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Translational tuberculosis research: immune profile as biomarker of tuberculosis infection

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CHAPTER 1: INTRODUCTION TO TUBERCULOSIS

1.1 Tuberculosis: past and present

Tuberculosis (TB) is one of the ancient and deadliest human communicable disease of world, still representing a huge health, social and economic problem (1). TB is a global pandemic for many reason: the lack of an effective vaccine, the long and expensive therapy and the higher incidence especially in low and middle income countries. Although TB is one of most studied infectious disease, since the study of R. Koch more than 100 years ago, we have not still completely understood the mechanisms of TB pathogenesis and which factors can protect from developing the disease. The TB disease has been endemic in Europe throughout the Middle Ages and during the 18th and 19th centuries a list of cofactors such as lack of treatment, overcrowding and poor hygiene increased the spread of the infection and the mortality (2). The first attempts to treat the disease were based on recovery of patients in sanatoria and alleviation of symptoms by pneumothorax without knowing the real etiology of the disease (2). In the 1882 Robert Koch finally identified the *Mycobacterium tuberculosis* (Mtb) as the bacteria responsible of TB (3). In the following years the development of the vaccine based on the immunity against the Bacillus Calmette-Guérin (BCG) (1921) (4), the use of antibiotics to fight the infection, streptomycin in 1943 and isoniazid and the rifamycins later (2), deeply improved the protection against the disease. However, still today TB remains a major cause of morbidity and mortality.

The specie of *Mycobacterium tuberculosis* complex (MTBC) are the etiologic agents of TB. MTBC includes: *Mycobacterium tuberculosis,* determining TB in humans; *M. africanum*, (human TB only in certain regions of Africa); *M. bovis*, *M. caprae* and *M. pinnipedii*, (TB in wild and domesticated mammals); *M. microti*, (TB only in voles). *Mycobacterium tuberculosis* emerged as a human pathogen in Africa 75-150 thousand years ago and spread by clonal expansion among human communities, following the migration flux, since then, giving rise to seven phylogeographic lineages with limited genetic variability, consisting of large sequence polymorphisms (LSPs) and single nucleotide polymorphisms (SNPs) (5-7). The ancient Mtb strains evolved to persist in low density populations, causing disease reactivation following long period of latent infection (8). The animal domestication permitted the transmission of tuberculosis from human to animal and *M. bovis* became the agent of TB in domestic and wild animals (7). Civilization and consequently higher density of human population have contributed to select modern Mtb strains with increased virulence and transmissibility (5,9). The present pandemics in sub-saharan Africa, South-East Asia and Eastern Europe are caused by the modern lineages 2, 3 and 4, whereas the ancient lineage 1 are responsible of the TB cases mainly in the Indo-Oceanic regions and the Philippines (10).

In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children. People

living with HIV accounted for 1.2 million (11%) of all new TB cases. Six countries accounted for 60% of the new cases: India, Indonesia, China, Nigeria, Pakistan and South Africa. Worldwide, the rate of decline in TB incidence remained at only 1.5% from 2014 to 2015. In 2015, there were an estimated 480 000 new cases of multidrug-resistant TB (MDR-TB) and an additional 100 000 people with rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment. India, China and the Russian Federation accounted for 45% of the combined total of 580 000 cases. There were an estimated 1.4 million TB deaths in 2015, and an additional 0.4 million deaths resulting from TB disease among people living with HIV. Although the number of TB deaths fell by 22% between 2000 and 2015, TB remained one of the top 10 causes of death worldwide in 2015 (11). Considering that with an early diagnosis and an appropriate therapy, TB disease can be treated, the number of deaths is unacceptably high. Moreover, it is estimated that a quarter of the global population is latently (asymptomatically) infected with Mtb (12), and approximately 3 to 10% of these infected individuals could develop active TB disease during their life (13-15). For all these reasons, all efforts to fight TB must be intensified.

1.2 Mycobcaterium tuberculosis

Mtb is an obligate intracellular pathogen able to infect several animal species, although human population represents the main hosts. It has the following microbiological characteristics: aerobic, acid-fast, non-motile, non-encapsulated and non-spore forming bacillus, it needs 15-20 h to complete a replication cycle. It grows most successfully in high oxygen environment , such as in the lung tissue. Mtb is neither gram positive nor gram negative, it is described as acid-fast because after the staining it resists to the acidified organic solvents used for the decolourisation process (3).

Mycobacteria possess an external membrane consisting of an asymmetric lipid bilayer made of long fatty acids in the inner leaflet (mycolic acids) and of glycolipids and waxy components on the outer layer.

The outer and inner membrane form a periplasmic space, with the presence of a thin layer of peptidoglycan in the innermost side covalently linked to arabinogalactan and lipoarabinomannan which in turn are bound to mycolic acids. The targeting of mycolic acids and arabinogalactan synthesis is at the base of two of the most successful anti-TB drugs isoniazid and ethambutol (16).

The main virulence factors of Mb are the protein secretion systems (ESX1-5) (17). ESX1 is necessary to translocate the bacteria from the phagosome into the cytosol of macrophages (18-20) and it secretes two small and highly immunogenic proteins, ESAT-6 and CFP-10, used in the interferon-gamma release assays (IGRAs) for the immunological diagnosis of Mtb infection (21). ESX3 is implicated in the acquisition of iron and zinc whereas ESX5, found only in MTBC, *M. marinum* and *M. ulcerans,* interacts with host immune system (16).

The dormancy survival regulon (Dos), controls expression of more than 50 genes responsible for the Mtb response to hypoxic conditions and for the survival of the bacteria in the host (22,23). The Mtb resides in macrophages and ganuloma characterized by low oxygen and nutrient depletion, in response to this particular milieu the Mtb induce a dormant state of the bacilli, inhibiting the replication, down-regulating metabolism and activating an anaerobic metabolism (24).

1.3 Pathogenesis

TB is a communicable disease, transmitted exclusively when few tubercle bacilli dispersed in the air from a patient with active pulmonary TB, reach the alveoli of the host. The risk of infection depends on the bacillary load present in the cough aerosol of the index case, the duration and intensity of exposure, Mtb strain-specific virulence factors and the immune status of the contact (25). TB is characterized pathologically by necrotizing granulomatous inflammation usually in the lung, although almost any extra-pulmonary site can be involved. Upon inhalation, the droplet nuclei travel through the respiratory tract, escaping the mucosal barriers in the bronchi, and eventually end up in the terminal alveoli where macrophages, dendritic cells (DCs) and neutrophils engulf the bacteria (26-28). During the early stages of infection, if the bacilli evade the initial host innate immune mechanisms, it starts actively replicating in macrophages, diffuse to nearby cells including epithelial and endothelial cells, causing a high bacterial burden with few weeks of replication (29). Infected DCs and macrophages also travel to the local lymph node where T-cells are primed and clonally expand in response to Mtb antigens. These antigen specific T cells then migrate back to the site of infection in 2-6 weeks after initial infection (30). After the activation of adaptive immune response, the migration to the site of primary infection of neutrophils, lymphocytes and monocytes create a typical and organized cellular infiltrate called granuloma (31,32). Fibrotic components around the granuloma leads to its calcification, therefore the bacilli remain isolated inside it and at the same time protected by the host immune response.
Although tuberculosis predominantly affects the lung, it can cause disease in any organ and must be included within the differential diagnosis of a vast range of clinical presentations. Symptoms and signs include those associated with the specific disease site as well as non-specific constitutional symptoms such as fever, weight loss, and night sweats (3). However, in the early stages of disease, symptoms might be absent as shown by community-based active case finding studies in Asia in which about one in four culture-confirmed cases of pulmonary tuberculosis were reported to be asymptomatic (3).
In the majority (~95%) of infected individuals, pathogenesis is characterized by a period of asymptomatic subclinical infection, defined broadly as latent tuberculosis infection (LTBI), in this state the bacilli are effectively contained within the granuloma (33,34). In this primary lesion, Mtb persists in a dormant, non-metabolically active state, for years, decades, or lifetime. LTBI, defined as a state of persistent immune

response to prior-acquired Mycobacterium tuberculosis antigens without evidence of clinically manifested active TB, affects about a quarter of the global population (12). Approximately 5-10% of people with LTBI will develop active TB disease in their lifetime, with the majority developing it within the first five years after initial infection (35). It could be detected either by the tuberculin skin test (TST) or by interferon (IFN)-γ release assay (IGRA) without signs of clinically TB (14). Based on this definition, subjects with LTBI carry a risk to develop the active TB disease. However, the majority of LTBI individuals will not develop TB, probably because their immune system persistently inhibits the mycobacterial replication or because the bacilli has been totally eradicated. Until recently, LTBI was thought to represent a uniform state, however, it has become clear that LTBI has to be considered a broad spectrum of infection states that differ by the degree of the pathogen replication, host resistance and inflammation (36). In vivo studies on non-human primate have demonstrated that during latent infection, Mtb is metabolically active and replicates in host tissues even though the absence of any clinical signs of TB disease (37). To note that a single monkey with active TB shows different type of lesions: liquefied cavities with high bacterial load, necrotic or caseous hypoxic lesions with variable number of bacilli and sterilized lesions (36). Similarly, TB disease presentation is diverse and heterogeneous. This heterogeneity includes the type of pathology developed in the lungs (pulmonary TB) or outside the lungs (extrapulmonary TB), the spread of the lesions, the characteristics of immune activation and inflammation, Mtb replication and bacterial load (37), the capacity of the TB lesions to differently respond to chemotherapy (36). An increasing body of evidence supports the hypothesis that the genetic background of the host influences TB disease manifestations and the success of the bacterium in infecting large numbers of susceptible individuals (38). The active TB patients represent the primary source of disease transmission. The concept of TB spectrum emphasize the risk that a latent infection condition, controlled by the host immune response with no clinical signs or symptoms, can reactivate if the balance between host and Mtb is interrupted. In fact, the risk of Mtb reactivation is significantly increased in LTBI individuals with a concomitant immune suppression, due to HIV co-infection or therapy with tumour necrosis factor (TNF)-α inhibitors, or other immune regulators used for inflammatory diseases and transplantation or compromised immunity due to non communicable diseases, such as type 2 diabetes (30,39,40). Therefore, it is very important to develop effective diagnostic tools to detect latent infection in these high risk individuals and very sensitive assays to identify new TB cases.

Animal models such as mice, guinea pigs, rabbits, macaques, and zebrafish have been used to study the pathogenesis and treatment of latent tuberculosis. Common limit to all models is the absence of pathological, clinical, and therapeutic consistency with human infection and disease. Therefore, different models could be used to study particular aspect similar to the human infection (35).

In conclusion, Mtb infection can result in a spectrum of disease phenotypes characterized by different immunodiagnostic, clinical and radiological profiles. Host immunity certainly plays an important role in the

host-pathogen interaction to determine the outcome of infection but how the immune response controls the infection or contribute to the development of active TB remains poorly understood.

1.4 Diagnosis of active tuberculosis

Accurate and early diagnosis is essential to permit TB control. The detection of Mtb in sputum, correlates with the presence of necrotic infection lesion in proximity to the airways. Although the microscopy is largely available and highly specific, it shows low sensitivity, missing the diagnosis in over one third of patients (41,42). Mycobacterial culture is the gold standard for TB diagnosis, due to its superior sensitivity, although detection can take weeks resulting in significant delays in treatment initiation (16).

Since many TB patients, such as HIV-coinfected individuals, diabetes patients, and children, do not have a Mtb-positive sputum, these test is useless in these particular populations (13,43,44). By definition, sputum diagnostics are not useful in extra-pulmonary disease. Non-pulmonary forms of TB may be more problematic to diagnose because of the difficulties in identifying the proper specimens (tissue or biological fluids as pleural-, cerebral-, synovial-fluids collected by invasive procedures) and the lower sensitivity of the microbiological assays, probably due to a lower bacterial concentration (14). In conclusions, definitive diagnosis of TB needs the detection of Mtb from the biological sample by at least one of the following techniques: microscopical analysis, culture isolation or molecular methods. The introduction of new, highly sensitive, fully automated molecular assays, is the major success of the TB diagnosis (14). In fact the Gene Xpert MTB/RIF assay, a cartridge based real-time PCR system that can detect Mtb DNA and genotypic rifampicin resistance in ~2 hours (45), reducing the time to diagnosis and treatment initiation.

1.5 Immunological diagnosis of latent tuberculosis infection

With a quarter of the world population infected with Mtb (12), it is important to identify latently infected individuals who may progress to active TB, such as those who are HIV infected. LTBI is defined by the presence of a specific immune response detected by an IFNγ release assay (IGRA) or the tuberculin skin test (TST) in the absence of signs or symptoms of TB and without lung lesions of active TB in xRay images (35). Diagnosis of LTBI is difficult because it is based on the presence of immunological memory to Mtb antigens rather than the direct assessment of mycobacterial load.

IGRA, such as QuantiFERON®-TB Gold Plus (QFT-Plus) (Qiagen, Hilden, Germany) and T-SPOT.TB (Oxford Immunotec, Marlborough, MA, USA) measure *in vitro* IFNγ production by whole blood ELISA or an enzyme-linked immunospot (ELISPOT) assay on peripheral blood mononuclear cells (PBMC), respectively (14,46). Blood cells are stimulated with Mtb-specific antigens (47), which are deleted from the genome of Bacille

Calmette and Guerin (BCG) vaccine (the attenuated strain of *M.bovis*), and are not present in most environmental mycobacteria (48,49). Since the vaccinal strain does not express ESX1 and ESAT-6 and CFP-10, IGRAs can be used to detect Mtb infection even in vaccinated population (14). TST, in use for over 100 years, is based on skin infiltration, through intradermal injection of purified protein derivative (PPD), which is a crude mixture of antigens many of which are shared by Mtb, *M. bovis*, BCG and several species of environmental mycobacteria. The TST elicits a delayed type hypersensitivity immune reaction at the site of injection visible between 48 and 72 hours after administration. When placed correctly, the injection should produce a pale elevation of the skin (a wheal) 6 to 10 mm in diameter (50). However, the test suffers from poor specificity due to previous BCG vaccination and exposure to environmental mycobacteria (14).

A particular benefit of *in vitro* testing is that there is a laboratory test with negative and positive controls, and that one visit suffices. In contrast to the TST, these *in vitro* tests may discriminate true negative responses from energy (14).

However all these tests have limitations as they cannot distinguish between latent infection with viable microorganisms and healed/treated infections; they also poorly predict who will progress to active TB (14).

1.6 Therapy

To cure TB and reduce disease transmission, patients should be placed on effective treatment soon after diagnosis. TB disease can be treated by taking several drugs for 6 to 9 months (50). There are 10 drugs currently approved by the U.S. Food and Drug Administration (FDA) for treating TB. Of the approved drugs, the first-line anti-TB agents that form the core of treatment regimens are: isoniazid (INH), rifampin (RIF),ethambutol (EMB) and pyrazinamide (PZA). Drug-resistant TB can occur when the drugs used to treat TB are misused or mismanaged. Examples of misuse or mismanagement include the following situations: people do not complete a full course of TB treatment; health care providers prescribe the wrong treatment (the wrong dose or length of time), drugs for proper treatment are not available, drugs are of poor quality. Drug-resistant TB is more common in people who: do not take their TB drugs regularly; do not take all of their TB drugs; develop TB disease again, after being treated for TB disease in the past; come from areas of the world where drug-resistant TB is common; have spent time with someone known to have drug-resistant TB disease (50). Multidrug-resistant TB (MDR TB) is caused by TB bacteria that is resistant to at least isoniazid and rifampin, the two most potent TB drugs. Extensively drug-resistant TB (XDR TB) is a rare type of MDR TB that is resistant to isoniazid and rifampin, plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). Because XDR TB is resistant to the most potent TB drugs, patients are left with treatment options that are much less effective (50).

According WHO, LTBI (1) can be effectively treated in order to prevent progression to active TB, thus resulting in a substantial benefit for both the individual and the community. Currently, available treatments for subjects with LTBI have an efficacy ranging from 60% to 90%. Systematic testing and treatment of LTBI

in at-risk populations is a critical component of WHO's eight-point framework adapted from the End TB Strategy to target pre-elimination and, ultimately, elimination in low incidence countries. The risk of progression to active disease is considerably higher in infected individuals who belong to specific high risk populations. Major risk factors for TB activation include:

- HIV infection
- recent contact with an infectious patient
- initiation of an anti-tumour necrosis factor (TNF) treatment
- receiving dialysis
- receiving an organ or hematologic transplantation
- silicosis
- being in prison
- being an immigrant from high TB burden countries
- being a homeless person
- being an illicit drug user

The following regimens are recommended by WHO for the treatment of LTBI:

- 6-month or 9-month isoniazid daily
- 3-month rifapentine plus isoniazid weekly
- 3- or 4-month isoniazid plus rifampicin daily
- 3– or 4-month rifampicin alone daily

1.7 The immune response to Mycobacterium tuberculosis infection

Inhalation of a small number of *M. tuberculosis* organisms carrying by droplet nuclei from an individuals with active TB culminate with the phagocytosis by alveolar macrophages in the alveolar space (51).
The first interaction between Mtb and the host is with the innate immune system and seems to be mediated by pattern recognition receptors. Recognition by macrophages and dendritic cells of Mtb through mannosylated lipoarabinomannan, trehalose dimycolate, and N-glycolyl muramyl dipeptide triggers innate responses and might be important in establishing subsequent host–pathogen interactions (52-54).
Following infection, different phagocytic cells, such as alveolar macrophages, neutrophils, and dendritic cells (DCs), arrive to the infected site and start to build the organize structure of granuloma (55,56).
It has been hypothesized that the different host- pathogen interactions in the early step of the infection determine a spectrum of granulomas with various capacities to contain and kill the bacteria. Consequently, the clinical outcome is strongly influenced by the characteristics of the granuloma (36,37).

Within granulomas, Mtb might hides itself from immune-based killing mechanisms and drugs, potentially promoting the emergence of drug-resistant strains. The macrophage might also have two mutually contradictory roles: activated macrophages are capable of killing or controlling the growth of Mtb, and they also provide the primary growth niche for this intracellular organism (33,34). The destiny of infected macrophages is to protect the host from Mtb by regulating innate and adaptive immunity. Virulent strains of Mtb inhibit apoptosis and trigger macrophage necrosis, thereby evading innate immunity and delaying the start of adaptive immune responses (57).

Recently, several gene expression studies have focused the attention on the neutrophil activity during the Mtb infection. It has been demonstrated the presence of a IFN-β signature dependent by neutrophil in patients with active TB disease (58,59). Neutrophils were the main cells infected with Mtb in samples of human BAL fluid, sputum, and pulmonary cavities from patients with active TB disease (26). Neutrophil recruitment plays a causal role in disease progression, with potentially both protective and destructive properties, that is likely dependent on their timing and magnitude of response (37).

Three to eight weeks after initial infection with Mtb, cell-mediated immunity develops. The underlying immunology of protection against TB is not fully understood and certainly involves both innate and adaptive responses; however, T helper 1 (Th1) cell-mediated immune responses appear to play a major role (31,32,60). The initial infection is dominated by a Th1-type immune response; however, if the infection is not contained, a gradual shift toward T helper 2 responses occurs (61). Th1 cells are activated and proliferate in response to interleukin-12 (IL-12) produced by Mtb-infected macrophages, as well as other cytokines released by antigen-presenting cells such as IL-18 and IL-23 (62). Activated Th1 cells are the major source of IFNγ, which is crucial for macrophage activation and containment of the infection. At this stage, elimination of Mtb before establishment of adaptive immunity is possible; however, Mtb has evolved immune evasion mechanisms that allow the bacteria to survive in the host for prolonged periods (32,55).

Despite the importance of IFN-γ, this cytokine alone is not sufficient for protection. Tumor necrosis factor α (TNFα), which induces chemokine secretion from macrophages, is equally important in controlling primary infection by supporting the formation of granulomas. Individuals treated with TNFα receptor antagonists for rheumatologic diseases frequently suffer from reactivation TB, highlighting the importance of this cytokine in containment of mycobacteria (63). Although TNF-α is required for protection, high IL-4 levels together with TNF-α appear to promote immunopathology (63). Similarly, IFN-γ and IL-2Rα are present at higher levels in bronchoalveolar lavages (BALs) from TB patients with severe disease than in those from moderate TB disease cases (64).

Other T-cell subsets playing an important role during Mtb infection, are Th17 cells, which facilitate the recruitment of Th1 cells to the site of disease (65) and regulatory T cells (Tregs), which control excessive immunopathology (66,67).

Our understanding of immune responses against TB, however, remains incomplete, because, for example, the importance of B cells is not well-defined, despite their presence in granulomas (43). Apart from B and T cells, other cell subsets also play a role during Mtb infection including myeloid-derived cells. Myeloid-derived suppressor cells (MDSCs) suppress T-cell function in cancer biology (68) and infectious diseases (69), including TB (70). MDSCs are present in higher frequencies and suppress T-cell activation and trafficking in TB patients and in individuals recently infected with *Mtb* (70).

In summary, there are a variety of cells, cytokines, and molecules present in airways that can modulate the initial response of the host to Mtb infection. These factors may prevent infection completely, limit initial establishment of granulomas, modulate the local environment of newly emerging granulomas, or increase the induction of T cell responses against Mtb. Changes in these factors could increase susceptibility to initial infection as well.

CHAPTER 2: TUBERCULOSIS BIOMARKERS: FROM DIAGNOSIS TO PROTECTION

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Tuberculosis Biomarkers: From Diagnosis to Protection

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2.1 Abstract

New approaches to control tuberculosis (TB) worldwide are needed. In particular, new tools for diagnosis and new biomarkers are required to evaluate both pathogen and host key elements of the response to infection. Non-sputum based diagnostic tests, biomarkers predictive of adequate responsiveness to treatment, and biomarkers of risk of developing active TB disease are major goals. Here, we review the current state of the field. Although reports on new candidate biomarkers are numerous, validation and independent confirmation are rare. Efforts are needed to reduce the gap between the exploratory up-stream identification of candidate biomarkers, and the validation of biomarkers against clear clinical endpoints in different populations. This will need a major commitment from both scientists and funding bodies.

2.2 Introduction

Tuberculosis (TB) is a communicable infectious disease, spread almost exclusively by coughed aerosols carrying pathogens from the Mycobacterium tuberculosis (Mtb) complex. TB is characterized pathologically by necrotizing granulomatous inflammation usually in the lung, although almost any extra-pulmonary site can be involved. TB remains one of the most significant infectious causes of mortality and morbidity worldwide. As reported by the World Health Organization (WHO) it causes disease among 9.6 million people each year and ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death

worldwide (1). In 2014, 1.5 million TB deaths were reported and among them approximately 140,000 were children. The number of TB deaths is unacceptably high because with a well-timed diagnosis and appropriate treatment, almost all people with TB can be cured. Therefore all efforts to fight TB must be intensified. Additionally, it is estimated that one-third of the world's population is latently (asymptomatically) infected with Mtb, and approximately 3 to 10% of these infected individuals are likely to progress to active disease during their life. The risk of reactivation and subsequent disease and mortality is significantly increased in individuals with HIV coinfection (2,3) and therapy with TNFa inhibitors (4,5). Approaches to decrease TB morbidity and mortality, along with Mtb transmission, rely on effective treatment, correct diagnosis, and prevention of infection and disease.

Effective therapy is central to any strategy for controlling TB and biomarkers that indicate initiation of successful treatment could facilitate development of alternative treatment strategies. There is a need for shorter treatment regimens to increase compliance. Unfortunately, recent studies have not shown definitive results (6-8). In fact, in the REMoxTB Clinical Trials experience, despite early effectiveness (superior early bactericidal activity and 2-month culture conversion rates in patients treated with moxifloxacin compared to the standard regimen), the 4-month-regimen was less effective than the standard 6-month regimen in preventing TB recurrence (6,8). Moreover, correct and efficacious treatment is also needed to avoid multidrug-resistant (MDR)-TB. Globally, an estimated 3.3% of new TB cases and 20% of previously treated cases had MDR-TB in 2014 (1). This translates into an estimated 480,000 people having developed MDR-TB in 2014 with a treatment success rate of only 48%. (1). Patients with MDR-TB urgently require treatments that quickly eradicate active infection while preventing emergence of additional resistance, which otherwise causes treatment failure and death.

Accurate diagnosis is also a cornerstone of TB control. Active TB diagnosis is based on the detection of Mtb in sputum, which depends on the presence of necrotic infection foci in proximity to the airways. The diagnosis then is based on sputum smear and culture, (9-12) and more recently positive GeneXpert MTB/RIF tests (13). Microscopy is largely available and highly specific, but lacks sensitivity, missing the diagnosis in over one third of patients seeking care (9,14). Mycobacterial culture remains the gold standard for TB diagnosis, but provides results only after considerable delay (3-4 weeks). All these diagnostic tests require a Mtb-positive sputum while many active TB patients, including HIV-coinfected individuals, diabetes patients, and children, often do not present with Mtb positive sputum (15,16). In pulmonary TB a positive microbiological diagnosis inevitably means the presence of Mtb in the airway secretions, such that in all likelihood Mtb has been already transmitted to others. By definition, sputum diagnostics are not useful in extra-pulmonary disease, the diagnosis of which relies on samples (tissue or biological fluids as pleural-, cerebral-, synovial-fluids) collected by invasive procedures. For all of these reasons, there is need for the development of highly sensitive and specific diagnostic tests for TB to rapidly identify - or rule out - the presence of active disease. These tests need to perform in endemic settings with limited laboratory

facilities, at low cost, using easily accessible non-sputum based samples such as blood, urine, or breath. These are the four high priority target product profiles (TPPs) recently published by the WHO as a result of a consensus meeting on new TB diagnostics (17). Therefore the urgent need to search for biomarkers (defined as measurable characteristics that indicate normal or pathogenic biological processes, or pharmacological responses to therapeutic intervention) (18) needs to be highlighted: biomarkers can serve as surrogate endpoints in clinical trials, and can be used to improve treatment outcome by informing therapeutic decisions for individual patients (19). Sputum culture conversion using solid medium is the best-characterized TB biomarker for successful treatment, having been examined in many studies either as a simple measure (e.g. month 2 culture status) or in more complex forms requiring subsequent negative cultures (e.g. stable culture conversion). However, as reported above, in the REMoxTB trial patients developed recurrent TB despite negative sputum cultures at month 2. Thus better biomarkers predictive of TB treatment outcome are needed (20,21). This is a priority for the TB research field and has the potential to impact not only research but also clinical practice globally (22-24).

In this paper we will review most of the recent advances in research into TB biomarkers for the diagnosis of active TB, latent TB infection (LTBI) and prevention of TB disease.

2.3 Biomarkers for diagnosing active tuberculosis

We may distinguish biomarkers related to the pathogen and to the host (Figure 1). From the pathogen perspective, Mtb products could be detected directly in blood, sputum or urine. Mtb DNA can be detected in blood and urine of pulmonary TB patients with a better sensitivity than Mtb culture from the same biological fluid (25-27). The Mtb cell wall component lipoarabinomannan (LAM) has been proposed as TB biomarker; however the available commercial test on urine has a poor sensitivity (28). This can be partly enhanced by other LAM assays (29-31). Although unsatisfactory as yet, in HIV-infected patients the Mtb DNA and LAM detection in urine may be an important tool to consider especially for those advanced cases with low CD4 T-cell counts (32-34). The Mtb Ag85 complex is a 30-32 kD family of three proteins (Ag85A, Ag85B, and Ag85C) with enzymatic mycolyl transferase activity involved in the coupling of mycolic acids to the arabinogalactan of the cell wall and in the biogenesis of the cord factor (35,36). The detection of Ag85 in blood and urine, however, shows highly variable performance in different studies (29,37,38).

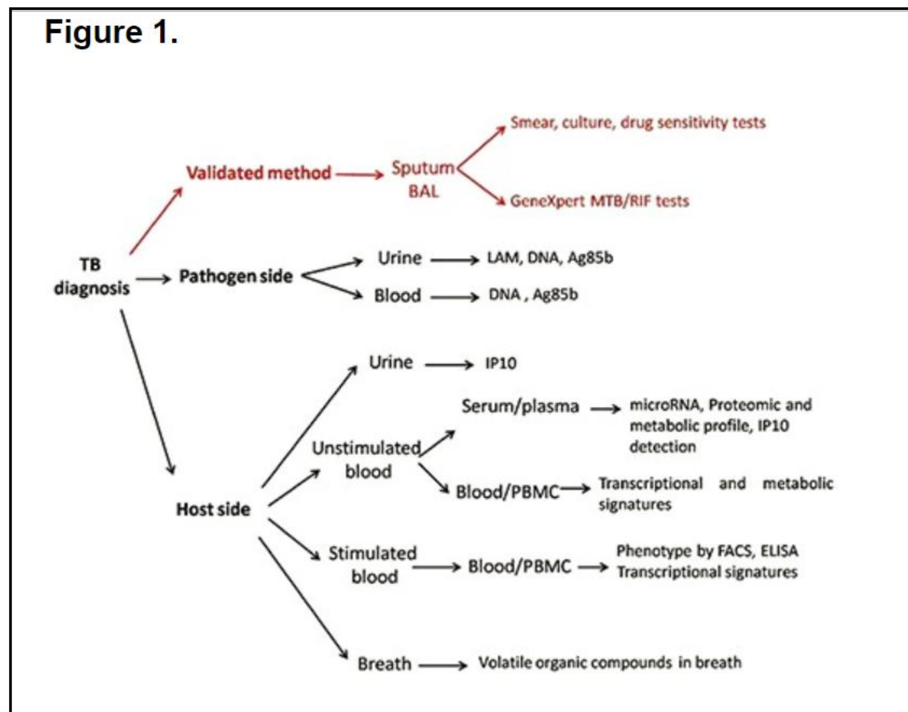

Figure 1 Flow chart of the biomarkers for active tuberculosis diagnosis. TB: tuberculosis; Ag: antigen; LAM: lipoarabinomannan; BAL: broncholavage; IP: Interferon-γ inducible protein; FACS: Fluorescence-activated cell sorting.

Among the host biomarkers, there are various non-sputum based-assays for active TB diagnosis, relying on serum, plasma, urine or stimulated or unstimulated blood. Considering serum or plasma products, Mtb specific antibody detection is not a promising diagnostic approach due to heterogeneity of the response to Mtb (11,39). Moreover WHO negatively advised on the use of such tests for diagnosing active TB disease (40). The evaluation of serum micro-RNAs has shown different levels of accuracy for diagnosing active TB in drug-sensitive and drug resistant TB (41-44).

A broad range of potential transcriptional TB biomarkers has been reported. Modular and pathway analysis revealed that the neutrophil driven interferon (IFN)-inducible gene profile, consisting of both Type 2 (IFNγ) and Type I (IFNαβ) IFN signaling represented a significant TB signature detectable in the peripheral blood from pulmonary TB patients (45). These findings have been also validated in other populations, (21,46-50) and in several studies could differentiate TB from other respiratory infections and inflammatory diseases (24,45,49,51). Moreover it has been shown that disease activity increased the signature whereas treatment decreased it (21,22,49). Integrated analysis of gene expression signatures obtained in eight independent studies revealed additional pathways that are likely to contribute to discrimination of TB disease from other diseases (52). Diagnostic signatures to distinguish TB from other diseases and from LTBI were also found in children from South Africa, Malawi and Kenya (53). However one of the major challenges in the evaluation of new childhood TB diagnostic is the lack of a reference, due to the difficulty of microbiological diagnosis of active disease. Taking all these studies together it is important to mention that the minimum TPP requirements are not yet satisfied in terms of sensitivity and specificity. The complexity of the analysis and

the expensive molecular techniques related to the transcriptional profiles make it currently difficult to be used as routine diagnostic tests unless easier technologies are developed (52). However, all studies reported above are important for our comprehension of TB pathogenesis.

The interferon (IFN)γ inducible protein 10 (IP10) was found to be increased in the unstimulated plasma of children and adults with active TB, (54-58) and has been evaluated by different methodologies including also innovative technologies based on lateral flow assays using the interference-free, fluorescent up converting phosphor (UCP) labels in multicenter studies conducted in Africa (59-63). Interestingly, IP10 can be also detected in the urine of adult patients (64), Ugandan children with active TB (58), and IP10 levels decreased after efficacious therapy (64). In comparison with blood, urine biomarkers offer the advantage of non-invasive sample collection, especially in children, and also pose lower bio safety risks for health care workers.

Flow-cytometry has been proposed as a potential tool to help improving TB diagnosis. Advancement in multiparametric flow cytometry allows the simultaneous evaluation of several immune functions in single cells such as cytokine production and memory status. Polyfunctional T-cells, cells able to produce more than one cytokine simultaneously, have been described as part of immune response to different pathogens such as viruses, bacteria and worms (65-68). Moreover T-cells coproducing IFNγ, TNFα and IL2 have been associated with protective T-cell immune responses in HIV non-progressors subjects (67). Studies evaluating the role of polyfunctional T-cells in TB did not show consistent results. Active TB has been associated with either monofunctional TNFα+CD4+ T-cells (69), or double functional IFNγ+TNFa+ CD4+ T-cells(70-73), or triple functional IFNγ+TNFα+IL2+ CD4+ T-cells (74). By contrast, studies on activation and memory status of Mtb-specific T-cells seem to be more consistent, even when comparing patient populations enrolled at different sites or using different experimental settings. Effector T-cells expand during active Mtb replication, whereas memory cells associate with control and eradication of Mtb infection (71,72,75-78). In particular, it has been shown that active TB is associated with a decrease in CD27 surface expression on circulating Mtb-antigen stimulated CD4+ T-cells (70,77,79-81). Recently, a novel T-cell activation marker-TB (TAM-TB) assay was described for diagnosis of active TB in children (80). The TAM-TB assay has been validated in an adult population in Tanzania and is based on the ratio of the median fluorescence intensity of all CD4+CD27+ T-cells over the median fluorescence intensity of Mtb-specific CD4+CD27+ T-cells (CD27 MFI ratio). This approach has also been tested in an adult population from a low TB endemic country and confirmed discrimination between different stages of TB infection (77).

Another interesting blood-based study showed that the expression of immune activation markers CD38 and HLA-DR and proliferation marker Ki-67 on Mtb-specific CD4+ T-cells associated with Mtb load. The modulation of these markers accurately distinguishes active from LTBI with 100% specificity and over 96% sensitivity. These markers also correctly classified individuals who had successfully completed TB therapy, indicating a correlation with the decrease in mycobacterial load following treatment (82). Interestingly,

recently the T-cell activation has been described also as an immune correlate of risk for TB development in BCG-vaccinated infants (83).

Among the untargeted discovery approaches to identify new markers for TB patients' stratification, the transcriptomic, proteomic, or metabolomic approaches have been used (43,84). In particular Tientchieu et al. evaluated the transcriptomic and metabolic profiles of subjects infected with two different lineages of Mtb, the M. africanum (Maf) and Mtb before and after anti-TB therapy to access the differences in host factors and/or biological processes associated with disease pathology and response to treatment. Peripheral blood gene expression profiles were not different between Maf- and Mtb-infected patients pre-treatment but differed significantly post-treatment, and these were mainly associated with immune responses and metabolic diseases. Notably, the upstream regulator hepatocyte nuclear factor 4-α regulated about 15% of the genes differentially expressed between the groups post-treatment. The serum metabolic profiles were similar between Maf- and Mtb-infected patients both pre- and post-treatment, but significantly different between pre- and post-treatment, particularly in Mtb- than in Maf-infected groups. Using different approaches, as the mass spectrometry or protein chip technology, it is possible to have a proteomic profiling of many peptides when comparing TB patients and healthy subjects. Analysis of sera for host markers showed that transthyretin, C-reactive protein and neopterin might discriminate TB patients from subjects with other infectious and inflammatory conditions with high accuracy (85). Similarly, sputum may also be used to analyze proteomic profiles, as data on smear-negative vs. smear-positive TB patients were significantly different from those found in control subjects (86).

Volatile organic compounds (VOCs) in breath may contain biomarkers of active pulmonary TB derived directly from the infectious organism (e.g. metabolites of Mtb) and/or from the infected host (e.g. products of oxidative stress). A breath test based on the detection and quantification of VOCs identified potential biomarkers of active pulmonary TB with 85% accuracy in symptomatic high-risk subjects (87). However, detection of VOCs is technically difficult because most breath VOCs is excreted in picomolar concentrations (parts per trillion), and most analytical instruments currently used cannot detect VOCs at such low concentrations.

2.4 Biomarkers to monitor tuberculosis therapy efficacy

The absence of satisfactory tools for monitoring TB therapy efficacy impedes optimal clinical management of patients, especially for extra-pulmonary TB where it is not possible to detect Mtb in sputum, (88,89) precluding the possibility to make a link between sputum culture and clinical outcome. The majority of publications investigating treatment response biomarkers failed to articulate the intended use and underlying TPP. Furthermore, most studies compared changes in proposed biomarkers over time during treatment without testing, or being powered to test, the correlation with patient outcome, i.e., relapse-free cure (Figure 2). Study comparisons of Xpert MTB/RIF, smear microscopy and culture using both solid

and liquid media have shown that the Xpert MTB/RIF assay has high sensitivity (97%) but poor specificity (49%) to identify culture positive specimens when Xpert is used as a binary readout. The quantitative measurement from the Xpert MTB/RIF assay, showed that the change in quantitative sputum bacterial load correlated with smear grades, solid culture grades, and time to liquid culture positivity (90). This quantitative data may be used in the future to predict clinical outcome of patients. Considering that the Xpert MTB/RIF test detects DNA from dead as well as live Mtb, a recent study proposed to perform the quenching of DNA detection from dead mycobacteria by adding propidium monoazide (which quenches PCR-mediated detection of DNA from dead mycobacteria) to specifically detect only viable bacilli (91). This test seems promising since a positive correlation with time to positivity of Mtb cultures on liquid media was reported. Concerning the host side, the development of biomarkers able to monitor Mtb load is still far from reality. Recently, Cliff et al. reported that cytotoxic cell gene expression signatures, expressed at diagnosis might predict disease relapse after initial successful cure (sputum conversion), indicating that host factors are important indicators of treatment success (22). The first profiles of a response to TB therapy were reported by Joosten et al (23). These promising data need to be confirmed in large-scale studies. Many efforts have evaluated different proteins in e.g. serum or plasma samples. It has been shown that plasma vascular endothelial growth factor (VEGF) (92) concentrations at 2 weeks of therapy correlated positively with time to sputum conversion. Similarly, hemeoxygenase-1 (HO-1) and matrix metalloproteinases (MMPs) levels correlated with clinical outcome of pulmonary TB, although contrasting findings were reported (93). This is likely due to the inhibition of MMP by CO, a product of Mtb-induced HO-1 activity, observed in vitro after infection with Mtb in human macrophages (94). Other factors as IL11 receptor antagonist, 2-antiplasmin, proteasome activator complex subunit 1, and serum amyloid A predicted sputum conversion with an estimated 80% sensitivity and specificity (95).

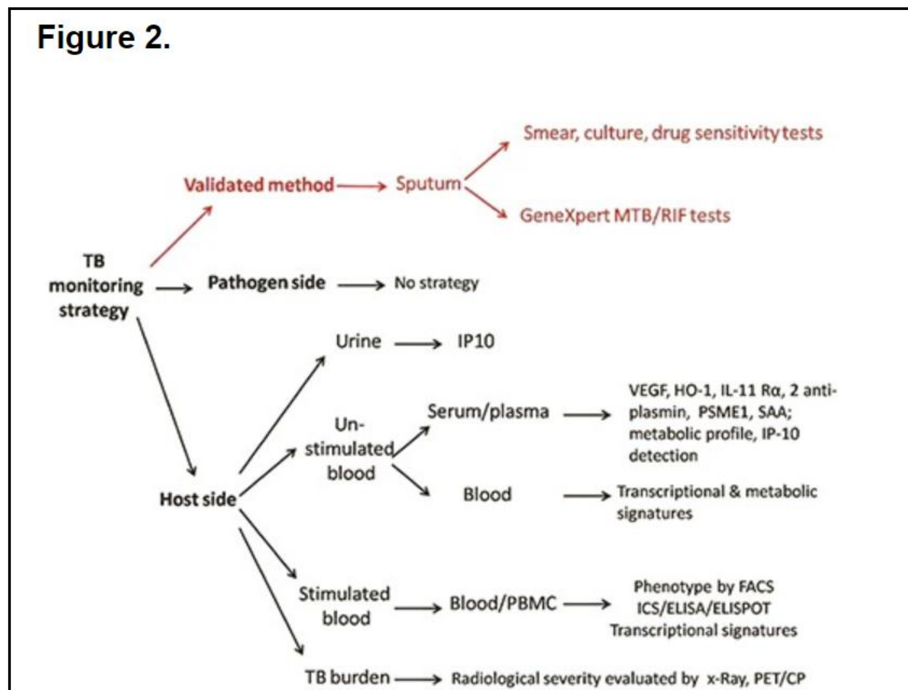

Figure 2: Flow chart of the biomarkers to monitor TB therapy efficacy. TB: tuberculosis; Ag: antigen; LAM: lipoarabinomannan; VEGF: vascular endothelial growth factor; BAL: broncholavage; IP: Interferon-γ inducible protein; FACS: Fluorescence-activated cell sorting; IL: interleukin; PSME1: proteasome activator complex subunit 1; SAA: serum amyloid A; PET/CP: positron emission tomography/computed tomography.

The chemokine IP10 that has increased in the unstimulated plasma of children and adults with active TB has also been measured in dry plasma spots as biomarker for therapy response (54-58,96). In addition, IP10 kinetics in the first week of TB therapy has been proposed as a tool to confirm a clinical diagnosis and guide specific therapy (97).

Radiological severity has been classified with different scores to predict treatment outcome in adults with pulmonary TB using different tools (98,99). High-resolution 3-dimensional imaging helps evaluating the pulmonary TB burden during therapy (100). In the lungs, a quantitative volumetric change in the uptake of 2-deoxy-2-[18F)-fluro-D-glucose (FDG) after 2 and 6 months of TB therapy has been detected by positron emission tomography/computed tomography (PET/CT) quantification and this modulation could be correlated with treatment outcome. However, due to machine equipment, complexity, cost and radiation exposure, the use of PET/CT approach is still restricted to clinical trials. As reported above modulation of CD27 evaluated by flow cytometric studies may be a novel marker not only for active TB diagnosis but also therapy monitoring (70,77,80,81). The same may hold true for the modulation of CD38, HLA-DR and proliferation marker Ki-67(82).

In analogy to cancer, an expansion of myeloid derived suppressor cells (MDSCs), which have a remarkable ability to suppress T-cell responses (101,102), has been observed in the lung and blood of patients with active TB whereas a contraction is reported after efficacious anti-TB therapy (102).

TB therapy significantly decreased the in vitro IFNγ response induced by peptides selected from ESAT-6 and CFP-10 in patients with active TB in studies conducted in Europe (103,104), Uganda (105) and India (106), suggesting that this response can be a tool to monitor anti-TB treatment efficacy. The results have been confirmed using IP10 instead of IFNγ (106,107).

2.5 Biomarkers for latent tuberculosis infection identification

Using a clinically pragmatic approach, LTBI is defined by the presence of a specific immune response detected by an IFNγ release assay (IGRA) or the tuberculin skin test (TST) (Figure 3) in the absence of lung lesions of active TB in xRay images, in individuals from whom it is not possible to isolate Mtb (108), IGRA(QuantiFERON TB Gold in tubes (Qiagen, Venio, the Netherlands; QFT-GIT) and T-SPOT.TB (Oxford Immunotec, Marlborough, MA, USA)) measure in vitro IFNγ production by whole blood ELISA (109) or an enzyme-linked immunospot (ELISPOT) (110) assay on peripheral blood mononuclear cells (PBMC), respectively (109,110). Blood is stimulated with Mtb-specific antigens (111), which are deleted from the genome of M. bovis BCG and are not present in most environmental mycobacteria (112-115). TST is based on skin infiltration caused by intradermal injection of purified protein derivative (PPD), which is a crude mixture of antigens many of which are shared by Mtb, M. bovis, BCG and several species of environmental mycobacteria. A particular benefit of in vitro testing is that there is a laboratory test with negative and positive controls, and that one visit suffices. In contrast to the TST, these in vitro tests may discriminate true negative responses from energy (116). Recently an updated version of the QFT-GIT has been launched (https://www.qiagen.com/it/about-us/press-releases/pressreleaseview?id=%7Bc861949e-df50-475b-8148-b4c70034c49e%7D&lang=en).

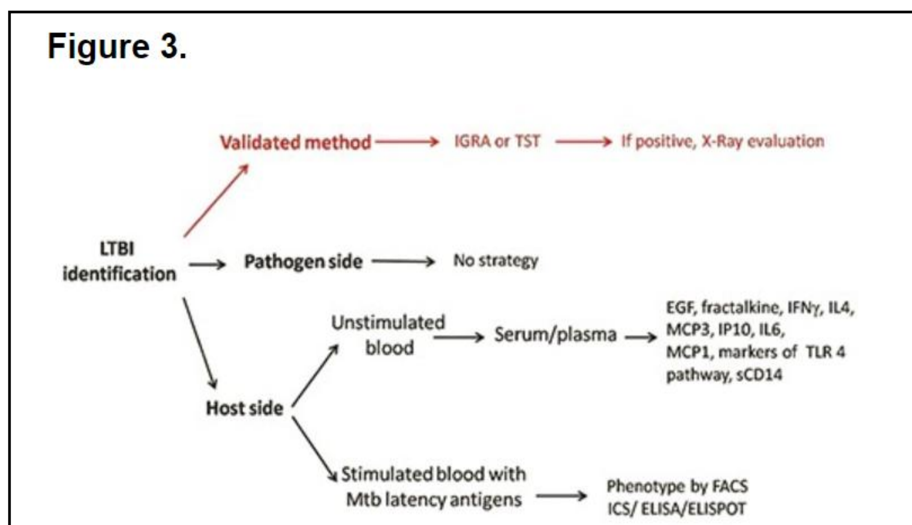

Figure 3: Flow chart of the biomarkers for LTBI identification: LTBI: latent tuberculosis infection; TST: tuberculin skin test; IGRA: IFNγ release assay; Interferon-γ inducible protein; IL: interleukin; PSME1: proteasome activator complex subunit 1; EGF: endothelial growth factor; MCP: monocyte chemoattractant protein; TLR: toll like recepetors; sCD14: soluble CD14; FACS: Fluorescence-activated cell sorting; ICS: intracellular staining.

Results from ongoing studies will show if the test has a better accuracy compared to the old QFT-GIT(117). It should be noted that both TST and IGRAs share limitations: a low accuracy in immune-compromised patients, impossibility to distinguish between LTBI and active disease, which is a major issue in TB endemic areas, and low predicting values for active TB diagnosis (118-119).

Several efforts have been undertaken to distinguish LTBI from active TB, with no clear success probably due to the fact that LTBI is characterized by a high heterogeneity of TB lesions that may depict as a broad spectrum of conditions that overlap in part with those seen in active disease (120). Some subjects show only the remnant of a waning infection, while others show a slowly progressing form of disease, or a chronic non-progressing infection (120).

In the QFT-GIT format the chemokine IP10 has been suggested as an alternative marker for IFNγ (106,121-123) with high accuracy in the HIV-infected patients (122,124,125).

Besides IGRA, several approaches for LTBI identification have been proposed: evaluation of plasma concentrations of epidermal growth factor fractalkine, IFNγ, IL4, monocyte chemoattractant protein (MCP)3, IP10 (126); evaluation of serum pro-inflammatory cytokines IL6, IP10, MCP1 (127); detection of plasma levels of markers involved in the Toll-like receptor 4 pathway, like soluble CD14 and myeloid differentiation-2(128). A recent study on LTBI subjects demonstrated that Mtb-specific CD4+ T-cells have a characteristic chemokine expression signature (CCR6+CXCR3+CCR4-), and that the frequency of these cells is increased in LTBI subjects compared with healthy donors (129). This study suggested a possible role of specific subsets of CD4+ T-cells in the containment of Mtb and raises interesting questions on the possible role of these cells. In particular the transcriptional profile of CCR6+CXCR3+CCR4-CD4+ T cells revealed characteristics important for TB containment, since gene expression profiles correlated with TB susceptibility genes, enhanced T-cell activation, cell survival and cytotoxic response (129). Stimulation with the so called Mtb latency antigens, such as Rv1733c, Rv2029c, Rv2628 and HBHA (130-136) seem promising tools to identify LTBI subjects and distinguish recent LTBI from remote LTBI (130,137,138). If confirmed in larger studies, these results may have important implications for risk stratification when deciding to initiate preventive therarpy (130,138,139).

2.6 Biomarkers for prevention: vaccine studies

As discussed above, among the T-cell based biomarkers, polyfunctional T-cells have been explored as potential biomarkers by multiparametric flow technology. In animal models, i.e. mice, vaccine-induced

protection against Mtb infection strongly correlated with a high frequency of polyfunctional CD4+ T-cells (140,141). However the correlation of this polyfunctional cytokine profile with protective efficacy of BCG vaccination was absent in humans, as reported in a cohort of BCG-immunized infants monitored for 2 years (142). Similar results were obtained in a TB vaccine study based on MVA85A (modified vaccinia virus Ankara expressing antigen 85A) (143). In addition, polyfunctional T-cells have been reported at increased frequencies in active TB (74). These studies suggest that polyfunctional T-cells play a role in vaccine induced protection against TB in animal models, but do not represent a correlate of BCG-induced or natural protection in humans, as they are also present in active TB (74,144) Th17 cells are capable of providing protection in immunization and cellular transfer mouse models (145-147). Th17 cells are long lived and can become memory cells, despite expressing markers characteristic of terminally differentiated cells (148) and have self-renewal capacities (149). Th17 cells preserve the molecular signature that is characteristic of T stem cell memory (TSCM)(150-152). IL17 seems to play an important role in Mtb protection. It has been shown that mice lacking IL17A receptor, despite being able to control acute infection, are unable to stably maintain long-term control of Mtb infection (153). This is due to decreased early neutrophil recruitment, more than IFNg deficiency. Recently it has been shown that the requirement for IL17 in host protection against Mtb in the mouse model is Mtb strain dependent. IL17 was dispensable for protective immunity against the lab-adapted strain H37Rv while necessary for protection against Mtb HN878, a hypervirulent Mtb strain (154). IL17 is important in vaccine-mediated protection in TB. Following BCG and ESAT-6 peptide immunization (155,156), antigen-specific Th17 cells localized in the lungs and were critical for the recruitment of Th1 cells to the lung after Mtb challenge. Innate immune responses are conventionally thought to provide immediate protection before the adaptive immune response is generated, thus contributing towards early containment of the pathogen. However, a growing number of studies suggests their involvement in the recall response and protection during secondary challenge, as shown by the generation and long-term maintenance of NK cells in response to viral infections such as those with cytomegalovirus (CMV) and hepatitis C virus (HCV) (157).There are studies ongoing to evaluate the role of NK memory cells in Mtb protection. γδT-cells recognize a variety of unrestricted, unprocessed and small phosphate antigens (158). In the mouse model, during the early phase of infection with Mtb, γδT-cells secreting IFNγ and IL17 with cytotoxic effector functions are recruited to the lungs (159). Expansion of γδT-cells in response to BCG vaccination and their presence in Mtb-specific recall response are also reported in the nonhuman primate macaque model.160 In addition, γδT-cells reduce the viability of intracellular Mtb via mechanisms dependent on perforin or granulysin (161,162). These data, together, indicate not only that γδT-cells are present during Mtb infection and following BCG vaccination but that, in humans, they are capable of restricting Mtb growth. Also many other components of the innate immune system participate in the control of Mtb infection, but this is beyond the scope of this brief review (144).

2.7 Conclusions

There is a pressing need for new biomarkers in TB at all different levels discussed above (144,163). Though studies on new candidate biomarkers are numerous, validation and independent confirmation are rare, unfortunately. Efforts are needed to reduce the gap between the exploratory up-stream identification of candidate biomarkers, the validation of biomarkers against clear clinical endpoints in different populations, and the development of simple point of care tests for use in low resourced settings (20). This needs important commitment from both researchers and economic funders.

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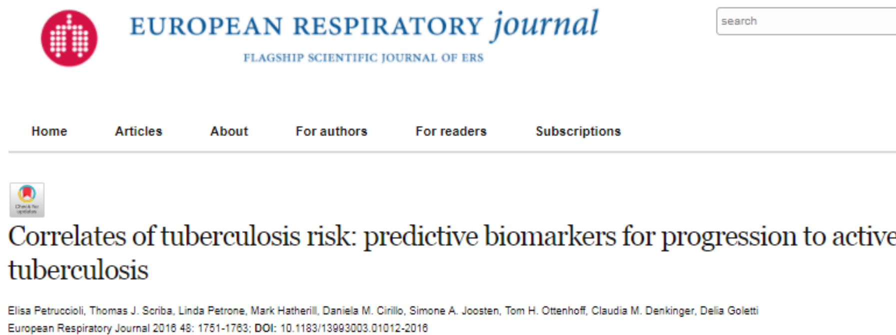
CHAPTER 3: CORRELATES OF TUBERCULOSIS RISK: PREDICTIVE BIOMARKERS FOR PROGRESSION TO ACTIVE TUBERCULOSIS



3.1 Abstract

New approaches to control the spread of tuberculosis (TB) are needed, including tools to predict development of active TB from latent TB infection (LTBI). Recent studies have described potential correlates of risk, in order to inform the development of prognostic tests for TB disease progression. These efforts have included unbiased approaches employing “omics” technologies, as well as more directed, hypothesis-driven approaches assessing a small set or even individual selected markers as candidate correlates of TB risk. Unbiased high-throughput screening of blood RNAseq profiles identified signatures of active TB risk in individuals with LTBI, ≥1 year before diagnosis. A recent infant vaccination study identified enhanced expression of T-cell activation markers as a correlate of risk prior to developing TB; conversely, high levels of Ag85A antibodies and high frequencies of interferon (IFN)-γ specific T-cells were associated with reduced risk of disease. Others have described CD27−IFN-γ+CD4+ T-cells as possibly predictive markers of TB disease. T-cell responses to TB latency antigens, including heparin-binding haemagglutinin and DosR-regulon-encoded antigens have also been correlated with protection.

Further studies are needed to determine whether correlates of risk can be used to prevent active TB through targeted prophylactic treatment, or to allow targeted enrolment into efficacy trials of new TB vaccines and therapeutic drugs.

3.2 Introduction

Tuberculosis (TB) is the most significant infectious cause of mortality and morbidity worldwide. As reported by the World Health Organization (WHO), there are 10.4 million new cases of TB each year(1). TB is a communicable disease, transmitted almost exclusively by cough aerosols carrying pathogens of the Mycobacterium tuberculosis complex. Pathogenesis is characterised by a period of asymptomatic subclinical infection, defined broadly as latent tuberculosis infection (LTBI), which might last for weeks or

decades. From an operational point of view, LTBI may best be defined as a state of persistent immune response to M. tuberculosis antigens detected either by the tuberculin skin test (TST) or by interferon (IFN)-γ release assay (IGRA) without evidence of clinically manifest TB. Based on this definition, individuals with LTBI carry an increased risk of progression to TB. However, an unknown but large proportion of those with LTBI will not develop TB, either because their immune system persistently controls mycobacterial replication or because they are no longer infected with live bacteria. The risk of reactivation and subsequent disease and mortality is significantly increased in M. tuberculosis-infected individuals with immune suppression, due to HIV co-infection(2) or therapy with tumour necrosis factor (TNF)-α inhibitors(3–6), or other immune regulators used for inflammatory diseases and transplantation(7) or compromised immunity due to noncommunicable diseases, such as type 2 diabetes(8, 9). Approaches to decrease TB morbidity and mortality, along with M. tuberculosis transmission, rely on correct diagnosis, effective treatment and prevention of infection and disease. Until recently, LTBI was thought to represent a uniform state(10). However, it has become clear that LTBI has to be considered a broad spectrum of infection states that differ by the degree of the pathogen replication, host resistance and inflammation(10–13). Similarly, TB disease presentation is similarly diverse and heterogeneous. This heterogeneity includes the type of pathology developed in the lungs (pulmonary TB) or outside the lungs (extrapulmonary TB), the spread of the lesions, the characteristics of immune activation and inflammation, M. tuberculosis replication and bacterial load. An increasing body of evidence supports the hypothesis that the genetic background of the host influences TB disease manifestations and the success of the bacterium in infecting large numbers of susceptible individuals(14–17). An important hurdle in the field of TB research is that the bacteria cannot be directly detected in vivo during latent, asymptomatic M. tuberculosis infection. A test that could quantify bacterial numbers or levels of replication during LTBI would advance our ability to define the stages of infection, and allow more detailed studies of pathogenesis and immunity during asymptomatic M. tuberculosis infection. Outcome of M. tuberculosis infection is therefore not a simple two-state distribution represented by either LTBI or active TB, but rather represents a continuous spectrum of states that differ by pathogen and host “activity”, which require different diagnostic and treatment strategies (figures 1 and 2).

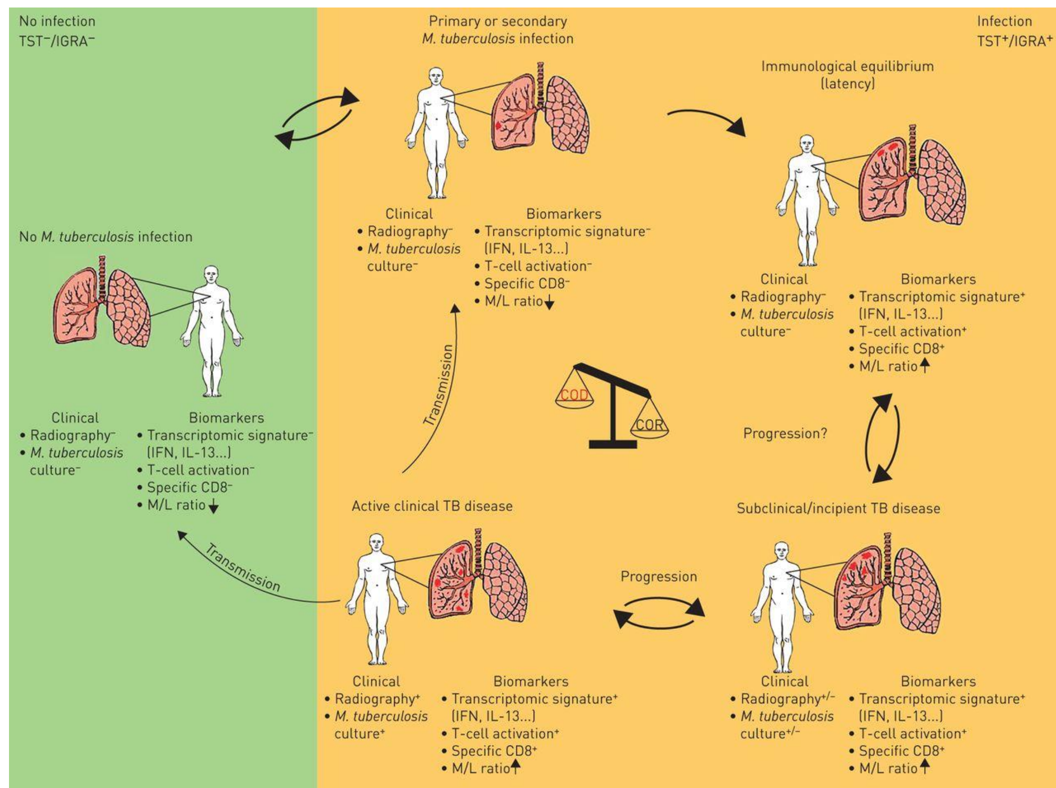


FIGURE 1 Outcome of *Mycobacterium tuberculosis* transmission and establishment of infection or disease based on the correlates of disease and correlates of risk. The outcome of a primary or secondary *M. tuberculosis* infection is not a simple two-state distribution represented by either active tuberculosis (TB) or latent TB infection, but rather represents a continuous spectrum of states that differ by the degree of the pathogen replication, host resistance and inflammatory markers. The identification of *M. tuberculosis* infection is complex, due to the absence of clinical signs, correlates of disease (COD), lung lesions detected by chest radiography or *M. tuberculosis* in the sputum culture. The latency state is characterised by an immunological equilibrium and by presumed control of the bacterial replication. As the infection advances, this balance is lost, resulting in increased bacterial burden and/or increased pathology. This state can be identified as subclinical or incipient TB disease, in which CODs may still be poorly informative. In contrast, correlates of risk (COR) may potentially allow the identification of those at risk, for preventive treatment. Indeed, upregulation of interleukin (IL)-13 and type I and II interferon (IFN)-related gene expression, elevated activation markers on T-cells (*e.g.* expression of D-related human leukocyte antigen and loss of CD27 expression), as well as an elevated monocyte/lymphocyte (M/L) ratio, have been shown to be predictive of TB disease development. The progression of subclinical TB to clinical TB is likely to be associated with a further increase in bacterial burden and/or pathology. Therefore, active TB diagnosis is based on CODs, including chest radiography findings such as lung lesions indicative of disease, detection of *M. tuberculosis* in sputum and positive COR tests. Transmission of *M. tuberculosis* from active TB patients may lead to a primary or secondary *M. tuberculosis* infection. Primary *M. tuberculosis* infection is defined by IFN-γ release assay (IGRA)/tuberculin skin test (TST) conversion and absence of radiological lung lesions and sputum negative for *M. tuberculosis*. ↓: downregulation; ↑: upregulation; +: presence of a modulation based on current knowledge; −: absence of a modulation based on current knowledge.

Disease progression; *M. tuberculosis* transmission; TB treatment

Phase of *M. tuberculosis* infection	Support	Test					
		TST/IGRA	*M. tuberculosis* culture	COR signature (mRNA, 16-gene)	T-cell activation	Ag-specific CD8 T-cells	M/L ratio
Active clinical TB disease	+	+	+	+	+	+	↑
Subclinical TB disease	–	+	+	+	+	?	↑
Incipient TB disease	–	+	–	+	+/–	?	↑
M. tuberculosis infection	–	+	–	–	–	–	↓
Cleared infection	–	+/–	–	–	–	–	↓
No infection	–	–	–	–	–	–	↓

FIGURE 2 Correlates of tuberculosis (TB) disease, infection and risk of disease. Evaluation of tests to detect active TB disease, subclinical TB disease, incipient TB disease, infection and cleared infection. *M. tuberculosis*: *Mycobacterium tuberculosis*; TST: tuberculin skin test; IGRA: interferon-γ release assay; COR: correlates of risk; M/L: monocyte/lymphocyte

From both a clinical and a research point of view, it would be important to correctly identify those M. tuberculosis-infected individuals who are most likely to progress to active disease in order to target them precisely with preventive treatment(18, 19). Prevention of TB disease arising from latent infection is key to achieving WHO elimination targets(18, 20, 21), yet mass preventive therapy based on IGRA/TST screening in TB-endemic countries would need to treat 50–80% of the population, most of them unnecessarily, since 85–95% of latently infected persons will never develop disease in their lifetime(22–24). The positive predictive value of IGRA/TST testing is thus too low, and better prognostic tests are required. The TST and IGRA tests are based on immunological sensitisation to mycobacterial antigens. In the case of the TST this is quantified by the transverse diameter of skin induration resulting from intradermal injection of purified protein derivative, a crude mixture of antigens, many of which are shared by M. tuberculosis, M. bovis, bacille Calmette–Guérin (BCG) and several species of environmental mycobacteria. Blood-based IGRA, including QuantiFERON TB Gold in-tube (QFT-GIT; Qiagen, Hilden, Germany) and T-SPOT.TB (Oxford Immunotec, Abingdon, UK), measure in vitro IFN-γ production upon antigen stimulation of whole blood using ELISA, or peripheral blood mononuclear cells (PBMCs) using enzyme-linked immunospot (ELISPOT) assay, respectively(25). In these assays, specificity for M. tuberculosis is derived from stimulation with peptides spanning the M. tuberculosis antigens ESAT-6, CFP-10 (and TB7.7 for QFT-GIT) that are restricted

to a region of the M. tuberculosis genome deleted from M. bovis BCG and which is not present in most environmental mycobacteria(26–29). A practical benefit of IGRA tests is that they require only a single laboratory test with negative and positive controls, and only one visit. Moreover, the in vitro tests may discriminate true negative responses from anergy(25). An updated version of the QFT-GIT has been launched(25, 30): the QuantiFERON TB Plus includes an additional antigen tube to QFT-GIT, which contains peptides that are intended to specifically induce a CD8+ T-cell response in addition to the CD4+ T-cell response(31) detected with the original QFT-GIT assay(32). The rationale for the inclusion of these new CD8-specific peptides is derived from the growing evidence that M. tuberculosis-specific CD8+ T-cells are more frequently detected in subjects with active TB disease compared to LTBI(33–36), they are associated with a recent exposure to TB(37) and they decline when patients receive anti-TB treatment(38). The first data on performance of QuantiFERON TB Plus in a multicentre European study were reported recently(39, 40). Additional promising tests for LTBI detection should be mentioned. C-Tb is a skin test(41) that measures hypersensitivity to recombinant ESAT-6 and CFP-10 proteins following intradermal administration. The authors claim that it combines the strengths and advantages of TST and IGRA technologies: low cost and for ease of use, like the TST, and high specificity analogous to IGRAs. Another test, based on Rv3615c encoded outside the RD1 region, has shown potential as a new T-cell based immunodiagnostic(42). However, it should be noted that the TST and IGRAs share limitations. They both suffer from low accuracy in immune-compromised patients and cannot distinguish between LTBI and active TB disease(25). The latter is a major issue in TB-endemic areas and leads to poor predictive value for the development of TB in persons with LTBI(25, 43, 44). These limitations provide a compelling reason why a simple measure of IFN-γ response does not allow correct evaluation of risk of progression from latency to active disease. It follows that the discovery of biomarkers that can differentiate between active and quiescent bacterial replication in persons with LTBI, or host markers that identify those with LTBI who are at risk of developing active disease(11, 45, 46) is of great importance. Moreover, it is critical that newly identified markers and signatures are validated in different geographic settings, because human populations with different genetic backgrounds and environmental influences, as well as different circulating M. tuberculosis lineages may be associated with variable rates of progression to TB disease(47). The characteristics of new prognostic tests that predict progression from latent to active TB disease have recently received much attention. In 2015, the WHO convened an expert group to discuss the target product profile (TPP) of such a test under the lead of FIND, based on currently available knowledge. For such a test to have utility in high TB-burden settings, it should ideally be based on a sample type more easily accessible than sputum. While a high positive predictive value (PPV; >95%) for progression from infection to active TB (figures 3 and 4) would be ideal, such a high value is probably unachievable in the short term, and a more realistic goal has to be considered. The performance characteristics presented here and in figures 3 and 4 represent expert opinion (a draft version of the TPP is published on the FIND website:

www.finddx.org/wp-content/uploads/2016/05/TPP-LTBIprogression.pdf) that is undergoing validation in a survey of global stakeholders. A sensitivity and specificity of >90% (and minimally 75% for both) were considered an appropriate target to obtain acceptable positive and negative predictive values.

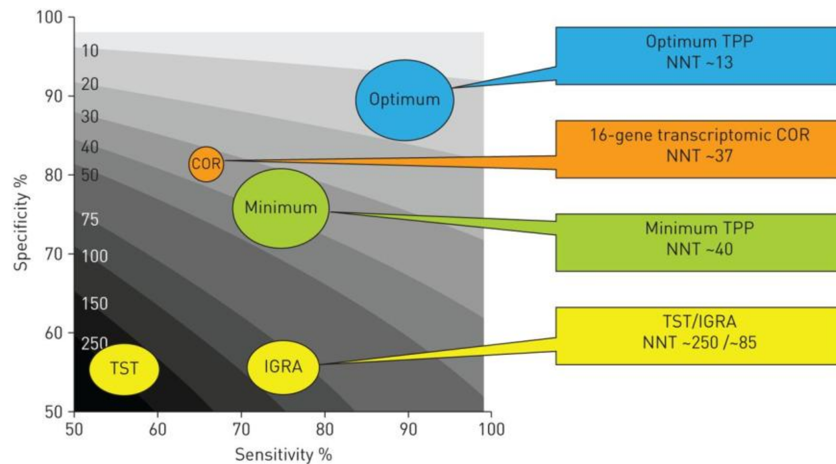


FIGURE 3 Number of patients needed to treat (NNT) to prevent one case of tuberculosis (TB) for the currently available, novel and envisioned diagnostic tests. If we assume a 2-year cumulative TB incidence of 2% and an effectiveness of isoniazid preventive treatment of 50%, for the optimal target the NNT would be 13. Similarly, in the same scenario, for the minimal target the NNT would be 40. If we evaluate the same parameters for the current generations of interferon-γ release assays (IGRA) (based on performance characteristics of IGRA for predicting progression of TB as outlined in World Health Organization latent TB infection guidelines(48, 49)), the NNT is 85. TST: tuberculin skin test; COR: correlate of risk for TB development; TPP: target product profile.

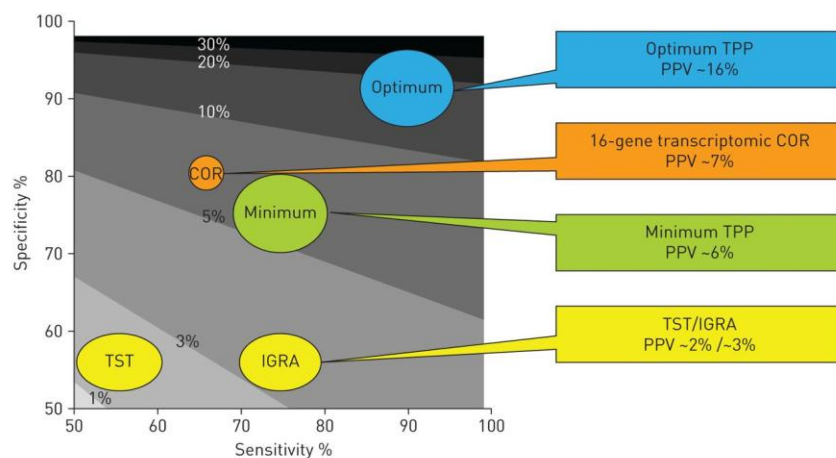


FIGURE 4 Positive predictive value (PPV) to identify cases who have latent tuberculosis (TB) and will develop active TB identified by currently available, novel and envisioned diagnostic tests. If we assume a 2-year cumulative TB incidence of 2% and an effectiveness of isoniazid preventive treatment of 50% the optimal PPV is 16%. Similarly, in the same scenario, for the minimal target the PPV is 6%. If we evaluate the same parameters for the current generations

of interferon-γ release assays (IGRAs) (based on performance characteristics of IGRA for predicting progression of TB as outlined in World Health Organization latent TB infection guidelines(48, 49)), the PPV is 2–3%. In parallel to the development of the target product profile (TPP), a framework for the validation of such tests is being formulated(50). TST: tuberculin skin test; COR: correlates of risk for TB development.

If we assume a 2-year cumulative TB incidence of 2% and an effectiveness of isoniazid preventive treatment of 50% (figures 3 and 4) for the optimal target the number of patients needed to treat (NNT) would be 13 with a PPV of 16%. Similarly, in the same scenario, for the minimal target the NNT would be 40 (figure 3) with a PPV of 6% (figure 4). If we evaluate the same parameters for the current generations of IGRAs (based on performance characteristics of IGRAs for predicting progression of TB as outlined in WHO LTBI guidelines(48, 49), the PPV is 2–3% and NNT is 85). In parallel to the development of the TPP, a framework for the validation of such tests is being formulated(50). Of course it is important to recognise that the performance characteristics of such new prognostic tests (i.e. PPV and NPV) will depend on the underlying prevalence of M. tuberculosis infection, the incidence of TB disease and the risk group. Recent research activities have aimed at developing correlates of risk of TB, which are likely to inform the future development of prognostic tests. These efforts have included unbiased approaches employing “omics” technologies, as well as more directed, hypothesis-driven approaches that assessed either a small set or even individual selected markers as candidate correlates of risk (COR) of TB (table 1). In the following sections we describe some of the most promising approaches.

TABLE 1

Candidate correlates of tuberculosis (TB) risk

	Biomarker	Neonates, children, adolescent or adult population	Location	[Ref.]
Validated correlates of TB risk				
Commercial or traditional tests for LTBI diagnosis	RD1-specific immune response in IGRA, immune sensitisation to PPD in TST	Children and adults	Global	[22, 44, 45]
Molecular tests	mRNA expression signature of 16 IFN response genes	Adolescents	Africa	[52]
Cell activation markers	Increased HLA-DR-expressing CD4⁺ T-cells	Infants	Africa	[63]
Blood cell counts	Elevated monocyte/lymphocyte ratio	Adults	Africa	[111, 114]
Unvalidated correlates of TB risk				
Molecular tests	IL-13 and AIRE mRNA expression signature	Adults	Europe	[58–60]
	Elevated expression signatures of IFN response and T-cell genes	Infants with strong response to BCG vaccination	Africa	[115]
	Elevated expression signatures of inflammation, myeloid and glucose metabolism genes	Infants with weak response to BCG vaccination	Africa	[115]
Antigen-specific T-cells	Increased IFN-γ-expressing Ag85A-specific T-cells	Infants	Africa	[63]
	Increased Th1-cytokine-expressing BCG-specific CD4⁺ T-cells	Infants with strong response to BCG vaccination	Africa	[115]
Cell differentiation markers	Downmodulation of CD27 in CD4⁺ T-cells	Adults	Africa	[76]
Serum/plasma cytokine tests	Increased levels of IP-10	Adults	Africa	[86, 87]
Antigen-specific antibodies	Elevated levels of anti-Ag85A-binding IgG	Infants	Africa	[63]
Responses to latency antigens	IFN-γ response to *in vitro* stimulation of PBMCs using HBHA	Adults	Europe	[95]
	IFN-γ response to *in vitro* stimulation of whole blood using Rv2628	Adults	Europe	[103]
CD8⁺ T-cell response	IFN-γ response to *in vitro* RD1 stimulation of PBMCs	Children and adults	Africa Europe	[37]

LTBI: latent TB infection; RD: region of difference; IGRA: interferon (IFN)-γ release assays; PPD: purified protein derivative; TST: tuberculin skin test; HLA-DR: D-related human leukocyte antigen; IL: interleukin; AIRE: autoimmune regulator; BCG: bacille Calmette–Guérin; Ag: antigen; Th: T-helper cell; IP: IFN-γ-inducible protein; Ig: immunoglobulin; PBMCs: peripheral blood mononuclear cells; HBHA: heparin-binding haemagglutinin.

3.3 Blood transcriptomic correlates of risk

The COR of TB in the most advanced stages of development is a whole-blood transcriptomic mRNA expression signature that was identified by mining RNA-sequencing data in a large prospective cohort of adolescents with LTBI from South Africa(51). The RNA signature was discovered in a set of 46 progressors and 107 controls, and is based on a classifier that computes a TB risk score from relative expression levels of 63 mRNA transcripts from 16 genes in whole blood(52). The 16-gene transcriptomic COR signature predicted progression from infection to TB disease with a sensitivity of 66% and a specificity of 81% in the

12 months preceding diagnosis of incident TB disease in the test cohort. Measurement of the 16-gene transcriptomic COR was transferred from measurement of mRNA expression by RNA sequencing (HiSeq2000; Illumina, San Diego, CA, USA) to a high-throughput, microfluidic, real-time PCR platform to allow cheaper and simpler measurement of gene expression. The PCR-based 16-gene transcriptomic COR signature was validated by blind prediction in two independent cohorts of South African and Gambian progressors and controls from a prospective household TB contact study, GC6–74 (the Biomarkers for TB consortium)(52). In the validation cohorts the sensitivity was 54% and the specificity 83%. Ongoing analyses are under way to explore the biological processes that underlie progression in the adolescent study. However, it is notable that the 16 genes that comprise the COR signature are all regulated by type I and II IFNs, suggesting that chronic peripheral activation of the IFN response, previously shown to be associated with active TB disease at the time of diagnosis(53–56), also precedes the onset of active TB disease.

3.4 Application of correlates to clinical trials and prevention strategies

Further development of this PCR-based 16-gene transcriptomic COR included assessment of diagnostic performance against published microarray data from TB patients and healthy controls. In a set of HIV-uninfected South African adults including 130 prevalent TB cases and 230 controls, combined from four published studies(53–56), the COR discriminated between active TB and healthy controls with 87% sensitivity and 97% specificity. Therefore, although the prognostic 16-gene transcriptomic COR was not originally developed for diagnostic purposes, these performance characteristics suggest the signature has excellent potential as a nonsputum-based triage test to trigger investigation for undiagnosed TB disease. Thus, the 16-gene transcriptomic COR has potential to identify individuals at high risk of undiagnosed TB disease at the time of screening and in addition, if active disease has been excluded, to identify individuals at high risk for progression to incident TB disease within 12–18 months of screening. These performance characteristics would be ideal for a nonsputum-based triage test to identify persons for definitive, sputum-based investigation; and to identify those persons who would benefit maximally from targeted preventive therapy, thus avoiding unnecessary treatment of persons with LTBI who would remain healthy. Prevalence of COR+ status in young HIV-uninfected South Africans is ∼12%, and is not different between IGRA-negative and IGRA-positive persons (Penn-Nicholson A and Scriba TJ, Cape Town, South Africa; personal communication). Given these criteria, screening with the 16-gene transcriptomic COR would allow several-fold reduction in the number of people requiring COR-targeted preventive therapy in high TB burden settings, compared to IGRA-targeted prevention strategies (figures 2 and 3). The ideal COR-targeted preventive therapy regimen would be short, safe, sterilising and able to be rolled out rapidly with high coverage in serial mass campaigns. A clinical trial to test the efficacy of a 3-month short-course, once-weekly, high-dose, directly-observed regimen of isoniazid and rifapentine (3HP) for 16-gene transcriptomic COR-positive persons without active TB disease started in October 2016 (clinicaltrials.gov/NCT02735590).

In parallel, efforts are ongoing to transfer COR testing to a point-of-care device that would allow mass COR screening by local healthcare providers. Translation to a point-of-care test may require a more parsimonious signature and efforts are underway to reduce the 16-gene transcriptomic COR to a smaller set of mRNA transcripts (Scriba TJ et al., Cape Town, South Africa; personal communication). Whole-blood transcriptomic signatures for diagnosis of TB disease comprising either three(57) or four genes(58) have recently been validated, providing proof of concept that very small sets of genes have potential for promising diagnostic utility. If successful, the CORTIS trial will provide proof of concept for the potential of community-wide COR screening campaigns to target curative and preventive therapy for those individuals at highest risk of TB disease, and impact the global epidemic through early diagnosis and interruption of transmission (table 1). Prediction of TB disease months before the onset of clinical symptoms in HIV-infected persons using host biomarker profiles was first demonstrated in a unique longitudinal cohort as part of the Amsterdam cohort studies(59–61). HIV-infected, intravenous drug users had regular blood collections at municipal health services and the archive of these samples allowed selection of PBMCs 6 months to 1 year before the clinical diagnosis of TB. Gene expression profiling using dual-color reverse transcriptase multiplex ligation-dependent probe amplification(62) revealed a predictive signature that discriminated those who did develop TB in the next period from those that remained free of disease for at least 2 years following sample collection(60, 61). Expression of interleukin (IL)-13 in the absence of AIRE (APECED, autoimmune regulator) was predictive for TB disease development within the next year in this high-risk population. Interestingly, when analysing individuals from the IL-13/AIRE group who progressed to TB in the following couple of months, there was also abundant expression of type I IFN-related genes, indicating detection of early disease markers in those individuals. Although this needs to be repeated in larger, longitudinal and independent cohorts, preferably also HIV-uninfected groups, it is an important proof of principle, illustrating the power of predictive TB biomarkers (table 1).

3.5 Immune activation-based COR tests

T-cell activation-based CORs of TB have also been the subject of investigation. A recent study, performed in BCG-vaccinated infants who participated in the recent phase IIb efficacy trial of MVA85A(63), compared a number of immunological outcomes as potential correlates of risk at trial baseline and after administration of the MVA85A boosting vaccine versus placebo. 53 infants who developed active TB disease and 205 controls who remained healthy were evaluated. An intriguing result was that infants who developed TB disease during follow-up had significantly higher levels of activated CD4+ T-cells, expressing D-related human leukocyte antigen at study baseline than infants who remained healthy(64, 65). Importantly, this result was validated independently in the cohort of M. tuberculosis-infected adolescents discussed earlier, in whom elevated CD4+ T-cell activation was also found to correlate with risk of TB(64). Elevated T-cell activation may thus reflect the same immunopathogenesis that underlies the observed upregulated IFN

response in TB progressors from the adolescent study (table 1). Taken together, the infant, adolescent and the GC6-74 household contact studies all imply that persons at risk of developing active TB have evidence of inflammation and/or immune activation. The source of this immune activation is not definitive and requires further study. In adolescents and household contacts, M. tuberculosis replication may be the primary driver of increased inflammation, but this cannot be the cause in the infants, who were all IGRA-negative at sample collection. A notable result in the infant study was that T-cell responses to cytomegalovirus were significantly higher in infants who progressed to TB disease(64). This raises the hypothesis that underlying co-infections may drive immune activation(66, 67), which may be associated with risk of TB. Genetic predisposition to immune activation and other environmental factors may also be involved. Two other candidate COR of TB were identified in the 4–6-month-old infant cohort from the phase IIb MVA85A trial(63). Higher levels of Ag85A-binding immunoglobulin (Ig)G antibodies were observed in controls than in progressors. Ag85A-specific IgG was thus associated with reduced risk of developing TB disease(64). In addition, elevated frequencies of BCG-specific IFN-γ secreting T-cells, measured by ELISPOT assay at trial baseline, were also associated with reduced risk of developing TB disease. The latter result was surprising in light of the previous immune correlates study in BCG-vaccinated infants, which aimed to determine whether frequencies or cytokine co-expression patterns of BCG-specific CD4 or CD8 cells were associated with subsequent risk of TB disease(68). This study, which measured CD4+ and CD8+ T-cell expression of IFN-γ, TNF-α, IL-2 and IL-17 at 10 weeks of age, found no associations between frequencies or cytokine-expression patterns of BCG-specific CD4+ and CD8+ T-cells and subsequent risk of TB(68). Several other studies that also addressed T-cell activation status have shown that phenotypic markers of T-cell activation and/or differentiation can discriminate between distinct clinical presentations of TB infection. During antigen-driven differentiation T-lymphocytes pass through several stages (early, late and terminally differentiated effector cells), and each stage can be characterised by a set of cell surface markers. Since the differentiation process depends on antigenic stimulation, markers of T-cell differentiation may serve as indicators of M. tuberculosis replication or antigen load. T-cell expression of CD27 has been observed to show promise as a COR. CD27 is a member of the TNF receptor superfamily, which is constitutively expressed by the naive T-cells and early effector lymphocytes, but downregulated during late stages of effector cell differentiation. Therefore, late effector lymphocytes exhibit low to no CD27 expression(69–70). Several studies have demonstrated that significantly higher proportions of M. tuberculosis-specific IFN-γ-producing CD4+ T-cells do not express CD27 (CD27−IFN-γ+ CD4+) in persons with active TB disease, compared with healthy controls. In addition, it has been shown that frequencies of CD27−IFN-γ+ CD4+ cells strongly correlated with the degree of pulmonary destruction(71) and TB treatment success(73, 76). Schuetz et al.(77) reported that HIV-infected patients without TB disease have higher proportions of CD27−IFN-γ+ CD4+ cells than HIV-negative persons without TB disease and suggested that accumulation of M. tuberculosis-specific CD27−CD4+ cells may reflect the degree of M. tuberculosis replication, thus

possibly identifying subclinical M. tuberculosis infection. The loss of CD27 expression on specific CD4+ T-cells was shown to proceed the development of active TB disease in one HIV-infected patient who had recently HIV-seroconverted, lending further support to this hypothesis(77) (table 1). The accuracy of assays based on the modulation of CD27 may be increased by combining several tests either based on CD27 or on cytokine expression(78); however, further studies are needed to confirm better accuracy compared to IGRA. IFN-γ inducible protein-10 (IP-10) is a chemokine secreted by multiple cell types, including monocytes, endothelial cells and fibroblasts, in response to IFN-γ. It acts as a chemoattractant for monocytes/macrophages, T-cells, natural killer cells, and dendritic cells and promotes T-cell adhesion(79, 80). IP-10 concentrations, measured after M. tuberculosis antigen exposure or in unstimulated blood, are elevated in patients with TB either with or without HIV co-infection either in blood(81–84) or urine(85, 86). High levels of plasma IP-10 was associated with incident TB in both HIV-uninfected(87) and HIV-infected subjects(88) (table 1).

3.6 HBHA and other M. tuberculosis latency antigens

Heparin-binding haemagglutinin (HBHA) antigen is expressed at the surface of a variety of mycobacterial species and promotes binding to host epithelial cells(89). This feature increases M. tuberculosis pathogenicity and facilitates the extrapulmonary dissemination of mycobacteria. Detection of T-cell responses specific to HBHA has been associated with LTBI, suggesting a protective role for the HBHA-specific responses(90–93). This is supported by the presence of HBHA-specific CD8+ T-cells in LTBI and the proposed role of such cells in maintaining the latent state(94, 95). However, as previously stated, LTBI is considered a heterogeneous entity which may include spontaneous cure, stable and persistent asymptomatic infection and, as discussed earlier, early progression towards active disease(10). CORBIERE et al.(96) have suggested that measuring IFN-γ responses to two different mycobacterial antigens, ESAT-6 and HBHA, may allow stratification of LTBI subjects into several groups. In this model, those who score negative to both antigens represent successful elimination of the M. tuberculosis infection, those who score positive to both antigens are in a stage of true latency able to control M. tuberculosis replication and those who are positive only to ESAT-6 have actively replicating M. tuberculosis (as in active TB disease). The latter is informed by the demonstration that ESAT-6 is produced at high levels during mycobacterial growth(97). Based on this model, the authors report that loss of the in vitro IFN-γ response to HBHA preceded development of TB in a dialysis patient(96). Other M. tuberculosis latency antigens have also been reported(98). Adaptation of M. tuberculosis to a state of non- or slow replicating persistence during LTBI, with downregulated metabolic activity and concomitant altered gene expression patterns have been suggested(99, 100). In this state, M. tuberculosis has been shown to induce the expression of the 48-gene-encoding DosR regulon(101). T-cell responses to M. tuberculosis DosR-encoded latency antigens were particularly dominant in TST+ LTBI individuals, compared to successfully treated or active TB patients. T-cell

proliferation and IFN-γ production driven by these latency antigens was consistently seen in LTBI in many diverse genetic and geographic populations(102–104). Responses to one of these antigens, Rv2628, discriminated recent from remote LTBI in a cross-sectional study(104). Moreover, the ratio of responses between M. tuberculosis DosR-encoded antigens and ESAT6, in analogy with the results found for HBHA and ESAT6, correlated with low risk of TB disease(105). These results suggest that the evaluation of this response may be useful for identification of LTBI subjects who are more likely to develop active TB within 2 years and may benefit from preventive treatment (QFT-IT-positive and Rv2628-negative) (table 1). In addition, it has been shown that the specific response to Rv2628 is found at the site of TB disease(106), emphasising the concept of TB as a spectrum of different stages of infection(10).

3.7 Elevated proportion of peripheral monocytes as COR for identifying contacts of TB patients at highest risk of developing active TB

SABIN and colleagues(107–109) showed in the 1920s that the ratio between monocyte and lymphocyte numbers in peripheral blood, or monocyte/lymphocyte (M/L) ratio, was associated with progressive outcome of mycobacterial infections in rabbits. Rediscovering this experimental study, a series of prospective cohort evaluations have recently been performed in adults, pregnant women and infants in sub-Saharan Africa(110–112). In each study an elevated M/L ratio was associated with risk of TB disease before the appearance of symptoms. The biological underpinning of this finding may be related to activation of myelopoiesis in the bone marrow induced by elevated expression of IFN-γ, which is involved in the antimycobacterial response, thus leading to increased myeloid cellularity in the blood(113, 114). Since lymphopoiesis is not activated in a similar manner, and peripheral lymphoid cells, including T- and B-cells, are recruited to the site of M. tuberculosis replication, this may lead to a decrease in peripheral blood lymphoid cells. The higher M/L ratio is thus likely to be a product of both these processes. Interestingly, in addition to this hypothesis, it was shown that in healthy donors a high M/L ratio was associated with antimycobacterial activity and with transcriptomic profiles characterised by an enrichment of IFN-associated transcripts in monocytes. These data suggest that the M/L ratio may be associated with subclinical disease by acting as a marker of monocyte function(115) (table 1).

3.8 Distinct host immune responses to BCG vaccination can obscure identification of COR

Another TB COR study was recently completed in 10-week-old BCG-vaccinated infants from South Africa, who during 2 years of follow-up either progressed to TB disease (progressors) or remained healthy (controls) (116). Comprehensive comparisons of global gene expression and cellular responses to BCG between progressors and controls did not lead to identification of COR in these infants. However, gene expression profiles revealed two distinct clusters of infants, each of which contained both progressors and

controls. One cluster had enrichment of differentially expressed genes in biological pathways including IFN responses and T-cell activation, while the other cluster had enrichment of myeloid cell and glucose metabolism pathways. Importantly, within cluster 1 infants, those who progressed to TB had elevated monocyte to T-cell ratios and frequencies of BCG-specific CD4+ T-cells expressing IFN-γ, among other T-helper type 1 cytokines. It was striking that the former finding is consistent with the M/L ratio associations with risk of TB (115, 116), while the latter is consistent with the observed immune activation and frequencies of IFN-γ-expressing Ag85B-specific T-cells identified as COR in the 4–6-month-old infant cohort from the phase IIb MVA85A trial.

3.9 Conclusions

Although only a handful of studies have identified biomarkers of progression to TB, a picture is emerging from the common biological processes, including immune activation, IFN responses and changes in peripheral blood myeloid and lymphoid cells which have been clinically validated and are associated with risk of TB. Promising biomarkers and biomarker signatures are being discovered that may allow accurate prediction of progression from infection to active TB disease. Several of these biomarkers are being taken further towards test development, evaluation and possible use in clinical settings. However, despite this great progress, no biomarker signature has yet been identified that fully meets the performance goals for a prognostic test of progression from latent infection to TB disease set forward by the global health community led by the WHO and other stakeholders. Further intensified research efforts are required for ongoing biomarker discovery, evaluation and test development. In addition, it is critical that different markers and signatures are validated in different geographic settings (and thus different host genetic backgrounds and circulating pathogen lineages) to ensure that accuracy in different high-burden settings is sufficiently acceptable to enable a “screen and treat” strategy. Utility of such biomarkers is also highly dependent on translation of the test to a near-patient platform to allow testing in settings where patients at highest risk for progression present for care (e.g. HIV clinics in TB-endemic populations). Without further progress on the development of tests that better predict the development of active TB, we will not be able to eradicate the seedbeds of TB and achieve the end-TB strategy of the WHO.

3.10 References

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CHAPTER 4: OBJECTIVES OF THE STUDY

Mtb is able to establish a chronic asintomatically infection mainly in the lung and the balance between host immune response and the mycobacteria plays a fundamental role in the control of the Mtb replication (3,36). Although the immune response to Mtb has been deeply studied, as described in the previously chapters, the host factors that leads to the development to active TB disease are not fully understood. However, on the base of immunogical findings on Mtb infection, it is possible to speculate that the containment of the latent Mtb needs an acquired cellular response with specific characteristics of immune surveillance, differently contrasting the replicating Mtb requires effector and cytotoxic proprierty.

Objective of the present study is to take a picture of the immunological status of patients with Mtb infection, characterizing their Mtb specific immune response, in order to find a correlation with the different stages of Mtb infection.

In the chapter 5 it is described the use of several cytometric approaches to evaluate the surface expression of the activation marker CD27 on Mtb-specific CD4+ T-cells, as a tool to diagnose active TB and LTBI.

The chapter 6 is focused on flow cytometric characterization of the specific CD4 and CD8 T-cell responses to Mtb antigens contained within the QuantiFERON®-TB Gold Plus (QFT-Plus). QFT-Plus is the new generation of QuantiFERON-TB Gold In-Tube test (QFT-GIT) to identify the latent tuberculosis infection, it includes two tubes called TB1 and TB2 tubes which contain selected Mtb peptides designed to stimulate both CD4 and CD8 T-cells. Aim of the study was to analyze if the immune response to TB1 and TB2 stimulation could or not highlight differences between different TB stages.

In the chapter 7, QFT-Plus performance was compared with that one of QFT-GIT in a cross sectional study of individuals with LTBI, active TB or treated for TB in the past. In this study, we wanted also to evaluate if the different ability to respond to TB1 and TB2 stimulation could associate or not with latency, TB disease or cured TB status.

CHAPTER 5: ASSESSMENT OF CD27 EXPRESSION AS A TOOL FOR ACTIVE AND LATENT TUBERCULOSIS DIAGNOSIS

5.1 Abstract

There are still no reliable tests to distinguish active tuberculosis (TB) from latent TB infection (LTBI). Assessment of CD27 modulation on CD4+ T-cells has been suggested as a tool to diagnose different TB stages.

Objectives: To use several cytometric approaches to evaluate CD27 expression on Mycobacterium tuberculosis (Mtb)-specific CD4+ T-cells to differentiate TB stages.

Methods: 55 HIV-uninfected subjects were enrolled: 13 active TB; 12 cured TB; 30 LTBI. Whole blood was stimulated with RD1-proteins or Cytomegalovirus-lysate (CMV). Interferon (IFN)-γ response was evaluated by cytometry. The proportion of CD27−/+ within the IFN-γ+ CD4+ T-cells or RATIO of the CD27-median fluorescence intensity (MFI) of CD4+ T-cells over the CD27 MFI of IFN-γ+ CD4+ T-cells was evaluated.

Results: The greatest diagnostic accuracy in discriminating active TB vs. LTBI or cured TB was reached by evaluating the CD27+ CD45RA− cells within the IFN-γ+ CD4+ T-cell subset (76.92 sensitivity for both, and 90% and 91.67% specificity, respectively), although the use of the CD27 MFI RATIO allows for stricter data analysis, independent of the operator.

Conclusions: the study of CD27 expression using different approaches, whether it involves evaluation of CD45RA expression or not, is a robust biomarker for discriminating TB stages.

5.2 Introduction

The global tuberculosis (TB) epidemic is still not under control.[1] Diagnosis of pulmonary active TB relies on the evaluation of clinical symptoms, radiological images and detection of *Mycobacterium tuberculosis* (Mtb) in patient respiratory samples, such as sputum. Microscopic detection of Mtb in sputum smears is the most

commonly used approach for diagnosing pulmonary TB and monitoring response to treatment (2,4).However, sputum smears have poor sensitivity, and a high proportion (20%–66%) of tuberculosis (TB) cases is smear-negative (2). Nucleic acid amplification–based tests are more sensitive for diagnosing ATB (2),but do not differentiate between live and dead Mtb, thus are not useful for monitoring treatment-mediated clearance of Mtb. Although it takes a long time to obtain microbiological isolation and culture of Mtb from sputum, it remains the TB diagnostic gold standard (2). Blood-based host biomarkers for diagnosing TB are attractive alternatives to tests that rely on detecting mycobacteria.

Interferon (IFN)-γ release assays (IGRAs) are new tools for latent TB infection (LTBI) diagnosis. These tests are based on the IFN-γ response to Mtb antigens as ESAT-6 and CFP-10 (3,4). They represent a breakthrough, however, they do not discriminate between active TB disease and LTBI (1,3-7). IGRA accuracy for LTBI diagnosis may be enhanced using other Mtb-specific antigens (8,9) or peptides selected from ESAT-6/CFP-10 (10-12), evaluating the response at the site of TB disease (13,14) or investigating host biomarkers other than IFN-γ in whole blood or peripheral blood mononuclear cells (PBMC) (10,14-21).

Cytometry has been proposed as a potential tool to improve TB diagnosis by phenotypical and functional characterization of antigen-specific T-cells. The cytokine profile of Mtb-specific T-cells has been studied in depth with the aim of finding a correlation with TB status (20,22-29). However, existing data are currently inconclusive due to contrasting findings on the distribution of the various cytokine-producing $CD4^+$ and $CD8^+$ T-cell subsets.

T-cell expression of surface molecules such as CD45RA, CD27 and CCR7 identifies different T-cell subsets that reflect different stages of cell differentiation (30-34). Interestingly, the effector T-cells are expanded during active Mtb replication, whereas the memory cells associate with control and eradication of Mtb infection (14,35-37). Moreover, several reports demonstrate that the decrease of CD27 surface expression on circulating Mtb-specific CD4 T-cells associates with the status of active TB disease (22,38-43).

Studies in mice have demonstrated that the lack of CD27 expression on T lymphocytes identifies the functionally mature highly differentiated effector T-cells (44). *In vivo* studies show that in the lungs of Mtb-infected mice, the effector $CD4^+$ T-cells with a low CD27 expression differentiate from effector $CD4^+$ T-cell precursors with a high CD27 expression (45). Moreover, it is shown that the Mtb infection leads to the accumulation of effector $CD4^+$ T lymphocytes with low CD27 expression in the lungs, blood, and other organs (44,45). However no consequences on the clinical outcome have been observed in CD27 KO mice infected with Mtb (46).

Interestingly, $CD4^+$ T-cells with a low CD27 surface expression have also been described as a marker of lung tissue destruction during pulmonary TB, suggesting its use as an immune assay to monitor the efficacy of TB therapy (38). The studies on CD27 expression on Mtb-specific T-cells show similar and reproducible results,

despite the differences of the experimental settings and clinical characteristics of the enrolled patients (22,38-43,47). Therefore, the phenotype data of the $CD4^+$ T-cells are more consistent compared to the cytokine profile studies, suggesting that CD27 evaluation on Mtb specific-$CD4^+$ T-cells may be a useful tool for diagnosing TB stages. Recently, a new assay, the T-cell activation marker of TB (TAM-TB), has been proposed (39). Different from the other approaches, it evaluates the ratio of the median fluorescence intensity (MFI) of CD27 within the $CD4^+$ T-cells over the MFI of CD27 in the Mtb-specific CD4 T-cells (39). The TAM-TB assay differentiates active TB from LTBI in children (39).

In the present study, we used several concomitant approaches to evaluate whether detection of the CD27 surface expression on Mtb specific-$CD4^+$ T-cells is a useful marker to discriminate active disease from LTBI in adult individuals from a low TB-endemic country such as Italy.

5.3 Materials and methods

Study population and sample collection

This study was approved by the Ethical Committee of the L. Spallanzani National Institute of Infectious Diseases (INMI), approval number 02/2007. Informed written consent was required to participate in the study that was conducted at INMI.

Active TB was defined based on Mtb isolation from sputum culture. Mtb was drug-sensitive to the first line of TB drugs; patients were enrolled within 7 days of starting the specific treatment. Cured TB was defined as microbiological pulmonary TB after a 6-month course of treatment and when sputum resulted Mtb culture-negative upon the end of therapy. Cured TB patients were evaluated one year after therapy completion. LTBI was defined based on a positive score to QuantiFERON TB Gold In-Tube (QFT-IT) (Qiagen, Hilden, Germany) in the absence of clinical, microbiological and radiological signs of active TB. Within the LTBI group, remote or recent contacts with smear-positive pulmonary TB patients were reported. A portion of the recent contacts underwent prophylaxis. All these LTBI subjects were considered as a whole group because no significant differences were found in terms of the immune responses evaluated (data not shown).

All enrolled subjects tested negative for HIV and none of the subjects enrolled had previously undergone treatment with immunosuppressive drugs. Demographic and epidemiological information were collected at enrollment (Table 1).

Stimuli and antibodies

Whole blood or peripheral blood mononuclear cells (PBMC) (in a small subgroup) were stimulated with recombinant proteins ESAT-6 and CFP-10 (Lionex, Braunschweig, Germany) (hereafter referred to as RD1 proteins or RD1) at 4 ug/ml with a contamination of lipolisaccaride reported by the manufacturer of less than 0.05 IU/mg for ESAT-6 and equal to 66.7 IU/mg for CFP-10. RD1 proteins were used as a stimulus to

evaluate Mtb-specific response by intracellular staining assay (ICS), as previously described (22). Cytomegalovirus (CMV) lysate of the human CMV strain AD169 propagated in human foreskin fibroblast (Experteam, Venice, Italy) at 5 ug/ml and staphylococcal enterotoxin B (SEB) (Sigma, St Louis, MO, USA) at 200 ng/ml were used as an uncorrelated antigen and positive control, respectively. Whole blood cells were co-stimulated with anti-CD28 and anti-CD49d monoclonal antibody (mAb) at 2 ug/ml each (BD Biosciences, San Jose, USA). Brefeldin A (BFA) (SERVA Electrophoresis GmbH, Heidelberg, Germany) was added at 50 ug/ml to whole blood cells to prevent cytokine secretion.

The following fluorescently conjugated mAb were used in this study: anti-CD8 Pacific Blue, anti-CD4 peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated, anti-CD45RA phycoerythrin (PE)-Cy7-conjugated, anti-CD27 Horizon V500, anti–IFN-γ allophycocyanin (APC)-conjugated (all from BD Biosciences).

Whole blood intracellular staining assay

Heparinized peripheral blood was collected and 100 µl of whole blood were incubated with stimuli for 16 h at 37 °C within 2 h of blood collection. BFA was added after one hour of stimulation. Unstimulated whole blood cells served as a negative control of ICS. Whole blood cells were stained with mAb for surface markers and red cells were lysed with FACS Lysing Solution (BD Biosciences). Whole blood cells were permeabilized with PBS-1% BSA-0.5% saponin-0.1% NaN_3 and stained with mAb for intracellular cytokines. At least 100,000 lymphocytes were acquired using a FACSCanto II flow cytometer (BD Biosciences).

Flow cytometry data analysis

Multiple-parameter flow cytometry data were analyzed using FlowJo (Tree Star Inc., San Carlos, CA) and SPICE software (provided by Dr. Roederer, Vaccine Research Center, NIAID, NIH, USA) (48). Cells were gated according to forward and side scatter plots and the frequency of single, double and triple cytokines producing $CD4^+$ T-cells was evaluated using Boolean combination gates. Background cytokine production in the negative control of the ICS assay was subtracted from each stimulated condition. To score a positive $CD4^+$ T-cell response we defined a detection limit of 0.03% of IFN-γ producing $CD4^+$ T-cells, corresponding to at least 30 analyzed events. All donors were responsive to the SEB positive control in the ICS assay. The phenotypical analysis of RD1-response of $CD4^+$ T-cells was evaluated by flow cytometry according to the expression of surface markers CD45RA and CD27 in the gate of IFN-γ^+ $CD4^+$ T-cells. The FACS results were generated by EP and blindly re-evaluated by a co-author, LP. The agreement of the results was high (k = 0.9) and the discrepancies were solved by discussion.

Definition of the CD27 MFI RATIO

For each sample, the ratio of the MFI of CD27 in the $CD4^+$ T-cells over the MFI of CD27 in the $CD4^+$ IFN-γ^+ T-cells was calculated as suggested by Portevin (39) (hereafter referred to as the CD27 MFI RATIO).

Statistical analysis

Data were analyzed using SPSS software (Version 19 for Windows, Italy SRL, Bologna, Italy), and Stata (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). For continuous measures, medians and interquartile ranges (IQR) were calculated; the Kruskal–Wallis test was used for comparisons among several groups and the Mann–Whitney U test with Bonferroni correction for comparisons between more than 2 groups. The Fisher exact test was used for categorical variables. P values ≤0.05 or ≤0.016 (if 3 groups were analyzed) after the Bonferroni correction were considered significant.

A receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic performances of the tests evaluated, and for scoring purpose we chose a cut-off point to maximize the sum of sensitivity and specificity(49,51). Test equality of ROC areas of the different cytometric approaches was performed to compare areas under the curves (AUC), using a test based on an algorithm suggested by DeLong et al., implemented by STATA software.[52]

5.4 Results

Characteristics of the patient population

We enrolled 55 subjects, 13 active TB, 12 cured TB and 30 LTBI, selected to score positive to the QFT-IT to have better chances of obtaining a positive cytometric response after RD1-specific stimulation. The majority was male and BCG-unvaccinated and 50.9% were from Western Europe. Significant differences were found for origin ($p < 0.0001$) and BCG vaccination ($p = 0.001$) (Table 1).

Table 1: Demographic and clinical characteristics of the subjects enrolled in the study

	active TB	cured TB	LTBI	TOTAL	p value
Enrolled subjects	13	12	30	55	
Median age (IQR)	31 (22-36.5)	30.5 (22.2-33.7)	35 (21-53.75)	31 (22-47)	0.566[a]
Female gender (%)	3 (23.1)	4 (33.3)	12 (40)	19 (34.5)	0.603[b]
Origin (%)					<0.0001[b]
West Europe	1 (7.7)	5 (41.7)	22 (73.3)	28 (50.9)	
Est Europe	9 (69.2)	4 (33.3)	6 (20)	19 (34.5)	
Asia	2(15.4)	0 (0)	2 (6.7)	4 (7.3)	
Africa	0(0)	1(8.3)	0 (0)	1 (1.8)	
Sud America	1(7.7)	2 (16.7)	0 (0)	3 (5.5)	
BCG					0.001[b]
Vaccinated	11 (84.6)	6 (50)	8 (73.3)	25 (45.5)	
unvaccinated	2 (15.4)	6 (50)	22 (26.7)	30 (54.5)	

Footnotes: TB: tuberculosis, LTBI: latent TB infection, BCG: Bacillus Calmette et Guérin, IQR: Inter quartile range

[a] Kruskal Wallis test

[b] Fisher exact test

Active TB associates with down modulation of CD27 expression

$CD4^+$ T-cells can be phenotypically divided into at least four different populations, based on the surface expression of CD45RA and CD27 (Fig. 1). Compared to active TB, a significantly higher proportion of $CD45RA^-$ $CD27^+$ T-cells was associated with LTBI ($p < 0.0001$) and cured TB ($p = 0.0005$) (Fig. 1A). Conversely, active TB was characterized by a significantly higher proportion of $CD45RA^-$ $CD27^-$ T-cells compared to LTBI and cured TB ($p = 0.0003$ and $p = 0.005$ respectively) (Fig. 1B). The pie charts represent the proportion of $CD45RA^{+/-}$and $CD27^{+/-}$ in the different groups (Fig. 1C).

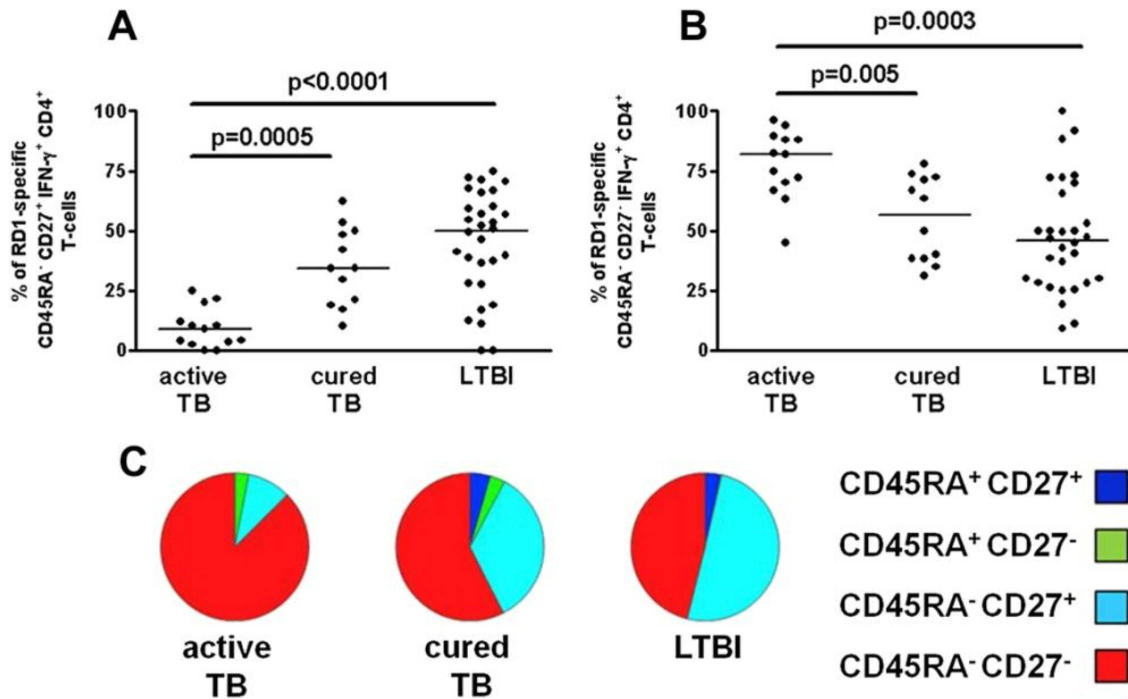

Figure 1 A $CD45RA^-CD27^+$phenotype significantly associates with LTBI, whereas active TB associates with a $CD45RA^-CD27^-$phenotype within the IFN-γ^+CD4^+T-cells. Whole blood was stimulated overnight with RD1 recombinant proteins (ESAT-6 and CFP-10) and the phenotype of $CD4^+$IFN-γ^+ T-cells was evaluated by flow cytometry according to the surface expression of CD27 and $CD45RA^-$. Analysis of $CD45RA^-$ $CD27^+$ IFN-γ^+ $CD4^+$ T-cells among groups (A). Analysis of $CD45RA^-CD27^-$ IFN-γ^+ $CD4^+$ T-cells among groups (B). The horizontal lines represent the median; statistical analysis was performed using the Mann–Whitney test with Bonferroni correction and p value was considered significant if < 0.016 (A–B). Pie charts represent the proportion of $CD45RA^{+/-}$ $CD27^{+/-}$ $CD4^+$IFN-γ^+ T-cells in the different groups in response to overnight stimulation with RD1 recombinant proteins (C). Footnotes: TB: tuberculosis, LTBI: latent TB infection, IFN: interferon.

RD1-specific T-cell response may also be analyzed evaluating only the CD27+/− within the IFNγ+ CD4+ T-cells. Compared to active TB, we showed a higher frequency of CD4+ IFNγ+CD27+ T-cells in LTBI

(p < 0.0001) and cured TB (p = 0.001) (Fig. 2A) indicating that increased CD27 expression is a marker of Mtb containment. Conversely, CD4+ IFN-γ+CD27− T-cells associated with active TB in comparison with LTBI (p < 0.0001) and cured TB (p = 0.001) (Fig. 2B), indicating that loss of CD27 expression is a marker of Mtb uncontrolled replication.

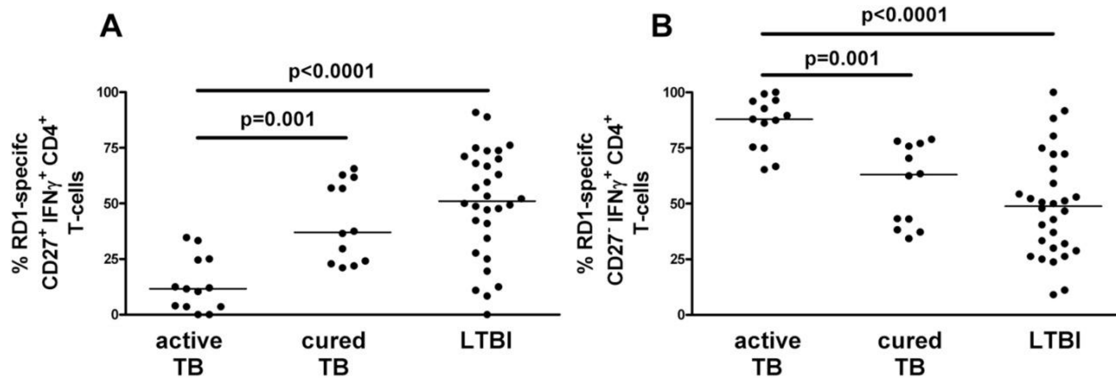

Figure 2 LTBI significantly associates with a CD27$^+$phenotype, whereas active TB associates with a CD27$^-$phenotype within the IFN-γ$^+$CD4$^+$T-cells. Whole blood was stimulated overnight with RD1 recombinant proteins (ESAT-6 and CFP-10) and the phenotype of IFN-γ$^+$ CD4$^+$ T-cells was evaluated by flow cytometry according to the surface expression of CD27. Analysis of CD27$^+$ IFN-γ$^+$ CD4$^+$ T-cells among groups (A). Analysis of CD27$^-$ IFN-γ$^+$ CD4$^+$ T-cells among groups (B). The horizontal lines represent the median; statistical analysis was performed using the Mann–Whitney test with Bonferroni correction and p value was considered significant if < 0.016 (A–B). Footnotes: TB: tuberculosis, LTBI: latent TB infection, IFN: interferon.

A higher CD27 MFI RATIO associates with TB disease:

Since modulation of the surface expression of CD27 allows for discriminating TB stages, we analyzed the data using an additional cytometric approach based on the MFI evaluation of CD27 in the CD4$^+$ T-cells. This methodology permits the operator to work without having the responsibility (or bias) to select the CD27$^{+/-}$ gate, therefore leading to a more accurate and stringent analysis. The data were analyzed evaluating the ratio of the MFI of CD27 in the CD4$^+$ T-cells over the MFI of CD27 in the CD4$^+$ IFN-γ$^+$ T-cells, as suggested by Portevin (39).

In a subgroup of subjects, the evaluation of the CD27 MFI RATIO was concomitantly conducted in PBMC and whole blood, and the analysis showed a higher CD27 MFI RATIO in active TB compared to LTBI, independent of the biological sample used (data not shown). Consequently, to simplify and facilitate the experimental procedure, the study was further conducted in whole blood samples (Fig. 3). We found a higher CD27 MFI RATIO in active TB compared to the cured TB (p = 0.03) and to LTBI (p = 0.0005) which was highly significant (Fig. 3A).

To evaluate the specificity of the result obtained, we analyzed the CD27 MFI RATIO in response to the uncorrelated CMV antigen. No significant differences were found among the groups analyzed (Fig. 3B). Therefore, a higher CD27 MFI RATIO in response to the RD1 antigen stimulation is a consequence of a lower CD27 expression only after Mtb-specific stimulation.

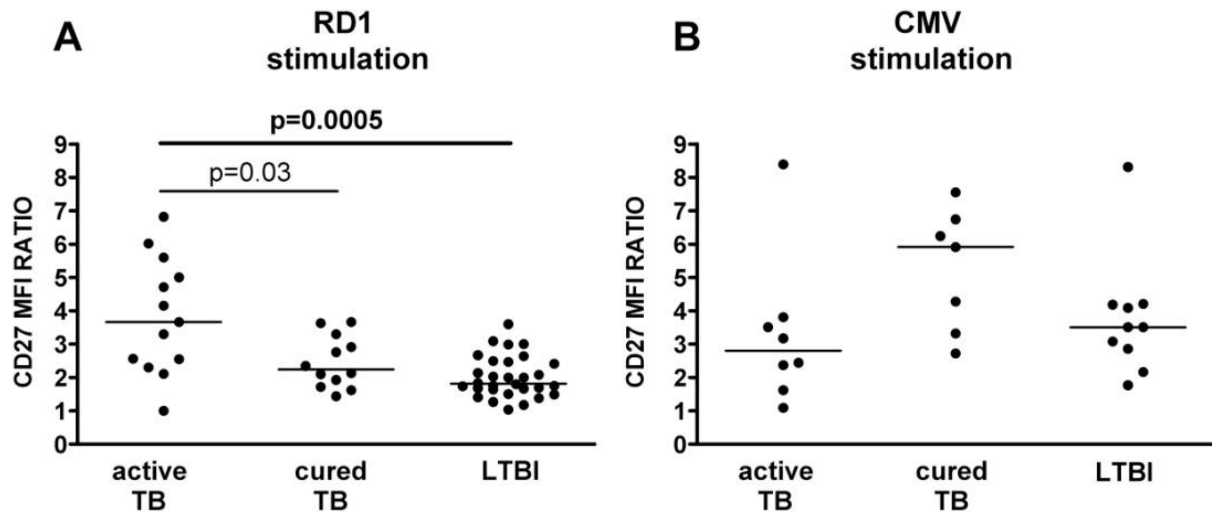

Figure 3 A higher CD27 MFI RATIO significantly associates with active TB disease. Whole blood was stimulated overnight with RD1 recombinant proteins (ESAT-6 and CFP-10) and the phenotype of IFN-γ$^+$ CD4$^+$ T-cells was evaluated by flow cytometry according to the surface expression of CD27. The CD27 MFI RATIO is represented by the MFI of CD27 in the CD4$^+$ T-cells over the MFI of CD27 in the IFN-γ$^+$ CD4$^+$ T-cells. The CD27 MFI RATIO referred to Mtb-specific T-cell response (A). The CD27 ratio referred to CMV-specific T-cell response (B). The horizontal lines represent the median; statistical analysis was performed using the Mann–Whitney test with Bonferroni correction and p value was considered significant if <0.016 (A–B). Footnotes: TB: tuberculosis, LTBI: latent TB infection, IFN: interferon, CMV: Cytomegalovirus, MFI: median fluorescence intensity.

Accuracy of the different cytometric approaches to evaluate CD27 expression

Considering the significant differences found on the modulation of the CD27 in Mtb-specific CD4$^+$ T-cells with the different cytometric approaches used, we performed the ROC analysis for scoring purposes (Table 2). AUC analysis showed significant results with good diagnostic accuracy for TB diagnosis (Table 2). Interestingly, the evaluation of the CD27$^+$CD45RA$^-$IFN-γ$^+$CD4$^+$ T-cells led to the highest accuracy for the discrimination of active TB from LTBI or cured TB with a sensitivity of 76.92% for both and specificity of 90.00% and 91.67 respectively (Table 2). The method based on the CD27 MFI RATIO showed a higher specificity to distinguish active TB from LTBI, probably due to the use of MFI which reduces the risk of any possible bias in the data analysis (Table 2).

We also assessed if there were differences among the AUC obtained from the different tests evaluated. No significant differences were found comparing the AUC, either for discriminating active TB from LTBI (p = 0.512) or active TB from cured TB (p = 0.536).

Table 2: ROC curve analysis of the different tests

	active TB vs. LTBI				active TB vs. cured TB			
Test	AUC 95% CI p value	Cut-off point	Sensitivity (95% CI) Number of positive response/active TB	Specificity (95% CI) Number of negative response/ LTBI	AUC 95% CI p value	Cut-off point	Sensitivity (95% CI) Number of positive response/active TB	Specificity (95% CI) Number of negative response/ cured TB
$CD4^+$ IFN-γ^+ $CD45RA^-$ $CD27^+$ T-cells	0.91 0.81-1.00 p<0.0001	12.25	76.92 (46.19-94.96) 10/13	90.00 (73.47-97.89) 27/30	0.92 0.81-1.02 p=0.0004	14.55	76.92 (46.19-94.96) 10/13	91.67 (61.52-99.79) 11/12
$CD4^+$IFN-γ^+ $CD45RA^-$ $CD27^-$ T-cells	0.85 0.73-0.97 p=0.0003	72.25	69.23 (38.57-90.91) 9/13	86.67 (69.28-96.24) 26/30	0.84 0.68-0.99 p=0.0043	74.35	61.54 (31.58-86.14) 8/13	91.67 (61.52-99.79) 11/12
$CD4^+$ IFN-γ^+ $CD27^+$ T-cells	0.90 0.80-0.99 p<0.0001	25.03	84.62 (54.55-98.08) 11/13	83.33 (65.28-94.36) 25/30	0.89 0.75-1.01 p=0.0011	21.51	69.23 (38.57-90.91) 9/13	91.67 (61.52-99.79) 11/12
$CD4^+$ IFN-γ^+ $CD27^-$ T-cells	0.90 0.81-0.99 p<0.0001	83.30	69.23 (38.57-90.91) 9/13	90.00 (73.47-97.89) 27/30	0.88 0.76-1.01 p=0.0011	82.58	69.23 (38.57-90.91) 9/13	100.0 (73.54-100.0) 12/12
CD27 MFI RATIO	0.84 0.68-1.00 p=0.0004	3.19	61.54 (31.58-86.14) 8/13	96.67 (82.78-99.92) 29/30	0.76 0.56-0.95 p=0.0296	3.64	53.85 (25.13-80.78) 7/13	91.67 (61.52-99.79) 11/12

Footnotes: TB: tuberculosis, LTBI: latent TB infection, AUC: area under the curve, IFN: interferon, MFI: median fluorescence intensity, CD27 MFI RATIO: CD27 MFI of CD4+ T-cells over CD27 MFI of CD4+ IFN-γ+ T-cells, CI: confidence interval

5.5 Discussion

In this study, among the IGRA-responders, we show that: (i) active TB significantly associates with an increase of the $CD27^-$ subset of Mtb-specific CD4 T-cells in whole blood; (ii) this result is Mtb-specific, as shown by the absence of CD27 modulation among the groups studied after *in vitro* CMV stimulation; (iii) the most accurate results for TB diagnosis are reached by evaluating the CD27 surface expression in the $CD4^+$ IFN-γ^+ T-cell subset (within the total IFN-γ^+ or IFN-γ^+ $CD45RA^-$ T-cells), although the use of the CD27 MFI RATIO may allow for a stricter data analysis, independent of the operator. In conclusion, we show that the modulation of the CD27 expression is a robust biomarker for TB diagnosis, as shown by the use of different cytometric approaches.

We found that the evaluation of the $CD27^+$ $CD45RA^-$ IFN-γ^+ $CD4^+$ T-cells subset has a greater diagnostic accuracy for discriminating active TB from LTBI or cured TB, compared to the other cytometric approaches employed here to evaluate CD27 modulation, with a sensitivity for active TB diagnosis of 76.92% for both and a specificity of 90.00% and 91.67% respectively. This method is based on the gate selection of $CD27^{+/-}$ T-cells by the operator, therefore, the choice of the negative or positive CD27 cell population may

constitute a bias of the test. Differently, the CD27 MFI RATIO approach allows for overcoming this potential problem because it analyzes the MFI of CD27 on the $CD4^+$ IFN-γ^+ T-cell population directly. Moreover, the calculation of the ratio allows for normalization of the results, avoiding possible discrepancies in the MFI of CD27 in the $CD4^+$ IFN-γ^+ T-cell subset due to the influence of the total surface expression of CD27 on $CD4^+$ T-cells. Furthermore, here we confirm the results reported in a high TB-endemic country as Tanzania (39) in a low TB-endemic country as Italy (1), demonstrating that it is possible to reproduce the experimental setting using whole blood instead of PBMC (39), allowing for easier manipulation of the samples for routine use.

CD27 is a member of the TNF-receptor superfamily (53). Therefore, the decrease of CD27 expression on $CD4^+$ T-cells indicates the presence of highly differentiated effector T-cells that are able to produce cytokines (38,44,45,54). TB is a chronic disease that leads to the increase of terminally differentiated T-cells (14,22,43,55). In the present study, we show that one year after completion of TB therapy the level of CD27 expression increases in cured TB patients, indicating that the lack of a sustained inflammation leads the T-cells toward a less differentiated phenotype, probably with memory features. Our data are supported by the finding that 2 months of TB therapy are enough to observe a lower proportion of $CD27^{low}CD4^+$ IFN-γ^+ T-cells (38). This evidence suggests that CD27 may also be a useful tool for monitoring TB therapy efficacy.

Potential limitations of the present study should be considered. First, the study was performed among the IGRA-responders, not considering that the IGRA-negative subjects among the active TB cases can be between 10 and 15% (6). However, this is a limitation of all the immune-experimental approaches tested in the literature (20,56) that indeed need to be overcome. Then, we performed a cross-sectional study, analyzing a relatively small number of subjects within each group. Although a larger patient population is needed to validate our findings, the evaluation of the surface CD27 expression on T-specific cells may have the required characteristics to exploit it as a TB biomarker for routine use. In addition, considering the total population analyzed, the sensitivity of the assay for active TB detection needs to be increased. This can probably be achieved by modifying the type of antigen (other than RD1), its composition (peptides instead of proteins) and incubation time (increasing from 16 h to 24 h). Finally, the decision to use a cytometric approach may generate doubts about the consistency of the assays for routine use due to reproducibility issues (dependent on the characteristics of the lasers, the type of instrument employed, the antibodies which may have been produced from different clones or labeled with different fluorochromes, or inconsistency due to the lack of quality controls) and the need to generate standard operating protocols (57,58). However, in this study using the CD27 MFI RATIO, we obtained an accuracy for TB diagnosis similar to that reported by Portevin who described a slightly higher sensitivity and lower specificity, probably due to the use of two antigen stimulations [purified protein derivative (PPD) and RD1] (39) instead of only one used in the present study.

In summary, the evaluation of the surface CD27 expression on T-specific cells may have the required characteristics to exploit it as a TB biomarker for routine use, although for the moment, this is only feasible in the IGRA-responders. Larger patient populations are needed to validate these findings.

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CHAPTER 6: FIRST CHARACTERIZATION OF THE CD4 AND CD8 T-CELL RESPONSES TO QUANTIFERON-TB PLUS

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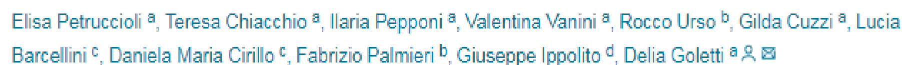
First characterization of the CD4 and CD8 T-cell responses to QuantiFERON-TB Plus



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6.1 Abstract

Introduction

QuantiFERON®-TB Gold Plus (QFT-Plus) is the new generation of QuantiFERON-TB Gold In-Tube test to identify latent tuberculosis infection (LTBI). QFT-Plus includes TB1 and TB2 tubes which contain selected *Mycobacterium tuberculosis* (Mtb) peptides designed to stimulate both CD4 and CD8 T-cells. Aim of this study is the flow cytometric characterization of the specific CD4 and CD8 T-cell responses to Mtb antigens contained within QFT-Plus.

Methods

We enrolled 27 active tuberculosis (TB) patients and 30 LTBI individuals. Following stimulation with TB1 and TB2, antigen-specific T-cells were characterized by flow cytometry. Data were also correlated with the grade of TB severity.

Results

TB1 mainly elicited a CD4 T-cell response while TB2 induced both CD4 and CD8 responses. Moreover, the TB2-specific CD4 response was detected for both active TB and LTBI patients, whereas the TB2-specific CD8 response was primarily associated with active TB ($p = 0.01$).

Conclusions

To our knowledge, we report the first characterization of the CD4 and CD8 T-cell response to QFT-Plus. CD8 T-cell response is mainly due to TB2 stimulation which is largely associated to active TB. These results provide a better knowledge on the use of this assay.

6.2 Introduction

Tuberculosis (TB), being responsible for 9.6 million cases and 1.5 million deaths annually, represents a major public health problem (1).Moreover, latent TB infection (LTBI), which is estimated to affect one-third of the world's population, may progress to active disease in about 3–15% of the LTBI individuals during their lifetimes (2,3). Considering that LTBI subjects are the seedbed of TB disease, diagnosing and treating LTBI is one of the main goals to control the TB epidemic (4-7). Tuberculin skin test (TST) and T-cell interferon-γ release assays (IGRAs) are the routine diagnostic tools to identify LTBI. Two IGRAs are commercially available: the QuantiFERON®-TB Gold In-Tube (QFT-GIT) (Qiagen, Hilden, Germany) and the T-SPOT.TB (Oxford Immunotec, Abingdon, UK). IGRAs have several advantages: the results are not affected by Bacille Calmette–Guérin (BCG)-vaccination(4-7) and by the majority of environmental mycobacteria; moreover, only one patient-visit is required. However, since these assays are based on an immune response detection, they have a poor sensitivity in children and in immune-compromised subjects (7-9) furthermore, they do not discriminate between active TB and LTBI (5) and poorly correlate with the risk of developing active disease (3,10,11).

In the last few years several studies have described the role of CD8 T-cell responses in TB. Mtb-specific CD8 T cells have been associated with active TB, both in HIV-uninfected and infected patients (12-15). And to recent infections, in adults and young children recently exposed to a smear-positive active TB case (16,17). An increase of the CD8 T-cell responses associates with Mtb load, as found for both humans and animal models (12,18-20). Importantly, longitudinal studies have shown a decrease of the CD8 T-cell response during anti-TB treatment (12,13,21).

Interestingly, a study on QFT-GIT performance has shown that the addition of peptides for eliciting CD8 T-cell responses to QFT-GIT tubes increases the sensitivity of the test for LTBI detection (22). On the base of this evidence, recently QuantiFERON®-TB Gold Plus (QFT-Plus) (23-28), has been proposed as a new generation of QFT-GIT. QFT-Plus includes two tubes, called TB1 and TB2 respectively, with *Mycobacterium tuberculosis* (Mtb) antigens to elicit a specific immune response. The TB1 tube, contains peptides derived from ESAT-6 and CFP-10 (TB-7.7, present in QFT-GIT, has been removed), and it is designed to induce a specific CD4 T cells response. TB2 contains newly designed peptides stimulating interferon (IFN)-γ production by both CD4 and CD8 T cells (29). However, as previously described, due to the lack of a gold

standard for LTBI detection, active TB cases are used as surrogate reference standard for evaluating test accuracy (5).

At present, the specific response to QFT-Plus TB1 and TB2 tubes has not been characterized. Therefore, the main aim of this study is to evaluate by flow cytometry the specific CD4 and CD8 T-cell responses to the Mtb antigens contained within the QFT-Plus test in patients with active TB and LTBI.

6.3 Materials and methods

Population characteristics

This study was approved by the Ethical Committee of "L. Spallanzani" National Institute of Infectious Diseases (INMI), approval number 72/2015. Written informed consent was required to participate in the study that was conducted at INMI. We prospectively enrolled HIV-uninfected patients with pulmonary active TB and LTBI. Active TB microbiologically diagnosed was defined based on the Mtb isolation from sputum culture. Active TB clinically diagnosed was defined based on the clinical and radiological lung lesions associated with TB in the absence of Mtb isolated in the sputum that completely recovered after TB-specific treatment for 6 months. Microbiological TB was characterized by first line Mtb drug-sensitive isolates. Patients were enrolled within 7 days of starting the specific treatment.

In the absence of clinical, microbiological and radiological signs of active TB, LTBI was defined based on a positive score to QFT-GIT (Qiagen, Hilden, Germany). The LTBI group included subjects with a remote infection (reported contact with a smear-positive pulmonary TB patient at least 3 years before the enrollment) and subjects reporting a recent contact (no more than 3 months), LTBI subjects reporting the time of exposure between "more than 3 months" and "3 years" were not enrolled (30,31). None of the subjects enrolled had previously undergone treatment with immunosuppressive drugs. Demographic and epidemiological information were collected at enrollment and are reported in Table 1.

Chest X-ray evaluation

All chest X-rays (rendered anonymous) were evaluated for the presence of nodules, fibrosis, infiltrates, cavitation, bronchial spread, miliarity, pleural effusion and adenopathy, as previously reported (32). Cavity size in centimeters was recorded (<4 cm, ≥4 cm). The proportion of the affected lung was analyzed by a visual estimate of the extent of parenchymal infiltrates; a proportion of 30% of affected lung was used as our internal cut-off value to grade TB severity. In agreement with literature data (33) and on the basis of experience, the disease was graded (by DG, FP, RU) using a sliding scale of severity as follows: 0: normal chest X-rays; 1: mild grade (nodules and or infiltrates with proportion of lung affected <30%); 2: intermediate grade (infiltrates with proportion of lung affected >30% and/or cavitation <4 cm in diameter); 3: high grade (an infiltrate of any percentage of extension with cavitation >4 cm in diameter and/or

bronchial spread and/or miliarity and/or pleural effusion and/or adenopathy). All subjects underwent standard chest X-rays at the time of TB diagnosis.

QFT-GIT and QFT-Plus

QFT-GIT and QFT-Plus assay was performed for each patient. QFT-Plus kits were donated by Qiagen and used according to manufacturer's instructions (29).

Levels of IFN-γ were quantified by ELISA and the QFT-Plus Analysis Software (available from www.quantiFERON.com) was used to analyze raw data and to calculate the results in international units per milliliter (IU/ml). The software performs a quality control assessment of the assay, generates a standard curve and provides a test result for each subject. Test results were interpreted according to manufacturer's criteria (29).

Intracellular staining assay

Intracellular staining (ICS) was performed, concomitantly to QFT Plus, for each patient. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll density gradient centrifugation and resuspended in complete RPMI-16-40 medium (Gibco, CA, USA) with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria). To characterize by flow cytometry the Mtb-specific T-cell response, 1×10^6 PBMC resuspended in 1 ml of medium, were dispensed in TB1, TB2, Mitogen and Nil tubes of QFT-Plus kit. After a 1-h incubation, PBMC were transferred in polystyrene round-bottom tubes, 1 µl/ml of Golgi plug (BD Biosciences San Josè , USA) was added to inhibit cytokine secretion and anti-CD28 and anti-CD49d monoclonal antibodies (mAb) at 2 µg/ml each, were added to co-stimulate cells.

Following an incubation of 16–24 h, the ICS was performed. As previously described (14,34-36), PBMC were stained with anti-CD4 peridinin chlorophylprotein (PerCp)-Cy5.5 conjugate, anti-CD8 allophycocyanin (APC)-H7 conjugate, anti-CD3 Horizon V500 conjugate and anti-IFN-γ Pacific Blue (PB) conjugate (all from BD Bioscences). The Mtb-specific T-cell response was characterized evaluating the frequencies of IFN-γ CD4 and IFN-γ CD8 T cells (Fig. 1). At least 200,000 lymphocytes were acquired with a FACS CANTO II (BD, Bioscences). Cytometry data were analyzed using FloJo software. Background cytokine production in the Nil tube was subtracted from each stimulated condition. If the background was higher than half of the antigen-specific response, the results were scored as negative. A frequency of IFN-γ-producing T cells of at least 0.03% was considered as positive response.

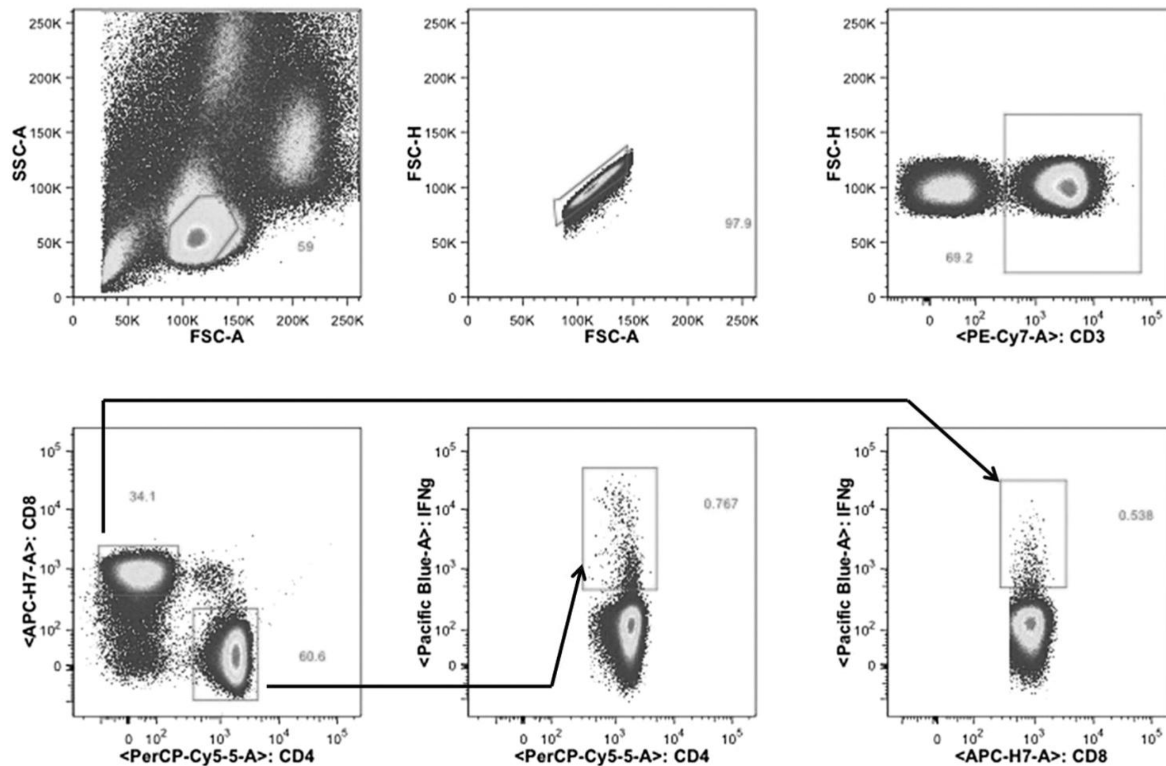

Figure 1. Representative dot plots of the TB2-induced T-cell response of an active TB patient. PBMC were stimulated overnight with TB1 and TB2 antigens and analyzed by flow cytometry for the intracellular production of IFN-γ. The frequency of Mtb-specific T cells was calculated from the proportions of CD4 IFN-γ T cells and CD8 IFN-γ T cells.

Statistical analysis

Data were analyzed using SPSS software (Version 19 FOR Windows, Italy SRL, Bologna, Italy). The median and interquartile ranges (IQRs) were calculated for continuous measures. The Chi-Square test was used for proportions. The Kruskal–Wallis test was used for comparison among several groups and the Mann–Whitney U test was used for pairwise comparison. The Spearman rank correlation was used to correlate continuous variables; $r_s \geq 0.7$ was considered a high correlation, $0.7 < r_s > 0.5$ was considered a moderate correlation and $r_s \leq 0.5$ was considered a low correlation. The Fisher exact test was used for categorical variables.

6.4 Results

Features of the population

A total of 57 participants were enrolled: 27 with active pulmonary TB (23 microbiologically diagnosed and 4 clinically diagnosed) and 30 with LTBI (18 remote LTBI and 12 recent LTBI). Fifty-five percent of the enrolled

subjects were from Western Europe; no significant differences were found for sex, age, origin and BCG vaccination among the different groups (Table 1).

Table 1: Demographic characteristics of enrolled patients

	ACTIVE TB	REMOTE LTBI	RECENT LTBI	TOTAL
N (%)	27 (47)	18 (32)	12 (21)	57 (100)
Age Median (IQR)	38 (28-44)	41 (35.7-60.7)	47 (28.5-60.5)	40 (32-51)
Sex				
Female N (%)	13 (48)	11 (61)	8 (67)	32 (56)
Origin				
West Europe (%)	10 (37)	11 (61)	9 (75)	30 (53)
East Europe (%)	10 (37)	6 (33)	2 (17)	18 (31)
Asia (%)	4 (15)	0 (0)	0 (0)	4 (7)
Africa (%)	2 (7)	1 (6)	1 (8)	4 (7)
South America	1 (4)			
BCG				
Vaccinated (%)	17 (63)	7 (39)	3 (25)	27 (47)

Footnotes: TB: tuberculosis; LTBI: latent tuberculosis infection; BCG: bacillus Calmette-Guérin , IQR: interquartile range

QFT-Plus evaluation among active TB and LTBI subjects

QFT-GIT and QFT-Plus assay were performed for each patient (Fig. 2 and Table 2). To evaluate the sensitivity of the test we used as a surrogate for Mtb infection, the data generated on active TB patients. Therefore the sensibility of the tests for Mtb infection in the active TB group was similar: 89% for QFT-GIT and 85% for QFT-Plus. Among the active TB patients, the proportion of TB1-responders was 78% whereas for TB2 it was 85% (Table 2). For LTBI subjects, the proportion of QFT-Plus responders to TB1 and TB2 was 100%, both for recent and remote infection (Table 2). No significant differences were found comparing the IFN-γ production to TB1 or TB2 among groups (Fig. 2). All samples scored positive to the mitogen stimulation.

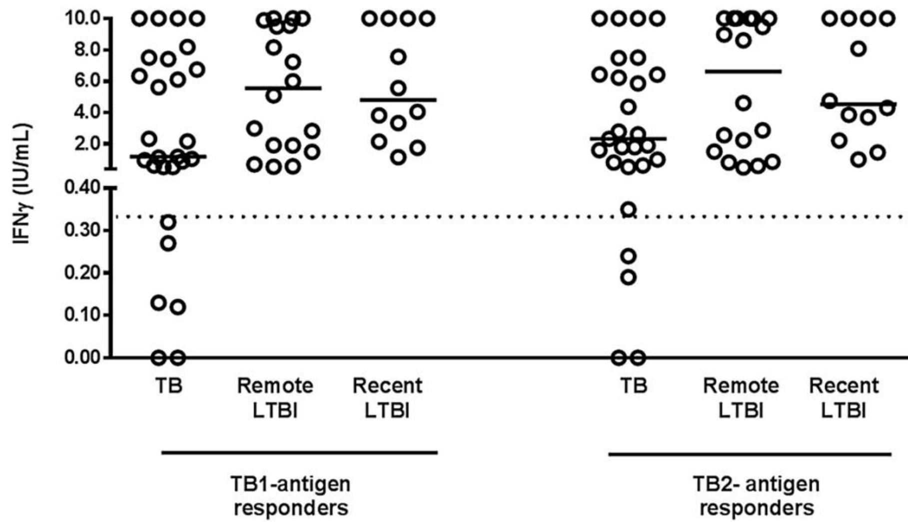

Figure 2. Evaluation of IFN-γ production using QFT-Plus kit. The IFN-γ T-cell response was evaluated in active TB patients and in LTBI subjects with recent and remote infection, one day after whole blood incubation in TB1 and TB2 tubes of the QFT-Plus kit. Horizontal lines indicate the median. The dotted line represents the cut-off value of 0.35 IU/ml. TB: tuberculosis, LTBI: latent tuberculosis infection; IU: international unit; IFN: interferon; QFT: QuantiFERON.

Table 2: Mtb specific response to TB1 and TB2 stimulation in the different groups

		Antigen response to	N (%) of responders ACTIVE TB 27 (47)	REMOTE LTBI 18 (32)	RECENT LTBI 12 (21)	TOTAL 57 (100)
QFT-GIT		TB antigen	24 (89)	18 (100)	12 (100)	54 (95)
QFT-PLUS		TB1	21 (78)	18 (100)	12 (100)	51 (89)
		TB2	23 (85)	18 (100)	12 (100)	53 (93)
		Concomitant TB1 and TB2	21 (78)	18 (100)	12 (100)	51 (89)
		TB1 only	0 (0)	0 (0)	0 (0)	0 (0)
		TB2 only	2 (7)	0 (0)	0 (0)	2 (4)
		TB1 or TB2	23 (85)	18 (100)	12 (100)	53 (93)
ICS	CD4	TB1	22 (81)	17 (94)	12 (100)	51 (89)
		TB2	24 (89)	15 (83)	11 (92)	50 (88)
		Concomitant TB1 and TB2	22 (82)	15 (83)	11 (92)	48 (84)
		TB1 only	0 (0)	2 (11)	1 (8)	3(5)
		TB2 only	2 (7)	0 (0)	0 (0)	2(4)
	CD8	TB1	4 (15)	3(18)	4 (33)	11 (19)
		TB2	12 (44)	3 (18)	3 (25)	18 (32)
		Concomitant TB1 and TB2	4 (15)	3 (18)	3 (25)	10(18)
		TB1 only	0 (0)	0 (0)	1 (8)	1(2)
		TB2 only	8 (30)	0 (0)	0 (0)	8(14)

Footnotes: Mtb*: Mycobacterium tuberculosis*; QFT: QuantiFERON; TB: tuberculosis; LTBI: latent tuberculosis infection; ICS: intracellular staining N:number

Characterization of the CD4 and CD8 T-cell responses: evaluation of the proportion of responders to QFT-Plus by flow cytometry

Our first goal was to compare the CD4 and CD8 T-cell responses elicited by the same antigen stimulation (Fig. 3). To assess the specificity of the ICS assay, 10 healthy donors not exposed to Mtb and scored negative to QFT-Plus, were enrolled. We found that the specificity of the ICS assay to detect a TB infection (latent or active TB status) was 100% (data not shown).

Among those with active disease, TB1 induced a CD4-specific T-cell response in 81% of subjects and a CD8-specific response in only 15% of them. On the other hand, TB2 induced a CD4-specific T-cell response in 89% of subjects and a CD8-specific response in 44% of those (Fig. 3, Table 2).

Among the remote LTBI subjects, TB1 induced a CD4-specific T-cell response in 94% and a CD8-specific T cell response in only 18% of them (Table 2). The TB2 induced a CD4-specific T-cell response in 83% of the sample evaluated and a CD8-specific T cell response in 18% of them (Table 2).

Lastly, for the recent LTBI subjects, TB1 induced a CD4-specific T-cell response in 100% and a CD8 T-cell response in 33%. After TB2 stimulation we found a CD4-specific T-cell response in 92% and a CD8 T-cell response in 25% for both, CD4 and CD8 T-cell subsets (Table 2). Interestingly a selected CD8 response to TB2 stimulation and not to TB1 was found only in patients with active TB (8 of them) (Table 2).

These data indicate that TB1 stimulation induces mainly a CD4 T-cell response in all studied groups, while TB2 stimulation elicits a CD4 response in all groups and a selective CD8 T-cell response in subjects with active Mtb replication, either with active disease or with a recent Mtb exposure.

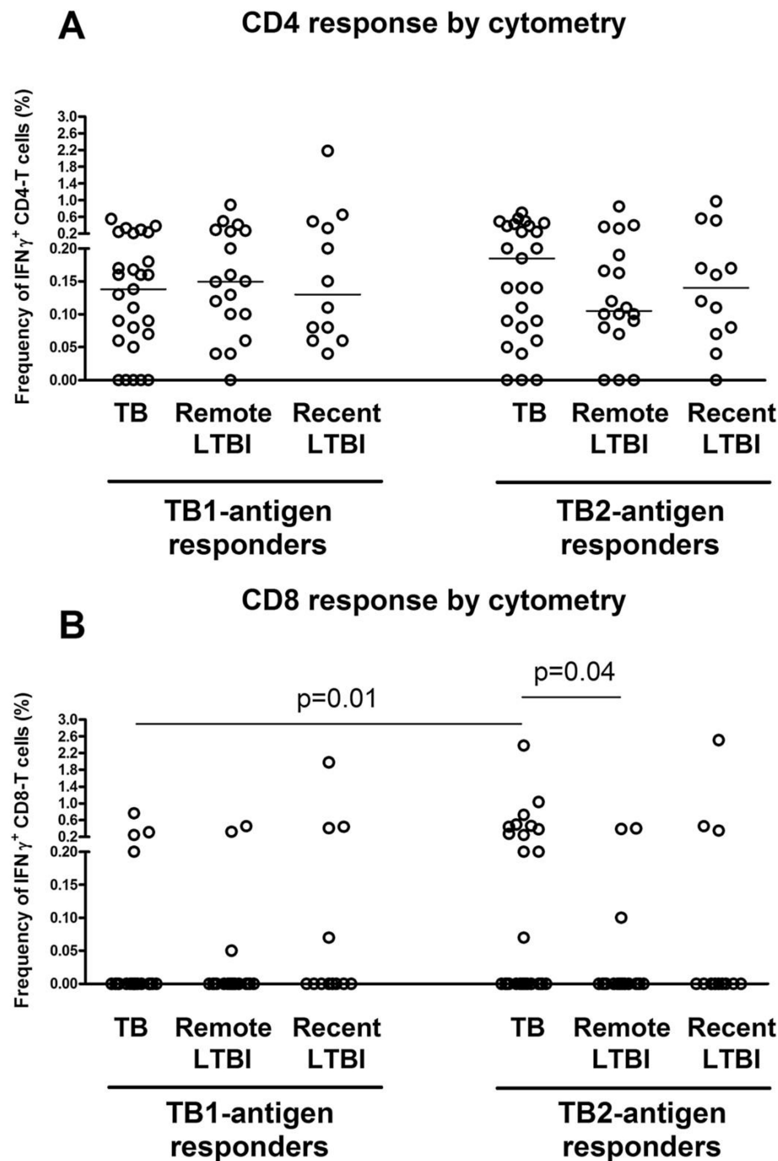

Figure 3. TB2 stimulation induces a CD8 T-cell response in active TB patients. Flow cytometric evaluation of CD4 (A) and CD8 (B) T-cell to TB1 and TB2 antigens in active TB patients and LTBI subjects with recent and remote infection. The response was scored positive if the frequency of IFN-γ producing T cells was at least 0.03%. The horizontal lines represent the medians. Statistical analysis was performed using the Mann–Whitney U test and the p value was considered significant if ≤0.05. TB: tuberculosis, LTBI: latent tuberculosis infection.

Evaluation of CD4 and CD8 T-cell frequencies to TB1 and TB2 by flow cytometry

To better characterize the CD8-specific T cells, we evaluated the frequency of the responses to TB1 and TB2. Among active TB patients, the CD8 response to TB2 was significantly higher than that one generated by TB1 ($p = 0.01$; Fig. 3B). Moreover, the active TB group has a frequency of CD8 response to TB2 stimulation significantly higher compared to that one elicited in the remote LTBI subjects ($p = 0.04$). These

data demonstrate that the Mtb-specific CD8 T-cell response associates with TB2 stimulation in active TB patients.

Then we compared the frequency of antigen-specific CD4 and CD8 T cells in response to the same stimulation. In LTBI subjects, a significantly higher frequency of the CD4 response to TB1 ($p < 0.0001$) and TB2 ($p = 0.0005$) was found in comparison to that one generated by CD8 T cells (Table 3). These data indicate that TB2 stimulation seldom induces a CD8 response in remote LTBI subjects.

Active TB patients showed a significantly higher frequency ($p < 0.0001$) of TB1-induced CD4 T cells compared to the levels found for the CD8 T cells. Since TB2 stimulation induced both a CD4 and a CD8 T-cell response we did not find significant differences comparing the frequency of the two antigen specific T-cell subsets. Interestingly, in active TB patients we found a positive and significant correlation between the frequencies of CD4 T cells and that one of the CD8 T cells in response to TB2 stimulation ($r_s = 0.76$, $p < 0.0001$). These results highlight the capacity of the cells from active TB patients to respond with both CD4 and CD8 T-cell subsets to TB2 stimulation.

Table 3: Comparison of the frequencies of the CD4 and CD8 T-cell response to TB1 and TB2 in the different groups

	Frequency of the CD4 vs CD8 T-cell response					
	TB1			**TB2**		
	ACTIVE TB	**REMOTE LTBI**	**RECENT LTBI**	**ACTIVE TB**	**REMOTE LTBI**	**RECENT LTBI**
	p*	***p****	***p****	***p****	***p****	***p****
ACTIVE TB	**<0.0001**	**-**	**-**	0.1	-	-
REMOTE LTBI	**-**	**<0.0001**	**-**	-	**0.0005**	-
RECENT LTBI	**-**	**-**	**0.02**	-	-	**0.02**

Footnotes: Mtb: Mycobacterium tuberculosis; TB: tuberculosis; LTBI: latent tuberculosis infection; *p**= significance according the Mann-Whitney test.

Impact of the severity of TB disease and mycobacterial load on QFT-Plus results

Finally, we evaluated whether the severity of TB disease may influence the response to QFT-Plus. Among the parameters used to estimate the clinical severity we used the evaluation of the lung lesions based on the radiology findings. To better analyze the results, the data from patients with intermediate/high severity (grades 2 and 3) were combined and compared to those from patients with low severity (grade 1). As shown in Table 4 the patients with intermediate/high severity TB had a similar proportion of responders (84%) to QFT-Plus assay compared to patients with low TB severity (88%). Analyzing the specific immune results, CD8 T-cell response to TB2 was found in 53% (10 out 19) of patients with intermediate/high severity TB and only in 25% (2 out 8) of the low severity TB group (Table 4, Fig. 4). Moreover stratifying the active TB patients according to the microbiological diagnosis, we found that the TB2-induced CD8 response was associated with a microbiological diagnosis of TB more than to a clinical TB diagnosis (48% vs 25%) (Table 4). To note that among the active TB, the CD8 responders either classified as severe TB (8/10) or by a microbiological diagnosis (8/11), a selective TB2 response was observed (Table 4). These results suggest that CD8 T cell response associate with the radiological severity of TB disease and with the mycobacterial load.

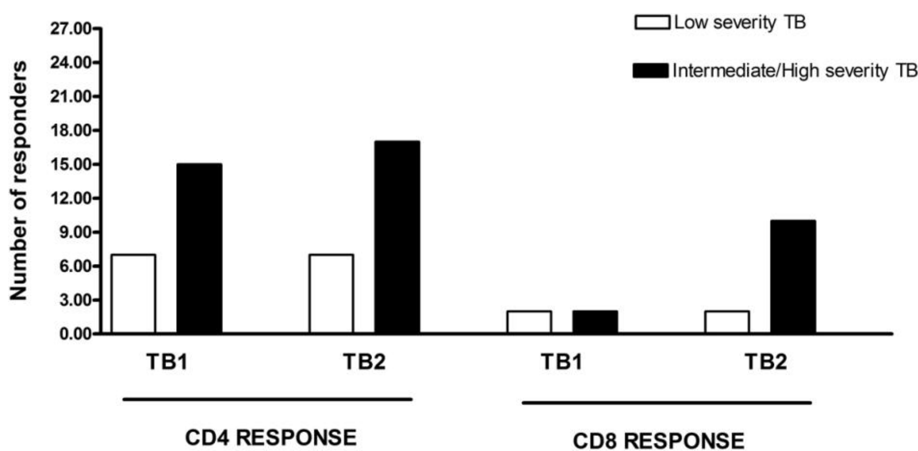

Figure 4. Impact of TB disease severity on CD4 and CD8 T-cell responses. Flow cytometric evaluation of CD4 and CD8 responses to TB1 and TB2 antigens in active TB patients. Patients were stratified according the grade of TB severity: low and intermediate/high severity. The bars represent the number of patients responding to TB1 and TB2 stimulation. TB: tuberculosis.

Table 4: Response to TB1 and TB2 stimulation according to the severity of active TB and the positivity of the mycobateria culture

		Antigen response to	ACTIVE TB N (%) of responders Radiological Classification Low severity TB 8 (30)	Intermediate High severity TB 19 (70)	Microbiological Classification Microbiological confirmed TB 23 (85)	Clinical TB 4 (15)
QFT-GIT		**TB antigen**	8 (100)	16 (84)	20 (87)	4 (100)
QFT-PLUS		**TB1**	7 (88)	14(74)	18 (78)	3 (75)
		TB2	7 (88)	16(84)	20 (87)	3 (75)
		Concomitant TB1 and TB2	7 (88)	14 (74)	18 (78)	3 (75)
		TB1 only	0 (0)	0(0)	0 (0)	0 (0)
		TB2 only	0 (0)	2 (11)	2 (9)	0 (0)
		TB1 or TB2	7 (88)	16(84)	20 (87)	3 (75)
ICS	**CD4**	**TB1**	7 (88)	15 (79)	19 (83)	3 (75)
		TB2	7 (88)	17 (89)	21 (91)	3 (75)
		Concomitant TB1 and TB2	7 (88)	15 (79)	19 (83)	3 (75)
		TB1 only	0 (0)	0 (0)	0 (0)	0 (0)
		TB2 only	0 (0)	2 (11)	2 (9)	0 (0)
	CD8	**TB1**	2 (25)	2 (11)	3 (13)	1 (25)
		TB2	2 (25)	10 (53)	11 (48)	1 (25)
		Concomitant TB1 and TB2	2 (25)	2 (11)	3 (13)	1 (25)
		TB1 only	0 (0)	0 (0)	0 (0)	0 (0)
		TB2 only	0 (0)	8 (42)	8 (35)	0 (0)

Footnotes: Mtb*: Mycobacterium tuberculosis*; QFT: QuantiFERON; TB: tuberculosis; LTBI: latent tuberculosis infection; ICS: intracellular staining, N:number

6.5 Discussion

This is the first characterization of the CD4 and CD8 T-cell responses to TB1 and TB2 tubes of QFT-Plus assay in a cohort of subjects with active TB disease and LTBI enrolled in a low TB endemic country such as Italy.

We demonstrated that both, TB1 and TB2 induce a CD4 T-cell response. On the other hand, CD8 T-cell response is mainly due to TB2 stimulation which is largely associated to active TB.

In an effort to find additional tools for performing better diagnosis using biomarkers (37), the QFT-Plus is a new generation IGRA designed to offer high sensitivity and specificity for LTBI diagnosis (29).

In the absence of a gold standard for the diagnosis of LTBI, active TB patients are used as a surrogate to validate the test. In line with this, a recent study demonstrated that QFT-Plus, in comparison to QFT-GIT, improves the sensitivity for active TB detection maintaining a high specificity (23). Other two studies performed in low TB endemic country demonstrated that the performance of QFT-Plus is as accurate as that of QFT-GIT (27,28). Here we confirm that QFT-GIT and QFT-Plus have similar sensitivity for active TB diagnosis. Moreover in the LTBI population we confirmed by QFT-Plus the data generated by QFT-GIT.

Several studies have described that CD8 T cells play a unique function in the recognition and containment of intracellular infection with Mtb, recognizing and eliminating heavily infected cells (38) including cells which do not belong to the immune system, such as the infected lung epithelial cells (39). Studies in the mouse model demonstrated that depletion of CD8 T cells in the chronic stage of Mtb infection results in increased bacterial burden (40). In vitro studies have shown that CD8 T cells may kill Mtb-infected human cells through granule-mediated functions such as granulysin (41). All these data together indicate that CD8 T cells are actively involved in the immune response to Mtb and they are necessary for the control of TB infection.

In the present study only few LTBI subjects showed a CD8 T-cell response. This is in accordance to the literature and to the concept that latent TB represents a spectrum of different stages in which the immune system and the mycobacteria find a host-pathogen equilibrium (42). In this environment, CD8 T cells are important players to control the Mtb bacterial load by emerging in the presence of replicating Mtb. This limits bacterial survival (41), but produces tissue damage. Interestingly, the decline of CD8 T-cell response in parallel with a decrease of Mtb replication has been described in active TB patients during TB treatment (12,21,43,44). In line with these results, we found lower CD8-specific responses in clinical TB compared to microbiologically diagnosed TB. This highlights that the monitoring of the CD8 T-cell response to TB2 by flow cytometry can be a tool to specifically evaluate treatment efficacy. Moreover we also show that TB2-induced CD8 T-cell responses associate to the grade of TB severity.

According to the manufacturer, TB1 peptides have been designed to stimulate CD4 T cells while TB2 should elicit both CD4 and CD8 responses. Unexpectedly, in the present study we showed that in a few subjects the CD8 response was detected also following TB1 stimulation. These subjects, equally spread between all evaluated groups, showed a concomitant TB2-dependent CD8 T cell-response. This is probably due to the

internalization and processing of the TB1 peptides by antigen presenting cells and their consequent presentation by the MHC I molecules to CD8 T cells.

Recently, studies using QFT-Plus kit showed that the difference in IFN-γ production between TB2 and TB1 stimulation may provide a surrogate marker of the CD8 T-cell response magnitude. This difference has been associated with smear positivity in active TB patients or with a recent exposure in TB contacts (23). In the present study, using the same approach, we did not find any association between these two parameters, probably due to the low number of recent LTBI subjects enrolled.

In conclusion this is the first report on the characterization of the CD4 and CD8 T-cell response to TB1 and TB2 tubes of the QFT-Plus assay. We demonstrated that CD8 T-cell responses are preferentially induced by TB2 which are mainly associated to active TB. This assay has the potential to be very useful in conditions of immune depression resulting from CD4 T-cell impairments.

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CHAPTER 7: ANALYTICAL EVALUATION OF QUANTIFERON- PLUS AND QUANTIFERON- GOLD IN-TUBE ASSAYS IN SUBJECTS WITH OR WITHOUT TUBERCULOSIS.

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Analytical evaluation of QuantiFERON- Plus and QuantiFERON-Gold In-tube assays in subjects with or without tuberculosis

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7.1 Abstract

The QuantiFERON-TB Gold Plus (QFT-Plus) represents the new QuantiFERON-TB Gold In-tube (QFT-GIT) to identify latent tuberculosis infection (LTBI). The main differences is the addition of a new tube containing shorter peptides stimulating CD8 T-cells. Aim of this study is to evaluate the accuracy of QFT-Plus compared with QFT-GIT in a cross sectional study of individuals with or without tuberculosis (TB).

We enrolled 179 participants: 19 healthy donors, 58 LTBI, 33 cured TB and 69 active TB. QFT-Plus and QFT-GIT were performed.

The two tests showed a substantial agreement. Moreover we found a similar sensitivity in active TB and same specificity in healthy donors. A higher proportion of the LTBI subjects responded to both TB1 and TB2 compared to those with active TB (97% vs 81%). Moreover, a selective response to TB2 was associated with active TB (9%) and with a severe TB disease, suggesting that TB2 stimulation induces a CD8 T-cell response in absence of a CD4-response.

In conclusion, QFT-Plus and QFT-GIT assays showed a substantial agreement and similar accuracy for active TB detection. Interestingly, a higher proportion of the LTBI subjects responded concomitantly to TB1 and TB2 compared to those with active TB, whereas a selective TB2 response associated with active TB.

7.2 Introduction

Tuberculosis (TB), being responsible for 10.4 million cases and 1.4 million deaths annually, represents a major public health problem(1). Moreover, latent TB infection (LTBI), which is estimated to affect one-fifth of the world's population, may progress to active disease in about 3–15% of the LTBI individuals during

their lifetimes (2–4). Considering that LTBI subjects are the reservoir of TB disease, diagnosing and treating LTBI is one of the main goals to control and eliminate the TB epidemic (5–10). Tuberculin skin test (TST) and T-cell interferon-γ release assays (IGRAs) are the routine diagnostic tools to identify LTBI (6). Two IGRAs are commercially available: the QuantiFERON_-TB Gold In-Tube (QFT-GIT) (Qiagen, Hilden, Germany) and the T-SPOT.TB (Oxford Immunotec, Abingdon, UK). IGRAs have several advantages: the results are not affected by Bacillus Calmette–Guérin (BCG)-vaccination (5–8) and by the majority of environmental mycobacteria; moreover, only one patient-visit is required. However, since these assays are based on detection of a Mycobacterium tuberculosis (Mtb) -specific immune response, they have a poor sensitivity in children where the immune system is immature and in immune-compromised subjects (8,11,12) furthermore IGRAs do not discriminate between active TB and LTBI (6) and poorly correlate with the presence of viable bacteria and the risk of developing active disease (4,13–15).

Recently, QFT Gold Plus (QFT-Plus) (16–21), has been proposed as a new generation of QFT-GIT. QFT-Plus contains two TB-specific antigen tubes, called TB1 and TB2, for the incubation of the whole blood with Mtb antigens. The TB1 tube, contains long peptides derived from ESAT-6 and CFP-10 (TB-7.7, present in QFT-GIT, has been removed), and it is designed to induce a specific CD4 T cells response. TB2 contains both the same long peptides of TB1 and newly designed shorter peptides to induce interferon (IFN)-γ production by both CD4 and CD8 T-cells (22). IGRA are designed to diagnose LTBI. However, there is not a gold standard for LTBI detection, therefore active TB is used as a surrogate reference standard for evaluating test accuracy (6).

Compared to QFT-GIT, it has been reported that the accuracy of QFT-Plus is similar (18,23), or that the sensitivity for active TB or LTBI detection is higher (16,17). Moreover, in a low-incidence setting the occurrence of conversions and reversions for the new QFT-Plus in serial testing of a high-TB risk cohort (19) has been described similar to that observed for QFT-GIT (24).

Therefore, the aim of this study is to evaluate the accuracy of the QFT-Plus assay compared with the QFT-GIT in a cross sectional study of individuals enrolled as healthy donors, subjects with active TB disease, cured TB or LTBI. The response to QFT-Plus is selectively evaluated in terms of single or combined response to TB1 and TB2.

7.3 Material and methods

Population characteristics

This study was approved by the Ethical Committee of "L. Spallanzani" National Institute of Infectious Diseases (INMI), approval number 72/2015. Written informed consent was required to participate in the study that was conducted at INMI. We prospectively enrolled HIV-uninfected patients with pulmonary and

extra-pulmonary active TB, cured TB subjects and LTBI. Enrolled patients were classified as “confirmed TB” if the diagnosis was based: i) in those with pulmonary TB by a positive culture for Mtb from the sputum or broncholavage; ii) in those with extrapulmonary TB by a) positive Mtb -specific RNA amplification (TRCReady M.TB, Tosoh, Japan) and/or Mtb -specific NAT (Home-made PCR (IS6110) GeneXpert, Cepheid; Genotype MTBDRPlus Hain Lifescience) from biological specimens or b) by histo-pathological findings consistent with TB and presence of acid fast bacilli (AFB) in a tissue sample or c) by positive culture for Mtb in clinical samples (pleural fluid and abscesses). Conversely, patients were classified as “clinical TB” if the diagnosis was based on clinical and radiologic criteria (having excluded other diseases) including appropriate response to standard anti-TB therapy. TB patients were enrolled within 7 days of starting the specific treatment.

Cured TB patients were defined as those who had completed a 6-month course of treatment for culture-positive (drug-susceptible) pulmonary TB and who resulted Mtb culture negative upon treatment completion.

In the absence of clinical, microbiological and radiological signs of active TB, LTBI was defined based on a positive score to QFT-GIT (Qiagen, Hilden, Germany). Finally, we enrolled 19 healthy control subjects with low risk of TB infection. Demographic and epidemiological information were collected at enrollment (Table 1).

Chest X-ray evaluation

All chest X-rays were evaluated blind to operators for the presence of nodules, fibrosis, infiltrates, cavitation, bronchial spread, miliarity, pleural effusion and adenopathy, as previously reported(25). Cavity size in centimeters was recorded (<4 cm or >4 cm). The proportion of the affected lung was analyzed by a visual estimate of the extent of parenchymal infiltrates; a proportion of 30% of affected lung was used as our internal cut-off value to grade TB severity. In agreement with literature data(25) and on the basis of experience, the disease was graded (by DG, FP) using a sliding scale of severity as follows: 0: normal chest X-rays; 1: mild grade (nodules and or infiltrates with proportion of lung affected <30%); 2: intermediate grade (infiltrates with proportion of lung affected >30% and/or cavitation <4 cm in diameter); 3: high grade (an infiltrate of any percentage of extension with cavitation >4 cm in diameter and/or bronchial spread and/or miliarity and/or pleural effusion and/or adenopathy). All subjects underwent standard chest X-rays at the time of TB diagnosis.

QFT-GIT and QFT-Plus

QFT-GIT and QFT-Plus assays were performed for each subject enrolled. For 11 patients the QFT-GIT value of IFN-γ production was not available because the assay was done in another hospital and only the score of the test was provided. QFT-Plus kits were donated by Qiagen and used according to manufacturer's instructions(22). Levels of IFN-γ were quantified by ELISA. The results were analyzed by a QFT-Plus Analysis Software (available from www.quantiFERON.com). The software performs a quality control assessment of the assay, generates a standard curve and provides a test result for each subject. Test results were analyzed according to manufacturer's criteria for both assays(22). All patients resulted positive to mitogen stimulation.

Statistical analysis

Data were analyzed using SPSS software (Version 19 FORWindows, Italy SRL, Bologna, Italy). The median and interquartile ranges (IQRs) were calculated for continuous measures. Chi square was used for categorical variables. The Kruskall Wallis test was used for comparisons among several groups and the Mann Whitney U test was used for pairwise comparisons. Test concordance was assessed by k-statistics where $k \leq 0.20$ was considered 'slight', $0.20 < k \leq 0.40$ 'fair', $0.40 < k \leq 0.60$ 'moderate', $0.60 < k \leq 0.80$ 'substantial' and $0.80 < k \leq 1.00$ 'optimal'.

7.4 Results

Population characteristics

We enrolled 179 participants: 19 healthy donors, 58 LTBI subjects, 33 cured TB and 69 active TB patients. Among the active TB patients, 49 were microbiologically confirmed (among them two patients had an extra-pulmonary form) and 20 clinically diagnosed (4 patients had an extra-pulmonary form). Forty-seven percent of the enrolled subjects were from Western Europe and female. The majority of TB and cured TB patients were from countries other than west Europe and they were BCG-vaccinated, consequently we found significant differences for BCG vaccination and origin among the different groups (Table 1).

Table 1: Demographic characteristic of enrolled patients

	Active TB	LTBI	Cured TB	Healthy donors	Total	p
N (%)	69 (38)	58 (32)	33 (18)	19 (10.5)	179	
Sex female N (%)	28(41)	30 (52)	15(45)	10(53)	84(47)	0.6 §
Age median (IQR)	35 (28-44)	42(31.75-57)	35 (28.5-42.5)	43 (33-48)	38 (29-47)	0.02 #
BCG-vaccinated N(%)	49 (71)	*23 (40)	24 (73)	1 (5)	98 (54)	≤0.0001 #
Origin (%)						≤0.0001 #
West Europe	20 (29)	36 (62)	9 (27)	19(100)	84 (47)	
East Europe	26 (38)	17 (29)	13 (39)	0 (0)	55 (30.5)	
Asian	11 (16)	1 (2)	5(15)	0 (0)	19 (10.5)	
Africa	7 (10)	3 (5)	4(12)	0 (0)	14 (8)	
South America	5 (7)	1 (2)	2(6)	0 (0)	7 (4)	
Central America	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	

Footnotes: TB: tuberculosis; LTBI: latent tuberculosis infection; BCG: Bacillus Calmette et Guérin;
§ Kruskal Wallis test ; # Chi Square test ; *For one patient the BCG-vaccination status is not available

Concordance between QFT-GIT and QFT-Plus assays

First, we evaluated the accuracy of the QFT-Plus and QFT-GIT assays. The sensitivity of QFT-Plus in active TB cases, based on the response to either TB1 or TB2 (“either TB1 or TB2”), was 90% (62/69) (Table 2), the specificity calculated on the low TB risk population of healthy donors was 100% (19/19); similarly the sensitivity of the QFT-GIT assay was 88% whereas the specificity 100% (Table 2). The proportion of response to QFT-Plus were significantly different comparing TB vs LTBI group (p = 0.007) and LTBI vs cured TB (p = 0.02) (Table 3).

Agreement between QFT-Plus and QFT-GIT results was evaluated (Table 2). The concordance among all samples evaluated was substantial (k = 0.8). In active TB, a moderate agreement (k = 0.5) was achieved whereas for cured TB it was substantial (k = 0.7). In the LTBI group one patient scored positive by QFT-GIT resulted negative by QFT-Plus; it was not possible to evaluate the agreement because the QFT-GIT score was a constant.

Table 2: Concordance of QFT-GIT and QFT-Plus results

QFT-GIT vs QFT-Plus			
Groups of subjects	**Positive within the group over total N (%)**	**k**	**#p**
Active TB	61/69 (88) vs 62/69 (90)	0.5	<0.0001
LTBI	58/58 (100) vs 57/58 (98)	na	na
Cured TB	24/33 (73) vs 27/33 (82)	0.7	<0.0001
Healthy donors	0/19 (0) vs 0/19 (0)	na	na
Total patients	143/179 (80) vs 146/179 (81)	0.8	<0.0001

Footnotes: QFT: quantiferon, IT: in tube; k= Cohen's kappa coefficient; na: not available because the QFT-IT score is a costant; N:number; #Chi Square test.

Table 3: QFT-Plus response among the different groups of patients with or without TB.

QFT-Plus response to:	TB status						Comparisons		
	TB (N. 69)		LTBI (N. 58)		Cured TB (N. 33)		TB vs LTBI	TB vs cured TB	LTBI vs cured TB
	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	#p	#p	#p
either TB1 or TB2	7 (10)	62 (90)	1 (2)	57 (98)	6 (18)	27(82)	-	-	0.005
TB1 and TB2	13 (19)	56 (81)	2 (3)	56 (97)	6 (18)	27 (82)	0.007	-	0.02
only TB1	69 (100)	0 (0)	57 (98)	1 (2)	33 (100)	0 (0)	-	-	-
only TB2	63 (91)	6 (9)	58 (100)	0 (0)	33 (100)	0 (0)	0.02	-	-
TB1	13 (19)	56 (81)	1 (2)	57 (98)	6 (18)	27 (82)	0.002	-	0.005
TB2	7 (10)	62 (90)	2 (3)	56 (97)	6 (18)	27 (82)	-	-	0.02

Footnotes: TB : tuberculosis; LTBI: latent TB infection; # Chi Square test TB1: tube 1, TB2: tube 2; N:number

Analysis of the QFT-Plus results based on the response to TB1 and TB2 tubes

To analytically evaluate the response to the peptides contained in TB1 and TB2 tubes, we stratified the QFT-Plus results according to the ability of subjects to respond to both TB1 and TB2 (“TB1 and TB2”), only to TB1 (“only TB1”) or only to TB2 (“only TB2”). We found that almost all LTBI subjects (97%) responded to both “TB1 and TB2” while among TB patients and cured TB only 81% and 82% respectively responded. These proportions were significantly different comparing TB vs LTBI group (p = 0.007) and LTBI vs cured TB (p = 0.02) (Table 3).

Interestingly, a selective response to TB2 was found only among those with active TB (6/69, 9%).

Regarding the stimulation to TB1, the responders were: 81% within TB patients, 98% within LTBI and 82% within cure TB. These proportions were significantly different comparing TB vs LTBI group (p = 0.002) and LTBI vs cured TB (p = 0.005) (Table 3).

Regarding the cumulative response to TB2, the responders were: 90% within the TB patients, 97% in LTBI and 82% in the cured TB. These proportions were significantly different comparing LTBI vs cured TB group (p = 0.02) (Table 3).

Interestingly, in the active TB group a higher proportion of patients responded to TB2 compared to TB1 (90% vs 81%). The higher sensitivity of the TB2 stimulation was likely due to the CD8-specific peptides contained in the TB2 tube and consequently to the presence of Mtb-specific CD8 T-cells(26).

Impact of mycobacterial load and severity of TB disease on the QFT-Plus assay results

To evaluate the impact of the mycobacterial load on the immunological response to QFT-Plus, we stratified the active TB patients according to the microbiological diagnosis. Interestingly we found that the six patients showing an “only TB2” response had a microbiological diagnosis (Table 4).

Moreover, we investigated whether the severity of pulmonary TB disease had an impact on the response to QFT-Plus. Clinical severity was estimated evaluating the lung lesions based on the radiological findings. The radiological data from 63 pulmonary TB patients were analyzed: patients characterized by an intermediate/high radiological severity (grades 2 and 3) were combined and compared with those from patients with low radiological severity (grade 1). As shown in Table 5 the patients with intermediate/high radiological severity TB had a similar proportion of responders (91%) to either TB1 or TB2 compared to the patients with low radiological severity (88%). Interestingly, we found that the six TB patients showing “only TB2” response (Table 3) were classified as: 5 with high/intermediate radiological severity and only 1 with low radiological severity (Table 5).

These results suggest that CD8 T-cell response associates with the radiological severity of TB disease (Table 5) and with the mycobacterial load (Table 4).

Table 4: QFT-Plus response in active TB patients according to the microbiological results

QFT-Plus response to:	Clinical TB (N. 20)		Microbiologically confirmed TB (N. 49)	
	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)
either TB1 or TB2	4 (20)	16 (80)	3 (6)	46 (94)
TB1 and TB2	4 (20)	16 (80)	9 (18)	40 (82)
only TB1	20 (100)	0 (0)	49 (100)	0 (0)
only TB2	20 (100)	0 (0)	43 (88)	6 (12)
TB1	4 (20)	16 (80)	9 (18)	40 (82)
TB2	4 (20)	16 (80)	3 (6)	46 (94)

Footnotes: TB: tuberculosis; TB1: tube 1, TB2: tube 2; N:number

Table 5: QFT-Plus response in active pulmonary TB patients according to lung lesions severity

QFT-Plus response to:	Low severity TB (N. 17)		Intermediate/high severity TB (N. 46)	
	Negative response N (%)	Positive response N (%)	Negative response N (%)	Positive response N (%)
either TB1 or TB2	2 (12)	15 (88)	4 (9)	42 (91)
TB1 and TB2	3 (18)	14 (82)	9 (20)	37 (80)
only TB1	17 (100)	0 (0)	46 (100)	0 (0)
only TB2	16 (94)	1 (6)	41 (89)	5 (11)
TB1	3 (18)	14 (82)	9 (20)	37 (80)
TB2	2 (12)	15 (88)	4 (9)	42 (91)

Footnotes: TB : tuberculosis; grade 0: low severity; grade 1: intermediate and high severity; TB1: tube 1, TB2: tube 2; N: number

Comparison of IFN-γ production using QFT-Plus and QFT-GIT

We evaluated the results also by quantitative means (Fig. 1). Using the QFT-Plus, we found that in the TB group the median of TB1 response (1.9 IU/mL, IQR: 0.7–6.8) was significantly lower than that observed in LTBI (5.6 IU/mL, IQR: 2–10) ($p = 0.0007$). Similar results were obtained in response to TB2, the median in active TB (2.5 IU/mL, IQR: 0.9–7.5) was significantly lower than in LTBI (7.3 IU/mL, IQR: 1.9–10) ($p = 0.003$).

The cured TB group showed levels of IFN-γ production similar to that reported in the active TB (TB1: 1.9 UI/ml, IQR: 0.6–8.2 and TB2: 2.3 UI/ml, IQR: 0.5–8.2). Comparing the cured TB group with LTBI we found significant differences in response to TB1 (p = 0.016). These results suggest that LTBI subjects have a higher immunological ability to respond to Mtb stimulation compared to those that experienced a higher Mtb load. Regarding the QFT-IT, we found that the median production of IFN-γ in response to AgTB stimulation was higher, although not significant, in the LTBI (5.6 UI/mL, IQR: 2–10) than in active TB (2.6 UI/mL, IQR: 1–8) and significantly higher compared to cured TB subjects (1.6 UI/mL, IQR: 0.2–7) (p = 0.01).

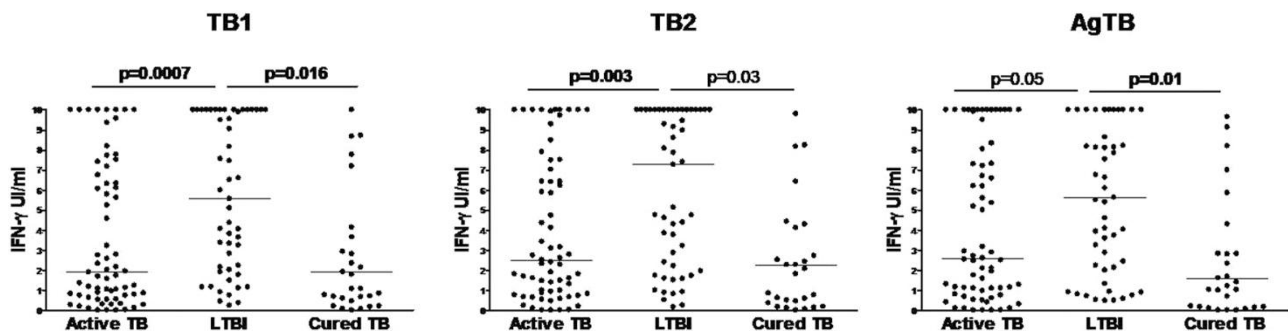

Figure. 1. Quantitative IFN-γ response to stimulation with QFT-Plus antigen TB1 and TB2 and QFT-GIT antigen AgTB. Horizontal lines indicate the median production. A $p \leq 0.016$ was considered significant after Bonferroni correction. The data are presented as IU/ml. Footnotes: IFN: interferon; IU: international unit.

7.5 Discussion

We evaluated in a low TB endemic country such as Italy, the accuracy of QFT-Plus in comparison to QFT-GIT, dissecting the response to TB1, TB2 and AgTB in a cohort of subjects with LTBI, active TB, cured TB and healthy donors. The accuracy for active TB detection of QFT-Plus was similar to that found for QFT-GIT. The two tests showed a substantial agreement and similar sensitivity in active TB patients and same specificity in the healthy donors. Interestingly, the majority of the LTBI subjects responded concomitantly to both QFT-Plus antigens TB1 and TB2 compared to the active TB (97% vs 81%); moreover, the response "only to TB2" was associated to active TB.

Based on the product information (22), TB1 contains long peptides eliciting a CD4 T-cell response whereas TB2, beside the same long peptides, contains additional short peptides specific for the CD8 T-cells. Therefore, when a selective response to TB2 is found, it is plausible to assume that the CD8 T-cells played a role in this antigen recognition otherwise, the response should have been observed also in response to TB1. Differently, if a simultaneously response to "either TB1 or TB2", is found, the reasonable scenario is that CD4 T-cells recognize the CD4 peptides present in TB1 and TB2 tubes. Therefore stratifying the QFT-Plus results according to the ability of subjects to respond or not simultaneously to TB1 and TB2, we found that among the LTBI subjects the majority of them responded to both TB1 and TB2 stimulation (97%). Based on the assay format, it is unknown whether the response to TB2 is mediated by the CD8 or CD4 T-cells.

Conversely, among the active TB patients showing “only TB2” response, it can reasonably be assumed that this response is mediated by CD8 T-cells. By cytometry this result could have been more refined, as we recently showed that the CD8 T-cells are mainly induced by TB2 and are associated to active TB (in 44%) although it may be found at a lower proportion also in LTBI (in 11%) (26).

Several studies have described that CD8 T cells play a unique function in the recognition and containment of intracellular infection with Mtb. CD8 T cells are important players to control the Mtb bacterial load, emerging in the presence of replicating Mtb and declining during TB treatment (15). Interestingly, in our study we did not observe any “only TB2” response after the completion of TB therapy, suggesting a loss of the CD8 T-cell response in parallel with the decrease of mycobacterial load. Therefore, investigation of the TB1 and TB2 response could be a springboard to find new tools to monitor the efficacy of TB therapy.

In line with the literature reporting a correlation between mycobacterial load and CD8 T-cell response (15), we found that patients showing a selective “only TB2” response had a microbiologically diagnosed TB. Interestingly, we showed that the majority of them had an intermediate/high level of TB severity, supporting the concept that CD8 T-cells limit bacterial survival and at the same time produce tissue damage(27). These data confirm recent findings generated by cytometry(26). Altogether, these results suggest that the Mtb load and consequently the lung damage may influence the ability to respond to the peptides specific for the CD8 T-cells, contained in the TB2 tube.

In those with active TB and cured TB a proportion of patients was scored as negative to QFT-GIT and QFT-Plus. This can be due in those with active TB group to a higher amount of specific cells at site of TB disease compared to peripheral blood (28,29); differently, in the cured TB to a decrease of Mtb load after therapy.

Looking at the quantitative levels of the IFN-γ produced, the LTBI subjects showed higher IFNγ levels in response to either TB1, or TB2 stimulation compared to the active TB patients. These results are mainly due to the absence of IFN-γ production in 7 patients with active TB.

We found that within the group of patients evaluated, the stimulation with either TB1, or TB2 or AgTB induced similar level of IFN-γ. Therefore, the absence of TB7 peptides in the QFT-Plus did not reduce the IFN-γ response. This finding is different from what recently reported in two studies performed in Japan and Germany in which it was described that the QFT-GIT induced higher level of IFN-γ compared to QFT-Plus in active TB patients (18,23) and the QFT-Plus induced higher IFN-γ amount in LTBI subjects (23). Regarding the German study, the data of QFT-Plus and QFT-GIT are related to the total cohort of patients with LTBI and TB, making difficult the comparison with our findings (18).

Using the QFT-Plus, we indirectly demonstrated that TB2 stimulation induces a CD8 T-cell response in absence of a CD4 T-cell response in the active TB patients. This ability to selectively induce a TB2 response could be potentially very useful in conditions of immune depression resulting from CD4 T-cell impairments. In line with this, it has been recently published a study demonstrating that human immunodeficiency virus (HIV) infection did not reduce the sensitivity of the QFT-Plus for active TB detection (30). Moreover, the

comparison of QFT-Plus and QFT-IT results in co-infected HIV-TB subjects, demonstrated the higher sensitivity of the QFT-Plus compare to QFT-IT (30,31).

Future studies on patients with different stages of Mtb infection, followed overtime during TB treatment could be useful to characterize distinct profile of Mtb-specific response to distinguish LTBI and active TB patients and to monitor TB therapy efficacy as previously suggested with different experimental settings (32–35) and as recently proposed with QFT-Plus assay (36).

In conclusion, this is the first report of the characterization of TB1, TB2 and AgTB response of the QFT-Plus and QFT-GIT assays respectively done in healthy donors and subjects with active TB disease, cured TB and LTBI. We demonstrated that the two tests have similar accuracy. Moreover, we indirectly demonstrated that TB2 stimulation induces a CD8 T-cell response in absence of a CD4 T-cell response in the active TB patients.

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CHAPTER 8: GENERAL DISCUSSION

New approaches to control tuberculosis worldwide are needed. In particular, new tools for diagnosis and new biomarkers are required to evaluate pathogen stage of infection and host response. Non-sputum based diagnostic tests, biomarkers predictive of adequate responsiveness to treatment, and biomarkers of risk of developing active TB disease are major goals. These tests need to perform in endemic settings with limited laboratory facilities, at low cost, using easily accessible non-sputum based samples such as blood, urine, or breath. These are the four high priority target product profiles (TPPs) recently published by the WHO as a result of a consensus meeting on new TB diagnostics (71). Therefore the urgent need to search for biomarkers (defined as measurable characteristics that indicate normal or pathogenic biological processes, or pharmacological responses to therapeutic intervention) (72) needs to be highlighted: biomarkers can serve as surrogate endpoints in clinical trials, and can be used to improve treatment outcome by informing therapeutic decisions for individual patients (73). By contrast, a diagnostic test classifies patients at a single time point as having active tuberculosis, latent infection with Mtb, or neither. Biomarkers can be either host or pathogen specific and tuberculosis-specific ones are needed to serve as surrogate endpoints, assisting candidate selection during drug discovery, accelerating dose selection in early clinical studies, shortening the time to licensing of new drugs, and development and assessment of new vaccines for tuberculosis (13).

Mtb is able to establish a chronic asymptomatically infection mainly in the lung and the balance between host immune response and the mycobacteria plays a fundamental role in the control of the Mtb replication (31,36). Although the immune response to Mtb has been deeply studied, as described in the previously chapters, the host factors that lead to the development of active TB disease are not fully understood. However, on the base of immunological findings on Mtb infection, it is possible to speculate that the containment of the latent Mtb needs an acquired cellular response with specific characteristics of immune surveillance, differently, contrasting the replicating Mtb requires effector and cytotoxic proprierty.

Moving from these hypothesis the studies reported in this manuscript have demonstrated the following findings:

- The loss of the CD27 significantly associates with active TB patients. This modulation has been described also in other studies with different experimental settings and in different countries (74,75). Therefore, this activation marker of Mtb specific T- cells has the required characteristics to be exploit as a TB biomarker for routine use.
- The QFT-Plus has a new tube containing peptides to stimulates both CD4 and CD8 T-cell response. Using a cytometer approach, we found that TB2 stimulation induced the CD8 T-cell response

preferentially in active TB patients. This assay has the potential to be very useful in conditions of immune depression resulting from CD4 T-cell impairments. Moreover, we found lower CD8-specific response in clinical TB compared to microbiologically diagnosed TB. This result suggests that the monitoring of the CD8 T-cell response to TB2 by flow cytometry can be a tool to specifically evaluate treatment efficacy.

- The comparison between QFT-Plus performance and that one of QFT-GIT has shown similar accuracy. Moreover, TB2 stimulation induces a CD8 T-cell response in absence of a CD4 T-cell response in the active TB patients as demonstrated by the presence of responders only to TB2 stimulation. In line with the literature reporting a correlation between mycobacterial load and CD8 T-cell response (76), we found that patients showing a selective "only TB2" response had a microbiologically diagnosed TB. Interestingly, we showed also that the majority of them had an intermediate/high level of TB severity, supporting the concept that CD8 T-cells limit bacterial survival and at the same time produce tissue damage (77). These data suggest that the TB2 response could be used to monitor the therapy and therefore the lung damage.

Even though the immunological studies have the limit to be useful only in the IGRA positive individuals, they could quickly describe an immunological profile possibly associated to TB and they could be used in patients usually tested negative to traditional sputum test (such as HIV positive subjects and children). As demonstrated by the study of Portevin, the CD27 modulation is able to diagnose TB in children in a high TB endemic country such as Tanzania (75). Moreover, monitoring CD27 expression has been used also to evaluate Mtb activity in HIV-1 infected individuals in vivo (78).
The available tools to monitor the efficacy of the TB therapy are the Mtb culture and the xRay evaluation, whereas there are not known correlates of protection to evaluate the efficacy of prophylaxis in LTBI subjects. Effective therapy is central to any strategy for controlling TB and biomarkers that indicate initiation of successful treatment could facilitate development of alternative treatment strategies. Considering the lack of correlates of protection, quick and easy to evaluate, to track the TB evolution, longitudinal study in patients followed overtime could be useful to characterize distinct immunological profile to monitor TB therapy and prophylaxis.

Although studies on new candidate biomarkers are numerous, validation and independent confirmation are rare. Efforts are needed to reduce the gap between the exploratory up-stream identification of candidate biomarkers, the validation of biomarkers against clear clinical endpoints in different populations, and the development of simple point of care tests for use in low resourced settings. This needs important commitment from both researchers and economic funders (15).

It is becoming clear that biomarkers, and the associations they generate, are probably specific and restricted to the population under study and might not necessarily be broadly applicable (3). Therefore, natural implication of biomarkers studies is the needed to validate the test in a larger population and in different geographical settings. In fact, patients from high TB endemic country could be exposed many times to Mtb, reflecting a different immune response to a repeated antigen stimulation. Moreover, different host genetic backgrounds and circulating pathogen lineages could influence the accuracy of the test (3).

To compare data from immunological test performed in different laboratory it is necessary to uniform the experimental settings. The choice of type of antigen (protein or peptide), the time of stimulation, the T-cell source (PBMC versus whole blood), the use of daily quality control to check the cytometer performance are all factors to be considered. Customized antibody specifically produced for the study could be useful to eliminate the bias due to the use of different fluorochrome to detect the antigen specific T cells.

The QFT-Plus is routinely used for the diagnosis of latent infection, the addition of peptides to elicit the CD8 response gives the possibility to deeply study the immune response to Mtb with a standardize IGRA. The QFT-Plus has been developed to improve the sensitivity of the QFT-IT stimulating also the CD8 T-cells, therefore the TB2 contains peptides able to induce IFN-γ production from both CD4 and CD8 T- cells. This characteristic of the test is an intrinsic limit for research purpose studying only the CD8 response, however it puts the accent on the needed of new antigen formulation to dissect the Mtb immune response. Moreover, the data obtained in PBMC stimulated with TB2 highlight the importance of CD8 T-cell subset during the Mtb infection, showing an association with the active TB disease that should be deeply studied in future studies evaluating also the T cell phenotype with activation and memory markers.

The study of biomarkers of TB is a topic of translational research of great interest for the scientific community. The NIH gives the following definition of translational research:

Translational research includes two areas of translation. One is the process of applying discoveries generated during research in the laboratory, and in preclinical studies, to the development of trials and studies in humans. The second area of translation concerns research aimed at enhancing the adoption of best practices in the community. Cost-effectiveness of prevention and treatment strategies is also an important part of translational science (79).

According to this definition, translational research is part of a unidirectional continuum in which research findings are moved from the researcher’s bench to the patient’s bedside and community. In the continuum, the first stage of translational research (T1) transfers knowledge from basic research to clinical research, while the second stage (T2) transfers findings from clinical studies or clinical trials to practice settings and communities, where the findings improve health (79).

The study of immunological profile of Mtb infected subjects belongs to the first stage of translational research. A recent example of the application of translational research to a routine test is the development of the QFT-Plus and QFT-IT. In fact, the studies on the CD8 response (76,80) have suggested to stimulate the CD8 response to improve the accuracy of the QFT-IT and studies on Mtb specific response have inspired the development of the IGRA (49).

In conclusions, further intensified research efforts are required for ongoing biomarker discovery, evaluation and test development and translation of the test to a near-patient platform.

CHAPTER 9: REFERENCES OF CHAPTER 1, 4 AND 8

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