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The NKp46 Receptor Contributes to NK Cell Lysis of Mononuclear Phagocytes Infected with an Intracellular Bacterium

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We used human tuberculosis as a model to investigate the role of NK cytotoxic mechanisms in the immune response to intracellular infection. Freshly isolated NK cells and NK cell lines from healthy donors lysed Mycobacterium tuberculosis-infected monocytes to a greater extent than uninfected monocytes. Lysis of infected monocytes was associated with increased expression of mRNA for the NKp46 receptor, but not the NKp44 receptor. Antisera to NKp46 markedly inhibited lysis of infected monocytes. NK cell-mediated lysis was not due to reduced expression of MHC class I molecules on the surface of infected monocytes or to enhanced production of IL-18 or IFN-γ. NK cell lytic activity against M. tuberculosis-infected monocytes and NKp46 mRNA expression were reduced in tuberculosis patients with ineffective immunity to M. tuberculosis compared with findings in healthy donors. These observations suggest that 1) the NKp46 receptor participates in NK cell-mediated lysis of cells infected with an intracellular pathogen, and 2) the reduced functional capacity of NK cells is associated with severe manifestations of infectious disease. The Journal of Immunology, 2002, 168: 3451–3457.

The innate and adaptive immune systems both contribute to host defenses against intracellular infections. An effective adaptive immune response depends on Ag presentation, interactions with cells of the innate immune system, and clonal expansion of lymphocytes. Because these processes require days to weeks, effective innate immunity is required to protect the host during this time. Prominent components of the innate immune response are NK cells, which are large granular lymphocytes that can kill autologous infected cells and tumor cells without prior sensitization. NK cells are best known for their contribution to antiviral immunity (1), but they can also lyse targets infected with bacteria and inhibit bacterial growth in vitro (2–4). In addition, production of IFN-γ by NK cells is critical for activation of macrophages and resistance to intracellular bacterial infections (1, 5, 6).

Human infection with Mycobacterium tuberculosis provides a model for study of the innate immune response to intracellular pathogens. Many persons who have worked with infectious tuberculosis patients for prolonged periods remain healthy and never develop a positive tuberculin skin test. This suggests that innate immunity controls the infection without the requirement for T cells to recognize M. tuberculosis Ags and mount a delayed hypersensitivity skin test response. To investigate the role of NK cytotoxic mechanisms in the human immune response to intracellular infection, we studied the capacity of freshly isolated NK cells from normal donors and tuberculosis patients to lyse monocytes infected with M. tuberculosis. We also evaluated cytokine production and expression of activating receptors by NK cells in response to M. tuberculosis-infected monocytes.

Materials and Methods

Patient population

Blood was obtained from 15 healthy tuberculin reactors, four healthy tuberculin-negative subjects, and 15 HIV-seronegative patients with culture-proven pulmonary tuberculosis who had received <4 wk of antituberculosis therapy. Informed consent was obtained from all patients. These studies were approved by the institutional review board of the University of Texas Health Center (Tyler, TX).

Cytofluorometric analysis

To measure the percentages of PBMC that expressed CD3 and CD56, we used FITC-conjugated anti-CD3 and PE-conjugated anti-CD56 (both from BD PharMingen, San Diego, CA), and immunostaining was performed by standard techniques (7). Based on forward and side scatter characteristics, we gated on lymphocytes, and the percentages of single- and double-stained cells were determined with an EPICS flow cytometer (Coulter, Hialeah, FL). For measurement of MHC class I expression, indirect immunostaining was performed, using a mouse anti-human MHC class I first Ab (W6/32; DAKO, Carpinteria, CA) and a FITC goat anti-mouse secondary Ab (Southern Biotechnology Associates, Birmingham, AL). After gating on monocytes, the mean fluorescence intensity of stained cells was measured.

To detect intracellular IFN-γ in cocultures of NK cells and M. tuberculosis-infected monocytes, we used the Cytotox/Cytoperm Plus kit (BD PharMingen). To induce intracellular accumulation of newly synthesized proteins, Golgi Stop (0.7 μl/ml; BD PharMingen) was added for 6 h to the cells in culture. Cells were then harvested, and immunostaining was performed. For double staining, PE-conjugated anti-CD56 or anti-CD14 (BD PharMingen) was added for 6 h to the cells in culture. Cells were then harvested, and immunostaining was performed.

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permeabilization/wash solution. FITC anti-IFN-γ (BD Pharmingen) was then added in staining buffer and 1× permeabilization/wash solution. After incubation at 4°C for 40 min, cells were washed in PBS and 5% FCS, and analyzed by flow cytometry.

Isolation of mononuclear phagocytes and M. tuberculosis infection

PBMC were isolated from blood by differential centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ). PBMC were centrifuged on a Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). Monocyte fractions (1.2 × 10⁶/well) were plated in 12-well plates (BD Labware, Franklin Lakes, NJ) in 1 ml RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% heat-inactivated human serum. Some monocytes were uninfected, and others were infected with single-cell suspensions of 5 × 10³ live M. tuberculosis (H37Ra or H37Rv), as previously described (8). Approximately 25–40% of the monocytes were infected. Cells were incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere, washed three times, and 5 × 10³ anti-CD3 (Miltenyi Biotec, Auburn, CA), and from the negative cell fractions of 5 × 10³ H11002–H9262 were collected after 10 and 20 h and stored at −80°C until cytokine concentrations were measured by ELISA. Paired Abs were used to detect IFN-γ (BD Pharmingen), IL-15, and IL-12 p70 (both from R&D Systems, Minneapolis, MN). ELISA kits were used to measure levels of IL-18 (MBL International, Nagoya, Japan). The lower limits of detection were 5 pg/ml for IL-12 and IFN-γ, 8 pg/ml for IL-15, and 13 pg/ml for IL-18. Cytokine concentrations were minimal when cells were cultured in medium alone, and results are expressed as the cytokine concentration in supernatants from NK cells and infected monocytes minus that in supernatants from NK cells and uninfected monocytes.

Real-time PCR for quantification of NKp44 and NKp46 mRNA

Total RNA was extracted from 1 × 10⁶ NK cells 10 h after culture with infected or uninfected monocytes, using TRIzol reagent (Life Technologies). cDNA was synthesized using the oligo(dT)15 primer (Promega) and Omniscript reverse transcriptase (Qiagen, Valley, CA). Primers and probes for the NKp44 and NKp46 cDNA were designed using Primer Express software (PE Applied Biosystems, Foster City, CA) and were synthesized by Operon Technologies (Alameda, CA). The forward and reverse primers for NKp44 were 5'-TCAAGGGTCAGG TACTTCAAAG-3' and 5'-GGGGGGGTACTGGCATT-3', respectively. The probe was 5'-ACGGTTAGGCCTCCGCTGCCG-3'. The forward and reverse primers for NKp46 were 5'-ACGGGACTCCA GAAAGACAT-3' and 5'-CAGGCCTACTCCGAGGA-3', respectively. Both probes were 5'-fluorescein phosphoramidite labeled and 3'-TAMRA labeled. The TaqMan β-actin control reagents (PE Applied Biosystems) were used as internal standards. Both sets of primers and probes spanned an intron, so that they detected NKp44 and NKp46 cDNA, but not genomic DNA. Real-time PCR assays were performed in a sealed 96-well microtiter plate (PE Applied Biosystems) on a spectrofluorometric thermal cycler (7700 PRISM; PE Applied Biosystems). Each cDNA sample was aliquoted into wells containing 1× TaqMan Universal PCR master mix (PE Applied Biosystems), 5 pmol of either the NKp44 or NKp46 β-actin primers, and 1 pmol of the corresponding probe in a total volume of 25 µL. Standard curves were constructed using serial 10-fold dilutions of cDNA in purified NK cells stimulated with IL-12 and IL-2 for 48 h. PCR reactions were performed in triplicate as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent signal was measured and plotted during each 60°C annealing and extension step for all samples. Using the cycle threshold (the number of PCR cycles required for the fluorescent dye to be detectable) and the constructed standard curve for each cDNA, the relative amounts of β-actin, NKp44, and NKp46 cDNA were generated using the corresponding primers and probes to amplify serial 10-fold dilutions of cDNA in purified NK cells stimulated with IL-12 and IL-2 for 48 h. PCR reactions were performed in triplicate as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent signal was measured and plotted during each 60°C annealing and extension step for all samples. Using the cycle threshold (the number of PCR cycles required for the fluorescent dye to be detectable) and the constructed standard curve for each cDNA, the relative amounts of β-actin, NKp44, and NKp46 cDNA in each sample were determined.

Statistical analysis

Results are shown as the mean ± SE. For data that were normally distributed, comparisons between groups were performed using a paired or unpaired t test, as appropriate. For data that were not normally distributed, the Wilcoxon rank-sum test was used. Values of p < 0.05 were considered statistically significant.

Results

NK cell-mediated cytotoxicity against M. tuberculosis-infected mononuclear phagocytes in healthy donors

To determine whether NK cells contributed to lysis of M. tuberculosis-infected mononuclear phagocytes, we first identified the most appropriate target cells. We obtained freshly isolated NK cells from two healthy tuberculin reactors and cultured them with uninfected and H37Ra-infected autologous monocytes, THP-1, and MonoMac6 cells at different time points ranging from 4–10 h. Lysis of infected targets was greater than that of uninfected targets for autologous monocytes, but not for THP-1 or MonoMac6 cells, and maximum lytic activity was observed at 10 h, so this time point and autologous monocytes were used for subsequent experiments.

To determine whether the capacity of NK cells to lyse M. tuberculosis-infected monocytes was affected by prior tuberculosis infection, we obtained freshly isolated NK cells from four healthy tuberculin-negative persons and 15 healthy tuberculin reactors. NK
cells from healthy tuberculin reactors lysed a higher percentage of H37Ra-infected monocytes than uninfected monocytes (59 ± 7% vs 23 ± 5%, respectively; p < 0.001), similar to findings in tuberculin-negative persons (77 ± 11% vs 41 ± 13%, respectively; p = 0.002; Fig. 1). To determine whether NK cells also lysed monocytes infected with virulent M. tuberculosis, we measured cytotoxicity of NK cells from three healthy tuberculin reactors against H37Ra- and H37Rv-infected autologous monocytes. There was no significant difference in the cytotoxicity of NK cells against H37Ra- and H37Rv-infected autologous monocytes (70 ± 17% vs 79 ± 15%, respectively; p = 0.59; Fig. 2).

Cytotoxicity of NK cell lines against infected monocytes

To confirm that the cytotoxicity observed above was due to NK cells and not due to contaminating T cells, we tested the cytotoxic potential of a homogeneous population of NK cells by generating NK cell lines from PBMC of four tuberculin reactors. These cells were 99% CD56+ and <1% CD3+, and all showed more cytotoxicity against infected than uninfected monocytes (67 ± 15% vs 34 ± 9%, respectively; p < 0.01; Fig. 3).

Cytokines produced by NK cells cultured with infected monocytes

Monocytes are an important source of cytokines that activate NK cells (9). To evaluate the involvement of these monokines in NK cell-mediated cytotoxicity, we cocultured NK cells with either infected or uninfected autologous monocyte from six healthy tuberculin reactors. Higher levels of IL-18 were detected in supernatants of infected monocyte and NK cells compared with those of control monocytes and NK cells (107 ± 31 vs 26 ± 9 pg/ml, respectively; p = 0.05; Fig. 4). IL-15 and IL-12 concentrations were slightly higher in cocultures containing infected than uninfected monocytes, but the differences were not statistically significant (Fig. 4). IFN-γ is produced by activated NK cells and is essential for NK-mediated defenses against CMV infection (10). Therefore, we measured IFN-γ concentrations, which were increased 12-fold when NK cells were cocultured with infected vs uninfected monocytes (p < 0.001; Fig. 4), and these levels were further increased after 20 h to 5222 ± 1624 vs 145 ± 29 pg/ml (p < 0.001). Because IFN-γ can be produced by macrophages, we combined surface immunolabeling with CD14 or CD56 and intracellular staining to detect IFN-γ in cocultures of NK cells and monocytes; 8.2% of the cells were IFN-γ+CD56+, and none was IFN-γ+CD14+, indicating that NK cells were the source of IFN-γ.

Effect of anti-cytokine Abs on NK cell-mediated cytotoxicity

Because NK cells and infected monocytes produced high levels of IL-18 and IFN-γ, we determined whether these cytokines contributed to lysis of infected monocytes. In cells obtained from three healthy tuberculin reactors, Abs to IL-18 and IFN-γ, alone or in combination, did not reduce NK cell-mediated specific lysis against M. tuberculosis-infected monocytes (Fig. 5).

MHC class I expression on infected monocytes

NK cells lyse autologous cells with reduced MHC class I expression because class I molecules engage inhibitory receptors on NK cells (11, 12). To determine whether MHC class I expression was reduced on M. tuberculosis-infected monocytes, we performed immunostaining with an anti-MHC class I Ab. In cells obtained from

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**FIGURE 1.** Cytotoxicity of freshly isolated NK cells against autologous M. tuberculosis-infected monocytes. Freshly isolated NK cells from 15 healthy tuberculin reactors (PPD+) and four healthy tuberculin-negative subjects (PPD−) were cultured with uninfected (control) and H37Ra-infected autologous monocytes at an E:T cell ratio of 40:1. Mean values and SEs for the percentage of cytotoxicity are shown.

**FIGURE 2.** Cytotoxicity of NK cells against H37Ra- and H37Rv-infected autologous monocytes. Freshly isolated NK cells from three healthy tuberculin reactors were cultured with H37Ra- or H37Rv-infected autologous monocytes or with uninfected (control) cells, and the percentage of cytotoxicity was determined. The mean of triplicate values is shown.

**FIGURE 3.** Cytotoxicity of NK cell lines against infected monocytes. CD56+CD3− NK cell lines were generated from four healthy tuberculin reactors and cocultured with autologous uninfected (control) and H37Ra-infected monocytes for 10 h, and the percentage of cytotoxicity was determined. Mean values and SEs are shown.
six healthy tuberculin reactors, the mean fluorescence intensities of infected and control monocytes were similar (44 ± 9 vs 45 ± 6).

**Nkp44 and Nkp46 mRNA expression**

Because lysis of monocytes by NK cells was not mediated by type 1 cytokines or by reduced MHC class I expression on monocytes, we next evaluated the expression of Nkp44 and Nkp46, receptors, which are present exclusively on NK cells and are associated with activation and cytolysis (13, 14). We cocultured infected or uninfected monocytes with NK cells from seven healthy tuberculin reactors and quantified Nkp44 and Nkp46 mRNA expression using real-time PCR. To normalize for the efficiency of RNA extraction and RT in different samples, data were expressed as the ratio of Nkp46 mRNA to β-actin mRNA, expressed in arbitrary units. Nkp46 mRNA expression was significantly up-regulated by coculturing NK cells with infected monocytes compared with uninfected monocytes (14 ± 3 vs 2 ± 1 arbitrary units; p = 0.006; Fig. 6A). Nkp44 mRNA expression was not up-regulated in NK cells exposed to infected vs uninfected monocytes (3 ± 1 vs 4 ± 2 arbitrary units, respectively; Fig. 6B).

**Effect of neutralization of Nkp46 on NK cell-mediated cytotoxicity**

To more definitively determine whether NK cell-mediated cytotoxicity of infected monocytes was mediated by Nkp46, we incubated freshly isolated NK cells from five healthy tuberculin reactors with a mouse antiserum raised against Nkp46-Ig (15), before coculture with *M. tuberculosis*-infected monocytes. The Nkp46 antiserum reduced net percent specific lysis of infected monocytes from 33 ± 8% to 2 ± 1% (p = 0.01; Fig. 7). Treatment with the...
NKp46 antiserum had no effect on the viability of the NK cells, as assessed by trypan blue exclusion (data not shown).

**NK cell activity in tuberculosis patients**

The above experiments demonstrated that freshly isolated NK cells from healthy donors can kill *M. tuberculosis*-infected monocytes. To determine the relevance of these findings to the clinical manifestations of *M. tuberculosis* infection, we evaluated NK cell number and function in tuberculosis patients, who have an ineffective immune response to infection. By flow cytometry, the percentages of CD56*+* cells in PBMC were similar in tuberculosis patients, healthy tuberculin reactors, and tuberculin-negative subjects (10.5 ± 2.8%, 8.2 ± 2.2%, and 13.0 ± 0.3%, respectively). The percentages of CD56*+CD3*−* and CD56*−CD3*+* cells were also similar in all three groups (data not shown).

We next compared the functional activities of NK cells from 15 healthy tuberculin reactors and 10 tuberculosis patients. Values were expressed as net percent specific lysis, which was the percent lysis of *M. tuberculosis*-infected monocytes, minus that of uninfected monocytes. The net percent specific lysis of NK cells was significantly reduced in tuberculosis patients compared with that in healthy tuberculin reactors (16 ± 6% vs 39 ± 6%, respectively; *p* = 0.01; Fig. 8). Lysis of the classic NK targets, K562 cells, by NK cells was also reduced in tuberculosis patients compared with that in healthy tuberculin reactors (7 ± 1% and 50 ± 5%, respectively; *p* < 0.001; Fig. 8).

Because the capacity of NK cells to mediate specific lysis of *M. tuberculosis*-infected monocytes was reduced in tuberculosis patients compared with healthy tuberculin reactors, we next evaluated the expression of NKp46 mRNA. In contrast to findings for healthy tuberculin reactors, NK cells from tuberculosis patients did not up-regulate NKp46 mRNA expression in response to *M. tuberculosis*-infected monocytes (Fig. 9).

**Discussion**

The present study demonstrated that freshly isolated NK cells and NK cell lines from healthy donors lysed *M. tuberculosis*-infected monocytes to a greater extent than uninfected monocytes. Lysis of infected monocytes was associated with increased expression of mRNA for the NKp46 receptor and was abrogated by anti-NKp46 antisera. NK cell-mediated lysis was not due to reduced expression of MHC class I molecules on the surface of infected monocytes or to enhanced production of IL-18 or IFN-γ. These findings are relevant to the clinical manifestations of infection with *M. tuberculosis*, because NK cell lytic activity against *M. tuberculosis*-infected monocytes and NKp46 expression were reduced in tuberculosis patients compared with findings in healthy donors.

NK cells are believed to play a central role in the innate immune response to microbial pathogens, both through destruction of infected cells and by secretion of cytokines that shape the subsequent immune response.
adaptive immune response. The lytic capacity of NK cells is controlled by a balance between inhibitory and activating receptors (11, 12). All normal nucleated cells express MHC class I molecules on their surface, which bind to inhibitory NK cell receptors and prevent triggering of the lytic machinery. NK cells also bear a variety of activating receptors, including some that are restricted to NK cells. These include the NKp30, NKp44, and NKp46 receptors, the enhanced expression of which is associated with lysis of tumor cells and virally infected cells (13, 14, 16).

Most studies of NK cells in infectious diseases have focused on the response to viruses. Viral infection of target cells inhibits MHC class I expression, leading to reduced ligation of the NK cell inhibitory receptors and lysis of infected cells. Limited studies have suggested that NK cells can destroy cells infected with intracellular bacteria, such as Listeria, Salmonella, and Legionella, but these studies used lymphokine-activated killer cells rather than highly purified NK cells (3, 17, 18). In mycobacterial infection, depletion of NK cells in C57BL/6 mice enhances the growth of Mycobacterium avium (19), and IL-12-stimulated human NK cells activate macrophages to inhibit the growth of M. avium in macrophages (20). Denis (21) first showed that freshly isolated NK cells from healthy donors can lyse M. tuberculosis-infected monocytes and reduce the rate of intracellular bacillary growth, but the mechanisms underlying these effects were not investigated. One study found that growth inhibition was mediated by soluble factors produced by NK cells, whereas another suggested that growth inhibition required contact between NK cells and monocytes and was associated with the induction of monocyte apoptosis (22, 23).

We found that freshly isolated NK cells from both tuberculin-positive and tuberculin-negative healthy subjects specifically lyse M. tuberculosis-infected monocytes, indicating that this activity was innate and independent of prior exposure to M. tuberculosis. Lysis of infected monocytes is associated with 7-fold enhanced expression of mRNA for NKp46, and antisera to NKp46 markedly decreased NKp46 mRNA expression. These findings suggest that the NK cell response to M. tuberculosis infection correlates with the clinical manifestations of infection, and that NKp46 contributes to destruction of infected cells by freshly isolated NK cells. NKp44 expression was not enhanced in NK cells exposed to infected monocytes, perhaps reflecting the fact that freshly isolated NK cells bear NKp46, but not NKp44, receptors (13, 14). NK cells lysed monocytes infected with both virulent and avirulent strains of M. tuberculosis, suggesting that immunotherapeutic strategies that enhance NK cell function may be useful in selected patients with tuberculosis.

Hemagglutinin of the influenza virus and hemagglutinin-neuraminidase of the Sendai virus have recently been identified as the first microbial ligands for NKp46 (15). A basic local alignment search tool search did not reveal any M. tuberculosis proteins that are likely to be expressed on the cell surface and that share significant homology with these viral proteins. However, it is possible that glycosylated mycobacterial proteins may interact with sialic acid residues on NKp46, which were found to be required for binding to viral hemagglutinin (15). It is intriguing to speculate that one of the mechanisms by which NK cells contribute to innate immunity is through recognition by NKp46 of conserved conformational molecular structures of intracellular pathogens that are expressed on the surface of infected cells, in a manner analogous to that of Toll-like receptors on macrophages.

The dominant mechanisms that mediate NK cell cytotoxicity against cells infected with different organisms are likely to vary. Viruses and several intracellular bacteria, such as Salmonella, Yersinia, and Chlamydia pneumoniae, may induce NK cell lytic activity by lowering MHC class I expression on mononuclear phagocytes (24–26). In contrast, we found that M. tuberculosis did not reduce MHC class I expression, confirming prior reports (27, 28). These findings are also consistent with studies showing that NK cells expressing NKp46 can lyse target cells, independent of the presence of MHC class I molecules (11).

NK cells produce cytokines that are likely to shape the subsequent adaptive immune response by T cells in response to intracellular pathogens. We found that NK cells produced larger amounts of IFN-γ in response to M. tuberculosis-infected monocytes than in response to uninfected monocytes. However, IFN-γ was not required for cytotoxicity, which was unaffected by addition of anti-IFN-γ Abs. Similarly, IFN-γ does not mediate the capacity of NK cells to kill intracellular M. tuberculosis (23). Although IFN-γ produced by NK cells in response to M. tuberculosis-infected macrophages does not directly mediate cytotoxicity, it may contribute to immune defenses by favoring the development of a protective type 1 response, as IFN-γ can enhance the production of both IL-12 and IL-18 in response to M. tuberculosis (29, 30). We and others have previously shown that M. tuberculosis-induced IFN-γ production is reduced in PBMC of tuberculosis patients, compared with healthy tuberculin reactors (31, 32), and depletion of NK cells from PBMC markedly reduces Ag-induced IFN-γ production (K. Vankayalapati, unpublished observations), suggesting that NK cells affect subsequent cytokine production by T cells stimulated with mycobacterial Ags. Further studies are needed to understand the mechanisms by which the innate immune response modulates adaptive T cell immunity to M. tuberculosis and other intracellular pathogens.

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