Neutrophil activation by *Candida glabrata* but not *Candida albicans* promotes fungal uptake by monocytes

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Summary

*Candida albicans* and *Candida glabrata* account for the majority of candidiasis cases worldwide. Although both species are in the same genus, they differ in key virulence attributes. Within this work, live cell imaging was used to examine the dynamics of neutrophil activation after confrontation with either *C. albicans* or *C. glabrata*. Analyses revealed higher phagocytosis rates of *C. albicans* than *C. glabrata* that resulted in stronger PMN (polymorphonuclear cells) activation by *C. albicans*. Furthermore, we observed differences in the secretion of chemokines, indicating chemotactic differences in PMN signalling towards recruitment of further immune cells upon confrontation with *Candida* spp. Supernatants from co-incubations of neutrophils with *C. glabrata* primarily attracted monocytes and increased the phagocytosis of *C. glabrata* by monocytes. In contrast, PMN activation by *C. albicans* resulted in recruitment of more neutrophils. Two complex infection models confirmed distinct targeting of immune cell populations by the two *Candida* spp.: In a human whole blood infection model, *C. glabrata* was more effectively taken up by monocytes than *C. albicans* and histopathological analyses of murine model infections confirmed primarily monocyctic infiltrates in *C. glabrata* kidney infection in contrast to PMN-dominated infiltrates in *C. albicans* infection. Taken together, our data demonstrate that the human opportunistic fungi *C. albicans* and *C. glabrata* are differentially recognized by neutrophils and one outcome of this differential recognition is the preferential uptake of *C. glabrata* by monocytes.

Introduction

*Candida albicans* and *Candida glabrata* are the two most prevalent pathogens in the genus *Candida* and account for the majority of cases of candidiasis worldwide (Perlroth et al., 2007; Pfaller and Diekema, 2007). Although the two species harbour some of the same virulence factors, i.e. adhesion properties (Cormack et al., 1999; Hoyer et al., 2008), glutathione biosynthesis (Yadav et al., 2011) and expression of extracellular enzymes (Hube et al., 1997; Kaur et al., 2007), they are phylogenetically quite distinct (Dujon et al., 2004; Brunke and Hube, 2013). This is most evidently recognized by the fact that both species differ on the key attribute of morphological plasticity: *C. albicans* can respond to changes in environmental conditions by alternating between yeast and hyphal growth. This morphological change is accompanied by specific expression of virulence-related surface proteins in filamentous forms and can directly contribute to host cell damage and initiation of immune responses (Kumamoto and Vinces, 2005; Wozniok et al., 2008; Sudbery, 2011; Martin et al., 2013). In contrast, *C. glabrata* exists exclusively in the yeast form during human infection. This, as well as other
fundamental differences in the biology of both species, suggests that virulence properties have evolved independently in both species (Brunke and Hube, 2013). Consequently, there is also evidence that the interplay of C. albicans and C. glabrata with the human immune system differs considerably. In murine i.v. (intravenous) injection models, C. albicans can cause systemic hyperinflammation, closely resembling clinical sepsis (Arendrup et al., 2002; Kollef et al., 2012; Voigt et al., 2014), whereas C. glabrata is less virulent and may lead to persistent infections (Jacobsen et al., 2010). In concordance with this observation, it has been shown that C. albicans and C. glabrata have evolved different mechanisms for escaping cellular immune responses (Brunke and Hube, 2013). Strategies of C. albicans to escape phagocytosis and killing by immune cells are mainly related to induction of filamentous growth. Inside host cells, C. albicans differentiates into the filamentous form, which penetrates the host cell and enables escape and continuous proliferation of the fungus (Kurzai et al., 2005; Vylkova and Lorenz, 2014). Recently, it has been shown that C. albicans actively modulates phagosomal pH to induce its filamentation (Vylkova and Lorenz, 2014), and thus host cell escape. Indeed, the dominant function of polymorphonuclear cells (PMN) in the response against C. albicans is also related to the fact that these cells are the only known type of host cells capable of actively preventing intracellular filamentation of C. albicans (Wozniok et al., 2008; Hünstiger et al., 2014). Rather than actively escaping immune cells, C. glabrata is capable of intracellular survival and replication in monocytic cells. Despite intracellular replication of C. glabrata, macrophages do not undergo apoptosis or necrosis and show little signs of activation (Kaur et al., 2007; Seider et al., 2011). However, phagosomes containing viable C. glabrata failed to recruit cathepsin D and were only weakly acidified; thus generating an intracellular environment permissive to fungal survival and proliferation (Seider et al., 2011).

In our study, we directly compared activation of human PMN by C. albicans and C. glabrata. Our data show that PMN activation triggered by the two species differs considerably. Whereas PMN are more efficiently able to eliminate C. glabrata, this species induces a low-grade inflammatory response, recruiting and activating monocytes rather than PMN. In contrast, PMN activation by C. albicans is rapid and pronounced, primarily recruiting and activating further PMN. Consequently, C. glabrata triggers human PMN to actively promote its uptake into a cell type where it can survive and replicate intracellularly. These in vitro data are reflected by a shift of fungal association from PMN to monocytes in human blood for C. glabrata and histopathological data from murine infection experiments.

Results

Different levels of PMN activation are induced by C. albicans and C. glabrata

To compare PMN activation triggered by C. albicans and C. glabrata, we confronted primary human PMN and quantified induced PMN effector mechanisms after confrontation with the two species. Both species induced neutrophil oxidative burst over time (Fig. 1A). However, confrontation with C. glabrata led to a slower initiation of oxidative burst than C. albicans. Formation of reactive oxygen species (ROS) was induced by C. albicans after 60 min of co-incubation (248.2 ± 67.8%) compared with the mock-infected control (set to 100%). At this time point, ROI formation was barely detectable in response to C. glabrata (118.9 ± 7.0%, P < 0.01 compared with PMN + C. albicans) and remained significantly lower than that for C. albicans at 120 min (324.0 ± 77.0% and 614.2 ± 203.9%, respectively, P < 0.05) (Fig. 1A). Analyses of the surface phenotype of PMN revealed that activation was largely restricted to those cells that had phagocytosed either C. albicans or C. glabrata. Increased surface levels of degranulation markers CD63 (496.2 ± 77.1%) and CD66b (354.4 ± 78.3%) and the down-regulation of CD16 (44.2 ± 5.5%) could be detected 60 min after inoculation of C. albicans for PMN associated with fungi (Fig. 1B). In comparison, C. glabrata induced a less pronounced response after phagocytosis (CD63: 247.3 ± 45.5%, CD66b: 150.7 ± 41.2%, CD16: 89.4 ± 6.8%). Differences in PMN surface exposure of degranulation markers CD63 and CD66b after contact to both fungi were also reflected by concentrations of secreted anti-microbial effector proteins in the supernatants of confrontation assays (Fig. 1C). Release of elastase 2 and myeloperoxidase (MPO) from primary granules was increased upon confrontation with both Candida spp. compared with mock-infected control. In each case, C. albicans confrontation increased effector protein concentration more than C. glabrata (Fig. 1C).

Similarly, protein secretion from the secondary granules showed that C. albicans induced a higher concentration of lactoferrin (1448.6 ± 825.6 ng ml⁻¹) than C. glabrata (581.3 ± 130.6 ng ml⁻¹). To determine the effect of this differential activation of PMN on fungal viability, we performed an XTT assay. Our data show that in the presence of PMN, C. glabrata (23.2 ± 3.8%) had lower metabolic activity than C. albicans (42.5 ± 12.5%, P < 0.01) (Fig. 1D). In line with this, live cell imaging revealed that after phagocytosis C. glabrata was more efficiently killed by PMN than C. albicans (Supporting Information Fig. S1). Following a 2 h confrontation, 69.2% (9 of 13 events) of phagocytosed C. glabrata was killed, whereas only 10.3% (3 of 29 events) of phagocytosed C. albicans was killed. Free Candida largely remained viable. To exclude
Different levels of PMN activation are induced by Candida spp. Primary PMN were either mock infected or confronted with C. albicans and C. glabrata at an MOI of 0.5 and analysed for generation of intracellular ROS (neutrophil oxidative burst), surface expression of PMN activation markers and secretion of neutrophil granule contents after various time points. In general, PMN activation was stronger during co-incubation with C. albicans than with C. glabrata.

A. The percentages of ROS formation in samples inoculated with C. albicans (dark grey bars) and C. glabrata (light grey bars), measured as median DCF fluorescence intensity, were calculated relative to basal levels in the mock-infected sample (set to 100%, white bars) and shown for 20, 60 and 120 min of infection. The bars show means ± SD of three independent experiments with cells from different donors, *P < 0.05, **P < 0.01.

B. Changes in the surface expression levels of PMN activation markers CD63, CD66b and CD16 are shown in representative histograms after 60 min of confrontation with C. albicans (dotted line) and C. glabrata (dashed line). Filled histograms indicate basal expression of PMN from mock-infected sample, lines represent PMN associated with Candida. Data from one of four independent experiments using cells from different donors with virtually identical results are shown. Quantitative analysis was performed for each surface marker. Data shown are means ± SD and normalized to basal levels of mock-infected PMN (set to 100%, white bars), **P < 0.01, ***P < 0.001. All activation markers revealed significantly different surface levels after phagocytosis of either C. albicans (dark grey bars) or C. glabrata (light grey bars).

C. Supernatants from mock-infected PMN (white bars) or immune cells co-incubated with C. albicans (dark grey bars) and C. glabrata (light grey bars) were analysed for the presence of MPO, elastase 2 and lactoferrin following a 4 h confrontation. Bars show means ± SD of at least three independent experiments with cells from different donors, *P < 0.05.

D. The metabolic activity of fungal cells in the absence or presence of PMN was determined via the XTT assay. Confrontation with PMN for 60 min resulted in a significantly greater metabolic activity of C. albicans (dark grey bars) than of C. glabrata (light grey bars). Values were normalized to 100% metabolic activity of control fungi in media (striped bars) and correspond to the means ± SD of at least three independent experiments with PMN isolated from blood of different donors, **P < 0.01.

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C. albicans induces higher motility in human PMN than C. glabrata

Manual cell tracks of PMN were characterized based on local migration behaviour to investigate pathogen-triggered motility. Mean square displacement (MSD) curves (see Experimental procedures) were analysed as a function of the time lag for phagocytic PMN before phagocytosis of C. albicans or C. glabrata. Here, the time lag is associated with all possible segments over a specific time interval that can be generated from the cell tracks (Michalet, 2010). Analysis was restricted to phagocytically active PMN before the phagocytic event took place, as PMN were reducing their motility dramatically after phagocytosis had occurred. This analysis allowed us to compare motility independent of phagocytosis rates (Fig. 3). Data were fitted by Fürth’s formula of persistent random walk migration to estimate the corresponding persistence times and diffusion constants. For the confrontation assay with C. albicans, persistence time \( t_p \) and diffusion constant \( D \) of PMN were \( t_p = 1.1 \text{ min} \) and \( D = 95 \mu \text{m}^2 \text{ min}^{-1} \), whereas for the confrontation assay with C. glabrata we found \( t_p = 0.7 \text{ min} \) and \( D = 41 \mu \text{m}^2 \text{ min}^{-1} \). This quantitative difference in the diffusion constants was confirmed to be significant by a Wilcoxon rank sum test comparing per time lag the sets of data points that contribute to the two MSD curves. We also estimated a lower and upper limit in the variation of the diffusion constant by fitting Fürth’s formula to the mean values ± the standard deviations (SDs) in the MSD.
The variation of the diffusion constant was found to be within $67 \mu m^2 min^{-1} \leq D \leq 135 \mu m^2 min^{-1}$ for PMN with *C. albicans* and this was clearly different from its variation between $26 \mu m^2 min^{-1} \leq D \leq 57 \mu m^2 min^{-1}$ for PMN with *C. glabrata*. Thus, in contrast to PMN confronted with *C. glabrata* ($D = 41 \mu m^2 min^{-1}$), confrontation with *C. albicans* resulted in a significant higher PMN diffusion constant ($D = 95 \mu m^2 min^{-1}$), which reveals that *C. albicans* induces significantly more PMN migration than *C. glabrata*.

In contrast to *C. albicans*, *C. glabrata* is frequently touched but not phagocytosed by PMN

The frequency of PMN–*Candida* spp. encounters was studied to analyse the physical interaction and its consequences (Fig. 4). PMN, which were associated with *C. albicans*, touched the fungal cells on average $1.31 \pm 0.15$ times per hour (Fig. 4A), which resulted in phagocytosis in $69.7 \pm 8.7\%$ of the events (Fig. 4B). Co-incubations of PMN and *C. glabrata* revealed that physical contact between PMN and *C. glabrata* is less likely to result in uptake of fungal cells by PMN. However, low phagocytosis of *C. glabrata* was not related to a lack of contact between fungi and PMN. In fact, *C. glabrata* was contacted significantly more often ($2.16 \pm 0.18$ times per *Candida* per hour, $P < 0.01$) than *C. albicans* (Fig. 4A). However, the probability that contact would result in phagocytosis was only $11.7 \pm 1.7\%$ for *C. glabrata* (Fig. 4B). The contact-to-phagocytosis ratio was independent of the multiplicity of infection (MOI):

Despite a higher number of phagocytosis events during confrontation of PMN and *C. glabrata* at an MOI of 5 (Supporting Information Video Clip S5), the frequency of touches by PMN was also enhanced and occurred on average $2.26 \pm 0.09$ times per fungal cell during 1 h (Fig. 4A). Hence, the phagocytosis probability after contact ($13.2 \pm 2.1\%$) was equal to PMN–*C. glabrata* confrontation at an MOI of 1 (Fig. 4B). These data indicate that the probability of phagocytosis after contact is significantly lower for *C. glabrata* than for *C. albicans*.

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**Fig. 3.** *C. albicans* induces higher motility in human PMN than *C. glabrata*. The MSD computed from cell tracks of PMN before phagocytosis of *C. albicans* (red) or *C. glabrata* (green) has a linear dependence on the time lag. Only PMN, which did phagocytose fungi during the confrontation, were included in the analysis. Error bars correspond to the standard deviation of the MSD and are shown at selected time points with the dashed grey curves being a guide for the eye. Theoretical MSD curves (blue) are based on a persistent random walk model.

**Fig. 4.** *C. glabrata* was frequently touched but not phagocytosed by PMN. Live cell imaging data of co-incubations with PMN and *C. albicans* at an MOI of 1 (dark grey filled bars), *C. glabrata* at an MOI of 1 (light grey filled bars) and MOI of 5 (light grey striped bars) were analysed manually for touching and uptake of the fungal cells by PMN.

A. Data represent the number of contacts between PMN with either *C. albicans* or *C. glabrata* per *Candida* per hour. Fungi, which were not contacted during co-incubation, were excluded.

B. Bars show the frequencies of *C. albicans* or *C. glabrata* that have been successfully phagocytosed relative to the total number of *Candida* contacts by PMN (set to 100%). *C. glabrata* was touched significantly more often by PMN than *C. albicans*, but the probability that contact would result in phagocytosis was significantly higher for *C. albicans*. Values correspond to the means ± SD of each three independent experiments with primary PMN isolated from different donors, **$P < 0.01$, ***$P < 0.001$.**
Differential cytokine secretion by PMN influences cell recruitment in response to Candida spp.

In addition to their direct effector mechanisms, PMN secrete inflammatory mediators that can influence other immune cells. We measured a set of 18 cytokines/chemokines (see Experimental procedures), which have been shown to be expressed and released by PMN upon activation (reviewed in Mantovani et al., 2011) and found six of them (IL-8, MIF, GROα, MIP-1α, MIP-1β and TNFα) to be differentially induced by the two Candida spp. (Fig. 5A). Secretion of IL-8 and GROα, both chemoattractants and activating factors for PMN and MIF, a pro-inflammatory factor that may inhibit migration of monocyctic cells, was more induced by C. albicans, although IL-8 and GROα concentrations during C. glabrata infection were also markedly increased compared with PMN alone. Interestingly, confrontation with C. glabrata induced secretion of chemokines MIP-1α and MIP-1β in significantly higher concentrations than with C. albicans. Furthermore, PMN confronted with C. glabrata released more TNFα than when confronted with C. albicans. Taken together, these data indicated chemotactic differences in PMN signalling towards recruitment of further immune cells upon confrontation with Candida spp. To investigate this, we tested the ability of PMN–Candida co-incubations to recruit either PMN or monocytes by using a transwell migration assay (Fig. 5B). These data showed that PMN migrate at a higher rate towards co-incubations of PMN with C. albicans than towards co-incubations with C. glabrata, which is in accordance with the reduced induction of IL-8 and GROα release by C. glabrata. As increased MIP-1α and MIP-1β and low levels of MIF were secreted by PMN in response to C. glabrata confrontation, we also determined monocyte migration towards PMN–Candida co-incubations. In contrast to further recruitment of PMN, a greater migration of monocytes towards PMN–C. glabrata co-incubations than towards PMN–C. albicans co-incubations was observed. These data indicate that C. glabrata actively enhances migration of monocyctic cells to a site of infection, whereas C. albicans – as shown in previous studies – induces a neutrophil-centred response (Hünniger et al., 2014).

Soluble factors secreted during PMN–C. glabrata confrontation enhance the monocyte response to C. glabrata

Based on the finding that PMN secrete chemokines, which may promote the chemotaxis of macrophages and monocytes (Kasama et al., 1993) upon confrontation with C. glabrata (Fig. 5), we examined the interaction of monocytes and C. glabrata in live cell imaging (Fig. 6, Supporting Information Video Clips S6–S8). Throughout the experiment in the presence of mock-conditioned media, monocytes were motile and although they made contact with C. glabrata, this rarely resulted in phagocytosis (6.8 ± 6.4% of total monocytes in the field of view) (Fig. 6B, left graph, Supporting Information Video Clip S6). Strikingly, when confrontation was performed in the presence of supernatant from a PMN and C. glabrata co-incubation, monocyte phagocytosis increased in occurrence and frequency (30.0 ± 20.2% of total monocytes in the field of view, P < 0.05) (Fig. 6B, left graph, Supporting Information Video Clip S7). These data indicate that the PMN-secreted factors not only act to recruit monocytes but also impact on their anti-fungal activity via increasing uptake of fungal cells. To determine if the effect of Candida–PMN conditioned media was general or specific for C. glabrata, we also performed live cell imaging on monocytes confronted by C. glabrata in the presence of supernatant from PMN confronted with C. albicans (Supporting Information Video Clip S8). As expected, monocyte activity was not increased in the presence of C. albicans-conditioned media (Fig. 6B, right graph).

C. glabrata shows enhanced association to monocytes in a human whole blood model and predominantly recruits mononuclear cells in a model of murine systemic infection

So far, experiments were performed with primary human immune cells. However, experiments with isolated cells may not necessarily reflect in vivo conditions as interaction of the purified cells with other components of the host response is abrogated (Hünniger et al., 2015). To further substantiate our findings in a more complex array of immune cells and soluble factors, we used a previously established whole blood infection model (Hünniger et al., 2014), which closely represents the environment in vivo.

In agreement with the results obtained for primary PMN confrontation assays, C. albicans resulted in a stronger PMN activation than C. glabrata during whole blood infection. Decrease in CD16 surface levels as well as up-regulation of degranulation marker CD66b on PMN that ingested Candida and, consequently, the release of anti-microbial effectors proteins were significantly more pronounced in the presence of C. albicans (Fig. 7A and B). However, following a 60 min confrontation with human blood, 35.2 ± 10.4% C. albicans versus 16.4 ± 9.2% C. glabrata remained viable, which is in line with the evidence from primary cell assays that C. albicans is more efficient in withstanding the PMN attack than C. glabrata (Fig. 7C). Although we could not detect an overall difference in the MIP-1α and MIP-1β secretion profile of whole blood after 4 h of infection, differences in IL-8 and MIF.
release induced by the two Candida spp. were clearly present. In accordance with data obtained for purified PMN, C. glabrata induced significantly lower levels of both chemokines than C. albicans (Fig. 7D). In addition, GRO\(\alpha\) levels in response to C. albicans and C. glabrata were similar to results obtained for primary PMN confrontation. To test whether C. glabrata would target monocytes more efficiently than C. albicans, we determined the distribution of C. albicans and C. glabrata to immune cells during whole blood infections. Relative to all
fungal cells, \textit{C. glabrata} associated with monocytes (11.0 ± 3.3\% of all fungal cells) significantly more than \textit{C. albicans} (6.6 ± 2.0\%, \(P < 0.001\)). In line with this, the association to PMN was higher for \textit{C. albicans} (72.6 ± 10.9\%) than for \textit{C. glabrata} (65.2 ± 10.7\%, \(P < 0.05\)) (Fig. 7E).

For further quantification, we applied a previously developed virtual infection model which facilitates the calculation of quantitative predictions of specific transition rates between \textit{Candida} states (Hünniger \textit{et al.}, 2014). The virtual infection model of the whole blood experiments confirmed our experimental data from the live cell imaging. While the difference between the intracellular killing rates of monocytes (\(\kappa_M\)) for \textit{C. glabrata} and \textit{C. albicans} was found to be negligible, a 2.4-fold higher intracellular killing rate of PMN (\(\kappa_G\)) was obtained for \textit{C. glabrata} compared with \textit{C. albicans} for the whole blood assay. Furthermore, a 2.5-fold higher phagocytosis rate was predicted by the virtual infection model for monocyte phagocytosis (\(\phi_M\)) of \textit{C. glabrata} compared with \textit{C. albicans}.

To determine whether the recruitment of innate immune cells also differs between \textit{C. albicans} and \textit{C. glabrata in vivo}, we analysed histopathological sections from kidneys of intravenously infected mice (Supporting Information Fig. S3A). In line with previous observations (Jacobsen \textit{et al.}, 2010), histological evidence clearly suggested PMN-dominated infiltrates for \textit{C. albicans} infection, whereas \textit{C. glabrata}-induced infiltrates mainly consisted of monocyctic cells. In addition, quantification of the PMN marker enzyme MPO in kidney homogenates...
revealed much higher levels in response to C. albicans (16.11 ± 1.12 μg g⁻¹; 24 h post infection and 17.03 ± 3.39 μg g⁻¹; 72 h post infection) than during C. glabrata infection (0.27 ± 0.11 μg g⁻¹; 48 h post infection), further supporting the high PMN infiltration into the kidney in response to C. albicans (Supporting Information Fig. S3B).

Taken together, the data from virtual and both complex infection models further confirm that C. glabrata induces predominantly a monocytic response whereas the innate response towards C. albicans is centred on neutrophils.

Discussion

C. albicans is habitually employed as a model organism to investigate the immune response to Candida although literature clearly suggests different immune responses to distinct species (Arendrup et al., 2002). This is especially true for C. albicans and C. glabrata. Although grouped in the same genus, they are only distantly related (Dujon et al., 2004; Brunke and Hube, 2013). Despite this, these two species are the most frequently isolated pathogens from cases of invasive candidiasis. Both can reside in similar niches and affect similar patient cohorts. However, there clearly is a disparity in both species’ virulence, which is evidenced by significant differences in morphological plasticity and regulatory responses to stress conditions (Fidel, 1999; Brunke and Hube, 2013; Cuellar-Cruz et al., 2014; Linde et al., 2015). In this study, we comparatively studied activation of human PMN by C. albicans and C. glabrata. Several lines of evidence suggest that these cells are of outstanding importance in the response against invasive Candida infections. PMN govern the transcriptional response of C. albicans to incubation in human blood (Fradin et al., 2005) and induce various stress responses (Rubin-Bejerano et al., 2003; Enjalbert et al., 2007; Jandric and Schuller, 2011; Fukuda et al., 2013). Primary PMN confrontation assays have shown that PMN can differentiate between yeast and filamentous forms of C. albicans and PMN are the only host cell type known so far that can effectively inhibit filamentation of C. albicans after phagocytosis (Wozniok et al., 2008). Polymorphonuclear cells have been shown to trigger several forms of stress response in C. albicans and these stresses may act synergistically in killing of the pathogen (Kaloriti et al., 2012; 2014; Miramon et al., 2012; 2014). Related stress responses are induced by PMN in C. glabrata, resulting in the up-regulation of gluconeogenesis, glyoxylate cycle and long-chain fatty acid metabolism (Fukuda et al., 2013). Here we show that activation of human PMN is markedly different for C. albicans and C. glabrata (Fig. 8). Our data indicate that C. albicans elicits a quick and strong neutrophil response resulting in rapid phagocytosis, highly up-regulated surface expression of activation markers and efficient secretion of anti-microbial compounds. One hallmark of this response is the recruitment of other PMN by potent chemoattractants like IL-8 and GROα. In contrast, the response of PMN against C. glabrata is characterized by a slower onset and reduced amplitude. Surface marker expression, release of the primary and secondary granule contents, and oxidative burst are all notably lower in response to C. glabrata than C. albicans. Interestingly, we find that C. glabrata, but not C. albicans, elicits a cytokine profile, which promotes involvement of monocytes rather than additional PMN. C. glabrata elicits higher levels of MIP-1α and MIP-1β and barely induces MIF secretion by PMN. Furthermore, IL-8 and GROα levels induced by C. glabrata are considerably lower than those found for C. albicans. Whereas IL-8 and GROα are potent PMN...
Chemoattractants and activators, MIP-1α and MIP-1β, induce increased migration predominantly in monocytes, while neutrophils are far less responsive (Menten et al., 2002; Maurer and von Stebut, 2004). Consequently, C. glabrata activation of PMN induces monocyte migration to the site of PMN–C. glabrata confrontation and internalization of the fungal cells by monocytes. This was confirmed in transwell migration assays and live cell imaging, clearly demonstrating that C. glabrata but not C. albicans prompts a response engaging monocytes.

Concomitant recruitment of monocytes during C. glabrata infection may serve as an aid to the neutrophil in clearance or a hideout for the facultative intracellular yeast pathogen. Indeed, our data show that C. glabrata is highly susceptible to PMN attack and killed more efficiently than C. albicans. In contrast, it has been previously reported that C. glabrata can survive and replicate within the phagosome of macrophages (Seider et al., 2011) but not of neutrophils (Seider et al., 2014). Therefore, monocytic cells could act as 'Trojan horses' during C. glabrata infection to gain protection from the extracellular defences of the human host.

Experiments with isolated human immune cells bear the disadvantage of analysing cells that may have been perturbed by the isolation procedure in an artificial environment. Clearly, there is evidence that neutrophil function can be modulated by the isolation procedure (Glasser and Fiederlein, 1990; Watson et al., 1992; Hasenberg et al., 2011). To show that the observed effects may be of relevance in a situation close to in vivo, we analysed data from two complex infection models. In a recently described human whole blood model of infection (Hünniger et al., 2014), the secretion patterns of IL-8, GROα and MIF mirrored those of primary PMN. In contrast, other than in the purified PMN confrontation, same MIP-1α and MIP-1β levels were detected for both Candida spp. This may be due to additional sources of both chemokines in whole blood or related to additional pathways of immune cell activation, e.g. activation of the complement system (Hünniger et al., 2015). Despite these differences, our data clearly showed a greater association of C. glabrata to monocytes in the whole blood assay. Bio-mathematical modelling of pathogen distribution in whole blood further confirmed a greater preference of association to monocytes for C. glabrata than for C. albicans. Finally, we analysed histopathological sections from in vivo infection experiments in the mouse i.v. injection model. Although blood cells differ substantially between mice and humans both in number and function, data confirmed earlier indications that C. glabrata elicits a
monocytic inflammatory infiltrate whereas *C. albicans*-triggered inflammation is governed by PMN.

Taken together, a combination of live cell imaging analysis, mathematical modelling and *in vitro* assays has aided in the elucidation of a mechanism by which *C. glabrata* may promote its own survival within the host. Our data provide a new line of evidence for the fact that different clinical presentation of *C. albicans* and *C. glabrata* infection is linked not only to different virulence attributes of the two species, but also depends on variable activation of innate immunity resulting in different effector mechanisms.

**Experimental procedures**

**Ethics statement**

Human peripheral blood was collected from healthy volunteers with written informed consent. This study was conducted in accordance with the Declaration of Helsinki and all protocols were approved by the Ethics Committee of the University Hospital Jena (permit number: 273-12-09).

**Isolation of primary human immune cells**

Venous blood of healthy volunteers was collected in EDTA monovettes. PMN were subsequently purified as described elsewhere (Wozniok et al., 2008). For monocyte purification, peripheral blood mononuclear cells (PBMCs) were obtained via density centrifugation with Biocoll separating solution (Biochrome). CD14 positive cells were purified from the PBMC fraction using MACS technology (Miltenyi). Immune cell fractions were resuspended in RPMI1640 (Gibco) containing 5% heat-inactivated human serum (type AB, PAA). Heat inactivation of human serum was performed by incubation at 56°C for 60 min.

**Fungal cells and culture**

*C. albicans* wild-type strain SC5314 (Gillum et al., 1984), GFP-expressing *C. albicans* strain (constructed as described in Hünniger et al., 2014), BFP-expressing *C. albicans* strain (see below), *C. glabrata* wild-type strain ATCC2001 (Dujon et al., 2004) and *C. glabrata*-expressing GFP (Seider et al., 2011) were routinely grown overnight in M199 medium (9.8 g l⁻¹ M199, 35.7 g l⁻¹ HEPES, 2.2 g l⁻¹ NaHCO₃), pH 4, at 37°C in a stationary phase in a shaking incubator. Cells were then reseeded in M199 medium, pH 8, and cultured for 1 h at 37°C, which induced filamentous growth in *C. albicans*. To investigate *C. albicans* yeasts in confrontation with PMN, cells from stationary culture were reseeded in M199, pH 4. In addition, we used *C. albicans* strain ATCC18804 (Marakalala et al., 2013) as well as three *C. albicans* clinical isolates (CABI29, CABI33, CABI39) and five *C. glabrata* clinical isolates (CABII8a, CABI22, NRZ#51, NRZ#52, NRZ#64), kindly provided by the German National Reference Center for Invasive Fungal Infections (NRZMyk). For subsequent differentiation between PMN and *Candida* via FACS, fungal cells were labelled by incubation for 15 min with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) dissolved in HBSS.

For construction of the BFP-expressing *C. albicans* strain (ADH1/adh1::BFP-SAT1), we transformed a cassette including ADH1 homology regions, a yeast-optimized TagBFP (Evrogen) and SAT1 as selection marker into the CaADH1 locus of wild-type strain SC5314, using the lithium acetate protocol (Walther and Wendland, 2003). Transformants were grown for 2 days on yeast-peptone-dextrose (YPD) agar with 200 µg ml⁻¹ of nourseothricine and verified by polymerase chain reaction (PCR) and microscopy.

**Time lapse microscopy of confrontation assays**

In most experiments, 2 × 10⁵ *C. albicans*- or *C. glabrata*-expressing GFP was seeded in a μGrid dish (MoBiTec GmbH) and confronted with 2 × 10⁵ PMN or monocytes (*Candida* : immune cell ratio of 1:1; MOI = 1) in a total volume of 2 ml of RPMI1640 containing 5% heat-inactivated human serum and 2.5 ng ml⁻¹ of propidium iodide (Sigma). Propidium iodide is widely used as a vital dye that labels the nucleus in dying cells, which lack an intact plasma membrane. Co-infection experiments with both *Candida* species were performed by co-incubation of 1 × 10⁵ BFP-expressing *C. albicans*, 1 × 10⁵ GFP-expressing *C. glabrata* and 2 × 10⁵ PMN. Confrontation assays were incubated in an environmental control chamber at 37°C and 5% CO₂. Images were acquired every 10 s with an LSM 780 confocal microscope, which was focused on the bottom of the imaging dish. Fluorescence and cell interactions were monitored with a 20× microscope objective (Plan-APOCHROMAT 20×/0.8NA) and processed using the ZEN 2012 Software (Carl Zeiss).

**Analysis of live cell imaging**

Live cell time lapse microscopy images were recorded with time resolution Δt = 0.17 min and analysed for immune cell activity and motility. Tracking of PMN was performed using open source software ImageJ (Mokhtari et al., 2013) in combination with the MTrackJ plug-in (Meijering et al., 2012). After movie import, the centroid of a cell under consideration was highlighted to retrieve the x- and y-coordinates of the cell position in the first frame, the cell was subsequently followed through all frames of the movie, and the procedure was repeated to generate a track file for each cell containing its positions as a function of time. Neglecting cells that did not fully enter the field of view, the total number of considered cell tracks was 162 (PMN–*C. albicans*) and 169 (PMN–*C. glabrata*) in the six videos. The mean number recorded cell tracks were 197.4 ± 137.4 and 189.9 ± 128.1 time steps. The accuracy in the manual determination of the cell centroid was checked by repeating the procedure for more than 10³ different cell positions by two different human experts. We found the differences in the centroid positions of neutrophils, which have diameters of about 11 μm, to be as small as 1.27 ± 0.75 μm. In addition to the cell position, we recorded for each immune cell its activity in the track file, i.e. the time points at which phagocytosis and touching events occurred within the field of view were registered. Live cell time lapse microscopy images were analysed for immune cell motility. PMN migration was characterized by the MSD which is computed by an ensemble analysis of cell track data (Michalet, 2010). For cell track i,
we obtained the square displacement for all possible combinations of time intervals denoted by $n = 1, \ldots, N_t - 1$ from

$$d^2_t(n) = \frac{1}{N_t - n} \sum_{i=1}^{N_t - n} (r_i(m + n) - r_i(m))^2.$$  

Here, $r_i(m)$ indicates the position vector of the cell with identification number $i$ at time point $m$ and $N_t$ denotes the number of this track’s time points. Averaging over the total number of cell tracks $I$ yields the MSD:

$$d^2(n) = \langle d^2_t(n) \rangle = \frac{1}{I} \sum_{I=1}^{I} \frac{1}{N_t - n} \sum_{i=1}^{N_t - n} (r_i(m + n) - r_i(m))^2.$$  

The measured MSD can be compared with a migration model assuming that PMN perform a persistent random walk, i.e. cells change direction randomly but only after a typical time span during which the direction of migration persists. Using Fürth’s formula for a two-dimensional system (Selmeczi et al., 2005),

$$d^2(t) = 4D \left( t - t_p \right) \left[ 1 - e^{-\frac{t-t_p}{t_p}} \right],$$

the diffusion constant $D$ and the persistence $t_p$ can be estimated from a fit to the experimental data.

### Primary PMN confrontation assay

Hanks’ balanced salt solution (HBSS) (for mock-infected control) or FITC-labelled Candida was added to neutrophils in RPMI1640 with 5% heat-inactivated human serum (Candida : PMN ratio of 1:2; MOI = 0.5) and incubated at 37°C under gentle rotation for various time points (as indicated). Following the incubation period, cells were immediately maintained at 4°C and subjected to flow cytometric analyses or collection of supernatant by centrifugation.

### Flow cytometry

Expression of PMN surface markers was analysed via differential FACS staining and subsequent measurement with the FACSCanto II (BD). Changes in surface expression were investigated for Fcy receptor III (mouse anti-human CD16-APC, clone 3G8) and degranulation markers CD66b (mouse anti-human CD66b-V450, clone G10F5) and CD63 (mouse anti-human CD63-PE, clone H5C6) as described previously (Hünniger et al., 2015). FlowJo 7.6.4 software was used for analysis.

### Quantification of secreted proteins

The concentrations of secreted proteins within cell culture supernatants or plasma samples were determined using Luminex technology (MILLIPLEX MAP Human Sepsis Magnetic Bead Panel 2 (IL-8), MILLIPLEX MAP Human Sepsis Magnetic Bead Panel 3 (lactoferrin, elastase 2), MILLIPLEX MAP Human Cardiovascular Disease Magnetic Bead Panel 2 (MPO); Millipore and Procarta Immunoassay Kit – Magnetic Beads, Human (GROα [CXCL1], ENA-78 [CXCL5], MIG [CXCL9], IP-10 [CXCL10], I-TAC [CXCL11], MCP-1 [CCL2], MIP-1α [CCL3], MIP-1β [CCL4], MIP-3α [CCL20], IL-1α, IL-1β, IL-6, IL-12p70, IL-23, MIF, TNFα, VEGF-A; Affymetrix). The analyses were performed according to the instructions from the manufacturer.

### Oxidative burst

Oxidative burst was determined by means of the DCF assay, whereby the oxidation of non-fluorescent DCFH-DA (Sigma) by intracellular reactive oxygen intermediates results in its conversion to fluorescent DCF. PMN were pre-incubated with 1 μM DCFH-DA at room temperature for 30 min. In a 96-well flat-bottom black optical plate (Greiner), 50 μl of 4 × 10^6 PMN/ml was confronted with 50 μl of 2 × 10^6 fungal cells/ml in RPMI1640 with 5% heat-inactivated FCS (Biochrome). Polymorphonuclear cells in media alone served as negative control. Fluorescence was measured using a Tecan Infinite 200 Plate Reader at 37°C, over a 3 h period, with excitation at 485 nm and emission at 535 nm.

### Metabolic activity assay

The metabolic activity of Candida following confrontation with neutrophils was determined via the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-5-[ phenylaminio]-carbonyl]-2H-tetrazolium hydroxide) (Sigma) assay as described previously (Wozniok et al., 2008; Voigt et al., 2014). The absorbance of supernatants was measured at 450 nm in a TECAN Infinite 200 Plate Reader. Fungal metabolic activity of Candida confronted with neutrophils was determined as a percentage of the metabolic activity of Candida in media.

### Immune cell migration

Immune cell migration assays were performed in Costar 24-well plates (Corning Life Sciences) with 3 μm pore polycarbonate membranes. Polymorphonuclear cells and monocytes were labelled with 2 μM BCECF-AM (Invitrogen) and allowed to migrate towards PMN confronted with either C. albicans or C. glabrata. The relative fluorescence of the basal chamber, representing migrated immune cells, was measured using a Tecan Infinite 200 Plate Reader with excitation and emission filters of 485 and 535 nm.

### Whole blood infection model

A human whole blood model was used as described previously (Hünniger et al., 2014). Briefly, HBSS (for mock-infected control) or 1 × 10^6 FITC-labelled Candida cells were added to 1 ml of anti-coagulated blood and incubated at 37°C with gentle rotation for time points indicated. Following the incubation, cells were maintained at 4°C and subject to flow cytometric analyses or collection of plasma by centrifugation (10 min, 13,200 r.p.m., 4°C). Association to specific immune cell populations was determined as previously described (Hünniger et al., 2014) and data were analysed using FlowJo 7.6.4 software.

### Parameter estimation from state-based virtual infection model

The virtual infection model allows detailed and quantitative predictions on the dynamics of host–pathogen interaction by combining a state-based modelling approach with the Monte Carlo method of simulated annealing to quantify a priori unknown transition rates. Time-resolved data from whole blood experiments were generated as described and simulated using the recently developed state-based virtual infection model (Hünniger et al., 2014).
Mouse models, histology and determination of MPO content

All animal experiments were performed in accordance with the national and European regulations and protocols approved by the Thuringian state authorities. Female BALB/c mice 8–10 weeks old (18–20 g; Charles River) were housed in groups of five in individually ventilated cages with free access to water and food. For infection, C. albicans SC5314 and C. glabrata ATCC2001 were grown for 12 h at 30°C in YPD medium. Harvested cells were washed three times in sterile phosphate-buffered saline (PBS) and diluted to a final concentration of 2.5 × 10^6 ml^-1 (C. albicans) or 2.5 × 10^6 ml^-1 (C. glabrata) prior to inoculation. On day 0, mice were infected intravenously via the lateral tail vein with 2.5 × 10^7 C. albicans cells/g body weight or 5 × 10^7 C. glabrata cells/g body weight respectively. PBS-mock infected mice served as controls.

On the indicated time points, mice were humanely sacrificed, kidneys were removed aseptically and longitudinally cut in half. One-half was fixed with buffered formalin, paraffin embedded, and 4 μm sections were stained with Periodic acid–Schiff according to standard protocols. The second half was rinsed with sterile PBS, weighed, and homogenized in ice-cold lysis buffer (200 mM NaCl, 5 mM EDTA, pH 7.4, 10% glycerin, 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^-1 of leupeptid, 28 μg ml^-1 of aprotinin) on ice using an Ika T10 basic Ultra-Turrax homogenizer (Ika). Tissue homogenates were centrifuged at 1,500 × g, 4°C for 15 min. The first supernatants were centrifuged again and the obtained final supernatants were stored at -80°C. Myeloperoxidase levels were determined using the MPO ELISA Kit (Hycult Biotechnology) according to the manufacturer’s recommendations.

Statistical analyses

For all experiments, at least three independent replicates using cells from non-identical donors were used. Data are presented as arithmetic means ± SD and statistical significance was calculated using a two-sided t-test for unpaired data, shown as *P < 0.05, **P < 0.01, ***P < 0.001.

Acknowledgements

We are grateful to all anonymous blood donors for supporting our study. Cindy Reichmann contributed to the work with expert technical assistance and Sören Totzauer helped in manually tracking live cell imaging videos. We thank Ines Leonhardt for fruitful discussions and advice on the figures. Many thanks to Bernhard Hube for the gift of GFP-expressing Candida glabrata.

Funding

SD was supported by the Excellence Graduate School, Jean School for Microbial Communication (JSMC, Jena), FE received a stipend from the Center for Sepsis Control and Care (CSCC, Jena), and SB was financially supported by the International Leibniz Research School (ILRS), Hans Knöll Institute, Jena. In addition, this work was supported by the Deutsche Forschungsgemeinschaft (DFG) within the Collaborative Research Center CRC124 FungiNet (projects B4 to MTF, C3 to OK, an C5 to IDJ) and the German Ministry for Education and Science in the Program Unternehmen Region (BMBF 03Z2JN21 to OK).

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** C. *glabrata* was more susceptible for killing after phagocytosis by PMN than C. *albicans*. Representative snapshots from live cell video microscopy experiments of PMN (unstained) confronted with either GFP-expressing C. *albicans* (A) or C. *glabrata* (B) after 2 h co-incubation demonstrate that C. *albicans* is more phagocytosed than C. *glabrata*. Propidium iodide (PI) stains the DNA of dead cells, indicated as red fluorescence, and thus allows distinction between live and dead fungi and host cells. The majority of phagocytosed C. *albicans* remained viable inside the PMN. C. *glabrata* were intracellularly killed to a higher extent. Numbers represent individual phagocytosis events; black numbers identify viable fungi and white numbers identify killed fungi.

**Fig. S2.** Analysis of PMN effector mechanisms in response to different isolates of both Candida spp. Activation of primary PMN in confrontation assays with five different C. *albicans* and six different C. *glabrata* strains was analysed to exclude strain-specific effects on ROS formation and surface exposure of activation markers. (A) The percentages of ROS formation in samples inoculated with C. *albicans* (dark grey bars) and C. *glabrata* (light grey bars) were calculated relative to basal levels in mock-infected sample (set to 100%, white bars) and shown for 60 and 120 min of infection. The bars show means ± SD of three independent experiments with cells from different donors, *P* < 0.05, ***P* < 0.01. (B) Changes in the surface expression levels of PMN activation markers CD63, CD66b and CD16 are shown in representative histograms after 60 min of confrontation with C. *albicans* (dotted line) and C. *glabrata* (dashed line). Filled histograms indicate basal expression of PMN from mock-infected sample, lines represent PMN associated with Candida. Colouring of the lines helps differentiate between C. *albicans* and C. *glabrata* strains respectively. Data from one of three independent experiments using cells from different donors with virtually identical results are shown.

**Fig. S3.** Different immune cell infiltration in kidneys of mice infected with C. *albicans* and C. *glabrata*. Mice were intravenously infected with 2.5 × 10⁴ C. *albicans* and 5 × 10⁴ C. *glabrata* per gram body weight respectively on day 0. (A) Formalin-fixed, paraffin-embedded histological sections of kidney tissues on day 7 post infection were stained with Periodic acid–Schiff (PAS) and microscopically analysed (bright pink: fungal cells); magnifications: ×4 (left), ×20 (middle) and ×100 (right). Whereas C. *albicans*-infected kidneys were predominantly infiltrated by PMN (upper panel), mainly monocytic cells were found in C. *glabrata*-induced infiltrates (lower panel). (B) Myeloperoxidase (MPO) levels in kidney homogenates of control mice (white bars) and mice infected with either C. *albicans* (light grey bar; 24 h *post infection*) or C. *glabrata* (dark grey bar; 48 h *post infection*) are shown. MPO contents in kidneys of both C. *glabrata*- and C. *albicans*-infected mice were increased compared with PBS controls but MPO is significantly higher in C. *albicans*-infected kidneys compared with C. *glabrata* (*P* < 0.0001). Each bar is the mean ± SD from five animals per group. ***P* < 0.001.

**Video Clip S1.** Confrontation of PMN with Candida *albicans* filaments.

PMN (unstained) and GFP-expressing C. *albicans* filaments (green) were co-incubated at an MOI of 1 for 1 h. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). PMN interact with and rapidly phagocytose free C. *albicans*.

**Video Clip S2.** Confrontation of PMN with C. *glabrata* yeasts.

PMN (unstained) and GFP-expressing C. *albicans* yeast cells (green) were co-incubated at an MOI of 1 for 1 h. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). Whereas yeast cells were not taken up by PMN, phagocytosis was induced immediately with germination of C. *albicans*, resulting in a large number of phagocytosis events.

**Video Clip S3.** Confrontation of PMN with C. *glabrata*.

PMN (unstained) and GFP-expressing C. *glabrata* (green) were co-incubated at an MOI of 1 for 1 h. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). PMN also interact with C. *glabrata*, but during 60 min of co-incubation only few PMN phagocytose C. *glabrata*.

**Video Clip S4.** Co-incubation of PMN with C. *albicans* and C. *glabrata*.

PMN (unstained), GFP-expressing C. *glabrata* yeasts (green) and BFP-expressing C. *albicans* filaments (blue) were co-incubated at a ratio of PMN to total Candida cells of 1 for 1 h. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). During co-incubation of PMN with both Candida spp., C. *albicans* cells were frequently taken up by PMN, whereas majority of C. *glabrata* was only contacted and remained extracellular.

**Video Clip S5.** Confrontation of PMN with C. *glabrata*.

PMN (unstained) and GFP-expressing C. *glabrata* (green) were co-incubated at an MOI of 5 for 1 h. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). An increased number of C. *glabrata* cells relative to PMN resulted in more touching and phagocytosis events, leading to the same phagocytosis probability like MOI of 1.
Video Clip S6. Confrontation of monocytes with *C. glabrata* at a higher MOI.
Monocytes (unstained) and GFP-expressing *C. glabrata* (green) were co-incubated at an MOI of 1 for 1 h. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). Monocytes are motile, but interaction with *C. glabrata* rarely results in phagocytosis.

Video Clip S7. Confrontation of monocytes with *C. glabrata* in PMN/*C. glabrata*-conditioned medium.
Monocytes (unstained) and GFP-expressing *C. glabrata* (green) were confronted at an MOI of 1 for 1 h in medium generated from a 2 h co-incubation of PMN with *C. glabrata*. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). The presence of PMN/*C. glabrata*-conditioned medium results in an increased occurrence and frequency of phagocytosis of *C. glabrata* by monocytes.

Video Clip S8. Confrontation of monocytes with *C. glabrata* in PMN/*C. albicans*-conditioned medium.
Monocytes (unstained) and GFP-expressing *C. glabrata* (green) were confronted at an MOI of 1 for 1 h in medium generated from a 2 h co-incubation of PMN with *C. albicans*. Propidium iodide was added to the live cell imaging experiments to selectively stain dead cells (red fluorescence). Monocyte activity in response to *C. glabrata* was not changed in the presence of PMN/*C. albicans*-conditioned medium.