Short Communication

Polymorphonuclear neutrophils and granulocytic myeloid-derived suppressor cells inhibit natural killer cell activity toward *Aspergillus fumigatus*

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Abstract

Invasive aspergillosis is a devastating infectious disease in immunocompromised patients. Besides neutrophils and macrophages, natural killer (NK) cells have recently emerged as important players in immunity to this infection. It was shown that NK cells comprise an essential role in the clearance of *Aspergillus fumigatus* (A. fumigatus) in neutropenic but not in nonneutropenic mice. However, the antifungal activity of NK cells and their regulation have not been fully characterized. In this study, we investigated the interplay between polymorphonuclear neutrophils (PMNs) or granulocyte myeloid-derived suppressor cells (Gr-MDSCs) with NK cells. Both cell types exhibited an equal inhibitory effect on NK cell activation through downregulation of NKp30 expression on the cell surface and cytotoxicity towards the cell line K562. Furthermore, we showed that NK cell activation and antifungal cytotoxicity were impaired when NK cells had been cultured in the presence of PMNs or Gr-MDSCs before fungal stimulation. Besides the reduced cytotoxicity a decreased release of interferon gamma (IFNγ), a key player in the clearance of an *A. fumigatus* infection, was observed. Thus, inhibition of NK cell activity by PMNs or Gr-MDSCs might impair an effective anti-fungal immune response during recovery from conditions such as hematopoietic stem cell transplantation.

Key words: *Aspergillus fumigatus*, invasive aspergillosis, natural killer cells, polymorphonuclear neutrophils, granulocytic myeloid-derived suppressor cells, Interferon γ.

Introduction

Invasive aspergillosis (IA) is a devastating infectious disease in immunocompromised patients and is associated with a mortality rate of up to 90% in hematopoietic stem cell transplant recipients [1]. Many consider neutropenia as one of the most important risk factors for IA, because polymorphonuclear neutrophils (PMNs) comprise an important immune defense directed against germinating
conidia and infiltrating hyphae of *Aspergillus fumigatus* (*A. fumigatus*) [2,3]. However, clinical data demonstrate that recovery from neutropenia is negatively associated with the progress of IA [4–6], suggesting an inhibitory role of PMNs on other immune cell types in this setting. Furthermore, it was observed that the mortality rate in non-neutropenic cases was significantly higher compared to neutropenic patients [7], an observation that strengthens the hypothesis that also other immune cells like natural killer (NK) cells are involved in the clearance of *Aspergillus* infection.

It was shown that NK cells exhibit an antifungal cytotoxicity against *A. fumigatus* germ tubes and hyphae via both degranulation [8] and interferon γ (IFNγ) release [9]. They are essential for improved disease outcome in a neutropenic mouse model of IA but not in non-neutropenic mice [10]. In addition, administration of IFNγ has shown beneficial effects for patients in both prevention and treatment of IA [11]. Based on these findings and the understanding that NK cell activation can be inhibited by other immune cells including granulocytes [12–14], we hypothesize that granulocytic cells might impair NK cell activity during an *A. fumigatus* infection. Therefore, we included two distinct groups of granulocytes in this study: 1) PMNs as the classic neutrophils and 2) the granulocytic myeloid-derived suppressor cells (Gr-MDSCs). The latter have been defined as immature, low-molecular weight CD66b+ granulocytic cells [15,16] that expand in inflammatory conditions such as malignancies as well as in fungal infections, where they suppress an efficient T-cell response [17,18]. In addition, Gr-MDSCs are able to inhibit NK cell cytotoxicity directed against tumor cells [19,20].

This is the first study to our knowledge to show that both PMNs and Gr-MDSCs dampen the cytotoxic effect of NK cells on *A. fumigatus* germ tubes by inhibiting IFNγ release, thus providing new insights into antifungal immunity and NK cell regulation by granulocytic cells in general.

**Materials and methods**

**Cell culture**

NK cells, PMNs, and Gr-MDSCs were isolated from fresh blood of healthy volunteers. Usage of the blood specimens was approved by the Ethical Committee of the University Hospital Wuerzburg.

PMNs were isolated by performing a Ficoll standard density gradient centrifugation (Biochrom AG) and erythrocyte lysis (Qiagen) following the manufacturer’s instructions.

NK cells and Gr-MDSCs were isolated from peripheral blood mononuclear cells (PBMCs) after performing a Ficoll standard density gradient centrifugation (Biochrom AG). PBMCs were depleted of CD3+ cells using anti-CD3 positive MACS selection (Miltenyi Biotec). Then, NK cells were isolated from CD3-depleted PBMCs using the MACS NK cell negative selection kit (Miltenyi Biotec) according to the manufacturer’s instructions. For 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assays and IFNγ-enzyme linked immunosorbent assays (ELISAs), NK cells were activated with 1000 IU/ml interleukin 2 (IL-2) (Miltenyi Biotec) for 24 h. Gr-MDSCs were isolated from CD3-depleted PBMCs using MACS positive selection with FITC-labeled anti-CD66b (Biolegend) and anti-FITC magnetic microbeads (Miltenyi Biotec) according to previous studies [15,16]. If not stated otherwise, 1 × 10⁶ cells/ml were cultured in RPMI 1640 (Invitrogen) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich) and 120 μg/ml gentamicin (Refobacin; Merck) at 37°C and 5 % CO₂.

**Co-culture conditions**

NK cells were either cultured alone or with PMNs or Gr-MDSCs (ratio 1:1) for 16 h. NK cells stimulated with 1000 IU/ml interleukin 15 (IL-15) served as a positive control for NK cell activation, cytokine production and cytotoxicity. After co-incubation, granulocytic cells were depleted from the co-culture with NK cells using the NK cell isolation kit (MACS negative selection, Miltenyi Biotec), followed by an additional selection of the CD66b+ cells using FITC-labeled anti-CD66b (Biolegend) and anti-FITC microbeads (Miltenyi Biotec). After depletion, purity (>95%) of NK cells was confirmed by flow cytometry. Co-cultures of NK cells and the cancer cell line K562 were performed at an effector to target (E:T) ratio of 10:1 for 5 h.

**Infection conditions**

Viable *A. fumigatus* (ATCC 46645) conidia and germ tubes were prepared in RPMI supplemented with 120 μg/ml gentamicin (Refobacin, Merck) as previously described [21], except inactivation. After depletion of Gr-MDSCs and PMNs, NK cells were incubated with viable germ tubes at a multiplicity of infection (MOI) of 1 at 37°C for 5 h.

**Flow cytometry**

Purity of all isolated cell types and expression of surface molecules was verified by flow cytometry in a FACS Calibur (BD). Cells were incubated with mouse anti-human fluorochrome-coupled antibodies to determine NK cell purity (anti-CD3 PerCP or PE, anti-CD56 APC or FITC or anti-NKp46 PE, BD Biosciences); purity of Gr-MDSCs and PMNs (anti-CD66b FITC, Biolegend); NK cell activation (anti-CD69 FITC, BD Bioscience, or APC, Miltenyi Biotec).
and anti-CD137 APC, Miltenyi Biotec); and NKp30 expression (anti-NKp30 PE, Biolegend). To evaluate NK cell cytotoxicity against K562 cells, expression of the degranulation marker CD107a (anti-CD107a APC, BD Biosciences) was measured. Data were analyzed with FlowJo software (Tree Star Inc.).

Enzyme Linked Immunosorbent Assay (ELISA)

To quantify the IFN\(\gamma\) concentration in co-culture supernatants, an ELISA was performed according to the manufacturer’s instructions (Biolegend).

Determination of fungal damage

The 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay was performed to determine fungal metabolic activity. Briefly, IL-2 primed NK cells were co-cultured with PMNs or Gr-MDSCs or left untreated before NK cells were stimulated with germ tubes at a multiplicity of infection (MOI) 1 for 5 h. Germ tubes cultured in medium alone served as negative control. After co-culture, NK cells were lysed with ice-cold water and the XTT-Assay was performed as previously described [22].

Statistical analysis

A two-tailed, paired Student \(t\)-test was used to evaluate statistical significance. \(P\)-values <.1 were considered statistically significant.

Results

PMNs and Gr-MDSCs equally reduced cell surface expression of NKp30 on NK cells

Cell surface expression of the NK cell activating receptors NKp46, NKp44, NKp30, and NKG2D was analysed, to investigate whether PMNs and Gr-MDSCs inhibit NK cell activity. Inhibition of these receptors leads to a reduced NK cell cytotoxicity directed against tumor cells [23–26]. NK cells were cultured in the presence of PMNs or Gr-MDSCs and then the cell surface expression of NKG2D, NKp46, NKp44 and NKp30 was determined by flow cytometry. NK cells cultured in the presence of Gr-MDSCs, showed a significant decrease in NKp30 expression compared to control NK cells (Fig. 1A). In parallel, down-regulation of NKp30 expression was observed to the same extent when NK cells were cultured together with PMNs compared to the down-regulation of NK cells cultured in the presence of Gr-MDSCs (Fig. 1A). However, no alterations in the expression of NKp46, NKp44, and NKG2D on NK cells were detected (data not shown).

PMNs and Gr-MDSCs impair NK cell activation and cytotoxicity directed against K562

To further confirm the equivalent inhibitory effect of PMNs and Gr-MDSCs, NK cells that were precultured with PMNs or Gr-MDSCs were co-cultivated with the erythroleukemia cell line K562. In contact with K562 cells, NK cells become activated as determined by increased expression of CD69 [27] and exhibit cytotoxicity against K562 target cells [28]. Therefore, CD69 expression on NK cells targeting K562 cells was monitored. NK cells precultured with PMNs or Gr-MDSCs showed a significant reduction in the expression of CD69 compared to non-pretreated NK cells in the presence of K562 (Fig. 1B). Furthermore, to determine the cytotoxicity of NK cells against K562, the expression of the degranulation marker CD107a was monitored by flow cytometry. CD107a is a marker of NK cell activation and its up-regulation correlates with the lysis of target cells [29]. NK cells were again cultured in the presence of PMNs or Gr-MDSCs and after the depletion of the granulocytic cell types, NK cells were confronted with K562 cells. The percentage of CD107a positive NK cells precultured with PMNs or Gr-MDSCs was markedly decreased compared to NK cells which had been precultured alone (Fig. 1C). Importantly, viability of NK cells was not decreased by the presence of PMNs and Gr-MDSCs (data not shown).

PMNs and Gr-MDSCs inhibit NK cell activation toward A. fumigatus

NK cells have a fundamental role in the clearance of A. fumigatus infection in neutropenic mice [10], and our group demonstrated that NK cells induce fungal damage via IFN\(\gamma\) [9]. Based on these findings, we analysed whether the inhibitory effect of PMNs and Gr-MDSCs on NK cell activation also influences the NK cell response towards A. fumigatus germ tubes. Besides CD69, an additional marker CD137 was measured for NK cell activation [30]. NK cells displayed an up-regulation of CD69 and CD137 when co-cultured with germ tubes (Fig. 2A and B). However, germ tube-induced activation was significantly decreased when NK cells were precultured with PMNs or Gr-MDSCs (Fig. 2A and B). The expression of the activation markers CD69 and CD137, decreased even below the expression levels of untreated NK cells. Interestingly, down-regulation
PMNs and Gr-MDSCs impair NK cell cytotoxicity toward *A. fumigatus* and inhibit IFNγ release

To investigate whether the decreased NK cell activation had also an impact on IFNγ release, we quantified the IFNγ concentration in the supernatant of NK cell co-cultures. NK cells were precultured either with PMNs or Gr-MDSCs, or left untreated respectively, followed by the depletion of PMNs and Gr-MDSCs after 16 h. Pretreated NK cells were then infected with *A. fumigatus* germ tubes. Compared to untreated NK cells, IFNγ concentrations were significantly decreased when NK cells had been precultured in the presence of PMNs or Gr-MDSCs (Fig. 3A).

Previously it was shown that IFNγ, released by NK cells, induces fungal damage and displays an anti-*Aspergillus* effect [9,31]. To analyse whether the decreased IFNγ release caused a reduced anti-fungal reactivity, we measured the fungal metabolic activity of *A. fumigatus* using the XTT-Assay. Therefore, NK cells were precultured with PMNs, Gr-MDSCs, or left untreated, and after depletion of granulocytic cells, NK cells were confronted with *A. fumigatus*. Untreated NK cells reduced the fungal metabolic activity to 45% compared to the control in which *A. fumigatus* was cultured alone in medium. Preculture of NK cells with PMNs and Gr-MDSCs significantly dampened this reduction and increased the fungal metabolic activity.
Figure 2. PMNs and Gr-MDSCs inhibit NK cell activation induced by A. fumigatus. NK cells were cultured alone, with PMNs or Gr-MDSCs at a ratio of 1:1 for 16 h. After co-cultivation, PMNs and Gr-MDSCs were depleted and NK cells were cultured with A. fumigatus at MOI 1 for 5 h. After A. fumigatus treatment, expression of the activation markers CD69 (A) and CD137 (B) was analyzed by flow cytometry. (A) n = 7, (B) n = 7. Mean ± SEM is displayed in the figure. Significant differences between NK cells and PMNs or Gr-MDSCs pretreated NK cells are indicated by an asterisk (*P < .1, **P < .05, ***P < .01, two-tailed, paired Student’s t-test).

Figure 3. PMNs and Gr-MDSCs reduce IFNγ release and suppression of fungal metabolic activity. NK cells were stimulated for 24 h with IL-2 prior to co-cultivation with PMNs and Gr-MDSCs. Then, Gr-MDSCs and PMNs were depleted after 16 h co-cultivation with NK cells and these NK cells were then stimulated with A. fumigatus at MOI 1 for 5 h. IFNγ concentrations were determined in culture supernatants using ELISA (A) and fungal metabolic activity was examined performing XTT-assays (B). (A) n = 5, (B) n = 3. Mean ± SEM is displayed in the figure. Significant differences between NK cells and PMNs or Gr-MDSCs pretreated NK cells are indicated by an asterisk (*P < .1, **P < .05, ***P < .01, two-tailed, paired Student’s t-test).

activity to 65% compared to the control (Fig. 3B). Hence, we demonstrated that NK cell dependent IFNγ release and the anti-Aspergillus effect is reduced in the presence of PMNs and Gr-MDSCs, suggesting a crucial role of PMNs and Gr-MDSCs in the immune response of NK cells directed against A. fumigatus.

Discussion

NK cells have emerged as potentially important players in IA [10]. It was shown that they are one of the major IFNγ producers during IA in a neutropenic mouse model [31] and directly damage A. fumigatus via IFNγ release and degranulation [8,9] suggesting an important role of NK cell cytotoxicity in the pathology of IA. However, the antifungal activity of NK cells and their regulation have not been fully characterized. NK cell activity is known to be regulated by other immune cell types, including PMNs and Gr-MDSCs. Both display an inhibitory effect on NK cell activation and cytotoxicity directed against tumor cells [13,19,20].

In our co-culture experiments, we observed a moderate significant down-regulation of NKp30 expression on the NK cell surface following culture with either PMNs or Gr-MDSCs (Fig. 1A), while the expression of the other
NCRs NKp46 and NKp44, and the activating receptor NKG2D was unaffected (data not shown). This result is in line with the finding of Hoechst et al. who showed that activation on NK cells is reduced in the presence of MDSCs due to a moderately decreased expression of NKp30 and suggested that MDSCs inhibit NK cell function via engagement of the NKp30 receptor [32]. The finding that PMNs also suppress the expression of NKp30 is novel and of great interest since NKp30 represents an important receptor for NK cell activation and for the interaction of NK cells with dendritic cells [33]. Furthermore, NK cell activation during viral and bacterial infections is mediated by NKp30 [34,35] suggesting that this is an essential recognition receptor on NK cells. Thus, together with published data, our results indicate that modulation of NKp30 expression might be a mechanism of both PMNs and Gr-MDSCs mediated immunomodulation dampening excess inflammation.

Furthermore, we confirmed the inhibitory effects of PMNs and Gr-MDSCs on NK cell function. Both, activation and cytotoxicity towards the classical NK cell target K562 were impaired when NK cells had been pretreated with PMNs or Gr-MDSCs. By directly comparing the effects of PMNs and Gr-MDSCs on the NK cell function, we demonstrated for the first time an equal inhibitory effect of both cell types on NK cell activity directed against tumor cells.

Previously, we reported an increased IFN-γ release by NK cells activated after in vitro stimulation with A. fumigatus, resulting in damage of A. fumigatus germ tubes [9]. These results are supported by the finding of Park et al. who demonstrated an important role of NK cell secreted IFN-γ for the host immune defence in neutropenic IA [31]. Here, we showed for the first time that germ tubes-dependent NK cell activation, IFN-γ release and anti-fungal cytotoxicity were significantly inhibited when NK cells were pretreated with PMNs or Gr-MDSCs. The strong reduction of IFN-γ release after the combination of NK cells with PMNs and Gr-MDSCs, together with an inhibition of antifungal activity of NK cells supports previous published data that IFN-γ release is involved in the impairment of the fungal metabolic activity [9]. Besides our investigation that PMNs inhibit anti-fungal activity of NK cells, it was shown that PMNs prevented degranulation and cytokine release of NK cells in the presence of C. albicans and that on the other hand PMNs were activated by NK cells [14]. These results suggest a strong interplay between PMNs and NK cells during fungal infections in vivo. Furthermore, additional inhibitory mechanisms of PMNs and Gr-MDSCs have already been proposed in other models, including arginase 1 [13], ROS [36] and the regulation of NKp30 [20,32], which is further supported by our investigations. It was shown, that upon depletion of the amino acid arginine by arginase I of PMNs, NK cell proliferation is diminished and that IFN-γ secretion is post transcriptionally suppressed [13]. Thus, further experiments are necessary to validate whether the availability of arginine is responsible for the reduced IFN-γ release of NK cells in the presence of A. fumigatus.

The results of this study partly explain the previous observation of Morrison et al. that NK cells mediate antifungal activity and improved the outcome of IA in neutropenic mice, but not in animals with normal neutrophil counts [10]. Although many patients developing IA are neutropenic, approximately 40% of IA patients are nonneutropenic and exhibit suppression of different immune cell types [7]. The suppressive activity of PMNs in these patients might hamper efficient NK cell activity and impair the antifungal immune response. It is known that patients receiving hematopoietic stem cell transplantation represent a major high risk cohort for IA [37] and that NK cells are the first innate immune cell type that reconstitutes after transplantation [38], which can trigger an immune response against A. fumigatus. Importantly, based on the ability of Gr-MDSCs to suppress NK cell activity and the knowledge that they accumulate during malignancies, chemotherapies and infections [39]; Gr-MDSCs might favour IA in the setting of hematopoietic stem cell transplantsations.

Taken together, in this study we describe an inhibitory effect of both PMNs and Gr-MDSCs on NK cell activation and cytotoxicity during stimulation with A. fumigatus in vitro. Although PMNs and NK cells are both known players in anti-fungal defence, the exact mechanism of their interaction remains unknown. Further investigations are necessary to elucidate the complete mechanisms during A. fumigatus infections. Despite the development of new tools for diagnosis and treatment, management of IA still remains challenging. However, increasing knowledge about the immunological mechanisms and immunopathology of this infection might lead to alternative treatment options.

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**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.
References


36. Harlin H, Hanson M, Johansson CC et al. The CD16-CD56(bright) NK cell subset is resistant to reactive oxygen species produced by activated granulocytes and has higher antioxidative capacity than the CD16+CD56(dim) subset. *J Immunol* 2007; 179: 4513–4519.

