Aspergillus nidulans and Chronic Granulomatous Disease
A unique interaction

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General introduction and outline of the thesis
Infectious diseases are the result of a tight interplay between microbial characteristics and host response. Whether this interaction results in microbial clearance, colonization or disease, defined as host damage, depends on both the virulence traits of the pathogen and the capacity of the host to respond in an appropriate manner (1). The filamentous fungus *Aspergillus* rarely causes disease in healthy immune competent hosts. The host defense against *Aspergillus* relies on the ability of the immune system to restrict spore germination and to prevent invasive hyphal growth, thereby limiting fungus-induced and/or inflammation-induced damage in infected tissues.

Consequently, the development of invasive disease caused by *Aspergillus* species points out a specific defect in the host defense. These specific defects can be caused either by an inherited inborn error of immunity (primary immunodeficiency) or by an external predisposing factor interfering with a normal function of the immune system (secondary immunodeficiency). Chronic granulomatous disease (CGD) is a primary immunodeficiency of the phagocytic cell characterized by the highest lifetime incidence of invasive aspergillosis among all cases of immuno-compromised patients (2). Chronic granulomatous disease is a well-described disease in terms of the functional immune defect as well as the underlying gene mutations. In its response, the CGD host offers a genuine ‘experiment of nature’, highlighting the importance of a specific component of the immune system in human antifungal immunity.

**CHRONIC GRANULOMATOUS DISEASE**

Chronic granulomatous disease is a genetically heterogeneous disease caused by a defect in any of the five structural components of the NADPH-oxidase complex including the granule or plasma membrane-bound glycoprotein gp91phox (phagocyte oxidase) and p22phox, and the cytoplasmatic components p47phox, p67phox and p40phox (3). Up to now, 5 genes are responsible for all known cases of CGD: the X-linked inherited CYBB, and the autosomal recessive CYBA, NCF1, NCF2 and NCF4, respectively (4). Birth prevalence ranges from 1/450,000 in Sweden up to 1/120,000 in the United Kingdom and Ireland (5-7). Most of the patients are diagnosed in early childhood; however, some patients remain undiagnosed until adolescence. The presenting symptom of the underlying primary immunodeficiency is often a localized invasive infection caused either by a relatively narrow spectrum of bacteria (e.g. *Staphylococcus aureus*, *Burkholderia cepacia*, *Serratia marcescens*, *Salmonella species*) or by fungi (*Aspergillus* species and other molds). In addition to the recurrent life-threatening bacterial and fungal infections, patients suffering from CGD are characterized by abnormal exuberant inflammatory responses leading to colitis.
and granuloma formation in liver, spleen, lung and lymph nodes (6)(9). These clinical manifestations are either a direct or indirect consequence of the defect in the NADPH-oxidase complex in phagocytes (12). Activation of the phagocytic NADPH-oxidase complex results in the production of the reactive oxidant superoxide anion and the downstream reactive oxygen species (ROS) hydrogen peroxide (H2O2), hydroxyl anion (HO·) and hypochlorous acid (HOCl). The NADPH oxidase complex mediates host defense via direct antimicrobial activity of the ROS, but is also coupled to intracellular and extracellular release of a variety of antimicrobial proteases. The lack of ROS leads to an impaired alkalization of the phagolysosomes and interferes with the release and activation of granule-derived enzymes within the phagosome impairing microbial killing (8). Neutrophil NADPH oxidase activation is furthermore linked to the formation of neutrophil extracellular traps (NETs) able to target and kill bacteria and fungi (9, 10).

In addition to the anti-microbial effects, NADPH-oxidase has shown to be essential in dampening the inflammation. Its absence leads to both an increased neutrophil influx at sites of infection driven by increased IL-8 production, and a diminished apoptosis and clearance of those neutrophils resulting in uncontrolled inflammation (11, 12). In addition, NADPH-oxidase is involved in the modulation of redox-sensitive transcription factors such as NF-κB, and leads to a dysregulated pro-inflammatory cytokine profile when absent.

Overall, the NADPH-oxidase complex is a critical regulator in the innate immunity and acts as anti-microbial effector complex as well as an important regulator of inflammation (Figure 1).

**INVASIVE ASPERGILLOSIS**

*Aspergillus* spp. are known to cause invasive diseases primarily in immunocompromised patients with quantitative or qualitative neutrophil defects. The *Aspergilli* are a ubiquitous group of over 185 filamentous fungi belonging to the Phylum Ascomycota. *Aspergillus* spp. are saprophytic fungi abundantly present in soil and decaying plant material. Their conidia are widely dispersed in the air and inhalation is the first event in establishing invasive disease by immunocompromised patients. One of the most common etiological agents in human invasive aspergillosis is *Aspergillus fumigatus*, being responsible for approximately 90% of all human filamentous fungal infections (2, 13), followed by *A. flavus*, *A. terreus* and *A. niger*. Much less attention had been given to *A. nidulans* as an opportunistic pathogen in humans until recent years, when it was recognized especially in patients with CGD. Scrutinizing the microbiology database of CGD at the National Institutes of Health (NIH; Bethesda, MD) revealed six *A. nidulans* infections compared to seventeen caused by *A. fumigatus*. In patients with fungal osteomyelitis, *A. nidulans* has been isolated in up to 50% of the cases (14, 15). In an extensive review of all PubMed database notated papers from 1970 to 2010 relating to invasive fungal infections in patients suffering from CGD, *A. nidulans* was the second most encountered species (23 cases out of the 116; 18%) only preceded by *A. fumigatus* (44 cases, 35%) (16). *Aspergillus nidulans* seems to have a remarkable unique interaction with CGD patients, as it remains a rare pathogen among other risk groups. In the Transplant-Associated Infections Surveillance Network (TRANSNET) report, *A. fumigatus* was isolated in 187 (44%) of the 425 cases of IA. No notification of *A. nidulans* was made (17). In a cohort of 139 cases of pediatric IA, including seven CGD patients, *A. fumigatus* was isolated in 67 cases (52.8%) compared to *A. nidulans* in only 1 patient (0.8%) (18).

Those clinical observations and the published epidemiological data suggest that CGD patients are at greater risk to develop *A. nidulans* infection than other immunocompromised patient populations and that *A. nidulans* is more virulent than *A. fumigatus* based on mortality rates and propensity to spread (15). As disease pathology and progression are the results of the complex interaction between the pathogen and the host, this striking observation of the unique epidemiology and clinical features in CGD patients was the starting point of the research presented in this thesis.

*Aspergillus nidulans*

Microscopically, *Aspergillus* spp. are characterized by the presence of a narrow, dichotomously branched septated hyphae and a conidiophore composed of a foot.
cell ending into a vesicle. Identification of the different Aspergillus spp. is based predominantly upon the morphology of these conidiophores with differences in conidial heads, phialides, color, shape and morphology of the conidia (Figure 2). Aspergillus nidulans is characterized by a very evident foot cell with short conidial heads. The conidiogenous cells are biseriate, with metulae and phialides covering the upper half of the vesicle. The conidia are spherical, green in mass and 3-4 µm in diameter. Colonies are often brown due to the conidiophores and the presence of cleistothecia and Hulle cells. In contrast, Aspergillus fumigatus has short hyaline or green conidiophores with a pear- or club-shaped vesicle. The conidiophore is uniseriate, with often green phialides, originating directly on the upper half of the vesicle. The conidial heads have a columnar disposition. The conidia are globose (2-3.5 µm), green, and can be either smooth or rough or echinulate, produced in long, basipetal chains.

Unfortunately, this morphology-based identification of clinically relevant aspergilli has often led to misidentification. In an era of increasing antifungal resistance and multiple classes of antifungal agents, it is crucial to correctly identify the causative fungi to species level as it is of utmost importance to tailor therapy appropriately. Use of comparative sequence-based methods in conjunction with the traditional phenotype-based methods will offer increased resolution in identification of these Aspergillus species. Recently, the taxonomy of Aspergillus spp. has been revised by incorporating sequence-based information. Following the recommendations of the international group of experts gathered for a workshop “Aspergillus Systematics in the Genomic Era”, sequence analyses of the internal transcribed spacer (ITS) region appears to be appropriate for identification of Aspergillus isolates to the subgenus/section level. The Partial β-tubulin or calmodulin are the most promising loci for Aspergillus identification to the species level (19). The use of molecular techniques for species identification has already impacted on the epidemiology of fungal diseases in CGD patients (20), and will definitely increase our insight into specific pathogens and host-pathogen interactions in the near future.

RESEARCH QUESTIONS

The aim of the studies presented in this thesis is to explore in depth the host-pathogen interaction between A. nidulans and patients suffering from CGD. We question whether the higher incidence of A. nidulans infections in the CGD patients may be explained by the presence of specific defects in the innate immune system, having more consequences for an efficient host defense against A. nidulans compared to A. fumigatus. We want to get more insight in the role of the deficient NADPH-oxidase complex as direct and indirect anti-fungal effector complex against A. nidulans and A. fumigatus. We aim to identify the role of the dysregulated inflammation in the pathogenesis of invasive Aspergillus infections in the CGD host and question whether this inflammatory response is Aspergillus-species dependent. And ultimately, we investigate to which extent an improved insight in the molecular interaction between Aspergillus species and the CGD host may result in the design of new management strategies.

OUTLINE OF THIS THESIS

In chapter 2 a comprehensive overview of all published invasive fungal infections in patients suffering from chronic granulomatous disease is presented. To investigate whether the current concepts of the increased susceptibility to A. nidulans infections are related to the direct antimicrobial effect of the NADPH-oxidase complex, which is defective in CGD patients, we investigate the susceptibility of A. nidulans and A. fumigatus to ROS, the ability of those two species to activate the production of ROS in phagocytes, and we determine antifungal killing by various, both human and murine, CGD cell populations. The results of these studies are described in chapter 3.

In chapter 4 the function of the NADPH-oxidase and its role in intracellular pathways is investigated with respect to the tryptophan metabolism by which tryptophan is converted to L-kynurenicine and its relationship to the Th17 response. The results of the studies presented in chapters 3 and 4 support the hypothesis that the occurrence of A. nidulans infections in the CGD host is possibly more related to the dysregulated inflammation causing damage to the host, than to an intrinsic impaired fungal killing ability of the CGD phagocyte. NADPH-oxidase is
well known to play a key role in modulating inflammation that is distinct from its antimicrobial function. Chapter 5 summarizes our current insights with respect to the interaction of \textit{A. nidulans} and the CGD host, leading to a new hypothesis concerning the pathophysiology of invasive \textit{A. nidulans} infections in the CGD patient.

In chapter 6 the results are described of the studies performed to assess if specific \textit{A. nidulans} fungal cell wall components might be responsible for the extent of inflammation, playing a differential role in the observed clinical pathogenesis of \textit{A. nidulans} infections in the CGD patient.

In the search for new management strategies to improve the outcome of invasive \textit{A. nidulans} infections in the CGD patient, we hypothesize that an adjunctive therapeutic agent should target both functions of the NADPH-oxidase. The ideal agent should be characterized by having both an antifungal and an immuno-modulatory effect, and by being active in the CGD phagolysosome known to display an abnormal pH regulation. Based on this consideration, we explore in chapter 6 a new application of the ‘old drug’ chloroquine.

Finally, all the studies presented in this thesis are discussed and framed into future perspectives in chapter 7.

REFERENCES


Invasive fungal infections in patients with chronic granulomatous disease

S. Henriet, P.E. Verweij, S.M. Holland, A. Warris

Invasive fungal infections are a major threat for chronic granulomatous disease (CGD) patients. The present study provides a comprehensive overview of published invasive fungal infections in the CGD host through an extensive review of epidemiological, clinical, diagnostic and therapeutic data. In addition to the often mild clinical presentation, the currently used diagnostics of invasive aspergillosis have low sensitivity in CGD patients and cannot be easily translated to this non-neutropenic host. *Aspergillus fumigatus* and *A. nidulans* are the most commonly isolated species. *A. nidulans* infections are seldom reported in other immunocompromised patients, indicating a unique interaction between this fungus and the CGD host. The occurrence of mucormycosis is mainly noted in the setting of treatment of inflammatory complications with immunosuppressive drugs. *Candida* infections are infrequently seen and do not cause mucocutaneous disease but do show an age-dependent clinical presentation. The CGD patient is susceptible to a wide range of fungal pathogens, indicating the need to determine the causative fungus, often by invasive diagnostics, to guide optimal and rational treatment. This review summarizes current understanding of invasive fungal infections in patients with CGD and will serve as a starting point to guide optimal treatment strategies and to direct further research aimed at improving outcomes.

**INTRODUCTION**

Patients suffering from chronic granulomatous disease (CGD) are well known to be prone to invasive fungal infection, thereby providing a unique opportunity to examine host susceptibility to fungal invasion within the framework of a well-defined immune defect. CGD is a rare inherited disorder of the NADPH oxidase in which phagocytes fail to generate the microbicidal reactive oxidant superoxide anion and its metabolites, hydrogen peroxide, hydroxyl anion, and hypohalous acid. Clinically, as a result of the defect in this key innate host defense pathway, CGD patients suffer from recurrent life-threatening bacterial and fungal infections and inflammatory sequelae. The molecular basis of CGD is well understood: CGD is a genetically heterogeneous disease caused by mutations in any of the five structural components of NADPH oxidase, including the membrane-bound glycoprotein p91<sub>phox</sub> (phagocyte oxidase) and p22<sub>phox</sub> and the cytoplasmic components p40<sub>phox</sub>, p47<sub>phox</sub> and p67<sub>phox</sub>. Birth prevalence ranges from 1/450,000 in Sweden, 1/250,000 in the United States and Japan, up to 1/120,000 in the United Kingdom and Ireland (1-4). Although the majority of CGD patients are diagnosed in early infancy, fungal infection as presenting symptom in adult patients has been reported (5-9). The suspicion of the presence of CGD as an underlying condition should arise whenever the diagnosis of invasive aspergillosis (IA) is made in the absence of another known risk factor.

*Aspergillus* is one of the most important encountered pathogens (10, 11), and the life-time incidence of IA in children with CGD before the advent of specific antifungal prophylaxis varied between 25 % and 40 % (11, 12); although since the introduction of azole prophylaxis the incidence seems to decrease (13). In comparison, the incidence of aspergillosis in children undergoing allogeneic hematopoietic stem cell transplantation (HSCT) or who were treated for hematological malignancy, who typically have a very limited period of susceptibility, lies between 2 % and 6.8% (13-17).

In more detailed information extracted from the published data of CGD patient registries, the percentage of patients who had at least one infectious episode caused by *Aspergillus* spp. ranges from 26 % in Europe (18) up to 46 % in Japan (19). More importantly, independent of the epidemiology of fungal infections in the published data, in the cases of pulmonary infection, brain abscesses and osteomyelitis, *Aspergillus* spp. have been the most commonly isolated organisms (1, 4, 18, 20) (Table 1).

The most common *Aspergillus* spp. affecting CGD patients is *A. fumigatus* (10, 21, 22) followed by *A. nidulans* (23). Much less attention has been paid to *A. nidulans* as an opportunistic pathogen in humans until recent years, when it was recognized especially in patients with CGD or with histories suggestive of CGD. White et al.
(24) suggested in 1988 that CGD patients were at greater risk for *A. nidulans* than other immunocompromised patient populations. Scrutiny of the microbiology database of CGD patients at the National Institute of Health (NIH, Bethesda, MD) over a 10-year-period revealed six cases of *A. nidulans*, compared to 17 cases of *A. fumigatus* (23). In cases of osteomyelitis, *A. nidulans* was isolated in up to 50% of the cases (23, 25). Although *A. nidulans* behaves more virulently in CGD patients than *A. fumigatus*, solid information on the relative frequency of IA due to *A. nidulans* in CGD patients is lacking. *Candida* is a less frequently encountered pathogen in CGD and is reported as being responsible for invasive fungal infection in 6% of all isolated microorganisms among CGD patients registered in the European database, in 10% in a Swedish CGD population, and up to 14% in the Italian CGD study (3, 18, 20).

Invasive mucormycosis has not been recognized in patients with CGD as frequently as IA (26, 27). A review of 929 pediatric and adult cases of mucormycosis revealed 81% of the individuals having an impaired immune status, but none of these were associated with CGD (28). In a systematic review of 157 cases of mucormycosis in children, 86% were associated with risk factors as neutropenia, prematurity, diabetes mellitus and ketoacidosis; but no underlying primary immunodeficiency was noted (29). However, when CGD patients are subjected to further immune compromising conditions affecting T cell function, such as methotrexate or steroids, typically in the setting of management of their inflammatory complications, mucormycosis has been encountered (30).

Globally, specific data regarding the epidemiology of non-*Aspergillus*, non-*Candida* IFD in CGD patients are scarce or described as “Fungi: not identified”. Little detailed information is given on the identification of the causative fungus, and available data are almost exclusively found as individual case reports, making it difficult to assess any epidemiological burden of those fungi within the CGD population.

As a cause of death, fungal infections caused by *Aspergillus* spp. have been responsible for one-third to half of all deaths in CGD patients (2, 4, 20). Mortality rates due to IFD caused by other fungi are unknown but, since case reports are more common with severe infections, may be overestimated from their true incidence.

Overall, as disease pathology and progression are the results of the complex interaction between the fungal pathogen and the host defense, exploring these host-fungus interfaces of the different fungal pathogens in relation to the host, gives us insight and detailed understanding of this challenging frontline, in order to optimize diagnostic and therapeutic tools.

We reviewed the literature of invasive fungal infections in CGD through topics such as epidemiological data, mycological characterization and identification, clinical manifestations, diagnostic features and in vitro susceptibility data in order to assess these findings within a framework of our current understanding of IFD in CGD.

<table>
<thead>
<tr>
<th>Geographical region (ref.)</th>
<th>No. of patients</th>
<th>Aspergillus infections</th>
<th>Lung</th>
<th>Skin</th>
<th>Liver</th>
<th>Brain</th>
<th>Bone</th>
<th>Septicemia</th>
<th>Death (Aspergillus)</th>
<th>AF prophylaxis</th>
<th>Death (AF prophylaxis)</th>
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<tbody>
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<td>Europe (18)</td>
<td>409</td>
<td>26</td>
<td>61</td>
<td>5</td>
<td>16</td>
<td>2</td>
<td>16</td>
<td>7</td>
<td>53</td>
<td>28</td>
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<td>78</td>
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<td>4</td>
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<td>12</td>
<td>10</td>
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<td>85</td>
<td>NA</td>
<td>0</td>
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<td>0</td>
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<td>Italy (20)</td>
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<td>24</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>NA</td>
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<td>0</td>
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</tbody>
</table>

AF, antifungal; NA, not available.
in CGD patients with a positive culture from a specimen obtained by an invasive procedure showing histological evidence of growing fungi. Cases were considered probable if radiological features were considered to be consistent with fungal infection by the radiologist and mycological evidence was obtained, including a positive culture or detection of circulating galactomannan or 1,3-beta D glucan. Cases without mycological evidence were not included in this review. In case of a negative culture during the patient’s lifetime, inclusion required the demonstration of infection at autopsy by culture of the causative agent from the affected organs.

Localisation of infection

Documentation of the primary site of the infection was required. The definitions used were the following. (i) Localized infections were defined by isolation of the fungus from a clinically infected site without positive blood cultures or evidence of dissemination to distant organs; (ii) Disseminated infections were defined by the isolation of the fungal isolate from two or more noncontiguous sites showing clinical or histological evidence of infection, and/or positive blood cultures; (iii) Infections with contiguous spread were defined by the isolation of the fungal isolate from at least one contiguous site showing clinical or histological evidence of infection; (iv) Fungemia indicated the presence of the fungus in the blood.

RESULTS

We identified 116 reported cases fulfilling the inclusion criteria of invasive fungal infections in CGD patients between 1970 and 2010. The demographic and clinical characteristics are summarized in Table 2.

One hundred twenty-seven fungal species were isolated: 44 (35 %) A. fumigatus, 23 (18 %) A. nidulans, 5 (4 %) A. niger, 11 (9 %) A. flavus, 1 (8 %) Candida spp., 10 (8 %) Trichosporon spp., 6 (5 %) Paecilomyces spp., 5 (4 %) Scedosporium spp., 4 (3 %) Penicillium spp., 2 Acremonium spp., 2 Alternaria spp., 1 Inonotus spp., 1 Exophiala, 1 Chrysosporium spp., 1 Fusarium spp., 1 Microascus spp. and 1 Hansela spp. (Figure 1). No reports of endemic mycoses in patients suffering from CGD were found following the above described search strategies.
Seventy-two percent of the reported fungal infections in CGD had lung-involvement. Some species were more prone to cause pulmonary infection, e.g. Zygomycetes (91%), *A. nidulans* (78%), followed by *A. fumigatus* (72%). The majority of the trichosporiosis patients suffered from lung infections as did half of the patients infected with *Paecilomyces* spp. Among the patients with candidiasis, only two out of ten mentioned pulmonary involvement, which can be explained by the route of infection being the result of disruption of skin or mucosal membrane (endogenous) instead of inhalation of spores (exogenous).

Symptoms include cough, usually non-productive, fever, chest discomfort and progressive dyspnea. However, some species have the propensity to be quite indolent, e.g. the clinical onset of *A. nidulans* infections was, compared to *A. fumigatus*, often very mild with nonspecific symptoms. *Trichosporon inkin* infections have also been described with an asymptomatic or mild presentation. However, the masking of the symptoms in those cases might have been at least partially explained by concomitant immunosuppressive drug use. Hemoptysis, a relatively common symptom of angioinvasive aspergillosis or mucormycosis in the immunocompromised host, has not been reported in CGD patients. In line with this, the typical lung lesions observed in neutropenic patients, i.e., the air crescent and

### Table 2 Characteristics of invasive fungal infections in 116 CGD patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases, n (%)</th>
<th>Mortality, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in years (range)</td>
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<td></td>
</tr>
<tr>
<td>Male/Female/unknown</td>
<td>103/10/3</td>
<td>24/4/2</td>
</tr>
<tr>
<td>X-linked</td>
<td>65 (56)</td>
<td>16 (25)</td>
</tr>
<tr>
<td>AR</td>
<td>22 (19)</td>
<td>6 (27)</td>
</tr>
<tr>
<td>p47phox</td>
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</tr>
<tr>
<td>p67phox</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>p22phox</td>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>Genetics unknown</td>
<td>29 (25)</td>
<td>8 (28)</td>
</tr>
<tr>
<td>Mono-fungal infections</td>
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<td>26 (24)</td>
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<td>Mixed-fungal infections</td>
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<td>4 (50)</td>
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<tr>
<td>Antifungal prophylactic treatment</td>
<td>22</td>
<td></td>
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<tr>
<td>INF- prophylactic treatment</td>
<td>28</td>
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<tr>
<td>Lung involvement</td>
<td>83</td>
<td>25 (30)</td>
</tr>
<tr>
<td>Bone involvement</td>
<td>47</td>
<td>10 (21)</td>
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<td>Infecting organism (n=127)</td>
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<tr>
<td><em>A. fumigatus</em></td>
<td>44</td>
<td>13 (30)</td>
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<td><em>A. nidulans</em></td>
<td>23</td>
<td>6 (27)</td>
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<td>Other Aspergillus spp.</td>
<td>6</td>
<td>3 (50)</td>
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<td>Zygomycetes</td>
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<td><em>Paecilomyces</em></td>
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<td><em>Candida</em></td>
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<tr>
<td>Miscellaneous group</td>
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<td>3 (21)</td>
</tr>
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</table>

### FUNGAL INFECTIONS IN CGD: BY CLINICAL MANIFESTATION

**Pneumonia**

Fungal involvement of the lung can result in 2 major clinical manifestations: the first is the result of direct infection of the pulmonary tissue or the infection of lung cavities, the second is the ability to trigger an immunological reaction when the fungus is inhaled. The CGD patient can suffer from both clinical identities. However, the clinical course and diagnostic signs and symptoms of invasive fungal pulmonary involvement differ remarkably from the classical clinical spectrum derived from cancer patients.
CHAPTER 2  Fungal Infections in CGD

Fungal Infections in CGD

IFD, was responsible for a femoral osteomyelitis (46) and the presence of halo signs, have never been reported in CGD cases. Nevertheless, A. fumigatus can hematogenously disseminate to liver, skin, brain. In contrast, A. nidulans tends to spread by contiguity.

A distinct pulmonary fungal infection syndrome in CGD is acute fulminant fungal pneumonia, “mulch pneumonitis”, a medical emergency highly associated with and probably pathognomonic for CGD. High-level exposure to aerosolized fungi, such as that which occurs during spreading of mulch, lawn care, gardening, haying or digging in soil, leads to rather stereotypical signs and symptoms of fungal pneumonia with hypoxia and diffuse infiltrates on chest radiographs. This condition can deteriorate rapidly leading to acute hypoxic respiratory failure, mechanical ventilation, and death. Since the first report in 1986 (36), 11 cases have been described (36-40). Mean age was 23 years, ranging from 7 - 64 years, 4 out of 11 patients were previously well and mulch pneumonitis was the initial presentation leading to the diagnosis of CGD. In more than half of these cases multiple organisms were isolated, including Aspergillus spp. in association with members of the phylum Zygomycota and/or Penicillium spp.. A. fumigatus and A. nidulans were the only causative agents isolated in all cases of mulch pneumonitis. The mortality rate was high (8/11, 73%) despite aggressive antifungal treatment, mechanical ventilation, extra-corporal membrane oxygenation and pulse corticosteroids (82 %) for the acute respiratory distress syndrome. However, it is obvious that with early recognition and prompt institution of antifungals to control the infection and steroids to regulate inflammation that response rates will improve. More recently developed immune regulatory agents might be of interest to further improve outcome. The pathological role of the agents of mucormycosis in this syndrome is unclear, and treatment directed at the Aspergillus component alone is probably adequate.

Aspergilloma and chronic necrotizing pulmonary aspergillosis have not been reported in CGD patients. Allergic bronchopulmonary aspergillosis (ABPA) in the CGD patient is rare, but has been reported (41). However, it seems much more likely that what was described as ABPA in a CGD patient, acute respiratory failure after heavily mulching, should be more appropriately classified as “mulch pneumonitis”.

Osteomyelitis

Bone-involvement was reported in almost half of the cases (41 %) of IFD in CGD, being the second most commonly encountered organ. A. fumigatus and A. nidulans were almost equally frequent (Table 3). However, in case of an A. nidulans IFD, osteomyelitis occurred in 74 % (n=17/23), whereas A. fumigatus had a lower propensity to spread to the bone (n=18/44, 41 %). It is very important to recognize that speciation of Aspergillus is an evolving field that has been transformed by molecular biological approaches, just as the other fields in microbiology has. The designation of A. fumigatus in most clinical laboratories is insensitive to subtle distinctions between A. fumigatus sensu strictu and organisms that have roughly similar growth and color characteristics but different clinical features, such as A. viridinutans (42).

As previously described by Sponseller et al. (43), fungal osteomyelitis is often the result of direct spread from a pulmonary focus towards adjacent chest-wall structures (64 %). Localized bone infection, without other organ involvement, was seen in only 4 subjects. Of these, A. fumigatus was isolated twice: a localized osteomyelitis of the midfoot (44) and an osteomyelitis of the patella and arthritis with positive culture of both tissue and the synovial fluid (45). A. nidulans, later identified as E. rugulosa, was responsible for a femoral osteomyelitis (46) and the basiomycetous mold Inonotus tropicalis for a destructive sacral bone osteomyelitis with soft tissue abscesses (47). None of these infections were preceded by trauma or apparent direct inoculation. The mortality rate was 21 %. Surgery was the cornerstone of treatment (72 %) and in combination with antifungal therapy the cure rate was 62 %. However, more prospective research is needed to define optimal treatment strategies.

Few data are available about the clinical, histopathology and radiologic characteristics of fungal bone infections in CGD patients. In a brief report published by Galluzzo et al. (48), 14 CGD patients were compared with control subjects to determine osteomyelitis-related markers suggestive of CGD. Generally, multifocal osteomyelitis and/or simultaneous other organ involvement were the most relevant and statistically significant clinical findings suggesting CGD as an underlying disease. Histopathologic findings of chronic inflammation plus granulomata, multinucleated giant cells, histiocytes, or necrosis were significantly over-represented among CGD patients. In contrast, acute or chronic inflammation plus granulation tissue, remodeled bone, or lymphocytes were significantly under-represented and infrequently found in CGD patients. The presence of extensive destruction in association with minimum sclerosis on the X-ray, almost invariably noted at the time of diagnosis, was also described previously by Wolfsen et al. (49). Whether the particular histopathologic and radiologic patterns described in osteomyelitis occurring in CGD patients are fungus dependent or CGD host dependent is unclear. Overall, the lack of early clinical signs of osteomyelitis in association with advanced destructive lesions - often multi-focal – and the isolation of a likely opportunistic pathogen, is highly suggestive for the presence of CGD as an underlying condition, especially if the osteomyelitis is associated with other organ involvement. or if there are histopathologic signs of chronic inflammation and granulomata-related features.
malaise and fever. The responsible pathogens are listed in Table 3. One case of linear IgA dermatosis has been described during IFD caused by Paecilomyces spp. (50).

Half of fungal skin lesions were preceded by a superficial abrasion of the skin or a minor trauma. The extremities were the most common locations. Primary cutaneous fungal infections can be the first manifestation of CGD. Mansoory et al. (9) described a chronic cutaneous Fusarium infection in a 54-year-old women with undiagnosed CGD. In such patients, milder CGD phenotypes likely due to residual levels of superoxide production are considered to be the reason for the unusually late manifestation (51). Secondary cutaneous fungal infection, defined as the skin being an end-organ of hematogenous dissemination, is a much more aggressive clinical feature, resulting in massive dissemination and a very high case-fatality rate.

Fungal invasive gastrointestinal (GI) involvement in CGD is very rare, especially when compared to the extensive granulomatous involvement of the GI tract (52). Etiologic agents are listed in Table 3. The clinical picture is that of hematogenous dissemination with abscess formation in liver, spleen and kidney, with exception of one isolated case of gastrointestinal mucormycosis that presented with an abdominal mass in a 10-month-old prematurely born infant (53). In this boy, the diagnosis of gastrointestinal zygomycosis served as the presenting symptom of the underlying CGD.

Lymphadenitis, one of the classical presenting signs of CGD, has been rarely attributed to infection by fungi. Nevertheless, Candida lymphadenitis in the infant is very suspicious for CGD (54-56). In case of Aspergillus infections, the lymph nodes involved are often those draining the site of infection.

Infection of the central nervous system was mainly observed as the consequence of a spread by contiguity from a primary pulmonary focus to the spinal cord. Localized brain abscesses are extremely rare but have been reported caused by A. fumigatus, S. prolificans and Alternaria infectoria (7, 57-59). Candida spp. were responsible as causative agents in meningitis or ventriculitis (60, 61).

### Fungal Infections in CGD: By Organism

#### Aspergillus fumigatus

Forty-four invasive A. fumigatus infections in CGD patients were reviewed. According to our criteria, 40 cases were proven IA and four were categorized as probable. Three out of the four patients with a probable IA presented with signs of symptoms of a mulch pneumonitis (37). The majority of infections (37/44), were caused by a single fungus. In mixed fungal infections, the associated organisms were, Rhizopus spp., A. niger, Penicillium spp., Absidia corymbifera and C. dubliniensis (37, 52).

### Table 3: Fungal species distribution and localization of invasive infections in CGD patients

<table>
<thead>
<tr>
<th>Organ involvement</th>
<th>Cases (%)</th>
<th>Species involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>83(72)</td>
<td>A. fumigatus (n=31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. nidulans (n=25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zygomycetes (n=9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trichosporon spp. (n=7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scedosporium spp., A. fumigatus other (n=4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paecilomyces spp. (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillium spp., Candida spp., Acremonium spp. (n=2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exophilia dermatitis, Chrysosporium zonatum, Hansenula polymorpha (n=1)</td>
</tr>
<tr>
<td>Bone</td>
<td>47(41)</td>
<td>A. nidulans (n=17)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. fumigatus (n=18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scedosporium spp. (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. niger, Trichosporon spp. (n=2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paecilomyces spp., Penicillium spp., Cryosporium zonatum, Inonotus tropicalis, Exophilia dermatitis (n=1)</td>
</tr>
<tr>
<td>Brain</td>
<td>17(15)</td>
<td>A. fumigatus (n=9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. nidulans (n=2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zygomycosis (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scedosporium prolificans, Exophilia dermatitidis, Alternaria infectoria (n=1)</td>
</tr>
<tr>
<td>Skin</td>
<td>11(10)</td>
<td>A. fumigatus, Paecilomyces spp. (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fusarium solani, Mucoraceae cinerea, Alternaria alternate, A. nidulans and T. pullulans (n=1)</td>
</tr>
<tr>
<td>Gastro-intestinal</td>
<td>11(10)</td>
<td>Rhizopus spp. (n=4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. fumigatus, C. glabrata (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paecilomyces (n=1)</td>
</tr>
<tr>
<td>Lymphadenitis</td>
<td>11(10)</td>
<td>A. fumigatus (n=4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. lusitaniae (n=2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. flavus, A. nidulans, T. glabrata, C. albicans, H. polymorphae (n=1)</td>
</tr>
<tr>
<td>Meningitis</td>
<td>1</td>
<td>C. lusitaniae (n=1)</td>
</tr>
</tbody>
</table>

Other organ involvement

The skin is less commonly infected by fungal than bacterial pathogens. The pathology is diverse, ranging from erythematous plaques, crusted, papulous, pustulous to purulent ulcers in the absence or presence of generalized symptoms as
Interestingly, in five out of the seven multiple-fungal infections a clear temporal relationship between massive exposure to molds, especially mulch, and the clinical presentation of pneumonia, was found (37, 38, 40). The age, sex distribution and genetic profile did not differ from the overall demographic characteristics. Interferon-γ had been administered prophylactically to one third of the patients. However, prophylactic use of itraconazole was documented in only two cases. All of the invasive fungal infections caused by *A. fumigatus* were symptomatic, and accompanied by fever in 61%. Detailed information on the individual clinical pictures is illustrated in Table 4.

Almost three-quarters had a history suggestive for primary lung invasion (30/44). Of these, half remained localized in the lungs (22, 36-38, 63-66) and more than one-third were disseminated, causing metastatic foci in bone, skin and/or the central nervous system (22, 67-74). In 14% of the *A. fumigatus* infections, the fungus had spread by contiguity from the lung to adjacent chest-wall structures (22, 70, 75-77).

The brain and the skin were the most common extra-pulmonary sites affected by *A. fumigatus*. Primary cutaneous aspergillosis due to *A. fumigatus* occurred in three cases, all of them in relation to direct inoculation of disrupted skin (78-80). In four patients *A. fumigatus* caused isolated brain abscesses (7, 22, 57, 73). It should be emphasized that the symptoms can be very indolent ranging from mild fever and headache in the absence of any localizing neurologic signs (57), up to isolated seizures mimicking intracranial tumor (58). Isolated osteomyelitis without evidence for pulmonary involvement was reported twice (44, 45).

Susceptibility data were rarely provided. An elevated MIC for amphotericin B of 1.2 mg/l was found in an *A. fumigatus* isolate causing pneumonia and secondary osteomyelitis of the foot (69) and a MIC of 1.5 mg/l in a dual pulmonary infection with *A. fumigatus* and *C. dubliensis* (63). A multiazole resistant *A. fumigatus* isolate (MIC itraconazole > 16 mg/l, voriconazole > 16 mg/l, posaconazole 0.25 mg/l) causing osteomyelitis of the midfoot of a patient who received itraconazole prophylaxis, was treated successfully with extensive surgery and long-term oral posaconazole (44). Patients with CGD may be at risk for azole-resistant fungi as they usually receive life-long azole antifungal prophylaxis (81-84). Amphotericin B was the most commonly used first line treatment, followed by voriconazole. Half of the cases also underwent surgical interventions. If mentioned, adjuvant immunomodulatory therapy, in the form of INF-γ and/or granulocyte transfusion was given in 27%. The overall mortality rate was 30%, mulch pneumonitis being responsible for more than half of the case-fatalities. However, as *A. fumigatus* has been the most commonly encountered fungal pathogen in CGD, it might be that less severe cases are not published, leading to overrepresentation of severe cases.

**Aspergillus nidulans**

Twenty-three cases of invasive fungal infections due to *A. nidulans* in CGD patients were previously reported and fulfilled the inclusion criteria of proven infection (n=22) or probable infection (n=1). In one patient, *A. nidulans*, *A. fumigatus* and *A. restrictus* were detected in sputum. However, no histological confirmation of multiple fungal infections was obtained (85).

The major clinical features of invasive *A. nidulans* infections in CGD patients are summarized in Table 5. Twenty-two patients were male, and the median age of the population was 8 years (range 3-21 years). Interestingly, of those patients whose genetic pattern was reported (n=19), 90% were X-linked gp91phox, compared to 75% in all documented cases and 74% in those caused by *A. fumigatus*. One boy had a defect in p22phox and one girl had a defect in the cytoplasmic factor p67phox. Four patients had suffered from previous fungal infections by the time the invasive *A. nidulans* infection occurred.

The most common localization (74%) of the reported *A. nidulans* infections in CGD patient was the lung, with direct spread to adjacent chest-wall structures (Figure 2).

In contrast to IFD caused by *A. fumigatus*, the presenting signs and symptoms were often mild with low grade fever, local pain or swelling, malaise and cough, but could even be completely asymptomatic, with undetected lung infiltrates. Vertebrae, mostly thoracic, were the most frequent sites of bone involvement (47% of cases), while the ribs and skull were involved in 39% and 9% of the cases, respectively. Invasion of the vertebrae was accompanied in 45% by clinical signs and symptoms of spinal cord involvement. Two more exclusive cases were noted: Casson et al. (86) reported an *A. nidulans* endocarditis in a three-year-old girl with CGD who also had an atrial septal defect. Extensive prior investigation by local biopsy and repeated fungal antibody titers were negative and diagnosis was made on a second culture in which the organism grew from a mass obtained in the right atrium. Subsequently, *A. nidulans* was grown from embolic skin lesions and the site of the sternotomy. Despite extensive surgery and antifungal treatment, blood cultures grew *A. nidulans* and she died 24 days post-surgery. In 2003, an unusual mild and localized course of the disease was described in a 16-year-old male with CGD presenting with femoral osteomyelitis, without pulmonary involvement (46). No blunt or penetrating trauma was noted. Treatment with liposomal amphotericin B and granulocyte colony-stimulating factor as well as extensive surgical debridement, followed by prolonged treatment with itraconazole resulted in an excellent clinical response. Microscopic characteristics include very short conidiophores and the presence of ascospores with two equatorial crests. However, after molecular typing in 2008, this showed to be caused by *A. rugulosa*, originally identified as *A. nidulans* based on microscopic features (87).
Table 4 The major clinical features of *A. fumigatus* infections in CGD

<table>
<thead>
<tr>
<th>Case (Ref.)</th>
<th>Sex</th>
<th>Genetic Type</th>
<th>Age (years)</th>
<th>Site of Disease</th>
<th>Prophylaxis</th>
<th>Treatment</th>
<th>Surgery</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (22)</td>
<td>NM</td>
<td>AR</td>
<td>8</td>
<td>Lung, chest wall</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>2 (22)</td>
<td>NM</td>
<td>AR</td>
<td>6</td>
<td>Lung, liver, brain, ribs</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>3 (22)</td>
<td>M</td>
<td>X-CGD</td>
<td>2</td>
<td>Brain</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB, 5-FC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>4 (22)</td>
<td>M</td>
<td>X-CGD</td>
<td>5</td>
<td>Hepatic abscess, chest wall, rib osteomyelitis</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB, ITC</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>5 (22)</td>
<td>NM</td>
<td>AR</td>
<td>4.5</td>
<td>Lung</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB, ITC</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>6 (62)</td>
<td>M</td>
<td>NA</td>
<td>5</td>
<td>Chest-wall, thoracic vertebrae, spinal cord</td>
<td>NM</td>
<td>AMB</td>
<td>NA</td>
<td>Survived</td>
</tr>
<tr>
<td>7 (62)</td>
<td>M</td>
<td>NA</td>
<td>5</td>
<td>Lung, chest wall, thoracic vertebrae</td>
<td>No</td>
<td>AMB, 5-FC, MIC</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>8 (36)</td>
<td>M</td>
<td>X-CGD</td>
<td>20</td>
<td>Mulch pneumonitis</td>
<td>No</td>
<td>AMB, 5-FC</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>9 (67)</td>
<td>M</td>
<td>X-CGD</td>
<td>10</td>
<td>Lung, lymphadenitis, femoral osteomyelitis</td>
<td>TMP-SMX</td>
<td>AMB, ITC</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>10 (68)</td>
<td>M</td>
<td>X-CGD</td>
<td>11</td>
<td>Lung, brain, bone (mastoïd)</td>
<td>TMP, rifampicin</td>
<td>AMB, 5-FC, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>11 (126)</td>
<td>M</td>
<td>NA</td>
<td>18</td>
<td>Anterior chest wall, sternum osteomyelitis, mediastinum, extensive vascular invasement with mycotic pseudoaneurysm</td>
<td>NM</td>
<td>NM</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>12 (63)</td>
<td>M</td>
<td>X-CGD</td>
<td>7</td>
<td>Lung, osteomyelitis foot, lymphadenitis draining Iam</td>
<td>Ampicilline</td>
<td>AMB</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>13 (70)</td>
<td>M</td>
<td>X-CGD</td>
<td>18</td>
<td>Lung, ribs, humerus, chest wall</td>
<td>TMP-SMX</td>
<td>AMB, 5-FC, ITC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>14 (71)</td>
<td>M</td>
<td>NA</td>
<td>15</td>
<td>Lung, humerus</td>
<td>No</td>
<td>AMB, 5-FC, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>15 (75)</td>
<td>M</td>
<td>X-CGD</td>
<td>10</td>
<td>Lung, rib, vertebrae, epidural soft tissue</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB, ABL, 5-FC, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>16 (64)</td>
<td>M</td>
<td>X-CGD</td>
<td>30</td>
<td>Lung</td>
<td>TMP-SMX, INF-γ</td>
<td>VOR</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>17 (65)</td>
<td>M</td>
<td>X-CGD</td>
<td>0</td>
<td>Lung</td>
<td>No</td>
<td>AMB</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>18 (57)</td>
<td>M</td>
<td>X-CGD</td>
<td>11</td>
<td>Brain abscess</td>
<td>TMP-SMX</td>
<td>AMB, ITC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>19 (44)</td>
<td>M</td>
<td>X-CGD</td>
<td>18</td>
<td>Osteomyelitis midfoot</td>
<td>TMP-SMX, INF-γ</td>
<td>VOR, CAS, POS</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>20 (76)</td>
<td>F</td>
<td>AR</td>
<td>14</td>
<td>Lung, pericard, myocard, endocard</td>
<td>No</td>
<td>AMBL, VOR, CAS, gran Tx</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>21 (166)</td>
<td>M</td>
<td>X-CGD</td>
<td>3</td>
<td>Soft-tissue skull, parietal bone, brain parenchym</td>
<td>No</td>
<td>NM</td>
<td>Yes</td>
<td>NA</td>
</tr>
<tr>
<td>22 (80)</td>
<td>M</td>
<td>X-CGD</td>
<td>3</td>
<td>Skin “sporotrichoid Aspergillosis”, lung</td>
<td>No</td>
<td>AMB, VOR, ITC,</td>
<td>Yes</td>
<td>Still not under control</td>
</tr>
<tr>
<td>23 (79)</td>
<td>M</td>
<td>X-CGD</td>
<td>13</td>
<td>Skin</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB, ITC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>24 (72)</td>
<td>M</td>
<td>NA</td>
<td>7</td>
<td>Lung, skin</td>
<td>NM</td>
<td>AMB</td>
<td>NA</td>
<td>Died</td>
</tr>
<tr>
<td>25 (78)</td>
<td>M</td>
<td>X-CGD</td>
<td>19</td>
<td>Skin</td>
<td>TMP-SMX</td>
<td>ITC</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>26 (37, 38, 40)</td>
<td>M</td>
<td>p47phox</td>
<td>14</td>
<td>Mulch pneumonitis</td>
<td>No</td>
<td>VOR, gran Tx</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>27 (38)</td>
<td>M</td>
<td>X-CGD</td>
<td>7</td>
<td>Mulch pneumonitis</td>
<td>No</td>
<td>AMBL, VOR, CAS</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>28 (37)</td>
<td>F</td>
<td>p47phox</td>
<td>23</td>
<td>Mulch pneumonitis</td>
<td>No</td>
<td>VOR, CAS</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>29 (37)</td>
<td>M</td>
<td>X-CGD</td>
<td>20</td>
<td>Mulch pneumonitis</td>
<td>TMP-SMX</td>
<td>AMB</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>30 (37)</td>
<td>M</td>
<td>X-CGD</td>
<td>23</td>
<td>Mulch pneumonitis</td>
<td>TMP-SMX</td>
<td>ITC</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>31 (37)</td>
<td>M</td>
<td>p47phox</td>
<td>64</td>
<td>Mulch pneumonitis</td>
<td>TMP-SMX, INF-γ, ITC</td>
<td>AMB</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>32 (37)</td>
<td>M</td>
<td>X-CGD</td>
<td>10</td>
<td>Mulch pneumonitis</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>33 (66)</td>
<td>M</td>
<td>NA</td>
<td>3</td>
<td>Lung</td>
<td>NA</td>
<td>AMB</td>
<td>No</td>
<td>Survived</td>
</tr>
</tbody>
</table>
A. nidulans has a unique predilection for CGD. It remains a rare pathogen among all other immunosuppressed patients at high risk for IA (88,89,90).

**Other Aspergillus species**

*A. fumigatus* is the primary agent of invasive human fungal infections, followed by *A. flavus, A. terreus,* and *A. niger* (12, 91-93). In contrast, among CGD patients, *A. fumigatus* and *A. nidulans* are by far the most commonly isolated *Aspergillus* spp., followed by *A. niger* (n=5) and *A. flavus* (n=1) (22, 37, 62, 94).

Most of the non-fumigatus non-nidulans *Aspergillus* spp. were isolated in mixed fungal infections, and associated to *A. fumigatus* or *Rhizopus* spp. (37, 62). The clinical picture related to multiple fungal species was that of mulch pneumonitis in half of the cases.

Kaltenis et al. (94) described a ten-year old child suffering from disseminated aspergillosis associated with systemic amyloidosis. The causative agent was *A. niger.* Despite aggressive treatment with amphotericin B and surgical drainage, intravenous leukocyte transfusions, INF-γ and immunoglobulin infusions, the

**Table 4 Continued**

<table>
<thead>
<tr>
<th>Case (Ref.)</th>
<th>Sex</th>
<th>Genetic Type</th>
<th>Age (years)</th>
<th>Site of Disease</th>
<th>Prophylaxis</th>
<th>Treatment</th>
<th>Surgery</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 (66)</td>
<td>M</td>
<td>NA</td>
<td>8</td>
<td>Lung</td>
<td>NA</td>
<td>AMB</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>35 (66)</td>
<td>M</td>
<td>NA</td>
<td>9</td>
<td>Lung</td>
<td>NA</td>
<td>AMB</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>36 (39)</td>
<td>M</td>
<td>X-CGD suspected FA</td>
<td>32</td>
<td>Mulch pneumonitis</td>
<td>NA</td>
<td>gran Tx</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>37 (167)</td>
<td>M</td>
<td>AR suspected, sister AR CGD</td>
<td>17</td>
<td>Vertebrae, ribs, cervical sinus, spinal cord</td>
<td>No</td>
<td>AMB, AMBL, ITC, CAS, ABLC, VOR</td>
<td>Drainage abscess, vertebral surgery not possible</td>
<td></td>
</tr>
<tr>
<td>38 (58)</td>
<td>M</td>
<td>p47&lt;sup&gt;fluc&lt;/sup&gt;</td>
<td>16</td>
<td>Brain abscess</td>
<td>No</td>
<td>AMBL, VOR</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>39 (7)</td>
<td>M</td>
<td>X-CGD</td>
<td>8</td>
<td>Brain abscesses, multiple</td>
<td>No</td>
<td>AMBL, AMBL, VOR</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>40 (73)</td>
<td>M</td>
<td>X-CGD</td>
<td>20</td>
<td>Lung, osteomyelitis skull and subcutaneous abscess</td>
<td>TMP-SMX</td>
<td>AMB, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>41 (74)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>Lung, osteomyelitis skull, epidural and intracerebral abscesses</td>
<td>No</td>
<td>AMB, 5-FC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>42 (45)</td>
<td>M</td>
<td>NA</td>
<td>17</td>
<td>Arthritis, osteomyelitis patella</td>
<td>No</td>
<td>AMB, ITC</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>43 (63)</td>
<td>F</td>
<td>NA</td>
<td>6</td>
<td>Lung</td>
<td>No</td>
<td>AMB, VOR</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>44 (77)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>Lung, rib, subphrenic abscess</td>
<td>NA</td>
<td>AMB, ITR</td>
<td>NA</td>
<td>Survived</td>
</tr>
</tbody>
</table>

AMB, amphotericin B deoxycholate; AMBL, amphotericin B liposomal; ABLC, amphotericin B lipid complex; ITC, itraconazole; VOR, voriconazole; POS, posaconazole; KTC, ketoconazole; MIC, miconazole; CAS, caspofungin; 5-FC, 5-flucytosine; NA, not available

**Figure 2** Computed tomography scan of *A. nidulans* infection in a patient with chronic granulomatous disease. (The extensive chest wall invasion and subcutaneous infiltration (arrow)).
### Table 5 The major clinical features of *A. nidulans* infections in CGD

<table>
<thead>
<tr>
<th>Case (Ref)</th>
<th>Sex</th>
<th>Genetic Type</th>
<th>Age (years)</th>
<th>Site of Disease</th>
<th>Prophylaxis</th>
<th>Treatment</th>
<th>Surgery</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (46)</td>
<td>M</td>
<td>NA</td>
<td>16</td>
<td>Osteomyelitis, long bone</td>
<td>INF-γ</td>
<td>ABL, ABL, ITC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>2 (168)</td>
<td>M</td>
<td>NA</td>
<td>6</td>
<td>Lung, chest wall, vertebrae</td>
<td>TMP-SMX</td>
<td>AM, ABL, ITC</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>3 (135)</td>
<td>M</td>
<td>X-CGD</td>
<td>20</td>
<td>Lung, 3rd rib, femur, skull</td>
<td>NA</td>
<td>AM, ITC, 5-FC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>4 (43)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>Lung, 8th-9th ribs, T6-L1 vertebrae</td>
<td>NA</td>
<td>AM, ABL, gran Tx</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>5 (43)</td>
<td>M</td>
<td>X-CGD</td>
<td>9</td>
<td>Lung, 4th rib</td>
<td>NA</td>
<td>AM</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>6 (44)</td>
<td>M</td>
<td>X-CGD</td>
<td>13</td>
<td>Progression of 4th rib lesion, T3-T4 with paraparesis</td>
<td>NA</td>
<td>AM</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>7 (23)</td>
<td>M</td>
<td>X-CGD</td>
<td>6</td>
<td>Lung, pleura</td>
<td>no INF-γ</td>
<td>AM, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>8 (23)</td>
<td>M</td>
<td>X-CGD</td>
<td>19</td>
<td>Lung, pleura, chest wall, vertebrae, skin, skull, brain</td>
<td>No</td>
<td>AM, ABL, ITC, 5-FC</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>9 (23)</td>
<td>M</td>
<td>X-CGD</td>
<td>16</td>
<td>Lung, pleura, vertebrae, chest wall</td>
<td>KTC</td>
<td>AM, ABL, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>10 (23)</td>
<td>M</td>
<td>X-CGD</td>
<td>7</td>
<td>Lung, pleura, vertebrae, chest wall, sinuses, brain</td>
<td>NA</td>
<td>AM, ABL, ITC</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>11 (23)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>Lung</td>
<td>INF-γ until 1 mo before <em>A. nidulans</em> infection</td>
<td>AMB, ITC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>12 (169)</td>
<td>M</td>
<td>X-CGD</td>
<td>8</td>
<td>Lung, rib</td>
<td>Clindamycin</td>
<td>AMB, gran Tx</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>13 (170)</td>
<td>M</td>
<td>NA</td>
<td>10</td>
<td>Lung, pleura, axillary abscess, 2nd-3rd ribs, vertebrae</td>
<td>No</td>
<td>AM</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>14 (86)</td>
<td>F</td>
<td>p67phox</td>
<td>3</td>
<td>Endocarditis, skin lesions, blood</td>
<td>TMP</td>
<td>AM</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>15 (128)</td>
<td>M</td>
<td>X-CGD</td>
<td>21</td>
<td>Lung, chest wall, spinal cord, T5-T7 vertebrae, popliteal abscess</td>
<td>TMP-SMX, ITC, INF-γ</td>
<td>AMB, ABL, VOR, CAS, gran Tx</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>16 (129)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>Lung, T2-T5 vertebrae, spinal cord</td>
<td>TMP-SMX, ITC</td>
<td>AMB, ABL, VOR, POS, CAS, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>17 (85)</td>
<td>M</td>
<td>X-CGD</td>
<td>13</td>
<td>Lung</td>
<td>TMP-SMX, ITC but diarrhea and serum levels (1)</td>
<td>AMB, VOR, CAS</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>18 (130)</td>
<td>M</td>
<td>p22phox</td>
<td>5</td>
<td>Lung, chest wall cutaneous abscess</td>
<td>NA</td>
<td>AMB, VOR, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>19 (149)</td>
<td>M</td>
<td>X-CGD</td>
<td>8</td>
<td>Lung, 6th rib, chest wall fistula over the rib, psoas abscess</td>
<td>TMP-SMX, ITC stopped</td>
<td>AMB, ABL, gran Tx</td>
<td>Yes</td>
<td>Survived (BM Tx)</td>
</tr>
<tr>
<td>20 (24)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>Lung, chest wall, T8-T11 vertebrae, 7th rib</td>
<td>NA</td>
<td>AM, 5-FC, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>21 (171)</td>
<td>M</td>
<td>NA</td>
<td>5</td>
<td>Lung, chest wall, vertebrae, spinal cord spondylosis</td>
<td>NA</td>
<td>VOR</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>22 (37)</td>
<td>M</td>
<td>X-CGD</td>
<td>16</td>
<td>Lung, MULCH pneumonitis</td>
<td>TMP-SMX, INF-γ</td>
<td>AMB, ITC</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>23 (150)</td>
<td>M</td>
<td>X-CGD</td>
<td>18</td>
<td>Lung, osteomyelitis rib, vertebra</td>
<td>TMP-SMX, ITC</td>
<td>AMB, VOR, CAS</td>
<td>Yes</td>
<td>Survived (BM Tx)</td>
</tr>
</tbody>
</table>

AMB, amphotericin B deoxycholate; ABL, amphotericin B liposomal; ABL, amphotericin B lipid complex; ITC, itraconazole; VOR, voriconazole; POS, posaconazole; KTC, ketoconazole; CAS, caspofungin; 5-FC, 5-flucytosine; NA, not available; gran Tx, granulocyte transfusion; BM Tx, bone marrow transplantation
Fungal infections in CGD

was the only causative agent in a
CHAPTER 2  FUNGAL INFECTIONS IN CGD

spp. were

but the course of the disease is distinctive from typical aspergillosis, being chronic and spreading in a contiguous manner across anatomical planes, and relative refractoriness to antifungal drugs. Although encountered in the CGD patient, fumigati-mimetic molds are not confined to CGD; unlike A. nidulans that is essentially restricted to this host. Correct genotypic-based identification of fumigati-mimetic fungi may have implications for clinical course and management (42, 95).

Non-Aspergillus species

Zygomycetes

Mucormycosis refers to a group of opportunistic invasive fungal infections caused by fungi of the order Mucorales, predominantly of the genus Rhizopus, Mucor and Absidia.

Eleven cases of mucormycosis in CGD patients have been reported. Among them, nine Rhizopus isolates were identified (30, 37, 53), one A. corymbifera (40), and in one report, published in 1976, the clinical manifestation was described as “Phycomycosis”, indicating the causative species as belonging to the phylum Zygomycota (96).

Two major clinical identities were noted: “mulch pneumonitis” (n=5) (37, 38, 40) and “invasive pulmonary mucormycosis” (n=5) (30, 96). Dissemination occurred in three patients. Mucorales are the second most commonly occurring species found in mulch pneumonitis, mostly in association with Aspergillus spp. The association of mucorales and mulch pneumonitis in CGD seems to be related to the fact that both mucorales and aspergilli are found in the same demographic or environmental niche.

Invasive pulmonary mucormycosis can progress to thoracic wall abscess or nodular infiltrates of lung, spleen and brain. Attention has to be drawn to the occurrence of fumigati-mimetic molds in unusual, chronic or refractory IA. Neosartorya udagawae and A. viridins have been reported in CGD patients. Phenotypically they resemble A. fumigatus, but the course of the disease is distinctive from typical aspergillosis, being chronic and spreading in a contiguous manner across anatomical planes, and relative refractoriness to antifungal drugs. Although encountered in the CGD patient, fumigati-mimetic molds are not confined to CGD; unlike A. nidulans that is essentially restricted to this host. Correct genotypic-based identification of fumigati-mimetic fungi may have implications for clinical course and management (42, 95).

Candida species

The clinical spectrum of Candida infections in the immunocompromised host ranges from mucocutaneous disease to systemic life-threatening infections. Mucocutaneous involvement is remarkably rare in CGD. One report mentions an achalasia-like esophageal motility disorder as a result of a previously experienced Candida esophagitis (97), although this has not been observed in other diseases. Overall, in the CGD patient, Candida was the most common cause of meningitis, fungemia and lymphadenitis (54-56, 61, 98), but is not a causative agent of pneumonia, keratitis, hepatic abscesses and disseminated disease (10, 60, 63, 99, 100).

Young children appear more prone to develop Candida lymphangitis, blood stream infections and meningitis than older patients. Among the proven cases of reported meningitis, candidemia and lymphadenitis with or without dissemination, the age ranged from 8 weeks to 4 months. Clinical signs were those of a septic infant, febrile, irritable, sometimes associated with local signs of organ involvement like lymphadenopathy, hepatosplenomegaly or signs of CNS involvement. The isolation of Candida spp. in an “otherwise healthy infant” was often the clue to diagnosis of a primary immunodeficiency defect. A possible explanation might be the immature mucosal immunity of the infant in the absence of a well-established tolerance for the Candida pathogen superposed to a defect in the phagocytic defense mechanisms.

The second risk group includes older children and adults in whom the “classic” risk factors, such as prolonged broad spectrum antimicrobial drug use (3/5 for having bacterial co-infection), use of intravascular catheters and prolonged intensive care stay or hospitalization, may be present.
Detailed information on Candida spp. distribution in CGD patients is not available. According to the individual reports, C. glabrata and C. lusitaniae were each isolated three times (10, 54, 55, 61, 98, 100). C. albicans was suspected twice according to microscopic features or positivity of antigen testing and the occurrence of antibodies (56, 99); in one case no species identification was given (60). Susceptibility testing was rarely performed. Species identification is important with respect to the intrinsic resistance profiles of various Candida spp., most notably the non-albicans Candida spp. Amphotericin B resistance has been reported in cases of C. lusitaniae infections. Four of the 11 CGD patients with invasive Candida infections died, three of them in the neonatal period due to a severely disseminated disease.

*Paecilomyces species*

In CGD patients, Paecilomyces spp. have been reported as the third most common cause of osteomyelitis after Serratia spp. and Aspergillus spp. (4). Cultured-proven infections caused by Paecilomyces spp. in CGD have been nicely reviewed by Wang et al. (8) in 2005. One additional probable pulmonary infection was described in a 12-year-old boy receiving cyclosporine treatment for severe colitis and peri-rectal disease (101). In total six cases have been reported of which four showed primary extrapulmonary disease. One of the extra-pulmonary cases was related to traumatic inoculation through a minor trauma. All of them were symptomatic and the clinical picture did not differ from those reported in the non-CGD host.

*P. variotii* has been shown to be the causative agent in four cases: two cases of soft-tissue infection of which one was associated with multifocal osteomyelitis (102, 103), one case developed isolated splenic abscess (8) and one pneumonia (101). *P. lilacinus* was reported only once in a soft-tissue infection of the abdominal wall (104). Sillevis Smitt (50) implicated a *Paecilomyces* spp. in a pulmonary infection of a CGD patient also suffering from bullous Ig-A dermatosis.

Differentiation between the two species is extremely important, since *P. lilacinus* and *P. variotii* seem to present marked differences in their in vitro susceptibilities. Amphotericin B, itraconazole, and echinocandins showed poor activity against *P. lilacinus*, while the new triazoles, voriconazole and posaconazole show in vitro activity. In contrast, *P. variotii* was susceptible to most antifungal agents apart from voriconazole and ravuconazole (105). All of the patients recovered; however, two were still under treatment by the time their report was published and one patient died after four months due to an *Aspergillus* pneumonia.

De Ravin et al. (106) raise the possibility that some of the *Paecilomyces* infection in CGD patients in previously published reports may have been caused by the emerging pathogen Geosmithia argillacea. When *P. variotii* is identified phenotypically, molecular approaches should be used to exclude *G. argillacea*, as infections observed in CGD are often aggressive, refractory to medical treatment and associated with a high fatality rate.

*Scedosporium species*

*S. apiospermum* (previously known as *Pseudoallescheria boydii*) and *S. prolificans* represent important multidrug-resistant opportunistic pathogens. Both pathogens have been described in CGD patients as breakthrough infections during treatment with amphotericin B and voriconazole, or after long-term treatment for *Aspergillus* infections (107, 108).

In these patients, *S. apiospermum* caused pulmonary infections with both contiguous spread to adjacent bone and central nervous system, and/or hematogenous dissemination. Outcome was favorable after extensive surgery of the infected tissues and antifungal therapy including azole derivatives, itraconazole and voriconazole, and INF-γ. *S. apiospermum* is generally considered resistant to amphotericin B, while extended-spectrum triazoles are active in vitro and voriconazole as well as posaconazole have been used successfully for the treatment of CNS abscesses (109-111).

Bhat et al. (107) described the development of a *S. prolificans* brain abscess in a 23-year-old CGD patient with a history of recurrent pulmonary aspergillosis who was on maintenance voriconazole and interferon-γ. MRI revealed a right frontal lobe lesion and CT-guided biopsy yielded non-specific yeast forms without hyphal elements. Cultures identified *S. prolificans*. This pathogen is considered resistant to virtually all antifungal agents, including the extended-spectrum triazoles and the echinocandins (109). The isolate showed high MICs for amphotericin B (>16 mg/l) and 5-FC (>16 mg/l). Although terbinafine does not appear to be active alone (112), the combination of voriconazole and terbinafine appeared synergistic and was used to treat the case, resulting in complete resolution of the brain abscess (107).

*Trichosporon species*

The eight cases of *Trichosporon* infection described in patients suffering from CGD include three due to *T. pullulans*, although one isolate was subsequently identified as *Cryptococcus albinus*, a likely contaminant (113, 114). *T. pullulans* has been reported as being a human pathogen, but controversy exists regarding whether *T. pullulans* causes invasive infection in patients with CGD (114). It is unlikely that *T. pullulans* causes invasive disease in patients with CGD, nor is it a significant invasive infectious agent in other immune compromised humans.

The case described by Lestini et al. (115), highlighted how difficult it may be to treat CGD colitis with a high dose of immunosuppressive drugs, including prednisone and infliximab: opportunistic organisms with high mortality and morbidity soon occur in such patients.
The four remaining Trichosporon spp. were identified as T. inkin, based on microscopic characteristics and inoculation on commercially available yeast identification systems. Clinically, T. inkin infection presented with pulmonary infiltrates. In three cases, the individuals were asymptomatic; the pulmonary infiltrates were found on routine chest radiographs. In one case, the lung lesions spread directly to adjacent ribs and penetrated the chest wall. Variable susceptibility to amphotericin B has described, and in general this agent lacks fungicidal activity against Trichosporon. The outcomes with voriconazole have been curative (S. M. Holland, unpublished).

Miscellaneous
A few other species of fungi have also been reported to cause invasive infections in CGD patients. Among these species, Penicillium spp. have been reported most commonly (n=4) (37, 116, 117) However, it is now clear that many if not most of the isolates identified as invasive Penicillium in CGD are in fact due to the morphologically similar but treatment resistant Geosmithia argillacea (106). Acremonium spp. (n=2) (118, 119) and Alternaria spp. (n=2) (59, 120) cause occasional infections as well. Inonotus, Chrysosporium, Exophiala, Fusarium, Microascus and Hansenula spp. have been reported once (9, 47, 121-125). All of these cases fulfilled the criteria of a proven IFD, with the exception of one mulch pneumonitis, in which a bronchoscopic specimen grew A. fumigatus, A. niger and Penicillium spp. (37).

The majority of patients presented with localized infections, two of them with spread to adjacent structures. The lung was the most commonly involved organ, skin and soft-tissue infections were observed in three cases, osteomyelitis and cerebral abscess were each reported once. With exception of a 4-month-old infant who suffered from a localized pneumonia caused by Acremonium kiliense (119), the age at onset was quite high. Half of the patients were adults (median 18, range 0 – 69). Seven patients had X-linked CGD, four were diagnosed with autosomal recessive CGD and in three patients the genetic mutation was not reported. Five patients each had received itraconazole prophylaxis and interferon-gamma prophylaxis. Amphotericin B was used in 12/14 cases as first line treatment, two of them also received miconazole or voriconazole combination therapy. One patient received itraconazole. The mortality rate was 21 %. Two patients died of multiple organ failure related to mulch pneumonitis and one patient succumbed to cerebral alternariosis (59).

Diagnosis
Culture and biopsy of clinical specimens
One hundred and eight of the 116 patients (93 %) reviewed in this study fulfilled our criteria for proven IFD and 8 patients for probable IFD. The proportion of proven cases was much higher than observed in other risk groups, such as patients with hematological malignancy. The tendency to prefer proven cases for publication might cause a bias leading to a high proportion of proven cases. Invasive procedures may be higher in CGD patients compared to other patient groups because they are relatively in a better clinical condition and rarely have thrombocytopenia.

Recovery of fungi by culture of sterile material in association with consistent radiologic or clinical infectious disease process made the diagnosis in 101/108 cases. Blood cultures were positive in one mycotic pseudoaneurysm due to A. fumigatus (126), one A. nidulans endocarditis (86), fungemia due to C. lusitaniae (54) and T. pullulans (115). Histopathologic and direct microscopic examination of a biopsy taken during open surgery was conclusive in the 7 additional proven cases (39, 59, 60, 85, 96, 108, 113). Percutaneous fine needle biopsy was performed in 16 % (n = 19), including 11 fine needle lung biopsies. Of these lung biopsies, all were conclusive, either on microscopic examination or culture. Molecular studies were helpful in diagnosing Inonotus tropicalis osteomyelitis of the sacrum using ultrasound guided bone biopsy (47).

In an era of multiple classes of antifungal agents, it is crucial to identify causative fungi to tailor therapy appropriately. Since 1970 numerous changes in the taxonomy of fungi have been implemented, especially through the use of molecular sequence information. Multilocus sequence-based phylogenetic analyses have emerged as the primary tool for inferring phylogenetic species boundaries and relationships within subgenera and sections. Recently, an international group of experts gathered for a workshop entitled “Aspergillus Systematics in the Genomic Era” and reviewed research data presented from research groups worldwide on recent genomic investigations, secondary metabolite analyses, multi locus phylogenetic analyses of the genus Aspergillus, and sequence based identification schemes for previously recognized human pathogens within the genus (127).

Sequence analyses of the ITS region appear to be appropriate for identification of Aspergillus isolates to the subgenus/section level (127). Use of the ITS sequence will not provide sufficient sensitivity to discriminate among individual species within the section. At present partial β-tubulin or calmodulin are the most promising loci for Aspergillus identification to the species level. For the section Nidulanti the identification of clinical isolates by use of β-tubulin sequencing
revealed that clinical isolates were commonly misidentified when solely relied on morphologic characteristics (87). Differences in drug activity against the different species of the section Nidulanti were shown, underscoring the need for identification to the species level (87). In addition to Aspergillus spp, the taxonomy of many other genera involved in fungal diseases in CGD has changed including Candida, Paecilomyces, Scedosporium, and Trichosporon. These changes will have impact on the epidemiology of fungal diseases in CGD patients, and the use of molecular techniques for fungal strain identification will increase our insight into specific pathogens and their susceptibilities in the near future.

**Nucleic acid amplification, antigens and serology**

In seven of the reported CGD cases, information about circulating Aspergillus antigen galactomannan could be extracted (46, 65, 77, 99, 128-130). In 5 of 7 patients (71%) circulating galactomannan could not be detected despite extensive invasive infection (46, 65, 77, 128, 129). Interestingly, Verweij et al. (77) reported a CGD patient who developed invasive pulmonary aspergillosis and a subphrenic abscess. Fine needle aspiration of the subphrenic abscess recovered A. fumigatus as the etiologic agent. During treatment, high levels of Aspergillus antigen were detected in the abscess, but circulating antigen and Aspergillus DNA were undetectable in the serum.

van ’t Hek et al. (130) described circulating galactomannan in a five-year-old boy with X-linked CGD suffering from invasive A. nidulans infection and chest-wall invasion in whom treatment with amphotericin B failed. The galactomannan ratio in serum remained high during amphotericin B treatment but started to decline six weeks after therapy with voriconazole was initiated and finally became negative after four months of treatment.

There has been a progressive increase in the understanding of the diagnostic utility of galactomannan which has enabled its incorporation into the EORTC/MSG consensus definitions and the ECIL guidelines (35). Excellent performance characteristics in patients with hematological malignancies have been reported, with sensitivity up to 92.6% and specificity of 95.4% when serial monitoring of galactomannan was performed (131, 132), but the kinetics and predictive values of the antigen as a diagnostic marker in non-neutropenic hosts have not yet been defined (133). The performance of galactomannan detection is the highest in the neutropenic host, while in non-neutropenic patients circulating galactomannan is detected in only a quarter of patients (134). Possibly the rapid angio-invasive growth of Aspergillus in neutropenic patients facilitates the release of galactomannan into the blood. The poor performance of galactomannan detection in patients with CGD may be due to the fact that they are not neutropenic and the well-formed abscesses may preclude the release of the antigen into the blood (46, 77).

Furthermore, since galactomannan is an immunogenic molecule, it could be rapidly removed from the circulation in the non-neutropenic host.

In our review of IFD in CGD, information on diagnostic PCR was retrieved in six proven cases (46, 47, 59, 77, 85, 129). Nucleic acid amplification of plasma and cerebrospinal fluid samples were all negative. In contrast, PCR performed on material obtained by biopsy of a brain lesion (59) and in the case of an osteomyelitis (47) revealed the etiological fungus.

Six cases mentioned strongly positive anti-Aspergillus antibodies (24, 64, 69, 77, 128, 135), even while circulating galactomannan was undetectable in repeated serum samples (128). Except for the latter, no information was given about the assays used or about the epitopes recognized by these antibodies or corresponding antigen testing. In addition, antibody detection against Scedosporium spp. was helpful in two diagnoses (108), and once in a C. albicans sepsis (99). Antibody responses to Aspergillus spp. may result from environmental exposure in the absence of disease, occur in the course of either aspergilloma or invasive disease, or be detected as part of a hypersensitivity syndrome. Secerted, cell wall and cytosolic fractions of A. fumigatus hyphae have been reported as potent B cell antigens (136). Furthermore, work using the recombinant antigen mitogillin (Aspergillus protein toxin) suggests that specific antibody may be more prevalent in severely immunocompromised patients than has been described previously (137). However at the moment, antibody detection is not considered useful for the diagnosis of IFD. Combined detection of Candida mannanemia and anti-mannan antibodies may substantially contribute to the early diagnosis of candidosis in high-risk patients (138). Nevertheless, little is known about the sensitivity of diagnostic modalities for IFD within specific immunocompromised patient groups as CGD. More work is required to define which diagnostic methods – or combination of methods – confer optimal predictive values in diagnosing IFD at an early stage in the CGD patient.

**Radiologic findings**

Although pulmonary involvement was present in 72% of the reported cases, beyond the description of “pulmonary consolidation” and “lung infiltrates”, no specific signs as nodules, air crescent formation, halo signs, necrosis or cavitations were described or could be detected in the published images. Computed tomography and magnetic resonance scanning established extra-pulmonary extension to soft tissues, spinal cord injury and the extension of the osteolytic lesions. Local extension of disease from lung parenchyma to adjacent structures and osteomyelitis of the thoracic cage have been found particularly associated with underlying CGD. Unfortunately, not a single feature was pathogen-specific (139). The absence of the characteristic diagnostic radiologic findings of IA in pediatric patients has been
previously described by Burgos (93) in a multicentre retrospective analysis of 139
children with malignancy with or without hematopoietic stem cell transplantation.
Thomas (139) performed a retrospective review of imaging performed in 27
consecutive patients with documented invasive aspergillosis, 12 of whom had
primary immunodeficiency syndromes (including six CGD patients). Different
pathophysiology in children compared to adults or, the exuberant granuloma
formation in CGD in response to infection might explain the observed radiographic
differences (64).

Pulmonary involvement is a common clinical feature of invasive fungal
infections. This highlights the importance of identifying early, host-specific
radiologic features on CT scan early in the course of disease. In the case of new or
rapidly changing lesions, it is usually necessary and highly desirable to move to
invasive diagnostic methods to identify the causative organisms.

TREATMENT

Overall, the combination of antifungal treatment and extensive and early surgical
debridement was used in most patients. Almost all of the patients received
presumptive initial treatment with amphotericin B (86 %). However, it is important
to keep in mind that this series is accumulated from the era well before the advent
of azole antifungals, and therefore is heavily weighted to amphotericin as the sole
antifungal agent available. In general, the treatment guidelines for patients with
aspergillosis and candidiasis can be followed in patients with CGD (140, 141)
keeping in mind that non-fumigatus Aspergillus species, in particular A. nidulans,
may require a different antifungal treatment. Furthermore, the use of adjunctive
immunomodulatory treatment options as INF-γ and granulocyte transfusions,
while conceptually robust in CGD is unproven and remains quite anecdotal.

Amphotericin B is active against the majority of clinically relevant yeasts and
moulds isolated in the CGD patient, including Candida spp., Aspergillus spp. and
Zygomycetes. However, the susceptibility of Trichosporon spp. to amphotericin B is
variable and Scedosporium spp. are generally considered resistant (109).

Acquired and intrinsic resistance of Aspergillus spp. has been documented.
Acquired azole-resistance is an emerging problem in A. fumigatus and may develop
during azole therapy. This observation is of particular interest for patients, as
they should receive life-long itraconazole prophylaxis (82-84). Development of
resistance in A. fumigatus during azole therapy has recently been described in a
patient suffering from CGD (81). Non-fumigatus Aspergillus species, especially A.
terreus and A. nidulans, may be intrinsically resistant to specific classes of antifungal
agents (142). Of particular interest is the resistance of A. nidulans to amphotericin B
which might contribute to the high rate of treatment failure of these IFD in the era
before azole antifungals (143). In a clinical CGD study, the total dose of amphotericin
B used in patients with A. nidulans infections was not only higher than for those
with A. fumigatus infections (231 mg versus 56 mg, respectively), but also the
duration of amphotericin B therapy was substantially longer, 220 days for A.
idulans infections compared to 65 days for A. fumigatus infections (23).

Voriconazole has recently been introduced as drug of choice of invasive
aspergillosis in children and has been shown as successful salvage therapy in
patients with CGD (144). Among the described cases, use of voriconazole was first
reported in 1998 and prescribed in 23 % of the cases. In comparison, 86 % of the
cases received amphotericin B.

In an open-label trial of oral posaconazole, Segal et al. evaluated the effect in
eight CGD patients suffering from refractory invasive mold infection including P.
varistii, S. apiospermum and A. fumigatus infections. Posaconazole led to complete
response in seven of the eight infections. In addition, Notheis et al. (129) described
the beneficial effect in a deteriorating A. nidulans infection refractory to
voriconazole and caspofungin treatment. Posaconazole shows promising results as
safe and effective salvage therapy in CGD patients (145, 146).

Granulocyte transfusions were prescribed in 22 % of our cases, and/or 28 %
received INF-γ. The use of granulocyte transfusions in CGD is supported by the
observation that in vitro a small number of normal phagocytes may complement
the oxidative defect and restore the killing ability towards A. fumigatus hyphae
(147). However, the efficacy of granulocyte transfusions is poorly documented and
limited to case reports. The use of INF-γ as adjunctive therapy of IFD in CGD
patients has not been investigated by controlled studies and remains controversial
(148). Three uncontrolled A. nidulans invasive fungal infections were cured either
by ex vivo gene therapy (129) or by BMT (149, 150).

The utility of in vitro susceptibility testing for filamentous fungi is poorly
defined, as acquired resistance was uncommon and species identification was
sufficient to guide antifungal therapy. However, both CLSI and EUCAST are
developing and validating reference methods for in vitro susceptibility testing of
Aspergillus and other conidium-forming fungi (151). For A. fumigatus breakpoints
have been proposed, while awaiting the recommendations by CLSI and EUCAST
(152). With the emergence of acquired resistance in A. fumigatus the need to
determine the susceptibility in order to guide antifungal therapy has increased.
In general, voriconazole is appropriate first line therapy for most IFD in CGD, with
the important exception of mucormycosis, for which lipid formulations of
amphotericin are first line and posaconazole a possible oral substitute.

In 104 of the 116 cases information on prophylactic treatment was retrieved.
Itraconazole or ketoconazole was prophylactically prescribed in 19 cases (18 %). In
addition, three patients were still under antifungal treatment at the onset of a new IFD: two developed a Scedosporium breakthrough infection (S. prolificans during voriconazole and S. apiospermum during amphotericin B treatment) (107, 108) and one Trichosporon sp. was cultured during amphotericin B, caspofungin, and voriconazole treatment for active A. nidulans infection (113). Twenty-eight patients received INF-γ (27 %), in almost half in combination with antifungal prophylaxis.

Interferon-gamma (INF-γ) is a macrophage-activating cytokine produced by T cells and natural killer cells and has a key role in the innate and adaptive host response against fungi (153). A subgroup of variant X-linked CGD patients (i.e., with very low, but detectable, baseline superoxide-generating activity), who have splice site mutations, has been shown to be responsive to INF-γ (154, 155). Treatment resulted in improved splicing efficiency and an increase in cytochrome b expression, allowing near normal levels of superoxide production and bactericidal activity of neutrophils and monocytes (156-158). However, subsequent reports have not confirmed the enhancement of superoxide production as the principal mechanism of INF-γ activity in CGD individuals (159-161). To date placebo-controlled studies are still limited and controversy remains about the routine prophylactic administration in CGD patients. INF-γ prophylaxis is offered only in selected CGD cases by most European physicians, while it is more universally prescribed in the USA and Japan (162, 163). In contrast to the multiple studies confirming value for the use of INF-γ prophylaxis to reduce infections in CGD, there are no prospective data to show any benefit of INF-γ treatment during acute infection.

One of the cornerstones of clinical care for CGD patients is the lifelong prophylactic treatment with intracellular active microbicidal agents. After the introduction of antibacterial prophylaxis, fungal infections persisted with an incidence of 0.15 episodes per patient year (4). In a prospective, open-label study with 30 patients, administration of intravenous amphotericin B reduced the rate of Aspergillus infections to one-third in comparison with historical controls (164). Gallin et al. (165) performed a randomized, double-blind, placebo-controlled crossover study in 39 CGD patients, finding only one serious fungal infection in the itraconazole group compared to seven cases in the placebo arm. Serious fungal infections developing during long-term itraconazole prophylaxis may well be due to molds that are resistant to itraconazole. Further studies are warranted to evaluate potential alternatives to the currently used antifungal prophylactic strategies.

**CLINICAL OUTCOME**

Unambiguous data on clinical outcome in accordance to the definition of complete response (defined as resolution of all clinical signs and symptoms attributable to IFD and complete or nearly complete radiological resolution) or a partial response (improvement or resolution of all clinical signs and symptoms attributable to IFD and ≥50 % improvement in radiological findings) were lacking and follow-up ranged from “still under treatment” to nine years after treatment. At the time cases were published, the mortality rate related to the fungal infection was 26 % (Table 1). Mixed fungal infections (50 %) and mucormycosis (55 %) seem to be responsible for the highest mortality rates, followed by Candida spp. (40 %), A. fumigatus (30 %) and A. nidulans (27 %). However, the reported small numbers of the individual groups, the occurrence of particular species like A. nidulans, or severe clinical presentations of the more commonly encountered A. fumigatus may substantially influence these mortality rates. This underscores again the need of more prospective surveillance cohort studies of invasive fungal infections in this unique host.

**CONCLUSIONS**

To summarize our current understanding of fungal epidemiology and clinical management of invasive fungal disease in CGD patients we have to conclude that there is a paucity of data. Consequently, current management strategies may not be optimal to protect CGC patients from these life-threatening infectious complications.

Host defense mechanisms influence the manifestations and severity of infections; the clinical presentation of fungal disease depends on the patient’s immune response. As a result, there are important clinical and diagnostic differences between IFD in CGD patients compared to patients with other immunocompromised conditions.

First of all, the CGD patient is a non-neutropenic host suffering from impaired phagocytic killing and a dysregulated inflammatory response. As a result, IFD can present with a huge variety of often non-specific symptoms, ranging from indolent and asymptomatic up to acute onset of hypoxia and respiratory failure, or isolated symptoms of specific organ involvement. Timely diagnosis of IFD in CGD requires a high degree of suspicion. This is in contrast to the neutropenic host, in whom the risk depends mainly on the duration of the neutropenia and in whom persistent febrile neutropenia is one of the early signs of a possible invasive fungal infection.

Secondly, invasive mould infections in neutropenic patients are characterized by hyphal angioinvasion, necrosis and paucity of inflammatory cells. In contrast,
directed antifungal therapy will improve outcome without the need for aggressive surgery. Adjunctive immunomodulatory therapy was used in about 25% of the patients. The role for immunomodulatory agents in addition to antifungal therapy is not well studied. Due to lack of a profound understanding of the pathogenesis of invasive fungal infections in the CGD host, it is currently hard to define to what extent specific immunomodulatory therapies will be of benefit for this specific patient population.

Antifungal prophylaxis is not universally prescribed and has been associated with invasive infections caused by intrinsically or acquired resistant fungal organisms. However, since the introduction of azole prophylaxis the incidence of invasive aspergillosis seems to have decreased.

An infection-related mortality of 26% was noted, but underestimation cannot be ruled out because of the huge variability in follow-up. Furthermore, large variability in the mortality was noted based on the clinical presentation and the causative fungus.

Management and outcome data should be gathered to guide optimal treatment decisions for future patients. At present, international and national databases are used to collect clinical data prospectively in children suffering from invasive fungal infections. Although not specifically designed for patients with CGD, it will be a first step to improve our knowledge about these devastating infections.

IA caused by *A. nidulans* is virtually unique to CGD patients and needs more aggressive antifungal therapy than the more commonly encountered *A. fumigatus*. Invasive infections by *A. nidulans* are very seldomly reported in other immunocompromised patients and indicate a unique interaction between this fungus and the CGD host. The clinical entity of “mulch pneumonitis” is a characteristic presentation of fungal pneumonia in the CGD patient in which multiple causative fungi may be recovered. The occurrence of infectious complications by Zygomycetes is mainly noted in the setting of inflammatory complications being treated with immunosuppressive drugs. *Candida* infections are less frequently seen but show an age-dependent clinical presentation; meningitis, fungemia and lymphadenitis with or without dissemination, were reported in neonates and young infants, and were not observed in older patients.

Details regarding strain identification and in vitro susceptibility testing are scarce in CGD patients. Accurate identification of the causative fungus to species level, due to a species dependent susceptibility to the currently used antifungal drugs for treatment and prophylaxis will definitely affect patient management. In vitro susceptibility testing may also have a role, but is as yet undefined.

Extensive and early surgery has been the cornerstone of treatment and amphotericin B was mostly used as the first-line drug. Since the introduction of the new triazoles, the occurrence of invasive fungal infections as a cause of death seems to decrease dramatically. It remains to be seen if the combination of an early diagnosis with an accurate specification of the causative fungus and subsequently
REFERENCES


CHAPTER 2  FUNGAL INFECTIONS IN CGD


FUNGAL INFECTIONS IN CGD


The NADPH-oxidase as antifungal effector complex

Human Leukocytes Kill *Aspergillus nidulans* by Reactive Oxygen Species-Independent Mechanisms

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ABSTRACT

Invasive aspergillosis is a major threat for patients suffering from chronic granulomatous disease (CGD). Although Aspergillus fumigatus is the most commonly encountered Aspergillus species, the presence of A. nidulans appears to be disproportionately high in CGD patients. The purpose of this study was to investigate the involvement of the NADPH-oxidase and the resulting reactive oxygen species (ROS) in host defense against fungi and to clarify their relationship towards A. nidulans.

Murine CGD alveolar macrophages (AM) and polymorphonuclear leucocytes (PMNs) and peripheral blood mononuclear cells (PBMCs) from healthy controls and CGD patients were challenged with either A. fumigatus or A. nidulans. Analysis of the antifungal effects of ROS revealed that A. nidulans, in contrast to A. fumigatus, is not susceptible to ROS. In addition, infection with live A. nidulans did not result in any measurable ROS release. Remarkably, human CGD PMNs and PBMCs and murine CGD AM were at least equipotent at arresting conidial germination compared to healthy controls. Blocking of the NADPH-oxidase resulted in significantly reduced damage of A. fumigatus but did not affect A. nidulans hyphae. Furthermore, the microbicidal activity of CGD PMNs was maintained towards A. nidulans but not A. fumigatus. In summary, antifungal resistance to A. nidulans is not directly ROS related. The etiology of A. nidulans infections in CGD cannot be explained by the simple absence of the direct microbicidal effect of ROS. In vivo, the NADPH-oxidase is a critical regulator of innate immunity whose unraveling will improve our understanding of fungal pathogenesis in CGD.

INTRODUCTION

Chronic granulomatous disease (CGD) is a rare (prevalence, 1/250,000 individuals) inherited immunodeficiency disorder of the NADPH-oxidase in which phagocytes fail to generate the microbicidal reactive oxidant superoxide anion and its metabolites, due to a mutation in any of the five structural components of the NADPH-oxidase complex. As a result, CGD patients are characterized by recurrent life-threatening bacterial and fungal infections as well as chronic inflammatory diseases due to dysregulated inflammatory pathways. The lifetime incidence of invasive aspergillosis (IA) in children with CGD varies between 25 % and 40 % and IA has been the main cause of death (35 %) (7,24).

The etiology of IA differs remarkably between patients with CGD and those with other underlying immunodeficiencies. Although Aspergillus fumigatus is by far the most frequently encountered pathogen, A. nidulans causes up to 50 % of cases of osteomyelitis and is overrepresented in cases of invasive aspergillosis in CGD patients, with a five- to tenfold higher mortality rate (8,22). Differences in either fungal virulence factors or antifungal mechanisms mounted by the host to kill these pathogens could explain why A. nidulans causes such a disproportionately high infection rate in CGD patients versus other immunocompromised patients (1,2,5,14,31).

Most studies focusing on innate immune responses against Aspergillus spp. have used A. fumigatus; knowledge of the host response against other Aspergillus species is scarce, in particular with respect to A. nidulans. The almost exclusive contribution of NADPH-oxidase to microbial killing has been debated, with studies showing contradictory results. On the one hand, the absence of NADPH-oxidase in CGD cells indicates its essential role in effective microbial killing. Investigators have focused attention on the products of the oxidase themselves as the microbicidal agents. In addition, clinical epidemiological data for infections occurring in CGD patients suggest a direct link between catalase-positive microorganisms as causative organisms and the absence of reactive oxygen species (ROS) in CGD patients. Species such as Staphylococcus aureus, Burkholderia cepacia, Serratia marcescens, Aspergillus species, and Nocardia species are able to destroy their own hydrogen peroxide radicals. This makes microbe-produced H$_2$O$_2$ unavailable to the phagocytic cells for conversion into more potent and potentially toxic microbialicidal reactive oxygen intermediates. Several in vitro studies have proposed a direct link between an increased oxidative response and enhanced fungal damage (19,20).

On the other hand, in vivo and in vitro studies have reported unaltered virulence of catalase-deficient A. nidulans and S. aureus in the CGD host (5,16), and experimental studies with alveolar macrophages (AM) from C57Bl/6 and gp91phox-deficient mice...
showed that conidial germination was equally inhibited in vitro. These results suggest that NADPH-oxidase independent mechanisms in murine alveolar macrophages contribute to the inhibition of conidial germination (6).

In order to unravel the unique interaction of the CGD host and A. nidulans and to evaluate the role of the NADPH-oxidase complex and ROS in resistance to Aspergillus spp., we investigated the ability of the various phagocytic cells to generate ROS in response to fungal invasion. This was carried out with the CGD-specific pathogen A. nidulans and compared to the more general pathogen A. fumigatus.

MATERIALS AND METHODS

Human PBMCs and PMNs
Venous blood was drawn from healthy volunteers and two gp91phox-deficient CGD patients after informed consent. Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leucocytes (PMNs) were isolated from peripheral blood by use of lymphoprep (Axis-Shield) and density gradient centrifugation. Briefly, blood was anticoagulated by use of lithium heparin (BD Vacutainer) and then diluted with an equal volume of phosphate-buffered saline (PBS). The diluted blood was carefully added to the top of the lymphoprep and centrifuged at 800xg to separate the plasma from the PBMCs fraction and the PMNs. PBMCs were harvested, washed twice in PBS, and counted by use of a hemocytometer. To remove the erythrocytes from the PMNs, the pellet was shocked at least twice with an ice-cold lysing reagent (NH4Cl, Na2EDTA, KHCO3). Subsequently cells were washed and resuspended in PBS. When the experiment required cells in ‘Hanks’ buffered salt solution (HBSS; Invitrogen Life Technologies) or culture medium (CM), the last step was adjusted with the corresponding medium. RPMI 1640 GlutaMAX-I medium (Invitrogen Life Technologies) plus 10 % heat inactivated fetal calf serum (FCS) was used as CM for all cell experiments.

Animal handling and AM sample collection
Wild-type 8- to 16-weeks-old male and female C57BL/6 mice and CYBB/C57BL/6 mice, which have a homozygous mutation in the CYBB gene, were used. The animals were maintained in sterilized microisoler cages in a pathogen-free environment at the animal facility of the National Institute of Allergy and Infectious Diseases, Bethesda, MD. Alveolar macrophages were isolated from bronchoalveolar lavage fluid (17). Lavage was performed by using 0.75 ml warm PBS (37°C) plus 3 mM EDTA. In total, 10 lavages were done four each mouse to increase the yield of AM. The bronchoalveolar lavage fluid was spun down at 400xg for 12 min and washed once with warm PBS. If many red blood cells were observed, a lysis buffer was added and an extra washing step was performed. The AM were resuspended in warm RPMI plus 10 % fetal bovine serum (FBS) in a total volume of 1 ml. The cells were counted with the use of a Bürker chamber and adjusted to a concentration of 5 x 10⁶/ml. Cell viability was checked by trypan blue exclusion. Aliquots of 100 µl/well (5 x 10⁴ AM) were added to 96-well plate and incubated overnight at 37°C. After 18h, the nonadherent cells were washed away and the medium (RPMI plus 10 % FBS) was refreshed.

Fungal strains
In all experiments, we used fully molecularly characterized A. nidulans and A. fumigatus strains originally isolated from patients with CGD who were suffering from invasive aspergillosis. The cell-free assay and the assays for the assessment of the interaction with human PMNs and PBMCs were performed with A. fumigatus strain V45-07 and A. nidulans strain V44-46. A. fumigatus strain R5233 and the A. nidulans strain RYCB were used for the animal experiments. The growth and killing assays of the Aspergillus isolates were prepared in a lipopolysaccharide (LPS)-free fashion. A. fumigatus strains were grown on Sabouraud glucose agar supplemented with chloramphenicol for 4 to 7 days at 37°C. Abundant conidia were produced under these conditions. Conidia were harvested by gently scraping the surfaces of agar slants and suspending the conidia in PBS with 0.05 % Tween 80. To remove hyphal debris, the conidial suspension was filtered twice through five layers of sterile gauze. Conidia were washed twice in PBS, resuspended in HBSS without phenol red, calcium, or magnesium [HBSS(−)], and stored in individual aliquots of 1 x 10⁶/ml at −80°C. Due to the tendency of A. nidulans strains to produce cleistothecia in their sexual cycle, the growth conditions used for A. fumigatus had to be modified. After growth on Sabouraud glucose agar, conidia were plated on water-agar for 3 days, followed by incubation on malt extract agar for an additional 3 days. Under these conditions, pure asexual growth was obtained. The harvest protocol was identical to that described for A. fumigatus.

To collect hyphal fragments, conidia were added to 5 ml of yeast nitrogen base medium (YNB) (Difco) at a final concentration of 1 x 10⁶/ml. After 18 h of incubation at 37°C, the cultures were centrifuged at 3,000xg for 15 min, and the pellet, containing almost exclusively mycelia, was washed twice in PBS and resuspended in 1 ml of HBSS(−).

Aliquots of the conidial suspension were heat killed for 15 min at 121°C. Nonviable conidia were centrifuged at 3,000xg for 15 min, resuspended, and vortexed vigorously. Finally, the suspension was washed three times with HBSS(−) to remove released Aspergillus components and then kept frozen at −80°C until they were used. The viability of the fungi was checked by culture at 37°C on Sabouraud glucose agar. No growth was observed following heat treatment.

CHAPTER 3
Cell-free effect of reactive oxygen species

The antifungal activity of ROS was evaluated by the growth rate and the amount of cell-free hydrogen peroxide-induced damage. Resting conidia, swollen conidia, or hyphae (1 x 10⁶/ml) were incubated at 37°C in the presence of hydrogen peroxide at three different concentrations, namely 10⁻³ M, 10⁻⁴ M, and 10⁻⁵ M H₂O₂. Swollen conidia were obtained by incubation in YNB at a density of 1x10⁵ conidia/ml. Growth was measured for 48h by spectrophotometry (optical density at 450 nm [OD₄₅₀]) and compared to that of the negative control. The cultures were observed under a microscope during the first 5h. All experiments were performed in triplicate under the same condition and were repeated twice.

Measurement of superoxide production/reactive oxygen intermediates

Oxygen radical production was evaluated by luminol-enhanced chemiluminescence, as measured in an automated LB96V Microlumat Plus luminometer (EG & G Berthold, Bad Wildberg, Germany). Luminol (Sigma-Aldrich) is an organic dye which reacts with both extracellular and intracellular oxygen species. As a result, the luminol molecule is excited and when the luminol returns from the excited state to the steady state, the energy is released in the form of light, which can be detected by the luminometer. Briefly, 100 µl of PMNs suspension (2 x 10⁶ cells/ml) in HBSS (150 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.5 mM MgCl₂, 5.6 mM glucose, 0.16 mg/ml BSA, 20 mM HEPES-Na, 2 mM sodium pyruvate, and 120 mM NaHCO₃) was added to a 96-well nontransparent white microtiter plate. The cells were then challenged with 0.5 µg/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 1 mg/ml opsonised zymosan (Sigma-Aldrich), or fungal cells. Either resting or heat-killed conidia of A. nidulans or A. fumigatus were tested at a cell-to-conidium ratio of 1:20 (100 µl of 4 x 10⁷ cells/ml per well). Control wells received 100 µl of medium. Luminol (10⁻² M [1.77 mg/ml]) in dimethylsulfoxide (DMSO) was diluted 10-fold in HBSS-0.5 % bovine serum albumin (BSA) prior to use. The reaction was started by the addition of 20 µl of luminol solution. Each measurement was performed in quintuplicate. Chemiluminescence was measured using a 1-s integration time at intervals of 228 s over a 3-hour incubation period at 37°C and a 0.5-s integration time over a 10-hour incubation. Luminescence was expressed as relative light units per second (RLU/s). The area under the concentration-time curve (AUC), representing the overall oxygen radical production during incubation, was calculated. To evaluate the effect of gamma interferon (INF-γ), conidia were added with or without 100 U or 500 U of commercially available human INF-γ (ImmuKine; Boehringer Ingelheim BV). Blocking of the NADPH-complex was performed by use of diphenyleneiodonium chloride (DPI; Sigma-Aldrich).

XTT assay

The XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide sodium salt] assay of fungal viability described by Meletiadis et al. (15) was modified as follows. Each well of a 96-well plate was filled with 100 µl of live resting conidia of A. nidulans or A. fumigatus at 1 x 10⁶/ml CM. Hyphae were obtained directly in the test well by overnight incubation (13h) of the conidia (1 x 10⁶/ml) at 37°C. Subsequently, CM was refreshed, and 100 µl of isolated PMNs or PBMCs was added at a concentration of 5 x 10⁶/ml. Conidial viability was assayed after 18h of incubation at 37°C in 5 % CO₂, while hyphal viability was assayed after 90 min of incubation to avoid overgrowth of hyphae. At the indicated times, the plates were centrifuged at 3,000g for 10 min and the supernatant was removed. Cells were lysed by addition of ice-cold MilliQ with 0.025 % Triton for 5 to 10 min on ice, after which each well was washed to remove cell debris. One hundred microliters of PBS and 100 µl of XTT-menadione solution was added to each well in order to obtain a final concentration of 200 µg/ml XTT (Sigma-Aldrich) and 25 µM (4.3 µg/ml) menadione (Sigma-Aldrich). Incubation was continued at 37°C for at least 2 h in the dark to allow conversion of XTT to its formazan derivative. Control wells containing PBMCs, PMNs, or AM alone were used as negative controls, while wells containing conidia or hyphae alone were used as positive controls. Plates were centrifuged, and the absorbance of the supernatant was read at 450 nm in a spectrophotometer. Fungal damage was defined as the percent reduction in metabolic activity compared to that of controls and was calculated as follows: [1 - (X/C)] x 100, where X is the mean absorbance in the test wells and C is the mean absorbance in the control wells. In addition, the potential enhancement of the antifungal effect by INF-γ was tested in 2 different concentrations: 100 U and 500 U of INF-γ/ml. This process was followed as indicated above. Ninety-six-well clear-bottom plates were used for the killing experiments with healthy host cells. To minimize loss of fungi, the killing experiments with and without DPI and the comparison between healthy and CGD cells were performed with a 96-well 0.45-µm-filter-bottom plate (Millipore Multiscreen-HIV).

Statistical analysis

Each experiment was performed with the cells of one particular donor and by their use in triplicate (growthrate), quadruplicate (XTT assay) or quintuplicate (chemiluminescence) wells. The mean value of these replicate wells was taken as the value for the particular donor and/or experiment. All cell-mediated experiments were performed in quadruplicate unless otherwise indicated. The means were then used in the data analysis to calculate the means ± standard errors of the means (SEM) for all the experiments under the same conditions. Comparisons between two means were tested by t tests, calculated using GraphPad Prism 4.0.
Chapter 3

ROS-Independent Killing of Aspergillus nidulans

A. nidulans conidia are poor stimulators of ROS

Since phagocytosis is associated with the activation of NADPH-oxidase, resulting in the release of ROS, the release of ROS was assessed in real time and during the whole process of phagocytosis by use of luminol-enhanced chemiluminescence. Human polymorphonuclear cells and PBMCs produced ROS in response to live resting A. fumigatus conidia. In contrast, no significant NADPH-oxidase activity could be measured in response to live resting A. nidulans conidia, either by PBMCs or in PMNs. Furthermore, the global respiratory burst activities of the PBMCs and PMNs during stimulation with live A. nidulans conidia (5,161 ± 547 RLU/sec and 4,512 ± 227 RLU/s, respectively), were similar to that of unstimulated cells (4,224 ± 428 RLU/s and 4,129 ± 225 RLU/s, respectively) (Figure 2).

Live and heat killed A. nidulans conidia are poor stimulators of ROS

To determine whether the lack of ROS production after A. nidulans conidial stimulation was dependent on the metabolic activity of the fungus, we exposed the PMNs and PBMCs to swollen conidia (4 h of incubation prior to stimulation). However, no additional germination-induced ROS production was observed (data not shown). Increasing the time of measurement up to 10 h, thereby covering the entire process of germination, from resting conidia to swollen conidia and then to hyphae, confirmed that infection with live A. nidulans did not result in any significant increase of ROS production by phagocytic cells. In contrast, live A. nidulans conidia and swollen conidia were resistant to H₂O₂. The 10⁻³ M H₂O₂-induced damage to swollen A. fumigatus conidia was significantly greater than to A. nidulans conidia and was sustained for up to 18 h of incubation (P<0.01). Growth curves for A. nidulans at 10⁻³ M H₂O₂ were similar to those for their negative controls, suggesting resistance of swollen A. nidulans swollen to H₂O₂ (Figure 1). No differences in growth inhibition were found between resting conidia or hyphae of both species at either 10⁻³ M H₂O₂ or 10⁻¹ M H₂O₂ (complete inhibition) or at 10⁻⁵ M H₂O₂ (no inhibition).

Figure 1. Cell-free damage of H₂O₂ on swollen conidia. Growth-curves of A. nidulans at 10⁻³ M H₂O₂ (dashed line) were similar to the negative controls (solid line), reflecting a complete H₂O₂ resistance of A. nidulans swollen conidia.

Figure 2. Live resting A. nidulans conidia are extremely poor stimulators of ROS. Data represent mean relative light units (RLU) per second as measured by luminol-enhanced chemiluminescence. Bar heights indicate the mean area under the curve (AUC) performed in 5 healthy donors. Error bars are the SEM of the 5 results. *, P < 0.05; **, P < 0.01.
Since both species have a slightly different green color, it is possible that differences in light absorption between the species could interfere with chemiluminescence. Therefore, live and heat killed A. nidulans or A. fumigatus were incubated in a cell-free ROS generation system, ammonium persulfate (APS) (Bio-Rad) and light emission was measured by luminol-enhanced luminescence. Only live A. fumigatus conidia absorbed light significantly more than their negative controls (40% ± 3%), further accentuating the observed differences between live A. nidulans and A. fumigatus.

Live A. nidulans hyphae are extremely poor stimulators of ROS. Whether ROS are important for conidial host defense is somewhat controversial, but it is widely accepted that once the conidia germinate into hyphae, degranulation of PMNs and release of ROS induce hyphal damage. Interestingly, after incubation of A. nidulans hyphae with healthy PMNs, no significant amount of ROS could be measured compared to that in unstimulated cells (9,922 ± 1,131 RLU/s versus 20,100 ± 8,616 RLU/s). In contrast, A. fumigatus hyphae induced a rapid and robust response (51,360 ± 6083 RLU/s), with a peak at approximately 20 min and completion within 1 h. PBMCs were also able to mount a significant respiratory burst in response to A. fumigatus hyphae. Interestingly, although the reaction was much less

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**Figure 3** Germinated A. nidulans conidia do not stimulate the respiratory burst. Data from a representative experiment with each cell type are shown. ROS produced by unstimulated cells and resting or swollen A. nidulans- or A. fumigatus-stimulated PBMC (A) and PMN (B) were measured by luminol-enhanced chemiluminescence for 10 h.

A. fumigatus conidia were able to generate a secondary peak during germination, but this was observed only for PBMCs (Figure 3). The fungal morphology was checked microscopically, and the viability of PMNs and PBMCs was tested by trypan blue exclusion at the 10 h time point. Both celltypes reached similar levels of viability.

To determine whether the lack of ROS production in response to live A. nidulans was due to active processes suppressing the respiratory burst or, alternatively, to a failure to stimulate the phagocytic cells, we exposed PMNs and PBMCs to heat-killed A. fumigatus and A. nidulans conidia. No significant difference in ROS induction was observed for either cell type between heat killed and live A. fumigatus stimulation (7,338 ± 452 RLU/s and 6,914 ± 590 RLU/s, respectively, for PMNs; and 15,477 ± 2,869 RLU/s and 12,632 ± 2,869 RLU/s, respectively, for PBMCs). Although PMNs were unable to respond to live A. nidulans, they did respond to heat-killed A. nidulans conidia (P < 0.05), suggesting some active response to the killed conidia (4,512 ± 227 RLU/s versus 6,558 ± 463 RLU/sec). The cells were still able to mount an ROS reaction in response to PMA or zymosan after incubation with the conidia, indicating maintained cellviability and NADPH activity.

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**Figure 4** Oxidative burst activity in phagocytes. (A) Data for one representative experiment with PMNs. (B) Overall ROS production. Data represent mean relative light units (RLU) per second, as measured by luminescence. Bar heights indicate the mean AUC (n=4). ***, P < 0.01; **, P < 0.001.
Blocking of the NADPH-oxidase does not affect germination and hyphal damage of *A. nidulans*

To further investigate the antifungal effect of the NADPH-oxidase on *A. fumigatus* and *A. nidulans*, we assessed the fungal damage to *A. nidulans* induced by PMNs and PBMCs in the presence of the NADPH-oxidase inhibitor DPI (10µM). Inhibition of NADPH-oxidase activity was controlled by serum-opsonized zymosan stimulation of the phagocytic cells after 1 h of DPI preincubation. NADPH-oxidase blocking by DPI resulted in a 98 % reduction of superoxide production by granulocytes as measured by chemiluminescence. Since DPI has an inhibitory effect on fungal germination, PMNs and PBMCs were preincubated with DPI for 1 h at 37 °C prior to addition of fungi. Cells were washed, resuspended in fresh CM, and incubated with the hyphae. No differences were found in the inhibition of germination, either towards *A. nidulans* or towards *A. fumigatus*. Accordingly, *A. nidulans* hyphae were equally damaged by PMNs in the absence and presence of the NADPH-oxidase blocker DPI (35 % ± 4 % versus 32 % ± 5 %, respectively) (P < 0.05) (Figure 5).
ROS-INDEPENDENT KILLING OF ASPERGILLUS NIDULANS-deficient

CHAPTER 3

**Antifungal activity of gp91phox-deficient PMNs towards A. nidulans hyphae is maintained**

Having characterized ROS release after stimulation of phagocytic cells with either *A. fumigatus* or *A. nidulans* hyphae as well as the antifungal activity after blocking of the NADPH-oxidase by DPI, we tested the extent to which PMNs of a gp91phox-deficient patient were able to damage either *A. nidulans* or *A. fumigatus*. Interestingly, in line with previous results obtained after blocking the NADPH-complex with DPI, in vitro X-CGD cells are perfectly able to damage *A. nidulans* hyphae (97% versus 95% ± 1% for CGD and NADPH blocking respectively). In contrast, the ability of the gp91phox-deficient PMNs to induce hyphal damage to *A. fumigatus* was between that of healthy controls and that after NADPH blocking (Figure 6).

**DISCUSSION**

We have investigated the effect of the NADPH-oxidase and its products on fungal host defense. Specifically, we attempted to clarify the host resistance of the unique CGD fungal pathogen *A. nidulans*. We observed striking differences between reactivities toward *A. nidulans* and *A. fumigatus*. Unexpectedly, we conclude that human phagocytes appear to kill *A. nidulans* by NADPH-independent mechanisms. This surprising conclusion is based on the following observations: (i) *A. nidulans* is not susceptible to the direct effect of ROS, (ii) *A. nidulans* does not induce a measurably different ROS release by PMNs or PBMCs of healthy donors, (iii) human gp91phox-deficient PMNs and PBMCs and murine gp91phox-deficient AM inhibit conidial germination as efficiently as healthy cells do, (iv) blocking of the NADPH-oxidase complex results in a significantly reduced ability to damage *A. fumigatus* but does not affect the damage to of *A. nidulans* hyphae, and (v) the antifungal activity of human gp91phox-deficient PMNs toward *A. nidulans* hyphae is maintained.

Roilides et al. (19,20) showed that superoxide is important for efficient host defense and is produced through NADPH-dependent mechanisms. Stimulation of PMNs and PBMCs with either conidia or hyphae of *A. fumigatus* resulted in increased ROS production, leading to inhibition of germination and to fungicidal activity. However, we found *A. nidulans* to be a weak inducer of ROS in both cell types. Surprisingly, even in the absence of any NADPH activity, as in CGD cells, *A. nidulans* growth was inhibited just as in normal cells, indicating that mechanisms other than ROS must be responsible for the predilection of CGD patients for *A. nidulans* infection. Since CGD phagocytes lack the ability to produce ROS, recent studies have focused on the role of ROS-independent factors in arresting the growth of *Aspergillus* conidia (9,27,32). Zarembner et al. demonstrated that neutrophils from healthy as well as CGD donors were able to arrest conidial growth with equal efficiencies and that lactoferrin, a major protein of neutrophil granules, contributes

**gp91phox-deficient PMNs and PBMCs inhibit conidial germination as efficiently as control cells do**

CGD patients having a defect in the gp91phox component of the NADPH-complex are unable to produce microbicidal superoxide anions and its metabolites. In order to test the hypothesis that CGD patients suffer from invasive aspergillosis due to the absence of microbicidal ROS, we analyzed the antifungal capacity of human CGD cells towards *A. fumigatus* and *A. nidulans*. PBMCs and PMNs were isolated from patients which have a defect in the gp91phox component of the NADPH-complex (X-CGD PBMCs and PMNs) and were incubated with either conidia or hyphae. The capacity of X-CGD PMNs to inhibit germination of either *A. nidulans* conidia (72% ± 3% versus 67% ± 2%) or *A. fumigatus* conidia (65% ± 4% versus 60% ± 2%) did not show a significant difference compared to that of healthy control cells. Accordingly, no differences were found in the ability of X-CGD and healthy PBMCs to cope with either *A. nidulans* (86% ± 3% versus 95% ± 2%) or *A. fumigatus* (74% ± 9% versus 73% ± 16%).

Furthermore, to test whether INF-γ could affect ROS production or enhance antifungal capacity, we added INF-γ (Immukine; Boehringer Ingelheim BV) to reaction mixtures to assess both processes. Neither of the tested concentrations (100 U/ml and 500 U/ml) enhanced the antifungal effect or the respiratory burst activity.

**Figure 6**: Percentage hyphal damage of wild type PMNs with (gray bar) and without (white bar) NADPH-oxidase blocked by 10µM DPI, and of gp91phox-deficient PMNs (black bar) to *A. nidulans* and *A. fumigatus* hyphae. Bar heights indicate % fungal damage. Error bars indicate SEM of 4 healthy controls. **, P < 0.01

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to reduced conidial growth (32). These data were subsequently confirmed by transcriptional responses of Aspergillus conidia to healthy and CGD PMNs (27). The role of ROS-independent antifungal mechanisms was underscored by Shibuya et al. (23). They found that the mycelium of the double cat1 cat2 mutant of A. fumigatus, which has no catalase activity at all, has only a slightly increased sensitivity to H2O2 and is as sensitive to killing by PMNs as the wild-type strain but shows delayed infection in an experimental rat model of invasive aspergillosis. Chang et al. (5) showed previously shown that the virulence of catalase-deficient A. nidulans was unimpaired in p47phox−/− mice, indicating that the catalase activity of fungi is not a virulence factor for CGD pathogenicity.

Cornish et al. showed that the in vitro inhibition of germination of A. fumigatus conidia by C57BL/6 and gp91phox−/− deficient mouse AM was comparable. Furthermore, they could not find evidence of the importance of NADPH-oxidase in AM by using transcriptional microarray analysis, luminometry or nitroblue tetrazolium (NBT) staining (6). Instead, they found the most strongly upregulated transcripts in vivo encoded chemokines and additional factors that play critical roles in neutrophil and monocyte recruitment. Indirect evidence of NADPH-independent resistance in AM was also found by Shibuya et al., who found that killing of catA conidia by AM and conidial virulence in animals were similar to those with wild-type conidia (25). In contrast, AM from p47phox−/− mice were unable to kill A. fumigatus conidia in vivo as shown by Philippe et al. (18). In addition, inhibitors of NADPH-oxidase that decreased the production of reactive oxidant intermediates inhibited the killing of A. fumigatus conidia in vitro, suggesting an NADPH-oxidase dependence. However, the inoculation time was limited to 6 h, in contrast to the 24 h inoculation time in vivo, probably reflecting a more fungistatic effect than a fungicidal effect. Our XTT assays with murine AM underlined the ability of conserved primary defense mechanisms toward Aspergillus conidia. Even in the absence of a functional NADPH-oxidase, germination of both A. fumigatus and A. nidulans were inhibited, with the latter inhibited even better by a gp91phox−/− deficient AM than by those derived from C57BL/6 mice.

With respect to the antifungal host defense mechanisms against hyphae, several studies have investigated the ability of Aspergillus species to modulate ROS in phagocytes relative to their fungicidal activity (1,10). Akpogheneta et al. (1) evaluated the activity of human PMNs and mononuclear phagocytes against hyphae of non-fumigatus Aspergillus species and compared it to that against A. fumigatus hyphae. They concluded that nonopsonized hyphae suppress the oxidative burst of PMNs and that these cells respond with a less vigorous oxidative burst in the presence of serumopsonized hyphae of non-fumigatus Aspergillus species. Similarly, PMNs induced less hyphal damage to non-fumigatus species, particularly A. flavus and A. nidulans, than to A. fumigates, as assessed by XTT colorimetric assay.

They concluded that non-fumigatus aspergilli are generally more resistant to mononuclear and polymorphonuclear phagocytes than A. fumigatus aspergilli. These results suggest a direct link between the induced oxidative response and hyphal damage. In contrast, we found that nonopsonized hyphae of A. fumigatus, being potent ROS inducers, were less damaged than those of A. nidulans, which were not able to induce any measurable respiratory burst activity. Akpogheneta et al. monitored the ROS at the 1-h time point, and concluded that nonopsonized hyphae suppress the oxidative burst compared to that in non-stimulated cells. By using luminol-enhanced chemiluminescence, which gave us the ability to follow the amount of ROS produced over time, we observed a high peak of ROS, with a maximum at 20 min and completion at 60 min. We could indeed confirm that the NADPH-oxidase activity at 60 min decreased below the basal activity.

The unique compositions of the cellwalls of A. fumigatus and A. nidulans might be responsible for the observed differences with respect to ROS stimulation and fungicidal activity of host immune cells. Melanin is known to be an important virulence factor by protecting fungal cells from ROS produced by phagocytotic cells (13.28) and by modulating the host immune response (4). The biosynthesis of melanin, and consequently its composition, differs between A. nidulans and A. fumigatus (3.29). Furthermore, during germination, the hydrophobic rodlet layer is removed and fungal pathogen-associated molecular patterns (PAMP) are exposed, mediating the immune response. Girardin et al. (11) found clear physiochemical differences in the composition of the cell wall between A. nidulans and A. fumigatus after they removed this rodlet layer. Analysis and comparison of the chemical characteristics of both species would definitively give new insights into this complex interface and are the subjects of future studies.

The question remains of whether the pathogenicity of aspergillosis in CGD patients is due to the loss of a direct antifungal effect of superoxide and its metabolites or to loss of more downstream effects of NADPH-activation. We attempted to clarify some of these points by examining the opportunistic pathogen A. nidulans, which is found almost exclusively in patients suffering from CGD. Our findings are robust but unexpected: first, A. nidulans is not susceptible to the direct fungicidal effect of ROS; second, gp91phox−/− deficient AM are able to inhibit A. nidulans more efficiently than controls; third, PMNs and PBMCs from healthy donors inhibit germination and induce hyphal damage to A. nidulans, even in the absence of significant ROS production, distinct from A. fumigatus; fourth, blocking the NADPH-oxidase does not affect the ability to kill A. nidulans hyphae, in stark contrast to the killing of A. fumigatus; and fifth, gp91phox−/− deficient neutrophils, which have complete NADPH deficiency, A. nidulans kill adequately in vitro, suggesting that at least the early antifungal effector activity of the innate response is maintained in the absence of a functional NADPH-oxidase.
Despite these robust findings, CGD patients are the most susceptible hosts for invasive aspergillosis, in particular that caused by *A. nidulans*. The central role of neutrophils in optimal microbial killing and regulation of tissue damage has been discussed extensively by Smith (26). Smith describes the complex function and balance of stimulatory and inhibitory pathways in the neutrophil, which are regulated by a plethora of local and systemic mediators. Superoxide is an important signal transduction intermediate (12). Romani et al. (21) indicated that the absence of ROS leads to defective tryptophan catabolism, dominant overproduction of interleukin-17 (IL-17), defective regulatory T-cell activity, and excessive inflammation. The role of ROS as mediator/regulator in inflammation has also been underscored by van de Veerdonk et al. (30). They showed that ROS appear to dampen inflammasome activation. As a consequence, the absence of ROS in CGD monocytes may in partly explain the inflammatory complications seen in CGD patients. A recent study by Segal et al. (23) supports the occurrence of NADPH-oxidase dependent, redox-mediated signaling which is critical for termination of lung inflammation. By challenging NADPH-oxidase deficient p47phox-/- mice and gp91phox-deficient mice with intratracheal zymosan, they showed that NADPH-oxidase limits lung inflammation by attenuating the proinflammatory transcription factor NF-κB and by activating Nrf2, a key redox-sensitive anti-inflammatory regulatory transcription factor. Results for X-linked CGD patients were consistent with these findings (26). It may well be that the increased inflammation in CGD patients leads to the observed pathogenicity of *Aspergillus* spp, instead of being due to the result of direct deficient innate immune responses against these fungi.

In summary, these in vitro studies do not support a direct role of ROS in the antifungal resistance to *A. nidulans* and cannot explain the etiology of *A. nidulans* infections in CGD patients by the simple absence of the direct microbicidal reactive oxidant superoxide anion and its metabolites. The NADPH-oxidase is a critical regulator of certain limbs of innate immunity, and its unraveling will improve our understanding of fungal pathogenesis in CGD.

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The NADPH-oxidase and its role in intracellular pathway signaling

Low Interleukin-17A Production in Response to Fungal Pathogens in Patients with Chronic Granulomatous Disease

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ABSTRACT

Patients with chronic granulomatous disease (CGD) cannot produce reactive oxygen species (ROS) due to a genetic defect in the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase system. Dysregulation of the L-tryptophan metabolism in mice with defects in NADPH-oxidase, resulting in overproduction of interleukin (IL) -17, has been proposed to link ROS defects with hyperinflammation and susceptibility to pulmonary aspergillosis. In this study, we assessed the L-tryptophan metabolism and cytokine profiles in response to fungal pathogens in CGD patients.

Peripheral blood mononuclear cells (PBMCs) from CGD patients showed increased production of IL-6, tumor necrosis factor-α and interferon-γ upon stimulation with Aspergillus or Candida species, while IL-17A production was strikingly low compared with healthy controls. Indoleamine 2,3-dioxygenase expression was similar in PBMCs and neutrophils from CGD patients compared with healthy controls. Conversion of L-tryptophan to L-kynurenine, as measured by high-performance liquid chromatography, did not differ between CGD patients and healthy controls. Moreover, adding L-kynurenine to the cell cultures did not suppress fungal-induced IL-17A production. Although PBMCs of CGD patients produced more proinflammatory cytokines after stimulation, IL-17A production was strikingly low in response to fungal pathogens when compared with healthy controls. In addition, cells from CGD patients did not display a defective L-tryptophan metabolism.

INTRODUCTION

Chronic granulomatous disease (CGD) is a rare (incidence 1/250,000 live births) inherited immunodeficiency disorder due to a defect in any of the structural components of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH-oxidase is a key player in innate host defense, and its activation results in production of superoxide anion and downstream reactive oxygen species (ROS). Clinically, CGD patients suffer from life-threatening bacterial and fungal infections and are characterized by abnormal inflammatory responses leading to granuloma formation. The precise “microbial” pathogenesis in CGD patients is unclear.

While the central role of ROS as direct antimicrobial effectors is still under discussion, ROS are also linked to key intracellular signaling events, such as the activation of transcription factors (1, 2), among which nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (3, 4), induction of mitogenesis (5, 6), and activation of proteases (7). ROS can also act as a substrate or cofactor for the catabolic indoleamine 2,3-dioxygenase enzyme (IDO) (8,11). IDO is the rate-limiting enzyme in the tryptophan metabolism (12). Activation of IDO results in decreased levels of L-tryptophan, thus actively depriving invading microorganisms of an essential amino acid (9, 13, 14). IDO is expressed in several tissues throughout the body and in immune cells like dendritic cells (DCs) (15), macrophages (16, 17), and eosinophils (18). IDO expression can be induced by mediators of immunity, such as interferon (IFN)γ (19, 20) and tumor necrosis factor (TNF)α (21). In addition, activation of tryptophan catabolism induces maternal immune tolerance (22), indicating that IDO can regulate T-cell responses. IDO can suppress the Th1 response (23-25), while in DC’s IDO is able to induce a Th2 response (26). In contrast, pulmonary IDO decreased Th2-driven experimental asthma (27). The immunomodulatory functions of IDO have made it an important target of research in many inflammatory conditions.

The tryptophan metabolism has also been linked to Th17 responses. In a collagen-induced arthritis mice model, L-kynurenine dampened the Th17 response (28). Furthermore, in a murine candidiasis model, retinoic acid receptor-related orphan receptorler C transcription was decreased by the induction of IDO (29). In a recent article, Romani and others (30) suggested that p47phox-deficient mice that have a phenotype reminiscent of CGD have a defective tryptophan metabolism, leading to an increased interleukin (IL)-17 production. It has thus been proposed that the activation of IDO and subsequently conversion of L-tryptophan to L-kynurenine suppresses the Th17 response.

A robust Th17 response is necessary for an adequate defense against Candida (31). Th17 cells are characterized by the production of IL-17A (32), which is important for attracting neutrophils to the tissues (33). The importance of IL-17A
in antifungal host defense is reflected by the fact that IL-17A receptor antagonist-deficient mice have an increased susceptibility to disseminated candidiasis (31). Furthermore, in a murine oropharyngeal candidiasis model, IL-17A- or IL-23p19-deficient animals showed increased severity of oropharyngeal candidiasis (34). Also humans with chronic mucocutaneous candidiasis (CMC) and hyper-IgE syndrome (HIES) have decreased Th17 responses that are believed to be responsible for their susceptibility to fungal infections (35-40).

CGD is the primary immunodeficiency with the highest incidence of invasive fungal infections (41), mainly due to Aspergillus spp. (lifetime incidence 25 %-40 %). Candida is commonly encountered in cases of fungal meningitis, fungemia and lymphadenitis (41-43). It is currently not clear why patients with CGD are specifically susceptible to fungal infections, although infections with bacterial pathogens such as Staphylococcus aureus are also often encountered. The absence of ROS has been shown not to be the direct explanation of the increased susceptibility (44). In the present study, the tryptophan metabolism and IL-17A response in human CGD patients were investigated. We demonstrate that CGD patients, in contrast to the p47phox-deficient mice, are able to convert L-tryptophan to L-kynurenine, and that they have a lower IL-17A production in response to stimulation with the fungal pathogen Candida albicans, which might explain their incapacity to clear fungal infections.

**MATERIALS AND METHODS**

**Patients and healthy volunteers**

Blood was collected from healthy volunteers who did not suffer from infectious or inflammatory diseases, and from eight CGD patients, harboring homozygous mutations in the NCF1 gene (p47-phox) or CYBB gene (p91-phox). After informed consent was given, blood was collected by venipuncture into 10 mL ethylenediaminetetraacetic acid tubes (367525; BD). The clinical characteristics of all patients are presented in Table 1.

**Reagents**

The following study materials were used: recombinant IFN-γ (Boehringer Ingelheim BV); L-kynurenine, L-tryptophan, 5-OH-tryptophan, serotonin, melatonin, niacin and resveratrol were purchased from Sigma-Aldrich; αCD3αCD28 beads were prepared from a T-cell activation/expansion kit (130-091-441, MACS, Miltenyi Biotec); lipopolysaccharide (LPS) (Escherichia coli serotype 055:B5) was purchased from Sigma and an extra purification step was performed as described previously (45). Purified LPS was tested in Toll-like receptor (TLR) 4-/- mice for the presence of

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Disease history</th>
<th>Active injection at time of exp</th>
<th>Mutation</th>
<th>Interferon therapy at time of exp</th>
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<td>No</td>
<td>CYBB (g91-phox)</td>
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</table>

| Table 1 Clinical characteristics of Chronic Granulomatous Disease patients |
contaminants and did not have any TLR4-independent activity (46). As culture medium, RPMI 1640 Dutch modifications (Sigma-Aldrich) was used, supplemented with 1% gentamicin, 1% f-glutamine and 1% pyruvate (Life Technologies).

Microorganisms
C. albicans ATCC MYA-3573 (UC 820) (47) 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 1h at 100°C; S. aureus (ATCC 25923) was heat-killed for 30 min at 100 °C; heat-killed conidia and hyphae of Aspergillus fumigatus (V4507) and Aspergillus nidulans (V44-46) were prepared as described previously (44, 48).

Human mononuclear cells and neutrophils
Peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated from peripheral blood using density gradient centrifugation (800g) of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech) (49). PBMCs were harvested, washed twice in phosphate buffered saline (PBS), and resuspended in culture medium. To remove the erythrocytes from the neutrophils, the pellet was shocked at least twice with an ice-cold lysing reagent (2.75 g NH4Cl + 0.25 g KHCO3) for 15 min. Subsequently, cells were washed in PBS and resuspended in culture medium. The PBMCs and neutrophils were counted in a hemocytometer, and their concentration was adjusted to 5 x 10^6 cells/ml.

About 5x10^5 PBMCs or neutrophils in a total volume of 200 µL per well were incubated at 37°C in round-bottomed 96-well plates (Greiner) with the different stimuli, as indicated in the figure legends. After 24 h, 48 h or seven days of incubation, supernatants were collected and stored at –20°C until assayed. When cells were incubated at 37°C; cells were harvested by centrifugation, washed twice, and resuspended in culture medium.

Enzyme-linked immunosorbant assay
The concentration of IFN-γ, IL-6, IL-17A, and TNF-α was measured in cell culture supernatants using enzyme-linked immunosorbent assay (ELISA) (IL-17A and TNF-α: R&D Systems; IFN-γ and IL-6: Sanquin), according to the instructions of the manufacturer.

Real-time polymerase chain reaction
One million freshly isolated PBMCs or neutrophils were incubated with the various stimuli. After 24 h of incubation at 37°C, total ribonucleic acid (RNA) was extracted in 400 µl of TRIzol reagent (Invitrogen). Isolated RNA was being reverse transcribed into complementary DNA using oligo(dT) primers and MMLV reverse transcriptase. Polymerase chain reaction (PCR) was performed using a 7300 realtime PCR system (Applied Biosystems). The primer sequences for human IDO are as follows: 5-GGT-CAT-GGA-GAT-GTC-CGT-AA-3 (forward) and 5-ACC-AAT-AGA-AGG-ACC-AGG-AA-3 (reverse). β2M was used as a reference gene, for which the primers were 5-ATG-AGT-CCT-GCC-GTC-TG-3 (forward) and 5-CCA-AAT-GCG-GCA-TCF-CAA-AAC-3 (reverse). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of PCR at 95°C for 15 seconds, and 60°C for 1 min. Data are expressed as fold increase compared to the unstimulated sample.

High-performance liquid chromatography
To compare the amount of L-tryptophan metabolized and the amount of L-kynurenine produced upon stimulation with fungal pathogens between CGD patients and healthy controls, cell culture supernatants were subjected to high-performance liquid chromatography (HPLC) analysis. HPLC was performed on a Spectra-SYSTEM autosampler and pump (Thermo Separation Products). Chromatographic separation was performed using an Inertsil 5 ODS-2 column (100 mm x 3.0 ID; Varian Inc.). Absorbance was monitored with a diode-array detector (UV6000LP; Thermo Separation Products) at wavelength of 280 nm for tryptophan and 360 nm for kynurenine. The mobile phase for isocratic elution was made by dissolving 40 mM sodium acetate. The pH of the eluent was adjusted to pH 4.5 with a solution of 40 mM citric acid and 2% acetonitrile of the total volume buffer was added. The continuous flow rate was 0.3 mL per minute (51). Data are presented as area under the curve.

Statistical analysis
Experiments were performed in duplicate, and supernatants were pooled. The differences between groups of 3 or more subjects were analyzed using the Mann-Whitney U test or the Wilcoxon signed rank test, for unpaired and paired data, respectively. The level of significance between groups was set at P < 0.05 (*) and P < 0.01 (**). Data are presented as mean ± standard error of the mean.

RESULTS
CGD patients have an increased production of proinflammatory cytokines in vitro
The cytokine profile of CGD patients upon stimulation with several stimuli was investigated. PBMCs of CGD patients produced more IL-6 and TNF-α upon stimulation with the specific TLR4 ligand LPS (Figure 1). Similarly, heat-killed C. albicans induced more IL-6 and TNF-α production in PBMCs isolated from CGD patients (Figure 1). In line with this, stimulation with live A. nidulans and A.
fumigatus conidia, two commonly encountered species in CGD patients, resulted in a higher cytokine production by PBMCs of CGD patients compared with healthy controls (Figure 1). Heat-killed Aspergillus conidia and hyphae showed to be poor stimulators of the proinflammatory cytokines.

**CGD patients have an intact L-tryptophan metabolism**

Romani et al. (30) showed that p47phox-deficient mice have a hyperinflammatory phenotype due to a defect in the conversion of L-tryptophan into L-kynurenine. Therefore we assessed whether the increased production of proinflammatory cytokines in patients with CGD could also be explained by an incapability to convert L-tryptophan into L-kynurenine. Cells isolated from CGD patients had a higher transcription of IDO upon stimulation with Candida and IFN-γ in both PBMCs and neutrophils, compared with healthy controls (Figure 2A). Moreover, upon stimulation with Candida, both healthy controls and CGD patients were able to convert L-tryptophan into L-kynurenine (Figure 2B). The conversion of L-tryptophan to L-kynurenine was independent of which fungal pathogen was used for stimulation (data not shown). This indicates that IDO functions normally in human CGD patients.

The tryptophan metabolite L-kynurenine has been suggested to be responsible for dampening the proinflammatory cytokine response, and has especially been associated with a lowering of IL-17A production by murine cells (28, 52). To determine whether L-kynurenine is able to influence the IL-17A response in human cells *in vitro*, we tested Candida-induced IL-17A production in healthy volunteers in the absence or presence of tryptophan metabolites. PBMCs from healthy controls were also stimulated with Candida in the absence or presence of increasing concentrations of L-kynurenine. There was no clear dose-response relationship between L-kynurenine and IL-17A (Figure 2C). Furthermore, L-tryptophan significantly increased Candida-induced IL-17A production (Figure 2D), while the other metabolites (5-OH-tryptophan, serotonin, melatonin and resveratrol) did not influence Candida-induced IL-17 production.

**CGD patients have a low IL-17A and high IFNγ production upon stimulation with fungal pathogens**

To investigate whether IL-17A production is affected in cells from CGD patients, PBMCs from CGD patients and healthy controls were stimulated for 7 days with αCD3αCD28-coated beads, LPS, different morphotypes of *A. nidulans* and *A. fumigatus*, and heat-killed *C. albicans*. There was no difference in αCD3αCD28-induced IL-17 production between CGD patients and healthy controls (Figure 3A). It is well established that especially in hyphal antifungal host-defense, rapid and efficient recruitment of neutrophils is important (53). Here we found that in response to *A.
Figure 2  CGD patients do not have a defective L-tryptophan metabolism. (A) PBMCs and neutrophils of 2 healthy volunteers and of 3 CGD patients were stimulated for 48 h with heat-killed *C. albicans* (1x10⁶/mL) or IFN-γ (50 ng/mL). IDO expression was measured using qPCR. Bars represent mean + SEM. (B) PBMCs of healthy volunteers and CGD patients were cultured for 48 h in the presence of L-tryptophan (50 µg/mL), with or without heat-killed *C. albicans* (1x10⁶/mL). L-Tryptophan (285 nm; black lines and arrows) and L-kynurenine (360 nm; grey lines and arrows) were measured using HPLC. (C) PBMCs of healthy volunteers were stimulated for 7 days in the presence of 10% human pool serum with heat-killed *C. albicans* yeast (1x10⁶/mL), in the absence or presence of L-kynurenine (200 ng/mL, 1 µg/mL or 10 µg/mL). IL-17 was measured in cell culture supernatants using ELISA. Experiments were performed in duplicate and cell culture supernatants were pooled. Data represent at least 7 healthy volunteers from at least 3 different experiments. Data were analyzed using the Wilcoxon signed rank test (* P < 0.05). Bars represent mean + SEM. (D) PBMCs were stimulated for 7 days in the presence of 10% human pool serum with heat-killed *C. albicans* yeast (1x10⁶/mL), in the absence or presence of L-tryptophan (50 µg/mL), 5-OH-tryptophan (200 ng/mL), serotonin (1 µg/mL), melatonin (200 µg/mL), or resveratrol (10 µg/mL). IL-17A was measured in cell culture supernatants using ELISA. Experiments were performed in duplicate and cell culture supernatants were pooled. Data represent at least 5 healthy volunteers from at least 2 different experiments. Data were analyzed using the Wilcoxon signed rank test (** P < 0.01). Bars represent mean + SEM. IDO, indoleamine 2,3-dioxygenase; qPCR, quantitative polymerase chain reaction; HPLC, high-performance liquid chromatography.
Figure 3 CGD patients have a low IL-17A and high IFN-γ production upon stimulation with fungal pathogens. (A) PBMCs of healthy controls and CGD patients were stimulated for 7 days in the presence of 10% serum with RPMI or αCD3αCD28-coated beads. IL-17A was measured in cell culture supernatants using ELISA. Data represent 4 CGD patients and 5 healthy controls from 3 different experiments. (B) PBMCs of healthy controls and CGD patients were stimulated for 7 days in the presence of 10% serum with RPMI, heat-killed *A. nidulans* (AN) and *A. fumigatus* (AF) conidia or hyphae, or LPS (10 ng/mL). IL-17A and IFN-γ were measured in cell culture supernatants using ELISA. Data represent at least 5 CGD patients and 6 healthy controls from 4 different experiments. (C) PBMCs of healthy controls and CGD patients were stimulated for 7 days in the presence of 10% serum with RPMI or heat-killed *C. albicans* (1x10⁶/mL). IL-17A was measured in cell culture supernatants using ELISA. Data represent 8 CGD patients and 8 healthy controls from 7 different experiments. Data were analyzed using the Mann-Whitney U test (*P < 0.05). Data are presented separately, and as mean ± SEM.
midals hyphae and A. fumigates hyphae, the IL-17A production was relatively low in CGD patients although this was not significant (Figure 3B). CGD patients produced more IFN-γ in response to all stimuli (Figure 3B). PBMCs of CGD patients produced significantly less IL-17A upon stimulation with C. albicans (Figure 3C).

**DISCUSSION**

In the current study we demonstrate that human CGD patients produce markedly lower IL-17A concentrations upon stimulation with the fungal pathogen C. albicans compared with healthy controls, despite an overall higher proinflammatory cytokine production. CGD patients are able to convert L-tryptophan into L-kynurenine, and ROS are not crucial for the IDO activity, which is needed to facilitate this conversion in humans. Furthermore, L-kynurenine did not decrease Candida-induced IL-17A production. These data suggest that NADPH-oxidase deficiency in humans is not associated with a defective tryptophan metabolism, but with a lower Th17 response against fungal pathogens that might explain their susceptibility to fungal infections.

Th17 responses have recently been described as a new T helper subset (54). In recent years it has become apparent that the Th17 response is essential for antifungal host defense. The recurrent mucosal Candida infections in patients with primary immunodeficiency syndromes such as HIES and CMC have been specifically linked to a deficiency in their Th17 response (35, 38, 40).

The role of the Th17 response in the host defense against Aspergillus is controversial. On the one hand, IL-17 deficiency is associated with a higher susceptibility to Aspergillus infection in mice (55, 56). On the other hand, studies suggest that a high IL-17 production is linked to a higher susceptibility to Aspergillus infection due to detrimental effects caused by a hyperinflammatory response (30, 57). These observations do not need to be contradictory, and they might support the concept that there is an optimal window for IL-17 response in the host defense against Aspergillus.

The decreased IL-17 production in response to fungal PAMPs was rather unanticipated. We have previously shown that CGD patients have relatively high IL-18 production in response to fungal stimuli (58). IL-18 is a driver of Th17 responses (59) and therefore we predicted a high Th17 response. Furthermore, it has been reported that T cells from CGD patients have increased IL-17 and IFN-γ production in response to mitogenic stimulation, because NADPH-oxidase-deficient macrophages cannot sufficiently induce Tregs that are needed to control pro-inflammatory T helper responses (60). Interestingly we observed no difference in uCD3αCD28 induced IL-17 production between CGD patients and healthy controls, indicating that the decreased IL-17 production observed in CGD patients is only present in the presence of a specific pathogen. This might explain why the rest of the phenotype of CGD patients is not similar to HIES or CMC.

A difference in proportion of γδ T-cells in PBMCs from CGD patients compared with healthy controls could be an explanation for the observed differences in IL-17A production (61). Therefore we have determined the relative contribution of the γδ T-cells to IL-17A production in response to Candida in healthy volunteers. We found that a very low amount of γδ T-cells was IL-17 positive compared to CD4+ T cells (data not shown). These data suggest that the large proportion of IL-17 in response to C. albicans is from the CD4+ T-cell population. Since CD4 lymphopenia is not described in CGD patients it is unlikely that differential T-cell counts are responsible for the low IL-17A production in CGD patients.

It is interesting to observe that both p47phox−/− deficient mice and patients with CGD are susceptible to Aspergillus infections, but the underlying mechanisms seem to differ. In mice it was suggested that the tryptophan metabolism was defective, which led to an increased level of IL-17 production that was detrimental for the mice. Here we confirm that in CGD patients the tryptophan metabolism is intact (62), and moreover we observed that they had a lower IL-17 production in response to fungal stimulation. In addition, L-kynurenine did not decrease IL-17A production induced by C. albicans in PBMCs of healthy controls, indicating that in human primary cells L-kynurenine does not suppress the Th17 response. However, L-tryptophan itself could increase Candida-induced IL-17A production in healthy controls. In line with this, Veldhoen and others (63) previously demonstrated that L-tryptophan is one of the aryl hydrocarbon receptor (AHR) agonists that can increase IL-17A production. The role of the tryptophan/kynurenine metabolism of the different human leucocytes was recently evaluated by De Ravin and others (62). They compared cultured DC, monocytes and isolated PMNs. In line with our data obtained from monocytes, De Ravin and others (62) found that highly purified PMN could indeed upregulate IDO expression after IFN-γ treatment.

In contrast to a low IL-17A production, we observed that the cells from CGD patients have an increased IFN-γ production compared with healthy controls in response to fungal stimuli. IFN-γ stimulates intracellular killing of C. albicans conidia by PMN in vitro (64, 65), and in a recent overview it has been reported that a large proportion of patients with defects in IL-12Rβ1 experience fungal infections (66). However, both IFN-γ and IL-17 are required for an adequate antifungal host defense. Although there is no defect in IFN-γ production, the low IL-17A production might cause a sensitivity to fungal infections. CGD patients also have an increased susceptibility to S. aureus infection. Susceptibility to S. aureus has also been associated with a defective IL-17A response (67, 68), which is in line with the data presented here.
In conclusion, human CGD patients have a specific lower production of fungal pathogen-induced IL-17A compared with healthy controls, while mitogenic stimulation of T-cells isolated from CGD patients does not result in an impaired IL-17 production. This might explain their inability to clear fungal infections. Furthermore, in contrast to a mouse CGD model, human CGD patients do not have a defect in their L-tryptophan metabolism to explain their susceptibility to fungal infections and their hyperinflammatory status.

Acknowledgements

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REFERENCES


Aspergillus nidulans and Chronic Granulomatous Disease: a Unique Host-Pathogen Interaction

Stefanie S.V. Henriët, Paul E. Verweij, Adilia Warris

ABSTRACT

Invasive fungal infections are a major threat for patients suffering from chronic granulomatous disease (CGD), a primary immunodeficiency caused by a defect in the nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-oxidase. Interestingly, Aspergillus (Emericella) nidulans is the second most encountered mold in CGD patients, causing almost exclusively invasive infections in this specific host, and is characterized by its aggressive behavior. A proper diagnosis is complicated by the often-mild clinical presentation, the low sensitivity of the currently used diagnostic tools, and the difficulties in accurate identification of the Emericella species. According to the hitherto accepted view on the role of the NADPH-oxidase in the innate host-defense pathway, the pathogenesis of A. nidulans in CGD cannot be explained. This synopsis covers the current understanding of invasive infections caused by A. nidulans in the CGD patient and is intended to direct further research by indicating gaps in our knowledge and to guide optimal management strategies.

INTRODUCTION

Aspergillus nidulans (Teleomorph Emericella nidulans) has been an important research organism for studying eukaryotic cell biology for over half a century. It has contributed to our understanding of cell cycle control, DNA repair, mutation, recombination, cytoskeletal function, mitochondrial DNA structure and human genetic disease (1). Much less attention had been given to A. nidulans as an opportunistic pathogen in humans until recently, when it was recognized as a major cause of invasive aspergillosis (IA) in patients with chronic granulomatous disease (CGD). CGD is a rare (birth prevalence 1:200,000) inherited immunodeficiency disorder of the NADPH-oxidase in which phagocytes fail to generate superoxide anion and downstream reactive oxygen species (ROS) (2, 3). CGD is a genetically heterogeneous disease caused by mutations in any of the 5 structural components of NADPH-oxidase, including the membrane-bound glycoproteins gp91phox (phagocyte oxidase), p22phox, and the cytoplasmatic components p47phox, p67phox and p40phox (4). As a result of the defect in the key innate host defense pathway, CGD patients suffer from life-threatening bacterial and fungal infections and inflammatory sequelae. Invasive fungal infections are often the first manifestation, revealing the underlying disease.

Aspergillus spp. are the most important encountered fungal pathogens (4). In more detailed information extracted from the published data of CGD registries, the percentage of patients who had at least one infectious episode caused by Aspergillus spp. ranges from 26% in Europe (5) up to 46% in Japan (6). Furthermore, Aspergillus spp. are the most common isolated causative pathogens in cases of pulmonary infections, brain abscesses, and osteomyelitis (2, 3, 5, 7) (Table 1). As a cause of death, fungal infections stand at the top with Aspergillus spp. being responsible for one-third to half of all deaths. Suggestion was made that CGD patients were at greater risk of A. nidulans infection than other immune-compromised patient populations and that A. nidulans was more virulent than A. fumigatus based on mortality rates and propensity to spread (8, 9). Although A. nidulans behaves more virulent in the CGD patient compared to A. fumigatus, clear incidences about IA due to A. nidulans are lacking. Scrutinizing the microbiology database of CGD at the National Institute of Health (NIH, Bethesda, MD) revealed six A. nidulans infections compared to 17 caused by A. fumigatus. In patients with fungal osteomyelitis, A. nidulans has been isolated in up to 50% of the cases (8, 10). Recently, we performed an extensive review of all PubMed database notated papers from 1970 to 2010, regarding invasive fungal infections in patients suffering from CGD. In the 116 reported cases, 127 fungal species were isolated: A. nidulans was the second most encountered species (23 cases, 18%) preceded by A. fumigatus (44 cases, 35%). Zygomycotes isolates were found in 9%, whereas Candida species in only 6% (10).
Focusing on the Aspergillus species, A. fumigatus was found in 55% followed by A. nidulans in 35%.

Aspergillus nidulans seems to have a remarkable unique interaction with CGD patients as it remains a rare pathogen among other risk groups. In the neutropenic host only three cases have been described: two pulmonary IA with highly resistant strains (13, 14) and a primary cutaneous infection associated with a Hickman catheter (15). In the Transplant-Associated Infections Surveillance Network (TRANSNET) report, A. fumigatus was isolated in 187 (44%) of the 425 cases of IA. Thirteen (3%) infections were caused by “other Aspergillus species”, however, no notification of A. nidulans was made (16). In a cohort of 139 cases of pediatric IA, including seven CGD patients, A. fumigatus was isolated in 67 cases (52.8%) compared to A. nidulans in only one patient (0.8%) (17).

Overall, as disease pathology and progression are the results of the complex interaction between the pathogen and the host, exploring the host-fungus interface will result in more insight and detailed understanding of this challenging frontline, in order to optimize diagnostic and therapeutic strategies. This synopsis covers the current understanding of invasive infections caused by A. nidulans in the CGD patient and addresses important areas for future research aiming at optimizing patient care.

### ASPERGILLUS NIDULANS

**Species identification and molecular characterizations**

Identification of A. nidulans is based predominantly upon the morphology of the conidia and conidiophores. Aspergillus nidulans is a homothallic species capable of producing the teleomorph (sexual stage) without mating studies. The dual nomenclature of members of the Aspergillus section Nidulanti may be confusing for the clinician, as the ability of the fungus to produce a sexual state depends on the culture conditions.

The application of molecular tools has had major impact on the taxonomy of fungi. Multilocus sequence-based phylogenetic analyses have emerged as the primary tool for inferring phylogenetic species boundaries and relationships within subgenera and sections. Sequence analyses of the internal transcribed spacer region appears to be appropriate for identification of Aspergillus isolates to the subgenus/section level (18). Partial β-tubulin or calmodulin are the most promising loci for Aspergillus identification to the species level.

### In vitro susceptibility testing

The efficacy of antifungal agents is different for the various Aspergillus spp., and A. nidulans reveals to be more resistant to amphotericin B compared to A. fumigatus.

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<td>Italy (7)</td>
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<td>23</td>
<td>60</td>
<td>23</td>
<td>0</td>
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<td>0</td>
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<td>Spain (11)</td>
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<td>Sweden (12)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
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</tbody>
</table>

Abbreviations: CGD, chronic granulomatous disease; NA, not available.
CHAPTER 5

ASPERGILLUS NIDULANS IN CGD

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Diagnosis

Information on diagnostic tools was provided in 22 of the 25 patients reviewed in this study. Twenty-one of them fulfilled the criteria for proven invasive mold infection. Histopathology and direct microscopy or culture of a biopsy taken during open surgery was conclusive in 67% (n=14) of the proven cases. Pulmonary radiologic features were present in 75%. Interestingly, beyond description of “pulmonary consolidation” and “lung infiltrates”, no specific signs as nodules, air crescent formation, halo signs or cavitations were described or could be detected on the published images. Computed tomography and magnetic resonance established extra-pulmonary extension to soft tissues, bones and spinal cord injury. Local extension of disease from lung parenchyma to adjacent structures and osteomyelitis of the thoracic skeleton have been found particularly associated with underlying CGD (40).

In four of the reported CGD cases, information about circulating antigens in serum could be extracted (25, 33, 34, 36). Three of them were negative despite the extensiveness of the disease. Only van ‘t Hek described positive galactomannan ratios in a X-linked CGD patient suffering from invasive A. nidulans infection and chest-wall invasion (36). Information on diagnostic polymerase chain reaction was retrieved in two proven cases and one probable; however, results were negative or inconclusive (25, 34, 35). Four cases mentioned strongly positive anti-Aspergillus antibodies (27, 29, 33, 38).

Treatment and Outcome

Combined antifungal treatment and extensive and early surgical debridement was used in most patients (83%). All patients except two received presumptive treatment with amphotericin B. Conventional amphotericin B deoxycholate (range 0.6-1.5 mg/kg/day intravenously) was used in 91%. Usually, treatment was initiated as a monotherapy (71%); combination therapy was started only in six cases by the addition of itraconazole, 5-flucytosine or caspofungin. Use of voriconazole was first reported in 1998 and used in five cases, only once as a first-line treatment (41). Twelve patients (50%) received granulocytes, and 7 patients (30%) received interferon (INF)-γ in addition to the antifungal therapy. The use of INF-γ as adjunctive therapy of IA in CGD patients has not been investigated by controlled studies and remains controversial. In those who did not receive surgery and survived, infection was limited to the lung or with minimal involvement of adjacent structures (30, 35).

Unambiguous data on clinical outcome was lacking, and follow-up ranged from “still under treatment” to eight years. At the time cases were published, the mortality rate was 32%. The exact mortality rate of A. nidulans invasive infection in the CGD patient is difficult to determine, and an underestimation cannot be ruled out because of the huge variability in follow-up.

INVASIVE A. NIDULANS INFECTIONS

Twenty-five cases of IA due to A. nidulans in CGD patients were previously reported. The major clinical features are summarized in Table 2. Twenty-three were male, and median age was 7.5 years (range, 3-21 years). Of those whose genetic pattern was reported (n=19), 89% were X-linked. The most common localization (72%) is lung invasion with direct spread to adjacent chest-wall structures (Figure 1). The presenting signs and symptoms were often mild with low-grade fever, local pain or swelling, malaise and cough, but could be completely silent with asymptomatic new lung infiltrates detected during a routine visit.

Figure 1 Computed tomography scan of A. nidulans infection in a patient with chronic granulomatous disease. Note the extensive chest wall invasion and subcutaneous infiltration (arrow).
### Table 2 The Major Clinical Features of Invasive *A. nidulans* Infections in CGD Patients

<table>
<thead>
<tr>
<th>Case (Ref.)</th>
<th>Sex</th>
<th>Genetic Type</th>
<th>Age, y</th>
<th>Previous Fungal Infection</th>
<th>Site of Disease</th>
<th>Mechanism of Spread</th>
<th>Prophylaxis</th>
<th>Treatment</th>
<th>Surgery</th>
<th>Outcome</th>
</tr>
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<tbody>
<tr>
<td>1(25)</td>
<td>M</td>
<td>NA</td>
<td>16</td>
<td>No</td>
<td>Osteomyelitis, long bone</td>
<td>No spread</td>
<td>INF-γ</td>
<td>AMB, AMBL, ITC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>2(26)</td>
<td>M</td>
<td>NA</td>
<td>6</td>
<td>No</td>
<td>Lung, chest wall, vertebrae</td>
<td>Direct</td>
<td>TMP-SMX</td>
<td>AMB, AMBL, ITC</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>3(27)</td>
<td>M</td>
<td>X-CGD</td>
<td>20</td>
<td>No</td>
<td>Lung, 3rd rib, femur, skull</td>
<td>Direct</td>
<td>NA</td>
<td>AMB, ITC, 5-FC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>4(28)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>No</td>
<td>Lung, 8th-9th ribs, T6-L1 vertebrae</td>
<td>Direct</td>
<td>NA</td>
<td>AMB, AMBL, gran Tx</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>5(28)</td>
<td>M</td>
<td>X-CGD</td>
<td>9</td>
<td>No</td>
<td>Lung, 4th rib</td>
<td>Direct</td>
<td>NA</td>
<td>AMB</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>6(28)</td>
<td>M</td>
<td>X-CGD</td>
<td>13</td>
<td>Yes, <em>A. nidulans</em> pneumonia and osteomyelitis 4th rib at the age of 9</td>
<td>Progression of 4th rib lesion, T3-T4 with paraparesis</td>
<td>Direct</td>
<td>NA</td>
<td>AMB</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>7(9)</td>
<td>M</td>
<td>X-CGD</td>
<td>6</td>
<td>Yes, <em>Aspergillus</em> spp.</td>
<td>Lung, pleura</td>
<td>No spread</td>
<td>INF-γ</td>
<td>AMB, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>8(9)</td>
<td>M</td>
<td>X-CGD</td>
<td>19</td>
<td>No</td>
<td>Lung, pleura, chest wall, vertebrae, skin, skull, brain</td>
<td>Direct</td>
<td>INF-γ after first event but stopped - relapse &lt;1y</td>
<td>AMB, ITC, 5-FC, gran Tx</td>
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<td>Died</td>
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<tr>
<td>9(9)</td>
<td>M</td>
<td>X-CGD</td>
<td>16</td>
<td>Yes, <em>Aspergillus</em> spp.</td>
<td>Lung, pleura, vertebrae, chest wall</td>
<td>Direct</td>
<td>KTC</td>
<td>AMB, AMBL, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>10(9)</td>
<td>M</td>
<td>X-CGD</td>
<td>7</td>
<td>No</td>
<td>Lung, pleura, vertebrae, chest wall, sinuses, brain</td>
<td>Direct</td>
<td>NA</td>
<td>AMB, ITC, gran Tx</td>
<td>Yes</td>
<td>Died</td>
</tr>
<tr>
<td>11(9)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>No</td>
<td>Lung</td>
<td>No spread</td>
<td>INF-γ until 1 mo before <em>A. nidulans</em> infection</td>
<td>AMB, ITC</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>12(9)</td>
<td>M</td>
<td>NA</td>
<td>6</td>
<td>No</td>
<td>Lung, ribs, vertebrae T1-T8, spinal cord</td>
<td>Direct</td>
<td>No</td>
<td>AMB</td>
<td>Yes</td>
<td>Died</td>
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<tr>
<td>13(30)</td>
<td>M</td>
<td>X-CGD</td>
<td>8</td>
<td>No</td>
<td>Lung, rib</td>
<td>Direct</td>
<td>Clindamycin</td>
<td>AMB, gran Tx</td>
<td>No</td>
<td>Survived</td>
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<tr>
<td>14(31)</td>
<td>M</td>
<td>NA</td>
<td>10</td>
<td>No, but pneumonia not responding to antimicrobial therapy, including tuberculosis drugs: subtotal right upper lobeectomy at 6</td>
<td>Lung, pleura, axillary abscess, 2nd-3th ribs, vertebrae</td>
<td>Direct</td>
<td>No</td>
<td>AMB</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>X-CGD</td>
<td>NA</td>
<td>NA</td>
<td>Lung, brain</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Survived</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>NA</td>
<td>4</td>
<td>NA</td>
<td>Lung</td>
<td>No spread</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Survived</td>
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<tr>
<td>17</td>
<td>M</td>
<td>X-CGD</td>
<td>19</td>
<td>NA</td>
<td>Lung, chest wall, brain</td>
<td>NA</td>
<td>NA</td>
<td>AMB, AMBL, ITC, 5-FC, FLC, gran Tx</td>
<td>No</td>
<td>Died</td>
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<tr>
<td>18(32)</td>
<td>F</td>
<td>p67&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>3</td>
<td>No, but borderline positive Mantoux test, consolidation on RX, no improvement with tuberculosis therapy or antibiotics</td>
<td>Endocarditis, skin lesions, blood</td>
<td>Hematogenous</td>
<td>TMP</td>
<td>AMB</td>
<td>Yes</td>
<td>Died</td>
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<tr>
<td>19(33)</td>
<td>M</td>
<td>X-CGD</td>
<td>21</td>
<td>Yes, <em>A. fumigatus</em> pneumonia at the age of 10, brain focus at the age of 13</td>
<td>Lung, popliteal abscess, soft tissues hemithorax, spinal cord, T5-T7 vertebrae</td>
<td>Direct/Hematogenous</td>
<td>TMP-SMX, ITC, INF-γ, poor compliance</td>
<td>AMB, AMBL, VOR, CAS, gran Tx</td>
<td>Yes</td>
<td>Died</td>
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<td>20(34)</td>
<td>M</td>
<td>X-CGD</td>
<td>4</td>
<td>No</td>
<td>Lung, T2/T5 vertebrae, spinal cord</td>
<td>Direct</td>
<td>TMP-SMX, ITC</td>
<td>AMB, AMBL, VOR, POS, CAS, gran Tx</td>
<td>Yes</td>
<td>Survived (ex vivo gene therapy)</td>
</tr>
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</table>
Studies on the role of NADPH-oxidase activity in killing A. fumigatus conidia by alveolar macrophages (AM) have produced contrasting interpretations. Experimental studies with AM from gp91phox−/− mice showed phagocytosis and killing rates comparable to AM from normal mice, indicating that NADPH-oxidase-independent mechanisms in murine AM are pivotal to the inhibition of conidial germination (41, 45, 46). In contrast, AM from p47phox−/− mice were unable to kill A. fumigatus conidia (47). Furthermore, inhibitors of NADPH-oxidase that decreased the production of reactive oxidant intermediates inhibited the killing of A. fumigatus in normal murine AM (48). By comparing the killing ratio of A. fumigatus and A. nidulans by gp91phox−/− AM and healthy AM, we confirmed that gp91phox−/− AM were at least as efficient in killing these two species as healthy AM are (49). Although differences in animal strains, morphotypes, cell sources, and methods to assay the fungal damage might be responsible for this discrepancy, this heterogeneity underscores the complexity of fungal resistance and the fact that other mechanisms than killing by ROS must be involved as well.

Reactive-oxygen species like H$_2$O$_2$ seem to act as chemoattractants (50). Furthermore, by studying IA in an experimental murine model, it was suggested that early polymorphonuclear neutrophil (PMN) recruitment is crucial. PMN recruitment to the lungs shows to be slower in gp91phox−/− mice, resulting in increased germination. More extensive hyphal proliferation and tissue invasion were observed in the lungs of gp91phox−/− mice, indicating that when the lungs are exposed to large numbers of conidia, early PMN recruitment and formation of

### Table 2 Continued

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Genetic Type</th>
<th>Age, y</th>
<th>Previous Fungal Infection</th>
<th>Site of Disease</th>
<th>Mechanism of Spread</th>
<th>Prophylaxis</th>
<th>Treatment</th>
<th>Surgery</th>
<th>Outcome</th>
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<td>21(35)</td>
<td>M</td>
<td>XCGD</td>
<td>13</td>
<td>No</td>
<td>Lung</td>
<td>No spread</td>
<td>TMP-SMX, ITC but diarrhea and serum levels</td>
<td>AMB, VOR, CAS</td>
<td>No</td>
<td>Survived</td>
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<tr>
<td>22(36)</td>
<td>M</td>
<td>p22phox</td>
<td>5</td>
<td>No</td>
<td>Lung, chest wall cutaneous abscess</td>
<td>Direct</td>
<td>NA</td>
<td>AMB, VOR, ITC, gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>23(37)</td>
<td>M</td>
<td>XCGD</td>
<td>8</td>
<td>No</td>
<td>Lung, 6th rib, chest wall fistula over the rib, psoas abscess</td>
<td>Direct</td>
<td>TMP-SMX, ITC; stopped 4 wk prior to A. nidulans infection</td>
<td>AMB, AMBL, gran Tx</td>
<td>Yes</td>
<td>Survived (BMT)</td>
</tr>
<tr>
<td>24(38)</td>
<td>M</td>
<td>XCGD</td>
<td>8</td>
<td>No</td>
<td>Lung, chest wall, T8-T11 vertebrae, 7th rib</td>
<td>Direct</td>
<td>NA</td>
<td>AMB, 5-FC, ITC gran Tx</td>
<td>Yes</td>
<td>Survived</td>
</tr>
<tr>
<td>25(39)</td>
<td>M</td>
<td>NA</td>
<td>5</td>
<td>NA</td>
<td>Lung, chest wall, vertebrae, spinal cord syringomyelia</td>
<td>Direct</td>
<td>NA</td>
<td>VOR</td>
<td>Yes</td>
<td>Survived</td>
</tr>
</tbody>
</table>

Abbreviations: 5-FC, 5-flucytosine; ABLC, amphotericin B lipid complex; AMB, amphotericin B deoxycholate; AMBL, amphotericin B liposomal; BMT, Bone marrow transplantation; CAS, caspofungin; gran Tx, granulocyte transfusion; INF-γ, interferon-gamma; ITC, itraconazole; KTC, ketoconazole; TBF, terbinafine; TMP-SMX, trimethoprim-sulfamethoxazole; NA, not available; PCZ, pozaconazole; VOR, voriconazole; FLIC, fluconazole;

### PATHOGENESIS AND HOST DEFENSE

By comparing invasive A. nidulans infections in the CGD host (n=25) with those caused by A. fumigatus (n=44), A. nidulans infections are often asymptomatic, behave more aggressively, and are significantly more likely to result in death. Primary lung involvement was followed in 75% (versus 14% for A. fumigatus) by extensive tissue destruction and direct spread to adjacent chest-wall structures (8-10). Only two studies with p47phox−/− mice provide histopathology data on A. nidulans infections, but no direct comparison was made to A. fumigatus infections (42, 43). Besides the fact that comparable fatal A. fumigatus infections were observed in the X-linked murine CGD model and that the histopathology data do support a role for aberrant inflammation, not more can be concluded from these studies.

Most studies focusing on innate immune responses against Aspergillus spp. have used A. fumigatus due to the fact that this species is the most commonly encountered causative agent of IA. Knowledge of the host response against other Aspergillus spp. is scarce, in particular with respect to A. nidulans. The first line of host-defense is directed against conidia, the infective form of the filamentous fungi, and consists of macrophages. The macrophages will kill the germinating spores intracellularly by mainly non-oxidative processes. The second line of defense against mold infections is superoxide production by neutrophils, a powerful mechanism to kill the invasive hyphal structures of filamentous fungi such as Aspergillus spp., and is missing in CGD (44) (Figure 2).
immunological response induced by A. nidulans is subject to debate, as earlier virulence studies of A. nidulans in neutropenic mice showed a significant later killing of conidia (45). These data were confirmed by microarray data of murine C57Bl/6 and gp91phox−/− AM exposed to A. fumigatus conidia in vivo: the most marked early transcriptional changes did not indicate obvious NADPH-oxidase involvement but occur in genes involved in PMN recruitment (46).

We recently explored the central role of the NADPH-oxidase, and the resulting ROS, in direct antifungal host defense. In vitro infection of circulating human leucocytes revealed that resistance to A. nidulans is not directly ROS related. We showed that A. nidulans, in contrast to A. fumigatus, is not susceptible to ROS. Infection of healthy PMN and peripheral blood mononuclear cells (PBMCs) by live A. nidulans did not result in any measurable ROS release, and the microbicidal activity of CGD PMN was maintained toward A. nidulans but not to A. fumigatus (49). These results indicate that the etiology of A. nidulans infections in CGD cannot be explained by the simple absence of the direct microbicidal effect of ROS.

In the early 1980s, it was suggested that abnormal pH regulation within the phagosome of CGD phagocytes might have a role in defective killing (51). The basis of this assumption was that the initiation of superoxide production is normally accompanied by phagosomal alkalization as a result of the proton-acceptor function of superoxide anions. This pH change was proposed to be essential for the activation of granule-derived enzymes within the phagosome. Later on, this scheme was adjusted by showing that it is the pH-dependent, compensatory potassium surge across the vacuolar membrane, which is responsible for the release and activation of cationic granule proteins, form the anionic sulphated proteoglycan matrix (47). In patients with CGD, however, the NADPH-oxidase function, alkalisation, and potassium influx is absent, resulting in impairment of these killing mechanisms. Whether this plays a significant role in the pathogenesis of A. nidulans is subject to debate, as earlier virulence studies of A. nidulans mutants in p47phox−/− mice indicate that pathogenicity was not influenced by fungal virulence factors as catalases and pH responsiveness (42, 43). Interestingly, the use of pH response mutants of A. nidulans in neutrophic mice showed a dramatic attenuation of ability to cause invasive disease (52). This observation shows that extrapolation of data from neutropenic mouse models of IA is insufficient to understand the pathophysiology of IA in CGD patients.

Phagocytic cells possess several non-oxidative fungal mechanisms, including antimicrobial peptides (eg, defensins, histatin 5) and hydrolases, which are effective in preventing germination or at killing intra- and extracellular fungi (53). Drosomycin-like defensin shows antifungal activity (54). This synthetic drosomycin-like defensin was designated based on a putative human homologue of the Drosophila-derived drosomycin, known for its antifungal properties. In this in vitro study, the susceptibility of A. fumigatus and A. nidulans to drosomycin-like

oxidative-active aggregates are essential in preventing germination of A. fumigatus conidia (45). These were confirmed by microarray data of murine C57Bl/6 and gp91phox−/− AM exposed to A. fumigatus conidia in vivo: the most marked early transcriptional changes did not indicate obvious NADPH-oxidase involvement but occur in genes involved in PMN recruitment (46).

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defensin were remarkably different; the growth of *A. nidulans* was inhibited by the synthetic drosomycin-like defensin, but not by drosomycin. In contrast, *A. fumigatus* was susceptible to both of these defensins. The role of defensins and other cationic proteins stored in the granules of phagocytes from CGD patients in the host defense against filamentous fungi is not yet known and needs to be investigated.

It is clear that the almost exclusive contribution of NADPH-oxidase to microbial killing is a justified subject of debate, and recent studies indicate a critical role of the NADPH-oxidase as regulator of the immune homeostasis at multiple levels (53-57).

We have observed that the absence of the respiratory burst is associated with a dysregulated production of pro- and anti-inflammatory cytokines and further contributes to the pathogenesis of IA in CGD patients (58). A more pro-inflammatory cytokine response was shown after stimulation with *A. fumigatus* conidia, while the balance shifted to an anti-inflammatory response after hyphal stimulation. The opposite was seen in healthy controls. In general, the induction of a Th-helper 1 (Th1)-type response, characterized by IFN-γ, tumor necrosis factor (TNF)-α and Interleukin (IL)-12 production, is protective against the development of IA. In contrast, defense against IA is impaired by IL-4 and IL-10. The respiratory burst in phagocytes is differentially regulated by Th1- and Th2-type cytokines: TNF-α enhances superoxide production by neutrophils, while IL-10 impairs the respiratory burst in macrophages (44). In a paper by Romani et al. (55), dysregulation of the L-tryptophan metabolism in mice with defects in NADPH-oxidase, resulting in overproduction of IL-17, has been proposed to link ROS defects with hyper-inflammation and susceptibility to pulmonary aspergillosis. However, in humans with CGD, tryptophan metabolism was shown to be intact, indicating that the mechanism of fungal susceptibility is different in CGD humans from CGD mice (59, 60). We evaluated in both gp91^phox−/−^ and p47^phox−/−^ CGD patients the L-tryptophan metabolism and cytokine profiles in response to Candida albicans, *A. fumigatus* and *A. nidulans*. Indeed, in contrast to mice, both CGD genotypes display a normal tryptophan metabolism. PBMCs of CGD patients produced more pro-inflammatory cytokines after stimulation, and IL-17A production was strikingly low in response to fungal pathogens when compared to healthy controls (61). Although it seems that an efficient anti-Aspergillus defense relies more on a Th1 immune response (62), the absence of an adequate IL-17 response might contribute to their inability to clear fungal infections.

Recently, Segal et al. (56) showed that NADPH-oxidase-deficient, redox-mediated signaling is critical for termination of lung inflammation. By challenging NADPH-oxidase-deficient p47^phox−/−^ mice and gp91^phox−/−^ mice with intratracheal zymosan, they showed that NADPH-oxidase limits lung inflammation by attenuating the pro-inflammatory transcription factor NfκB and by activating Nrf2, a key redox-sensitive anti-inflammatory regulatory transcription factor. Data from mononuclear cells from X-linked CGD patients were consistent with these findings.

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. PMN from CGD patients show lower expression levels of TLR3, TLR9, CD11b, CD18, CD35, and CXCXR1 compared to those from healthy controls, whereas similar or increased receptor expressions were found in patients without CGD but with bacterial pneumonia (63). *A. fumigatus* is able to modulate the host TLR responses by directly decreasing the capacity of the host cells to respond to TLR2 and TLR4 ligation, a mechanism that can be interpreted as a means to evade the host immune system or to interfere with the resultant signaling pathways (64). Whether differences in immune receptor expression and regulation or modulation during invasive fungal infections in CGD patients are relevant for the observed epidemiology has still to be elucidated.

Overall, it is clear that the innate response to fungal pathogens serves two main purposes: a direct antifungal activity and a regulatory function. Cellular mediators may serve both functions, allowing a certain degree of redundancy and compensation under specific conditions of either infection or other causes of inflammation. Inhibitors of TNF-α are commonly used to control severe inflammatory bowel disease in CGD, but these have been complicated by severe and sometimes fatal occurrence of fungal infections (65). These observations suggest that TNF-α is playing a more prominent role in the host defense in the absence of superoxide formation. The absence of ROS and the inflammasome activation, resulting in IL-1β production, is another proposed mechanisms for hyper-inflammation in CGD patients. ROS likely dampen inflammasome activation and NADPH-oxidase-defective human PBMCs are a source of elevated IL-1β (66). Infection of human CGD leucocytes with *A. nidulans* has shown significantly more IL-1β secretion compared to *A. fumigatus* (unpublished data). Current studies are conducted to unravel the IL-1β processing in CGD patients infected by *A. nidulans* and *A. fumigatus*. Targeting the IL-1β secretion provides new potential therapeutic options for inflammatory conditions associated in CGD. Besides anti-fungal treatment, targeted damping of inflammation during an *A. nidulans* infection in the CGD patient definitely needs further investigations.

**CONCLUSIONS**

This synopsis summarizes our current understanding of the unique interaction between *A. nidulans* and its preferred host, the CGD patient. The clinical epidemiology points out to a specific disease pathology being the result of the complex interaction between the pathogen and the host.

Fungal pathogenesis is a continuum between infection and inflammation. In the CGD host, the absence of a functional NADPH-oxidase complex has both an
impact on displaying an efficient antimicrobial effect as well as a controlled inflammatory response. The defective NADPH-oxidase results in an impaired direct antimicrobial function but cannot explain the etiology of A. nidulans infections in CGD patients. Dysregulated cytokine production, the L-tryptophan metabolism, induction of Th17 cells, differential expression of innate immune receptors, impaired NrF2 activity and inflammasome activation are discussed as immunological mechanisms underlying the dysregulated inflammatory response as observed in CGD upon interaction with A. nidulans. More extensive and in-depth analyses of these mechanisms in the unique interaction of A. nidulans in the CGD patient will definitely improve the current understanding of the role of the NADPH-oxidase in the host-immune response. More insight and detailed analyses of these mechanisms in the unique interaction of A. nidulans in the CGD patient. In summary, invasive A. nidulans infections are a major threat for patients suffering from CGD, while the molecular interaction between A. nidulans and the immune cells of the CGD host are hardly explored.

Footnote page

Potential conflicts of interest. All authors: No reported conflicts.

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The NADPH-oxidase as regulator of inflammation

Decreased Cell Wall Galactosaminogalactan in Aspergillus nidulans Mediates Dysregulated Inflammation in the CGD Host

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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.

INTRODUCTION

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 (II-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, Lee et al., submitted), 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 five CGD patients (three 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 PBMCs in a total volume of 200 µL per well were incubated at 37°C and 5% CO_2 in round-bottom 96-wells plates (Nunc, Roskilde, Denmark) with either 10 ng/ml LPS (derived from E. coli serotype O55: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 (Δalbl, kind gift of K.J. Kwon-Chung, NIH, Bethesda, USA). The alb1 gene codes for a polyketide synthase (pksP) in the 1,8-dihydroyphanalthene (DHN)-melanin pathway, involved in the biosynthesis of conidal 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 ugc3 (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 10^9/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, 1x10^5 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). 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 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 (p<0.05) (Figure 1A, 1B). 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 1C).
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 and TNF-α than *A. fumigatus*.

**Immunomodulatory potential of bound *A. nidulans* 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 *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 *A. nidulans* conidial melanin in the hyper-inflammation 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* (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 *Aspergillus*.

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**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).
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* (12, 24), 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 pro-inflammatory 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 melanins 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 (Figure 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. Both CGD and healthy PBMCs were stimulated with an increasing concentration of isolated cell wall polysaccharides (0.01 up to 25 µg/ml) from both *A. nidulans* or *A. fumigatus*. Cell wall polysaccharides of *A. nidulans* induced higher levels of IL-1β secretion by both healthy and CGD PBMCs as compared to *A. fumigatus* cell wall polysaccharides (Figure 4A, 4B). CGD PBMCs secreted higher levels of IL-1β than did healthy PBMCs upon stimulation with either cell wall polysaccharide preparation. TNF-α release was also higher by CGD PBMCs as compared to healthy PBMCs when stimulated with *A. fumigatus* derived cell wall polysaccharides. However, for *A. nidulans* derived cell wall polysaccharides, CGD PBMCs released higher levels of TNF-α than healthy PBMCs only at lower concentrations (< 3.13 µg/ml, Figure 4C, 4D). 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 (Figure 4E, 4F). In addition, a dose-response relationship was observed for TNF-α and IL-1β release by healthy PBMCs in response to cell wall polysaccharides starting from a concentration of 0.39 µg/ml. In CGD PBMCs, a dose-response relationship was observed for both IL-1β and IL-10 secretion and polysaccharide stimulation, but not for TNF-α release.

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.
Figure 4 The IL-1β (A,B), TNF-α (C,D) and IL-10 (E,F) cytokine response 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*. Bars indicate SEM.
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 *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

![Figure 5](image)

**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 (B) The IL-1β cytokine and IL1-Ra release of healthy PBMCs (white bars) and CGD PBMC with live *A. nidulans* live *A. nidulans* strains and live *A. nidulans* strains overexpressing *A. fumigatus* uge3 (*A. nidulans* + uge 3). The cytokine release after stimulation with WT live *A. nidulans* strains was set to 100 %. Bars represent mean ± SEM (CGD, n=2). The cytokine release after stimulation with WT live *A. nidulans* strains was set to 100 %. Bars represent mean ± SEM (CGD, n=2).

![Figure 6](image)

**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.
induction. The decrease of IL-1β 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-1β secretion were not likely due to differences in β-glucan exposure, as immunofluorescence studies using recombinant Fc-Dectin-1 demonstrated no difference in β-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 *A. fumigatus* stimulated higher levels of IL-1Ra release by PBMCs than did 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 to *A. nidulans* cell-wall polysaccharides) as CGD patients (n.s). Surprisingly however, overexpression of GAG by *ugg3*-complemented *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 *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 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-recognized phenomenon characterized by prolonged inflammatory reactions and granuloma 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 TolH-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 NADPH-oxidase independent mechanisms and that *A. nidulans* is more sensitive to extracellular killing by NADPH-oxidase dependent NET-osis compared to *A. fumigatus* (6, Lee et al., submitted). Experiments in CGD mice have demonstrated that *A. nidulans* strains induce excessive inflammation and death from invasive pulmonary aspergillosis (33). 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
CHAPTER 6 DYSREGULATED INFLAMMATION BY A. NIDULANS IN CGD

Dysregulated inflammation by A. nidulans in CGD, we were able to observe isolates increased the stimulation of cytokines contributing to the dysregulated inflammation during invasive aspergillosis in the CGD host. Low amounts of GAG in the Aspergillus 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.

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.

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 α,1,4-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, 37). 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.

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.

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REFERENCES


## Table 1 Clinical characteristics of the chronic granulomatous disease patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Disease history</th>
<th>Active infection at time of exp.?</th>
<th>Interferon-γ at time of exp.?</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>16</td>
<td><em>Aspergillus fumigatus</em> infection (at age 5 years), no inflammatory complications</td>
<td>No</td>
<td>No</td>
<td>CYBB (gp91-phox)</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>9</td>
<td>Mucormycosis (at age of 10 months), no inflammatory complications</td>
<td>No</td>
<td>No</td>
<td>NCF1 (p47-phox)</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>9</td>
<td>No history of invasive fungal disease, no inflammatory complications</td>
<td>No</td>
<td>No</td>
<td>NCF1 (p47-phox)</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>19</td>
<td><em>Aspergillus nidulans</em> infection (at age of 5 years), <em>A. fumigatus</em> infection (8 years), no inflammatory complications</td>
<td>No</td>
<td>Yes</td>
<td>CYBB (gp91-phox)</td>
</tr>
</tbody>
</table>
New therapeutic strategies for invasive fungal disease in chronic granulomatous disease

Chloroquine Modulates the Fungal Immune Response in Phagocytic Cells From Patients With Chronic Granulomatous Disease
Stefanie S.V. Henriët, Jop Jans, Elles Simonetti, Kyung J. Kwon-Chung, Antonius J.M.M. Rijs, Peter W.M. Hermans, Steve M. Holland, Marien I. de Jonge, and Adilia Warris

ABSTRACT

Invasive aspergillosis is a major threat to patients with chronic granulomatous disease (CGD). Fungal pathogenesis is the result of a diminished antifungal capacity and dysregulated inflammation. A deficient NADPH-oxidase complex results in defective phagolysosomal alkalization. To investigate the contribution of defective pH regulation in phagocytes among patients with CGD during fungal pathogenesis, we evaluated the effect of the acidotropic, antimalarial drug chloroquine (CQ) on the antifungal capacity of polymorphonuclear cells (PMNs) and on the inflammatory response of peripheral blood mononuclear cells (PBMCs).

Chloroquine exerted a direct pH-dependent antifungal effect on Aspergillus fumigatus and Aspergillus nidulans; it increased the antifungal activity of PMN from patients with CGD at a significantly lower concentration, compared with healthy individuals; and decreased the hyperinflammatory state of PBMCs from patients with CGD, as observed by decreased tumor necrosis factor α and interleukin 1β release. Chloroquine targets both limbs of fungal pathogenesis and might be of great value in the clearance of invasive aspergillosis in patients with CGD.

INTRODUCTION

Chronic granulomatous disease (CGD) is a rare inherited immunodeficiency disorder of the NADPH-oxidase complex. Patients are at increased risk of life-threatening invasive fungal infections, particularly invasive aspergillosis (1). Aspergillus fumigatus is the most commonly encountered species, followed by Aspergillus nidulans, with the former detected in approximately one-third of all cases of invasive aspergillosis in patients with CGD (2). A. nidulans causes invasive infections almost exclusively in this specific host and is characterized by its aggressive behavior and high case-fatality rate (3). Since the NADPH-oxidase complex acts both as an antimicrobial effector molecule and a regulator of inflammation, fungal pathogenesis in patients with CGD is the result of an imbalance of antifungal capacity and regulation of inflammation. The defective NADPH-oxidase complex results in a lack of superoxide production, an accumulation of protons, and impaired alkalization of the phagolysosomes. In the early 1980s, it was suggested that abnormal pH regulation within phagosomes from patients with CGD might have a role in defective killing capacities (4). The basis of this assumption is that the initiation of superoxide production is accompanied by an influx of K+ and a rise in pH as a result of the proton-acceptor function of superoxide anions. This pH change is proposed to be essential for the release and activation of granule-derived enzymes within the phagosome (5).

In patients with CGD, this pH dysregulation is of clinical importance, as supported by the fact that upon normalization of the phagocytic pH in CGD phagocytes, the ability to kill Staphylococcus aureus is restored (4).

In addition to this impaired antifungal defense system, the absence of the oxidative burst in phagocytes from patients with CGD leads to a dysregulated inflammatory response (6). Several studies indicate hyperresponsive production of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in patients with CGD, both upon encountering A. fumigatus and without any obvious stimulus (6-8). The excessive inflammation is thought to play a role in the outcome of invasive aspergillosis in patients with CGD; limiting this exaggerated inflammatory response might decrease tissue damage and improve outcome.

The antimalarial drug chloroquine (CQ) is an acidotropic agent that passively diffuses into acidic organelles. It has shown to have a direct antifungal effect on Histoplasma capsulatum and Cryptococcus neoformans, possibly by accumulation in the fungal vacuole (9-11). CQ, being a weak base, is able to raise the endocytic and lysosomal pH of eukaryotic cells (12, 13). On entering the phagocytic cell, it diffuses into the acidic endosomes and can reach a concentration that is up to 10 000-fold higher than its extracellular levels (14). Earlier studies have shown that the addition of CQ to human mononuclear cells has an additive effect on the killing of
both H. capsulatum and C. neoformans (11, 15). In addition to the antifungal effect of CQ, this drug has been effective in the treatment of diseases associated with increased release of proinflammatory cytokines, such as rheumatoid arthritis (16). CQ is able to reduce the proinflammatory cytokine release of mononuclear phagocytes stimulated by lipopolysaccharide (LPS), and to antagonize TNF-α expression induced by fungi (such as C. neoformans and Candida albicans) by a mechanism suggested to be dependent on alkalization of endolysosomes (17-19).

To clarify the contribution of defective alkalization of phagolysosome during fungal pathogenesis in patients with CGD, we evaluated the in vitro effect of CQ on fungicidal effector mechanisms and cytokine release by phagocytes from healthy volunteers and patients with CGD stimulated with A. fumigatus and A. nidulans. We hypothesized that the addition of CQ would enhance the antifungal activity of phagocytes from patients with CGD by raising the pH in phagolysosomes and downregulating the exaggerated proinflammatory response.

METHODS

Human leukocytes

Venous blood was drawn from healthy volunteers, three patient with CGD and gp91phox-deficiency and two patients with CGD and p47phox-deficiency after informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) were isolated by use of Lymphoprep (Axis-Shield, Oslo, Norway). Briefly, blood was anticoagulated with lithium heparin (BD Vacutainer) and diluted with an equal volume of phosphate-buffered saline (PBS). Venous blood was drawn from healthy volunteers, three patient with CGD and gp91phox-deficiency and two patients with CGD and p47phox-deficiency after informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) were isolated by use of Lymphoprep (Axis-Shield, Oslo, Norway). Briefly, blood was anticoagulated with lithium heparin (BD Vacutainer) and diluted with an equal volume of phosphate-buffered saline (PBS).

The diluted blood was carefully added to the top of the Lymphoprep and centrifuged at 800 x g. PBMCs were harvested, washed twice in PBS, and counted by use of a hemocytometer. To remove the erythrocytes from the PMNs, the pellet was shocked at least twice with an ice-cold lysing reagent (NH4Cl, Na2EDTA, KHCO3). Subsequently, cells were washed and resuspended in PBS. Roswell Park Memorial Institute 1640 GlutaMAX-I medium (Invitrogen Life Technologies) plus 10% heat-inactivated human serum was used as culture medium for all cell experiments.

Fungal strains

Fully molecularly characterized A. nidulans (V44-46) and A. fumigatus (V45-07) strains, originally isolated from patients with CGD who had invasive aspergillosis, were used. The Aspergillus spp. were grown and prepared in a LPS-free fashion as described previously (20).

XXT assay

The XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide sodium salt) assay of fungal viability described previously was modified as follows (20). Each well of a 96-well plate was filled with 100 µl of CQ (chloroquine dihydrogen phosphate salt; Sigma-Aldrich, St. Louis, MO) in different concentrations and 100 µl of live, resting conidia at concentrations of 2 x 10⁶/ml. CQ was diluted in MilliQ and sterilized through a 0.2-µm filter membrane. Conidial viability was analyzed after 18 hours of incubation at 37°C in 5% CO₂ (20).

Effect of pH on the antifungal activity of CQ

To analyze the effect of pH on the antifungal activity of CQ, the pH of the medium was set at pH 6, pH 7 and pH 8. The medium containing different concentrations of CQ was buffered with 50 mM MES for pH 6, 50 mM HEPES for pH 7, and 50 mM Tris for pH 8. By titration of hydrochloric acid or sodium hydroxide the specific pH values were obtained. Each well of a 96-well plate was filled with 100 µl of CQ solution in buffered medium with different pH values and 100 µl of live, resting conidia at a concentration of 2 x 10⁶ conidia/ml. Fungal damage was analyzed by the XTT assay.

In addition, 100 µl of the supernatant was transferred to a new 96-well plate to determine effective maintenance of the pH value during the experiment. The OD of the supernatant was measured at 560 nm. Since the growth medium contains the acid-base indicator phenol red, the absorption properties of phenol red were correlated with the pH of the growth medium, as described by Lancz et al. (21).

Antifungal activity of PMNs in the presence of CQ

Each well of a 96-well plate was filled with 100 µl of PMNs from healthy volunteers (hereafter, “healthy PMNs”) or patients with CGD (hereafter, “CGD PMNs”) at a concentration of 1 x 10⁶ PMNs/ml. Cells were preincubated with 50 µl of CQ for 1 hour to allow uptake of CQ, and 50 µl of live, resting conidia at a concentration of 4 x 10⁶ conidia/ml. CQ concentrations of up to 100 µM (51.6 µg/ml) were evaluated based on the basis of the drug’s correlation with the immunomodulatory therapeutic dosage of 400 mg daily given to patients with rheumatoid arthritis (22). After incubation for 18 hours, PMNs were lysed by addition of 100 µl of saponin 0.005% for 30 minutes at 37 °C and underwent centrifugation at 3000 x g for 10 minutes. Cell lysis was confirmed microscopically. Conidial viability was analyzed by the XTT assay. Wells containing PMNs or CQ alone were used as negative controls, while wells containing conidia alone were used as positive controls.

Cytokine-stimulation assays

PBMCs from healthy volunteers (hereafter, “healthy PBMCs”) or from patients with CGD (hereafter, “CGD PBMCs”) were stimulated with LPS (derived from Escherichia
coli serotype 055:B5; Sigma-Aldrich) after incubation for 1 hour with CQ at concentrations up to 100 µM. An extra LPS-purification step was performed before use (23). Each 96-well plate was filled with 100 µl of healthy or CGD PBMCs at a concentration of 1 x 10⁶ PBMCs/ml. Fifty microliters of LPS were added to obtain a final concentration of 1 ng/ml. The plates were incubated for 18 hours at 37 °C and centrifuged at 3000 x g. Finally, 180 µl of culture supernatant was removed for measurement of cytokines. TNF-α and interleukin 1β (IL-1β) were measured by specific enzyme-linked immunosorbent assays (Sanquin Blood Supply, Amsterdam, the Netherlands, and R&D Systems, Oxon, United Kingdom), using specific monoclonal antibodies according to the manufacturer’s instruction. Wells containing only PBMCs were used as negative controls, and LPS-stimulated cytokine release without the addition of CQ was set to 100%. The release of lactate dehydrogenase was measured to assess cell viability for each condition.

Statistical analysis
Each experiment with human cells was performed in quadruplicate for the XTT assay and in duplicate for the cytokine stimulation assay. Mean values ± standard errors of the means were calculated for all of the experiments under the same conditions. Comparisons between > 2 mean values were analyzed by analysis of variance, using a Bonferroni-adjusted level of significance. P values of <0.05 were considered statistically significant.

RESULTS

CQ Has a direct antifungal activity against A. fumigatus and A. nidulans
We first tested the direct antifungal activity of CQ at concentrations ranging from 0.1 mM to 100 mM. We observed a direct dose-response relationship between CQ concentration and fungal damage for both Aspergillus species. A CQ concentration of ≥ 0.25 mM resulted in significant fungal damage of A. fumigatus (P < 0.0001), while fungal damage of A. nidulans occurred at ≥ 0.5 mM (P <0.05), a two-fold higher concentration (Figure 1). There was significantly more damage to A. fumigatus than to A. nidulans (P < 0.0001) in the range of 0.25 mM – 1 mM CQ (Figure 1).

Antifungal activity of CQ is pH dependent
We next assessed the influence of the environmental pH on the antifungal activity of CQ. An alkaline environment (pH 8) increased the antifungal activity at a fixed concentration (10 mM) against A. fumigatus, as well as against A. nidulans (P < 0.0001) (Figure 2).

Figure 1 Antifungal activity of chloroquine assessed by the XTT assay. A chloroquine concentration of ≥ 0.25 mM has significant antifungal activity against Aspergillus fumigatus (P <0.0001). Significant fungal damage of Aspergillus nidulans occurred at a chloroquine concentration of ≥ 0.5 mM (P <0.05). A. fumigatus is more susceptible than A. nidulans to chloroquine concentrations of ≥ 0.25 mM to ≤ 1 mM. Error bars indicate standard error of the mean (n=12). * P <0.0001.

Figure 2 The antifungal activity of a fixed concentration (10 mM) of chloroquine increases in an alkaline environment. An increase of fungal damage at pH 8 for Aspergillus fumigatus and Aspergillus nidulans was observed. Error bars indicate standard error of the mean (n=3). * P <0.0001.

In a constant acidic environment (pH 6) no difference in fungal damage due to CQ was observed between the Aspergillus species (Figure 3A). However, increasing the pH of the medium to 8 resulted in more-exaggerated differences in the susceptibility
of the two species to CQ. A. fumigatus was significantly more susceptible to the antifungal effect of CQ than A. nidulans (P < 0.001) in a pH 8 environment (Figure 3B). Changing the pH of the culture medium in the absence of CQ did not itself result in fungal damage (data not shown). Moreover, the pH of the growth media remained stable, with only very minor deviations (pH ± 0.3) over the incubation period in the different experiments performed (data not shown).

Figure 3 Antifungal activity of chloroquine in media with different pH values. A, At pH 6, no significant difference in antifungal activity against Aspergillus fumigatus versus Aspergillus nidulans is seen. B, At pH 8, A. fumigatus is significantly more susceptible than A. nidulans to the antifungal activity of chloroquine. Error bars indicate standard error of the mean (n=3). * P <0.01, ** P <0.001.

CQ Increases the antifungal activity of healthy and CGD PMNs
To further investigate the antifungal activity of CQ as a lysosomotropic and acidotropic agent, we assessed its effect in the presence of healthy and CGD PMNs. The addition of 100 µM CQ increased the antifungal activity of healthy PMNs against A. fumigatus by 75% (P < 0.05) (Figure 4A). The same trend was seen for A. nidulans, although these results were not significant (P = 0.23). Remarkably, a 20-fold lower concentration of CQ (5 µM) increased the antifungal activity of CGD PMNs against A. fumigatus by 63% (P < 0.0001) (Figure 4B). CQ did not influence the antifungal activity of CGD PMNs against A. nidulans.

Figure 4 The effect of chloroquine on the antifungal activity of polymorphonuclear leukocytes (PMNs) from healthy volunteers (hereafter, "healthy PMNs") and PMNs from patients with chronic granulomatous disease (hereafter, "CGD PMNs"). Antifungal activity of PMNs without chloroquine was set to 1. Chloroquine increases the antifungal activity against A. fumigatus for healthy PMNs at 100 µM (75% ± 31%; n=8; A) and for CGD PMNs at 5 µM (63% ± 10%; n=5; B). Error bars indicate standard error of the mean. * P <0.05. ** P <0.0001.

CQ Inhibits LPS-induced TNF-α and IL-1β release by healthy and CGD PBMCs
To investigate the immunomodulating effect of CQ, we assessed its effect on cytokine release by PBMCs. We observed a higher LPS-induced release of TNF-α and IL-1β by CGD PBMCs compared to healthy PBMCs (data not shown). We showed a dose-dependent decrease of IL-1β release by healthy and CGD PBMCs at a CQ concentration of ≥ 5 µM (P < 0.0001). A decrease in TNF-α release by healthy PBMCs was observed in the presence of ≥ 10 µM CQ (P < 0.001), while higher concentrations of CQ (≥ 50 µM) were needed to inhibit TNF-α release by CGD PBMCs (P < 0.0001) (Figure 5). No loss of cell viability in the presence of the different concentrations of CQ used was observed, as indicated by lactate dehydrogenase concentrations in the media. These results are consistent with previous cell-viability studies (19, 24).
observed in the presence of ≥ 5 µM \((P < 0.01)\) and ≥ 10 µM \((P < 0.0001)\) CQ respectively, while higher concentrations of CQ (≥ 50 µM) were needed to significantly inhibit TNF-α and IL-1β release by CGD PBMCs \((P < 0.01)\) for both. In contrast, significant inhibition of TNF-α and IL-1β was already observed with 5 µM CQ upon \(A.\ nidulans\) infection of both healthy PBMCs \((P < 0.01\) and \(P < 0.0001\), respectively) and CGD PMBCs \((P < 0.05\) and \(P < 0.0001\), respectively).

**Figure 5** The effect of chloroquine on cytokine release by peripheral blood mononuclear cells (PBMCs) from healthy volunteers (hereafter, “healthy PBMCs”) and PBMCs from patients with chronic granulomatous disease (hereafter, “CGD PBMCs”). Bars indicating 0 on the x-axis are lipopolysaccharide (LPS)-stimulated PBMCs without chloroquine. The LPS-stimulated cytokine release without chloroquine was set to 100%. Chloroquine reduces LPS-induced tumor necrosis factor α (TNF-α; A) and interleukin 1β (IL-1β; B) release by healthy PBMCs \((n = 6)\). Chloroquine reduces IL-1β release by CGD PBMCs \((n = 4–5; D)\), whereas higher concentrations are necessary to reduce the release of TNF-α \((C)\). Error bars indicate standard error of the mean. *\(P < 0.001\), **\(P < 0.0001\).

**Figure 6** The effect of chloroquine on cytokine release by peripheral blood mononuclear cells (PBMCs) from healthy volunteers (hereafter, “healthy PBMCs”) and PBMCs from patients with chronic granulomatous disease (hereafter, “CGD PBMCs”) after Aspergillus fumigatus and Aspergillus nidulans stimulation. Bars indicating 0 on the x-axis are PBMCs without chloroquine. The LPS-stimulated cytokine release without chloroquine was set to 100%. Chloroquine reduces \(A.\ fumigatus\)- and \(A.\ nidulans\)-induced TNF-α \(A\) and IL-1β \(B\) release by healthy PBMCs \((n = 6; *P < 0.01, **P < 0.0001)\). Chloroquine reduces \(A.\ fumigatus\)- and \(A.\ nidulans\)-induced TNF-α \(C\) and IL-1β \(D\) release by CGD PMBCs \((n = 3; *P < 0.05, **P < 0.01, ***P < 0.0001)\). Error bars indicate standard error of the mean.
DISCUSSION

In this study, we demonstrate that CQ has antifungal properties and downregulates the exaggerated proinflammatory cytokine response, as seen in CGD PBMCs. A direct antifungal effect of CQ on both A. fumigatus and A. nidulans was found. This effect is enhanced in an alkaline environment. A. fumigatus is more susceptible than A. nidulans to CQ, and this difference is even more pronounced in an alkaline environment. Furthermore, CQ increases the antifungal activity of healthy and CGD PBMCs. Interestingly, this enhanced antifungal activity occurs at a significant lower CQ concentration in association with CGD PMNs as compared to healthy PMNs. Finally, CQ decreases both LPS-induced and A. fumigatus- and A. nidulans-induced release of TNF-α and IL-1β by healthy and CGD PBMCs.

Previous studies showed accumulation of CQ into the acidic food vacuole of the parasite Plasmodium falciparum (25). Accumulation of quinacrine, an antifungal activity of CQ at a given concentration. In an acidic environment, less antifungal effect, and an alkaline environment would lead to an increased concentration of CQ and an extended incubation period are needed to observe the pH-neutral or acidic environment (27). These data do not conflict with ours. Higher antifungal effect on conidial viability was observed at CQ concentrations of ≤ 1 mM in a non-oxidative mechanism. The enhanced antifungal activity of CQ involves alkalizing the neutrophil lysosome, it is suggested that CQ activation of granule-derived anti-microbial enzymes (5). Considering that one property of CQ involves alkalizing the neutrophil lysosome, it is suggested that CQ improves the antimicrobial function of neutrophils via pH change, mainly by non-oxidative mechanisms. The enhanced antifungal activity and the differences between healthy and CGD PMNs, suggest a clear relationship with the alkalizing properties of CQ.

We observed a direct pH-dependent antifungal effect of CQ on both A. fumigatus and A. nidulans. A higher extracellular pH resulted in increased fungal damage at a constant concentration of CQ, suggesting an increased diffusion rate of CQ into the fungal organisms. Direct antifungal activity of CQ against A. fumigatus conidia has only been investigated in a study evaluating the contribution of the pkS gene of A. fumigatus in intracellular processing of conidia. At a 6-hour time point, no effect on conidial viability was observed at CQ concentrations of ≤ 1 mM in a pH-neutral or acidic environment (27). These data do not conflict with ours. Higher concentrations of CQ and an extended incubation period are needed to observe the antifungal effect, and an alkaline environment would lead to an increased antifungal activity of CQ at a given concentration. In an acidic environment, less CQ would diffuse into the Aspergillus species and, consequently, less killing would be observed.

Interestingly, a higher concentration of CQ was necessary to cause fungal damage of A. nidulans. We hypothesize that the difference might be explained by two mechanisms: variation in diffusion rates of CQ into the fungus or/and different intracellular resistance against the activity of CQ. Since diffusion of CQ is pH dependent, a lower intracellular pH of A. fumigatus as compared to that for A. nidulans might result in different diffusion rates of CQ and, consequently, antifungal activity. No study has compared the intracellular pH of A. fumigatus and A. nidulans, but findings from study a study could strengthen this hypothesis. Once accumulated, CQ has an antifungal effect through yet unknown mechanisms. Differences in alkalization of the fungus or inhibition of the activity of proteins and enzymes may result in the different susceptibility between A. fumigatus and A. nidulans. Future research is required to investigate the mechanisms underlying the antifungal activity of CQ and the increased susceptibility of A. fumigatus, compared with A. nidulans.

The next step was to investigate the additional antifungal activity of PMNs on Aspergillus species in the presence of CQ. We found an enhanced antifungal activity of both healthy and CGD PMNs. Remarkably, for healthy PMNs this increase occurred at 100 µM CQ, while for CGD PMNs this increase was already seen at 5 µM CQ, a 20-fold lower concentration. The absence of phagolysosomal alkalization in CGD PMNs might be responsible for an increased diffusion of CQ into the relative acidic environment of the CGD cell. Consequently, the same extracellular concentration of CQ would result in a higher intracellular concentration in the CGD PMNs as compared to the healthy PMNs, resulting in increased antifungal activity of CGD PMNs at lower CQ concentrations.

Lack of phagolysosomal alkalization in the CGD phagocytic cell is suggested to play a key role in the direct antimicrobial activities of the phagocyte. Reeves et al. demonstrated that killing activity of PMNs is not directly related to reactive oxygen species, but is mediated through activation of proteases by a K+ flux that crosses the membrane in a pH-dependent manner, followed by release and activation of granule-derived anti-microbial enzymes (5). Considering that one property of CQ involves alkalizing the neutrophil lysosome, it is suggested that CQ improves the antimicrobial function of neutrophils via pH change, mainly by non-oxidative mechanisms. The enhanced antifungal activity and the differences between healthy and CGD PMNs, suggest a clear relationship with the alkalizing properties of CQ.

An additional proposed mechanism of enhanced fungal killing through CQ is by limiting iron availability (28-30). Iron acquisition is critical for the survival of numerous pathogenic fungi in mammalian hosts (31). Siderophore-mediated iron uptake is essential to the survival of A. fumigatus and A. nidulans (32, 33). PMNs are able to provide iron uptake by the reduction of Fe+3, resulting in the release of iron from transferrin. Iron restriction has been proposed to cause increased antifungal activity of monocyte-derived macrophages against C. neoformans (11). Considering the iron-dependent survival of Aspergillus species, CQ might reduce its growth via restricting the iron release from transferrin of PMN.

Absence of the respiratory burst is associated with dysregulated cytokine production, resulting in more proinflammatory cytokine release, which further contributes to the pathogenesis of invasive aspergillosis in patients with CGD (8).
Reactive oxygen species likely dampen inflammasome activation, and NADPH-oxidase complex defective PBMCs are a source of elevated IL-1β release (34, 35). Therefore, immunomodulating agents, besides antifungal treatment, that target the dampening of inflammation during invasive aspergillosis in patients with CGD may be worthwhile to explore. CQ is known for its immunomodulatory properties and is used in patients with rheumatoid arthritis and lupus (16, 36). Furthermore, CQ has been shown to antagonize the proinflammatory cytokine response to opportunistic fungi such as C. neoformans and C. albicans by alkalizing the fungal phagolysosome. The therapeutic efficiency of hydroxychloroquine was reported in treatment of granulomatous complications in CGD (37). Our results show that IL-1β and TNF-α release, upon stimulation with LPS and Aspergillus species, by both healthy and CGD PBMCs can be inhibited by CQ. Interestingly, during A. nidulans infection a significantly decrease of both TNF-α and IL-1β release by healthy and CGD PBMCs was already seen at lower concentrations, compared with observations during A. fumigatus infection.

Jang et al. have studied the mechanisms by which CQ leads to a diminished TNF-α and IL-1β production in human monocytes and showed that different mechanisms were involved for specific cytokines (17). In LPS-stimulated monocytes, CQ blocks the conversion of cell-associated TNF-α precursor to mature soluble protein, whereas it reduced the levels of IL-1β messenger RNA, at least in part, by decreasing their stability and by a pH-dependent mechanism. The alkalizing effect of CQ as a weak base was a more important factor responsible for the decrease in IL-1β release, compared with TNF-α release. The more pronounced effect on IL-1β release might be especially beneficial in patients with CGD, since infection of human CGD leucocytes with A. fumigatus or A. nidulans leads to exaggerated IL-1β secretion. The increased immunomodulatory effect of CQ on A. nidulans-induced IL-1β release is remarkable, especially because we observed a significantly higher level of IL-1β release after infection with A. nidulans, compared with infection with A. fumigatus (unpublished data). Studies to unravel the IL-1β processing in patients with CGD infected by A. nidulans and A. fumigatus and the influence of CQ on this process are ongoing.

In summary, CQ has antifungal properties and damps the inflammatory response of CGD cells in vitro. The antifungal activity of CQ might be beneficial in the clearance of fungal infections in patients with CGD. More importantly, the immunomodulating effect of CQ should be considered for dampening the hyper-inflammatory state in patients with CGD, and thereby reducing their inflammatory complications and further improve life expectancy and quality of life. Future research is necessary to translate these promising findings to clinical practice.

**Note**

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REFERENCES

General discussion and future perspectives
The aim of the studies presented in this thesis was to explore the unique host-pathogen interaction and pathogenicity of the generally considered non-pathogenic fungus *Aspergillus nidulans* in patients suffering from chronic granulomatous disease (CGD). CGD is a genetically heterogeneous disease caused by a defect in any of the five structural components of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex. In vivo, the NADPH-oxidase is a critical regulator of the innate immunity. This thesis contributes to a further understanding of the central role of the NADPH-oxidase in fungal pathogenesis by detailed unravelling of the interplay between *A. nidulans* and the CGD host and opens a new dimension in treatment possibilities.

The syndrome “fatal granulomatous disease of childhood” (1) was firstly described in 1957. Only 7 years later, and before the underlying cellular mechanism causing CGD was discovered, Redmond and colleagues published the first unique observation of *A. nidulans* infection causing fatal granulomatous pulmonary infection and extensive osteomyelitis in a 6-year old boy (2). It was Segal in 1998 who recognized *A. nidulans* as a distinct, but frequently occurring pathogen in CGD and who made doctors aware that its isolation carries more severe implications than that of *Aspergillus fumigatus* (3). In the following years, epidemiological studies have further established the observation that *A. nidulans* is more frequently the causative agent of invasive aspergillosis in patients suffering from CGD compared to the classical risk group, the haemato-oncological patients during neutropenia. In view of such a particular observation, explanations might be found and hypotheses can be risen, taking into account 3 aspects relevant to the establishment and diagnosis of infection: (1) exposure, are there any differences in environmental factors and exposure to *A. nidulans* between these two groups; (2) identification, are there any differences in recovery and identification of *A. nidulans* from clinical samples from both patient groups in comparison with *A. fumigatus*; or, (3) host-pathogen interaction, is the higher incidence of *A. nidulans* infections in CGD patients explained by a specific defect in the innate immune system, resulting in a uniquely higher susceptibility of CGD patients for *A. nidulans* in addition compared to that for *A. fumigatus*. This point is the key-question addressed by this thesis in which we strengthen and add new insights to the hitherto accepted view on fungal pathogenesis in patients suffering from CGD.

Before discussing our recent in vitro observations concerning the unique interaction between *A. nidulans* and its preferred host, the CGD patient, the aspects of exposure (environmental epidemiology) and identification (diagnostic issues) need to be addressed.
Aspergillus species are ubiquitous airborne saprophytic fungi. Their natural niche is the soil and decaying vegetation. Aspergillus spp. sporulate abundantly and environmental surveys indicate that all humans inhale at least several hundred Aspergillus conidia per day. Consequently, transmission by air is the main route of the conidia infecting the lungs. Information on the ‘normal’ conidial air load of non-fumigatus Aspergillus species is difficult to retrieve as most environmental surveys are focused on A. fumigatus due to its clinical relevance. In the few, geographically widespread studies, substantial variation and seasonality are noted. A study about environmental levels of Aspergillus spp. isolated in air samples in the province of Madrid, revealed A. fumigatus in 54% of the total Aspergillus isolates, compared to only 5% of A. nidulans (4). The investigation of airborne fungi inside and outside the homes of asthmatic children and control subjects in Delhi, India, showed a low overall prevalence of 11.0% and 8.2% respectively of Aspergillus species, with comparable A. nidulans and A. fumigatus occurrences (10% to 15% of the Aspergillus species recovered both indoor and outdoor) (5). In a study performed in Kolkata, India, evaluating the composition and variability of airborne fungal spores in an outdoor environment in Kolkata suburb, the predominant fungal genus was Aspergillus spp (40%) of which 20% was identified as A. fumigatus compared to 15% A. nidulans (6). These studies suggest that although important geographic variations exist in Aspergillus spp. airtypes, the environmental conidial concentration of A. nidulans is less than that of A. fumigatus.

The lifetime incidence of Aspergillus infections in CGD before the advent of specific antifungal prophylaxis varied between 25 and 40% (7, 8). In comparison, the incidence of invasive aspergillosis in children undergoing allogeneic haematopoietic stem cell transplantation (HSCT) or those treated for a haematological malignancy varies between 2% and 16% (9-13).

Aspergillus fumigatus is the most commonly isolated species in CGD, closely followed by A. nidulans. In the haematopoietic patients, A. nidulans is rarely reported. CGD patients spend most of their lifetime outside of the hospital while haematopoietic patients do spend a substantial amount of their ‘vulnerable’ time during their disease period in a hospital ward. The current recommendation for use of high efficiency air filters (HEPA) in bone marrow and haematology units reduces the risk of nosocomial aspergillosis to almost zero (14). Previous colonisation of the lower respiratory tract can lead to invasive pulmonary aspergillosis during hospital admission in haematopoietic patients, as shown by Einsele and colleagues (15). This observation is coherent with molecular strain typing studies, indicating that a significant number of Aspergillus infections are acquired outside the health care setting (16). Prevention of exposure to abundant fungal spores in the air is an important aspect in the care of both CGD and haematopoietic patients. The CGD trust has developed specific recommendations to prevent exposure to fungal spores and thereby prevention of disease [http://www.cgdsociety.org].

Occasionally, fungal spores are released in clouds, causing large variations in conidial counts. Construction works within a hospital building or on adjacent sites may consequently lead to micro-epidemics of invasive aspergillosis, but have never been observed to be caused by A. nidulans (17) [http://www.outbreak-database.com].

Unfortunately, no data is available about fungal colonization in the CGD host. Fungal respiratory tract colonization has been relatively frequently evaluated in the cystic fibrosis patient. Interestingly, A. nidulans is seldom reported. Of the 3336 respiratory samples from 287 Danish CF patients, A. fumigatus was the most frequently isolated species in almost 40% while A. nidulans was isolated only twice (18). These results are coherent with those of the Dutch National Cystic Fibrosis Fungal Bank, which collects all fungal sputum isolates of CF patients in the Netherlands: only one A. nidulans isolate has been reported in the 4267 positive fungal sputum samples, while A. fumigatus was isolated in the majority of samples (2971 samples) (personal communication). A study of fungal colonization in cattle and donkey handlers published in 1979 showed A. flavus being the pre-dominant species of pharyngeal colonization (11 isolates out of the 300 pharyngeal swabs), followed by A. fumigatus. A. nidulans was isolated once. Although no data could be retrieved about the A. nidulans air concentration, this study shows that in an environment of high fungal exposure, A. fumigatus is definitely more commonly isolated in healthy hosts compared to A. nidulans (19).

In order to understand the process and relationship of exposure, colonization, infection and disease in the CGD host, longitudinal studies are needed, to test the hypothesis that Aspergillus species colonization is host-specific, and differs from what would have been anticipated from its profusion in the air (20).

In conclusion, the CGD patient and the classical chemotherapy induced neutropenic host are exposed to the same environmental niche of Aspergillus spp. The distribution of A. nidulans in the environment is less than A. fumigatus. The exposure, qualitatively speaking, of both the CGD patient and the haematopoietic patient is the same. Quantitatively, there is a huge difference as the CGD patient has a lifelong exposure associated with a high risk developing invasive aspergillosis, compared to a relatively short exposure of the haematopoietic host with neutropenia. More studies are needed to evaluate the role of the host environment of the lung in colonization and whether fungal colonization of the CGD lung is dominated by A. nidulans.
DISCUSSION

The second suggestion for the unique epidemiological observation of *A. nidulans* in the CGD host, is that invasive diagnostic measures and consequently the recovery and identification of *A. nidulans* is less feasible in other risk groups.

The reported proportion of proven invasive fungal infection (IFI) versus probable and possible disease, as defined by the EORTC/MSG criteria (21) applying to patients with cancer, is in patients suffering from chronic granulomatous disease disproportionally high. In our literature review (chapter 2) we described 116 CGD patients with invasive fungal disease of which more than 90% were proven infections (22). Invasive fungal infection among transplant patients as reported in the Transplant-Associated Infection Surveillance Network (TRANSNET) showed that in the cohort of 2001-2006, only 42% of the IFI were proven (23). The question may be asked if among those probable infections, the incidence of non-*fumigatus* *Aspergillus* species, including *A. nidulans* is substantially higher. Although we cannot verify this, the distinct clinical phenotypes of invasive aspergillosis caused by *A. nidulans* compared to *A. fumigatus* in the CGD host clearly points out to a unique host-pathogen interaction rather than an external factor as possible differences in recovery and identification. We conclude that, although clear reporting biases exist, the differences in recovery and identification are not the explanation of the disproportionally high prevalence of *A. nidulans* infections in CGD patients.

HOST-PATHOGEN INTERACTION

The key question addressing the specific immune defect of the CGD host and its susceptibility to *A. nidulans* is the central theme of this thesis: in particular, is the higher incidence of *A. nidulans* infections in the CGD patients explained by the absence of the NADPH-oxidase complex, which would have more consequences, direct or indirect for an efficient host defence against *A. nidulans* compared to *A. fumigatus*?

The NADPH-oxidase as antimicrobial effector complex

The mechanisms by which the NADPH-oxidase and it metabolites kill pathogens are complex and incompletely understood. We concluded in chapter 3 that the antifungal effector mechanisms against *A. nidulans* are distinctive from those against *A. fumigatus* and, surprisingly, that human leukocytes without a functional NADPH-oxidase efficiently kill *A. nidulans* suggesting fungicidal activity by NADPH-independent mechanism. These important findings are in contrast to the hitherto accepted antimicrobial role of the NADPH-oxidase displaying a direct microbicidal activity by the resultant reactive oxygen species.

Activation of the NADPH-oxidase leads to the production of superoxide anions. Superoxide anions are unstable and will convert either spontaneously or by the superoxide dismutase enzyme to hydrogen peroxide. Myeloperoxidase converts hydrogen peroxide in turn to the highly microbicidal hypochlorous acid. Myeloperoxidase (MPO) deficiency is common (1/2000-4000 in general population) but in contrast to CGD it is usually asymptomatic. Increased susceptibility to *Candida* infections may be observed (less than 5%), especially if other predisposing conditions such as diabetes are present (24). In MPO deficient mice, antifungal activity against *Aspergillus* spp. is only slightly impaired (24). In humans, no *Aspergillus* spp. infections in MPO deficient patients have been published. In contrast to the crucial role of the NADPH-oxidase and the production of superoxide anions itself, the microbicidal endproducts of MPO and SOD activity are redundant for normal fungal host-response in an otherwise healthy hosts.

Activated NADPH-oxidase is the major source for reactive oxygen species in phagocytes, but other ROS generating systems do exist. Mitochondria do have a respiratory chain. Question remains whether mitochondria can modulate host defence and whether a functional mitochondrial respiratory chain can contribute to the antimicrobial effector function of phagocytes. Wiese et al. showed that the mitochondrial respiratory chain contributed to the antimicrobial activity of macrophages to a different extent depending on the microbial species (*E. coli* versus *S. aureus*) (25). They found that the effect was independent of the phagocytic NADPH-oxidase or the inducible NO synthase, another reacting oxygen intermediate generating system. Furthermore, West et al. demonstrated that activation of specific toll-like receptors in macrophages leads to recruitment of mitochondria to phagosomes and to augmentation of mitochondrial reactive oxygen species production (26). These results suggest that mitochondrial ROS is an important component of species-specific antimicrobial responses and further establish mitochondria as hubs for innate immune signaling. The role of mitochondrial ROS in CGD acting as a fungicidal agent towards *A. nidulans* needs to be explored.

Reactive oxygen species join nitrogen intermediate products, the result of inducible nitric oxide synthase pathway (iNOS), to form reactive metabolites like peroxynitrito anions, with highly microbicidal and inflammatory properties. In vitro. cell-Free iNOS generating systems show to have antimicrobial properties against *Burkholderia cepacia* and *Chromobacterium violaceum*, both CGD pathogens. Peroxinitrito, the resultant of NO and superoxide anion, shows merely fungistatic properties against *Aspergillus* spp. but is candidacidal (27). In human iNOS deficiency has not yet been demonstrated. Knockout mice lacking iNOS have increased susceptibility to various infections, including *Mycobacterium tuberculosis*,
Leishmania spp. and Salmonella enterica. iNOS deficient mice are resistant to candidiasis. The role of iNOS in invasive aspergillosis has not been studied so far. Mice deficient in both NADPH oxidase and iNOS activities are far more susceptible to infections than single gene-deficient host models, which supports distinct but interacting roles of these two pathways (28). As such, mice deficient in iNOS and for phagocyte oxidase (gp91phox) show increased susceptibility to candidiasis, however, this was not related to decreased killing ability but to excessive inflammation (29). Overall, unraveling the contribution of nitrogen intermediate products and mitochondrial reactive oxygen species in fungal pathogenesis of the CGD host seems a challenging future research topic.

Beyond the direct microbicidal activity of the products of the NADPH-oxidase, its activation is coupled to extra- and intracellular release of preformed antimicrobial proteases. Adequate phagolysosomal alkalization and the pH-dependent compensatory potassium surge across the vacuolar membrane are essential steps for the release and activation of cationic granule proteins from the anionic sulfated proteoglycan matrix (30). Results of in vitro studies and murine studies are currently conflicting about the exact role of the absent NADPH-oxidase function and its relation with a defective alkalization and absent potassium influx in the microbial pathogenesis. The absence of the rodlet layer, a process occurring during germination, resulted for A. nidulans, in contrast to A. fumigatus, in the induction of a basic character, a marked decrease in hydrophobicity and in electronegativity of conidia (31). Question is whether this might be responsible for the conserved killing ability of the CGD phagocytic cell towards A. nidulans, through binding of the cationic granule proteins on the remaining negatively charged A. nidulans.

Neutrophil NADPH oxidase activation is linked to extracellular release of granule proteins. DNA and chromatin forming the neutrophil extracellular traps (NETs), NETs target fungi in a nonspecific way and its role in arresting fungal growth, fungal killing or protecting the fungi against further neutrophil attack is still under debate. Reconstitution of the NADPH-oxidase activity by gene therapy resulted in a restored protection against A. nidulans infections (32). The authors linked this effect to the restoration of the NET formation by calprotectin-mediated mechanisms. Question remains whether reconstitution of other NADPH-dependent mechanisms, such as direct effects or indirectly via IDO activity, autophagy or counteracting the hyperinflammation, rather than the restoration of the capability of NET formation, will lead to resistance against Aspergillus infections (33).

The NADPH-oxidase as regulator of inflammation

In chapter 4 we strengthen the hypothesis that hyperinflammation is a major accomplice in the pathogenesis of invasive aspergillosis in the CGD host rather than a distinct clinical characteristic of the CGD patient e.g. as sterile colitis or granulomatous formation.

Dysregulation of the L-tryptophan metabolism in mice with defects in NADPH oxidase, resulting in overproduction of interleukin-17, has been proposed to link ROS defects with hyperinflammation and susceptibility to pulmonary aspergillosis (34). In this thesis we assessed the L-tryptophan metabolism and cytokine profiles in response to different fungal pathogens in CGD patients. We show that CGD patients display a normal L-tryptophan metabolism and although PBMCs of CGD patients produce more proinflammatory cytokines after stimulation, fungal (Candida albicans, A. fumigatus and A. nidulans) induced IL-17A production is strikingly low compared with healthy controls. Mitogenic stimulation of T-cells isolated from CGD patients did not result in an impaired IL-17 production supporting a pathogen specific mechanism. At present, it is not clear if the impaired IL-17 response to fungal pathogens in CGD patients plays an additional role in the occurrence and pathogenesis of invasive fungal infections.

The recurrent mucosal Candida infections in patients with primary immunodeficiency syndromes like hyper-IgE syndrome (HIES) and chronic mucocutaneous candidiasis (CMC) have been specifically linked to a deficiency in the Th17 response. Current knowledge points to a differential role of IL-17: it is of importance in the resistance to mucocutaneous fungal infections, but in invasive fungal infections it is less well defined. Further studies are needed to unravel the exact mechanisms by which the lower IL-17 production contributes to the increased susceptibility of the human CGD host to invasive Aspergillus infections. Influence on the microbiome and colonisation pattern, and in turn the effect of the microbiome on the host defence, as shown for CMC and HIES (35), both characterized by impaired Th17 responses, is an interesting concept. Further attention may be given to the role of IL-17 in maintaining mucosal barrier integrity in the lung and protection to pathogenic insults through preserving tissue integrity by enhancing synthesis of tight junction proteins (36). It is an attracting hypothesis that a first hit is needed, before A. nidulans becomes invasive. Old studies in plant pathology support this hypothesis (37). Another mechanism of the protective effect of IL-17 is the induction of antimicrobial peptides secreted by bronchial epithelial cells such as β-defensins, which in turn have been shown to exert Aspergillus spp. specific activities (38, 39). Inversely, studying the direct effect of IL-17 on the fungi itself is an intriguing topic. IL-17 is able to bind on the conidial surfaces, in a way of “sensing host immunity” followed by significant interference on host-pathogen interplay by affecting fungal morphology and virulence (40).

The observed hyperinflammatory phenotype of the CGD patient is thought to be the consequence of the absence of ROS-mediated downregulation of intracellular signalling pathways and inflammatory cell recruitment. One could argue that any
fungal morphology has been shown to influence the host-pathogen interface of *A. nidulans* and *A. fumigatus* in front of CGD host cells. We suggest that the decreased galactosaminogalactan content in the *A. nidulans* cell wall, which is a known immune-suppressive polysaccharide of *A. fumigatus*, is responsible for the exaggerated inflammatory response. Investigation of the influence of individual polysaccharides, or the expression of other pathogen associated molecular patterns (PAMPs) by the individual *Aspergillus* species, and investigation of the differences in pathogen recognition receptor (PRR) expression between healthy and CGD cells is a crucial next step to take. The observation of a higher pro-inflammatory cytokine release by *A. nidulans* in the CGD host is robust and persistent. Hyperinflammation is the Achilles tendon of the CGD host, and invasive *A. nidulans* infections in the CGD host seem to be much more the result of host damage due to exaggerated host response rather than a defective clearing of the pathogen. These findings do have major implications in the direction of new treatment strategies. They need to target the dysregulated inflammation being at least partly responsible for the induced host damage and detrimental outcome of invasive *A. nidulans* infections in CGD host.

We show that the hyperinflammation induced by *A. nidulans* is IL-1β mediated. Further studies are needed to unravel the IL-1β processing in patients with CGD infected by *A. nidulans* and *A. fumigatus*. Preliminary data indicate post-transcriptional differences in pro-IL-1β processing between *A. nidulans* and *A. fumigatus*. IL-1β-dependent mechanisms, such as decreased autophagy and increased inflammasome activation, are linked to the pathological conditions in CGD and may play a role in the pathogenesis of invasive aspergillosis in CGD. The improved insight in the molecular pathophysiology has provided us, and others, with new targets for treatment. The alkalizing effect of the old malarian drug chloroquine, being a weak base, and its well-known immunomodulating effect as observed in patients with rheumatoid arthritis, led us to investigate its effect on the hyperinflammation caused by *A. nidulans*. We observed a potent decrease of the fungal induced IL-1β release. Others have shown that the decreased autophagy and increased inflammasome activation can be restored by IL-1β receptor blockade in CGD patients (41).

### THE PATHOGEN

In this thesis, we investigated the host-immune interaction between *A. nidulans* compared to *A. fumigatus* and polymorphonuclear cells or PBMCs of patients suffering from CGD. Most of current knowledge on fungal host-pathogenesis with regard to inflammatory responses is based on infection models with inactivated, e.g. heat-killed or formaldehyde inactivated, microorganisms. Use of fixed, killed conidia and or hyphae gives the researcher the possibility to study static interactions between the host and the pathogen. Inactivated pathogens have been taken as inert opponents full of PAMPs. This is a rather unidirectional view on the host-pathogen “interaction” in which we evaluate in a controlled way the effect of the pathogen, in a fixed state of his development, on the immune system. It completely ignores the influence of the immune system on the pathogen and how the pathogen itself is able to counteract the “attack” of the immune system.

In vivo, resting conidia are metabolically quiescent. Once they are in a suitable environment swelling of the conidia begins within hours and is followed by the germination and elongation of hyphae. The conidial maturation triggers a profound morphological change that involves the loss of the proteinaceous hydrophobic layer and the exposure of the inner cell wall. This cell wall is composed mainly of polysaccharides consisting of β-glucan, mannan, chitin and galactomannan (42). It is important to recognize that the morphological state of the *Aspergillus* spp. is critical to its recognition by the host. Our in vitro observations of fungal growth show a substantially slower germination rate of *A. fumigatus* (1x10⁶/ml at 8h shows 20-30% germination, with hyphal length ranging from 8.7 to 11.6 µm) compared to *A. nidulans* (1x10⁶/ml at 8h shows > 90% germination with hyphal length ranging from 17.4 to 46.4 µm). Striking differences in individual cell wall, which may become significant in host recognition and host defence as indicated in our study, do exist. *A. nidulans* and *A. fumigatus* conidia are covered by a different melanin type, and their rodlet layer and individual sugar cell-wall polysaccharide composition differ (43-45). Nevertheless, hardly any data is available about the individual PAMPs present on those two *Aspergillus* species playing a role in the host-pathogen interaction.

From the host site of view, reactive oxygen species play important roles in antimicrobial killing, in signalling and in regulation of inflammatory processes. But from the pathogen’s perspective, ROS do trigger oxidative stress responses and regulate morphogenesis in fungal cells, which in turn might have huge implications in the pathogenesis. *Candida albicans* morphology has been shown to be influenced by hydrogen peroxide, and intracellular ROS is important for maintaining polarized growth of *A. nidulans* (46). As fungal growth and morphogenesis are crucial steps in host-pathogen recognition, to understand the

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virulence of \textit{A. nidulans} in the CGD host, more research is needed to understand the ability and how those fungi react to these different oxidative stress responses. Infections by opportunistic fungi have traditionally been viewed as microbial insults towards a weakened host. Fungal sensing of a host's immune environment might render this process more elaborate than previously thought. Studying the pathogen's response to being recognized by the host cells, e.g. the ability to respond to antifungal mechanisms by the host, will create insights into new and potentially important dimensions of the specific host-pathogen interaction.

**NEW TREATMENT STRATEGIES**

Ultimately, an improved insight in molecular interaction of \textit{Aspergillus} species and CGD host should result in the design of new management strategies.

Up to now, the treatment guidelines for haemat-oncology patients with invasive aspergillosis can be followed in patients with CGD keeping in mind that non-	extit{fumigatus} \textit{Aspergillus} species, like \textit{A. nidulans}, may require a different antifungal treatment (31, 47). The use of adjunctive immunomodulatory treatment options as INF-γ and granulocyte transfusions, while conceptually robust in CGD, is unproven and remains anecdotal (48-50). In this thesis we concluded that \textit{A. nidulans} pathogenicity and disease is the result of imbalanced inflammation rather than impaired direct antifungal host mechanisms. Consequently, medical treatments aiming at killing the fungus (e.g. anti-fungal drugs) are expected to incompletely target the disease process. In chapter 7 we translated our new insights into the pathogenesis of \textit{A. nidulans} infections in the CGD host into promising new treatment options. We show that the old malarial drug chloroquine has antifungal properties and dampens the inflammatory response of CGD cells in vitro. Chloroquine targets both limbs of fungal pathogenesis and may be of great value in the treatment of invasive aspergillosis in patients with CGD. A first step in implementation of these findings is the evaluation of antifungal and anti-inflammatory potential of the less toxic compounds e.g. hydroxychloroquine. Prolonged treatment with chloroquine has been complicated by severe retinopathy. Hydroxychloroquine is an antimalarial agent licensed for the treatment of rheumatoid arthritis juvenile idiopathic arthritis, discoid and systemic lupus erythematosus. It is reported as being half as toxic as chloroquine, yet equally active against \textit{Plasmodium falciparum} (51, 52). The in vivo studies aiming at implementation of the beneficial use of chloroquine in the CGD host need to focus on chloroquine as adjuvant therapy during invasive fungal infections in the CGD patient, as well as on its use as a prophylactic drug. Hydroxychloroquine shows to be of promising value in the treatment of granulomatous complications in chronic granulomatous disease (53) probably by reduction of the inflammatory mediators implicated in the granuloma formation. Moreover, in contrast to corticosteroids commonly used in the treatment of granulomata, hydroxychloroquine is a drug that does not increase the risk of infectious adverse events. It is definitely worthwhile further studying the role of chloroquine, and other chloroquine compounds, as a prophylactic drug in chronic granulomatous disease as it harbours a great potential targeting both infectious and inflammatory complications.

In line with the above-mentioned concept, the next step is the evaluation of IL-1β receptor blocking, e.g. Anakinra®, as adjuvant treatment during invasive \textit{A. nidulans} infections in the CGD patient. Beneficial effects of blocking IL-1β by dampening the inflammasome activation and restoring the defective autophagy, have been observed in patients suffering from CGD colitis treated with the IL-1R antagonist Anakinra® (41). The possible beneficial role of IL-1R blocking as additional immunomodulatory treatment in invasive aspergillosis, specifically due to \textit{A. nidulans}, is an intriguing domain of further research.

To date, haematopoietic stem-cell transplantation (HSCT) is the only curative option for patient with chronic granulomatous disease (54), and has also been used to treat refractory invasive fungal infections in CGD with variable outcome (55). The complexities of the clinical phenotype of chronic granulomatous disease, on one hand, and the perceived and real risks associated with HSCT, on the other, have prevented many patients with this devastating disease from getting this curative treatment. Gene therapy, looking promising in several single gene primary immunoodeficiencies such as severe combined immunodeficiencies (SCID), is currently not an available treatment option due to the need for improved and safer vectors. Although functional correction of only a minor fraction of cells (approximately 10%) provides complete clinical protection, as learned from many X-linked CGD carriers, the absence of growth or survival advantage of the transduced stem cells in the marrow or in the tissue is a great point of concern as well (56).

As long as a cure is not feasible for the majority of the patients with CGD, supportive treatment, e.g. the management of invasive fungal complications, the main cause of premature death in CGD patients, needs to be improved.

**CONCLUSION**

The studies presented in this thesis have provided us with new insights in the understanding of invasive aspergillosis in the CGD patient and have opened new avenues in the treatment of those, often fatal, fungal infections.

More specifically, the absence of a functional NADPH-oxidase leads to exaggerated inflammation and harmful IL-1β release while fungal killing is only
slightly impaired. These results shed new light on the hitherto paradigm that the CGD host is not able to efficiently kill Aspergillus spp. Mechanisms responsible for fungicidal activity in the CGD host need to be explored in more detail, as well as the mechanisms leading to the observed hyperinflammation. Differential PAMPs on A. nidulans and A. fumigatus recognized by as yet unknown PRRs on the CGD phagocytes need to be assessed in association with intracellular signalling pathways. These new findings point to a novel strategy for the prevention and treatment of invasive fungal infections in the CGD host. New treatment strategies need to be viewed in terms of targeting aberrant and inefficient consequences of the host response rather than the pathogen itself. In this regard, chloroquine and IL-1β blockade in addition to the classical antifungal drugs may prove to be potent additional regulators, capable of dampening the excessive inflammatory response in addition to the beneficial eradication of A. nidulans by NADPH-oxidase independent mechanisms. Murine studies in which experimental infections can be modified by chloroquine and IL-1β blockade and its influence on the pathophysiology are needed.

REFERENCES


9

Summary | Samenvatting
Invasive fungal infections are a major treat for patients suffering from chronic granulomatous disease (CGD). Chronic granulomatous disease is a primary immuno-deficiency characterized by the highest lifetime incidence of invasive aspergillosis among all immunocompromised patients. It is a genetically heterogeneous disease caused by a defect of the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) system in which phagocytes fail to produce the microbicidal reactive oxidant superoxide anion and its metabolites. The NADPH-oxidase is a key complex in the innate immunity and plays an essential role as antimicrobial effector complex and as regulator of inflammation. Clinically, as a result of the defect in this key innate host defence pathway, CGD patients suffer from recurrent life-threatening bacterial and fungal infections and inflammatory sequelae leading to granulomata formation. The life-time incidence of invasive fungal infections in children with CGD varies between 25% and 40%, and is the major cause of premature death.

The basis of the fundamental research work presented in this thesis is the observation of the unique epidemiology of invasive fungal infections in patients suffering from CGD. *Aspergillus fumigatus* is the most commonly encountered species. Interestingly, *Aspergillus (Emericella) nidulans* is the second most isolated species and is seldom reported to cause disease in other immunocompromised patients, indicating a unique interaction between this fungus and the CGD host. Furthermore, *A. nidulans* infections in the CGD host are more virulent and associated with a five-to ten-fold higher mortality rate compared to invasive infections caused by *A. fumigatus*.

The aim of my research was to explore the unique host-pathogen interaction between *A. nidulans* and patients suffering from CGD. The studies presented in this thesis addresses the following research-questions:

- Is the incidence of *A. nidulans* infections in the CGD patient explained by the presence of specific defects in the innate immune system, resulting in the higher susceptibility for invasive infections caused by *A. nidulans*.
- What are the direct and indirect consequences on anti-fungal effector mechanisms against *A. nidulans* and *A. fumigatus* of a deficient NADPH-oxidase complex.
- What is the mechanism of the dysregulated inflammation and how does this contribute to the pathogenesis of species-specific invasive *Aspergillus* infections in the CGD host.
- And ultimately, it is possible to translate the improved insight in the molecular interaction between *Aspergillus* species and the CGD host into the design of new management strategies.
RESULTS

In chapter 2, a general introduction is given in the form of a comprehensive review on the current understanding of epidemiological data, mycological characterization and identification, clinical manifestations, diagnostic features and therapeutic strategies of all invasive fungal infections in patients with CGD.

In chapter 3, we investigated the involvement of the NADPH-oxidase and the resulting reactive oxygen species (ROS) in antifungal host defence focused on differences between A. fumigatus and A. nidulans. In contrast with current concepts, we showed that A. nidulans, in contrast to A. fumigatus, is not susceptible to the direct antimicrobial effect of ROS. Stimulation of healthy peripheral blood leukocytes with live A. nidulans did not result in any measurable ROS release, this in contrast to live A. fumigatus. Evaluating the ability of the phagocytic cells to inhibit fungal germination showed that human CGD leukocytes and murine CGD alveolar macrophages were at least equipotent at arresting conidial germination compared to healthy controls. Blocking of the NADPH-oxidase resulted in significantly reduced damage of A. fumigatus but did not affect the damage to A. nidulans hyphae. Furthermore, the microbicidal activity of CGD neutrophils was maintained toward A. nidulans but not A. fumigatus. These in-vitro studies show that antifungal resistance to A. nidulans is not directly ROS related and as a consequence, the etiology of A. nidulans infections in CGD cannot be explained by the simple absence of the direct microbicidal effect of ROS.

The results of the investigations on the role of the NADPH-oxidase complex in intracellular pathway signalling are described in chapter 4. In CGD mice, dysregulation of the L-tryptophan metabolism resulting in overproduction of interleukin-17 has been proposed to link ROS defects with hyperinflammation and susceptibility to pulmonary aspergillosis. We showed that, in contrast to CGD mice, A. fumigatus and A. nidulans induced interleukin-17 production by CGD blood mononuclear cells was strikingly low compared to healthy controls. Reactive oxygen species can act as co-factors for the catabolic indoleamine 2,3-dioxygenase enzyme (IDO). IDO is a rate-limiting enzyme of the L-tryptophan metabolism. Assessment of the L-tryptophan metabolism showed that the indoleamine 2,3-dioxygenase enzyme expression was similar in blood mononuclear cells and neutrophils from CGD patients compared with healthy controls. Conversions of L-tryptophan to L-kynurenine, mediated by IDO, did not differ between CGD patients and healthy controls. Moreover, adding L-kynurenine to the cell cultures did not suppress fungal-induced interleukin-17 production. We concluded that in contrast to the mouse model, CGD patients do not display a defective L-tryptophan metabolism to explain their hyperinflammation and fungal susceptibility.

As the obtained results so far, shed new light on the pathogenesis of Aspergillus infections in CGD host, we summarized our current insights in the position paper entitled “Aspergillus nidulans and chronic granulomatous disease: a unique host-pathogen interaction” (chapter 5). This paper intends to direct further research by indication the gaps in current knowledge and to guide optimal treatment strategies.

In chapter 6 we evaluated the role of the individual cell-wall composition of A. nidulans compared to A. fumigatus and hypothesized that differences in cell-wall components could lead to differences in recognition and pro-inflammatory cytokine release. CGD patients displayed evidence for a chronic hyperinflammatory state as indicated by elevated plasma IL-1β and TNF-α. Peripheral blood monocytes from CGD patients secreted higher levels of IL-1β and TNF-α in response to A. nidulans compared to 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 blood mononuclear cells. Purified cell wall polysaccharides of A. nidulans induced a much higher level of IL-1β secretion by CGD blood mononuclear cells 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 blood mononuclear cells in response to A. nidulans are a consequence of low levels of cell-wall associated galactosaminogalactan.

Based on the above-described new insight in host-pathogen interaction of Aspergillus species, more precisely the interaction between A. nidulans and its preferred host the CGD patient, new treatment strategies for invasive aspergillosis in CGD should target the dysregulated inflammation in addition to fungicidal agents. In chapter 7, we showed that an ‘old drug’, chloroquine, might become of great importance in the treatment of invasive aspergillosis in patients suffering from CGD. The antimalarial drug chloroquine (CQ) is an acidotropic agent that passively diffuses and accumulates into acidic organelles.

Beyond its known antimalarial potential, CQ has shown to have a direct antifungal effect (Histoplasma capsulatum, Cryptococcus neoformans) and has been effective in the treatment of diseases associated with increased release of pro-inflammatory cytokines such as rheumatoid arthritis.

We hypothesized that the addition of CQ would enhance the antifungal activity of phagocytes from patients with CGD by increasing the phagolysosomal pH and by downregulating the exaggerated proinflammatory response. Chloroquine exerted a direct pH-dependent antifungal effect on A. fumigatus and A. nidulans and increased the antifungal activity of CGD neutrophils at a significantly lower concentration compared with the healthy neutrophils. In addition, CQ decreased the TNF-α and IL-1β release after infection with A. nidulans and A. fumigatus.
Significant inhibition of the *A. nidulans*-induced TNF-β and IL-1β release was reached in a ten-fold lower concentration of CQ compared to *A. fumigatus*-induced release. In conclusion, CQ targets both limbs of fungal pathogenesis and might be of great value in the clearance of invasive aspergillosis in patients with CGD.

**OVERALL CONCLUSION**

The studies in this thesis show that the observed clinical epidemiology of the CGD host is the results of a unique interaction between the pathogen and the host. The pathogenesis of invasive fungal infections is a continuum between infection and inflammation. Since the NADPH-oxidase complex acts both as an antimicrobial effector molecule and a regulator of inflammation, fungal pathogenesis in patients with CGD is the result of an imbalance of antifungal capacity and regulation of inflammation.

*A. nidulans* infections in the CGD host are characterized by a distinctive clinical phenotype and hyperinflammation. The hitherto accepted view, invasive fungal infections are the results of a defective antimicrobial function of the NADPH-oxidase, is, justified by sound of this research work, no longer appropriate.

This research gives us new, important insights in the host-pathogen interaction between filamentous fungi and the CGD host. It opens new perspectives in current treatment possibilities. It emphasizes the role of inflammation in infection diseases and the urgent need to focus on this topic if we want to make miles in the treatment and the survival of children with this devastating primary immuno-deficiency.

**NEDERLANDSE SAMENVATTING**

**INTRODUCTIE**

Invasieve schimmelinfecties vormen een ernstige bedreiging voor patiënten met chronische granulomateuze ziekte (CGD). Chronische granulomateuze ziekte is een genetisch heterogene ziekte veroorzaakt door een stoornis in het nicotinamide adenine dinucleotide fosfaat-oxidase (NADPH-oxidase) waardoor geen superoxide, en de hieraan gerelateerde zuurstofradicalen, geproduceerd worden. Het NADPH-oxidase is een sleutel-complex in de aangeboren immuïteit en speelt een centrale rol als antimicrobiële effector, en als regulator van de inflammatoire reactie. Klinisch zijn deze patiënten dan ook gekenmerkt door zowel invasieve bacteriële en schimmel-infecties, als door gestoorde ontstekingsreacties leidend tot granulomen. De ‘lifetime’ incidentie van invasieve aspergillose in patiënten met CGD varieert tussen de 25% en de 50%, en is de belangrijkste oorzaak van vroegtijdig overlijden.

De aanleiding van het fundamentele onderzoek leidend tot de kern van dit proefschrift is de observatie van een unieke epidemiologie van invasieve schimmelinfecties in CGD patiënten.

*Aspergillus fumigatus* is globaal gezien de meest frequente verwekker van invasieve filamenteuze schimmelinfecties. Opmerkelijk is het feit dat in de CGD-patiënt, *Aspergillus (Emericella) nidulans* de tweede meest voorkomende schimmel is, dit in tegenstelling tot het ogenschijnlijke non-virulente karakter in de hemato-oncologische patiënt waarbij infecties door *A. nidulans* slechts zeer sporadisch gerapporteerd zijn. Naast het uniek voorkomen van *A. nidulans* in deze gastheer, zijn deze infecties gekenmerkt door hun agressief karakter met uitgebreide weefseldestructie van de omliggende weefsels. De mortaliteit van invasive aspergillose veroorzaakt door *A. nidulans* is een vijf- tot tienvoud hoger dan invasieve infecties veroorzaakt door *A. fumigatus*.

Het doel van dit onderzoek was het ontrafelen van de unieke gastheer-pathogeene interactie tussen *A. nidulans* en patiënten met CGD. De studies beschreven in dit proefschrift omvatten de volgende fundamentele onderzoeksvragen:

- Kan de hogere incidentie van *A. nidulans* infecties in CGD patiënten verklaard worden door het feit dat het specifieke defect in de aangeboren immuïteit meer consequenties heeft voor een efficiënte afweerreactie ten aanzien van *A. nidulans* in vergelijking met *A. fumigatus*?
- Wat is de rol van het deficiënte NADPH-oxidase als directe en indirecte antimicrobiële effector in de afweer tegen *A. nidulans* en *A. fumigatus*?
- Wat is het onderliggende mechanisme van de gedysreguleerde inflammatoire respons, en hoe beïnvloed dit de species-specifieke pathogenese van invasieve *Aspergillus* infecties in CGD?

**OVERHEF CONCLUSION**

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- Wat is het onderliggende mechanisme van de gedysreguleerde inflammatoire respons, en hoe beïnvloed dit de species-specifieke pathogenese van invasieve *Aspergillus* infecties in CGD?
· Is het mogelijk om de verkregen vernieuwde moleculaire inzichten in de interactie tussen deze verschillende Aspergillus species en de CGD gastheer te vertalen naar nieuwe therapeutische strategieën?

RESULTATEN

In hoofdstuk 2 beschrijven we in een overzichtsartikel de huidige epidemiologische, klinische, diagnostische en therapeutische inzichten en gegevens met betrekking tot invasieve schimmelinfecties in de CGD patiënt.

In hoofdstuk 3 onderzochten we de centrale rol van het NADPH-oxidase en de geproduceerde zuurstofradicalen als antimicrobiële effector in de interactie met A. fumigatus en A. nidulans. In tegenstelling tot alle heersende concepten, ontdekten we dat A. nidulans, in tegenstelling tot A. fumigatus, niet gevoelig is voor het directe effect van zuurstofradicalen. Het stimuleren van 2 soorten witte bloed cellen, de polymorfe neutrofielen en de mononucleaire cellen, van gezonde controles met A. nidulans leidde niet tot inductie van zuurstofradicalen, in tegenstelling tot stimulatie met A. fumigatus. Vervolgens bestudeerden wij het remmen van de ontkieming van Aspergillus conidia en zagen dat humane CGD polymorfe neutrofielen en mononucleaire cellen, en alveolaire macrofagen geïsoleerd uit CGD muizen deze ontkieming even efficiënt remmen als gezonde cellen dit konden. Het blokkeren van het NADPH-oxidase complex in gezonde polymorfe neutrofielen resulteerde in een significante vermindering in het vermogen tot ontkieming opmerkelijk vergelijkbaar met de activiteit van gezonde polymorfe neutrofielen en mononucleaire cellen, en alveolaire macrofagen geïsoleerd uit CGD muizen deze ontkieming even efficiënt remmen als gezonde cellen dit konden. Het blokkeren van het NADPH-oxidase complex in gezonde polymorfe neutrofielen resulteerde in een significante vermindering in het vermogen tot ontkieming van schade aan de A. fumigatus, maar had geen enkele invloed op de groei van de A. nidulans hyfen. Ook de antifungale activiteit van humane CGD polymorfe neutrofielen tegen A. nidulans hyfen bleek vergelijkbaar met de activiteit van gezonde polymorfe neutrofielen. Deze in vitro resultaten gaan in tegen de algemeen geaccepteerde belangrijke rol van het NADPH-oxidase en de zuurstofradicalen in de afweer tegen schimmels, waaronder Aspergillus spp. Ook is middels deze experimenten duidelijk geworden dat het voorkomen van infecties door A. nidulans in de patiënt met CGD niet verklaard kan worden door het directe effect van de afwezigheid van het NAPDH-oxidase.

De onderzoeksresultaten met betrekking tot de rol van het NADPH-oxidase complex in intracellulaire signaleringsprocessen zijn beschreven in hoofdstuk 4. In CGD muizen zijn aanwijzingen geworden dat dysregulatie van het L-tryptofaan metabolisme resulteert in een overproductie van interleukine(IL)-17. Deze overproductie zou de ontbrekende schakel vormen tussen de geobserveerde hyperinflammatie en de gevoeligheid voor invasieve aspergillose in CGD. Wij toonden aan dat in tegenstelling tot het onderzoek gedaan in CGD muizen, de IL-17 productie bij CGD patiënten opmerkelijk laag was na stimulatie met A. nidulans en A. fumigatus. Dit ondanks het pro-inflammatoire profiel van de CGD fagocyt. Zuurstofradicalen zijn een belangrijke co-factor van het enzym indoleamine 2,3-dioxygenase (IDO). IDO is een belangrijk enzym in het L-tryptofaan metabolisme. Evaluatie van het L-tryptofaan metabolisme toonde aan dat ondanks de afwezigheid van zuurstofradicalen, de expressie van het enzym IDO in CGD polymorfe neutrofielen en mononucleaire cellen vergelijkbaar is met deze van gezonde controles. De door IDO gemedieerde conversie van L-tryptofaan tot L-kynurenine, verscheiden niet tussen CGD patiënten en gezonde controles. Opmerkelijk, toewijzing van L-kynurenine resulteerde niet in een onderdrukking van de geïnduceerde IL-17 productie. In tegenstelling tot de observatie gedaan in muizen, is de hyperinflammatie in CGD patiënten niet het gevolg van een gestoorde L-tryptofaan metabolisme.

Gezien het feit dat onze bevindingen nieuwe aspecten in de pathogenese van Aspergillus infecties in de CGD gastheer hebben laten zien, waarbij bestaande dogma’s werden weerlegd, bundelden we deze samen met de bestaande kennis in een opinie artikel getiteld “Aspergillus nidulans en Chronische Granulomateuze Ziekte: een unieke gastheer-pathogen interactie” (hoofdstuk 5).

De celwand is het belangrijkste grensvlak in de interactie tussen gastheer en pathogen en een belangrijk doelwit voor behandeling. In hoofdstuk 6 bestudeerden we de rol van de individuele celwand van A. nidulans en A. fumigatus en onderzochten we de hypothese dat verschillen in componenten van de individuele celwand, waaronder polysacchariden en melanine, resulteren in verschillen in herkenning en de resulterende pro-inflammatoire cytokine release. CGD patiënten zijn gekenmerkt door een intrinsiek pro-inflammatoire fenotype zoals to uiting kwam in verhoogde tumour necrosis factor (TNF)-α en IL-1β basale cytokine concentraties in het plasma van CGD patiënten. Stimulatie van mononucleaire cellen van CGD patiënten met A. nidulans resulteerde in hogere IL-1β en TNF-α cytokine release in vergelijking met A. fumigatus stimulatie. Wij toonden aan dat zowel de cytokine productie van gezonde mononucleaire cellen als die van CGD patiënten gestimuleerd door A. nidulans conidia onafhankelijk is van de aanwezigheid van melanine in de celwand. Geïsoleerde polysacchariden uit de A. nidulans celwand stimuleren een opmerkelijk uitgesproken IL-β afgifte ten opzichte van polysacchariden uit de A. fumigatus celwand. Verschillen in de composietie van de individuele polysacchariden celwand van A. fumigatus en A. nidulans is duidelijk aangetoond. De meest opmerkelijke hierin ligt in de afwezigheid van galactosaminogalactan (GAG) in de celwand van A. nidulans. Door middel van gemanipuleerde A. nidulans stammen, welke galactosaminogalactan tot overexpressie brachten, waren we in staat om aan te tonen dat de toegenomen secretie van IL-1β door CGD mononucleaire cellen in antwoord op A. nidulans stimulatie het gevolg is van de lagere hoeveelheid van celwand geassocieerde galactosaminogalactan.
Het ideaal therapeutisch middel voor CGD patiënten met invasieve Aspergillus infecties zou, gebaseerd op de resultaten van de voorgaande besproken studies, dus niet alleen direct antifungaal moeten werken, maar ook immunomodulair. In hoofdstuk 7 beschrijven we waarom het stokoude antimalaria middel chloroquine wel eens belangrijk zou kunnen worden in de behandeling van invasieve aspergillose in CGD patiënten. Chloroquine dankt zijn werkzaamheid aan de passieve diffusie in zure organellen. Het bevat antischimmel activiteit zoals eerder werd aangetoond bij o.a. *Histoplasma capsulatum*, een zogenaamde dimorfe schimmel, en wordt gebruikt bij patiënten met inflammatoire aandoeningen zoals reumaartse artritis op basis van haar immunomodelerende werking. In dit hoofdstuk onderzochten we de hypothese dat het toevoegen van chloroquine aan de CGD fagocyt zou resulteren in zowel een toename van antischimmel activiteit als een demping van de inflammatoire respons, dit door herstel van de alkalinisatie van het phagolysosoom. Chloroquine vertoont een pH-afhankelijke, directe antifungale activiteit ten aanzien van *A. fumigatus* en *A. nidulans*. Bovendien versterkt chloroquine de antifungale activiteit van de CGD fagocyt en dit bij een twintigvoudig lagere concentratie dan in gezonde controles. Chloroquine remt de TNF-α en IL-1β secretie na stimulatie met *A. fumigatus* en *A. nidulans*. Significante inhibtie van de *A. nidulans*-geïnduceerde TNF-α en IL-1β secretie werd geobserveerd in een tienvoudig lagere concentratie van chloroquine ten opzichte van *A. fumigatus* geïnduceerde cytokine secretie. Concluderend heeft chloroquine een positieve invloed op de twee effector mechanismen die gestoord verlopen in de afweer tegen invasieve aspergillose in CGD patiënten ten gevolge van het defecte NADPH-oxidase.

CONCLUSIE EN BESCHOUWING

Samenvattend kan gezegd worden dat de geobserveerde klinische epidemiologie in de CGD patiënt het resultaat is van een unieke pathogenese welke het resultaat is van een complexe interactie tussen gastheer en pathogeen. In de CGD gastheer heeft de afwezigheid van een functioneel NADPH-oxidase zowel consequenties voor de direct antimicrobiële capaciteit als voor de functie als regulator van inflammatie.

*Aspergillus nidulans* infecties in de CGD patiënt zijn gekenmerkt door een zich onderscheidend van *A. fumigatus* infecties klinisch fenotype en hyperinflammatie. Het heersende concept dat schimmelinfecties in de CGD patiënt simpelweg het gevolg zijn van de afwezigheid van zuurstofradicalen is door toedoen van ons onderzoek niet meer staande. Ons onderzoek heeft geleid tot belangrijke nieuwe inzichten in de gastheer-pathogeen interactie tussen *Aspergillus* en de CGD gastheer. Het opent nieuwe perspectieven voor therapeutisch handelen en benadrukt de noodzaak tot uiteenrafelings van de unieke gastheer-pathogeen interactie met focus op de rol van inflammatie indien men verdere vooruitgang wenst te boeken in de behandeling en een betere overleving van deze patiënten met een ernstige aangeboren afweerstoornis.
Dankwoord

Curriculum Vitae

List of Publications
DANKWOORD

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**CURRICULUM VITAE**

Stefanie Henriet was born on the 4th of December 1976 in Ghent, Belgium. She attended secondary school at the Koninklijk Atheneum Voskenslaan and subsequently undertook medical studies at the University of Ghent, Faculty of Medicine, where she graduated in 2002 cum laude.


The ESPID-Wyeth Fellowship Award granted to her in 2008 provided the opportunity to start a PhD on invasive fungal infections in patients suffering from chronic granulomatous disease. This research was carried out at the Radboud University Medical Center, Nijmegen, The Netherlands, under supervision of Dr. Adilia Warris, Prof. Peter Hermans and Prof. Ronald de Groot. She undertook a clinical fellowship in pediatric infectious diseases and immunology in 2011 at the same institute, which was completed in August 2013. Two clinical attachments in those years contributed to widen her horizon and experience: one at the Department of Pediatric Bone Marrow Transplantation, University Medical Center Utrecht, Wilhelmina Children's Hospital, under supervision of M. Berings (2012), and one at the Department of Pediatric Immunology, Great North Children's Hospital, Royal Vicotoria Infirmary, Newcastle upon Tyne Hospital NHS Foundation Trust, UK, under supervision of Prof. A. J. Cant and Prof. A. Gennery (2013).

Concurrently she studied at the University of Oxford, UK, where she obtained the Postgraduate Diploma in Pediatric Infectious Diseases in 2013.

In the period 2010-2011 she attended the TULIPS (Training Upcoming Leaders in Pediatric Science) PhD Curriculum. Since 2014 she serves as board member of the TULIPS programme. TULIPS has the mission to improve child health by empowering young clinician scientists to become international competitive researchers. It provides PhD and post-doctoral curriculae and weekend educational retreats, to create opportunities for collaboration in all fields related to child health and to enhance competences required to become successful in clinical science.
At present she is working as specialist in pediatric infectious diseases and immunology at the Amalia Children’s Hospital of the Radboud University Medical Centre, Nijmegen, were she combines clinical work in close collaboration with the Laboratory of Pediatric Infectious Diseases, Radboud Institute for Molecular Life Sciences.

Stefanie lives in Nijmegen, with her partner Gert van Bergen.

LIST OF PUBLICATIONS


