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Dynamic Host-Pathogen Interactions Result in Fungal Epitope Unmasking

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DYNAMIC HOST-PATHOGEN INTERACTIONS RESULT IN FUNGAL EPITOPE UNMASKING

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By Alex Hopke

Dissertation Advisor: Dr. Robert Wheeler

An Abstract of the Dissertation Presented
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Molecular camouflage is used by a diverse set of pathogens to disguise their identity and avoid recognition by protective host receptors. The opportunistic fungal pathogen *Candida albicans* is a good example, as it masks the inflammatory component β-glucan in its cell wall to evade detection by the immune receptor Dectin-1. Interestingly, it has been seen that β-glucan becomes unmasked during infection *in vivo*, though the underlying mechanisms remained unclear. Exposure levels of this epitope may be important, as Dectin-1 mediates protection from some strains of *C. albicans* and alterations in the organization and composition of the *Candida* cell wall can influence host responses.

This research sought to understand *C. albicans* cell wall dynamics, particularly within the context of host-pathogen interactions. Special attention was paid to elucidating mechanisms of β-glucan unmasking and we have revealed a novel and dynamic interaction in which neutrophils damage the fungal cell wall via a mechanism involving neutrophil extracellular traps. This damage provoked the disruption of fungal cell wall architecture including increased chitin deposition and β-glucan unmasking at sites of immune attack. Surprisingly, these cell wall changes were also dependent on an active fungal response, which required cell wall integrity signaling and involved relocalization of cell wall remodeling components. Importantly, neutrophil mediated β-glucan unmasking could result in enhanced immune responses to fungi

from macrophages, suggesting that this epitope unmasking could have functional consequences during infection. Work we participated in helped elucidate mechanisms involved in baseline fungal epitope masking in the form of Cho1 and phosphatidylserine biosynthesis and also demonstrated that changes to cell wall composition and architecture influence the importance of β-glucan exposure to host responses to *C. albicans*.

Overall, this work helps elucidate the importance of host-pathogen interactions in influencing fungal cell wall dynamics during disseminated candidiasis. Given the importance of the cell wall as a drug target, understanding how this fungus maintains integrity and epitope masking during attack may identify therapeutic targets to aid the treatment of candidiasis. This work also highlights an important concept, which is that microbial cell walls can change dynamically during infection with important consequences for host recognition and immunity.

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CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Host-Pathogen Interactions: Shaping the Outcome of Infection

While humanity may like to think itself the masters of this planet, the world is truly dominated by microbes. In fact, the estimated 10^{12} bacteria on our skin, 10^{10} in our mouths and 10^{14} in our GI tract leave the estimated 3.72 x 10^{13} trillion cells that compose our body hopelessly outnumbered before even considering the vast reservoirs of microbes in the environment (1, 2). The human body is therefore forced to exist in a state of eternal readiness, as we are constantly on the brink of being overrun by a horde of microbial invaders. When restricted to the appropriate locations, many microbes are benign or can even be beneficial, as the importance of the microbiota to nutrition and proper immune system development has begun to be elucidated (3). Unfortunately some microbes are pathogens which, upon successful invasion, can cause debilitating or lethal disease in their host. Indeed, even normally benign microbes cause harm if allowed to roam freely into normally sterile sites. In order to survive, a host must therefore be able to restrict where microbes are able to access and be ready to confront those which breach such barriers. The immune system is evolution's answer to such a demanding request, representing a complex network which allows the host to maintain its vigil against foreign microbes and then decide how best to deal with any invaders which slip past those walls (4). This system is typically broken down into two main parts, the adaptive and the innate immune system. Adaptive immunity is usually associated with the development of a highly specific responses, with activation of immune cells like lymphocytes which include T cells and B cells. This arm of the immune system is primarily responsible for immunological memory and has been highly studied in relation to vaccine development. The innate immune system is usually thought of as providing less specific responses and is comprised of professional

phagocytes like macrophages and neutrophils as well as epithelial cells which provide an important front line as a barrier at mucosal surfaces (5). These two arms of immunity are a highly connected network, with phagocytes from innate immunity responsible for activating adaptive responses via antigen presentation and cytokine secretion (6). Activated adaptive responses can then direct innate immunity via further cytokine and chemokine production. Discoveries in recent years have begun to blur the lines between innate and adaptive immunity even further, with innate lymphoid cells that produce cytokines characteristic of adaptive lymphoid cells and "trained immunity" in which innate cells show limited immunological memory (7, 8).

1.2 Host Recognition of Infection

Every immune response begins with the host having to solve one seemingly simple question "What's attacking me?" Unfortunately, the vast diversity of potential pathogens which the host may encounter, including bacteria, viruses, protozoans, helminths and fungi, make devising a system capable of correctly answering that question a daunting task. The stakes are quite high as individual pathogens often require a specific immune response to effectively clear them and mechanisms which function against one pathogen may be ineffective or worse, cause collateral damage or promote infection with another. The immune system must therefore have a toolset that allows for both exquisite sensitivity and the rapid initiation of a response. Evolution has provided this toolset in the form of pathogen recognition receptors (PRRs), which recognize a wide variety of pathogen associated molecular patterns (PAMPs) present in or on microbes. These PRRs include many receptor families such as the Toll-like receptors (TLRs), the C-type lectins receptors (CTLs), the nucleotide-binding oligomerization domain like receptors (NLRs), and the retinoic acid inducible gene (RIG-I) like receptors (9). Upon binding of their specific ligand, which can include PAMPs as diverse as bacterial lipopolysaccharide, nucleic acids

such as DNA or RNA, or carbohydrates like fungal β-glucan, these receptors activate signaling pathways which can induce important and protective immune functions (9). This includes responses like phagocytosis, the production and mobilization of antimicrobials (including reactive oxygen or nitrogen species, proteases and antimicrobial peptides), and cytokine and chemokine production which act together to help directly clear the pathogen and to activate adaptive immunity (9, 10).

1.3 Fungal Infections

Most people are familiar with the big name infections that stalk humanity. Malaria. Influenza. Tuberculosis. These infections represent a major global health burden and have, deservingly, received a great deal of attention. Unfortunately, while these type of infections hold everyone's attention, few seem to be aware that another type of microbial predator has emerged from the shadows in the form of fungal infections. Fungi are an emerging threat to biodiversity, agriculture and human health on a global scale with examples of both primary and opportunistic pathogenic potential seen threatening plants, arthropods, amphibians, reptiles, fish, birds and mammals, including humans (11).

Recent years have provided front row seats to the destructive power fungal infections can exert on biodiversity with Chytridiomycosis providing a particularly chilling example. This disease is caused by the fungus *Batrachochytrium dendrobatidis* and has an enormous host range, being shown to be able to infect over 500 different species often with a high degree of virulence. Chytridiomycosis has been decimating global amphibian populations with some areas of Central America losing over 40% of their amphibian species and causing nearly half of amphibian species worldwide to see population declines, representing one of the greatest disease driven losses of biodiversity ever documented (11). Chytridiomycosis is unfortunately not the only example of an emerging fungal infection wreaking havoc in the natural world.

Geomyces destructans, which causes white nose syndrome, has been devastating North American bat populations with afflicted sites showing up to 70% population decline and at least one species predicted to have a 99% chance of local extinction because of this disease. Beyond the cost to biodiversity and ecosystem health, it has been estimated that this decline in bat populations costs US agriculture more than $3.7 billion per year. Fungal infections also represent a significant direct agricultural threat as wheat stem rust and rice blast disease are estimated to account for loss of food for 414-2155 million people on an annual basis (11). During a survey of published literature it was determined that fungi were responsible for 72% of animal and 57.1% of plant extinctions or extirpations due to infectious disease, observations that may actually be underestimations due to poor surveillance of infectious disease in natural systems, especially in the tropics (11).

Facing the global disruption of ecosystems and agriculture caused by fungal infection would be daunting enough, but fungi are also highly capable of preying directly on humans where they can act as either primary or opportunistic pathogens. Superficial infections of the skin and nails such as athlete's foot and ringworm are incredibly common and are thought to affect up to 25% of the world's population. Mucosal infections, such as oral thrush and vaginal yeast infections are also quite common, with up to 50-75% of women in their childbearing years experiencing at least one episode of vulvovaginitis (12). These superficial infections, while not usually life-threatening, represent a major public health burden and can be the cause of significant discomfort. The more urgent issue however, is that fungi can also cause invasive and lethal infection. *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Coccidioides immitis* and *Blastomyces dermatitidis* can all act as primary pathogens with life threatening consequences for those afflicted and opportunistic fungal pathogens are targeting the substantial immunocompromised populations which have emerged due to modern medical interventions or

the rise of immune suppressing infections like HIV/AIDS. These opportunistic fungi include *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Rhizopus oryzae* and *Pneumocystis jirovecii*. It is thought that, globally, there are greater than 400,000 life threatening infections with *C. albicans*, greater than 200,000 with *A. fumigatis* and nearly 1 million with *C. neoformans* on an annual basis. The mortality rate with these infections is currently unacceptably high, with *C. albicans* estimated at 28-46% and *A. fumigatis* estimated between 30-95% despite modern antifungal therapy. *Cryptococcus* infection also has a high mortality rate, with 15-20% seen in the United States despite medical treatment and even higher rates, up to 50-70%, in Latin America and sub-Saharan Africa where treatment is not as readily available. Out of the nearly 1 million estimated global cases of cryptoccocal meningitis that occur annually, over 620,000 deaths were estimated to have occurred in sub-Saharan Africa alone (12). In fact, there is an equal or greater number of deaths caused by the top 10 invasive fungal infections than by tuberculosis or malaria, demonstrating the devastating burden these fungal infections have on human health. The number of fungal outbreaks investigated each year by the Centers for Disease Control has increased considerably since the 1990s and, as modern medical procedures and the global AIDS pandemic continue to increase the number of susceptible immunocompromised individuals, this trend is not likely to reverse (13). Many fungal infections have limited therapeutic options and resistance to antifungal drugs has emerged which makes a greater understanding of these infections, especially the interactions which occur between host immunity and the fungus, a necessity if we are going to improve patient outcomes (12).

1.4 *Candida albicans*

C. albicans is a polymorphic fungus which can grow in the ovoid yeast form, the elongated hyphal form or as pseudohyphae (14). While often found as a commensal on the skin

and mucosal surfaces of healthy individuals, it can act as an opportunistic pathogen causing superficial mucosal diseases like oropharyngeal candidiasis and the vulvovaginal candidiasis as well as life threatening disseminated candidiasis, especially in the immunocompromised (12). Neutropenia, corticosteroid use, antibiotic use, invasive medical procedures such as indwelling catheters, major gut surgery or liver transplants and extended hospital stays in intensive care represent risk factors for developing disease (12). *Candida* species represent the 4th most common nosocomial cause of bloodstream infections and while there are over a dozen *Candida* species capable of infecting humans, *C. albicans* is by far the most common and mortality rates for invasive disease caused by *C. albicans* are estimated to extend as high as 46% even with modern drug treatments, demonstrating the severe burden that this fungus places on the health care system and individual patients (12). *C. albicans* has a number of abilities which influence virulence and allow it to thrive as a pathogen. Morphogenic switching, the ability to switch from growing as yeast to hyphae or vice versa, is thought to be of prominent importance for virulence and both morphotypes seem to play important roles as strains locked either as yeast or as hyphae show defects (15, 16, 14). Adhesins and numerous other factors allow *C. albicans* to bind effectively to host and environmental surfaces and establish biofilms or invade tissues (17, 18, 19). *C. albicans* is highly capable of sensing and adapting to the stress from its environment, a key trait for surviving the highly varied and dynamic niches within a host. Important stresses include nutrient availability as well as pH and the presence of reactive oxygen and nitrogen species. Sensing of these stresses is typically done through cell wall integrity pathways. These pathways, which include Hog1, Mkc1 and Cek1 are all part of map kinase signal transduction pathways and can lead to the regulation of fungal responses at both the transcriptional and post-transcriptional level to specific stressors (20). Hog1 is important for adaption to stress including osmotic stress, oxidative stress as well as chemical cell wall stressors and antifungal

drugs (20, 21). Importantly, *hog1* deficiency results in increased susceptibility to killing by immune cells, suggesting this pathway would be of great importance during interaction with the host (22). Mkc1 is also activated following numerous stresses including osmotic and oxidative stress as well as antifungal drugs (20, 23). The Cek1 pathway is important for cell wall growth and construction and *cek1* deficient mutants show altered cell wall morphology (24) as well as susceptibility to cell wall stressors like antifungals (25). *cek1* deficiency increases resistance to killing by some phagocytes (22), but decreases it against others (24). Unsurprisingly *C. albicans* deficient in *hog1*, *mkc1* or *cek1* show decreased virulence in infection models demonstrating the importance of all these pathways in adapting to the dynamic environments in a living host (26, 27, 28). Finally, the ability to regulate cell wall architecture to hide immunogenic epitopes in the fungal cell wall from host recognition can contribute to immune evasion and virulence of this organism. Masking of these epitopes, particularly the carbohydrate β-glucan, requires a complex network of genes including those involved in cell wall integrity signaling, phospholipid biosynthesis and numerous other functions which will be explored in more detail later on (29, 24, 30).

1.5 Host Recognition of *C. albicans* Infection

As alluded to above, host immune responses begin with recognition of PAMPs from the pathogen. The *C. albicans* cell wall consists of an outer layer of mannan and mannoproteins connected by β-1,6 glucan linkers to inner layers of β-1,3 glucan and chitin (31). Many of these components are capable of being recognized by the host and eliciting immune responses by a multitude of host PRRs including members of the TLR, CLR and NLR families (10). As carbohydrates make up the majority of the cell wall (90%), they tend to dominate host recognition, however proteins also represent important antigens (31).

1.5.1 Toll-like Receptors

Fungal mannan can be recognized by members of the TLR family like TLR2, which recognizes phospholipomannans and TLR4 which recognizes O-linked mannans. TLR4 is important for host defense, as it induces inflammatory cytokines and both TLR2 and TLR4 deficient mice are more susceptible to disseminated candidiasis (32, 33), though this depends on the *C. albicans* strain (34). TLR9 has been found to be able to collaborate with NOD2 and Mannose receptor (MR) to induce IL-10 in response to fungal chitin (35). Mice deficient in MyD88, the adaptor used by most TLR signaling, are also more susceptible to disseminated candidiasis (36), supporting the importance of TLRs for host defense in this model. The importance of TLRs in human defense against *C. albicans* is less clear, while some studies have found associations between polymorphisms in TLR2 or TLR4 and risk for candidemia, a larger study of patients found no association. TLR1 polymorphisms have also been linked with susceptibility to candidemia, but these findings have not yet been replicated. Polymorphisms in TLR3 have been suggested to be related to chronic mucocutaneous candidiasis (CMC) (37). Interestingly, humans with defects in MyD88 don't appear particularly susceptible to candidiasis (38, 39), suggesting that in humans other PRR pathways play more prominent roles in organizing antifungal immunity.

1.5.2 C-Type Lectin Receptors

Members of the CLR family, which includes Dectin-1, Dectin-2, Dectin-3, Mincle, Mannose receptor (MR) and dendritic cell specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), are thought to play critical roles in host detection of fungi. This is supported by the fact that many patients deficient in caspase recruitment domain containing protein 9 (CARD9), an important adaptor for many CTL signaling pathways, show increased

susceptibility to both CMC and invasive fungal disease (40). This stands in contrast to patients deficient in MyD88, the primary adaptor for most TLR signaling, who don't show increased susceptibility to fungal infection (38, 39).

1.5.2.1 Dectin-1

The fungal cell wall component β-glucan is recognized by a wide variety of organisms, including invertebrates, plants and animals suggesting that it represents an evolutionarily important fungal PAMP. In mammalian systems like mice and humans, the major β-glucan receptors are Dectin-1 and Complement receptor 3 (CR3), though other receptors have also been reported to recognize this PAMP (41, 42). Dectin-1 activation results in direct signaling but can also influence the outcome of signaling by other PRRs like TLR2 and TLR6. Activation of Dectin-1 by fungal β-glucan signals via the spleen tyrosine kinase through CARD9-BCL10-MALT1 and eventually to NF-κB (42). It can also result in signaling through other pathways that include Raf1 (43). Dectin-1 activation leads to immune responses including phagocytosis, activation of the oxidative burst, activation of both classical and non-classical inflammasomes (44, 45) and the production of cytokines including TNF-α, IL-1β, IFN-β, IL-6, IL-12 and Il-23 and CXCL2 (42, 43). This cytokine production can help shape adaptive immunity, as activation by the selective ligand curdlan (a β-glucan polymer) is sufficient for promoting the induction of Th1 and Th17 responses. It may not be absolutely required however, since Dectin-1 deficient mice still developed Th17 responses during *C. albicans* infection (46), with another CTL, Dectin-2, able to compensate for its absence (47). Dectin-1 has been demonstrated to play critical roles in anti-fungal defense in both humans and mice, though its importance during defense against candidiasis is still under debate (48, 49). Recent data suggests that the importance of Dectin-1 for defense against disseminated candidiasis in a murine model depends on the *C. albicans* strain used (50). Humans with a Dectin-1 polymorphism are more susceptible to chronic

mucocutaneous candidiasis (CMC) and recurrent vulvovaginal candidiasis (51) and this polymorphism has been associated with *C. albicans* colonization in stem cell transplant patients (52). Dectin-1 polymorphism has also been linked to a severe form of ulcerative colitis (53). No link between Dectin-1 polymorphism and susceptibility to disseminated candidiasis in humans has been found yet however, suggesting other receptors play important roles in protection from this form of infection.

1.5.2.2 Dectin-2 and Dectin-3

Dectin-2, which recognizes α-mannan, can induce the production of cytokines like TNF-α, Il-6, IL-1β and IL-23, and is thought to play a major role in signaling adaptive immunity to develop a Th17 response after *C. albicans* infection (10). These responses are important for host defense, as Dectin-2 deficient mice have been seen to be more susceptible to *C. albicans* infection, though its requirement is controversial (54, 55, 47). Recent work has demonstrated that the CLR Dectin-3 collaborates with Dectin-2 to mediate N-linked mannan recognition and proinflammatory responses. Dectin-3 is also important for host defense, as blocking Dectin-3 makes mice more susceptible to disseminated candidiasis (55).

1.5.2.3 Mincle and Mannose Receptor

Mannan is also recognized by the CTL Mincle, and fungal recognition by this receptor can lead to phagocytosis, fungal killing, induction of cytokines like TNF-α and IL-6 and chemokines MIP-2 and KC (10). Mice deficient in Mincle have been seen to be more susceptible to disseminated candidiasis, though other studies have reported that Mincle isn't required to recognize C. albicans (56, 57). This may be due to strain dependent differences in recognition, which has been seen for other C-type lectins like Dectin-1 (57, 50). The role of Mincle in human infection is not clear, as no defects in Mincle linked to susceptibility to fungal infection have been found yet (10). MR recognizes N-linked mannan in *C. albicans* and has been shown to be

able to promote IL-17 production (58). MR has also been demonstrated to play a role in the recognition of *C. albicans* chitin which induces IL-10 production in conjunction with TLR9 and NOD2 (35). MR deficient mice are not more susceptible to disseminated candidiasis however, suggesting either that there are differences between human and mouse MR, or that MR plays a redundant role which can be compensated for by other receptors (10).

1.5.2.4 DC-SIGN

DC-SIGN recognizes mannose and fucose carbohydrate structures. This receptor can activate Raf-1 signaling which allows it to influence signaling induced by receptors like the TLRs and other CLRs (43). DC-SIGN has also been shown to be bind to *C. albicans* N-linked mannan with high affinity (59) and can mediate its uptake by dendritic cells (60, 61). The influence of DC-SIGN on the adaptive response during *C. albicans* infection is unclear, though DC-SIGN can inhibit Dectin-1 mediated Th17 responses in a tuberculosis model (62) and those deficient in DC-SIGN have increased susceptibility to invasive pulmonary aspergillosis during stem cell transplant of chemotherapy suggesting it can play a role during human infection (10).

1.5.3 NOD-like Receptors

The NOD like receptors (NLRs) are a group of cytosolic receptors that can recognize internalized PAMPs and include both the NOD and the NOD leucine rich repeat and pyrin domain containing protein (NLRP) subfamily. Members of the NOD family include NOD1 and NOD2. Recognition of chitin and the resulting induction of IL-10 was shown to depend on NOD2, in collaboration with TLR9 and MR (35). The importance of NOD1 and NOD2 in *C. albicans* recognition remain to be defined as only in vitro studies have been done. One study found no association between NOD2 polymorphisms and increased susceptibility to *C. albicans* in humans, suggesting that it may not play a critical role (63). NLRPs family members like NLRP3 and NLRC4 do play an important role in antifungal defense. Mice deficient in NLRP3 are more

susceptible to disseminated candidiasis and NLRC4 is important for defense against oral infections (64, 65). Interestingly, NLRP3 and NLRC4 show compartment specific requirements, with NLRP3 being required in hematopoietic cells and NLRC4 in the mucosal stroma demonstrating the niche specific nature of host defenses (10). NLRP3 has also been implicated as contributing to host responses during vaginal infection of the mouse model (66). Polymorphisms in NLRP3 has been associated with increased risk for recurrent vulvovaginal candidiasis (RVVC) in humans, suggesting that these receptors do play a role in defense against *C. albicans* in humans (67).

1.5.4 RIG-I like Receptors

The RIG-I like receptor family are well characterized for their role in mediating antiviral responses. This family, which includes RIG-I and MDA5, are helicases which recognize nucleic acids and initiate signaling that leads to an immune response, often centering on type-1 interferon, which is important for defense against viruses (68). A recent study has demonstrated that MDA5 is also capable of influencing the immune response against *C. albicans* infection in mice, and that polymorphisms in MDA5 may be associated with infection in humans as well, though what fungal component activates this receptor was not identified (69). Interestingly, a role for type 1 interferon in human responses to *C. albicans* has been suggested (37) which, given the central role RLRs can play in the induction of IFN responses to viral infection, suggests a possible mechanism for how MDA5 influences human responses to *Candida* infection.

1.6 Host Defense against *C. albicans* Infection

Once initiated by PAMP recognition, host defense against *C. albicans* involves a complex array of factors which vary depending on both the site and timing of the infection. Innate immunity plays a particularly important role, as defects in this arm of immunity are primary risk

factors for severe disseminated disease while defects in adaptive immunity are associated with mucosal disease (12, 63).

1.6.1 Neutrophils

The innate immune cell known as the neutrophil is well equipped to defend against invading microbes. They are usually recruited rapidly to the site of infection where they can deploy a highly diverse arsenal to contain or kill microbes including fungi (70). Neutrophils are one of the most critical innate immune cells in defense against invasive fungal infections including disseminated candidiasis, as neutropenia is a primary risk factor for developing this disease and is associated with poor patient outcomes from invasive fungal infection (71).

1.6.1.1 Neutrophil Antimicrobial Mechanisms

Neutrophils play a critical role in innate defenses and they are armed to the teeth with antimicrobial weapons to use against any invaders they encounter. The NADPH phagocyte oxidase plays a central role in these antimicrobial functions as it allows neutrophils to produce reactive oxygen species (ROS). These ROS can be used to generate reactive nitrogen species (RNS) or further processed by superoxide dismutase to create the antimicrobial hydrogen peroxide, which in turn can be used by myeloperoxidase (MPO) to generate the potently antimicrobial hypochlorite (70). Neutrophils also have antimicrobial peptides and proteases, which could also contribute to fungal killing (70, 72). The critical nature of neutrophils to defense against disseminated candidiasis can be seen in the highly susceptible nature of neutropenic humans or mice to this disease (73, 74, 75). When the neutrophils phagocytose a *C. albicans yeast*, ROS, hypochlorite, RNS, antimicrobial peptides and proteases combine to create an extremely hostile environment in the phagolysosome that is important for fungal killing (76). When confronted by particles too big for phagocytosis, such as the hyphal form of the fungus, neutrophils can attempt to deploy those same weapons by extruding them onto the hyphae via

frustrated phagocytosis or the can use a weapon known as neutrophil extracellular traps (NETs) (77, 78, 79). NETs occur when a neutrophil extrudes its DNA, which is both antimicrobial in itself and is coated in potent antimicrobials like citrullinated histones and MPO, out onto a pathogen. This creates a trap that is thought to contain the spread of infection as well as have direct antimicrobial function (80, 70). NETosis was initially thought to be a form of cell death that ended with the destruction of the neutrophil, however examples of NETosis have been seen which do not kill the cell, allowing the neutrophil to create a NET while remaining viable (81, 82). NETs have been shown to be deployed against *C. albicans* and are capable of killing both yeast and hyphae *in vitro* (79). It has also been demonstrated that they are preferentially deployed against *C. albicans* hyphae where phagocytosis is not an option due to the size of hyphal cells (83). The components required for NET formation appear to be highly context dependent, and could involve the NADPH oxidase, MPO and neutrophil proteases depending on the stimuli and conditions used (84, 83, 85). Unfortunately, the highly potent weapons neutrophils deploy against pathogens are equally destructive to host tissue and this collateral damage can sabotage efforts to survive infection. Aberrant recruitment and accumulation of neutrophils to the kidney of mice with disseminated candidiasis has been shown to be responsible for significant immunopathology during infection and is one of the primary factors driving the decline of the animal's health (86). Therefore neutrophil responses, while absolutely critical for host defense, also need to be tightly regulated to minimize collateral damage to tissues and ensure the successful survival of the host.

1.6.2 Mononuclear Phagocytes

Mononuclear phagocytes, which include cells like monocytes, macrophages and dendritic cells are also involved in host defense against candidiasis. The ability of macrophages to control *C. albicans* is debated, as it has long been seen that *C. albicans* yeast can use

germination into hyphae as a mechanism to kill the immune cell and escape *in vitro* though many other studies have indicated their importance to defense against candidiasis (87). Macrophages have been demonstrated to be able to contain *C. albicans in vivo* in a zebrafish model however, raising the question of whether the *in vitro* environment has been providing an accurate reflection of macrophage's abilities *in vivo* (88). Furthermore, mononuclear phagocytes have been shown to be critical for defense during disseminated candidiasis in a murine model, where they were required to limit fungal growth in the kidney at early timepoints (71, 89). This was dependent on Cx3cr1, as Cx3cr1 deficient mice had less macrophages in the kidney and were much more susceptible to disease. Humans with the dysfunctional *CX3CR1-M280* allele show increased susceptibility to disseminated candidiasis as well, supporting the clinical relevance of these findings. Interestingly, Cx3cr1 does not seem to play a role in defense against mucosal candidiasis, demonstrating how unique factors are involved in host defense at different sites of infection (71). Mechanistically, mononuclear phagocytes can directly contain the fungus, assist other immune cells or act as bridges to activate adaptive immunity. Macrophage extracellular traps (METs) have been shown not to be able to control or kill *C. albicans in vitro* (90), however this work was done with cell culture and thioglycollate elicited macrophages which are different than macrophages from other sources (i.e. murine macrophages derived from bone marrow, resident macrophages from different organs or those from humans) suggesting that more work needs to be done to fully elucidate a definitive answer on the role of METs and macrophages in host defense. Dendritic cells have also been shown to be important for orchestrating both adaptive and innate immune responses against *C. albicans*. Human dendritic cells are critical for the expansion of an IL-17 producing subset of γδ T cells in response to *C. albicans* (91). Dendritic cell and Syk signaling are also involved in organizing natural killer cell (NK) mediated GM-CSF secretion, a process that enhances neutrophil antifungal function

(92). Furthermore, a dendritic cell mediated type-I INF-β response was also critical for host immunity to *C. albicans* (93), demonstrating the multifaceted role these cells can play.

1.6.3 The Inflammasome

The inflammasome is the term for a complex of proteins that are critical for the processing of immature cytokines like pro-IL-1β and pro-IL-18 into their mature functional forms. The classical inflammasome consists of sensor molecules like the NLRP family member NLRP3, the ASC adaptor protein and caspase-1. Upon recognition of microbial ligands, NLRPs will associate with ASC. ASC then assembles into a large complex of ASC dimer multimers, which can interact with pro-caspase-1. Bringing pro-caspase-1 monomers into proximity allows for self-cleavage and the formation of active caspase-1 heterodimers. Caspase-1 can then proteolytically process many proteins, including pro-IL-1β and pro-IL-18, which are then active and can be released to influence immunity (94). The inflammasome is known to be activated by *C. albicans* infection, with inflammasome mediated processing of IL-1β shown to be downstream of TLR2 and Dectin-1 signaling (44). Non-classical inflammasome activation of IL-1β in response to *C. albicans* can also occur, with ASC and caspase-8 responding directly to the CARD9-BCL10-MALT1 complex following Dectin-1 signaling (45). The inflammasome plays an important role in defense against *C. albicans*, as mice deficient in inflammasome components NLRP3, NLRC4, ASC or caspase-1 are more susceptible to infection, though it makes them less susceptible to the immunopathology associated with vaginitis (10, 66). As mentioned above, NLRP3 polymorphism has been found to be associated with RVVC, suggesting the inflammasome can play a role in human infection (67).

1.6.4 Natural Killer Cells

NK cells, while largely appreciated for their roles in host defense against virus and other intracellular pathogens, have also been shown to play critical roles in protection against

disseminated candidiasis, where they act on neutrophils by secretion of GM-CSF, IFN-γ and TNF-α (95, 96). NK cells can also phagocytose *C. albicans*, though this didn't prevent hyphal growth suggesting that this ability may not be of primary importance for their antifungal contributions. Extracellular perforin from NK cells did have antifungal activity against *C. albicans* however, suggesting that NK cells can play both direct and indirect antifungal roles (96).

1.6.5 Adaptive Immunity

While innate immunity is primarily responsible for defense against disseminated candidiasis, adaptive immunity also responds to *Candida* infection and is important for defense against mucosal infection. The T-helper 17 (Th17) response in particular is thought to play an important role in antifungal responses, including those against *C. albicans* (97, 63). The cytokines IL-1β, IL-23, IL-6 and TGF-β help skew T-cells towards Th17 differentiation and these Th17 cells then produce the characteristic cytokines IL-17A, IL-17F and IL-22. Both IL-17A and IL-17F signal through a common receptor composed of the subunits IL-17RA and IL-17RC (98). IL-17 is thought to act largely through neutrophils to promote antifungal immunity. IL-17 is a potent inducer of neutrophil granulopoiesis and chemotaxis, able to induce mucosal epithelial cells and stroma to produce chemokines to promote neutrophil recruitment and cytokines like G-CSF which promotes granulopoiesis. IL-17 and IL-22 are also capable of inducing antimicrobial peptides such as β-defensin 2, β-defensin 3, S100A7, S100A8, S100A9 and histatin at the skin and mucosal surfaces including the lung, oral and vaginal cavities (63). There is also a report that IL-17 can directly bind *C. albicans* and induce nutrient starvation, suggesting it can act directly on the pathogen (99). IL-17 can act on NK cells as well, inducing them to produce GM-CSF which in turn promotes the function of neutrophils as outlined above. Humans with mutations in Th17 related signaling or function including CARD9, STAT3, STAT1, RORC, IL17RA or IL-17F all show increased susceptibility to mucosal candidiasis, demonstrating the importance of this adaptive

response in defending against mucosal infection (100, 101). The importance of this response against disseminated candidiasis is less clear, as many patients with defective TH17 responses are not seen to be more susceptible to this disease. While CARD9 deficiency is linked to susceptibility to systemic disease and this deficiency does result in a lack of TH17 differentiation and response, CARD9 also plays critical roles in innate immunity making it impossible to attribute the increased susceptibility just to lack of Th17. Polymorphism in IL-12B, which is shared between IL-12 and IL-23, has been shown to be related to persisting candidemia however, suggesting either IL-12 (which promotes Th1 responses) and/or IL-23 (which promotes Th17 responses) may play roles in this form of infection (102). Taken together, these results demonstrate that TH17 responses play critical roles in host defense against *C. albicans* infection, but potentially in a tissue specific manner. Interestingly, polymorphisms in IL-4, a characteristic Th2 cytokine, have been associated with chronic disseminated candidiasis and RVVC suggesting important antifungal roles for adaptive responses beyond Th17 may exist (103).

1.7 Pathogen Epitope Masking

Pathogens, in an effort to persist and cause infection, have evolved ways to avoid detection by the host or suppress immune responses. Masking of epitopes the host could use to recognize the pathogen is a common strategy of immune evasion, having been demonstrated in bacteria, viruses, protozoans, helminths and fungi.

1.7.1 Parasite Epitope Masking

Both protozoan and helminth parasites are well known manipulators of host responses. One of the best characterized is the use of variable surface glycoproteins (VSG) by trypanosomes. These VSGs serve many purposes as they can provide protection from complement, the soluble form is capable of altering host immune responses and they mask the constant epitopes in the trypanosome cell membrane from host recognition (104, 105). While

the host eventually raises enough antibody against a particular VSG for antibody mediated killing to occur, trypanosomes are capable of antigenic switching with their VSG coat, allowing them to present an extremely diverse number of VSG epitopes within a population (an individual parasite will only express one VSG at a time). This means that, while many parasites will be killed by host antibody responses, those within the population that switched to different VSG coats will persist thereby allowing the population to stay one step ahead of host antibody responses. Malaria is also capable of masking its presence from the immune system. When it infects erythrocytes, *Plasmodium falciparum*, which causes the most virulent form of malaria, forces the expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) on the membrane. This protein plays important roles in virulence and can be targeted by IgG1 for protective host immunity (106). *P. falciparum* has evolved a mechanism to mask PfEMP1 from protective IgG1 responses, as it has an affinity for non-specific and non-protective IgM binding which then blocks access of protective IgG1 (107). It has also been shown that the circumsporozoite protein possessed by *P. falciparum* is capable of undergoing reversible conformational changes to allow masking its own epitopes (108).

Helminths are also well known as master manipulators of their hosts, an essential skill for pathogens that can persist for years or even decades (109). While the epitopes important for recognition and host responses against helminths are poorly understood, some examples of epitope masking have been discovered. N-deglycoslation of *Echinostoma caproni* excretory/secretory products has been shown to increase IgM responses, suggesting that these carbohydrates mask epitopes important for IgM recognition (110). It is also thought that *Taenia solium* cysts, which are responsible for neurocysticercosis, mask themselves from host immunity and only elicit an immune response when they begin to degenerate and leak cyst fluid. This theory is supported by the observation that only cyst fluid, but not cyst cell wall or crude cyst

lysate, resulted in a proinflammatory response in peripheral blood monocytes and that viable cysts were associated with regulatory and anti-inflammatory responses instead (111, 112). It is also known that surface epitopes are inaccessible on schistosomes in the lung-stage and this masking was resistant to manipulation of nutrient availability and pH. Depletion of cholesterol lead to exposure of surface antigens on *Schistosoma mansoni*, suggesting that this molecule plays a crucial rule in epitope masking for this helminth. Interestingly, cholesterol depletion did not alter epitope availability for the related *Schistosoma haematobium*, suggesting it has distinct methods for epitope masking (113). Incubation with arachidonic acid or corn oil has also been shown to result in exposure of these surface epitopes on both *S. mansoni* and *S. haematobium* via a mechanism hypothesized to involve a parasite associated neutral sphingomyelinase and its ability to alter a tight hydrogen bond barrier around the worm that would otherwise shield proteins from outside access (113, 114).

1.7.2 Bacterial Epitope Masking

Despite being single celled prokaryotes, bacteria have evolved immune masking mechanisms just as intricate as their eukaryotic counterparts. In *Neisseria meningitidis* killing by complement in human serum was found to be related to the amount of available lacto-N-neotetraose in its cell membrane. It resists this killing by sialylating its lipopolysaccharides which masks lacto-N-neotetraose (115).

Capsule formation offers another mechanism by which bacteria can evade with host immunity, as the capsule can interfere with opsonization, phagocytosis and mask antigenic epitopes from the host and can be found in a diverse group of bacteria including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Escherchia coli K1* and *N. meningitidis* (116). Pathogenic mycobacteria have been shown to evade TLR mediated immunity by masking PAMPs with cell surface associated pthiocerol dimycoceroserate (PDIM). PDIM deficient mycobacteria activated

MyD88 dependent signaling and recruited iNOS positive macrophages which were rarely seen to be recruited to wild type infection (117).

1.7.3 Viral Epitope Masking

Viruses are such a "simple" life form that it is debated whether or not they should even be considered as alive, having to hijack host cell systems in order to replicate. This obligate parasitic lifestyle has resulted in the evolution of numerous host immune evasion mechanisms in viruses, including epitope masking. The Hepatitis B preS1 domain of the large hepatitis B surface protein is N-myristolated and this N-myristolation masks important epitopes in the preS1 domain from binding by neutralizing antibodies (118).

Ebola virus also makes extensive use of masking to evade host immunity. Ebola virus glycoproteins are highly glycosylated and these glycans shield critical components like the receptor binding site from being targeted for antibody generation by the host. These glycans instead promote the generation of antibodies against more variable (and dispensable) regions at the viral surface. Furthermore, when these Ebola virus glycoproteins are expressed at the membrane of infected host cells, they mask important host molecules like major histocompatibility class I and β-integrins from the rest of the immune system preventing their anti-viral functions (119). Glycan shielding is actually a common epitope masking strategy used by viruses and has been seen in hepatitis C, bovine herpes virus, influenza A, Nipah virus and HIV-1 (119).

1.7.4 Fungal Epitope Masking

Considering the massive burden fungal infections present worldwide (11, 12), it is not surprising that they too have evolved ways to subvert or avoid detection by the immune systems of their hosts. It has been seen that *Magnaporthe oryzae*, a famine causing rice pathogen, uses α-glucan to shield itself from plant immune recognition and responses (120, 121)

and that conversion of surface exposed chitin to chitosan by a number of fungal pathogens helps shield them from plant immune attack and detection (122). The fungus *Normuraea rileyi*, a pathogen of insects, seems to be able to have its surface epitopes mimic those found on host molecules, thereby masking its presence and escaping immune surveillance (123). In fact, some fungal pathogens of insects mask themselves by forgoing a cell wall altogether, forming protoplasts within the host which don't elicit a significant host response (124).

In terms of PAMP mediated recognition, one of the most attractive epitopes that fungi often contain in their cell wall is the sugar β-glucan, as this epitope is highly immunostimulatory to a wide variety of hosts including plants, arthropods, fish and mammals (41). It is therefore not surprising that many fungal pathogens go to great lengths to mask their β-glucan to avoid provoking robust immune responses against them and many examples of this are known in clinically relevant fungi. *Histoplasma capsulatum* masks its β-glucan with non-inflammatory α-glucan and mutants which lack this α-glucan shield elicit increased Dectin-1 mediated responses from immune cells (125). The mold *Aspergillus fumigatus* also masks its β-glucan, which becomes exposed during germination or in hypoxic host environments resulting in increased Dectin-1 mediated responses *in vitro* and *in vivo* (126, 127). This basal masking depends on a cell surface protein, the hydrophobin RodA, as mutants deficient in RodA have increased β-glucan exposure and elicit increased Dectin-1 mediated responses both *in vitro* and *in vivo* (128). It also requires α-glucan, as aspergillus mutants with a triple knockout of the α-1,3 glucan synthases show cell wall architecture disruptions including increased β-glucan and chitin exposure. Epitope masking has also been shown to involve the pigment protein pskP as mutants deficient in pskP have increased conidial β-glucan exposure and are phagocytosed at a much higher rate than wild type strains (129). Interestingly, *A. fumigatus* RodA also appears important for masking α-mannose and blocking Dectin-2 mediated responses as well. *Paracoccidioides*

brasiliensis can convert its β-1,3 glucans to α-1,3 glucan while transitioning into pathogenic yeast in the host. Greater inflammatory responses to an avirulent strain of P. brasiliensis has been tied to greater β-glucan content in their cell wall, suggesting that the switch to α-1,3 glucan helps mask the fungus from eliciting an immune reaction from the host (130). *Fonsecaea pedrosoi*, a major agent of chromoblastomycosis, masks β-glucan in the cell wall of its sclerotic cells with a chitin-like component (131). *Cryptococcus neoformans* masks PAMPs, including β-glucan, behind a thick capsule.

C. albicans also generally masks its β-glucan from Dectin-1, with limited exposure at sites like bud scars. It is known that a complex network controls masking of this epitope (29) and it is generally thought that the outer layer of mannan in its cell wall shields the inner β-glucan from Dectin-1 recognition. In agreement with this, it has been shown that disruptions of mannose structure and organization by targeting genes in the *MNN2* family or *MNT1* and *MNT2* can result in unmasking of β-glucan (132, 133) and that this unmasking can result in increased Dectin-1 mediated responses. Furthermore, disruption of proper GPI anchor synthesis by targeting genes like *GWT1* or *GPI7* as well as disruptions of the phosphotidylserine biosynthesis by targeting *CHO1* results in increased unmasking of β-glucan along with increased Dectin-1 dependent responses by the host (134, 135, 30). Exposure to echinocandins like caspofungin, which inhibits the β-glucan synthase, also results in β-glucan unmasking (136). Interestingly, β-glucan becomes unmasked at later timepoints during disseminated infection *in vivo*, thereby allowing for increased Dectin-1 recognition, however the mechanism(s) for this unmasking remained unclear (136). Beyond epitope masking, *C. albicans* also appears to be able to actively suppress IL-17 production by PBMCs, a mechanism that involves regulation of tryptophan metabolism (137).

1.8 Summary

Invasive fungal infections, including *C. albicans*, represent a significant and growing health problem, one for which current treatments are increasingly inadequate as demonstrated by the high mortality rate for disseminated candidiasis. Understanding the complex interactions between the host and *C. albicans* during infection, especially in relation to the immune response, holds the promise of highlighting targets for developing new, more effective therapies. Immunity to *C. albicans* begins with host recognition via many PRRs with the CLRs thought to play a primary role. A prominent CLR, Dectin-1, acts as the primary receptor for β-glucan which is a component in the fungal cell wall. Recognition of fungi leads to a complex immune response that involves both innate immunity, of which the neutrophil plays a primary role, and adaptive immunity including Th17 mediated responses. Importantly, both innate and adaptive immune responses can be mediated via recognition of fungal β-glucan by Dectin-1 (10). As a fungal immune evasion tactic, β-glucan is often masked in order to avoid host recognition but it becomes unmasked later during infection (136). The mechanism by which this unmasking occurred in the host remained unclear and, given the potential importance of Dectin-1 mediated responses to host defense, this represents an important gap in our knowledge of host-pathogen interactions during candidiasis. Alterations in epitope availability are known to have consequences for immune responses during fungal infection both in animals and plants (50, 138, 139, 140) but are poorly understood, so investigating this mechanism of β-glucan unmasking could not only help the design of novel therapies to assist the host during candidiasis but will also broadly benefit our understanding of an underappreciated concept, which is how host-pathogen interactions can alter epitope availability dynamically during infection.

CHAPTER 2

NEUTROPHIL ATTACK TRIGGERS EXTRACELLULAR TRAP-DEPENDENT *CANDIDA ALBICANS* CELL WALL REMODELING AND ALTERED IMMUNE RECOGNITION

2.1 Summary

Pathogens hide immunogenic epitopes from the host to evade immunity, persist and cause infection. The opportunistic human fungal pathogen *Candida albicans*, which can cause fatal disease in immunocompromised patient populations, offers a good example as it masks the inflammatory epitope β-glucan in its cell wall from host recognition. It has been demonstrated previously that β-glucan becomes exposed during infection *in vivo* but the mechanism behind this exposure was unknown. Here, we show that this unmasking involves neutrophil extracellular trap (NET) mediated attack, which triggers changes in fungal cell wall architecture that enhance recognition by the Dectin-1 β-glucan receptor *in vitro*. Furthermore, using a mouse model of disseminated candidiasis, we demonstrate the requirement for neutrophils in triggering these fungal cell wall changes *in vivo.* Importantly, we found that fungal epitope unmasking requires an active fungal response in addition to the stimulus provided by neutrophil attack. NET-mediated damage initiates fungal MAP kinase-driven responses, particularly by Hog1, that dynamically relocalize cell wall remodeling machinery including Chs3, Phr1 and Sur7. Neutrophil-initiated cell wall disruptions augment some macrophage cytokine responses to attacked fungi. This work provides insight into host-pathogen interactions during disseminated candidiasis, including valuable information about how the *C. albicans* cell wall responds to the biotic stress of immune attack. Our results highlight the important but underappreciated concept that pattern recognition during infection is dynamic and depends on the host-pathogen dialog.

2.2 Introduction

Innate immune recognition of pathogen-specific patterns plays a crucial role in initial infection control and activation of appropriate adaptive immune responses (141, 142). Recognition though Toll-like, C-type lectin, Nod-like and Rig-I-like receptors elicits production of autocrine, paracrine and endocrine immunity. This includes activities as varied as deployment of neutrophil extracellular traps to directly attack pathogens and production of proinflammatory cytokines that recruit, activate and polarize additional innate and adaptive immune cells.

Pattern recognition receptors have evolved over millions of generations, and pathogens have concurrently developed creative ways to avoid these receptors by hiding specific epitopes. Epitope masking is practiced by many pathogens including bacteria, viruses, fungi, protozoans and helminths (117, 143, 128, 144, 29, 145, 146). Work from a number of groups, including ours, has described how fungal cell wall architecture limits recognition of the β-glucan sugar by immune receptors that include Dectin-1, a C-type lectin crucial for resistance to fungal infections (128, 31, 144). This epitope masking can be observed in *Candida albicans*, an opportunistic human pathogen which can cause both superficial mucosal and life threatening disseminated disease, particularly in immune compromised patients. However, *C. albicans* β-glucan epitope availability increases dramatically *in vivo* during a phase of neutrophilic influx in experimental murine candidemia (147, 136). Although the dynamics of immune recognition during infection have implications for the trajectory of the immune response, the fungal and host mechanisms that lead to eventual β-glucan masking *in vivo* are unknown.

It is possible that the host, the fungus or both contribute to these changes in immune recognition during infection. On the fungal side, the cell wall integrity (CWI) pathway is critical in maintaining this organelle in response to abiotic stresses, but we still don't understand how it functions in the context of immune attack in the challenging host environment (148). We have

previously described how a highly interconnected cell wall remodeling network creates and maintains the cell wall architecture that masks β-glucan from Dectin-1 under steady-state conditions, and this network may also act *in vivo* (29). On the host side, cell-mediated immune attack by neutrophils can kill or incapacitate pathogens using reactive oxygen and nitrogen species, antimicrobial peptides, proteases, glycosidases, and extracellular traps (ETs) (70, 149). Proteases and glycosidases could act on the outer mannan layer to directly expose underlying β-glucan, or phagocyte attack could indirectly trigger active fungal cell wall remodeling that unmasks underlying epitopes.

Changes in *C. albicans* cell wall β-glucan exposure due to early host-pathogen interaction during infection may sufficiently alter availability of cell wall epitopes to affect subsequent immune responses. However, the complexity of *in vivo* systems has limited our understanding of whether immune attack regulates subsequent immune cytokine elicitation. Here, we use a combination of *in vitro* and *in vivo* tools to show that neutrophils counter β-glucan masking by creating NETs that are required to trigger fungi to actively remodel local cell wall architecture. These disruptions of cell wall epitope masking alter recognition of the fungi and could enhance subsequent secondary immune responses.

2.3 Materials and Methods

2.3.1 *C. albicans* strains and Growth Conditions

C. albicans strains used in this study are listed in the Table 1. *C. albicans* was maintained on YPD 37˚C. Single colonies were picked to 5 mL YPD liquid and grown at 30˚C overnight on a rotator wheel. For hyphal cells, a defined number of yeast cells were transferred into RPMI and grown in 5 mL tubes at 30˚C overnight on a rotator wheel. JC-94-2 was constructed from the JC-94 parent (150). *PHR1-GFP*, along with 1kb upstream and 0.5 kb downstream regulatory sequence, was amplified from JC94 genomic DNA with primers PHR1-CIp20-FOR (5'-

ATATTCGACTGA**AAGCTT**GATTACAAGTGGGATGCAAAA-3'), and PHR1-CIP20-REV (5'-TCGTCGGGCTCA**AAGCTT**CGTTGAAAAAGCATAAGAAGG-3') and cloned into pCIp20 (151) with HinDIII, after which it was sequence verified. Integration of Sal1-cut Cip20-PHR1-GFP at the RP10 locus was confirmed by PCR and three independent clones had similar phenotypes.

Table 2.1. Fungal Strains

Strain names used	Parental Strain	Source or reference	Genotype
SC5314-GFP	SC5314	(136)	*Peno1*::*Peno1-EGFP-NAT*^{R}
hog1Δ/Δ-dTom	JC50	(152)	*ura3*:: λ *imm434/ura3*::*limm434*, *his1*::*hisG/his1*::*hisG*, *hog1*::l*oxP-ura3-loxP*, *hog1*::*loxP-HIS-loxP* Clp20 (*URA3, HIS1*), *Peno1*::*Peno1-dTom-NAT*^{R}
hog1/HOG1-dTom	JC52	(152)	*ura3*:: λ *imm434/ura3*::*limm434*, *his1*::*hisG/his1*::*hisG*, *hog1*::*loxP-ura3-loxP*, *hog1*::l*oxP-HIS-loxP* Clp20-*HOG1* (*URA3*, *HIS1*), *Peno1*::*Peno1-dTom-NAT*^{R}
WT-dTom (for *hog1* strains)	JC21	(152)	*ura3*:: λ *imm434/ura3*::*limm434*, *his1*::*hisG/his1*::*hisG* Clp20 (*URA3*, *HIS1*), *Peno1*::*Peno1-dTom-NAT*^{R}
cap1/CAP1-dTom	JC807	(153)	*cap1*::*loxP-HIS1-loxP/cap1*::*loxP-ARG4-loxP*,Clp20-*CAP1* (*URA3, HIS1*), *Peno1*::*Peno1-dTom-NAT*^{R}
WT-dTom (for *cap1* strains)	JC747	(154)	SN148 + Clp30 (*URA3 HIS1 ARG4*), *Peno1*::*Peno1-dTom-NAT*^{R}
cap1Δ/Δ-dTom	JC842	(154)	*cap1*::*loxP-HIS1-loxP/cap1*::*loxP-ARG4-loxP*, Clp20 (URA3), *Peno1*::*Peno1-dTom-NAT*^{R}
Phr1-GFP	CAS22	(150)	*phr1*Δ::*hisG/PHR1-GFP ura3*Δ::*imm434/ura3*Δ::*imm434* + Clp20 *PHR1-GFP*
	JC-94	(150)	*PHR1/PHR1-GFP ura3Δ::imm434/ura3Δ::imm434*
JC-94-2	JC-94	This work	*PHR1/PHR1-GFP ura3Δ::imm434/ura3Δ::imm434* + Clp20 *PHR1-GFP*
CAI4-dTom	CAI-4	(155)	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*, *Peno1*::*Peno1-dTom-NAT*^{R}
chs3Δ/Δ-dTom	chs3Δ/Δ	(156)	*chs3*Δ::*hisG/chs3*Δ::*hisG*, *Peno1*::*Peno1-dTom-NAT*^{R}
chs2Δ/Δ chs8Δ/Δ -dTom	NGY138	(157)	*chs2*Δ::*hisG/chs2*Δ::*hisG*, *chs8*Δ::*hisG/chs8*Δ::*hisG*, *Peno1*::*Peno1-dTom-NAT*^{R}
Sur7-GFP	YHXW4	(158)	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG SUR7-GFPγ*::*URA3*
Hwp1-GFP	YJB8250	(159)	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG Hwp1/Hwp1-GFP*
SC5314-FarRed	SC5314	This work	*Peno1*::*Peno1-FarRed670-NAT*^{R}
Chs3-YFP	NGY477	(160)	*ura3*::*imm434/ura3*::*imm434*, *his1*::*hisG/his1*::*hisG*, *arg4*::*hisG/arg4*::*hisG*, *CHS3/CHS3–YFP*:*URA3*, *RPS1/RPS1*::Clp30

2.3.2 Incubation of *C. albicans* and neutrophils *ex vivo*

Neutrophils from 6-12 week old female C57BL/6J or gp91$^{phox-/-}$ (B6.129S-*Cybb*^{tm1Din}/J (Jackson Laboratories) (161) mice were purified from bone marrow using biotin anti-Ly6G antibody (eBioscience) and AutoMACS separation (Miltenyi). Bone marrow from sex- and age-matched C57BL/6-backcrossed DPPI -/- and control mice was extracted after overnight shipment on cold packs (162). *C. albicans* hyphae at a concentration of 3x10^8 cells/mL were labeled with Biotin-XX-SSE (Molecular Probes; 0.01 µg/µL). Cells were then labeled with Alexa Fluor®647-conjugated Streptavidin (Jackson Immunoresearch; 36 µg/mL). 3 x10^7 hyphal cells were then incubated with or without 7 x 10^6 neutrophils in RPMI + 5% FBS for 2.5 hours. Neutrophils were lysed with 0.02% Triton X-100. *C. albicans* hyphae were then stained with sDectin-1-Fc (17 µg/ml) followed by donkey anti-human IgG Cy3 antibody (Jackson Immunoresearch; 0.8 mg/ml) and Calcofluor White (Sigma Chemicals; 25 ng/mL). Purified sDectin-1-Fc was prepared from stably transfected HEK293T cells as previously described {Graham, 2006 #163}. Cells were visualized by optical sectioning fluorescence microscopy using a Zeiss Axiovision Vivotome microscope (Carl Zeiss Microscopy, LLC). Fields of view were chosen randomly and an equal number of images were obtained for each sample. Maximum image projections were used to score the percentage of cells with increased chitin deposition, β-glucan exposure and the overlap of both phenotypes.

For post-challenge labeling experiments, hyphae were biotinylated, and incubated with neutrophils; after neutrophil lysis and staining, Alexa Fluor®647-conjugated Streptavidin was included along with the secondary antibody. For chemical inhibition, neutrophils were pre-incubated with either the vehicle DMSO, 10 µM DPI, 300 µM Apocynin, 100 µM ABAH or 500 µM ABAH for 10 minutes before addition to *C. albicans* and samples were treated for the entire 2.5 hours. For UV inactivation experiments, hyphal cells were UV inactivated as described (29). To

ensure that lack of damage was not due to altered neutrophil attack rates, staining in experiments with the *hog1*Δ/Δ, *cap1*Δ/Δ and *chs2*Δ/Δ *chs8*Δ/Δ and *chs3*Δ/Δ mutant strains was performed without neutrophil lysis with Triton X-100 treatment. To examine NETs, sDectin-1-Fc and CFW staining procedures were carried out as described above, with Sytox Green (Molecular Probes; 156 nM) added along with the secondary antibody and CFW. We used Anti-Histone H3 citrulline R2+R8+R17 (abcam; 0.014 mg/mL) and donkey anti-rabbit IgG Cy3 (Jackson Immunoresearch; 0.0075 mg/mL) as well as Anti-MPO (R&D Systems; 0.1 mg/mL) with Donkey anti-goat Cy3 (Jackson Immunoresearch; 0.007 mg/mL). In experiments with DNase 1, the RPMI used for the incubation was supplemented with 100 mM $CaCl_2$ and 100 mM $MgCl_2$.

2.3.3 Imaging Dish Experiments

Streptavidin-labeled hyphae of the indicated strain, at a concentration of $6x10^6$ cells, were added to a Delta T imaging dish (Bioptechs Inc) with $8x10^5$ neutrophils in 1 mL of Phenol red-free RPMI + 5%FBS (Lonza). The Chs3-YFP strain was not labeled and imaged in 1 mL of PBS with 5% FBS and 5.5 mM glucose. Imaging dishes were then either incubated at 37˚C in an incubator for the indicated amount of time or immediately imaged on the Zeiss Axiovision Vivotome microscope (Carl Zeiss Microscopy, LLC) or Nikon PerfectFocus microscope (Nikon) with a heated stage (Bioptechs, Inc) at 37˚C. Chs3-YFP timelapses were instead taken on a Nikon Ti-E PFS live cell microscope (Nikon, Inc). For staining in dishes, the sDectin-1-Fc and CFW staining was done as described in the above section except $4.25x10^6$ neutrophils were added, they were not lysed and the process was carried out in the dish.

2.3.4 *In vivo* infections and organ *ex vivo* fluorescence

Six week old female Balb/cJ, C57BL/6J or $gp91^{phox-/-}$ mice (Jackson Laboratories) were infected via tail vein. WT, isotype controls (Rat IgG2a for 1A8 from Bio X cell) and RB6-8C5 (Bio X cell) treated mice received $1x10^5$ cfu, those receiving 1A8 received $2.5x10^4$ cfu and $gp91^{phox-/-}$

mice received 500 cfu. Mice were treated with isotype, RB6-8C5 or 1A8 antibody (Bio X cell; 100 µg in 200 µL of PBS) via i.p. injection on day 2 post-infection. On day 5 post-infection mice were sacrificed via CO_2 inhalation followed by cervical dislocation. Neutropenia was confirmed by Wright staining of blood obtained by cardiac puncture. Organs were harvested and homogenized as described (136). For some experiments, kidneys were bisected with a razor and half was processed for histology. Homogenates were stained with sDectin-1-Fc (17 µg/ml) then donkey anti-human IgG Cy3 (0.8 mg/ml) and Calcofluor White (25 ng/mL). Alternatively, homogenates were stained with anti-β-glucan antibody (Biosupplies, Inc., Australia; 1.7 mg/mL) then with goat anti-mouse Cy3 antibody (Jackson Immunoresearch; 3.8 mg/mL). Cells were visualized by optical sectioning fluorescence microscopy using a Zeiss Axiovision Vivotome microscope (Carl Zeiss Microscopy, LLC). Maximum projection images were quantified using Cellprofiler (www.cellprofiler.org) as described (136).

2.3.5 Macrophage cytokine elicitation

RAW-blue macrophages (Invivogen) were maintained in DMEM + 10% FBS supplemented with sodium pyruvate, gentamicin and zeocin. For detection of cytokines by ELISA, unlabeled $3.0x10^7$ *C. albicans* hyphae were incubated with or without $7x10^6$ neutrophils overnight in RPMI+5%FBS. A neutrophil alone group was also included. The next morning RAW-blue macrophages were harvested. Macrophages were resuspended at $2.77x10^6$ cells/mL and pre-incubated with Anti-mDectin-1 (Bio-Rad; 10 µg) or IgG2b isotype control (Invivogen; 10 µg) for 90 minutes. *Candida*-neutrophil mixtures were treated with 0.05% Triton X-100 solution for 5 minutes. As control for the impact of neutrophil debris in the context of fungal stimulation, one of the neutrophil alone samples was added to the *C. albicans* alone sample just before lysis. Samples were washed extensively and then incubated with 200 units of DNase1 for 1 hour. Samples were then washed extensively again and resuspended in 500 µL PBS. 25 µL of sample

was added to the wells of a 96 well plate in duplicate before UV inactivation by treatment with 5 x 100,000 μJ/cm^2. For a positive control, depleted zymosan (Invivogen) was added and for a negative control sterile water was added. RAW-blue cells, either Anti-mDectin-1 treated or not, were then added to the UV-inactivated fungal cells at 5x10^5 cells per well. Supernatants were harvested after 6 hours. ELISA was performed using Mouse TNF-α and IL-6 DuoSets (R&D Systems) according to manufacturer's instructions and detected using Supersignal ELISA Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) using a Biotek Synergy 2 plate reader (Biotek Instruments, Inc).

2.3.6 Statistical Analysis

Statistics were performed as described in figure legends. For normally distributed data, Student's t test or one or two way ANOVA analysis with Tukey's post-test were used. For non-parametric data, Kruskal-Wallis with Dunn's post-test was applied. ANOVA and Kruskal-Wallis were done using Prism software (Graphpad Software). $p < 0.05$ was considered significant.

2.4 Results

2.4.1 Neutrophils disrupt cell wall organization and cause β-glucan unmasking *in vitro*

Changes to the cell wall during infection alter *C. albicans* recognition by pattern recognition receptors, but the mechanisms driving these changes are unknown (50, 136). Host defense against invasive candidiasis relies critically on neutrophils, evidenced by the increased susceptibility of neutropenic patients to candidemia (71). We reasoned that they may disrupt the fungal cell wall and mediate β-glucan unmasking because neutrophils can damage the *C. albicans* cell wall and are present in high numbers during infection when β-glucan unmasking appears (164, 147, 136). To determine the spatiotemporal dynamics of neutrophilic damage, we labeled biotinylated fungi with streptavidin-Alexa 647 and incubated with neutrophils. Time-

lapse imaging shows that streptavidin fluorescence is lost rapidly at sites of neutrophil attack (Figure 2.1A-D, 2.1A-B Movie). Controls demonstrate that there is also a loss of labeled protein (Figure 2.2). Fluorescence of an Hwp1-GFP fusion protein, present in the hyphal cell wall, is also rapidly reduced upon neutrophil attack (Figure 2.3, 2.2A-B Movie). Overall, this suggests that neutrophils rapidly damage cell wall protein at sites of attack, in agreement with and extending previous reports (164).

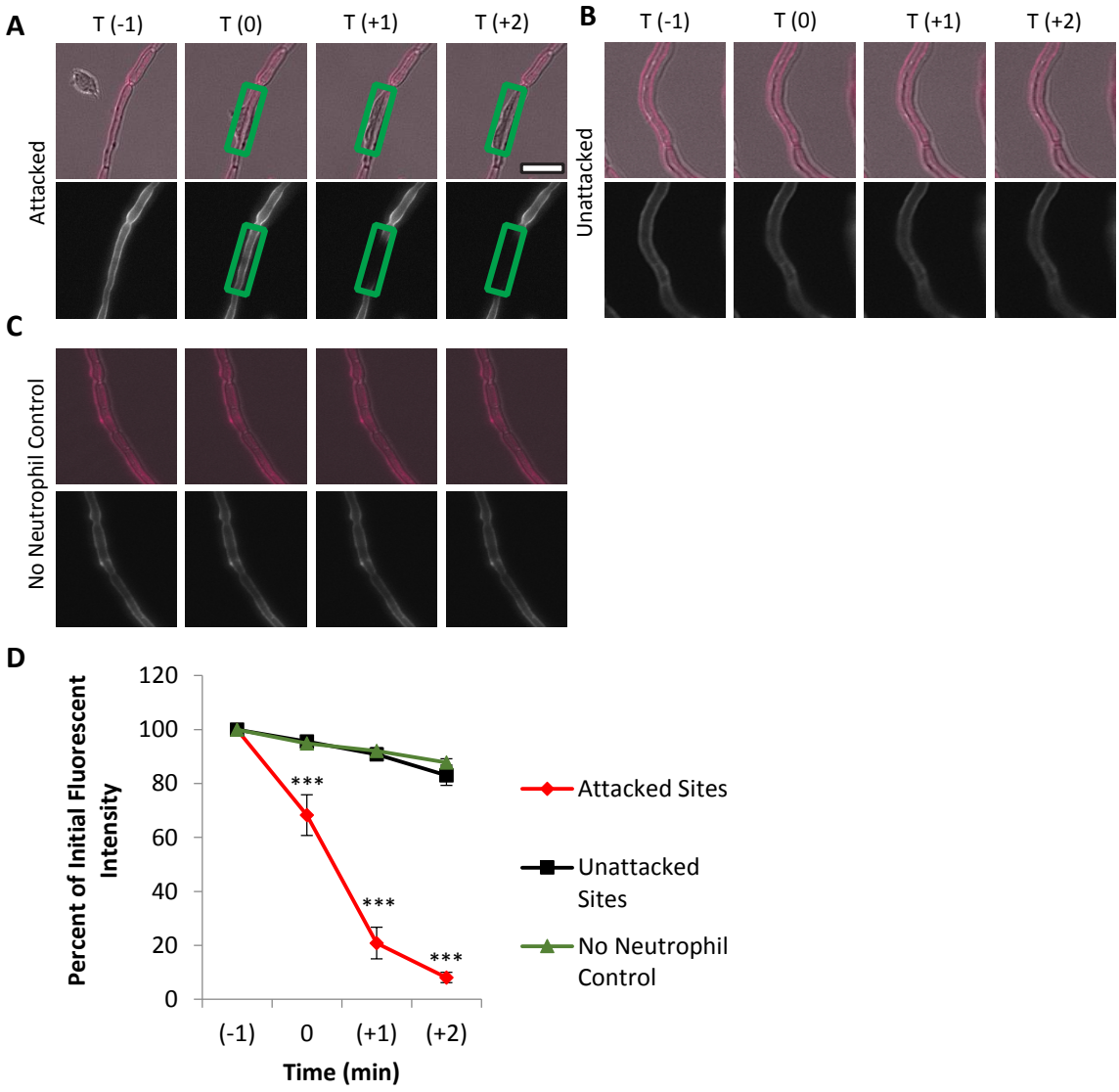
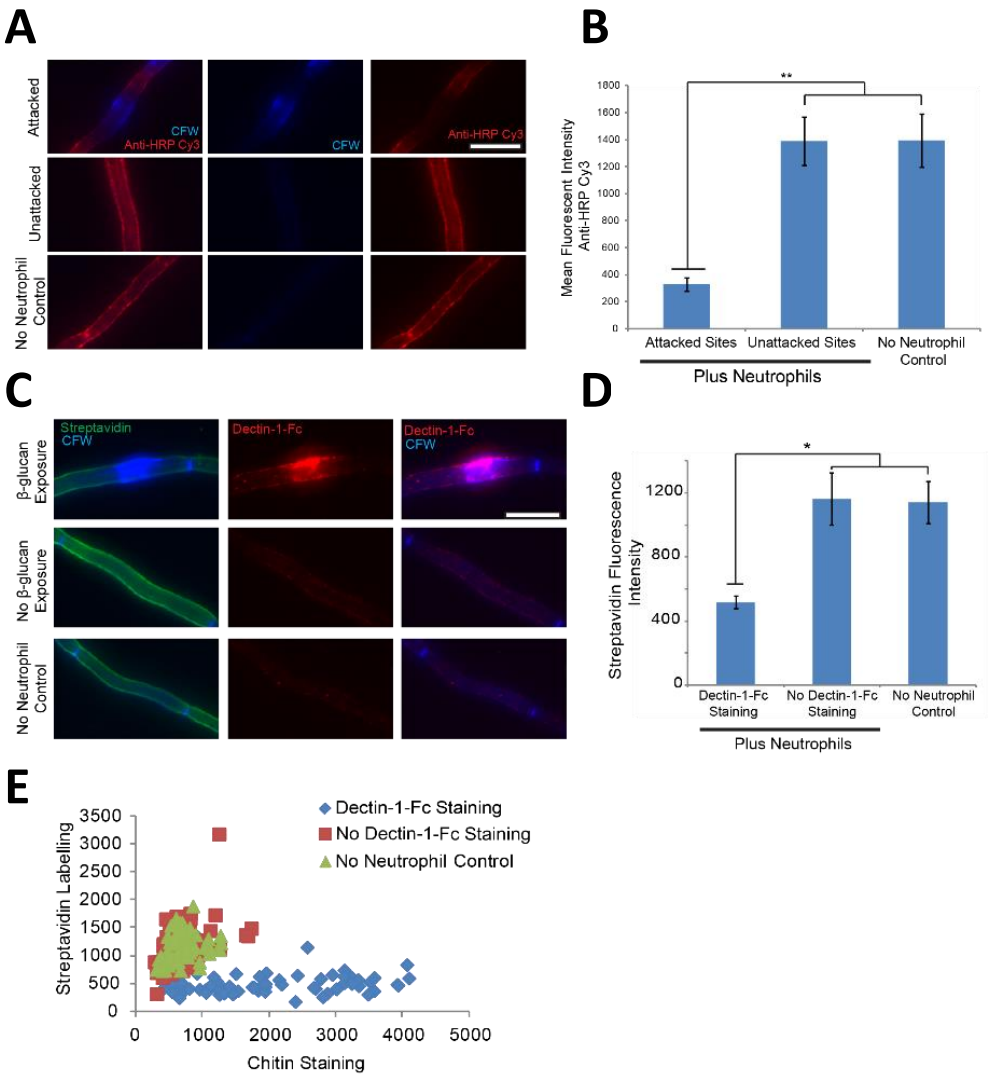


Figure 2.1. Neutrophil attachment results in rapid cell wall damage to *C.albicans*

(A-D) Streptavidin-Alexa 647-labeled SC5314-GFP was incubated with or without neutrophils. (A-C) Representative timelapse images at one minute intervals for (A) attacked, (B) non-attacked and (C) control hyphal segments. (D) Relative streptavidin mean fluorescence intensity (MFI) at attacked and unattacked sites. Data represents the pooled average of thirteen cells measured in three experiments ± SEM. Scale bar = 10 µm. *** $p \leq 0.001$ (one-way ANOVA with Tukey's post-test).

Figure 2.2. Neutrophil attack results in cell wall damage and protein loss

(A-B) Streptavidin-HRP labeled SC5314-GFP was incubated with neutrophils or alone. Neutrophils were lysed and fungi were then stained with an anti-HRP Cy3 antibody and CFW. Representative images for each group are shown (A). Images were analysed by obtaining the mean fluorescent intensity at sites with and without chitin deposition and data is presented as the mean ± SEM of three independent experiments (B). (C-E) SC5314-GFP was pre-labeled with biotin and then incubated with neutrophils. After incubation, neutrophils were lysed and hyphae were stained with Streptavidin-A647, Dectin-1-fc and CFW. Images were analyzed by obtaining the mean fluorescent intensity in the blue, red and far red channels at sites of β-glucan exposure, at sites without β-glucan exposure and from sites in the no neutrophil control, limited to viable cell segments. (D) Streptavidin fluorescence at sites with sDectin-1 staining or no sDectin-1 staining. Mean MFI ± SEM of three independent experiments. (E) Streptavidin versus CFW fluorescence for individual sites from three pooled experiments, broken down into sites with sDectin-1 staining or not. Cell viability was confirmed based on the characteristic cytoplasmic EGFP expression of live cells (not shown here). * $p \leq 0.05$ ** p-value ≤ 0.01 (one way ANOVA with Tukey's post-test). Scale bar represents 10 µm.


A
Attacked
Unattacked
No Neutrophil Control
CFW
Anti-HRP Cy3
CFW
Anti-HRP Cy3
B
Mean Fluorescent Intensity Anti-HRP Cy3
1800
1600
1400
1200
1000
800
600
400
200
0
**
Attacked Sites
Unattacked Sites
No Neutrophil Control
Plus Neutrophils
C
β-glucan Exposure
No β-glucan Exposure
No Neutrophil Control
Streptavidin
CFW
Dectin-1-Fc
Dectin-1-Fc
CFW
D
Streptavidin Fluorescence Intensity
1200
800
400
0
*
Dectin-1-Fc Staining
No Dectin-1-Fc Staining
No Neutrophil Control
Plus Neutrophils
E
Streptavidin Labelling
3500
3000
2500
2000
1500
1000
500
0
0
1000
2000
3000
4000
5000
Chitin Staining
Dectin-1-Fc Staining
No Dectin-1-Fc Staining
No Neutrophil Control

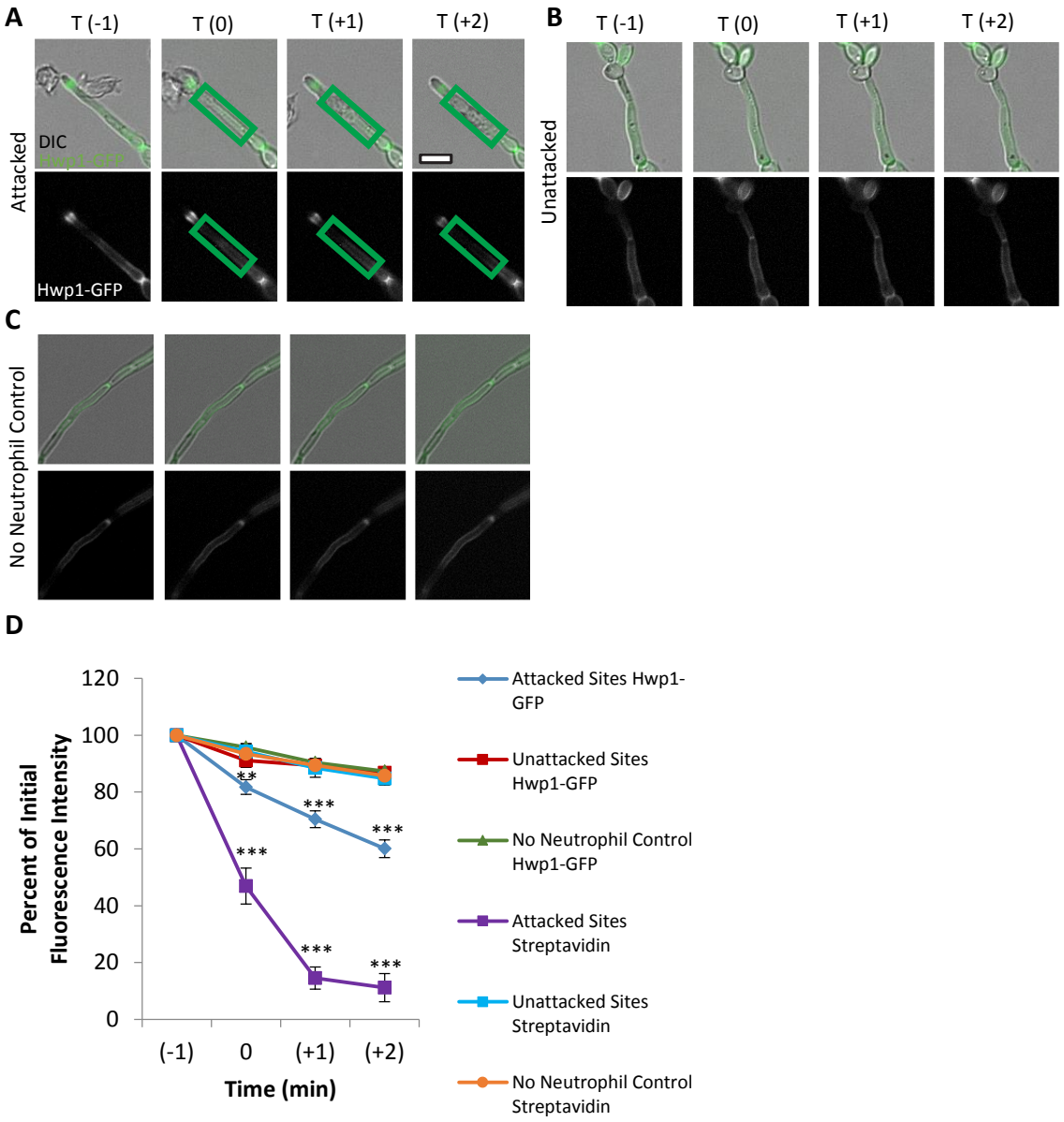
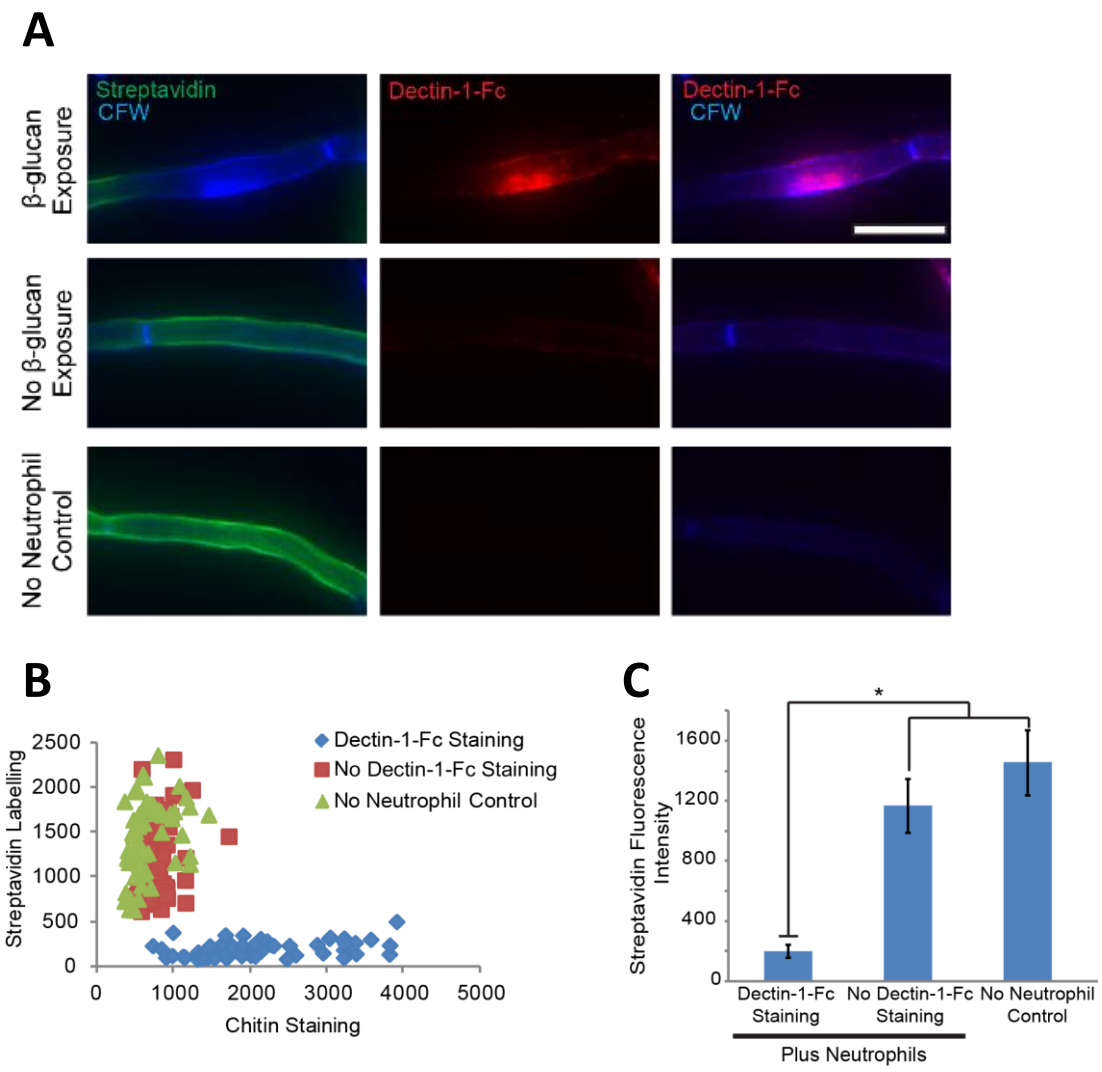


Figure 2.3 Neutrophil attachment results in rapid damage to cell wall proteins like Hwp1-GFP (A-D) Representative time-lapse images for (A) attacked, (B) unattacked and (C) control hyphal segments. (D) Relative streptavidin and Hwp1-GFP intensities for thirteen cells imaged in three independent experiments were measured and the pooled average ± SEM is shown. Scale bar = 10 µm. ** p ≤0.01 and *** p≤0.001 (one-way ANOVA with Tukey's post-test).

To determine if neutrophil attack disrupts other aspects of cell wall architecture to alter immune recognition, we stained attacked filaments with soluble Dectin-1-Fc (sDectin-1-Fc) to quantify β-glucan availability and Calcofluor white (CFW) to quantify chitin deposition. This revealed areas of the lateral cell wall with β-glucan unmasking and increased chitin deposition at sites with cell wall protein loss (Figure 2.4). These overlapping sites of cell wall disruption occur uniquely in the neutrophil challenged samples but not in the absence of neutrophils, demonstrating that they are a direct or indirect result of immune activity. Taken together, these results show that neutrophil attack can result in the disruption of *C. albicans*' cell wall architecture and β-glucan unmasking *in vitro*.

Figure 2.4. Neutrophils cause β-glucan unmasking and disrupt cell wall architecture

(A-C) SC5314-GFP cells were biotinylated, labeled with Streptavidin-Alexa647 and incubated with neutrophils or alone. Neutrophils were lysed and fungi were stained with sDectin-1-Fc and Calcofluor White. (A) A representative set of images for pre-challenge streptavidin labeling. (B-C) Images were analyzed by obtaining the mean fluorescent intensity in the blue, red and far red channels at sites of β-glucan exposure, at sites without β-glucan exposure and from sites in the no neutrophil control, limited to viable cell segments. (B) Streptavidin versus CFW fluorescence for individual sites from three pooled experiments, broken down into sites with sDectin-1 staining or not. (C) Streptavidin fluorescence at sites with sDectin-1 staining or no sDectin-1 staining. Mean MFI ± SEM of three independent experiments. Cell viability was confirmed based on the characteristic cytoplasmic EGFP expression of live cells (not shown here). Scale bar represents 10 μm. * $p \leq 0.05$ (one-way ANOVA with Tukey's post-test).


A
Streptavidin
CFW
Dectin-1-Fc
Dectin-1-Fc
CFW
β-glucan Exposure
No β-glucan Exposure
No Neutrophil Control
B
Dectin-1-Fc Staining
No Dectin-1-Fc Staining
No Neutrophil Control
Streptavidin Labelling
Chitin Staining
2500
2000
1500
1000
500
0
0
1000
2000
3000
4000
5000
C
Streptavidin Fluorescence Intensity
1600
1200
800
400
0
*
Dectin-1-Fc Staining
No Dectin-1-Fc Staining
No Neutrophil Control
Plus Neutrophils

2.4.2 Neutrophils are critical for β-glucan unmasking during disseminated candidaisis

We have previously shown β-glucan unmasking occurs during infection and our *in vitro* data suggests that neutrophils can mediate this exposure (Wheeler et al 2008 #136). To test if neutrophils are required for these fungal cell wall changes *in vivo*, we examined *C. albicans* epitope exposure in neutropenic mice at day 5 post-infection, when there is normally β-glucan unmasking. To interrogate the native state of the *C. albicans* cell surface we used the *ex vivo* fluorescence method, which involves no fixation or permeabilization (136). There is a significant reduction in β-glucan unmasking in neutropenic mice, demonstrating that neutrophils are critical for β-glucan unmasking *in vivo* (Figure 2.5 A-E). Similar results are seen in a second model of neutropenia (Figure 2.6). Levels of β-glucan unmasking were similar when detected via either anti-β-glucan antibody or sDectin-1-Fc staining, demonstrating that this is not an artifact of a specific probe (Figure 2.6). Further, chitin staining revealed that fungi from control mice have significantly stronger chitin deposition than neutropenic mice, suggesting that neutrophil attack is also important for increased chitin deposition *in vivo* (Figure 2.5 A-D). Fungi from neutropenic mice have slightly increased β-glucan unmasking and chitin levels as compared to *in vitro* RPMI-grown control cells, suggesting that growth in the host or possibly attack by other immune cells may also yield minor but significant cell wall changes even without neutrophil attack. Taken together, these results demonstrate that neutrophils are critical drivers of β-glucan unmasking and increased chitin deposition during disseminated infection *in vivo*.

Figure 2.5. Neutrophils are critical for β-glucan unmasking *in vivo* in the kidney

(A-E) BALB/cJ mice were injected in the tail vein with SC5314-GFP and were treated with either IgG2a isotype control or 1A8 antibody via i.p. injection before being sacrificed at day five post infection. (A-B) Representative images of kidney homogenates stained with sDectin-1-Fc and Calcofluor White. Bottom panels show homogenates treated with secondary antibody only as a control. (C) Representative images of an overnight culture of SC5314-GFP grown in RPMI. Scale bar represents 10 μm. (D-E) Quantification of chitin staining (D) and sDectin-1-Fc staining (E). Data is presented as the mean ± SEM from three pooled experiments. ** p-value ≤0.01 and *** p-value ≤0.001 (Kruskal-Wallis with Dunn's post-test).

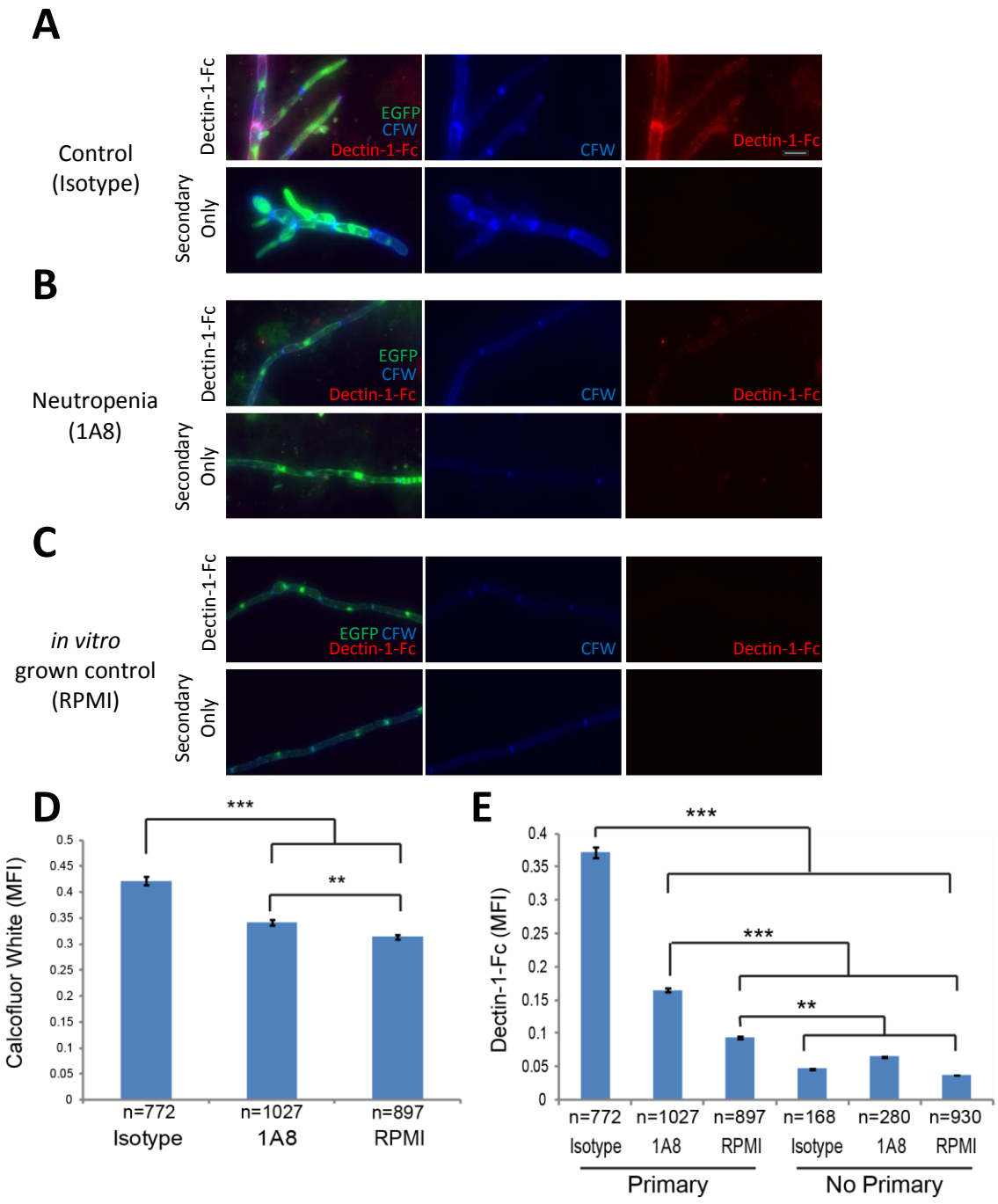
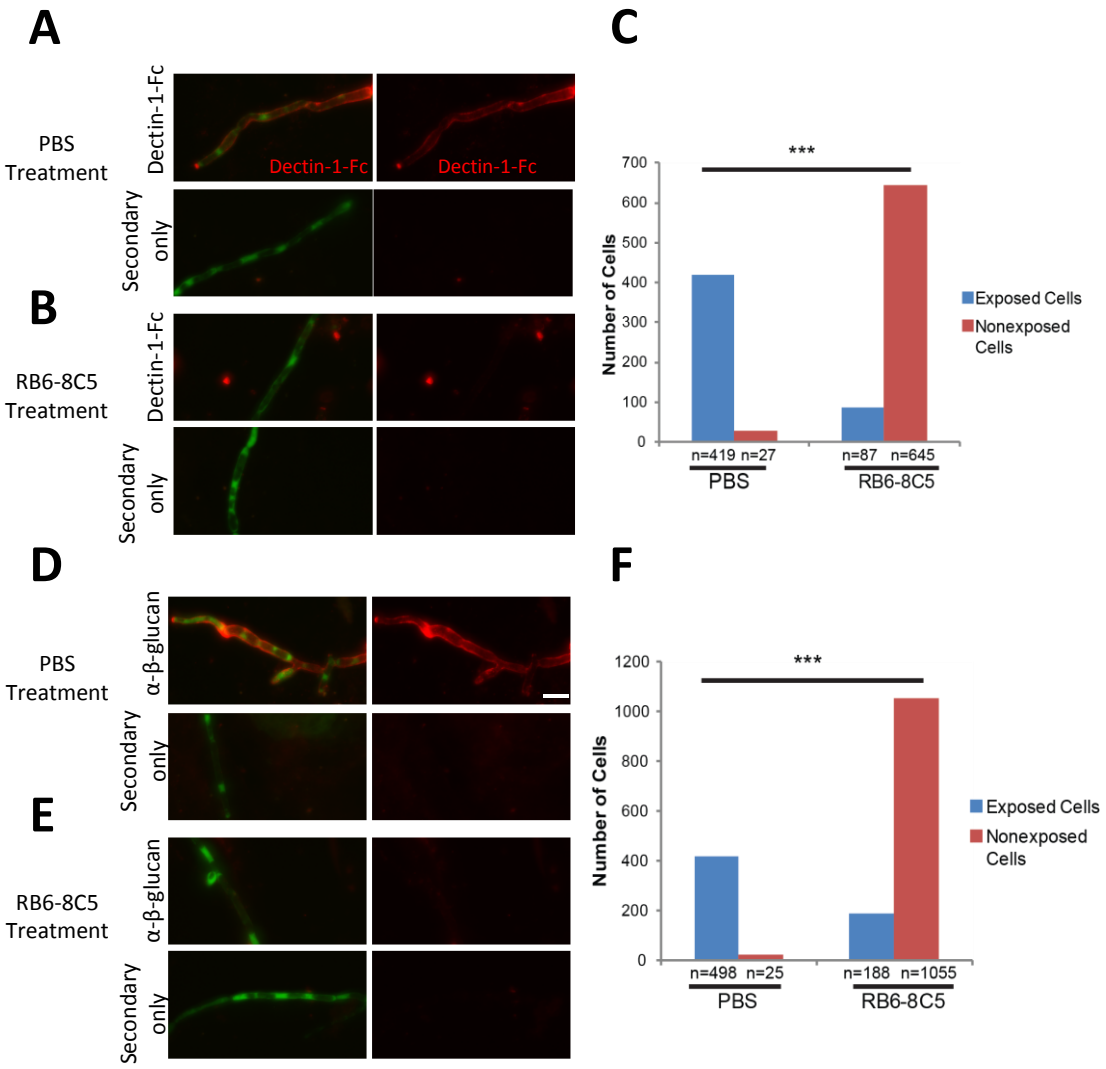

A
Control
(Isotype)
Dectin-1-Fc
Secondary Only
EGFP
CFW
Dectin-1-Fc
CFW
Dectin-1-Fc
B
Neutropenia
(1A8)
C
in vitro
grown control
(RPMI)
D
Calcofluor White (MFI)
n=772
Isotype
n=1027
1A8
n=897
RPMI
E
Dectin-1-Fc (MFI)
n=772
Isotype
n=1027
1A8
n=897
RPMI
n=168
Isotype
n=280
1A8
n=930
RPMI
Primary
No Primary

Figure 2.6. Requirement of neutrophils for the development of β-glucan unmasking is independent of the method of neutropenia or β-glucan staining

BALB/cJ mice were injected in the tail vein with SC5314-GFP and were treated with either PBS or RB6-8C5 antibody via i.p. injection before being sacrificed at day five post infection. (A-B) Representative images of kidney homogenates stained with sDectin-1-Fc. Bottom panels show homogenates treated with secondary antibody only. (C) Images were quantified by scoring *Candida* cell segments for β-glucan exposure (either exposed or non-exposed). Total number of cells found in each category were presented according to mouse treatment group. Three independent experiments were performed. A significant association between PBS treatment and exposed cells and between RB6-8C5 treatment and non-exposed cells was seen, p-value<0.0001 (Fisher's Exact test). (D-E) Representative images of kidney homogenates stained with anti-1,3-β-glucan antibody. Bottom panels show homogenates treated with secondary antibody only. (F) Images were quantified by scoring as described for (E). A significant association between PBS treatment and exposed cells and between RB6-8C5 treatment and non-exposed cells was seen, p-value<0.0001 (Fisher's Exact test). Scale bar represents 10 μm.


A
PBS
Treatment
Dectin-1-Fc
Dectin-1-Fc
Dectin-1-Fc
Secondary
only
B
RB6-8C5
Treatment
Dectin-1-Fc
Secondary
only
C
***
Number of Cells
700
600
500
400
300
200
100
0
n=419 n=27
n=87 n=645
PBS
RB6-8C5
Exposed Cells
Nonexposed
Cells
D
PBS
Treatment
α-β-glucan
Secondary
only
E
RB6-8C5
Treatment
α-β-glucan
Secondary
only
F
***
Number of Cells
1200
1000
800
600
400
200
0
n=498 n=25
n=188 n=1055
PBS
RB6-8C5
Exposed Cells
Nonexposed
Cells

2.4.3 NET attack results in fungal cell wall disruption

It was not previously known that neutrophils alter innate pattern recognition of fungi *in vivo*, so we sought to characterize the mechanisms required to alter epitope unmasking. NET production, in which neutrophils create traps out of DNA and numerous antimicrobial factors, is a means of NADPH oxidase-dependent neutrophil attack against *C. albicans* and other fungi *in vivo* and *in vitro* (83, 165). Despite the poor NET production of mouse neutrophils relative to human neutrophils, we find strong evidence of NET formation *in vitro*. These NETs stain positive with the membrane impermeant Sytox green DNA dye, anti-citrullinated histone antibody, and anti-myeloperoxidase (MPO) antibody (Figure 2.7). Furthermore, we observed that neutrophils could create ETs without dying (Figure 2.8, 2.3A-B Movie) Strikingly, treatment with DNase I to degrade extracellular DNA and prevent the establishment of NETs blocks both chitin deposition and β-glucan unmasking, functionally implicating NETs in driving this interaction (Figure 2.9A). In further support of NET-triggered changes during attack, inhibition of myeloperoxidase (MPO) with 4-aminobenzoic acid hydrazide (ABAH) prevents neutrophil attack from resulting in chitin deposition or β-glucan unmasking (Figure 2.9B). Taken together, these data provide strong evidence that NETs provide the initial stimulus that results in fungal cell wall changes including β-glucan exposure.

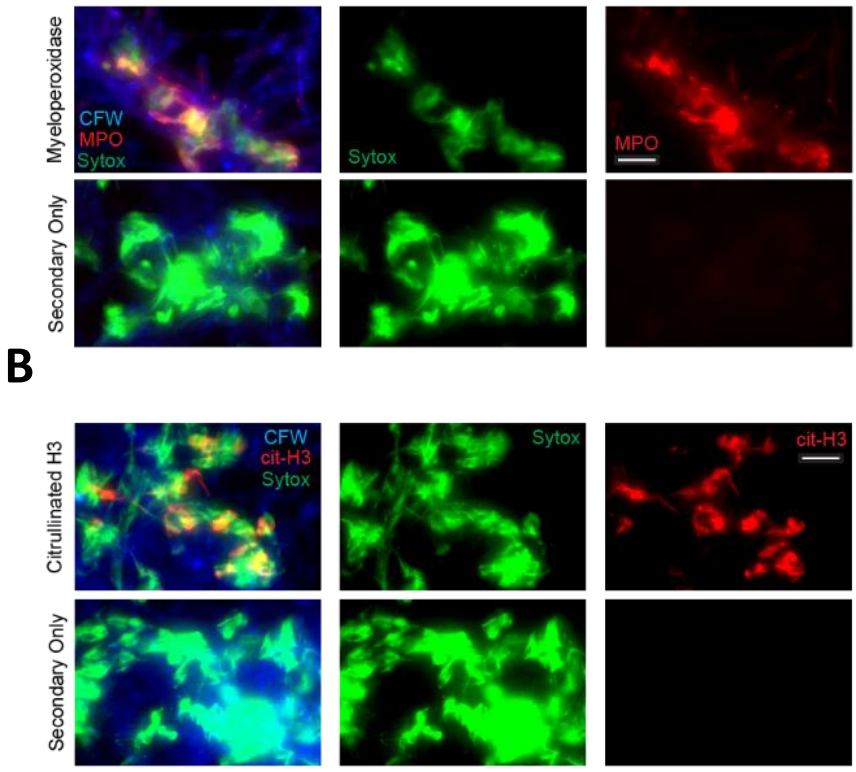


Figure 2.7. Neutrophil extracellular traps are deployed against *C. albicans*

Neutrophils were incubated with SC5314-FarRed hyphae for 2.5 hours and then samples were stained to probe for the presence of specific NET components. (A-B) Representative images of MPO (A) and citrullinated H3 staining (B). Scale bar represents 20 µm.

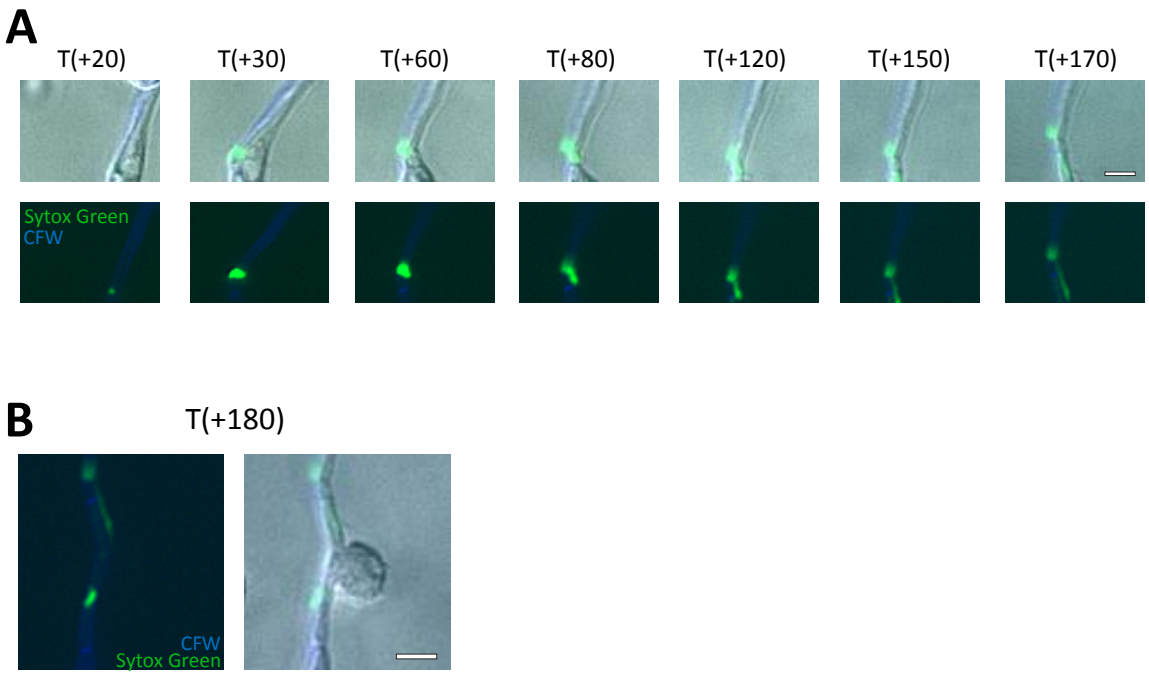


Figure 2.8. Extracellular trap deployment by live neutrophils

(A-B) SC5314-FarRed hyphae were incubated with neutrophils in an imaging dish with CFW and Sytox Green. Timelapses were obtained using binning 2x2 and 10 minute intervals. (A) Individual panels from the timelapse showing neutrophil ET deployment. (B) Image taken after the end of the timelapse of the area of ET deployment. Exclusion of Sytox Green from the neutrophil demonstrates it is still viable and that ETs can be deployed by live neutrophils in our assays. Scale bar represents 5 μm.

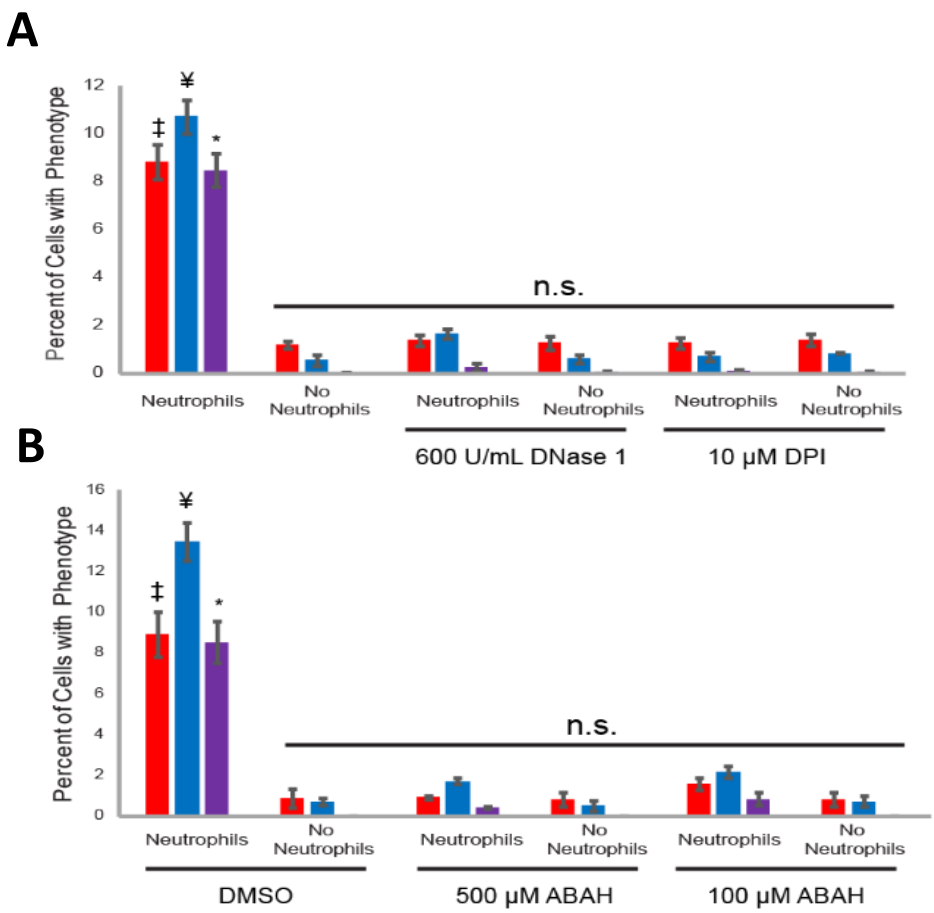


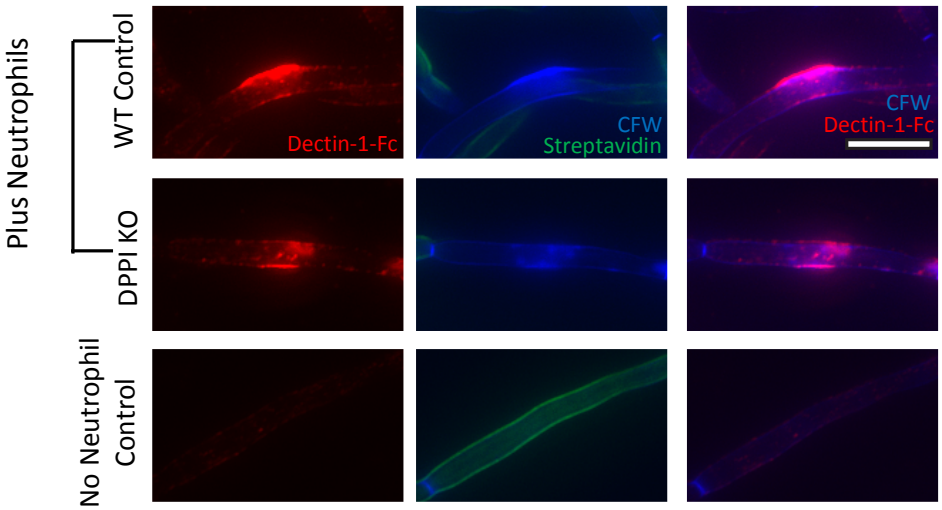
Figure 2.9. Neutrophil extracellular traps are critical for β-glucan unmasking

(A-B) Neutrophils were pretreated with DNase I, DPI (A) or ABAH (B) and then incubated with SC5314-FarRed for 2.5 hours. Neutrophils were not lysed and samples were stained with CFW, sDectin-1-Fc and Sytox Green. Images were analyzed by scoring viable cell segments for the presence of the indicated phenotypes and results are presented as the percentage of total cell segments counted with the phenotype for each group. Data is presented as the mean ± SEM from three experiments. Cell viability was determined using characteristic cytoplasmic far red expression (not shown here). ‡ p-value ≤ 0.01 when comparing the sDectin-1-Fc group from WT to other groups (Red). ¥ p-value ≤ 0.01 when comparing the chitin deposition group from WT to other groups (Blue). * p-value ≤ 0.01 when comparing the overlap group from WT to other groups (Purple). Comparisons done by one way ANOVA and Tukey's post-test.

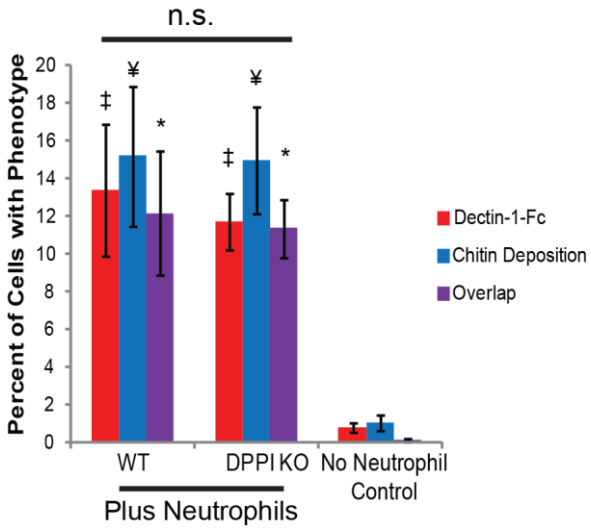
Neutrophil proteases are thought to be an important component of NET formation in some contexts and neutrophil elastase trafficking is regulated during NETosis against *C. albicans* (83, 166, 167). However, neutrophils from mice deficient in the dipeptidyl peptidase (DPPI), which is required for the activation of the three major neutrophil proteases: elastase, cathepsin G and proteinase 3 (168), show no defect in their ability to cause β-glucan unmasking, chitin deposition or streptavidin loss (Figure 2.10, Figure 2.11).Thus, these three proteases do not appear to play an important role in the downstream cell wall remodeling triggered by neutrophil attack of *C. albicans* in this system.

Figure 2.10. The major neutrophil proteases are not required for *C. albicans* cell wall disruption Neutrophils from WT age and sex matched controls or DPPI KO mice were incubated with Streptavidin-Alexa 647 labelled SC5314-GFP hyphae. Neutrophils were lysed and the fungi were stained with sDectin-1-Fc and Calcofluor White. (A) Representative set of images for each group. (B) Images were analyzed by scoring all viable cell segments for the presence of the indicated phenotype and the results are presented as the percentage of total cell segments counted. Data is presented as the mean ± SEM from three experiments. Cell viability was determined using characteristic cytoplasmic GFP expression (not shown here). ‡ p-value ≤ 0.05 for comparing the sDectin-1-Fc group from WT or DPPI KO to the no neutrophil group. ¥ p-value ≤ 0.05 for comparing the chitin deposition group from WT or DPPI KO to the no neutrophil group. * p-value ≤ 0.05 for comparing the overlap group from WT or DPPI KO to the no neutrophil group. Comparisons done by one way ANOVA with Tukey's post-test. Scale bar represents 10 µm.

A


Plus Neutrophils
WT Control
DPPI KO
No Neutrophil Control
Dectin-1-Fc
CFW
Streptavidin
CFW
Dectin-1-Fc

B


n.s.
Percent of Cells with Phenotype
0
2
4
6
8
10
12
14
16
18
20
‡
¥
*
‡
¥
*
WT
DPPI KO
No Neutrophil Control
Plus Neutrophils
Dectin-1-Fc
Chitin Deposition
Overlap

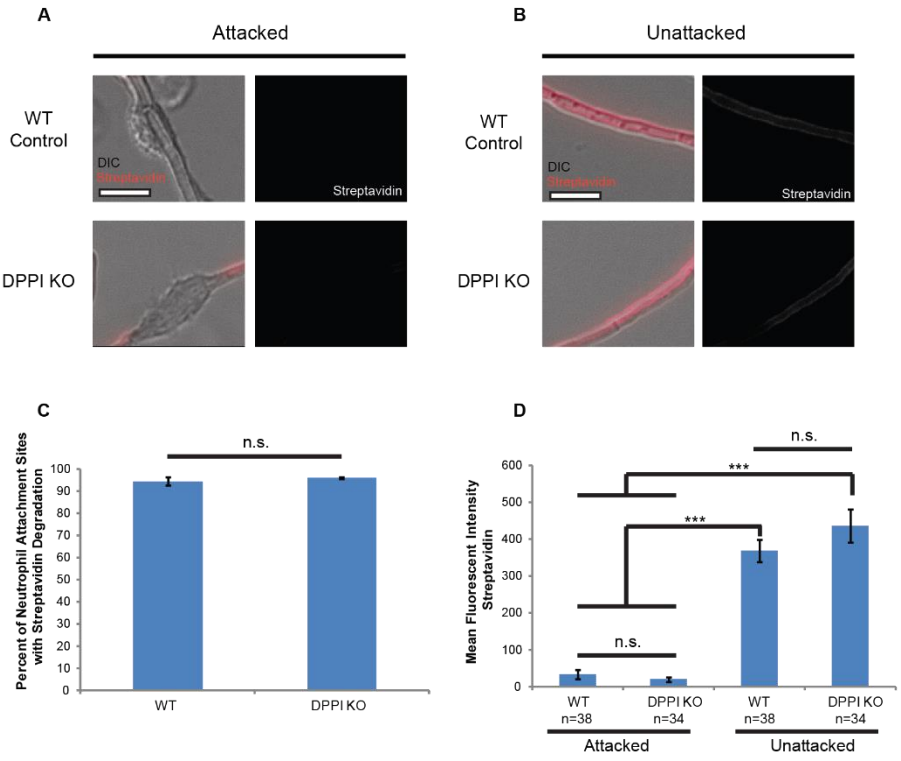


Figure 2.11. The major neutrophil proteases aren't required for causing cell wall damage

(A-D) Streptavidin-Alexa 647 labeled hyphae were incubated with either WT or DPPI KO neutrophils in an imaging dish for 30 minutes. (A-B) Representative images for each group. (C) The percent of neutrophil sites with streptavidin degradation for each group. Data represents the mean ± SEM from three independent experiments. (D) The average MFI in the far red channel at sites of neutrophil attachment for each group. Data represents the mean ± SEM from three pooled experiments. *** p-value ≤ 0.001 (one way ANOVA with Tukey's post-test). n.s. means non-significant by Student's Ttest (E) or one way ANOVA with Tukey's post-test (B,F). Scale bar represents 10 µm.

Phagocyte NADPH oxidase is important in defense against candidemia and plays an important role in NET formation under many conditions, including in response to fungi (169, 83, 170). We find that chemical or genetic disruption of NADPH oxidase function decreases cell wall damage and prevents immune attack from resulting in β-glucan unmasking or chitin deposition *in vitro* (Figure 2.12., Figure 2.13). This was not due to a complete lack of neutrophil attack on the hyphae (Figure 2.13). Similarly, fungi from the kidneys of gp91$^{phox-/-}$ mice had significantly less chitin staining and β-glucan unmasking, demonstrating the importance of phagocyte oxidase for causing cell wall remodeling *in vivo* (Figure 2.14). As expected, WT mice were able to control fungal growth while gp91$^{phox-/-}$ mice were unable to do so (Figure 2.15). Importantly, immune cells including many neutrophils were found surrounding hyphae in gp91$^{phox-/-}$ mice (Figure 2.15), suggesting that the loss of β-glucan unmasking was not due to lack of immune cell recruitment. Taken together, these data suggest that NETs also trigger *C. albicans* cell wall remodeling and enhanced Dectin-1 recognition *in vivo* and reveal a new way that immune cells counter fungal immune evasion.

Figure 2.12. The phagocyte NADPH oxidase is critical for fungal cell wall disruption *in vitro*

(A-F) Neutrophils from C57BL/6J or gp91$^{phox-/-}$ mice were pretreated with the NADPH oxidase inhibitors DPI (10 µM) or Apocynin (300 µM), empty vehicle (DMSO) or nothing for 10 minutes before being incubated with Streptavidin-Alexa 647 labelled SC5314-GFP hyphae. Neutrophils were lysed and fungi were stained with sDectin-1-Fc and Calcofluor White. (A) A representative set of images are shown for each group. (C,E) Representative images of drug treatment experiments are shown. (B, D, F) Images were analyzed by scoring viable cells for the phenotype indicated and data is presented as the percent of total cells with the mean ± SEM for three independent experiments. . ‡ p-value ≤ 0.01 for comparing the sDectin-1-Fc group from WT or DMSO to the gp91$^{phox-/-}$, 10 µM DPI, 300 µM Apocynin or either no neutrophil groups. ¥ p-value ≤ 0.01 for comparing the chitin deposition group from WT or DMSO to the gp91$^{phox-/-}$,10 µM DPI, 300 µM Apocynin or either no neutrophil groups. * p-value ≤ 0.01 for comparing the overlap group from WT or DMSO to the gp91$^{phox-/-}$, 10 µM DPI, 300 µM Apocynin or either no neutrophil groups. n.s. means non-significant. Comparisons done by one way ANOVA with Tukey's post-test. Scale bar represents 10 µm.

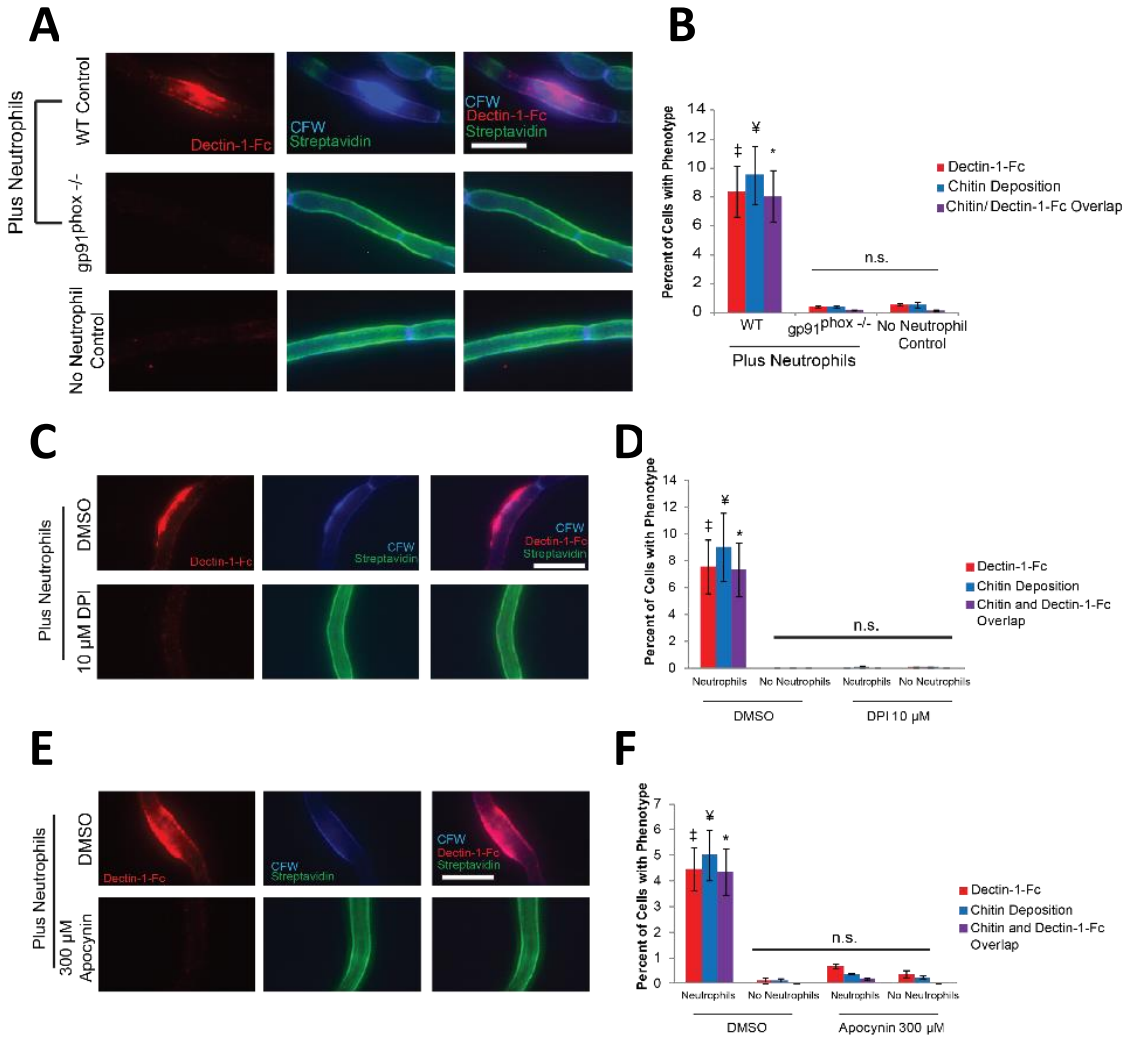

A
Plus Neutrophils
WT Control
gp91phox -/-
No Neutrophil Control
Dectin-1-Fc
CFW
Streptavidin
CFW
Dectin-1-Fc
Streptavidin
B
Percent of Cells with Phenotype
Dectin-1-Fc
Chitin Deposition
Chitin/ Dectin-1-Fc Overlap
n.s.
WT
gp91phox -/-
No Neutrophil Control
Plus Neutrophils
C
Plus Neutrophils
DMSO
10 µM DPI
D
Percent of Cells with Phenotype
Dectin-1-Fc
Chitin Deposition
Chitin and Dectin-1-Fc Overlap
n.s.
Neutrophils
No Neutrophils
DMSO
DPI 10 µM
E
Plus Neutrophils
DMSO
300 µM Apocynin
F
Percent of Cells with Phenotype
Dectin-1-Fc
Chitin Deposition
Chitin and Dectin-1-Fc Overlap
n.s.
Neutrophils
No Neutrophils
DMSO
Apocynin 300 µM

Figure 2.13. Phagocyte NADPH oxidase is important for streptavidin fluorescence loss *in vitro*

(A-E) Streptavidin-Alexa 647 labeled SC5314-GFP was incubated with either C57BL/6J or gp91$^{phox-/-}$ neutrophils in an imaging dish for 30 minutes. (A-B) Representative images for each group. (C) The percent of neutrophil sites with degradation for each group. Data represents the mean ± SEM from three independent experiments, with between 130 and 275 sites scored per experiment. (D) The average MFI in the far red channel at sites of neutrophil attachment. Data represents the mean ± SEM from three pooled experiments where n represents the number of neutrophil attachment sites where MFI was measured. (E) The average MFI for each group, including a no neutrophil control, from a single experiment. * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤0.001 by Student's Ttest (C) or one way ANOVA with Tukey's post-test (D). Scale bar represents 10 μm.

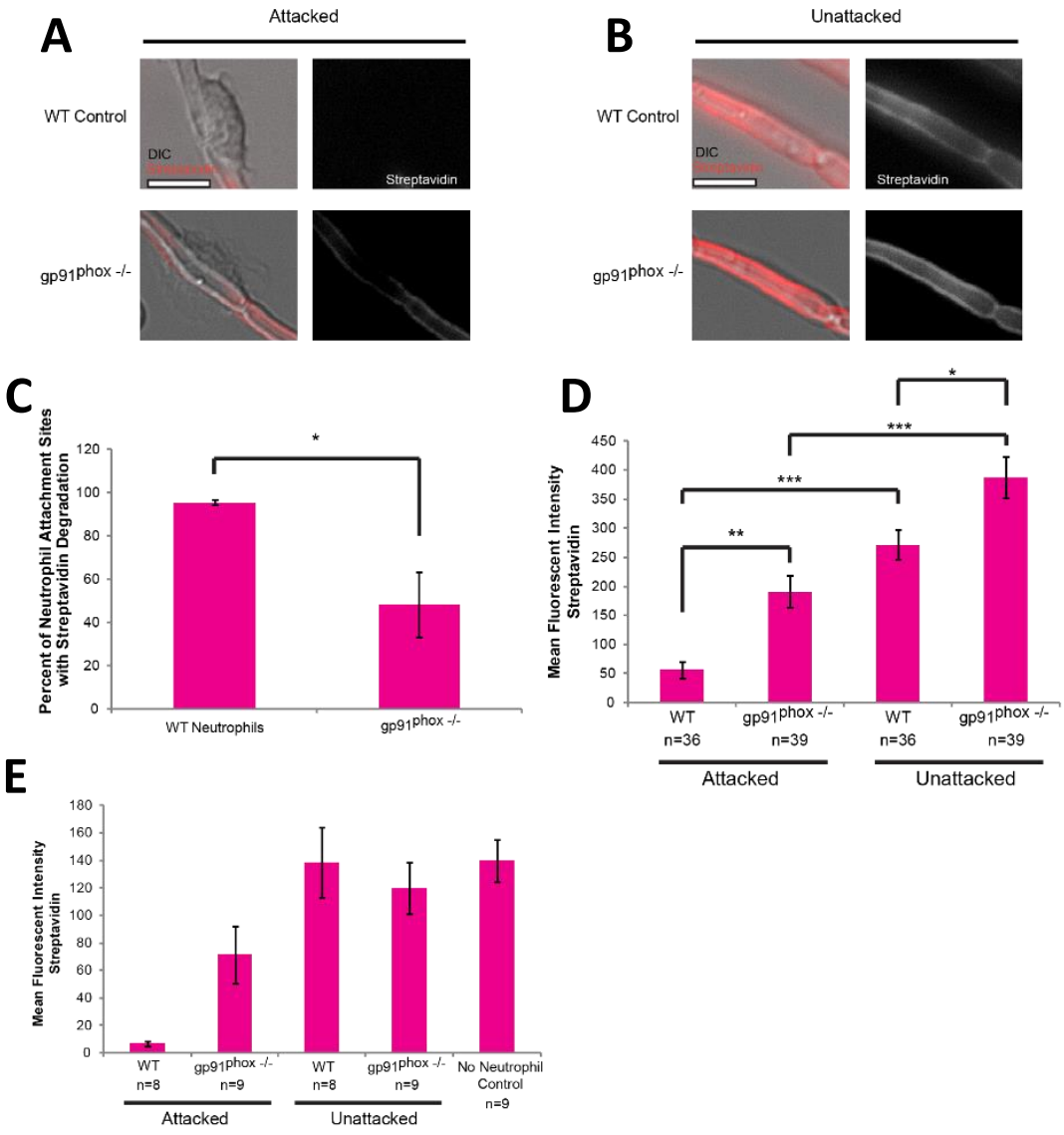
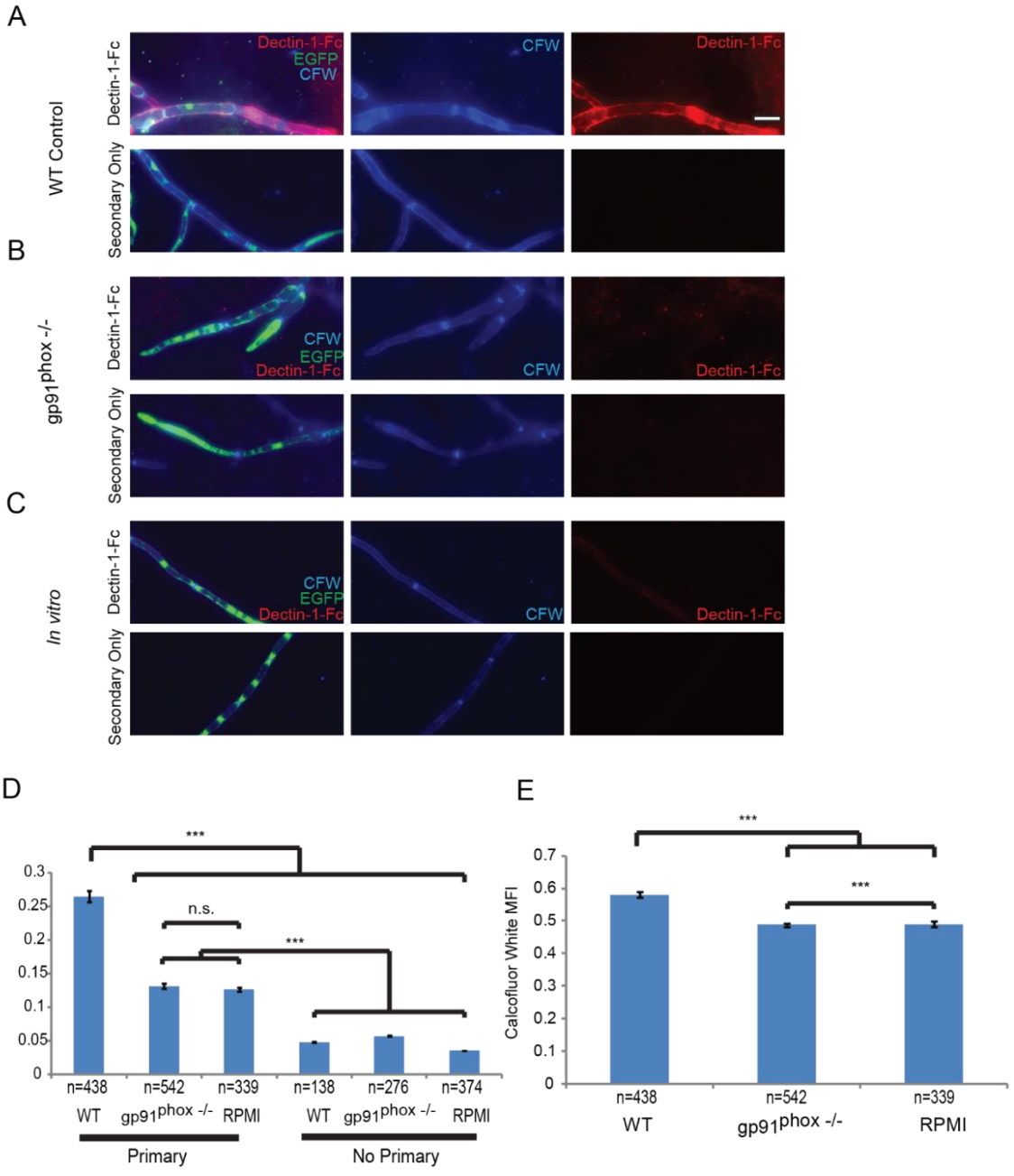

A
Attacked
WT Control
DIC
Streptavidin
gp91phox -/-
B
Unattacked
WT Control
DIC
Streptavidin
gp91phox -/-
C
Percent of Neutrophil Attachment Sites with Streptavidin Degradation
120
100
80
60
40
20
0
*
WT Neutrophils
gp91phox -/-
D
Mean Fluorescent Intensity Streptavidin
450
400
350
300
250
200
150
100
50
0
*
***
***
**
WT
n=36
gp91phox -/-
n=39
WT
n=36
gp91phox -/-
n=39
Attacked
Unattacked
E
Mean Fluorescent Intensity Streptavidin
180
160
140
120
100
80
60
40
20
0
WT
n=8
gp91phox -/-
n=9
WT
n=8
gp91phox -/-
n=9
No Neutrophil Control
n=9
Attacked
Unattacked

Figure 2.14. The phagocyte NADPH oxidase is critical for β-glucan unmasking *in vivo*

(A-E) C57BL/6J mice were injected in the tail vein with SC5314-GFP and kidneys were harvested on day 5 post infection. (A-B) Representative images of kidney homogenates stained with sDectin-1-Fc and CFW. Bottom panels show homogenates treated with secondary antibody only as a control. (C) Representative images of an overnight culture of SC5314-GFP grown in RPMI and then stained with Dectin-1-Fc and CFW. (D-E) Quantification of chitin staining (E) and sDectin-1-Fc staining (D). Data is presented as the mean ± SEM from two pooled experiments, except for the RPMI group which represents a single experiment. *** p-value ≤0.001 (Kruskal-Wallis with Dunn's post-test). n.s means non-significant. Scale bar represents 10 μm.


A
WT Control
Dectin-1-Fc
Secondary Only
Dectin-1-Fc
EGFP
CFW
CFW
Dectin-1-Fc
B
gp91phox -/-
Dectin-1-Fc
Secondary Only
CFW
EGFP
Dectin-1-Fc
CFW
Dectin-1-Fc
C
In vitro
Dectin-1-Fc
Secondary Only
CFW
EGFP
Dectin-1-Fc
CFW
Dectin-1-Fc
D
Dectin-1-Fc MFI
0.3
0.25
0.2
0.15
0.1
0.05
0
***
n.s.
***
n=438
n=542
n=339
n=138
n=276
n=374
WT
gp91phox -/-
RPMI
WT
gp91phox -/-
RPMI
Primary
No Primary
E
Calcofluor White MFI
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
***
n=438
n=542
n=339
WT
gp91phox -/-
RPMI

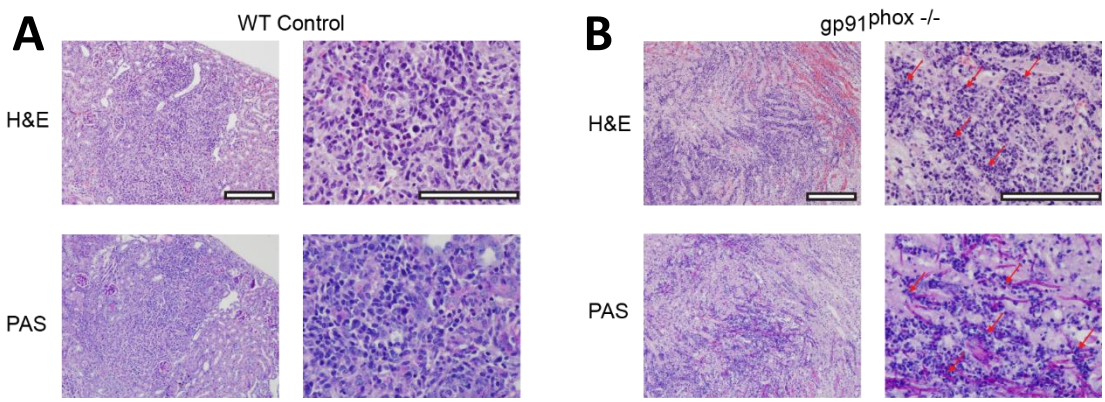


Figure 2.15. Loss of β-glucan unmasking in gp91$^{phox-/-}$ mice is not due to lack of immune cell recruitment

(A-B) Representative images of serial kidney sections from either C57BL/6J (A) or gp91$^{-/-}$phox mice (B) after staining with hematoxylin and eosin or periodic acid-Schiff. The scale bar in the first column of each panel represents 200 µm and in the second column represents 100 µm. (B) Images were examined by a pathologist and example areas containing neutrophils are highlighted with red arrows.

2.4.4 Chitin deposition and β-glucan unmasking are an active fungal response to neutrophil attack

Although NET attack could directly cause these cell wall changes, NET damage could also initiate conserved fungal stress signaling pathways that are known to both respond to cell wall insults and mask β-glucan in steady-state (24, 29). We reasoned that if neutrophil-triggered changes are passive from the fungal perspective, they should occur rapidly and simultaneously, and should also occur in inactivated fungi. Surprisingly, although initial cell wall protein damage occurs within seconds (Figure 2.1), chitin deposition is not apparent until 30 minutes post-challenge, and enhanced Dectin-1 recognition lags even further (Figure 2.16).

The nature of these sequential changes over hours suggests that unmasking results from an active fungal response rather than by direct immune mediated damage. In support of this hypothesis, UV-inactivated fungi lose streptavidin at attack sites but fail to develop sites of chitin deposition or β-glucan unmasking (Figure 2.16, Figure 2.17). UV inactivation is a minimally invasive means of killing fungi, so these results indicate that only initial cell wall damage is a direct result of immune attack (Figure 2.17, 2.4A-B Movie). Thus, it appears that immune attack triggers β-glucan unmasking and chitin deposition only indirectly, by promoting active fungal signaling in response to immune mediated attack.

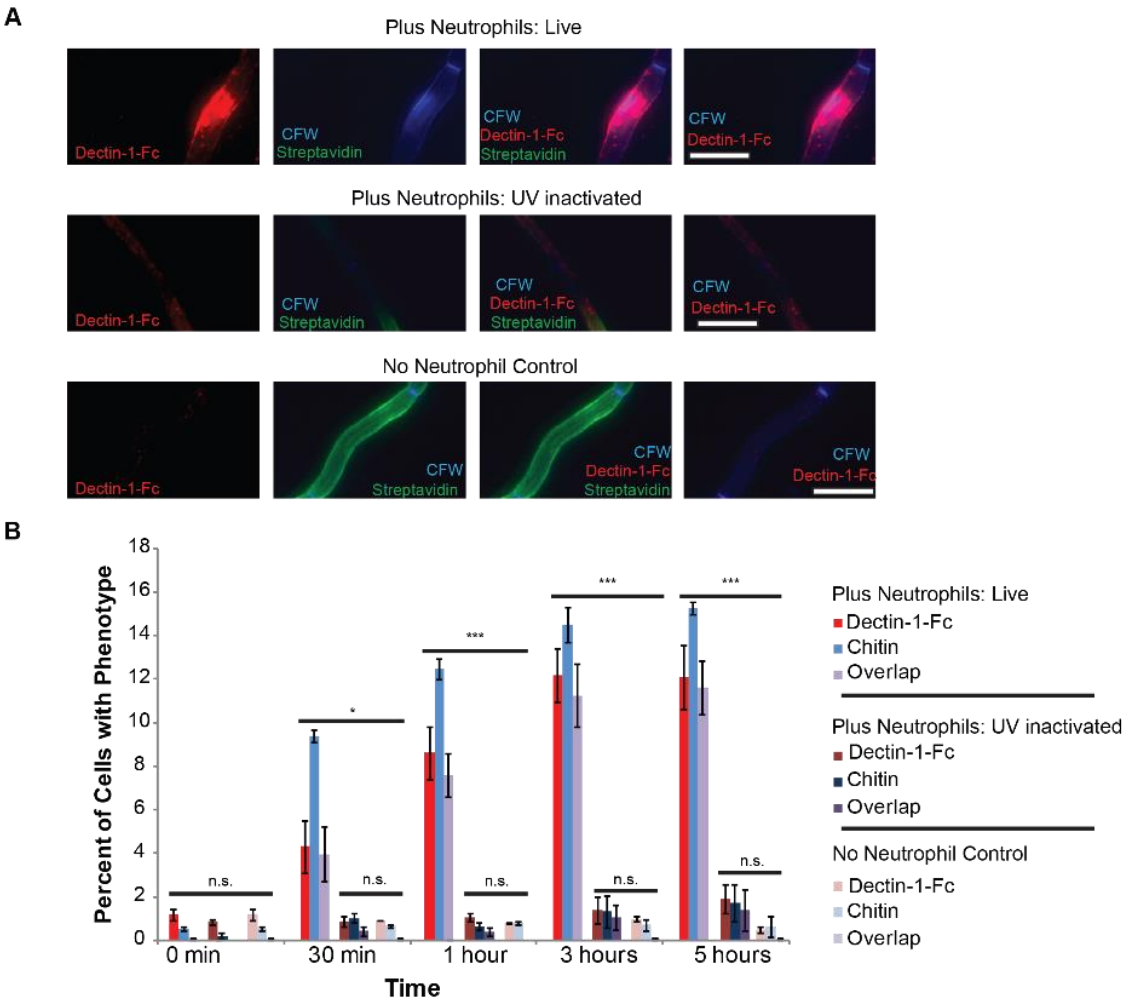


Figure 2.16. Chitin deposition and β-glucan unmasking are the result of an active fungal process (A-B) Streptavidin-Alexa 647 labeled SC5314-GFP hyphae were either UV inactivated or not before being incubated with neutrophils or alone for the amount of time indicated. (A) Representative images of cells from each group at the three hour timepoint are shown. (B) Quantitation of cells for the phenotype indicated over the timecourse. Cells were scored for the indicated phenotype and results are presented as the percent of total cells for each time point. The data represents the mean ± SEM of three independent experiments. Scale bars represent 10 µm. * p-value of < 0.05 and *** p-value of ≤ 0.001 (one way ANOVA with Tukey's post-test). n.s. means non-significant.

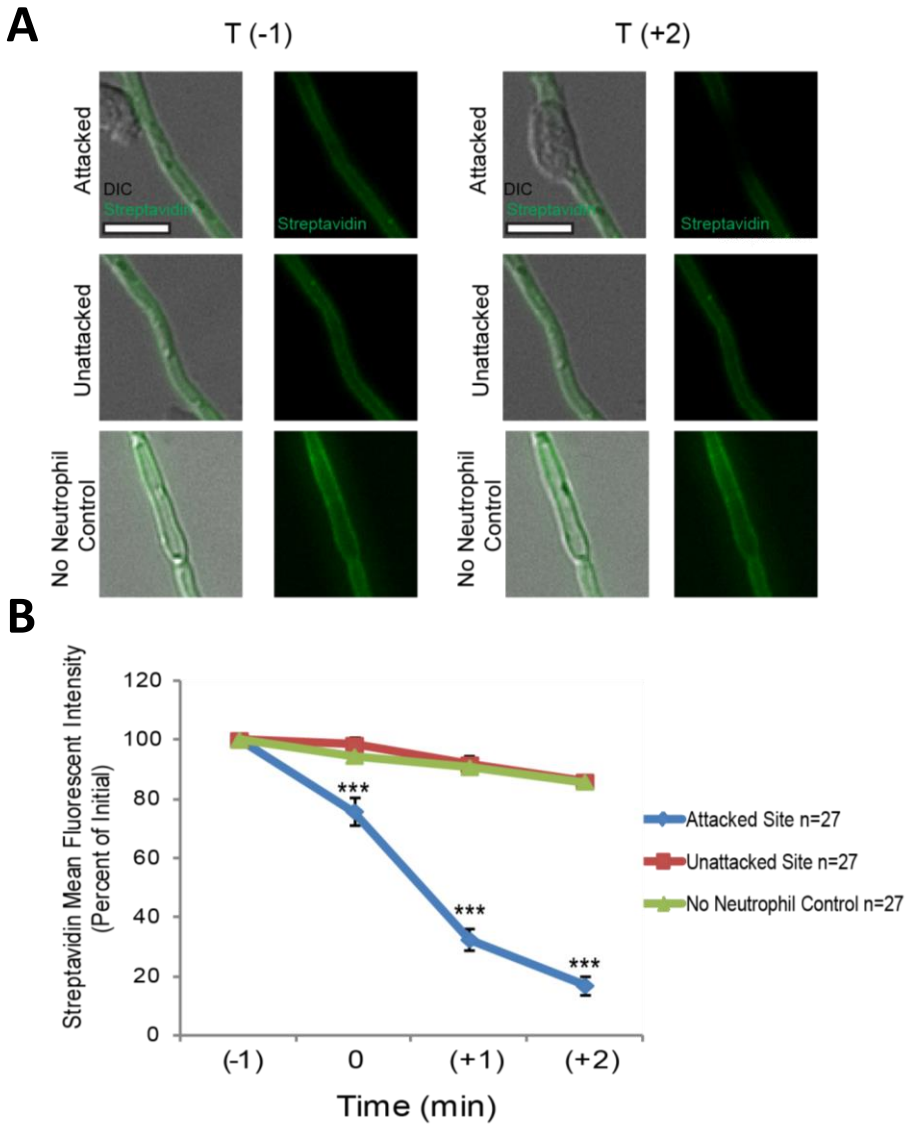


Figure 2.17. Fungal cell wall protein loss is due to direct neutrophil mediated damage

(A-B) Streptavidin-Alexa 647-labeled SC5314-GFP was UV inactivated and incubated with or without neutrophils. (A) Representative images of the T(-1) and T(+2) timepoints from timelapses of UV inactivated *Candida*. (B) Results represent the pooled average MFI at sites from individual frames in timelapses. *** p-value of ≤ 0.001 (one way ANOVA with Tukey's post-test). Scale bars represent 10 µm.

2.4.5 The fungal response to neutrophil attack includes cell wall integrity signaling and remodeling

The fungal cell wall integrity (CWI) signaling pathway plays a key role in stress responses and in maintaining the normal cell wall architecture that masks β-glucan (24, 29). However, it is not known how *C. albicans* responds to immune-mediated cell wall damage, so we sought to identify which signaling pathway(s) drives localized cell wall remodeling. Targeted screening of mutants deficient in individual CWI signaling components for defects in responding to neutrophil attack revealed that *HOG1* is important for this process. The *HOG1* deficient strain has a significantly decreased ability to respond to neutrophil attack with chitin deposition (Figure 2.18). This defect is not due to differences in fungal cell viability or attack rates between strains (Figure 2.19). Interestingly, *C. albicans* deficient in *CAP1*, which is involved in responding to some types of oxidative stress (153, 171), is not required for this response (Figure 2.18, Figure 2.19). This primary dependence on Hog1p suggests that chitin deposition and enhanced Dectin-1 binding result from post-transcriptional activities, as Hog1p plays a limited role in regulating stress-mediated transcription (148).

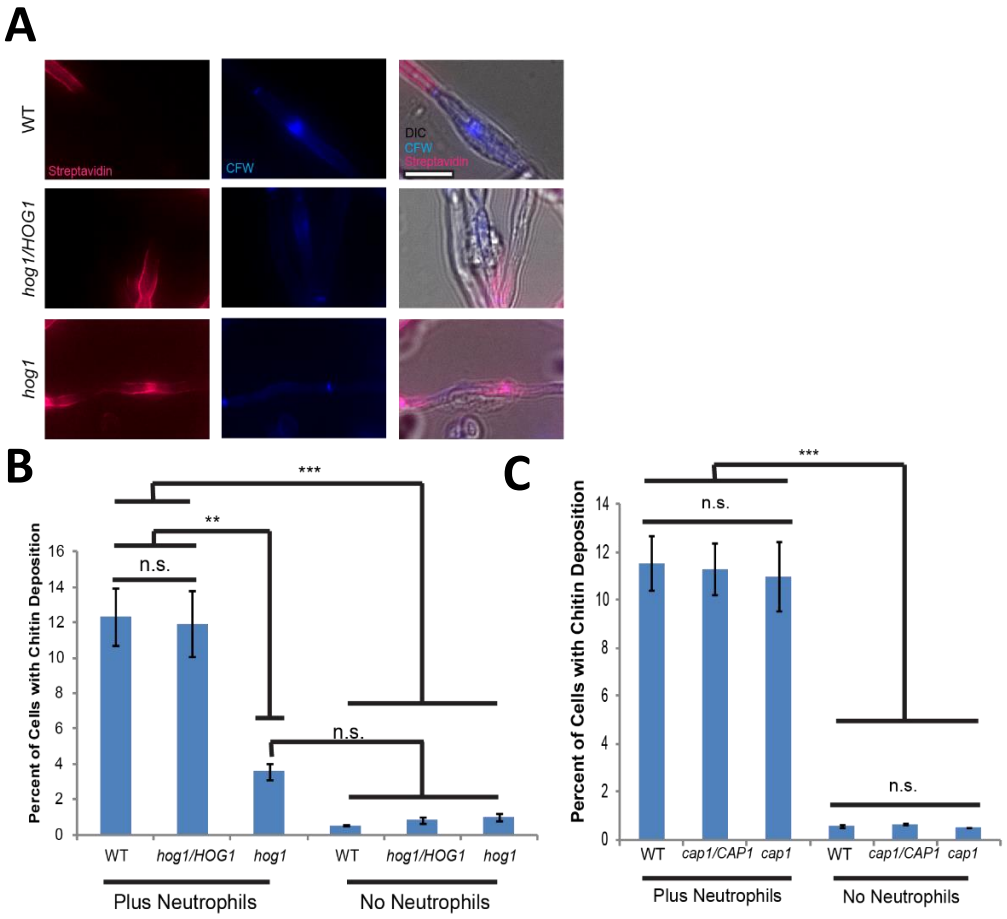


Figure 2.18. The Hog1 cell wall integrity sensing pathway is critical in the fungal response to neutrophil attack

(A-C) Streptavidin-Alexa 647 labeled *C. albicans* of the indicated strains were incubated with neutrophils or alone. Neutrophils were not lysed and samples were stained with CFW. (A) Representative images of the Hog1 strain set. (B-C) Images were analyzed by scoring cells for localized chitin deposition. Data is presented as the percent of total cells with the phenotype and represents the mean ± SEM from three independent experiments. (C) ** p-value of ≤0.01 and *** p-value of ≤0.001 (one way ANOVA with Tukey's post-test). n.s. means non-significant. Scale bar represents 10 µm.

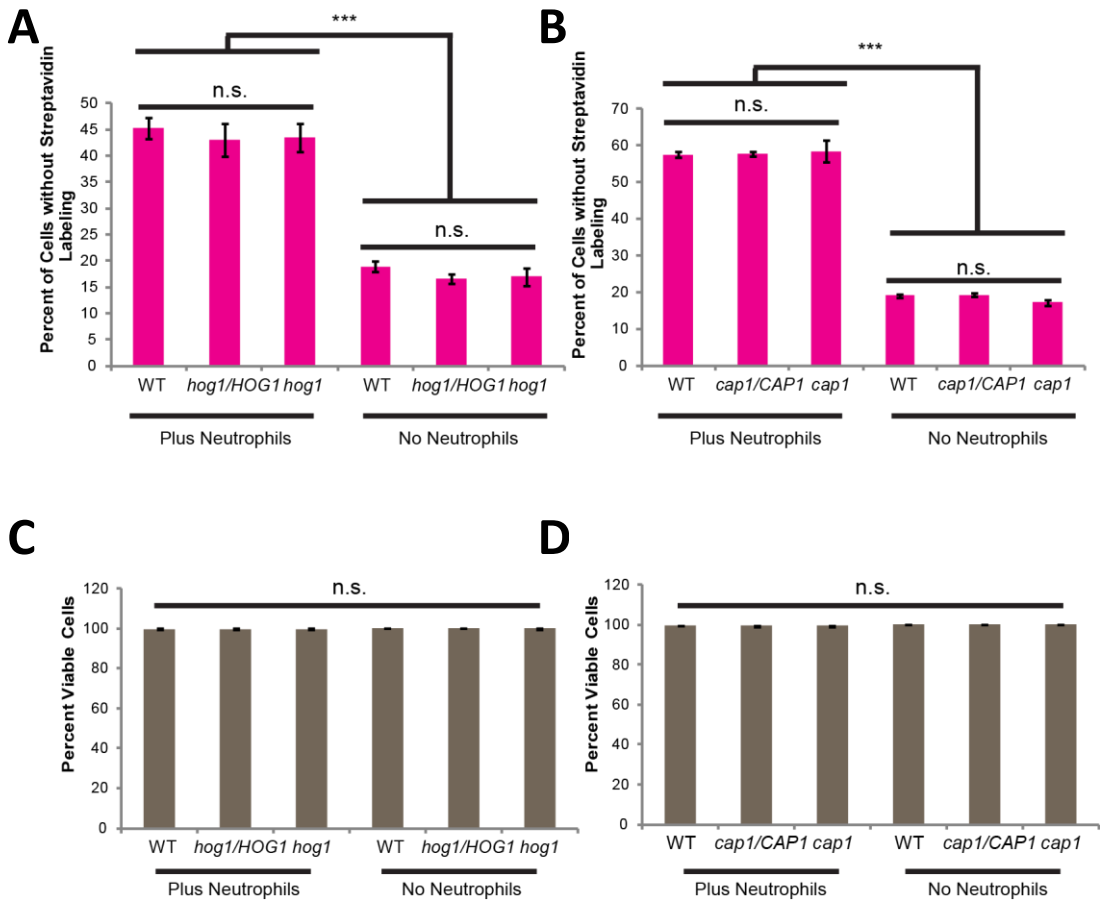


Figure 2.19. Lack of cell wall chitin deposition in *hog1*Δ/Δ mutants is not due to differences in viability or amount of neutrophil attack

(A-D) *hog1*Δ/Δ and *cap1*Δ/Δ strain experiments had all cells scored for the indicated phenotype. Data is presented as the mean ± SEM for three experiments. ***p-value ≤0.001. n.s. means non-significant (one way ANOVA with Tukey's post-test).

Increased chitin levels can rescue *C. albicans* from stress, including antifungal treatment (172). To implicate a specific synthase in enhanced chitin deposition at attack sites, we examined post-attack chitin deposition in mutants in either the major chitin synthase, *CHS3*, or both stress-activated synthases *CHS2* and *CHS8* (160). Both WT and *chs2*Δ/Δ *chs8*Δ/Δ strains have dramatic increases in areas with localized chitin deposition following interaction with neutrophils when compared to their no neutrophil controls (Figure 2.20). However, while the abnormal morphology of the *chs3*Δ/Δ deletion mutant results in a high baseline number of areas with increased chitin deposition, there is very little increase in localized chitin deposition after neutrophil attack. This defect was not due to differences in cell viability, number of attacked sites, or lack of cell wall damage (Figure 2.21). Quantitative analysis of the intensity of chitin staining is consistent with a major role for Chs3p in driving neutrophil-triggered chitin deposition (Figure 2.21). The *chs3*Δ/Δ mutant was not completely deficient in responding to attack with chitin deposition, however, suggesting that other chitin synthases may play a limited role in this process. In support of the idea that Chs3p is the major synthase in the response to neutrophil damage, timelapse of a Chs3-YFP fusion strain demonstrates recruitment of Chs3p-YFP to most sites of increased chitin deposition (Figure 2.20, 2.5 Movie).

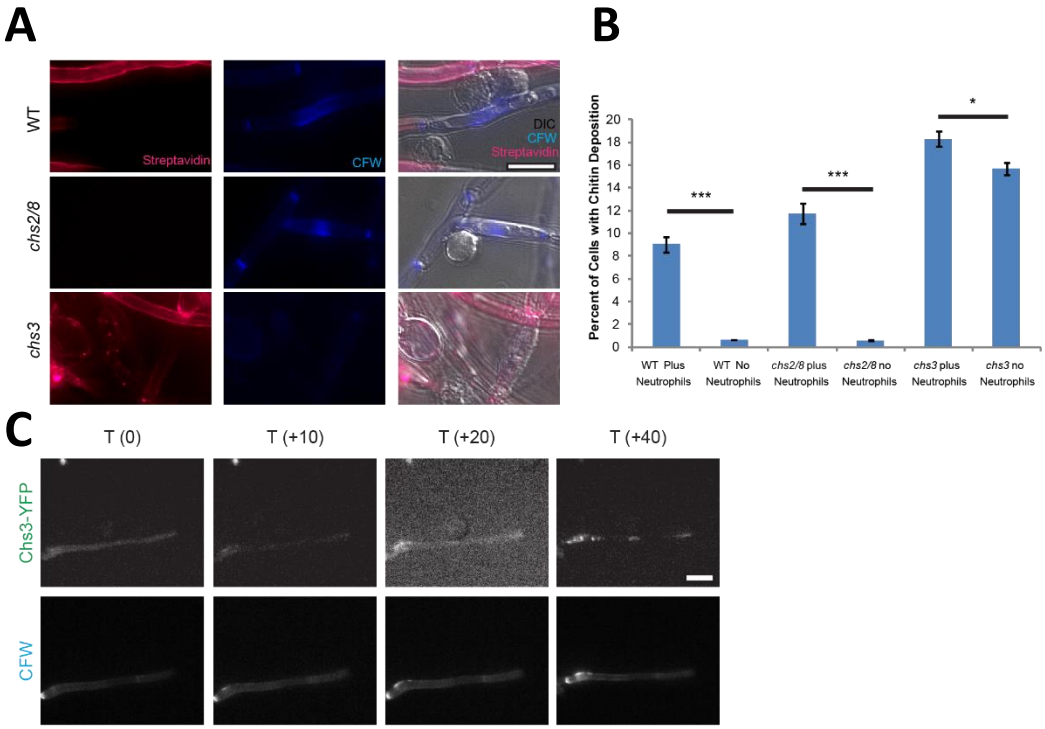
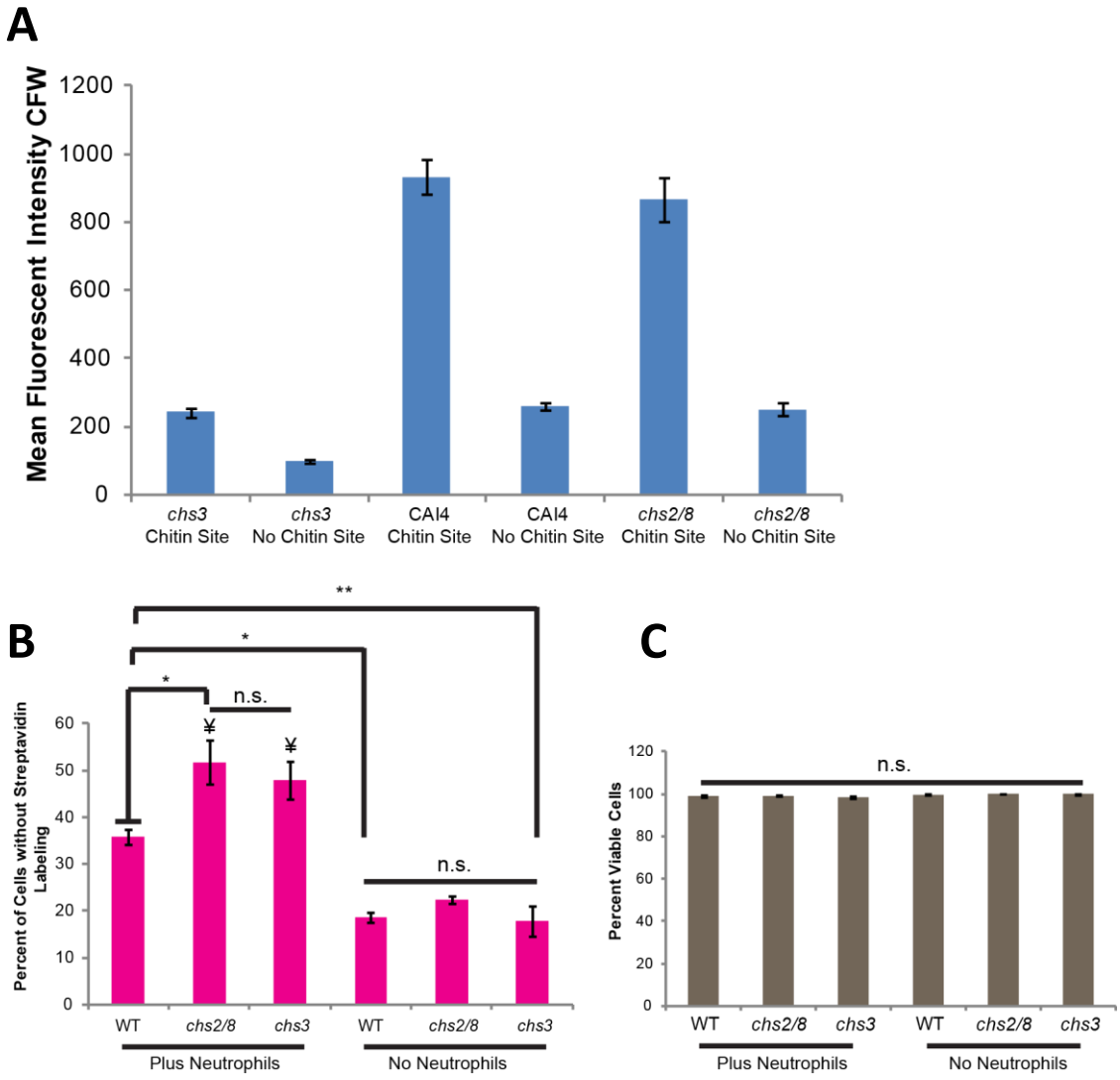


Figure 2.20. Chitin synthase 3 plays the major role in chitin deposition following neutrophil attack

(A-C) Streptavidin-Alexa 647 labeled *C. albicans* of the indicated strains were incubated with neutrophils or alone. Neutrophils were not lysed and samples were stained with CFW. (A) Representative images of the chitin synthase strain set. Images were analyzed by scoring cells for increased chitin deposition. (B) Data is presented as the percent of total cells with the phenotype and represents the mean ± SEM from three independent experiments. The Chs3-YFP strain was incubated with neutrophils in an imaging dish and timelapses were taken. (C) Panels taken from a timelapse of the Chs3-YFP strain after neutrophil attack. * p-value of ≤0.05 and *** p-value of ≤0.001. (Student's Ttest). n.s. means non-significant. Scale bar represents 10 µm.

Figure 2.21. The requirement for Chs3 is not due to differences in viability or decreased neutrophil attack

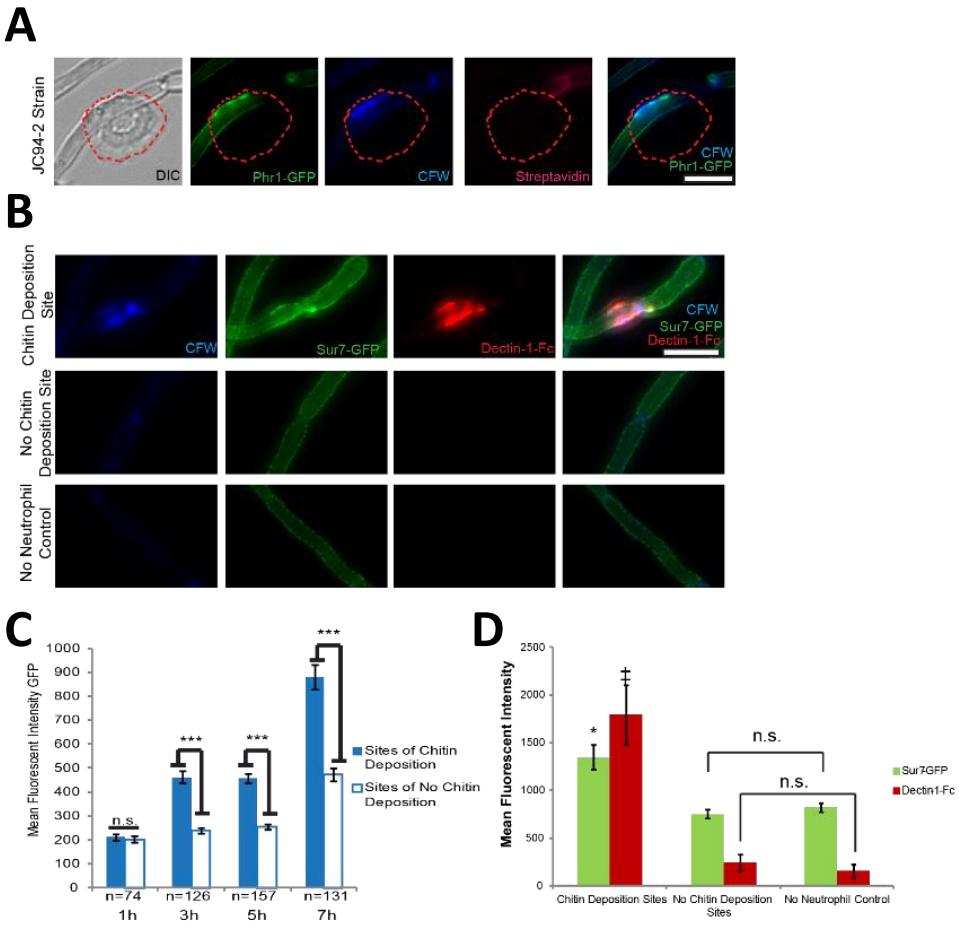
(A-C) Streptavidin-Alexa 647 labeled *C. albicans* of the indicated strains were incubated with neutrophils or alone. Neutrophils were not lysed and samples were stained with CFW. (A) The MFI at equal numbers of sites with and without chitin deposition were obtained for all images from a representative chitin synthase experiment. Results are presented as the average MFI for the pooled sites from that experiment (B) *chs3*Δ/Δ and *chs2*Δ/Δ *chs8*Δ/Δ chitin synthase strain experiments had all cells scored for the indicated phenotype. Data is presented as the mean ± SEM for three experiments. *p-value ≤0.05 and ** p-value ≤0.01. ¥ p-value ≤0.001 when compared to the respective no neutrophil control. n.s. means non-significant (one way ANOVA with Tukey's post-test).


A
Mean Fluorescent Intensity CFW
1200
1000
800
600
400
200
0
chs3 Chitin Site
chs3 No Chitin Site
CAI4 Chitin Site
CAI4 No Chitin Site
chs2/8 Chitin Site
chs2/8 No Chitin Site
B
Percent of Cells without Streptavidin Labeling
60
50
40
30
20
10
0
**
*
*
n.s.
n.s.
¥
¥
WT
chs2/8
chs3
WT
chs2/8
chs3
Plus Neutrophils
No Neutrophils
C
Percent Viable Cells
120
100
80
60
40
20
0
n.s.
WT
chs2/8
chs3
WT
chs2/8
chs3
Plus Neutrophils
No Neutrophils

Cell wall remodeling is also crucial for response to stress, but we know little about the spatiotemporal dynamics of these responses, especially in the context of immune attack. We therefore characterized the post-attack movement of cell wall remodeling and biogenesis proteins, including Sur7p and Phr1p. Sur7p is deposited in new cell wall and marks eisosomes, and Phr1p is a glucan remodeling enzyme crucial to cell wall integrity (173, 150). Both Phr1p and Sur7p are recruited to sites of neutrophil attack. Sur7p was recruited early and coincident with chitin deposition while Phr1p accumulated at later times (Figure 2.22, 2.6A-B Movie). Overall, these results help elucidate important components of the fungal response to immune cell attack, suggesting Hog1p is important for the initial signaling response which leads to chitin deposition mainly through Chs3p localization and the later cell wall remodeling possibly involving Phr1p and Sur7p.

Figure 2.22. Sur7 and Phr1 are recruited to localized areas of immune attack

The JC94-2 strain was incubated with neutrophils for the indicated time in an imaging dish before being imaged. (F) Representative images from the 3 hour timepoint. The red dotted line represents the outline of a neutrophil. (G) Images were analyzed by obtaining the mean fluorescent intensity of GFP at sites with or without chitin deposition and the data is presented as the mean MFI ± SEM at sites from three pooled experiments except the 1h timepoint, which represents two pooled experiments. (H-I) The Sur7-GFP strain was incubated with neutrophils. Following incubation, neutrophils were lysed and fungi were stained with sDectin-1-Fc and CFW. Data represents the MFI of GFP or Cy3 staining at sites with or without chitin deposition or from the no neutrophil control and is presented as the mean MFI ± SEM from three pooled experiments. * p-value of ≤0.05 and *** p-value of ≤0.001. For D, * p≤0.05 comparing Sur7-GFP at chitin deposition sites vs no chitin deposition or no PMN control. ‡ p≤ 0.01 comparing sDectin-1-Fc at chitin deposition sites vs no chitin deposition sites or no PMN control. (Student's Ttest for C and one way ANOVA with Tukey's post-test for D). n.s. means non-significant. Scale bar represents 10 μm.


A
JC94-2 Strain
DIC
Phr1-GFP
CFW
Streptavidin
CFW
Phr1-GFP
B
Chitin Deposition Site
No Chitin Deposition Site
No Neutrophil Control
CFW
Sur7-GFP
Dectin-1-Fc
CFW
Sur7-GFP
Dectin-1-Fc
C
Mean Fluorescent Intensity GFP
1000
900
800
700
600
500
400
300
200
100
0
n.s.
***
***
***
n=74
n=126
n=157
n=131
1h
3h
5h
7h
Sites of Chitin Deposition
Sites of No Chitin Deposition
D
Mean Fluorescent Intensity
2500
2000
1500
1000
500
0
*
n.s.
n.s.
Chitin Deposition Sites
No Chitin Deposition Sites
No Neutrophil Control
Sur7GFP
Dectin1-Fc

2.4.6 Neutrophil-mediated cell wall disruption results in enhanced immune responses

Neutrophil-triggered enhancement of Dectin-1 binding may result in an altered secondary immune response to *C. albicans* or have no impact due to redundant recognition modalities. To assay secondary immune responses, we challenged *C. albicans* hyphae with neutrophils, then lysed the neutrophils. We treated the samples with DNase1 to reduce activation of macrophages by neutrophil debris before UV-inactivating the fungi and adding them to murine macrophages. A mixture of unattacked fungi with neutrophil lysate served as a control for activation by remaining neutrophil debris in the context of fungal stimulation (174). A schematic diagram of the experiments can be seen below (Figure 2.23). ELISA assays revealed that attacked fungi induced higher production of the proinflammatory cytokine IL-6 when compared to any of several controls (Figure 2.24). Interestingly, this increased cytokine production was not completely dampened by Dectin-1 inhibition suggesting other receptors may also be involved in this response. These results indicate that neutrophil attack and the resulting cell wall changes, including β-glucan unmasking, can lead to enhanced recognition and responses by other immune cells.

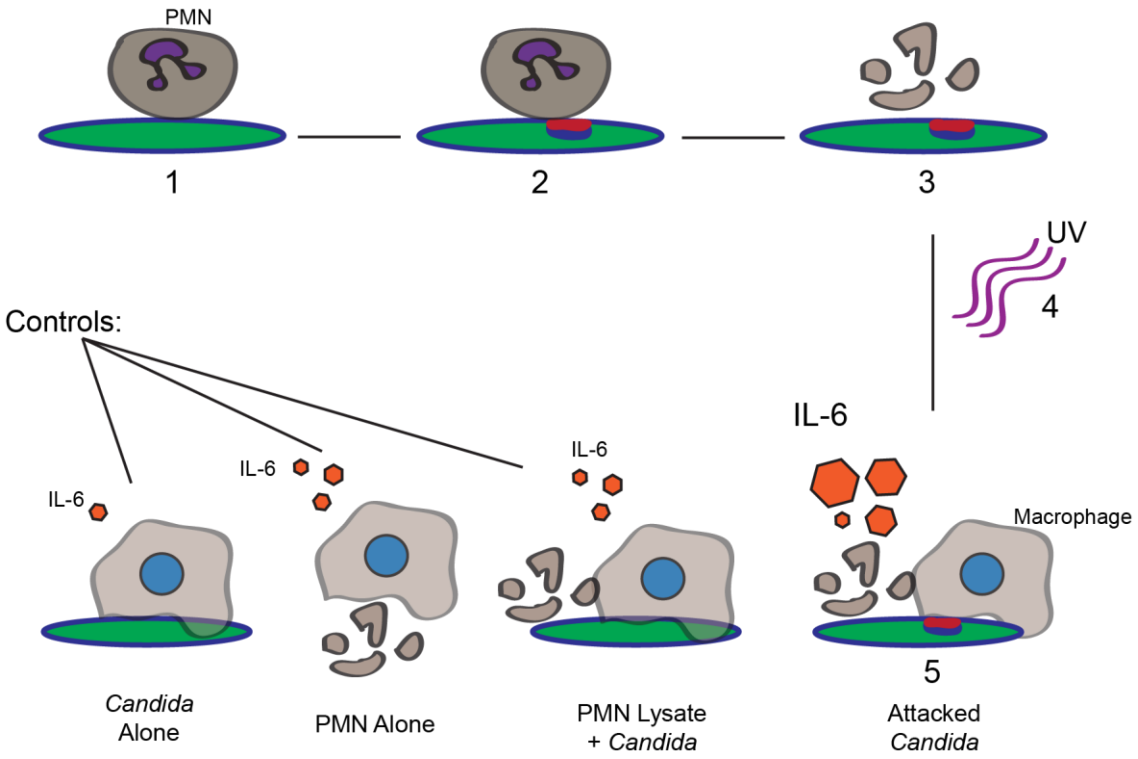


Figure 2.23. Schematic representation of RAW-Blue macrophage experiments

1.) Neutrophils are incubated with *C. albicans* overnight. 2.) During this incubation, neutrophils attack *C. albicans*, initiating cell wall remodeling and resulting in β-glucan unmasking and chitin deposition. 3.) Following incubation, neutrophils are lysed and samples are treated with DNase 1 to reduce activation by neutrophil debris. 4.) Samples are UV inactivated. 5.) Samples are then incubated with macrophages. Macrophages recognize the exposed fungal epitopes and produce cytokines in response. The attacked fungi with increased epitope unmasking elicit more IL-6 in comparison to the controls.

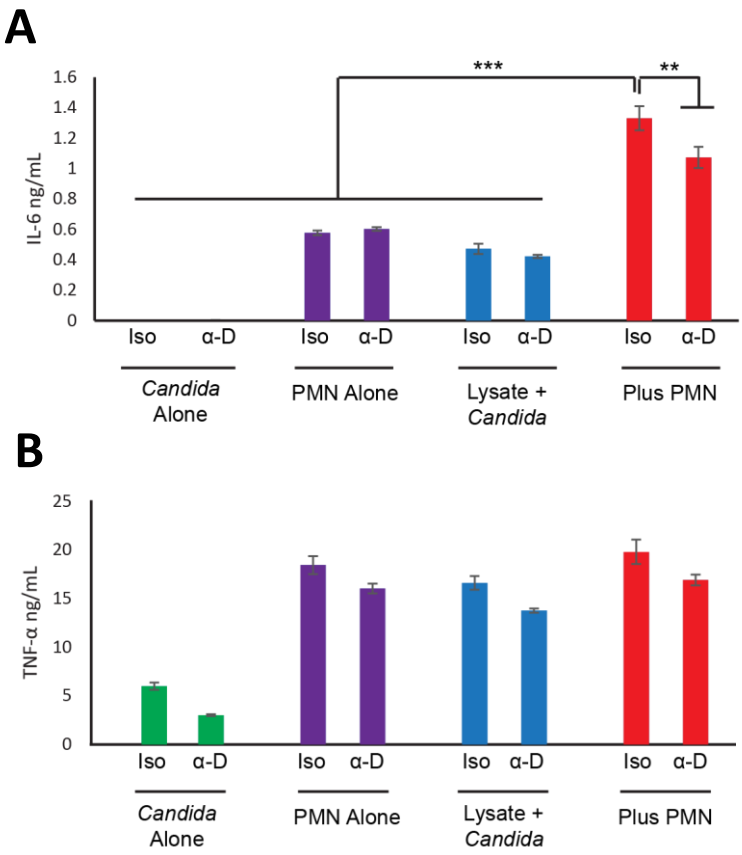


Figure 2.24. *C. albicans* induces an enhanced IL-6 response after neutrophil attack

RAW-blue cells were treated as indicated and incubated for 6 hours. (A-B) ELISA was conducted on supernatants from RAW-Blue cells to detect (A) IL-6 or (B) TNF-α. A representative experiment is shown for each cytokine. Comparisons were done by two way ANOVA with Tukey's post-test. ** p-value of ≤0.01 and *** p-value of ≤0.001.

2.5 Discussion

Recognition of pathogens based on conserved molecular patterns is a cornerstone of innate immunity but it is a dynamic battlefield between host and pathogen. The host's task is complicated because pathogens conceal essential molecular patterns from detection, thereby denying the host the knowledge it needs to initiate a response. Here, we build on previous work to show that although *C. albicans* masks β-glucan during infection, host immune cells can damage the invader and trigger the disruption of cell wall architecture in a manner that could enhance innate immune recognition, including the unmasking of β-glucan. Our findings echo work in diverse animal and plant hosts that suggest pathogen recognition and responses are dynamic and can impact immunity during infection (175, 138, 176, 50). Other types of "unmasking" might take place in a number of infections, as masking of epitopes has been demonstrated for bacteria, viruses, fungi, protozoans and helminths (117, 143, 128, 144, 29, 145, 146). While the microbial cell wall is an adaptable landscape that is capable of responding to numerous stimuli, we still have a limited understanding of how cell wall architecture changes *in vivo* and how host-pathogen interactions influence PAMP availability during infection.

We describe here how the host subverts a fungal evasion strategy, unmasking *C. albicans* to reveal fungal-specific epitopes like β-glucan. Surprisingly, this is a two-step process where NET-dependent neutrophil attack results in β-glucan unmasking via an active fungal process. The fungal response to localized cell wall stress includes a cascade of events, with chitin deposition mediated by Hog1p signaling and the major chitin synthase Chs3p. Remodeling of cell wall architecture enhanced recognition and could enhance responses by the host, but is also likely to protect the fungus, as is the case for plant cell walls that remodel upon fungal attack (140, 96). These mechanisms of cell wall architecture control during fungal infection are likely relevant for other fungi that hide immunogenic β-glucan from the host and thereby limit

immune responses (128, 177, 144). Given the importance of Dectin-1 signaling in anti-fungal defense, and the fact that NETs are deployed against other fungi, it seems likely that immune mediated unmasking may take place during other fungal infections (178, 179, 180).

The ability of a host to recognize and respond to microbe-specific components is a key determinant to mounting an effective defense. Neutrophil unmasking of hyphal β-glucan, which is structurally distinct and elicits greater inflammatory cytokine responses than yeast β-glucan, could help the host discriminate between commensalism and opportunistic disease (181), especially since *C. albicans* hyphae are typically associated with invasion and greater recognition could enhance the "danger" response to invading hyphae (182). Unmasked epitopes could also assist in "trained immunity" for other innate immune cells like monocytes, which has been shown to depend on fungal β-glucan and Dectin-1 signaling for protection against *C. albicans* (183). Our results demonstrate that cell wall changes following neutrophil attack do increase recognition of β-glucan and also specifically elicit more IL-6, but not TNF-α, from macrophages when compared to controls. Elevated IL-6 production, in particular, may be important as it participates in the induction of Th17 responses which are important for antifungal immunity (184, 185, 186). Interestingly, some of the increased macrophage response was not dependent on Dectin-1, suggesting that other cell wall changes beyond β-glucan unmasking may play a role. In addition to enhanced Dectin-1 recognition, it is likely that post-attack disruption of cell wall architecture results in alterations to other fungal cell wall epitopes. Our preliminary data using wheat germ agglutinin as a probe suggests there is also increased availability of chitin at attack sites (data not shown). Because fungal chitin recognition can modulate inflammation, altered chitin recognition may contribute to secondary immune response (187, 35).

The impact these epitope changes might have on the outcome of infection remains to be explored. While elevated IL-6 could contribute to the development of protective Th17

response after recognition of exposed fungal epitopes, hyperinflammation of the IL-17 axis and excessive neutrophilic influx are also risk factors, and recent work suggests that myeloid-derived suppressor cell-mediated immunomodulation is protective during this phase around 4-7 days post-infection (188, 189, 86, 190, 191, 192). Whether for protection or pathogenesis, the potential of immune attack to alter subsequent immune response suggests that immune dynamics may play an important regulatory role.

Extracellular traps (ETs) function in pathogen containment and killing, and we find that they can also influence pathogen epitope exposure. The specific requirements for neutrophils and phagocyte oxidase to damage *C. albicans* and initiate unmasking *in vivo* fits with previous *in vitro* findings that ETs made by macrophages don't damage *Candida* (Liu et al 2014 #90) and our own preliminary observations that macrophage attack doesn't elicit the same changes to the *C. albicans* cell wall (data not shown). NADPH oxidase and MPO are known players in NET formation (84, 170), but NET formation has also been shown to occur in an ROS-independent manner so it is still unknown if or how these components contribute to the fungal damage that induces cell wall remodeling. While some NETs were still produced upon NADPH oxidase inhibition (data not shown), our data demonstrates the requirement for functional NADPH oxidase, MPO and extracellular DNA in NETs for inducing fungal cell wall changes following immune attack. This suggests that the role of the NADPH oxidase and MPO is not primarily in NET creation but instead may contribute to decorating NETs with damaging components which provoke fungal integrity responses (with the role of the NETs themselves to hold these components in close proximity to the fungal cell wall). Further experiments will be required to fully understand the exact role NETs and their components are playing. The MPO requirement suggests this is not strictly due to the neutrophil respiratory burst causing localized hypoxia, which is an environmental condition previously associated with fungal cell wall changes (127).

Surprisingly, our experiments suggest that neutrophil proteases are not required for NET-dependent unmasking in our system, although they have been previously implicated in human and mouse NET formation (83, 166, 167). We tested this by using neutrophils deficient in DPPI, in which the three major neutrophil proteases couldn't be processed properly (168). Interestingly, it has also been seen that DPPI deficiency limits PMA and ROS-induced NET production (85). As more research emerges, the requirement for different components in NET production has been found to be highly context and stimulus dependent, even for elements like the phagocyte NAPDH oxidase which were previously thought to be absolutely critical (170, 84). It is therefore possible that we have identified a situation in which these proteases do not play a critical role in NET formation or that the defects which result are not severe enough to compromise their function in the unmasking process. Further work will be required to determine if this lack of a requirement is because other proteases can fill in during this situation or if no protease activity is required at all.

The early initiation of NET-dependent *C. albicans* cell wall remodeling suggests a rapid deployment of NETs, which occurs in a subset of neutrophils in response to certain stimuli (166). Intriguingly, rapid NET deployment leaves neutrophils intact, consistent with our preliminary observations of early NETs and "live" NET production (82). A better understanding of neutrophil and NET function during infection could have clinical benefits, as defects in either result in increased susceptibility to many infections, including those caused by *Candida* (193, 71).

The mechanism whereby neutrophil attack reveals fungal epitopes is unexpected, as cell wall changes are not a direct result of immune attack but rather are initiated by signaling in the fungus. The importance of the Hog1p MAPK in response to neutrophil attack is consistent with its established roles in interactions with phagocytes and host immunity both *in vitro* and *in vivo* (26, 22). Further, our data suggest that *hog1*Δ/Δ hypersensitivity to neutrophil-mediated

killing may be due to a failure to deposit chitin and reinforce their cell wall, a process that rescues *C. albicans* from other stresses (172). The requirement for Hog1p, but not Cap1p, in sensing and responding to NET attack suggests that the neutrophil attack response is not simply a reaction to oxidative stress, and implies that Hog1p responds to immune attack in addition to its previously described roles in osmotic and oxidative stress (20).

The localized cell wall stress caused by neutrophil attack provides an advantageous situation to dynamically model how *C. albicans* hyphae mobilize their cell wall machinery in response to neutrophil attack *in vivo*. Genetic deletion mutants show that Chs3 is responsible for the majority of this localized lateral cell wall chitin deposition, while Chs2 and Chs8 are not required. Time-lapse microscopy also demonstrated accumulation of Chs3-YFP at attack sites with chitin deposition. These observations suggest a new role of Chs3 in stress response, in addition to its responsibility for producing the majority of chitin in *C. albicans* (194). The requirement of individual synthases may be context dependent, as recent reports show Chs2 and Chs8 are involved in maintaining cellular integrity during some forms of stress *in vitro* (195). This data, combined with that showing the importance of Hog1p signaling in responding with chitin deposition, supports previous observations that Hog1p can regulate and activate chitin synthesis (196). The recruitment of cell wall regulatory and remodeling enzymes Sur7p and Phr1p suggests a multistep process of remodeling post-attack. While Phr1p is known to be recruited to apical growth sites and septa and to respond to other stresses, this is the first time it has been found enriched in a localized section of lateral cell wall (197, 150). Sur7p is important in regulating cell wall organization and integrity (173). Early Sur7p enrichment at sites of chitin deposition and β-glucan unmasking suggests it plays an early role in cell wall reorganization at sites of neutrophil attack, in contrast with the later role for Phr1p. Beyond providing detailed insight into how *C. albicans* responds to neutrophil attack, this model of localized cell wall stress

offers a powerful new method to image the multistep dynamics of stress-stimulated cell wall remodeling.

β-glucan recognition is relevant beyond mammalian immunity, as it has been demonstrated that both invertebrates and plants sense and respond to β-glucan, with important implications for antifungal immunity (41, 198). Indeed, dynamic host-pathogen interactions revolving around fungal β-glucan masking and host recognition also occur during infections in plants, suggesting parallels with important agricultural fungal infections (138, 139, 140). The game of pathogen camouflage and host-mediated unmasking has been played out over generations throughout the animal, fungal and plant kingdoms. The localized fungal cell wall remodeling we observe upon immune-mediated stress represents a novel model to probe basic mechanisms of cell wall dynamics and may identify novel therapeutic targets or strategies especially relevant to the *in vivo* infection environment.

CHAPTER 3

PHOSPHATIDYLSERINE PLAYS A CRITICAL ROLE IN β-GLUCAN MASKING

3.1 Introduction

The maintenance of the fungal cell wall, including masking of β-glucan from the host, requires a complex network of genes and pathways (29) in *C. albicans*. The role of the plasma membrane in this process is not well understood. Phospholipids like phosphatidylethanolamine (PE) and phosphatidylserine (PS) are found in the plasma membrane and are known to be required for virulence in a systemic mouse model, though their importance to the cell wall and mechanisms of action are unknown (199). PS synthesis begins with Cho1p, the PS synthase and mutants deficient in Cho1p also lack virulence in a systemic infection model, suggesting a critical role for PS in the survival of the fungi in the host. PS also serves as the substrate for the *de novo* synthesis of PE and can be acted upon by Psd1p or Psd2p, which are phosphatidylserine decarboxylases, to convert PS to PE. This pathway also plays an important role in fungal survival in the host, as double mutants deficient in Psd1p and Psd2p have highly attenuated virulence (199). PS and PE are also involved in fungal extracellular vesicle formation and function as the *cho1* Δ/Δ and the *psd1* Δ/Δ *psd2* Δ/Δ mutants showed altered vesicle cargo with decreased secreted protease and phospholipase activity. The *cho1* Δ/Δ mutant vesicles also didn't activate NF-κB in macrophages like wild type vesicles (200). Work we have published in collaboration with others (30) aimed to further elucidate the functions of these phospholipids and examined what role they might play in maintaining fungal cell wall architecture.

3.2 Materials and Methods

C. albicans strains were streaked onto YPD agar plates and left at 37°C overnight. A single colony per strain was picked and transferred into 5 ml YPD liquid, which was put into a rotator wheel and left overnight at 37°C. A sample of culture was centrifuged and washed three

times with PBS. Samples were blocked in PBS plus 2% bovine serum albumin for 1 h at room temperature. After blocking, samples were stained with sDectin-1–Fc at 16.5 μg/ml for 1.5 h on ice. Samples were washed with PBS five times and then stained with donkey anti-human IgG DyLight 488 antibody (Jackson ImmunoResearch) at 0.83 μg/ml for 20 min on ice. Samples were washed five times with PBS and then resuspended in 500 μl PBS for flow cytometry. Flow cytometry data were obtained for 10,000 gated events per strain, and statistics were calculated with the paired Student *t* test. For imaging, *Candida* cells were prepared as outlined above but were resuspended in 50 μl PBS and visualized with a Zeiss AxioVision Vivotome microscope (Carl Zeiss Microscopy, LLC). This experiment was repeated twice.

3.3 Results and Discussion

To investigate the role PS and PE may play in β-glucan masking, we took a wild type strain, *cho1* Δ/Δ or *psd1* Δ/Δ *psd2* Δ/Δ mutants, and their reintegrants, grew them in YPD to examine their yeast form and stained with sDectin-1-Fc. Fungal cells were then examined by both flow cytometry and fluorescent microscopy, revealing that the *cho1* Δ/Δ mutant had significantly increased β-glucan unmasking when compared with the wild type strain, its reintegrant and all other strains examined (Figure 3.1). Further work done by Davis et al demonstrated that the cho1Δ/Δ mutant also displayed this phenotype in the hyphal form and therefore that unmasking was not restricted to a particular fungal morphotype. Importantly, it was demonstrated that the unmasking on the *cho1* Δ/Δ mutant resulted in increased Dectin-1 mediated recognition by macrophages and increased TNF-α production (30). These results illustrate the importance of *cho1* and presumably PS to the masking of β-glucan in the fungal cell wall, adding another pathway to the complex network involved in maintaining proper fungal cell wall architecture. As *cho1* is necessary for virulence during systemic infection (199), plays a role in the function of extracellular vesicles carrying potential virulence factors like proteases and

phospholipases (200, 201), is required for efficient β-glucan masking (30) and does not have a mammalian homologue (202), it represents an attractive target for the development of novel therapeutics for candidiasis.

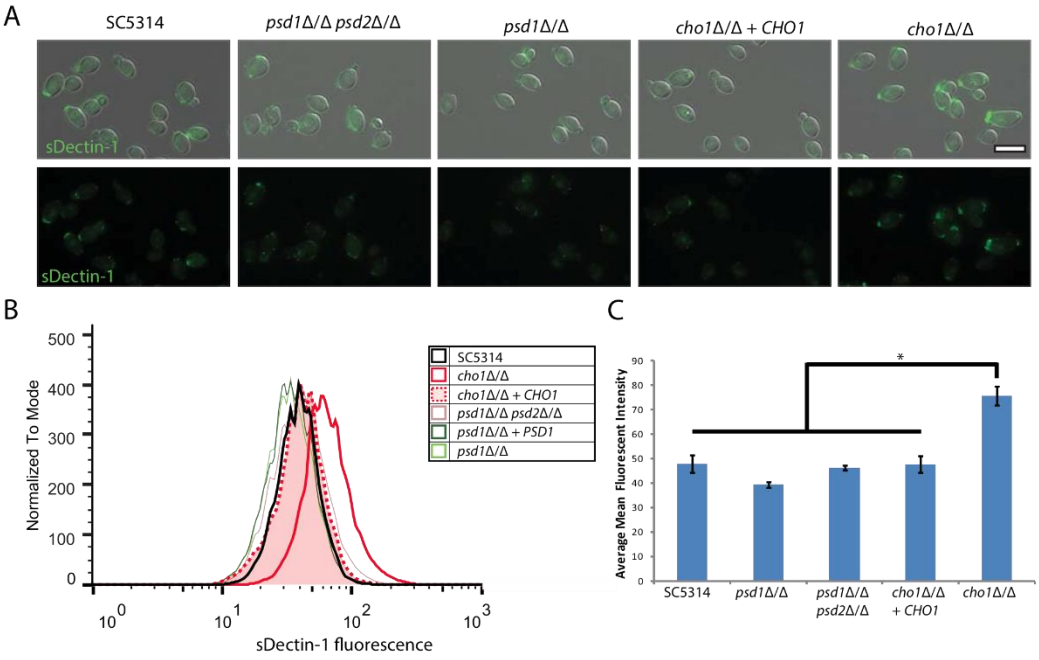


Figure 3.1. Cho1 plays an important role in β-glucan masking

The *cho1Δ/Δ* mutant has increased binding to the Dectin1 receptor. (A) *C. albicans* strains grown overnight in YPD were stained with sDectin-1–Fc and a fluorescently labeled secondary antibody, showing that the *cho1Δ/Δ* mutant exhibits greater staining than all of the other strains. (B) Flow cytometry reveals that the *cho1Δ/Δ* mutant (solid red line) has greater binding to the Dectin-1 receptor than the other strains. (C) A graph of the relative mean staining intensity of each sample reveals that the *cho1Δ/Δ* mutant exhibits significantly greater staining with sDectin-1–Fc than the other strains. * $P \leq 0.05$ (adapted from 30).

CHAPTER 4

FUNGAL CELL WALL COMPOSITION INFLUENCES THE REQUIREMENT FOR SPECIFIC HOST IMMUNE RECEPTORS

4.1 Introduction

The host receptor Dectin-1 is well known to be the major β-glucan recognition receptor and is capable of inducing immune responses to many fungi. The importance of Dectin-1 for host defense against *C. albicans* has been the subject of debate however, as one group showed it was critical for survival in a mouse model of disseminated infection while another group showed that it was dispensable for protection (48, 49). These research groups used different mouse backgrounds and fungal strains, either of which could have contributed to these differences in requirement for Dectin-1. In published work that we contributed to, the cause for these conflicting results was investigated (50).

4.2 Materials and Methods

4.2.1 Strain Creation

SC5314-GFP and ATCC18804-GFP strains were created by transformation with the pENO1-yEGFP3-NAT plasmid and verified by PCR as described previously (136).

4.2.2 *Ex vivo* Staining

C57BL/6J mice were injected in the tail vein with 5.2 x 10^4 cfu of either SC5314-GFP or ATCC18804-GFP. After nine days, mice were sacrificed and the kidneys were harvested, homogenized, and processed as described (136). Homogenates were stained with anti-β-glucan antibody (Biosupplies, Inc., Australia) at a concentration of 1.7 μg/ml, then stained with goat anti-mouse Cy3 antibody (Jackson Immunoresearch) at a concentration of 3.8 μg/ml. For soluble Dectin-1-Fc staining, homogenates were instead stained with Alexa647-labeled Dectin-1-Fc

(163) at a concentration of 17 µg/ml and then with donkey anti-human IgG Cy3 antibody (Jackson Immunoresearch) at a concentration of 0.8 µg/ml. Cells were visualized by optical sectioning fluorescence microscopy using a Ziess Axiovision Vivotome microscope (Carl Zeiss Microscopy, LLC). Live cells were identified based on characteristic EGFP fluorescence. Maximum projection images were quantified using Cellprofiler (www.cellprofiler.org) as described (136). Briefly, EGFP fluorescence was used to manually define individual cell segments and average fluorescence intensity of β-glucan or Dectin-1-CRD fluorescence was measured for the whole cell segment. Cells labeled without primary antibody or Dectin-1-CRD were used as negative controls.

4.3 Results and Discussion

It was discovered that the altered requirement of Dectin-1 for host protection from disseminated candidiasis was not related to different mouse genetic backgrounds, instead it was entirely dependent on fungal strain (50). In pursuit of the mechanism behind this, we investigated if it was due to differences between strains in β-glucan unmasking *in vivo* during infection. When examined, both displayed staining with anti-β-glucan antibodies and with Dectin-1-Fc at 9 days post infection. There was no significant differences in staining between strains seen with the antibody, but a significant difference was seen with Dectin-1-Fc. The Dectin-1-Fc probe is smaller than the antibody, which could allow it access to areas of the cell wall that the antibody is too large to access and suggests that these probes have different levels of sensitivity, something that should be considered in future studies looking to assay β-glucan availability. Surprisingly, the strain for which Dectin-1 was not required for protection (ATCC18804) showed significantly higher levels of soluble Dectin-1-Fc staining than the strain for which host defense depended on Dectin-1 (SC5314) suggesting that factors beyond just β-glucan availability are influencing the importance of Dectin-1 for host survival (Figure 4.1). In line with

this, it was found that differences in another fungal cell wall component, chitin, could be correlated with the strain dependent Dectin-1 importance (50). Indeed, manipulation of chitin content could make protection from the strain which is normally dependent on Dectin-1 become Dectin-1 independent. These observations further demonstrate the ability of fungal cell wall composition and architecture to influence host immunity which, when combined with our observations of *C. albicans*' rapid and dynamic cell wall changes in responses to immune cells, highlight the importance of studying these interactions to fully understand host immunity to this pathogen.

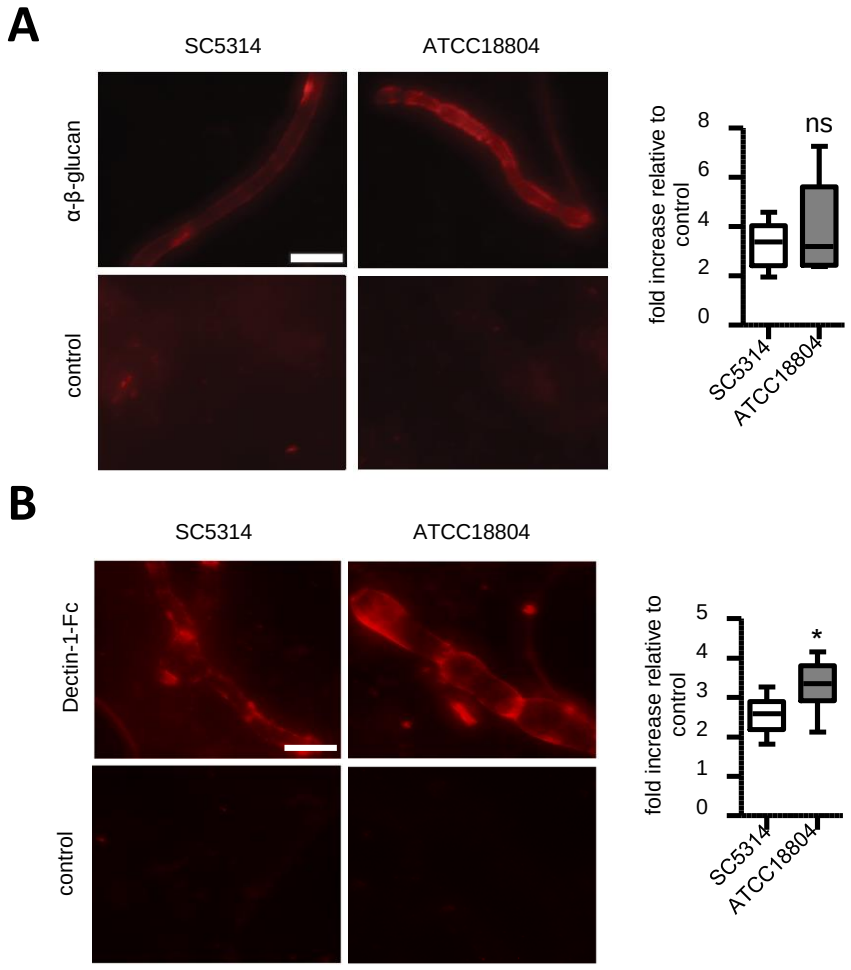


Figure 4.1 β-glucan unmasking does not explain the strain specific requirement for Dectin-1 C57BL/6J mice were infected with the indicated strain via the tail vein. Fungal cells were isolated from the kidney at day 9 post infection and stained *ex vivo* with (A) anti-β-glucan antibodies or (B) soluble Dectin-1 to examine β-glucan unmasking. Control cells were stained with secondary antibody only. Data shown are from a representative experiment. Bar indicates the mean.* $p<0.05$. ns, not significant. Scale bar represents 10 μm (adapted from 50).

CHAPTER 5

INTERACTIONS BETWEEN *C. ALBICANS* AND MACROPHAGES DIFFER BETWEEN *IN VITRO* AND *IN VIVO* ENVIRONMENTS

5.1 Introduction

In vitro modeling has long been used in an attempt to elucidate the contributions of different host immune cells to control and killing of *C. albicans*. These systems lead to the observations that, *in vitro*, neutrophils are much better at controlling the fungus than macrophages. Dramatic timelapses have shown for years that *C. albicans* yeast which are phagocytosed by murine macrophages *in vitro* will often germinate, eventually causing lysis of the macrophage and allowing escape from immune containment. The *in vitro* environment lacks many important cues that would be found *in vivo* and could enhance immune function however, which suggests that this interaction may not reflect what actually happens in the host. Indeed addition of cytokines like IFN-γ, IL-3, GM-CSF, CSF, M-CSF (87) or other factors like myeloperoxidase (203) can greatly increase the ability of macrophages to contain *C. albicans*. Recent work in an *in vivo* model, the zebrafish, demonstrates that macrophages can contain *Candida* during infection (204) with no evidence of germination by fungi contained within the immune cells over long periods of time. As these are fish, the question was raised as to whether or not this was simply due to some intrinsic differences between fish and mammalian macrophages. We therefore isolated macrophages from the zebrafish and incubated them with *C. albicans in vitro* to see if the interaction was similar to that observed with mammalian macrophages.

5.2 Materials and Methods

Zebrafish were spawned to create Tg(*mpeg1*:mcherry/UAS:Kaede) embryos for these experiments. Zebrafish were kept for 7 days before use. Macrophages were obtained via

homogenization as follows: Two frosted glass slides were obtained and cleaned with ethanol. Fish were placed on the "frosted" portion of one of the frosted glass slides. The other slide was then brought down on top of it (with the frosted portion facing the fish) and moved back and forth to achieve homogenization. Fish homogenate was washed into a 15 mL conical tube with a total of 2.5 mL media (L15 with glutamine and 5%FBS). The homogenate was then centrifuged @ 300xg for 15 minutes. The supernatant was discarded, resuspended in L15 with glutamine, 1mM MgCl2, 1mM CaCl2 and 5% FBS. A solution of 100 mg/mL collagenase/dispase was added to get a final concentration of 0.1 mg/mL. This mixture was agitated at medium speed for 20 minutes. An equal volume of stop solution (L15 with glutamine and EDTA with a final concentration of 0.01M) was added. The homogenate was centrifuged @ 1500xg for 5 minutes. The supernatant was discarded and the pellet was resuspended in 2 mL media (L15 with glutamine and 5% FBS). This was layered on top of 3mL of 1077 Histopaque in a 15mL conical tube. Centrifuge @ 400xg for 45 minutes. Collect the interphase of interest, dilute in media (L15 with glutamine and 5% FBS). Centrifuge @ 300xg for 15 minutes. The pellet was resuspended in 50 µL of media and transferred to an imaging dish and allowed to sit at 28°C for 4 hours. SC5314-GFP *C. albicans* yeast from an overnight YPD culture was prepared by collecting 500 µL and washing three times with 1x PBS. After the 4 hours, the macrophages were washed and 1 mL fresh media was added. The *C.albicans* was added at a final dilution of 1:1000 and mixed. Fields of view containing both *Candida* and macrophages were found and timelapses were taken on the Zeiss Vivatome Axiovision microscope with 2 minute intervals. The imaging dish was maintained at 28°C for the duration of imaging.

5.3 Results and Discussion

Timelapse microscopy revealed that zebrafish macrophages are less able to control *C. albicans in vitro*, with examples of germination, budding and macrophage killing observed

(Figure 5.1). The relatively rare process of vomitocytosis was also seen. These observations demonstrate that zebrafish macrophages are less able to contain *C. albicans in vitro* than was seen *in vivo* and suggest that the ability of macrophages to contain *C. albicans* in the zebrafish model are more due to differences between the *in vitro* and *in vivo* environments as opposed to some intrinsic difference between fish and mammalian macrophages. This highlights the limitations of *in vitro* work in understanding complex host pathogen interactions and the importance of using *in vivo* models whenever possible.

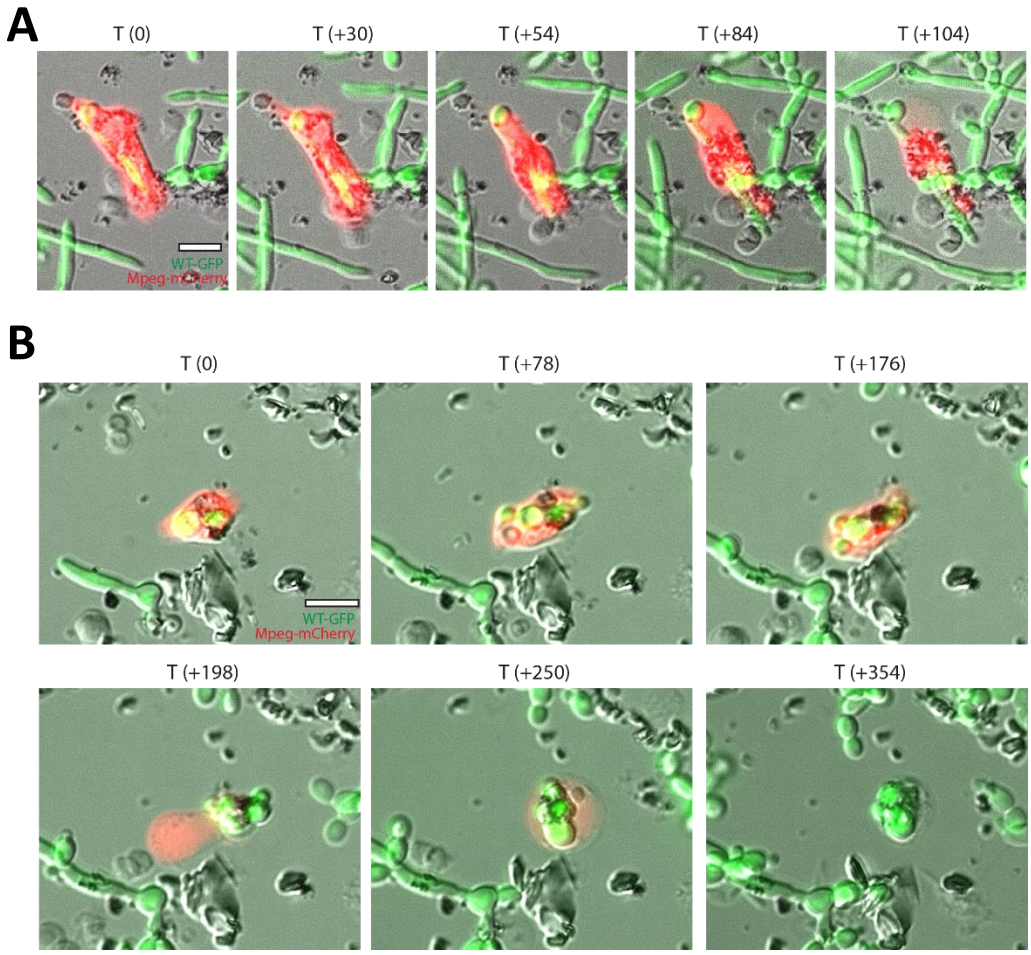


Figure 5.1 Zebrafish Macrophages are unable to efficiently contain *C.albicans in vitro*

Macrophages were harvested from Tg(*mpeg1*:mcherry/UAS:Kaede) zebrafish and incubated with SC5314-GFP in an imaging dish at 28°C. Timelapses with 2 minute intervals were obtained using a Zeiss Axiovision Vivatome microscope. (A) Panels from a timelapse of fungal germination and killing are shown. (B) Panels from a timelapse showing yeast budding, vomitocytosis and macrophage killing are shown. Scale bar represents 10 µm.

CHAPTER 6

DISCUSSION AND FUTURE DIRECTIONS

In the evolutionary arms race between host immunity and invading pathogens, many microbes have found that it is easier to avoid detection than to evolve ways to survive the full force of an immune response. Immune evasion is therefore a very common and frequently complex tactic used by a diverse array of pathogens to persist in their hosts. The opportunistic fungus *C. albicans* is a prime example, masking the inflammatory β-glucan in its cell wall from recognition by the host receptor Dectin-1. We have elucidated a novel host-pathogen interaction in which *C. albicans* actively responds to immune attack mediated by neutrophil extracellular traps, resulting in localized changes to the fungal cell wall composition and architecture including increased chitin deposition and β-glucan unmasking. In addition to this dynamic interaction, work in collaboration with others has revealed further pathways involved in the maintenance of baseline β-glucan masking (30) in the form of *cho1* and phosphatidylserine synthesis as well as uncovering the ability of changes in cell wall composition and architecture beyond straight β-glucan availability, specifically in chitin content, to influence the importance of specific host components for defense against the fungi (50). These insights have important implications for our understanding of host immunity during candidiasis, as they indicate a far more dynamic interplay between host and pathogen than is currently characterized. The fungus induces a diverse cascade of responses after suffering the biotic stress of immune attack, resulting in numerous changes to normal cell wall architecture. These changes have important implications for host immunity, as increased β-glucan unmasking could result in increased Dectin-1 mediated host responses but it has been shown that other changes in the cell wall, like increased chitin, can change the importance of this host receptor (50). The ability of fungal cell wall changes to influence the importance of other host receptors beyond

Dectin-1 remains to be explored. This leaves the complex interplay of the effects these cell wall changes could have on host immunity and even the specific nature of these cell wall changes themselves largely unknown, though their elucidation will be instrumental if we are going to be able to design effective therapies for patients with candidiasis. Furthermore the fungal signaling pathways we have identified likely play a role in maintaining cell wall integrity during immune attack (22) and, as disruption of cell wall integrity is the mechanism of action for some existing antifungal drugs, these pathways could yield attractive targets for developing new standalone or combination therapeutics.

While we have elucidated many components of this complex interaction between neutrophils and *C. albicans*, much remains to be explored. There are many neutrophil attack sites evident from areas with streptavidin fluorescence loss, though only a percentage of these sites go on to develop β-glucan unmasking. The reasons for this are currently unknown though, given that neutrophils can be a heterogeneous population with a variety of ages and activation states, it seems possible that attack by some will result in more damage than attack by others. Perhaps there is a threshold level of cell wall damage required before the fungus responds. The exact stimulus required to provoke fungal cell wall rearrangement is also still unclear. The requirement for the NADPH oxidase and MPO could suggest that this stimulus could involve damage mediated by MPO products like hypochlorite however, as both NADPH oxidase and MPO can be important for NETosis (84, 170), it may be required simply to regulate proper NET formation and another element provides the damaging stimulus. Furthermore, while many elements involved on the fungal side have been elucidated, many more steps in these pathways remain unknown. The upstream elements that initially sense NET mediated damage and activate Hog1p in this context remain to be discovered. Additionally, while we have begun to identify possibilities in the form of Phr1p and Sur7p, the specific elements actually required for cell wall

remodeling and eventual β-glucan unmasking to occur after attack remain to be identified. The model we have described here will serve as an important tool to further elucidate these elements and will therefore be invaluable in the dissection of fungal cell wall dynamics in response to immune stress. Beyond this narrow focus, our system is innovative as it allows interrogation of highly localized cell wall dynamics as opposed to cell wide responses to environmental stress which were typical of previous *in vitro* studies.

This work has important implications beyond expanding our understanding of candidiasis. Epitope masking, which allows the pathogen to avoid detection during infection, represents a highly desirable advantage in the evolutionary arms race with the host, and shielding of β-glucan is a common strategy seen in numerous fungi (128, 177, 144). We demonstrate here that host-pathogen interactions can trigger the unmasking of fungal epitopes in *C. albicans* but these type of interactions may also occur during infections with other fungi and this remains to be explored. The work outlined here could serve as the starting point for identifying evolutionarily conserved pathways which are critical for fungal cell wall integrity and remodeling following immune stress. Furthermore, this could identify both conserved and unique changes to fungal cell walls following interaction with host immunity which could have important implications for the outcome of immune responses. Alternatively, research into these areas could highlight novel ways in which fungi have evolved to escape host immunity, including methods of circumventing attempts at epitope unmasking.

The idea that the cell wall of pathogens can change dynamically during infection has implications which extend beyond the sphere of mycology. In addition to fungi, bacteria, protozoans, helminths and viruses can all manipulate how the host perceives them by altering epitope availability so, in order to truly understand infection, we will need to gain a more complete understanding of how these pathogens are adapting to face the dynamic

environments presented in a living host (117, 143, 128, 144, 29, 145, 146). Cell surface changes could benefit pathogens by allowing adaption to different host stresses, to attempt to salvage cellular integrity following damage, to evade recognition by host immunity or to manipulate host systems toward non-protective responses. The host could benefit from triggering cell wall changes if they expose important epitopes for furthering immune recognition and responses or make it more susceptible to sequential attack by immune stresses. In practice, it is likely that microbial cell wall changes could simultaneously benefit both the pathogen and the host and in order to understand the true implications of these changes to the overall outcome of infection we will need to examine them *in vivo*. This line of investigation could also identify previously unknown epitopes which only become exposed under certain conditions or *in vivo*, thereby providing novel targets for vaccine and therapeutic design.

It is important to remember that, as demonstrated by our work with zebrafish macrophages, *in vitro* environments are not always the best systems for understanding complex immune cell interactions with pathogens. It will therefore be important to validate our results in vivo whenever possible. We have demonstrated the critical nature of neutrophils through ex vivo work however much, especially the requirement of different fungal signaling pathways to cell wall changes remain to be interrogated *in vivo*. The mouse model of disseminated candidiasis would continue to serve as a great system to interrogate these interactions and combining different *C. albicans* signaling mutants with the *ex vivo* fluorescence technique used here and described in (136) can allow us to probe the requirement of numerous fungal signaling elements in initiating cell wall changes in a mammalian host. Unfortunately, even when using advanced systems like 2-photon microscopy, the opaque nature of the mouse makes direct observation of host-*C. albicans* interactions *in vivo* extremely difficult. As an alternative, the zebrafish model offers many advantages, one of the most important being that they are optically transparent

and therefore provide a nearly unparalleled opportunity for the direct imaging of events *in vivo* in a vertebrate host. The zebrafish has many of the basic elements of mammalian immunity, including innate immune cells like neutrophils and macrophages, increasing the likelihood that observations made in this system will be translational as compared to other transparent models like the nematode *Caenorhabditis elegans*, which lacks many important immune features seen in vertebrates (205, 206). Multiple models of *C. albicans* infection have already been described in the zebrafish including mucosal infections and disseminated infection (88, 207, 208). Preliminary results suggest that β-glucan does become unmasked during *C. albicans* infection of the zebrafish (data not shown), meaning the interactions we have characterized with murine immune cells may be at least partially conserved in fish. This model system could therefore provide a powerful platform to observe neutrophil/*C. albicans* interactions directly in an *in vivo* environment. The zebrafish can also serve as a model for numerous other infections including bacteria, viruses and other fungi (209, 210, 211). In fact, an example of bacterial epitope masking was elucidated in the zebrafish model, demonstrating this system can be a powerful tool in probing the implications for changes in epitope availability during infection (117). The zebrafish is not a complete replacement for the mouse however, and its deficiencies must be taken into account when deciding what experiments to conduct with the model. Of particular importance to our work, zebrafish pattern recognition receptors are not nearly as well characterized as those in mice or man and it appears that zebrafish don't have orthologs of many characterized mammalian pattern recognition receptors, particularly with respect to the CLRs including Dectin-1 (data not shown). This makes experiments centered on specific ligand-receptor interactions better suited for the mouse model, where there is both better characterization and homology with what is seen in humans. With careful consideration, future

studies done with the mouse and zebrafish should be able to fill in many of the gaps in our understanding of the events which occur during candidiasis.

Overall, this work has elucidated a novel and dynamic host pathogen interaction between the neutrophil and *C. albicans*, in which immune attack results in an active fungal response with cell wall remodeling and during which masked fungal epitopes become exposed. The elucidation of some of the pathways involved in this interaction has provided valuable insight into host-pathogen interactions during candidiasis and our work has provided a model which can be used as the foundation for probing the dynamic and localized changes in the fungal cell wall during stress. As most antifungal drugs target the integrity of the fungal cell wall, this system can be leveraged to identify novel targets for drug design that, when combined with insights into how the host is interacting with *C. albicans*, can improve patient outcomes in the future. Finally, this work can serve as a spark to ignite interest in seeking these kinds of dynamic host-pathogen interactions during infections caused not only by other fungi but with all types of pathogens, research that could have a great impact by increasing our general understanding of the dynamic events that occur during infection and hopefully result in improved therapeutic options for numerous diseases.

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Biography of the Author

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