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A Polysaccharide Virulence Factor of a Human Fungal Pathogen Induces Neutrophil Apoptosis via NK Cells

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Aspergillus fumigatus is an opportunistic human fungal pathogen that sheds galactosaminogalactan (GG) into the environment. Polymorphonuclear neutrophils (PMNs) and NK cells are both part of the first line of defense against pathogens. We recently reported that GG induces PMN apoptosis. In this study, we show that PMN apoptosis occurs via a new NK cell–dependent mechanism. Reactive oxygen species, induced by the presence of GG, play an indispensable role in this apoptotic effect by increasing MHC class I chain–related molecule A expression at the PMN surface. This increased expression enables interaction between MHC class I chain–related molecule A and NKG2D, leading to NK cell activation, which in turn generates a Fas-dependent apoptosis-promoting signal in PMNs. Taken together, our results demonstrate that the crosstalk between PMNs and NK cells is essential to GG-induced PMN apoptosis. NK cells might thus play a role in the induction of PMN apoptosis in situations such as unexplained neutropenia or autoimmune diseases. The Journal of Immunology, 2014, 192: 000–000.

Polymorphonuclear neutrophils (PMNs) are the most abundant blood leukocytes and are key components of the early innate response to bacterial and fungal pathogens. In response to pathogens, PMNs rapidly migrate from the blood to inflamed tissues, where their activation triggers microbicidal mechanisms such as the release of proteolytic enzymes and antimicrobial peptides, and rapid production of reactive oxygen species (ROS) in an “oxidative burst.” After microbial killing, PMNs die spontaneously, mainly by apoptosis. Although PMNs have a very short lifespan, their activation by circulating bacterial and fungal products as well as by proinflammatory mediators promotes their survival and is a critical mechanism in their effectiveness against pathogens (1). Less is known, however, about the factors that promote PMN apoptosis during infections.

NK cells are also part of the innate immune system, typically recognized for their role in cancer immunosurveillance and pathogen clearance. NK cell effector functions include direct cytotoxicity as well as the production of cytokines and chemokines involved in regulating immune responses. Although NK cells can directly recognize some pathogens, it is becoming increasingly clear that NK cell activation requires the presence of such accessory cells as monocytes, macrophages, and dendritic cells (DCs) (2–5). In addition, previous studies have described the interaction between PMNs and NK cells and its role in the regulation of their separate activities (6, 7), but few have addressed the receptors involved in this cross-talk. Moreover, the consequence of this crosstalk during microbial infections has never been investigated.

Aspergillus fumigatus is an opportunistic human fungal pathogen known to cause a spectrum of human diseases, including allergic syndromes, noninvasive infections, and invasive aspergillosis. The morbidity and mortality associated with the latter remain high despite recent advances in prevention, diagnosis, and treatment (8, 9). Long recognized as critical to host defense against Aspergillus species, particularly when conidia escape macrophage phagocytosis (10), PMNs provide essential anti-conidia defense and prevent conidial germination by releasing proteolytic enzymes and rapidly producing ROS in the so-called oxidative burst (11, 12).

A. fumigatus uses various strategies to evade immune recognition, however. Some of the toxins and enzymes it produces may facilitate its growth within the host and impair the host’s immune response. In particular, toxins, such as gliotoxin and fumagillin, disrupt the formation of a functional NADPH oxidase complex in PMNs and inhibit both PMN degranulation and F-actin formation (13, 14). In addition, the rodlet layer of the conidial surface silences the PMN response (15). We previously showed that galactosaminogalactan (GG), a polysaccharide secreted by the fungus during early growth in vivo, can modulate immune response in a mouse model by increasing PMN apoptosis and favoring A. fumigatus infection (16).

In this study, analyzing the mechanisms of GG-induced PMN apoptosis, we show that the cross-talk between PMN and NK cells is essential to it. These findings point to a new mechanism developed by a fungal pathogen to escape the innate system and promote its survival. Moreover, our data underline the role of NK cells in the induction of PMN apoptosis and the role of these cellular interactions in pathological situations.
Materials and Methods

Polysaccharides from A. fumigatus

GG was isolated and purified as described previously (16). Briefly, GG was produced by A. fumigatus (strain CBS 144-89) in a Bionorm 1 fermentor. The culture supernatant was precipitated with 2.5 vol ethanol overnight at 4°C. The ethanolic pellet was collected by centrifugation (3000 × g, 10 min), washed twice with 150 mM NaCl, and finally extracted with 8 M urea (2× twice at room temperature under shaking). This latter extraction was extensively dialyzed against water and freeze-dried. The 8 M urea-soluble GG fraction was re-suspended in 10 mM HCl before use. HCl was used at a final concentration of 100 μM (mock). In addition, as gliotoxin has been reported to induce cell apoptosis (17, 18), we used chloroform to extract any gliotoxin from the GG preparation by HPLC and quantify it, as described previously (19). Briefly, 10 mg GG fraction was re-suspended in 10 mM HCl (pH 2) and extracted twice with chloroform. Chloroform phases were pooled and dried under an N2 stream. Samples were analyzed by reversed-phase HPLC, with a C18 column (Uptisphere 3HDO, 150 × 4.6 mm, Interchim; Montluçon, France) under the following conditions: flow 1 ml/min; elution gradient, 0–20 min isotropic condition with 25% acetonitril in 1% acetic acid, 20–25 min linear gradient until 75% acetonitril in 1% acetic acid was reached, with UV detection at 254 nm. Standard gliotoxin was a gift from S. Doyle (National University of Ireland, Maynooth, Kildare, Ireland). The absence of detectable gliotoxin in this procedure demonstrated that the GG was not contaminated by this toxin.

Galogtomanan, α-1-3 glucans, and β-1-3glucans were purified as described previously (20).

Isolation of cells and coculture of PMN and NK cells

Whole-blood samples collected from healthy donors after written consent were obtained from Hôpital Pitié Salpêtrière through the Etablissement Français du Sang (Paris, France), after approval for the use of this material by the ethics committees of INSERM and the Etablissement Français du Sang (conversion 12/EPS/079). Whole-blood samples collected in accordance with the Helsinki Declaration from three chronic granulomatous disease (CGD) patients were obtained from the Study Center for Primary Immunodeficiencies, Necker Hospital (Paris, France). The study was approved by the local ethics committee of Necker-Enfants Malades Hospital (Paris, France). Human PMNs were isolated as described previously (21).

CD56-depleted whole-blood samples were obtained with human CD56 microbeads and a Whole Blood Column Kit (Miltenyi Biotec). The NK cell content of depleted whole-blood samples was <1%, confirmed by flow cytometry.

The NK Cell Isolation Kit (human; Miltenyi Biotec) was used to obtain pure populations of NK cells from PBMCs, in accordance with the manufacturer’s instructions. The purity of NK cells (defined as CD56+/CD3−) was >95%.

Isolated PMNs (5 × 10⁶/ml) were cocultured with NK cells at a 1:1 ratio in RPMI 1640 + Glutamax medium (Life Technologies) supplemented with 1% penicillin-streptomycin (Life Technologies) and 10% FBS (Life Technologies). In selected experiments, isolated PMNs were cultured either above or below a 0.4-μm-pore Transwell membrane (Corning Costar).

Measurement of PMN apoptosis

Fresh whole-blood samples from healthy donors, depleted of CD56+ cells or not, and isolated PMNs alone or cocultured with NK cells were treated in 24-well tissue culture plates at 37°C with 5% CO2 for various periods of time with PBS and mock (1–20 μg/ml) solutions. Cycloheximide (3 μg/ml, Sigma-Aldrich), and LPS from Escherichia coli 055:B5 (10 ng/ml; Sigma-Aldrich) were used as proapoptotic and antiapoptotic controls, respectively (21). In some experiments, samples were pretreated for 1 h with 30 μM diphenyleneiodonium (DPI) (Sigma-Aldrich), an inhibitor of NADPH oxidase, 100 μM benzyloxyacarbonyl-Val-Ala-Asp (OMe) fluoromethylkette (z-VAD-FMK) (BD Pharmingen), an inhibitor of caspases, 50 μM Z-IETD, a caspase-8 inhibitor (BD Pharmingen), 100 μM Z-LEHD-CHO, a caspase-3 inhibitor (Millipore, Sigma-Aldrich), and 100 μM Z-Val-Ala-Asp-CHO, a calpain inhibitor III (Calbiochem-Millipore). In some experiments, we also used neutralizing Abs against Fas ligand (FasL) (10 μg/ml), clone 10F2 (Serotec), against TNF-α (5 μg/ml; R&D Systems), against TRAIL (10 μg/ml; R&D Systems), against MHC class I chain–related (MIC) molecules A (MIC-A) (50 μg/ml, clone 159227; R&D Systems) as well as against NKG2D (50 μg/ml, clone 1D11; BioLegend) and their isotype controls, anti-MIC-A-PE (clone 159227; R&D Systems) and anti-IgG1 (clone MOPC-21; BioLegend). To block non-specific binding through FcRs, cells were pretreated with Fc-blocking reagent (Miltenyi Biotec) before adding specific Abs against MIC mole-
cules A/B (MIC-A/B) as well as against NKG2D and their isotype controls. Whole-blood PMN apoptosis was also performed in vitro in blood from patients with CGD.

FITC-Annexin V (BD Pharmingen) and the impermeant nuclear dye 7-aminoactinomycin D (7-AAD; BD Via-Probe) were used to quantify apoptosis, as described previously (21, 22). After pretreatment of whole blood with PBS, GG, CHX, or LPS for 24 h, samples (100 μl) were washed twice in PBS, incubated with APC-anti-CD15 (clone H91B; BD Pharmingen) for 15 min, and then incubated with FITC-annexin V for 15 min. After detergent in PBS (500 μl), the samples were incubated with 7-AAD at room temperature for 10 min and analyzed immediately by flow cytometry.

Measurement of the PMN oxidative burst

Superoxide anion (O2−) production was measured with a flow cytometric assay derived from the hydroethidine oxidation technique (23, 24). Whole-blood samples were loaded for 15 min with 1500 ng/ml hydroethidine (Sigma-Aldrich) at 37°C and then incubated with PBS, GG (0.005–20 μg/ml), or LPS (10 ng/ml), used as a positive control, for 45 min. In some experiments, DPI (30 μM) was used to inhibit NADPH oxidation produce. Samples were then treated with PBS, PMA (1 mg/ml; Sigma-Aldrich), or N-Formyl-Met-Leu-Phe (MLP, 10−6 M; Sigma-Aldrich) for 5 min. Red cells were lysed with 1× BD FACS Lysing Solution (BD FACS), and white cells were resuspended in 1× BD cell fix (BD FACS).

Determination of NK receptor ligands and Fas expression at the PMN surface

Whole-blood samples were pretreated with PBS, LPS (10 ng/ml), GG (1–20 μg/ml), or mock solutions for 20 h. One hundred microliters of this stimulated blood was then incubated with either 10 μg/ml NCR ligands from R&D (NKp46-Fc, NKp44-Fc, and NKp30-Fc, and their isotype control Rh-IgG1-Fc) or 10 μg/ml NKG2 ligand Abs (anti-UL16 binding protein [ULBP1] [clone 170818; R&D Systems], anti-ULBP2/5/6 [clone 165903; R&D Systems], anti-ULBP3 [clone 166510; R&D Systems], and their isotype control anti-IgG2a [10 μg/ml, clone 20102; R&D Systems]) for 2 h at 4°C, washed, and then stained with secondary Abs PE-IgG1 or PE-IgG2a (1/100; Jackson ImmunoResearch Laboratories) for 1 h at 4°C. To evaluate the expression of MIC-A and MIC-B, samples were incubated with anti Systems MIC-A-PE (clone 159227; R&D Systems), anti–MIC-B-PE (clone 236511; R&D Systems) and their isotype control anti-IgG2b-PE (clone 133303; R&D Systems) for 1 h at 4°C. Cells were then washed and stained with APC-CD15 Abs for 30 min at 4°C. RBCs were lysed, and cells resuspended in 1× BD cell fix. FITC-conjugated Annexin V and TO-PRO-3 iodide were added to exclude dead cells from the analysis.

Determination of NK-cell activation

PBMCs or NK cells cocultured with PMN were incubated for 20 h with mock, GG (1–20 μg/ml), or IL-12 (10 ng/ml) + IL-18 (100 ng/ml) solutions (the latter used as a positive control); then 100 μl of this stimulated blood or stimulated 105 PMBCs were stained with anti–CD69-APC (clone 553; BD Biosciences), anti–CD69-PE (clone 2H7; BioLegend), and anti–CD56-PE-C7 Abs (clone n901; BD) for 30 min at 4°C. Cells were fixed and analyzed by BD flow cytometry.

NK cell degranulation and intracellular analysis of cytokine production by NK cells

Whole blood or NK cells were pretreated with mock or GG (1 and 20 μg/ml) solutions for 20 h and then cocultured (ratio 1:1) with isolated PMNs or K562 and incubated for 1 h in the presence of FITC-conjugated anti-human CD107a (clone H4A3; BD Pharmingen). After 1 h, monensin (GolgiStop; BD Biosciences) and brefeldin A (GolgiPlug; BD Biosciences) were added. Cells were then washed and stained with APC-CD15 Abs for 30 min at 4°C. RBCs were lysed, and cells resuspended in 1× BD cell fix. FITC-conjugated Annexin V and TO-PRO-3 iodide were added to exclude dead cells from the analysis.

Determination of FasL expression at the NK cell surface

Cocultures of PMN and NK cells were pretreated with GG (1–20 μg/ml) or mock solutions for 20 h. One hundred μl of this stimulated blood was then incubated with anti–CD3-APC-eFluor780 (clone UCHT1; eBioscience), anti–CD69-PE (clone 2H7; BioLegend) or its isotype control anti-IgG1k (clone MOPC-21; BioLegend) for 30 min at 4°C. Cells were labeled and analyzed by BD flow cytometry.

Downloaded from http://www.jimmunol.org/ on April 19, 2017
Flow cytometry

A Gallios (Beckman Coulter) flow cytometer was used. PMNs were analyzed with Kaluza software. To measure apoptosis in whole blood, PMNs were defined as CD15<sup>high</sup> cells and 2 x 10<sup>6</sup> events were counted per sample. A gate was drawn around the PMN population. Fluorescence analysis was performed on this gate. The combination of FITC-annexin V and 7-AAD was used to distinguish early apoptotic cells (annexin V<sup>+</sup>/7-AAD<sup>+</sup>), from late apoptotic cells (annexin V<sup>+</sup>/7-AAD<sup>−</sup>), necrotic cells (annexin V<sup>−</sup>/7-AAD<sup>−</sup>), and viable cells (unstained). In other experiments, forward and side scatter were used to identify the PMN population and to gate out other cells and debris; 10,000 events were counted per sample. Mean fluorescence intensity was used to quantify the responses.

Statistical analysis

Data are reported as means ± SEM. Comparisons, performed with Prism 3.0 software (Graph Pad software), used ANOVA, Tukey’s posthoc tests, and Student t tests. Values are means ± SEM (significantly different from sample incubated with PBS [p < 0.05], */p < 0.01, **p < 0.001). *significantly different from mock [p < 0.05], ** p < 0.01, ***p < 0.001).

Results

GG induces PMN apoptosis via NK cells

PMNs survive in the circulation for ~1 or 2 d before undergoing apoptosis (25). Cultured control whole-blood PMNs died rapidly by apoptosis: 30% were annexin V<sup>+</sup> after 20 h (Fig. 1A). This percentage of apoptotic PMNs in whole-blood samples increased significantly in the presence of GG, compared with control (mock) solution (Fig. 1A, 1B), as we previously reported (16). The dose-response relation is shown in Supplemental Fig. 1. In contrast, other purified fungal polysaccharides extracted from A. fumigatus (i.e., galactomannan, α1-3 glucan, and β1-3 glucan) did not induce such cellular apoptosis (Fig. 1B). We also tested the effect of the intact fungus and observed that A. fumigatus germinating conidia promoted PMNs apoptosis, a result that might be related to GG-induced effect (Supplemental Fig. 2). To determine the mechanism involved in GG-induced PMN apoptosis, we first tested the effect of GG on isolated PMNs. The antiapoptotic and proapoptotic effects of LPS and CHX, respectively, were still detectable, but the proapoptotic effect of GG was not (Fig. 1C), a result that indicates the necessity of the blood environment for triggering GG-induced PMN apoptosis.

Because NK cells have recently been reported to accelerate spontaneous PMN apoptosis (26), we investigated the effect of GG on whole-blood samples depleted of CD56<sup>+</sup> cells (Fig. 1D). The proapoptotic effect of GG on whole-blood PMNs was no longer detectable after this depletion. We next evaluated whether addition of either resting autologous or allogeneic NK cells to freshly isolated PMNs could trigger GG-induced apoptosis. When PMNs were cocultured in the presence of highly purified NK cells for 16 h, GG exerted a proapoptotic effect on isolated PMNs similar to that reported in whole-blood conditions (Fig. 1E). Similar results were observed in the presence of autologous and allogeneic NK cells, findings consistent with the low expression by PMNs of surface HLA class I molecules, which GG treatment did not change (Fig. 1F).

We next examined whether the role of NK cells in triggering GG-induced PMN apoptosis depended on soluble factors or required cell–cell contact. Conditioned medium, obtained from GG-activated NK cells, did not trigger GG-induced PMN apoptosis (Fig. 1G). Moreover, the disappearance of the proapoptotic effect when the PMNs were physically separated from the NK cells (Transwell coculture system) (Fig. 1H) suggests that cell–cell contact might be necessary to trigger this GG proapoptotic signal.

**GG increases MIC-A expression on PMN surfaces and thereby promotes PMN apoptosis**

The role of NK cells in the clearance of tumor or virus-infected cells (27) is implemented by the interaction between activating NK receptors and their specific ligands, such as the natural cytotoxicity receptors (NCRs: Nkp30, Nkp44, and Nkp46) and NKG2D, which interacts with stress-inducible ligands such as MIC-A/B, and the UL16-binding protein (ULBP) 1–6 molecules (28, 29). As shown in Fig. 2A, we did not observe any significant binding of the NCR fusion proteins (Nkp30-Fc, Nkp44-Fc, or Nkp46-Fc) to either resting PMNs or GG-treated PMNs, compared with samples stained with isotype controls. This finding shows that GG did not induce the expression of any NCR ligands on PMN surfaces. Notably, treatment of whole-blood samples with GG for 20 h dose-dependently increased the expression of MIC-A at the PMN surface (Fig. 2B, 2C, Supplemental Fig. 3) but did not modify the expression of any ULBPs or MIC-B (Fig. 2B). In contrast, LPS did not significantly modulate NK ligand expression on PMN surfaces compared with isotype control (Fig. 2A, 2B). More importantly, in the presence of NK cells, pretreatment with either anti–MIC-A or independently, with anti-NKG2D Abs significantly inhibited GG-induced PMN apoptosis (Fig. 2D), whereas incubation with isotype controls (Fig. 2D) or with Abs directed at ULBPs (Fig. 2E) did not exert any significant effect. These results demonstrate that GG-induced PMN apoptosis depends on MIC-A/NKG2D interaction.

**MIC-A expression on PMN surface and PMN apoptosis both depend on ROS production**

ROS are involved in modulating NK-ligand expression on different targets (30, 31) and especially in inducing surface MIC-A/B expression on airway epithelial cells without modifying this ligand’s intracellular levels (28). These data strongly suggest that ROS are key mediators in the translocation of NKG2D ligands to the surface of stressed cells that are flagged for death by NK cells. We analyzed the effect of GG on ROS generation by PMNs: pretreatment of whole-blood samples with GG resulted in a rapid increase of intracellular ROS production by PMNs in response to N-formylated peptides (fMLP) (Fig. 3A, Supplemental Fig. 4) and separately to PMA (data not shown). The maximum ROS level was significantly higher even than that observed after LPS priming (Fig. 3A).

We next investigated whether GG-induced MIC-A expression on PMNs depended on ROS production. ROS in PMNs are generated mostly by the inducible NADPH oxidase system, which can be blocked by DPI (32). Pretreatment of whole-blood samples with DPI inhibited GG-induced ROS production by PMNs in response to fMLP (Fig. 3B). GG dose-dependent MIC-A expression was totally inhibited after preincubation with DPI (Fig. 3C). Importantly, and in line with these data, PMNs from patients with CGD, who have a deficiency in NADPH oxidase activity (33), did not produce ROS in response to fMLP after GG-stimulation (Fig. 3D), and no increase in MIC-A expression was observed after GG stimulation (Fig. 3E). Finally, we analyzed the potential link between the GG-induced PMN oxidative burst and the GG-induced PMN apoptosis. PMNs from whole-blood samples of healthy donors and treated with DPI were resistant to GG-induced apoptosis (Fig. 3F). This result was also confirmed in PMNs from CGD patients (Fig. 3G).

Thus, ROS production is essential to GG-induced PMN apoptosis by increasing MIC-A expression on PMNs.

**PMN apoptosis mechanisms triggered by NK cells**

NKG2D binding to the neutrophil-encoded ligand MIC-A/B has been reported to trigger NK cell functions (34). Accordingly, we investigated the effect of GG on NK cell activation by evaluating
FIGURE 1. GG-induced PMN apoptosis occurs via contact with NK cells. Apoptosis was quantified by counting annexin V+/7-AAD− cells. (A) Dot plot of flow cytometric whole-blood PMN apoptosis analysis after 20 h stimulation. Data are from one representative donor (of 10). (B–E) Quantification of PMN apoptosis: in whole blood stimulated for 20 h (B), in isolated PMNs stimulated for 20 h (C), in CD56+ cell–depleted whole blood stimulated for 20 h (D), in autologous and allogenic PMN-NK cells cocultured for 16 h (E). (F) Absence of HLA molecules expression on GG-activated PMN. Data are from one representative donor of five. White histograms represent the fluorescence of cells stained with matching isotype control. (G and H) Absence of GG-induced PMN apoptosis when PMN cells were cultivated with supernatant from NK cells stimulated by GG for 20 h (G) or when PMNs were separated from NK cells by a Transwell membrane (H). Values are means ± SEM [n = 10, except n = 5 (F) and n = 4 (G and H)]. Blank bars indicate samples incubated with PBS, LPS or CHX; black bars indicate GG-incubated samples referenced with mock control, in gray bar. In (H), results are expressed as the percentage acceleration of PMN apoptosis (1− [% of annexin V+ PMNs in PBS or mock-treated sample/percentage of annexin V+ PMNs] × 100) (Figure legend continues)
these cells’ level of CD69 expression, which is an early activation marker (35) (36). Although treatment of PBMCs, which are free of PMNs, with GG did not induce any activation (Fig. 4A), incubation of cocultured NK cells and PMNs, from healthy controls, with GG significantly increased CD69 expression on NK cells (Fig. 4B). These data suggest that GG-induced NK cell activation results from a MIC-A/NKG2D interaction that may subsequently generate an apoptosis-promoting signal in PMNs.

We next examined GG’s effect on CD107a expression on NK cells as a surrogate marker for granule mobilization (37). As shown in Fig. 4B, NK cells incubated with K562 cells, the standard NK-susceptible target, showed significant degranulation in CHX- or GG-treated sample

\[ \times 100. \] \( *p < 0.05, **p < 0.01, ***p < 0.001; \) significantly different from sample incubated with PBS. \( *p < 0.05, **p < 0.01, ***p < 0.001; \) significantly different from sample incubated with mock. AG, α1-3 glucan; BG, β1-3 glucan; GG, galactosaminogalactan; GM, galactomannan.
which was not modified by GG stimulation. Moreover, we observed no increase in CD107a surface expression on NK cells cocultured with untreated or GG-treated PMNs (Fig. 4C). Results with whole blood were similar (data not shown). Taken together, these data suggest that GG does not act directly on NK-cell cytotoxicity. Upon activation, NK cells have also been reported to release TNF-α (38), which might also trigger death receptor-induced apoptosis in PMNs. We therefore determined intracellular levels of TNF-α and IFN-γ in NK cells cocultured with untreated versus GG-treated PMNs. Neither GG-treated NK cells alone nor NK cells cocultured with GG-treated PMNs produced TNF-α (Fig. 4D) or IFN-γ (Fig. 4E). Furthermore, GG had no effect on TNF-α (Fig. 4D) or IFN-γ (Fig. 4E) production by NK cells incubated with their target K562 cells.

More importantly, blocking FasL by its neutralizing Ab (compared with use of its isotype control) significantly decreased GG’s effect on the apoptosis of PMNs cocultured with NK cells (Fig. 5A). In contrast, blocking Trail or TNF-α did not modify PMN apoptosis (Fig. 5B, 5C). In addition, Fas (CD95) was barely expressed on resting PMN cells, and treatment by GG, compared with LPS, significantly and dose-dependently increased its expression (Fig 5D). GG also promoted FasL expression on the surface of CD56dim NK cells, which is in accordance with the GG-activated NK cells population (Fig. 5E). Because ROS production might induce Fas expression (39), we investigated DPI’s effect on Fas expression at the PMN surface and observed a significant decrease (Fig. 5F). Consistently with these findings, both Z-V AD-FMK, a general caspase inhibitor, and ZIETD-FMK, a specific inhibitor of caspase-8, significantly inhibited GG-induced PMN apoptosis (Fig. 4G), although inhibitors of calpains and cathepsins had no effect on it (Fig. 5H, 5I).

**Discussion**

In this study, intended to identify the mechanism of GG-induced PMN apoptosis, we investigated the role of NK cells in this process. Our study clearly deciphers the sequence of the interactions between these two major cell groups involved in innate immunity and shows that the crosstalk between them is essential to this apoptosis. The presence of GG induced PMNs to produce ROS, which then increased MIC-A expression at the PMN surface. This expression in turn enabled PMNs to interact with NK cells and promoted NK-cell activation. Once activated, NK cells triggered PMN apoptosis through the Fas and caspase pathways (Fig. 6).

Alveolar macrophages are primary immunological cells resident in the respiratory tract and a critical component of host defense against *Aspergillus* conidia. However, alveolar macrophages also
induce the initiation of a proinflammatory response that recruits PMNs to infection site. Conidia that evade macrophage killing and germinate become the target of infiltrating PMNs that are able to destroy hyphae. Consequently, neutropenia is among the most important risk factors for invasive aspergillosis. Furthermore, invasive aspergillosis has been associated with early recruitment of

FIGURE 4. Promotion of PMN apoptosis by GG-induced NK cell activation is not due to either degranulation or cytokine release. (A) GG did not induce NK cell activation in GG-stimulated culture of PBMCs. (B) GG-stimulated-cocultured of PMN and NK cells upregulates CD69 on the surface of NK cells. Dot plots are from one representative donor (of five). Significance as described in Fig. 1 legend. (C) GG did not trigger degranulation by NK cells cocultured with PMNs. (D) and (E) NK cells cocultured with GG-treated K562 or GG-treated PMNs did not produce TNF-α (D) or IFN-γ (E). The data represent one experiment of three.
NK cells in the lungs (40); NK cells present in the infected area might thus interact with PMNs. Previous studies have addressed the reciprocal crosstalk of NK cells with PMNs, focusing mainly on the effects of PMNs on NK cell functions and demonstrating that PMNs favor the terminal maturation of NK cells and the acquisition of their full effector functions (6). Furthermore, it has been suggested that soluble factors (IFN-γ or GM-CSF) released by cytokine-activated NK cells (i.e., IL-2–treated NK) send survival signals to PMNs and may promote their accumulation for extended time periods at the inflammation site and support their functions there (7, 41). In contrast, Thore´ n et al. (26) recently reported that NK cells accelerate spontaneous apoptosis of isolated PMNs. In this study, to avoid the PMN isolation procedures that have been reported to alter PMN responses (21, 42, 43), we analyzed PMN apoptosis by flow cytometry in whole-blood conditions. In these conditions, with whole-blood PMN activation by GG, a polysaccharide extracted from A. fumigatus, we demonstrated that NK cells are a critical trigger of PMN apoptosis. In addition, the proapoptotic effect of GG on PMNs was observed at a concentration of 20 μg/ml, which is a relevant concentration in vivo, because Aspergillus can secrete GG in a concentration of 50 μg/ml (44).

Lung DCs have been reported to recognize, ingest, and kill both swollen conidia and hyphae and initiate protective T cell immunity against Aspergillus. Several lines of evidence argue for a cross-talk between PMN and DCs. In particular, in response to Aspergillus, neutropenia enhances lung inflammatory response and is associated with an increase in inflammatory DCs in the lungs, a mechanism protective of the host (45). The GG-induced PMN apoptosis might thus, on one hand, lead to a decrease in fungal clearance by PMNs and on the other hand confer protection on the host via the accumulation of DCs in the lungs.

Our findings also show that these GG-activated PMNs did not stimulate NK cells either to produce TNF-α and IFN-γ or to degranulate. They strongly suggest that GG-induced PMN–NK cell interaction triggers a Fas-dependent apoptosis pathway in NK cells in the lungs (40); NK cells present in the infected area might thus interact with PMNs. Previous studies have addressed the reciprocal crosstalk of NK cells with PMNs, focusing mainly on the effects of PMNs on NK cell functions and demonstrating that PMNs favor the terminal maturation of NK cells and the acquisition of their full effector functions (6). Furthermore, it has been suggested that soluble factors (IFN-γ or GM-CSF) released by cytokine-activated NK cells (i.e., IL-2–treated NK) send survival signals to PMNs and may promote their accumulation for extended time periods at the inflammation site and support their functions there (7, 41). In contrast, Thore´ n et al. (26) recently reported that NK cells accelerate spontaneous apoptosis of isolated PMNs. In this study, to avoid the PMN isolation procedures that have been reported to alter PMN responses (21, 42, 43), we analyzed PMN apoptosis by flow cytometry in whole-blood conditions. In these conditions, with whole-blood PMN activation by GG, a polysaccharide extracted from A. fumigatus, we demonstrated that NK cells are a critical trigger of PMN apoptosis. In addition, the proapoptotic effect of GG on PMNs was observed at a concentration of 20 μg/ml, which is a relevant concentration in vivo, because Aspergillus can secrete GG in a concentration of 50 μg/ml (44).

Lung DCs have been reported to recognize, ingest, and kill both swollen conidia and hyphae and initiate protective T cell immunity against Aspergillus. Several lines of evidence argue for a cross-talk between PMN and DCs. In particular, in response to Aspergillus, neutropenia enhances lung inflammatory response and is associated with an increase in inflammatory DCs in the lungs, a mechanism protective of the host (45). The GG-induced PMN apoptosis might thus, on one hand, lead to a decrease in fungal clearance by PMNs and on the other hand confer protection on the host via the accumulation of DCs in the lungs.

Our findings also show that these GG-activated PMNs did not stimulate NK cells either to produce TNF-α and IFN-γ or to degranulate. They strongly suggest that GG-induced PMN–NK cell interaction triggers a Fas-dependent apoptosis pathway in

FIGURE 5. GG-induced PMN apoptosis by NK-cell activation is due to a Fas and caspase-8 dependent mechanism. (A–C) Blocking FasL by its neutralizing Ab (compared with use of its isotype control) inhibited GG-induced PMN apoptosis (A) whereas blocking TNF-α (B) or TRAIL (C) did not. (D) GG increased Fas (CD95) expression on PMN surfaces; results are quantified in percentage of CD95+ cells. (E) FasL is expressed on NK cell surfaces after GG stimulation. Increase in Fas expression on GG-treated PMNs was inhibited by the NADPH oxidase inhibitor DPI. (F) Involvement of caspases (Z-VAD-FMK) and especially caspase-8 (ZIETD-FMK) pathway in GG-induced PMN apoptosis. (H and I) Inhibition of calpains (H) or cathepsins (I) did not modify GG-induced PMN apoptosis. Values are means ± SEM (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001; significantly different from sample incubated with PBS. *p < 0.05, **p < 0.01, ***p < 0.001; significantly different from sample incubated with mock.
PMN but does not affect other NK cell functions. These results are in line with the data reported by Kloss et al. (46) showing that LPS-treated monocytes stimulate IFN-γ production by activated NK cells, and that this stimulation depends substantially on MICA-NKG2D interaction, even though MICA expression does not make the monocytes susceptible to NK cell cytotoxicity. It may seem surprising that various functions of NK cells are differentially affected by MICA/B-NKG2D interaction. Nonetheless, we must remember that the different aspects of NK cell reactivity are mediated by tightly regulated and at least partially independent signaling pathways involving various kinases, phosphatases, and transcription factors. Although activation of ERK is crucial for various signaling pathways involving various kinases, phosphatases, and transcription factors. Although activation of ERK is crucial for NK cell–mediated lytic functions (47), IFN-γ production is influenced by various pathways that lead to the activation of transcription factors such as NF-κB or members of the STAT family (48).

PMN apoptosis depends on a balance between the expression of proapoptotic and antiapoptotic molecules, modulated by various mediators, including cytokines (49). We provide direct evidence here that interaction between GG-activated PMNs and NK cells leads to NK activation but does not increase the production of intracellular cytokines such as IFN-γ, which should have been able to trigger antiapoptotic pathways in PMNs (50). GG seems to inactivate the PMN antiapoptotic pathways at the same time as it activates PMN proapoptotic pathways, thus optimizing induction of PMN apoptosis.

Most studies indicate that pathogen-activated PMNs delay PMN apoptosis to resolve inflammation (51–54), but others report the opposite. They underline the role of the pathogen in inducing PMN apoptosis to escape destruction by immune system. For example, Kobayashi et al. (55) have shown that *Streptococcus pyogenes* induces PMN apoptosis by modulating PMN gene expression, whereas Spinner et al. (56) have demonstrated that *Mycobacterium tuberculosis* accelerates PMN apoptosis through contact between dendritic cells and PMNs.

Moreover, PMN apoptosis is correlated with ROS production in these studies (57). In our study, GG-induced ROS production by PMNs, because of increased expression of both MIC-A and Fas expression on the PMN surface, was the key factor in promoting PMN apoptosis. Our results also suggest that GG-induced PMN apoptosis depends on a ROS threshold. Lower concentrations of GG (1 μg/ml), as well as LPS, induce ROS production without increasing MICA/B or Fas expression and without inducing PMN apoptosis. In contrast, high GG concentrations (>5 μg/ml) lead to greater ROS production and do induce PMN apoptosis. This result is consistent with the report by Zhang et al. (58) that phagocytosis-inducing cell death depends on a ROS threshold in PMNs. Yet another study, this one in airway epithelial cells, describes a dose-dependent relation between ROS exposure and NKG2D ligand expression (28).

To promote its survival, *A. fumigatus* is able to impair its host’s immune responses by secreting mycotoxins such as gliotoxins (59). These toxins are known to inhibit phagocytosis and induce apoptosis of monocytes and neutrophils (60, 61). Although GG is a polysaccharide from the cell wall of *A. fumigatus*, it can be secreted in vivo during early growth and can have an immunosuppressive effect (16). We show in this paper that, in addition to promoting *in vivo* *A. fumigatus* growth in a mouse model, GG may also be one of these mycotoxins, through its NK-mediated promotion of PMN apoptosis.

Thus, our results point to a new escape mechanism in which a polysaccharide produced by *A. fumigatus* and involved in fungal virulence appears to reverse a host immune response by inducing cross-talk between these two major types of immune cells. Shortening the programmed PMN lifespan by abnormally accelerating PMN apoptosis can exacerbate infections (62, 63). For example, HIV-1 gp41 induces H2O2-dependent NKp44L expression on CD4+ T cells, rendering them susceptible to autologous NK killing (31). A better understanding of PMN and NK regulation has the potential to lead to novel immune-based therapies. Further studies are thus necessary to investigate the role of NK cells in the induction of PMN apoptosis in these infectious situations as well as in conditions such as autoimmune diseases or unexplained neutropenia.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Material

A polysaccharide virulence factor of a human fungal pathogen induces neutrophil apoptosis via NK cells

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Supplemental Figures

**Supplemental Figure S1. GG-induced PMNs apoptosis in a dose-dependent manner**

Quantification of PMNs apoptosis was performed by flow cytometry by counting annexin V+/7-AAD− cells in whole blood stimulated for 20 h with different concentrations of GG, as indicated. LPS and CHX were respectively used as antiapoptotic and proapoptotic controls. Values are means ± SEM, n=10.

Blank bars indicate samples incubated with PBS, LPS or CHX; black bars indicate GG-incubated samples referenced with mock control, in gray bar. Values are means ± SEM.

* Significantly different from sample incubated with PBS (p< 0.05), ** p< 0.01, *** p< 0.001
* Significantly different from sample incubated with mock (p<0.05), ** p< 0.01, *** p< 0.001
Supplemental Figure S2. *Aspergillus fumigatus* germling conidia induced PMN apoptosis

Quantification of PMNs apoptosis was performed by flow cytometry by counting annexin V+/7-AAD− cells in whole blood stimulated for 20 h with different concentrations of germling conidia, as indicated. LPS and CHX were respectively used as antiapoptotic and proapoptotic controls. Values are means ± SEM, n=4.

Blank bars indicate samples incubated with PBS, LPS or CHX; black bars indicate germling conidia-incubated samples referenced with PBS. Values are means ± SEM.

* Significantly different from sample incubated with PBS (p< 0.05), ** p< 0.01, *** p< 0.001
Supplemental Figure S3. GG-induced dose-dependent MICA/B expression on PMN surfaces

Quantification of MICA/B expression on PMN surfaces was performed by flow cytometry, in resting state and in whole-blood samples stimulated for 20 h with different concentrations of GG.

Values are means ± SEM, n=8.

Blank bars indicate samples incubated with PBS, LPS or CHX; black bars indicate GG-incubated samples referenced with mock control, in gray bar.

* Significantly different from sample incubated with PBS (p<0.05), ** p<0.01, *** p<0.001
Supplemental Figure S4. GG-induced dose-dependent ROS production by PMNs

Whole-blood samples were stained with hydroethydine and then pretreated with different concentrations of GG for 45 min. LPS was used as positive control. Quantification of intracellular ROS production by PMNs in response to N-formylated peptides (fMLP) was performed by flow cytometry.

Values are means ± SEM, n=10.