

IL-17 and IFN- γ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease

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ABSTRACT

Th1 lymphocytes are crucial in the immune response against *Mycobacterium tuberculosis*. Nevertheless, IFN- γ alone is not sufficient in the complete eradication of the bacteria, suggesting that other cytokines might be required for pathogen removal. Th17 cells have been associated with *M. tuberculosis* infection, but the role of IL-17-producing cells in human TB remains to be understood. Therefore, we investigated the induction and regulation of IFN- γ and IL-17 during the active disease. TB patients were classified as High and Low Responder individuals according to their T cell responses against the antigen, and cytokine expression upon *M. tuberculosis* stimulation was investigated in peripheral blood and pleural fluid. Afterwards, the potential correlation among the proportions of cytokine-producing cells and clinical parameters was analyzed. In TB patients, *M. tuberculosis* induced IFN- γ and IL-17, but in comparison with BCG-vaccinated healthy donors, IFN- γ results were reduced significantly, and IL-17 was markedly augmented. Moreover, the main source of IL-17 was represented by CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes, a Th1/Th17 subset regulated by IFN- γ . Interestingly, the ratio of antigen-expanded CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes, in peripheral blood and pleural fluid from TB patients, was correlated directly with clinical parameters associated with disease severity. Indeed, the highest proportion of CD4⁺IFN- γ ⁺IL-17⁺ cells was detected in Low Responder TB patients, individuals displaying se-

vere pulmonary lesions, and longest length of disease evolution. Taken together, the present findings suggest that analysis of the expansion of CD4⁺IFN- γ ⁺IL-17⁺ T lymphocytes in peripheral blood of TB patients might be used as an indicator of the clinical outcome in active TB. *J. Leukoc. Biol.* 91: 991-1002; 2012.

Introduction

The immune response elicited after *M. tuberculosis* infection is critically dependent on CD4⁺ T cells. In particular, Th1 cells have been shown to play an important role in granuloma formation and clearance of *M. tuberculosis* infection [1-3]. In fact, deficiencies in the IL-12-IFN- γ -STAT1 signaling pathway lead to the dissemination of mycobacterial infections [4, 5]. However, the fact of how *M. tuberculosis* is able to evade host immune surveillance and persist, particularly inside macrophages, remains to be understood. It has been proposed that CD4⁺ T cells producing IL-17 and IL-22 might contribute to adaptive immunity to *M. tuberculosis* [6]. Indeed, early cellular responses to *M. tuberculosis* induce IL-17 production, contributing to granuloma formation and control of bacterial growth [7]. Nevertheless, excessive IL-17 levels exacerbated inflammation, increasing neutrophil recruitment and tissue damage [8]. Despite the great strides made in the characterization of the acquired cellular response in TB patients, it remains to be elucidated what exactly constitutes a protective response [7].

During the chronic phase of TB, an equilibrium between Th1 and Th17 responses needs to be accomplished to control bacterial growth and constrain immunopathology [9]. Accordingly, the balance between IL-23/IL-17 and IL-12/IFN- γ pathways might define the resolution of *M. tuberculosis* infection [10], making such equilibrium crucial for the definition of the

Abbreviations: AFB=acid-fast bacilli, BCG=bacillus Calmette-Guérin, HD=healthy donors, HR=high-responder tuberculosis patients, I=isoniazid, LR=low-responder tuberculosis patients, Mtb-Ag=Mycobacterium tuberculosis antigen, PD-1=programmed cell death-1, PFMCS=pleural fluid mononuclear cells, RORC/ γ T=retinoic acid receptor-related orphan receptor C/ γ T, SLAM=signaling lymphocytic activation molecule, T-bet=T-box-containing protein expressed in T cells, TB=tuberculosis

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disease outcome [9]. Nevertheless, although the role of IFN- γ in the defense against *M. tuberculosis* and its ability to inhibit IL-17 production have been addressed [11, 12], the participation of IL-17-producing cells during human *M. tuberculosis* infection remains controversial. In the present report, we investigated the role of IL-17-secreting cells and their relationship with IFN- γ -expressing lymphocytes in patients with TB. Furthermore, we analyzed the potential correlation between the proportion of cytokine expression in peripheral blood and pleural fluid lymphocytes and the severity of the disease.

MATERIALS AND METHODS

Patients

HIV-uninfected patients with active TB were evaluated at the Hospital Muñiz (Buenos Aires, Argentina). Diagnosis of disease was established based on clinical and radiological data, together with the identification of AFB in sputum. Patients included in this study had received less than 1 week of anti-TB therapy. In some cases, tuberculous pleural effusions were obtained by thoracentesis. TB patients were classified as reported previously [13], based on in vitro lymphocyte responses to *Mtb*-Ag. Briefly, High Responder (HR) patients are individuals displaying significant proliferative responses, IFN- γ production, and an increased SLAM expression against the antigen, whereas Low Responder (LR) patients exhibit low proliferative responses, IFN- γ secretion, and percentages of SLAM⁺ cells [13]. Healthy donors (HD), who had received BCG vaccination at birth and lacked a history of TB, participated in the study, excluding those subjects with latent TB (determined by Quantiferon TB Gold test, Cellectis, Valencia, CA, USA). The control group included individuals who were matched in terms of sex, age, and ethnicity with TB patients included in the study. All participants provided a written, informed consent for the collection of samples and subsequent analysis. The protocols conducted in this work were approved by the Ethical Committee of the Hospital Muñiz and by the International Review Board Fundación Huésped.

Antigen

In vitro stimulation of cells throughout the study was performed with a cell lysate from the virulent *M. tuberculosis* H37Rv strain prepared by probe sonication (*Mtb*-Ag), obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA): *M. tuberculosis*, Strain H37Rv, whole cell lysate, NR-14822.

Cell preparation and reagents

PBMCs or PFMCs were isolated by centrifugation over Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ, USA) and cultured (1×10^6 cells/ml) with/without *Mtb*-Ag (10 μ g/ml) with RPMI 1640 (Gibco, Gaithersburg, MD, USA), supplemented with L-glutamine, gentamicin, and 10% human serum (Sigma-Aldrich, St. Louis, MO, USA), during 16 h, 48 h, or 5 days. In different experiments, cells were incubated with/without blocking anti-PD-1 mAb (5 μ g/ml, J116, eBioscience, San Diego, CA, USA); agonistic anti-SLAM mAb (10 μ g/ml, A12, eBioscience); blocking IFN- γ mAb (10 μ g/ml, NIB42, eBioscience); or isotype controls, in the presence/absence of *Mtb*-Ag (during 5 days) or PMA (25 ng/ml) plus Io (10 μ g/ml; for 16 h). Then, IL-17A and IFN- γ expression was determined by ELISA (eBioscience) and flow cytometry.

Flow cytometry

PBMCs or PFMCs were stimulated with *Mtb*-Ag for 5 days and incubated with Golgi Stop reagent containing monensin (1 μ l/ml; BD Biosciences, San Diego, CA, USA) for the final 5 h of culture.

Cells were then stained with specific fluorophore-marked antibodies against CD4 (PerCPCy5.5, OKT4, eBioscience), CD45RO (PECy7, UCHL1, Becton Dickinson, Franklin Lakes, NJ, USA), CCR7 (PE, 3D12, Becton

Dickinson), SLAM (PE, A12, Becton Dickinson), and/or PD-1 (FITC, MIH4, eBioscience). Intracellular staining was performed to determine IL-17A (PE or Alexa Fluor 647, eBio64CAP17, eBioscience), IFN- γ (FITC or allophycocyanin, 4S.B3, Becton Dickinson), T-bet (PE, eBio4B10, eBioscience), and RORC (PE, AFKJS-9, eBioscience) expression. For cytokine staining, BD Cytotfix/Cytoperm and BD Perm/Wash buffers were used following the manufacturer's instructions. Forkhead box p3 fixation/permeabilization buffers (eBioscience) were used when staining of transcription factors (T-bet and RORC) was performed. Negative control samples were incubated with irrelevant isotype-matched mAb in parallel with experimental samples, which were analyzed on a FACSaria II flow cytometer (BD Biosciences).

Statistical analysis

The Mann-Whitney and the Wilcoxon rank sum tests were used to analyze differences between unpaired or paired samples, respectively. Fisher's exact test and χ^2 for trend test were used for categorical variables. Correlation analyses were performed using the nonparametric Spearman correlation test. One-way ANOVA was used to compare the differences among multiple groups. Values of $P < 0.05$ were considered significant.

RESULTS

Patient population

No differences regarding age distribution, sex, ethnicity, AFB in sputum, or frequency of extra pulmonary forms of TB were found in HR and LR TB patients (Table 1). However, significant differences were detected regarding X-ray radiography severity [14], leukocyte count, and time of disease evolution (days previous to hospital admission, during which the patient displays clinical symptoms; Table 1). In particular, a pattern of considerable severity was detected in LR patients, who presented significant lower leukocytes, lymphocytes, and monocytes counts, evidenced severe pulmonary lesions, and had been ill longer than HR individuals (Table 1).

M. tuberculosis promoted IL-17 secretion from CD4⁺IFN- γ ⁺IL-17⁺ cells

PBMCs from TB patients and HD were stimulated with *Mtb*-Ag and IL-17, and IFN- γ expression was analyzed. A significant increase in the levels of both cytokines was detected at 5 days (Fig. 1A). Among TB patients, both groups of individuals secreted similar amounts of IL-17, whereas antigen stimulation induced significantly lower production of IL-17 in HD (Fig. 1A). In fact, *M. tuberculosis* induced IFN- γ and IL-17 in TB patients, but when compared with BCG-vaccinated HD, IFN- γ was reduced significantly, whereas IL-17 was markedly augmented in patients bearing active TB, indicating that the pattern of IL-17 secretion against *Mtb*-Ag would be opposite to the pattern of IFN- γ produced by the same individuals.

We next evaluated the potential relationship between IFN- γ and IL-17 in TB by analyzing the coexpression of both cytokines. Surprisingly, the majority IL-17-producing cells expanded by *Mtb*-Ag was IFN- γ ⁺ (Fig. 1B). In contrast, PMA/Io treatment markedly expanded CD4⁺IFN- γ ⁺IL-17⁻ and CD4⁺IFN- γ ⁻IL-17⁺ lymphocytes, whereas it did not increase CD4⁺IFN- γ ⁺IL-17⁺ cells (Fig. 1C). Given that CD4⁺IFN- γ ⁺IL-17⁺ cells, expanded by *Mtb*-Ag, coexpressed RORC and T-bet (Supplemental Fig. 1), present data demonstrated that the

TABLE 1. Epidemiological and Demographic Characteristics of TB Patients

Characteristic	HR TB patients (n = 49)	LR TB patients (n = 32)	P value
Proliferative responses (proliferation index)	10.96 \pm 10	2.31 \pm 0.37	0.009 ^a
IFN- γ production (fold-stimulation)	231.1 \pm 51.60	29.63 \pm 4.82	0.0001 ^a
Increase in the percentage of SLAM-positive cells	11.21 \pm 2.17	3.97 \pm 1.11	0.0093 ^a
Age	32.1 \pm 3.5	33.2 \pm 4.2	0.9669 ^b
Sex			
Male	40 (81.6%)	26 (81.25%)	
Female	9 (18.4%)	7 (18.75%)	0.7819 ^c
Ethnicity			
White (from European lineages)	13 (26.5%)	7 (21.9%)	
American Indian	36 (73.5%)	25 (78.1%)	0.7933 ^c
Hematologic studies			
Leukocytes (cells/mm ³)	10746 \pm 488	8394 \pm 432	0.0025 ^{b,d}
Lymphocytes (cells/mm ³)	1870 \pm 103	1410 \pm 115	0.0049 ^{b,d}
Monocytes (cells/mm ³)	997 \pm 55	673 \pm 58	0.0002 ^{b,c}
AFB in sputum smear			
Positive	44 (89.8%)	28 (87.5%)	
Negative	5 (10.2%)	4 (12.5%)	0.7341 ^c
Radiological lesions			
Severe	13 (26.5%)	18 (56.3%)	
Moderate	26 (53.1%)	13 (40.6%)	
Mild	10 (20.4%)	1 (3.1%)	0.0022 ^{d,f}
Time of disease evolution (days)	66.2 \pm 7.1	135.0 \pm 23.4	0.0004 ^{b,c}
Extrapulmonary TB			
Pleural effusion	9 (18.4%)	7 (21.9%)	
Milliary	4 (8.2%)	2 (6.3%)	
Other	2 (4.1%)	0 (0.0%)	0.9255 ^f

Continuous data are expressed as mean \pm SEM, and categorical data are expressed as number (percentages). HR and LR TB patients were classified according to proliferation [proliferation index: (cpm after sonicated *Mtb*-Ag stimulation)/(cpm after culturing with medium)]; IFN- γ production [fold-stimulation: (ng/mL after sonicated *Mtb*-Ag stimulation)/(ng/mL after culturing with medium)]; and increase in the percentage of SLAM-positive cells in response to sonicated *Mtb*-Ag stimulation, as previously reported (see Materials and Methods). HR, Proliferation index \geq 4, IFN- γ production $>$ 34, increase in % SLAM-positive cells \geq 8; LR proliferation index $<$ 4, IFN- γ production $<$ 34, increase in % SLAM-positive cells $<$ 8. If a patient fulfilled two of three of these criteria, the patient was assigned to that group. Radiological lesions: mild, patients with a single lobe involved and without visible cavities; moderate, patients presenting unilateral involvement of two or more lobes with cavities, if present, reaching a total diameter no greater than 4 cm; severe, bilateral disease with massive affectation and multiple cavities. Clinical symptoms previous to hospital admission analyzed in TB patients to establish the time of disease evolution: weight loss, night sweats, symptoms of malaise or weakness, persistent fever, presence of cough, history of shortness of breath, and hemoptysis. ^aP values were calculated by the Mann-Whitney U test for unpaired samples. ^bP values were calculated by the Mann-Whitney test for continuous variables. ^cP values were calculated by Fisher's exact test for categorical variables. ^dP $<$ 0.01. ^eP $<$ 0.001. ^fP values were calculated by the χ^2 for trend test for categorical variables.

main population involved in the secretion of IL-17 during active TB belonged to the Th1/Th17 subset.

M. tuberculosis induced Th1/Th17 lymphocytes with a memory-like phenotype in patients with active disease

Memory T lymphocytes include different cell populations characterized by distinct homing capacity and effectors' functions [15]. To further characterize the Th1/Th17 CD4⁺ subset involved in the immune response to *Mtb*-Ag, the proportion of central and effector memory lymphocytes among CD4⁺IL-17⁺IFN- γ ⁺ lymphocytes was investigated. We observed that after in vitro antigen stimulation, the majority of CD4 lymphocytes (56% \pm 6.5) displayed an effector memory-like phenotype (CCR7⁻CD45RO⁺) in TB patients (Fig. 2A), whereas in HD, 38% \pm 4.2 of T lymphocytes displayed a central memory-like phenotype (CCR7⁺CD45RO⁺; Fig. 2B). The proportions of cells with effector and central memory-like phenotypes detected in LR TB patients were similar to those observed in HR

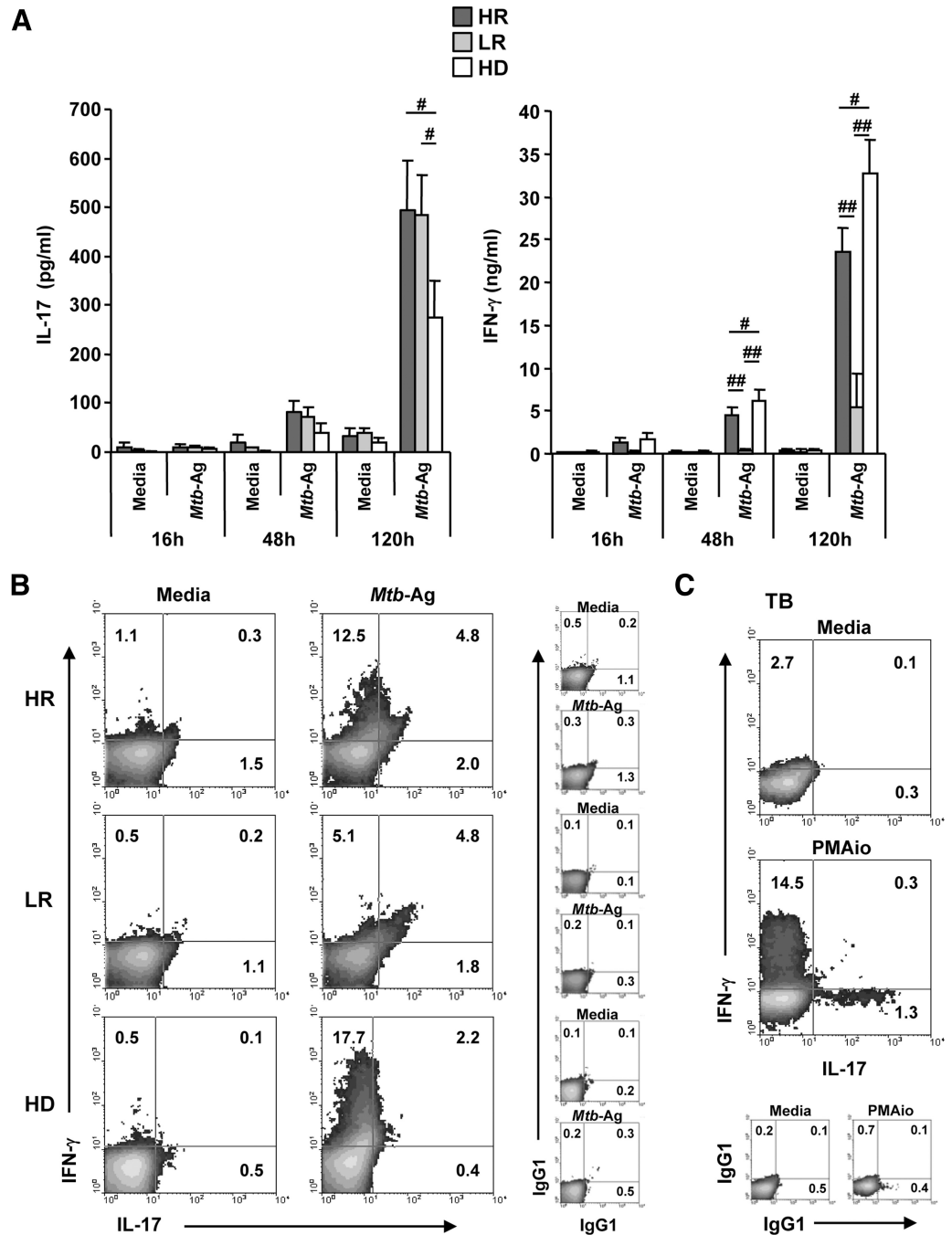
patients (data not shown), although LR patients displayed a lower expansion of antigen-specific cells [13].

When the effector and memory-like phenotypes on *Mtb*-Ag-expanded Th1/Th17 cells from TB patients were analyzed, it was observed that CD4⁺IFN- γ ⁺IL-17⁺ T lymphocytes displayed central and effector memory-like phenotypes (Fig. 2A and C). Conversely, in control individuals, central memory-like T cells were the main producer of IL-17 and IFN- γ (Fig. 2B and C). These results further confirm that CD4⁺IFN- γ ⁺IL-17⁺ T cells are specifically induced by *Mtb*-Ag, as they display a memory-like phenotype (central memory or effector memory).

The cytokine microenvironment influenced the expansion of IFN- γ - and IL-17-producing lymphocytes against *M. tuberculosis*

The cytokine milieu plays a main role during the differentiation of CD4⁺Th effectors [16]. For instance, IFN- γ and IL-4 negatively regulate the generation of IL-17-producing cells [17,

Figure 1. IL-17 and IFN- γ production in response to *Mtb*-Ag stimulation. (A) PBMCs from HR and LR TB patients and HD were stimulated with sonicated *Mtb*-Ag for 16 h, 48 h, and 120 h. IL-17 and IFN- γ levels were then determined by ELISA. Each bar represents the mean \pm SEM of IL-17 or IFN- γ secretion for each group ($n=18$ for each group). Statistical differences were calculated using the nonparametric Mann-Whitney test for unpaired samples. # $P < 0.05$; ## $P < 0.01$. (B) PBMCs from HR and LR TB patients and HD were stimulated with *Mtb*-Ag for 5 days, and then, IL-17 and IFN- γ coexpression was determined by flow cytometry, first, gating on lymphocytes by light scatter and then, gating on CD4⁺ T cells (HR, $n=9$; LR, $n=8$; HD, $n=9$). A representative density plot for each group is shown. Isotype controls are shown on the right. (C) PBMCs from TB patients were stimulated with PMA + Io (PMAio) for 16 h, and then, IL-17 and IFN- γ coexpression was determined by flow cytometry, first, gating on lymphocytes by light scatter and then, gating on CD4⁺ T cells. A representative density plot out of seven is shown. Isotype controls are shown (lower).



18]. In this respect, we reported previously that the addition of exogenous IL-17 significantly diminished the levels of IFN- γ secreted by TB patients in response to *Mtb*-Ag [19]. Therefore, we here analyzed whether the blockage of endogenous IFN- γ influenced the number of IL-17-secreting lymphocytes. Surprisingly, we observed that the blocking of IFN- γ in the presence of antigen significantly reduced the amount of IL-17 produced by both groups of TB patients, whereas it did not modify the levels of IL-17 secreted by HD (Fig. 3A). Taking into account the ample evidence demonstrating that IFN- γ negatively regulated Th17 cells [11, 12], the present data were quite unexpected. In fact, blockage of IFN- γ in cells stimulated with

PMA/Io significantly augmented the levels of IL-17 in TB patients and HD (Supplemental Fig. 2), confirming the specificity of the findings observed using *Mtb*-Ag. Indeed, the results indicate that *Mtb*-induced IFN- γ contributes to IL-17 secretion in patients with active disease.

To further gain insight into why IFN- γ blockage decreased *Mtb*-Ag-induced IL-17 in TB patients, the effects of IFN- γ blockage on antigen-expanded CD4⁺Th subsets were examined. As expected, blockage of endogenous IFN- γ significantly decreased the percentage of CD4⁺IFN- γ ⁺IL-17⁻ lymphocytes in the three groups of individuals studied (Fig. 3B). Moreover, anti-IFN- γ treatment slightly increased the number of

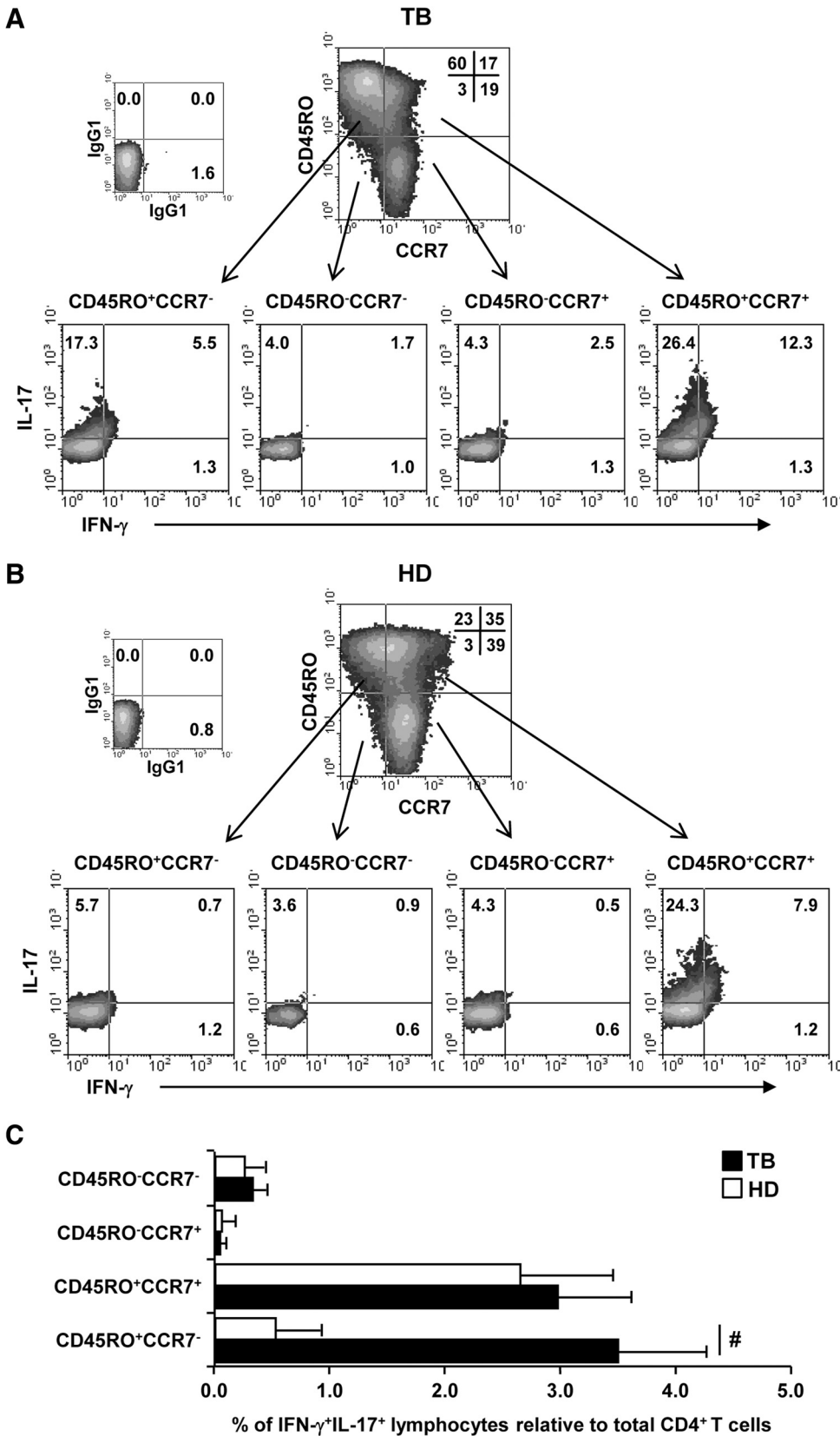


Figure 2. Phenotype profile of *Mtb*-Ag-induced CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes. PBMCs from TB patients and HD were stimulated with sonicated *Mtb*-Ag for 5 days. IL-17 and IFN- γ coexpression was determined by flow cytometry, first, gating on lymphocytes by light scatter; then, gating on CD4⁺ T cells; and finally, gating according to CD45RO/CCR7 expression. (A) A representative dot plot of a HR patient out of seven patients analyzed is shown. (B) A representative dot plot out of seven individuals analyzed is shown for HD. Isotype controls for CD45RO and CCR7 are shown on the left. (C) Each bar represents the mean \pm SEM of the percentage of CD4⁺ cells that are IFN- γ ⁺IL-17⁺ for each memory phenotype (CD45RO⁺CCR7⁺, central memory T cells; CD45RO⁺CCR7⁻, effector memory T cells; CD45RO⁻CCR7⁺, naïve T cells; or CD45RO⁻CCR7⁻, effector T cells). Values were calculated as (% of CD45RO⁺/⁻CCR7⁺/⁻ cells \times % of IFN- γ ⁺IL-17⁺ cells/100). Statistical differences were calculated using the non-parametric Mann-Whitney test for unpaired samples. #*P* < 0.05.

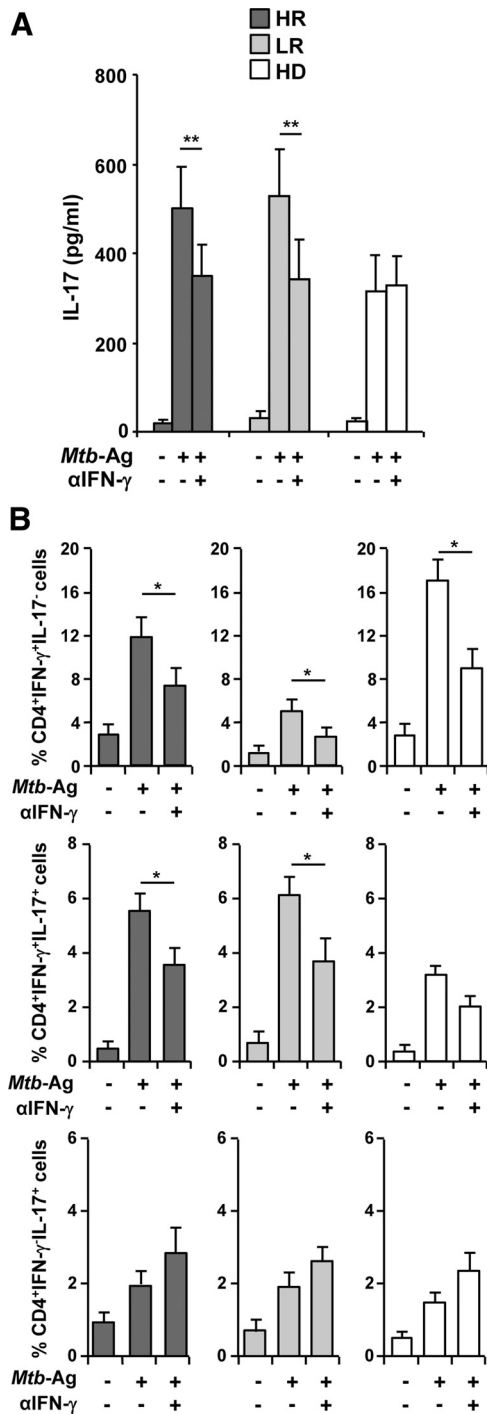


Figure 3. The expansion of Th1/Th17 lymphocytes in response to *Mtb-Ag* was regulated by IFN- γ . PBMCs from HR and LR TB patients and HD were stimulated with *Mtb-Ag* for 5 days in the presence or absence of α IFN- γ mAb. The percentages of CD4⁺IFN- γ ⁺IL-17⁻, CD4⁺IFN- γ ⁺IL-17⁺, and CD4⁺IFN- γ ⁻IL-17⁺ cells and the production of IL-17 were then analyzed. (A) Each bar represents the mean \pm SEM of IL-17 secretion for each group. (B) IL-17 and IFN- γ coexpression was determined by flow cytometry, first, gating on lymphocytes by light scatter and then, gating on CD4⁺ T cells. The bars represent the mean \pm SEM of the percentages of CD4⁺IFN- γ ⁺IL-17⁻, CD4⁺IFN- γ ⁺IL-17⁺, and CD4⁺IFN- γ ⁻IL-17⁺ cells for each condition. Statistical differences were calculated using the nonparametric Wilcoxon rank sum test for paired samples. ** $P < 0.01$; * $P < 0.05$.

CD4⁺IFN- γ ⁻IL-17⁺ T cells (Fig. 3B). However, IFN- γ blockage markedly diminished the percentage of CD4⁺IFN- γ ⁺IL-17⁺ (Fig. 3B). These data demonstrate that not only Th1 but also Th1/Th17 lymphocytes are positively regulated by IFN- γ during active disease.

***M. tuberculosis*-expanded Th1/Th17 lymphocytes were regulated by costimulatory molecules**

Several signaling proteins are known to modulate the level and pattern of cytokines produced by T cells upon antigen stimulation [20, 21]. In fact, as we reported previously, SLAM activation increased Th1 lymphocytes against *Mtb-Ag* [13], whereas PD-1 inhibited this population [22]. Although nearly 70% of CD4⁺IFN- γ ⁺IL-17⁺ cells expressed PD-1 or SLAM in TB patients, only very low levels of SLAM or PD-1 were detected on IFN- γ ⁻IL-17⁺ lymphocytes (Fig. 4A). The PD-1 blockage or signaling through SLAM in antigen-stimulated cells from TB patients significantly increased not only CD4⁺IFN- γ ⁺IL-17⁻ but also CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes (Fig. 4C). The number of CD4⁺IFN- γ ⁻IL-17⁺ cells was unaffected by PD-1 blockage or by SLAM activation. However, both treatments significantly increased IL-17 production against *Mtb-Ag* in TB patients and HD (Fig. 4B). The present findings suggest that signaling through PD-1 inhibited the expansion of CD4⁺IFN- γ ⁺IL-17⁺ cells, whereas activation of SLAM increased Th1/Th17 lymphocytes against the pathogen.

***M. tuberculosis* expanded Th1/Th17 cells at the site of infection**

We next investigated the induction of Th1, Th1/Th17, and Th17 lymphocytes in TB pleural effusions. As shown in Fig. 5, *M. tuberculosis* strikingly enhanced the production of IL-17 and IFN- γ by PFMCs of HR and LR TB patients (Fig. 5A). In contrast to the findings in peripheral blood, PFMCs from LR individuals secreted significantly higher levels of IL-17 compared with HR patients (Fig. 5A). In addition, PFMCs from LR patients produced markedly lower amounts of antigen-specific IFN- γ (Fig. 5A), as we described previously [22], and the majority IL-17-producing cells was IFN- γ ⁺ in both groups of patients. IFN- γ blockage significantly reduced the percentages of CD4⁺IFN- γ ⁺IL-17⁻ and CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes (Fig. 5B) and the production of IL-17 (Fig. 5C). Besides, PD-1 blockage or SLAM activation significantly increased the secretion of IL-17 by PFMCs (Fig. 5D). It is plausible that at the site of infection, *M. tuberculosis* expanded and regulated CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes in the same way as in peripheral blood.

The proportion of IL-17-secreting lymphocytes induced by *Mtb-Ag* was inversely correlated with IFN- γ production against the pathogen

The subtle balance between Th1 and Th17 responses seems to be critical in defining the outcome of *M. tuberculosis* infection [9]. We next evaluated the proportion of Th1, Th17, and Th1/Th17 lymphocytes expanded by *Mtb-Ag*. As shown in Fig. 6, striking differences in the proportions of CD4⁺Th subsets were detected among the three groups of individuals. Indeed, whereas HD displayed the highest ratios of CD4⁺IFN-

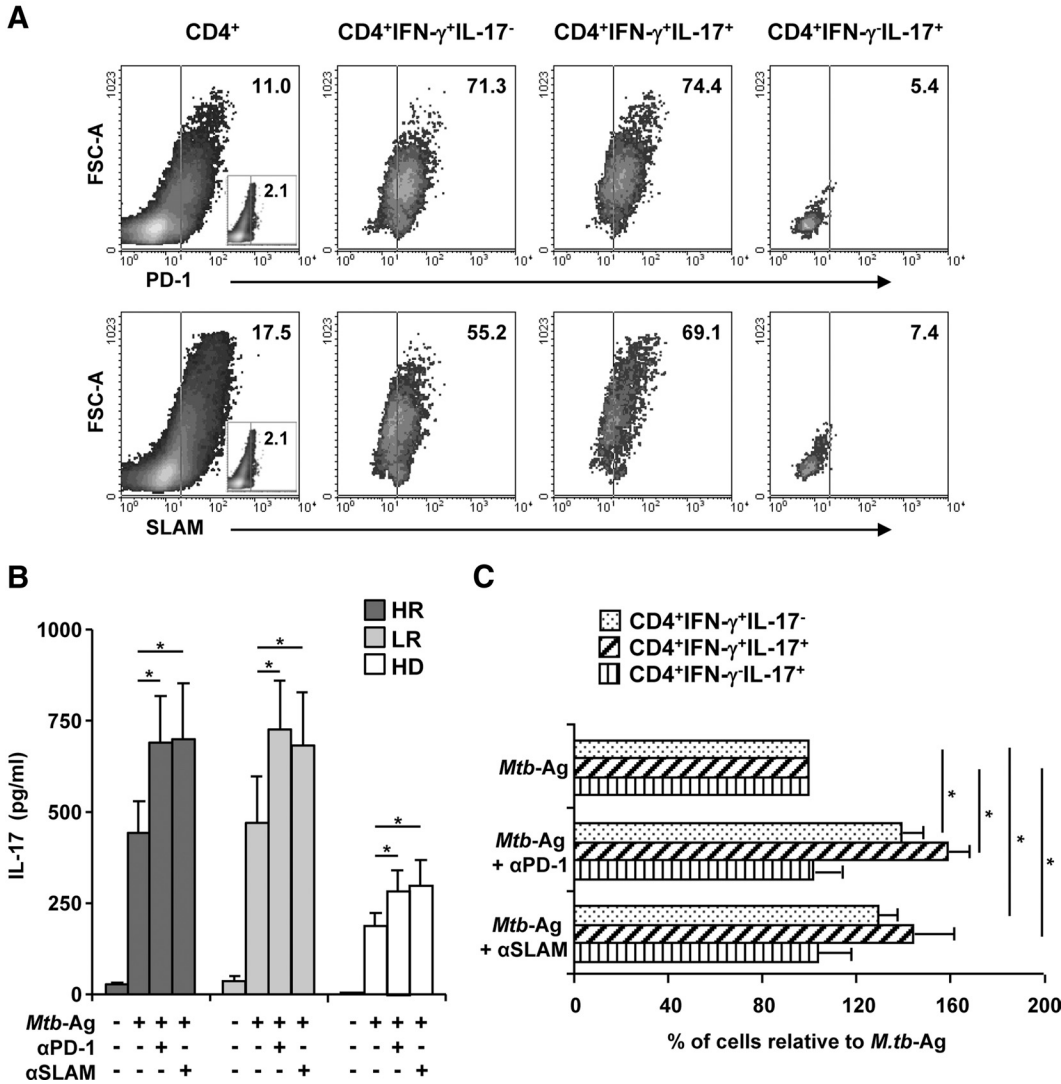


Figure 4. Expression and function of costimulatory molecules on CD4⁺IFN- γ ⁺IL-17⁻, CD4⁺IFN- γ ⁻IL-17⁺, and CD4⁺IFN- γ ⁺IL-17⁺ T cells from TB patients. (A) PBMCs from HR TB patients were stimulated with *Mtb*-Ag, and after 5 days, IL-17 and IFN- γ coexpression in PD-1⁺ (upper) or SLAM⁺ (lower) cells was determined by flow cytometry, first, gating on lymphocytes by light scatter; then, gating on CD4⁺ T cells; and finally, gating on CD4⁺IFN- γ ⁺IL-17⁻, CD4⁺IFN- γ ⁻IL-17⁺, or CD4⁺IFN- γ ⁺IL-17⁺ cells. A representative density plot for HR patients analyzed is shown. FSC-A, Forward-scatter-area. (B and C) PBMCs from HR patients were stimulated with *Mtb*-Ag in the presence or absence of blocking α PD-1 mAb or agonistic α SLAM mAb. After 5 days of culture, IL-17 production was determined by ELISA (B), and intracellular IL-17 and IFN- γ coexpression was determined by flow cytometry (C), first, gating on lymphocytes by light scatter and then, gating on CD4⁺ T cells. (C) Each bar represents the mean \pm SEM of the percentage of cells and was calculated as: (% CD4⁺IL-17⁻IFN- γ ⁺, CD4⁺IL-17⁺IFN- γ ⁺, or CD4⁺IL-17⁺IFN- γ ⁻ *Mtb* + α PD-1 or *Mtb* + α SLAM-stimulated cells) / (% CD4⁺IL-17⁻IFN- γ ⁺, CD4⁺IL-17⁺IFN- γ ⁺,

or CD4⁺IL-17⁺IFN- γ ⁻ *Mtb*-stimulated cells) \times 100 ($n=8$ for all groups). Statistical differences were calculated using the nonparametric Wilcoxon rank sum test for paired samples. * $P < 0.05$.

γ IL-17⁻ cells, HR presented an intermediate proportion, and LR individuals showed the lowest ratios of CD4⁺IFN- γ ⁺IL-17⁻ lymphocytes (Fig. 6A). On the other hand, LR patients exhibited the highest proportion of CD4⁺IFN- γ ⁻IL-17⁺ cells compared with the other groups. Furthermore, the highest ratio of CD4⁺IFN- γ ⁺IL-17⁺ cells was found in LR patients, intermediate proportions were detected in HR patients, and the lowest ratio of those lymphocytes was measured in HD (Fig. 6A). Consequent to PBMCs, PFMCs from LR patients bared a superior proportion of CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes expanded against *Mtb*-Ag as compared with HR individuals (Fig. 6B).

As the levels of *Mtb*-induced IFN- γ represented a criterion for patient classification [13], the probable correlation between the amount of IFN- γ secreted and the proportions of each CD4⁺Th subset expanded was investigated. As expected, a positive correlation between IFN- γ levels and the ratio of CD4⁺IFN- γ ⁺IL-17⁻ lymphocytes expanded by *Mtb*-Ag (Fig. 6C) was found. In contrast, a striking negative association was iden-

tified regarding the amount of IFN- γ secreted by each group of individuals and the proportion of IL-17-secreting lymphocytes (CD4⁺IFN- γ ⁺IL-17⁺ or CD4⁺IFN- γ ⁻IL-17⁺; Fig. 6C). Hence, the induction of IFN- γ responses is critically required in immunity against mycobacteria [1], whereas the expansion of IL-17-secreting lymphocytes might be associated with an impaired immunity.

Clinical parameters measured in TB patients were associated with the proportion of *Mtb*-Ag-expanded CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes

At diagnosis, TB patients display elevated absolute neutrophil and monocyte counts and low lymphocyte subset counts [23]. Accordingly, we demonstrated previously that immunological features paralleled common clinical parameters analyzed in patients with TB in Argentina: HR patients had significant higher percentages of total lymphocytes compared with LR patients; HR patients exhibited higher purified protein deriva-

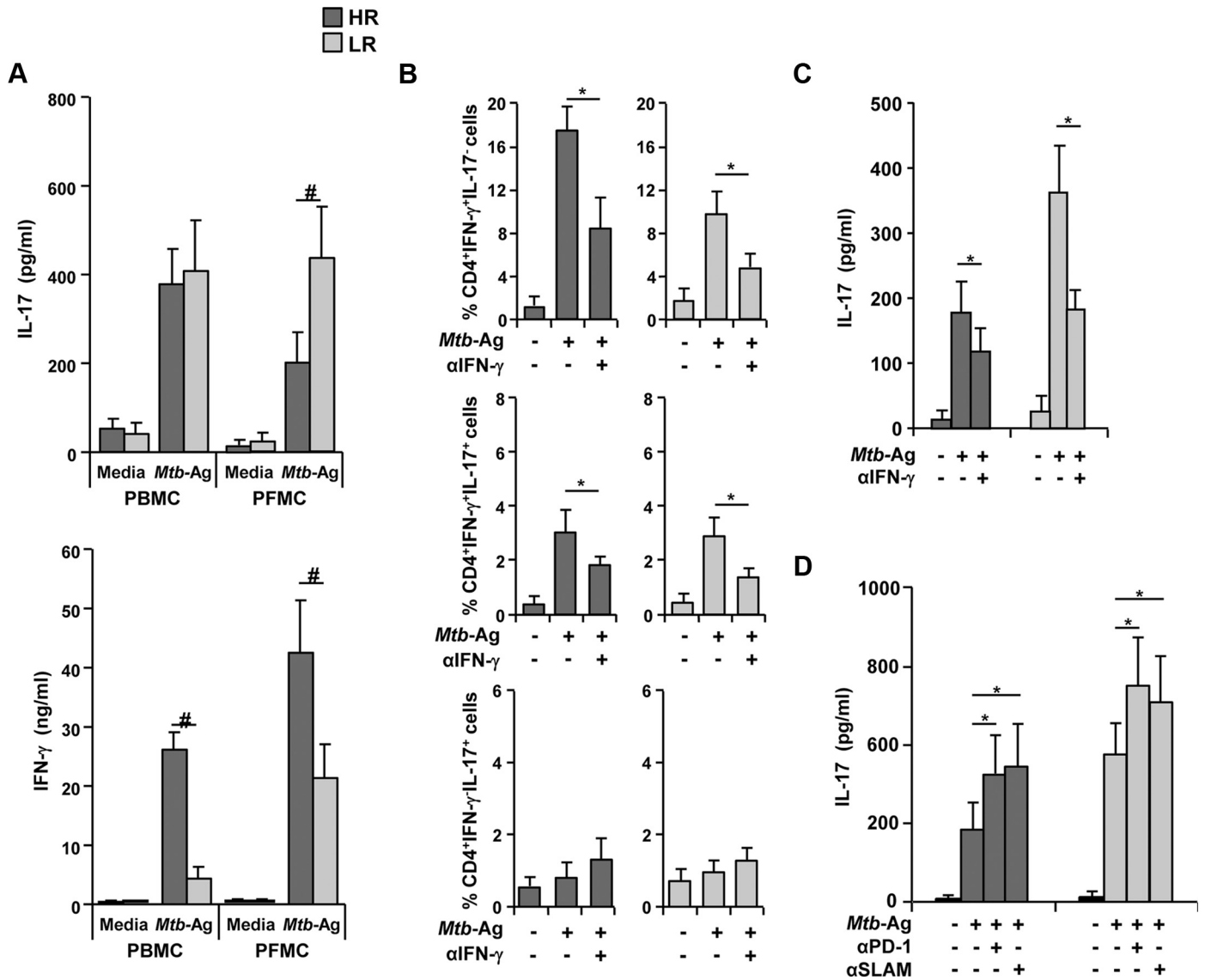


Figure 5. *M. tuberculosis* induced IL-17 production at the site of infection. (A) PFMCs and PBMCs from HR and LR TB patients were stimulated with *Mtb*-Ag for 5 days. Afterwards, IL-17 and IFN- γ production was determined by ELISA. Each bar represents the mean \pm SEM of IL-17 or IFN- γ secretion for each group (HR, $n=11$; LR, $n=8$). (B) PFMCs from HR and LR TB patients were stimulated with *Mtb*-Ag for 5 days in the presence or absence of α IFN- γ mAb. Then, IL-17 and IFN- γ coexpression was determined by flow cytometry, first, gating on lymphocytes by light scatter and then, gating on CD4⁺ T cells. Bars represent the mean \pm SEM of the percentages of CD4⁺IFN- γ ⁺IL-17⁻, CD4⁺IFN- γ ⁺IL-17⁺, and CD4⁺IFN- γ ⁺IL-17⁺ cells. (C and D) PFMCs from HR and LR were stimulated with *Mtb*-Ag for 5 days in the presence or absence of blocking α IFN- γ mAb (C) and blocking α PD-1 mAb or agonistic α SLAM mAb (D), and IL-17 production was determined by ELISA. Each bar represents the mean \pm SEM of IL-17 production for each group ($n=12$ for all groups). Statistical differences were calculated using the nonparametric Mann-Whitney test for unpaired samples ($\#P<0.05$) and the Wilcoxon rank sum test for paired samples ($*P<0.05$).

tive diameters than LR patients; and LR individuals had severe pulmonary lesions, a striking loss of weight, and had been ill longer than HR individuals [13]. We further expand our previous studies by finding that LR patients exhibited significantly lower numbers of leukocytes as compared with HR individuals (Table 1). The leukocyte count was correlated directly with the proportion of CD4⁺IFN- γ ⁺IL-17⁻ lymphocytes and inversely associated with the ratio of CD4⁺IFN- γ ⁺IL-17⁺ cells (Fig. 7A). Furthermore, higher proportions of CD4⁺IFN- γ ⁺IL-17⁺ cells were directly associated with longer times of disease

evolution, whereas elevated ratios of CD4⁺IFN- γ ⁺IL-17⁻ were correlated with shorter length of disease progress (Fig. 7B). In fact, the presence of higher proportions of CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes was correlated directly with more extensive lung affection and a higher number of pulmonary lesions (Fig. 7C), but no association between the ratio of CD4⁺IFN- γ ⁺IL-17⁺ cells and the clinical parameters investigated was found (Fig. 7). These findings demonstrate that the proportion of CD4⁺IFN- γ ⁺IL-17⁺ cells was elevated in patients who exhibit weak cell-mediated immunity against *M. tuberculosis*. Further-

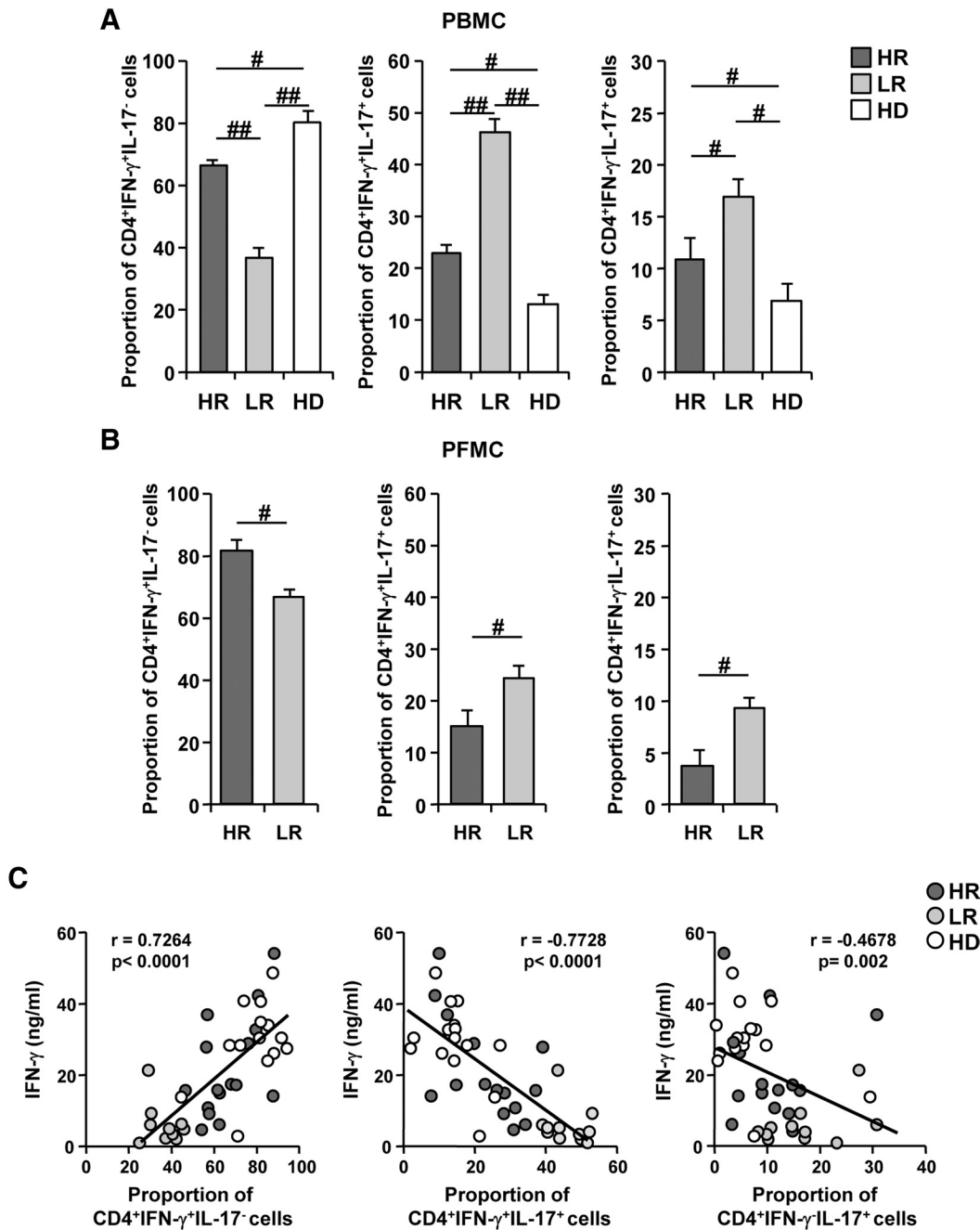


Figure 6. *Mtb*-Ag differentially expanded CD4⁺IFN- γ ⁺IL-17⁻, CD4⁺IFN- γ ⁻IL-17⁺, and CD4⁺IFN- γ ⁻IL-17⁺ lymphocytes. PBMCs (A) and PFMCS (B) from HR and LR TB patients and PBMCs (A) from HD were stimulated with *Mtb*-Ag for 5 days. Afterwards, IL-17 and IFN- γ coexpression was determined by flow cytometry, first, gating on lymphocytes by light scatter and then, gating on CD4⁺ T cells. The proportions of Th1, Th1/Th17, and Th17 cells induced by *Mtb*-Ag were calculated as the ratio of IFN- γ ⁺IL-17⁻ (Th1), IFN- γ ⁺IL-17⁺ (Th1/Th17), or IFN- γ ⁻IL-17⁺ (Th17) cells relative to the total number of cytokine-producing cells (100% of cells = IFN- γ ⁺IL-17⁻ + IFN- γ ⁺IL-17⁺ + IFN- γ ⁻IL-17⁺). Statistical differences were calculated using the nonparametric Mann-Whitney test for unpaired samples. #*P* < 0.05; ##*P* < 0.01. (C) Correlation between IFN- γ production and the proportion of Th1, Th1/Th17, or Th17 lymphocytes. Values are expressed as IFN- γ production (IFN- γ produced by *Mtb*-Ag-stimulated cells - IFN- γ produced by cells cultured with media) versus the proportion of Th1, Th1/Th17, or Th17 cells for each group of individuals. Correlation factor (*r*) and *P* values were calculated using the nonparametric Spearman correlation test. Proportions were calculated as described in A. HR, *n* = 15; LR, *n* = 11.

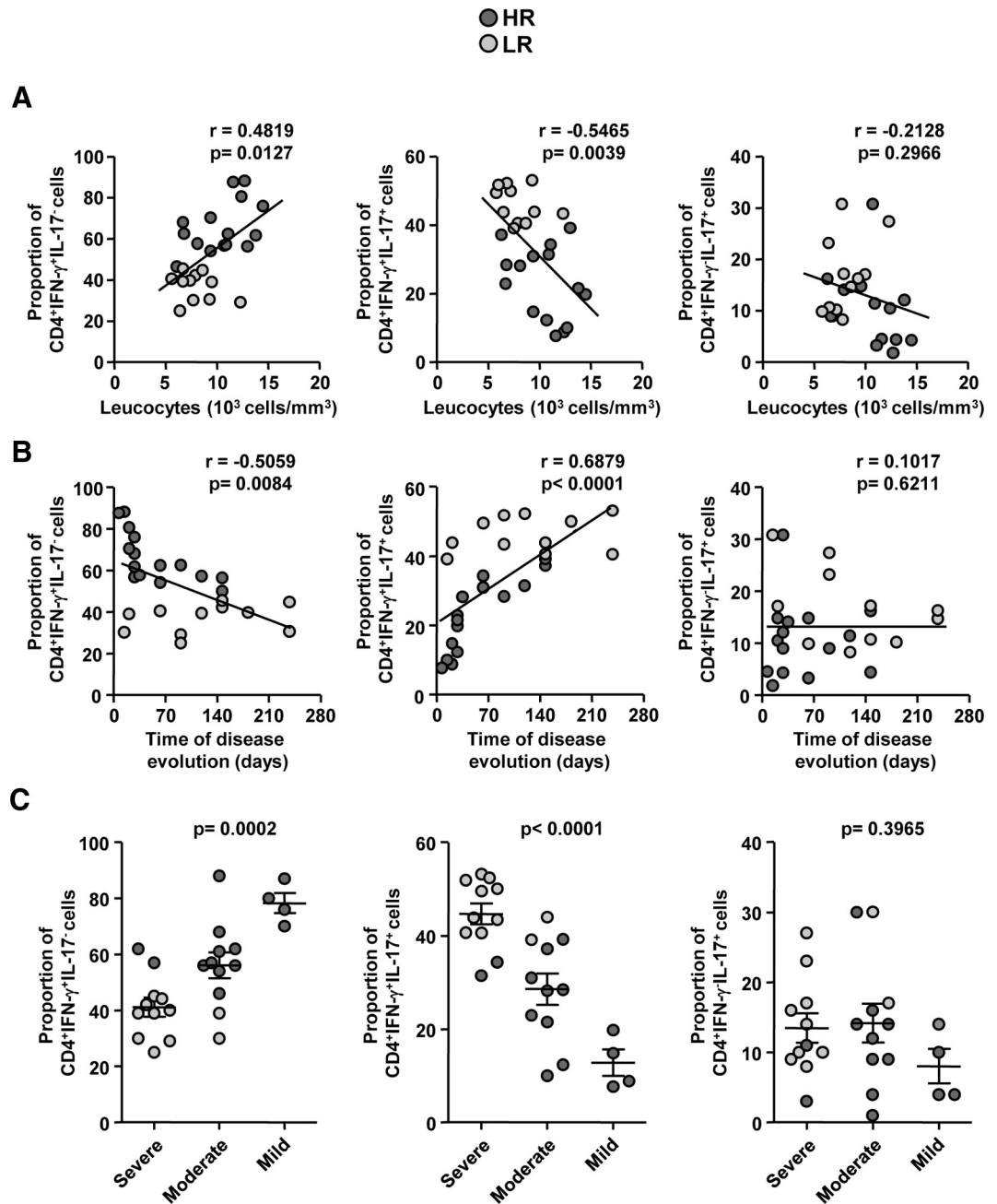
more, CD4⁺IFN- γ ⁺IL-17⁺ lymphocyte ratio was directly related to clinical parameters, indicating disease severity.

DISCUSSION

IFN- γ -producing Th1 cells are essential to control mycobacterial replication [24–26]. Indeed, reduced IFN- γ production is a marker of severe disease [27]. Hence, we previously identified HR and LR TB patients based on their T cell responses to *M. tuberculosis* [13]. In spite of that, Th1 cells alone do not explain the resistance/susceptibility to infection and disease [28], suggesting that other cytokines might be required during

the immune regulation of TB [6, 29]. Besides Th1 lymphocytes, Th17 cells have also been associated with *M. tuberculosis* infection [6, 10]. IL-17-producing lymphocytes constitute a significant proportion of *M. tuberculosis*-activated cells in PBMCs of healthy tuberculin reactors [6]. We have demonstrated that the number of Th17 cells was increased significantly in TB patients compared with BCG-vaccinated controls [19]. Furthermore, we have shown previously that IL-17 inhibited IFN- γ production during human TB by decreasing the expression and function of stimulatory signaling proteins [19]. As a result of the fact that IFN- γ and Th17 cells possess antagonistic effects [11, 12, 19], we speculate that the increase in

Figure 7. The ratio of Th1-Th1/Th17 lymphocytes correlated with clinical parameters in TB patients. PBMCs from HR and LR TB patients were stimulated with *Mib*-Ag for 5 days. Afterwards, IL-17 and IFN- γ coexpression was determined by flow cytometry, first, gating on lymphocytes by light scatter and then, gating on CD4⁺ T cells. (A) Each dot represents the proportion of CD4⁺IFN- γ ⁺IL-17⁻ cells versus the number of leukocytes (10^3 cells/mm³) for each patient analyzed. (B) Each dot represents the proportion of CD4⁺IFN- γ ⁺IL-17⁻, CD4⁺IFN- γ ⁻IL-17⁺, and CD4⁺IFN- γ ⁺IL-17⁺ cells versus the time of disease evolution (days) for each patient analyzed. (A and B) Correlation factor (r) and P were calculated by the nonparametric Spearman correlation test. (C) Each dot represents the proportion of Th1, Th1/Th17, or Th17 cells versus the severity of the radiological lesions (severe, moderate, and mild; see Table 1) for each patient analyzed. Statistical differences were calculated using the ANOVA test. (A–C) Proportions were calculated as described in Fig. 6. HR, $n = 15$; LR, $n = 11$.



IL-17 production might regulate the capacity of T cells from patients with TB to produce IFN- γ in response to mycobacteria. Therefore, we aimed to elucidate the role of IL-17-secreting cells during human active disease.

Several studies have recently profiled the phenotype and plasticity of human Th17 cells, providing novel insights into the relationship and functionality of the Th17 and Th1 subpopulations [16, 30, 31]. Accordingly, IFN- γ ⁺IL-17⁺ lymphocytes were shown to maintain their propensity to produce IL-17 and IFN- γ or become IL-17 or IFN- γ single-producing cells, suggesting a potential pathogenic role for this subset [16, 30, 31]. Moreover, individual Th1/Th17 cells, stably coexpressing T-bet and ROR γ T, define a functionally distinct popula-

tion of lymphocytes that combines the proinflammatory potential of Th1 and Th17 cells [31–33]. Herein and to our knowledge, we demonstrated for the first time, that CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes were expanded during human active TB in correlation with the disease severity.

As mentioned already in Results, IFN- γ blockage markedly diminished the IL-17 secretion induced upon antigen stimulation, a decrease related to a significant down-regulation of IFN- γ ⁺IL-17⁺ lymphocytes. In fact, anti-IFN- γ treatment only slightly augmented IFN- γ ⁻IL-17⁺ T cells, without modifying the resulting IL-17 reduction in the cellular milieu. In contrast, the overall effect of the blockage of IFN- γ on the production of IL-17 in control individuals was not significant. The

regulation of IFN- γ on IL-17 secretion by TB patients was detected after stimulation with the specific antigen but not with a polyclonal stimulus, indicating that *M. tuberculosis*-induced IFN- γ participated in the induction of Th1/Th17 cells during active disease. In fact, costimulation through molecules that modulate IFN- γ responses in TB [13, 22] also regulated IL-17 production during active disease.

As we reported previously, common clinical parameters analyzed in patients with active TB in Argentina paralleled immunological parameters studied in those individuals, allowing the classification of patients in HR and LR individuals [13]. Our present results extend those findings, demonstrating a positive association between two clinical parameters (time of disease evolution and pulmonary lesions) and the proportion of CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes expanded in response to *Mtb*-Ag. In fact, LR TB patients, whose severity parameters were described above, showed higher ratios of Th1/Th17 cells in pleural fluids and peripheral blood compared with HR patients. Therefore, our findings provide evidence that the proportion of CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes in patients with active TB might be associated with the severity of the disease.

An important aspect of this report was to evaluate the induction of Th1/Th17 lymphocytes in pleural fluids from TB patients. Our results in PFCMs confirmed that the majority of IL-17-producing lymphocytes against *Mtb*-Ag also coproduced IFN- γ and displayed elevated levels of SLAM and PD-1. Furthermore, patients with weak immunity to *M. tuberculosis* presented the highest proportions of IFN- γ ⁺IL-17⁺ T lymphocytes at the site of infection, strongly supporting the fact that this subset of CD4⁺ T cells might play a potential, detrimental role during *M. tuberculosis* infection. In contrast to us, Chen et al. [34] did not detect IFN- γ ⁺IL-17⁺ T cells in patients with active TB and reported that the magnitude of the Th17 response was associated with the clinical outcome of *M. tuberculosis* infection. Nevertheless, the discrepancies may be attributed to differences in the experimental design and/or stimulation protocols used. Basically, Chen et al. [34] stimulated fresh blood and PBMCs/PFCMs with PMA/ionomycin for 6 h or PBMCs/PFCMs with H37Rv lysate during 3 days. By performing those protocols, the authors reported significantly lower frequency of nonspecific Th17 responses in TB patients as compared with HD and suggested that those nonspecific responses might involve Th17 cells derived from naïve CD4⁺ T cells [34]. Regarding the stimulation with the specific antigen, the authors obtained undetectable Th17 cell responses [34]. In the present work, we investigated the frequency of antigen-specific, IL-17-secreting lymphocytes in PBMCs and PFCMs from TB patients. As demonstrated in Fig. 1, significant levels of IL-17 were detected at 5 days of stimulation with sonicated H37Rv but not earlier. Furthermore, we showed that *M. tuberculosis* induced significant secretion of IL-17 from CD4⁺IFN- γ ⁺IL-17⁺ T cells and that these lymphocytes displayed a memory-like phenotype.

It is worth noticing that the majority of our results obtained in peripheral blood of TB patients was replicated in PFCMs of the same individuals, which strongly suggests that the determinations in PBMCs reflect what is occurring at the site of infection. Taken together, the analysis of Th1-Th1/Th17-Th17 lymphocyte ratios in peripheral blood might be used as an indicator of the clinical manifestations of the disease.

phocyte ratios in peripheral blood might be used as an indicator of the clinical manifestations of the disease.

In conclusion, we demonstrated that during human TB, IL-17 was secreted mainly by Th1/Th17 cells, a population of CD4⁺IFN- γ ⁺IL-17⁺ lymphocytes regulated by IFN- γ and present in proportions directly associated with the time of disease evolution and pulmonary lesions. The association of the ratio of *Mtb*-expanded Th1/Th17 cells with disease severity suggests that these lymphocytes might comprise a potentially pathogenic population during active TB. In this line, although blocking and agonistic mAb have shown promise for their use in therapies for chronic viral diseases and malignancies [35], caution should be taken in designing ways to promote Th1 responses during chronic *M. tuberculosis* infection, as the production of IFN- γ could also result in an inappropriate expansion of Th1/Th17 lymphocytes and pathology. According to our present findings, although IFN- γ is required to achieve a protective response to *M. tuberculosis*, therapeutically, addition of exogenous IFN- γ would not be a solution to achieve a strong cell-mediated immunity response to *M. tuberculosis*. It is plausible that the balance between Th1 and Th1/Th17 cells, with higher numbers of Th1 over Th1/Th17 lymphocytes, would contribute to a protective response against *M. tuberculosis*. Further studies are required to elucidate the pathogenicity of Th1/Th17 lymphocytes during human active TB.

AUTHORSHIP

J.O.J., V.P., and V.E.G. conceived of and designed the experiments. J.O.J., V.P., I.B.A., and A.I.R. performed the experiments. J.O.J. and V.E.G. analyzed the data. J.O.J., A.I.R., N.L.T., R.M.M., D. Palmero, and H.E.C. contributed reagents/materials/analysis tools. J.O.J., D. Peña, H.E.R., and V.E.G. wrote the paper.

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KEY WORDS:

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