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Mycobacterium tuberculosis-Induced Gamma Interferon Production by Natural Killer Cells Requires Cross Talk with Antigen-Presenting Cells Involving Toll-Like Receptors 2 and 4 and the Mannose Receptor in Tuberculous Pleurisy▽

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Tuberculous pleurisy allows the study of human cells at the site of active *Mycobacterium tuberculosis* infection. In this study, we found that among pleural fluid (PF) lymphocytes, natural killer (NK) cells are a major source of early gamma interferon (IFN-γ) upon *M. tuberculosis* stimulation, leading us to investigate the mechanisms and molecules involved in this process. We show that the whole bacterium is the best inducer of IFN-γ, although a high-molecular-weight fraction of culture filtrate proteins from *M. tuberculosis* H37Rv and the whole-cell lysate also induce its expression. The mannose receptor seems to mediate the inhibitory effect of mannosylated lipoarabinomannan, and Toll-like receptor 2 and 4 agonists activate NK cells but do not induce IFN-γ like *M. tuberculosis* does. Antigen-presenting cells (APC) and NK cells bind *M. tuberculosis*, and although interleukin-12 is required, it is not sufficient to induce IFN-γ expression, indicating that NK cell-APC contact takes place. Indeed, major histocompatibility complex class I, adhesion, and costimulatory molecules as well as NK receptors regulate IFN-γ induction. The signaling pathway is partially inhibited by dexamethasone and sensitive to Ca^{2+} flux and cyclosporine. Inhibition of p38 and extracellular-regulated kinase mitogen-activated protein kinase pathways reduces the number of IFN-γ^+ NK cells. Phosphorylated p38 (p-p38) is detected in ex vivo PF-NK cells, and *M. tuberculosis* triggers p-p38 in PF-NK cells at the same time that binding between NK and *M. tuberculosis* reaches its maximum value. Thus, interplay between *M. tuberculosis* and NK cells/APC triggering IFN-γ would be expected to play a beneficial role in tuberculous pleurisy by helping to maintain a type 1 profile.

Natural killer (NK) cells are $CD56^+$ $CD16^{+/-}$ $CD3^-$ lymphocytes that are critical components of the innate immune system and have a number of effector functions, including recognition and lysis of infected, stressed, or transformed cells and production of cytokines, particularly gamma interferon (IFN-γ) (11). Their activity is regulated by activating and inhibitory signals from a wide range of cell surface receptors (27, 45). Two subsets of NK cells have been identified in humans according to their CD56 expression. They differ in phenotype and function and also in terms of chemokine receptors and adhesion molecule expression, suggesting that they have different homing properties (7, 19). Indeed, $CD56^{bright}$ NK cells have been found to be enriched in human secondary lymphoid organs and in chronic inflammatory sites (13, 15, 17, 40). Functionally, $CD56^{bright}$ NK cells are more effective at producing cytokines in response to monokine costimulation, while $CD56^{dim}$ NK cells are efficient effectors of natural and antibody-dependent target cell lysis (11, 49).

IFN-γ production is an early and critical event in the immune response to intracellular pathogens because, as a pleiotropic cytokine, IFN-γ influences both the innate and acquired arms of the immune response. At early time points following infection, the expression of IFN-γ is detected mainly in $CD56^{bright}$ NK cells via the secretion of proinflammatory monokines, such as interleukin-12 (IL-12), IL-15, and IL-18, by professional antigen-presenting cells (APC) (8, 24), whose functional receptor complexes are constitutively expressed by NK cells (15, 48). Macrophages, dendritic cells, and some epithelial cells recognize pathogen-associated molecular patterns (PAMPs) expressed by microorganisms through cell surface receptors (pattern recognition receptors [PRRs]) involved in endocytosis or in cell activation, such as the Toll-like receptors (TLRs) and C-type lectins (10, 37, 42, 52). Interestingly, recent evidence suggests that NK cells can recognize microorganisms by themselves and can be activated directly by PRRs (9, 36, 41).

Among the many clinical manifestations of tuberculosis (TB), pleuritis is of particular interest since it may be resolved without therapy and patients are known to undergo a relatively effective immune response against *Mycobacterium tuberculosis*. Tuberculous pleuritis is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of *M. tuberculosis* infection, but it may also be developed as a complication of primary pulmonary TB (3, 28). We recently demonstrated that in tuberculous pleurisy, $CD56^{bright}$ NK cells are activated and, upon *M. tuberculosis* stimulation, produce large amounts of IFN-γ without the need for acces-

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sory $CD3^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes/macrophages (40). In line with this, a protective role of NK cell-derived IFN-γ in *M. tuberculosis* infection was recently addressed in a T-cell-deficient mouse model (16). However, the signaling pathways triggered by *M. tuberculosis* in human NK cells have not yet been studied in a relevant physiological system (29).

In the present study, we describe that despite the reduced frequency of NK cells in tuberculous pleurisy, these cells are a major source of IFN-γ. Therefore, we have investigated regulatory signals and intracellular pathways accountable for *M. tuberculosis*-induced IFN-γ production in NK cells from tuberculous pleurisy. Herein we show that pleural fluid NK cells (PF-NK cells) are able to directly recognize *M. tuberculosis* antigens and to be activated to produce IFN-γ in a mechanism enhanced by IL-12, dependent on calcineurin, p38, and extracellular signal-regulated kinase (ERK) pathways, and modulated by extracellular signals coming from PRRs and NK cell-APC cross talk.

MATERIALS AND METHODS

Patients. Patients with newly diagnosed moderate and large pleural effusions were identified at the Servicio de Tisioneumonología, Hospital F. J. Muñiz, Buenos Aires, Argentina. Informed consent was obtained from patients according to the Ethics Commission of the Hospital F. J. Muñiz. Patients were evaluated by history, physical examination, complete blood cell count, determination of electrolyte levels, chest X-ray, and human immunodeficiency virus testing. PF and peripheral blood (PB) were obtained from the patients during diagnostic thoracentesis before the initiation of chemotherapy. Exclusion criteria included a positive test for human immunodeficiency virus and the presence of concurrent infectious diseases. A total of 40 patients with tuberculous PF were studied (average age, 32 years; range, 20 to 65 years). Among patients with pleural effusions, 24 had pulmonary disease, which was classified according to the extent and type of chest X-ray findings as moderate ($n = 15$), advanced ($n = 7$), or miliary ($n = 2$), and the remaining 16 patients did not have pulmonary disease. The effusions were classified as exudates according to at least one of the criteria of Light et al. (28). No correlation was found between *M. tuberculosis*-induced IFN-γ^+ PF-NK cells or IL-12 secretion and clinical classification, as we have previously reported for a smaller sample (40).

Thoracentesis and mononuclear cells. PF was collected by therapeutic thoracentesis. Briefly, following local anesthesia of the skin and subcutaneous tissue, 100 ml of PF was aspirated under sterile conditions, using an 18-gauge needle (Abrams). Biochemical analysis, bacterial cultures, and cytologic examinations were performed on all PF samples in the Central Laboratory of Hospital F. J. Muñiz. A second sample of the PF was dispensed into a 50-ml polystyrene tube (Corning, NY) containing heparin. Peripheral blood mononuclear cells (PBMC) and PF mononuclear cells (PFMC) were isolated from heparinized blood and PF by Ficoll-Hypaque gradient centrifugation and suspended in RPMI 1640 tissue culture medium (Gibco Laboratories, NY) containing gentamicin (85 μg/ml) and 10% heat-inactivated fetal calf serum (FCS; Gibco Laboratories) (complete medium). Purity and viability were tested using trypan blue exclusion.

Isolation of NK cells from PFMC. To isolate NK cells in APC depletion experiments (see Fig. 6), PFMC (10×10^6 to 20×10^6 cells in a pellet) were treated with anti-CD3 hybridoma culture supernatant (145-2C) and purified anti-CD20 (Ancell Corp., Bayport, MN) monoclonal antibody (MAb) to remove T and B cells, respectively, and with anti-CD14 (Immunotech, Marseille, France), -CD1a, -CD1b, -CD1c (Ancell Corp., Bayport, MN), and -dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) (R&D Systems Inc., Minneapolis, MN) to remove APC populations (monocytes, macrophages, and DC). PFMC were incubated for 30 min at 4°C and washed, and then goat anti-mouse immunoglobulin G (IgG)-coated beads (Dynal, Oslo, Norway) were added. In general, one cycle of treatment was sufficient for effective depletion (<5% $CD3^+$ cells and <0.05% APC), as assessed by flow cytometry.

Antigens. The γ-irradiated *M. tuberculosis* H37Rv strain and mannosylated lipoarabinomannan (ManLAM), peptidoglycan (PGN), whole-cell lysate (WCL), and culture filtrate protein (CFP) from *M. tuberculosis* H37Rv were kindly provided by J. Belisle (Colorado State University, Fort Collins, CO). Mycobacteria were suspended in pyrogen-free phosphate-buffered saline (PBS), sonicated, and adjusted to a concentration of $\approx 1 \times 10^8$ bacteria/ml (optical density at 600 nm = 1). The protein content of $\approx 1 \times 10^6$ bacteria used to stimulate PFMC was 86 ± 8 ng (Bradford assay; Sigma Chemical Co., St. Louis, MO). The synthetic lipoprotein Pam_3Cys-SK4 (Pam_3Cys) and *Escherichia coli* O111:B4 lipopolysaccharide (LPS) were purchased from Sigma. To rule out LPS contamination of *M. tuberculosis* preparations, polymyxin B (0.1 to 1 μg/ml; Sigma) was employed in control experiments (data not shown). For molecular size fractionation of CFP, aliquots of 0.5 ml of 1-mg/ml protein were subjected to centrifugation at $10{,}000 \times g$ for 10 min at 4°C in an Ultrafree-0.5 centrifugal filter device following the manufacturer's instructions (Millipore, MA). The nominal molecular weight (NMW) limits used were 10, 30, 50, and 100. At least two independent fractionation procedures for each NMW product were analyzed for NK cell stimulation and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%) band pattern, with the same results (data not shown).

PFMC cultures and treatments. PFMC (1×10^6 cells/ml) were incubated in Falcon 2063 tubes (Becton Dickinson, Lincoln, NJ) for 18 to 24 h at 37°C in a humidified 5% CO_2 atmosphere in complete medium, with or without *M. tuberculosis* (1×10^6 bacteria/ml; PFMC/*M. tuberculosis* ratio, 1:1), Pam_3Cys (100 ng/ml), LPS (1 μg/ml), ManLAM (0.1 μg/ml), PGN (50 ng/ml), and different amounts of WCL and CFP. To determine the role of monokines, a neutralizing MAb to IL-12, IL-15, IL-18 (1 μg/ml), or recombinant human IL-12 (rhIL-12; 10 ng/ml) (Peprotech, Rocky Hill, NJ) was added. Pharmacological inhibition of the *M. tuberculosis*-induced response was performed by pretreatment of PFMC for 30 min at 37°C with the following drugs before culture: SB203580 (20 μM), PD98059 (50 μM), wortmannin (200 nM), JUN1 (200 nM) (all from Calbiochem-Behring, La Jolla, CA), dexamethasone (Dex; 1 μM), cyclosporine (CsA) (500 ng/ml), EDTA (5 mM), and EGTA (1 mM) (all from Sigma). When required, a 1:1,000 dilution of dimethyl sulfoxide (DMSO; Mallinckrodt Baker, Phillipsburg, NJ) was employed as a vehicle control. Blockade of surface molecules was done at room temperature for 30 min with cell pellets prior to antigen stimulation, and the specific MAb or isotype-matched controls were present throughout the subsequent culture period. Functional-grade purified MAbs (5 μg/ml) against the following molecules were employed: CD2 (RPA-2.10), TLR2 (TL2.1), TLR4 (HTA125), CD54/ICAM-1 (HA58), CD86 (IT2.2) (all from *e*Bioscience, San Diego, CA), NKG2D (1D11; Becton Dickinson, Mountain View, CA), DC-SIGN (120507; R&D), CD6 (Tü33; Clonab, Germany), CD161 (B199.2; Ancell), and mannose receptor (MR) (15-2; HyCutl Biotechnology, The Netherlands). Hybridoma supernatants (30 μg/ml) against major histocompatibility complex class I (MHC-I; W6/32) and CD16 (3G8) were also used.

Immunofluorescence analysis. (i) Expression of surface markers on $CD3^-$ $CD56^+$ lymphocytes and APC. To evaluate the expression of surface markers on freshly isolated or cultured PBMC and PFMC, fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and Cy5-PE-labeled MAbs against CD3, CD56, CD69, CD14, CD86 (*e*Bioscience), CD56 (Immunotech, Marseille, France), and HLA-II (DR/DQ/DP; Ancell) and isotype-matched MAbs were used. Stained cells were analyzed in a FACScan cytometer using Cellquest software (Becton Dickinson) by acquiring 30,000 to 80,000 events. Analysis gates were set on mononuclear phagocytes and on lymphocytes according to their forward- and side-scatter properties. NK cells were defined as $CD3^-$ $CD56^+$ lymphocytes, and APC were defined as mononuclear phagocytes with high expression of HLA-II. Surface expression of molecules analyzed in blocking experiments is shown in Table A1.

(ii) Intracytoplasmic detection of IFN-γ on NK cells. PFMC (1×10^6 cells/ml) were cultured for 24 h with or without *M. tuberculosis* antigens, with the addition of brefeldin A (5 μg/ml; Sigma) for the final 4 h, washed, and stained with anti-CD3 and anti-CD56 MAbs for 20 min at 4°C. Thereafter, the cells were fixed according to the manufacturer's instructions (IntraPrep; Immunotech), washed with PBS supplemented with 1% FCS and 0.01% azide (PBS-FCS-azide), and suspended in 100 μl of PBS-FCS-azide. FITC–anti-IFN-γ (Caltag, Burlingame, CA) was added together with 100 μl of permeabilizing solution (IntraPrep), and the cells were incubated for 30 min at 4°C, washed with PBS-FCS-azide, suspended in Isoflow (Becton Dickinson), and analyzed by flow cytometry. Given the variability among samples, results were expressed as relative percentages of IFN-γ-positive cells among the $CD3^-$ $CD56^+$ cells, as follows: relative % NK IFN-γ^+ cells = % IFN-γ^+ NK cells (PFMC control or different treatments) × 100/% IFN-γ^+ NK cells (PFMC + *M. tuberculosis*).

(iii) Intracytoplasmic detection of IL-12 on APC. PFMC (1×10^6 cells/ml) were cultured with or without *M. tuberculosis* for 24 h, with the addition of brefeldin A (5 μg/ml; Sigma) for the final 6 h, and the intracellular expression of IL-12 (PE–anti-IL-$12_{p40/p70}$/IL-23_{p40}, IgG1 κ chain; *e*-Bioscience) was determined as described above. The analysis gate was set on mononuclear

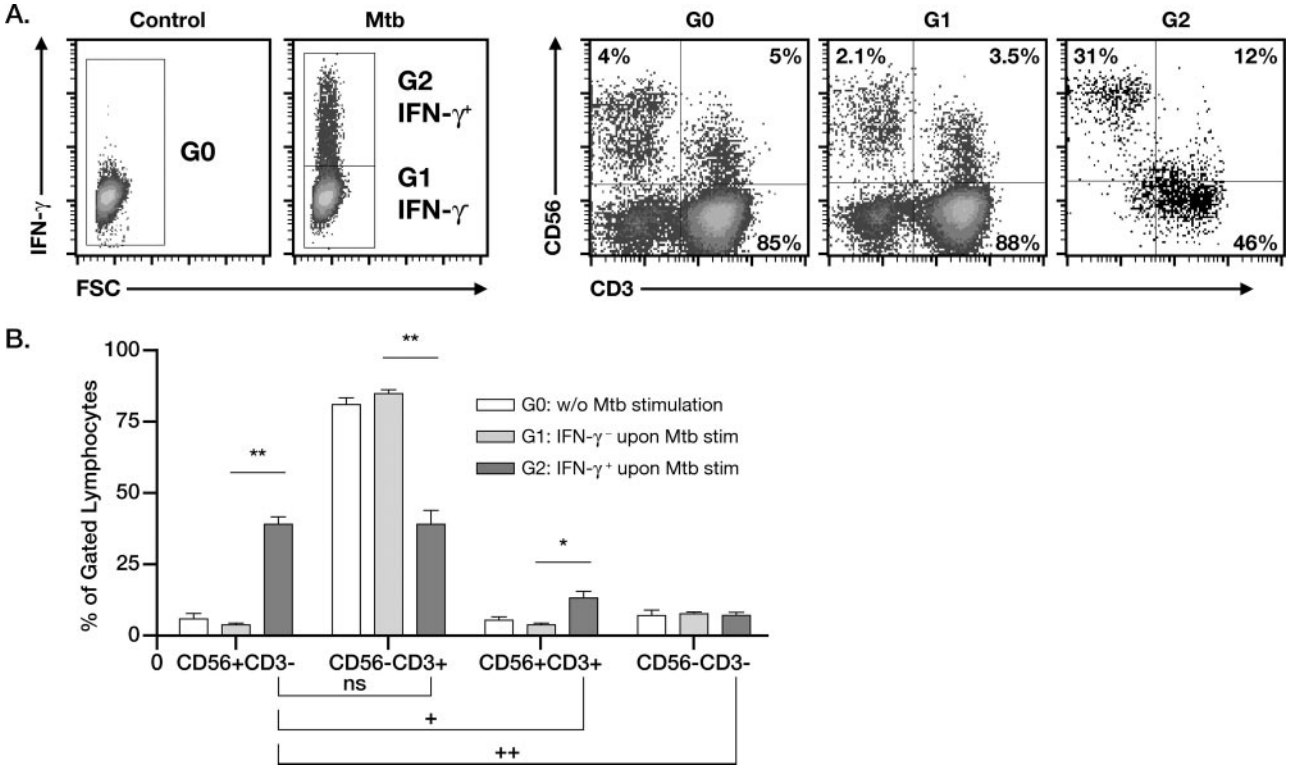

FIG. 1. NK cells are a major source of IFN-γ among PFMC. (A and B) PFMC were stimulated or not with *M. tuberculosis* for 24 h. After that, intracellular expression of IFN-γ was determined among lymphocyte populations (A, left panel). Three gates were defined as follows: G0, unstimulated lymphocytes; G1, *M. tuberculosis*-stimulated IFN-γ-negative lymphocytes; and G2, *M. tuberculosis*-stimulated IFN-γ-positive lymphocytes. The cell phenotype of each gate was analyzed according to CD56 and CD3 expression (A, right panel). (A) Representative analysis. (B) Percentages of gated lymphocytes (means ± standard errors of the means [SEM]). *, $P < 0.01$; **, $P < 0.001$ (G2 versus G1). ns, not significant; +, $P < 0.01$; ++, $P < 0.0005$ ($G2_{NK}$ versus $G2_{other\ phenotypes}$) ($n = 10$). Mtb, *M. tuberculosis*.

phagocytes according to their forward- and side-scatter properties. Results are expressed as percentages of positive cells.

(iv) Determination of p38 mitogen-activated protein kinase (MAPK) activation. The phosphorylated form of p38 (p-p38) was measured in PF-NK cells as follows. PFMC (1×10^6/ml) were incubated alone or with *M. tuberculosis* for 15 min to 2 h. Thereafter, cells were placed on ice, fixed with 1% paraformaldehyde for 15 min, and washed twice with cold PBS. Fixed cells were stained with PE–anti-CD56 and Cy5-PE–anti-CD3 as previously indicated. After that, intracellular staining with phospho-specific anti-p-p38–FITC (Santa Cruz Biotechnology, Santa Cruz, CA) was performed as indicated above. An isotype-matched IgM control was used to determine nonspecific staining. Anisomycin (1 μM; Sigma) (15 min at 37°C) was employed as a positive control of p38 tyrosine phosphorylation. Results are expressed as relative median fluorescence intensities (MFIs) with respect to that of basal control cells (time zero).

IL-12 ELISA. IL-12 secretion was measured by enzyme-linked immunosorbent assay (ELISA), employing a commercial kit according to the manufacturer's instructions (Peprotech). Supernatants from 2×10^6 PFMC/ml, stimulated or not with *M. tuberculosis* for 18 h, were analyzed without dilution. The sensitivity of the assay was 32 pg/ml. For correlation analysis, the percentage of APC present in each PF sample ($n = 7$) was determined by flow cytometry as indicated above.

Detection of *M. tuberculosis*–NK-cell binding. The γ-irradiated *M. tuberculosis* H37Rv strain was covalently stained with FITC. Briefly, 1×10^9 bacteria were washed twice with 0.1 M $NaCO_3$ (pH 9) and resuspended in 1 ml of the same solution. FITC (isomer I; Sigma) dissolved in DMSO (20 mg/ml) was added to a final concentration of 1 mg/ml and incubated in the dark for 2 h at 37°C. Bacteria were washed gently until unbound colorant was eliminated, and labeling was confirmed by flow cytometry and fluorescence microscopy. To detect *M. tuberculosis*–NK-cell conjugates, 1×10^6 *M. tuberculosis*-FITC cells were added to 1×10^6 PBMC or PFMC and centrifuged for 1 min at $500 \times g$ to favor cell contact. Thereafter, cells were incubated for 5 min to 2 h, and 2% paraformaldehyde was added to fix conjugates as previously described (4). Binding was determined by flow cytometry of gated $CD3^-$ $CD56^+$ cells. To rule out nonspecific binding, competition experiments were performed in the presence of 10^7 unlabeled *M. tuberculosis* cells together with 10^6 *M. tuberculosis*-FITC cells. A reduction of NK-cell–*M. tuberculosis*–FITC conjugates was observed in the presence of a 10-fold excess of unlabeled *M. tuberculosis*, which is consistent with specific binding. The formation of NK-cell ($CD56^+$)–*M. tuberculosis*–FITC conjugates was confirmed by indirect immunofluorescence microscopy examination of cytospin preparations (data not shown).

Statistical analysis. Comparisons of paired PB and PF samples and of different treatments of the same sample were performed using the Wilcoxon (nonparametric) paired test. The Spearman two-tailed (nonparametric) test was used for correlation analysis. *P* values of <0.05 were assumed to be significant.

RESULTS

PF-NK cells are a major source of IFN-γ after short times of *M. tuberculosis* stimulation. We have previously shown that *M. tuberculosis* induces strong IFN-γ production in PF-NK cells (40). Given the remarkable predominance of T lymphocytes observed in tuberculous pleural effusions (~80 to 90%), we wanted to determine the contribution of lymphocyte subsets to IFN-γ production upon *M. tuberculosis* stimulation. To this end, PFMC were stimulated with or without *M. tuberculosis* for 24 h, and then the phenotypes of lymphocytes among IFN-γ-positive and -negative gates were determined. As shown in Fig. 1A and B, although $CD3^+$ $CD56^-$ T cells were the most abundant subset within the control (G0) or *M. tuberculosis*-stimulated IFN-γ^- (G1) gate, $CD3^-$ $CD56^+$ NK cells were

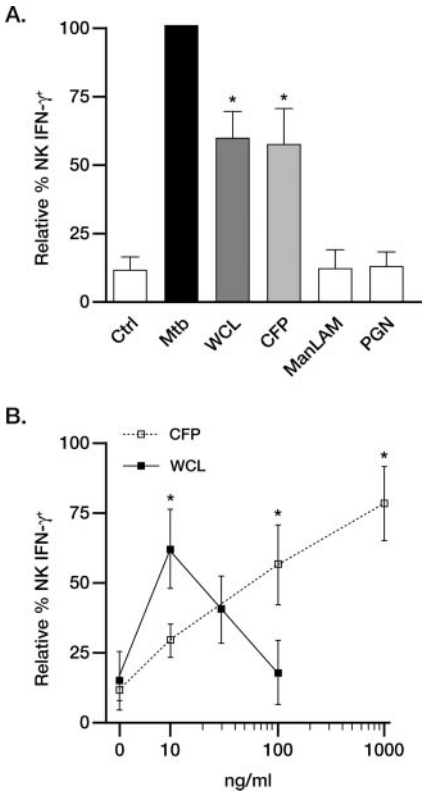

FIG. 2. WCL and CFP induce IFN-γ production by PF-NK cells. PFMC were stimulated with *M. tuberculosis*-derived antigens for 24 h, and the intracellular expression of IFN-γ was determined on gated $CD3^-$ $CD56^+$ cells. (A) Data for medium alone (Ctrl), *M. tuberculosis* (*M. tuberculosis*/PFMC ratio, 1:1; 10^6 *M. tuberculosis* cells ≈ 90 ng), WCL (10 ng/ml), CFP (100 ng/ml), ManLAM (100 ng/ml), and *M. tuberculosis*-derived PGN (50 ng/ml). Relative percentages of IFN-γ^+ NK cells are shown (means ± SEM). *, $P < 0.05$ (treatment versus control) ($n = 6$). (B) PFMC stimulated with different amounts of WCL (■) or CFP (□). Relative percentages of IFN-γ^+ NK cells (means ± SEM) are shown. *, $P < 0.05$ (treatment versus control) ($n = 4$).

one of the most frequent populations in the IFN-γ^+ gate (G2). This result shows that NK cells represent a major source of IFN-γ after short times of in vitro *M. tuberculosis* stimulation, suggesting an important role in the immunity of tuberculous pleurisy.

Whole *M. tuberculosis* induces the highest expression level of IFN-γ in PF-NK cells. To discern whether bacterial components can also trigger IFN-γ production by PF-NK cells, PFMC were stimulated with *M. tuberculosis*-derived antigens, including WCL, which includes the cellular wall, cytosol, and membranes; CFP, which includes most of the excreted/secreted proteins; and the mycobacterial cell wall components PGN and ManLAM. PFMC were incubated with the antigens for 24 h, and the percentage of IFN-γ^+ NK cells was determined by flow cytometry. Among the tested *M. tuberculosis* antigens, significant levels of IFN-γ^+ cells were observed by stimulation with WCL and CFP, but neither ManLAM nor PGN induced IFN-γ^+ NK cells over the level for control cells (Fig. 2A). In addition, neither WCL nor CFP was a better inducer of IFN-γ than whole *M. tuberculosis*. To investigate whether the production of IFN-γ was related to the amounts of antigens, PFMC were stimulated with different WCL and CFP concentrations. As shown in Fig. 2B, higher concentrations of WCL inhibited IFN-γ^+ PF-NK cells, and in contrast, larger amounts of CFP induced higher percentages of PF-NK cells expressing IFN-γ.

To evaluate whether the inhibitory effect observed with high doses of WCL on IFN-γ^+ PF-NK cells could be mediated by the major *M. tuberculosis* cell wall glycolipid, ManLAM, PFMC were preincubated with ManLAM for 30 min at 37°C, and thereafter, *M. tuberculosis* was added for 24 h and activation was evaluated in terms of CD69 expression and IFN-γ^+ PF-NK cells. As shown in Fig. 3A and B, pretreatment with ManLAM inhibited the activation and the number of *M. tuberculosis*-induced IFN-γ^+ PF-NK cells. In addition, to discern whether low- or high-molecular-mass proteins present in H37Rv CFP could induce IFN-γ^+, CFP was fractionated by employing centrifugal filter units with different molecular size cutoffs, and the upper and lower fractions were employed to stimulate PFMC. As shown in Fig. 3C and D, CFP proteins (or native protein complexes) with molecular masses above 50 kDa induced IFN-γ in PF-NK cells, while within the same samples, proteins/complexes below this molecular mass were better inducers of IFN-γ production in $CD3^+$ T cells (data not shown).

Involvement of PRRs in *M. tuberculosis*-induced IFN-γ production. We previously found that $CD14^+$ cells were not involved in the production of IFN-γ by PF-NK cells (40). To characterize APC within cells from PF, PFMC were stained with anti-MHC class II, -CD14, and -CD86 surface markers. The APC population was defined by forward- and side-scatter properties and MHC class II^{high} expression. We determined that 2.74% ± 1.5% of cells could be considered APC and that, among them, 43% ± 10% were $CD14^+$ and 5.3% ± 3.2% were $CD14^-$ $CD86^+$ ($n = 10$) (data not shown). It is well known that *M. tuberculosis* is recognized by different PRRs on APC (52). Among them, MR and DC-SIGN have been associated with *M. tuberculosis* recognition in bronchoalveolar lavage fluid and lymph nodes from TB patients (46, 47), and furthermore, we have also observed an increased percentage of PF-APC expressing DC-SIGN (see Table A1). In addition, functional relevance for TLR2 and -4 in *M. tuberculosis* recognition by neutrophils and mononuclear phagocytes has also been observed (1, 2, 23). Moreover, recent reports have demonstrated that viral and bacterial PAMPs can directly activate human NK cells to produce IFN-γ via several TLRs (9, 36, 41).

To evaluate the role of PRRs involved in the production of IFN-γ by PF-NK cells, blocking assays were performed by preincubation of PFMC with purified anti-DC-SIGN, anti-MR, anti-TLR2, and/or anti-TLR4; thereafter, cells were stimulated or not with *M. tuberculosis*, and numbers of $CD69^+$ and IFN-γ^+ NK cells were determined. The expression of PRRs is shown in Table A1. As shown in Fig. 4A, although the blockade of DC-SIGN did not modify IFN-γ expression, a small increase was found when MR was blocked. In addition, inhibition of IFN-γ^+ NK cells was observed when TLR2 and, more significantly, TLR4 were blocked. Hence, we evaluated whether specific TLR2 (Pam_3Cys) and TLR4 (LPS) agonists were able to induce IFN-γ and CD69 expression in PF-NK cells. As shown in Fig. 4B and C, activation of PF-NK cells, as measured by CD69 expression, was observed upon Pam_3Cys and LPS stimulation, but while Pam_3Cys did not induce IFN-γ, LPS was not as good at inducing *M. tuberculosis*. Notably, while

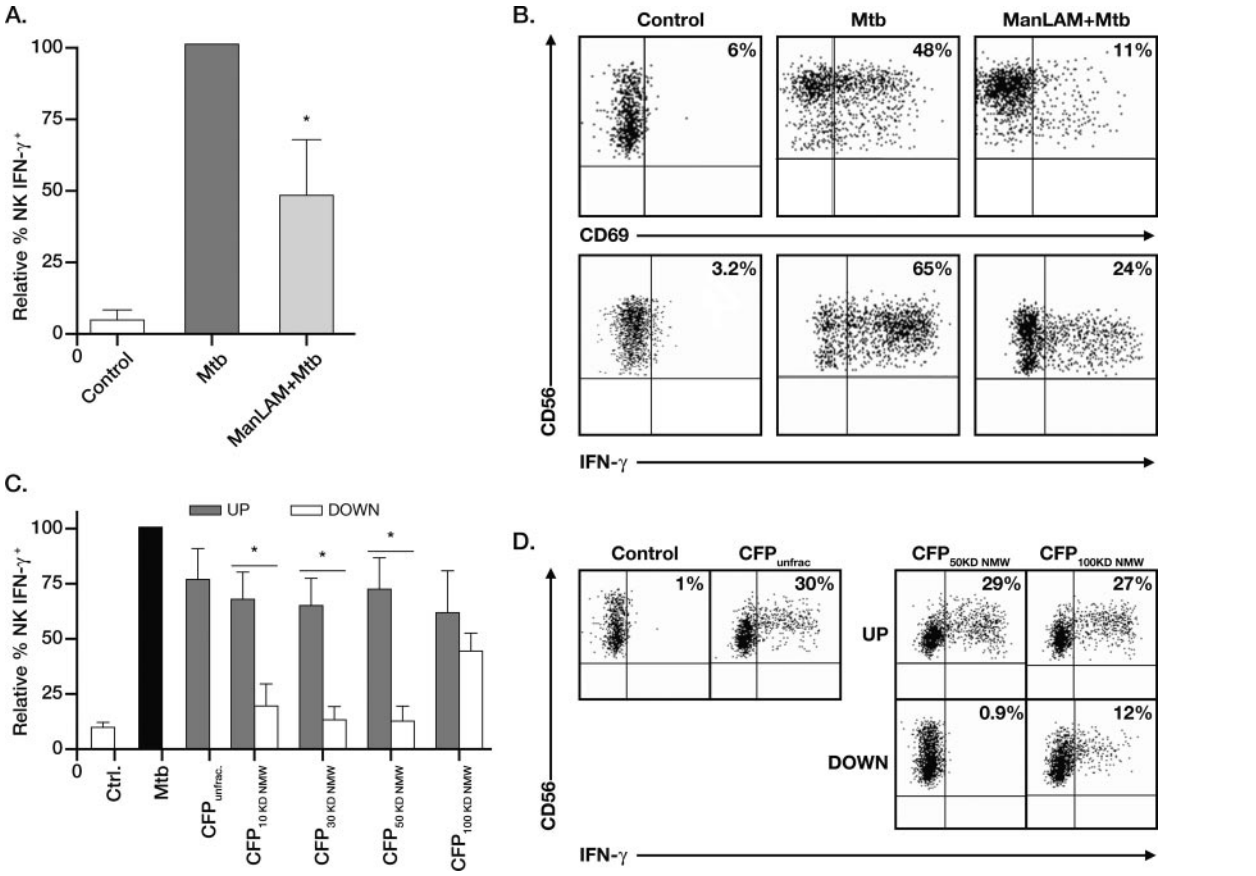

FIG. 3. *M. tuberculosis* antigens differentially modulate PF-NK cell response. (A and B) PFMC were stimulated with medium alone (control) or with *M. tuberculosis* or were pretreated with ManLAM before *M. tuberculosis* stimulation (ManLAM+Mtb); thereafter, CD69$^+$ and IFN-γ$^+$ cells among gated CD3$^-$ CD56$^+$ cells cultured for 18 h or 24 h (respectively) were evaluated. (A) Relative percentages of IFN-γ$^+$ NK cells (means ± SEM) are shown. *, $P < 0.05$ (*M. tuberculosis* versus ManLAM + *M. tuberculosis*) ($n = 6$). (B) Dot plots of a representative experiment showing CD69 and IFN-γ expression on gated PF-CD3$^-$ CD56$^+$ cells. (C) Data for PFMC stimulated with *M. tuberculosis*, CFP (1 μg/ml), or the upper (up; gray bars) or lower (down; white bars) size fractions of 10, 30, 50, and 100 NMW samples from CFP. Relative percentages of IFN-γ$^+$ NK cells (means ± SEM) are shown. *, $P < 0.05$ (upper versus lower fraction) ($n = 4$). (D) Dot plots of a representative experiment with the upper and lower fractions showing IFN-γ expression on gated PF-CD3$^-$ CD56$^+$ cells.

the CD56^{bright} NK cell subset was preferentially activated by *M. tuberculosis*, both subsets were activated to the same extent by TLR agonists. Altogether, these results suggest that both TLR2 and -4 are implicated in the activation of PF-NK cells but that other factors are necessary to exceed the threshold for IFN-γ production.

***M. tuberculosis*-triggered IFN-γ production by PF-NK cells is dependent on endogenous IL-12.** Given the presence of APC in PFMC, we evaluated the role of monokines, which have been detected in increased amounts in PF from TB patients (44, 54) and which may stimulate IFN-γ production via constitutive receptors on NK cells (8, 32, 49). Thus, PFMC were stimulated with *M. tuberculosis* in the presence or absence of antibodies to IL-18, IL-15, and IL-12 for 24 h, and IFN-γ$^+$ NK cells were then evaluated. As shown in Fig. 5A and B, neither anti-IL-18 nor anti-IL-15 significantly modified the number of IFN-γ$^+$ cells, but their number was decreased by IL-12 neutralization. Furthermore, we determined whether APC could be the source of IL-12. As shown in Fig. 5C, *M. tuberculosis* was able to induce IL-12 expression in a fraction of PF-APC (% IL-12$^+$ PF-APC for control cells, 4% ± 3%; % IL-12$^+$ PF-APC for *M. tuberculosis*-stimulated cells, 15% ± 7% [$n = 5$]; $P < 0.05$). Consistently, IL-12 secretion upon *M. tuberculosis* stimulation was enhanced in four of seven PFMC samples studied (Fig. 5D, left graph). Given the variability observed in *M. tuberculosis*-induced IL-12 secretion among PF samples, we wanted to determine whether this fact could be ascribed to the number of APC present in PFMC. As shown in Fig. 5D (right panel), a clear correlation between *M. tuberculosis*-induced IL-12 secretion and the percentage of APC was observed. This result suggests that APC are a main source of IL-12 in tuberculous pleurisy and may explain why IL-12 remains undetectable in those samples with low percentages of APC. However, no clear correlation was found between IL-12 secretion and IFN-γ expression by PF-NK cells upon *M. tuberculosis* stimulation (data not shown), suggesting that other signals intervene in the latter process.

NK and APC cell contact is required for *M. tuberculosis*-induced IFN-γ production by PF-NK cells. Considering that endogenous IL-12 contributes to IFN-γ production, we further investigated whether *M. tuberculosis* and/or IL-12 was sufficient to induce its expression. To do this, PFMC and PF-NK cells,

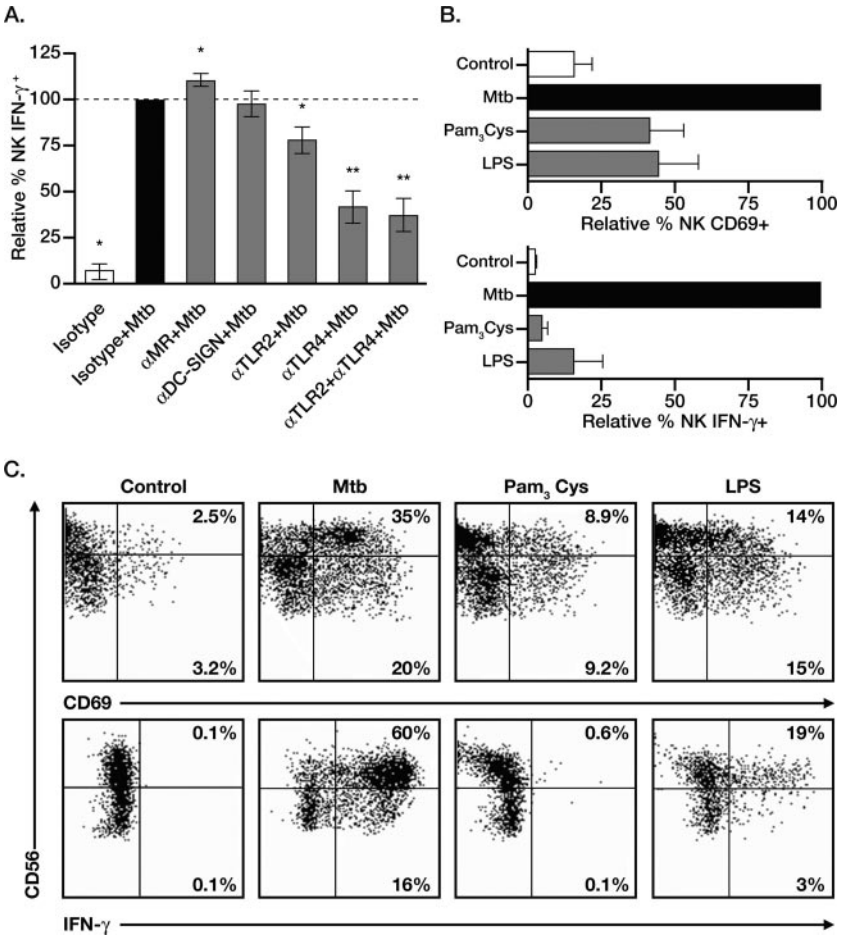

FIG. 4. PRRs regulate *M. tuberculosis*-induced PF-NK cell response. (A) PFMC were pretreated with 5 μg/ml of the following anti-human receptor antibodies: anti-MR, anti-DC-SIGN, anti-TLR2, anti-TLR4, anti-TLR2 plus anti-TLR4, and isotype-matched antibodies. They were then stimulated with *M. tuberculosis* for 24 h. IFN-γ^+ cells were evaluated among gated $CD3^-$ $CD56^+$ cells. Relative percentages of IFN-γ^+ NK cells (means ± SEM) are shown. *, $P < 0.05$; **, $P < 0.005$ (treatment versus isotype) ($n = 5$). (B) PFMC were stimulated with *M. tuberculosis*, Pam_3Cys (0.5 μg/ml), or LPS (1 μg/ml) for 24 h. Relative percentages of $CD69^+$ NK cells (upper graph; $n = 4$) and IFN-γ^+ NK cells (lower graph; $n = 5$) (means ± SEM) are shown. (C) NK cell subsets respond differentially to TLR agonists. Quadrants denote $CD56^{dim}$ and $CD56^{bright}$ NK cells. Representative dot plots are depicted.

enriched by negative selection employing magnetic methods (APC-depleted PFMC), were incubated in the presence or not of *M. tuberculosis* and/or exogenously added rhIL-12 for 24 h, and then the number of IFN-γ^+ cells was determined. As shown in Fig. 6A and B, IL-12 alone allowed slight IFN-γ induction in NK cells from PFMC, despite the fact that the amount of IL-12 added was considerably higher than the endogenous production level (Fig. 5D). However, a synergistic effect was observed for IL-12 and *M. tuberculosis* costimulation. Interestingly, in NK cells from APC-depleted PFMC, neither *M. tuberculosis* alone, IL-12 alone, nor IL-12 plus *M. tuberculosis* stimulated the number of IFN-γ^+ NK cells obtained with *M. tuberculosis*-stimulated PFMC.

It is known that NK cell effector function is the result of a balance between signals coming from activating and inhibitory receptors (45). The above results showed that IL-12 alone is not enough to induce IFN-γ upon *M. tuberculosis* stimulation, implicating the existence of additional APC-derived signals. Therefore, we addressed the role of cell-to-cell contact by inhibiting the interaction between molecules known to be involved in the NK-cell–APC immune synapse (22, 27, 39, 48). To do this, blocking MAbs or isotype controls were added before *M. tuberculosis* stimulation. As shown in Fig. 6C and D, a modest but significant inhibition of IFN-γ^+ and $CD69^+$ NK cells was observed by blocking the NK receptors CD2, NKG2D, CD6, and CD161 and the costimulatory molecules CD54 (ICAM-1) and CD86. Consistently, neither IFN-γ nor CD69 (not shown) expression was altered by blocking CD16, which is an APC-independent NK-activating receptor. Importantly, blockade of MHC-I, which is the ligand of several NK cell inhibitory receptors, gave an increase in IFN-γ and CD69 expression (Fig. 6D). The expression of these molecules on PBMC, PF-NK cells, and/or APC is shown in Table A1. Altogether, these results suggest that a direct NK-cell–APC interaction takes place in PFMC, where NK cell function is controlled by inhibitory and activating signals that ultimately lead to IFN-γ production.

***M. tuberculosis* binds to NK cells.** Given that even in the absence of APC and IL-12, *M. tuberculosis* is able to induce significant increases in IFN-γ^+ PF-NK cells (Fig. 6A and B),

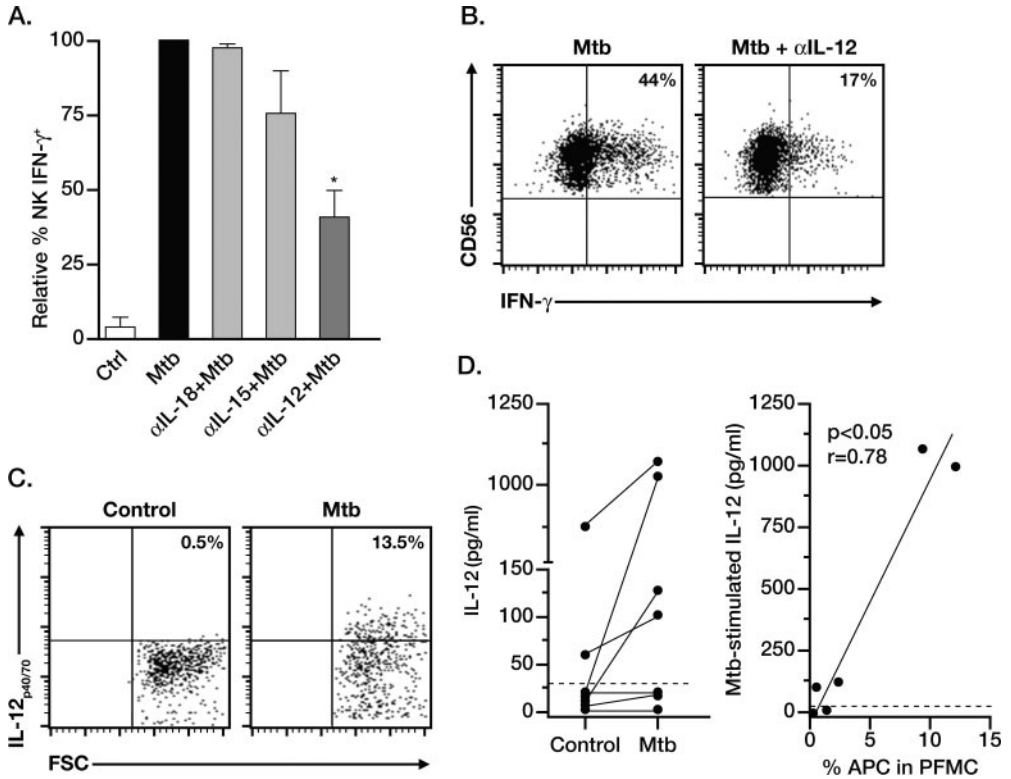

FIG. 5. IFN-γ production by PF-NK cells is partially dependent on APC-derived IL-12. PFMC were treated or not with 1 μg/ml of neutralizing anti-IL-18, anti-IL-15, or anti-IL-12 and then stimulated for 24 h with *M. tuberculosis*. IFN-γ^+ cells were evaluated among gated $CD3^-$ $CD56^+$ cells. (A) Relative percentages of IFN-γ^+ NK cells (means ± SEM) are shown. *, $P < 0.05$ (*M. tuberculosis* versus anti-IL-12 plus *M. tuberculosis*) ($n = 5$). (B) Dot plots for one representative experiment. (C) Intracellular IL-12 expression was determined by flow cytometry of APC-gated PFMC incubated alone (control; left) or with *M. tuberculosis* (right) for 18 h. Dots plots of a representative experiment of five are shown. (D) IL-12 secretion was determined by ELISA with supernatants from 2×10^6 PFMC/ml incubated alone (control) or with 1×10^6 *M. tuberculosis* cells (left graph; Mtb). The right graph shows the correlation between *M. tuberculosis*-induced IL-12 and the percentage of APC present in the corresponding sample before the assay. The horizontal dashed line represents the detection limit of the assay (~30 pg/ml) ($r = 0.78$; $P < 0.05$ [Spearman two-tailed correlation test]) ($n = 7$).

we investigated whether a direct contact between NK cells and *M. tuberculosis* surface components could occur. Furthermore, considering that PF-NK cells are already activated in comparison with their peripheral counterparts (40), we also wanted to discern whether an association with the activation state could be established. For this purpose, paired samples of PBMC and PFMC were incubated with *M. tuberculosis*-FITC for 5, 20, 60, and 120 min at 37°C, and binding was determined by flow cytometry on $CD3^-$ $CD56^+$ cells. As shown in Fig. 7A, while a small proportion of PB-NK cells bound *M. tuberculosis*, the proportion increased significantly as soon as 5 min and reached the maximum value at 20 min (Fig. 7B). Besides, the decrease in NK-cell–*M. tuberculosis*-FITC conjugates in competition assays with unlabeled *M. tuberculosis* rules out a nonspecific interaction. In addition, $CD56^{bright}$ and $CD56^{dim}$ subsets from PF-NK cells bound *M. tuberculosis* to the same extent. Overall, these results reinforce the observation that a direct recognition of *M. tuberculosis* by PF-NK cells takes place but that the interaction with APC is necessary to fulfill IFN-γ production.

Intracellular pathways involved in IFN-γ production by PF-NK cells. It has been demonstrated that several intracellular signaling cascades, such as phosphoinositide 3-kinase (PI3K), MAPK cascades (ERK1/2 and p38 kinase), and stress-activated protein kinases (e.g., c-Jun N-terminal kinase) (43), are triggered by mycobacteria in macrophages upon receptor ligation. Moreover, activation of these signaling cascades culminates in the induction of multiple proinflammatory cytokines as well as in the regulation of costimulatory molecules in many cell types (2, 3, 18, 24, 29, 32, 36, 53).

Our results described above indicate that in our system, NK activation occurs by direct *M. tuberculosis* binding and, in a bystander fashion, by release of IL-12 and contact with APC. Thus, we wanted to evaluate the signaling pathways involved in the *M. tuberculosis*-induced PF-NK cell response. To this end, PFMC were incubated with EDTA, EGTA, Dex, CsA, and pharmacological inhibitors of PI3K (wortmannin), MAPK kinase (MEK) (PD98059), p38 (SB203580), and JNK1/2 (JUN1) for 30 min at 37°C. Thereafter, PFMC were stimulated with *M. tuberculosis* for 18 and 24 h, and then CD69 and IFN-γ expression was evaluated. As shown in Fig. 8A, the level of *M. tuberculosis*-induced IFN-γ production by PF-NK cells was completely abrogated by CsA and partially inhibited by Dex, and furthermore, Ca^{2+} mobilization was required. Pretreatment of PFMC with highly specific inhibitors of the kinases p38 (SB203580) and MEK (PD98059) diminished the production of IFN-γ, while neither wortmannin nor JUN1 modified the

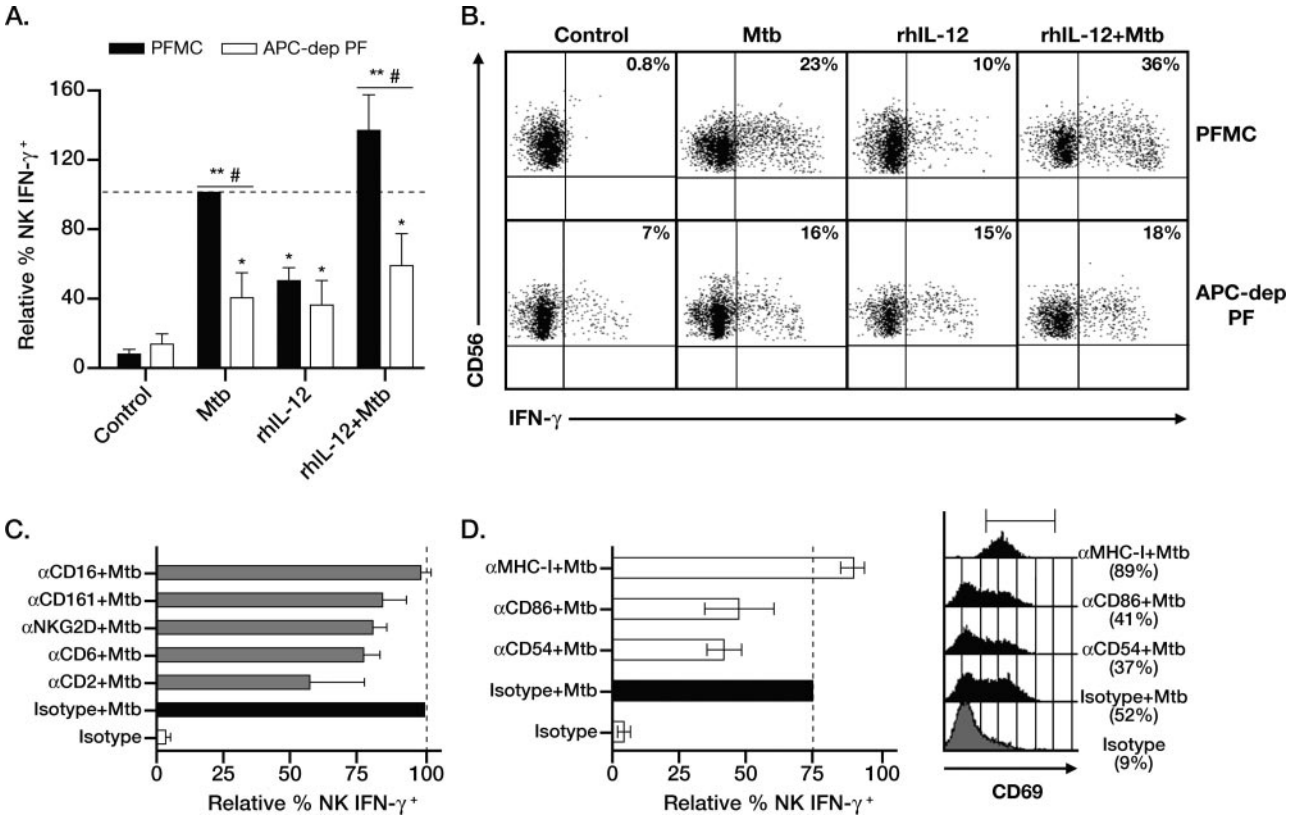

FIG. 6. APC contact-dependent stimulation modulates *M. tuberculosis*-induced PF-NK cell response. (A and B) PFMC or $CD20^+$ $CD1_{a\text{-}b\text{-}c}{}^+$ $CD14^+$ DC-$SIGN^+$ $CD3^+$ cell-depleted PFMC (APC-deplet PF) were cultured with medium alone (control), *M. tuberculosis*, rhIL-12, or rhIL-12 plus *M. tuberculosis* for 24 h. IFN-γ^+ cells were evaluated among gated $CD3^-$ $CD56^+$ cells. (A) Relative percentages of IFN-γ^+ NK cells (means ± SEM) in PFMC (black bars) or APC-depleted PFMC (white bars). *, $P < 0.05$; **, $P < 0.005$ (treatment versus control) ($n = 5$). #, $P < 0.05$ (PFMC versus APC-depleted PFMC) ($n = 6$). (B) Representative dot plots of IFN-γ expression on gated PF-$CD3^-$ $CD56^+$ cells from PFMC and APC-depleted PFMC. (C and D) PFMC were pretreated for 30 min with blocking MAbs against NK cell receptors (anti-CD2, anti-CD161, anti-CD6, anti-NKG2D, and anti-CD16) (C) or APC ligands (anti-CD86, anti-CD54, and anti-MHC-I) (D) or with isotype-matched control MAbs (IgG1κ, IgG2a, and IgG2b) and then stimulated with *M. tuberculosis* for 24 h. (C and D, left) Relative percentages of IFN-γ^+ NK cells (means ± SEM; $n = 3$). (D, right) Representative histogram plot of PF-NK cell CD69 expression.

capacity of *M. tuberculosis* to induce IFN-γ (Fig. 8B). In addition, when the CD69 activation marker was measured under the same conditions, a striking correlation between CD69 expression and the production of IFN-γ was observed. Despite treatment with Dex, wortmannin, and JUN1 having only minimal effects, in the presence of CsA PF-NK cells did not upregulate CD69 upon *M. tuberculosis* stimulation, while a slight up-regulation was observed in cells treated with PD98059 or SB203580 (Fig. 8C).

Because the inhibition of IFN-γ and CD69 expression observed in PF-NK cells could also be determined by an indirect effect exerted by the inhibition of these pathways on bystander cells and given that we have detected *M. tuberculosis*–NK-cell conjugates, we wanted to evaluate whether the activation of p38 on PF-NK cells could be induced upon *M. tuberculosis* stimulation, as has been observed with other stimuli (24, 32, 36). Hence, the kinetics of p38 phosphorylation was followed on gated $CD3^-$ $CD56^+$ PF-NK cells for 15, 30, and 120 min of incubation of PFMC stimulated or not with *M. tuberculosis* and anisomycin as a positive control of p38 activation. Thereafter, cells were stained with FITC–anti-phospho-p38 antibody or the corresponding isotype. As shown in Fig. 9A and B, the expression of p-p38 was detected in untreated PF-NK cells at 15 min, and it was enhanced by *M. tuberculosis* stimulation to the levels observed with anisomycin. In addition, the kinetics of p-p38 showed an increase at 15 min of *M. tuberculosis* stimulation, remaining detectable for up to 30 min and declining afterwards, while in control cells the p-p38 expression level was unaffected during incubation. We wanted to address whether p-p38 could be induced by IL-12 (32) and inhibited by CsA treatment (31). To do this, PFMC were incubated for 15 min with IL-12 alone or pretreated with CsA for 30 min before *M. tuberculosis* stimulation. As shown in Fig. 9C, pretreatment with CsA prevented *M. tuberculosis* induction of p-p38 and IL-12 alone did not affect its expression over the basal level.

DISCUSSION

Along with the development of an inflammatory exudate, which is the major and immediate consequence of infection, tuberculous pleurisy is characterized by a strong granulomatous inflammatory response to *M. tuberculosis* antigens. In the current study, we have identified the $CD3^-$ $CD56^+$ lymphocyte population present in tuberculous pleural effusions

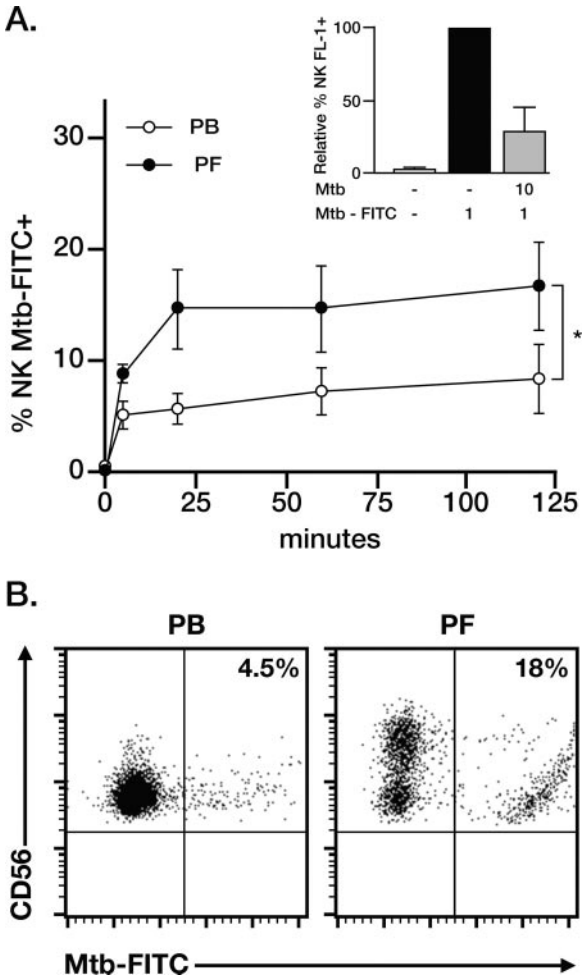

FIG. 7. Conjugate formation between PF-NK cells and *M. tuberculosis*. PBMC (PB) and PFMC (PF) were incubated with *M. tuberculosis*-FITC (1:1 ratio) for 5, 20, 60, and 120 min, and the conjugates between $CD3^-$ $CD56^+$ cells and *M. tuberculosis*-FITC were determined by flow cytometry. (A) Percentages of NK-cell–*M. tuberculosis*-FITC conjugates (means ± SEM). *, $P < 0.05$ (PF versus PB) ($n = 5$). (Inset) Specific binding was confirmed in competition experiments by coincubation of PFMC ($n = 3$) with *M. tuberculosis*-FITC and a 10-fold overload of unlabeled *M. tuberculosis* for 20 min. (B) Representative patient dot plot analysis among five similar analyses done at 20 min.

(PF-NK cells) as a main IFN-γ producer upon *M. tuberculosis* stimulation, suggesting that these cells play a beneficial role in tuberculous pleurisy by helping to maintain a type 1 profile. In line with this, we have investigated the mechanisms triggered by *M. tuberculosis* to induce cellular activation and IFN-γ expression in PF-NK cells. We demonstrate that at least three signals are required to reach a maximal response, including direct recognition of *M. tuberculosis* by NK cells, IL-12 induction upon *M. tuberculosis* stimulation, and the interaction between APC and NK cells.

Herein we show that the whole bacterium is the best inducer of IFN-γ expression in PF-NK cells, although proteins present in CFP and *M. tuberculosis* cell wall components regulate its production. In this context, it has been shown that low-molecular-mass proteins (<40 kDa) are prominently recognized by T cells (5, 12, 14), while high-molecular-mass proteins are the major targets of B cells (26). It has been demonstrated that low-molecular-mass secreted polypeptides from culture filtrates contain many targets for T-cell recognition from PBMC and PFMC (38). Furthermore, considerable homogeneity in the recognition of multiple polypeptides below 15 kDa by T cells from pleural effusions and PB from immune patients has been observed (38). In this study, we observed that a protein(s) or protein complexes with high molecular masses (>50 kDa) present in CFP are responsible for IFN-γ production in PF-NK cells. In contrast, CFP fractions below 30 kDa seemed to mediate the induction of IFN-γ when PF-$CD3^+$ T cells were analyzed in the same samples (data not shown). Although we cannot rule out if proteins below 50 kDa are involved in *M. tuberculosis*-induced IFN-γ production by PF-NK cells because we did not evaluate purified polypeptides, these results suggest the requirement of different targets among CFP proteins for the induction of IFN-γ in NK and $CD3^+$ T cells from tuberculous PF.

It is well known that several PRRs are associated with *M. tuberculosis* recognition by APC (52), and among them, DC-SIGN and MR recognize the major *M. tuberculosis* cell wall component ManLAM. Recent studies have demonstrated the expression of DC-SIGN and binding of *M. tuberculosis* to this receptor in alveolar macrophages (46) and the colocalization of DC-$SIGN^+$ cells and *M. tuberculosis* in lymph nodes from TB patients (47). On the other hand, it has been demonstrated that MR cross-linking activates an anti-inflammatory response in dendritic cells by inhibiting IL-12 and inducing IL-10 production (10, 34). Our blocking experiments indicate that the recognition of whole *M. tuberculosis* by MR delivers inhibitory signals for *M. tuberculosis*-induced IFN-γ production by PF-NK cells. In addition, we show that purified ManLAM does not induce IFN-γ in PF-NK cells, although it has an inhibitory role in *M. tuberculosis*-induced IFN-γ production. The inhibitory role of ManLAM has been ascribed to its capacity to modulate the immune response by dampening IL-12 production and down-regulating costimulatory molecules on APC (20, 34). Hence, the recognition of the *M. tuberculosis* cell wall component ManLAM through MR may regulate the inflammatory response at the site of active *M. tuberculosis* infection. The relevant roles for TLR2 and -4 in recognition as well as in cell activation and signaling pathways mediated by *M. tuberculosis* and *Mycobacterium bovis* BCG are well known (1, 2, 6, 23, 33, 37, 50, 51). In addition, it has been demonstrated that PAMPs can directly activate human NK cells to produce IFN-γ via several TLRs (9, 36). By employing blocking assays, we show that at the site of *M. tuberculosis* infection, both TLR2 and TLR4 trigger activation signals for expression of IFN-γ by PF-NK cells. However, stimulation with specific TLR agonists demonstrates that Pam_3Cys and LPS activate PF-NK cells but that only the latter induces weak expression of IFN-γ. Our result showing that IL-12 is involved in *M. tuberculosis*-induced IFN-γ production is in accordance with a previous report demonstrating that LPS induces IFN-γ in NK cells by an IL-12-dependent mechanism (21). In line with this, the requirement of IL-12 for IFN-γ production is not surprising because IL-12, as well as IL-15 and IL-18, has been shown to mediate bystander activation of NK cells in response to a number of different pathogens (4, 22). However, as we show with APC-depleted PFMC, IL-12 alone does not allow the same level of

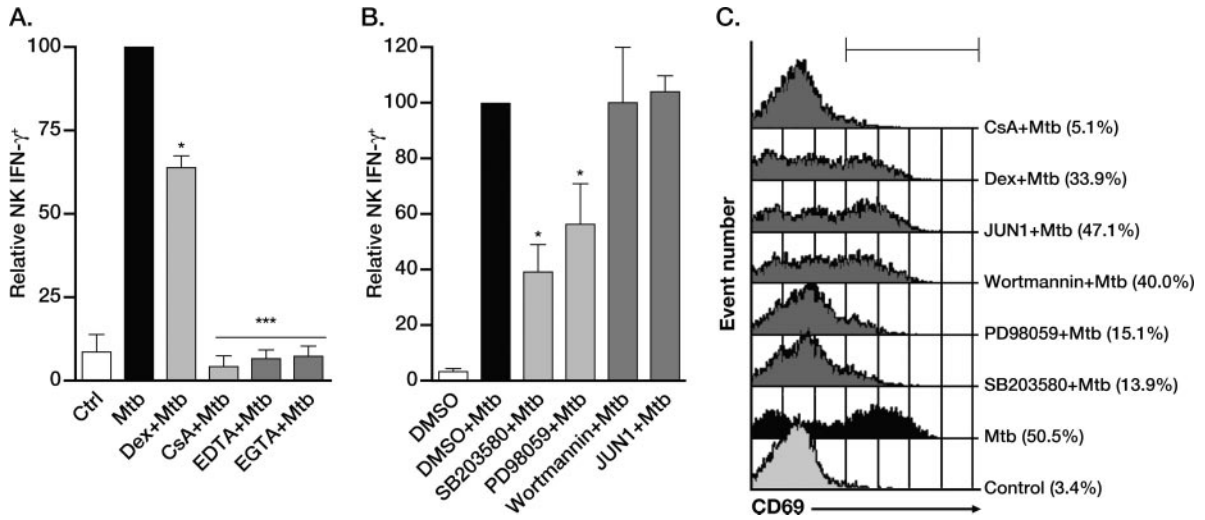

FIG. 8. Calcineurin, ERK, and p38 MAPK pathways are involved in cellular activation and IFN-γ expression by PF-NK cells. PFMC were stimulated with medium alone (Ctrol) or *M. tuberculosis* or were pretreated with the following drugs or kinase inhibitors before *M. tuberculosis* stimulation: Dex, CsA, EDTA, EGTA, DMSO (vehicle alone; 1:1,000), DMSO plus *M. tuberculosis*, SB203580, PD98059, wortmannin, and JUN1. Thereafter, IFN-γ^+ and CD69$^+$ cells were determined at 24 or 18 h (respectively) among gated CD3$^-$ CD56$^+$ cells. (A) Relative percentages of IFN-γ^+ NK cells (means ± SEM). *, $P < 0.05$; ***, $P < 0.001$ (*M. tuberculosis*- versus drug-pretreated cells) ($n = 5$). (B) Relative percentages of IFN-γ^+ NK cells (means ± SEM). *, $P < 0.05$ (DMSO-plus-*M. tuberculosis*- versus kinase inhibitor-pretreated cells) ($n = 5$). (C) Percentages of CD69$^+$ cells among control and PF-NK cells pretreated or not with CsA, Dex, SB203580, PD98059, wortmannin, or JUN1 before *M. tuberculosis* stimulation. A representative histogram plot among those done for four patients is depicted.

IFN-γ^+ NK cells as that observed with PFMC alone, suggesting that PF-APC or other PF cells activate other signals.

Signaling through TLRs not only mediates IL-12 production (53) but also up-regulates costimulatory molecules in APC (35, 42, 50). Accordingly, our results indicate that costimulatory signals delivered by APC, such as receptor-ligand interactions, are required. Indeed, herein we show that CD54/ICAM-I and CD86 as well as NK receptor engagement are necessary for *M. tuberculosis*-induced IFN-γ production. Costimulation through CD40, CD80, and CD86 is required for IFN-γ production by IL-2-activated NK cells (22, 48). Consistently, we have demonstrated that ex vivo NK cells from tuberculous PF are already activated (40). In addition, an impaired CD11a and ICAM-1 interaction has been associated with reduced NK cell activity in PB from TB patients (39). Interestingly, the inhibitory role played by MHC class I on activation and production of IFN-γ could be ascribed to the higher expression of CD94/NKG2A that we previously found in PF-NK cells (40). Thus, IFN-γ production is controlled by a balance between activating and inhibitory receptors that could limit the inflammatory response at the site of *M. tuberculosis* infection.

Herein we provide evidence that a direct interaction be-

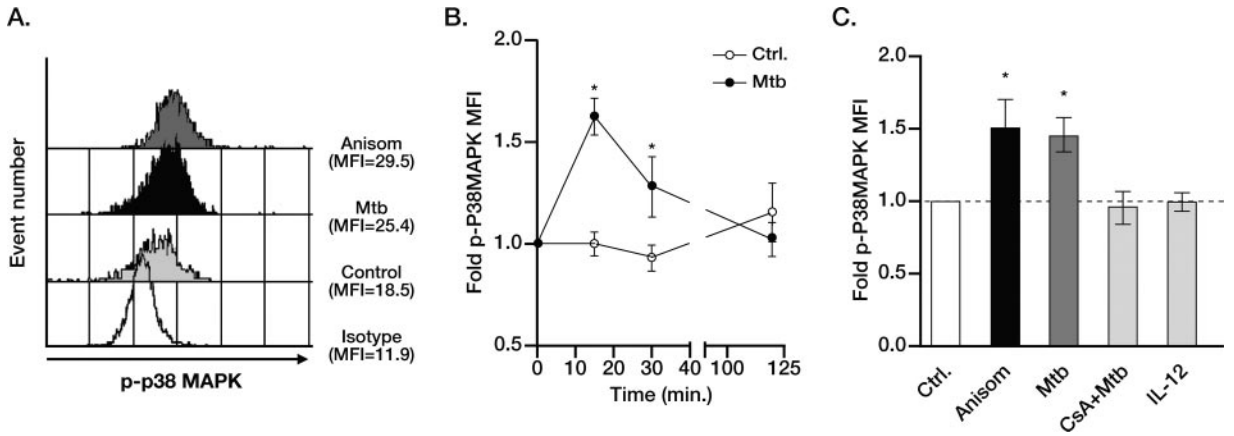

FIG. 9. p38 MAPK phosphorylation is induced by *M. tuberculosis* in PF-NK cells. PFMC were stimulated with medium alone (Ctrl), anisomycin (Anisom), IL-12, *M. tuberculosis* (Mtb), or CsA pretreatment plus *M. tuberculosis* (CsA+Mtb), and intracellular expression of p-p38 MAPK was determined on gated CD3$^-$ CD56$^+$ PFMC at 0, 15, 30, and 120 min. (A) MFIs in a representative histogram plot of p-p38 among five done at 15 min. (B) Kinetics of p-p38. Data are expressed as *x*-fold increases in p-p38 MAPK MFI for intracellular expression over basal expression (means ± SEM). *, $P < 0.05$ (*M. tuberculosis*-stimulated versus control cells) ($n = 4$). (C) *x*-Fold MFI increase for p-p38 MAPK at 15 min. *, $P < 0.05$ (treatment versus control). #, $P < 0.05$ (*M. tuberculosis* versus CsA plus *M. tuberculosis*) ($n = 5$).

TABLE A1. Surface expression analysis of molecules evaluated in neutralization experiments

MAb[b]	Receptor	% NK cells[a]		No. of paired samples tested	*P* value[c]	% APC[a]		No. of paired samples tested	*P* value[c]
		PBMC	PFMC			PBMC	PFMC		
TL2.1[1]	TLR2	3 ± 4	19 ± 7	8	<0.05	75 ± 4	67 ± 6	10	NS
HTA125[1]	TLR4	ND	ND	5		86 ± 3	61 ± 7	8	<0.05
120507[2]	DC-SIGN	ND	ND	3		9 ± 5	41 ± 6	10	<0.01
PAM-1*[6]	MR	ND	ND	3		19 ± 8	45 ± 7	5	<0.05
HA58[1]	ICAM-1	NM	NM			94 ± 5	90 ± 7	5	NS
IT2.2[1]	CD86	NM	NM			50 ± 4	45 ± 6	5	NS
3F10[3]	MHC-I	NM	NM			100 ± 1	98 ± 2	4	NS
D7*[6]	MICA	NM	NM			19 ± 5	55 ± 7	6	<0.05
149810[2]	NKG2D	95 ± 2	89 ± 6	6	NS	NM	NM		
39 C1.5[4]	CD2	85 ± 4	82 ± 6	4	NS	NM	NM		
Tü33*[5]	CD6	86 ± 4	79 ± 7	4	NS	NM	NM		
B199.2[3]	CD161	70 ± 6	83 ± 12	5	NS	NM	NM		
G25.2[4]	CD11a	99 ± 1	98 ± 3	5	NS	NM	NM		

[a] Values are means ± SEM. NK cells, cells were gated on $CD3^-$ $CD56^+$ lymphocytes from PBMC or PFMC; APC, cells were gated on MHC-II^{high} mononuclear phagocytes from PBMC or PFMC. ND, not detected; NM, not measured.

[b] FITC- or PE-labeled or unlabeled (*) MAbs were employed. MAbs were obtained from the following sources, as indicated by superscript numbers: 1, eBioscience; 2, R & D Systems; 3, Ancell; 4, Immunotech; 5, Clonab; and 6, M. Vulcano and N. Zwirner.

[c] *P* value for paired nonparametric Wilcoxon test (PBMC versus PFMC). <0.05, significant; NS, not significant.

tween *M. tuberculosis* and NK cells takes place. Moreover, the increased binding that we observed in PF-NK cells compared to that in PB-NK cells could be ascribed to the activation state of NK cells found in tuberculous pleurisy (40). In addition, the interaction between *M. tuberculosis* and PF-NK cells occurs in both $CD56^{dim}$ and $CD56^{bright}$ PF-NK cell subsets, and these subsets have predetermined effector functions. Therefore, the binding of *M. tuberculosis* could trigger different pathways in each subset (11, 19, 49).

Infection with dead but not live *M. tuberculosis* results in a transient Ca^{2+} flux within macrophages (30). In line with this, we show that Ca^{2+} flux is also necessary, and given that CsA abrogates IFN-γ production, our results support the involvement of the calcineurin pathway (31). Besides, since Dex does not abrogate IFN-γ production like CsA does, our results strongly support the hypothesis that signals other than IL-12 are involved in *M. tuberculosis*-induced IFN-γ production by PF-NK cells, as demonstrated in T cells (25). The production of proinflammatory cytokines by *M. tuberculosis* has been associated with MAPK activation (43, 52, 53). In this context, it has been shown that monocytes from active pulmonary TB patients have greater secretion of cytokines and activation of both ERK1/2 and p38 MAPK dependent on a ligand that signals through TLR2 and TLR4 (23). Accordingly, we show that the signaling pathway does not involve the activation of JUNK1/2 and PI3K but does involve p38 and ERK MAPK. In addition, we observed the expression of activated p38 in ex vivo PF-NK cells and *M. tuberculosis*-triggered p38 phosphorylation in all PF-NK cells when binding of *M. tuberculosis* and NK cells had reached the maximum value. IL-12 induces a weak but prolonged activation of p38 in resting NK cells (32), and given that in our system the addition of IL-12 did not increase the expression of p38 over that observed in ex vivo PF-NK cells, our results suggest that the activated form of p38 in ex vivo cells could be induced in vivo by IL-12, a cytokine that has been found to be increased at the site of *M. tuberculosis* infection (54). Interestingly, pretreatment with CsA inhibited *M. tuberculosis*-induced p38 activation suggesting upstream regulatory steps in the signaling cascade (31).

In summary, we have shown that PF-NK cells receive at least three stimulatory signals that act in concert to fulfill IFN-γ production, including (i) direct NK-cell–*M. tuberculosis* interaction, (ii) IL-12, and (iii) direct APC contact. At the same time, these activation signals can be modulated by C-type lectins and MHC class I ligands limiting the inflammatory response. In addition, at the site of infection, the signaling pathways triggered by *M. tuberculosis* to induce IFN-γ production by PF-NK cells involve p38, ERK, and calcineurin. Hence, the interplay between *M. tuberculosis* and NK cell/APC triggering of IFN-γ production by NK cells would be expected to play a beneficial role in tuberculous pleurisy by helping to maintain a type 1 profile.

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APPENDIX

Receptors and/or counterreceptors functionally tested in neutralization assays (Fig. 4A and 6C and D) were analyzed for surface expression in selected cellular populations (APC or NK cells) from PBMC and PFMC. We previously reported CD16 (FcγRIII) and CD95/NKG2A (an inhibitory heterodimeric counterreceptor for MHC-I) expression (40).

As Table A1 shows, the percentage of cells expressing TLR2 was up-regulated in PF-NK cells, but not in PF-APC, in comparison with their PB counterparts. In contrast, TLR4 was undetectable in NK cells and down-modulated in PF-APC. The C-type lectins MR and DC-SIGN were both up-regulated in PF-APC and were absent in PB-NK and PF-NK cells. Neither costimulatory molecules (ICAM-1 and CD86) nor MHC-I was significantly altered in PF-APC. Interestingly, increased numbers of APC expressing the stress-induced MHC class I-related chain A were found in PF, which is important because this molecule is one of the ligands of the NKG2D activating receptor (45). Finally, none of the NK cell-activating receptors analyzed here (NKG2D, CD2, CD6, CD161, and CD11a) were differentially expressed in PF or PB, which is in accordance with their constitutive expression levels (27).

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