

Programmed Death (PD)-1:PD-Ligand 1/PD-Ligand 2 Pathway Inhibits T Cell Effector Functions during Human Tuberculosis¹

Javier O. Jurado,^{*†} Ivana B. Alvarez,^{*†} Virginia Pasquinelli,^{*†} Gustavo J. Martínez,[†] María F. Quiroga,^{*†} Eduardo Abbate,[‡] Rosa M. Musella,[‡] H. Eduardo Chuluyan,[†] and Verónica E. García^{2*†}

Protective immunity against *Mycobacterium tuberculosis* requires the generation of cell-mediated immunity. We investigated the expression and role of programmed death 1 (PD-1) and its ligands, molecules known to modulate T cell activation, in the regulation of IFN- γ production and lytic degranulation during human tuberculosis. We demonstrated that specific Ag-stimulation increased CD3⁺PD-1⁺ lymphocytes in peripheral blood and pleural fluid from tuberculosis patients in direct correlation with IFN- γ production from these individuals. Moreover, *M. tuberculosis*-induced IFN- γ participated in the up-regulation of PD-1 expression. Blockage of PD-1 or PD-1 and its ligands (PD-Ls: PD-L1, PD-L2) enhanced the specific degranulation of CD8⁺ T cells and the percentage of specific IFN- γ -producing lymphocytes against the pathogen, demonstrating that the PD-1:PD-Ls pathway inhibits T cell effector functions during active *M. tuberculosis* infection. Furthermore, the simultaneous blockage of the inhibitory receptor PD-1 together with the activation of the costimulatory protein signaling lymphocytic activation molecule led to the promotion of protective IFN- γ responses to *M. tuberculosis*, even in patients with weak cell-mediated immunity against the bacteria. Together, we demonstrated that PD-1 interferes with T cell effector functions against *M. tuberculosis*, suggesting that PD-1 has a key regulatory role during the immune response of the host to the pathogen. *The Journal of Immunology*, 2008, 181: 116–125.

Protective immunity against *Mycobacterium tuberculosis* requires the production of IFN- γ by CD4⁺ and CD8⁺ T cells (1). In addition, CD8⁺ T lymphocytes also contribute to protection by lysing infected cells (2). Thus, impaired immunity in tuberculosis infection is associated with impaired T cell activation and reduced production of IFN- γ (3, 4).

The strength of the signal transduced by the TCR is influenced by the cellular context of Ag recognition, and one variable that provides this context is the expression of activating and inhibitory receptors on T cells (5). A balance between activating and inhibitory signals ensures the development of an effective immune response. In fact, several signaling proteins contribute to the active up- or down-regulation during the priming of a T cell (6, 7), modulating the level and pattern of cytokines produced by T cells upon Ag stimulation. For example, during the immune response of the host against *M. tuberculosis*, we demonstrated that signaling lym-

phocytic activation molecule (SLAM)³ and ICOS enhanced IFN- γ secretion (8, 9), whereas the SLAM-associated protein and CD31 interfered with Th1 responses in tuberculosis (8, 10). Another signaling molecule that participates in the regulation of T cell effector functions is programmed death 1 (PD-1), an activation-induced inhibitory receptor expressed on lymphocytes and monocytes (11, 12). In vitro studies have shown that engagement of PD-1 by its ligands, PD-L1 and PD-L2, inhibited TCR-mediated T cell proliferation and cytokine production, indicating that the cross-linking of PD-1 by its PD-Ls leads to down-regulation of T cell responses (13, 14), although not all the studies support the inhibitory role for the PD-1:PD-Ls pathway (15). Given that some microorganisms that cause chronic infection exploit the PD-1:PD-Ls pathway to evade host immune effector mechanisms (16), we investigated the role of these signaling molecules during the immune response to persisting *M. tuberculosis* infection. Our specific aim was to elucidate whether PD-1 and its ligands participated in the regulation of cell-mediated immunity (CMI) responses to *M. tuberculosis*, which is critical for host defense against this pathogen.

Materials and Methods

Patients

Patients with active pulmonary tuberculosis were evaluated at the Hospital Muñiz. The diagnosis of tuberculosis was established based on clinical and radiological data together with the identification of acid-fast bacilli in

*Department of Biological Chemistry, School of Sciences, University of Buenos Aires, Buenos Aires, Argentina; [†]Laboratorio de Inmunogenética, Hospital de Clínicas José de San Martín, University of Buenos Aires, Buenos Aires, Argentina; and [‡]División de Tisiopneumología, Hospital F. J. Muñiz, Buenos Aires, Argentina

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² Address correspondence and reprint requests to Dr. Verónica E. García, Department of Biological Chemistry, School of Sciences, University of Buenos Aires, Intendente Guiraldes 2160, Pabellón II, 4th floor, Capital Federal, 1428, Buenos Aires, Argentina. E-mail address: vgarcia@qb.fcen.uba.ar

³ Abbreviations used in this paper: SLAM, signaling lymphocytic activation molecule; PD-1, programmed death-1; PD-L, PD-1 ligand; CMI, cell-mediated immunity; HD, healthy donor; PB, peripheral blood; HR, high responder; LR, low responder; PF, pleural fluid; PFM, PF mononuclear cell.

sputum. All participating patients had received <1 wk of antituberculosis therapy. Bacillus Calmette-Guérin-vaccinated healthy control donors (HD) from the community participated in this study. Peripheral blood (PB) samples were collected in heparinized tubes from all individuals after receiving informed consent. In some cases, pleural effusions were obtained by thoracentesis. The local ethical committee approved all the studies performed.

All tuberculosis patients (with or without tuberculous pleuritis) were classified according to our previously published criteria (8, 9). Briefly, two groups were identified, based on *in vitro* T cell responses to an extract of virulent *M. tuberculosis* Ag: high responder (HR) patients are individuals displaying significant proliferative responses (proliferation index ≥ 4), IFN- γ production (fold stimulation ≥ 34), and an increase ≥ 8 in the percentage of SLAM⁺ cells in response to the Ag; low responder (LR) patients are individuals exhibiting low proliferative responses (proliferation index <4), IFN- γ secretion (fold stimulation <34), and an increase <8 in the percentage of SLAM⁺ cells. If a patient fulfilled two of three of these criteria, the patient was assigned to that group. Interestingly, and as we previously reported (8), proliferation and IFN- γ production paralleled common clinical parameters analyzed in patients with active tuberculosis in Argentina: HR patients displayed markedly higher levels of the percentage of total lymphocytes compared with LR patients and showed higher purified protein derivative diameters than LR patients; LR individuals had severe pulmonary lesions, a striking loss of weight, and had been ill longer (days since symptoms begin until patient hospital admission) than HR individuals.

Antigen

In vitro stimulation of cells throughout the present study was performed with an extract from the virulent *M. tuberculosis* strain H37Rv provided by Mycobacteria Research Laboratories (Colorado State University, Fort Collins, CO) prepared by probe sonication.

Cell preparation and culture conditions

PBMC and pleural fluid (PF) mononuclear cells (PFMC) were isolated by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences) and cultured (1×10^6 cells/ml) with or without *M. tuberculosis* Ag (10 μ g/ml) in flat-bottom 24-well (TPP Renner) or 96-well (Cellstar; Greiner Bio-One) plates with RPMI 1640 (Invitrogen Life Technologies) supplemented with L-glutamine (2 mM; Sigma-Aldrich), gentamicin, and 10% human serum. After 5 days, cells were pulsed with [³H]TdR (1 μ Ci/well), harvested 16 h later, and [³H]TdR incorporation was measured in a liquid scintillation counter. IFN- γ production was evaluated in all the patients studied by ELISA (eBioscience) after 48 h of stimulation with *M. tuberculosis* Ag. In different experiments, cells were incubated with rIFN- γ (7.5 ng/ml; eBioscience), anti-IFN- γ (15 μ g/ml, MD-1; eBioscience) or isotype control, in the presence or absence of *M. tuberculosis* Ag. After 5 days PD-1, PD-L1, or PD-L2 expression was determined by flow cytometry (see below). In other experiments, cells were incubated with or without blocking Abs against PD-1 (5 μ g/ml, J116; eBioscience), and/or PD-L1 (2 μ g/ml, MIH1; eBioscience) and PD-L2 (2 μ g/ml, MIH18; eBioscience), or isotype control, in the presence or absence of *M. tuberculosis* Ag. After 24 h or 4 days, cytotoxicity and the percentage of IFN- γ -secreting cells were determined by flow cytometry, respectively (see below). In separated experiments, mAbs anti-SLAM (10 μ g/ml, A12), anti-ICOS (12.5 μ g/ml, ISA-3), anti-PD-1, or isotype control were added alone or together to cells previously stimulated for 5 days with the specific Ag. After 48 h, IFN- γ production was measured by ELISA (eBioscience).

Flow cytometry

PBMC were cultured with *M. tuberculosis* and stained for CD3, CD4, CD8, SLAM, PD-1, PD-L1, and PD-L2 expression using specific mAbs (eBioscience), before and after culture with the Ag. Intracellular cytokine staining was performed to determine IFN- γ production at the single-cell level as previously described (9, 17). Negative control samples were incubated with irrelevant, isotype-matched mAbs in parallel with experimental samples. All the analyses were performed excluding dead cells using propidium iodide following the manufacturer's instructions (Caltag Laboratories). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Lytic degranulation

CD107a/b lysosome-associated membrane protein-1/2 expression was used to measure CD8⁺ T lymphocyte degranulation, as previously described (18). Briefly, PBMC and PFMC were incubated with or without *M. tuberculosis* Ag \pm PD-1/PD-Ls blocking mAbs as described in the

"Cell preparation and culture conditions" section. Then, CD107a/b-FITC Abs (H4A3/H4B4, 20 μ l/ml; BD Biosciences) were added directly to the cells in culture (for 6 h) together with GolgiStop reagent (BD Biosciences) (during the last 5 h), according to the manufacturer's instructions. Finally, cells were stained with anti-CD8 and anti-CD3, and FACS analysis was performed. Negative control samples were incubated with irrelevant, isotype-matched Abs in parallel with all experimental samples.

Statistical analysis

Statistical analysis was performed using the nonparametric Wilcoxon rank sum test for paired samples and the Mann-Whitney *U* test for nonpaired samples. Values of $p < 0.05$ were considered significant.

Results

PD-1 expression is associated with *M. tuberculosis*-induced IFN- γ in patients with active disease

PD-1 is tightly regulated and induced upon TCR signals (12, 19). Moreover, the amount of PD-1 expression and the extent of engagement of PD-1 by its ligands regulate the threshold for T cell activation and quantities of cytokine production (16). Because protection against *M. tuberculosis* requires T cell activation and IFN- γ production (20–22), we investigated Ag-induced PD-1 expression in T cells from patients with active tuberculosis. Minimal levels of PD-1 were detected on resting T cells from PBMC (data not shown), as previously reported in human T cells (23). However, *M. tuberculosis* Ag stimulation induced a significant increase in PD-1 expression on the surface of T cells from HR patients and HD (Fig. 1A, $p < 0.0001$, Wilcoxon rank sum test). Moreover, PD-1 expression was up-regulated both on CD4⁺ and CD8⁺ T lymphocytes from responder patients (Fig. 1B, left panel). In contrast, no significant differences in T cell expression of PD-1 were detected after Ag stimulation in LR patients (Fig. 1, A and B, right panel). Given that HR individuals secreted IL-10 but produced high levels of IFN- γ against *M. tuberculosis* (9), whereas in LR patients, *M. tuberculosis* induced IL-10 production but very low levels of IFN- γ , the differential regulation of PD-1 expression in PBMC from tuberculosis patients suggested to us that PD-1 expression might be associated with the cytokine microenvironment produced by the differential number of specific T cells induced by the Ag in each group. Then, we investigated the association between PD-1 expression and IFN- γ production in tuberculosis patients by performing statistical analysis, which indicated a significant correlation between both parameters (Spearman coefficient, $r = 0.5807$, $p < 0.0001$, Fig. 1C). These data demonstrate that PD-1 expression in tuberculosis patients correlates with IFN- γ secretion and that PD-1 is expressed in the group of patients who displayed CMI against the pathogen. To further define the relationship between PD-1 and IFN- γ production, we measured PD-1 expression and IFN- γ production by flow cytometry after *M. tuberculosis* stimulation. We found that virtually all (96%) of the IFN- γ -producing T cells expressed PD-1 (Fig. 1D). T cells that expressed PD-1 but did not express detectable IFN- γ may have taken up IFN- γ from neighboring T cells. Together, these data suggested to us that PD-1 could be regulated by Th1 cytokines.

To finally confirm that PD-1 expression could be regulated by IFN- γ , PBMC from tuberculosis patients were stimulated with Ag in the presence or absence of neutralizing anti-IFN- γ mAb or rIFN- γ and the expression of PD-1 was determined. Fig. 1E shows that the levels of PD-1 induced in response to the Ag were markedly inhibited by anti-IFN- γ in HR tuberculosis patients. We next examined whether PD-1 expression could be enhanced in *M. tuberculosis*-unresponsive patients through the addition of rIFN- γ .

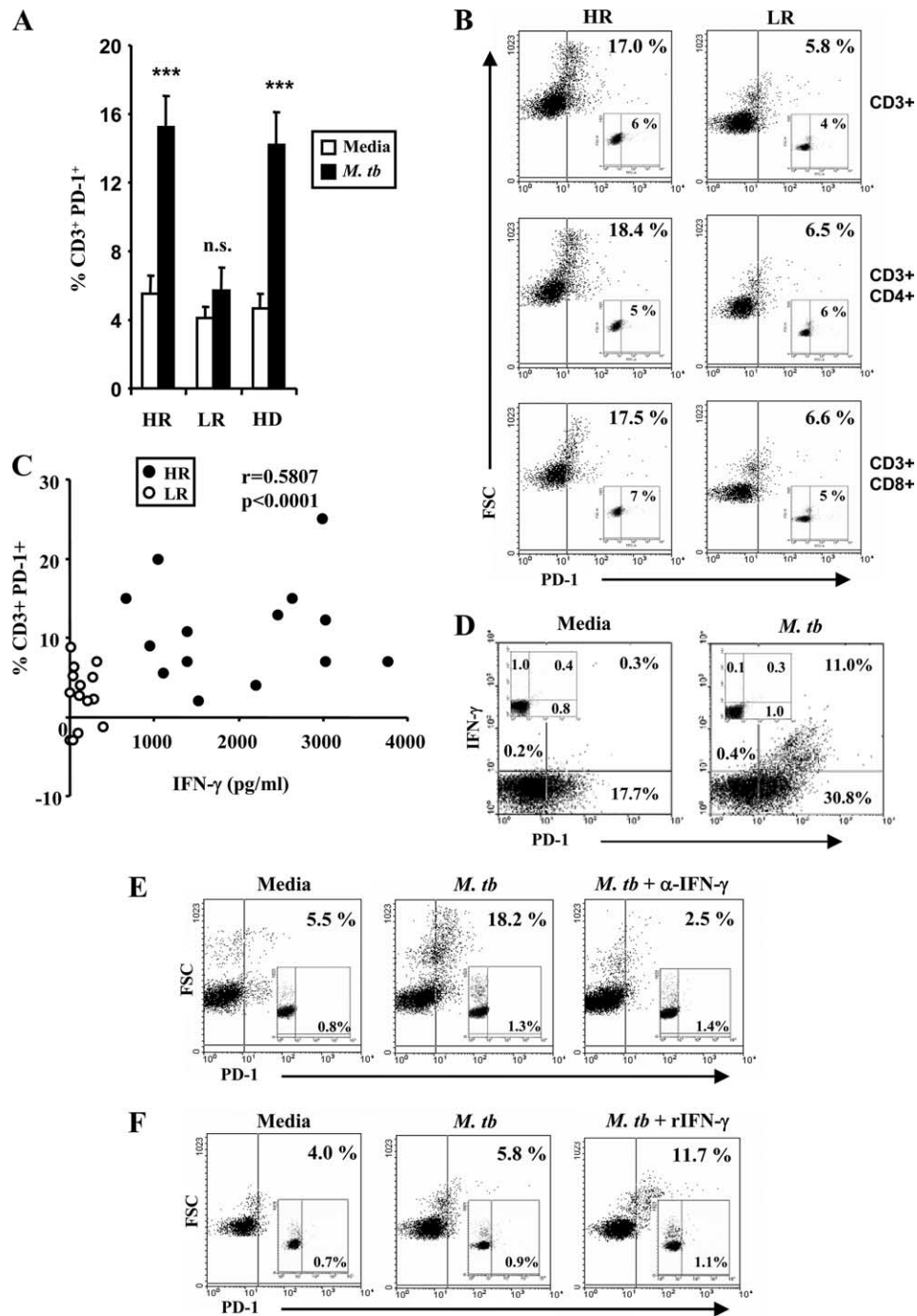


FIGURE 1. T cells up-regulate PD-1 after Ag stimulation in an IFN- γ -dependent manner. **A**, PBMC from HR, LR tuberculosis patients and HD were stimulated with sonicated *M. tuberculosis* Ag for 5 days. PD-1 expression was determined by flow cytometry, first gating on lymphocytes by light scatter, then by gating on CD3⁺ T cells. Each bar represents the mean \pm SEM from each group (14 individuals/group). ***, $p < 0.0001$; n.s., differences not significant. **B**, PD-1 expression on CD4 and CD8 cells as determined by flow cytometry. PBMC were stimulated with the Ag for 5 days and the expression of PD-1 on *M. tuberculosis*-stimulated T cells was examined on CD4 and CD8 cells by flow cytometry, first gating on lymphocytes by light scatter properties, then by gating on CD3⁺ (upper panel), CD3⁺CD4⁺ (middle panel), or CD3⁺CD8⁺ (lower panel) T cells. One representative HR and LR tuberculosis patient of five is shown for each group. The percentage of PD-1⁺ cells after culturing with *M. tuberculosis* Ag (large quadrant) is expressed in the right portion of the panels. Cells cultured with medium are shown in the inset. **C**, Correlation between PD-1 expression and IFN- γ production in tuberculosis patients. Statistical analysis was performed using the Spearman test to determine the association between PD-1 levels and IFN- γ production. Each symbol represents the percentage of PD-1⁺CD3⁺ T cells and the IFN- γ secretion in *M. tuberculosis*-stimulated cells for each tuberculosis patient. Spearman coefficient, $r = 0.5807$; ***, $p < 0.0001$. **D**, PBMC from HR tuberculosis patients were stimulated with *M. tuberculosis* Ag for 4 days and the expression of PD-1 on *M. tuberculosis*-reactive IFN- γ -producing T cells was examined by flow cytometry. T cells were identified first gating on blast lymphocytes by light scatter properties, then by gating on CD3⁺ cells, and finally evaluating PD-1 and IFN- γ expression. One representative HR patient of five is shown. The percentage of PD-1⁺IFN- γ ⁺ T cells after culturing with *M. tuberculosis* Ag or medium (large quadrants) is expressed in the right portion of the panels. Isotype controls (inset) are shown. **E** and **F**, PBMC from tuberculosis patients were stimulated with *M. tuberculosis* for 5 days in the presence or absence of anti-IFN- γ mAb (**E**) or rIFN- γ (**F**), and the expression of PD-1 was determined by flow cytometry. One representative HR (**E**) and LR (**F**) tuberculosis patient of five is shown for each group. The percentage of PD-1⁺CD3⁺ cells after culturing with *M. tuberculosis* Ag \pm anti-IFN- γ (**E**) or \pm rIFN- γ (**F**) (large quadrants) is expressed in the right portion of the panels. Isotype controls (inset) are shown.

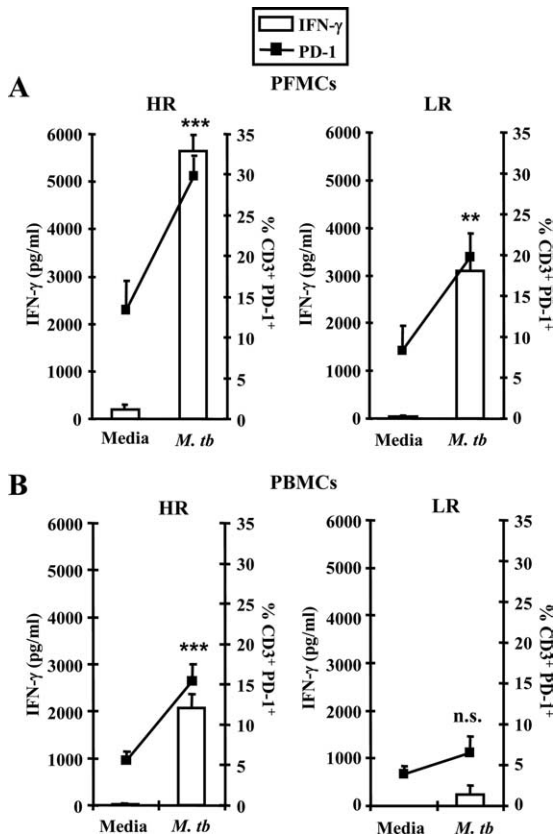


FIGURE 2. PD-1 expression and IFN- γ production in pleural effusions from tuberculosis patients. PFMCS (A) or PBMC (B) from tuberculosis patients were cultured in the presence or absence of sonicated *M. tuberculosis* Ag. IFN- γ production (2 days) and PD-1 expression (5 days) were determined by ELISA and flow cytometry, respectively. The mean \pm SEM of IFN- γ secretion or PD-1 levels is shown for each group (A, 6 individuals/group; B, 14 individuals/group). **, $p < 0.001$; ***, $p < 0.0001$; n.s., differences not significant.

When added alone, IFN- γ did not alter the expression of PD-1 (data not shown). However, when the cells were stimulated with *M. tuberculosis*, IFN- γ significantly increased the expression of PD-1 on T cells from LR patients when compared with cells stimulated with *M. tuberculosis* alone (Fig. 1F). As expected, IFN- γ treatment also increased PD-1 expression on *M. tuberculosis*-stimulated T cells from HR tuberculosis patients and HDs (data not shown). Taken together, these results suggest that *M. tuberculosis*-induced IFN- γ participates in the up-regulation of PD-1 expression.

Tuberculosis PFs are characterized by a T lymphocyte predominance (24). To further investigate the relationship between PD-1 and IFN- γ production in tuberculosis, we evaluated the levels of both parameters at the site of active infection. In HR patients, *M. tuberculosis* markedly augmented PD-1 expression and IFN- γ secretion from PFMCS (Fig. 2A, left panel), similar to our results in PBMC, but at higher levels (Fig. 2B, left panel). In PFMCS from LR tuberculosis patients, Ag stimulation significantly increased IFN- γ and PD-1 levels (Fig. 2A, right panel), in sharp contrast to the results obtained in PB of these patients (Fig. 2B, right panel), likely because at the site of infection, higher numbers of Ag-specific T cells are present.

Although in PFMCS from LR patients we detected lower levels of PD-1 and IFN- γ compared with HR individuals, these results, together with our observation that anti-IFN- γ mAb blocked the ability of the Ag to increase PD-1, confirmed that the up-regulation

of PD-1 expression in T cells from tuberculosis patients in response to *M. tuberculosis* is mediated by IFN- γ .

Ag activation of T cells from tuberculosis patients induces expression of PD-Ls

Both PD-1 ligands, PD-L1 and PD-L2, have been implicated in the regulation of effector T cell functions (25). Moreover, it was reported that PD-L1 and PD-L2 might have different functions in regulating type 1 and type 2 cytokine responses (25). Therefore, we next investigated the expression of PD-Ls in PBMC from tuberculosis patients. Similar to our data on PD-1, minimal expression of PD-L1 and PD-L2 was detected on resting PB T cells (data not shown). However, *M. tuberculosis* stimulation significantly augmented the levels of PD-L1 and PD-L2 on T cells from both groups of patients, though significantly higher levels of the receptors were detected in HR patients compared with LR individuals (Fig. 3A, $p < 0.01$, Mann-Whitney U test). Furthermore, while it was described that PD-L2 expression was restricted to macrophages and dendritic cells (26), we found that *M. tuberculosis* induced significant levels of this ligand on T cells from tuberculosis patients (Fig. 3A, right panel). Moreover, elevated levels of PD-L1 and PD-L2 were measured on PF T cells from HR and LR tuberculosis patients (data not shown). Furthermore, the levels of both PD-Ls induced in response to Ag were markedly inhibited by anti-IFN- γ , an effect shown even in LR patients, individuals who produce very low levels of IFN- γ to *M. tuberculosis* (Fig. 3B). Thus, these results suggest that endogenous *M. tuberculosis*-induced IFN- γ participates in the up-regulation of PD-Ls expression.

PD-1:PD-Ls interaction regulates T cell effector functions in tuberculosis

Our present data showed that *M. tuberculosis*-induced IFN- γ modulates PD-1 and PD-Ls levels in patients with active tuberculosis. We next sought to investigate whether the PD-1:PD-Ls pathway participated in the regulation of T cell effector functions during active disease. Initially, we analyzed the function of PD-1: PD-Ls interactions on CD8⁺ T cell-specific lytic degranulation against *M. tuberculosis*. PBMC or PFMCS from tuberculosis patients were incubated in the presence or absence of blocking mAbs against PD-1, and/or PD-L1 and PD-L2. Cells were then stimulated with or without *M. tuberculosis* Ag and, after 24 h, lytic degranulation of CD8⁺ T cells was measured by analyzing CD107a/b expression as described in *Materials and Methods*. In HR patients, PB CD8⁺ T lymphocytes displayed a striking degranulation against *M. tuberculosis* (Fig. 4A, left panel). Interestingly, blockade of PD-1 significantly augmented the percentage of CD3⁺CD8⁺ T cells expressing CD107a/b (Fig. 4A, left panel), suggesting that PD-1 might be involved in lytic degranulation against the pathogen. Ample evidence suggests that both PD-1 ligands, PD-L1 and PD-L2, bind a second, as yet unidentified, receptor(s) (27). In fact, it has been recently described in mice that PD-L1 interacts specifically with B7-1 (28). However, to date, only PD-1 has been reported as the receptor for PD-L1 and PD-L2 in humans. Therefore, to confirm that the observed inhibition of the lytic degranulation of CD8⁺ T cells by PD-1 was through the binding of the ligands to PD-1 and not through the interaction of PD-L1 and/or PD-L2 to an unknown receptor, we blocked the entire PD-1:PD-L1/PD-L2 pathway. As shown in Fig. 4A, left panel, no differences in the percentage of CD3⁺CD8⁺ T cells expressing CD107a/b were detected when we simultaneously blocked PD-1, PD-L1, and PD-L2 molecules, indicating that PD-1:PD-Ls interactions inhibit PB CD8⁺ T cell degranulation in HR tuberculosis patients. In contrast, PB CD8⁺ T cells from LR tuberculosis patients showed no

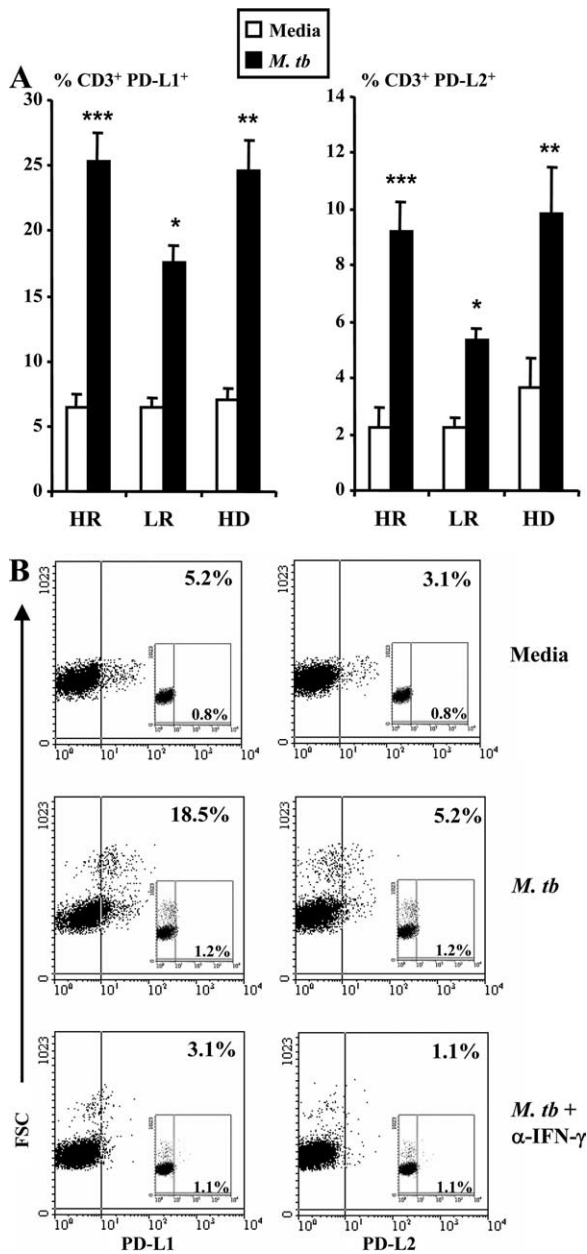


FIGURE 3. PD-Ls expression is up-regulated on T cells from tuberculosis patients after *M. tuberculosis* stimulation. **A**, PBMC from tuberculosis patients (HR and LR) and HD were stimulated with *M. tuberculosis* Ag for 5 days and PD-L1 and PD-L2 expression was determined by flow cytometry first gating on lymphocytes by light scatter, then by gating on CD3⁺ T cells. Each bar represents the mean \pm SEM from each group (11 individuals/group). *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$. **B**, PBMC from LR tuberculosis patients were stimulated with medium alone or with *M. tuberculosis* Ag in the presence or absence of anti-IFN- γ mAb. After 5 days, the expression of PD-L1 and PD-L2 was determined by flow cytometry. A representative patient of six is shown. The percentage of PD-L1⁺CD3⁺ and PD-L2⁺ CD3⁺ cells after culturing with medium, *M. tuberculosis* Ag, or *M. tuberculosis* Ag \pm anti-IFN- γ (large quadrants) is expressed in the right portion of the panels. Isotype controls (inset) are shown.

lytic degranulation either against *M. tuberculosis* or after blockage of the PD-1:PD-Ls pathway (Fig. 4A, left panel). However, and in striking contrast to our results in PB, both HR and LR tuberculosis patients' PF lymphocytes displayed a strong increase in the percentage of CD3⁺CD8⁺ T cells expressing CD107a/b against *M.*

tuberculosis at the site of infection, which was augmented by blocking the PD-1:PD-Ls pathway (Fig. 4A, right panel). These data indicate that the interaction of PD-1 through its ligands inhibits the cell degranulation of CD3⁺CD8⁺ lymphocytes after *M. tuberculosis* stimulation.

Given that production of IFN- γ by T cells is crucial for immunity against *M. tuberculosis* infection, we next determine whether the PD-1:PD-Ls pathway was involved in the modulation of the signaling pathway that controls IFN- γ in tuberculosis. Thus, we analyzed the percentage of *M. tuberculosis*-induced CD3⁺IFN- γ ⁺ T cells before and after blocking PD-1 and/or the complete PD-1:PD-Ls pathway. PBMC from tuberculosis patients were stimulated \pm *M. tuberculosis* \pm PD-1 and/or PD-Ls mAbs and the percentage of IFN- γ -producing cells was determined. As shown in Fig. 4B (left panel), HR patients produced significant numbers of PB CD3⁺IFN- γ ⁺ T cells against the Ag, and PD-1 blockade strikingly augmented the percentage of specific IFN- γ -producing lymphocytes. Moreover, the simultaneous blockage of PD-1 and its ligands demonstrated that PD-1, through the interaction with PD-L1 and PD-L2, inhibited IFN- γ production in these patients (Fig. 4B, left panel). This observed increase in IFN- γ secretion by effect of blocking the PD-1:PD-Ls pathway was due to an enhancement in the number of T cells producing the cytokine (as indicated by the percentage of CD3⁺IFN- γ ⁺ lymphocytes) but not to an increased production from already activated cells (as determined by analyzing the mean fluorescence intensity (MFI \pm SEM): *M. tuberculosis* 146 \pm 14; *M. tuberculosis* plus anti-PD-1: 157 \pm 19, *M. tuberculosis* plus anti-PD-1 plus anti-PD-Ls: 159 \pm 24 differences not significant among the groups). Thus, our results indicate that more effector cells are secreting IFN- γ by effect of the blockage of the PD-1 pathway, in contrast to our previous data showing that the increase in IFN- γ production after costimulation through ICOS-enhanced production from already activated cells (9). In this way, we demonstrate the differences in the role of distinct costimulatory pathways on IFN- γ production in tuberculosis. In contrast, *M. tuberculosis* stimulation did not induce significant IFN- γ -producing T cells in PB from LR patients, and blocking PD-1 and/or its ligands did not modify the percentage of IFN- γ -secreting cells (Fig. 4B, left panel). However, at the site of infection, both HR and LR tuberculosis patients produced IFN- γ against *M. tuberculosis*, and blocking PD-1 and the complete PD-1:PD-Ls pathway, significantly increased the number of CD3⁺IFN- γ ⁺ T cells in both groups of patients (Fig. 4B, right panel, $p < 0.01$, Wilcoxon rank sum test). Together, the present data suggest that PD-1 and its ligands would participate in the regulation of type 1 immune responses against *M. tuberculosis*. Cells cultured with medium in the presence of blocking mAbs against PD-1, and/or PD-Ls did not induce any changes in T cell effector functions studied (data not shown).

Previously, we demonstrated that SLAM engagement on *M. tuberculosis*-stimulated cells from HR and LR tuberculosis patients significantly augmented the levels of IFN- γ (8). Our present results showed that blockage of PD-1 augmented the levels of IFN- γ produced by PF T cells from LR patients, although they did not reach the levels produced by HR patients (Fig. 4B, right panel). Then, we next investigated whether blocking PD-1 after Ag stimulation might induce higher levels of IFN- γ from unresponsive LR patients. PBMC from tuberculosis patients were stimulated with *M. tuberculosis* Ag and, after 5 days, cells were cultured in the presence or absence of anti-PD-1 mAb. Cell-free supernatants were collected at 48 h and assayed for IFN- γ by ELISA. As shown in Fig. 4C, blocking PD-1 significantly increased *M. tuberculosis*-induced IFN- γ

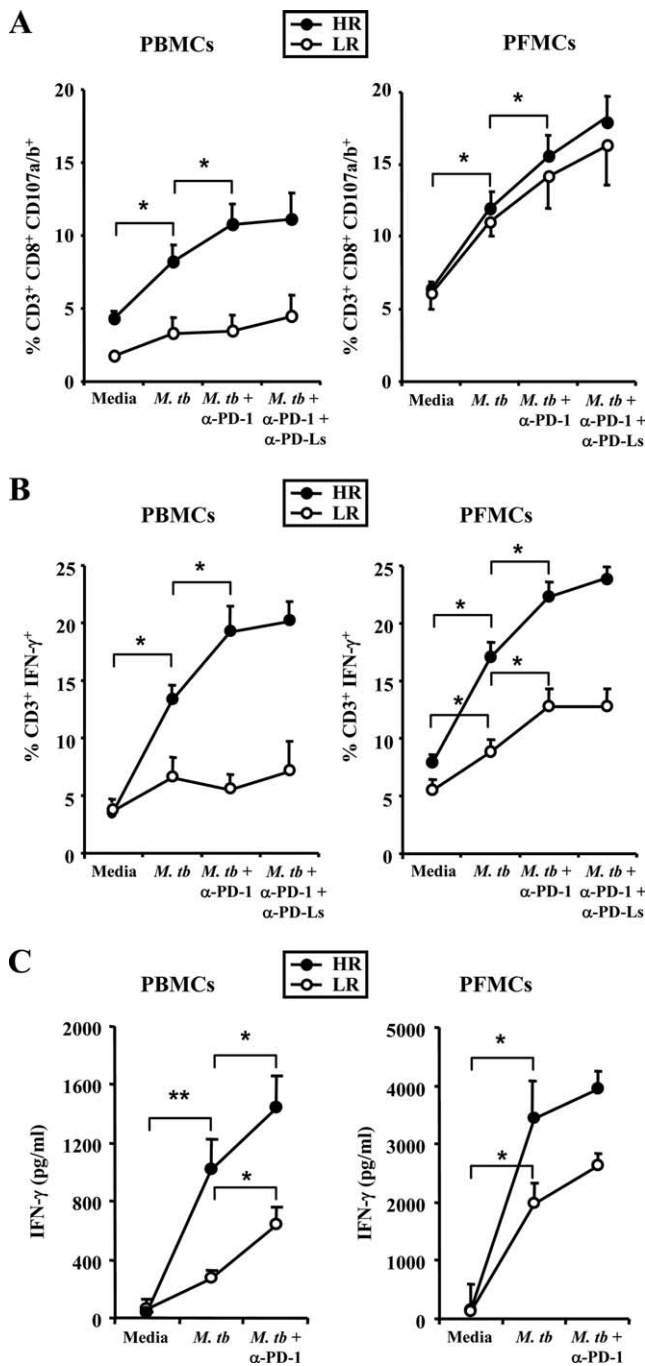


FIGURE 4. Effect of PD-1:PD-Ls interactions on T cell effector functions in tuberculosis patients. **A**, PBMC and PFMC from tuberculosis patients were stimulated with *M. tuberculosis* in the presence or absence of blocking anti-PD-1 mAb and/or PD-L1 and PD-L2 mAbs. After 24 h, the expression of CD107a and CD107b was determined on CD3⁺ CD8⁺ T cells by flow cytometry. Each point represents the mean ± SEM of the percentage of CD3⁺ CD107a/b⁺ cells in the entire groups (PBMC: 11 patients/group; PFMC: 7 patients/group). *, *p* < 0.01, Wilcoxon rank sum test. **B**, PBMC (8 patients/group) and PFMC (6 patients/group) from tuberculosis patients were stimulated with *M. tuberculosis* for 4 days in the presence or absence of anti-PD-1 mAb or anti-PD-1 plus anti-PD-L1 and anti-PD-L2 and intracellular IFN-γ expression was determined by flow cytometry. Lymphocytes were gated based on their forward scatter channel/side scatter channel (FSC/SSC) profile and gated further based on CD3⁺ expression. Each point represents the mean ± SEM from each group. *, *p* < 0.01. **C**, PBMC and PFMC from tuberculosis patients were stimulated with *M. tuberculosis* and after 5 days the cells were washed and cultured in the presence or absence of anti-PD-1. Cell-free supernatants were

production from Ag-stimulated cells from both groups of tuberculosis patients, even in PB from LR patients, although at lower levels compared with HR individuals. Blocking PD-1 had no effect on IL-10 production from HR and LR tuberculosis patients (data not shown). Together, these results further indicate that the blockage of PD-1 leads to the induction of type 1 responses in tuberculosis patients in response to the specific Ag.

Enhancement of IFN-γ production against M. tuberculosis by simultaneous PD-1 blockage and costimulation through SLAM

Cooperation between costimulatory and coinhibitory molecules are required for driving T cell responses into immunity or tolerance (27). It was suggested that the combinatory immunotherapy might improve T cell responses in cancer and in chronic viral infections (29–31), either through the dual costimulation with costimulatory molecules or through the blockage of an inhibitory receptor and the activation of a costimulatory molecule (32). We therefore investigated whether cooperation between costimulatory and coinhibitory pathways could enhance IFN-γ responses against *M. tuberculosis*. Data presented here showed that PD-1 blockage significantly augmented *M. tuberculosis*-induced IFN-γ production from unresponsive patients (Fig. 4C), although at lower levels than those secreted by HR individuals. Besides, we previously demonstrated that engagement through SLAM strikingly increased Ag-induced IFN-γ secretion from HR and LR tuberculosis patients, and similar to PD-1 blockage, the levels of the cytokine produced by LR patients did not reach those produced by HR patients (8, 9). Therefore, we sought to investigate whether cooperation between costimulatory molecules in tuberculosis might display additive/synergistic effects or show a functional dichotomy in modulating effector T cell responses as it was described in other pathologies (28–30). PBMC or PFMC from HR and LR tuberculosis patients were stimulated with sonicated *M. tuberculosis* Ag and, after 5 days, cells were cultured in the presence or absence of anti-SLAM, -PD-1, or -SLAM plus anti-PD-1 mAbs. Cell-free supernatants were collected at 48 h and assayed for IFN-γ by ELISA. As shown in Fig. 5A, and in accordance with our previous and present results, SLAM ligation or PD-1 blockage significantly increased *M. tuberculosis*-induced IFN-γ production from HR patients (Ref. 8 and Fig. 4C, respectively). Furthermore, the simultaneous addition of both mAbs increased the secretion of IFN-γ by PB T cells from responder individuals even more (Fig. 5A, upper panel). Interestingly, simultaneous blockage of PD-1 and activation of SLAM significantly enhanced the production of IFN-γ by PB T lymphocytes from LR tuberculosis patients, up to the levels of the cytokine secreted by HR individuals to *M. tuberculosis* Ag (Fig. 5A, lower panel). At the site of infection, where *M. tuberculosis* stimulation induced high PD-1 expression (Fig. 2A) and elevated SLAM levels (data not shown) in both groups of patients, the combination of anti-SLAM and -PD-1 treatment in Ag-stimulated PFMC induced higher levels of IFN-γ compared with PB (Fig. 5B). Together, our present data indicate that costimulation through SLAM and blocking PD-1 cooperate to produce a synergistic effect on IFN-γ production against *M. tuberculosis*, even in unresponsive tuberculosis patients.

collected at 48 h and assayed for IFN-γ production by ELISA. Each point represents the mean ± SEM from each group. *, *p* < 0.01; **, *p* < 0.001 (PBMC: 11 patients/group; PFMC: 7 patients/group).

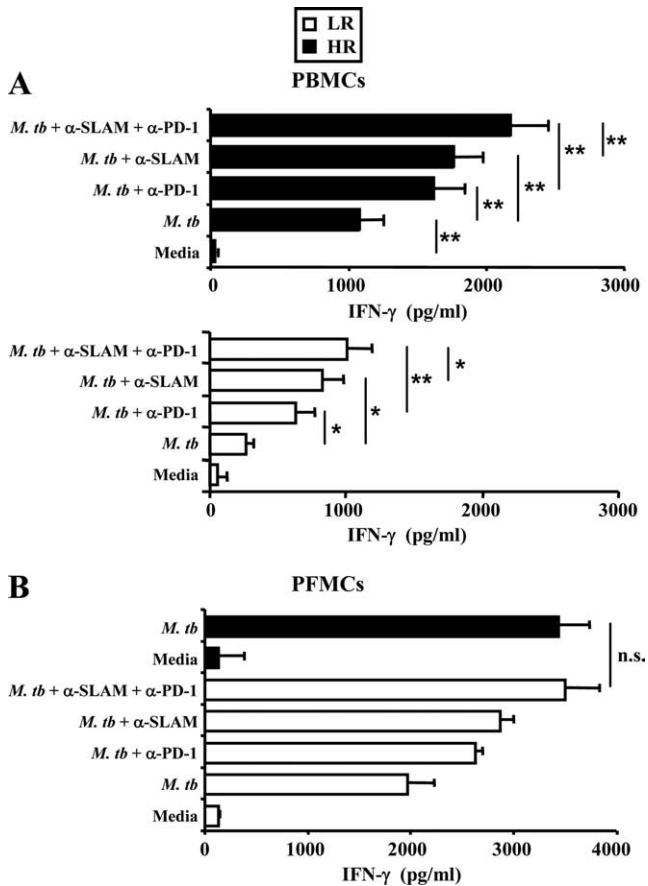


FIGURE 5. Effect of simultaneous SLAM ligation and PD-1 blocking on IFN- γ production by tuberculosis patients. *A* and *B*, PBMC and PFMC from HR and LR tuberculosis patients were stimulated with *M. tuberculosis* Ag and, after 5 days, the cells were washed and cultured in the presence or absence of anti-SLAM, anti-PD-1, or anti-SLAM + anti-PD-1 mAbs. Cell-free supernatants were collected at 48 h and assayed for IFN- γ production by ELISA. Each bar represents the mean \pm SEM from each group (*A*, 11 individuals/group; *B*, 6 individuals/group). *, $p < 0.01$; **, $p < 0.001$; n.s., differences not significant.

Discussion

Cosignals are required to mount effective Ag-specific immune responses during T cell activation. These cosignals are costimulatory pathways that can be either positive or negative and consequently determine the nature of the immune response. Whereas the classical interactions B7-1/B7-2:CD28/CTLA-4 serve as the main switches regulating the clonal composition of activated naive T cells, other newly discovered costimulatory receptors, like ICOS and PD-1, fine-tune the expansion and properties of activated T cells (33). ICOS:ICOSL interactions might promote expansion of effector cells that would traffic into inflamed tissues, interact with APC there, and be regulated by PD-1 and its ligands (33). Moreover, it has been shown that PD-1:PD-Ls interactions can regulate T cell proliferation, cytokine production (34), the balance among tolerance, autoimmunity, infection, and immunopathology (16). Previously, we reported that ICOS activation promoted the induction of protective Th1 cytokine responses against *M. tuberculosis* (8, 9). Thus, to further investigate the regulation of T cell activation in tuberculosis, in this work we analyzed the role of the PD-1:PD-Ls pathway.

PD-1:PD-Ls is a central pathway in the interaction between host defenses aimed at eradicating pathogenic microbes and microbial strategies that evolved to resist immune responses. As a result,

several reports demonstrated that PD-1:PD-Ls interactions regulate immune-mediated tissue damage during chronic viral infection (16), whereas it was also stated that many microorganisms that cause chronic infection exploit the PD-1:PD-Ls pathway to evade host immune effector mechanisms (16). Hence, it was shown that the PD-1:PD-Ls pathway contributes directly to T cell dysfunction and lack of viral control in established chronic infection both in mice (35) and humans (36–38). Similarly, persisting infection with pathogens other than viruses, like *Helicobacter pylori* and *Porphyromonas gingivalis*, showed elevated expression of PD-L1 on gastric epithelial cells (39) and monocytes (40), respectively, suggesting a potential involvement of PD-L1 in promoting chronic infection (39). However, the function of the PD-1:PD-Ls interactions during the regulatory pathways that contribute to persistence of intracellular bacteria has not been investigated. Therefore, in this study, we evaluated the function of the PD-1:PD-Ls pathway in patients with active *M. tuberculosis* infection.

Production of IFN- γ and a shift toward a Th1 cytokine profile are crucial mediators of the protection against *M. tuberculosis* (41). PD-1 expression is induced by TCR signaling and can be augmented by proinflammatory cytokines (42). Then, we studied the levels of PD-1 upon *M. tuberculosis* Ag stimulation. Given that both circulating T cell responses and lymphocyte responses at the site of infection are considered important during the immune response of the host to *M. tuberculosis*, PD-1 expression was investigated in T cells from PB and PFs from tuberculosis patients. Our results showed a direct association between PD-1 and IFN- γ at the site of infection as well as in PB from tuberculosis patients. These findings indicate that T cells from patients that display potent T cell responses against the pathogen (HR) were systemically activated by the specific Ag, inducing IFN- γ and generating a Th1-like microenvironment that in turn led to heightened PD-1 expression. Moreover, virtually all of the IFN- γ -secreting T cells in these patients expressed PD-1, similarly to our published results with SLAM (43). In contrast, systemic T cells from unresponsive tuberculosis patients (LR) were weakly activated by *M. tuberculosis* Ag, produced IL-10 but very low levels of IFN- γ (9), creating a predominant Th2-like environment that impaired the increase in PD-1 levels on their T cells. This impairment in PD-1 up-regulation in these patients might be related to the lower frequency of *M. tuberculosis*-specific T cells in PB compared with HR patients. In fact, at the site of infection, where unresponsive patients have elevated numbers of Ag-specific lymphocytes, they secreted higher levels of IFN- γ against the Ag compared with PB, which led to an increase in PD-1 levels. These data are in line with previous reports showing that PF T lymphocytes from tuberculosis patients are polarized toward a Th1 cytokine phenotype (44). Moreover, *M. tuberculosis*-induced PD-1 expression was markedly inhibited by anti-IFN- γ and addition of exogenous IFN- γ enhanced expression of PD-1 on PB T cells from tuberculosis patients. Together, we demonstrated that stimulation with *M. tuberculosis* induced IFN- γ by T cells from tuberculosis patients, and this cytokine activates T lymphocytes, which increased PD-1 expression in an Ag-specific manner.

Because PD-1 interacts with PD-L1 and/or PD-L2, we next analyzed the expression of PD-1 ligands on T cells from tuberculosis patients. It is widely known that the two PD-1 ligands differ in their expression patterns: PD-L1 is expressed on T cells, B cells, dendritic cells, macrophages, nonhematopoietic and other cell types, while expression of PD-L2 is much more restricted to dendritic cells, B1 cells, and macrophages (16, 26). Here, we found that both PD-1 ligands were inducibly expressed on T cells from tuberculosis patients in response to *M. tuberculosis*, demonstrating

for the first time that PD-L2 is expressed on human T cells. Furthermore, both PD-L1 and PD-L2 levels were significantly up-regulated by Ag-induced IFN- γ produced by tuberculosis patients, even by the low levels of the cytokine secreted by PB T cells from LR individuals. Accordingly, it was demonstrated that PD-L1 expression might be induced by low doses of IFN- γ in monocytes/macrophages (25, 45). Moreover, in accord with our data, previous reports showed that PD-L1 expression on macrophages could be induced by IFN- γ (25), indicating that Th1 cells as well as microbial products could enhance PD-L1 levels on many different cell types, both in humans and mice (25, 46). Although others suggested that PD-L2 might be induced on mouse macrophages by activation via IL-4 (25), our findings demonstrated that in tuberculosis infection, even low levels of IFN- γ might enhance PD-L2 expression on T cells. Together, our data indicate that *M. tuberculosis* induced both PD-1 ligands on T cells from patients with active disease.

We next evaluated the physiological role of the interaction of PD-1 and its ligands in terms of IFN- γ -producing cells and the lytic degranulation against the pathogen. Studies with blocking Abs and knockout mice indicate important inhibitory functions for the PD-1:PD-Ls pathway in vivo (47–49). However, data suggest that PD-L1 and/or PD-L2 may signal bidirectionally (50, 51). In this study, by blocking PD-1 and/or the complete PD-1:PD-Ls pathway, we demonstrated a significant increase in the number of CD3⁺IFN- γ ⁺ T cells and CD8⁺CD107a/b⁺ T lymphocytes against *M. tuberculosis*. In PB, PD-1:PD-Ls interactions showed an active coinhibition of IFN- γ production and lytic degranulation only in patients with robust CMI against the pathogen. However, at the site of infection, blockage of the PD-1:PD-Ls pathways clearly augmented T cell effector functions in both groups of tuberculosis patients. Because tuberculous pleurisy is an intense immune response to mycobacteria that results in the clearance of organisms from the pleural space, the PD-1:PD-Ls pathway might have a role at the site of *M. tuberculosis* infection, where both inflammation and immune-mediated pathology require containment by the host.

Considering that LR individuals displayed very low IFN- γ and lytic degranulation responses to *M. tuberculosis* (Fig. 4), and their PB T cells expressed low levels of PD-1, blocking the pathway did not affect the systemic T cell effector functions in these patients. However, previously, we demonstrated that SLAM ligation did affect systemic effector functions of LR subject, increasing IFN- γ production, whereas signaling through the ICOS-ICOSL pathway had no effect on effector functions of LR patients (8, 9). Thus, our present and previous data might suggest that LR tuberculosis patients' effector responses would depend either on the absence of an effect of the particular costimulatory pathway or on the absence of enough numbers of Ag-specific reactive T cells.

To date, literature regarding the effects of PD-1:PD-Ls interactions on the cytolytic capability of CTLs has been contradictory, with reports suggesting inhibition of cytotoxicity (52) and others suggesting no effect (53). Our data clearly support that the PD-1:PD-Ls interactions function as an inhibitory pathway on the lytic degranulation of Ag-specific CD8⁺ T cells against *M. tuberculosis*, as illustrated by acquisition of CD107a/b expression. Given that the expression of CD107a/b has been described as a surrogate marker for cytolytic activity (18, 54, 55), we conclude that the PD-1 pathway would inhibit the cytotoxic activity against the pathogen. Accordingly, several groups have demonstrated that blocking PD-1:PD-Ls interactions in vitro reverses the exhaustion of HIV and hepatitis C virus-specific CD8⁺ and CD4⁺ T cells and restores cytokine production and proliferation (36, 38, 56, 57). Moreover, administration of PD-L1-specific blocking mAbs leads

to exacerbated keratitis-increased HSV-specific effector CD4⁺ T cell proliferation, IFN- γ production, and survival, suggesting that PD-L1 may participate in limiting immune-mediated tissue damage in herpes stromal keratitis infection (58). Besides, during hepatitis B virus infection, PD-1:PD-L1 interactions were shown to contribute to the suppression of IFN- γ secretion following Ag recognition in the liver (59). Furthermore, in infection with *H. pylori*, anti-PD-L1 was shown to increase T cell proliferation and IL-12 secretion (39). Accordingly, in this work we demonstrated that the PD-1:PD-Ls pathway displayed a coinhibitory role on IFN- γ production during chronic *M. tuberculosis* infection.

Compared with unresponsive LR tuberculosis individuals, HR patients showed higher expression of PD-1, PD-L1, and PD-L2, and stronger T cell effector functions upon blocking the PD-1:PD-Ls pathway. Recent studies in mice suggested the presence of bacteria in a compartment that cannot be mobilized from the lungs to the lymph node (60). Thus, based on those results, a potential hypothesis to explain the differences in the immune response of HR and LR patients might be that variations in the transport of bacteria from the lungs to the local lymph node impairs the complete activation of T cells from unresponsive patients. However, additional studies need to be performed to probe an immunological mechanism to explain the weak T cell responses of LR patients.

In any case, and even at the site of infection, LR tuberculosis patients produced lower levels of IFN- γ , the crucial mediator of the protection against *M. tuberculosis* (20–22). Given that we previously demonstrated that immunological parameters correlated with disease severity (8), we next investigated whether IFN- γ production from LR tuberculosis patients could be enhanced up to the levels produced by HR individuals. Because it was demonstrated that blocking of an inhibitory pathway simultaneously with the stimulation of an activation pathway might improve T cell effector functions (61), we blocked PD-1 and costimulated through SLAM in patients with weak and strong CMI against *M. tuberculosis*. Interestingly, we observed that the simultaneous treatment augmented the levels of IFN- γ secreted from unresponsive LR patients up to the levels of the cytokine produced by HR patients against the Ag, both in PB and at the site of infection. Moreover, similar results were found by blocking PD-1 together with ICOS activation (data not shown).

In conclusion, in this report we demonstrated the inhibitory function of the PD-1:PD-Ls pathway during *M. tuberculosis* active disease. Furthermore, combining our present data with our previous findings on the positive role of SLAM and ICOS in tuberculosis, we showed that the use of blocking and agonistic mAbs might have important implications for potential therapies for chronic bacterial infections like tuberculosis, as it was reported for chronic viral diseases and malignancies (61). This study further suggests that PD-1 might be a focal point for therapeutic modulation of T cell cytokine responses in tuberculosis.

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Disclosures

The authors have no financial conflict of interest.

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