuberculosis Fact sheet N°104 (2014), www.who.int/mediacentre/factsheets/fs104/es/

71. Turner J, Gonzalez-Juarrero M, Ellis D L, Basarba R J, A Kipnis, *et al.* (2002), *"In vivo* IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice", *J. Immunol.,* Vol. 169, pp. 6343–6351.

72. Van Crevel R, Ottenhoff T H and van der Meer J W (2002), " Innate immunity to *Mycobacterium tuberculosis*", *Clin Microbiol Rev,* Vol. 15, pp. 294-309.

73. World Health Organization (2012), Global Tuberculosis Report 2012. WHO/HTM/TB/2012.6.

74. Zhang H, Shi C H, Xue Y, Bai Y L, Wang L M, *et al.* (2006), "Immune response and protective efficacy induced by fusion protein ESAT6-CFP10 of *M. tuberculosis* in mice", Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi, Vol. 22, pp. 443-446.

75. Zuñiga J, Torres-García D, Santos-Mendoza T, Rodriguez-Reyna T S, Granados J, *et al*. (2012), "Cellular and humoral mechanisms involved in the control of tuberculosis", *Clin Dev Immunol.*, pp. 193923.