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***Mycobacterium tuberculosis*-specific CD8⁺ T cells are functionally and phenotypically different between latent infection and active disease**

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Abstract

Protective immunity to *Mycobacterium tuberculosis* (*Mtb*) remains poorly understood and the role of *Mtb*-specific CD8⁺ T cells is controversial. Here we performed a broad phenotypic and functional characterization of *Mtb*-specific CD8⁺ T cells in 326 subjects with latent *Mtb* infection (LTBI) or active TB disease (TB). *Mtb*-specific CD8⁺ T cells were detected in most (60%) TB patients and few (15%) LTBI subjects but were of similar magnitude. *Mtb*-specific CD8⁺ T cells in LTBI subjects were mostly T_{EMRA} cells (CD45RA⁺ CCR7⁻), coexpressing 2B4 and CD160, and in TB patients were mostly T_{EM} cells (CD45RA⁻ CCR7⁻), expressing 2B4 but lacking PD-1 and CD160. The cytokine profile was not significantly different in both groups. Furthermore, *Mtb*-specific CD8⁺ T cells expressed low levels of perforin and granulysin but contained granzymes A and B. However, in vitro-expanded *Mtb*-specific CD8⁺ T cells expressed perforin and granulysin. Finally, *Mtb*-specific CD8⁺ T-cell responses were less frequently detected in extrapulmonary TB compared with pulmonary TB patients. *Mtb*-specific CD8⁺ T-cell proliferation was also greater in patients with extrapulmonary compared with pulmonary TB. Thus, the activity of *Mtb* infection and clinical presentation are associated with distinct profiles of *Mtb*-specific CD8⁺ T-cell responses. These results provide new insights in the interaction between *Mtb* and the host immune response.

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Keywords

Active TB disease; Cytotoxicity; Functional profile; Latent *Mtb* infection; *Mtb*-specific CD8⁺ T cells

Introduction

One-third of the world's population is believed to be latently infected with *Mycobacterium tuberculosis* (*Mtb*) and two million people die of tuberculosis (TB) every year [1], thus underscoring the tremendous need for protective vaccines, new diagnostic tools, and medications.

T lymphocytes are thought to play an important role in the control of TB and *Mtb* may reactivate under certain conditions of immunodeficiency such as in elderly or secondary to coinfection with HIV or to immunosuppressive therapy [2, 3]. Several studies have underscored the essential role of CD4⁺ T cells in protection against *Mtb*, since CD4⁺ T-cell depletion is also associated with *Mtb* reactivation in HIV-infected individuals [4] and uncontrolled bacilli growth [5, 6]. The protective *Mtb*-specific CD4⁺ T-cell response is considered to be a typical T_H1 response with CD4⁺ T cells producing cytokines such as IFN- γ or TNF- α that contribute to the recruitment of monocytes and granulocytes and activate the antimicrobial activity of macrophages [7, 8]. Of interest, we recently demonstrated that *Mtb*-specific CD4⁺ T-cell responses were functionally different in patients with active TB disease as compared with those in subjects with latent *Mtb* infection (LTBI) [9]. Several studies also suggested a role of T_H17 cells in the control of TB [10, 11].

The importance and the role of *Mtb*-specific CD8⁺ T cells in the control of *Mtb* and their mechanism of action remain highly controversial. A number of secreted immunodominant *Mtb* antigens can be processed by cytosolic pathways and presented by MHC class I molecules [12–14]. Several studies performed in mice and nonhuman models have proposed a role of *Mtb*-specific CD8⁺ T cells in the control of *Mtb* infection [15–17]. In these models, IFN- γ and perforin production by *Mtb*-specific CD8⁺ T cells was necessary to protect mice from *Mtb* infection [15, 18]. Other studies performed in humans supported these conclusions [19, 20]. Although many in vitro studies indicated that perforin- and/or granulysin-containing *Mtb*-specific CD8⁺ T-cell lines were able to kill *Mtb*-infected macrophages or free bacteria [21–23], *Mtb*-specific CD8⁺ T cells from lung-associated tissues generally lacked expression of these effector molecules [24, 25].

In the present study, we performed broad phenotypic (T-cell differentiation and exhaustion) and functional (cytokines production, proliferation capacity, and cytotoxic potential) characterizations of *Mtb*-specific CD8⁺ T-cell responses in 326 TB and LTBI subjects and evaluated their correlation with different clinical presentations of *Mtb* infection. In particular, we hypothesized that the detection of *Mtb*-specific CD8⁺ T cells and their phenotype and function may vary in active TB versus latent infection.

Our results have shown differences in the prevalence, frequency, and phenotypic and functional profiles of *Mtb*-specific CD8⁺ T cells in active disease versus latent infection and

between pulmonary TB (PTB) and extrapulmonary TB (ETB). These findings provide new insights in the role of CD8⁺ T cells in *Mtb* infection and disease.

Results

Identification and frequency of *Mtb*-specific CD8⁺ T cells in TB and LTBI subjects

We have studied 326 individuals with either active TB disease (TB) or LTBI.

Mtb-specific CD8⁺ T-cell responses were assessed using polychromatic flow cytometry following stimulation with ESAT-6 and/or CFP-10 peptide pools. The flow cytometry panel included a viability marker, CD3, CD4, and CD8 to determine T-cell lineage and IFN- γ , TNF- α , and IL-2 antibodies.

Mtb-specific CD8⁺ T-cell responses were detected in 52 out of the 86 (60%) TB patients, and in 37 out of the 240 (15%) LTBI subjects ($p < 0.0001$; Fig. 1A). Overall, 50 and 58 *Mtb*-specific CD8⁺ T-cell responses (directed against CFP-10 or ESAT-6) were observed in the 37 and 52 LTBI and TB individuals with detectable *Mtb*-specific CD8⁺ T-cell responses, respectively. These *Mtb*-specific CD8⁺ T-cell responses in both groups were directed against CFP-10, ESAT-6 or both peptide pools in 50–60%, 20–25%, and 20–30% of cases, respectively (data not shown).

Of interest, the magnitude of *Mtb*-specific CD8⁺ T-cell responses, as determined by the frequency of IFN- γ -producing CD8⁺ T cells following ESAT-6 or CFP-10 stimulation, was not significantly different between LTBI and TB subjects ($p > 0.05$; Fig. 1B). Therefore, these results indicate that *Mtb*-specific CD8⁺ T-cell responses are a component of the host immune response during active TB disease and also potentially, to some extent, during latent infection.

T-cell differentiation of *Mtb*-specific CD8⁺ T cells in LTBI and TB subjects

We then investigated the level of T-cell differentiation of *Mtb*-specific CD8⁺ T cells in a portion of the LTBI and TB subjects. Subjects were randomly selected based on the availability of cryopre-served PBMC. For this purpose, *Mtb*-specific CD8⁺ T-cell responses were evaluated for the expression of CD45RA and CCR7. As shown in the representative subject LTBI#910, most *Mtb*-specific CD8⁺ T cells were CD45RA⁺ CCR7⁻ (i.e. terminally differentiated; T_{EMRA}), whereas in the representative patient TB#TB-3, the majority of *Mtb*-specific CD8⁺ T cells were CD45RA⁻ CCR7⁻ (i.e. effector memory; T_{EM}) (Fig. 2A). Cumulative analyses confirmed that the majority of *Mtb*-specific CD8⁺ T cells were composed of T_{EMRA} and T_{EM} in LTBI and TB subjects, respectively (both $p < 0.00001$; Fig. 2B).

Therefore, these results indicate that *Mtb*-specific CD8⁺ T cells in TB and LTBI subjects have distinct stages of differentiation.

Expression of regulatory receptors in *Mtb*-specific CD8⁺ T cells

We then investigated the expression of regulatory receptors in *Mtb*-specific CD8⁺ T cells in a portion of LTBI and TB individuals randomly selected based on the availability of

cryopreserved PBMC. For this purpose, *Mtb*-specific CD8⁺ T-cell responses were evaluated for the expression of PD-1, 2B4, and CD160, which are three relevant coinhibitory molecules whose expression is associated to functional defects [26]. As shown in the representative flow cytometric profiles and confirmed in the cumulative analyses (Fig. 2C and D), the majority of *Mtb*-specific CD8⁺ T cells in TB patients expressed 2B4 but mostly lacked PD-1 and CD160, that is, were 2B4⁺ PD1⁻ CD160⁻. Differently, in LTBI subjects, the *Mtb*-specific 2B4⁺ PD1⁻ CD160⁻ CD8⁺ T-cell population was significantly lower ($p < 0.0001$); moreover a substantial proportion (about 50%) of *Mtb*-specific CD8⁺ T cells coexpressed CD160 and/or PD-1 in addition to 2B4 (Fig. 2C and D).

Therefore, *Mtb*-specific CD8⁺ T cells from LTBI and TB subjects showed significant differences regarding the expression of regulatory receptors ($p < 0.0001$; Fig. 2D).

Functional profile of *Mtb*-specific CD8⁺ T cells in LTBI and TB subjects

We then performed a broad characterization of the functional profile of *Mtb*-specific CD8⁺ T cells. Consistently with previous studies [27,28], we observed that in both LTBI and TB patients, the majority of *Mtb*-specific CD8⁺ T cells were composed of dual IFN- γ /TNF- α - or single IFN- γ -producing cells (Supporting Information Fig. 2). Still, *Mtb*-specific CD8⁺ T cells of LTBI subjects contained a greater ($p = 0.03$) proportion of triple cytokine producing CD8⁺ T cells (i.e. IFN- γ ⁺ TNF- α ⁺ IL-2⁺) whereas a higher proportion of single TNF- α -producing CD8⁺ T cells was found in TB patients ($p = 0.03$; Supporting Information Fig. 2). Considering the large number of individuals analyzed ($n = 35$ *Mtb*-specific CD8⁺ T-cell responses in each group), these data indicate that the cytokines profile of *Mtb*-specific CD8⁺ T cells is only slightly different between TB and LTBI subjects.

Cytotoxic potential of *Mtb*-specific CD8⁺ T cells

Previous studies suggesting a direct role of *Mtb*-specific CD8⁺ T cells in the control of *Mtb* infection proposed expression of perforin as a key mechanism [15, 18, 20]. We therefore analyzed perforin expression in *Mtb*-specific CD8⁺ T cells in a subset of TB and LTBI subjects randomly selected based on the availability of cryopreserved PBMCs. We found that about 10% of *Mtb*-specific CD8⁺ T cells contained perforin in TB and LTBI subjects *ex vivo* (Fig. 3A and B). However, a consistent proportion (20–30%) of total CD8⁺ T cells and of IFN- γ -producing CD8⁺ T cells following polyclonal stimulation (positive control) expressed perforin (Fig. 3A and B).

We then investigated the expression of additional cytotoxic granules such as granzyme (Grm)B, GrmA, and granulysin (Grlz) known to be also associated with the cytotoxic capacity of CD8⁺ T cells [22, 23]. *Mtb*-specific CD8⁺ T cells from LTBI subjects lacked expression of all cytotoxic markers in about 40% of cells and only contained GrmB in about 30% of cells. Interestingly, the majority (>55%) of *Mtb*-specific CD8⁺ T cells from TB patients were composed of cells coexpressing GrmB and GrmA ($p < 0.0001$; Fig. 3C and D) whereas less than 10% contained Grlz (Fig. 3A and D).

Since perforin, GrmB, and GrmA are upregulated upon T-cell stimulation and proliferation [29], CFSE-labeled mononuclear cells from LTBI subjects or TB patients were stimulated with the cognate antigens for 6 days and assessed for the expression of the cytotoxic

granules after in vitro expansion. No significant difference in the proliferation capacity (i.e. the percentage of CFSE^{low} CD8⁺ T cells) was observed between LTBI subjects and TB patients (data not shown) and *Mtb*-specific CD8⁺ T cells coexpressed perforin, granulysin, and granzymes in both TB and LTBI individuals (Fig. 3E and F).

Overall, these results indicate that *Mtb*-specific CD8⁺ T cells from TB and LTBI subjects express distinct patterns of cytotoxic granules ex vivo and that expression of perforin and Grz2 can be induced after antigen-specific in vitro T-cell expansion.

Association between *Mtb*-specific CD8⁺ T-cell responses and clinical presentation

Since phenotypic and functional differences in *Mtb*-specific CD8⁺ T cells were observed between subjects with LTBI versus active TB disease, it was possible that the type of clinical presentation could play a role in the distinct phenotypic and functional profiles observed. Of note, among the 86 TB patients enrolled in this study, and based on WHO classification [30], 67 patients had PTB whereas 19 patients had ETB.

Mtb-specific CD8⁺ T cells were significantly more frequently detected in PTB as compared with ETB (67 versus 37%, respectively; $p = 0.017$; Fig. 4A). Furthermore, differently from PTB, a broader range of magnitude of *Mtb*-specific CD8⁺ T-cell responses was observed in ETB (Fig. 4B). PTB patients were also stratified according to the smear test that is commonly considered as a reflection of the bacterial burden [31]. Within PTB patients, *Mtb*-specific CD8⁺ T-cell responses were significantly greater in smear-positive as compared with those of smear-negative patients ($p = 0.01$; Fig. 4C). Furthermore, we also separately analyzed the two patients who were not microbiologically confirmed but were clear clinical PTB. Of interest, the magnitude of *Mtb*-specific CD8⁺ T cells from these two patients was typically in the range of that of the other smear-negative microbiologically confirmed PTB patients and thus was lower as compared with the responses from smear-positive PTB patients (Supporting Information Fig. 3). Of note, the cytokines and perforin profiles were not different between smear-positive and smear-negative, or between PTB and ETB patients, or between microbiologically confirmed and clinical TB (data not shown).

We also compared the proliferation capacity of *Mtb*-specific CD8⁺ T cells between ETB and PTB patients in a subset of patients randomly selected on the basis of the availability of cryopreserved PBMC. The proliferation capacity of *Mtb*-specific CD8⁺ T cells, as determined by the frequency of CFSE^{low} CD8⁺ T cells following stimulation with ESAT-6 or CFP-10, was significantly higher in ETB patients as compared with those in PTB patients ($p = 0.005$) and to LTBI subjects ($p = 0.016$; Fig. 4D and E). Of note, the proliferation capacity of *Mtb*-specific CD4⁺ T cells was not different between PTB and ETB patients (Supporting Information Fig. 4) and thus did not skew *Mtb*-specific CD8⁺ T-cell responses.

These data indicate significant associations between the clinical presentation of TB and profiles of *Mtb*-specific CD8⁺ T cells.

Discussion

Although growing evidence suggests that CD8⁺ T cells contribute to the control of *Mtb* [15–20, 32, 33], the results obtained in humans and in animal models remain controversial and the discrepancies likely result from the difficulty to compare investigations performed by in vitro studies versus in vivo observations or from the antigens utilized to discriminate between T-cell responses induced by infection versus vaccination [34, 35].

Mtb-specific CD8⁺ T-cell responses against ESAT-6 or CFP-10 were detected predominantly in patients with active TB disease as compared with LTBI subjects, consistently with a previous report by Day and colleagues performed on whole blood [28]. Previous studies have shown that ESAT-6 and CFP-10 antigens can identify *Mtb*-specific T-cell responses in 99% of LTBI and TB patients [36]. However, since *Mtb* expresses approximately 4000 proteins [37], we cannot exclude that additional *Mtb*-specific CD8⁺ T-cell responses may be directed particularly against latency antigens [38].

One potential mechanism to explain the greater frequency of *Mtb*-specific CD8⁺ T-cell responses in TB patients is that these responses are predominantly stimulated in the presence of higher antigen load [39]. This hypothesis is supported by a recent study performed in children showing that *Mtb*-specific CD8⁺ T cells were detected in active TB disease but not in healthy children recently exposed to *Mtb*, despite that similar frequencies of *Mtb*-specific CD4⁺ T-cell responses were present in both groups [40]. Along the same lines, the higher number of granulomas found in TB patients as compared with that in LTBI subjects may also be a significant determinant of the reduced proportion of *Mtb*-specific CD8⁺ T-cell responses in LTBI subjects.

Major phenotypic and functional differences were observed between TB and LTBI subjects, consistently with previous studies [27, 28]. *Mtb*-specific CD8⁺ T cells were mostly composed of T_{EMRA} in LTBI and of T_{EM} in TB patients. This is consistent with the observation that TNF- α blockade induced a decrease of T_{EMRA} CD8⁺ T cells, underscoring the potential role of *Mtb*-specific T_{EMRA} CD8⁺ T cells in *Mtb* control [19]. These results are also consistent with current models of antiviral immunity suggesting that T_{EMRA} and T_{EM} are associated with chronic controlled and uncontrolled virus infection, respectively [41]. Our results suggest that this may be also the case for *Mtb*-specific CD8⁺ T cells in LTBI and active disease.

It is well established that coregulatory molecules are upregulated upon activation and that coexpression of these receptors is associated with a state of T-cell exhaustion (reviewed in [26]). We have found little coexpression of PD-1, CD160, and 2B4 in *Mtb*-specific CD8⁺ T cells from TB patients. Significant differences were observed in the expression of CD160 between TB and LTBI; however PD-1 was always expressed at low levels thus questioning the hypothesis of exhaustion of *Mtb*-specific CD8⁺ T cells. The findings that *Mtb*-specific CD8⁺ T cells retained proliferation capacity and had a polyfunctional cytokines profile are also not supporting the exhaustion hypothesis.

Furthermore, there were no major differences in the cytokines profile of *Mtb*-specific CD8⁺ T-cell responses between LTBI subjects and TB patients. Still, it is interesting to note that

Mtb-specific CD8⁺ T cells were more polyfunctional (i.e. IFN- γ ⁺ TNF- α ⁺ IL-2⁺) in LTBI, consistently with the current paradigm in antiviral immunity (reviewed in [41]). Also, *Mtb*-specific CD8⁺ T cells were enriched in single TNF- α -producing CD8⁺ T cells in TB patients, thus following the same trend than CD4⁺ T cells [9].

The current model for the protective role of CD8⁺ T cells in TB is based on the ability to lyse infected cells (in addition to cytokines release). Differently from many animal studies [42, 43], we found that *Mtb*-specific CD8⁺ T cells expressed little perforin and granulysin *ex vivo*. These results are consistent with *in situ* analyses of human tissues [25, 44]. We observed, however, that *Mtb*-specific CD8⁺ T cells expressed significant levels of GrmB and GrmA. Taken together, these observations suggest a potential perforin-independent cytotoxic mechanism of action of *Mtb*-specific CD8⁺ T cells. Furthermore, the majority of *Mtb*-specific CD8⁺ T cells expressed perforin, GrmA, GrmB, and GrIz after antigen-specific *in vitro* T-cell expansion, consistently with studies performed on T-cell lines [22], activated T cells [31], and animal models [15, 18].

Next we investigated whether the bacterial load (i.e. smear-positive versus smear-negative TB) or the clinical presentation of TB disease (i.e. PTB versus ETB) correlated with distinct profiles of *Mtb*-specific CD8⁺ T-cell responses. We observed a higher prevalence of *Mtb*-specific CD8⁺ T-cell responses in PTB compared with ETB and a higher magnitude of these responses in smear-positive versus smear-negative PTB patients. We also observed limited proliferation capacity of *Mtb*-specific CD8⁺ T cells from PTB patients compared with ETB patients. This is consistent with the current paradigm associating CD8⁺ T-cell responses to high antigen burden [28, 39, 40]. Furthermore, as mentioned above, these functional differences may reflect different conditions in the stimulation of the immune responses in the different anatomic sites, to the tropism of responding T cells or to distinct stages of disease.

Overall, we have identified several phenotypic and functional differences in the profiles of *Mtb*-specific CD8⁺ T cells between patients with active disease and LTBI subjects.

Notwithstanding the key role of CD4⁺ T cells in the control of *Mtb* infection, we report here several associations between profiles of *Mtb*-specific CD8⁺ T cells and distinct clinical presentations. In particular, we found significant differences in the phenotypic (e.g. T-cell differentiation) and functional (e.g. GrmA expression) profiles between patients with active TB disease and subjects with latent infection. Whether these phenotypic and functional profiles reflect different levels of immune control remains to be determined.

Recent studies [45, 46] also questioned whether LTBI subjects represent a model of efficient control of *Mtb*. In this regard, there is growing evidence that LTBI corresponds to a broad spectrum of subjects with *Mtb* infection ranging from exposed uninfected subjects to subjects with subclinical TB [46]. One could speculate that the presence of CD8⁺ T cells (found in 15% of LTBI) may represent a marker of truly chronically infected subjects and potentially identify subjects at risk of reactivation (occurring in about 10% of subjects) that would benefit from chemoprophylaxis. Longitudinal studies are needed to confirm this hypothesis.

In conclusion, our descriptive study allowed us to identify major differences in the prevalence, function, and phenotype of *Mtb*-specific CD8⁺ T-cell responses in active TB and LTBI. Additional studies are needed to clarify the mechanisms driving these differences. Still, our results represent a step forward in the understanding of the role of CD8⁺ T cells in TB pathogenesis and provide new insights on the use of distinct phenotypic and functional profiles of CD8⁺ T cells as markers of *Mtb* activity and different clinical presentation of TB disease.

Materials and methods

Study groups

The majority of the 240 LTBI subjects and 86 TB patients were recruited at the Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland and few patients were also recruited at the INMI, Rome, Italy or in Cape Town and Worcester, South Africa. All TB patients had a diagnosis based on laboratory isolation of *Mtb* on mycobacterial culture from sputum, BALF, or biopsies and/or tuberculin skin test and/or ELISPOT and/or PCR as described [9]. The final diagnosis was given by a clinician after validation of these criteria associated with clinical symptoms. Furthermore, based on WHO classification [30], the 86 TB patients included 67 patients with PTB and 19 patients with ETB. Of note, a subset of 52 TB patients was further investigated for immunological assessments and these patients were randomly selected based on the availability of cryopreserved PBMCs (which was dependent upon the volume of blood collected). Furthermore, the selection of the type of immunological measures performed in each patient was random thus excluding any selection bias. Demographic and clinical data on the 52 TB patients are described in Supporting Information Table 1. Also, four TB patients were coinfecting with HIV. Since *Mtb*-specific CD8⁺ T-cell responses from these patients were similar to those from the HIV-seronegative patients (clearly identified with red dots in all analyses), they were not excluded from the analyses.

All LTBI subjects were asymptomatic and were either healthcare workers routinely screened or were investigated for *Mtb* infection prior to the initiation of anti-TNF- α antibody treatment and had negative chest radiographs. All LTBI subjects were IGRA-positive, that is, had *Mtb*-specific T-cell responses against ESAT-6 or CFP-10 using IFN- γ ELISPOT (Supporting Information Fig. 5). Of note, *Mtb*-specific CD8⁺ T-cell responses were identified in only 37 of the 240 LTBI subjects tested. As for TB patients, a subset of the 37 LTBI subjects was further investigated for immunological measures and the selection was based on the availability of cryopreserved PBMC (which was depending upon the volume of blood collected) and the selection of immunological measures performed in each patient was random thus excluding any selection bias. None of these subjects (TB or LTBI) was under antimycobacterial treatment for more than 1 week at the time of the enrollment and analysis. These studies were approved by the Institutional Review Boards of the different Centers and informed written consent was obtained from each volunteer.

Peptides

Mtb-derived peptides covering ESAT-6 and CFP-10 proteins were pools of HPLC-purified (>80% purity) 15-mers peptides overlapping by 11 amino acids as described [9].

Intracellular cytokine staining

For intracellular cytokine staining, cryopreserved blood mononuclear cells (1×10^6) were rested for 6 h and then stimulated overnight in Brefeldin A (1 μ L/mL, BD) and anti-CD28 antibodies (0.5 μ g/mL, BD) containing media as described [47]. Monensin (1 μ L/mL, BD) was also added in cell cultures for the assessment of cytotoxicity as described [48]. For cell stimulations, peptide pools were used at 1 μ g/mL for each peptide. SEB stimulation (200 ng/mL) served as positive control. After stimulation, cells were stained for dead cells (LIVE/DEAD kit, Invitrogen), permeabilized (Cytotfix/Cytoperm, BD), and stained with various combinations of antibodies depending on the analysis.

Antigen-specific in vitro T-cell expansion

Cryopreserved cells were labeled with 5,6-CFSE (Molecular Probes) as described [49]. Subsequently, cells were cultured in 6% human AB serum (Institut de Biotechnologies Jacques Boy) RPMI. For cell stimulation, peptide pools were used at 1 μ g/mL for each peptide. SEB stimulation (200 ng/mL) served as positive control. After 6 days of in vitro T-cell expansion, cells were washed and stained for dead cells (LIVE/DEAD kit, Invitrogen) and with the antibody panel described below. The percentage of proliferating CD8⁺ T cells, that is, CFSE^{low} cells, was determined in the live CD8⁺ CD4⁻ T-cell population.

Flow cytometry analyses

The following antihuman monoclonal antibodies were used in various combinations: CD3, CD4, CD8, IFN- γ , TNF- α , IL-2, granzyme B, PD-1, and CD45RA were purchased from BD; granzyme A and CD160 from Lucerna Chem; CD4 and 2B4 from BioLegend; granulysin from Bender MedSystems GmbH; Perforin from Biotest AG and CCR7 from R&D System. Data were acquired on an LSRII four laser (405, 488, 532, and 633 nm) and analyzed using FlowJo version 8.8.6 (Tree Star Inc.). Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from <http://exon.niaid.nih.gov/spice/> [50].

Statistical analyses

Comparisons of categorical variables were performed using Fisher's exact test. Statistical significance (*P* values) of the magnitude of responses was calculated with unpaired two-tailed Student's *t*-test using GraphPad Prism 5 version 5.04. Mann-Whitney test (two tailed) was used as nonparametric test. Bonferroni correction for multiples analyses was applied. Regarding SPICE analyses of the flow cytometry data, comparison of distributions was performed using a Student's *t*-test and a partial permutation test as described [50].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ETB	extrapulmonary TB
LTBI	latent Mtb infection
Mtb	<i>Mycobacterium tuberculosis</i>
PTB	pulmonary TB
TB	tuberculosis

References

1. Koul A, Arnoult E, Lounis N, Guillemont J and Andries K, The challenge of new drug discovery for tuberculosis. *Nature* 2011 469: 483–490.
2. Winthrop KL, Risk and prevention of tuberculosis and other serious opportunistic infections associated with the inhibition of tumor necrosis factor. *Nat. Clin. Pract. Rheumatol* 2006 2: 602–610. [PubMed: 17075599]
3. Bumbacea D, Arend SM, Eyuboglu F, Fishman JA, Goletti D, Ison MG, Jones CE et al., The risk of tuberculosis in transplant candidates and recipients: a TBNET consensus statement. *Eur. Respir. J* 2012 40: 990–1013. [PubMed: 22496318]
4. Granich R, Akolo C, Gunneberg C, Getahun H, Williams P and Williams B, Prevention of tuberculosis in people living with HIV. *Clin. Infect. Dis* 2010 50(Suppl 3): S215–S222. [PubMed: 20397951]
5. Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, Chan J and Flynn JL, Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. *J. Exp. Med* 2000 192: 347–358.
6. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR and Flynn JL, Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J. Immunol* 1999 162: 5407–5416. [PubMed: 10228018]
7. Walzl G, Ronacher K, Hanekom W, Scriba TJ and Zumla A, Immunological biomarkers of tuberculosis. *Nat. Rev. Immunol* 2011 11: 343–354. [PubMed: 21475309]
8. Flynn JL and Chan J, Tuberculosis: latency and reactivation. *Infect. Immun* 2001 69: 4195–4201. [PubMed: 11401954]
9. Harari A, Rozot V, Enders FB, Perreau M, Stalder JM, Nicod LP, Cavassini M et al., Dominant TNF-alpha+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. *Nat. Med* 2011 17: 372–376. [PubMed: 21336285]
10. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA and Bloom BR, An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J. Exp. Med* 1993 178: 2249–2254. [PubMed: 7504064]
11. Scriba TJ, Kalsdorf B, Abrahams DA, Isaacs F, Hofmeister J, Black G, Hassan HY et al., Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J. Immunol* 2008 180: 1962–1970. [PubMed: 18209095]
12. Lewinsohn DM, Grotzke JE, Heinzl AS, Zhu L, Ovendale PJ, Johnson M and Alderson MR, Secreted proteins from Mycobacterium tuberculosis gain access to the cytosolic MHC class-I antigen-processing pathway. *J. Immunol* 2006 177: 437–442. [PubMed: 16785540]

13. Woodworth JS, Fortune SM and Behar SM, Bacterial protein secretion is required for priming of CD8+ T cells specific for the Mycobacterium tuberculosis antigen CFP10. *Infect. Immun* 2008 76: 4199–4205. [PubMed: 18591224]
14. Grotzke JE, Siler AC, Lewinsohn DA and Lewinsohn DM, Secreted immunodominant Mycobacterium tuberculosis antigens are processed by the cytosolic pathway. *J. Immunol* 2010 185: 4336–4343. [PubMed: 20802151]
15. Brighenti S and Andersson J, Induction and regulation of CD8+ cytolytic T cells in human tuberculosis and HIV infection. *Biochem. Biophys. Res. Commun* 2010 396: 50–57.
16. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, Dockrell H et al., Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. USA* 1998 95: 270–275. [PubMed: 9419365]
17. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, Shen Y et al., A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS Pathog* 2009 5: e1000392. [PubMed: 19381260]
18. Mazzaccaro RJ, Stenger S, Rock KL, Porcelli SA, Brenner MB, Modlin RL and Bloom BR, Cytotoxic T lymphocytes in resistance to tuberculosis. *Adv. Exp. Med. Biol* 1998 452: 85–101.
19. Bruns H, Meinken C, Schauenberg P, Harter G, Kern P, Modlin RL, Antoni C et al., Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J. Clin. Invest* 2009 119: 1167–1177. [PubMed: 19381021]
20. Cooper AM, Cell-mediated immune responses in tuberculosis. *Annu. Rev. Immunol* 2009 27: 393–422. [PubMed: 19302046]
21. Stenger S, Mazzaccaro RJ, Uyemura K, Cho S, Barnes PF, Rosat JP, Sette A et al., Differential effects of cytolytic T cell subsets on intracellular infection. *Science* 1997 276: 1684–1687.
22. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T et al., An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 1998 282: 121–125. [PubMed: 9756476]
23. Semple PL, Watkins M, Davids V, Krensky AM, Hanekom WA, Kaplan G and Ress S, Induction of granulysin and perforin cytolytic mediator expression in 10-week-old infants vaccinated with BCG at birth. *Clin. Dev. Immunol* 2011 2011: 438463. [PubMed: 21234358]
24. Rahman S, Gudetta B, Fink J, Granath A, Ashenafi S, Aseffa A, Derbew M et al., Compartmentalization of immune responses in human tuberculosis: few CD8+ effector T cells but elevated levels of FoxP3+ regulatory T cells in the granulomatous lesions. *Am. J. Pathol* 2009 174: 2211–2224. [PubMed: 19435796]
25. Andersson J, Samarina A, Fink J, Rahman S and Grundstrom S, Impaired expression of perforin and granulysin in CD8+ T cells at the site of infection in human chronic pulmonary tuberculosis. *Infect. Immun* 2007 75: 5210–5222. [PubMed: 17664265]
26. Vigano S, Perreau M, Pantaleo G and Harari A, Positive and negative regulation of cellular immune responses in physiologic conditions and diseases. *Clin. Dev. Immunol* 2012 2012: 485781. [PubMed: 22548114]
27. Caccamo N, Guggino G, Meraviglia S, Gelsomino G, Di Carlo P, Titone L, Bocchino M et al., Analysis of Mycobacterium tuberculosis-specific CD8 T-cells in patients with active tuberculosis and in individuals with latent infection. *PLoS One* 2009 4: e5528. [PubMed: 19436760]
28. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, O'Rie T, Pienaar B et al., Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load. *J. Immunol* 2011 187: 2222–2232. [PubMed: 21775682]
29. Harari A, Enders FB, Cellerai C, Bart PA and Pantaleo G, Distinct profiles of cytotoxic granules in memory CD8 T cells correlate with function, differentiation stage, and antigen exposure. *J. Virol* 2009 83: 2862–2871. [PubMed: 19176626]
30. Global tuberculosis control: WHO (World Health Organization) Report 2011. http://www.who.int/tb/publications/global_report/archive/en/index.html.
31. Helke KL, Mankowski JL and Manabe YC, Animal models of cavitation in pulmonary tuberculosis. *Tuberculosis (Edinb.)* 2006 86: 337–348. [PubMed: 16359922]

32. Behar SM, Dascher CC, Grusby MJ, Wang CR and Brenner MB, Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. *J. Exp. Med* 1999 189: 1973–1980. [PubMed: 10377193]
33. Flynn JL, Goldstein MM, Triebold KJ, Koller B and Bloom BR, Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proc. Natl. Acad. Sci. USA* 1992 89: 12013–12017. [PubMed: 1465432]
34. Heinzel AS, Grotzke JE, Lines RA, Lewinsohn DA, McNabb AL, Streblow DN, Braud VM et al., HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J. Exp. Med* 2002 196: 1473–1481. [PubMed: 12461082]
35. Woodworth JS and Behar SM, Mycobacterium tuberculosis-specific CD8+ T cells and their role in immunity. *Crit. Rev. Immunol* 2006 26: 317–352. [PubMed: 17073557]
36. Lalvani A, Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. *Chest* 2007 131: 1898–1906.
37. Ernst JD, Lewinsohn DM, Behar S, Blythe M, Schlesinger LS, Kornfeld H and Sette A, Meeting Report: NIH Workshop on the Tuberculosis Immune Epitope Database. *Tuberculosis (Edinb.)* 2008 88: 366–370. [PubMed: 18068490]
38. Dosanjh DP, Bakir M, Millington KA, Soysal A, Aslan Y, Efee S, Deeks JJ et al., Novel M tuberculosis antigen-specific T-cells are early markers of infection and disease progression. *PLoS One* 2011 6: e28754. [PubMed: 22216109]
39. Lewinsohn DA, Heinzel AS, Gardner JM, Zhu L, Alderson MR and Lewinsohn DM, Mycobacterium tuberculosis-specific CD8+ T cells preferentially recognize heavily infected cells. *Am. J. Respir. Crit. Care Med* 2003 168: 1346–1352. [PubMed: 12969871]
40. Lancioni C, Nyendak M, Kiguli S, Zalwango S, Mori T, Mayanja-Kizza H, Balyejusa S et al., CD8+ T cells provide an immunologic signature of tuberculosis in young children. *Am. J. Respir. Crit. Care Med* 2012 185: 206–212.
41. Harari A, Dutoit V, Cellerai C, Bart PA, Du Pasquier RA and Pantaleo G, Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol. Rev* 2006 211: 236–254. [PubMed: 16824132]
42. Serbina NV, Liu CC, Scanga CA and Flynn JL, CD8 +CTL from lungs of Mycobacterium tuberculosis-infected mice express perforin in vivo and lyse infected macrophages. *J. Immunol* 2000 165: 353–363. [PubMed: 10861072]
43. Rook GA, Lowrie DB and Hernandez-Pando R, Immunotherapeutics for tuberculosis in experimental animals: is there a common pathway activated by effective protocols? *J. Infect. Dis* 2007 196: 191–198. [PubMed: 17570105]
44. Murray RA, Mansoor N, Harbacheuski R, Soler J, Davids V, Soares A, Hawkrigde A et al., Bacillus Calmette Guerin vaccination of human newborns induces a specific, functional CD8+ T cell response. *J. Immunol* 2006 177: 5647–5651. [PubMed: 17015753]
45. Young DB, Gideon HP and Wilkinson RJ, Eliminating latent tuberculosis. *Trends Microbiol* 2009 17: 183–188. [PubMed: 19375916]
46. Barry CE 3rd, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, Schnappinger D et al., The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat. Rev. Microbiol* 2009 7: 845–855. [PubMed: 19855401]
47. Zimmerli SC, Harari A, Cellerai C, Vallelian F, Bart PA and Pantaleo G, HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc. Natl. Acad. Sci. USA* 2005 102: 7239–7244. [PubMed: 15872023]
48. Hersperger AR, Martin JN, Shin LY, Sheth PM, Kovacs CM, Cosma GL, Makedonas G et al., Increased HIV-specific CD8+ T-cell cytotoxic potential in HIV elite controllers is associated with T-bet expression. *Blood* 2011 117: 3799–3808. [PubMed: 21289310]
49. Harari A, Cellerai C, Enders FB, Kostler J, Codarri L, Tapia G, Boyman O et al., Skewed association of polyfunctional antigen-specific CD8 T cell populations with HLA-B genotype. *Proc. Natl. Acad. Sci. USA* 2007 104: 16233–16238. [PubMed: 17911249]
50. Roederer M, Nozzi JL and Nason MC, SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* 2011 79A: 167–174. [PubMed: 21265010]

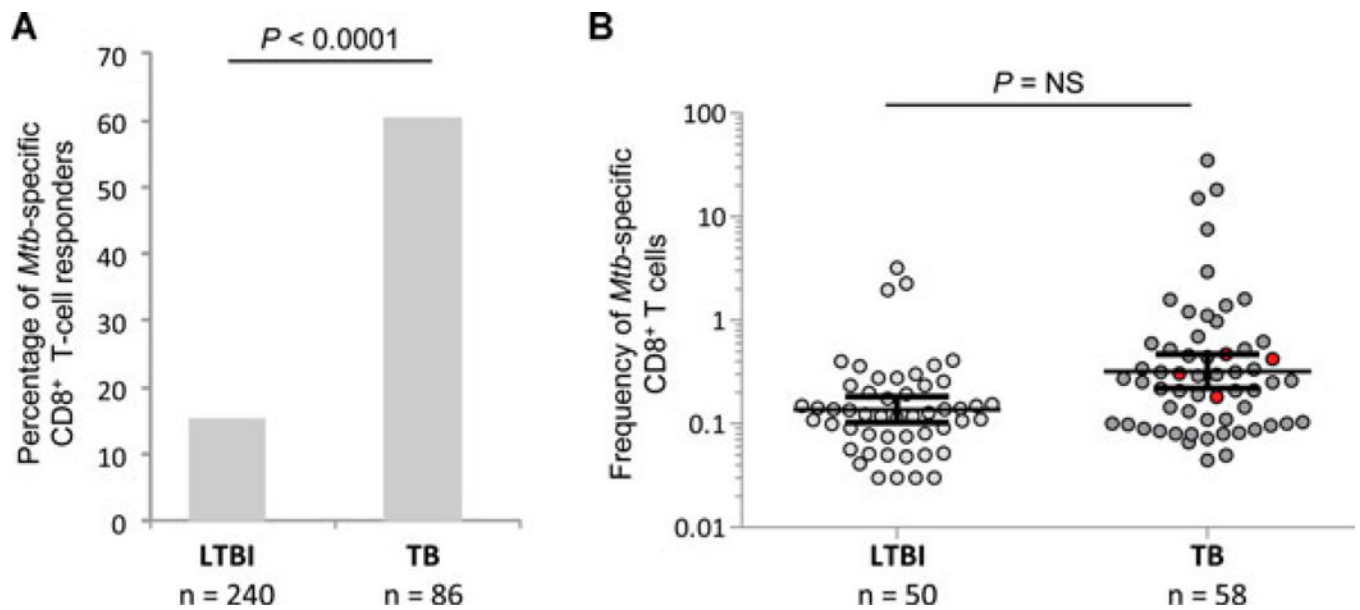


Figure 1.

Detection and magnitude of *Mtb*-specific CD8⁺ T-cell responses in LTBI subjects and TB patients: (A) Proportion of LTBI subjects and TB patients with *Mtb*-specific CD8⁺ T-cell responses. Statistical significance was calculated using two-tailed Fisher's exact test. CD8⁺ T cells were gated as shown in Supporting Information Fig. 1A. (B) Magnitude (mean with 95% CI) of *Mtb*-specific CD8⁺ T-cell responses in the 37 LTBI and 52 TB patients with *Mtb*-specific CD8⁺ T-cell responses. *Mtb*-specific CD8⁺ T-cell responses were defined by the presence of IFN- γ -producing CD8⁺ CD4⁻ CD3⁺ T cells following stimulation with ESAT-6 and/or CFP-10 peptide pools. Red points identify *Mtb*-specific CD8⁺ T-cell responses from HIV-coinfected subjects. An unpaired two-tailed Student's *t*-test was performed.

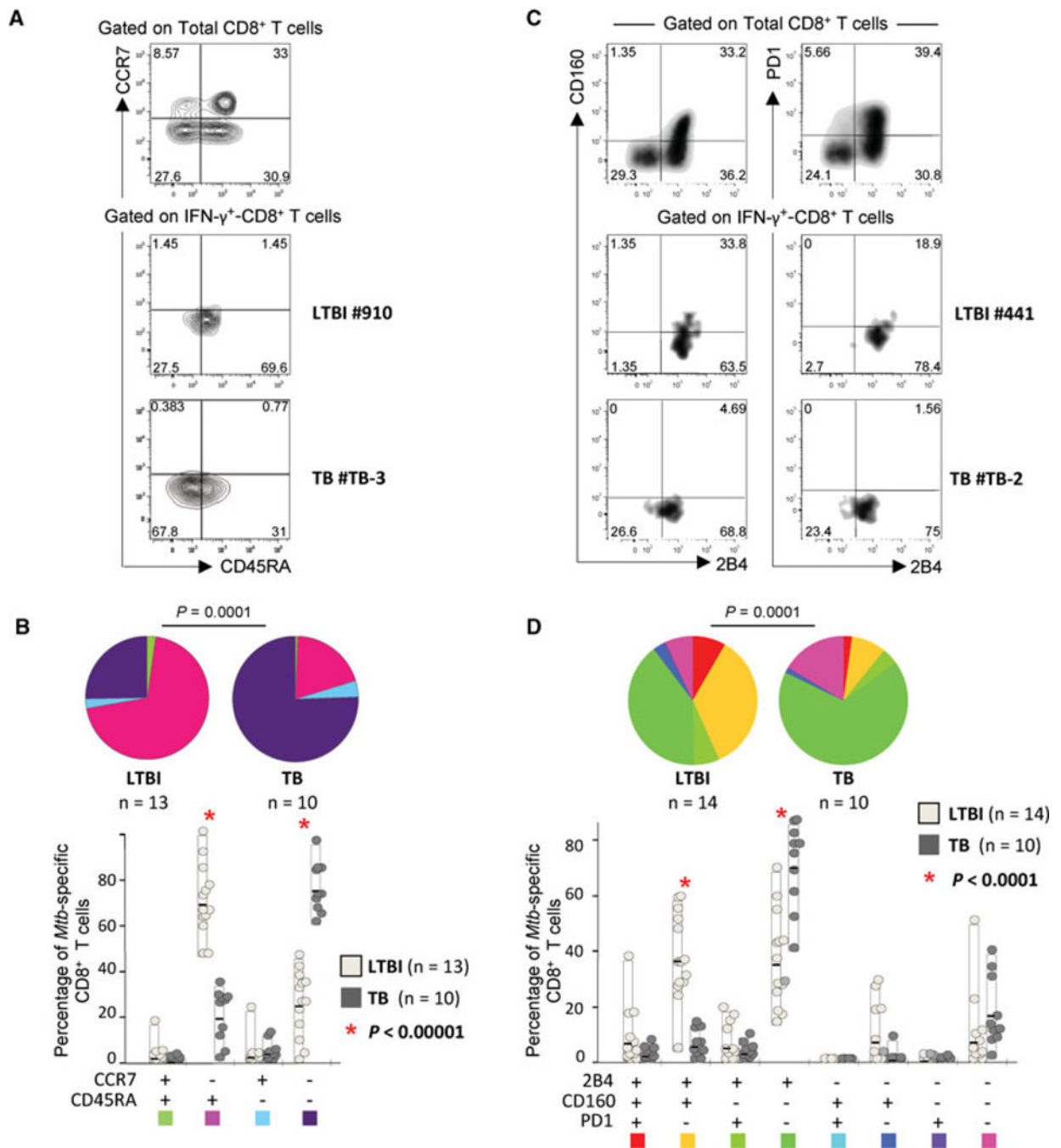


Figure 2. T-cell differentiation and exhaustion of *Mtb*-specific CD8⁺ T cells in LTBI subjects and TB patients. (A) Representative flow cytometry examples and (B) cumulative analyses of the expression of CCR7 and CD45RA on *Mtb*-specific CD8⁺ T cells from LTBI subjects and TB patients are shown. CD8⁺ T cells were gated as shown in Supporting Information Fig. 1A. As in all other flow cytometry analyses from this study, the gating is based on the distribution of the different markers on bulk CD8⁺ T cells (A; top) and it is then conserved in the analyses of *Mtb*-specific CD8⁺ T cells (A; bottom). (C) Representative flow cytometry

examples and (D) cumulative analyses of the expression of PD-1, 2B4, and CD160 on *Mtb*-specific CD8⁺ T cells from LTBI subjects and TB patients are shown. (A, C) Flow cytometry profiles are gated on live CD3⁺ CD4⁻ CD8⁺ T cells and *Mtb*-specific CD8⁺ T-cell responses were defined as IFN- γ -producing cells following stimulation with ESAT-6 and/or CFP-10 peptide pools. (B, D) For cumulative analyses, all the possible combinations of the different markers are shown on the *x*-axis whereas the percentages of the distinct T-cell subsets within *Mtb*-specific CD8⁺ T cells are shown on the *y*-axis. The pie charts summarize the data, and each slice corresponds to the mean proportion of *Mtb*-specific CD8⁺ T cells positive for a certain combination of markers. (B, D) Comparisons of markers distribution were performed using a Student's *t*-test and a partial permutation test as described [50].

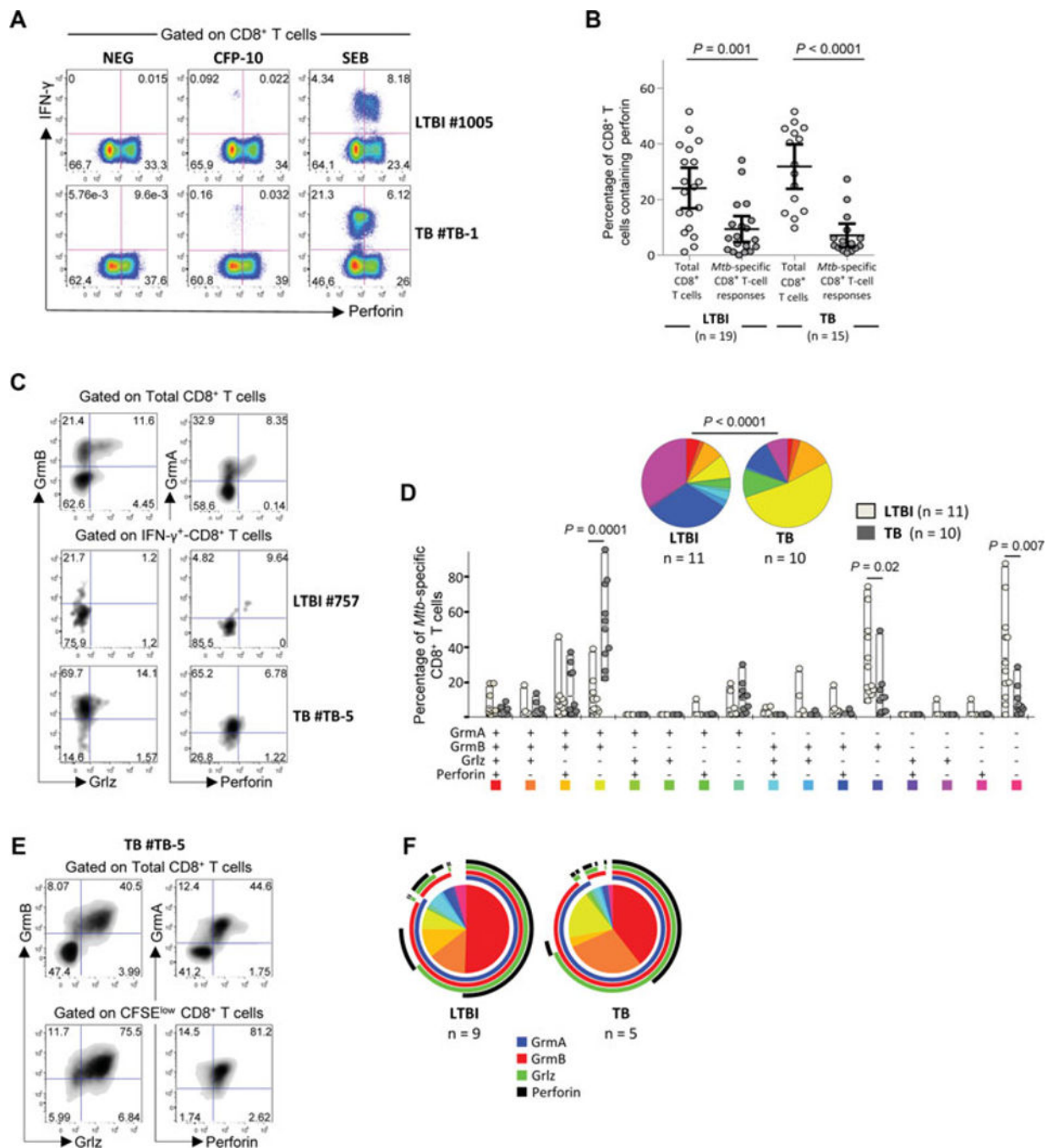


Figure 3. Cytotoxic potential of *Mtb*-specific CD8⁺ T-cell responses in LTBI subjects and TB patients. (A) Flow cytometric profiles showing perforin expression on *Mtb*-specific IFN- γ -producing CD8⁺ T cells in representative LTBI subjects and TB patients. CD8⁺ T cells were gated as shown in Supporting Information Fig. 1A. The flow cytometric profiles of unstimulated cells (negative control) and cells stimulated with a polyclonal stimulation (positive control) are also shown. Flow cytometry profiles are gated on live CD3⁺ CD4⁻ CD8⁺ T cells and *Mtb*-specific CD8⁺ T-cell responses were defined as IFN- γ -producing cells following stimulation

with ESAT-6 and/or CFP-10 peptide pools. (B) Percentages (mean with 95% CI) of perforin expression in total- and *Mtb*-specific CD8⁺ T cells from LTBI ($n = 19$) or TB ($n = 15$) patients. Unpaired two-tailed Student's *t*-tests were performed. (C) Representative flow cytometry examples and (D) cumulative analyses of the expression of perforin, granzyme (Grm) B, GrmA, and granulysin (Grlz) on *Mtb*-specific CD8⁺ T cells from LTBI subjects and TB patients. All the possible combinations of the different markers are shown on the *x*-axis whereas the percentages of the distinct T-cell subsets within *Mtb*-specific CD8⁺ T cells are shown on the *y*-axis. (E) Representative flow cytometry example and (F) cumulative analyses of the expression of perforin, GrmB, GrmA, and Grlz on *Mtb*-specific CD8⁺ T cells from LTBI subjects and TB patients after 6 days of antigen-specific in vitro T-cell expansion. (D, F) The pie charts summarize the data, and each slice corresponds to the mean proportion of *Mtb*-specific CD8⁺ T cells positive for a certain combination of markers identified by the respective arcs. Regarding SPICE analyses, comparison of distributions (D) was performed using a Student's *t*-test and a partial permutation test as described [50].

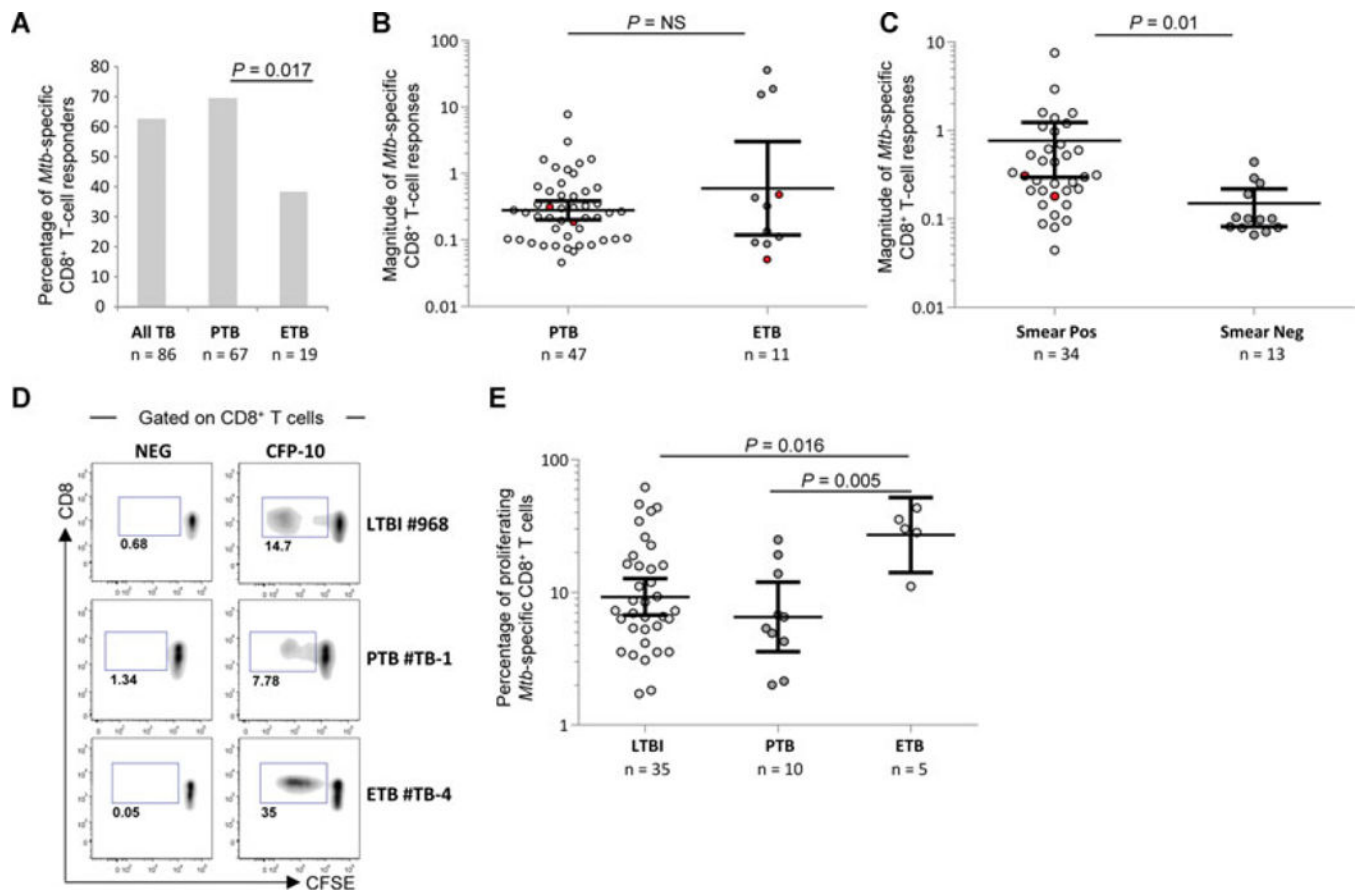


Figure 4.

Associations between *Mtb*-specific CD8⁺ T-cell responses and clinical presentation. (A) Proportion of patients with *Mtb*-specific CD8⁺ T-cell responses in all TB patients ($n = 86$) and in patients with pulmonary TB (PTB; $n = 67$) or extrapulmonary TB (ETB; $n = 19$). Statistical significance was calculated using two-tailed Fisher's exact test. (B) Magnitude (mean with 95% CI) of the frequency of *Mtb*-specific IFN- γ -producing CD8⁺ T-cell responses in PTB ($n = 47$) and ETB ($n = 11$) patients with detectable *Mtb*-specific CD8⁺ T-cell responses (two-tailed Mann-Whitney test). Red points identify *Mtb*-specific CD8⁺ T-cell responses from HIV-coinfected subjects. (C) Magnitude (mean with 95% CI) of the frequency of *Mtb*-specific IFN- γ -producing CD8⁺ T-cell responses within PTB patients subdivided into smear-positive ($n = 34$) and smear-negative ($n = 13$) patients. Red points identify *Mtb*-specific CD8⁺ T-cell responses from HIV-coinfected subjects. (D) Representative flow cytometry examples and (E) cumulative analyses (mean with 95% CI) of the frequency of *Mtb*-specific CD8⁺ T cells endowed with proliferation capacity in LTBI ($n = 35$), PTB ($n = 10$) and ETB ($n = 5$) patients. T-cell proliferation was determined using the CFSE dilution assay and profiles are gated on live CD3⁺ CD8⁺ CD4⁻ T cells as shown in Supporting Information Fig. 1B. Two-tailed Mann-Whitney tests were performed.