

Human Natural Killer Cells Mediate Killing of Intracellular *Mycobacterium tuberculosis* H37Rv via Granule-Independent Mechanisms

KEVIN J. BRILL,¹ QING LI,¹ RHONDA LARKIN,¹ DAVID H. CANADAY,^{2,3} DAVID R. KAPLAN,⁴
W. HENRY BOOM,^{2,3} AND RICHARD F. SILVER^{1,2,3*}

Divisions of Pulmonary and Critical Care Medicine¹ and Infectious Diseases,² Department of Medicine, and Department of Pathology,⁴ Case Western Reserve University School of Medicine, and University Hospitals of Cleveland,³ Cleveland, Ohio 44106

Received 4 April 2000/Returned for modification 9 May 2000/Accepted 23 November 2000

Despite the continued importance of tuberculosis as a world-wide threat to public health, little is known about the mechanisms used by human lymphocytes to contain and kill the intracellular pathogen *Mycobacterium tuberculosis*. We previously described an in vitro model of infection of human monocytes (MN) with virulent *M. tuberculosis* strain H37Rv in which the ability of peripheral blood lymphocytes to limit intracellular growth of the organism could be measured. In the current study, we determined that lymphocyte-mediated killing of intracellular *M. tuberculosis* occurs within the first 24 h of coculture with infected MN. Natural killer (NK) cells isolated from both purified protein derivative (PPD)-positive and PPD-negative subjects were capable of mediating this early killing of intracellular H37Rv. NK cell-mediated killing of intracellular *M. tuberculosis* was not associated with the production of gamma interferon. Transferred supernatants of cocultured NK cells and *M. tuberculosis*-infected MN could not mediate the killing of intracellular *M. tuberculosis*, and Transwell studies indicated that direct cell-to-cell contact was required for NK cells to mediate the killing of the organism. Killing was not dependent upon exocytosis of NK cell cytotoxic granules. NK cells induced apoptosis of mycobacterium-infected MN, but neither killing of intracellular *M. tuberculosis* by NK cells nor NK cell-induced apoptosis of infected MN was inhibited by blocking the interaction of FasL and Fas. Thus, human NK cells may mediate killing of intracellular *M. tuberculosis* via alternative apoptotic pathways.

Tuberculosis remains a major international health problem which is likely to become even more significant in coming years because of the high prevalence of human immunodeficiency virus (HIV) disease in regions where infection with the intracellular pathogen *Mycobacterium tuberculosis* is endemic. Although it is estimated that one-third of the world's population is currently infected with *M. tuberculosis* (40), the great majority of these individuals never develop active disease, indicating the ability of human immune responses to contain the organism. Despite the importance of *M. tuberculosis* infection, little is known about the mechanisms that serve to contain this pathogen. Lymphocytes are thought to activate *M. tuberculosis*-infected mononuclear phagocytes to mediate the killing of intracellular bacteria, but the killing of *M. tuberculosis* has not been widely studied as an indicator of protective immunity. Although CD4, CD8, and $\gamma\delta$ lymphocytes exhibit proliferative, cytokine, and cytotoxic responses following stimulation by whole *M. tuberculosis* or its antigens (4, 8, 12, 41, 42), these responses have generally not been correlated with bacterial killing. On the other hand, killing of intracellular *Mycobacterium bovis* BCG and *M. tuberculosis* has been assessed in response to various chemical mediators of cell death. These studies have indicated that necrotic death of infected mono-

cytes does not result in killing of intracellular *M. tuberculosis*, whereas apoptotic cell death does result in bacterial killing (21, 25). However, with the exception of one report involving cell lines restricted to the unusual CD1 antigen-presenting pathway (39), these cytotoxic mechanisms have not been investigated in the context of specific lymphocyte populations capable of mediating killing of intracellular *M. tuberculosis*.

We previously described an in vitro model of reproducible infection of human monocytes (MN) with virulent *M. tuberculosis* strain H37Rv in which the ability of peripheral blood lymphocytes (PBL) to limit intracellular growth of the organism could be demonstrated (36). In the current study, we determined that killing of intracellular *M. tuberculosis* occurred within the first 24 h of coculture of infected MN with unstimulated PBL, which implied a role for innate immune responses in containment of intracellular *M. tuberculosis*. Natural killer (NK) cells represent a population of lymphocytes which could mediate innate protection against *M. tuberculosis*. NK cells have been implicated in early immune responses to viruses and to a variety of intracellular pathogens and are capable of rapidly producing gamma interferon (IFN- γ) as well as lysing specific target cells in the absence of prior activation (2, 6, 31, 32). More recently, NK cells also have been shown to express several surface ligands capable of initiating apoptosis (9, 29, 46). We therefore investigated the ability of NK cells to mediate the killing of intracellular *M. tuberculosis* following 24 h of coculture with infected MN, and we sought to correlate this killing with the production of IFN- γ , granule-mediated lysis of infected MN, and the induction of MN apoptosis.

* Corresponding author. Mailing address: Divisions of Pulmonary and Critical Care Medicine and Infectious Diseases, Biomedical Research Bldg., Rm. 1030, Case Western Reserve University School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106-4984. Phone: (216) 368-1151. Fax: (216) 368-2034. E-mail: rfs4@po.cwru.edu.

MATERIALS AND METHODS

Donors. Subjects were paid healthy volunteers between the ages of 22 and 50. For some studies, purified protein derivative (PPD)-positive subjects were specifically recruited. All protocols were approved by the Institutional Review Board of University Hospitals of Cleveland. Informed consent was obtained from each subject.

Cultivation and processing of mycobacteria. Broth cultures of *M. tuberculosis* strain H37Rv and the attenuated vaccine strain *M. bovis* BCG Pasteur (strains 25618 and 35734, respectively, American Tissue Type Collection [ATCC], Rockville, Md.), were grown in sterile Middlebrook 7H9 medium with 10% Middlebrook ADC enrichment and 0.2% glycerol. Plated cultures were grown on Middlebrook 7H10 agar with 10% Middlebrook OADC enrichment (Difco, Detroit, Mich.) and 0.5% glycerol.

H37Rv and BCG were initially grown to mid-log phase as broth cultures in 1.7-liter rolling bottles (no. 2528-1700; Corning, Corning, N.Y.) at 37°C. Cultures were divided into aliquots and immediately stored at -70°C. These aliquots were used to inoculate all subsequent roller bottle cultures for use in the infection of MN so that all infections were performed with organisms which had undergone only one previous laboratory passage. In preparation for the infection of MN, mycobacteria were processed using a series of mechanical disruptions and centrifugations based on the methods of Schlesinger (33) and previously described in detail (36), which served to minimize clumping and to provide for accurate quantification of the inoculum.

Isolation of blood MN and lymphocytes. Peripheral blood was obtained by venipuncture from healthy individuals and, mononuclear cells were isolated by density sedimentation using Ficoll-Hypaque (Ficoll-Paque, Pharmacia, Uppsala, Sweden) and washed three times in RPMI 1640 (BioWhittaker, Walkersville, Md.). Peripheral blood mononuclear cells (PBMC) were incubated in tissue culture-grade 100-mm polystyrene petri dishes (Falcon 3003; Becton Dickinson Labware, Lincoln, N.J.) to separate adherent MN and nonadherent PBL populations as previously described (36).

Isolation of human NK cells. NK cells were isolated from blood lymphocytes using a two-step method. First, CD56⁺ cells were positively selected using anti-CD56 antibodies in a MACS magnetic column separation system (Miltenyi Biotec, Auburn, Calif.). Each portion of 10⁸ PBL was resuspended in 800 μ l of MACS buffer (phosphate-buffered saline with 0.5% bovine serum albumin and 2 mM EDTA) and incubated with 200 μ l of MACS CD56 MicroBeads (no. 504-01) for 15 min at 4°C. Cells were washed with 10 to 20 ml of MACS buffer and resuspended in 1 ml of buffer. Blood lymphocytes were then added to a MACS MS separation column (no. 422-01) on a MiniMACS magnet that had been precooled for 20 min at 4°C and primed with 500 μ l of buffer. Lymphocytes were allowed to run through the column, which was then washed three times with 500 μ l of buffer. The column was then removed from the magnet. After the addition of 1 ml of buffer, a plunger was used to expel positively selected cells into 5-ml polystyrene tubes.

The selected population of CD56⁺ lymphocytes was further purified by removing residual CD3⁺ cells using Dynabeads M-450 CD3 (no. 111.13; Dynal). Beads were washed with medium and added to the CD56⁺ population for a 45-min incubation on a rotating platform at 4°C. CD3⁺ cells were removed using a Dynal magnetic particle separator. Two-color fluorescence-activated cell sorter analysis using CD3-fluorescein isothiocyanate (FITC) and CD56-Phycoerythrin (PE) (no. 340542 and no. 347747, respectively; Becton Dickinson) confirmed that the resulting cell populations consistently contained >95% CD3⁻ CD56⁺ NK cells.

Infection of human MN with *M. tuberculosis* and assessment of intracellular growth. Isolated MN were resuspended at a density of 10⁶/ml in Iscove's modified Dulbecco's medium with NaHCO₃, 25 mM HEPES, 1% L-glutamine (subsequently referred to here as IMDM; BioWhittaker 12-722F) with 5% fresh autologous serum (without antibiotics), and 100 μ l (10⁵ monocytes) were divided into aliquots in triplicate wells of round-bottomed 96-well plates (Corning) for CFU assessment for each time point to be studied. Following overnight incubation to allow for readherence, the supernatants were removed and H37Rv was added, in a 1:1 bacterium-to-MN infecting ratio, in 100 μ l of IMDM per well with 30% autologous serum. Plates were returned to a 37°C incubator for 1 h, at which time the supernatants were aspirated, and each well was washed three times with RPMI 1640 and 10% fetal calf serum and 1% HEPES to remove noningested mycobacteria. Wells were then refilled with 200 μ l of IMDM and 10% non-heat-inactivated autologous serum.

At selected time points, supernatants from the appropriate triplicate wells were aspirated and saved. Cell pellets were lysed with 0.067% sodium dodecyl sulfate (SDS), pooled, and diluted for plating onto 7H10-OADC plates for CFU

determination as previously described in detail (36). The results were expressed as CFU per milliliter of lysate, which corresponded to CFU/10⁶ cultured MN.

Addition of blood lymphocytes and NK cells to infected MN. PBL were resuspended in IMDM containing 10% fresh autologous serum and stored in a 37°C CO₂ incubator during the readherence of MN into microtiter wells. The next morning, the lymphocytes were washed and resuspended at a density of 5 \times 10⁶/ml in IMDM with 10% autologous serum. Lymphocytes were then added to autologous MN cultures immediately following the rinsing of nonphagocytosed bacteria. The addition of 200 μ l of PBL (for a 10:1 PBL/monocyte ratio) was utilized in order to reconstitute the approximate composition of the PBMC. Various ratios of NK cells were added to autologous infected MN to determine dose-response effects as described in Results.

***M. tuberculosis*-induced production of IFN- γ by lymphocyte populations and blocking of IFN- γ .** Supernatants were collected from cocultured *M. tuberculosis*-infected human MN and PBL or NK cells after 24 and 96 h of culture. Samples were frozen at -70°C until use, at which time they were thawed and filtered through 0.22- μ m (pore-size) filters to remove residual organisms. IFN- γ concentrations in supernatants were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (EH-IFN γ ; Endogen, Cambridge, Mass.). ELISA plates were read using an automated plate reader (Molecular Devices Corp., Menlo Park, Calif.) and a dual-wavelength reading at λ = 450/570. In blocking studies, neutralizing polyclonal antibody to human IFN- γ (Endogen P-700) was added to cultures at a concentration of 10 μ g/ml.

Supernatant transfer studies. Supernatants were collected from 96-well microtiter cocultures of NK cell, and *M. tuberculosis*-infected human monocytes were established in the fashion described above. Supernatants removed after 6, 18, and 24 h of coculture were filtered to remove residual organisms and added to freshly infected MN from the same subjects. The CFU counts were determined at 24 h of culture, and the supernatant-mediated reduction in intracellular *M. tuberculosis* was compared to that observed in cocultures of *M. tuberculosis*-infected MN cocultured with NK cells.

Assessment of the requirement for direct contact between NK cells and *M. tuberculosis*-infected MN in killing of intracellular bacilli. Dual-chamber studies were established using 24-well Transwell plates (no. 3413; Costar, Cambridge, Mass.). A total of 6 \times 10⁵ MN was added to the lower chamber of each well and infected with *M. tuberculosis* using a 1:1 bacterium-to-cell ratio as described above. For each subject, 3 \times 10⁶ NK cells were then added to the upper chamber of one well (separated from the infected MN by a 0.4- μ m [pore size] membrane) and directly to infected MN in the lower chamber of another well. After 24 h of coculture, lower-chamber cells were lysed to allow for CFU determination. Intracellular *M. tuberculosis* levels in cultures of MN alone were compared to those within cultures in which NK cells were added in direct contact with infected MN and to wells in which NK cells were separated by membrane filters from the MN.

Assessment of NK cell-mediated cytotoxicity. NK cell activity was measured using the standard target K562 human leukemia cell line (no. 45506; ATCC) in a chromium release assay using radioactive ⁵¹Cr. K562 cells were labeled with 100 μ Ci of ⁵¹Cr (1 mCi/ml; ICN Radiochemicals, Irvine, Calif.) for 2 h at 37°C, washed four times, and resuspended in 10% fetal calf serum at 10⁵ cells/ml, of which 3,000 to 5,000 cell aliquots were plated into 96-well round-bottomed plates. NK cells were added to the wells in various effector/target ratios (1:1, 5:1, and 20:1). Plates were centrifuged at 200 \times g for 30 s and incubated at 37°C for 4 h. Supernatants were harvested and ⁵¹Cr release measured by using a gamma counter. The spontaneous release of ⁵¹Cr was measured in wells containing target cells alone. The maximum release was determined from target cells which had been lysed with 3% SDS. The percent specific ⁵¹Cr release was calculated for each experimental group by using the following equation: percent specific release = [(cpm experimental - cpm spontaneous release)/(cpm maximum release - cpm spontaneous release)] \times 100.

Inhibition of exocytosis of NK cell cytotoxic granules. For some studies, NK cell granule release was inhibited using the calcium chelator EGTA (E 0396; Sigma, St. Louis, Mo.). For cytotoxicity studies, NK cells were resuspended in medium containing 5 mM EGTA prior to the addition to K562 cells, and the specific ⁵¹Cr release determined as described above. In CFU studies, NK cells were resuspended in IMDM containing 5 mM EGTA prior to the addition to *M. tuberculosis*-infected MN. Control studies adding IMDM with EGTA to infected MN alone were also performed.

Measurement of NK cell-induced MN apoptosis. NK-cell mediated apoptosis of mycobacterium-infected MN was measured using FITC labeling of DNA strand breaks with a commercially available TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay (In Situ Cell Death Detection Kit 1684795; Boehringer-Mannheim, Indianapolis, Ind.). In order to accurately quantify apoptosis in a mixed cell system, MN were also labeled using

CD14-PE (no. 347497; Becton Dickinson) and analyzed by two-color flow cytometry. Because of the need to perform flow cytometry outside of a biosafety level 3 facility, MN were infected with *M. bovis* BCG for these studies. MN were placed in polypropylene tubes and incubated for 1 h with BCG in IMDM with 30% autologous serum in a 10:1 bacterium-to-cell ratio. Infected MN were then pelleted by centrifugation at $480 \times g$ and resuspended in IMDM with 10% serum. NK cells were added to infected autologous MN in a 5:1 ratio and incubated for 24 h. Uninfected MN, BCG-infected MN, and uninfected MN cocultured with NK cells were incubated as well for use as controls. Cells were labeled with anti-CD14 and fixed with 2% paraformaldehyde prior to permeabilization and labeling for apoptosis with the TUNEL reagent according to the manufacturer's protocol.

To assess apoptosis within MN cultures infected with virulent *M. tuberculosis*, in situ TUNEL assays were performed using three-color fluorescent microscopy. To allow for recognition of infected cells stained with both CD14-PE and TUNEL-FITC, H37Rv was labeled with the amine-reactive fluorescent blue dye AlexaFluor 350 carboxylic acid, succinimidyl ester (A10168; Molecular Probes, Eugene, Oreg.). Bacilli were washed and resuspended in PBS (pH 8.7; adjusted by the addition of 1 M sodium bicarbonate buffer). AlexaFluor 350 solution was prepared according to the manufacturer's recommendations by dissolving 5 g of the dye in 500 μ l of dimethyl sulfoxide. Then, 25 μ l of this solution was added to 450 μ l of H37Rv. Following a 30-min incubation at room temperature, H37Rv was washed three times and diluted to the appropriate concentration for infection of MN in the same manner as that described for BCG above. NK cells were then added to cultures in a 5:1 ratio relative to the number of infected MN. Following 24 h of coculture, this preparation was incubated with CD14-PE, washed, and fixed by overnight incubation with 2% paraformaldehyde. The next day, cells were counted, and cytospin slides were prepared using 50,000 cells per slide. In situ TUNEL assessment of apoptosis was performed according to the manufacturer's protocol (Boehringer-Mannheim). Fluorescent micrograph images using appropriate filters for each color were obtained using a digital camera (Spot Digital Camera; Diagnostic Instruments, Sterling Heights, Mich.), and composite three-color micrographs were assembled using Spot Advanced Software (Diagnostic Instruments).

Blocking of Fas-FasL interaction. Blocking anti-FasL monoclonal antibody NOK1 (Pharmingen, San Diego, Calif.) was added in final concentration of 10 μ g/ml to assays of both NK cell-mediated killing of intracellular *M. tuberculosis* H37Rv and NK cell-induced apoptosis of BCG-infected MN. Results were determined by CFU assay and TUNEL assay, respectively, as described above. As a control for the efficacy of blocking of FasL by NOK1, the antibody was also added to cultures in which a previously described FasL-expressing transfectant KFL9 (35) was incubated with Jurkat target cells. The ability of KFL9 to induce apoptosis of Jurkat cells in the presence of medium alone and with the addition of 10 μ g of NOK1 per ml was assessed by the TUNEL method. Cells were incubated with anti-CD54-PE prior to the TUNEL procedure in order to distinguish the two cell populations. Apoptosis of Jurkat was calculated as percentage of CD54-negative cells that stained positively with the TUNEL reagent.

Assessment of the effects of blocking multiple NK cell effector pathways simultaneously. To evaluate the possibility that multiple effector functions of NK cells function cooperatively to result in the killing of intracellular *M. tuberculosis*, we assessed the results of simultaneously blocking IFN- γ with the antibody P-700 (again at concentration 10 μ g/ml), granule release with 5 mM EGTA, and Fas-FasL interactions with the anti-FasL antibody NOK1 (10 μ g/ml). The effects of each pair of these inhibitors upon NK cell-mediated killing of intracellular *M. tuberculosis* was determined at 24 h of coculture, as was the effect of simultaneous addition of all three inhibitors to the cultures.

Statistics. All statistical comparisons were made using paired *t* tests and calculated on Prism software (GraphPad Software, San Diego, Calif.).

RESULTS

PBL mediate killing of intracellular *M. tuberculosis* within the first 24 h of coculture. Standard assays of intracellular growth of *M. tuberculosis* have measured the CFU at days 4 and 7 following infection. Because our previous study suggested PBL-mediated killing of intracellular *M. tuberculosis* within human MN occurred prior to the day 4 time point, we sought to clarify the kinetics of early PBL-mediated limitation of growth of *M. tuberculosis*. The CFU of intracellular *M. tuberculosis* was determined on a daily basis during days 0 to 4 of cultures of infected MN alone and cocultures of infected

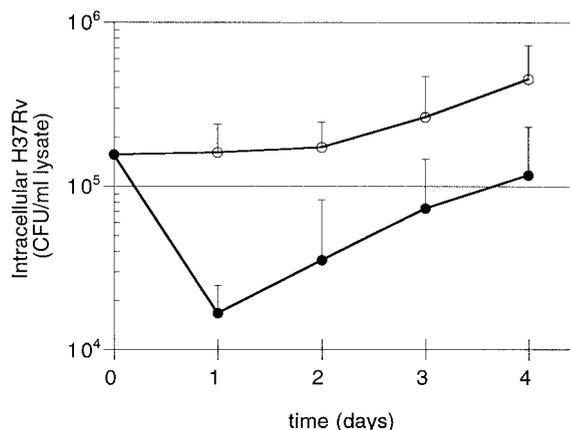


FIG. 1. PBL-mediated reduction in intracellular *M. tuberculosis* occurs within the first 24 h of coculture with infected MN. The CFU count of intracellular *M. tuberculosis* H37Rv within infected MN was determined at 24 h intervals for cultures of MN alone (○) and infected MN cocultured with PBL in a 10:1 PBL MN ratio (●). As illustrated, PBL mediated a drop in intracellular *M. tuberculosis* of approximately 1 log within the first 24 h of coculture, whereas in days 2 to 4 the rates of intracellular growth of the organism in the two culture conditions were comparable. The results represent the mean and the standard deviation (SD) from five donors.

MN plus PBL. Figure 1 shows the results for studies of five subjects and indicates consistent reduction of intracellular *M. tuberculosis* within the first 24 h after the addition of PBL to infected MN ($P = 0.006$ compared to CFU within MN alone). Following this 9.6-fold early reduction, growth of *M. tuberculosis* both within MN alone and within MN cocultured with PBL were essentially the same through days 2 to 4 (2.6-fold growth versus 3.3-fold growth, respectively). The finding of killing within 24 h of the addition of unprimed lymphocytes suggested a role for innate immune responses such as those mediated by NK cells in the early inhibition of intracellular *M. tuberculosis*.

Isolated human NK cells can mediate early killing of intracellular *M. tuberculosis*. Flow cytometry confirmed that the two-step enrichment procedure described above consistently yielded a cell population composed of greater than 95% CD3⁻CD56⁺ lymphocytes, which are subsequently referred to as NK cells.

In initial studies, NK cells were added in ratios of 1:1, 3:1, and 5:1 to *M. tuberculosis*-infected MN. As illustrated in Fig. 2, NK cells were able to mediate killing of intracellular *M. tuberculosis* within 24 h of coculture, and the magnitude of this effect increased with increasing ratios of NK cells relative to *M. tuberculosis*-infected MN. The reductions in intracellular *M. tuberculosis*-mediated by NK cells in both 3:1 and 5:1 ratios were statistically significant ($P = 0.006$ and $P = 0.003$, respectively). In order to maximize our ability to assess the mechanisms involved in this killing, the 5:1 ratio of NK cells to infected MN was used in all subsequent studies. Donor-to-donor variability can be substantial in human studies of mycobacteria, as indicated by large standard deviations, particularly of CFU of infected MN alone. Nevertheless, we found that relatively low numbers of subjects were required to demon-

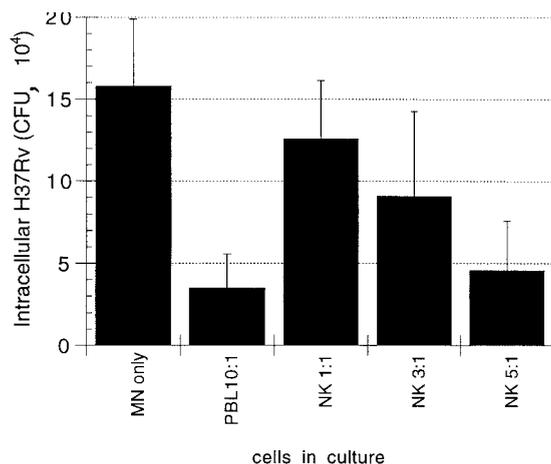


FIG. 2. NK cells mediate the killing of intracellular *M. tuberculosis* within 24 h of coculture with infected MN. CFU of intracellular *M. tuberculosis* was assessed in MN alone and following the addition of PBL (in a 10:1 ratio relative to MN) and of isolated CD3⁻ CD56⁺ NK cells (in ratios of 1:1, 3:1, and 5:1 relative to infected MN). The results indicate the mean and SD for four subjects studied. As illustrated, NK cells were capable of killing intracellular *M. tuberculosis*. The magnitude of this effect increased with the use of higher ratios of NK to infected MN and was statistically significant following the addition of NK cells in both a 3:1 ratio ($P = 0.006$) and a 5:1 ratio ($P = 0.003$).

strate statistically significant effects of NK cells on the viability of intracellular *M. tuberculosis* in the various studies described.

To confirm that our NK cell-enriched cell populations were effectively depleted of antigen-specific immune responses to *M. tuberculosis*, we compared the effects of the addition of NK cells obtained from PPD-positive and PPD-negative donors to *M. tuberculosis*-infected MN. As shown in Fig. 3, NK cells from both subject groups mediated killing of intracellular H37Rv. The magnitude of *M. tuberculosis* killing following the addition of NK cells from the two groups was similar, since NK cells of PPD-positive subjects mediated 63% reduction in intracellular *M. tuberculosis* compared to CFU within infected MN alone ($P = 0.036$, Fig. 3A), whereas NK cells from PPD-negative subjects mediated 70% reduction in CFU ($P = 0.013$, Fig. 3B).

Killing of intracellular *M. tuberculosis* by NK cells is not associated with IFN- γ production. Because early production of IFN- γ has been demonstrated to be essential for NK cell-mediated protection against other pathogens, we sought to determine whether IFN- γ played a role in the ability of these cells to mediate killing of intracellular *M. tuberculosis*. IFN- γ was measured in supernatants collected at 24 and 96 h from cultures of *M. tuberculosis*-infected MN alone and of infected MN cocultured with either PBL or isolated NK cells. As illustrated in Fig. 4A, none of the cell populations studied produced detectable quantities of IFN- γ following 24 h of coculture with *M. tuberculosis*-infected MN. NK cells also produced only minimal amounts of IFN- γ at 96 h of coculture with *M. tuberculosis*-infected MN (37 ± 36 pg/ml), whereas PBL produced substantial amounts of the cytokine (683 ± 149 pg/ml, $P = 0.0002$ compared to infected MN alone).

To rule out the possibility that NK-cell mediated killing of intracellular *M. tuberculosis* could be mediated by local IFN- γ production not detectable by ELISA, we added anti-IFN- γ

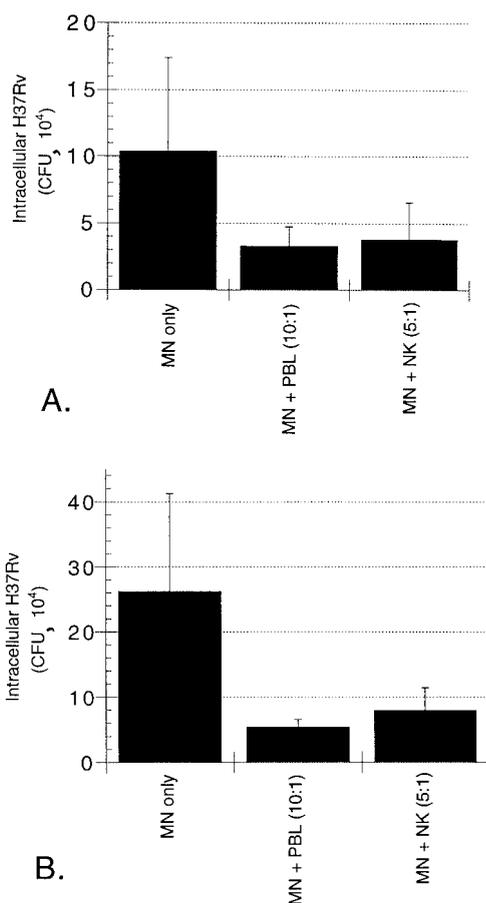


FIG. 3. NK cells from both PPD-positive (A) and PPD-negative (B) subjects mediate a significant reduction in intracellular *M. tuberculosis* at 24 h. The addition of NK cells from PPD-positive donors to *M. tuberculosis*-infected MN resulted in a 63% decrease in intracellular H37Rv at 24 h, whereas the addition of NK cells from PPD-negative donors mediated a 70% reduction in the organism. The NK cell-mediated reduction in intracellular *M. tuberculosis* compared to CFU within MN alone was significant for both groups of subjects ($P = 0.036$ for PPD-positive subjects; $P = 0.013$ for PPD-negative subjects). The graphs illustrate the mean and SD for five subjects in each group.

blocking antibody to cultures. As indicated in Fig. 4B, the addition of anti-IFN- γ had no impact on the ability of NK cells to mediate the killing of intracellular *M. tuberculosis*. Killing by NK cells both in medium alone and in the presence of anti-IFN- γ was statistically significant ($P = 0.011$ and 0.012, respectively, compared to CFU within MN alone)

Transferred supernatants of cocultured NK cells and *M. tuberculosis*-infected MN do not have the capacity to mediate killing of intracellular *M. tuberculosis*. To assess the possibility that NK cell-mediated killing of intracellular *M. tuberculosis* results from the actions of cytokines other than IFN- γ or of combinations of cytokines, we studied the ability of supernatants collected from cocultured NK cells and *M. tuberculosis*-infected MN to alter intracellular growth of *M. tuberculosis* within freshly infected MN. The CFU levels of intracellular *M. tuberculosis* were assessed 24 h after the addition of the supernatants and compared to CFU within MN alone and within MN to which NK cells were added. As illustrated in Fig. 5, NK

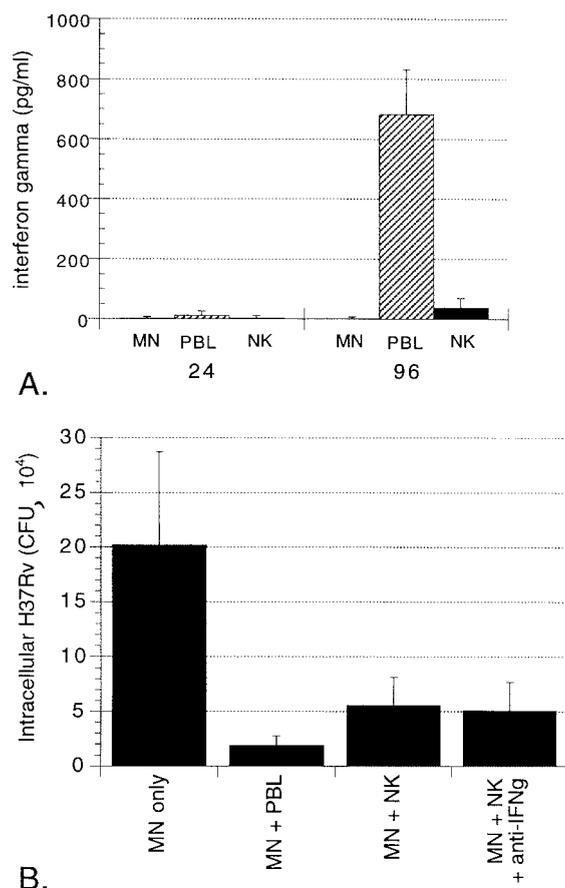


FIG. 4. Killing of intracellular *M. tuberculosis* by NK cells is not mediated by IFN- γ . (A) Results of the measurement of IFN- γ within 24- and 96-h culture supernatants of *M. tuberculosis*-infected MN alone and infected MN cocultured with PBL or NK cells. As illustrated, none of the 24-h supernatants contained appreciable amounts of IFN- γ . At 96 h, PBL had produced significant amounts of IFN- γ in response to *M. tuberculosis*-infected MN. IFN- γ production by NK cells remained minimal, however, and was not significantly greater than within supernatants of infected MN alone. The data represent the mean and SD values for five subjects studied. (B) Effects of addition of blocking anti-IFN- γ polyclonal antibody (10 μ g/ml) to cultures. As shown, blocking antibody had no effect on the NK cell-mediated killing of intracellular *M. tuberculosis*. NK cells in medium alone induced a 72% reduction in intracellular *M. tuberculosis*, whereas in the presence of anti-IFN- γ , NK cells mediated a 75% reduction in intracellular H37Rv ($P = 0.252$ compared to NK cells in medium alone). The results represent the mean and SD of studies of cells obtained from five subjects.

cells mediated significant killing of intracellular *M. tuberculosis* ($P = 0.012$) at 24 h. In contrast, the addition of supernatants collected at 6, 18, and 24 h of coculture did not result in significant reduction of intracellular *M. tuberculosis* compared to that observed in infected MN alone ($P = 0.136$, $P = 0.410$, and $P = 0.471$, respectively).

NK cell-mediated killing of intracellular *M. tuberculosis* requires direct contact between NK cells and *M. tuberculosis*-infected MN. To further assess the role of soluble factors as opposed to direct cell-to-cell contact in killing of *M. tuberculosis* by NK cells, we established cultures of NK cells and *M. tuberculosis*-infected MN within a dual-chamber culture system.

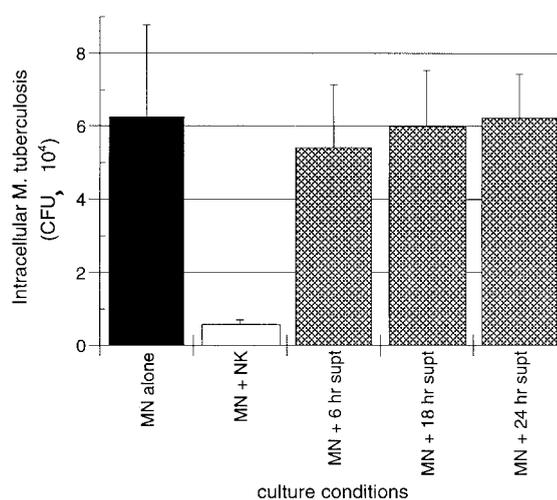


FIG. 5. Supernatants of cocultured NK cells and *M. tuberculosis*-infected MN alone cannot mediate the killing of intracellular *M. tuberculosis*. As illustrated, the 92% reduction in intracellular *M. tuberculosis* mediated by the addition of NK cells (compared to the CFU level within infected MN alone) was much greater than that mediated by the addition of supernatants of cocultured NK cells and infected MN. Supernatants collected after 6, 18, and 24 h of coculture resulted in reductions in intracellular *M. tuberculosis* of only 13, 4, and 1.2%, respectively. The results represent the mean and SD studies from four subjects.

Comparison was made between the CFU levels of intracellular *M. tuberculosis* in cultures in which NK cells were added to infected MN within the same chamber of Transwell culture plates and those in which the same number of NK cells were placed in an upper chamber separated from infected MN by a 4- μ m (pore-size) filter. As shown in Fig. 6, NK cells directly cocultured with infected MN mediated a significant reduction in the intracellular *M. tuberculosis* compared to CFU levels at 24 h within MN alone ($P = 0.011$). In contrast, NK cells placed within the upper chamber did not significantly alter CFU of *M. tuberculosis* within MN on the other side of the filter ($P = 0.248$).

Inhibition of granule release does not affect the ability of human NK cells to kill intracellular *M. tuberculosis*. Because a major effector mechanism of NK cells is that of granule-mediated cytotoxicity, we sought to determine whether this pathway was involved in NK cell-mediated killing of intracellular *M. tuberculosis*. The calcium chelator EGTA inhibits granule release from NK cells and was therefore used to investigate the role of granules in the killing of H37Rv. As illustrated in Fig. 7A, CD3⁻ CD56⁺ NK cells lyse the standard NK target K562 line in a dose-dependent fashion. In the presence of 5 mM EGTA, granule-dependent lysis was completely inhibited, indicating that this concentration of EGTA effectively blocked calcium-dependent granule release and activity. Parallel CFU studies were performed simultaneously using NK cells from the same subjects in order to assess the effects of blocking of granule release on the ability of NK cells to kill intracellular *M. tuberculosis* H37Rv (Fig. 7B). We found that 5 mM EGTA itself had no direct effect on the intracellular growth of *M. tuberculosis*, as illustrated. NK cells in medium reduced the CFU level of intracellular H37Rv by 83% compared to the

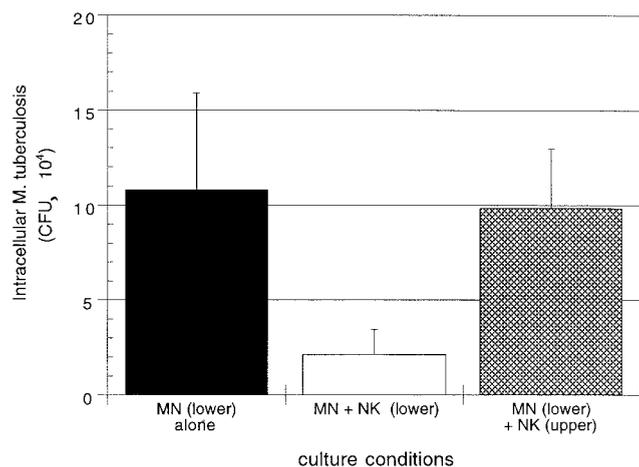
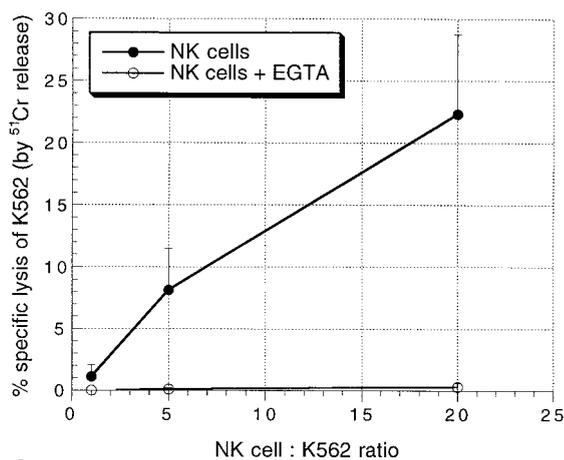


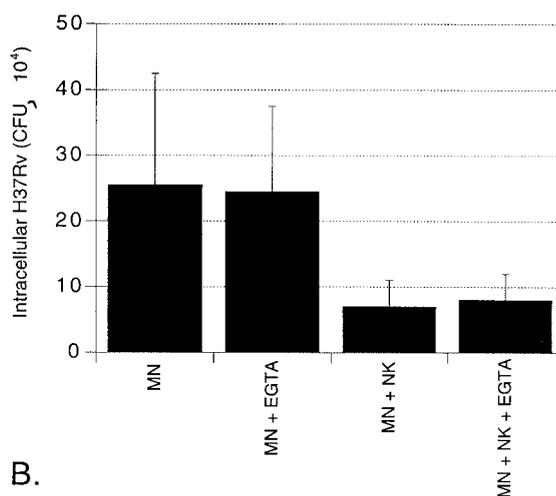
FIG. 6. NK cell-mediated killing of intracellular *M. tuberculosis* requires direct contact between NK cells and infected MN. The killing assay was modified to fit a 24-well dual-chamber Transwell plate format. The addition of NK cells directly to *M. tuberculosis*-infected MN within the lower chamber resulted in an 81% reduction in intracellular *M. tuberculosis* compared to the CFU count within MN alone. In contrast, the addition of NK cells to the top chamber of the Transwell plates, in which these effector cells were separated from infected MN by a 4- μ m membrane, resulted in a nonsignificant 9.5% reduction in intracellular *M. tuberculosis*. The results represent the mean and SD of studies of four subjects.

CFU level found within MN alone. In the presence of 5 mM EGTA, NK cells still mediated 82% reduction of the intracellular *M. tuberculosis*. These studies thus indicated that the killing of intracellular *M. tuberculosis* by NK cells is independent of the release of cytotoxic granules.

NK cells induce apoptosis of *M. bovis* BCG-infected autologous MN. Since previous investigations have demonstrated that chemically induced apoptosis of MN infected with *M. bovis* BCG and virulent *M. tuberculosis* results in the killing of intracellular bacteria, we assessed whether NK cells mediate apoptosis of infected MN. MN apoptosis was measured by the TUNEL method with analysis by flow cytometry to determine the percentage of CD14⁺ MN which were undergoing apoptosis. Because flow cytometry cannot be performed in our biosafety level 3 facility, we infected MN with the attenuated strain *M. bovis* BCG rather than H37Rv for these studies. Representative results are displayed in Fig. 8. MN apoptosis, represented by dual staining with CD14-PE and TUNEL-FITC, was minimal within BCG-infected MN alone at 24 h after infection (Fig. 8A). The addition of NK cells, however, increased the percentage of apoptotic MN from <1% to 18.9% in this subject (Fig. 8B). Table 1 summarizes the mean results of TUNEL assays from separate experiments using NK cells from four donors. As shown, apoptosis was minimal (1.4%) in uninfected MN and was not significantly increased in BCG-infected MN (3.5%, $P = 0.100$). The addition of NK cells to uninfected MN significantly increased apoptosis to 11.5% of MN ($P = 0.014$ compared to MN alone), and apoptosis of BCG-infected MN following the addition of NK cells increased to 21.4% ($P = 0.009$ compared to BCG-infected MN alone, $P = 0.005$ compared to uninfected MN incubated with NK cells).



A.



B.

FIG. 7. NK cell-mediated killing of intracellular *M. tuberculosis* is independent of the release of cytotoxic granules. (A) Purified NK cells in medium alone mediate the lysis of the standard K562 target leukemia cell line in a dose-dependent fashion (●), whereas in the presence of 5 mM EGTA this granule-dependent lysis is completely eliminated (○). The data represent the mean and SD of the percent specific lysis for four subjects studied. (B) Results of parallel studies of the effects of inhibition of granule release on the killing of intracellular *M. tuberculosis*. The reduction in intracellular *M. tuberculosis* by NK cells both in medium alone and in the presence of EGTA was statistically significant ($P = 0.047$ and $P = 0.043$, respectively). The figure illustrates the mean and SD for four subjects studied.

Because it has been reported that virulent *M. tuberculosis*, in comparison to BCG, renders infected cells resistant to apoptosis (1), we made use of an in situ TUNEL assay to confirm that NK cells also induced the apoptosis of H37Rv-infected MN. As the fluorescent micrograph shown in Fig. 9 illustrates, *M. tuberculosis*-infected MN did undergo apoptosis following coculture with human NK cells.

NK cell-mediated killing of intracellular *M. tuberculosis* and apoptosis of infected MN are both independent of FasL-Fas interactions. The best characterized of many known pathways of apoptosis induction is that mediated by the interaction of FasL with Fas. Because NK cells can express FasL, we investigated the role of this interaction in NK cell-mediated killing

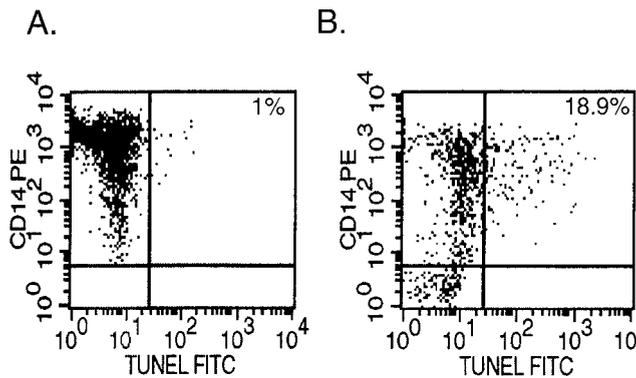


FIG. 8. Representative TUNEL assay showing ability of NK cells to induce apoptosis of BCG-infected human MN. MN apoptosis is indicated as percentage of CD 14-PE positive cells which are also TUNEL-FITC-positive. As illustrated in this example, BCG-infected MN alone display minimal apoptosis (A), whereas 18.9% of CD14+ cells become apoptotic during 24 hours of co-culture with NK cells (B). (Both dot plots represent data gated for monocytes from FSC/SSC images.)

of intracellular *M. tuberculosis* using the neutralizing anti-FasL antibody NOK1 (10 µg/ml). As shown in Fig. 10A, the addition of NOK1 had no effect on the ability of NK cells to kill intracellular H37Rv. For the studies of the four subjects illustrated, NK cells alone mediated 51.1% reduction in intracellular H37Rv compared to MN alone ($P = 0.018$). In the presence of NOK1, The CFU of intracellular *M. tuberculosis* was reduced by 56.2% by NK cells ($P = 0.014$). Figure 10B illustrates the effects of NOK1 on apoptosis. Induction of apoptosis of Jurkat cells by the FasL transfectant KFL9 was significantly inhibited by the addition of NOK1, since the percentage of TUNEL-positive Jurkat cells decreased from 45.3 to 17.1% ($P = 0.039$). In contrast, NK cell-mediated apoptosis of BCG-infected MN was not inhibited by NOK1 (27.7% in medium alone versus 25.8% with NOK1; $P = 0.227$). Thus, both apoptosis of BCG-infected MN and killing of intracellular *M. tuberculosis* by NK cells are mediated by pathways other than those initiated by the interaction of FasL and Fas.

NK cell-mediated killing of intracellular *M. tuberculosis* is not blocked by the simultaneous blocking of NK cell granule release, IFN-γ, and Fas-FasL. To rule out the possibility that multiple mechanisms contributing to NK cell-mediated killing of *M. tuberculosis* must be blocked simultaneously in order to

inhibit the observed killing, we studied the effects of EGTA treatment and the use of neutralizing antibodies to IFN-γ and FasL in various combinations (Fig. 11). In the presence of the various combinations of two of the three inhibitors, as well as the simultaneous blockage of all three pathways, NK cells retained their ability to mediate significant reduction in intracellular *M. tuberculosis* compared to CFU within MN alone, as illustrated ($P < 0.001$ in each case).

DISCUSSION

Human cell-mediated immunity to *M. tuberculosis* is assumed to involve the ability of lymphocytes to activate infected mononuclear phagocytes, resulting in the killing of intracellular bacilli. Nevertheless, studies of the role of lymphocyte responses to *M. tuberculosis* and its antigens have generally not addressed the correlation between the various responses of these cells and bacterial killing. In this study, we demonstrated that human NK cells can mediate the early killing of intracellular *M. tuberculosis*, and we examined the relationship between the various innate immune functions of NK cells and this killing.

Studies of protective human responses to *M. tuberculosis* have largely focused on healthy PPD-positive individuals who are considered to have developed specific immunity to *M. tuberculosis*. PPD-positive individuals display resistance to reinfection upon subsequent exposure to *M. tuberculosis* (37), and the remarkable susceptibility of HIV-infected individuals with dysfunction and depletion of CD4+ T cells provides strong support for the concept that antigen-specific responses play a major role in resistance to *M. tuberculosis* (15, 24). However, a role for innate immune responses in protection against *M. tuberculosis* has been suggested as well by clinical scenarios in which heavily exposed individuals do not develop PPD reactivity (16) and by the observation that some PPD-negative individuals display in vitro responses to *M. tuberculosis* (18, 30). Our finding that unprimed human PBL mediated the killing of the intracellular *M. tuberculosis* within 24 h of coculture with infected MN implied a role for innate immunity in this process. Several effector functions of NK cells may serve to mediate innate immune responses. The “NK activity” which led to the initial identification of this cell population specifically refers to the ability of nonprimed cells to lyse target cells in an HLA-unrestricted manner (31). Furthermore, NK cells can be activated by infected MN independently of specific antigen recognition by cytokines such as interleukin-12 (IL-12), IL-15, and IL-18 (10, 20, 43). Activated NK cells can serve as early sources of IFN-γ (28, 32, 38) and of proinflammatory chemokines such as MIP1α (7).

We observed that unstimulated NK cells could mediate killing of intracellular H37Rv at 24 h of coculture in a dose-dependent fashion. Previous studies have reported that NK cells can inhibit intracellular growth of *M. avium* (5) and the avirulent *M. tuberculosis* strain H37Ra (45). Although killing of a recent clinical isolate of *M. tuberculosis* by IL-12-stimulated NK cells has also been observed (13), the current findings provide the first report of the ability of unstimulated NK cells to mediate the killing of a well-characterized virulent strain of *M. tuberculosis*. Our subsequent studies were aimed at identifying the mechanisms by which this killing occurred.

TABLE 1. NK cells induce apoptosis of BCG-infected human MN

Cells in culture	% Apoptosis of CD14+ MN ^a (SD)
Uninfected alone	1.4 (1.7)
BCG-infected MN	3.5 (1.5)
Uninfected MN plus NK cells	11.5 (5.0) ^b
BCG-infected MN plus NK cells.....	21.4 (8.2) ^c

^a Values represent the mean and SD of the percentage of CD14+ monocytes undergoing apoptosis (as measured by the TUNEL assay) for studies of NK cells from four subjects.

^b $P = 0.014$ compared to uninfected MN alone as calculated by the paired *t* test.

^c $P = 0.009$ compared to BCG-infected MN alone; $P = 0.005$ compared to uninfected MN plus NK cells.

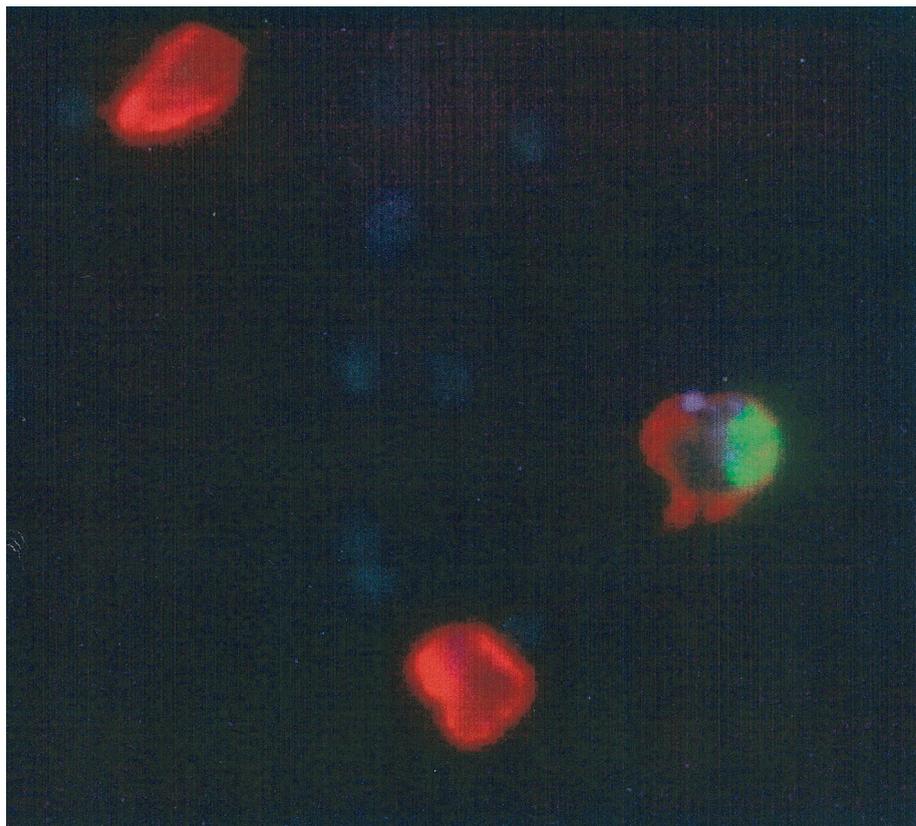


FIG. 9. MN infected with virulent *M. tuberculosis* H37Rv undergo apoptosis in the presence of NK cells. H37Rv was stained with the fluorescent blue dye AlexaFluor 350 prior to infection. After 24 h of coculture with NK cells, MN were labeled with anti-CD14-PE, and cytospin slides were prepared for the in situ determination of apoptosis by using an FITC-labeled TUNEL reagent. As shown in this composite image, the addition of the smaller, unstained NK cells resulted in apoptosis (green) of the red-stained MN infected with (blue) AlexaFluor 350-stained H37Rv.

The antimicrobial functions of NK cells have been studied in murine models of infection with a wide variety of intracellular pathogens. The production of IFN- γ by NK cells plays an essential role in the containment of murine infection with cytomegalovirus, listeria, and various intracellular parasites (3, 28, 32). In contrast, we observed that the killing of intracellular H37Rv by human NK cells was not associated with the production of IFN- γ . The finding that neutralizing antibodies to IFN- γ had no effect on NK cell-mediated killing of *M. tuberculosis* further rules out a role for IFN- γ in this process. These observations are consistent with an earlier report that PPD does not induce human NK cells to produce IFN- γ unless activated T cells are present as well (19). Our subsequent studies involving both the transfer of supernatants of cocultured NK cells and *M. tuberculosis*-infected MN and the assessment of NK cell activity in Transwell cultures further indicate that NK cell-mediated killing of intracellular *M. tuberculosis* cannot be attributed to the effects of soluble mediators. We therefore investigated the role of the cytotoxic effector functions of human NK cells in the killing of intracellular *M. tuberculosis*.

Because NK cell cytotoxicity has classically been attributed to the release of cytotoxic granules containing perforin and other effector molecules (17), we next sought to determine whether granule release was essential for NK cell-mediated killing of intracellular *M. tuberculosis*. Free calcium is required

for the exocytosis of NK cell cytotoxic granules, and the calcium chelator EGTA has therefore been utilized to demonstrate granule-independent effects of NK cells (44). The addition of EGTA to our cultures blocked the granule-mediated lysis of K562 targets by NK cells but had no effect on the ability of NK cells to kill intracellular H37Rv. These findings indicate that the killing of *M. tuberculosis* by NK cells is independent of granule exocytosis. Our observations contrast with those reported in studies of CD1-restricted cytotoxic T-cell lines recognizing lipid and lipoglycan antigens of *M. tuberculosis* (39). The CD1-restricted lymphocytes were found to comprise two distinct populations that exhibited differing patterns of cytotoxicity. CD4⁻ CD8⁺ lymphocytes killed infected MN by granule-mediated cytotoxicity that resulted in the killing of intracellular *M. tuberculosis*, whereas CD4⁻ CD8⁻ lymphocytes killed infected MN via Fas-FasL-induced apoptosis and had no effect on the viability of the organism. The clinical significance of CD1-restricted lymphocytes remains uncertain, however, and it has recently been suggested that the CD1 pathway may in fact be coopted by *M. tuberculosis* in order to facilitate bacterial survival (34). Our assessment that cytotoxic granules do not play a role in the killing of *M. tuberculosis* by NK cells is consistent with two studies which reported that the course of *M. tuberculosis* infection in perforin knockout mice does not differ from that observed in wild-type animals (11, 22), as well as with the observation that chemically induced necrosis of

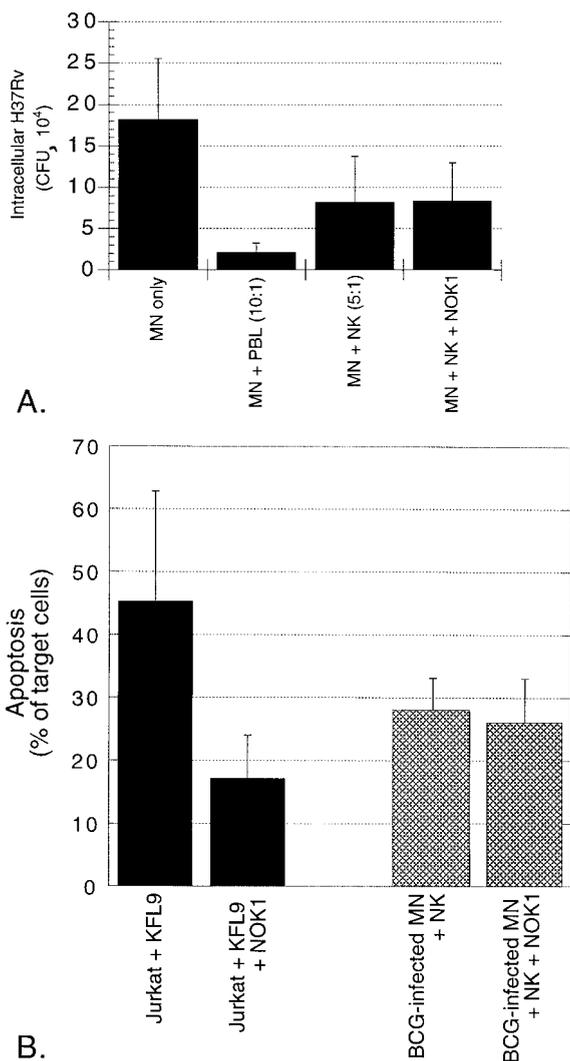


FIG. 10. Both NK cell-mediated apoptosis of BCG-infected MN and killing of intracellular *M. tuberculosis* are independent of Fas-FasL interactions. Anti-FasL blocking antibody NOK1 (10 µg/ml) was added to cultures containing infected MN and NK cells. Panel A shows the mean and the SD for CFU studies of five subjects. As illustrated, the addition of NOK1 had no effect on the ability of NK cells to kill intracellular H37Rv. Likewise, although NOK1 did inhibit the FasL-dependent induction of apoptosis of Jurkat cells by the FasL-transfectant KFL9 cell line, NK cell-mediated apoptosis of MN-infected *M. bovis* BCG was not affected by the addition of NOK1, as shown in panel B. Jurkat-KFL9 results represent the mean and SD from triplicate experiments. MN-NK data is presented as mean and SD from studies of four subjects.

BCG-infected human MN does not result in the killing of intracellular organisms (25). An earlier report by Molloy et al. found that human LAK cells lysed BCG-infected MN but that lysis did not result in the killing of the organism (26). Although a large proportion LAK precursors are CD56⁺ CD3⁻ NK cells, the authors' observation that the cells with lytic activity specific for mycobacterium-infected cells were CD56⁻ CD3⁺ emphasizes the fact that an IL-2-stimulated LAK population is not equivalent to the isolated NK cells we studied. In addition, IL-2 stimulation may not enhance the other activities of LAK

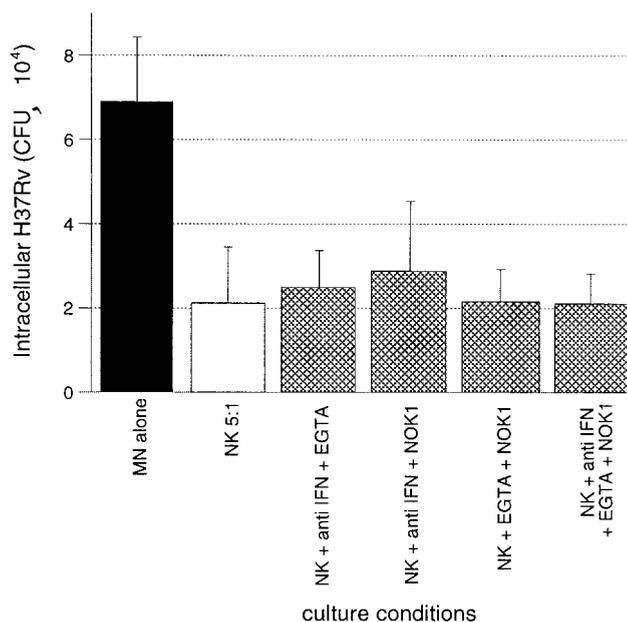


FIG. 11. NK cell-mediated killing of intracellular *M. tuberculosis* cannot be accounted for by interactions of IFN-γ, cytotoxic granule release, and Fas-FasL interactions. To assess the possibility that the additive effects of these pathways were required in the killing of *M. tuberculosis* by NK cells, neutralizing anti-IFN-γ monoclonal antibody, EGTA, and NOK1 (anti-FasL) were added to cocultures of NK cells and infected MN in combination. As shown, none of the combinations of two or all three of the inhibitors led to a significant decrease in the ability of NK cells to kill intracellular *M. tuberculosis*. The results represent the mean and SD for five subjects studied.

precursor cells as it does their capacity for cytolysis. The culture conditions used in that study also differed from ours in that LAK cells were added to MN 6 days after infection, at which time the infected cells were reported to contain an average of 10 to 20 bacilli. This level of infection could have resulted in a model system more conducive to cell lysis and less so to bacterial killing than the early, low-level infection model we studied.

The finding that NK cells induce apoptosis of BCG-infected MN is intriguing as a possible mechanism of NK cell-mediated bacterial killing in light of previous studies of cytotoxicity directed at MN infected with both *M. bovis* BCG (21, 25) and *M. tuberculosis* (27). These investigations showed that chemical- or cytokine-induced apoptosis of the infected cells was associated with decreased viability of the mycobacteria within the target MN. Although the protocol used in these studies resulted in the infection of 65 to 75% of monocytes, only 21% of monocytes were found to be apoptotic by TUNEL analysis after 24 h of coculture with NK cells. Nevertheless, the addition of NK cells mediated a 60 to 80% reduction in the CFU level of intracellular *M. tuberculosis* at the same time point. This discrepancy could indicate that the killing of intracellular *M. tuberculosis* is not tied to NK cell-mediated monocyte apoptosis. Alternatively, it may be that bacterial killing is a more rapid effect of the relevant apoptotic pathways than the DNA splicing which results in positive TUNEL staining. In this case, the timing of our assays could underestimate the ultimate extent of MN apoptosis occurring within the cultures. An ad-

ditional possibility is that the apoptosis of some cells serves to activate effector functions of nonapoptotic MN, resulting in bacterial killing. This latter mechanism has been described in *in vitro* studies of MN infection with *M. avium* (14). Previous studies had indicated that infection with virulent *M. tuberculosis* increases the resistance of isolated MN to apoptosis and that this resistance is based on increased production of soluble tumor necrosis factor (TNF) receptor by the infected cells (1). Our fluorescent microscopy studies indicate that MN infected with virulent H37Rv are susceptible to NK cell-mediated apoptosis. Because of the apparent specificity of these previous observations for TNF-mediated apoptosis, however, our findings do not necessarily contradict this earlier report.

Because of the possible relationship of the observed NK cell-mediated apoptosis to the killing of intracellular *M. tuberculosis*, we investigated a possible role of the interaction of FasL and Fas in this killing. Our studies showed that blocking of this apoptotic pathway had no effect on the ability of NK cells to mediate the killing of intracellular *M. tuberculosis* or upon NK cell-mediated apoptosis of BCG-infected MN. In addition to FasL, however, NK cells have recently been shown to express other apoptosis-inducing surface ligands, including membrane-bound TNF (23), TRAIL (TNF-related apoptosis-inducing ligand) (46), and CD40L (9). Our findings suggest that NK cell-induced apoptosis of infected MN is mediated by these or other as-yet-undescribed pathways and may be linked to the killing of intracellular *M. tuberculosis*.

In summary, unstimulated NK cells from both PPD-positive and PPD-negative subjects can mediate the early killing of intracellular *M. tuberculosis* H37Rv. This killing, which takes place within the first 24 h of coculture, is not associated with the production of IFN- γ by NK cells and does not require the release of NK cell cytotoxic granules, but it is associated with the induction of NK cell-mediated MN apoptosis. Both the killing of intracellular *M. tuberculosis* and the induction of MN apoptosis by NK cells are independent of the interaction of FasL and Fas. Further clarification of the mechanisms by which NK cells mediate the killing of intracellular *M. tuberculosis* may facilitate the development of strategies for augmenting innate immunity as a means of prevention and treatment of tuberculosis. Such interventions may be particularly relevant for HIV-infected individuals in whom antigen-specific CD4⁺ T-cell responses to *M. tuberculosis* are impaired. In addition, understanding of the mechanisms by which unstimulated NK cells mediate the killing of intracellular *M. tuberculosis* may help illuminate the pathways by which stimulated antigen-specific lymphocyte populations contain this important pathogen.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants HL59851, AI35027, AI27243, AI95383, AI36219, and AI01581 and by American Lung Association Research grant RG-1489-N. R. F. Silver was also supported by a Parker B. Francis Fellowship in Pulmonary Research sponsored by the Francis Families Foundation.

We thank Abhay Patki and Roxana Rojas of Case Western Reserve University for assistance with the protocols for the TUNEL assays and the MACS sorting system, respectively. We also thank Laurie Hall and Eric Pearlman of Case Western Reserve University for critical review of the manuscript.

REFERENCES

- Balcewicz-Sablinska, M. K., J. Keane, H. Kornfeld, and H. G. Remold. 1998. Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF α . *J. Immunol.* **161**:2636–2641.
- Bancroft, G. 1993. The role of natural killer cells in innate resistance to infection. *Curr. Opin. Immunol.* **5**:503–510.
- Bancroft, G. J., M. J. Bosma, G. C. Bosma, and E. R. Unanue. 1986. Regulation of macrophage Ia expression in mice with severe combined immunodeficiency: induction of Ia expression by a T cell independent mechanism. *J. Immunol.* **137**:4–9.
- Barnes, P. F., C. L. Grisso, J. S. Abranms, H. Band, T. H. Rea, and R. L. Modlin. 1992. $\gamma\delta$ T lymphocytes in human tuberculosis. *J. Infect. Dis.* **165**:506–512.
- Bermudez, L. E., M. Wu, and L. S. Young. 1995. Interleukin-12 stimulated natural killer cells can activate human macrophages to inhibit growth of *Mycobacterium avium*. *Infect. Immun.* **63**:4099–4104.
- Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* **17**:189–220.
- Bluman, E. M., K. J. Bartynski, B. R. Avalos, and M. A. Caligiuri. 1996. Human natural killer cells produce abundant macrophage inflammatory protein 1- α in response to monocyte-derived cytokines. *J. Clin. Investig.* **97**:2722–2727.
- Boom, W. H., R. S. Wallis, and K. A. Chervenak. 1991. Human *Mycobacterium tuberculosis*-reactive CD4⁺ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. *Infect. Immun.* **59**:2737–2743.
- Carbone, E., G. Ruggiero, G. Terrazzano, C. Palomba, C. Manzo, S. Fontana, H. Spits, K. Karre, and S. Zappacosta. 1997. A new mechanism of NK cell cytotoxicity activation: The CD40-CD40 ligand interaction. *J. Exp. Med.* **185**:2053–2060.
- Carson, W. E., M. E. Ross, R. A. Baiocchi, M. J. Marien, N. Boiani, K. Grabstein, and M. A. Caligiuri. 1995. Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon- γ by natural killer cells *in vitro*. *J. Clin. Investig.* **96**:2578–2582.
- Cooper, A. M., C. D'Souza, A. A. Frank, and I. M. Orme. 1997. The course of *Mycobacterium tuberculosis* infection in the lungs of mice lacking expression of either perforin- or granzyme-mediated cytolytic mechanisms. *Infect. Immun.* **65**:1317–1320.
- De Libero, G., I. Flesch, and S. H. E. Kaufmann. 1988. Mycobacteria-reactive Lyt-2⁺ T cell lines. *Eur. J. Immunol.* **18**:59–66.
- Denis, M. 1994. Interleukin-12 augments cytolytic activity of natural killer cells toward *Mycobacterium tuberculosis*-infected human monocytes. *Cell. Immunol.* **156**:529–536.
- Fratuzzi, C., R. D. Arbeit, C. Carini, and H. G. Remold. 1997. Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. *J. Immunol.* **158**:4320–4327.
- Havir, D. V., and P. F. Barnes. 1999. Tuberculosis in patients with human immunodeficiency virus infection. *N. Engl. J. Med.* **340**:367–373.
- Houk, V. N., J. H. Baker, K. Sorensen, and D. C. Kent. 1968. The epidemiology of tuberculosis infection in a closed environment. *Arch. Environ. Health.* **16**:26.
- Kagi, D., B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis *in vivo*. *Annu. Rev. Immunol.* **14**:207–232.
- Kemp, E. B., R. B. Belshe, and D. F. Hoft. 1996. Immune responses stimulated by percutaneous and intradermal Bacille Calmette-Guerin. *J. Infect. Dis.* **174**:113–119.
- Kemp, K., L. Hviid, A. Kharazmi, and M. Kemp. 1997. Interferon γ production by human T-cells and natural killer cells *in vitro* in response to antigens from the two intracellular pathogens *Mycobacterium tuberculosis* and *Leishmania major*. *Scand. J. Immunol.* **46**:495–499.
- Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* **170**:827–837.
- Lammass, D. A., C. Stober, C. J. Harvey, N. Kendrick, S. Panchalingam, and D. S. Kumararatne. 1997. ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. *Immunity* **7**:433–444.
- Laochumroonvorapong, P., J. Wang, C.-C. Liu, W. Ye, A. L. Moriera, K. B. Elkon, V. H. Freedman, and G. Kaplan. 1997. Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for early control of mycobacterial infection in mice. *Infect. Immun.* **65**:127–132.
- Lee, R. K., J. Spielman, D. Y. Zhao, K. J. Olsen, and E. R. Podack. 1996. Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells. *J. Immunol.* **157**:1919–1925.
- Markowitz, N., N. I. Hansen, P. C. Hopewell, J. Glassroth, P. A. Kvale, B. T. Mangura, T. C. Wilcosky, J. M. Wallace, M. J. Rosen, and L. B. Reichman. 1997. Incidence of tuberculosis in the United States among HIV-infected

- persons. *Ann. Intern. Med.* **126**:123–32.
25. **Molloy, A., P. Laochumroonvorapong, G. Kaplan.** 1994. Apoptosis, but not necrosis, of infected monocytes in coupled with killing of intracellular *Bacillus Calmette-Guerin*. *J. Exp. Med.* **180**:1499–1509.
 26. **Molloy, A., P. A. Meyn, K. D. Smith, and G. Kaplan.** 1993. Recognition and destruction of *Bacillus Calmette-Guerin*-infected human monocytes. *J. Exp. Med.* **177**:1691–1698.
 27. **Oddo, M., T. Renno, A. Attinger, T. Bakker, H. R. MacDonald, and P. A. R. Meylan.** 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. *J. Immunol.* **160**:5448–5454.
 28. **Orange, J. S., B. P. Wang, C. Terhorst, and C. A. Biron.** 1995. Requirement for natural killer cell-produced interferon γ in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J. Exp. Med.* **182**:1045–1056.
 29. **Oshimi, Y., S. Oda, Y. Honda, S. Nagata, and S. Miyazki.** 1996. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. *J. Immunol.* **157**:2909–2915.
 30. **Ravn, P., H. Boesen, B. K. Pedersen, and P. Andersen.** 1997. Human T cell responses induced by vaccination with *Mycobacterium bovis* Bacillus Calmette-Guerin. *J. Immunol.* **158**:1949–1955.
 31. **Robertson, M. J., and J. Ritz.** 1990. Biology and clinical relevance of human natural killer cells. *J. Am. Soc. Hematol.* **76**:2421–2438.
 32. **Scharton-Kersten, T. M., and A. Sher.** 1997. Role of natural killer cells in innate resistance to protozoan infections. *Curr. Opin. Immunol.* **9**:44–51.
 33. **Schlesinger, L.** 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *J. Immunol.* **150**:2920–2930.
 34. **Shinkai, K., and R. M. Locksley.** 2000. CD1, tuberculosis, and the evolution of major histocompatibility complex molecules. *J. Exp. Med.* **191**:907–914.
 35. **Sieg, S., D. Smith, and D. Kaplan.** 1999. Differential activity of soluble versus cellular Fas ligand: regulation by an accessory molecule. *Cell. Immunol.* **195**:89–95.
 36. **Silver, R. F., Q. Li, W. H. Boom, and J. J. Ellner.** 1998. Lymphocyte-dependent inhibition of growth of virulent *Mycobacterium tuberculosis* H37Rv within human monocytes: requirement for CD4⁺ T-cells in PPD-positive but not in PPD-negative subjects. *J. Immunol.* **160**:2408–2417.
 37. **Stead, W. W.** 1995. Management of health care workers after inadvertent exposure to tuberculosis: a guide for the use of preventative therapy. *Ann. Intern. Med.* **122**:906–912.
 38. **Stein-Streilein, J., and J. Guffee.** 1986. In vivo treatment of mice and hamsters with antibodies to asialo GM1 increased morbidity and mortality to pulmonary influenza infection. *J. Virol.* **136**:1435–1441.
 39. **Stenger, S., R. J. Mazzaccaro, K. Uyemura, C. Cho, P. F. Barnes, J.-P. Rosat, A. Sette, M. B. Brenner, S. A. Porcelli, B. R. Bloom, and R. L. Modlin.** 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science* **276**:1684–1687.
 40. **Sudre, P., G. ten Dam, and A. Kochi.** 1992. Tuberculosis: A global overview of the situation today. *Bull. W. H. O.* **70**:149–159.
 41. **Tan, J. S., D. H. Canaday, W. H. Boom, K. N. Balaji, S. K. Schwander, and E. A. Rich.** 1997. Human alveolar T lymphocyte responses to *Mycobacterium tuberculosis* antigens: role for CD4⁺ and CD8⁺ cytotoxic T cells and relative resistance of alveolar macrophages to lysis. *J. Immunol.* **159**:290–297.
 42. **Tsukaguchi, K., K. Balaji, and W. Boom.** 1995. CD4 $\alpha\beta$ T cell and $\gamma\delta$ T-cell responses to *Mycobacterium tuberculosis*: similarities and differences in Ag recognition, cytotoxic effector function, and cytokine production. *J. Immunol.* **154**:1786–1796.
 43. **Ushio, S., M. Namba, T. Okura, K. Hattori, Y. Nukada, K. Akita, F. Tanabe, K. Konishi, M. Micalled, M. Fujii, K. Torigoe, T. Tanimoto, S. Fukuda, M. Ikeda, H. Okamura, and M. Kurimoto.** 1996. Cloning of the cDNA for human IFN γ -inducing factor, expression in *Escherichia coli*, and studies on the biologic activities of the protein. *J. Immunol.* **156**:4274–4279.
 44. **Vujanovic, N. L., S. Nagashima, R. B. Herberman, and T. L. Whiteside.** 1996. Nonsecretory apoptotic killing by human NK cells. *J. Immunol.* **157**:1117–1126.
 45. **Yoneda, T., and J. J. Ellner.** 1998. CD4⁺ T cell and natural killer cell-dependent killing of *Mycobacterium tuberculosis* by human monocytes. *Am. J. Respir. Crit. Care Med.* **158**:395–403.
 46. **Zamai, L., M. Ahman, I. M. Bennett, L. Azzoni, E. S. Alnemri, and B. Perussia.** 1998. Natural killer cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *J. Exp. Med.* **188**:2375–2380.

Editor: S. H. E. Kaufmann