Evaluation of research techniques to study fungal evasion in vitro

Author: Tamar Grevelink (3517179)
Supervisor: Dr. Pieter-Jan Haas

Master thesis Infection & Immunity
Department of Medical Microbiology
UMC Utrecht, the Netherlands
25 November 2013 –10 January 2014

Index

Abstract

The incidence of patients with fungal infections is rising due to increased numbers of immunosuppresed individuals. Opportunistic fungi are a major cause of death under vulnerable patients with a compromised immune system. In order to better understand how fungi are able to infect humans, much research has been done to study the interplay between fungi and the human immune system and how fungi are able to escape from the immune defence. Interestingly, fungi that cause endemic as well as fungi causing opportunistic infections have developed immune evasion mechanisms. These evasion strategies involve escape from recognition by pattern recognition receptors, modulation immune responses, interference with intracellular trafficking and resistance against oxidative stress and antimicrobial components. To investigate these evasion mechanisms, many different specific assays can be used. General techniques, such as, genomics, transcriptomics, proteomics and secretomics are useful to reveal many genes and proteins that are involved in the fungal escape from the immune defence. Moreover, chromatin immunoprecipitation, gene modifications and RNA interference can be used as a tool to identify the binding site of DNA binding proteins (such as transcription factors and repressors) and the functions of genes that involved in evasion mechanisms.

Introduction

The incidence of fungal infections is increasing and to combat these infections more knowledge is needed about the pathogenesis of fungal disease. Therefore, more studies are conducted to investigate the interplay between fungi and the immune system and how fungi are able to evade from an immune attack. In this thesis I discuss fungi in general, the natural immune defence against fungi, the evasion mechanisms of fungi and methods to study the involved proteins and pathways.

Fungi

Fungi are similar as mammalians eukaryotes, however, their cell membranes differ because they contain ergosterol, whereas mammalian cell membranes are comprised of cholesterol. Furthermore, fungi have a cell wall that consists of chitin, mannan and β-glucans. Fungi can be morphologically distinguished into two forms: a unicellular form, referred as yeast and a multicellular filamentous form called moulds. Yeast are oval or spherical cells that reproduce asexually by blastoconidia formation (budding) or by fission (figure 1A and 1B). The branched, filamentous structures of the moulds are termed hyphae and when it forms a mass of intertwining strands it is referred as mycelium (Figure 1C). Some fungi can both grow as moulds or yeast and this phenomenon is called dimorphism[1].

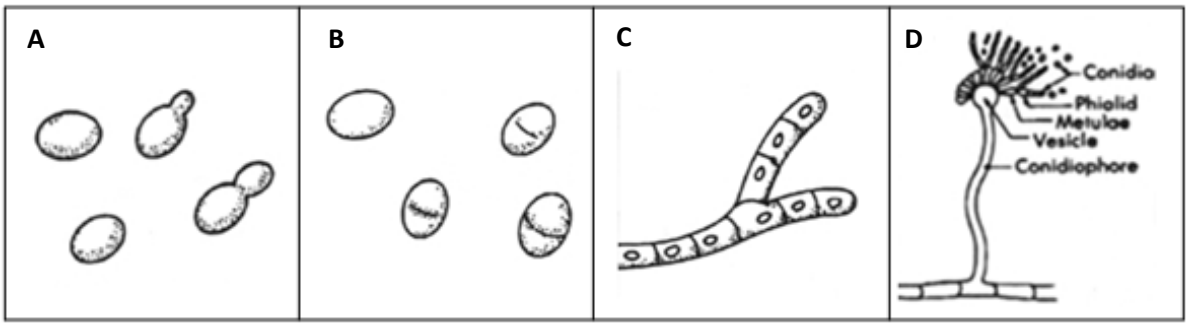

Figure 1 Morphology of fungi. Fungi can assume two forms: yeast and moulds. Different morphologies of yeast and moulds are depicted: A) Blastoconidia formation; B) Yeast divinding by fission C) Septate hyphea of mould; D) Formation of conidia by *Aspergillus*. Figures from Medical Microbiology, 1990, Murray et al. p299-300 Fig 28-1 and Fig 28-2.

Pathogenic fungi that cause systemic infections are commonly found in yeast form while the mould is more seen in the environment. The reproduction of fungi can be asexual or sexual resulting in the production of conidia or spores (Figure 1D). Inhaling or ingestion of conidia, spores and small yeast cells can expose humans to environmental fungi. When those fungi have entered the body they are able to cause infections in the immunocompromised host. Examples of these fungi are *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Pneumocytis jiroveci* and *pneumocytis carinii*. However, in immunocompetent individuals infections are efficiently cleared, except for endemic mycosis. Endemic mycoses are caused by e.g. *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides immitis*. Moreover, fungi in the normal human flora can also become pathogenic and cause infections in immunocompromised individuals as is seen with *Candida albicans*. Infections can be superficial, cutaneous, subcutaneous and systemic[1].

The four most important species that cause 90% of the fungi-related deaths in human are: *Aspergillis*, *Candida, Cryptococcus and Pneumocytis*[2]. The incidence of fungal diseases in humans has been increased due to the larger number of immunosuppressed individuals. Pathogenic fungi are a major cause of morbidity and mortality under vulnerable patients, such as, hospitalised patients in

intensive care units, transplantation patients, AIDS patients, patients that receive chemotherapy and patients with immunodeficiencies and autoimmune diseases[3]. In these patients their immune system is partially compromised and not able to fight fungal infections. Though, fungi that cause opportunistic infections as well as fungi that cause endemic infections have developed immune evasion strategies. Only in endemic mycosis the immune evasion mechanisms result in effective infection in immunocompetent individuals, while for opportunistic fungal pathogens this is not the case.

Immune responses to fungi

The first line of defence against fungi is provided by the epithelial cells present at sites of the body that continuously encounter pathogens and include the skin and mucosal epithelial surfaces of the gastrointestinal, genitourinary and respiratory tract. These cells present an anatomical barrier, secrete anti-microbial peptides, prevent adherence of fungi by ciliary clearance and are considered as part of the innate immune defence[4].

However, some fungi can get passed this defence and infiltrate the tissues. In the tissues, the fungus can be recognized by pattern recognition receptors on residual tissue macrophages and other phagocytes. Activated tissue macrophages secrete inflammatory mediators including cytokines and chemokines inducing leukocyte extravasation. Moreover, complement can be activated via the alternative or lectin pathway that is induced by fungal surfaces or spontaneous C3 cleavage. When complement is activated it can opsonise fungi and chemoattract immune cells. Furthermore, antibodies are also involved and are able to activate complement via the classical pathway, opsonise and neutralise fungal pathogens. Neutrophils, monocytes and lymphocyte infiltrate the tissues and help to clear the infection[5].

With pattern recognition receptors (PRRs) phagocytes are able to sense pathogen-associated molecular patterns (PAMPs) of pathogenic fungi. The main source of PAMPs that are recognised by PRRs are the β-glucans, chitin and mannans of the fungal cell wall (Figure 2, pag. 7). There are two main PRR groups that recognise fungal PAMPS: Toll-like receptors and C-type lectin receptors. Toll-like receptors (TLR2, TLR4 and TLR9) are able to recognize both fungal cell wall components, such as O-linked mannans, phospholipomannan and zymosan as well as fungal DNA. C-type lectin receptors, including dectin-1, dectin-2, mincle, dendritic cell specific ICAM3-grabbing non-integrin (DC-SIGN), mannose receptor, langerin and mannose-binding lectin recognize N-linked mannan, α- and β-glucans and chitin. Recognition of PAMPs by PPRs of phagocytes will lead to downstream signalling that induces production of defensins, chemokines and cytokines. Macrophages, monocytes and neutrophils as well as non-phagocytic endothelial and epithelial cells bear PRRs and are able to kill the fungi by phagocytosis or by direct pathogen killing. Dendritic cells are also able to recognise fungal PAMPs with PRRs and can phagocytose the fungus[4].

Phagocytosis can be mediated by complement induced opsonisation. Activation of complement can be triggered by three different pathways: classical, lectin and alternative pathway. All pathways will lead to the proteolytic cleavage of C3 in C3a and C3b resulting in the formation of C5a and C5b and components to form the membrane attack complex. However, pore formation by C9 polymers of the membrane attack complex is blocked by the thick fungal cell wall. C3a and C5a function as chemoattractants to recruit neutrophils and macrophages and C3b opsonise pathogens which will ultimately result in phagocytosis and destruction of invading fungi[6-8].

Fungal pathogens that are directed into the endocytic pathway of professional antigen presenting cells (APCs) such as dendritic cells and macrophages are processed and the fungal antigens are

presented to T cells. Upon antigen presentation, naive T cells will differentiate into different types of effector T helper (T_H) cell subsets. For protective immunity against fungi T_{H1} responses are induced, which differentiation is IL-12 driven. T_{H1} promotes fungal clearance by inducing inflammation through production of IFNγ that activates phagocytes at site of infection. Furthermore, T_{H1} cells providing costimulatory signals for B-cells needed the production of opsonising antibodies. Fungal specific T_{H17} immune responses can also be induced. T_{H17} cells are known to induce and effective immune response to extracellular pathogens. It is thought that T_{H17} cells promote fungus specific T_{H1} type immune responses and restrain T_{H2} responses. Besides T_{H1} and T_{H17} also T_{H2} and T_{REG} responses can be induced. T_{REG} can be beneficial because they limited pathology however they also reduce the efficacy of the protective immune response. T_{H2} responses are driven by IL-4 and associated with diminished IFNγ production, production of non-protective antibodies (IgA, IgG4 and IgE) and eosinophilia resulting in inhibition of fungal clearance[4,5].

The adaptive as well as the innate immune system thus collaborate to clear the infection. However, different fungi require different responses to clear the infection. For instance, the main host defence against invasion of mucosa and dissemination of *C. albicans* are neutrophils. Less important is phagocytosis by macrophages and monocytes and T cell mediated immunity is only important to prevent proliferation of *Candida* in mucosal surfaces. In contrast, T lymphocytes are crucial for clearing *C. neoformans* infections. Though neutrophils and macrophages are important in phagocytosis and killing, they are inhibited in their capacity to phagocytose *C. neoformans* due to increased capsule size. For *Aspergillus*, neutrophils and macrophages are the main host defence to clear infection. Macrophages are able to kill conidia and neutrophils secrete reactive oxygen species to kill hyphea[1,9]. The most important host defences against *Pneumocystis* are macrophages in the alveoli and T cell immunity. Moreover, evidence has been found that *Pneumocytis* specific antibodies also play a major role to clear the infection[10].

For the endemic mycosis caused by *H. capulatum*, *B. dermatitidis* and *C. immitis* is T cell immunity the most important host response[11-13]. Furthermore, activated macrophages are more important than neutrophils to fight infections with *H. capulatum*, *B. dermatitidis* and *C. immitis* [9].

Immune evasion

For effective fungal invasion and colonisation the fungus has to resist the first line of defence that includes the ciliary clearance by the epithelical cells. Therefore, *Candida spp*. express surface proteins that are covalently linked to the β-glucans in the cell wall, referred as adhesions, to attach to epithelial surfaces[14]. Besides adherence to epithelial cells, the adhesions such as hyphal wall protein-1 (HWP1) and agglutinin-like sequence (ALS) facilitate cell-to-cell attachment and thereby contribute to biofilm formation[15,16]. Not only *Candida* is able to form biofilms but also *Aspergillus*, *Coccidioides*, *Cryptococcus*, *Pneumocystis* for instance. Biofilm formation shield the embedded fungi from anti-fungal therapeutics and host immune responses. Moreover, biofilm formation offers a safe environment for genetic variation which may leads to resistance[17].

Avoiding recognition by PRRs

Another strategy of the fungus to evade the immune system is to avoid recognition by phagocytic cells. By hiding its cell wall components, which are glucans, mannans and chitin (Figure 2), from PRR they protect themselves from being killed by phagocytosis. *Candida albicans* uses mannoproteins to mask its β-glucan and avoid recognition by dectin-1 and phagocytosis into macrophages and neutrophils[18]. Only when the cell buds, a scar of the cell wall exposes β-glucans[19]. However, for

recognition and uptake by phagocytes mannoproteins are also important mediators. The state of glycosylation is an important determinant for recognition and uptake by macrophages and neutrophils. O-linked and N-linked mannosylation prevents recognition and ingestion of the fungus by macrophages, while phosphomannan increases phagocytosis of the fungus by macrophages[20]. In contrast, neutrophils are able to recognize and phagocyte N- and O-linked mannoproteins and phosphomannan plays only a minor role for recognition and phagocytosis by neutrophils[21]. This suggests that there is a trade-off among hiding epitopes from different immune effectors.

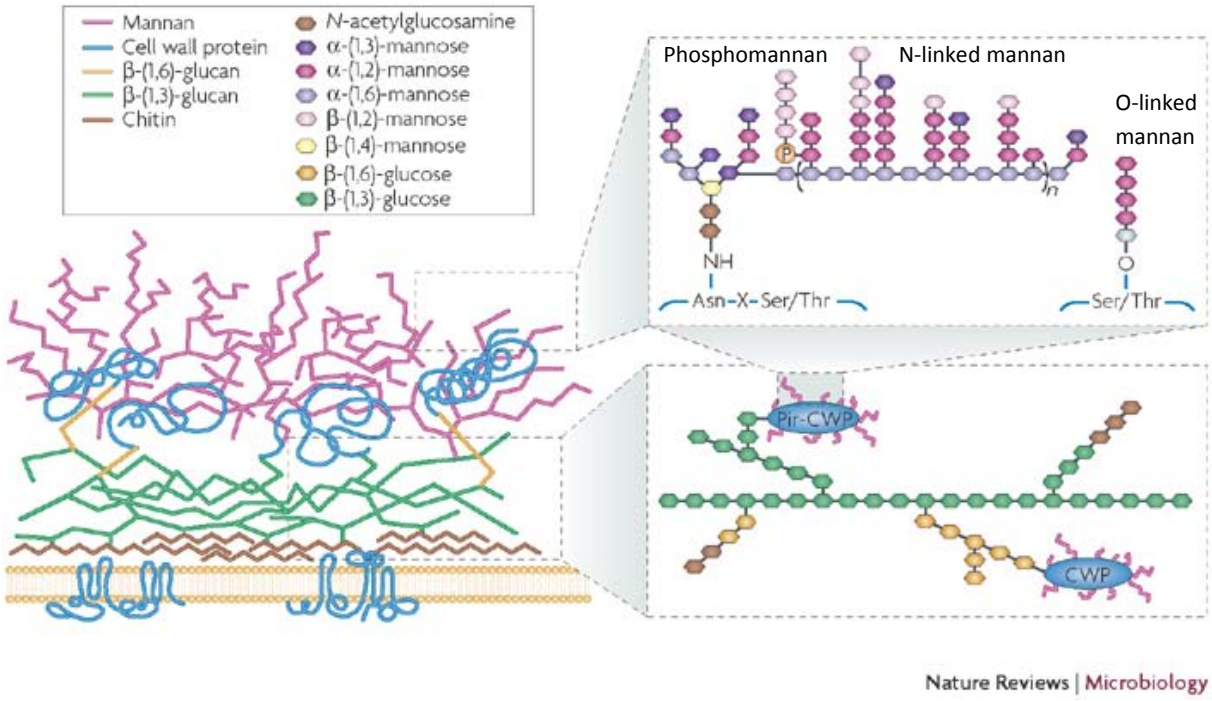

Figure 2 Fungal cell wall composition. The fungal cell wall composes a inner layer of chitin and β-glucans, which are the two main components of the cell wall. Cell wall proteins are attached to the β-glucans. Mannosylation of the cell wall proteins with phosphomannan, N-linked mannan or O-linked mannan can shield the β-glucans from recognition by pattern recognition receptors. Figure provided from Netea *et al.,* 2008, Nature reviews microbiology.

Another option to conceal the β-glucans is to cover it with α-(1,3)-glucans. The chemotype II yeast *Histoplasma capsulum* produces α-(1,3)-glucans which is important for its virulence. Suggested is that the α-1,3-glucans might mask the β-glucans and therefore less undesired immunostimulatory effects are induced[22]. Similarly, *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* switch to α-glucans in the yeast phase[18]. *H. capsulum* chemotype I yeast hide its β-glucans and escape dectin-1 detection only without the use of α-glucans, though this factor has not been characterised[23].
As well as *H. capsulum*, *B. dermatitidis* can mask its β-glucan. However, *B. dermatitidis* uses binding of mannose-binding lectin, which is a host molecule used for opsonisation, to hide its β-glucans[24].
Cryptococcus neoformans uses an extracellular capsule of glucuronoxylomannan (GXM) to shield its surface PAMPs. For the attachment of the capsule, α-glucans are needed, which also may play a role in the coverage of cell wand immunogenic β-glucans and chitin[25]. *Aspergillus fumigatus* expresses RodA, a hydrophobin that is bound to the cell wall polysaccharides and can form a rodlet layer around the conidia cell surface. In the RodA mutant of *A. fumigatus* the surface β-glucan and α-mannose were more exposed resulting in increased recognition by the PRRs dectin-1 and dectin-2 and enhanced neutrophil recruitment[26].

Coccidioides posadasii has another immunogenic surface protein, namely spherule outer wall glycoprotein (SOWgp). To avoid immune recognition, the fungus degrades the SOWgp by a metalloproteinase, Mep1, that is secreted during endospore differentiation. This way, Mep1 secretion helps the fungus to evade phagocytosis and killing and increases it virulence[27].

Modulation of inflammatory signals

Activation of TLRs by PAMPs can result in pro as well as anti-inflammatory cytokine production depending on which TLR is activated. For example TLR2 stimulation induces an anti-inflammatory T_{H2} response while TLR4 induce a pro-inflammatory T_{H1} response. The balance between T_{H1}/T_{H2} response determines the outcome of infection. When a strong T_{H1} response is induced, pro-inflammatory cytokines, mainly IFN-γ activate phagocytes resulting in phagocytosis and killing of the fungus. On the other hand T_{H2} responses characterised with the production of IL4, IL5 and IL-10 induces a humoral response and inhibit the cell-mediated T_{H1} response[28].

Some fungi modulate these inflammatory signals and inducing a T_{H2} response beneficial to their survival. *C. albicans* for instance is able to escape from the host defence by inducing TLR2 derived signals. It was shown that TLR2 deleted macrophages had increased anti-candidal capabilities and in vivo, TLR2 knockout mice were more resistant to *Candida* infection. This suggest that by inducing a T_{H2} response macrophage activation and oxidative burst is inhibited[29,30]. Additionally, it was shown that *C. albicans* can inhibit IL-12 and IFNγ production and upregulated IL-10 production, skewing the immune response from T_{H1} to T_{H2} which is essential for *C. albicans* survival. Besides the T_{H1} response it was recently shown that T_{H17} responses are also important for the host defense against *C. albicans*. However, by altering host tryptophan metabolism *C. albicans* can actively inhibit IL17 production and escape from the immune defence[31].

Similarly, *A. fumigatus* is able to modulate the TLR induced signalling for immune responses. Conidida of *A. fumigatus* induce the internalisation of TLR2 due to uptake of conidida in the phagosome which result in a lower expression level of TLR2 on the surfaces. However, hyphea of *A. fumigatus* instead down-modulate the TLR4 immune response[32]. Later, it was shown that the cell wall components have immunomodulatory effects. B-glucan and galactomannan were able to suppress the TLR4 immune response. Galactomannan had a limited inhibitory effect on the TLR2 response while α-glucan inhibited IL6 induced through TLR2 and TLR4 stimulation. These immunomodulatory effects of *A. fumigatus* may contribute to its virulence[33].

The main component of the capsule of *C. neoformans*, GXM is able to induce anti-inflammatory cytokine IL10 production and induces apoptosis in mononuclear cells to suppress the immune defence[34]. Moreover, melanin pigment of *C. neoformans* seems to skew the immune system towards a T_{H2} response by production of high levels of IL4[35].

The fungus *B. dermatitidis* uses a different mechanism to down-modulate the immune response. The virulence factor BAD1 of *Blastomyces* binds to the complement receptor (CR) 3 on macrophages resulting in suppression of TNFα release which is beneficial for survival of *B. dermatitidis*[36].

Inhibition of complement activity

Besides pattern recognition that subsequently results in inflammatory signals, the complement system plays also an important role in the host defence against fungi. To evade from the complement system *A. fumigatus* has for example bluish-green pigmentation on the conidial surface which limit the C3 complement deposition suggested by masking the C3 binding sites[7]. Normally, C3 deposition leads to opsonisation of the fungus and the production of the chemoattractant C5a that recruits leukocytes. When this is limited, less neutrophils are activated and the virulence of the

fungus is increased[7]. It was shown when disrupting the gene arp1 or alb1, which are both involved in the pigmentation, there was increased C3 deposition on the conidial surface[37,38]. Later, it was shown that melanin is important for the correct assembly of cell wall layers. Atomic force microscopy examination showed that the melanin mutant conidia did not have a rodlet layer whereas the reference did show to have rodlet layer[39]. The rodlet layer might mask C3 bindings which might explain why melanin mutants have a increased C3 deposition. However, the exact mechanism how melanin causes less C3 deposition is still unknown.

Additionally, *A. fumigatus* as well as *C. albicans* acquire complement regulatory proteins on their surface such as Factor H, Factor H like protein 1 (FHL-1) and C4b binding protein (C4BP) to down-modulate the complement cascade[6,7](Figure 3). Factor H, FHL-1, and C4BP are a cofactors for Factor I that inactivates C3b and C4b by degradation[40]. For *C. albicans* glycerol-3-phosphate dehydrogenase, the high affinity glucose transporter, pH-regulated antigen 1 (Pra-1) and phosphoglycerate mutase, have been identified as complement regulator binding surface proteins while for *A. fumigatus* the complement regulator binding surface proteins are not yet known[41-44]. The *C. albicans* Pra1 not only serve as complement binding surface protein but can also be secreted by *C. albicans*. When secreted, it binds C3 and blocks the conversion of C3 to C3a and C3b and thereby inhibits complement activation[45](Figure 3).

Plasminogen is another complement regulatory protein and is bound both by *C. albicans*, as *P. brasiliensis*, *Pneumocystis jiroveci* and *C. neoformans*[18] (Figure 3). Plasminogen enhances Factor H and Factor I mediated inactivation of C3b and activated plasmin is able to cleave and degrade C3b and C5, thereby inhibiting complement activity[46].

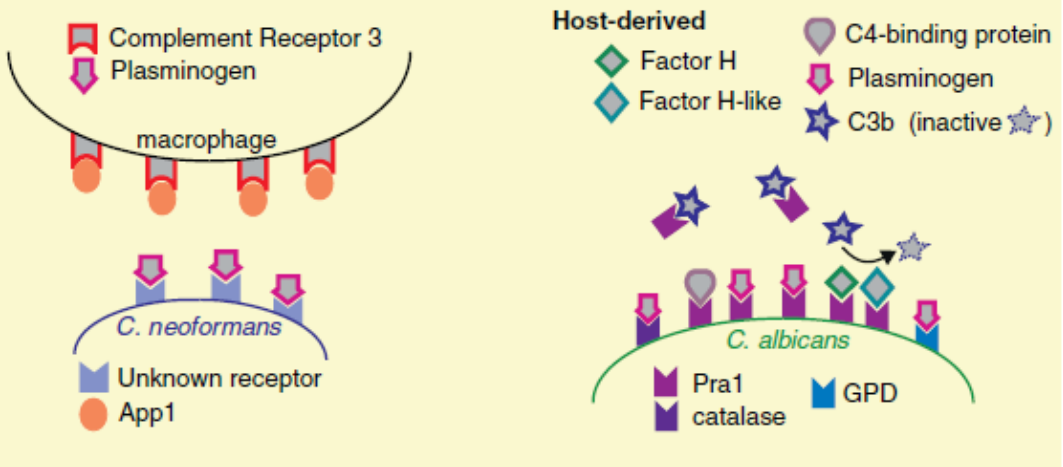

Figure 3 Inactivation of complement factors by *C. neoformans* and *C. albicans*. *C. albicans* is able to acquire complement regulatory proteins, such as Factor H, Factor H like and C4-binding protein on its cell surface to down-modulate the complement response. The complement regulatory proteins are bound by glycerol-3-phosphate dehydrogenase GDP and pH-regulated antigen 1 (Pra-1) for instance. Both *C. neoformans* and *C. albicans* are able to bind plasminogen. Plasminogen enhances Factor H and Factor I mediated inactivation of C3b and thereby inhibiting complement activity. Soluble Pra-1 of C. albicans is able to bind C3 and thereby blocks the conversion of C3 to C3a and C3b and thereby inhibits complement activation. *C. neoformans* also secretes a protein called App1. App1 bind the complement receptor 3 of macrophages and thereby blocks phagocytosis of the fungus by macrophages. Figure provided from Collete and Lorenz, 2011, Current Opinion in Microbiology.

Instead of using human inhibitors of complement some fungi secrete their own proteases to degrade complement. For example, *C. albicans* Secreted Aspartyl Proteases (SAPs) 1, 2 and 3 degrade the complement components C3b, C4b and C5 and inhibit the formation of the membrane attack

complex[47]. Similarly, *A. fumigatus* secretes a serine protease, Alp1, that degrades these complement proteins[48].
Besides direct inhibition of complement, *C. neoformans* uses a strategy that involves the blockage of complement receptors to inhibit complement mediated phagocytosis. By secreting the small protein App1 that binds CR3 and CR2, it inhibits phagocytosis of opsonised cells by alveolar macrophages and increases it virulence[49](Figure 3). Moreover, the *C. albicans* secreted Pra-1 seems to have a similar function. Pra-1 can be bound by the CR3 and soluble Pra-1 reduces neutrophil activation counteracting the activation of neutrophils by cell-wall bound Pra-1[50].
In addition, the thick fungal cell wall is largely resistant to the membrane attack complex lysis[14]. However, the membrane attack complex formation can also be inhibited by vitronectin that is bound by αvβ3 integrin like protein expressed by *C. albicans*[6].

Antiphagocytic mechanisms

When fungi are detected by the PRRs of the immune system or opsonised by complement they are subsequently phagocytosed. One way to evade phagocytosis is to have a large cell size. For example, internalisation of multinucleate hyphe of *Aspergillus fumigatus* and *C. albicans* is not efficient and mature spherules of *Coccidioides* which are 30-80µm in diameter and too large to be phagocytosed[18,51].
C. neoformans large capsule increases the cell size and is a deterrent to phagocytosis. Furthermore, another morphological form of *Cryptococcus* is identified, referred as giant or titan cells that can be 50-100µm in diameter. This titan cells have a thickened cell wall and a different capsule which is effective to dampen host immune responses[18,25]. *C. neoformans* has also a different anti-phagocytic strategy that is independent of the capsule. With a gene disruption approach two genes, GAT204 and BLP1 regulated by the transcription factor GAT201 were identified that are involved in evasion of phagocytosis[52]. However, the exact mechanisms for the anti-phagocytotic capacity is not yet clear.
Another way to evade from phagocytosis is to hide in non-phagocytic tissues. *A. fumigatus* conidia can become endocytosed by human epithelial cell lines, airway epithelial cells and type II pneumocytes[14,53]. Inside the epithelial cells of the airway the fungus limit the secretion of proinflammatory cytokines and in type II pneumocytes the fungus survives and germinates in the late endosome and have the potential to spread[54,54]. Besides epithelial cells, endothelial cells may also be used to hide from the immune system. *C. albicans* hyphea invade cells by inducing their endocytosis by binding of invasins Als3 or Ssa1 to catherin of endothelial cells as well as epithelial cells which contributes to its virulence[55,56]. Furthermore, *C. neoformans* yeast cells can induce internalisation by endothelial cells which is an important step to cross the blood brain barrier and cause meningitis[57].

Inhibit intracellular trafficking

When fungi are phagocytosed by immune cells they are normally killed inside the cell. However, some fungi can persist within the phagocytes. For example, *C. albicans* can develop hyphal forms inside the macrophage which resist killing and can escape from the cell[58]. When *C. albicans* is phagocytosed, endosomal and lysosomal organelles are rapidly recruited nonetheless *C. albicans* is able to form germ tubes. This suggest that *C. albicans* might exploit the intracellular trafficking pathways for own survival[59]. A more recent study showed that the intracellular trafficking is disordered. *C. albicans* containing phagosomes had lower level of actin, which is associated with a reduced late endocytic organelle fusion, are able to prevent acidification, inhibit phagosome maturation by the recycling of lysosome associated membrane protein 1 (LAMP-1) the fusion with hydrolytic components is prevented, and are able to interact with the ER. However the mechanisms

how the trafficking and pH is altered by *C. albicans* is still unknown[60]. Another C*andida* species, *C. krusei*, is also able to evade macrophage killing. A recent study showed that macrophages infected with *C. krusei* had a defect in phagolysosome maturation and yeast extrusion and yeast transfer among macrophages occurred which are thought to be evade mechanisms of the fungus[61]. Similar as *C. krusei*, *C. glabrata* has the ability to disrupt phagolysosome maturation. *C. glabrata* is able to inhibit acidification of the vesicle which helps the fungus to survive and replicate inside the macrophage. *C. glabrata* escapes from the macrophage when the macrophage is lysed by a non-apoptotic mechanism, which is suggested to be either by increased fungal load leading to mechanical rupture or by secretion of fungal factors that disturbs cellular processes resulting in cell lysis[62]. Moreover, the yeast *C. neoformans* is able to survive and reproduce in phagocytic cells. The yeast is able to resist intracellular killing by inducing aberrant lysosomal trafficking. Phagosomes are fused with lysosomes however a leaky phagolysosome is formed due to permeabilisation of the membrane, impairing its function. Furthermore, the capsule of *C. neoformans* plays an important role in inhibition of intracellular killing. For instance, the capsular polysaccharide can interfere with macrophage metabolism because it can bind glycolytic enzymes. Additionally, the capsule can increase its size thereby it becomes less sensitive to free radicals and anti-microbial peptides. Capsule enlargement inside the phagolysosome causes dilution of the lysosomal content and helps to escape lysosomal killing. Besides resistance to intracellular killing, *C. neoformans* is also able to escape from the cell. By multiple divisions of the yeast, macrophage lysis occur and the yeast cells can exit the cell[63]. Conversely, there is another mechanism that *C. neoformans* uses to escape from the macrophage that remains the cell alive. This process is called vomocytosis, extrusion or non-lytic exocytosis and proceeds after phagosome maturation. Vomocytosis is inhibited by actin polymerisation induced by activate Arp2/3 while an essential factor is the secretion phospholipase B1[64-66]. However, the detailed mechanisms remain to be elucidated.
H. capsulum can persist for a long time in macrophages and can become re-activated when host immunity wanes. To do so, it inhibits phagolysosome formation and modulates the acidification of phagolysosomes[67,68]. Similarly, *A. fumigatus* interferes with the acidification of the phagolysosome, a process in which its melanin plays a role[69].

Resist oxidative stress and antimicrobial mechanisms

Within the phagosome the fungus is exposed to toxic reactive oxygen, nitrogen and chloride species produced by the phagocyte in order to induce DNA, protein and lipid damage which leads to fungal cell death. To survive the fungi have developed different mechanisms to detoxify or inhibit production of oxidants and resist the oxidative burst. For example, *C. albicans* has an anti-nitric oxide defence mechanisms to protect itself from reactive nitrogen species, such as nitric oxide. Hyphea of *C. albicans* are able to suppress the production of nitric oxide by the inhibition of the inducible nitric oxide synthase enzyme activity and protein expression[70]. Furthermore, a flavohemoglobin gene, YHB1 is involved in an anti-nitric oxide mechanism that cause the scavenging and the convertion of nitric oxide into less toxic molecules. This process is regulated by the transcription factor CTA4 that transcribes the YHB1 gene under nitrosative stress conditions[71,72]. To protect itself against reactive oxygen species *C. albicans* expresses, for instance, superoxidase dismutase (SOD) 4 and SOD5. These are glycophosphatidylinositol (GPI) anchored cell surface enzymes that are able to degrade extracellular reactive oxygen species[73]. *C. glabrata* is also able to repress reactive oxygen species production, mainly by detoxification. Both *C. albicans* and *C. glabrate* expresses the catalase, CTA1, which is involved in the protection against reactive oxygens species[62,74]. Multiple transcription

factors, including YAP1, SKN7, MSN2 and MSN4 control CTA1 and thus the oxidative stress response and mediate protection against hydrogen peroxide and other oxidants[75]. Similarly, *Histoplasma* yeast express two catalases, CatB and CatP which are both involved in protection against hydrogen peroxide and from antimicrobial reactive oxygen produced by macrophages and neutrophils[76]. Furtermore, *Histoplasma* yeast produces the superoxidase dismutase 3 (SOD3) which is associated with the cell surface. SOD3 is able to convert extracellular superoxide produced by neutrophils and macrophages to hydrogen peroxide and enables *Histoplasma* to survive[77]. *A. fumigatus* also expresses catalases. In hyphea, Cat1 and Cat2 are expressed and in yeast the CatA. However, the catalases do not play an essential role in the protection against the oxidative burst. This is probably because the main reactive oxygen species involved in *Aspergillus* killing is not hydrogen peroxide[78]. In contrast to detoxification, *B. dermatitidis* yeast cells interfere with the enzyme activity of the inducible nitric oxide synthase and thereby suppress production of nitric oxide in alveolar macrophages[79]. Additionally, *Coccidioides* spherules, but not arthroconidia, are able to suppress nitric oxide production of macrophages. Nevertheless, the mechanism of this morphotype-specific inhibition has not been determined[80].

Another fungus, *C. neoformans*, protects itself against reactive oxygen species with anti-oxidant enzymes, mainly of the gluthatione system. Besides anti-oxidant enzymes, sphingiolipid and mannitol production by *C. neoformans* also plays a role in its resistance against anti-microbial mechanisms[63].

A different oxidative stress protection mechanism is used by *A. fumigatus*. This fungus uses melanin pigmentation to protect from oxidative killing by phagocytes[69]. Moreover, it synthesise gliotoxin which can inhibit the NADPH oxidase and thereby suppress the generation of reactive oxygen[81]. The *A. fumigatus* GliK protein is involved in the synthesis of gliotoxin and protects against hydrogen peroxide induced oxidative stress[52,76,81,82].

Research techniques to study fungal evasion in vitro

Many different techniques can be used to investigate fungal evasion. In this chapter I discuss the use of "-omics", gene manipulation, RNA interference and chromatin immunoprecipitation and specific assays used to study evasion of immune recognition, complement attack, phagocytotic trafficking, oxidative bursts and inflammatory responses.

The use of "–omics"

Sequencing is a valuable tool which already has provided much more information about pathogenic fungi. One of the best studied fungus is *C. albicans*. With the revealing of the genome sequence of *C. albicans* in 2004, it was possible to initiate thorough research to expand our knowledge of this pathogenic fungi[83]. Comparing the genome of pathogenic fungi with related non-pathogenic species revealed pathogen specific genes that are required for effective infection of humans. Revealing these pathogen specific genes can help to gain more inside in the pathogenesis of different fungi species[84].

Besides sequencing of the genome, the RNA of the fungi, referred as the transcriptome, can be sequenced. Sequencing of the transcriptome involves the isolation of total RNA, subsequently convertion into library cDNA fragments which than can be sequenced with high-throughput sequencing technologies. With the results of the RNA sequencing the functional elements of the genome can be determined and help to understand and interpret the molecular details of cellular constituents involved in different process such as immune evasion[85]. For example, sequencing of the transcriptome was used as research approach to examine the oxidative stress response of *P.*

brasiliensis. Results revealed that *P. brasiliensis* transcribes multiple gene-coding proteins and transcription factors that are involved in the anti-oxidant responses[86].
After revealing the transcriptomes of fungi, the next step could be to study proteomics of the fungi. The two main methods for proteomics are two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) based proteomics and gel-free proteomics. With 2D-PAGE proteomics the proteins are separated on gel and the individual spots can be analysed by mass spectrometry (MS). However for membrane proteins, very hydrophobic proteins and proteins with a high pI or molecular mass it there were some difficulties with 2D separation. A solution therefore was to use gel-free proteomics. With gel-free proteomics multidimensional liquid chromatography (LC) is used for separation and tandem mass spectrometry (MS/MS) to identify the proteins. Combined LC-MS/MS can identify all type of proteins and overcome the problems with 2D-PAGE proteomics. Proteomics have been used to identify virulence factors of *C. albicans* and *A. fumigatus*. For example, proteomics studies have revealed that both in *A. fumigatus* as well as *C. albicans* the level of many proteins with antioxidant functions, proteins involved in the trehalose biosynthesis (a stress protectant) and heat shock proteins are increased during the oxidative stress response[83,87,88].
A specific type of proteome, referred as the secretome, can be determined to gain more knowledge about fungal evasion strategies. The predicted secretome contains all the proteins that have an N-terminal sequence (which is essential for protein secretion), while, the measured secretome contains all the proteins that are secreted and detected in medium under certain conditions. Interestingly, in the measured secretome are also cytosolic proteins present of which is suggested that they are actively secreted in membranous vesicles called “virulence bags”. Unravelling the secretome of pathogenic fungi can reveal secreted proteins that are important for the virulence of the fungi, for instance by evading the immune evasion[88]. The secretome approach has been used to identify the serine protease Alp1, that is able to cleave complement and helps *A. fumigatus* to evade from complement attacks[48].
Once a protein or gene involved in immune evasion has been identified the function can be further studied using chromatin immunopreciptiation when it regards DNA binding proteins. Furthermore, gene manipulation and RNA interference techniques can be used to create mutants lacking the gene or protein of interest. Subsequently, these mutants can be used in specific assays to investigate if the gene or protein is involved in evasion of pattern recognition, inflammatory responses, complement defence, phagocytic trafficking and the oxidative burst.

Chromatin immunoprecipitation, gene manipulation and RNA interference.

To understand the molecular mechanisms of immune evasion pathways that involve DNA-protein interactions chromatin immunoprecipitation (ChIp) can be used as research technique. In short, for ChIp DNA and proteins are reversibly cross-linked, than fragments of chromatins are created and immunoprecipitated with an specific antibody against the protein of interest. After release of crosslinkages, the DNA is purified and sequenced. With this method the sequence of the DNA binding site of posttranslationally modified histone proteins, chromatin modifying enzymes, chromosome associated proteins, and transcription factors and repressors can be identified[85]. This method was for instance used to reveal the targets of the transcription factor GAT201 that is involved in the capsule independent anti-phagocytosis strategy of C*. neoformans*[52].
Another valuable tool to study immune evasion is to genetically manipulate genes that are suggested to play a role in this process. With homologous recombination by DNA-mediated transformation a knock-out or knock in transformant can be generated. In yeast homologous recombination occurs

frequently which is beneficial for gene manipulation. However, the yeast *C. albicans* requires more complex methods for gene disruption because of its diploid genome. In knock-out transformants the gene of interest is no longer expressed because of a substitution with a marker gene. A commonly used nutritional selection marker gene for *C. albicans* is the URA3 gene. Besides nutritional markers, drug markers and gfp or luciferase reporter genes can be used for selection. The first develop method that use the URA3 gene is known as the "URA blaster method" and an alternative method as "URA flipper method". Further development led to the use of the UAU1 cassette to disrupt both copies of the gene in a single transformation step, a Cre-Lox based system with increased efficiency and the Gene Replacement And Conditional Expression (GRACE) method and tetracycline-regulatable system for conditional expression of essential genes in *C. albicans*[89,90]. Mutants of *C. albicans*, with defects in glycosylation are for example used to investigate evasion of recognition by PPRs[20].
In filamentous fungi homologous recombination is rare and which causes technical difficulties with gene manipulation. To overcome problems with filamentous fungi another method called split-marker technology was developed and applied to make knock-outs. With split marker technology a mixture of at least two DNA fragments covering the selectable marker gene are used for transformation (Figure 4A).

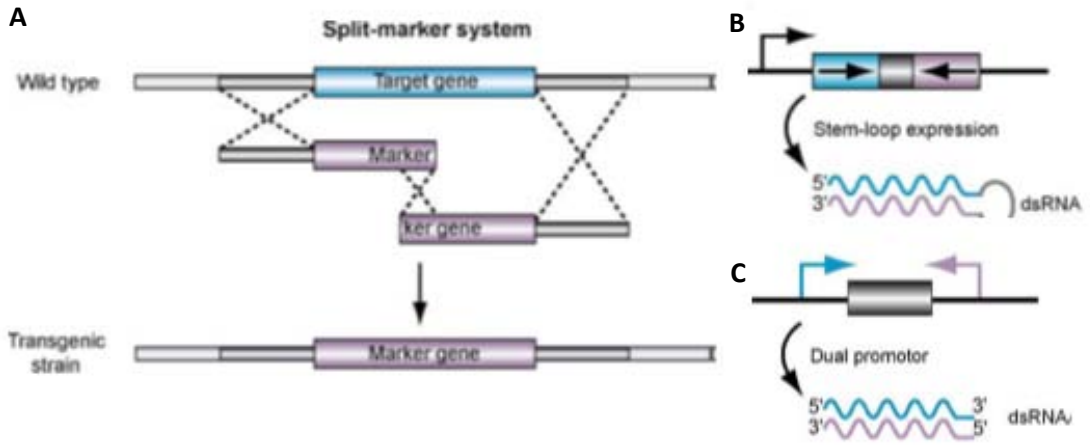

Figure 4 DNA fragments used for gene manipulation and RNA interference.
A) Split marker technology; B) Stem-loop vector for RNAi; C) Dual promotor vector for RNAi to create dsRNA. FIgure provided from Kück and Hoff, 2010, Applied Microbiology and Biotechnology

Another option to increase the frequency of homologous recombination needed for transformation is to use strains that are deficient in non-homologous end joining. Strains that have a disruption in the ku70, ku80 or lig4 gene (involved in non-homologous end joining process) have been generated. These strains have an increased homologous recombination frequency which is beneficial for gene manipulation[89].
RNA interference (RNAi) can also be used to silence genes. When in the nucleus double stranded RNA (dsRNA) is formed of mRNA transcribed by the RNA dependent RNA polymerase, the dsRNA is transported to the cytoplasm via nuclear pores. This cytosolic dsRNA is fragmented by dicer into small interfering RNAs (siRNA). SiRNA is recognized and bound by the RNA-induced silencing complex (RISC). The RISC contains the catalytic component argonaute that cleaves the complementary mRNA that is bound by RISC resulting in post transcriptional silencing of the targeted gene. To use RNA interference, stem loop vectors are used that contain spacer or intron sequences between two fragments that are inversely orientated. When transcribed, a double stranded RNA with a hairpin

structure is formed (Figure 4B). Another way to form the double stranded RNA needed for RNAi is to use a plasmid with a dual promotor which transcribe the target gene from both sides (Figure 4C). To be sure that the target gene is silenced properly a reporter gene is simultaneously silenced[89]. RNA interference has been used for example by a study to demonstrate the role of α-1,3 glucans in the virulence of *H. capsulatum*[22]. RNA interference can also be used in many other fungi, for instance *A. fumigatus*, *B. dermatitidis* and *C. neoformans*, however for *C. albicans* it has yet to be proven successful[89,90].

Assays to study evasion of recognition

A main evasion mechanism of fungi is to evade dectin-1 recognition by masking its β-glucans. To analyse recognition by dectin-1, a fluorescently labelled soluble dectin-1 can be generated to observe binding of dectin-1 to fungal cells with confocal fluorescence microscopy or flow cytometry[19]. Similarly, in another study a dectin-Fc fusion protein and ConA were fluorescently labelled and used to detect surface β-glucans and α-mannose by fluorescent microscopy[26]. Dectin-1 binding can also be quantified with an enzyme-linked dectin-1 binding assay. Additionally, activation of dectin-1 can be analysed by phosphotyrosine immunoblotting. Stable epitope-tagged dectin-1 transfected cells from a mouse macrophage cell line are incubated with the yeast cells and hyphe of the fungus. Subsequently, cells are lysed and immunoprecipitated using the dectin-1 tag. With anti-phosphotyrosine antibodies, activation of the receptor was detected by immunoblotting[19]. To examine if for example glycosylation is used to mask epitopes from recognition by dectin-1, mutants with defects in glycosylation can be used. To determine if the mutants are still recognized and phagocytosed by macrophages a yeast cell phagocytosis assay can be performed. Wild-type and mutant strains are coincubated with macrophages. After coincubation cells are stained and fixed and the uptake of yeast cell can be assessed by light microscopy[20]. Similarly, this type of assay can be used to study if recognition by neutrophils and thus phagocytosis is inhibited. To do so freshly isolated neutrophils as well as the fungi, *C. albicans* in this case, are stained fluorescently. After coincubation, phagocytosis can be analysed by confocal fluorescent microscopy and flow cytometry[21]. To investigate if the hidden β-glucans and/or α-mannoses prevent recognition by PPRs and subsequently cytokine and chemokine production of neutrophils or macrophages, cytokine and chemokine assays can be used. After coincubation of macrophages or neutrophils with the wild type or mutant strain, the cytokine and chemokine production in the supernatant can be measured with ELISA based assays[21,26].

Assays to study modulation of the immune response

To examine effect of fungal components on modulating the immune response, stimulation assays with peripheral blood mononuclear cells (PBMCs), macrophages or spleen mononuclear cells can be performed. First, cells are incubated with the fungal component. After incubation, supernatant is replaced for fresh medium with the secondary stimulus, for instance TLR2 ligand or TLR4 ligands. With ELISA based cytokine measurements can be determined if the fungus modulate TLR2 or TLR4 responses[29,33,34]. To determine if a TLR2 or TLR4 response is induced by the fungus to evade from the immune system, knock macrophages were tested at their phagocytose, anti-fungal and cytokine production ability. For anti-fungal assays, macrophages are incubated with for instance *C. albicans*. After incubation, anti-fungal activity can be determined with a colony forming unit (CFU) inhibition assay[29]. Other methods to determine inhibition of fungal growth are for instance mycelium dry weight measurements and calcofluor white staining measurements[91,92]. Phagocytose and cytokine measurements can be performed as described above[29,30,32]. The TLR response can also be modulated

by the fungi at transcriptional level. To measure TLR2 and TLR4 transcription and expression quantitative PCR and flow cytometry can be used[32].

Assays to study complement evasion

Different assays can be used to investigate if the fungus is able to evade the complement attack. For instance a complement component C3 bindings assay can be used to study if the C3 deposition on the fungal cell surface is inhibited. Complement component C3 can be isolated from human plasma and radioactively labelled. After incubation of radioactive C3 with the conidia of the fungus, the fraction of covalently bound C3 can be measured with a gamma counter[37,38]. More recently, flow cytometry and confocal fluorescents microscopy were used to examine C3 deposition[45,47].
Additionally, complement mediated phagocytosis assays with the secreted fungal protein or fungal mutants lacking the gene involved C3 deposition inhibition can be performed to analyse its effect phagocytosis[38,45,47]. Furthermore, the deposition of MAC on fungi can be measured. This can be done by flow cytometry of fungi that were incubated with serum and afterwards labelled for the MAC or with an ELISA based method[42,47].
To determine if a secreted protein by a fungus is a complement inhibitor, binding with C3 and C3b, generation of C3a and C5a, and interactions with C3 convertases can be analysed. Binding of the protein with C3 or C3b can be determined with an ELISA. Plates coated with the recombinant protein can be incubated with C3 or C3b. Binding can be detected by using an primary antibody against C3 or C3b and a secondary HRP-coupled antibody and coloration can be measured after the HRP substrate was added. To determine if the fungal protein inhibits C3a and C5a generation the alternative complement pathway can be activated by incubation of human serum with heat treated *C. albicans* or zymosan in presence or absence of the protein. Subsequently generation of C3a and C5b can be determined with ELISA or western blotting[45,47]. To determine if the C3 convertase formation is blocked, the secreted fungal protein is incubated with C3b-coated zymosan and factor B, D, and P. After incubation, C3 covertase formation can be detected on the surface of the zymosan particles with flow cytometry. Moreover, it is possible that the fungal protein dissociate a preformed C3 covertase. To investigate this, plates coated with C3 covertases are incubated with the secreted protein. After incubation, remaining C3 covertase can be detected with polyclonal anti-factor B serum[45].
Another way to evade from complement attacks is to degrade the components. To study if the fungus secretes proteases that degrade complement factors the culture supernatant can be incubated with human complement purified C3, C4b and C5. Additionally, to identify the type of protease specific inhibitors can be added to the mixture. After incubation, the proteins can be separated with SDS-PAGE and degradation of the complement components can be visualised with immunoblotting[47,48]. To determine if the protease mediated cleavage inhibit complement activity a haemolytic assay can be used. For the haemolytic assay, human complement and culture medium containing the proteases are added to rabbit erythrocytes. On the erythrocytes is complement normally activated resulting in lysis of the cells[47].
Complement can also be inhibited by fungi that bind human complement regulatory proteins, for instance factor H. To determine molecules that bind complement regulatory proteins, an expression library can be probed with serum and subsequently an anti-factor H antibody, for example, and a secondary antibody for detection. Positive clones that are detected can be sequenced to identify the factor H binding molecule[42,44]. Another method to identify complement regulatory protein binding molecules, makes use of chromatography. For example, to a factor H coupled column cell extracts

are loaded and afterwards bound protein is eluated and identified by mass spectrometry[43]. Additionally, the proteome of the yeast *saccharomyces cerevisiae* can be used. Recombinant proteins of the yeast are attached to a glass slide and are probed to factor H. Bound proteins are detected with a fluorescent labelled antibody when the array is scanned with a fluorescent microarray scanner. Data can be analysed with corresponding software to reveal the binding proteins. Homologous of these proteins can be found in other species as was done for *C. albicans*[41]. When the factor H binding molecule is identified a knock-out mutant can be generated and tested with immunofluorescence microscopy for binding of factor H[42]. Another way to measure binding is with ligand affinity blotting. For ligand affinity blotting, factor H is subjected to SDS-PAGE and transferred to a western blot membrane. After blocking of non-specific binding sites, the recombinant factor H binding protein is added. With immunoblotting, binding of the factor H binding protein to factor H can be detected[43,44]. Binding to the factor H protein can also be analysed with ELISA, similarly as described above [41,43,44]. Flow cytometry can be used to test binding of human complement regulatory factors[42,44]. To determine if the fungal protein that binds a complement regulatory factor does not influence its activity a cofactor assay can be performed. Plates are coated with the recombinant fungal protein and subsequently the complement regulatory protein is added. Then the plate is incubated with C3b and factor I and C3 degradation can be analysed by western blotting[41,44,45,47,48]. For studying the blockage of complement receptors to inhibit complement mediated phagocytosis CR3 and CR2 bindings assays can be performed. By binding of the CR3 and CR2 with a secreted factor, the receptor is blocked and unable to bind the fungus and induce phagocytosis. To study this, cells expressing CR3 are coincubated with the secreted protein of interest. After removal of unbound proteins by washing, cells are lysed and binding of the protein can be analysed with western blotting. An ELISA, similarly as described above, can be used to determine CR2 binding. Furthermore, phagocytosis assays can be performed to analyse effects on complement mediated phagocytosis with mutants that lack the production of the secreted protein[49].

Assays to study interference with phagocytic trafficking

Uptake of the fungus by macrophages can be monitored with live cell imaging. Phagocytosis and intracellular killing can be observed with the use of the adherent monolayer method. Briefly, the monolayer of macrophages is incubated with the fungus. To discern ingested fungi from those remaining outside the macrophage after incubation, different fluorescent labelling is used[60]. Moreover, with FACS analysis using propidium iodide to discriminate killed cells from ingested cells, fungal cell killing by macrophages can be addressed. With conventional transmission electron microscopy the development of phagosomes containing the fungus can be analysed[59-62,64]. The involvement of actin polarimerisation during phagosomal trafficking can be investigated with light or fluorescent microscopy using rhodamine-phalloidin staining or fluorescently labelled phalloidin[60,64]. Some fungi are able to interfere with the phagocytic trafficking including the fusion of the phagosome with early endosomes, late endosomes and lysosomes. To study this, different markers of those compartments can be used. A good marker for the early endosome is the transferrin receptor in complex with transferrin or the early endosome antigen 1 that co-localise with this receptor. The transferrin receptor is present on the cell surface and only traffics through the early endosome and can therefore be used to examine recycling and sorting of early endosomes[60,62]. For late endosomes and lysosome compartments there is not one ideal marker due to complexity and heterogeneity of the different markers. Markers that can be used are: lysobisphosphatidic acid (LBPA), which is a lipid that is present in late endosomes; colloidal gold conjugated to rhodamine,

which is a fluid phase marker that when internalised labels both late endosomes and lysosomes; LAMP-1, which is also a marker for both lysosomes and late endosomes; the lysosomal protease cathepsin D; LysoTracker that labels acidificated compartments (pH below 5.5-6) and the vATPase subunit, that acidifies the phagosomes, can be used as marker[59-62,64]. Sometimes the phagosomes are able to communicate with the host cell biosynthetic pathway. To investigate if engagement with the endoplasmatic reticulum (ER) is important to evade from the host defense, fungal phagosomes can be labelled for calnexin, which is an ER-specific protein[60]. Furthermore, to see if acidification, actin assembly and fusion of phagosomes with lysosomes are important in evasion strategies of the fungus studies with inhibitors can also be performed[59,60,64].

Assays to study inhibition of the oxidative stress response

Inside the phagosome the fungus is exposed to a low pH and microbiocidal compounds such as reactive oxygen and nitric oxide species for instance. Many fungi are able to evade this oxidative stress response. To examine if nitric oxide release is influenced by the fungus, nitric oxide production in cell supernatants can be determined with the Griess reagent and in living cells with diaminofluorescein-2 diacetate (DAF-2DA). Additionally, inhibitors for nitric oxide species can be used in the assays. A reduction of nitric oxide production can be due to inhibition of the inducible nitric oxide synthesase. To investigate if a fungus inhibits the upregulation of this protein real time PCR can be used to determine RNA transcript levels and western blotting for protein levels. Moreover, nitric oxide production can be inhibited due to limitation of the availability of the arginine substrate. This can be achieved by inducing expression of the macrophage arginase that competes for arginine with the nitric oxide synthesase. To determine if expression is upregulated Real-time PCR can be used. Another possibility is that the fungus produces its own arginine degrading enzymes and this can be studied by adding exogenous arginine and observe if nitric oxide production is restored[60,70,79,80]. Reactive oxygen species are generated by activation of the NADPH oxidase complex that generates superoxides. In the supernatant of cells reactive oxygen species can be measured with chemiluminescence assays. Intracellular reactive oxygen species can be determined with FACS analysis using a specific dye for hydrogen peroxide[62,73]. Some fungi are able to produce superoxide dismutase enzymes that are able to destroy reactive oxygen species. To examine dismutase activity, superoxide reduction can be measured with use of an tetrazolium dye WST-1[77]. Additionally, fungi can secrete catalases which are able to detoxify hydrogen peroxide and evade from this host defence. Catalase activity can be determined by photospectometrically measuring the decrease in hydrogen peroxide over time. To observe sensitivity to hydrogen peroxide the fungi can be incubated with different concentrations of hydrogen peroxide, plated on agar and colonies can be counted or the minimum inhibitory concentration can be determined[75,76,78].

Conclusion and discussion

The need to understand the pathogenesis of fungal diseases is arising as the incidence of infected patients is increasing. To this end, much research has been done to study the interplay between fungi and the human immune system and how fungi are able to escape from the immune defence. Fungi that cause endemic as well as fungi causing opportunistic infections have developed immune evasion mechanisms. These mechanisms involve escape from recognition by PRRs, immune modulation, interference with intracellular trafficking and resistance against oxidative stress and antimicrobial components. To investigate this evasion strategies, specific assays have been used. Furthermore, genomics, transcriptomics, proteomics and secretomics have revealed many genes and

proteins that are involved in the fungal escape from the immune defence. Moreover, ChIP, gene modifications and RNA interference have been used as a tool to identify the bindingsite of DNA binding proteins (such as transcription factors and repressors) and the functions of genes that involved in evasion mechanisms.

When performing these assays there are some aspects which has to be considered. For instance, the type of phagocytes is used in a phagocytosis assay should be rightly chosen. Neutrophills have to be freshly isolated while for macrophages also cell lines can be used. The use of a macrophage cell line can be beneficial because it they remain longer viable and they do not need to be isolated, which saves time and makes the assay easier to perform. However, it should be considered that for instance for *C. albicans* and A. fumigatus neutrophils form a major host defence against infection. Moreover, when phagocytic trafficking is studied it is important to choose late endosome and lysosomal markers wisely. Many different markers have been developed and sometimes conclusions based on only one marker can be conflicting. This can be illustrated by a study with *C. albicans* that suggested that that there was no phagolysosome fusion based on acidophilic markers while another study using LAMP-1 concluded that phagolysosome fusion did occur[59,93]. A solution is to combine different markers to examine the intracellular fate of the fungus and to use live cell imaging to analyse the process in time as was done by of Fernandez-Arenas and colleagues[60] .

Furthermore, when the function of a protein or gene is studied, gene manipulation or RNAi can be used as method. However, when a diploid or filamentous fungus is studied gene manipulation might be more complex and time consuming than for instance RNAi. On the other side, with RNAi the gene is still present and the downregulation of gene expression can be variable between transformants[89].

Recently, a similar system as is used with RNAi has been described, namely the CRISPR/cas system. This system forms a prokaryotic defence mechanism against foreign nucleic acids, such as that from bacteriophages. Interestingly, this system can be exploited to target specifically DNA sequences resulting in genome editing and transcriptional repression and suggested is that this system might also target RNA sequences. Therefore, applying the CRISPRS/Cas system for genome editing, transcriptional repression and RNA interference can serve as alternative for gene manipulation and RNAi to study fungal evasion[94].

References

1. Engleberg, N. C., DiRita, V. & Dermody, T. S. in *Schaechter's Mechanisms of Microbial Disease* 761 (Lippincott Williams&Wilkins, 2007).
2. Brown, G. D. *et al*. Hidden killers: human fungal infections. *Sci. Transl. Med.* **4**, 165rv13 (2012).
3. Pappas, P. G. Opportunistic fungi: a view to the future. *Am. J. Med. Sci.* **340**, 253-257 (2010).
4. Romani, L. Immunity to fungal infections. *Nat. Rev. Immunol.* **11**, 275-288 (2011).
5. Hamad, M. Innate and adaptive antifungal immune responses: partners on an equal footing. *Mycoses* **55**, 205-217 (2012).
6. Luo, S., Skerka, C., Kurzai, O. & Zipfel, P. F. Complement and innate immune evasion strategies of the human pathogenic fungus Candida albicans. *Mol. Immunol.* **56**, 161-169 (2013).
7. Speth, C. & Rambach, G. Complement Attack against Aspergillus and Corresponding Evasion Mechanisms. *Interdiscip. Perspect. Infect. Dis.* **2012**, 463794 (2012).
8. Kozel, T. R. Activation of the complement system by pathogenic fungi. *Clin. Microbiol. Rev.* **9**, 34-46 (1996).
9. Lehmann, P. F. Immunology of fungal infections in animals. *Vet. Immunol. Immunopathol.* **10**, 33-69 (1985).
10. Kelly, M. N. & Shellito, J. E. Current understanding of Pneumocystis immunology. *Future Microbiol.* **5**, 43-65 (2010).

11. Beaman, L. V., Pappagianis, D. & Benjamini, E. Mechanisms of resistance to infection with Coccidioides immitis in mice. *Infect. Immun.* **23**, 681-685 (1979).
12. Tewari, R. P., Sharma, D. K. & Mathur, A. Significance of thymus-derived lymphocytes in immunity elicited by immunization with ribosomes or live yeast cells of Histoplasma capsulatum. *J. Infect. Dis.* **138**, 605-613 (1978).
13. Brummer, E., Morozumi, P. A., Vo, P. T. & Stevens, D. A. Protection against pulmonary blastomycosis: adoptive transfer with T lymphocytes, but not serum, from resistant mice. *Cell. Immunol.* **73**, 349-359 (1982).
14. Chai, L. Y., Netea, M. G., Vonk, A. G. & Kullberg, B. J. Fungal strategies for overcoming host innate immune response. *Med. Mycol.* **47**, 227-236 (2009).
15. Nobile, C. J. *et al*. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. *PLoS Pathog.* **2**, e63 (2006).
16. Nobile, C. J., Nett, J. E., Andes, D. R. & Mitchell, A. P. Function of Candida albicans adhesin Hwp1 in biofilm formation. *Eukaryot. Cell.* **5**, 1604-1610 (2006).
17. Ramage, G., Mowat, E., Jones, B., Williams, C. & Lopez-Ribot, J. Our current understanding of fungal biofilms. *Crit. Rev. Microbiol.* **35**, 340-355 (2009).
18. Collette, J. R. & Lorenz, M. C. Mechanisms of immune evasion in fungal pathogens. *Curr. Opin. Microbiol.* **14**, 668-675 (2011).
19. Gantner, B. N., Simmons, R. M. & Underhill, D. M. Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. *EMBO J.* **24**, 1277-1286 (2005).
20. McKenzie, C. G. *et al*. Contribution of Candida albicans cell wall components to recognition by and escape from murine macrophages. *Infect. Immun.* **78**, 1650-1658 (2010).
21. Sheth, C. C. *et al*. Glycosylation status of the C. albicans cell wall affects the efficiency of neutrophil phagocytosis and killing but not cytokine signaling. *Med. Mycol.* **49**, 513-524 (2011).
22. Rappleye, C. A., Engle, J. T. & Goldman, W. E. RNA interference in Histoplasma capsulatum demonstrates a role for alpha-(1,3)-glucan in virulence. *Mol. Microbiol.* **53**, 153-165 (2004).
23. Edwards, J. A., Alore, E. A. & Rappleye, C. A. The yeast-phase virulence requirement for alpha-glucan synthase differs among Histoplasma capsulatum chemotypes. *Eukaryot. Cell.* **10**, 87-97 (2011).
24. Koneti, A., Linke, M. J., Brummer, E. & Stevens, D. A. Evasion of innate immune responses: evidence for mannose binding lectin inhibition of tumor necrosis factor alpha production by macrophages in response to Blastomyces dermatitidis. *Infect. Immun.* **76**, 994-1002 (2008).
25. O'Meara, T. R. & Alspaugh, J. A. The Cryptococcus neoformans capsule: a sword and a shield. *Clin. Microbiol. Rev.* **25**, 387-408 (2012).
26. Carrion Sde, J. *et al*. The RodA hydrophobin on Aspergillus fumigatus spores masks dectin-1- and dectin-2-dependent responses and enhances fungal survival in vivo. *J. Immunol.* **191**, 2581-2588 (2013).
27. Hung, C. Y., Xue, J. & Cole, G. T. Virulence mechanisms of coccidioides. *Ann. N. Y. Acad. Sci.* **1111**, 225-235 (2007).
28. Netea, M. G., Van der Meer, J. W., Sutmuller, R. P., Adema, G. J. & Kullberg, B. J. From the Th1/Th2 paradigm towards a Toll-like receptor/T-helper bias. *Antimicrob. Agents Chemother.* **49**, 3991-3996 (2005).
29. Blasi, E. *et al*. Biological importance of the two Toll-like receptors, TLR2 and TLR4, in macrophage response to infection with Candida albicans. *FEMS Immunol. Med. Microbiol.* **44**, 69-79 (2005).
30. Netea, M. G. *et al*. Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. *J. Immunol.* **172**, 3712-3718 (2004).
31. Cheng, S. C., Joosten, L. A., Kullberg, B. J. & Netea, M. G. Interplay between Candida albicans and the mammalian innate host defense. *Infect. Immun.* **80**, 1304-1313 (2012).
32. Chai, L. Y. *et al*. Modulation of Toll-like receptor 2 (TLR2) and TLR4 responses by Aspergillus fumigatus. *Infect. Immun.* **77**, 2184-2192 (2009).
33. Chai, L. Y. *et al*. Aspergillus fumigatus cell wall components differentially modulate host TLR2 and TLR4 responses. *Microbes Infect.* **13**, 151-159 (2011).

34. Chiapello, L. S. *et al*. Immunosuppression, interleukin-10 synthesis and apoptosis are induced in rats inoculated with Cryptococcus neoformans glucuronoxylomannan. *Immunology* **113**, 392-400 (2004).
35. Mednick, A. J., Nosanchuk, J. D. & Casadevall, A. Melanization of Cryptococcus neoformans affects lung inflammatory responses during cryptococcal infection. *Infect. Immun.* **73**, 2012-2019 (2005).
36. Brandhorst, T. T., Wuthrich, M., Finkel-Jimenez, B., Warner, T. & Klein, B. S. Exploiting type 3 complement receptor for TNF-alpha suppression, immune evasion, and progressive pulmonary fungal infection. *J. Immunol.* **173**, 7444-7453 (2004).
37. Tsai, H. F., Washburn, R. G., Chang, Y. C. & Kwon-Chung, K. J. Aspergillus fumigatus arp1 modulates conidial pigmentation and complement deposition. *Mol. Microbiol.* **26**, 175-183 (1997).
38. Tsai, H. F., Chang, Y. C., Washburn, R. G., Wheeler, M. H. & Kwon-Chung, K. J. The developmentally regulated alb1 gene of Aspergillus fumigatus: its role in modulation of conidial morphology and virulence. *J. Bacteriol.* **180**, 3031-3038 (1998).
39. Pihet, M. *et al*. Melanin is an essential component for the integrity of the cell wall of Aspergillus fumigatus conidia. *BMC Microbiol.* **9**, 177-2180-9-177 (2009).
40. Zipfel, P. F. & Skerka, C. Complement regulators and inhibitory proteins. *Nat. Rev. Immunol.* **9**, 729-740 (2009).
41. Poltermann, S. *et al*. Gpm1p is a factor H-, FHL-1-, and plasminogen-binding surface protein of Candida albicans. *J. Biol. Chem.* **282**, 37537-37544 (2007).
42. Lesiak-Markowicz, I. *et al*. Candida albicans Hgt1p, a multifunctional evasion molecule: complement inhibitor, CR3 analogue, and human immunodeficiency virus-binding molecule. *J. Infect. Dis.* **204**, 802-809 (2011).
43. Luo, S., Hoffmann, R., Skerka, C. & Zipfel, P. F. Glycerol-3-phosphate dehydrogenase 2 is a novel factor H-, factor H-like protein 1-, and plasminogen-binding surface protein of Candida albicans. *J. Infect. Dis.* **207**, 594-603 (2013).
44. Luo, S., Poltermann, S., Kunert, A., Rupp, S. & Zipfel, P. F. Immune evasion of the human pathogenic yeast Candida albicans: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. *Mol. Immunol.* **47**, 541-550 (2009).
45. Luo, S., Hartmann, A., Dahse, H. M., Skerka, C. & Zipfel, P. F. Secreted pH-regulated antigen 1 of Candida albicans blocks activation and conversion of complement C3. *J. Immunol.* **185**, 2164-2173 (2010).
46. Barthel, D., Schindler, S. & Zipfel, P. F. Plasminogen is a complement inhibitor. *J. Biol. Chem.* **287**, 18831-18842 (2012).
47. Gropp, K. *et al*. The yeast Candida albicans evades human complement attack by secretion of aspartic proteases. *Mol. Immunol.* **47**, 465-475 (2009).
48. Behnsen, J. *et al*. Secreted Aspergillus fumigatus protease Alp1 degrades human complement proteins C3, C4, and C5. *Infect. Immun.* **78**, 3585-3594 (2010).
49. Stano, P. *et al*. App1: an antiphagocytic protein that binds to complement receptors 3 and 2. *J. Immunol.* **182**, 84-91 (2009).
50. Losse, J. *et al*. Role of pH-regulated antigen 1 of Candida albicans in the fungal recognition and antifungal response of human neutrophils. *Mol. Immunol.* **48**, 2135-2143 (2011).
51. Drutz, D. J. & Huppert, M. Coccidioidomycosis: factors affecting the host-parasite interaction. *J. Infect. Dis.* **147**, 372-390 (1983).
52. Chun, C. D., Brown, J. C. & Madhani, H. D. A major role for capsule-independent phagocytosis-inhibitory mechanisms in mammalian infection by Cryptococcus neoformans. *Cell. Host Microbe* **9**, 243-251 (2011).
53. Filler, S. G. & Sheppard, D. C. Fungal invasion of normally non-phagocytic host cells. *PLoS Pathog.* **2**, e129 (2006).
54. Zhang, Z., Liu, R., Noordhoek, J. A. & Kauffman, H. F. Interaction of airway epithelial cells (A549) with spores and mycelium of Aspergillus fumigatus. *J. Infect.* **51**, 375-382 (2005).

55. Park, H. *et al*. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. *Cell. Microbiol.* **7**, 499-510 (2005).
56. Phan, Q. T. *et al*. Als3 is a Candida albicans invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol.* **5**, e64 (2007).
57. Chang, Y. C. *et al*. Cryptococcal yeast cells invade the central nervous system via transcellular penetration of the blood-brain barrier. *Infect. Immun.* **72**, 4985-4995 (2004).
58. Tavanti, A. *et al*. Candida albicans isolates with different genomic backgrounds display a differential response to macrophage infection. *Microbes Infect.* **8**, 791-800 (2006).
59. Kaposzta, R., Marodi, L., Hollinshead, M., Gordon, S. & da Silva, R. P. Rapid recruitment of late endosomes and lysosomes in mouse macrophages ingesting Candida albicans. *J. Cell. Sci.* **112 (Pt 19)**, 3237-3248 (1999).
60. Fernandez-Arenas, E. *et al*. Candida albicans actively modulates intracellular membrane trafficking in mouse macrophage phagosomes. *Cell. Microbiol.* **11**, 560-589 (2009).
61. Garcia-Rodas, R., Gonzalez-Camacho, F., Rodriguez-Tudela, J. L., Cuenca-Estrella, M. & Zaragoza, O. The interaction between Candida krusei and murine macrophages results in multiple outcomes, including intracellular survival and escape from killing. *Infect. Immun.* **79**, 2136-2144 (2011).
62. Seider, K. *et al*. The facultative intracellular pathogen Candida glabrata subverts macrophage cytokine production and phagolysosome maturation. *J. Immunol.* **187**, 3072-3086 (2011).
63. Garcia-Rodas, R. & Zaragoza, O. Catch me if you can: phagocytosis and killing avoidance by Cryptococcus neoformans. *FEMS Immunol. Med. Microbiol.* **64**, 147-161 (2012).
64. Johnston, S. A. & May, R. C. The human fungal pathogen Cryptococcus neoformans escapes macrophages by a phagosome emptying mechanism that is inhibited by Arp2/3 complex-mediated actin polymerisation. *PLoS Pathog.* **6**, e1001041 (2010).
65. Alvarez, M. & Casadevall, A. Phagosome extrusion and host-cell survival after Cryptococcus neoformans phagocytosis by macrophages. *Curr. Biol.* **16**, 2161-2165 (2006).
66. Chayakulkeeree, M. *et al*. SEC14 is a specific requirement for secretion of phospholipase B1 and pathogenicity of Cryptococcus neoformans. *Mol. Microbiol.* **80**, 1088-1101 (2011).
67. Eissenberg, L. G., Goldman, W. E. & Schlesinger, P. H. Histoplasma capsulatum modulates the acidification of phagolysosomes. *J. Exp. Med.* **177**, 1605-1611 (1993).
68. Strasser, J. E. *et al*. Regulation of the macrophage vacuolar ATPase and phagosome-lysosome fusion by Histoplasma capsulatum. *J. Immunol.* **162**, 6148-6154 (1999).
69. Heinekamp, T. *et al*. Aspergillus fumigatus melanins: interference with the host endocytosis pathway and impact on virulence. *Front. Microbiol.* **3**, 440 (2012).
70. Schroppel, K. *et al*. Suppression of type 2 NO-synthase activity in macrophages by Candida albicans. *Int. J. Med. Microbiol.* **290**, 659-668 (2001).
71. Chiranand, W. *et al*. CTA4 transcription factor mediates induction of nitrosative stress response in Candida albicans. *Eukaryot. Cell.* **7**, 268-278 (2008).
72. Ullmann, B. D. *et al*. Inducible defense mechanism against nitric oxide in Candida albicans. *Eukaryot. Cell.* **3**, 715-723 (2004).
73. Frohner, I. E., Bourgeois, C., Yatsyk, K., Majer, O. & Kuchler, K. Candida albicans cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol. Microbiol.* **71**, 240-252 (2009).
74. Chauhan, N., Latge, J. P. & Calderone, R. Signalling and oxidant adaptation in Candida albicans and Aspergillus fumigatus. *Nat. Rev. Microbiol.* **4**, 435-444 (2006).
75. Cuellar-Cruz, M. *et al*. High resistance to oxidative stress in the fungal pathogen Candida glabrata is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot. Cell.* **7**, 814-825 (2008).
76. Holbrook, E. D., Smolnycki, K. A., Youseff, B. H. & Rappleye, C. A. Redundant catalases detoxify phagocyte reactive oxygen and facilitate Histoplasma capsulatum pathogenesis. *Infect. Immun.* **81**, 2334-2346 (2013).

77. Youseff, B. H., Holbrook, E. D., Smolnycki, K. A. & Rappleye, C. A. Extracellular superoxide dismutase protects Histoplasma yeast cells from host-derived oxidative stress. *PLoS Pathog.* **8**, e1002713 (2012).
78. Paris, S. *et al*. Catalases of Aspergillus fumigatus. *Infect. Immun.* **71**, 3551-3562 (2003).
79. Rocco, N. M., Carmen, J. C. & Klein, B. S. Blastomyces dermatitidis yeast cells inhibit nitric oxide production by alveolar macrophage inducible nitric oxide synthase. *Infect. Immun.* **79**, 2385-2395 (2011).
80. Gonzalez, A., Hung, C. Y. & Cole, G. T. Coccidioides releases a soluble factor that suppresses nitric oxide production by murine primary macrophages. *Microb. Pathog.* **50**, 100-108 (2011).
81. Tsunawaki, S., Yoshida, L. S., Nishida, S., Kobayashi, T. & Shimoyama, T. Fungal metabolite gliotoxin inhibits assembly of the human respiratory burst NADPH oxidase. *Infect. Immun.* **72**, 3373-3382 (2004).
82. Gallagher, L. *et al*. The Aspergillus fumigatus protein GliK protects against oxidative stress and is essential for gliotoxin biosynthesis. *Eukaryot. Cell.* **11**, 1226-1238 (2012).
83. Jones, T. *et al*. The diploid genome sequence of Candida albicans. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 7329-7334 (2004).
84. Moran, G. P., Coleman, D. C. & Sullivan, D. J. Comparative genomics and the evolution of pathogenicity in human pathogenic fungi. *Eukaryot. Cell.* **10**, 34-42 (2011).
85. Kabir, M. A., Hussain, M. A. & Ahmad, Z. Candida albicans: A Model Organism for Studying Fungal Pathogens. *ISRN Microbiol.* **2012**, 538694 (2012).
86. Campos, E. G., Jesuino, R. S., Dantas Ada, S., Brigido Mde, M. & Felipe, M. S. Oxidative stress response in Paracoccidioides brasiliensis. *Genet. Mol. Res.* **4**, 409-429 (2005).
87. Kniemeyer, O., Schmidt, A. D., Vodisch, M., Wartenberg, D. & Brakhage, A. A. Identification of virulence determinants of the human pathogenic fungi Aspergillus fumigatus and Candida albicans by proteomics. *Int. J. Med. Microbiol.* **301**, 368-377 (2011).
88. Sorgo, A. G., Heilmann, C. J., Brul, S., de Koster, C. G. & Klis, F. M. Beyond the wall: Candida albicans secret(e)s to survive. *FEMS Microbiol. Lett.* **338**, 10-17 (2013).
89. Kuck, U. & Hoff, B. New tools for the genetic manipulation of filamentous fungi. *Appl. Microbiol. Biotechnol.* **86**, 51-62 (2010).
90. Samaranayake, D. P. & Hanes, S. D. Milestones in Candida albicans gene manipulation. *Fungal Genet. Biol.* **48**, 858-865 (2011).
91. Sutton, L. M.,3rd & Starzyk, M. J. Procedure and analysis of a useful method in determining mycelial dry weights from agar plates. *Appl. Microbiol.* **24**, 1011-1012 (1972).
92. Stagoj, M. N., Komel, R. & Comino, A. Microtiter plate assay of yeast cell number using the fluorescent dye calcofluor white M2R. *BioTechniques* **36**, 380-382 (2004).
93. Mor, N. & Goren, M. B. Discrepancy in assessment of phagosome-lysosome fusion with two lysosomal markers in murine macrophages infected with Candida albicans. *Infect. Immun.* **55**, 1663-1667 (1987).
94. Sampson, T. R. & Weiss, D. S. Exploiting CRISPR/Cas systems for biotechnology. *Bioessays* **36**, 34-38 (2014).