Outbreaks of *Mycobacterium Tuberculosis* MDR Strains Induce High IL-17 T-Cell Response in Patients With MDR Tuberculosis That Is Closely Associated With High Antigen Load

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Background. The proinflammatory cytokine interleukin 17 (IL-17) plays an important role in immune responses but it is also associated with tissue-damaging inflammation. So, we evaluated the ability of *Mycobacterium tuberculosis* clinical isolates to induce IL-17 in tuberculosis (TB) patients and in healthy human tuberculin reactors (PPD$^+$HD).

Methods. IL-17, interferon γ (IFN-γ), and interleukin 23 (IL-23) receptor expression were evaluated ex vivo and cultured peripheral blood mononuclear cells from TB and PPD$^+$HD stimulated with irradiated clinical isolates from multidrug resistant (MDR) outbreaks M (Haarlem family) and Ra (Latin American–Mediterranean family), as well as drug-susceptible isolates belonging to the same families and laboratory strain H37Rv for 48 hours in T-cell subsets by flow cytometry.

Results. We observed that: (1) MDR strains M and Ra are stronger IL-17 inducers than drug-susceptible *Mtb* strains of the Haarlem and Latin American–Mediterranean families, (2) MDR-TB patients show the highest IL-17 expression that is independent on the strain, (3) IL-17 expression is dependent on CD4$^+$ and CD8$^+$ T cells associates with persistently high antigen load.

Conclusions. IL-17–producing T cells could play an immunopathological role in MDR-TB promoting severe tissue damage, which may be associated with the low effectiveness of the second-line drugs employed in the treatment.

Interleukin 17 (IL-17) is a proinflammatory cytokine that plays an important role in autoimmunity [1], graft rejection [2], and immune responses against intracellular pathogens [3]. By inducing the recruitment and activation of polymorphonuclear neutrophils (PMN) and triggering the production of proinflammatory cytokines and chemokines [4], IL-17 is also associated with tissue-damaging inflammation [5]. Although CD4$^+$ T cells are the main source of IL-17 [4, 6–10], CD8$^+$ T, γδT, natural killer T (NKT), and natural killer (NK) cells also produce IL-17 [11–13]. Upon challenge with *Mycobacterium tuberculosis* (*Mtb*), an expansion of CD4$^+$ T and γδT cells producing IL-17 occurs in the lungs of vaccinated mice together with a recruitment of interferon γ$^+$ (IFNγ$^+$)-producing T cells [11, 14, 15], all indicating that IL-17 plays a role in the containment of *Mtb* infection. In healthy human tuberculin reactors (PPD$^+$HD), circulating CD4$^+$ T cells are the major source of IL-17 [10, 16, 17]. On the other hand, patients with drug-susceptible tuberculosis (S-TB) show an increase in peripheral IL-17–producing γδ T cells [18], suggesting that these cells might be involved in the immunity to *Mtb* infection or in the pathological development of pulmonary TB.

In the early 1990s, outbreaks of multidrug resistant tuberculosis (MDR-TB) caused by *Mtb* strains showing resistance to at least isoniazid and rifampin [19] took place in the cities of Buenos Aires and Rosario, Argentina [20, 21]. Although these strains had been isolated from human immunodeficiency virus (HIV)-infected patients, they disseminated to immunocompetent individuals [22–24]. Epidemiological, bacteriological, and genotyping data allowed the identification of the outbreak strains M (Haarlem family) and Ra (Latin American–Mediterranean) families. 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.

Recently, we have demonstrated that MDR strains M and Ra induce an altered T-helper 1/T-helper 2 (Th1/Th2) profile in T cells from PPD$^+$HD and from patients with TB, strain M being a weaker inducer of IFNγ and of CD8-dependent cytotoxic activity [25]. In the present work, we evaluated the ability of strains M and Ra to stimulate IL-17 in TB patients and in PPD$^+$HD. We showed that strains M and Ra significantly increase the level of IL-17$^+$ cells. The highest CD4$^+$ and CD8$^+$ T-cell–dependent IL-17 response was observed in MDR-TB patients, and it was strongly associated with persistently high antigen load.

MATERIAL AND METHODS

Patients
Blood samples were obtained from patients with TB hospitalized in F. J. Muñiz Hospital, Buenos Aires, Argentina. Informed consent was obtained from patients according to the guidelines of the ethics committee of the hospital. All patients were diagnosed by the presence of recent clinical respiratory symptoms, abnormal chest radiography, a sputum smear positive for acid-fast bacilli (AFB), and the identification of *Mtb* in culture. Exclusion criteria included a positive test for HIV and the presence of concurrent infectious diseases or noninfectious conditions (cancer, diabetes, or steroid therapy). Sputum smear examination, mycobacterial culture, strain identification, and drug susceptibility testing were performed according to standard procedures. Susceptibility to isoniazid, rifampin, and to any other specific anti-TB drugs was tested as previously described [26]. Available MDR *Mtb* isolates were genotyped by IS*6110* DNA fingerprinting and spoligotyping using standardized protocols [27, 28]. A total of 30 MDR-TB patients (14 men and 16 women; median age 26 [25th to 75th percentiles: 23 to 40] years) and 15 S-TB patients (9 men and 6 women; 31 [22 to 51] years) were included. All MDR-TB and S-TB patients showed radiological advanced pulmonary disease (MDR-TB: 85% showed bilateral and 15% unilateral cavities; S-TB: 57% with bilateral cavities, 43% unilateral cavities) and 21/30 MDR-TB and all S-TB were AFB-smear-positive at the time of the study. The median number of AFB/field for MDR-TB was 4 (25th to 75th percentiles: 0.7 to 10); and for S-TB, 6 [1 to 10]). Ten PPD$^+$HD

(4 men and 6 women; 30 [27 to 46] years) were included as controls.

Mononuclear Cells
Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque centrifugation and suspended in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, Thermo Scientific) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal calf serum (Gibco, Invitrogen).

Antigens
MDR strains M and Ra, as well as clinical isolates from drug-susceptible strains 10406 (Ls) of the Latin American–Mediterranean family and 10475 (Hs) of the Haarlem family and the laboratory strain H37Rv, were grown in Middlebrook 7H9 broth (Difco Laboratories) at 37°C in 5% CO_2. Mycobacteria were harvested in the log phase, washed 3 times, and the bacterial pellets were inactivated by gamma irradiation. Thereafter, pellets were suspended in pyrogen-free phosphate-buffered saline (PBS), sonicated, and suspended at an optical density of 1 at 600 nm ($\approx 10^8$ bacteria/mL), and stored at -20°C until use.

PBMC Cultures
PBMCs (2×10^6 cells/mL) were cultured for 48 hours or 5 days in polystyrene tubes (BD Falcon) at 37°C in a humidified 5% CO_2 atmosphere with strains M, Ra, Hs, Ls, or H37Rv at a 2:1 *Mtb* to PBMC ratio. Then, cells were tested for IL-23R, IFNγ, or IL-17 expression by flow cytometry.

Immunofluorescence Analysis
For surface staining, ex vivo or cultured PBMCs were incubated with PE-Cy5/FITC-anti-CD4 or anti-CD8, and FITC-anti-gamma-delta TCR (all from BD Bioscience) and PE-anti-IL-23 receptor (IL-23R, R&D System Inc.) or the corresponding isotype monoclonal antibodies (mAb) for 20 minutes at 4°C.

Prior to intracellular cytokine staining, Brefeldin A (5 µg/mL; Sigma Chemical Co.) was added for the last 4 hours of culture to block cytokine secretion. Then cells were surface-stained for CD4, CD8, or γδTCR expression, fixed and permeabilized according to the manufacturer's instructions (Perm2, BD Bioscience) before PE-anti-IL-17 and FITC-anti-IFNγ (BD Bioscience) or the corresponding isotypes were added.

Stained cells were washed, fixed with 0.5% paraformaldehyde, and suspended in Isoflow (BD Bioscience) for acquisition. Sixty-thousand events were acquired using a FACSCan flow cytometer (BD Bioscience). FCS Express software (De Novo Software) was used for 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 the lymphocyte population or within CD4$^+$, CD8$^+$, or γδTCR$^+$ cells.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 (Graphpad Software Inc.). Results were expressed as medians and 25th to 75th percentiles. Nonparametric Kruskal-Wallis test was used to compare data from TB patients and healthy individuals, followed by Mann–Whitney U test to compare 2 groups. Friedman test was performed to compare data within each group, followed by Wilcoxon rank sum test. Correlations were done by the nonparametric Spearman rank correlation test. All statistical analyses were 2-sided, and the significance level adopted was $P < .05$.

RESULTS

MDR Strains M and Ra Expand More IL-17$^+$ Cells Than Drug-Susceptible Strains Hs and Ls

The ability of MDR strains M and Ra to expand IL-17$^+$ cells upon stimulation of PBMC from S-TB and MDR-TB patients and PPD$^+$HD was assessed using strain H37Rv as a reference control. The percentages of CD4$^+$ (%CD4$^+$IL-17$^+$), CD8$^+$ (%CD8$^+$IL-17$^+$), and γδTCR$^+$ cells (%γδTCR$^+$IL-17$^+$) expressing IL-17 were determined in the lymphocyte gate. Given that in our experimental conditions the expression of IL-17 was consistently higher at 48 hours compared with 5 days, all experiments were performed only at 48 hours.

As shown in Figure 1, nonstimulated CD4$^+$ and γδTCR$^+$ cells from TB patients showed higher percentages of IL-17$^+$ cells than PPD$^+$HD. In TB patients and PPD$^+$HD, the %CD4$^+$IL-17$^+$ and %γδTCR$^+$IL-17$^+$ were enhanced upon stimulation with strains M, Ra, and H37Rv; H37Rv being the lowest IL-17 values detected, and strain M the highest (Figure 1A, C, and D). In addition, a greater increase of %CD8$^+$IL-17$^+$ cells by strains M and Ra than H37Rv was observed in TB and PPD$^+$HD (Figure 1B). Interestingly, the levels of IL-17$^+$ cells expanded by all 3 strains were remarkably increased in MDR-TB patients, CD4$^+$ and CD8$^+$ cells being the main Th17 subsets in TB patients.

Having observed the high capacity of strains M and Ra to induce IL-17, we wanted to determine whether it was a characteristic of these particular outbreak MDR strains, or whether it was shared with drug-susceptible *Mtb* strains belonging to their respective families. For this purpose, 2 fully drug-susceptible clinical isolates were selected, strain 10406 (Ls), a representative of the Latin American–Mediterranean family related to strain Ra; and 10475 (Hs), a representative of the Haarlem family related to strain M. As observed in Figure 2, Hs and Ls expanded a lower percentage of IL-17$^+$ cells than M and Ra in MDR-TB, while in PPD+HD, they did not (data not shown).

CD4$^+$, CD8$^+$, and γδTCR$^+$ Cells From TB Patients Show Increased Expression of IL-23 Receptor

Given that human Th17 cells constitutively express the receptor for IL-23 (IL-23R) [29], its expression was determined in PBMCs as shown in Supplementary Figure 2 (online only). As

shown in Table 1, ex vivo PBMCs from TB patients showed a higher percentage of IL-23R$^+$ cells than PPD$^+$HD, the highest values being detected in MDR-TB patients. In addition, γδTCR$^+$ cells from TB expressed more IL-23R than CD4$^+$ cells, but no differences were observed between these 2 subsets in PPD$^+$HD. Furthermore, upon stimulation with *Mtb* strains, IL-23R expression increased in cells from TB patients, strain M being the highest inducer and the only one that induced IL-23R expression in PPD$^+$HD. These results indicate that TB patients show a high proportion of circulating IL-23R$^+$ cells that can be expanded upon stimulation with *Mtb* strains.

MDR-TB Patients Show an Altered Proportion of Single IL-17$^+$IFNγ$^-$ Cells

IFNγ controls the differentiation of Th17 cells, and the existence of a remarkable number of IL-17$^+$IFNγ$^+$-double-positive cells, named "Th17-Th1" cells, has been reported in circulating T cells [30]. To explore the importance of this double-positive subset, we determined the proportions of IL-17$^+$ and IFNγ$^+$ as well as of IL-17$^+$IFNγ$^-$ and IL-17$^+$IFNγ$^+$ T cells in antigen-stimulated CD4$^+$ and CD8$^+$ cells from TB and PPD$^+$HD. As shown in Figure 3, an inverse correlation between the %IL-17$^+$ and the %IFNγ$^+$ cells was found for all strains. Interestingly, the drop in IFNγ$^+$ cells was observed up to 2% of IL-17$^+$ cells, whereupon no further reduction in IFNγ$^+$ cells was seen. The percentage of single IL-17$^+$IFNγ$^-$ cells was higher in nonstimulated CD4$^+$ and CD8$^+$ cells from TB patients, the highest values found being in MDR-TB (Figure 4). Upon *Mtb* stimulation, the proportion of IL-17$^+$IFNγ$^-$ cells increased in CD4$^+$ and CD8$^+$ cells from TB, while in PPD$^+$HD this proportion was slightly enhanced in CD8 only by strain M (Figure 4A and C). Besides, among patients with TB, MDR-TB showed the highest levels of IL-17$^+$IFNγ$^-$ in CD4$^+$ cells (Figure 4A), but differences with S-TB were not found in the CD8 subset (Figure 4C). Regarding the Th17-Th1 cells, IL-17$^+$IFNγ$^+$ cells did not differ between TB and PPD$^+$HD in nonstimulated cultures (Figure 4B and D); however, upon *Mtb* stimulation, H37Rv was the highest stimulus for IL-17$^+$IFNγ$^+$ in cells from TB and PPD$^+$HD, and strain M was the lowest. Additionally, S-TB and MDR-TB showed similar %IL-17$^+$IFNγ$^+$ within the CD4$^+$ subset (Figure 4B), but a diminished proportion within *Mtb*-stimulated CD8$^+$ T cells was observed in MDR-TB (Figure 4D).

Correlation Between IL-17 Expression and Clinical Features

In MDR-TB patients, we evaluated whether in vitro *Mtb*-stimulated IL-17 expression correlated with clinical data such as presence/absence of AFB in sputum using Ziehl–Neelsen stained smears, antigen load (number of bacilli/field), disease severity, primary and acquired MDR-TB (primary MDR-TB was defined as that diagnosed in a patient without previous TB treatment; acquired MDR-TB was defined as MDR-TB patients with a history of previous TB chemotherapy), days of treatment at the time
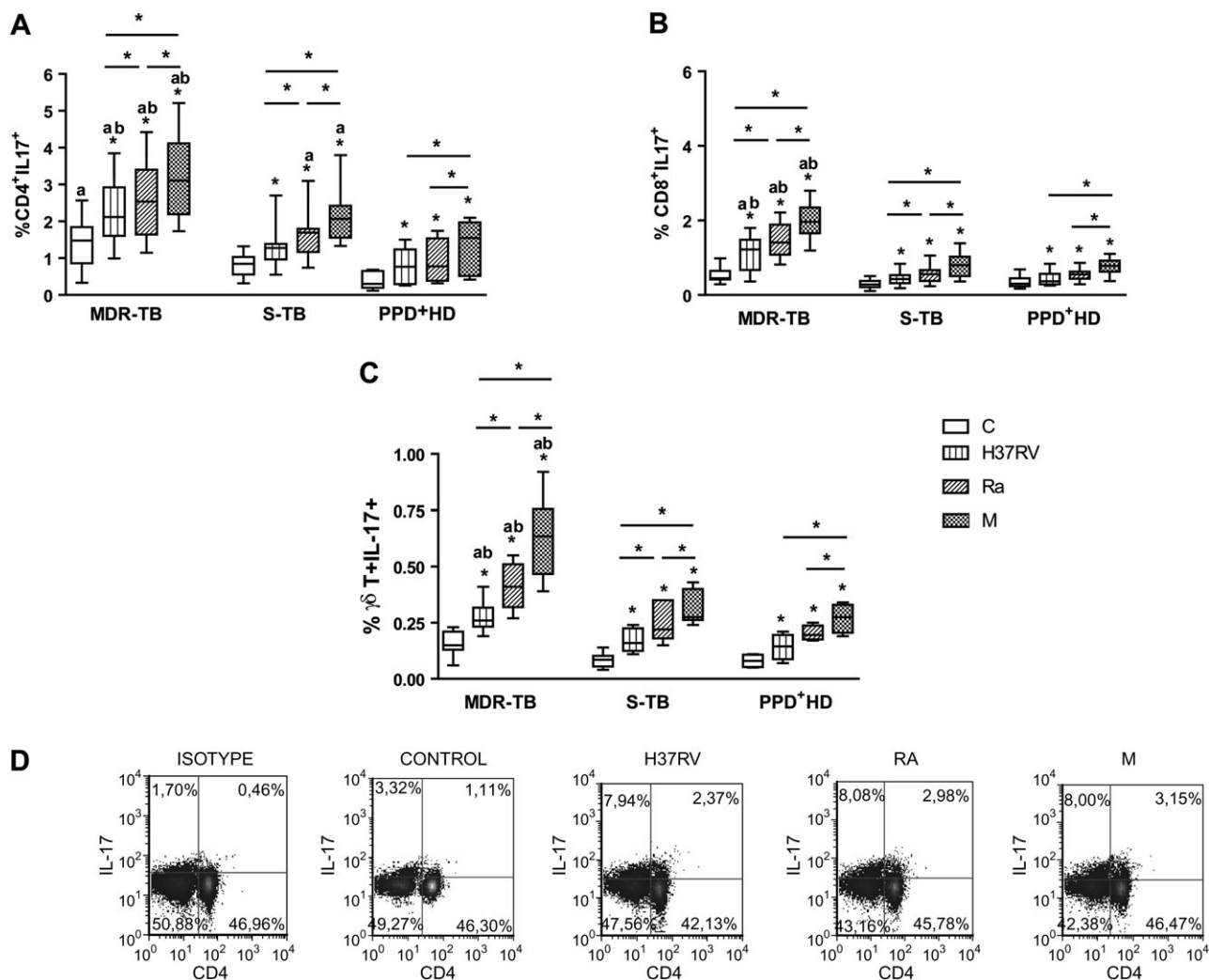
Figure 1. Strains of *M tuberculosis* (*Mtb*) expand IL-17$^+$ cells from patients with tuberculosis and healthy individuals. PBMCs from 30 MDR-TB and 15 S-TB patients and 10 PPD$^+$ healthy individuals (PPD$^+$HD) were cultured alone (control) or with the laboratory strain H37Rv or the MDR strains M and Ra for 48 hours. Then, the percentages of CD4$^+$, CD8$^+$, and γδT$^+$ cells expressing IL-17 were determined by flow cytometry (FACS). Results are expressed as (*A*) %CD4$^+$IL-17$^+$, (*B*) %CD8$^+$IL-17$^+$, and (*C*) %γδT$^+$ IL-17$^+$ cells in the lymphocyte gate (medians and 25th to 75th percentiles are shown). * = *P* < .05 for *Mtb*-stimulated vs control PBMCs; * = *P* < .05 for differences between *Mtb* strains; a = *P* < .05 for MDR-TB vs S-TB patients; b = *P* < .05 for MDR-TB or S-TB patients vs PPD$^+$HD. *D*, Intracellular IL-17 staining in nonstimulated and *Mtb*-stimulated CD4$^+$ cells; dot plots from 1 MDR-TB patient are shown and are representative of 30 patients studied. Numbers in upper-right quadrant represent the percentage of CD4$^+$IL-17$^+$ cells within live lymphocyte gate.

of the study, and days to reach AFB-smear-negative sputum (AFB$^-$). At the time of the study, we observed that MDR-TB with AFB-smear-positive sputum (AFB$^+$) showed higher proportions of CD4$^+$, CD8$^+$, and γδTCR$^+$ cells expressing IL-17 than that of AFB$^-$ patients (Figure 5*A*). Besides, the levels of spontaneous and M-stimulated γδTCR$^+$IL-17$^+$ cells directly correlated with the antigen load (%γδTCR$^+$IL-17$^+$ cells vs number of bacilli/field; Spearman rank correlation, C: $r = 0.8494$, $P = .0019$; M: $r = 0.6567$, $P = .0391$). AFB$^+$ MDR-TB also showed higher levels of IL-17$^+$IFNγ$^-$ and lower IL-17$^+$IFNγ$^+$ cells within CD4$^+$ and CD8$^+$ subsets than AFB$^-$ patients (Figure 5*B*). Notably, 70% of primary MDR-TB were AFB$^-$ and 67% of acquired MDR-TB were AFB$^+$. Thus, when patients were grouped into primary and

acquired MDR-TB, the distribution of IL-17$^+$IFNγ$^-$ and IL-17$^+$IFNγ$^+$ cells was similar to that observed with AFB smear status (Figure 5*C*). Remarkably, at the time of the study, all S-TB patients showed a primary disease and were AFB$^+$, but the levels of *Mtb*-stimulated IL-17$^+$ cells in these patients were lower than in AFB$^+$ MDR-TB patients (Figure 4).

We also observed that during TB treatment, the levels of CD4$^+$IL-17$^+$ (Figure 6*A*) and IL-17$^+$IFNγ$^-$ within CD4$^+$ cells decreased (Figure 6*B*). Besides, we observed that the fewer the days to reach a negative AFB, the higher the IFNγ-producing cells; that is, the %IL-17$^+$IFNγ$^+$ within CD4$^+$ (Figure 6*C*) and the %CD4$^+$IFNγ$^+$ (Figure 6*D*). The same pattern was detected for CD8$^+$IL-17$^+$ and CD8$^+$IFNγ$^+$ cells (data not shown).
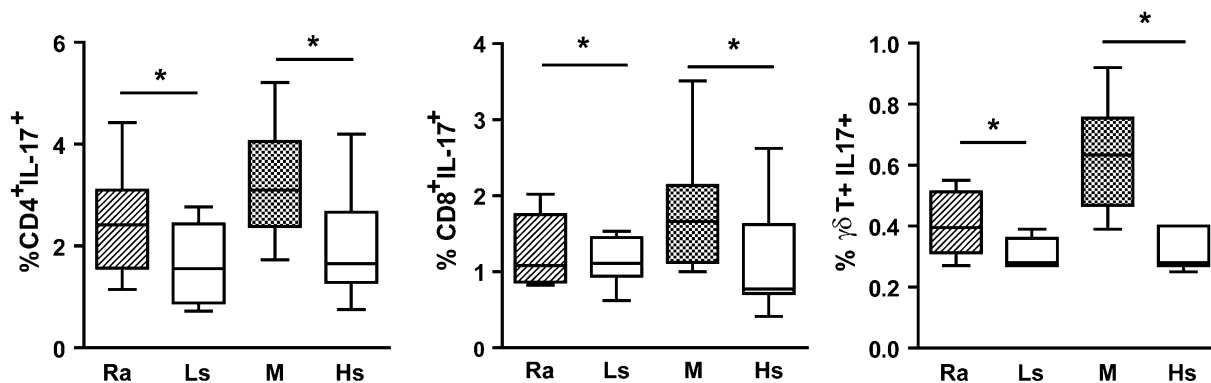
Figure 2. MDR M and Ra strains expand more IL-17$^+$ cells than drug-susceptible strains of the Haarlem and the Latin American–Mediterranean family. PBMCs from 10 MDR-TB patients were cultured for 48 hours with MDR strains M and Ra and 2 drug-susceptible strains belonging to Haarlem (Hs) and Latin American–Mediterranean (Ls) families. The %CD4$^+$IL-17$^+$, %CD8$^+$IL-17$^+$, and %γδT$^+$IL-17$^+$ cells were determined by FACS. Medians and 25th to 75th percentiles are shown. * = $P < .05$ for Ra vs Ls and M vs Hs.

DISCUSSION

Recent studies in animal models have demonstrated that IL-17 is essential for the development of a protective response in mycobacterial infections [31, 32]; however, its role in human tuberculosis is not clearly understood. In this work, we have found

Table 1. MDR-TB Express High Proportion of IL-23R$^±$ Cells in Lymphocytes

	MDR-TB	S-TB	PPD$^+$HD
CD4$^+$ T cells			
Ex vivo	2.3 (2.1–3.7)[a,b]	1.1 (0.9–1.6)[a]	0.6 (0.5–1.2)
Control	2.4 (2.3–2.6)[a,b]	1.4 (1.0–1.5)[a]	0.6 (0.4–0.7)
H37Rv	2.7 (2.5–2.9)[c]	1.8 (1.4–2.2)[c]	0.8 (0.6–1.0)
Ra	3.1 (3.1–3.3)[c]	2.1 (2.9–3.1)[c]	0.8 (0.6–1.1)
M	4.3 (4.2–4.5)[c]	3.3 (2.8–3.6)[c]	1.0 (0.9–1.7)[c]
CD8$^+$ T cells			
Ex vivo	1.0 (0.9–2.3)	1.0 (0.8–1.2)	1.0 (0.6–1.8)
Control	1.04 (1.01–1.06)	0.9 (0.86–0.91)	0.6 (0.5–0.9)
H37Rv	1.3 (1.2–1.9)[c]	1.13 (1.1–1.2)[c]	0.7 (0.5–0.9)
Ra	1.75 (1.7–1.8)[c]	1.6 (1.5–1.7)[c]	0.8 (0.6–0.9)
M	1.86 (1.8–1.9)[c]	2.2 (2.1–2.5)[c]	1.3 (0.9–2.3)[c]
γδT cells			
Ex vivo	4.5 (4.1–4.7)[a,b]	2.3 (1.6–2.5)[a]	0.8 (0.6–1.6)
Control	4.1 (4.0–4.3)[a,b]	2.3 (2.2–2.5)[a]	1.2 (0.8–1.3)
H37Rv	4.9 (4.6–5.0)[c]	2.9 (2.7–3.0)[c]	1.3 (0.9–3.5)
Ra	5.8 (5.4–6.0)[c]	3.2 (3.1–3.4)[c]	1.3 (0.9–3.6)
M	7.2 (6.4–7.9)[c]	3.8 (3.4–4.2)[c]	1.6 (1.2–4.9)[c]

NOTE. IL-23 receptor (IL-23R) expression was tested in recently isolated PBMCs (ex vivo) or cultured for 48 hours without control or with MDR strains H37Rv, Ra, and M. Results are expressed as percentage of IL-23R$^+$ cells within CD4$^+$, CD8$^+$, and γδT$^+$ cells. Medians and 25th to 75th percentiles are shown. Statistical differences: MDR-TB or S-TB vs PPD$^+$HD:

[a] $P < .05$.

[b] MDR-TB vs S-TB patients: $P < .05$.

[c] Control vs *Mtb*-stimulated cells: $P < .05$.

that MDR strains M and Ra are stronger stimuli of IL-17 expression than drug-susceptible strains of the same families in T cells from patients with TB and PPD$^+$HD. TB patients also showed an increased proportion of circulating T cells expressing IL-17 that was associated with high IL-23R levels on T cells, and showed the presence of AFB in sputum smears.

It has been demonstrated that *M. bovis* BCG vaccine and purified protein derivative (PPD) are able to expand memory CD4$^+$IL-17$^+$ cells [10, 16]. Also, dendritic cells (DCs) matured with H37Rv induce IL-17 expression in isolated CD4$^+$ T cells from healthy donors [33]. Herein we demonstrated that MDR strain M expands larger numbers of IL-17$^+$ cells than MDR strain RA and H37Rv in T cells from PPD$^+$HD. In healthy individuals, IL-17 is highly expressed in CD4$^+$ cells and poorly expressed in CD8$^+$ and γδT$^+$ cells, which is consistent with previous studies [16]. Also, we demonstrated that drug-susceptible Haarlem and Latin American–Mediterranean clinical isolates do not expand IL-17$^+$ cells at all in PPD$^+$HD, but develop lesser IL-17$^+$ cells than MDR isolates in TB. These discrepancies might likely be ascribed to variations in cell-wall structure among *Mtb* strains and consequently to different recognition of *Mtb* by pattern recognition receptors on antigen-presenting cells. In this line, Toll-like receptors (TLRs), C-type lectins, and nucleotide-binding oligomerization domain (NOD)-like and Dectin-1 receptors have been implicated in *Mtb* recognition by DCs and the triggering of IL-12 and IL-23 [33–37].

We found large numbers of IL-17$^+$ T cells in TB patients, particularly in MDR-TB patients. Both MDR strains were better IL-17 producers than H37Rv, and strain M was the strongest stimulus for an already-induced response in CD4$^+$ but also to a lesser extent in CD8$^+$ and γδTCR$^+$ cells from TB patients. These results contrast with the reportedly low ability of BCG [16], 6-kDa early secretory

antigenic target (ESAT-6), and 10-kDa culture filtrate protein (CFP-10) [38] to expand IL-17$^+$-expressing CD4$^+$ T cells in
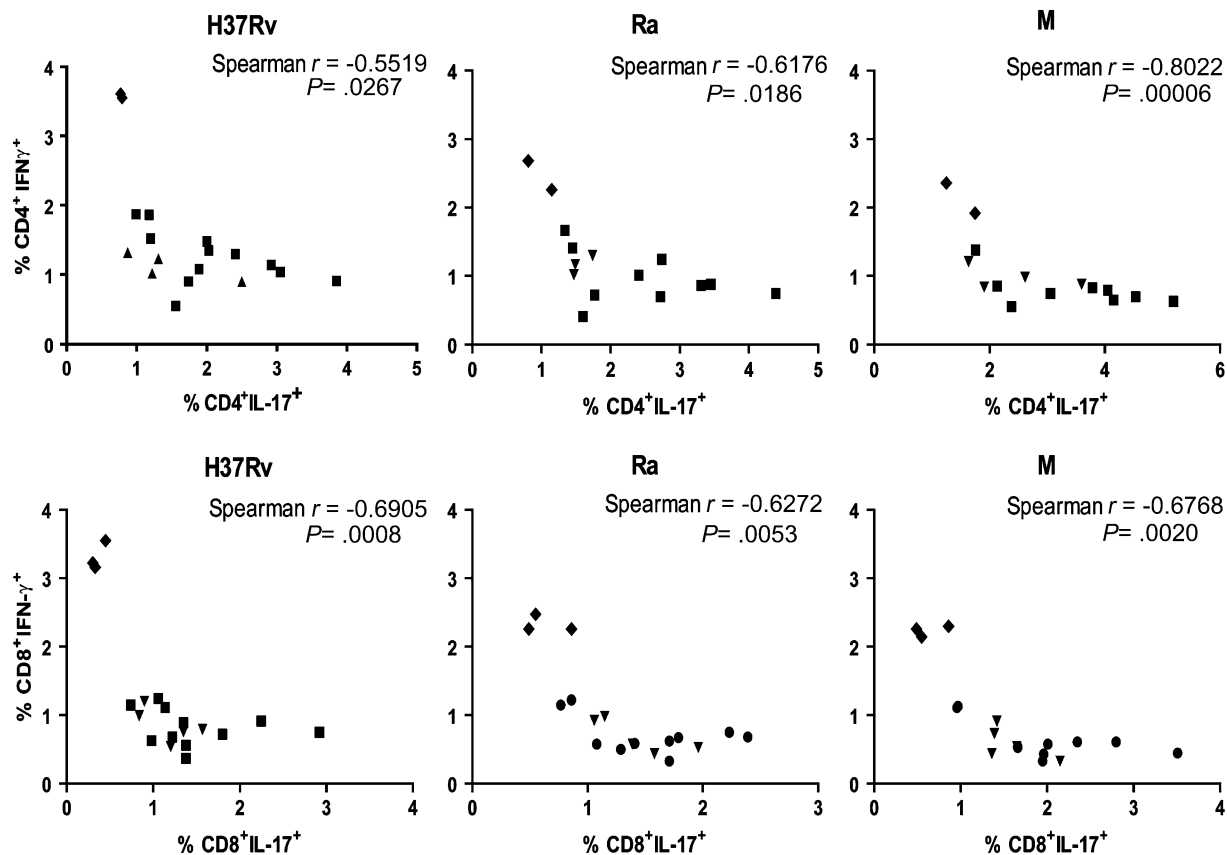
Figure 3. The levels of IL-17$^+$ cells negatively correlate with the expression of IFNγ. Correlation among the percentages of *Mtb*-stimulated CD4$^+$/CD8$^+$ IL-17$^+$ cells and CD4$^+$/CD8$^+$ IFNγ^+ cells from MDR-TB (□) and S-TB (■) patients and PPD$^+$HD (▲) was calculated. Individual data and Spearman rho coefficients are shown.

S-TB patients. The discrepancy may be due to differences in assay conditions, use of purified CD4$^+$ cells instead of PBMCs, and antigens chosen. Although CD8$^+$IL-17$^+$ cells have not been previously detected in TB patients, mouse-naive T cells were experimentally differentiated into CD8$^+$IL-17$^+$ cells by developmental conditions [39]. Thereby, it is tempting to hypothesize that MDR strains promote Th17-inducing conditions. The expansion of $\gamma\delta$TCR$^+$IL-17$^+$ cells by *Mtb* observed in TB, and particularly in MDR-TB, are in accordance with previous reports in humans as well as in mouse models [11, 18, 32]. In this context, the higher IL-23R expression on lymphocytes may also explain the greater IL-17$^+$ cell levels found in TB patients. It is known that IL-23 is required for Th17 development because mice deficient in IL-23p19 are unable to hold a proper Th17 response; although upon *Mtb* infection, IL-23 is not required for initial differentiation of IL-17-producing cells, its expression is required to sustain IL-17 production [14, 17]. So, both MDR strains might be stronger IL-23 producers, tipping IL-23/IL-12 balance to Th17 response. It is known that IL-12p40 is shared by both IL-12p70 and IL-23 [40, 41] so the relative expression of IL-23p19 and IL-12p35 subunits would balance the secretion of bioactive IL-12p70 and IL-23. Although this is not well

understood in TB, it is likely that further cytokine signaling would be important to sustain a Th1 or a Th17 response. IFNγ has been demonstrated to negatively regulate the production of IL-17 in a mouse model of mycobacterial infection [42], while the levels of IFNγ-producing $\gamma\delta$T cells were reciprocally associated with IL-17–producing $\gamma\delta$T cells in TB patients [18]. In this line, CD4$^+$IL-17$^+$ and CD8$^+$IL-17$^+$ levels were inversely correlated with IFNγ levels, and the highest expansion of IL-17 proportions were obtained upon M and Ra stimulation (which are poor IFNγ producers [25]), indicating that the altered Th1 profile could heighten Th17 response. Human Th17 cells are a heterogeneous population composed of IL-17$^+$-single- and IL-17$^+$IFNγ^+-double-positive cells, as observed in T cells differentiated in vitro [4, 10, 43, 44] as well as memory CD4$^+$ T cells with distinctive properties from Th1 lineage [43]. Herein we showed that the higher the levels of IL-17$^+$IFNγ^+, the lower the proportion of IL-17$^+$IFNγ^- cells observed in CD4$^+$ and CD8$^+$ cells from TB patients and from PPD$^+$HD upon *Mtb* stimulation. However, the strains showed differences in the expansion of IL-17$^+$IFNγ^+ cells. H37Rv was the best stimulus in PPD$^+$HD, which is in accordance with other studies [30]. Consistently with the low ability to stimulate IFNγ production
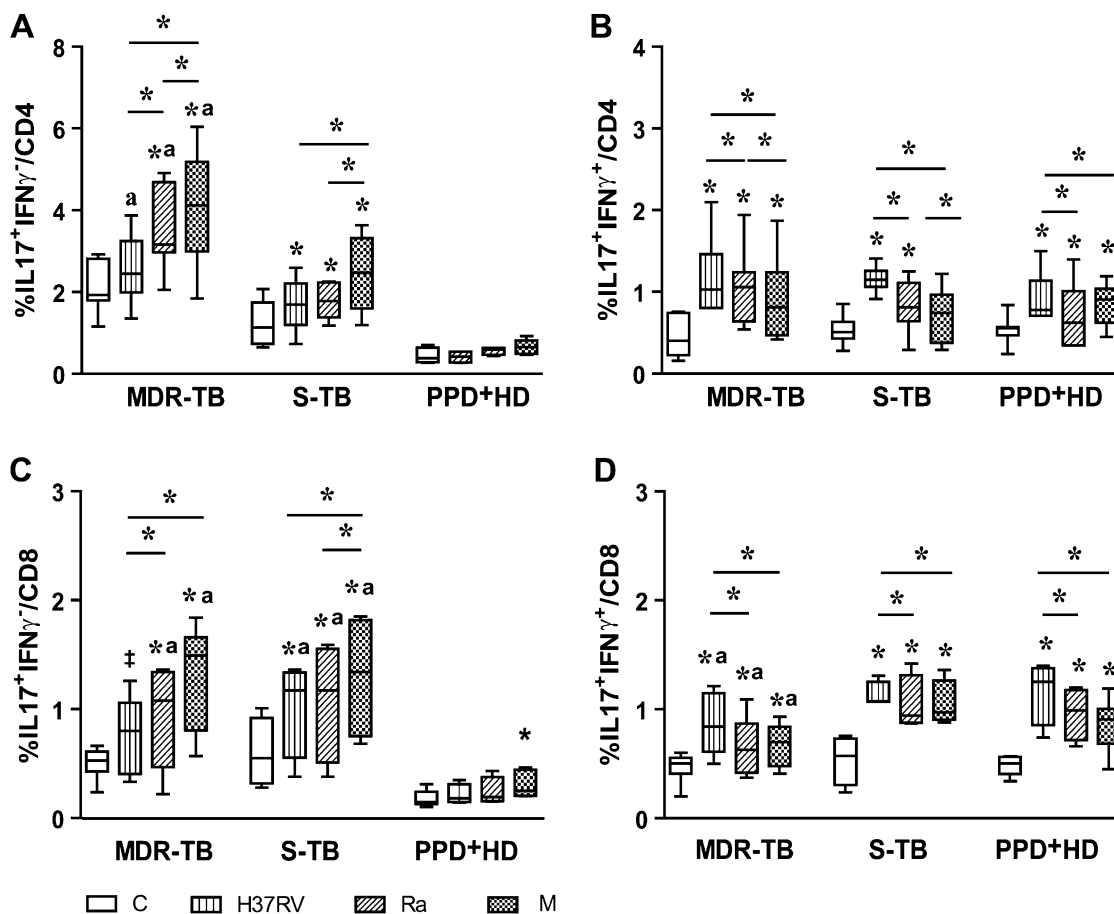
Figure 4. MDR strains M and Ra increase the levels of IL-17$^+$IFNγ$^-$ cells in CD4$^+$ and CD8$^+$ cells from MDR-TB patients. PBMCs from 15 MDR-TB patients and 8 PPD$^+$HD were cultured for 48 hours without control (C) or with strains H37Rv, Ra, and M. Then, cells were tested for their IL-17 and IFNγ expression by flow cytometry. Results are expressed as %IL-17$^+$IFNγ$^-$ and %IL-17$^+$IFNγ$^+$ cells within CD4$^+$ (*A* and *B*, respectively) and CD8$^+$ (*C* and *D*, respectively) cells. Medians and 25th to 75th percentiles are shown. * indicates $P < .05$ for *Mtb*-stimulated vs control PBMC; * indicates $P < .05$ for differences among strains; a = MDR-TB vs PPD$^+$HD individuals.

[25], Ra and M strains expanded lower double-positive cells, but they stimulated IL-17$^+$IFNγ$^-$ CD4$^+$ and CD8$^+$ cells in MDR-TB patients. Considering that IL-12 [30] and IFNγ [45] drive CD4$^+$IL-17$^+$ cells to produce IFNγ, it is tempting to speculate that the low proportion of double-positive cells in MDR-TB could also be associated with the impaired *Mtb*-induced Th1 response observed in these patients [25, 45, 46]. However, we cannot exclude anti-inflammatory cytokines, such as IL-4 or transforming growth factor β (TGF-β), which could also be contributing to the differential activation of these 2 Th17 subsets. Current studies are being performed to evaluate the role of cytokines related to the IFNγ/IL-12 and IL-17/IL-23 axes and anti-inflammatory cytokines on Th17 response induced by MDR M and Ra strains.

H37Rv-infected mice subjected to repeated BCG vaccination display an increased Th17 response with increased PMN influx and lung tissue damage [47]. As immunopathology is a central feature of *Mtb* lung infection and its regulation during chronic *Mtb* infection is essential for host survival, IL-17 and Th17 cells may play a role by inducing the recruitment and activation of PMN and triggering the production of proinflammatory cytokines and chemokines [4]. Neutrophil recruitment and survival may be one of the mechanisms by which IL-17 promotes granuloma organization. However, this response must be regulated because large PMN accumulation in the lung is generally associated with a bad prognosis and increased lung pathology. It has recently been asserted that PMN are the predominant infected cells in active TB patients, suggesting that these cells provide a permissive environment for bacillary replication [48]. In this study, we showed that bacterial load is associated with increased IL-17-producing CD4$^+$ and CD8$^+$ cells in vivo and, upon *Mtb* stimulation, IL-17 expression was further enhanced in AFB$^+$ and AFB$^-$ MDR-TB; however, the latter showed less IL-17 expression. In addition, the decreased levels of CD4$^+$IL-17$^+$ cells during treatment can also be explained by either the diminution in CD4$^+$IL-17$^+$IFNγ$^-$ levels or by the acquisition of an AFB$^-$ status in which low proportions of single IL-17$^+$ cells were observed. Though all S-TB patients had an advanced disease and were
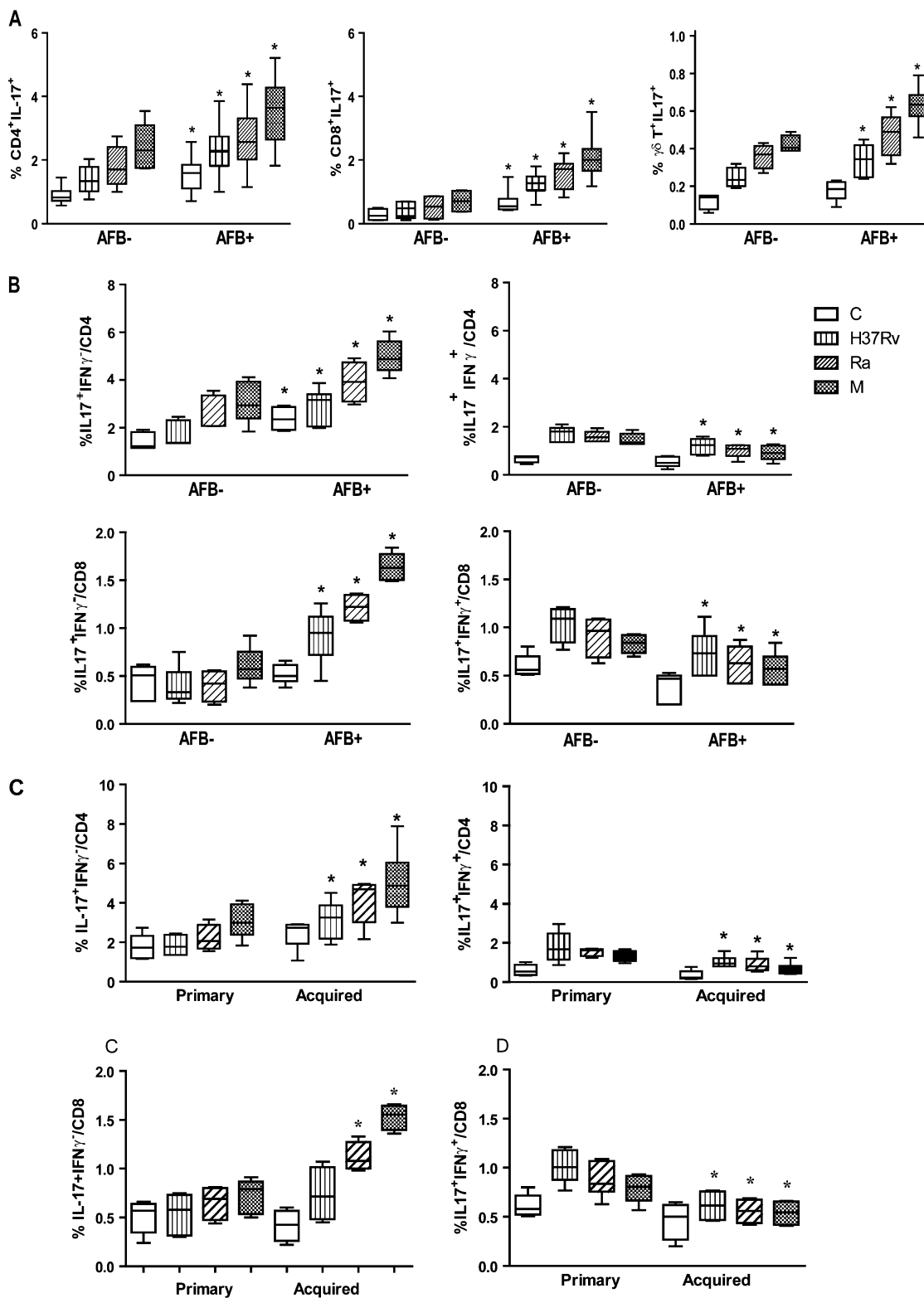
Figure 5. IL-17$^+$IFNγ^- and IL-17$^+$IFNγ^+ cells distribute differently in MDR-TB patients. MDR-TB patients were grouped according to the presence or absence of acid-fast bacilli in sputum smear (AFB$^+$ and AFB$^-$). Then, the medians and 25th to 75th percentiles of (*A*) IL-17$^+$ within CD4$^+$, CD8$^+$ and γdT$^+$ cells and (*B*) %IL-17$^+$IFNγ^- and %IL-17$^+$IFNγ^+ cells in CD4$^+$ and CD8$^+$ subsets were calculated for each group. * = $P < .05$ for AFB$^+$ vs AFB$^-$ patients. *C*, MDR-TB patients were grouped in primary and acquired tuberculosis. Then, the medians and 25th to 75th percentiles of %IL-17$^+$IFNγ^- cells and %IL-17$^+$IFNγ^+ cells in CD4$^+$ and CD8$^+$ T cells were calculated for each group. * = $P < .05$ for primary vs acquired MDR-TB patients.
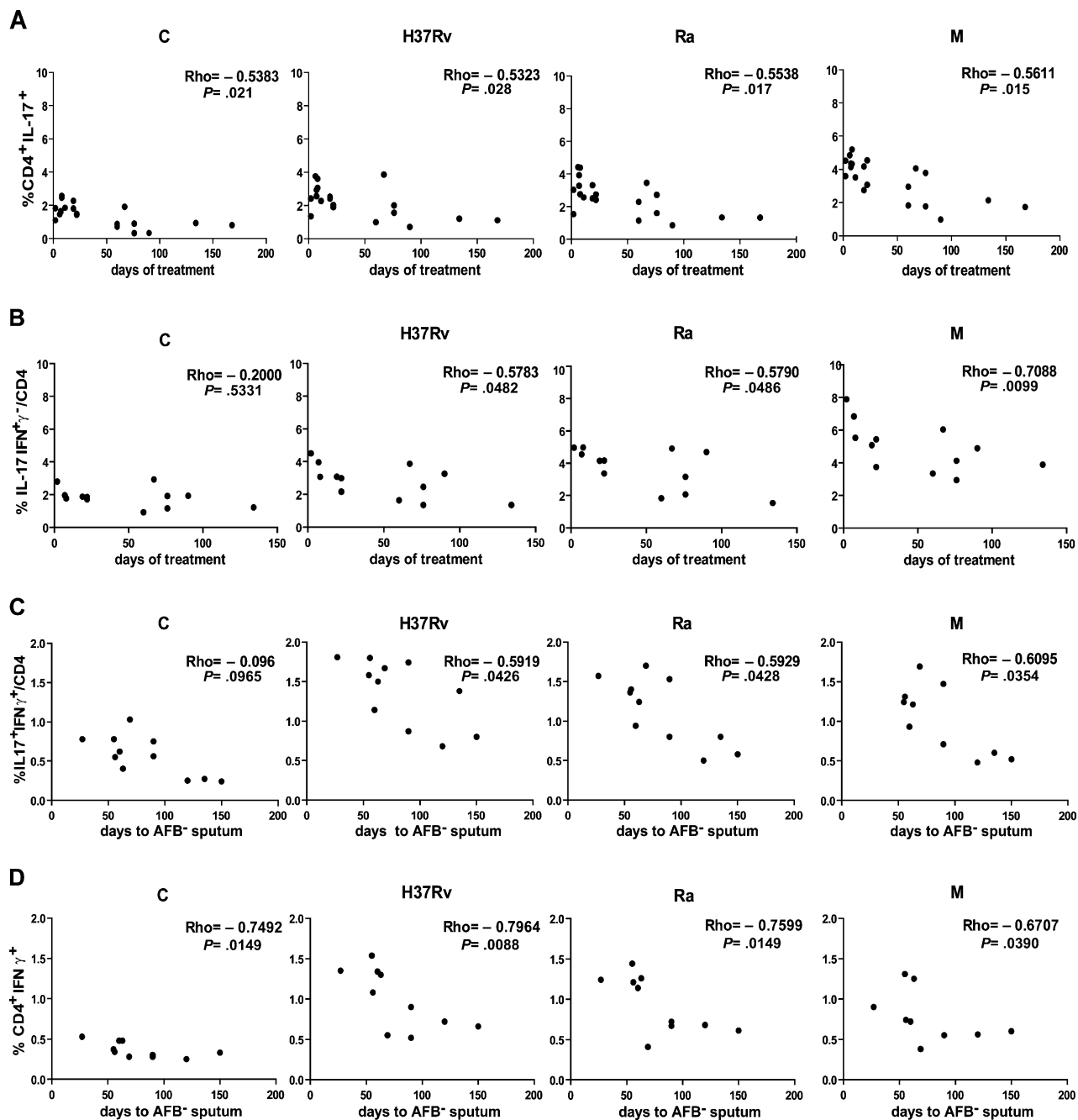
Figure 6. Correlation between CD4$^+$IL-17$^+$ and CD4$^+$IFNγ^+ cells with clinical features from MDR-TB. Correlation between i) the days of TB treatment and the percentage of *Mtb*-stimulated CD4$^+$IL-17$^+$ cells (*A*) and %IL-17$^+$IFNγ^- cells within CD4$^+$ (*B*); ii) the days to reach a negative sputum smear (days for AFB$^-$) and the %IL-17$^+$IFNγ^+ (*C*) and % CD4$^+$IFNγ^+ cells (*D*) in MDR-TB patients are depicted. Individual data and Spearman rho coefficients are shown.

AFB$^+$, they showed lower IL-17$^+$ cells than MDR-TB. Thus, other host factors could be implicated in Th17 response in MDR-TB. Besides, the lower proportion of CD4$^+$IL-17$^+$IFNγ^+ cells observed in patients who took longer to become AFB$^-$ could be associated with a decreased ability of CD4$^+$ cells to mount a Th1 response. Hence, all these results might suggest an immunopathological role of IL-17–producing cells in MDR-TB for the maintenance of high PMN influx that promotes severe tissue damage which, in turn, is associated with the low effectiveness of the second-line drugs employed in the treatment. In line with this, IL-17 or IL-23 can influence the homeostasis and phenotype of PMN, making them more prone to cause immunopathology [49]. Therefore, IL-17 may have beneficial effects on early stages of infection by recruiting PMN and promoting secretion of

tissue-specific homing chemokines, but prolonged the exposure of PMN to IL-17 or IL-23 can cause immunopathology.

In summary, we have observed that MDR strains M and Ra significantly increase the level of IL-17$^+$ cells more so than drug-susceptible *Mtb* strains of the Haarlem and Latin American–Mediterranean families. Remarkably, MDR-TB patients show the highest CD4$^+$ and CD8$^+$ cell-dependent IL-17 response that is independent on the strain but is closely associated with persistent and high antigen load, suggesting an immunopathological role in MDR-TB.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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