Università degli Studi di Palermo
INTERNATIONAL PhD COURSE IN IMMUNOPHARMACOLOGY

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HLA-E-restricted CD8^{+} T lymphocytes as a new player in the adaptive immune response to *Mycobacterium tuberculosis*

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Ciclo XXVI - SSD MED/04 Patologia Generale - a.a. 2015-16

To Capitano

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Introduction

Mycobacterium tuberculosis infection and disease: an overview

Mycobacteria belongs to *Mycobacterium tuberculosis complex* (MTBC), which includes the species *Mycobacterium tuberculosis* (Mtb), *M.bovis, M.bovis BCG, M. africanum* (1). These species are the causative agents of tuberculosis (TB), one of the most prevalent and severe infectious diseases worldwide. Potentially, TB is a lethal disease, but curable if properly treated. Factors that lead to a higher risk of transmission among people include HIV. Moreover, HIV co-infection dramatically increases the risk of transition from latent infection to active TB at least 20-30 times. Other factors for TB incidence include: country of birth, malnutrition, active and passive smoking, the use of immunosuppressive drugs (2) and type-II diabetes mellitus have a 1.5-fold increased risk of developing TB (3). In the poorest country HIV represent the strongest risk factor, whereas the increased prevalence of type-II diabetes mellitus mainly contributes to TB epidemics in developing countries (4).
According to WHO, in 2014 6 million new cases of TB were reported (5). An estimated 9.6 million people developed TB (5.4 million men, 3.2 million women and 1.0 million children) and 63% (fewer than two-thirds) of these people became ill. Based on this report we can conclude that 37% of new cases were undiagnosed or were not reported. Furthermore, in the group of 9,6 million people who developed TB in 2014, more than half (58%) were infected in the South-East Asia and Western Pacific Regions. One quarter (28%) of patients in the African Region also had the highest rates of lethal cases relative to population (**Figure I1**). In 2014 TB killed 1.5 million people. Among them 1.1 million people (12% of the 9.6 million TB cases) were HIV-negative and 0.4 million HIV-positive. In Africa about four out of five TB patients (75%) were HIV-positive. As stated in WHO report 2015, TB now ranks alongside HIV as a leading cause of death worldwide (**Figure I2**).

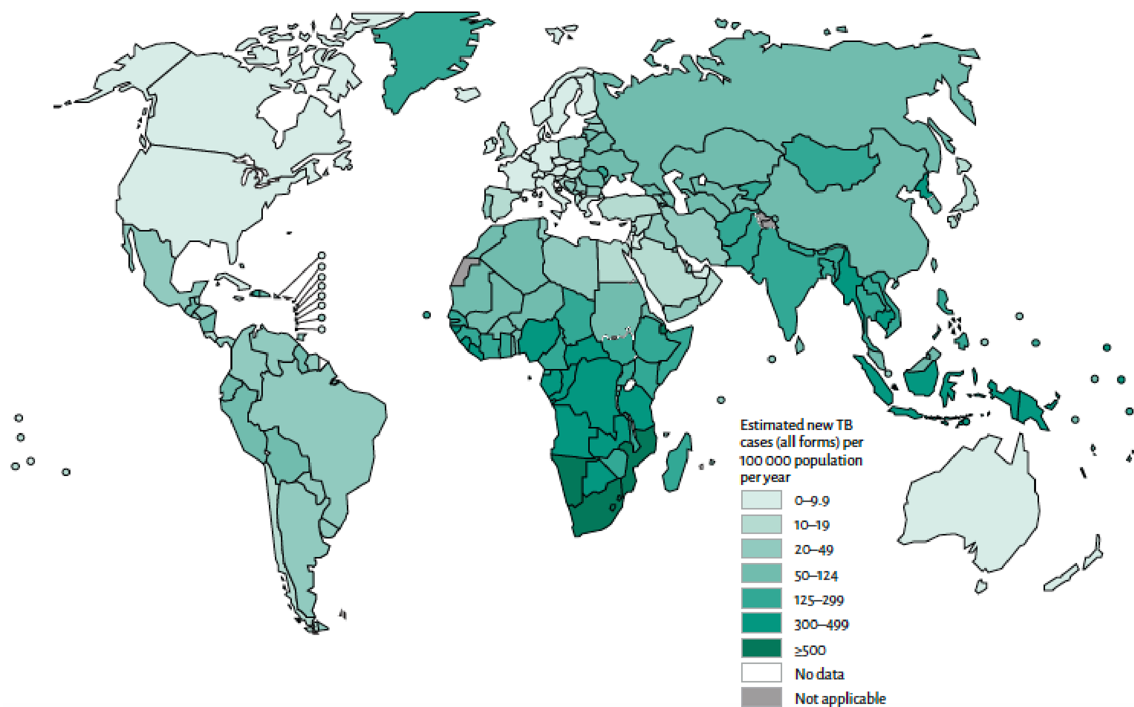

Figure I1: Estimated TB incidence rates in 2014. The number of incident TB cases relative to population size varies widely among countries. The lowest rates were found predominantly in high-income countries including most countries in western Europe, Canada, the United States of America, Australia and New Zealand. Oppositely, the higher rates was found in African regions (WHO 2015).

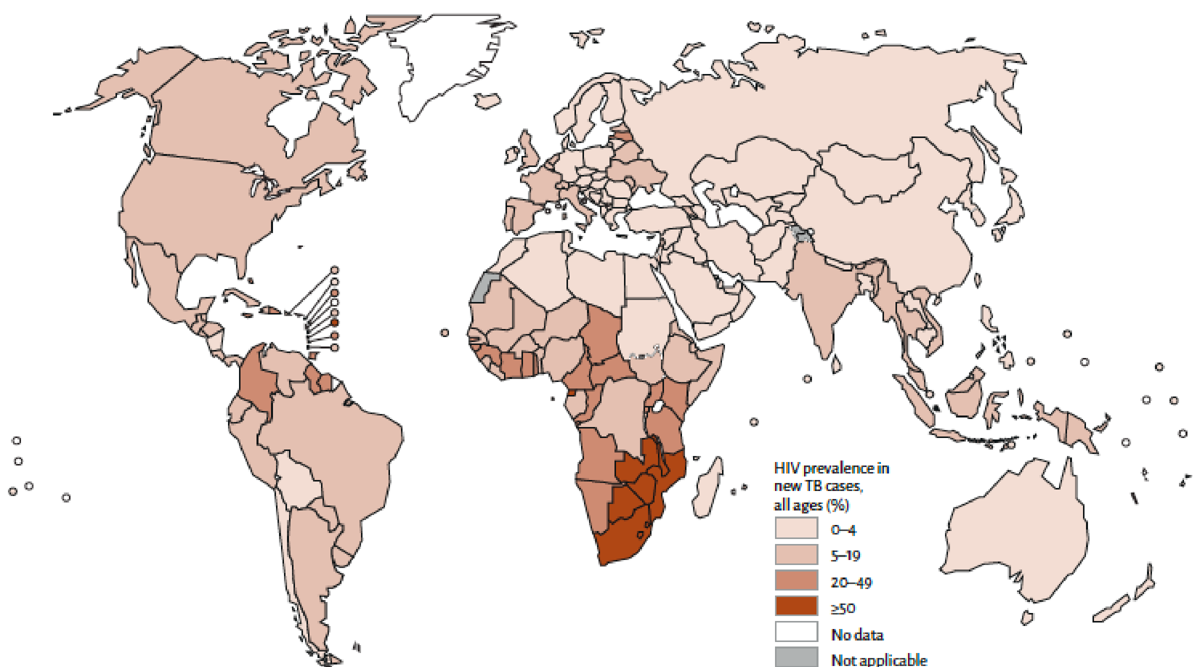

Figure I2: HIV prevalence in new and relapse TB cases in 2014. The proportion of TB cases co-infected with HIV was highest in Africa. Notable, more than 50% of TB-, HIV-positive patients were diagnosed in the Southern Africa (WHO 2015).

The TB disease originates in the ancient past. According to an article of The American Journal of Physical Anthropology published in 2008 by Kappelman J. et al., the earliest evidences of TB dates back to almost 500,000 years ago, although it is generally believed that the TB disease broke out only several thousand years ago (6). The researchers were able to characterize small lesion in an ancient fossilized skull belonging to a young Homo erectus found in western Turkeyas typical for *Leptomeningitis tuberculosa*, a form of TB that attacks the meningeal structures. With respect to TB epidemiology it should be pointed out that Homo erectus was the first human species to migrate out of Africa, gradually adapting to different climatic conditions.
Comas et al. in an article published in Nature Genetics in 2013 analyzed the genomes of 259 MTBC strains and indicated that TB disease emerged about 70,000 years ago in Africa and expanded as a consequence of increases in human population density during the Neolithic period (7). Avicenna in the 10th century was the first physician who identified pulmonary TB as an infectious disease, recognized the association with diabetes and suggested that TB could spread through contact with soil and water. He also developed the method of quarantine to limit the spread of tuberculosis. TB became epidemic in medieval Europe and was known as “white plague” or “consuption” because it seemed to consume people with bleeding from the mouth, fever, pale skin and a long decay. Due to the variety of symptoms, TB was not identified as a single disease until about 1820 (8). The causative agent of TB, *Mycobacterium tuberculosis,* was identified and described in 1882 by Robert Koch who was awarded the nobel prize in 1905 for this discovery.

Pathogenesis of tuberculosis

TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within alveolar macrophages. The common transmission is by direct human-to-human spread usually by inhalation of mucous droplets that become airborne when infected individual coughs, sneezes, speaks, or even breathes. The bacilli can remain in the air for prolonged periods of time, and can even survive for days to months if protected from sunlight (9, 10). Cutaneous-mucous contact of skin lesions or mucous membranes with infectious material as well as the indirect transmission through contaminated objects is another way of infection. The infection is possible as long as the bacilli continue to be present in the secretions of the infected patient. Sometimes in patients untreated or inadequately treated, the infectious period can last for years. The severity of infectiousness depends essentially on the number of tubercle bacilli issued (infectious load) and their virulence.
The Mtb that entered the body through the respiratory route, initially forms the primary complex which leads to a local outbreak with exudative character. Primary TB establishes in the parenchyma of the lung where the organisms lodge the inflammatory reaction. Immediately, the released bacteria are taken up by activated resident lung alveolar macrophages which try to engulf and destroy them. Mtb-infected macrophages can be themself engulfed by uninfected macrophages through a dedicated apoptotic cell engulfment process called efferocytosis (11). This prevent dissemination of infection and reduce viability of intracellular mycobacteria.

Lung dendritic cells (DC) that encountered Mtb become infected as well. When they engulf pathogens they migrate to draining lymph nodes to prime or boost specific T cells, and this way leads to the production and secretion of antimicrobial peptides, cytokines, chemokines. On the other hand, the tubercle bacillus has the ability to evade the innate response through release of the chordal factor to inhibit macrophages activities. The chordal factor is a particular derivative of mycolic acids present in the mycobacterium's membrane, which blocks the merger of the phagosome with the lysosome, the action of the lytic enzymes contained in it, and the activation of the enzyme complexes that produce highly reactive molecules able to eliminate the foreign agent. As a consequence, the macrophage won't be able to eliminate the pathogen, but it will enhance its cytocidal activity.
The bacterial persistence within macrophages results in the activation of a cell-mediated immune response. Both $CD4^+$ T helper 1 (Th1) cells and $CD8^+$ T cells are fundamental in protection against TB (12). In particular, $CD4^+$ T lymphocytes activated by MHC class II molecules expressed by DC produce cytokines as IFNγ, TNF, IL-2, lymphotoxin (LT) and chemokines that are capable of activating other cellular components. IL-2 acts as an autocrine growth factor, whereas LT and TNF induce the activation of neutrophils. IFNγ enables macrophages to enhance phagocytosis and microbicidal activity in the phagolysosome. Finally, chemokines recruit other cells, including monocytes, at the site of inflammation.
Additionally, $CD8^+$ T cells are also activated after recognition of peptides associated with MHC class I molecules. The activated $CD8^+$ T lymphocytes differentiate to cytotoxic lymphocytes (CTL) and are able to produce cytokines and cytolytic molecules. Thus, they selectively eliminate any cell expressing the foreign antigen, including phagocytic cells containing replicating mycobacteria.
Inflammatory cells involved in immune response against Mtb evolve into a well organized structure known as granuloma. The central part of the granuloma is formed by bacilli, epithelioid cells and death macrophages with Langhans giant cells that form an epitelioid layer, similar to the epithelium. More externally, T and B lymphocytes, fibroblasts and connective tissue surround this specific structure and form a kind of capsular layer. γδT cells and CD1-restricted αβT cells are also involved (13, 14). Adhesion to the extracellular matrix (ECM) is essential for the recruitment and retention of T cell, B cells and macrophages during formation of granuloma. T cells use integrins for adhesion while fibronectin is a major component of ECM engaged in the process (15). The main function of the granuloma is to avoid the dissemination of bacteria by isolation of the mycobacteria from the rest of the lung, and to localize bacteria within this structure for the action of the immune system. However, granuloma formation can cause tissue damage that might lead to the loss of function in involved organ. Therefore, on the one side the established immune response is important to stop the dissemination of bacteria, but on the other side an excessive and uncontrolled activation of the immune system cause tissue damage that results in the impaired body's function.
Infected individuals usually develop active TB one or two years after infection. Infection does not necessarily lead to active pulmonary TB. In more than 90% of subjects infection remains in a subclinical stage known as latent TB infection (LTBI). During LTBI the pathogen lasts in a quiescent state in which only non- or slowly replicating Mtb bacilli are

present (16). Mtb may persist for decades in the lymph nodes and in some cases in latent infected individuals a reactivation of TB infection can occur. Risk factors for reactivation of TB are socio-economic or behavioral factors, age, gender, the concomitant presence of other disease such as diabetes mellitus, HIV, or diseases in which patients need immunosuppressive anti-TNF antibodies treatment such as rheumatoid arthritis, psoriasis and Crohn's disease. This emphasizes the important role of the immune system in suppressing reactivation and replication of mycobacteria (17). There is a constant battle between Mtb and host immune system.

In rare cases, tubercle bacilli from the primary complex spread via the bloodstream and lymphatic system to many parts of the body, especially if a pulmonary TB is not properly treated or in immunodeficiency conditions. This form of TB disease is known as extrapulmunary TB. In this case, bacteria most commonly resides in the apices of the lungs, liver, spleen, meninges, peritoneum, lymph nodes, pleura, and bone. Extrapulmonary TB can develop either in localized or disseminated forms (miliary TB, see **Figure I3**). The most severe form of extrapulmonary TB is TB meningitis that is lethal in most of the cases.

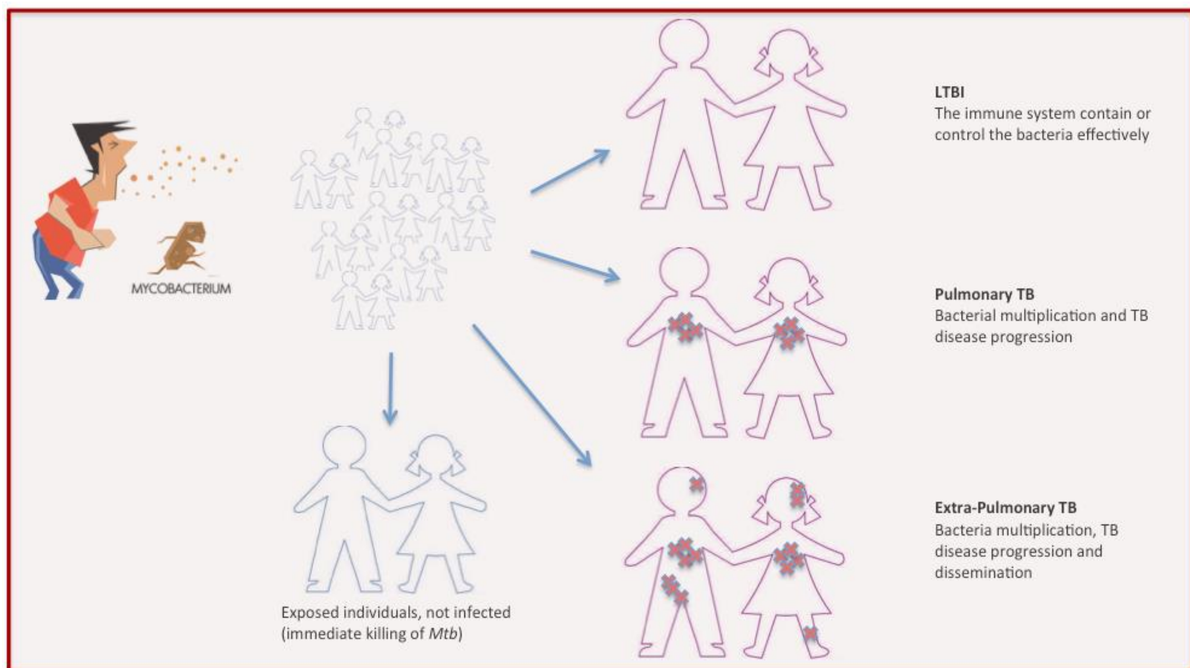

Figure I3: Mtbtransmission and outcome.TB is transmitted through airborne particles containing the "droplet nuclei" of the mycobacteria, which are released by a person with infectious TB during coughing, sneezing or simply talking. Host can respond to Mtb infection in several ways.If the immune system of infected individual manages immediate killing of Mtb disease is asymptomatic. An about 10% of individuals infected withMtb develop active TB (primary TB). A significant proportion of affected peopleharbors Mtband develops latent TB infection (LTBI). These individual have about 5-10% risk of TB reactivation form the latent state. The mycobacteria can however attack any organ via bloodstream resulting in extrapulmonary TB.

Tuberculosis therapy and vaccination

Efficient drugs against TB were first developed in the 1940s (5, 8). The most effective first-line anti-TB drug, rifampicin, became available inthe 1960s. The currently recommended therapeutic strategy by WHO for new cases of drug-susceptible TB isa combination of four

first-line drugs:isoniazid, rifampicin, ethambutol and pyrazinamide. The course of TB treatment usually takes four/six months in which rapidly killing of massive number of bacteria occurs. The prolonged therapy prevents the development of multi-drug resistent mutants and effectively sterilizesof the local infection site. Most recently, WHO reportedsuccessful treatment in 85% of new TB cases (5).

Treatment for multidrug-resistant TB (TB resistant to the two most powerful anti-TB drugs isoniazid and rifampicin;MDR-TB) requires more expensive and more toxic drugs. This is they reason why patients often fail to complete the therapy. Globally, in 2014, an estimated 190 000 people died of MDR-TB. More TB patients were diagnosed for drug resistance in 2014 than ever before. A total of 111 000 people started alternative MDR-TB treatment in 2014, which gives an increaseof 14% compared with previous year (**Figure I4**).

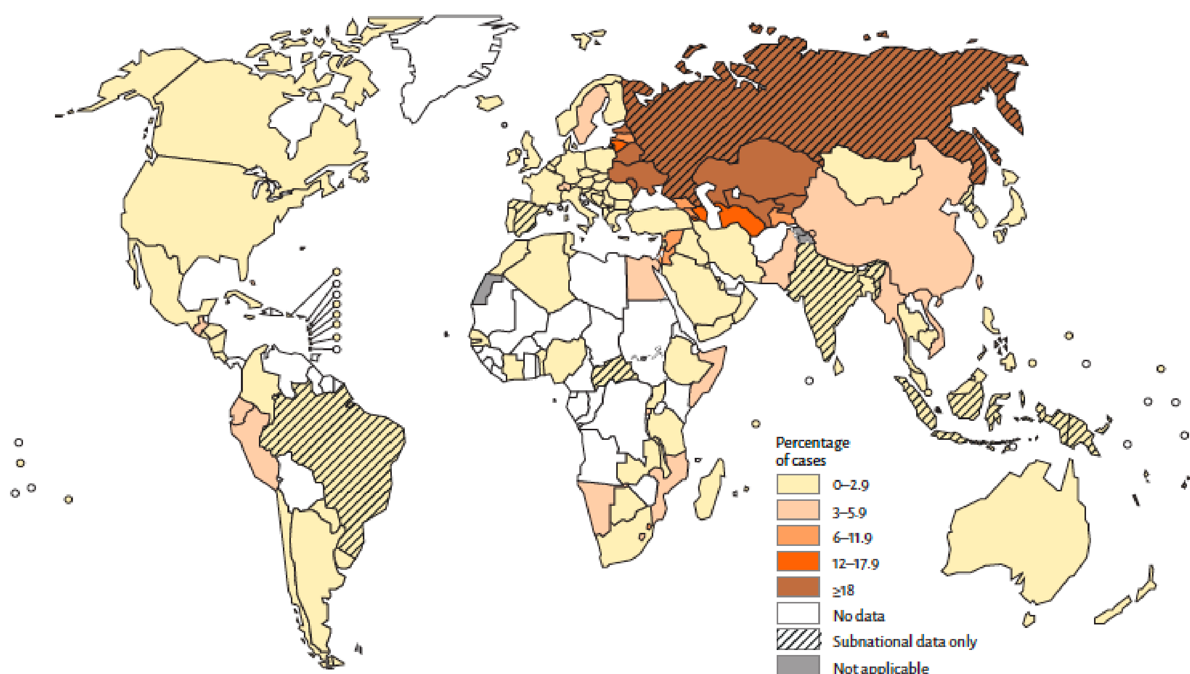

Figure I4: New TB cases with MDR-TB. Eastern European and Central Asian countries have the highest levels of MDR-TB. Levels of drug resistance among new cases remain low (<3%) in many parts of the world, including many countries in Americas, majority of African countries, most of the South-East Asia Region, most of Western Europe, several countries in the Western Pacific Region (WHO 2015).

According to the previously reported WHO analysis, it's clear that although effective drug treatments are avalaible in tackling TB disease, TB nevertheless kills about 4000 people every day so a vaccine that would prevent infection and/or disease is an essential component in the fight against TB with a view to both the containment of infection and the complete elimination of the pathogen worldwide.

Historically, the major purpose of most immunization programs was to obtain high antibody titers in vaccinated subjects.The first attempts to TB control through vaccination started at the end of 1800, just after the identification of the bacillus by Robert Koch, but it was the live attenuated vaccine by Albert Calmette and Camille Guérin (BCG) to dominate in next century.

In 1908, Guérin and Calmette were dedicated to the finding of an effective vaccine against TB from a virulent strain of *Mycobacterium bovis,* the etiologic agent of TB in cattle, which is closely related to Mtb, having a homology greater than 90%. Thirteen years later they created an attenuated bacillus which was proved safe and effective in preventing the development of the TB disease. Since 1921 the BCG vaccine has been introduced for human purpose. At the beginning the result of BCG vaccination in the human population was controversial. Up to date *M. bovis* BCG-vaccination protects infants from dissemination of TB, particularly TB-meningitis (18,19) but no efficacy was demonstrated against pulmonary TB in adults.

In addition, like all live-attenuated vaccines, its use is not adviced in immunosuppressed patients, in particular in HIV^+ subjects for which there is an increased risk of developing severe clinical forms of tuberculosis. Despite the many negative aspects related to the vaccine, the BCG is still widely used. Until now, no new effective vaccine against TB are avalaible.

There have been several attempts to explain the variable protective efficacy of BCG.The insufficient level of protection obtained with BCG may be explained in part by the lack of effective stimulation of T lymphocytes, in particular CTLs by the attenuated bacillus. The heterogeneity of the strains used was suggested as another possible explanation in the variability of the results obtained in different clinical trials.

Behr and Small have shown that, during the process of attenuation of *M. bovis* numerous genes potentially relevant in the induction of an effective immune response directed against the Mtb are lost (20). In particular, they identified a region of about 9.5 kb named RD1 (Delete Region 1)in the genome of the virulent strain, that is absent in the bacillus Calmette-Guerin. The genes present in this region are responsible for the synthesis of antigenic determinants important for the activation of T lymphocytes such as the immunodominant protein ESAT-6.

In conclusion, one fourth of the global population is infected with Mtb, and the vast majority of them do not develop active TB disease indicating a stable coexistence between Mtb and human host. The disease outcome depends on pathogen, host and environmental factors. Although effective TB drugs and vaccination are avalaible, MDR-TB strain, HIV co-infection, the lack of BCG efficacy make TB control still far from reach. An effective TB vaccine development is necessary and depends on a detailed understanding of the mechanism underlying the host immune response to Mtb.

Outline of this thesis

Protection against Mtb infection depends on the cellular immune system, involving many subsets both of innate and adaptative immunity. Upon exposure to Mtb in the airways, a series of immune responces are triggered. At first macrophages become activated. Then, DCs migrate to draining lymph nodes where they prime and boost specific T cell. Finally, several subsets of T cells get involved such as: Th1 and Th17 $CD4^+$ T cells, regulatory T cells (Tregs), $CD8^+$ T cells, γδ T cells and CD1-restricted T cells. These subsets cooperate and/or interfere with each other to control infection, and carry out inflammatory as well as regulatory activities that maintain immunological homeostasis.
This thesis aims to analyze the specific contribution of different T cell subsets in the control of TB infection, with particular attention to HLA-E restricted CD8 T cells which are a new player in the adaptive immune response to Mtb.

Chapter 1 summarizes our current knowledge on the immune response in human TB and focuses on the role of different functional signatures of T cell subsets in the immune response to Mtb infection. The role of the "conventional" subsets of CD4 and CD8 T cells will be discussed as well as the role of other "non coventional" subsets. The protective role of Mtb-specific-$CD4^+$ Th1-type polyfunctional T cell responses will be discussed.
Antigens specific $CD8^+$ T cells may also produce Th1-type cytokines as well and cytotoxic molecules that directly kill mycobacteria causing apoptosis of target cells. Moreover, it has been recently reported that CD8 T cells with a "multifunctional"profile can be a better markers of protection than $CD4^+$ T cells. Although, they cannot compensate for the lack of $CD4^+$ T cells, there is a special interest in studying CD8 T cells that are either MHC-class Ia or MHC-class Ib-restricted. These studies include the role of HLA-E-restricted cells, lung mucosal-associated invariant T cells (MAIT), Stem Memory T cell (Tscm) and CD1-restricted cells.

In **Chapter 2** HLA-E restricted $CD8^+$ T cells were studied in detail. We have characterized this specific subset as anadditional type of effector cells playing a role in immune response to Mtb during active infection. HLA-E restricted $CD8^+$ T cells from TB patients recognize Mtb-peptides in a CD3/TCR αβ mediated and CD8-dependent manner. HLA-E-restricted recognition of Mtb peptides is detectableby a significant enhanced *ex vivo* frequency of tetramer-specific circulating CD8 T cellsduring active TB. These CD8 T cells produce type 2 cytokines upon antigenic stimulation *in vitro*, help B cells for antibody production, and mediate limited TRAIL-dependent cytolytic and microbicidal activity toward Mtb-infected target cells.

The **Chapter 3** reports part of the results collected during the one-year internship period carried out at the Department of Infectious Disease of the Leiden University Medical Centrum (LUMC) directed by Prof. Dr Tom H. M. Ottenhoff. The project is still in progress.

Recently the Ottenhoff's group demonstrated that HLA-E restricted $CD8^+$ T cell clones obtained from PBMCs of healthy PPD^+donors and recognizing Mtb peptides produce Th2 cytokines (in particular IL-4, IL-5 and IL-13) instead of the usual Th1 cytokines, exert cytolitic or suppressive functions, inhibit mycobacterial growth and are able to activate B-cells (224). These results as well demonstrate the unique capability of this subset to respond in a specific manner after stimulation with the selected HLA-E peptides. The data were obtained using different techniques such as intracellular cytokine staining (ICS) for the detection of Ag-specific cytokine production, tetramer assay and T cell cloning for evaluation of the *ex vivo* frequency and the functional activities of HLA-E antigen-specific $CD8^+$ T cells, in patients in active TB with or without HIV co-infection and in LTBI subjects. PPD^- healthy donors were used as control.
The *ex-vivo* frequency of MAIT cells was also analyzed.

Chapter 4 reports in detail the material and methods used to perform the experiments presented in Chapter 3. Chapter 4 is followed by a brief **discussion** analyzing all obtained results.

Chapter 1

Functional signatures of human CD4 and CD8 T cell responces to *Mycobacterium tuberculosis*

Prezzemolo T., *Guggino G., La Manna M.P., Di Liberto D., Dieli F., Caccamo N.*
Front Immunol. 2014 Apr 22;5:180.

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Introduction

TB, with approximately 9 million cases annually, determines a world-wide mortality and morbidity, especially in low-income countries (21-23). Mtb, the causative agent of TB, is transmitted via aerosol droplets that are suspended in the air for prolonged periods of time (24), determining a risk of infection to people who inhalate these droplets. However, infection does not necessarily lead to TB disease; in fact, as reported in several studies, only 3–10% of immunocompetent individuals that are infected will develop the disease during their life-time (25), while more than 90% of infected subjects contain infection in a subclinical stage known as latent TB infection (LTBI), in which the pathogen remains in a quiescent state (24). One of the important aspects that can contribute to reactivation depends on the immune system of each individual that can be perturbed by several factors during life-time, such as chronic diseases: diabetes, alcoholic liver disease, HIV co-infection, and in some circumstances, the use of steroids or other immunosuppressive drugs. Another occurrence of active disease in later life is attributable to reactivation of latent Mtb bacilli or to a new infection with another Mtb strain. However, this huge reservoir contributes to fuel the high numbers of new active TB disease (23, 26); therefore, in order to diminish the risk of new active TB disease, it is important to treat LTBI cases by chemoprophylaxis, successfully eradicating the infection in the majority of cases. LTBI subjects, due to the increasing use of biological drugs, such as tumor necrosis factor-α (TNF-α)/Interleukin (IL)-12/IL-23 blockers for the treatment of inflammatory diseases like rheumatoid arthritis, Crohn’s disease, and psoriasis, have major risk to progress toward active disease more than other subjects (23, 27). Diagnosis of LTBI remains a priority for TB control within high income, low TB prevalence countries (28, 29), where a high proportion of TB cases occurs in immigrants from countries with high TB incidence (30, 31).
The study of subjects that are able to control Mtb infection in the long-term may be particularly informative in this respect. Despite two decades of intensified research, the mechanisms involved in the protective immune response against Mtb are not well understood. So, the comprehension of the pathways involved in protection in the host could represent biomarkers useful as correlates of protection, while the inhibition of the pathways involved in the surviving of host pathogens, could represent a biological target to contrast the bacilli growth and replication (32, 33).
Mtb involves several conventional and unconventional T cell subsets that are characterized by distinct effector functions and surface phenotype markers (34). Th1 CD4 T cells activate effector functions in macrophages that control intracellular Mtb, and their role has been

correlated with protection (34). Moreover, several studies have reported that Th17 cells, which are able to produce IL-17, are involved in immune protection against Mtb, primarily due to the effect of this cytokine in attracting and activating neutrophils (34, 35). Th17 cells have been involved in protection against TB at early stages (35, 36), for their capacity to recruit monocytes and Th1 lymphocytes to the site of granuloma formation (34, 35, 37). On the contrary, several studies have demonstrated that unrestricted Th17 stimulation determines an exaggerated inflammation mediated by neutrophils and inflammatory monocytes that rush to the site of disease causing tissue damage (34, 38–40).

CD4 T cells recognize antigenic peptides derived from the phagosomal compartment in the context of MHC-class II molecules (41). Mtb preferentially resides in the phagosome, where mycobacterial Ags can be processed and assembled to MHC-class II molecules (34, 42, 43). Another conventional lymphocytes subset, CD8 T cells, contributes to immune protection against TB (44): upon specific Ag recognition, CD8 T cells differentiate into effector cells, which produce cytolytic molecules and cytokines that kill both host cells and the intracellular Mtb (34, 45).

CD8 T lymphocytes recognize antigenic peptides, which are generally loaded in the cytosolic compartment in the context of MHC-class I molecules (41). MHC-class I loading can occur because of the intracellular pathogen or Mtb proteins diversification from the phagosome to the cytosol (34, 46). Moreover, apoptotic vesicles coming from infected macrophages and dendritic cells (DCs) can be uptaken by DCs (47, 48), which, in turn, will process and shuttled peptides into the canonical MHC-class I presentation pathway, a process termed cross-presentation (49).

Other cells play a role in the control or in the suppression of immune responses during Mtb infection such as Th2 cells, which counter-regulate Th1 cells and likely impair protective immunity against TB (50, 51), and regulatory T (Treg) cells (52, 53), which also contribute to the down modulation of the immune response to the pathogen (34) and to TB reactivation (34, 52–54).

The so-called unconventional T cells are activated during TB; these cells are able to recognize lipids that are abundant in the mycobacterial cell wall, in the context of non-polymorphic CD1 molecules (55). Very recently, mucosal-associated invariant T cells (MAIT) have been found to recognize protein Mtb (Ags) presented by the non-classical molecule MR1 (56). γδ T cells, recognize “phosphor Ags” of host or bacterial origin and may also contribute to the immune response to Mtb as well (34, 57). **Figure F1** shows the different cell population involved in the immunopathology of TB.

In the last years, the potential role of distinct T cell subsets as biomarkers of active TB and/or LTBI has been studied. Functional CD4 and CD8 T cell subsets have been defined on the bases of cytokine production as single, double, or triple producer cells. These different cytokine signatures have been differently associated with disease stage, mycobacterial load or treatment, and several studies, mostly derived from vaccination in animals, have highlighted that polyfunctional CD4 T cells are associated with protective immunity. In contrast, more recent studies have suggested that these cells may be not correlated with protection, but rather with TB disease activity (58, 59).

In this review, we will analyze the complexity of the immune response of conventional CD4 and CD8 T cells widely described by recent studies in patients with pulmonary and extra-

pulmonary disease and in subjects with LTBI, in order to better define the potential of different functional signatures of T cells as potential biomarkers.

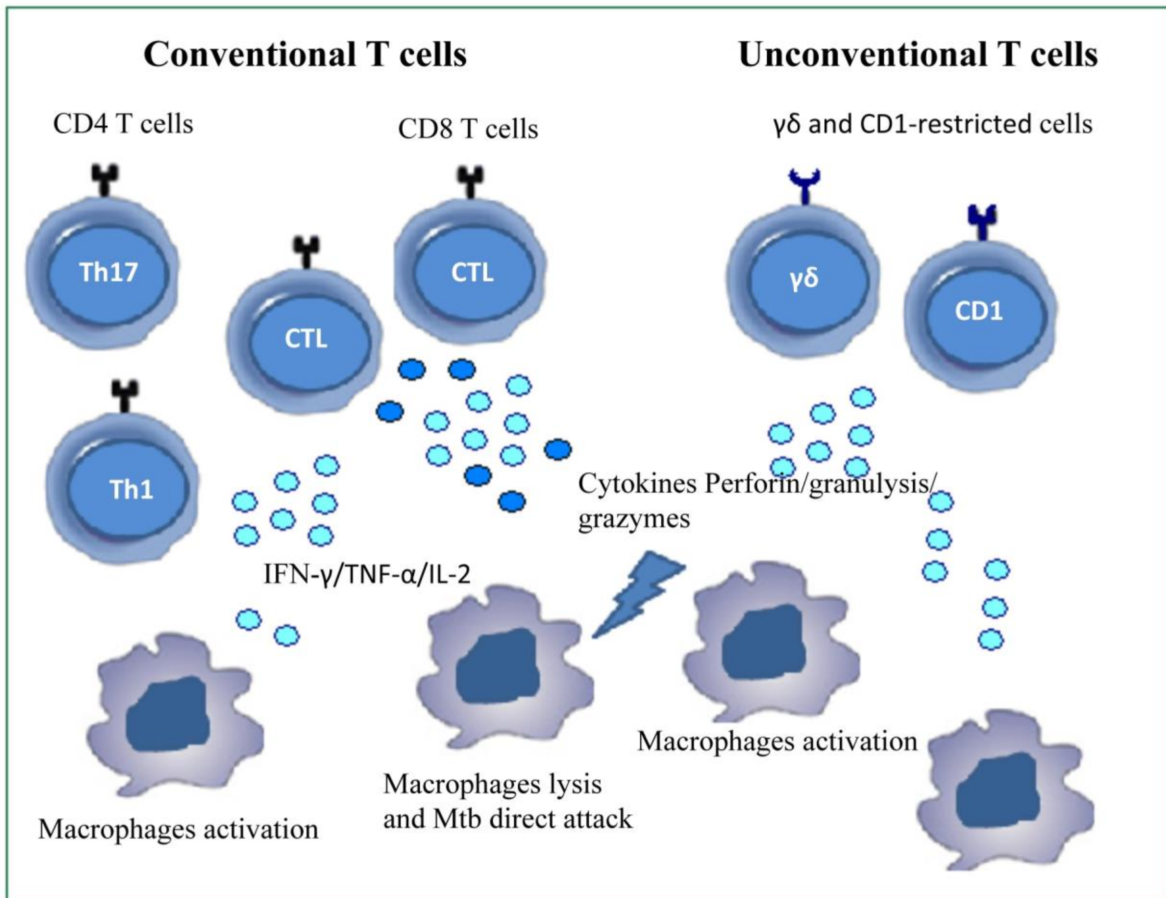

Figure F1: Cells involved in immune response during Mtb infection. The figure shows conventional and unconventional T cell subsets that contribute to the immune response against Mtb.

Population of human memory T cells

Individuals that have encountered a pathogen, develop an adaptive immune response with the induction of memory cells that will recognize the same Ag, upon the second encounter, dictating the type of immune response. Several studies have delineated that the quality of the memory response is important to dissect the real difference between protection and immunopathology, and to design strategies for vaccination (60).

Generally, the generation of memory T cells is characterized by different phases (61). The first encounter with an Ag, defined priming, determines a massive proliferation and clonal expansion of Ag-specific T cells followed by a phase of contraction, where the majority of these cells, named effector cells, are eliminated by apoptosis (62, 63). During this primary response, memory T cells develop and are maintained for extended periods due to several mechanisms such as the retention of Ag, stimulation/boosters, or homeostatic proliferation, that will insure the maintenance of a pool of cells that can rapidly respond to subsequent encounters with the pathogen.

The induction of memory T cells by vaccination against intracellular pathogens has definitively led a major challenge for the development of new subunit vaccines (60).
In humans, the functional properties of memory T and B cells can be defined, at least for those cells circulating in the blood, using techniques that detect typical surface markers (64). The combinatorial expression of surface markers such as adhesion molecules, chemokine receptors, and memory markers, allows for tissue specific homing of memory and effector lymphocytes and thus provides full characterization of that particular subsets of memory T cells, in terms of preferential residence inside tissues (60, 65, 66).
At least dozens of subsets can be identified and enumerated on the basis of distinct cellular functions that express unique combinations of surface and intracellular markers (67).
Memory T cells could be divided into $CD62L^+$ and $CD62L^-$ subsets; moreover some surface markers are specific for T cells homing to mucosa and skin that are confined to the $CD62L^-$ subset (68, 69). The development of techniques that allow to measure cytokines production at the single-cell level and the analysis of several surface markers has permitted to correlate the functional properties of T cells with their phenotype (70). $CCR7^+$ memory cells are named central memory (T_{CM}) cells: they are able to home to secondary lymphoid tissues, produce high amounts of IL-2 but low levels of other effector cytokines (71), while their $CCR7^-$ counter parts, named effector memory (T_{EM}) cells, are able to produce high levels of cytokines, exert rapid effector functions and home to peripheral tissues (71). It has been established a relationship between T_{CM} and T_{EM} cells suggested by the analysis of the telomeres that are longer in T_{CM} than T_{EM} cells and T_{CM} cells are capable of generating T_{EM} cells *in vitro*, but not *vice versa* (71). Studies performed in humans and rhesus macaques both *in vitro* and *in vivo* have led to the identification of T cells with multiple stem cell-like properties, termed memory T stem cells (T_{SCM}). These cells constitute a relatively rare memory population having a largely T naive (T_N) phenotype, while overexpressing CD95 (71, 72), which is usually expressed at high levels by all memory cells (73, 74). T_{SCM}cells, precede T_{CM} cells in differentiation. These type of cells are capable of generating all memory subsets, including T_{CM} cells (71, 72); no other memory subset thus far has been found to regenerate T_{SCM} cells (64).
Another subset of "transitional" memory T cells (T_{TM}) has been defined, mostly of which were isolated in the peripheral blood of healthy individuals (75, 76). These T_{TM} cells are more differentiated than T_{CM} cells but not as fully differentiated as T_{EM} cells in terms of phenotype (75, 76) and ability to expand in response to IL-15 *in vivo* (77, 78).
Very recently, Mahnke et al. propose that the phenotypic, functional, and gene expression properties of human memory T cell differentiation follow a linear progression along a continuum of major clusters (T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells) (64). According to this linear progression, memory T cells, progressively acquire or lose their specific functions (**Figure F2**).

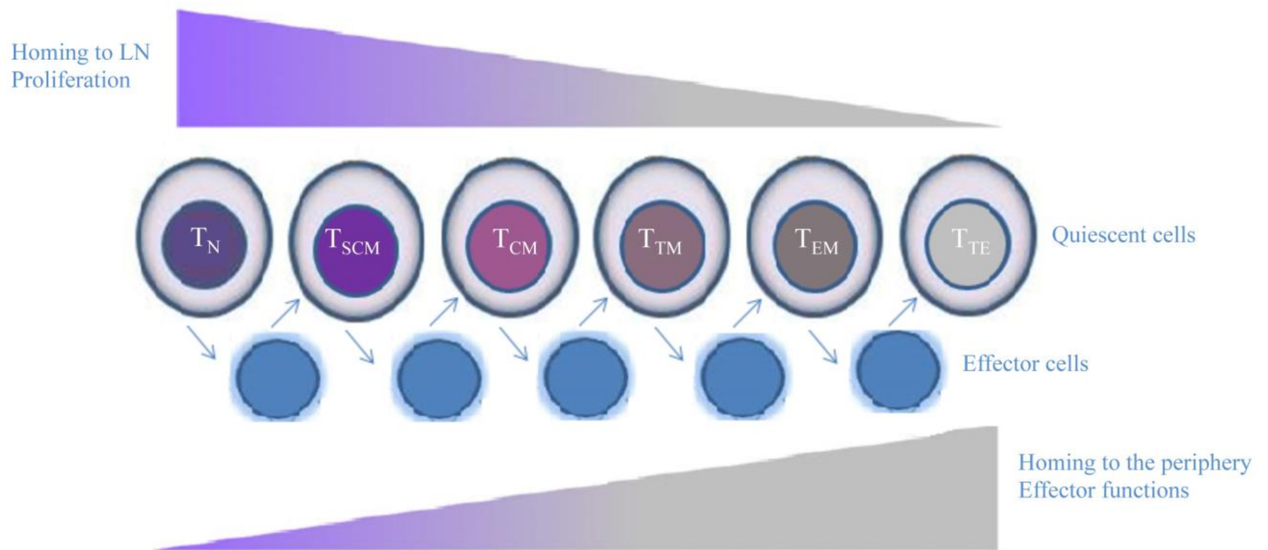

Figure F2: Human memory T cell subsets. Following encounter with Ag, quiescent T cells develop into effectors, whose phenotype is highly dynamic and largely unpredictable. When the Ag is cleared, effector T cells that survive return to a quiescent memory state. Cells differentiate from T_N to T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and culminating in T_{TE} cells. Memory T cells progressively lose or acquire specific functions, such as the ability to migrate to peripheral tissues or to proliferate or produce effector molecules.

Other molecules that mediate lymphocyte functions, including markers of migration, co-stimulation, and cytotoxic molecules and adhesion markers can better define these different T cell subsets (**Table F1**).

Seder et al. have proposed that T cells progressively acquire their functions with further differentiation, until they reach the phase that is adequate for their effector function (such as the production of cytokines or cytotoxic activity) (64, 79). The authors have demonstrated that the continued antigenic stimulation led to progressive loss of memory potential as well as the ability to produce cytokines, until the last step of the differentiation pathway represented by effector cells that are able to produce only IFN-γ and are short-lived, named terminally differentiated effector cells (TEMRA) (79). Another aspect that can optimize this linear differentiation process will depend on the amount of initial Ag exposure or the different conditions that are present in the microenvironment, which will dictate the extent of differentiation (64, 79).

Hierarchical expression of cytolytic molecules and surface markers, such as CD27, CD28, and CD57, has been delineated for CD8 T cell subsets. Granzyme (GrA) is the first cytotoxic molecule detected in memory cells, followed by GrB (GrB) and subsequently by perforin (80–82). GrB is always expressed in the presence of GrA, while, perforin^{+} cells are primarily positive for GrA and GrB, making it a choice indicator for cytolytic cells (82). Usually, perforin is present in cells that are CD27^{-} and CD28^{-} (83), while this molecule is always associated with the expression of the senescence marker CD57, which can be used as marker for T cells with high cytolytic potential (64, 82). Finally, the identification of the

different subsets of human memory T cells, through the analysis of the expression of exclusive markers in that particular population could have a potential implications in T cell-based immunotherapy for infectious disease or other immune pathological conditions. Several studies have evaluated the different distribution of Ag-specific memory T cells subsets as good model of correlate of protection; for example, in response to chronic infectious agents such as HIV-1, hepatitis C virus (HCV), and Mtb, the increase of the frequency of Ag-specific TCM cells, which produce high levels of IL-2, is associated with individuals' ability to control the viral load (84–88).
Moreover, the response to cytokines used to differentiate or to maintain the different human memory T cells has been characterized (89). It has been shown that T_{EM} cells can proliferate in response to IL-7 and IL-15 *in vitro* but do not expand because of spontaneous apoptosis; conversely, T_{CM} proliferate and differentiate to T_{EM} cells, in the absence of these cytokines (90, 91).
Therefore, the quality of T cell responses can be modulated by several factors, and it is crucial for establishing the disease outcome in the context of various infections or pathologies.
In summary, the definition of the different subsets of memory T cells can be used to delineate the quality of a given T cell response, and this can be achieved by the combination of cell-surface phenotype, functional properties, and the capacity to traffic to lymphoid and non-lymphoid tissues: such a complex analysis should confer more intuition if an immune response will be protective or not.

Subsets	TN	TSCM	TCM	TTM	TEM	TTE	Category	Antigen	Function
	+	++	++	++	-	-	Costimulation/ Survival	CD28	Costimulation
	++	+	+	+	-/+	-		CD27	Costimulation
	++	+++	+++	++	-/+	-		CD127	IL-7 signaling
	-	-/+	+	++	+	+		PD-1	Inhibition of effector function
	-	+	++	+++	+++	+++		CD122	IL-2/IL-15 signaling
	+	+	+	+	+	+		CD132	γc cytokine signaling
	-	ND	-/+	+	++	+++		KLRG-1	Inhibition of effector function
	+	++	++	+++	+++	+++	Adhesion	CD11a	Adhesion to APC/endothelium
	-	+	++	+++	+++	+++		CD58	Adhesion to APC
	-/+	+	++	++	++	++		CD99	Transendothelial migration
	+	+	+	-	-	-	Migration	CD62 L	Secondary lymphoid tissues homing
	-	-	-	-	+	-		CD103	Gut homing
	-/+	+	++	+++	+++	-/+		CCR4	Chemokine response/Th2 associated
	-	-	+	++	+++	++		CCR5	Homing to inflamed tissues
	-	-	++	+++	+++	-		CCR6	Chemokine response/Th17 associated
CD4	-	ND	+	-	-	-		CCR9	Gut homing
CD8	-	ND	+	++	++	-			
	-	-	+	ND	++	-		CCR10	Skin homing
CD4	-	-/+	+	++	+++	+++		CXCR3	Homing to inflamed tissues
CD8	++	+++	+++	++	+	+			
	+	++	+++	+++	++	++		CXCR4	Homing to Bone Marrow
	-	ND	+	ND	++	ND		CLA	Skin homing
CD4	-	-	-	-	-/+	+	Cytolitic molecules	Granzyme A	Cleavage of cellular proteins
CD8	-	-	-/+	++	+++	+++			
CD4	-	-	-	-	-/+	-/+		Granzyme B	Cleavage of cellular proteins
CD8	-	-	-	+	++	+++			
CD4	-	-	-	-	-/+	-/+		Perforin	Pore forming
CD8	-	-	-/+	+	++	+++			
Combination of + and – indicate the expression level respect to TN cells. ND= not determined.									

Table F1: Expression of functional molecules by circulatingT cell subsets.

Subsets of Memory CD4 T cells in tuberculosis

Mtb-specific-CD4$^+$ T cell protective response is typically due to Th1 cells and is mediated by IFN-γ and TNF-α that recruit monocytes and granulocytes and promote their anti-microbial activities (92–94).

Recent studies have shown that polyfunctional T cells (i.e., T cells equipped with multiple effector functions) (64, 95), could exert immune protection toward viral infections such as HIV (96, 97), models of TB vaccine (98–101), or in murine models of leishmania (56). However, the role of polyfunctional T cells during Mtb infection is controversial and different from that observed in chronic viral infections (56, 60, 101).

The definition of polyfunctional T cells was attributed to their ability to proliferate and to secrete multiple cytokines and these cells were found to play a protective role in antiviral immunity in chronic infections (when Ag load is low). Conversely, single IFN-γ-secreting CD4 and CD8 T cells typically predominate in acute infections (when Ag load is high), and in chronic infection characterized by the failure of immune control: in the case of HIV-1 infection, in fact, the response is dominated by HIV-1-specific-CD4 and -CD8 T cells that are able to produce only IFN-γ in both the primary and chronic phases of infection. On the

other hand, the distinct cytokines profile during intracellular pathogens infection, comprises a very wide spectrum of T cell subpopulations (95).
Several authors have recently shown that polyfunctional T cells release multiple cytokines simultaneously in a relatively short period. The analysis of different aspects that could contribute to the release of cytokines, such as the methodologies used to stimulate the cells, peptides, or proteins used, the different cohort groups included in the study, should be taken into account, considering that very often the results obtained are controversial (95, 102).
Earlier studies in human TB have investigated on the role of polyfunctional T cells able to produce IFN-γ in combination with IL-2 (95, 103–106), and later on, a subset of cells able to simultaneously produce IFN-γ, TNF-α, and/or IL-2 was detected in patient with active TB disease compared to latently infected individuals (107–110), whose frequency decreased after anti-TB treatment. In another study, high frequencies of CD4 T cells expressing three cytokines simultaneously (IFN-γ, TNF-α, and IL-2) was found in adults with active TB disease, as compared to the frequency found in LTBI subjects, in which IFN-γ single and IFN-γ/IL-2 dual secreting CD4 T cells dominated the anti-mycobacterial response. Therefore, the presence of multifunctional CD4 T cells in TB patients was associated with the bacterial loads, as suggested by their decrease after completion of anti-TB chemotherapy (102, 111). This implies that multifunctional CD4 T cells are indicative of active TB rather than assuming a protective role. However, during these years, several contrasting findings have been reported, which do not allow a clear-cut conclusion on the role of polyfunctional CD4 T cells (60). In fact, some authors have found a reduced frequency of polyfunctional T cells in patients with active TB disease compared to latently infected individuals, which is recovered with the anti-TB therapy (95, 112, 113). Similar recovery of dual IFN-γ/IL-2-producing cells with the anti-TB therapy was also previously reported (102, 114).
Finally, a higher proportion of Ag-specific effector memory T_{EM} cells and a decreased frequency of T_{CM} $CD4^+$ T cells has been found in patients with active TB (115, 116), as compared to the distribution found in LTBI individuals (95).
Since it is not possible to associate any specific cytokine profile with protection against active TB, recent studies have tried to find a correlation between functional signatures of CD4 or CD8 T cells and the state of infection/disease.
Marin et al. have analyzed the Th1 and Th17 responses through the counts of IFN-γ and IL-17 producing T cells by elispot assay, the frequencies of polyfunctional T cells producing IFN-γ, TNF-α, IL-2, and IL-17 by ICS, and the amounts of the above cited cytokines released after 1 day (short term) and 6 days (long-term) of *in vitro* stimulation using different Ags (CFP-10, PPD, or Mtb) (95) by ELISA. The evaluation of different T cell subsets after short- and long-term *in vitro* stimulation with different Ags has permitted to find a significant increase in single and double producer $CD4^+$cells in long-term *in vitro* stimulation compared to short term *in vitro* stimulation in LTBI subjects and a significant increase of the frequency of single producer cells in patients with active disease (95). Mtb stimulation determined an increase in the frequency of single and triple producer T cells in LTBI subjects in 6 days compared to the frequency found in 1 day *in vitro* stimulated cells, with a significant value found for the frequency of double producer T cells in patients with active disease (95). These results suggest that the use of different mycobacterial Ags could induce distinct T cell functional signatures in LTBI subjects and in patients with active disease, highlighting that it is possible to define "functional signatures" of CD4 T cells

correlated with the state of infection and that could be used as indicators of the clinical activity of the disease (102).
Very recently, Petruccioli et al. have correlated bifunctional “RD1-proteins”-specific-CD4 T cells with effector memory phenotype with active TB disease, while “RD1-proteins”-specific-CD4 T cells with a central memory phenotype were associated with cured TB and LTBI subjects (102). According to this study, the EM phenotype should be associated with inactive TB due to the presence of live and replicating bacteria, whereas the contraction of this phenotype and the further differentiation toward CM T cells in LTBI and cured TB subjects could indicate Mtb control, suggesting that the different expression of the memory/effector status may be used to monitor treatment efficacy, as previously suggested in patients with active TB with HIV co-infection (102, 117, 118).
A more detailed study on the role of Ag-specific T cell phenotype and function has been carried out by Lalvani et al. who delineated the association of TB disease stage with Mtb-specific cellular immunity. The authors have found the same trend of functional signature demonstrated by Petruccioli, but in response to different antigenic stimulation, namely PPD and RD1-peptides: in fact, Ag-specific-CD4 T cells were principally of the CM phenotype in subjects with latent infection compared to EM cells predominantly found in patients with active disease. Combined measurement of both functional profile and differentiation phenotype, in this study, reflects a discriminatory immunological status in the different cohort groups studied (patients with active disease vs. LTBI) (119). Moreover, HIV infection did not influence the number of Mtb-specific-CD4 effector cells, which instead was influenced by TB disease stage. This last aspect could be intriguing for the fact that assessment of cellular changes could be used also for immune compromised patients; in fact, it is known that HIV and active TB both impact Mtb-specific T cell immunity, such as skin test anergy, and therefore, dissection of distinct subsets as biomarkers could have an impact also in HIV co-infection.
Altogether, the above studies highlight the concept that the protective immune response against mycobacterial infection seems to depend more on the quality of CD4 T cell response assessed as the capacity to exert multiple functions, than on their magnitude, which is due to their Ag-specific frequency (64, 95). Finally, several methodologies used for the evaluation of the profiles of Mtb-specific-CD4 T cells in the reported studies led to different results: these include Ag specificity and type, *in vitro* stimulation conditions (short- or long-term *in vitro* stimulation), variability of the study cohort characteristics and at least, the monoclonal antibodies used to distinguish the subsets of CD4 T cells or intracellular cytokines content (60).
Thus, further studies are necessary to define particular phenotypes of Mtb-specific-CD4 T cells, assessing several functional properties such as activation, memory, migratory and inhibitory receptors, and ligands.

Subsets of CD8 T cells in tuberculosis

$CD8^+$ T cells contribute to protective response against TB (120, 121). $CD8^+$ T cells recognize Ags derived from an intracellular environment and could serve as sensors of bacterial burden. In fact, human $CD8^+$ T cells preferentially recognize cells heavily infected

with *Mtb* (122) and in animal models, the magnitude of the CD8 response correlates with bacterial load (123–125).
The mechanisms involved in $CD8^+$ T cell activation during Mtb infection are incompletely defined. DCs possess several pathways to load MHC-class I molecules, such as classical cytosolic processing, or alternative processing of phagosome located pathogens and endosome-located Ags. The recent evidences that virulent mycobacteria can escape from the phagosome into the cytoplasm and the possibility to direct access MHC-class I processing/presentation pathway provide a new mechanism (47). DCs also can take up vesicles derived from apoptotic Mtb-infected cells, after which the Ags are cross-presented through MHC-class I and class II molecules (48, 49). Finally, autophagy, which has a prominent role in cellular homeostasis and bacterial sequestration into vacuolar organelles, is involved in Ag presentation and cross-priming of T cells in response to intracellular pathogens, including Mtb (126, 127).
It has been demonstrated that several pathways are used in order to activate $CD8^+$ T cells by phagosomal Ags, and, very recently, MHC-class Ib-restricted $CD8^+$ T cells have received attention, including a role for HLA-E, which presents peptides from a wide range of mycobacterial Ags (54, 128). CD1-restricted CD8 T cells recognize lipids such as mycolic acids and lipoarabinomannan from the bacterial cell wall (54) and lung MAIT recognize Mtb Ags in the context of the non-classical MR1 molecule (129).
Thus, $CD8^+$ T cell immunity offers evidences of their clear synergy of action and complementarities in association with $CD4^+$ T cell immunity, for the fact that $CD8^+$ T cells display other direct effector functions such as the secretion of granules that contain cytotoxic molecules as perforin, granzymes, and granulysin. These molecules can lyse host cells, or can have a direct killing toward Mtb and other bacteria. Moreover, $CD8^+$ T cells can induce apoptosis of infected target cells through molecules such as Fas or TNF-R family-related cell-death receptors. Finally, $CD8^+$ T cells release, upon activation, cytokines such as IFN-γ, TNF-α, and in many cases also IL-2. These functions are also used by MHC-class Ib-restricted $CD8^+$ T cells, suggesting a role for classical as well as non-classical $CD8^+$ T cells in TB protection.
From the functional point of view, different studies conducted in mice and non-human models have delineated a role for Mtb-specific $CD8^+$ T cells in the control of Mtb infection (122–124). In these studies, it has been demonstrated that IFN-γ and perforin released by Mtb-specific $CD8^+$ T cells were necessary to induce protection in Mtb-infected mice (122, 125). The role of these molecules has been efforted in humans' studies that have reported the same conclusions (41, 130).
Hence, other *in vitro* studies have indicated that perforin- and/or granulysin-containing Mtb-specific $CD8^+$ T cell lines were able to kill Mtb-infected macrophages or even free bacteria (45, 131, 132), other studies have found the complete absence of these molecules released by Mtb-specific $CD8^+$ T cells from lung-associated tissues (133, 134).
Though it is not still possible to attribute a role to polyfunctional T cells as marker of protective immunity or of disease activity, multi-, or polyfunctionality of CD8 T cells is referred to the simultaneous production of several cytokines (IFN-γ, IL-2, TNF-α) and/or the expression of multiple effector functions (perforin, granulysin, cytolysis, etc.). However, contrary to initial expectations, these cells do not appear to correlate with BCG-induced protection in infants (135) and adults (136). Moreover, they are also present in active TB,

although they may nevertheless be part of the protective host response attempting to limit infection rather than contributing to active disease.
Previously, we have correlated the frequency of Mtb-Ag85A-specific $CD8^+$ T cells with the efficacy of anti-mycobacterial therapy in children. In particular, we found that Ag85A epitope-specific $CD8^+$ T cells in children with active disease were able to produce low levels of IFN-γ and perforin, which recovered after successful therapy (137). In a later study, the analysis of the *ex vivo* frequencies, cytokine production, and memory phenotype of circulating CD8 T cells specific for different non-amers of Mtb proteins was performed in adult HLA-A*0201 different cohorts (107).
We found a lower percentage of circulating tetramer specific CD8 T cells in TB patients before therapy respect to LTBI subjects, but values increased after 4 months of anti-mycobacterial therapy to those found in subjects with LTBI. In this study, we also found high percentages of $IL\text{-}2^+/IFN\text{-}\gamma^+$ and single $IFN\text{-}\gamma^+$ in subjects with LTBI, and a reduction of $IL\text{-}2^+/IFN\text{-}\gamma^+$ population in TB patients, suggesting a restricted functional profile of Mtb-specific CD8 Tcells during active disease (107).
Many studies have focused on the response to different Mtb Ags expressed in the early phase of infection such as ESAT6, CFP-10, and Ag85B proteins but further studies should also incorporate those Ags expressed at different phases of infection (60).
Another study, using defined cohorts of individuals with smear-positive and smear-negative TB and LTBI subjects, evaluated Mtb-specific responses in correlation to mycobacterial load (113). The authors found, in individuals with high mycobacterial load smear-positive TB, a decrease of polyfunctional and IL-2-producing cells, and an increase of $TNF\text{-}\alpha^+$ Mtb-specific-CD4 T cells and CD8 T cells, both of which had an impaired proliferative capacity (60). These patients were followed during the anti-mycobacterial therapy and it was shown that the percentage of triple positive CD8 T cells (producing IFN-γ, IL-2, and TNF-α) increased over time in 7 out of 13 patients and this increase was paralleled by decrease of the frequency of $IFN\text{-}\gamma^+$ T cells, providing another evidence that the cytokine production capacity of Mtb-specific CD8 T cells is associated with mycobacterial load.
In children or immunocompromised individuals, where it is very difficult to distinguish Mtb infection from disease, and in people that are at high risk to develop active disease, the increase of polyfuntional CD8 T cells and the reduction of single IFN-γ or TNF-α producing cells may be used to correlate these CD8 T cell subsets with TB disease progression, highlighting a new possible role as indicator of successful response to treatment.
Mtb DosR-regulon encoded Ags (138) expressed by Mtb during *in vitro* conditions, represent rational targets for TB vaccination because they mimic intracellular infection. It has been shown that LTBI individuals are able to recognize Mtb DosR-regulon encoded Ags belonging to different ethnically and geographically distinct populations (60, 131, 138, 139). Moreover, Mtb DosR Ag-specific-$CD4^+$ and -$CD8^+$ polyfunctional T cells were found in LTBI subjects. In detail, a hierarchy of response, in terms of the ability of Ag-specific CD8 T cells to produce one or more cytokines, was found. The highest response was observed among single cytokine producing $CD4^+$ and $CD8^+$ T cell subsets, followed by double producing $CD4^+$ and particularly $CD8^+$ T cells. In particular, the most frequent multiple-cytokine producing T cells were $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ $CD8^+$ T cells. These cells were effector memory ($CCR7^-$ and $CD45RA^-$) or terminally differentiated effector memory ($CCR7^-$ and $CD45RA^+$) T cells, both phenotypes associated with the protective role of $CD8^+$ T cells in

Mtb infection (60, 131, 140). Another important observation was the number of epitopes identified, in accordance with their immunogenicity and recognition by a wide variety of HLA backgrounds (141, 142).
Therefore, the role of Mtb DosR-regulon encoded peptide Ag-specific single and double functional $CD4^+$ and $CD8^+$ T cell responses in LTBI, significantly improves the understanding of the immune response to Mtb phase-dependent Ags in the control of infection, and suggests a possible role for using Mtb DosR-Ag and/or peptide based diagnostic tests or vaccination approaches to TB.
Several studies have tried to correlate the frequency, the phenotype, and the effector functions of CD8 T cells in patients with disease and subjects with latent infection. Here, we report other additional recent studies aimed at identify biological indicators useful to discriminate between patients with active disease, subjects with latent infection and patients that recovery after successful therapy.
Niendak et al. have observed that specific $CD8^+$ T cell response decreased by 58.4% at 24 weeks, with the majority of the decrease (38.7%) noted at 8 weeks in subjects receiving successful anti-TB treatment (143); decrease of the $CD8^+$ T cell response was relatively unaffected by malnutrition, supporting the hypothesis that the frequency of Mtb-specific $CD8^+$ T cells declines with anti-tuberculosis therapy potentially as consequence of decreasing intracellular mycobacterial Ags, and may prove to be a surrogate marker of response to therapy (54, 144). The authors postulate that each individual has a CD8 “set point,” which reflects the complex interplay of antigenic exposure, in conjunction with host factors such as the HLA background. Nonetheless, these findings are concordant with the observation that removal of Ag results in decreasing T cell frequencies, and help to explain the observed reduction in $CD8^+$ T cell frequency following anti-tuberculosis therapy.
Another recent study of Harari et al. (112) highlighted phenotypic and functional properties of Mtb-specific CD8 T cell responses in 326 TB patients and LTBI subjects in order to correlate their presence with different clinical form of Mtb infection (94). Authors found a higher frequency of Mtb-specific CD8 T cell responses in TB patients, which was correlated with the presence of higher Ag load (94, 112). These results were confirmed by two different studies, the first performed in children with active disease, where Mtb-specific CD8 T cells were detected in active TB disease but not in healthy children recently exposed to Mtb (112), and the second that demonstrated the presence of higher number of granulomas in TB patients as compared with those in LTBI subjects (94). Moreover, major phenotypic and functional differences were observed between TB and LTBI subjects, as Mtb-specific $CD8^+$ T cells were mostly represented by terminally differentiated effector memory cells (TEMRA) in LTBI and of T_{EM} cells in TB patients. These results also suggests that TEMRA and T_{EM} cell subsets, are involved in the control of Mtb infection, as already demonstrated in chronic controlled and uncontrolled virus infection, respectively (94, 145).
The authors did not find any statistically significant difference in the cytokines profile of Mtb-specific $CD8^+$ T cell responses between LTBI subjects and TB patients, while they found that Mtb-specific $CD8^+$ T cells were more polyfunctional (i.e., IFN-γ^+TNF- α^+IL-2^+) in LTBI subjects, according to the role that these cells play in anti-viral immunity (94, 145). Instead, it was found that Mtb-specific $CD8^+$ T cells have a higher frequency as single TNF-α-producer cells in TB patients, as occurred for $CD4^+$ T cells (145). Further analysis of the

functional properties of these Mtb-specific CD8^{+} T cells, permitted to detect significant high levels of GrB and GrA, but low level of perforin, suggesting a mechanism of action of Mtb-specific CD8^{+} T cells that is independent on the expression of perforin (94).
Another intriguing aspect of that study was the finding of a higher prevalence of Mtb-specific CD8^{+} T cell responses in pulmonary TB patients compared with extra-pulmonary TB patients and the higher magnitude of these responses in smear-positive versus smear-negative pulmonary TB patients (94). Moreover, Mtb-specific CD8^{+} T cells from pulmonary TB patients were not able to proliferate compared to CD8 T cells from extra-pulmonary TB patients (94). These functional differences of the CD8 T cell responses, in term of cytokines release or proliferation, most likely depend on antigenic stimulation that occur at different anatomic sites, that could be correlated with high Ag burden (108, 146, 147), attributing to tropism of responding T cells (94).
In conclusion, Mtb-specific CD8 T cell response, as defined by the qualitative and the quantitative aspects above cited, could have significance in understand how the immune system fails to control the progression of TB, or how the quality of the response could facilitate early diagnosis in order to reduce TB associated morbidity and mortality and to individuate subjects that are at high risk to develop active disease (60).

Role of T Cells in TB-HIV Co-infection

HIV infection has led to an increase in the incidence of TB, and TB-HIV co-infection has determined not easy decisions in both the diagnosis and treatment. The treatment of co-infected patients requires anti-tuberculosis and antiretroviral drugs to be administered together. The therapeutic treatment leads to different results, according to patient compliance, drug toxic effects, and, finally to a syndrome that appears following the initiation of antiretroviral therapy (ART) named immune reconstitution inflammatory syndrome (IRIS).
Several studies have provided to clarify the relationship that exists between HIV and Mtb pathogens and how they interact both *in vitro* and *in vivo*, highlighting how HIV infection could increase the risk of TB and how Mtb infection may accelerate the evolution of HIV infection. Flynn et al., very recently, have summarized the results obtained from different studies, discerning the several hypotheses on the role of the immune system in the co-infection (148).
It is well known that TB-HIV co-infection is destructive (149–151), but nowadays the mechanisms involved in the impairment of the immune system, guiding to the morbidity and mortality of co-infected subjects, remain to be elucidated (152). In countries with low rates of TB and, of course, with high-burden TB, the identification of LTBI within individuals co-infected with HIV is important due to the high risk to develop active TB. One of the control strategy adopted by the WHO is the use of preventive therapy of LTBI with isoniazid (INH) treatment (153). HIV-infected individuals are at high risk to develop active TB for the progressive CD4 depletion in the first few years after infection, even if the number of peripheral CD4 T cells is still high at the beginning (154–156). Although, the ART could restore absolute CD4 T cell numbers, it does not reduce the risk of TB progression in HIV patients (157). Conversely, TB infection has a negative impact on clinical progression of HIV infection (158).

Studies of human disease have characterized functional defects in CD4 T cells in TB-HIV co-infection by the analysis of cytokine production (e.g., IFN-γ) by CD4 cells in response to Mtb Ags (159–162) and by the analysis of phenotype distribution of CD4 T cells in lymphoid tissue, peripheral blood, and at the sites of disease (159, 163, 164). The correlation of different phenotypes of Ag-specific-CD4 T cells, and their role on the protection or susceptibility to infection, has been clearly demonstrated by the emerging characterization of polyfunctional CD4 T cells in TB-HIV co-infection. In the peripheral blood of TB-HIV-infected people, CD4 T cells are less able to secrete more than one cytokine when the viral load is high (165). Kalsdorf et al. have demonstrated that polyfunctional T cells specific for mycobacterial Ags are reduced in BAL from latent TB-HIV-infected subjects with no symptoms of active TB. The impairment of mycobacterial specific T cells could contribute to develop active TB, suggesting that HIV infection affects the frequency of Ag-specific polyfunctional T cells in the BAL of people with latent TB-HIV (160). Therefore, several studies have tried to correlate the presence of these cells in blood or in fluids recovered at the site of infection, highlighting how their presence can be reduced or increased, in term of absolute number. In fact, some authors have found a reduction of polyfunctional CD4 T cells in the peripheral blood of HIV-infected infants, in response to restimulation with BCG, compared with HIV-uninfected infants, or in BAL samples from HIV-infected subjects compared with HIV-uninfected healthy subjects, and finally, an increase in pericardial fluid of TB-HIV patients, with a terminally effector phenotype (163). Matthews et al. have found a lower proportions of Ag-specific polyfunctional T cells, with the less mature phenotype of CD4 T memory, at the site of disease of both HIV-infected and uninfected TB patients, supporting the hypothesis that their presence could correlate with Ag load and disease status, instead than with protection (163). Finally, understanding how the immune system contributes to TB-HIV co-infection could provide the basis for the discovery and development of new drugs and vaccines that can prevent or cure TB in co-infected people. At the moment, an early ART treatment still represents the gold standard in the control of TB-HIV co-infection.

Concluding Remarks

Tuberculosis research in the field of vaccine and diagnostic tests development suffers from lack of rigorous correlates of protection in order to better understand the basic mechanisms underlying pathophysiology. Therefore, the identification of biosignatures that predict risk of disease, but also vaccine efficacy would be important.

Studies of human T cell responses, using different protocols of *in vitro* stimulation, have made possible to delineate some functional signatures indicative of the immunological status of each studied individual (60).

From the above cited studies, it has clearly emerged that, for TB diagnosis it is necessary to investigate on several biomarkers. The different expression levels of several cytokines, evaluated *ex vivo* in cells obtained from blood samples, comparing uninfected subjects, LTBI individuals, and patients with active disease, led to not unique results. This issue, therefore, requires further investigation by different analytical platforms. In particular, we believe that TB biomarkers research may continue to generate signatures with clinical

applicability and additionally provides novel hypotheses related to disease pathophysiology (166).
Finally, the identification of such functional T cell signatures could help to better make diagnosis of different stages of TB, including also the cases of risk of reactivation and/or progression to active disease such as occurs in HIV patients (166).

Acknowledgments.
This work was supported by grants from the European Commission within the 7th Framework Program, NEWTBVAC contract no. HEALTH-F3-2009-241745. The text represents the authors' views and does not necessarily represent a position of the Commission who will not be liable for the use made of such information.

Chapter 2

Human CD8+ T lymphocytes recognize Mycobacterium tuberculosis antigens presented by HLA-E during active tuberculosis and express type 2 cytokines

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Eur J Immunol. 2015 Apr;45(4):1069-81

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T-cell mediated immune responses play a fundamental role in fighting against Mtb (167, 168). Traditionally, this response is mediated by Th1-type CD4 T cells secreting IFN-γ (168, 169), but it has become clear that CD8 T cells also contributeto protective immunity against Mtb (170) by the releaseof Th1-type cytokines and cytotoxic and microbicidal molecules that promote killing of Mtb infected macrophages and restrict the growth of intracellular bacilli (168). In humans, Mtb reactive CD8 T cells recognize peptides associated to classical HLA-A, HLA-B, and HLA-C class I (class Ia) molecules, glycolipids associated to group 1 CD1 molecules (171, 172), and mycobacterial Ag associated to MHC class I related molecule (MR1) (173). However, there is little information on the role that these cells play during infection. In mice, the MHC class Ib molecule H2-M3 binds formylated peptides derived from Mtb and induces H2-M3-restricted CD8 T cells (174, 175) that are protective against Mtb infection (176).

In humans, CD8 T cells restricted by class Ib molecules comprise the very large majority of the overall Mtb specific CD8 T cell response (177) and CD8 T cells recognizing Mtb Ags in the context of the class Ib molecule HLA-E have been isolated from subjects with latent Mtb infection (178, 179). However, the functions of this HLA-E-restricted population, as well as its contribution to the host response to Mtb during infection and disease, remain unknown. HLA-E is the least polymorphic of all the HLA molecules (180) with only two alleles in the Caucasian population, which differ at one aa position located outside the peptide binding groove (181). Physiologically, HLA-E binds nonamer peptides derived from the signal sequence of other HLA class I molecules (182, 183), and the HLA-E/peptide complex in

turn binds to CD94/NKG2A (inhibitory) and CD94/NKG2C (activating) NK receptors (183-185). However, HLA-E also binds peptides derived from self-Ags (186, 187), tumors (188), and pathogenic microorganisms (179, 189-192), including Mtb (177, 179), and presents them to CD8 T cells. The lack of allelic variation in the peptide-binding groove maybe advantageous to design peptide-based vaccines against TB. In fact, while the polymorphism of MHC class Ia molecules is an obstacle for clinical application of these peptide based vaccination strategies, vaccines based on peptides presented by HLA-E are suitable for the global heterogenic population. In addition to its limited polymorphism, HLA-E offers another potential advantage in relation to Mtb infection. As opposed to HLA-A and HLA-B molecules, HLA-E is resistant to HIV-nef-mediated downregulation (193, 194). This is particularly relevant in view of the high prevalence of HIV/Mtb co-infection in countries such as South Africa where approximately 70% of TB patients are also HIV infected (195). Thus, while HIV might affect recognition of Mtb coinfected macrophages (196) by HLA class Ia molecules, HLA-E-dependent Ag presentation and target-cell recognition are likely to be less or not affected at all by HIV co-infection. Therefore, targeting Mtb specific HLA-E-restricted responses by vaccination or immunotherapy might be a novel and advantageous approach to combat TB.

In this article we have studied the epitope specificity, functional attributes, and *ex vivo* frequencies of HLA-E-restricted CD8 T cells specific for Mtb peptides in patients with active TB. We show here that HLA-E-restricted CD8 T cells recognize Mtb peptides in a CD3/TCR αβ mediated and CD8-dependent manner. Moreover, we provide evidence that during active TB disease, these cells produce type 2 (Tc2) cytokines, help B cells for Ab production, and have very poor TRAIL-mediated cytolytic activity and microbicidal potential. Finally, using HLA-E/Mtb peptide tetramers we show that the frequency of such tetramer specific CD8 T cells declines after successful chemotherapy, indicating that this novel human T-cell population participates inimmune response in TB.

Results:

Identification of predicted HLA-E-binding Mtb peptides

A recent study using a combination of several bioinformatic methods has led to the identification of 69 potential HLA-E-binding peptidesfrom the total Mtb H37Rv genome (179). Despite differences in their binding affinities to recombinant human HLA-E, 79% peptides were recognized by at least one healthy purified protein derivative (PPD) responder, in a CD8 T cell proliferation assay (179). Thus, and as found for other HLA class I molecules as well (197), actual affinities determined in this biochemical cell-free binding assay do not fully correlate with epitope recognition by CD8 T cells. Therefore 18 peptides (**Supporting Information Table 1**) were chosen to represent groups of six peptides each that display high, intermediate, and low binding affinity to HLA-E molecule in a cell-free assay, and these peptides were screened for their capability to induce HLA-E cell surface expression and to elicit peptide-specific response of CD8 T cells from at least 30% of patients with active TB disease.

To test the capacity of the Mtb peptides to bind HLA-E, we performed a stabilization assay (183, 184, 198) in which TAP deficient RMA-S (TAP2 mutant Rauscher Murine Leukemia virus induced T cell lymphoma) cells transfected with HLA-E (RMAS/HLA-E) were

cultured in the presence or absence of peptide at 37°C overnight and cell-surface expression of HLA-E analyzed by flow cytometry. In the absence of peptide, there was no evidence of HLA-E expression (**Supporting Information Figure 1A**).

The canonical VMAPRTLIL sequence (used as a positive control) identical to that present in the leader sequence of most HLA-C alleles (199) was able to stabilize HLA-E expression. Interestingly, culture with Mtb peptide 44 resulted in the highest levels of HLA-E expression. Peptide 34 also stabilized HLA-E cell surface expression although not to the extent of peptide 44. Co-culture with peptides 62 and 68 resulted in somewhat lower, but significant surface levels of HLA-E expression compared to peptide 44, while peptide 55 stabilized only poorly HLA-E molecules on RMA-S/HLA-E cells.

All other tested Mtb peptides failed to stabilize HLA molecules on transfected RMA-S cells. Together the data demonstrated that 5 of the 18 tested Mtb peptides (i.e. peptides 34, 44, 55, 62, and 68) were able to stabilize surface expression of HLA-E, albeit to varying degrees. In parallel with the analysis of HLA-E surface expression, the 18 Mtb peptides were tested for their capability to induce proliferative responses of CD8 T cells from ten patients with active TB. Peptide-induced CD8 T cell responses were scored positive when exceeding 10% proliferation in the absence of peptide (see Materials and Methods and (179), in at least 30% of the tested TB patients. According to these criteria, a total of 4 of the 18 tested peptides (i.e. peptides 34, 55, 62, and 68) were found to elicit a CD8 T cell response (**Supporting Information Fig. 1B**).

When HLA-E surface expression and CD8 T cell response results were compared, peptides 34, 62, and 68 were found capable of stabilizing HLA-E surface expression and eliciting a CD8 T cell response; peptide 44 caused the highest HLA-E surface expression but a very low CD8 T cell response and conversely, peptide 55 induced relatively low HLA-E surface expression but significant CD8 T cell response. Therefore, because HLA-E cell surface stabilization did not fully correlate with the CD8 T cell response, in subsequent experiments we decided to use the five peptides that gave a positive result in either assay.

HLA-E/Mtb peptide complexes are not recognized by CD94/NKG2 receptors

The C-type lectin receptors CD94/NKG2A and CD94/NKG2C specifically interact with HLA-E, resulting either in inhibition or activation of NK-cell function (183-185). Therefore we tested theability of Mtb peptides to affect target-cell lysis by NK cell clones expressing either CD94/NKG2A or CD94/NKG2C.

In order to address whether complexes of HLA-E and Mtb peptides inhibit lysis mediated by CD94/NKG2A^{+} NK cells, the HLA class I negative K562 cells were transfected with HLA-E (K562/HLA-E) and were incubated overnight at 37°C with either Mtb peptides or the VMAPRTLIL peptide (used as control) and tested as targets in a 51Cr release assay (**Figure H1A**). As expected, K562/HLA-E cells were efficiently killed by CD94/NKG2A^{+} NK-cell clones (e.g. 26.3 and 7.1 clones) and incubation of target cells with the VMAPRTLIL reference peptide was able to protect them from killing (**Figure H1A).** Pre-incubation of NK cells with anti-CD94 mAb reversed inhibition of cytolysis, demonstrating recognition of HLA-E/VMAPRTLIL complexes by the inhibitory CD94/NKG2A receptor (**Figure H1A**). In contrast, all tested Mtb peptides failed to protect K562/HLA-E cells from killing by

CD94/NKG2A+ NK-cell clones and cytotoxicity was not (or was only minimally) altered by the presence of anti-CD94 mAb (**Figure H1A**).

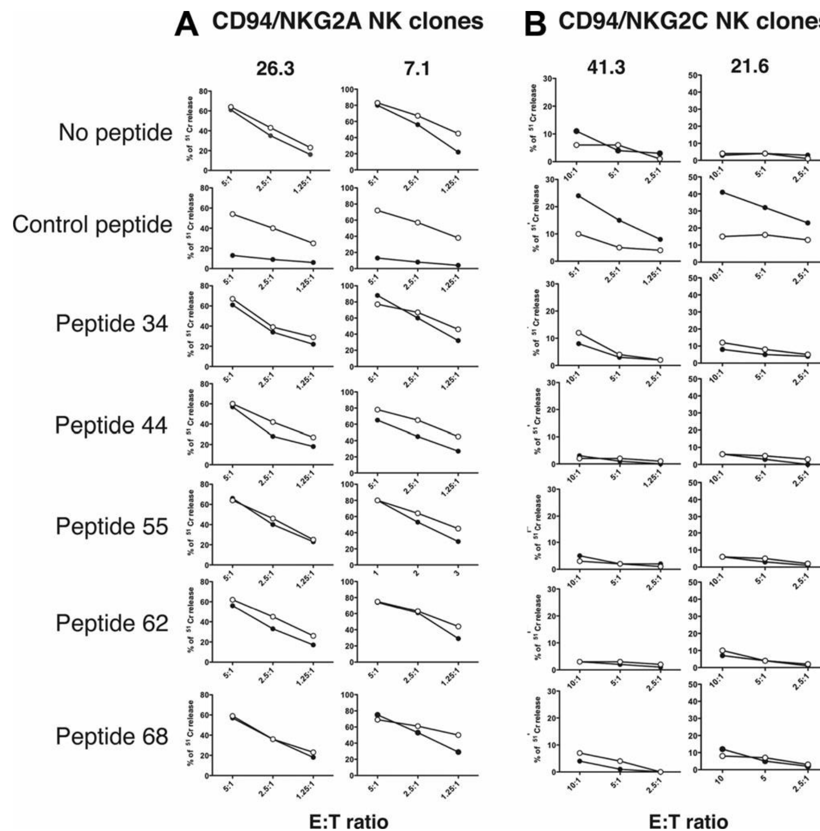


Figure H1: Mtb derived peptides are not recognized by NKG2A+ by NKG2C+ NK-cell clones.(A) 51Cr-labeled K562/HLA-E cells were incubated either alone (no peptide) or in the presence of the VMAPRTLIL peptide (Control peptide) or the indicated Mtb peptides and were tested for susceptibility to lysis by NKG2A+ NK-cell clones (26.3 and 7.1) at different E:T ratios in the absence (black) or presence (white) of anti-CD94 mAb Y9 (IgM). **(B)** 51Cr-labeled RMA-S/HLA-E cells were incubated as in (A) and were tested for susceptibility to lysis by NKG2C+ NK-cell clones (41.3 and 21.6) at different E:T ratios in the absence (black) or presence (white) of anti-CD94 mAb Y9 (IgM). (A and B) Data are from one experiment representative of five independent experiments, each performed in duplicate.

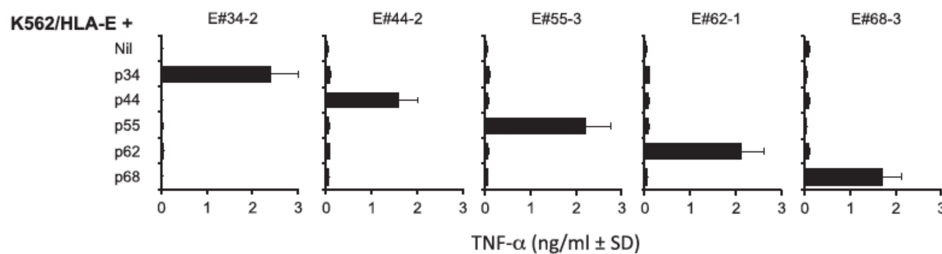
We then investigated whether HLA-E molecules loaded with Mtb peptides are recognized by NK-cell clones expressing a functional activatory CD94/NKG2C receptor. To this end, RMA-S/HLA-E cells incubated with either Mtb peptides or the VMAPRTLFL reference peptide (used as control) were tested as targets in a 51Cr release assay with the CD94/NKG2C+ NK cell clones 41.3 and 21.6 as effectors. VMAPRTLFL-loaded RMAS/HLA-E cells were killed more efficiently than control target cells (i.e. unpulsed RMA-S/HLA-E) and lysis of VMAPRTLFL-pulsed RMA-S/HLA-E cells was inhibited on mAb-mediated masking of CD94 (**Figure H1B**). In contrast, incubation of RMA-S/HLA-E cells with the Mtb peptides had no impact on target-cell recognition by CD94/NKG2C+ NK-cell clones and the presence of anti-CD94 mAb did not alter target-cell lysis. Taken together, these results indicate that complexes of HLA-E and Mtb peptides do not engage inhibitory or activatoryCD94/NKG2 receptors.

Recognition of HLA-E/Mtb peptides requires the TCR αβ and is CD8 dependent

To further investigate the Ag-recognition requirements of the HLA-E-binding Mtb peptide-responding T cells in more detail, we examined responses in three TB patients against five peptides using peptide-specific polyclonal CD8 T cell lines generated by peptide stimulation of PBMCs. Donors were selected on the grounds that (i) there was peptide-dependent CD8 T cell-proliferative response in that specific donor and (ii) sufficient PBMCs were available. Phenotype characteristics of the lines are summarized in **Supporting Information Table 2** and representative data with the E#68-1 cell line are shown in **Supporting Information Figure 2**.

T-cell lines displayed a $CD3^{+}$ $CD8^{+}$ $TCR\alpha\beta^{+}$ phenotype and did not express either CD56 or CD16. CD94 was expressed by 8 of the 15 lines on an average of 22% of the cells. Similarly, the activatory NKG2C receptor was expressed by less than 15% of the cells in only three lines, and the inhibitory NKG2A receptor was expressed by 2 of the 15 tested cell lines, on less than 20% of their cells. Three lines expressed granzyme B and TRAIL on the vast majority of cells, but none expressed perforin and granulysin. All the tested CD8 T cell lines exhibited fine Ag specificity and strict HLA-E dependence, as demonstrated by their capability to produce TNF-α only when stimulated by K562/HLA-E cells loaded with the specific peptide. **Figure H2A** shows primary data obtained with five representative T cell lines. In no case TNF-α productionwas detected by stimulation of CD8 T cell lines with peptide pulsed K562 cells (data not shown). To assess the contribution of the TCR or NK receptors andHLA-E interaction to target-cell recognition, we performed mAbblocking experiments, in which peptide-loaded K562/HLA-E cellswere used to stimulate TNF-α production by CD8 T cell lines. As shown in **Figure H2B**, significant inhibition of TNF-α production was observed in the presence of blocking mAbs to CD3, TCR αβ,CD8, pan-HLA class I, or HLA-E molecules. Blocking mAbs specific for NKG2D, NKG2C, CD94, or HLA-A/B/C molecules had no inhibitory effect on this process, while capable to inhibit killing of target cells by NK or HLA-A*0201-restricted CD8 T cell clones (**Supporting Information Figure 3**).

A



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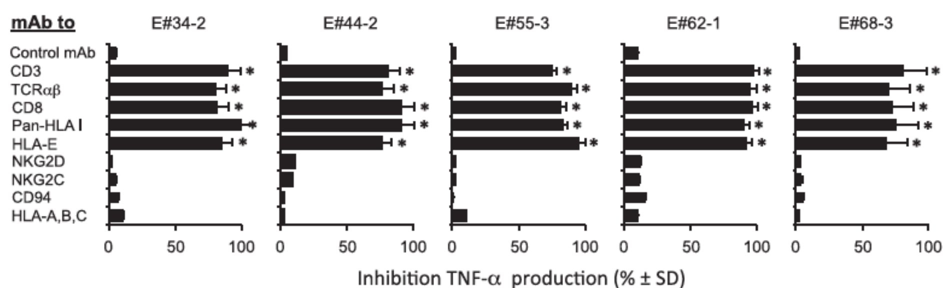


Figure H2. Peptide specificity and recognition requirements of Mtb specific HLA-E-restricted CD8 T cell lines.(A) CD8 T cell lines ($5x10^4$) were cultured for 24 h with an equal number of irradiated K562/HLA-E cells pulsed or unpulsed with peptides, and TNF-α production assessed by ELISA. **(B)** CD8 T cells were stimulates as in (A) but in the presence of the indicated blocking mAbs. After 24 h, the supernatants were collected and TNF-α levels were assessed by ELISA. Data are shown as mean ±SD (n=3) and are pooled from two independent experiments, each performed in triplicate. *p< 0.001 when compared to control, as calculated by the Mann–Whitney U test.

Together, these data unveil the requirement for TCR αβ mediated and CD8-dependent recognition of HLA-E-binding Mtb peptides.

Functional properties of HLA-E-restricted and Mtb specific CD8 T cells

To assess the cytokine profile of HLA-E-restricted and Mtb specific CD8 T cells, we stimulated T cell lines with specific peptides and tested cytokine production by ELISA in 24-h supernatants. All 15 CD8 T cell lines produced TNF-α and 2 lines also produced low yet detectable levels of IL-2 (**Figure H3A**). Additionally, 9 of the 15 tested cell lines produced significantly IL-4 and IL-13, either alone or in combination with, IL-5, IL-10, and/or TGF-β, while we failed to detect production of IFN-γ, IL-17, and IL-22. Cytokine production was strictly peptide specific as no significant cytokine production was detected with T cell lines stimulated by peptides different than those used to generate the line. **Figure H3B** shows representative specificity results with two different CD8 T cell lines, E#34-1 and E#68-3. Even if the generation of HLA-E-restricted CD8 T cell lines was carried out under neutral culture conditions (i.e. with IL-2 and in the absence of polarizing cytokines), we cannot exclud that their cytokine pattern may be biased by prolonged *in vitro* stimulation. Therefore, we decided to define the cytokine produced by CD8 T cells directly *ex vivo* upon stimulation of PBMCs from seven patients with active TB disease with Mtb peptides. Intracellular cytokine staining confirmed the dominant IL-4 and IL-13 cytokine production by the $CD8^+$ T cells and the lack of IFN-γ secretion. Primary data from seven TB patients (with PBMCs) stimulated with peptide 68 are shown in **Supporting Information Figure 4**.

Thus, and differently from CD8 T cells recognizing Mtb peptides in the context of HLA-class Ia molecules (197, 200–203), CD8 T cells recognizing Mtb peptides presented by HLA-E have an unorthodox Tc2-like, multifunctional phenotype. Therefore, we looked into whether these cells support B cells to secrete Ig. To that end, CD8 T cell lines were cultured in the presence of sorted tonsillar $CD19^+$ B cells and Ab production assessed by ELISA. B cells produced comparable low amounts of IgG and IgM when cultured for 10 days without CD8 T cell lines. In contrast, coculture of B cells with 9 of the 15 HLA-E-restricted CD8 T cell lines resulted in a significant increase in the production of IgG and IgM. As a control, coculture of tonsillar B cells with sorted $CD8^+$ T cells isolated from the PBMCs of healthy donors did not increase significantly Ab production. **Figure H3C** shows representative experiments with three different HLA-E-restricted CD8 T cell lines. These data indicate that CD8 T cells recognizing Mtb peptides presented by HLA-E supply B cells with thesignals required for Ig production.

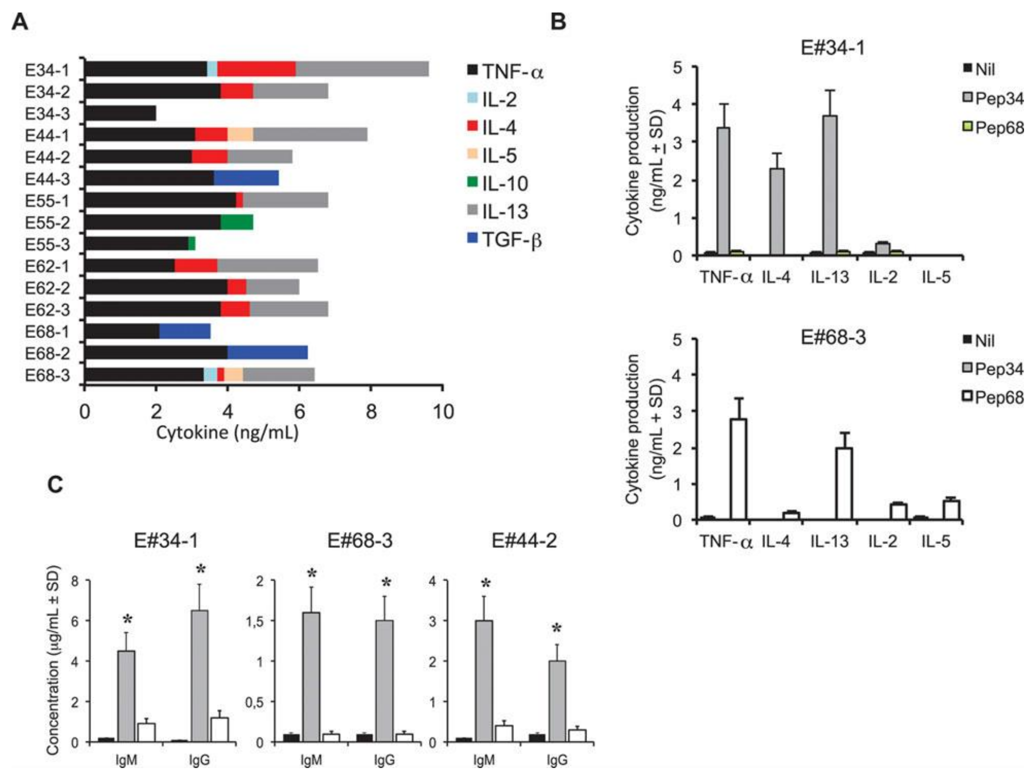


Figure H3. Cytokine production and induction of IgG synthesis by Mtb specific HLA-E-restricted CD8 Tcell lines.(A and B) CD8 T cell lines were stimulated with specific peptide as described above for 24 h, the supernatants were collected and cytokine levels were assessed by ELISA. Data are mean of three independent experiments, each performed in triplicate (SD < 10% of the means). **(B)** Data shown are from two representative CD8 T cell lines. **(C)** Tonsillar B cells were cultured for 10 days either alone (black-filled columns) or in the presence of HLA-E-restricted CD8 T cell lines (gray-filled columns) or normal CD8 T cells sorted from PBMCs (white columns). IgG and IgM concentrations in the culture supernatants were determined by ELISA. A representative example with three different CD8 Tcell lines is shown. Data are shown as mean ±SD (n = 3) and are representative of four independent experiments *p < 0.001 when compared to B cells cultured alone, as calculated by the Mann–Whitney U test.

With regard to their potential ability to develop cytotoxic responses, only 3 of the 15 tested CD8 T cell lines were equipped with specific lytic activity, E#44-1, E#55-2, and E#68-1 (**Figure H4A**), as shown by their ability to kill K562/HLA-E cells pulsed with the specific peptide (**Figure H4B**). Most notably, the three cytotoxic CD8 T cell lines were also able to kill K562/HLA-E or human monocytic THP-1 target-cell lines infected with virulent Mtb (**Figure H4C**), indicating that the HLA-E-restricted peptides they recognize are generated during natural infection. However, HLA-E-restricted CD8 T cell lines only minimally reduced the viability of intracellular Mtb (**Figure H4D**) in K562 and THP-1 targe lines. As a control, an HLA-A*0201-restricted CD8 T cell clone specific for Mtb Ag 16-kDa 120–128 was fully able to reduce the viability of intracellular Mtb.

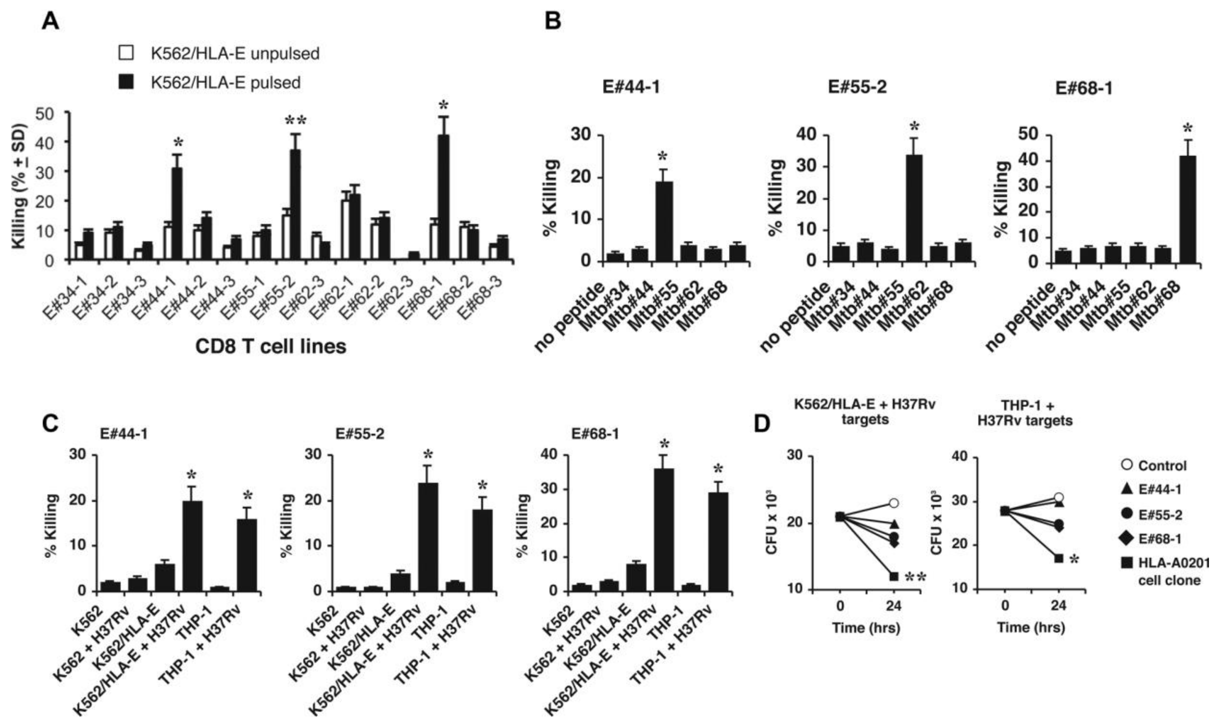


Figure H4. Cytotoxic activity of Mtb specific HLA-E-restricted CD8 T cell lines.(A) Cytotoxic activity of 15 different HLA-E/Mtb peptide specific CD8 T cell lines toward peptide-pulsed or unpulsed K562/HLA-E target cells**. (B)** Peptide specificity in the killing activity of three different CD8 T cell lines toward K562/HLA-E target-cell lines pulsed with the specific or irrelevant peptides. **(C, D)** Three different HLA-E/Mtb peptide specific CD8 T cell lines were coincubated with uninfected or Mtb H37Rv infected K562, K562/HLA-E, and THP-1 cell line target cells. Cytotoxic activity toward targets was assessed after 6-h coculture, while Mtb CFUs were estimated after 20-h coculture. The human HLA-A*0201-restricted CD8 T cell clone specific for Mtb Ag 16-kDa120-128 (218) was used as a positive control. **(A–D)** Bars represent mean ±SD (n = 3) and data are from one experiment representative of five independent experiments. (A) *p< 0.005 and **p < 0.01 when compared to unpulsed K562/HLA-E target cells, as calculated by the Mann–Whitney U test. (B) *p< 0.001 when compared to unpulsed target cells. (C) *p< 0.002 when compared to uninfected K562 and THP-1 target cells, as calculated by the Mann–Whitney U test. (D) *p< 0.005 and **p < 0.01 when compared to control, as calculated by the Mann–Whitney U test.

To further elucidate the mechanisms responsible for killing of CD8 T cells recognizing Mtb peptides in the context of HLA-E, we individually inhibited the granule exocytosis-, TRAIL-, TNF-α-, and FasL-mediated (FasL is Fas ligand) pathways. In agreement with flow cytometry data (**Supporting InformationTable 2**), treatment of CD8 T cells with concanamycin A (CMA), an inhibitor of vacuolar type H^+-ATPase that blocks perforin based cytotoxic activity, had no effect on killing of peptide-treated K562/HLA-E-cell targets (**Supporting Information Figure 5**), thus excluding involvement of the perforin pathway.
Addition of mAbs against TRAIL-R1 and TRAIL-R2 caused 82–86% inhibition of target-cell killing, indicating that TRAIL played a major role in the killing activity of HLA-E-restricted CD8 T cell lines. Finally, mAbs to FasL and TNF-α failed to significantly inhibit the cytotoxicity of all three tested HLA-E-restricted CD8 T cell lines.

Validation of HLA-E-restricted and Mtb-specific CD8 T cells using tetramers

Using HLA-E/peptide tetramers for four HLA-E-binding peptides, direct *ex vivo* recognition by TB patients CD8 T cells was demonstrated for all four Mtb epitopes (**Figure H5A and B**). In all tested individuals, specificity of tetramer staining was confirmed by the absence of staining among PBMCs from ten normal, uninfected donors using the same Mtb tetramers (**Figure H5A**).

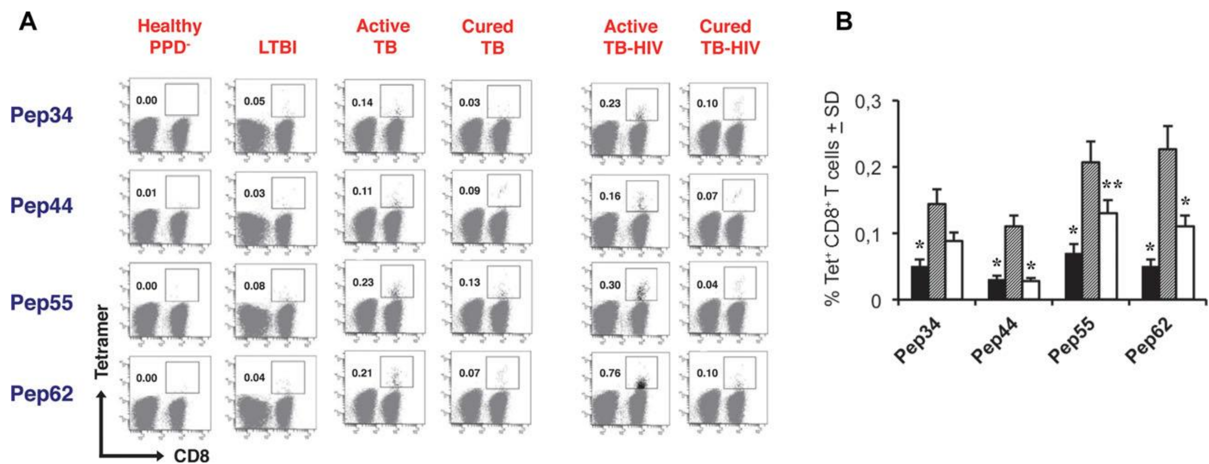


Figure H5. Ex vivo analysis of the frequencies of HLA-E/Mtb peptide tetramer^{+} CD8 T cells.(A) Dot plot analysis of HLA-E/peptide tetramer^{+} CD8^{+} T cell populations of one representative healthy tuberculin-negative subject, LTBI subject, and TB patient with active or cured TB disease, with or without HIV co-infection. Shown in the upper left of each FACS plot are the percentages of tetramer^{+} CD8^{+} T cells, for all four tested Mtb peptide/HLA-E tetramers. Data shown are representative of three independent experiments. **(B)** Cumulative data on the frequencies of the tetramer-specific CD8 T cells in peripheral blood of 11 LTBI subjects (black columns) and 24 TB patients with active (gray columns) or cured (white columns) TB disease. Data are shown as mean ±SD (n = 3) and are representative of three independent experiments. *$p< 0.01$ and **$p < 0.02$ when compared to patients with active TB disease, as calculated by the Mann–Whitney U test.

Although there was considerable variability in the proportions of CD8 T cells that bound to single tetramers, the *ex vivo* frequencyof tetramer-specific CD8 T cells was higher in TB patients than in latent tuberculosis infection (LTBI) subjects and this difference attained statistical significance with all epitopes. Significant differences in frequencies of epitope-specific CD8 T cells were observed in TB patients before and after chemotherapy: in all instances mean frequencies of epitope-specific CD8 T cells significantly decreased after therapy and differences attained statistical significance with most of the studied epitopes (i.e. peptides 44, 55,and 62). Finally, and most notably, the highest mean frequency of tetramer-specific CD8 T cells was found in three TB patients who were coinfected by HIV, which declined after antituberculous therapy as well. **Figure H5A** shows FACS analysis of the CD8^{+} tetramer^{+} T cells of one individual from any tested group and **Figure H5B** shows cumulative data from 24 patients with active TB disease before and after therapy, and 11 LTBI individuals.

As five TB patients were typed as HLA-A*0201^{+} and we had available HLA-A*0201/Mtb peptide tetramers, we followed up the CD8 T cell response to HLA-A*0201^{-} and HLA-E restricted epitopes in the same patients during therapy. As shown in **Figure H6**, the frequencies of HLA-E-epitope-restricted CD8 T cells significantly decreased after 6-month therapy, while mean frequencies of HLA-A*0201-restricted, epitope-specific (ESAT-

6,Rv1490, Ag85, and 16-kDa Ags) CD8 T cells increased after therapy. Thus, the opposite trend of HLA-E- and HLA-A*0201-restricted responses strongly suggests these two CD8 T cell populations may play different roles during Mtb infection and disease.

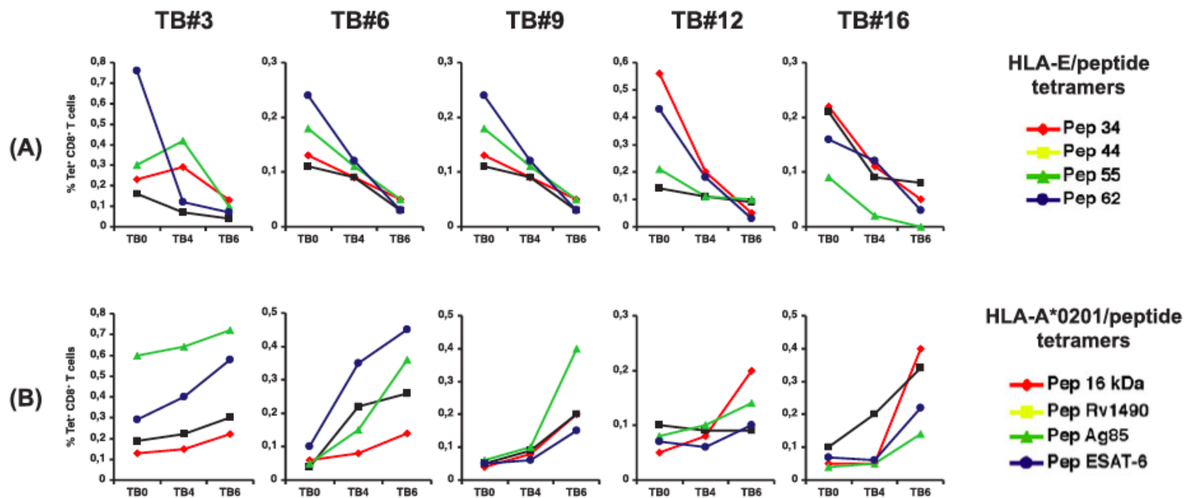


Figure H6. Comparison of the frequencies of HLA-A*0201/Mtbpeptide and HLA-E/Mtbpeptide tetramer+ CD8 T cells during antimycobacterial therapy. Frequency of **(A)** HLA-E/Mtb and **(B)** HLA-A*0201/Mtb tetramer+ CD8+ T cells in PBMCs of five HLA-A*0201-positive TB patients (patients) before (TB0) chemotherapy and 4 (TB4) and 6 (TB6) months after chemotherapy. Data shown are representative of three independent experiments.

Discussion

Within recent years, it has become clear that HLA-E can present peptides from several bacteria and viruses to CD8+ T cells in a manner comparable to peptides presented by classical MHC class I molecules (180, 190, 204–208). Indeed, HLA-E-restricted CD8+ T cell recognition of peptides from pathogens may be an important mechanism of immune regulation during infections, as was first demonstrated for Mtb (178, 179). HLA-E is enriched in the Mtb phagosome compared to regular HLA class I molecules (209), suggesting that HLA-E may have unique functions in presenting phagosomal Ags. Because Mtb resides in immature phagosome, the probability that some peptides derived from Mtb could be presented to CD8 T cells during active infection in association with HLA-E molecules is intriguing. Accordingly, careful examination of HLA-E-restricted CD8+ T cell responses to peptides from Mtb found such responses to a number of these peptides (179). Therefore in our study, we were interested in examining whether TB patients had a detectable CD8+ T cell response to peptides derived from Mtb, which have been shown capable to bind to HLA-E.

In this article, using a selected pool of Mtb peptides capable to stabilize the HLA-E molecule on the surface of transfected cells and to induce proliferation of CD8 T cells in PBMCs from at least 30% TB patients, we provide direct evidence that during active TB HLA-E-restricted CD8 T cells can recognize such Mtb peptides, thus representing an additional type of effector cells playing a role in immune response to active infection with Mtb. Usually, HLA-E/peptide complexes may serve as a ligand for CD94/NKG2A or C, which are NK-cell-inhibitory and NK-cell activating receptors, respectively (183, 184).

CD94/NKG2A appears to bind ligand with higher affinity, which has led to the notion that HLA-E/peptide complexes may protect target cells from NK-cell-mediated killing (210, 211). Conversely, engagement of CD94/NKG2C promotes NK-cell killing (210, 211). In theory, presentation of the Mtb derived peptides by HLA-E molecule may have similar consequences. However, and in contrast to the HLA-E/self-peptide complexes, we did not find recognition of HLAE/Mtb peptide complexes by NK-cell clones selectively expressing inhibitory (NKG2A) or activatory (NKG2C) receptors. Thus, we propose that HLA-E/Mtb peptide complexes may have very low or even impaired affinity for CD94/NKG2A or C receptors. Moreover, using T cell lines generated from PBMCs of TB patients by long-term peptide stimulation in vitro, we demonstrate that $CD8^+$ T cells recognize HLA-E/Mtbpeptide complexes. Importantly, HLA-E/Mtb peptide complexes are recognized differently from HLA-E/self-peptides: whereas the latter are predominantly recognized by CD8-negativecells (subset of NK cells) in a CD94-dependent manner, HLA-E/Mtb peptides are specifically recognized by $CD8^+$ T cells in a CD3/TCR αβ mediated and CD8-dependent manner. Finally, HLA-E/Mtb peptide tetramers did not bind to CD8-negative cells, although this subset is expected to containan NK-cell subset expressing CD94/NKG2. Altogether, the above reported results strongly indicate that HLA-E-restricted CD8T cells recognize Mtb peptides differently than NK cells but in a similar manner as HLA-class I-restricted CD8 T cells.

Human $CD8^+$ Tcell lines derived from TB patients efficiently recognize HLA-E-binding Mtb peptides, resulting in cell proliferation, but the responding cells had very poor cytotoxic activity to HLA-E-expressing cells (K562 and THP-1). This was initially suspected by the lack of expression of perforin and granulysin, and confirmed by the finding that only 3 of the 15 tested HLA-E-restricted Mtb specific CD8 T cell lines lysed these target cells. Nonetheless, killing of targets occurred in a peptide-specific and HLA-E-dependent fashion, strongly suggesting peptide/HLA-E cognate recognition via the TCR. Moreover, these epitopes are also likely recognized during natural infection, since live Mtbinfected K562/HLA-E and THP-1 cell lines were lysed by HLA-E/peptide-specific effector CD8 T cells. However, despite their capability to kill peptide-pulsed and Mtb infected target cells, all three HLA-E-restricted CD8 T cell lines caused only minimal, if any inhibition of Mtb outgrowth from these infected human cell lines, indicating that they are equipped with cytolytic potential but lack microbicidal activities. The latter finding could indicate that HLA-E-restricted Mtb peptide specific CD8 T cells during active TB have an impaired ability to inhibit Mtb outgrowth, since in another study (submitted) we find that T cell clones from latently infected individuals were able to inhibit Mtb outgrowth from infected primary human macrophages. Alternatively, experimental differences (e.g. the use of cell lines here versus primarycells in our other study) might account for this seeming difference.

In any case, the lack of correlation between target-cell apoptosis and antimicrobial activity by Mtb specific CD8 T cells is not surprising and was first reported by Modlin and co-workers (171).

In addition to their poor cytolytic capacity, HLA-E/peptide reactiveCD8 T cell lines from TB patients also had an unusual Tc2-type pattern of cytokine production: they secreted TNF-α and two lines also produced low, yet detectable, levels of IL-2. However, and surprisingly, 9 of the 15 tested cell lines secreted IL-4 and IL-13, either alone or in combination with IL-5, IL-10, and/or TGF-β, while very low or no production of IFN-γ, IL-

17, and IL-22 was detected. The cytokine production profile of HLA-E-restricted CD8 Tcell lines was confirmed by intracellular staining of freshly isolated CD8 T cells stimulated with Mtb peptides. Thus, HLA-E/Mtb peptide specific CD8 T cells more closely resemble Tc2 T cells and accordingly they are capable to provide help to B lymphocytes for Ab production. Moreover, the finding that some CD8 T cell lines also secrete TGF-β suggests that a fraction of these cells might play regulatory activities, as shown by previous studies in autoimmune disease models (212) and response to microorganisms (213), including TB (179). Overall, data reported here differ from previously described HLA-E-restricted, TB-specific CD8 T cells that produce IFN-γ and are cytolytic (178, 179). However, we would like to point that in the Heinzel paper (178) only two HLA-E-restricted and TB-specific CD8 T cell clones were studied, both derived from one healthy LTBI subject, and cytotoxic activity of the clones was not assessed.

Similarly, in the Joosten paper (179) only two of four tested HLA-E restricted CD8 T cell lines produced IFN-γ and all four lines killed BCG-infected targets but also exerted TGF-β-mediated suppressive activities. None of the studies investigated the microbicidal properties of HLA-E-restricted CD8 T cells. In our studies, CD8 T cell lines were generated from patients with active TB and a total of 15 CD8 T cell lines were tested. Moreover, the cytokine producing profile of the T cell lines was also evident when CD8 T cells were stimulated ex vivo with HLA-E-binding Mtb peptides.

Using HLA-E/peptide tetramers for four recognized HLA-E binding Mtb derived peptides, direct ex vivo recognition by TB patients' CD8 T cells was demonstrated for all four epitopes and frequencies of peptide/tetramers in TB patients declined 6 months after the antimycobacterial therapy. One may speculate that expansion of HLA-E-restricted and Mtb specific CD8 T cells during active TB disease might be the consequence of active replication of Mtb residing in immature phagosomes. Since HLA-E is enriched within the Mtb phagosome, as compared to HLA class I molecules, the probability that endogenous peptides derived from Mtb could be presented to CD8 T cells in association with HLA-E molecules is very high during active TB disease but diminishes after successful therapy. In this case, the frequency of HLA-E-restricted/Mtb peptide specific CD8 T cells may be a TB-specific biomarker of bacterial load during active infection, which is also rapidly regulated during successful chemotherapy.

In conclusion, our studies have identified CD8 T cells from TB patients, which recognize Mtb derived peptides presented by HLA-E molecules, produce type 2 cytokines upon antigenic in vitro stimulation, mediate B cell help for Ab production, and have very limited TRAIL-dependent cytolytic activity and microbicidal potential toward Mtb infected human cell lines expressing HLA-E. These data, together with the finding that the frequency of HLA-E/Mtb peptide specific CD8 T cells declines after successful chemotherapy in TB patients withor without HIV co-infection, suggest that this is a new human T cell population involved in TB.

Future studies are then needed to better dissect this HLA-E restricted response and to precisely determine the relative importance of effector/protective versus immunoregulatory/pathogenic mechanisms within this response during Mtb infection and disease.

Material and methods

Human subjects

Peripheral blood was obtained from 24 adults with TB disease (15 men, 9 women, age range 42–61 years) from the Dipartimento di Medicina Clinica e delle Patologie Emergenti, University Hospital, Palermo, 15 LTBI subjects (10 men, 5 women, age range 38–59 years), and 10 tuberculin-negative healthy subjects (7 men and 3 women, age range 40–57 years). TB-infected patients had clinical and radiological findings consistent with active pulmonary TB (214). Diagnosis was confirmed by bacteriological isolation of Mtb in 14 patients. Other patients were classified as having highly probable pulmonary TB on the basis of clinical and radiological features that were highly suggestive of TB and unlikely to be caused by any other disease; the decision was made by the attending physician to initiate anti-TB chemotherapy, which resulted in an appropriate response to therapy. All patients were treated in accordance with Italian guidelines and received therapy for 6 months. Treatment was successful in all participants all of whom completed the full course of anti-TB chemotherapy, as shown by the absence of any clinical or radiographic evidence of recurrent disease and sterile mycobacterial cultures. Peripheral blood was collected before (TB0) and 4 (TB4) and 6 months after completion of chemotherapy (TB6). None of the TB patients had been vaccinated with BCG, or were being treated with steroid or other immunosuppressive or antitubercular drugs at the time of their first sampling. Three patients had evidence of HIV infection. Tuberculin (PPD) skin tests were considered positive when the in duration diameter was larger than 10 mm at 72 h since injection of 5 U of PPD (Statens Serum institut, Copenhagen, Denmark). Individuals with LTBI were defined as healthy people with apositive tuberculin (PPD) skin test and no symptoms and signs of active TB (215). However, because the response to QuantiFERON TB Gold test was found in 73% (11/15) of PPD-positive LTBI subjects, only those 11 subjects positive to QuantiFERON TB Gold test were considered as being latently infected and were included in the study. All of the LTBI subjects were health-care workers, and thus very likely to be close contacts of TB index cases. Moreover, none of the LTBI subjects included in this study had been vaccinated with BCG.

Ethics statement

The study was approved by the Ethical Committee of the University Hospital, Palermo, where the patients were recruited. The study was performed in accordance to the principles of the Helsinki Declaration and those of the "Good Clinical Practices," and all individuals gave written informed consent to participate.

Peptide-HLA-E binding assays

Murine TAP2-deficient T cell lymphoma RMA-S-cell line co-transfected with human β2-microglobulin and HLA-E*01033 allele (RMA-S/HLA-E, kindly provided by J. E. Coligan, Laboratory ofImmunogenetics, NIAID, Rockville, MD, USA) was used in the experiments. RMA-S/HLA-E cells were resuspended in complete Roswell Park Memorial Institute medium at 1 x 10^6 cells/mL and incubated either alone or in the presence of the synthetic peptides (200 μM). The Mtb derived peptides used in these experiments are derived from

(179) and are listed in Table 1. The canonical VMAPRTLIL peptide sequence, identical to that present in most HLA-C alleles (199), was used as a positive control in binding experiments. After an overnight incubation at 37°C, cells were washed with PBS to remove free peptides. Next, HLA-E surface expression was monitored after staining with the anti-HLA class I mAb A6.136 (IgM, produced in our laboratory) followed by appropriate PE-conjugated goat antimouse Ab.

CD8 T cell proliferation induced by Mtb peptides

PBMCs were labelled with CFSE (5 mM, Molecular Probes, Eugene, OR, USA) and 10^5 cells were stimulated with Mtb derived HLA-E-binding peptides at a concentration of 10 μg/mL in complete RPMI 1640 medium (Euroclone, Devon,UK) supplemented with 10% heat-inactivated pooled human AB^+ serum, 2 mM L-glutamine, 20 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol (complete medium) in the presence of 5 ng/mL IL-7 (Peprotech,Rocky Hill, NJ, USA), as described in (179). Positive (PHA, 1μg/mL; Life Technologies, Paisley, UK) and negative (medium only) controls were included in each assay. On day 7 of culture, cells were harvested, replicates (n = 6) pooled and stained using CD3-PerCP, CD8-APC, and CD56-PE (BD Biosciences, San Jos´e,CA, USA) before acquisition on a FACSCalibur using CellQuestPro software (BD Biosciences). To analyze proliferation, cells were gated on lymphocytes, followed by gating on $CD3^+CD8^+CD56^-$ cells. The percentage ofproliferation was calculated using geometric means by subtracting the geometric mean of all cells from the geometric mean of the undivided population. Subsequently, the percentage was calculated by the following: (delta geo mean of sample − delta geo mean of negative control)/delta geo mean of maximal proliferation (179).

Isolation and culture of NK cells

NK cells from healthy donors were isolated using the Rosette Sepmethod (Stem Cell Technologies, Vancouver, BC, Canada). Only those populations displaying >95% of $CD56^+CD3^-$ NK cells were selected. Purified NK cells were then cultured on irradiated feeder cells in the presence of 2 μg/mL PHA and 100 U/mL IL-2 (Proleukin, Novartis Pharma AG, Basel, Switzerland) to obtain, after limiting dilution, clonal NK cells. Proliferating NK-cell clones were selected on the basis of the expression of either CD94/NKG2A or CD94/NKG2C receptors as determined by flow cytometric analysis with the Z270 mAb (IgG1, anti-NKG2A produced in our laboratory) and the 1381 mAb (IgG2b, anti-NKG2C, R&D Systems, Minneapolis, MN, USA). To analyze the surface markers of NK-cell clones, cells were incubated with mAbs followed by PE-conjugated isotype-specific goat antimouse second reagents (Southern Biotechnology Associates, Birmingham, AL, USA). Samples were analyzed on a FACSCalibur with the CellQuest program (BD Biosciences).

NK-cell cytolytic activity

NK-cell clones were tested for cytolytic activity in a 4-h 51Cr release assay as described (216) in the presence or absence of anti-CD94 mAb Y9 (IgM, 10μg/mL). Target cells used in these experiments were RMA-S/HLA-E (described above) and the human erythroleukemic cell line K562 transfected with HLA-E*0103(K562/HLA-E, kindly

provided by E. H. Weiss, Department of Biology, Anthropology and Human Genetics, Ludwig-Maximilians-Universitat, Munich, Germany) (217). Target-cell lines were previously incubated overnight at 37°C, either alone or in the presenceof the synthetic peptides (200 μM). Peptides were kept throughout the assay to assure higher levels of HLA-E expression. E:T ratios used in experiments are indicated in figures.

Generation of CD8 T cell lines and functional assays

PBMCs from TB patients were cultured with 10 μg/mL of Mtb derived peptides at 3 x 10^6 cells/well in complete medium and human recombinant IL-2 (40 U/mL) (218). Every 3 days, the culture was refreshed by medium plus IL-2 (40 U/mL). After 15 days, cultures were restimulated weekly with an equal number of peptide-pulsed irradiated (120 Gy from a Caesium source) K562/HLA-E cells, in the presence of 40 U/mL IL-2 and 15ng/mL IL-15. After four to five cycles of restimulation, the enriched population contained >80% $CD8^+$ T cells (also see Table 2). Cells were restimulated weekly as described earlier. CD8 T cell lines were analyzed in detail by flow cytometry. Fluorochrome-conjugated antibodies used for staining included CD3 (HIT3a or SK-7, BD Biosciences), CD8 (RPA-TB or SK1, BD Biosciences), CD45 (2D1, BD Biosciences), CD56 (B159, BD Biosciences), CD94 (HP-3D9, BD Biosciences), CD16 (3GB, BD Biosciences),TCRαβ (WT31, BD Biosciences), NKG2A (134111, R&DSystems), NKG2C (1381, R&D Systems), Granzyme B (GB11, BDBiosciences), rabbit anti-human granulysin (a kind gift of Dr. A.Krensky, Stanford, CA, USA) in combination with goat-anti-rabbit FITC (BD Biosciences), TRAIL (RIK-2, eBioscience), perforin (dG9, eBiosciences), or isotype-control mAbs.

Samples were acquired on a FACSCanto and analyzed using FACSDiva software (BD Biosciences). Scoring was based on the percentage of cells that expressed the particular marker, and markers expressed by 10% of the cells, or more, were considered positive.

The gating strategy is shown in **Supporting Information Figure 6**.

For functional assays, cells were maintained for 1 week without restimulation (i.e. only with IL-2 and IL-15) before assays were done. $CD8^+$ T cells (5 x 10^4) were incubated with K562 orcK562/HLA-E cells in the presence or absence of synthetic peptides. After 24 h, the supernatants were collected and stored at −70° C until testing. Cytokine levels (TNF-α, IFN-γ, TGF-β, IL-2, IL-4, IL-5, IL-10, IL-13, IL17, and IL-22) were assessed by ELISA (R&D Systems). In some experiments, cocultures were carried out inthe presence of blocking mAbs to HLA-E (3D12, BioLegend, San Diego, CA, USA), HLA-A/B/C (G46-2.6, BD Biosciences), anti-HLA Class I monomorphic (MEM-147, from Prof. V. Horejsi, Institute of Molecular Genetics, Prague, Czech republic), anti-CD3 (blocking, MEM-57 from Prof. V. Horejsi), anti-TCR αβ (WT31,BD Biosciences), CD8 (RPA-TB, BD Biosciences), NKG2D (1D11, eBioscience, San Diego, CA), NKG2C (1381, R&D Systems), CD94 (HP-3D9, BD Biosciences), or isotype-matched mAbs at 10 μg/mLfinal concentrations. The cytotoxic activity of CD8 T cell lines was assessed following 6-h incubation with unpulsed or peptide-pulsed K562 or K562/HLA-E target cells at an E:T ratio of 10:1. Evaluation of dead cells was performed by flow cytometry after incubation with annexin-V-FLUOS staining kit (Roche Diagnostics, Milan, Italy) for the detection and quantification of apoptosis and differentiation from necrosis at single cell level, based on Annexin-V-labeling. In some experiments, cytotoxic assay was carried out in the presence mAbs to FasL (CD95L, 2C101, Vinci Biochem, Firenze, Italy), TNF-α (Infliximab, a gift of Prof. G. Triolo, Dipartimento

Biomedico di Medicina Interna e Specialistica, Università di Palermo, Palermo, Italy), or TRAIL receptors 1 (DR4) and 2 (DR5), both provided by Dr. H. Walczak (Tumor Immunology Unit, Division of Medicine, Imperial College, London, UK). To inhibit perforin-mediated cytotoxicity CD8 T cell lines were incubated with CMA (Sigma, St. Louis, MO, 15 nM final concentration) for 30 min at 37°C prior to coculture, without further washing. Pre-treatment of CD8 T cell lines with CMA at the concentration used in this study did not have any cytotoxic effect. Cytotoxicity of Mtb cells and assessment of viability of Mtb THP-1-, K562-, and K562/HLA-E-cell lines were infected for 24 at 37°C, 5% CO2 with single-cell suspensions of Mtb H37Rv strain at a multiplicity of infection of 10. The infection was carried out in the absence of antibiotics, and post treatment, cells were washed with PBS to eliminate extracellular bacteria. Viability of infected cells was determined by trypan blue exclusion. Infected or uninfected THP-1-cell line and K562/HLA-E cells were extensively washed and added to peptide-specific CD8 T cell linesat an E:T ratio of 10:1. After 6h of co-culture, cytotoxicity of target cells was assessed by flow cytometry, as previously described, after incubation with annexin-V-FLUOS staining kit (Roche Diagnostics). Mtb infected THP-1-, K562-, and K562/HLA-E-celllines were incubated with peptide-specific CD8 T cell lines for 20 h at 37°C as described above, washed three times to eliminate bacteria that were not cell associated, lysed with 0.1% saponin, and sonicated for 20 s. Serial tenfold dilutions were made in 7H9 broth and plated on 7H10 agar plates. Plates were sealed in plastic, kept at 37°C, and the number of colonies (CFUs) was counted after14–21 days (219).
A human HLA-A*0201-restricted CD8 T cell clone specific for Mtb Ag 16-kDa120-128 (218) was used as a positive control.

Intracellular cytokine staining
PBMCs (10^6/mL) were stimulated with peptides in the presence of monensin for 6 h at 37°C in 5% CO_2. The cells were harvested, washed, and stained with anti-CD8 mAb (RPA-TB, BDBiosciences) in incubation buffer (PBS-1% FCS-0.1% Na azide) for 30 min at 4°C. The cells were washed twice in PBS-1% FCS and fixed with PBS-4% paraformaldehyde overnight at 4°C. Fixation was followed by permeabilization with 1% PBS, 0.3% FCS, 0.1% saponin, and Na azide for 15 min at 4°C. Staining of intracellular cytokines was performed by incubation of fixed permeabilized cells with anti-IFN-γ (25723.11, BD Biosciences), anti-IL-2 (MQ1-17H12, BD Biosciences), anti-TNF-α (MAb11, BD Biosciences), anti-IL10 (BT-10, eBioscience), anti-IL-17A (eBio64DEC17, eBioscience), anti-IL-4 (BD Biosciences, 3010.211), anti-IL13 (Biolegend,JES10-5A2) mAbs or isotype-matched control mAbs. Cells were acquired and analyzed by FACS as described above. Analysis was performed on a minimum of 100 000 CD8 events acquired for each sample.
Negative controls were obtained with CD8 T cell lines incubated with medium, in the absence of any stimulant. Cut-off values for a positive response were predetermined to be in excess of 0.01% responsive cells (197, 203). Results below this value were considered negative and set to zero (197, 203).

Tetramer staining
Tetramer staining was carried out as described in detail previously (197, 203, 220). PBMCs (106/mL) were incubated in U-bottom 96-well plates, washed twice in PBS containing 1%

FCS (Sigma), and stained for 30 min at 4°C with PE-labeled tetramers (5 μLeach) prepared as previously described (197, 203, 220); they were washed and subsequently stained with FITC-labeled anti-CD8 mAb (RPA-TB, BD Biosciences) and analyzed by flow cytometry on a FACSCanto. Data were analyzed with the use of FACSDiva (BDBiosciences). Viable lymphocytes were gated by forward and side scatter and the analysis was performed on 100000 CD8 events acquired for each sample. A cutoff of 0.01% was used as described previously (197, 203); values below this were set to zero.

Ab production *in vitro*.

CD8 T cell help in Ab production was studied according to (221). HLA-E-restricted CD8 T cell lines or CD8 T cells isolated from the PBMCs of healthy donors were cocultured with sorted tonsillar B cells in 96-well plates at 105 cells/well each of T and B cells, in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 20 nM HEPES, and 100 U/mL penicillin/streptomycin. Ten days later IgG and IgM levels in the culture supernatants were determined by ELISA.

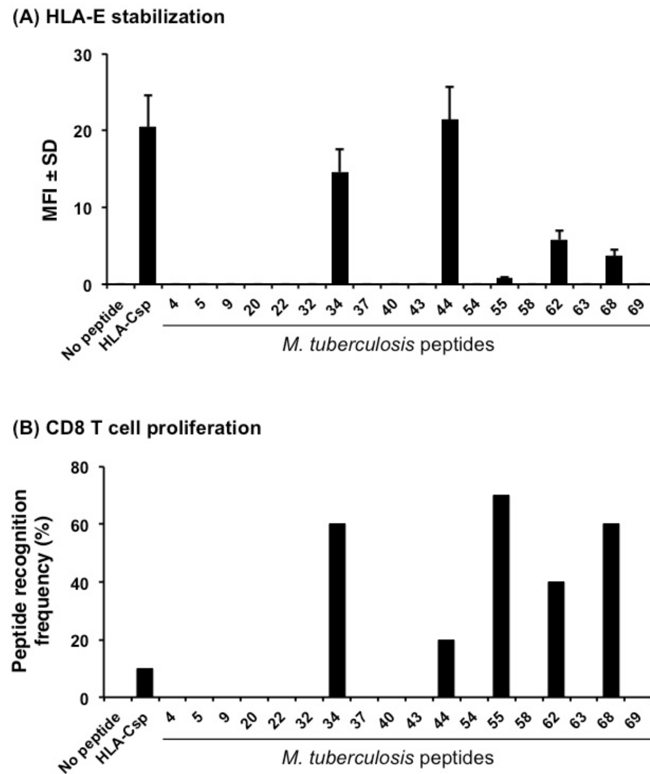
Statistics

Nonparametric Mann–Whitney U test was used to determine statistical differences in the distribution of the results. Values of $p < 0.05$ were considered significant. Data were analyzed using statistical software SYSTAT 11 (Systat Software).

Supporting Informations

Peptide #	Amino acid sequence	Derived from	Amino acid position	Accession number[1]	Binding (in µM)[2]	Stabilization (in MFI)[3]
4	KMNAKAATL	Rv2911	145-153	Q7D6F2	15	<0,1
5	QMKFYAVAL	Rv2119	180-188	O33254	1,5	<0,1
9	WMCDRAVDL	Rv2954c	126-134	Q50461	>50	<0,1
20	PMAPLAPLL	Rv3871	490-487	O69736	3	<0,1
22	PMQQLTQPL	Rv3873	218-226	Q79F92	>50	<0,1
32	VMMSEIAGL	Rv1813c	42-50	P64889	13	<0,1
34	**VMTTVLATL**	**Rv1734c**	**42-50**	**P71992**	**1,8**	**14,6**
37	EMLTSRGLL	Rv1997	498-506	P63687	>50	<0,1
40	SMFAAVQAL	Rv2030c	153-181	O53475	5	<0,1
43	EMGRAPLDL	Rv2627c	186-193	O06185	>50	<0,1
44	**RLPAKAPLL**	**Rv1484**	**53-61**	**P0A5Y6**	**0,03**	**21,4**
54	FLLPRGLAI	Rv0056	29-37	P66315	>50	<0,1
55	**VMATRRNVL**	**Rv1518**	**240-248**	**Q50590**	**16**	**0,8**
58	DLPSRLGKI	Rv3087	187-195	Q53304	>50	<0.1
62	**RMPPLGHEL**	**Rv2997**	**470-478**	**O53244**	**4**	**5,8**
63	VMAPDAAVRI	Rv0324	183-191	O08446	31	<0.1
68	**VLRPGGHFL**	**Rv1523**	**251-259**	**Q50584**	**1,8**	**3,7**
69	WLPPLLTNL	Rv0294c	32-40	O53671	39	<0.1

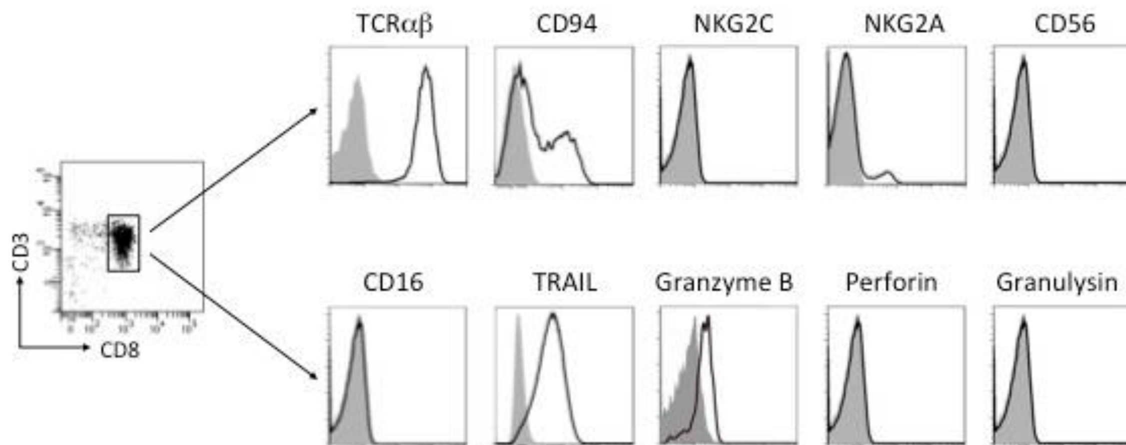
Supporting Information Table 1. Characteristics of HLA-E-binding Mtb derived peptides. Mtb-derived peptides containing HLA-E binding motifs were selected from (179). Peptide sequences are indicated, as well as their originating proteins (Mtb Rv numbers and their Swissprot accession numbers). Peptides further selected in this study are indicated in bold.[1]UniProtKB/ Swissprot accession number. [2]Binding was calculated as the concentration (µM) of peptide required to reduce fluorescence intensity of the standard peptide with 50% (IC50, (177)). [3]Stabilization is expressed as MFI (see Materials and Methods).



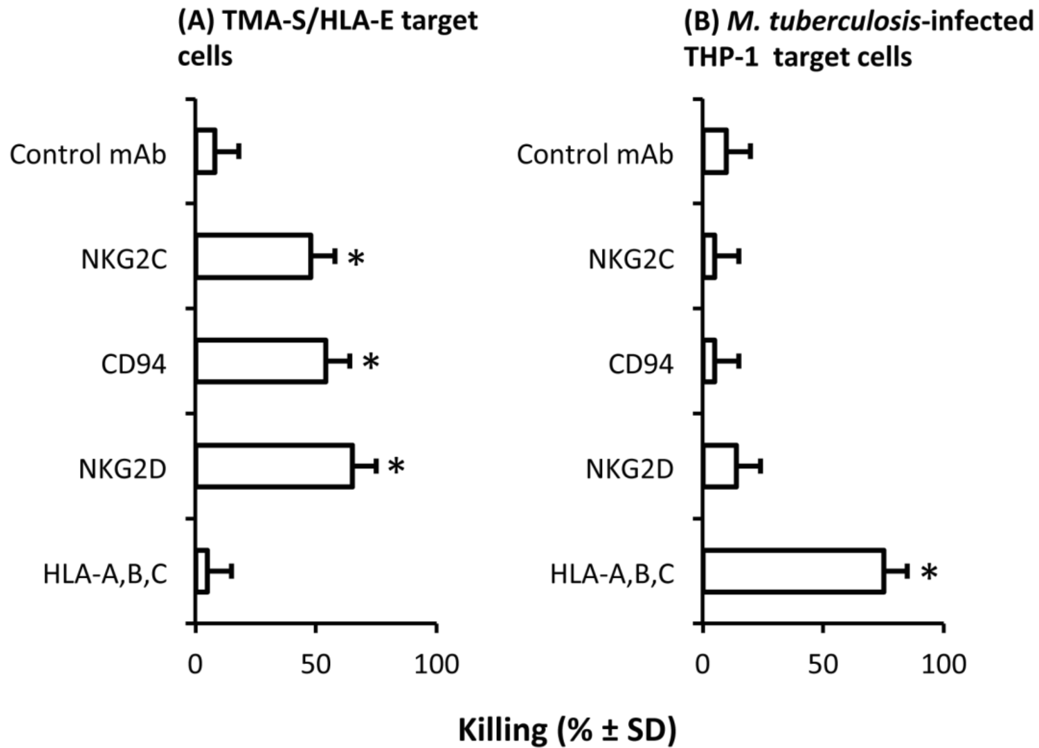
Supporting Information Figure 1. Ability of Mtb peptides to stabilize HLA-E cell surface expression and to induce CD8 T cell proliferation. In **(A)** RMA-S/HLA-E cells were pulsed with the indicated peptides and were analyzed by flow cytometry for cell surface HLA-E expression. Each column shows the difference in MFI between anti-HLA class I A6.136 mAb and isotype-control mAb. Each value represents the mean ± SEM of three experiments. Peptide numbers are listed in Table 1. Negative control consisted of unpulsed RMA-S/HLA-E cells and positive controls consisted of RMA-S/HLA-E cells pulsed with the HLA-C signal peptide (HLA-Csp) VMAPRTLIL. In **(B)**, PBMC from 10 patients with active TB were stimulated with Mtb-derived peptides, as indicated in Materials and Methods. Peptide-induced CD8 T cell responses were scored positive when exceeding 10% proliferation in the absence of peptide. The frequency (%) of responder TB patients is shown. *$p < 0.001$ when compared to the No peptide group, as calculated by the Mann-Whitney U test.

T cell line	CD3	TCRαβ	CD8	CD56	CD16	CD94	NKG2C	NKG2A	GrB*	TRAIL	Per*	Gra*
E#34-1	**90**	**88**	**85**	<1	<1	**24**	**15**	<1	2	<1	<1	<1
E#34-2	**92**	**85**	**85**	<1	<1	<1	<1	<1	3	4	2	<1
E#34-3	**85**	**80**	**80**	<1	<1	1	<1	<1	2	<1	<1	<1
E#44-1	**94**	**90**	**86**	<1	<1	<1	<1	<1	**95**	**90**	<1	<1
E#44-2	**92**	**90**	**85**	<1	<1	**15**	<1	<1	1	6	<1	<1
E#44-3	**87**	**85**	**85**	<1	<1	**34**	<1	**18**	2	3	<1	<1
E#55-1	**85**	**85**	**84**	<1	<1	**12**	<1	<1	<1	2	4	3
E#55-2	**92**	**86**	**81**	<1	<1	<1	<1	<1	**90**	**88**	<1	<1
E#55-3	**90**	**92**	**87**	<1	<1	<1	<1	<1	3	<1	<1	<1
E#62-1	**94**	**90**	**85**	<1	<1	**16**	**14**	<1	5	<1	<1	<1
E#62-2	**88**	**80**	**86**	<1	<1	**15**	**12**	<1	<1	<1	<1	<1
E#62-3	**92**	**82**	**92**	<1	<1	<1	<1	<1	2	<1	5	<1
E#68-1	**88**	**85**	**85**	<1	<1	**48**	<1	**14**	**97**	**95**	<1	<1
E#68-2	**89**	**88**	**80**	<1	<1	<1	<1	<1	4	<1	<1	2
E#68-3	**92**	**90**	**90**	<1	<1	**15**	<1	<1	2	4	<1	<1

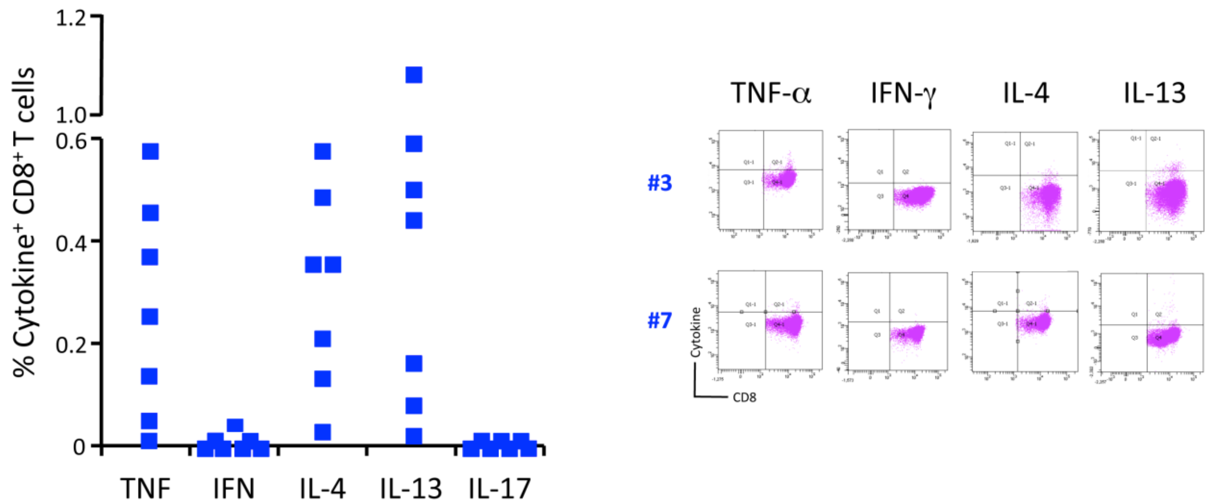
Supporting Information Table 2. Phenotype of HLA-E-binding Mtb peptide-specific T cell lines. Numbers indicate the percentage (SD less that 15%) of positive cells. Markers expressed by more than 10% of cells are indicated in bold. *GrB = Granzyme B; Per = Perforin; Gra = Granulysin.



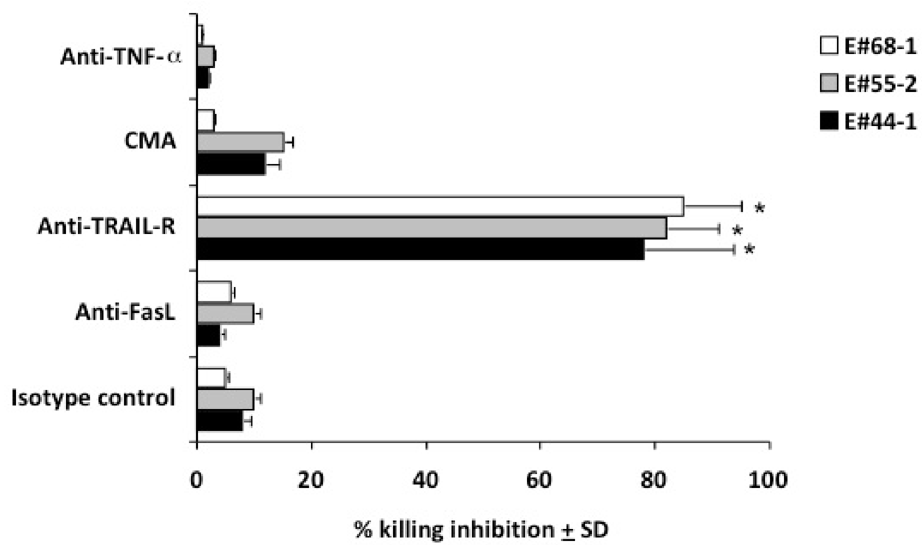
Supporting Information Figure 2. Surface phenotype of Mtb peptide specific and HLA-E-restricted T cell lines. T cell lines were generated as described in Materials and Methods and cells were surface stained for several different markers. Representative overlay histograms showing surface markers expression on gating on $CD3^{+}$ $CD8^{+}$ T cells (open histograms) against appropriate control Ig isotypes (filled histograms).



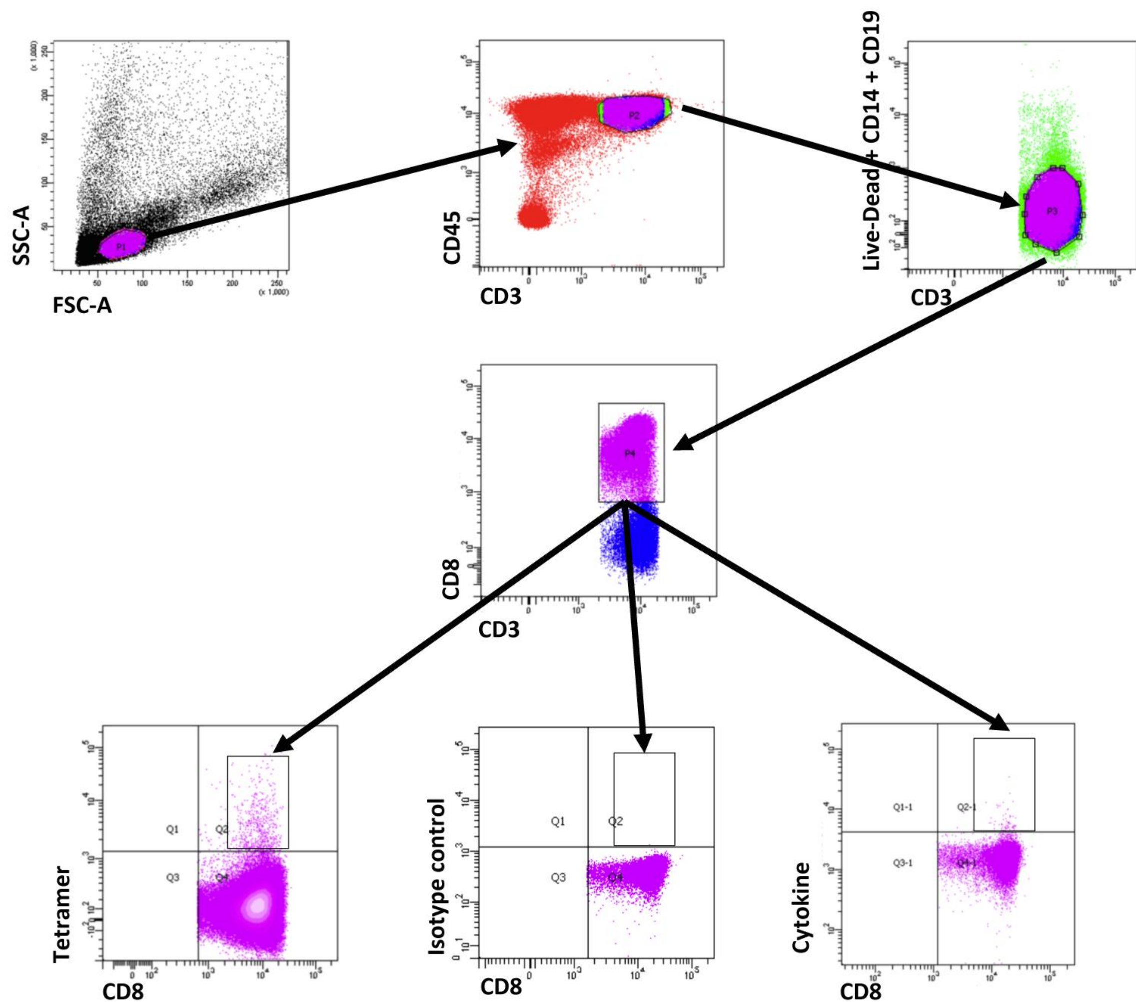
Supporting Information Figure 3. Control for antibody inhibition activity. In **(A),** the NKG2C+ NK clone 41.3 was tested for killing of RMA-S/HLA-E target cells in the presence of the indicted mAbs or isotype control mAb. In **(B)** the HLA-A*0201-restricted CD8 T cell clone specific for M. tuberculosis antigen 16-kDa$_{120-128}$, was tested for killing of Mtb infected THP-1 macrophage cell line, in the presence of the indicated mAbs or isotype control mAb. Data are mean ± SD of two different experiments carried out in triplicate. *p < 0.001 when compared to control mAb, as calculated by the Mann-Whitney U test.



Supporting Information Figure 4. Cytokine production by PBMC from TB patients stimulated *ex vivo* with HLA-E-binding Mtb peptides. PBMC (10^6/mL) from TB patients were stimulated with the Mtb peptide 68 for 6 hrs, and stained for intracellular cytokines, as described under Materials and Methods. Shown are data from 7 different TB patients (left) and FACS plot from two TB patients (right).



Supporting Information Figure 5. Mechanism of killing of CD8 T cells recognizing Mtb peptides in the context of HLA-E. Three different HLA-E-restricted Mtb peptide-specific CD8 T cell lines were incubated with specific peptide-pulsed K562/HLA-E target cells. Cytotoxic activity toward targets was assessed after 6 hrs coculture, in the presence of blocking mAbs to TNF-α, FasL, TRAIL-R1 and -R2, or CMA. Data are shown as mean ±SD of five different experiments carried out in triplicate. *p < 0.001 when compared with cytotoxicity carried out in the absence of inhibitors (isotype control mAb), as calculated by the Mann-Whitney U test.



Supporting Information Figure 6. Gating strategy used to analyze expression of surface markers, tetramer binding and intracellular cytokines in populations of CD8 T cells. Starting from the upper left, arrows indicate directionality of subgates. Markers are indicated to the left and bottom of each dot plot.

Acknowledgments: The authors acknowledge J. E. Coligan, E. H. Weiss, V. Horejsi, G. Triolo, H. Walczak, and A. Krensky for providing reagents. This work was supported by grants from the European Commission within the 6th Framework Programme, TBVAC contract no. LSHP-CT-2003-503367, and the 7th Framework Programme, NEWTBVAC contract no. HEALTH-F3-2009-241745, to T. H. M. O. and F. D. The text represents the authors' views and does not necessarily represent a position of the Commission, which will not be liable for the use made of such information.

Abbreviations: CMA: concanamycin A. **FasL:** Fas ligand. **K562/HLA-E**: K562 cells transfected with HLA-E. **LTBI**: latent tuberculosis infection. **PPD:** purified protein derivative. **RMA-S:** TAP2 mutant Rauscher Murine Leukemia virus-induced T cell lymphoma. **RMA-S/HLA-E**: RMA-S cells transfected with HLA-E. **TB**: tuberculosis. **Tc2:** type 2 $CD8^+$ T cells.

Chapter 3

Results

Frequency of HLA-E-restricted CD8$^+$ T cells before and after Mtb peptide-specific stimulation

Recently Van Meijgaarden et al. (222) have demonstrated that Mtb-specific and HLA-E restricted CD8$^+$ T cell clones derived from PBMC of healthy PPD$^+$donors uniquely produce Th2(IL-4, IL-5, IL-13) cytokines following peptide stimulation, confirming the previously discussed data (Chapter 2).The presence of HLA-E-restricted Mtb peptide-specific CD8$^+$ T cells was confirmed in the peripheral blood of TB patients during active disease. HLA-E tetramers containing peptide 62 and peptide 68 were recognized by an average of 0.28% and 0.32% CD8$^+$ T cells, respectively (**Figure R1A**). Moreover, PBMC obtained from TB patients showed little or no TNF-α and IFN-γ production, but produced significant levels of IL-4 and IL-13 after an overnight of Mtb-specific peptide stimulation *in vitro* (**Figure R1B**).

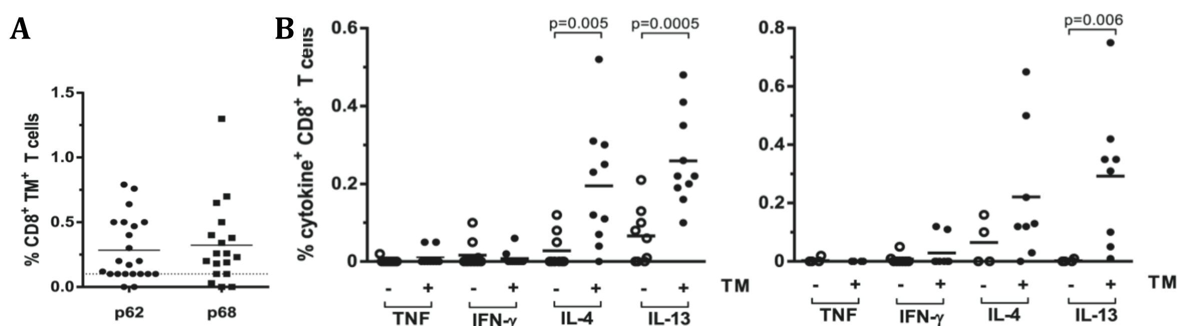


Figure R1: CD8$^+$ T cells from TB patients bind HLA-E/peptide tetramers and produce Th2 cytokines following peptide stimulation (from Van Meijgaarden et al., 2015).
PBMCs from patients with pulmonary TB were stained directly *ex vivo* with HLA-E/peptide tetramers and analysed by flow cytometry. Data are presented as the percentage tetramer positive cells within the CD8$^+$ population. PBMCs were stimulated with either peptide 62 or peptide 68 for 16 hours in the presence of monensin. Cytokines were stained by intracellular staining followed by flow cytometric analysis. These data are depicted as percentage of CD8$^+$ T cells. (**A**) Results of combined TM staining on PBMCs from TB patients for both tetramers containing P62 or P68, data are presented as percentage of CD8$^+$ T cells. (**B**) Cytokine production by CD8$^+$ T cells following stimulation with peptide 62 (left) and peptide 68 (right). Open circles represent patients with tetramer staining $<0.1\%$, close circles represent patients with tetramer staining $>0.1\%$. Groups were compared using a Mann-Whitney U test and obtained $p<0.05$ value was significant.

Based on these results and given that HLA-E tetramers loaded with Mtb-derived peptides p62 and p68 were avalaible, direct *ex vivo* recognition by CD8$^+$ T cells was analysed in 7 tuberculin-negative (PPD$^-$) healthy subjects, 3 latent tuberculosis infection (LTBI) subjects, 10 patients with active TB and 3 HIV-TB co-infected patients (for the characteristics of recruited subjects see Materials and Methods, **Table M1**). The analysis was performed for both p62 and p68 tetramers in the same staining, therefore the frequency of tetramer$^+$ CD8$^+$ T cells refers to the T cell subsets recognizing one or the other Mtb-specific peptide. Moreover, we successfully developed an innovative protocol which combines surface staining with HLA-E tetramers and intracellular staining (ICS) to analyse the functional property of

antigen-specific HLA-E-restricted $CD8^+$ T cells through a gating strategy. This novel method also allowed us to specifically select and analyze the $tetramer^+$ $CD8^+$ T cell subset (**Figure R2, A**).
The *ex-vivo* frequency of tetramers-specific $CD8^+$ T cell was higher in patients with TB active than in healthy PPD^-control donors and this difference was statistically significant, despite the fact that relevant variability in the proportion of $tetramer^+CD8^+$ T cells was observed. As expected, the highest mean frequency of tetramer-specific $CD8^+$ T cells was found in the 3 HIV-TB patients (**Figure R2, B**).
Given the low frequency of HLA-E-restricted and Mtb-specific $CD8^+$ T, we performed a preliminary polyclonal activation with PHA to amplify the size of this $CD8^+$ T cell pool.
After 6 days of PHA stimulation, $CD8^+$ T cells were sorted and were stimulated for one week in the presence of irradiated allogeneic feeder cells, the p62 and the p68 Mtb-specific peptides, and IL-2, IL-7 and IL15. Using this method, the size of Mtb peptide-specific $CD8^+$ T cells escalated after Mtb-specific peptides stimulation, as shown by the large increase of the number of $tetramer^+$ events. However, the *ex vivo* mean frequency of tetramer specific $CD8^+$ T cells remained unchanged upon stimulationwith Mtb-specific peptides (0.52% versus 0.5%), clearly indicating that the *in vitro* stimulation with PHA and specific peptides did not introduce any bias in the obtained $CD8^+$ T cell population (**Figure R3, A and B and Table R1**).

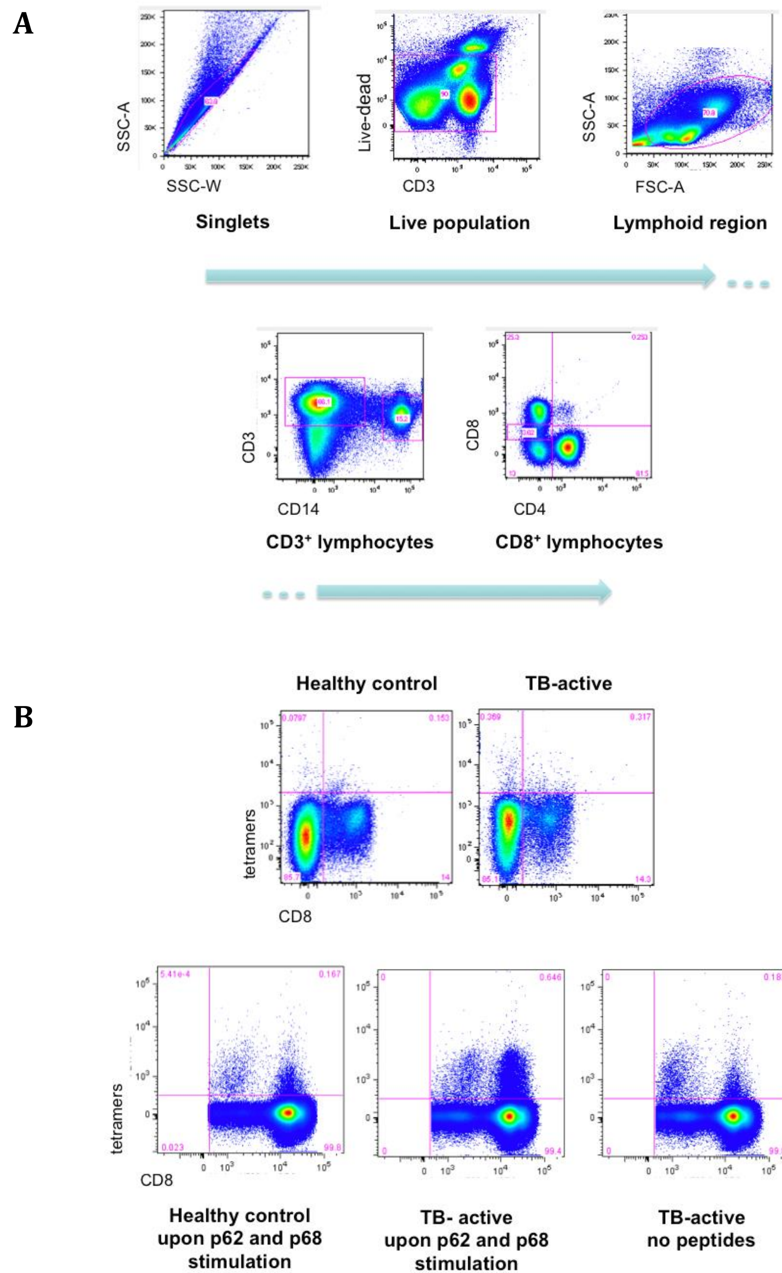


Figure R2: FACS dot plots of CD8⁺ tetramer⁺ T cells *ex vivo* and after Mtb-specific peptides stimulation. **(A)** Gating strategy was used to analyze expression of surface markers, tetramer binding and intracellular cytokines in population of tetramer⁺ CD8⁺ T cells. The dot plots represent each step of subgating. **(B)** Representative flow cytometry dot plots of a single active TB patient compared to a single PPD⁻ healthy subject following staining with HLA-E tetramers containing both peptide 62 and peptide 68. Cells were gated on CD8⁺ T cells and showed before (on the top) and after Mtb-specific peptides stimulation (on the bottom).

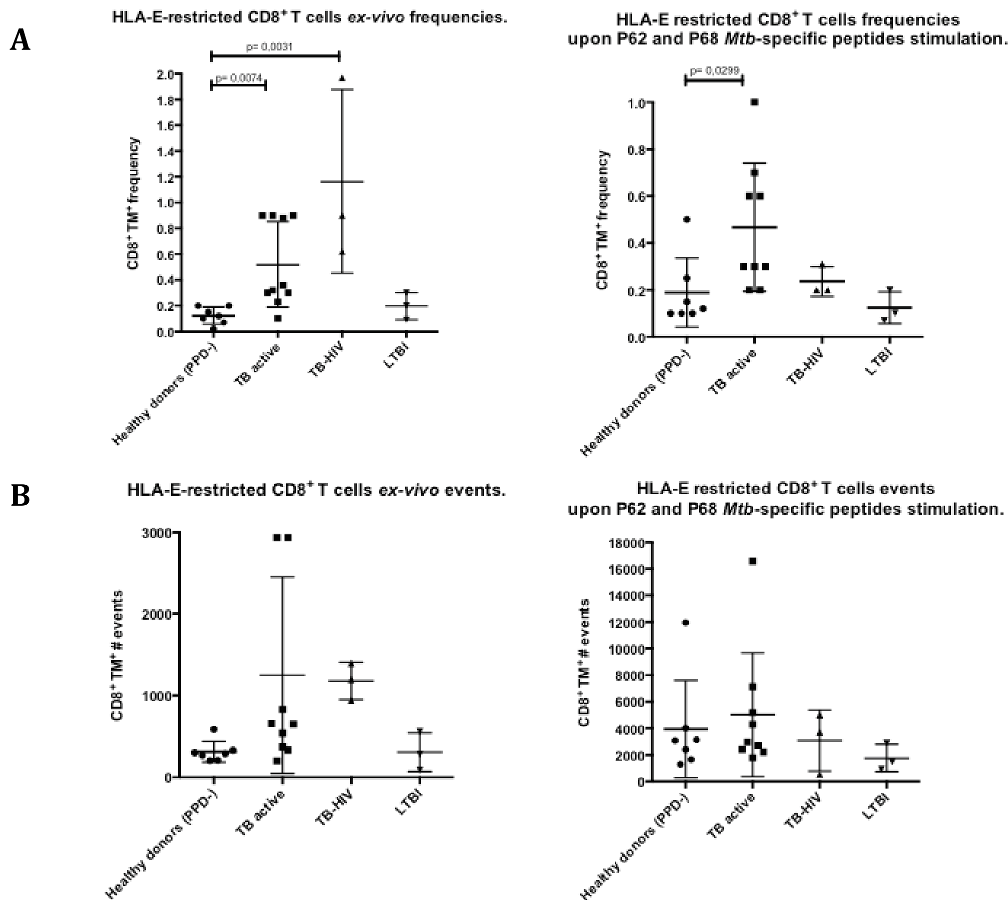


Figure R3: $CD8^+$ tetramer$^+$ T cells frequency *ex vivo* and after Mtb-specific peptides stimulation.
(A and B): HLA-E-restricted $CD8^+$ T cells displayed by frequency **(A)** and number of tetramer$^+$ $CD8^+$ events **(B)** and showed before (on the left-hand side) and after Mtb-specific peptides stimulation (on the right-hand side). Data are depicted as the percentage tetramer positive cells within the $CD8^+$ population, as shown in Figure R2A.

The mean frequency of tetramer specific $CD8^+$ T cells in TB-HIV co-infected patients was 1.16% and decreased to 0.23% after Mtb-specific peptides stimulation: exhaustion of $CD8^+$ T cells in HIV-infected patients most likely accounts for the loss of CD8 T cells. The phenomenon of T cell exhaustion is well know in chronic viral infections such as HIV in which the lack of $CD4^+$ T cells and the persistence of antigens can induce defects in memory T cell responses.

Moreover the higher mean frequency of tetramers-specific $CD8^+$ T cells in patients with active TB compared to LBTI subjects and healthy donors was confirmed, as previously described. The *ex vivo* frequency found in LTBI subjects was 0.19% decreasing to 0.12% after Mtb-peptide specific stimulation (**Table R1**).

Condition	Healthy Donors	LTBI	Active TB	HIV-TB
ex-vivo	0.12	0.19	0.52	1.16
After Mtb-peptides *in vitro* stimulation	0.18	0.12	0.5	0.23

Table R1:The mean frequency of tetramer specific $CD8^+$ T cells. The table shows mean frequency of tetramer specific $CD8^+$ T cells analyzed in the peripheral blood of 7 tuberculin-negative (PPD^-) healthy subjects, 3 latent tuberculosis infection (LTBI) subjects, 10 active TB and 3 HIV-TB co-infected patients.

Phenotypic analysis of HLA-E-restricted $CD8^+$ T cells before and after Mtb-specific peptidestimulation

The phenotype of the total $CD8^+$ T cells as well as of HLA-E/Mtb peptide $tetramer^+$ $CD8^+$ T cells was determined by analysis of CCR7 and CD45RA expression in 7 PPD^- healthy subjects, 3 LTBI subjects, 10 patients with active TB and 3 HIV-TB co-infected patients by flow cytometry.

Ex vivo analysis of total $CD8^+$ T cells showed a consistent proportion of naïve cells in LTBI, active TB samples and healthy control donors, whereas cells with a TEMRA phenotype dominated the $CD8^+$ population in TB-HIV co-infected patients.

In all tested groups the effector memory compartment was most abundant upon stimulation by Mtb-specific HLA-E-restricted peptides, indicating progressive differentiation of $CD8^+$ T cells after stimulation (**Figure R4**).

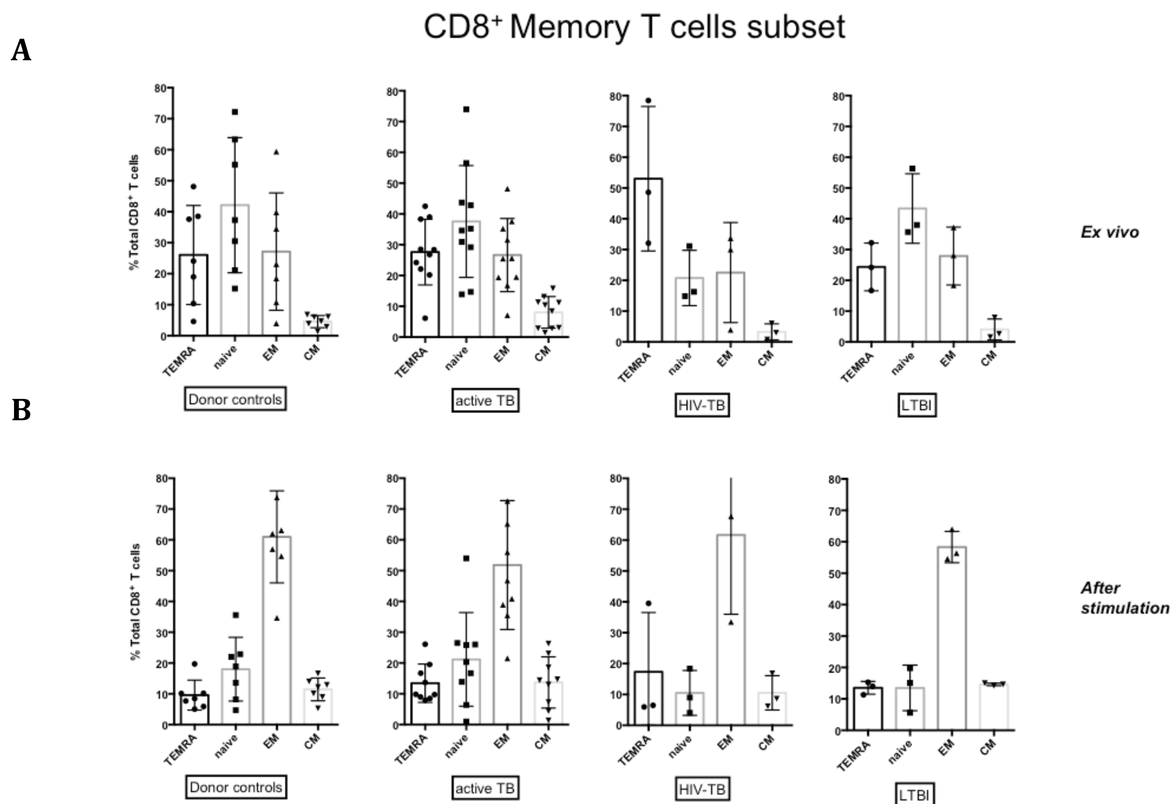


Figure R4: CD8$^+$ T Memory T cell subset phenotype. (A) *Ex vivo* profile gated on total CD8$^+$ T cells revealed a consistent proportion of precursor cells in active TB and LTBI samples and healthy control donors, whereas a TEMRA profile was the main phenotype of the TB-HIV co-infected CD8$^+$ T cells. (**B)** The CD8$^+$T effector memory compartment was the most representative subset upon p62 and p68 Mtb-specific peptides stimulation.

Ex vivo analysis of tetramer-specific CD8$^+$ T cells revealed a higher percentage of naïve subset in patients with active TB, LTBI subjects and healthy donors, while tetramer$^+$ CD8$^+$ T cell with a TEMRA phenotype were the main population in TB-HIV co-infected patients.
Stimulation with Mtb-specific peptides caused a decrease of antigen-specific naïve CD8$^+$ T cells in patients with active TB and healthy control donors, an increase in the effector memory compartment, in control donors and LTBI subjects and an increase in the TEMRA compartment in patients with active TB.
Finally, in HIV-TB co-infected patients Mtb peptide stimulation *in vitro* caused an increase in effector memory and central memory compartments (**Figure R5**).

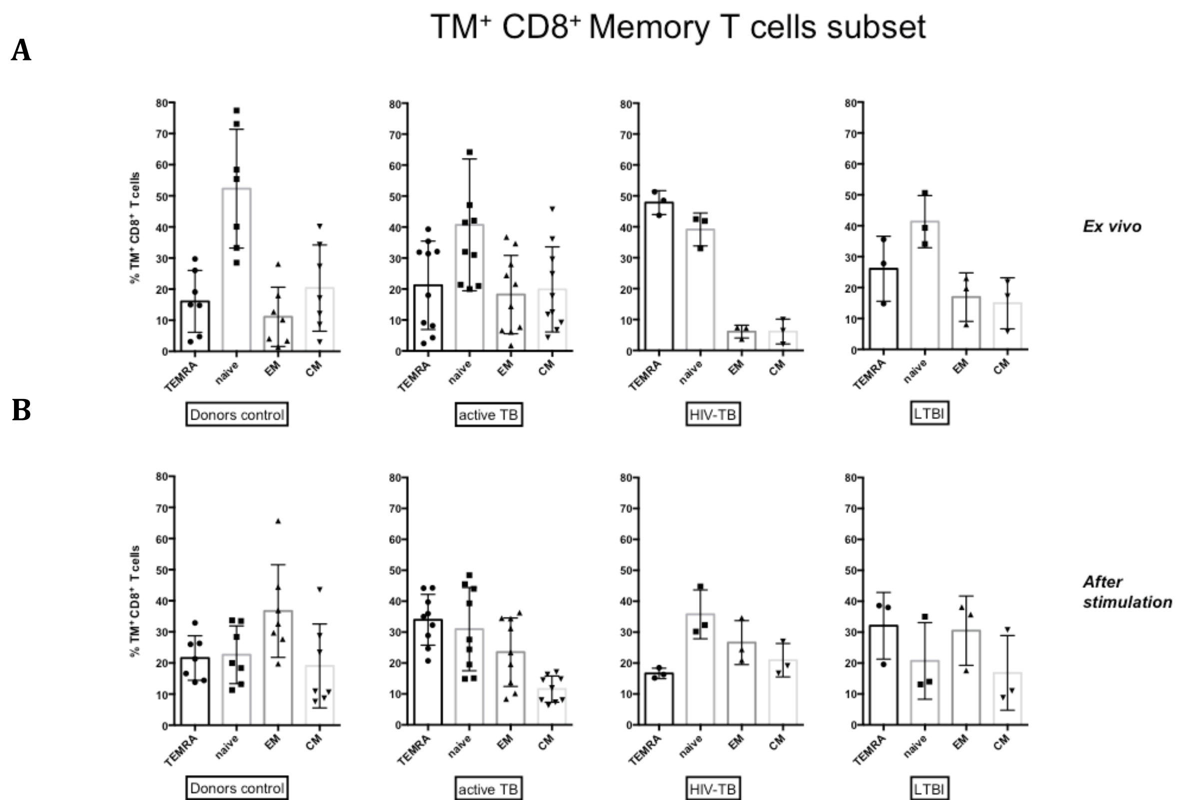


Figure R5: Mtb-specific HLA-E-restricted CD8$^+$ T cells memory phenotype. (**A**)The *ex vivo* analysis tetramers of specific CD8$^+$ T cells phenotype revealed a higher percentage in naïve compartment for all active TB, LTBI, healthy control donors samples. In TB-HIV co-infected samples the main population is represented by a TEMRA phenotype. (**B**) An increase in the effector memory compartment was identified in active TB, LTBI samples and healthy control donors, while the active TB's tetramer$^+$ CD8$^+$ T cells exhibited a related increase in the TEMRA compartment. A trend of increase in both effector memory and central memory compartments was also identified in HIV-TB co-infected samples.

Functional properties of HLA-E-restricted and Mtb-specific CD8$^+$ T cells

To assess the functional profile of HLA-E-restricted and Mtb-specific CD8$^+$ T cells, we stimulated the T cell lines with either p62 and p68 Mtb-specific peptides and tested their cytokines profile as well as cytotoxic molecules production by intracellular staining **(see Figure R6**).

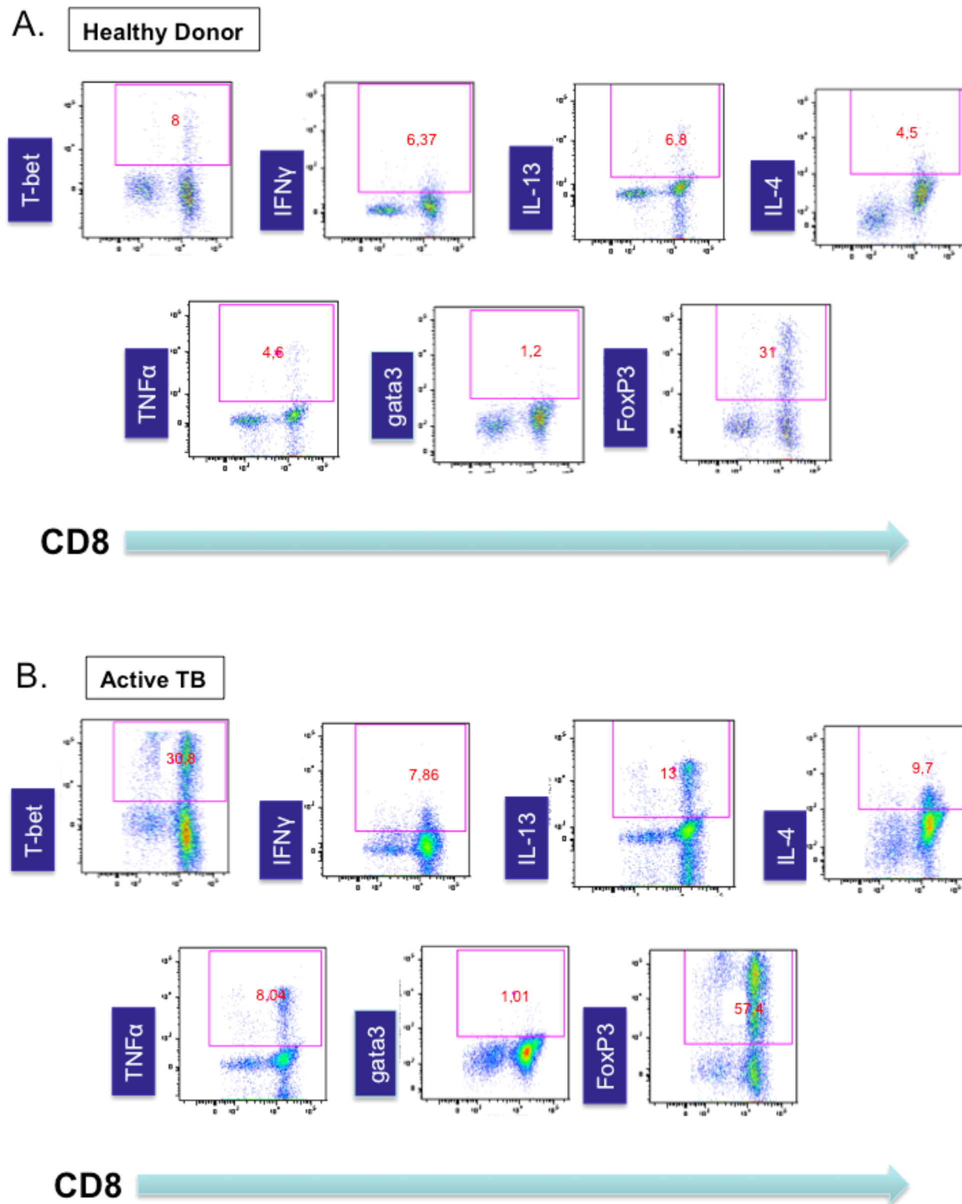
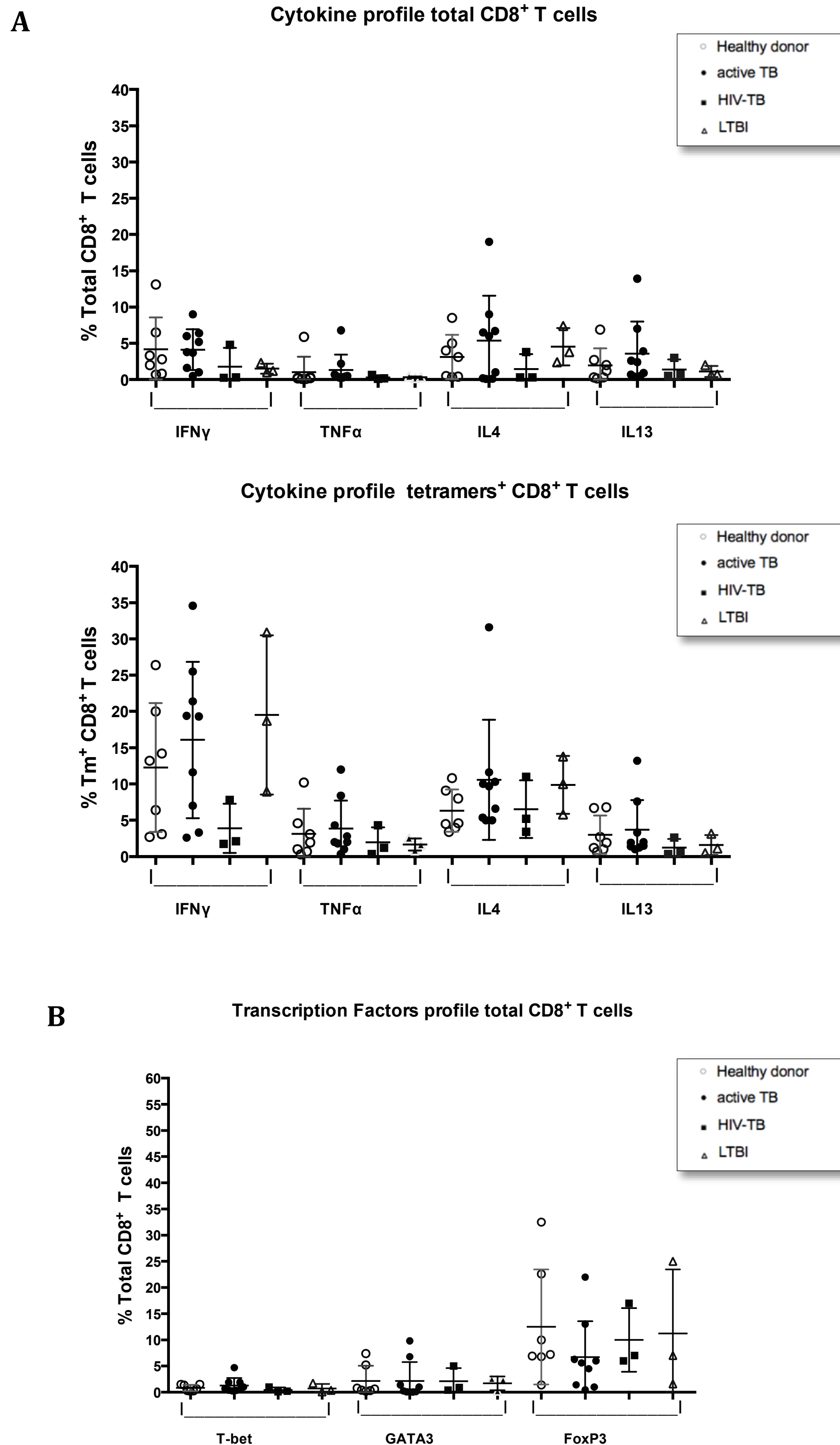


Figure R6: FACS dot plots of cytokine and transcription factors profile of HLA-E-restricted and Mtbspecific CD8⁺ T cells. Dot plot analysis of a healthy control donor **(A)** and an active TB sample **(B)** stimulated with p62 and p68 HLA-E-specific peptides. The percentages of tetramer⁺ CD8⁺ T cells expressing IFNγ, IL13, IL4, TNFα, T-bet, gata-3 or FoxP3 are shown into the red quadrants of each FACS gate.

Tetramer-specific CD8⁺ T cells produced Th2 cytokines, (particularly IL-4) **(Figure R7)**. These results are consistent with the data reported in the study of Van Meijgaarden et al., in which peptide-specific HLA-E-restricted CD8⁺ T cell clones were characterized by IL-4 and IL-13 production following overnight stimulation with peptides p62 or p68.
However, an unexpected production of IFNγ by tetramer specific CD8⁺ T cells was observed.
We also assessed the expression of Th1-function associated T-bet, Th2-function associated GATA3 and regulatory-function associated FoxP3 transcription factors. Significative expression of the Treg specific transcription factor FoxP3 was found in tetramer⁺ CD8⁺ T cells from patients with active TB and LTBI subjects suggesting a possible regolatory role of

these cells. Notably, no expression of GATA3 was detected. Thus, some alternative mechanism has to be involved in IL- 4 and IL-13 expression that needs further investigation.

In agreement with FNγ production, higher expression of the transcription factor T-bet (which is associated with Th1-function) was found in tetramer$^+$ CD8$^+$ T cells from patients with active TB compared to healthy controls.



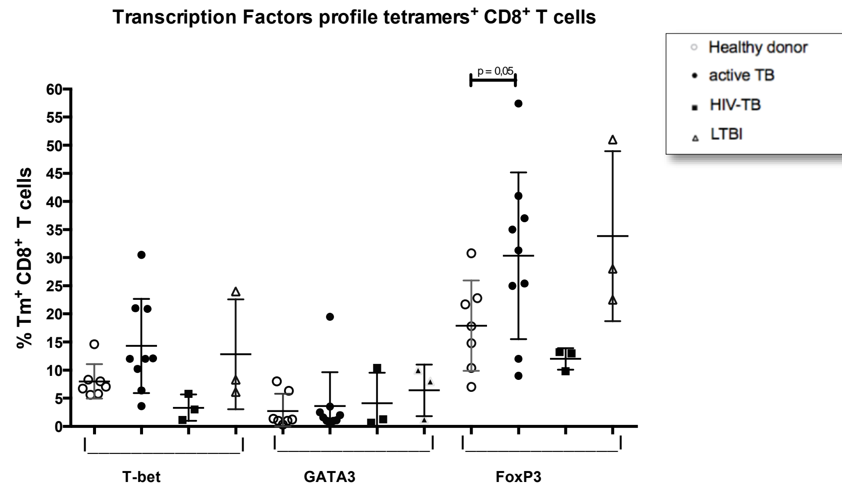
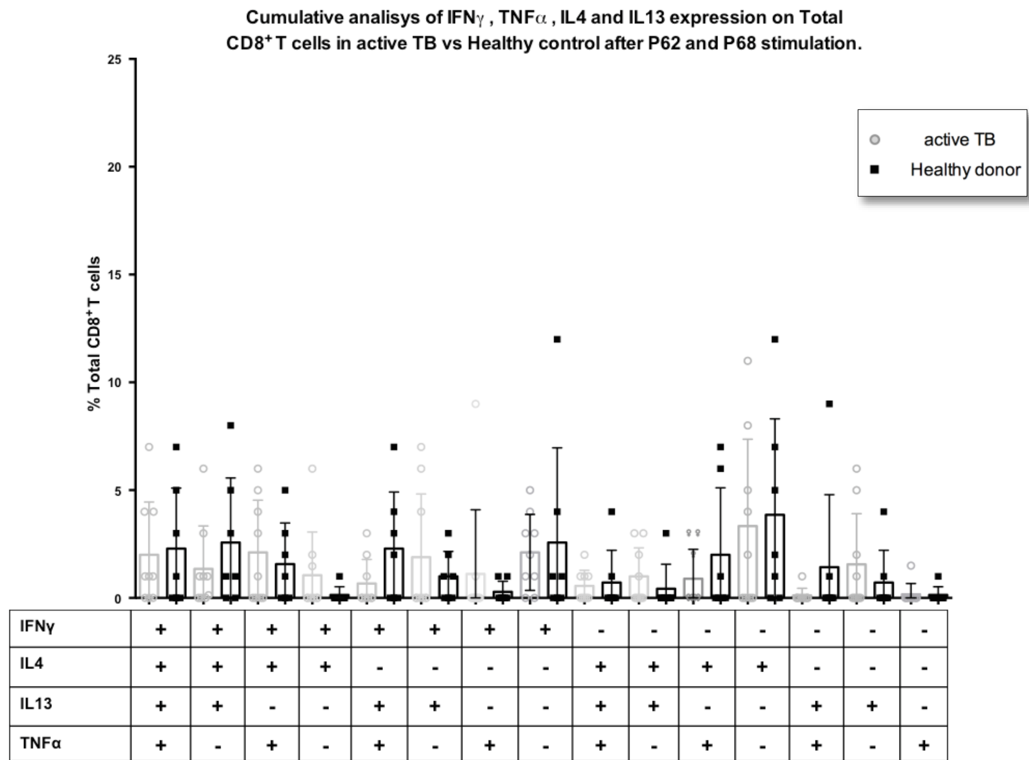


Figure R7: Cytokines and Transcription factors profile of total CD8⁺ T cell compartment and of Mtb-specific HLA-E restricted CD8⁺ T cells. An intracellular staining for IFNγ, IL13, IL4, TNFα and T-bet, gata-3 or FoxP3 was assessed. In **(A)** is shown the cytokine profile evaluated in total CD8 and tetramer-specific CD8 T cells and in **(B)** the transcription factors profile is evaluated as previously mentioned.

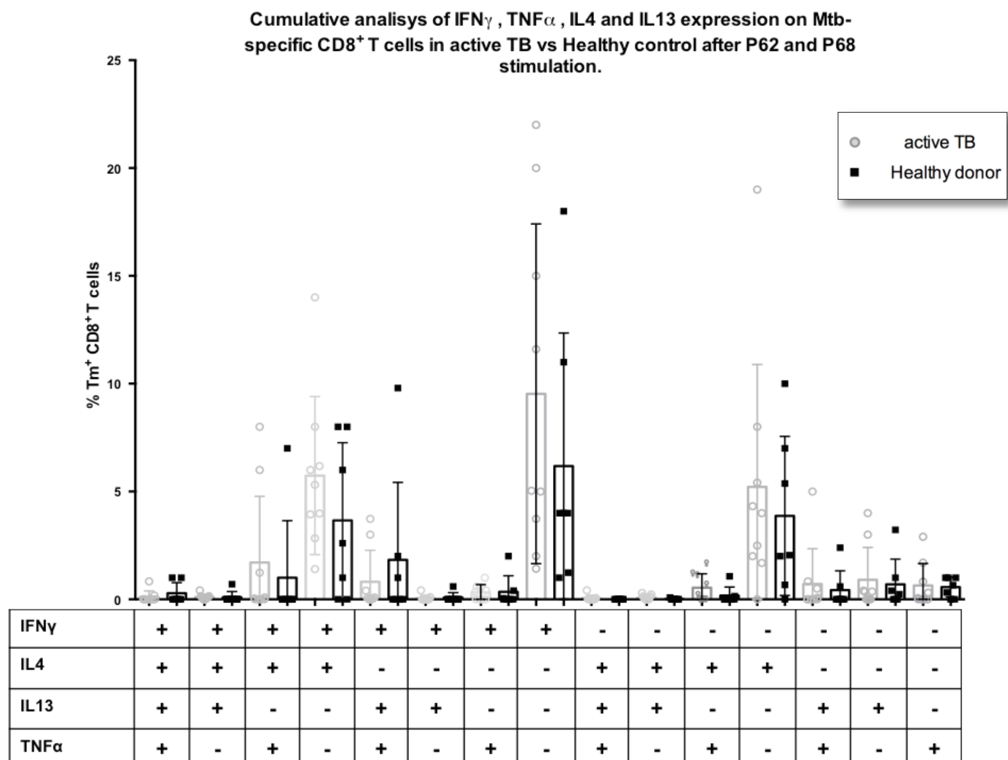
Further analysis of cells with a polyfunctional profile showed no significative difference between healthy controls and patients with active TB either in the total CD8⁺ T cell compartment or in the tetramer⁺ CD8⁺ T cells (**Figure R8**).

A



IFNγ	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
IL4	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-
IL13	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
TNFα	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+

B



IFNγ	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
IL4	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-
IL13	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
TNFα	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+

Figure R8: Polyfunctional cytokine profile of total CD8$^+$ T cell compartment and of Mtb-specific HLA-E restricted CD8$^+$ T cells. The simultaneous analysis of IFNγ, IL13, IL4, TNFα was assessed for total CD8$^+$ T cells **(A)** and of tetramer$^+$ CD8$^+$ T cells **(B)** using FlowJo' Boolean gating tool. No evident differences between healthy control and active TB CD8$^+$ T cells in multiple cytokines expression were observed, although in the tetramer$^+$ CD8$^+$ T subset an insignificant increase of T cells producing both IFNγ and IL-4 was detected in active TB subjects.

Several cytolitic molecules are produced by CD8 T cells in responce to Mtb infection, promoting host protection. These include Perforin and other molecules stored in granules such as Granzyme A, Granzyme B and Granulysin (223-227). Expression of Perforin, Granzyme A, Granzyme B and Granulysin was then analysed in the cohort of healthy subjects, LTBI subjects, patients with active TB and HIV-TB co-infected patients (**Figure R9**).

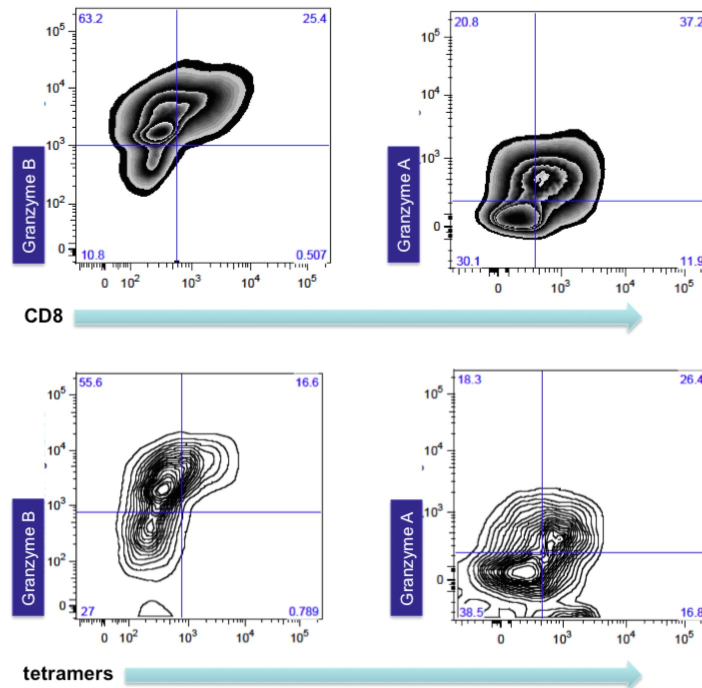
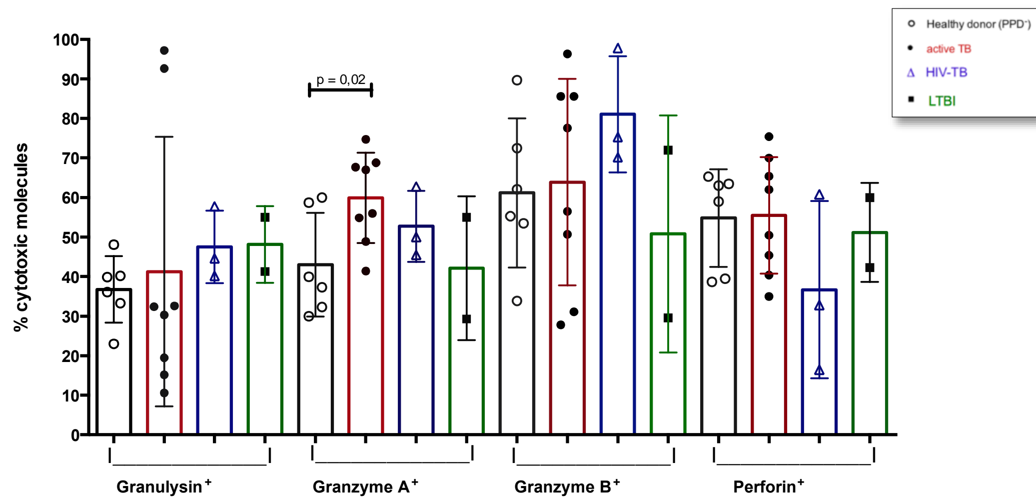


Figure R9: FACS dot plots of cytotoxic molecules profile of total $CD8^+$ T cell subset and HLA-E-restricted- Mtb specific $CD8^+$ T cells. $CD8^+$ T cells were stimulated for seven days with p62 and p68 HLA-E specific Mtb peptides and the expression of Perforin, Granzyme A, Granzyme B and Granulysin was assessed by flow cytometry. The dot plots are gated on total $CD8^+$ T cell subset (on the top) and HLA-E-restricted- Mtb specific $CD8^+$ T cells (on the bottom).

In all tested samples, the cells expressed no cytotoxic molecule and no significant differences were found between healthy control, patients with active TB, LTBI subjects and HIV-TB coinfected patients, with the exception of Granzyme A production which was significantly increased in total $CD8^+$ T cells from patients with active TB compared to healthy controls ($p<0.02$, **Figure R10 A and B**).

A



B

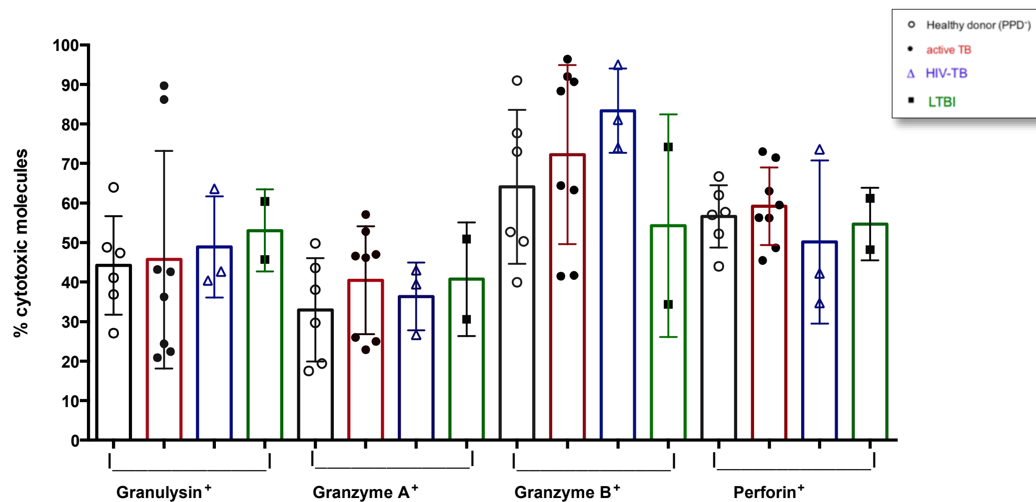
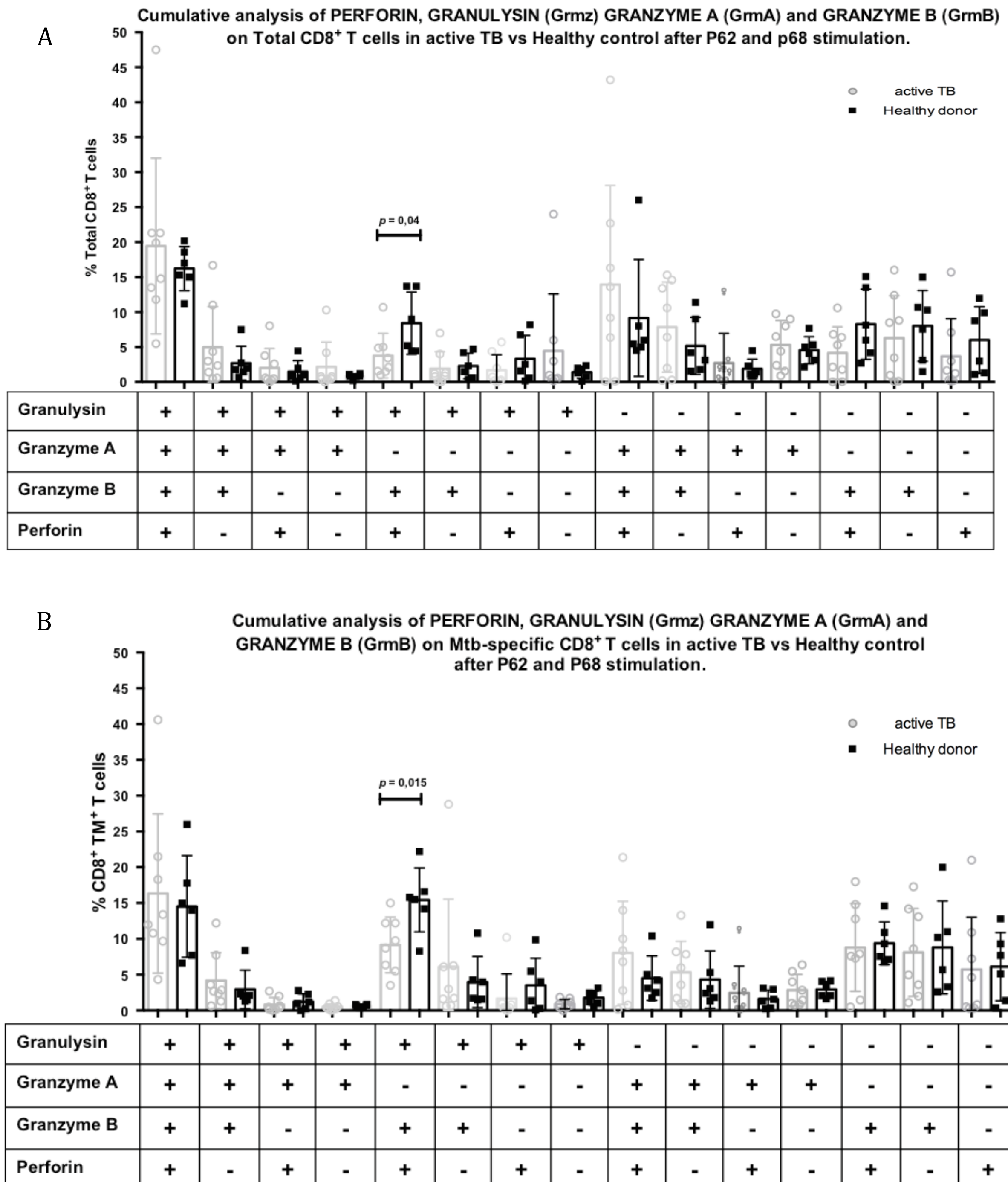


Figure R10: Cytotoxic potential of total CD8$^+$ T cell compartment and of Mtb-specific HLA-E restricted CD8$^+$ T cells. The histogram shows total CD8$^+$ T cell subset (**A**) and HLA-E-restricted- Mtbspecific CD8$^+$ T cells (**B**) expressing Perforin, Granzyme A, Granzyme B and Granulysin in healthy control, active TB, LTBI, HIV-TB samples, after p62 and p68 HLA-E Mtb peptides stimulation.

Moreover, and interestingly, a decrease of total and Mtb-specific CD8$^+$ T cells co-expressing Perforin, Granzyme B and Granulysin was detected in patients with active TB as compared to healthy controls ($p<0.04$ for total CD8$^+$ T cells and $p<0.015$ for Mtb-specific CD8$^+$ T cells; **Figure R11 A and B** respectively). In contrast, CD8$^+$ T cells containing Granzyme A alone or in combination with other cytotoxic molecules were more represented in patients with active TB than in healthy control, although differences were not statistically significant.



Granulysin	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Granzyme A	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-
Granzyme B	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
Perforin	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+

Granulysin	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Granzyme A	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-
Granzyme B	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
Perforin	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+

Figure R11: Cumulative analysis of Perforin, Granulysin, Granzyme A and Granzyme B in total CD8⁺ T cell and Mtb-specific CD8⁺ T cell compartments. The cumulative analysis of all cytotoxic markers in both total CD8⁺ (**A**) and Mtb-specific CD8⁺T cell compartments (**B**) indicated a decrease of T cells co-expressing Perforin, Granzyme B and Granulysin ($p<0.04$ for total CD8⁺ T cells and $p<0.015$ for Mtb-specific CD8⁺ T cells respectively).

Generation of HLA-E-restricted Mtb-specific CD8 T cell clones

To study in detail the function of HLA-E-restricted Mtb-specific CD8⁺ T cells, we generated clones from the CD8⁺ T cell lines previously described. In particular, CD8⁺ T cell lines from

two patients with active TB were selected on the basis of the best response/expression of Th2 cytokines and were stained with PE-labeled tetramers and sorted by positive selection using anti-PE magnetic beads. This procedure enabled us to select and separate the TM^+ cells from total $CD8^+$ T cells (**Figure R12**).
Sorted $tetramer^+$ cells were used to generate clones by the limited dilution technique. A total 12 clones were isolated from the two cell lines and stored in liquid nitrogen for future studies.

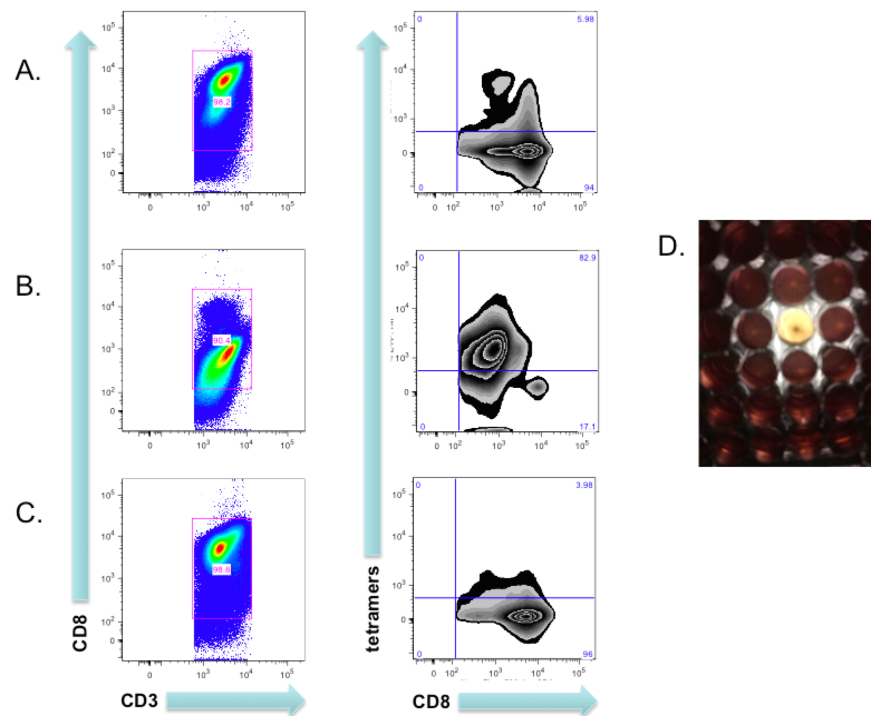


Figure R12: FACS dot plots of HLA-E-$tetramer^+$ $CD8^+$ T cells. Cultures of purified $CD8^+$ T cells were stained with PE-labeled tetramers and sorted by positive selection using anti-PE magnetic beads (MACS, Miltenyi Biotec). Dot plots in A, B and C show the $tetramer^+$ $CD8^+$ T subset before **(A)** and after MACS sorting, in particular the selected $tetramer^+$ $CD8^+$ T cells **(B)** and the leftover **(C)**. In total 12 clones from two cell lines were isolated and frozen in nitrogen. **(D)** Image of one representative T cell clone obtained upon limiting dilution assay culture at 0.3 cell/well.

Analysis of *ex-vivo* frequency of MAIT cells

Recently there is a great deal of interest in studing Mucosal-Associated Invariant T (MAIT) cells because they exert an important antimicrobial role in infection models (228, 229). MAIT cells are characterized by the expression of a unique Vα7.2-Jα33/12/20 TCR together with additional surface markers such as CD26, CD161, IL-18Rα, and a range of chemokine receptors such as CCR6, CCR5, CCR9 and CXCR6, that promote their localization to gut, lungs and liver (230, 231, 232). MAIT cells are absent in the peripheral blood of HCV- and HIV-infected patients (233, 234) and in these latter MAIT loss persists despite the successful anti-retroviral therapy (235, 236).
Similarly, the frequency of MAIT cells is lower in the peripheral blood of patients with active TB, both with or without HIV co-infection, than in healthy controls (237, 238). A reduction in

circulating MAIT cells is also associated with aging and with alterations in cytokine profile (238).

Using flow cytometry, we compared the *ex vivo* frequency of MAIT cells in 7 tuberculin-negative (PPD^-) healthy subjects, 3 LTBI subjects, 10 patients with active TB and 3 HIV-TB co-infected patients. MAIT cells were indentified by the simultaneous expression of Vα7.2 TCR, CD161 and CD26, upon gating on total $CD8^+$ T cells using the FlowJo's Boolean gating tool which allows to see all possible Boolean combination gates of a population thanks to the possibility to select more than one specific cell marker at the same time (**Figure R13A and B**). As expected, MAIT cells are less represented in the peripheral blood of patients with active TB and TB-HIV co-infection, in comparison to healthy donors. The same finding was detected in LTBI subjects, although only three subjects were analysed (**Figure R13 C**).

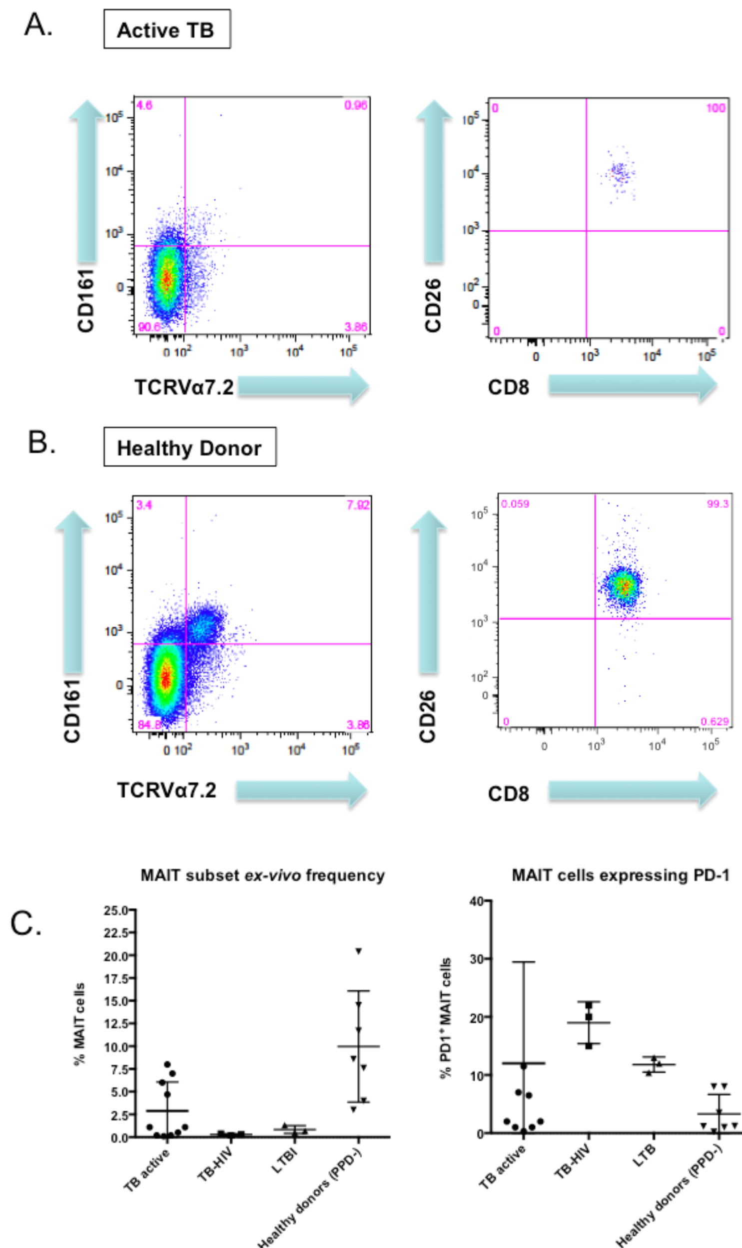


Figure R13: *Ex-vivo* analysis of MAIT compartment. Dot plots show the percentage of $CD8^+$, $CD161^+$ and Vα7.2 TCR^+ T MAIT cells in one active TB patient **(A)** compared with a healthy donor **(B)**. **(C)** *Ex vivo* MAIT frequency in 7 healthy control donors, 3 LTBI subjects, 10 active TB and 3 HIV-TB co-infected patients (graph

on the left) and amount of MAIT cells expressing PD-1 (graph on the right). MAIT cells were identified as $CD8^+$, Vα7.2 TCR^+, $CD161^+$ and $CD26^{++}$ using the FlowJo's Boolean gating tool.

T cell exhaustion is a hallmark of $CD8^+$ T cells engaged in immune response in several chronic infections such as HIV, HCV and HBV (239). In addition, programmed cell death protein 1 (PD-1) has an important role in the exhaustion of classical $CD8^+$ T cells in HIV infection (240). Therefore we should understand if there is an association between MAIT cells depletion in patients with TB and HIV/TB co-infections and the phenomenon of exhaustion of $CD8^+$ T cells. However, we did not find significant increase in PD-1 expression on MAIT cells in patients with TB and HIV-TB co-infection (**Figure 13C).**

Materials and Methods

Recruited patients

Peripheral blood was collected from 10 adults with TB disease, 3 adults with TB-HIV co-infection and 3 LTBI subjects, from the Dipartimento di Medicina Clinica e delle Patologie Emergenti, University Hospital, Palermo whereas 7 tuberculin-negative PPD^- healthy subjects were recruited from anonymous bloodbank donors in Netherlands (Sanquin Bloodbank, the Netherlands) (**Table M1**). The PBMCs of each patients was stored in nitrogen and, upon reaching a reasonable number of samples, the frozen samples were sent to the Department of Infectious Disease at Leiden University Medical Center (LUMC) in Leiden in which the experiments described below were assessed.

Stage of disease	Enrolled subjects	Gender	Year of birth	Clinical characteristics	Other information
Active TB	TB1	F	/	pulmonary TB	mantoux positive
	TB2	M	1972	pulmonary TB	mantoux positive
	TB3	M	1996	pulmonary TB	mantoux positive
	TB4	M	1996	miliary TB	mantoux positive
	TB5	F	1979	pulmonary TB	mantoux positive
	TB6	F	1982	pulmonary TB	mantoux positive
	TB7	M	1986	pulmonary TB	mantoux positive
	TB8	M	1970	extra-pulmonary TB	mantoux positive
	TB9	F	1972	pulmonary TB	mantoux positive
	TB10	F	1982	pulmonary TB	mantoux positive
TB-HIV co-infection	TB-HIV1	M	1983	miliary TB, HIV and HCV co-infection	mantoux positive
	TB-HIV2	M	1969	miliary TB, HIV co-infection	mantoux positive
	TB-HIV3	M	1979	miliary TB, HIV co-infection	mantoux positive
Latent TB	LTBI-1	F	1954	No disease, LTBI	mantoux positive
	LTBI-2	M	1960	No disease, LTBI	mantoux positive
	LTBI-3	M	1960	No disease, LTBI	mantoux positive
Healthy donors (PPD^-)	HD-1				PPD-
	HD-2				PPD-
	HD-3				PPD-
	HD-4				PPD-
	HD-5				PPD-
	HD-6				PPD-
	HD-7				PPD-

Table M1: Clinical characteristics of the subjects enrolled in the study. Peripheral blood was collected from a total of 16 subjects, 10 adults with active TB disease, 3 adults with TB-HIV co-infection and 3 adults with LTBI from the Dipartimento di Medicina Clinica e delle Patologie Emergenti, University Hospital, Palermo.The 7 tuberculin-negative healthy donors included in the study were obtained from the anonymous Dutch bloodbank donors (Sanquin Bloodbank, the Netherlands), therefore no information about age, sex and clinical characteristics of the anonymous samples was available. For each healthy donors control the PPD- responce was assessed by IFNγ ELISA.

Selection of PPD^- healthy controls from bloodbank donor.

Lymphocyte stimulation assays were performed stimulating PBMCs from the healthy donors ($0,5x10^6$/w) in 48-well plates with 5 μg/mL final concentration of PPD (Staten Serum Institute, Copenhagen, Denmark) in an incubator at 37°C with 5% CO_2. Phytohemagglutinin (PHA, 5 μg/mL final concentration, Remel Europe), and unstimulated samples were used as

positive and negative controls, respectively. After 24hours and 7 days the supernatant were collected and then used to assess an IFNγ-ELISA (U-CyTech, Utrecht, the Netherland).

Ex vivo surface tetramer staining

HLA-E/Mtb peptide tetramers were used to assess the *ex vivo* frequency of HLA-E/peptide $CD8^+$ T cells in the peripheral blood of 7 tuberculin-negative (PPD^-) healthy donors, 3 LTBI subjects, 10 active TB patients and 3 HIV-TB co-infected patients.

Peripheral blood mononuclear cells (PBMC, 10^6/mL) were isolated from heparinized blood samples by Ficoll-Hypaque (Pharmacy LUMC, the Netherland) density centrifugation, washed twice in phosphate buffered saline (PBS, Pharmacy LUMC, the Netherland) containing 0,1% BSA (Sigma), stained for 10 min at RT with a purified anti-CD94 mAb and then stained for 30 min at 4°C with PE-labeled tetramers in 5ml FACS tubes. For the characteristics of peptides used see **Table M2**. After the incubation the samples were washed with PBS (Pharmacy LUMC, the Netherland) containing 0,1% BSA (Sigma), and subsequently characterized by 11 different surface markers plus live/dead dye, with a combination of 12 colour fluorochromes (also see **Table M3** in which the characteristics of mAb used are summarized) collected in the same panel. Live/dead stain (Vivid fixable violet reactive dye, Invitrogen, Thermo Fisher Scientific Inc.) was used for all samples according to the manufacturer's protocol. Fluorochrome-conjugated antibodies used for staining included anti-CD3 (Horizon V500, UCHT1), anti-CD8 (AlexaFluor700, RPA-T8), anti-CD4 (PerCp-Cy5.5, RPAT4) anti-CD45RA (BV570, MI100), anti-CCR7 (PE-Cy7, 3D12), anti-CD95 (AlexaFluor647, DX2), anti-CD62L (Qdot605, *DREG-56*), anti-CD14 (Qdot655, *TüK4*),anti-TCR Vα7.2 (APC-Cy7, 3C10), anti-CD161 (PE-Cy5, DX12), anti-CD26 (PE-C594, M-A261) mAbs. The samples were stained with for 30 min at 4°C, washed twice in PBS containing 0,1% BSA and then aquired on a LSRFortezza flow cytometer with Diva software (v6.2, BD Biosciences).

# Peptide [1]	Amino acid sequence	Derived from	Amino acid position	Protein name	Accession number [2]	Binding (inμ M) [3]
62	RMPPLGHEL	Rv2997	470-478	P49 protein	O53244	4
68	VLRPGGHFL	Rv1523	251-259	Methyltransferase	Q50584	1,8

Table M2: HLA-E-binding Mtb-derived peptides properties.
[1] Peptides containing HLA-E binding motifs were selected from Joosten et al., 2010. [2] UniProtKB/ Swissprot accession number. (www.uniprot.org) [3] Binding was calculated as the concentration (mM) of peptide required to reduce fluorescence intensity of the standard peptide with 50% (IC50, Joosten et al., 2001).

ex-vivo PANEL				
SURFACE MARKERS	FLUOROCHROMES	CLONES	BRAND	SUBSET
CD4	*PerCp-Cy5.5*	***RPA-T4***	**BD Pharmigen**	CD4+ T cells
PD1	*AlexaFluor488*	***MIH4.1***	**BD Pharmigen**	Exaustion surface marker
TCRVα7.2	*APC-Cy7*	***3C10***	**BioLegend**	MAIT
CD8	*AlexaFluor700*	***RPA-T8***	**BD Pharmigen**	CD8+ T cells
CD14	*Qdot655*	***TüK4***	**INVITROGEN**	Monocytes
CD45RA	*BV570*	***MI100***	**BioLegend**	Phenotype analysis
CD3	*HorizonV500*	***UCHT1***	**BD Horizon**	Lymphoid region
Vivid	*Pacific Blue*		**INVITROGEN**	live cells
CCR7	*PE-Cy7*	***3D12***	**BD Pharmigen**	Phenotype analysis
CD161	*PE-Cy5*	***DX12***	**BD Pharmigen**	MAIT
CD26	*PE-C594*	***M-A261***	**BD Horizon**	MAIT
P62 and P68 Tetramers	*PE*			HLA-E resctricted CD8+ T cells

Table M3: FACS *ex vivo* analysis panel reporting surface markers, fluorochromes, clones, brand and related subset.

Collected data were analyzed with the use of FlowJo software (version 9.7.3 Treestar Inc., Ashland, OR, USA). Scoring was based on the percentage of cells that expressed the particular marker. The analysis of each specific subset was performed using the gating strategy shown in **Figure M1**. After a previously selection of the singlet events, viable lymphocytes were gated by forward and side scatter and selected by the T cell co-receptor CD3. The analysis was performed on 100 000 $CD8^+$ events acquired for each sample.

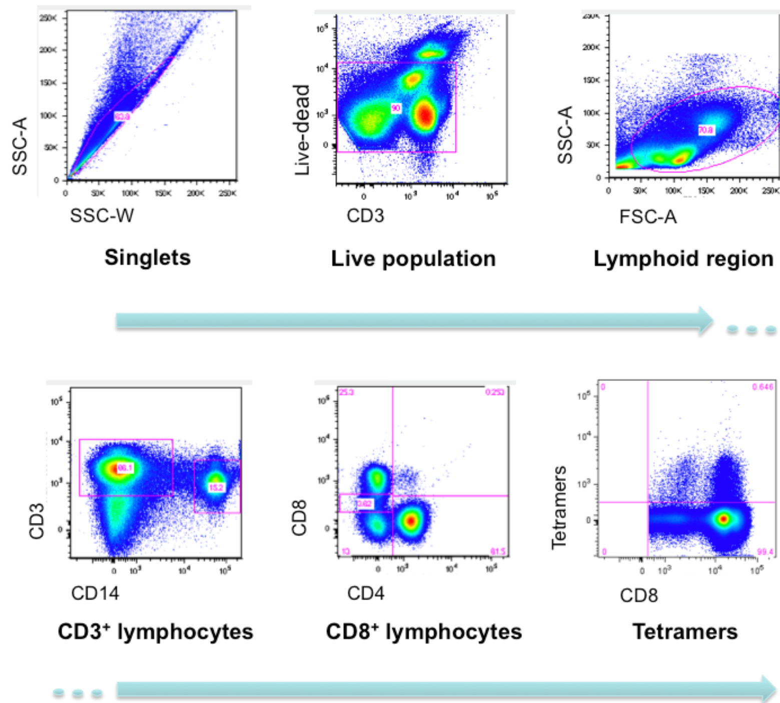


Figure M1: FACS *ex vivo* dot plots showing the gating strategy used for the identification of Total CD8$^+$ T cells and Tetramers$^+$ CD8$^+$ T cells analysed.

Generation of HLA-E restricted CD8$^+$T cell lines

PBMCs from 7 tuberculin-negative (PPD$^-$) healthy donors, 3 LTBI individual, 10 active TB patients and3 HIV-TB co-infected patients were thawed (80% median viable cell yield), counted (CASY cell counter, Roche) and cultured in 24-well plates with Phytohemagglutinin (PHA, 5 µg/mL final concentration, Remel Europe), in complete Iscove's modified Dulbecco's medium (IMDM, Life Technologies-Invitrogen) added with 10% of human serum (pooled serum from blood bank donors) in an incubator at 37°C with 5% CO_2. After 3 days, the growing cells were splitted and refreshed with human recombinant IL-2 (Proleukin, Chiron, Amsterdam, the Netherlands 25 U/mL final concentration). After 7 days of PHA polyclonal stimulation, the CD8$^+$ T cells were sorted by positive selection using magnetic beads (MACS, Miltenyi Biotec BV, Leiden, the Netherlands). Purity of all cells sorts was checked by flow cytometry and resulted in $\geq$ 98% of purity. CD8$^+$ T cells sorted were subsequently cultured in presence of MtbP62- (**RMPPLGHEL**) and P68- (**VLRPGGHFL**) specific peptides (for peptide's charactheristics see **Table M1**). Human recombinant IL-15 and IL-7 (Peprotech, Rocky Hill, NJ both 5 ng/mL final concentration) have also been added as well as irradiated feeders cells (3600 rads for 4 minutes). After 24 h, the supernatants were collected and stored at −80°C. After 3 days, the culture was refreshed by complete IMDM medium plus IL-2 (50 U/mL). After 5 days, the cultures were splitted and refreshed with complete IMDM plus IL-2 (100 U/mL). After 7 days, the supernatants were collected and stored at −80°C.Starting with a number of 3-4x10^6 PBMCs, the yield of Mtb-specific CD8$^+$ T cells after this procedure was about 80-100x10^6.

Intracellular staining (ICS)

Cytokine, trascriptional factor and cytotoxic molecules profiles of the $CD8^+$ T cells stimulated with Mtb-specific peptides were analysed followed by 16 hours incubation with BrefeldinA (3 μg/ml, Sigma-Aldrich) and monensin (1:1000, BioLegend) at 37°C in 5% CO^2. The cells were harvested, washed, and stained for 10 min at RT with a purified anti-CD94 mAb and then were stained with PE-labeled tetramers in 5ml FACS tubes and with anti-CCR7 (PE-Cy7, 3D12) for 15 min at 37°C. After the incubation, the cells were washed once in PBS (Pharmacy LUMC, the Netherland) containing 0,1% BSA (Sigma) and subsequently characterized by 24 different markers with a combination of 15 colour fluorochromes (see **Table M3 and M4** for the characteristics of mAb used) collected in two different panels of 14 and 10 markers respectively for the analysis of cytokines/trascriptional factor, and cytotoxic molecules profiles.

Fluorochrome-conjugated antibodies used for surface marker staining in the cytokine and trascriptional factors analysis comprised anti-CD3 (BV570, UCHT1), anti-CD8 (Horizon V500, RPA-T8), anti-CD25 (APC-H7, M-A251) and anti-CD45RA (BV570, MI100) mAbs, whereas in the cytotoxic molecules profile analysis were included anti-CD3 (BV570, UCHT1), anti-CD8 (Horizon V500, RPA-T8) and anti-CD45RA (BV570, MI100) mAbs. Live/dead stain (Vivid fixable violet reactive dye, Invitrogen, Thermo Fisher Scientific Inc.) was used for all samples according to the manufacturer's protocol.

Staining of intracellular cytokines was performed by fixing cells using FIX and PERM KIT according to the manufacturer's protocol (BD Bioscence) and then by staining with anti-IFN-γ (AlexaFluor700, B27), anti-IL-13 (APC, JES10-5A2), anti-TNF-α (PerCP-Cy5.5, MAb11), anti-IL4 (PE-C594), anti-GATA3 (AlexaFluor488, L50-823), anti-T-bet (BV605, 4BIO), anti-FoxP3 (PE-Cy5, PCH101) mAbs. The samples were stained with for 30 min at 4°C, washed twice in PBS containing 0,1% BSA and then aquired on a FACS LSRFortezza flow cytometer with Diva software (v6.2, BD Biosciences).

Transciptional Factors and Cytokines PANEL				
SURFACE MARKERS	**FLUOROCHROMES**	**CLONES**	**BRAND**	**SUBSET**
TNFα	*PerCp-Cy5.5*	***MAb11***	**BD Pharmigen**	Th1 -function associated
GATA3	*AlexaFluor488*	***L50-823***	**BD Pharmigen**	Th2-function associated
CD25	*APC-H7*	***M-A251***	**BD Pharmigen**	Treg-function associated
IFNγ	*AlexaFluor700*	***B27***	**BD Pharmigen**	Th1-function associated
IL13	*APC*	***JES10-5A2***	**BD Pharmigen**	Th2-function associated
CD45RA	*Qdot655*	***MEM-56***	**INVITROGEN**	Phenotype analysis
T-bet	*BV605*	***4BIO***	**BioLegend**	Th1-function associated
CD3	*BV570*	***UCHT1***	**BioLegend**	Lymphoid region
CD8	*HorizonV500*	***RPA-T8***	**BD Horizon**	$CD8^+$ T cells
Vivid	*Pacific Blue*		**INVITROGEN**	live cells
CCR7	*PE-Cy7*	***3D12***	**BD Pharmigen**	Phenotype analysis
FoxP3	*PE-Cy5*	***PCH101***	**Ebioscience**	Treg-function associated
IL4	*PE-C594*	***MP4-25D2***	**BD Horizon**	Th2-function associated
P62 and P68 Tetramers	*PE*			HLA-E resctricted $CD8^+$ T cells

Table M3: Trascriptional factor and cytokines analysis FACS panel reporting surface markers, fluorochromes, clones brand and related subsets.

After the fixation and permeabilzation steps, the cells engaged for cytolytic molecules expression analysis were stained with rabbit-anti-human Granulysin (kind gift of Dr. A. Krensky, Stanford, CA) followed by Goat-anti-Rabbit-FITC, anti-Perforin (PEC594, δG9),anti-GranzymeB-(AlexaFluor700, GB11) anti-GranzymeA (PerCPCy5.5, CB9) mAbs and also an anti-IL10 (BV605, JES3-9D7) mAb was included.

Cytotoxic T lymphocytes PANEL				
SURFACE MARKERS	**FLUOROCHROMES**	**CLONES**	**BRAND**	**SUBSET**
Granzyme A	*PerCp-Cy5.5*	***CB9***	**BioLegend**	CTL -function associated
Granulysin	*FITC*			CTL-function associated
Granzyme B	*AlexaFluor700*	***GB11***	**BD Pharmigen**	CTL-function associated
CD45RA	*Qdot655*	***MEM-56***	**INVITROGEN**	Phenotype analysis
IL10	*BV605*	***JES3-9D7***	**BD Horizon**	anti-inflammatory function associated
CD3	*BV570*	***UCHT1***	**BioLegend**	Lymphoid region
CD8	*HorizonV500*	***RPA-T8***	**BD Horizon**	$CD8^+$ T cells
Vivid	*PB*		**INVITROGEN**	live cells
CCR7	*PE-Cy7*	***3D12***	**BD Pharmigen**	Phenotype analysis
Perforin	*PE-C594*	***δG9***	**BD Horizon**	CTL-function associated
P62 and P68 Tetramers	*PE*			HLA-E resctricted $CD8^+$ T cells

Table M4: Cytotoxic molecules analysis FACS panel reporting surface markers, fluorochromes, clones brand and related subsets.

Cells were acquired by FACS LSRFortezza flow cytometer as described above. Analysis of data was performed with FlowJo software (version 9.7.3 Treestar Inc., Ashland, OR, USA). Analysis was performed on a minimum of $2x10^6$ $CD8^+$ events acquired for each sample. Negative controls were obtained with $CD8^+$ T cell lines incubated with medium without stimulant.

Generation of HLA-E restricted CD8+ T cell clones

$CD8^+$ T cell lines from two active TB individual, generated as previously described were cultured overnight in complete Iscove's modified Dulbecco's medium (IMDM, Gibco Life technologies, Thermo Fisher Scientific Inc), supplemented with 10% pooled human serum and were restimulated in 24 well plates with P62 and P68 Mtb-specific HLA-E-restricted peptides (5 μg/ml final concentration). The two $CD8^+$ T cell lines were selected on the basis on the best responce/expression of Th2 cytokines profile. Cultures of purified $CD8^+$ T cells were subsequently harvested and stained with PE-labeled tetramers (tetramers were folded at the facilities of the LUMC, Leiden) and were sorted by positive selection using anti-PE magnetic beads (MACS, Miltenyi Biotec BV, Leiden, the Netherlands). This procedure enabled us to select and separate the TM^+ cells. Purity of all cell sorts was checked by flow cytometry and resulted in$\geq$ 98% of purity.

A limiting diluition assay was assessed with the sorted TM^+ cells that were cultured in 96 well round bottom plates with descending cell density/well and with a feeder mixture providing irradiated feeders cells (3600 rads for 4 minutes, $5x10^4$c/w), P62 and P68 Mtb-specific peptides (1μg/ml final concentration), in the presence of IL-7, IL-15 (Peprotech, Rocky Hill, NJ, both 5 ng/mL final concentration) and IL-2 (Proleukin, Chiron, Amsterdam, the Netherlands 50 U/mL final concentration). Every other day cells were splitted and freshed, IL-2 (100U/ml) was added.

After two weeks of culture, growing clones were selected from the 0.3 c/well cultures and expanded as described above with alternating peptide pulsed irradiated feeders or T cell expander αCD3/CD28 beads (Gibco Life Technologies, Thermo Fisher Scientifics Inc.). In total 12 clones from two cell lines were isolated and stored in nitrogen.

Discussion

HLA-E-restricted $CD8^+$ T lymphocytes as a new player in the adaptive immune response to *Mycobacterium tuberculosis*

Over recent years it has become evident that many cellular subsets are involved in ensuring immune protection against pathogenic agents. Within adaptative immunity, the greatest group of $CD4^+$ helper and $CD8^+$ cytotoxic T cells are subdivided in several subsets each exerting different roles which includes pro-, anti- inflammatory and regulatory activities. These subsets can also differ significantly depending on tissue distribution. The homeostatic immune responses that work properly are implemented to maintain stability, expecially in case of chronic infections (241).

As already discussed in Chapter 2, non-classical MHC class I molecules such as HLA-E, can present peptides derived from several viruses and bacteria, including Mtb (178, 179) to $CD8^+$ T cells, similarly to classical MHC class I molecules (180, 190, 204–208).

Using a pool of Mtb peptides selected from a list previously characterized by Joosten et al. (179) we further screened these peptides for their capability to stabilize the HLA-E molecule on the surface of transfected cells, and to elicit a $CD8^+$ T cells response. Five out of eighteen Mtb peptides were selected and were used in subsequent studies. HLA-E molecules are usually recognized by NK cells through the two CD94-NKG2A (inhibitory) or CD94-NKG2C (activatory) NK receptors. In order to exclude the recognition of HLA-E-Mtb peptide complexes by NK cells, experiments were conducted using NK cell clones selectively expressing the CD94-NKG2A inhibitory or CD94-NKG2C activatory receptors and target cells expressing HLA-E/Mtb peptides. Results obtained showed that there was no recognition of HLA-E/Mtb peptide complexes by NK-cell clones indicating that, although HLA-E/peptide complexes usually interact with the CD94-NKG2 NK receptors, HLA-E Mtb/peptide complexes do not engage NK receptors. Conversely, the five HLA-E/Mtb peptides are specifically recognized by $CD8^+$ T cells in a CD3/TCR αβ mediated and CD8-dependent manner.

$CD8^+$ T cell lines derived from PBMC of patients with active TB secreted TNF-α, IL-4 and IL-13, either alone or in combination with IL-5, IL-10, and/or TGF-β, upon *in vitro* stimulation with specific Mtb peptides. The secretion of TGF-β suggests that a fraction of these cells might play regulatory activities, as shown by previous studies in TB (179). Very low or even no production of IFN-γ, IL-17, and IL-22 was detected.

These results have been confirmed using an innovative protocol which combines HLA-E tetramer staining and intracellular staining (ICS) in $CD8^+$ T cell lines obtained from PBMC of patients with active TB and TB-HIV co-infection, LTBI subjects and healthy donors, stimulated with two out of five Mtb peptides, p62 and p68. The ICS of tetramer-specific $CD8^+$ T cells also revealed and confirmed the presence of an unusual Th2-type pattern of cytokine production.

In a previous study Van Meijgaarden et al. found that HLA-E restricted $CD8^+$ T cell clones derived from PBMC of healthy PPD^+donors had a Th2 cytokine profile associated with the expression of the lineage-specifying transcription factor GATA3 (222). In contrast to these

data, in our experiments no expression of GATA 3 was detected. Furhter investigation is thus required to uncover mechanism responsible for this unexpected result.
However, an unexpected production of IFNγ by tetramer specific $CD8^+$ T cells was found. Again, at present we do not have any possible explanation for this finding and additional experiments are thus needed. Along with IFNγ production, also the lineage-specifying trascription factor T-bet associated with Th1 function had a greater expression in $tetramer^+$ $CD8^+$ T cells from patients with active TB, as compared to expression levels found in healthy controls. Surprisingly we also found a significant expression of the Treg specific transcription factor FoxP3 in HLA-E/Mtb peptide-reactive $CD8^+$ T cell lines. These results imply a possible regolatory role of these cells.

In addition to their unusual Th2 cytokine profile, HLA-E restricted $CD8^+$ T cells have very poor cytolytic capacity. In particular, we found that only 3 of the 15 tested HLA-E-restricted Mtb-specific $CD8^+$ T cell lines from patients with active TB were able to lyse target cells, at the same time demonstrating their impaired cytotoxic activity and their inability to inhibit Mtb outgrowth.
An additional analysis of cytotoxic molecules profile was assessed in the same group of samples using ICS combined with tetramer staining. The simultaneous expression of perforin, granulysin, granzyme A and granzyme B was analysed. A significant increate expression of granzyme A was evident in total $CD8^+$ T cells from patients with active TB compared to healthy donors. Conversely, HLA-E/Mtb-specific $CD8^+$ T cells from different groups of patients and controls show similar granzyme A expression. A cumulative analysis of all cytotoxic markers in both total $CD8^+$ and Mtb-specific $CD8^+$ T cells revealed a significant decrease of T cells co-expressing perforin, granzyme B and granulysin in patients with active TB, compared to healthy controls.

Finally, we studied *ex vivo* phenotypic analysis of tetramer-specific $CD8^+$ T cells in the four tested groups. We found a higher percentages of naïve cells in patients with active TB, and in LTBI subjects and healthy donors, while in TB-HIV co-infected patients the main population was represented by $tetramer^+$ $CD8^+$ T cells with a TEMRA phenotype.
Moreover, we have evaluated the phenotype of these tetramer-specific $CD8^+$ T cells upon stimulation with the peptides p62 and p68 for 7 days. The analysis of stimulated cells displayed an increase of cells with a TEMRA phenotype in patients with active TB and an increase of cells with effector memory and central memory phenotypes in the other tested groups.

In conclusion, HLA-E-restricted $CD8^+$ T cells specific for Mtb peptides represent a new effector T cells population in human TB, playing a role in immune response to active infection with Mtb. HLA-E/Mtb peptide-complexes are stable on the surface of transfected cells, induce proliferation of $CD8^+$ T cells, and are specifically recognized by $CD8^+$ T cells in a CD3/TCR αβ mediated and CD8-dependent manner. HLA-E-restricted $CD8^+$ T cells are important part of the homeostatic immune response against Mtb. They elicit a Th2 response and possess potential regulatory function, demostrating that not only the canonical Th1 responce is involved in the control of Mtb infection. Moreover, these cells have poor

cytolytic activity and microbicidal potential toward Mtbi nfected human cell lines expressing HLA-E molecule.

Finally, the analysis of a new cellular subset such as MAIT cells was also investigated in our system. The innate anti-microbial activities exerted by these cells were already demostrated in murine model as well as in human (242). The comparison of the frequency of MAIT cells in peripheral blood of patients with active TB alone or in association with HIV co-infection revealed that MAIT cells are depleted from the blood in subjects with active TB or TB-HIV co-infected. This last aspect could be explained by a mechanism of cell exhaustion of constantly activated cells or their specific recruitment at the sites of infection.

Acknowledgements

The Ph.D study and related research included in this thesis could not have been performed without the experience, patience, support and immense knowledge of many individuals.

I would like to extend my gratitude first and foremost to my dean Prof. Francesco Dieli and my thesis advisors Prof. Nadia Caccamo for their confidence in me. They guided me every step of the way, following me in the realization of this dissertation.

Besides my advisor, I would like to thank all my wonderful group of the CLADIBIOR, Central Laboratory of Advanced Diagnosis and Biomedical Research. In particular, I'm sincerely thank to Dr. Marco La Manna and Dr. Diana Di Liberto,who introduced me to my Ph.D project and followed me throughout the period of Ph.D course. They helped me in all the time and for that I sincerely thank to them.

My sincere thanks goes to Prof. Tom H. M. Ottenhoff, who gaved me the unique opportunity to join his team as intern for one year internship, and to Dr. Simone Joosten, and Dr. Krista Van Meijgaarden. I could not have imagined having better mentors for my Ph.D studies. I would like to express my sincere thanks to entire group of the Department of Infectious Disease of the Leiden University Medical Center who have taken me in and made me feel at home. Thanks for having made the last year one of the best years of my life.

Moreover, I express my deep gratitude to Prof. Masaru Taniguchi for the great experience I lived inthe Laboratory of Immune Regulation at RIKEN Center for Integrative Medical Sciences (Yokohama), both personally and professionally. I'll never forget this experience.

Last but not least, I would thanks my family and my closest friends for their support and for always believing in me.

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Introduction

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