Determining the roles of *Candida albicans* secreted aspartyl proteases (SAPs) and macrophage pattern recognition receptors (PRRs) in host-pathogen interactions

A Major Qualifying Project submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the degree of Bachelor of Science in Biology & Biotechnology

Submitted by: Jeffrey Letourneau

Submitted to: Professor Reeta Rao, PhD

Date: April 26th, 2017

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

There are multiple people whose support and expertise made this project possible. First, I would like to thank Dr. Reeta Rao, my MQP advisor, for providing continuous guidance on this project and in my career as a microbiologist. It was her Medical Microbiology course that first got me interested in the field, and I will now be pursuing my PhD in Molecular Genetics and Microbiology at Duke University starting this fall, also thanks in part to her advice throughout my graduate school application process. Thank you to my lab mates, Toni Delorey, Pablo Reyes-Gutierrez, Meredith Rioux, and Bo Yang, for sharing their expertise, offering assistance on lab techniques, and making coming into lab and attending lab meetings fun. I would also like to thank Dr. Stu Levitz and Dr. Chrono Lee at the University of Massachusetts Medical School for providing the macrophage cell lines used in this study as well as Dr. Gerald Fink and Dr. Valmik Vyas at the Whitehead Institute for providing the plasmids used for the CRISPR experiments.

ABSTRACT

Candida albicans is an opportunistic yeast that causes diseases such as yeast infections, oral thrush, and systemic blood infections. The host-pathogen interactions between *C. albicans* and host immune cells such as macrophages are complex and dynamic, with pattern-recognition receptors (PRRs) such as Dectin-1 and the mannose receptor playing a key role in yeast phagocytosis via recognition of structures on the cell surface. Previous studies have yielded conflicting results about the relative importance of these and other PRRs, making further research necessary. Once internalized by macrophages, *C. albicans* is often able to survive phagocytosis by employing virulence factors such as secreted aspartyl proteases (SAPs). The goal of this project was to further elucidate the roles of host PRRs and yeast SAPs by using an *in vitro* macrophage invasion assay to quantify *C. albicans* survival following exposure to murine macrophages. The results indicate that murine macrophages lacking either the mannose receptor or Dectin-1 are impaired in their ability to eliminate *C. albicans*. However, simultaneous knockout of both receptors led to an enhanced ability to eliminate *C. albicans,* likely pointing to involvement of additional receptors, a phenomenon that merits further study. Single gene deletion of SAPs 1-6 in *C. albicans* was shown to cause decreased survival and may provide evidence for the potential use of SAPs as a target for novel antifungal therapy.

INTRODUCTION

Emerging fungal diseases

Opportunistic fungal infections have grown in frequency in recent years. These infections are caused by agents including common fungi such as *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* as well as rare and emerging pathogens. While many of these fungi are members of a healthy microbiome, any fungus has the potential to become invasive and cause a lethal infection if the individual is sufficiently immunocompromised. Populations at risk for these types of infection include organ transplant recipients, individuals with AIDS, patients undergoing immunosuppressive therapy, the elderly, and children born prematurely, among others (Pfaller & Diekema, 2004).

C. auris is one intriguing example of an emerging fungal pathogen of the *Candida* genus. *C. auris* was first identified in 2009, the causative agent of an ear infection of a patient in Japan. Since then, it has been associated with life-threatening, primarily nosocomial, infections at the international level, with outbreaks in regions such as Asia and South America. Like *C. albicans*, *C. auris* forms biofilms. However, one distinguishing feature of *C. auris* is its well documented high resistance to antifungals, including reduced susceptibility to azoles and amphotericin B (Sherry et al., 2016). A study by Chowdhary et al. (2014) examined the drug susceptibility of *C. auris* using 15 clinical isolates from north India, Japan, and Korea. Of these 15, all were resistant to fluconazole, 73% were resistant to voriconazole, 47% were resistant to flucytosine, and 40% had high minimum inhibitory concentrations (MICs) of caspofungin. Not surprisingly, this same study observed breakthrough fungemia in 29% of patients and failure of antifungal therapies in 67% of these. While it should be noted that individuals with healthy immune systems are at low risk for *C. auris* infections, the high resistance is poses a serious concern for immunocompromised patients (Sherry et al., 2016). Additionally, *C. auris* has been isolated from a number of tissue types and bodily fluids,

including pus and broncheoalveolar lavage (BAL) fluid (Chowdhary et al., 2014). This indicates that, while first discovered in an ear infection, *C. auris* may have the potential to cause various diseases.

Members of the *Candida* genus are some of the most common fungal pathogens. Over 17 *Candida* species have been shown to cause bloodstream infections. However, just four species cause around 95% of these infections: *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (Pfaller & Diekema, 2004). In this study, I will focus on *C. albicans*. Other opportunistic fungi include *Trichosporon spp.*, *Rhodotorula spp.*, and *Geotrichum capitatum*, and less common but increasingly drug-resistant species such as *Aspergillus terreus* (Pfaller & Diekema, 2004). The landscape of fungal infections is changing, as new species such as *C. auris* emerge and well-characterized species such as *C. albicans* develop drug resistance. Because of this, it is vital to continue to study these organisms in order to understand how they cause a disease state and how infections can best be treated.

Candida albicans **as a human pathogen**

Candida albicans is an opportunistic dimorphic fungus that causes disease in humans. A member of the healthy microbiota, *C. albicans* lives commensally in most humans, asymptomatically colonizing the reproductive tract, oral cavity, gastrointestinal tract, and skin. Other members of the host microbiota keep it in check and help prevent infection. However, this balance can be disrupted by changes in environmental pH, immunosuppressant therapy, or alterations to the microbiome ecology often caused, for example, by antibiotics. Frequently, these opportunistic infections are superficial mucosal and dermal infections, including vaginal yeast infections, thrush, and diaper rash (Nobile & Johnson, 2015). Three quarters of healthy women experience at least one vaginal yeast infection over the course of their lives and 5% suffer recurrent infections (Naglik, Challacombe, & Hube, 2003).

In addition to these superficial infections, *Candida* infections have the potential to be life-threatening, especially when the pathogen enters the bloodstream. Sepsis infections have significant mortality rates and species in the CTG clade including closely related species but primarily *C. albicans* cause 15% of hospital-acquired sepsis infections (Nobile & Johnson, 2015). Overall, *C. albicans* is the fourth leading cause of nosocomial infections, and the incidence of such infections has increased greatly over the past 20-30 years (Naglik et al., 2003).

One property of *C. albicans* that makes it an especially effective pathogen is its ability to reversibly switch between two morphologies: yeast and hyphal. Strains permanently locked into either morphology have been shown to be avirulent (Gantner, Simmons, & Underhill, 2005). A study by Saville, Lazzell, Monteagudo, and Lopez-Ribot (2003) demonstrated that the yeast form is necessary for systematic dispersal, while the hyphal form is required for pathogenicity via infiltration of host cells. Additionally, *C. albicans* acts as an effective pathogen through the formation of biofilms, which are comprised of multiple cell types (both yeast and hyphal as well as an intermediate pseudohyphal form) encased in a highly structured extracellular matrix (ECM). This poses a particular danger in medical devices, such as catheters and dentures, as biofilm accumulation on implanted devices can lead to easy access to the bloodstream for the pathogen (Nett, Sanchez, Cain, Ross, & Andes, 2011). While there are a number of antifungal medications currently available to treat these infections, systemic *C. albicans* infections are fatal 42% of the time even with proper treatment (Wisplinghoff et al., 2003).

Antifungals and antifungal resistance

The three most common classes of antifungals are azoles, polyenes, and echinocandins. All target components of the cell wall. Azoles and polyenes both work by targeting ergosterol, a sterol in the fungal cell membrane (Ford et al., 2015). Azoles such as fluconazole inhibit the enzyme

lanosterol 14-α-sterol demethylase, which converts lanosterol to ergosterol, resulting in a reduced ergosterol content. By reducing ergosterol content, these drugs alter the structure and function of the cell membrane in such a way that fungal growth is suppressed (Sanguinetti, Postarero, & Lass-Flörl, 2015). Polyenes, which include Amphotericin B, exhibit high toxicity and are therefore used sparingly. By contrast, azoles have relatively few side effects and is easily bioavailable orally, making them more widely used (Ford et al., 2015). Echinocandins like casponfungin are fungicidal, and act by inhibiting β(1,3)D-glucan synthase. This leads to a deformed cell wall, which results in cell death. The Infectious Diseases Society of America (ISDA) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) generally recommend echinocandins as the first line of treatment, with ISDA suggesting the use of azoles for patients with mild infections and who have not had prior azole exposure (Sanguinetti et al., 2015)

Many commonly used antifungals such as fluconazole are fungistatic, meaning they do not kill the fungus, but merely stops it from growing. The downside to this is that it gives the fungus time to potentially develop drug resistance (Ford et al., 2015). There are a number of ways fungi such as *C. albicans* can exhibit drug resistance. Some strains do have a natural resistance, but it is also common for strains that are initially susceptible to develop resistance. Resistance to fluconazole in *C. albicans* is rare at approximately 1.4%. Mechanisms of azole resistance include induction of multi-drug efflux pumps, upregulation of the target enzyme, and the development of bypass pathways. Resistance to echinocandins may come about by mutations in the target enzyme and initiation of adaptive stress response. Specifically, *Candida* species have been shown to increase chitin content in the cell wall in order to overcome the lack of β-glucans, as evidenced by strains that grow at supra-MIC concentrations of caspofungin and have significantly higher chitin content. Cross-resistance has been well documented between different azoles, and while cross-resistance among azoles and echinocandins is less common, there have been reports of multi-drug resistant *C. glabrata*. Polyenes

remain a class of drug for which resistance is very uncommon (Sanguinetti et al., 2015). Poorer clinical outcomes and increased treatment costs are direct consequences of drug resistance. Given the increase in rates of drug resistance, it is incredibly important to understand the molecular mechanisms of pathogenesis in fungi such as *C. albicans* in order to work toward the development of novel antifungals.

The yeast cell wall

While fungi such as *C. albicans* share considerable homology with humans, the fungal cell wall is an entire organelle not present in human cells, making it an ideal target for antifungal agents (Kitamura, Someya, Hata, Nakajima, & Takemura, 2009). The *C. albicans* cell wall is approximately 90% carbohydrate (25-30% mannans, 40% β-1,3-glucan, 20% β-1,6-glucan, and 1-2% chitin) and 10% protein (Gow & Hube, 2012; Klis, Groot, & Hellingwerf, 2001). The carbohydrates are typically the components recognized by the host immune system, while the proteins are involved in surface adhesion and other interactions with the host cell surfaces. There are three polysaccharide forms present in the cell wall: mannans, β-glucans, and chitin. Mannoproteins form the outer layer of and some mannan may exist in a free state within the cell wall. β-glucans, the most abundant

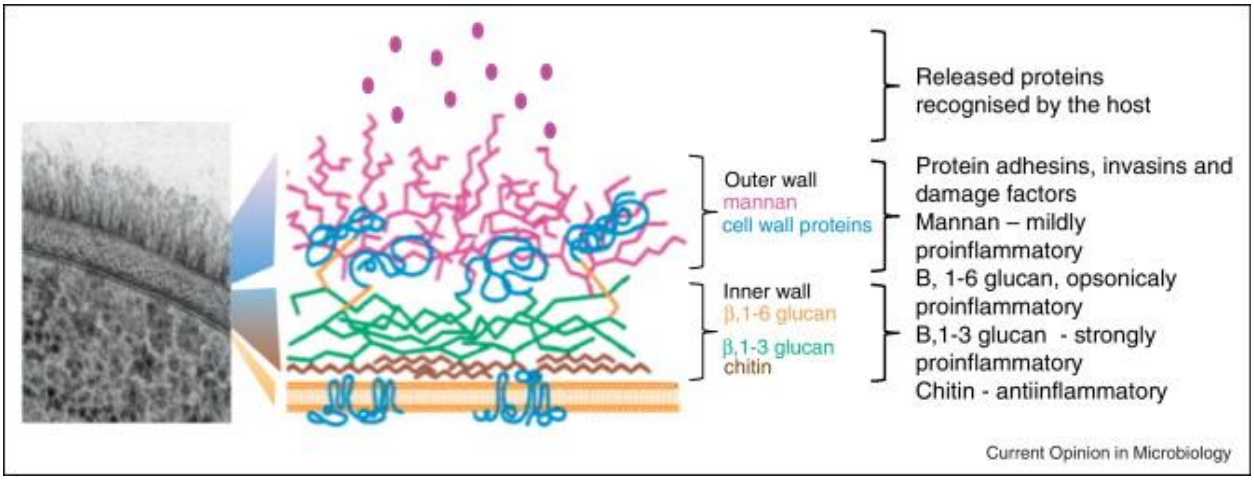

Figure 1. Transmission electron microscope image and diagram of the *C. albicans* cell wall (Gow & Hube, 2012).

component of the yeast cell wall, exist as either β-1,3 glucans, which are strongly proinflammatory and form a more inner layer, and β-1,6 glucans, which act as a linker molecule between this layer and the mannan layer (Cid et al., 1995). Together with chitin (and its deacetylated form chitosan) the β-glucans provide the rigid structure of the cell wall. Mannans (the polymerized form of individual mannose residues) are less structured, but have low permeability and porosity. Therefore, while they do not influence cell shape, the mannans influence the resistance of the wall to attack by host molecules (Gow & Hube, 2012). A diagram of the cell wall is shown in Figure 1.

The pathway by which β-glucans are synthesized in *C. albicans* has yet to be fully elucidated. Fortunately, cell wall pathways are relatively conserved among fungi and putative pathways have been developed for *C. albicans* based off studies in *Saccharomyces cerevisiae* as well as additional work

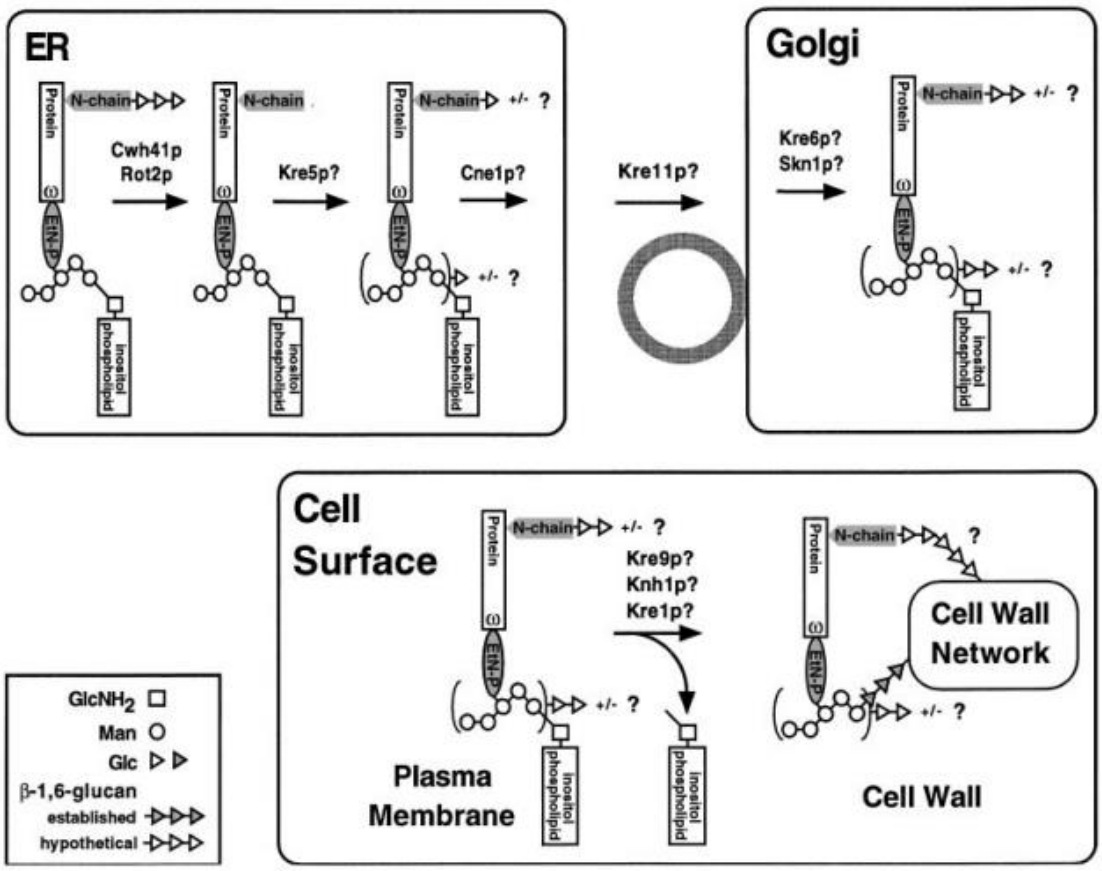

Figure 2. Proposed pathway of β-1, 6 glucan synthesis (Shahinian & Bussey, 2000).

conducted in *C. albicans* itself (Nett et al., 2011). Shahinian and Bussey (2000) developed such a pathway describing the synthesis of β-1,6-glucan, shown in Figure 2. Though β-1, 6 and β-1, 3 glucans are synthesized in different pathways, there may be some commonalities, including the fact that both polysaccharides are believed to be synthesized from UDP-glucose (Nett et al., 2011).

The pathway for β-1,3-glucan synthesis is less clear, though four particular downstream components of the protein kinase C (PKC) pathway have been implicated. *FKS1* and *FKS2* are believed to act redundantly on UDP-glucose. In *S.* cerevisiae, disruption of either gene is viable, but disruption of both is lethal. In C. *albicans*, disruption of *FKS1* is lethal, but disruption of *FKS2* is not. These genes are regulated by way of transcription factor *RLM1*, which is thought to be activated by *SMI1*. The function of *SMI1* is not fully known, but there is evidence that it plays a role in both β-glucan production and deposition. The *SMI1* knockout has enhanced antifungal susceptibility, thinner cell walls, a 4-fold reduction in biofilm β-glucans, and a 50% reduction in *FKS1* transcripts. The study of β-glucans is particularly important as these cell wall components have been shown to play a role in biofilm-specific drug resistance, possibly by sequestering drugs, thus preventing them from reaching the pathogen cells (Nett et al., 2011).

Pattern-recognition receptors (PRRs)

In order to combat pathogens such as *C. albicans*, immune cells employ pattern recognition receptors (PRRs) to identify conserved structures in microbes. These structures are termed pathogen-associated molecular patterns (PAMPs). PRRs may also recognize damage-associated molecular patterns (DAMPs), endogenous molecules released by damaged host cells that signal infection (Takeuchi & Akira, 2010). Recognition of *C. albicans* is primarily based on cell wall components (Jouault et al., 2009). Defense against organisms such as *C. albicans* is complex, and multiple cell types, PRRs, and signaling pathways all play a role (Netea & Maródi, 2010). PRRs may

be expressed in a number of immune cells, including macrophages and dendritic cells (Takeuchi & Akira, 2010).

The PRRs known to play a major role in yeast recognition are shown in Figure 3, along with the structures they recognize. Note that both Dectin-1 and MR interact with TLR2 and induce a pro-inflammatory response when activated (Jouault et al., 2009). Interestingly, the pro-inflammatory response induced by recognition of C. albicans is necessary for the establishment of infection (Jouault et al., 2009). There are four families of PRRs: toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG I-like receptors (RLRs), and NOD-like receptors (Takeuchi & Akira, 2010). This study focuses primarily on two CLRs: Dectin-1 and the mannose receptor (MR), which are encoded by the genes *CLEC7A* and *MRC1*, respectively. CLRs recognize polysaccharide structures on pathogens. These receptors play a central role in the recognition of fungal pathogens and help to induce the innate immune response (Netea & Maródi, 2010).

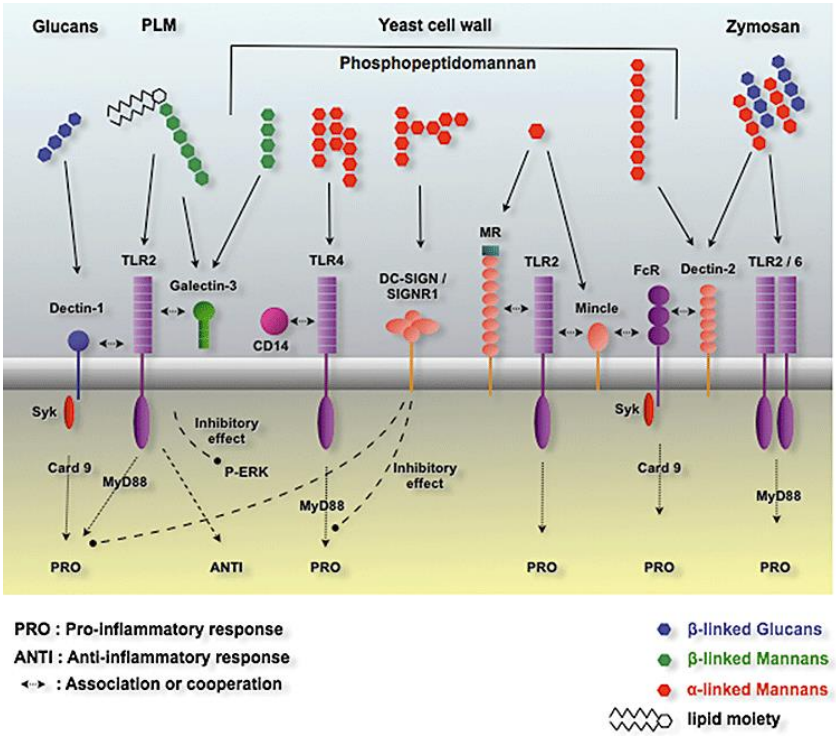

Figure 3. Diagram of major host PRRs and the yeast structures they recognize (Jouault et al., 2009).

The primary PRR for β-glucans is Dectin-1, which is mostly expressed in myeloid cells such as monocytes and macrophages (Saijo et al., 2007; Netea & Maródi, 2010). The precise role of β-glucans in antifungal immunity is unclear. However, interactions between Dectin-1 and β-glucans are known to trigger the inflammasome complexes in host cells (Gow & Hube, 2012). Following

recognition of β-glucans by Dectin-1, the host cell engulfs the pathogen via phagocytosis and signaling pathways trigger the release of pro-inflammatory cytokines and the production of reactive oxygen species (Huang et al., 2012; Gantner, Simmons, & Underhill, 2005). Specifically, Dectin-1 signals via Syk kinase and CARD9, which in turn induces pro-inflammatory signaling via IL-2 and IL-10 (Netea & Maródi, 2010). The Dectin-1 pathway is nonredundant and not compensated by other PRRs (Gow et al., 2007). There are two conflicting models for how Dectin-1 triggers phagocytosis. In the "trigger model," Dectin-1 activation directly initiates phagocytosis, and this is the model most supported by present data. However, the alternative "zipper model" suggests that additional receptors are involved to coordinate phagocytosis following pathogen recognition by Dectin-1 (Gantner et al., 2005).

Various studies have attempted to elucidate the role of Dectin-1 using *in vitro* and *in vivo* models. Dectin-1 deficient leukocytes have been shown to demonstrate a significantly impaired response to fungal pathogens (Taylor et al., 2007). Dectin-1 knockout mouse models have yielded conflicting results, with mutant mice demonstrating increased mortality in some models but not others (Huang et al., 2012). In a study by Taylor et al. (2007), Dectin-1 knockout mice demonstrated decreased survival and higher fungal burden in response to *C. albicans* infection. However, a subsequent study by Saijo et al. (2007) using a different mouse line found the knockout mice equally susceptible to infection by *C. albicans*, but did find a greater susceptibility to *Pneumocystis* infection (Netea & Maródi, 2010). These conflicting results reflect the complex nature of pathogen recognition and indicate the need for further studies into Dectin-1 and other PRRs.

There is an interesting relationship between C. albicans morphology and activation of Dectin-1. As shown in Figure 1 and described previously in the text, β-glucans are largely shielded by outer cell wall components such as mannans. However, yeast budding and cell separation results in "bud and birth scars" which expose β-glucans. In the hyphal form, filamentous growth results in

no such separation and as a result, Dectin-1 is unable to recognize the pathogen (Gantner et al., 2005).

MR is another C-type lectin receptor that recognizes molecules with terminal mannose residues, including mannans (Dan et al., 2008). MR mediates phagocytosis by macrophages following recognition of mannan (Jouault et al., 2009). This activation also results in production of cytokines such as IL-17, which controls the neutrophil-mediated inflammatory response and induces protective Th17 response (van de Veerdonk et al., 2009; Netea & Maródi, 2010). In contrast to Dectin-1, the expression of mannans at the cell surface means that MR recognition of *C. albicans* is unlikely to be dependent on cellular morphology (van de Veerdonk et al., 2009). Furthermore, a study by Gow et al. (2007) found that in recognition of live *C. albicans*, mannans have the dominant role in immune stimulation, not β-glucans. This is rather logical, given that mannans are more external and more easily accessible by host PRRs.

As with Dectin-1, multiple studies have been performed to understand the role of MR *in vivo*. An *in vivo* infection experiment conducted by Lee, Zheng, Clavijo, & Nussenzweig (2003) used MR-deficient mice and found no significant difference in mortality between WT and MR -/- mice. Furthermore, this study found that MR -/- murine macrophages were not impeded in their ability to uptake *C. albicans* via phagocytosis. Interestingly, the authors found that addition of β-glucan could block phagocytosis. Based on these data, they concluded that MR is not required for host defense and that a β-glucan receptor likely plays a more prominent role, in opposition to the findings by Gow et al. (2007) that MR plays the dominant role in yeast recognition. However, Lee et al. (2003) did observe higher fungal burdens in some organs of the mice, despite the comparable mortality. Others have questioned the methods used in this study, as the *C. albicans* challenge was administered intraperitoneally, a method with little clinical relevance. Additionally, the inhibition of *Candida* uptake caused by mannans is stronger than β–glucan-induced inhibition (Netea & Maródi, 2010). A

similar study by Dan, Kelly, Lee, & Levitz (2008) subjected MR -/- mice to a pulmonary *Cryptococcus neoformans* challenge and observed higher mortality and higher pulmonary fungal burden in the MR -/- mice. However, no difference was found with infection by *Pneumocystic carinii*. It is possible that this difference is due in part to soluble mannosylated ligands released from *C. neoformans*, initiating a more robust response. Given the conflicting nature of some of these findings, further research is still necessary in order to determine how significant a role MR plays in *C. albicans* infection.

Dectin-1 and MR may interact during fungal recognition and phagocytosis, though this mechanism has not been studied in depth. One way in which MR activity may actually assist in Dectin-1 activation is by helping to expose β–glucans. Researchers have speculated that the initial host inflammatory response brought about by MR activation likely helps to degrade the outer mannan layer, at which point β–glucans become exposed, resulting in a further amplified immune response (Netea & Maródi, 2010). The Dectin-1-TLR2 complex has also been found to augment MR-induced IL-17 secretion (van de Veerdonk et al., 2009). Because of this, it would be valuable to conduct further research examining both receptors at once, such as using a double knockout.

As seen in Figure 3, there are a number of receptors in addition to Dectin-1 and MR that play a role in recognition and phagocytosis of *C. albicans*. Other CLRs include Dectin-2, Galcetin-3, DC-SIGN, and DC-SIGNR. Dectin-2 recognizes structures with high mannose content and induces TNF production. It likely plays a role in hyphal recognition (Netea & Maródi, 2010). In dendritic cells, activation of Dectin-1 or Dectin-2 causes the cell to interact with T cells and instruct them to confer immunity against *C. albicans* (Takeuchi & Akira, 2010). Galectin-3 recognizes specific β-1,2 mannosides specific to *Candida* (Jouault et al., 2009). DC-SIGN and DC-SIGNR are two related receptors expressed mainly in dendritic cells that also detect structures with high mannose content (Netea & Maródi, 2010).

In addition to CLRs, multiple TLRs including TLR2, TLR4, and TLR9 are involved in *C. albicans* PAMP recognition. These TLRs have also been shown to interact with other receptors such as Dectin-1 and MR (Netea & Maródi, 2010). The two TLRs most involved in *C. albicans* recognition are TLR2 and TLR4 (Jouault et al., 2009). Blocking TLR2 leads to decreased production of TNF, IL-10, and IL-1β as well as increased production of IL-12 and IFNγ following stimulation by *C. albicans*. Contrary to expectations, TLR2 -/- mice demonstrate increased resistance to *C. albicans* and TLR2 -/- macrophages are enhanced in their ability to contain *C. albicans in vivo*. These results indicate that TLR2 activation induces an immunosuppressive effect (Netea & Maródi, 2010). TLR4 recognizes mannans in yeast as well as lipopolysaccharides in bacteria. However, not all strains of *C.* albicans are recognizable to TLR4. TLR4-defective mice are more susceptible to infection, produce fewer chemokines, and demonstrate impaired recruitment of neutrophils (Netea & Maródi, 2010).

TLRs have also been shown to form complexes with other PRRs. Dectin-1 most likely forms a co-receptor complex with TLR2 (Gow & Hube, 2012). Several studies have indicated that collaboration with other PRRs, primarily TLR2, is required for proper signaling via Dectin-1 (Netea & Maródi, 2010). Dectin-1 interacts with TLRs in order to induce pro-inflammatory signaling. Simultaneous activation of Dectin-1 and TLR2 leads to a significant increase in the production of cytokines such as TNFα and IL-12 (Gantner et al., 2005). MR has also been shown to associate with TLR2 following stimulation by certain fungi such as *Pneumocystis* and Galectin-3 associates with TLR2 during pathogen recognition as well (Jouault et al., 2009). The number of receptors involved in yeast recognition and the complexity of interactions between different PRRs makes understanding host-pathogen interactions of *C. albicans* a difficult but important task.

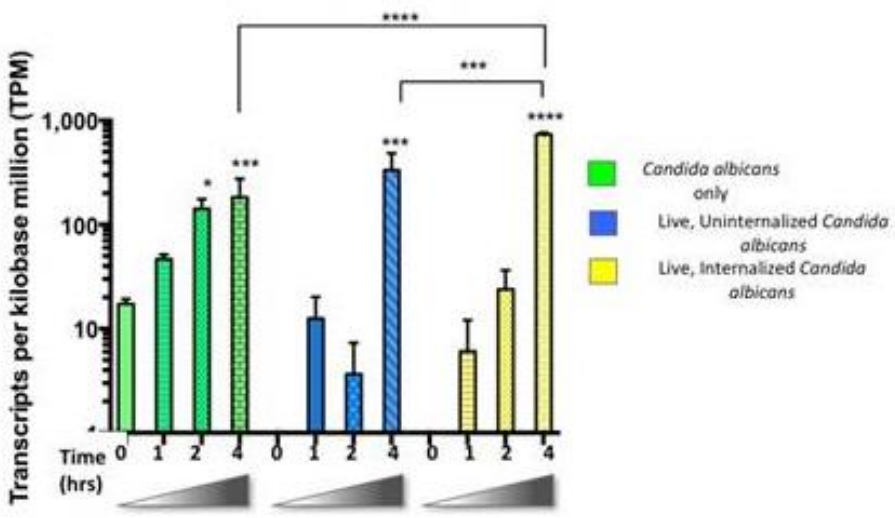

Figure 4. SAP5 transcription in live *C. albicans* over time. Mean of 3 biological replicates and standard error of mean (SEM) plotted. Significance within groups is calculated by comparing each sample to the ten minute control for that group. One-way ANOVA, multiple comparisons. * = p ≤ .05, ** = p ≤ .01, *** = p ≤ 0.001, **** = p ≤ 0.001 (Delorey, data not published).

Secreted aspartyl proteases (SAPs)

While host immune cells use PRRs to identify *C.* albicans, C. *albicans* in turn uses numerous virulence factors to invade and damage host cells. These are represented as pink circles in Figure 1. Previous work in our lab and a collaborator lab at the Broad Institute has focused on one family of virulence factors called secreted aspartyl proteases (SAPs). RNA-seq data has demonstrated that SAP genes, especially SAPs 5 and 6, are upregulated in *C. albicans* cells which successfully overcome phagocytosis by macrophages (Delorey, data not published). These results point toward a key role played by SAPs 4-6 in pathogenesis by directly preventing macrophage killing. Figure 4 is a graph of transcriptional data for SAP5 in live *C. albicans* and shows that SAP5 expression is significantly upregulated at 4 hours in cells that have been internalized by macrophages. Working off of this and other data, a logical next step would be to quantify survival of *C. albicans* lacking SAP genes in an infection assay.

In *C. albicans,* ten genes encode for SAP preproenzymes which gain approximately 60 amino acids when processed in the secretory pathway, which occurs concurrently with transportation. These proteins facilitate adhesion and invasion by distorting host cell membranes, digest foreign molecules for nutrient acquisition, and digest immune cells to evade attack. SAPs are produced by

post pathogenic *Candida* species, including *C. tropicalis* and *C. parapsilosis*, though many of these have significantly fewer SAP genes than *C. albicans*. Non-pathogenic or minimally pathogenic *Candida* species produce insignificant quantities of protease, even though many possess SAP genes. Even within *C. albicans*, protease production is associated with virulence, as clinical isolates from patients with vaginal candidiasis were 1.5- to 2-fold more proteolytic than isolates from asymptomatic carriers. Additionally, more proteolytic strains have been showed to adhere more strongly to human epithelial cells (Naglik et al., 2003)

While there are four types of protease (the other three being serine, cysteine, and metalloproteases), SAPs are the only secreted protease in *Candida*. Interestingly, these SAP genes are among the mere 6-7% of genes in *C. albicans* with no homologs in *S. cerevisiae*. Figure 3 shows a dendogram of the 10 *C. albicans* SAP genes. The two clusters at the top, SAPs 1-3 and SAPs 4-6, have historically been the most studied. Simplistically speaking, yeast cells predominantly express SAPs 1-3, whereas hyphal cells predominantly express SAPs 4-6. Expression of SAPs 4-6 is not only associated with hyphal formation, but these processes have been found to be coordinately regulated. These three SAPs are up to 89% identical, and the optimal pH for their activity is 5.0. This is an important characteristic, as the pH of macrophage phagolysosomes is typically between 4.7-4.8 (Naglik et al., 2003).

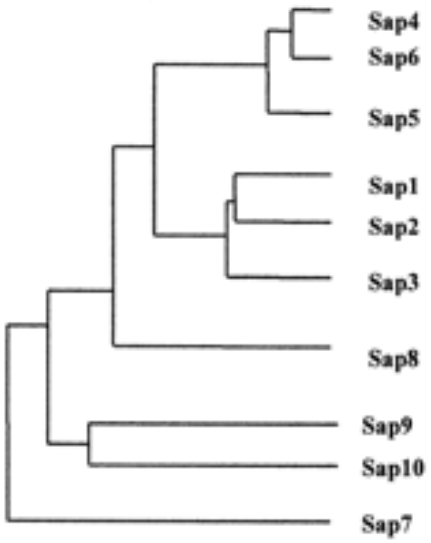

Figure 5. Dendogram of SAP genes in *C. albicans* (Naglik et al., 2003).

Additional studies have provided evidence for the role of SAPs 4-6 in pathogenesis. The *sap456* -/- triple mutant has been shown to exhibit reduced invasiveness, though it is still able to form hyphae (Naglik et al., 2003). After phagocytosis by murine peritoneal macrophages, SAPs 4-6 but not SAPs 1-3 are upregulated

and the *sap456* -/- mutant is significantly more susceptible to phagocytosis than the wild-type, with 53% more mutant cells eliminated in killing assays. It is currently unknown whether these proteins act directly on phagolysosomal enzymes involved in microbial killing or on other key enzymes involved in macrophage metabolism (Borg-von Zepelin, Beggah, Boggian, Sanglard, & Monod, 1998). In this project, these results are expanded upon using single SAP knockout strains and triple knockout *sap123* -/- and *sap456* -/- strains.

Further elucidating host-pathogen interactions

In this Major Qualifying Project, the roles of yeast SAPs and macrophage receptors in host-pathogen interactions were explored by using mutants of both *C. albicans* and murine macrophages. First, wild-type SC5314 was tested in a macrophage invasion assay along with four macrophage cell lines: wild-type (WT), Dectin-1 (D1) knockout, MR knockout, and the double knockout of both Dectin-1 and MR. *C. albicans* was expected to have a higher percent survival when plated with macrophages lacking these receptors, as they would be impaired in their ability to recognize and phagocytize the pathogen (Fig. 6C).

Next, to provide further evidence for the role of *SMI1* in production of β-glucans and to reveal how β-glucan levels affect pathogenesis, engineering of a *SMI1* knockout strain of *C. albicans* was attempted in order to assay this new strain with the four macrophage cell lines. Since the macrophage would have significantly fewer β-glucans to bind to, this *C. albicans* strain was expected to exhibit a higher survival rate than the wild-type. Percent survival against the D1 mutant should be comparable between WT and *smi1* -/- *C. albicans*, since the macrophage cannot recognize the pathogen by β-glucans either way. Similarly, the highest survival is expected with the MR knockout macrophage line and the D1 MR double knockout, since both will be unable to recognize either mannans or β-glucans.

Finally, *C. albicans* knockout strains of SAPs 1-6 individually and the triple mutants *sap123* -/- and *sap456* -/- were assayed against the same macrophage cell line set. *C. albicans* lacking SAP genes were hypothesized to exhibit decreased survival since they will be impaired in their ability to degrade macrophage host proteins and thereby escape phagocytosis (Fig. 6B). Based on prior RNA-seq data, we expected SAPs 5 and 6 to play the greatest role in overcoming phagocytosis, since these genes were the most upregulated in live *C. albicans* exposed to macrophages. Furthermore, the triple mutants were expected to have the poorest survival, as the lack of multiple SAP proteins would further reduce their defenses. Overall, the results of this project will provide important insight into how *C. albicans* interacts with host immune cells. Understanding such mechanisms is integral if we are to develop novel therapeutics to treat infections caused by this pathogen.

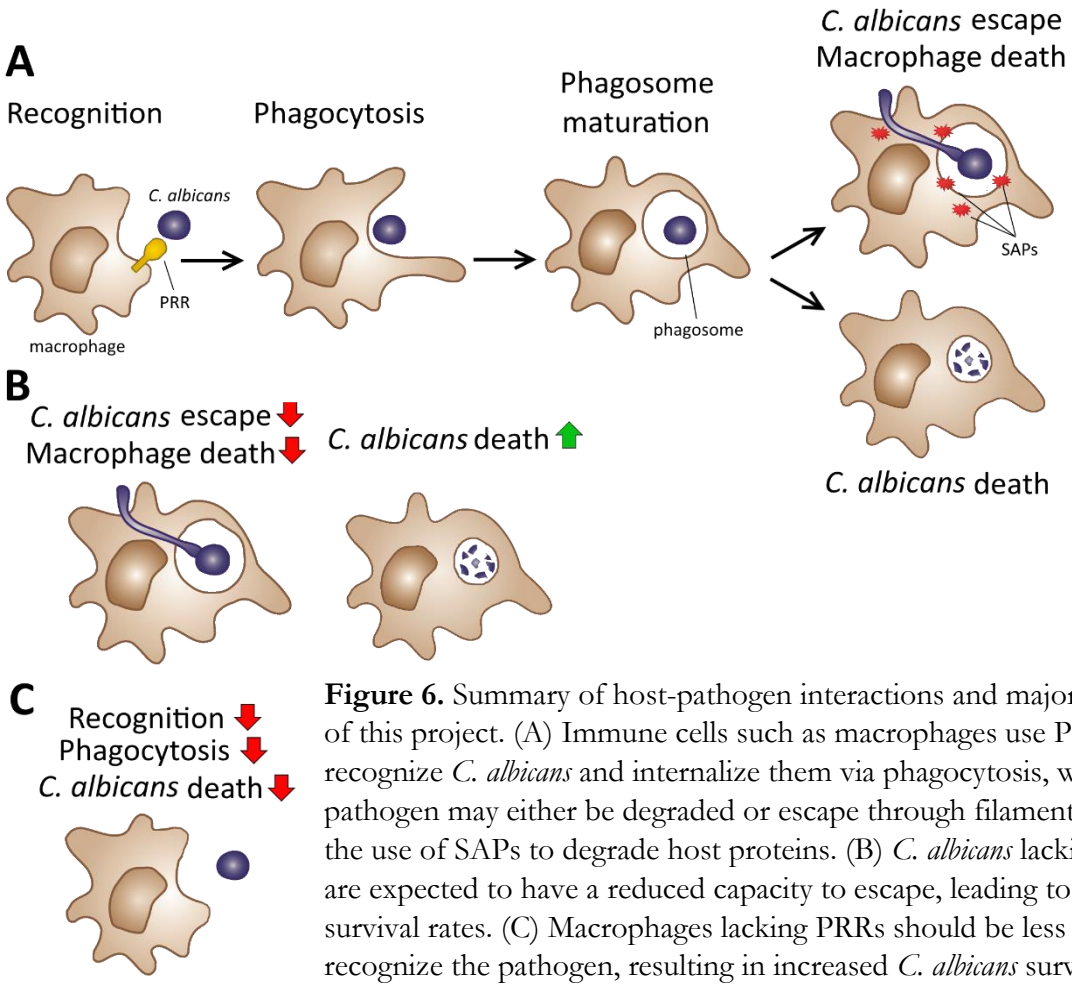

Figure 6. Summary of host-pathogen interactions and major hypotheses of this project. (A) Immune cells such as macrophages use PRRs to recognize *C. albicans* and internalize them via phagocytosis, where the pathogen may either be degraded or escape through filamentation and the use of SAPs to degrade host proteins. (B) *C. albicans* lacking SAPs are expected to have a reduced capacity to escape, leading to decreased survival rates. (C) Macrophages lacking PRRs should be less able to recognize the pathogen, resulting in increased *C. albicans* survival.

METHODS

Macrophage invasion assay

In order to investigate host-pathogen interactions, a macrophage invasion assay was used to quantify survival rates of *C. albicans* when plated alongside murine macrophages. Macrophages were received from the Levitz Lab at UMass Medical School and included the wild-type NR-9456 (WT) as well as mutants lacking the Dectin-1 receptor (D1), the mannose receptor (MR), or both (D1 MR). RAW 264.7 cells were also used as an additional control. Strains of *C. albicans* used included wild-type SC5314 as well as mutants lacking individual SAP genes 1-6 (*sap1* -/- through *sap6* -/-) as well as triple knockouts *sap123* -/- and *sap456* -/-.

The assay was performed according to a protocol adapted from da Rosa, Boyartchuk, Zhu, & Kaufman (2010). Macrophages were grown in DMEM containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37° C and 5% CO_2. These cells were plated at a density of 2×10^6 cells/well in 9.6 cm^2 wells using six-well plates. After one hour of incubation so that the cells could adhere, *C. albicans* was added at a 1:15 ratio (1.33×10^5 cells/well) for a final volume of 2 ml per well. Plates were spun down for three minutes at 3000 rpm to further facilitate cell adhesion, since we initially observed PRR mutants failing to adhere as well as the WT macrophages. Plates were incubated for approximately 8 hours and cells were scraped and collected in 8 ml 0.02% Triton X-100 to lyse the macrophages. Finally, a 10^{-2} dilution of the Triton X-100 cell suspension was plated on YPD and incubated for 24 hours at 37° C. The number of colony forming units (CFU) was counted and percent survival was calculated based on the number of CFU present for the *C. albicans* samples grown in parallel without macrophages. All conditions were done in triplicate (n=3) and some macrophage-*C. albicans* combinations were assayed a second time (n=6) to verify results.

Results gathered on different days were compared by normalizing all data to a "WT/WT" control of SC5314 (WT) *C. albicans* with WT macrophages. For each sample, normalized survival was calculated as the ratio of the observed raw percent survival to the percent survival for the WT/WT control on that day. Statistical significance was calculated using a two-tailed Student's t-test.

β-glucan modified ELISA

In order to measure the total cellular levels of β-glucan in the various *C. albicans* strains used, a modified ELISA was used, following a protocol from Pablo Reyes-Gutierrez. All spin-down steps were 5 min at 4200 rpm (3000g) unless otherwise stated and were followed by discarding the supernatant. Yeast cultures, grown overnight in a 96-well plate, were killed by incubation in boiling water for 5 min. Plates were spun down, cells were resuspended in 50 µl PBS containing 2% BSA, and plates were incubated at room temperature for one hour using a swinging mixer. Plates were spun down again and cells were resuspended in 50 µl of the antibody solution (antibody against either 1,3- or 1,6-β-glucan), then incubated at 37º for one hour on a swinging mixer. Plates were spun down and wells were washed three times with PBS containing 0.05% Tween-20 (PBS-T), with cells being resuspended and spun down for 3 min at 4200 rpm after each wash. Cells were then resuspended in 50 µl of the secondary antibody solution (2 µg/ml goat anti-human HRP-conjugate antibody for 1,6-β-glucan or 1 µg/ml rabbit anti-mouse HRP-conjugate antibody for 1,3-β-glucan). Plates were incubated at 37º for one hour on a swinging mixer and again spun down. All wells were washed three times with PBS-T and finally resuspended in 50 µl/well chemiluminescent peroxidase substrate. Luminescence was measured in a microplate reader.

CRISPR/Cas9 gene knockout

The protocol used for the attempted CRISPR-mediated knockout of *SMI1* was adapted from Vyas, Barrasa, & Fink (2015). The *SMI1* gene was selected since it is putatively involved in β-glucan production and deposition, and knocking it out still produces a viable organism (Nett et al., 2011). Guide RNAs were identified by searching the database provided in the Vyas et al. (2015) supplement for sequences specific to both alleles of *SMI1* and containing the NGG protospacer adjacent motif (PAM). In addition to *SMI1*, guide sequences for *ADE2* were also used as a positive control, as *ADE2* knockout was successfully achieved in the study by Vyas et al. (2015). Primers were then designed based on the sequence of the pV1393 plasmid used in this study, such that the top strand was of the format $ATTTGN_{20}G$ and the bottom strand was of the format $AAAACN_{20}C$, where N_{20} is the guide sequence (not including the PAM). Repair templates were made by identifying a 100 bp region centered on the cut site, designing two 60 nt oligos with a 20 bp overlap, and extending these oligos using PCR. In these repair templates, the PAM site was mutated to add a unique restriction site (EcoRI) and a stop codon was added as well to knock out the gene. All primers and oligos used in this study are given in Table S1.

DH5α competent *Escherichia coli* were transformed with the plasmid obtained from Valmik Vyas at the Whitehead Institute Fink Lab in order to grow a stock of the plasmid. Successful transformants were selected for on LB plates containing 50 µg/ml nourseothricin (Nat) and 200 µg/ml ampicillin (Amp). Liquid cultures were grown from single colonies and the plasmid was obtained by miniprep using a QIAprep Spin Miniprep Kit. Concentration was obtained using a Nanodrop machine.

Guide sequences were next cloned into plasmid pV1393, the map of which is shown in Figure S1. 2 µg of plasmid was digested per reaction by combining it with 5 µl 10x NEB3.1 buffer, 1 µl BsmBI, and water to 50 µl. This mixture was incubated for 20 min at 55° C, cooled to room

temperature, spun down, 1 µl CIP was added, and then it was incubated at 37º C for one hour. To purify this sample, a QIAquick Gel Extraction Column was used, eluting in 30 µl buffer EB and again using the Nanodrop to measure the concentration. Guide oligos were phosphorylated and annealed by adding to a PCR tube 0.5 µl of each the top and bottom strands (stock concentration 100 µM), 5 µl 10x T4 ligase buffer, 1 µl T4 polynucleotide kinase, and 43 µl water. This was incubated in a thermocycler for 30 min at 37° C then 5 min at 95º C and finally cooled to 16° C on the slowest ramp available. These guide oligos were then ligated into the cut plasmid by combining in a PCR tube 1 µl 10x T4 ligase buffer, 0.5 µl ligase, 0.5 µl annealed oligos, 20-40 ng pV1393, and water to 10µl, then incubating in a thermocycler for 30 min at 16° C, 10 min at 65º C, then cooling to 25º C. DH5α *E. coli* was transformed with this recombinant plasmid and successful transformants were selected for as described above.

Selected colonies were miniprepped as described above, and successful cloning was verified by performing two diagnostic digests, an EcoRI digest and a double digest with BsmBI and BglII. The digested plasmid was then subject to gel electrophoresis. Plasmids extracted from colonies that were determined to have the guide sequence successfully cloned in were then used to transform *C. albicans* using a lithium acetate transformation protocol. pV1393 was linearized by SacI-HF and KpnI-HF double digest, and 1-2 µg of the linearized plasmid was used in the transformation along with 6-8 µg of repair template. *C. albicans* transformants were selected for on YPD plates containing 200 µg/ml Nat.

RESULTS

Comparison of cell lines used in this study

In order to verify that the NR-9456 macrophage cell line (WT) is a viable alternative to the more commonly used RAW cells, cell morphology and ability to clear *C. albicans* were compared between the two cell lines. Under the microscope, WT and RAW cells appear similar in morphology, with cells taking on a variety of shapes ranging from circular to spread out with visible pseudopods, as shown in Figure 7. However, it is noteworthy that, over time, the WT macrophages displayed a greater range of morphologies, such as forming dense microcolonies and highly elongated cells, whereas RAW morphology was more or less constant over time. The mutant cell lines exhibited traits similar to the isogenic WT, including temporal variations, but overall had a higher frequency of

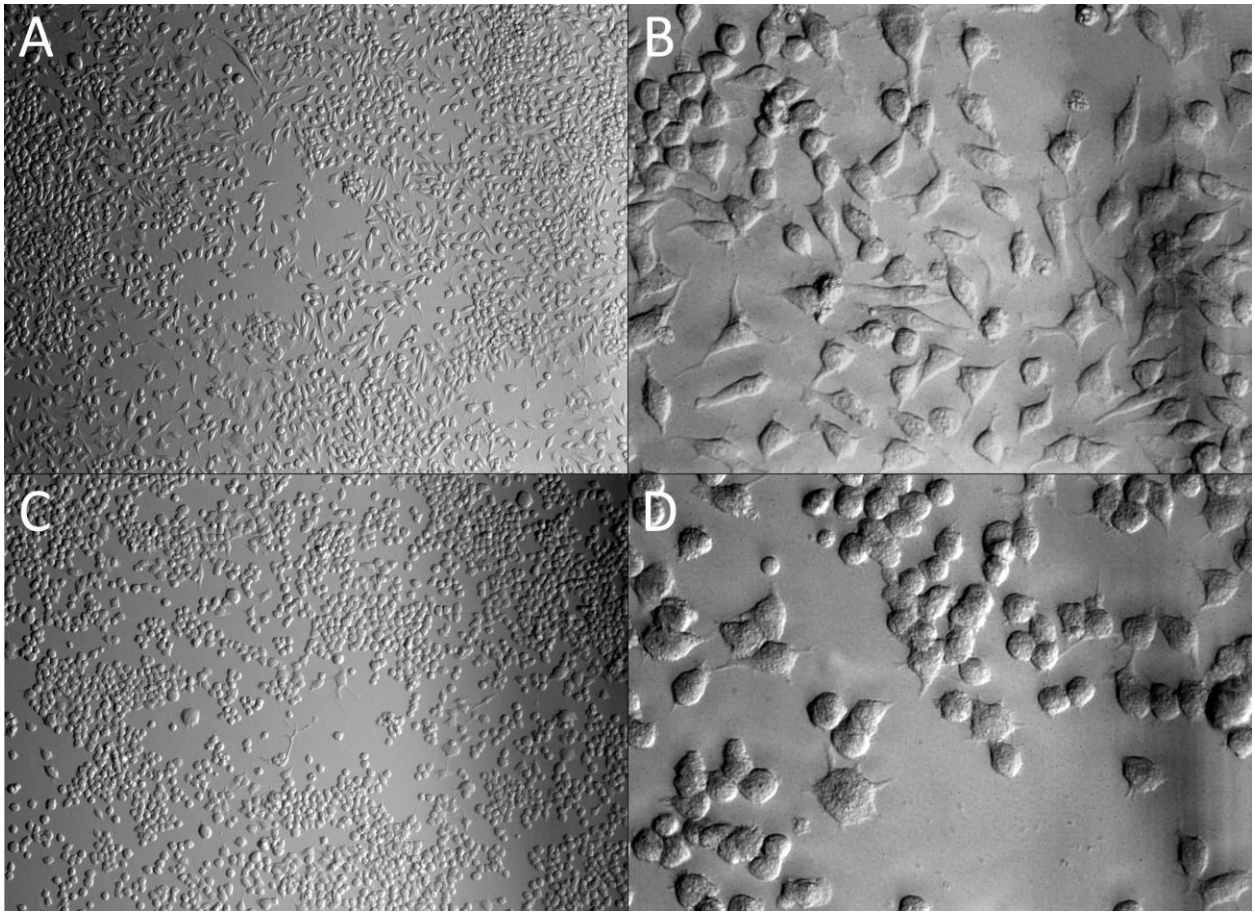

Figure 7. Morphological comparison of macrophage cell lines used in this study. Microscope images of RAW cells at 100x (A) and 400x magnification (B) and WT NR-9456 cells at 100x (C) and 400x (D) magnification.

the circular morphology. Next, survival rates of each *C. albicans* strain used in the study were compared between the two cell lines. As shown in Figure 8, there was no significant difference between the two cell lines for all *C. albicans* strains except SC5314 and the *sap456* -/- triple knockout isolate B. Overall, the trends in *C. albicans* survival increases and decreases in survival were comparable for both WT and RAW cells.

The macrophage invasion assay protocol was used to measure the survival of each *C. albicans* strain when plated with each macrophage cell line. As described in the methods section, percent survival was calculated by determining the ratio of the number of CFUs for each sample to the number of CFUs of *C. albicans* of that strain grown in parallel without macrophages. These data were used in two ways: to determine the effect of SAP gene knockout on *C. albicans* survival for each macrophage cell line and to determine the effect of macrophage PRR knockout on survival for each individual *C. albicans* strain.

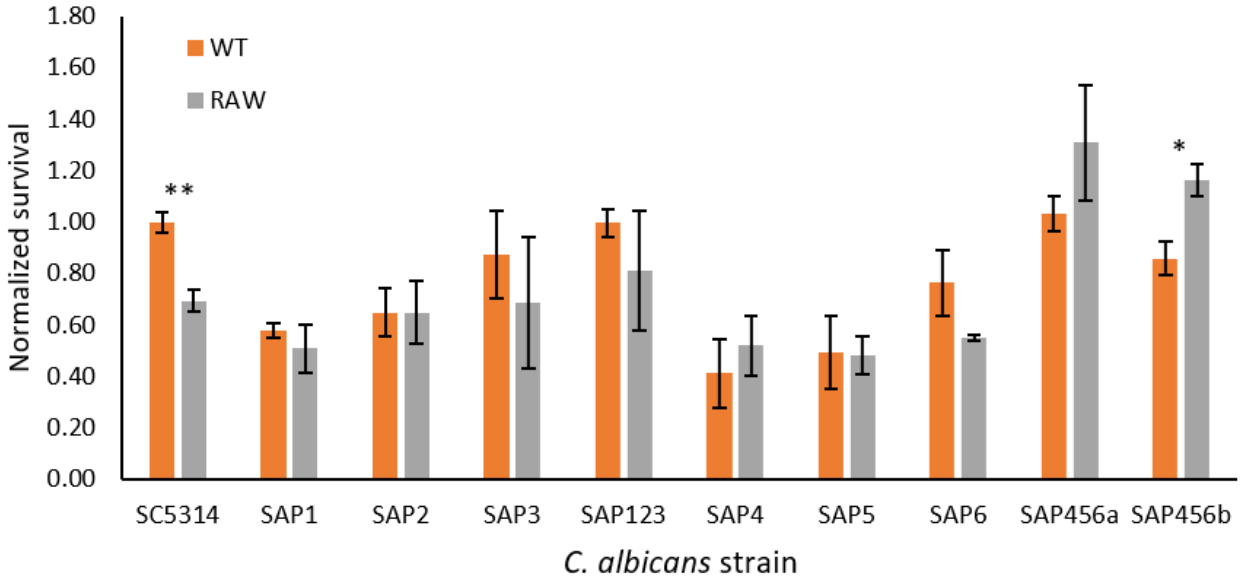

Figure 8. Comparison of WT macrophage (orange) and RAW 264.7 (gray) profiles with respect to the effect of SAP gene knockout on *C. albicans* survival after 8 hours macrophage-*C. albicans* co-culture. Mean of 3 biological replicates (exceptions: n=6 for RAW SC5314 and n=28 for WT SC5314) and SEM plotted. Significance within groups calculated by 2-tailed t-test comparing WT and RAW cell lines for that *C. albicans* strain. * = p<.05, ** = p<.01, *** = p<.001.

Effect of *C. albicans* SAP knockout

In Figure 9, the survival for each SAP mutant is compared to the survival of SC5314 for each macrophage cell line to test the hypothesis that *C. albicans* lacking SAPs will be impaired in their ability to defend against phagocytosis and thereby exhibit decreased survival. For WT macrophages, knocking out SAPs 1, 2, 4, and 5 significantly reduced *C. albicans* survival and this decreased was greatest for *sap4* -/- and *sap5* -/-. There was also an observed decrease for SAPs 3 and 6, though it was not significant. Interestingly, the survival rate for *sap123* -/- and *sap456* -/- isolate A were approximately equal to the survival of SC5314 (Fig. 9A). For RAW cells, there was a significant decrease in survival for *sap5* -/- and a significant increase for both *sap456* -/- isolates (Fig. 9B.) The D1 knockout macrophages had decreases in survival for all SAP single mutants, which was significant in *sap3* -/- and *sap4* -/-. Survival increases were observed for *sap123* -/- and *sap456* -/- isolate A (Fig. 9C). All SAP mutants exhibited decreased survival when exposed to the MR knockout macrophages. This decrease was statistically significant for *sap1* -/-, *sap2* -/-, *sap3* -/-, *sap123* -/-, *sap6* -/- and both *sap456* -/- isolates and was greatest for knockouts of SAPs 1-3 (Fig. 9D). For the D1 MR double knockout cell line, only *sap3* -/- showed a significant decrease in survival (Fig. 9E). Note that, in general, the survival rates of the triple mutants were inconsistent with those of the single mutants for the same macrophage type, and the two isolates of *sap456* -/- exhibited profiles inconsistent with each other.

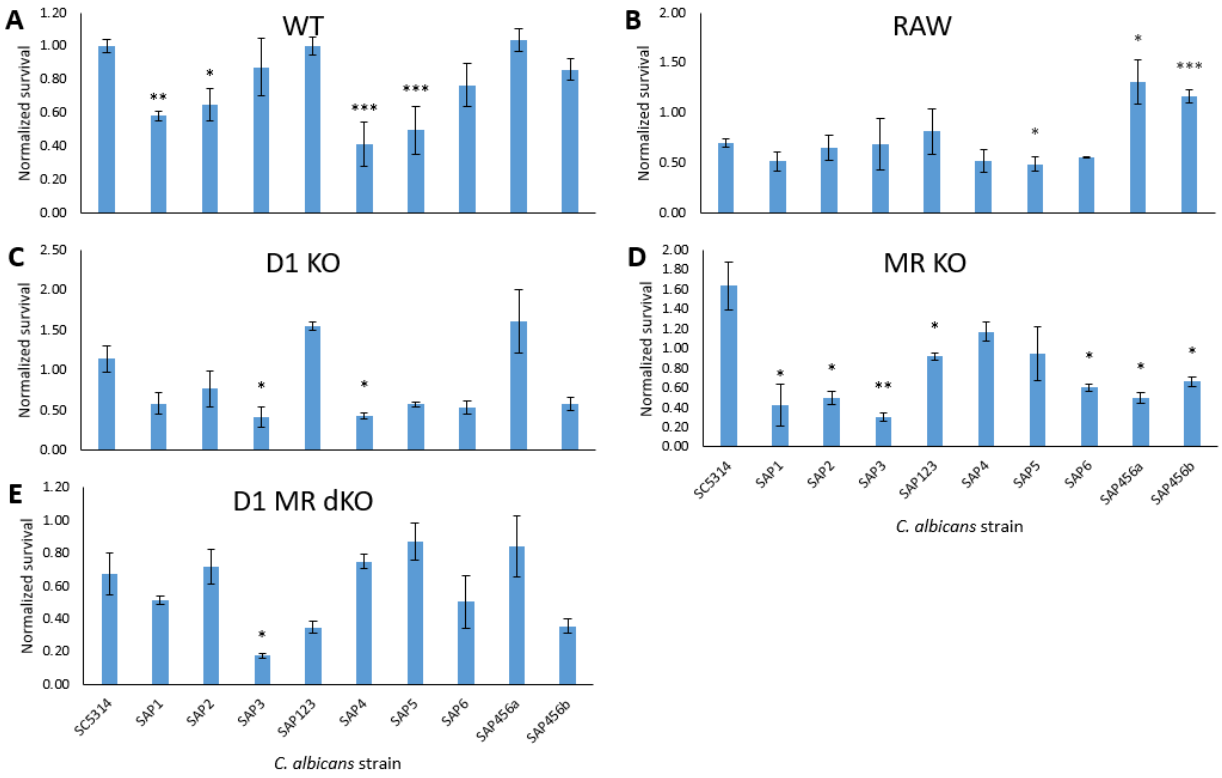

Figure 9. Effect of SAP gene knockout on *C. albicans* survival when grown for 8 hours with WT macrophages (A), RAW 264.7 cells (B), D1 KO macrophages (C), MR KO macrophages (D), and D1 MR dKO macrophages (E). Mean of 3 biological replicates (exceptions: n=6 for WT SAP456a, RAW SC5314, RAW SAP456a, D1 SAP456a, and D1 MR dKO SC5314; n=9 for D1 SC5314; n=28 for WT SC5314) and SEM plotted. Significance calculated by 2-tailed t-test comparing each sample to the SC5314 sample for that macrophage cell line. * = p<.05, ** = p<.01, *** = p<.001.

Effect of macrophage PRR knockout

The same data from Figure 9 was analyzed again to compare survival for each *C. albicans* strain plated with various macrophage line to test the hypothesis that macrophages lacking the PRRs D1 and MR are less effective at clearing *C. albicans*. This comparison is shown graphically in Figure 10. For wild-type *C. albicans* SC5314, survival was increased significantly with the MR knockout macrophage, and slightly (not significantly) for the D1 knockout as compared to WT (Fig. 10A). A survival increase was also observed for the MR in *sap4* -/- and *sap5* -/-, which was significant in the former and, interestingly, there was a decrease in MR for *sap3* -/- and *sap456* -/- isolate A (Fig. 10D, F, G, I). Unexpectedly, there was a significant survival decrease for the D1 MR double knockout cell

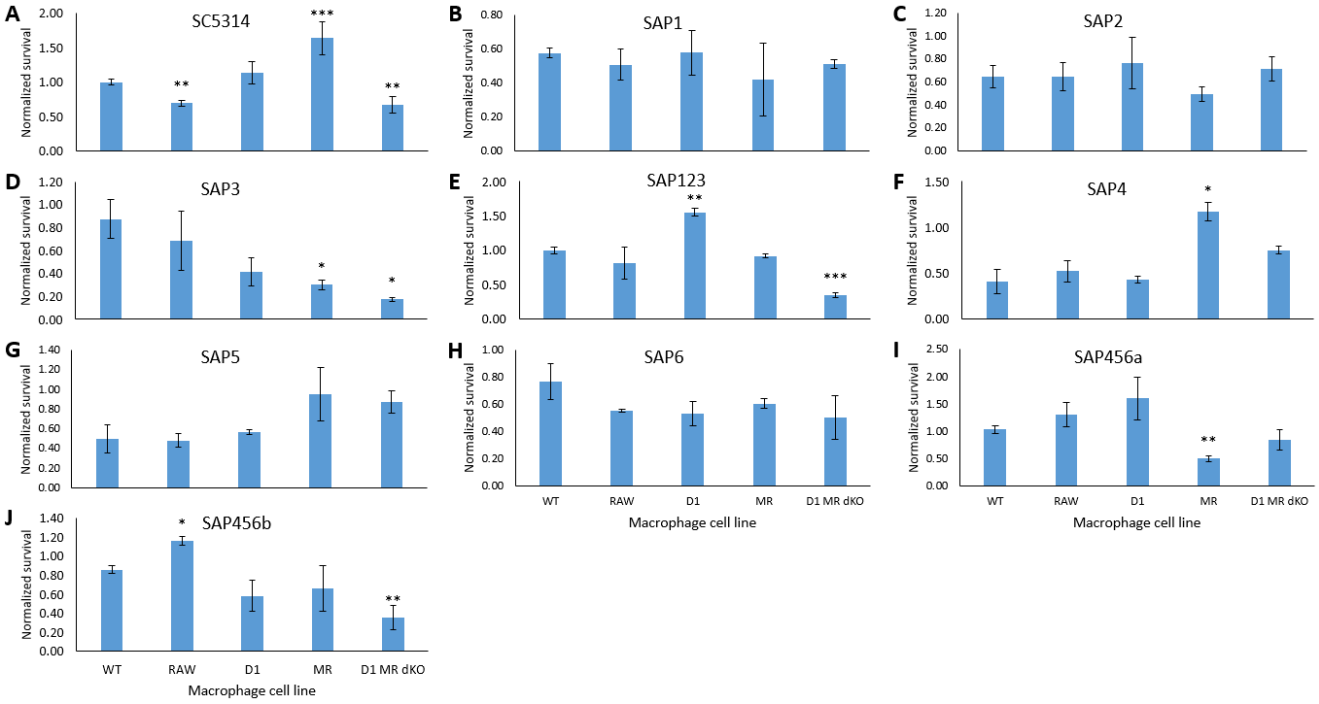

Figure 10. Effect of macrophage PRR knockout on *C. albicans* survival after 8 hours growth with macrophages, for the wild-type *C. albicans* strain SC5314 (A) and knockouts of SAP1 (B), SAP2 (C), SAP3 (D), SAP123 (E), SAP4 (F), SAP5 (G), SAP6 (H), and SAP456 isolates A (I) and B (J). Mean of 3 biological replicates (exceptions: n=6 for SC5314 RAW, SC5314 D1 MR dKO, SAP456a WT, SAP456a RAW, and SAP456a D1; n=9 for SC5314 D1; n=28 for SC5314 WT) and SEM plotted. Significance calculated by 2-tailed t-test comparing each sample to the WT macrophage sample for that *C. albicans* strain. * = p<.05, ** = p<.01, *** = p<.001.

line in SC5314 as well as *sap3* -/-, *sap123* -/-, and *sap456* -/- isolate B (Fig. 10A, D, E, J). There were no significant differences in survival between the five macrophage cell lines used in this study for *sap1* -/-, *sap2* -/-, *sap5* -/-, and *sap6* -/- (Fig. 10B, C, G, H).

Quantification of total β-glucan in SAP mutants

As a control to validate the assumption that β-glucan levels would be approximately equal between the SAP knockout strains and SC5314, a modified ELISA was used to quantify the levels of β-1,3-glucan and β-1,6-glucan for each *C. albicans* strains used in this study. Contrary to expectations, while levels of β-1,3-glucan remained approximately equal for the single SAP mutants as compared with SC5314, the levels of β-1,6-glucan increased for knockouts of SAPs 2-6, and this increase was

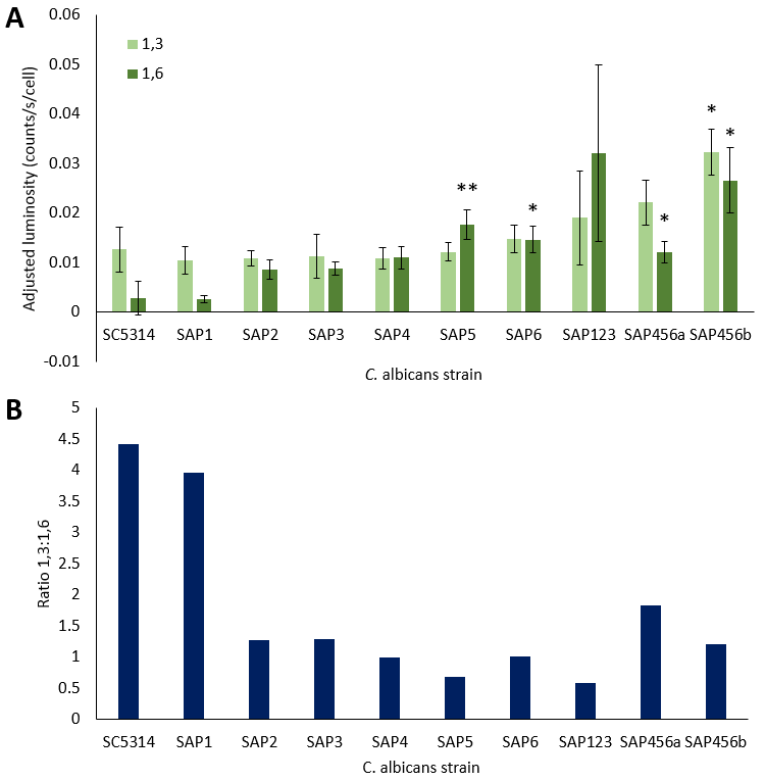

Figure 11. β-glucan ELISA results for all *C. albicans* strains used in this study. (A) Levels of β-1,3-glucan (light green) and β-1,6-glucan (dark green) per cell as determined by modified ELISA. Mean of 6 biological replicates and SEM plotted. Significance calculated by 2-tailed t-test comparing each sample to the level of either β-1,3-glucan or β-1,6-glucan for SC5314. * = p<.05, ** = p<.01. (B) Ratio of β-1,3-glucan to β-1,6-glucan for each *C. albicans* strain.

significant for *sap5* -/- and *sap6* -/-. The levels of both β-glucans were elevated for the triple mutants, significantly so for β-1,3-glucan in *sap456* -/- isolate A and β-1,6-glucan in both *sap456* -/- isolates (Fig. 11A). These data were used to calculate the ratio of β-1,3-glucan to β-1,6-glucan for each strain. The 1,3:1,6 ratio for SC5314 was approximately 4.5 and there were noticeable decreases in this ratio for all strains except *sap1* -/- (Fig. 11B).

Attempted SMI1 knockout

In order to knock out the SMI1 gene in *C. albicans*, plasmid pV1393 was grown up in *E. coli* and extracted by miniprep. Following digestion with BsmBI, guide sequences for either SMI1 or ADE2 (a positive control that turns *C. albicans* colonies red when knocked out) were cloned into pV1393, as described in greater detail in the Methods section. These plasmids with guide sequences were then again transformed to *E. coli* to grow a sufficient stock and plasmids were again extracted

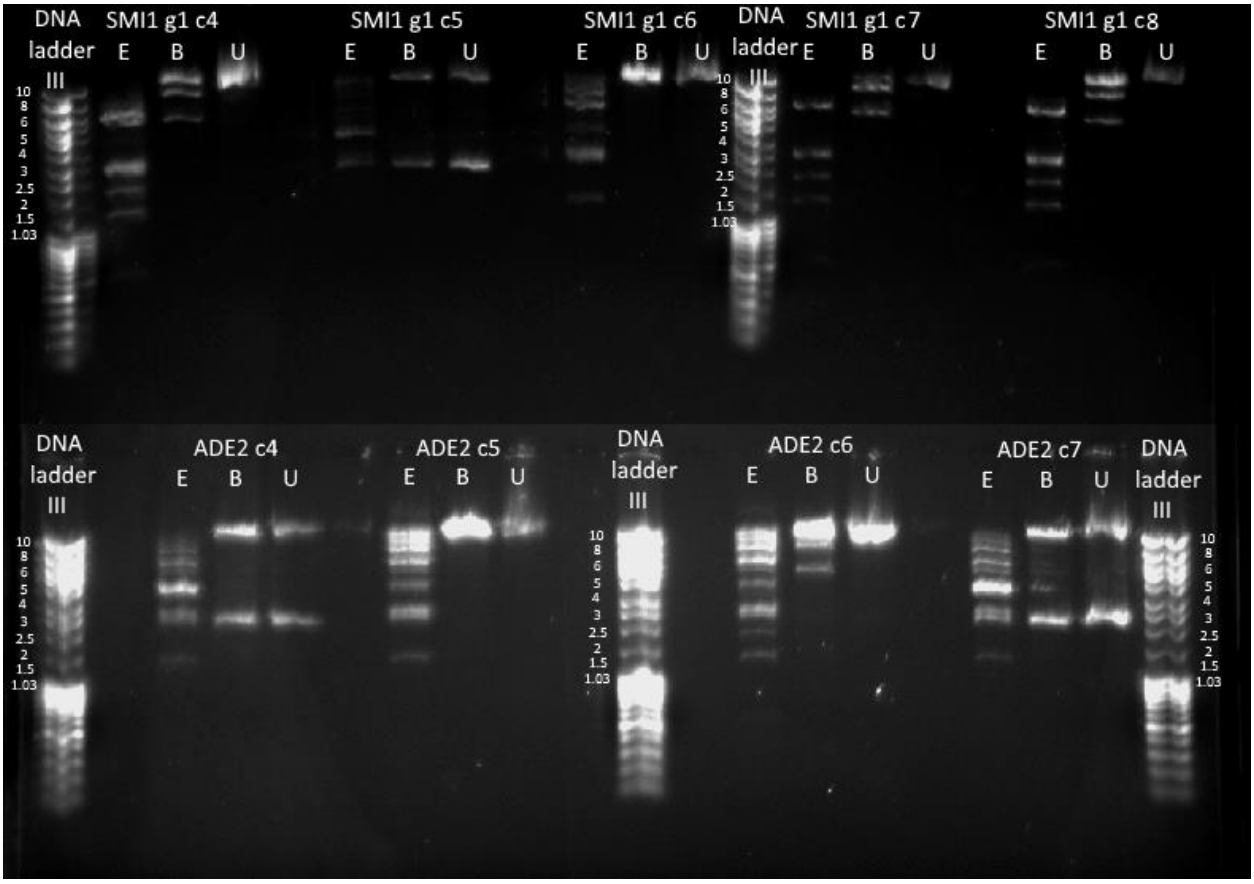

Figure 12. Gel electrophoresis of digested CRISPR plasmid following insertion of guide sequences specific to SMI1 (top) and ADE2 (bottom). For each colony isolate, extracted plasmid was run on the gel following EcoRI digest (E), following BsmBI + BglII double digest (B), and uncut (U). DNA ladder III run in parallel with labeled fragment sizes in kilobase pairs.

by miniprep. In order to confirm successful cloning, miniprep products were subject to digest by EcoRI or double digest by BsmBI and BglII (Fig. 12). Based on plasmid sequence, EcoRI was expected to digest pV1393 into fragments of 6.24, 2.605, 2.483, 1.812, and 0.493 kbp. Since a small portion of the plasmid is lost following successful cloning of a guide sequence, EcoRI should digest guide-containing pV1393 into fragments of 6.24, 2.605, 2.483, 2.307, and 1.335 kbp (Fig. S2). The adjacent BsmBI cut sites are also lost following insertion of the guide sequences, meaning that double digest by BsmBI and BglII should linearize the plasmid if the guide sequence has been cloned in correctly. However, if the guides were not cloned in, the BsmBI sites will still be present, resulting in two fragments of about 5.2 and 9.7 kbp. The total length of pV1393 is 15.0 kbp. Note

that the EcoRI digest and BsmBI/BglII digest are two methods of verifying the same result: whether or not guides have been cloned in.

As shown in Figure 12, the plasmids extracted from SMI1 colonies 4, 7, and 8 as well as ADE2 colony 6 showed three bands, two at approximately 5 kbp and 10 kbp, and a third above 10 kbp. This likely indicates a mixture of pV1393 with and without guide sequences cloned in present in the sample. These samples also had a band between 1.5 and 2 kbp for the EcoRI digest, as well as a band at approximately 0.5 kbp, both of which are characteristic of plasmids without guide sequences inserted. SMI1 colony 6 and ADE2 colony 5 had only one band in the B lane and lacked the 1.8 and 0.5 kbp bands in the E lane, characteristic of plasmids with the guide sequences. SMI1 colony 5 and ADE2 colonies 4 and 7 also had this profile, but with an additional band in all three lanes (include uncut) between 2.5-3 kbp. Because of the presence of this unaccounted for DNA, only SMI1 c6 and ADE2 c5 were selected to move forward for transformation of *C. albicans*.

Unfortunately, four attempts at transformation of *C. albicans* yielded no positive results, as either no colonies grew or an approximately equal number of colonies grew for *C. albicans* transformed with these plasmids and corresponding repair templates as with *C. albicans* transformed in parallel with only the repair template. Additionally, the red colonies characteristic of *ade2* -/- mutants were not observed (although following one transformation, pink colonies were observed on all plates and determined to be bacterial contamination). Possible reasons for the failure of this transformation and additional strategies to circumvent these issues are described in the discussion section.

DISCUSSION

The results of this Major Qualifying Project provide new evidence regarding the roles of *C. albicans* SAPs and the macrophage PRRs Dectin-1 and mannose receptor in phagocytosis and yeast survival. The use of a panel of macrophages with different mutations to assay multiple strains of *C. albicans* against was novel, and therefore it was necessary to verify that our isogenic wild-type was equivalent to a cell line commonly used in similar infection assays. RAW 264.7 cells are frequently used as an immortalized macrophage line. These cells were originally derived by Raschke, Baird, Ralph, & Nakoinz (1978) from tumors that had been induced in mice with the Abelson leukemia virus. By contrast, the NR-9456 line used in this study is an immortalized cell line derived from wild-type mouse primary bone marrow cells (BEI Resources, 2014). We chose to use this cell line because primary bone marrow-derived cells should in theory be closer to macrophages *in vivo* encountered by *C. albicans* than tumor-derived cells. In support of this idea, there are substantial documented differences between RAWs and bone-marrow derived macrophages (BMDMs). Proteomic analysis comparing the two found that RAWs are greatly reduced in phagosomal functions including acidification and proteolysis. RAWs also expressed more Dectin-1 and less MR than BMDMs (Guo et al., 2015)

WT NR-9456 and RAW 264.7 macrophages were found to have similar morphology and similar rates of clearance of the various *C. albicans* strains used in this study, indicating that NR-9456 is a reasonable substitute (Fig. 7, Fig. 8). However, one important distinction was that *C. albicans* survived significantly less when plated with RAW as compared to WT (Fig. 8, Fig. 10A). As a consequence, only SAP5 exhibited a significant decrease in survival as compared with SC5314 for RAW cells, whereas multiple SAP mutants exhibited decreased survival with WT, described in more detail below (Fig. 9A-B). This difference is likely due to differences in cytokine production and

receptor expression in RAW cells and may raise some concerns about the accuracy of RAW cells in infection assays (Guo et al., 2015).

This study is the first to show that BMDMs lacking either Dectin-1 or MR were impaired in their ability to eliminate *C. albicans*. Taylor et al. (2007) found that peritoneal macrophages lacking Dectin-1 were decreased in their ability to recognize, but not kill, *C. albicans*, though they did find a decrease in killing efficiency in D1-deficient leukocytes. Lee et al. (2003) found that MR -/- murine peritoneal macrophages phagocytosed *C. albicans* as efficiently as the wild-type, but did not measure *C. albicans* survival. As in the case of RAW cells, there are significant phenotypic differences between BMDMs and other macrophage types such as peritoneal macrophages (PEMs). A recent study found that PEMs and BMDMs are phenotypically distinct, and differ from macrophages in lesions with respect to expression of M1/M2 markers and lipid metabolism genes. (Bisgaard et al., 2016). Additionally, BMDMs were found to be more effective at phagocytosis and proliferated more robustly than PEMs or splenic macrophages (SPMs), and all three populations exhibited very different profiles of cytokine production. For example, PEMs expressed more of the pro-inflammatory IL-12 (Wang et al., 2013). At present, it is unknown whether one cell line is more representative of the conditions encountered by pathogens *in vivo*, and it is therefore necessary to conduct *in vitro* studies using multiple lines as well as *in vivo* experiments in order to gain a more thorough understanding of these interactions.

In addition to considering various macrophage cell lines, there are also variations within a given macrophage population. Macrophages are not phenotypically constant, but instead exhibit plasticity, able to polarize into activated subtypes. M1 (classically activated) macrophages are pro-inflammatory whereas M2 (alternatively activated) macrophages are anti-inflammatory and play a role in clearing damaged tissue. Interestingly, while M1 macrophages demonstrate an enhanced ability to clear microbes, their phagosomes fail to acidify, while M2 macrophages reach a pH of less

than 5.0 (Canton, Khezri, Glogauer, & Grinstein, 2014). M2 macrophages have also been shown to have increased expression of both Dectin-1 and the mannose receptor (Martinez, Gordon, Locati, & Mantovani, 2006). It is important to note that tumor-derived macrophages such as RAWs are highly biased towards the M2 phenotype (Mantovani, Sozzani, Locati, Allavena, & Sica, 2002). This is another reason BMDMs may yield results with greater *in vivo* relevance, as they retain the potential to differentiate in a way lost by RAWs.

In this study, macrophages lacking Dectin-1 and/or the mannose receptor were used to test the hypothesis that disruption of these receptors inhibits the macrophages' ability to phagocytose yeast, leading to greater *C. albicans* survival. For wild-type *C. albicans* (SC5314), loss of Dectin-1 led to slightly increased survival (not significant, $p=0.23$) and loss of the mannose receptor led to significantly increased survival ($p=1.4x10^{-4}$) (Fig. 10A). This may indicate that the mannose receptor plays a greater role in yeast recognition, which is somewhat logical given that mannans are on the outer surface of the cell wall (Fig. 1). However, past studies have yielded conflicting results as to which of these receptors play a greater role, and it is impossible to say with certainty to what extent these *in vitro* results translate to pathogen recognition *in vivo*, and some authors have cautioned against drawing such conclusions (Lee et al., 2003; Gow et al., 2007; Bisgaard et al., 2016).

The D1 MR double knockout line was expected to produce the greatest yeast survival since it lacks both key receptors. However, survival of SC5314 with this line was significantly decreased ($p=4.4x10^{-3}$) as compared with WT macrophages (Fig. 10A). As with the triple SAP mutants, this unexpected result may indicate a mistake in the cell line's genetic engineering or a compensatory mechanism. However, I hypothesize that this decrease in survival stems from the relationships between D1, MR, and TLR2, as both D1 and MR are known to interact and form co-receptor complexes with TLR2 (Fig. 3; van de Veerdonk et al., 2009; Jouault et al., 2009). TLR2 binds phospholipomannans on *C. albicans* and multiple studies have found that macrophages lacking TLR2

are enhanced in their ability to contain *C. albicans in vitro* and *in vivo* (Blasi et al., 2005; Netea & Maródi, 2010). Blasi et al. (2005) posited that TLR2 inhibits the ability of macrophages to phagocytose *C. albicans*, possibly by interfering with other receptors. I argue that this inhibition is not caused by TLR2 alone, but by these known interactions between TLR2, D1, and MR. Thus, by knocking out both D1 and MR, we are essentially recreating the effect observed in TLR2 -/- macrophages. Further studies will be needed to confirm this relationship. Particularly, it would be interesting to see whether the cytokine production for the D1 MR dKO macrophages mirrors the differential cytokine production that has been observed for TLR2 -/- macrophages, including reduced IL-10 and increased IL-12 and INFγ production (Blasi et al., 2005; Netea & Maródi, 2010; Gantner et al., 2005).

With respect to SAP genes, our initial hypothesis was that *C. albicans* lacking SAPs would exhibit decreased survival as compared with wild-type *C. albicans* (SC5314) when plated with macrophages and the results of this study generally supported this hypothesis. When plated with WT macrophages, *C. albicans* strains lacking SAPs 1, 2, 4, and 5 were significantly decreased in their survival rates ($p=3.0x10^{-3}$, $1.3x10^{-2}$, $1.5x10^{-4}$, and $8.3x10^{-4}$, respectively) and SAPs 3 and 6 were insignificantly decreased (Fig. 9A). This indicates that these SAPs play an important *role* in helping *C. albicans* escape phagocytosis. Multiple SAP mutants also exhibited decreased survival with the mutant macrophages, indicating that a significant proportion of *C. albicans* are still phagocytosed by these receptor-lacking macrophages but are able to overcome phagocytosis through expression of SAPs (Fig. 9C-E). Overall, SAPs 1-3 and 4-6 exhibited similar trends within those two groups, which makes sense given that SAPs 1-3 share high homology, as do SAPs 4-6 (Fig. 5, Fig. 9).

While survival was expected to be lowest for the *sap123* -/- and *sap456* -/- triple knockouts, the survival rates for these *C. albicans* strains were in fact generally greater than those of the single knockouts and greater than or equal to that of SC5314 for the same macrophage cell line (Fig. 9).

This may be evidence of a compensatory mechanism in which disruption of multiple SAP genes causes leads to an upregulation of other SAP genes or other virulence factors, or off-target effects caused by the genetic engineering. To further explore this outcome and verify results, two different isolates of *sap456* -/- were used, deemed SAP456a and SAP456b. Use of these two strains frequently yielded conflicting results, most notably with the D1 KO and D1 MR dKO macrophages, where SAP456a had increased survival compared to SC5314, yet the survival of SAP456b was decreased (Fig. 9, Fig.10I-J). This difference makes it more likely that the cause of the unexpected results for the triple mutants is an issue in genetic engineering. Furthermore, the observed increase in survival is in oppositition to the findings by Borg-von Zepelin et al. (1998) that showed a 53% decrease in survival of their *sap456* -/- strain as compared to SC5314, though there were some key differences in the protocol used. Specifically, the authors used PEMs instead of BMDMs, plated at a 5:1 yeast:macropage ratio in contrast to the 1:15 ratio used in this study, and incubated the yeast and macrophages together for 90 minutes instead of the 8 hours used here. These differences make it difficult to directly compare results, and genome sequencing and transcriptional analysis of the triple knockout isolates used in this study would be useful to identify or rule out off-target mutations or differential gene expression that may be altering survival.

In order to confirm that the results for the SAP mutants were not due to differences in the cell wall composition between strains, a modified ELISA was used to detect the levels of β-1,3- and β-1,6-glucan in each strain. While the levels of β-1,3-glucan did not increase in the single SAP mutants, β-1,6-glucan did increase for all single mutants except *sap1* -/-, although this was only significant for *sap5* -/- and *sap6* -/-. Levels of both types of β-glucan were increased in the triple mutants (Fig. 11A). The calculated ratio of β-1,3- to β-1,6-glucan in SC5314 was approximately 4.5, consistent with the finding by Bowman & Free (2006) that 65-90% of the glucan content in the cell wall by dry weight is β-1,3-glucan. This ratio was greatly decreased in all SAP mutants except *sap1* -/-

(Fig. 11B). However, these variations did not correlate with differences in yeast survival with D1-deficient macrophages between *C. albicans* strains. Since β-1,3-glucan is the predominant glucan type and did not vary significantly between SC5314 and the single mutants, this is not surprising. Ideally, a similar assay will be used to quantify mannan levels across strains to make sure this is constant as well and not a cause of the results for the MR-deficient macrophages. It is also notable that levels of both β-glucans were highest for the triple mutants and the profiles of *sap456* -/- isolates A and B were very different, giving further credence to the possibility that genetic engineering issues in these triple mutants may at least partially explain the unexpected results in these strains (Fig. 11A).

One important future experiment will be to repeat the macrophage invasion assay for the SAP1-6 mutants in WT macrophages in order to determine the role of SAPs at time points earlier than 8 hours. If knocking out SAP genes has a significant effect on *C. albicans* survival at early time points (30 minutes, 1 hour), this may provide evidence that SAP proteins play a role in recognition. Furthermore, it is possible that SAPs 1-3 play a role later on in infection than SAPs 4-6, as SAPs 1-3 are more active at a lower pH (3.2-4.5), which is what they are likely to encounter in acidified phagolysosomes, whereas SAPs 4-6 may work to degrade macrophage proteins even before being taken up by the macrophage (Naglik et al., 2003). Time course experiments would be valuable for exploring these hypotheses.

It is worth noting that initial attempts at the macrophage invasion assay resulted in high error and general decreases in *C. albicans* SC5314 survival for the mutant macrophage lines. It was observed that these mutant macrophages adhered poorly to the plates and retained a spherical morphology, whereas WT macrophages spread out flat along the plate. Since macrophages are highly motile, it was hypothesized that the observed decreases in survival were caused by the mutant cell lines being able to move about on the plate more freely and encountering more *C. albicans* as a result. To level the playing field between all macrophage cell lines, a spin down step was added to

the protocol following the addition of *C. albicans*. The addition of this step was key in obtaining the results of this study, and this is something future researchers may wish to consider when using macrophages with mutations of proteins on the cell wall or other components that may potentially alter their adherence.

Unfortunately, *smi1* -/- *C. albicans* was unable to be tested using the macrophage invasion assay as the CRISPR genome editing was unsuccessful. Diagnostic digests indicated that, at least in certain miniprep samples, the guide sequence had been successfully cloned in (Fig. 12). However, these diagnoses only reveal that the 20 bp region between the two BsmBI cut sites had been excised from the plasmid and it is possible that the plasmid re-ligated. In this case, the *C. albicans* transformation would have resulted in nourseothricin-resistant colonies, but the intended mutation would not have occurred, meaning an absence of red *ade2* -/- colonies for the control. Instead, there was no instance in which there were significantly more colonies on the transformation plates than the control plates in which the transformation protocol was followed with no plasmid. This indicates that the most likely issue is with the transformation protocol used, which was the lithium acetate method. Electroporation, alternative versions of the lithium acetate protocol, or hybrid lithium acetate-electroporation should be tried next, and additional controls should be implemented to verify the transformation protocol is working. Finally, the plasmids taken from the transformed *E. coli* may be sequenced to confirm that the proper guide sequences have, in fact, been inserted into the plasmid.

Understanding the host-pathogen interactions between *C. albicans* and immune cells is of great importance for designing novel antifungals and vaccines. A vaccine against SAP2 using a truncated version of the protein is currently in clinical trials, and SAPs 1 and 2 have been found to be highly upregulated in vaginal and oral candidiasis (De Bernardis et al., 2012). SAPs are an ideal vaccine target because they share minimal homology with any human proteins. However, this

vaccine will only allow the host to produce anti-SAP2 antibodies, and some have advocated for an approach targeting multiple SAPs at once. A past doctoral student in our lab previously found that knocking out SAPs reduced symptoms of *C. albicans* infection in the nematode *C. elegans* and identified a 35 residue domain common to all secreted SAPs but sufficiently divergent from even the most closely related human proteins. Furthermore, this domain lies on a highly exposed region of the protein, making it a perfect target for antibody binding (Issi, 2014). Understanding the relevance of SAPs in *C. albicans* infection is integral to their utilization as a vaccine target.

Additionally, understanding the role PRRs play in infection is important, as 3-5% of women suffer from chronic yeast infections and the underlying genetic factors that predispose individuals to this type of infection are poorly understood (Naglik et al., 2003). Loss-of-function mutations in PRRs such as Dectin-1 or the mannose receptor may help explain this phenomenon. Overall, the results of this study have helped shed light on the roles of PRRs and SAPs in *C. albicans* infection. Further studies are still needed to understand the interactions between different PRRs and the effects of knocking out multiple SAPs at once.

REFERENCES

BEI Resources. (2014). NR-9456 Mus musculus, Macrophage Cell Line Derived from Wild Type Mice (Cell Banks). Retrieved April 17, 2017, from https://www.beiresources.org/Catalog/cellBanks/NR-9456.aspx

Bisgaard, L. S., Mogensen, C. K., Rosendahl, A., Cucak, H., Nielsen, L. B., Rasmussen, S. E., & Pedersen, T. X. (2016). Bone marrow-derived and peritoneal macrophages have different inflammatory response to oxLDL and M1/M2 marker expression–implications for atherosclerosis research. *Scientific reports*, 6.

Blasi, E., Mucci, A., Neglia, R., Pezzini, F., Colombari, B., Radzioch, D., ... & Peppoloni, S. (2005). Biological importance of the two Toll-like receptors, TLR2 and TLR4, in macrophage response to infection with Candida albicans. *Pathogens and Disease*, 44(1), 69-79.

Borg-von Zepelin, M., Beggah, S., Boggian, K., Sanglard, D., & Monod, M. (1998). The expression of the secreted aspartyl proteinases Sap4 to Sap6 from Candida albicans in murine macrophages. *Molecular microbiology*, 28(3), 543-554.

Bowman, S. M., & Free, S. J. (2006). The structure and synthesis of the fungal cell wall. *Bioessays*, 28(8), 799-808.

Canton, J., Khezri, R., Glogauer, M., & Grinstein, S. (2014). Contrasting phagosome pH regulation and maturation in human M1 and M2 macrophages. Molecular biology of the cell, 25(21), 3330-3341.

Cid, V. J., A. Duran, F. Rey, M. P. Snyder, C. Nombela, and M. Sanchez. 1995. Molecular basis of cell integrity and morphogenesis in Saccharomyces cerevisiae. *Microbiol. Rev.* 59:345-386.

Chowdhary, A., Kumar, V. A., Sharma, C., Prakash, A., Agarwal, K., Babu, R., ... & Meis, J. F. (2014). Multidrug-resistant endemic clonal strain of Candida auris in India. *European journal of clinical microbiology & infectious diseases*, 33(6), 919-926.

da Rosa, J. L., Boyartchuk, V. L., Zhu, L. J., & Kaufman, P. D. (2010). Histone acetyltransferase Rtt109 is required for Candida albicans pathogenesis. *Proceedings of the National Academy of Sciences*, 107(4), 1594-1599.

Dan, J. M., Kelly, R. M., Lee, C. K., & Levitz, S. M. (2008). Role of the mannose receptor in a murine model of Cryptococcus neoformans infection. *Infection and immunity*, 76(6), 2362-2367.

De Bernardis, F., Amacker, M., Arancia, S., Sandini, S., Gremion, C., Zurbriggen, R., ... & Cassone, A. (2012). A virosomal vaccine against candidal vaginitis: immunogenicity, efficacy and safety profile in animal models. *Vaccine*, 30(30), 4490-4498.

Ford, C. B., Funt, J. M., Abbey, D., Issi, L., Guiducci, C., Martinez, D. A., ... & Rao, R. P. (2015). The evolution of drug resistance in clinical isolates of Candida albicans. *Elife*, 4, e00662.

Gantner, B. N., Simmons, R. M., & Underhill, D. M. (2005). Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. *The EMBO journal*, *24*(6), 1277-1286.

Gow, N. A., & Hube, B. (2012). Importance of the Candida albicans cell wall during commensalism and infection. *Current opinion in microbiology*, *15*(4), 406-412.

Gow, N. A., Netea, M. G., Munro, C. A., Ferwerda, G., Bates, S., Mora-Montes, H. M., ... & Brown, G. D. (2007). Immune recognition of Candida albicans β-glucan by dectin-1. *Journal of Infectious Diseases*, 196(10), 1565-1571.

Guo, M., Härtlova, A., Dill, B. D., Prescott, A. R., Gierliński, M., & Trost, M. (2015). High-resolution quantitative proteome analysis reveals substantial differences between phagosomes of RAW 264.7 and bone marrow derived macrophages. *Proteomics*, 15(18), 3169-3174.

Huang, H., Ostroff, G. R., Lee, C. K., Agarwal, S., Ram, S., Rice, P. A., ... & Levitz, S. M. (2012). Relative contributions of dectin-1 and complement to immune responses to particulate β-glucans. *The Journal of Immunology*, 189(1), 312-317.

Issi, L. (2014). Identification and characterization of novel putative virulence factors in *Candida albicans* (Doctoral dissertation). Retrieved from WPI Electronic Theses and Dissertations database.

Jain, C. (2012). Development of a fungal virulence assay using *Caenorhabditis elegans* as a model host to identify mechanisms of host pathogen interactions (Doctoral dissertation). Retrieved from WPI Electronic Theses and Dissertations database.

Jouault, T., Sarazin, A., Martinez-Esparza, M., Fradin, C., Sendid, B., & Poulain, D. (2009). Host responses to a versatile commensal: PAMPs and PRRs interplay leading to tolerance or infection by Candida albicans. *Cellular microbiology*, 11(7), 1007-1015.

Klis, F. M., Groot, P. D., & Hellingwerf, K. (2001). Molecular organization of the cell wall of Candida albicans. *Medical mycology*, 39(1), 1-8.

Lee, S. J., Zheng, N. Y., Clavijo, M., & Nussenzweig, M. C. (2003). Normal host defense during systemic candidiasis in mannose receptor-deficient mice. *Infection and immunity*, 71(1), 437-445.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., & Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in immunology*, 23(11), 549-555.

Martinez, F. O., Gordon, S., Locati, M., & Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *The Journal of Immunology*, 177(10), 7303-7311.

Naglik, J. R., Challacombe, S. J., & Hube, B. (2003). Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. *Microbiology and molecular biology reviews*, 67(3), 400-428.

Netea, M. G., & Maródi, L. (2010). Innate immune mechanisms for recognition and uptake of Candida species. Trends in immunology, 31(9), 346-353.

Nett, J. E., Sanchez, H., Cain, M. T., Ross, K. M., & Andes, D. R. (2011). Interface of Candida albicans biofilm matrix-associated drug resistance and cell wall integrity regulation. *Eukaryotic cell*, 10(12), 1660-1669.

Nobile, C. J., & Johnson, A. D. (2015). Candida albicans biofilms and human disease. *Annual review of microbiology*, 69, 71-92.

Pfaller, M. A., & Diekema, D. J. (2004). Rare and emerging opportunistic fungal pathogens: concern for resistance beyond Candida albicans and Aspergillus fumigatus. *Journal of clinical microbiology*, 42(10), 4419-4431.

Raschke, W. C., Baird, S., Ralph, P., & Nakoinz, I. (1978). Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell*, 15(1), 261-267.

Saijo, S., Fujikado, N., Furuta, T., Chung, S. H., Kotaki, H., Seki, K., ... & Kinjo, T. (2007). Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. *Nature immunology*, 8(1), 39-46.

Sanguinetti, M., Posteraro, B., & Lass-Flörl, C. (2015). Antifungal drug resistance among Candida species: mechanisms and clinical impact. *Mycoses*, 58(S2), 2-13.

Saville, S. P., Lazzell, A. L., Monteagudo, C., & Lopez-Ribot, J. L. (2003). Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection. *Eukaryotic cell*, 2(5), 1053-1060.

Sherry, L., Ramage, G., Kean, R., Borman, A., Johnson, E. M., Richardson, M. D., & Rautemaa-Richardson, R. (2016). Candida auris is highly virulent and resistant, and capable of forming biofilms. *Emerging Infectious Diseases*.

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, 140(6), 805-820.

Taylor, P. R., Tsoni, S. V., Willment, J. A., Dennehy, K. M., Rosas, M., Findon, H., ... & Brown, G. D. (2007). Dectin-1 is required for β-glucan recognition and control of fungal infection. *Nature immunology*, 8(1), 31-38.

van de Veerdonk, F. L., Marijnissen, R. J., Kullberg, B. J., Koenen, H. J., Cheng, S. C., Joosten, I., ... & Netea, M. G. (2009). The macrophage mannose receptor induces IL-17 in response to Candida albicans. *Cell host & microbe*, 5(4), 329-340.

Vyas, V. K., Barrasa, M. I., & Fink, G. R. (2015). A Candida albicans CRISPR system permits genetic engineering of essential genes and gene families. *Science advances*, 1(3), e1500248.

Wang, C., Yu, X., Cao, Q., Wang, Y., Zheng, G., Tan, T. K., ... & Harris, D. C. (2013). Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC immunology*, 14(1), 6.

Wisplinghoff, H., Seifert, H., Tallent, S. M., Bischoff, T., Wenzel, R. P., & Edmond, M. B. (2003). Nosocomial bloodstream infections in pediatric patients in United States hospitals: epidemiology, clinical features and susceptibilities. *The Pediatric infectious disease journal*, 22(8), 686-691.

APPENDIX A: Supplemental data

The plasmid used for the CRISPR experiments, pV1393, was provided by Valmik Vyas and is a modified version of the Solo System plasmids used by Vyas et al. (2015). The map below shows the major features of the plasmid. The Cas9 gene encodes the protein used to target specific genomic sequences and make a double strand break at that location, which has been optimized for use in *C. albicans*. This protein is under the control of the ENO1 promoter. The NatR gene provides nourseothricin resistance and is used as a selection marker in *C. albicans* and *E. coli*, and AmpR provides resistance to ampicillin in *E. coli*. NatR may be excised by FLP-FRT recombination so that the system can be reused for multiple iterations of genome editing. The guide sequence specific to the gene of interest can be cloned in at the location labeled sgRNA on this map, and this is under the control of the SNR52 promoter. Relevant restriction sites are also shown. Double digest by KpnI and SacI is used to linearize the plasmid so that it may be integrated into the *C. albicans*

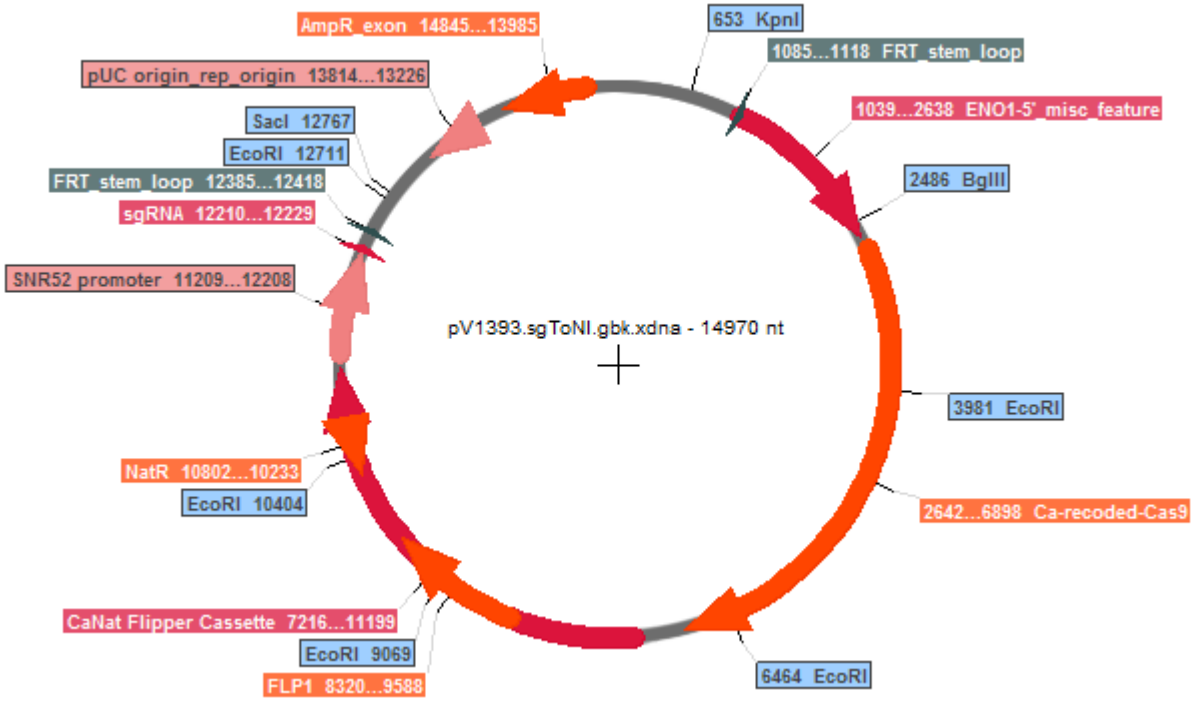

Figure S1. Map of pV1393 containing inserted guide sequence with select features labeled, created using Serial Cloner.

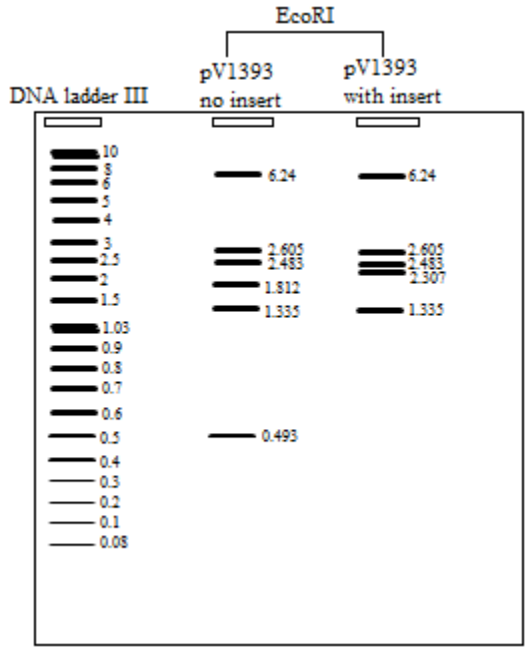

Figure S2. Diagram of expected results for diagnostic digest with EcoRI. Fragment lengths in kbp.

genome. EcoRI, BglII, and BsmBI were used for diagnostic digests to determine successful insertion of the guide sites. For the EcoRI digest, the bands at 1.812 and 0.493 kbp were used as a way to identify unsuccessful clones (Fig. 12, Fig. S2). Note that in pV1393 prior to insertion of the guide sequence, the sgRNA site contains two BsmBI cut sites and an EcoRI cut site.

The oligonucleotides used as guide sequences, repair templates, and PCR primers were selected as described in the methods section. In brief, guide sequences unique to both alleles of SMI1 were selected from the supplement of Vyas et al. (2015) and three guides were chosen that were closer to the start of the open reading frame to more likely result in functional gene knockout. Repair templates were 100 bp strands centered on the planned double stranded break. In order to more likely ensure gene knockout, three guide sequences and three corresponding repair templates were designed for SMI1. The guide and repair template used for ADE2 were taken from Vyas et al. (2015). After multiple attempts at cloning each guide oligo into pV1393 and transforming *E. coli* with the recombinant plasmids, successful transformation was observed only for ADE2 and SMI1 guide 1. The full list of oligos and primers used is given in Table S1.

Table S1. Oligonucleotides and primers used in this study.

Gene target	Purpose	Direction	Sequence
ADE2	guide	forward	atttgcaacaatcatacgacctaatg
ADE2	guide	reverse	aaaacattaggtcgtatgattgttgc
SMI1	guide (1)	forward	atttgtcattatcgtcatttctatag
SMI1	guide (1)	reverse	aaaactatagaaatgacgataatgac
SMI1	guide (2)	forward	atttgtattcattagataattctacg
SMI1	guide (2)	reverse	aaaacgtagaattatctaatgaatac
SMI1	guide (3)	forward	atttgcaattttataaacgtcatgag
SMI1	guide (3)	reverse	aaaactcatgacgtttataaaattgc
ADE2	repair template	forward	atggatagcaaaactgttggtattttaggaggttaatgattaggtcgtatgattgttgaa
ADE2	repair template	reverse	ttacggtcttgatattcaatctatgtgctgcttcaacaatcatacgaccttaatcattaac
SMI1	repair template (1)	forward	ttcattcaattacaacagaagatcattatgcttcatatgattcataagaattcaatgacg
SMI1	repair template (1)	reverse	gttgatgacctatgagaattggtaccaattggttcattatcgtcattgaattcttatgaa
SMI1	repair template (2)	forward	aatggaaatttgttgctaaatttgttaagagttataagtaatctattaagatgaattctt
SMI1	repair template (2)	reverse	gaacaactctactaaagtatggaccagatattttgggcttaagaattcatcttaatagat
SMI1	repair template (3)	forward	aatattcattagataattctactggtgcagcagggttttatagataagaattcaaatcat
SMI1	repair template (3)	reverse	ccattggcactaaaattttgtaatggtaaatcttgttgagatgatttgaattcttatcta
ADE2	PCR primer	forward	aacacccccccaccaaaaagaatc
ADE2	PCR primer	reverse	acaagtcatcgactgtgttgg
SMI1	PCR primer	forward	ttgaaaaccagccactagtctcg
SMI1	PCR primer	reverse	aagggctacccttctattggt

APPENDIX B: Protocol for CRISPR gene editing in *C. albicans*
Adapted from Vyas et al. (2015).

1. **Select gene to target**
2. **Design guide sequences and repair templates**
    a. Guide sequence should be 23 bp unique to gene, including PAM (NGG)
        i. Order sequence minus PAM, plus nts to ligate into plasmid
            1. Forward strand: ATTTG[sequence minus PAM]G
            2. Reverse strand: AAAAC[reverse complement of sequence minus PAM]C
        ii. If going for knockout, select sequences which affect protein closer to N terminus
        iii. If going for knockout, make sure to find sequence that affects both alleles!
    b. Repair template is 100 bp region centered on cut site in gene
        i. Order two 60 nt oligos with 20 bp overlap around cut site, extend using PCR
        ii. Mutate PAM site and add unique restriction site to confirm transformation
        iii. Add stop codon for knockout
        iv. May be necessary to try more than one guide sequence/repair template combination to mutagenize a given gene
3. **Prepare plates**
4. **Transform competent bacteria to make more plasmid**
5. **Prepare large culture of transformed cells and miniprep**
    a. Use QIAprep Spin Miniprep Kit.
6. **Clone guides**
    a. Cut pV1393 with BsmBI
        i. Prepare heat block at 55° C
        ii. Digest 2 µg plasmid DNA
            1. 2 µg DNA
            2. 5 µl 10x NEB3.1
            3. 1 µl BsmBI
            4. Add water to 50 µl
        iii. Incubate in heat block for 20 min
        iv. Cool to RT and spin down
        v. Add 1 µl CIP
        vi. Incubate at 37° C for 1 hour
        vii. Purify using QIAquick Gel Column.
            1. Start with isopropanol step.
            2. Elute in 30 µl buffer EB.
            3. Nanodrop to get concentration.
    b. Phosphorylate and anneal sgRNA oligos
        i. Add to a PCR tube:
            1. 0.5 µl 100 µM guide oligo top strand
            2. 0.5 µl 100 µM guide oligo bottom strand
            3. 5 µl 10x T4 Ligase Buffer
            4. 1µl T4 polynucleotide kinase
            5. 43 µl water
        ii. Incubate in a thermocycler
            1. 37° C for 30 min

2. 95° C for 5 min
3. Cool to 16° C at slowest ramp rate available

c. Ligate annealed guide oligos into plasmid
   i. Add to a PCR tube:
      1. 1µl 10x T4 ligase buffer
      2. 0.5 µl ligase
      3. 0.5 µl annealed oligos (include negative control with no oligos)
      4. 20-40 ng vector
      5. Add water to 10 µl
   ii. Incubate in thermocycler
      1. 16° C for 30 min
      2. 65° C for 10 min
      3. Cool to 25° C
d. Transform 5µl to chemically competent DH5 alpha *E. coli*
e. Select on 50 µg/ml Nat + 200 µg/ml Amp LB agar plates
f. Identify correct clones by sequencing 3-4 colonies. (If tandem insertion a problem, reduce amount of annealed oligos added to ligation reaction)

7. Prepare repair template

a. PCR repair template oligos
   i. 4 x 50 µl reactions, pooled
   ii. Purify using QIAquick Gel extraction column (no gel)
   iii. Elute in 50 µl EB
   iv. Nanodrop

8. Transform *Candida*

a. Linearize plasmid by KpnI and SacI double digest
b. Use lithium acetate or hybrid lithium acetate/electroporation to make *Candida* competent
c. Use minimal plasmid and lots of repair template
   i. 1-2µg of linearized plasmid with 6+µg of repair
   ii. Try varying repair template amount while holding plasmid constant if needed
   iii. Include no repair template control
d. Include pV1081 + repair template as control for mutagenesis efficiency
   i. Positive control to knockout ADE2 gene

9. Verify transformants

a. Select on 200 µg/ml Nat plates
   i. Try 400 µg/ml if needed
   ii. Include a no DNA control
b. Streak for singles.
c. Use PCR to amplify flanking region and digest PCR product, then run on gel (we added EcoRI cut site to repair templates)