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# Patients with Multidrug-Resistant Tuberculosis Display Impaired Th1 Responses and Enhanced Regulatory T-Cell Levels in Response to an Outbreak of Multidrug-Resistant *Mycobacterium tuberculosis* M and Ra Strains▽

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**In Argentina, multidrug-resistant tuberculosis (MDR-TB) outbreaks emerged among hospitalized patients with AIDS in the early 1990s and thereafter disseminated to the immunocompetent community. Epidemiological, bacteriological, and genotyping data allowed the identification of certain MDR *Mycobacterium tuberculosis* outbreak strains, such as the so-called strain M of the Haarlem lineage and strain Ra of the Latin America and Mediterranean lineage. In the current study, we evaluated the immune responses induced by strains M and Ra in peripheral blood mononuclear cells from patients with active MDR-TB or fully drug-susceptible tuberculosis (S-TB) and in purified protein derivative-positive healthy controls (group N). Our results demonstrated that strain M was a weaker gamma interferon (IFN-γ) inducer than H37Rv for group N. Strain M induced the highest interleukin-4 expression in $CD4^+$ and $CD8^+$ T cells from MDR- and S-TB patients, along with the lowest cytotoxic T-lymphocyte (CTL) activity in patients and controls. Hence, impairment of CTL activity is a hallmark of strain M and could be an evasion mechanism employed by this strain to avoid the killing of macrophages by M-specific CTL effectors. In addition, MDR-TB patients had an increased proportion of circulating regulatory T cells (Treg cells), and these cells were further expanded upon in vitro *M. tuberculosis* stimulation. Experimental Treg cell depletion increased IFN-γ expression and CTL activity in TB patients, with M- and Ra-induced CTL responses remaining low in MDR-TB patients. Altogether, these results suggest that immunity to MDR strains might depend upon a balance between the individual host response and the ability of different *M. tuberculosis* genotypes to drive Th1 or Th2 profiles.**

Human interventions, namely, mistreatment of tuberculosis (TB) and poor patient compliance, selectively favor the multiplication of drug-resistant *Mycobacterium tuberculosis* mutants over drug-susceptible bacilli in tuberculous lesions. *M. tuberculosis* isolates are considered to be multidrug resistant (MDR) when showing resistance to isoniazid and rifampin (rifampicin), the most effective drugs for TB treatment; they become extensively drug resistant when showing additional resistance to key second-line drugs (32, 36). MDR-TB and extensively drug-resistant TB are very difficult to treat, their prognosis is somber, and mortality is high (14, 27).

In Argentina, a total of 11,464 new cases of TB were reported in 2006, with an incidence of 29.1 per 100,000 inhabitants. MDR-TB occurred in 4.5% of the cases. MDR-TB outbreaks emerged in Argentina among hospitalized patients with AIDS in the early 1990s (1, 45) and thereafter disseminated to immunocompetent individuals (37–39). Epidemiological, bacteriological, and genotyping data allowed the identification of certain MDR *M. tuberculosis* outbreak strains, such as the so-called strain M of the Haarlem family and strain Ra of the Latin America and Mediterranean (LAM) family. Each of these two strains managed to perpetuate in its geographical niche, i.e., Buenos Aires and the Rosario City area, respectively. In particular, strain M appears to be highly prosperous in the country and is able to build up further drug resistance without impairing its ability to spread (29).

TB development depends not only on the host immune response and on its natural resistance/susceptibility to *M. tuberculosis* infection but also on differences in transmissibility, virulence, and immunogenicity among *M. tuberculosis* strains, determined by the genetic background of the organisms. It is becoming evident that certain strains of *M. tuberculosis* with special transmission potential are able to manipulate host immunity by inducing Th1 or Th2 responses which could impact disease outcome and/or evolution (5, 28, 30, 31, 40–42, 51). Protective immunity against TB is mediated by a Th1-type immune response characterized by high levels of interleukin-12

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(IL-12) from infected macrophages and gamma interferon (IFN-γ) from antigen-specific T cells, which control and contain infection in the lungs (24). Peripheral blood mononuclear cells (PBMC) from MDR-TB patients have been shown to poorly respond in vitro to H37Ra whole bacilli, purified protein derivative (PPD), and specific antigens, such as ESAT-6 and the 30-kDa protein (16, 25, 26, 33). Furthermore, increased IL-4 secretion by $CD4^+$ T cells after H37Rv total lipid stimulation was observed in MDR-TB patients (50).

Although a Th1 profile is necessary for a protective response, it may also cause immunopathologic damage; for this reason, either regulatory T cells (Treg cells) or a Th2 response might play important regulatory functions protecting the patient from collateral host tissue damage. Nevertheless, an excessive Th1 downregulation might favor disease progression. In this context, increased levels of $CD4^+$ $CD25^{high}$ $Foxp3^+$ Treg cells have been detected in PBMC from drug-susceptible tuberculosis (S-TB) patients compared to those from PPD-positive ($PPD^+$) healthy donors (18–20, 46). In addition, the results of studies involving in vitro depletion of $CD4^+$ $CD25^{high}$ T lymphocytes suggest a role of Treg cells in TB pathogenesis (18–20, 43).

In the current study, we examined immune profiles induced by two MDR *M. tuberculosis* strains disseminated in Argentina, namely, strains M and Ra. Our results demonstrate that strain M is a weak inducer of IFN-γ and elicits a remarkably low cytotoxic T-cell (CTL) activity. Also, in vitro expansion of Treg cells in PBMC from TB patients is not *M. tuberculosis* strain dependent and efficiently suppresses antigen-induced IFN-γ and CTL responses.

## MATERIALS AND METHODS

**Patients and isolates.** Blood samples were obtained from MDR-TB and S-TB patients hospitalized in the Phthisio-Pneumonology Institute University of Buenos Aires, placed in the F. J. Muñiz Hospital, Buenos Aires, Argentina. Informed consent was obtained from patients according to the guidelines of the ethics committee of the F. J. Muñiz Hospital. All patients were diagnosed by the presence of recent clinical respiratory symptoms, abnormal chest radiography, a positive sputum smear test for acid-fast bacilli, and the identification of *M. tuberculosis* in culture. Exclusion criteria included a positive test for human immunodeficiency virus (HIV) and the presence of concurrent infectious diseases or noninfectious conditions (cancer, diabetes, or steroid therapy).

Sputum smear examination, mycobacterial culture, species identification, and drug susceptibility testing were performed according to standard procedures. Susceptibility to isoniazid, rifampin, ethambutol, and streptomycin was determined according to World Health Organization standards. Susceptibility to kanamycin, *p*-aminosalicylic acid, and cycloserine was tested according to the Canetti, Rist, and Grosset method, whereas the pyrazinamidase test was used to infer pyrazinamide susceptibility (59). Available MDR *M. tuberculosis* isolates were genotyped by IS*6110* DNA fingerprinting and spoligotyping, using standardized protocols (23, 58).

A total of 25 MDR-TB patients (8 males and 17 females; median age [25th to 75th percentiles], 28 [24 to 39] years) and 20 S-TB patients (14 males and 4 females; median age [25th to 75th percentiles], 26 [21 to 54] years) were included. All MDR-TB and S-TB patients had radiological advanced pulmonary disease and were sputum smear positive at the time of the study (median number of acid-fast bacilli/field [25th to 75th percentiles] for MDR-TB, 5 [1 to 10]; that for S-TB, 5 [0.5 to 10]). Percentages of different *M. tuberculosis* lineages among MDR-TB patients in this study were as follows: LAM, 54%; Haarlem, 33% (70% of whom were infected with strain M); T, 8%; and other, 4%. Those among S-TB patients were as follows: Haarlem, 35%; T, 30%; LAM, 27%; and other, 8%. Ten $PPD^+$ healthy volunteers (4 males and 6 females; median age [25th to 75th percentiles], 30 [27 to 46] years) (group N) were included as controls.

**Mononuclear cells.** PBMC were isolated from heparinized blood by Ficoll-Triyosom gradient centrifugation (3) and suspended in RPMI 1640 (HyClone; Thermo Scientific, UT) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Invitrogen, Gibco) (complete medium).

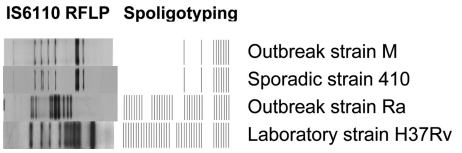


FIG. 1. Spoligotyping and IS*6110*-restriction fragment length polymorphism pattern profiles of *M. tuberculosis* strains used as antigens in this study, including reference virulent strain H37Rv and local MDR strains M, 410, and Ra.

**CD25 cell depletion.** PBMC ($1 \times 10^7$) were incubated with 1 μg anti-CD25 monoclonal antibody (MAb; eBioscience, San Diego, CA) for 30 min at 4°C, washed with phosphate-buffered saline (PBS), and mixed with goat anti-mouse immunoglobulin G-coated magnetic beads (Invitrogen Dynal, Oslo, Norway) by gentle rolling at 4°C for 30 min. Nonrosetted cells (CD25-depleted PBMC) were enriched using a magnet. Generally, one cycle of treatment was sufficient for an effective depletion, as assessed by flow cytometry: approximately 80 to 98% of $CD4^+$ $CD25^{high}$ T cells were eliminated after depletion, and a reduction of $CD4^+$ $CD25^{low}$ T cells was observed (27 to 45%) in CD25-depleted PBMC. $CD8^+$ $CD25^+$ T cells were slightly reduced (5 to 28%) after depletion. CD25-depleted PBMC were suspended in complete medium, ensuring that the number of cells/ml of each subset was the same as in total cultured PBMC in order to compare their cytokine production and CD107 expression.

**Antigens.** MDR outbreak strains M (Haarlem family) and Ra (LAM family), as well as laboratory strain H37Rv (T family), were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) at 37°C in 5% $CO_2$ until log phase. Strain 410, an MDR strain of the Haarlem family that is highly related but not identical to strain M, was included in some experiments (Fig. 1). Mycobacteria were harvested, washed three times, and suspended in PBS that was free of pyrogens. Bacteria were killed by heating at 80°C for 1 h, suspended in PBS at an optical density at 600 nm of 1 ($\approx 10^8$ bacteria/ml), and stored at −20°C until use. These mycobacterial suspensions contained soluble as well as particulate antigens.

**PBMC cultures.** Total or CD25-depleted PBMC ($2 \times 10^6$ cells/ml) were cultured in polystyrene tubes (BD Falcon, Franklin Lakes, NJ) at 37°C in a humidified 5% $CO_2$ atmosphere in complete medium in the presence of heat-killed bacilli (suspensions of *M. tuberculosis* strain M, Ra, 410, or H37Rv at a 2:1 ratio of *M. tuberculosis* to PBMC).

**$^{51}$Cr release cytotoxic assay.** The ability of PBMC stimulated by M, Ra, 410, or H37Rv killed bacilli (effector cells) to lyse autologous *M. tuberculosis*-pulsed macrophages was examined using a standard chromium release cytotoxicity assay as described previously (12).

**Immunofluorescence analysis. (i) Surface membrane expression.** The following MAbs were used to evaluate surface marker expression in fresh and 5-day-cultured PBMC: anti-CD4 (Cy5-phycoerythrin [Cy5-PE] or fluorescein isothiocyanate [FITC] conjugated), anti-CD8 (Cy5-PE or PE conjugated) (both from BD Bioscience, San Jose, CA), and PE-conjugated anti-CD25 (eBioscience).

**(ii) Intracellular expression of Foxp3 transcription factor.** Foxp3 expression was detected using a FITC-conjugated anti-human Foxp3 staining set (eBioscience) according to the manufacturer's instructions. An isotype-matched antibody was used as a control (eBioscience).

**(iii) CD107 surface expression.** To evaluate the frequency of CD4 and CD8 T cells undergoing recent degranulation, PBMC ($2 \times 10^6$ cells/ml, in complete medium) were cultured for 5 days, with or without *M. tuberculosis* (2:1 ratio of *M. tuberculosis* to PBMC). FITC-labeled anti-CD107 MAb (BD Pharmingen) was then added directly to tubes, and cells were incubated for a further 4 h at 37°C in a 5% $CO_2$ incubator. After that, cells were washed and stained for CD4 and CD8 expression.

**(iv) Intracellular cytokine expression.** Intracellular IL-10, IL-4, and IFN-γ expression was determined in 5-day-old PBMC cultures. Briefly, brefeldin A (5 μg/ml; Sigma Chemical Co., St. Louis, MO) was added for the last 4 h of culture to block cytokine secretion, and cells were surface stained with anti-CD4 and anti-CD8. They then were fixed with 0.5% paraformaldehyde and permeabilized with fluorescence-activated cell sorter permeabilizing solution 2 (BD Bioscience) before FITC- or PE-conjugated anti-IL-10, anti-IL-4 (both from BD Bioscience), or anti-IFN-γ (Caltag, Burlingame, CA) was added.

Stained cells were analyzed by flow cytometry. Twenty thousand events were

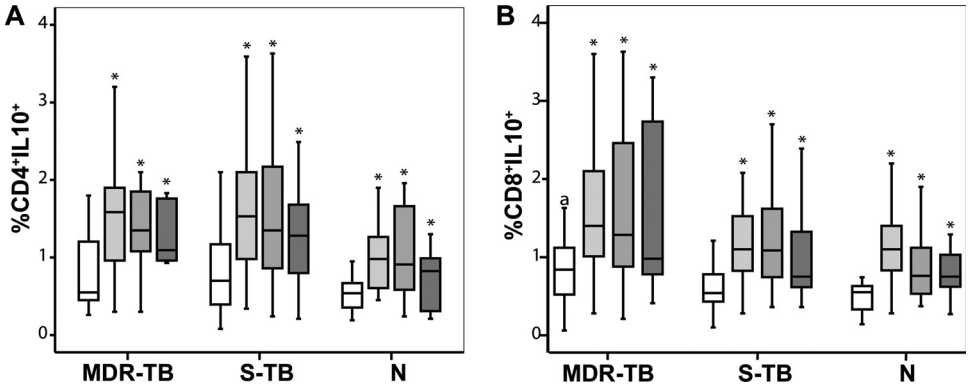


FIG. 2. H37Rv, M, and Ra induce IL-10 in $CD4^+$ and $CD8^+$ T cells. PBMC from 25 MDR-TB and 20 S-TB patients and from 10 $PPD^+$ healthy individuals (N) were cultured for 5 days alone (white bars) or with H37Rv (lightest gray bars), M (light gray bars), or Ra (dark gray bars). The proportions of $CD4^+$ and $CD8^+$ T cells expressing IL-10 were determined by flow cytometry. Results are expressed as percentages of $CD4^+$ $IL\text{-}10^+$ and $CD8^+$ $IL\text{-}10^+$ cells in the lymphocyte gate; medians and 25th to 75th percentiles are shown. *, $P < 0.05$ for *M. tuberculosis*-stimulated versus nonstimulated PBMC; a, $P < 0.05$ for MDR-TB patients versus group N controls.

acquired for each cell preparation, using a FACSCan flow cytometer (BD Bioscience) with CellQuest. FCS Express software (De Novo Software, Los Angeles, CA) was used for the analysis. Lymphocyte gates were set according to forward- and side-scatter parameters, excluding cell debris and apoptotic cells. Results were expressed as percentages of positive cells in a lymphocyte population or percentages of positive cells within $CD4^+$ or $CD8^+$ T cells.

**Statistical analysis.** Data were expressed as medians and 25th to 75th percentiles. The analysis was performed using the nonparametric Kruskal-Wallis test to compare responses of MDR-TB and S-TB patients and healthy individuals, followed by the Mann-Whitney U test to compare two groups. The Friedman test was performed to compare responses to different treatments within each group, followed by the Wilcoxon test. Correlations between CD107 and cytokine expression were analyzed by the nonparametric Spearman's rank test. All statistical analyses were two sided, and the significance level adopted was for *P* values of <0.05. The analysis was performed using the statistical software SPSS 15.0 for Windows (SPSS Inc., IL) and Graphpad Prism 4.0 (Graphpad Software Inc., CA).

## RESULTS

**Strains M and Ra induce differential IL-10, IL-4, and IFN-γ expression in $CD4^+$ and $CD8^+$ T cells.** To assess the immune profiles induced by *M. tuberculosis*, intracellular expression of IL-10, IL-4, and IFN-γ was determined in PBMC from MDR-TB and S-TB patients and healthy controls (group N) after stimulation with or without strain H37Rv, M, or Ra for 5 days. As shown in Fig. 2, higher spontaneous IL-10 expression in $CD8^+$ cells was observed in MDR-TB patients than in group N controls. In addition, although the percentage of IL-10-positive ($IL\text{-}10^+$) cells increased above basal levels upon H37Rv, M, and Ra stimulation in $CD4^+$ and $CD8^+$ cells from TB patients and group N controls, no significant differences were observed among them. In healthy $PPD^+$ controls, none of the strains induced IL-4 in $CD4^+$ cells; however, H37Rv did induce $CD8^+$ $IL\text{-}4^+$ cells. In TB patients, IL-4 was induced in $CD4^+$ and $CD8^+$ cells upon stimulation with *M. tuberculosis* strains (Fig. 3), with strain M being the highest IL-4 inducer. Remarkably, when MDR-TB patients were grouped according to the infecting *M. tuberculosis* genotype, those patients infected with Haarlem strains showed a higher proportion of M-induced $IL\text{-}4^+$ cells (mean [25th to 75th percentiles] for CD4, 3.0 [1.6 to 3.2] [$P < 0.05$]; that for CD8, 2.5 [2.0 to 3.2] [$P < 0.05$]) than did patients infected with LAM strains (mean [25th to 75th percentiles] for CD4, 1.5 [0.8 to 1.6]; that for

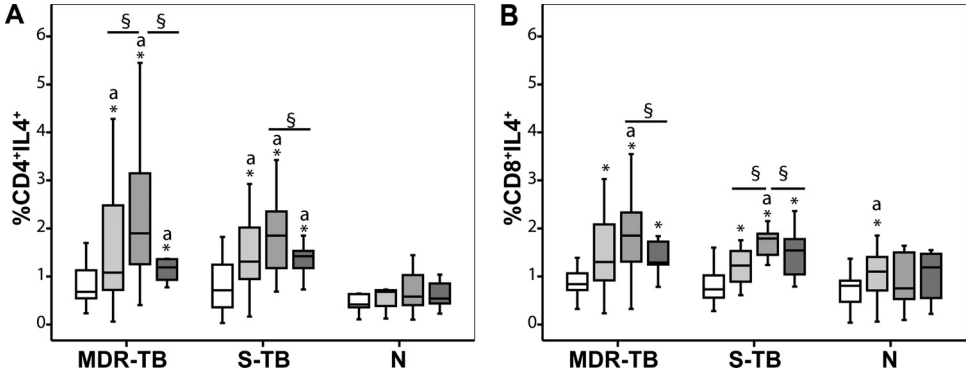


FIG. 3. H37Rv, M, and Ra induce different proportions of IL-4 in $CD4^+$ and $CD8^+$ T cells from TB patients. PBMC from 25 MDR-TB and 20 S-TB patients and from 10 group N controls were cultured for 5 days alone (white bars) or with H37Rv (lightest gray bars), M (light gray bars), or Ra (dark gray bars). The percentages of $CD4^+$ $IL\text{-}4^+$ and $CD8^+$ $IL\text{-}4^+$ cells were determined by flow cytometry, and results are expressed as medians and 25th to 75th percentiles. *, $P < 0.05$ for *M. tuberculosis*-stimulated versus nonstimulated PBMC; §, $P < 0.05$ for M versus H37Rv or Ra; a, $P < 0.05$ for MDR-TB or S-TB patients versus group N controls.

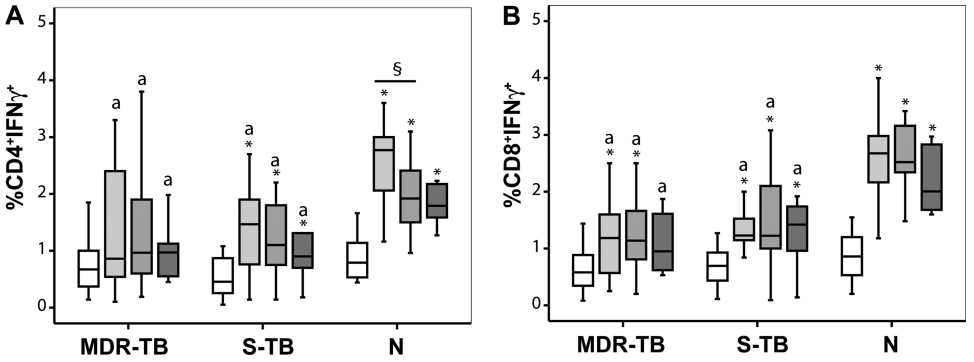


FIG. 4. H37Rv, M, and Ra induce different proportions of IFN-γ in $CD4^+$ and $CD8^+$ T cells in TB patients and group N controls. PBMC from 25 MDR-TB and 20 S-TB patients and from 10 group N controls were cultured for 5 days alone (white bars) or with H37Rv (lightest gray bars), M (light gray bars), or Ra (dark gray bars). The percentages of $CD4^+$ IFN-$\gamma^+$ and $CD8^+$ IFN-$\gamma^+$ cells were determined by flow cytometry, and results are expressed as medians and 25th to 75th percentiles. *, $P < 0.05$ for *M. tuberculosis*-stimulated versus nonstimulated PBMC; a, $P < 0.05$ for patients versus group N controls; §, $P < 0.05$ for M versus H37Rv or Ra.

CD8, 1.8 [1.4 to 2.3]). Conversely, no differences were observed between both groups of MDR-TB patients for H37Rv- or Ra-induced IL-$4^+$ cells. Thus, the ability to induce high IL-4 levels in TB patients seems to be an intrinsic characteristic of strain M.

$CD4^+$ and $CD8^+$ cells expressing IFN-γ were increased above basal levels upon PBMC stimulation with H37Rv, M, and Ra in S-TB patients and group N controls, whereas in MDR-TB patients, $CD8^+$ IFN-$\gamma^+$ cell enhancement was achieved only by H37Rv and M stimulation (Fig. 4). Moreover, a reduction in the number of *M. tuberculosis*-induced IFN-$\gamma^+$ cells was observed in TB patients compared to that in $PPD^+$ healthy controls. Furthermore, strain H37Rv was the best IFN-γ inducer in $CD4^+$ cells from healthy $PPD^+$ controls, whereas the strain made no difference in the induction of $CD8^+$ IFN-$\gamma^+$ cells.

**Strain M induces low CD107 expression in $CD8^+$ T cells.** CD107a expression on the cell surface has been described as a marker of cytotoxic $CD8^+$ T-cell degranulation/activation (2). CD107a and -b are intracellular proteins normally found in lysosomes that are transiently expressed on CTL surfaces upon exocytosis of cytotoxic granules (60). Considering that in our system $CD8^+$ T cells are in close contact with antigen-presenting cells, we evaluated the presence of $CD8^+$ T cells having undergone recent granule exocytosis in 5-day-old PBMC cultures by employing a CD107 mobilization assay. As shown in Fig. 5A, the proportion of $CD107^+$ cells in $CD8^+$ T cells increased in TB patients and group N controls upon H37Rv, M, and Ra stimulation. However, TB patients showed fewer $CD107^+$ cells than did group N controls. Moreover, strain M induced the lowest CD107 expression not only in TB patients but also in group N controls, suggesting that this strain has an impaired ability to evoke a CTL response. These results were confirmed in group N controls by employing a standard $^{51}Cr$ release cytotoxicity assay (Fig. 5B).

Considering that CD107 upregulation has been demonstrated to be in concordance with perforin loss and enhanced IFN-γ production (2), we compared the proportions of IFN-γ and CD107 on antigen-stimulated $CD8^+$ T cells from MDR-TB and S-TB patients and group N controls. A significant correlation between both markers was observed, with that for strains H37Rv and M being weaker. This suggests that impaired CD107 expression could be related to diminished IFN-γ expression in $CD8^+$ T cells from TB patients (Fig. 5C). In contrast, an inverse correlation between the proportions of $CD107^+$ and IL-$4^+$ or IL-$10^+$ cells in the $CD8^+$ cell subset was found.

Having observed that strain M induced weak IFN-γ expression in group N controls, low CTL activity in group N controls and TB patients, and strong IL-4 expression in TB patients, we wondered whether these characteristic patterns were strain specific or, in contrast, were shared with other related MDR strains of the Haarlem family. For this purpose, we evaluated the same effector functions, but using strain 410, a Haarlem strain that is highly related to strain M but has rarely caused disease since the latter started clonal expansion. A decreased percentage of $CD4^+$ IL-$4^+$ cells was indeed detected with strain 410 in MDR-TB patients (median [25th to 75th percentiles], 2.6% [1.7 to 5.6%] [$P < 0.05$]), while neither the IL-10 nor IFN-γ level was modified. Remarkably, strain 410 induced a higher % $CD107^+$ cells among $CD8^+$ T cells in group N controls (6.9% [6.4 to 7.6%]) and MDR-TB (4.8% [2.8 to 6.4%]) and S-TB (7.0% [5.2 to 7.4%]) ($P < 0.05$) patients than did strain M. These results were confirmed in group N controls by a $^{51}Cr$ release assay (% cytotoxicity for strain M, 38 [14 to 42]; that for strain 410, 61 [51 to 70] [$P < 0.05$]).

**$CD4^+$ and $CD8^+$ Treg cells are increased ex vivo in PBMC from MDR-TB patients.** Considering that Treg cells affect Th1 and Th2 responses (21) and that $CD4^+$ $CD25^+$ $Foxp3^+$ T cells are increased in S-TB patients (18, 19, 46), we wondered whether Treg cells were involved in the impaired *M. tuberculosis*-induced T-cell response observed in MDR-TB patients. We first evaluated the percentages of $CD4^+$ and $CD8^+$ T cells ex vivo in PBMC from MDR-TB and S-TB patients and group N controls. As shown in Table 1, a lower percentage of $CD4^+$ T cells was detected in TB patients than in group N controls, while no differences were observed in the proportion of $CD8^+$ cells. Although the absolute number of total $CD4^+$ T cells from MDR-TB patients (856 [725 to 1,218] cells/$mm^3$) was similar to that from group N controls (1,061 [943 to 1,166] cells/$mm^3$), a lower value was found for S-TB patients (527 [436 to 646] cells/$mm^3$) ($P < 0.05$). In addition, absolute $CD8^+$

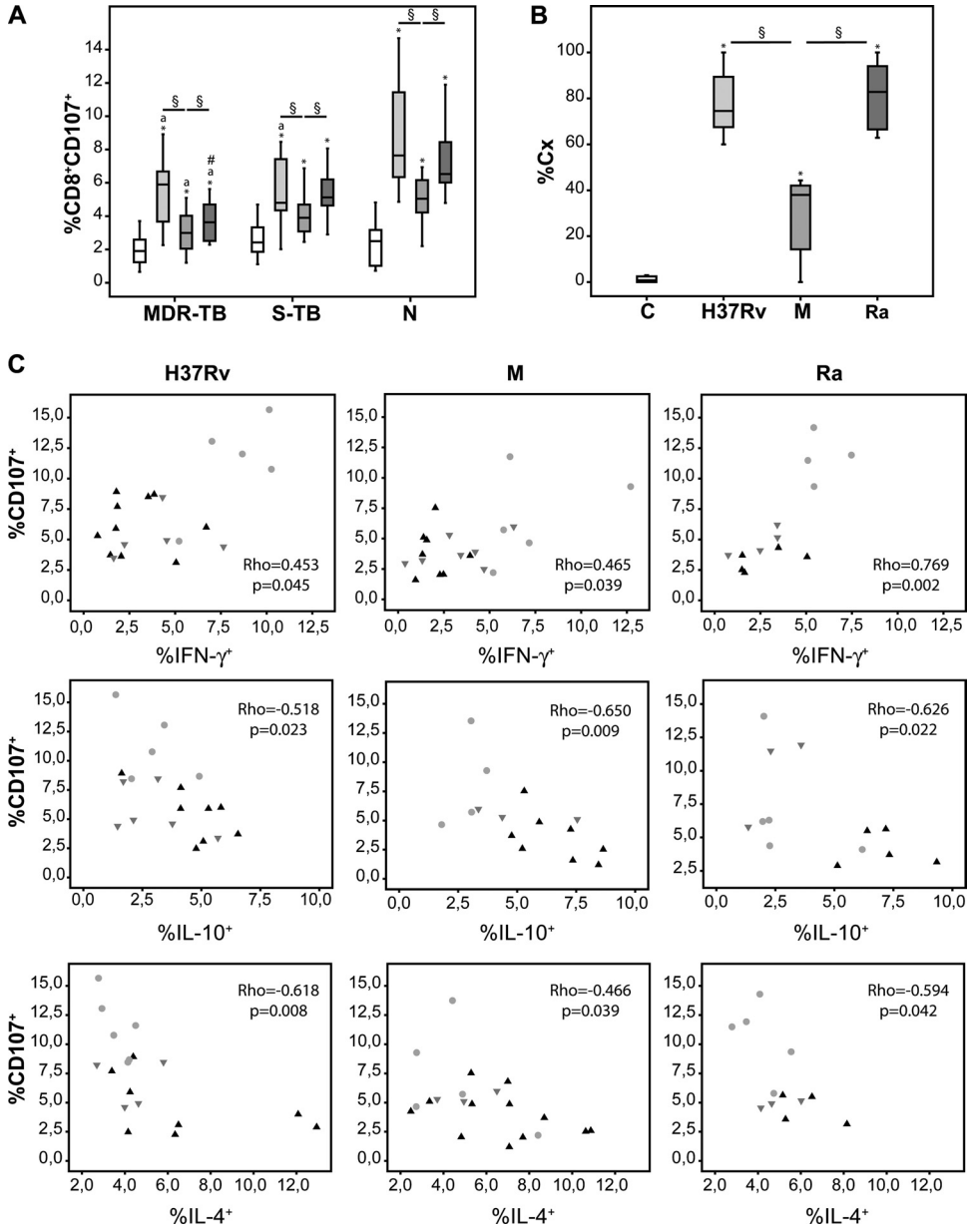


FIG. 5. $CD8^+$ T cells from TB patients express low levels of CD107 surface molecules. PBMC from 20 MDR-TB and 16 S-TB patients and 10 group N controls were cultured for 5 days alone (C; white bars) or with H37Rv (lightest gray bars), M (light gray bars), or Ra (dark gray bars). (A) Control and *M. tuberculosis*-stimulated $CD8^+$ T cells were tested for CD107 surface expression by flow cytometry. Results are expressed as percentages of $CD107^+$ cells in the $CD8^+$ lymphocyte gate (medians and 25th to 75th percentiles). ∗, $P < 0.05$ for *M. tuberculosis*-stimulated versus nonstimulated PBMC; §, $P < 0.05$ for M versus H37Rv or Ra; #, $P < 0.05$ for MDR-TB versus S-TB patients; a, $P < 0.05$ for MDR-TB patients versus group N controls. (B) Control and *M. tuberculosis*-stimulated PBMC from 10 group N controls were tested for lytic ability against autologous *M. tuberculosis*-pulsed macrophages, employing a $^{51}Cr$ release assay. Results are expressed as percentages of cytotoxicity (%Cx) (medians and 25th to 75th percentiles). ∗, $P < 0.05$ for *M. tuberculosis*-stimulated versus control PBMC; §, $P < 0.05$ for M versus H37Rv or Ra. (C) Correlation between % $CD107^+$ $CD8^+$ cells and % $IL\text{-}10^+$, $IL\text{-}4^+$, or $IFN\text{-}\gamma^+$ cells in *M. tuberculosis*-stimulated $CD8^+$ T cells from MDR-TB (triangles) and S-TB (inverted triangles) patients and group N controls (circles). Individual data and Spearman rho coefficients are shown.

T-cell counts in MDR-TB and S-TB patients (for MDR-TB patients, 462 [404 to 668] cells/$mm^3$ [$P < 0.05$]; for S-TB patients, 405 [282 to 556] cells/$mm^3$ [$P < 0.01$]) were lower than those in group N controls (688 [633 to 788] cells/$mm^3$). The percentage of $CD25^+$ cells was significantly increased within the total $CD4^+$ T-cell population from TB patients compared to that from group N controls. In contrast, the proportions of $CD25^+$ cells within $CD8^+$ T cells were similar among TB patients and group N controls (Table 1).

The proportions of conventional activated $CD4^+$ T cells and natural Treg cells were analyzed by discriminating low or high CD25 expression on the basis of CD25 mean fluorescence intensity within the $CD4^+$ cell population. As shown in Table 1, higher proportions of both $CD25^{low}$ and $CD25^{high}$ cells

TABLE 1. Increased percentage of $CD25^+$ $Foxp3^+$ cells in $CD4^+$ and $CD8^+$ T cells from MDR-TB patients

| Cell population[b] | % of cells (median [25th to 75th percentiles])[a] | | |
|---|---|---|---|
| | MDR-TB patients | S-TB patients | N (controls) |
| Total $CD4^+$ T cells | 45.4 (40.0–50.8)* | 38.0 (31.0–45.9)* | 50.5 (44.9–55.5) |
| $CD25^+$ $CD4^+$ cells | 15.9 (10.4–22.3)* | 14.0 (16.6–30.2)* | 9.3 (6.3–12.9) |
| $CD25^{low}$ $CD4^+$ cells | 11.3 (8.3–16.9)* | 15.2 (9.0–22)* | 7.7 (6.1–9.4) |
| $CD25^{high}$ $CD4^+$ cells | 4.0 (2.2–6)* | 4.8 (2–7.2)* | 2.4 (1.4–3.1) |
| $CD25^{high}$ $Foxp3^+$ CD4 cells | 4.3 (1.4–5.4)* | 3.7 (1.9–5.5)* | 2.0 (1.2–2.4) |
| Total $CD8^+$ T cells | 26.3 (22.6–30.9) | 28.8 (20.1–39.5) | 32.8 (30.1–37.5) |
| $CD25^+$ $CD8^+$ cells | 2.7 (1.8–4.7) | 2.0 (1.1–4.1) | 1.2 (0.7–2.7) |
| $Foxp3^+$ $CD25^+$ $CD8^+$ cells | 0.7 (0.4–2.3)* | 0.4 (0.2–1.1) | 0.3 (0.27–0.47) |

[a] *, $P < 0.05$ for MDR-TB or S-TB patients versus group N.
[b] Populations were determined in $CD4^+$ or $CD8^+$ lymphocyte gates.

within $CD4^+$ cells were detected in TB patients than in group N controls. The Foxp3 transcription factor is considered a molecular marker of natural Treg cells (15); hence, we evaluated its intracellular expression in $CD4^+$ $CD25^{high}$ and $CD8^+$ $CD25^+$ T cells. A higher proportion of $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells was observed in TB patients (Table 1); however, Foxp3 mean fluorescence intensities were similar for all groups (data not shown). Furthermore, the proportion of ex vivo Treg cells strongly correlated with antigen load in S-TB patients (Spearman test; $r = 8{,}198$; $P = 0.0119$), while no correlation was observed for MDR-TB patients. This difference may be ascribed to the fact that MDR-TB patients constituted a much more heterogeneous population in terms of previous duration of disease and anti-TB treatment. Recently, $CD8^+$ Treg cells that are able to inhibit T-cell proliferation and cytokine production have also been described, and human $CD8^+$ $CD25^+$ $Foxp3^+$ cells have been observed in adult PBMC and in neonatal thymus (8, 52). In line with this, we detected an increased percentage of $CD8^+$ $CD25^+$ $Foxp3^+$ cells only in MDR-TB patients (Table 1). Altogether, our results indicate that $CD4^+$ and $CD8^+$ Treg cells are expanded in vivo in MDR-TB patients and confirm previous reports that detected $CD4^+$ Treg cell expansion in S-TB patients (6, 18, 19, 46).

**High levels of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells are induced by *M. tuberculosis* strains in MDR-TB patients.** We next wanted to determine whether local MDR strains were able to induce an in vitro expansion of $CD4^+$ Treg cells. To do this, PBMC from MDR-TB and S-TB patients and group N controls were stimulated for 5 days with strain H37Rv, M, or Ra, and the proportion of $CD25^{high}$ $Foxp3^+$ cells in the $CD4^+$ T-cell population was evaluated. As shown in Fig. 6, H37Rv, M, and Ra enhanced $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells in TB patients and group N controls; however, MDR-TB patients showed higher percentages of H37Rv- and M-induced Treg cells than did group N controls. Thus, outbreak MDR strains M and Ra induce Treg cells, like H37Rv does, with the highest Treg cell expansion observed in MDR-TB patients.

**Treg cells suppress effector functions of PBMC from TB patients.** Finally, we evaluated whether circulating Treg cells suppressed *M. tuberculosis*-induced responses, such as cytokine production and CD107a degranulation, in TB patients. Depletion of $CD25^+$ cells enhanced the percentages of H37Rv-, M-, or Ra-induced $CD4^+$ IFN-$\gamma^+$ and $CD8^+$ IFN-$\gamma^+$ cells (Fig. 7) and decreased the proportions of $CD4^+$ IL-$10^+$ and $CD8^+$ IL-$10^+$ cells (Fig. 8) in TB patients, whereas CD25 depletion did not modify the level of IL-$4^+$ cells (data not shown). In addition, CD25 depletion also increased *M. tuberculosis*-induced $CD8^+$ $CD107^+$ T cells in TB patients (Fig. 9), suggesting that $CD25^+$ Treg cells are functionally active in TB, upregulating IL-10 and inhibiting IFN-γ expression and CTL degranulation. In spite of CD25 depletion, degranulation induced by the M and Ra strains was still lower in MDR-TB patients than in S-TB patients and group N controls. Moreover, strain M induced lower CD107 expression than did H37Rv in MDR-TB patients ($P < 0.05$) and group N controls ($P < 0.05$), and Ra also induced less degranulation than did H37Rv in MDR-TB patients ($P < 0.05$), even in the absence of Treg cells.

## DISCUSSION

The development of TB disease depends on a subtle balance between host genetic factors involved in susceptibility and re-

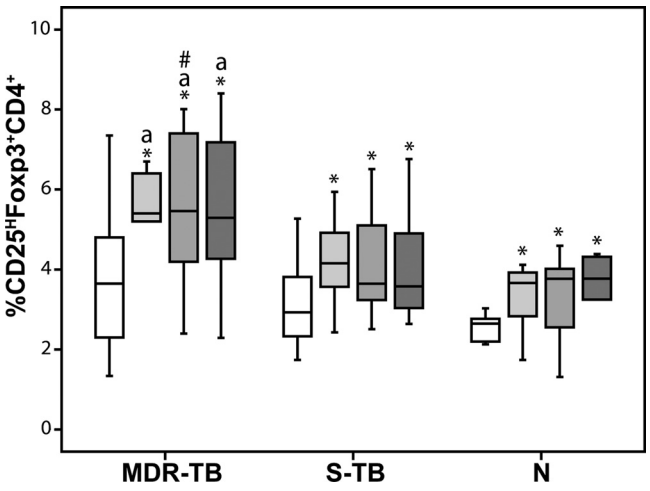


FIG. 6. Expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ cells is not dependent on *M. tuberculosis* strain stimulation. PBMC from 25 MDR-TB and 20 S-TB patients and 10 group N controls were cultured for 5 days alone (white bars) or with H37Rv (lightest gray bars), M (light gray bars), or Ra (dark gray bars). The percentages of $CD25^+$ $Foxp3^+$ cells in $CD4^+$ T cells were determined by flow cytometry. Results are expressed as medians and 25th to 75th percentiles. *, $P < 0.05$ for *M. tuberculosis*-stimulated versus nonstimulated PBMC; a, $P < 0.05$ for MDR-TB or S-TB patients versus group N controls; #, $P < 0.05$ for MDR-TB versus S-TB patients.

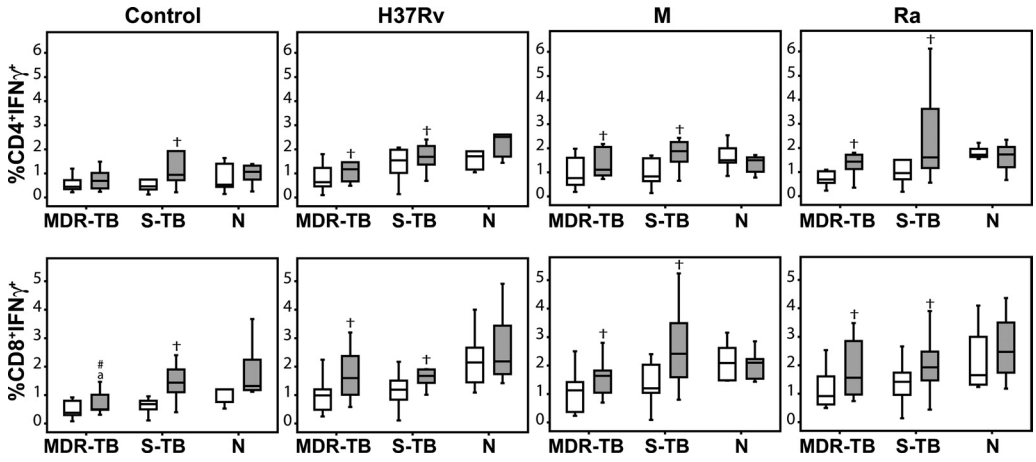


FIG. 7. CD25 depletion enhances IFN-γ expression on antigen-stimulated $CD4^+$ and $CD8^+$ T cells. PBMC from 10 MDR-TB and 8 S-TB patients and 6 group N controls were depleted of $CD25^+$ cells by magnetic methods. PBMC (white bars) and CD25-depleted cells (gray bars) were cultured for 5 days with *M. tuberculosis* strains and tested for IFN-γ expression. Results are expressed as % $CD4^+$ IFN-$\gamma^+$ or $CD8^+$ IFN-$\gamma^+$ cells (medians and 25th to 75th percentiles). †, $P < 0.05$ for PBMC versus CD25-depleted PBMC; a, $P < 0.05$ for MDR-TB patients versus group N controls; #, $P < 0.05$ for MDR-TB versus S-TB patients.

sistance to TB following infection (9) and the ability of different *M. tuberculosis* genotypes to induce a strong or a weak Th1 protective immune response (30, 41, 42, 51). Th1 responses, such as IFN-γ production and CTL activity, have been associated not only with bacterial growth control and lysis of infected macrophages but also with tissue damage, as observed in TB.

Herein we showed that our outbreak MDR strains M and Ra, and even the nonprosperous MDR strain 410, induced lower IFN-γ expression than H37Rv did in $CD4^+$ cells from healthy $PPD^+$ individuals. This result may be related to the lack of host selective pressure suffered by the laboratory strain H37Rv. Low in vitro Th1 responses to different *M. tuberculosis* antigens have been observed in MDR-TB patients, such as impaired IFN-γ production (4, 16, 25, 26, 33) and increased IL-4 production by $CD4^+$ T cells stimulated by a lipid extract of H37Rv (50). Likewise, in our study, both MDR-TB and S-TB patients showed an impaired expression of IFN-γ in $CD4^+$ and $CD8^+$ T cells, irrespective of the tested strain; therefore, this might be ascribed to the altered Th1/Th2 profile characteristic of advanced disease (13). In contrast to the work of McDyer et al. (33), we found diminished IFN-γ expression in MDR-TB patients, in spite of their normal $CD4^+$ counts of >500/μl. Moreover, high IL-4 levels were observed in $CD4^+$ and $CD8^+$ cells from MDR- and S-TB patients, with M being the best inducer, suggesting that this strain exploits and enhances the preexisting tendency of TB patients to mount a Th2 response. Moreover, the fact that patients infected with strain M showed more $CD4^+$ and $CD8^+$ IL-$4^+$ cells highlights the peculiar ability of this strain to bias the Th1 response through IL-4 induction. High IL-4 levels have been associated with progression from latent infection to active disease (35, 54), advanced radiological disease, and cavitary TB (49, 57). In this context, IFN-γ deficiency in TB patients could be due to a shift

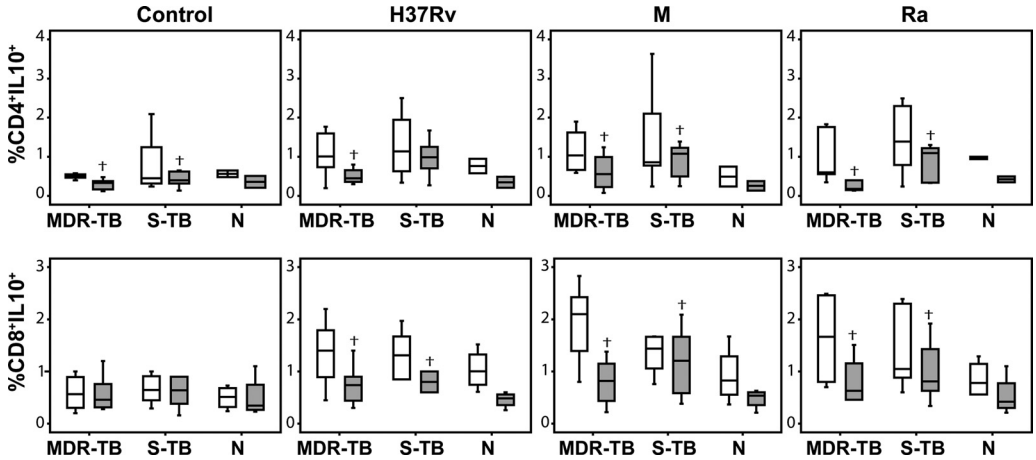


FIG. 8. CD25 depletion decreases IL-10 expression on antigen-stimulated $CD4^+$ and $CD8^+$ T cells. PBMC (white bars) and CD25-depleted cells (gray bars) from 10 MDR-TB and 8 S-TB patients and 6 group N controls were cultured for 5 days with *M. tuberculosis* strains and tested for IL-10 expression. Results are expressed as % $CD4^+$ IL-$10^+$ or % $CD8^+$ IL-$10^+$ cells (medians and 25th to 75th percentiles). †, $P < 0.05$ for PBMC versus CD25-depleted PBMC.

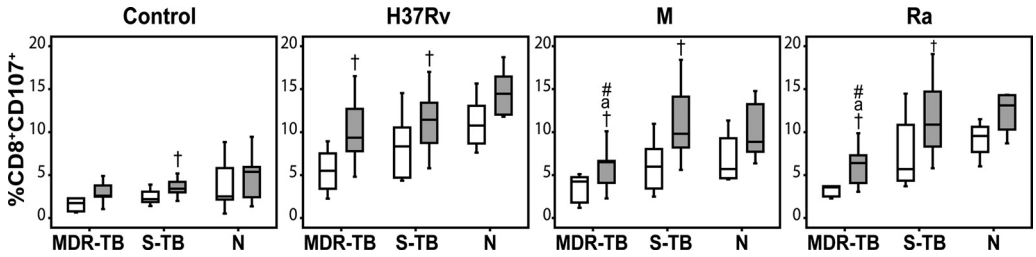


FIG. 9. CD25 depletion enhances CD107 expression on antigen-stimulated $CD4^+$ and $CD8^+$ T cells. PBMC from 10 MDR-TB and 8 S-TB patients and 6 group N controls were depleted of $CD25^+$ cells by magnetic methods. PBMC (white bars) and CD25-depleted cells (gray bars) were cultured for 5 days with H37Rv, M, or Ra and tested for CD107 expression. Results are expressed as % $CD107^+$ cells in $CD8^+$ T cells (medians and 25th to 75th percentiles). †, $P < 0.05$ for PBMC versus CD25-depleted PBMC; a, $P < 0.05$ for MDR-TB patients versus group N controls; #, $P < 0.05$ for MDR-TB versus S-TB patients.

to a Th2 cytokine profile, undermining the efficacy of Th1-mediated immunity and causing immunopathology (47). Similarly, IL-4 and IL-13 were found to be induced in human monocytes by virulent Beijing strains (30), and differences in Th1/Th2 profile have been observed in healthy individuals with single-copy IS*6110*-carrying strains from South India (41, 42) and in TB patients infected with non-Beijing or Beijing strains (56). Hence, the overall T-cell response to *M. tuberculosis* strains seems to be dependent on the host ability to mount Th1/Th2 responses and the potential of each strain to increase the Th2 cytokine profile in susceptible individuals.

CTL activity has been associated with lysis of *M. tuberculosis*-infected macrophages and direct killing of mycobacteria (12, 55). Here we observed that MDR- and S-TB patients failed to induce degranulation of $CD8^+$ cells and to evoke a CTL response, likely due to the high Th2 profile induced in $CD4^+$ and $CD8^+$ cells from these patients. In line with this, a gradual loss of CD8-mediated CTL activity against autologous H37Rv-pulsed macrophages dependent on IL-10 production has been observed in patients with active S-TB (10–12), and modulation of *Mycobacterium leprae* Hsp65-induced CTL activity by IL-10 and IL-4 has also been observed in leprosy patients (11). In addition, IL-4 leads to development of a $CD8^+$ T-cell subset that fails to upregulate granzyme B, a potent apoptosis-inducing protease of CTLs (44). A Th2 cytokine profile is a suitable explanation for the weak cytotoxic activity detected in MDR- and S-TB patients but does not account for the inability of strain M to induce cytotoxicity in healthy controls. To shed further light on this finding, we examined another MDR-TB strain with a close genetic link with M, but which is epidemiologically incompetent, namely, strain 410. In healthy controls, these two highly related Haarlem strains showed similar IL-10, IL-4, and IFN-γ expression patterns; however, they differed in cytotoxic activity. Thus, CTL impairment is a hallmark of strain M, and deficient lysis of infected macrophages could be one of multiple factors involved in strain M fitness. Current studies are being performed to evaluate the mechanisms employed by strain M to hamper the CTL response.

It has been speculated that Treg cells might play important regulatory functions in the immune response (48). Herein, we observed an increase in circulating $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells in MDR-TB and S-TB patients, as previously demonstrated (6, 18, 19, 46). In addition, we also showed an increase in $CD8^+$ $CD25^+$ $Foxp3^+$ cells in MDR-TB patients, although their levels were threefold lower than those of $CD4^+$ Treg cells. Accordingly, a $CD8^+$ regulatory T-cell subset was recently described as capable of mediating suppression of *Mycobacterium bovis* BCG-induced PBMC proliferation in $PPD^+$ healthy individuals and in *M. tuberculosis*-infected lymph nodes and tonsils (22). Furthermore, as demonstrated with heat-killed strain Erdman and BCG (17, 20), strains M and Ra expanded Treg cells in PBMC from MDR-TB and S-TB patients and healthy tuberculin reactors, like H37Rv did, showing that this expansion is not *M. tuberculosis* genotype dependent. The marked Treg cell expansion observed in MDR- and S-TB patients could be related to the high IL-4 levels induced by *M. tuberculosis*, which is in accordance with $CD4^+$ $CD25^+$ Treg cell induction from $CD25^-$ peripheral blood T cells by IL-4 (53). Treg cell depletion decreased IL-10 and enhanced IFN-γ levels in $CD4^+$ and $CD8^+$ T cells from TB patients, suggesting that Treg cells may suppress effector functions. Indeed, Treg cell depletion also enhanced $CD107^+$ $CD8^+$ cells in MDR- and S-TB patients, as also observed in mice (34). Furthermore, low M- and Ra-induced CD107 expression and IFN-γ upregulation were still observed in MDR-TB patients in the absence of Treg cells, suggesting that additional factors could inhibit the Th1 response. Despite the presence of Treg cells, high proportions of $CD4^+$ and $CD8^+$ $IL\text{-}4^+$ cells were induced by *M. tuberculosis* strains in MDR-TB patients; therefore, it is likely that this cytokine could interfere with CTL differentiation or functionality. In line with this, Th2 T-cell clones have been shown to be less susceptible to the suppressive activity of $CD25^+$ regulatory thymocytes (7). The fact that Treg cell depletion did not restore either Ra- or M-induced CD107 to group N levels in MDR-TB patients suggests that the impairment of CTL activity and cytokine production could not be ascribed exclusively to Treg cell suppression.

In summary, we have demonstrated an increased frequency of circulating Treg cells in MDR-TB patients that are expanded in vitro by *M. tuberculosis* stimulation independent of the strain and that suppress antigen-induced IFN-γ and CTL responses. Outbreak MDR strain M is a weak IFN-γ inducer. Remarkably, the inability to develop CTL activity is a hallmark of strain M, regardless of its ability to induce IFN-γ in $CD8^+$ cells in healthy $PPD^+$ controls. Hence, impairment of CTL activity may be an evasion mechanism employed by strain M to avoid macrophage lysis by antigen-specific CTLs. Altogether, these results suggest that immune responses to M and Ra outbreak strains are the result of the host's ability to mount an

efficient immune response and the potential of different *M. tuberculosis* genotypes to drive Th1 or Th2 profiles.

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