MAJOR ARTICLE

Role Played by the Programmed Death-1–Programmed Death Ligand Pathway during Innate Immunity against *Mycobacterium tuberculosis*

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Tuberculous pleurisy allows the study of specific cells at the site of *Mycobacterium tuberculosis* infection. Among pleural lymphocytes, natural killer (NK) cells are a major source of interferon γ (IFN-γ), and their functions are regulated by activating and inhibitory receptors. Programmed death-1 (PD-1), programmed death ligand 1 (PD-L1), and programmed death ligand 2 (PD-L2) are recognized inhibitory receptors in adaptive immunity, but their role during innate immunity remains poorly understood. We investigated the PD-1:PD-L1/PD-L2 pathway on NK cell effector functions in peripheral blood and pleural fluid from patients with tuberculosis. *M. tuberculosis* stimulation significantly up-regulated PD-1, PD-L1, and PD-L2 levels on NK cells. Interestingly, a direct correlation between PD-1 and IFN-γ expression on NK cells was observed. Moreover, blockade of the PD-1 pathway markedly augmented lytic degranulation and IFN-γ production of NK cells against *M. tuberculosis*. Furthermore, PD-1$^+$ NK cells displayed a diminished IFN-γ mean fluorescence intensity, denoting the relevance of PD-1 on IFN-γ regulation. Together, we described a novel inhibitory role played by PD-1:PD-L interactions in innate immunity in tuberculosis.

Tuberculous pleurisy, one of the most common manifestations of extrapulmonary tuberculosis, is a severe delayed-type hypersensitivity reaction against the rupture of a subpleural focus of *Mycobacterium tuberculosis* infection [1, 2]. Natural killer (NK) cells present in tuberculous fluids (PFNK cells) express a differential pattern of molecules that participates in their migration to the lung and in the modulation of the immune response and T cell activation [3], addressing the importance of innate immunity to fight this pathogen.

NK cell activity is regulated by a balance between activating and inhibitory receptors [4, 5], and this regulation can be differential among subsets of NK cells. Two major subsets of NK cells have been recognized in peripheral blood based on the differential expression of CD56 receptor [6]. Thus, >90% of circulating NK cells are CD56^{dim} NK cells, efficient effectors of natural and antibody-dependent target cell lysis, whereas CD56^{bright} NK cells are more effective in producing cytokines [7–9]. In human secondary lymphoid organs and inflammatory sites, there is an enrichment in CD56^{bright} NK cells [10–13] as a consequence of the different repertoires of chemokine receptors and adhesion molecules that provide these cells divergent migratory properties. Moreover, it was described an increment in CD56^{bright} NK cells and a major susceptibility of CD56^{dim} NK cells to apoptosis in human tuberculosis pleural effusions [14]. Among pleural fluid lymphocytes, CD56^{bright} NK cells are the major producers of

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interferon γ (IFN-γ) after *M. tuberculosis* stimulation through a mechanism that involves NK cell–APC interaction and *M. tuberculosis*–NK direct contact [14, 15].

As mentioned, NK cell activity is regulated by activating and inhibitory receptors [4, 5], and programmed death-1 (PD-1) has been recently described to be expressed on NK cells [16], T lymphocytes, NK T cells, monocytes [17–19], macrophages [20], and mouse dendritic cells. In fact, $CD56^{bright}$ NK cells were shown to express high levels of PD-1 during human chronic hepatitis C virus infection [20]. Moreover, antiviral therapy was demonstrated to be associated with down-regulation of PD-1 on NK cells [20]. The 2 PD-1 ligands, PD-L1 and PD-L2, differ in their expression patterns: PD-L1 is expressed on T cells, B cells, dendritic cells, macrophages, nonhematopoietic cells, NK cells, and other cell types [21, 22], whereas expression of PD-L2 is much more restricted to dendritic cells, B1 cells, and macrophages [21, 23]. There is ample evidence of the role played by PD-1:PD-L interactions in the inhibition of adaptive immune responses. Several microorganisms that cause chronic infection exploit the PD-1:PD-L pathway to evade host immune effector mechanisms [23]. In fact, we recently demonstrated that the PD-1:PD-L pathway inhibits T cell effector functions during human tuberculosis [24]. However, little is known about this pathway in human innate immune responses. During *Listeria monocytogenes* infection, PD-L1 is induced on splenic dendritic cells and inhibits innate immune responses [25]. Therefore, on the basis of the importance of innate immunity linking to adaptive immunity, here we investigated whether PD-1:PD-L interactions regulated innate immunity during human active tuberculosis.

MATERIALS AND METHODS

Patients. Patients with active tuberculosis were evaluated at the Hospital F. J. Muñiz, Buenos Aires, Argentina. The diagnosis was established on the basis of clinical and radiological data, together with the identification of acid-fast bacilli in sputum. Physical examination, complete blood cell count, electrolyte, chest X-ray, and HIV test were performed for each patient. Exclusion criteria included a positive HIV test result or the presence of concurrent infectious diseases. Pleural effusions were obtained by thoracentesis and classified as exudates according to at least 1 of Light's criteria [2]. We studied a total of 17 pleural fluids from patients with pulmonary disease according to chest X-ray findings (patients' average age, 25 years; range, 19–37 years). All participating patients had received <1 week of antituberculosis therapy. BCG-vaccinated healthy control donors from the community participated in this study. Peripheral blood samples were collected in heparinized tubes from all individuals after receiving informed consent. The local ethics committee approved all the studies performed.

Antigen. In vitro stimulation of cells throughout the present study was performed with the γ-irradiated virulent *M. tuberculosis* strain H37Rv generously provided by the TB Vaccine Testing and Research Materials program from Colorado State University, Fort Collins, Colarado. Mycobacteria were suspended in free pyrogen phosphate-buffered saline, sonicated, and adjusted to $\sim 1 \times 10^8$ bacteria per mL (optical density value at 600 nm = 1).

Cell preparations and culture conditions. After sample collection, peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) were freshly isolated by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences). The cells were then cultured at 1×10^6 cells per mL ± *M. tuberculosis* (10 μg/mL) in 24 or 96 well plates (Greiner Bio-One) with RPMI 1640 medium (Gibco BRL), supplemented with L-glutamine (2 mmol/L; Sigma-Aldrich), gentamicin, and 10% human serum, in a CO_2 incubator at 37°C. In some experiments, cells were incubated with or without blocking antibodies against PD-1 (2.5 μg/mL, J116, eBioscience), PD-L1 (2 μg/mL, MIH1, eBioscience), PD-L2 (2 μg/mL, MIH18, eBioscience), or isotype control, for 30 min. Then, *M. tuberculosis* Ag was added. After 24 h, IFN-γ production was evaluated by enzyme-linked immunosorbent assay (ELISA; eBioscience), whereas cytotoxicity and the percentage of IFN-γ–producing cells were determined by flow cytometry (see below).

Flow cytometry. PBMCs or PFMCs were stained for CD56, CD3, PD-1, PD-L1, PD-L2 expression, before and after 24 h culture with *M. tuberculosis* ± human recombinant interleukin 4 (IL-4) (5 ng/mL; eBioscience), recombinant interleukin 17 (IL-17) (5 ng/mL; eBioscience), recombinant IFN-γ (7.5 ng/mL; eBioscience) or neutralizing anti–IFN-γ monoclonal antibodies (mAbs) (10 ng/mL, NIB42; eBioscience). Coexpression of PD-1 and IFN-γ in NK cell was performed as described elsewhere [26]. In separated experiments, PBMCs and PFMCs were incubated ± *M. tuberculosis* ± anti–PD-1 and/or anti–PD-Ls blocking mAbs and IFN-γ production was measured as described elsewhere [24]. In all cases, negative control samples were incubated with irrelevant isotype-matched mAbs (eBioscience) in parallel with experimental samples. All samples were analyzed on a FACS Aria II cell sorter (BD).

Confocal microscopy. PBMCs were incubated 2 h in complete media to allow adherence of monocytes to plastic. Nonadherent cells were recovered and $CD19^-CD3^-CD56^+$ cells were obtained after 2 rounds of negative selection with magnetic beads (Dynal beads; Dynal Biotech) conjugated with anti-CD3 and anti-CD19 mAbs (OKT3 and HIB19, eBioscience). The purity of the cell fractions was evaluated by flow cytometry (>95% for the $CD3^-CD56^+$ fraction). Negative selected $CD56^+$ NK cells were then stained for CD56 and PD-L1/L2 expression. Afterward, the cells were added onto a poly-lysine (Sigma-Aldrich) coated slide and examined by confocal microscopy in an Olympus FV300 model.

Lytic degranulation. CD107a (Lysosome-Associated Membrane Protein 1, LAMP-1) expression was performed to measure NK cell degranulation, as described elsewhere [27, 28]. Briefly, PBMCs and PFMCs were incubated for 24 h ± *M. tuberculosis* ± anti–PD-1 and/or anti–PD-Ls blocking mAbs. Then CD107a FITC antibody (H4A3, 20 μL/mL; BD Biosciences) was added directly to the cells in culture for 6 h, and 1 h later the GolgiStop reagent was incorporated (1 μL/mL; BD Biosciences) for 5 h, according to the manufacturer's instructions. Finally, cells were stained with anti-CD56 and anti-CD3, and FACS analysis was performed. Negative control samples were incubated with irrelevant isotype-matched Abs in parallel with all experimental samples. All samples were analyzed on a FACS Aria II cell sorter.

Statistical analysis. Statistical analysis was performed using the nonparametric Wilcoxon rank sum test for paired samples and the Mann-Whitney test for unpaired samples. Values of $P < .05$ were considered significant.

RESULTS

Expression of PD-1 on NK cells from patients with tuberculosis. We first investigated whether *M. tuberculosis* stimulation could modify PD-1 expression on NK cells from patients with tuberculosis. We observed that PD-1 basal levels were significantly higher on peripheral blood NK cells compared to pleural fluid (mean ± standard error of mean [SEM] %PD-1$^+$ NK cells, peripheral blood: 11 ± 3; pleural fluid: 6 ± 0.9, $^{**}P < .01$, Mann-Whitney test). However, when PBMCs and PFMCs were stimulated with *M. tuberculosis* we detected a significant increase in the receptor levels on NK cells, which were markedly higher in pleural fluid compared to peripheral blood (Figure 1*A*). The expression of PD-1 on NK cells from healthy donors was significantly lower than that from patients with tuberculosis, even after *M. tuberculosis* stimulation (Figure 1*A*).

We next investigated whether the expression of PD-1 might be modulated on NK cells by the cytokine microenvironment. Thus, we studied whether Th1, Th2, or Th17 cytokines might regulate PD-1 expression on pleural fluid NK cells from patients with tuberculosis. After *M. tuberculosis* stimulation, both IL-4 and IL-17 markedly decreased PD-1 expression on NK cells (Figure 1*B*). In contrast, addition of recombinant IFN-γ markedly augmented PD-1 levels on pleural fluid NK cells (Figure 1*B*). Furthermore, the levels of PD-1 were significantly diminished by anti–IFN-γ (Figure 1*B*).

Correlation between PD-1 expression and IFN-γ production on NK cells from patients with tuberculosis. To further investigate the relationship between PD-1 expression and IFN-γ secretion by pleural fluid NK cells, we determined simultaneously the IFN-γ production and PD-1 expression after *M. tuberculosis* stimulation. Figure 1*C* shows that the increment in the secretion of IFN-γ by NK cells was accomplished by a significant augment of PD-1. Because IFN-γ expression is detected mainly in CD56^{bright} NK cells in response to proinflammatory cytokines [11, 29, 30], we analyzed the expression of PD-1 among the 2 subsets of NK cells. We observed that *M. tuberculosis* stimulation induced markedly higher levels of PD-1 on CD56^{bright} NK cells compared with those detected on CD56^{dim} NK cells (Table 1), in agreement with previous reports [16]. Next, we evaluated the simultaneous expression of PD-1 and IFN-γ. Figure 1*D* shows that at the site of infection, among PD-1$^+$CD56^{bright} NK cells, the majority were IFN-γ$^+$ (mean ± SEM, 73.7 ± 6.7). Therefore, we performed statistical analysis to assess the relationship between PD-1 expression and IFN-γ production. We observed a significant correlation between the expression of PD-1 on NK cells and the number of IFN-γ$^+$CD56^{bright} cells (Figure 1*E*). Together, these results suggest that the IFN-γ secreted by NK cells against *M. tuberculosis* might be associated with the up-regulation of PD-1.

Expression of PD-1 ligands on NK cells from patients with tuberculosis. Although the expression of PD-L1 on NK cells has been reported elsewhere [22, 31], little information is available regarding PD-Ls function on NK cells. Thus, we evaluated the expression of the PD-Ls on NK cells from patients with tuberculosis. Constitutive higher levels of PD-L1 were detected on pleural fluid NK cells compared with peripheral blood (mean ± SEM [percentage of PD-L1$^+$ NK cells]; peripheral blood, 16.4 ± 1; pleural fluid, 30.25 ± 3; $P < .005$; Mann-Whitney test). Regarding PD-L2, we detected low constitutive expression in peripheral blood and pleural fluid NK cells from patients with tuberculosis (mean ± SEM [%PD-L2$^+$ NK cells]; peripheral blood, 3.4 ± 0.4; pleural fluid, 6.24 ± 0.8, n.s). Moreover, we observed that like PD-1, the highest levels of PD-L1 were detected on CD56^{bright} NK cells (Table 1). Furthermore, pleural fluid CD56^{bright} NK cells showed markedly elevated expression of PD-L1 compared with peripheral blood CD56^{bright} NK cells (Table 1). In contrast, PD-L2 was mainly expressed on CD56^{dim} NK cells (Table 1). Even more, pleural fluid CD56^{bright} NK cells displayed lower levels of PD-L2 than did peripheral blood CD56^{bright} NK cells from patients with tuberculosis (Table 1). Given that PD-L2 expression on NK cells has not been described elsewhere, we sought to confirm this result by confocal microscopy. As shown in Figure 2*A*, our data clearly demonstrate for the first time to our knowledge that PD-L2 is expressed on human NK cells. We then investigated whether *M. tuberculosis* modulated the PD-Ls expression on NK cells. *M. tuberculosis* significantly enhanced the percentage of PD-L1 and PD-L2 on NK cells, in both peripheral blood and pleural fluid (Figure 2*B*). Furthermore, we observed significant differences regarding PD-Ls expression among peripheral blood NK cells from patients with tuberculosis and healthy donors, and, like PD-1, the highest levels of both PD-Ls were induced on

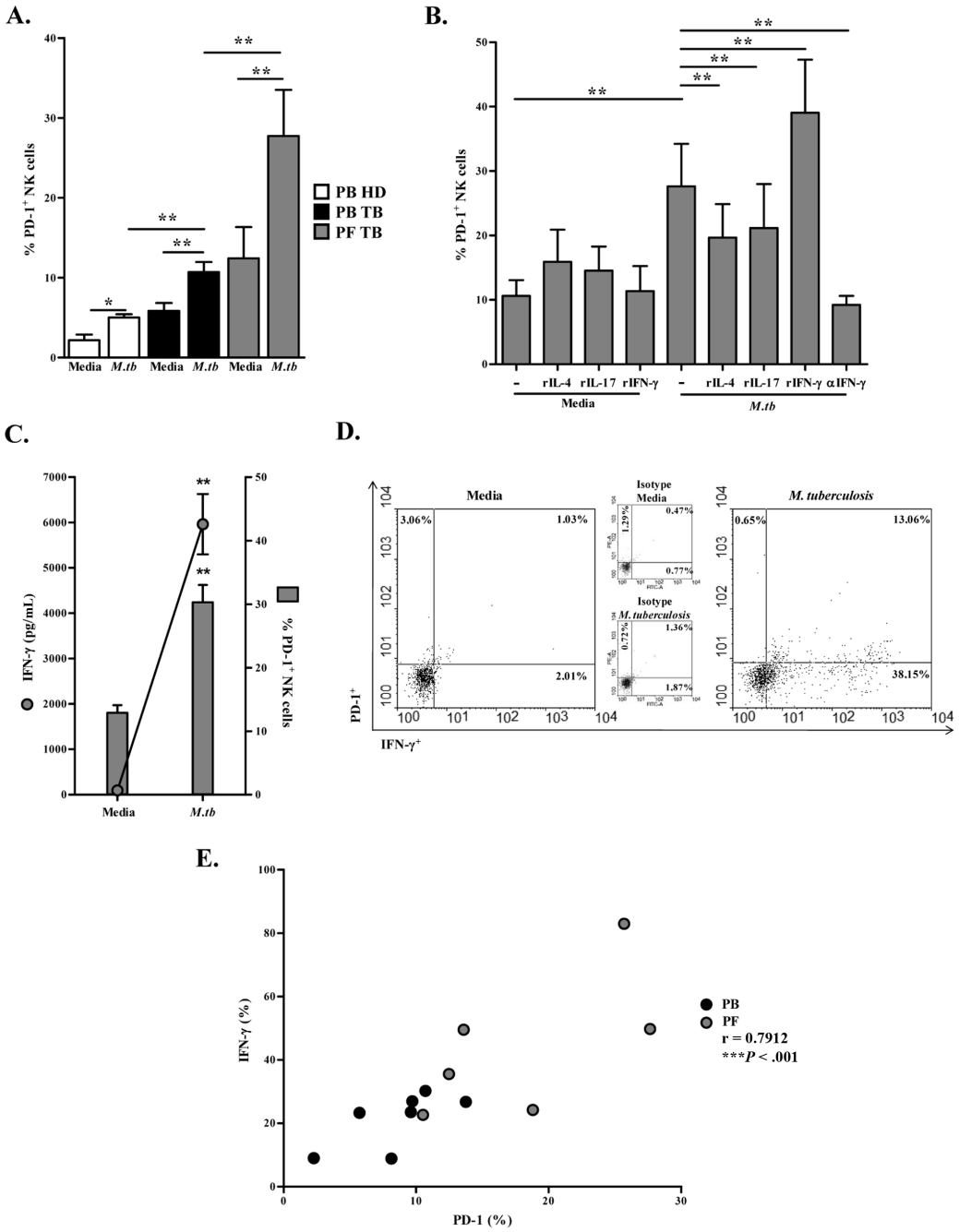

Figure 1. Expression of programmed death 1 (PD-1) on natural killer (NK) cells. *A*, Peripheral blood mononuclear cells (PBMCs) from patients with tuberculosis (TB) and healthy donors (HDs) and pleural fluid mononuclear cells (PFMCs) from TB were cultured with *Mycobacterium tuberculosis* (*M. tb*) for 24 h, and PD-1 expression on NK cells was determined by flow cytometry. Cells were first gated on lymphocytes by light scatter, then on $CD3^-$ cells, and finally on $CD56^+$ cells. The bars represent the mean ± standard error of mean (SEM) (HD, $n = 11$; PB TB, $n = 12$; PF, $n = 10$). *B*, PFMCs were stimulated with or without *M. tb* ± recombinant human interleukin 4 (rIL-4), recombinant IL 17 (rIL-17), recombinant interferon γ (IFN-γ) or *M. tb* + anti–IFN-γ (α-IFN-γ) for 24 h, and the expression of PD-1 on NK cells was determined by flow cytometry ($n = 8$). *C*, PFMCs were stimulated ± *M. tb* for 24 h, and IFN-γ production and PD-1 expression were evaluated by ELISA and flow cytometry, respectively. Bars and circles represent the mean ± SEM. *D*, Coexpression of PD-1 and IFN-γ on PF NK cells. PFMCs were stimulated ± *M. tb* for 24 h, and PD-1 expression on NK cells was determined by flow cytometry. Cells were first gated on lymphocytes by light scatter, then on $CD3^-$ cells, and finally on $CD56^{bright}$ NK cells. A representative dot plot of 1 of 7 patients is shown. *E*, Correlation between PD-1 and IFN-γ expression on $CD56^{bright}$ NK cells was determined by flow cytometry. Values are expressed as percentage of PD-1 or IFN-γ in *M. tb*–stimulated cells − % of PD-1 or IFN-γ cells cultured with media. Spearman coefficient, $r = 0.7912$, $^{***}P < .001$ (PB, $n = 7$; PF, $n = 7$). *A–E*, $^{*}P < .05$; $^{**}P < .01$, Wilcoxon rank sum test for paired samples and Mann-Whitney test for unpaired samples.

Table 1. Constitutive Programmed Death-1 (PD-1) and PD-1 Ligand (PD-L) Expression on CD56^{dim} and CD56^{bright} Natural Killer (NK) Cell Subpopulations

	% PD-1$^+$ NK cells		% PD-L1$^+$ NK cells		% PD-L2$^+$ NK cells	
Sample	CD56^{dim}	CD56^{bright}	CD56^{dim}	CD56^{bright}	CD56^{dim}	CD56^{bright}
PB HD	2.89 ± 1.30	8.29 ± 1.24	3.26 ± 1.16	8.72 ± 1.14	4.21 ± 0.65	1.03 ± 0.28
PB TB	2.94 ± 3.43	11.83 ± 3.37	3.57 ± 2.71	10.52 ± 2.34	4.38 ± 0.81	2.49 ± 0.71
PF TB	4.82 ± 2.49	17.61 ± 2.63	5.72 ± 4.19	14.01 ± 3.82	4.69 ± 1.05	1.98 ± 0.94

NOTE. HD, healthy donor; PB, peripheral blood; PF, pleural fluid; TB, tuberculosis.

pleural fluid NK cells (Figure 2*B* and 2*C*). Moreover, the expression of PD-L1 and PD-L2 was modulated by Th2 and Th17 cytokines, similar to our results on PD-1 (Figure 2*C*). In contrast, although PD-Ls expression was diminished after neutralizing endogenous IFN-γ, addition of this cytokine did not increase the levels of the PD-Ls (Figure 2*C*).

PD-1:PD-L interaction regulates NK cell effector functions in tuberculosis. We next sought to study whether the PD-1:PD-L pathway might participate in the regulation of NK cell effector functions. Initially, we analyzed PD-1:PD-Ls interactions on *M. tuberculosis*–induced NK cell degranulation. Then, PD-1 and/or the PD-Ls were blocked with specific mAbs and stimulated with or without *M. tuberculosis*. After 24 h, NK cell lytic degranulation was measured by analyzing CD107a expression. After *M. tuberculosis* stimulation, CD107a was mainly expressed on CD56^{dim} pleural fluid NK cells (data not shown). In peripheral blood, *M. tuberculosis* significantly enhanced the percentage of CD107a$^+$CD56^{dim} NK cells (Figure 3*A*). Blockade of PD-1 and/or PD-Ls significantly augmented the number of CD107a$^+$CD56^{dim} peripheral blood NK cells compared with *M. tuberculosis* alone (Figure 3*A*). However, in pleural fluid, stimulation of NK cells with *M. tuberculosis* led to a strong increase in the percentage of CD107a$^+$CD56^{dim} and CD107a$^+$CD56^{bright} NK cells, with significantly higher numbers of CD107a$^+$CD56^{dim} NK cells ($P < .01$; Mann-Whitney test; data not shown). Furthermore, blockade of PD-1, PD-Ls, or the complete pathway significantly augmented the percentage of CD56^{dim} NK cells expressing CD107a, whereas blocking the PD-1 pathway did not modify the percentage of CD107a$^+$CD56^{bright} pleural fluid NK cells (data not shown). In healthy donors, *M. tuberculosis* increased the percentage of CD107a$^+$CD56^{dim} NK cells, but the PD-1:PD-L interaction did not regulate lytic degranulation (data not shown). Thus, these results suggest that PD-1:PD-L interactions inhibit CD56^{dim} NK lytic cell degranulation against the pathogen.

We next investigated whether PD-1 and its ligands modulated the IFN-γ produced by NK cells after antigen stimulation. First, PBMCs or PFMCs from patients with tuberculosis were stimulated with *M. tuberculosis* in the presence or absence of anti–PD-1 or anti–PD-Ls mAbs. After 24 h, cell-free supernatants were collected and assayed for IFN-γ by ELISA. PFMCs produced significantly higher levels of IFN-γ after *M. tuberculosis* stimulation than did PBMCs from patients with tuberculosis (Figure 3*B*, $^{**}P < .01$; Mann-Whitney test), indicating a clear increase in the Th1 profile at the site of infection. Furthermore, the blockage of PD-1 and/or the whole pathway significantly increased the IFN-γ production by PBMCs and PFMCs (Figure 3*B*), denoting that PD-1:PD-L interactions inhibits the secretion of IFN-γ against *M. tuberculosis* in an early immune response. In view of these results, we next evaluated whether the blockage of the PD-1 pathway modulated IFN-γ expression in NK cells at a single cell level. We then analyzed the percentage of IFN-γ$^+$ NK cells in PBMCs and PFMCs from patients with tuberculosis after *M. tuberculosis* stimulation (Figure 3*C*). In accord with previous reports [6, 9, 14, 15], we observed that CD56^{bright} NK cells were the main source of IFN-γ production by PBMCs and PFMCs. Furthermore, we detected higher percentages of IFN-γ$^+$CD56^{bright} NK cells in PBMCs from patients with tuberculosis than in those from healthy donors (data not shown). Moreover, in PFMCs, CD56^{bright} IFN-γ–secreting NK cells were significantly elevated compared with peripheral blood ($^{**}P < .01$, Mann-Whitney test; data not shown). As shown in Figure 3*C*, an increase in the percentage of IFN-γ$^+$CD56^{bright} NK cells was observed by PD-1 blockage. Moreover, the simultaneous blockage of PD-L1 and PD-L2 or of PD-1 and its ligands also enhanced the percentage of IFN-γ$^+$CD56^{bright} cells after *M. tuberculosis* stimulation. Together, our data demonstrate that PD-1, through the interaction with PD-L1 and PD-L2, inhibited IFN-γ production by CD56^{bright} NK cells in tuberculosis.

In view of our results on the coexpression of PD-1 and IFN-γ, the correlation found between both molecules after *M. tuberculosis* stimulation on NK cells from patients with tuberculosis, and considering that the PD-1 pathway inhibited IFN-γ production by NK cells, we then analyzed the mean fluorescence intensity of IFN-γ$^+$ cells in PD-1$^+$ and PD-1$^-$CD56^{bright} NK cells. Our aim was to elucidate whether the inhibition of IFN-γ by PD-1 signaling would be the result of a direct signaling on the NK cell. Interestingly, our data showed a significant decrease of the IFN-γ$^+$ mean fluorescence intensity on PD-1$^+$CD56^{bright} NK cells compared with PD-1$^-$CD56^{bright} NK cells (Figure 3*D*). These results might be suggesting that the

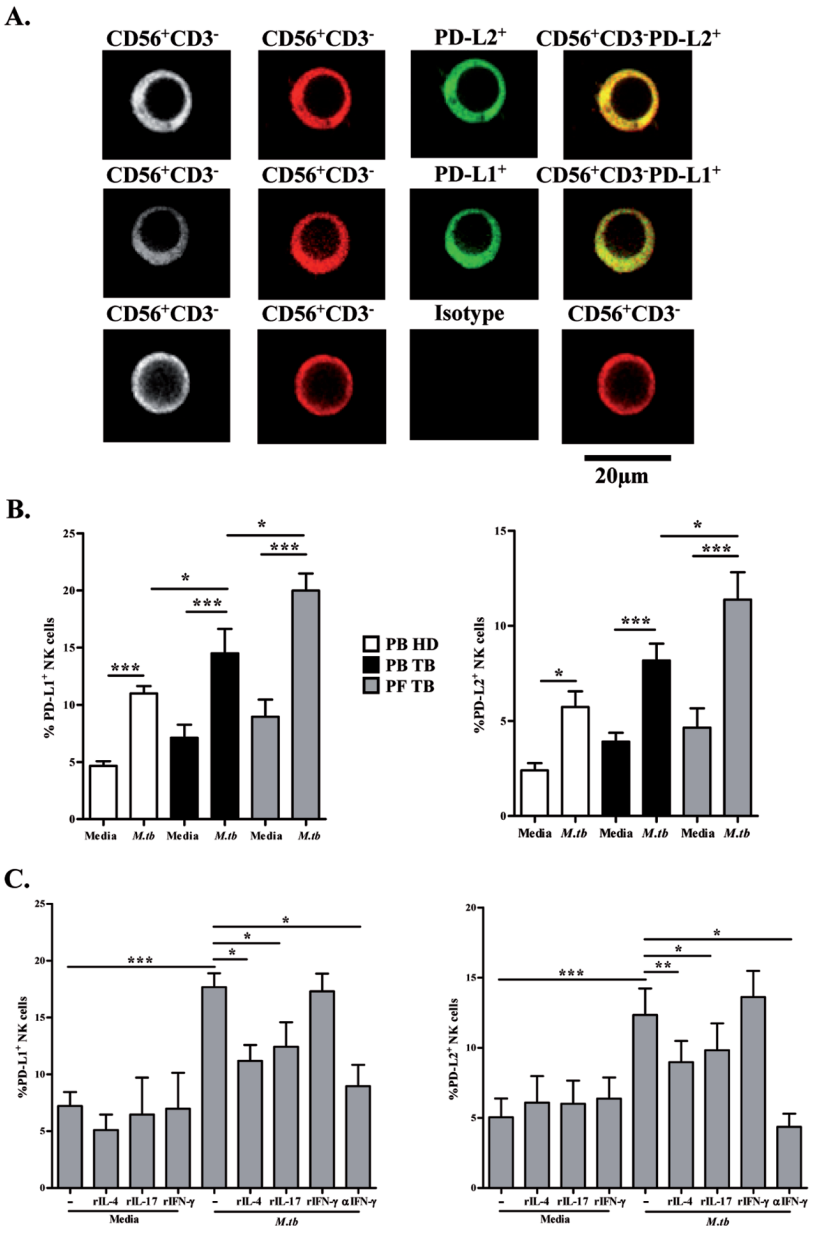

Figure 2. Expression of programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) on human natural killer (NK) cells. *A*, Constitutive coexpression of PD-L2/CD56 and PD-L1/CD56 on CD3/CD19/CD14–depleted NK cells was determined by confocal microscopy. PD-L2 or PD-L1 expression (*green*) was detected on $CD56^+$ (*red*) NK cells. The third column shows the colocalization (*yellow*) of CD56 with PD-L2 (*top row*) and PD-L1 (*middle row*). One representative patient of 6 is shown. The pictures show an augment of 60× (1.4) with a 2.5× zoom. *B*, Peripheral blood mononuclear cells (PBMCs) from patients with tuberculosis (TB) and healthy donors (HDs) and peripheral fluid mononuclear cells (PFMCs) from patients with tuberculosis (TB) were cultured with *Mycobacterium tuberculosis* (*M. tb*) for 24 h, and PD-L1 or PD-L2 expression on NK cells was determined by flow cytometry. Bars represent the mean ± standard error of mean (SEM) (PB HD, $n = 12$; PB TB, $n = 12$; PF TB, $n = 12$). *C*, PFMCs were stimulated with or without *M. tb* ± recombinant human interleukin 4 (rIL-4), recombinant interleukin 17 (IL-17), interferon γ (IFN-γ), or anti–IFN-γ (α-IFN-γ) for 24 h, and the expression of PD-Ls on NK cells was determined by flow cytometry ($n = 7$). $^*P < .05$; $^{**}P < .01$, $^{***}P < .001$. Wilcoxon rank sum test for paired samples; Mann-Whitney test for unpaired samples.

IFN-γ secreted by NK cells against *M. tuberculosis* up-regulates PD-1 expression on these cells, and then PD-1 would down-regulate IFN-γ production by NK cells in an autocrine way.

DISCUSSION

In recent years, it has become clear that NK cells are capable

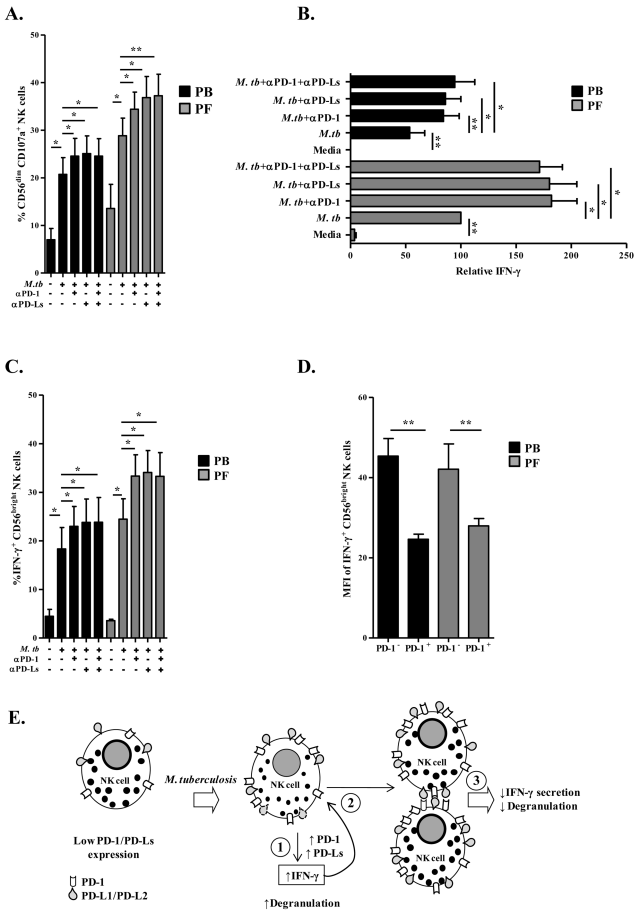

Figure 3. Signaling through the programmed death 1 (PD-1) pathway regulates degranulation and interferon γ (IFN-γ) production by *Mycobacterium tuberculosis*–stimulated natural killer (NK) cells. *A*, Peripheral blood mononuclear cells (PBMCs) and peripheral fluid mononuclear cells (PFMCs) from patients with tuberculosis were stimulated with *M. tuberculosis* (*M. tb*) ± blocking monoclonal antibodies (mAbs) anti–PD-1 (αPD-1), anti–PD-L1 (αPD-L1), and/or anti–PD-L2 (αPD-L2) for 24 h, and the expression of CD107a was determined by flow cytometry by gating on $CD56^{dim}$ NK cells. Each bar represents the mean ± standard error of mean (SEM) of the percentage of $CD3^{-}CD56^{+}CD107a^{+}CD56^{dim}$ NK cells (PB and PF, $n = 6$). *B*, PBMCs and PFMCs from patients with tuberculosis were stimulated ± *M. tb* ± blocking mAbs αPD-1 and/or αPD-Ls for 24 h, and IFN-γ production was measured by enzyme-linked immunosorbent assay (ELISA) (PB and PF, $n = 6$). Each bar shows the mean ± SEM of the production of IFN-γ by cells stimulated in each condition relative to the production by PFMCs stimulated with *M. tb*. The values were calculated as follows: [(pg/mL of IFN-γ secreted by PFMCs or PBMCs cultured in each condition)/(pg/mL of IFN-γ secreted by PFMCs cultured with *M. tb* alone)] × 100. *C*, PFMCs from patients with tuberculosis were stimulated ± *M. tb* ± αPD-1 or PD-Ls blocking mAbs for 24 h, and IFN-γ expression was determined by intracellular flow cytometry on $CD56^{bright}$ NK cells. Each bar represents the mean ± SEM of the percentage of $IFN\text{-}\gamma^{+}CD56^{bright}$ NK cells (PB and PF, $n = 7$). *D*, PBMCs and PFMCs were stimulated with *M. tb* for 24 h, and the coexpression of PD-1 and IFN-γ was determined by flow cytometry. The mean fluorescence intensity (MFI) of IFN-γ was determined for $PD\text{-}1^{+}$ and $PD\text{-}1^{-}$ populations by gating on $CD56^{bright}IFN\text{-}\gamma^{+}PD\text{-}1^{+}$ or $CD56^{bright}IFN\text{-}\gamma^{+}PD\text{-}1^{-}$ NK cells. * $P < .05$, ** $P < .01$ Wilcoxon rank sum test. *E*, Schematic diagram summarizing the role played by the PD-1:PD-L pathway on NK cell effector functions during human tuberculosis. (1) After *M. tuberculosis* stimulation, inhibitory and activating receptors are up-regulated on NK cells from peripheral blood and pleural effusions, including PD-1 and its ligands PD-L1 and PD-L2. In the presence of the pathogen, NK cells lyse *M. tuberculosis*–infected cells and produce large amounts of IFN-γ. (2) Then the secreted IFN-γ significantly increases the levels of PD-1 and the PD-Ls expression. (3) In turn, the interaction of PD-1:PD-L on NK cells and/or between NK cells and other cells expressing PD-1:PD-L inhibits the effector functions of NK cells, modulating the innate immune response of the host against the bacteria.

of mounting a vigorous antituberculosis protective response, but their exact function in vivo remains unclear. Moreover, whereas plenty of evidence supports the role played by PD-1 in negative regulation of adaptive immune responses [31], the function of PD-1 and its PD-Ls in human innate immunity remains poorly investigated. Here we investigated the role played by the PD-1:PD-L pathway during NK cell responses to *M. tuberculosis*.

We demonstrated that, after *M. tuberculosis* stimulation, the 3 receptors were expressed on peripheral blood and pleural fluid NK cells, but higher levels of the molecules were detected at the site of active disease. In contrast to PD-L1, the expression

of PD-L2 on human NK cells had not been previously reported. Although we observed low levels of PD-L2, several recognition receptors expressed at very low percentages on NK cells effectively modulate their activity [32]. Interestingly, we also showed that Th2 and Th17 cytokines—molecules known to diminish Th1 responses [31]—lead to a reduction in PD-1 levels of pleural fluid NK cells, whereas the IFN-γ present in the cellular microenvironment increases the expression of PD-1 on these cells, demonstrating that PD-1 expression can be modulated on NK cells by cytokines from the environment. Furthermore, not only most of the PD-1^{+}CD56^{bright} NK cells were IFN-γ^{+}, but a direct correlation between PD-1 and IFN-γ was also observed. Together, our data show that IFN-γ produced by NK cells after *M. tuberculosis* stimulation regulates the expression of PD-1. Pleural fluid NK cells from patients with tuberculosis display an activated phenotype and are major IFN-γ producers [15]. Thus, the potent IFN-γ response produced by NK cells against *M. tuberculosis* would generate a Th1 microenvironment that in turn would heighten the levels of PD-1.

To date, no direct evidence exists implicating PD-1 in the regulation of NK cell responses [20]. Therefore, we analyzed the PD-1:PD-L interactions on NK cell effector functions in tuberculosis. The cytotoxic activity of CD56^{dim} NK cells is significantly higher than that of CD56^{bright} cells [6, 33], and CD56^{dim} NK cells contain much more perforin, granzyme, and cytolytic granules [34]. Accordingly, we demonstrated that after *M. tuberculosis* stimulation, CD107a, a marker of NK and CTL degranulation [27], was mainly expressed on the CD56^{dim} subset of NK cells from both peripheral blood and pleural fluid, indicating that this subpopulation of cells displays a strong degranulation to *M. tuberculosis*. Interestingly, blockade of the PD-1:PD-L pathway significantly enhanced *M. tuberculosis*–induced CD107a expression on CD56^{dim} NK cells at the periphery and at the site of active disease. In fact, the present results on pleural fluid NK cells are in line with our recently published data demonstrating that the PD-1:PD-L pathway regulates the lytic activity of CD8 T cells in pleural effusions [24].

The early production of IFN-γ by cells of the innate immune response within the inflammatory site regulates innate resistance and shifts adaptive immunity toward a Th1 response [35, 36]. Moreover, a protective role played by NK cell-derived IFN-γ in *M. tuberculosis* infection was recently addressed in a T cell–deficient mouse model [37]. Thus, we evaluated the role played by PD-1 and its ligands on IFN-γ production by NK cells against *M. tuberculosis*. Among the 2 subpopulations of NK cells, CD56^{bright} cells are the most efficient cytokine producers [6]. In accord with previous reports [15], we found that circulating and pleural CD56^{bright} NK cells were the main producers of the IFN-γ secreted against *M. tuberculosis*. When we blocked PD-1, the PD-Ls, or the complete pathway, we detected a significant augment in IFN-γ levels and in the number of IFN-γ^{+}CD56^{bright} NK cells, indicating that the interaction of PD-1 with its ligands would be a negative regulator of IFN-γ production.

In summary, we demonstrated that *M. tuberculosis* modulated the PD-1 and the PD-L expression on NK cells from patients with tuberculosis at the periphery and at the site of infection. Furthermore, the inhibition of IFN-γ down-regulated the expression of PD-1 on NK cells from patients with tuberculosis. In addition, we showed that the PD-1 pathway inhibited NK cell effector functions during active tuberculosis. Moreover, PD-1^{+} NK cells displayed a diminished IFN-γ mean fluorescence intensity, highlighting the relevance of PD-1 on the regulation of IFN-γ secretion by NK cells. Thus, as shown in Figure 3*E*, we propose that *M. tuberculosis* stimulation would up-regulate PD-1 and its PD-Ls on NK cells and would induce them to lyse infected cells and to secrete IFN-γ. This IFN-γ would then lead to a higher increase in the expression of PD-1 and PD-Ls. Subsequently, PD-1:PD-L interaction between NK cells and/or between NK cells and other cells expressing PD-1 or the PD-Ls would inhibit the effector functions of NK cells, modulating the innate immune response of the host against the bacteria (Figure 3*E*).

An important aspect of our study was the ability to investigate the expression and role played by PD-1 at the site of a human infection. Furthermore, the present data contribute with significant information about the role played by PD-1:PD-L interaction, a well-recognized inhibitory pathway of adaptive immune responses, on the innate immunity against *M. tuberculosis*. Tuberculous pleurisy is an intense immune response to mycobacteria that results in the clearance of organisms from the pleural space. Therefore, on the basis of our findings, we hypothesize that PD-1 signaling might be modulating the innate immunity by inhibiting NK cell responses to the pathogen, contributing to avoidance of immune-mediated pathology caused by excessive host response to the infection (Figure 3*E*).

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