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## Immunological Diagnosis of Active and Latent TB

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### 1. Introduction

Immunological diagnosis of tuberculosis (TB) is based on the immune responses against *Mycobacterium tuberculosis* (MTB). Immunological diagnosis can detect both active and latent TB, and can detect not only pulmonary TB but also extra-pulmonary TB. Compared to conventional diagnosis, immunological diagnostic tests have eminent advantages. On the other hand, there are still some limitations. As is known, various mycobacteria share homologous proteins, that lead to immunological cross reaction. To correctly detect TB infection, we need to choose a method that initiates the anti-MTB immune response properly. Human immunodeficiency virus (HIV) infection weakens the immune response, which may lead to false-negative results. HIV infection accompanied by TB is another urgent issue in global health. In this chapter, we will explain the immunological responses to MTB and the immunological interaction between HIV and TB. We will then introduce each diagnosis from the immunological point of view, and describe novel assays which we are now developing.

### 2. Immunological response to MTB

In this section, we describe the immune response to MTB and MTB/HIV co-infection.

#### 2.1 Innate immunity and adaptive immunity

When the immune system encounters foreign organisms, it works to eliminate them and both innate immunity and adaptive immunity are engaged in this process (Murphy, 2011). In this section, we will briefly describe the immune responses to give the theoretical basis for the immunological diagnosis of TB.

Innate immunity is a non-specific response to pathogen and is the first line of defence against microorganisms. When macrophages recognize foreign organisms, the cells ingest and digest them. Receptors on the cell surface, especially those of toll-like receptor family,

are involved in this process. Then macrophages express digested fragments on their surface promoting the initiation of the secondary response, the adaptive immunity.

Adaptive immunity is antigen specific and creates immunological memory. Responding T cells are functionally divided into T-helper cells 1 (Th1) and T-helper cells 2 (Th2), which are activated by antigen presentation through major histocompatibility complex class II (MHC II) on macrophages. Th1 cells stimulate T cell populations that secrete interferon gamma (IFN- $\gamma$ ) and interleukin-2 (IL-2) to activate cytotoxic T cells and, finally, to eliminate foreign organisms. This response is known as cell mediated immunity. Th2 cells secrete interleukin-4 (IL-4) to stimulate B cells, which produce antibody against the pathogen. This response is known as humoral immunity. Immunological diagnosis is based on the adaptive immune response to a targeted pathogen. If we detect or measure the activation of the immune response induced by MTB, we can diagnose MTB infection.

Recently, Th17 cells, which are responsible for inflammation, and T regulatory (Treg) populations, which suppress a variety of immune responses, have received much attention.

## 2.2 Immunological responses to MTB

MTB enters our body through the airway in droplet nuclei and is phagocytosed by alveolar macrophages. Macrophages digest MTB to connect MHC II molecules in the cell and fragments of MTB, then present their complex on the cell surface. Antigen presentation generates adaptive immunity (Walzl et al., 2011).

Th1 cells are activated and produce cytokines, such as IFN- $\gamma$  and IL-2. These cytokines activate cytotoxic T cells and macrophages. IFN- $\gamma$  enhances the anti-microbial activity of macrophages.

Th2 cells, producing IL-4, promote TB specific antibody production by B cells. These interactions lead to the generation of memory cells. There are two main populations of memory cells, effector memory T cells (which may be transiently present in the blood if bacteria are cleared) and central memory T cells (which may remain for life but may not provide protection in all individuals). The recently developed IGRA test measures IFN- $\gamma$  produced by effector memory T cells (Horsburgh & Rubin, 2011).

Despite these sophisticated immune responses, they often fail to eliminate MTB from the body and the bacteria may exist in a quiescent state for a prolonged period. Such a state is called latent tuberculosis infection (LTBI). In 10% of infected individuals, active TB develops and more than 80% of new cases of TB result from reactivation of the primary infection. The increase of HIV rates facilitates the reactivation of TB due to the immunosuppression.

## 2.3 TB with HIV infection

This lethal combination of TB/HIV is anything but rare; demographic analyses have estimated that over 60 % of the population in the sub-Saharan region has been infected by MTB, which has become a leading cause of mortality among HIV patients. At the end of 2009, TB infection was reported to be responsible for 13% of HIV deaths (Science Daily, 2009). Retrospective studies concluded that, among HIV/TB patients, 7% to 45% of them

probably develop symptoms homogeneous with Immune Reconstitution Inflammatory Syndrome (IRIS) (Murdoch et al., 2007; Shelburne et al., 2005). Therefore, exploring the relationship between TB and HIV become necessary.

Since HIV virus can weaken the immune system, LTBI can be activated resulting in pulmonary or extrapulmonary TB. A variety of immune cells and immune cytokines are involved in the reactivation of LTBI. Some cytokines, such as interleukin-8 (IL-8) and interleukin-12 $\beta$  (IL-12 $\beta$ ), may be used as biomarkers to monitor the immune reaction to LTBI (Wu et al., 2007) and possibly shed light on preventing of LTBI progression in HIV patients (Walzl et al., 2011). So far, various biomarkers to characterize LTBI and TB in HIV patients have been proposed. Without medical intervention, HIV infection will progress to acquired immune deficiency syndrome (AIDS), accompanied by multi-microbial infections, including TB infection, which often proves lethal.

### 2.3.1 Mechanism of immunological interaction of HIV and TB

Infection with HIV enhances the susceptibility to MTB infection. Because the occurrence of these two diseases is heavily dependent on the immune system, their interactions are more complex than previously understood. Previously, we studied the plasma levels of two matricellular proteins such as galectin-9 (GAL-9) and osteopontin (OPN) in AIDS patients complicated with various opportunistic infections (Chagan-Yasutan et al., 2009). The levels of both molecules were high in all the patients but only the level of GAL-9 decreased and that of OPN remained high after Highly Active Antiretroviral Therapy (HAART). Also, As Figure 1 shows, it was noted that the GAL-9 level was exceptionally high in acute HIV infected individuals (Chagan-Yasutan et al., 2009). The cellular receptor for GAL-9 is the T-cell immunoglobulin domain and mucin domain 3 (Tim-3) and Tim-3 is expressed on Th1 cells. Mycobacterium tuberculosis-infected macrophages express GAL-9 and the Tim-3 GAL-9 interaction leads to macrophage activation and stimulates the bactericidal activity by inducing caspase-1-dependent interleukin-1 $\beta$  (IL-1 $\beta$ ) secretion. Therefore, Th1 cell surface molecule Tim-3 may have evolved to inhibit the growth of intracellular pathogens via its ligand GAL-9, which is also known to inhibit the expansion of effector Th1 cells (Jayaraman et al., 2010). Therefore, only one case of MTB associated with acute HIV was reported (Crowley et al., 2011).

In contrast, chronic HIV infected individuals succumb to various opportunistic infections and pulmonary TB is known to occur when CD4<sup>+</sup> T cell numbers are still high, indicating that the immune system plays a role in the development of pulmonary TB (Holmes et al., 2005). Similarly, T cell epitopes of different strains representative of global diversity are highly conserved in MTB (Comas et al., 2010). Due to the conserved epitopes, the host can maintain MTB for a long time as latent infection and can transmit it to the next generation. It is also suspected that CD4<sup>+</sup> T cells have an essential role in tissue damage that results in cavity formation, which enhances aerosol infection. In HIV endemic areas, the situation becomes more complex if the CD4<sup>+</sup> T cells numbers increase after HAART and then recovery of the immune system is variable (Fig 2).

### 2.3.2 Network among LTBI and HIV

A. Healthy Individuals. For immunologically potent people, the reaction to invading microbes consists of innate and adaptive immune mechanisms. Genome research has

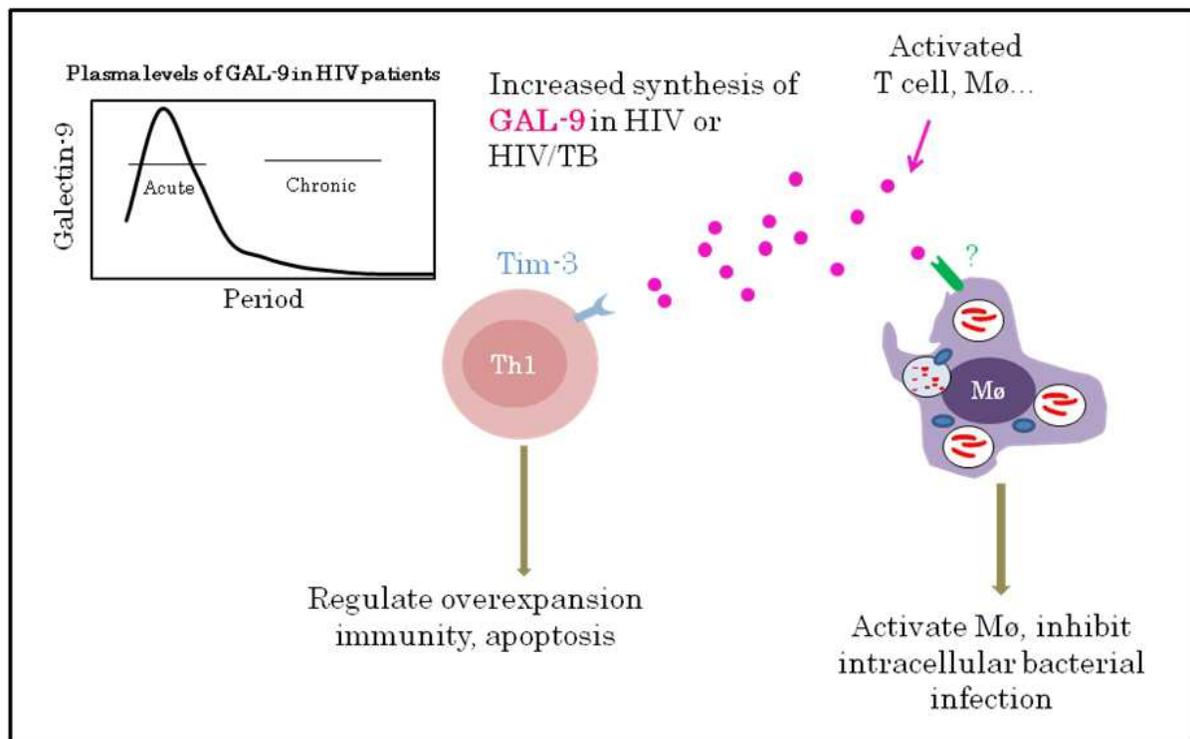


Fig. 1. Proposed biological effects of GAL-9 in HIV or HIV/TB infection.

The plasma levels of GAL-9 were elevated in chronic AIDS patients as well as in TB patients (non-published data), but were exceptionally high in acute HIV infection (Chagan-Yasutan et al., 2009). It was reported that GAL-9 interacts with its Tim-3 ligand to regulate the overexpansion of Th1 cells to induce apoptosis (Zhu et al., 2005; Kashio et al., 2003). In TB, however it was speculated that GAL-9 contributes to the activation of macrophage cells (Mφ) and then inhibits intracellular bacterial infection by caspase-1 dependent IL-1 $\beta$  production (Jayaraman et al., 2010).

revealed that TB epitopes binding to human CD4 + T cells are conserved (Comas et al., 2010). Accordingly, during the long history of fighting against TB, human beings have evolved a spectrum of potential TB-specific naive CD4+ T cells, which can be activated as soon as TB invades and transformed to TB effective CD4+ T cells to fight against TB bacilli, some of which would transform into central memory T cells (CMT) after TB is controlled. Such CMT cells are capable of quick proliferation once they confront TB. During infection, these TB antigen specific CD4 + T cells make up a certain percentage of CD4+ T cells; the percentage could be affected by the volume of CMT and individual differences in gene expression profiles (Maertzdorf et al., 2011).

B. LTBI (Latent tuberculosis infection) Individuals. Thirty percents of the population in the world is infected by MTB. However, effector CD4+ T cells protect LTBI individuals from developing active TB. In this case CMT act as a backup to proliferate of effector CD4+ T cells. Observation of this TB-specific reaction can be simplified by monitoring the IFN- $\gamma$  level in IGRA (Rueda et al., 2010). Apart from IFN- $\gamma$ , other biomarkers including CD154 and CD107 also indicate a TB-specific reaction (Streitz et al., 2011). IFN- $\gamma$  has multiple effects on the immune system to control TB. As IFN- $\gamma$  is secreted by TB effector CD4+ Th1 cells, it could mediate cytotoxic T cells to recognize and damage TB-infected macrophage cells. IFN-

$\gamma$  also enhances MHC II on macrophages. Other cytokines such as IL-2 and IL-4 could act in synergy with IFN- $\gamma$  to control LTBI. However, 10% of them eventually progress to active TB (Day et al., 2011).

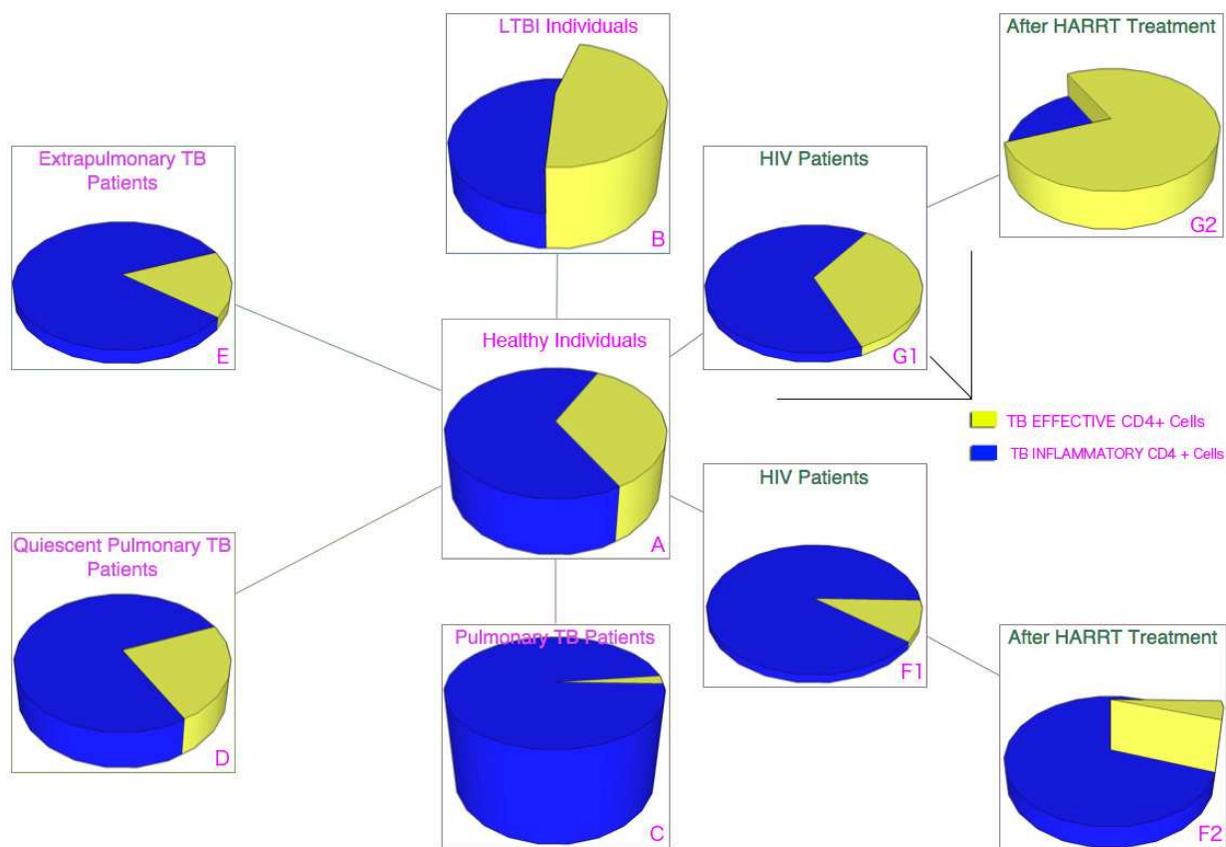


Fig. 2. Schematic network among LTBI and HIV.

The horizontal plane indicates the make up of inflammatory CD4+ cells and TB effective CD4 + T cells (Effector memory T cells), which are coloured in blue and yellow, respectively. The overall reaction of different cells to TB is described according to the volumes of the different colours, the vertical lengths of which indicate biomarkers that rise up or descend resulting from TB reaction. Such schema and many exceptional cases are present in HIV/AIDS and tuberculosis.

C. Pulmonary TB. A pulmonary cavity is a typical sign of pulmonary TB. A recent paper reported that CD4+ T cells could be an essential factor for TB cavity formation (Russell et al., 2010). Patients of pulmonary TB have been reported to have mainstream TNF- $\alpha$  TB specific CD4+ T cells, which can lead to an inflammatory reaction (Indicated by blue colour in vertical direction of C) in active TB patients. A recent study based on 101 TB and LTBI individuals described that TNF- $\alpha$  specific CD4+ T cells might be an important biomarker for diagnosing active TB. In the majority of active TB patients TNF- $\alpha$  can be detected in the MTB antigen stimulated cells. Flow-cytometry showed that inflammatory-related CD4+ T cells represent 37.4% of total CD4+ T cells (indicated by area between blue and yellow colour in C) is the cut-off of LTBI becoming active TB (Harari et al., 2011). And such TNF- $\alpha$  TB specific CD4+ T cells activate other inflammatory cells. However, IFN-inducible neutrophils may also play an essential role in eliciting inflammatory CD4+ T cells.

Neutrophils corresponding to IFN- $\alpha$ , $\beta$  and  $\gamma$  modulate an inflammatory reaction in active TB patients (Berry et al., 2010).

D. Extrapulmonary TB. Due to the small number of inflammatory CD4+ T cells, TB lacks the ability to form cavities in the lungs and lead to multi-organ or systemic infection. Such patients are frequently found among those with AIDS and patients on immunosuppressive therapy. Interestingly, extrapulmonary TB is a prominent risk factor for IRIS. (Manosuthi et al., 2006)

E. The stage between LTBI and active TB infection. It features opacities in the lungs, but sputum and IGRAs test are negative.

F1, G1. HIV-infected Patients. In Sub-Saharan region, frequently seen cases are LTBI individuals infected by HIV. Asymptomatic HIV can last by 2 years to 10 years in normal individuals after infection. As a result of HIV infection, CD4+ cells drop progressively. After HIV infection, the virus targets all CD4+ T cells including effector, inflammatory and CMT CD4+ T cells and anti-HIV drugs restores their function. F1 indicates those patients who didn't carry larger number of CMT cells and could not produce enough effector CD4+ T cells after TB stimulation. G1 indicates that those patients with a large pool of TB CMTs or who has been infected by LTBI prior to HIV infection and then carried more LTBI stimulating specific effector CD4+ T cells or memory T cells (Mueller et al., 2008). F1, G1 will finally process to extrapulmonary TB (indicated by E), if no medication intervenes.

F1-F2. HIV Patients during HAART treatment. In macaque experiment, SHIV was found to preferentially infect CMT cells (He et al., 2011). When HAART treatment is applied, CD4+ T cells count will rise. Occasionally, such rise causes IRIS, because CMT cell count will bounce up and effector CD4+ T cells will show increased activity. However MTB specific inflammatory CD4+ T cells will dominate their large number. (Indicated by proportional volume between blue and yellow). As TB Inflammatory CD4+ T cells lead the reaction, patients have similar prognoses as pulmonary TB (Indicated by C) (Worodria et al., 2011).

G1-G2. HIV Patients after HAART treatment. HAART will rapidly reconstitute the immune surveillance (indicated by elevation of yellow column) (Hua et al., 2011). The occurrence of TB therefore decreases (Sant'Anna et al., 2009).

#### Net work

C-F1-F2. TB and HIV stimulate specific inflammatory T cells to produce TNF- $\alpha$  which, in turn, help them to progress at faster rate (Sorathiya et al., 2010). The patients can be treated by anti-TB therapy followed by HAART. Occasionally, paradoxical TB IRIS<sup>1</sup> occurs, probably caused by MTB specific inflammatory CD4+ T cells. MCP-1 (monocyte chemotactic protein 1) was found to be a reliable candidate biomarker to screen patients who may develop to paradoxical IRIS (Haddow et al., 2011). B-G1-G2. HAART strengthen immune responses against MTB and can decrease the occurrence of TB (Middelkoop et al., 2011, Hua

<sup>1</sup> Definition of TB IRIS: 'paradoxical' worsening of symptoms of known disease, either at a new body site or at the original body site, with an incidence of 8–43% of TB-co-infected individuals starting ART; or 'unmasking' of occult Mycobacterium tuberculosis infection, in which infection was not clinically apparent prior to ART but presents floridly during ART, affecting around 5% among those starting ART without known TB infection in South Africa.

et al., 2011). Notably, HAART unmasking TB manifestation can be found in some cases and C-reactive protein (CRP) was reported to be helpful to detect the development of unmasking TB-IRIS cases (Haddow et al., 2011).

### 3. Immunological diagnosis

Over decades, there have been attempts to find new diagnostic tools, that are sensitive and specific, simple, inexpensive and able to distinguish latent tuberculosis from active tuberculosis as well as MTB infected individuals from uninfected ones.

#### 3.1 Tuberculin skin test

Tuberculin skin test, also called as Mantoux skin test, has been used for the diagnosis of tuberculosis for more than a century. Despite the numbers of logistic and performance problems and poor specificity, TST is still performed as a routine diagnostic method. The purified protein derivative (PPD) antigens, that are used for TST are highly homologous to antigens of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine and non-tuberculosis mycobacteria (NTM) antigens. These and other factors may lead to false-positive or false negative TST results.

Although other antigens have been evaluated as a skin test reagents e.g. molybdopterin-64 (MPT-64) and molybdopterin-59 (MPT-59), none of them proved superior to tuberculin skin test (Wilcke et al., 1996).

False-positive reactions	False-negative reactions
✓ Infection with NTM	✓ Cutaneous anergy
✓ Previous BCG vaccination	✓ Recent TB infection
✓ Incorrect method of TST administration	✓ Very old TB infection
✓ Incorrect interpretation of reaction	✓ Very young age (less than 6 months old)
✓ Incorrect bottle of antigen used	✓ Recent live-virus vaccination (e.g., measles and chicken pox)
	✓ Incorrect method of TST administration
	✓ Incorrect interpretation of reaction

Table 1. False-positive and false-negative TST reactions. (CDC, 2010)

#### 3.2 Interferon Gamma Releasing Assay (IGRA)

Because IFN- $\gamma$  is a cytokine that plays a critical role in resistance to *Mycobacterium tuberculosis* infection and MTB infected individuals respond to MTB antigen stimulation by releasing increased amounts of this cytokine from effector memory cells, methods based on measuring the IFN- $\gamma$  production by antigen stimulated human T lymphocytes have been developed. There are two new blood tests on the market, the enzyme-linked immunospot assay (ELISpot) (T-SPOT<sup>®</sup>.TB, Oxford Immunotec, Oxford, UK) and the enzyme-linked immunosorbent assay (ELISA) (QuantiFERON-TB Gold In-Tube, QFT-GIT, Cellestis, Carnegie, Australia). Both IGRAs have high sensitivity and specificity, for QFT-GIT 81%-

92.6% and 98.8%-99.2% and for T-SPOT<sup>®</sup>.TB 87.5%-95.6% and 86.3%-99.9%, respectively (Diel et al., 2008; Harada et al., 2008; Oxford Immunotec, 2011). Firstly, it should be mentioned that using IGRA it is impossible to distinguish between latent and active TB for which no such a method yet exists. However the detection of both latent and active TB has been markedly improved by employing IGRA methods. The factor, that increased the sensitivity and specificity of IGRA was the discovery and use of antigens encoded by Regions of Difference 1 (RD1) in the MTB genome, which is absent in BCG vaccination or NTB. Among the nine antigens encoded by RD1, early secreted antigenic target 6kDa (ESAT-6) and culture filtrate protein 10kDa (CFP-10) are used as a stimulatory antigens. However, ESAT-6 and CFP-10 antigens are also present in NTM, namely *M. leprae*, wild type *M. bovis*, *M. marium*, *M. kansasii*, *M. sulgai*, *M. flavescens*, in NTM endemic areas, IGRA results might be false positive and make it difficult to distinguish MTB between NTM.

### 3.2.1 QuantiFERON-TB Gold In Tube assay

In comparison to the previous form of QuantiFERON Gold, the QuantiFERON Gold In Tube (QFT-GIT) version enables immediate antigen stimulation of lymphocytes within whole blood. In addition to ESAT-6 and CFP-10, QFT-GIT contains a peptide from the internal section of TB 7.7 (Rv2654), which may increase the sensitivity of the test (Aagaard et al. 2004; Brock et al., 2004), though it is arguable whether specificity is improved. All three antigens are present on the wall surface of the blood collection tubes. Besides the immunity status and the absolute and relative lymphocyte number there are external factors that may influence the QFT results, e.g. drawing an adequate volume of blood, appropriate attachment of lymphocytes to the antigens, sample handling and the ELISA assay procedure. Another important issue is the interpretation of the results. There are two cut-offs given by the manufacture, 0.35 IU/l and 0.1 IU/l. A value of more than 0.35 IU/l seems to be appropriate for good discrimination of truly infected individuals (Harada et al., 2008). On the other hand, the values between 0.35 IU/l and 0.1 IU/l, the intermediate result, should take into account the individual patient's condition (Harada et al., 2008; Liote H & Liote F, 2011). In Japan, the interpretation criteria differ from those anywhere else. Intermediate results in Japanese people are suspected to be positive and are flagged for follow-up observation (Prevention Committee, Japanese Society of Tuberculosis, 2006). Depending on the MTB infection prevalence, it has been suggested to use different cut-offs (Harada et al., 2008). In areas where the MTB infection prevalence is low, the specificity is probably of great importance. However, in high-risk TB screening situations, identification of LTBI is likely to be more important than the potential side effects of the MTB treatment and a cut-off value of 0.1 IU/l should be employed (Yew & Leung, 2006).

### 3.2.2 T-SPOT<sup>®</sup>.TB test (ELISpot)

Using the ELISpot assay it is possible to visualize and count MTB-specific memory T cells producing IFN- $\gamma$ . The great advantage of this test is that each test well contains the same number of peripheral blood mononuclear cells (PBMCs). Especially in the patients with low T cell counts from HIV or other immune disorders, it enables the objective evaluation by adjusting the designed cell number. While in QFT-GIT all antigens are present in the same tube, in the ELISpot assay ESAT-6 and CFP-10 antigens are added and read separately. The manufacture claims that this assay has very low cross-reactivity with NTM (Oxford

Immunotec, 2011). The important step is the accurate adjustment of the cell count. However, if the cells are not well adjusted and the nil control contains more spots than indicated in the instructions, the test needs to be repeated. What is more, to run the ELISpot assay, a trained professional should be engaged and other special tools such as a plate reader are needed. Exact interpretation of the results is crucial, so the manufacturer prepared a training manual for distinguishing between valid and invalid spots. The ELISpot assay's intermediate result rate is 3-4% (Dosanjh et al., 2008), which is significantly lower than that for QFT assays (11-21%) (Ferrara et al., 2006; Piana et al., 2006).

QFT-GIT test has recently become routinely used as a diagnostic tool for MTB, but the T-SPOT®.TB test is employed only in few countries, mostly because of the high cost of this assay. In conclusion, IGRA seems to be beneficial tool for TB diagnosis, especially for people with a high-risk of developing active TB.

### 3.2.3 Other approaches of LTB diagnosis

In addition to ESAT-6 and CFP-10, other antigens have been proposed for ELISpot assay but have not been implemented for commercial use. Addition of the novel antigen Rv3879c increased the diagnostic sensitivity of the standard ELISpot assay and, in combination with TST, reached a sensitivity of 99% (Dosanjh et al., 2008). Other researchers showed that antigen heparin-binding-hemagglutinin was significantly more sensitive than ESAT-6 and more specific than PPD for the detection of LTBI (Hougardy et al., 2007a).

### 3.2.4 Conditions altering IGRA results

There are various conditions such as oncologic disease, HIV infection, anti-TNF- $\alpha$ , corticoid or other immunomodulatory therapy, diabetes mellitus, renal failure or other immunocompromising conditions that are responsible for intermediate or false negative results (Schoepfer et al., 2008; Matulis et al., 2008; Kim et al., 2009). Particularly, the anti-TNF- $\alpha$  treatment has increased recently, which hampers the activation of the innate immune responses, T cell mediated adaptive immune response and production of protective IFN- $\gamma$ . Similarly, metabolic diseases such as diabetes mellitus are known to affect chemotaxis, phagocytosis, activation, and antigen presentation by phagocytes in response to MTB, and this defect does not improve with insulin (Moutschen et al., 1992). Importantly, IFN- $\gamma$  production was found to be impaired in hyperglycemia (Yamashiro et al., 2005), but another study showed that both IGRA results were not affected in diabetes mellitus patients (Walsh et al., 2011). Intermediate results have been found to be also associated with lower serum albumin and double immunomodulatory therapy (Papay et al., 2011).

## 3.3 Role of regulatory T cells in diagnosis of MTB

Since TB is a chronic disease in which bacilli evade the immune system to persist in the host organism, scientists are trying to find and understand the mechanism of MTB immunopathology. It is still unclear what conditions prone to MTB infection, what factors are involved in TB latency, activation or masking of the disease and what causes the imbalance of the immune responses that finally lead to the failure of MTB eradication. The immune system poses a regulatory mechanism in which Treg cells play essential roles in

establishing and sustaining self tolerance and immune homeostasis as well as regulate the host response to infection.

### 3.3.1 Role of Treg in mycobacterium tuberculosis infection

The first demonstration of the suppressive capacity of T cells was performed in 1973 in an animal model of bacillus BCG vaccination. Thymocytes from BCG-injected rats were harvested and transfected to alive normal recipients. Subsequently, recipients were challenged with the same antigen and the inhibition of their skin reaction was observed (Ha & Waksman, 1973).

The number of CD4+CD25+FoxP3+ Treg cells was found to be increased in the blood or at the site of infection in active tuberculosis patients (Guyot-Revol et al., 2005) and the frequency of CD4+CD25+FoxP3+ T lymphocytes was inversely collated with the local MTB-specific immunity, and both blood and pleural Treg cells were able to suppress IFN- $\gamma$  and IL-10 production in TB patients (Chen et al., 2007). This mechanism is thought to contribute to the pathogenesis of human TB (Guyot-Revol et al., 2005; Chen et al., 2007). Treg cell expansion is believed to predispose or be a marker of the progression of latent TB to active disease (Hougardy et al., 2007). What is more, it was found that depletion of CD4+CD25+ T cells enhanced the protective IFN- $\gamma$  production in TB patients (Guyot-Revol et al., 2005) and transiently reduced the bacterial load and granuloma formation (Ozeki et al., 2010).

### 3.3.2 Objectives of our Treg study

The majority of individuals vaccinated with BCG or infected with MTB develop a delayed-type hypersensitivity which is manifested as a positive response of intradermal injection to a purified protein derivative from MTB. But about 15% of active TB patients show false negative results and are considered to be anergic TB (Bloom & Small, 1998). Similarly, in HIV infected individuals, TST results are often found to be false-negative. Concerning the high frequencies of Treg cells in both diseases (Chen et al., 2007; Bi et al., 2009), it is highly probable that Treg cells may play central role in the anergy mechanism. Cutaneous anergy in active TB is associated with the absence of granuloma formation and poor clinical outcome (Boussiotis et al., 2000). Another study showed that, in an anergic patient, sustained MTB stimulation led to enhanced IL-10 production and the generation of anergic MTB-specific T regulatory cells with the Tr-1 phenotype (Boussitis, 2000). In certain closed populations, a high percentage of TB anergy and high prevalence of active TB were observed, which led to the speculation that innate genetic factors may play a role (Delgado & Ganea, 2001). We observed several cases of anergy in health care workers (HCW), who had been in close contact with active TB patients. Therefore, we questioned whether Treg cell may mask latent TB infection.

### 3.3.3 Materials and methods

#### Human subjects and samples

According to previously obtained TST results, one TST positive (27 years old) and one TST negative (42 years old) healthy (X-ray and sputum smear negative) health care individuals with no history of previous MTB infection or other chronic disease were recruited in this

study. A TST result  $\geq 10$  mm was considered to be positive. Both individuals were vaccinated with BCG when young. The protocol was approved by the ethical committee of Tohoku University Medical School. Written informed consent was obtained. Both individuals were HIV, hepatitis B and C virus negative.

From each donor 20 ml of Ethylenediamine tetraacetic acid (EDTA) treated peripheral venous blood was obtained, centrifuged and the peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll separation (Ficoll-Paque PLUS, GE Healthcare Bio-Science AB, Uppsala, Sweden). After washing twice in Phosphate Buffered Saline (PBS), PBMCs were resuspended in complete RPMI 1640 medium (consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2% glutamine and 1% penicillin/streptomycin) at a concentration  $5 \times 10^6$  cells/ml.

### **Depletion of CD4+CD25+ cells**

The cells designed for depletion were centrifuged and resuspended in Running buffer (MACS Separation Buffer, Miltenyi Biotec). To avoid unspecific binding of the antibody, 100  $\mu$ l of FcR Blocking Reagent (Miltenyi Biotec) was added to the PBMCs which were then incubated for 15 minutes on ice. After adding 25  $\mu$ l of CD25-Biotin monoclonal antibody (Miltenyi Biotec) and 15 minutes incubation on ice, the cells were washed with Running buffer. To cells resuspended in Running buffer, 25  $\mu$ l of anti-Biotin Microbeads (Miltenyi Biotec) were added, followed by incubation for 15 minutes on ice. Subsequently, the cells were washed and resuspended in Rising Solution (Miltenyi Biotec) and CD4+CD25+ T cells were depleted by positive selection using a magnetic-activated cell sorter (MACS)-assisted cell sorting system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

### **Cell phenotype determination**

To visualize the biotinylated mAb on CD25+ cells, streptavidin-allophycocyanin (BD PharMingen) was used and the CD4+ cells were stained by anti-CD4 FITC antibody. Flow cytometric analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson) using the CELL quest program (Becton Dickinson). More than 90% of CD4+CD25+ cells were depleted from the PMBCs of both individuals.

### **Cultivation and cytokine determination**

Freshly isolated, undepleted and Treg depleted PBMCs were cultured in triplicate in 96-well plates at a concentration of  $2 \times 10^5$  cells per well in 200  $\mu$ l of complete RPMI 1640 medium and incubated for 24 hours at 37 °C with 5% CO<sub>2</sub>. The cells were stimulated with 1  $\mu$ g/ml of PPD (Statens Serum Institute, Copenhagen, Denmark), 500nM of recombinant CFP-10 and ESAT-6 protein antigen. The cell culture supernatant was harvested from each well for ELISA analysis. IFN- $\gamma$  and IL-10 production was determined by using human IFN- $\gamma$  and IL-10 BD Opt EIA™ Set according to the manufacturer's instructions (BD Bioscience). Optical densities were read at 450 nm on an ELISA plate reader (VersaMax-KT, Molecular Devices Corp., CA, USA) and the concentrations were calculated from standard curves using the Soft Max Pro program (Molecular Devices Corp.).

### **Statistical analysis**

Two-sided paired *t*-test was used to analyze the effect of Treg on cytokine production. Differences were considered significant when the *p* value was less than 0.05.

### 3.3.4 Results

To investigate the immunosuppressive effect of Treg cells on the anti-TB immune response, CD25<sup>+</sup> T cells were depleted from PBMCs of TST positive and TST negative healthy HCW. IFN- $\gamma$  and IL-10 production upon PPD, CFP-10 and ESAT-6 stimulation was assayed.

The positive response to PPD stimulation in the TST-positive individual agreed with the TST result. Similarly, the low IFN- $\gamma$  response to PPD in the TST anergic person supported the unresponsiveness of PBMCs to PPD stimulation. Both individuals had low levels of IFN- $\gamma$  on CFP-10 stimulation, but the TST anergic person, in contrast to the TST positive one, responded to ESAT-6 stimulation, what is suspicious for LTBI. Depletion of Treg cells significantly enhanced the IFN- $\gamma$  production in the TST anergic person, but in the TST positive person it influenced the IFN- $\gamma$  level only after PPD stimulation (Fig. 3A). Depletion of Treg cells significantly influenced the IL-10 production only by PPD and CFP-10, but not by ESAT-6 stimulation (Fig. 3B).

### 3.3.5 Discussion

PPD is the prototypical mycobacterial antigen, which is included also in the Mantoux skin test antigens. The result with PPD stimulation demonstrated a T cell antigen recognition level consistent with TST results. PPD unresponsiveness in anergic TB patients might be due to the inability of their antigen presenting cells to present antigens or to the inability of their T cells to respond to antigen-specific stimulation (Boussiotis et al., 2000). Our results showed that PPD anergy might be due to an impaired T cell response as an effect of the Treg cell activity.

Recombinant antigens such as ESAT-6 and CFP-10 have been reported to be strong IFN- $\gamma$  inducers. CFP-10 was reported to be less reactive in comparison to ESAT-6 in TB patients as well as in healthy controls (Oliveira et al., 2007), which was also observed in our assay. The anergic subject in our study showed a strong response to ESAT-6 stimulation, which made him highly suspected to be TB infected.

CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells have been found to be increased in MTB infection and to suppress the MTB-specific immunity (Hourgady et al., 2007; Chen et al., 2007). It has been found that elimination of CD4<sup>+</sup>CD25<sup>+</sup> T cells significantly increased the BCG-induced production of IFN- $\gamma$  and IL-10 by PBMCs from patients with active TB, but not by those from healthy volunteers (Chen et al. 2007), but there is no report of Treg functions in TST anergic LTBI. We have demonstrated for the first time that Treg cell depletion in anergic individual led to enhanced IFN- $\gamma$  production upon MTB specific ESAT-6 and CFP-10 antigen stimulation, while in the TST positive healthy individual we did not observe such a phenomenon. These results support the argument that CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress the Th1 immune response.

IL-10 is considered a soluble factor that plays a central role in controlling inflammatory processes, suppressing T cell responses, and maintaining immunological tolerance (Moore et al., 2001). In the condition of MTB infection, mycobacteria-induced IL-10 production by macrophages allow mycobacteria-infected cells to elude immune surveillance (Larsen et al., 2007).

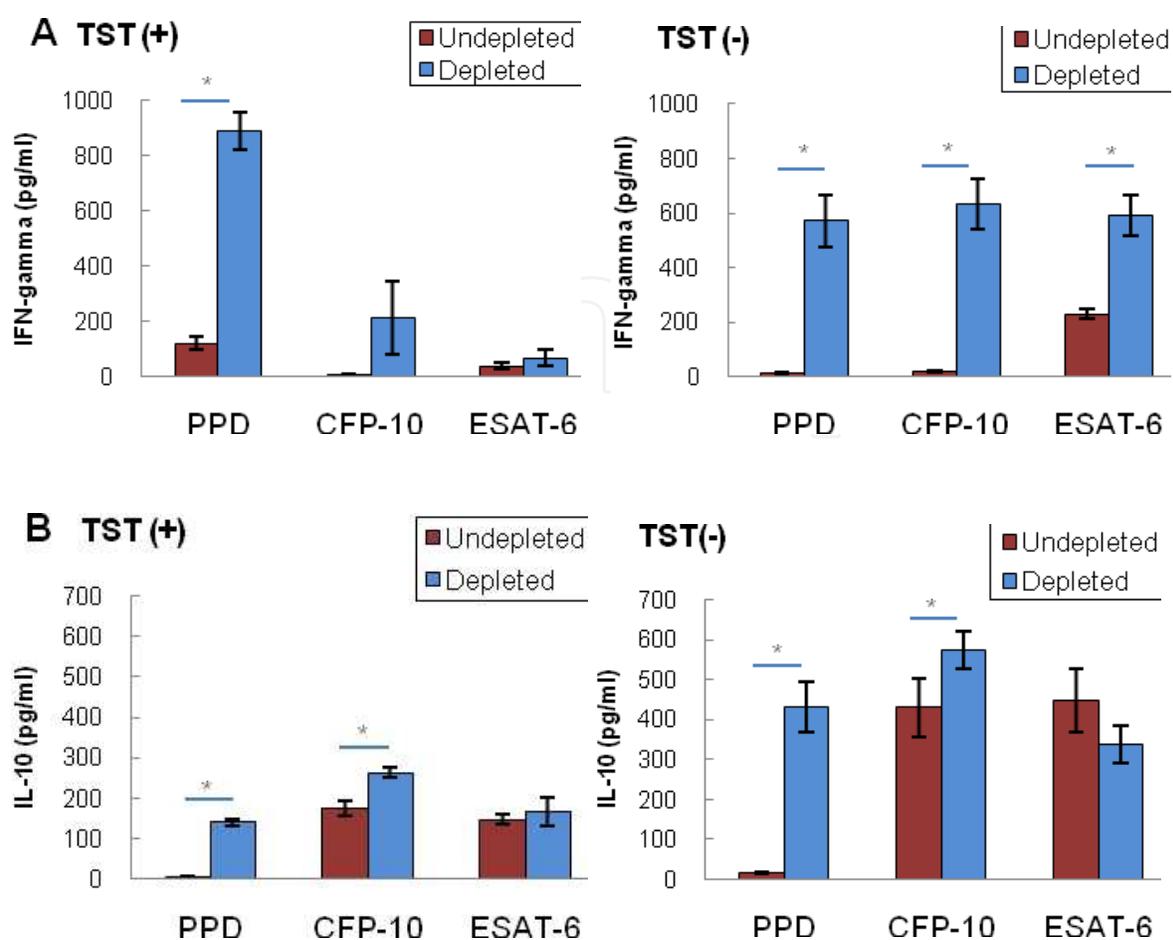


Fig. 3. Immunossuppressive effect of Treg on (A) IFN- $\gamma$  production and (B) IL-10 production. Statistically significant (\*)  $p < 0.05$

Antigen presenting cells matured in the presence of BCG are able to instruct naive T cells to develop into cytokine-producing T cells that can be categorized into Th1 (IFN- $\gamma$  producing), Th2 (IL-4-producing) or Tr1 (IL-10-producing) cells (Larsen et al. 2007). We can speculate that upon first contact with BCG vaccine in early childhood, the naive T cell development may lead to polarization of the immune response in favor of Th2 and Tr1 IL-10-producing cells. An anergic individual, in comparison to a TST positive person, have elevated levels of IL-10 upon MTB antigen stimulation and it might be possible that his immune responses are switched towards an IL-10 immune response. The anergizing effect and antiinflammatory properties of IL-10 might be one of the factors maintaining the anergy and LTBI chronicity.

Even if this is a sporadic finding, we believe that these results enable a better understanding of the immune mechanisms involved in anergy and LTBI in adult, healthy, BCG-vaccinated individuals. It is necessary to confirm these data in larger numbers of volunteers. It might be disputed whether the in vitro conditions mimic MTB infection in vivo.

In summary, Treg cells play a role in masking LTBI by suppressing the specific MTB immune response through altering IFN- $\gamma$  and IL-10 production.

#### 4. Serological diagnosis of tuberculosis

The diagnosis of tuberculosis infection remain unchanged or with very limited progress for many decades. Until recent years, diagnosis of tuberculosis primarily depends on traditional sputum microscopy for acid fast bacilli (AFB) in low and middle income countries where the disease is heavily concentrated, although the sensitivity of the method is variable (20~60%) (Steingart et al., 2006). In HIV/AIDS infection, frequent occurrence of non-cavitary pulmonary lesion can cause sputum negative tuberculosis disease. Extra-pulmonary involvement is 10-20% of all tuberculosis case and can occur relatively frequently in children than adults and in HIV/AIDS infection than healthy. Since enhancement of B cell immunity and production of antibody along with protective cell-mediated immunity may play an important role in the immunopathogenesis of tuberculosis, detection of specific antibodies against various mycobacterial derived antigens could also play a significant role in the diagnosis of tuberculosis. The value of various mycobacterial native or recombinant protein, lipid or different combinations of purified antigens or commercially available kits as a potential candidate of active tuberculosis sero-marker was evaluated by the ELISA method in many attempts (Abebe et al., 2007; Verma & Jain, 2007; Steingart et al., 2009). Development of a serological test with sufficient diagnostic efficacy for tuberculosis diagnosis could be very much useful tool in resource-limited countries, as the procedures are simple, relatively cost effective and can be performed rapidly.

Characteristics of commonly used antigens have been listed in the following table (Table 2). Until recently, various mycobacterial culture filtrate and surface exposed proteins including 38kDa, Ag85B, MPT51, Ag60 antigens, malate synthase, heat shock protein, RD1 antigens were investigated to determine their diagnostic efficacy.

Target antigen	Type of antigen	Mycobacterial location/Rv region	Biological effects
LAM	Lipoglycan	Cell wall	Immunomodulation, Anti-inflammatory
TDM	Glycolipid	Cell wall	Immunomodulation, Enhance inflammation and granuloma formation
DAT	Glycolipid	Cell wall	Immunomodulation
TAT	Glycolipid	Cell wall	Immunomodulation
SL-I	Glycolipid	Cell wall	Related to MTB virulence
38kDa	Protein	Rv0934	Immunogenic protein
Ag85B	Protein	Rv1886c	Immunogenic protein
Malate synthase	Protein	Rv1837c	Immunogenic protein
MPT51	Protein	3803c	Immunogenic protein
ESAT-6	Protein	3875	Immunogenic protein
CFP-10	Protein	3874	Immunogenic protein
Antigen 60	Glycopeptidolipid		Immunomodulation

Table 2. Characteristic of mycobacterial antigens commonly assessed for the serodiagnosis of tuberculosis.

LAM: lipoarabinomannan; TDM: trehalose 6,6 dimycolate; DAT: 2,3-diacyl trehalose; TAT: 2,3,6-triacyl trehalose; SL-I: 2,3,6,6-tetraacyl trehalose 2'-sulphate (Sulfolipid-I); Ag85B: Antigen85B; ESAT-6: early secreted antigenic target-6; CFP-10: culture filtrate protein-10

#### 4.1 Serodiagnostic markers

**38kDa antigen:** The 38-kDa antigen (also known as Antigen 5) is a major lipoprotein antigen of *M. tuberculosis*. As reviewed by Steigart et al. in a meta-analysis, it yielded a sensitivity of 47% and a specificity of 94% in smear positive tuberculosis patients. The increased sensitivity is found to be associated with smear-positive than negative tuberculosis (Wilkinson et al. 1997; Julian et al., 2000). Use of native or recombinant protein did not show any difference in term of diagnostic efficacy. But, the sensitivity of IgG detection was relatively higher than that of IgA against 38 kDa antigen (Verma & Jain, 2007). Several commercially available antibody detection kits were also developed using this antigen including Pathozyme Myco (LAM+38kDa), Pathozyme TB complex plus (38kDa+16kDa). The sensitivity varies in both Pathozyme Myco (21-46%) and Pathozyme TB complex plus (29-76%). However, the tests are highly specific (94-100%) for tuberculosis diagnosis (Steingart et al., 2007). The sensitivity was less than 70% and the specificity varies from 70% to 94.9% in different studies for the diagnosis of smear-positive tuberculosis patients co-infected with HIV (Abebe et al., 2007). The sensitivity of Pathozyme Myco and Pathozyme TB complex plus varies from 11% to 51% respectively, although the specificities were more than 90% by both the kits for the diagnosis of extra-pulmonary tuberculosis (Steigart et al., 2007).

**Antigen 85B:** It is a member of a family of Ag85 protein complex (Ag85A, Ag85B and Ag85C). It is a major fraction of secreted proteins in the MTB culture filtrate and cell wall. A relatively higher sensitivity in HIV-positive tuberculosis patients (62%) than HIV-negative tuberculosis patients (53%) with a high specificity (>95%) in both groups were reported for Ag85B (Steingart et al., 2009). MPT51 is an antigen also related to the protein family of Ag85 complex. MPT51 provided comparable diagnostic efficacy in both HIV-negative (sensitivity: 59%) and HIV-positive tuberculosis patients (sensitivity: 58%) and the specificities of 94% and 97% respectively (Steingart et al., 2009).

**ESAT-6 and CFP-10:** These are two low molecular weight secreted proteins, encoded within the RD1 region of *M. tuberculosis* and highly associated with the virulence of the organism. It is known to induce strong cell mediated immune response. Although the use of ESAT-6 and CFP-10 in IFN- $\gamma$  based immunological methods for tuberculosis is widely acceptable for the diagnosis of active and latent tuberculosis infection, the potential use of this antigens in antibody-based diagnostic methods were also evaluated. Association of antibody responses against ESAT-6 was described to be related with inactive stage of tuberculosis (Davidow et al., 2005; Doherty et al., 2002), although increased antibody in progressive tuberculosis was also demonstrated in other report (Demissie et al., 2006). In addition, CFP-10 showed a sensitivity of 48% and a specificity of 96% for the diagnosis of active pulmonary tuberculosis. The use of CFP-10/ESAT-6 fusion antigen obtained a relatively higher sensitivity of 60.4% and a specificity of 73.8% in HIV-seronegative tuberculosis patients (Wu et al., 2010).

**Antigen 60 (A60):** It is a heat-stable component of PPD extracted from BCG that can be recognized by the sera of tuberculosis patients (Abebe et al., 2007). Anda TB (Anda Biologicals, Strasbourg, France), a commercially available ELISA kit was developed using A60 and its diagnostic ability was evaluated by many investigators for the diagnosis of pulmonary and extra-pulmonary tuberculosis. The sensitivity was variable in pulmonary

tuberculosis (29%-85%) as well as in extra-pulmonary tuberculosis (0%-100%). However, the specificities ranges 70%-100% for both types of tuberculosis (Steingart et al., 2007).

Non-peptidic antigens from the mycobacterial cell wall grasp the main focus of comprehensive research for the determination of their potential role in the protective immunity or marker of TB disease.

**Lipoarabinomannan (LAM):** LAM, a complex glycolipid antigen forming is a major part of cell wall of MTB. Evaluation of anti-LAM-IgG against purified LAM from MTB for the serodiagnosis of tuberculosis was reported to have good diagnostic ability (sensitivity: 91%, specificity: 72%) in detection of both pulmonary, pleural and miliary tuberculosis as well as tubercular lymphadenitis (Sada et al., 1990). However, lower rate of sensitivity (50.5%) and comparable specificity (78.3%) for the diagnosis of pulmonary tuberculosis patients were reported by Tessema et al. (2002). In addition, MycoDot, (Genelabs, Switzerland), a commercially available kit for the detection of antibodies against MTB specific LAM was also evaluated in many reports (Steingart et al., 2007; Verma & Jain, 2007). Although the specificities were high (84-100%), low rate of sensitivities (16-56%) were obtained and low sensitivities were mostly related to HIV/TB co-infection (Verma & Jain, 2007)

**DAT, TAT and SL-I:** Assessment of antibody responses using DAT, TAT, and SL-I antigens in ELISA for serodiagnosis of tuberculosis revealed variable results in terms of diagnostic efficacy. Widely variable ranges of sensitivity of 11 to 88% by DAT antigen were reported in several studies. A similar rate of sensitivity by MTB (44.5%) and *M. fortuitum* (48.6%) infection were also demonstrated. In addition, the test sensitivities of TAT antigen also vary from 51-93% (Julian et al., 2002). Julian et. al. reported the best performance of IgG (Sensitivity: 81% and specificity: 77.6%) and IgA (sensitivity: 66% and specificity: 87%) antibodies by SL-I among four trehalose contacting glycolipids (DAT, TAT, SL-I, TDM). Although, several reasons including variation in the ELISA protocol, using of different antigen concentrations and population from different subgroups were described as possible reason of such variability, the effectiveness of these antigens are still uncertain.

**TDM (also known as cord factor):** It composes a major part of the mycobacterial cell wall, was identified as the most immunogenic glycolipid. Clinical evaluation of serodiagnosis of pulmonary tuberculosis using of TDM purified from *Mycobacterium tuberculosis* H37Rv, in ELISA reported its sensitivity of 81% and its specificity of 96% (Mizusawa et. al. 2008). Best performance by TDM (sensitivity 69%, specificity: 91%) among other lipid antigens including DAT, TAT, SL-I for the serodiagnosis of tuberculosis were also reviewed by Steingart et al. (2009). IgG antibody against TDM can also recognize mycolic acid sub-classes and highly active against methoxy-mycolic acid in the cord factor of *M. tuberculosis* than keto-mycolic acid in *M. avium* complex (Pan et al., 1999). By combining TDM with more hydrophobic glycolipids, a new tuberculous glycolipid (TBGL) antigen was designed and a more sensitive serodiagnostic kit for TB, an anti-TBGL IgG test was developed (Kyowa Medex Co, Japan). Anti-TBGL IgG antibody (TBGL IgG) has been proposed as a useful serodiagnostic marker of active pulmonary tuberculosis (PTB) (sensitivity: 84% and specificity: 95% in young adults) in Japan (Maekura et al., 2001). Strong association between TBGL IgG and IgA was also revealed in active pulmonary tuberculosis cases and increased TBGL IgG and IgA was found to be associated with CRP and cavity formation indicating their involvement in the disease pathogenesis (Mizusawa et al., 2008). Elevated titers of TBGL IgG

were found in aged (>40 yrs) healthy control people (17%) in Japan (Maekura et al., 2001) and high titers of both TBGL IgG (46%) and IgA (36%) in healthy adults was also observed in our recent study in Thailand (Siddiqi et. al.; in press). But, elevated titers of TBGL IgG are not related to BCG vaccination (Nabeshima et al., 2005). Very recently, significant association between the TBGL IgG and Quatiferon-TB Gold IT assay responses in diagnosis of latent tuberculosis infection in healthy healthcare workers was also found (Siddiqi et. al., unpublished data) that represents enhancement of humoral immune responses along with T cell mediated immunity in latent tuberculosis infection. Increased synthesis of TBGL IgA titers that was related to serum IgA were observed in HIV carriers with low CD4+ T cells counts (less than 350/ $\mu$ l) compared to high CD4+ T cells counts (more than 350/ $\mu$ l). However, the sensitivities of TBGL IgG and IgA were very low (10% and 8% respectively) although their specificities were more than 90% for the diagnosis of tuberculosis in pediatric cases (Siddiqi et. al., 2009, unpublished data).

#### 4.2 Discussion

The performance of various purified antigens and commercially available kits for the sero-diagnosis of active pulmonary tuberculosis with or without associated HIV/AIDS co-infection were evaluated in many studies and were reviewed extensively (Abebe et al., 2007; Steingart et al., 2006, 2007, 2009; Varma & Jain, 2007). Protein antigens were reported to have high specificity (>95%) than lipid antigens. In relation to use of single antigen, relatively higher sensitivities can be achieved by using multiple antigens. Cord factor (TDM), among the lipid antigens had the best overall performance. In addition, higher rate of sensitivity can be obtained by evaluation of IgG and/or IgA than IgM. Maes and colleagues has been conceptualized the human immune responses against mycobacteria into four different stages based on BCG vaccination and tuberculosis disease and treatment, initiated from innate response followed by intermingled innate and adaptive response against low molecular weight oligopeptidic and nonpeptidic, as muramyl dipeptide and trehalose 6,6 dimycolate and high molecular weight nonpeptidic antigens such as lipoarabinomannan. The final response is directed against protein antigens (Maes et al., 1999). Although it is not clearly understood, enhancement of humoral immune response can be dependent on disease pathogenesis and different stage of infection can influence different subclasses of immunoglobulins. Enhanced IgM expression can usually occur in the early stage of infection that can subsequently be diminished on progression of disease. Therefore, detection of IgM may show limited value in the sero-diagnostic assay. The reason of low sensitivity of IgA antibody is not clear. It is possible that the generation of IgA antibody needs larger amounts of antigens and related with degree of disease pathogenesis than do IgG responses and indicate the heterogeneity of tuberculosis infection.

However, until now, any performance was not successful to show the, stable, consistent and acceptable sero-diagnostic efficacy with a sensitivity of at least more than 85% and a specificity of more than 95% and to replace the traditional sputum microscopy as a reliable diagnostic tool in different groups of tuberculosis patients including HIV-positive, -negative, extra-pulmonary tuberculosis or in pediatric tuberculosis detection. However, extensive study for evaluation of humoral immune responses in different stages of tuberculosis infection and disease and their association with the disease pathogenesis should be consider to clarify the variable antibody responses against different antigens. As

most of the investigations for the determination of sero-diagnostic ability of various antigens were carried out in tuberculosis endemic countries, determination of antibody responses in latent tuberculosis infection could be helpful to some extent for explaining the reason of low specificities and the possibility of influence by tuberculosis endemicity in such countries. The immune response in HIV/AIDS patients co-infected with tuberculosis is more complex than single infection. Antibodies against several single or multiple antigens were detected in HIV/AIDS patients with active tuberculosis and even months to year's prior development of tuberculosis related symptoms in some prospective studies. More investigation with diverse antigens for the sero-diagnosis of subclinical and active tuberculosis particularly sputum-negative and extra-pulmonary tuberculosis especially in those with HIV/AIDS co-infection and in pediatric cases is an urgent necessity.

It is generally believed that, T helper immunity and elaboration of IFN- $\gamma$  offer vital role in protective immunity to clear or containment of the intracellular MTB infection. However, BCG-induced antibodies was shown to potentiate IFN- $\gamma$  production by mycobacterium-specific CD4+ T cells and also can cause enhancement of mycobacterial phagocytosis probably by inducing opsonization by antibodies and that might be related to mucosal immunity (Abebe & Bjune, 2009). Protective role of antibodies against several TB antigens in mice model was also review by Glatman-Freedman (2009). Therefore, antibody-mediated immunity against diverse mycobacterial antigens in synergy with cell mediated immunity can play a vital role in the protection and immunopathogenesis of tuberculosis infection and disease. Frequent detection of antibodies in latent or progressive stages of latent to active tuberculosis and their relation to immune responses especially with mucosal immunity needs to be clarified further.

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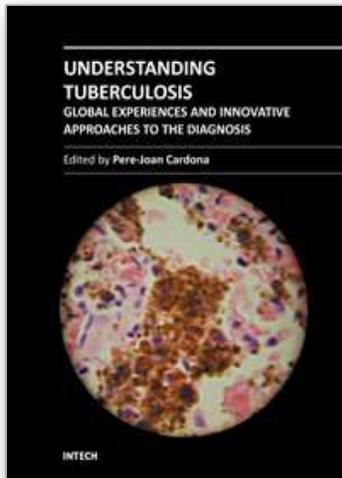
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## **Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis**

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Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

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