Human Natural Killer Cells Acting as Phagocytes Against *Candida albicans* and Mounting an Inflammatory Response That Modulates Neutrophil Antifungal Activity

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Background. Natural killer (NK) cells are innate lymphocytes with potent cytotoxic activity. Whereas activity of NK cells has been demonstrated against the fungal pathogens *Aspergillus fumigatus* and *Cryptococcus neoformans*, little was known about their interaction with *Candida albicans*.

Methods. Primary human NK cells were isolated from buffy coats, primed with a cytokine cocktail and used for confrontation assays with *C. albicans*. Interaction was monitored and quantified using live cell imaging, confocal microscopy, flow cytometry, and enzyme-linked immunosorbent assay.

Results. Human NK cells actively recognized *C. albicans*, resulting in degranulation and secretion of granulocyte-macrophage colony-stimulating factor, interferon γ, and tumor necrosis factor α. Uniquely, activation of NK cells was triggered by actin-dependent phagocytosis. Antifungal activity of NK cells against *C. albicans* could be detected and mainly attributed to secreted perforin. However, NK cells were unable to inhibit filamentation of *C. albicans*. Human polymorphonuclear neutrophils (PMNs) counteracted the proinflammatory reaction of NK cells by preventing direct contact between NK cells and the fungal pathogen. Activation of PMNs was enhanced in the presence of NK cells, resulting in increased fungicidal activity.

Conclusions. Our results show a unique pattern of NK cell interaction with *C. albicans*, which involves direct proinflammatory activation and modulation of PMN activity. For the first time, phagocytosis of a pathogen is shown to contribute to NK cell activation.

Keywords. NK cells; *C. albicans*; phagocytosis; neutrophils; TNF-α; IFN-γ; perforin.
effectors in bacterial, parasitic and also fungal infections [2]. For *Cryptococcus neoformans*, NK cells exhibit a direct antifungal activity in vitro and confer enhanced organ clearance in vivo [4, 5]. This effect is mainly mediated by perforin [6]. Similar effects have been described for the dimorphic fungal pathogens *Paracoccidioides brasiliensis* and *Coccidioides immitis* [7, 8].

Research into the role of NK cells in invasive aspergillosis was driven by an increasing interest in immunotherapy. The tolerogenic potential of NK cells after adoptive transfer and a potential antileukemia activity favor their use in such approaches [9]. In neutropenic mice, CCL2 induction in pulmonary tissue during invasive aspergillosis led to a recruitment of NK cells, which favored fungal clearance and survival [10]. Production of IFN-γ by activated NK cells has been shown to be a major prerequisite for these protective effects [11]. A direct antifungal activity against *Aspergillus fumigatus* hyphae was shown for human NK cells [12, 13].

For *Candida albicans*, several groups have reported interaction with human NK cells but minor or no direct antifungal activity [14–16]. In contrast, murine NK cells were able to inhibit growth of *C. albicans* [17]. Mice with combined T-cell and NK cell deficiency were highly susceptible to *C. albicans* mucosal infection, whereas T-cell deficiency alone did not confer susceptibility [18]. NK cells have been shown to be a potential source of IFN-γ and TNF-α during invasive *Candida* infection [19, 20]. IFN-γ enhances candidacidal activity of macrophages and PMNs, and TNF-α may potentiate PMN activity [21–23]. Consequently, NK cell depletion suppressed the phagocytic activity of splenic macrophages against *C. albicans* [24, 25].

In the current study, we investigated the antifungal activity of human NK cells against *C. albicans*. Cytokine-primed human NK cells were activated by direct contact with *C. albicans*, which was established after actin-dependent engulfment of fungal cells. NK cell activation resulted in degranulation and the release of proinflammatory cytokines and induced fungal damage. Human PMNs were hyperactivated in the presence of NK cells and counteracted the proinflammatory activation of NK cells by sequestering the fungal pathogen. These data suggest distinct functions of NK cells in *C. albicans* infection, dependent on the clinical setting

**METHODS**

**Ethics Statement**

This study was conducted according to the principles expressed in the Declaration of Helsinki. All protocols were approved by the Ethics Committee of the University Hospital Jena (permit number: 2734–12/09). Written informed consent was obtained from all blood donors.

**Isolation of Human Immune Cells**

Primary human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by gradient centrifugation (Biochrom). Untouched NK cells were separated by magnetic-activated cell sorting using the NK cell negative isolation kit (Miltenyi Biotec). The purity of the cells was checked with fluorescence-activated cell sorting analysis, with results of >95% CD56+/CD16+/CD3–/CD14– NK cells. Isolated NK cells were cultivated at a concentration of 2 × 10^6/mL in Stem Cell Growth Medium (SCGM; Cell Genix) plus 10% human serum (PAA) and 100 U/mL interleukin 2 (Immunotools). To generate stimulated NK cells, half of the medium was replaced after 3 d by fresh SCGM plus 10% human serum supplemented by 100 U/mL interleukin 2, 50 ng/mL interleukin 15, 1000 U/mL IFN-α, and 2000 U/mL IFN-β (Immunotools). Activation of primary human NK cells with this cytokine cocktail resulted in development of a larger and more granular subpopulation [26]. PMNs were isolated from whole blood of healthy donors using Polymorph-Prep (PROGEN Biotechnik), as described elsewhere [27].

**Fungal Strains and Culture Conditions**

*C. albicans* (SC5314) was cultured overnight in yeast peptone D-glucose medium (YPD) (1% yeast extract, 2% bactopeptone, 2% D-glucose) at 30°C and diluted 1:10 in fresh medium for a 1.5-hour induction culture at 30°C on a shaker before the experiments. Harvested cells were washed 3 times in phosphate-buffered saline (PBS) and resuspended in SCGM plus 10% human serum. *C. albicans* expressing green fluorescent protein (codon optimized for *C. albicans*) under the control of the ADH1-promotor, was kindly provided by Peter Staib.

**In Vitro Infection Experiments**

NK cells were cocultured with fungi at a multiplicity of infection of 0.5 for 4 hours at 37°C with 5% carbon dioxide (CO2) in SCGM medium plus 10% human serum. Confrontation experiments involving PMNs were performed at a ratio of 2:2:1 (NK cells/PMNs/*C. albicans*). To gain conditioned medium, the supernatant of *C. albicans* infected cells (multiplicity of infection, 0.5; 4 hours, 37°C, and 5% CO2) was sterile filtered. When indicated, Transwell systems (0.4-µm pores; Corning) were used to prevent direct contact between fungi and NK cells. Fungal viability was quantified using an XTT-based assay, as described elsewhere [27].

**Fluorescence-Activated Cell Sorting Analysis**

NK cells were washed with CellWash (BD) and centrifuged at 300 g for 5 minutes. Staining was performed at 4°C for 30 minutes, and cells were analyzed using a BD FACSCanto II flow cytometer. The following antibodies were used: CD282 (TLR2; Clone TL2.1; NatuTec); Dectin-1 (Clone GE2, Mor-phosys AbD); CD16 (Clone 3G8, Biozol); CD56 (Clone B159; BD); CD69 (Clone FN50; BD) CD335 (Clone 9E2; Biozol); CD314 (Clone 1D11; AbD Serotec); CD107a (Clone H4A3; BD), CD66b (Clone G10F5; Biozol). Cells were gated in forward and side scatter. To discriminate between intra- and extracellular fungal cells, we used *C. albicans* green fluorescent
protein and counterstained extracellular fungi with rabbit anti-Candida antibody (Acris Antibodies) before cell permeabilization.

Quantification of Secreted Proteins
Cytokine concentrations within the supernatant of coinfections were determined by means of enzyme-linked immunosorbent

Figure 1. Contact of NK cells with Candida albicans results in cellular activation. A, Innate immune receptors such as CD16, as well as the adhesion marker CD56 or immunoreceptor tyrosine-based activating motif (ITAM)-bearing receptors such as CD335 and CD314 were down-regulated on natural killer (NK) cells after interaction with the fungus for 4 hours. In contrast, surface exposure of the degranulation marker CD107a (4 hours) and activation marker CD69 (3 hours) were markedly up-regulated. White bars show basal expression on primed NK cells (set to 100%); black bars, expression levels after contact with C. albicans normalized against the uninfected control. Each bar represents the arithmetic median (± standard deviation [SD]) of 3 independent experiments with cells from 3 donors. Differential regulation of receptor exposure is further shown in histograms including isotype controls in a representative experiment. B, Microscopic staining verified a relocation of CD107a, which is present in the granules (left) before contact with C. albicans to the surface after contact with the fungus (right; red represents anti-CD107a-AF649; green, calcofluor; scale bar, 10 µm). C, D, Supernatants from confrontation assays were analyzed for the release of perforin and granzyme B (C) and cytokines/chemokines (D). White bars indicate basal levels of cytokine-primed NK cells; black bars, levels 4 hours after contact with C. albicans. C, Both granzyme B and perforin were significantly increased after contact with the fungus. Bars represent arithmetic means ± SDs from independent experiments with cells from 8 donors for granzyme B and 13 donors for perforin. *P < .05. D, Release of proinflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN) γ, and tumor necrosis factor (TNF) α, and the chemokine macrophage inflammatory protein (MIP)-1β was significantly increased after confrontation with C. albicans for 4 hours. Bars show arithmetic means ± SDs of ≥5 independent experiments from cells of different donors for uninfected (white) and infected (black) NK cells. *P < .05; ***P < .001.
assay (perforin, granzyme B; Abcam) or Bio-Plex assay (all other markers; BioRad), according to the manufacturers’ instructions. Supernatants were harvested at the indicated time points and stored at −80°C before the analysis.

Fluorescence Microscopy
NK cells were seeded on gelatin-coated glass coverslips and confronted with C. albicans for 1 hour at 37°C and 5% CO2. Cells were fixed (Histofix, 4%) and stained for 30 minutes at 4°C with primary antibody (CD107a) alone or in combination with phalloidin-AF568. Then cells were washed 3 times in PBS and stained with secondary antibody (goat-anti-mouse-AF649) and calcofluor white (Sigma; 350 µg/mL). For differential staining, cells were fixed and extracellular C. albicans cells were stained with calcofluor white (Sigma; 350 µg/mL) for 30 minutes at 4°C followed by washing in PBS. After 5 minutes permeabilization (0.5% Triton X) all C. albicans were stained using rabbit anti-Candida antibody (Acris Antibodies), NK cells were stained with anti-CD56 (mouse; BD) and phalloidin-AF488 (Invitrogen). Afterward, cells were washed 3 times in PBS and stained with secondary antibodies (goat anti-mouse AF649, goat anti-rabbit AF568) for 1 hour at 4°C. Slides were embedded in Mowiol-Dabco and analyzed using a Zeiss AxioObserver microscope.

Live Cell Imaging
NK cells and C. albicans alone or in combination with PMNs were seeded in an optical dish (MoBiTec) and monitored for 2 hours. PMNs were stained with 2’,7’-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF-AM, 3.2 µmol/L; Invitrogen) before the experiment. Pictures were taken every 10 seconds with a Zeiss LSM 780 confocal microscope.

Cytotoxic Impact of Purified Perforin, Purified Granzyme B, Recombinant TNF-α, and Recombinant IFN-γ on C. albicans and Blocking Experiments
Perforin and granzyme B isolated from the YT cell line (Enzo Life Science) or recombinant TNF-α or IFN-γ (Immunotools) were added to the fungus either alone or in combination for 4 hours at 37°C and 5% CO2, and fungal viability was determined afterward. In all cases, dilution buffers of the agents were used for control experiments. For blocking experiments, antibodies against TNF-related apoptosis-inducing ligand (TRAIL) (10 ng/mL) and Fas ligand (FasL; 1.6 ng/mL; R&D Systems) were added. To block actin or microtubule function, cytochalasin D (1 µmol/L) or colchicine (1 µmol/L) was added (Sigma). As an activation control, beads coated with anti-NKp46 and anti-CD2 (Miltenyi Biotec) were used. To induce degranulation, NK cells were incubated for 24 hours with 25 mmol/L strontium chloride (SrCl2). To specifically block perforin, NK cells were incubated with 10 nmol/L concanamycin A 24 hours before the experiment. Cells were washed 3 times in SCGM plus 10% human serum, and viability was checked with trypan blue staining.

Statistics
Estimation of P values was performed with unpaired, 2-sided Student t test.

Figure 2. Natural killer (NK) cells completely engulf Candida albicans. To confirm internalization of C. albicans by human NK cells, we performed differential staining of extracellular and intracellular fungal cells. Extracellular C. albicans cells were stained with calcofluor white before permeabilization (blue). After permeabilization, both intra- and extracellular C. albicans were stained using a specific antibody (red). Immune cells were identified as NK cells by CD56 positivity (orange), and staining of the actin cytoskeleton (green) further confirmed embedding of the fungal cells within the NK cells. Images are representative of cells from 3 independent experiments using cells from different donors (630× magnification).
RESULTS

Human NK cell degranulation and cytokine release triggered by C. albicans

Cytokine-activated human NK cells were coincubated for 4 hours at 37°C/5% CO₂ with C. albicans at a ratio of 1 fungal cell to 2 immune cells. Live cell imaging of the interaction revealed phagocytosis-like events within the first 30 minutes of coincubation, although phagocytic activity of NK cells was lower than that of professional phagocytes under comparable conditions [27]. Up to 20% of NK cells actively interacted with fungal cells within 20–60 minutes of coincubation. Viability of the engulfed fungi appeared to be largely unaffected because continuous filamentation could be observed, and within 80 minutes fungi started to outgrow NK cells by hyphae formation. Contact with C. albicans resulted in activation of NK cells, as shown by increased surface exposure of activation marker CD69 (141% ± 43% within 3 hours of contact, compared with uninfected control). In addition, surface expression of several receptors involved in innate immune recognition was markedly down-regulated on all NK cells after contact with C. albicans (Figure 1A). The cellular adhesin CD56 and ITAM-bearing receptors CD314 and CD335 were down-regulated after confrontation. A strong increase in CD107a surface exposure after 4 hours indicated an active release of cytotoxic granule content (Figure 1B). Along with the up-regulation of CD107a, a >3-fold increase in granzyme B release and significantly increased levels of perforin were detected compared with an uninfected control (Figure 1C).

Coincubation of human NK cells and C. albicans also resulted in an ≥3-fold increased release of granulocyte-macrophage colony-stimulating factor, INF-γ, and TNF-α compared with uninfected controls (Figure 1D). Although no cytokine production was detected after 1 hour of coincubation, the release of cytokines increased within 3 hours and further at 6 hours after coincubation. We further analyzed unstimulated primary human NK cells after confrontation with C. albicans. Importantly, phagocytosis of fungal cells could also be observed for primary NK cells. In addition, and similarly to the preactivated NK cells, primary cells showed down-regulation of CD16 and degranulation (up-regulation of CD107a) after contact with C. albicans. All signs of activation occurred at a lower level in primary NK cells than in preactivated NK cells (Supplementary Figure 1).

Phagocytosis of C. albicans by Human NK cells

To clarify whether the phagocytosislike interactions of NK cells and C. albicans observed in live-cell imaging resulted in full internalization of the fungus, we performed in-depth microscopic analysis of the interaction. Differential staining was used to discriminate extracellular fungi stained with the cell-wall dye calcofluor white before permeabilization from intracellular fungi stained by an anti-Candida antibody after a permeabilizing step. This clearly revealed intracellular fungal cells, protected from calcofluor white staining by the NK cell membrane (Figure 2). Corresponding immune cells covering the fungi were identified...
as NK cells by CD56 positivity, thus excluding potential phagocytosis by remaining contaminating monocytes. Additional staining of the actin skeleton and 3-dimensional reconstruction of z-stacks using confocal microscopy unequivocally confirmed embedding of the fungus within the actin network of the CD56+ cells, thus proving engulfment and uptake of *C. albicans* by human NK cells (Supplementary videos 2 and 3). Identical interactions were observed for primary human NK cells.

Contact-dependent activation of Human NK cells

Direct contact as well as adhesion have been shown to be essential for NK cell activation [28]. To determine whether contact between the fungal pathogen and NK cells was required for activation we used a Transwell system (0.4-µm pores), allowing physical separation of fungi and NK cells while permitting the flow of soluble mediators. Physical separation completely prevented activation, TNF-α release, CD16 down-regulation and degranulation of NK cells during a 4-hour coinoculation (Figure 3). In live cell imaging, the establishment of direct contact was largely dependent on formation of pseudopodia and NK cell motility. To investigate whether actin-dependent formation of pseudopodia and motility of NK cells was required for activation we used cytochalasin D to block actin polymerization (Figure 3). No phagocytosis-like interactions could be observed when NK cells were pretreated with cytochalasin D (Figure 4). Although cytochalasin D by itself induced some degree of degranulation and consequently a higher basal surface exposure of CD107a on NK cells and low-level secretion of cytokines, we clearly demonstrated that blocking of actin reorganization resulted in significantly lower response to *C. albicans* than in untreated cells. This effect was specific, because cytochalasin D–treated cells could still be activated by activating beads coated with anti-NKp46 and anti-CD2 (Figure 4). In contrast, blocking by colchicine of microtubule polymerization,
which is required for granule transport, prevented degranulation of NK cells in response to activating beads (Figure 4).

**Perforin-mediated Damage of C. albicans**

After confrontation of C. albicans and human NK cells, plating assays demonstrated a growth inhibition of 20% ± 9% compared with controls. In contrast to the experimental limitations of plating filamentous morphotypes of C. albicans, the XTT assay provides a culture-independent quantification of fungal metabolic activity. In this assay, the metabolic activity of C. albicans was reduced by 32% ± 10% after confrontation with human NK cells for 4 hours (Figure 5A). To analyze the mechanism of NK

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**Figure 5.** Perforin has antifungal activity against Candida albicans. A, Coincubation of natural killer (NK) cells and C. albicans results in reduced metabolic activity of C. albicans measured by the reduction of a dye in the XTT assay. This effect could be assigned to soluble mediators by use of conditioned medium (cM; supernatant of a 4-hour coincubation of NK cells and C. albicans). Metabolic activity was normalized against the C. albicans control. B, Purified perforin and granzyme B were incubated with C. albicans, either alone or in combination. Perforin was able to mediate cytotoxic effects in a concentration dependent manner while granzyme B did not show any effects. Combination of both substances did not lead to enhanced effects. C, Metabolic activity quantified by XTT assay of C. albicans alone, or after confrontation with NK cells. Degranulation was induced by strontium chloride (SrCl2)-treatment, perforin was inhibited by concanamycin A (ConA)-treatment of the NK cells. Data shows arithmetic means of ≥5 independent experiments. Metabolic activity was normalized against the C. albicans control. **P < .01, ***P < .001. (Not shown: solvent control.) Abbreviations: C, ConA, concanamycin A; SrCl2, strontium chloride. *P < .05.
Neutrophils show enhanced activation in the presence of natural killer (NK) cells and counteract the proinflammatory response of NK cells. Coincubation of NK cells and Candida albicans in the presence of polymorphonuclear neutrophils (PMNs) resulted in significantly reduced tumor necrosis factor (TNF-α) release compared with confrontation in the absence of PMNs. Proteolysis of TNF-α by PMN products could be excluded by performing confrontation of PMNs and C. albicans in the presence of conditioned medium (cM; supernatant of a 4-hour coincubation of NK cells and C. albicans), which did not result in degradation of TNF-α from the conditioned medium [31]. **$P<.01$. B, NK cells significantly up-regulate CD107a in response to phagocytosis of C. albicans.

Figure 6. Neutrophils show enhanced activation in the presence of natural killer (NK) cells and counteract the proinflammatory response of NK cells. A, Coincubation of NK cells and Candida albicans in the presence of polymorphonuclear neutrophils (PMNs) resulted in significantly reduced tumor necrosis factor (TNF) $\alpha$ release compared with confrontation in the absence of PMNs. Proteolysis of TNF-α by PMN products could be excluded by performing confrontation of PMNs and C. albicans in the presence of conditioned medium (cM; supernatant of a 4-hour coincubation of NK cells and C. albicans), which did not result in degradation of TNF-α from the conditioned medium [31]. **$P<.01$. B, NK cells significantly up-regulate CD107a in response to phagocytosis of C. albicans.
cell-mediated antifungal activity, cells were treated with blocking antibodies against the death receptor ligands FasL and TRAIL to exclude receptor-induced apoptosis. Inhibition of both NK cell surface proteins did not have any effect on antifungal activity (data not shown). On the other hand, conditioned supernatant from a 4-hour coincubation of NK cells and C. albicans was able to mediate a decrease of metabolic activity of 20% ± 15% in the XTT assay, indicating that soluble factors may play an important role (Figure 5A).

To further identify the soluble mediator for the observed effect, we exposed the fungus to purified perforin and granzyme B, as well as recombinant IFN-γ and TNF-α. Although we observed no impact of recombinant IFN-γ, TNF-α (data not shown), or granzyme B on C. albicans, purified perforin inhibited the metabolic activity of the fungus up to 35% ± 17% in a dose-dependent manner (Figure 5B). Degranulation of NK cells was induced by SrCl2 24 hours before the experiment, whereas perforin inhibition was achieved by concanamycin A treatment [29]. The SrCl2- and concanamycin A–treated NK cells induced significantly reduced fungal damage, supporting a central role for perforin in NK cell–mediated antifungal activity (Figure 5C).

**Interaction of PMNs and Human NK cells in the presence of C. albicans**

PMNs have been shown to interact with activated NK cells, resulting in a modulation of activation markers and survival of neutrophils [30]. We performed coincubation assays of NK cells and C. albicans in the presence of PMNs to analyze their effects on NK cell activation in the context of C. albicans infection. The release of cytokines in response to C. albicans was strongly diminished in the presence of PMNs; TNF-α is shown as an example (Figure 6A). The presence of PMNs also prevented degranulation, as indicated by CD107a up-regulation on the NK cells (Figure 6B). In parallel, increased up-regulation of the activation marker CD66b on PMNs in response to C. albicans in the presence of conditioned medium (supernatant of a 4-hour coincubation of NK cells and C. albicans), indicating enhanced PMN activation (Figure 6B). A modulatory effect of PMNs on the cytokine response of PBMCs mediated by a secreted protease has recently been described [31]. However, cytokine levels did not decrease in a coculture of C. albicans, PMNs, and conditioned supernatant harvested from NK cells after a 4-hour coincubation with C. albicans (Figure 6A). Therefore, a protease-independent mechanism was likely to be responsible for the diminished TNF-α levels.

Given the different kinetics of C. albicans recognition by PMNs and NK cells and the importance of C. albicans phagocytosis for full NK cell activation, we hypothesized that rapid phagocytosis of the fungus by PMNs could prevent physical contact between NK cells and the fungus, which is required for full NK cell activation. Indeed, live cell imaging revealed a rapid uptake of C. albicans by PMNs preventing engulfment of the fungus by NK cells in a mixed-cell confrontation assay, whereas the control video shows an active interaction of NK cells and C. albicans in the absence of PMNs during the same time period (Supplementary videos 4 and 5). We observed an association rate of up to 26% ± 8% (phagocytosis rate, 8% ± 0.5%) for NK cells alone within the first 2 hours of interaction with C. albicans (Figure 6D). This interaction was completely abolished in the presence of PMNs (Figure 6D). Furthermore, PMNs showed a stronger association of 52% ± 2% (control, 37% ± 2%) and a higher phagocytosis rate of 41% ± 7% (control, 28% ± 1.5%) in the presence of NK cells (Figure 6D). To quantify fungicidal activity of PMNs with or without activation by NK cells derived supernatants, we performed XTT assays. NK cell–activated PMNs showed enhanced antifungal activity, indicating that NK cell enhancement of PMN function is functionally relevant for fungicidal activity (Figure 6E).

**DISCUSSION**

To analyze the interaction of human NK cells with C. albicans, we used activated primary human NK cells. Similarly, in current clinical approaches, activated rather than naïve NK cells are used, and novel protocols for NK cell expansion and activation are being developed [32–35]. However, primary human NK cells were found to display similar responses, albeit at lower levels. Human NK cells not only establish contact with C. albicans but are also engaged in interactions resembling phagocytosis. The fungus can be completely engulfed by both cytokine-primed and primary NK cells, resulting in NK cell activation and fungal damage. In contrast to the professional phagocytic activity of neutrophils, phagocytosis by NK cells did
not inhibit the further elongation of *C. albicans* filaments. This ultimately resulted in destruction of NK cells, similar to what has been observed elsewhere for other host cells [36]. Formation of NK cell pseudopodia was required for engulfment of the fungus, and blocking of actin polymerization prevented both interaction with the fungus and degranulation. Therefore activation of NK cells after contact with *C. albicans* is initially similar to the formation of an immunological synapse in contact with a target cell. Although trafficking of lytic granules depends on microtubule polymerization, the initial forming of the NK cell synapse depends solely on rearrangement of the actin cytoskeleton [37–39].

Physical interaction with the fungus resulted in NK cell activation, as shown by up-regulation of CD107a, down-regulation of several receptors, and secretion of cytokines and granule content [28, 40, 41]. We could demonstrate secretion of granulocyte-macrophage colony-stimulating factor, IFN-γ, and TNF-α by human NK cells after contact with *C. albicans*. Thus, NK cells may contribute significantly to the proinflammatory response in invasive candidiasis by recruiting and activating other immune cells. Furthermore, after *C. albicans* induced degranulation of NK cells, high levels of perforin and granzyme B were detected. Our data clearly show that the antifungal effects of NK cells against *C. albicans* are mediated at least in part by perforin; this finding, together with available data for *A. fumigatus* and *C. neoformans*, suggests a potential role of perforin in antifungal immune responses in general [6, 12, 42–44].

Interestingly, activation of NK cells was prevented in the presence of PMNs, known to be important players during invasive candidiasis [21, 45, 46]. In coinfection assays of human NK cells and *C. albicans* in the presence of PMNs, degranulation and the secretion of cytokines by NK cells was prevented. A similar effect of PMNs has recently been shown to reduce the amounts of proinflammatory cytokines secreted by PBMCs [31]. However, in this case a PMN-derived protease actively degraded proinflammatory cytokines, whereas our data show a new mechanism, which is based on the prevention of direct contact between pathogen and NK cells through the rapid engulfment of fungal cells by PMNs. Therefore, PMNs may counteract a proinflammatory response in several ways. Because we also observed an increased activation of PMNs during interaction with NK cells and *C. albicans*, which resulted in enhanced phagocytosis and killing of the fungal pathogen, it is likely that communication between PMNs and NK cells may be relevant in vivo [30, 47].

Our results suggest distinct functions of NK cells in the response to *C. albicans* infection in vivo, which may largely depend on the clinical setting (eg, neutropenic vs nonneutropenic patient). Whereas NK cells may contribute to protective immunity by recruitment of other immune cells via cytokine release, they may also enhance proinflammatory activities without efficiently inactivating the fungal pathogen. The latter was recently implied by an in vivo study wherein depletion of NK cells resulted in a mitigated course of *C. albicans* sepsis [48]. However, the role of NK cells in vivo needs to be further analyzed, especially in light of their communication with neutrophils. Our data imply that the impaired prognosis of systemic candidiasis in a neutropenic setting may not only result from the absence of the most potent antifungal immune cell type but also from demasking of nonprotective, hyperinflammatory reactions. On the other hand, NK cells may be a prerequisite for full PMN antifungal activity in vivo.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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