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Natural Killer Cells and Antifungal Host Response

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As a result of improved experimental methodologies and a better understanding of the immune system, there is increasing insight into the antifungal activity of natural killer (NK) cells. Murine and human NK cells are able to damage fungi of different genera and species *in vitro*, and they exert both direct and indirect antifungal activity through cytotoxic molecules such as perforin and through cytokines and interferons, respectively. On the other hand, recent data suggest that fungi exhibit immunosuppressive effects on NK cells. Whereas clear *in vivo* data are lacking in humans, the importance of NK cells in the host response against fungi has been demonstrated in animal models. Further knowledge of the interaction of NK cells with fungi might help to better understand the pathogenesis of invasive fungal infections and to improve treatment strategies.

Natural killer (NK) cells are usually defined as $CD56^+$ $CD3^-$ cells and represent 5 to 20% of peripheral blood mononuclear cells (1). NK cells are lymphocytes of the innate immune system and are able to kill their target by molecules such as perforin or granzyme B or by death receptor-mediated apoptosis. In addition, NK cells influence other arms of the host immune response through chemokines and cytokines, which enhance the activity of professional phagocytes, dendritic cells (DCs), and T cells (2). Historically, the term of NK cells came from their natural ability to kill tumor cells *in vitro*, for example Ewing sarcoma or rhabdomyosarcoma (3). Importantly, these data are supported by clinical observations such as an association of low NK cell activity and an increased risk of cancer (1). NK cells also exhibit cytotoxicity against virus-infected cells, and data provide evidence of NK cell activity against bacteria such as *Staphylococcus aureus* and against various parasites (4, 5, 6). Regarding the antifungal activity of NK cells, the majority of *in vitro* data and animal studies were published in the 1980s and 1990s. However, the significant improvement of experimental methods and the exciting expansion in the understanding of the immune system have given new impetus for revisiting the role of NK cells in the host response to fungi. This minireview will focus on the available data concerning the interaction of NK cells and fungi and will highlight the specific research gaps that must be addressed in order to improve our understanding of the pathogenesis of invasive fungal infections.

NK CELLS DAMAGE A VARIETY OF FUNGI *IN VITRO*

The majority of studies demonstrate that both murine and human NK cells exhibit *in vitro* activity against various fungi, such as *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, or *Rhizopus oryzae* (7–18). However, at the same time, one has to recognize that the reported results are inhomogeneous and sometimes seemingly contradictory. For example, Ma et al. reported that unstimulated primary NK cells constitutively express anticryptococcal activity at an effector-to-target cell (E:T) ratio of 25:1 to 500:1 (12), whereas no fungicidal activity against *Cryptococcus neoformans* was observed when isolated NK cells prestimulated with interleukin-12 (IL-12) and IL-18 were used at an E:T ratio of 1,000:1 (19, 20). In this respect, it is noteworthy that IL-12 predominantly stimulates $CD56^{bright}$ $CD16^-$ NK cells, a subpopulation of NK cells classically designated immunoregulatory, which produce significantly lower levels of perforin and granulysin and display less cytotoxicity than $CD56^{dim}$ $CD16^+$ NK cells (21–24). These findings corroborate the observation that IL-12 and IL-18 synergistically induce fungicidal activity of murine peritoneal exudate cells against *C. neoformans* through the production of gamma interferon (IFN-γ) by NK cells (25). On the other hand, an early study reported that the addition of exogenous IL-12 to highly purified NK cells of HIV-infected patients restores the impaired anticryptococcal activity of the NK cells *in vitro* (26). In addition, IL-12 in combination with IL-2 as well as IL-2 alone induce both production of perforin and granzyme and an increased expression of natural cytotoxicity receptors (NCR) in freshly isolated human NK cells, which results in an enhanced antitumor and antifungal activity (13, 17, 18, 27, 28). Therefore, further studies have to evaluate which cytokines, alone or in combination, result in a large and long-lasting antifungal effect of NK cells *in vivo*, as it was recently demonstrated for the antitumor effect of NK cells prestimulated with different cytokines such as IL-12, IL-15, and IL-18 (29). In addition to the different dosages, schedules, and combinations of cytokines for prestimulation, other reasons may account for differences in study results. Thus, the NK cell populations investigated differ significantly across the studies, not only regarding their origin (human versus mouse) but also in purity. In the more recent studies, NK cells were isolated using antibodies, which results in highly homogeneous cell fractions (e.g., percentage of NK cells >90), whereas many early studies used NK cell-enriched cell populations that were obtained by a passage through a nylon-wool column. This procedure results in cell populations that consist not only of NK cells but also of a considerable number of $CD4^+$ and $CD8^+$ T cells, as well as antigen-presenting cells (30).

In addition to the experimental differences on the effector side, namely, the NK cells, differences in the fungal target might have had an important impact on the reported results. For example, the use of different strains of the same fungal species resulted in differences in the gene expression of perforin or in differences in

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fungal damage (10, 13, 15). Additionally, human NK cells may respond differently to distinct stages of hyphae developing fungi, as demonstrated in *Aspergillus fumigatus*. Whereas hyphae and germlings are damaged by both freshly isolated and IL-2-prestimulated NK cells, conidia of *A. fumigatus* are not affected by NK cell populations (7, 17, 18). This might be due, at least in part, to the fact that the conidia of fungi are often protected by capsule formations, melanin pigments, and hydrophobic layers, also known to prevent recognition by immune cells (31–34). In this respect, *C. neoformans* strain CAP67, which lacks a capsule, induces higher perforin expression by NK cells than the encapsulated strain B3501 does (13).

In conclusion, despite considerable differences in the experimental settings, *in vitro* data indicate that NK cells are able to damage fungi. However, studies using similar experimental conditions (e.g., isolation of NK cells, E:T ratio, and prestimulation) would allow a better insight in NK cell activity against fungi of different strains, species, and genera.

MECHANISMS OF DIRECT FUNGAL DAMAGE BY NK CELLS

Although NK cells are able to kill their target by a variety of different mechanisms, NK cells primarily exhibit cytotoxicity through release of their granule content, including perforin, granzymes, and granulysin, proteins which are constitutively expressed (12, 35). Perforin forms pores in the target cell membrane, which results in the loss of intracellular compounds and in massive influx of water, thus leading to the lysis of the target cell (36, 37). As a granzyme, granzyme B triggers apoptosis in the target cell, but it is not essential for cytotoxicity, as cells lacking granzymes may still be cytotoxic. Similar to perforin, granulysin can perforate the cell membrane independently of any receptor, but it also acts as a chemoattractant (36, 38–41). An early study demonstrated the importance of granules in the antifungal activity of NK cells against *Cryptococcus neoformans* (11). In this study, enriched human NK cell populations inhibited growth of *C. neoformans*, and this effect was partially abrogated by the use of monensin, which is an inhibitor of granule secretion. Interestingly, the anticryptococcal effect seems to be mediated by perforin rather than granulysin, since inhibition of perforin by concanamycin A or by small interfering RNA decreased antifungal activity of NK cells, whereas inhibition of granulysin did not (12). Corroborating the findings in the NK cell-mediated antifungal activity against *Cryptococcus* (12), the importance of perforin for NK cell-mediated antifungal activity was also demonstrated for *A. fumigatus* and *Rhizopus oryzae* (17, 18). For both fungi, inhibition of perforin by concanamycin A resulted in a significant decrease of fungal damage, although this effect is not specific for perforin and was not abrogated. These facts indicate that other molecules and/or mechanisms might be involved in the NK cell-mediated antifungal activity.

In striking contrast to the studies mentioned above, one study suggested that the antifungal activity of NK cells against *Aspergillus fumigatus* is not mediated through degranulation of their cytotoxic proteins but only via an alternative mechanism involving IFN-γ (7). Although it is well-known that IFN-γ increases the host response against fungal pathogens (e.g., by increasing the antifungal activity of professional phagocytes), this is the first report of a direct antifungal activity by IFN-γ. The authors propose that IFN-γ might cooperate with fungal ribotoxins secreted by *A. fumigatus* and transform them into suicide molecules for the fungus. However, further studies are needed to prove this interesting hypothesis.

To date, it is not clear whether direct contact of NK cells with the fungal target is necessary for damaging the pathogen. The Epstein-Barr virus (EBV)-positive, IL-2-independent human NK cell line YT exhibits anticryptococcal activity only in direct contact with the fungus (12), and rearming the perforin stores of NK cells requires direct contact of NK cells and *Cryptococcus neoformans* (13). In contrast, recent work suggested that direct contact of NK cells with *Aspergillus fumigatus* and *Rhizopus oryzae* is not mandatory for fungal damage, since cell-free supernatant exhibited antifungal activity (7, 17, 18). It remains unclear whether this difference is due to the pathogens investigated (e.g., *Cryptococcus* versus *Aspergillus*) or due to differences in the experimental settings (e.g., NK cell line versus prestimulated isolated human NK cells).

In addition to NK cell cytotoxicity mediated by the release of granule proteins, NK cells are able to induce death receptor-mediated apoptosis in many targets via the Fas ligand or tumor necrosis factor (TNF) family ligands. Although yeast cells are capable of apoptosis, this process is little understood in other fungal pathogens, and to date, no components of the apoptotic pathway, including death receptors and their ligands, have been found on fungal cells (42). Therefore, it remains unclear whether NK cell-induced apoptosis plays a role in fungal damage.

An early study demonstrated that NK cell cytotoxicity against *C. neoformans* was enhanced by the presence of specific antibodies, which bind to the activating NK cell receptor CD16 (43). Unfortunately, the importance of this pathway, which has also been reported for NK cell antitumor activity (44), is unclear for other fungal pathogens, but it may help to better define the role of antibodies in the host response to fungi.

ROLE OF NK CELLS IN THE COMPLEX WEB OF ANTIFUNGAL HOST RESPONSE

Although NK cells have the ability to directly damage their targets, they also interact directly (e.g., via cell surface receptors) or indirectly (via cytokines and interferons) with a variety of cells of the innate and adaptive immunity systems (Fig. 1), thus modulating the immune response. For example, NK cells produce GM-CSF (granulocyte-macrophage colony-stimulating factor) and RANTES (regulated upon activation, normal T-cell expressed, and secreted; chemokine ligand 5), which augment the immune response by phagocytes and T cells, respectively (45, 46, 47). Gamma interferon (IFN-γ), which is constitutively produced by NK cells, plays a central role in the cross talk between NK cell and other immune cells, since it stimulates migration, adherence, phagocytosis, and oxidative killing by neutrophils and macrophages and enhances maturation of dendritic cells (DCs) (48, 49). The importance of DCs in orchestrating the antifungal host response has been recognized over the last decade. DCs are able to kill fungal pathogens directly (50), to directly upregulate tumor necrosis factor alpha (TNF-α) and IFN-γ production of NK cells via triggering the natural cytotoxicity receptor NKp30 on NK cells (51), and more importantly, to shape the antifungal T cell response via priming and expansion of different specific T cell subsets (52). Notably, in *Aspergillus*-infected neutropenic mice, depletion of DCs resulted in impaired clearance of the fungus (53), and the number of DCs in lungs correlated with survival (54). The

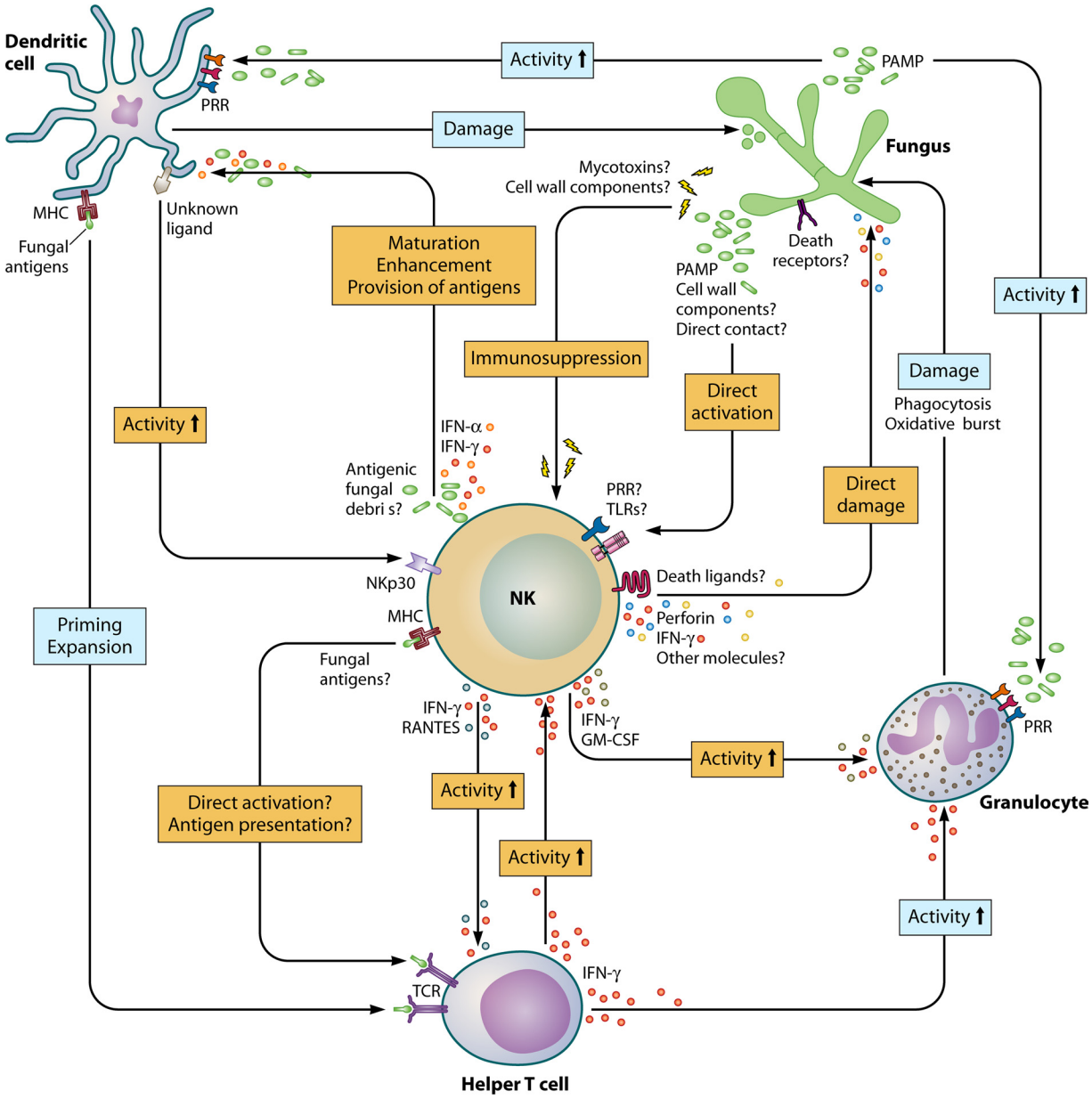

FIG 1 role of NK cells in the antifungal host response. NK cells are able to damage fungi directly, e.g., via soluble factors. In addition, NK cells produce a number of cytokines to influence other cells of the innate and adaptive immunity systems, such as granulocytes, dendritic cells, and T cell subsets. Conversely, these immune cells produce cytokines, by which they regulate NK cell activity. Fungi activate host immunity by pathogen-associated molecule patterns, but they are also able to suppress immune cells, e.g., by the secretion of mycotoxins. Brown boxes indicate effects directly induced by or affecting NK cells, and blue boxes indicate indirect effects induced by NK cells. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-α, alpha interferon; IFN-γ, gamma interferon; MHC, major histocompatibility complex; NK, natural killer cell; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TCR, T cell receptor; TLR, Toll-like receptor; RANTES, regulated upon activation, normal T-cell expressed, and secreted.

induction of specific antifungal T helper cell responses through DCs may also be influenced by NK cells, since NK cells provide antigenic cellular debris, which is internalized by maturing DCs and presented to T cells in lymph nodes (55). Interestingly, NK cells also have the capability to directly induce $CD4^+$ T cell responses by antigen presentation in a class II HLA-restricted manner, as was demonstrated for NK cells presenting tetanus toxoid to tetanus-specific T cell clones (56).

FUNGI INFLUENCE NK CELL IMMUNOREGULATION

In order to ensure their survival, fungi are able to manipulate the regulatory network of the host, for example by the secretion of mycotoxins such as gliotoxin (57). This toxin is produced by *Aspergillus*, and inhibits the phagocytic activity of macrophages, induces apoptosis of monocytes, decreases the activation of NADPH oxidase in neutrophils, and impairs functional T cell responses (58–62). Similarly, fungi are able to exert a

negative effect on the immunoregulatory function of NK cells (17, 18, 63, 64). For example, *C. neoformans* downregulates the production of GM-CSF and TNF-α of unstimulated human NK cells, as assessed by gene expression and supernatant protein levels (64). Similarly, *A. fumigatus* hyphae and *C. albicans* germ tubes downregulate the levels of IFN-γ, measured in the supernatant of IL-2-prestimulated NK cells (17, 63). Additionally, *R. oryzae* decreases the production of RANTES, which plays an important role in adaptive immunity (18). Interestingly, the effects of *A. fumigatus* and *R. oryzae* on NK cells were seen only for hyphae, but not for conidia, which corroborates the findings on NK cell-mediated fungal damage.

In contrast to the aforementioned studies which demonstrated an immunosuppressive effect of fungi on NK cells, one study reported upregulation of GM-CSF, IFN-γ, and TNF-α gene expression when coincubating *Candida albicans* germ tubes and IL-2-prestimulated NK cells (65). This difference, however, may be due to the different periods of coincubation: whereas in the study by Arancia et al. the upregulation of TNF-α and GM-CSF mRNA levels was observed after 2 h (65), Murphy et al. found decreased mRNA levels of TNF-α and GM-CSF after 6 and 18 h, respectively (64). This is supported by time course experiments demonstrating that the gene expression of TNF-α by NK cells increases after 3 and 6 h, before it decreases after 12 h of coincubation with *A. fumigatus* (64).

Taken together, current data suggest that the interaction of fungi and NK cells results in an impairment of the immunoregulatory activity of NK cells. However, future experiments have to address several points: first, since data have suggested differences in the immunosuppressive effect of *R. oryzae* compared to *A. fumigatus*, the effects of different fungal strains and species on NK cells have to be analyzed. In this regard, it has to be noted that the specific effects of different mycotoxins on NK cell activity have not been addressed at all thus far. Second, and more importantly, *in vivo* experiments have to clarify whether and to what extent the immunosuppressive effect of fungi on NK cells may have a clinical relevance. This might be the basis for further research investigating which interleukins or cytokines are able to restore the antifungal activity of NK cells, which ultimately could result in a better outcome of invasive fungal infection.

RECOGNITION OF FUNGI BY NK CELLS

Triggering of NK cells is the result of a complex balance between inhibitory and activating signals and requires not only deficient major histocompatibility complex class I (MHC-I) expression on target cells but also the expression of inducible ligands of activating NK cell receptors (for details, see reviews by Langers et al. [1] and by Lanier [66], respectively). Cells of the innate immunity system recognize fungi by pattern recognition receptors (PRRs), which sense pathogen-associated molecular patterns (PAMPs) and induce downstream cell-specific responses (52). The best described PRRs are the mannose receptors, c-type lectin receptors (CLR) including dectin-1 and DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; CD209), and the Toll-like receptors (TLRs). Among the TLRs, mainly Toll-like receptor 2 (TLR2), TLR4, and TLR9 are associated with the detection of fungal antigens like zymosan, phospholipomannans, or *O*-linked mannans and fungal DNA (52, 67–72). Interestingly, recent data demonstrate that functional TLR2, TLR4, and TLR9 can be detected on the surfaces of NK cells (68, 73, 74, 75), and activation of NK cells via TLR2 has been reported for *Leishmania major* lipophosphoglycan and *Klebsiella pneumoniae* outer membrane protein A (68, 75). Although it seems plausible that NK cells are triggered, at least in part, by fungi via the TLRs, the direct involvement of these receptors in NK cell activation by fungi has not been demonstrated thus far. In addition, it is unclear whether fungal PAMPs may be recognized by other NK cell-activating receptors; for many of them, the ligands have not been identified. In conclusion, little is known on the recognition of fungi by NK cells, which clearly should be a focus of future research.

ROLE OF NK CELLS IN ANTIFUNGAL HOST DEFENSE

(i) Animal studies. Similar to the *in vitro* data, *in vivo* studies demonstrate that NK cells interact with fungi. For example, an early study shows that NK cells proliferate in mice experimentally infected with *Aspergillus niger*, which was associated with an inhibition of the fungal growth (76). The importance of NK cells in the antifungal host response was also demonstrated in mice inoculated with *C. neoformans* (77). Depletion of NK cells by antibodies resulted in a significant higher fungal burden in the lungs compared to untreated controls, although this did not result in a difference in survival. In contrast, the adoptive transfer of NK cell-enriched cell populations to cyclophosphamide-pretreated mice suffering from cryptococcosis led to an enhanced clearance of the fungus compared to controls receiving NK cell-depleted grafts (78, 79). Similarly, studies in NK cell-depleted mice revealed the pivotal role of NK cells in the host response against *A. fumigatus*, *C. albicans*, and *Histoplasma capsulatum* (80–83). Interestingly, in the lungs of neutropenic mice with invasive aspergillosis, NK cells were the major population of cells capable of generating IFN-γ (84). Depletion of NK cells reduced lung IFN-γ levels and subsequently increased the fungal load, whereas the transfer of activated NK cells from wild-type, but not from IFN-γ-deficient, mice resulted in greater pathogen clearance from the lungs. These data corroborate the findings that in mice with systemic *Candida* infection, NK cells were the main inducers of phagocytic activity of splenic macrophages and mediated protection by secretion of IFN-γ (80). On the other hand, it was demonstrated that deficiency of perforin increased fungal burden and mortality in mice with *Histoplasma capsulatum* infection, although the authors of this study did not address the question whether NK cells were the source of perforin (85). Future studies will need to clarify to what extent the antifungal activity of NK cells *in vivo* is due to the secretion of cytotoxic molecules (e.g., perforin) or to the modulation of the host response (e.g., by IFN-γ). In addition, *in vivo* studies have to better clarify whether other populations of immune cells are necessary for a significant antifungal effect of NK cells, as recently demonstrated for the role of $CD4^+$ T cells in the antitumor effect of NK cells (29). To this end, the preliminary data suggesting a benefit of transferring NK cells to animals with invasive fungal infection definitely needs to be explored in more detail (e.g., safety data, efficacy of different approaches such as prophylaxis or treatment), which might help to better define the role of NK cells as an immunotherapeutic tool in the antifungal armamentarium.

(ii) Clinical data. The abundance of *in vitro* and animal data is in sharp contrast to the lack of clinical evidence that NK cells are important in the antifungal host defense in humans. However, the

complexity and redundancy of the different arms of the immune system make it difficult to define the role of NK cells in protection against fungal pathogens in the clinical setting. For example, one case report described a patient, who received corticosteroids for therapy of systemic lupus erythematosus (SLE) (86). This patient developed *Trichophyton rubrum* infection, which did not resolve after cessation of immunosuppressive therapy. Since further examination revealed that this patient had both reduced NK cell numbers and NK cell activity, the authors speculated that the impaired host defense by NK cells increased the risk for developing fungal infection. On the other hand, reduced NK cell numbers and NK cell activity have also been demonstrated in patients with SLE who did not develop fungal infection (87). Similarly, another case report described a patient with invasive aspergillosis, who was found to have significantly reduced NK cell activity (88). However, as discussed above in detail, infections due to *Aspergillus* may decrease NK cell activity, and therefore, it remains unclear whether the observed NK cell impairment was a risk factor for or an effect of invasive aspergillosis. The rare cases of isolated NK cell deficiencies described have not been associated with increased susceptibility to fungi (89, 90), but again, the complexity of the immune system does not allow a firm conclusion to be drawn at the moment.

Due to the cytotoxic activities of NK cells against a variety of tumors, there is increasing interest in using NK cells as adoptive immunotherapy in hematopoietic stem cell transplant recipients. In this regard, it was recently reported that transplants from NK cell-alloreactive donors were associated with a significantly lower relapse rate and better event-free survival (91). Although there are no data available regarding infectious complications of this approach, adoptively transferred NK cells could be an attractive strategy in the prophylaxis or treatment of invasive fungal infections in allogeneic hematopoietic stem cell transplant recipients. However, as outlined above in the section on animal studies, the safety and efficacy of this approach have to be thoroughly evaluated in the animal model before clinical studies can be performed.

CONCLUSIONS AND PERSPECTIVES

Although there is no clear clinical evidence in humans, *in vitro* and animal data demonstrate that NK cells play an important role in the antifungal host response. The antifungal activity is mediated via direct damage of fungi and via immunomodulation by cytokines and interferons. Despite the fact that NK cells are active against various clinically important fungi such as *Aspergillus* spp., *Candida* spp., and mucormycetes, at this time, it remains unclear how NK cells recognize fungal pathogens. In addition, further studies have to evaluate how and to what extent fungi exert an immunosuppressive effect on NK cells. Should animal studies demonstrate a benefit of adoptively transferring NK cells into an immunocompromised host suffering from invasive fungal disease, NK cells may become an interesting tool in adoptive immunotherapeutic strategies, in particular in hematopoietic stem cell transplant recipients.

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