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Human NK Cells Display Important Antifungal Activity against Aspergillus fumigatus, Which Is Directly Mediated by IFN-γ Release

Maria Bouzani,* Michael Ok,* Allison McCormick,† Frank Ebel,† Oliver Kurzai,‡§ C. Oliver Morton,§ Hermann Einsele,* and Juergen Loeffler*

Despite the strong interest in the NK cell-mediated immunity toward malignant cells and viruses, there is a relative lack of data on the interplay between NK cells and filamentous fungi, especially Aspergillus fumigatus, which is the major cause of invasive aspergillosis. By studying the in vitro interaction between human NK cells and A. fumigatus, we found only germinated morphologies to be highly immunogenic, able to induce a Th1-like response, and capable of upregulating cytokines such as IFN-γ and TNF-α. Moreover, priming NK cells with human rIL-2 and stimulating NK cells by direct NK cell–pathogen contact were essential to induce damage against A. fumigatus. However, the most interesting finding was that NK cells did not mediate anti-Aspergillus cytotoxicity through degranulation of their cytotoxic proteins (perforin, granzymes, granulysine), but via an alternative mechanism involving soluble factor(s). To our knowledge, our study is the first to demonstrate that IFN-γ, released by NK cells, directly damages A. fumigatus, attributing new properties to both human NK cells and IFN-γ and suggesting them as possible therapeutic tools against IA. The Journal of Immunology, 2011, 187: 1369–1376.

N atural killer cells are CD56+CD3− lymphocytes that are cytotoxic against neoplastic and virus-infected host cells (1). They possess a sophisticated repertoire of activating and inhibitory receptors through which they perceive their environment. In response to stimuli, they produce and release cytokines and chemokines, such as IFN-γ and TNF-α, which shape the host’s immunity (2, 3). Moreover, they exert a strong, cytolytic effect via the perforin–granzyme, Fas ligand (FasL), and TRAIL pathways (1). Increasing data provide evidence of direct NK cell action against extracellular pathogens, such as bacteria (4), parasites (5), and yeast (6). A role for NK cells and IFN against Aspergillus fumigatus in mice has been suggested (7). This was recently confirmed by Morrison et al. (8), who showed that, in neutropenic mice with invasive aspergillosis (IA), the recruitment of NK cells was a critical host defense mechanism. In a similar animal model, it was shown that NK cell-derived IFN-γ was the protective factor against IA (8, 9). Clinical data confirmed an antifungal and, more specifically, the anti-Aspergillus activity of IFN-γ (7, 10–14). These studies attributed the beneficial effect of IFN-γ to its immunoregulatory role with phagocytes of the innate immune system, which are conventionally involved in the host defense against A. fumigatus (11, 15).

In our study, we investigated the largely unknown interplay between human NK cells and A. fumigatus. We showed that human NK cells interact only with germinated morphotypes of the fungus, and direct physical contact is necessary to induce an NK cell response. This response involves expression of Th1-like cytokines and release of a soluble factor that has direct antifungal properties, which we identified as IFN-γ. These data provide new insights into NK cell biology demonstrating that A. fumigatus, an extracellular pathogen, is a direct target of these cells. Moreover, to our knowledge our results identify, for the first time, unrecognized properties of a key IL and designate IFN-γ as the mediator of a novel NK cell cytotoxic mechanism.

Materials and Methods

Cells

Institutional approval was obtained to isolate cells from the buffy coat of peripheral blood from healthy volunteer donors. After layering over a Ficoll standard density gradient (Biochrom AG), untouched NK (CD56+CD3−) cells were isolated by MACS negative selection procedure, using the NK cell isolation kit (Miltenyi Biotec), according to the manufacturer’s protocol, and were resuspended at a concentration of 1 × 10⁶ cells/ml in culture media (RPMI 1640 with 2 mM L-glutamine [Invitrogen] supplemented with 10% heat-inactivated FCS [Sigma-Aldrich] and 100 μg/ml gentamicin [Refobacin; Merck]). The human erythroleukemia cell line K562 (provided by R. Seggewiss, Medizinische Klinik II, Würzburg, Germany) was cultured in the same culture medium and at the same density as NK cells. In all experiments, except those performed with resting NK cells, NK cells were pulsed with 500 U/ml recombinant human (rh)IL-2 (Proluekin; Novartis) for 24 h before being used.

Fungal strains

A. fumigatus resting conidia (ATCC 46645) were cultivated for 3 d on beer mash plates at 28°C. Conidia were detached from the plate using endotoxin-free sterile water and were filtered through a cell strainer, 40-μm nylon mesh pore membrane, to obtain a single-fungal cell suspension. Swelling and synchronization of fungal growth were achieved by culturing conidia in culture medium at room temperature and under continuous shaking at 200 rpm overnight. Germlings were obtained after an...
additional 5-h incubation at 37°C in the shaker. Fresh swollen conidia and germlings were used directly in cocultures with NK cells.

Infection experiments

After priming, NK cells were applied to 24-well plates at a density of 1×10³ cells/ml, 1 ml/well, and were cocultured with swollen conidia or germlings at an E:T ratio of 1, at 37°C and 5% CO₂ humidified air. Cocultures with the K562 cell line were established as positive controls. Evaluation of cell viability using trypan blue dye preceded all experiments. To estimate the effect of NK cell–fungal cell contact, cocultures were performed in the presence of transwell permeable membranes (Corning) with pores (0.4 μm) small enough to prohibit the contact of cells placed on opposite sides of the insert, but large enough to enable the free diffusion of molecules between the basal and apical compartments of the well. Cultures were harvested at 3, 6, and 12 h and were centrifuged at 5400 rpm for 5 min. Culture supernatants were stored at −80°C. RNA was extracted from the cell pellets with the RNeasy mini kit and QiaShredder spin columns, according to Qiagen’s protocol. QuantiTect reverse transcription kit (Qiagen) was used for the reverse transcription of 500 ng total RNA into cDNA following the manufacturer’s protocol. To evaluate the importance of exocytosis of cytotoxic lysosomal proteins in fungal damage, NK cells were treated for 12 h with 5 mM EGTA (IDRANAL VI; Sigma-Aldrich). EGTA is a calcium (Ca²⁺) chelating agent, inhibiting the Ca ²⁺ flux required for degranulation. NK cells were also treated with 25 mM SrCl₂ (Sigma-Aldrich) for 24 h, to induce degranulation and thereby to eliminate cytotoxic granules (6). The cells were then washed three times in culture media before being used in the experiments. NK cell viability was not affected by such treatments (trypan blue exclusion).

Real-time quantitative PCR assays

Reactions were performed with a LightCycler 1.5 (Roche). Real-time PCR master mix was made with 10 μl Qiagen Quantifast system supplemented with 0.75 μl primers (5 μM; Tib-molbiol), 1 μl probes (3 μM; Tib-molbiol), and 4.5 μl RNAse-free water per reaction, and 2 μl cDNA added as template. We investigated the expression of two genes, as follows: IFN-γ (primers, 5′-GCATCCTAAAGGTGGAG-3′, 5′-GCAGGCGAGGACACCATAC-3′; probes, 5′-LC640-TCAAAGTATGGCTGGTAACTGTCG-PH-3′, 5′-TCCACCGCAAGAACATATGACCT-FL-3′), TNF-α (primers, 5′-CTCTGCGCAAGGTACGTAGA-3′, 5′-GGGCTTGAGGAATGTGTA-3′; probes, 5′-GCATTGCGGCCTGGCT-FL-3′, 5′-LC640-CCATGAGGTCGGCCTCAGCT-FL-3′). Real-time PCR was normalized against the housekeeping gene 5-aminolevulinic synthase expression (primers, 5′-CAGCTCCGCGTCTAATGCGA-3′, 5′-AATGGTGCAGCCCA-CCCCAC-3′; probes, 5′-CCTGCCGCCAGCACCACATTGT-FL-3′, 5′-LC640GTCATACGGCCGACACACC-PH-3′) of the unstimulated control at each time point.

ELISA

IFN-γ and TNF-α release were quantified in culture supernatants, according to the manufacturer’s instructions (BioSource International).

FACS analysis

The purity of NK cells was assessed by FACS analysis, consistently showing >95% CD56⁶CD³⁰ NK cells. The average proportion of CD56⁶CD³⁰ cells, evaluated immediately after isolation from PBMCs, was 96.53%. Twenty-four hours later, after priming with rhIL-2, the CD56⁶CD³⁰ cells accounted for 97.9%, and these cells were used in subsequent experiments. In detail, the contaminating WBC populations were: CD3⁺ T cells, 1.8%; CD20⁺ B cells, 0.19%; CD14⁺ monocytes, 0.15%; CD1c⁺CD19⁺CD14⁻ myeloid dendritic cells, 0.36%; CD56⁻PE, CD3-FTTC, CD20-allophycocyanin, CD14-allophycocyanin, CD11c-PE, CD19-FTTC (BD Biosciences). The presence of cytokine molecules was assessed by the detection of degranulation markers CD107a/b on CD56⁶CD³⁰ NK cells (CD107a-FTTC, CD107b-FTTC, CD56-allophycocyanin, CD3-PerCP [BD Biosciences]).

Plating assays

Plating assays were performed by coculturing 4×10⁵ NK cells with different morphologies of A. fumigatus at an E:T ratio of 1. The assay was performed for 3 and 6 h in 600 μl medium, at 37°C and 5% CO₂ humidified air. In similar experiments, 4×10⁵ germlings were incubated for 24 h, to induce degranulation and thereby to eliminate cytotoxic granules (6). The cells were then washed three times in culture media before being used in the experiments. NK cell viability was not affected by such treatments (trypan blue exclusion).

Analysis of fungal damage

A total of 2×10⁵ fungal germlings was cultured in 150 μl NK cell supernatant (prepared after IFN-γ partially depleted supernatant, or culture medium supplemented with rhIFN-γ) and at 37°C. IFN-γ depletion was performed magnetically, using the IFN-γ secretion assay cell enrichment and detection kit (Milenyi Biotec). IFN-γ was depleted by 85%. The mean concentration of IFN-γ in IFN-γ partially depleted supernatants, was 2.5 pg/ml (as determined by ELISA). To mimic fungal damage caused by IFN-γ, we used rhIFN-γ. rhIFN-γ was diluted in culture medium to a final concentration equal to that in the undepleted supernatant of each experiment (as determined by ELISA). To evaluate the dose response, rhIFN-γ was diluted in RPMI 1640 without any supplements. This medium was inoculated with germlings and conidia, as described above. After a 3- to 6-h incubation period, the cultures were washed three times with cold water. A total of 400 μl PBS with 0.5 mg/ml 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]2H-tetrazolium-5-carboxanilide (XTT) (Sigma-Aldrich) and 50 μg/ml coenzyme Qo (Sigma-Aldrich) was added to fungus, which was then incubated for 1.5 h at 37°C. Duplicate 100 μl samples of supernatant were transferred to an ELISA plate, and the OD was measured at 450 nm with 655 nm reference filter. Anti-fungal effect was calculated according to the following formula: percentage of damage = (1 − s/y) × 100, where x is the OD of the different culture conditions and y is the OD of the negative control (fungus alone).

Germings growth inhibition assay

Germings of the A. fumigatus strain ATCC 46645 (generated as described before) were resuspended in 0.1 M sodium carbonate buffer (pH 10.0) and 0.3 mg/ml FITC (Sigma-Aldrich) and incubated for 30 min at 37°C in the dark with shaking. Germings were washed twice in sterile deionized water and resuspended in fresh medium at 4×10⁶ germings/ml. Germing solution (100 μl) was used to inoculate each well of a 24-well plate containing 1.5-300 μl/well NK supernatant, rhIFN-γ–enriched medium, or supplemented medium and a glass coverslip. The mean concentration of IFN-γ was 5.5 pg/ml. The plate was incubated at 37°C, 5% CO₂, and, after 6-h incubation, the cells were fixed in 3.7% formaldehyde for 5 min at room temperature. Germing growth beyond the FITC labeling was visualized by immunofluorescence using hybridoma supernatant containing the mAb 1L0-3 (500 ng/ml) diluted in partially depleted medium, or supplemented medium and a glass coverslip. Coverslips were mounted to glass slides in Fluoroprep, and germing elongation was measured using the software package provided with the Leica SP-S confocal laser scanning microscope (Leica Microsystems). At least 100 germings were measured for each growth condition, and a Student t test was used to calculate significance.

Statistics

Estimation of p values was performed with the unpaired, two-sided Student t test; p > 0.1 NS, *p < 0.1, **p < 0.05, ***p < 0.001. Bars show arithmetic means of the values of the independent experiments ± SEM.

Results

Human NK cells express Th1-like cytokines during interaction with A. fumigatus germlings

Both animal and clinical studies have demonstrated the protective effect of Th1 immunity against IA, with IFN-γ and TNF-α being the major mediators of this response (9, 17–22). To evaluate whether human NK cells express these cytokines upon incubation with A. fumigatus, we performed in vitro infection experiments with resting (conidia) and germinated (germlings) fungal morphologies. We observed that NK cells, challenged by germlings, upregulated the genes IFN-γ and TNF-α, in a time-dependent pattern, reaching a maximum between 6 and 12 h after stimulation (Fig. 1A, 1B). A similar temporal profile was also observed for the release of these cytokines, with the maximum being detected after 12 h of stimulation (Fig. 1C, 1D). Interestingly,
resting conidia induced no effect on NK cells; however, cytokine release occurred following germination (Fig. 1C). Focusing further on the mechanism of this recognition, NF-κB transcription factor translocation to the nucleus was demonstrated in the presence of germlings, but not by conidia, suggesting the involvement of pattern distinct recognition receptors in the interaction of NK cells with *A. fumigatus* (data not shown).

Human NK cells primed with rhIL-2 exhibit cytotoxicity against *A. fumigatus* germlings

To investigate whether NK cells interfere with *A. fumigatus* growth, we performed plating assays. When NK cells were co-incubated with conidia, there was no fungal damage observed, at all time points (Fig. 2A). However, when they were confronted for 3 h with germlings, there was a significant reduction of 40–60% in the number of fungal colonies (Fig. 2B). To evaluate the influence of priming with rhIL-2 on the NK cell cytotoxicity, we repeated the previous experiments cocultivating germlings with either rhIL-2-primed NK or unprimed NK cells (Fig. 2C). Resting NK cells were unable to damage *A. fumigatus*, highlighting the importance of priming in the induction of cytotoxicity (Fig. 2C).

NK cell stimulation by *A. fumigatus* is contact dependent, but antifungal activity is not

To investigate whether the interaction of NK cells with *Aspergillus* was contact dependent or mediated by soluble factors, the previous infection experiments were repeated using permeable transwell inserts (///) (Fig. 3A, 3B). These allowed the free circulation of soluble molecules, but prohibited direct contact between cells placed in opposite compartments. The compartments were inoculated as follows: NK /// germlings and NK + germlings /// germlings. NK + germlings and germlings alone served as positive and negative controls, respectively (Fig. 3B). Cytokine release was determined as before, and plating assays were performed after 3-h incubation. In the presence of the transwell membrane, without direct fungal contact (NK /// Germlings), cytokine release was abolished as compared with contact-stimulated NK cells (Fig. 3A, NK + Germlings). NK cells showed no antifungal activity across the membrane (NK /// Germlings), permitting unhindered growth of *A. fumigatus* (Fig. 3B). However, when NK cells were coincubated with the fungus at one side of the insert and germlings alone were at the other side (NK + Germlings /// Germlings), the CFU of germlings cultured alone was equivalent to the CFU of the positive control, where the germlings were cultured in contact with NK cells (Fig. 3B). These data show that direct contact is required as a first step in the stimulation of NK cells by *A. fumigatus*. NK cells then release soluble factors with antifungal properties. Thus, NK cell signaling induced by *A. fumigatus* is contact dependent, but the resultant antifungal effect is not.

Soluble factors mediate the anti-Aspergillus effect of NK cells

To confirm the effectiveness of the released soluble factors against the fungus, we performed plating assays where germlings were incubated, for 3 h, in culture supernatants. Incubation in supernatant caused a reduction in CFU development to 60% of that observed in culture medium alone (Fig. 4A). The antifungal activity of the supernatant was also assessed by the XTT assay. Interestingly, 40% fungal damage was observed after exposing germlings to culture supernatant for 3 h (Fig. 4B). These findings suggest that NK cells exert cytotoxicity against *A. fumigatus*.
through soluble molecules released by NK cells after direct contact with the fungus. *A. fumigatus* might be damaged by these factors without being in physical contact with the NK cells.

NK cell anti-*Aspergillus* cytotoxicity is independent of NK cell degranulation

Cytotoxic protein degranulation represents one of the two major killing mechanisms of NK cells against malignant and virus-infected cells (2). To determine whether this mechanism was responsible for *A. fumigatus* damage, we evaluated the expression of the lysosomal associated membrane proteins LAMP-1 and LAMP-2 (CD107a,b), which appear on the cell surface when degranulation occurs (23). CD107a,b–FITC Abs were incubated

FIGURE 2. RhIL-2–primed NK cells exert direct cytotoxicity against *A. fumigatus* germlings. Plating assays were performed with NK cells primed with rhIL-2 and challenged with *A. fumigatus* conidia or germlings for the indicated times. Coincubation of resting conidia with NK cells for 3 and 6 h had no impact on conidial growth, compared with controls (A). In contrast, growth of germlings was clearly reduced after coincubation with NK cells (B). Without rhIL-2 priming, NK/ko rhIL-2 cells failed to damage germlings (C), demonstrating that a pretreatment with rhIL-2 is essential to render NK cells capable of exerting cytotoxicity toward *A. fumigatus*. Experiments were performed independently, on different days, with two to four donors each time. Data from four experiments studying the NK cell effect on conidia and eight experiments studying the impact on germlings.

FIGURE 3. NK cell stimulation by *A. fumigatus* requires direct cell-to-cell contact. NK cells and germlings were placed in the two compartments of transwell chambers (NK /// Germlings). The insert prohibited cell contact between the two populations, but allowed free circulation of molecules between the compartments. Without direct contact to the fungus, NK cells showed no enhanced cytokine release, as quantified by ELISA (A), as well as no cytotoxicity, measured by plating assays (B). To determine whether soluble factors released by NK cells upon stimulation by the fungus are sufficient to trigger the observed cytotoxic effect, germlings were coincubated with NK cells in one compartment and germlings were placed in the other compartment (NK + Germlings /// Germlings). The antifungal effect across the membrane was normalized against a control in which germlings were cultured alone (B). Data from five independent experiments with one donor each. *p < 0.1, **p < 0.05.

Three experiments compared the cytotoxicity of primed and resting NK cells. The cultures were performed in duplicates. Each duplicate was plated twice on Sabouraud agar plates. *p < 0.1, **p < 0.05.
with unstimulated NK cells (negative control), fungal germlings–NK cell cocultures, or K562 cell–NK cell cocultures (positive control) for 4 h and measured by flow cytometry. Surprisingly, *A. fumigatus* did not induce the surface expression of CD107a, suggesting that exocytosis of cytotoxic proteins (perforin, granzymes, granulysine) is not the mechanism mediating *A. fumigatus* damage (Fig. 5A).

It has previously been shown that mobilization and increase of the intracellular Ca\(^{2+}\) are essential for exocytosis of the lytic granules (24). Moreover, the Ca\(^{2+}\) flux correlates with the surface expression of CD107a (25). To determine whether exocytosis is required for the observed antifungal activity, NK cells were treated with EGTA, a Ca\(^{2+}\) chelating agent (6), and thereafter challenged with *A. fumigatus* germlings or K562 cells (control) for 3 h. The antifungal activity of NK cells was not influenced by EGTA pretreatment (Fig. 5B), whereas the same treatment abolished their ability to kill K562 cells (trypan blue staining) (Fig. 5C). Depletion of cytotoxic proteins from NK cells mediated by SrCl\(_2\) that triggers degranulation (6) did not affect the antifungal activity of NK cells (Fig. 5B). However, the same treatment abolished the cytotoxicity of NK cells against the K562 cells (Fig. 5C).

**FIGURE 5.** Anti-*A. fumigatus* activity is degranulation process independent. NK cells were coincubated for 4 h with FITC-labeled Abs directed against the degranulation markers CD107a,b, which are exposed on the NK cell surface upon release of cytotoxic proteins. In the presence of germlings, the expression of CD107a,b was equivalent to the unstimulated control (negative control). However, NK cells exposed these markers upon confrontation with *A. fumigatus* germlings or K562 cells (control) for 3 h. The antifungal activity of NK cells was not influenced by EGTA pretreatment (Fig. 5B), whereas the same treatment abolished their ability to kill K562 cells (trypan blue staining) (Fig. 5C). Data from three independent experiments with two donors each. *p < 0.1, **p < 0.05, ***p < 0.001.
The second major cytotoxic pathway engaged by NK cells involves the interaction of FasL and TRAIL expressed on NK cells, with their cognate receptors on target cells (1). We repeated our plating assays after treating NK cells with blocking Abs against FasL and TRAIL. Treated NK cells were equally as effective as untreated cells against *A. fumigatus*. This finding suggests that FasL and TRAIL do not mediate an antifungal response (data not shown). Collectively, these data showed that the two major cytotoxic mechanisms of NK cells are not involved in the anti-*Aspergillus* activity.

**IFN-γ demonstrates direct anti-*A. fumigatus* activity**

To date, IFN-γ has been recognized as a factor exhibiting indirect antifungal properties, via an increase in the antifungal activity of innate immune effector cells (26, 27). In our study, we investigated whether IFN-γ also acts directly against *A. fumigatus*, as a soluble factor released by NK cells to damage fungal germ-lings. XTT assays were performed after 3-h incubation of germ-lings in NK cell supernatants, partially depleted of IFN-γ (mean concentration of IFN-γ, 2.5 pg/ml) and undepleted supernatant (mean concentration of IFN-γ, 18 pg/ml) (Fig. 6B). This demonstrated that the depletion of IFN-γ decreased the anti-*Aspergillus* effect of the supernatant (Fig. 6A). To confirm the direct antifungal properties of IFN-γ, XTT assays were repeated after incubation of the fungus in culture medium supplemented with rhIFN-γ at concentrations equal to those detected in the complete supernatants by ELISA (Fig. 6B). In fact, damage in the presence of rhIFN-γ was equivalent to damage in undepleted supernatant, confirming the anti-*Aspergillus* properties of this cytokine (Fig. 6A). To quantify this observation and to investigate the presence of a potential, alternative anti-*Aspergillus* molecule, the hyphal length was additionally measured after 6-h incubation of germ-lings with low IFN-γ concentration NK supernatants and rhIFN-γ-enriched medium (mean concentration of IFN-γ, 5.5 pg/ml). Both NK supernatants and rhIFN-γ medium inhibited the hyphal elongation similarly. However, a trend of a higher inhibition caused by the NK supernatants was observed (Fig. 6C). It is worth noting the morphological alterations of the fungus caused by NK supernatant (Supplemental Fig. 1, Image 3) and rhIFN-γ medium (Supplemental Fig. 1, Image 2). Both culture conditions provoked similar structural distortions of the germ-lings, which acquired an uncommon flattened and twisted conformation. To provide further evidence for a direct role of IFN-γ against *A. fumigatus* and to exclude any synergistic effect with the supplements of the culture medium, we measured by XTT assay the effect of different concentrations of rhIFN-γ in unconditioned RPMI 1640 medium. A dose-dependent toxicity was observed toward germ-lings (Fig. 6D).

![FIGURE 6. IFN-γ directly mediates NK cell damage against *A. fumigatus*. We compared the antifungal effect of undepleted and partially IFN-γ-depleted supernatants on germ-lings, after 3-h incubation using an XTT assay. Data from 10 independent experiments with one donor each. The bar on the right shows the fungal damage caused by the culture medium supplemented with rhIFN-γ at concentrations equal to those detected in NK cell supernatants (mean concentration, 17.63 pg/ml). This inhibition resembled that obtained with undepleted supernatants (left bar), whereas partial depletion of IFN-γ reduced the fungal growth inhibition (middle bar). Data from four independent experiments (A). The concentrations of IFN-γ of the experimental conditions of A are shown here (B). We confirmed the antifungal effect of IFN-γ, even at low concentrations, by measuring the hyphal elongation under the effect of NK supernatants and rhIFN-γ-supplemented medium with same amounts (mean, 5.5 pg/ml) of the cytokine, respectively. After 6-h incubation, both conditions caused an equivalent reduction of hyphal elongation, which was statistically significant compared with the growth control. Data from three independent experiments (C). A dose-damage relationship was established between the different concentrations of rhIFN-γ in unconditioned RPMI 1640 medium toward germ-lings. Data from eight independent experiments (D). *p < 0.1, **p < 0.05, ***p < 0.001.](http://www.jimmunol.org/issue.download)
In contrast, RPMI 1640 supplemented with rhIFN-γ demonstrated no effect against conidia (data not shown).

These results indicate the direct anti-\textit{A. fumigatus} properties of IFN-γ, and show that IFN-γ is a factor mediating NK cell cytotoxicity toward the fungus.

**Discussion**

Since their discovery, NK cells have proven to be a useful tool in the fight against neoplasia and viral infections. NK cells are both potent effectors of innate immunity, exerting direct cytotoxicity without prior sensitization (1), and immunoregulatory mediators, shaping the response of other immune cells, such as dendritic cells and T cells (28–30). Interestingly, their role toward fungal pathogens, especially \textit{A. fumigatus}, has been poorly elucidated. Our work aimed to study the direct interaction of human NK cells with \textit{A. fumigatus}.

Studies in both humans and animals have documented the favorable effect of the Th1 immune response against IA. Proinflammatory cytokines such as IFN-γ and TNF-α are associated with increased antifungal activity of phagocytes, higher clearance of \textit{A. fumigatus}, and better response to antifungal treatment (9, 17–22). Our results reveal that human NK cells challenged with \textit{A. fumigatus} mediate a Th1-like response secreting IFN-γ and TNF-α. Interestingly, the release of these cytokines is morphotype dependent and is induced only by germinals and not by resting conidia. Moreover, in accordance with data in animals showing the importance of mouse NK cells for the clearance of \textit{A. fumigatus} from the lungs (8), we demonstrated that human NK cells exert direct cytotoxicity against germinals; however, this is not the case with conidia. Both cytokine induction and antifungal activity suggest that NK cells perceive only germinated morphologies of \textit{A. fumigatus}, recognizing that the fungus has escaped the first line of host defense, provided by alveolar macrophages and neutrophils (31–33). Priming of NK cells with rhIL-2 seems to be a prerequisite for NK cell–fungus interaction, suggesting an important role for cytokine-producing immune cells in the interplay of NK cells with \textit{A. fumigatus}. We next questioned whether physical contact or soluble molecules mediated NK cell signaling and cytotoxicity. Separating the immune cells from the fungus, using transwell membranes, we observed a complete abrogation of both NK cell stimulation and fungal damage. However, when NK cells were cocultured with \textit{A. fumigatus} on one side of the membrane, a condition allowing NK cell signaling, the antifungal effect on the other side was equivalent to the effect observed when NK cells and \textit{A. fumigatus} were cultured in direct contact. These results indicate a two-step mechanism of cytotoxicity. First, germinals stimulate NK cells when in direct contact. In response, NK cells release soluble molecules able to harm the fungus not in physical contact with the immune cells.

The two major killing pathways of NK cells have been the object of extensive studies during the last two decades. Contact-dependent exocytosis of highly cytotoxic proteins and the activation of death receptors on target cells by TNF and FasL are the main means of attack by NK cells against tumor- and virus-transformed cells (1). Our results revealed that neither of these mechanisms is specifically involved in the anti-\textit{Aspergillus} activity of NK cells. Instead, we demonstrate that IFN-γ released by NK cells in the presence of \textit{A. fumigatus} germinals causes the observed antifungal effect. Moreover, we revealed that the anti-\textit{Aspergillus} activity of IFN-γ is concentration dependent. This surprising finding is consistent with the previous report that NK-derived IFN-γ is essential to the host defense in a murine model of IA (9). It was shown that NK cell depletion and IFN-γ deficiency augmented similarly the severity of IA, suggesting that the contribution of NK cells to the anti-\textit{A. fumigatus} effect is confined to IFN-γ production. Moreover, depletion of NK cells from IFN-γ knockout mice had no adverse effect on the outcome of infection. This argues against an involvement of other effectors, apart from IFN-γ (e.g., cytotoxic proteins, perforin, granzymes, granulysine) in the NK cell-mediated anti-\textit{A. fumigatus} activity (9).

Recent data on the human NK cell–\textit{A. fumigatus} interaction recognized an activity of NK cells against germinals. Although we found that IFN-γ can directly inhibit the fungal growth, Schmidt et al. (34) implicated perforin as a major effector molecule of NK cell-mediated anti-\textit{Aspergillus} activity. We believe that the discrepancies between their data and ours can be explained by the different study designs. Our study does not rule out that perforin, granzymes, granulysine, or other proteins of the NK cell granules have antifungal activity. However, our data demonstrate that the NK cell–\textit{A. fumigatus} interaction does not involve substantial degranulation, which argues against a functional importance of the above-mentioned proteins in the antifungal activity of NK cells (34). It is worth noting that our data cannot exclude the involvement of an additional factor, induced by the fungus and secreted by an unknown process. This might explain the minor, although significant decrease of the antifungal effect caused by the partially IFN-γ-depleted supernatant (Fig. 6A) and the trend toward an increased growth inhibition induced by the NK cell supernatant (Fig. 6C). Moreover, lower levels of hyphal damage caused by rhIFN-γ in unconditioned medium (Fig. 6D) compared with FCS and rebofacin-supplemented medium (Fig. 6A) might reflect effects of the supplements.

Experimental infections in mice and clinical trials in humans have recognized the ability of IFN-γ to increase the phagocytic activity of innate immune effector cells against different species of fungi and especially \textit{Aspergillus} (9–11, 13, 14, 17, 35). Our data suggest that in addition to acting as an immunoregulatory molecule, IFN-γ acts directly against \textit{Aspergillus}, further explaining the antifungal effect observed in the previous studies. The concept of human proteins and peptides capable of eradicating pathogens is not new. Indeed, there is increasing evidence regarding the antibacterial and antifungal activity of chemokines, defensins, cathelicidins, and histatins (36–39). It is worth noting that, in our experiments, low concentrations of IFN-γ proved to be sufficient for anti-\textit{Aspergillus} activity. Moreover, the finding that rhIFN-γ alone damages \textit{A. fumigatus} underlines that the fungus is directly targeted by this major cytokine. If another factor is involved, it should originate from fungal germinals. Interestingly, Hu et al. (40) reported a synergistic cytotoxicity against hepatoma cell lines, resulting from the interaction of IFN-γ with a RNAse. IFN-γ might cooperate with fungal ribotoxins, secreted by \textit{A. fumigatus} (41), transforming them into suicide molecules for fungus.

In conclusion, despite the progress in antifungal diagnostics and treatment, IA remains a leading cause of morbidity and mortality in immunosuppressed patients. Our study sheds new light on the host defense against the most common cause of IA, \textit{A. fumigatus}, and provides a rational framework for the use of NK cells and IFN-γ as therapeutic tools against IA.

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**Disclosures**

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