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Mycobacteria-specific cytokine responses detect TB infection and distinguish latent from active TB

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At a glance commentary:

Scientific knowledge on the subject

Current immunodiagnostic tests for tuberculosis (TB), including the tuberculin skin test and interferon-gamma release assays (IGRA), have significant limitations. These include the inability to distinguish between latent TB infection (LTBI) and active TB, a distinction that is critical for clinical management.

What this study adds to the field

This study identified mycobacteria-specific cytokine responses that allow the distinction between TB-infected and TB-uninfected individuals, as well as between LTBI and active TB. Several of the cytokine biomarkers showed better performance characteristics than interferon-gamma, which forms the basis of IGRA. Addition of these biomarkers into future immunodiagnostic assays for TB is likely to result in higher assay sensitivity while retaining high specificity, and could potentially allow the distinction between LTBI and active TB based on a blood test alone.

ABSTRACT

Rationale: Current immunodiagnostic tests for tuberculosis (TB), including the tuberculin skin test (TST) and interferon-gamma (IFN-γ) release assays (IGRA), have significant limitations, which include their inability to distinguish between latent TB infection (LTBI) and active TB, a distinction critical for clinical management.

Objectives: To identify mycobacteria-specific cytokine biomarkers that characterize TB infection, to determine their diagnostic performance characteristics, and to establish whether these biomarkers can distinguish between LTBI and active TB.

Measurements and main results: 149 children investigated for TB infection were recruited; all participants underwent a TST and QuantiFERON-TB Gold assay. In parallel, whole blood assays using ESAT-6, CFP-10 and PPD as stimulatory antigens were undertaken, and cytokine responses were determined by xMAP multiplex assays. IFN-γ, IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β responses were significantly higher in LTBI and active TB cases than in TB-uninfected individuals, irrespective of the stimulant. Receiver operating characteristic analyses showed that IP-10, TNF-α, and IL-2 responses achieved high sensitivity and specificity for the distinction between TB-uninfected and TB-infected individuals. TNF-α, IL-1ra, and IL-10 responses had the greatest ability to distinguish between LTBI and active TB cases; the combinations of TNF-α/IL-1ra and TNF-α/IL-10 achieved correct classification of 95.5% and 100% of cases, respectively.

Conclusions: We identified several mycobacteria-specific cytokine biomarkers with the potential to be exploited for immunodiagnosis. Incorporation of these biomarkers

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into future immunodiagnostic assays for TB could result in substantial gains in sensitivity, and allow the distinction between LTBI and active TB based on a blood test alone.

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INTRODUCTION

Approximately one third of the world's population is infected with *Mycobacterium tuberculosis* (MTB), and there are more than 8 million new cases of active TB (ie TB disease) resulting in an estimated 1.3 million deaths each year.[1, 2] Children account for an increasing proportion of TB cases, in both high-resource and low-resource settings.[3]

Despite advances in diagnostics, microbiological confirmation of TB in childhood remains challenging, primarily because most children have paucibacillary disease, reducing the yield of conventional microbiological methods. Newer molecular diagnostic techniques, including the Xpert MTB/RIF assay, show promise, but also perform considerably less well in patients with paucibacillary disease.[4]

Existing immunodiagnostic tests for TB also have considerable limitations.[5, 6] The tuberculin skin test (TST) is operator-dependent and has limited specificity. False-positive results can occur as a result of boosting from repeated TST, prior Bacille Calmette-Guérin (BCG) vaccination, or infection with non-tuberculous mycobacteria (NTM).[7] The comparatively poor specificity of the TST intrinsically results from the use of purified protein derivative (PPD), a heterogeneous mixture of more than 200 mycobacterial peptides, many of which are expressed by BCG vaccine strains and NTM.[8] Interferon-γ (IFN-γ) release assays (IGRA), which rely on the detection of IFN-γ produced by sensitized T cells, are thought to have better specificity than the TST, as mycobacterial peptides that are absent from all BCG vaccine strains and most NTM are used as the stimulatory antigens in these assays.[8] Currently licensed IGRA (the QuantiFERON-TB Gold (QFT) assay and the T-SPOT.*TB* assay) incorporate the

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MTB-specific RD1 peptide antigens early secretory antigenic target 6 (ESAT-6) and 10 kDa culture filtrate protein (CFP-10). However, IGRA have other, important limitations. Although IGRA are solely licensed for the diagnosis of latent TB infection (LTBI), in clinical practice these assays are frequently used to support a presumptive diagnosis of active TB. However, a recent meta-analysis shows that the sensitivity of both commercial IGRA barely exceeds 80% in patients with active TB.[9] Furthermore, IGRA perform considerably worse in children, and significant rates of indeterminate results are an additional problem in this age group.[10-14]

A further significant limitation of both TST and IGRA is their inability to discriminate between LTBI and active TB, a distinction that is highly relevant in geographic regions where TB prevalence is high and a large proportion of the population has LTBI.[6] This distinction is critical, as the treatment of LTBI and active TB differs. Consequently, an immunodiagnostic test that can discriminate between these two infection states would be a major advance for clinical care.

A large body of evidence supports the critical role of IFN-γ in the immune response to mycobacterial infections.[15, 16] However, mounting data highlight the importance of other cytokines in the immune response to MTB, which may also have a potential to be used for immunodiagnosis.[15-17] This study aimed to identify mycobacteria-specific cytokine biomarkers that characterize TB infection, to determine their diagnostic performance characteristics, and to establish whether any of the identified biomarkers can be used to distinguish between LTBI and active TB.

METHODS

Participants

Children and adolescents up to 18 years of age were recruited at the Royal Children's Hospital (RCH) Melbourne between January 2010 and February 2011. Eligible for participation were all children undergoing screening for suspected LTBI or active TB, comprising the following: i) children with symptoms and signs suggestive of active TB, ii) children with known contact with a case of active TB, iii) children who had recently migrated from countries with a high TB prevalence (defined by incidence ≥ 40 TB cases/100,000 population). The exclusion criteria comprised the following: i) known immunodeficiency, ii) current immunosuppressive treatment (including oral corticosteroids), and iii) TST in the previous 6 to 52 weeks. The last criterion was chosen, because at commencement of the study it was thought that a TST undertaken more than 6 weeks prior to an IGRA may induce boosting, thereby causing a false-positive IGRA result.[18] We have subsequently shown this not to be the case.[19]

Prior to participation, informed consent was obtained from the child's parent and/or guardian. The study was approved by the RCH Human Research Ethics Committee (HREC 29040A). Demographics, history and clinical findings were recorded on a standardized data collection sheet. A chest x-ray was obtained in all cases with a positive TST and/or positive IGRA result. Histological, conventional and molecular microbiological tests were performed in all children with suspected active TB as clinically indicated.

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Diagnostic tests

All participants had a TST placed by specifically-trained nurses by intradermal injection of 0.1 ml of Tubersol (Sanofi Pasteur; Toronto, Canada; bioequivalent to 5 Tuberculin Units PPD-S) into the volar surface of the lower arm, and any resulting induration was recorded after 48 to 72 hours. In addition, blood was obtained for the QFT In-Tube (QFT-GIT) assay, and an additional 10 ml for whole blood assays were collected in heparinized tubes. The QFT-GIT was processed and interpreted at the Victorian Infectious Diseases Reference Laboratories (VIDRL) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) testing for *M. tuberculosis,* for which a Taqman real-time PCR (Applied Biosystems; Waltham, MA) targeting the insertion sequence IS6110 was used, was also performed at VIDRL using previously described methods.[20]

Categorization of participants and definitions

Participants were categorized into seven categories according to their clinical features, TST, IGRA and microbiological results as detailed in Table 1. Active TB was defined as either i) microbiological confirmation of infection with MTB by culture or polymerase chain reaction (PCR), or ii) a symptomatic patient fulfilling at least two of the following three criteria in conjunction with response to treatment with anti-tuberculous therapy: a) symptoms and signs consistent with active TB (chronic cough, persistent fever, night sweats, unexplained weight loss), b) radiological findings suggestive of active TB, c) presence of risk factors for TB infection (known TB contact, birth or previous residence in a high TB prevalence country). These stringent criteria exceed those proposed by the American Thoracic Society and the Centers for

Disease Control and Prevention.[21] In this manuscript, the term 'TB-infected' is used as a collective term for participants with LTBI or active TB.

Whole blood assays

Whole blood was incubated with ESAT-6, CFP-10 (each at a concentration of 10 μg/ml; JPT Peptide Technologies, Berlin, Germany), PPD (20 μg/ml; RT50; Statens Serum Institut, Copenhagen, Denmark), staphylococcal enterotoxin B (SEB) (5 μg/ml; positive control; Sigma-Aldrich, St. Louis, MO), or without stimulant (negative control) in the presence of co-stimulatory antibodies, anti-CD28 and anti-CD49d (each 1 μg/ml; BD Biosciences, San Jose, CA). Mycobacterial antigens were added at the beginning of the assay; SEB was only added for the last 5 hours of the incubation period based on previous optimization experiments (data not shown). Following incubation at 37°C for 20 to 24 hours, supernatants were harvested and cryopreserved at -80°C for batched analysis.

Cytokine analysis

Cytokine concentrations in supernatants were measured using Milliplex human cytokine/chemokine kits (Millipore Corp., Billerica, MA) according to the manufacturer's instruction, with analyses conducted on a Luminex 200 analyzer (Luminex Corp., Austin, TX). Based on previous optimization experiments (data not shown), IFN-γ, tumor necrosis factor-alpha (TNF-α), IL-1ra, IL-2, IL-10, IL-12(p40), IL-13, IL-15, IL-17, and granulocyte-macrophage colony stimulating factor (GM-CSF) were analyzed in undiluted samples with a 10-plex assay, while interferon-inducible protein-10 (IP-10), IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), monocyte chemotactic protein-3 (MCP-3), macrophage inflammatory protein-1-beta

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(MIP-1β), and CCL5 (RANTES) were analyzed in 1:20 diluted samples with a 7-plex assay. The laboratory scientists performing the sample analysis were blinded to the clinical data and the results of the TST, the QFT-GIT and the microbiological investigations.

Statistical analysis

Only those participants with an unambiguous diagnosis ('uninfected', 'common discordance', 'LTBI' and 'active TB') were included in the analysis. Participants in the 'probable uninfected' and 'possible discordance' categories were deliberately not included *a priori* to avoid potential contamination of data.

Comparisons of continuous variables between multiple groups were done using Kruskal Wallis tests. In instances where the Kruskal Wallis p-value was < 0.05, indicating a difference between the groups, additional two-group comparisons were done using Mann Whitney *U* tests. Categorical variables were compared using two-tailed chi-square tests. A p-value < 0.05 was considered significant. Cytokine concentrations were background-corrected prior to analysis (ie by subtracting the concentration measured in the negative control sample). Analyses were done using Stata (V11; StataCorp, College Station, TX) and Prism (V5; GraphPad Software Inc., La Jolla, CA). Receiver operating characteristic (ROC) analyses were performed with Prism; the optimal cut-offs for each of the stimulant/cytokine combinations were determined by tabulation of sensitivity against specificity at every threshold in the data set. The study was conducted and is reported in accordance with Quality Assessment of Diagnostic Accuracy Studies (QADAS) criteria.[22]

RESULTS

A total of 149 patients were recruited. Nine participants were excluded: three did not return for TST reading, insufficient blood was obtained in three, a laboratory error occurred during the QFT-GIT processing in one, and the QFT-GIT result was indeterminate in two (both failed positive controls). Therefore, a total of 140 patients were included in the analysis.

The six participants with active TB comprised three patients with microbiologically-confirmed TB (one case with intra-thoracic TB; two cases with lymph node TB), and three patients without microbiological confirmation who fulfilled the study criteria for active TB (two cases with pulmonary TB; one case with spinal TB). All six patients had both a positive TST (range: 13 to 25 mm induration) and a positive QFT-GIT result, as well as resolution of symptoms with anti-tuberculous therapy. Additional patient data are shown in Supplementary Table 1.

Table 2 shows the demographic and other details of the participants in the four major diagnostic categories (uninfected, common discordance, LTBI and active TB). In the remaining three diagnostic categories there were 6 ‘probable uninfected’, 5 ‘possible discordance’ and 4 ‘reverse discordance’ cases.

Mycobacteria-specific cytokine responses in supernatants

Significant differences in the background-corrected cytokine concentrations measured in supernatants between the four major diagnostic groups in response to all three antigenic stimulants were detected for IFN-γ, IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β (Figure 1A-C). Overall, median concentrations of these cytokines were

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highest in the active TB group, followed by the LTBI group. For all seven cytokines the lowest median concentrations were observed in the uninfected group, irrespective of the antigenic stimulant.

Table 3 shows the results of the two-group comparisons of cytokine responses in the four diagnostic groups. Median concentrations of IFN-γ, IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β were all significantly higher in the LTBI and the active TB groups, compared with the uninfected group, with all three antigenic stimulants, with the single exception of IL-1ra in PPD-stimulated samples. Most of these comparisons were highly statistically significant with a Mann Whitney *U* p-value below 0.0001. In contrast, few comparisons between the LTBI and the active TB group reached statistical significance: TNF-α in ESAT-6- and CFP-10-stimulated samples, IL-1ra in ESAT-6- and PPD-stimulated samples, and IP-10, IL-6 and IL-10 in PPD-stimulated samples. In all instances median concentrations were higher in the active TB group than in the LTBI group (Figure 1), with the exception of IP-10 in PPD-stimulated samples. In addition, a number of significant differences in median cytokine concentrations between the uninfected group and the group with common discordance were detected (Table 3). This was the case not only in response to stimulation with PPD, but also in response to stimulation with ESAT-6 and CFP-10.

Receiver operating characteristic analyses

To determine the potential for diagnostic use of the seven cytokine responses that were found to have discriminatory ability in the previous analyses, ROC analyses were performed. For this purpose the data from patients with LTBI and active TB were grouped together (TB-infected group; case values), and compared with the data

from the uninfected group (control values). At their optimal cut-off values, IP-10, TNF-α, and IL-2 achieved sensitivity and specificity values close to, or exceeding those of IFN-γ (Table 4). The best performance characteristics were observed with IL-2 in PPD-stimulated samples (sensitivity: 100%; specificity: 96%).

In addition, ROC curves were constructed for each cytokine/stimulant combination (Figure 2). The very high area under curve (AUC) values for IFN-γ, IP-10, TNF-α, and IL-2 (irrespective of the stimulant used) further support the potential of these cytokines to discriminate between TB-uninfected and TB-infected individuals. Notably, the AUC values of IP-10, TNF-α and IL-2 in ESAT-6- and CFP-10-stimulated samples universally exceeded those of IFN-γ.

Ability of cytokine responses to discriminate between LTBI and active TB

Among the cytokines investigated TNF-α, IL-1ra and IL-10 responses were found to have the greatest ability to discriminate between LTBI and active TB (Figure 3). At a cut-off of 80 pg/ml in ESAT-6-stimulated samples, and 40 pg/ml in CFP-10-stimulated samples, TNF-α responses correctly classified 81.8% and 86.4% of the participants, respectively. In PPD-stimulated samples IL-1ra responses (cut-off: 450 pg/ml) and IL-10 responses (cut-off: 100 pg/ml) correctly classified 90.9% and 100% of the participants, respectively. Figure 4 shows that combining TNF-α with either of these two cytokines results in very high levels of correct classification. The combination TNF-α/IL-1ra only classified one participant with LTBI falsely as ‘active TB’ (95.5% correct classification); the combination TNF-α/IL-10 classified all participants correctly.

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DISCUSSION

Our data show that in addition to IFN-γ, which forms the basis of existing immunodiagnostic tests for TB, several other MTB-specific cytokine responses, namely IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β responses, differ significantly between individuals infected with MTB and those with no evidence of infection, indicating their potential as diagnostic biomarkers of TB infection. Notably, the performance characteristics of some of these cytokine responses, including IP-10, TNF-α, and IL-2, were similar to, or exceeded those of IFN-γ.

Importantly, our data also show that certain cytokine responses, including TNF-α, IL-1ra and IL-10, in addition to identifying TB infection, may simultaneously allow the distinction between LTBI and active TB. Further, we have shown that high levels of discriminatory accuracy can be achieved by combining these biomarkers. This potentially represents a significant advance, as current immunodiagnostic tests (i.e. TST and IGRA) are unable to make this distinction.[5] From a clinical perspective, the ability to discriminate between LTBI and active TB based on a blood test alone, which can provide a result within two days, would be an important advantage, as this would allow clinicians to make timely management decisions, rather than having to wait for culture results, which can take several weeks.

Several recent studies have aimed to identify biomarkers of TB infection, and some have also attempted to identify biomarkers that differ between cases with LTBI and active TB. Harari *et al.*, who used multi-color flow cytometry to analyze responses to stimulation with RD1 antigens (ESAT-6 and CFP-10), reported that MTB-specific CD4+ T cells producing only TNF-α are the hallmark of active TB, while

polyfunctional CD4+ T cells producing IFN-γ, IL-2 and TNF-α are characteristic of LTBI.[23] Based on their findings, the authors suggested measurement of single-positive TNF-α+ CD4+ T cells could be used in the diagnostic setting to distinguish between both infection states. However, the high cost of flow cytometry and the need for highly-trained personnel limit its usefulness in resource-limited, high TB prevalence settings where better TB diagnostics are needed most. Our data suggest that phenotypic analysis of the cellular origin of TNF-α may not be necessary, and that cytokine measurement in supernatant, which could be achieved with much simpler methods, is likely to be sufficient.

A number of studies have previously highlighted the diagnostic potential of IP-10 (CXCL10).[24-30] In agreement with our findings, those studies showed that MTB-specific IP-10 responses in TB-infected individuals are of greater magnitude than IFN-γ responses, and that this biomarker lacks the ability to distinguish between LTBI and active TB.[24-28] This is not surprising as IP-10 production in polymorphonuclear neutrophils and monocytes is primarily induced by IFN-γ that also lacks this discriminatory ability (as reflected by IGRA lacking this ability).[31] MTB-specific IP-10 responses likely represent an amplified read-out of IFN-γ responses, meaning they differ quantitatively, but not qualitatively.

In our study, IL-2 responses had greater sensitivity and specificity than IFN-γ responses for distinguishing between TB-uninfected and TB-infected individuals. Animal models show that IL-2 plays an important role in the anti-mycobacterial host immune response,[32, 33] but few studies have investigated the diagnostic potential of MTB-specific IL-2 responses.[25, 26, 34-39] Similar to our findings, two of these studies

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reported that RD1 antigen-induced IL-2 responses differ between TB-uninfected and TB-infected individuals, but not between LTBI and active TB.[26, 36] Interestingly, data from one study using ELISpot assays suggested that combining the measurement of IFN-γ responses with IL-2 responses results in increased sensitivity for detecting TB infection.[37] However, ELISpot assays are labor-intensive and difficult to integrate into a routine diagnostic laboratory setting. Our data show that measuring IL-2 in supernatants from whole blood assays is a suitable, more practicable alternative.

Few studies have investigated MTB-specific IL-1ra responses as a diagnostic tool.[26, 40, 41] Similar to our findings, one study found that RD1 antigen-induced IL-1ra responses were significantly higher in active TB than in LTBI.[26] However, there was a substantial overlap between the IL-1ra responses in both groups, suggesting that a categorical separation between infection states based on this biomarker alone would be difficult. Interestingly, we found that while there was considerable overlap between the magnitude of IL-1ra responses in LTBI and active TB cases when RD1 antigens were used for stimulation, there was very little overlap when PPD was used. The likely explanation for this is that PPD-stimulation resulted in several-fold higher IL-1ra responses in cases with active TB compared with RD1 antigen-stimulation (Figure 1), thereby resulting in greater differences between the two groups.

We also found that combining cytokine biomarkers resulted in accurate discrimination between LTBI and active TB. The combination of TNF-α and IL-1ra responses correctly classified 95.5% of cases, while the combination of TNF-α and IL-10 responses resulted in correct classification of all cases. The concept of combining two or more cytokine biomarkers to improve the distinction between

infection states has been explored previously. Frahm *et al.* used a model based on MCP-1 and IL-15 responses, but only achieved 86.4% correct classification with this approach.[26] Wang *et al.* used an IL-2/IFN-γ ratio, which achieved a sensitivity and specificity of 77.2% and 87.2%, respectively.[39] A study by Sutherland *et al.* that compared anti-mycobacterial cytokine responses in patients with LTBI (defined as TST-positive individuals) with active TB patients reported that in PPD-stimulated samples a combination of TNF-α, IL-12(p40) and IL-13 responses correctly classified 81% of cases.[42]

This study has also produced interesting data in relation to patients with discordance, who remain a significant management dilemma in clinical practice.[5, 7, 10, 11] It has been postulated that a TST+/IGRA- discordant result constellation is predominately the result of false-positive TST results, primarily resulting from prior BCG vaccination. However, while BCG vaccination can produce false-positive TST results due to cross-reactivity of antigens present in PPD and antigens produced by BCG vaccine strains, solid evidence to support that this accounts for the majority of cases with discordance is lacking. Notably, the results of a meta-analysis, involving more than 200,000 children in 24 studies, suggest that only 8.5% of individuals BCG-vaccinated in infancy develop a false-positive TST (defined as ≥ 10 mm induration),[43] indicating that BCG vaccination alone is unlikely to account for the large proportions of discordance reported in most pediatric IGRA studies.[44-46] In our study, we observed multiple statistically significant differences between the cytokine responses in the uninfected group and the common discordance group. Importantly, these differences were also detected in samples stimulated with ESAT-6 and CFP-10, which cannot be explained by antigenic cross-reactivity, as both peptides are absent from all BCG

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vaccine strains.[8] However, there was also considerable overlap between the responses observed in both groups, with some patients with common discordance showing no response to antigenic stimulation, highlighting that these patients are likely a heterogeneous group comprising both TB-infected and TB-uninfected individuals.

For all cytokines identified as potential TB biomarkers in this study there are convincing data indicating that they play an important role in the anti-mycobacterial host immune response. The central role of TNF-α in the human immune response to mycobacterial infections is undisputed.[15] Multiple studies have shown that patients treated with TNF-α inhibitors for autoimmune conditions are at significant risk of TB progression.[47, 48] The importance of IL-1ra in the anti-mycobacterial immune response, particularly related to granuloma formation, has been extensively documented in animal models.[49] Recent reports of TB reactivation in patients treated with monoclonal IL-1ra further highlight the importance of this cytokine in this setting.[48]. Infection of murine macrophages with MTB has been shown to result in upregulation of MIP-1β expression.[50] Also, progression of TB disease is associated with increased MIP-1β expression in murine lung tissue.[50] *In vitro* studies in humans have shown that infection of macrophages with MTB results in increased production of MIP-1β, which suppresses intracellular growth of MTB.[51] IL-13 is a key cytokine in the alternative activation of macrophages, which is associated with macrophage fusion and granuloma formation, a critical event in mycobacterial infections.[52, 53]

The main limitation of this study is the inclusion of a limited number of patients with active TB, a limitation shared by many other studies in this area.[23, 26, 27, 29, 34, 38] Nonetheless the differences in cytokine responses were sufficiently large to enable the

detection of statistical differences between the diagnostic groups. An important strength of this study is the use of unambiguous diagnostic groups in the analyses. Many previous immunodiagnostic studies have included patients with uncertain TB infection status (eg solely based on TST results) or uncertain active TB cases (eg 'possible' and 'probable TB' cases based on clinical features alone), and therefore have an inherent risk for data contamination to occur. One limitation of all diagnostic biomarker studies is that cut-offs and performance characteristics are based on the data from the study population, thus potentially overestimating their performance. Our study population did not include cases at the most 'severe' end of the disease spectrum (i.e. miliary TB or TB meningitis); therefore the performance of our cytokine biomarkers in these patients remains uncertain. In addition, the majority of children with active TB were older than 10 years of age and it is therefore uncertain whether their performance is equally robust in infants and young children. Consequently, further evaluation of these biomarkers is required in larger cohorts with a broad range of disease manifestations. Further studies in both children and in adults are currently ongoing.

In conclusion, our study shows that a number of MTB-specific cytokine responses, including IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β, allow the distinction between individuals infected with TB and those without TB infection. Importantly, some of these biomarkers had better performance characteristics than IFN-γ. In addition, we have identified biomarkers that distinguish between LTBI and active TB potentially with high levels of accuracy. Incorporation of these biomarkers in future immunodiagnostic assays for TB could result in substantial gains in assay sensitivity,

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and may allow the distinction between LTBI and active TB based on a blood test alone.

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Table 1. Criteria for categorization of study participants

Diagnostic category	TST induration	QFT-GIT assay result
Uninfected	0 mm	Negative
Probable uninfected	1 – 4 mm	Negative
Possible discordance	5 – 9 mm	Negative
Common discordance	≥ 10 mm	Negative
LTBI	≥ 10 mm	Positive
Active TB	*	*
Reverse discordance	< 10 mm	Positive

* Microbiological confirmation OR presence of 2 of 3 diagnostic criteria in conjunction with response to anti-tuberculous treatment (irrespective of TST and IGRA result; see methods section).

IGRA: interferon-gamma release assays; LTBI: latent TB infection; QFT-GIT: QuantiFERON-TB Gold In-Tube assay; TST: tuberculin skin test.

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Table 2. Demographic and other details of study participants in each diagnostic category

	Total cohort (n=140)	Uninfected (n=75)	Common discordance (n=28)	LTBI (n=16)	Active TB (n=6)
Median age, years (IQR)	8.3 (3.7 – 12.8)	6.3 (2.8 – 10.9)	12.1 (6.0 – 14.5)	11.6 (6.0 – 14.2)	15.0 (12.1 – 16.2)
Ethnic origin, no. (%)					
Africa	60 (42.8)	31 (41.3)	9 (32.1)	10 (62.5)	5 (83.3)
Asia	53 (37.9)	24 (32.0)	13 (46.4)	5 (31.3)	1 (16.7)
Middle East	9 (6.4)	5 (6.7)	4 (14.3)	0	0
Australia/New Zealand	18 (12.9)	15 (20)	2 (7.1)	1 (6.3)	0
Country of birth, no. (%)					
Africa	41 (29.3)	14 (18.7)	8 (28.6)	10 (62.5)	5 (83.3)
Asia	29 (20.7)	10 (13.3)	7 (25.0)	5 (31.3)	1 (16.7)
Middle East	9 (6.4)	4 (5.3)	4 (14.3)	0	0
Australia/New Zealand	59 (42.1)	47 (62.7)	8 (28.6)	1 (6.3)	0
Europe	2 (1.4)	0	1 (3.6)	0	0
Migration background*, no. (%)	81 (57.9)	28 (37.3)	20 (71.4)	15 (93.8)	6 (100)
Time of migration to Australia*, months (IQR)	8.0 (3.5 – 36.0)	5.5 (2.0 – 33.0)	17.0 (6.0 – 36.0)	7.0 (4.0 – 36.0)	21.0 (3.0 – 69.0)
BCG vaccination history, no. (%)					
Yes	75 (53.6)	23 (30.7)	21 (75.0)	14 (87.5)	5 (83.3)
No	58 (42.4)	49 (65.3)	4 (14.2)	1 (6.3)	1 (16.7)
Unknown	7 (5.0)	3 (4.0)	3 (10.7)	1 (6.3)	0
BCG scar, no. (%)					
Yes	67 (47.9)	22 (29.3)	18 (64.3)	12 (75.0)	5 (83.3)
No	73 (52.1)	53 (70.7)	10 (35.7)	4 (25.0)	1 (16.7)
Known TB contact, no. (%)					
Yes	89 (63.6)	54 (72.0)	14 (50.0)	8 (50.0)	1 (16.7)
No	51 (36.4)	21 (28.0)	14 (50.0)	8 (50.0)	5 (83.3)
Type of TB contact, no. (%)					
Parent	34 (24.3)	14 (18.6)	9 (32.1)	4 (25.0)	1 (16.7)
Other household member	33 (23.6)	23 (30.7)	3 (10.7)	3 (18.8)	0
Other contact	22 (15.7)	17 (22.7)	2 (7.1)	1 (6.3)	0

* excludes migrants from New Zealand. Abbreviations: no.: number; IQR: interquartile range

Table 3. Comparisons of median cytokine responses (p-values) from the data shown in figure 1. Statistically significant p-values are highlighted in bold.

Stimulant	Cytokine	Kruskal Wallis p-value	Uninfected vs. CD	Uninfected vs. LTBI	Uninfected vs. Active TB	CD vs. LTBI	CD vs. Active TB	LTBI vs. Active TB
ESAT-6	**IFN-γ**	**< 0.0001**	**0.0147**	**< 0.0001***	**< 0.0001***	**0.0001***	**0.0008***	0.3020
	IP-10	**< 0.0001**	**0.0026**	**< 0.0001***	**< 0.0001***	**< 0.0001***	**0.0007***	0.8828
	TNF-α	**< 0.0001**	0.0504	**< 0.0001***	**< 0.0001***	**< 0.0001***	**0.0004***	**0.0183**
	IL-1ra	**< 0.0001**	0.2925	**0.0005***	**< 0.0001***	**0.0180**	**0.0007***	**0.0183**
	IL-2	**< 0.0001**	**< 0.0001***	**< 0.0001***	**< 0.0001***	**< 0.0001***	**0.0010***	0.5070
	IL-6	0.0642	-	-	-	-	-	-
	IL-8	0.7564	-	-	-	-	-	-
	IL-10	0.1042	-	-	-	-	-	-
	IL-12(p40)	**0.0061**	**0.0080**	**0.0255**	0.0540	0.6258	0.2433	0.3451
	IL-13	**< 0.0001**	**0.0269**	**< 0.0001***	**< 0.0001***	**0.0127**	**0.0006***	0.0650
	IL-15	0.4083	-	-	-	-	-	-
	IL-17	0.1647	-	-	-	-	-	-
	GM-CSF	**< 0.0001**	0.6012	**< 0.0001***	**0.0058**	**< 0.0001***	**0.0129**	0.7124
	MCP-1	0.1701	-	-	-	-	-	-
	MCP-3	0.1038	-	-	-	-	-	-
	MIP-1β	**< 0.0001**	0.1883	**< 0.0001***	**< 0.0001***	**0.0005***	**0.0006***	0.0900
	RANTES	0.3249	-	-	-	-	-	-
CFP-10	**IFN-γ**	**< 0.0001**	0.1588	**< 0.0001***	**< 0.0001***	**0.0128**	**0.0011***	0.0900
	IP-10	**< 0.0001**	0.2630	**< 0.0001***	**< 0.0001***	**0.0013***	**0.0018**	0.6058
	TNF-α	**< 0.0001**	0.2908	**< 0.0001***	**< 0.0001***	**0.0029**	**0.0005***	**0.0121**
	IL-1ra	**< 0.0001**	0.4653	**0.0013***	**0.0003***	**0.0338**	**0.0025**	0.1048
	IL-2	**< 0.0001**	**0.0202**	**< 0.0001***	**< 0.0001***	**0.0007***	**0.0021**	0.4174
	IL-6	0.1354	-	-	-	-	-	-
	IL-8	0.8776	-	-	-	-	-	-
	IL-10	**0.0367**	**0.0279**	**0.0250**	0.2635	0.7325	0.8037	0.9412
	IL-12(p40)	0.1895	-	-	-	-	-	-
	IL-13	**< 0.0001**	**0.0556**	**0.0066**	**< 0.0001***	**0.1368**	**0.0004***	0.0765
	IL-15	0.9146	-	-	-	-	-	-
	IL-17	0.4539	-	-	-	-	-	-
	GM-CSF	**0.0013**	0.4064	**0.0012***	**0.0104**	**0.0192**	**0.0377**	0.7681
	MCP-1	0.1643	-	-	-	-	-	-
	MCP-3	**0.0155**	**0.0149**	**0.0120**	0.2948	0.5310	1	0.9105
	MIP-1β	**< 0.0001**	0.4630	**0.0041**	**< 0.0001***	**0.0404**	**0.0018**	0.1404
	RANTES	0.1390	-	-	-	-	-	-
PPD	**IFN-γ**	**< 0.0001**	**< 0.0001***	**< 0.0001***	**< 0.0001***	0.0832	**0.0025**	0.1845
	IP-10	**< 0.0001**	**< 0.0001***	**< 0.0001***	**0.0007***	0.2134	0.5877	**0.0270**
	TNF-α	**< 0.0001**	**< 0.0001***	**< 0.0001***	**< 0.0001***	**0.0180**	**0.0016***	0.0553
	IL-1ra	**0.0040**	**0.0154**	0.6843	**0.0044**	0.0570	0.3203	**0.0032**
	IL-2	**< 0.0001**	**< 0.0001***	**< 0.0001***	**< 0.0001***	**0.0043**	**0.0025**	0.4610
	IL-6	**< 0.0001**	**< 0.0001***	**< 0.0001***	**0.0001***	0.5747	**0.0100**	**0.0122**
	IL-8	0.0719	-	-	-	-	-	-
	IL-10	**0.0007**	0.3737	**0.0022**	**0.0058**	0.0790	**0.0239**	**0.0004***
	IL-12(p40)	**< 0.0001**	**< 0.0001***	**0.0002***	**< 0.0001***	0.3602	0.0377	0.5070
	IL-13	**0.0003**	**0.0007***	**0.0061**	**0.0173**	0.8073	0.8922	0.3763
	IL-15	0.2734	-	-	-	-	-	-
	IL-17	0.3771	-	-	-	-	-	-
	GM-CSF	**< 0.0001**	**< 0.0001***	**< 0.0001***	**0.0002***	0.4068	0.1245	0.4610
	MCP-1	0.4549	-	-	-	-	-	-
	MCP-3	0.2129	-	-	-	-	-	-
	MIP-1β	**< 0.0001**	**0.0004***	**0.0002***	**0.0024***	0.3667	0.3907	0.7681
	RANTES	0.3126	-	-	-	-	-	-

CD: common discordance (TST+/IGRA-); LTBI: latent TB infection

* Significant at Bonferroni-corrected significance level of 0.0016

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Table 4. Results of the receiver operating characteristic analyses of the seven mycobacteria-specific cytokine responses with the potential ability to discriminate between TB-uninfected and TB-infected individuals

	IFN-γ			IP-10			TNF-α			IL-1ra			IL-2			IL-13			MIP-1β		
	cut-off	sens. (%)	spec. (%)	cut-off	sens. (%)	spec. (%)	cut-off	sens. (%)	spec. (%)	cut-off	sens. (%)	spec. (%)	cut-off	sens. (%)	spec. (%)	cut-off	sens. (%)	spec. (%)	cut-off	sens. (%)	spec. (%)
ESAT-6	10.2	**90.9**	**97.3**	10820.0	**95.5**	**96.0**	5.99	**95.5**	**88.0**	-28.5	81.8	69.3	16.7	**95.5**	**97.3**	0.32	77.3	89.3	158.6	90.9	70.7
CFP-10	0.8	**81.8**	**89.3**	2204.0	**86.4**	**93.3**	1.9	**86.4**	**85.3**	-23.6	77.3	78.7	4.7	**90.9**	**94.7**	0.05	63.6	86.7	50.2	77.3	68.0
PPD	659.4	**95.5**	**97.3**	46571.0	**95.5**	**81.3**	125.3	**95.5**	**84.0**	279.7	50.0	46.7	383.7	**100.0**	**96.0**	103.5	77.3	69.3	9618.0	81.8	60.0

All cut-offs are in pg/ml. Bold figures indicate that both sensitivity and specificity exceed 80%. Abbreviations: sens.: sensitivity; spec.: specificity.

Figure 1. Box plot with Tukey whiskers showing background-corrected cytokine concentrations in uninfected participants (white) and participants with common discordance (yellow), LTBI (orange) and active TB (red) in ESAT-6 (Part A), CFP-10 (Part B) and PPD (Part C) stimulated samples. Statistical differences were analyzed using Kruskal Wallis tests; the corresponding p-values are shown at the top of each graph. The results of additional two-group comparisons are detailed in Table 3.

Figure 2. Receiver operating characteristic curves for IFN-γ, IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β responses according to antigenic stimulant (ESAT-6, CFP-10, PPD). Case values comprised background-corrected cytokine concentrations in participants with LTBI or active TB (TB-infected group); control values comprised cytokine concentrations in uninfected participants.

Figure 3. Background-corrected TNF-α, IL-1ra and IL-10 concentrations in participants with LTBI (circles) and active TB (squares) in ESAT-6, CFP-10 and PPD stimulated samples. The bars indicate the median concentrations. The dotted lines indicate the optimal cut-offs for the distinction between participants with LTBI and those with active TB. Statistical differences were analyzed using Mann-Whitney *U* tests; the corresponding p-values are shown above each bracket.

Figure 4. Background-corrected TNF-α, IL-1ra and IL-10 concentrations in uninfected participants (diamonds), participants with LTBI (circles) and active TB (squares) in PPD stimulated samples. Part A shows the combination of TNF-α/IL-1ra, part B the combination of TNF-α/IL-10. The dotted lines indicate the optimal cut-offs for the distinction between participants with LTBI and those with active TB (TNF-α: 800 pg/ml; IL-1ra: 450 pg/ml; IL-10: 100 pg/ml).

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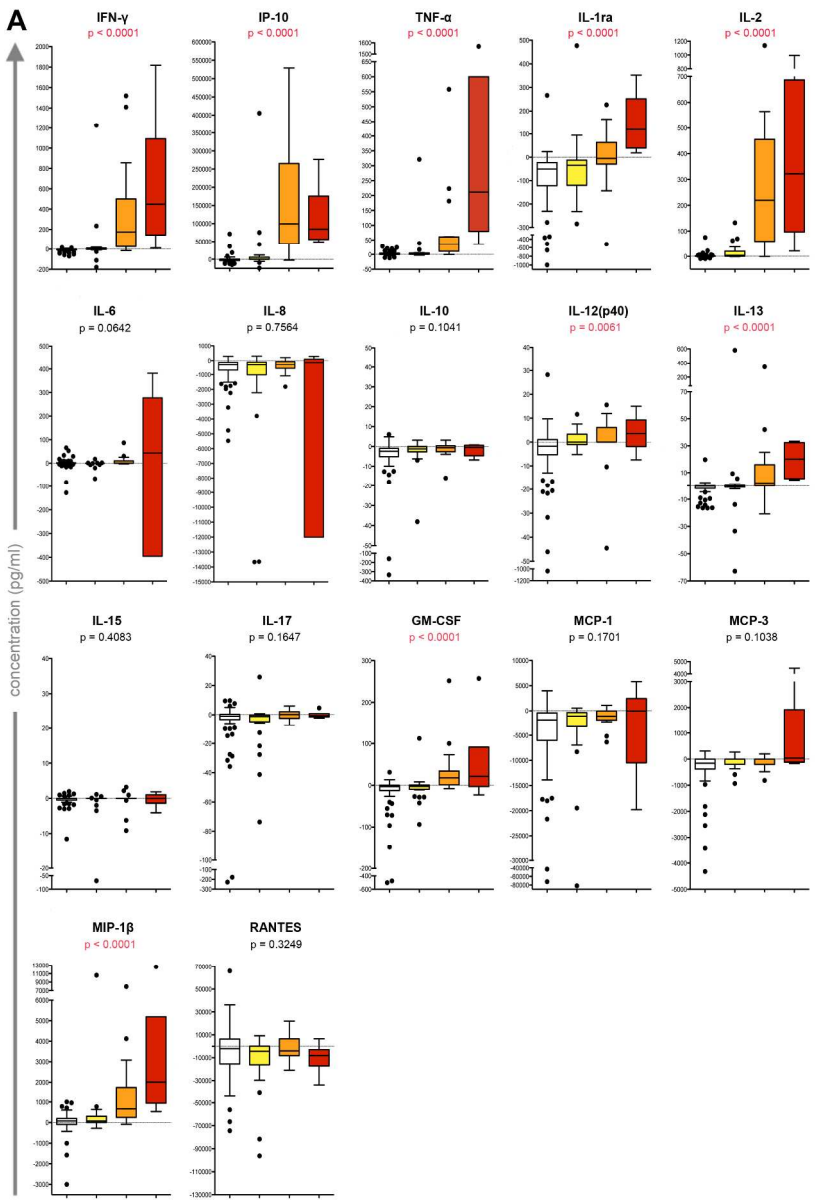

Figure 1. Box plot with Tukey whiskers showing background-corrected cytokine concentrations in uninfected participants (white) and participants with common discordance (yellow), LTBI (orange) and active TB (red) in ESAT-6 (Part A), CFP-10 (Part B) and PPD (Part C) stimulated samples. Statistical differences were analyzed using Kruskal Wallis tests; the corresponding p-values are shown at the top of each graph. The results of additional two-group comparisons are detailed in Table 3.
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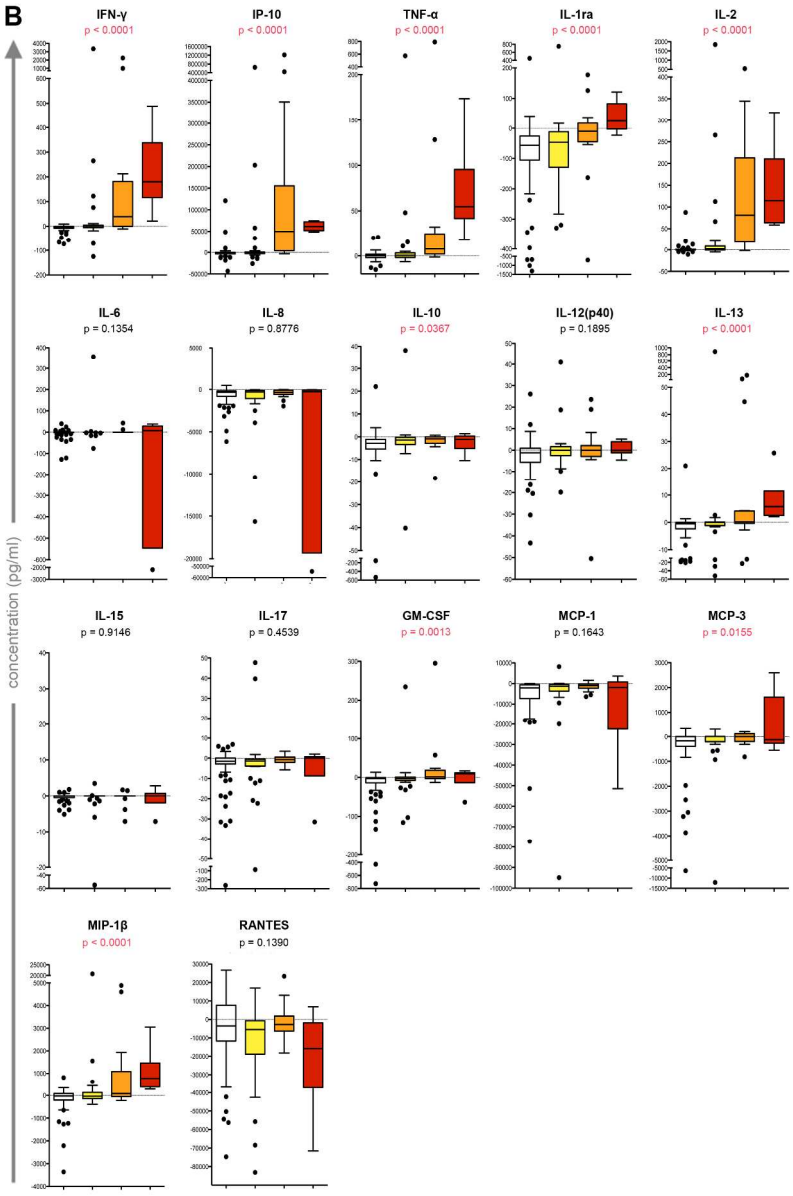
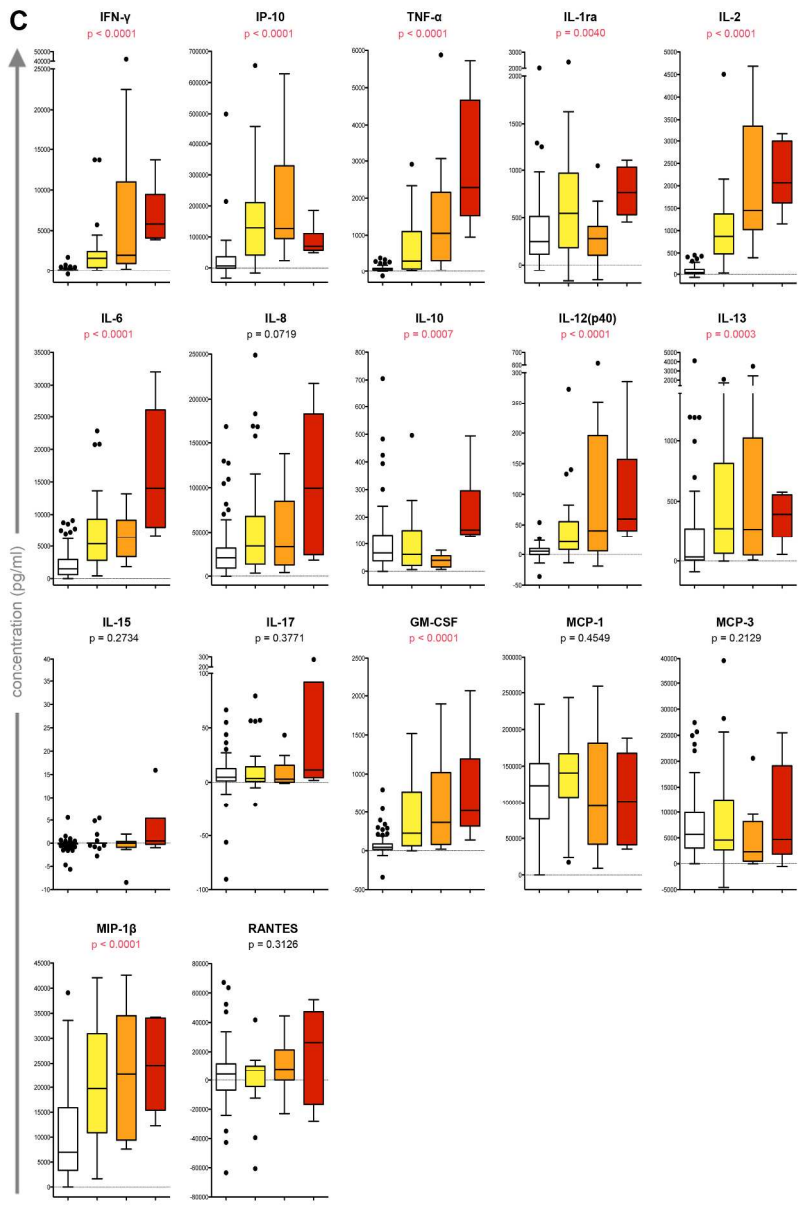


Figure 1. Box plot with Tukey whiskers showing background-corrected cytokine concentrations in uninfected participants (white) and participants with common discordance (yellow), LTBI (orange) and active TB (red) in ESAT-6 (Part A), CFP-10 (Part B) and PPD (Part C) stimulated samples. Statistical differences were analyzed using Kruskal Wallis tests; the corresponding p-values are shown at the top of each graph. The results of additional two-group comparisons are detailed in Table 3.
191x284mm (300 x 300 DPI)



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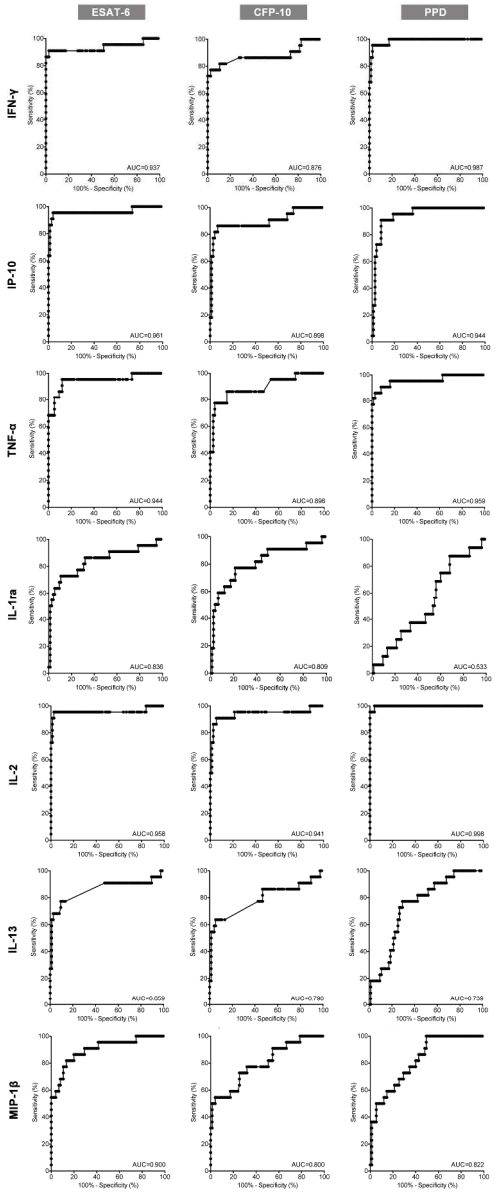

Figure 2. Receiver operating characteristic curves for IFN-γ, IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β responses according to antigenic stimulant (ESAT-6, CFP-10, PPD). Case values comprised background-corrected cytokine concentrations in participants with LTBI or active TB (TB-infected group); control values comprised cytokine concentrations in uninfected participants.
118x285mm (300 x 300 DPI)

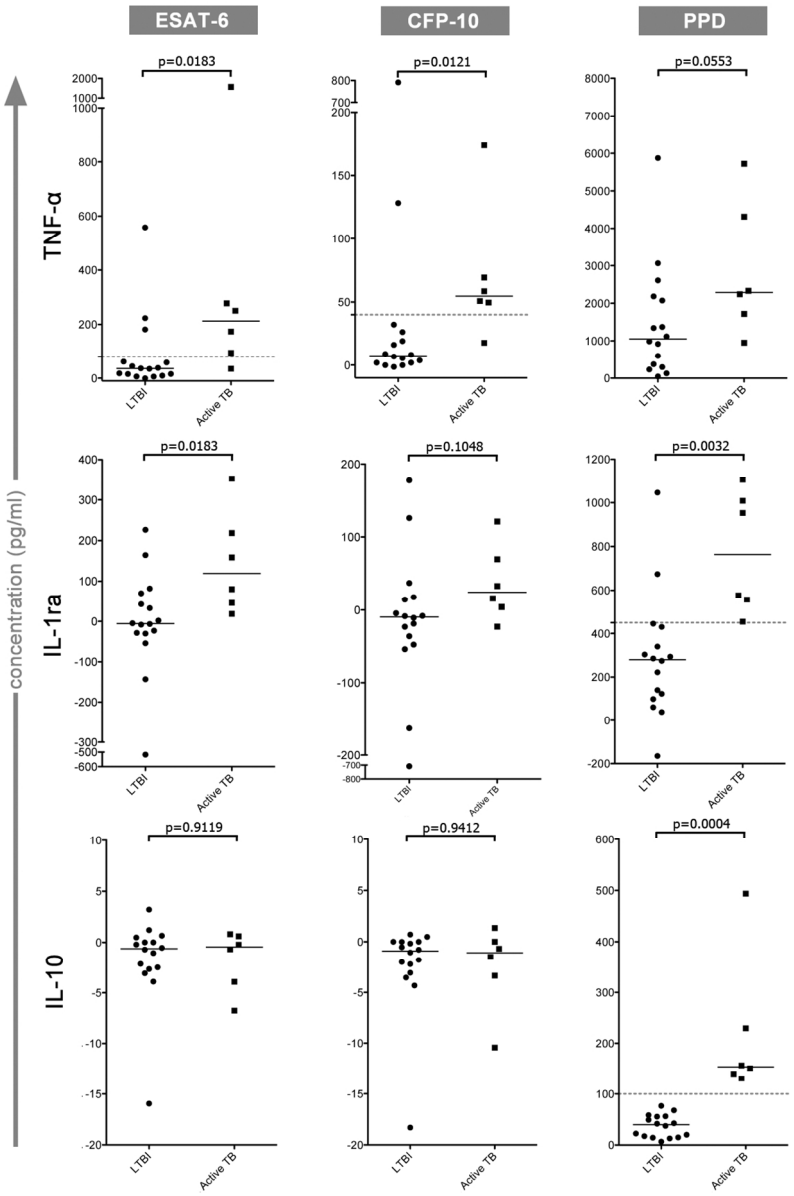

Figure 3. Background-corrected TNF-α, IL-1ra and IL-10 concentrations in participants with LTBI (circles) and active TB (squares) in ESAT-6, CFP-10 and PPD stimulated samples. The bars indicate the median concentrations. The dotted lines indicate the optimal cut-offs for the distinction between participants with LTBI and those with active TB. Statistical differences were analyzed using Mann-Whitney U tests; the corresponding p-values are shown above each bracket.
100x148mm (300 x 300 DPI)

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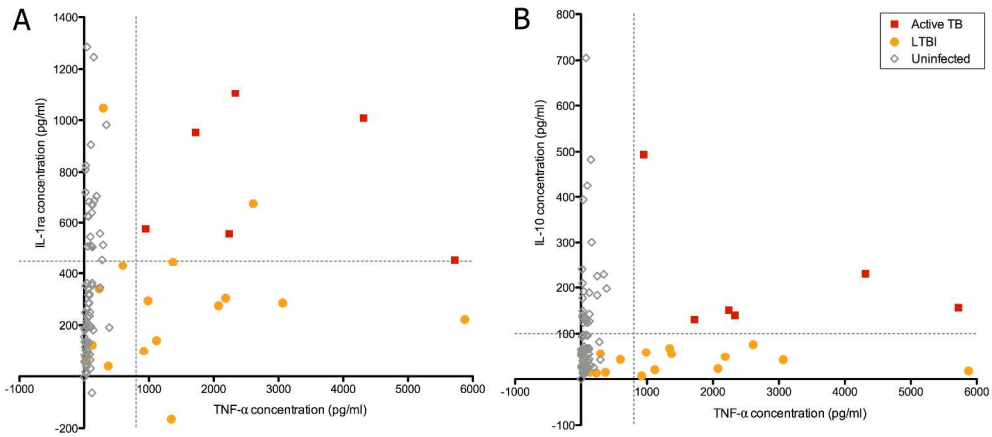

Figure 4. Background-corrected TNF-α, IL-1ra and IL-10 concentrations in uninfected participants (diamonds), participants with LTBI (circles) and active TB (squares) in PPD stimulated samples. Part A shows the combination of TNF-α/IL-1ra, part B the combination of TNF-α/IL-10. The dotted lines indicate the optimal cut-offs for the distinction between participants with LTBI and those with active TB (TNF-α: 800 pg/ml; IL-1ra: 450 pg/ml; IL-10: 100 pg/ml).
339x149mm (300 x 300 DPI)

Supplementary Table 1. Demographic details, clinical findings and investigations in study participants with active TB

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age (years)	7.3	13.8	14.7	15.3	15.9	17.0
TB risk factors	Birth in high TB prevalence country (Kenya) Household TB contact (parent sputum-smear positive)	Birth in high TB prevalence country (India)	Birth in high TB prevalence country (Sudan)	Birth in high TB prevalenc e country (Somalia)	Birth in high TB prevalence country (Somalia)	Birth in high TB prevalence country (Somalia)
History of BCG vaccination / scar	Yes / Yes	Yes / Yes	Yes / Yes	Yes / Yes	No / No	Yes / Yes
Disease manifestation	Pulmonary TB	Cervical lymphadeni tis	Cervical & mediastinal lymphadenitis	Chest wall / pleural mass	Pulmonary TB	Spinal TB
Symptoms	Chronic cough	Persistent fever, night sweats, weight loss, enlarged, non-tender lymph nodes	Enlarged, non-tender lymph nodes	Chest wall lesion	Chronic cough, persistent fever, night sweats, weight loss	Weight loss, back pain, spinal deformity
TST result (mm induration)	Positive (25 mm)	Positive (13 mm)	Positive (14 mm)	Positive (18 mm)	Positive (20 mm)	Positive (22 mm)
QFT-GIT result	Positive	Positive	Positive	Positive	Positive	Positive
Radiological findings	CXR: hilar lymphadenopat hy and pulmonary infiltrates	CXR: calcified apical pulmonary focus	CXR: mediastinal lymphadenopat hy	Chest CT: pleural mass with extension into chest wall	CXR: hilar lymphadenopat hy, atelectasis, pulmonary infiltrates Chest USS: pleural effusion	CXR: hilar lymphadenopat hy Spinal MRI: vertebral body destruction and contrast enhancement
Sample(s) taken for microbiologi cal tests	Sputum samples	Lymph node tissue	Lymph node tissue	Biopsy from lesion	Sputum samples	BAL
Culture result	Negative	Positive	Positive	Positive	Negative	Negative
PCR result	-	Positive	Positive	Positive	Negative	Negative
Histology	-	Caseating granulomat a	Caseating granulomata	Caseating granuloma ta	-	-
Response to anti-TB treatment	Yes	Yes	Yes	Yes	Yes	Yes

BAL: bronchoalveolar lavage; CT: computed tomography; CXR: chest x-ray; MRI: magnetic resonance imaging; QFT-GIT: QuantiFERON-TB Gold in-Tube assay;
TB: tuberculosis; USS: ultrasound scan