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Detailed characterization of human *Mycobacterium tuberculosis* specific HLA-E restricted CD8⁺ T cells

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Abstract

HLA-E presented antigens are interesting targets for vaccination given HLA-Es' essentially monomorphic nature. We have shown previously that *Mycobacterium tuberculosis* (Mtb) peptides are presented by HLA-E to CD8⁺ effector T cells, but the precise phenotype and functional capacity of these cells remains poorly characterized. We have developed and utilized in this study a new protocol combining HLA-E tetramer with intracellular staining for cytokines, transcription factors and cytotoxic molecules to characterize these cells in depth. We confirm in this study the significantly increased ex vivo frequency of Mtb-peptide/HLA-E-TM⁺ CD8⁺ T cells in the circulation of patients with active tuberculosis (TB). HLA-E restricted CD8⁺ T cells from TB patients produced more IL-13 than cells from controls or subjects with latent tuberculosis infection (LTBI). Compared to total CD8⁺ T cells, HLA-E restricted cells produced more IFN γ , IL-4, IL-10, and granzyme-A. Moreover, compared to "classical" Mtb specific HLA-A2 restricted CD8⁺ T cells, HLA-E restricted CD8⁺ T cells produced less TNF α and perforin, but more IL-4. In conclusion, HLA-E restricted- Mtb specific cells can produce Th2 cytokines directly.

Keywords

Active TB; CD8⁺ T cells; Cytokines; Tetramers; Th2

Introduction

An estimated one fourth of the global population is infected with *Mycobacterium tuberculosis* (Mtb) [1]. According to WHO Global Tuberculosis control report 2016, there

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Conflict of interest:

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were 10.4 million new tuberculosis (TB) cases and 1.8 million TB deaths in 2015, including deaths resulting from TB disease among HIV-positive people [2].

TB is a potentially lethal disease, but curable if properly treated[2]. *M. bovis* BCG-vaccination protects infants from severe and disseminating TB, particularly TB-meningitis and military TB [3] but has poor efficacy against pulmonary TB in adults. A novel, effective TB vaccine is urgently required and its design depends on a detailed understanding of what exactly controls host immune protection.

Over recent years it has become evident that many cell subsets are involved in immune protection. Antigen-specific CD8⁺ effector T cells are detectable in most Mtb infected subjects and animals [4]. Depletion of CD8⁺ T cells in mice reduced survival after infection with Mtb, demonstrating their essential role in protection against TB [5]. Similarly, also MHC class I knockout mice showed an enhanced sensitivity to Mtb infection [6]. In humans, both “classical” class Ia (HLA-A,-B and -C) and “non-classical” class Ib (HLA-E,-F and -G) HLA molecules are known to activate CD8⁺ T cells by presenting cognate peptide to the TCR. MHC class Ia molecules can present endogenous, cytoplasmic antigens as well as exogenous antigens which are “cross-presented” by dendritic cells [7], including antigens derived from intracellular bacteria or viruses [8–11]. Mtb-specific CD8⁺ T cells in the blood of Mtb infected individuals differ in frequency, phenotype and functional activities in patients with active TB compared to subjects with latent tuberculosis infection (LTBI) [12, 13].

HLA-E is a highly conserved MHC class Ib molecule with rather unique properties. Primarily HLA-E is involved in prevention of lysis by NK cells through ligation with NKG2/CD94 complex. Moreover, HLA-E can also present antigens to CD8⁺ T cells and thus plays a role in both innate and adaptive immunity [11]. Its low allelic variability positions it as an interesting candidate antigen presenting molecule for peptide based vaccination [11,14]. HLA-E comprises four alleles (E*01:01; E*01:03; E*01:04; E*01:05), but only the first two are expressed as functional proteins. HLA-E*01:01 and *01:03 differ in a single amino acid outside the peptide binding groove, therefore they are anticipated to have a very similar peptide binding profile [15–17]. HLA-E is enriched in Mtb phagosomes compared to class Ia molecules and accessible for loading with Mtb peptides [18]. In addition to HLA-E’s low allelic variation, another advantage is that HLA-E is not down-regulated by the HIV-nef protein, as opposed to HLA class Ia molecules [19, 20]. In fact, certain components of HIV may even stabilize HLA-E cell surface expression to prevent NK mediated lysis of HIV infected cells [21].

In a first study we showed that Mtb peptides were presented by HLA-E to CD8⁺ T cells from LTBI as well as BCG-vaccinated donors. These T cells had both cytolytic and immunoregulatory activities [14]. In subsequent work we demonstrated that HLA-E-restricted clonal CD8⁺ T cells were able to inhibit Mtb outgrowth in infected human macrophages [22]. Qa-1, the mouse homolog of HLA-E, can also present Mtb derived antigens during infection [23]. Mice that lack Qa-1, are more susceptible to Mtb infection and this increased susceptibility to progress to disease was due to dysregulated T-cell responses [23].

HLA-E-restricted CD8⁺ T cells circulated in higher frequencies in the blood of active TB patients [14, 22, 24] compared to LTBI [15, 22, 24] and were associated with a Th2 cytokine profile, notably IL-13. However, although we detected production of Th2 cytokines in response to specific Mtb peptide stimulation, and Mtb peptide driven type-2 cytokine production was most abundant in donors with strong tetramer staining [22, 24], we were unable to demonstrate that HLA-E restricted CD8⁺ T cells were the source of these cytokines. In the current study combine Mtb-peptide/HLA-E tetramer staining with intracellular cytokine staining in active TB and LTBI subjects, to demonstrate that HLA-E-restricted CD8⁺ T cells actively produce type-2 cytokines. These results provide important new evidence on the role of HLA-E restricted CD8⁺ T cells in human infectious disease.

Results

Ex vivo analysis

Direct ex vivo binding of HLA-E tetramers (TM) loaded with p62 and p68 Mtb- peptides to CD8⁺ T cells was analyzed in peripheral blood mononuclear cells (PBMCs) of 26 tuberculin (PPD)-negative healthy subjects (HDs), 14 LTBI subjects, 24 patients with active TB and 5 HIV-TB co-infected patients (for detailed characteristics see Table 1).

The gating strategy used to select TM⁺ CD8⁺ T cells is shown in Fig. 1A and comprised of sequential gating on single cells, live cells, lymphocytes, CD3⁺ cells and CD8⁺/CD4⁻ cells. The CD94 blocking mAb was always included in the staining procedure to prevent HLA-E tetramer binding to the NKG2A/CD94 complex. Furthermore, an HLA-E CMV TM was included to exclude cells with aspecific binding. By definition T cells have single specificities and thus cells binding different TMs cannot do so through cognate TCR interactions and thus all double positive cells were excluded and only single Mtb-HLA-E TM⁺ cells were analyzed. Examples of TM staining for HD, LTBI, TB, and TB-HIV are shown and illustrate the variation in HLA-E TM binding (Fig. 1B).

Confirming our own previous work, the ex vivo frequency of HLA-E-/Mtb-peptide TM⁺ CD8⁺ T cells was higher in patients with active TB than in HDs ($p < 0.0001$) (Fig. 1C). As expected, the highest frequency of TM⁺ CD8⁺ T cells was found in the 5 HIV-TB patients ($p = 0.0004$ versus HD). Donors with LTBI showed a wide array of TM staining, but only a small proportion had a high frequency of HLA-E/Mtb TM⁺ cells. The median values of TM⁺ CD8⁺ T-cell frequencies were 0.22, 0.23, 0.89, and 1.9% in HDs, LTBI subjects, active TB and HIV-TB co-infected patients, respectively (Fig. 1C).

Thus, Mtb-peptide TM binding was highest to CD8⁺ T cells of patients with active TB and even increased with concomitant HIV infection.

Memory phenotypes of HLA-E-/Mtb-peptide TM⁺ CD8⁺ T cells

Next, the memory phenotype of HLA-E-/Mtb-peptide TM⁺ CD8⁺ T cells was analyzed using CCR7 and CD45RA. To assess discrete populations rather than single cells, only samples with at least 100 TM⁺ events were included for memory subset analysis. Application of this cut-off permitted analysis of 16 out 26 HDs, 11 out 14 LTBI, 20 out 24 TB and 5 out 5 HIV-TB patients. The majority of circulating HLA-E-/Mtb-peptide TM⁺ CD8⁺ T cells had an

effector-memory profile, both T_{EMRA} and T_{EM} ($p = 0.049$) phenotypes were prominent in TB patients compared to HDs. In contrast, HLA-E/Mtb-peptide $TM^+ CD8^+$ T cells with a T_{EMRA} phenotype were the most abundant in TB-HIV co-infected patients compared to other groups (Fig. 1D).

To characterize HLA-E restricted cells functionally in more depth, $CD8^+$ T cells were expanded and stained with specific TMs combined with intracellular markers. Only few samples could not be included due to low cell yields or poor expansion; 26 out of 26 HDs, 12 out of 14 LTBI and 17 out of 24 active TB were included. Of note but not unexpectedly, cells from the TB-HIV group were relatively impaired in their growth in culture, therefore only TM frequencies are shown but not the functional characteristics of these cells.

The median HLA-E $TM^+ CD8^+$ T-cell frequencies were 0.13, 0.14, 0.34, and 0.23% in the blood of HDs, LTBI subjects, TB and HIV-TB co-infected patients, respectively. Thus, following Mtb peptide expansion the frequencies of HLA-E- TM^+ Mtb-specific $CD8^+$ T cells remained higher in active TB compared to LTBI and HDs. However it considerably decreased in TB-HIV co-infected patients, probably due to the poor expansion and survival of the cells in culture (Fig. 2A and B). In general, expansion resulted in a significant amplification of the number of TM^+ cells to allow detailed characterization of their functional properties (Fig. 2C). The average TM^+ cell counts increased from 750 directly ex vivo (for 24 active TB patients) to 4418 after expansion (for 17 TB patients), leading to an average expansion of 4.89-fold.

Phenotype after expansion of HLA-E-/Mtb-peptide $TM^+ CD8^+$ T cells

Previous characterization of T-cell receptors (TCR) recognizing the CMV UL40 epitope in HLA-E had revealed a strong preferential usage of the TCR variable β -region 16 ($V\beta 16$) and it was suggested that expression of the $V\beta 16$ TCR fragment was a hallmark of HLA-E restricted T cells [25]. Therefore we assessed the expression of $V\beta 16$ on HLA-E- Mtb-specific $CD8^+$ T cells. Although $V\beta 16$ staining was observed in the total $CD8^+$ T-cell population, we did not observe any preferential $V\beta 16$ usage by $TM^+ CD8^+$ T cells in any of the study groups (Fig. 2D), showing that HLA-E/Mtb-specific $CD8^+$ T cells have a clearly different TCR- $V\beta$ repertoire compared to the previously published HLA-E/CMV-specific $CD8^+$ T cells.

We next evaluated the memory phenotypes of the expanded HLA-E $TM^+ CD8^+$ T cells of 14 out of 26 HDs (10 Dutch and 4 Italian), 6 out of 13 LTBI, 15 out of 17 TB, that had more than >1000 TM^+ events. Memory profile analysis of $TM^+ CD8^+$ T cells upon stimulation was not significantly different between T_{EMRA} , naïve (T_N), effector memory (T_{EM}) and central memory (T_{CM}) compartments. HLA-E $TM^+ CD8^+$ T cells with an effector phenotype were the main population in all groups, consistent with the in vitro expansion (Fig. 2E). When compared to the ex vivo memory distribution we observed consistently more T_{EM} and less T_N , which fits with the expansion observed in the cultures. T_{EMRA} and T_{CM} frequencies were not different from ex vivo after the in vitro expansion.

Cytokine profile of HLA-E/Mtb- specific CD8⁺ T cells

We evaluated expression of cytokine as well as cytotoxic molecules by intracellular staining of HLA-E/Mtb TM⁺ CD8⁺ T cells (Fig. 3A). Peptide stimulated cells are shown in comparison with unstimulated, but expanded cells. In general, TM⁺ cells produced both Th2 and Th1 cytokines as well as IL-10, and TM⁺ cells from the different clinical groups appeared relatively similar. TM⁺ CD8⁺ T cells from active TB patients produced more TNF- α and IL-13 compared to healthy controls (Fig. 3B). Approximately 30% of TM specific CD8⁺ T cells produced IFN γ in HDs, LTBI, active TB groups. Also transcription factors for the different lineages were expressed by HLA-E restricted CD8⁺ T cells. GATA3 was expressed more frequently in donors with LTBI ($p = 0.042$; Fig. 3C). Cytolytic molecules were expressed in most TM⁺ cells, however the range of expression between individuals was quite large and different combinations of cytolytic molecules were expressed between different individuals (Fig. 3D).

To investigate the relative specific properties of HLA-E-Mtb TM⁺ CD8⁺ T cells we compared them to the total pool of CD8⁺ T cells in terms of functional profiles and across all groups (Fig. 4); TB patients only are shown in Supporting Information Fig. 1 to show the relative abundance of specific cytokines in this group. HLA-E TM⁺ cells expressed significantly more IFN- γ compared to total CD8⁺ T cells in all samples ($p < 0.0001$; TB patients only $p = 0.0127$). Also IL-4 was detected at a higher frequency in the HLA-E TM⁺ populations compared to the total CD8⁺ T cells ($p < 0.0101$), as was IL-10 ($p < 0.0001$; TB patients only $p = 0.0001$) (Fig. 4A). TNF- α and IL-13 were not significantly differently expressed in the expanded TM⁺ cells compared to the total CD8⁺ T-cell population.

In agreement with these data, the frequency of TM⁺ CD8⁺ T cells expressing T-bet, Gata3 and FoxP3 was higher compared to the total CD8⁺ T cells (T-bet $p < 0.0001$, TB samples only $p = 0.0003$; Gata3 $p = 0.0009$, TB samples only $p = 0.0012$; FoxP3 $p < 0.0001$, TB samples only $p = 0.0012$) (Fig. 4B).

CD8⁺ T cells producing Granulysin (Gnly) were significantly increased in the TM⁺ population compared to total CD8⁺ T cells, while a decrease occurred in Granzyme A (GzmA) in TM⁺ CD8⁺ T cells (Gnly $p < 0.0001$, TB samples only $p = 0.049$; GzmA $p = 0.0091$, TB samples only $p = 0.0002$) (Fig. 4C). Granzyme B (GzmB) and perforin (PRF) levels were not different between total and TM⁺ CD8⁺ T cells.

Comparison of HLA-E restricted CD8⁺ T cells to HLA-A2 restricted Mtb specific CD8⁺ T cells

Finally, we compared the functional phenotypes of “non-classical” HLA-E restricted Mtb-specific CD8⁺ T cells to that of “classical” HLA-A2 restricted, Mtb-specific CD8⁺ T cells in the same samples (Fig. 5). All donors were typed for HLA-A*0201 using specific antibody staining on PBMCs: 3 out 26 HDs, 3 out 13 LTBI and 7 out 17 TB patients were HLA-A*0201⁺. For Panel A (including cytokines) 7 out 17, and for Panel B (including cytotoxic molecules and IL-10) 6 out 17 active TB patients were analyzed after re-stimulation with HLA-E presented Mtb p62 and p68 peptides or with HLA-A2 presented Mtb-Ag85B peptides p74 and p79 (characteristics of Mtb-peptides: Table 2). The frequency of HLA-E-

restricted CD8⁺ T cells producing TNF- α was lower than that of HLA-A2-restricted CD8⁺ T cells (all samples, $p = 0.0342$) (Fig. 5A). Th2 cytokines were more abundantly expressed in HLA-E restricted T cells (IL-4, all samples $p = 0.0327$) (Fig. 5A), especially in TB patients (Supporting Information Fig. 2), but this did not reach statistical significance due to the low number of HLA-A2⁺ TB patients that could be analyzed. In contrast, Tbet and FoxP3 expression was higher in HLA-E-restricted CD8⁺ T cells compared to HLA-A2-restricted CD8⁺ T cells (T-bet, all samples, $p = 0.0017$; FoxP3, all samples, $p = 0.0012$; FoxP3, TB patients only, $p = 0.0156$) (Fig. 5B). CD8⁺ T cells expressing Perforin (Prf) were higher and lower, respectively, in frequency among HLA-E restricted than HLA-A2- restricted Mtb specific CD8⁺ T cells (Prf $p = 0.0161$) (Fig. 5C). Together these data demonstrate that HLA-E restricted Mtb specific cells display unique functional properties compared to either total CD8⁺ T cells or HLA-A2 restricted Mtb specific T cells.

Discussion

This is the first comprehensive analysis of human HLA-E restricted CD8⁺ T cells, permitting detailed characterization of functional capacities of these antigen specific cells. As we have previously shown [22, 24], PBMCs from TB patients produced significant levels of IL-4 and IL-13 following stimulation with HLA-E presented Mtb-peptides. In addition, HLA-E restricted Mtb-specific CD8⁺ T-cell clones displayed a unique Th2-like profile (secreting IL-4, IL-5, IL-13) upon peptide stimulation [22]. Although these studies strongly suggested an association between HLA-E restricted T cells and the production of Th2 cytokines, they did not formally prove that circulating HLA-E restricted Mtb specific T cells from TB patients produced Th2 cytokines. In this study, we developed a protocol that combined tetramer with intracellular (cytokine) staining and that allowed us to describe for the first time the characteristics of these unconventional, HLA-E restricted Mtb-specific CD8⁺ T cells in cohorts of active TB patients and LTBI subjects.

Firstly, the direct *ex-vivo* frequency of HLA-E restricted Mtb-TM⁺ CD8⁺ T cells was higher in patients with active TB compared to HDs. The majority of these cells had an effector-memory profile, suggesting that these cells were activated in the circulation of active TB patients, in contrast to healthy donors in which these cells mostly had a naïve phenotype. Expansion of cells allowed assessment of intracellular cytokines within the TM⁺ population. Most cytokines were expressed at similar levels by HLA-E restricted T cells among the different clinical groups. TNF α and IL-13 were abundantly expressed by TM⁺ CD8⁺ T cells from active TB patients compared to healthy controls. While the lineage-specifying transcription factor Tbet associated with Th1 function was expressed by the majority of HLA-E- TM⁺ CD8⁺ T cells from each group, a higher frequency of Gata3 expression was observed in TM⁺ CD8⁺ T cells from LTBI subjects. Gata3 expression was also detected previously in healthy PPD⁺ donors' HLA-E restricted Mtb specific T-cell clones [22]. FoxP3 was detected in TM⁺ CD8⁺ T cells from all groups. CD4⁺ and CD8⁺ Tregs were present at the site of infection [26–28], and were increased in the circulation of patients with active TB compared to healthy controls [26–28]. Tregs were found to suppress T-cell IFN- γ production during active TB, which normalized after treatment [29]. In our previous work, *in vitro* stimulation with HLA-E Mtb-peptides p62 and p68 resulted in the induction of functionally active regulatory T cells as well as cytolytic effector cells [14]. This dualism of

effector versus immunoregulatory activities of HLA-E restricted T cells was also found for the T-cell clones [14, 22]. Not only the cytolytic effector functions but also the immunoregulatory properties of HLA-E restricted cells may be important in relation to protective immunity, by controlling pathogenic inflammation [23]. A negative consequence may be that immunoregulation contributes to pathogen persistence and chronic infection [11, 30].

A specific TCR fragment, V β 16, has been associated with the recognition of the CMV UL40 epitope in the context of HLA-E [25]. However, in contrast to previous suggestions, V β 16 was not universally used in TCR mediated recognition of pathogen derived peptides presented by HLA-E, since none of our donors preferentially utilized the V β 16 TCR fragment in their Mtb specific HLA-E restricted response. HLA-E-restricted, Mtb-specific CD8⁺ T cells were compared for expression of secreted molecules and transcription factors relative to the total CD8⁺ T-cell population within the same donor. HLA-E TM⁺ cells produced significantly more IFN γ , IL-4 and IL-10 compared to total CD8⁺ T cells. Increased expression of IFN γ and IL-10 in active TB patients may reflect recent activation. The frequency of TM⁺ CD8⁺ T cells expressing T-bet, Gata3 and FoxP3 was higher than in total CD8⁺ T cells. CD8⁺ T cells producing Granulysin were significantly higher in the HLA-E restricted population compared to total CD8⁺ T cells, contrasting with the decreased frequency of Granzyme A producing TM⁺ CD8⁺ T cells. Moreover, Mtb specific CD8⁺ T cells with classical (HLA-A2) and non-classical (HLA-E) restriction were compared. This revealed reduced TNF- α and perforin expression in HLA-E restricted T cells, but increased expression of T-bet, FoxP3 and IL-4.

During the first period of Mtb infection, key cytokines associated with protection are IFN- γ and TNF- α [31, 32]. After clearance or successful containment of Mtb, activated immune responses must be ceased to avoid inflammatory damage. This may be mediated by anti-inflammatory cytokines such as IL-10, IL-13, or by a Th2 environment. This Th1/Th2 'switch' may be antigen-specific, depending on HLA restriction or the stage, early or late in infection, in which the antigens are presented [33]. The ex vivo frequency of HLA-E-restricted CD8⁺ T cells is higher in active TB but significantly decreased after 6 months of chemotherapy whereas the frequencies of HLA-A2-restricted CD8⁺ T cells increased after therapy [24]. This opposite trend of HLA-E and HLA-A2 restricted responses may be related to the different roles exerted by the two HLA molecules and by the different set of antigens presented during Mtb disease stages. This indicates that although both HLA-A2 restricted as well as HLA-E restricted CD8⁺ T cells are Mtb-specific, their restriction correlated with different functional properties. Future studies need to resolve the relative significance of effector versus immunoregulatory activities within the HLA-E based antigen presentation system in infection and other human diseases.

Materials and methods

Recruited patients

Peripheral blood was collected from a total of 69 adults; 13 healthy donors, 14 with LTBI (age range 36–60), 24 with active TB disease (age range 22–38), and 5 with TB-HIV co-infection (age range 28–40) from the Dipartimento di Medicina Clinica e delle Patologie

Emergenti, University Hospital, Palermo, Italy (for patients' characteristics see Table 1). The study was approved by the Ethical Committee of the University Hospital, Palermo, where patients were recruited. All patients signed an informed consent and the study was performed according to the guidelines of the local ethics committee and in accordance with the principles of the Helsinki Declaration and those of "Good Clinical Practices". All TB patients had clinical and radiological findings consistent with active pulmonary TB, the diagnosis was confirmed by bacteriological isolation of Mtb. None of the active TB patients had been vaccinated with BCG, or were treated with steroid or other immunosuppressive or anti-tubercular drugs at the time of sampling.

LTBI subjects were health-care workers with a positive tuber-culin (PPD) skin test and no symptoms and signs of active TB [34]. Skin tests were considered positive when the induration was larger than 10 mm at 72 h after injection of 5 U of PPD (Statens Serum Institut, Copenhagen, Denmark).

Besides the 13 healthy donors mentioned above, an additional 13 PPD negative anonymous bloodbank donors (Sanquin Bloodbank, the Netherlands) were recruited; These donors were tested for PPD responses in a 6 day lymphocyte-stimulation-assay [35, 36] with IFN- γ measurement by ELISA (U-CyTech, Utrecht, the Netherlands) as readout. Donors that produced less than 100 pg/mL IFN- γ in response to PPD were considered negative.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by Ficoll-Hypaque (Sigma) density centrifugation, frozen and stored in liquid nitrogen. Frozen samples were sent to the Department of Infectious Diseases at Leiden University Medical Center (LUMC) where the experiments were performed.

Preparation of MHC class I tetramers

Extracellular HLA class I, with a C-terminal BirA recognition site, and β 2-microglobulin were expressed in *Escherichia coli* as insoluble aggregates in the form of inclusion bodies. Purified inclusion bodies were solubilized in urea and folded with peptide by dilution. Monomers were biotinylated using the BirA enzyme and purified by gelfiltration on a Hiload 16/60 Superdex 75 prep grade.

HLA class I tetramers were generated by adding SA-phycoerythrin (PE) or SA-allophycocyanin (APC) at an SA:MHC class I molar ratio of 1[2236]6 [37]. To ensure maximum saturation of the SA with peptide-MHC monomers (i.e. that tetramers are formed), SA was sequentially added in four steps at 20-min intervals. The resulting tetramers were stored in PBS, with a final concentration of 16% glycerol, 0,6% BSA, 0,02% azide and Complete protease inhibitors (Roche) at -80°C . Tetramers were generated for HLA-E*01:03 and HLA-A2*02:01.

The following peptides were used for in vitro stimulation of T cells and/or for TM generation: the HLA-E restricted Mtb peptide p62 (RMPPLGHEL), p68 (VLRPGGHFL), and CMV UL40 (VMAPRTLLL); the HLA-A2 restricted Ag85 Mtb peptides (GLPVEYLQV), (KLVANNTRL), and HIV Gag (SLYNTVATL) [14, 25, 38]. All peptides were synthesized by Peptide2.0 Inc (Chantilly, VA, USA).

Ex-vivo tetramer staining

PBMCs were thawed, counted (CASY cell counter, Roche, Woerden, The Netherlands) and rested for one hour in an incubator at 37°C with 5% CO₂. Approximately 10⁶ cells per sample were blocked for 10 min at RT with 10 µg/mL purified CD94 mAb (Clone HP-3D9, BD Bioscience) to block NKG2A/CD94 binding of HLA-E TMs, and then stained with HLA-E TMs for 15 minutes at 37°C. Both p62 and p68 HLA-E- TMs were PE-conjugated and merged in the same colour channel. CMV-specific-HLA-E- TM (APC-conjugated) was used as control. The CMV-TM was added in the same staining step as the Mtb-specific TMs. Table 2 summarizes the peptides and TMs used.

After incubation, samples were washed with PBS/0.1% BSA (Pharmacy LUMC, the Netherlands, Sigma-Aldrich, Zwijndrecht, the Netherlands), and stained with live/dead marker (Vivid fixable violet dye, Invitrogen, Thermo Fisher Scientific Inc., Bleiswijk, the Netherlands) according to the manufacturer's protocol. Followed by Fluorochrome-conjugated antibodies: CD3 (Horizon-V500, clone UCHT1), CD8 (AlexaFluor700, clone RPA-T8), CD4 (PerCp-Cy5.5, clone RPAT4), CCR7 (PE-Cy7, clone 3D12) (all BDBio-sciences), and CD45RA (BV570, clone MI100), CD14 (Qdot655, clone Tük4) (both Invitrogen). Samples were stained for 30 min at 4°C, washed twice in PBS/0.1% BSA and then acquired on a LSRFortessa flow cytometer with Diva software (v6.2, BDBio-sciences). A minimum of 1 × 10⁶ PBMC were acquired and analysis was performed on at least 100 000 CD8⁺ events for each sample.

Data were analyzed with FlowJo software (version 9.9.6 Treestar Inc., Ashland, OR, USA). The analysis of each specific subset was performed using the gating strategy shown in Fig. 1A in compliance with the MIATA guidelines [39].

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

PBMCs were thawed, counted and 2 × 10⁶ PBMCs were expanded in 24-well plates using 5 µg/mL PHA (Remel, Thermo Fisher Scientific Inc.), in Iscove's modified Dulbecco's medium (IMDM, Life Technologies-Invitrogen) supplemented with 10% human serum (Heat inactivated pooled serum from bloodbank donors) and incubated for 3 days at 37°C, 5% CO₂. Medium was refreshed with 25 CU/mL IL-2 (Proleukin, Novartis Pharma, Amsterdam, the Netherlands). After 6 days, CD8⁺ T cells were sorted by positive bead selection (MACS, Miltenyi Biotec BV, Leiden, the Netherlands). Purity of all cells sorts was >98% as checked by flow cytometry (FACS Accuri Cytometer, BD Bioscience). Purified CD8⁺ T cells were subsequently stimulated with 10 µg/ml Mtb p62 (RMPPLGHFL) and p68 (VLRPGGHFL) peptides, recombinant human IL-15 and IL-7 (both 5 ng/mL final concentration Peprotech, Rocky Hill, NJ, USA), as well as irradiated (30 Gy) allogeneic feeder cells. At day 3, the culture was refreshed with complete IMDM medium with IL-2 (50 CU/ml). After 5 days, cultures were splitted and refreshed with complete IMDM with IL-2 (100 CU/mL). At day 7, cells were harvested and analyzed for tetramers, cytokines and surface markers by flowcytometry. Starting with a number of 3 – 4 × 10⁶ PBMCs, the yield of CD8⁺ T cells after this procedure was about 80–100 × 10⁶.

Intracellular staining (ICS) for flow cytometry

HLA-A2 TM analysis was only performed on donors that were HLA-A*0201, therefore 10^5 cells of all samples were typed with an anti-HLA-A2 antibody (FITC, clone BB7.2, BDBiosciences).

Expanded CD8⁺ T cells were stimulated for 6 h with 10 µg/mL Mtb- peptides and analyzed after 16 h incubation with BrefeldinA (3 µg/mL, Sigma-Aldrich) and monensin (1:1000, BioLegend) at 37°C in 5% CO₂. Cells were harvested, washed with PBS/0.1% BSA, and blocked for 10 min at RT with a purified CD94 mAb and then stained with tetramers and CCR7 (PE-Cy7, clone 3D12, BDBiosciences) for 15 min at 37°C. After incubation, cells were washed once in PBS/0.1% BSA and subsequently stained for surface markers (30 min, 4°C) CD3 (BV570, clone UCHT1, BioLegend), CD8 (Alexa700, clone RPA-T8), CD45RA (Qdot655, clone MEM-56, Invitrogen) followed by a fix/perm procedure according to manufacturer's instruction (BDBiosciences). Two different panels for intracellular markers were used. Panel A included: T-bet (BV605, clone 4BIO), IL-13 (PerCP-Cy5.5, clone JES10-5A2) (both BioLegend); IFN-γ (AlexaFluor700, clone B27), TNF-α (APC-H7, clone MAb11), IL4 (PE-C594, clone MP4-25D2), GATA3 (AlexaFluor488, clone L50-823) (all BDBiosciences); FoxP3 (PE-Cy5, clone PCH101) (Ebioscience). Panel B: rabbit-anti-human Granulysin (kind gift of Dr. A. Krensky, Stanford, CA) followed by Goat-anti-Rabbit-FITC, Perforin (PEC594, clone δG9), Granzyme B (AlexaFluor700, clone GB11), Granzyme A (PerCP-Cy5.5, clone CB9) (BioLegend), IL10 (BV605, clone JES3-9D7) (BDBio-sciences). Cells were acquired on a LSRFortessa flow cytometer with Diva software (v6.2, BDBiosciences). A minimum of 3×10^6 events were acquired for all samples except for the cultures of the HIV-TB samples, there only 1×10^6 events could be acquired. Analysis of data was performed with FlowJo software (version9.9.6 Treestar Inc., Ashland, OR, USA) on a minimum of 2×10^6 CD8⁺ cells acquired for HD, LTBI and TB samples whereas only 5×10^5 CD8⁺ cells were analyzed in HIV-TB subjects.

Statistical analysis

Most data shown are individual patient data, lines indicate the median of the population. Data were analyzed using Kruskal–Wallis including multiple test correction, Wilcoxon-Signed Rank testing and 2-way ANOVA with Dunnet's multiple comparisons. *P*-values < 0.05 were considered significant.

Supplementary Material

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Abbreviations

LTBI	latent tuberculosis infection
PPD	purified protein derivative
Mtb	<i>Mycobacterium tuberculosis</i>
TB	tuberculosis
CMV	Cytomegalovirus
HIV	Human Immunodeficiency Virus
Th1	T helper 1 cytokines
Th2	T helper 2 cytokines
TM	tetramers

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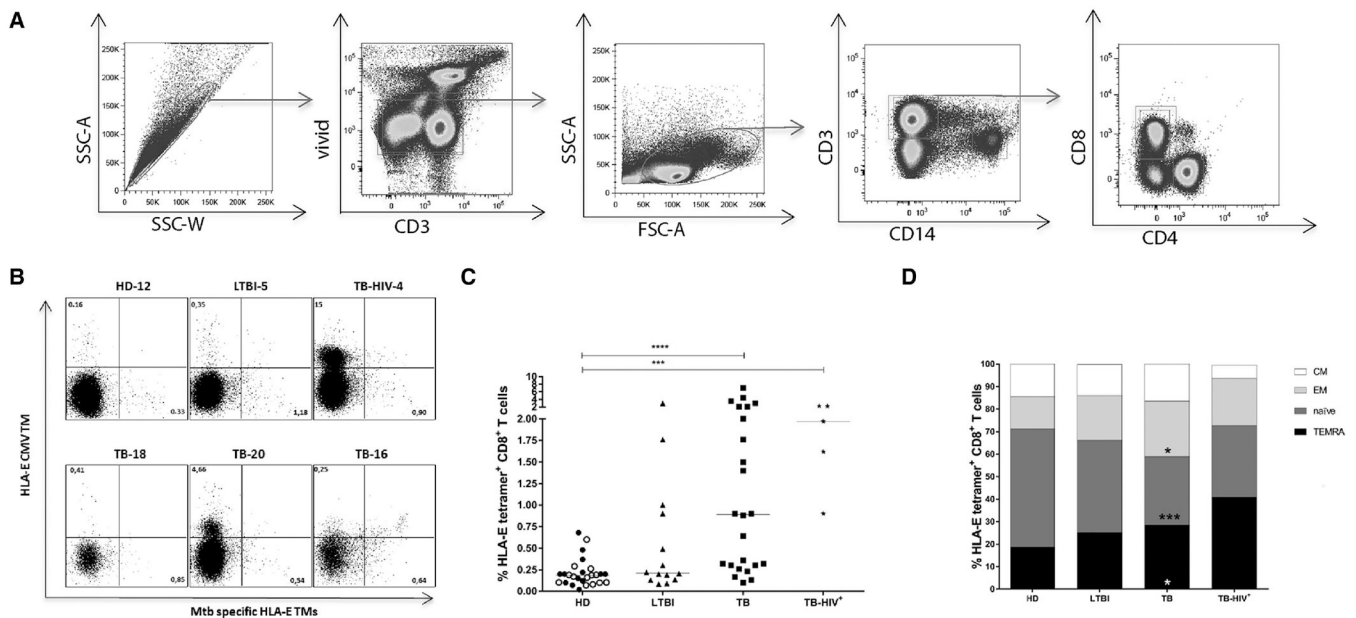


Figure 1.

Ex vivo frequency of HLA-E restricted CD8⁺ T cells in HDs, LTBI, TB, and TB-HIV subjects. PBMCs were stained using TMs followed by viability staining and cell surface marker staining. Each experiment contained samples from different clinical groups, a total of 21 experiments were performed to analyze all samples. (A) Gating strategy: Initial gate was on single cells, followed by selection of live cells using VIVID cell viability dye and gating on lymphocytes on the basis of forward-side scatters. CD3⁺ cells were gated as T cells and further selected for CD8, but not CD4 expression. (B) Representative dot plot of ex vivo TM staining, with a control (CMV) TM on the y-axis and the combined Mtb TMs on the x-axis. Dot plots show results of one HD, one LTBI, one TB-HIV coinfecting patient and 3 TB patients. (C) Frequency and (D) memory profile analysis of ex vivo HLA-E restricted CD8⁺ T cells in HDs, LTBI, TB, and TB-HIV patients. Memory populations were defined based on the expression of CCR7 and CD45RA. N = naïve T cells; EM = effector memory T cells; CM = central memory T cells; TEMRA = T effector memory recently activated cells. For ex vivo analysis, event counts below <100 were not considered a TM⁺ population and therefore not included in the downstream analysis of the memory compartment. 16 out of 26 HDs (11 Dutch and 5 Italian HDs), 11 out of 14 LTBI, 20 out of 24 TB and 5 out of 5 HIV-TB subjects were included in the analysis of memory subsets. Horizontal lines (C) represent median values. Black circles: Dutch HDs, open circles: Italian HDs, black triangles: LTBI; black squares: TB patients, black stars: HIV-TB. Bars represent mean values. Data shown in A-B is representative of 21 independent experiments with in total 71 samples and data shown in C and D are pooled from 21 independent experiments with —three to four donors per experiment. *P*-values were calculated using the Kruskal-Wallis test, including multiple test correction, ****p*<0.001, *****p*<0.0001. TB, LTBI and TB-HIV groups were each compared to the HD control group.

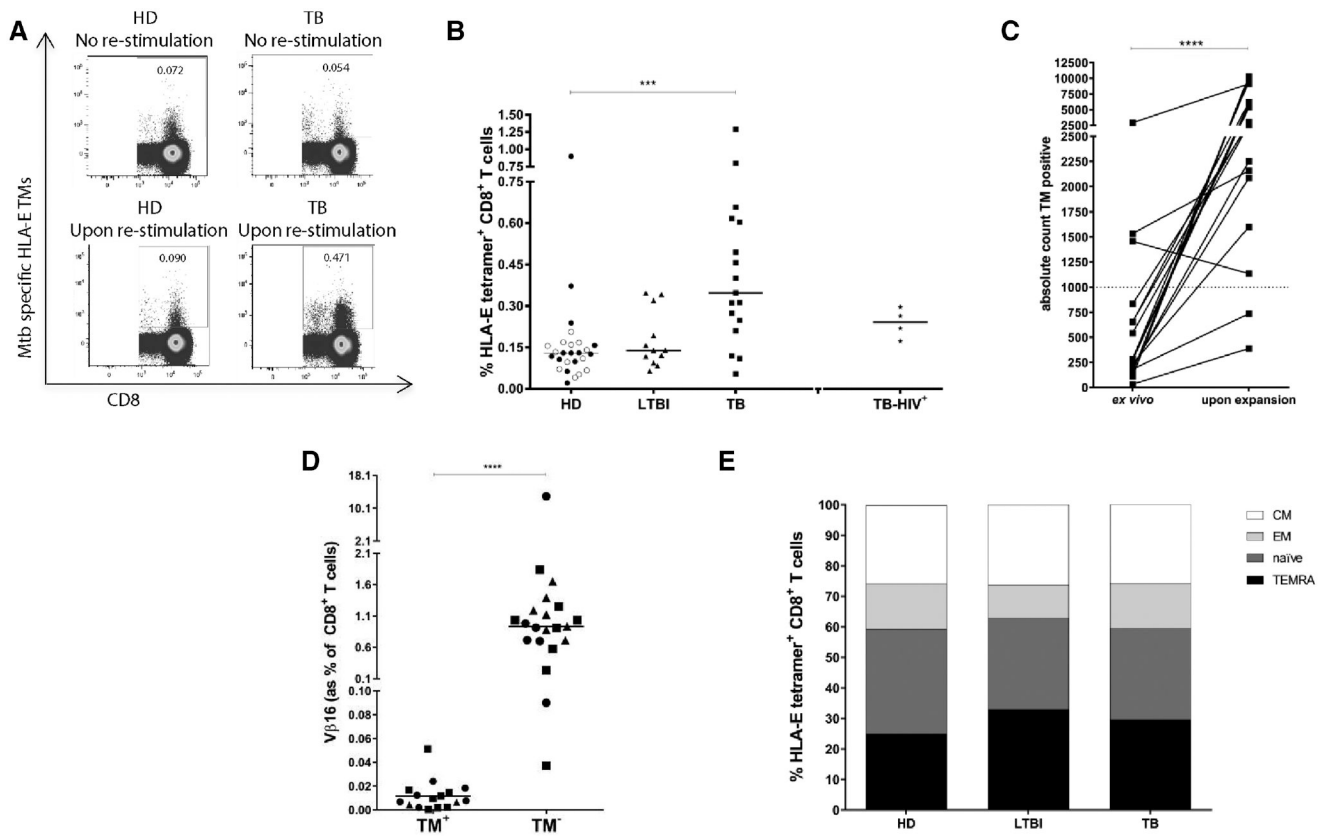


Figure 2.

HLA-E/Mtb TM^+ $CD8^+$ T-cell frequency after expansion. PBMCs were expanded using mitogenic stimulation, followed by magnetic bead separation of $CD8^+$ T cells and specific peptide stimulation. Cells were stained with TMs first before staining with cell surface markers and intracellular cytokine staining. (A) Representative plots of TM staining of $CD8^+$ T cells after control or combined p62 and p68 stimulation. Dot plots are representative of one HD and one TB patient that were analyzed in the same experiment. (B) Frequency of HLA-E/Mtb TM^+ $CD8^+$ T cells after PHA expansion and p62 and p68 peptide re-stimulation. 26 out of 26 HDs (13 Dutch and 13 Italian HDs), 12 out of 14 LTBI, 17 out of 24 active TB and 4 out of 5 HIV-TB had sufficient cells for in vitro expansion. Black circles: Dutch HDs, open circles: Italian HDs, black triangles: LTBI; black squares: TB patients, black stars: HIV-TB. Each symbol represents one patient. *P*-values were calculated using Kruskal–Wallis test including multiple-test correction. *** $p < 0.001$. TB, LTBI and TB-HIV groups were compared to HD control group. —Two to three donors from different clinical groups were included per experiment. (C) Absolute count of TM^+ events from TB patients before (ex vivo) and after PHA expansion and p62 and p68 re-stimulation. An average fold increase of 4.89 was achieved after expansion. Data were pooled from 13 culture experiments. The dashed horizontal line represents the cut off of 1000 cells required for in-depth analysis of the TM^+ population, the *p*-value was calculated using a Wilcoxon signed rank test. **** $p < 0.0001$. (D) HLA-E restricted $CD8^+$ T cells expanded with PHA and with p62 and p68 Mtb- specific peptides were characterized for expression of TCR- $V\beta 16$. These results were obtained from six independent experiments in which 6 HDs (all Dutch), 7 LTBI

and 8 active TB patients were included. Each symbol represents one sample and the p -value was calculated using a Wilcoxon-signed-rank-test. **** $p < 0.0001$. (E) Memory profile analysis of HLA-E/Mtb TM⁺ CD8⁺ T cells upon PHA expansion and p62 and p68 re-stimulation. Samples with TM⁺ counts <1000 were not considered for analysis of the memory compartment: 14 out 26 HDs (10 Dutch and 4 Italian), 6 out 13 LTBI, 15 out 17 TB subjects were considered in the memory analysis. Bars represent mean values.

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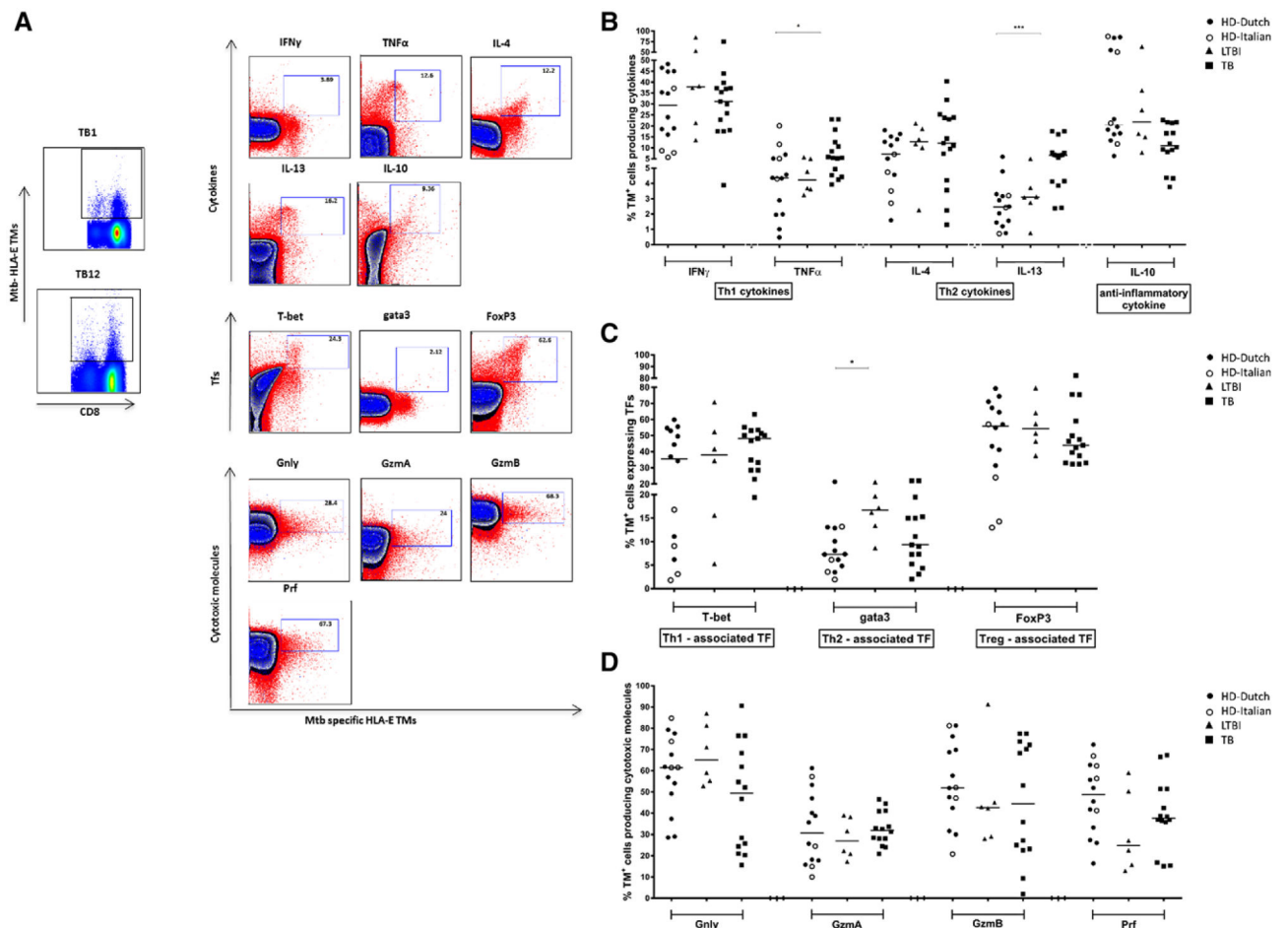
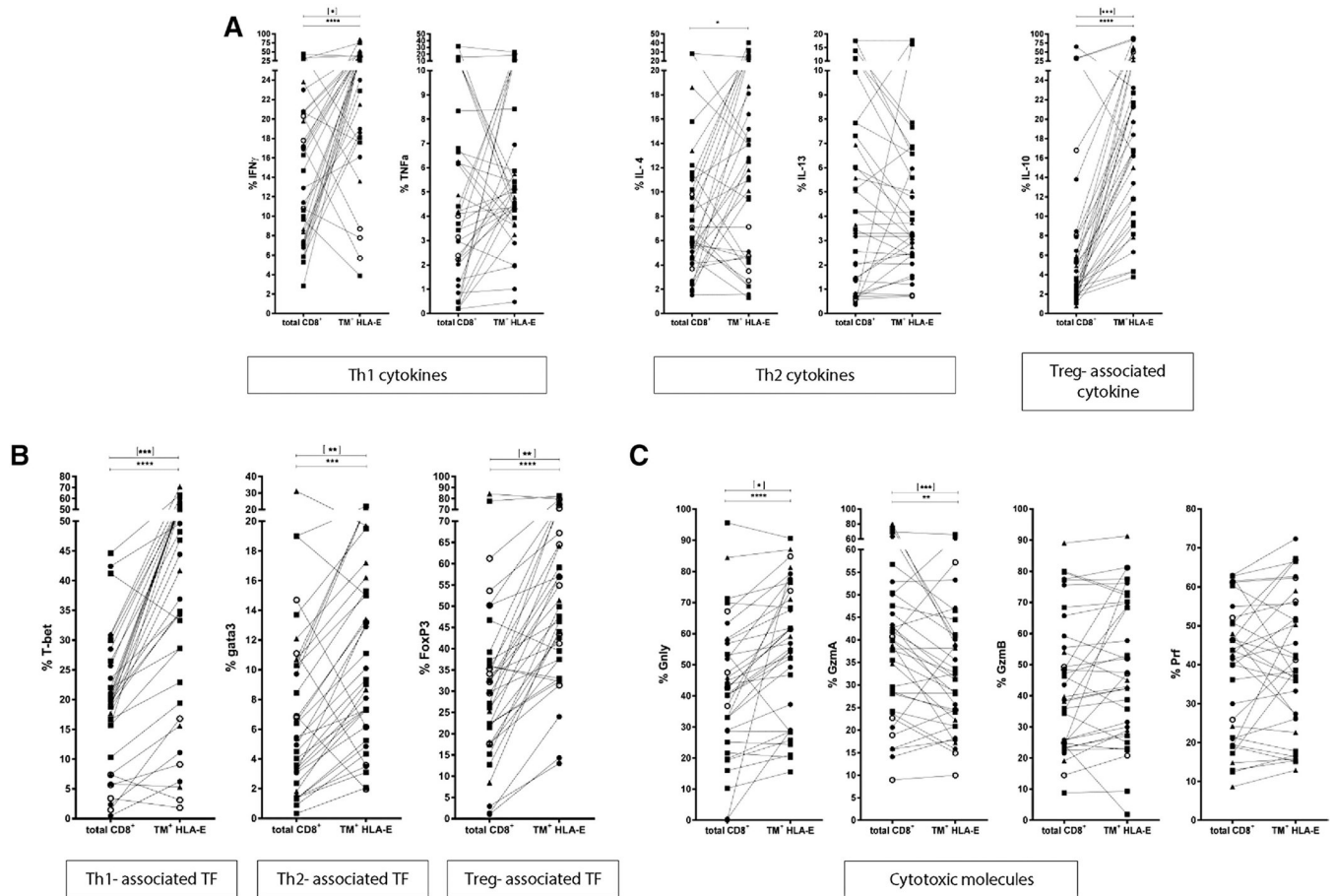
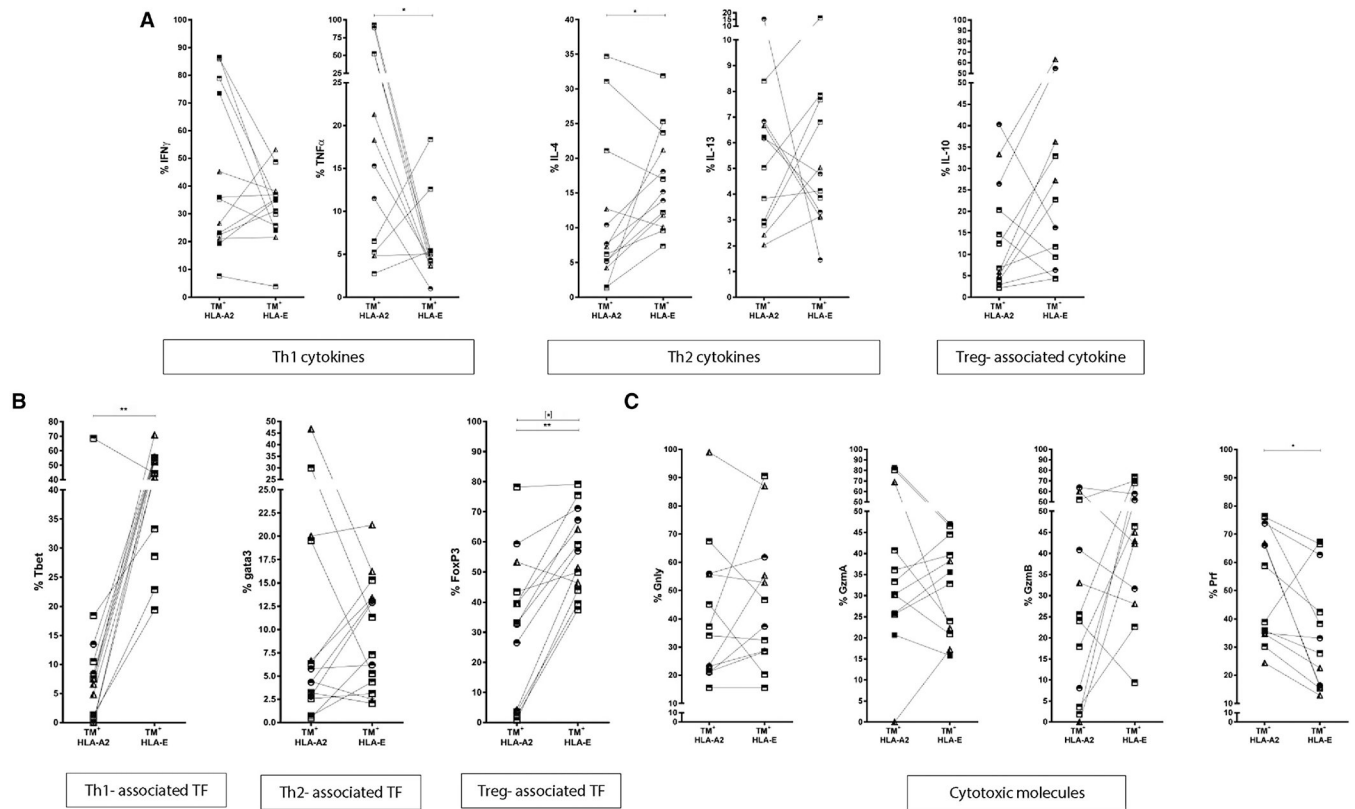


Figure 3. HLA-E/Mtb TM⁺ CD8⁺ T cells cytokine production, transcription factors, and cytolytic molecules. PBMCs were expanded using mitogenic stimulation, followed by magnetic bead separation of CD8⁺ T cells and specific peptide stimulation. Cells were stained with TMs first before staining with cell surface markers and intracellular cytokine staining. Events count below <1000 were not included for analysis of HLA-E restricted CD8⁺ T cells' properties; 14 out of 26 HDs (10 Dutch, 4 Italian), 6 out of 13 LTBI, 15 out of 17 active TB subjects were analyzed. *P*-values were calculated using a two-way ANOVA non-parametric Dunnet's test multiple comparison of LTBI and TB with HD control group. Black circles: Dutch HD; Open circles: Italian HD; black triangles: LTBI; black squares: TB patients. (A) A representative plot from 21 independent experiments with —two to four samples per experiment show HLA-E restricted CD8⁺ T cells upon p62 and p68 peptide stimulation in a TB patient, dot plots show TM⁺ cells producing cytokines; transcription factors and cytotoxic molecules. Blue indicates expanded cells not re-stimulated with specific peptides (unstimulated control), red is the sample following overnight peptide stimulation. Data are shown for total CD8⁺ T cells, with the tetramer positive population on the x-axis. Donor TB1 is shown for IFN- γ , TNF- α , IL-4, IL-13 and the transcription factors, whereas donor TB12 is shown for IL-10 and the cytotoxic molecules. (B) Frequency of TM⁺ CD8⁺ T cells producing IFN γ , TNF α , IL-4, IL-13, and IL-10 upon p62 and p68 peptide stimulation in

HDs, LTBI and TB samples. 15 out 17 TB patients were analyzed, only for IL-10 (included in Panel B) 14 out 17 TB patients were evaluated. (C) Frequency of TM⁺ CD8⁺ T cells expressing T-bet, gata3 and FoxP3 transcription factors upon expansion in HDs, LTBI and TB subjects. (D) Frequency of TM⁺ CD8⁺ T cells producing Granulysin (Gnly), Granzyme A (GzmA), Granzyme B (GzmB) and Perforin (Prf). upon expansion in HDs, LTBI and TB samples. In Panel B (including cytotoxic molecules) 14 out 17 active TB patients were analyzed. Each symbol represents one sample. Data shown in B and D are pooled from 21 independent experiments with —two to four patients per experiment. the *p*-value was calculated using a two-way ANOVA with Dunnett's multiple comparison correction **p*<0.05, ***p*<0.01, ****p*<0.001.

**Figure 4.**

Comparison of HLA-E TM⁺ with total CD8⁺ T cells. PBMCs were expanded using mitogenic stimulation, followed by magnetic bead separation of CD8⁺ T cells and peptide stimulation. Cells were stained with TMs before staining cell surface markers and intracellular cytokine staining. Total CD8⁺ T-cell production of IFN- γ , TNF- α , IL-4, IL-13, and IL-10 (A), expression of T-bet, Gata3 and FoxP3 transcription factors (B), and Granulysin, Granzyme A, Granzyme B and Perforin (C) was compared with the proportion of cells within the HLA-E/Mtb TM⁺ CD8⁺ T cells upon p62 and p68 stimulation. Lines connect cytokine/transcription factor expression in the same patient sample. Data are pooled from 21 independent experiments with —two to four patients per experiment, *p*-values were calculated using the Wilcoxon-signed-rank-test. Black stars indicate *p* values calculated for all samples ($n = 37$) **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001; stars in square brackets are *p*-values calculated for TB subjects only ($n = 15$). Circles: HD; squares: TB patients; triangles: LTBI.

**Figure 5.**

Comparison of HLA-A*0201 and HLA-E TM⁺ CD8⁺ T cells. PBMCs were expanded using mitogenic stimulation, followed by magnetic bead separation of CD8⁺ T cells and specific peptide stimulation. Cells were stained with TMs first before staining with cell surface markers and intracellular cytokine staining. Frequency of HLA-A*0201/Mtb and HLA-E/Mtb TM⁺ CD8⁺ T cells producing IFN γ , TNF α , IL-4, IL-13, and IL-10 (A), expressing Tbet, Gata3 and FoxP3 transcription factors (B) and producing Granzyme A, Granzyme B and Perforin (C). 3 out of 26 HDs (all Dutch), 3 out of 13 LTBI, 7 out of 17 active TB patients for panel A and 6 out of 17 active TB patients for panel B were HLA-A*0201⁺. Data are pooled from 10 independent experiments with 1–3 HLA-A2⁺ samples per experiment. *P*-values were calculated using a Wilcoxon-signed-rank-test. *p*-values calculated for all samples ($n = 14$; except for cytotoxic molecules & IL-10 $n = 13$) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; stars in square brackets: *p*-values for TB subjects ($n = 7$). Circles: HD; squares: TB patients; triangles: LTBI.

Table 1.

Characteristics of subjects enrolled in the study

Variable	HD	LTBI	TB	TB-HIV	Total
Total subjects, <i>n</i>	26	14	24	5	69
Age (range)	<i>a)</i> 34–60		22–38	28–40	
Clinical characteristics	<i>a)</i> No disease, Mantoux positive		Pulmonary TB (22) Military TB (1) Extra-pulmonary TB (1) Military TB (1)	Military TB and HIV co-infection	
Nationality	<i>b)</i> Italian		Italian (6) North African (18)	Italian (2) North African (3)	
Subjects used for ex vivo analysis, <i>n</i>	26/26	14/14	24/24	5/5	69
Subjects included in ex vivo analysis of memory compartment (TM ⁺ events count >100)	16/26	11/14	20/24	5/5	52
Subjects used for expansion with PHA and HLA-E peptides	26/26	12/14	17/24	4/5	60
Subjects included in functional analysis upon expansion (TM ⁺ events count >1000)	14/26	6/13	15/17 panel A 14/17 panel B	-	35
Subjects used for expansion with PHA and HLA-A2 peptides	3/26	3/13	7/17 panel A 6/17 panel B	-	13
Subjects used for TCR Vβ16 analysis	6/26	7/13	8/17	-	21

a) No information of the anonymous samples was available.

b) 13 samples were obtained from anonymous bloodbank donors (Sanquin Bloodbank, the Netherlands); 13 samples were collected from the Dipartimento di Medicina Clinica e delle Patologie Emergenti, University Hospital, Palermo.

HD, Healthy Donor; LTBI, Latently TB Infected subjects; TB, active TB patients TB-HIV, TB-HIV co-infected patients.

Characteristics of peptides used in the study

Epitope ID	144950	144971	101382	32213	21078	59612
Name code	p62	p68	TM-E control	p79	p74	TM-A2 control
Linear sequence	RMPPLGHEL	VLRRGGHFL	VMAPRLLL	KLVANNTRL	GLPVEYLQV	SLYNTVATL
Source organism	MTB H37RV	MTB H37RV	CMV	MTB H37RV	MTB H37RV	HIV-1
Source antigen	P49 protein, possible alanine rich dehydrogenase	Probable methyltransferase	hCMV UL40-derived peptide	Antigen 85-B precursor (Ag85B), 30 kDa extracellular protein, Mycolyl transferase 85B, Fibronectin-binding protein B	Antigen 85-A precursor Mycolyl transferase 85A, Fibronectin-binding protein A	p17 Gag (77–85) epitope
On-line source	http://www.iedb.org/epitope/144950	http://www.iedb.org/epitope/144971	http://www.iedb.org/epitope/101382	http://www.iedb.org/epitope/32213	http://www.iedb.org/epitope/21078	http://www.iedb.org/epitope/59612
MHC restriction	HLA-E*01:03	HLA-E*01:03	HLA-E*01:03	HLA-A2*02:01	HLA-A2*02:01	HLA-A2*02:01
Tetramers	PE	PE	APC	APC and PE	APC and PE	PE

CMV: Cytomegalovirus; HIV-1: human immunodeficiency virus type 1; MTB H37RV: *Mycobacterium tuberculosis* H37Rv strain.