Beyond the IFN-γ horizon: biomarkers for immunodiagnosis of infection with *Mycobacterium tuberculosis*

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ABSTRACT Latent infection with *Mycobacterium tuberculosis* (LTBI) is defined by the presence of *M. tuberculosis*-specific immunity in the absence of active tuberculosis. LTBI is detected using interferon-γ release assays (IGRAs) or the tuberculin-skin-test (TST). In clinical practice, IGRAs and the TSTs have failed to distinguish between active tuberculosis and LTBI and their predictive value to identify individuals at risk for the future development of tuberculosis is limited.

There is an urgent need to identify biomarkers that improve the clinical performance of current immunodiagnostic methods for tuberculosis prevention, diagnosis and treatment monitoring. Here, we review the landscape of potential alternative biomarkers useful for detection of infection with *M. tuberculosis*. We describe what individual markers add in terms of specificity for active/latent infection, prediction of progression to active tuberculosis and immunodiagnostic potential in high-risk groups' such as HIV-infected individuals and children.

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Introduction

Tuberculosis remains a leading cause of morbidity and mortality worldwide with 7 million annual cases and 2 million deaths [1]. An estimated 2 billion individuals have immune reactivity towards *Mycobacterium tuberculosis* without clinical, radiological or microbiological disease. These persons are, per definition, considered to have subclinical infection, traditionally referred to as latent tuberculosis infection (LTBI), and provide an enormous potential reservoir of persons with a future risk of tuberculosis [2, 3]. However, the majority will remain healthy in spite of a positive immunodiagnostic test. It is thus unclear if the immunodiagnostic tests and herewith the concept of latency, actually reflect true infection or immunological memory [4, 5].

For almost a century, immune diagnosis of LTBI was performed using the tuberculin skin test (TST) [6]. Approximately ten years ago, an *in vitro* alternative to the TST, the interferon (IFN)-γ release assays (IGRAs), was introduced [7]. IGRAs were designed to address the problem of low specificity of the TST, thus providing more accurate diagnosis and better prediction of progression to active TB. However, it is now apparent that IGRAs only perform marginally better in this respect [8]. New initiatives are needed.

The advent of simple and rapid bead-based multiplex assays has allowed for quantification of multiple cytokines and chemokines as alternative immunodiagnostic markers to IFN-γ. Several new markers are suggested to be specific for tuberculosis or LTBI, and to indicate a high risk of progression to active tuberculosis, but these data are preliminary.

This review describes the concept of LTBI and current methods for the detection of immune responses to *M. tuberculosis* and indicators for risk of active TB; we provide an overview of the landscape of alternative immunodiagnostic markers and explore the potential of these markers to serve as tools in the management of TB.

References for this review were identified through PubMed and Google Scholar searches using the following terms: ''tuberculosis'', ''IGRA'', ''interferon release assay'', ''cytokine'', ''chemokine'', ''multiplex'', ''ESAT-6'', ''CFP10'' and in-depth searches relating to the individual cytokines.

Immunodiagnosis of *M. tuberculosis* infection

The interaction between *M. tuberculosis* and the infected host is complex and incompletely understood. During LTBI, the host immune system is able to contain the live bacilli within the granuloma structure, but it is unknown whether all persons with a positive immunodiagnostic test actually harbour live bacilli [5]. Recently, it was proposed that the concept of latent tuberculosis should be considered a continuous spectrum ranging from near active tuberculosis with obvious lesions containing live bacilli, to cleared infection with no or only minimal risk of developing disease [4, 9]. As is reflected in their poor predictive value, neither IGRA nor TST is able to differentiate the various underlying subgroups of this spectrum [4, 10].

Protective immunity and mycobacterial containment depends on a wide range of innate and adaptive immune mechanisms [11, 12]. Pro-inflammatory T-helper (Th) type 1 cells are essential for phagocyte activation to promote killing of intracellular *M. tuberculosis* and for chemo-attraction of immunocompetent cells to the site of infection [13, 14]. Regulatory and anti-inflammatory responses dampen excessive tissue destruction, and play an essential role in the establishment of protection and infection containment within granuloma [15, 16]. *M. Tuberculosis*-specific T-cells representing both pro- and anti-inflammatory aspects of infection control are readily detectable in peripheral blood, and provide the basis of the immunodiagnostic tests [17].

For almost a century, the TST was the only available diagnostic modality to assess presence of *M. tuberculosis* infection and prediction of risk of progression to active tuberculosis [6, 18]. This immunodiagnostic test is based on delayed-type hypersensitivity skin reaction to tuberculin, a mixture of antigenic compounds in extracts of mycobacterial culture filtrates [19, 20]. A major drawback to the TST is low specificity in certain groups of patients. Antigens in tuberculin are also recognised in Bacille Calmette–Guérin (BCG)-vaccinated individuals and persons with previous sensitisation to non-tuberculous mycobacteria (NTM), potentially leading to false-positive reactions [6]. Additionally, completing the TST requires two visits by the patient and measurement of reaction size is subjective [21].

The identification of a set of *M. tuberculosis* genes that are deleted in BCG and most NTMs pathogenic to humans and, at the same time, highly recognised by most presumed infected humans, led to the development of the IGRAs. These *in vitro* tests utilise *M. Tuberculosis*-specific T-cells present in a blood sample capable of responding by the secretion of cytokines during incubation with the *M. Tuberculosis*-specific gene products. Two IGRAs are commercially available today: the whole-blood and ELISA-based QuantiFERON-Gold In Tube test (QFT; Qiagen, Düsseldorf, Germany) and the peripheral blood mononucleated cell (PBMC)- and ELISPOT-based T-SPOT.TB test (Oxford Immunotec, Abingdon, UK).

Both IGRAs incorporate the region of difference 1 (RD1)-encoded 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP10) antigens, whereas an additional single peptide from TB7.7, encoded in RD11, is added to the QFT [22–26].

IFN-γ and the immunology of IGRA

IFN-γ is the archetypical readout for cell-mediated immune response (CMI) assays [27], and has been recognised as the defining cytokine of Th1 cells. In the IGRAs, IFN-γ primarily derives from specific Th1 cells recognising their peptide presented on monocytes which act as antigen presenting cells (APC) [28]. IFN-γ release is augmented by APC-derived tumour necrosis factor (TNF)-α and interleukin (IL)-12 and autocrine IFN-γ [29]. The IFN-γ response reaches a plateau 10–72 h after stimulation, depending on sample, assay and type of stimulating antigen/mitogen [30–32]. IFN-γ is central in immune activation, mediating transcriptional regulation of >200 genes through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. The multiple actions of IFN-γ include increased bactericidal activity of phagocytes, stimulation of antigen presentation, B cell isotype switching, cellular proliferation and apoptosis [33–35]. *In vivo*, IFN-γ is crucial in the orchestration of the leukocyte–endothelial interactions and attraction of immunocompetent cells to sites of inflammation. IFN-γ synchronises this process by upregulating the expression of adhesion molecules and secretion of multiple chemokines including IFN-γ-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), monokine induced by IFN-γ (MIG), macrophage inflammatory protein (MIP)-1α/β and regulated on activation, normal T-cell expressed and secreted (RANTES) [35], many of which seem promising IFN-γ substitutes in immunodiagnostic tests.

Detection of a specific immune response as correlate of infection

When evaluating the performance of immunodiagnostic tests, it is essential to distinguish between the detection of a specific immune response to the pathogen and the clinically more important prediction of future progression to active disease.

IGRAs were designed to provide a more specific measure of an immune response against *M. tuberculosis* compared with TST. The diagnostic algorithms guiding IGRA interpretation were developed from case–control studies comparing patients with confirmed active tuberculosis to unexposed healthy contacts and cutoffs for positive tests were set at the IFN-γ release level that best separated cases from controls [36]. Meta-analyses have established that IGRAs are indeed very specific for detection of *M. tuberculosis* infection also in BCG-vaccinated individuals, and that IGRAs detect approximately four out of five people with confirmed active tuberculosis [37]. In exposed individuals with no symptoms of active disease, IGRAs appear comparable or better associated with surrogate measures of infection compared with TST [38]. Patients with immunosuppression are clinically difficult groups, as the immune system necessary for good test performance is compromised or immature, and risk of developing disease is high for the same reason. In HIV-infected people, a decrease in CD4 cell count compromises IGRA and TST accuracy, but IGRAs appear more robust than TST in this population [39–41]. In young children, IGRA test results are frequently indeterminate, but several studies suggest that the IGRA results can be positive in TST-negative children indicative of better sensitivity [42, 43].

Immunodiagnostics for early detection of active TB

The main clinical application of immunodiagnostic tests is to identify individuals at risk for the future development of tuberculosis [44, 45]. The risk of progression depends on the age and immune status of the person at risk, time since exposure, virulence of the mycobacterial strain, *etc.* For example, the progression rate in QFT-positive untreated individuals was 12.5% among close contacts from the UK with low probability of infection prior to exposure [46]; but only 2.8% in recently exposed immigrant close contacts from The Netherlands, many of whom presumably had a well-controlled latent infection from earlier in life [47]. In contrast, the negative predictive value of an immunodiagnostic test is very high (>98% in most studies [38]), suggesting that although many individuals with positive tests never progress, the tests do classify the persons without risk correctly.

IGRAs detect infection more accurately than TSTs, mainly by reducing the number of false-positive results, due to BCG vaccination [44], but it is now apparent that the IGRAs do not add much as indicators for risk of active tuberculosis [10]. Therefore, the immunodiagnostic tests cannot stand alone and prophylactic treatment decisions must take into account the person's immune status and pretest probability of infection.

IGRA: a blueprint for next generation tests

The concept of immunodiagnosis based on *in vitro* cell-mediated immune recognition has been a popular blueprint for the development of possible next generation tests based on new antigens and new biomarkers.

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The nature of the antigen used for stimulation is central for test sensitivity, specificity and possibly also the predictive potential. Natural immunity to *M. tuberculosis* is highly individual, multi-epitopic and multi-antigenic, and more than 80 antigens are necessary to capture 80% of the *M. Tuberculosis*-specific T-cell response [13]. The currently used antigens ESAT-6, CFP10 and TB7.7 were selected for their high immunogenicity and specificity for *M. tuberculosis* infection, not for their predictive potential [26, 36]. ESAT-6 is considered among the most immunogenic proteins, but it is secreted in the whole spectrum of latency and also in active stages of the infection, thus strongly suggesting that disease stage-specific diagnosis is impossible using ESAT-6 [13, 22, 48]. New immunogenic and specific antigens, *e.g.* associated with *M. tuberculosis* infection phases, have been described as well as antigens that could render ESAT-6 nonessential in the antigen cocktail [13, 49–56]. Tests based on new antigens are needed if a vaccine based on ESAT-6 proves to be efficacious in humans [48, 57, 58]. However, it remains to be shown if stage-specific antigens have potential for diagnostic applications. Although the choice of antigen is central to immunodiagnosis of *M. tuberculosis* infection, it is beyond the scope of this review to discuss details in depth.

The search for and reliance on highly immunogenic antigens for IGRA diagnostics is, at least in part, driven by the need for strong IFN-γ responses for reliable analytical accuracy in the measurements. It is now clear that multiple cytokine and chemokine markers are expressed in concert with IFN-γ, some of which at 10–1000-fold higher levels. High levels of biomarker suggest improved detection of immune recognition, *e.g.* of subdominant antigens potentially with better predictive power for development of TB or for use in vaccine immunogenicity studies [59].

The landscape of potential immunodiagnostic biomarkers

Before addressing the question of whether potential novel immunodiagnostic markers can improve the management of individuals with presumed LTBI, we explored which alternative cytokine and chemokines are consistently and specifically expressed in response to IGRA-peptide antigen stimulation in whole blood or PBMCs from cases with confirmed tuberculosis.

Table 1 summarises the results from a literature search of potential immunodiagnostic biomarkers expressed in whole blood and PBMC culture. Across studies we found a panel of cytokine and chemokine markers associated to Th1 cell activity and IFN-γ mediated signalling consistently upregulated in patients with confirmed tuberculosis. Markers associated with Th2 cell activity or general inflammation are expressed at lower magnitude and show poorer association with confirmed infection.

In the following subsections, we describe the underlying immunology of the most consistently expressed and most explored immunodiagnostic markers. We discuss their potential to detect a CMI response in patients with confirmed tuberculosis or presumed LTBI.

IL-2

IL-2 is mainly produced by antigen-activated T-cells, but also by natural killer and dendritic cells. IL-2 is cardinal for adaptive immune activity. Binding of antigen to the T-cell receptor stimulates IL-2 secretion and the expression of the IL-2 receptor [99], and IL-2 receptor ligation activates the JAK/STAT pathway leading to growth, proliferation, T-cell differentiation to effector T-cells and establishment of T-cell memory [100].

The kinetics of IL-2 release are comparable to IFN-γ, but the magnitude of response is lower [60, 62, 101, 102]. Case–control studies comparing adult patients with active tuberculosis to healthy controls, suggest that IL-2 has comparable sensitivity for active TB and specificity in unexposed controls as IFN-γ and IP-10 [60, 82, 102, 103]. A similar ability has also been shown for presumed latent infection defined by both IGRA/TST response [69, 94] and exposure gradient [102, 103]. In contrast, other studies suggest that IL-2 expression is lower in patients with tuberculosis compared to latently infected individuals and controls [98, 104], this will be discussed in detail later.

IP-10

IP-10 (chemokine (C-X-C motif) ligand (CXCL)10) is a chemokine secreted by APCs upon stimulation by multiple cytokines; mainly IFN-γ and TNF-α, as well as IFN-α/β, IL-2, IL-17, IL-27 and IL-1β. IP-10 is also induced through cell-surface receptor interaction with T-cells [105–110]. IP-10 shares the chemokine (C-X-C motif) receptor (CXCR)3 with MIG and IFN-inducible T-cell α chemoattractant (I-TAC), an important receptor involved in the regulation of innate and adaptive immune responses through chemotaxis, cell growth and angiostasis [108, 111–113]. In IP-10 release assays, IP-10 is secreted by monocytes directly interacting with the antigen specific T-cell, and from bystander cells responding to the T-cell derived cytokines [66, 70, 114]. IP-10 mRNA expression and protein release follow the same kinetics as IFN-γ, but at levels 100-fold higher than IFN-γ [66, 107, 115] (T. Blauenfeldt and M. Ruhwald, Dept of Infectious Disease Immunology, Statens Serum Institut, Copenhagen; unpublished data).

TABLE 1 The landscape of potential immunodiagnostic biomarkers expressed in whole blood and peripheral blood mononucleated cell culture

Potential immunodiagnostic biomarkers	>10 fold induction	<10 fold induction	No induction
Chemokine			
CXCL8/IL-8	[60]	[61]	[62–65]
CXCL9/MIG	[62, 66–68]	[69, 70]	[71, 72]
CXCL10/IP-10	[51, 60, 62, 63, 65, 66, 69, 72–85]		[61, 72]
CCL2/MCP1	[60, 62]	[63, 71, 78]	[65, 69, 72, 86, 87]
CCL3/MIP-1α	[60, 88]		[62, 65, 69, 71, 86,]
CCL4/MIP-1β	[60, 62, 85, 89]		[65, 69]
CCL7/MCP3		[78]	
CCL8/MCP2	[78, 90, 91]	[91]	[77]
Interferon			
IFN-α			[62, 69]
Tumour necrosis factor			
TNF-α	[92, 93]	[51, 63, 64]	[60–62, 65, 69, 86, 89, 94, 95]
TNF-β		[64]	[86]
Interleukin			
IL-1β		[64, 86]	[60–62, 65]
IL-2	[60, 62, 63, 65, 82, 83, 85, 86, 94]	[69, 91]	[64, 77]
IL-4	[60]	[63]	[51, 60, 62, 64, 69, 85, 86]
IL-5	[93]	[65, 78, 86]	[64, 69, 85]
IL-6	[60, 85]	[61, 64]	[62, 65, 69]
IL-12(p70)/(p40)	[62]	[61]	[51, 60, 61, 63, 65, 69, 85, 86, 92]
IL-13	[85, 93]	[65, 94]	[60, 62, 69, 92, 95]
IL-15	[62]	[63, 69]	[65]
IL-17	[96]		[62, 65, 69, 85, 92, 95]
IL-22	[96]		
Growth factor			
EGF	[51]	[89]	[65]
TGF-β		[51]	[63, 86]
VEGF		[89]	[51, 60, 65]
Soluble receptor			
sCD40L		[89]	[65]
IL-1RA	[62]	[78]	[69]

CXCL: chemokine (C-X-C motif) ligand; IL: interleukin; MIG: monokine induced by interferon-γ; IP-10: interferon-γ-induced protein 10; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein; IFN: interferon; TNF: tumour necrosis factor; EGF: epidermal growth factor; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; sCD40L: soluble CD40 ligand; IL-1RA: IL-1 receptor antagonist. Several markers showed no or only little diagnostic potential including GRO (CXCL1) [60], stromal cell-derived factor 1 (CXCL12) [97], Fractalkine (CXC3CL1) [65, 89], I-309 (chemokine (C-C motif) ligand (CCL) 1) [97], regulated on activation, normal T-cell expressed and secreted (CCL5) [62, 69, 86], eotaxin (CCL11) [62, 65, 69], IFN-α [51, 62, 69, 98], IL-1α [65, 89], IL-7 [62, 65, 69], IL-10 [51, 60–65, 68, 69, 71, 72, 92, 94, 95, 97], IL-18 [63, 86, 92], granulocyte colony-stimulating factor [65], granulocyte–macrophage colony-stimulating factor [60, 62, 65, 69, 86], TGFα [65, 89], insulin-like growth factor-1β [65], soluble IL-2 receptor [62], soluble IL-2 receptor agonist [65], soluble IL-6 receptor [86], matrix metallopeptidase 9 [86], triggering receptor expressed on myeloid cells 1 [86].

IP-10 is the most extensively investigated alternative immunodiagnostic biomarker. Studies in patients with active tuberculosis and unexposed controls find comparable sensitivity and specificity between IP-10 release assays and IGRAs [62, 67, 73–76, 84, 107, 116–120]. Several studies show that IFN-γ and IP-10 can be combined, to significantly improve sensitivity for active tuberculosis (2–11% increase) without a compromise in the rate of false-positive responders [73–75, 90, 120]. Two studies in adult household contacts compared with cases with active tuberculosis concluded that IP-10 detects a similar number of exposed individuals as IGRAs [117, 118] and have comparable increases in test positivity with increasing age in the population [118]. A French study in healthcare workers, found IP-10 positive in all eight QFT positive, and in 32% of 41 healthcare workers with negative QFT and positive TST [69]. Similar discordance was observed in a Chinese study of 73 healthy household contacts. In this study, IP-10 classified 56%

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contacts as positive compared with 38–40% positive with QFT and IL-2, and IP-10 showed a stronger association with risk factors for LTBI [102].

MIG-γ

MIG-γ (CXCL9) is mainly expressed by monocytes and macrophages. MIG is strongly induced by IFN-γ, but not IFN-α/β or other T-cell cytokines involved in IP-10 release. TNF-α is incapable of inducing MIG alone, but does synergise with IFN-γ [109, 121]. MIG binds the CXCR3 receptor and induces the similar downstream immune effector functions as IP-10 and I-TAC. It thereby participates in a complex collaborative network of which MIG is the only agonist exclusively mediating the signal of adaptive immune activation [108, 121].

BRICE *et al.* [122] introduced MIG as an amplified correlate of IFN-γ in CMI assays. MIG is induced specifically to *M. tuberculosis* antigen stimulation *in vitro*, and secretion follows a similar pattern and shows a high degree of correlation to IFN-γ and IP-10 [62, 70]. MIG is released at high levels; although not as impressive as seen for other chemokines [62, 66, 69, 123], and responses are more variable compared to IL-2, IFN-γ or IP-10 [62, 67, 71]. KASPROWICZ *et al.* [66] compared MIG and IP-10 detected with real-time quantitative PCR (RT-qPCR) and found IP-10 10-fold more sensitive than MIG to detect cytomegalovirus-antigen immunorecognition. ABRAMO *et al.* [70] explored the diagnostic potential using ESAT-6/CFP10 stimulated PBMCs, and found it less sensitive for active tuberculosis compared to IFN-γ; similar results were recently shown in a whole blood model [62]. Other studies demonstrated comparable performance to IP-10 and IFN-γ in patients with active tuberculosis compared to controls [67] and in healthcare workers with presumed LTBI [69].

MIP-1β

MIP-1β (chemokine (C-C motif) ligand (CCL)4) is produced by activated macrophages, dendritic cells, natural killer cells, T-cells [124] and is chemoattractive to mainly activated T helper cells and macrophages [125]. MIP-1β is inducible by TNF-α, IFN-γ and IL-1 whereas anti-inflammatory cytokines including IL-4 and IL-10 downregulate expression [125, 126]. CHEGOU *et al.* [89] evaluated the potential of MIP-1β in QFT test supernatants of 23 pulmonary TB patients and 34 household contacts. Higher levels of MIP-1β were observed in the household contacts compared with the tuberculosis patients, and antigen-specific levels of MIP-1β ascertained the presence of active tuberculosis with a sensitivity of 85% and specificity of 61%, but this protein showed the most potential when used in combination with other markers [89]. Similar results were obtained in a low tuberculosis-burden setting, where MIP-1β showed perfect sensitivity and specificity in a set of confirmed tuberculosis cases compared with presumably uninfected controls [60], but other studies show only little potential of MIP-1β for diagnosis of active tuberculosis or latent infection as defined by positive IGRA [62].

MCP-2

MCP-2 (CCL8) is a chemokine secreted from antigen presenting cells after stimulation by IFN-γ, IFN-α and IL-1 [127]. MCP-2 is chemoattractive to granulocytes, monocytes and T-cells [127, 128]. MCP-2 is produced at >10-fold higher levels than IFN-γ, but the immunodiagnostic potential for active tuberculosis has been found significantly lower than both IFN-γ and IP-10 [78, 90]. GOLETTI *et al.* [129] evaluated MCP-2 responses against selected RD1 peptides in tuberculosis cases and controls and found significantly higher responses in patients with active tuberculosis than in controls, but not between the cases and household contacts [77]. These data and data in HIV-infected people suggest that MCP-2 is less suited as a standalone immunodiagnostic marker [91] (M. Ruhwald; unpublished data).

MCP-1

MCP-1 (CCL-2) is released in response to TNF-α and IL-1 stimulation by antigen presenting cells. The actions of MCP-1 include chemotaxis of monocytes and basophiles and after N-terminal cleavage also eosinophils [130]. *In vivo* MCP-1 expression is variable and has been associated with severity of pulmonary tuberculosis [68]. Case–control studies suggest that MCP-1 is secreted in response to antigen stimulation in patients with culture-confirmed tuberculosis, but not in healthy controls [60, 62, 63, 71, 78]. Two studies find MCP-1 expression inconsistent in people with LTBI, suggesting a differential diagnostic potential in combination with, for example, an IGRA test [62, 69]. Antigen MCP-1 expression is heterogeneous and can be of very high magnitude, also in unstimulated samples. This poses technical challenges in the measurements and renders this marker less attractive [60, 62, 78].

IL-1 receptor antagonist

IL-1 receptor antagonist (IL-1RA) is a naturally occurring competitive inhibitor of IL-1α and IL-1β. IL-1RA is secreted by monocytes, neutrophils, epithelial cells and adipocytes in response to granulocyte–macrophage colony-stimulating factor, IL-1β and TNF-α stimulation [131, 132]. IL-1RA has been suggested as a plasma biomarker in many inflammatory and infectious diseases including TB, and serum levels decline with treatment [133]. IL-1RA has shown potential as an immunodiagnostic biomarker for tuberculosis [62, 78, 134], and as a discriminatory marker between active tuberculosis and LTBI as inducible levels in samples from presumed LTBI infected are lower [62, 69]. IL-1RA is an attractive potential biomarker as the responses in reactive samples are high, but IL-1RA responses levels are variable [78]. More studies using assays optimised for the relevant range of IL-1RA response are needed to substantiate these findings.

Summary

In this section we explored biomarker-responses in patients with confirmed tuberculosis or presumed LTBI from whole blood or PBMC culture. Across studies, we identified a similar pattern of markers expressed by immune-competent cells from infected patients, strongly suggesting that antigen-specific immune recognition is detectable with markers expressed not only by T-cells, but also APCs and even adjacent immune-competent cells responding to the cytokines produced in the T-cell–APC interaction (fig. 1). These findings are in line with expression patterns seen in other CMI assays *e.g.* following phytohaemagglutinin stimulation of whole blood from presumed healthy donors [32, 60] and *Mycobacterium leprae*-specific peptide stimulation of whole blood from *M. leprae*-infected patients [135].

Biomarkers for prediction of development of active TB

Despite the obvious clinical need for improved tests; only one study has assessed the development of active tuberculosis in exposed individuals using an immunodiagnostic test based on another marker than IFN-γ. TUUMINEN *et al.* [136] followed 60 school children exposed to a case of active tuberculosis and found that QFT and an IP-10 release assay had perfect concordance and of 58 children with negative tests, none had developed active tuberculosis at 4 years of follow-up. This study renders no information on the predictive value for development of tuberculosis, and is underpowered to conclude on the predictive value for remaining tuberculosis free given the test is negative.

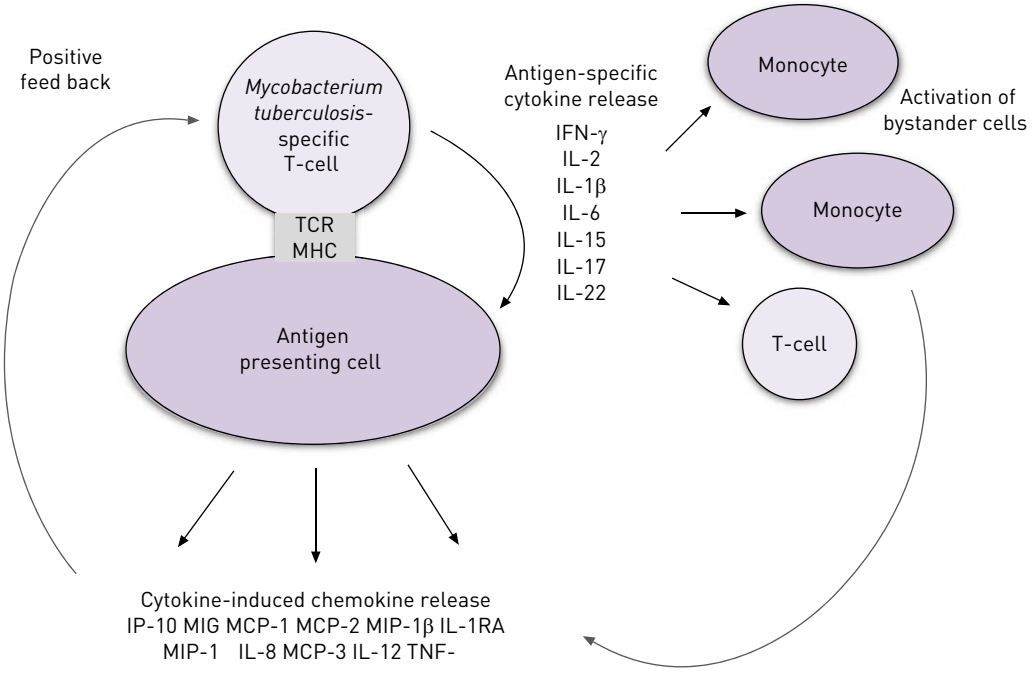

FIGURE 1 Schematic overview of key cells and cytokines involved in the immune response towards *Mycobacterium tuberculosis*-specific antigens in immunodiagnostic tests. IFN: interferon; IL: interleukin; TCR: T-cell receptor; MHC: major histocompatibility complex; IP-10: IFN-γ-induced protein 10; MIG: monokine induced by IFN-γ; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein; IL-1RA: IL-1 receptor antagonist; TNF: tumour necrosis factor.

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More studies are needed to investigate the predictive value of alternative biomarkers. Such studies should be specifically designed with the aim to adjust cut offs at the point that best separates individuals who progress to active tuberculosis from those who remain disease free.

Biomarkers for the differentiation of active tuberculosis *versus* LTBI

In high-burden settings, IGRAs are currently not recommended for clinical management due to the high prevalence of latent cases, and the inability of the tests to discriminate between LTBI and active tuberculosis [137] Different approaches have been attempted in the search for biomarkers for this end. These include measurement of alternative biomarkers in *M. tuberculosis*-specific antigen-stimulated supernatants [89], the use of ratios of intracellular expression of different cytokines [138], T-cell phenotype as well as cytokine expression profiles of specific T-cells [139–141], and transcriptomic approaches to identify genes or gene signatures, which could be characteristic for latent or active disease [142–144].

Several panels of secreted immunodiagnostic biomarkers from QFT supernatants have been suggested as potential differential markers. One study identified EGF, sCD40L, MIP-1β, TGF-α, and VEGF as potential candidates [89]. A follow-up study using 7-day culture, confirmed EGF and TGF-α as potential discriminating markers [51]. Another small study including 76 children identified unstimulated levels IL-1RA, IP-10 and stimulated levels of VEGF as potential discriminatory markers [145]. Two studies suggest that IL-2 adds discriminatory power to IFN-γ [98, 104], although other studies have not been able to show this association [62, 102]. Similar ability has been proposed when combining IL-15 and MCP-1[62]; TNF-α, IL-12p40 and IL-17 [92]; or EGF, MIP-1β, sCD40L and IL-1α [89] but no clear pattern has emerged, and larger confirmatory studies are needed to validate these reports.

Enumeration of cells secreting IFN-γ and/or IL-2 by flow cytometry or immunospot is an area that is actively being explored [101, 141, 146]. Several recent reports suggest that LTBI and infection control are dominated by central memory T-cells with potential of IL-2 and optionally IFN-γ co-secretion; whereas active tuberculosis is characterised by loss of IL-2 production and T-cells with effector memory T-cell phenotype [101, 104, 139, 141, 146–148]. Along these lines, HARARI *et al.* [149] evaluated CD4 T-cells producing TNF-α, IFN-γ and IL-2 by flow cytometry in subjects with active tuberculosis or LTBI. The proportion of *M. tuberculosis* antigen-specific CD4+ TNF-α single-positive T-cells was found to provide the best discrimination between tuberculosis disease and LTBI, with sensitivity and specificity of 100% and 96%, respectively, in the test cohort (eight active tuberculosis and 48 LTBI), and sensitivity of 67% and specificity of 94% when patients from a South African validation cohort were included in the analysis. In countries of low tuberculosis prevalence, local immunodiagnostic by IFN-γ ELISPOT on mononuclear cells from the bronchoalveolar lavage is suitable to discriminate active tuberculosis from LTBI with a high diagnostic accuracy but requires bronchoscopy [150–152]. At present, no large confirmatory biomarker studies for discriminating LTBI from active tuberculosis from peripheral blood exist.

Prediction of tuberculosis in special populations

Immunocompromised patients

Immunocompromised patients are at higher risk developing tuberculosis [153–156]. HIV-infected patients, patients receiving immune suppressive medication (*e.g.* prednisolone or TNF-α inhibitors) and patients with chronic renal failure, are currently considered candidates for screening and targeted treatment, although the risk is highly dependent on prevalence [157]. Alternative immunodiagnostic markers expressed in higher levels and through other signalling pathways than IFN-γ, could have potential to improve the management of immunocompromised patients.

IP-10 remains the most investigated marker in these patient groups. In HIV-infected patients with tuberculosis, three studies found IP-10 sensitivity for infection higher than QFT [76, 77, 116], and one study showed no difference [73]; all agreed that IP-10 appear less affected by a low CD4 cell count than IFN-γ. In otherwise healthy HIV-infected people from India, IP-10 rendered higher rates of positive responders compared with QFT in individuals at high risk of LTBI, but no assessment of later development of disease was done. Comparable results were seen in an Italian cohort with lower *a priori* risk of tuberculosis [116, 158]. In a cohort of patients suspected of active tuberculosis in whom another diagnosis was subsequently made (*e.g.* cancer or infection), it was found that patients with pneumonia and other infections had significantly reduced IFN-γ responsiveness to mitogen challenge and a lower rate of positive responders, compared to IP-10 [75]. In HIV-infected individuals, MCP-2 responses against RD-1 selected peptides were not associated with TB disease [91], a finding reproduced using QFT supernatants in a set of 68 HIV-infected patients from Tanzania where the sensitivity of MCP-2 using predefined cut offs was very low at 42% (M. Ruhwald; unpublished data). One study compared IP-10 and QFT responses in patients with rheumatoid arthritis before anti-TNF-α

treatment, and found IP-10 at least comparable to QFT for the detection of LTBI [79]. Other markers have not been explored for this group of patients.

Performance of biomarkers in children

The diagnosis of LTBI and tuberculosis in children is difficult, microbiological confirmation of infection is often not obtained and treatment is directed by the clinical presentation alone [159]. In both active and presumed latently infected young children, the immune system is immature, and is the likely cause of lower cytokine release and compromised IGRA performance [43, 65, 159–161].

We found no studies assessing alternative markers as indicators for risk of active tuberculosis, and again we can only assess the immunodiagnostic potential. In children with active tuberculosis, IP-10 sensitivity is reported variable but comparable to IFN-γ [80, 81, 141, 162]. In children with a tuberculosis household contact, IP-10 correlates with the degree of exposure comparable to QFT [65, 80, 81, 103, 118, 162–165]. IP-10 appears less influenced by young age and HIV infection in children [65, 80, 162, 163], but larger studies are needed to confirm the findings. In line with the studies in adults, IL-2 holds diagnostic potential in children. Two studies found comparable performance of IL-2 and IFN-γ for both tuberculosis and LTBI, but IL-2 expression levels were very low [65, 93]. IL-2 levels may differentiate active and LTBI [65], although two the two studies that reported on this showed discrepant results [166]. Other markers which have been reported to show potential for diagnosis of tuberculosis disease in children include IFN-α2, IL-1RA, sCD40L and VEGF but observations are yet to be validated in other cohorts [145].

New biomarkers, new assays

Several of the markers including IP-10, MIG, MCP-1, MCP-2, IL-1RA and MIP-1β are expressed at levels many fold higher than IFN-γ. This opens possibilities for both simpler detection assays and higher analytical accuracy when detecting weaker responses.

An emerging application of RT-qPCR detection of cytokines and chemokines at the mRNA level is in the diagnostic field. mRNA is the precursor for protein, wherefore molecular detection would allow for shorter incubation time [138, 167]; and provide a method suitable for multiplexing and high-throughput automation [63, 138, 168]. Case–control studies have established proof of concept for IFN-γ, IL-2, IP-10 and MIG detection using RT-qPCR, and it seems that the differences in magnitude of biomarker release is reflected also at the mRNA level [63, 66, 138, 167, 169, 170].

Flow cytometry allows for single cell investigation of multiple markers. This technology allows for identification of cellular subsets associated with active TB and subclinical infection [101, 141, 147, 149], but the laborious set-up of antibody panels and assay reproducibility is a main challenge for flow cytometry (MIATA (Minimal Information About T-cell Assays) reporting framework http://miataproject.org/). Duo-colour fluorescence-linked immunospot captures a simplified view of the information obtained from flow cytometry, and has shown promise for IL-2/IFN-γ co-expression analysis [147].

Lateral-flow assays are an attractive platform for patient-near analysis in resource restraint settings [171, 172]. These assays generate results in minutes, though often requiring a reader for quantitative readings. Recently a lateral flow assay for IP-10 demonstrated proof of concept for the diagnosis of tuberculosis and is currently under further testing (B. Lange and D. Wagner, Centre for Infectious Diseases, Travel Medicine and Centre for Chronic Immune Deficiencies, University Hospital Freiburg, Germany; personal communication).

Biomarker detection from dried blood spots is another emerging technology applicable for field use. Drying of blood on filter paper stabilises proteins and allows for long-distance letter-based transport [173, 174]. SKOGSTRAND *et al.* [86] demonstrated proof of concept for the method in a Luminex-based assay, and subsequent studies have shown that IP-10 performs equally well in dried blood spots and in plasma [115, 119, 136]. A limitation to this method is the low sample volume extractable from dried blood spots, which renders the lower expressed markers less suitable [175]. Lateral flow and dried blood spot methods will likely not lead to improved diagnostic precision, but allow for dissemination of IGRA-like tests in resource restraint settings where BCG vaccination is universal and high rates of false-positive TST responses compromise its performance.

Summary and conclusion

In this review we assessed cytokine and chemokine markers expressed in response to *M. tuberculosis*-specific antigen stimulation *in vitro* and their potential for the early detection of active tuberculosis. We did not identify studies addressing risk for developing active tuberculosis. Nevertheless, several biomarkers possess a potential to monitor specific immunity to *M. tuberculosis*, among which, IP-10, IL-2, MCP-1, MCP-2, IL-1RA and MIP-1β are strong markers, most of which are induced at high levels.

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Interestingly, these markers are associated with IFN-γ through interlinked and seemingly redundant inflammatory signalling cascades that involve activation of multiple subsets of cells in concert. This implies that the likelihood of identifying a highly expressed marker specific for a certain risk of infection or clinically well-defined state is low. But, as several of these markers are highly expressed, it should allow for a new generation of IGRA-like tests based on less immunogenic but potentially better predictive antigens.

No single biomarker or biomarker combination was identified as specific for LTBI or active tuberculosis. But several recent studies suggest that subpopulations of cells with distinct cytokine secretion patterns correlated with active tuberculosis or LTBI. These data need to be confirmed in relevant clinical studies, but suggest that this approach has potential for further development and validation.

In immunocompromised patients and children, there is a need for improved sensitivity of the current IGRA, as these groups of individuals have high risk of developing tuberculosis, and false-negative immunodiagnostic test results occur. IP-10 was identified as a more robust marker for detection of tuberculosis-specific immunity in HIV-infected patients and perhaps also in children. More studies on markers other than IP-10 are needed.

In conclusion, our review identified several interesting markers with potential for detection of *M. tuberculosis*-specific immune responses. Many of these potential biomarkers were expressed at very high levels allowing for field-friendly detection assays, simple sample transport and, potentially, detection of responses from new antigens with lower immunogenicity. IP-10 remains the most investigated alternative immunodiagnostic marker, seemingly showing higher accuracy for diagnosing infection in HIV-infected individuals and children.

Future studies should evaluate not only the diagnostic accuracy of the proposed markers discussed but also their utility within routine clinical practice and accuracy for prediction of risk of tuberculosis.

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