Human Natural Killer Cells Exhibit Direct Activity Against *Aspergillus fumigatus* Hyphae, But Not Against Resting Conidia

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Because natural killer (NK) cells kill tumor cells and combat infections, there is growing interest in adoptively transferring NK cells to hematopoietic stem cell recipients. Unfortunately, in humans, the activity of NK cells against *Aspergillus* species, the major cause of invasive fungal infection in stem cell recipients, are poorly characterized. Our results show that unstimulated and interleukin-2 prestimulated human NK cells kill *Aspergillus fumigatus* hyphae but do not affect resting conidia. Killing is also induced by the supernatant of prestimulated NK cells and human perforin. The high levels of interferon-γ and granulocyte macrophage colony-stimulating factor produced by prestimulated NK cells are significantly reduced by *Aspergillus*, indicating an immunosuppressive effect of the fungus. Whereas *Aspergillus* hyphae activate NK cells, resting, and germinating, conidia and conidia of ΔrodA mutants lacking the hydrophobic surface layer do not. Our results suggest that adoptively transferred human NK cells may be a potential antifungal tool in the transplantation context.

Despite improved chemotherapeutic regimens, relapse and infectious complications remain major causes of morbidity and mortality in the context of allogeneic hematopoietic stem cell transplantation [1, 2]. To improve outcome in transplant recipients, there is growing interest in adoptive immunotherapeutic strategies, such as the administration of antigen-specific T cells and natural killer (NK) cells. Whereas the administration of T cells is associated with the risk of potentially lethal graft-versus-host disease, NK cells are usually well tolerated and may even mitigate graft-versus-host disease [3]. In vitro data demonstrate that human NK cells are cytotoxic against a variety of tumor types, including acute lymphoblastic and myelogenous leukemia [4]. The potential clinical benefit of adoptive NK cell therapy is supported by the observation that patients with high-risk acute myelogenous leukemia who received a transplant from an NK-cell alloreactive donor had a significantly lower relapse rate and a better event-free survival than did patients who received a transplant from a non–NK-alloreactive donor [5]. In addition to the killing of tumor cells, NK cells play a crucial role in combating infections due to a variety of pathogens [6]. To date, NK cells have been examined in a neutropenic mouse model. It was demonstrated that, in mice with pulmonary aspergillosis, the chemokine-mediated recruitment of NK cells is an important antifungal host defense mechanism, and the transfer of activated NK cells results in greater pathogen clearance [7, 8]. Unfortunately, in humans, the activity and antifungal mechanisms of NK cells against *Aspergillus* species are poorly characterized, although *Aspergillus* is the major cause of invasive fungal infection in allogeneic stem cell
recipients, with a mortality up to 80% [9]. We therefore investigated the effect of human NK cells on various Aspergillus morphotypes, and our results suggest that adoptively transferred human NK cells may be a potential antifungal tool in hematopoietic transplant recipients at high risk of or experiencing invasive aspergillosis.

**MATERIALS AND METHODS**

**Isolation and Cultivation of Primary Human NK Cells**

Primary human NK cells were isolated from peripheral blood according to a protocol previously described with some modifications [10]. In brief, blood was obtained from healthy volunteers who had no evidence of previous invasive fungal infection. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Paque density gradient, harvested, and washed twice. NK cells were isolated using a 2-step method; a negative selection using anti-CD3 MicroBeads was followed by a positive selection of CD56+CD3- cells using anti-CD56 antibodies (MACS magnetic column separation system; Miltenyi Biotech). The purity and viability of the isolated human CD56+CD3- NK cells were ≥95% and ≥98%, respectively, as determined by flow cytometry (FC-500, Beckmann Coulter) using the following antibodies: anti-CD3, CD14, CD45, and CD56, labeled with FITC, PE, ECD, and PC7, respectively, and 7-amino-actinomycin D for testing viability (all Beckmann Coulter). Anti-CD69 labeled with ECD was used for assessment of NK cell activation (Beckman Coulter). After isolation, NK cells were resuspended in X-VIVO 10 medium (Lonza) supplemented with 5% human frozen plasma (German Red Cross Blood Donor Service Baden-Wuerttemberg – Hessen). The NK cells were used immediately or further cultivated for 7–10 days in the presence of Escherichia coli–derived recombinant human interleukin (IL)–2 (1000 U/mL added every third day; Novartis). The protocol was approved by the local ethics committee.

**Preparation of A. fumigatus**

The A. fumigatus strain AF4215 (MYA 1163; American Type Culture Collection) was grown on Sabouraud glucose agar plates at 25°C for 1 week and prepared as described above [12].

**Assessment of Antifungal Activity**

Activity against Aspergillus hyphae was assessed by means of the colorimetric assay with (2,3-bis[2-methoxy-4-nitro-5sulphenyl] 2H-tetrazolium-5-carboxyanilide)sodium salt (XTT; Sigma-Aldrich) plus coenzyme Qo (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma-Aldrich) using A. fumigatus as described previously with some modifications [13]. In brief, NK cells were added to A. fumigatus hyphae in different effector-to-target ratios and incubated for up to 6 h. Then, wells were washed twice with sterile water to lyse NK cells, and XTT wasdissolved in phosphate-buffered saline at a concentration of 0.25 mg/mL and 40 µg/mL coenzyme Qo were added. After incubation for 1 h at 37 C with 5% carbon dioxide, 100 µL were transferred to a 96-well plate, and the change in color (absorbance) was assessed spectrophotometrically at 450 nm with use of a 690-nm reference. Antihyphal activity was calculated according to the formula: percent hyphal damage = (1-X/C) × 100, where X is the absorbance of experimental wells and C is the absorbance of control wells with hyphae only. For some experiments, a polycarbonate cell culture insert (Greiner Bio-One; pore size, 0.4 µm) was used to separate A. fumigatus hyphae and NK cells.

In addition to the XTT test, viability staining with 5-carboxyfluorescein diacetate/propidium iodide was performed (FungaLight; Molecular Probes).

For the evaluation of the activity of NK cells against Aspergillus conidia, resting conidia were incubated with or without NK cells for 24 h. A total of 100 µL of the suspension was spread onto Sabouraud glucose agar plates and incubated at 37°C for 24 h. After 24 h, the conidial damage was assessed by determination of colony-forming units (CFUs) per mL. The comparison of the number of CFUs with and the number of CFUs without co-incubated NK cells indicated the antifungal activity of NK cells against resting conidia of A. fumigatus.

**Measurement of Perforin, Interferon (IFN)-γ, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and RANTES (CCL5)**

The concentration of perforin, IFN-γ, GM-CSF, and RANTES in the supernatant was measured using commercially available enzyme-linked immunoabsorbent assays (ELISAs). According to the manufacturers’ instruction, the detection limit for perforin was 40 pg/mL (Diaclone); for IFN-γ, 4 pg/mL (eBiosciences); for GM-CSF, 7.8 pg/mL; and for RANTES, 31.2 pg/mL (GM-CSF and RANTES: R&D Systems).

**Evaluation of the Effect of Perforin**

To block perforin-mediated cytotoxicity of the NK cells, NK cells were treated with 10 nM concanamycin A for 2 h (Sigma Aldrich) [14]. NK cells were washed 3 times in X-VIVO 10.
medium, assessed for viability by flow-cytometry, and placed for the experiment.

Human perforin isolated from cytotoxic granules of YT cells (Alexis Biochemicals) was used at a concentration of 100 ng/mL.

**Statistical Analysis**

Data were analyzed using GraphPad Prism (GraphPad Software). Comparisons between experimental groups were performed by unpaired Student’s *t* test. Pearson’s correlation was used to analyze the degree of linear relationship between 2 variables. A 2-sided *P* value <.05 was considered to be statistically significant.

**RESULTS**

**Activation of Primary Human NK Cells by *A. fumigatus***

To evaluate whether different morphotypes of *A. fumigatus* are able to activate human NK cells, we assessed the up-regulation of CD69 on unstimulated human NK cells when co-incubated with *A. fumigatus* conidia, resting conidia, and germinating conidia, respectively. Whereas CD69 on NK cells is up-regulated by *A. fumigatus* hyphae indicating activation, wild-type resting conidia and germinating conidia do not affect CD69 up-regulation of unstimulated NK cells (Figure 1). Similarly, conidia of *ΔrodA* mutants lacking the hydrophobic surface layer are not able to activate NK cells (Figure 1). In addition, the activation of NK cells by *A. fumigatus* hyphae is prevented by a membrane with a pore size of 0.4 μm, which separates human NK cells and the fungus, suggesting that close vicinity or direct NK cell–fungus interaction is responsible for CD69 up-regulation (data not shown).

**Unstimulated and IL-2–Stimulated Primary Human NK Cells Kill *A. fumigatus* Hyphae, But Do Not Affect Resting *A. fumigatus* Conidia**

Our results demonstrate that both unstimulated and IL-2 prestimulated human NK cells kill *A. fumigatus* hyphae, as revealed by means of the XTT assay (Figure 2) and viability staining with 5-carboxyfluorescein diacetate /propidium iodide (Figure S1). The extent of hyphal killing of IL-2 prestimulated NK cells is significantly higher, compared with unstimulated NK cells (*P* = .026). In addition, increasing effector-to-target ratios result in higher hyphal damage. For example, when coincubating NK cells and *Aspergillus* hyphae for 6 h at effector-to-target ratios of 10:1, 20:1, and 50:1, the mean hyphal killing of unstimulated NK cells ranges from 10% (standard deviation [SD], 4%) to 24% (SD, 4%) and 34% (SD, 13%), respectively (each test performed at least 3 times). Similarly, for IL-2 prestimulated NK cells, hyphal killing ranges from 15% (SD, 7%) to 24% (SD, 6%) and 43% (SD, 7%), respectively (each test performed at least 3 times). In contrast to *A. fumigatus* hyphae, resting conidia are not affected by NK cells, whether unstimulated or prestimulated with IL-2 (Figure 2).

**The Perforin-Pathway is Involved in Aspergillus Hyphal Killing by NK Cells**

The levels of perforin, which is constitutively expressed by NK cells, are significantly higher in the supernatant of IL-2 prestimulated than in unstimulated NK cells (median, 18,434 pg/mL [range, 13,185–43,113 pg/mL]; and 3808 pg/mL [range, 2271–5208 pg/mL], respectively; *P* = .013; *n* = 5 each). Higher perforin levels correlate with higher killing activity of *Aspergillus* hyphae by both unstimulated (*r* = 0.94; *P* < .0002; data from 9 tests, each performed in triplicates) and IL-2 prestimulated human NK cells (*r* = 0.96; *P* < .0001; data from 9 tests, each performed in triplicates). When separating IL-2 prestimulated human NK cells and *A. fumigatus* hyphae by a membrane (pore size, 0.4 μm), a similar extent of hyphal damage is seen, compared with the context without membrane, indicating that hyphal damage does not depend on direct NK cell–fungal interaction (Figure 3A). This is further supported by the fact that killing of *A. fumigatus* hyphae is induced by the supernatant of
IL-2 prestimulated NK cells (Figure 3A) and by purified human perforin (Figure 3B). Blocking of perforin-mediated cytotoxicity via concanamycin A results in a significant decrease of anti-Aspergillus activity of IL-2 prestimulated NK cells (Figure 3B), indicating that perforin plays an important role in the antifungal activity of NK cells.

**Influence of A. fumigatus Morphotypes on IFN-γ, GM-CSF, and RANTES Production by Human NK Cells**

Because IFN-γ and GM-CSF play an important role in the host response to invasive aspergillosis, we assessed the influence of different morphotypes of *A. fumigatus* on the production of these molecules by human NK cells. Without adding *A. fumigatus* hyphae, higher levels of IFN-γ are measured in the supernatant of IL-2 prestimulated, compared with unstimulated NK cells (data not shown). Whereas coincubation with *A. fumigatus* hyphae does not significantly influence the level of IFN-γ in the supernatant of unstimulated NK cells, the concentration of IFN-γ in the supernatant of IL-2 prestimulated NK cells is significantly reduced by the addition of *A. fumigatus* hyphae (*P = .013*) and of germinating conidia (*P = .016*) (Figure 4A). Similarly, the levels of GM-CSF are reduced in the supernatant of IL-2 prestimulated NK cells compared with unstimulated NK cells, which is also seen upon addition of *A. fumigatus* hyphae (*P = .037*) and of germinating conidia (*P = .016*) (Figure 4B). In contrast, resting conidia of *A. fumigatus* do not affect the levels of both IFN-γ and GM-CSF (Figure 4A and 4B). The production of RANTES, which plays a major role in inflammatory processes and is highly produced by IL-2 prestimulated NK cells, is not altered by any of the *A. fumigatus* morphotypes (Figure 4C).

**DISCUSSION**

Here, we report for the first time to our knowledge that both unstimulated and IL-2 prestimulated human NK cells kill *A. fumigatus* hyphae but are not active against resting *A. fumigatus* conidia. These results might have an important impact on the strategy of adoptive NK cell immunotherapy in hematopoietic transplant recipients. Whereas the cytotoxic effect of NK cells against malignant cells is well described and is already being evaluated in clinical trials, there are only few data on the effect of human NK cells against fungi, in particular against *A. fumigatus*, the most important fungal pathogen in transplantation. Our results show that IL-2 prestimulated NK cells have a higher antifungal activity in vitro against *A. fumigatus* than unstimulated NK cells, which corroborates previous studies investigating the activity of human NK cells against various malignancies [15–17]. Of note, IL-2 was used in these clinical trials either for prestimulation of NK cells ex vivo or as NK cell activation in vivo. In contrast to the killing of *Aspergillus* hyphae, we did not observe that unstimulated or IL-2 prestimulated human NK cells damaged resting *A. fumigatus* conidia. In addition, neither resting nor germinating conidia had an effect in...
activating unstimulated NK cells. This is in line with a recent report that dendritic cells and alveolar macrophages are not activated by resting Aspergillus conidia resulting from the surface hydrophobic layer on the fungus [12]. When removing the hydrophobic layer chemically or genetically (ΔrodA mutant), the altered conidial morphotype was able to activate dendritic cells and alveolar macrophages. In contrast, our results show that the ΔrodA mutant did not induce NK cell activation, indicating that different mechanisms are responsible for the activation of dendritic cells, alveolar macrophages, and NK cells by Aspergillus, which is the focus of our current research.

We further evaluated potential mechanisms by which NK cells kill A. fumigatus hyphae. Because perforin is known as a key mediator of NK cell-mediated cytotoxicity against malignant cells and a variety of microbes, we focused on this molecule. Corroborating previous reports on NK cell activity against Cryptococcus neoformans, our data indicate that NK cells constitutively express activity against A. fumigatus [14]. In addition, similar to NK cell antitumor and anticytotoxic activity, the activity against Aspergillus can be up-regulated by pre-activation of NK cells with IL-2 [15, 18]. Because perforin production is up-regulated in IL-2 prestimulated NK cells, purified human perforin exhibits killing of A. fumigatus hyphae, and blocking of perforin-mediated cytotoxicity via concanamycin A results in a significant decrease of anti-Aspergillus activity, our data suggest that perforin plays a significant role in the killing of Aspergillus. Of note, NK cell–mediated killing of Cryptococcus requires contact of NK cells with the fungus, whereas the damage of Aspergillus does not depend on direct NK cell–fungal interaction. Further studies have to evaluate whether other molecules released by NK cells are also involved in anti-Aspergillus activity.

In addition to the direct cytotoxic activity of NK cells, which is mediated at least in part by perforin, the secretion of soluble factors such as IFN-γ, GM-CSF, and RANTES up-regulates the activity of phagocytes and has an important impact on the cellular immune response [19, 20]. For example, NK cells potentiate, via secreted IFN-γ, the fungicidal activity of macrophages, which leads to a higher killing of C. neoformans [21]. In addition, the transfer of activated NK cells from wild-type, but not from IFN-γ-deficient mice, results in greater pathogen clearance in mice with pulmonary aspergillosis [8]. However, we observed that the IFN-γ release of IL-2 prestimulated NK cells is down-regulated by A. fumigatus hyphae and germinating conidia, but not by resting conidia, indicating an immunosuppressive effect depending on the morphotype of the fungus. A similar effect was previously reported in mice, in which Candida albicans inhibited the IFN-γ secretion by NK cells [22]. Whereas the production of GM-CSF is also reduced by Aspergillus hyphae and by germinating conidia, the production of RANTES is not affected, indicating specific immunosuppressive effects of the fungus. Of note, the viability of the NK cells was not significantly affected when co-incubated with A. fumigatus hyphae or germinating conidia (data not shown). Because Aspergillus mycotoxins, such as gliotoxin or citrinin, are known to affect IFN-γ production by T cells, one is prompted to speculate that these toxins are also responsible for the down-regulation of IFN-γ or GM-CSF secretion by human NK cells, which might have important clinical implications [23]. On the other hand, no significant improvement of outcome has been demonstrated by the intravenous administration of IFN-γ to patients with invasive aspergillosis, indicating that the IFN-γ effect is complex and might depend on the microenvironment and timing [24].

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Figure 4. Aspergillus hyphae and germinating conidia decrease interferon (IFN)-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) levels in the supernatant of IL-2 prestimulated natural killer (NK) cells, but do not affect the concentration of RANTES. Co-incubation with Aspergillus fumigatus hyphae (light gray columns) or A. fumigatus germinating conidia (black columns) leads to a significant reduction of IFN-γ and GM-CSF production (Figure 4A: \( * P = .013; ** P = .016 \), and Figure 4B: \( * P = .037; ** P = .016 \)), whereas the production of RANTES remains unaffected (Figure 4C). Co-incubation with A. fumigatus resting conidia (dark gray columns) has no significant effect on the production of IFN-γ, GM-CSF, and RANTES. Columns represent mean and standard deviation of at least 3 separate experiments in all figures.
Taken together, our data demonstrate that human NK cells exhibit direct and, via IFN-γ, also indirect killing activity against A. fumigatus hyphae. Therefore, in the context of transplantation, adoptive immunotherapy involving NK cells may be a potential tool in the antifungal armamentarium, administered either alone or in combination with other antifungal therapies. However, prophylactic strategies using NK cells might be limited because of the lack of activity against infecting conidia. Before evaluating the antifungal activity of NK cells in the clinical setting, future studies have to address several questions, including the exact mechanisms of NK cell activation by the fungus and how Aspergillus suppresses the activity of NK cells.

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**References**