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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

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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

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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

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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 Grlz 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%, respec-tively; $p = 0.017$; 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.

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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

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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 Grlz 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.

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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 coinfected 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.

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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 μL/mL, BD) and anti-CD28 antibodies (0.5 μg/mL, BD) containing media as described [47]. Monensin (1 μL/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 μg/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 (Cytofix/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 μg/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

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Abbreviations:

ETB	extrapulmonary TB
LTBI	latent Mtb infection
Mtb	*Mycobacterium tuberculosis*
PTB	pulmonary TB
TB	tuberculosis

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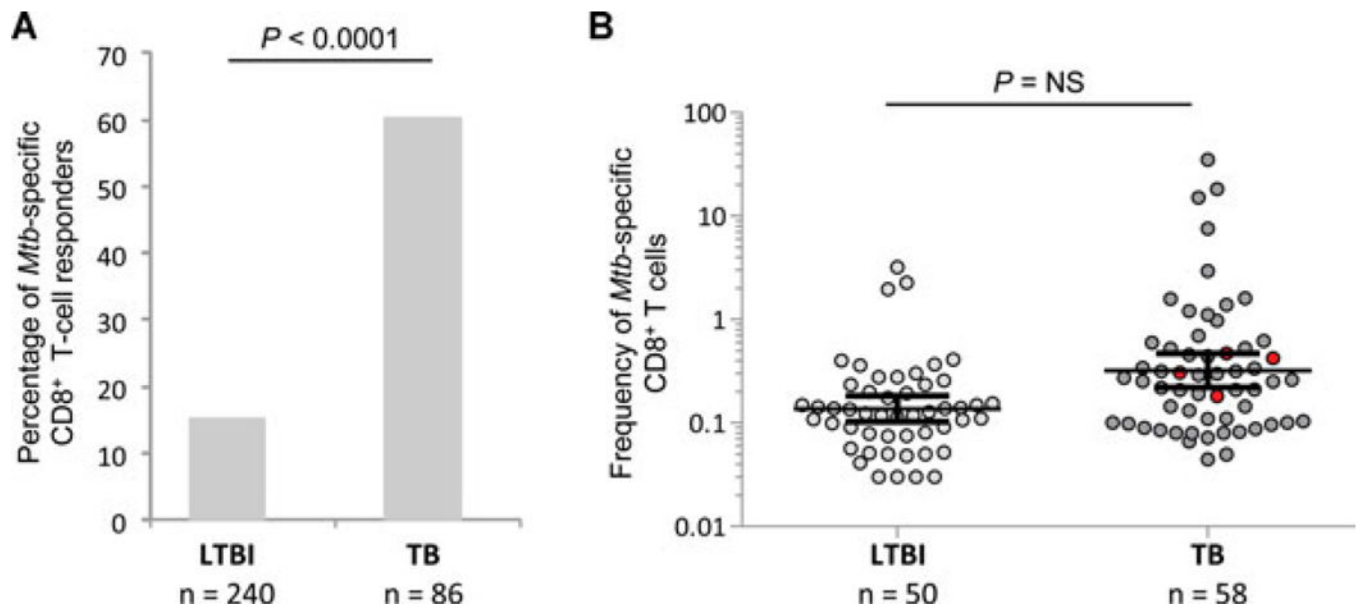

Figure 1.

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.

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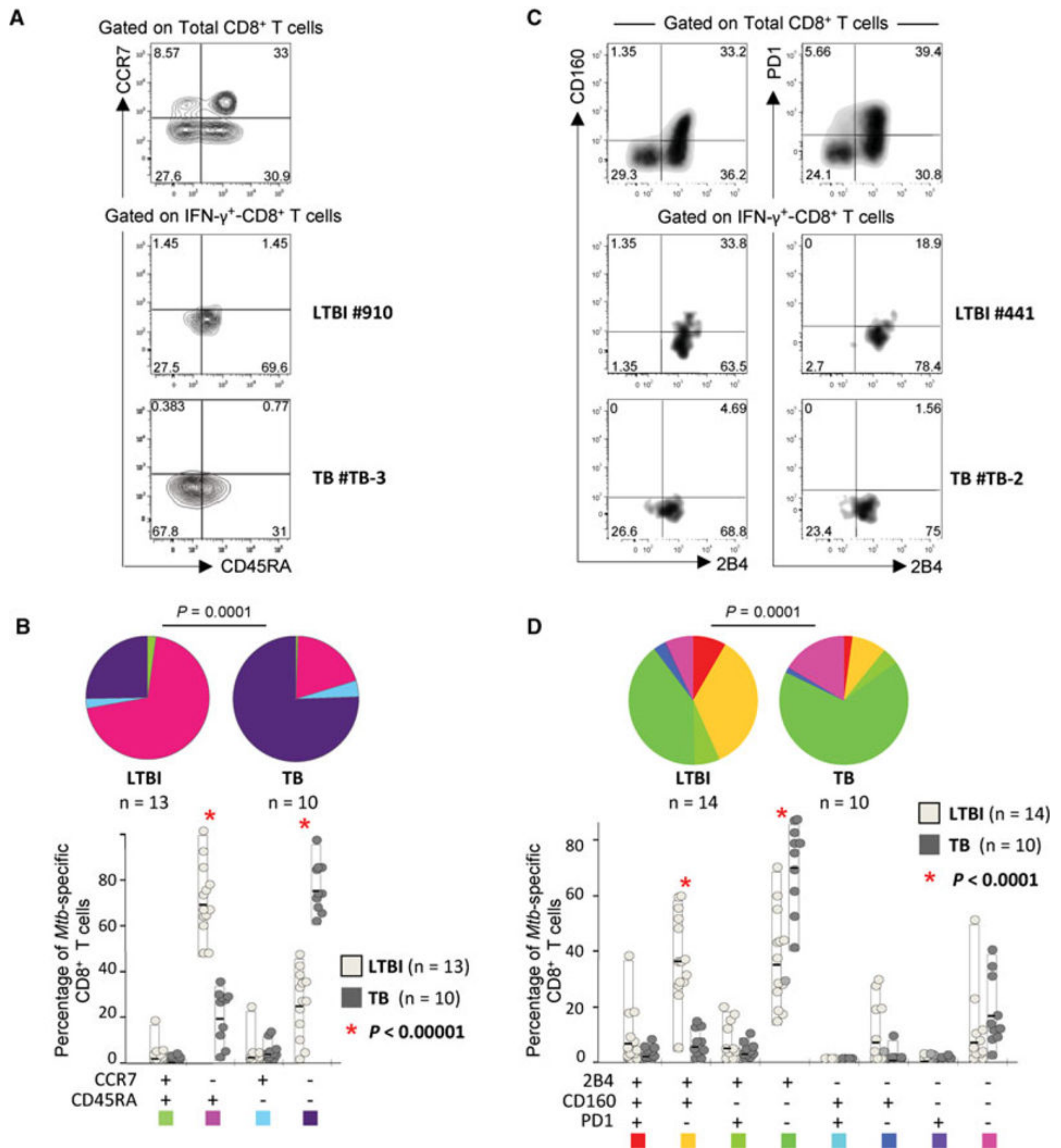

Figure 2.

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].

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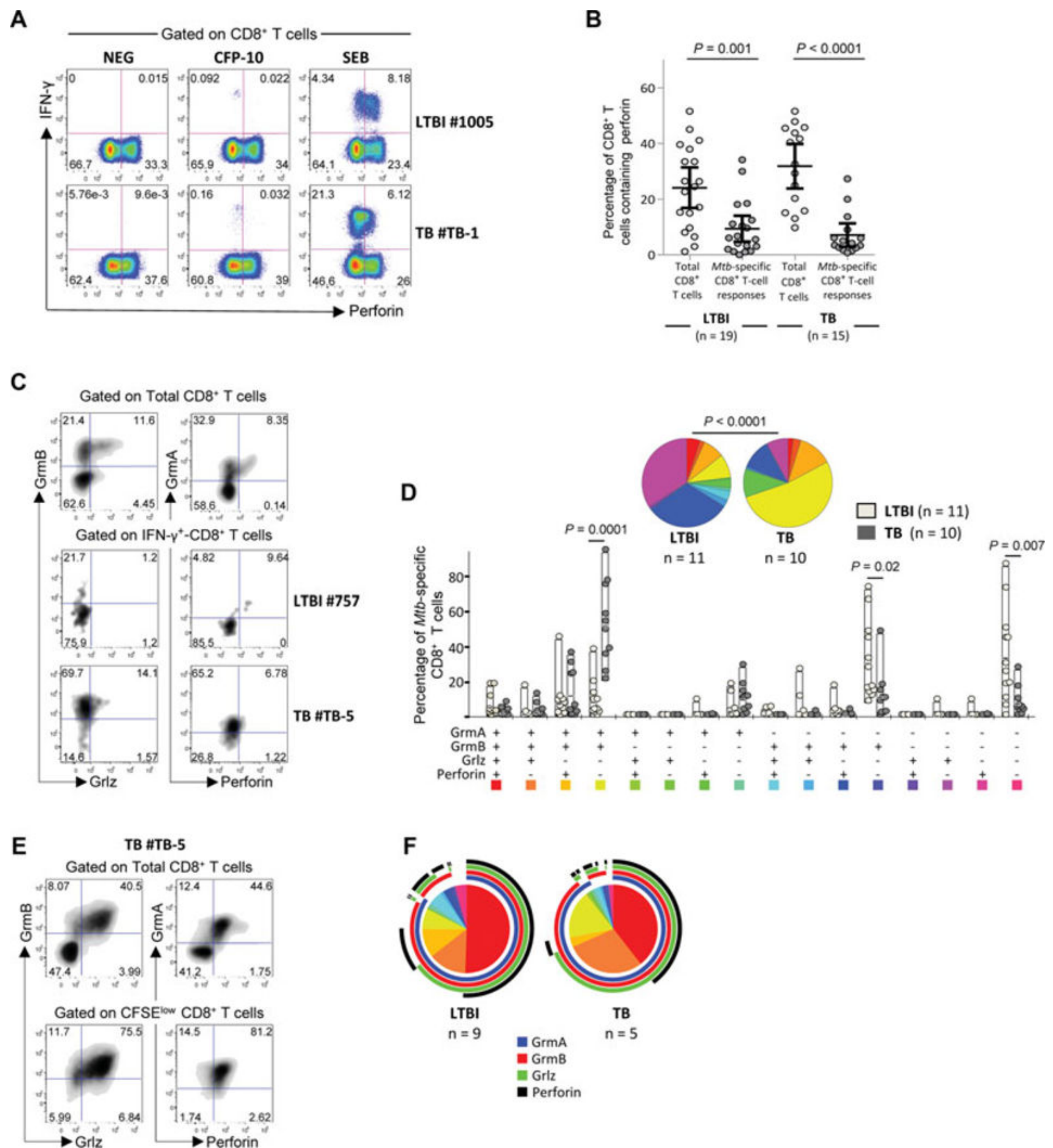

Figure 3.

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

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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].

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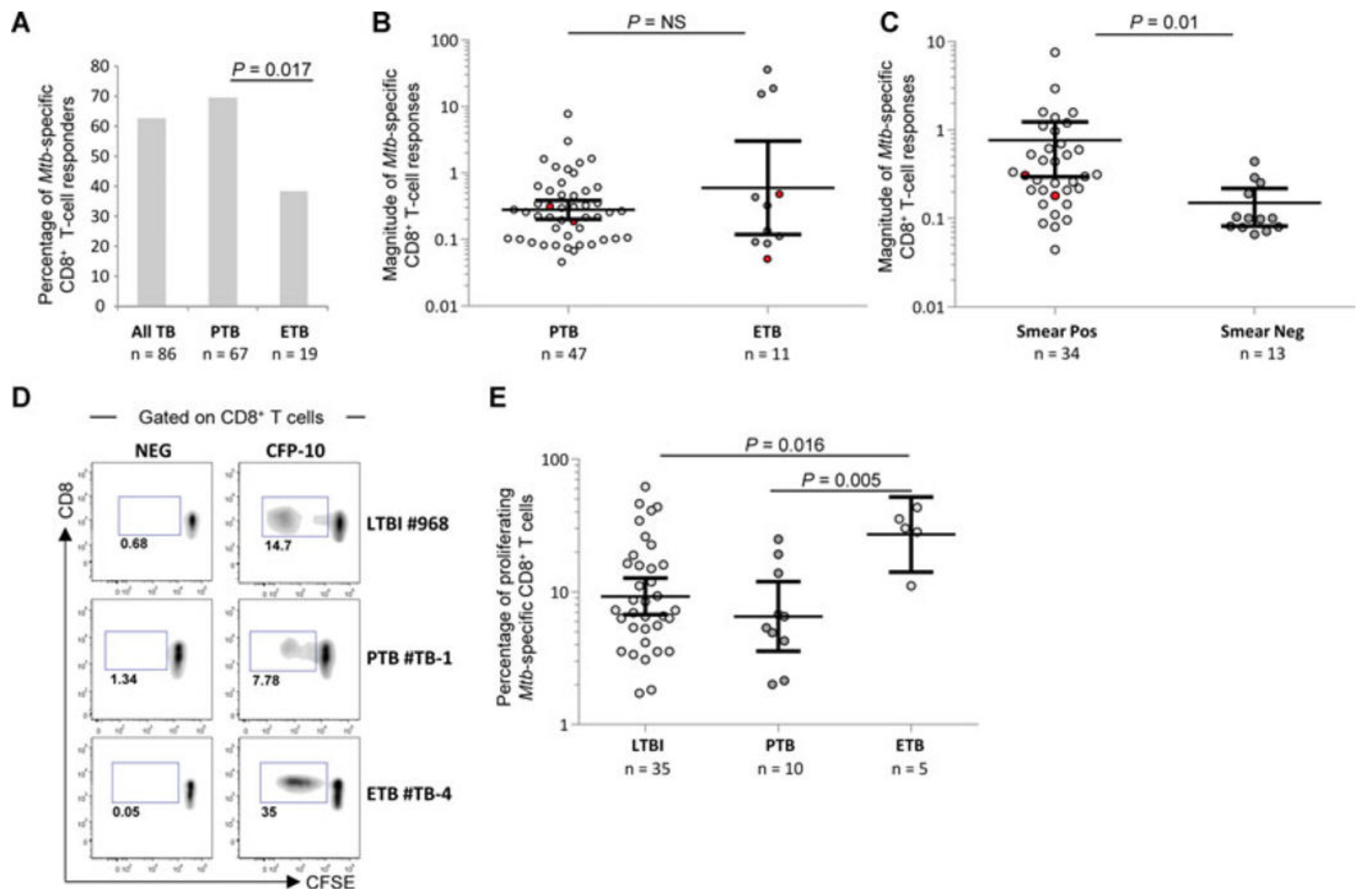

Figure 4.

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.