

NK cells kill mycobacteria directly by releasing perforin and granulysin

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ABSTRACT

Although the mechanisms underlying the cytotoxic effect of NK cells on tumor cells and intracellular bacteria have been studied extensively, it remains unclear how these cells kill extracellular bacterial pathogens. In this study, we examine how human NK cells kill *Mycobacterium kansasii* and *M.tb*. The underlying mechanism is contact dependent and requires two cytolytic proteins: perforin and granulysin. Mycobacteria induce enhanced expression of the cytolytic proteins via activation of the NKG2D/NCR cell-surface receptors and intracellular signaling pathways involving ERK, JNK, and p38 MAPKs. These results suggest that NK cells use similar cellular mechanisms to kill both bacterial pathogens and target host cells. This report reveals a novel role for NK cells, perforin, and granulysin in killing mycobacteria and highlights a potential alternative defense mechanism that the immune system can use against mycobacterial infection. *J. Leukoc. Biol.* 96: 1119–1129; 2014.

Introduction

NK cells play an important role in the host innate immune system by killing tumor and virus-infected cells through contact-induced apoptosis [1, 2]. NK cells recognize tumor and virus-infected cells through specific interactions between NCRs and cell-surface antigens expressed on target cells [3–5]. Activation of NCRs (e.g., NKp30, NKp44, and NKp46) and the

NKG2D receptor stimulates several MAPKs, including ERK, JNK, and p38 kinase [6–8]. These intracellular signaling events lead to reorientation of microtubule organizing centers toward the target cell and polarization of secretory granules containing the cytolytic proteins perforin, granulysin, and granzymes [9, 10]. Perforin is a pore-forming protein that allows delivery of the proapoptotic proteins granzyme and granulysin into the cytoplasm of the target cells, therefore inducing target cell death by apoptosis [11–14].

Whereas previous reports have suggested that NK cells may possess antibacterial activity [15, 16], it remains unclear how NK cells directly kill extracellular bacterial pathogens, such as mycobacteria. In addition, the cellular mechanism responsible for the antibacterial activity of NK cells has not been characterized. With the use of the tuberculosis vaccine strain of *Mycobacterium bovis* BCG as a model, Esin et al. [17] demonstrated that NK cells can interact directly with *M. bovis* BCG, inducing NK cell cytotoxic activity without the need for accessory host cells [18]. Besides bacteria, NK cells also showed a direct killing effect on the eukaryotic yeast pathogen, *Cryptococcus neoformans* [19]. In spite of these studies, the possibility that NK cells may directly bind and kill mycobacteria through a mechanism similar to the one used to kill tumor and virus-infected cells has not been investigated.

Here, we examine the interaction between human pNK cells and the pathogens *M. kansasii* and *M.tb*, which represent the cause of severe pulmonary and systemic infections in humans [20–23]. We show that pNK cells bind directly to the mycobacteria using nanotube-like structures, redistribute their cytoplasmic organelles, and kill the bacteria using the cytotoxins per-

Abbreviations: BCG=bacillus Calmette-Guérin, DIC=differential interference contrast, M.tb=Mycobacterium tuberculosis, NCR=natural cytotoxicity receptor, NKG2D=NK group 2D, NLR=nucleotide-binding oligomerization domain-like receptor, pNK=primary NK, SEM=scanning electron microscopy, siRNA=small interfering RNA

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forin and granulysin. The pNK cells are stimulated through the activating receptors, NCRs and NKG2D, followed by activation of MAPKs and enhanced production of perforin and granulysin. These findings thus reveal a novel function for NK cells during the immune response against mycobacterial infection.

MATERIALS AND METHODS

Cells and cell culture

The human NK cell line NK92 (ATCC CRL-2407) was derived from a case of non-Hodgkin's lymphoma, as described previously [24]. NK92 cells were maintained in culture with 100 IU/ml IL-2 (PeproTech, Rocky Hill, NJ, USA). Human pNK cells were collected from healthy individuals using a published protocol [25]. The use of human samples in this study was approved by the Institutional Review Board of Linkou Chang Gung Memorial Hospital, and written, informed consent was obtained from the volunteers who provided blood samples. Briefly, pNK cells were isolated from peripheral blood using the EasySep NK cell enrichment kit, as described by the manufacturer (Stemcell Technologies, Grenoble, France). The purity of pNK cells was verified systematically by flow cytometry, based on CD16, CD56, and CD3 expression. pNK cells were used only when cell preparations contained >90% CD16⁺CD56⁺ cells and were devoid of CD3⁺ cells. pNK cells were activated by adding 200 IU/ml IL-2 (PeproTech) to the culture medium for 36–48 h. All cell lines were cultured in α MEM (Gibco, Carlsbad, CA, USA), containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 12.5% horse serum, and 100 IU/ml IL-2. As a negative control, B cells were purified with a negative selection protocol using EasySep (Stemcell Technologies) and were routinely >98% pure, as revealed by CD19 expression.

Culture of mycobacteria

The *M. kansasii*-type strain (ATCC 12478) and *M.tb* H37Rv were obtained from American Type Culture Collection (Manassas, VA, USA). Bacteria were grown at 37°C on Middlebrook 7H11 agar or in 7H9 broth (Difco, Franklin Lakes, NJ, USA), supplemented with 0.5% glycerol and 10% oleic acid-bovine albumin-dextrose-catalase (Becton Dickinson, Franklin Lakes, NJ, USA). Bacteria were transferred into 7H9 broth media (Difco), containing 10% glycerol, adjusted to optical density = 0.8 (absorbance at 600 nm), and stored at –80°C.

Mycobacterial killing assay

For the mycobacterial killing assay by perforin or granulysin, mycobacteria were resuspended in PBS or grown in 7H9 broth, in which different concentrations of commercially available human perforin (50 μ g/ml; ALX-200-604; Enzo Life Sciences, Farmingdale, NY, USA) or granulysin (30 μ g/ml; 3138-GN/CF; R&D Systems, Minneapolis, MN, USA) were added. After a given time period, CFUs were determined by preparation of serial bacterial dilutions in 7H9 broth, followed by plating on Middlebrook 7H11 plates. Bacterial colonies were counted after incubation at 37°C for 14–18 days. Bacteria were prepared in parallel for SEM. Experiments using *M.tb* were performed in a P3-level laboratory at Linkou Chang Gung Memorial Hospital. For evaluation of bacteriostatic or killing effect by NK cells, the Live/Dead BacLight bacterial viability kit (Invitrogen, Carlsbad, CA, USA) was used.

Transwell assay

A device containing a polycarbonate membrane with a pore size of 0.45 μ m (Becton Dickinson) was inserted into the wells of 24-well culture plates to determine whether direct cell contact is required for killing of *M. kansasii* or *M.tb*. Mycobacteria were cultured in the lower chamber and pNK cells in the upper chamber. After 0, 24, or 48 h, mycobacterial growth from the various fractions was examined.

SEM

NK cells were incubated with *M. kansasii* and *M.tb*, respectively, for the indicated times in a cell-culture incubator at 37°C. For SEM, NK cells were infected in tubes and then transferred onto polylysine-coated glass coverslips. Samples were fixed and dehydrated using standard procedures and then critical-point dried in CO₂ before examination under a Hitachi S-5000 microscope (Tokyo, Japan).

Immunofluorescence staining and confocal microscopy

NK cells were incubated with mycobacteria for 72 h in an incubator at 37°C. For confocal microscopy, the cells were fixed, permeabilized, and blocked, as described previously [26]. Mycobacteria were stained with the Live/Dead BacLight kit (1:20 dilution, L7012; Invitrogen), followed by incubation with NK cells at an E:T of 100. After incubation for 24 h, NK cells were labeled with mouse anti-human perforin (labeled as red dots), followed by Alexa 555-conjugated goat anti-mouse IgG and phalloidin-Alexa 350 for staining F-actin (labeled as blue dots). Cells were washed, mounted on slides, and visualized by DIC microscopy using the DeltaVision microscope (Applied Precision, Issaquah, WA, USA). DIC and fluorescent images represent one deconvolved Z-stack, obtained using the digital deconvolution program, SoftWoRx (Applied Precision). To quantify nanotube formation, NK cells were labeled with mouse anti-human perforin IgG, followed by Alexa 555-conjugated goat anti-mouse IgG and phalloidin-Alexa 350 for staining F-actin and mixed with an equal number of mycobacteria before coincubation on a fibronectin-coated glass surface and visualization under confocal microscopy.

siRNA transfection

The siRNAs against human p38 (sc-29433), JNK-1 (sc-29380), ERK-2 (sc-35335), NKG2D (sc-42948), NKp44 (sc-72170), NKp46 (sc-63344), NKp30 (sc-42950), and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NK92 cells were counted, cell density was determined, and the required number of cells (5 \times 10⁶ cells/sample) was centrifuged at 1200 rpm for 5 min at room temperature and rinsed with PBS. Cell pellets were resuspended in 100 μ l human cell nucleofector solution (82 μ l nucleofector solution and 18 μ l supplement; Amaxa, Gaithersburg, MD, USA), according to the manufacturer's instructions. Cell suspension (100 μ l) was combined with 300 nM siRNA and transferred into a certified cuvette. The electroporation program A-024 was used. Culture medium (1900 μ l) was added to the cuvette. The mixture was gently transferred into a six-well plate and analyzed after 24 h.

Quantitative RT-PCR

Transcription of NCRs and NKG2D, granulysin, perforin, and β -actin was assayed by reverse transcription, followed by PCR analysis. Total cellular RNA was extracted using the TRIzol reagent kit (Invitrogen). Oligo-deoxythymines were used to prime cDNA synthesis. The reaction mixture was then cooled rapidly on ice, and 4 μ l 5 \times RT buffer containing 10 mM DTT, 100 μ M dNTP, and 200 U Superscript III RT (Invitrogen) was added. This reaction mixture was incubated for 1 h at 50°C, and the reaction was stopped by heating at 70°C for 15 min. The PCR reaction was performed for 1 min each at 95°C, 60°C, and 72°C in a 2 \times Extensor Hi-Fidelity PCR Master Mix (consisting of 350 μ M dNTP, 2.25 μ M MgCl₂, and 2.5 U AB-gene extensor PCR enzyme; Thermo Fisher Scientific, Loughborough, UK) for 25–30 cycles.

Western blot analysis

NK cells (10⁶/flask) were plated for 48 h in IL-2-free medium containing 12.5% horse serum and 12.5% FBS. Cells were then treated with the indicated inhibitors for 30 min, followed by treatment with mycobacteria for 24 and 48 h. Washed cells were lysed in Mammalian Protein Extraction Reagent (Pierce Chemical, Rockford, IL, USA). Total lysate protein samples

(40 $\mu\text{g}/\text{lane}$) were fractionated on a 10% SDS polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat milk for 1 h at room temperature in TBS (Tris 10 mM, NaCl 150 mM, pH 7.6), containing 0.1% Tween 20 (TBST) and probed with primary antibodies (1:1000 for anti-perforin, anti-NKG2D, anti-ERK, anti-JNK, anti-p38, anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, and 1:10,000 for anti-GAPDH) overnight at 4°C. Membranes were then incubated with appropriate HRP-conjugated secondary antibodies (1:5000). Immunoreactive protein bands were developed using the ECL detection kit (Amersham Pharmacia Biotech, Amersham, UK).

Statistical analysis

Data obtained from three replicate experiments are shown as means \pm SE. Statistical analysis was performed using unpaired *t*-tests. A difference between results of two assay conditions that gave $P < 0.05$ was considered to be significant.

RESULTS

Contact-dependent killing of mycobacteria by pNK cells

Previous studies have shown that NK cells can directly kill some pathogens, such as the yeast *C. neoformans* [19, 27]. To examine the mycobacterial killing activity of NK cells, we cultured pNK cells with *M.tb* at different E:T ratios of 10:1, 20:1, and 100:1, respectively, for 24 and 48 h (Fig. 1). In contrast to the B cell control, the number of viable mycobacteria was reduced significantly up to 30% and 60% at the ratio of 20:1 and 100:1, respectively [Fig. 1; pNK+*M.tb* (E:T)]. Given that these observations may be a result of bacteriostatic or killing effects, we used the BacLight staining assay to characterize the viability of the treated mycobacteria. Approximately 50–70% of mycobacteria were dead after 24 and 48 h of incubation with pNK cells (data not shown). Below the E:T ratio of 100:1, this phenomenon was not observed when bacteria were cultured, separated from pNK cells, using a transwell system that allowed exchange of the culture supernatants but prevented direct bacteria-cell contact [Fig. 1; pNK/(/)*M.tb*]. Similarly, under different E:T ratios, no killing was observed when the bacteria were separated from pNK cells (Fig. 1; pNK/*M.tb*). Coculture of pNK cells with mycobacteria also failed to kill the bacteria cultured separately in the transwell system (Fig. 1;

pNK+*M.tb*/*M.tb*). These results indicate that direct contact is required for mycobacterial killing by pNK cells. Similar results were observed when the NK92 cell line was treated with *M. kansasii* (Supplemental Fig. 1). NK cells can thus kill mycobacteria in the absence of other immunity-related cells, and direct contact is required.

Mycobacteria killing by NK cells requires perforin or granulysin

We next examined the role of NK cell cytotoxic proteins, in particular, perforin and granulysin, on mycobacterial killing. To see whether the expression level of perforin and granulysin is affected by mycobacteria challenge, we incubated pNK and/or NK92 cells with *M.tb* and *M. kansasii*, respectively. Although NK cells produced a certain amount of proteins, the level of perforin and granulysin increased up to 1.7- and 2.2-fold, respectively, after mycobacterial challenge (Fig. 2A and Supplemental Fig. 2A), indicating that mycobacterial contact enhances expression of perforin and granulysin.

To assess whether the cytotoxic granules of pNK cells are required for antimycobacterial activity, we used SrCl₂ to deplete the intracellular granules [28]. After treatment with 25 mM SrCl₂ for 24 h, perforin and granulysin were significantly depleted (Fig. 2B and Supplemental Fig. 2B). Concomitantly, *M. kansasii* or *M.tb* killing activity was reduced 2.5-fold compared with controls (Fig. 2C and Supplemental Fig. 2C). Consistent with these results, when perforin and granulysin mRNA levels were depleted in the NK92 cells, the ability of NK cells to kill *M. kansasii* or *M.tb* was reduced at least threefold compared with the mock siRNA control (Fig. 2D and E and Supplemental Fig. 2D and E). When perforin and granulysin were depleted simultaneously, mycobacterial killing efficacy was reduced further (Fig. 2F and Supplemental Fig. 2F). These data suggest that perforin and granulysin are involved in mycobacterial killing.

The effect of perforin and/or granulysin depletion on mycobacterial killing was next examined. Depletion of perforin or granulysin in NK92 cells reduced mycobacterial killing significantly in broth culture (Fig. 2G and Supplemental Fig. 2G). When the coculture was complemented with exogenously added perforin or granulysin, the killing effect was restored

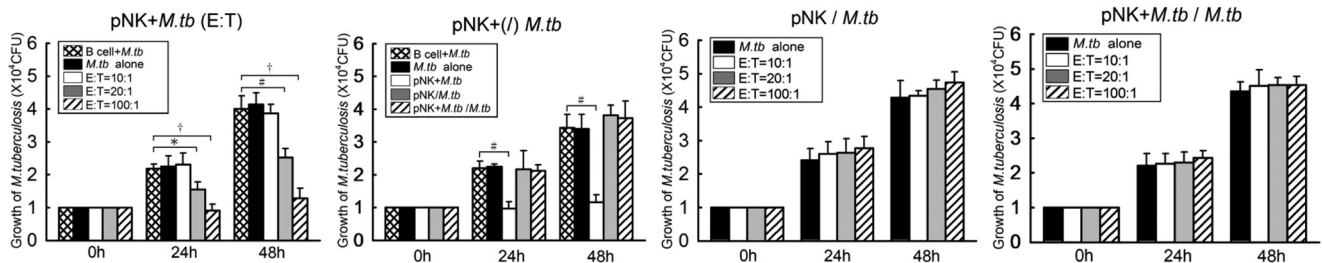


Figure 1. Human pNK cells kill mycobacteria in a contact-dependent manner. The pNK cells were cocultured with mycobacteria (pNK+*M.tb*) at the E:T ratios indicated (10:1, 20:1, and 100:1). *M.tb* (1×10^4 CFU) were cultured alone, cocultured with pNK cells [pNK+(/)*M.tb*], cultured separately from pNK cells in the same well by transwell assay (pNK/*M.tb*), or cultured separately from pNK + *M.tb* cells in the same well (pNK+*M.tb*/*M.tb*) for 24 and 48 h, respectively. The mycobacteria CFU were then determined using the plate assay. The experiments were performed independently in triplicate. * $P < 0.05$; # $P < 0.01$; † $P < 0.001$.

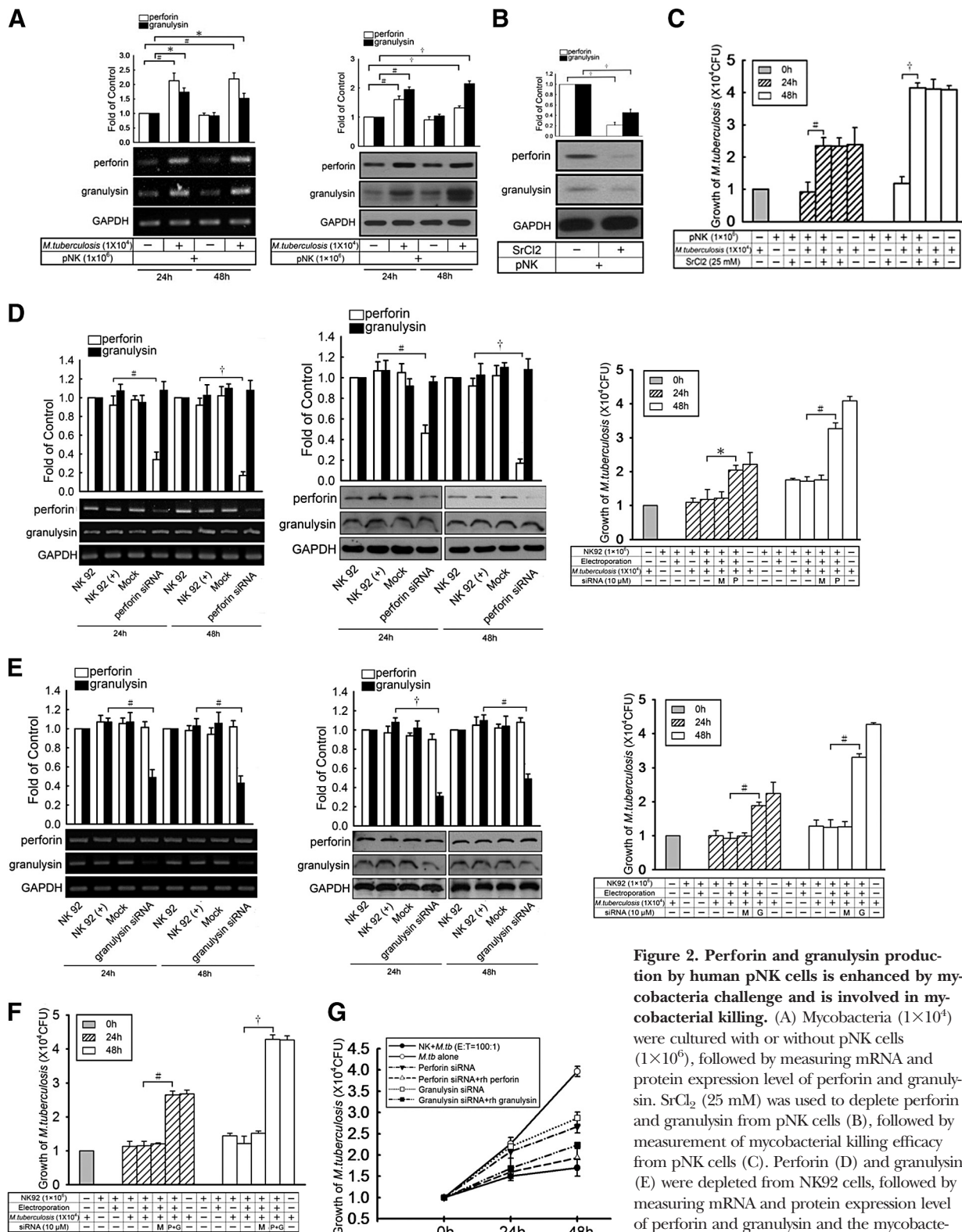


Figure 2. Perforin and granulysin production by human pNK cells is enhanced by mycobacteria challenge and is involved in mycobacterial killing. (A) Mycobacteria (1×10^6) were cultured with or without pNK cells (1×10^6), followed by measuring mRNA and protein expression level of perforin and granulysin. SrCl_2 (25 mM) was used to deplete perforin and granulysin from pNK cells (B), followed by measurement of mycobacterial killing efficacy from pNK cells (C). Perforin (D) and granulysin (E) were depleted from NK92 cells, followed by measuring mRNA and protein expression level of perforin and granulysin and the mycobacterial killing efficacy. RT-PCR was used to quantify

the mRNA transcriptional level, and Western blot analysis was used for protein quantification. P, perforin depletion; G, granulysin depletion; M, scramble control. (F) Both perforin and granulysin were depleted from NK92 cells (P+G), followed by evaluation of NK92 cell-killing efficacy. Recombinant human (rh) perforin (50 $\mu\text{g}/\text{ml}$) or granulysin (30 $\mu\text{g}/\text{ml}$) was added to perforin- or granulysin-depleted cells to evaluate mycobacterial killing efficacy (G). Samples were harvested at 0, 24, and 48 h. The experiments were performed independently in triplicate. * $P < 0.05$; # $P < 0.01$; + $P < 0.001$.

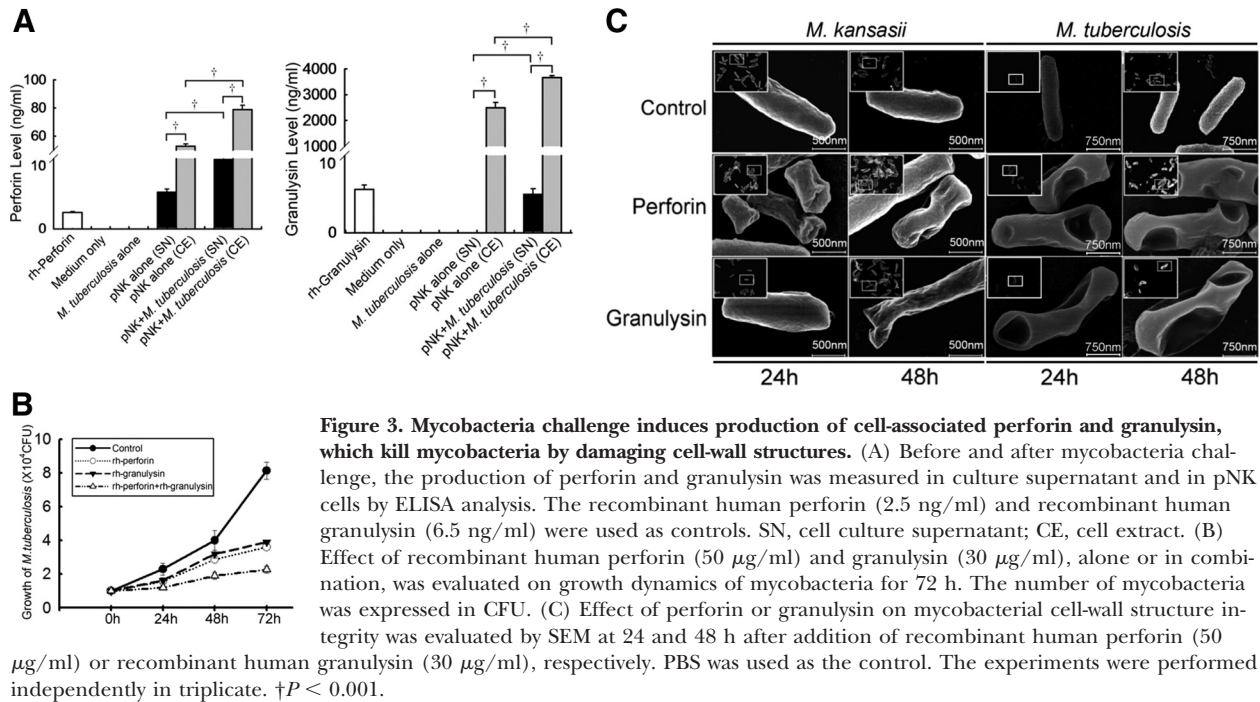


Figure 3. Mycobacteria challenge induces production of cell-associated perforin and granulysin, which kill mycobacteria by damaging cell-wall structures. (A) Before and after mycobacteria challenge, the production of perforin and granulysin was measured in culture supernatant and in pNK cells by ELISA analysis. The recombinant human perforin (2.5 ng/ml) and recombinant human granulysin (6.5 ng/ml) were used as controls. SN, cell culture supernatant; CE, cell extract. (B) Effect of recombinant human perforin (50 μ g/ml) and granulysin (30 μ g/ml), alone or in combination, was evaluated on growth dynamics of mycobacteria for 72 h. The number of mycobacteria was expressed in CFU. (C) Effect of perforin or granulysin on mycobacterial cell-wall structure integrity was evaluated by SEM at 24 and 48 h after addition of recombinant human perforin (50 μ g/ml) or recombinant human granulysin (30 μ g/ml), respectively. PBS was used as the control. The experiments were performed independently in triplicate. $\dagger P < 0.001$.

(Fig. 2G and Supplemental Fig. 2G). These results indicate that perforin and granulysin play a major role in NK cell-mediated killing of mycobacteria.

Cell-associated perforin and granulysin disrupt mycobacterial cell-wall integrity

The expression of perforin and granulysin in mycobacteria-treated pNK cells was next investigated. Before incubation with mycobacteria, pNK cells (1×10^6 cultured in 1 ml) produced ~50 ng perforin and 2500 ng granulysin. After challenge with mycobacteria, the expression of cytotoxic proteins increased to 65–80 ng for perforin and to 3000–3500 ng for granulysin, with both proteins located mostly within pNK cells (Fig. 3A and Supplemental Fig. 3A). These results indicate that the production of perforin and granulysin increased after mycobacterial challenge, but instead of being secreted, these proteins remained mainly associated with the pNK cells. The ability of perforin (50 μ g/ml) or granulysin (30 μ g/ml) to kill *M. kansasii* or *M.tb* in broth cultures was monitored. Perforin or granulysin individually killed the two mycobacterial species, with an additive effect observed when both proteins were added together (Fig. 3B and Supplemental Fig. 3B).

Bacterial morphology was visualized by SEM after incubation with the two cytotoxic proteins. After perforin (50 μ g/ml) treatment for 24 h, the cell-wall structure of *M. kansasii* and *M.tb* was clearly damaged (Fig. 3C). The treated bacteria showed abnormal shapes, and the cell envelopes were often perforated, with the cells appearing to lose their cytoplasmic contents (Fig. 3C). In contrast, an aberrant cell-wall structure was observed after 48 h of granulysin treatment (30 μ g/ml) for *M. kansasii* and after 24 h for *M.tb*. In brief, NK cell-associated perforin and granulysin perforate and destroy the mycobacterial cell-wall structure.

Mycobacteria induce the formation of nanotube-like structures on NK cells and activate perforin redistribution in treated NK cells

SEM was used to visualize morphological changes in NK cells responding to mycobacterial treatment. Significantly, in contrast to resting NK92 and pNK cells, nanotube-like structures were commonly seen in mycobacteria-treated NK92 and pNK cells (Fig. 4A). These structures were previously reported to facilitate the interaction between NK cells and target cells [29]. Interestingly, mycobacteria were often observed in direct contact with NK cells, apparently tethered by the nanotube-like structures (Fig. 4A). A quantitative analysis of nanotube formation showed that the level of nanotubes on NK cells treated with mycobacteria for 24 or 48 h was at least twice as high as the level observed for untreated NK cells (Fig. 4D).

NK cell morphology and perforin distribution was also monitored after the NK cells were challenged with fluorescent *M. kansasii* (observed as green dots). As shown in Fig. 4B, the level of perforin (labeled as red dots) increased significantly, and this protein was redistributed toward regions of the cell that interacted with mycobacteria, whereas little perforin production or trafficking to the cell membrane was observed in untreated NK92 cells (Fig. 4B). The degree of perforin polarization was quantified in Fig. 4C. In brief, mycobacterial challenge induced a redistribution of perforin granules toward mycobacteria in NK cells.

Enhanced perforin and granulysin production through NKG2D/NCR and MAPK signaling pathways is involved in mycobacterial killing

As mycobacteria could enhance the expression of perforin and granulysin tentatively through ligation of the activating

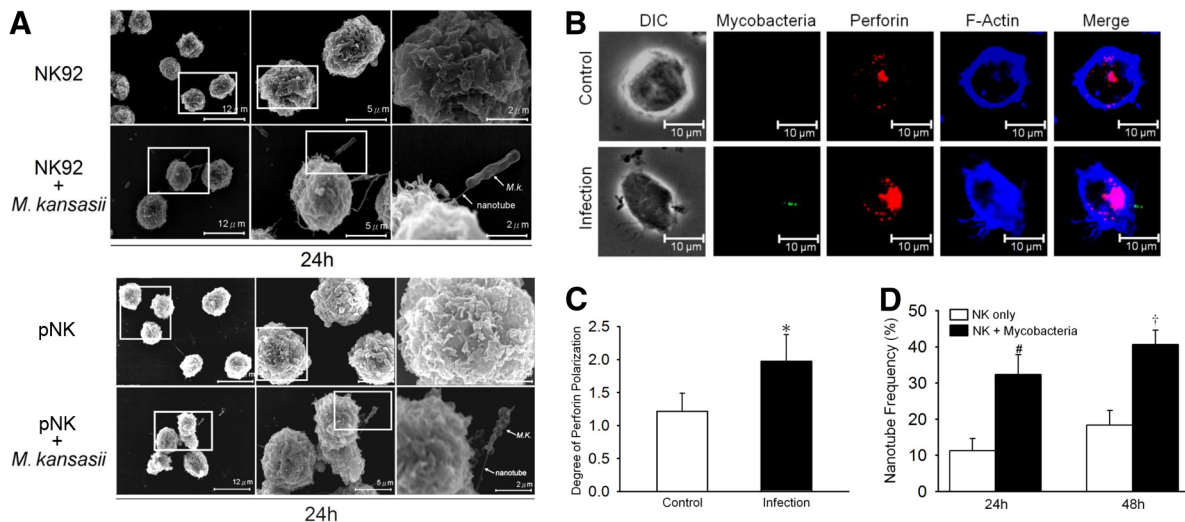


Figure 4. Mycobacteria challenge induces the formation of protruding nanotube-like structures on NK cells and results in redistribution of perforin granules in NK cells. (A) After *M. kansasii* (*M.k.*) challenge for 24 h, SEM analysis showed formation of nanotube structures in NK92 and pNK cells for direct interaction with mycobacteria. (B) Redistribution of perforin granules in pNK cells after *M. kansasii* challenge. The F-actin (stained in blue), perforin (red), and *M. kansasii* (green) were observed by fluorescence microscopy. The experiments were performed independently in triplicate. (C) Movement of perforin to the synapse/polymerized F-actin was quantified by assigning a score from zero to three as follows: Score 0 was assigned when perforin granules were located predominately in the cell half that was distal to the synapse; Score 1 was assigned for granules predominantly distributed in the cell half proximal to the synapse; Score 2 for granules located near the synapse; and Score 3 for granules in close association with the synapse. * $P < 0.05$. The data shown are representative of 10 established conjugates from two independent experiments. (D) Quantification of nanotube formation in mycobacteria-treated NK cells, which were labeled with mouse anti-human perforin IgG followed by Alexa 555-conjugated goat anti-mouse IgG and phalloidin-Alexa 350 for staining F-actin before mixing with an equal number of mycobacteria and incubation for 24 and 48 h on a fibronectin-coated glass surface. The cells were observed under confocal microscopy. # $P < 0.01$; † $P < 0.001$.

NKG2D and NCR (NKp30, NKp44, and NKp46) signaling pathways, the effect of mycobacterial challenge on the expression level of these receptors was evaluated. As shown in Fig. 5A and Supplemental Fig. 4A, the levels of mRNA and protein from the NKG2D, NKp44, and NKp46 receptor genes in NK cells increased significantly after challenge with *M. kansasii* or *M.tb*. In contrast, the expression of NKp30 was not affected significantly.

To determine whether NKG2D, NKp30, NKp44, and NKp46 are involved in mycobacteria-induced perforin or granzysin production, the RNA levels of these receptors were, respectively, depleted in NK92 cells, and perforin and granzysin production was measured following incubation with the bacteria. As shown in Fig. 5B, the mRNA and protein levels of NKG2D and the three NCRs were reduced significantly by the respective RNA interference treatments, as compared with the scramble control groups. Simultaneously, expression of perforin and granzysin was also attenuated significantly. When the four receptors were depleted concurrently, perforin and granzysin expression was reduced further (Fig. 5Ci). Concurrently, the killing efficacy of the receptor-depleted NK cells against mycobacteria was abrogated dramatically (Fig. 5Cii and Supplemental Fig. 4B). These data indicate that NKG2D and the three NCRs are involved in mycobacterial stimulation of perforin and granzysin production and subsequent mycobacterial killing.

MAPKs are important mediators of the NKG2D and NCR signaling pathways. We therefore evaluated whether activation of MAPKs is involved in NK cell killing of *M. kansasii* or *M.tb*. After mycobacterial challenge, phosphorylation levels of JNK, ERK, and p38 were measured in pNK cells, with or without treatment with specific chemical inhibitors. As shown in Fig. 6A–C and Supplemental Fig. 5A–C, phosphorylation levels of JNK, ERK, and p38 kinases were enhanced significantly by mycobacterial challenge and were reduced by the chemical inhibitors, especially after 48 h of treatment. Consistent with these results, mycobacteria-stimulated perforin and granzysin production was also reduced (Fig. 6A–C). The chemical inhibitors used had no significant effects on cell viability (Supplemental Fig. 6).

The results obtained with chemical inhibitors were confirmed further by depleting mRNA levels of the three kinases through RNA interference. Depletion of the kinases using siRNA reduced perforin and granzysin mRNA and protein levels in a manner similar to the chemical inhibitors (Fig. 6D–F). In all cases, the ability of NK cells to kill mycobacteria was reduced (Fig. 6D–F and Supplemental Fig. 5D). As shown for the chemical inhibitors described above, the siRNA treatments produced no significant effects on cell viability (Supplemental Fig. 6). These data indicate that JNK, ERK, and p38 kinases play a role in mycobacteria-stimulated perforin and granzysin production, resulting, in turn, in enhanced antibacterial killing efficacy.

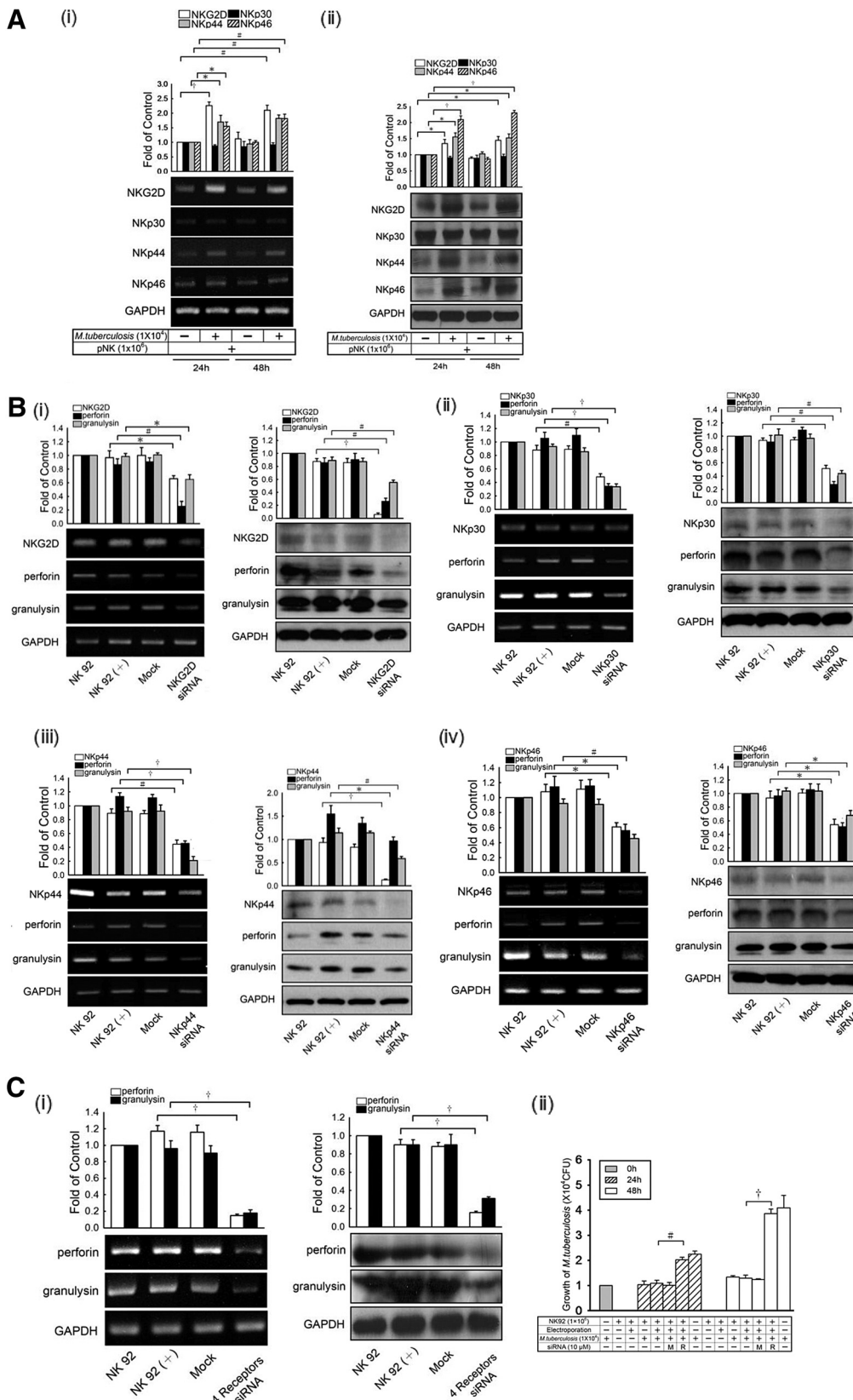


Figure 5. NKG2D and NCRs are involved in mycobacteria-dependent perforin and granulysin production and mycobacterial killing. (A) pNK cells (1×10^6) were cultured with or without mycobacteria (1×10^4) for 24 and 48 h. The levels of mRNA and protein of NKG2D and NCRs (Nkp30, Nkp44, and Nkp46) were measured by RT-PCR (i) and Western blot analysis (ii) 24 and 48 h after infection. (B) NKG2D (Bi), Nkp30 (Bii), Nkp44 (Biii), and Nkp46 (Biv) were depleted by siRNA in NK92 cells, followed by confirmation of mRNA and protein amount reduction of each receptor and perforin and granulysin production. (C) NKG2D and the three NCRs were depleted simultaneously by siRNA, followed by confirmation of reduction of mRNA and protein amount of each receptor and perforin and granulysin production in NK92 cells (Ci). The effect of four receptor depletion on killing efficacy was then evaluated 24 and 48 h after infection (Cii). NK92 (+), NK92 cells challenged with *M.tb*; Mock, NK92 (+) scramble control; R, knockdown of four receptors; Electroporation, NK92 cells were sham electroporated without siRNA. The experiments were performed independently in triplicate. * $P < 0.05$; # $P < 0.01$; † $P < 0.001$.

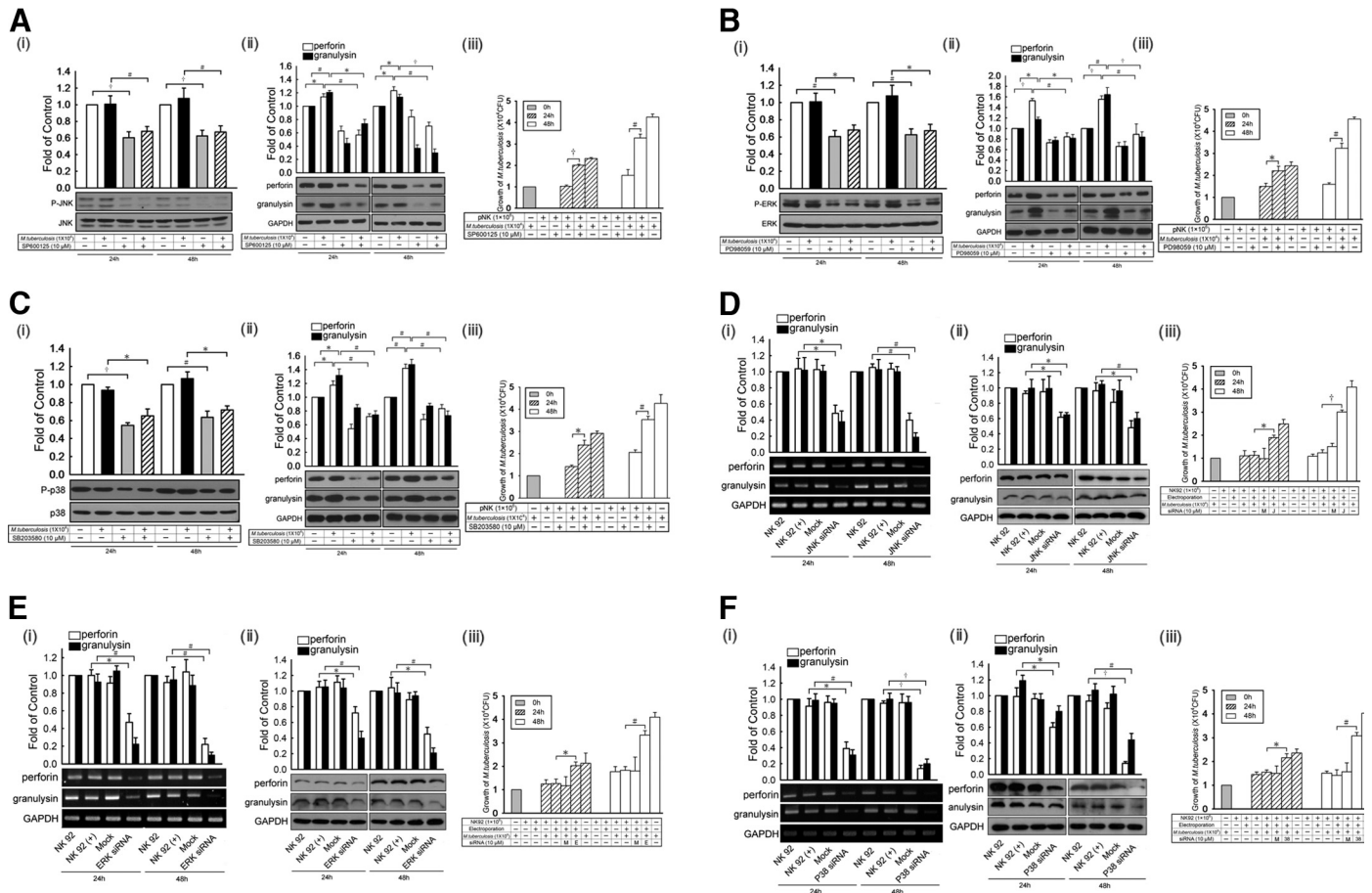


Figure 6. JNK, ERK, and p38 MAPKs are involved in mycobacteria-stimulated perforin and granulysin production and mycobacterial killing. Specific chemical inhibitors for JNK (SP600125; A), ERK (PD98059; B), and p38 (SB203580; C), each at 10 μ M, were incubated with NK92 cells (1×10^6), cultured with or without *M.tb* (A–C; 1×10^4) for 24 and 48 h. The phosphorylation (P) status of JNK (Ai), ERK (Bi), and p38 (Ci), together with the production of perforin and granulysin after inhibition of JNK (Aii), ERK (Bii), and p38 (Cii), was measured. The effect of chemical inhibition of JNK (Aiii), ERK (Biii), and p38 (Ciii) on killing efficacy of mycobacteria, 24 and 48 h after infection, was then measured. For specific evaluation of the role of each kinase activity on perforin and granulysin production, siRNA was used to deplete JNK (D), ERK (E), and p38 (F), followed by measurement of mRNA (Di, Ei, and Fi) and protein (Dii, Eii, and Fii) production of perforin and granulysin. The effect of depletion of JNK (Diii), ERK (Eiii), or p38 (Fiii) on killing efficacy of *M.tb* was measured. J, E, and 38 in the graph legends denote knockdown of JNK, ERK, and p38, respectively. The experiments were performed independently in triplicate. * $P < 0.05$; # $P < 0.01$; † $P < 0.001$.

DISCUSSION

In this study, we describe the mechanism underlying the anti-mycobacterial activity of NK cells. Namely, we show that NK cells kill mycobacteria directly in a contact-dependent manner, independently of the presence of an infected host cell. Shortly after mycobacterial challenge, NK cells produce nanotube-like structures (that can tether mycobacteria), express higher levels of the activating receptors NKG2D and NCRs, and show enhanced activation of the p38, ERK and JNK MAPKs, resulting in increased production of the cytotoxic proteins perforin and granulysin. Increased production of the cytotoxic proteins coincides with preferential rearrangement of perforin-rich vesicles toward the interacting mycobacteria, although the vesicles do not localize at the cell-to-bacteria contact synapse, a phenomenon observed during interaction of NK cells with tumor or virus-infected target cells.

At the concentration of perforin (50 μ g/ml) or granulysin (30 μ g/ml) used, we observed that mycobacteria, grown in broth culture, are killed efficiently. SEM observations indicate that the bacterial cell wall is damaged by both cytolytic proteins, suggesting that disruption of cell-wall integrity may be the main mechanism leading to bacterial death. Previously, Gonzales et al. [30] reported that human and murine NK cells show antibacterial activity against the bacterial pathogen, *Bacillus anthracis*. Our results indicate that NK cells use relatively high concentrations of perforin and granulysin to kill mycobacteria and that the killing activity of these cytolytic proteins requires direct contact with the pathogen. Moreover, the recognition and killing of mycobacteria by NK cells do not require accessory cells, as no macrophages or dendritic cells acting as APCs were added in our experiments. Besides killing *M. kansasii*, NK cells could also kill *M.tb*, the dominant pathogen

responsible for human tuberculosis, indicating that a conserved killing mechanism may be used by NK cells for lysing all mycobacterial species.

The work of Vankayalapati et al. [31, 32] demonstrated that Nkp46, Nkp30, Nkp44, and NKG2D are involved in recognition of *M.tb*-infected macrophages through interaction with specific ligands. However, the bacterial ligands that activate NK cells remain to be identified. A previous study indicated that several unidentified surface molecules of *M. bovis* BCG may be recognized by Nkp44 in human NK cells [17]. In addition, NKG2D on NK cells is involved in clearance of extracellular pathogens, such as *Pseudomonas aeruginosa*, in mouse models [33]. Furthermore, although human proteins, such as MHC class I chain-related proteins A and B and UL16-binding proteins, have also been identified as activating ligands of NKG2D [34, 35], no bacterial ligand has been characterized so far. On the other hand, whereas the expression of Nkp30 on NK cells was not increased by mycobacterial challenge, we found that depletion of Nkp30 still resulted in reduced killing of mycobacteria by NK cells, suggesting that Nkp30 may be constitutively involved in defense against these bacteria. The depletion of each of the four NK cell receptors separately reduced killing, and the depletion of all four receptors at the same time further reduced killing efficiency, suggesting that all four receptors may be used in concert by NK cells for mycobacterial recognition. Alternatively, as NK cells also express pattern recognition receptors, such as TLRs and NLRs, which may recognize bacterial products, activation of the NKG2D and Nkp44 and Nkp46 receptors may be an indirect effect, resulting from activation of TLRs or NLRs.

Previous studies had reported that granulysin reduces the viability of a broad spectrum of pathogenic bacteria, fungi, and parasites in vitro [36, 37]. Granulysin is a cytolytic protein present in the granules of activated human CD4⁺ and cytotoxic CD8⁺ CTLs, as well as NK cells [38, 39], and is a member of the saposin-like protein family [39]. The underlying mechanism of granulysin's cytotoxic effects may involve the insertion of its positively charged domain into the negatively charged surface of target microbes or cells, resulting in alteration of membrane permeability.

In addition to granulysin, our results indicate that perforin alone kills extracellular *M. kansasii* and *M.tb*. Perforin is a pore-forming protein [40–42] that can directly damage the cytoplasmic membrane of cancer cells or virus-infected cells [10, 43]. However, before the present study, there was no report of direct bacterial killing by perforin. Previous studies had shown that upon secretion by CTLs or NK cells, perforin binds and inserts itself into the phospholipid bilayer of the target cell plasma membrane and polymerizes to form a pore of ~16 nm in diameter that spans the target cell membrane [27]. Perforin can synergize with other lytic granule components, such as granzyme B, to induce apoptosis in target cells [44]. As mycobacteria harbor a thick and waxy cell wall, which is considerably different from the cellular membrane of eukaryotic cells, whether perforin can interact directly with cell-wall components of mycobacteria or the bacterial membrane remains to be examined.

The effects of granulysin on the *M.tb* membrane have been observed previously by SEM by Stenger et al. [36], whereas the effect of perforin was not reported. The membrane morphology observed by this group appears to be different from our results. In addition, these authors observed that human perforin was ineffective in reducing the viability of *M.tb* in culture or intracellularly in macrophages [36]. These differences could be a result of the variable susceptibility of the mycobacterial species or strains used in the two studies or of the different duration of treatment. Previous studies have shown that NK-lysin, the pig homologue of granulysin, can permeabilize lipid bilayers and the bacterial cell membrane by interacting with lipid components [45, 46], providing a possible mechanism for the antibacterial effect of this protein.

CTLs and NK cells have been reported to kill *C. neoformans* directly. However, the underlying mechanism used by NK cells to kill this pathogen may be different from the one used to kill mycobacteria. The antifungal activity of NK cells is mediated by a perforin-dependent mechanism under the control of the PI3K-dependent ERK1/2 signaling pathway [27]. Moreover, granulysin is not involved in killing of *C. neoformans*. Intriguingly, it has been reported that granulysin but not perforin is involved in CD8⁺ T cell-mediated killing of *C. neoformans* [47]. The differential requirements for killing of yeast and bacteria by perforin and granulysin remain to be investigated.

Various E:T ratios have been used in previous studies. For instance, Gonzales and colleagues [30] demonstrated that human NK cells used at a E:T ratio of 5:1 efficiently reduce *B. anthracis* CFU, whereas the cells were not active against spores. In another study, NK cells showed perforin-mediated anticytotoxic activity at E:T ratios of 100:1, 200:1, and 500:1 [28]. These differences in E:T ratios may be explained by different killing mechanisms that depend on the composition of the tested bacteria. For instance, mycobacteria possess a thick layer of lipid in their cell wall, which may require a larger number of effector NK cells compared with other bacteria.

Although the nanotube-like membrane structures were rarely observed when NK cells were cultured alone, these structures were observed protruding from NK cells following challenge with *M. kansasii*. The mycobacteria appeared to be tethered by the nanotubes, and many bacteria were seen to be trapped and in close contact with NK cells via the nanotubes. Notably, nanotube-like membrane structures were observed previously when NK cells were coincubated with other immune cells or tumor cells [29], where the nanotubes were shown to facilitate long-distance interactions between NK cells and their target cells [29]. It was reported previously that these membrane structures could transfer cell-surface proteins across the NK cell-immune synapse [48]. It is thus possible that NK cells may use nanotubes to deliver perforin and granulysin to their mycobacterial targets for direct killing. The use of nanotubes would also explain the requirement for a contact-dependent mechanism for bacterial killing.

A possible limitation of the present study is the use of the NK92 cell line for the experiments requiring transfection, as pNK cells are not suitable for this purpose. NK92 cells maintained in culture require treatment with rIL-2, which is an activation signal for perforin and granulysin. This process may be

responsible for the inability of mycobacteria to induce perforin and granulysin secretion in some experiments (see, for instance, Figs. 5 and 6).

In conclusion, we describe here a novel innate-immune function for NK cells—the direct killing of mycobacteria. The underlying mechanism requires activation of the signaling pathways downstream from NKG2D and NCRs. Perforin and granulysin are involved in the killing process, and loss of the integrity of the bacterial cell wall appears to be the major cause of bacterial death. Modulation of NK cell-signaling pathways may thus be an alternative strategy for enhancing host defense against mycobacterial infection.

AUTHORSHIP

C-C.L. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. Y-J.H., C-J.C., and C-S.L. performed experiments. T-S.W., J-H.C., and T-L.W. performed experiments in P3 laboratories. T-T.H., D.M.O., and J.M. assisted with the experimental design and manuscript writing. J.D.Y. and H-C.L. conceived of the study, supervised experiments, and composed the manuscript.

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