Clinical & Experimental Immunology
The Journal of Translational Immunology

British Society for immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2009.04000.x

Mycobacterial antigen-induced T helper type 1 (Th1) and Th2 reactivity of peripheral blood mononuclear cells from diabetic and non-diabetic tuberculosis patients and *Mycobacterium bovis* bacilli Calmette–Guérin (BCG)-vaccinated healthy subjects

R. J. Al-Attiyah and A. S. Mustafa
Department of Microbiology, Faculty of Medicine, Kuwait University, Safat, Kuwait

Summary

Patients with diabetes mellitus are more susceptible to tuberculosis (TB), and the clinical conditions of diabetic TB patients deteriorate faster than non-diabetic TB patients, but the immunological basis for this phenomenon is not understood clearly. Given the role of cell-mediated immunity (CMI) in providing protection against TB, we investigated whether CMI responses in diabetic TB patients are compromised. Peripheral blood mononuclear cells (PBMC) obtained from diabetic TB patients, non-diabetic TB patients and *Mycobacterium bovis* bacilli Calmette–Guérin (BCG)-vaccinated healthy subjects were cultured in the presence of complex mycobacterial antigens and pools of *M. tuberculosis* regions of difference (RD)1, RD4, RD6 and RD10 peptides. The PBMC were assessed for antigen-induced cell proliferation and secretion of T helper 1 (Th1) [interferon (IFN)-γ, interleukin (IL)-2, tumour necrosis factor (TNF)-β], and Th2 (IL-4, IL-5, IL-10) cytokines as CMI parameters. All the complex mycobacterial antigens and $RD1_{pool}$ stimulated strong proliferation of PBMC of all groups, except moderate responses to $RD1_{pool}$ in healthy subjects. In response to complex mycobacterial antigens, both IFN-γ and TNF-β were secreted by PBMC of all groups whereas diabetic TB patients secreted IL-10 with concentrations higher than the other two groups. Furthermore, in response to RD peptides, IFN-γ and IL-10 were secreted by PBMC of diabetic TB patients only. The analyses of data in relation to relative cytokine concentrations showed that diabetic TB patients had lower Th1 : Th2 cytokines ratios, and a higher Th2 bias. The results demonstrate a shift towards Th2 bias in diabetic TB patients which may explain, at least in part, a faster deterioration in their clinical conditions.

Keywords: diabetes, peripheral blood mononuclear cells, Th1 and Th2 cytokines, tuberculosis

Accepted for publication 7 July 2009
Correspondence: R. J. Al-Attiyah, Department of Microbiology, Faculty of Medicine, Kuwait University, PO Box 24923, Safat 13110, Kuwait.
E-mail: rj.alattiyah@hsc.edu.kw

Introduction

Tuberculosis (TB) and diabetes are among the most important diseases of global concern. TB ranks among the top 10 causes of world mortality [1]. It is estimated that approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*; 8 million people develop active disease, leading to about 2 million deaths each year [1]. With respect to diabetes, the statistics released by the World Health Organization and the International Diabetes Federation shows that the number of diabetics in the world is expected to increase from 194 million in 2003 to 330 million in 2030, with 75% diabetic patients living in developing countries [2].

In addition to being two independent major global health problems of infectious and non-infectious nature, there is a strong association between TB and diabetes [3]. Patients with diabetes are more susceptible to bacterial infections, including pulmonary TB, and both types 1 and 2 diabetes are linked to TB [3]. The prevalence of TB is two to five times higher in diabetic patients than in non-diabetic patients and the disease is more aggressive in poorly controlled diabetes [4,5,6]. In diabetic patients, pulmonary TB may progress rapidly and thus a high index of suspicion is required [7]. Before the discovery of insulin a diagnosis of diabetes was a death sentence within 5 years, and the usual cause of that death was TB [3]. Although the frequent association of diabetes mellitus and TB has been observed for more than 2000

 © 2009 British Society for Immunology,

years, the reasons for this association are largely unknown [3].

It is now well established that cellular immune responses mediated by T cells and macrophages play the major role in defence against TB [8]. The few studies that investigated the cellular immunity in diabetic TB patients have shown that, compared to non-diabetic TB patients, diabetic TB patients had depressed cellular immunity as evidenced by a decrease in the number of T cells and their ability to proliferate *in vitro* in response to antigenic stimuli using a T cell transformation test as indicator [9]. These immunological parameters became normal following successful therapies [9]. In addition, macrophage functions including bacterial phagocytic capacity, resistance to tubercle bacilli and T cell function in delayed type hypersensitivity, were also depressed in mice with experimentally induced diabetes [10]. These studies suggest that the functions of both T cells and macrophages are compromised in diabetes.

Resistance to mycobacterial infections is mediated largely by T helper type 1 (Th1) cells and their cytokines, whereas Th2 cells and their cytokines correlate with disease susceptibility and pathology in TB [11–14]. In particular, the Th1 cytokine interferon (IFN)-γ is considered a principal mediator of protective immunity against TB [11,13]. Individuals with mutations in IFN-γ receptors or IFN-γ intracellular pathway components have experienced fatal disseminated mycobacterial infections during childhood [15], and inclusion of aerosolized IFN-γ in the treatment regimen of multi-drug-resistant pulmonary TB patients cured the disease [16]. In addition, the secretion of IFN-γ in response to *M. tuberculosis*-specific region of difference (RD)1 antigens has been shown to have diagnostic significance [8,17–19]. Moreover, Th1 cells also produce tumour necrosis factor (TNF)-β (lymphotoxin-alpha), a cytokine that is essential for the control of pulmonary TB [20]. TNF-β has a critical role in the activation of T cells and macrophages and local organization of the granulomatous response. On the other hand, the Th2 responses, characterized by the secretion of interleukin (IL)-4, IL-5 and IL-10, are associated with the lack of protection in TB [12,13]. In particular, IL-10 is associated with reduced resistance and chronic progressive TB [14]. Furthermore, IL-10 deactivates macrophages and down-regulates the secretion of Th1 cytokines [14], and some human populations exhibit increased expression of IL-10, which correlates with ineffective bacilli Calmette–Guérin (BCG) vaccination [21]. In addition, *in vitro* secretion of IL-4 in response to *M. tuberculosis* by $CD8^+$ and γ/δ T cells of health care workers was associated with the development of progressive TB within 2–4 years [22].

In relation to diabetes mellitus, impaired host resistance against *M. tuberculosis* infection in diabetic mice is associated with reduced production of Th1-related cytokines IL-12 and IFN-γ and impaired development of Th1 cells [23]. In humans, conflicting reports have appeared, which suggest no difference [24], and lower [4] or higher production [25] of IFN-γ by diabetic than non-diabetic TB patients. However, to our knowledge, a systematic analysis of cell-mediated immunity (CMI) responses in diabetic TB patients, and in particular the profile of Th1 and Th2 cytokines in response to *M. tuberculosis*-specific RD antigens, is lacking in the published literature.

In this study, to explore further the immunological mechanism of reduced resistance to TB in diabetic patients, we have studied CMI parameters that included *in vitro* cell proliferation and secretion of a battery of Th1 (IFN-γ, IL-2, TNF-β), Th2 (IL-4, IL-5, IL-10) cytokines by peripheral blood mononuclear cells (PBMC) obtained from diabetic and non-diabetic pulmonary TB patients and *M. bovis* BCG-vaccinated healthy subjects. PBMC were cultured in the absence and presence of complex mycobacterial antigens (whole-cell *M. bovis* BCG and whole cells, culture filtrate and cell walls of *M. tuberculosis*), and pools of synthetic peptides corresponding to *M. tuberculosis*-specific open reading frames (ORFs) of RD1, RD4, RD6 and RD10 that are deleted in all vaccine strains of *M. bovis* BCG [26,27]. The CMI responses were determined by evaluating antigen-induced cell proliferation and the secretion of Th1 (IFN-γ, IL-2, TNF-β), Th2 (IL-4, IL-5, IL-10) cytokines and the responses were compared among the three groups of donors.

Materials and methods

Donor groups

Heparinized venous blood was collected from newly diagnosed and culture-confirmed non-diabetic pulmonary TB patients ($n = 18$) and those suffering from pulmonary TB and type II diabetes ($n = 11$) attending the Chest Diseases Hospital, Kuwait. In addition, buffy coats were obtained from *M. bovis* BCG-vaccinated and purified protein derivative (PPD)-positive healthy subjects ($n = 20$) donating blood at the Central Blood Bank, Kuwait. All the patients and healthy subjects were residing in Kuwait at the time of the study. The patients were diagnosed on the basis of clinical and radiological features and positive cultures for *M. tuberculosis*. TB patients were diagnosed for having diabetes mellitus based on two blood glucose measurements in accordance with World Health Organization (WHO) criteria. The diabetic patients had uncontrolled diabetes for a period of 1–10 years. In addition, quality of glycaemic control was assessed by measuring haemoglobin A1c (HbA1c) levels in the peripheral blood. Demographic data of the three study groups are detailed in Table 1. The groups of healthy donors, non-diabetic TB patients and diabetic TB patients were all serologically negative for HIV infection. Informed consent was obtained from all the subjects and the study was approved by the Ethical Committee of the Faculty of Medicine, Kuwait University, Kuwait.

Table 1. Demographic and clinical data of non-diabetic tuberculosis (TB) patients, diabetic TB patients and bacille Calmette–Guérin (BCG)-vaccinated healthy subjects.

Parameter	Diabetic TB patients	Non-diabetic TB patients	BCG-vaccinated healthy subjects
Ethnicity [no. (%) of patients]			
Kuwaiti	0 (0·0)	0 (0·0)	2 (10)
Indian subcontinent	6 (54·5)	8 (44·4)	7 (35)
Asian	5 (45·5)	10 (55·6)	11 (55)
Age range (years)	20–57	28–57	20–47
Sex (male : female)	10:1	17:1	18:2

Complex antigens and synthetic peptides of *M. tuberculosis*

The complex mycobacterial antigens used in this study were irradiated whole cell *M. tuberculosis* H37Rv and *M. bovis* BCG [28], and *M. tuberculosis* culture filtrate (MT-CF) and cell walls (MT-CW) (provided by J. T. Belisle, Fort Collins, CO, USA and produced under NIH contract HHSN266200400091C/ADB contract NO-AI40092, 'Tuberculosis Vaccine Testing and Research Materials Contract'). A total of 607 peptides (25-mers overlapping with neighbouring peptides by 10 aa) spanning the sequence of putative proteins encoded by genes in RD1, RD4, RD6 and RD10 genomic regions were designed based on the amino acid sequence deduced from the nucleotide sequence of the respective genes (Table 2). These peptides were synthesized commercially by Thermo Hybaid GmBH (Ulm, Germany) using fluonerylmethoxycarbonyl chemistry, as described previously [29]. The stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0·9%) by vigorous pipetting, and the working concentrations were prepared by further dilution in tissue culture medium RPMI-1640, as described previously [30].

Isolation of PBMC and antigenic stimulation

PBMC were isolated from blood of TB patients and healthy subjects by flotation on Lymphoprep gradients using standard procedures [28,29]. The cells were finally suspended in complete tissue culture medium [RPMI-1640 + 10% human AB serum + penicillin (100 U/ml) + streptomycin (100 μg/ml) + gentamycin (40 μg/ml) + fungizone (2·5 μg/ml)] and counted in a Coulter Counter (Coulter Electronics Ltd, Luton, UK).

Antigenic stimulation of PBMC was performed according to standard procedures [28,31]. In brief, 2×10^5 PBMC suspended in 50 μl were seeded into the wells of 96-well tissue culture plates (Nunc, Roskilde, Denmark) and stimulated with complex mycobacterial antigens and peptide pools of RD1, RD4, RD6 and RD10, as described previously [31,32]. The cells in the control wells were cultured in the absence of exogenously added mycobacterial antigens. The final volume of the culture wells was adjusted to 200 μl. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. On day 6, culture supernatants (100 μl) were collected from each well and frozen at −70°C until used to determine cytokine concentrations. The remaining cultures were pulsed with 1 μci [^{3}H]-thymidine (Amersham Life Science, Amersham, UK) and harvested (Skatron Instruments As, Oslo, Norway) according to standard procedures [28,29].

Interpretation of proliferation results

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 presented as stimulation index (SI), which is defined as follows: Sl = cpm in antigen or peptide-stimulated cultures/cpm in cultures lacking antigen or peptide. A patient was considered

Table 2. Synthetic peptides corresponding to open reading frames (ORFs) predicted in the regions of difference (RDs) present in *M. tuberculosis* H37Rv but absent in all strains of *Mycobacterium bovis* bacille Calmette–Guérin (BCG).

Region deleted*	ORF designation†	No. of ORFs	No. of synthetic peptides
RD1	ORF2-ORF11, ORF14, ORF15	12	220
RD4	Rv0221-Rv0223c	3	80
RD6	Rv1506c-Rv1516c	11	236
RD10	Rv1255c-Rv1257c	3	71
Total		29	607

*RD designations are according to Behr *et al.* [27]. †ORFs designations for RD1 are according to Amoudy *et al.* [26], whereas for other RDs, the ORF designations are according to Behr *et al.* [27].

 © 2009 British Society for Immunology,

responder to a given antigen if the PBMC yielded SI ≥ 3 [28]. Based on the median SI and percentages of responders in antigen-induced proliferation assays, the antigens were considered strong (median SI > 10 with > 70% responders), moderate (median SI > 3·0 but < 10 with 50–70% responders) or weak (median SI < 3·0 with < 50% responders) stimulators of PBMC.

Immunoassays for the quantification of cytokines

Supernatants (0·1 ml) from each well of 96-well plates were collected from the cultures of PBMC before [^{3}H]-thymidine pulse. The supernatants were kept frozen at –20°C until assayed for cytokine concentration using FlowCytomix kits (Bender Medsystems Inc., Vienna, Austria), which allows for the simultaneous quantification of several analytes in one sample using a low sample volume (25 μl), as described previously [31]. The cytokines tested included Th1 (IFN-γ, IL-2, TNF-β) and Th2 (IL-4, IL-5, IL-10) cytokines. The test samples were analysed by flow cytometry using Coulter EPICS FC500 (Beckman Coulter Inc., Fullerton, CA, USA). For each analysis, up to 10 000 events were acquired. The mean concentration of each cytokine was expressed as pg/ml. By using the above kits, the minimum detectable concentrations of IL-2, IL-4, IL-5, IL-10, TNF-β and IFN-γ were 8·9 pg/ml, 6·4 pg/ml, 5·3 pg/ml, 6·9 pg/ml, 3·2 pg/ml and 7·0 pg/ml, respectively. In response to antigenic stimuli, experimental/control (E/C) values above 3 were considered a positive response [11]. E/C is the cytokine concentration in the supernatants of PBMC cultured in the presence of *M. tuberculosis* antigens/cytokine concentration in the supernatants of PBMC cultured in the absence of *M. tuberculosis* antigens. The antigen-induced positive responses were considered weak, moderate and strong when E/C-values were > 3–5, > 5–10 and > 10, respectively [31].

Statistical analysis

Statistical analyses were performed using SPSS 16·0 for Windows software package. The results of cytokine secretion were analysed statistically for significant differences between cultures of patients with TB and diabetes, patients with TB alone and healthy subjects using the non-parametric Mann–Whitney *U*-test. *P*-values of < 0·05 were considered significant.

Results

Antigen-induced proliferation of PBMC in response to complex mycobacterial antigens and RD peptides

All complex mycobacterial antigens were strong stimulators of PBMC proliferation, as indicated by high median SI in diabetic TB patients, non-diabetic TB patients and healthy subjects (median SI = 23–45, 13·5–76·5 and 15·6–29, respectively) and high percentage of responders (82–100%, 88–100% and 76–100%, respectively) (Table 3). In response to RD peptides, the proliferation of PBMC from diabetic TB and non-diabetic TB patients was strong in response to $RD1_{pool}$ (median SI of 13 and 16·5, respectively, and 100% responders) whereas, in healthy subjects, $RD1_{pool}$ induced weak responses (median SI of 4·5 and 57% responders) (Table 3). All other RD peptides ($RD4_{pool}$, $RD6_{pool}$ and $RD10_{pool}$) induced weak proliferation responses in all of the tested groups (Table 3). The statistical analysis showed that none of the complex mycobacterial antigens induced proliferation responses that were significantly different in TB patients than in healthy subjects ($P > 0·05$), whereas $RD1_{pool}$ induced significantly higher responses in both the diabetic and non-diabetic TB patients than in healthy subjects ($P < 0·05$). Furthermore, RD1-induced proliferation responses were significantly higher than the responses

Table 3. Antigen-induced proliferation of peripheral blood mononuclear cells of diabetic tuberculosis (TB) patients, non-diabetic TB patients and healthy subjects in response to complex mycobacterial antigens and peptide pools of regions of difference (RD1), RD4, RD6 and RD10.

	Diabetic TB patients		Non-diabeticTB patients		Healthy subjects	
Antigen	SI*	P/T†(%)	SI	P/T (%)	SI	P/T (%)
M. bovis BCG	**45** (< 1–280)	10/11 (91)	**32** (< 1–672)	15/17 (88)	**23** (< 1–64)	17/20 (85)
M. tuberculosis	**23** (< 1–346)	9/11 (82)	**13·5** (1–464)	17/18 (94)	**15·6** (4–91)	20/20 (100)
MT-CF	**32** (8–281)	7/7 (100)	**76·5** (10–505)	13/13 (100)	**29** (3–328)	7/7 (100)
MT-CW	**38** (3–342)	11/11 (100)	**39** (4–3233)	18/18 (100)	**20** (< 1–179)§	13/17 (76)
$RD1_{pool}$	**13** (6–78)	7/7 (100)	**16·5** (7–250)	13/13 (100)	**4·5** (< 1–29)‡,§	4/7 (57)
$RD4_{pool}$	1·7 (1–8·5)	2/7 (28·5)	2·3 (1–8)	4/12 (33)	2 (< 1–27)	1/7 (14)
$RD6_{pool}$	1 (< 1–7)	1/7 (14)	1·2 (< 1–8)	2/12 (17)	1 (< 1–2)	0/7 (0)
$RD10_{pool}$	1 (< 1–3)	1/7 (14)	1 (< 1–5)	3/12 (25)	1 (< 1–7)	1/7 (14)

*Stimulation index (SI) is presented as median (range) and is significant if ≥ 3 (shown in bold type). †P/T: positive responders per total tested (% = percentage of positive responders). ‡$P < 0·05$ when diabetic TB compared with healthy subjects; §$P < 0·05$ when non-diabetic TB compared with healthy subjects. *M. bovis*, *Mycobacterium bovis*; BCG, bacille Calmette–Guérin; MT-CF, *Mycobacterium tuberculosis* culture filtrate; MT-CW, *M. tuberculosis* cell walls.

induced by $RD4_{pool}$, $RD6_{pool}$ and $RD10_{pool}$ in both the diabetic and non-diabetic TB patients ($P < 0·05$), but not the healthy subjects ($P > 0·05$) (Table 3).

Cytokine secretion by PBMC in the absence of mycobacterial antigens

Only PBMC of diabetic TB patients demonstrated spontaneous secretion of detectable levels of all the tested Th1, except for IL-2 (median concentrations: IFN-γ = 48, TNF-β = 35 and IL-2 < 8·9 pg/ml), and Th2 (median concentrations: IL-4 = 82, IL-5 = 95 and IL-10 = 29 pg/ml) cytokines (data not shown). However, compared to the other two groups, PBMC of diabetic TB patients produced significantly higher concentrations of IL-4 alone ($P < 0·05$) (data not shown).

Th1/Th2 cytokine secretion by PBMC stimulated with complex mycobacterial antigens and RD peptides

PBMC from all groups of subjects showed strong (E/C > 10) secretion of Th1 cytokine IFN-γ in response to all the complex mycobacterial antigens with comparable concentrations in diabetic and non-diabetic TB patients ($P > 0·05$) (Table 4). In response to RD peptides, only $RD1_{pool}$ induced strong IFN-γ secretion by all the tested groups but the diabetic TB patients secreted significantly more IFN-γ than non-diabetic TB patients and healthy subjects ($P < 0·05$) (Table 4). However, the peptide pools of the other RDs induced significantly higher IFN-γ by PBMC of diabetic TB patients than non-diabetic TB patients and healthy subjects ($P < 0·05$) (Table 4). Statistical analysis of the data showed that higher levels of IFN-γ were induced by the $RD1_{pool}$ compared to the peptide pools of other RDs in both diabetic and non-diabetic TB patients ($P < 0·05$) (Table 4).

The results of quantification of other Th1 cytokines in the culture supernatants showed that none of the antigens induced secretion of IL-2 in any group of subjects tested (data not shown), whereas all the complex mycobacterial antigens induced the secretion of TNF-β (E/C ≥ 3) by PBMC of all the groups, except for whole cells of *M. bovis* BCG and *M. tuberculosis* in the diabetic TB patients (Table 5). The median concentrations of TNF-β in response to complex mycobacterial antigens in the tested groups of subjects were comparable ($P > 0·05$) (Table 5), except for *M. bovis* BCG, which induced significantly less TNF-β in PBMC cultures of healthy subjects compared to the diabetic patients (median concentration $n = 21$ pg/ml and 90 pg/ml, respectively). However, the non-diabetic TB patients and healthy subjects showed higher values of E/C (9·4–20 and 4·6–21·5, respectively) than the diabetic TB patients (E/C = 1·5–6·4). Among the RD peptides, only $RD1_{pool}$ induced weak secretion of TNF-β by PBMC of diabetic and non-diabetic TB patients (E/C = 3·4, 4·4, respectively) but, as expected, none of the RD peptide pools induced positive responses in BCG-vaccinated healthy subjects (E/C < 3) (Table 5).

With respect to Th2 cytokines, none of the complex mycobacterial antigens and RD peptide pools induced the secretion of IL-4 and IL-5 by PBMC of all tested groups (E/C < 3, data not shown), whereas IL-10 was secreted in response to all complex mycobacterial antigens in the three groups of subjects, except MT-CF which did not induce IL-10 secretion in healthy subjects (E/C = 1·4) (Table 6). Statistical analysis of the data showed that in response to *M. bovis* BCG, PBMC of diabetic TB patients secreted significantly higher concentrations of IL-10 than the non-diabetic TB patients and healthy subjects ($P < 0·05$) (Table 6). In addition, the diabetic TB patients secreted significantly higher concentrations of IL-10 than the healthy subjects in response to whole cells of *M. tuberculosis* and MT-CW ($P < 0·05$) (Table 5). In

Table 4. Interferon (IFN)-γ secretion by peripheral blood mononuclear cells (PBMC) of diabetic tuberculosis (TB) patients, non-diabetic TB patients and healthy subjects in response to complex mycobacterial antigens and peptide pools of regions of difference (RD1), RD4, RD6 and RD10.

	Concentration of IFN-γ (pg/ml) in PBMC culture supernatants of					
	Diabetic TB patients		Non-diabetic TB patients		Healthy subjects	
Antigen	Median (range)	E/C*	Median (range)	E/C	Median (range)	E/C
No antigen	48·5 (≤ 7–434)		≤ 7 (≤ 7–434)		12 (≤ 7–334)	
M. bovis BCG	2248 (273–10408)	**46**	2157 (38–10507)	**308**	787 (≤ 7–18279)‡	**65·5**
M. tuberculosis	1997 (253–7439)	**41**	1479 (174–14188)	**211**	972 (29–10914)	**81**
MT-CF	2275 (1143–19322)	**47**	2692 (836–13421)	**384**	3990 (202–15945)	**332**
MT-CW	2474 (1672–6467)	**51**	2277 (343–14785)	**325**	1868 (170–9112)	**156**
$RD1_{pool}$	1789 (251–11194)	**37**	405 (≤ 7–10507·3)†	**58**	164 (≤ 7–15870)‡	**13·6**
$RD4_{pool}$	368 (23–790)	**7·5**	5·5 (≤ 7–562)†	0·7	12·6 (≤ 7–2270)‡	1
$RD6_{pool}$	233 (64–1276)	**4·7**	7·6 (≤ 7–1254)†	1	61 (≤ 7–3699)‡	**5**
$RD10_{pool}$	23 (≤ 7–386)	0·4	≤ 7 (≤ 7–160)†	1	14·5 (≤ 7–229)‡	1·1

*E/C: cytokine concentration in antigen stimulated cultures/cytokine concentration in cultures lacking antigen, and is considered positive if ≥ 3 (shown in bold type). †$P < 0·05$ when diabetic TB compared with non-diabetic TB patients; ‡$P < 0·05$ when diabetic TB compared with healthy subjects. BCG, bacille Calmette–Guérin; MT-CF, *Mycobacterium tuberculosis* culture filtrate; MT-CW, *M. tuberculosis* cell walls.

© 2009 British Society for Immunology, *Clinical and Experimental Immunology*, **158:** 64–73

Table 5. Tumour necrosis factor (TNF)-β secretion by peripheral blood mononuclear cells (PBMC) of diabetic patients, non-diabetic tuberculosis (TB) patients and healthy subjects in response to complex mycobacterial antigens and peptide pools of regions of difference (RD1), RD4, RD6 and RD10.

	Concentration of TNF-β (pg/ml) in PBMC culture supernatants of					
	Diabetic TB patients		Non-diabetic TB patients		Healthy subjects	
Antigen	Median (range)	E/C*	Median (range)	E/C	Median (range)	E/C
No antigen	35 (≤ 3·2–70 283)		≤ 3·2 (≤ 3·2–153)		≤ 3·2 (≤ 3·2–147)	
M. bovis BCG	90 (≤ 3·2–138 445)	2·5	30 (≤ 3·2–221)	**9·4**	21 (≤ 3·2–450)	**6·6**
M. tuberculosis	54 (≤ 3·2–68 537)	1·5	33 (4–238)	**10·4**	15 (≤ 3·2–343)	**4·6**
MT-CF	225 (≤ 3·2–212 980)	**6·4**	64 (8–647)	**20**	69 (≤ 3·2–151)	**21·5**
MT-CW	140 (≤ 3·2–124 181)	**4**	59 (≤ 3·2–283)	**18·4**	31 (≤ 3·2–398)	**9·7**
$RD1_{pool}$	120 (≤ 3·2–141 116)	**3·4**	14 (≤ 3·2–225)	**4·4**	≤ 3·2 (≤ 3·2–398)§	1
$RD4_{pool}$	12 (≤ 3·2–47 277)	0·3	≤ 3·2 (≤ 3·2–38)	1	≤ 3·2 (≤ 3·2–41)‡	1
$RD6_{pool}$	37 (≤ 3·2–55 723)	1	≤ 3·2 (≤ 3·2)†	1	≤ 3·2 (≤ 3·2–15)	1
$RD10_{pool}$	≤ 3·2 (≤ 3·2–43 632)	0·1	≤ 3·2 (≤ 3·2–17)	1	≤ 3·2 (≤ 3·2–5)	1

*E/C: cytokine concentration in antigen stimulated cultures/cytokine concentration in cultures lacking antigen, and is considered positive if ≥ 3 (shown in bold type). †$P < 0·05$ when diabetic TB compared with non-diabetic TB patients; ‡$P < 0·05$ when diabetic TB compared with healthy subjects; §$P < 0·05$ when non-diabetic TB compared with healthy subjects. BCG, bacille Calmette–Guérin; MT-CF, *Mycobacterium tuberculosis* culture filtrate; MT-CW, *M. tuberculosis* cell walls.

response to RD peptide pools, $RD1_{pool}$, $RD4_{pool}$ and $RD10_{pool}$ induced secretion of IL-10 by PBMC of diabetic patients only (E/C = 7, 4, 5·6, respectively), and the levels of IL-10 were significantly higher in all RD peptide pools in diabetic TB patients than in non-diabetic TB patients and healthy subjects ($P < 0·05$) (Table 6).

Th1 : Th2 cytokine ratios

To determine Th1 *versus* Th2 cytokine bias in response to various mycobacterial antigens in the tested groups of subjects, Th1 : Th2 cytokine ratios were calculated and compared between groups (Table 7). The results showed that IFN-γ : IL-4 ratios to all complex mycobacterial antigens and $RD1_{pool}$ were lower in diabetic TB patients compared to non-diabetic TB patient and healthy subjects (Table 7). A similar observation was made regarding IFN-γ : IL-5 and IFN-γ : IL-10 ratios (Table 7). Furthermore, and in general, ratios of TNF-β : IL-4, TNF-β : IL-5 and TNF-β : IL-10 to various antigens were also lower in the diabetic TB patients compared to the other two groups (Table 7). A similar analysis for $RD4_{pool}$, $RD6_{pool}$ and $RD10_{pool}$ was not possible because they were weak stimulators of Th1 and Th2 cytokines, as reported above. The overall analysis showed that Th1 : Th2 cytokine ratios were consistently lower in diabetic TB patients than non-diabetic TB patients and healthy subjects (Table 7), thus suggesting a Th2 cytokine bias in diabetic TB patients.

Discussion

To understand the immunological mechanisms involved in increased pathogenesis of TB in diabetic patients, we have analysed CMI responses using PBMC from pulmonary TB

Table 6. Interleukin (IL)-10 secretion by peripheral blood mononuclear cells (PBMC) of diabetic patients, non-diabetic tuberculosis (TB) patients and healthy subjects in response to complex mycobacterial antigens and peptide pools of regions of difference (RD1), RD4, RD6 and RD10.

	Concentration of IL-10 (pg/ml) in PBMC culture supernatants of					
	Diabetic TB patients		Non-diabetic TB patients		Healthy subjects	
Antigen	Median (range)	E/C*	Median (range)	E/C	Median (range)	E/C
No antigen	29 (≤ 6·9–837)		≤ 6·9 (≤ 6·9–666)		≤ 6·9 (≤ 6·9–297)	
M. bovis BCG	601 (162–1740)	**20·4**	169 (≤ 6·9–2427)†	**24·4**	38 (≤ 6·9–1109)‡	**5·5**
M. tuberculosis	974 (250–6536)	**33**	221 (≤ 6·9–4934)	**32**	330 (≤ 6·9–2186)‡	**47·8**
MT-CF	157 (≤ 6·9–1063)	**5·3**	81 (≤ 6·9–430)	**12**	10 (≤ 6·9–273)	1·4
MT-CW	747 (54–10 949)	**25·3**	104 (≤ 6·9–3390)	**15**	25 (≤ 6·9–2389)‡	**3·6**
$RD1_{pool}$	207 (13–519)	**7**	≤ 6·9 (≤ 6·9–329)†	1	≤ 6·9 (≤ 6·9–198)‡	1
$RD4_{pool}$	120 (14–352)	**4**	≤ 6·9 (≤ 6·9–20)†	1	≤ 6·9 (≤ 6·9–166)‡	1
$RD6_{pool}$	67 (≤ 6·9–1222)	2·2	≤ 6·9 (≤ 6·9–21)†	1	≤ 6·9 (≤ 6·9–62)‡	1
$RD10_{pool}$	167 (≤ 6·9–561)	**5·6**	≤ 6·9 (≤ 6·9–14)†	1	≤ 6·9 (≤ 6·9–116)‡	1

*E/C: cytokine concentration in antigen stimulated cultures/cytokine concentration in cultures lacking antigen, and is considered positive if ≥ 3 (shown in bold type). †$P < 0·05$ when diabetic TB compared with non-diabetic TB patients; ‡$P < 0·05$ when diabetic TB compared with healthy subjects. BCG, bacille Calmette–Guérin; MT-CF, *Mycobacterium tuberculosis* culture filtrate; MT-CW, *M. tuberculosis* cell walls.

Table 7. T helper type 1 (Th1) : Th2 type cytokine ratios in culture supernatants of peripheral blood mononuclear cells (PBMC) obtained from diabetic and non-diabetic tuberculosis (TB) patients and *Mycobacterium bovis* bacille Calmette–Guérin (BCG)-vaccinated healthy subjects in the presence of complex mycobacterial antigens and region of difference (RD)1_{pool}.

Antigen Th1 : Th2 cytokine	Cytokine ratios in culture supernatants of Diabetic TB patients	Non-diabetic TB patients	Healthy subjects
M. bovis BCG			
IFN-γ : IL-4	31	337	123
IFN-γ : IL-5	16·7	240	148
IFN-γ : IL-10	3·7	13	21
TNF-β : IL-4	1·3	5	3·3
TNF-β : IL-5	0·7	3·3	4
TNF-β : IL-10	0·2	0·2	0·6
M. tuberculosis			
IFN-γ : IL-4	27	231	152
IFN-γ : IL-5	10·5	134	183†
IFN-γ : IL-10	2	6·6	3
TNF-β : IL-4	0·7	5	2·3
TNF-β : IL-5	0·3	3	2·8
TNF-β : IL-10	0·1	0·2	0·1
MT-CF			
IFN-γ : IL-4	103	420*	623†
IFN-γ : IL-5	30	448*	752†
IFN-γ : IL-10	14·5	33	399†
TNF-β : IL-4	10	10	11
TNF-β : IL-5	3	11	13
TNF-β : IL-10	1·4	0·8	7
MT-CW			
IFN-γ : IL-4	28	355	291
IFN-γ : IL-5	12	189*	352†
IFN-γ : IL-10	3·3	22	74
TNF-β : IL-4	1·6	9	5
TNF-β : IL-5	0·7	5	6
TNF-β : IL-10	0·2	0·6	1·2
$RD1_{pool}$			
IFN-γ : IL-4	17	63	25·6
IFN-γ : IL-5	15·3	58	31
IFN-γ : IL-10	8·6	58*	23‡
TNF-β : IL-4	1·1	2·2	0·5
TNF-β : IL-5	1	2	0·6
TNF-β : IL-10	0·6	2	0·5

**P* < 0·05 when diabetic TB compared with non-diabetic TB patients; †*P* < 0·05 when diabetic TB compared with healthy subjects. ‡*P* < 0·05 when non-diabetic TB compared with healthy subjects. IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; MT-CF, *Mycobacterium tuberculosis* culture filtrate; MT-CW, *M. tuberculosis* cell walls.

patients with and without type II diabetes and *M. bovis* BCG-vaccinated healthy subjects. The assays used have been shown previously to correlate with protective immunity (antigen-induced cell proliferation and secretion of Th1 cytokines IL-2, IFN-γ and TNF-β) and susceptibility/pathogenesis in TB (secretion of Th2 cytokines IL-4, IL-5 and IL-10) [11–14]. A number of complex mycobacterial antigen preparations and peptides corresponding to *M. tuberculosis*-specific regions RD1, RD4, RD6 and RD10 were used, because they may induce immune responses that vary in type and magnitude based on antigenic differences [33]. To study antigen-induced cellular responses the cultures were harvested on day 6, because previous studies have shown that this time period is optimal for mycobacterial antigen-induced cellular proliferation and cytokine responses, e.g. IFN-γ, of human PBMC [31,34].

The results of antigen-induced cell proliferation studies showed that all the complex mycobacterial antigen preparations were strong stimulators of PBMC of diabetic and non-diabetic TB patients and *M. bovis* BCG-vaccinated healthy subjects (Table 3). These findings are in agreement with our recent study using various preparations of complex antigens of *M. tuberculosis* and defined secreted antigens of cross-reactive nature, i.e. MPT59 and MPT64 [33]. However, among RD peptides, only $RD1_{pool}$ induced strong responses in diabetic and non-diabetic TB patients and moderate responses in *M. bovis* BCG-vaccinated healthy subjects (Table 3). These results suggest that diabetic and non-diabetic TB patients respond in a similar way to both cross-reactive and specific antigens of *M. tuberculosis*, and only RD1 contains immunodominant antigens of *M. tuberculosis*. By using one of the antigens constituting the RD1 peptide pool, i.e. early secreted antigenic target 6 (ESAT6), we have shown previously that ESAT6 induces equally strong responses in diabetic and non-diabetic TB patients but weak responses in *M. bovis* BCG-vaccinated healthy subjects [33], due most probably to latent TB [11,28,31,36,37]. The moderate responses observed to $RD1_{pool}$ in this study in *M. bovis* BCG-vaccinated healthy subjects could also have been due to the presence of Pro-Pro-Glu 68 (PPE68), because the sequence of the immunodominant peptide of PPE68 is also present in PPE-family proteins of *M. bovis* BCG [32,35]. To study the profile of Th1 and Th2 cytokines, we first determined their spontaneous *ex vivo* secretion by PBMC, which implies prior *in vivo* stimulation, and thus represents cell-specific activity in the context of current infection [38,39]. The results showed that PBMC of only diabetic TB patients spontaneously secreted detectable concentrations of Th1 (IFN-γ and TNF-β) and Th2 cytokines (IL-4, IL-5 and IL-10), but the concentration of IL-4 alone was significantly higher in diabetic TB patients compared to non-diabetic TB patients and healthy subjects. The elevated IL-4 secretion may contribute to increased pathogenesis in diabetic TB patients because IL-4 impairs anti-microbial activity of infected cells, increases availability of iron to intracellular *M. tuberculosis* and drives pulmonary fibrosis, etc. [22].

The present work also included studies to assess mycobacterial antigen-induced secretion of Th1 and Th2 cytokines by PBMC of various groups of subjects, which may be useful as early predictors of disease activity and contribute to the understanding of the disease pathogenesis [11–14]. The

results showed that among the Th1 cytokines studied, large quantities of IFN-γ were secreted by PBMC of all groups tested in response to complex mycobacterial antigens, without statistical differences between the groups, except for significantly higher levels of IFN-γ produced by diabetic patients, compared to healthy subjects, in response to *M. bovis* BCG (Table 4). In response to RD peptides, i.e. $RD1_{pool}$, $RD4_{pool}$ and $RD6_{pool}$, significantly higher concentrations of IFN-γ were secreted by PBMC of diabetic TB patients compared to non-diabetic TB patients and *M. bovis* BCG-vaccinated healthy subjects ($P < 0{\cdot}05$). However, strong responses ($E/C \geq 10$) were observed with RD1 only.

TNF-β was secreted by all groups in response to the complex mycobacterial antigens and/or $RD1_{pool}$ (Table 5). However, in spite of positive antigen-induced proliferation responses to complex mycobacterial antigens and $RD1_{pool}$, IL-2 was not detected in any of the antigen-stimulated cultures (data not shown). This could have been due to utilization of IL-2 by T cells proliferating in response to antigenic stimuli [40]. Thus, the overall results suggest that the increased pathogenesis observed in diabetic patients may not be due to decreased mycobacterial antigen-induced secretion of Th1 cytokines in these patients.

Among the Th2 cytokines, mycobacterial antigen-induced secretions of IL-4 and IL-5 were not observed in any group of subjects included in this study (data not shown), thus supporting previous observations reported for non-diabetic TB patients and healthy subjects [11,31,41]. However, in response to the complex mycobacterial antigens and/or RD peptide pools, significantly higher concentrations of IL-10 were secreted by PBMC of diabetic TB patients than non-diabetic TB patients and healthy subjects (Table 6). Despite the abundant production of Th1 cytokines, secretion of IL-10 interferes with their protective function [42], and helps mycobacteria to survive intracellularly [43]. Therefore, our results suggest that, in spite of large quantities of IFN-γ secretion in response to mycobacterial antigens, the elevated secretion of IL-10 may contribute to increased pathogenesis in diabetic TB patients.

Because Th1 and Th2 cytokines regulate the secretion of each other, their net biological effect can be evaluated more clearly by comparing the cytokine ratios [44–46]. A higher Th1/Th2 ratio indicates a protective type 1 cytokine bias, while a lower ratio suggests a bias towards pathological type 2 cytokines in TB [44–50]. In this study, we found that the overall Th1/Th2 cytokine ratios (IFN-γ : IL-4, IFN-γ : IL-5 and IFN-γ : IL-10; and TNF-β : IL-4, TNF-β : IL-5 and TNF-β : IL-10) were lower in the diabetic TB patients than non-diabetic TB patients and healthy subjects (Table 7). The lower Th1 : Th2 ratios may also contribute to hampered resistance to *M. tuberculosis* infection under diabetic conditions. This study is restricted to selected Th1 and Th2 cytokines; however, it should be stressed that the failure to eradicate *M. tuberculosis* probably involves sophisticated immune evasion strategies of the pathogen as well as the immune regulatory mechanisms of the host [51]. In the context of regulatory mechanisms, several subsets of regulatory T cells (T_{reg}) have been identified such as IL-10 (Tr1) or TGF-β-secreting T_{reg} (Th3) and $CD4^+CD25^+$ forkhead box P3 $(FoxP3)^+$ T_{reg} cells [22,51,52]. It has been shown that $CD4^+CD25^+FoxP3^+$ T_{reg} cells capable of suppressing Th1 responses are present in high frequency in TB patients [52]. Furthermore, previous studies have shown a correlation of IL-4 antagonist IL-4δ2 with protective immunity in TB by demonstrating high levels of IL-4δ2 in healthy infected subjects compared to TB patients and increase in the levels of IL-4δ2 after chemotherapy of TB patients [53–55]. Therefore, in future, studies may be carried out to determine the role of T_{reg} cells and IL-4δ2 in understanding the immunological mechanisms responsible for the increased pathogenesis of TB in diabetic patients.

Acknowledgements

The work was supported by the Kuwait Foundation for the Advancement of Sciences (KFAS) grant no. 2002-1302-04. The supply of blood from diabetic and non-diabetic TB patients from the Chest Diseases Hospital, and buffy coats from the Central Blood Bank, Kuwait are gratefully acknowledged.

Disclosure

None of the authors has any conflict of interest to declare.

References

1 Dye C. Global epidemiology of tuberculosis. Lancet 2006; **36**:938–40.

2 Boutayeb A. The double burden of communicable and non-communicable diseases in developing countries. Trans R Soc Trop Med Hyg 2006; **100**:191–9.

3 Broxmeyer L. Diabetes mellitus, tuberculosis and the mycobacteria: two millennia of enigma. Med Hypotheses 2005; **65**:433–9.

4 Tsukaguchi K, Okamura H, Matsuzawa K *et al.* Longitudinal assessment of IFN-gamma production in patients with pulmonary tuberculosis complicated with diabetes mellitus. Kekkaku 2002; **77**:409–13 [in Japanese].

5 Alisjahbana B, van Crevel R, Sahiratmadja E *et al.* Diabetes mellitus is strongly associated with tuberculosis in Indonesia. Int J Tuberc Lung Dis 2006; **10**:696–700.

6 Dyck RF, Klomp H, Marciniuk DD *et al.* The relationship between diabetes and tuberculosis in Saskatchewan: comparison of registered Indians and other Saskatchewan people. Can J Public Health 2007; **98**:55–9.

7 Swai AB, McLarty DG, Mugusi F. Tuberculosis in diabetic patients in Tanzania. Trop Doct 1990; **20**:147–50.

8 Mustafa AS. Recombinant and synthetic peptides to identify *Mycobacterium tuberculosis* antigens and epitopes of diagnostic and vaccine relevance. Tuberculosis (Edinb) 2005; **85**:367–76.

9 Karachunskii MA, Gerget Via, Iakovleva OB. Specific features of

cellular immunity of pulmonary tuberculosis in patients with diabetes mellitus. Probl Tuberk 1997; **6**:59–60 [in Russian].

10 Saiki O, Negoro S, Tsuyuguchi I, Yamamura Y. Depressed immunological defence mechanisms in mice with experimentally induced diabetes. Infect Immun 1980; **28**:127–31.

11 Al-Attiyah R, Madi N, El-Shamy AS, Wiker H, Andersen P, Mustafa AS. Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of *Mycobacterium tuberculosis*. FEMS Immunol Med Microbiol 2006; **47**:254–61.

12 Bai X, Wilson SE, Chmura K, Feldman NE, Chan ED. Morphometric analysis of Th(1) and Th(2) cytokine expression in human pulmonary tuberculosis. Tuberculosis 2004; **84**:375–85.

13 Flynn J. Immunology of tuberculosis and implications in vaccine development. Tuberculosis 2004; **4**:93–101.

14 Turner J, Gonzalez-Juarrero M, Ellis DL *et al. In vivo* IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice. J Immunol 2002; **169**:6343–51.

15 Ottenhoff TH, Verreck FA, Hoeve MA, van de Vosse E. Control of human host immunity to mycobacteria. Tuberculosis (Edinb) 2005; **85**:53–64.

16 Grahmann PR, Braun RK. A new protocol for multiple inhalation of IFN-gamma successfully treats MDR-TB: a case study. Int J Tuberc Lung Dis 2008; **12**:636–44.

17 Arend SM, Andersen P, van Meijgaarden KE *et al.* Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. J Infect Dis 2001; **181**:1850–4.

18 Ferrara G, Losi M, D'Amico R *et al.* Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. Lancet 2006; **367**:1328–34.

19 Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA, Lalvani A. Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. Lancet 2004; **364**:2196–203.

20 Roach DR, Briscoe H, Saunders B *et al.* Secreted lymphotoxin-alpha is essential for the control of an intracellular bacterial infection. J Exp Med 2001; **193**:239–46.

21 Weir RE, Black GF, Dockrell HM *et al.* Mycobacterial purified protein derivatives stimulate innate immunity: Malawians show enhanced tumor necrosis factor alpha, interleukin-1beta (IL-1beta), and IL-10 responses compared to those of adolescents in the United Kingdom. Infect Immun 2004; **72**:1807–11.

22 Rook GAW. Th2 cytokines in susceptibility to tuberculosis. Curr Mol Med 2007; **7**:327–37.

23 Yamashiro S, Kawakami K, Uezu K *et al.* Lower expression of Th1-related cytokines and inducible nitric oxide synthase in mice with streptozotocin-induced diabetes mellitus infected with *Mycobacterium tuberculosis*. Clin Exp Immunol 2005; **139**:57–64.

24 Stalenhoef JE, Alisjahbana B, Nelwan EJ *et al.* The role of interferon-gamma in the increased tuberculosis risk in type 2 diabetes mellitus. Eur J Clin Microbiol Infect Dis 2008; **27**:97–103.

25 Restrepo BI, Fisher-Hoch SP, Pino PA *et al.* Tuberculosis in poorly controlled type 2 diabetes: altered cytokine expression in peripheral white blood cells. Clin Infect Dis 2008; **47**:634–41.

26 Amoudy HA, Al-Turab MB, Mustafa AS. Identification of transcriptionally active open reading frames within the RD1 genomic segment of *Mycobacterium tuberculosis*. Med Princ Pract 2006; **15**:137–44.

27 Behr MA, Wilson MA, Gill WP *et al.* Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999; **284**:1520–3.

28 Al-Attiyah R, Shaban FA, Wiker HG, Oftung F, Mustafa AS. Synthetic peptides identify promiscuous human Th1 cell epitopes of the secreted mycobacterial antigen MPB70. Infect Immun 2003; **71**:1953–60.

29 Mustafa AS, Shaban FA. ProPred analysis and experimental evaluation of promiscuous T-cell epitopes of three major secreted antigens of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2006; **86**:115–24.

30 Hanif SNM, El-Shammy AM, Al-Attiyah R, Mustafa AS. Whole blood assays to identify Th1 cell antigens and peptides encoded by *Mycobacterium tuberculosis*-specific RD1 genes. Med Princ Pract 2008; **17**:244–9.

31 Al-Attiyah R, Mustafa AS. Characterization of human cellular immune responses to novel *Mycobacterium tuberculosis* antigens encoded by genomic regions absent in *Mycobacterium bovis* BCG. Infect Immun 2008; **76**:4190–8.

32 Mustafa AS, Al-Attiyah R, Hanif SNM, Shaban FA. 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 2008; **15**:916–24.

33 Mustafa AS, El-Shamy AM, Madi NM, Amoudy HA, Al-Attiyah R. Cell mediated immune responses to complex and single mycobacterial antigens in tuberculosis patients with diabetes. Med Princ Pract 2008; **17**:325–30.

34 Leyten EM, Arend SM, Prins C, Cobelens FG, Ottenhoff TH, van Dissel JT. Discrepancy between *Mycobacterium tuberculosis*-specific gamma interferon release assays using short and prolonged *in vitro* incubation. Clin Vaccine Immunol 2007; **14**:880–5.

35 Okkels LM, Brock I, Follmann F *et al.* PPE protein (Rv3873) from DNA segment RD1 of *Mycobacterium tuberculosis*: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. Infect Immun 2003; **71**:6116–23.

36 Lalvani A, Nagvenkar P, Udwadia Z *et al.* Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium tuberculosis* infection in healthy urban Indians. J Infect Dis 2001; **183**:469–77.

37 Cardoso FL, Antas PR, Milagres AS *et al.* T-cell responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 in Brazilian tuberculosis patients. Infect Immun 2002; **70**:6707–14.

38 Bhattacharyya S, Singla R, Dey AB, Prasad HK. Dichotomy of cytokine profiles in patients and high-risk healthy subjects exposed to tuberculosis. Infect Immun 1999; **67**:5597–603.

39 Walker D, Jason J, Wallace K *et al.* Spontaneous cytokine production and its effect on induced production. Clin Diagn Lab Immunol 2002; **9**:1049–56.

40 Stadler BM, Dougherty SF, Farrar JJ, Oppenheim JJ. Relationship of cell cycle to recovery of IL-2 activity from human mononuclear cells, human and mouse T cell lines. J Immunol 1981; **127**:1936–40.

41 Morosini M, Meloni F, Uccelli M, Marone Bianco A, Solari N, Fietta AM. *Ex vivo* evaluation of PPD-specific IFN-gamma or IL-5 secreting cells in the peripheral blood and lungs of patients with tuberculosis. Int J Tuberc Lung Dis 2005; **9**:753–9.

42 van Crevel R, Ottenhoff THM, van der Meer JWM. Innate immunity to *Mycobacterium tuberculosis*. Clin Microbiol Rev 2002; **15**: 294–309.

43 Murray PJ, Wang L, Onufryk C, Tepper RI, Young RA. T cell-

derived IL-10 antagonizes macrophage function in mycobacterial infection. J Immunol 1997; **158**:315–21.

44 Katsikis PD, Cohen SB, Londei M, Feldmann M. Are CD4+ Th1 cells pro-inflammatory or anti-inflammatory? The ratio of IL-10 to IFN-gamma or IL-2 determines their function. Int Immunol 1995; **7**:1287–94.

45 Jamil B, Shahid F, Hasa Z *et al.* Interferon γ/IL-10 ratio defines the disease severity in pulmonary and extra pulmonary tuberculosis. Tuberculosis 2007; **87**:279–87.

46 Sahiratmadja E, Alisjahbana B, de Boer T *et al.* Dynamic changes in pro- and anti-inflammatory cytokine profiles and gamma interferon receptor signaling integrity correlate with tuberculosis disease activity and response to curative treatment. Infect Immun 2007; **75**:820–9.

47 Mutis T, Cornelisse YE, Ottenhoff TH. Mycobacteria induce CD4+ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. Eur J Immunol 1993; **23**:2189–95.

48 Hussey GD, Watkins ML, Goddard EA *et al.* Neonatal mycobacterial specific cytotoxic T-lymphocyte and cytokine profiles in response to distinct BCG vaccination strategies. Immunology 2002; **105**:314–24.

49 Seah GT, Scott GM, Rook GA. Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. J Infect Dis 2000; **181**:385–9.

50 Lienhardt C, Azzurri A, Amedei A *et al.* Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity *in vivo*. Eur J Immunol 2002; **32**:1605–13.

51 Baumann S, Nasser Eddine A, Kaufmann SH. Progress in tuberculosis vaccine development. Curr Opin Immunol 2006; **18**:438–48.

52 Chen X, Zhou B, Li M *et al.* CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress *Mycobacterium tuberculosis* immunity in patients with active disease. Clin Immunol 2007; **123**:50–9.

53 Demissie A, Abebe M, Aseffa A *et al.* Healthy individuals that control a latent infection with *Mycobacterium tuberculosis* express high levels of Th1 cytokines and the IL-4 antagonist IL-4delta2. J Immunol 2004; **172**:6938–43.

54 Dheda K, Chang JS, Breen RA *et al. In vivo* and *in vitro* studies of a novel cytokine, interleukin 4delta2, in pulmonary tuberculosis. Am J Respir Crit Care Med 2005; **172**:501–8.

55 Wassie L, Demissie A, Aseffa A *et al. Ex vivo* cytokine mRNA levels correlate with changing clinical status of Ethiopian TB patients and their contacts over time. PLoS ONE 2008; **3**:e1522.