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Research Paper

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

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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%

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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-Attayah *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-1 β , 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 mononuclear 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% CO₂ and 95% air. On day 6, the culture supernatants (100 μ L) were collected from each well and used for cytokine estimations, as given below. The remaining cultures were pulsed with 1 μ Ci [³H]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 [³H]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-1 β), Th1 (IFN- γ , TNF- β), Th2 (IL-4 and IL-5) and anti-inflammatory (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-1 β , 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 ≥ 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 proinflammatory cytokine IL-6 in response to all complex mycobacterial antigens and RD peptides, whereas IL-1 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 MTCTF, RD1, RD5, RD7 and RD9 (Table 1), and anti-inflammatory 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- γ 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			% responders for 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			% responders for 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- γ 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-Attayah, 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-Attayah, 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-Attayah *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-Attayah *et al.*, 2012; Mustafa and Godal, 1983; 1985), and practically convenient for all other cytokines reported in this study (Al-Attayah *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 cytometry assay (Al-Attayah and Mustafa, 2008; 2009; Mustafa and Al-Attayah, 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 flow cytometry assays are multiplex and allow quantification of multiple cytokines using very low sample volume, i.e., 25 μ L (Al-Attayah and Mustafa, 2008; 2009; Mustafa and Al-Attayah, 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.

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