Differential *In Vitro* Cytokine Induction by the Species of *Cryptococcus gattii* Complex

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ABSTRACT Cryptococcal species vary in capsule and cell size, thermotolerance, geographic distribution, and affected populations. *Cryptococcus gattii sensu stricto* and *C. deuterogattii* affect mainly immunocompetent hosts; however, *C. bacillisporus*, *C. decagattii*, and *C. tetragattii* cause infections mainly in immunocompromised hosts. This study aimed to compare the capacities of different species of the *C. gattii* species complex to induce cytokines and antimicrobial molecules in human peripheral blood mononuclear cells (PBMCs). *Cryptococcus bacillisporus* and *C. deuterogattii* induced the lowest levels of tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and IL-6 among the five species of the *C. gattii* complex. *Cryptococcus deuterogattii* induced higher levels of IL-22 than those induced by *C. tetragattii* and the environmental species *C. flavescens*. In addition, *C. bacillisporus* and *C. gattii sensu stricto* proliferated inside human monocyte-derived macrophages after 24 h of infection. All *Cryptococcus* species were able to generate reactive oxygen species (ROS) in human PBMCs, with *C. bacillisporus* and *C. deuterogattii* being more efficient than the other species. In conclusion, *C. bacillisporus* and *C. deuterogattii* induce lower levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 and higher ROS levels than those induced by the other species. Species of the *Cryptococcus gattii* complex have different abilities to induce cytokine and ROS production by human PBMCs.

KEYWORDS *Cryptococcus*, *Cryptococcus gattii*, cryptococcosis, cytokines, proinflammatory

Cryptococcosis is a systemic fungal disease caused by the basidiomycetous yeasts belonging to the *Cryptococcus neoformans*/*C. gattii* species complexes (1). The infection is acquired from the environment by inhalation of desiccated yeast cells or basidiospores, which establish either an asymptomatic latent infection or pneumonia and meningoencephalitis (2). The taxonomy of the polyphyletic genus *Cryptococcus* was recently revised (1, 3, 4). The two *C. neoformans* varieties were raised to the species level, as *C. neoformans sensu stricto* (formerly *C. neoformans* var. *grubii*) and *C. deneoformans* (formerly *C. neoformans* var. *neoformans*) (1). In addition, the five genotypes within the *C. gattii* species complex were raised to the species level, as *C. gattii sensu*

Received 2 January 2018 Accepted 4 January 2018

Accepted manuscript posted online 8 January 2018

Citation Herkert PF, dos Santos JC, Hagen F, Ribeiro-Dias F, Queiroz-Telles F, Netea MG, Meis JF, Joosten LAB. 2018. Differential *in vitro* cytokine induction by the species of *Cryptococcus gattii* complex. Infect Immun 86:e00958-17. <https://doi.org/10.1128/IAI.00958-17>.

Editor George S. Deepe, University of Cincinnati

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stricto (AFLP4/VGI), *C. bacillisporus* (AFLP5/VGIII), *C. deuterogattii* (AFLP6/VGII), *C. tetragattii* (AFLP7/VGIV), and *C. decagattii* (AFLP10/VGIV) (1). Although the taxonomic revision was established after >15 years of debate, part of the community prefers the use of the term “species complex” (5) despite the accumulating evidence for the revised taxonomy (6).

Species discrimination has clinical and epidemiological importance, as it has been observed that there are differences in disease presentation and in susceptibility profiles for antifungal drugs (1). Infections by members of the *C. gattii* species complex mainly cause pneumonia, meningoencephalitis, skin lesions, pulmonary or cerebral cryptococcomas, and central nervous system (CNS) disease associated with neurological sequelae. These infections typically require prolonged antifungal therapy (7–11). In contrast, severe meningitis is the main infection caused by *C. neoformans sensu lato* (12). In addition, the species *C. gattii sensu stricto* and *C. deuterogattii* affect mainly immunocompetent hosts (1, 13), whereas *C. bacillisporus*, *C. decagattii*, and *C. tetragattii* are more commonly found in immunocompromised hosts, mostly HIV/AIDS patients, similar to *C. neoformans sensu lato* (1, 7, 14, 15). However, fatal cryptococcosis by *C. bacillisporus* in immunocompetent hosts has been reported in the literature (16, 17).

In addition, phenotypic diversity supports the differences among the species within the *C. gattii* species complex. Capsule and cell sizes vary: *C. gattii sensu stricto* (AFLP4/VGI) has the largest capsules but smaller cells than those of other species, whereas *C. deuterogattii* (AFLP6/VGII) has the largest cells but smaller capsules (18). All species have the ability to grow at 25, 30, and 35°C, but they have various levels of tolerance to 37°C (1, 18). *Cryptococcus deuterogattii* (AFLP6/VGII) has the highest thermotolerance at 37°C, while *C. gattii sensu stricto* (AFLP4/VGI), *C. bacillisporus* (AFLP/VGIII), and *C. tetragattii* (AFLP7/VGIV) have less growth at 37°C than at 30°C (1, 18, 19). There are no significant differences in tolerance to oxidative (19) or osmotic (18) stresses among species.

An important aspect of cryptococcal infections is how *Cryptococcus* species evade the host immune system and establish infection. To reach the CNS, cryptococcal cells use paracytosis to move between tight junctions of the brain endothelium (20), transcytosis to move directly through the endothelial cells (21), and hitchhiking within phagocytes (22, 23). Typically, these fungi avoid killing by host phagocytic cells due to virulence factors, such as the polysaccharide capsule, melanin, and urease production, in addition to other traits, such as the ability to escape phagocytosis by inducing nonlytic exocytosis and by producing titan cells (24–26).

Cryptococcus gattii sensu lato is also able to disturb the inflammatory process, inducing low levels of proinflammatory cytokine tumor necrosis factor alpha (TNF- α) production *in vitro* (27). The cryptococcal capsule components glucuronoxylomannan and galactoxylomannan dampen inflammation by suppressing the NF- κ B pathway and blocking surface antigen recognition (27, 28). In consequence, dendritic cell maturation is compromised, leading to release of TNF- α , interleukin-12 (IL-12), and IL-23 at low levels and to decreased major histocompatibility complex class II molecule expression. Thus, suboptimal T-cell responses and weak proinflammatory responses are induced, inhibiting adequate cryptococcal clearance (27–29).

Most knowledge about the immune response patterns against *Cryptococcus* species is derived from studies of *C. neoformans* species complex isolates performed with animal models and human cells (30–34). However, the interest in studying immune response patterns against the members of the *C. gattii* species complex has increased recently (28, 29, 35–38) due to evidence of genetic diversity, phenetic differences, and epidemiologic particularities among the species (1, 18, 39–41). Based on these aspects, the present study hypothesized that the five species within the *C. gattii* complex have different abilities to induce cytokine production in human cells, a crucial step for the activation of host defense. In addition, macrophage infection and antimicrobial molecules were evaluated.

FIG 1 Monocyte-derived cytokine production induced by members of the *C. gattii* species complex. Human PBMCs ($n = 6$) from healthy volunteers (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml) or live yeast forms of *Cryptococcus* species (2.5×10^6 cells/ml). After 24 h of incubation, TNF- α , IL-1 α , IL-1 β , IL-6, IL-8, and IL-10 production in supernatants was determined by ELISA. Mean values with SEM for three independent experiments done in duplicate are presented. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. s.s., *sensu stricto*.

RESULTS

***Cryptococcus bacillisporus* and *C. deuterogattii* induced lower levels of TNF- α , IL-1 β , and IL-6 than those induced by the other species.** The quantitative concentrations of cytokines produced by human peripheral blood mononuclear cells (PBMCs) from healthy volunteers after stimulation with different *Cryptococcus* species are shown in Fig. 1. *C. neoformans sensu stricto*, *C. bacillisporus*, and *C. deuterogattii* induced lower

FIG 2 T-cell-derived cytokine production induced by members of the *C. gattii* species complex. Human PBMCs ($n = 6$) from healthy volunteers (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml), live yeast forms of *Cryptococcus* species (2.5×10^5 cells/ml), or heat-killed *Candida albicans* (1×10^7 cells/ml), as a positive control. After 7 days of incubation, IL-17, IL-22, and IFN- γ production in supernatants was determined by ELISA. Mean values ($n = 6$) with SEM for three independent experiments done in duplicate are presented. *, $P \leq 0.05$; **, $P \leq 0.01$. Δ , *Candida albicans*.

levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 than those induced by the other species. *Cryptococcus gattii sensu stricto* and the control species *C. flavescens* induced higher levels of IL-10 than those observed with the other species, and no differences in IL-8 levels were observed.

***Cryptococcus deuterogattii* induced higher levels of IL-22 than those induced by *C. tetragattii* and *C. flavescens*.** A comparison of quantitative T-cell-derived cytokine production stimulated by *Cryptococcus* species is shown in Fig. 2. *Cryptococcus* species induced low levels of gamma interferon (IFN- γ) production by human PBMCs, without significant differences among the species. The same was observed for IL-17, with all species tested inducing small amounts of IL-17. For IL-22, *C. bacillisporus*, *C. decagattii*, *C. deuterogattii*, and *C. gattii sensu stricto* induced similar levels. However, *C. deuterogattii* induced higher levels than those induced by *C. tetragattii* ($P < 0.01$) and *C. flavescens* ($P < 0.01$).

***Cryptococcus bacillisporus* and *C. gattii sensu stricto* proliferated inside macrophages after 24 h of infection.** Human primary granulocyte-macrophage colony-stimulating factor (GM-CSF)-derived macrophages were used to evaluate the rates of phagocytosis of three *Cryptococcus* species. *Cryptococcus neoformans sensu stricto* was included as a control; *C. bacillisporus* and *C. gattii sensu stricto* were included because the first induced low and the latter large amounts of proinflammatory cytokines. The rates of phagocytosis for macrophages infected with *C. neoformans sensu stricto*, *C. bacillisporus*, and *C. gattii sensu stricto* are shown in Fig. 3. After infection, 60 to 80% of the total number of macrophages were infected with cryptococci. No significant differences in the percentage of infection were observed after 3 or 24 h of incubation. After preactivation of macrophages with IFN- γ (50 U/ml), no effect on phagocytosis rates was observed (Fig. 3A), and no differences in numbers of intracellular cryptococci per preactivated or nonpreactivated macrophage were found. However, after 24 h, there was a significant increase in the number of intracellular *C. neoformans*, *C. bacillisporus*, or *C. gattii sensu stricto* organisms per macrophage ($P < 0.05$), independent of the absence or presence of IFN- γ . The infection index after 24 h was lower for *C. gattii sensu stricto* ($P < 0.05$) than for *C. neoformans* and *C. bacillisporus* in preactivated and nonpreactivated macrophages. Photomicrographs of macrophages infected with different cryptococcal species are presented in Fig. 3B to D.

The phagocytosed cryptococci were recovered by macrophage lysis, and their viability was investigated by CFU counting (Fig. 3E). The numbers of viable *Cryptococcus neoformans sensu stricto* and *C. bacillisporus* organisms were higher after 24 h than after 3 h of infection for nonpreactivated macrophages. For preactivated macrophages, there was an increase in the number of viable cells only for *C. bacillisporus* after 24 h of infection compared to 3 h of infection. In contrast, *C. gattii sensu stricto* had more

FIG 3 Macrophage infection indexes and cryptococcal viability. Primary human GM-CSF-derived macrophages ($n = 6$) (2×10^5 cells/ml), pretreated or not with IFN- γ (50 U/ml), were infected with one of three cryptococcal species (1×10^6 cells/ml). After 3 and 24 h, the lysates of cells diluted $100\times$ in PBS were seeded onto Sabouraud dextrose medium and incubated for 48 to 72 h, and the numbers of CFU were determined. (A) Primary GM-CSF-derived macrophages (2×10^5 cells/ml) were infected with cryptococcal species (1×10^6 cells/ml) on coverslips under the conditions described above. After the incubation time, cells were stained, and the percentage of infected macrophages, number of intracellular cryptococci per macrophage, and infection index were determined. (B to D) *C. neoformans sensu stricto* (B), *C. bacillisporus* (C), and *C. gattii sensu stricto* (D) internalized by macrophages. (E) Total numbers of live cryptococcal cells recovered from macrophages after 3 and 24 h of incubation. N, macrophage nucleus; Y, yeast cell. *, $P \leq 0.05$.

viability than that of *C. bacillisporus* 3 h after infecting nonpreactivated macrophages. Nevertheless, the viability of *C. gattii* decreased from 3 h to 24 h. Comparing the viabilities among species, *C. neoformans* had more viability than *C. bacillisporus* and *C. gattii sensu stricto* after 24 h of infection in preactivated or nonpreactivated macrophages ($P < 0.05$).

Macrophage cytokine production was measured in the supernatants 3 and 24 h after infection. After 3 h of infection, there was no production of IL-1 β , IL-6, and IL-10 by macrophages. All tested cryptococcal species induced IL-8 and TNF- α production in macrophages after 24 h; *C. gattii sensu stricto* induced higher levels of IL-8 than those induced by *C. bacillisporus* ($P < 0.05$) (see Fig. S1 in the supplemental material). Preactivated macrophages with IFN- γ produced higher levels of TNF- α after 24 h of infection with *C. neoformans sensu stricto* than those produced by nonpreactivated macrophages infected with *C. neoformans sensu stricto* ($P < 0.05$); however, no differences were found among other species.

***Cryptococcus bacillisporus* and *C. deuterogattii* induced higher levels of ROS production than those induced by the other species.** All species within the *C. gattii*

FIG 4 ROS production induced by cryptococcal species. Human PBMCs (1×10^6 cells/ml) from healthy volunteers were stimulated with zymosan (positive control; 1 mg/ml), live yeast forms of *Cryptococcus* species (5×10^6 cells/ml), or heat-killed *Candida albicans* (as a control; 1×10^7 cells/ml), and ROS production was measured by chemiluminescence assay. Data represent mean RLU ($n = 6$ [in quadruplicate]) for two independent experiments, with data collected every 2 min 23 s for 1 h. (A) Integral levels of PBMC ROS production induced by controls and *Cryptococcus* species. *, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant. (B) Kinetics of PBMC ROS production induced by *Cryptococcus* species. (C) Kinetics of PBMC ROS production induced by controls. Δ , *Candida albicans*.

complex induced smaller amounts of reactive oxygen species (ROS) than those seen with *Candida albicans*. *Cryptococcus decagattii*, *C. gattii sensu stricto*, *C. tetragattii*, and an interspecies hybrid (*C. neoformans sensu stricto* $\times$ *C. gattii sensu stricto*) showed the lowest levels of ROS. On the other hand, human PBMCs produced high levels of ROS when stimulated with *C. bacillisporus*, *C. deuterogattii*, and *C. flavesceus*, with peak ROS production at 11 to 19 min (Fig. 4B).

The mRNA expression levels of inducible nitric oxide synthase (iNOS) and the antimicrobial peptides β -defensin 2 and cathelicidin were measured quantitatively (Fig. S2). *Cryptococcus neoformans sensu stricto* downregulated and the *C. gattii* species complex did not change iNOS expression (Fig. S2A). Similarly, no change in β -defensin 2 mRNA expression was detected (Fig. S2B). Overall, the interspecies hybrid, *C. bacillisporus*, *C. decagattii*, and *C. gattii sensu stricto* downregulated the expression of cathelicidin in human PMBCs *in vitro* (Fig. S2C). The main results found are summarized in Fig. 5 and Table 1.

DISCUSSION

In this study, we demonstrate that species of the *C. gattii* complex present different abilities to induce cytokines in human cells. *Cryptococcus bacillisporus* and *C. deuterogattii* induced lower levels of production of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 by human PBMCs than those induced by the other species. *Cryptococcus gattii sensu stricto* induced higher levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 and higher levels of the anti-inflammatory cytokine IL-10 than those observed with other species of the *C. gattii* complex. The interspecies hybrid, *C. decagattii*, and *C. gattii sensu stricto* induced high levels of the proinflammatory cytokines TNF- α and IL-6. All cryptococcal species induced low levels of IFN- γ and IL-17 compared to those induced by another medically important yeast, *Candida albicans*, used as a positive control.

The decrease of proinflammatory cytokines may favor an environment that disturbs maturation and epitope presentation by dendritic cells, leading to suboptimal levels of the Th1 and Th17 responses, which are protective against cryptococcosis and provide fungal clearance (29, 30, 42, 43). IL-1 α and IL-1 β are important in the host defense against fungal infections, activating innate immune cells and modulating adaptive immunity (44, 45). In addition, high levels of the proinflammatory cytokines IL-6, IL-8, IFN- γ , and TNF- α in serum have been associated with cryptococcal meningitis survival in HIV patients (46). Schoffelen and colleagues observed that heat-killed *C. gattii* species complex members induced higher concentrations of the proinflammatory cytokines IL-1 β , IL-6, TNF- α , and IL-17/22 than those seen with the *C. neoformans* species complex (47). However, they did not investigate differences among species of the *C. gattii* complex. Only *C. deuterogattii* induced significant production of IL-22, with clear

FIG 5 Representative scheme of cytokine profile and antimicrobial molecule production in human PBMCs induced by cryptococcal species. *C. bacillisporus* and *C. deuterogattii* induced low levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6, and *C. deuterogattii* also induced IL-22. In contrast, *C. gattii sensu stricto* induced higher levels of TNF- α , IL-1 β , IL-6, and IL-10 than those induced by the other species. ROS are important for controlling *C. bacillisporus* infection in the beginning, but nonkilled *C. bacillisporus* cells are able to proliferate inside macrophages. *Cryptococcus gattii sensu stricto* infection control may be mediated by the combination of ROS and proinflammatory cytokine production. Red arrow, inhibition; dark blue arrow, induction.

differences among the species. IL-22 is a proinflammatory cytokine that promotes antimicrobial molecule production by epithelial cells and may have a protective role against infections (48), including cryptococcal infections. Meningitis caused by members of the *C. gattii* species complex in non-HIV-infected patients has been associated with low levels of the proinflammatory cytokines IFN- γ , TNF- α , and IL-6 and high levels of the anti-inflammatory cytokine IL-10 (49). In the present study, *C. gattii sensu stricto* and the predominantly environmental species *C. flavescens* induced high levels of IL-10, while other species of the *C. gattii* complex induced low levels of IL-10. According to Angkasekwina and coworkers, *C. gattii sensu stricto* and *C. deuterogattii* downregulate pulmonary chemokine expression, leading to a failure to mount protective immunity in immunocompetent hosts (29) and thus contributing to the disease process. It may be that differences in immune responses or clinical aspects of immunocompetent hosts preferentially infected by *C. gattii sensu stricto* or *C. deuterogattii* are due to the

TABLE 1 Summary of observed data

Species, host	Regulation ^a					
	Cytokines	IL-22	IL-10	ROS	Cathelicidin	Proliferation in macrophages
<i>C. gattii sensu stricto</i> , immunocompetent host	↑		↑	↓	↓	↓
<i>C. deuterogattii</i> , immunocompetent host	↓	↑		↑		
<i>C. bacillisporus</i> , immunocompromised host	↓			↑		↑
<i>C. decagattii</i> , immunocompromised host	↑			↓		
<i>C. tetragattii</i> , immunocompromised host	↑			↓		
<i>C. neoformans</i> , immunocompromised host				↓		
<i>C. flavescens</i> , environmental host			↑	↑		

^a ↓, downregulation; ↑, upregulation.

differential levels of cytokines induced in PBMCs, as we showed that these species lead to production of different amounts of cytokines.

Our results showed that all cryptococcal species induced low levels of IFN- γ , and the use of IFN- γ in macrophages preactivation did not improve the phagocytosis rate or reduce the infection, even for *C. neoformans sensu stricto*. However, this cytokine increased TNF- α production in *C. neoformans sensu stricto*-infected human macrophages (see Fig. S1 in the supplemental material). Ikeda-Dantsuji and colleagues also showed that preactivation with IFN- γ did not promote phagocytosis of *C. deuterogattii* but that IFN- γ increased the phagocytic rate of *C. neoformans sensu stricto* (50). Since IFN- γ has been associated with a good prognosis for cryptococcosis (46, 51, 52), these findings may be important for understanding the pathogenesis of cryptococcosis. In our experiments, IFN- γ did not significantly increase the TNF- α production of macrophages after infection with *C. bacillisporus* or *C. gattii sensu stricto*, which would affect the efficiency of macrophages at controlling cryptococcal growth. Wang and colleagues observed low levels of serum IFN- γ in immunocompetent patients with pulmonary cryptococcosis, and after antifungal treatment the serum levels of IFN- γ increased and the protective inflammatory response was restored. They suggested that cryptococcal infection may suppress the immune system and that its elimination helps to establish the immune system again (52). In addition, the use of IFN- γ in combination with standard therapy has been related to successful fungal clearance, resolution of symptoms, and restoration of immunological parameters (51, 53). Improvements in general condition were also observed among non-HIV/nontransplant patients after administration of IFN- γ as adjuvant therapy (54). Meningitis caused by *C. gattii sensu lato* in non-HIV-infected patients has been associated with low levels of IFN- γ and other proinflammatory cytokines in parallel with high levels of the anti-inflammatory cytokine IL-10 (49). Thus, according to *in vivo* results, IFN- γ is important for controlling cryptococcal disease. The results obtained in *in vitro* experiments in the present study may have been affected by the assay conditions, such as the IFN- γ concentration and the time of macrophage exposure to this cytokine. Despite that possibility, for PBMCs, no differences in IFN- γ induction could be detected among the species of the *C. gattii* species complex.

Cryptococcus bacillisporus induced the highest production of ROS, induced small amounts of cytokines, and downregulated cathelicidin compared to those observed with the other species. In contrast, *C. gattii sensu stricto* induced low levels of ROS and high levels of cytokines and downregulated cathelicidin expression. We speculate that ROS are important for controlling *C. bacillisporus* infection during the first hours of infection, as fewer viable *C. bacillisporus* cells than *C. gattii sensu stricto* cells were recovered after 3 h of infection. However, after the initial killing by ROS, the nonkilled *C. bacillisporus* cells were able to proliferate inside macrophages, which might explain the increased number of intracellular cryptococci after 24 h of infection (Fig. 3E). It might be that the production of cytokines by the different *Cryptococcus* species does not correlate with the virulence of the particular species. However, the combination of cytokine production and ROS production may be important in the host-microbe interaction that finally results in control of the microorganism (in this case *C. bacillisporus*). Although *C. gattii sensu stricto* had more viable cells after 3 h of infection, we observed a decrease in viable intracellular cryptococci after 24 h. We speculate that nonlytic exocytosis may have happened, as cryptococcal cells were observed outside macrophages after 24 h of incubation (27). For this species, the combination of ROS and proinflammatory cytokines may increase the capacity of macrophages to control infection. The environmental species *C. flavescentis* induced high concentrations of ROS and cytokine production by human PBMCs, suggesting that the host immune response is effective at eliminating this nonhuman pathogen; this may explain the small number of cases of cryptococcosis caused by this species (55). However, *C. neoformans* and *C. deuterogattii* induced small amounts of cytokines and large amounts of ROS in the first hour of infection, showing the ability to escape and survive inside macrophages, in accordance with previous studies (56, 57–61). Hole and coworkers observed that ROS

TABLE 2 Details of cryptococcal strains used to conduct human PBMC and macrophage stimulation

Isolate code	Code(s) in other collection(s)	Species	Mating serotype	Genotype	Origin	Reference (source) ^a
CBS 10515	H99, WM 04.15	<i>C. neoformans sensu stricto</i>	αA	AFLP1/VNI	Clinical (USA)	1 (CBS)
CBS 10085	WM148	<i>C. neoformans sensu stricto</i>		AFLP1/VNI	Clinical (Australia)	
CBS 996		<i>C. neoformans sensu stricto</i>		AFLP1/VNI	Clinical (Argentina)	
CBS 10496	LSPQ#308	<i>C. neoformans sensu stricto</i> × <i>C. gattii sensu stricto</i>	A × B	AFLP1/VNI × AFLP4/VGI	Clinical (Canada)	63 (CBS)
CBS 10081	WM161, TP 0689, D1.13H	<i>C. bacillisporus</i>	αB	AFLP5/VGIII	Clinical (USA)	1 (CBS)
CBS 6993		<i>C. bacillisporus</i>		AFLP5/VGIII	Clinical (USA)	
CBS 8755		<i>C. bacillisporus</i>		AFLP5/VGIII	Environmental (Colombia)	
CBS 11687	IHEM14941, IHEM14941S	<i>C. decagattii</i>		AFLP10/VGIV	Clinical (Mexico)	1 (CBS)
IHEM14941W		<i>C. decagattii</i>		AFLP10	Clinical (Mexico)	
CBS 10082	WM 178, IFM 50894	<i>C. deuterogattii</i>	αB	AFLP6/VGII	Clinical (Australia)	1 (CBS)
CBS 6956		<i>C. deuterogattii</i>		AFLP6/VGII	Clinical (USA)	
CBS 10090		<i>C. deuterogattii</i>		AFLP6/VGII	Clinical (Greece)	
CBS 10078	WM 179, H33.1, MH56	<i>C. gattii sensu stricto</i>	αB	AFLP4/VGI	Clinical (Australia)	1 (CBS)
CBS 919		<i>C. gattii sensu stricto</i>		AFLP4/VGI	Clinical (USA)	
CBS 6290		<i>C. gattii sensu stricto</i>		AFLP4/VGI	Clinical (Democratic Republic of Congo)	
CBS 10101	WM 779, IFM 50896	<i>C. tetragattii</i>	αC	AFLP7/VGIV	Veterinary (South Africa)	1 (CBS)
B5472		<i>C. tetragattii</i>		AFLP7/VGIV	Clinical (India)	
B5478		<i>C. tetragattii</i>		AFLP7/VGIV	Clinical (India)	
CBS 8645	DBVPG 7166	<i>C. flavescens</i>			Clinical (Greece)	CBS
UC820	ATCC MYA-3573	<i>Candida albicans</i>			Clinical (USA)	ATCC

^aCBS, Westerdijk Fungal Biodiversity Institute (<http://www.westerdijknstitute.nl>); ATCC, American Type Culture Collection (<https://www.lgcstandards-atcc.org>).

produced by human plasmacytoid dendritic cells are required for *C. neoformans sensu stricto* growth inhibition but are not the only mechanism used to control *C. neoformans sensu stricto* (62). The control of the interspecies hybrid, *C. gattii sensu stricto*, and *C. decagattii* may be mediated by cytokine pathways in an ROS-independent manner. These species induced high concentrations of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) which are essential for protection against cryptococcosis (43, 44, 46). According to our results, neither iNOS nor β-defensin 2 seems to be involved in *C. gattii* species complex infection control, but other antimicrobial molecules might be involved.

In conclusion, *C. bacillisporus* and *C. deuterogattii* induced low levels of production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6, and *C. deuterogattii* induced more IL-22 than that induced by *C. tetragattii*. In contrast, *C. gattii sensu stricto* stimulated high levels of production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 and high levels of the anti-inflammatory cytokine IL-10. Despite ROS being important for controlling *C. bacillisporus* infection at the beginning of infection, non-killed *C. bacillisporus* cells are able to proliferate inside macrophages, maintaining the infection. On the other hand, for *C. gattii sensu stricto*, the combination of ROS and proinflammatory cytokine production may be involved in infection control. Overall, the species within the *C. gattii* complex have different abilities to induce cytokine production and ROS by human PBMCs. We have to be aware that host variability is an important factor in determining disease processes, and we cannot exclude the possibility that differences in exposure time or concentrations of fungi or the variation in the host defense systems of the human donors may have affected the outcomes of the comparison experiments. Our results with regard to differences in cytokine and ROS production in response to the members of the *C. gattii* species complex might contribute to the understanding of the cryptococcosis disease process.

MATERIALS AND METHODS

Cryptococcus isolates. Eight cryptococcal strains were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). The details of these isolates are provided in Table 2. The yeast isolates were grown on Sabouraud dextrose agar plates, and a suspension of each isolate was prepared in sterile phosphate-buffered saline (PBS), pH 7.4 (1,000 × g, 10 min, 10°C). The cell viability was checked by dilution in 0.01% trypan blue in PBS, and cells were counted by use of a hemocytometer. *Cryptococcus neoformans sensu stricto* was used as a control instead of *C. deneoformans* because it is predominant

worldwide and is related to the majority of cryptococcosis cases caused by *C. neoformans* species complex infection (39, 64).

Candida isolate. Heat-killed *Candida albicans* ATCC MYA-3573 (UC 820) diluted in sterile PBS was used as a positive control for cytokine stimulation.

PBMC isolation and stimulation. Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Human peripheral blood mononuclear cell (PBMC) isolation was performed by dilution of blood in PBS and differential density centrifugation over Ficoll-Paque density gradient medium (GE Healthcare, Uppsala, Sweden). Cells were washed three times in sterile PBS and then suspended and cultured in RPMI 1640 medium (Gibco-Life Technologies, Carlsbad, CA, USA) supplemented with 10 mM pyruvate, 10 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). Subsequently, cells were counted in a Coulter ZH counter (Beckman Coulter, Fullerton, CA, USA) and adjusted to 5×10^6 cells/ml. Thereafter, 100- μ l aliquots of PBMCs (5×10^5 cells) were added to a round-bottomed 96-well plate and incubated with either 100 μ l live *Cryptococcus* (2.5×10^6 cells/ml) isolate, 100 μ l of ultrapure *Escherichia coli* lipopolysaccharide (LPS) (O111:B4; Sigma-Aldrich) (10 ng/ml), or 100 μ l of RPMI medium alone at 37°C and 5% CO₂. All stimulations were performed in medium containing 10% human serum, which was obtained from a serum pool from healthy volunteers. After 24 h or 7 days, supernatants were collected and stored at -20°C until they were used for assays. After 24 h, the cell monolayers were collected by adding 100 μ l of 0.5% Triton X-100 to measure intracellular cytokine production and 200 μ l of TRIzol and were stored at -80°C until mRNA extraction.

Cytokine measurements. Monocyte-derived TNF- α , IL-6, IL-1 β , IL-8, and IL-10 levels in culture supernatants after 24 h of incubation were determined by use of commercial enzyme-linked immunosorbent assay (ELISA) kits (Sanquin, Amsterdam, the Netherlands [IL-6, IL-8, and IL-10], and R&D Systems, Minneapolis, MN, USA [TNF- α and IL-1 β]), and intracellular IL-1 α protein in cell lysates collected by use of Triton X-100 was measured by another ELISA (R&D Systems) according to the manufacturer's instructions. In preliminary experiments, we determined that 24 h was the time of maximum levels of monocyte-derived cytokine production for each species (data not shown). The levels of T-cell-derived cytokines IFN- γ , IL-17, and IL-22 in culture supernatants after 7 days of incubation were determined using ELISA kits (Sanquin [IFN- γ] and R&D Systems [IL-17 and IL-22]). Results are presented in picograms per milliliter.

Macrophage infection assay. Primary monocytes were obtained by hyperosmotic Percoll (Sigma-Aldrich) density gradient centrifugation of PBMCs. Afterward, cells were washed once with PBS and seeded in tissue culture plates at 37°C and 5% CO₂ in the presence of RPMI medium supplemented with 10% human pooled serum and GM-CSF (50 ng/ml) (R&D Systems). After 6 days of differentiation, macrophages were harvested by use of cold PBS and adjusted to 5×10^6 cells/ml. Thereafter, 200- μ l aliquots of macrophage suspension (2×10^5 cells/ml) were added to 24-well plates, with or without glass coverslips. Macrophages were left to adhere for 30 min and preincubated with or without recombinant human IFN- γ (rhIFN- γ) (50 U/ml; Boehringer Ingelheim, Alkmaar, the Netherlands) for 1 h. Thereafter, cultures were infected with 1×10^6 (multiplicity of infection [MOI] = 5:1) live yeast forms of *C. neoformans sensu stricto*, *C. bacillisporus*, or *C. gattii sensu stricto*. After 3 h, noninternalized fungi were washed off, the medium was replaced, and cultures were incubated for 24 h. For wells without coverslips, after the incubation time (3 h or 24 h), macrophages were lysed with water and mechanical lysis, diluted 100 $\times$ in PBS, and seeded onto Sabouraud dextrose medium. Plates were incubated for 48 to 72 h at 37°C for CFU quantification. For wells with coverslips, cells were fixed, stained with Giemsa stain (Merck Millipore, Billerica, MA, USA), and analyzed by light microscopy (magnification, $\times 1,000$) to determine the infection index. Three hundred cells were analyzed, and the percentage of infected cells and the mean number of intracellular cryptococci per infected cell were determined. The infection index was calculated as follows: infection index = percentage of infected macrophages $\times$ mean number of intracellular cryptococci per macrophage.

ROS measurement. Human PBMCs (1×10^6 cells/ml) were suspended in Hanks' balanced salt solution and exposed to different concentrations of live yeast forms of *Cryptococcus* species (5×10^6 cells/ml). Heat-killed *C. albicans* and zymosan (1 mg/ml) (InvivoGen, Toulouse, France) were used as positive controls. ROS formation was measured by a chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (5 mM; Sigma-Aldrich). The luminometer measured chemiluminescence in the integration mode at 37°C for 1 h after luminol was added. Results are presented as numbers of relative light units (RLU) per second.

mRNA expression by qPCR. RNA isolation was carried out as reported previously (65). RNA was precipitated with isopropanol and washed with 75% ethanol, followed by reconstitution in RNase-free water. Subsequently, RNA was reverse transcribed into cDNA by use of an iScript kit (Bio-Rad, Hercules, CA, USA). Diluted cDNA was used for quantitative real-time PCR (qPCR) analysis, which was done by using a StepOne Plus sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR green master mix (Applied Biosystems). Primer sequences (see Table S1 in the supplemental material) for inducible nitric oxide synthase (iNOS), β -defensin 2, and cathelicidin were obtained from the Harvard Primerbank database. Primers were purchased from Biolegio (Nijmegen, the Netherlands). The mRNA analysis was done by the $2^{-\Delta\Delta CT}$ method, and the results were normalized against those for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

Statistical analysis. Data are given as mean values with standard errors of the means (SEM). The Mann-Whitney U test for unpaired, nonparametric data was used to compare differences in cytokine production between two groups. Two-way analysis of variance (ANOVA) with Bonferroni's multiple-

comparison test was used when more than two groups were compared. GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) was used to tabulate and analyze the data.

Ethics statement. The donation of blood by healthy volunteers was performed after written consent and was approved by the Ethical Committee of the Arnhem-Nijmegen Region, the Netherlands.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00958-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

We thank CNPq for financial support (F.R.-D. and L.A.B.J.) (grant 465771/2014-9 via the INCT [National Institute of Science and Technology], Brazil, for strategies in host-pathogen interaction). F.R.-D. is a research fellow of CNPq. CAPES and CNPq, Brazil, financially supported the work of P.F.H. and J.C.D.S. M.G.N. was supported by an ERC consolidator grant (grant 310372) and a POC-FUSE grant of the Romanian National Agency for Scientific Research.

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