Cellular Immune responses during latent tuberculosis : immunodiagnosis and correlates of protection

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Chapter 1

Introduction &
Outline of the thesis

INTRODUCTION

TUBERCULOSIS

Tuberculosis (TB) is a major threat to global health, with a conservative estimate of 3 persons dying of TB every minute (1). TB is a communicable disease caused by an acid-fast, rod-shaped bacillus called *Mycobacterium tuberculosis.* Most individuals who become infected with *M. tuberculosis* contain the primary infection and do not develop active disease. Pulmonary disease is the most common clinical manifestation of active TB. Frequent symptoms are persistent cough, fever, night sweats, weight loss and haemoptysis. Pulmonary infiltrates, sometimes with typical cavitations, are seen on a chest x-ray. However, TB can affect practically every organ, e.g. lymph nodes, spine, central nervous system, abdomen and kidneys. Extrapulmonary TB is found in approximately 20% of the cases. Untreated active TB has a high mortality; a large study in 1923 indicated that the mortality of untreated sputum-positive patients was 61% for those "treated" in a sanatorium and 81% for those who stayed at home (2).

IMMUNITY TO *M. TUBERCULOSIS* AND LATENT TUBERCULOSIS INFECTION

The infection is acquired through inhalation of small droplets, containing *M. tuberculosis.* In the lung *M. tuberculosis* is phagocytosed by alveolar macrophages. *M. tuberculosis* has the capacity to survive and even replicate in the phagosome of resting macrophages, by preventing phagosome maturation and fusion with the lysosome (3). Upon entry of *M. tuberculosis* the macrophage starts to produce cytokines, including interleukine-12 (IL-12) and IL-18, IL23, and mycobacterial antigen will be presented on the cell-surface by human leukocyte antigen (HLA)-class II and also by HLA-class I and CD1 molecules. Recognition of antigens by CD4 T cells in the presence of IL12 will drive development of a T-helper 1 (Th1) response with production of interferon-gamma (IFN-γ). IFN-γ will activate macrophages, and, together with tumor necrosis factor alpha (TNF-α), mainly produced by IFN-γ activated macrophages, will mediate microbacterial killing. This IL-12-/IFN-γ cascade is crucial in the protection against mycobacteria, as has been clearly demonstrated by studies in gene-knockout mice and by the increased susceptibility to (non) tuberculous mycobacteria in individuals with genetic defects in the IFN-γ/IL-12/IL-23 pathway (4;5). Activated T-cells and macrophages form granulomas in which *M. tuberculosis* is contained. Some infected macrophages migrate to the lymph nodes and from there can spread to other parts of the body. In the lymph nodes the antigen specific host immune response is initiated.

After establishment of the primary infection, only an estimated 10% of the infected individuals develop active TB disease during their lifetime. The risk of development of active TB varies according to the time since infection, age at time of infection and host immunity. From large 20-year follow-up studies it is known that roughly half of the cases of active TB develop during the first 2 years after infection and 68% within 5 years (6;7). The availability of DNA fingerprinting recently provided evidence of reactivation of *M. tuberculosis* as long as 7 to 53 years after the primary infection (8;9).

The term latent tuberculosis was first introduced in 1909 by Von Pirquet, the 'godfather' of the tuberculin skin test (TST), when he observed tuberculin skin reactions of ≥ 5mm in children who did not manifest tuberculosis (10). Thus a latent tuberculosis infection indicates the presence of *M. tuberculosis* without signs and symptoms of disease. The table lists hallmark discoveries in the battle against tuberculosis (Table 1).

EPIDEMIOLOGY AND CONTROL OF TUBERCULOSIS

Epidemiology

Tuberculosis is still the world's second leading cause of death due to infectious agents, after HIV/AIDS, despite the availability of effective drugs since half a century now. In 2004 the estimated number of new TB cases yearly was 9 million. More than 80% of all TB patients live in sub-Saharan Africa and Asia. Each year approximately 2 million people die from TB. Current mortality rates strongly differ by geographic region with estimates by the WHO ranging from 7% in industrialized countries to as high as 35% in sub-Saharan Africa. These difference dependent on the percentages of cases treated and prevalence of underlying diseases such as HIV. For example, in Africa 33% of the new TB cases is HIV infected, with a markedly higher mortality rate of 24% compared to 2.7 % of non-HIV infected individuals with active TB (1).

In the Netherlands, the incidence of TB has remained quite stable over the past years with about 1200 new cases per year. In 2005 the total incidence was 7.1 per 100.000; varying from 2.4 among native Dutch to 47.6 among first generation immigrants (11). In the Netherlands every year 7% of the registered TB patients in the Netherlands dies, of which 2% directly due to TB. Nearly half of those who died of TB were not diagnosed with TB and therefore not treated. The mortality rate is highest among older and immunocompromised patients.

The exact prevalence of latent TB is not known, but surveys with the TST suggest that one-third of the world's population is latently infected with *M. tuberculosis*, corresponding to 2 billion persons worldwide (12;13).

Table 1. DISCOVERIES IN THE FIELD OF TUBERCULOSIS

When	*Who*	*What*
1865	Villemin	**Infectious nature of tuberculosis.** Inoculation of a rabbit with purulent liquid from a tuberculous cavity resulted in extensive TB.
1882	Koch	Discovery of **tubercle bacillus** (Koch-Henle postulates)
1883	Ziehl & Neelsen	Development of Ziehl-Neelsen (ZN) stain based on the acid-fastness of the tubercle bacilli
1890	Koch	Developed **tuberculin,** a substance from tubercle bacilli, to cure TB. Proved not to be successful as a cure; "Koch reaction"
1907	Von Pirquet	Developed **tuberculin skin test (TST);** intracutaneous injection of tuberculin for diagnosis of TB
1908	Mantoux	Cannulated needle and syringe to inject tuberculin intracutaneously
	Seibert	Developed purified protein derivative (**PPD**), used for the TST
1909	Von Pirquet	Introduced the term **latent tuberculosis**; positive tuberculin reactions in children who did not manifest tuberculosis.
1921	Calmette & Guérin	Developed a **vaccine Bacille Calmette-Guérin (BCG)** through attenuating *M. bovis*
1943	Lehmann	First **therapeutic agent:** para-amino salicylic acid (**PAS**)
1943	Waksman & Schatz	First **mycobactericidal** agent: **streptomycin**
1952		First **oral mycobactericidal** drug: **isoniazid (INH)**
1957		**Rifampycins**
1998	Cole et al.[1]	Deciphering of the **Genome sequence of *M. tuberculosis***
2000	Andersen et al. [2]	Development of *in vitro* ***M. tuberculosis*-specific immuno-diagnostic assays**
2007		Several **new TB vaccines candidates** in phase one and two clinical trials.

1. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998;393(6685):537-44.
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Control of tuberculosis

Over a century ago Robert Koch already declaimed: "This is the time to control TB" (14). However, after a strong decline in the incidence of tuberculosis in the developed world, the incidence decreased only little in the past decades mostly due to reactivation of latent TB in immigrant from TB endemic regions. In the developing world the incidence had only slightly decreased, when the emerging HIV pandemic resulted in a significant increase in incidence again. Thus, 100 years later, it became clear that control and eradication of TB were not imminent. In 1993, TB was declared "a global emergency"

by the World Health Organisation (WHO) and "control of TB" became part of the United Nations Millennium Development Goals.

International TB control programs focused almost entirely on early detection of active tuberculosis and effective treatment. Direct observed therapy (DOTS) is being used to improve compliance to therapy and cure rate. There are two important factors that preclude the success of this TB control strategy (15). First, in many areas of the world where TB is highly endemic, there is also a high prevalence of HIV-infection. In HIV and *M. tuberculosis* co-infected individuals, the risk of progression to active TB is significantly higher, up to 8-10% per year, which greatly increases the number of active TB cases (16;17). Secondly, effective TB treatment is complicated by the increasing presence of multidrug-resistant TB and the emerging of extensively drug-resistant TB. Thus, in order to be able to effectively control or even eradicate TB in the future, other strategies, focussing on latent *M. tuberculosis* infection are needed.

Control of latent tuberculosis infection

New cases of active TB are in part attributed to ongoing community transmission, including reinfections, but a substantial number of new cases will arise from the enormous reservoir of individuals with a latent *M. tuberculosis* infection, which is estimated to consist of 2 billion persons. Therefore, efforts to control and finally eradicate TB should also be directed towards the prevention of progression from latent infection to active disease. This could be achieved through two different strategies: 1. adequate/accurate diagnosis and treatment of individuals with latent infection and 2. an immunotherapeutic/post-exposure vaccine which prevents reactivation from latent infection. With regard to the first, the currently available methods to detect persons with a latent *M. tuberculosis* infection and select those with an increased risk to develop TB disease are insufficient. Contact tracing and treatment of latent infection is only achievable in a setting where most persons are tuberculin skin test negative, this being only the case in industrialized countries (without standard BCG vaccination) where TB incidence is already low. Even in this setting, the effectiveness of the currently available regimens used for the treatment of latent *M. tuberculosis* infection is limited, due to problems with low compliance to treatment, drug toxicity mainly in the elderly, and prevalence of antibiotic resistant strains (9,4% in the Netherlands in 2005; multi-drug resistant strains: 0,8%). Further, *in vitro* studies demonstrated that dormant *M. tuberculosis* are moderately to highly resistant to commonly used drugs such as rifampin and isoniazid (INH) that are bactericidal to replicating bacilli (18;19).

With regard to vaccination, the only currently available vaccine against TB is *M. bovis* bacillus Calmette-Guérin (BCG) that affords limited and highly variable protection against pulmonary TB in adults, which is mostly due to reactivation of latent tuberculosis infection (20;21).

DIAGNOSIS OF LATENT TUBERCULOSIS INFECTION

Identification of individuals who are latently infected with *M. tuberculosis* is complicated by the fact that there is no gold standard for the diagnosis of latent tuberculosis. Since it was discovered, the TST has thus far remained the only available method to determine whether an individual has become infected with *M. tuberculosis* after exposure to a patient with contagious tuberculosis. The closest proxy to proof of infection consists of an observed conversion of the TST from negative to positive.

Tuberculin Skin Test

The TST measures the size of induration of the skin after intradermal injection of purified protein derivative (PPD). PPD consist of precipitated filtrate of heat treated *M. tuberculosis*. It contains primarily small (≤ 10kDa) denatured proteins. The TST is based on a delay-type hypersensitive (type IV) response to the *M. tuberculosis* antigens present in PPD. When a person has been infected with *M. tuberculosis*, *M. tuberculosis*-specific T cells will be present. These antigen-sensitized T cells will release lymphokines following contact with PPD. These lymphokines activate and attract macrophages and finally induce an inflammatory reaction which leads to induration of skin. The Mantoux test is the most used skin-testing method. It is performed by injecting 0.1 ml PPD tuberculin (1 or 2 TU) intradermally on the volar side of the forearm, with a specific gauge needle, creating a pale bleb in the superficial layer of the skin. After 48-72 hours the size of the induration is measured by palpation and is expressed in millimetres. Cut-off values for a positive test result depend on the population tested. Commonly used cut-off values are ≥ 15 mm in BCG vaccinated persons, ≥ 5mm in children and immunocompromised persons and in others ≥ 10mm is regarded as positive.

Advantages and limitations of TST

Since the TST has been the only available diagnostic tool for detection of latent *M. tuberculosis* infection for the past century, there is an extensive experience with this assay worldwide. The predictive value of a positive TST for the chance of developing active TB has been well established in the past (22). Moreover, in those studies that demonstrated the beneficial effect of INH prophylaxis for the reduction of the number of secondary TB cases, the TST was used to select exposed individuals for INH treatment (23-25). Further, the TST is cheap and does not require access to laboratory facilities except a refrigerator.

However, the TST also has several important limitations. The main limitation is its impaired specificity in BCG-vaccinated individuals. As BCG shares many antigens with *M. tuberculosis* (and thus with PPD), cross-reactive immune responses to BCG can cause false positive TST results. The WHO reported a global BCG coverage at birth of 80-85% for the past 15 years, indicating that the overall worldwide coverage of BCG vaccination is

very high. The specificity of TST can also be impaired due to cross-reactivity to environmental mycobacteria or when repeated skin testing is done (26-28). Another limitation of the TST is that the sensitivity can reduced in various circumstances, e.g. in immunocompromised individuals, in the elderly and in patients with extensive, active TB. In addition, there are several practical limitations regarding the performance of the TST. The TST needs to be done by well-trained personnel and inter-individual variation in administration and reading can cause variability in the test result. Moreover, two visits are needed and compliance to return for TST reading has been estimated at 50%.

Another characteristic of the TST is that repeated testing can result in an increase in tuberculin reaction which is believed to result from boosting or immunologic recall of pre-existing delayed type hypersensitivity to mycobacterial antigens (28). This can be of use when it is relevant to detect remotely acquired *M. tuberculosis* infections in elderly persons. However repeated TST testing can also give rise to false a positive TST results due to boosting of cross-reactive immune responses to BCG.

Development of *M. tuberculosis*-specific immunodiagnostic assays

Recently, a novel method of more specific and therefore potentially more reliable detection of infection with *M. tuberculosis* has come in scope. These new assays have been designed to overcome the most important drawback of the TST; its poor specificity. This poor specificity results from the fact that PPD contains many protein components that are shared between mycobacterial species. Therefore, research has focussed on the identification of antigens that are unique to *M. tuberculosis.* This lead to the identification of an early secreted antigenic target of 6 kDa (ESAT-6); an antigen from the culture filtrate which was found because of its strong recognition in *M. tuberculosis* infected mice (29-31). The gene of ESAT-6 was shown to be absent in BCG and in most environmental mycobacteria (31). Further, molecular analysis revealed that stretches of the genome of *M. bovis* were deleted in BCG (32). Since the deciphering of the complete genome of *M. tuberculosis* in 1998 (33) and of BCG in 1999, more genomic regions of difference could be identified that are present in *M. tuberculosis* but absent from BCG and most environmental mycobacterial species (34). The gene of ESAT-6 resides in one of these genomic RDs, which was defined as RD1. RD1 also encodes for another potent T-cell antigen: culture filtrate protein-10 (CFP-10 encoded by Rv3874). These two *M. tuberculosis*-specific antigens were evaluated using *in vitro* immunodiagnostic assays that measure responses of antigen-specific T-cells in peripheral blood to these antigens. Such assays are based on culture of peripheral blood mononuclear cells (PBMC) and measure the release of IFN-γ in response to specific antigens. These assays are often referred to as "interferon-gamma release assays" (IGRA). The diagnostic potential of ESAT-6 was first shown in cattle (35). Around 2000 the potential value of ESAT-6 and CFP-10 for specific diagnosis of tuberculosis was demonstrated in human (36-39).

Subsequently, several different formats of IGRA, mostly based on ESAT-6 and/or CFP-10, have been developed (40). These IGRA differ with respect to the type of cells cultured (whole blood or PBMC), *in vitro* incubation period and the method to detect the IFN-γ response either by ELISA or enzyme-linked immunospot assay (ELISPOT).

M. tuberculosis-specific IGRA were first evaluated in individuals with active TB and healthy controls from regions where TB is low endemic. In this setting IGRA results could be compared to the gold standard for the diagnosis of TB, namely culture of *M. tuberculosis*. These studies showed that a 6-day lymphocyte stimulation assay based on ESAT-6 and CFP-10, *M. tuberculosis*-specific secreted antigens, were found to be sensitive as well as specific for the detection of active TB (41;42). Also when tested in a mainly BCG-vaccinated population with a low prevalence of TB infection, the specificity of these assays was very high, up to 98% (43). This is in contrast to the TST which is known to frequently cause false positive test results in healthy BCG-vaccinated individuals.

ESAT-6 and CFP-10 were not only found to be recognized by TB patients but also by healthy, *M. tuberculosis* exposed, TST positive individuals, indicating that IGRA based these antigens could be used for diagnosis of latent tuberculosis infection(38;39;44;45). However, evaluation of the new *M. tuberculosis*-specific IGRA for the detection of latent TB is more complicated due to the lack of a gold standard for the diagnosis of latent TB. Until now, what has been done is to compare the level of agreement between the novel IGRA's and the TST as the only other available assay for the diagnosis of latent TB. However, due to its limited specificity, a positive TST is not suitable to be used as surrogate marker for latent TB while there is no alternative diagnostic test. Therefore, the calculation of the sensitivity and specificity of IGRA for the diagnosis of latent TB is formally not possible.

CELLULAR IMMUNITY DURING LATENCY AND CORRELATES OF PROTECTION

BCG

The attenuated strain of *M. bovis,* bacillus Calmette-Guérin (BCG), is currently the only available vaccine against *M. tuberculosis.* The vaccine confers significant protection against severe childhood tuberculosis, which is the main reason why it is part of standard childhood vaccination programmes in many parts of the world. However, the protective effect of BCG against adult/pulmonary TB varies considerably between different clinical trials and geographically distinct populations, which is thought to be related to the level of exposure to environmental mycobacteria (20). In agreement with this line of thought is the observations in mice that prior sensitization with environmental mycobacteria inhibits BCG multiplication and thereby prevents the development of a protective immune

response against TB (46). Further, the relative ineffectiveness of vaccination with BCG, which actually is an infection with a low virulence strain, can partially be explained by the observation that even natural TB disease fails to protect against reinfection disease with another strain at a later point (47). Furthermore, BCG does not effectively prevent the establishment of *M. tuberculosis* infection, nor does it seem to protect against reactivation from latent infection to TB disease. Thus far, only one recent study suggests that BCG does confer a certain level of protection against establishing (a latent) *M. tuberculosis* infection, as indicated by the presence of a *M. tuberculosis*-specific (ESAT-6/CFP-10) immune response, but this study had several important limitations and needs to be confirmed (48).

Besides the limited efficacy of BCG, another disadvantage of the vaccine is that it can cause disseminated BCG disease in immunocompromised patients, including HIV infected infants (49;50). Vaccine safety is of particular importance as in many regions of the world the TB epidemic coincides with the HIV/AIDS epidemic. The above underscores the needs for a more effective as well as safe vaccine.

Vaccine development

Several approaches to develop novel TB vaccines are being followed, e.g. construction of attenuated *M. tuberculosis* or BCG over-expressing *M. tuberculosis* antigens, the use of recombinant antigens based subunit vaccines or DNA vaccines. Recent efforts towards the development of such an improved vaccine have mainly focused on prophylactic vaccines that are intended to be administered before infection with *M. tuberculosis* has occurred, and these have been evaluated in animal models of acute primary infection. These prophylactic vaccine candidates were ineffective or even deleterious when used in a post-exposure setting using animal models of chronic infection or mimicking latent infection (51-53). For example, the *M. leprae* Hsp65-DNA vaccine was reported to induce dramatic reductions in both pulmonary and splenic bacterial loads in mice with established TB infection (54), as well as for prophylactic immunization (55). However, no studies have been able to reproduce the therapeutic efficiency of the Hsp65 DNA vaccine; in contrast it was shown that the vaccine could critically exacerbate the infection (56). Further, vaccination of chronically *M. tuberculosis*-infected mice with CFP-10 subunit vaccine or a DNA vaccine encoding antigen 85B did not modulate the course of infection, while immune activation through vaccination with BCG was even associated with increased pathology without decreased bacterial load (51;52). The latter recent observations are in line with an observation 100 years earlier, when treatment of TB patients with an extract of *M. tuberculosis* resulted in a deteriorated clinical condition in a part of the treated patients, which became known as the "Koch phenomenon" (57). This characteristic, together with the WHO estimate of 2 billion people already harbouring *M. tuberculosis,* underlines the need for a post-exposure vaccine specifically designed

to prevent reactivation of TB and which can be safely administered to already latently infected individuals. The antigens to be included in such a post-exposure vaccine should enhance the immune response against *M. tuberculosis* during latency, without causing hyper-inflammatory immune reactivity.

The secret of latency

M. tuberculosis latency is a well kept secret. Surprisingly little is known about the physiological state of *M. tuberculosis* in latently infected individuals, and the specific host immunity that is responsible for the establishment and maintenance of latency is poorly understood (58-62). The study of latent *M. tuberculosis* infection is hampered by the absence of an accurate animal model in which *M. tuberculosis* can persist without signs and symptoms of disease, similar to the situation during latent infection in humans. Important insight into the immune mechanisms involved in controlling latent infection in humans is derived from a few specific clinical conditions which are associated with an increased risk of reactivation of TB. First, the high risk of developing active TB in HIV infected individuals points towards an important role of CD_4 T-cells for protective immunity. Secondly, it has been shown that individuals with a genetic defect in the IL-12 – IFN-γ pathway are highly susceptible to severe mycobacterial infections (4;5;63). Thirdly, individuals receiving anti-TNF therapy, e.g. as treatment of Crohn's disease or rheumatoid arthritis, have an increased risk of developing TB. Based on the predominant occurrence of TB within months after starting anti-TNF treatment, this suggests reactivation from latent infection (64). In agreement with these observations are results from experimental studies which indicate that CD_4 T-cells and IFN-γ play an essential role in the resistance to *M. tuberculosis*. Several studies indicate that CD8 T-cells are important in maintaining latency as well (65;66).

During latent infection tubercle bacilli are most likely contained within granulomas, mainly consisting of differentiated macrophages with peripheral aggregates of infiltrating lymphocytes (67;68). However, it is not precisely known where *M. tuberculosis* resides during latent infection in humans. During autopsies of latently infected individuals, microscopic examination of pulmonary specimens, such as old granulomatous lesions, rarely revealed acid-fast bacilli and even when microscopically visible they could often not be cultured. Nonetheless, it has been demonstrated that viable *M. tuberculosis* are present, as homogenized pulmonary lesions were capable of causing infection when injected into animals. These observations raise questions such as whether the bacilli replicate and whether *M. tuberculosis* has an altered metabolic state during latency. A recent study indeed showed that tubercle bacilli undergo distinct cell-wall alterations during persistence (69). During active infection in mice or humans, *M. tuberculosis* could be stained using the Ziehl-Neelsen stain (ZN) for acid-fast bacilli.*M. tuberculosis* could not be visualized using ZN staining during chronic/persistent infection, yet a staining

method for *M. tuberculosis* that is independent of the cell-wall composition was still positive (69). Another example which indicates that *M. tuberculosis* adapts its metabolism in response to the host environment is the observation that *M. tuberculosis* within activated macrophages increases expression of isocitrate lyase (ICL), an enzyme essential for the metabolism of fatty acids. An ICL delta mutant was markedly attenuated for survival in activated but not resting macrophages. In IFN-γ knockout mice the ICL delta mutant was not attenuated, indicating a link between the host immune response and the activity of ICL (70). These findings indicate that the metabolic state of *M. tuberculosis* differs during different stages of the infection.

In vitro models of latency

With the aim to evaluate possible metabolic or physiological changes of *M. tuberculosis* during latency and to study the host immune mechanisms responsible for inducing and maintaining latency, much research has focused on the development of animal- and *in vitro*-models that accurately mimic latent infection in humans. Although conditions of persistence are poorly defined, it is generally thought that persisting bacilli will mostly reside in granulomas where they are subjected to nutrient and/or oxygen deprivation, to microbicidal molecules such as reactive oxygen and nitrogen intermediates and to immune-mediated killing (reviewed in (61;62;71)). The conditions that have been used in *in vitro*-models included mainly adaptation to nutrient depletion or hypoxia, of which the latter has been studied most extensively. Wayne et al. established a model of latency by growing *M. tuberculosis* in sealed containers which causes a gradually decreasing oxygen tension and resulted in growth arrest of the bacilli, a state which was named non-replicating persistence (NRP). Reintroduction of oxygen to these bacilli in NRP led to resumption of growth. During NRP, *M. tuberculosis* was found to have an altered metabolic state, which for example was indicated by the observation that during NRP bacilli were sensitive to metronidazol and resistant to ciprofloxacin (a DNA gyrase inhibitor), while during the exponential growth phase the opposite was seen (19;72). Others used constant hypoxic culture conditions to study the metabolic changes of *M. tuberculosis* and later also the changes in gene expression during *in vitro* induced persistence (73-77).

16kDa, α-crystallin protein

Using proteomics it was shown with *in vitro* models of latency inducing NRP that the expression of a 16kDa α-crystallin-like small heat shock protein encoded by Rv2031c and referred to as α-crystallin(*acr)* or heat shock protein X (HspX) was strongly upregulated during hypoxia (73;77) . α-Crystallin was first identified by Lee et al. who isolated the protein from the membrane of the virulent Erdman strain of *M. tuberculosis* and showed the presence of antibodies against this 16kDa major membrane protein in serum of active TB patients (78). α-Crystallin has not been identified outside the *M. tuberculosis*-complex,

which includes BCG, which suggests a unique role in the pathogenesis of TB. Its expression was also induced during *in vitro* infection in macrophages and a 16kDa-α-crystallin-protein knock-out strain of *M. tuberculosis* showed reduced growth in macrophages (79). Other authors recently indicated, however, that the mutant was associated with increased growth *in vivo* and increased pathology (80;81). This α-crystallin protein is known to elicit antibody responses in patients with active TB disease and to induce T-cell proliferation *in vitro* after stimulation of PBMC of TB patients, healthy PPD-positive persons or BCG vaccinated persons (82-86). Others identified CD8-specific epitopes and showed that α-crystallin specific CD8 T-cells had a cytotoxic effect against *M. tuberculosis* infected macrophages (87;88). In conclusion, these observations indicate that 16kDa specific immunity could be of importance in the protection against *M. tuberculosis* infection.

SCOPE AND OUTLINE OF THE THESIS

This thesis focuses on latent tuberculosis infection, which is the main Achilles' heel for control of TB. The studies described here address various aspects of cellular immunity during latent tuberculosis. Improved insight into the specific cellular immune responses during latency can be employed for accurate diagnosis of latent TB and can ultimately contribute to the development of successful post-exposure vaccines.

The **first part** of the thesis focuses on the development and evaluation of new immunodiagnostic assays for the detection of latent infection with *M.tuberculosis*.

The **second part** describes the search for, and immunological evaluation of, antigens that are specifically targeted by the immune system during latency, with the ultimate aim to identify latency associated antigens that are correlated with protection.

PART I: IMMUNODIAGNOSIS

Recent studies indicate that immunodiagnostic assays, based on the *M.tuberculosis*-specific antigens ESAT-6 and CFP-10, are highly specific for detection of *M. tuberculosis* infections. However, the sensitivity of such assays based on single antigens could be further optimised by discovery of additional *M. tuberculosis*-specific antigens. Therefore we evaluated in **chapter 2** the diagnostic potential of four other proteins, Rv2653, Rv2654 (TB7.7), Rv3873 (TB37.6) and Rv3878, which are encoded in regions of *M. tuberculosis* that are deleted in BCG and are lacking in most nontuberculous mycobacteria. Overlapping peptide pools of these antigens were screened for recognition by PBMC of BCG-vaccinated individuals, which enabled the selection of highly *M. tuberculosis* specific peptide stretches. Subsequently the sensitivity and specificity of these peptides were evaluated using PBMC of TB patients and BCG vaccinated individuals.

The study described in **chapter 3** illustrates the value of an in-house IFN-γ-ELISPOT using peptides of ESAT-6, CFP-10, TB37.6 and TB7.7, for the detection of recently acquired latent infection after accidental exposure to *Mycobacterium tuberculosis* in a microbiological laboratory.

According to recently issued U.K. guidelines, the method of choice to screen for latent *M. tuberculosis* infection is a two-step procedure using TST followed by an IFN-γ release assays (IGRA) in case the TST is positive. However, from a two step TST it is known that boosting of pre-existing immune response to mycobacterial antigens can occur. The study presented in **chapter 4** aimed to evaluate the effect of TST administration on the result of an IGRA, QuantiFERON-TB Gold in-tube (QFT-GIT), when performed on the day of reading of the TST.

In 2005 a large sized contact investigation was organized in the Netherlands when a supermarket employee with a diagnosis of highly contagious pulmonary TB was detected. The study reported in **chapter 5** was embedded within this large scale contact investigation and assessed the performance of two at that time just recently commercially available IGRA, QFT-GIT and T-SPOT.*TB,* for the diagnosis of latent TB. The IGRA were directly compared with each other and with the TST, and the association with measures of exposure was assessed.

Results from the studies described in chapters 4 and 5 indicated that a substantial group of *M. tuberculosis* exposed, BCG-unvaccinated persons with TST result of ≥ 15 mm had negative results in QFT-GIT and/or T-SPOT™.*TB*. This rather unexpected finding was further evaluated in **chapter 6** by studying the effect of varying IGRA formats and *in vitro* incubation periods on test outcome. For that aim, we compared the performance of the TST with two short-incubation IGRA, QFT-GIT and an in-house ELISPOT, as well as with a "classic" 6-day cell culture method, using identical *M. tuberculosis* specific peptides for the detection of past latent *M. tuberculosis* infection.

The clinical case presented in **Chapter 7** illustrates the potential value of IGRA for the diagnosis of active TB in an immunocompromised patient and the dissociation between *in vivo* immune responses and disease activity.

PART II: CELLULAR IMMUNITY DURING LATENCY

From *in vitro* models of latency it has become evident the *M. tuberculosis* undergoes specific metabolic changes, accompanied with rather different gene and protein expression profiles. In this part of the thesis we aimed to assess whether such changes in expression profile of *M. tuberculosis* result in a different repertoire of *M. tuberculosis* antigens available for T cell recognition during latent infection in humans. We hypothesize that immune responses to latency antigens might contribute to the control of persistent *M. tuberculosis* infection.

In **chapter 8** we investigated possible differences in antigen recognition patterns of responding T cells from active TB patients, healthy *M. tuberculosis* exposed persons and community controls from TB high- and low-endemic regions. For this purpose, T-cell responses to early secreted antigens ESAT-6 and CFP-10 were compared with responses to the16kDa, α-crystallin protein, which was found to be strongly expressed by *M. tuberculosis* in *in vitro* models of persistence.

The study described in **Chapter 9** was conducted to assess whether proteins encoded by the "dormancy regulon", a set 48 genes which is up-regulated by *M. tuberculosis in vitro* during exposure to nitric oxide or decreased oxygen tension, can be targeted by the

immune system during latent infection in humans. The 25 most strongly induced genes of the dormancy regulon were cloned and expressed in *Escherichia coli*. Subsequently, we evaluated the T cell responses to these 25 "*M. tuberculosis* latency antigens" in TB patients, TST-positive latently infected persons, and healthy uninfected controls.

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