MINIREVIEW

Intracellular survival of Candida glabrata in macrophages: immune evasion and persistence

Lydia Kasper1,*, Katja Seider1 and Bernhard Hube1,2,3

1Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology–Hans Knöll Institute, 07745 Jena, Germany, 2Integrated Research and Treatment Center, Sepsis und Sepsisfolgen, Center for Sepsis Control and Care (CSCC), University Hospital, 07747 Jena, Germany and 3Friedrich Schiller University, 07743 Jena, Germany

*Corresponding author: Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology–Hans-Knoell-Institute (HKI), Beutenbergstrasse 11a, 07745 Jena, Germany. Tel: +49-3641-5321219; Fax: +49-3641-5320810; E-mail: lydia.kasper@leibniz-hki.de

One sentence summary: The authors review the strategies of Candida glabrata that permit intracellular survival and replication within macrophages.

Editor: Monique Bolotin-Fukuhara

ABSTRACT

Candida glabrata is a successful human opportunistic pathogen which causes superficial but also life-threatening systemic infections. During infection, C. glabrata has to cope with cells of the innate immune system such as macrophages, which belong to the first line of defense against invading pathogens. Candida glabrata is able to survive and even replicate inside macrophages while causing surprisingly low damage and cytokine release. Here, we present an overview of recent studies dealing with the interaction of C. glabrata with macrophages, from phagocytosis to intracellular growth and escape. We review the strategies of C. glabrata that permit intracellular survival and replication, including poor host cell activation, modification of phagosome maturation and phagosome pH, adaptation to antimicrobial activities, and mechanisms to overcome the nutrient limitations within the phagosome. In summary, these studies suggest that survival within macrophages may be an immune evasion and persistence strategy of C. glabrata during infection.

Keywords: Candida glabrata; macrophage; survival; replication; immune evasion; persistence

INTRODUCTION

Candida glabrata is a human commensal which asymptptomatically colonizes mucosal surfaces such as the oral cavity and the gastrointestinal tract of the majority of healthy individuals. This yeast is also a successful opportunistic human pathogen, causing both superficial and systemic infections under certain predisposing conditions. Although phylogenetically more closely related to the baker’s yeast Saccharomyces cerevisiae than to the most important pathogenic Candida species, C. albicans (Dujon et al. 2004; Bolotin-Fukuhara and Fairhead 2014), C. glabrata is the second most frequent cause of invasive candidiasis in the USA and most of Europe, accounting for 15–20% of cases (Perlroth, Choi and Spellberg 2007; Pfaller and Diekema 2007). Risk factors for candidemia include the disturbance of normal microbiota (e.g. by antibiotic treatments), immunodeficiency such as neutropenia (e.g. cancer and transplant patients), severe illness and ICU stay and, especially for C. glabrata, high age (Perlroth, Choi and Spellberg 2007; Pfaller and Diekema 2007; Pfaller et al. 2010). Candida glabrata is an emerging pathogen, and infections are associated with high mortality (Fidel, Vazquez and Sobel 1999; Silva et al. 2012). The increasing incidence of C. glabrata infections over the last decades can likely be attributed (a) to higher numbers of immunocompromised patients and an increasingly aged population, (b) to improved diagnostic approaches, but (c) also to difficulties in therapeutic treatments of infected patients.
due to a high intrinsic and often further acquired resistance of this fungus to frequently used antifungal drugs, such as azoles (Pfaller 2012; Silva et al. 2012).

Candida glabrata is a haploid yeast, which is not able to switch to true hyphal growth but strictly grows in the yeast form under most conditions (Caanck and Haynes 2000; Kaur et al. 2005). This is in strong contrast to C. albicans, where the dimorphic switch is considered as one of the most important virulence factors (Jacobsen et al. 2012). Despite the lack of this morphological flexibility (and several other virulence attributes which are crucial for C. albicans; Brunke and Hube 2013), C. glabrata is a successful pathogen and well adapted to the human host. The yeast is able to attach to host cells and to form biofilms, partly mediated by a large family of glycosylphosphatidylinositol-linked epithelial adhesins (Epa proteins), but likely also by other adhesin-like cell-wall-anchored proteins (Cormack, Ghori and Falkow 1999; de Groot et al. 2008; Kranefeld et al. 2011). Besides, C. glabrata is equipped with a high intrinsic stress tolerance, enabling the fungus, for example, to sustain prolonged starvation periods and to withstand oxidative stress (Kramer, Schwebke and Kampf 2006; Cuellar-Cruz et al. 2008). At least in vitro, C. glabrata can resist the confrontation with host immune cells, especially macrophages, as a significant fraction of phagocytes C. glabrata cells is able to survive and replicate inside human and murine macrophages (Fig. 1) (Otto and Howard 1976; Kaur, Ma and Cormack 2007; Roetzer et al. 2010; Seider et al. 2011).

In this review, we summarize the current knowledge about the interaction of C. glabrata with macrophages, focusing on the mechanisms which facilitate survival in these immune cells in vitro, but also discussing intracellular survival, immune evasion and persistence as a possible in vivo infection strategy of C. glabrata.

MACROPHAGES AS INNATE IMMUNE CELLS IN ANTIMICROBIAL DEFENSE

Macrophages are professional phagocytes of the monocytic lineage that act as part of the innate immune system in the first line of defense against invading microbes. They can be found in almost all tissues and are abundant at mucosal surfaces (Pollard 2009). The first contact between a phagocytic cell and a microbe is mediated by host receptors. These include pattern recognition receptors (PRRs) which detect conserved basic molecular components of microorganisms, the pathogen-associated patterns (PAMPs) and opsonic receptors which recognize opsonized microbes (Medzhitov and Janeway 1997; Gordon 2002).

The details of fungal recognition and macrophage downstream signaling pathways have been extensively reviewed elsewhere (Netea et al. 2008; Bourgeois et al. 2010; Romani 2011; Cheng et al. 2012; Lionakis and Netea 2013). Especially for C. albicans, a number of immune cell surface receptors have been identified to be responsible for non-opsonized fungal recognition, including toll-like receptors TLR-2 and TLR-4 (Netea et al. 2002; Jouault et al. 2003), C-type lectin receptors dectin-1 and dectin-2 (Brown et al. 2003; Saijo et al. 2010a), the macrophage mannose receptor (Ezekowitz et al. 1999) and the complement receptor CR3 (Soloviev, Jawhara and Fonzi 2011).

Recognition of microbial ligands by macrophage receptors activates a series of intracellular signaling pathways that lead to reorganization of the actin cytoskeleton and phagocytosis. Engulfed microorganisms are trapped in a plasma-membrane-derived vacuole, the phagosome. This premature compartment lacks the ability to degrade particles or to kill pathogens. However, during a maturation process, in a series of fusion events with compartments of the endosomal pathway, phagosomes acquire these properties by becoming more acidic and hydrolytically active (Vieira, Botelho and Grinstein 2002; Underhill 2005; Haas 2007). Oxidative and non-oxidative antimicrobial mechanisms as well as nutritional limitation contribute to killing and growth restriction of microbes. Furthermore, the activation of subsequent innate and adaptive immune reactions is promoted by antigen presentation and the production of immunomodulatory proteins such as cytokines.

SURVIVAL AND IMMUNE EVASION STRATEGIES OF C. GLABRATA

Immune evasion strategies are critical for the survival of successful microbial pathogens within a host. Since the host defense mechanisms of innate immune effector cells such as macrophages are multifaceted, a number of different evasion strategies have evolved among bacterial and fungal pathogens. These include avoidance of contact with macrophages, a rapid escape from host cells, the ability to withstand macrophage antimicrobial activities, but also the use of macrophages as an intracellular niche for protection from other immune cells (Flanagan, Cosio and Grinstein 2009; Seider et al. 2010; Smith and May 2013; Gilbert, Wheeler and May 2014).

In general, pathogens that survive macrophage phagocytosis and consequently undergo a shorter or longer intracellular period pass through four phases: entry, survival, replication and exit from the host cell (Casadevall 2008). Following, we will discuss the current knowledge about C. glabrata–macrophage
interaction and possible mechanisms behind intracellular survival and replication.

**Getting inside—recognition, phagocytosis and the role of the fungal cell surface**

A common strategy of fungal pathogens during interaction with immune cells is the masking of immunostimulatory cell wall components to avoid recognition and macrophage activation and/or phagocytosis (Seider et al. 2010; Gilbert, Wheeler and May 2014). The cell walls of yeasts are normally composed of an inner skeletal layer consisting of chitin, β-1,3-glucan and β-1,6-glucan, linked to an outer layer, which is made up of heavily mannosylated proteins and phospholipomannan (Klis, Boorsma and Groot 2006; Netea et al. 2008). β-1,3-Glucan plays a key role in immune recognition, mediated by the lectin-receptor on macrophages (Brown et al. 2003). Intact yeast cells of C. albicans, however, have low reactivity with lectin-1, as β-1,3-glucan is shielded by the outer mannan layer. When the integrity of the cell wall is perturbed and β-1,3-glucan is exposed, binding of lectin-1 facilitates recognition and elicits macrophage activation and production of cytokines such as TNF-α (Wheeler and Fink 2006).

Concerning fungal PAMPs, similar observations have been made for C. glabrata. Deletion mutants with disturbed cell wall integrity and altered accessibility of immunostimulatory cell wall components (β-1,3-glucan and/or chitin) caused a stronger inflammatory response by macrophages. This was indicated by elevated TNF-α secretion, often coupled with a higher oxidative burst, and the mutants were in most cases also killed more efficiently by macrophages. This was true for deletion mutants lacking cell surface-associated C. glabrata proteases (yapsins) (Kaur, Ma and Cormack 2007) or for mutants with defects in protein glycosylation (West et al. 2013; Seider et al. 2014), but also for mutants lacking genes with predicted functions not primarily related to cell surface integrity (Seider et al. 2014). In conclusion, as seen for C. albicans, an intact outer (mannan) network probably shields the inner layer of the C. glabrata cell wall and prevents macrophage activation and fungal killing. Still, the C. glabrata cell surface composition triggers efficient phagocytosis by human monocyte-derived macrophages (MDMs) and murine macrophages in vitro (Keppeler-Ross et al. 2010; Seider et al. 2011). Binding to macrophages is partially mediated by the yeast adhesin Epa1 (Kuhn and Vyas 2012).

Concerning PRRs responsible for recognition of C. glabrata by macrophages, not much is known to date. Recently, the C-type lectin receptor lectin-2 has been shown to be important for host defense against C. glabrata (Ifrim et al. 2014). Lectin-2−/− knockout mice were more susceptible to C. glabrata infection, and phagocytosis of fungal cells by lectin-2−/− macrophages was decreased. Referring to signaling pathways downstream of PRRs, we found that the fungus does not induce substantial phosphorylation of the MAP kinases Erk1/2 (extracellular signal-related kinases), SAPK/JNK (stress-activated protein kinases/c-jun amino-terminal kinases) and p38, or NFκB signaling (Kasper et al. 2014). In contrast, spleen tyrosine kinase (Syk), which signals downstream of C-type lectin receptors lectin-1, but also lectin-2 (Drummond et al. 2011), was activated upon C. glabrata infection of macrophages, again pointing to an importance of C-type lectin receptors in C. glabrata recognition (Kasper et al. 2014).

In line with these findings, induction of proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8 and IFN-γ) upon infection of macrophages was low. The only cytokine considerably induced by C. glabrata was GM-CSF (Seider et al. 2011). This is in agreement with studies on C. glabrata interaction with epithelial cells or systemic mouse infection (discussed below), where also overall cytokine induction was low but GM-CSF was induced upon C. glabrata infection (Schaller et al. 2002; Li and Dongari-Bagtzoglou 2007, 2009; Jacobsen et al. 2010). GM-CSF is an activator of macrophages and induces macrophage recruitment. Because of the remarkable ability of C. glabrata to survive and replicate in macrophages, it is tempting to speculate that attracting more macrophages to the site of infection in vivo may even be beneficial for the fungus and may constitute part of its immune evasion strategy (Seider et al. 2011).

**Countering macrophage antimicrobial action—modification of phagosome maturation and oxidative stress resistance**

After phagocytosis, the delivery of the ingested microorganism to a phagolysosome is one central antimicrobial mechanism of macrophages. Many intracellular pathogens, including fungi, have evolved strategies to arrest phagosome maturation, thus creating a less hostile macrophage intracellular environment (Flannagan, Cosio and Grinstein 2009; Seider et al. 2010; Smith and May 2013; Gilbert, Wheeler and May 2014).

We have shown that C. glabrata resides in a modified phagosomal compartment, preventing full phagosome maturation and acidification. Candida glabrata-containing phagosomes acquire markers of the early and late endosomal stage (early endosomal antigen 1, EEAl and lysosomal-associated membrane protein 1, LAMP-1, respectively), but not of the phagolysosomal stage (cathepsin D, proteolytic activity, lysosomal fusion). Correspondingly, although the phagosomal proton pump V-ATPase seems to be present in these compartments, phagosomes containing viable yeasts are only weakly acidified (Seider et al. 2011; Kasper et al. 2014). Candida albicans has similarly been found to subvert the lysosomal system of macrophages. Experiments with murine macrophages indicate that C. albicans actively recycles cathepsin D and LAMP-1 out of phagosomes (Fernandez-Arenas et al. 2009). Live cell imaging revealed that C. albicans delays phagosome maturation and acidification—a process depending on fungal cell wall composition and morphology (Bain et al. 2014; Bain, Gow and Erwig 2015).

In contrast to viable C. glabrata cells, heat-killed yeasts reach an acidified compartment with phagolysosomal properties, indicating that either a heat-labile surface factor or a fungal activity is required for phagosomal modification (Seider et al. 2011; Kasper et al. 2014). Recently, the metabolization of amino acids as carbon sources by C. albicans and the resulting ammonia excretion has been implicated in environmental alkalization and neutralization of phagosome pH by this Candida species (Vylkova et al. 2011; Vylkova and Lorenz 2014). Candida glabrata is similarly able to alkalize its environment in vitro when metabolizing amino acids in the absence of glucose (Kasper et al. 2014). The putative C. glabrata α-1,6-mannosyltransferase Mnn10 and the related mannosyltransferase Mnn11 are important for both in vitro environmental alkalization and prevention of phagosome acidification (West et al. 2013; Kasper et al. 2014), suggesting that (Mnn10/11-dependent) alkalization is a fungal strategy to raise phagosome pH. Further work is needed to clarify (a) whether active pH modulation is indeed involved in phagosome modification by C. glabrata and (b) how Mnn10/11 and other fungal factors contribute to these processes.

For bacterial pathogens, many species-specific effector molecules are known which interfere, for example, with
phagosomal membrane fusion events or integrity (Fiannagan, Cosio and Grinstein 2009). Surprisingly, while mutant screens or analyses of single gene deletion mutants have identified many genes that are important for C. glabrata survival in macrophages (see below), the genes and mechanisms underlying phagosome modification by this fungus are still poorly understood. A recent study has implicated a role of phosphatidylinositol 3-kinase-mediated signaling in phagosome modification. Macrophages infected with C. glabrata mutants that lack the two class III phosphoinositide 3-kinase (PI3K) subunit-encoding genes VPS15 and VPS34 showed a slightly increased number of acidified phagosomes (Rai et al. 2015). The authors suggest that PI3K-dependent cellular trafficking events in C. glabrata may be involved in modification of phagosome maturation by a so far unknown mechanism.

Future work will also help to clarify the role of host factors in phagosome maturation of C. glabrata-containing phagosomes. Dectin-1-mediated Syk signaling, for example, has recently been implicated in the control of phagosome maturation following β-1,3-glucan recognition (Mansour et al. 2013). Viable C. glabrata cells induce a less pronounced Syk activation in macrophages than heat-killed cells, suggesting that reduced Syk-mediated signaling may be involved in triggering delivery of viable yeasts to non-matured phagosomes (Kasper et al. 2014).

To date, it is not entirely clear whether C. glabrata needs to arrest phagosome maturation or to modify phagosome pH to survive. In vitro, C. glabrata growth is unaffected by low pH values, even at pH 2.0 (Bairwa and Kaur 2011). However, the combination of low pH and the full arsenal of macrophage antimicrobial properties in a mature phagosome would likely strongly restrict C. glabrata survival. In general, combinatorial stress has been shown to have far more antifungal potential than single stress conditions (Kaloriti et al. 2012). The exposure to a combination of low pH and carbon starvation, for example, caused partial loss of viability of C. glabrata in vitro (Roetzer et al. 2010). Thus, the energy-demanding equilibration of extracellular pH combined with limited energy resources might be the challenging environment C. glabrata is exposed to in the phagosome (Roetzer, Gabeldon and Schuller 2011).

The production of toxic reactive oxygen species (ROS) is one central aspect of the macrophage anti-microbial response. ROS production is driven by the macrophage NADPH oxidase complex which is assembled at the cytoplasmic and phagosomal membrane and generates superoxide, hydroxyl anions and hydroxyl radicals (Segal et al. 2012). However, microorganisms have developed different strategies to survive and counteract the oxidative burst. Often, the production of antioxidant defense enzymes plays an important role (for C. albicans, see for example Miramon, Kasper and Hube 2013). Candida glabrata has a high intrinsic oxidative stress resistance compared to C. albicans and S. cerevisiae. This is partly mediated by expression of a highly active catalase, Cta1; however, this enzyme is dispensable during mouse systemic infection (Cuellar-Cruz et al. 2008). Besides, the core response to oxidative stress in vitro includes thioredoxin peroxidases (Tsa1 and Tsa2), thioredoxin reductases (Trt1, Trt2), thioredoxin cofactor (Trx2), glutathione peroxidase (Gpx2) and superoxide dismutases (Sod1 and Sod2) (reviewed in Jandric and Schuller 2011; Cuellar-Cruz et al. 2014). A recent study has shown that Sods are not only required for protection against oxidative damage, but are also important for metabolism, lysine biosynthesis, DNA protection and aging in C. glabrata (Briones-Martin-Del-Campo et al. 2015). During interaction with macrophages in vitro, C. glabrata Sod1 confers resistance to killing in the absence of Yap1-dependent signaling (Roetzer, Klopf and Gratz 2011). Further, a CTA1-reporter gene is induced after macrophage phagocytosis, indicating both, exposure of C. glabrata to oxidative stress in macrophages and a role of Cta1 in oxidative stress response (Roetzer et al. 2010).

Studies on human and murine macrophages have shown that C. glabrata suppresses ROS production by these phagocytes (Wellington, Dolan and Krysan 2009; Seider et al. 2011). The mechanisms involved in this process are not entirely clear. NADPH oxidase activation is triggered upon binding and phagocytosis of microbes even before the phagosome is sealed (Nunes, Demaurex and Dinauer 2013). It is therefore not necessarily to be expected that incomplete maturation of C. glabrata-containing phagosomes will result in less ROS induction. ROS suppression is currently rather seen as an active downregulation of macrophage ROS production by the fungus as an immune evasion strategy (Wellington, Dolan and Krysan 2009; Seider et al. 2011).

While a correlation between phagocytosis-associated oxidative metabolism and fungal killing in vitro has long been shown for C. albicans (Sasada and Johnston 1980), the role of ROS for killing of C. glabrata is unclear. The above-mentioned high intrinsic resistance of C. glabrata to oxidative stress together with the fact that experimental inhibition of ROS production in macrophages does not increase fungal survival (Seider et al. 2014) may suggest that ROS plays a relatively minor role in direct killing of C. glabrata. In line with this, none of the mutants lacking genes with known functions in ROS detoxification (genes encoding catalase, superoxide dismutases, peroxidases or thioredoxin proteins) were detected in a screen of a gene deletion library for reduced survival in human macrophages (Seider et al. 2014). However, some of the mutants with reduced macrophage survival identified in this screen showed higher susceptibility to oxidative stress in vitro. The currently available data thus suggest that C. glabrata possesses robust and redundant antioxidant systems, and that ROS may act in combination with other stresses inside the phagosome on killing of this fungus (Kaloriti et al. 2012; Seider et al. 2014). In fact, C. glabrata infections are lethal in p47phox−/− mice which lack a NADPH oxidase component, showing that the capacity of phagocytes to produce ROS is indeed relevant for controlling fungal infection in vivo (Ju et al. 2002).

In addition to ROS, reactive nitrogen species may contribute to the antifungal defense of macrophages. Nitric oxide is, for example, important for macrophage activity against C. albicans (Cenci et al. 1993). However, not much is known about the contribution of nitrosative stress to the macrophage defense against C. glabrata, except that the yeast induces only low NO production by murine macrophages (Kaur, Ma and Cormack 2007).

**Metabolic adaptation to the intracellular milieu**

To survive and replicate in the phagosome, C. glabrata has to adapt its metabolism to the nutritional conditions in this compartment. This includes adaptation to the use of alternative carbon sources other than glucose and to nitrogen deprivation. The transcriptional response of C. glabrata to phagocytosis by macrophages reflects such reprogramming processes: the upregulation of genes coding for enzymes of glyoxylate cycle, gluconeogenesis and β-oxidation of fatty acids and the downregulation of glycolysis indicate a switch to the use of alternative carbon sources (Kaur, Ma and Cormack 2007; Rai et al. 2012). Downregulation of protein synthesis as well as upregulation of amino acid biosynthetic pathways and amino acid and ammonium transport genes suggests that the fungus experiences nitrogen...
deprivation. Further, genes of the methylcitrate cycle, which is important for the degradation of fatty acid chains and which allows the use of lipids as alternative carbon sources, are up-regulated (Kaur, Ma and Cormack 2007; Rai et al. 2012). This response is very similar to the response of C. albicans to phagocytosis (Lorenz, Bender and Fink 2004; Miramon, Kasper and Hube 2013).

Pexophagy, a specialized form of autophagy, is needed for C. glabrata to overcome glucose starvation in the phagosome. Macrophage phagocytosis induces an increase in peroxisome numbers in C. glabrata cells, likely as a strategy to switch to alternative carbon source utilization. In later stages of phagocytosis, peroxisomes are degraded via pexophagy. Correspondingly, deletion of genes required for pexophagy causes a reduction in macrophage survival (Roetzer et al. 2010).

An expression analysis of selected C. glabrata genes during infection of the mouse spleen showed upregulation of alternative carbon source utilization through gluconeogenesis, glyoxylate cycle and long-chain fatty acid metabolism, while protein and ergosterol biosynthesis was downregulated (Fukuda et al. 2013). The similarity of this metabolic adaptation to that observed after macrophage phagocytosis indicates that similar metabolic changes are required for growth in a carbohydrate-limited host environment, potentially including subpopulations of phagocytosed C. glabrata cells in vivo.

Chromatin remodeling processes are suggested to play a central role in reprogramming the cellular energy metabolism inside the phagosome (Rai et al. 2012). Mutants of C. glabrata defective in chromatin organization were reduced in macrophage survival, and internalized wild-type C. glabrata cells showed a differentially modified chromatin and elevated cellular lysine deacetylase activity, as compared to non-phagocytosed cells (Rai et al. 2012). The importance of the tail subunit of the RNA polymerase II complex Med2 for proliferation in macrophages also underlines the requirement of general regulatory processes for intracellular fitness (Borah et al. 2014).

Besides carbon and nitrogen, trace elements such as iron are important for yeast growth. Iron is an essential transitional metal, serving as a cofactor for several enzymes and being required for numerous biochemical processes including cellular respiration and metabolism, oxygen transport and DNA synthesis (Nevitt 2011). Consequently, there is a constant competition for iron between host and pathogen. It is therefore not surprising that the host actively sequesters iron from extracellular spaces to challenge invading microorganisms with micronutrient limitation, a process called nutritional immunity (Hood and Skaar 2012). The phagosomal environment is thought to be limited for iron, but pathogenic microorganisms have evolved strategies to accumulate this transition metal in the phagosome (Wagner et al. 2005). In contrast to C. albicans, C. glabrata has not been shown to use the host iron-binding proteins ferritin or transferrin as iron sources, and C. glabrata is not able to efficiently utilize heme or hemoglobin (Weissman and Kornitzer 2004; Nevitt and Thiele 2011). However, the fungus expresses one siderophore iron transporter, Sit1, which is able to bind hydroxamate-type xenosiderophores of fungal origin, such as ferrichrome, ferrirubin or coprogen, but not bacterial siderophores. Sit1-mediated binding of siderophore-bound iron increases fitness and survival of C. glabrata, when subsequently exposed to macrophages (Nevitt and Thiele 2011). A recent study has further shown the importance of the reductive iron uptake genes FTR1, FET3 and CCC2 as well as the iron storage and utilization genes FETS and YFH1 for intracellular survival in macrophages (Srivastava, Suneetha and Kaur 2014). These two studies indicate that iron availability and iron acquisition are indeed important determinants of intracellular survival and replication of C. glabrata in macrophages. In line with this, 11 out of 23 mutants identified in a mutant screen for reduced survival within macrophages showed defects in growth under iron limitation (Seider et al. 2014).

In addition to nutritional adaptation and oxidative stress response, there are more adaptive processes required for full fitness inside macrophages. For example, mutants defective in stress signaling were found to be attenuated for survival within macrophages. These include mutants lacking the calcium transporter-encoding gene CCH1 (involved in calcium-mediated signaling), the TOR pathway component-encoding gene SLM1, the high osmolarity glycerol (HOG) pathway component-encoding genes SHO1 and HOG1 and two genes involved in phosphatidylinositol 3-kinase-mediated signaling, VPS15 and VPS34 (Seider et al. 2014; Rai et al. 2015; Srivastava, Suneetha and Kaur 2015). The mechanisms behind the role of these pathways in macrophage survival remain to be studied in more detail.

**Escape after long-term containment in macrophages**

The exit from the intracellular niche at a certain stage of infection is an essential step for many pathogens, for example to facilitate transmission or dissemination in the host. An early escape from the phagosome into the cytoplasm is also a common microbial strategy to avoid the hostile phagosomal conditions. Intracellular pathogens have evolved numerous strategies to escape from host cells (reviewed in Hybiske and Stephens 2008; Smith and May 2013). Strategies employed by fungal pathogens include non-lytic exocytosis, observed for Cryptococcus neoformans and C. albicans (Alvarez and Casadevall 2006; Bain et al. 2012), but also the induction of host cell death by apoptosis (C. neoformans; Villena et al. 2008) or pyroptosis, a caspase-dependent early lytic proinflammatory cell death (C. albicans; Uwamahoro et al. 2014; Wellington et al. 2014). After phagocytosis of C. albicans, rapid hyphal formation is triggered, which seems to be important for both early inflammatory and late mechanical damage and escape (Uwamahoro et al. 2014; Wellington et al. 2014). Candida glabrata, as mentioned above, grows mainly in the yeast form and only forms pseudohypha under certain low nitrogen conditions (Csank and Haynes 2000). This growth form is routinely not observed during macrophage infection; however, microevolution experiments with permanent exposure of C. glabrata with macrophages for months have selected for genetic alterations, which caused pseudohypha-like growth, higher macrophage damage and faster escape (Brunke et al. 2014).

During interaction with human MDMs, C. glabrata remains intracellular for 2–3 days without causing obvious damage to the macrophages or inducing apoptosis. Replicating yeasts are still surrounded by a membrane and are not collocated to the cytoplasm, as indicated by detection of LAMP-1 around the ingested yeast cells after 24 h. After 2–3 days, individual macrophages with high fungal loads were observed to lyse and release fungal cells into the medium (Seider et al. 2011). The time of macrophage burst is dependent on the initial yeast-to-macrophage ratio and the macrophage cell type. A higher infection dose for murine macrophages resulted for example in macrophage lysis already after 24 h (Dementhon, El-Kirat-Chatel and Noel 2012).
INTRACELLULAR SURVIVAL IN MACROPHAGES AS A MICROBIAL STRATEGY

Microbes that share intracellular survival in macrophages as a part of their life cycle are widespread among bacteria, fungi and protozoa (Smith and May 2013). The intracellular life style requires some general adaptation mechanisms for all microbes, independent from their phylogenetic relation. In fact, common patterns can be observed for most of these pathogens, suggesting convergent evolution (Casadevall 2008). The general strategies described so far for C. glabrata survival in macrophages, namely modification of the cell surface to avoid recognition by host receptors, detoxification of ROS, modification of phagosome maturation as well as metabolic adaptation to ensure nutrient acquisition in the phagosome are shared with other successful fungal and bacterial pathogens such as C. albicans, Histoplasma capsulatum, C. neoformans or Aspergillus fumigatus, but also Mycobacterium tuberculosis or Legionella spp. (Flannagan, Cosio and Grinstein 2009; Seider et al. 2010; Gilbert, Wheeler and May 2014).

Many of these survival strategies are not necessarily a specific adaptation to mammalian phagocytes. This is particularly true for environmental fungi. For example, intracellular survival mechanisms of soil-borne pathogens, such as H. capsulatum or C. neoformans in macrophages, have probably been developed due to frequent contact with soil amoebae, which use similar mechanisms to kill their microbial prey as compared to macrophages (environmental virulence school; Casadevall 2008; Bliska and Casadevall 2009). In the case of endogenous pathogens such as C. glabrata or C. albicans, the commensal life style is thought to shape the virulence potential (‘commensal virulence school’; Hube 2009). During commensal growth, C. glabrata cells probably form biofilms on mucosal surfaces and may therefore be exposed to nutrient limitation, osmotic and oxidative stress, have competition with other organisms and may occasionally encounter immune cells such as phagocytes (Jandric and Schuller 2011; Roetzer, Gabaldon and Schuller 2011). Many of the adaptations needed in the commensal environment are therefore likely beneficial for intracellular survival in macrophages.

Despite the similarity of approaches to survive inside macrophages, specific strategies of the single pathogens differ, as seen when comparing C. albicans and C. glabrata. While the intracellular phase of C. albicans within macrophages in vitro is short and phagocytosis is rapidly followed by hyphal formation, damage and escape, C. glabrata seems to be adapted to long-term containment within macrophage phagosomes. In addition, and in contrast to S. cerevisiae or C. albicans, C. glabrata has undergone frequent gene losses, resulting in various auxotrophies, such as the inability to synthesize pyridoxine, nicotinic acid and thiamine and the inability to catabolize galactose (Dujon et al. 2004; Kaur et al. 2005; Bolotin-Fukuhara and Fairhead 2014). Such loss of certain metabolic genes or even pathways (reductive evolution, genome reduction) has been discussed as an adaptation mechanism of commensals or pathogens to their specific host niches or even as a hallmark of intracellular pathogens (Casadevall 2008). In this context, gene loss may indicate that the corresponding gene products are dispensable in the host environment, e.g. due to uptake of metabolites from the host. On the other hand, the gene’s absence may be selected during evolution because it increases the fitness of the microbe in the host (concept of antivirulence genes) (Casadevall 2008; Bliven and Maurelli 2012).

A yet unsolved question is the route of C. glabrata invasion, leading to dissemination and systemic infection. When growing on epithelia, C. glabrata yeast cells exert low cytotoxicity, low invasion rates and a low cytokine response (Jayatilake et al. 2006; Li, Kashleva and Dongari-Bagtzoglou 2007; Li, Redding, Dongari-Bagtzoglou 2007; Westwater et al. 2007; Silva et al. 2011). Despite low damage and the absence of hyphal formation, C. glabrata is able to reach the bloodstream and to disseminate to different organs in a mouse model of intragastrointestinal infections (Atanasova et al. 2013) and also in a chorioallantoic membrane chicken embryo model of fungal infection (Jacobsen et al. 2011). In humans, the predominant origin of C. glabrata bloodstream infection seems to be the gut (Fidel, Vazquez and Sobel 1999). A possible passive route from the gut to the bloodstream can be generated by the destruction of natural barriers via catheters, injury, surgery or parenteral nutrition (Perillo, Choi and Spellberg 2007). Similarly, in a mouse model, immune suppression and malnutrition, leading to disturbance of the intestinal barrier, promote C. glabrata dissemination (Atanasova et al. 2013). In the absence of such barrier disruptions, C. glabrata may rely on epithelial endocytosis instead of active penetration to cross epithelia (Li and Dongari-Bagtzoglou 2007). Also, coinfection with other fungi or bacteria is likely to promote tissue invasion and destruction and possibly even access to the bloodstream by C. glabrata. In oral candidosis for example, coinfections with C. glabrata and C. albicans are commonly found (Coco et al. 2008). In vitro, such coinfection can lead to enhanced invasiveness and cytotoxicity of C. glabrata (Silva et al. 2011), although not observed in every setting (Li, Kashleva and Dongari-Bagtzoglou 2007).

During colonization of mucosal surfaces, frequent contact of C. glabrata to host immune cells is likely, for example with resident macrophages in the gastrointestinal mucosa (reviewed in Smith et al. 2011). It is thus also possible that C. glabrata uses phagocytosis by macrophages as a ‘trojan horse mechanism’ to overcome epithelial barriers and to disseminate, similar to the strategy postulated for C. neoformans (Charlier et al. 2009). In this scenario, the intracellular life style in macrophages may allow dissemination and escape from other immune cells in vivo.

CANDIDA GLABRATA INFECTION MODELS

Fungal genes important for in vivo fitness and virulence

Different mouse models of oral, vaginal, gastrointestinal, intraperitoneal and intravenous bloodstream infections have been developed for C. glabrata (extensively reviewed in Maccallum 2012). In addition, non-vertebrate models based on Drosophila melanogaster and Galleria mellonella have been established (Cotter, Doyle and Kavanagh 2000; Junqueira et al. 2011; Quintin et al. 2013). In most of these models, C. glabrata only shows moderate virulence. During systemic mouse infection, fungal dissemination to various organs of the body, including kidney, liver, brain, spleen, lung and heart was observed, underlining that C. glabrata is highly adaptable and able to colonize many niches in the body. Although C. glabrata was present at high levels in the mouse kidneys, there was little inflammation, low tissue damage, no clinical signs of disease observed and mice did not die. Immunosuppression led to increased fungal burden, but mouse survival was only decreased in some types of C. glabrata infections (Brieland et al. 2001; Arendrup, Horn and Frimodt-Moller 2002; Calcagno et al. 2003; Kamran et al. 2004; Jacobsen et al. 2010).

Different mouse models have been applied to study the virulence potential of C. glabrata gene deletion mutants. These
studies have associated certain fungal genes with virulence, predominantly during systemic infection. Importantly, as many of these studies analyzed fungal burden and not host mortality as read out (due to low mortality of mice after C. glabrata infection, see above), the respective genes should be termed ‘fitness factors’ rather than ‘virulence factors’ of C. glabrata. Genes-encoding proteins associated with cell wall integrity, such as the cell-wall-associated yapsins Yps1-11 (Kaur, Ma and Cormack 2007), and the MAPK Sh2 (Miyazaki et al. 2010a) were crucial for fungal fitness during systemic infection. Also, genes coding for mannosyltransferases with functions in protein glycosylation were associated with infection. Deletion of the β-mannosyltransferase-encoding genes BMT2–6 led to reduced colonization and inflammation in a mouse colitis model (Jawhara et al. 2012), while the deletion of α-mannosyltransferase-encoding genes ANP1 and MNN2 caused increased virulence as monitored by mouse mortality (West et al. 2013). The deletion of ACE2, encoding a transcription factor involved in chitin biosynthesis regulation, resulted in hypervirulence and host death in immunosuppressed but not in immunocompetent mice (Kamran et al. 2004; MacCallum et al. 2006).

In addition to cell wall integrity, stress signaling and regulatory processes are central aspects for in vivo fitness of C. glabrata in mice: deletion of genes coding for the calcineurin signaling components Cnb1 and Crz1 (Miyazaki et al. 2010b), the transcription factor Skn7 which regulates oxidative stress response (Saio et al. 2010b), the putative protein kinase Ire1 involved in ER stress response (Miyazaki et al. 2013), factors involved in chromatin structure and DNA damage repair (Rai et al. 2012) and the PI3K subunits Vps15 and Vps34 involved in cellular trafficking (Borah et al. 2014) all caused a reduction in fungal burden in mice. Furthermore, components of the HOG pathway have been found to be related to mouse mortality (Ste11 and Ste20; Calcagno et al. 2004, 2005), mouse fungal burden (Hog1; Srivastava, Suneetha and Kaur 2015) and host mortality in a D. melanogaster ‘minihost’ model (Sho1, Pbs2; Quintin et al. 2013). In the same Drosophila model, deletion of the above-mentioned yapsin-encoding genes YPS1–11 led to reduced virulence of C. glabrata (Quintin et al. 2013).

Other C. glabrata genes associated with infection include CYB2, coding for lactate dehydrogenase, which is required for lactate assimilation and colonization of the mouse intestine (Ueno et al. 2011). Further, deletion of genes with (putative) functions in high-affinity iron uptake, hemolysis and hem binding led to reduced fungal burden in the systemic mouse model (Srivastava, Suneetha and Kaur 2014). Deletion of STE12, involved in nitrogen limitation-induced filamentation, caused a reduction in mouse mortality (Calcagno et al. 2003). In addition, the transcription factor Pdr1, involved in resistance to azole antifungals, has been associated with infection in the absence of azoles: while deletion of Pdr1-regulated genes led to reduced mortality induced by azole-resistant C. glabrata strains, PDR1 overexpression caused enhanced mortality (Ferrari et al. 2009, 2011). Lastly, the RAM2-encoded prenyltransferase was critical for fungal survival during systemic mouse infection (Nakayama et al. 2011).

Until now, mostly single gene deletion C. glabrata mutants have been studied in complex in vivo infection models. Recently, we have investigated a larger collection of mutants (Schwarzmuller et al. 2014) using the non-vertebrate Drosophila model and a pooled mouse fitness model (Brunke et al. 2015). Interestingly, this study has shown that surveying Drosophila survival after infection with C. glabrata mutants is a suitable model to predict outcome of murine infections, especially following infection with severely attenuated mutants. This study further underlines the importance of cell wall integrity for fly infection as well as the requirement of HOG pathway components for both fly virulence and relative fitness in mice (Brunke et al. 2015). This and similar approaches, based on currently available mutant collections, including a targeted library (Schwarzmuller et al. 2014) and a random transposon-based library (Castano et al. 2003; Rai et al. 2012), can be the basis for future identification of fungal genes and pathways involved in virulence. In addition, the C. glabrata genome has been recently shown to harbor 49 yet unknown loci coding for proteins which could be new potential infection-relevant fungal factors (Linde et al. 2015).

### Immune evasion and persistence strategies in vivo

In line with the above-described low activation of macrophages upon C. glabrata phagocytosis in vitro, C. glabrata infection in mice does not lead to a significant proinflammatory cytokine response. TNF-α, IFN-γ and IL-12 levels were increased only within the first 24 h after infection (Brieland et al. 2001), and at later stages, the amounts of the proinflammatory cytokines IL-1α, IL-1β, IL-2, IL-6 and TNF-α in the kidneys, livers and brains of infected immunocompetent mice were either unaltered or only marginally increased over those in uninfected controls (Jacobsen et al. 2010). In agreement with this, chicken embryo infections with C. glabrata resulted in only a transient proinflammatory cytokine response and only a minor influx of immune cells (Jacobsen et al. 2011). These data together with the in vitro observations during macrophage interaction strongly suggest that C. glabrata uses an immune evasion strategy to survive in the host. This is in contrast to C. albicans which, once invasive, follows a (often hypoxia-mediated) destructive strategy, causing more host cell damage and inflammation (Brunke and Hube 2013).

For invasive C. albicans infections, neutrophils are well recognized as key players in murine and human defense, while the importance of macrophages in the immunity to candidiasis has been discussed controversially (van ’t Wout et al. 1988; Qian et al. 1994; Vazquez-Torres and Balish 1997; Brown 2011). Only recently, recruited inflammatory monocytes and resident kidney macrophages have been correlated to innate immune control of systemic candidiasis (Lionakis et al. 2013; Ngo et al. 2014). Interestingly, while a strong neutrophil infiltration is characteristic for C. albicans infections (Spelberg et al. 2005; MacCallum et al. 2009), C. glabrata does not seem to strongly attract neutrophils. Instead, C. glabrata is associated with mononuclear infiltrates in infected tissues (Brieland et al. 2001; Westwater et al. 2007; Jacobsen et al. 2010; Cheng et al. 2014). These data suggest that neutrophil recruitment does not play a major role during immune response to C. glabrata in mice. As neutrophil infiltration is believed to contribute to host tissue destruction, the absence of recruitment of these immune cells might be one crucial factor contributing to low virulence of C. glabrata in murine models (Jacobsen et al. 2010). The only cytokine found to be upregulated in early stages of C. glabrata mouse infections was GM-CSF (Jacobsen et al. 2010). As GM-CSF is an activator of macrophages and an inducer of macrophage recruitment, the induction of this cytokine may explain the enhanced infiltration of mononuclear cells, but not neutrophils in vivo (Jacobsen et al. 2010).

Of note, even immunocompetent mice do not fully clear C. glabrata after infection (Brieland et al. 2001; Jacobsen et al. 2010; Cheng et al. 2014). Viable fungal cells can be reisolated from infected organs for up to 28 days (Jacobsen et al. 2010). Similarly, C. glabrata persists for up to 14 days in infected fruit flies. In this model, the Drosophila Toll pathway is
important for restraining fungal growth, as flies deficient for Toll pathway activation succumb to the C. glabrata infection (Quintin et al. 2013).

Very little data are known which would elucidate the mechanisms enabling C. glabrata to persist in host tissues. Host interferon type I signaling seems to promote persistence in a mouse model of disseminated candidiasis (Bourgeois et al. 2011). The authors suggest that this response is triggered by containment of C. glabrata in host immune cells. Supporting phagocytosis of C. glabrata induces an IFN-β release by dendritic cells that is much stronger than in response to C. albicans (Bourgeois et al. 2011).

It remains to be elucidated whether intracellular containment of C. glabrata in macrophages indeed supports fungal persistence in vivo. During Drosophila infection, C. glabrata was found in hemocytes, phagocytic cells in the hemolymph (Quintin et al. 2013). However, phagocytosis was not required for fungal persistence, arguing against hemocytes as a niche for C. glabrata proliferation in this model. Persistence of C. glabrata, however, may indicate that the fungus is able to withstand the host immune response, or a dynamic equilibrium between fungal proliferation and killing by the host immune system may be reached (Quintin et al. 2013).

Persistence in macrophages after a primary infection can lead to reinfection and symptomatic disease upon decrease of host immunity, a problem commonly associated with airborne fungal pathogens such as Cryptococcus and Histoplasma species (Woods 2003; Ma and May 2009). Persistence of C. glabrata may similarly allow reinfection; however, this is not yet experimentally proven. In addition, persistence of C. glabrata in the host may open up possibilities for microevolutionary processes that allow the adaptation to specific host niches and may even increase the pathogenic potential of C. glabrata. As discussed above, microevolution in macrophages has recently been shown to lead to a hypervirulent C. glabrata strain with pseudohyphal-like growth due to a point mutation in the chitin synthase gene CHS2 (Brunke et al. 2014).

It has to be kept in mind that animal infection experiments are models and never ideal systems to study pathogenesis of human candidiasis. Candida glabrata causes lethal infections in humans but is only moderately virulent in most infection models (Perlroth, Choi and Spellberg 2007; Pfaller and Diekema 2007; Maccallum 2012). In addition, C. glabrata seems not to be a common natural commensal of mice (Wells et al. 2007; Iliev et al. 2012) and there are many differences between the mouse and the human immune system (Mestas and Hughes 2004). However, infection models such as the mouse model are valuable tools which will likely provide more insights in the general strategies that C. glabrata may use in vivo to benefit from immune evasion strategies.

CONCLUSIONS

Candida glabrata is well adapted to intracellular survival in macrophages. Similar to other successful human pathogens, C. glabrata is able to modify its phagosomal compartment, avoiding full maturation and acidification and thus not reaching the hostile phagolysosomal environment. Further, suppression of ROS production as well as efficient metabolic and stress adaptation to the phagosomal environment supports intracellular survival. Candida glabrata is able to persist in macrophages for days without causing substantial damage, until the host cells finally burst. Although efficiently phagocytosed, the fungus does not induce a strong proinflammatory cytokine response. These in vitro data suggest containment in macrophages as an immune evasion and persistence strategy of C. glabrata (Fig. 2). In vivo
infection of different model hosts showed similar characteristics: dissemination in the host coupled to persistence with low immune activation, inflammation and host tissue damage. Future work is needed to elucidate whether fungal containment in macrophages is part of the in vivo infection strategy of C. glabrata.

**FUNDING**

Our own work was supported by the Deutsche Forschungsgemeinschaft within the priority program ‘Intracellular compartments as places of pathogen–host interactions’ SPP1580 (Hu 528/16–2).

**Conflict of interest.** None declared.

**REFERENCES**


Sasada M, Johnston RB, Jr. Macrophage microbicidal activity. Correlation between phagocytosis-associated oxidative


