Tuberculosis 114 (2019) 91–102

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: www.elsevier.com/locate/tube

Diagnostics

Cytokine biomarkers for the diagnosis of tuberculosis infection and disease in adults in a low prevalence setting

Vanessa Clifford[a,b], Marc Tebruegge[a,b,c], Christel Zufferey[b], Susie Germano[b], Ben Forbes[b], Lucy Cosentino[f], Elizabeth Matchett[d], Emma McBryde[d], Damon Eisen[d], Roy Robins-Browne[b,e], Alan Street[d], Justin Denholm[d,e,f], Nigel Curtis[a,b,*]

[a] Department of Paediatrics, The University of Melbourne, Parkville, VIC 3052, Australia
[b] Murdoch Children's Research Institute, Royal Children's Hospital Melbourne, Parkville, VIC 3052, Australia
[c] Department of Infection, Immunity and Inflammation, UCL Great Ormond Street Institute of Child Health, University College London, London, UK
[d] Victorian Infectious Diseases Service, Royal Melbourne Hospital, Parkville, VIC 3052, Australia
[e] Department of Microbiology and Immunology, University of Melbourne, VIC 3052, Australia
[f] Victorian Tuberculosis Program, Peter Doherty Institute, Parkville, VIC 3052, Australia

ARTICLE INFO

Keywords:
Tuberculosis
Cytokines
Biomarkers
LTBI
Active TB
Diagnostics

ABSTRACT

Objective: Accurate and timely diagnosis of tuberculosis (TB) is essential to control the global pandemic. Currently available immunodiagnostic tests cannot discriminate between latent tuberculosis infection (LTBI) and active tuberculosis. This study aimed to determine whether candidate mycobacterial antigen-stimulated cytokine biomarkers can discriminate between TB-uninfected and TB-infected adults, and additionally between LTBI and active TB disease.

Methods: 193 adults were recruited, and categorised into four unambiguous diagnostic groups: microbiologically-proven active TB, LTBI, sick controls (non-TB lower respiratory tract infections) and healthy controls. Whole blood assays were used to determine mycobacterial antigen (CFP-10, ESAT-6, PPD)-stimulated cytokine (IL-1ra, IL-2, IL-10, IL-13, TNF-α, IFN-γ, IP-10 and MIP-1β) responses, measured by Luminex multiplex immunoassay.

Results: The background-corrected mycobacterial antigen-stimulated cytokine responses of all eight cytokines were significantly higher in TB-infected participants compared with TB-uninfected individuals, with IL-2 showing the best performance characteristics. In addition, mycobacterial antigen-stimulated responses with IL-1ra, IL-10 and TNF-α were higher in participants with active TB compared those with LTBI, reaching statistical significance with PPD stimulation, although there was a degree of overlap between the two groups.

Conclusion: Mycobacterial antigen-stimulated cytokine responses may prove useful in future immunodiagnostic tests to discriminate between tuberculosis-infected and tuberculosis-uninfected individual, and potentially between LTBI and active tuberculosis.

1. Introduction

Accurate diagnosis of tuberculosis (TB) is essential to control the global pandemic [1–3]. The only currently available *in vitro* immunodiagnostic tests, interferon-gamma release assays (IGRAs), have high specificity but relatively low sensitivity for active TB [4,5] particularly in immunocompromised hosts [6] and children [7,8]. They also lack the ability to discriminate between active TB disease and latent TB infection (LTBI) [9]. Although it is claimed that the fourth generation QuantiFERON Plus assay offers advantages in discriminating active TB and LTBI [10], evidence for this remains weak [11,12].

Several previous studies, including our own, have reported promising results regarding the diagnostic potential of *Mycobacterium tuberculosis* (MTB)-antigen stimulated cytokine biomarkers to identify TB-infected individuals [5,13–15]. A small number of studies have also identified cytokine biomarkers that may discriminate between LTBI and active TB [15–18]. Our own recently published study in children identified IL-1ra, IL-10 and TNF-α as the most promising biomarkers for this distinction [15]. Distinguishing between these infection states is important for clinical care, as they require different treatment

* Corresponding author. Department of Paediatrics, The University of Melbourne, Royal Children's Hospital Melbourne, Flemington Road, Parkville, VIC 3052, Australia.
E-mail address: nigel.curtis@rch.org.au (N. Curtis).

https://doi.org/10.1016/j.tube.2018.08.011
Received 24 April 2018; Received in revised form 19 August 2018; Accepted 22 August 2018

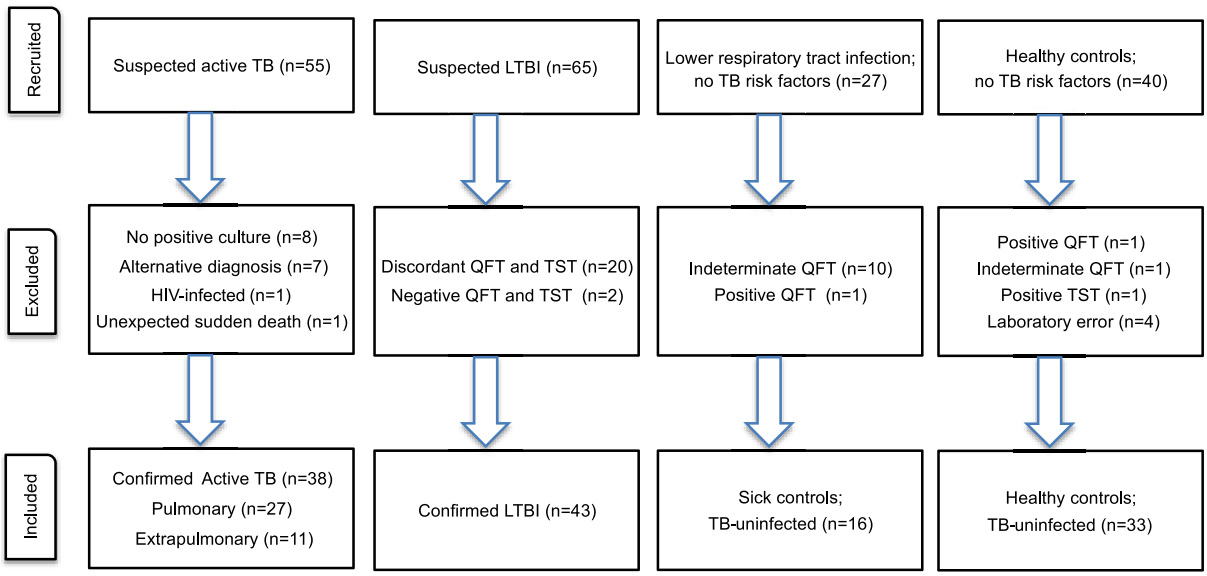

Fig. 1. Outline of participant categorisation.

strategies. Therefore, an immune-based blood test that can make this distinction would be a major advance.

The dynamic nature of the interaction between MTB and the host immune system suggests that a combination of biomarkers may prove to be more sensitive and specific than a single biomarker alone [19].

Many TB studies have focussed on biomarker sensitivity, often overlooking the importance of specificity [20,21]. This is an important distinction in highly endemic settings, where many patients presenting with lower respiratory tract infection have co-existing LTBI, making it difficult to discriminate pulmonary TB from other bacterial causes of pneumonia [22]. Determining the specificity of new biomarkers in the setting of suspected active TB requires the inclusion of a 'sick' control group, which most previous TB diagnostic studies have lacked.

This study aimed to determine whether candidate MTB antigen-stimulated cytokine biomarkers previously identified in our recent biomarker study in children also reliably discriminate between TB-infected and TB-uninfected adults and, secondly, adults with LTBI and active TB.

2. Methods

2.1. Participants

Adults with suspected active TB or LTBI were recruited at the Royal Melbourne Hospital (Melbourne Health, Victoria, Australia), a tertiary referral center, over a 3-year-period (March 2012–November 2014). These patients were either admitted to the hospital whilst undergoing investigation for active TB, or were referred to the hospital outpatient TB clinic with suspected LTBI (e.g. positive QFT) or suspected active TB. Australia is a low TB prevalence setting, with an estimated annual TB incidence of 5–6/100,000 population; the large majority (> 85%) of notified new TB cases are overseas-born [23]. In addition, hospitalised individuals with clinical and/or radiological evidence of lower respiratory tract infection (LRTI) without known risk factors for TB were recruited as potential 'sick controls'. Asymptomatic volunteers without known risk factors for TB were recruited as potential 'healthy controls'. Individuals who were receiving immunosuppressive medication or were known to have a primary or secondary immunodeficiency were excluded, as were participants who had received anti-tuberculous therapy for more than one week. Individuals unable to provide informed consent and pregnant women were also excluded.

Following written informed consent, data on demographic and clinical characteristics were recorded on a standardised case report form. Details included country and date of birth, TB exposure history, BCG immunisation status (including presence of BCG scar), results of tuberculin skin tests (TST) in the past 8 weeks, and history of previous TB treatment. In addition, clinical symptoms, past medical history, current medications, physical examination, and radiological and HIV test results were recorded.

2.2. Tuberculin skin tests and interferon-gamma release assays

Participants had blood samples taken for QuantiFERON-TB Gold-in-Tube (QFT-GIT) assays and into sodium heparin tubes for the whole blood stimulation assays detailed below. All QFT-GIT assays were processed in a fully-accredited diagnostic laboratory, at either the Royal Melbourne Hospital or the Victorian Infectious Diseases Reference Laboratory, following the manufacturer's instructions.

All participants had a TST (5 Tuberculin Units PPD; Tubersol, Sanofi Pasteur, Toronto, Canada) done by a trained healthcare professional, with the exception of participants who had microbiologically-confirmed active TB at recruitment. A positive TST result was defined as an induration greater than or equal to 10 mm at 48–72 h. For the purpose of this study, a negative TST was defined as an induration of 0 mm.

2.3. Definitions and categorisation of participants

Based on TST, QFT-GIT and microbiological results, participants were classified into the following unambiguous diagnostic groups (Fig. 1): Group A - active TB (defined as microbiologically-confirmed TB based on culture or polymerase chain reaction), Group B - LTBI (defined as asymptomatic participants with positive TST and positive IGRA result), Group C - sick controls (LRTI caused by a pathogen other than MTB), and Group D - healthy controls (asymptomatic volunteers without risk factors for TB with a negative TST result (absence of any induration) and a negative IGRA result). Risk factors for TB were defined as known TB contact or travel to a high TB prevalence country (with a TB incidence ≥40/100,000 per year). Participants who did not fulfill the criteria for any of these four distinct diagnostic categories were excluded from the analyses.

2.4. Whole blood assays

Whole blood samples were stimulated with the MTB-specific

antigens ESAT-6 (10 μg/mL; JPT Peptide Technologies, Berlin, Germany) and CFP-10 (10 μg/mL; JPT), and PPD (20 μg/mL; RT50, Statens Serum Institut, Copenhagen, Denmark)) or left unstimulated (negative control). All stimulation assays were done in the presence of co-stimulatory antibodies anti-CD28 and anti-CD49 (both BD Biosciences, San Jose, CA, USA) [15]. Following 19 h of incubation at 37 °C, supernatants were harvested and immediately cryopreserved at −80 °C for later analysis.

2.5. Cytokine analysis

Cytokines were measured using *Biorad* human cytokine kits (Biorad, Gladesville, Australia) in batched analyses, following the manufacturer's instructions. In brief, standards, controls and samples were added to a 96-well filter-plate and incubated with pre-mixed detection beads, followed by detection antibodies, and then streptavidin-PE. The mean fluorescence intensity was read for each cytokine using a xMAP Luminex 200 Bioanalyzer. Based on our previous experience that the concentrations of certain cytokines exceed the dynamic detection range of the *Biorad* assays [15], each sample was analysed using two separate plates: (i) undiluted samples for IFN-γ, IL-1ra, IL-2, IL-10, IL-13, and TNF-α, and (ii) 1:20 diluted samples for IP-10 and MIP-1β. The manufacturer stated limit of detection of the Biorad human cytokine assay is ≤1 pg/mL.

2.6. Statistical analysis

We estimated that with n = 40 in each group we could establish a sensitivity of 88%–100%, based on large sample normal approximation, such that a two-sided 95.0% confidence interval for a single proportion will extend 0.068 from the observed proportion for an expected proportion of 0.950.

All cytokine responses were background-corrected prior to analysis (i.e. by subtracting the concentration measured in the negative control sample). Non-parametric tests were used to compare cytokine responses between the four diagnostic groups: Kruskal-Wallis tests for multiple groups and Mann Whitney *U* tests for two-group comparisons (in instances where Kruskal Wallis p-values were significant). Categorical variables were compared using two tailed chi-square tests. A p-value < 0.05 was considered significant. Receiver operating characteristics (ROC) curve analysis was used to determine the analytic sensitivity and specificity of each cytokine biomarker. Optimal sensitivity and specificity were estimated using Youden's index. All statistical analyses were done using Prism v5 (GraphPad Software Inc; La Jolla, CA, USA) and Stata V14 (StatCorp, College Station, TX, USA). The study was conducted in accordance with Quality Assessment of Diagnostic Accuracy Studies (QUADAS) criteria and is reported according to Standards for Reporting Diagnostic Accuracy Studies (STARD) guidelines [24].

2.7. Ethical approval

The study was reviewed and formally approved by the RMH human research ethics committee (HREC approval number 2011.128). Research was conducted in accordance with Good Clinical Practice guidelines and the principles of the Declaration of Helsinki.

2.8. Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

3. Results

3.1. Participants

A total of 193 participants were recruited, six of whom were excluded prior to determination of diagnostic category (three participants did not have a blood sample collected, one had blood sent in unlabelled tubes so QFT-GIT could not be performed, one did not have a QFT-GIT done, and one declined to have a TST done).

Based on the results of QFT-GIT and TST, 22 participants with suspected LTBI were excluded: 20 participants had discordant QFT-GIT and TST results (five participants QFT-GIT positive, TST < 10 mm and 15 participants TST ≥ 10 mm, QFT-GIT negative), and two participants were both TST and QFT-GIT negative (Fig. 1). Amongst participants with suspected active TB, 17 were excluded: eight did not have a positive TB culture, one had a diagnosis of adenocarcinoma, one had *M. kansasii* infection, five had an alternative diagnosis of LRTI with negative mycobacterial cultures, one died shortly after diagnostic bronchoscopy and one was diagnosed with HIV infection shortly after recruitment. Amongst participants with suspected non-TB LRTI, 11 were excluded: one had a positive QFT-GIT and 10 had indeterminate QFT-GIT results. Amongst healthy volunteers without risk factors for TB, seven were excluded: one had a positive TST, one had a positive QFT-GIT, one had an indeterminate QFT-GIT result and four were excluded due to an error during sample processing.

The remaining 130 participants were classified into the following distinct diagnostic groups: Group A - active TB (n = 38); Group B - LTBI (n = 43); Group C - sick controls (n = 16); Group D - healthy controls (n = 33). Baseline demographic characteristics for participants included in the final analyses are summarised in Table 1. The majority of participants in the active TB and LTBI group were BCG vaccinated and born overseas, whereas most participants in the two control groups were not BCG vaccinated and were born in Australia. In the active TB group, 27 participants had pulmonary TB and 11 had extrapulmonary

Table 1
Baseline characteristics of participants included in the final analyses.

	Active TB (n = 38)	LTBI (n = 43)	Sick controls (n = 16)	Healthy controls (n = 33)
Age (median [IQR]) in years	28 [25,44]	26 [24,31]	53 [28,58]	26 [24,33]
Male	19/38 (50%)	21/43 (49%)	6/16 (38%)	13/33 (39%)
BCG-vaccinated	22/38 (58%)	33/43 (75%)	5/16 (31%)	4/33 (12%)
Born overseas	36/38 (95%)	42/43 (98%)	4/16 (25%)	5/33 (15%)
Region of birth				
Africa	7 (18%)	6 (14%)	0	0
Asia	27 (71%)	34 (79%)	1 (6%)	1 (3%)
Middle East	1 (3%)	0	0	0
Australasia	2 (5%)	1 (2%)	12 (75%)	28 (85%)
Europe	0	1 (2%)	3 (19%)	2 (6%)
North America	1 (2%)	1 (2%)	0	2 (6%)
Time since arrival in Australia (median [range]) in years[a]	6.0 [0,29]	4.0 [0.5,30]	23 [0,49]	15 [0,23]

LTBI: latent tuberculosis infection; TB: tuberculosis; IQR: interquartile range.
[a] Overseas-born participants only.

Table 2
MTB antigen-induced cytokine responses in active TB, LTBI and controls with significance testing for differences between groups.

	Active TB (n = 38)	LTBI (n = 43)	Sick controls (n = 16)	Healthy controls (n = 33)	4-group test	LTBI vs healthy controls	TB-infected (LTBI & Active TB) vs TB-uninfected (healthy & sick controls)	Active TB vs sick controls	Active TB vs LTBI
	Median [IQR] (pg/mL)				Kruskal-Wallis	Mann Whitney *U*			
IFN-γ									
CFP-10	292.7 [77.8, 1089]	223.2 [29.9, 779.2]	−12.4 [−39.5, 7.9]	−12.7 [−56.5, 2.2]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.25
ESAT-6	105.5 [29.1, 665.1]	100.6 [16.5, 581.0]	−3.8 [−29.4, 16.4]	2.2 [−65.1, 17.7]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.42
PPD	3705 [1925, 6211]	3195 [1892, 12958]	447 [96,972]	717 [274,1369]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.74
IL-1ra									
CFP-10	290.3 [-121.0, 703.0]	56.4 [−79, 364.4]	−226.6 [−459.8, 0]	−203.5 [−392.1, −37.6]	< 0.0001	< 0.0001	< 0.0001	< 0.001	0.30
ESAT-6	87.3 [−169.2, 631.2]	152.4 [−9.1, 476.3]	−7.2 [−396.5, 34]	−34.3 [−300.4, 105.4]	< 0.001	< 0.001	< 0.0001	0.08	0.42
PPD	2674 [1310, 4159]	1817 [1023, 2785]	1174 [268.6,2741]	979 [453.3,1699]	< 0.001	0.01	< 0.001	0.02	0.04
IL-2									
CFP-10	102.5 [40.6, 312.2]	101.9 [23.6, 183.5]	0.0 [−2.1, 0]	0.0 [−1.6, 0]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.42
ESAT-6	60.0 [19.8, 182.2]	52.8 [10.6, 230.3]	0.0 [−1.39, 0.66]	0.0 [−1.5, 0.5]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.90
PPD	837.3 [456.8, 1468]	889.4 [548.2, 1334]	175.2 [73.8, 711.9]	148.0 [97, 297.5]	< 0.0001	< 0.0001	< 0.0001	< 0.001	0.97
IL-10									
CFP-10	−2.0 [−14.5, −0.6]	−0.8 [−2.7, −0.3]	−7.9 [−10.1, −2.3]	−6.7 [−19.8, −0.7]					
ESAT-6	−2.2 [−14.7, −0.5]	−0.3 [−2.12, 0.41]	−3.9 [−7.9, −1.7]	−3.4 [−12.3, −0.2]					
PPD	49.3 [26.2, 81.2]	26.3 [14.3, 48]	17.2 [10.4, 37.4]	16.0 [5.9, 45.8]	< 0.01	0.20	< 0.01	0.02	0.009
IL-13									
CFP-10	2.4 [0.6, 9.1]	0.6 [0, 3.1]	−0.1 [−1.8, 0]	−0.6 [−2.4, −0.1]					
ESAT-6	1.0 [0, 3.4]	0.6 [−0.1, 2.8]	0.0 [−0.9, 0]	−0.2 [−2.4, 0.1]					
PPD	51.1 [21.0, 113.9]	66.1 [44.5, 117.9]	25.5 [3.4, 48.8]	40.1 [18.1, 71]	< 0.01	< 0.01	< 0.001	0.02	0.23
TNF-α									
CFP-10	46.8 [9.6, 183.1]	17.0 [2.7, 55.8]	0.0 [−9.8, 4.4]	−3.1 [−36.8, 1.0]	< 0.0001	< 0.0001	< 0.0001	< 0.01	0.06
ESAT-6	29.0 [5.2, 135.8]	15.3 [1.5, 50.1]	−2.2 [10.2, 3.3]	2.1 [−5.4, 30.4]	< 0.001	0.02	< 0.0001	< 0.001	0.47
PPD	1125 [647.9, 1691]	634.0 [203.8, 1642]	129.8 [17.6, 274.9]	103.6 [23, 202.9]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.01
IP-10									
CFP-10	84857 [50346,178530]	83960 [24095,129339]	−476.5 [−1580, 1004]	0.0 [−3207, 1479]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.15
ESAT-6	45126 [16741,130091]	51884 [13139,127257]	−698 [−3444, −126]	1058 [−1655, 11077]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.80
PPD	66784 [31604,137844]	82175 [38227,112108]	20843 [6610, 48889]	47938 [24794, 73935]	< 0.01	0.01	< 0.001	< 0.01	0.60
MIP-1β									
CFP-10	4406 [1988, 9393]	3230 [925, 6904]	−113 [−940, 821]	306 [−750, 940]	< 0.0001	< 0.0001	< 0.0001	0.0001	0.24
ESAT-6	2645 [1448, 7432]	4220 [1563,7033]	611 [−65,1843]	1414 [737,4135]	< 0.001	< 0.01	< 0.001	< 0.01	0.60
PPD	75518 [30114,125997]	59803 [42202,114488]	14850 [6981, 43757]	12954 [7747, 19876]	< 0.0001	< 0.0001	< 0.0001	< 0.001	1.00

TB (disseminated (n = 3), intra-abdominal (n = 2), lymph node (n = 6)). The most common comorbidity in patients with TB infection was vitamin D deficiency (82% in active TB patients); no participant had a parasitic infection.

3.2. MTB antigen-stimulated cytokine responses

In participants with LTBI and active TB median responses of all cytokines stimulated by CFP-10 were higher than those stimulated by ESAT-6, particularly in the active TB group where they were up to 2-fold higher. Responses stimulated by PPD were approximately 10-fold higher than those stimulated by MTB-specific antigens (Table 2).

CFP-10 and ESAT-6-stimulated median IL-10 and IL-13 responses were below the manufacturer stated detection limit of the Luminex assay and were therefore not included in the ROC analysis.

3.3. TB-infected vs TB-uninfected

Cytokine responses stimulated by CFP-10, ESAT-6, and PPD were significantly higher in TB-infected participants (LTBI and active TB combined) compared with TB-uninfected participants (healthy and sick controls combined) for IFN-γ, IL-1ra, IL-2, IL-10, IL-13, TNF-α, IP-10 and MIP-1β (Table 2 and Fig. 2). The median concentrations of these cytokines were also all higher in participants with active TB compared to sick controls, irrespective of the stimulant used. This was statistically significant for all MTB antigen/cytokine combinations, except for IL-1ra in ESAT-6-stimulated samples and IL-10 in CFP-10- and ESAT-6-stimulated samples. In addition, the median concentrations of IFN-γ, IL-1ra, IL-2, IL-10, IL-13, TNF-α, IP-10 and MIP-1β were all higher in participants with LTBI than in healthy controls. This was statistically significant for all MTB antigen/cytokine combinations, with the exception of IL-10 in PPD-stimulated samples.

The ROC analyses show that IL-2 and IP-10 responses, in addition to IFN-γ responses, are sensitive and specific markers of TB infection (Fig. 3). IL-2 was the most sensitive and specific cytokine to discriminate TB-infected from TB-uninfected participants (Fig. 3). Notably, IL-2 had higher area under the curve (AUC) values than IFN-γ, regardless of the antigen used for stimulation. In CFP-10- and ESAT-6-stimulated samples IP-10 achieved AUC values that were very similar to IFN-γ.

3.4. Active TB vs LTBI

Irrespective of the stimulant used, IFN-γ lacked the ability to discriminate between LTBI and active TB whereas median PPD-stimulated TNF-α, IL-1ra and IL-10 responses were significantly higher in the group of participants with active TB than in either LTBI cases or sick controls (Table 2 and Fig. 2c). Both in CFP-10- and ESAT-6-stimulated samples, TNF-α responses were also considerably higher in the active TB group compared with the LTBI group (46.8 vs 17.0 pg/mL and 29.0 vs. 15.3 pg/mL, respectively), although this did not achieve statistical significance. In CFP-10-stimulated samples, median IL-1ra responses were 5-fold higher in the active TB group compared with the LTBI group (290.3 vs 56.4 pg/mL, respectively), although this difference was not statistically significant (see Table 2). We did not find a difference in IL-1ra responses between those groups after ESAT-6 stimulation.

3.5. Active TB vs sick controls

Cytokine responses stimulated by CFP-10, ESAT-6, and PPD were significantly higher in participants with active TB compared to sick controls for all cytokines investigated, except for ESAT-6 and CFP-10 stimulated IL-10 responses, where concentrations in both groups were below the manufacturer stated detection limit.

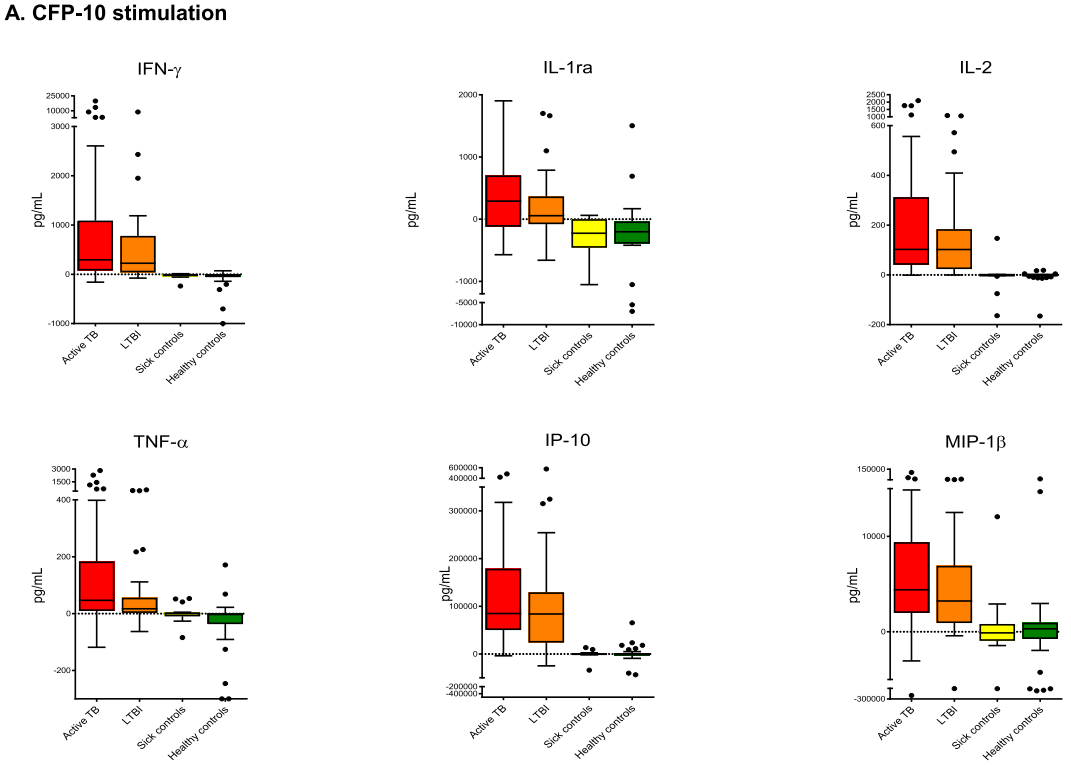

Fig. 2. Background-corrected MTB antigen-induced cytokine responses according to diagnostic group and stimulant. Box plot with Tukey whiskers; the horizontal lines represent the medians. For cytokines which discriminated between the active TB and LTBI groups, p-values are shown for the difference between both the active TB and LTBI groups and between the active TB and sick control groups.

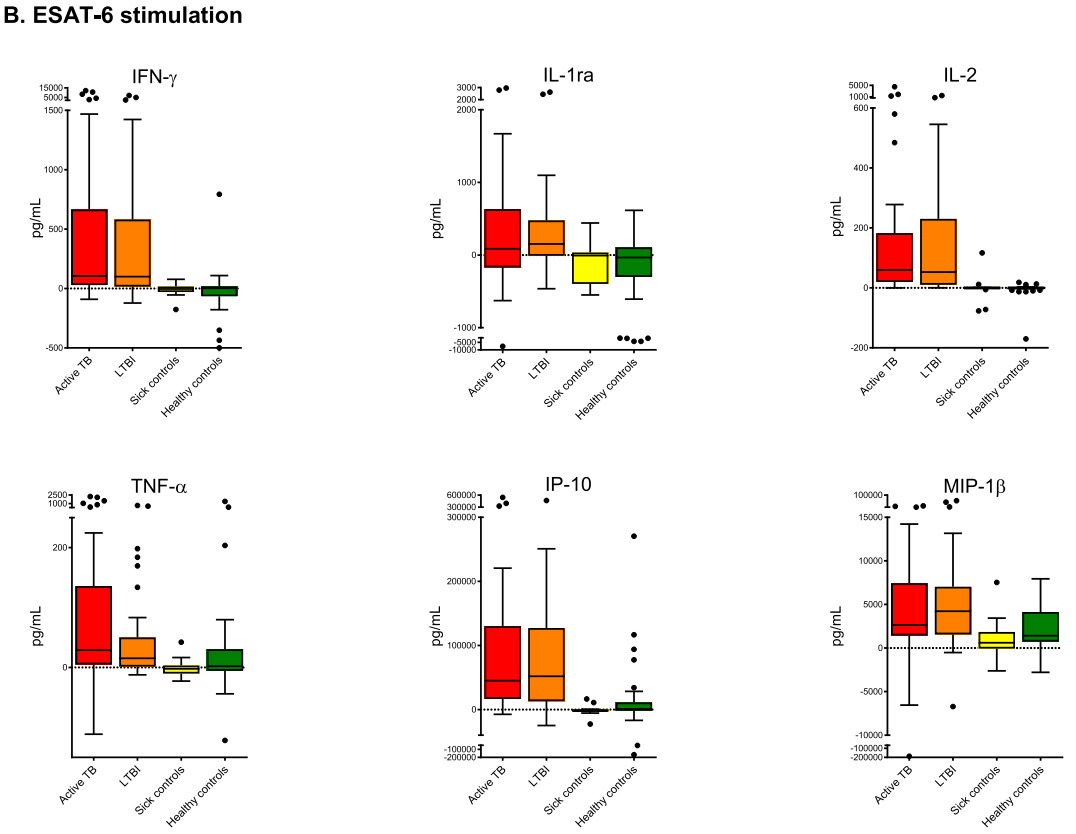

Fig. 2. (*continued*)

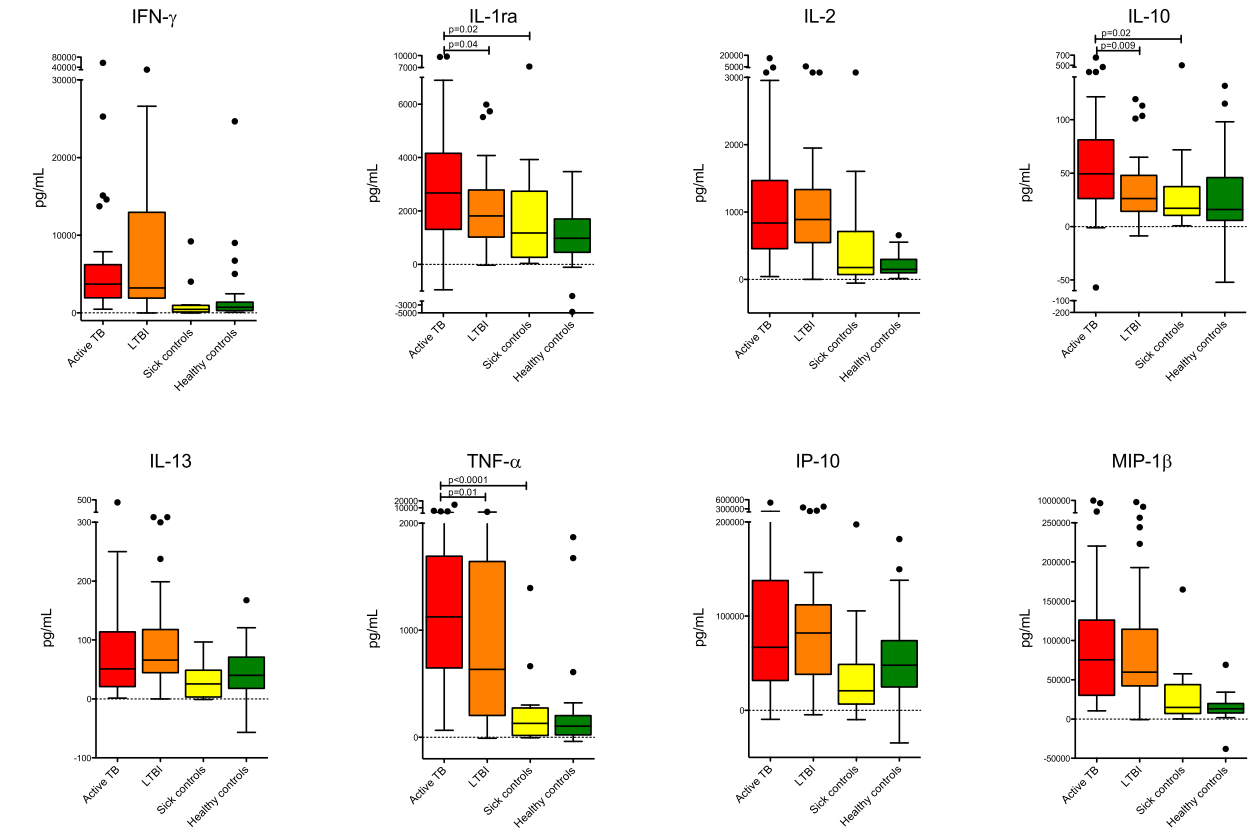

Fig. 2. (*continued*)

A. CFP-10 stimulated samples

	IFN-γ	IP-10	IL-1ra	IL-2	TNF-α	MIP-1β
Area under the curve [95% CI]	0.92 [0.87,0.97]	0.92 [0.87,0.97]	0.81 [0.73,0.88]	0.95 [0.91,0.98]	0.81 [0.73,0.88]	0.82 [0.75,0.90]
Standard error	0.02	0.02	0.04	0.02	0.04	0.04
p-value	< 0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Optimal cut off (pg/mL)	12.62	19301	5.7	6.1	2.4	1466
Sensitivity (%) [95% CI]	88.0	81.9	64.2	90.4	78.3	75.3
Specificity (%) [95% CI]	93.9	95.9	87.8	93.9	77.6	85.7
Positive likelihood ratio	14.4	20.1	5.2	14.8	3.5	5.3
Youden's index	81.8	77.9	52.0	84.2	55.9	61.0

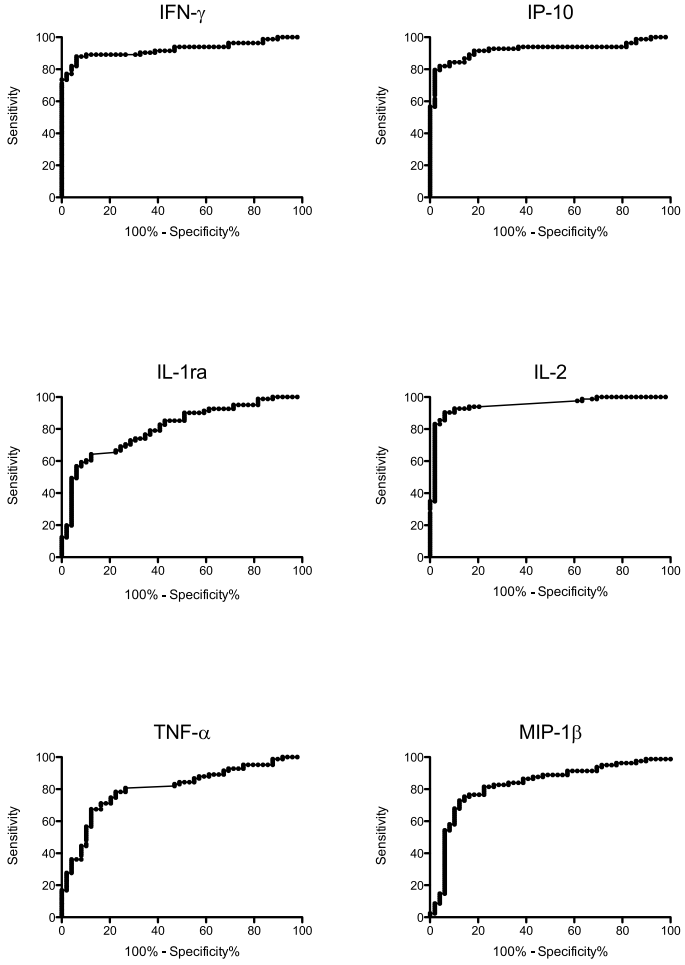

Fig. 3. Receiver operated characteristic curves for the discrimination between TB-infected and TB-uninfected individuals according to cytokine and stimulant: (A) CFP-10, (B) ESAT-6 and (C) PPD.

3.6. Further subgroup analysis according to BCG vaccination status and site of disease

There were no statistically significant differences in PPD-stimulated cytokine responses between BCG-vaccinated and BCG-non-vaccinated participants in all four diagnostic groups (Supplementary Fig. 1), indicating that prior BCG vaccination has no significant impact on these responses.

We also analysed whether there were significant differences in the cytokine responses to CFP-10, ESAT-6 and PPD in participants with

B. ESAT-6 stimulated samples

	IFN-γ	IP-10	IL-1ra	IL-2	TNF-α	MIP-1β
Area [95% CI]	0.84 [0.77,0.91]	0.85 [0.77,0.92]	0.71 [0.62,0.79]	0.94 [0.90,0.98]	0.71 [0.62,0.81]	0.69 [0.60,0.79]
Standard error	0.03	0.04	0.04	0.02	0.05	0.05
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
Optimal cut off (pg/mL)	20.9	12383	47.1	2.1	3.1	1507
Sensitivity (%) [95% CI]	76.5	79.0	63.0	92.6	77.8	77.8
Specificity (%) [95% CI]	81.6	83.7	71.4	87.8	61.2	57.1
Positive likelihood ratio	4.2	4.8	2.2	7.6	2.0	1.8
Youden's index	58.2	62.7	34.4	80.4	39	34.9

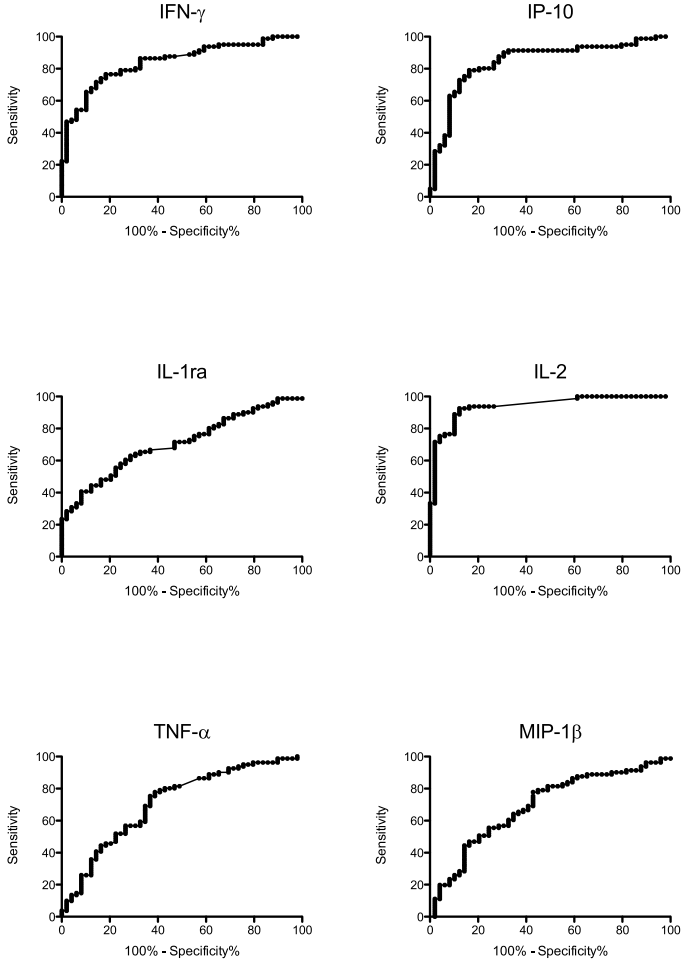

Fig. 3. (*continued*)

pulmonary compared with extrapulmonary active TB (Supplementary Fig. 2). Although overall there was a tendency for cytokine responses to be higher in the group with extrapulmonary TB, this was only statistically significant for IP-10 in ESAT-6 stimulated samples (median concentration 153445 pg/mL versus 40194 pg/mL) and for TNF-α in PPD-stimulated samples (median concentration 1364 pg/mL versus 789 pg/mL). For all other stimulant/cytokine combinations, differences were not statistically significant.

4. Discussion

In this study, we evaluated MTB antigen-stimulated cytokine

C. PPD stimulated samples

	IFN-γ	IP-10	IL-1ra	IL-2	TNF-α	MIP-1β
Area [95% CI]	0.86 [0.78,0.93]	0.68 [0.59,0.78]	0.69 [0.60,0.78]	0.88 [0.81,0.94]	0.86 [0.80,0.93]	0.88 [0.82,0.94]
Standard error	0.04	0.05	0.05	0.03	0.03	0.03
p-value	<0.0001	0.0004	0.0003	<0.0001	<0.0001	<0.0001
Optimal cut off (pg/mL)	1215	92987	1784	398	332	36536
Sensitivity (%) [95% CI]	90.1	42.0	56.8	87.7	74.1	75.3
Specificity (%) [95% CI]	79.6	89.8	77.6	81.6	89.8	89.8
Positive likelihood ratio	4.4	4.1	2.5	4.8	7.3	7.4
Youden's index	69.7	31.8	34.3	69.3	63.9	65.1

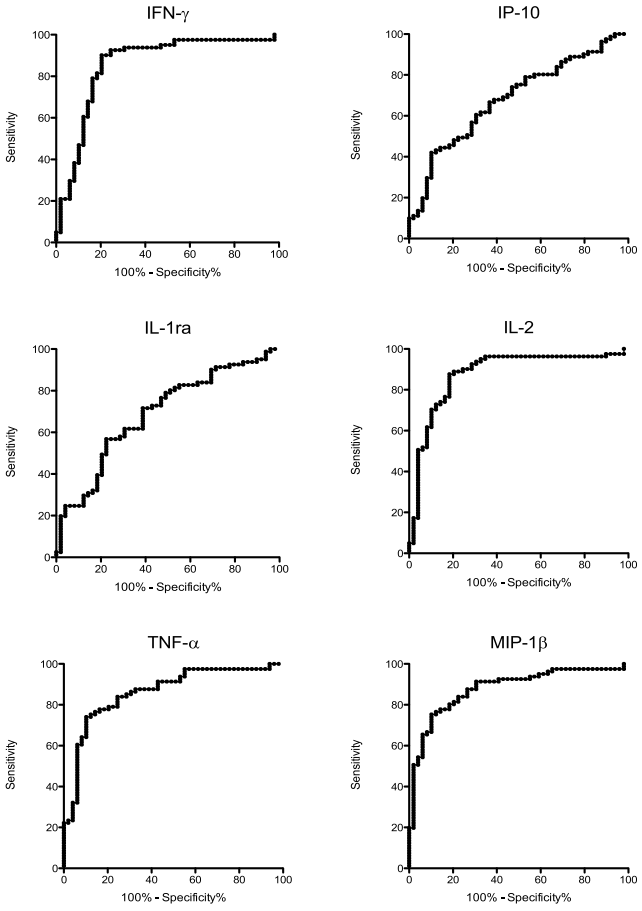

Fig. 3. (*continued*)

biomarker responses that had been shown to discriminate between TB-infected and TB-uninfected individuals in our previous study in children in a low TB prevalence setting. In that study, we identified that some of these biomarkers, specifically IL-1ra, IL-10 and TNF-α, were also significantly higher in active TB than LTBI [15].

The findings of our current study in adults confirm that all eight cytokines investigated discriminate between TB-infected and TB-uninfected individuals. In contrast to our previous study in children, we were able to include a sick control group. Importantly, depending on the antigen used for stimulation, some of these cytokines showed better performance characteristics than IFN-γ, which forms the basis of commercial IGRAs.

IL-2 had greater AUC values, and higher sensitivity and specificity than IFN-γ, irrespective of the stimulant used. Our finding that IL-2 is the most sensitive and specific marker of TB infection is consistent with results from other recent studies [16,25–28], including our own [15]. This finding supports recent suggestions that assays combining IL-2 and IFN-γ may achieve greater sensitivity for the detection of TB infection [26,29]. Although not investigated in our study, the role of IL-2 as a diagnostic test in immunocompromised patients deserves further study. Several investigators have also suggested that IL-2, or IL-2/IFN-γ ratio [30,31] may discriminate between active TB and LTBI [29], but other studies have not confirmed this finding [16,25,32]. We found that IL-2 responses, irrespective of the stimulant used, did not differ significantly between participants with active TB and those with LTBI, suggesting that this cytokine lacks the ability to discriminate between infection states.

We found that, after PPD stimulation, IL-1ra, IL-10 and TNF-α responses were higher in individuals with active TB compared to those with LTBI. This is consistent with our previous study in children [15,33–35] although the reported discriminatory ability of these cytokines has been variable in other published studies (particularly for TNF-α and IL-10) [36–38].

IL-1ra is a competitive inhibitor of IL-1α and IL-1β, and is thought to play an important part in granuloma formation, an event crucial for containing MTB [39,40]. The few previous studies investigating the ability of IL-1ra to discriminate between active TB and LTBI have all reported that IL-1ra responses discriminate between LTBI and active TB [15,16,41]. One of these studies was done by Frahm et al., and included 32 adults and children with LTBI and 12 with active TB, but no sick control group [16]. Similar to our study, there was considerable overlap between the IL-1ra responses observed in both groups, indicating that a test based on this marker alone would not reliably discriminate between these infection states.

Previous studies investigating mycobacterial-antigen stimulated TNF-α responses to discriminate between active TB and LTBI have yielded variable results. Of 14 previous studies, five reported that TNF-α responses were increased in active TB compared to LTBI [15,27,34,42,43], one found that TNF-α responses were decreased [44] and nine found no significant difference [33,37,38,45–50].

IL-10 is an anti-inflammatory cytokine that exerts both protective and detrimental effects in the host response to MTB infection [51] Our finding that IL-10 was higher in active TB compared to LTBI is consistent with some previous studies [15,33,37,42], however other studies have found no significant difference [27,34,36,44,48].

There are several possible explanations for heterogeneity of reported findings between studies of the same cytokines: these include heterogeneity of study inclusion criteria, genetic differences in patient populations [52–54] and differences in severity of illness at the time of testing. Lower cytokine responses in active TB are more commonly reported in low resource settings [44,55], where patients are likely to be more severely unwell at the time of presentation and thus may have impaired cell-mediated immunity.

It is important to note that as a result of the overlap in cytokine concentrations between LTBI and active TB groups, none of the cytokine biomarkers in our study in the current test format reliably discriminate active TB and LTBI. Combining multiple cytokines did not improve their discriminatory ability. In addition, PPD contains antigens that are present in BCG vaccine strains, thereby potentially reducing its diagnostic accuracy in BCG-vaccinated individuals. However, our results indicate that prior BCG vaccination does not have a significant impact on the cytokine responses measured in our assays, even if PPD is used as the stimulant.

The MTB-specific antigen used for stimulation and the duration of incubation impacts on measured cytokine responses [56]. We used two of the region of difference 1 (RD1)-encoded antigens (ESAT-6 and CFP-10) that are included in the commercial QFT-GIT assay, in addition to PPD. ESAT-6 and CFP-10 are known to be highly immunogenic across different ethnic groups [57,58], although responses may vary according to the strain type within the *M. tuberculosis* complex [59]. Cytokine responses varied with these different MTB antigens both in magnitude and direction of response. Our finding that, in active TB in particular, CFP-10-stimulated cytokine responses in adults were higher than those stimulated by ESAT-6 contrasts with our recent study in children, but is consistent with other published studies in adults [54,60,61].

The pattern of antigen expression is thought to vary across the spectrum from LTBI and active TB [5,62]. It is known that ESAT-6 is secreted across the spectrum of MTB infection and may therefore be less useful in discriminating LTBI and active TB [5,51,63,64]. It is possible that alternative phase-specific antigens may result in a test with superior discriminatory ability for TB infection states [5,65]. Early *M. tuberculosis* infection stage antigens include Rv0203, Rv0642, Rv1196 and LTBI phase antigens include Rv1284, Rv2031, Rv2244, Rv2659 and Rv2660 [66]. Antigens that are phase-specific, such as heparin binding haemagglutinin [67–69] and other latency associated antigens (Rv1733, Rv2628 and Rv2029) have been investigated for their potential use in cytokine-based assays [70]. Identification of an ideal antigen stimulant has proved elusive to date as immune responses may vary significantly both between individuals, as well as between populations of different ethnicities [52,71,72]. A recent longitudinal study from Greenland found significant changes in population response to various *M. tuberculosis* antigens (replicative, latency-associated and constitutive antigens) over time [66] and that none of the antigens were consistently associated with a specific infection state.

An alternative approach, using whole blood transcriptomic mRNA expression signatures [73,74], has also shown promise in recent years. A South African study in adolescents with LTBI found that a 16-gene transcriptomic signature predicted risk of progression from LTBI to active TB with 66% sensitivity and 81% specificity [73], although the sensitivity was considerably lower in the validation cohorts at 54% (specificity 83%).

Our study shows that the diagnostic cytokine biomarkers investigated can discriminate between active TB and respiratory tract infection caused by other bacteria and viruses, as shown by the fact that all cytokine responses were significantly higher in the active TB group than in the sick control group, with the exception of IL-10 responses. This finding shows that background inflammation does not interfere with the interpretation of our cytokine biomarker responses.

The strengths of our study include the careful selection of participants for inclusion, resulting in unambiguous diagnostic groups. In addition, the inclusion of sick controls (i.e. individuals with respiratory infections caused by pathogens other than MTB) allowed assessment of the specificity of the MTB antigen-stimulated cytokine biomarkers. A limitation of many previous biomarker studies is the absence of a control group with non-TB respiratory tract infections, which is important to establish specificity of any candidate biomarker in the context of suspected active TB. The sick control group in our study was smaller than the remaining three groups as many participants with suspected non-TB respiratory tract infection had to be excluded due to indeterminate QFT-GIT results, as coincidental, co-existing LTBI could not be ruled out. In addition, many previous studies have included 'healthy control' subjects with known TB exposure (but negative TST and/or IGRA result). This approach is problematic since both TST and IGRA have imperfect sensitivity, meaning that some of those individuals may in fact have had LTBI. Therefore, the inclusion of a healthy, non-TB exposed control group, as done in our study, is vital to establish the true specificity of any biomarker in the setting of suspected LTBI.

A limitation of our findings is that whilst cytokine responses were generally higher in TB-infected than LTBI infected participants, there were outliers with high mycobacterial antigen-induced cytokine responses in each group, including the healthy control group. This may have been due to unknown or undisclosed prior TB exposure, exposure to atypical mycobacteria [75,76], prior BCG vaccination (for PPD

responses) or possibly intercurrent infection.

In conclusion, in addition to IFN-γ, several other MTB antigen-stimulated cytokine responses, including IL-1ra, IL-2, IL-10, IL-13, TNF-α, IP-10 and MIP-1β, can distinguish between TB-infected and TB-uninfected individuals. Irrespective of the antigen stimulant used, IL-2 responses performed better in discriminating between TB-infected and TB-uninfected individuals than IFN-γ responses, which form the basis of IGRA. In addition, our data confirms our previous finding that PPD-stimulated IL-1ra, IL-10 and TNF-α responses are significantly higher in individuals with active TB than in those with LTBI, although there was a degree of overlap between the two groups. These biomarkers should be investigated in future studies using alternative MTB phase-specific antigens, with the aim of developing future TB immunoassays that facilitate the distinction between infection states.

Support

This study was supported by a grant from the John Burge trust. MT was supported by a Clinical Lectureship provided by the U.K. National Institute for Health Research.

Conflicts of interest and financial disclosure

The author(s) declare no competing financial interests.

Authors' contribution

VC, MT, NC, AS, JD, EMc, RRB and DE were involved in the conception and design of the study. NC led the study. VC, MT, CZ, SG, NC, EM, LC, JD and BF made a substantial contribution to the acquisition, analysis and interpretation of data. VC, NC, MT and JD were involved in drafting the manuscript and all other authors revised it critically for important intellectual content and provided final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Acknowledgements

The authors thank Ms Snezana Spillane and Ms Vi Dang (Murdoch Children's Research Institute) for their help with the cytokine stimulation assays and harvesting of supernatants, and Ms Paulette Manton (Victorian Infectious Diseases Department of the Royal Melbourne Hospital) for administrative assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tube.2018.08.011.

References

[1] Perez-Velez CM. Pediatric tuberculosis: new guidelines and recommendations. Curr Opin Pediatr 2012;24(3):319–28.
[2] Lawn SD, Zumla AI. Tuberculosis. Lancet 2011;378(9785):57–72.
[3] Wallis RS, Maeurer M, Mwaba P, Chakaya J, Rustomjee R, Migliori GB, et al. Tuberculosis-advances in development of new drugs, treatment regimens, host-directed therapies, and biomarkers. Lancet Infect Dis 2016;16(4):e34–46.
[4] Metcalfe JZ, Everett CK, Steingart KR, Cattamanchi A, Huang L, Hopewell PC, et al. Interferon-gamma release assays for active pulmonary tuberculosis diagnosis in adults in low- and middle-income countries: systematic review and meta-analysis. J Infect Dis 2011;204(Suppl 4):S1120–9.
[5] Chegou NN, Heyckendorf J, Walzl G, Lange C, Ruhwald M. Beyond the IFN-gamma horizon: biomarkers for immunodiagnosis of infection with Mycobacterium tuberculosis. Eur Respir J 2014;43(5):1472–86.
[6] Denkinger CM, Dheda K, Pai M. Guidelines on interferon-gamma release assays for tuberculosis infection: concordance, discordance or confusion? Clin Microbiol Infect 2011;17(6):806–14.
[7] Tebruegge M, de Graaf H, Sukhtankar P, Elkington P, Marshall B, Schuster H, et al. Extremes of age are associated with indeterminate QuantiFERON-TB gold assay results. J Clin Microbiol 2014;52(7):2694–7.
[8] Connell T, Tebruegge M, Ritz N, Curtis N. Interferon-gamma release assays for the diagnosis of tuberculosis. Pediatr Infect Dis J 2009;28(8):758–9.
[9] Horvat RT, Pentella M. From the arm to the test tube: laboratory's new role in tuberculosis testing. Clin Microbiol Newsl 2012;34(15):117–25.
[10] Qiagen QuantiFERON®-TB Gold Plus (QFT®-Plus). ELISA package insert Germany 2015.
[11] Cirillo DM, Barcellini L, Goletti D. Preliminary data on precision of QuantiFERON-TB Plus performance. Eur Respir J 2016;48(3):955–6.
[12] Hoffmann H, Avsar K, Gores R, Mavi SC, Hofmann-Thiel S. Equal sensitivity of the new generation QuantiFERON-TB Gold plus in direct comparison with the previous test version QuantiFERON-TB Gold IT. Clin Microbiol Infect 2016;22(8):701–3.
[13] John SH, Kenneth J, Gandhe AS. Host biomarkers of clinical relevance in tuberculosis: review of gene and protein expression studies. Biomarkers 2012;17(1):1–8.
[14] Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. Nat Rev Immunol 2011;11(5):343–54.
[15] Tebruegge M, Dutta B, Donath S, Ritz N, Forbes B, Camacho-Badilla K, et al. Mycobacteria-specific cytokine responses detect tuberculosis infection and distinguish latent from active tuberculosis. Am J Respir Crit Care Med 2015;192(4):485–99.
[16] Frahm M, Goswami ND, Owzar K, Hecker E, Mosher A, Cadogan E, et al. Discriminating between latent and active tuberculosis with multiple biomarker responses. Tuberculosis 2011;91(3):250–6.
[17] Harari A, Rozot V, Enders FB, Perreau M, Stalder JM, Nicod LP, et al. Dominant TNF-+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. Nat Med 2011;17(3):372–6.
[18] Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MOC. Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. Eur J Immunol 2009;39(3):723–9.
[19] Wang X, Jiang J, Cao Z, Yang B, Zhang J, Cheng X. Diagnostic performance of multiplex cytokine and chemokine assay for tuberculosis. Tuberculosis (Edinb) 2012 Nov;92(6):513–20.
[20] Wergeland I, Pullar N, Assmus J, Ueland T, Tonby K, Feruglio S, et al. IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy. J Infect 2015;70(4):381–91.
[21] Clifford V, Tebruegge M, Zufferey C, Germano S, Denholm J, Street A, et al. Serum IP-10 in the diagnosis of latent and active tuberculosis. J Infect 2015;71(6):696–8.
[22] Wallis RS. Infectious complications of tumor necrosis factor blockade. Curr Opin Infect Dis 2009;22(4):403–9.
[23] Toms C, Stapledon R, Waring J, Douglas P, the National tuberculosis Advisory committee ftCDNA, the Australian Mycobacterium reference laboratory Network. Tuberculosis notifications in Australia, 2012 and 2013. Comm Dis Intell 2015;39(2).
[24] Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig L, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. BMJ (Clin Res Ed) 2015;351. h5527.
[25] Armand M, Chhor V, de Lauzanne A, Guerin-El Khourouj V, Pedron B, Jeljeli M, et al. Cytokine responses to quantiferon peptides in pediatric tuberculosis: a pilot study. J Infect 2014 Jan;68(1):62–70.
[26] Biselli R, Mariotti S, Sargentini V, Sauzullo I, Lastilla M, Mengoni F, et al. Detection of interleukin-2 in addition to interferon-gamma discriminates active tuberculosis patients, latently infected individuals, and controls. Clin Microbiol Infect 2010;16(8):1282–4.
[27] Borgstrom E, Andersen P, Atterfelt F, Julander I, Kallenius G, Maeurer M, et al. Immune responses to ESAT-6 and CFP-10 by FASCIA and multiplex technology for diagnosis of M. tuberculosis infection; IP-10 is a promising marker. PLoS One 2012;7(11):e43438.
[28] Chiappini E, Della Bella C, Bonsignori F, Sollai S, Amedei A, Galli L, et al. Potential role of M. tuberculosis specific IFN- and IL-2 ELISPOT assays in discriminating children with active or latent tuberculosis. PLoS One [Electron Resource] 2012;7(9):e46041.
[29] Chesov D, Lange C, Daduna F, Crudu V, Preyer R, Ernst M, et al. Combined antigen-specific interferon-gamma and interleukin-2 release assay (FluoroSpot) for the diagnosis of Mycobacterium tuberculosis infection. PLoS One 2015;10(3):e0120006.
[30] Kim SY, Park MS, Kim YS, Kim SK, Chang J, Lee HJ, et al. The responses of multiple cytokines following incubation of whole blood from TB patients, latently infected individuals and controls with the TB antigens ESAT-6, CFP-10 and TB7.7. Scand J Immunol 2012;76(6):580–6.
[31] Wang X, Jiang J, Cao Z, Yang B, Zhang J, Cheng X. Diagnostic performance of multiplex cytokine and chemokine assay for tuberculosis. Tuberculosis (Edinb). 2012;92(6):513–20.
[32] Wang S, Diao N, Lu C, Wu J, Gao Y, Chen J, et al. Evaluation of the diagnostic potential of IP-10 and IL-2 as biomarkers for the diagnosis of active and latent tuberculosis in a BCG-vaccinated population. PLoS One 2012;7(12).
[33] Hur YG, Gorak-Stolinska P, Ben-Smith A, Lalor MK, Chaguluka S, Dacombe R, et al. Combination of cytokine responses indicative of latent TB and active TB in Malawian adults. PLoS One 2013;8(11):e79742.
[34] Sutherland JS, de Jong BC, Jeffries DJ, Adetifa IM, Ota MO. Production of TNF-alpha, IL-12(p40) and IL-17 can discriminate between active TB disease and latent infection in a West African cohort. PLoS One 2010;5(8):e12365.
[35] Wergeland I, Assmus J, Dyrhol-Riise AM. Cytokine patterns in tuberculosis infection; IL-1ra, IL-2 and IP-10 differentiate borderline quantiferon-TB samples from uninfected controls. PLoS One 2016;11(9):e0163848.

[36] Hur YG, Kang YA, Jang SH, Hong JY, Kim A, Lee SA, et al. Adjunctive biomarkers for improving diagnosis of tuberculosis and monitoring therapeutic effects. J Infect 2015;70(4):346–55.
[37] Jamil B, Shahid F, Hasan Z, Nasir N, Razzaki T, Dawood G, et al. Interferon gamma/IL10 ratio defines the disease severity in pulmonary and extra pulmonary tuberculosis. Tuberculosis 2007;87(4):279–87.
[38] Marin ND, Paris SC, Rojas M, Garcia LF. Functional profile of CD4+ and CD8+ T cells in latently infected individuals and patients with active TB. Tuberculosis 2013;93(2):155–66.
[39] Ruth JH, Bienkowski M, Warmington KS, Lincoln PM, Kunkel SL, Chensue SW. IL-1 receptor antagonist (IL-1ra) expression, function, and cytokine-mediated regulation during mycobacterial and schistosomal antigen-elicited granuloma formation. J Immunol 1996;156(7):2503–9.
[40] Demangel C, Bertolino P, Britton WJ. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. Eur J Immunol 2002;32(4):994–1002.
[41] Suzukawa M, Akashi S, Nagai H, Nagase H, Nakamura H, Matsui H, et al. Combined analysis of IFN-gamma, IL-2, IL-5, IL-10, IL-1RA and MCP-1 in QFT supernatant is useful for distinguishing active tuberculosis from latent infection. PLoS One 2016;11(4):e0152483.
[42] Jeong YH, Hur YG, Lee H, Kim S, Cho JE, Chang J, et al. Discrimination between active and latent tuberculosis based on ratio of antigen-specific to mitogen-induced IP-10 production. J Clin Microbiol 2015;53(2):504–10.
[43] Gourgouillon N, de Lauzanne A, Cottart CH, Curis E, Debord C, Guerin-El Khourouj V, et al. TNF-alpha/IL-2 ratio discriminates latent from active tuberculosis in immunocompetent children: a pilot study. Pediatr Res 2012;72(4):370–4.
[44] Kumar NP, Gopinath V, Sridhar R, Hanna LE, Banurekha VV, Jawahar MS, et al. IL-10 dependent suppression of type 1, type 2 and type 17 cytokines in active pulmonary tuberculosis. PLoS One [Electron Resource] 2013;8(3):e59572.
[45] Armand M, Chhor V, de Lauzanne A, Guerin-El Khourouj V, Pedron B, Jeljeli M, et al. Cytokine responses to quantiferon peptides in pediatric tuberculosis: a pilot study. J Infect 2014;68(1):62–70.
[46] Dhanasekaran S, Jenum S, Stavrum R, Ritz C, Faurholt-Jepsen D, Kenneth J, et al. Identification of biomarkers for Mycobacterium tuberculosis infection and disease in BCG-vaccinated young children in Southern India. Gene Immun 2013;14(6):356–64.
[47] Frahm M, Goswami ND, Owzar K, Hecker E, Mosher A, Cadogan E, et al. Discriminating between latent and active tuberculosis with multiple biomarker responses. Tuberculosis (Edinb). 2011;91(3):250–6.
[48] Kim SY, Park MS, Kim YS, Kim SK, Chang J, Lee HJ, et al. The responses of multiple cytokines following incubation of whole blood from TB patients, latently infected individuals, and controls with the TB antigens ESAT-6, CFP-10, and TB7.7. Scand J Immunol 2012 Dec;76(6):580–6.
[49] Nausch M, Lundtoft C, Schulz G, Henckel H, Mayatepek E, Fleischer B, et al. Multiple cytokines for the detection of Mycobacterium tuberculosis infection in children with tuberculosis. Int J Tubercul Lung Dis 2017;21(3):270–7.
[50] Suzukawa M, Akashi S, Nagai H, Nagase H, Nakamura H, Matsui H, et al. Combined analysis of IFN-gamma, IL-2, IL-5, IL-10, IL-1RA and MCP-1 in QFT supernatant is useful for distinguishing active tuberculosis from latent infection. PLoS One [Electron Resource] 2016;11(4):e0152483.
[51] Etna MP, Giacomini E, Severa M, Coccia EM. Pro- and anti-inflammatory cytokines in tuberculosis: a two-edged sword in TB pathogenesis. Semin Immunol 2014;26(6):543–51.
[52] Coppola M, van Meijgaarden KE, Franken KL, Commandeur S, Dolganov G, Kramnik I, et al. New genome-wide algorithm identifies novel in-vivo expressed Mycobacterium Tuberculosis antigens inducing human T-cell responses with classical and unconventional cytokine profiles. Sci Rep 2016;6:37793. https://doi.org/10.1038/srep37793.
[53] Awoniyi DO, Teuchert A, Sutherland JS, Mayanja-Kizza H, Howe R, Mihret A, et al. Evaluation of cytokine responses against novel Mtb antigens as diagnostic markers for TB disease. J Infect 2016 Sep;73(3):219–30.
[54] Lindestam Arlehamn CS, McKinney DM, Carpenter C, Paul S, Rozot V, Makgotlho E, et al. A quantitative analysis of complexity of human pathogen-specific CD4 T cell responses in healthy M. tuberculosis infected South africans. PLoS Pathog 2016;12(7):e1005760.
[55] Kassa D, de Jager W, Gebremichael G, Alemayehu Y, Ran L, Fransen J, et al. The effect of HIV coinfection, HAART and TB treatment on cytokine/chemokine responses to Mycobacterium tuberculosis (Mtb) antigens in active TB patients and latently Mtb infected individuals. Tuberculosis 2016;96:131–40.
[56] Chegou NN, Essone PN, Loxton AG, Stanley K, Black GF, van der Spuy GD, et al. Potential of host markers produced by infection phase-dependent antigen-stimulated cells for the diagnosis of tuberculosis in a highly endemic area. Erratum Appears PLoS One 2012;7(8). https://doi.org/10.1371/annotation/bc36a9c6-d5c0-4d55-bc92-9ce4a07b4f70. PLoS One [Electronic Resource]. 2012;7(6):e38501.
[57] Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet 2000;356(9235):1099–104.
[58] Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, et al. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am J Respir Crit Care Med 2004;170(1):59–64. Epub 2004 Apr 1.
[59] Tientcheu LD, Haks MC, Agbla SC, Sutherland JS, Adetifa IM, Donkor S, et al. Host immune responses differ between M. africanum- and M. tuberculosis-Infected patients following standard anti-tuberculosis treatment. PLoS Neglected Trop Dis 2016;10(5):e0004701.
[60] Kellar KL, Gehrke J, Weis SE, Mahmutovic-Mayhew A, Davila B, Zajdowicz MJ, et al. Multiple cytokines are released when blood from patients with tuberculosis is stimulated with Mycobacterium tuberculosis antigens. PLoS One 2011;6(11):e26545.
[61] Singh SB, Biswas D, Rawat J, Sindhwani G, Patras A, Devrani S, et al. Ethnicity-tailored novel set of ESAT-6 peptides for differentiating active and latent tuberculosis. Tuberculosis 2013;93(6):618–24.
[62] Pai M, Nicol MP, Boehme CC. Tuberculosis diagnostics: state of the art and future directions. Microbiol Spectr 2016;4(5). https://doi.org/10.1128/microbiolspec.TBTB2-0019-2016.
[63] Andersen P, Askgaard D, Ljungqvist L, Bennedsen J, Heron I. Proteins released from Mycobacterium tuberculosis during growth. Infect Immun 1991;59(6):1905–10.
[64] Aagaard C, Hoang T, Dietrich J, Cardona PJ, Izzo A, Dolganov G, et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat Med 2011;17(2):189–94.
[65] Losi M, Knights AJ, Mariani F, Altieri AM, Paone G, Loxton AG, et al. QuantiFERON-TB performance enhanced by novel Mycobacterium tuberculosis-specific antigens. Eur Respir J 2016;47(2):660–4.
[66] Michelsen SW, Soborg B, Diaz LJ, Hoff ST, Agger EM, Koch A, et al. The dynamics of immune responses to Mycobacterium tuberculosis during different stages of natural infection: a longitudinal study among greenlanders. PLoS One 2017;12(6):e0177906.
[67] Wyndham-Thomas C, Dirix V, Schepers K, Martin C, Hildebrand M, Goffard JC, et al. Contribution of a heparin-binding haemagglutinin interferon-gamma release assay to the detection of Mycobacterium tuberculosis infection in HIV-infected patients: comparison with the tuberculin skin test and the QuantiFERON-TB Gold In-tube. BMC Infect Dis 2015;15:59.
[68] Loxton AG, Black GF, Stanley K, Walzl G. Heparin-binding hemagglutinin induces IFN-gamma(+) IL-2(+) IL-17(+) multifunctional CD4(+) T cells during latent but not active tuberculosis disease. Clin Vaccine Immunol CVI 2012;19(5):746–51.
[69] Delogu G, Chiacchio T, Vanini V, Butera O, Cuzzi G, Bua A, et al. Methylated HBHA produced in M. smegmatis discriminates between active and non-active tuberculosis disease among RD1-responders. PLoS One 2011;6(3):e18315.
[70] Mensah GI, Addo KK, Tetteh JA, Sowah S, Loescher T, Geldmacher C, et al. Cytokine response to selected MTB antigens in Ghanaian TB patients, before and at 2 weeks of anti-TB therapy is characterized by high expression of IFN-gamma and Granzyme B and inter- individual variation. BMC Infect Dis 2014;14:495.
[71] Awoniyi DO, Teuchert A, Sutherland JS, Mayanja-Kizza H, Howe R, Mihret A, et al. Evaluation of cytokine responses against novel Mtb antigens as diagnostic markers for TB disease. J Infect 2016;73(3):219–30. https://doi.org/10.1016/j.jinf.2016.04.036. Epub Jun 14.
[72] Lindestam Arlehamn CS, McKinney DM, Carpenter C, Paul S, Rozot V, Makgotlho E, et al. A quantitative analysis of complexity of human pathogen-specific CD4 T cell responses in healthy M. tuberculosis infected South africans. PLoS Pathog 2016;12(7):e1005760https://doi.org/10.1371/journal.ppat. eCollection 2016 Jul.
[73] Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. Lancet 2016;387(10035):2312–22. https://doi.org/10.1016/S0140-6736(15)01316-1. Epub 2016 Mar 24.
[74] Anderson ST, Kaforou M, Brent AJ, Wright VJ, Banwell CM, Chagaluka G, et al. Diagnosis of childhood tuberculosis and host RNA expression in Africa. N Engl J Med 2014;370(18):1712–23. https://doi.org/10.056/NEJMoa1303657.
[75] Hur YG, Crampin AC, Chisambo C, Kanyika J, Houben R, Ndhlovu R, et al. Identification of immunological biomarkers which may differentiate latent tuberculosis from exposure to environmental nontuberculous mycobacteria in children. Clin Vaccine Immunol CVI 2014;21(2):133–42.
[76] Hermansen TS, Thomsen VO, Lillebaek T, Ravn P. Non-tuberculous mycobacteria and the performance of interferon gamma release assays in Denmark. PLoS One 2014;9(4):e93986.