Decreased Cell Wall Galactosaminogalactan in *Aspergillus nidulans* Mediates Dysregulated Inflammation in the Chronic Granulomatous Disease Host

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Invasive aspergillosis is a major threat to patients suffering from impaired neutrophil function, with *Aspergillus fumigatus* being the most common species causing this life-threatening condition. Patients with chronic granulomatous disease (CGD) not only develop infections with *A. fumigatus*, but also exhibit a unique susceptibility to infection with the normally nonpathogenic species *Aspergillus nidulans*. In this study, we compared the inflammatory cytokine response of peripheral blood mononuclear cells (PBMCs) from healthy and CGD patients to these two fungal species. CGD patients displayed evidence for a chronic hyperinflammatory state as indicated by elevated plasma IL-1β and TNF-α levels. PBMCs isolated from CGD patients secreted higher levels of IL-1β and TNF-α in response to *A. nidulans* as compared with *A. fumigatus*. The presence or absence of melanin in the cell wall of *A. nidulans* did not alter the cytokine release by healthy or CGD PBMCs. In contrast, *A. fumigatus* mutants lacking melanin stimulated higher levels of proinflammatory cytokine release from healthy, but not CGD PBMCs. Purified cell wall polysaccharides of *A. nidulans* induced a much higher level of IL-1β secretion by CGD PBMCs than did cell wall polysaccharides isolated from *A. fumigatus*. Using modified *A. nidulans* strains overexpressing galactosaminogalactan, we were able to show that the increased secretion of inflammatory cytokines by CGD PBMCs in response to *A. nidulans* are a consequence of low levels of cell wall-associated galactosaminogalactan in this species.

Introduction

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency (1:125,000) that is associated with an intrinsic susceptibility to invasive aspergillosis (Henriet and others 2013). The molecular basis of CGD is well understood: CGD is a group of heterogeneous diseases caused by a defect in any of the five structural components of the NADPH-oxidase (gp91phox, p47phox, p67phox, p22phox, and p40phox). As a result, the CGD phagocytic cell is unable to produce reactive oxygen species (ROS). Generation of ROS by a functional NADPH-oxidase is important in at least two major functions of the innate immune system, such as antimicrobial killing and the regulation of inflammation (Segal and others 2012). As such, clinically, CGD patients are characterized by recurrent life-threatening infections and inflammatory complications such as colitis-like syndromes and formation of granulomata.

The lifetime incidence of invasive aspergillosis in CGD patients varies between 25% and 40%, and is a primary cause of death (Denning 1998; Segal and others 1998). While *Aspergillus fumigatus* is the most commonly encountered species, patients with CGD are uniquely susceptible to invasive infections with *Aspergillus nidulans*, a nonvirulent fungus that rarely causes disease in other immunocompromised patient populations. *A. nidulans* infections in patients with CGD have a greater propensity to disseminate and a higher mortality rate than those caused by *A. fumigatus* (Segal and others 1998).

To date, the immune mechanisms underlying the pathogenesis of invasive *A. nidulans* infections remain poorly understood.
understood. Importantly, peripheral blood mononuclear cells (PBMCs) from CGD patients produce higher levels of the proinflammatory cytokine interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ) upon stimulation with Aspergillus species, whereas IL-17A production is strikingly low compared with healthy controls (Smeekens and others 2012). These differences in cytokine production are not related to differences in leukocyte viability as CGD phagocytes do not undergo cell death more rapidly than healthy cells in response to A. nidulans infection (Henriet and others 2011). The fungal factors underlying this hyperinflammatory response to A. nidulans have not been elucidated.

The constituents of the fungal cell wall play an important role in mediating the immune response to Aspergillus. Cell wall polysaccharides, such as galactomannan, β-glucan, and the recently discovered galactosaminogalactan (GAG), are important immune ligands that can modulate cytokine expression (Latgé 2010). Comparative analyses of the cell wall composition of A. fumigatus and A. nidulans have demonstrated differences between these organisms. The most striking of these differences is that the A. nidulans cell wall contains only low levels of the heteropolysaccharide galactosaminogalactan (Guest and Momany 2000; Lee and others 2015), which has recently been found to mediate immunosuppression during infection with A. fumigatus (Fontaine and others 2011; Gravelat and others 2013).

In addition to cell wall polysaccharides, conidial melanin of A. fumigatus, that is, 1,8-dihydroxy naphthalene (DHN), is another important fungal cell wall structure that influences the immune response to fungi (Chai and others 2010; Thy- wielen and others 2011). A. nidulans produces melanin of the 3,4-dihydroxyphenylalanine (DOPA) type rather than the DHN type found in A. fumigatus (Gonçalves and others 2011). The implications of these differences in fungal cell wall composition on fungal pathogenesis in the CGD host are currently an unexplored domain.

We hypothesized that differences in cell wall composition between A. nidulans and A. fumigatus may lead to altered inflammatory responses in CGD leukocytes. Therefore, we evaluated the cytokine response of CGD PBMCs to A. nidulans as compared with A. fumigatus, as well as to cell wall components of these organisms.

Materials and Methods

Human PBMCs

After informed consent was given, venous blood was drawn from healthy volunteers and four CGD patients (two gp91phox deficient and two p47phox deficient). All patients were below the age of 18 and free from any infectious or inflammatory diseases. The clinical characteristics of all patients are presented in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/jir). Blood was collected by venepuncture into 10-mL ethylenediaminetetraacetic acid (EDTA) tubes (367525; BD). PBMCs were isolated using lymphoprep (Axis-Shield), by density gradient centrifugation as previously described (Henriet and others 2011). Briefly, blood was diluted with an equal volume of phosphate-buffered saline (PBS). The lymphoprep was carefully added below the diluted blood and centrifuged at 800 × g to separate the plasma from the peripheral blood mononuclear cells (PBMC) fraction and the polymorphonuclear leukocytes (PMN). PBMCs were harvested and counted by hemocytometer.

For the stimulation assays, 5 × 10^5 PBMCs in a total volume of 200 μL per well were incubated at 37°C and 5% CO₂ in round-bottom 96-well plates (Nunc) with 10 ng/mL lipopolysaccharide (LPS) (derived from E. coli serotype O55:B5; Sigma-Aldrich), Pami3Cys (10 μg/mL), heat-killed Candida albicans, live or heat-killed (HK) A. fumigatus or A. nidulans in specified concentrations. An extra purification step of LPS was performed before use (Hirschfeld and others 2000). RPMI 1640 Glutamax-I medium (Invitrogen Life Technologies) +10% heat-inactivated human serum (H6914; Sigma) was used as culture medium (CM). After 24 h, supernatants were collected and stored at −20°C until assayed.

To exclude a role of cellular toxicity during stimulation, the lactate dehydrogenase (LDH) concentrations were measured in the supernatants of the PBMCs. The stimuli as used in our experiments did not affect cell viability after 24 h of coinoculation compared with cells cultured without any stimulus.

Fungal strains

A. nidulans (V44-46) and A. fumigatus strains (V45-07; B-5233) are wild-type strains, originally isolated from patients suffering from invasive aspergillosis. The strain RG-12, which produces albino conidia devoid of melanin, was obtained by deletion of the gene alb1 in the strain B-5233 (Δalb1, kind gift of K.J. Kwon-Chung, NIH, Bethesda, MD). The alb1 gene codes for a polyketide synthase (pksP) in the 1,8-dihydroxynaphthalene (DHN)-melanin pathway, involved in the biosynthesis of conidial pigment (Tsai and others 1998, 1999). The A. nidulans A191 strain, was obtained by deletion of the wa gene, resulting in white conidia (Mayorga and Timberlake 1990; Tilburn and others 1990) (Δwa, kind gift of K.J. Kwon-Chung, NIH, Bethesda, MD).

The A. nidulans strains overexpressing heterologous uge3 (derived from A. fumigatus), and necessary for the production of GAG in the cell wall, were constructed as previously described (Lee and others 2014, 2015). These strains were grown on minimal media supplemented with biotin (50 μL per 1 L of medium of 0.5 mg/mL biotin stock solution). All other Aspergillus strains were initially grown on a Sabouraud glucose agar supplemented with chloramphenicol for 4–7 days at 37°C and subsequently plated on a 1:10 diluted Sabouraud agar. Conidia were harvested, filtered, and washed as previously described (Henriet and others 2011). They were stored in individual aliquots of 1 × 10^7/mL at −80°C. To obtain heat-killed (HK) conidia, the conidial suspension was HK for 15 min at 121°C. The growth and killing of the Aspergillus isolates were carried out in an LPS-free fashion.

C. albicans ATCC MYA-3573 (UC 820) (Lehrer and Cline 1969) was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in CM. C. albicans was heat killed for 1 h at 100°C. The viability of the fungi was checked by culturing at 37°C on fungal agar. No growth was observed following heat treatment.

Extraction of melanin from A. fumigatus and A. nidulans conidia

Melanin was extracted from the strains V44-46 (A. nidulans) and V45-07 (A. fumigatus) as previously described
Further dilutions were made in CM.

Cell wall sugars were suspended in sterile and endotoxin-free media and mechanically homogenized in a vacuum. The mycelia were washed several times to remove traces of media and obtained by gravity filtration. In summary, both species were cultured in liquid media for isolated as previously described (Guest and Momany 2000).

A. fumigatus conidia have been reported to induce less inflammatory cytokines in CGD patients, plasma was isolated from four pediatric CGD patients free from any infectious or inflammatory complications and compared with healthy controls, repeated at least at three independent time points. Significant increased levels of IL-1β (86.96 ± 27.8 pg/mL vs. 8.0 ± 0.1 pg/mL in healthy controls) and TNF-α (124.2 ± 47.3 pg/mL vs. 8.3 ± 0.5 pg/mL in healthy controls) were found in the plasma of CGD patients (P < 0.01 for IL-1β and TNF-α), indicating an intrinsic hyperinflammatory state. Interleukin-10 levels in plasma from CGD patients were not significantly different from those in healthy controls (139.7 ± 67.3 pg/mL vs. 42.4 ± 34.6 pg/mL) (Fig. 1A).

PBMCs isolated from these CGD patients also produced an exaggerated proinflammatory response upon ex vivo stimulation. Compared with PBMCs isolated from healthy controls, CGD PBMCs secreted higher levels of IL-1β and TNF-α after stimulation by the selective TLR4 ligand LPS (P < 0.01 for IL-1β and TNF-α), TLR2 ligand Pam3Cys (P < 0.01 for TNF-α), and C-type lectin agonist C. albicans (n.s.) (Fig. 1B, C). Interleukin-10 release by CGD PBMCs was also increased after stimulation by those ligands although the absolute amount of IL-10 secretion was much lower (Fig. 1D).

A. nidulans induces higher levels of proinflammatory cytokine production than A. fumigatus in CGD cells

Coculture experiments were performed to evaluate the ability of A. fumigatus and A. nidulans to induce cytokine production in healthy and CGD PBMCs. Both species of Aspergillus induced an increased release of IL-1β and TNF-α by CGD PBMCs as compared with healthy PBMCs (Fig. 2A, B). This induction of proinflammatory cytokine production was much higher when PBMCs were incubated with live organisms as compared with heat-killed organisms for both species. In both CGD and healthy PBMCs, heat-killed A. nidulans conidia led to significantly increased levels of IL-1β release than A. fumigatus conidia (P < 0.05 and P < 0.01, respectively). Live A. nidulans conidia acted as the most potent inducer of IL-1β and TNF-α by CGD PBMCs and levels were significantly higher compared with healthy PBMCs (P < 0.01 and P < 0.05, respectively) (Fig. 2A, B).

Secretion of the anti-inflammatory cytokine IL-10 by CGD PBMCs upon stimulation with both fungi was also increased, however, the absolute amount of IL-10 release was low in all conditions (range 2.3–162 pg/mL). A. nidulans induced higher levels of IL-10 secretion compared with A. fumigatus and this difference was significant when heat-killed conidia were used (Fig. 2C). No interleukin-10 release by healthy PBMCs was observed upon stimulation by the two Aspergillus species. Collectively, these results suggest that in the absence of a functional NADPH-oxidase, the proinflammatory state of the CGD PBMCs is increased by both Aspergillus species as compared with healthy PBMCs, and that A. nidulans is a more potent inducer of IL-1β than A. fumigatus.

Immunomodulatory potential of bound A. nidulans melanin differs from A. fumigatus melanin in healthy PBMCs

Fungal melanin is a well-known virulence factor influencing host–pathogen interactions. Heat-killed melanized A. fumigatus conidia have been reported to induce less...
FIG. 1. (A) Intrinsic hyperinflammatory state of CGD patient as reflected by significant higher amounts of circulating plasma IL-1β and TNF-α (n=4) compared with healthy controls (n=9). IL-1β (B), TNF-α (C), and IL-10 (D) cytokine responses of healthy (n=5–7) and CGD (n=4) PBMCs after stimulation with 1 ng/mL LPS, 10 μg/mL Pam3Cys, and 1×10⁶/mL Candida albicans. Bar represent mean + SEM. *P < 0.05, **P < 0.01. CGD, chronic granulomatous disease; SEM, standard error of the mean; PBMCs, peripheral blood mononuclear cells.

FIG. 2. IL-1β, TNF-α, and IL-10 cytokine responses of healthy (n = 5) and CGD (n = 4) PBMC after stimulation by either 5×10⁶/mL heat-killed or live A. nidulans, and A. fumigatus conidia. Bar represent mean + SEM. *P < 0.05, **P < 0.01.
proinflammatory cytokine release from healthy PBMCs than do albino conidia (Chai and others 2010). In agreement with this report, we found that healthy PBMCs secreted significantly increased levels of the proinflammatory cytokines TNF-α (P < 0.01) and IL-1β (P = 0.05) in response to live A. fumigatus conidia devoid of melanin (albino) as compared with wild-type (WT) A. fumigatus conidia. A similar trend toward increased TNF-α and IL-1β secretion by healthy PBMCs in response to albino as compared with WT conidia was seen in A. nidulans, however, this difference was not statistically significant (Fig. 3A).

The presence of melanin does not influence the proinflammatory response of the CGD PBMCs upon fungal stimulation

To assess the role of A. nidulans conidial melanin in the hyperinflammation observed in CGD PBMCs, these cells were stimulated with either WT or albino live A. nidulans and A. fumigatus conidia. No differences in IL-1β and TNF-α release by CGD PBMCs were observed in response to WT or albino A. nidulans or between WT and albino A. fumigatus (Fig. 3B). Thus, conidial melanin does not play a significant role in modulating the release of proinflammatory cytokines by CGD cells in response to either species of Aspergillus.

Melanin isolated from A. nidulans or A. fumigatus does not induce or modulate cytokine response by PBMCs from healthy individuals or CGD patients

Since fungal melanin has been shown previously to modulate the immune response toward C. neoformans and A. fumigatus (Mednick and others 2005; Chai and others 2010) we next compared the immunogenic properties of isolated A. nidulans and A. fumigatus conidial melanin. Isolated melanin of both Aspergillus spp. in concentrations up to 1 mg/mL were extremely poor stimulators of proinflammatory cytokine release by both healthy and CGD PBMCs as indicated by the lack of significant IL-1β and TNF-α release in the supernatant (data not shown). Isolated melamins from both Aspergillus species were not able to modulate the IL-1β and TNF-α release by both healthy and CGD PBMCs stimulated with LPS (data not shown) or the albino A. fumigatus and A. nidulans conidia (Fig. 3C).

A. nidulans cell wall polysaccharides are responsible for the enhanced IL-1β release by CGD PBMCs

We next questioned whether differences in polysaccharide cell wall composition between A. fumigatus and A. nidulans could be responsible for the differences in inflammatory cytokine production. Therefore, we compared the cytokine release induced by increasing concentration of isolated cell wall polysaccharides (0.01–25 μg/mL) from A. nidulans and A. fumigatus by CGD and healthy PBMCs (Fig. 4).

Stimulating CGD PBMCs resulted in a dose–response relationship of IL-1β and IL-10 in response to A. fumigatus as well as A. nidulans cell wall polysaccharides, but was not observed for TNF-α secretion. In healthy PBMCs, a dose–response relationship in response to both cell wall polysaccharides was observed for IL-1β and TNF-α, whereas IL-10 secretion was minimal. Cell wall polysaccharides of

FIG. 3. The cytokine response of (A) human healthy PBMCs and (B) CGD PBMCs to 1×10⁶/mL live albino (ALB, gray bars) or live wild-type (WT, black bars) A. fumigatus and A. nidulans conidia (n = 6–9). (C) The IL-1β release by CGD PBMCs in response to albino A. nidulans (ΔanΔA) (A. nidulans ALB, white bars) or A. fumigatus (Δalb1) (A. fumigatus ALB, black bars) stimulation in the presence of increasing concentration (mg/mL) of isolated A. nidulans (AN) conidal melanin or A. fumigatus (AF) conidal melanin. The albino conidial-stimulated cytokine release without isolated melanin was set to 100% (n = 2). Bar represent mean ± SEM. *P = 0.05, **P < 0.01.
FIG. 4. The IL-1β (A, B), TNF-α (C, D), and IL-10 (E, F) release of healthy PBMCs (n = 4) and CGD PBMCs (n = 3) after stimulation with increasing concentrations of isolated cell wall sugars of A. fumigatus or A. nidulans. The IL-1β, TNF-α, and IL-10 cytokine response of healthy PBMCs compared with CGD PBMCs after stimulation with 12.5 μg/mL isolated A. nidulans cell wall sugars (G) or A. fumigatus cell wall sugars (H). Bars indicate SEM.
A. nidulans induced higher levels of IL-1β secretion as compared with A. fumigatus cell wall polysaccharides both by healthy and CGD PMBCs, this difference was not observed for the induction of TNF-α and IL-10.

Comparing the cytokine responses between CGD and healthy PMBCs, the CGD PMBCs secreted substantially higher levels of IL-1β than the healthy controls upon stimulation with the individual cell wall polysaccharides (Fig. 4G, H). Stimulation by A. nidulans cell wall polysaccharides did not lead to differences in TNF-α release by CGD and healthy PMBCs in contrast to A. fumigatus cell wall polysaccharides. IL-10 production by healthy PMBCs was minimal, whereas CGD PMBCs released a significant amount of this cytokine in response to stimulation by both A. fumigatus- or A. nidulans-derived cell wall polysaccharides and no differences between both species were observed.

**GAG deficiency in the cell wall of A. nidulans leads to increased IL-1β release**

Finally, we tested the hypothesis that the low levels of GAG in the cell wall of A. nidulans could be responsible for the increased IL-1β release by PMBCs in response to this organism. The reduced expression of GAG by A. nidulans is due to decreased expression of ugeB, encoding an epimerase required for the synthesis of N-acetylgalactosamine (GalNAc) and the subsequent production of GAG. Heterologous expression of the orthologous A. fumigatus uge3, increases cell wall GAG production in A. nidulans to levels similar to A. fumigatus (Lee and others 2015). Therefore, to analyze the contribution of GAG to inflammatory cytokine production by PMBCs, A. nidulans strains overexpressing GAG (uge3-complemented A. nidulans strain) were compared with wild-type A. nidulans for their ability to induce IL-1β secretion by these cells.

Both healthy PMBCs and PBMCs treated with the NADPH-oxidase inhibitor DPI (10μM) were stimulated with this A. nidulans strain. Overexpression of GAG in the A. nidulans cell wall resulted in a significant (P<0.01) decrease of IL-1β release mimicking the A. fumigatus profile of cytokine induction. The decrease of IL-1β release was more pronounced in the absence of a functional NADPH-oxidase complex as compared with healthy PMBCs (Fig. 5A). Importantly, the differences in induction of IL-1β secretion were not likely due to differences in β-glucan exposure, as immunofluorescence studies using recombinant

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**FIG. 5.** (A) The IL-1β release of healthy PMBCs (white bars) and PMBCs treated with the NADPH-oxidase inhibitor DPI and stimulation with live A. nidulans strains, live A. nidulans strains overexpressing A. fumigatus uge3 (A. nidulans + uge3), and A. fumigatus. Bars represent mean ± SEM (n = 6). **P < 0.01. The IL-1β (B) and IL-1Ra (C) release of healthy PMBCs (white bars) and CGD PMBC with live A. nidulans strains and A. nidulans strains overexpressing A. fumigatus uge3 (A. nidulans + uge 3). Bars represent mean ± SEM (CGD, n = 2).
Fc-Dectin-1 demonstrated no difference in β-glucan exposure between these strains (Fig. 6).

Recently, the anti-inflammatory property of GAG has been linked to the ability of soluble GAG-related induction of interleukin-1 receptor antagonist (IL-1Ra) (Liu and Nizet 2009). Consistent with these reports, cell wall polysaccharides isolated from *A. fumigatus* stimulated higher levels of IL-1Ra release by PBMCs than did the cell wall polysaccharides isolated from *A. nidulans*. This increase in IL-1Ra release was seen with PBMCs from both healthy controls (*P* < 0.05 compared with *A. nidulans* cell wall polysaccharides) as CGD patients (n.s). Surprisingly, however, overexpression of GAG by uge3-complemented *A. nidulans* strains did not result in increased IL-1Ra induction by healthy PBMCs or NADPH-oxidase-defective PBMCs isolated from CGD patients (Fig. 5C).

Collectively, these results support our hypothesis that the low levels of GAG in the *A. nidulans* cell wall lead to the dysregulated inflammation by CGD PBMCs upon interaction with *A. nidulans* and to increased IL-1β release. Furthermore, these differences in IL-1β release are not the result of GAG-mediated induction of IL-1Ra secretion.

**Discussion**

The results of this study demonstrate that CGD patients are characterized by a state of hyperinflammation, as shown *in vitro* by specific pattern recognition receptor (PRR) stimulation, and *in vivo* by increased concentrations of plasma proinflammatory cytokines in the absence of infection. *A. nidulans* enhances the inflammation in the CGD patient to a greater extent than *A. fumigatus* as illustrated by
a significantly higher release of IL-1β. Furthermore, we observed that differences in cell wall GAG content underlies the differences in the inflammatory response of CGD PBMCs to these species and may contribute to the unique pathogenicity of *A. nidulans* in the CGD host.

Hyperinflammation in the CGD patient is a clinically well-recognized phenomenon characterized by prolonged inflammatory reactions and granulomata formation (Schäppi and others 2008; Kuipers and Lutter 2012; Rieber and others 2012). The exaggerated cytokine response to *Aspergillus* as observed in our study is consistent with this phenomenon. Furthermore, elevated cytokine levels were found in the plasma of young CGD patients in the absence of clinically apparent infections or other inflammatory complications. To the best of our knowledge, this finding has not been described, and underscores that CGD patients display intrinsic dysregulated inflammation even in the absence of infection (Segal and others 2012).

The mechanism underlying the hyperinflammatory response of CGD PBMCs to *Aspergillus* species remains unknown, although there are multiple possible explanations for these observations. Differences in PRR expression between healthy and CGD cells might influence the observed cytokine profiles. Innate immune receptors, such as toll-like receptors (TLRs) and complement receptors, are important in orchestrating the host defense and may be modulated by pathogens during the course of infection (Chai and others 2009). PMN from CGD patients show lower expression levels of TLR5, TLR9, CD11b, CD18, CD35, and CXCR1 compared with those from healthy controls (Hartl and others 2008). The distribution and expression of Dectin-1 and the mannose-receptor on the CGD phagocyte is as yet unknown.

Whether differences in immune receptor expression and regulation or modulation during invasive fungal infections in CGD patients are relevant for the observed cytokine profiles between both species, and as such the unique fungal epidemiology in the CGD host has still to be elucidated.

An alternate hypothesis is that the intracellular pathways leading to proinflammatory cytokine release are differentially activated in the absence of a functional NADPH-oxidase. The innate immune response against *A. fumigatus* involves both expression and synthesis of pro-IL-1β, as well as the Syk-induced activation of the NLRP3 inflammasome and caspase-1, allowing processing and secretion of the mature cytokine (Said-Sadier and others 2010). The activity of these pathways in the context of CGD remains unexplored.

Invasive aspergillosis in the CGD host is the result of impaired direct antifungal effector function, as well as defective modulation of inflammation in response to fungal products (Segal and Romani 2009). These two functions of the NADPH-oxidase complex are stimulus dependent and stimulus specific as shown by the unique interaction of *A. nidulans* with the CGD host (Henriet and others 2012). Previous work from our group indicates that killing of *A. nidulans* is largely determined by NADPH-oxidase-independent mechanisms and that *A. nidulans* is more sensitive to extracellular killing by NADPH-oxidase-dependent NET-osis compared with *A. fumigatus* (Henriet and others 2011; Lee and others 2015).

Experiments in CGD mice have demonstrated that *A. nidulans* strains induce excessive inflammation and death from invasive pulmonary aspergillosis (Bignell and others 2005). These results suggest that excessive inflammation may play an important role in the pathogenesis of *A. nidulans* infections in the CGD host. Our data support this hypothesis as we observed that while CGD PBMCs responded with the production of high levels of inflammatory cytokines in response to both *Aspergillus* species, the induction of IL-1β and TNF-α was more dramatic with *A. nidulans* compared with *A. fumigatus*.

The fungal cell wall consists of polysaccharides (eg, galactomannan, chitin, β(1–3)glucan, α(1–3)glucan), peptides (eg, hydrophobins), and melanin, and harbors many of the fungal pathogen-associated molecular pattern molecules (PAMPs) recognized by host pattern recognition receptors (PPR) (Latgé 2010). Cell wall melanin protects microbial cells from oxidative stress, modulates immune responses, and has been linked to virulence in several human pathogenic fungi (Jacobson 2000; Gómez and Nosanchuk 2003; Mednick and others 2005; Liu and Nizet 2009). Melanin of *A. fumigatus* cloaks conidial PAMPs from recognition by host PRR resulting in impaired cytokine response (Chai and others 2010). Surprisingly, however, we found that the presence or absence of melanin in either *A. fumigatus* or *A. nidulans* did not affect the release of TNF-α and IL-1β by CGD PBMCs.

These findings suggest that the recognition of *A. fumigatus* and *A. nidulans* by CGD PBMCs leading to proinflammatory cytokine release is not influenced by the presence of conidial melanin and suggests that hyphal factors may play a more important role in governing the cytokine response by CGD PBMCs.

In light of these findings we tested the hypothesis that hyphal cell wall polysaccharides play a key role in inducing inflammatory cytokine release by CGD PBMCs. Consistent with this hypothesis, exposure of PBMCs to hyphal cell wall polysaccharide preparations resulted in the induction of proinflammatory cytokine release. Both live organisms and cell wall polysaccharides induced higher levels of IL-1β secretion by CGD PBMCs as compared with healthy cells. Additionally, stimulation with cell wall polysaccharides isolated from *A. nidulans* resulted in the secretion of higher levels of this cytokine in both types of PBMCs, suggesting that differences in cell wall polysaccharide composition may contribute to the hyperinflammatory response of PBMCs to *A. nidulans*.

Recent studies have found that the cell wall of *A. nidulans* contains low levels of GAG, a heteropolysaccharide composed of α1,4-linked galactose and N-acetylgalactosamine. To test if the higher levels of IL-1β release by PBMCs could be related to this low level of GAG, we utilized strains of *A. nidulans* engineered to express cell wall-associated GAG to levels similar to *A. fumigatus*. Increasing the cell wall GAG content of *A. nidulans* resulted in similar levels of IL-1β expression by PBMCs to that induced by *A. fumigatus*. Collectively, these data suggest that the low amount of GAG produced by *A. nidulans* underlies the increased secretion of IL-1β by PBMCs.

There are multiple possible mechanisms whereby cell wall GAG mediates the suppression of IL-1β expression by PBMCs. Galactosaminogalactan has been reported to mediate immunosuppression through a number of mechanisms, including masking of cell wall β-glucans, induction of IL-1Ra secretion, and NK-cell-dependent leukocyte apoptosis (Fontaine and others 2011; Gravelat and others 2013; Gresnigt and others 2014). It is unlikely that GAG-mediated cloaking of β-glucans plays a significant role in the differences in cytokine release by PBMCs observed in this study.
as immunofluorescence studies using Fc-Dectin-1 showed no differences in the degree of β-glucan exposure between *A. fumigatus*, *A. nidulans*, and the *A. nidulans* expressing increased amounts of GAG. Similarly, no differences in the degree of cell injury were observed in PBMCs infected with *A. fumigatus* and *A. nidulans*. Although it would be interesting to compare the *A. fumigatus uge3* deletion mutant directly with WT *A. nidulans*, the fact that β-glucans are more exposed in the *A. fumigatus uge3* deletion mutant has been shown to confound the cytokine release (Gravelat and others 2013). The effects of the quantity of GAG contents on cytokine release is, therefore, best studied by using the *A. nidulans* mutant strains in which there is no secondary effect of differences in β-glucan exposure.

Finally, while others have reported that the addition of 10 µg/mL soluble GAG in combination with heat-killed *Aspergillus* isolates increased the stimulation of IL-1Ra release by PBMCs (Liu and Nizet 2009), overexpression of *Aspergillus* in the CGD host. Low amounts of GAG in the dysregulated inflammation during invasive aspergillosis in the CGD host remains unclear and need to be further elucidated. GAG has been shown to protects CGD mice from experimental colitis (Liu and Nizet 2009), and the possibility that soluble GAG may be beneficial for invasive aspergillosis by *A. nidulans* will be the subject of future studies.

In conclusion, we found that the unique polysaccharide wall composition of *A. nidulans* contributes to the dysregulated inflammation during invasive aspergillosis in the CGD host. Low amounts of GAG in the *A. nidulans* cell wall seem to result in enhanced pathogenesis of invasive infections as observed in the CGD host. Further studies are urgently needed to unravel the specific interaction of the fungal cell wall components with their corresponding PRR on the various immune cells from specific hosts.

**Author Disclosure Statement**

No competing financial interests exist.

**References**


as immunofluorescence studies using Fc-Dectin-1 showed no differences in the degree of β-glucan exposure between *A. fumigatus*, *A. nidulans*, and the *A. nidulans* expressing increased amounts of GAG. Similarly, no differences in the degree of cell injury were observed in PBMCs infected with *A. fumigatus* and *A. nidulans*. Although it would be interesting to compare the *A. fumigatus uge3* deletion mutant directly with WT *A. nidulans*, the fact that β-glucans are more exposed in the *A. fumigatus uge3* deletion mutant has been shown to confound the cytokine release (Gravelat and others 2013). The effects of the quantity of GAG contents on cytokine release is, therefore, best studied by using the *A. nidulans* mutant strains in which there is no secondary effect of differences in β-glucan exposure.

Finally, while others have reported that the addition of 10 µg/mL soluble GAG in combination with heat-killed *Aspergillus* isolates increased the stimulation of IL-1Ra release by PBMCs (Liu and Nizet 2009), overexpression of GAG in *A. nidulans* did not result in increased induction of IL-1Ra secretion by PBMCs. Only by using isolated cell wall polysaccharides from both *A. fumigatus* and *A. nidulans*, we were able to observe differences in the amounts of IL-1Ra secretion by PBMCs. It is possible that differences in the final concentrations achieved by fungal shedding of soluble GAG versus the addition of purified carbohydrate, and/or differences in the activities of the extracted soluble GAG alone versus native GAG may explain the observed differences in PBMC response.

The exact immunomodulating mechanisms of soluble GAG and cell wall-associated GAG in the fungal pathogenesis, in particular, the CGD host remains unclear and need to be further elucidated. GAG has been shown to protect CGD mice from experimental colitis (Liu and Nizet 2009), and the possibility that soluble GAG may be beneficial to dampen the hyperinflammation *in vivo* during invasive aspergillosis by *A. nidulans* will be the subject of future studies.

In conclusion, we found that the unique polysaccharide cell wall composition of *A. nidulans* contributes to the dysregulated inflammation during invasive aspergillosis in the CGD host. Low amounts of GAG in the *A. nidulans* cell wall seem to result in enhanced pathogenesis of invasive infections as observed in the CGD host. Further studies are urgently needed to unravel the specific interaction of the fungal cell wall components with their corresponding PRR on the various immune cells from specific hosts.

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