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Different phenotypes of CD8+ T cells associated with bacterial load in active tuberculosis

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ABSTRACT

Tuberculosis is an infectious disease that affects millions of people worldwide with an annual mortality rate of 1.3 million. The mechanisms contributing to the loss of balance of immune responses and progression to active tuberculosis disease are unknown. Although CD4+ and CD8+ T cells and the cytokines they produce are crucial for protection against tuberculosis they have different roles in tuberculosis immunology. The function of CD4+ T cells has been extensively studied; however, less is known about the phenotype and function of CD8+ T cells. This study evaluated the specific expression of IFN-γ, IL-17, IL-10, and TGF-β and *ex vivo* expression of perforin and granzyme-B by CD8+ T cells from active tuberculosis individuals compared with latent infected individuals and non-latent infected individuals. Tuberculosis responses were correlated with the baciloscopy score. We observed that the presence of IL-10 and TGF-β expression and down-expression of granzyme-B in CD8+ T cells correlated with increased sputum bacillary load in active tuberculosis individuals. These findings provide new insights into the role of CD8+ T cells in *Mycobacterium tuberculosis* disease.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb) that affects millions of people worldwide with a mortality rate of 1.3 million people. The dissemination of multi-drug resistant Mtb and the continuing HIV/AIDS outbreak has led to the perpetuation of a global epidemic of TB. According to the World Health Organization, one-third of the world's population is latently infected with Mtb (LTBI), of which 5–10% develop active TB [1]. However, the exact mechanisms contributing to the loss of balance of immune responses and the progression to active TB disease are unknown.

T-cell immunity is crucial for Mtb infection control and both CD4+ and CD8+ T cells play important roles in host protection. Although IFN-γ has a vital function against Mtb infection [2–4], a complex network of other cytokines, such as IL-17 [3–6], IL-6 [4,7,8], IL-10 [4,9,10] and TGF-β [11] may play a role in the immunopathogenesis of Mtb infection. Additionally, Harari et al. [12] and Rozot et al. [13] demonstrated that CD4+ and CD8+ T-cell subpopulations were functionally different in patients with active TB and subjects with LTBI, respectively, suggesting a different role of these cells in the immunology of TB [12,13].

While most studies have focused on the characterization of CD4+ T cells in TB, less is known about the phenotype and function of CD8+ T cells and their mechanisms of action, which remain highly controversial. Different roles of Mtb-specific CD8+ T cells in immunity to human Mtb infection have been proposed [13–16]. The main function ascribed to CD8+ T cells is cytolytic activity to kill infected cells mediated by cytotoxic granules such as perforin, granzyme B and granulysin, with residual Fas-L and TNFR-dependent cytolytic activity [17]. In addition, CD8+ T-cell-produced cytokines have also been the subject of much research [6,13,14]. IL-17-secreting CD8 (Tc17) effector cells have been described in healthy subjects [18] and in tuberculous pleural effusion [6] and have been associated with the pathogenesis of many diseases in mice [19–21]. However, their role in pulmonary tuberculosis is unknown. Recently, many studies have described and established CD8+ T cells with regulatory functions [13,16,22,23], but their involvement in the pathogenesis

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of TB has not been clarified. Highlighting novel functions of CD8+ T cells in the immunology of TB may help elucidate the mechanisms involved in disease pathogenesis.

Active pulmonary TB is the most common clinical manifestation of TB and is characterized by high levels of bacterial replication and the presence of acid-fast bacilli (AFB) in sputum. Although there is no direct quantitative measurement of bacillary load, previous clinical studies indicated that sputum smear grading at the time of diagnosis (negative, scanty, 1+, 2+, or 3+) is predictive of relapse and recurrence of TB disease [24,25]. Moreover, sputum smear grading correlates with disease severity by chest radiograph scoring [26]. Recently, studies correlated cytokine production from lymphocytes and disease severity. TB severity was associated with increased levels of CD4+ IFN-γ+IL-17+ and CD8+ T cells and reduced levels of polyfunctional or IL-2-producing Mtb-specific CD4+ T cells [3,27]. The aim of this study was to evaluate CD8+ T-cell-specific cytokine and perforin/granzyme expression in active TB and correlate its response with the baciloscopy score.

2. Materials and methods

2.1. Study populations

This was a transversal descriptive study composed of three groups: TB, composed of active pulmonary patients; LTBI, composed of latent Mtb infected individuals, and CS, composed of non-latent infected individuals. TB patients were consecutively recruited at the Hospital de Doenças Tropicais and Hospital das Clínicas in Goiânia, and Centro de Atenção Integral a Saúde do Jardim Nova Era (CAIS Jd. Nova Era) in Aparecida de Goiânia, Goiás, Brazil and 36 patients who presented with inclusion criteria agreed to participate in this study. TB patients were recruited independent of sex and race and inclusion criteria for TB patients were pulmonary TB diagnosis based on epidemiologic history, signs, and symptoms, and chest X-ray findings consistent with TB. All included patients had positive sputum smear microscopy within 15 days of TB treatment. The number of AFB in sputum smears was counted according to the World Health Organization guidelines [28], and three groups of TB patients were enrolled: 1+ (10–99 AFB per 100 fields); 2+ (1–10 AFB per field in 50 fields), or 3+ (more than 10 AFB per field in 20 fields). During the same period, healthy individuals with no TB and without symptoms of other investigative diseases were recruited at the Universidade Federal de Goiás. Healthy individuals were recruited, matched by sex and age to TB patients, and classified according to the tuberculin skin test (TST). Exclusion criteria for all individuals were pregnancy, positive serology for HIV, and aged younger than 18 years. All studies were approved by the Hospital of the Federal University of Goiás Ethics Committee (protocol number: 055/2009), and informed consent and a clinical questionnaire were obtained from all participating subjects.

2.2. Tuberculin skin test

Healthy individuals ($n = 36$) were administered the TST to characterize latent Mtb infection. All individuals received 0.1 mL of purified protein derivative (PPD-RT 23 Mtb-2 UT, Copenhagen, Denmark) intradermally (Mantoux technique) in the left forearm. The size of skin induration was measured along the longer transverse axis 72 h later and the result was expressed in millimeters. Individuals with TST ≤ 9 mm were classified as TST− and were included in the CS group ($n = 23$). Individuals with TST > 10 mm were classified as TST+ and were included in the LTBI group ($n = 13$) according to the World Health Organization classification [29].

2.3. Peripheral blood mononuclear cells and culture

Sixteen milliliters of peripheral blood was collected in heparinized tubes from each individual and peripheral blood mononuclear cells (PBMCs) were isolated by gradient density centrifugation using Ficoll (Ficoll-Paque Plus, GE Healthcare Bio-Sciences AB). The cells were washed twice in saline and a total of 2×10^5 cells/well were cultured in 96-well culture plates for 96 h at 37 °C with 5% CO_2 and recombinant Mtb antigen (rMPT51, 2 μg/mL) in the presence of anti-CD3 (eBioscience) and RPMI 1640 supplemented medium as previously reported [30]. PBMCs cultured in the medium or phytohemagglutinin (PHA, 2 μg/mL) were used for negative and positive controls, respectively.

2.4. Flow cytometry

Cell surface and intracellular staining was performed using the following monoclonal antibodies: IFN-γ-FITC, CD8-APC, granzyme-B-PE (BD Pharmingen, San Jose, CA, USA), IL-10-PE, IL-17-PE (eBioscience), TGF-β-PE (IQ Products), and IgG1-FITC and IgG1-PE (BD Pharmingen) as an isotype control according to the manufacturer's instructions. Cells were labeled with CD8-APC and with rat IgG1-PE isotype control to set up a gate for the IL-17, granzyme-B, IL-10, and TGF-β positive cells. Cells were also labeled with CD8-APC and rat IgG1-FITC isotype control to set up a gate for IFN-γ and perforin-positive cells. Briefly, cells cultured with medium alone, PHA, or TB antigen were treated with Golgi Stop Solution (containing monensin, BD Pharmingen) and after 4–6 h of further incubation, they were harvested for analysis. The cells were treated with PBS containing 0.05% azide for 20 min prior to surface and intracellular staining. After centrifugation 800 × *g* 10 min, cells were stained at 4 °C for 18 min with CD8-APC. Subsequently, the plates were washed twice with PBS containing 0.05% azide and treated with PermFix (BD Pharmingen) for 18 min. For intracellular staining, cells were permeabilized with Perm Wash buffer (BD Pharmingen) and incubated at 4 °C for 18 min with the following specific antibodies: IFN-γ-FITC, Perforin-FITC, granzyme-B-PE (BD Pharmingen), IL-10-PE, IL-17-PE (eBioscience), and TGF-β-PE (IQ Products). After washing, the samples were immediately analyzed on a FACSCanto II apparatus (Becton Dickinson) at the FARMATEC/UFG (Goiás, Brazil). At least 50,000 events were acquired per sample. Data analysis was performed using FACSDiva software (BD Becton Dickinson).

2.5. IL-6 detection

Levels of IL-6 in the plasma were measured using standard Human IL-6 ELISA Ready-SET-Go! Kit (eBioscience) according to the manufacturer's instructions. Concentrations of IL-6 in the samples were calculated with a standard curve generated from recombinant IL-6, and results were expressed as pg/mL. The sensitivity of the assay was 2 pg/mL.

2.6. Statistical analysis

The mean and standard error of the mean (SEM) were calculated. After assessing normality of all data by quantile plots, one-way analysis of variance was used to compare variances between the groups. As the variance magnitude from each group was similar, an *F*-test and *p*-values were calculated to predict the differences between the mean results of each group. A *post hoc* test (Kruskal–Wallis test) was used to evaluate whether each specific group pair was different from each other. A p value less than < 0.05 was considered statistically significant. To calculate the relationship between IL-10 and TGF-β T CD8 expression, the mean fluoresce

Table 1
Characteristics of subjects.

Characteristics	Tuberculosis ($n = 36$)	LTBI TST+ ($n = 13$)	Control subjects ($n = 23$)
Male/female, *n* (%)	22/14 (61.1/38.9)	6/7 (46.2/53.8)	16/7 (69.5/30.4)
Mean age (minimum–maximum)	41.8 (20–71)	38.9 (22–72)	35.4 (22–69)
BCG, *n* (%)	27 (75)	11 (84.6)	23 (100)
History of contact with TB, *n* (%)	13 (36.1)	9 (69.2)	7 (30.4)
Current smokers, *n* (%)	18 (50)	5 (38.4)	6 (26.1)
Concomitant diseases, *n* (%)			
Hypertension	5 (16.6)	3 (23.7)	1 (4.3)
Diabetes mellitus	3 (8.3)	0 (0)	0 (0)
Anemia	2 (5.5)	0 (0)	0 (0)
Gout	2 (5.5)	0 (0)	0 (0)
Pneumonia	2 (5.5)	0 (0)	2 (8.6)
Cardiovascular	2 (5.5)	1 (7.6)	0 (0)
Gastrointestinal	1 (2.7)	0 (0)	3 (13)
Autoimmune	2 (5.5)	0 (0)	0 (0)
Other diseases	3 (8.3)	1 (7.6)	4 (17.3)
Presence of cavity	10 (27.7)	ng	ng
Smear grade of bacillary load, *n* (%)			
1+	14 (38.9)	ng	ng
2+	6 (16.6)	ng	ng
3+	16 (44.5)	ng	ng
[a]Percentage of CD8+ T cells (min – max)	37.2 ± 4.2 (31–44.8)	30.4 ± 3.9 (24.3–37.4)	30.9 ± 6.9 (18.5–40.5)
[a]Total number of CD8+ T cells × 10^5 (min – max)	4.0 ± 0.7 (3.1–5.3)	2.2 ± 0.3 (1.8–2.6)	1.6 ± 0.4 (1.0–2.3)

TB: tuberculosis; TST: tuberculin skin test; BCG: Bacillus Calmette Guérin; ng: negative. TB patients were divided according to bacillary load: 1+ (10–99 acid-fast bacilli (AFB) per 100 fields); 2+ (1–10 AFB per field in 50 fields), or 3+ (>10 AFB per field in 20 fields).
[a] PBMC CD8T cells.

for each cytokines for an individual were plotted in a *xy* graph and a linear regression was determined.

3. Results

3.1. Characteristics of study participants

The characteristics of TB patients and healthy individuals are shown in Table 1. A total of 72 individuals (36 patients with TB and 36 healthy individuals in LTBI ($n = 13$) and CS ($n = 23$) groups) were included in the study. Recently diagnosed pulmonary TB patients with a mean age of 41.8 years were mainly male (61.1%), the majority of whom were BCG vaccinated. Only 36.1% of TB patients had previous contact with active TB. The majority of TB patients ($n = 16$) presented with a high bacillary load and 27.7% ($n = 10$) presented with a cavity by X-ray but did not present with multi-drug-resistant TB. Concomitant predominant diseases in these patients were hypertension and diabetes mellitus. Fifty percent of TB patients were smokers (Table 1). Among LTBI individuals ($n = 13$) the mean age was 38.9 years and 69.2% had previous contact with TB. The majority was BCG vaccinated and three individuals presented with hypertension. The control subject group (non-infected individuals) had a mean age of 35.4 years and all were vaccinated with BCG before 6 months of age. Around 30% of CS individuals had previous contact with TB. A large percentage of the healthy individuals were smokers (LTBI = 38.4% and CS = 26.1%).

3.2. Specific Tc1 and Tc17 cells and IL-6 in active tuberculosis

PBMCs from TB, LTBI, and CS were analyzed for IFN-γ (Tc1) and IL-17 (Tc17) expression after rMPT51 stimulus (Fig. 1A). Specific Tc1 (Fig. 1B) and Tc17 (Fig. 1C) cells were induced in active TB but not in LTBI or CS individuals ($p < 0.05$). No differences were observed in double-positive (IFN-γ+IL-17+) CD8+ T cells between all groups (Fig. 1A and data not shown). The specific Tc1/Tc17 ratio was significantly higher in TB patients (Fig. 1D) compared with LTBI and CS individuals ($p < 0.05$). Therefore, during active TB, specific Tc1 and Tc17 cells were generated.

Levels of IL-6 in the plasma of all individuals involved in this study were quantified. We observed that TB patients had higher levels compared with LTBI or CS individuals (Fig. 1E; $p < 0.05$). Thus, active TB infection induced Tc1 and Tc17 cells and increased IL-6 levels.

3.3. Specific expression of IL-10 and TGF-β by CD8+ T cells in active tuberculosis

In addition to their proinflammatory functions, we investigated whether CD8+ T cells had an anti-inflammatory role in active TB. Expression of IL-10 and TGF-β is shown in Fig. 2. Both cytokines (IL-10; Fig. 2A and C and TGF-β; Fig. 2B and D) were induced in CD8+ T cells from TB patients in response to the Mtb antigen, when compared with LTBI or CS individuals ($p < 0.05$). Consequently, these results indicate that CD8+ T cells from active TB patients produce regulatory cytokines.

3.4. Cytotoxic granules of CD8+ T cells in tuberculosis

Next, we investigated whether CD8+ T cells from TB patients had a cytolytic molecules. We analyzed perforin and granzyme-B expression *ex vivo* in CD8+ T cells from patients with active TB, LTBI, and CS individuals. No difference was observed when perforin expression was analyzed (Fig. 3A and B). However, granzyme-B expression was down-regulated in CD8+ T cells from active TB patients (Fig. 3A, and C, $p < 0.05$). Overall, these results indicate that TB patients contain CD8+ T cells with cytolytic properties, although granzyme-B expression was impaired. In order to discard if this phenomenal was associated with a reduced CD8+ T cell population among pulmonary TB patients, the percentages of CD8+ T cells in the peripheral blood among TB, LTBI, and CS and the total numbers of CD8+ T cells were evaluated. TB patients presented higher numbers of CD8+ T cells (Table 1) when compared with LTBI or CS ($p < 0.05$) while the percentages were similar.

3.5. Bacillary load is associated with CD8+ T-cell phenotype

The bacillary load in the sputum smear of TB patients was used to classify the patients according to the different CD8+ T-cell

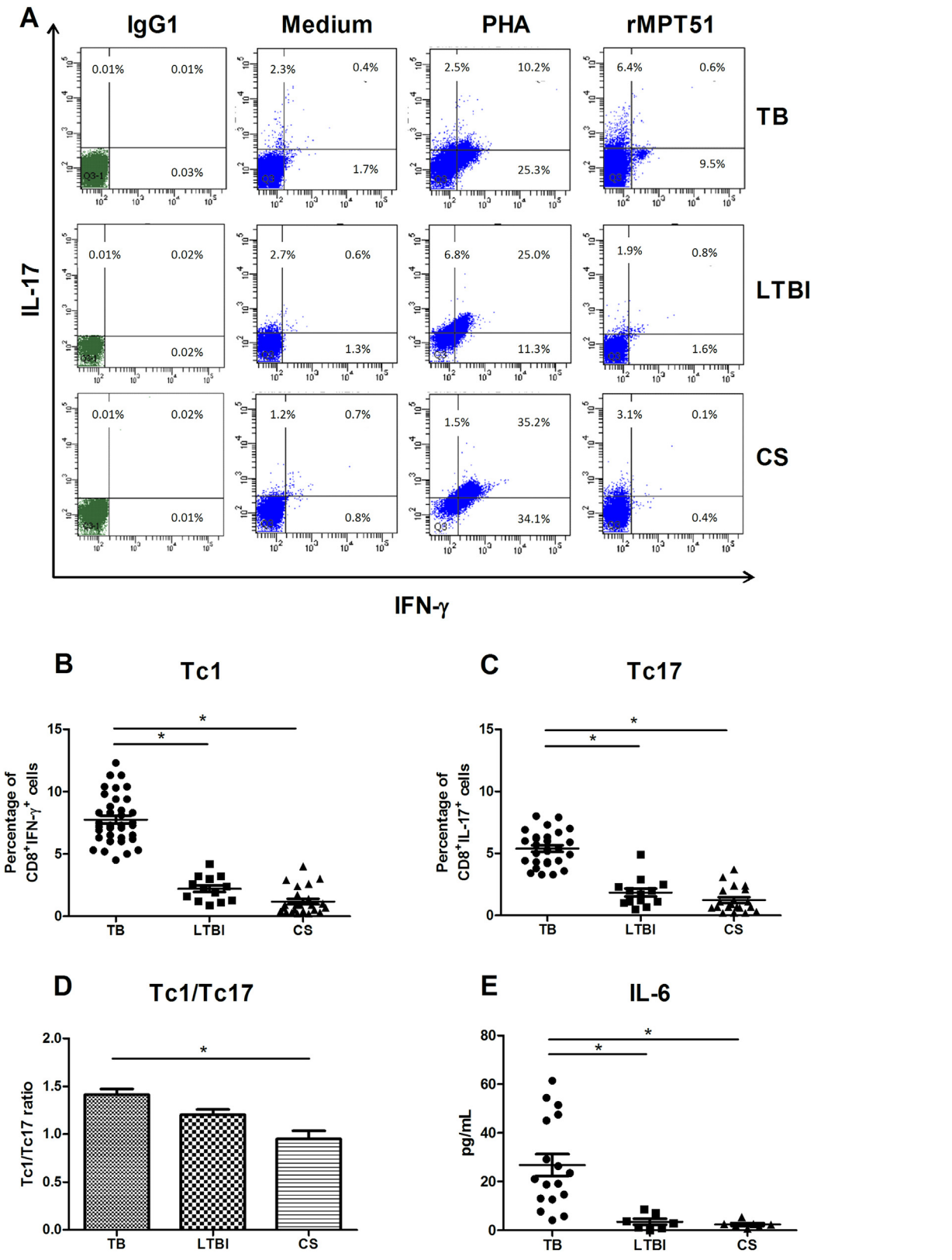

Fig. 1. Analysis of CD8+ T cells expressing IFN-γ and IL-17 and plasma levels of IL-6 in individuals with TB, LTBI, and CS. PBMC were cultured with medium alone, PHA (2 μg/mL) or rMPT-51 (2 μg/mL) for 96 h and the expression of cytokines were analyzed by flow cytometry. (A) Representative FACS plot of IFN-γ and IL-17 expression in CD8+ T cells after stimulation with medium alone, PHA or rMPT51. IgG1 was used as an isotype control. (B) Percentage of specific Tc1 (CD8+ IFN-γ+) cells among TB, LTBI, and CS individuals. (C) Percentage of specific Tc17 (CD8+ IL-17+) cells among TB, LTBI, and CS individuals. (D) Ratio of Tc1/Tc17 cells. (E) Plasma levels of IL-6 among TB, LTBI, and CS individuals. Data are expressed as the mean ± SEM. Significant differences between the groups (*$p < 0.05$).

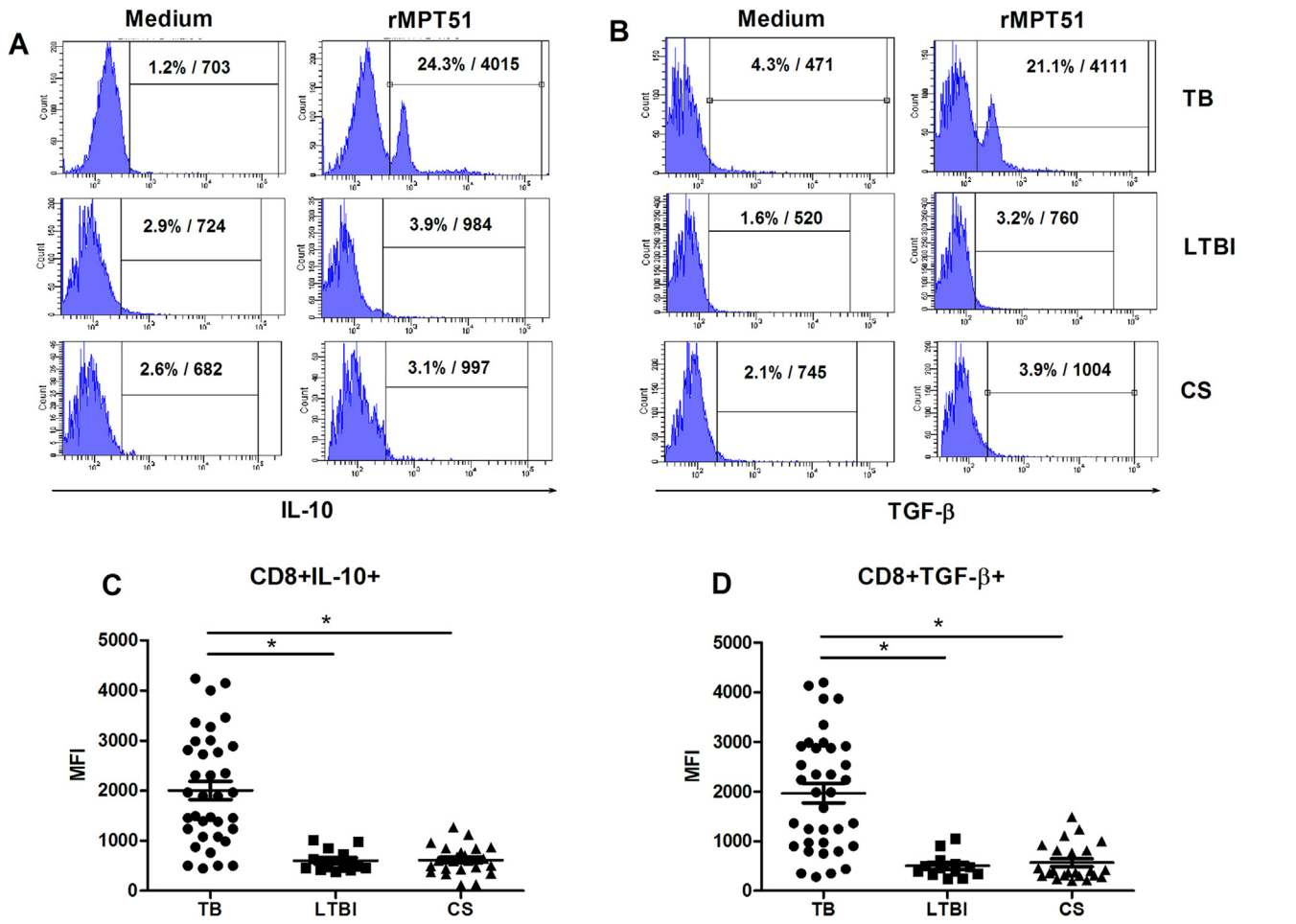

Fig. 2. Specific expression of IL-10 and TGF-β by CD8+ T cells in TB, LTBI, and CS. PBMC were cultured with medium alone, PHA (2 μg/mL) or rMPT-51 (2 μg/mL) for 96 h and the expression of cytokines was analyzed by flow cytometry. Representative FACS plot of mean fluorescence intensity (MFI) of IL-10 (A) and TGF-β (B) expression from CD8+ T cells of TB patients and LTBI or CS individuals stimulated with medium and rMPT51 are shown. The range gate was set using PBMC cultures without stimulation (medium) for each subject. The numbers are the percentage of CD8+ T cells positive for each cytokine and the mean fluorescence intensity of this population. (C) Specific IL-10 MFI of CD8+ T cells from TB patients and LTBI and CS individuals. (D) Specific TGF-β MFI of CD8+ T cells from TB patients and LTBI and CS individuals. Data are expressed as the mean ± SEM. Bars show significant differences between the groups (*$p < 0.05$).

phenotypes described in this study. For this, TB patients were stratified according to sputum smear grade of low (1+), medium (2+), or high (3+) bacillary load. The CD8+ T-cell expression of IL-10 (Figs. 4A and 5A) and TGF-β (Figs. 4B and 5B) was proportional to the bacillus quantity in the smear from TB patients. A positive correlation between IL-10 and TGF-β expression was observed ($r^2 = 0.60$) among all TB individuals. There was also a clear positive association of the bacillary load and the expression of those anti-inflammatory cytokines production by CD8+ T cells (Fig. 5C). Conversely, IFN-γ+ or IL-17+, CD8 T-cell numbers were similar among the studied groups, independent of the sputum bacillary load (Fig. 5D and E). Paradoxically, the expression of granzyme-B in CD8+ T cells diminished as the bacillary load increased in TB patients (Fig. 5F). These data indicate that patients with higher bacillary load in the sputum presented CD8+ T cells with a regulatory phenotype and diminished granzyme-B expression.

4. Discussion

In this study, we analyzed the specific expression of IFN-γ, IL-17, IL-10, and TGF-β cytokines and the *ex vivo* expression of perforin and granzyme-B in CD8+ T cells from TB, LTBI, and CS individuals. The phenotypes observed in CD8+ T cells were associated with bacillary load of Mtb in sputum smears from TB patients. Our results demonstrate that specific Tc1 and Tc17 cells are induced in active TB but not in LTBI or CS. In addition, TB patients presented with a higher expression of IL-10 and TGF-β from CD8+ T cells in contrast to the lower expression of granzyme-B. When these phenotypes were evaluated according to smear grade of bacillary load, TB patients with a higher smear grade had increased IL-10 and TGF-β expression and reduced expression of granzyme-B in CD8+ T cells. This is the first study, to the best of our knowledge, to demonstrate specific CD8+ T cells producing IL-17 in the peripheral blood of active TB patients and the direct association of different CD8+ T-cell phenotypes with the smear grade of bacillary load in TB patients.

The specific immunity to different Mtb antigens has been studied thoroughly. Some of those antigens, as for example MPT51, was shown to discriminate patients with active TB from healthy individuals by cellular and/or humoral responses [22,31], specially in HIV infected TB patients [32,33]. Previously we have shown that stimulating PBMC from TB patients with MPT51, CD8+ T cells were induced to produce both IL-10 and IFN-γ in similar levels as other antigens, like ESAT-6 and Ag85 [22]. Therefore justifying its use to evaluate CD8+ T cell cytolytic response. The MPT51 antigen is a protein of 27 kDa characterized as a non-catalytic α/β hydrolase that is involved in *M. tuberculosis* adhesion mechanisms contributing to the virulence, being produced in both active and latent phases of the disease [34].

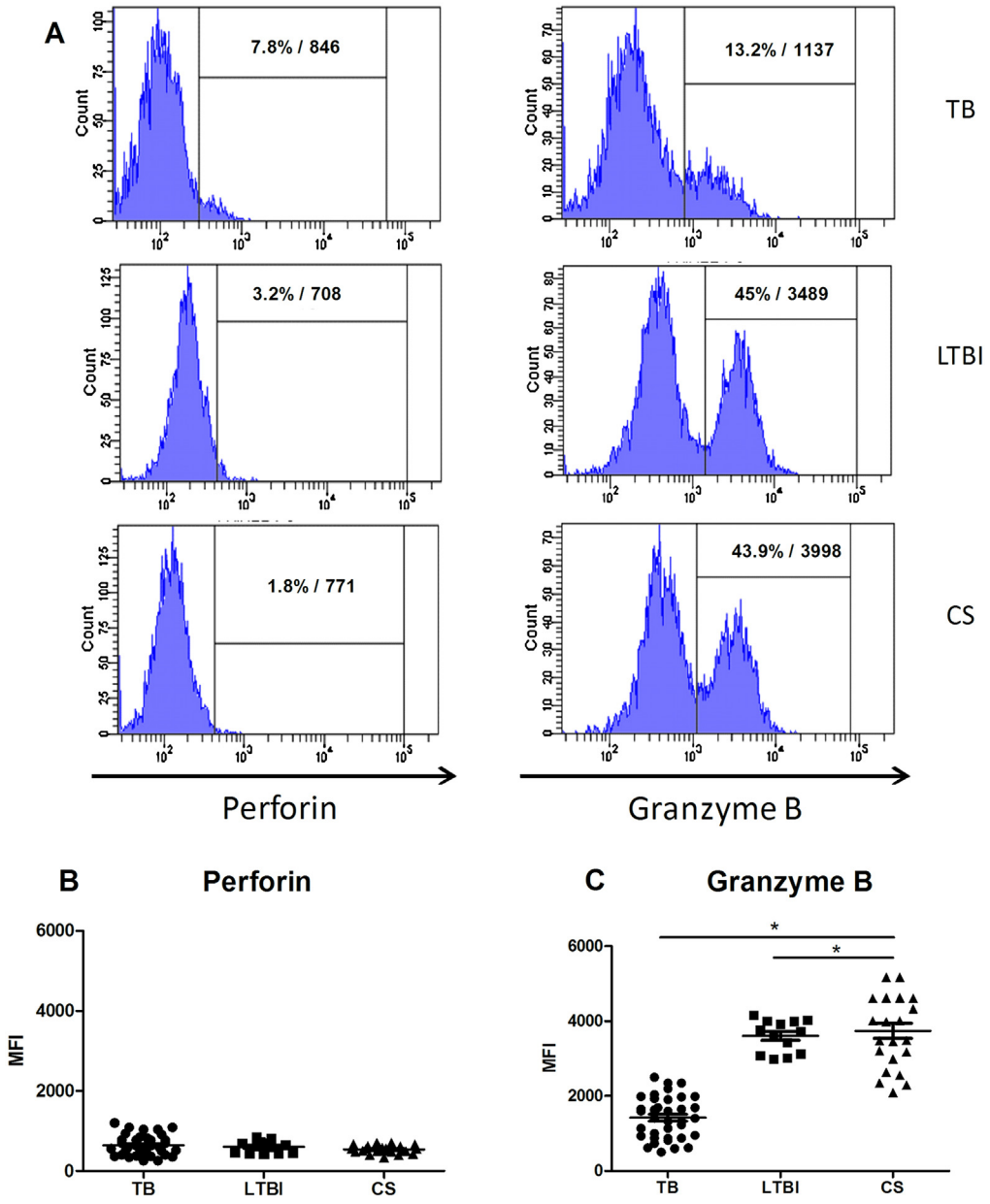

Fig. 3. *Ex vivo* expression of perforin and granzyme-B from CD8+ T cells in TB, LTBI, and CS individuals. PBMC were obtained from all individuals and the expression of cytokines was analyzed by flow cytometry. (A) Representative FACS plot of perforin and granzyme-B expression from CD8+ T cells. The numbers are the percentage of CD8+ T cells positive for each cytokine and the mean fluorescence intensity of this population. (B) *Ex vivo* perforin mean fluorescence intensity (MFI) of CD8+ T cells from TB patients and LTBI or CS individuals. (C) *Ex vivo* MFI of granzyme-B in CD8+ T cells from TB patients and LTBI or CS individuals. Data are expressed as the mean ± SEM. Bars show significant differences between the groups (*$p < 0.05$).

IFN-γ expression from CD8+ T cells of patients with active TB reinforced the evidence that IFN-γ is crucial for immune responses to control Mtb during the active phase of the disease [2–4,8] and can be produced by different cells [2,35]. In addition, CD8+ T cells from TB patients were positive for IFN-γ independently of the sputum bacterial load or the expression of IL-17 (Figs. 1 and 5A). However, TB severity was not associated with IFN-γ+ or IL-17+ by CD8+ T cells different from what was observed with CD4+ IFN-γ+IL-17+ T cells that increased levels in active TB patients and correlates with the severity of the disease [3].

The presence of CD8+ T cells expressing IL-17 in patients with active TB may be explained by high levels of IL-6 in the plasma of TB patients, which contributes to Th17 and Tc17 cell differentiation [36–38], which is highly prevalent in TB [39]. During the active phase of TB infection, a large number of cells are activated, leading to high levels of inflammatory cytokines that contribute to the activation of cell subpopulations that produce IL-17. This might be specific to patients with active TB. The presence of Tc17 cells in active TB suggests they might be involved in the immunopathogenesis of TB, as has been demonstrated for Th17 cells [40] and indicates that CD8+ T cells are involved in immune responses against Mtb.

Increased numbers of Tc17 and IL-6 cells in TB patients together with Tc1 cells can promote the development of other cell populations that produce regulatory cytokines to compensate for the inflammatory process, as shown for CD4+ T cells [41,42]. Importantly, effective suppression by regulatory T cells was only reported in IFN-γ+ cells, but the suppression of IL-17 by Treg cells has not been shown [36–38]. The presence of regulatory cytokines in TB immunology generally correlates with an attempt to control

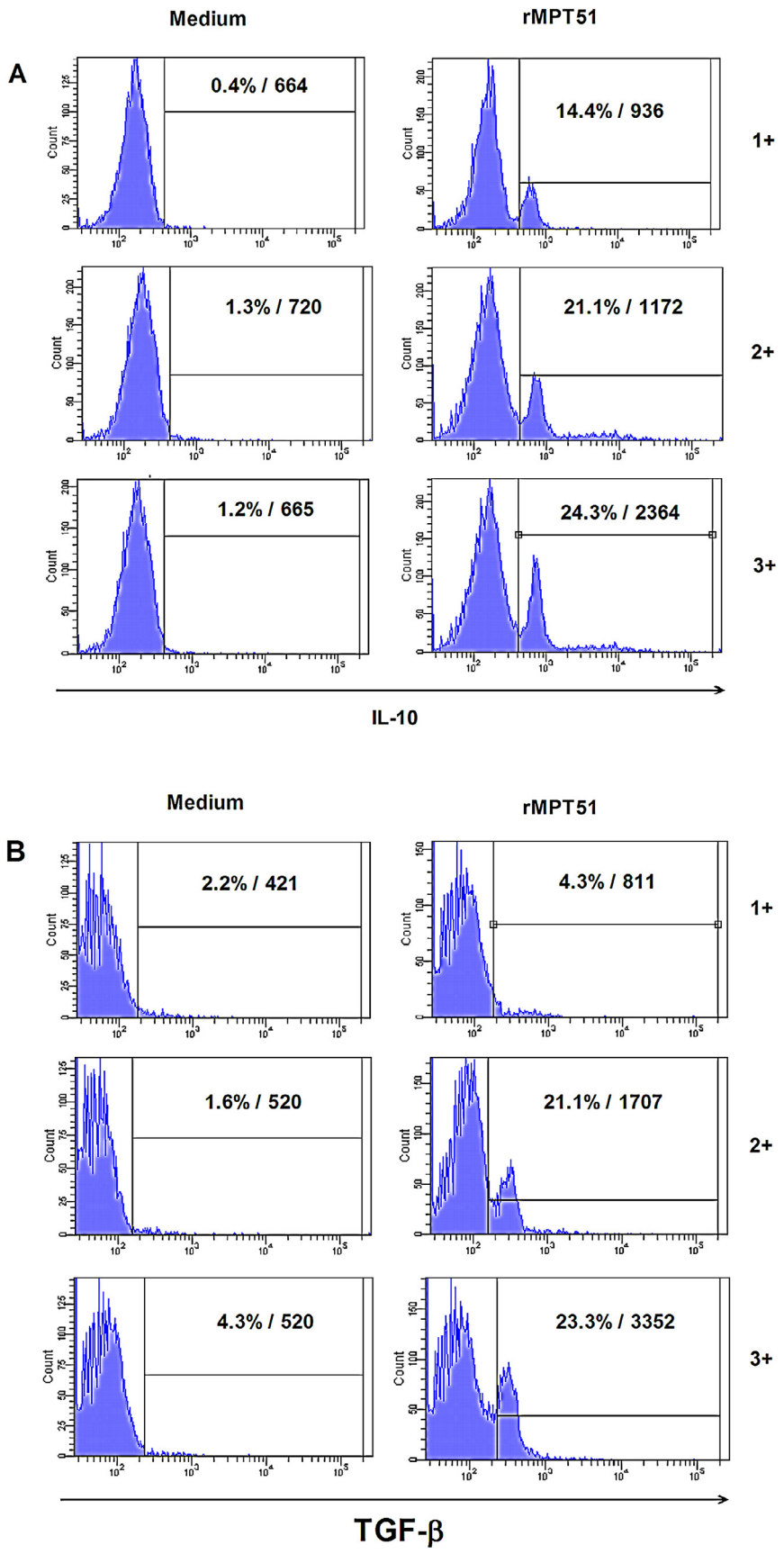

Fig. 4. Representative histogram flow plots of CD8+ T cells expressing IL-10 and TGF-β from TB patients stratified according to the bacillary load. TB patients were stratified according to sputum smear grade of low (1+), medium (2+), or high (3+) bacillary load. The range gate was set using PBMC cultures without stimulation (medium) for each subject. The numbers are the percentage of CD8+ T cells positive for each cytokine and the mean fluorescence intensity of this population. (A) Representative flow plots of CD8+ T cells expressing IL-10. (B) Representative flow plots of CD8+ T cells expressing TGF-β.

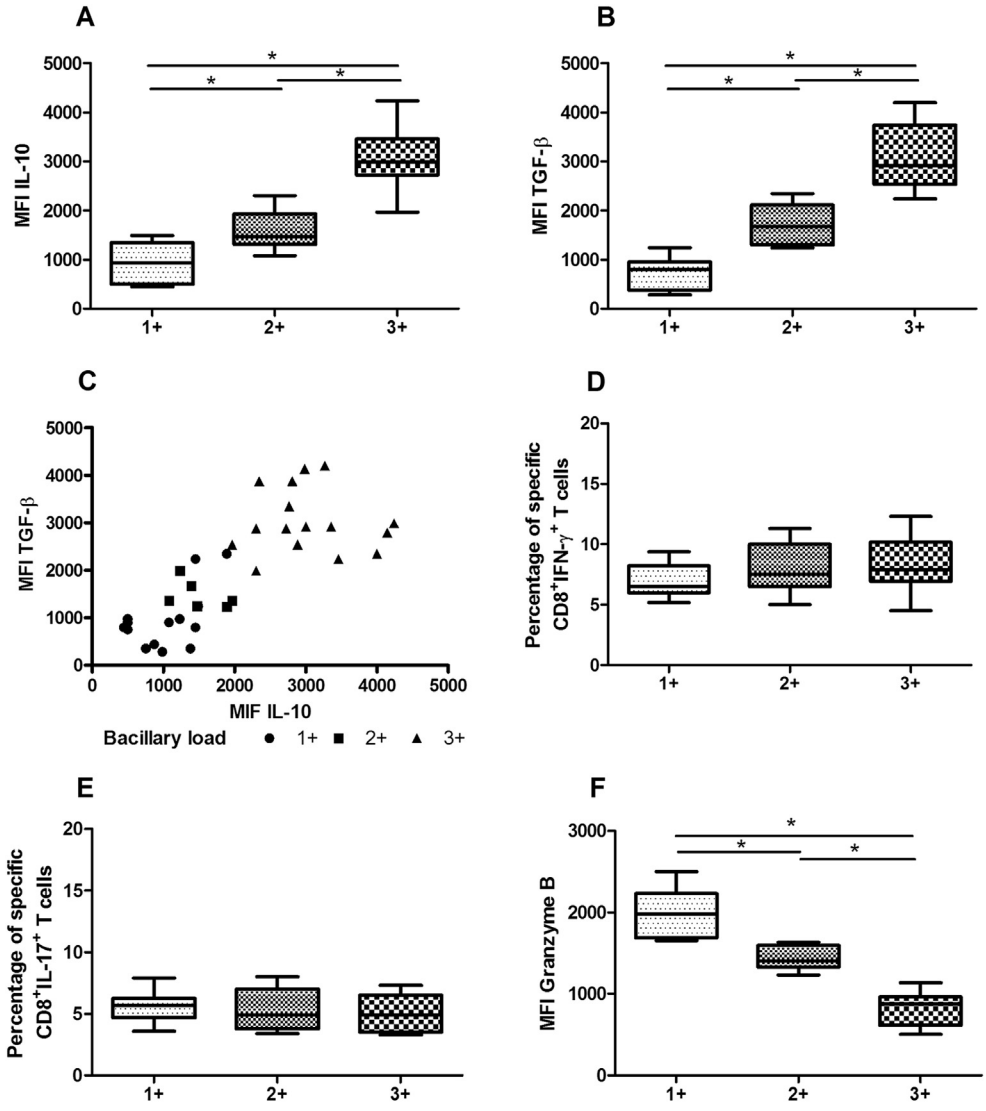

Fig. 5. Expression of IL-10, TGF-β, IFN-γ, IL-17, and granzyme-B from CD8+ T cells of TB patients according to bacillary load. TB patients were divided according to sputum smear grading: 1+ ($n = 14$), 2+ ($n = 6$), and 3+ ($n = 16$). The phenotypes of CD8+ T cells were associated with the sputum smear grade of bacillary load (A – IL-10, B – TGF-β, C – relationship between IL-10 and TGF-β T CD8+ expression: expressed as the mean fluoresce intensity (MFI), D – Tc1, E – Tc17, F – granzyme-B). Box plots show the mean ± SEM. Bars show significant differences between the groups (*$p < 0.05$).

tissue damage caused by inflammatory cytokines in response to Mtb [42].

Currently, the role of CD8+ T cells in TB is thought to involve their cytolytic activity mediated by cytotoxic granules [13,17]. The reduced level of granzyme-B granules observed here was associated with an increase in the bacillary load and this fact may contribute to the multiplication of the bacillus because granzyme-B activates caspases such as caspase-3 and the mitochondrial infected cell death pathways [43]. This observation suggests that during active TB, CD8+ T cells develop a potential granzyme-B-independent cytotoxic mechanism involving Fas-L and/or TNFR pathways [17]. Rozot et al. [13] observed different results, but they analyzed the expression of granzyme-B in Mtb-specific CD8+ T cells producing IFN-γ, which may have generated these discordant results [13]. Confirming our results, the down-regulation of cytotoxic granules in CD8+ T cells was associated with *Chlamydia* infection [44].

Next, we investigated whether the bacillary load in sputum smears from TB patients is correlated with the phenotypes of CD8+ T cells described in this study. The mechanism of cytotoxicity of CD8+ T cells is dependent on perforin and we did not find significant differences in the expression of perforin by CD8+ T cells between analyzed groups because it was not correlated with the bacterial load, since it has been demonstrated that this molecule is involved in the response against Mtb in humans [13]. Independent of the severity of disease, the numbers of CD8+ T cells producing IFN-γ and IL-17 was not altered, reinforcing the idea that these cytokines participate in the pathogenesis of TB [3]. However, the specific expression of IL-10 and TGF-β by CD8+ T cells was significantly increased in TB patients with a high bacillary load. Although a direct correlation between categorical and numerical variables is difficult to evaluate, a relationship between the increase of MFI from both cytokines was associated to the bacillary load, suggesting that those cytokines expression on CD8+ T cells might be correlated with the immune exhaustion discussed by others researchers (Fig. 5) [3,17]. The functional capacity of Mtb-specific T-cell responses in humans is associated with the mycobacterial load when observed for polyfunctional T cells. However, this was not observed for IL-17, IL-10, TGF-β, and granzyme-B expression in CD8+ T cells [27]. The expression of granzyme-B in CD8+ T cells was decreased while the bacillary load increased. The down-regulation of cytotoxic granules in CD8+ T cells is associated with the severity of *Chlamydia*

infection [44] similar to what might happen in active TB. The presence of cavitations observed in 62.5% ($n = 10$) of patients with a high bacillary load (3+) indicates that disease severity contributes to the generation of different CD8+ T-cell phenotypes in active TB [24–26].

It is important to emphasize that the results observed here were directly related with the type of TB (pulmonary), the bacillary load, and the presence of cavities in the lungs of available patients, but not to the generation of necrosis and/or apoptosis in lung tissue. Thus, the appearance of CD8+ T-cell subpopulations might be to counterbalance inflammation generated in response to Mtb at the tissue level.

Our study has several limitations including the small number of enrolled patients that voluntarily accepted to participate, corresponding to about 60% of the consecutively attending patients at the outpatient clinic of Hospital das Clínicas, HDTAA and CAIS Nova Era, governmental hospitals, and medical centers during the recruitment period. However, we believe this did not influence the outcome of our analysis.

In conclusion, our study suggests that during active TB, many CD8+ T-cell phenotypes are induced and that the bacillary load in sputum smears from TB patients can be associated with these phenotypes. Thus, our results represent a step forward in understanding the role of CD8+ T cells in the pathogenesis of tuberculosis and provide new insights on different functional and phenotypic profiles of CD8+ T cells induced in an active disease. However, to clarify the mechanisms involved, further studies are required.

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