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NEW MOLECULAR DIAGNOSTIC AND IMMUNOLOGICAL TOOLS FOR TUBERCULOSIS RESEARCH

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Abbreviations

AFB: acid fast bacilli

APC: antigen presenting cells

BCG: bacillus Calmette-Guérin

CFP: culture filtrate protein

CMV: cytomegalovirus

ELISA: enzyme-linked immunosorbent assay

ELISPOT: enzyme-linked immunospot assay

ESAT: early secreted antigenic target

FoxP3: family transcriptional repressor p3

GITC: guanidine isothiocyanate

HIV: human immunodeficiency virus infection

IFN: interferon

IGRAs: interferon-gamma release assays

IL : interleukin

IP: inducible protein

LST: lymphocyte stimulation test

LTBI: latent tuberculosis infection

MCP-2: macrophage chemotactic protein 2

MIP: macrophage inflammatory protein

MoAb: monoclonal antibodies

NAA: nucleic acid amplification

NFAT: nuclear transcription factors of activated T-cells

PBMC: peripheral blood mononuclear cells

PHA: phytohaemagglutinin

PPD: purified protein derivative

Pt: patient

QF: QuantiFERON-TB Gold in-tube

RD: region of difference

ROC: receiver operator characteristic

TB: tuberculosis

TGF: transforming growth factor

Th: T helper

TNF: tumor necrosis factor

Tr-DNA: transrenal DNA

Treg: T regulatory cells

TST: tuberculin skin test

WBA: whole blood assay

WBE: whole blood ELISA

Abstract

Tuberculosis (TB) remains one of the world's leading causes of mortality due to a single infectious agent, with approximately 1.5 million deaths and 9.2 million new cases per year as estimated in 2006. It is assumed that about 5-10% of individuals infected with *M. tuberculosis* develop TB and the remaining 90-95% contain *M. tuberculosis* through their immune systems, but have a latent tuberculosis infection (LTBI).

To effectively control TB, it is essential to detect individuals with LTBI and to reliably diagnose active TB.
Conventional TB diagnosis continues to rely on smear microscopy and culture that have several known limitations in terms of both speed and sensitivity that delay the diagnosis and, consequently, hold-up TB treatment and increase the spread of infection in the community.

M. tuberculosis infection remains widespread, but the disease is generally limited to the primary infection stage. Patients with an immune defect or impaired immunity are more prone to develop the disease. In LTBI, the host immune response is capable of controlling the infection by the release of chemokines and cytokines produced by T helper (Th) cells, critical for the outcome of the infection. Several cells of the immune system are involved in the control of TB, from the macrophages and dendritic cells, called antigen presenting cells (APC) to the T cells, CD4, CD8, γδ T cells. Activation of these cells with excessive pro inflammatory responses can lead to tissue damage, with the need of mechanisms to counteract this, such as Th2 and T regulatory cells (Treg)-mediated responses. The optimal scenario would therefore seem to have balanced Th1, Th2 and Treg response, suited to the immune challenge. The balance between these types of response is reflected in the resultant host resistance against infection.

Therefore the aims of the thesis were to find new approaches for diagnosis of active TB (First Part) and LTBI (Second Part). In this work we wanted to explore the immune mechanisms of TB pathogenesis with particular focus on the impact of Treg on suppressing *M. tuberculosis*-specific response (Third Part).

For the diagnosis of active TB, we describe an alternative PCR methodology based on the amplification of small DNA fragments, originated from cells dying throughout the body (transrenal DNA; Tr-DNA) and detected in urine. It was found that small *M. tuberculosis* DNA fragments were specifically detected in the cell-free fraction of urine specimens from pulmonary TB patients.

To detect LTBI, we compared the performances of two short-incubation interferon (IFN)-γ release assays (IGRAs), the commercial QuantiFERON TB-Gold and the in-house whole blood stimulation with region of difference (RD)-1 proteins, with those of a 7-day whole blood stimulation and tuberculin skin test (TST). In an effort to find new markers for LTBI diagnosis, we also evaluated the production of pro-inflammatory cytokines [interleukin (IL)-1β, IL-2, IL-6 and Tumor Necrosis Factor (TNF)-α], anti-inflammatory cytokines (IL-4, IL-10, IL-13) and chemokines [inducible protein (IP)-10, Macrophage Inflammatory Protein (MIP)-α, MIP-1β, IL-8] after specific stimulation. The results raise the hypothesis that short-incubation IGRAs mainly detect recent or ongoing infection with *M. tuberculosis*, while prolonged-incubation IGRAs seem to be more sensitive for the diagnosis of past latent infection. Moreover we found that IL-2 and IP-10 may be additional markers for TB infection after RD1 specific stimulation.

Finally we wanted to evaluate the impact of Treg on suppressing *M. tuberculosis*-specific response.
Using classical markers for Treg recognition, discordant results were found in terms of Treg expansion during active TB disease. Recently CD39 has been shown to be an accurate marker for Treg detection. Objectives of this part of the thesis were: 1) to identify Treg expressing CD39 in patients with TB and to compare the results with those obtained by the standard phenotypic markers; 2) to evaluate if Treg are expanded *in vitro* by exogenous IL-2 or by antigen-specific stimulation; 3) to characterize Treg function on the modulation of antigen-specific responses. In this study we demonstrated that CD39 is a useful marker to detect Treg because within $CD4^{+}CD25^{high}$ cells, it identifies a cell subset characterized by high production of transforming growth factor (TGF)-β1 and the absence of IFN-γ expression. Moreover, we showed that $CD39^{+}$ Treg are expanded by *M. tuberculosis*-specific stimulation in patients with active TB disease.

Introduction

Tuberculosis (TB) remains one of the world's leading causes of mortality due to a single infectious agent, with approximately 1.5 million deaths and 9.2 million new cases per year as estimated in 2006 [1]. It is estimated that worldwide one third of the human population is infected with the causative agent *Mycobacterium tuberculosis* and is therefore at risk of developing the disease. Once infected, active disease develops in about 5-10% of cases, usually within 1–2 years after exposure. Remaining individuals enter into a state of latentcy [latent tuberculosis infection (LTBI)], which can reactivate at a later stage, particularly if the individual becomes immunocompromised [2].

M. tuberculosis usually attacks the lungs (pulmonary TB) but can also affect the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, the gastrointestinal system, bones, joints, and even the skin. Other mycobacteria such as *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, and *Mycobacterium microti* also cause TB, but these species are less common [3].

The classic symptoms of TB are a chronic cough with blood-tinged sputum, fever, night sweats, and weight loss. Infection of other organs causes a wide range of symptoms. The diagnosis relies on radiology (commonly chest X-rays), a tuberculin skin test (TST), blood tests, as well as microscopic examination and microbiological culture of bodily fluids. TB treatment is difficult and requires long courses of multiple antibiotics. Contacts are also screened and treated if necessary. Antibiotic resistance is a growing problem in multi-drug resistant TB. Prevention relies on screening programs and vaccination, usually with bacillus Calmette-Guérin (BCG) vaccine [3].

The distribution of TB is not uniform across the globe with about 80% of the population in many Asian and African countries testing positive in tuberculin tests, while only 5-10% of the US population testing positive [1].

Diagnosis of TB

Active TB disease

Given the infectious nature of pulmonary TB, fast and accurate diagnosis is an important element for TB treatment and control.

TB control is based on the consequent use of preventive chemotherapy in individuals LTBI who are at risk of developing active disease and on the rapid diagnosis and effective treatment of infectious cases [4-6]. Conventional TB diagnosis continues to rely on smear microscopy, culture and chest radiography. These tests have several known limitations [7]:

- rapid alcohol acid fast bacilli (AFB) detection can be performed on sputum smears, but diagnosis of infectious cases by sputum microscopy is only possible in approximately 50% of cases, both field and high technology-based laboratories [2]
- culture may require a long period up to 6–8 weeks for the final results
- chest radiography is not available everywhere.

The sub-optimal performances of existing diagnostic tools in terms of both speed and sensitivity [8], delays diagnosis and, consequently, treatment of active TB.

For diagnosis of active TB tests are being developed that offer the hope of cheap, fast and more accurate TB testing. These include newer versions of nucleic acid amplification (NAA) tests, immune-based assays and rapid culture systems. Although the ideal test for TB is still not in sight, substantial progress has been made in the past decade [7].

NAA tests have emerged with the intended goal of enabling clinicians to make a rapid and accurate diagnosis. PCR is the best-known NAA test. All NAA tests amplify target nucleic acid regions that uniquely identify the *M. tuberculosis* complex. An important advantage of NAA tests is the rapidity by which the results can be obtained, about 3–6 h from receipt of specimen. Using PCR in pulmonary TB, specificity of diagnostic tests based on direct identification of *M. tuberculosis* in the sputum, generally exceeds 98% and sensitivity is also high in patients whose sputum smear is positive for acid-fast bacilli on microscopic examination. However, the sensitivity of such tests may be less than 50% for patients with negative sputum smear [9,10]. On the other hand, a highly sensitive molecular-based test for the detection of *M. tuberculosis* remains a goal of great importance for the control and eventual eradication of the disease worldwide.

Advances in knowledge of the genetic structure of tubercle bacillus have recently contributed to the development of several new molecular methods for detection and identification of *M. tuberculosis* from cultures or directly from biological specimens [9].

In an attempt to develop a more rapid, sensitive and reliable diagnostic test, some studies have been performed to evaluate the possibility of identifying the bacteria in the urine of patients with pulmonary TB by testing for the

presence of specific DNA sequences. This approach appears to be attractive because urine specimens are easier and safer to collect than sputum which can generate infectious aerosols and prove to be difficult to obtain especially in children.
Recently, Botezatu et al. [11] reported small cell-free DNA fragments (150 to 200 bp) in urine that originating from cells dying throughout the body. Since all evidence suggests that this DNA appears in the urine by crossing the renal barrier, it was named transrenal DNA (Tr-DNA) in order to differentiate these DNA fragments from those of high molecular weight that are known to be associated with urine sediment [12].
We report here the first demonstration that *M. tuberculosis*-specific DNA sequences detected as short fragments of less than 200 bp in the soluble fraction of urine specimens from patients with pulmonary TB. The evidence presented further demonstrates that these specific DNA fragments disappear following successful TB treatment.

Latent TB infection

Until recently, the only available assay for LTBI identification was TST. It is based on a delayed-type hypersensitivity response to purified protein derivative (PPD), a rough culture supernatant of *M. tuberculosis* and false-positive results can occur due to cross-reactive immune responses to homologous proteins in *M. bovis* BCG or environmental mycobacteria.
Several immunodiagnostic assays, based on interferon (IFN)-γ release (IGRAs), have been developed for the diagnosis of *M. tuberculosis* infection. Their high specificity is based on the use of region of difference (RD)-1 gene products that are specific for *M. tuberculosis* [Early Secreted Antigenic Target (ESAT)-6 and Culture Filtrate Protein (CFP)-10] and which are absent in BCG and most environmental mycobacteria. This reagents' specificity of IGRAs overcomes the problem of cross-reactive immune responses. The first demonstration of the performance of IGRA was described using in a 6-day lymphocyte stimulation test (LST) the IFN-γ response to ESAT-6 and CFP-10 [13-16]. Afterward other IGRAs were developed that differed from the classical LST with respect to the in vitro incubation period, the type of cells cultured (whole blood, frozen or fresh peripheral blood mononuclear cells [PBMCs]), and the way that the IFN-γ response is detected (by enzyme-linked immunosorbent assay [ELISA] or enzyme-linked immunospot assay [ELISPOT]).

The evaluation and comparison of new diagnostic assays for the detection of LTBI have been hampered by the lack of a "gold standard" and, therefore, the inability to reliably calculate their sensitivities and specificities. Most studies used the level of exposure as a surrogate marker for infection, and discrepancies between TST and IGRAs were mostly attributed to prior BCG vaccination [17-19]. However, data from recent studies [20-22] indicate that this explanation may not account for all discrepant results, as a substantial group of BCG-unvaccinated persons with TST indurations of >15 mm had negative results by commercially available IGRAs, the QuantiFERON-TB Gold in-tube (QF) test and/or the T-SPOT. TB test (Oxford Immunotec, Abingdon, United Kingdom) [23,24].
Moreover recently it has been shown that IFN-γ, inducible protein 10 (IP-10) and macrophage chemotactic protein 2 (MCP-2) can be considered as additional markers for LTBI detection after RD1-specific stimulation [21,22,25].
In the present study I further evaluated the latter observation by comparing the performances of two short-incubation IGRAs, the commercial QF and the in-house whole blood stimulation with RD1 proteins, with those of a 7-day whole blood stimulation and TST for the diagnosis of LTBI. Moreover in an effort to find new markers to detect LTBI I evaluated the production of pro-inflammatory cytokines [interleukin (IL)-1β, IL-2, IL-6 and Tumor Necrosis Factor (TNF)-α], anti-inflammatory cytokines (IL-4, IL-10, IL-13) and chemokines [IP-10, Macrophage Inflammatory Protein (MIP)-α, MIP-1β, IL-8) after specific stimulation.

Pathogenesis of TB

Mycobacterium tuberculosis is an obligatory aerobic, intracellular pathogen, which has a predilection for the lung tissue rich in oxygen supply. The tubercle bacilli enter the body via the respiratory route.
TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within the endosomes of alveolar macrophages. The primary site of infection in the lungs is called the Ghon focus, and is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe. Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymph nodes. Further spread is through the bloodstream to other tissues and organs where secondary TB lesions can

develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone. All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid [3].
TB is classified as one of the granulomatous inflammatory conditions. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma, with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the mycobacteria, but also provides a local environment for communication of cells of the immune system. Within the granuloma, T lymphocytes ($CD4^+$) secrete cytokines such as IFN-γ, which activates macrophages to destroy the intracellular bacteria. T lymphocytes ($CD8^+$) can also directly kill infected cells [26].
Importantly, bacteria are not always eliminated within the granuloma, but can become dormant, resulting in a latent infection. Another feature of the granulomas of human TB is the development of cell death, also called necrosis, in the center of tubercles. To the naked eye this has the texture of soft white cheese and was termed caseous necrosis.
If *M. tuberculosis* bacteria gain entry to the bloodstream from an area of damaged tissue they spread through the body and set up many foci of infection, all appearing as tiny white tubercles in the tissues. This severe form of TB disease is most common in infants and the elderly and is called miliary TB. Patients with this disseminated TB have a fatality rate of approximately 20%, even with intensive treatment. In many patients the infection waxes and wanes. Tissue destruction and necrosis are balanced by healing and fibrosis. Affected tissue is replaced by scarring and cavities filled with cheese-like white necrotic material. During active disease, some of these cavities are joined to the air passages bronchi and this material can be coughed up. It contains living bacteria and can therefore pass on infection. Treatment with appropriate antibiotics kills bacteria and allows healing to take place. Upon cure, affected areas are eventually replaced by scar tissue [3].

About 90-95% of the people infected with *M. tuberculosis* have asymptomatic, latent TB infection, with only a 10% lifetime chance that a latent infection will progress to TB disease. However, if untreated, the death rate for these active TB cases is more than 50% [27].
Host immune responses are necessary to control *M tuberculosis* growth by the release of chemokines and cytokines produced by T helper (Th) cells.

Several cells of the immune system are involved in the control of TB, from the macrophages and dendritic cells, called antigen presenting cells (APC) to the T cells, CD4, CD8, γδ cells. Activation of these cells with excessive pro inflammatory responses can lead to uncontrolled tissue damage, with the need of mechanisms to counteract this, such as Th2 and T regulatory cells (Treg)-mediated responses. The optimal scenario would therefore seem to be that humans should produce a well balanced Th1, Th2 and Treg response, suited to the immune challenge. The balance between these types of response is reflected in the resultant host resistance against infection. The mechanisms responsible for insufficient T cell-dependent protection *M. tuberculosis* infection remain unclear, but Treg may be involved.

Impact of Treg on suppressing *M. tuberculosis*-specific response

Treg play an important role in immune regulation to prevent autoimmunity diseases and to control the immune responses by down-regulating the function of effector $CD4^+$ or $CD8^+$ T-cells [28]. The role of Treg in TB infection and persistence is inadequately documented. Therefore, the current study was designed to evaluate the impact of Treg on suppressing *M. tuberculosis*-specific response. The result of this study can be important to understand the pathogenesis of *M. tuberculosis* and potentially to find new tools for TB immune diagnosis that may overcome suppressed immune responses.

It is believed that Treg down-modulate immune responses after pathogen eradication to avoid exacerbated pathology. Although this mechanism is generally to the benefit of the host in acute infections, it poses problems in chronic infections, notably when pathogen persistence is sustained in the face of an active immune response.

Treg comprise multiple subsets: naturally occurring thymus-derived $CD4^+CD25^{high}$ T-cells, that have the ability of cell-contact dependent suppression of immune response, and different subsets of adaptive Treg, such as Tr1 cells secreting high levels of IL-10, and Th3 cells which produce high levels of transforming growth factor (TGF)-β1 [29,30]. It has been shown that several factors such IL-2, IL-10 and TGF-βı are involved in the generation and maintenance of Treg [31].

Natural Treg constitutively express CD25 [α chain of IL-2 receptor], they are identified as CD4 T-cells with high expression of CD25 and they have regulatory properties. This fact introduced certain difficulties and confusion

in distinguishing Treg from conventional non-regulatory activated $CD4^+CD25^+$ T-cells. At present, the intracellular forkhead winged-helix family transcriptional repressor p3 (FoxP3) is described as the most specific marker of Treg. FoxP3 expression well correlates with regulatory activity, it is expressed in $CD4^+CD25^+$ Treg and is considered a key player for their development and function [32]. FoxP3 represses IL-2, IL-4 and IFN-γ gene expression and interacts with nuclear transcription factors of activated T-cells (NF-kB, NFAT) resulting in poor cytokine production and impaired proliferation [33]. However, the FoxP3 marker is present also in activated T-cells [34]. Moreover, recent studies have shown that lack or low expression of CD127 (the α chain of the IL-7 receptor), is linked with Treg identification similar to $CD4^+CD25^+FoxP3^+$ T-cells [35]. Recently CD39, an ectoenzyme that degrades ATP to AMP, has been shown to be specifically associated to Treg, as described in inflammatory autoimmune diseases [36].
Treg have been implicated in infectious diseases, particularly in chronic or persistent infections [29,37]. Discordant results were found *ex vivo* in terms of Treg expansion during active TB disease, some authors reporting an increase of $CD4^+CD25^+FoxP3^+$ T-cells whereas others reporting absence of modulation of this T-cell subset [28,38-41]. Thus, the objectives of this study were to: 1) identify Treg expressing CD39 in patients with TB and compare the results with those obtained by the standard phenotypic markers; 2) to evaluate if Treg can be expanded *in vitro* by exogenous IL-2 or by antigen-specific stimulation with RD-1 proteins; 3) to characterize Treg functions in the modulation of antigen-specific responses.

Aims

The aims of the thesis were to find new approaches for diagnosis of active TB (First Part) and LTBI (Second Part). Moreover, we wanted to explore the immune mechanisms of TB pathogenesis with particular focus on the impact of Treg on suppressing *M. tuberculosis*-specific response (Third Part).

Materials and methods

First Part: New tools to detect active tuberculosis infection: *Mycobacterium tuberculosis* DNA in soluble fraction of urine specimens

Study population

All study participants were enrolled at the National Institute for Infectious Diseases (INMI) "L. Spallanzani". In this study we included patients who had been diagnosed with pulmonary TB as confirmed by the tuberculin skin test and isolation of *M. tuberculosis* from sputum culture. Nineteen of the 20 patients had sputum smear positive for acid-fast bacilli, whereas, only one was HIV positive. No patient exhibited clinical evidence of extra-pulmonary involvement, and all urine cultures for *M. tuberculosis* performed were negative. Urine specimens were obtained either before or within one week of initiation of anti-TB therapy. Information regarding age, sex, ethnicity, *M. bovis* BCG vaccination, and results of laboratory examinations for TB were collected. In order to determine whether subsequent therapy had an influence on the ability to detect *M. tuberculosis* DNA, 8 of the patients with pulmonary TB were asked to return to donate urine specimens two months after initiating anti-TB therapy. In addition, ten healthy individuals were included as controls in the study.
This study was approved by the Institutional Review Board at INMI and all study participants gave written informed consent in accordance with applicable guidelines.

Urine specimen preparation

Urine specimen preparation for Tr-DNA analysis was carried out at ambient temperature. Approximately 50-60 ml of urine was collected from each individual in the study. To minimize degradation of soluble DNA by nucleases that may be present in urine, 0.5M EDTA-0.5M Tris-HCl, pH 8.5, was added to a final concentration of 10 mM within 30 minutes of collection. The stabilized urine specimens were then stored in 5 ml aliquots at -80^{o}C. In instances where it was necessary to separate the soluble urine

fraction from sediment, fresh specimens were centrifuged for 20 min at 4000xG and the supernatant and pellet stored separately at -80^{0}C.

DNA isolation

DNA isolation from urine was carried out according to the protocol developed in our laboratory [11,42] specifically developed to optimize isolation of small DNA fragments. Briefly, 2 volumes of 6 M guanidine isothiocyanate (GITC) were added to 5ml of un-fractionated urine or separately to supernatant and pellet fractions, and mixed vigorously. DNA was captured by Wizard Resin (Promega, Milano, Italy) and after extensive washing was eluted with water or 10 mM Tris-HCl, pH 7.5-1mM EDTA. In comparison with other techniques and commercially available kits this protocol was found to efficiently isolate low molecular weight DNA fragments (<200 bp).

PCR primer design

PCR primers for the detection of *M. tuberculosis* complex species DNA were selected for the region of IS6110 shown to be highly specific [43]. For primer design FastPCR software was used (see http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm). Nested primers were designed using Primer 3 package available online (see also http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers were screened against the complete sequence of human genome. Primers used in this study are presented in Table 1.

ID	Sequence	Location*	Product (bp)
F-785	**ACCAGCACCTAACCGGCTGTGG**	**785**	**129**
R-913	**CATCGTGGAAGCGACCCGCCAG**	**913**	
Rn-851	**GTAGGCGAACCCTGCCCAGGTC**	**851**	**67**
F-489	**GCCCCATCGACCTACTACG**	**485**	**330**
R-819	**TGAGGTCTGCTACCCACAGC**	**819**	
Fn-627	**CCCTGAACCGTGAGGGCATCG**	**627**	**69**
Rn-690	**ACAGGCCGAGTTTGGTCATCAGC**	**690**	

Table 1: Design of primers employed in the study. (*) Nucleotide numbering is based on the consensus created from the alignment of IS6110 elements nucleotide sequences.

PCR amplification and electrophoresis analysis

Twenty cycles of PCR amplification were performed as following: isolated DNA equivalent to that contained in 300 µl of urine was added to a 25 µl mixture containing 0.2 µmol/liter of outer primers F-785 and R-913, 5 µl 5X PCR buffer (Promega), 200 µmol/liter dNTP each, and 1 U GoTaq Polymerase (Promega), denatured at 94°C for 5 minutes followed by 20 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute, and 1 cycle of 72°C for 5 minutes. 1 µl of the product from this amplification was diluted 1:10 and 1 µl of the dilution was re-amplified 35 cycles using primers F-785 and Rn-851 under the same conditions as in the first reaction. Identical setting was used for the amplification of large, 330 bp fragment.
The products from the second amplification were resolved by electrophoresis in 7% polyacrylamide gel, and visualized by ethidium bromide staining.

Second Part: New tools to detect latent tuberculosis infection: evaluation of memory responses and new biomarkers in response to RD1 antigens in TST$^+$ subjects exposed to *Mycobacterium tuberculosis*

Study population

In a four-years period we enrolled in National Institute for Infectious Diseases (INMI) "L. Spallanzani" a heterogeneous group of persons with presumed recent or more remotely acquired latent TB infection: a group of close contacts of patients with a sputum culture positive for *M. tuberculosis* and a group of TST$^+$ homeless known to have been exposed to *M. tuberculosis* in the past sent to a screening for LTBI to the pneumology division of the National Institute for Infectious Disease "L. Spallanzani" in collaboration with the Institute of Hygiene, Catholic University, for a project of TB surveillance in Rome.
Participants underwent a TST on the day of blood sampling (see below). We included in the present analysis individuals who tested TST$^+$ that provided a written consent to the study. Individuals who were known to have human immunodeficiency virus infection (HIV-1) or who had received treatment with immunosuppressive drugs were not eligible for inclusion in the study. Upon enrolment demographic and epidemiological information were collected by the physician through a structured questionnaire, including information about BCG vaccination.

TST

TST was administered by the Mantoux procedure using 5 IU of PPD (Chiron, Siena, Italy). Results were read after 72 hours. Induration of at least 5 mm was considered a positive response for group of close contacts and a reaction ≥10mm was considered positive for homeless.

Whole blood Assay (WBA) based on proteins at day 1 and day 7: Effector and Memory response

Effector response

For detecting an effector response, 0.5 ml per well of heparinized whole blood was seeded in a 48-well plate and treated with PPD at 5 μg/ml (batch RT 47, Staten Serum Institut, Copenhagen, Denmark), RD1 intact proteins at 0.2 μg/ml (Lionex, Braunschweig, Germany) and Phytohaemagglutinin (PHA) at 5 μg/ml (Sigma, St Louis, MO, USA). Samples were then incubated for 24 hours. On day 1 the plasma was harvested and cold storaged until they were tested (+4°C or if used after 15 days at -20°C).

Memory response

For detecting a whole blood memory response we used a previously reported methodology with some modification [44]. Briefly at the day of sampling an aliquot of blood was diluted 5-fold using RPMI 1640 supplemented with penicillin, streptomycin and 2mM L-glutamine and was plated into 48-well plates [44] and stimulated as above described. The day-7 diluted plasma was harvested following incubation at 37°C.

Cytokine and chemokine determination

IFN-γ

IFN-γ results from day-1 and day-7 plasma were evaluated by a commercial ELISA (CMI, Cellestis Limited, Carnegie, Victoria, Australia) and are presented as IU/ml after subtraction of the appropriate control. Cut-off values were previously determined by constructing a Receiver Operator Characteristic (ROC) curve by means of LABROC-1 software and were 0.7 IU/mL for all stimuli [45, 46]. For day-7 diluted plasma the same IFN-γ cut-off value was used and the data are shown considering the dilution factor. Moreover for IFN-γ values above 10 IU/ml serial dilutions of plasma were performed.

IL-1β, IL-2, IL-6, TNF-α, IL-4, IL-10, IL-13, IP-10, IL-8, MIP-1 α, MIP-1β

IL-1β, IL-2, IL-6, TNF-α, IL-4, IL-10, IL-13, IP-10, IL-8, MIP-1 α, MIP-1β levels in plasma from day 1-cultured whole blood was evaluated by

xMAP multiplex technology on the Luminex platform (Luminex, Austin TX, USA), using Biosource reagents (Biosource, Camarillo, USA) acquired and analyzed with the STarStation v2.0 software (Applied Cytometry Systems, United Kingdom). Responses were scored positive if the value was 2 fold over the negative control.

Commercially available assay

QF (Cellestis Limited, Carnegie, Victoria, Australia) was performed and its results were scored as indicated by the manufacturer (cut-off value for a positive test was 0.35 IU/ml). For IFN-γ values above 10 IU/ml serial dilutions of plasma were performed.

Statistical analysis

The main outcome of the study was the evaluation of IFN-γ production in response to mitogen (PHA) and antigenic stimulation in the QF and whole blood ELISA (WBE), expressed as dichotomous (positive/negative) and continuous (IU/ mL) measures. IFN-γ median and range were calculated. The Mann-Whitney U test was used to compare continuous variables, and Chi square or McNemar tests were used for categorical variables. Analysis was carried out with SPSS v 14 for Windows (SPSS Italia srl, Bologna, Italy).

Third Part: Characterization of regulatory T-cells identified as $CD4^{+}CD25^{high}CD39^{+}$ in patients with active tuberculosis

Study population

Thirteen patients with newly diagnosed pulmonary TB and twelve healthy control subjects were recruited at the National Institute for Infectious Diseases (INMI) "Lazzaro Spallanzani". The study was approved by the Ethics Committee of the Institute, and all enrolled individuals provided written informed consent. All the patients included in the study were sputum culture positive for *M. tuberculosis*, tested negative for HIV infection and

did not receive immunosuppressive drugs. Healthy subjects were chosen if: i) no exposure to *M. tuberculosis* was reported; ii) negative response to the QuantiFERON-TB Gold was shown; iii) negative TST scoring. Characteristics of all participants are shown in Table 6.
Patients with active TB were studied within 7 days of admission and before they started anti-TB therapy. For each enrolled participant, a blood sample was drawn into tubes containing heparin.
TST was administered by the Mantoux procedure using 5 IU of PPD (Chiron, Siena, Italy). Results were read after 72 hours. Induration of at least 10 mm was considered a positive response [47] .
QuantiFERON-TB Gold In-Tube (Cellestis Limited, Carnegie, Victoria, Australia) was performed and its results were scored as indicated by the manufacturer (cut-off value for a positive test was 0.35 IU/mL).
In a group of patients we also evaluated Cytomegalovirus serology by VIDAS CMV IgG and IgM (Biomerieux, Marcy l'Etoile, France).

Isolation of peripheral blood mononuclear cells and cultures

PBMC were isolated from whole blood using Ficoll density gradient centrifugation. After washing, PBMC at a concentration of $1x10^6$ cells/mL were cultured for 6 days at 37°C and 5% CO_2 in a complete medium (RPMI, 10% heat-inactivated human serum, 10mM HEPES, 2mM *L*-glutamine and 10 U/mL penicillin-streptomycin, all from Euroclone Ltd, United Kingdom). Cultures were prepared in 48-well tissue-culture plates (Costar, Corning Inc, NY, USA) in 1 mL/well. PBMC were stimulated with *M. tuberculosis*-specific antigens identified as ESAT-6 and CFP-10 (RD1) intact proteins (Lionex, Braunschweig, Germany) at 4 μg⁄mL in the presence or absence of IL-2 (Chiron) at 5 U/mL.

Depletion of $CD4^+CD25^+$ T-cells

In some experiments, $CD4^+$ T-cells were isolated from PBMC by a negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany), then $CD4^+CD25^+$ T-cells were positively selected from $CD4^+$ T-cells using a human $CD4^+CD25^+$ regulatory T-cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Approximately 90% of Treg were eliminated after depletion, as determined by flow cytometry analysis.

The cell fractions "$CD4^-$"and "$CD4^+CD25^-$"were pooled and used as Treg-depleted PBMC.

Cytokine detection

From PBMC different cell subsets were sorted (MoFlo, Beckman Coulter, Fullerton, CA) based on the expression of CD39 on $CD25^{high/low}$ $CD4^+$ T-cells, and were used to determine TGF-β1 production; B cells ($CD19^+$ cells) and monocytes ($CD14^+CD3^-$ cells) were used as control. The different cell subsets were *in vitro* stimulated for 1 day with anti-CD3 and anti-CD28 antibodies (Becton Dickinson). TGF-β1 and IL-10 release was evaluated by ELISA (R&D Systems, Minneapolis, MN, USA).
IFN-γ in the cell cultures' supernatants was evaluated by ELISA according to the manufacturer (QuantiFERON-CMI, Cellestis Limited, South Melbourne, Australia). Cytokines results were expressed as pg/mL.

Antigen-specific response evaluation.

Freshly isolated Treg-undepleted PBMC and Treg-depleted PBMC from TB patients were cultured in 96-well plates at a concentration of 2×10^5 cells per well in 250 μL of complete medium. The cells were stimulated with *M. tuberculosis*-specific RD1 intact proteins (Lionex), phytohemagglutinin (PHA) at 5 μg/mL (Sigma Aldrich, St Louis, MO, USA), and Cytomegalovirus (CMV) lysate (StrainAD 169, ABI Inc, Columbia, MD, USA) at 2 μg/ml and cultured for 3 days.

Phenotypic analysis

Phenotypic analysis of PBMC ($1x10^6$ cells/mL) was performed by flow cytometry either *ex-vivo* or after 1 and 6 days of culture in complete medium in the presence or absence of the stimuli as indicated above. Expression of different markers was assessed by staining with appropriate combinations of monoclonal antibodies (MoAb) directly conjugated to fluorochromes: fluorescein isothiocyanate (FITC)-conjugated anti-CD39 (Ancell, Bayport, MN, USA), phycoerythrin (PE)-conjugated anti-CD25 (Becton Dickinson), peridinin chlorophyll-protein complex (PerCP)-conjugated anti-CD4 (Becton Dickinson) and Alexa Fluor 647-conjugated anti-FoxP3 (Becton Dickinson).

To detect intracellular expression of IFN-γ, 10 μg/mL of brefeldin A (Sigma Aldrich) was used, as described previously [48]. Phorbol-12-myristate-13-acetate (PMA) plus ionomycin (Sigma Aldrich) were used as positive controls at 3 nM and 1,5 μM, respectively. Briefly, production of IFN-γ was assessed by staining with appropriate combinations of MoAb conjugated directly to fluorochromes. Data acquisition and analysis were performed on FACSCalibur flow cytometer (Becton Dickinson) using CellQUEST software (version 3.1; Becton Dickinson). For all staining procedures, an isotype-matched negative control was processed in parallel.

Statistical analysis

Statistical significance of results was determined with the statistics program included in the GraphPad Prism software (GraphPad). Statistical analysis was performed using Student's *t*-test to assess differences between the different study groups. The Wilcoxon matched pair *t*-test was used to analyze cytokines production by Treg. Differences were considered significant when the *p* value was less than 0.05.

Results

First Part: New tools to detect active tuberculosis infection: *Mycobacterium tuberculosis* DNA in soluble fraction of urine specimens

Characteristics of patients with TB included in this study are shown in Table 2. DNA was extracted from the urine of the patients as described in materials and methods, and examined to assess the presence of small cell-free *M. tuberculosis* DNA fragments using semi-nested PCR.

	Pulmonary tuberculosis n.20	Controls n.10
Mean age ± SE	34±4	28±1
Sex		
Females	7	5
Males	13	5
Ethnicity		
Western Europe	4	10
Eastern Europe	13	0
Africa	1	0
South America	2	0
BCG Vaccination	15	0
TST positive/ performed	12/16	0/0
Urine culture for MTB, positive/ performed	0/8	0/0

Footnotes: MTB : *M. tuberculosis*; TST: tuberculin skin test; SE: standard error; BCG: bacillus Calmette et Guerin.

Table 2: Epidemiological and demographic characteristics of patients with active pulmonary TB microbiologically diagnosed by sputum culture and healthy controls.

Results from semi-nested amplification are shown in Figure 1. The *M. tuberculosis*-specific product of 67 bp was found in urine specimens from all 20 patients analyzed. Ten healthy individuals used as controls were all found to be negative for the presence of *M. tuberculosis*-specific sequences.

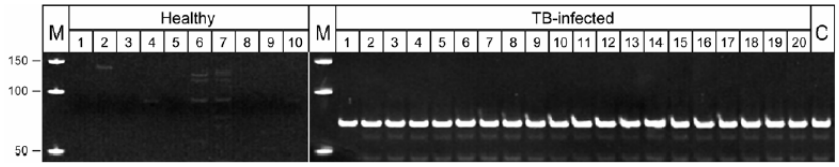

Figure 1: Semi-nested PCR products of DNA extracted from urine of 8 healthy individuals and 20 patients diagnosed with active pulmonary *M. tuberculosis* infection. Product is 67 base pairs, and semi-nested PCR was performed with species-specific primers. The last lane in both gels is genomic DNA from H37RV strain

In order to determine whether *M. tuberculosis*-specific DNA sequences were still detectable after completion of the initial phase of specific treatment, out of 8 patients with TB who had been found to be *M. tuberculosis* Tr-DNA positive at enrolment were asked to return to donate urine samples approximately two months following initiation of chemotherapy. At the time of the second test clinical symptoms had resolved in these patients and all sputum cultures were negative. Tr-DNA extracted from these samples was analyzed and no product corresponding to 67 bp specific *M. tuberculosis* sequence was observed (Figure 2).

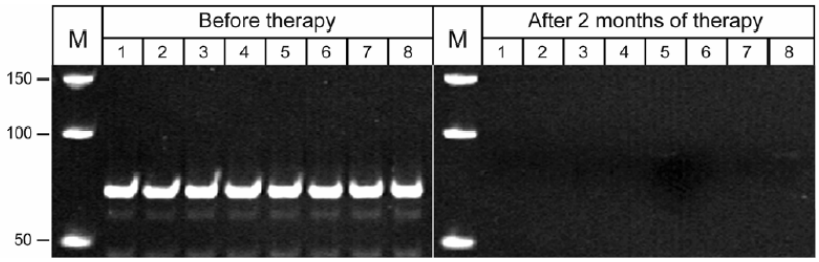

Figure 2: Semi-nested PCR products of DNA extracted from urine of 8 patients with active pulmonary TB performed before and after two months of drug therapy. *M. tuberculosis*-specific product is 67 base pairs as indicated

Previous reports on detection of *M. tuberculosis* DNA in urine employed isolation methods designed to extract high molecular weight DNA from whole urine or urinary sediment. Furthermore, in those studies large amplicons >200 bp were used in PCR reactions [49]. Therefore, it was important to further characterize the nature of the *M. tuberculosis* Tr-DNA found in our study, and to compare results with larger amplicon sizes similar to those used by others.

In order to address this question, two experimental designs were employed. In the first, specimens were centrifuged as described above and DNA was isolated separately from urine sediment and supernatant. The urine specimens from eight pulmonary TB patients were centrifuged at 4000xg and the supernatant was separated from the insoluble sediment. As shown in Figure 3, it was found that the 67 bp *M. tuberculosis*-specific bands were present in 7 of the 8 supernatants, whereas, only 2 of the 8 matched sediments exhibited positive bands.

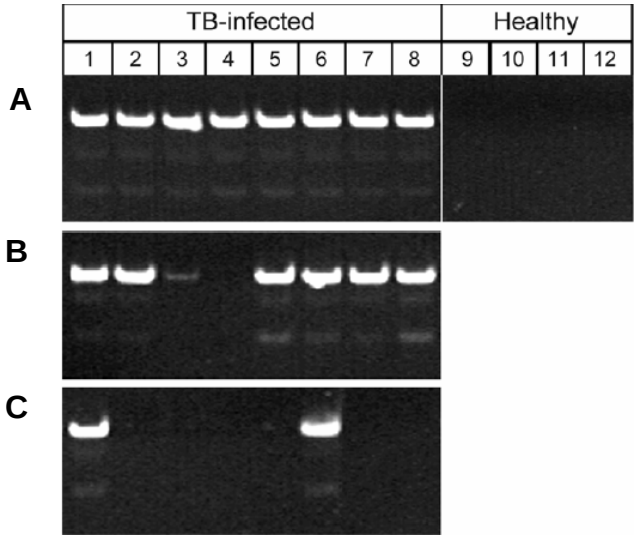

Figure 3: Semi-nested PCR for *M. tuberculosis* in whole urine (A) compared with products from DNA extracted from matched supernatant (B) and sediment (C) following centrifugation at 4000xg, 20 min at ambient temperature. The right 4 lanes are healthy individuals. Amplification product is 67 base pairs.

A second experimental design was based on the comparison of PCR results obtained with primers specific for a larger amplicon size used by several other investigators. Results presented in Figure 4 demonstrate that *M. tuberculosis* DNA sequences again were detected in all 7 samples of DNA that had been isolated from urine of pulmonary TB patients with primers for 129/67 amplicons. However, no PCR products were detected using primers for 330/69 amplicons. These data confirmed that bacterial DNA fragments extracted from urine are relatively short. Furthermore, these results strongly suggest that it is unlikely that *M. tuberculosis* DNA can be reproducibly detected in urine specimens of pulmonary TB patients using urine sediment PCR analysis combined with large amplicon sizes.

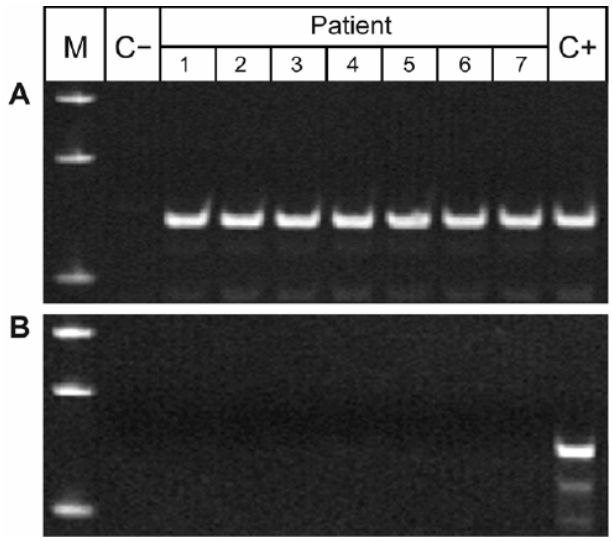

Figure 4: Semi-nested PCR performed on aliquots of urine DNA isolated from seven TB infected patients (Lanes 3-9) with primers designed for short 129/67 (A) and large 330/69 (B) amplicons (see Materials and methods). Lanes 1 in both gels shows the DNA standards, Lanes 2 are negative controls, and Lanes 10 are positive genomic DNA controls.

Second Part: New tools to detect latent tuberculosis infection: evaluation of memory responses and new biomarkers in response to RD1 antigens in TST$^+$ subjects exposed to *Mycobacterium tuberculosis*

Characteristics of the population.

We prospectively recruited 410 subjects to be screened for LTBI. Among them 337 were healthy contacts of patients with sputum smear positive pulmonary TB and 73 were TST$^+$ homeless subjects. Among the healthy contacts 209 resulted TST$^+$. Among the all TST$^+$ individuals studied, the laboratory testing was incomplete in 155 healthy contacts and in 21 homeless subjects. Therefore we analyzed the data only in 54 healthy contacts and 52 homeless (Figure 5 study flow diagram).

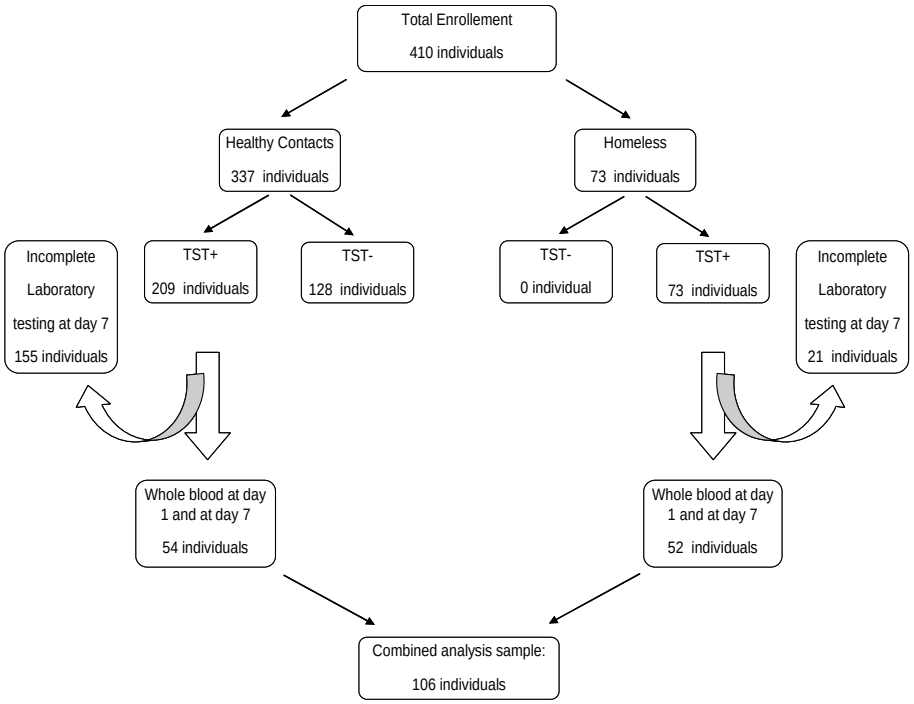

Figure 5: Flow chart of the subjects enclosed in the study.

The two study populations were statically different with regard to demographic factors as age ($p<0.05$) and sex ($p<0.0001$). Conversely, no significant differences were observed for the other features considered as origin, BCG vaccination and size of TST induration (Table 3).

	Healthy contacts	**Homeless**	**Total**
	N. (%)	**N. (%)**	**N. (%)**
	54 (51)	**52 (49)**	**106 (100)**
Female sex	31 (57.4)	2 (3.8)	33 (31.1)
Age Median	26	41.5	34
Origin			
Italy	32 (59.3)	17 (32.7)	49 (46.2)
Abroad	22 (40.7)	35 (67.3)	57 (53.8)
BCG vaccinated	19 (35.2)	19 (36)	38 (35.8)
TST Median in mm	25	17	20

Table 3: Characteristics of study subjects

In vitro responses to PPD, RD1 proteins and QF at day 1 post-*in vitro* stimulation

Among the 54 healthy contacts IFN-γ production in response to PPD was present in 100% (54/54), to RD1 proteins in 58.6% (34/54) and to QF in 72.2% (39/54). Median of IFN-γ production in response to PPD was 19.75 IU/mL (CI, 0.7-56.8), to RD1 proteins 7.1 IU/mL (CI, 1-34.50) and 7.7 IU/mL (CI, 0.6-52.80) to QF (Table 4).

In vitro response to:		Healthy contacts N. (%)	Homeless N. (%)	Total N. (%)
		54 (51)	**52 (49)**	**106 (100)**
PPD				
	Positive N. (%)	54 (100)	51 (98)	105 (99)
	IFN-γ (IU/ml) median	19.8	23.4	21.3
RD1 proteins				
	Positive N. (%)	34 (58.6)	30 (57.7)	64 (60)
	IFN-γ (IU/ml) median	7.1	8.5	7.6
QFGold In tube				
	Positive N. (%)	39 (72.2)	31 (59.6)	70 (66)
	IFN-γ (IU/ml) median	7.7	9.8	8.1

Table 4: *In vitro* responses to *M. tuberculosis* antigens in the study groups populations.

Among the homeless, response to PPD was observed in 98% (51/52), to RD1 proteins in 57.7% (30/52) and to QF in 59.6% (31/52). Median of IFN-γ production in response to PPD was 23.4 IU/mL (CI, 0.7-63.2), to RD1 proteins was 8.5 IU/mL (CI, 0.7-49.3) and 9.8 IU/mL to QF (CI, 0.4-50.4). Since no statistical difference was found between the single tests' results obtained in these two groups from here after we pooled the data together. Therefore considering whole data, the response to PPD was observed in 99% (105/106), to RD1 proteins in 60% (64/106) CI, 0.7-49.3) and to QF in 66% (70/106) CI, 0.4-52,80) (Table 4). Median of IFN-γ production in response to PPD was 21.3 IU/mL (CI, 0.7-63.2), to RD1 proteins 7.6 IU/mL (CI, 0.7-

49.3) and to QF 8.1 IU/mL (CI, 0.4-52.80). These data indicate that stimulation with RD1 antigens is more restricted than that with PPD, as expected [45]. Moreover, the results obtained by RD1 proteins stimulation are similar to those obtained by QF test, as we previously reported [46]. Therefore from here after we used either stimuli to detect RD1 responses.

Memory Response to RD1 proteins

In an effort to find new tools to detect LTBI, we evaluated IFN-γ response to RD1 proteins at day 7 (memory response), as previously shown [44]. Among these 42 TST^+ individuals known to have been exposed to *M. tuberculosis* and to have negative results either to QF or to day-1 RD1 protein stimulation, 8 subjects (8/42; 19%) resulted positive to the 7-day whole blood test and this difference was statistically significant ($p<0.005$). Moreover, based on the response detected at day 1 and day 7, we divided the population studied in 4 groups: the first group enclosed 54 subjects classified as "responders over time" (Figure 6A), if IFN-γ response was positive over time; the second group comprised 34 subjects defined as "no responders over time" if no specific response was detected at both time points (Figure 6B); the third group enclosed 8 individuals that were categorized as "memory responders" if IFN-γ response was undetectable at day 1 and recovered at day 7 (Figure 6C) and finally the forth group enclosed "the effector responders" if IFN-γ response was detectable at day 1 and absent at day 7 (Figure 6D). In the "responders over time" we found a statistical significant difference ($p<0.0001$) between IFN-γ release at day 1 (median=8.3 IU/ml, range, 0.7-49.3) compared to that at day 7 (median= 107.5 IU/ml; range, 4-330.5). In contrast, no difference was found in the group of the 34 subjects classified as "no responders over time" between IFN-γ release at day 1 (median=0.1 IU/ml, range, 0-0.5) compared to that at day 7 (median= 0 IU/ml; range, 0-0.6). Interestingly in the 8 subjects defined as "memory responders" a statistical significant difference ($p<0.04$) between IFN-γ release at day 1 (median=0.3 IU/ml, range, 0-0.6) compared to that at day 7 (median= 26 IU/ml; range, 4-143.5) was found. To note that in the 10 subjects showing a selective effector response without memory response, a statistical significant difference ($p<0.05$) between IFN-γ release at day 1 (median=2.4 IU/ml, range 1-25.1) compared to that at day 7 (median= 0.2 IU/ml; range 0-0.6) was found. The majority of these subjects were healthy contacts (7/10).

All these data together indicate that in 8/42 (19%) a memory response to RD1 proteins can be detected in TST^+ subjects enrolled with a suspect of LTBI. Moreover, the generation of a memory response can be lost in 10/64 (15.6%) especially if the subjects have been recently exposed to *M. tuberculosis*.

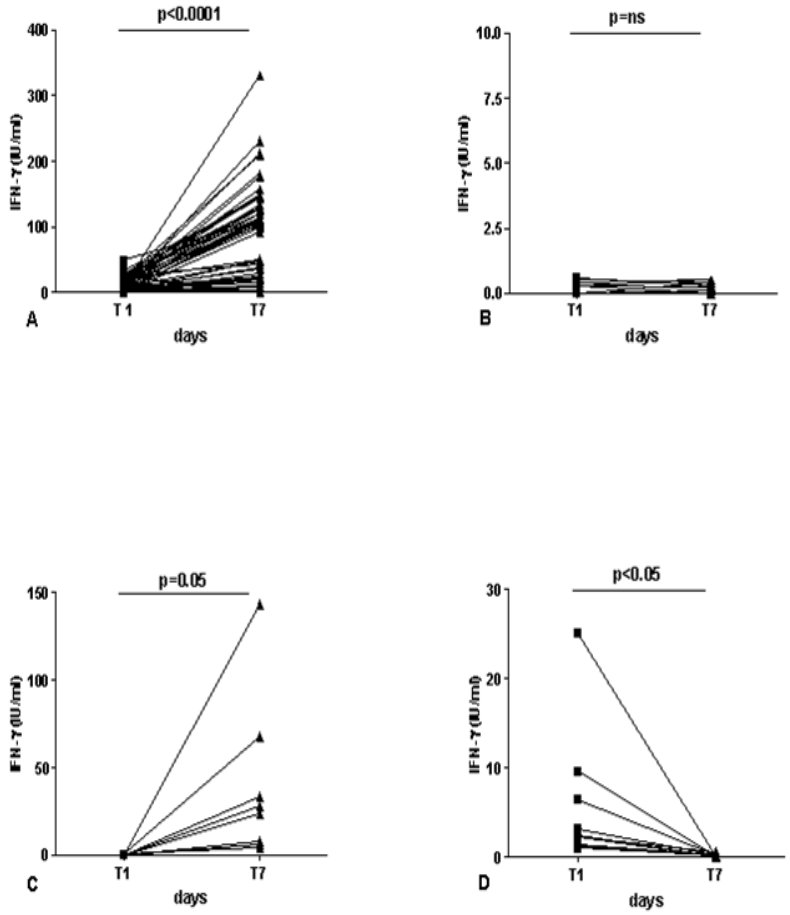

Figure 6: Effector and memory response in TST^+ subjects enrolled with a suspect of LTBI. IFN-γ secretion in response to RD1 proteins was evaluated at day 1 (effector response) and at day 7 (memory response). Based on these responses, we divided the population studied in 4 groups: the first group enclosed 54 subjects classified as "responders over time" (6A), if IFN-γ response was positive over time; the second group comprised 34 subjects that were defined as "no responders over time" if no specific response was detected at both time points

(6B); the third group enclosed 8 individuals that were categorized as "memory responders" if IFN-γ response was undetectable at day 1 and recovered at day 7 (6C) and finally the forth group enclosed "the effector responders" if IFN-γ response was detectable at day 1 and absent at day 7 (6D).
Abbreviations: IFN: interferon; T1: day 1; T2: day 7; ns: no significant.

Cytokines different from IFN-γ that are induced by RD1 proteins

It has been recently shown that cytokines and chemokines different from IFN-γ can be additional biomarkers to detect *M. tuberculosis* infection as they appear to be both sensitive and specific for *M. tuberculosis* exposure [21]. Based on these observations we evaluated at day 1 in a subgroup of "responders over time" the production of pro-inflammatory cytokines (IL-1β, IL-2, IL-6 and TNF-α), anti-inflammatory cytokines (IL-4, IL-10, IL-13) and chemokines (IP-10, MIP-1α, MIP-1β, IL-8) after stimulation with RD1 proteins (Table 5A-C).

		Pro-inflammatory cytokines			
		IL-1β	**IL-6**	**IL-2**	**TNF-α**
		Median(range)			
"Responders over time" N.9	**PHA***	72(0-175)	559(0-1153)	37(0-202)	260(0-353)
	Ag TB*	18(0-137)	61(0-140)	142(91-313)	52(0-156)
	Nihil	179(40-278)	241(27-365)	37(18-195)	281(12-402)
"No responders over time" N. 9	**PHA***	78(0-199)	649(154-2178)	44(18-105)	205(0-540)
	Ag TB*	44(0-72)	52(0-611)	0(0-57)	17(0-186)
	Nihil	187(81-462)	228(81-1181)	39(3-355)	210(49-1007)
"Memory Responders" N. 5	**PHA***	18(0-134)	519(82-1490)	57(8-126)	92(0-342)
	Ag TB*	29(8-62)	59(31-256)	14(0-24)	0(0-67)
	Nihil	140(99-200)	259(112-740)	35(3-115)	251(198-302)

Table 5A

Legend: PHA: phytohaemoagglutinin; Ag TB: antigen *M. tuberculosis*; IL: interleukin; IP: interferon-inducible protein; MIP: macrophage inflammatory protein; TNF: tumor necrosis factor; * indicates median value after subtraction of the nihil value.

		Anti-inflammatory cytokines		
		IL-4	**IL-10**	**IL-13**
		Median(range)		
"Responders over time" N.9	**PHA***	10(0-12)	147(54-241)	117(2-284)
	Ag TB*	2(0-3)	0(0-10)	32(4-69)
	Nihil	10(6-30)	26(19-49)	13(8-48)
"No responders over time" N. 9	**PHA***	5(0-13)	132(22-401)	100(11-218)
	Ag TB*	1(0-6)	0(0-55)	2(0-20)
	Nihil	10(6-63)	29(16-460)	10(8-345)
"Memory Responders" N. 5	**PHA***	4(1-10)	105(0-453)	129(4-152)
	Ag TB*	0(0-3)	0(0-21)	0(0-8)
	Nihil	11(8-16)	25(18-58)	14(10-17)

Table 5B

Legend: PHA: phytohaemoagglutinin; Ag TB: antigen *M. tuberculosis*; IL: interleukin; IP: interferon-inducible protein; MIP: macrophage inflammatory protein; TNF: tumor necrosis factor; * indicates median value after subtraction of the nihil value.

		Chemokines			
		IL-8	**MIP-1α**	**MIP-1β**	**IP-10**
		Median(range)			
"Responders over time" N.9	**PHA***	0(0-25)	555(0-1418)	1299(0-4573)	2387(1175-3542)
	Ag TB*	0(0-3846)	27(0-596)	407(0-1345)	3065(1357-3839)
	Nihil	2474(496-6955)	162(24-303)	522(137-954)	564(309-1034)
"No responders over time" N. 9	**PHA***	0(0-13930)	163(0-1250)	238(0-1325)	1526(0-2259)
	Ag TB*	0(0-3246)	0(0-187)	0(0-3344)	0(0-0)
	Nihil	1471(288-12840)	215(53-596)	726(208-7369)	625(375-2756)
"Memory Responders" N. 5	**PHA***	303(0-16392)	384(11-2021)	642(0-2338)	1560(947-2581)
	Ag TB*	167(0-2895)	50(0-103)	258(0-733)	373(7-1538)
	Nihil	1204(288-3041)	179(135-207)	583(515-969)	799(406-1729)

Table 5C

Legend: PHA: phytohaemoagglutinin; Ag TB: antigen *M. tuberculosis*; IL: interleukin; IP: interferon-inducible protein; MIP: macrophage inflammatory protein; TNF: tumor necrosis factor; * indicates median value after subtraction of the nihil value.

In the tables, median and range of the factors detected are reported. As shown, we found a statistical significant difference between the release of IL-2 ($p<0.006$) and IP10 ($p<0.0001$) in response to RD1 proteins compared to control. Therefore we evaluated in the other groups if these factors were modulated by specific stimulation. In the subgroup of "no responders overtime" we found that 2/9 (22%) responded to IL-2 and 1/9 (11%) to IP-10, whereas in the subgroup of "memory responders", we detected a response to IL-2 in 2/8 (25%) and to IP-10 in 1/5 (20%). These data together indicate that IL-2 and IP-10 may be additional markers of TB infection after RD1 specific stimulation.

Third Part: Characterization of regulatory T-cells identified as $CD4^{+}CD25^{high}CD39^{+}$ in patients with active tuberculosis

Epidemiological and demographic characteristics of the subjects enrolled.

Demographic and clinical characteristics of the 13 patients with active TB and 12 healthy control subjects are summarized in Table 6. Among the 13 patients with active TB, 9 (69%) had TST-positive results. All TB subjects were bacillus Calmette et Guerin (BCG)-vaccinated. Among the 12 control subjects, all were TST-negative, BCG-unvaccinated and none was infected with *M. tuberculosis* (negative result to QuantiFERON-TB Gold).

	Active TB N. 13 (%)	Healthy subjects N. 12 (%)
Median age in years (range)	33 (21-65)	34 (27-45)
Female (positive over total)	4 (30.7)	7 (58.3)
Origin (positive over total)		
West Europe	1 (7.7)	12 (100)
East Europe	6 (46.1)	0
Asia	3 (23.1)	0
Africa	3 (23.1)	0
TST (positive over total)	9 (69.2)	0
BCG-vaccinated (positive over total)	13 (100)	0
QuantiFERON-TB Gold In-Tube (positive over total)	11 (84.6)	0

Table 6: Epidemiological and demographic characteristics of the subjects enrolled

Phenotypic Analysis of Treg.

To compare the classical markers for Treg (CD25, FoxP3 and CD127), with the recently identified CD39 marker, we stained PBMC *ex-vivo* with the appropriate combination of MoAbs.
As shown in Figure 7A, CD25 identified 3 distinct populations of $CD4^+$ T-cells (High, Low and Negative), that also differently express FoxP3 (Figure 7B, C, D), CD127 (Figure 7E, F, G) and CD39 (Figure 7H, I, J). Interestingly, the CD39 marker is expressed almost exclusively by the $CD4^+CD25^{high}$ population (median 40±20% Figure 7H), similar to FoxP3 (median 86±14% Figure 7B). On the contrary, CD127 is expressed at very low levels by $CD4^+CD25^{high}$ compared to the $CD4^+CD25^{low}$ and $CD4^+CD25^{neg}$ cells.

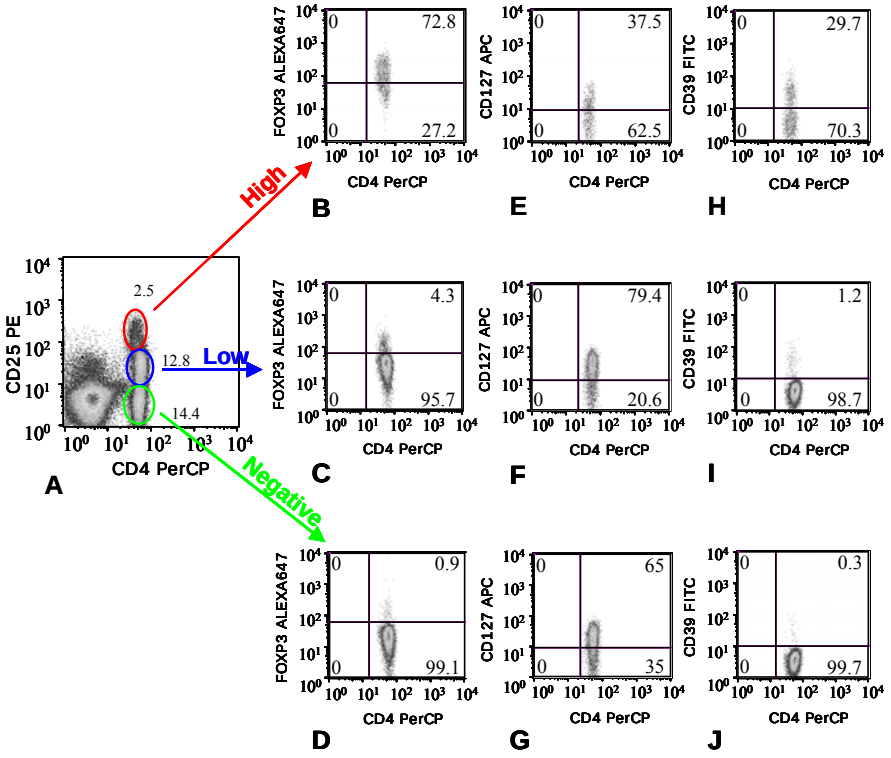

Figure 7: Phenotypic analysis of Treg. Classical markers for CD4 Treg (CD25, FoxP3 and CD127) were compared with the innovative CD39 marker. PBMC from healthy control subjects were stained with appropriate combinations of monoclonal antibodies and FACS analysis was performed, as described in the Materials and methods section. The expression of FoxP3 (7B-D), CD127 (7E-G) or CD39 (7H-J) was evaluated among $CD4^{+}CD25^{high}$, $CD4^{+}CD25^{low}$ and $CD4^{+}CD25^{neg}$ T-cells (7A). Representative flow cytometric panels from one donor out of 5 are shown.

Characterization of $CD39^{+}$ T-cells

In order to evaluate whether $CD39^{+}$ T-cells have the functional characteristics of Treg, intracellular IFN-γ cytokine expression was evaluated. As previously shown, the CD4 marker is down-modulated after PMA stimulation [50,51], therefore we analyzed IFN-γ cytokine production by $CD8^{-}CD3^{+}CD39^{+}$ T-cells. Among the gated $CD3^{+}CD8^{-}$ T-cells (Figure 8A), we found that CD39 expression correlates with the inability to produce

IFN-γ (Figure 8B), consistent with a regulatory phenotype. Conversely, IFN-γ was significantly produced by $CD8^{-}CD3^{+}CD39^{-}$ cells. TGF-β1 production further characterizes Treg function; however it cannot be evaluated by intracellular staining. Therefore its release was evaluated by immunoenzimatic methods in different sorted-cell subsets according to the expression of CD39 on $CD25^{high/low}$ $CD4^{+}$ T-cells; B cells ($CD19^{+}$ cells) and monocytes ($CD14^{+}$ $CD3^{-}$ cells) were used as control (Figure 8C). The different cell subsets were stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies, with the exception of B cells and monocytes. As shown in Figure 8C, TGF-β1 release was significantly higher in $CD4^{+}CD25^{high}CD39^{+}$ ($p<0.05$) compared to the other cell subsets, substantiating the notion that these cells have Treg characteristics.

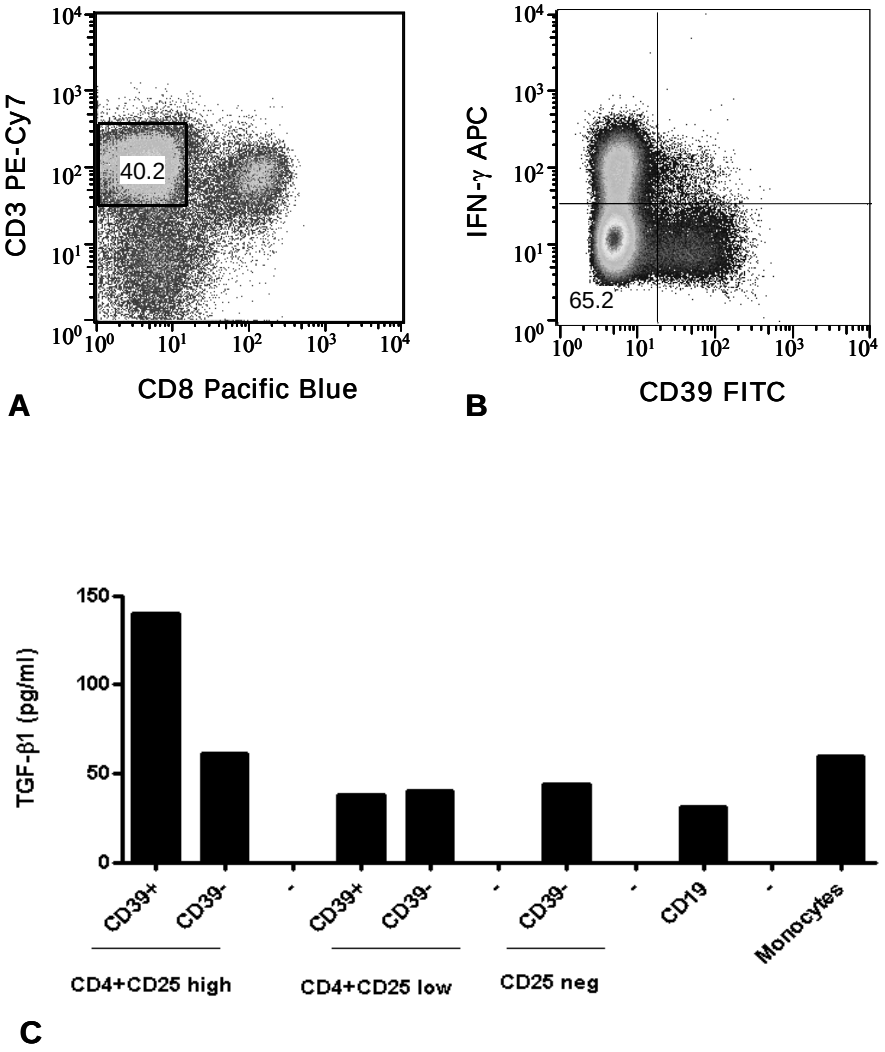

Figure 8: Characterization of CD39$^+$ T-cells. PBMC from a healthy control subjects out of 5 tested were stained with CD8 and CD3 MoAbs, as shown in panel 8A. IFN-γ cytokine expression on CD39 T-cells was evaluated among $CD3^+CD8^-$ gated T-cells by flow cytometer (8B). TGF-β1 production was evaluated in different cell subsets that were sorted based on the expression of CD39 on $CD25^{high/low}$ $CD4^+$ T-cells. Moreover B cells ($CD19^+$ cells) and monocytes were used as controls. Cells, were stimulated *in vitro* for 1 day with anti-CD3 and anti-CD28 MoAbs. TGF-β1 release was evaluated by ELISA. Results are shown as TGF-β1 production from a representative healthy subject out of 2 tested (8C). FACS analysis and sorting were performed as described in the Materials and methods section.

***Ex-vivo* and *in vitro* evaluation of CD4 Treg by different markers in healthy donors and TB patients.**

We then measured the frequency of $CD25^{high}FoxP3^{+}$, $CD25^{high}CD39^{+}$ and $CD25^{high}CD127^{-}$ on gated $CD4^{+}$ T-cells in *ex-vivo* PBMC of subjects with or without active TB (Figure 9A). The percentage of $CD4^{+}CD25^{high}CD39^{+}$ and $CD4^{+}CD25^{high}CD127^{-}$ cells was higher in TB patients than in healthy donors, although the difference was not statistically significant. The percentage of $CD4^{+}CD25^{high}FoxP3^{+}$ cells was comparable in TB vs healthy donors. Thus no increase of Treg was found in the *ex vivo* analysis in patients with active TB disease compared to controls.

Further, we evaluated if *in vitro* antigen-specific stimulation could expand Treg in TB patients. Concerning the $CD4^{+}CD25^{high}FoxP3^{+}$ T-cells, a significant difference was found in TB patients at day 1 ($p=0.01$) and day 6 ($p=0.01$) stimulated with RD1 proteins compared to healthy donors. Exogenous IL-2 significantly increased the expansion of Treg in RD1 protein-stimulated PBMC at day 1 ($p=0.04$) and day 6 ($p=0.04$) (Figure 9B).

Similarly, using CD39 as a Treg marker, a significantly higher increase of this cell population was found at day 1 ($p=0.0009$) and day 6 ($p=0.03$) in TB patients compared to healthy donors, after RD1 protein specific stimulation which was further increased by the addition of exogenous IL-2 at day 1 ($p=0.005$) and day 6 ($p=0.04$) (Figure 9C). Thus RD1-specific stimulation induces a significant expansion of Treg in patients with active TB.

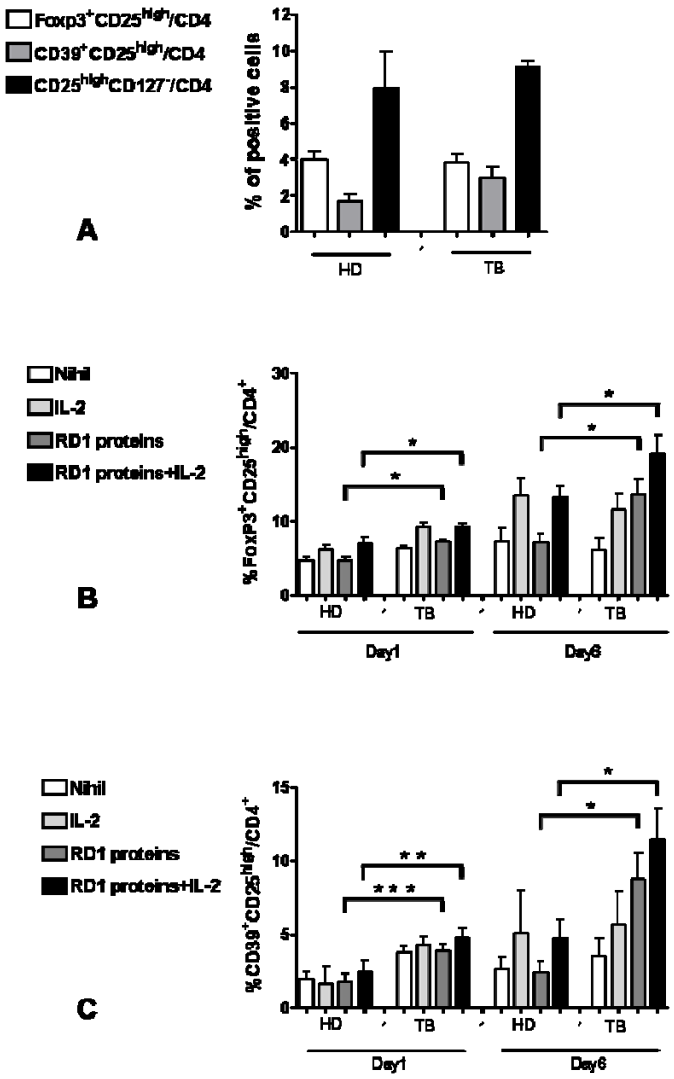

Figure 9: *Ex-vivo* and *in vitro* evaluation of CD4 Treg by different markers in healthy donors and patients TB.

***Ex-vivo* evaluation of Treg in patients with or without active TB (9A).** *Ex-vivo* expression of $CD25^{high}$ in combination with FoxP3 (white bar), CD39 (gray bar) or CD127 (black bar) on gated $CD4^+$ T lymphocytes, from healthy donors and TB patients, was evaluated by flow cytometry. **Overtime evaluation of CD25 and FoxP3 expression on gated CD4 T-cells after RD1-specific stimulation (9B)**. *In vitro* culture of PBMC stimulated for 1 or 6 days by RD1 proteins, in presence or absence of IL-2, was performed. $CD4^+CD25^{high}FoxP3^+$ cells

were significantly higher in TB patients at day 1 (p=0.01) and day 6 (p=0.01) in response to RD1 proteins compared to healthy donors. Exogenous IL-2 significantly increased the expansion of Treg in RD1 proteins-stimulated PBMC at day 1 (p=0.04) and day 6 (p=0.04).
Overtime evaluation of CD25 and CD39 expression on gated CD4 T-cells after RD1-specific stimulation (9C). $CD4^{+}CD25^{high}CD39^{+}$ T-cells were significantly increased at day 1 (p=0.0009) and day 6 in TB patients (p=0.03) after RD1 protein specific stimulation which was further increased by exogenous IL-2 at day 1 (p=0.0058) and day 6 (p=0.04).
Data are shown as the mean of 6 independent experiments in which 12 healthy donors and 13 patients with active TB were studied. Standard deviations are reported.

Cytokine analysis after RD1-specific stimulation.

Among patients with active TB we evaluated whether IL-10 and TGF-β1 were produced upon stimulation with RD1 proteins in the presence or absence of exogenous IL-2. The results show that at day 6, IL-10 production was increased in response to RD1 proteins in TB patients (median 298.7 pg/ml; range: 10.88-1058.26 pg/ml) as compared to healthy donors (median: 262.5 pg/ml; range: 48.21-493.6 pg/ml) whereas it was not statistically significant. On the other side TGF-β1 production was not modulated by *M. tuberculosis*-specific stimulation [TB patients (median 8189 pg/ml, range: 2080-18328 pg/ml) vs healthy donors (median 8460 pg/ml, range: 2378-14607 pg/ml)].

Depletion of $CD4^{+}CD25^{+}CD39^{+}$ T-cells from TB patients PBMC increases responses to recall antigens.

To investigate whether Treg may hinder anti-TB immune response, $CD4^{+}CD25^{high}CD39^{+}$ T-cells were depleted from TB patients' PBMC (Figure 10).
IFN-γ production in both cell fractions, Treg-undepleted PBMC and Treg-depleted PBMC, in response to RD1 proteins was evaluated after 3 days of cell culture. In those with detectable serum IgG antibodies to CMV, we used specific stimulation with CMV as control to evaluate the response to a recall antigen. In the 4 patients analyzed, $CD4^{+}CD25^{+}CD39^{+}$ depleted T-cells showed a significantly enhanced RD1- and CMV-mediated production of IFN-γ (p=0.001) compared to the Treg-undepleted PBMC fraction (Figure 11A). Data are also shown as fold of increase of IFN-γ responses to antigens in Treg-depleted PBMC/Treg-undepleted PBMC (Figure 11B).

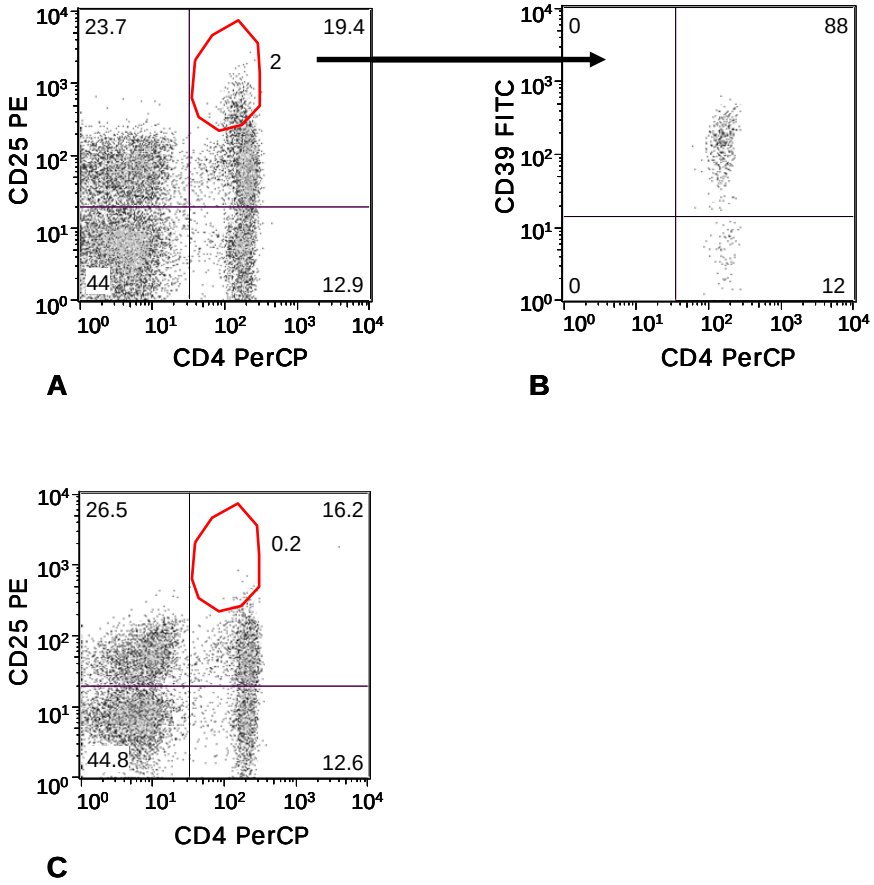

Figure 10: Flow cytometric analysis before and after depletion of Treg from TB patients' PBMC. TB patients' PBMC were stained with CD4 and CD25, before (10A) and after (10C) depletion of Treg. CD39 expression was evaluated on gated $CD4^{+}CD25^{high}$ T-cells (10B). FACS analysis was performed as described in the Materials and methods section. Representative panels from one patient are shown. The percentage of Treg evaluated as $CD4^{+}CD25^{high}$ in Treg-undepleted PBMC is 2% (10A). Among them 88% are $CD39^{+}$ (10B). After Treg depletion the percentage of Treg is 0.2% (10C).

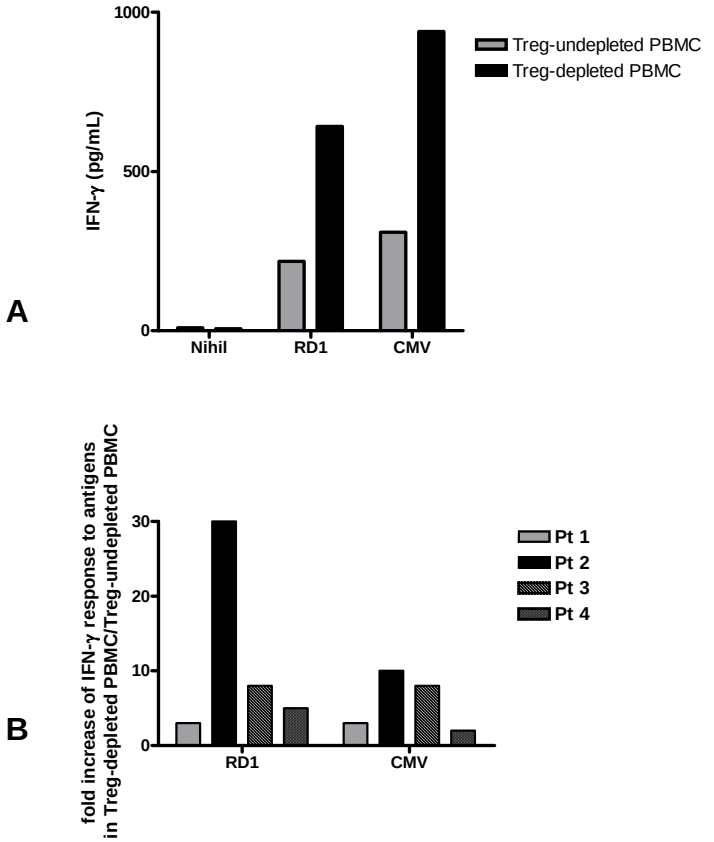

Figure 11: Depletion of $CD4^+CD25^+CD39^+$ T-cells from TB patients' PBMC increases IFN-γ production in response to recall antigens. PBMC from TB patients were cultured in the presence or absence of RD1-specific proteins and CMV. After 3 days of culture IFN-γ production was evaluated in the supernatants by ELISA in Treg-undepleted PBMC (gray bar) and Treg-depleted PBMC (black bar). IFN-γ production was increased in response to RD1 proteins and CMV in Treg-depleted PBMC. Results are shown as IFN-γ production from a representative TB patient (11A) and as fold of increase of IFN-γ responses to antigens in Treg-depleted PBMC/Treg-undepleted PBMC from 4 TB patients (11B).

Discussion

First Part: New tools to detect active tuberculosis infection: *Mycobacterium tuberculosis* DNA in soluble fraction of urine specimens

We hypothesized that small cell-free *M. tuberculosis* DNA fragments originating from sites of infection outside the urogenital tract also could be present in the urine. It is well known that metabolically active mycobacterium promotes apoptosis of infected cells [52]. One of the key features of apoptosis is the cleavage of dying cell's genomic DNA into small oligonucleosomes of approximately 180 bp which subsequently can appear in plasma [53]. However, prokaryotic DNA does not possess a nucleosomal structure and the fate of bacterial DNA has not been extensively studied. At the earliest stages of infection, the mycobacterium may be ingested by alveolar macrophages and monocytes and destroyed by phagolysosomes. The host cells themselves may die by apoptosis [52,54] and bacterial DNA could then be released from apoptotic cells. Such a process has been suggested by the observations of several investigators who report that infectious agents have been found within apoptotic bodies [55] and released bacterial DNA has been detected in the supernatant of infected cells [56]. Therefore, these results have suggested to us that bacterial DNA may appear in plasma and possibly be cleared through the kidneys as Tr-DNA in urine specimens in a manner similar to post-apoptotic DNA from the eukaryotic host cells.

In this part of my thesis, we have demonstrated for the first time that small cell-free *M. tuberculosis* DNA fragments can be detected in the urine of patients with active pulmonary TB. The data also show that the *M. tuberculosis*-specific DNA fragments were no longer detected following an effective treatment, and that they are absent from the urine of healthy controls. Overall, these data provide the first experimental evidence that the presence of *M. tuberculosis* DNA fragments in urine can be an accurate marker of active pulmonary TB.

PCR based tests have been used successfully to detect *M. tuberculosis* in the urine of patients with genitourinary TB [57] in which cases the infectious bacteria are present in the urinary tract. In these studies high sensitivity and specificity were achieved by amplifying large (245 to 1000 bp) fragments of mycobacterial DNA purified from urine sediment and the results of molecular test are strongly correlated with the presence of viable

mycobacteria which can be cultured from the urine [58]. Thus, presence of specific large DNA fragments in those patients appears to reflect urinary excretion of mycobacteria actively replicating in the urogenital tract.

Attempts also have been made to detect mycobacterial DNA in the urine of patients with pulmonary TB. One study found specific mycobacterial DNA sequences in the urine of 13 AIDS patients with pulmonary TB, two of whom had also positive urine culture for *M. tuberculosis*. In other larger studies, conducted on both HIV-infected and uninfected patients with pulmonary TB, sensitivity of PCR-based test for detection of *M. tuberculosis* DNA in the urine was found to be 20 to 60%, with higher sensitivity reported in HIV infected patients [49]. Again, in these studies the methods used were designed to detect large DNA fragments purified from urine pellet after centrifugation. By using these methods, detection of mycobacterial DNA in the urine appears to have little, if any, clinical usefulness in the diagnosis of pulmonary TB.

In this part of the thesis we took a different methodological approach to the detection of *M. tuberculosis* DNA sequences in the urine. Based on previous studies concerning transrenal DNA [59], we proposed that *M. tuberculosis* Tr-DNA may be detected in urine arising from sites remote from the urinary tract. We have previously reported that specific HIV-1 sequences can be found in the form of small cell-free DNA fragments in the urine of HIV-1 infected individuals [59]. Furthermore, we reported that small fetal DNA fragments originating from dying cells within the developing fetus can be detected in maternal urine specimens [13]. Similarly, we found that tumor-specific sequences are present in the Tr-DNA fraction of urine specimens from cancer patients [42].

Since we were aware based upon earlier studies that Tr-DNA consists of low molecular weight fragments of approximately 150-200 bp [60], it was necessary to modify DNA extraction techniques commonly employed by others, as well as to modify the PCR reaction conditions in order to efficiently target the relatively short Tr-DNA fragments. In our opinion the different techniques employed may explain the finding that all pulmonary TB patients tested before treatment were positive in the present study, in contrast with the 40-60% detection rate reported in previous studies [61,49].

The molecular basis that would account for the presence of mycobacterial DNA in the urine of patients with pulmonary TB is not known. It is possible that the presence of these DNA fragments reflects replication of mycobacteria in the kidney or urinary tract of patients although no genitourinary infection may be clinically apparent. It has been suggested that

approximately 5% of patients with pulmonary TB may have positive culture for mycobacteria in the absence of any sign of involvement of the genitourinary tract [62] and this proportion could be expected to be higher among HIV-infected patients since they have an increased risk of developing extrapulmonary dissemination of TB in the case of pulmonary infection [63,64]. However, in the present study all urine cultures performed were negative for mycobacteria and only one patient in our study was HIV positive. It also may be possible that these DNA fragments derive from circulating mycobacteria or perhaps even represent free DNA filtered into the urine. We speculate that transrenal *M. tuberculosis* DNA does actually represent free DNA originating from the pulmonary site of the infection. The small fragment size and the fact that they can mainly be found in the soluble portion of urine are in favor of this hypothesis. The survival of these DNA fragments further suggests that they arise through a mechanism that provide some degree of protection from nucleases that would otherwise degrade the fragments in the tissues and plasma.

It is important to account for the fact that other groups have failed to consistently detect *M. tuberculosis*-specific DNA in urine of patients with pulmonary infection. It is likely that the short *M. tuberculosis*-specific DNA fragments were not found by others since both DNA extraction techniques as well as amplicon sizes that were employed were capable of detecting primarily larger DNA commonly associated with urine sediment. Our hypothesis is supported by the results of the experiment in which urine specimens from seven patients, selected randomly from the twenty TB patients, were tested side by side with primers for short (129/67 bp) and long (330/69 bp) amplicons. Both sets of primers generated comparable amounts of DNA fragments with *M. tuberculosis* genomic DNA. However, no detectable product was generated with primers for the large amplicon. *M. tuberculosis*-specific product was only found using the short amplicon primers (129/67) (Figure 4).

The Tr-DNA protocol presented here has several noteworthy advantages over methods conventionally used in the diagnosis of pulmonary TB. First, the use of urine specimens avoids the necessity of elaborate procedures for potentially infectious sputum collection. This may be particularly important for pediatric patients or in general for patients from whom it is difficult to obtain adequate sputum samples, and for whom complex and/or potentially hazardous procedures such as gastric aspirate or broncho-alveolar lavage or sputum induction are otherwise required. The *M. tuberculosis* Tr-DNA test can provide a safer method not only for patients, but also for healthcare

workers who may otherwise be exposed to infectious aerosols under normal conditions in the clinic.
The data on *M. tuberculosis* Tr-DNA disappearance during the course of treatment supports the potential utility of this test in monitoring the clinical course of the disease. It is also clear that small urine specimens can be obtained in the field more easily and safely which is important when broad screening of large populations is of concern to public health. It is also likely that Tr-DNA testing for diagnosis of TB would have particular value for clinics located in regions having limited resources. Although PCR is currently the preferred method for detecting specific nucleic acid sequences, new emerging technologies can be expected to eventually provide the tools to perform the relatively simple Tr-DNA test in the field [65,66,67] offering practical advantages over those of PCR can be readily applied to the Tr-DNA test. All steps of the diagnostic process, from Tr-DNA purification to biomarker detection, can be automated and brought to the point of care in small hospitals or even in the field with greater facility than can be done with conventional microbiological testing.
In conclusion, if the results presented in this study are confirmed in larger clinical studies, the application of the Tr-DNA test could prove to be a significant advance in the diagnosis of TB and in the monitoring of its clinical course. The availability of new diagnostic tools that are more accurate and accessible may greatly benefit individual patients and significantly contribute to the control of the disease.

Second Part: New tools to detect latent tuberculosis infection: evaluation of memory responses and new biomarkers in response to RD1 antigens in TST$^+$ subjects exposed to *Mycobacterium tuberculosis*

In this part of the thesis we compared the sensitivities of three *M. tuberculosis*-specific IGRAs for the diagnosis of LTBI and found a remarkable consistency between the outcomes of the short-incubation IGRAs, i.e., the QF and the whole blood test based on RD1 proteins; however we found inconsistency between the outcomes of the short-incubation IGRAs, i.e., the whole blood test based on RD1 protein, on the one hand, and whole blood RD1 assay with a prolonged, 7-day incubation and TST, on the other. Among TST$^+$ individuals known to have been exposed to *M. tuberculosis* and to have negative results either to QF or to the 1-day RD1 proteins stimulation, the 7-day whole blood test was positive

significantly more often. Moreover, we demonstrated that besides IFN-γ, IL-2 and IP-10 can be additional markers for LTBI identification. Our findings indicate that that the short incubation assays may have limited sensitivities for the detection of past infection and that additional factors such as IL-2 and IP-10 can be used for identification of LTBI. Whether TST-positive persons with negative QF results but positive 7-day whole blood tests results or positive response to IL-2 and IP-10 short incubation test are at risk for the development of TB needs to be elucidated before short-incubation IGRAs can be used for the screening of individuals for latent TB. This is particular relevant for those that need to be screened before undergoing immunosuppressive treatment [68].

To give consistency to the study, we performed 1-day and 7-day whole blood tests using identical *M. tuberculosis* specific proteins and repeated a TST at the same time. Furthermore, we chose to study a group of persons documented to be positive by TST after exposure to *M. tuberculosis*. Some of these individuals were known to have been exposed to TB decades ago, and others were known to have been exposed more recently.

Although a significant difference in sensitivity between 7-day whole blood test and both 1-day WBA and the QF test could be observed, the study size was too small to correlate the observed discrepancy to factors such as the time that had elapsed since the *M. tuberculosis* infection had been acquired. The agreement between the QF test and RD1 proteins-based 1-day whole blood was high, but the outcomes of these assays showed poor agreement with those of both TST and 7-day whole blood test, assays whose results were highly concordant. Around 34-40% of the TST^{+} individuals had negative results by both the QF test and RD1 proteins-based 1-day whole blood while 19% of those scored negative were positive by the 7-day whole blood test. Of note, all three assays measured the levels of IFN-γ production in response to the same antigens that were found to be highly specific for *M. tuberculosis* [69,70]. Among the participants negative by the QF test and RD1 proteins-based 1-day whole blood test, high levels of IFN-γ could be produced by the 7-day whole blood test, indicating that the observed discrepancy was not simply explained by differences in the levels of detection of IFN-γ. A plausible explanation for the difference in sensitivity would be the differences in the in vitro incubation periods for the QF test and the RD1 proteins-based 1-day whole blood test, on the one hand, and that for 7-day whole blood test, on the other. We hypothesize that after 24 h incubation only circulating effector memory T cells have had sufficient time to produce IFN-γ, while central memory T cells first started producing IFN-γ

after a more prolonged incubation. In individuals who have been infected with *M. tuberculosis* in the past, the number of circulating effector cells could be low, causing negative results in a short-incubation assay but positive responses after a prolonged incubation. In accordance with this line of thought are findings from a recent study of hepatitis C virus showing that short-term ELISPOT responses were not influenced by depletion of lymphotropic chemokine receptor 7-positive T cells, representing memory cells, while the depletion of these memory cells did decrease the antigen-specific responses after prolonged culture [71]. Our findings suggest that prolonged incubation of the IGRAs, such as a 7-day whole blood test, might be the most sensitive method for screening for latent TB infection in persons with an increased risk of the development of a reactivation of TB, such as those eligible for transplantation or treatment with tumor necrosis factor alpha antagonists [68]. A recently published case of pulmonary TB in a liver transplant patient with a negative QF test result before transplantation illustrates that the results of the QF test must be interpreted with caution in this setting [72].

We also described a sub-group of patients characterized by an absence of memory response. The majority was composed by healthy contacts probably undergoing to an active *M. tuberculosis* replication and it can be hypothesized that their effector cells were highly activated and therefore prompt to dye instead undergoing a differentiation process leading to the generation of memory cells. This mechanism has been proposed in the pathogenesis of other infectious diseases, in which an elimination of effector T cells may occur when T cells confront high doses of antigens [73,74]. There is a lack of knowledge on the performance of IGRAs with a short incubation period compared to those of IGRAs with a more prolonged incubation period in relation to the detection of *M. tuberculosis* infection. Two studies that compared the overnight ELISPOT with either the 6-day LST or the 7-day whole blood incubation test reported that these prolonged tests performed better [20,44]. This is in agreement also with our previous data in which we reported that negative responses to a panel of RD1 peptides in an overnight ELISPOT became positive responses in a cultured ELISPOT in cured TB patients [75].

Several studies compared one short-incubation IGRA with TST for the detection of latent *M. tuberculosis* infection [17,19,23,25,76-83], but the levels of agreement between TST and the IGRA varied widely between studies. In line with the hypothesis that a short incubation IGRA might have a lower sensitivity for the detection of past latent infection are the

observations of several other studies [25,83,84]. In two cross-sectional studies performed in South Africa, approximately one-third of adults with a TST induration higher than 15 mm had a negative QF test result [25,84] and 38% had a negative T-SPOT. TB test result [84]. Another study noticed that in a mostly BCG-vaccinated Korean control population, 51% of the subjects were TST^+ and only 4% were QF assay positive, while the expected prevalence of *M. tuberculosis* infection was 33% [83].

Recent reports have shown that the detection rate of IGRA can potentially be enhanced by measuring alternative or additional biomarkers to IFN-γ [27,83], such as interferon gamma inducible protein 10 (IP-10). As part of our search for improved diagnostic measures we have screened >20 potential biomarkers and among those we have characterized IP-10 and IL-2 as potential in-vitro biomarkers for infection with *M. tuberculosis*. Using these markers the detection rate for LTBI in the TST^+ subjects increased although not significantly, probably due to the small size of the samples analyzed.

In conclusion, a discrepancy was observed between the results of two short-incubation IGRAs, i.e., the whole blood test based on RD1 protein, on the one hand, and whole blood RD1 assay with a prolonged, 7-day incubation and TST, on the other. This study raises the hypothesis that short-incubation IGRAs mainly detect recent or ongoing infection with *M. tuberculosis*, while prolonged-incubation IGRAs seem to be more sensitive for the diagnosis of past latent infection. Further studies are needed to confirm these data and evaluate the consequence of this hypothesis for the predictive value for the risk of TB. Moreover IL-2 and IP-10 were shown to be additional markers for LTBI detection. Whether TST-positive persons with positive prolonged tests results or positive IL-2 or IP-10 responses but negative QF results are at risk for the development of TB needs to be elucidated before short-incubation IGRAs can be used for the screening of individuals for latent TB before immunosuppressive treatment.

Third Part: Characterization of regulatory T-cells identified as $CD4^+CD25^{high}CD39^+$ in patients with active tuberculosis

We also evaluated whether Treg were expanded in active TB disease using different Treg markers, by *ex-vivo* and *in vitro* cell culture systems assessment. We demonstrated that CD39 is a useful marker to detect Treg because within $CD4^+CD25^{high}$ cells, it identifies a cell subset characterized by high production of TGF-β1 and the absence of IFN-γ expression.

Moreover, we show that *ex-vivo* evaluation of CD4 Treg, identified either by the expression of $CD25^{high}FoxP3^{+}CD127^{-}$ molecules or by the CD39 marker, did not show an increase in the peripheral blood of active TB patients compared with healthy donors. Conversely, in active TB patients, RD1 proteins-specific stimulation *in vitro* expanded Treg, evaluated by the classical and the new marker, which was further increased by IL-2. This Treg expansion was associated with an increase of endogenous IL-10, although it was not statistically significant. Depletion of $CD4^{+}CD25^{high}CD39^{+}$ increased *M. tuberculosis*-specific responses, as well as other recall antigens responses. In conclusion, for the first time to our knowledge, we demonstrated that CD39 is a good marker for Treg identification in an infectious disease like TB. This information can be useful for future studies to monitor *M. tuberculosis*-specific response during TB.

To validate CD39 as an appropriate marker for T-cells endowed with suppressive abilities, we measured the capability of $CD39^{+}$ T-cells to produce IFN-γ and TGF-β1. Interestingly, we found that $CD39^{+}$ T-cells do not produce IFN-γ and those within the $CD4^{+}CD25^{high}$ subset do produce TGF-β1. These data together indicate that the $CD4^{+}CD25^{high}CD39^{+}$ present Treg functions.

Discordant results were found *ex vivo* in terms of Treg expansion in those with TB disease, some authors reporting an increase of $CD4^{+}CD25^{+}FoxP3^{+}$ T-cells [28,38-40] whereas others showed absence of modulation of this cell subset [41]. In this study we did not observe any statistical difference between healthy and diseased subjects analyzing 3 different combinations of markers identifying Treg. Conversely, an increase of Treg after *in vitro* specific-stimulation in patients with active TB was found. The antigen-specific increase of Treg was amplified by exogenous IL-2 which is a well known factor for Treg generation [31]. Treg increase was demonstrated by the expression of classical (FoxP3, CD25 and CD127) and innovative (CD39) markers.

The mechanisms by which Treg control immune responses are incompletely understood, but there is evidence for a central role of the inhibitory cytokines IL-10 and TGF-β1. When these cytokines were evaluated in TB patients, we found an increase of IL-10 after specific stimulation, although not statistically significant. Differently, no modulation of TGF-β1 was found. These data are in line with those found by Roberts T et al. [41] that did not find any statistical difference between TB patients and LTBI controls in

terms of mRNA expression for IL-10 and TGF-β1 after *in vitro* BCG vaccination.

Depletion of $CD4^+CD25^{high}CD39^+$ increased *M. tuberculosis*-specific responses, as well as other recall antigens responses indicating that Treg broadly modulate antigen-specific immunity. This result is consistent with the findings by other authors [28,38,39] who reported an increase of IFN-γ response, specific either to BCG or RD1- or heparin-binding haemagglutinin adhesin -proteins after the depletion of $CD4^+CD25^+$ T-cells.

This study provides the first evidence for a role of Treg identified as $CD4^+CD25^{high}CD39^+$ in TB. The added value of this finding is that CD39 may allow an easier detection of Treg compared to FoxP3 which is an intracellular marker. Moreover, at least in humans, FoxP3 is upregulated during activation, and a fraction of $FoxP3^+$ cells produces IFN-γ (unpublished observations). On the contrary, $CD39^+$ cells do not produce IFN-γ and their role in the control of unwanted immune reactions (such as those that occur in autoimmune diseases) has been recently suggested. Indeed, in patients affected by multiple sclerosis, an autoimmune disease of the central nervous system, $CD39^+$ T-cells are greatly reduced compared to healthy subjects. Our results are consistent with the hypothesis that Treg help to control the critical balance between immune-mediated suppression of *M. tuberculosis* and immunopathology in patients with TB.

More work is required to delineate the role of these Treg in TB disease and infection. It will be interesting to analyze prospectively whether dynamic changes in Treg frequencies are associated with the paradoxical worsening of symptoms and tissue inflammation observed in a proportion of patients with TB during treatment. It is also important to ascertain whether or not Treg are expanded in latent TB infection, where pathogen and antigen load are low and immunopathology is absent.

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List of publications during the PhD course by Dr. Teresa Chiacchio

1. Cannas A, Goletti D, Girardi E, **Chiacchio T**, Calvo L, Cuzzi G, Piacentini M, Melkonyan H, Umansky SR, Lauria FN, Ippolito G, Tomei LD. Mycobacterium tuberculosis DNA detection in soluble fraction of urine from pulmonary tuberculosis patients. Int J Tuberc Lung Dis 2008;**12**:146-51.

2. **Chiacchio T**, Casetti R, Butera O, Vanini V, Carrara S, Girardi E, Di Mitri D, Battistini L, Martini F, Borsellino G, Goletti D. Characterization of regulatory T-cells identified as $CD4^{+}CD25^{high}CD39^{+}$ in patients with active tuberculosis. Clin Exp Immunol 2009; In Press.

3. Butera O, **Chiacchio T**, Casetti R, Vanini V, Meraviglia S, Guggino G, Dieli F, Vecchi M, Lauria F, Laurenti P, Caccamo N, Girardi E, Goletti D. New tools to detect latent tuberculosis infection: evaluation of memory response and new biomarkers. Tuberculosis 2009; Submitted.