# Characterization of diagnostic biomarkers for *Mycobacterium tuberculosis* infection

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**Academic Dissertation**

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## Contents

## Original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I **Savolainen L**, Pusa L, Kim HJ, Sillanpää H, Seppälä I, Tuuminen T. Pilot study of diagnostic potential of the *Mycobacterium tuberculosis* recombinant HBHA protein in a vaccinated population in Finland. PLoS ONE 2008, Sep;3(9):e3272.

II **Savolainen L**, Kantele A, Sandboge B, Sirén M, Valleala H, Tuompo R, Pusa L, Erkinjuntti-Pekkanen R, Knuuttila A, Ku CL, Chi CY, Vasankari T, Tuuminen T. Modification of Clearview tuberculosis (TB) enzyme-linked immunosorbent assay for TB patients not infected with HIV**.** Clin Vaccine Immunol 2013, 20: 1479-1482.

III **Savolainen LE**, Koskivirta P, Kantele A, Valleala H, Pusa L, Tuompo R, Westerlund-Wikström B, Tuuminen T. Cytotoxic response persists in subjects treated for tuberculosis decades ago. BMC Infect Dis 2013, 13: 573.

IV **Savolainen LE**, Kantele A, Knuuttila A, Pusa L, Karttunen R, Valleala H, Tuuminen T. Pilot investigation of the combined expression of IFN-γ, IL-17 and IL-4 mRNA for discriminating latent infection in patients with miscellaneous inflammatory conditions from active tuberculosis. Submitted.

## Abbreviations

| | |
|---|---|
| AFB | Acid-fast bacilli |
| AUC | Area under the curve |
| BCG | Bacillus Calmette-Guérin |
| CFP-10 | Culture filtrate protein-10 |
| CFU | Colony forming unit |
| CTLs | Cytotoxic T lymphocytes |
| DC | Dendritic cells |
| DosR | Dormancy regulon |
| ECDC | European Center for Disease Prevention and Control |
| ELISA | Enzyme-linked immunosorbent assay |
| ELISPOT | Enzyme-linked immune spot assay |
| EPTB | Extrapulmonary TB |
| ESAT-6 | Early secretory antigenic target-6 |
| FoxP3 | Forkhead box P3 |
| GrB | Granzyme B |
| HBHA | Heparin-binding hemagglutinin |
| HHC | Household contact |
| HIV | Human immunodeficiency virus |
| HRCT | High-resolution computed tomography |
| IFN-γ | Interferon-gamma |
| IGRA | Interferon-gamma release assay |
| IL | Interleukin |
| IP-10 | IFN-γ-inducible protein-10 |
| kDa | Kilo dalton |
| LAM | Lipoarabinomannan |
| LAMP | Loop-mediated isothermal amplification |
| LTBI | Latent tuberculosis infection |
| MDR | Multidrug resistant |
| Mod. | Treated with modern treatment for TB |
| mRNA | Messenger RNA |
| NAATs | Nucleic acid amplification tests |
| NK | Natural killer |
| nHBHA | Native HBHA |
| NTM | Non-tuberculous mycobacteria |
| Part. | Partially treated for TB |
| PBMCs | Peripheral blood mononuclear cells |
| Perc. | Percentile |
| PIMs | Phosphatidylinositolmannosides |
| PPD | Purified protein derivative |
| Prf | Perforin |
| PTB | Pulmonary TB |
| QFT-IT | QuantiFERON®-TB Gold In-Tube |
| rCFP-10 | Recombinant CFP-10 |
| RD | Region of difference |
| rESAT-6 | Recombinant ESAT-6 |

| | |
|---|---|
| rMS-HBHA | Recombinant HBHA produced in *M. smegmatis* |
| ROC | Receiver operating characteristic curve |
| RT-qPCR | Quantitative reverse transcriptase PCR |
| sICAM-1 | Soluble intercellular adhesion molecule 1 |
| suPAR | Serum urokinase plasminogen activator receptor |
| Surg. | Treated without chemotherapy for TB |
| TB | Tuberculosis |
| $TB^+HIV^-$ | TB patients without HIV co-infection |
| $TB^+HIV^+$ | TB patients with HIV co-infection |
| $T_{CM}$ | Central memory T cell |
| $T_{EM}$ | Effector memory T cell |
| $T_{EMRA}$ | Terminally-differentiated effector memory T cell |
| Th | T helper |
| $T_N$ | Naïve T cell |
| TNF-α | Tumor necrosis factor-α |
| Treg | Regulatory T cell |
| TST | Tuberculin skin test |
| uLAM | Urinary LAM |

## Abstract

Tuberculosis (TB) still ranks among the most lethal infectious diseases in the world. The traditional distinction between active tuberculosis and latent tuberculosis infection (LTBI) is changing; at the moment, tuberculosis infection is described as a continuum with different stages of infection. The development of TB prevention, treatment and diagnostics is hampered by the diverse pathogenesis of the disease and the ability of the bacteria to fight host defence mechanisms. Traditional culture methods are slow and staining methods not sufficiently sensitive. Nucleic acid amplification techniques (NAAT) tend to be costly and in some cases lack sensitivity. New methods are needed for rapid and inexpensive detection of active disease and to distinguish between stages of infection in cases where the patient presents with symptoms compatible with active TB and with a positive Interferon Gamma Release Assay (IGRA) result, but bacteriological confirmation is not yet available. In addition, methods are needed that enable us to predict the activation of infection or monitor the effects of treatment.

The aim of this thesis was to investigate: a) the possible diagnostic use of heparin-binding haemagglutinin (HBHA), a surface protein of *M. tuberculosis*, in distinguishing between stages of infection in a vaccinated population; b) the effect of sample concentration on the properties of Clearview® TB ELISA, a urine antigen test measuring lipoarabinomannan (LAM), a *M. tuberculosis* glycolipid, in the diagnosis of active TB; c) the suitability of granzyme B (GrB), perforin (Prf), and interferon-gamma (IFN-γ)-producing and CD107a-degranulation factor-expressing cytotoxic T lymphocytes (CTLs) for differentiating between stages of infection; d) the suitability of IFN-γ, interleukin (IL) -17, IL-4, IL-4δ2 and Forkhead box P3 (FoxP3) mRNA expression levels for differentiation between stages of infection.

The results showed that HBHA-specific cells producing IFN-γ were also found in the circulation of healthy subjects who had been given the Bacillus Calmette-Guérin vaccine. In view of this, use of the HBHA antigen for diagnostic purposes does not look promising in countries where a widespread vaccination programme has taken place. No differences were found in the numbers or phenotypic properties of CTLs between persons with active TB and those with LTBI, which is why they are not suited for the differential diagnosis of infection stages.

Functional antigen-specific CTLs were found in the circulation of persons who had been treated for TB. This most significant finding of this thesis demonstrates that the T cell memory generated by active TB maintains functionally active CTL populations decades after infection and despite adequate treatment with rifampicin.

Although no statistically significant differences were found between patients with active TB and LTBI in the quantitative detection of IFN-γ, IL-17, or IL-4 mRNA from cells stimulated with purified protein derivative (PPD), this approach revealed a trend towards discrimination. The usefulness of combined quantitative IFN-γ, IL-17, and IL-4 mRNA expression for the differential diagnosis of active TB and LTBI should be retested with a larger sample size enrolling more homogenous patients in the LTBI group.

The findings indicating that LAM may be detected with moderate sensitivity (57%) in the urine samples of TB patients show the greatest promise from a diagnostic point of view. The 100-fold concentration of urine used in this study improved the sensitivity of the Clearview® TB ELISA test from 7% to 57%, although specificity was decreased somewhat from 98% to 89%. Based on the results, demonstrating the presence of LAM in urine samples may be considered a potential diagnostic tool for detecting active TB. The method does require further development, however.

## Tiivistelmä

Tuberkuloosi (TB) kuuluu vielä nykyisinkin tappavimpiin tartuntatauteihin maailmassa. Perinteinen jako aktiiviseen ja latenttiin tuberkuloosiin (LTBI) on muuttumassa; nyttemmin tuberkuloosi-infektio kuvataan vaihteleva-asteisena jatkumona. Patogeneesin monimuotoisuus ja bakteerin kyky torjua isännän puolustusmekanismeja hankaloittavat ennaltaehkäisyn, hoitomenetelmien ja diagnostiikan kehitystä. Perinteisen TB-viljelyn heikkoutena on hitaus, ja värjäys tiedetään epäherkäksi. Geenimonistusmenetelmien käyttöä puolestaan rajoittaa hintavuus eikä niiden herkkyys aina riitä. Uusia keinoja kaivataan taudin nopeaan ja edulliseen osoittamiseen sekä infektion asteiden erotteluun esimerkiksi tilanteissa, joissa potilaalla on aktiiviseen tuberkuloosiin viittaavia oireita ja positiivinen gammainterferoni (IGRA) -tulos, mutta bakteriologinen varmistus puuttuu. Lisäksi tarvitaan menetelmiä, joilla voidaan ennustaa infektion aktivoitumista tai seurata hoidon vaikutuksia.

Tässä väitöskirjatyössä selvitettiin: a) *M. tuberculosis* -pintaproteiinin, heparin-binding hemagglutiniinin (HBHA), diagnostista käyttömahdollisuutta eroteltaessa rokotetussa väestössä infektion eri asteita; b) konsentroinnin vaikutusta *M. tuberculosis* -bakteerin glykolopidiä, lipoarabinomannaania (LAM), mittaavan virtsan antigeenitestin, Clearview® TB ELISA:n, ominaisuuksiin aktiivi-TB-diagnostiikassa; c) granzyme B:tä (GrB), perforiiniä (Prf) ja gammainterferonia (IFN-γ) tuottavien sekä CD107a-degranulaatiofaktoria ekspressoivien sytotoksisten T-lymfosyyttien soveltuvuutta infektion asteiden erotteluun; d) IFN-γ, interleukiini (IL) -17, IL-4, IL-4δ2 ja Forkhead box P3 (FoxP3) -mRNA-ekspressiotasojen toimivuutta infektion asteiden erottelussa.

Tutkimuksessa löydettiin myös terveiden Bacillus Calmette-Guérin (BCG) -rokotteen saaneiden verestä HBHA-spesifisiä, IFN-γ:aa tuottavia soluja. HBHA-antigeenia ei siis voitane hyödyntää diagnostiikassa maissa, joissa rokotusohjelmaan on kuulunut lasten BCG-rokotus. Aktiivi-TB- ja LTBI-ryhmien välillä ei havaittu määrällisiä eikä fenotyyppisiä eroavuuksia sytotoksissa T-lymfosyyteissä, joten ne eivät sovellu käytettäviksi infektion asteiden erotusdiagnostiikassa.

Toiminnallisia antigeenispesifisiä sytotoksisia T-lymfosyyttejä havaittiin myös tuberkuloosihoidon saaneiden verinäytteissä. Tämä on väitöskirjan arvokkain löydös. Se osoittaa, että aktiivi-TB:n aikaansaama T-solumuisti ylläpitää toiminnallisesti aktiivista sytotoksisten T-solujen populaatiota vuosikymmeniä, asianmukaisesta rifampisiinihoidosta huolimatta.

Vaikka IFN-γ-, IL-17- ja IL-4 mRNA -ekspressiotasoissa ei esiintynyt tilastollisesti merkitseviä eroja purified protein derivative (PPD) -stimuloiduissa soluissa, aktiivi-TB- ja LTBI-ryhmien välillä oli nähtävissä erotteleva suuntaus. IFN-γ-, IL-17- ja IL-4 mRNA- ekspression hyödyntämistä aktiivi-TB:n erottamiseksi LTBI:stä tulisikin tulevaisuudessa testata suuremmalla ja yhtenäisemmällä LTBI-aineistolla.

Diagnostiikan kannalta lupaavampia ovat tulokset, joiden perusteella LAM voidaan osoittaa tuberkuloosipotilaiden virtsanäytteestä kohtalaisella herkkyydellä (57%). Tässä työssä käytetty virtsan satakertainen konsentrointi paransi Clearview® TB ELISA -testin herkkyyttä 7%:sta 57%:iin; spesifisyys tosin samalla heikkeni 98%:sta 89%:iin. LAM:n osoittamista virtsanäytteestä voidaan mahdollisesti hyödyntää aktiivi-TB:n diagnosoinnissa, tosin menetelmä vaatisi vielä kehittämistä.

# 1. REVIEW OF THE LITERATURE

## 1.1. History and epidemiology of tuberculosis

The disease tuberculosis (TB) has been known for millennia, and the causative agent, *Mycobacterium tuberculosis* (*M. tuberculosis)*, is thought to have existed for 150 000 years [1]. *M. tuberculosis* has been found by PCR sequencing from a 17 000-year-old bone of a bison in Wyoming, USA [2]. In humans, *M. tuberculosis* has been detected from the bone of Neolithic infant and a woman in a 9000-year-old settlement in the Eastern Mediterranean [3]. The characteristic lesion of the disease, the tubercle, was first described by Laennec in 1819, and the disease was named tuberculosis by Johann Lukas Schönlein in 1834. A few decades later, Jean-Antoine Villemin demonstrated the transmissibility of the infection. After centuries of speculation, in 1882, the German scientist Robert Koch reported the isolation of tubercle bacillus, the causative agent of tuberculosis. One year later, the bacillus was named *M. tuberculosis*. [1, 4]

Worldwide, TB remains a major health problem. It has been ranked as the second leading cause of death by an infectious disease after the human immune deficiency virus (HIV) infection. Almost 9 million new TB cases and 1.3 million TB deaths (including 300 000 HIV-associated cases) were estimated in 2012. Most of the cases occurred in Asia (58%) and Africa (27%), with fewer cases in the Eastern Mediterranean region (8%), the European region (4%) and the region of the Americas (3%). Multidrug-resistant (MDR), extensively drug-resistant (XDR), and most recently, *M. tuberculosis* strains that are resistant to all anti-tuberculosis drugs have emerged over the past two decades. In 2012, there were 450 000 estimated MDR-TB cases among diagnosed pulmonary TB patients, from which the highest incidences were in Eastern European and Central Asian countries. [5]

In Finland, there were almost 9000 TB deaths per year in 1930, and TB was a dreaded national disease. The incidence and death rates began to decline in the 1940s due to the development of the first anti-tuberculous drugs and the establishment of national preventative programs with sanatorium activity. [6] In 2012, only 261 new TB cases and 3 MDR-TB cases were recorded in Finland [5]. However, increasing immigration and high incidence rates in neighboring countries, as well as the reactivation risk in persons infected in the 1940s and 1950s, are causes for concern at present.

Currently, the prevention, control, vaccination, diagnostics and treatment of TB are accurately monitored worldwide by the surveillance programs of WHO, the Centre for Disease Control and Prevention (CDC), and the European Centre for Disease Control and Prevention (ECDC). Although the incidence rates have decreased worldwide during the last decade, the challenges in tuberculosis control remain.

## 1.2. *Mycobacterium tuberculosis*

### 1.2.1. Taxonomy and characteristics

Mycobacteria are acid-fast, aerobic, non-motile, non-encapsulated and non-spore-forming bacilli with a high DNA GC content. The genus *Mycobacterium* is the only member of the *Mycobacteriacae* family, but forms a uniform group with a range of other mycolic acid-containing genera, namely *Gordonia*, *Tsukamurella*, *Nocardia*, and *Rhodococcus*. *M. tuberculosis* belongs to the *M. tuberculosis* complex, together with the species *M. bovis*, *M. africanum*, *M. microti*, *M. canettii*, and *M. pinnipedii*, forming a tight and discrete group with >95% DNA-DNA homology. [7] The complete genome of the first sequenced laboratory strain of *M. tuberculosis*, H37Rv, consists of 4.4 x $10^6$ base pairs and contains approximately 4000 genes, including regions that are deleted in other members of the complex [8].

*M. tuberculosis* is an obligate intracellular pathogen, which most successfully infects tissues with a high oxygen content, such as the lungs, but can infect almost any organ of the host. It principally infects humans, but can also infect several other animal species. *M. tuberculosis* divides extremely slowly (15–20 hours) compared to other bacteria and has the ability to persist in a latent state. Mycobacteria are highly resistant to desiccation, alkaline conditions, chemical disinfectants, and therapeutic agents. This resistance is related to the unusual structure and low permeability of the mycobacterial cell wall. [7, 9] *M. tuberculosis* lacks such virulence factors as toxins. However, important virulence determinants have been found, for example, in the categories of cell envelope proteins, lipid and fatty acid metabolism, proteases, gene expression regulators, and the proteins inhibiting antimicrobial response of macrophages, such as phagolysosome fusion and the production of reactive oxygen and nitrogen. [10]

### 1.2.2. Structure of the cell envelope

The peptidoglycan of mycobacteria contains a unique *meso*-diaminopimelic acid and an *N*-glycosylated muramic acid residue, in contrast to the *N*-acetylation of other bacteria. Together with its unique polysaccharide, arabinogalactan, supplemented by mycolic acids, peptidoglycan composes the core of the cell wall, the mycolylarabinogalactan-peptidoglycan (mAGP) complex. Phosphatidylinositolmannosides (PIMs) are the main plasma membrane components and form the lipid anchor of lipoarabinomannan (LAM) and lipomannan, which belong to the upper segment of the cell wall together with free lipids and proteins (Figure 1). [11, 12]

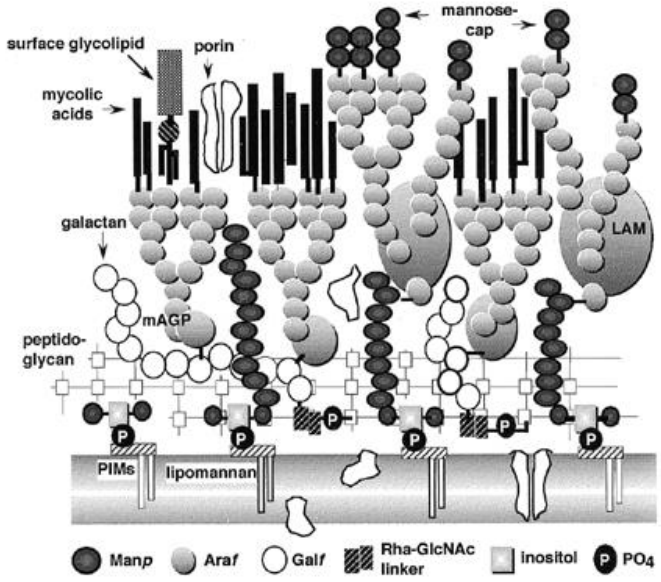


**Figure 1.** The mycobacterial cell wall. Reprinted with permission from Oxford University Press: Glycobiology [12], copyright 2013.

## 1.3. Tuberculosis

TB is an airborne infectious disease caused by mycobacteria, usually by *M. tuberculosis*. In most cases, it occurs in the lungs of the host. Infection outside the lung is called extrapulmonary TB (EPTB). The transmission of infection ensues when a susceptible person inhales *M. tuberculosis*-containing particles, or droplet nuclei, generated for example by coughing or sneezing of a patient with pulmonary tuberculosis (PTB) [7]. A person inhaling *M. tuberculosis* aerosols has a 5–10% risk of developing active TB, a rapidly progressive disease that can be diagnosed with microbiological, radiological, or histological findings. The risk of disease activation is the highest within the first few years after transmission. Most immunocompetent persons develop an effective immune response and succeed in eliminating *M. tuberculosis*, or remain asymptomatic and non-infectious, having a so-called latent tuberculosis infection (LTBI). The diagnosis of LTBI is based on the known history of TB exposure and the measurement of a host's immune response to *M. tuberculosis* antigens using either tuberculin skin test (TST) or interferon-γ (IFN-γ) release assay (IGRA). [9, 13] Nowadays, the traditional dichotomy of active TB and LTBI is changing to a continuum of infection, including a wide spectrum of infection stages. Presently, LTBI is defined to range from the completely cleared infection to the stage where replicating bacteria without clinical symptoms are present. Meanwhile, active TB can represent diverse presentations from a subclinical infection to a clinical disease with an extensive load of replicating bacteria. [13]

### 1.3.1. Pathogenesis

The interaction between *M. tuberculosis* and its host is complex and the pathogenesis and immunological processes are not yet well understood. For example, regulation of the transition from primary growth to persistence, further reactivation of LTBI, bacterial determinants required for persistence, and the metabolic state of *M. tuberculosis* during latency are currently under investigation. [14]

#### 1.3.1.1. Infection initiation and innate immunity

After *M. tuberculosis* is inhaled in respiratory droplets, it is ingested by airway antigen-presenting cells, such as monocyte-derived macrophages and dendritic cells (DCs) [15]. The recognition and binding of cell wall components, such as mannose-capped LAM (ManLAM) and PIMs, as well as bacterial uptake, are executed by numerous receptors, including C-type lectin receptors, complement receptors and scavenger receptors. Toll-like receptors and nucleotide-binding oligomerization receptors are important for the induction of synthesis of pro-inflammatory cytokines. [16] Upon *M. tuberculosis* recognition, pro-inflammatory cytokines, such as interleukin (IL)-6, IL-21, IL-1β, and IL-12p40, are expressed by phagocytic cells mentioned above [17]. Infected phagocytes migrate across the alveolar epithelium into the lung tissue, where granuloma formation is initiated by macrophages [18]. Infected macrophages express vimentin, which is recognized by the NKp46 receptor of natural killer (NK) cells. Activated NK cells lyse *M. tuberculosis*-infected macrophages with a help of antimicrobial granulysin. [19] In early mycobacterial infection in mice, γδ T cells have been reported to be a major source of proinflammatory IL-17. These cells get activated antigen-

independently by IL-23. IL-17 has a role in the initiation of granuloma formation and early neutrophil recruitment. [17] Neutrophils are rapidly recruited to the infection site after infection initiation. They directly internalize mycobacteria using pattern recognition receptors (PRRs) or by opsonization and lyse them with mechanisms such as human neutrophil proteins (HNPs), stored in azurophilic granules. Neutrophils also participate in the recruitment of macrophages. [20]

*M. tuberculosis* is transported by DCs to the local draining lymph nodes and antigens are presented to naïve T cells. $CD8^+$ and $CD4^+$ T cells recognize the bacterial peptide epitopes presented by classical MHC class I and II molecules, respectively. [21] Furthermore, non-classically restricted $CD8^+$ and $CD4^+$ T cells recognize lipid and glycolipid antigens presented by CD1 molecules [22]. Recruited T cells travel to the lung tissue and are present in the mature granuloma. Their role is crucial for the control of infection after the acute phase. After the onset of adaptive immunity, most humans become asymptomatic and are considered to have LTBI. [21, 23]

Approximately 5% of infected subjects rapidly develop progressive disease, active TB. Usually, most immunocompetent persons eliminate the invading bacteria or maintain the infection in a latent state. These subjects develop an effective adaptive immune response and remain free of symptoms. A proportion of these might later develop an active disease, a process called disease reactivation. [9, 13] The risk of disease reactivation is significantly increased in immune-compromised patients. However, only a few mechanisms explaining reactivation in humans have been consistently identified. These are the therapeutic neutralization of tumor necrosis factor-α (TNF-α), the quantitative and qualitative loss of $CD4^+$ T cells in HIV-infected persons [23], and a deficiency of vitamin D [19]. Other conditions and mechanisms associated with reactivation, such as diabetes mellitus, malnutrition, T-cell exhaustion and altered antigen expression, have also been suggested, but not thoroughly proven. [23]

#### 1.3.1.2. Immune evasion and the roles of granulomas

*M. tuberculosis* uses many immune evasion mechanisms during the innate immunity stage, such as the prevention of phagosome-lysosome fusion, the inhibition of host cell apoptosis, the neutralization of toxic oxygen and nitrogen intermediates, and the inhibition of IFN-γ receptor-mediated signaling [21]. Due to these mechanisms, pathogenic mycobacteria are able to resist or evade the host immune response during the innate immunity stage. This delayed initiation of adaptive immunity allows uncontrolled growth of bacteria in the lungs during the innate immunity stage and leads to the progression of infection. [23] The host-protective role of the granuloma, the well-organized hallmark structure of tuberculosis, has traditionally dominated in the literature. This hypothesis is supported by the observation that poorly formed granulomas and hypersusceptibilty to *M. tuberculosis* occur under TNF-α, IFN-γ, or IL-12 deficiency. [18] However, it has recently been demonstrated in a zebra fish embryo model that the early granuloma benefits the pathogenic mycobacteria. The ESX-1 type VII secretion system, encoded by the region of difference 1 (RD1) virulence locus, has been shown to promote the recruitment of macrophages to granulomas and drive the necrotic death of infected cells. This allows the release of intracellular bacteria and further expansion of the bacterial population into newly recruited macrophages. [24]

The tuberculous granuloma can be caseous, consisting of an acellular necrotic center with surrounded macrophages, neutrophils, DCs, natural killer cells, and B and T lymphocytes (Figure 2). Necrotic neutrophilic, fibrotic, and suppurative granulomas also occur. Ghon's complex, with a calcified granuloma in a single lung lobe and associated draining lymph node, has been related to LTBI. In this form of a granuloma, successful immune responses with fewer inflammatory cells are present. [15, 18, 25]

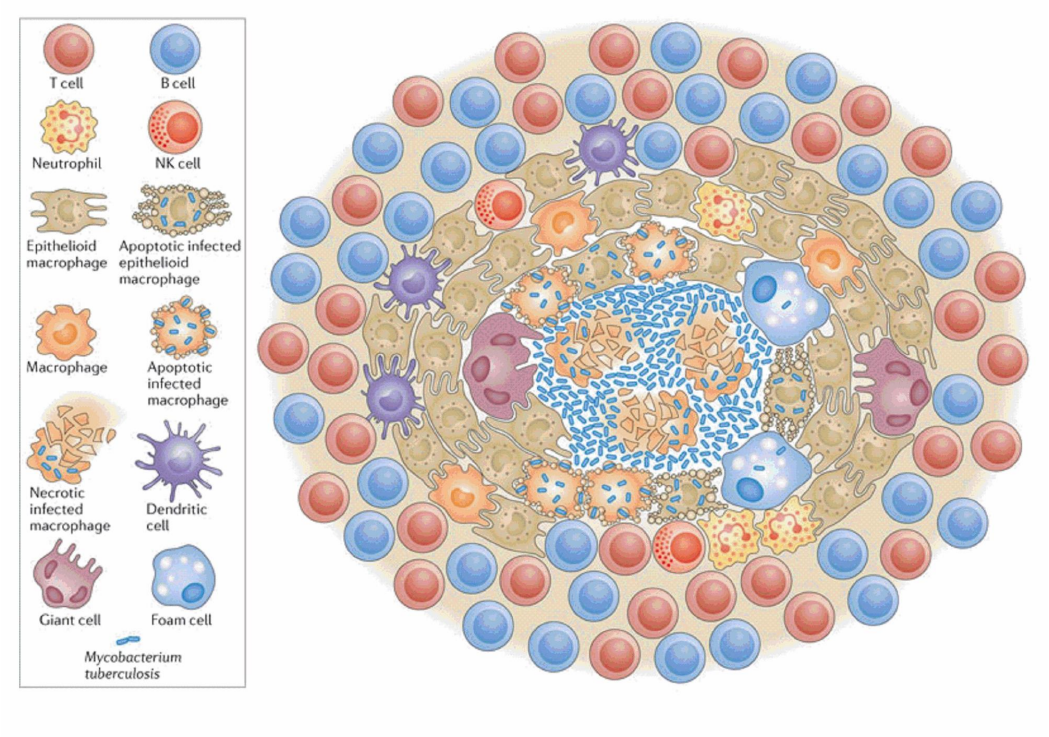


**Figure 2.** Cell organization of a mature tuberculous granuloma. The granuloma is a compact aggregate that is formed by linked specialized macrophages, epithelioid cells, and fused multinucleated giant cells. Foam cells are located on the edge of the necrotic center and bacteria are present in macrophages and other phagocytic cells, but also extracellularly in necrotic areas of the granuloma. The mature granuloma contains many other cell types, such as dendritic cells and T and B cells. Reprinted with permission from Nature Publishing Group: Nature Reviews Immunology [18], copyright 2014.

Recently, in models with cynomolgus macaques, the spectrum of lesions, including Ghon's complexes, was described in animals with active TB. Subclinical TB, in which macaques had no clinical symptoms but had positive bronchoalveolar lavage fluid cultures for *M. tuberculosis*, was demonstrated. [26, 27] Most recently, similar bacterial killing capacity in individual lesions of active TB and LTBI has been reported. In active TB, high variation in sterilizing activity between lesions has been recorded within a single macaque. [28]

In humans, a range of lesion types found in active TB, with replicating bacteria, have also been demonstrated in LTBI using the high-resolution computed tomography (HRCT) analysis [29]. On the other hand, a strong evidence for nonreplicating persistence of *M. tuberculosis* during latency has been reported. This study was performed with clinical isolates recovered during primary infection and reactivation of the disease in the same persons more than three decades later. No sequence variation between the pairs was detected [30]. Because a variety of granuloma types are found in active TB, LTBI, and in disease reactivation, the function of the granuloma, rather than its structure has been suggested to be essential for the determination of the infection stage. [15]

### 1.3.2. The cells of the adaptive immune response

#### 1.3.2.1. $CD4^+$ T cells

*T helper (Th)1 cells.* The specific immune response against *M. tuberculosis* consists of a variety of cell types. Best characterized are $CD4^+$ Th1 cells [21]. The development and maintenance of Th1 cells is induced by IL-12 [31]. After phagocytosis of *M. tuberculosis*, macrophages and DCs produce IL-12 which induces the effector functions of $CD4^+$ T cells, especially the production of IFN-γ. Together with TNF-α, IFN-γ induce the antimycobacterial effects of macrophages. In TB, IFN-γ is produced by most infected subjects and its secretion is induced by a variety of mycobacterial antigens. However, IFN-γ secretion correlates poorly with protection. [21] It has been suggested that $CD4^+$ T cells also have other functions in the control of TB, such as the promotion of Fas-L-mediated apoptosis of infected macrophages and the secretion of other cytokines, including IL-2 and TNF-α [14]. The high frequencies of polyfunctional $CD4^+$ T cells that secrete multiple cytokines have been suggested to correlate with protection in chronic virus infections with a low antigen load [32]. Recently, the same has also been proposed for TB. In a few studies, higher frequencies of $CD4^+$ T cells producing IFN-γ, IL-2, and TNF-α have been recorded in the blood of subjects with LTBI than in that of patients with active TB [33, 34]. Whereas, in some other studies polyfunctionality have been related to active TB [35, 36].

*Th2 cells.* The Th2 pathway assisting humoral immune response is more important for the protection against extracellular pathogens. It has a lesser impact against intracellular pathogens [37]. Th2 cells produce IL-4, IL-5, and IL-13, and regulate antibody-mediated immunity [38]. In TB, Th2 cells have been association with decreased immunity and increased immunopathology [38, 39]. Moreover, excess production of IL-4 is related to a depressed Th1 response [40] and increased disease severity [41]. IL-4δ2, the splice variant and antagonist of IL-4, has an opposite role and is related to the control of *M. tuberculosis* infection [42, 43].

*Regulatory T cells (Tregs).* Inducible Tregs arise in the periphery from conventional $CD4^+$ T cells in response to specific stimulatory signals, while natural Tregs develop in the thymus after a normal maturation process [44]. A specific marker and unique transcription factor of natural Tregs, forkhead box P3 (FoxP3), is required for the generation of these cells. The regulatory mechanisms exploited by Tregs suppress the effector functions of T cells, which limit immune-mediated tissue pathology but may also result in a failure to control an infection. [45] In TB, Tregs delay the priming of adaptive immune cells and their migration to the lungs, a phenomenon which allows uncontrolled replication of *M. tuberculosis*. The number of Tregs at the infection site and in the periphery is increased in active TB. That positively correlates with the bacterial burden and severity of the disease. However, it is not known whether the expansion of Tregs is a cause or a consequence of advanced TB. [44]

*Th17 cells.* A recently recognized cell subset, Th17 cells produce effector molecules IL-17, IL-17F, IL-21, and IL-22, which induce tissue inflammation. Th17 cells are linked to autoimmunity, but have also been associated with some bacterial infections, including TB. [46] Induced in draining lymph nodes, Th17 cells migrate to the lungs and execute effector functions [17]. In mice, this cell subset has been shown to respond rapidly after antigen challenge. They help to recruit neutrophils and effector cells, such as IFN-γ-producing cells, and that promotes granuloma formation [17, 47]. Whether these cells play a role in the protection against or the control of TB is not yet well known. Excessive production of IL-17 potentially mediates immunopathology and has detrimental effects on the lung tissue. The balance between Th1 and Th17 cells during TB is essential in promoting immunity and preventing immunopathological consequences. [17]

*Th9 cells.* Th9 cells are a recently identified cell population distinct from Th1, Th2, and Th17 subsets. They have been suggested to participate in inflammation and the development of allergic reactions. [48] Th9 cells have also been found in the pleural fluid of patients with TB pleurisy [49]. In the same study, constructed with cells isolated from the pleural effusion of patients with TB pleurisy, differentiation into the Th9 cell subset from $CD4^+$ T cells was shown to be induced by transforming growth factor-β and IL-4 and suppressed by IFN-γ [49]. In addition, a positive correlation between Th9 and Th17 cell frequencies and the contribution of IL-9 to Th17 differentiation has been reported [49]. The function of Th9 cells in the pathophysiology of TB is not yet known. [50]

#### 1.3.2.2. Cytotoxic $CD8^+$ T cells

$CD8^+$ T cells contribute to the control of *M. tuberculosis* infection. They recognize *M. tuberculosis* antigens and produce effector molecules, such as IFN-γ, TNF-α, and IL-2 [34, 51], but also lyse infected cells and kill intracellular bacteria via the granule exocytosis pathway [52]. A protective role of cytotoxic T lymphocytes (CTLs) has been suggested by human studies in which higher frequencies of *M. tuberculosis* -specific $CD8^+$ T cells producing perforin (Prf)- and granzyme B (GrB) were found in the household contacts (HHC) of TB patients and healthy vaccinated subjects compared to patients with active TB. In these studies, reduced cytotoxic activity in patients with active TB has also been reported. [53, 54] In addition, the lytic activity of $CD8^+$ T cells has been found to decrease gradually with increasing severity of the disease [55]. Furthermore, increases in the frequency of Antigen

85A peptide-specific Prf-producing effector cells have been observed after four months of anti-TB treatment [56].

#### 1.3.2.3. B cells and humoral immune response

Recently, also B cells and the humoral immune response have been suggested to possess a protective role against *M. tuberculosis*. For example, increased immunopathological consequences and susceptibility to *M. tuberculosis* have been shown in B cell-deficient mice [57]. Furthermore, monoclonal antibodies to *M. tuberculosis* surface components have been reported to increase survival times, and reduce disease dissemination and bacterial burden in experimentally infected mice [58]. The inhibition of Fcγ receptors, expressed by antigen-presenting cells, in the lungs of *M. tuberculosis*-infected mice limited the protection and suggests the beneficial role of humoral immunity [59]. In humans, a wide variety of *M. tuberculosis* antigens have been reported to induce the antibody response. BCG vaccination has been shown to induce IgG and IgM responses to several antigens, especially LAM. [58] LAM-specific IgG antibodies have been demonstrated to assist cell-mediated immunity by increasing phagocytosis by neutrophils and antigen processing and presentation by DCs [60].

## 1.4. Vaccination

The Bacillus Calmette-Guérin (BCG) vaccine contains a live attenuated strain of *M. bovis*, originally isolated from cattle. It was used for the first time in 1921, and following the WHO Expanded Program of Immunization in 1974, vaccination coverage rates soon exceeded 80% in TB endemic countries. However, BCG vaccination has a limited impact on TB transmission. It is only protective against childhood TB meningitis and disseminated TB. [61] In Finland, the BCG vaccination program for newborns began in the 1940s and a 98% vaccine coverage was subsequently achieved. In many low incidence countries, such as Sweden and Norway, only children in risk groups are vaccinated. After careful risk and cost-effectiveness assessments, since 2006 the vaccinations have also been targeted at risk groups in Finland. [62]

## 1.5. Antibiotic treatment of tuberculosis

*M. tuberculosis* is resistant to many common antibiotics [63]. Treatment comprises a combination of three or more anti-TB drugs for a period of at least 6 months. The first effective antimicrobial agents against clinical TB, streptomycin (SM) and para-amino salt of salicylic acid (PAS), were discovered in the 1940s. In the 1950s, 24-month triple therapy with isoniatzid (INH), SM and PAS resulted in a cure for 90–95% of patients. Later, ethambutol (EMB) replaced para-amino salt of salicylic acid. The first bactericidal antibiotic, rifampicin (RMP), was included in the treatment of TB in the 1970s. In the 1980s, a 6-month therapy with pyrazinamide (PYR), isoniazid, and rifampicin was found to be effective and is still used as the first-line anti-TB treatment. [64, 65] Following the emergence of drug-resistant strains, new antibiotic classes, such as fluoroquinolones have been used. The increasing rate of resistance necessitates a continuous search for new drug candidates. [66]

## 1.6. Diagnostics of tuberculosis

The diagnosis of active TB has traditionally been based on clinical and radiological findings, acid-fast bacilli (AFB) smear microscopy and *M. tuberculosis* culture. Since the last decade, nucleic acid amplification tests (NAATs) have also been widely used. LTBI can be diagnosed with the radiological findings, known history of *M. tuberculosis* exposure and TST or IGRAs. [9, 67, 68] The following sections introduce the conventional and some non-conventional methods and host- or pathogen-specific markers (Table 1) investigated during the last decades.

### 1.6.1. Conventional methods

AFB smear microscopy is still the most widely used rapid and inexpensive method for TB diagnosis, especially in developing countries [7]. The method is also used to determine the contagious level of the infection [127]. However, the sensitivity of smear microscopy varies from 20% to 80%, and in other than respiratory samples the diagnostic yield is lower. From $10^4$ to $10^6$ AFB/ml is usually required for a positive result. The specificity is high, being up to 94%, in high prevalence areas. [7] Improved sensitivity has been shown with fluorescence microscopy with auramine or acridine compared to conventional methods with carbolfuchsin [128], and also when a sample volume of more than 5 ml is concentrated by cytocentrifugation [129, 130]. In the future, smear microscopy could be improved, for example, by using ultra-bright light-emitting diodes (LED) in microscopy [131].

Culture is the gold standard for the laboratory confirmation of TB, but it requires 2–6 weeks of incubation. Since the 1990s, more rapid and sensitive semi-automated and automated culture systems using liquid media instead of Löwenstein-Jensen solid media have been developed. At present, systems such as the BACTEC 460TB (Becton Dickinson, NJ, USA), BACTEC MGIT 960 (Becton Dickinson) and MB/BacT ALERT 3D (bioMérieux, Craponne, France), are widely used. With these methods, in a smear-positive specimen the average detection time is 9 to 14 days for *M. tuberculosis*, and the limits of detection vary between 10–100 colony-forming units (CFU) per ml. [7] Isolation of bacteria is required for drug-susceptibility testing and genotyping [9]. Sputum culture and staining have also been used for the quantification of mycobacteria and the evaluation of treatment success. Moreover, a decline in the CFUs of *M. tuberculosis* in sputum has been shown to be an objective and reproducible method for measuring the early bactericidal activity of anti-TB agents. [132]

**Table 1. Diagnostic markers of TB under research.**

| Marker | Sample and/or method | Associated outcome | Reference |
|---|---|---|---|
| **Antigens for immunological assays** | | | |
| HBHA protein | PBMCs, IFN-γ concentration | LTBI ↑ | 69-71 |
| DosR proteins | PBMCs, IFN-γ concentration | LTBI ↑ | 72-76 |
| RD peptides and proteins | PBMCs, IFN-γ concentration / frequency of IFN-γ-producing cells | Contact with *M. tuberculosis*↑ | 77-79 |
| **Chemokines and cytokines** | | | |
| IP-10 | Plasma concentration | Contact with *M. tuberculosis*↑ | 80-88 |
| IL-4/IL-4δ2, IL-4/IFN-γ | mRNA from unstimulated PBMCs | Treatment response ↓<br>Infection reactivation ↑ | 41, 89 |
| **Antigen detection** | | | |
| LAM | Urine concentration | Active TB ↑/ treatment response ↓ | 90-107 |
| Antigen 85 | Serum/sputum concentration | Active TB ↑/ treatment response ↓ | 108-111 |
| Rv1681 | Urine concentration | Active TB ↑ | 112, 113 |
| **Molecular methods** | | | |
| Transrenal DNA | Urine | Active TB ↑ | 114, 115 |
| LAMP | Sputum | Active TB ↑ | 116, 117 |
| **Non-specific markers** | | | |
| Volatile organic compounds | Breath | Active PTB ↑ | 118, 119 |
| Neopterin | Serum concentration | Treatment response ↓/ relapse ↑ | 120, 121 |
| sICAM1 | Serum concentration | Treatment response ↓ | 122, 123 |
| suPAR | Serum concentration | Risk of mortality ↑ | 123-126 |

### 1.6.2. Nucleic acid amplification techniques

Reliable and rapid direct NAATs became routine, supplemental procedures in many settings, during the 1990s [133]. However, the sensitivity of commercial tests ranged from 81.8% to 100% in smear-positive and from 35.3% to 95.9% in smear-negative respiratory samples [134]. A recently evaluated cartridge-based, automated and rapid real-time PCR method, the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA), simultaneously detects *M. tuberculosis* and the *rpoB* mutation related to rifampicin resistance [135]. The method displayed 98.7% and 75.0% sensitivity in smear-positive and smear-negative PTB cohorts, respectively, and 98.4% overall specificity was found. In the same meta-analysis, an rifampicinresistance gene was found with 94.1% sensitivity and 97.0% specificity. [136] WHO has endorsed the use of Xpert MTB/RIF in diagnostic tests for persons suspected of MDR-TB or HIV-associated TB. However, this does not eliminate the need for conventional methods. [137]

A method developed in the last decade that is highly specific and simple to perform, i.e. the loop-mediated isothermal amplification (LAMP) technique using six primer sets, has been evaluated [116, 117, 138]. LAMPs have advantages over other NAATs in terms of their low cost and simplicity, and they have been introduced as promising platforms, especially for resource-poor settings. High specificity and sensitivity in the evaluated LAMPs has been shown with smear- and culture-positive samples. However, lower sensitivity has been found in smear-negative samples. [116, 117, 138]

### 1.6.3. Molecular line probe assays

The Inno-LiPA Rif.TB (Innogenetics, Zwijndrecht, Belgium), Genotype MTBDR, and Genotype MTBDR*plus* (Hain Lifescience, Gmbh, Germany) line probe assays can be used to detect the *M. tuberculosis* complex and mutations in the *rpoB* gene. Genotype MTBDR also identifies the mutation in the *katG* gene and Genotype MTBDR*plus* mutations in the *katG* and *inhA* genes, both related to isoniatsid resistance. Based on the WHO report with the pooled data from systemic reviews and meta-analysis, *rpoB* mutations can be detected from *M. tuberculosis* isolates with 97% sensitivity and 99% specificity using the Inno-LiPA Rif.TB. Moreover, 98.1% sensitivity and 98.7% specificity can be achieved from *M. tuberculosis* isolates using Genotype MTBDR assays, but also from the smear-positive pulmonary samples. Isoniatsid resistance can be detected with 90% sensitivity and 99.5% specificity using the MTBDR*plus* assay. [139]

### 1.6.4. Immunological methods

#### 1.6.4.1. Tuberculin skin test

In 1890, Dr Robert Koch prepared tuberculin, a glycerol extract of mycobacteria, for the cure for tuberculosis. However it caused a febrile reaction in patients. It was later used for diagnosis. In 1907, Dr. Clemens von Pirquet was the first to perform the tuberculin skin reaction test (TST) with an intracutaneous injection. Later, Dr. Charles Mantoux modified the method. He injected tuberculin with a needle but not with a help of skin incision. Since 1930s, a sterilized filtrate of *M. tuberculosis* culture, the purified protein derivative (PPD) has been used in the TST. [140] The TST measures a delayed-type hypersensitivity reaction to PPD after an intradermal injection [141]. A positive result indicates that a person has an immunological memory to mycobacterial antigens. The method does not discriminate between active TB and LTBI, and it should not be used for the diagnosis of active TB. [142] PPD contains protein components that are shared between all mycobacterial species [143]. Hence, exposure to non-tuberculous mycobacteria (NTM) and BCG vaccination reduces the specificity of the test. Moreover, the sensitivity of TST is limited, especially in patients with advanced TB, HIV-positive individuals, and children [144, 145]. TST requires a return visit for test reading to the clinic three days after the injection [141]. Despite many drawbacks, the method is still used, particularly in developing countries, probably due to the unavailability of accurate and cost-effective methods.

#### 1.6.4.2. Interferon-γ release assays

IGRAs are *in vitro* immune tests based on the detection of antigen-specific T cell immune responses. The methods have been introduced as an alternative to TST for the diagnosis of LTBI. They have been extensively described during the last decade [146]. Two commercial IGRA tests are available. QuantiFERON®-TB Gold In-Tube (QFT-IT) (Qiagen, Germany) quantitates released IFN-γ from the supernatant after whole-blood antigen stimulation using an enzyme-linked immunosorbent assay (ELISA) [147]. T-SPOT®.*TB* (Oxford Immunotec Ltd, Abingdon, UK) measures the frequencies of antigen-specific IFN-γ-producing cells from a purified lymphocyte fraction [148] with an enzyme-linked immune spot assay (ELISPOT) (Figure 3). Both assays use the following *M. tuberculosis*-specific antigens, the early secretory antigenic target-6 (ESAT-6), the culture filtrate protein-10 (CFP-10), and TB7.7 (p4) (only in QFT-IT), which all are absent in most of the NTMs and BCG strains [141, 149]. This feature is advantageous over PPD, especially in populations with a high NTM exposure and general BCG vaccination. Neither of the IGRAs can distinguish between active TB and LTBI [146].

The TB Network European Trials Group and ECDC have carried out systematic reviews and meta-analyses to assess the accuracy of IGRAs in the diagnosis of active TB and LTBI in different populations [150, 151]. As concluded in the ECDC guidance, no added value of IGRAs combined with standard methods for active TB diagnostics has been found. However, in certain clinical situations, including patients with EPTB or negative AFB staining or culture, children, or the differential diagnosis of infection with NTM, IGRAs can be used to supplement the diagnostic work-up. IGRAs should not be used as a rule-out test of active TB. For the diagnosis of LTBI in low-incidence countries, clear advantages over TST have been

shown, and IGRAs can be used in contact-tracing algorithms and in risk assessment to identify individuals for preventative treatment. A negative IGRA result does not rule out LTBI. [146] Only subjects who have an increased risk of developing active TB from LTBI, and would benefit from preventative therapy, should be tested by IGRA. [152]

The estimated sensitivity of IGRAs for the detection of LTBI is 80–90%, when culture-confirmed TB patients have been tested. In low TB settings, the specificity of over 95% has been identified. The reproducibility of the IGRAs is limited and highly susceptible to numerous factors related to manufacture, sample processing, analytical testing and immunological variability. [152]

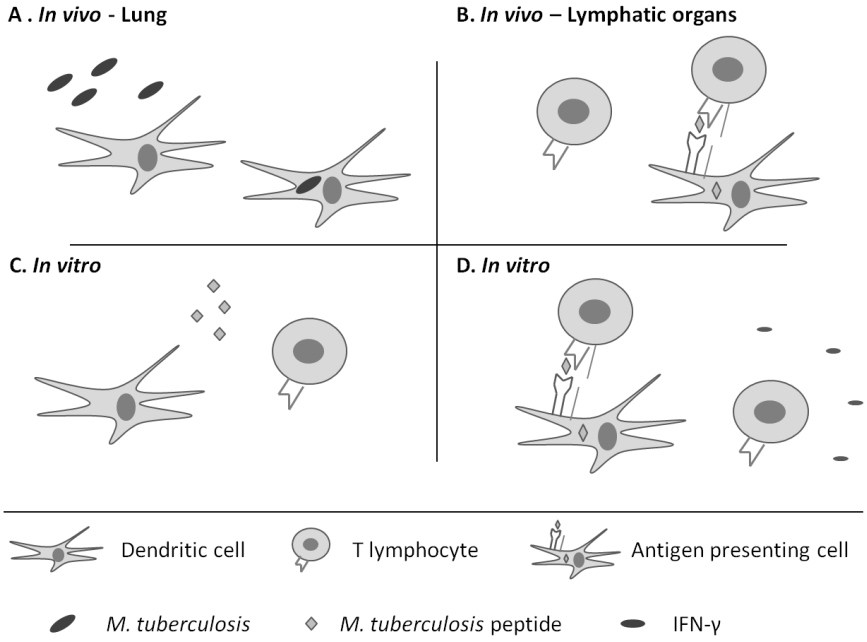


**Figure 3.** Immunological principle of IGRAs. A: Antigen-presenting cells recognize and take up invading *M. tuberculosis* bacteria in the lungs. B: Processed *M. tuberculosis* antigens are presented to naïve T cells in lymphatic organs, and they induce the differentiation of naïve T cells into effector T cells. C: *M. tuberculosis* peptides are incubated *in vitro* with peripheral blood mononuclear cells (PBMCs), including circulating *M. tuberculosis* sensitized effector T lymphocytes and DCs. D: DCs present peptides associated with MHC molecules to effector T lymphocytes , and the activated lymphocytes produce IFN-γ, which is measured with ELISA or ELISPOT.

### 1.6.4.3. Serodiagnostic tests

There are numerous commercial serological tests for TB diagnostics. WHO has reviewed 67 studies for PTB and 25 studies for EPTB in which serological tests have been evaluated. The sensitivity and specificity of serological tests have varied from 0% to 100% and from 31% to 100%, respectively. The tests are not recommended for use in TB diagnostics. [153]

### 1.6.4.4. Proposed antigens for immunological assays

#### 1.6.4.4.1. Heparin-binding hemagglutinin

Heparin-binding hemagglutinin (HBHA) is a 28-kilodalton (kDa) surface adhesin produced by all members of the *M. tuberculosis* complex, including *M. bovis* BCG, but not by nonpathogenic *M. smegmatis* [154, 155]. HBHA has hemagglutination and aggregation activity and it binds to the sulfated glycoconjugates of non-phagocytic cells, such as those of pulmonary epithelial cells [154]. This interaction is mediated by the lysine-rich C-terminal region of the protein [156]. It has been demonstrated that HBHA is involved in the extrapulmonary dissemination of *M. tuberculosis* [157]. HBHA has been shown to induce IFN-γ production and protective immunity in mice, however only when native HBHA (nHBHA), that has undergone a post-translational modification, was used in immunization [69]. The modified protein contains a complex methylation pattern in the C-terminal domain, which is absent from recombinant HBHA, produced in *Escherichia coli* [69, 155]. A similar methylation pattern and immunological properties compared to nHBHA were observed when recombinant protein was produced in *M. smegmatis* (rMS-HBHA) [158-160].

rMS-HBHA and nHBHA purified from *M. bovis* BCG have been used in several studies to assess the diagnostic potential of HBHA [70, 71, 159, 160]. In three studies, significantly higher concentrations of IFN-γ were found from LTBI subjects compared to the active TB group after the nHBHA or rMS-HBHA stimulation of PBMCs [70, 71, 160]. In one study, the nHBHA IFN-γ assay was shown to discriminate TST-positive LTBI subjects and active TB patients from healthy subjects, half of whom were vaccinated, with a respective sensitivity of 92% and 55%. A specificity of 94% was observed in both analyses [71]. The same study revealed a significant difference between LTBI and active TB groups. However, a high degree of overlap was present between LTBI and active TB groups, and the discriminative ability between these two groups was not estimated. [71] Comparable IFN-γ levels between persons with LTBI and active TB patients who had successfully completed anti-TB treatment was reported in one study [160].

Only a few of the BCG-vaccinated healthy controls were observed to produce low concentrations of IFN-γ after nHBHA stimulation [70, 71]. In a study in which rMS-HBHA was used, the BCG-vaccinated control group was not included [160].

When antibody responses to HBHA were examined, the majority of the active TB patients, but only a minority of those with LTBI, possessed IgG-class antibodies to nHBHA or rMS-HBHA. No IgG-class antibodies to nHBHA were found from BCG-vaccinated healthy subjects. [70, 159] The presence of rMS-HBHA-specific IgM- and IgG-class antibodies in the serum of treated TB patients has been reported in one study [161]. For the diagnostics, future studies with larger cohorts are needed.

#### 1.6.4.4.2. Latency-associated antigens

Little is known about the mechanisms by which *M. tuberculosis* survives in granulomas during the persistent stage of the infection. The most extensively studied is the oxygen depletion model, in which the gradual depletion of oxygen has been shown to lead to the nonreplicating persistence of bacteria displaying minimal metabolic, chromosomal and structural changes [162]. Besides hypoxia, bacterial respiration has also been shown to be inhibited by low levels of nitric oxide and carbon monoxide produced by immune cells [163, 164]. When *M. tuberculosis* was cultured under these conditions, the dormancy regulon (DosR), including 48 genes, was expressed and the bacteria were prepared for survival in *in vitro* dormancy in a non-replicating form [163, 164]. The DosR regulon has also been demonstrated to be crucial for the rapid resuscitation of growth when aerobic and non-nitric-oxide conditions have been restored [164].

It has been suggested that the DosR regulon plays a protective role in LTBI. The finding that subjects with LTBI, rather than active TB patients, bear specific T cell responses against recombinant DosR regulon antigens suggests that the regulon is expressed during latency [72]. When the diagnostic potential of DosR antigens was investigated with IFN-γ ELISA, large differences were observed in antigen recognition between different geographical populations [72-75]. In these studies, most of the recognized antigens in different TST-positive populations were a probable phosphohexokinase (Rv2029), a hypothetical protein (Rv2628), and a possible nitrate/nitrite transporter (Rv1737) [72-74]. Rv2628-specific IFN-γ production has also been associated with cured tuberculosis in an Italian study [165]. When resuscitation-associated antigen Rv3407 was used for the stimulation of PBMCs of individuals of German origin, none of the TB patients but 55% of subjects with LTBI had an IFN-γ response in an ELISA assay [76]. Furthermore, different LTBI patients appeared to recognize different epitopes of the antigen [76]. Variation in the recognized latency antigens reveals that the cumulative IFN-γ response to all known or a few of the most immunogenic antigens appears to be the most promising approach for differentiation between LTBI or HHCs and active TB [72, 75].

Interestingly, IFN-γ levels measured by ELISA after the stimulation of PBMCs with the resuscitation proteins Rv0867c and Rv2389c have been found to differ significantly between two groups exposed to *M. tuberculosis*, specifically household and community contacts with a positive IGRA result. HHCs with a more intense exposure had a stronger response to these antigens. The result suggests that Rv0867c and Rv2389c antigens distinguish different exposure levels and might predict future disease progression. [166]

1.6.4.4.3. Region of difference

The *M. tuberculosis* genome contains 16 regions, called regions of difference (RDs), that are lacking or deleted in some strains of *M. bovis* and *M. bovis* BCG. Eleven of these 16 RDs of the *M. tuberculosis* strain H37Rv are absent in all the currently used *M. bovis* BCG vaccine strains. [167] The proteins encoded by RDs offer the opportunity to avoid a cross-reaction between *M. tuberculosis* and BCG vaccine-induced immune responses. For example, the RD1-encoded ESAT-6, CFP-10, and TB7.7 are used in IGRA tests, as discussed earlier in this thesis [141].

The diagnostic ability of six proteins from the RD2 and RD11 regions was tested by IFN-γ ELISPOT, and an increase in sensitivity from 86.2% to 92.9% was shown when the IGRA was supplemented with a recombinant small subunit of ribonucleotide reductase antigen, Rv1981c. However, the analysis of samples from vaccinated healthy subjects revealed a decrease in specificity from 100% to 93.3%. [77] In another study, peptides of a putative transcriptional regulatory protein from the RD2 region, Rv1985, showed 96.2% sensitivity and 95.5% specificity when added to the antigen mixture of the QFT-IT assay [78]. These results indicate that the accuracy of the currently used IGRAs might be improved in the future with new antigens.

Antibody responses to RD proteins have also been investigated. In one study, IgG antibody levels to enoyl-CoA hydratase, Rv0222, from the RD4 region reached the cut-off in 98% and 90% of TB patients with or without HIV, respectively, and 90% specificity was reported when vaccinated healthy subjects were tested [79].

1.6.4.4.4. Proteome studies

Recently, genome-scale proteomic analysis has expanded the possibilities to identify antigenic proteins recognized by the antibodies of infected persons. Antibodies to *M. tuberculosis* were measured with high-throughput proteome microarray technology, using microarrays that carried 4099 proteins covering more than 99% of the open reading frames of *M. tuberculosis* H37Rv. The results of this study revealed that antibody targets vary between active TB patients, but the response is mainly focused on extracellular and membrane-associated proteins. [168] In another study involving antibodies to proteins encoded by 87% of the open reading frames of *M. tuberculosis*, 249 antigenic proteins were found, including regulatory proteins and proteins related to lipid transport and metabolism. When the diagnostic potential of 20 of the most antigenic proteins was assessed with serum from 96 active TB patients and 24 healthy controls, the specificity was found to range between 92% and 100%. However, sensitivity varied from 14% to 74%. [169]

### 1.6.4.5. Immune cells and their markers

#### 1.6.4.5.1. IFN-γ-inducible protein

IFN-γ-inducible protein (IP-10/CXCL-10) is a proinflammatory chemokine induced by IFN-γ and produced by antigen-presenting cells infected with microorganisms. The potential of IP-10 as an alternative or supplemental biomarker to IFN-γ for the diagnosis of TB infection has been introduced. [80] Like IFN-γ, IP-10 is produced *in vitro* in patients with active TB and subjects with LTBI, but not in unexposed controls when QFT-IT tubes were used for the stimulation [81, 82]. IP-10 is expressed at higher concentrations than IFN-γ [81-83], which may enable assay simplification, for example for dried blood or plasma spots [84]. The IP-10 was found stable in filter papers for at least four weeks, which enables sample delivery over long distances [84]. The IP-10 dried plasma spot method derived from QFT-IT tubes was evaluated in a cohort including 78 active TB patients and 98 healthy controls [85]. Increased sensitivity from 78% to 84%, with a slightly decreased specificity, from 100% to 97%, compared to IFN-γ QFT-IT ELISA was found [85]. In the studies with HIV-infected TB patients who had low $CD4^+$ T cell counts, slightly increased sensitivity and a lower number of indeterminate results were obtained in an IP-10 assay compared to IFN-γ [86, 87]. In children under five years old, reduced IFN-γ, but not IP-10 responses to *M. tuberculosis* antigens were found [88]. Contrary to IFN-γ, IP-10 is also expressed at high concentrations by unstimulated cells [82]. The variety of methodologies and cut-offs used complicates the interpretation and comparison of results [80].

#### 1.6.4.5.2. Ratios of IL-4 to IL-4δ2 and IL-4 to IFN-γ

The importance of an IL-4 response to *M. tuberculosis* was controversial before 2000 due to technical issues [170]. IL-4 has a low messenger RNA (mRNA) copy number and it is a labile cytokine that is produced in low concentrations. Therefore, it is difficult to detect by immunoassays and quantitative reverse transcriptase PCR (RT-qPCR). [171] In earlier studies, the methods did not distinguish between IL-4 and IL-4δ2, an alternatively spliced variant and antagonist of IL-4 [172, 173]. The later-developed RT-qPCR method has enabled the quantification of IL-4 and IL-4δ2 mRNA separately in unstimulated PBMCs [170]. IL-4 is expressed in higher quantities by the unstimulated PBMCs of active TB patients compared to TST-positive or TB-exposed controls in cohorts from developed countries [89, 174]. In contrast, no differences in IL-4 expression have been detected between patients and HHCs from a TB-endemic region, where elevated exposure to many pathogens, including helminths, is frequent [41]. IL-4δ2 expression is related to LTBI rather than to active TB [175]. In the follow-up studies, the ratio between IL-4 and IL-4δ2 mRNAs and also IL-4 and IFN-γ mRNAs has been reported to correlate with disease activity and cure. In these studies, the expression of IL-4δ2 and IFN-γ decreased relative to IL-4 in TB contacts when they developed active TB. During the TB treatment, the expression levels of IL-4δ2 and IFN-γ increased relative to IL-4. [41, 89] Variation in the expression levels of IL-4 and IL-4δ2 mRNAs in different studies and populations have hampered the use of these genes for diagnostics of active TB or LTBI. However, the ratio between expressions of the two genes may be useful in monitoring the cure or progression of the disease.

1.6.4.5.3. Cytotoxic T lymphocytes

CTLs are effector T lymphocytes that kill tumor cells or cells infected with intracellular pathogens. CTLs destroy their targets by granule exocytosis or by the FAS-ligand pathway. Cytotoxic granules contain membrane-disrupting and pore-forming protein, perforin, serine proteases, namely granzymes, and the antimicrobial agent granulysin. [52] The killing activity of CTLs has been historically measured by chromium ($^{51}Cr$) release assays and later by methods monitoring the release of enzymatic fluorescent dyes [176]. The frequencies of antigen-specific CTLs can be measured by tetramer staining. In addition, their ability to produce effector molecules can be analyzed by ELISPOT techniques, or by the flow-cytometric analysis of lysosomal-associated membrane proteins [177, 178]. These glycoproteins include CD107a, CD107b, and CD63, which surround the core of the lytic granules and are found on the surface of activated, degranulating lymphocytes [178]. mRNA expression analysis has also been used for the quantification of up-regulation of cytotoxic molecules [53, 179].

CTLs can be activated *in vitro*, resulting in the increased expression of cytotoxic molecules, such as Prf, GrB, and granulysin, but also in the cytolytic activity when stimulated with a variety of mycobacterial antigens, including live *M. bovis* or *M. tuberculosis*, or *M. tuberculosis*-specific peptides and recombinant proteins [53-55, 179, 180]. It appears that the enumeration of antigen-specific CTLs by methods, such as intracellular staining, is complicated, mainly because the cytotoxic effector molecules are expressed constitutively. It has also been shown that cells expressing effector molecules were not necessarily able to kill their targets. [181] However, antigen-specific GrB and Prf production measured by ELISPOT, or the degranulation factor CD107a-expression have been shown to correlate with cytotoxicity [177, 178, 182].

It has been shown in a mouse model that the cytotoxic potential of $CD8^+$ T cells decreased after 20 weeks of infection compared to the early stage of the disease when *M. tuberculosis* was used in an *in vitro* cell stimulation [183]. In another study with mice, cytolytic activity mediated by pulmonary $CD8^+$ T cells was still detectable 37 weeks after the initiation of infection when stimulation was performed with CFP-10 peptides [180]. In humans, *M. tuberculosis*-specific CTLs have also been found from HHCs and subjects with LTBI, which may suggest a protective role for CTLs [54, 179]. The diagnostic potential of CTLs has not yet been extensively studied.

1.6.4.5.4. Combinations of immunological markers

Because of the complex pathogenesis of TB, the analysis of only a single immunological marker might not be sufficient for the discrimination of active TB from LTBI. When multivariate analyses were used to assess the discriminative ability of TB disease stages with a combination of multiple immunological markers, high sensitivities and specificities were achieved [184-186]. For example, when the concentrations of seven cytokines were measured following the *in vitro* TB10.4 antigen stimulation of whole blood, the combination of TNF-α, IL-12(p40) and IL-17 resulted in the 61% sensitivity and 97% specificity for the differentiation of active TB patients and HHCs [185]. In another study a total of 45 immune-related genes were analyzed by RT-qPCR in the ESAT-6-stimulated PBMCs of active TB

patients and subjects with LTBI. A combination of IL-8, FoxP3, and IL-12β gene expression levels differentiated the tested groups with 97% sensitivity and 89% specificity. [184] When, the levels of 17 cytokines or chemokines in the unstimulated plasma samples of active TB patients and HHCs were studied using the Luminex assay, the combination of fractalkine, IFN-γ, IL-4, IP-10, and TNF-α discriminated active TB patients from HHCs, out of whom half were IGRA-positive, with 96.7% sensitivity and 100% specificity. [186]

1.6.4.5.5. High-throughput array techniques

Recently introduced high-throughput array technologies have enabled the searching of broader gene and protein profiles related, for example, to active TB and LTBI. In one study, a distinct 393-transcript signature was determined from the blood of active TB patients when the genome-wide transcription profile was assessed [187]. This signature pattern was found to discriminate between active TB and LTBI with 61.67% sensitivity and 97.75% specificity. However, a lower specificity of 86% was recorded when profiles from active TB patients were compared to patients with other bacterial or inflammatory diseases. [187] A neutrophil-driven IFN-inducible gene pattern was found to dominate in patients with the active TBin this study [187]. The dominance of the IFN-inducible gene pattern has also been reported in another study in which a whole-genome array was used. However, the results of this study demonstrated a notable similarity between the gene profiles of patients with active TB and sarcoidosis. [188] In a study in which the array technology was used for the identification of gene profiles of PPD-stimulated PBMCs, 229 transcripts for active TB were identified. The selected genes IP-10, adenolpyrophosphatase-10A, and Toll-like receptor-6 in RT-qPCR differentiated active TB from LTBI with 71% sensitivity and 89% specificity [189].

When the serum protein profiles of TB patients were compared to those of patients with sarcoidosis or other destructive pulmonary pathology by the surface-enhanced laser desorption ionisation time of flight mass spectrometry method (SELDI-TOF-MS), 95.5% sensitivity and 94.9% specificity was recorded. Two out of the 20 most informative peaks were identified and found to be serum amyloid A protein and transthyretin. [190] Later, active TB patients were compared to subjects with LTBI by the same method, and three peak areas overexpressed by active TB patients with 89% sensitivity and 82% specificity were observed [191].

### 1.6.5. Antigen detection

#### 1.6.5.1. Lipoarabinomannan

LAM is a 17.5 kDa, highly immunogenic and heterogeneous glycolipid associated with the mycobacterial cell wall. It is a virulence factor of *M. tuberculosis* [192]. Attachment in the bacterial cell wall is mediated by a PIMs anchor and extends to around 20 highly branched mannose residues that form the core of the LAM. The molecular termini of rapidly growing strains comprise linear or branched arabinan motifs. In the virulent species of mycobacteria, such as *M. tuberculosis* and *M. bovis,* arabinan motifs are extensively capped with mannan residues (Figure 4). LAM or related structures are also present in other mycobacterial species and other genera of *Actinomycotales,* such as *Corynebacterium.* [12, 193]

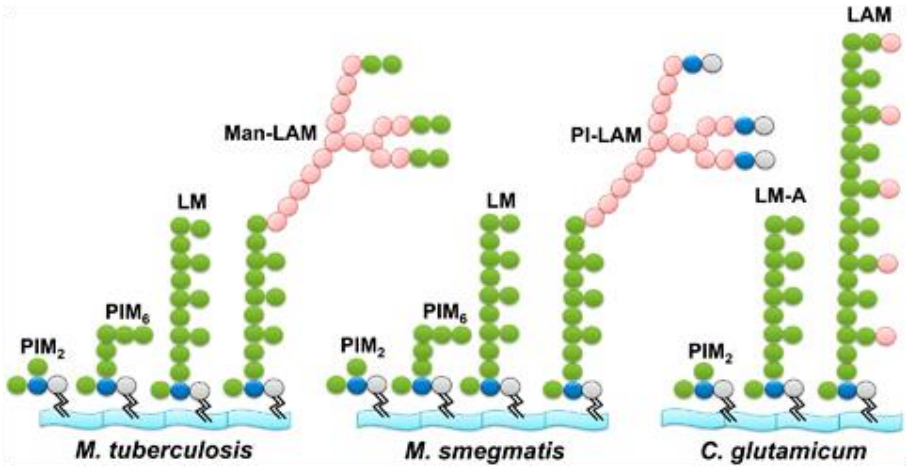


**Figure 4.** Cell-wall-anchored LAMs of *M. tuberculosis, M. smegmatis and C. glutamicum*. LAM of *M. tuberculosis* is terminated by mannan (green) residues resulting in Man-LAM, while Pi-LAM of *M. smegmatis* is capped by phosphoinositol (blue and gray) and LAM of *C. clutamicum* by arabinan (pink) units. Reprinted with permission from John Wiley and Sons: FEMS Microbiology Reviews [193], copyright 2014.

LAM is released by metabolically active or degrading bacterial cells [192] and it is believed to be filtered by the kidney and excreted into the urine (uLAM) of active TB patients [90]. LAM as an antigenic marker of TB was first described in the early 1990s, when it was detected in the sputum and serum of active TB patients [194, 195]. In 2001, Hamasur reported that mice injected with purified LAM excreted it into urine at ng/ml levels when detected by an ELISA method with polyclonal anti-LAM antibodies. Detectable levels of uLAM in the 50-fold concentrated urine of TB patients but not in that of healthy controls were also found. [91] When the method was evaluated with Ethiopian TB patients with positive AFB and healthy subjects, with an assay cut-off of 0.45 ng/ml, 81% sensitivity and 90% specificity was achieved. When AFB-negative samples were included in the TB group, the sensitivity decreased to 74%. [92] LAM has also been detected in the samples of serum [195, 196], cerebrospinal fluid [197], pleural effusion [198] and sputum [93]. However, urine is the most extensively studied biological sample and has been suggested as an attractive option for the point-of-care diagnostics of active TB. Urine has advantages over sputum, such as safety and ease of collection.

Two commercial kits for uLAM detection are now available, Clearview® TB ELISA (Alere inc., MA, USA) and the lateral flow method Determine TB LAM ag (Alere inc.). Based on evaluations performed primarily in Africa and Asia, the accuracy of these tests for clinical use is, however, inadequate. In most of the studies the sensitivity of the test in HIV-noninfected patients ($TB^+HIV^-$) has been found to be lower than 22% [94]. 74% sensitivity has been reported in one study [95]. The assay sensitivity is increased in HIV-infected patients ($TB^+HIV^+$) with a decreased blood $CD4^+$T cell count. Sensitivity up to 85% in patients with $CD4^+$ T cell counts <50 cells/µl has been reported. [94] In most of the studies, increased sensitivity has been reported in HIV-infected compared to non-infected populations [93, 96-100]. According to the manufacturer, these tests are only recommended for use in TB patients with HIV co-infection. However, in some studies, no differences in uLAM concentrations between $TB^+HIV^+$ and $TB^+HIV^-$ patients have been found [95, 101].

The increased assay sensitivity has been explained by immunosuppression due to the loss of $CD4^+$ T cells and a further increase in the bacterial burden and uLAM excretion. However, in one study, a positive correlation was found between HIV positivity, uLAM excretion, and a positive result in the Xpert MTB/RIF assay performed on urine. [99] This result raises a question about the relationship between disseminated genitourinary TB and increased uLAM assay sensitivity in HIV-infected patients. When the results of the LAM assay were compared to smear microscopy in PTB patients, concordant sensitivity, 37%, was only found in TB patients with a $CD4^+$ T cell count <200 cells/µl. In $TB^+HIV^-$ and $TB^+HIV^+$ groups with $CD4^+$ T cells >200 cells/µl, smear microscopy was more sensitive compared to the uLAM assay. However, smear microscopy and uLAM test identified partly different patients. [93] Increased sensitivity of the uLAM assay has been reported in patients with smear-positive PTB compared to a smear-negative disease [92, 95-97, 99, 102]. Very low sensitivity in the LAM assay was observed in patients with EPTB. [99, 102]

The concentration of uLAM has varied between studies from pg/ml levels to over 100 ng/ml. In these studies, the concentration of urine samples has not been used or no effect on assay accuracy has been found. [95, 103, 104] The kinetics of uLAM excretion is not well documented, but in one study in which 32 samples were tested, a positive uLAM result converted negative in approximately 50% of the patients between the second and eighth week of treatment. In all but one out of the 24 tested, the test was negative after 24 weeks of initial of treatment [99].

The specificity of commercial uLAM detection tests for active TB varies between 83% and 100% [90, 94, 105-107]. The cross-reactivity of antibodies used in commercial kits with other mycobacterial species or other respiratory pathogens has been investigated with artificial samples in which bacterial colonies have been inoculated in broth media [93, 95]. As expected, polyclonal antibodies recognized all tested mycobacterial species, but also some *Nocardia.* None of the tested Gram-negative bacteria or bacteria of *Staphylococcus* or *Streptococcus* families caused a cross-reaction. However, when monoclonal anti-LAM antibodies were used, no cross-reaction was observed with some pulmonary pathogens, including *Nocardia asteroids* [199]. When patients with a smear-negative pulmonary NTM infection were tested with a commercial LAM test, 12.2% showed a positive result [96]. No results have been reported regarding other disease control groups, such as patients with pneumonia or urinary tract infection.

#### 1.6.5.2. Antigen 85

The Antigen 85 complex contains three extracellular 30- to 32-kDa secreted proteins, mycolyltransferases, that are essential for the *M. tuberculosis* cell wall biosynthesis [200]. Expression of the Antigen 85 complex is induced by the anti-TB drug isoniatsid. This mechanism has been suggested to be a response of mycobacteria to compromised cell wall integrity [201]. In some studies, the expressed Antigen 85 complex proteins have been measured in the sputum of PTB patients as a marker of treatment outcome [108, 109]. Significantly higher amounts of Antigen 85 were found in patients with persistent infection compared to those who were rapidly cured. In these studies, infection was defined as persistent when the BACTEC culture became positive at or after 90 days of anti-TB treatment within 20 days of incubation [202]. The difference was only observed in samples collected 3–7 and 14 days after treatment initiation [109]. These reports, based on a small cohort, indicate that parameters measured during the first weeks of therapy may predict the future positivity of the BACTEC culture and disease outcome.

Antigen 85 has also been found in the peripheral blood of active TB patients [110, 111]. Bentley-Hibbert measured >30 µU/ml of Antigen 85 with a dot immunobinding assay in the sera of all 13 patients tested to have active TB, including patients with smear-negative PTB and patients with EPTB. All healthy controls, and also the groups with a positive TST, had Antigen 85 concentrations below 30 µU/ml. [110] Later, Antigen 85 was measured in serum by ELISA and 96% sensitivity was observed when bacteriologically confirmed TB patients were tested. Analysis of the disease control group including patients with acute or chronic non-TB diseases, such as asthma or pneumonia, and a healthy control group produced 80% and 100% specificity, respectively. [111] When Antigen 85 was measured in urine, lower concentrations compared to serum and no correlation with the diagnosis was found. A chromatographic analysis of serum showed that Antigen 85 exists in a complex with fibronectin or IgG, which may explain its absence in urine. [110]

#### 1.6.5.3. Rv1681 protein

To investigate the protein antigens excreted into the urine of PTB patients, liquid chromatography and mass spectrometry were used in one study [112]. From approximately 400 identified peptide sequences, four were found to be identical to *M. tuberculosis* proteins, and one of these, the 32 kDa, putative molybdopterin biosynthesis protein MoeX/MoeA, Rv1681, was unique to the *M. tuberculosis* complex. Importantly, the patients with renal or urinary tract abnormalities were excluded from the study, suggesting that the excreted protein did not originate from the kidney, but rather from the lungs of the PTB patients. [112] Recently, the diagnostic ability of Rv1681 was evaluated [113]. First, recombinant Rv1681 was expressed and used to produce rabbit antisera. The immunological detection of urinary Rv1681 was demonstrated by immunoaffinity purification with produced antisera. When the ELISA method with a limit of detection ranging from 20 pg/ml to 50 pg/ml was used, the protein was found in 11 of 25 tested non-concentrated urine samples from PTB patients. None of the samples from tested TST-positive healthy subjects, patients with urinary tract infection, or patients with tropical diseases, such as schistosomiasis, reacted in the test. Also, one of the 21 individuals with clinical symptoms consistent with TB, but without bacteriologic

confirmation, reacted. The researchers concluded that Rv1681 is an attractive candidate for the development of an accurate urine antigen detection method. [113]

### 1.6.6. Other methods

#### 1.6.6.1. Transrenal DNA

Cell-free nucleic acids, such as those from dying human cells and microorganisms, are degraded and released into the circulation [115, 203]. The size of the cell-free nucleic acids in the plasma is smaller than 300 bp, which enables DNA fragments to pass through the kidney barrier and be excreted into the urine as transrenal DNA [204, 205]. The presence of cell-free nucleic acids has been introduced as a disease-activity and stage-differential marker in areas such as oncology, organ transplantation, and infectious diseases. The advantage of the method compared to other DNA amplifications is that the determination of the infection site is not needed. However, the accuracy of the method, and especially the sensitivity of the developed clinical applications, is limited. [115, 203]

In 1997, *M. tuberculosis* DNA was reported in the culture negative urine of an active TB patient [114]. Green reviewed seven papers in which methods for detecting transrenal DNA from the urine of TB patients were evaluated. In these studies, the sensitivity of the assays varied from 7% to 100%. There is a lack of understanding of the nature of transrenal DNA, which has caused inconsistency in the methodology and sample preparation. For example, the low copy number and high degradation of urinary DNA and the low molecular weight of the fragments complicate sample storage, DNA isolation, and primer design. Because urine is an attractive material for TB diagnostics, further studies with optimized and standardized methods are needed for the detection of transrenal DNA. [115]

#### 1.6.6.2. Volatile organic compounds

Metabolic profiling by gas or liquid chromatography, nucleic magnetic resonance or mass spectrometry has increasingly been used in the classification and diagnostics of diseases such as cancer and diabetes. In contrast, only a few studies have shown specific metabolic profiles occurring in infectious diseases. [206] Organic compounds have been detected in the breath and urine of TB patients and have been proposed as diagnostic markers [119, 207]. An increased abundance of *o*-xylene and isopropyl acetate and reduced levels of 3-pentanol, dimethylstyrene, and cymol were observed by mass spectrometry in the urine of active TB patients compared to controls [207]. Volatile organic compounds were found in the breath of TB patients [119] and later described as a point-of-care test detecting derivatives of naphthalene, benzene, and alkanes for the diagnosis of active PTB. However, inadequate accuracy with 71.2% sensitivity and 72% specificity indicates that further advances are required. [118]

#### 1.6.6.3. Non-specific immune activation markers

Some non-specific markers of immune activation have been investigated as markers of active PTB, but also as predictors of the TB outcome or treatment response. Neopterin, a pteridine derivative of guanosine triphosphate, is produced by monocytes and macrophages upon stimulation with IFN-γ. The detection of neopterin concentrations in body fluids provides information on Th1-derived immune activation, for instance in viral and intracellular bacterial infections. [208] In TB, significantly higher serum and urine neopterin concentrations have been reported in patients with active PTB compared to healthy controls [120, 121, 209]. A significant decline in serum neopterin levels during treatment was also observed [120, 121]. In one study, an association was found between an increased serum neopterin concentration after unsuccesfull treatment and bacteriologically confirmed infection relapse [120].

Soluble intercellular adhesion molecule 1 (sICAM-1) is an adhesion molecule mainly expressed by endothelial cells. It participates in the trafficking of inflammatory cells and is up-regulated by TNF-α, IFN-γ, and IL-1 [210]. ICAM-1 has been shown to be necessary for the initiation of inflammatory granuloma formation [211]. Significantly higher levels of sICAM-1 have been found by ELISA in the serum of PTB patients compared to healthy subjects or TB patients who had completed 6 months of chemotherapy, and patients with pneumonia or lung cancer [122, 123, 212, 213]. sICAM-1 levels also correlated with disease severity and the disease extent, and decreased significantly during the first month of anti-TB treatment [123, 212]. However, in another study, only a modest decrease was found after 13 weeks of chemotherapy [123].

Elevated serum and plasma levels at the time of TB diagnosis and decreases during treatment were also described with the soluble urokinase plasminogen activator receptor (suPAR) [123, 124]. suPAR is involved in the adhesion and migration of monocytes and macrophages. Increased levels of suPAR in body fluids have been shown to positively correlate with the activation of the immune system [124, 125]. In TB, an association between the number of bacteria in sputum and suPAR levels in serum has been described [124]. High suPAR levels after one to four months of anti-TB treatment may predict increased mortality [124, 126].

## 2. AIMS OF THE STUDY

The aims of this study were to investigate the diagnostic potential of selected biomarkers of tuberculosis and to study the specific immune response in different stages of tuberculosis infection. The specific aims were as follows:

- To compare the immune responses to HBHA in patients with different stages of TB infection and healthy BCG-vaccinated subjects (study I)
- To improve the performance of Clearview® TB ELISA for the detection of LAM in the urine of TB patients without HIV co-infection (study II)
- To quantify the excreted LAM in urine samples (study II)
- To investigate the potential of CTLs producing GrB, Prf, and expressing CD107a to discriminate the TB infection stages (study III)
- To examine the phenotypic properties of functional CTLs in the different stages of TB (study III)
- To compare the IFN-γ, IL-4, IL-4δ2, IL-17, and FoxP3 mRNA expression levels in active TB and LTBI (study IV)

# 3. MATERIALS AND METHODS

## 3.1. Ethical statements

The study was approved by the ethical committees of the University Hospital of Helsinki (232/E5/07, 356/E5/07, 149/2010), the Hospital District of Southwest Finland (47/180/2009), the University Hospital of Kuopio (105/2010) and the Medical University Hospital of Taichung, Taiwan (DMR-99-IRB-075-2). Written informed consent was obtained from all participants. From some of the patients with active TB, who were in isolation, a verbal consent was obtained by phone.

## 3.2. Study population

Clinical samples were collected between 2007 and 2013. The patients with active TB and NTM infections were from the University Hospitals of Helsinki, Turku and Kuopio, and the Medical University Hospital of Taichung. Samples from persons with LTBI and a few of the treated persons were provided in collaboration with HUSLAB and rheumatologists from Helsinki University Hospital. The persons treated for TB decades ago were recruited and interviewed by a medical student, Pekka Tapaninen. Healthy volunteers were laboratory personnel and medical students.

Whole blood, serum and/or urine samples were collected from 62 patients with active PTB, 16 patients with active EPTB, 30 persons with LTBI, 49 treated for tuberculosis, 5 infected with NTM, and 124 healthy subjects. Serum samples were used in study I and urine samples in study II. Aliquots of isolated PBMCs were stored in liquid nitrogen and used in studies I, III, and IV. The demographic and clinical data are presented in Table 2.

The diagnosis for the majority of patients with active TB was bacteriologically confirmed by *M. tuberculosis* culture and NAAT. In two pulmonary TB patients, the diagnosis was based on clinical and radiological findings, defined according to standard operation procedures, and on the response to anti-TB treatment, and in one patient on NAAT only. One patient with TB spondylitis was diagnosed with magnetic resonance imaging and a positive IGRA result. Samples were taken no later than two weeks after the initiation of anti-TB treatment. However, one patient with miliary TB had received anti-TB treatment for three months. From one patient with PTB, three urine samples were taken on days 11, 14 and 15, and again nine months after treatment initiation.

The LTBI group consisted of persons with a positive IGRA result, a known history of TB exposure, and a lack of clinical signs of active TB. One person was diagnosed with Ghon's complex in the chest X-ray. The decision of the positive IGRA test was based on the standard operation procedures adopted in each clinical laboratory. Seventeen out of the 30 subjects in the LTBI group had rheumatic diseases and 13/30 had miscellaneous inflammatory conditions. Anti-TB treated persons were divided into three groups: seven persons were treated with surgery only (Surg.), 32 had been partially treated with anti-TB treatment before the rifampicin era (Part.), and 10 had undergone the modern three-drug anti-TB therapy

(Mod.). In most of the cases, the sampling was performed decades after the completion of treatment. One of the NTM subjects had recently been diagnosed and four of them had been treated and were outpatients at the time of sampling.

Studies in which cell-mediated immunity was tested (I, III, IV) included healthy subjects who were BCG-vaccinated (Vaccinated) and IGRA negative. For study II, the BCG vaccination status or IGRA result was not defined. In addition, three samples from persons who had not received BCG vaccination (Unvaccinated) were included. Samples from healthy subjects used in study I were collected anonymously; only data on the approximate age were collected.

**Table 2. Demographic and clinical data and number of included samples in each study.**

| Patient groups | n | Age range | Female n (%) | I Serum / PBMCs | II Urine | III PBMCs | IV PBMCs |
|---|---|---|---|---|---|---|---|
| **Active TB, pulmonary (PTB)** | 62 | 18 - 86 | 16 (26) | 3 / 4 | 45 | 18 | 22 |
| **Active TB, other organs (EPTB)** | 16 | 24 - 89 | 9 (57) | 1 / 2 | 12 | 3 | 3 |
| Lymphatic nodes | 7 | 24 - 80 | 4 (80) | 1 / 1 | 5 | 2 | 2 |
| Bones | 3 | 32 - 89 | 3 (100) | - / 1 | 2 | 1 | - |
| Abdomen | 2 | 26 - 32 | 1 (50) | - / - | 2 | - | - |
| Miliary TB | 2 | 81 | 0 (0) | - / - | 2 | - | - |
| Parotid gland | 1 | 82 | 0 (0) | - / - | 1 | - | - |
| Intestine | 1 | 24 | 1 (100) | - / - | - | - | 1 |
| **LTBI** | 30 | 30 - 77 | 15 (50) | - / - | 15 | 11 | 20 |
| Rheumatic diseases | 17 | 49 - 77 | 9 (53) | - / - | 9 | 8 | 13 |
| Miscellaneous conditions | 13 | 30 - 69 | 6 (46) | - / - | 6 | 3 | 7 |
| **Treated for TB** | 49 | 32 - 86 | 32 (65) | - / - | 4 | 37 | - |
| No chemotherapy (Surg.) | 7 | 49 - 82 | 3 (43) | - / - | 1 | 6 | - |
| Partial treatment (Part.) | 32 | 39 - 86 | 22 (69) | 9 / 5 | - | 23 | - |
| Modern treatment (Mod.) | 10 | 32 - 82 | 7 (70) | - / - | 3 | 8 | - |
| **NTM*** | 5 | 66 - 79 | 4 (80) | - / 4** | 1 | - | - |
| **Healthy controls** | 124 | n/a | n/a | 7 / 16 | 101 | 6 | 8 |

**M. avium* (n = 3), *M. intracellulare* (n = 1), *M. abscessus* (n = 1); **treated; n/a = not available

## 3.3. Sample preparation and storage

PBMCs were isolated (studies I, III, IV) from whole heparinized blood on a Ficoll Paque gradient (Amersham Biosciences AB, Uppsala, Sweden) and stored in the CryoABC Kit medium (CTL-Europe, GmbH, Bonn, Germany) in liquid nitrogen until use. Serum and urine samples were preserved at -20 °C until use.

For the ELISPOT assays (study I, III), thawed and washed PBMCs were diluted in the CTL Test Medium (CTL-Europe), and for the flow-cytometric (study III) and RT-qPCR assays (study IV), in the RPMI cell culture media (Haartbio, Helsinki, Finland/Sigma-Aldrich, Saint Louis, USA ) supplemented with 10% heat-inactivated fetal calf serum (Haartbio /Sigma-Aldrich). All media used were supplemented with glutamine (2 mM) and penicillin (100 µg/ml)/streptomycin (0.1 mg/ml) (Sigma-Aldrich). The cells were counted using a blood count analyzer (ADVIA-60, Bayer, Germany) (study I) or in Bürker chambers (study II, IV).

## 3.4. Antigens

### 3.4.1. Recombinant proteins

Recombinant rMS-HBHA used in study I was expressed in *M. smegmatis*, and provided by Dr Hwa-Jung Kim from Chungham National University, South Korea. The plasmid containing the full open reading frame of HBHA was obtained from Dr G. Delogu, University of Sassari, Italy [158].

Recombinant CFP-10 (rCFP-10) and ESAT-6 (rESAT-6) were used in study III. Primers encoding the sequence of *cfp-10* and *esat-6* genes were designed (study III). Purified chromosomal *M. tuberculosis* DNA was used as a template and PCR amplified genes were cloned into the pQE30 vector using the QIAexpress System (QIAGEN, GmbH, Germany). The resultant plasmids were transformed into *E. coli* M15 host cells and the transformation mixture was plated onto Luria-Bertani plates containing ampicillin and kanamycin. A single colony was cultured until growth reached the mid-log phase; optical density at 600 nm was 0.5–0.7. Isopropyl-β-thigalactoside was added and after 4 hours of incubation the cells were lysed using the QIAexpress System. The lysate was applied to nickel-nitrilotriacetic acid (QIAGEN) matrices, washed with isopropanol as described [214] and eluted with urea buffer. The molecular weight and purity of the expressed proteins were confirmed by SDS-PAGE gel electrophoresis and Western blotting with anti-His antibodies (Clontech Laboratories, CA, USA). The protein endotoxin level was determined by the *Limulus amebocyte* lysate assay (GenScriptinc., Piscataway, NY, USA), and was found to be below 0.1 EU/ml.

### 3.4.2. Synthetic peptides

ESAT-6 and CFP-10 synthetic peptides (study I, III, IV) were from the T-SPOT®-TB kit (Oxford Immunotec, Oxford, UK).

The genomic sequence of the *M. tuberculosis* H37Rv HBHA protein was used to design the 15-mer sequential peptides overlapping by nine amino acids (study I). A methyl residue was added to three peptides from the C-terminus of the protein and the peptides for serology were biotinylated (Proimmune, Oxford, UK; Alta Bioscience, Birmingham, UK). Controls for the ELISA method in study I were previously tested peptides [215] from the *Borrelia burgdorferi* VlsE-protein $IR_6$ region (Core Facility of Protein Chemistry, Haartman Institute, Helsinki, Finland).

### 3.4.3. Extracts

LAM used in study II was an extract from the *M. tuberculosis* Aoyama-B strain (NacalaiTesque, Inc., Kyoto, Japan).

PPD used in studies I, III, and IV was obtained from Statens Serum Institute, Denmark.

## 3.5. Methods

### 3.5.1. Heparin-binding hemagglutinin ELISA (study I)

IgG- and IgM-class antibodies to rMS-HBHA, synthetic peptides, and PPD were measured by in-house ELISAs. The microtiter plates were coated with 100 µl of rHBHA, PPD, or for biotinylated peptides with streptavidin, and were incubated overnight at +4 °C. After blocking the wells with 0.25% human serum albumin (Finnish Red Cross, Helsinki, Finland) buffer, the plates were washed. In the peptide assay, the synthetic peptides were added and the plates were incubated for two hours at RT and washed. Serum samples were diluted 1:100 in 0.5% human serum albumin-10% fetal calf serum buffer, added in duplicate into plates and incubated for two hours. Detection was performed with anti-human IgM or IgG antibodies (Jackson Immuno Research, W. Baltimore, USA) diluted 1:5000 in 0.5% human serum albumin. After two hours of incubation, 100 µl of 4-nitrophenylphosphate (Boehringer Mannheim, Mannheim, Germany) was added as a substrate. The plates were read at 405 nm with an iEMS Reader MF (Labsystems, Vantaa, Finland).

### 3.5.2. ELISPOT (study I, III)

T-cell reactivity was tested by an IFN-γ (study I, III), GrB and Prf (study III) ELISPOT assays. A total of 2.5 x $10^5$ PBMCs/well were stimulated in duplicate in ELISPOT plates (Mabtech, Nacka Strand, Sweden) with rMS-HBHA, HBHA peptides, PPD, and ESAT-6 and CFP-10 peptides (study I) or with PPD, or rESAT-6, rCFP-10, or ESAT-6-CFP-10 peptide pools (study III), in the CTL Test Media at +37 °C under 5% $CO_2$ for 48 and 22 hours, in respective studies. Cell

viability was tested by stimulating $5 \times 10^4$ cells with phytohemagglutinin. The CTL Test Medium was used as a negative control. After the stimulations, the assays were performed according to the manufacturer's instructions (Mabtech). The spots were counted with an ELISPOT reader (Biosys-GmbH, Lyngby, Germany) and the readings in media wells were subtracted for the calculation of the net values.

### 3.5.3. Lipoarabinomannan ELISA (study II)

Clearview® TB ELISA (Alere Inc., MA, USA) was used to detect uLAM in urine samples. Thawed samples were first boiled for 30 min according to the manufacturer's instructions, cooled to room temperature, and then centrifuged for 10 minutes at 3000 rpm. Part of the supernatant was concentrated 100-fold with Vivapore 10/20 concentrators (Sartorius Ag, Goettingen, Germany), allowing the passage of molecules less than 7.5 kDa. Clearview® TB ELISA was performed according to the manufacturer's instructions for concentrated and non-concentrated samples. A calibration curve was prepared with a commercial LAM (NacalaiTesque, Inc.), starting from 20 ng/ml and ending at 0.5 ng/ml. The ODs were read at 450 nm with an iEMS Reader MF (Labsystems).

To investigate interfering factors, urine samples from pneumonia patients that tested positive (n = 7) or negative (n = 7) in the Binax NOW *S. pneumoniae* ag test (Alere Inc.) and 45 samples from patients with urinary tract infection were obtained from the Eastern Finland Laboratory Centre Joint Authority Enterprise, Mikkeli, Finland. These samples were obtained anonymously. Urinary *S. pneumoniae* ag positive and negative samples were tested as non-concentrated and 75-fold concentrated. Urinary tract infection samples were pooled according to the characterized bacterial species and tested as non-concentrated and 100-fold concentrated. In addition, the urine of a healthy person was spiked with colonies of *E. coli*, *E. faecalis*, *C. albicans* and *Corynebacterium sp* and whole inactivated *M. tuberculosis* bacteria to achieve solutions of 10 to $10^6$ CFU/ml, incubated for one hour at room temperature, and processed as actual real samples.

### 3.5.4. Flow-cytometric analysis (study III)

IFN-γ and CD107a were determined from $CD4^+$ and $CD8^+$ T lymphocytes with flow cytometric assays, and the phenotypic profile of $CD8^+CD107a^+$ T cells was assessed. PBMCs were first incubated with PPD or with media alone for 22 hours. Anti-CD107a-FITC (eBioscience, Inc., CA, USA) was added to the cells before stimulation. After two hours of incubation, Brefeldin A was added (eBioscience). The cells were surface stained with anti-CD8-Horizon-V450 (Becton Dickinson) and anti-CD4-Alexa Fluor® 700 (Becton Dickinson) and fixed as instructed (eBioscience). Anti-IFN-γ-PE-Cy7 (BD) was added with permeabilization buffer (eBioscience) for the intracellular staining. For the characterization of surface marker expression, the cells were stimulated as mentioned above and surface stained with anti-CD8-Horizon-V450 (Becton Dickinson), anti-CD45RA-PE-Cy7 (eBioscience), and anti-CCR7-PE (eBioscience). At least $10^5$ lymphocytes were analyzed with a FACSAria (Becton Dickinson) flow cytometer and the results were processed with the FACSDiva Version 6.1.3 software (Becton Dickinson).

### 3.5.5. RT-qPCR (study IV)

RT-qPCR was performed for the mRNA expression analyses of IFN-γ, IL-4, IL-4δ2, IL-17, FoxP3, and the validated housekeeping gene human ribosomal protein, in the stimulated PBMCs. The specific primers [42, 216-219] for the amplification of the selected genes *IFN-γ*, *IL-4*, *IL-4δ2*, *IL-17*, *FoxP3*, and human ribosomal protein were purchased from Sigma Aldrich. The sizes of the amplified products were assessed once with gel electrophoresis and later with a melting curve analysis.

PBMCs were incubated with PPD, a pool of CFP-10 and ESAT-6 peptides, and without antigen for 22 hours. The cells were lysed with TRI Reagent® (Molecular Research Center, Inc., Cincinnati, USA) and mRNA was extracted with the RNeasy mini kit (Qiagen, Dusseldorf, Germany). Then, the AMV reverse transcriptase (New England Biolabs, Ipswich, England), Oligo$(dT)_{23}$ primers (Sigma-Aldrich), and dNTP Mix (Thermo Scientific, Waltham, MA) were used for the cDNA synthesis. Amplification was performed with a Power SYBR® Green PCR Master Mix (Life Technologies Ltd., Paisley, UK). Analysis was conducted with the iCycleriQ™ Real-time PCR detection system (Biorad, Hercules, CA) and the results were analyzed with the comparative $C_t$ method $2^{\Delta\Delta Ct}$ [220].

### 3.5.6. Statistical analyses

Statistical analyses were performed with GraphPad Prism (versions 4.0 and 6.0, San Diego, CA) and the MedCalc software (Ostend, Belgium).

Correlations between the tested parameters in studies I and III were analyzed by the non-parametric Spearman's rank correlation test and statistical differences between the tested groups by the non-parametric Kruskal-Wallis test with Dunn's multiple comparison or the Mann-Whitney U-test. A *p*-value of less than 0.05 was considered significant.

In studies I, II, and IV, sensitivities, specificities, and cut-offs of the assays were calculated using the receiver operating characteristic (ROC) curve and area under the curve (AUC). The 95% confidence intervals were used to assess the discriminative ability of each of the tests. In study II, the statistical differences in sensitivity and specificity between the tested methods, were analyzed by the Chi-square test and Fisher's exact test.

# 4. RESULTS

## 4.1. Ability of the immune response to HBHA to discriminate between the stages of TB (study I)

To investigate the diagnostic potential of HBHA to discriminate between the patients with active TB, subjects with treated TB and BCG-vaccinated healthy subjects, IFN-γ-producing cells were analyzed using the ELISPOT method in the groups TB, Part., Vaccinated, and NTM. In addition, IgG and IgM antibodies were measured in the groups TB, Part., Vaccinated, and Unvaccinated.

### 4.1.1. Cell-mediated immune response measured by ELISPOT

rMS-HBHA stimulation induced the production of IFN-γ in all the tested groups, including the persons with active TB, with a high level of inter-individual variation in the frequency of cells producing the cytokine (Table 3). The mean of the number of the spots in a negative control wells was 12. Stimulation with the HBHA peptides did not increase the frequency of IFN-γ-producing cells compared to the stimulation with media only in any of the tested groups (data not shown).

The frequency of rMS-HBHA-stimulated IFN-γ-producing cells was compared to that of cells stimulated with PPD the known T-cell stimulator of *M. tuberculosis* infected. A positive correlation was found *(r* = 0.469, $p < 0.01$) when the samples from all the tested groups were included in the analysis.

As expected, none of the Vaccinated persons responded to the *M. tuberculosis*-specific ESAT-6 and CFP-10 peptides, while a response was observed with samples from groups TB and Part. (Table 3). To investigate the ability of rMS-HBHA, PPD, CFP-10, and ESAT-6 to distinguish between the IFN-γ response primed by *M. tuberculosis* infection (TB and Part. groups) and BCG vaccination, the ROC curves were constructed for the infected (TB and Part.) and Vaccinated groups. The respective AUCs with 95% confidence intervals were 0.636 (0.391–0.886), 0.736 (0.531–0.941), 0.947 (0.842–1.052), and 0.972 (0.917–1.027). The result indicated a good discrimination only for the CFP-10 and ESAT-6 assays. As PPD, rMS-HBHA showed inability to discriminate TB infection from the immune response induced by BCG vaccination.

Waning of the immunological response to HBHA was studied by dividing the healthy vaccinees into two groups according to age. No difference in the frequencies of IFN-γ-producing cells was found between the groups with approximate mean ages of 25 (n = 7) and 50 years (n = 8). The result reveals that the immunological memory for HBHA and PPD is persistent.

**Table 3. Frequencies of antigen stimulated IFN-γ-producing cells/$10^6$ lymphocytes.**

| | | rMS-HBHA | PPD | ESAT-6 peptides | CFP-10 peptides |
|---|---|---|---|---|---|
| **Group (mean age)** | **n** | **Median (25th-75th perc.)** | **Median (25th-75th perc.)** | **Median (25th-75th perc.)** | **Median (25th-75th perc.)** |
| TB | 6 | 164 (17 - 309) | 550 (312 - 745) | 99 (71 - 111) | 267 (108 - 343) |
| Part. | 5 | 21 (11 - 325) | 563 (288 - 760) | 115 (40 - 229) | 160 (96 - 737) |
| Vaccinated | 15 | 147 (85 - 340) | 274 (151 - 468) | 0 (0 - 27)* | 14 (0 - 23)* |
| Vaccinated (25) | 7 | 233 (103 - 366) | 237 (120 - 459) | 4 (0 - 27)** | 7 (0 - 21)** |
| Vaccinated (50) | 8 | 123 (67 - 338) | 364 (184 - 525) | 29 (0 - 39)*** | 14 (0 - 25)*** |
| NTM | 4 | 84 (36 - 263) | 194 (62 - 374) | n/d | n/d |

*n = 13, **n = 6, ***n = 7, n/d = not defined

Also in the semiquantitative analysis of the amount of IFN-γ produced, estimated by the size and the intensity of the spots, a high inter-individual variation was observed in all the tested groups (data not shown).

### 4.1.2. IgG and IgM response

Specific IgG and IgM antibodies to rMS-HBHA, HBHA synthetic peptides, and PPD were measured using an in-house ELISA. IgG-class antibodies to rMS-HBHA were detected in the groups TB, Part., and Vaccinated (Table 4). A positive correlation with the IgG response to PPD ($r$ = 0.739, $p$ < 0.001) was observed. The result reveals that rMS-HBHA is not usable for the discrimination between persons infected with *M. tuberculosis* and healthy vaccinees. Almost all tested persons had IgM antibodies to rMS-HBHA, including one non-vaccinated infant. IgM-class antibodies to PPD were not found in the samples of any of the tested persons (Table 4).

**Table 4. IgG and IgM antibodies to rMS-HBHA and PPD presented as optical densities (405 nm).**

| | | rMS-HBHA | PPD |
|---|---|---|---|
| **Group** | **n** | **Median IgG (25th - 75th perc.)** | **Median IgG (25th - 75th perc.)** |
| TB | 4 | 1.260 (0.346 - 1.806) | 0.862 (0.286 - 1.556) |
| Part. | 9 | 0.779 (0.657 - 1.255) | 0.371 (0.215 - 0.669) |
| Vaccinated | 4 | 0.473 (0.329 - 0.668) | 0.197 (0.034 - 0.556) |
| Unvaccinated | 3 | 0.204 (0.130 - 0.342) | 0.024 (0.021 - 0.119) |
| **Group** | **n** | **Median IgM (25th - 75th perc.)** | **Median IgM (25th - 75th perc.)** |
| TB | 4 | 1.485 (0.799 - 2.267) | 0.077 (0.023 - 0.103) |
| Part. | 9 | 0.875 (0.647 - 1.442) | 0.071 (0.021 - 0.138) |
| Vaccinated | 4 | 1.481 (1.193 - 2.238) | 0.032 (0.170 - 0.136) |
| Unvaccinated | 3 | 0.449 (0.287 - 1.218) | 0.015 (0.040 - 0.026) |

IgM-class antibodies to three methylated peptides from the C-terminus of the HBHA protein out of the 23 peptides tested were found in all tested groups. Samples from two unvaccinated infants also had IgM-class antibodies to two methylated peptides. No antibodies were found to the other tested peptides. The result may point to a non-specific recognition by heterophilic IgM-class antibodies.

## 4.2. Lipoarabinomannan detection for the diagnostics of active TB (study II)

### 4.2.1. Comparison of the modified and the original method to detect uLAM

The effect of the 100-fold concentration of urine for the detection of active TB was investigated by the Clearview® TB ELISA (Alere Inc.) assay. All samples were analyzed non-concentrated and 100-fold concentrated. A calibration curve from 20 ng/ml to 0.5 ng/ml was run with each test. A statistically significant ($p < 0.001$) difference between the healthy and the PTB groups was detected with both methods. However, a wider dynamic range and improved discriminatory power were achieved with the modified method, where the urine was 100-fold concentrated (Figure 5A). The cut-off of the modified method was assessed to a level of 1.1 ng/ml by the ROC curve analysis. For the unconcentrated sample, this is approximately equivalent to 11 pg/ml, if all uLAM is recovered from the processed sample. The AUC was calculated and was found to be 0.767, which reveals a moderate discrimination between active TB and healthy subjects.

Figure 5B presents the range of uLAM concentrations in the concentrated samples. In the PTB group, a concentration of uLAM up to 17 ng/ml was detected. In intact urine, this concentration equals to 170 pg/ml. When the assay was performed as instructed by the manufacturer, 2/28 samples from PTB patients gave a positive result, and with the modified method the respective figure was 16/28 samples. In the healthy group, 3/101 and 13/101 false positives were observed in the non-concentrated and modified forms of the test, respectively.

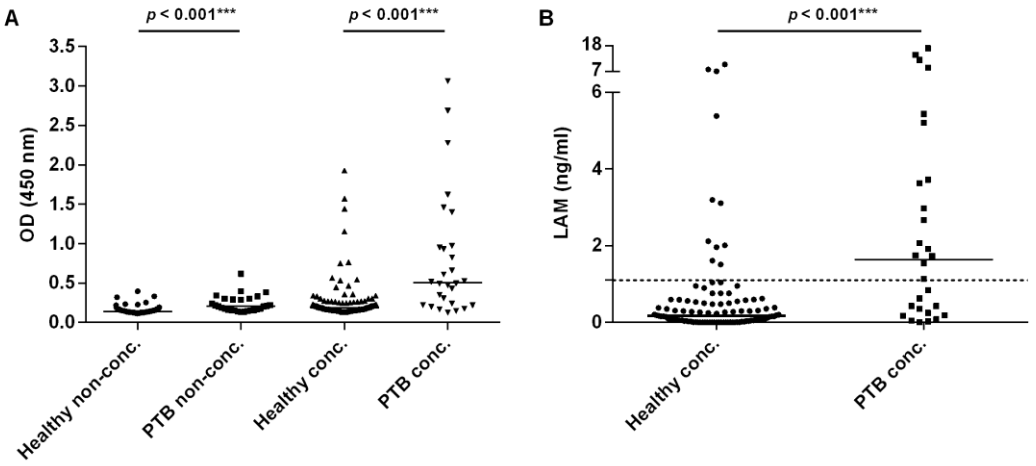


**Figure 5. Detection of uLAM from PTB patients (n = 28) and healthy subjects (n = 101).** (A) ODs of non-concentrated and concentrated samples. (B) Concentration of uLAM in the concentrated samples with the calculated cut-off (1.1 ng/ml) presented with a dashed line. Horizontal bars represent the medians of the groups.

As shown in Table 5, sample concentration improved the assay sensitivity from 7% to 57% ($p < 0.0001$), but decreased the specificity from 97% to 89% ($p < 0.05$). When the results were compared to sputum staining, some (16/24) but not all (12/24) patients were identified by uLAM detection.

**Table 5. Comparison of LAM detection with sputum staining.**

| Method | Positive (n) | Negative (n) | Sensitivity % | Specificity % |
|---|---|---|---|---|
| uLAM (as instructed) | 2 | 26 | 7 | 97 |
| uLAM (concentrated urine) | 16 | 12 | 57 | 89 |
| Sputum AFB | 24 | 4 | 86 | n/a |
| Sputum AFB + uLAM (concentrated urine) | 25 | 3 | 90 | n/a |

n/a = not available

When the modified method was used in the EPTB group, uLAM concentrations in 3/7 samples reached the cut-off. However, the highest concentration was only 1.4 ng/ml, while in the sample of a patient with miliary TB, 16.6 ng/ml of uLAM was found. This sample also gave a positive result when analyzed intact. Only one LTBI patient out of 15, and none of the 4 treated patients were positive after sample concentration. Samples from PTB (n = 17) and EPTB (n = 3) patients were also collected in Taiwan, and interestingly, only 2 samples in the PTB and none in the EPTB group gave a positive result. The difference was significant (PTB, $p < 0.05$; PTB+EPTB, $p < 0.01$) when compared to the samples collected in Finland (Savolainen et al. unpublished results). The numbers of positive samples when the original and modified methods were used are presented in Table 6.

**Table 6. The number of positive samples using the original and modified methods.**

| Group | n | Non-conc. n (%) | 100-fold n (%) |
|---|---|---|---|
| TB pulmonary (PTB) | 28 | 2 (7) | 16 (57) |
| TB of other organs (EPTB) | 7 | 0 (0) | 3 (43) |
| TB, miliary | 2 | 1 (50) | 2 (100) |
| TB pulmonary, Taiwan | 17 | 0 (0) | 2 (12) |
| TB of other organs, Taiwan | 3 | 0 (0) | 0 (0) |
| LTBI | 15 | 1 (7) | 1 (7) |
| Treated | 4 | 0 (0) | 0 (0) |
| Healthy | 101 | 3 (3) | 13 (13) |

### 4.2.2. Studies on factors interfering with the specificity of the assay

In order to examine the interfering factors, urine samples from patients with other infectious diseases (groups Urinary tract infection, Urinary *S. pneumoniae* Ag positive and Urinary *S. pneumoniae* Ag negative) were studied. Altogether, 12/15 samples in the Urinary tract infection group gave a false-positive result after sample concentration and 1/15 without concentration. However, when the urine from a healthy person was spiked with *E. coli*, *E. faecalis* and *C. albicans* in the range of $10^4$ – $10^6$ CFU/ml, no positive results were obtained. *Corynebacterium sp.* produced false-positive results in a dose-dependent manner when concentrated. Spiking with *M. tuberculosis* produced high ODs at low concentrations (10 CFU/ml). In the Urinary *S. pneumoniae* Ag positive and Urinary *S. pneumoniae* Ag negative groups, 2/7 and 3/7 of samples produced a false positive result when concentrated, and 0/7 and 1/7 without concentration, respectively. In a sample from a patient with a pulmonary *M. avium* infection, the concentration just above the cut-off, 1.2 ng/ml of uLAM, was detected in a concentrated sample. The results are provided in Table 7. In conclusion, urine concentration increased the number of the samples with a false positive result. The increase was most apparent, from 1/15 to 12/15, in the Urinary tract infection group.

**Table 7. The number of positive samples in the disease control groups.**

| Group | n | Non-conc. n (%) | 100-fold n (%) |
|---|---|---|---|
| *M. avium,* pulmonary | 1 | 0 (0) | 1 (100) |
| Urinary tract infection | 15* | 1 (7) | 12 (80) |
| Urinary *S. pneumoniae* Ag pos. | 7 | 0 (0) | 2 (29) |
| Pneumonia, but urinary *S. pneumoniae* Ag neg. | 7 | 1 (14) | 3 (43) |

* 45 Urinary tract infection samples pooled according to bacterial species.

## 4.3. Properties and function of cytotoxic T cells in different study groups (study III)

To assess the power of CTLs in differentiating the TB disease stages and to analyze their functional properties, GrB, Prf, and IFN-γ ELISPOT and CD107a and IFN-γ flow-cytometric analyses were performed for the antigen-stimulated PBMCs in groups TB, LTBI, Surg., Part., Mod and Vaccinated.

### 4.3.1. Production of granzyme B and perforin by antigen-stimulated cytotoxic T cells measured by ELISPOT

Stimulation with PPD, rCFP-10, rESAT-6, and CFP-10 and ESAT-6 peptide pools increased the frequency of effector molecule-producing T cells in all the tested groups (Table 8). In the Vaccinated group, *M. tuberculosis*-specific antigens produced only a minor increase in the frequency of antigen-specific cells compared to PPD, as expected. Table 8 shows the proportions of GrB-, Prf-, and IFN-γ-producing cells in the tested groups when PPD or rCFP-10 was used for stimulation. The proportions of rESAT-6-stimulated cells were quite similar to those stimulated with rCFP-10 (data not shown). The frequency of Prf-producing cells was low compared to GrB- or IFN-γ-producing cells with all tested antigens. In view of the high inter-individual variation in the frequencies of GrB-, and Prf-producing cells, and the absence of significant differences between the TB and LTBI groups, no added value for the IGRA methods in differentiating the TB infection stages was observed. We also found a high spontaneous release of GrB (mean, 56 spots/well) and Prf (mean 24 spots/well), which may complicate the interpretation of results and the diagnostic use of GrB and Prf ELISPOTs.

As shown in Table 8, antigen-specific GrB-, Prf, and IFN-γ-producing cells were also found in all the treated groups, including the subjects treated with modern three-drug therapy (Mod.). Significantly higher frequencies ($p < 0.05$) of GrB- and IFN-γ-producing cells were found in the Mod. group than in the Surg. group. However, the mean age was lower and the period from the experienced infection was shorter in the Mod. than in the Part. group.

**Table 8. Frequencies of GrB-, Prf-, and IFN-γ producing cells/$10^6$ PBMCs in the study groups.**

| | | PPD | PPD | PPD |
|---|---|---|---|---|
| **Group** | **n** | **Median GrB (25th - 75th perc.)** | **Median Prf (25th - 75th perc.)** | **Median IFN-γ (25th - 75th perc.)** |
| TB | 17 | 115 (59 - 359) | 23 (0 - 58) | 250 (152 - 550) |
| LTBI | 9 | 165 (118 - 439) | 23 (0 - 89) | 183 (68 - 557) |
| Surg. | 6 | 101 (0 - 337) | 16 (5 - 30) | 59 (14 - 121)* |
| Part. | 23 | 170 (68 - 261) | 26 (0 - 73) | 129 (59 - 328) |
| Mod. | 7 | 385 (107 - 457) | 15 (6 - 64)[a] | 410 (89 - 719)*[a] |
| Vaccinated | 6 | 248 (239 - 341)[b] | 13 (4 - 46)[b] | 220 (22 - 453) |
| | | **rCFP-10** | **rCFP-10** | **CFP-10 peptides** |
| **Group** | **n** | **Median GrB (25th - 75th perc.)** | **Median Prf (25th - 75th perc.)** | **Median IFN-γ (25th - 75th perc.)** |
| TB | 17 | 58 (0 - 395) | 31 (11 -53)[c] | 142 (23 - 267) |
| LTBI | 9 | 210 (144 - 434) | 15 (0 - 88)[d] | 192 (85 - 397) |
| Surg. | 6 | 0 (0 - 320)* | 25 (6 - 98)[b] | 24 (0 - 103) |
| Part. | 23 | 55 (5 - 111) | 8 (0 - 45)[e] | 19 (9 - 108) |
| Mod. | 7 | 344 (69 - 648)*[f] | 0 (0 - 0)[g] | 81 (40 - 360)[a] |
| Vaccinated | 6 | 73 (37 - 92)[b] | 0 (0 - 8)[g] | 15 (8 - 41) |

*$p < 0.05$; [a]n = 5, [b]n = 4, [c]n = 13, [d]n = 7, [e]n = 21, [f]n = 6, [g]n = 3

### 4.3.2. Flow-cytometric analysis of CD107a- and IFN-γ-expressing cells

The proportions of PPD-stimulated degranulation marker CD107a- and IFN-γ-positive $CD4^+$ and $CD8^+$ T cell populations were next analyzed in different subject groups. When all tested results (TB n = 13, LTBI n = 5, Surg. n = 5, Part. n = 5, Mod. n = 5 and Vaccinated n = 5) were pooled, significantly higher frequencies of antigen-specific CD107a-expressing cells were observed in the $CD8^+$ T cell subset ($p < 0.01$) compared to the $CD4^+$ T cell subset, whereas IFN-γ was mostly synthesized by $CD4^+$ T cells ($p < 0.0001$) (Figure 6). The frequencies of CD107a and IFN-γ double-positive cells were low in both cell subsets (data not shown).

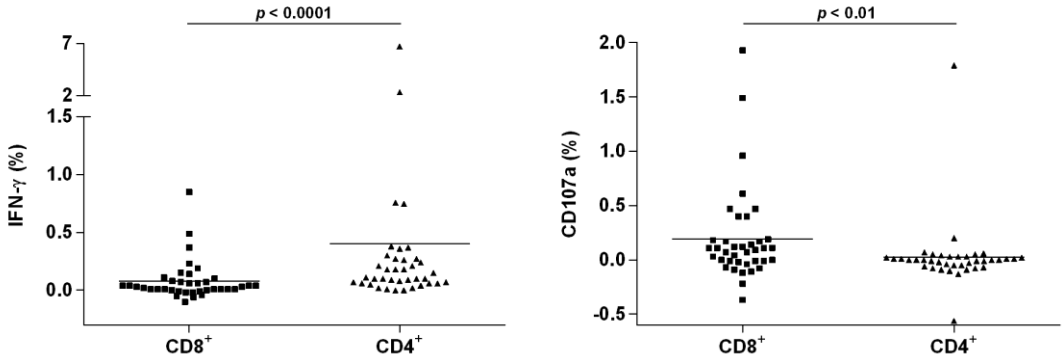


**Figure 6. Analysis of IFN-γ- and CD107a-expressing CD8⁺ and CD4⁺ T cells.** Unstimulated cells were subtracted from PPD-stimulated cells and the frequencies of IFN-γ- and CD107a-positive cells were calculated (n = 38). Medians of the cell frequencies are shown by horizontal bars.

As shown in Table 9, CD107a-expressing CD8⁺ T cells and IFN-γ-synthesizing CD4⁺ T cells were found in all the tested groups. No significant differences between the the groups were observed.

**Table 9. Frequencies of antigen-specific CD8⁺CD107a⁺ and CD4⁺IFN-γ⁺ T cells in the study groups.**

| | | CD8⁺CD107a⁺ | CD4⁺IFN-γ⁺ |
|---|---|---|---|
| Group | n | Median (25th - 75th perc.) | Median (25th - 75th perc.) |
| TB | 13 | 0.12 (0 - 0.54) | 0.18 (0.08 - 0.27) |
| LTBI | 5 | 0.07 (0 - 0.16) | 0.21 (0.05 - 0.56) |
| Surg. | 5 | 0.11 (0 - 0.83) | 0.06 (0.02 - 0.17) |
| Part. | 5 | 0.11 (0 - 0.16) | 0.15 (0.01 - 0.47) |
| Mod. | 5 | 0.07 (0 - 0.29) | 0.09 (0.06 - 3.48) |
| Vaccinated | 5 | 0.03 (0 - 0.25) | 0.11 (0.10 - 1.37) |

### 4.3.3. Correlation between the different markers of cytotoxicity

A significant positive correlation between GrB and Prf production was only obtained for the LTBI group ($r$ = 0.78, $p$ < 0.05). The frequency of CD107a-expressing CD8⁺ T cells correlated positively ($r$ = 0.41, $p$ < 0.05) with the GrB production, but not with the Prf production when all the tested samples from different subject groups were included in the analysis. The poor correlation between the different cytotoxicity markers might suggest that they are regulated independently or are short-lived.

### 4.3.4. Characterization of the surface marker expression of antigen-specific CTLs

The following classification was used to identify the differentiation stages of antigen-specific $CD8^+CD107a^+$ T cells: $CD45RA^+CCR7^+$ naive ($T_{N,}$), $CD45RA^-CCR7^+$ central memory ($T_{CM}$), $CD45RA^-CCR7^-$ effector memory ($T_{EM}$), and $CD45RA^+CCR7^-$ terminally-differentiated effector memory ($T_{EMRA}$) cells [51].

When three samples from each group were analyzed, all cell phenotypes were recorded in all the groups. The $T_{EM}$ phenotype was predominantly found in the $CD8^+$ T cell population in the groups TB, LTBI, Surg. and Part. $T_{EMRA}$ cells were the most frequent in the Mod. group, and $T_N$ cells in the Vaccinated group (Table 10). Interestingly, when the percentages of $CD107a^+$ cells were analyzed within each cell subpopulation, $T_{CM}$ had the highest proportions of antigen-specific cells compared to other cell subsets in all the tested groups (Table 11). The results may reveal that also $T_{CM}$ cells, which represent the minor cell population in periphery, have a role in a rapid recognition of *M. tuberculosis* and immunologic protection.

**Table 10. Cumulative frequencies of each phenotype in the test groups when cell subsets were analyzed within the $CD8^+CD107a^+$ T cell pool.**

| Group | $T_N$ | $T_{CM}$ | $T_{EM}$ | $T_{EMRA}$ |
|---|---|---|---|---|
| TB | 0.11 | 0.01 | **0.58** | 0.15 |
| LTBI | 0.03 | 0.03 | **0.10** | 0.07 |
| Surg. | 0.13 | 0.19 | **0.58** | 0.21 |
| Part. | 0.12 | 0.25 | **0.89** | 0.22 |
| Mod. | 0.08 | 0.05 | 0.16 | **0.24** |
| Vaccinated | **0.14** | 0.04 | 0.06 | 0.05 |

**Table 11. Cumulative frequencies of each phenotype in the test groups when cell subsets were analyzed within each phenotypic subpopulation.**

| Group | $T_N$ | $T_{CM}$ | $T_{EM}$ | $T_{EMRA}$ |
|---|---|---|---|---|
| TB | 0.61 | **16.41** | 6.15 | 0.17 |
| LTBI | 0.71 | **9.32** | 0.66 | 0.11 |
| Surg. | 0.48 | **5.32** | 1.77 | 0.78 |
| Part. | 0.42 | **8.08** | 3.05 | 0.57 |
| Mod. | 0.5 | **3.67** | 0.84 | 0.32 |
| Vaccinated | 1.43 | **5.47** | 0.29 | 0.14 |

## 4.4. Ability of selected immune-related genes to discriminate active TB from LTBI (study IV)

RT-qPCR was employed to analyze the ability of the expression of selected genes, *IFN-γ*, *IL-17*, *IL-4*, *IL-4δ2*, and *FoxP3* to discriminate the infection stages of TB. The fold increase in mRNA expression was analyzed by the comparative CT method ($2^{\Delta\Delta Ct}$), in which expression levels were compared with unstimulated samples and with a housekeeping gene, human ribosomal protein.

### 4.4.1. mRNA expression levels of IFN-γ, IL-17, IL-4, IL-4δ2, and FoxP3 after antigen stimulation

First, the genes were analyzed after PPD stimulation in five samples of the groups TB, LTBI, and Vaccinated. IFN-γ, IL-17, IL-4 and FoxP3 mRNA were expressed in the TB and in the LTBI group (data not shown). The IL-4δ2 mRNA expression levels were low in both groups. Next, *M. tuberculosis*-specific, CFP-10 and ESAT-6 peptide pool-stimulated IFN-γ, IL-17, IL-4 and FoxP3 mRNA expression levels were measured. The peptides stimulated IFN-γ and IL-4, but not IL-17 or FoxP3 mRNA expression (data not shown) in the groups TB (n = 11) and LTBI (n = 6). The respective *p*-values for peptide stimulated IFN-γ and IL-4, to discriminate between active TB and LTBI were 0.983 and 0.199. As expected, the peptide stimulations did not increase the expression of any tested genes in the Vaccinated group (n = 4).

### 4.4.2. IFN-γ, IL-17 and IL-4 mRNA expression profiles for the differentiation of active TB from LTBI

PPD-stimulated IFN-γ, IL-17, and IL-4 mRNA expressions were chosen for evaluation with larger TB (n = 25), LTBI (n = 20), and Vaccinated (n = 8) groups. In this setting, statistically significant discrimination between the active TB and LTBI groups was not achieved either with a single (Table 12) or with a combination of cytokines (data not shown).

**Table 12. The median $2^{\Delta\Delta Ct}$ and the ability of each cytokine to discriminate between active TB and LTBI.**

| | **TB*** **Median $2^{\Delta\Delta Ct}$ (25th - 75th perc.)** | **LTBI**** **Median $2^{\Delta\Delta Ct}$ (25th - 75th perc.)** | **Vaccinated***** **Median $2^{\Delta\Delta Ct}$ (25th - 75th perc.)** | ***p*-values** |
|---|---|---|---|---|
| IFN-γ | 22.6 (7.3 - 59.7) | 12.1 (6.5 - 25.2) | 11.1 (2.2 - 45.8) | 0.263 |
| IL-17 | 10.6 (3.3 - 30.0) | 8.6 (4.6 - 15.7) | 5.4 (1.4 - 13.0) | 0.698 |
| IL-4 | 4.6 (1.5 - 8.3) | 2.1 (0.3 - 4.0) | 1.7 (1.2 - 4.2) | 0.064 |

*n = 25; **n = 20; ***n = 8

# 5. DISCUSSION

A biomarker is an objectively defined indicator that describes the phases of normal or pathogenic processes, or pharmacological responses to a treatment intervention [221]. In the case of tuberculosis infection, this could mean host- or pathogen-specific markers for predicting the reactivation of infection or infection relapse, or to monitor response to treatment or elimination of the microbe from the body. [222] There is a particular need for markers that would enable us to assess, with just one measurement, whether the person in question has active TB, LTBI, or neither. At the moment, the greatest need globally is for rapid and affordable point-of-care tests for diagnosing active TB. One solution for this could be a non-invasive urine antigen test. [222] On the other hand, there is also a need for markers that could be used to distinguish active TB from LTBI in situations where the patient exhibits clinical symptoms compatible with active TB, possible pulmonary lesions, signs of inflammation and a positive IGRA result, but with no microbiological confirmation. Methods suited for predicting reactivation or assessing the need for preventive treatment are also lacking at the moment [67]. In cases such as these, with a low bacterial burden, potential markers could include a single host-specific markers, as well as combinations of several host-specific markers. Markers are also needed for monitoring a response to treatment. [67] In the event of large, rapid changes in bacterial load, such as in the initial stages of treatment, pathogen-specific markers, such as LAM and transrenal DNA, could be appropriate. Computed tomography, magnetic resonance imaging [223] and other imaging modalities are also needed to lend support to the exploitation pathogen- and host-specific markers in diagnosing TB.

Poor characteriztion of the patient groups studied and the likelihood of heterogeneity within groups is a weakness in the literature focusing on the search for new diagnostic biomarkers. As a rule, active TB is diagnosed based on bacteriological findings while LTBI is determined based on TST or IGRA positivity and the known history of TB exposure. It is likely that both groups, active TB and LTBI, comprise many different substages of infection, with differences such as the bacterial load, the number, type and location of granulomas, and the immunological response. In this thesis, the LTBI group comprised of persons who inflicts difficulties in terms of differential diagnosis between active TB and LTBI, i.e., persons who have some underlying disease, such as rheumatoid diseases or other miscellaneous inflammatory condition. Another feature that makes the population we have studied here unique is that we enrolled subjects who had been infected decades ago and had most likely not been exposed to tuberculosis since then, due to the low incidence of TB in Finland. The quite small groups of tested patients with heterogeneity in age and disease activity, and the use of the frozen PBMCs were the limitations of this study. The presence of patients with inflammatory diseases undergoing immunosuppressive treatment also hampered the interpretation of the results in the immunological studies.

## 5.1. Diagnostic uses of the HBHA antigen in the BCG-vaccinated population

Heparin-binding hemagglutinin (HBHA), is involved in the adhesion of *M. tuberculosis* bacteria to non-phagocytic cells, such as epithelial cells, thus contributing to the spread of tuberculosis outside the lungs [224]. Individuals with LTBI and those treated for TB have been shown to have a significantly higher HBHA-specific IFN-γ-response compared to patients with active TB [69-71, 160]. The HBHA antigen has therefore been considered as a potential tool for differentiating between active TB and LTBI. Even though statistically significant differences have been found between the groups, some patients with active TB have been shown to have a high IFN-γ-response to nHBHA or rMS-HBHA [71, 160]. In this thesis (study I), in addition to persons who had previously undergone treatment, some of the patients with active TB were also found to have rMS-HBHA-specific IFN-γ-producing cells in their peripheral circulation. It thus seems unlikely that the HBHA-specific IFN-γ production alone could permit sufficient differentiation between the active TB and LTBI for diagnostic purposes.

It has previously been shown that healthy BCG-vaccinated and unvaccinated persons do not exhibit IFN-γ response to nHBHA [69-71]. However, a recent study showed that BCG vaccine elicited an extensive cytokine response, including IFN-γ to nHBHA in PBMCs [225]. The same study also demonstrated that vaccinated adults had an IFN-γ response to nHBHA and that it does not wane over time [225]. In this thesis, rMS-HBHA-specific IFN-γ-producing cells were found in the peripheral circulation of healthy BCG-vaccinated persons as well (study I). In addition, the number of rMS-HBHA-specific cells was found to have a positive correlation ($r$ = 0.469, $p < 0.01$) with the number of PPD-specific cells. It thus appears that, similarly to PPD, the stimulation of PBMCs with rMS-HBHA does not distinguish the response elicited by the vaccine from that prompted by infection. One reason for the differences between the findings here and those reported previously may be that the vaccine strains used differ in their immune properties. The response to the vaccine may also be influenced by differences in vaccination schedules and vaccine manufacturing processeses. [61] The results of this thesis suggest that the possibilities of using HBHA as an antigen for distinguishing between the persons infected with *M. tuberculosis* and the healthy ones with BCG vaccination are limited in countries with a widespread vaccination programme.

There are few studies looking at the possibilities of using HBHA to monitor the TB treatment efficacy or TB reactivation. However, Masungi et al. have shown that the HBHA-specific IFN-γ response is increased significantly in patients with active TB during the first four weeks of treatment [70]. In a recently published study, changes in IFN-γ response to HBHA and ESAT-6 in persons with LTBI were followed up to 10 years [226]. The investigators summarised their findings as follows: a continuous, proportionally higher IFN-γ response to nHBHA, as compared to ESAT-6, might indicate a response that protects against active TB. In addition, a link between a continuously changing HBHA-ESAT-6 ratio, re-exposure to the pathogen, and reactivation of the disease has been described. [226] It is therefore possible that the nHBHA antigen could be utilised in the diagnosis of disease reactivation or monitoring of cure. Further studies on the subject are needed, however.

There are controversies between different studies concerning the disease stage-discriminating ability of HBHA. One reason for this may be the fact that posttranslationally modified, complex C-terminal methylation has been shown to be significant for T cell recognition and the generation of the IFN-γ response [69]. Even though the methylated

structures of HBHA isolated from the *M. bovis* BCG strain and produced in the *M. smegmatis* strain as a recombinant protein have been shown to be nearly identical, it is possible that there are differences in protein folding, and therefore also in immunological recognition. The fact that the IFN-γ response was measured in this study (study I) as frequencies of reactive cells while most results presented in the literature were based on the quantitative measurement of excreted IFN-γ may also partly contribute to the difference.

## 5.2. Lipoarabinomannan as a diagnostic marker for tuberculosis

Properties, such as non-invasiveness, robustness and inexpensiveness of urine antigen detection, and the moderate sensitivity and specificity of some tests currently in use, such as BinaxNOW® *Legionella pneumoniae* (Alere inc.) and BinaxNOW® *Streptococcus pneumoniae* (Alere inc.), are the basis for considering the use of antigen tests in the TB diagnostics as well. LAM, a heat-stabile glycolipid, forming part of the cell wall of *M. tuberculosis*, is excreted into urine and considered a potential candidate for the development of an antigen test. [227]

The sensitivity of the existing urine LAM tests, Clearview® TB ELISA (Alere inc.) and Determine TB (Alere inc.), has been reported to vary between 13% and 93% and the specificity between 87% and 99% [90, 94, 105]. In addition, higher clinical sensitivities reported are almost without an exception linked to the co-infection of HIV and TB and to a decreased frequency of $CD4^+$ T cells caused by HIV and the expected higher bacteria load in the body [93, 96-100]. In the second study of this thesis, the presence of LAM was demonstrated with the Clearview® TB ELISA in the 100-fold concentrated and unconcentrated urine samples from $TB^+HIV^-$ patients and healthy controls. Concentrating the urine increased the sensitivity of the method from 7% to 57% ($p < 0.0001$) while the specificity decreased from 97% to 89% ($p < 0.05$). Based on the results, it can be noted that concentrating the urine is necessary to obtain even moderate sensitivity in $TB^+HIV^-$ patients.

Besides the patients with HIV infection, higher sensitivity values with uLAM assays have also been linked to TB patients with a more severe disease [102]. Furthermore, uLAM tests showed higher sensitivity in PTB patients with a positive sputum smear compared to PTB patients with a negative smear [92, 95-97, 99, 102]. High uLAM levels were found in a sample of a patient with miliary TB in the present study as well (study II). The concentrated urine sample of one out of the four PTB patients with a negative sputum smear was positive for uLAM (Table 5). Therefore, the uLAM test and sputum smear could be used as complementary methods. In line with earlier findings [99], lower uLAM levels were found in patients with EPTB compared to those with PTB (Table 6). Furthermore, in LTBI group, uLAM level above the limit of detection were found only in one sample (7%) (Table 6). Based on the earlier and our findings, it seems that when the bacterial load in the body increases, the amount of uLAM increases as well. However, the previously demonstrated correlation between uLAM and *M. tuberculosis* DNA determined with the Xpert MTB/RIF method [99] raises a question whether the positive uLAM is a result of genitourinary TB infection or uLAM excreted via kidneys.

The results of this study show that in $TB^+HIV^-$ patients uLAM is excreted at pg/ml levels. Previous studies, most of which have included $HIV^+$ patients, have reported varying levels,

even up to hundreds of nanograms per millilitre [92, 95, 103, 104]. It is likely that the generally observed poor sensitivity of LAM tests is at least partly associated with the poor test performance. However, other factors, such as those related to immunological mechanisms cannot be excluded. For example, it has been speculated that if LAM is bound to the anti-LAM antibodies, the high molecular weight of the formed immune complexes cannot pass through the glomerular membrane and therefore LAM is not excreted into the urine. [99]. The speculation was supported by the results indicating that the sera of TB patients contain anti-LAM antibodies [228]. In addition, increased levels of immune complexes consisting of *M. tuberculosis* glycolipid antigens and antibodies have been detected in the circulation of TB patients [229].

In this study, the number of false positives was shown to be high in patients with urinary tract infection. In accordance with earlier studies [93, 95], common bacteria that cause urinary tract infections, such as *E. coli*, were not observed to react in the test when using a spiked sample. This allows speculation as to whether the lack of specificity might be associated with the inflammatory process itself. This hypothesis requires further research, however. The polyclonal antibodies used in the method were shown to recognise the bacteria in the family of *Corynebacterium,* which may be occasionally present as a part of the microbiota on mucous membranes. This may partly explain the false positive findings in healthy subjects. When developing potential new methods in the future, it is likely that the specificity of the method can be improved considerably by using monoclonal antibodies.

## 5.3. T cell memory

Functional CTLs are important for the protection against active TB. For example, higher numbers of CTLs producing GrB and Prf have been detected in persons with LTBI and those vaccinated with BCG compared to patients with active TB [53, 54]. It has been observed that the lytic activity of CTLs is decreased in patients with active TB [55]. On the other hand, it has also been suggested that the decreased frequencies of CTLs in the peripheral circulation is partly due to the cell compartmentalisation to inflamed tissues [56]. The third study in this thesis looked at the possibility of utilising antigen-specific functional GrB-, Prf-, or CD107a-synthesizing CTLs to differentiate between the stages of TB infection. However, we observed that both the patients with active TB and the persons with LTBI have in their peripheral circulation cell types with similar surface marker expression profiles and functional properties. These include the frequencies of the antigen-specific GrB and Prf-producing PBMCs (Table 8) and CD107a-degranulatioin factor-expressing $T_N$, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ cell subsets (Table 10). In addition, in line with previously published studies [181, 230], we observed that the production and release of cytolytic molecules is a dynamic process that is difficult to demonstrate repeatedly. In light of these results, a diagnostic approach based on the enumeration of GrB-, Prf-, or CD107a-producing CTLs does not appear promising.

Our results indicate that persons whose active TB was treated decades ago have functional CTLs in their peripheral blood in frequencies similar to those of subjects with active disease (Table 8). Furthermore, the CTLs of these persons were shown to represent mostly the short-lived effector-memory cells, $T_{EM}$ and $T_{EMRA}$ (Table 10). Using the same clinical material it was previously shown that IFN-γ-producing $CD4^+$ T cells also had the $T_{EM}$ phenotype [231]. What

maintains the short-lived $T_{EM}$ cell population in the periphery of treated patients remains unclear. Therefore, there is a possibility that even the modern anti-tuberculosis treatment does not eradicate the bacteria, which then could lead to the continuous stimulation and proliferation of antigen-specific T cell populations. On the other hand, the possibility of unspecific stimulation caused by other microbes with similar structures to those of *M. tuberculosis* cannot be excluded, either. It does, however, seem clear that once formed, the T cell memory that recognises *M. tuberculosis* antigens is sustained throughout life, albeit with some variation between individuals. This makes the differential T cell-based immunological diagnosis of stages of the TB infection difficult. In light of these findings, the suggestion put forth in the literature, to exploit the attenuation of IFN-γ response for the detection of treatment efficiency, is questionable. Even if the reversion of the IGRA result from positive to negative could be observed, the correlation with a stage of the disease is at present very unclear [152].

One third of the global population is infected with *M. tuberculosis* when the positive tuberculin skin test result is used as a criterion. However, the majority of the infected do not develop active TB during their lifetime. Nevertheless, the risk of reactivation is increased by a compromised immune defence, for example, when TNF-α blockers are used to treat various inflammatory disorders, or when $CD4^+$ T cell levels are reduced, e.g. due to the HIV infection. It was recently shown, in a review comprising 18 prospective cohort studies with 19,886 subjects, that the persons with LTBI diagnosed by TST, have a 79-percent lower risk of developing active tuberculosis as a result of being re-exposed to *M. tuberculosis*, as compared to healthy TST-negative persons [232]. This finding has caused speculation as to whether having LTBI or post-treatment “immunological scar” might have a protective effect against clinical disease.

The so-called “hygiene hypothesis" has been put forward to explain the growing rate of allergic conditions in the Western populations. This theory explains why the immune system needs to be primed in infants to shift their immune response from the predominance of the Th2 to the Th1 arm, which is required e.g. for the differentiation of Tregs. The factors responsible for this priming are not yet fully understood. However, the contact with bacteria belonging to the *Enterobacteriacae* family seems important. [233, 234] Early contact with mycobacterial antigens, even as LTBI or after vaccination, may be beneficial too. Thus, for example, a significant decrease in the prevalence of recent allergic rhinitis in the TST-positive children compared to the TST-negative ones was reported [235]. On the other hand, the systematic review of the literature showed a high variation in the results between studies, in which the correlation between *M. tuberculosis* infection or BCG vaccination and atopic diseases in children have been studied [236]. That is explained, e.g., by the variation in the BCG vaccination schemes, vaccine strain differences, differences in exposure to environmental mycobacteria and the lack of uniformity in the definition of atopy [236]. The benefits of latent infections to confer protection against more detrimental infections were also described in animal models. It has been recently shown in mice that the IFN-γ-mediated macrophage activation maintained by latent Epstein-Barr virus or cytomegalovirus infections has a protective role against some bacterial infections [237].

## 5.4. IFN-γ, IL-17 and IL-4 mRNA expression in the differential diagnosis of M. tuberculosis infection stages

It is possible that high-throughput array techniques are needed in order to find markers that distinguish the stages of TB infection [190]. Therefore, the development of robust diagnostic methods for the diagnostics of active TB calls for multiplex method formats, in which multiple markers can be detected cost-effectively in a single reaction [186].

The calculation of the combined expression, of IFN-γ, IL-17 and IL-4 mRNAs is justified on the basis of the following: *a)* when the immune response is evolved after the contact with the pathogen, $CD4^+$ T lymphocytes differentiate into distinct major lineages, Th1, Th17 or Th2, each expressing IFN-γ, IL-17 or IL-4, respectively; *b)* because the different cell lineages, Th1, Th17 and Th2, co-operate or interfere with each other during the inflammatory process, the dominant subset may differ. [38] Therefore, the combined analysis of cytokines produced by these cell subsets takes into account any shifts that might occur.

In this thesis, no statistically significant differences in IFN-γ, IL-17 and IL-4 mRNA expressions were found in patients with active TB and LTBI. However, a trend towards discrimination was shown. The method may prove useful if repeated on a larger sample size when more meticulously selected LTBI patients with a homogenous clinical background are enrolled

We observed that, compared to CFP-10 and ESAT-6 peptides ($p$ = 0.983), PPD ($p$ = 0.263) stimulation had a higher capacity to discriminate the active TB group from the LTBI group when IFN-γ was measured. As reported, PPD stimulation has resulted in the higher IFN-γ and IL-17 cytokine production compared to the stimulation with CFP-10 and ESAT-6 peptides [185, 238]. By definition, patients with active TB have a higher bacterial load, stronger inflammatory reaction and quantitative and qualitative differences in reactive T-cell pools compared to LTBI. Therefore, recognition by the $CD4^+$ T cells of TB patients of a higher variety of protein components of the PPD rather than CFP-10 and ESAT-6 peptides alone offers a distinct diagnostic advantage.

Earlier, significantly higher FoxP3 mRNA levels have been demonstrated in antigen-stimulated PBMCs of patients with active TB than in subjects with LTBI [184]. In this thesis study, no difference in FoxP3 mRNA levels was observed between these groups. FoxP3 mRNA is expressed low levels [184], and its expression might peak earlier than at 22 h, the time point used in this study. Consistently with earlier findings [89], antigen stimulation did not induce the expression of IL-4δ2 mRNA in this study.

Importantly, in other studies reporting the high discriminative ability of a variety of gene expression profiles, the LTBI group has consisted of healthy subjects, mainly household contacts [184, 189]. By contrast, our LTBI cohort consisted of patients with underlying diseases, such as rheumatic diseases or other inflammatory disorders. Similarly to active TB, these patients have disease manifestations compatible with active TB. Our LTBI patient group had dysbalanced cytokine production, and in the real clinical situation this LTBI cohort is therefore a challenge. To our knowledge, such an approach of enrolling an LTBI group with clear disease manifestations has not previously been attempted.

## 6. CONCLUSIONS AND FUTURE PROSPECTS

The urine LAM tests that are currently in use are not suitable as point-of-care test for the diagnostics of active TB in $TB^+HIV^+$ or $TB^+HIV^-$ patients. In this study, the sensitivity of the Clearview® TB ELISA method could be significantly improved by concentrating urine 100-fold. However, because only moderate sensitivity (57%) and specificity (89%) was achieved, the method is still inadequate for the diagnostic use. Compared to protein antigens, the glycolipid could be a preferred antigen for TB diagnostics, for example, due to its heat stability. This is an important aspect, because the infectivity of a urine can be reduced by heating. Furthermore, a urine sample of patients without genitourinary TB infection is presumably much less infectious than a sputum sample. It is obvious that many issues, such as those related to kinetics of the antigen excretion in active TB, call for further studies. In addition, in order to improve the specificity of the method, it is very likely that, in the future, the use of monoclonal antibodies and careful analysis of preanalytic factors will be necessary.

The most fascinating results of the current thesis include the finding that the memory of CTLs, generated by a previously treated active TB, is sustained for several decades, despite adequate treatment with bactericidal rifampicin. It is possible that long-lived T-cell memory may provide some protection against re-infection with mycobacteria, or even against other detrimental infections. As shown in this thesis, because of T-cell memory, assays based on host-related biomarkers are less useful for measuring treatment efficiency.

Based on the results, the ability of PPD-stimulated IFN-γ, IL-17 and IL-4 mRNA expression in PBMCs to discriminate the disease stage was not sufficient for diagnostic use. However, larger sample cohorts should be tested. Furthermore, the LTBI group should be more homogeneous in terms of clinical manifestations, the duration of the clinical course, and the duration and assortment of anti-rheumatic drugs, because each of these factors affects the basal level of cytokine production. It could be possible that by using a combination of several biomarkers, active TB could be distinguished from LTBI immunologically. New immunodiagnostic tests could provide a possibility for detecting the reactivation of TB infection, thus leading to earlier preventive treatment than by using microbiological or molecular methods. The search for new immunological markers should therefore continue. In our opinion, however, the search for biomarkers of tuberculosis infection should be more focused on pathogen-related than on host-related markers, because these offer a higher degree of specificity.

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