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The Facultative Intracellular Pathogen *Candida glabrata* Subverts Macrophage Cytokine Production and Phagolysosome Maturation

Katja Seider,* Sascha Brunke,*† Lydia Schild,* Nadja Jablonowski,* Duncan Wilson,* Olivia Majer,* Dagmar Barz,*§ Albert Haas,* Karl Kuchler,* Martin Schaller,‖ and Bernhard Hube*,#

Although *Candida glabrata* is an important human pathogenic yeast, its pathogenicity mechanisms are largely unknown. Immune evasion strategies seem to play key roles during infection, since very little inflammation is observed in mouse models. Furthermore, *C. glabrata* multiplies intracellularly after engulfment by macrophages. In this study, we sought to identify the strategies that enable *C. glabrata* to survive phagosome biogenesis and antimicrobial activities within human monocyte-derived macrophages. We show that, despite significant intracellular proliferation, macrophage damage or apoptosis was not apparent, and production of reactive oxygen species was inhibited. Additionally, with the exception of GM-CSF, levels of pro- and anti-inflammatory cytokines were only marginally increased. We demonstrate that adhesion to and internalization by macrophages occur within minutes, and recruitment of endosomal early endosomal Ag 1 and lysosomal-associated membrane protein 1 indicates phagosome maturation. However, phagosomes containing viable *C. glabrata*, but not heat-killed yeasts, failed to recruit cathepsin D and were only weakly acidified. This inhibition of acidification did not require fungal viability, but it had a heat-sensitive surface attribute. Therefore, *C. glabrata* modifies the phagosome into a nonacidified environment and multiplies until the host cells finally lyse and release the fungi. Our results suggest persistence of *C. glabrata* within macrophages as a possible immune evasion strategy. *The Journal of Immunology*, 2011, 187: 000–000.

*Candida glabrata* is an emerging pathogen that has become the second most frequent cause (15%) of candidiasis after *Candida albicans* (1, 2). Both species are usually commensals, existing as part of the normal microbial flora of human mucosal surfaces, but they are also successful opportunistic pathogens, causing either superficial or disseminated infections when the normal bacterial flora is disturbed, barriers are damaged, or when compromised host immunity permits disease. *C. glabrata* infections are especially difficult to treat due to high antifungal resistance (3).

Although translocation mechanisms remain unclear, *C. glabrata* is able to access the bloodstream, from where it can disseminate to internal organs in susceptible patients. Therefore, *C. glabrata* must have developed strategies to counteract or bypass mammalian host defense systems, enabling the fungus to cause systemic disease. Key virulence attributes of *C. albicans*, such as hyphal formation and secretion of hydrolases, are thought to be essential for tissue invasion and persistence (4–6). *C. glabrata* lacks these attributes (1), but it can still be reisolated from infected immunocompetent mice over long periods (7). Furthermore, colonization of organs in mice causes very little inflammation (7–9). Additionally, it has been shown that *C. glabrata* can survive attack by phagocytes and even replicate within macrophages after engulfment (10–12). This suggests that immune evasion strategies might play a key role during infection with *C. glabrata*.

Phagocytic cells of the innate immune system, such as macrophages, dendritic cells, and neutrophils, act to eliminate microbial pathogens from the bloodstream and tissue. Once recognized, a microbe is quickly engulfed by the phagocyte and enters the phagocytic pathway. The phagosome then matures via fusion events with endosomal vesicles, which in turn leads to the exchange of membrane proteins and finally yields the phagolysosome, an extremely hostile environment to microbes (13, 14). Inside the phagolysosome, certain nutrients and trace elements are severely restricted, accompanied by an increase in acidification. This activates acidic hydrolases, such as cathepsin D. Furthermore, during phagosome biogenesis a battery of reactive oxygen species (ROS) and reactive nitrogen species and antimicrobial peptides is

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Abbreviations used in this article: ADH, alcohol dehydrogenase promoter; EEA1, early endosomal Ag 1; hSOZ, human serum-opsin-zonulin; LAMP1, lysosomal-associated membrane protein 1; LDH, lactate dehydrogenase; MDM, monococyte-derived macrophage; MOL, multiplicity of infection; ROS, reactive oxygen species; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; VPD, 1% yeast extract, 2% peptone, 2% dextrose.

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Transported into the organelle. The combined action of all of these factors is normally sufficient to kill and degrade most phagocytosed microbes. Furthermore, phagocytic cells act as immune modulators by presenting Ags and secreting pro- and anti-inflammatory cytokines and chemokines, recruiting further immune cells and activating the adaptive arm of the immune system.

Nevertheless, pathogenic microbes have evolved strategies to counteract the phagocytic attack and to overcome killing by immune cells (14, 15). Many bacterial pathogens have been studied with great success; far less, however, is known about facultative intracellular fungi. *Histoplasma capsulatum* yeasts, for example, are able to inhibit phagosome acidification following uptake by macrophages (16–18). *C. albicans* inhibits phagosome maturation, preventing the production of ROS and reactive nitrogen species; this allows survival and final escape via lyphal formation (19–21). Additionally, *C. albicans* modulates the immune response indirectly via manipulating the host’s cytokine response (22, 23).

The capsule of *Cryptococcus neoformans* has been shown to mediate macrophage apoptosis and therefore induces a distinct anti-inflammatory mechanism to kill the phagocyte (24). *C. neoformans* can also escape macrophages via extrusion/expulsion (25, 26). The formation of massive vacuoles by the fungus is followed by extrusion of the phagosomes and the release of the pathogen. Macrophages and expelled yeast cells appear morphologically normal and remain alive.

Although interactions of these fungal pathogens with immune cells have been the subject of several studies, the ability of *C. glabrata* to survive the attack by phagocytic cells is less well described. Kaur et al. (11) showed that, despite the morphological differences, the transcriptional adaptation of *C. glabrata* within a murine macrophage-like cell line is highly similar to that of *C. albicans*, and they highlighted the requirement of a family of GPI-linked asparyl proteases (yapsins) for survival within macrophages and for virulence. Degradation and recycling of endogenous cellular components (autophagy) seem to be another intracellular survival strategy for *C. glabrata* within macrophages (12). However, the precise mechanisms by which *C. glabrata* subverts killing by macrophages are yet unknown and because *C. glabrata* mainly attracts mononuclear cells in vivo (7), we sought to elucidate the interactions of *C. glabrata* with the adaptive arm of the immune system.

**Materials and Methods**

**Fungal strains and yeast cell culture**

*C. glabrata* wild-type strain ATCC2001, a GFP-expressing *C. glabrata* strain (see below), *S. cerevisiae* strain ATCC9763, *C. albicans* wild-type clinical isolate SC5314 (28), and five clinical *C. glabrata* isolates (R. Rüchel, University of Göttingen, Göttingen, Germany) were routinely grown overnight in 1% yeast extract, 2% peptone, and 2% dextrose (YPD) at 37°C (C. glabrata) or 30°C (*S. cerevisiae* and *C. albicans*) in a shaking incubator at 180 rpm.

In selected experiments, serum-opsonized *C. glabrata* cells were used. YPD overnight cultures were washed with PBS and incubated with 50% nonimmune human serum at 37°C for 30 min under shaking (150 rpm) and washed again several times with PBS. For preparation of heat-killed yeast cells, 500 μl overnight culture was washed with PBS and incubated at 70°C for 10 min, followed by several washes with PBS.

**Plasmid and strain construction**

The GFP-*C. glabrata* strain was obtained by genomic integration of GFP (S65T) under the *C. glabrata* alcohol dehydrogenase promoter (ADHp) into the endogenous *TRP1* locus. Primers used for strain construction are listed in Table I. GFP-Act was cut out from pTD125 (29) and cloned via EcoRI/Sall into pADHpCg (30). A PCR fragment of the endogenous *TRP1* gene containing a 5’ untranslated region and a fragment of the 3’ untranslated region were amplified from ATCC2001 genomic DNA. ADHp-GFP-Act was PCR amplified from the new constructed plasmid with flanking regions to the 5’-Trp and 3’ fragments. For construction of the final transformation cassette, all three fragments (5’-Trp, 3’, and ADHp-GFP-Act) were fused in a separate PCR reaction and transformed into ATCC2001 *trp1Δ*. Correct genomic integration of all fragments was verified by genomic PCR.

**Macrophage culture and infection**

Human PBMCs were isolated with Histopaque-1077 (Sigma-Aldrich) density centrifugation from buffy coats donated by healthy volunteers. To differentiate PBMCs into monocyte-derived macrophages (MDMs), 0.1×10^6 PBMCs/ml were plated in RPMI 1640 media with 1-glutamine and 25 mM HEPES; PAA Laboratories) containing 10% heat-treated FBS (Life Technologies) in cell culture dishes. Ten nanograms per millilitre M-CSF (ImmunoTools) was added to the cultures to induce the differentiation of macrophages. After 5 d at 5% CO2 and 37°C, nonadherent cells were removed and the purity of MDMs was determined by staining with an anti-CD14 Ab and flow cytometry. The percentage of CD14-positive cells was ≥80%. All experiments were performed with cells isolated from at least three different donors.

For infection experiments, adherent MDMs were detached with 50 mM EDTA and plated in flat-bottom 96- or 24-well plates to give a final concentration of ~3×10^5 or 2×10^6 MDMs/well, respectively, in RPMI 1640 without serum. For microscopic analysis MDMs were allowed to adhere to cover slips within a 24-well plate. For some experiments MDMs were activated by adding 100 U/ml IFN-γ (ImmunoTools) 24 h prior to infection and during infection. If not stated otherwise, the overnight yeast culture (*C. glabrata, S. cerevisiae*, or *C. albicans*) was washed with PBS, counted using a hemacytometer, and adjusted to the desired concentration in RPMI 1640 without serum. MDMs were then infected with yeast cells at a multiplicity of infection (MOI) of 5 unless stated otherwise.

**Replication assay**

MDMs were infected with the GFP-expressing *C. glabrata* strain as described above and coincubated for 30 min. The culture was washed three times to remove unbound yeast cells, followed by an additional incubation step of 3, 8, 12, or 24 h at 5% CO2 and 37°C. Cells were fixed with 4% paraformaldehyde for 10 min at 37°C, stained for 30 min at 37°C with 25 mM Alexa Fluor 647-conjugated Con A (Molecular Probes) to visualize nonphagocytosed yeast cells, and mounted cell side down in ProLong Gold antifade reagent with DAPI (Molecular Probes). The phagocytic index (number of *C. glabrata* taken up per 100 MDMs) was assessed by fluorescence microscopy (Leica DM5500B, Leica DFC360) and results were obtained by analyzing a minimum of 200 MDMs/well.

To quantify yeast intracellular replication, *C. glabrata* cells were labeled with 100 μg/ml FITC (Sigma-Aldrich) in carbonate buffer (0.1 M Na2CO3, 0.15 M NaCl) (pH 9.0) for 30 min at 37°C, followed by washing with PBS. MDMs, either untreated or activated with 100 U/ml IFN-γ (ImmunoTools) for 24 h, were infected and incubated as described above, fixed, stained with Con A, and mounted. Because FITC is not transferred to daughter cells, differentiation of mother and daughter cells was possible and intracellular replication was quantified by counting at least 200 phagocytosed yeast cells and scoring for FITC staining or no staining.

**Damage assay**

The release of lactate dehydrogenase (LDH) into the culture supernatant was monitored as a measure of MDM damage. Experiments were performed in 96-well plates. For control samples, MDMs were incubated with medium only or yeast cells were seeded without MDMs. After 24 and 48 h coinfection, culture supernatants were collected and the amount of LDH was determined using a cytotoxicity detection kit (Roche Applied Science) according to the manufacturer’s instructions. LDH activity was analyzed spectrophotometrically at 492 nm and calculated from a standard curve obtained from dilutions of the LDH control. All experiments were performed in triplicate for each condition.
Apoptosis assay
Caspase-3 or caspase-7 activity was measured using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer’s instructions. MDMs were cultured in white 96-well plates and after 3, 8, or 24 h infection the Caspase-Glo 3/7 reagent was added containing a luminescent caspase-3/7 substrate. Upon caspase-3 or caspase-7 activity, the light emitted was detected in a microplate reader (Tecan Infinite 200) and was proportional to the amount of caspase-3 or caspase-7 activity. In positive control experiments, MDMs were treated with 1 μM staurosporine (Sigma-Aldrich). All samples, performed in triplicate, were normalized to samples treated with staurosporine for 8 h.

Additionally, induction of apoptosis was detected by Western blot. For this purpose, MDMs were seeded in 6-well plates at 1 × 10⁶ cells/well. After 8 h infection with C. glabrata (MOI of 1, 5, or 15) or treatment with 500 nM staurosporine or both, MDMs were lysed with 500 μl RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS [pH 8.0]). The protein concentration was measured with the DC protein assay kit (Bio-Rad) and 50 μg proteins were separated by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% milk powder at room temperature for 1 h and then incubated with the primary anti-caspase-3 Ab (Cell Signaling Technology) overnight at 4°C. Subsequently, the membrane was incubated with a HRP-conjugated anti-rabbit Ab (Abcam) blocked with 5% milk powder at room temperature, and blocked with Image-iT FX signal enhancer (Molecular Probes); finally, cover slips were mounted cell side down in ProLong Gold antifade reagent with DAPI (Molecular Probes). The perinuclear actin ring was visualized using a Leica DM5500B microscope.

Detection of ROS
ROS production was measured by luminol-ECL and all cells and reagents were prepared in RPMI 1640 without phenol red. MDMs were grown in white 96-well plates in 100 μl medium per well. Yeast cultures washed in RPMI 1640 were counted and 50 μl was added to MDMs (according to a MOI of 1, 10, and 50). For control experiments, MDMs were left untreated and indolent in 150 μl RPMI 1640, or 100 mM PMA (Sigma-Aldrich) was added to 50 μl RPMI 1640. For simultaneous addition of yeast cells and PMA to MDMs, PMA and yeasts were premixed. Fifty microliters of this premix was added to the MDMs at a final concentration of 100 nM PMA and a MOI of 10. All samples were prepared in triplicates. Fifty microliters of a mixture containing 200 μM luminol and 16 U horseradish peroxidase in RPMI 1640 was immediately added prior to quantification. Luminescence was measured every 3 min over a 3 h incubation period at 37°C using a microplate reader (Tecan Infinite 200) and maximum relative luminescence units were determined for each measurement. All experiments were performed in triplicates.

Immunoﬂuorescence microscopy
MDMs on cover slips were infected with GFP-expressing C. glabrata, opossumized C. glabrata, heat-killed C. glabrata, or FITC-labeled human serum-opsonized zymosan (hSOZ). Synchronization of phagocytosis was performed by placing the 24-well plate on ice for 15 min until yeast cells settled. Unbound yeast cells were removed by washing with prewarmed (37°C) medium, and phagocytosis was initiated by incubating at 37°C and 5% CO₂. Cover slips were fixed with 4% paraformaldehyde (10 min, 37°C) at indicated time points, stained with Alexa Fluor 647-conjugated Con A (30 min at 37°C), permeabilized in 0.2% Triton X-100 in PBS (5 min, room temperature), and blocked with Image-iT FX signal enhancer (Molecular Probes; 30 min at room temperature). Cells were then incubated with primary Ab (anti-lysosomal–associated membrane protein 1 [LAMP1] Ab, BD Biosciences; anti-lysosomal–associated membrane protein 1 [LAMP1] Ab, BD Biosciences; anti-cathepsin D Ab, Santa Cruz Biotechnology) diluted 1:500 in PBS with 2% BSA for 1 h at room temperature followed by incubation with the appropriate secondary Alexa Fluor 555-conjugated anti-mouse Ab (Molecular Probes); finally, cover slips were mounted cell side down in ProLong Gold antifade reagent with DAPI (Molecular Probes). The percentage of colocalization was calculated manually by analyzing a minimum of 200 yeast cells per well under fluorescent light (Leica DM5000B, Leica DFC360).

Transmission electron microscopy
For transmission electron microscopy, MDMs were grown in 24-well plates with a polystyrene plastic bottom. C. glabrata was added at a MOI of 5

Table I. Primers used for the GFP-C. glabrata strain construction

<table>
<thead>
<tr>
<th>Amplification of GFP-Act from pTDL25</th>
<th>Sequence (5’ to 3’)</th>
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<tr>
<td>OM04F</td>
<td>CTAGAAATTCCAGCCGGATCTAGGTTACCCGGTCGAAGTAGTAAAGGAGAAAC</td>
</tr>
<tr>
<td>OM04R</td>
<td>CGTTCGACGTRGAGGGGTTAGACGACCCG</td>
</tr>
<tr>
<td>OM05F</td>
<td>CGCCTGCCTGAGCCGGCGCTGATGACGCAACATTCAACACACACACAAAC</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>OM01R</td>
<td>TTAGACAGCGATATCAAATTACCGAC</td>
</tr>
<tr>
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<tr>
<td>OM11R</td>
<td>CTTCTCTCTGTTAATCATTGTTTCCTTG</td>
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</table>

Cytokine production
MDMs were infected in 24-well plates. LPS (Sigma-Aldrich) was used as a control and applied at concentration of 1 μg/ml. After 8 and 24 h, samples of surrounding medium were collected and centrifuged (10 min, 1000 × g). The amount of TNF-α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, and IFN-γ secreted by MDMs was determined by ELISA according to the manufacturer’s protocol (ImmuNoTools for IL-6, IL-8, IL-10, and IFN-γ, eBioscience for TNF-α, IL-1β, and GM-CSF). Cytokine levels were determined by measuring spectrophotometrically at 450 nm and calculated from a standard curve obtained from dilutions of recombinant proteins (supplied by manufacturer). All experiments were performed in triplicate and normalized to C. glabrata-infected samples collected after 24 h coincubation.

Detection of ROS
ROS production was measured by luminol-ECL and all cells and reagents were prepared in RPMI 1640 without phenol red. MDMs were grown in white 96-well plates in 100 μl medium per well. Yeast cultures washed in RPMI 1640 were counted and 50 μl was added to MDMs (according to a MOI of 1, 10, and 50). For control experiments, MDMs were left untreated and indolent in 150 μl RPMI 1640, or 100 mM PMA (Sigma-Aldrich) was added to 50 μl RPMI 1640. For simultaneous addition of yeast cells and PMA to MDMs, PMA and yeasts were premixed. Fifty microliters of this premix was added to the MDMs at a final concentration of 100 nM PMA and a MOI of 10. All samples were prepared in triplicates. Fifty microliters of a mixture containing 200 μM luminol and 16 U horseradish peroxidase in RPMI 1640 was immediately added prior to quantification. Luminescence was measured every 3 min over a 3 h incubation period at 37°C using a microplate reader (Tecan Infinite 200) and maximum relative luminescence units were determined for each measurement. All experiments were performed in triplicates.
and, after 3 h incubation, nonadherent cells were removed by rinsing three times with PBS. Samples were fixed with Carnovsky fixative (3% paraformaldehyde, 3.6% glutaraldehyde [pH 7.2]). After centrifugation, the sediment was embedded in 3.5% agarose at 37°C, coagulated at room temperature, and fixed again in Carnovsky fixative. Postfixed samples (1% OsO₄ containing 1.5% potassium ferrocyanide in aqua bidest, 2 h) were rinsed with distilled water, block stained with uranyl acetate (2% in distilled water), dehydrated in alcohol (stepwise 50-100%), immersed in propyleneoxide, and embedded in glycide ether (polymerized 48 h at 60°C; Serva). Ultrathin sections were examined with a LIBRA 120 transmission electron microscope (Carl Zeiss SMT) at 120 kV.

**Quantification of phagolysosomal fusion**

Acidification of the phagosomes was assessed with the acidotropic dye LysoTracker Red DND-99 (Molecular Probes). LysoTracker (diluted 1:10,000 in RPMI 1640) was added 1 h prior to infection and during the incubation with yeast cells. Phagocytosis was synchronized as described above, and control experiments were performed with uninfected cells, cells inoculated with hSOZ, or 3-mm latex beads (Sigma-Aldrich). After 10, 30, and 90 min, the cells were fixed, stained with Alexa Fluor 647-conjugated Con A, and mounted. Two hundred yeast-containing phagosomes were counted and scored for lysosomal fusion or no fusion.

**Phagosomal pH measurements**

Viable and heat-killed *C. glabrata* were FITC labeled (see above) and washed in PBS prior to infection of MDMS seeded in black 24-well plates (MOI of 10). After 1 h coincubation, nonadherent yeast cells were removed by gentle washing with prewarmed (37°C) PBS, and intracellular pH was calculated by linear regression analysis from the fluorescence intensity ratio at Ex550/EEm550 by comparison with a standard curve (31). Standard curves were generated with MDMS infected with viable or heat-killed yeasts incubated with buffer of varying pH (PBS/HCl with pH from 4 to 7) containing 50 µM nigericin (Sigma-Aldrich) and the fluorescence intensity ratio at Ex485/EEm550 was determined. All experiments were performed in triplicates.

**Microarray experiments**

*C. glabrata* cells from a YPD overnight culture were washed with PBS, counted, and either incubated in fresh YPD medium (pH 4.5 or 7.0) (1 × 10⁵ cells/ml) for 30 min at 37°C or added to MDMS in a 6-well plate (MOI of 5). Infected cells were placed on ice for 15 min (synchronization), washed with RPMI 1640 to remove unbound yeast cells, and phagocytosis was induced by incubating for 30 min at 37°C and 5% CO₂. For control samples, *C. glabrata* was treated the same way without MDMS. *C. glabrata* cells from liquid culture (YPD cultures and culture without MDMS) were then centrifuged and the pellet was resuspended in 500 µl AE buffer (50 mM sodium acetate [pH 5.3], 10 mM EDTA, 1% SDS). Infected MDMS were lysed by adding 500 µl AE buffer and centrifuged to pellet phagocytosed yeast cells and to remove host debris and host nucleic acids. The sample was washed and resuspended in 500 µl AE buffer. For RNA extraction, 500 µl 25:24:1 phenol/chloroform/isoamyl alcohol was added to each sample, vortexed, and heated for 5 min at 65°C. Samples were frozen at −80°C, rethawed again, and centrifuged. RNA was precipitated from the aqueous phase by isopropanol, and RNA quality was checked with an Agilent bioanalyzer. RNA labeling, microarray hybridization, and data analysis were performed as described previously (32). Data of phagocytosed *C. glabrata* were compared with data of samples obtained without MDMS to filter for genes upregulated upon phagocytosis and not upon any other condition (e.g., RPMI 1640 medium, CO₂). These genes were then compared with genes upregulated only at pH 4.5 and not at pH 7.0. Experiments were repeated twice. The microarray data are stored in the ArrayExpress database of transcription profiles (http://www.ebi.ac.uk/arrayexpress/; E-MEXP-2974, Interactions of *C. glabrata* with human macrophages; E-MEXP-2974, *C. glabrata* response to pH change).

**Inhibitor and killing studies**

An overnight culture of *C. glabrata* was washed and treated with thiolutin (5 µg/ml; AppliChem), cycloheximide (1 µg/ml; AppliChem), or tunicamycin (1 µg/ml; Sigma-Aldrich) for 2 h and, in the case of tunicamycin, also for 16 h in YPD. Additionally, yeast cells were killed by heating (10 min, 70°C in PBS), UVC light (0.1 J/cm²), or thimerosal (100 mM, 1 h in PBS; Sigma-Aldrich). Afterward, yeasts were extensively washed to remove excess thiolutin and added immediately to MDMS. After synchronization (see above), MDMS were allowed to phagocytose yeasts for 30 min at 37°C and 5% CO₂. Acidification of the phagosome was monitored by LysoTracker staining as described above. Two hundred yeast-containing phagosomes were counted and scored for lysosomal colocalization.

Killing and inhibition of growth was verified by plating yeast cells on YPD agar for at least 3 d and by measuring metabolic activity by the cleavage of the tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT). Briefly, 600 µl PBS containing 2 × 10⁵ yeast cells, 350 ng/ml XTT, and 350 ng/ml coenzyme Q were incubated 1 h at 37°C. Metabolic activity of dead yeast cells was analyzed spectrophotometrically at 451 nm and normalized to viable yeast cells. All experiments were performed in triplicate.

**Time-lapse microscopy**

MDMS were seeded in petri dishes (Ibidi) at a density of 8 × 10⁵ cells in RPMI 1640 with 1% FBS and infected with *C. glabrata*. After 30 min phagocytosis, cells were washed with prewarmed medium to remove excess yeast cells and further incubated in a plexiglas environment surrounding the microscope, which was kept constant at 37°C by a heated stage and heated air unit and 5% CO₂. Phase-contrast images were taken every 5 min with an Axio Observer.Z1 microscope (Zeiss).

**Statistical analysis**

All experiments were performed with cells isolated from at least three different donors. Unless otherwise stated, all data are reported as the mean ± SD. Data were analyzed using two-tailed, unpaired Student t test for intergroup comparisons. Statistically significant results are indicated as follows:* p < 0.05, **p < 0.01.

**Results**

*C. glabrata* survives macrophage phagocytosis and replicates intracellularly

*C. glabrata* prevents killing by macrophages and may therefore have evolved distinct strategies for intracellular survival (10, 11). To investigate this phenomenon in more detail, replication of *C. glabrata* in human MDMS was assessed. We infected macrophages with a GFP-expressing *C. glabrata* wild-type strain at a *C. glabrata*/macrophage ratio of 5:1 (MOI of 5) for 1 h. Unbound yeast cells were washed away and the number of intracellular yeasts was assessed microscopically after different incubation times using differential (inside/outside) staining. Within 3 h, almost all yeast cells were phagocytosed (>95%), resulting in a relatively stable phagocytic index (number of phagocytosed yeast cells per 100 macrophages) between 3 and 12 h (Fig. 1A). However, between 12 and 24 h, we observed a 3-fold increase in the number of internalized yeast cells, which was presumably due to replication, as virtually no extracellular yeasts were detectable. Similar results were obtained when macrophages were lysed and yeast cells were plated on YPD agar plates after each time period (CFU counting is not shown). To verify that the increase in the number of internalized yeast cells was indeed due to intracellular replication, we stained *C. glabrata* with FITC, a dye that is not transferred to daughter cells (Fig. 1C). This has proven to be an ideal tool to quantify replication within host cells. Over time, replication increased, and by 24 h, >80% of the population inside macrophages consisted of daughter cells (Fig. 1B). In contrast, we observed no intracellular replication of the nonpathogenic yeast *S. cerevisiae*. To determine whether additional activation of MDMS could influence replication of *C. glabrata*, we primed MDMS with IFN-γ (100 U/ml) prior to and during infection; however, this had no effect on replication within the 24-h time course (Fig. 1B).

*C. glabrata* and *S. cerevisiae* elicit similar levels of macrophage damage

Next, we determined whether uptake and intracellular replication of *C. glabrata* causes damage to macrophages. The ability of *C. glabrata*, *S. cerevisiae*, and *C. albicans* to damage MDMS was assayed by quantifying the release of LDH in the culture supernatant at 24 and 48 h postinfection (Fig. 2). Despite rapid phagocytosis and intracellular replication of *C. glabrata* within
MDMs, very little damage to macrophages was measurable after 24 h, and this was only slightly more than the damage caused by infection with *S. cerevisiae*. Serum opsonization of *C. glabrata* prior to infection did not influence damage. In contrast, *C. albicans*, which is known to escape macrophages by producing hyphae and pseudohyphae (19), induced a significant release of LDH after 24 h, whereas notable damage by *C. glabrata* and *S. cerevisiae* required 48 h coincubation. When compared with *C. albicans*, the damage caused by *C. glabrata* and *S. cerevisiae* at this time was still significantly lower. Microscopic analysis confirmed development of hyphae and escape of *C. albicans* at this stage of infection, whereas there was no evidence of escape for *C. glabrata* and *S. cerevisiae*.

**C. glabrata does not induce macrophage apoptosis**

The induction of host cell apoptosis is one mechanism used by some pathogenic microorganisms to facilitate intracellular survival, to escape from phagocytes, or to modulate the inflammatory response by phagocytes (33, 34). To test whether *C. glabrata* can cause apoptosis of MDMs, we determined the activity of the effector caspases 3 and 7. As a positive control, MDMs were stimulated with staurosporine, an ATP-competitive protein kinase inhibitor, which is known to induce apoptosis by activating caspase-3 (35). As expected, staurosporine-treated MDMs showed pronounced caspase-3 or caspase-7 activity after 3 h, resulting in a peak of activation after 8 h coincubation (Fig. 3A). However, neither *C. glabrata* (untreated or serum-opsonized) nor *S. cerevisiae* cells induced detectable caspase-3 or caspase-7 activation at 3, 8, or 24 h postinfection. In contrast, infection with *C. albicans* for 24 h was associated with high levels of caspase-3 or caspase-7 activity, which indicates induction of apoptosis by this fungus.

**During activation of the apoptotic pathway, caspases undergo proteolytic processing**

The active form of caspase-3 is generated by cleavage of the 32-kDa proenzyme into subunits of 17 and 12 kDa, which can be detected by Western blot analysis. The addition of staurosporine to MDMs resulted in the expected caspase-3 cleavage (Fig. 3B). This was not observed postinfection with *C. glabrata* or *S. cerevisiae*. The release of LDH into the supernatant of infected MDMs (MOI of 5) after 24 and 48 h was measured as a marker for host cell damage. *C. glabrata* (untreated or serum-opsonized) damaged MDMs to a low extent, similar to *S. cerevisiae*. The comparison with damage caused by *C. albicans* shows significantly lower LDH levels for *C. glabrata* and *S. cerevisiae* at indicated time points. Results shown are the means ± SD, n ≥ 3. *p < 0.05, **p < 0.01 compared with *C. albicans* at indicated time points (unpaired Student *t* test).

**FIGURE 1.** *C. glabrata* survives and replicates within macrophages. A, Microscopic quantification of *C. glabrata* (C.g.) phagocytosed by human MDMs. MDMs were infected with GFP-expressing *C. glabrata* for 30 min (MOI of 5), washed, and coincubated for indicated time points. The phagocytic index, which represents the number of internalized yeast cells per 100 macrophages, increased with time. For each time point a minimum of 200 macrophages from at least three separate experiments were analyzed. Results shown are the means ± SD; n ≥ 3. B, IFN-γ activation of MDMs has no impact on *C. glabrata* replication. Untreated and IFN-γ-treated (100 U/ml) MDMs were infected with FITC-labeled *C. glabrata* for 30 min (MOI of 5), washed to remove excess unbound yeasts, and coincubated for 3, 8, 12, and 24 h. Replication was analyzed microscopically (shown in C). For quantification, at least 200 yeast cells per time point were counted and scored for FITC staining or no staining. Results shown are the means ± SD; n ≥ 3. C, Representative fluorescent microscopy images of FITC-labeled *C. glabrata* (indicated in green) phagocytosed by MDMs after 8 h coincubation. FITC was not transferred to daughter cells, allowing subsequent identification of daughter cells and determination of yeast replication within MDMs. White arrows illustrate nonstained intracellular (daughter) yeast cells (outside staining with Con A is indicated in yellow, the merged image additionally shows the nucleus stained with DAPI in blue). Original magnification ×63.

**FIGURE 2.** Moderate macrophage damage by *C. glabrata*. The release of LDH into the supernatant of infected MDMs (MOI of 5) after 24 and 48 h was measured as a marker for host cell damage. *C. glabrata* (C.g.) (untreated or serum-opsonized [C.g. opson.]) damaged MDMs to a low extent, similar to *S. cerevisiae* (S.c.). The comparison with damage caused by *C. albicans* (C.a.) shows significantly lower LDH levels for *C. glabrata* and *S. cerevisiae* at indicated time points. Results shown are the means ± SD, n ≥ 3. *p < 0.05, **p < 0.01 compared with *C. albicans* at indicated time points (unpaired Student *t* test).
C. glabrata, providing further evidence that C. glabrata does not induce apoptosis in MDMs. To determine whether C. glabrata does not induce apoptosis, or whether the yeast is able to actively inhibit apoptotic cell death, MDMs were coincubated with C. glabrata and staurosporine. In this study, we used the lowest staurosporine concentration that still induced apoptosis (500 nM). Because the band pattern from MDMs coincubated with C. glabrata and stimulated with staurosporine was similar to the pattern induced by staurosporine alone (Fig 3), we concluded that C. glabrata is not capable of repressing staurosporine-induced apoptosis.

Finally, we determined whether C. glabrata induced DNA fragmentation of MDMs, another characteristic sign of apoptosis. Upon treatment with staurosporine, the typical DNA ladder indicative of apoptosis was observed (Supplemental Fig. 1). Untreated MDMs or MDMs infected with C. glabrata at an MOI of 1, 5, or 15 showed full-length caspase-3 only (indicated by arrows at 35 kDa), confirming the absence of an induced apoptosis of MDMs. Simultaneous treatment of MDMs with staurosporine and C. glabrata did not inhibit induction of apoptosis. Results are representative for at least three independent experiments.

Inhibition of proinflammatory cytokine production by C. glabrata

Cytokines are important mediators of the immune system and their production is crucial for determining the outcome of fungal infections (36, 37). Although cytokine release by epithelial cells coincubated with C. glabrata has been reported before (38, 39), the cytokine response of macrophages infected with C. glabrata has not yet been studied. We therefore examined the levels of proinflammatory cytokines (IL-1β, IL-6, IL-8, IFN-γ, TNF-α, GM-CSF), as well as the anti-inflammatory cytokine (IL-10), produced by MDMs infected with C. glabrata. LPS, a potentiator of most cytokines, was used as positive control. Although MDM populations from different donors produced different total cytokine levels (resulting in high SDs), the pattern of the cytokine profiles appeared the same. Therefore, all samples were normalized against supernatants collected from MDMs infected with C. glabrata for 24 h to facilitate interspecies comparison with C. albicans and S. cerevisiae (Fig. 4) as well as comparison with serum-opsonized or heat-killed C. glabrata or samples of IFN-γ-preactivated MDMs infected with C. glabrata in a separate experiment (Supplemental Fig. 2). Ranges of cytokine data are indicated in the legend of Fig. 4. As shown in Fig. 4, after prolonged incubation (24 h), C. glabrata-infected macrophages produced less proinflammatory TNF-α, IL-1β, IL-6, IL-8, and IFN-γ as compared with those infected with S. cerevisiae. Similar differences were observed in comparison with infection with C. albicans. Additionally, the predominantly anti-inflammatory cytokine IL-10 (40) was also poorly produced by C. glabrata-infected MDMs when compared with MDMs exposed to S. cerevisiae. Serum opsonization, IFN-γ treatment, or heat killing did not increase cytokine levels induced by C. glabrata after 24 h to those induced by S. cerevisiae or C. albicans, with the exception of IL-10 and IFN-γ (Supplemental Fig. 2). This indicates a generally reduced stimulation of MDMs by C. glabrata. In contrast, GM-CSF was most strongly produced by C. glabrata-infected MDMs. Preactivation of MDMs with IFN-γ or heat killing of C. glabrata abolished this effect (Supplemental Fig. 2). In summary, C. glabrata failed to induce a pronounced pro- or anti-inflammatory cytokine response in MDMs, with the notable exception of GM-CSF.

C. glabrata suppresses production of ROS by MDMs

Because cytokine production of MDMs infected with C. glabrata was low, we reasoned that antimicrobial activities such as ROS production may also be reduced. ROS production by untreated and C. glabrata-infected MDMs was measured in real time by luminol-ECL, a technique that measures intracellular and extracellular ROS (41). We determined differences in ROS production by MDMs exposed to PMA (a strong inducer of ROS), C. glabrata (untreated, serum-opsonized, or heat-killed), S. cerevisiae (untreated or serum-opsonized), or C. albicans during a time course of 3 h (Fig. 5A). Additionally, IFN-γ-preactivated MDMs infected with C. glabrata were also included. PMA induced a 2.5-fold increase in ROS production, whereas in comparison with untreated MDMs, significantly less ROS was released upon infection with C. glabrata, C. albicans, or S. cerevisiae. For each species, a dose-dependent decrease of ROS was observed. Neither preopsonization nor IFN-γ preactivation of MDMs had a significant effect on ROS repression by C. glabrata. However, heat inactivation of C. glabrata (although not statistically significant) as well as serum opsonization of S. cerevisiae (at a MOI of 10) hampered ROS inhibition. Overall, these data suggest that these yeast species may actively downregulate ROS production by MDMs.
To investigate this further, we analyzed ROS production upon coincubation of MDMs with PMA and yeast cells at an MOI of 10 (Fig. 5B). Coincubation of MDMs with C. glabrata reduced PMA-induced ROS production, albeit not significantly. Coincubation of MDMs with S. cerevisiae or C. albicans significantly inhibited PMA-induced ROS production (Fig. 5B). Taken together, these data suggest an ability of all tested yeast species, especially C. albicans, to actively suppress ROS production by MDMs.

C. glabrata alters phagosome maturation

Because C. glabrata cells are able to survive and replicate within macrophages (see above), this yeast must either inhibit or divert the normal process of phagosome maturation or withstand the hostile environment of the mature phagolysosome. We therefore analyzed C. glabrata phagocytosis and the C. glabrata–phagolysosome maturation process in detail. We first determined the presence of the EEA1 on C. glabrata-containing phagosomes, a marker protein for early endosomes. As shown in Fig. 6A and 6B, by 10 min postinfection, a small percentage of phagosomes containing C. glabrata (untreated, opsonized, or heat-killed) displayed EEA1, and this percentage had decreased by 30 min. In contrast, the percentage of hSOZ-containing phagosomes displaying EEA1 remained relatively constant between 10 and 30 min. Other early endocytic markers (Rab5, transferrin receptor) were not detectable at 10 min or later, even in positive control samples (data not shown), suggesting a very rapid phagosome maturation process in MDMs.

During phagosome maturation, early endocytic components are replaced with those associated with late endosomes and lysosomes (e.g., LAMP1). Following 30 and 90 min coincubation of MDMs with C. glabrata (either untreated, serum-opsonized, or heat-killed) or with hSOZ, ~80% of all phagosomes were LAMP1-positive (Fig. 6A, 6B). Even after 24 h, LAMP1 remained colocalized with C. glabrata-containing phagosomes (Fig. 6C), indicating that C. glabrata does not escape from the phagosome to the cytosol during this period.

Finally, the normal end point of phagosome maturation is fusion with lysosomes, which is associated with the acquisition of acid hydrolases such as cathepsin D. Despite constitutive LAMP1 localization on C. glabrata-containing phagosomes, we observed aberrant lysosome fusion: whereas 40 and 60% of phagosomes containing heat-killed C. glabrata or hSOZ were stained positively for cathepsin D, respectively, significantly fewer phagosomes containing viable C. glabrata cells displayed this lysosomal marker at both time points (Fig. 6A, 6B). This suggested that progression from the late endosomal to the lysosomal stage of C. glabrata-containing phagosomes was perturbed.

In addition to immunofluorescence microscopy, we also employed transmission electron microscopy to examine localization of C. glabrata within MDMs 3 h postinfection. The micrograph in Fig. 6D shows five representative yeast cells phagocytosed by an MDM and demonstrates the presence of intact phagosomal membranes tightly surrounding the yeast cells.

FIGURE 4. Cytokine release following yeast infection. With the exception of GM-CSF, the release of pro- and anti-inflammatory cytokines is generally lower by C. glabrata-infected macrophages when compared with S. cerevisiae- or C. albicans-infected macrophages. Primary MDMs from different donors were incubated with 1 µg/ml LPS, C. glabrata (C.g.), S. cerevisiae (S.c.), or C. albicans (C.a.) at an MOI of 5. After 8 and 24 h cytokines were measured in the supernatant by ELISA. Results shown are the means ± SD, n ≥ 3. Note that y-axes of IL-1β and IL-6 are on a logarithmic scale. Cytokine concentrations of MDMs coincubated for 24 h with yeast were in the following ranges (minimum and maximum in pg/ml): TNF-α, 1240.8 (C.g.)–2570.7 (C.a.); IL-1β, 57.9 (C.g.)–1064.3 (C.a.); IL-6, 804.7 (C.g.)–1446.9 (S.c.); IL-8, 2491.5 (C.g.)–6166.4 (C.a.); IL-10, 16.0 (C.g.)–63.1 (S.c.); GM-CSF, 60.3 (C.a.)–157.5 (C.g.); IFN-γ, 413.8 (C.g.)–730.3 (S.c.).
C. glabrata replicates within a nonacidic phagosome

Maturing phagosomes are characterized by a steady decrease in pH, ranging from 6.0 in early endosomes, 5.5–6.0 in late endosomes, to 4.5–5.5 in phagolysosomes. This decrease in pH contributes to the antimicrobial environment of phagolysosomes and is crucial for the activation of hydrolytic enzymes (13). Because C. glabrata appeared to inhibit phagolysosome formation (see above), we used the acidotropic dye LysoTracker Red (DND-99) to examine phagosome acidification. This membrane-diffusible dye is trapped within intracellular acidic compartments following protonation and can therefore be used to visualize phagosomes with a pH below 5.5–6.0 (42, 43). LysoTracker staining of phagosomes containing heat-killed C. glabrata yeasts, however, rarely colocalized with this marker (∼10% at 30 min, with increased background staining due to low signal intensity). When C. glabrata was preopsonized with human serum we observed a slightly higher percentage (∼30% after 30 min) of LysoTracker-positive phagosomes. To exclude a strain-specific effect, we tested five clinical isolates for LysoTracker colocalization. None of the clinical isolates differed significantly in the number of acidified phagosome (ATCC2001, 6.52%; isolate 1, 5.44%; isolate 2, 6.85%; isolate 3, 11.78%; isolate 4, 8.45%; isolate 5, 10.46%).

We next performed genome-wide transcriptional profiles of C. glabrata cells exposed to media with a pH of 4.5 or 7.0 and yeast cells ingested by MDMs. Genes upregulated at pH 4.5 versus pH 7.0 were compared with those upregulated in phagocytosed yeast cells. Only 5.5% (5 of 90) of pH 4.5 upregulated genes ($\geq$2-fold) were also induced in MDMs, providing further evidence that C. glabrata cells are not exposed to an acidic environment within macrophages (Supplemental Table I).

To confirm our LysoTracker staining and gene expression analyses, we sought to directly determine the phagosomal pH. For this approach we made use of the pH-dependent shift in the 495/450 nm fluorescent ratio of FITC (31, 44). MDMs were incubated with FITC-prestained C. glabrata yeasts cells (either viable or heat-
killed) for 1 h and the pH was calculated by comparison with a standard curve. The pH of phagosomes containing heat-killed *C. glabrata* was ∼5.3 (Fig. 7C), reflecting a mixture of ∼70% acidified phagosomes (with a pH probably <5.0) and 30% non-acidified phagosomes as quantified by LysoTracker colocalization (Fig. 7B). In contrast, the average pH of phagosomes containing viable yeasts was 6.1. Taken together, these data demonstrate that *C. glabrata*-containing phagosomes do not undergo normal acidification.

Inhibition of phagosome acidification is independent of fungal transcription and translation or glycosylation activities, but it requires a UV light-indestructible fungal attribute

We next sought to determine which fungal activities are required for suppression of phagosome maturation. *C. glabrata* was pretreated with a range of inhibitors to selectively block transcription (thiolutin), translation (cycloheximide), or glycosylation (tunicamycin) prior to incubation with MDMs. The effective concentrations were first determined by measuring growth in the presence of all three substances (Supplemental Fig. 3). Following pretreatment of *C. glabrata*, phagosome acidification was determined by assessing colocalization with the LysoTracker dye. Treatment with these different inhibitors did not increase the percentage of LysoTracker-positive phagosomes, suggesting that phagosome manipulation does not rely on transcriptional or translational changes in response to phagocytosis (Fig. 8). Furthermore, neither short- nor long-term inhibition of glycosylation had an effect on acidification inhibition.

We concluded that inherent attributes or properties of the fungus, rather than an active response, are more likely to be involved in acidification inhibition. Although heat killing eliminated acidification suppression, such thermal treatment is known to result in significant disruption to the fungal cell surface. Because such surface components may mediate phagosomal acidification inhib-

**FIGURE 6.** *C. glabrata* containing phagosomes acquire the early and late endosomal markers EEA1 and LAMP1, but lack the lysosomal marker cathepsin D. A. Representative fluorescent microscopy images 30 min postinfection show a colocalization (white arrows) of *C. glabrata*-containing phagosomes with the early endosomal marker EEA1 and the late endosomal/lysosomal marker LAMP1. Acquisition of the lysosomal enzyme cathepsin D is only partially detectable (white arrows mark colocalization, red arrows mark absence of colocalization). Marker proteins are indicated in red, while GFP-expressing *C. glabrata* is indicated in green. To detect nonphagocytosed yeasts, samples were stained with Con A (in yellow, marked with red arrows in upper row) prior to permeabilization. The merged image additionally shows the nucleus stained with DAPI in blue. B. Colocalization with EEA1 (upper panel), LAMP1 (middle panel), and cathepsin D (lower panel) was quantified in each experiment for at least 200 phagosomes containing *C. glabrata* (C.g), serum-opsonized *C. glabrata* (C.g. opson.), heat-killed *C. glabrata* (C.g. HK), or hSOZ at indicated time points. Statistical analysis was performed in comparison with viable *C. glabrata* at indicated time points. Results shown are the means ± SD; *n* ≥ 3. *p* < 0.05 by unpaired Student *t* test. C. Representative fluorescent microscopy images 24 h postinfection. *C. glabrata* containing phagosomes remains positive for LAMP1 after 24 h coincubation. D. Transmission electron micrographs of *C. glabrata* phagocytosed by primary MDMs 90 min postinfection. The left panel shows an MDM with five internalized yeasts (red arrows). The red arrow in the magnification (right panel) marks the phagosomal membrane closely surrounding the yeast cell. Original magnification ×2,000 (left panel), ×31,500 (right panel).
We used UVC light, which mainly damages DNA (45), and thimerosal, a substance known to inactivate cells without disrupting or changing the cell surface (46), to kill yeast cells. The absence of colonies on YPD agar and a residual metabolic activity of 4.9% in UVC light-killed yeasts and 3.7% in thimerosal-killed yeasts as compared with viable metabolic activity 4.7% in heat-killed yeasts in an XTT assay confirmed that both treatments blocked immersion, we investigated other methods of inactivating the fungus.

Phagocytosed thimerosal-killed C. glabrata stained with LysoTracker to the same extent as heat-killed yeasts (Fig. 8) and the dye was able to stain the entire phagosome (similar to heat-killed cells in Fig. 7A). In contrast, most UVC light-killed yeasts resigned in nonacidified phagosomes: only ~15% of phagosomes colocalized with LysoTracker. Therefore, the observed suppression of acidification does not require fungal viability and activity, but potentially a surface factor that is resistant to UVC light.

**Intracellular replication results in eventual macrophage lysis**

As described above, C. glabrata is able to suppress phagosome maturation and survives and replicates intracellularly without eliciting a strong proinflammatory response or causing extensive macrophage damage. This poses the question if and how C. glabrata finally escapes from macrophages.

To identify a possible escape mechanism, we investigated the course of infection by time-lapse microscopy. MDMs were allowed to phagocytose yeast cells for 30 min, followed by intensive washing to remove unbound yeasts. Subsequent interactions were monitored for 7 d in the presence of 1% FBS (Fig. 9). Our observations confirmed that C. glabrata survived and replicated within macrophages for days, with obvious damage to MDMs first visible after 2 to 3 d. At this stage, individual MDMs with high fungal loads tightened and contracted, until finally host cell lysis occurred, releasing fungal cells into the medium as a clumped mass, with loose yeasts floating away (Fig. 9A–D). MDMs in close proximity were still motile and phagocytic (Fig. 9E–G). As time progressed, MDM lysis continued and the released C. glabrata cells readily proliferated in the medium (Fig. 9H, 9I).

**Discussion**

Even though infections caused by C. glabrata are increasingly common causes of morbidity and mortality, especially in immunocompromised patients (47, 48), relatively little is known about the pathogenesis of diseases caused by this yeast, as compared with C. albicans. For example, few virulence attributes of C. glabrata have been described (1, 11, 12, 32, 49). One remarkable feature of C. glabrata is its ability to persist for long periods in mice after transient systemic infection (following i.v. infections), without killing the animal and without inducing a strong inflammatory response. Indeed, even immunocompetent mice do not clear such C. glabrata infections over the course of several weeks (7). This suggests that immune evasion is an important aspect of C. glabrata pathogenicity. This view is supported by observations...
that C. glabrata can survive attack by macrophages and replicates within these immune cells (10–12).

These studies, which were based on interactions of C. glabrata with a macrophage cell line, proposed that C. glabrata can adapt to the harsh internal environment of the phagocyte via transcriptional reprogramming (11) and mobilization of intracellular resources via autophagy (12). However, the mechanisms by which C. glabrata survives phagocytosis remained unknown. In the current study we used primary human MDMs to more closely reflect human infections and dissected the interactions of C. glabrata with these cells in detail. By analyzing multiple stages of C. glabrata–macrophage interactions, from initial contact, phagocytosis, and intracellular proliferation to escape from macrophages, to our knowledge we provide new insights into the immune evasion strategies of this important human fungal pathogen.

Intracellular proliferation of C. glabrata does not trigger cytotoxicity or apoptosis of macrophages

Following phagocytosis, most C. glabrata cells survived and went on to proliferate. Although a certain low level of killing of C. glabrata may occur, indicated by an increase in daughter cell number but stable phagocytic index between 3 and 12 h, intracellular survival and proliferation were evident at all time points analyzed. Priming of MDMs with IFN-γ, which is known to enhance candidacidal activity against C. albicans (50, 51), did not affect intracellular replication of C. glabrata. Furthermore, opsonization of C. glabrata with serum factors did not alter the course of infection, intracellular survival, or replication.

Although C. glabrata, the related but apathogenic ascomycete S. cerevisiae, and C. albicans were all phagocytosed by macrophages, the consequences of uptake were dramatically different among the three species: S. cerevisiae cells were efficiently killed by macrophages, whereas many cells of C. albicans and C. glabrata survived and replicated intracellularly. Intracellular growth of C. albicans is associated with hyphal formation, piercing of the host membrane by hyphae, and killing of macrophages as early as 3 h after phagocytosis (Refs. 19, 52, 53 and A. Luettich, K. Seider, S. Brunke, and B. Hube, unpublished observations). In sharp contrast, replication of C. glabrata within MDMs did not cause any significant cytotoxicity within 48 h, and macrophages remained viable for long periods. This suggests that C. glabrata can adapt to the normally harsh intracellular environment and has evolved an intracellular survival strategy, whereas C. albicans adopts a rapid escape strategy.

Moreover, despite intracellular replication of C. glabrata, we did not observe any sign of apoptosis of MDMs, as neither activation of effector caspases 3 or 7 nor fragmentation of DNA was detectable. Inhibition of apoptosis is a known strategy of facultative intracellular microbes, which allows them to persist intracellularly, concealed from other branches of the immune system, such as neutrophils and humoral immune components. For
example, the facultative intracellular dimorphic fungus *Histoplasma capsulatum* was shown to delay host cell death of monocytes by inhibiting apoptosis, thereby securing persistence in an intracellular niche (54). Even though an active repression of apoptosis was not apparent for intracellular *C. glabrata* cells, the absence of any apoptotic activity is in agreement with an intracellular persistence strategy.

**C. glabrata does not elicit production of the major proinflammatory cytokines**

In addition to killing pathogens by phagocytosis, the ability of macrophages to recruit other types of immune cells to the site of infection is a critical function. Therefore, many pathogens have evolved mechanisms to actively manipulate cytokine production of phagocytes to modify the activity of infected cells and their influence on noninfected host cells. For example, the morphological switch of *C. albicans* from yeast to hyphal growth abolishes TLR4 activation, the receptor responsible for IFN-γ production. Instead, hyphae bind to TLR2 on mononuclear cells and macrophages, which leads to an increased production of the anti-inflammatory cytokine IL-10 and thereby to fewer regulatory T cells (23). In vivo, however, following i.v. infection, *C. albicans* induces a fulminant immune response with high cytokine levels and subsequent death of the murine host (55). This is in striking contrast to infections with *C. glabrata*, which are characterized by a mild immune response and long-term survival of mice (7, 9). In this study, we made similar observations on the cellular level with purified macrophages with regard to the production of the proinflammatory cytokines IL-1β, IL-6, IL-8, TNF-α, and IFN-γ. These cytokines induce anti-*Candida* effector functions, including regulation of leukocyte trafficking, immune cell proliferation, and/or activating oxidative and nonoxidative metabolic responses by immune cells. All of these cytokines were produced considerably less by *C. glabrata*-infected macrophages, in comparison with MDMs coinoculated with *S. cerevisiae* or *C. albicans*. This is in agreement with a study of Aybay and Imam (56), which showed a reduced potency of *C. glabrata* to induce TNF-α from peritoneal macrophages when compared with various *Candida* species and *S. cerevisiae*. Furthermore, IL-10, an anti-inflammatory cytokine that inhibits the secretion of proinflammatory cytokines, was also not induced, suggestive of a generally low stimulation of MDMs by *C. glabrata*. The only cytokine that was induced by *C. glabrata* was GM-CSF, even though differences to uninfected controls were only marginal. These data are in agreement with experimental mice infections, where an upregulation of GM-CSF on day 2 postinfection was observed, although the overall cytokine response was low (7). Furthermore, findings of Li et al. (39, 57) and Schaller et al. (38) suggested that *C. glabrata* predominantly induces the release of GM-CSF from epithelial cells, whereas induction of other proinflammatory cytokines remained low, especially in comparison with *C. albicans*. GM-CSF is a potent activator of macrophages and induces differentiation of precursor cells as well as the recruitment of macrophages to sites of infection. This may explain the enhanced tissue infiltration of mononuclear cells, but not neutrophils, observed in vivo (7). Because of the remarkable ability of *C. glabrata* to survive and replicate in macrophages, it is tempting to speculate that luring more macrophages to the site of infection may even be beneficial for the fungus and may constitute part of its immune evasion strategy.

**C. glabrata cells repress the production of high ROS levels**

Among other antimicrobial activities, phagocytes use the NADPH oxidase complex-mediated oxidative burst to eliminate pathogens. A variety of bacterial and fungal pathogens, however, are equipped with antioxidant enzymes and molecules to avoid generation of or to counteract the production of ROS. *C. albicans*, for example, when exposed to neutrophils, expresses extracellular superoxide dismutases (Sod4–Sod6) (58). Accordingly, an inactivation of these detoxifying enzymes leads to severe loss in virulence and viability inside immune cells (59). The oxidative stress response of *C. glabrata* is controlled by the transcription factors Yap1, Skn7, Msn2, and Msn4 (30, 60, 61), which mediate resistance of *C. glabrata* to H₂O₂ and other oxidants. Additionally, *C. glabrata* possesses a catalase (Cta1p) that is required to survive oxidative stress (although this is dispensable in a mouse model of systemic infection) (60, 62). In agreement with a previous study, which showed that *C. glabrata* can suppress the release of oxidants during its interaction with a murine macrophage cell line (21), in this study we demonstrate repression of ROS production by primary human macrophages. Additionally, our experiments with MDMs prestimulated with PMA indicate that *C. glabrata* predominantly counteracts the oxidative burst by actively detoxifying ROS, rather than by downregulating its production. The detoxification of ROS might therefore be one mechanism that contributes to the intracellular survival of *C. glabrata* in macrophages. Similarly, *C. albicans* can reduce ROS production by macrophages or dendritic cells (21, 63). However, in our study, even *S. cerevisiae* was able to suppress ROS levels in macrophages, but not to survive, indicating that reduction of ROS alone is not sufficient for survival within macrophages, and may even be a general property of yeast. Interestingly, serum opsonization hampered ROS inhibition by *S. cerevisiae* and, to a lesser extent, *C. glabrata*, suggesting that receptor–ligand pairs involved in the uptake of serum-opsonized fungal cells may affect ROS levels. Additionally, patterns observed for heat-inactivated *C. glabrata* hint at a heat-labile factor or a yeast activity involved in ROS repression.

**C. glabrata blocks phagolysosome formation and phagosome acidification**

In addition to ROS repression, pathogens may disrupt normal phagosome maturation. Changing the phagosome fate has been described for bacteria such as *Mycobacterium*, *Legionella*, *Chlamydia*, and *Listeria* spp. and for pathogenic fungi such as *Aspergillus fumigatus*, *H. capsulatum*, *C. neoformans*, and *C. albicans* (reviewed in Refs. 14, 15). In this study, we discovered that *C. glabrata* controls the process of phagosome maturation and acidification in human primary macrophages at a distinct stage. Shortly after sealing, new phagosomes exchange material with early endosomes, rendering them fusogenic with late endocytic compartments. The recruitment of EEA1 and LAMP1 marker proteins to phagosomes containing *C. glabrata* indicated a normal progression to the early and late endosomal stages. The presence of a tight phagosome membrane enclosing the yeast cell was confirmed by electron microscopy, excluding the possibility of fungal escape into the cytoplasm, a survival strategy of bacteria such as *Listeria* and *Shigella* spp. (64). Phagosomes normally continue to interact with lysosomes, thereby acquiring a complex mixture of hydrolytic enzymes and an acidic pH of ∼4.5, finally resulting in activation of antimicrobial activities. Our results indicate that viable *C. glabrata* alter phagosomal biogenesis at this later stage and prevent fusion with lysosomes, as most phagosomes did not acquire the lysosomal enzyme cathepsin D at any time tested and did not acidify, when compared with heat-killed yeast cells or other controls. Inhibition of acidification is not a strain-specific ability, as five clinical isolates tested in this study also resided mainly in nonacidified phagosomes (88–95%).
Opsonization caused a slightly higher percentage of LysoTracker colocalization, suggesting that Fc and/or complement receptor activation partially restores normal maturation. Additionally, the measurement of phagosomal pH with a pH-dependent fluorescent dye confirmed a much higher pH for phagosomes containing viable yeasts (pH 6.1) than that for heat-killed yeasts (pH 5.3). These data are in line with transcriptional profiles that did not indicate any exposure of intracellular C. glabrata cells to acidic pH. Thus, C. glabrata resides and replicates in weakly acidified, cathepsin D-free vesicles, a status that probably contributes to its survival. A similar ability has been postulated for other pathogenic fungi, such as C. albicans and H. capsulatum (17, 20). Both species block the fusion of phagosomes with lysosomes, as lysosomal membrane proteins are actively recycled from, and hydrolytic compounds are reduced in, their phagosomes. In the case of H. capsulatum, it is known that reduced accumulation of the phagolysosomal proton pump, vacuolar ATPase, together with the activity of the fungal saposin-like calcium-binding protein, which potentially interacts with host lipids of the phagosome, contributes to altered phagosome trafficking (18, 65). As all anti-vacuolar ATPase Abs tested in this study (four in total) bound non-specifically to the surface of C. glabrata cells (and were thus not suitable for microscopic analyses; data not shown), it remains unclear whether C. glabrata may prevent acidification by down-regulation of vacuolar ATPase.

Because live C. glabrata, but not heat-killed yeasts, inhibited the acidification of phagosomes, we postulated that this is an active fungal-driven process. We therefore performed inhibitor studies to block transcription, translation, and glycosylation. Fungi were pretreated with appropriate inhibitors prior to infection of MDMs, and phagosomal acidification was monitored after 30 min coinoculation; this early time point was chosen to avoid a resumption of the blocked activity. However, no significant enhancement of LysoTracker colocalization (and thus prevention of acidification) was apparent when transcription, translation, or glycosylation was inhibited. Therefore, we propose that a heat-sensitive surface factor may disturb phagosomal maturation. To test this hypothesis, it was necessary to kill the fungus without damaging its cell surface. Killing C. glabrata with thimerosal turned out to be unsuitable, as the treatment had a negative effect on cell integrity. However, inactivation of C. glabrata with UVC light did not prevent inhibition of phagosomal acidification, suggesting that a heat-sensitive, UV light-destoryable surface attribute may be responsible for the observed phenotype.

Although inhibition of phagolysosome maturation has been observed for C. albicans, H. capsulatum, A. fumigatus, and C. neoformans, the mechanisms employed by these fungi remain largely unidentified. Although it is completely unknown how C. albicans causes incomplete phagosome maturation, it has been suggested that H. capsulatum and C. neoformans secrete proteins and polysaccharides that lead to the formation of abnormal phagosomes (65–67). A. fumigatus possesses a surface-bound structure (1,8-dihydroxynaphthalene–like melanin) that has been proposed to inhibit lysosomal fusion in human MDMs (68). Bacterial processes that lead to disruptions of phagosome maturation have been studied in greater detail and might provide hints about the nature of the UV light-destoryable factor of C. glabrata. For example, roles for glycolipids such as lipoarabinomannans and cord factor (trehalose dimycolate) of Mycobacterium tuberculosis in interference with phagosome maturation have been reported (69–71). Both glycolipids contain long hydrophobic mycolic acid chains, which might intercalate into the phagosomal membrane and change its membrane properties. This might disturb trafficking or, alternatively, inhibit the recruitment of factors needed for phagosome progression. Similar molecules may contribute to the ability of C. glabrata to arrest phagosome maturation at a prephagolysosomal stage.

Escape from macrophages

Facultative intracellular microbes have developed a number of strategies to escape from their transient intracellular environment. One mechanism is to induce apoptosis to kill the host cell without causing proinflammatory cytokine production, which itself would attract and activate immune cells. This has been reported for many bacteria (e.g., Salmonella or Chlamydia spp.; reviewed in Ref. 72) and also for fungal pathogens such as C. neoformans (24). However, we did not observe signs of apoptosis by C. glabrata-infected MDMs, indicating that, at least during the early stages (24 h) of interaction, C. glabrata does not induce apoptosis. C. albicans is known to switch from a yeast to a filamentous form, which is required for its escape from macrophages. Consequently, nonfilamentous mutants become trapped within macrophages, even though these cells are still able to replicate (53, 73). The higher destructive potential of C. albicans may result from several other virulence factors, such as extracellular hydrolytic enzymes, which may act as cytolysins in macrophages, directly inducing cytotoxicity by modifying host cell structures (74). C. glabrata, in contrast, does not exhibit significant levels of extracellular protease activity, at least in vitro, and a role for C. glabrata phospholipases for virulence has not been reported to date (75, 76). However, Kaur et al. (11) proposed a function of a family of GPI-linked aspartic proteases (yapsins) for interaction with host cells and showed their requirement for survival of C. glabrata within macrophages.

C. glabrata clearly pursues a different strategy than C. albicans, as escape, even following 48 h, was not observed. However, staining phagosomes for LAMP1 after 24 h revealed a diffuse and punctuate pattern. This is in contrast to the smooth and annular pattern seen for earlier time points and for killed yeasts, suggesting changes in phagosomal membrane integrity. Therefore, we investigated whether C. glabrata is capable of escaping MDMs at later stages to disseminate. We used time-lapse microscopy to follow the interaction of C. glabrata with MDMs in real time and observed bursting of MDMs and release of viable yeast cells after 3 d. The yeasts continued to replicate in the medium, and a fraction of the escaped population was rephagocytosed by so-far unaffected neighboring MDMs.

Although the exact mechanism of escape remains unclear at this time, it is possible that mechanical rupture of host MDMs occurs following a critical increase in fungal burden. Alternatively, secretion of fungal factors (such as hydrolases), which may either accumulate over time or which are triggered in a quorum-dependent mechanism, could disrupt macrophage cellular processes, leading to host cell lysis. C. glabrata may also reside within host cells until age-related death of the macrophage occurs, although this seems unlikely due to the remaining phagocytic activity of neighboring MDMs following C. glabrata escape.

We did not observe an extrusion or expulsion mechanism, identified for C. neoformans, by which fungal cells and macrophages remain alive even after the release of yeast cells (25, 26). MDMs clearly burst when releasing C. glabrata cells. The process is therefore more comparable to cytolysis, an escape mechanism used by bacterial species such as Chlamydia or Leishmania spp. (77, 78). In these cases, lysis of host cells is not only the consequence of the expanding bacterial vacuole, but also of the action of pore-forming proteins or proteolytic digestion rupturing host cell membranes. Whether C. glabrata produces such molecules (in addition to yapsins) inside of MDMs or whether tensioning of
phagosomal membranes is sufficient for fungal escape remains speculative.

In summary, our data demonstrate that \textit{C. glabrata} is able to subvert normal macrophage phagosome maturation, surviving and replicating within these immune cells for considerable periods of time without damaging the host cell or eliciting a proinflammatory immune response. Although possibly mutually beneficial during commensal carriage, such an interaction may subvert an effective immune response and clearance during invasive infections. We therefore propose that \textit{C. glabrata} exploits the intracellular niche of the macrophage phagosome as part of an immune evasion and persistence strategy.

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Disclosures
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