Chapter 1

Introduction
1.1 Candida and candidiasis: An introduction

1.1.1 Fungi

Fungi are large group of ubiquitous, eukaryotic and heterotrophic organisms which contribute to approximately 25% of the global biomass (Miller, 1992; Sorenson, 1999). Kingdom fungi consist of organisms displaying enormous diversity in ecological niches, size, morphology, reproduction and life styles (Blackwell, 2012). Reproduction in fungi occurs by two methods viz., asexual or clonal propagation and meiotic sexual reproduction (Taylor et al., 1999). Although, most of the fungi are multicellular and exhibit hyphal or mycelial growth, some species grow in unicellular yeast forms and reproduce by budding or binary fission (Taylor et al., 1999).

Fungi decompose complex organic matter to simple inorganic compounds and, thus, play essential role in maintaining ecosystem and recycling nutrients and minerals (Gadd, 2007). In addition, their enormous applications in industry for production of antibiotics and biopesticides, fermentation and food and recreational purposes make them invaluable resources (Blackwell, 2012). Although most of the fungal species are harmless to living organisms, a small number of them are infectious and cause diseases in plants, animals and humans (LeighAnne Olsen, 2011). Famous quote by an eminent mycologist Arturo Casadevall “Fungi are the only group of organisms that have been convincingly shown to cause extinction” emphasize potential impact of the fungal pathogen (LeighAnne Olsen, 2011). Despite the availability of advanced medical technologies, diagnosis and treatment of fungal infections remains a major challenge for clinicians.

1.1.2 Human fungal pathogens

Fungal species were historically considered as non-pathogenic commensals and not medically important organisms (Richardson, 1991). However, this assumption has changed during last three decades since fungal pathogens emerged as one of the leading cause of increased mortality and morbidity among hospitalized patients (Pfaller and Diekema, 2007; Richardson and Lass-Florl, 2008). Of existing 1.5 million species, approximately 300 fungal species are infectious to human (Hawksworth, 2001; Taylor et al., 1999). Species belonging to genus Candida, Aspergillus and Cryptococcus are the leading fungal pathogens across the world.
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(Fridkin and Jarvis, 1996; Pfaller and Diekema, 2007; Singh, 2001). Other endemic fungal pathogens, which are limited to a certain geographical region of the world, include species of *Histoplasma, Blastomyces, Paracoccidioides Coccidioides, Penicillium* and *Tinea* (Fridkin and Jarvis, 1996). Common fungal pathogens, associated infections and their sites of infection are listed in Table 1.1.

Table 1.1: Spectrum of the common fungal pathogens

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fungal pathogen</th>
<th>Infections</th>
<th>Sites of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Candida</em></td>
<td>Candidiasis</td>
<td>Oral cavity, Gastrointestinal (GI) tract, Liver, Spleen Vagina, Blood and Skin</td>
</tr>
<tr>
<td>2.</td>
<td><em>Aspergillus</em></td>
<td>Aspergillosis</td>
<td>Respiratory system, Lungs</td>
</tr>
<tr>
<td>3.</td>
<td><em>Cryptococcus</em></td>
<td>Meningitis</td>
<td>Brain, Spinal cord</td>
</tr>
<tr>
<td>4.</td>
<td><em>Histoplasma</em></td>
<td>Histoplasmosis</td>
<td>Brain, Spinal cord, Lungs</td>
</tr>
<tr>
<td>5.</td>
<td><em>Pneumocystis</em></td>
<td>Pneumonia</td>
<td>Lungs</td>
</tr>
<tr>
<td>6.</td>
<td><em>Coccidioides</em></td>
<td>Coccidiomycosis</td>
<td>Skin</td>
</tr>
</tbody>
</table>

1.1.3 *Candida* and candidiasis

*Candida* species are the predominant nosocomial fungal pathogens and account for 10-15% of total blood stream infections in hospitalised patients (Pfaller and Diekema, 2007; Richardson and Lass-Florl, 2008). Fungal infections caused by opportunistic fungal pathogens belonging to genus *Candida* are commonly known as candidiasis or candidosis (Li et al., 2007). *Candida albicans* is the most frequently isolated *Candida* species from blood stream infections (BSIs) and responsible for approximately 50-60% of candidiasis cases (Pfaller et al., 2010). Non *C. albicans* *Candida* (NCAC) species causing candidiasis include *C. glabrata, C. tropicalis, C. parapsilosis, C. dubliensis, C. kruzi, C. lusitaniae, C. guilliermondii, C. famata* and *C. rugosa* (Pfaller and Diekema, 2007). Sometimes, invasive candidiasis (IC) is caused by more than one species resulting in mixed infection (Richardson and Lass-Florl, 2008).
1.1.3.1 *Candida* species: general features

Genus *Candida* represents a group of around 150 asporogenous yeast species which are often classified in ‘Deuteromycetes’ due to their inability to form sexual spores (Barnes, 1979; Scully *et al*., 1994). Except *C. glabrata*, other *Candida* species display polymorphism *i.e.* ability to grow in more than one morphological form, *viz.*, unicellular yeast, multicellular pseudohyphae and hyphae (Fidel *et al*., 1999). Pseudohyphal and hyphal forms originate from yeast by budding and germ tube formation, respectively (Lodder, 1970). Pseudohyphae and hyphae are filamentous forms of growth, wherein former possess the constrictions at cell to cell junction while latter grow as smooth filaments (Lodder, 1970). *Candida* species reside as harmless commensals in human microbiota of gastrointestinal tract, oral mucosa and vagina (Shao *et al*., 2011). However, they are capable of causing superficial mucosal to life threatening systemic BSIs in immunocompromised patients (Eggimann *et al*., 2003b; Richardson and Lass-Florl, 2008).

1.1.3.2 Epidemiology of candidiasis

Advances in medical science and improved chemotherapy in last few years have led to the better treatment of fatal diseases which were previously difficult to treat or thought to be incurable (Fridkin and Jarvis, 1996; Richardson, 1991). However, progress in medical science has also expanded the population of severely ill and immunocompromised individuals including patients undergoing organ transplantation and suffering from cancer, neutropenia, acquired immunodeficiency syndrome (AIDS) and tuberculosis (Fridkin and Jarvis, 1996; Pfaller *et al*., 2011; Richardson, 2005). Immunocompromised patients are highly susceptible to nosocomial fungal infections which partially contributed to a global rise in the rate of *Candida* infections during last three decades (Fridkin and Jarvis, 1996; Pfaller *et al*., 2011; Richardson, 2005).

1.1.3.2.1 Types and sources of *Candida* infections

*Candida* infections can broadly be classified in two classes, *viz.*, haematogenous and non-haematogenous infections. Haematogenous infections are usually disseminated and associated with blood circulation while non-haematogenous infections are localized to tissues (Eggimann *et al*., 2003a). Haematogenous *Candida*
infections, also known as candidemia, are defined as the presence of Candida species in circulatory system (blood) leading to symptomatic systemic fungal infections (Eggimann et al., 2003a). Localized Candida infections (oral and vaginal candidiasis) in general are associated with tissue surfaces (Eggimann et al., 2003a). Organ involved in disseminated candidiasis may vary depending on the route of infection. For example, liver and splenic abscesses develop when Candida species inhabiting the gastrointestinal (GI) tract migrate to liver and spleen through a breakdown in mucosal or epithelial tissue barriers (Fridkin and Jarvis, 1996). Although candidemia is usually acquired from endogenous and prior-colonized sources, such as gastrointestinal tract, vagina, oral cavity and skin, sometimes infections can spread from exogenous sources like catheter and post-operative sites (Richardson and Lass-Florl, 2008). Manifestations and clinical features of Candida infections vary depending on the type, the site of infection and the species involved. Table 1.2 describes the prevalence of different Candida species and associated clinical manifestations.

### 1.1.3.2 Candida species: prevalence, risk factors and diseases

Candida species are ubiquitous organisms which usually constitute part of normal human microflora of oral cavity, gastrointestinal and urinary tracts (Eggimann et al., 2003a). Of more than 100 identified Candida species, only 17 are etiological agents in humans (Pfaller and Diekema, 2007). Importantly, 90-95% episodes of invasive candidiasis and candidemia are attributed to only five Candida species - C. albicans, C. glabrata, C. parapsilosis, C. tropicalis and C. krusei (Pfaller and Diekema, 2007; Richardson and Lass-Florl, 2008). The most common predisposing factors for Candida infections are immunosuppression, prolonged ICU stay and neutropenia (Richardson, 2005).

Recent time has witnessed a changing pattern in epidemiology of invasive candidiasis. Although C. albicans remains the most common cause of candidiasis, there has been a continuous decreasing trend in its infections over time (Pfaller and Diekema, 2007; Richardson and Lass-Florl, 2008). Incidences of C. albicans infections have fallen from 75 to 50% from 1980 to 2009, whereas infections due to NCAC species, C. glabrata, C. tropicalis and C. parapsilosis have increased during the same period (Pfaller et al., 2010; Richardson, 2005; Silva et al., 2012). This
dramatic shift in Candida epidemiology is believed to be due to excessive use of 
fluconazole antifungal for prophylaxis and treatment of invasive fungal infections
(Singh, 2001). It has been reported in many epidemiological studies that use of 
fluconazole in antifungal prophylaxis not only led to decrease in C. albicans 
infections but also raised the number of fluconazole-resistant NCAC isolates (Pfaller
et al., 2010; Pfaller and Diekema, 2007; Richardson and Lass-Florl, 2008; Singh,
2001). However, role of fluconazole prophylaxis in changing epidemiology of 
candidiasis remains controversial as many studies claim no correlation between 
fluconazole usage and incidences of NCAC infections (Lin et al., 2005; Marchetti et
al., 2004).

Change in epidemiology of candidiasis has also been observed in India
(Oberoi et al., 2012). A review of candidaemia between 1999 and 2008 in an Indian
hospital, revealed an increase in candidaemia due to NCAC species and a correlation
with fluconazole prophylaxis (Oberoi et al., 2012). At a level I trauma centre in New
Delhi, an incidence rate of 0.71 of candidaemia per 1000 patients was recorded (Singh
et al., 2011). More than 80% candidaemia was found to be due to NCAC species
(Singh et al., 2011). Notably, C. tropicalis (39%) and C. parapsilosis (22%) were
more commonly isolated species than C. albicans (14.7%) and C. glabrata (5.9%) in
this study (Singh et al., 2011).

C. glabrata is a fluconazole resistant Candida species and emerging as the
second most common cause of candidiasis in the United States of America (Pfaller
and Diekema, 2007). Occurrence of C. glabrata infections is more common in old age
and cancer patients (Pfaller and Diekema, 2007). Broad spectrum antibiotic treatment,
use of central venous catheters and prolonged stay in ICU are the most important risk
factors for C. glabrata infections (Malani et al., 2005). C. krusei is another
fluconazole-resistant species which displays a high mortality rate of 49% (Richardson
and Lass-Florl, 2008). Higher mortality rate of C. krusei is presumed to be due to its
lower susceptibility towards azole antifungal agents (Richardson and Lass-Florl,
2008). Neutropenic patients with hematologic malignancies are the high risk
population for C. krusei infections (Schuster et al., 2013). C. parapsilosis is most
prevalent Candida isolate in neonates and children (Richardson and Lass-Florl, 2008).
Infections owing to C. parapsilosis have a lower mortality rate compared to
candidiasis caused by other species (Richardson and Lass-Florl, 2008). *C. parapsilosis* is known to colonize on skin and hands of health care workers suggesting that it may be transmitted through contact (Richardson, 2005). *C. parapsilosis* can form biofilms on the surfaces of catheters and other implanted medical devices (Pfaller and Diekema, 2007). *C. tropicalis* is a common fungal pathogen among patients suffering from cancer, neutropenia and haematological malignancies (Pfaller and Diekema, 2007). Compared to *C. albicans*, *C. tropicalis* infections in patients with haematological malignancies exhibited higher mortality rate (Richardson and Lass-Florl, 2008).

### 1.1.3.2.3 Candidiasis: impact of underlying diseases and age

Patients undergoing stem cell or solid organ transplantation therapy are at high risk of acquiring *Candida* infections. A population-based multicenter study on patients undergoing hematopoietic stem cell transplantation revealed that 87.5% of *Candida* infections were caused by NCAC species (Gamaletsou et al., 2013). Strikingly, *C. parapsilosis* was responsible for more than 50% infections with a 45% crude mortality rate in this study (Gamaletsou et al., 2013). A ten year surveillance study performed in liver transplant patients revealed that 67.5% patients carrying fungal infections had developed *C. albicans* infections (Yang et al., 2012). Among patients receiving hemopoietic progenitor stem cell transplantation (HPCT) treatment, *C. albicans* and *C. glabrata* were the most prevalent *Candida* isolates recovered (Westbrook et al., 2013).

Protease inhibitor-based antiretroviral therapy predisposed HIV patients to *Candida* infections (Lin et al., 2013). *C. albicans*, *C. glabrata* and *C. tropicalis* were responsible for 73.5, 5.9 and 5.9% *Candida* infections, respectively, in this study (Lin et al., 2013). A study on oropharyngeal candidiasis revealed that 82% of HIV patients were positive for *Candida* colonization and 27% of them developed symptomatic oropharyngeal candidiasis (Patel et al., 2012).

Age is a critical predisposing factor for *Candida* infections. A multicenter study from 79 hospitals covering 1239 patients revealed that patients of 0-19, 69-79 and 80-99 year age groups displayed highest occurrence of *C. parapsilosis*, *C. albicans* and *C. glabrata* infections, respectively (Pfaller et al., 2010). Susceptibility
to *Candida* infections was found to be highest in neonates and children below one year age (Oeser *et al*., 2013). NCAC species were the predominant cause of candidiasis responsible for 52-56% infections in children (Steinbach *et al*., 2012).

**Table 1.2: Candida infection: Species distribution and associated clinical features**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Candida species</th>
<th>Prevalence (%)</th>
<th>Clinical features</th>
</tr>
</thead>
</table>
| 1.     | *C. albicans*   | 50-62.3        | Mucocutaneous infections: oropharyngeal, oesophagitis, vaginitis  
Deep seated infections: polynephritis, peritonitis  
Haematogenous infections: Candidaemia, meningitis, hepatosplenic |
| 2.     | *C. glabrata*   | 12-17.4        | Systemic candidiasis, candidaemia, urinary tract infections |
| 3.     | *C. parapsilosis* | 7.3-17.4     | Candidaemia, deep infection associated with implanted devices |
| 4.     | *C. tropicalis* | 7.5-9.8        | Candidaemia and systemic candidiasis in immunosuppressed patients |
| 5.     | *C. krusei*     | 1.8-2.7        | Candidaemia, endophthalmitis and diarrhoea in new borns |
| 6.     | Other species (including unidentified species) | 3.6-8.1 | Mucocutaneous or systemic infections |

(Compiled from Eggimann *et al*., 2003 (a, b); Pfaller and Diekema, 2007 and Pfaller *et al*., 2010)

1.1.3.2.4 Pathophysiology of invasive candidiasis

*Candida* species are natural commensal inhabitants of human microbiological flora. About 40-50% human beings are either temporary or permanent carriers of *Candida* species in GI tract (Cohen *et al*., 1969). *Candida* species switch from commensal to pathogenic state in immunocompromised individuals (Cohen *et al*., 1969). Adherence is the first step towards development of candidiasis followed by colonization which may arise either from exogenous or endogenous source (Figure 1.1). Subsequently, *Candida* species proliferate at the site of colonization and get translocated across the tissue surfaces after disrupting their integrity (Eggimann *et al*.,...
Exposure to the risk factors promotes further invasion and secondary haematogenous dissemination (Eggimann et al., 2003a). Carefully designed studies and molecular DNA typing of clinical isolates have indicated that endogenous sources are responsible for most of the severe invasive candidiasis episodes (Eggimann et al., 2003a). However, exogenous sources alone or in combination with endogenous sources in clinical settings can also lead to the development of invasive candidiasis (reviewed in Eggimann et al., 2003a). Figure 1.1 illustrates steps involved in the development of invasive candidiasis.

Figure 1.1. Pathophysiology of invasive candidiasis. Candida cells adhere to mucosal surfaces utilizing adhesin family of proteins. Next, they proliferate to colonize over mucosal lining and invade across tissues and blood vessels by disrupting the membrane integrity. Hematogenous dissemination helps Candida cells spread to different organs and distantly located tissues (Reviewed in Eggimann et al., 2003a).
1.1.3.3 Diagnosis

Symptoms of candidiasis are largely nonspecific which renders diagnosis difficult at early stage. Common manifestations of candidemia include fever, which remains unresponsive to antibiotic therapy, occasionally accompanied with macronodular skin lesions and decreased renal activity (Richardson, 2005). Identification of correct source and species responsible for Candida infections is crucial for suitable prophylactic treatment. Conventional microbiological diagnosis methods include culturing of isolates from blood and other sterile sites, microscopic observation and antifungal susceptibility testing (De Rosa et al., 2009).

In addition, chromogenic diagnostic methods, which detect species-specific enzyme activity of selected yeast species, upon addition of different chromogenic substrates, have also been developed to identify Candida isolates (Hospenthal et al., 2006). For this, CHROMagar medium is used which simultaneously detects C. albicans, C. krusei and C. tropicalis based on appearance and color of grown colonies (Hospenthal et al., 2006). CHROMagar test in conjunction with rapid trehalose test can be used to identify C. glabrata isolates owing to quick utilization of trehalose by C. glabrata (Lopez et al., 2001). Fluorescence in situ hybridization (FISH) methods, which utilize peptide nucleic acids (PNAs), have been developed for specific detection of C. albicans (Alexander et al., 2006). In recent times, molecular nucleic acid probes specific for target fungal r-RNA are being used for diagnosis and identification of Candida species (Alexander et al., 2006).

1.2 Virulence factors of Candida species

Virulence factors in primary bacterial pathogens are traditionally defined as proteins encoded by genes present on pathogenicity islands (Hacker et al., 1997). However, such widely-accepted definition of virulence factors in Candida species does not exist. Furman and Ahearn defined virulence factors as all traits required to establish disease while Odds used this term for molecules which interact directly with host factors (Furman and Ahearn, 1983; Odds, 1994). Ken Haynes has defined virulence traits in Candida species as a subset of genes, which are primarily expressed in vivo, promote survival, allow proliferation of the fungus and facilitate disease progression in the mammalian host (Haynes, 2001). As this definition covers wider
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aspects of Candida pathogenesis, we have accordingly categorized virulence factors as discussed below.

1.2.1 Adherence

Adherence is the first step towards establishing an infection after a pathogen encounters its host. To invade host tissues, Candida cells either adhere to host tissues or indwelling medical devices such as catheter (Eggimann et al., 2003a). Int1, an integrin-like protein, was first shown to facilitate the adhesion of C. albicans to epithelial and endothelial cells through binding to proteins containing tripeptide sequence arginine-glycine-aspartic acid (RGD) motif e.g. fibronectin (Gale et al., 1996). Deletion of INT1 resulted in diminished adherence to epithelial cells and attenuated virulence in murine model of systemic candidiasis (Gale et al., 1996; Gale et al., 1998). Consistent with this, another ALS (Agglutinin-like sequence) family of adhesin proteins has also been found to contribute to virulence in C. albicans (Hoyer et al., 2001).

In C. glabrata, a family of adhesins, encoded by 17-23 genes, regulates its attachment to epithelial cells and macrophages (Castano et al., 2005; Cormack et al., 1999; Kuhn and Vyas, 2012). Deletion of CgEPA1 led to 95% decrease in adherence of yeast cells to human epithelial cells (Cormack et al., 1999). However, CgEpa1Δ mutant did not show any defect in colonization and fungal burden in different body organs in mice suggesting functional redundancy among adhesins of Epa family (Cormack et al., 1999). In non pathogenic yeast S. cerevisiae, Epa1 was necessary and sufficient for adhesion to and phagocytosis by PBMC-derived macrophages (Kuhn and Vyas, 2012). Further, Epa1 expression in S. cerevisiae induced production of inflammatory cytokines IL-8 and TNF-α by human PBMC-derived macrophages. In contrast, although C. glabrata cells expressed Epa1 on cell surface, they were neither phagocytosed nor did they induce cytokines in human PBMC-derived macrophages (Kuhn and Vyas, 2012). Expression of EPA genes in C. glabrata is regulated by subtelomeric silencing (De Las Penas et al., 2003). CgEPA6, a member of Epa family, was found to be expressed in a UTI model of infection but not under in vitro conditions (Domergue et al., 2005). C. glabrata is nicotinic acid auxotroph and depends on host for its nicotinic acid requirements (Brunke and Hube, 2013; Kaur et al., 2005). In urinary tract infection, C. glabrata cells are deprived of nicotinic acid.
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(Domergue et al., 2005). Hence, NAD$^+$ synthesis and NAD$^+$-dependent Sir2 deacetylase activity goes down leading to derepression of Epa genes (Domergue et al., 2005). Thus, auxotrophy to nicotinic acid assists *C. glabrata* colonize in urinary tract (Domergue et al., 2005).

1.2.2 Hydrolytic activity

Hydrolytic enzymes represented by secreted aspartyl proteases (SAPs) and phospholipases are central to *Candida* pathogenesis. *C. albicans* possesses a family of 10 SAPs encoded by the genes *SAP1–10* (Naglik et al., 2004). Of ten Saps, Sap1-8 are secreted and Sap9-10 are GPI-linked proteins (Naglik et al., 2004). Saps are known to be implicated in virulence of *C. albicans* in several models of candidiasis (Naglik et al., 2003). Deletion of *SAP1*, *SAP2* and *SAP3* in *C. albicans* resulted in attenuated virulence in murine model of systemic candidiasis (Hube et al., 1997). Consistent with this, transcripts of genes encoding different SAPs were observed in oral, skin and vaginal candidiasis models (Calderone and Fonzi, 2001). Interestingly, *C. albicans* cells exposed to antifungal drugs at sub-MIC (minimum inhibitory concentration) and grown in biofilm mode secreted higher amount of secretory aspartyl proteinases (Mores et al., 2011). Notably, genes homologous to *C. albicans* SAPs are also present in *C. tropicalis, C. parapsilosis* and *C. guillermondii* (Parra-Ortega et al., 2009). Secreted aspartyl proteinases are presumed to degrade host proteins, such as haemoglobin and secreted immunoglobulins, at lesion sites, thus, helping in tissue invasion and colonization (Hube et al., 1998; Naglik et al., 2003).

Phospholipase B1 (Plb1) is a secretory glycoprotein which possesses both hydrolase and lysophospholipase-transacylase activity in *C. albicans* (Ghannoum, 2000). Phospholipase acitivity has been detected at hyphal tips and is postulated to help in tissue invasion (Ghannoum, 2000). Consistent with this, Plb1 has been implicated in virulence of *C. albicans* in animal model of systemic candidiasis (Ghannoum, 2000).

Similar to *C. albicans*, proteinase and phospholipase activity has also been implicated in virulence of *C. glabrata* (Ghannoum, 2000; Kaur et al., 2007). In *C. glabrata*, a family of eleven aspartyl proteases was found to be upregulated upon phagocytosis by murine macrophages (Kaur et al., 2007). Consistently, a protease
mutant lacking all eleven aspartyl proteases was unable to survive in murine macrophages and displayed diminished fungal burden in murine model of systemic candidiasis (Kaur et al., 2007). Extracellular phospholipase activity has been detected in several C. glabrata clinical isolates suggesting a role for phospholipases in virulence of C. glabrata (Ghannoum, 2000).

1.2.3 Morphological switching

*Candida* species are mostly polymorphic in nature *i.e.* they possess the ability to exist in three different forms, yeast, pseudohyphae and hyphae (Lo et al., 1997). Based on the environmental cues, yeast form switches to pseudohyphal and hyphal forms through an integrated transcriptional rewiring process (Lo et al., 1997). Although morphogenesis is considered as a key virulence trait since *C. albicans* mutants arrested in any one morphological form display attenuated virulence, recent studies suggest that morphogenetic switching and virulence are not always strictly coupled with each other in *C. albicans* (Lo et al., 1997; Noble et al., 2010).

1.2.4 Phenotypic switching and mating

*C. albicans* colonies can switch reversibly among different phenotypes including rough, smooth, star, stippled, wrinkle, white and opaque forms (Slutsky et al., 1985). Notably, white-opaque colony switching is more frequent among different colony morphology switch systems (Miller and Johnson, 2002). Opaque-phase cells display higher colonization than white-phase cells in cutaneous model of candidiasis (Kvaal et al., 1999). However, opaque-phase cells were attenuated in systemic model of candidiasis compared to white-phase cells (Miller and Johnson, 2002). Fresh clinical isolates associated with vaginal candidiasis and invasive infections display higher frequency of phenotypic switching (Jones et al., 1994; Soll et al., 1988). Phenotypic switching is directly connected with mating and tetraploid generation. *C. albicans* possesses two alleles of mating type locus *viz.*, *MTLa* and *MTLa* and undergo mating (Hull and Johnson, 1999). *C. albicans* strains, irrespective of homozygous or heterozygous state of MTL alleles, displayed similar level of virulence (Ibrahim et al., 2005). However, tetraploids displayed attenuated virulence in murine model of systemic candidiasis (Ibrahim et al., 2005).
Notably, *C. glabrata* also undergoes reversible phenotypic switching among dark brown (DB), light brown (LB), white (Wh) and irregular wrinkle (IWr) colony types. Dark brown phenotype displayed higher colonization in two major target organs, spleen and liver, in the mouse model and is the most predominant form in clinical *C. glabrata* isolates (Srikantha *et al.*, 2008).

### 1.2.5 Pigmentation

In fungi, pigment production is associated with presence of alternative carbon and nitrogen sources, cyclic AMP and oxygen availability (van Burik and Magee, 2001). Wild-type *C. glabrata* cells, when grown on pigment inducing medium, displayed elevated resistance to hydrogen peroxide (Brunke *et al.*, 2010). In addition, pigmented *C. glabrata* cells survived better upon coincubation with human neutrophils and damaged human epithelium monolayer. Lack of these effects in a mutant defective in pigment formation indicates a possible role of pigmentation in interactions with host cells (Brunke *et al.*, 2010).

### 1.2.6 Signal transduction cascades

Signaling cascades, which contribute to survival and proliferation of *Candida* cells in host environment, are considered as virulence determinants. In *C. albicans*, deletion of the gene encoding transcriptional factor, Tup1, caused morphogenetic aberrations and arrested *tup1* mutant in pseudohyphal form (Braun and Johnson, 1997). Accordingly, *tup1* deletion mutant displayed attenuated virulence in murine model of systemic candidiasis (Braun and Johnson, 1997). Another signaling pathway, conserved cAMP signaling cascade, is also known to regulate virulence of *C. albicans* and other fungal pathogens (reviewed in D’Souza and Hietman, 2001). In *C. albicans*, Tpk1, Tpk2 kinases and major transcription factor Efg1 are important constituent of Ras-protein kinase-A pathway and have been implicated in yeast-to-hyphal transition (Park *et al.*, 2005). Homozygous deletion of *TPK1*, *TPK2* and *EFG1* caused diminished virulence in murine models of oropharyngeal candidiasis (OPC) and haematogenously disseminated candidiasis (HDC) (Park *et al.*, 2005).

Similarly deletion of *STE12* and *STE20* in *C. glabrata*, whose counterparts in *S. cerevisiae* regulate mating, filamentation, cell wall biosynthesis and invasive growth (Dolan *et al.*, 1989; Leberer *et al.*, 1992), led to attenuated virulence in murine
model of systemic candidiasis compared to wild-type (Calcagno et al., 2004; Calcagno et al., 2003). Ire1, a transmembrane kinase and endoribonuclease, splice HAC1 mRNA to produce transcription factor Hac1, upon accumulation of misfolded proteins. Hac1 activates target genes which increase protein folding capacity of endoplasmic reticulum. Surprisingly, C. glabrata Ire1 did not display mRNA splicing activity (Miyazaki et al., 2013). Deletion of Ire1 in C. glabrata resulted in attenuated virulence in murine model of disseminated candidiasis (Miyazaki et al., 2013).

1.3 Immunity against candidiasis

*Candida* species reside as harmless commensals in healthy humans but can switch to pathogenic state in immunocompromised individuals (Brunke and Hube, 2013). Host immunity is a major determinant of the commensal to pathogenic transition and manifestation of *Candida* infections (Romani, 2004). Our understanding of anti-*Candida* host defense is largely limited to *C. albicans* and immune responses against other species are poorly investigated. Host defense against candidiasis ranges from protective, non-specific innate immune response to specifically induced adaptive immunity (Romani, 2004).

1.3.1 Role of innate immunity in resistance to candidiasis

Innate immunity is traditionally considered as the first line of host defense which is promptly activated in response to pathogen invasion (Kuby, 2000). Components of innate immune system include anatomical and physiological barriers, antimicrobial peptides (AMPs) and professional phagocytes *viz.*, macrophages, neutrophils, dendritic and natural killer (NK) cells (Kuby, 2000). Anatomical barriers such as skin and mucous membranes including mucosal lining of gastrointestinal, respiratory and urogenital tracts act as physical barricade while physiological barriers, saliva and mucous secretions, wash away the pathogens thereby preventing invasion (Kuby, 2000). Antimicrobial peptides are short and conserved peptides of 6-59 amino acids and produced by a variety of cells including neutrophils and paneth cells (Kuby, 2000). Antimicrobial peptides can kill microbes within minutes by disrupting microbial membranes and/or inhibiting synthesis of microbial DNA, RNA and proteins (Kuby, 2000).
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Despite being nonspecific, innate immune system is able to discriminate precisely between self (host) and non-self (pathogen) and activates adaptive immune mechanisms accordingly (Kuby, 2000). Innate effectors utilize molecular sensors, known as pattern recognition receptors (PRRs), to discriminate between pathogen and host (Figure 1.2). PRRs on cells of innate immune system recognize conserved structural motifs known as pathogen-associated molecular patterns (PAMPs) which are present in microbial species but absent in host (Kuby, 2000). In healthy humans, innate effectors ensure that proliferation of commensals including Candida species remains below the infection threshold (Netea et al., 2008a; Romani, 2004). Consistent with this, any defect in innate immunity such as neutropenia fails to control commensal growth leading eventually to an infection (Romani, 2004). Hence, cellular effectors of innate immunity play pivotal roles ranging from pathogen recognition, phagocytosis, antigen presentation to activation of acquired immune responses to thwart Candida infection as discussed below (Netea et al., 2008a; Romani, 2004).

1.3.1.1 Recognition of Candida species

Recognition of Candida species is mediated by the arsenal of PRRs present on surfaces of innate immune cells which bind to PAMPs expressed on Candida cell wall (Netea et al., 2008a). Cell wall in Candida cells is composed of four macromolecules viz., β 1,3-glucan, β 1,6-gluca, chitin and mannoproteins (Klis et al., 2001). β 1,3-glucan, β 1,6-gluca and chitin together constitute 60% of cell wall dry weight and form an inner core of the carbohydrates (Klis et al., 2001). Mannoproteins account for 40% of cell wall dry weight and make an outer covering over inner carbohydrate mesh (Klis et al., 2001). Carbohydrate chains in the inner core are crosslinked to mannoproteins to stabilize them on cell wall (Klis et al., 2001). Monocytes, macrophages and neutrophils are the key cell types involved in the detection of Candida species in infected tissues (Netea et al., 2008a) (Figure 1.2). These cells differ from each other in their expression of PRRs and, thus, initiate varying responses as discussed below (Netea et al., 2008a; van de Veerdonk et al., 2008a).

1.3.1.1.1 Toll like receptors

Toll like receptors (TLRs) are a family of conserved transmembrane receptor proteins which detect a broad range of microbial products (Figure 1.2) (Akira and
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Sato, 2003). Cell wall components of Candida species act as PAMPs and are recognized by specific TLRs (Netea et al., 2008a). Ligand binding to TLRs triggers signaling cascades leading to induction of gene expression and production of inflammatory cytokines (Akira, 2006). Toll like receptors, along with lectin receptors, recognize C. albicans through glucan and mannan PAMPs (Jouault et al., 2003; Netea et al., 2006). Netea et al. examined the roles of TLR2 and TLR4 in host defense against disseminated candidiasis. They demonstrated that TLR4-defective C3H/HeJ mice were more susceptible to C. albicans infection and displayed impaired expression of genes encoding chemokines and neutrophil recruitment (Netea et al., 2002). Additionally, they observed that antibody-blocking of TLR2 receptors resulted in decreased production of TNF-α and IL-1β in monocytes stimulated by C. albicans (Netea et al., 2002). Consistently, yeast and hyphal forms of C. albicans have been reported to induce less production of TNF-α and macrophage inhibitory protein-2 (MIP-2) in TLR2−/− mice (Villamon et al., 2004).

Roles of TLR1 and TLR6, which form heterodimers with TLR2, have also been examined in mice (Netea et al., 2008b). TLR1−/− mice did not show any altered susceptibility to disseminated candidiasis indicating that it may not be essential for recognition of Candida species (Netea et al., 2008b). However, TLR6−/− mice displayed decreased and increased release of IL-10 and IFN-γ, respectively, leading to perturbed Th1/Th2 cytokine balance (Netea et al., 2008b). Intriguingly, perturbed Th1/Th2 cytokine balance in TLR6−/− mice did not affect its susceptibility to C. albicans infection (Netea et al., 2008b). TLR9, a cytosolic receptor, which recognizes unmethylated CpG sequences present in microbial DNA, has also been implicated in recognition of Candida species as inhibition of TLR9, either in macrophages derived from TLR9−/− mice or blocking the TLR9 receptor in monocytes, resulted in reduced production of IL-10 (van de Veerdonk et al., 2008b). Importantly, TLRs are also involved in recognition of other fungal pathogens including Aspergillus and Cryptococcus (Reviewed in van de Veerdonk et al., 2008).

1.3.1.1.2 C-type lectin receptors

C-type lectin receptors (CLRs) are a large family of receptors containing carbohydrate recognition domains (CRDs) which are known to be involved in recognition of Candida species (Netea et al., 2008a). Important CLRs which detect
Candida species are dectin-1, dectin-2, macrophage mannose receptor (MR), complement receptor 3 (CR3), galectin-3 and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) (Netea et al., 2008a). Expression and distribution of these receptors vary in different cell types. For example, DC-SIGN is specifically expressed on cell membranes of dendritic cells (Figure 1.2) (Netea et al., 2008a).

β-glucan constitutes approximately 58% of the C. albicans cell wall and has been detected in blood circulation during systemic fungal infections (Klis et al., 2001; Obayashi et al., 1995). β-glucan is primarily recognized by CR3 and dectin-1 receptors (Netea et al., 2008a). Although dectin-1 is able to recognize yeast form of C. albicans, hyphal state remains undetected probably owing to masked β-glucan (Netea et al., 2008a). Wheeler and Fink showed that subinhibitory doses of antifungal drug caspofungin unmasked underlying β-glucan and elicited stronger immune response (Wheeler and Fink, 2006). Dectin-1 recognition of C. albicans stimulated production of Syk-dependent Th2 type anti-inflammatory cytokine IL-10 and TLR Myd88-dependent Th1 type proinflammatory cytokine TNF-α (Gow et al., 2007). Another study revealed that dectin-1 binding to yeast triggered production of IL-2 and IL-10 in dendritic cells through Syk kinase and adapter CARD9 signaling (LeibundGut-Landmann et al., 2007). Importantly, dectin-1−/− mice were found to be more susceptible to C. albicans infection (Taylor et al., 2007). Dectin-2 binds to mannose residues present in mannoproteins and phospholipomannan and interacts with FcγR to detect C. albicans (McGreal et al., 2006; Sato et al., 2006). Notably, dectin-2 has also been reported to recognize hyphal forms of other fungal pathogens (Neth et al., 2000). Another CLR, galectin-3, expressed on macrophages, binds specifically to β-1,2 mannosides present in C. albicans but not in S. cerevisiae and, thus, can discriminate between these two yeast species (Jouault et al., 2006).

1.3.1.1.3 Other receptors

Mannose receptors (MRs) detect oligosachharides through CRDs and are implicated in recognition of C. albicans and several other fungi (Netea et al., 2008a). N-linked mannans in C. albicans cell wall are recognized by mannose receptors present on monocytes and macrophages (Netea et al., 2008a). DC-SIGNs are
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primarily expressed on dendritic cells and involved in antigen uptake and presentation of C. albicans (Cambi et al., 2003).

Figure 1.2. Differential expression of pattern recognition receptors on surface of innate immune cells which are involved in recognition of Candida cells (Adapted from Netea et al., Nature Reviews Microbiology 6, 67-78; January 2008).

Though individual PRRs trigger specific downstream immune responses, final host response is governed by the complex interplay of all activated signaling cascades induced by different PRRs.

1.3.1.2 Role of innate immune cells in immunity against candidiasis

Monocytes, macrophages, neutrophils and dendritic cells together constitute cellular effectors of innate immune system (Kuby, 2000). These phagocytes are primarily involved in pathogen recognition, antigen presentation and activation of acquired immune responses as discussed below.

1.3.1.2.1 Macrophages

Macrophages recognize Candida species through an array of PRRs after initial infection. Although macrophages possess antigen presentation machinery, their main functions are phagocytosis and killing of the pathogen (Mansour and Levitz, 2002).
Macrophages are activated following *C. albicans* phagocytosis and produce different cytokines and chemokines (Mansour and Levitz, 2002). Activation of macrophages is considered to be an essential step for resistance to candidiasis (Bistoni et al., 1986). Macrophages from various origins and sources have been shown to phagocytose and kill *C. albicans* cells (Mansour and Levitz, 2002). Thompson and Wilton showed that human polymorphonuclear leukocytes, monocytes and monocyte-derived macrophages were able to kill *C. albicans* yeast cells under both aerobic and anaerobic conditions (Thompson and Wilton, 1992). Interestingly, candidacidal activity of macrophages was higher under aerobic conditions suggesting that macrophage-mediated killing of *C. albicans* cells involved a respiratory burst (Thompson and Wilton, 1992). Similar to macrophages, candidacidal activity of other phagocytes has also been shown to be both oxygen-dependent and –independent (Vazquez-Torres and Balish, 1997). However, role of phagocytic cells in resistance to candidiasis remains controversial as Danley and Polakoff failed to record any phagocytic cell-mediated killing of *C. albicans* cells (Danley and Polakoff, 1986). Similarly, depletion of alveolar macrophages has been shown to result in decreased mortality in a murine model of pulmonary candidiasis despite higher fungal burden (Kubota et al., 2001).

### 1.3.1.2.2 Dendritic cells

Dendritic cells are specialised antigen presenting cells (APCs) and act as link between innate and adaptive antifungal immunity (Kuby, 2000). Dendritic cells are important constituents of host defense against candidiasis since they are crucial for antigen processing and presentation to T cells, which further lead to activation of specific immunity (d'Ostiani et al., 2000). Dendritic cells recognize *C. albicans* through MR and DC-SIGN and can discriminate between the yeast and the hyphal forms (d'Ostiani et al., 2000). Importantly, exposure of yeast and hyphal forms of *C. albicans* led to differential immune response in dendritic cells (Romani, 2000). For example, dendritic cells induced Th1 and Th2 cytokine production upon exposure to yeast and hyphal forms, respectively (Romani, 2000). Additionally, dendritic cells have also been reported to exhibit candidacidal activity which is comparable to that of macrophages (Mansour and Levitz, 2002)
1.3.1.2.3 Neutrophils

Neutrophils are the most abundant phagocytes and generally the first cell type to be recruited to infection sites (Mansour and Levitz, 2002). They can phagocytose opsonised and unopsonized fungi and kill them by oxidative and non-oxidative mechanisms (Mansour and Levitz, 2002). The fact that neutropenia is associated with enhanced susceptibility to systemic candidiasis indicates a pivotal role for neutrophils in resistance against candidiasis (Fridkin and Jarvis, 1996; Richardson, 2005). Neutrophils trigger production of IL-10 and IL-12 cytokines depending upon the virulence of Candida strains (Romani, 1997). In response to less virulent C. albicans strain, neutrophils released IL-12 and initiate Th1 response while exposure to a virulent C. albicans strain led to the production of IL-10 (Romani, 1997). Because of large number of neutrophils in blood and at infection site, cytokine production by neutrophils may influence differentiation of helper T cells (Romani, 2000).

1.3.1.2.4 Natural killer (NK) cells

Natural killer cells are large granulocytes that recognize C. albicans through B2-integrin CD11b/CD18 and can bind to Candida germ tube (Forsyth et al., 1998). Further, IL-2 activated NK cells exhibited increased binding to Candida germ tube (Arancia et al., 1998). However, NK cells either as such or upon IL-2 activation, were unable to kill and inhibit germ tube/hyphal growth (Arancia et al., 1998). Greenfield et al. found no enhanced susceptibility of NK cells-depleted, severe combined immunodeficiency (SCID) mice to candidiasis and ruled out a direct role of NK cells in host defense against candidiasis (Greenfield et al., 1993). Intriguingly, killed yeast and hyphal forms of C. albicans inhibited IFN-γ secretion by murine NK cells (Murciano et al., 2006). Since NK cells are known to produce IFN-γ for activation of phagocytes and maturation of dendritic cells (Kuby, 2000; Murciano et al., 2006), it is likely that they contribute indirectly to resistance against candidiasis.

1.3.1.3 Role of soluble factors

Innate immune system produces a variety of soluble factors either at the site of infection, where they act locally (antimicrobial peptides), or at distant sites. Soluble factors produced at distantly located sites reach to target sites via blood circulation.
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(complement proteins) (Kuby, 2000). These soluble factors include antimicrobial peptides and complement proteins as detailed below.

1.3.1.3.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are known to neutralize broad range of pathogens including viruses, bacteria, protozoans and fungi (Peters et al., 2010; Shai, 2002). Candida species, despite being present in oral cavity, are unable to cause oral candidiasis in healthy humans (Helmerhorst et al., 2001; Scully et al., 1994). Saliva regulates growth of Candida cells through small antimicrobial peptide histatin-5 produced by ductal cells of salivary glands (Helmerhorst et al., 2001; Nikawa et al., 2001). Consistent with this, Mandel et al. observed diminished level of histatins in HIV patients suffering from oral candidiasis compared to healthy individuals (Mandel et al., 1992). Recently, it has been reported that salivary histatin-5 also provides protection to oral mucosal tissues against C. albicans infection (Peters et al., 2010). Mechanistically, histatins are presumed to compete with superoxide dismutases (SODs) for copper inside yeast cells, thereby, inhibiting yeast cell growth (Lupetti et al., 2002). Intriguingly, histatin-5 has also been reported to disrupt C. albicans surface and reach into the cytoplasm (Mochon and Liu, 2008). Notably, histatins display fungicidal activity for other fungal pathogens as well (Helmerhorst et al., 2001). Another antimicrobial peptide, LL-37, a member of cathelicidin family, inhibited the infectivity of C. albicans cells by affecting their adherence to oral and urinary bladder epithelial cells (Tsai et al., 2011). Owing to their inhibitory effects on microbial growth, antimicrobial peptides are currently being projected as potential antifungal therapeutic agents (Lupetti et al., 2002; Peters et al., 2010).

1.3.1.3.2 Complement system

Complement system consists of soluble proteins which, upon activation, aggregate surrounding the target pathogen and induce opsonization, inflammation and attract neutrophils and other phagocytes (Kuby, 2000). Activation of complement system can be achieved via one of the three known pathways viz., classical, alternative and lectin pathways (Kuby, 2000). C. albicans utilizes all three known pathways to activate complement system (Kozel, 1996; Zhang et al., 1998). Mannan-specific immunoglobulin activates classical pathway while C3b molecules directly bind to C. albicans to activate alternative pathway (Kozel et al., 1996; Zhang et al., 1998).
Different sugar moieties present in PAMPs on *C. albicans* cell surface bind directly to mannose-binding lectins resulting in the activation of lectin pathway (Neth *et al*., 2000). These activated-complement pathways help in opsonization and phagocyte-mediated killing of *Candida* cells (Kozel, 1996).

1.3.2. Role of acquired immunity in resistance to candidiasis

Innate immunity mediates recognition of *Candida* species, antigen presentation, and activates acquired immune responses (Kuby, 2000). Acquired immunity comprises of highly specialized cells and processes which generate immunological memory after an initial encounter with a pathogen and provide protection against secondary infections by that particular pathogen (Kuby, 2000). Here, we discuss the roles of humoral and cell-mediated immunity in resistance to candidiasis.

1.3.2.1. Humoral immunity

Roles of antibody-mediated protection against *Candida* infections are not well-understood and are still being investigated. Although, mice immunized with yeast displayed resistance towards candidiasis (Mourad and Friedman, 1968), similar level of resistance of B-cell defective CBA/N or SCID mice and immunocompetent mice towards candidiasis precluded any significant role of antibodies in host defense (Carrow *et al*., 1984; Jensen *et al*., 1993). However, several studies have demonstrated that both, vaccination and passive immunization with antimannan antibodies, provide protection to mice from disseminated candidiasis (Han and Cutler, 1995; Han *et al*., 2000; Zhang *et al*., 2006). Further, antibodies generated against secreted aspartyl protease-2 and β-glucan have been successfully used to provide protection from systemic candidiasis (Montagnoli *et al*., 2003). Similarly, another study demonstrated that a recombinant antibody against HSP-90 can be administered in combinatorial therapy for disseminated candidiasis (Matthews and Burnie, 2005).

1.3.2.2 Cell-mediated immunity

Cell-mediated immunity is a major determinant of host defense against disseminated candidiasis (Romani, 2000). Cytokines secreted by phagocytes induce differentiation of T cells into Th1 and Th2 cells (Romani, 1997). The ability of host
defense to prevent *Candida* infections relies mainly on the development of Th1 and Th2 responses (Puccetti *et al.*, 1995; Romani, 2000). Th1 cells produce cytokines, which induce the candidacidal activity of phagocytes and, thus, development of Th1 response correlate with acquired resistance to infections (Romani, 1997, 2000). Accordingly, Th1-deficient mice strain exhibited low fungal burden and did not develop disease (Conti *et al.*, 2009). In contrast, Th2 cytokines inhibit both development of Th1 response as well as phagocytic activity and are associated with susceptibility to disseminated candidiasis (Romani, 1997, 2000). Although protective Th1 and unprotective Th2 responses act antagonistically to each other, their integration is required to efficiently control *Candida* infections (Romani, 1997, 2000).

Protective anti-*Candida* Th1 responses include production of several cytokines including IFN-γ, TGF-β, TNF-α, IL-6 and IL-12 (Mencacci *et al.*, 1998; Romani *et al.*, 1992; Romani *et al.*, 1994; Spaccapelo *et al.*, 1995). Neutralization of Th1 cytokines IFN-γ and IL-12, early after infection, lead to the activation of Th2 response (Romani, 2000). Similarly, neutralization of Th2 cytokines IL-4 and IL-10 induced development of Th1 response (Romani, 2000). However, external administration of IL-4 did not nullify already developed Th1 response in susceptible mice (Tonnetti *et al.*, 1995). In addition to helper T cells, CD4+ T-regulatory (TReg) cells are also known to play a crucial role in anticandida immunity (Sakaguchi, 2000). TReg cells produce IL-10, IL-4 and TGF-β cytokines which are known to trigger induction of Th2 response (Sakaguchi, 2000). Unexpectedly, depletion of TReg cells in mice led to improved resistance to disseminated candidiasis (Netea *et al.*, 2004b). These observations suggest that a complex interplay between immunoregulatory Th1 and Th2 cytokine response underlies protection against disseminated candidiasis.

Secretion of IL-1β, IL-6 and IL-23 by antigen presenting cells lead to differentiation of CD4+ T cells into Th17 lineage cells (Romani, 2011; Smeekens *et al.*, 2011). Th17 cells produce closely related proinflammatory cytokines, IL-17A, IL-17F and IL-22, which signal through a common receptor IL-17R (Hernandez-Santos and Gaffen, 2012) and induce neutrophil-mediated protective immune response against *C. albicans* infection (Matsuzaki and Umemura, 2007). Depletion of Th17 cells and IL17R led to the development of severe oropharyngeal candidiasis in mice (Conti *et al.*, 2009).
Dectin-1 recognizes β(1-3)-glucan and induces development of Th17 response through PKCδ activation and CARD9 phosphorylation (LeibundGut-Landmann et al., 2007; Strasser et al., 2012). Consistent with this, dectin-1−/− mice displayed impaired IL-6 and granulocyte-colony stimulating factor (G-CSF) production which are required for IL-17-mediated signaling and induction of Th17 response (Gaffen, 2009; Taylor et al., 2007). However, the role of dectin-1 in anti-Candida immunity is not clear. While Taylor et al. showed that dectin-1−/− mice were more susceptible to disseminated candidiasis, Saijo et al. observed increased resistance to candidiasis in dectin-1−/− mice (Saijo et al., 2007; Taylor et al., 2007). It is plausible that use of different mice strains led to these inconsistent findings. Type1-interferons in human peripheral blood mononuclear cells (PBMCs) have recently been shown to play a crucial role in resistance to systemic candidiasis (Smeekens et al., 2013a). Consistently, polymorphisms in type1-interferon genes have been implicated in modulation of Candida-induced cytokine production and susceptibility to disseminated candidiasis (Smeekens et al., 2013b).

Overall, innate and adaptive immune systems collaborate with each other to provide protection against Candida infection. Innate immune cells identify, phagocytose and process Candida cells. Antigen presentation by dendritic cells activates panel of effectors viz., T helper cells, T_{Reg} and B cells, to initiate appropriate adaptive immune response as well as induce memory which protect from subsequent Candida infections. Figure 1.3 summarizes the key events leading to the induction of host immune response against Candida infection.

1.4 Treatment of fungal infections

1.4.1 Antifungal drugs

Incidences of systemic mycosis have increased during last few decades, partly, owing to improved diagnosis and rise in immunocompromised population. Due to this increase, higher antifungal prophylaxis is being used in clinical settings (Ghannoum and Rice, 1999). Further, exposure of fungal pathogens to a broad range of antifungal agents has led to a rise in drug resistant pathogenic isolates (Ghannoum and Rice, 1999). To combat this issue, search for novel, safer and more efficient antifungal agents has been intensified (Ghannoum and Rice, 1999). Currently available
antifungal drugs target ergosterol and its biosynthesis, fungal cell wall and nucleic acids as discussed below.

1.4.1.1 Antifungal drugs targeting ergosterol and its biosynthesis

This class of drugs interacts with enzymes involved in the biosynthesis of ergosterol from squalene. Ergosterol is usually found in two forms in fungal cells, a predominant “bulk” sterol present in fungal cell wall and a trace “sparking” sterol necessary for cells to complete cell cycle (Hitchcock, 1993). Ergosterol biosynthesis inhibitors arrest synthesis of both forms. These antifungals include allyl amines and thiocarbamates, azoles, morpholines and polyenes (Ghannoum and Rice, 1999).

![Image](image.png)

(Source: L. Romani, 2004)

**Figure 1.3** A co-operative effort of innate and adaptive immune system provides protection against *Candida* infections. Phagocytes recognize *Candida* cells within hours of infection by recruiting PRRs. Following pathogenic recognition, an early
induced inflammatory response gets activated and provides transient protection to the host. Macrophages process the antigens and present them to dendritic cells for activation of helper-T cells. Different Th-cell subsets release panels of cytokines which activate effector phagocytes leading to generation of appropriate immune response. Adaptive immune response generates antigen-specific T-helper, regulatory T (T\textsubscript{Reg}) and B-cells. These cells target the pathogen, induce memory cells and prevent subsequent infections. (Source: L. Romani, *Nature Reviews Immunology* 4, 11-24; January 2004).

### 1.4.1.1 Allyl amines and thiocarbamates

Allyl amines (terbinafine and noftifine) and thiocarbamates (tolnoftate) are non-competitive inhibitors of the enzyme squalene epoxidase (Erg1) which catalyses conversion of squalene to 2,3-oxidosqualene. Inhibition of squalene epoxidase results in the accumulation of squalene in fungal cells which is toxic to cells, and, thus, retards cell growth and proliferation (Favre and Ryder, 1996; Monk and Brogden, 1991).

### 1.4.1.2. Azoles

Azole antifungals are commonly used in either imidazole (ketoconazole and miconazole) or triazole (fluconazole, itraconazole and variconazole) forms (Hitchcock, 1993). Azole compounds inhibit enzyme lanosterol demethylase, a cytochrome P-450 enzyme, which is encoded by the *ERG11* gene, and contains a heme moiety in its active site (Hitchcock, 1991). Inhibition of Erg11 results in decreased ergosterol biosynthesis and accumulation of toxic sterol intermediates (Akins, 2005; Hitchcock, 1991). Importantly, higher concentration of azoles can bind directly to lipids present in membranes, thus, sequestering and rendering them unavailable to cells (Joseph-Horne and Hollomon, 1997). Currently, fluconazole is in wide-spread use for antifungal prophylaxis because of its clinical efficacy, oral availability and less toxicity to mammalian cells (White *et al.*, 1998).

### 1.4.1.3 Morpholines

Target enzymes of morpholines (amorolfine) are C-14 sterol reductase (Erg24) and C-8 sterol isomerase (Erg2) which catalyze synthesis of zymosterol and episterol, respectively, in ergosterol biosynthesis pathway (White *et al.*, 1998). Growth inhibitory effects of morpholines are presumed to be due to decreased ergosterol biosynthesis and accumulation of toxic intermediates (White *et al.*, 1998).
1.4.1.4. Polyenes

Polyenes antifungal agents (amphotericin B and nystatin) target membranes carrying ergosterol (Dixon and Walsh, 1996). Polyenes are amphipathic in nature i.e. contain both hydrophobic and hydrophilic moieties, and form pores in plasma membrane. Leakage of cellular ions through these pores destroys proton gradient which is crucial for maintenance of plasma membrane integrity and functions (Ghannoum and Rice, 1999; Vanden Bossche et al., 1994). Additionally, amphotericin B-mediated killing of C. albicans has also been attributed to oxidative stress (Kim et al., 2012). Although amphotericin B, the most commonly used polyene drug, shows fungicidal activity against yeasts, moulds and dimorphic fungi, its applications are limited owing to adverse side effects including renal toxicity (Ellis, 2002). Notably, amphotericin B formulated in liposomes (Amphotec and AmBisome) is shown to transfer higher dose with minimal toxicity to host cells (Ellis, 2002; Ghannoum and Rice, 1999).

1.4.1.2 Antifungal drugs targeting fungal cell wall

Chitin, glucan and mannan constitute major components of fungal cell wall and are uniquely present in fungi suggesting that compounds capable of affecting fungal cell wall composition can be potential targets for development of new antifungal drugs (Ghannoum and Rice, 1999). Although chitin synthase inhibitor, nikkomycin, has been explored broadly for its antifungal properties, it has not been commercially exploited (Ghannoum and Rice, 1999). Inhibitors of glucan synthesis have been utilized to develop effective antifungal drugs (Ghannoum and Rice, 1999). Echinocandins (caspofungin and micafungin), most common drugs of this class, are lipopeptides which display fungicidal activity against Candida and Aspergillus species through non competitive inhibition of β-glucan synthase (Cassone et al., 1981; Ghannoum and Rice, 1999; Hector, 1993). Notably, caspofungin is preferred for antifungal therapy over amphotericin B as it displays equivalent efficacy with lower toxicity to mammalian cells (Wingard et al., 2005).

1.4.1.3 Antifungal drugs inhibiting nucleic acid synthesis

This class of drugs targets biosynthesis of nucleic acids (Ghannoum and Rice, 1999). 5-Flucytosine, a fluorinated pyrimidine, enters into the fungal cell through a
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cytosine permease and gets converted to 5-fluorouracil by cytosine deaminase (White et al., 1998). Further, 5-fluorouracil is converted to 5-fluoro-dUMP and 5-fluoro-dUTP (Vanden Bossche et al., 1994) (Bossche et al., 1994). 5-fluoro-dUMP is a specific inhibitor of an essential enzyme, thymidylate synthetase, involved in DNA replication during cell division, (Vanden Bossche et al., 1987). 5-fluoro-dUTP is an analog of UTP which gets incorporated in RNA and, thus, terminates protein synthesis (Vanden Bossche et al., 1987). Currently, 5-flucytosine is being used in combinatorial therapy along with other antifungal drugs such as fluconazole and amphotericin B (White et al., 1998).

1.4.2 Immunotherapy for fungal infections

Despite the availability of many antifungals, an ever-increasing mortality rate associated with invasive mycosis suggests that modulation of host immunity can be an attractive alternative to control fungal infections and improve clinical outcome of the antifungal drugs (Pikman and Ben-Ami, 2012). Host immunity can be modulated by either passive or active immunization as described below.

1.4.2.1 Passive immunotherapy

Passive immunotherapy entails use of molecules which counter fungal infections passively without activating memory-based immune responses (Dan and Levitz, 2006; Pikman and Ben-Ami, 2012). Immunomodulators can be used as adjuvants in antifungal therapy i.e., can be administered in addition to primary therapy. Important passive immunomodulators and their roles in antifungal immunity are as follows.

1.4.2.1.1 Colony stimulating factors

Granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) induce differentiation of granulocytes as well as modulate antifungal activity of neutrophils, macrophages and monocytes (Kuhara et al., 2000; Kullberg et al., 1999). Notably, prophylactic use of GM-CSF in patients receiving induction chemotherapy for acute myeloid leukemia resulted in a significant decrease in the frequency of fatal fungal infections (Giles, 1998). Consistent with this, G-CSF and M-CSF, when administered in combination with antifungal drugs, fluconazole
and amphotericin-B, respectively, prolonged the survival of Candida-infected mice compared to drug alone (Kuhara et al., 2000; Kullberg et al., 1999). Similarly, pretreatment with human recombinant M-CSF protected the neutropenic rabbit against A. fumigatus infection (Gonzalez et al., 2001). Together, these studies indicate that CSFs have the potential to be used in adjunctive treatments for invasive mycosis in high-risk patients.

1.4.2.1.2 Cytokines

Proinflammatory cytokines are potential candidates of adjuvant therapy for invasive mycosis. Among these, IFN-γ has been used in several controlled and randomized clinical trials (Pikman and Ben-Ami, 2012). IFN-γ either alone or in combination of amphotericin-B was effective in controlling C. neoformans and H. capsulatum infections in animal cryptococcal infection and murine systemic histoplasmosis models, respectively (Clemons et al., 2001a, b). Consistent with this, Netea et al. reported that patients carrying cryptococcal infections displayed impaired IFN-γ production (Netea et al., 2004a). Importantly, defective cytokine production was reversed by recombinant interferon-gamma therapy (Netea et al., 2004a). However, IFN-γ treatment has not consistently been reported to provide protection to Candida-infected mice (Garner et al., 1989; Kullberg et al., 1993).

Additionally, proinflammatory cytokines, IL-12, IL-15 and TNF-α, have also been studied as adjuvant candidates in preclinical trials (Pikman and Ben-Ami, 2012). IL-12 was shown to be required for antifungal activity of monocytes against A. fumigatus infection and Candida-induced differentiation of Th1 cells (Roilides et al., 1999; Romani et al., 1995). However, concerns were raised over the use of IL-12 in adjuvant therapy since treatment with IL-12 resulted in death in non-neutropenic mice and increased fungal infection in patients undergoing stem cell transplantation (Pikman and Ben-Ami, 2012; Romani et al., 1995; Toren et al., 1997). IL-15 is known to induce production of superoxide ions and increase antifungal activity of human monocytes against C. albicans (Vazquez et al., 1998). Consistently, IL-15 treatment led to increased Paracoccidioides brasiliensis killing by human neutrophils (Tavian et al., 2008). Another proinflammatory cytokine, TNF-α, has also been reported to display protective functions against A. fumigatus infection (Mehrad et al.,
Despite all this information, further preclinical and clinical trials are required to develop IL-12, IL-15 and TNF-α as adjuvants in antifungal immunotherapy.

1.4.2.1.3. Antibodies

The first evidence of antibody-based immunotherapy surfaced in 1987 when Dromer et al. showed that monoclonal antibodies against capsular polysaccharide provided protection to mice from *C. neoformans* infection (Dromer et al., 1987). In recent times, two monoclonal antibodies Mycograb® and 18B7 have been tested in clinical trials for antifungal immunotherapy (Dan and Levitz, 2006). Mycograb® is a human recombinant antibody generated against immunodominant epitope HSP90 of *Candida* cell wall (Matthews et al., 2003). Mycograb® displayed antifungal activity against a wide range of *Candida* species and acted synergistically with antifungal drugs, amphotericin B and caspofungin (Hodgetts et al., 2008; Matthews et al., 2003). Notably, Mycograb® may also be useful for treatment of *Cryptococcus* and *Aspergillus* infections as both species possess HSP90 homologs (Matthews and Burnie, 2004). 18B7 monoclonal antibody, raised against capsular polysaccharide component glucuronylxylomannan (GXM) of *C. neoformans*, displayed protective functions in animal model of cryptococcosis (Mukherjee et al., 1993). Although 18B7 antibody has been found to be safe in phase-1 clinical trials, its adverse side effects including increased viral load in HIV patients and development of human anti-mouse antibodies in some patients restrict its applicability (Larsen et al., 2005). In addition, monoclonal antibodies raised against histone H2B and a surface-associated protein in *H. capsulatum* have been reported to promote killing of yeast cells (Nosanchuk et al., 2003).

Monoclonal antibody C7, designed against *Candida* cell wall mannoprotein, inhibited adherence of *C. albicans* to epithelial cells and displayed fungicidal activity (Moragues et al., 2003). Consistently, treatment with this antibody led to lower fungal burden and increased survival in mice infected with *C. albicans* (Sevilla et al., 2006). A novel approach to treat fungal infections is development of a “killer antibody” (Magliani et al., 2005b). Killer antibody is an anti-idiotype monoclonal antibody which exhibits antifungal activity towards *A. fumigatus* infection (Magliani et al., 2005b). Killer antibodies are directed to monoclonal antibodies against killer toxin (KT) of yeast *Pichia anomala* which interacts with β-glucan present in fungal cell
wall (Magliani et al., 2005a; Magliani et al., 2005b). Use of anti-idiotypic KT antibodies displayed protection against vaginal candidiasis (Magliani et al., 2005a; Pikman and Ben-Ami, 2012).

Another interesting approach of antibody-based therapy is radioimmunotherapy which utilizes radiolabelled antibodies, directed against fungal antigens, to deliver fungicidal radiation with low toxicity (Bryan et al., 2010). This approach has successfully been used to cure C. neoformans infection using a monoclonal antibody against capsular polysaccharide of C. neoformans (Bryan et al., 2010; Pikman and Ben-Ami, 2012).

1.4.2.2 Active immunotherapy

Active immunotherapy involves vaccinations which utilize memory of immune cells to induce secondary immune response in host upon confrontation with a fungal pathogen (Pikman and Ben-Ami, 2012). Antifungal vaccination may induce humoral or cell-mediated immune response as described below.

1.4.2.2.1 Antifungal vaccination to induce antibody-mediated immunity

The success of an antifungal vaccine depends on its ability to induce pathogen-specific immune response and develop memory which enable the immune system to provide long-term protection against infection (Carvalho et al., 2012). An intrinsic limitation of fungal vaccines lies in the immunocompromised host itself who fails to mount strong protective response to vaccines (Carvalho et al., 2012; Pikman and Ben-Ami, 2012). Phosphomannoprotein isolated from C. albicans cell wall has been reported to provide protection against candidiasis in the murine model of disseminated candidiasis (Han et al., 2001). Similarly, an antibody, raised against β-(1,3-glucan), was able to provide protection against three fungal pathogens, C. albicans, A. fumigatus and C. neoformans. (Torosantucci et al., 2009). Anti-C. albicans vaccines, composed of recombinant N-termini of surface-associated adhesins Als1 and Als3, reduced fungal burden and improved host survival in murine model of candidiasis (Spellberg et al., 2006; Spellberg et al., 2005). Intriguingly, Als3 vaccine was also able to confer resistance against Staphylococcus aureus infection (Spellberg et al., 2008). Currently, two anti-Candida vaccines, aimed to prevent recurrent vulvogenitis, are under clinical trials (Spellberg et al., 2006). These two vaccines are
targeted against specific Candida antigens Als3, a GPI anchored adhesin and Sap2, a secreted aspartic proteinase (Sandini et al., 2011; Spellberg et al., 2006). Other Candida cell wall antigenic components which were immunogenic includes a GPI-linked protein, Hyr1, which gets induced in response to hyphal development and confers resistance to phagocytic killing, and enolase, a cell-wall-associated glycolytic enzyme (Luo et al., 2010; Montagnoli et al., 2004).

In Aspergillus, identified vaccine candidates include secreted protein Pep1 and GPI-anchored proteins Gel1 and Crf1 (Bozza et al., 2009). Among these candidates, Crf1 is immunogenic in nature and confers cross resistance to C. albicans too (Stuehler et al., 2011). In C. neoformans, capsular GXM is a potential target for vaccine development. Mice immunized with GXM-diphtheria toxin generated human anti-GXM antibodies and protected mice from subsequent C. neoformans infections (Devi, 1996). In accord with this, GXM conjugated either with diphtheria or tetanus toxin displayed markedly enhanced immunogenicity (Datta and Pirofski, 2006). These studies indicate that potential targets can be translated to vaccines bringing them from benchside to bedside to control fungal infections.

1.4.2.2 Antifungal vaccination to induce cell-mediated immunity

Immunocompromised population defective in cell-mediated immunity, such as neutropenic patients, is more susceptible to systemic mycosis (Fridkin and Jarvis, 1996; Richardson, 2005). To address this, several vaccination strategies aimed to boost cell-mediated immunity have been tested in animal models of systemic mycosis (Dan and Levitz, 2006). For example, live attenuated bad1 mutant of Blastomyces dermatitidis provided protection to mice against a lethal dose of a virulent strain of this fungus (Klein, 2000). In addition, bad1 mutant induced sterilizing immunity i.e. a complete effective immune response capable of preventing infections, CD8+ T cells memory responses and pro-inflammatory cytokines (Wuthrich et al., 2003). Similarly, mice vaccinated subcutaneously with H. capsulatum followed by CD4+ T cell depletion displayed protection from subsequent infections (Wuthrich et al., 2003). These studies suggest that an antifungal vaccine targeting CD8+ T cells for protective immunity could be beneficial for AIDS patients as HIV infection is known to cause a decrease in CD4+ T cell count below 200 cells per µL (Dan and Levitz, 2006).
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Vaccination with recombinant *H. capsulatum* Hsp60 provided IL-12 and IFN-γ-dependent protection in mice challenged with a lethal intranasal inoculum (Deepe and Gibbons, 2002; Gomez et al., 1995). A 15 amino acid long synthetic peptide of *P. brasiliensis* glycoprotein 43 (gp43) provided IL-2 and IFN-γ-mediated protection against intratracheal infection in mice model (Taborda et al., 2004). Above studies suggest that modulation of cell-mediated acquired immunity is emerging as an alluring prospect to develop novel strategies to cure fungal infections.

1.5 *C. glabrata*: General features and interaction with host

*C. glabrata* is a haploid budding yeast and the second most prevalent yeast pathogen in humans (Pfaller et al., 2010). *C. glabrata* forms smooth, glistening, cream-colored colonies on YPD-agar medium. Size-wise, *C. glabrata* cells (1-4 μm) are comparably smaller than *C. albicans* cells (4-6 μm) (Figure 1.4) (Fidel et al., 1999). Among *Candida* species, *C. glabrata* is the only member which does not exhibit polymorphic growth (Fidel et al., 1999). Phylogenetically, *C. glabrata* is more closely related to *S. cerevisiae* than to other *Candida* species (Kaur et al., 2005). The AT-rich *C. glabrata* genome is 12.3 Mb in size and consists of 13 chromosomes which encode a total of 5283 ORFs with an average size of 493 codons (http://genolevures.org/cagl.html#). Though *C. glabrata* possesses mating apparatus, mating has not been reported under physiological conditions (Brunke and Hube, 2013; Kaur et al., 2005). *C. glabrata* infections are difficult to treat partly due to intrinsic resistance of *C. glabrata* to azole antifungals and, hence, associated with higher mortality among immunocompromised patients (Fidel et al., 1999; Richardson and Lass-Florl, 2008; Silva et al., 2012).

Successful dissemination of a pathogen depends partly on its ability to avoid clearance by host immune cells. Although strategies of *C. albicans* to evade immune response are well studied, interaction analysis of *C. glabrata* with host immune cells is still in infancy. Wells et al. examined the ability of *C. glabrata* to disseminate from the mouse intestinal tract and found that systemic *C. glabrata* infections are unlikely to spread from digestive tract (Wells et al., 2007). Additionally, trauma and immunosuppression, which are known to induce dissemination of *C. albicans* and other intestinal microbes, did not lead to *C. glabrata* dissemination in this study (Wells et al., 2007). Contrary to these findings, oral inoculation via drinking water
has recently been reported to result in hematogenous dissemination of \textit{C. glabrata} cells to liver and eventually death of immunosuppressed mice (Atanasova \textit{et al.}, 2013). These contradictory results underscore a need of further investigations to better understand dissemination mechanisms of \textit{C. glabrata} in mammalian host.

![Figure 1.4 A differential interference contrast (DIC) micrograph (63X magnification) depicting budding in \textit{C. glabrata} cells.](image)

### 1.5.1 Host immune responses against \textit{C. glabrata} infection

Unlike \textit{C. albicans}, \textit{C. glabrata} infection to immunocompetent mice does not lead to their killing (Jacobsen \textit{et al.}, 2010). Hence, \textit{C. glabrata} virulence is usually measured in terms of fungal burden in body organs including kidney, liver, spleen and brain (Jacobsen \textit{et al.}, 2010). Although \textit{C. glabrata}-infected mice survived and displayed rapid induction of proinflammatory cytokines TNF-\(\alpha\), IL-12, and IFN-\(\gamma\) both at RNA and protein levels, levels of anti-inflammatory cytokine IL-10 remained unchanged in these mice (Brieland \textit{et al.}, 2001). Further, TNF-\(\alpha\) is also known to play a pivotal role in mediating host defense against systemic candidiasis caused by \textit{C. glabrata} similar to \textit{C. albicans} (Brieland \textit{et al.}, 2001). Accordingly, deficiency of endogenous TNF-\(\alpha\) has been associated with elevated fungal burden in murine model of disseminated candidiasis (Brieland \textit{et al.}, 2001).

### 1.5.2 Intracellular behavior of \textit{C. glabrata}

The first study to delineate intracellular behavior of \textit{C. glabrata} (previously known as \textit{Torulopsis glabrata}) was carried out by Howard and Otto, who demonstrated that \textit{C. glabrata} growth was inhibited in mice histiocytes (Howard and Otto, 1967). However, this attenuated \textit{C. glabrata} growth in mice histiocytes was
partly attributed to the serum present in the culture medium as iron supplementation of medium rescued the growth defect (Otto and Howard, 1976). Since this report, survival and interaction of *C. glabrata* cells with host cells have remained largely unexplored. Summarized below are the recent studies elucidating interaction of *C. glabrata* cells with epithelial and endothelial cells, neutrophils, dendritic cells, PMNs and macrophages which act as first line of defense against *C. glabrata* infections.

1.5.2.1 *Interaction of C. glabrata with epithelial cells*  

*C. glabrata* is an important constituent of opportunist oral microflora and causes oropharyngeal candidiasis (Shao et al., 2011). Denture-wearing, aging and immunosuppression are major risk factors for oral candidiasis (Li and Dongari-Bagtzoglou, 2007). Compared to *C. albicans*, *C. glabrata* cells display lower adherence to oral keratinocytes but higher adherence to denture-surface (Li and Dongari-Bagtzoglou, 2007). A study comparing the immune response generated by oral epithelial cells upon *C. albicans* infection to that upon *C. glabrata* infection revealed that both species induced production of IL-1α (Li and Dongari-Bagtzoglou, 2007). However, unlike *C. albicans*, while *C. glabrata* cells were able to produce higher amount of GM-CSF, they were incapable of inducing IL-8 production (Li and Dongari-Bagtzoglou, 2007). Intriguingly, another study has reported moderate or weak upregulation of IL-1α, IL-1β, TNF-α, GM-CSF and IL-8 in epithelial cells in response to *C. glabrata* infection (Schaller et al., 2002). Overall, *C. glabrata* infection to oral epithelial cells is thought to generate a different pro-inflammatory immune response than that to *C. albicans* infection (Li and Dongari-Bagtzoglou, 2007; Li et al., 2007).

*Candida* species inhabit vagina and display adherence to vaginal epithelial cells similar to oral epithelial cells (Fidel et al., 1999). Vaginal *C. glabrata* infections are common in females suffering from uncontrolled diabetes mellitus (Fidel et al., 1999). Adherence of *Candida* species including *C. glabrata* with vaginal epithelial cells was found to be highest with epithelial cells collected from pregnant diabetic women (Nwobu et al., 1997). Lactoferrin, a globular glycoprotein present in various secreted fluids, acts synergistically with fluconazole against *Candida* species and prevent their growth (Lupetti et al., 2002; Lupetti et al., 2003). Consistent with this, activated lactoferrin inhibited adherence of *C. glabrata* to vaginal epithelium and was
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found to be effective against vaginal candidiasis (Naidu et al., 2004a; Naidu et al., 2004b).

1.5.2.2 Interaction of *C. glabrata* with endothelial cells

During systemic blood-stream infections, *Candida* species have to cross the blood vessel endothelial lining for deep-seated infections (Eggimann et al., 2003a). Thus, endothelial cells are among the first host cell types that encounter *Candida* cells during systemic candidiasis (Filler et al., 1996). *C. albicans* infection to endothelial cells induced expression of mRNAs encoding E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1) and inducible cyclooxygenase (Filler et al., 1996). However, no such response was observed in *C. glabrata*-treated endothelial cells indicating differential response of endothelial cells to *C. glabrata* and *C. albicans* infection (Filler et al., 1996). It is noteworthy here that a novel family of GPI-anchored proteins, comprised of Pwp7 (PA14 domain-containing Wall Protein) and Aed1 (Adherence to Endothelial cells), has been implicated in adherence of *C. glabrata* cells to endothelial cells as deletion of *PWP7* and *AED1* led to marked reduction in adherence to umbilical vein endothelial cells compared to the wild-type parental strain (Desai et al., 2011).

1.5.2.3 Interaction of *C. glabrata* with neutrophils

The fact that neutropenia is one of the major risk factors associated with *C. glabrata* infections underscores the significance of neutrophils to counteract *C. glabrata* infections (Horn et al., 2010). Several studies have shown that neutrophils are capable of engulfing and killing *C. glabrata* cells (Ferrante, 1989; Kan et al., 1996; Thong et al., 1979). Approximately 60-80% *C. glabrata* cells are killed by neutrophils within 3 h of infection (Ferrante, 1989; Kan et al., 1996; Thong et al., 1979). Fungicidal activity of neutrophils can be modulated by many factors. Thong et al. showed that bilirubin inhibits fungicidal capacity of neutrophils suggesting that neonates having jaundice are more susceptible to *C. glabrata* infections (Thong et al., 1979). Interestingly, TNF-α treatment to neutrophils, 30 min prior to coincubation with *C. glabrata* cells, enhanced their candidacidal activity by virtue of increased oxidative burst and higher production of lysosomal enzymes (Ferrante, 1989).
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Similarly, preincubation of neutrophils with GM-CSF caused increased ROS production leading to enhanced *C. glabrata* killing (Kowanko et al., 1991). Importantly, GM-CSF treatment to human monocyte-derived macrophages (MDMs) resulted in elevated antifungal activity of voriconazole and caspofungin (Baltch et al., 2008). These studies suggest that neutrophil-generated oxidative burst plays a major role in killing *C. glabrata* cells. *C. glabrata* like other *Candida* species, produces an antioxidant, 2,4-dihydroxyphenyl ethanol, to counter oxidative burst-mediated killing by neutrophils (Cremer et al., 1999). In contrast to *C. albicans*, *C. glabrata* infections do not lead to lethality in murine model of candidiasis even with higher initial inocula (Brieland et al., 2001). However, mice having defective phagocyte oxidative capacity (gp91phox−/− and gp47phox−/−) displayed progressive lethal infection with high fungal burden upon *C. glabrata* infection (Ju et al., 2003). These data suggest that oxidative burst is an important arsenal utilized by phagocytes to control *C. glabrata* infections.

Another salient feature of activated neutrophils is that they form neutrophil extracellular trap (NET) consisting of granular proteins and chromatin which degrades virulence factors and kills pathogenic bacteria (Brinkmann et al., 2004). Interestingly, *C. albicans* induces NET-formation and is susceptible to NET-mediated killing (Urban et al., 2006). Moreover, NET-mediated killing is equally effective against both hyphal and yeast forms suggesting that neutrophils are one of the key players in clearance of *C. albicans* infection (Urban et al., 2006). However, it remains to be investigated whether neutrophils employ such mechanisms to kill *C. glabrata* cells.

Recently transcriptional response of *C. glabrata* cells to neutrophil uptake has been examined (Fukuda et al., 2013). Microarray analysis from neutrophil-internalized *C. glabrata* cells revealed 519 and 360 genes to be up- and down-regulated, respectively (Fukuda et al., 2013). Induced gene sets included genes coding for enzymes involved in lysine biosynthesis, gluconeogenesis, glyoxylate cycle and trehalose utilization mirroring a carbon source depletion environment in neutrophils (Fukuda et al., 2013). Additionally, genes implicated in methionine metabolism, autophagy, oxidative stress response and nitrogen starvation response were also up-regulated (Fukuda et al., 2013). Down-regulated gene sets included genes involved in protein synthesis, ribosome biogenesis and sterol biosynthesis. These microarray
data reflected a nutrient-poor and ROS-enriched environment that *C. glabrata* cells encounter upon ingestion by neutrophils (Fukuda *et al*., 2013).

1.5.2.4 *Interaction of C. glabrata with dendritic cells*

Dendritic cells along with monocytes, macrophages and neutrophils perform the function of initial recognition of fungal pathogens including *Candida* species (Netea *et al*., 2008a). In addition, dendritic cells by virtue of their antigen presenting capacity are instrumental in relaying initial pathogenic information from innate immune system to components of adaptive immune system (Kuby, 2000). However, studies pertaining to interaction of *C. glabrata* with dendritic cells are lacking. Bourgeois *et al.* studied the behavior of bone marrow-derived conventional dendritic cells (BM-DCs) in response to *C. glabrata* infection and demonstrated that *C. glabrata*-induced release of IFN-β led to up-regulation of type-1 IFN-specific genes (Bourgeois *et al*., 2011). Further IFN-β release by BM-DCs was found to be dependent of Src/Syk kinase signaling and required phagocytosis, phagosome acidification and TLR7 and Myd-88 activation. Surprisingly, this release of IFN-β was independent of TLR2 and TLR4 receptors (Bourgeois *et al*., 2011). These results suggest that *C. glabrata* infection drives type-1 IFN host immune response.

1.5.2.5 *Interaction of C. glabrata with polymorphonuclear leukocytes and monocytes*

Polymorphonuclear (PMN) leukocytes are predominant cell types that provide protection against *Candida* infections (Fidel *et al*., 1999). Consistently, *C. glabrata* infections in immunocompetent leukopenic mice led to increased fungal burden (Jacobsen *et al*., 2010). Following infection, host immune signaling is known to cause infiltration of PMN leukocytes to the site of infection. Using PMN marker enzyme, myeloperoxidase (MPO), Jacobsen *et al.* showed increased PMN infiltration 7 days post *C. glabrata* infection (Jacobsen *et al*., 2010). Alternatively, PMN leukocytes may migrate towards the site of infection via chemotaxis. Notably, *Candida* species including *C. glabrata* secrete potent chemoattractants which attract PMN leukocytes (Geiger *et al*., 2004). Phagocytosis by PMN leukocytes is critical in initiating immune response against *Candida* species (Lyman and Walsh, 1994). PMN leukocytes phagocytose different *Candida* species including *C. albicans* and *C. glabrata* at
comparable phagocytic rates indicating that differential primary immune response against *Candida* species is independent of their uptake (Lyman and Walsh, 1994).

Epithelial cells have been reported to produce protective Th-1 cytokines in response to *C. albicans* infection in a PMN leukocytes supplemented model of reconstituted human oral epithelium (Schaller *et al.*, 2004). Notably, cytokine induction was found to be associated with chemoattraction of PMN leukocytes to the epithelial cells (Schaller *et al.*, 2004). Importantly, antifungal activity of PMN leukocytes can be modulated by different cytokines. Proinflammatory cytokines, IFN-γ and TNF-α, elevated candidacidal activity of phagocytic cells while anti-inflammatory cytokines, IL-10 and IL-4, displayed the opposite effect (Brieland *et al.*, 2001). In addition, antifungal drugs are also capable of modulating anti-candida activity of PMN leukocytes and monocytes. Human MDMs displayed significantly greater killing of *C. glabrata* in the presence of voriconazole and caspofungin either singularly or in combination (Baltch *et al.*, 2008). In accordance, micafungin, an echinocandin antifungal, inhibited production of TNF-α, IL-1β, IL-1Ra, IL-6 and IL-8 cytokines by human MDMs (Baltch *et al.*, 2008).

### 1.5.2.6 Interaction of *C. glabrata* with macrophages

Compared to *C. albicans* cells, *C. glabrata* cells interact differently with macrophages. For example, *C. albicans* cells, upon coinubcation with macrophages, switch their morphology from yeast to hyphal form and hyphal form protrudes out by damaging macrophages (Figure 1.5A). In contrast, *C. glabrata* cells do not undergo any morphological switching and are unable to damage macrophage monolayer (Figure 1.5B). However, they still manage to survive and undergo moderate intracellular replication (Kaur *et al.*, 2007; Seider *et al.*, 2011). Human MDMs, upon *C. glabrata* infection, displayed a moderate induction of proinflammatory cytokines, TNF-α, IL-6 and IL-8, and chemokines, MCP-1 and MIP-1β, in response to *C. glabrata* infection (Baltch *et al.*, 2012). Additionally, colonization of *C. glabrata* in mice organs did not exhibit inflammation suggesting that *C. glabrata* adapts an immune evasion strategy to survive in human macrophages (Brieland *et al.*, 2001).
Figure 1.5 Micrographs displaying uninfected THP-1 monocytes (A) and PMA-activated THP-1 macrophages infected with *C. albicans* (B) and *C. glabrata* (C). THP-1 monocytes cultured in RPMI medium were imaged using Olympus IX51 inverted microscope under 10X objective (A). PMA-differentiated THP-1 macrophages were infected with *C. albicans* and *C. glabrata* at a MOI of 1:1 and images were captured after 24 h coincubation using Olympus IX51 inverted microscope under 10X and 40X objectives, respectively (B & C).

1.5.2.6.1 *C. glabrata* modulates macrophage antimicrobial response for intracellular proliferation

*C. glabrata* triggers high induction of GM-CSF universally in all cell types including macrophages (Li and Dongari-Bagtzoglou, 2007; Schaller *et al.*, 2002; Seider *et al.*, 2011). Cytokines induced by *C. glabrata* infections modulate fungicidal activity of effector immune cells. GM-CSF-treated neutrophils exhibit elevated fungicidal activity through increased oxidative burst (Kowanko *et al.*, 1991). In addition, macrophage internalization of *C. glabrata* induces expression of cellular catalase CgCta1 suggesting that *C. glabrata* cells face elevated oxidative stress in macrophages (Roetzer *et al.*, 2010). To avoid host-generated oxidative burst, *Candida* species including *C. albicans* and *C. glabrata* are known to actively suppress generation of reactive oxygen species (ROS) (Wellington *et al.*, 2009). Notably, *S. cerevisiae* and heat-killed *Candida* cells were unable to prevent ROS generation in this study (Wellington *et al.*, 2009). Increased MOI of *C. glabrata* infection to human MDMs has also been reported to suppress ROS generation in a dose-dependent manner (Seider *et al.*, 2011).
Despite an inability to damage macrophages, *C. glabrata* cells undergo 5-7-fold replication in macrophages. One strategy employed by *C. glabrata* to evade macrophage killing and proliferate intracellularly is to prevent acidification of phagolysosomes (Seider *et al.*, 2011). Phagolysosomal maturation is known to result in a drop of pH of phagolysosome from 6 to 4.5 which helps in maintaining hydrolytic activity of lysosomal enzymes and, thus, kill pathogenic organisms (Geisow *et al.*, 1981; Levitz *et al.*, 1999). Immunostaining of *C. glabrata*-containing phagosomes by EEA-1 and LAMP-1 revealed that live *C. glabrata* cells prevented phagolysosomal acidification (Seider *et al.*, 2011). Positive staining of LAMP-1 even after 24 h coinubcation suggests that *C. glabrata* cells multiply within phagosome (Seider *et al.*, 2011). A comparison of genome-wide transcriptional analysis revealed that only 5.5% of *C. glabrata* genes up-regulated at pH 4.5 were common to those induced in response to macrophage internalization suggesting that *C. glabrata* cells were not exposed to low pH in phagolysosomes (Seider *et al.*, 2011). In addition, *C. glabrata* cells face altered iron content in phagosomes upon macrophage internalization (Nevitt and Thiele, 2011). Sit1, a sole siderophore-iron transporter, in *C. glabrata* cells has been reported to assist in iron acquisition and resist macrophage-mediated killing depending on iron availability in macrophages (Nevitt and Thiele, 2011).

### 1.5.2.6.2 Metabolic adaptation of *C. glabrata* cells to host environment

To understand the intracellular behavior of yeast pathogens, Lorentz and Fink utilized *S. cerevisiae* as a model system and examined global gene expression profiles of phagolysosome-harvested *S. cerevisiae* cells (Lorenz and Fink, 2001). Genes encoding enzymes involved in glyoxylate cycle, Icl1 (isocitrate lyase), Mls1 (malate synthase), Mdh1 (malate dehydrogenase) and Cit2 (citrate synthase) were found to be highly up-regulated upon macrophage-internalization (Lorenz and Fink, 2001). Similar to *S. cerevisiae*, macrophage-internalized *C. albicans* cells displayed elevated expression of *ICL1* and *MLS1* suggesting the essentiality of glyoxylate cycle in survival of *C. albicans* in host. Accordingly, deletion of *ICL1* led to markedly decreased virulence of *C. albicans* (Lorenz and Fink, 2001). These observations indicate that macrophage environment is poor in glucose and probably rich in acetyl CoA.
To examine if *C. glabrata* cells display a similar metabolic shift in response to macrophage internal milieu, Kaur *et al.* analyzed the transcriptional response of macrophage-internalized *C. glabrata* cells (Kaur *et al.*, 2007). Similar to *S. cerevisiae* and *C. albicans*, macrophage-internalized *C. glabrata* cells, 2 h post infection, displayed up-regulation of genes involved in glyoxylate cycle (*ICL1*, *ACO1* and *MLS1*), gluconeogenesis (*FBP1* and *PCK1*), β-oxidation of fatty acids (*FAA2*, *FOX2*, *POT1* and *POX1*) and methylcitrate cycle (*PDH1*, *CIT3* and *ICL2*) (Kaur *et al.*, 2007). Importantly, the above gene expression pattern existed till 6 h post infection (Kaur *et al.*, 2007). Genes related to protein synthesis, ribosomal protein genes, tRNA synthetases, translation initiation and elongation factors were down-regulated in response to macrophage internalization (Kaur *et al.*, 2007). Interestingly, macrophage-internalized *S. cerevisiae* cells did not exhibit downregulation of translational machinery (Lorenz *et al.*, 2004). Overall, macrophage-internalized *C. glabrata* and *C. albicans* cells displayed largely overlapping transcriptional response indicating a global shift in carbon metabolism and economical cellular translation (Kaur *et al.*, 2007).

Notably, *C. glabrata* cells upon phagocytosis by primary murine macrophages, display elevated biosynthesis of peroxisomes (Roetzer *et al.*, 2010). Furthermore, prolonged incubation of *C. glabrata* cells led to pexophagy-mediated degradation of peroxisomes (Roetzer *et al.*, 2010). Accordingly, *C. glabrata* mutant, *atg11Δ*, defective in pexophagy, displayed diminished survival in primary murine macrophages (Roetzer *et al.*, 2010). Importantly, inhibition of peroxisome biogenesis by Pex3 deletion rescued the survival defect of *atg11Δ* mutant suggesting that peroxisomes probably sequester cellular resources required for optimal survival of *C. glabrata* cells in macrophages (Roetzer *et al.*, 2010). Autophagy is another strategy employed by yeast and mammalian cells to survive during nutrient deprivation. Roetzer *et al.* noted that autophagy defective *C. glabrata* mutant, *atg17Δ*, exhibited diminished survival in murine macrophages indicating an important role for autophagy in intracellular survival of *C. glabrata* cells (Roetzer *et al.*, 2010). It has been postulated that autophagy assists *C. glabrata* cells mobilize cellular resources for their survival in macrophages (Roetzer *et al.*, 2010). Figure 1.6 summarizes the intracellular behavior of *C. glabrata* cells in macrophages.
Figure 1.6 A pictorial representation of interaction of *C. glabrata* cells with macrophages.
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Objectives of the present study

Invasive Candida infections are a major cause of mortality in Indian hospitals. Although C. albicans and C. tropicalis are the predominant species found in clinical settings, C. glabrata has also emerged as an important fungal pathogen in last decade. Despite an ever-increasing prevalence of C. glabrata infections in ICU patients, our knowledge about the unique strategies that C. glabrata has developed to multiply and avoid recognition by host phagocytic cells since it lacks key fungal virulence traits such as hyphal formation and secreted proteolytic activity, remains limited. Cells of innate immune system, viz., professional phagocytes, neutrophils and macrophages act as the first line of barrier against invading fungal pathogens. The current study is aimed at a genomic analysis of interactions of C. glabrata with human macrophages using an in vitro THP-1 cell culture model system via signature-tagged mutagenesis (STM) approach. By identifying genes specifically required for survival and/or replication of C. glabrata cells in cultured human macrophages, we have attempted to address types of nutritional and antimicrobial stresses, C. glabrata cells encounter in the intracellular milieu of macrophages. Specific objectives of the present study are listed below.

1. To establish an in vitro cell culture model system to study C. glabrata-macrophage interaction.

2. To screen C. glabrata Tn7 insertion mutant library for altered intracellular survival profiles via signature-tagged mutagenesis approach.

3. To examine the role of identified C. glabrata genes in survival in macrophages and virulence in a murine model of systemic candidiasis.