Decreased cell wall galactosaminogalactan in Aspergillus nidulans mediates dysregulated inflammation in the CGD host.

Running title: Dysregulated inflammation by A. nidulans in CGD

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ABSTRACT

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 hyper-inflammatory 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 pro-inflammatory 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.
Chronic granulomatous disease (CGD) is a rare primary immunodeficiency (1:125,000) that is associated with an intrinsic susceptibility to invasive aspergillosis (1). 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: antimicrobial killing and the regulation of inflammation (2). 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 (3, 4). 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* (4).

To date, the immune mechanisms underlying the pathogenesis of invasive *A. nidulans* infections remain poorly understood. Importantly, PBMCs from CGD patients produce higher levels of the pro-inflammatory cytokines interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) upon stimulation with *Aspergillus* species, while IL-17A production is strikingly low compared to healthy controls (5). 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 (6). 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 (7). 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 (8, 33), which has recently been found to mediate immunosuppression during infection with *A. fumigatus* (9, 10). In addition to cell wall polysaccharides, conidial melanin of *A. fumigatus*, i.e. 1,8-dihydroxy naphthalene (DHN), is another important fungal cell-wall structure that influences the immune response to fungi (11, 12). *A. nidulans* produces melanin of the 3,4-dihydroxyphenylalanine (DOPA)-type rather than the DHN-type found in *A. fumigatus* (13). 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 to *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 supplemental table 1 (supplemental data). Blood was collected by venepuncture into 10 mL ethylenediaminetetraacetic acid (EDTA) tubes (367525, BD, Plymouth, UK). PBMCs were isolated using lymphoprep (Axis-Shield), by density gradient centrifugation as previously described (6). 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 x g to separate the plasma from the PBMC fraction and the PMN. PBMCs were harvested and counted by hemocytometer. For the stimulation assays, 5 x 10^5 P BMCS in a total volume of 200 μL per well were incubated at 37°C and 5% CO2 in round-bottom 96-wells plates (Nunc, Roskilde, Denmark) with either 10 ng/ml LPS (derived from *E. coli* serotype 055:B5, Sigma-Aldrich St. Louis), Pam3Cys (10
μg/ml), heat-killed *C. albicans*, live or heat killed (HK) *A. fumigatus* or *A. nidulans* in specified concentrations. An extra purification step of LPS was performed before use (14). RPMI 1640 GlutaMAX-I medium (Invitrogen Life Technologies) + 10% heat inactivated human serum (Sigma, H6914) was used as culture medium (CM). After 24 hours, 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 hours of co-incubation compared to 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 RGD-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, USA). The *alb1* gene codes for a polyketide synthase (pksP) in the 1,8-dihydroynaphthalene (DHN)-melanin pathway, involved in the biosynthesis of conidial pigment (15, 16). The *A. nidulans* A191 strain, was obtained by deletion of the *wa* gene, resulting in white conidia (17, 18) (Δwa, kind gift of K.J. Kwon-Chung, NIH, Bethesda, USA).

The *A. nidulans* strains overexpressing heterologous *uge3* (derived from *A. fumigatus*), and necessary for the production of GAG in the cell-wall, was constructed as previously described (19, Lee et al., submitted). These strains were grown on minimal media supplemented with biotin (50 μL per 1L 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 to 7 days at 37°C and subsequently plated on a 1:10 diluted Sabouraud agar. Conidia were harvested, filtered and washed as previously described (6). They were stored in individual aliquots of 1 x 108/ml at -80°C. To obtain heat killed (HK) conidia, the conidial suspension was heat killed for 15 min at 121°C. The growth and killing of the *Aspergillus* isolates was carried out in a LPS-free fashion. *Candida albicans* ATCC MYA-3573 (UC 820) (20) was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium. *C. albicans* was heat-killed for one
hour 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 (21). In brief, conidia were enzymatically lysed to form protoplasts. The protoplasts were incubated with the chaotropic agent guanidine thiocyanate (4.0 M) to generate dark particles, which were treated with proteinase K to remove residual proteins. The pellet was incubated at 100°C in 6.0 M HCl for 1 h to obtain pure melanin. Melanin concentration was quantified by weighing the dried mass (22). The melanins were suspended in PBS at a stock concentration of 10 mg/ml, and sonicated to obtain a homogeneous solution.

**Isolated cell wall sugars**

Mycelial cell wall sugars of both *A. nidulans* (FGSC strain A28) and *A. fumigatus* (Clinical strain A237) were isolated as previously described (8). In summary, both species were cultured in liquid media for 48hrs and the mycelia were obtained by gravity filtration and vacuum filtration. The mycelia were washed several times to remove traces of media and mechanically homogenized in chilled Tris/EDTA buffer and lyophilized. The lyophilized cell wall sugars were suspended in sterile and endotoxin free deionized water (Braun, Germany) at a stock concentration of 2.5 mg/ml, and sonicated to obtain a homogeneous solution. Further dilutions were made in CM.

**Enzyme-linked immunosorbant assay**

The concentration of IL-10, IL-1β, IL-1Ra and TNF-α was measured in cell culture supernatants using enzyme-linked immunosorbant assay (ELISA) (IL-1β and IL-1Ra: R&D Systems, Abingdon, UK, and IL-10 and TNF-α: Sanquin, Amsterdam, the Netherlands), according to the instructions of the manufacturer.

**Fc-Dectin-1 staining**

Surface exposure of β-1,3-glucan was measured by immunostaining with an Fc-dectin-1 recombinant construct, as previously described (10). Briefly, 1x105 conidia of indicated strains were grown on glass coverslips in 24 well plates for 12 hours then fixed with 4% paraformaldehyde in PBS. The fixed samples were blocked with 3% bovine serum albumin supplemented with 0.2% sodium azide in PBS,
and labelled with 10 μg/mL of Fc-dectin-1 (23) followed by FITC-labeled AffiniPure F(ab’)-fragment donkey anti-human IgG, FCγ fragment specific (Jackson ImmunoResearch). Stained hyphae were imaged using confocal microscopy (IX81, Olympus), excitation 488 nm and emission 519 nm.

Statistical analysis. Experiments were performed in duplicate, the mean value of this duplicate was taken as the value for the particular donor. The differences between groups were analyzed using the Mann-Whitney U test. P values of < 0.05 were considered statistically significant. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

CGD patients exhibit increased baseline levels of inflammatory cytokines

One hallmark of CGD is increased inflammation. To analyse the basal levels of pro-inflammatory cytokines in CGD patients, plasma was isolated from 4 paediatric 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 versus 8.0 ± 0.1 pg/ml in healthy controls) and TNF-α (124.2 ± 47.3 pg/ml versus 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 hyper-inflammatory 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) (Figure 1A). PBMCs isolated from these CGD patients also produced an exaggerated pro-inflammatory response upon ex-vivo stimulation. Compared with PBMCs isolated from healthy controls, CGD PMBCs 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.) (Figure 1B, 1C). 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 (Figure 1D).
Aspergillus nidulans induces higher levels of pro-inflammatory cytokine production than A. fumigatus in CGD cells

Co-culture 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 to healthy PBMCs (Figure 2A, 2B). This induction of pro-inflammatory 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 to healthy PBMCs (p<0.01 and p<0.05 respectively) (Figure 2A, 2B). 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 pg/ml to 162 pg/ml). A. nidulans induced higher levels of IL-10 secretion compared to A. fumigatus and this difference was significant when heat-killed conidia were used (Figure 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 pro-inflammatory state of the CGD PBMCs is increased by both Aspergillus species as compared to 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 pro-inflammatory cytokine release from healthy PBMCs than do albino conidia (12). In agreement with this report, we found that healthy PBMCs secreted significantly increased levels of the pro-inflammatory 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 to wild-type (WT) A. fumigatus conidia. A similar trend towards increased TNF-α and IL-1β secretion
by healthy PBMCs in response to albino as compared to WT conidia was seen in \textit{A. nidulans}, however this difference was not statistically significant (Figure 3A).

**Presence of melanin does not influence the pro-inflammatory response of the CGD PBMCs upon fungal stimulation**

In order to assess the role of \textit{A. nidulans} conidial melanin in the hyper-inflammation observed in CGD PBMCs, these cells were stimulated with either WT or albino live \textit{A. nidulans} and \textit{A. fumigatus} conidia. No differences in IL-1\(\beta\) and TNF-\(\alpha\) release by CGD PBMCs were observed in response to WT or albino \textit{A. nidulans} or between WT and albino \textit{A. fumigatus} (Figure 3B). Thus, conidial melanin does not play a significant role in modulating the release of pro-inflammatory cytokines by CGD cells in response to either species of \textit{Aspergillus}.

**Melanin isolated from \textit{A. nidulans} or \textit{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 \textit{C. neoformans} and \textit{A. fumigatus} (12, 24) we next compared the immunogenic properties of isolated \textit{A. nidulans} and \textit{A. fumigatus} conidial melanin. Isolated melanin of both \textit{Aspergillus} spp. in concentrations up to 1 mg/ml were extremely poor stimulators of pro-inflammatory cytokine release by both healthy and CGD PBMCs as indicated by the lack of significant IL-1\(\beta\) and TNF-\(\alpha\) release in the supernatant (data not shown). Isolated melanins from both \textit{Aspergillus} species were not able to modulate the IL-1\(\beta\) and TNF-\(\alpha\) release by both healthy and CGD PBMCs stimulated with LPS (data not shown) or the albino \textit{A. fumigatus} and \textit{A. nidulans} conidia (Figure 3C).

**\textit{A. nidulans} cell wall polysaccharides are responsible for the enhanced IL-1\(\beta\) release by CGD PBMCs**

We next questioned whether differences in polysaccharide cell wall composition between \textit{A. fumigatus} and \textit{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 μg/ml - 25 μg/ml) from *A. nidulans* and *A. fumigatus* by CGD and healthy PBMCs (Figure 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 PBMC’s, a dose-response relationship in response to both cell wall polysaccharides was observed for IL-1β and TNF-α, while IL-10 secretion was minimal. Cell wall polysaccharides of *A. nidulans* induced higher levels of IL-1β secretion as compared to *A. fumigatus* cell wall polysaccharides both by healthy and CGD PBMCs, this difference was not observed for the induction of TNF-α and IL-10.

Comparing the cytokine responses between CGD and healthy PBMCs, the CGD PBMCs secreted substantial higher levels of IL-1β than the healthy controls upon stimulation with the individual cell wall polysaccharides (Figure 4G-H). Stimulation by *A. nidulans* cell wall polysaccharides did not lead to differences in TNF-α release by CGD and healthy PBMCs in contrast to *A. fumigatus* cell wall polysaccharides. IL-10 production by healthy PBMCs was minimal while CGD PBMCs 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 was 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 galactosaminogalactan (GAG) in the cell wall of *A. nidulans* could be responsible for the increased IL-1β release by PBMCs 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 et al, submitted]. Therefore to analyse the contribution of GAG to inflammatory cytokine production by PBMCs, *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 PBMCs and PBMCs treated with the NADPH-oxidase inhibitor DPI (10μM) were stimulated
with this \textit{A. nidulans} strain. Overexpression of GAG in the \textit{A. nidulans} cell wall resulted in a
significant (p<0.01) decrease of IL-1\(\beta\) release mimicking the \textit{A. fumigatus} profile of cytokine
induction. The decrease of IL-1\(\beta\) release was more pronounced in the absence of a functional
NADPH-oxidase complex as compared to healthy PBMCs (Figure 5A). Importantly, the differences in
induction of IL-1\(\beta\) secretion were not likely due to differences in \(\beta\)-glucan exposure, as
immunofluorescence studies using recombinant Fc-Dectin-1 demonstrated no difference in \(\beta\)-glucan
exposure between these strains (Figure 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)
(37). Consistent with these reports, cell wall polysaccharides isolated from \textit{A. fumigatus} stimulated
higher levels of IL-1Ra release by PBMCs than did cell wall polysaccharides isolated from \textit{A.
idulans}. This increase in IL-1Ra release was seen with PBMCs from both healthy controls (p < 0.05
compared to \textit{A. nidulans} cell-wall polysaccharides) as CGD patients (n.s). Surprisingly however,
overexpression of GAG by \textit{uge3}-complemented \textit{A. nidulans} strains did not result in increased IL-1Ra
induction by healthy PBMCs or NADPH-oxidase defective PBMCs isolated form CGD patients
(Figure 5B).

Collectively, these results support our hypothesis that the low levels of GAG in the \textit{A. nidulans} cell
wall lead to the dysregulated inflammation by CGD PBMCs upon interaction with \textit{A. nidulans} and t o
increased IL-1\(\beta\) release. Furthermore, these differences in IL-1\(\beta\) 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 hyper-inflammation, as shown in vitro by specific pathogen recognition receptor (PRR) stimulation, and in vivo by increased concentrations of plasma pro-inflammatory 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. Further, 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-recognised phenomenon characterized by prolonged inflammatory reactions and granulomata formation (25-27). The exaggerated cytokine response to *Aspergillus* as observed in our study, are consistent with this phenomenon. Further, 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 (2). 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 like 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 (28). PMN from CGD patients show lower expression levels of TLR5, TLR9, CD11b, CD18, CD35, and CXCR1 compared to those from healthy controls (29). 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 pro-inflammatory cytokine release are differentially activated in the absence of a functional NADPH-oxidase. The innate-immune response against *A. fumigatus* involves both the 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 (30). 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 (31). 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 (32). Previous work from our group indicates that killing
of A. nidulans is largely determined by NAPDH-oxidase independent mechanisms and that A.
nidulans is more sensitive to extracellular killing by NADPH-oxidase dependent NET-osis compared
to A. fumigatus (6, 33). Experiments in CGD mice have demonstrated that A. nidulans strains induce
excessive inflammation and death from invasive pulmonary aspergillosis (34). 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 (e.g. galactomannan, chitin, β(1-3)glucan, α(1-3)
glucan), peptides (e.g. hydrophobins) and melanin, and harbours many of the fungal pathogen
associated molecular pattern molecules (PAMPs) recognized by host pattern recognition receptors
(PRR) (7). Cell wall melanin protects microbial cells from oxidative stress, modulates immune
responses and has been linked to virulence in several human pathogenic fungi (24, 35-37). Melanin of
A. fumigatus cloaks conidial PAMPs from recognition by host PRR resulting in impaired cytokine
response (12). Surprisingly however we found that the presence or absence of melanin in either A.
fumigatus and A. nidulans did not affect the release of TNFα and IL-β by CGD PBMCs. These
findings suggest that the recognition of A. fumigatus and A. nidulans by CGD PBMCs leading to pro-
inflammatory 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 pro-inflammatory cytokine release. Both live organisms and cell wall polysaccharides induced higher levels of IL-1β secretion by CGD PBMCs as compared to 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 (9, 10, 38). 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 (10). 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 (37), 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 needs to be further elucidated. GAG has been shown to protect CGD mice from experimental colitis (37), and the possibility that soluble GAG may be beneficial to dampen the hyperinflammation in vivo during invasive aspergillosis by Aspergillus 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.
REFERENCES


Figure 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 to healthy controls (n=9). TNF-α (B), IL-1β (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 1x10^6/ml C. albicans. Bar represent means + SEM. * p<0.05, ** p< 0.01.
Figure 2 IL-1β, TNF-α and IL-10 cytokine responses of healthy (n=5) and CGD (n=4) PBMC after stimulation by either $5 \times 10^6$/ml heat-killed or live *A. nidulans* and *A. fumigatus* conidia. Bar represent means + SEM. * p<0.05, ** p<0.01.
Figure 3 The cytokine response of (A) human healthy PBMCs and (B) CGD PBMCs to 1x10^6/ml live albino (ALB, grey 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* (ΔwA) (*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) conidial melanin or *A. fumigatus* (AF) conidial melanin. The albino conidial stimulated cytokine release without isolated melanin was set to 100% (n=2). Bar represent means ± SEM. * p=0.05, ** p<0.01.
Figure 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 to CGD PBMCs after stimulation with 12.5ug/ml isolated *A. nidulans* cell wall sugars (G) or *A. fumigatus* cell wall sugars (H). Bars indicate SEM.
Figure 5 (A) The IL-1β release of healthy PBMCs (white bars) and PBMCs treated with the NADPH-oxidase inhibitor DPI and stimulation with live *A. nidulans* strains, live *A. nidulans* strains overexpressing *A. fumigatus uge3* (*A. nidulans* + uge 3), and *A. fumigatus*. Bars represent mean ± SEM (n=6). * p<0.05, ** p<0.01. The IL-1β (B) and IL-1Ra (C) release of healthy PBMCs (white bars) and CGD PBMC with live *A. nidulans* strains and *A. nidulans* strains overexpressing *A. fumigatus uge3* (*A. nidulans* + uge 3). Bars represent mean ± SEM (CGD, n=2).
Figure 6 Detection of β-1,3-glucan exposure on the hyphal surface of *A. fumigatus*, *A. nidulans*, *A. nidulans* overexpressing *A. fumigatus* uge3 (comparable amounts of galactosaminogalactan as *A. fumigatus*) and as a control Δuge3 *A. fumigatus* (no galactosaminogalactan on the outer cell wall) by immunostaining with Fc-dectin-1 antibody by fluorometry.