MUCOSAL IMMUNE DEFENSES TO THE FUNGAL PATHOGEN

Candida albicans

By

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Submitted in fulfillment of the requirements for the
degree of Doctor of Philosophy

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August 2013
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Dedication

I dedicate my collective works to those who have supported me in all endeavors in my life. The support of my family, friends, teachers, and colleagues has made this achievement possible. Without their guidance, care, and most importantly patience it would not have been possible to fulfill my dreams. Through time, and with their help, I have grown from a curious young man into a knowledgeable scientist with a thirst for discovery.
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blessed to have a great woman behind me that supports me when I need support and pushes me when I need motivation.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus inhibitor of apoptosis protein repeat</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type Lectin Receptor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>hBD</td>
<td>human β-defensin</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin-1-beta converting enzyme</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mBD</td>
<td>murine β-defensin</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B-cells</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal apoptosis inhibitory protein</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide, or NACHT, oligomerization domain</td>
</tr>
<tr>
<td>NLR</td>
<td>Nacht-like receptor</td>
</tr>
<tr>
<td>NLRC</td>
<td>Nacht-like receptor containing a CARD domain</td>
</tr>
<tr>
<td>NLRP</td>
<td>Nacht-like receptor containing a pyrin domain</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 receptor</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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Mucosal Immune Defenses to the Fungal Pathogen *Candida albicans*

**Abstract**

by

JEFFREY ALAN TOMALKA

Fungal pathogens represent one of the greatest emerging threats to human health. In spite of this, limited research has been conducted on the mechanisms utilized to clear fungi. We first discovered that IL-1β is critical for mounting an effective immune response to *Candida albicans*. It was recently discovered that proteolytic cleavage of IL-1β, a required step in its release and activity, can be mediated by intracellular protein complexes termed “inflammasomes” which consist, in part, of members of the NLR family of proteins. Given the critical role for IL-1β in our model, we next investigated the NLR(s) responsible for *Candida* induced IL-1β. We found that NLRP3 was indispensable for production of IL-1β from macrophages. *Nlrp3*−/− mice had elevated oral fungal loads early, with sustained increases in disseminated infection through the course of infection. Despite the absolute requirement for NLRP3 in IL-1β release from macrophages, levels of circulating IL-1β were still detectable in the absence of both NLRP3 and ASC. This led to the hypothesis that an additional CARD containing NLR was functioning to induce IL-1β release following live *Candida* infection. *Nlrc4*−/− macrophages exhibited no defect in IL-1β release however serum from infected mice did exhibit a substantial reduction in IL-1β. Mice deficient in NLRC4 were susceptible to sustained mucosal infection and early bloodstream dissemination.
Bone marrow chimeric mice revealed that WT bone marrow transplant was incapable of ameliorating disease in Nlrc4−/− hosts and Nlrp3−/− bone marrow induced susceptibility to infection in WT mice, demonstrating these two NLRs exert their function in different cells. Absence of either NLR was found to result in decreased neutrophil infiltration into infected tongues and decreased inflammatory gene expression in buccal tissue, including a substantial defect in β-defensin expression. The profound defect in β-defensins led to the investigation of the capacity for murine β-defensin 1 to protect from oral Candida infection. Mice deficient in this protein exhibited increased mucosal and disseminated fungal loads at early timepoints. Concurrent with this early susceptibility was decreased neutrophils and inflammatory gene expression in infected mucosal tissue. These data demonstrate the critical role of IL-1β during protective mucosal immune responses to Candida.
Chapter 1

Introduction to *Candida* and innate immunity
1.1 Fungi as a rising threat to human health

Fungi represent one of the largest threats to human health, with occurrence of infections rising in recent years. Fungi are ubiquitous in natural environments, constituting a significant portion of biomass in soils\(^1\). They differ from two other common pathogenic microorganisms, bacteria and viruses, due to the presence of a nucleus which allows them to actively respond to environmental stress. Fungi have evolved the capacity to derive carbon from numerous sources and their critical role as saprophyles recycling organic material from detritus has conferred a strong evolutionary advantage on these organisms\(^1\). Fungi can be single-cell or multi-cellular organisms, with the former dominating the species known to cause human diseases. Single-celled fungi can be characterized into three distinct subsets based upon morphological characteristics. Yeast-form fungi can be easily distinguished due to their round, punctate shape throughout their life-cycle. Filamentous fungi, on the other hand, form long tubular structures known as hyphae in nutrient replete environments. In conditions of nutrient depletion, filamentous fungi form conidia which are highly stable and resemble yeast fungi in shape and size but are metabolically inert until a carbon source is present again. Dimorphic fungi, as the name would suggest, exist in the ecosystem in both yeast and hyphal morphologies during their life-cycle. This capacity to alter cell morphology has important impacts on the ability of these fungi to infect human tissue and evade protective host immune responses.

The ability of filamentous and dimorphic fungi to form branched, connected networks of hyphae readily allow these organisms to form biofilms
both with other fungi as well as other micro-organisms including bacteria. These biofilms provide protection from environmental stresses, including shearing force from wind and water, as well as cooperative nutrient utilization amongst the organisms allowing these biofilms to persist for extended periods of time under stressful and/or nutrient poor conditions. Many fungi are also resistant to alterations in pH, dessication, oxidative stress, and have evolved special mechanisms to acquire essential metals from the environment, and to utilize multiple carbon sources. All of these combine to make fungi highly adaptable and durable, both in nature and in host bodies.

1.2 Overview of Candida

*Candida* is a genus of dimorphic fungi which are commonly found associated with mammalian hosts. *Candida* undergoes morphological transition at 37°C, allowing it to readily form biofilms on human host tissue. To date, a large number of *Candida* species have been found to colonize the skin and mucosal tract of humans. *Candida albicans* is the most common species isolated from human hosts and carriage rates in the healthy population have been found to be as high as 50%3-6. Like other fungi, *Candida* has a cell wall composed of repeating saccharide subunits and proteins. This cell wall provides protection from environmental stress and some immune functioning but also constitutes the major components recognized by innate immune cells, demonstrating the complex interplay that exists between hosts and pathogenic microorganisms. The first layer
of the cell wall is composed of chitin\textsuperscript{7} (Figure 1.1). Above this layer of chitin, are repeating $\beta_1$-$6$ and $\beta_1$-$3$ glucan residues which crosslink to provide structural stability to the cell wall. The final layer of the cell wall is composed of mannans which are associated with GPI-anchored surface proteins\textsuperscript{7}. A number of these proteins are virulence factors involved in adhesion to host cells or alteration of normal host functioning.

\textit{C. albicans} is a commensal microbe causing no observable alteration of host function but under conditions permissive to overgrowth \textit{Candida} can induce pathology. Overgrowth is normally prevented by competition for substrates and nutrients with other mucosal microorganisms as well as innate host immunity, including antimicrobial peptides, which prevent growth through direct killing of the fungus.

\subsection*{1.3 Predisposing conditions to \textit{Candida} infection}

If the normal oral flora is profoundly perturbed in an individual or a state of altered immune function occurs, fungal overgrowth is often seen. Oral and systemic antibiotics are a major predisposing factor to \textit{Candida} infections including oral and vaginal candidiasis\textsuperscript{8-13}. Discontinuation of the antibiotic in conjunction with antifungal treatment is often sufficient to ameliorate disease in these individuals and future infection is held in check due to continued immune function and restoration of the normal components of the mucosal flora. Interestingly, one recent study found that
Figure 1.1. Saccharide composition of the *Candida* cell wall.
Schematic representation of the saccharide residues present in the cell wall of *Candida*.
Red indicates Chitin. Blue represents β,1-3 glucan. Yellow is β,1-6 glucans. Green stands for mannose residues.
pretreatment with antibiotics led to increased occurrences of fluconazole-resistant
*Candida* strains in patients. This indicates that not only do antibiotics predispose
individuals to *Candida* infection but they may also impact the capacity to treat
subsequent fungal infections with traditional antifungal agents\(^\text{12}\). High
carbohydrate diets have also been posited as a predisposing factor for *Candida* by
increasing nutrient sources available for microbial growth/survival\(^\text{14}\). By
providing a substrate for fungal colonization, dentures can predispose an
individual to oral *Candida* infection. It was once believed that good oral hygiene
could prevent *Candida* overgrowth however a recent study found no correlation
between increased plaque index or gingival index, indicators of poor hygiene, and
*Candida* carriage/density\(^\text{3}\). This does not eliminate the potential for poor hygiene
to contribute to disease but it appears that it is not a major determining factor in
either prevalence or level of oral *Candida*.

For individuals suffering from an immunosuppressive condition, these
infections can be more difficult to deal with. This altered immune phenotype can
be the result of intrinsic defects or extrinsic medical treatments directed against a
different condition. Intrinsic defects include immature immune functioning in
infants, age-related reduction in immune functioning, impaired salivary gland
function, mucosal cancers, and inborn genetic defects in immune pathways.
Dentures\(^\text{15-20}\), inhaled or systemic steroids\(^\text{21-25}\), chemotherapeutics\(^\text{26-31}\), and
radiotherapy\(^\text{32-37}\) constitute the major medical treatments previously associated
with susceptibility to *Candida* infection. Corticosteroid treatment, for example,
has a profound impact on the occurrence of deep tissue *Candida* infection\(^\text{9}\).
 Patients with AIDS are also highly susceptible to *Candida* infection\(^4,38-42\). Recent advances in the field of immunotherapy, in particular TNFα neutralizing drugs\(^43-45\), have broadened the number of individuals undergoing medical treatments which leave them highly susceptible to *Candida* infections. It is likely that cases of candidiasis will continue to increase in the population as treatments to limit autoinflammatory conditions continue to be developed.

Since elimination of these predisposing conditions is not as simple as removing antibiotics, many of these patients rely solely on antifungal drugs to help reduce their symptoms. The recent spike in single and multi-drug resistant fungi in clinical isolates is resulting in less efficacious treatment with already established drugs\(^46-56\). Compounded with the fact many of these drugs show human toxicity after extended exposure\(^57-61\), the development of new therapies for these patients is essential to returning them to a normal quality of life.

### 1.4 Human *Candida* infections

*Candida* infections can be divided into two major categories: disseminated and mucosal. Disseminated infection is defined by the presence of detectable, viable fungus in the blood of patients. This dissemination, in humans, occurs rarely as a result of a distant mucosal infection. Rather it is highly associated with in-dwelling catheters and infections following deep tissue exposure, either due to trauma or surgery \(^11,13,62\). *Candida* is currently the 4\(^{th}\) most commonly isolated pathogen in the blood of hospital patients\(^63\). This makes disseminated *Candida*
infections a critical concern for healthcare facilities. The fact that many post-
surgical regimens involve immune suppression and/or antibiotics only exacerbates
the likelihood of disseminated candidiasis. With mortality rates rising as high as
50% following positive blood culture, prevention of disseminated candidiasis is
crucial in limiting mortality associated with surgery and hospital stays $^{63-65}$.

Mucosal Candida infections represent the majority of cases observed in
humans. These infections can occur in the oral cavity, all portions of the
gastrointestinal tract, and vaginal mucosa. Mucosal infections are normally
superficial, self-limiting infections which do not result in systemic disease
symptoms but can induce detrimental alterations in host functioning in infected
tissue. Mucosal infections are composed primarily of the hyphal form of Candida,
resulting in robust biofilms that can be difficult to eliminate without the use of
antifungal drugs. These fungal plaques induce local tissue inflammation which
results in symptoms including a burning sensation, altered nutrient intake, and
tissue erosion. Much of the inflammation and tissue damage early during
infections is due to the infiltration of neutrophils into infected sites. These
neutrophils are critical for the control of most mucosal infections due to the
capacity of granular components to degrade the fungus in the extracellular
environment. This is clearly demonstrated by neutropenia being identified as a
key predisposing component to murine and human Candida infections $^{66-69}$. The
large size of fungal hyphae can cause phagocytosis to become more difficult than
for the yeast form or for many other microbial pathogens $^{70}$. In such cases, the
release of granular components becomes essential in combating these infections.
One of the most common mucosal Candida infections is of particular interest to our lab. Oropharyngeal candidiasis (OPC), more commonly referred to as thrush, is an oral infection characterized by the presence of visible pseudomembranous patches of fungus\(^7\). These patches present diffusely in the oral cavity including the gums, palate, tongue, and pharynx. In addition to the associated burning sensation, patients often suffer from dysphagia which can result in dehydration and malnutrition\(^7\). These complications can be especially troubling for infants suffering from OPC. As with a number of mucosal infections, dissemination and mortality are infrequent however the significant impact this infection has on quality of life makes research into more effective treatments of great importance. While rises in drug resistance have spurred the development of novel antifungal drugs, development of therapies aimed at augmenting normal immune functioning would signify a large breakthrough in the treatment of fungal infections as such therapies would target pathways that have remained intact through thousands of years of co-evolution of humans and Candida, resulting in a lower likelihood that Candida is capable of developing resistance to these pathways.

1.5 Increasing recognition of the role of Innate Immunity

The innate immune system comprises the body’s first line of defense against pathogens, in particular mucosal pathogens. Originally, it was believed that innate immune cells derived only from the bone marrow, like those of the
adaptive immune system. It has become more evident that non-hematopoietic cells present in many tissues are indispensible in the generation of innate immune responses. In general, innate immune cells are defined by their capacity to respond to microbes through germ-line encoded receptors that recognize specific ligands present in microbial organisms. Innate immune cells can express multiple receptors allowing them to respond to multiple pathogens whereas adaptive immune cells express a receptor with a singular specificity. Epithelial cells are the first innate immune cells to encounter a mucosal pathogen. In addition to functioning as a physical barrier, epithelial cells contain pre-synthesized inflammatory mediators, including IL-1α, that become released upon cell lysis. One of the characteristics that separate non-pathogenic from pathogenic microbes is that pathogens cause tissue damage. In this manner, epithelial cells are the first cells capable of alerting other immune cells to the presence of a mucosal pathogen.

Epithelial cells can recognize the presence of microbes through innate immune receptors, called pattern recognition receptors or PRRs. These PRRs respond to conserved molecular patterns present in microbial species, termed pathogen-associated molecular patterns or PAMPs, that are largely absent in host cells. Specialized epithelial cells lining the salivary duct are potent secretors of antimicrobial peptides which function to limit microbial growth on mucosal surfaces. Following the release of early inflammatory mediators in tissue, endothelial cells lining nearby blood vessels become activated. This state of activation results in the upregulation of cell adhesion molecules including P-
selectin\textsuperscript{76-78}, E-selectin\textsuperscript{79}, ICAM\textsuperscript{78,80}, and VCAM-1\textsuperscript{81}. These adhesion molecules interact with surface ligands on leukocytes such as PSGL1\textsuperscript{82,83}, L-selectin\textsuperscript{77,82,83} and LFA1\textsuperscript{80}. The ultimate consequence of these interactions is the arrest of the leukocyte on the endothelium followed by diapedesis. Once out of the bloodstream, cells home to the sight of infection through the action of chemokines released by epithelial cells and other resident cells including fibroblasts and tissue macrophages. The first detected innate cells to infiltrate tissue from circulation are neutrophils. These are cells characterized by the presence of multi-lobular nuclei, resulting in their commonly being referred to as polymorphonuclear cells, and granules storing presynthesized components that are important to combating invading microbes. Granules are divided into three categories: primary (azurophil) granules which characterized by the presence of myeloperoxidase\textsuperscript{84,85}, secondary (specific) granules which contain proteins including lactoferrin\textsuperscript{86}, and tertiary granules with enzymes including heparanase and gelatinase\textsuperscript{87}. Neutrophil infiltration, in our model, begins as soon as a few hours following infection and often peaks during the first week of infection. Following neutrophils, the next major cell type to infiltrate infected tissue are macrophages. These cells derive from immature circulating monocytes in the bloodstream. In addition to the production of cytokines in response to activation of PRRs, macrophages can engulf foreign particles and microbes through a process called phagocytosis. This directed engulfment allows for rapid internalization of foreign particles into vesicles termed phagosomes, which can then be fused with lysosomes to mediate degradation. Lysosomes contain proteases capable of degrading organic material
and a low pH which is essential for protease function and can also directly lead to microbial death. Phagocytosis not only results in the degradation of foreign particles but also allows for peptide fragments to induce the activation of the adaptive immune response.

1.6 Role of Pattern Recognition Receptors: TLRs

In order to recognize the presence of infectious pathogens, immune cells express pattern recognize receptors (PRRs) which are capable of recognizing the presence of conserved molecular patterns in microbes. Toll-like receptors, or TLRs, were the first characterized PRRs involved in innate immune activation. These proteins share common structural components of an extracellular leucine-rich repeat domain of varying length and a cytoplasmic signaling domain called a Toll/Interleukin receptor (TIR) domain. TLRs can be found on the surface of immune cells, as well as in endosomal vesicles. The first such identified protein was discovered in a random mutagenesis of Drosophila aimed at determining genes involved in dorsal/ventral patterning\(^8\). Named toll, German for great or amazing, it was discovered that in addition to a noticeable phenotype in patterning these mutants would become engulfed by fungus. Further research identified a number of similar proteins present in mammals and that homologues of components of the toll pathway in Drosophila were members of a signaling cascade leading to NF-kB activation, a key transcription factor involved in transcription of immune genes. There have been 12 identified TLRs in mice and
10 in humans\textsuperscript{89}. In the decades since the discovery of\textit{toll}, a substantial amount of research has been aimed at determining what ligands are capable of activating these receptors. The first ligand identified was for TLR4, which was found to respond to lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. One report identified a mutation in TLR4, termed the LPS responsive gene, in C3HE/J mice which conferred the known resistance of this strain of mice to bacterial sepsis\textsuperscript{90}. Another report identified homologues of the\textit{toll} pathway in mice as being activated downstream of LPS treatment and resulting in NF-kB activation which was later shown to be the result of recruitment of MyD88\textsuperscript{91,92}. In mice, TLR2 is a cell surface receptor that has been shown to form heterodimers with either TLR1 or TLR6 for signaling, with TLR2/1 recognizing triacyl peptides and TLR2/6 diacyl peptides\textsuperscript{93}. TLR3 is an endosomal TLR responsible for recognition of double-stranded viral RNA\textsuperscript{94}. TLR5 recognizes the presence of bacterial flagellin\textsuperscript{95}. TLR7/8 are also present in endosomes and respond to single-stranded viral RNA\textsuperscript{96,97}. TLR9 is activated by unmethylated CpG DNA, which is present in many microbes but not common in mammals\textsuperscript{98}. Unlike other TLRs, TLR9 uses the adaptor molecule TRAM to mediate signaling rather than MyD88. In addition to being the first discovered TLR, TLR4 distinguishes itself by its use of both MyD88 and TRAM as signaling adaptors. The ability for this receptor to use both signaling adaptors remained unexplained until it was discovered that when bound to LPS on the cell surface, TLR4 utilizes MyD88 and upon internalization into an endosome functionally recruits TRAM to replace MyD88 and lead to downstream signaling\textsuperscript{99}. In this
capacity, it functions both as a cell surface PRR and endosomal PRR and signals for differential gene expression depending on the cellular location of the receptor. Shortly after the discovery of TLRs, it was found that TLR2 is capable of recognizing zymosan from the cell wall of *Saccharomyces cerevisiae*\textsuperscript{100}. It was later found that phospholipomannan from *Candida* was capable of activating TLR2\textsuperscript{101}. TLR4 was found to be involved in control of *Candida* dissemination\textsuperscript{102}, though the precise molecule recognized still remains somewhat controversial. Though TLRs have been found to respond to PAMPs present in all types of pathogens, they are not the only family of PRRs which have been found to be important in innate immune activation.

### 1.7 Role of Pattern Recognition Receptors: CLRs

Another class of cell surface PRRs was discovered that recognize the presence of saccharide motifs. These receptors were termed C-type lectin receptors, or CLRs. These receptors are especially important in immune recognition of fungi because of the saccharide rich cell wall. Dectin-1 was identified as a receptor for β-1,3 glucans, a key structural component of cell wall of many fungi including *Candida*\textsuperscript{103,104}. Dectin-1 initiates intracellular signaling through activation of Spleen Tyrosine Kinase, or Syk\textsuperscript{105,106}. Along with CARD9, Syk activation results in downstream transcriptional activation of NF-kB driven inflammatory genes\textsuperscript{107-109}. Another CLR named Dectin-2 has been shown to bind high mannose residues and α-mannose\textsuperscript{110-112}. Macrophage-inducible C-type Lectin, Mincle, and DC-SIGN have also been found to bind to mannose residues and the capacity for all three receptors to bind mannose residues may reflect
distinct cellular distributions of the receptors. In addition to α-mannose, Mincle binds mycobacterial glycolipid\textsuperscript{113} and is also activated endogenous ligands known to induce inflammation called danger-associated molecular patterns, or DAMPs, including SAP\textsubscript{130}\textsuperscript{113}. In addition to a role in recognition of fungi, DC-SIGN has been implicated in viral responses including HIV\textsuperscript{114}, HCV\textsuperscript{115}, and Ebola\textsuperscript{116} as well as the parasite Leishmania\textsuperscript{117}. Dectin-2 and Mincle mediate signaling through interaction with the common gamma chain of the Fc receptor, which not only highlights similarities between these two receptors but cross-talk between innate and adaptive signals\textsuperscript{111,113}

### 1.8 IL-1: Biology and disease implications

A major downstream effect of PRR engagement is the production of cytokines. These constitute the major mechanism of cell-cell communication during immune responses. One of the most potent cytokines involved in the induction of immune responses against infections, including those with \textit{Candida}, is Interleukin-1beta or IL-1β. This protein was originally isolated as a component of the serum and identified as a pyrogenic factor for its capacity to induce fever\textsuperscript{118}. Over a period of years, it was determined that many of the pyrogenic factors isolated from blood were in fact IL-1 and had been erroneously described as different proteins\textsuperscript{119}. Pyrogens identified as IL-1 were later divided into two cytokines, IL-1α and IL-1β, based upon differences in protein sequences. Despite differences in primary sequence, these proteins utilize the same receptor, IL-1R,
to mediate their biological effects. These cytokines are capable of functioning through all 3 pathways for signaling: autocrine, paracrine, and endocrine. Autocrine signaling occurs when IL-1 released from a cell binds to a receptor on that same cell, resulting in further activation of inflammatory gene expression. Paracrine signaling occurs when IL-1 released from one cell acts upon a neighboring cell, with similar effects as observed in autocrine signaling. When released in the bloodstream, IL-1 can act in an endocrine fashion on the hypothalamus to induce fever, cause loss of appetite, muscle lethargy, and the release of C-reactive protein from the liver. This endocrine ability separates IL-1 from many other immune cytokines that function locally and highlights its important function in relaying the presence of pathogenic infection to distant tissues.

Despite their similar biological actions, IL-1α and IL-1β differ in a number of key characteristics. IL-1α is often found presynthesized in the cytoplasm as compared to IL-1β which requires transcriptional upregulation following an inflammatory signal. This difference allows IL-1α to serve as an early sensor of cell damage and IL-1β as a sensor of the presence of PAMPs. Though both proteins are produced as pro-proteins which require proteolytic cleavage for optimal biological activity, IL-1α is capable of binding to IL-1R prior to proteolytic cleavage but remains intracellular until cleaved or released from a lysed cell. IL-1β, on the other hand, has no effect until it is cleaved into its active component. Recent research has identified a set of proteins which mediate this IL-1β cleavage intracellularly in response to tissue
dysfunction/damage, further distinguishing IL-1β as a sensor of PAMPs in the presence of tissue damage. The presence of PAMPs and tissue damage is an indication of pathogenic infection, identifying IL-1β as a key determining factor in identifying tissue infections.

The original work done on IL-1 was a result of its pyrogenic effects in blood, particularly in response to bacterial sepsis. Its role was initially downplayed due to the presumption at the time that TNFα was the critical cytokine involved in septic shock, due to its extremely high levels in blood during sepsis. It was later proven, through genetic manipulation of mice, that IL-1R signaling, downstream of either IL-1α or IL-1β, was the cause of septic shock and not TNFα signaling\textsuperscript{121}. This finding spurred a field of research aimed at determining what other disease characterized by inflammation involve IL-1. It is now known that IL-1 is important in the development of rheumatoid arthritis\textsuperscript{122-125}, gouty arthritis\textsuperscript{126}, myeloma\textsuperscript{127,128}, and a range of autoinflammatory disorders.

The role of IL-1 signaling in Candida remains a poorly researched subject, despite the important role of IL-1 in a wide range of disease pathologies. Early studies found that recombinant IL-1 was able to rescue mortality in neutropenic mice infected with Candida albicans\textsuperscript{129}. Additionally, recombinant IL-1β was found to protect mice from disseminated Candida albicans\textsuperscript{130} and following intracerebral injection\textsuperscript{131}. IL-1 has also been found to be released by oral epithelial cells following Candida stimulation and IL-1α was shown to increase the anticandidacidal capacity of macrophages\textsuperscript{73,132,133}. As mentioned before, in
order for biological activity, IL-1β requires a proteolytic cleavage event which is mediated by members of a recently discovered third family of PRRs called NLRs.

1.9 Role of Pattern Recognition Receptors: NLRs

Nacht-like receptors, or NLRs, share a similar structure across the family with a C-terminal leucine rich repeat domain believed to be involved in autorepression, a central nucleotide oligomerization domain (NOD) involved in protein-protein interaction, a variable N-terminal effector domain which mediates the biological function (Figure 1.2). Unlike TLRs and CLRs, NLRs are present in the cytoplasm and not restricted to the cell membrane or vesicles. This allows them to sense the presence of microbial components released into the host cell. Two members, NOD1 and NOD2, have been well characterized for their ability to activate gene transcription in response to the presence of bacterial cell wall products. NOD1 recognizes meso-diaminopimelic acid (meso-DAP) and NOD2 recognizes muramyl dipeptide (MDP)\textsuperscript{134,135}. Activation of these receptors results in degradation of the IKK inhibitory complex which leads to nuclear translocation of NF-kB subunits and activation of target genes\textsuperscript{136-139}.

A subset of these NLRs with similar structure to NOD1/2 but with distinct immunological function was identified. Rather than functioning primarily to activate gene transcription as other PRRs had been shown to do, members of this group also function to activate inflammatory caspases, canonically caspase-1, which are responsible for the proteolytic cleavage of IL-1β from its 37kD pro-
form into the 17kD active protein. The first NLR involved in the cleavage of IL-1β was discovered through identification of a gain of function mutation associated with patients with familial cold autoinflammatory syndrome, or FCAS\textsuperscript{140}. It was determined that a mutation on the long arm of Chromosome 1, 1q44, was associated with FCAS and this was later mapped to a gene termed cold induced autoinflammatory syndrome 1, or CIAS1 which has since been renamed NALP3, NACHT, LRR, and Pyrin containing protein 3. This gene has since been associated with other autoinflammatory disorders. It was not until it was discovered that the NALP3, further referred to as NLRP3 (NOD-like receptor family, pyrin domain containing 3), protein mediated caspase-1 cleavage and subsequent IL-1β cleavage that the role of this gene in inflammation was fully discovered\textsuperscript{141}. This discovery led to the term “inflammasome” due to the capacity for the multi-protein complex containing NLRP3 to be a potent mediator of inflammation through activation of IL-1β\textsuperscript{142}. To date, 16 human and 28 murine NLRS have been discovered which can become incorporated into inflammasomes\textsuperscript{143}. These members contain 1 of 3 effector domains: a pyrin domain (PYD) in the NLRP proteins, a caspase activation and recruitment domain (CARD) for the NLRC proteins, or a baculovirus inhibitor of apoptosis repeat domain (BIR) for NAIP. The name NAIP came about due to their recognition for the capacity as an apoptosis inducing protein. PYD containing inflammasomes recruit an adaptor molecule called Apoptosis-associated Speckle-like protein containing a CARD (ASC) which has a PYD domain to bind to the inflammasome and a CARD domain to recruit inflammatory caspases.
Inflammasomes containing a CARD domain can recruit inflammatory caspases without requiring the adaptor protein ASC. BIR containing inflammasomes are thought to be involved in control of cell death given the known function for BIR domains in inhibiting apoptosis. Many of these NLRs remain poorly characterized.

![NLR family structures and domains](image)

**Figure 1.2. NLR family structures and domains.**
Schematic representation of the various domains present in the different categories of NLRs. LRR=leucine rich repeat, NOD=NACHT oligomerization domain, PYD=pyrin domain, FIIND=function to find domain, CARD=caspase activation and recruitment domain, BIR= baculovirus inhibitor of apoptosis.
Though a great deal of research has been performed on the NLRP3 inflammasome, most has been directed at identifying activating ligands while little is known about the actual mechanistic functioning of inflammasomes. It is thought that these NLRs exist in an autorepressed state, wherein the LRR domains sterically prevent interaction between the NOD domains of multiple inflammasome proteins. Upon activation, this repression is lost which allows for oligomerization of NLRs, resulting in the formation of large multimeric complexes. This oligomerization allows for the association of scaffold and adaptor proteins, which recruit caspase-1 to the complex. Caspase-1 itself exists in an inactive form requiring autoproteolytic cleavage for activation. It is believed that upon association with a formed inflammasome, caspase-1 loses the repression of its catalytic domain which allows for self cleavage into its active components. These components, the p10 and p20 subunits, form a heterotetramer containing 2 p10 and 2 p20 subunits which constitutes the active interleukin converting enzyme, or ICE\textsuperscript{144}. ICE then associates with immature IL-1β, to mediate its cleavage and biological activity (Figure 1.3). Recently, researchers were able to visualize a formed inflammasome using electron microscopy. This revealed that the proteins oligomerize to eventually form a large circular complex that serves as an assembly line for quick activation of large amounts of caspase-1\textsuperscript{145}. It still remains to be determined the exact molecular steps involved in this oligomerization and the precise stoichiometry of the complex.
In addition to their role in IL-1 processing, it is now known that certain NLR inflammasome proteins can induce cell death by pyroptosis\textsuperscript{146}. This form of cell death shares characteristics of both apoptosis and necrosis. Apoptosis is a cell death process characterized by nuclear DNA fragmentation and the blebbing of membrane from the cell to form apoptotic vesicles. Because the cellular contents of an apoptotic cell remain largely contained with the apoptotic vesicles, surrounding phagocytes are able to degrade these vesicles without inducing high levels of inflammation. Necrosis, on the other hand, is the complete lysis of a cell. Since this lysis occurs rapidly and does not involve the formation of vesicles, cellular contents are released directly into the extracellular environment where they can induce inflammation. Pyroptosis shares the release of inflammatory components with necrosis while requiring the activation of cellular pathways similar to those seen in apoptosis. Pyroptosis can be thought of as directed necrosis, a state that occurs only after the presence of microbial products is detected in the cytoplasm of a cell and caspase-1 activation occurs.
Figure 1.3. Two steps required for inflammasome activation.
Illustration of the two step process in IL-1β cleavage. First, engagement of a pattern recognition receptor by microbial or host ligand induces upregulation of key inflammasome genes. Once expressed, proteins exist in the cytosol. Upon introduction of inflammasome activating ligand, autorepressed NLRP3 linearizes and inflammasome complex formation begins with recruitment of ASC and Caspase-1. Cleavage of Caspase-1 releases the P10 and P20 subunits with form a heterotetramer called Interleukin-1 Converting Enzyme (ICE) which functions to cleave IL-1β into its mature and biologically active form.
1.10 NLRP3: A central mediator of inflammation

Due to its discovery as the first NLR involved in caspase-1 cleavage, NLRP3 has been the most studied of all inflammasomes. Originally identified in autoinflammatory conditions, it has since been shown to be activated by a range of stimuli. These stimuli include microbial components as well as host derived molecules, since termed danger-associated molecular patterns or DAMPs. Many pathogenic microbes has been shown to be capable of activating NLRP3. Viruses including influenza A, rabies, herpes simplex virus 1 (HSV-1), and respiratory syncytial virus (RSV) have been shown to activate NLRP3. The bacteria Staphylococcus, Streptococcus, enterohaemorrhagic Escherichia, Mycobacteria, Listeria, Pneumococcus, and Chlamydia have all been identified as activators of NLRP3. One of the first identified endogenous activators of NLRP3 was extracellular ATP. This molecule is normally restricted to the cytoplasm but if released from damaged cells or microbes, it can bind to a surface receptor P2X7 which causes a massive potassium efflux from the cell. This potassium efflux also results in an alteration in intracellular calcium levels, a key signaling component, and this potassium efflux has been shown to be critical for this process by the observation that addition of high concentrations of potassium to the extracellular media abrogated ATP/P2X7R induced IL-1β cleavage. Uric acid crystals, the causative agent of gout and gouty arthritis, also activate NLRP3. Fibrillar β-amyloid and more recently oligomeric β-amyloid activate this protein, indicating a role for NLRP3 in the etiology of Alzheimer’s Disease as well as other
neurodegenerative disorders\textsuperscript{174,175}. Silica and asbestos are two examples of exogenous crystalline activators of NLRP3 and this activation may contribute to the etiology of disorders such as mesothelioma which are associated with asbestos exposure\textsuperscript{176-178}. Given the wide range of activating stimuli, NLRP3 does not appear to function as a traditional receptor identifying a specific ligand. Rather, it appears that NLRP3 functions as a central nexus for molecular events activated downstream of tissue damage or dysfunction. A number of the crystalline activators as well as some microbial activators require release of cathepsin B from phagolysosomes for activation\textsuperscript{176,179,180}. This has led to the hypothesis that one pathway leading to NLRP3 activation is phagosomal rupture due to swelling of the phagosomes when it is unable to degrade its contents. This could be used as a means to sense microbes, such as \textit{Candida}, that are known to be able to persist with phagosomes following acidification. Limited studies have also identified mitochondrial dysfunction, through production of ROS\textsuperscript{181-184}, as a possible mechanism leading to NLRP3 activation. A third mechanism that may result in NLRP3 activation is oligomerization with other NLRs. It is possible that some activators act upon other NLRs but that the recruitment of NLRP3 to that complex is required for proper functioning. That is to say, the wide range of stimuli found to involve NLRP3 actually activate different NLRs and NLRP3 is used as more of an effector NLR through recruitment to the complex and heightened caspase-1 activation.
1.11 NLRC4 sensing the presence of cytosolic bacterial proteins

In contrast to NLRP3, NLRC4 has been identified to recognize a more restricted set of molecules. Initially, it was discovered that flagellin was the ligand for NLRC4 activation\textsuperscript{185-187}. A role for this protein has been discovered in response to *Salmonella*\textsuperscript{186,188}, *Shigella*\textsuperscript{189}, *Legionella*\textsuperscript{185}, *Listeria*\textsuperscript{190}, and *Pseudomonas*\textsuperscript{191,192} bacteria. One of these studies was able to map the residues of flagellin responsible and transcription of flagellin in a host cell was shown to be sufficient for induction of NLRC4, indicating coinciding bacterial infection was not necessary. The discovery that flagellin activated NLRC4 was confounding as there did not appear to be a mechanism by which bacteria could translocate flagellin into a host cell. Flagellin is released from the basal body of the flagellum and assembles into the polar flagellum responsible for motility. Flagellin released into the extracellular matrix is known to activate TLR5 but is likely degraded by lysosomal actions upon internalization making it difficult to envision a way in which extracellular flagellin crosses the plasma membrane into the cytoplasm. It was then discovered that the Type III secretion system, a multi-protein complex used to directly puncture host membranes and translocate cytotoxic proteins into the host cytosol also translocates flagellin due to structural homology with numerous components of the Type III secretion system\textsuperscript{193-195}. This discovery identified the pathway by which flagellin is able to enter the cell cytoplasm and also led to identification of members of the Type III secretion system that activate NLRC4. The fact that flagellin activation of NLRC4 was mapped to a small region of the protein, it seemed plausible that NLRC4 functioned as a more
traditional receptor than NLRP3. That is until it was discovered that a different cytoplasmic protein, NAIP5, was responsible for conferring the flagellin-specific response of NLRC4. Generation of NAIP5 knockout mice eliminated flagellin induced NLRC4 activation and these cells failed to respond to *Legionella pneumophila* infection\(^ {196,197} \). The discovery of the importance of NAIP5 in NLRC4 activation demonstrates the functional capacity for NLRs to form heteromeric complexes and that these molecules may not act as traditional receptors. Further research will be needed to determine if the ability for NLRs, such as NLRP3, to respond to stimuli is dependent on the presence of other NLR family members.

1.12 Antimicrobial peptides: Killers of pathogens or more?

While much of our understanding of the role of innate immunity in combating microbial infections is attributed to cellular activation through PRRs and subsequent production of cytokines to mediate leukocyte infiltration and activation. Another mechanism utilized by the immune system to prevent and control infections is the release of antimicrobial peptides into the luminal space of mucosal tissues. AMPs were originally described for their capacity to directly cause microbial death. There are four major classes of AMPS: 1) Small, anionic peptides that require the cofactor zinc, 2) small α-helical cationic peptides (<40 amino acids) lacking cysteines, 3) cationic, linear peptides rich in proline or tryptophan but lacking in cysteines, and 4) defensins, which are β-sheeted
peptides containing disulfide bonds which are critical for function. The size of antimicrobial peptides can range from 6 amino acids to ~60 amino acids. One of the characteristics shared by these four types of defensins is the presence of a region enriched with charged amino acids. It is these charged residues that are believed to be critical for the antimicrobial functioning of these proteins. Anionic peptide sequences tend to be rich in aspartic and glutamic acid residues whereas cationic sequences are enriched for arginine and lysine. These peptides are often released into saliva or the luminal space of the oral and gastrointestinal tract where they function to limit microbial growth and promote normal population homeostasis. A subset of the β-sheeted cysteine containing AMPs belongs to a group of peptides called β-defensins. Research on these molecules has revealed both antimicrobial and chemotactic properties. In this manner, β-defensins constitute a key early immune modulator responsible not only for the control of pathogenic growth but also perhaps in augmenting the infiltration of leukocytes critical for proper immune activation.

The majority of research on β-defensins has focused on these peptides in antibacterial responses. Recently, focus has begun to shift on the role of these peptides in response to other mucosal pathogens, including *Candida*. Patients suffering from oral candidiasis often have reduced salivary levels of antimicrobial peptides and that this reduction promotes both fungal adhesion and survival. It was recently discovered that patients suffering from Hyper-IgE syndrome, a genetic defect in the STAT3 signaling pathway downstream of the IL-17R, had decreased AMP production including hBD2. Patients with this mutation were...
one of the first characterized groups with a genetic predisposition to mucosal candidiasis. Though initial research was focused on the roles of these peptides during the course of human infection, many findings have been corroborated in mice.

β-defensins from mice are similar to a number human defensins including mBD1, mBD4, and mBD14 which have been identified as orthologs of hBD1, hBD2, and hBD3 respectively. hBD1, previously described as having low antimicrobial activity, was found to be a potent antimicrobial peptide after reduction of the disulfide bonds. The murine ortholog of hBD1, mBD1, was first identified as a critical component of the antiviral response to *Haemophilus influenza*, with secretion detected from the epithelium of the lung and urinary tract. More recently recombinant mBD1, possibly lacking disulfide bond formation due to production in *E. coli*, had direct candidacidal activity via inhibition of germ tube formation and growth. This same report also found that mBD1 expression was high in tongues and kidneys, organs often harvested for the study of oral *Candida* due to the significant fungal colonization observed in these organs. Other reports have found augmented mBD3 and mBD4 expression in *Candida* infected tongues and stomachs. The increased expression of these peptides in organs commonly infected by *Candida* led to our investigation of the role of these proteins during mucosal immune responses to *Candida*.

The precise mechanism(s) by which β-defensins mediate their antimicrobial effects remains somewhat unclear. Many reports have found the activity of β-defensins is salt sensitive, likely due to the importance of cationic
residues in mediating antimicrobial activity\textsuperscript{211-213}. One of the earliest proposed mechanisms for the antimicrobial activity of β-defensins was membrane disruption. Pore formation was identified in one report as the mechanism by which mBD3 was capable of inducing \textit{Candida} death\textsuperscript{214}. Recently, it has been demonstrated that LL37, an α-defensin, reduced the adhesion and viability of \textit{Candida} by reducing membrane stability by augmenting the activity of Xog1p, a β-1,3-exogluconase\textsuperscript{215}. These reports demonstrate that two distinct AMPs both function to limit \textit{Candida} through membrane disruption. However the antifungal activity of the β-defensins hBD2 and hBD3 is not reliant on pore formation or membrane disruption\textsuperscript{212}. A subsequent report identified the high-osmolarity glycerol (HOG) MAP kinase pathway as the target for the antifungal activity of hBD2 and hBD3\textsuperscript{216}. These data indicate that despite similarities in structure among β-defensins, substantial differences exist in the precise mechanistic action of the peptides on microbes. hBD2 also requires Ssa1 for execution of its antifungal effects\textsuperscript{211}. Ssa1 is a heat shock protein that can be expressed on the surface of \textit{Candida} and this protein is involved in host cell invasion through binding of host cadherins and subsequent induction of endocytosis\textsuperscript{217}. The capacity for hBD2 to bind to a protein involved in adhesion demonstrates that AMPs can not only function to directly kill microbes but also prevent association with host tissues.

It has also been proposed that β-defensins can impact pathogenic clearance by directing leukocyte chemotaxis. Indeed, studies have found that hBD1 and hBD2 are capable of binding and activating the chemokine receptor CCR\textsubscript{6}\textsuperscript{203,218}. 
Interestingly MIP3α, a chemokine that binds to CCR6, was found to possess some antimicrobial properties and more recently direct anti-\textit{Candida} activity\textsuperscript{218,219}. Similar to these studies, CXCL9, CXCL10, and CXCL11 have also been identified as having antimicrobial activities\textsuperscript{220}. The capacity for select chemokines to have antimicrobial activity and for select defensins to bind a chemokine receptor highlights a possible evolutionary relationship between these two groups of proteins that were previously thought to function in completely independent manners.
Chapter 2

Characterization of the role of PRRs in mediating protective antifungal responses to the pathogenic fungus *Candida albicans*
SUMMARY

Candida infections represent a major threat to world health, both in developing and developed countries. A great deal of research has been conducted over the past two decades identifying receptors, and their cognate ligands, which are used by the innate immune system to sense pathogenic infection. Much of this work has focused on bacteria and viruses, leaving the role of PRRs in recognition of fungal species lacking. To enhance the understanding of innate immune functioning in fungal infections, we developed a novel murine model of oral candidiasis. Using this model, we identify TLR2 and Dectin-1 as innate receptors which function to control mucosal and disseminated Candida infections. Serum levels of IL-1β were significantly reduced in Tlr2⁻/⁻ mice and absent in Dect1⁻/⁻ and Tlr2⁻/⁻/Dect1⁻/⁻ mice. Stimulation of primary macrophages revealed single deficiency in either receptor resulted in loss of detectable IL-1β production. We next examined the role of the inflammasome protein NLRP3 on the generation of IL-1β. Macrophages deficient in NLRP3 or its adaptor ASC failed to release IL-1β in response to treatment with fixed Candida. Interestingly, IL-1β release from WT cells did not require prior priming with a PRR agonist, indicating Candida is sufficient to drive upregulation of IL-1β transcript and subsequent release. This release was found to be independent of the P2X7 receptor, indicating release of endogenous ATP is not required. Nlpr3⁻/⁻ mice exhibited increased oral and disseminated fungal levels, with Asc⁻/⁻ animals demonstrating a more severe disease phenotype. Serum from both strains showed significantly reduced IL-1β levels. In conclusion, these data demonstrate a crucial role for surface receptors
TLR2 and Dectin-1 on the initiation of innate immune signaling and a critical role for NLRP3 on the release of IL-1β from infiltrating macrophages. Activation of these receptors is required for proper control of *Candida* infections, both in mucosal organs as well as through prevention of dissemination.
**INTRODUCTION**

Recognition of microbes by receptors on the cell surface of immune cells represents one of the earliest, and most important, steps in controlling tissue infection. A set of these receptors have been termed pattern recognition receptors (PRR) for their capacity to bind conserved molecular structures on microbes. These molecules are often components of the cell wall or nucleic acids, meaning these structures are not readily mutated due to their essential requirements for normal functioning of the microbe. In spite of decades of research on the role of these receptors in mediating immunity, relatively little has been discovered as to how these receptors contribute to the recognition of a key fungal pathogen, *Candida albicans*. A number of PRRs have been implicated in response to fungal pathogens. Members of the toll-like receptor family, TLR2 and TLR4, have been shown to recognize phospholipomannan and O-linked mannans, respectively\textsuperscript{101,221}, which are both components of *Candida* cell wall. A breakthrough in fungal recognition occurred upon the discovery that Dectin-1 binds to β,1-3 glucans\textsuperscript{103,104}. These glucan residues are a key structural component of the cell wall of many fungi, including *Candida*. Dectin-1 is the most well characterized member of a group of innate receptors termed CLRs and all share the common ability to bind to saccharide residues. Given the substantial enrichment of saccharide residues in fungi, these receptors opened up a whole new avenue of research into innate immune recognition. One of the major downstream consequences of PRR activation is the transcriptional activation of key cytokine and immune effector genes which are critical for the induction of
protective immune responses. One such cytokine that has been found to be central in responses to both microbial and sterile injury is IL-1β. First discovered as the serum factor that causes pyrogenic fever, this protein has since been shown to be a potent inducer of tissue inflammation and can act both locally in tissues and systemically once released into the bloodstream\(^\text{119}\). A unique feature of IL-1β is that it is produced as an inactive pro-protein that requires cleavage in order to become biologically active. The second portion of this two step process can be mediated by a recently discovered group of proteins termed inflammasomes. These inflammasome proteins are members of the NACHT-like receptor family, a subset of PRRs, and were initially discovered because a gain of function mutation in the inflammasome protein NLRP3 was associated with a number of human autoinflammatory disorders\(^\text{222-226}\). Though the mechanistic details remain somewhat vague, it is known that inflammasomes mediate the cleavage of IL-1β by associating with caspase-1. Caspase-1 itself normally exists in an inactive form however following association with an inflammasome complex, caspase-1 becomes activated and is then capable of cleaving its substrates which includes IL-1β. The NLRP3 inflammasome has been found to be activated by viruses\(^\text{148,149,151,227}\), bacteria\(^\text{155,157,160}\), foreign particles such as asbestos and silica\(^\text{176-178}\), and by a range of host derived molecules including ATP\(^\text{164-166}\), uric acid\(^\text{172,173}\), and amyloid proteins\(^\text{174,175}\). It is believed that inflammasomes function as sensors of tissue damage/dysfunction and pathogenic infection, ensuring that the highly inflammatory and lethal, at high doses, IL-1β does not become improperly released in the absence of active infection. Given the limited research
on the role of PRRs in response to live *Candida* infection, we utilized a novel model of mucosal candidiasis developed in the lab to assess the role of various TLRs, CLRs, and NLRs in the induction of immune responses and eventual control of disease.
METHODS

Animals

Female C57BL/6 and Il1r−/− mice (ages 8–12 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME). Tlr2+/− mice were generated by Shizuo Akira (Osaka University, Osaka, Japan). Dectin1−/− mice were generated by Gordon Brown (University of Aberdeen, Aberdeen, Scotland). Nlrp3−/− and Asc−/− mice were generated by Millenium Pharmaceuticals (Cambridge, MA). Casp1−/− mice were generated by R. Flavell (Yale University, New Haven, CT). P2X7R−/− mice (C57BL/6 background) were originally provided by Pfizer Global Research and Development, Pfizer Inc. and then backcrossed into a pure C57BL/6 background for > 12 generations from a P2X7R−/− mouse strain by George Dubyak (Case Western Reserve University, Cleveland, OH). Animals were housed in filter-covered microisolator cages in ventilated racks. All animal studies have been approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and University of Massachusetts.

Fungal strains

For oral infection and cell culture stimulation, Candida albicans GHD2346 is utilized. GDH2346 is a Candida strain with a tropism for oral colonization that was originally isolated from a denture stomatitis patient at the Glasgow Dental Hospital. 15% glycerol stocks of GDH2346 are stored at -80°C. To generate master plates, a sterilized loop is used to isolate fungus from the
glycerol stock. The isolated fungus is grown in 25 mL of Sabaroud-Dextrose broth (Difco) overnight in a shaking incubator at 220 rpm and 37°C. 200 μL of the resulting suspension is pipetted, under sterile conditions in a Biosafety cabinet, onto prepared Sabaroud-Dextrose Agar plates (Difco). Solution is spread evenly using a plastic spreader. Plates are sealed with parafilm and placed into a 37°C incubator for 3 days, or until fungus has grown across the entire surface of the agar. Master plates are stored at 4°C in a coldroom and are used for up to one month after which preparation of a new master plate is performed from glycerol stocks. Preparation of new master plates from glycerol stocks, rather than fungus on previous master plates, is used to minimize genetic alterations in the fungal strain that may occur following multiple rounds of growth.

**Murine model of Oral candidiasis**

In order to study the role of innate immune processes on progression of fungal disease, the lab designed a novel murine model of oral candidiasis. Five days prior to challenge with *C. albicans*, the mice were placed on tetracycline (Amresco) containing drinking water (2.5 g/l) to reduce competing oral bacteria, allowing for conditions conducive to overgrowth. The mice were anesthetized by intra-peritoneal injection with a cocktail of Ketamine (Vedco) and Acepromazine (Boehringer Ingelheim), and the dorsal surface of the tongue was lightly scratched using a sterile #10 blade scalpel. Scratching is limited to the stratum corneum and performed in order to provide a rough substrate for initial fungal colonization. A sterile, uniform-sized cotton packing was placed in the oral cavity and saturated
with PBS to keep the mouth moist. After 3 hr, the cotton packing is removed, replaced with fresh sterile cotton packing, and saturated with a suspension of *Candida albicans* yeast in PBS. The yeast are grown for 16 hr in 50 mL of Sabaroud-Dextrose broth (Difco) in a shaking incubator at 220 rpm and 37°C. After growth, yeast are pelleted at 3000 rpm for 5 minutes. Cells are washed by resuspension in 20 mL PBS followed by centrifugation. Washing is performed twice and then yeast are resuspended in 10 mL PBS. Serial dilutions (1:10) of the yeast suspension are made and dilutions counted on a hemocytometer in order to obtain accurate CFU count. A yeast suspension of $5.0 \times 10^7$ CFU/mL is made and 100 uL placed onto the sterile cotton packing. The saturated cotton is left on the tongues for an additional 4 hr while the mice are sedated. Cotton packing is removed with forceps and mice allowed to recover overnight. The mice are maintained on tetracycline drinking water throughout the entire course of infection. After a 3–28 day infection period, the mice are euthanized by CO$_2$ asphyxiation. The dorsal surface of the tongue and the surrounding oral cavity are examined by dissecting microscope for visual presence of fungus. Each mouse is given a gross clinical score to assess the degree of oral infection the tongues. Clinical scores are as follows:

0 = no visible presence of fungus and normal light reflection off of tongue surface

1 = presence of isolated patches of fungus throughout the oral cavity with diminished light reflection

2 = confluent patches of fungus on the tongues and palates

3 = presence of a visible tissue damage on the tongue indicating severe infection.
Additionally, organs are harvested aseptically from the mouse for assessment by quantitative fungal burden. Sterilized forceps and scissors are used to excise organs. New scissors are used for each organ and soaked in 100% ethanol for 15 min following excision to re-sanitize for further use. Kidneys, Tongue, Esophagus, Stomach, Duodenum, Jejunum, and Ileum are harvested for CFU fungal loads. The stomach is bisected to expose lumen and any food particles are removed and fecal matter is removed from intestinal tissue. The organs are then weighed on an electric balance (Mettler-Toledo) and placed into sterile 2 mL nucleotide and pyrogen free tubes (Eppendorf) containing 1 mL of sterile, normal saline. Individual kidneys and stomach halves are placed into separate tubes, resulting in two tubes per organ per animal.

Each organ is then chopped using sterile scissors in a biosafety cabinet. Tissue chopping is performed to shorten homogenization time, promoting fungal survival. A sterilized metal bead (Glen Mills Inc.) is placed into each tube and tissue is homogenized using a TissueLyser bead-beater homogenizer (Retsch). Tongues and Stomachs are homogenized for 3 min at a frequency of 30 Hz. All other organs are homogenized for 2 min at 30 Hz. Increased homogenization times are used due to high levels of muscle and connective tissue in the tongues and stomach. Homogenized organs are transferred into labeled, sterile 15 mL conical tubes (Danville Scientific). For kidney and stomach, the two homogenized samples for each animal are pooled into one tube. For other organs, homogenate is mixed with 1 mL sterile, normal saline to create equal volumes of initial homogenate for all organs. Serial dilutions (1:10) of each organ, excluding
kidneys which remain undiluted, are made in 15 mL conical tubes containing 9 mL of sterile, normal saline for each organ. 3 serial dilutions are made for the Tongue, Esophagus, Duodenum, Jejunum, and Ileum while 4 serial dilutions are made for the Stomach. Dilutions are then plated on 100 mm petri dishes (Fisher Scientific). Each organ dilution is plated in triplicate. Sabaroud-Dextrose Agar solution is prepared (Difco). After autoclaving, solution is cooled to under 45°C and poured onto petri dishes containing tissue homogenates. Plates are swirled to evenly distribute tissue homogenate. Plates are left out to solidify, flipped to prevent accumulation of condensation on surface of the agar, and placed into a 37°C incubator. After 72 hours, CFU is determined for each organ by manual counting of visible, punctate colonies of fungus. Counts are recorded into an excel sheet and logCFU/organ is determined using the following equation: log(CFU value*dilution factor*2). Since 1 mL of 2 mL total organ homogenate is plated, the final counts are multiplied by a factor of 2 in order to obtain CFU for the entire organ.

**Fungal preparations**

To assess the impact of *C. albicans* on immune cell functioning *in vitro*, formalin-fixed preparations of GDH2346 were generated. GDH2346 yeast, isolated following overnight growth as described previously, were placed into RPMI1640 (Thermo Scientific) containing 10% FBS (Atlanta Biologicals) for 0, 2, 4, 6, 12, and 24 hrs. This media is permissive to hyphal formation and the timepoints utilized allow for the testing of *Candida* at its two morphological
stages, yeast (0 hr) and hyphae (24 hr) as well as intermediate germ tube stages. Cells were then centrifuged at 3000 rpm for 5 min in 50 mL conical tubes (Denville Scientific) and fixed in 10 mL of 10% formalin for 1 hr. Cell were then centrifuged a second time at 3000 rpm for 5 min, followed by washing in 20 mL of sterile PBS. Cells were again centrifuged at 3000 rpm for 5 min followed by resuspension in 5 mL of PBS. Fixed preparations were then stored at -20°C for long term storage and placed into 4°C for short term use.

**Primary macrophage cell culture**

To elucidate the role of innate immune receptors and pathways in macrophages in response to *Candida* treatment, primary peritoneal macrophages were isolated from WT, *Tlr2*−/−, *Dect1*−/−, *Nlrp3*−/−, *Asc*−/−, *P2X7R*−/−, and *Casp1*−/− mice. Experimental mice were injected with 3 mL of thioglycollate solution (Sigma-Aldrich) into their peritoneal cavity using a 3 mL syringe (Terumo) and 26 gauge needle (BD Biosciences). After 72 hours, mice were euthanized via CO₂ asphyxiation. Under a sterile BioSafety cabinet, the skin covering the peritoneal cavity was excised using sterile scissors. Once the peritoneal cavity was exposed, mice were injected with 10 mL of cold RPMI1640 (Thermo Scientific) containing 10% FBS (Atlanta Biologicals). Care is taken to ensure no organs or tissue are punctured during injection. The peritoneal cavity is agitated to suspend cells into the media and the solution is drawn back into the syringe. Care is taken to ensure the needle does not become clogged with fatty tissue floating in the cavity. Once the cell suspension is obtained, it is placed into a 50 mL conical tube (Denville
Scientific) and placed on ice. After all isolations are complete, cell suspensions are centrifuged at 1500 rpm for 10 min at 4°C. Supernatant is decanted off and cell pellets are resuspended in 2 mL of red cell lysis buffer (eBiosciences). Samples are placed on ice for 10 min. After incubation with cell lysis buffer, 20 mL of RPMI+10%FBS are added to each tube and centrifugation is performed as before. Supernatant is decanted off and cell pellets are resuspended in 5 mL of RPMI1640+10%FBS. 10 μL of cell suspensions are mixed with 10 μL of trypan blue (Sigma-Aldrich). Cell counts are obtained by hemocytometer. Suspensions are further diluted until a concentration of 2.0 x 10⁶ cells/mL is obtained. Cells are plated onto tissue culture plates (BD Falcon). 2 million cells are plated into 6 well plates and 100,000 cells are plated into 96 well plates. Plates are placed into a cell culture incubator (Thermo Scientific) at 37°C with 5% CO₂. After 4 hours, supernatants are removed to eliminate non-adherent cells. For select studies, macrophages are primed for 4 hr with 10 ng/mL LPS, to provide transcriptional upregulation of IL-1β, followed by overnight (O/N) treatment with formalin fixed Candida preps to assess the capacity for each morphological stage to induce IL-1β release. Alternatively, macrophages were stimulated O/N without prior LPS priming to determine the capacity for Candida preparations to drive both signal 1 and signal 2 of IL-1β processing. Supernatants of cells were harvested and dilutions assessed for cytokine release by ELISA (R&D Systems).

Serum collection
Serum was collected from mice infected with GDH2346 in our model of oral candidiasis to determine systemic cytokine levels and immune activation. Blood was collected by retro-orbital bleeding, a non-terminal procedure which allows for multiple blood isolations from the same animals during experimentation. Mice were put into a mild state of anesthesia by administration of CO\textsubscript{2}. A sterile, heparinized capillary tube is then inserted below the eye into the eye socket (Fisher Scientific). Physical grinding and disruption is performed until blood begins to fill the capillary tube. Blood is collected into non-heparinized tubes (Sarstedt). After coagulation occurs, blood samples are centrifuged at 5000 rpm for 5 min to separate cellular and serum contents. Serum supernatant is harvested. A minimum of 3 mouse serum samples from each strain at each time point are pooled and stored at -80°C for future use. Cytokine concentration in isolated samples are determined by DuoSet Enzyme-linked Immunosorbant Assays (R&D Systems).

**Statistical Analysis**

Statistical analysis were performed using GraphPad Prism software (GraphPad Software Inc.). Mann-Whitney U-tests were performed for quantitative fungal burdens and gross clinical score. Logrank tests were used for assessment of mortality. Student t-tests were used for analysis of in vitro macrophage supernatants.
RESULTS

Role of Toll-like receptors in early immune responses to oral *C. albicans* infection

In order to ascertain the role of Toll-like receptors in recognition of *Candida*, genetic knockout mice for *Tlr2, Tlr1, Tlr6*, and *Tlr4* were orally infected with GDH2346 and organs harvested for quantitative fungal burden. After 3 days of infection, none of the tested knockout mice exhibited significantly altered fungal loads on their tongues when compared to WT (Figure 2.1A). The gross clinical score, a measure of visual disease severity in the oral cavity, was significantly elevated by Day 3 in the *Tlr2*−/− animals (Figure 2.1C). *Tlr2*−/− mice have an average score of 1.94 indicating the presence of confluent patches of fungus on the tongue, a characteristic sign of oral candidiasis. WT and all other tested TLR knockout animals had average gross scores of between 1 and 1.5, indicating the presence of isolated patches of fungus. This demonstrates that while TLR2 may not be critical for initial prevention of infection or control of fungal CFU at Day 3, these mice are beginning to develop characteristic markers of OPC. Examination of the kidneys of WT mice revealed no detectable fungal growth at Day 3, demonstrating that immunocompetent mice do not disseminate at this early timepoint (Figure 2.1B). In contrast, *Tlr2*+/− mice possess significantly elevated fungal CFU in their kidneys at Day 3 (Figure 2.1B). A small, but statistically insignificant, increase in fungal load was found in the kidneys of *Tlr6*+/− mice with no detectable fungus in either *Tlr1*+/− or *Tlr4*+/−.
By Day 7 post-infection, \( Tlr2^{-/-} \) tongues had significantly elevated fungal loads when compared to WT (Figure 2.1D). While \( Tlr2^{-/-} \) animals demonstrated a 2 fold increase in oral fungal loads between Day 3 and Day 7, WT mice had a greater than 5 fold reduction in the number of viable CFU indicating that TLR2 is crucial for limiting fungal infection during early stages of immune activation. All other TLRs tested exhibited a similar trend of reduction as WT mice which shows they are dispensable for the control of mucosal fungal infections. In agreement with their tongue CFU, \( Tlr2^{-/-} \) mice maintained a significantly elevated gross clinical score compared to WT animals which, along with the other TLR knockout mice tested, had a gross score of ~1 (Figure 2.1F). As was observed at 3 day, minimal levels of bloodstream dissemination are observed in the WT cohort at Day 7 (Figure 2.1E). \( Tlr2^{-/-} \) kidneys had significantly elevated fungal presence with nearly a 1 log increase in the level of detectable fungus versus Day 3 values. Similar to Day 3, none of the other tested TLR deficient animals exhibited elevated kidney fungal loads.

Given the early role for TLR2 in mediating control of mucosal and disseminated candidiasis, we next tested the impact genetic deficiency of this receptor has on later stages of infection. No statistically significant differences in fungal loads were observed between the tongues of WT and \( Tlr2^{-/-} \) at Day 14 and Day 21 post infection (Figure 2.1G). In contrast to WT mice which had slightly decreased levels of fungus at Day 21 compared to Day 14, \( Tlr2^{-/-} \) exhibited elevated fungal loads between Days 14 and 21. These data suggest TLR2 deficiency may dispose mice to recurrent infection whereas immunocompetent
mice continue to control infection at later timepoints. The gross clinical score of $Tlr2^{-/-}$ mice remained elevated at Day 14 and 21, demonstrating that fungal CFU cannot be utilized as a sole determinant of infection status (Figure 2.1I). Viable fungus was still detectable in the kidneys of Tlr2-/- mice at both Day 14 and 21, with significantly elevated levels seen for Day 14 (Figure 2.1H).

**The crucial role of Dectin-1 on limiting oral fungal infection**

Although TLRs were the first discovered and most well studied innate immune receptors, other families of receptors exist to respond to classes of ligands unable to activate TLRs. One such group of receptors are the C-type lectin receptors which recognize saccharide structures present in the cell wall of fungi including *Candida*. It has been well established that Dectin-1 is activated by the presence of β-1,3 glucans, which are a key structural component in the cell wall of *Candida*.

To elucidate the role for Dectin-1 during oral infection, $Dect1^{-/-}$ mice were infected orally with GDH2346 and CFU determined at 3 and 7 days post-infection. The fungal loads on tongues of $Dect1^{-/-}$ mice were significantly elevated.
at both Day 3 and Day 7 (Figure 2.2A). At both timepoints the fungal loads were found to be elevated by ~1 log unit signifying almost 10 times the amount of fungus present on the tongues of Dect1−/− mice compared to WT. In agreement with these findings, Dectin-1 deficient animals also possessed elevated disease severities as measured by gross clinical score (Figure 2.2B). Dectin-1 is critical
for the prevention of disseminated candidiasis as knockout mice had significantly elevated fungal loads in their kidneys at both timepoints studied (Figure 2.2C). Given the substantial and sustained fungal colonization in the kidneys of Dect1−/− mice, the impact of Dectin-1 functioning on survival was ascertained. Dect1−/− mice exhibited nearly 20% mortality rate by Day 7, which is significantly elevated compared to WT mice which have no mortality over the same time period (Figure 2.2D). These finding contrast with Tlr2−/− mice which have no observable mortality at Day 7 despite increases in oral and disseminated fungal loads (data not shown).

Figure 2.2. Dectin-1 is critical for control of candidiasis and prevention of mortality. WT and Dect1−/− mice were infected orally with GDH2346 for 3 and 7 days. CFUs were determined for (A) Tongues and (C) Kidney. (B) Visual disease severity was assessed by Gross Clinical Score. (D) Kaplan-Meyer survival curve of mice following oral Candida infection. Data generated by Brian A. Hall. Statistical analysis was performed by Mann-Whitney U-test for CFU and Gross Score and Logrank test for survival (*p<0.05, **p<0.01, ***p<0.001)
The cytokine IL-1β is induced following TLR2 and Dectin-1 activation and IL-1 signaling is crucial for control of *Candida in vivo*

One of the major downstream events following PRR engagement is the increased transcription of cytokine genes involved in activating and directing inflammatory immune responses. One such cytokine that has been known to be critical for microbial infections, including fungal infections, is IL-1β. To assess the impact of TLR2 and Dectin-1 deficiency on production of IL-1β, primary peritoneal macrophages were isolated following thioglycollate injection. Macrophages were primed with LPS and then stimulated with formalin fixed preparations of GDH2346 grown to morphological stages ranging from yeast to germ-tube to hyphae. IL-1β release was readily detectable in the supernatants of WT macrophages by ELISA (Figure 2.3A). This release was reduced in both Tlr2−/− and Dect1−/− macrophages. LPS priming for 4 hours followed by 5mM ATP treatment for 30 min resulted in no significant difference in IL-1β release between WT and knockout macrophages indicating that Tlr2−/− and Dect1−/− mice are capable of releasing IL-1β in response to non-*Candida* stimuli (Figure 2.3A).

Due to the critical importance for TLR2 and Dectin-1 in the production of IL-1β from macrophages, we next assessed the impact deficiency in these two pathways has on the release of IL-1β *in vivo*. Serum was obtained via retro-orbital bleed and IL-1β levels assessed by ELISA. Tlr2−/− mice had a ~50% reduction in the level of circulating IL-1β when compared to WT mice (Figure 2.3B). IL-1β levels were undetectable in the serum Dect1−/− and Tlr2−/−/Dect1−/− mice. Although macrophages rely upon both receptors for the induction of IL-1β, Dectin-1
signaling is more critical \textit{in vivo} for IL-1β production which correlates with the exacerbated disease phenotype observed in Dect1\textsuperscript{-/-} mice compared to Tlr2\textsuperscript{-/-} mice.

To mediate its pleiotropic effects on host cells, IL-1β must bind to its cognate receptor IL-1R. Il1r1\textsuperscript{-/-} mice were infected in our model of OPC in order to assess the impact of IL-1 signaling \textit{in vivo}. Mice deficient in IL-1 signaling had significantly elevated oral fungal loads at all time points studied (Figure 2.3C). Unlike WT mice which were able to control infection and reduce the number of CFU through the course of infection, Il1r1\textsuperscript{-/-} maintained similar levels of CFU out to 21 days indicating a severe defect in fungal clearance. Accompanying this increase in levels of oral fungus, Il1r1\textsuperscript{-/-} mice had severely exacerbated signs of disease as assessed by gross clinical score (Figure 2.3D). As with the oral fungal loads, Il1r1\textsuperscript{-/-} mice maintained consistently high gross scores throughout the infection as opposed to the stark reduction observed in WT mice. Bloodstream dissemination followed the same trend as Tongue CFU and Gross score, with significantly elevated levels of fungus through 21 days of infection and minimal signs of disease amelioration (Figure 2.3E). Not surprisingly, IL-1R1 deficient hosts exhibited one of the most severe defects in mortality observed in our model, with ~40% mortality observed by Day 21 (Figure 2.3F). A limited analysis of oral fungal loads of Il1b\textsuperscript{-/-} mice at Day 7 reveals a significant increase in oral colonization compared to WT mice, strengthening our findings of the role for this cytokine in anti-\textit{Candida} immune defenses (Figure 2.3G)
Figure 2.3. IL-1 signaling is critical for prevention of candidiasis. (A) WT, Thr2-/-, and Dect1-/- macrophages were treated with 10 ng/mL LPS for 4 hr followed by overnight stimulation with formalin-fixed C. albicans morphological stages. (B) Circulating serum levels of IL-1β were determined by ELISA at 3 and 7 days WT, Thr2-/-, Dect1-/-, and Thr2/Dect1-/- mice. CFU loads of (C) Tongues and (D) Kidneys were determined for Il1r1-/- animals. (E) Visual disease severity of WT and Il1r1-/- mice. (F) Kaplan-Meier survival curve of Il1r1-/- following oral infection. (G) 7 day CFU loads on tongues of IL-1β-/- animals. Il1r1-/- data generated by Brian A Hall. Statistical analysis was performed by student t-test for macrophage ELISA data, Mann Whitney U-test for CFU/Gross Score, and logrank mean test for survival. (*p<0.05, **p<0.01, ***p<0.001)
NLRP3 plays a central role in *in vitro* and *in vivo* IL-1β release

Production of IL-1β is a two step process, requiring both transcriptional upregulation of the gene and post-translational proteolytic cleavage of the protein in order for it to become released from cells. Having established an important role for two innate immune receptors in the transcriptional upregulation of IL-1β, we next sought to determine which inflammasome(s) are mediating IL-1β release following *Candida* infection.

Due to its known role in response to a range of pathogenic and non-pathogenic stimuli, we initially investigated the role of NLRP3 and its adaptor protein ASC in *Candida*-induced IL-1β production. Expressed at low levels in many resting cells, NLRP3 must become transcriptionally upregulated before it is capable of mediating IL-1β cleavage. In order to study the capacity for *Candida* to induce inflammasome activation, we provided Signal 1 by treating primary peritoneal macrophages from WT, *Nlrp3*<sup>−/−</sup>, and *Asc*<sup>−/−</sup> mice with LPS for 4 hours. Subsequent treatment of these macrophages with fixed *Candida* revealed, as before, WT macrophages were capable of releasing substantial quantities of IL-1β in response to *Candida* and that this response peaked during germ-tube morphologies (Figure 2.4A). This response was completely dependent on NLRP3 and ASC, with minimal IL-1β released into the supernatants for all morphological stages tested. In order to ascertain whether *Candida* is sufficient to activate inflammasome gene expression (signal 1) and induce inflammasome functioning (signal 2), macrophage stimulations were repeated in the absence of LPS priming. IL-1β release was readily seen in supernatants from WT macrophages and nearly
abrogated in the supernatants of Nlrp3<sup>+/−</sup> and Asc3<sup>+/−</sup> macrophages (Figure 2.4B).

The inability for these knockout macrophages to release IL-1β in response to LPS priming followed by ATP, known to function through NLRP3/ASC, confirms these cells are deficient in these proteins (Figure 2.4A).

Having established an indispensible role for NLRP3 in Candida-induced IL-1β release from macrophages, we wanted to interrogate the role of other proteins implicated in IL-1β release. ATP released from necrotic cells has been implicated as a critical host-derived stimulus for NLRP3 activation. ATP activation of the NLRP3 inflammasome occurs via binding to a purinergic receptor, P2X7, which functions as a gated ion channel resulting in a net efflux of potassium from the cell. This potassium efflux can be demonstrated as necessary since addition of extracellular potassium abrogates ATP induced IL-1β processing<sup>167-169</sup>. P2x7<sup>−/−</sup> macrophages were found to have no observable defect in IL-1β production following stimulation with LPS and fixed morphological stages of Candida or fixed Candida alone, demonstrating release of endogenous ATP from cells is not required for Candida to activate the NLRP3 inflammasome (Figure 2.4C,D).

Having determined that ATP is not required for Candida induced IL-1β release, components of Candida needed to be tested for the capacity to activate the NLRP3 inflammasome. In order to assess the role of cell wall components on NLRP3 dependent IL-1β production, a Candida albicans water soluble (CAWS) extract was produced. Pretreatment of macrophages with the TLR2 ligand Pam3CSK4 followed by fixed Candida overnight resulted in IL-1β release in an
NLRP3 and ASC dependent fashion (Figure 2.4E). CAWS treatment alone was unable to induce the production of IL-1β from macrophages. Release of KC, a chemotactic cytokine, was similar between WT and NLR deficient cells indicating normal functioning following CAWS treatment (Figure 2.4F)

**The NLRP3 inflammasome is required for control of mucosal and disseminated Candidiasis**

Due to the critical role for IL-1 signaling in murine OPC and the indispensable role of NLRP3 in IL-1β release from macrophages, it was hypothesized that NLRP3 deficiency would result in susceptibility to oral and disseminated Candida infection. Oral infection with GDH2346 resulted in a statistically insignificant increase in the fungal loads on the tongues of Nlrp3−/− at 3 days after infection (Figure 2.5A). The oral fungal loads of Nlrp3−/− mice were significantly elevated at Day 7 and returned to WT levels by Days 14 and 21. NLRP3 deficient mice exhibited slightly increased disease severity by gross clinical score assessment at Days 3-14, with a return to WT levels at Day 21 (Figure 2.5B). In contrast with their oral fungal loads, Nlrp3−/− animals had significant increases in disseminated Candida levels at 3, 7, and 14 days after infection (Figure 2.5C). Bloodborne Candida levels began to drop in these mice at Day 21, though not to the levels seen in WT mice. The critical roles for NLRP3 and ASC on the release of IL-1β in vitro led us to investigate the impact these
proteins have on IL-1β release into circulating serum during mucosal *Candida* infection. IL-1β levels were substantially reduced in both NLRP3 and ASC deficient hosts at 7 days post infection (Figure 2.5D). NLRP3 deficient hosts have a mildly elevated mortality rate as compared to WT mice demonstrating that
sustained dissemination to kidneys does not necessarily lead to death (Figure 2.5E). Since *Candida* is able to cause mucosal infections throughout the gastrointestinal tract, quantitative fungal burdens were determined for other important mucosal organs. Fungal loads in the esophagi of *Nlrp3*<sup>−/−</sup> mice mimicked the trend seen in tongues, with fungal loads peaking at Day 7 followed by a return to WT levels of infection by Day 14 (Figure 2.5F). The stomachs and portions of the small intestines had increased fungal loads in *Nlrp3*<sup>−/−</sup> mice at Day 7, with sustained increases observed at Day 14 (Figure 2.5G-J). Interestingly, the gastrointestinal tissue WT mice exhibit a trend of decreased fungal colonization from Day 3 to Day 14, followed by a substantial increased at Day 21. *Nlrp3*<sup>−/−</sup> mice exhibit a similar pattern that begins at Day 7 demonstrating these mice may have temporal defects in immune activation that result in their disease phenotype.

In order to activate caspase-1, NLRP3 requires the molecular scaffolding protein ASC to recruit caspase-1 to an active inflammasome complex. ASC also functions as adaptor for other NLRs containing a pyrin effector domain, so we wanted to investigate the role this protein played in OPC. Given its potential to be invoked in multiple pathways following *Candida* stimulation, it was hypothesized that ASC deficiency would result in a more exacerbated disease phenotype than NLRP3 deficiency. Oral fungal loads revealed that *Asc*<sup>−/−</sup> mice have significantly
elevated infection at all timepoints studied (Figure 2.6A). These deficient animals have concurrent increases in visual disease severity at all times, with statistical
significance at Days 7 and 14 (Figure 2.6B). In line with the exacerbated oral phenotype, Asc−/− mice have significantly increased levels of dissemination out through 21 days of infection (Figure 2.6C). A modest decrease in survival was observed in ASC deficient mice (Figure 2.6D). The esophagi of infected mice exhibit heightened levels of fungal infection by Days 14 and 21 (Figure 2.6E). Enhanced fungal colonization of knockout stomachs was observed at Days 7 and 14 (Figure 2.6F). The small intestines of Asc−/− mice begin to develop increased fungal infection at Day 7 which peaks at Day 21 (Figure 2.6G-I). These intestinal tissues lack the pattern of diminishing fungal levels over time observed in Nlpr3−/−, identifying a possible NLRP3-independent role for ASC in the small intestines.

The action of the protease caspase-1 is required for IL-1β production and protection from oral candidiasis

Caspase-1 is the canonical inflammatory caspase involved in the proteolytic cleavage of immature IL-1β into its biologically active form. Treatment of Casp1−/− macrophages with fixed Candida resulted in no appreciable increases in IL-1β release over media treated cells alone identifying this caspase as the mediator of IL-1β cleavage following Candida stimulation (Figure 2.7A). Casp1−/− mice have a pronounced susceptibility to oral fungal infection, with CFUs peaking at Day 3 and returning to WT levels by Day 21, sharing this characteristic
Figure 2.6. ASC deficiency results in severe susceptibility to *Candida* infection.
WT and *Asc−/−* mice were infected orally with GDH2346 for 3-21 days and infected organs harvest. Quantitative fungal burdens for (A) Tongues, (C) Kidneys, (E) Esophagi, (F) Stomachs, (G) Duodenum, (H) Jejunum, and (I) Ileum were determined. (B) Gross Clinical Scores of disease severity. (D) Kaplan-Meier survival curve of WT and *Asc−/−* mice following oral infection. Select WT and *Asc−/−* CFU data generated by Brian A. Hall. Statistical analysis was performed by Mann Whitney U-test for CFU and Logrank test for survival (*p*<0.05, **p**<0.01, ***p***<0.001)

return to WT oral infection levels with NLRP3 deficient hosts (Figure 2.7B). Interestingly, despite significantly elevated oral fungal loads at Day 3 *Casp1+* mice have similar visual disease severity at this time point (Figure 2.7C). Disease severity increases in these mice at Day 7, then drops off while still maintaining an elevation over WT gross scores. A sustained susceptibility to disseminated candidiasis is also seen in caspase-1 deficient mice through the entire course of
infection (Figure 2.7D). Lack of a functional caspase-1 protein raises the susceptibility of mice to *Candida*-induced mortality, especially prior to 7 days of infection (Figure 2.7E). This early and robust mortality demonstrates the crucial role for early IL-1β production on the prevention of further tissue infection and damage.

**Figure 2.7. Caspase-1 is crucial for *in vitro* and *in vivo* control of *Candida* infection.**

(A) 2x10⁶ WT and Casp1⁻/⁻ peritoneal macrophages were stimulated O/N with fixed preparation of *C. albicans* GDH2346. WT and Casp1⁻/⁻ mice were infected orally with *C. albicans* GDH2346. (B) Tongues and (D) Kidneys were harvested after 3-21 days of infection. (C) Visual disease severity was analyzed by Gross Clinical Score. (E) Kaplan-Meyer survival curve of orally infection WT and Casp1⁻/⁻ mice. In vivo data generated by Brian A Hall. Statistical analysis was performed by student t-test for macrophages, Mann Whitney U-test for CFU, and Logrank test for survival (*p<0.05, **p<0.01, ***p<0.001)*
Fungal pathogens represent a significant emerging threat to global health, especially regions of economic strife. Unlike the majority of their pathogenic counterparts, fungi are composed of nucleated cells. This nucleus allows them to actively respond to environmental cues and stresses, including host immune mechanisms, and alter protein expression patterns to limit those stressors. This means that fungi are refractory, partially or fully, to a number key immune components involved in combating microbes. Fungi are also more evolutionarily related to humans than bacteria or viruses, meaning many critical metabolic pathways and intracellular signaling complexes share homology. Treatments that disrupt key features required for fungal viability can often have substantial host side effects due to blockade of these pathways in the host. These facts, added with increasing numbers of drug resistant fungal isolates, lend themselves to the notion that antifungal treatments need to shift from targeting the fungus to augmenting normal host immunity. By targeting augmentation of the host response, rather than the pathogen itself, identified treatments will not only clear fungal infection but also limit evolutionary resistance to these treatments. One of the key elements to innate immune responses is they target, or are activated by, conserved processes in pathogens that are not widely altered by evolution.

In order to develop effective immunology-based therapeutics for fungal infections, it is necessary to understand the precise mechanisms involved in fungal recognition. Despite the discovery of pattern recognition receptors over a decade ago and emergence of *Candida* as a key detriment to global health, limited
research has focused on how *Candida* is recognized by host innate immune cells. It was hypothesized that TLR2 and Dectin-1 would be key mediators of fungal immunity in our model of oral candidiasis given their known role in recognizing fungal cell wall components.

In order to delineate the role of Toll-like receptors during oral candidiasis, TLR genetic knockout mice were infected with GDH2346 and assessed for their capacity to combat infection. None of the TLR-deficient animals possessed increased oral fungal colonization early after infection. *Tlr2*−/− mice were the only animals with a demonstrable phenotype following *Candida* infection. Though oral fungal loads were similar to WT at Day 3, by Day 7 a significant increase in fungal colonization was observed. This corresponded with increased visual disease severity in these mice. Despite returning to WT levels of infection at Day 14, an increase in oral fungal loads was again visible at Day 21 demonstrating these mice may be susceptible to recurrent infection.

Disseminated fungal loads of *Tlr2*−/− mice were elevated at all time points tested. Interestingly, *Tlr2*−/− kidneys had elevated levels of fungal dissemination at Day 3 indicating that this receptor is crucial for preventing early tissue dissemination even if it is somewhat dispensable for control of oral infection at the same time point. Increased dissemination was observed throughout the experiment clearly demonstrating that control of oral infection does not directly correlate with protection from disseminated infection.
Another family of innate immune receptors were identified for their capacity to respond to saccharide residues present on microbes. Members of this family, the C-type lectin receptors, were a natural target for study with respect to *Candida* given the saccharide rich cell wall that has been identified as potent immune activator. Dectin-1 was the first characterized CLR and was shown to bind β-1,3 glucans. Given the β-glucan rich nature of the *Candida* cell wall, the role of this receptor in *in vivo* immune responses was tested. *Dect1*−/− mice had augmented levels of oral infection at Day 3 and 7 post infection, which corresponded with increased visual disease severity as well. These animals also had substantial disseminated infection, with nearly 2 log units of *Candida* at Day 7. A 20% reduction in survival was observed in the absence of Dectin-1 with no such defect occurring after TLR2 knockout, identifying Dectin-1 signaling as critical for the prevention of *Candida*-induced death.

One of the major downstream targets of innate immune receptor activation is the upregulation of key immune proteins called cytokines. One of the key cytokines produced in response to foreign infectious pathogens is Interleukin-1 beta, or IL-1β. To test the contribution of TLR2 and Dectin-1 signaling on IL-1β generation, macrophages were treated with LPS and then stimulated with fixed preparations of *Candida*. IL-1β was readily detectable in the supernatant of WT macrophages with substantially reduced levels in both *Tlr2*+/− and *Dect1*+/− mice. Dectin-1 deficiency resulted in greater reduction in IL-1β release confirming the more crucial role for this protein identified in early *in vivo* experiments. Stimulation of macrophages with LPS followed by ATP resulted in similar IL-1β
levels for all 3 strains, demonstrating $Tlr2^+$ and $Dect1^+$ mice are fully capable of releasing IL-1β in response to a non-*$Candida$ stimulus. Isolation of circulating serum from WT, $Tlr2^+$, and $Dect1^+$ revealed the *in vitro* defects in IL-1β correlate with diminished secretion levels in mice. The loss of TLR2 resulted in ~50% reduction in serum IL-1β levels while loss of Dectin-1 led to undetectable levels of circulating IL-1β. These findings, along with the exacerbated disease phenotype observed during OPC infection, identify Dectin-1 as the most critical PRR involved in responses to *Candida* infection.

Given the link between PRR expression, IL-1β release, and control of oral candidiasis it was necessary to determine the precise role of IL-1 signaling *in vivo*. In order to mediate its cellular effects, IL-1β must bind to the IL-1R on the surface of target cells. Genetic deficiency of the IL-1R1 subunit of IL-1R resulted in severe susceptibility to *Candida* infection. Oral fungal loads were elevated at all timepoints studied. Unlike WT mice which begin to clear fungal infections, $Il1r1^-$ mice had sustained levels of oral colonization indicating a drastic defect in fungal clearance and killing. These mice also had consistently elevated kidney dissemination levels that correlate with a marked increased mortality rate of ~40%. This mortality rate is the highest observed for any animal strain tested, clearly elucidating the indispensible role for IL-1 signaling in controlling infection and preventing death. The IL-1R is responsible for signaling downstream of both IL-1α and IL-1β, so to eliminate the contribution of IL-1α signaling in the disease mechanism $Il1b^-$ were infected with *Candida*. Significantly elevated fungal loads on the tongues of $Il1b^-$ confirm the role of
this protein during the generation of protective mucosal immune responses following *Candida* infection.

As stated previously, IL-1β release requires not only transcriptional upregulation but also a proteolytic cleavage in order to render a mature protein. This processing is performed by inflammatory caspases, canonically caspase-1, which themselves require an activation step in order to cleave their substrate. The activation of inflammatory caspases requires the formation of a large multiprotein complex termed the inflammasome. These inflammasomes are composed of NLR proteins, adaptor/scaffolding proteins, and inflammatory caspases. Originally discovered due to associations with autoinflammatory disorders, members of the inflammasome complexes have now been implicated in response to a wide range of microbial and non-microbial stimuli. The central role for IL-1β in host defenses against *Candida* led us to investigate which NLR(s) was responsible for mediating IL-1β release during the course of infection. The first inflammasome protein to be identified was NLRP3 and it was subsequently found to become activated by both bacterial and viral pathogens making it an excellent target gene to study in our model. To establish that *Candida*-induced IL-1β was NLRP3 dependent, peritoneal macrophages were primed with LPS followed by overnight stimulation with formalin fixed *Candida* preparations grown to various morphological stages. IL-1β release was readily evident in the supernatants of WT cells but nearly abrogated in *Nlrp3*−/− cells. ASC, the adaptor required by NLRP3 to recruit caspase-1, was also required for *Candida* induced IL-1β from macrophages. Germ-tube stage *Candida*, intermediate growth stage between yeast
and hyphae, were found to be the most immunostimulatory morphological stage which is consistent with prior reports demonstrating higher levels of β-glucan exposure in these stages. It is known that LPS is a potent inflammasome priming signal used in most studies however we wanted to determine if Candida itself is sufficient to drive both signal 1 and signal 2. To test this, peritoneal macrophages were again stimulated with fixed Candida overnight, in the absence of LPS priming, and the same robust, NLRP3-dependent IL-1β release was observed. These data indicate that not only does IL-1β release following Candida not require a viable organism, the fungus itself is sufficient for driving both priming and inflammasome activation. This was one of the first reports of a dead, whole pathogen providing both signals necessary for IL-1β production. ATP has been identified as a potent danger signal capable of driving NLRP3 activation via P2X7R signaling. Since Candida has been shown to be capable of inducing cell death and necrotic cells are considered a major source of extracellular ATP, the role for ATP in Candida induced IL-1β was investigated. Stimulation of P2X7R deficient macrophages revealed no discernable defect in IL-1β release in response to Candida preparations, however IL-1β release following ATP stimulation was completely abrogated. These findings demonstrate that ATP release is not a required step in Candida mediate NLRP3 activation.

Having eliminated ATP as a possible driver of IL-1β release, it was next investigated whether a component of the cell wall of Candida was capable of inducing inflammasome activation. A water soluble cell wall extract (CAWS) was found to be capable of inducing IL-1β release, but only after prior priming. This
IL-1β release was significantly dependent on the presence of NLRP3 and ASC. These data together identify a component of the cell wall of Candida as capable of inducing IL-1β release in an NLRP3 dependent, ATP independent manner.

The stark in vitro phenotype observed in Nlrp3<sup>−/−</sup> and Asc<sup>−/−</sup> macrophages prompted investigation into the role of these proteins during oral candidiasis. It was posited that NLRP3 deficiency would result in severe oral disease and a loss of detectable IL-1β in the serum. Circulating serum isolated from mice after 7 days of infection revealed a substantial reduction in circulating IL-1β levels in both NLRP3 and ASC deficient hosts. To my surprise, IL-1β was still detectable in the serum of both of these strains of mice demonstrating NLRP3 is not sufficient for in vivo IL-1β release, which contrasts what was observed in macrophages. Oral infection of Nlrp3<sup>−/−</sup> mice resulted in increased fungal colonization on the tongue by Day 3 that peaked at Day 7 and returned to WT levels by Day 14. Similar results were seen for other mucosal tissues including the esophagus, stomach, and small intestine. Disseminated Candida infection was augmented at all timepoints hinting at a more crucial role for NLRP3 in the control of disseminated candidiasis versus oral infection. Oral colonization was significantly elevated at all timepoints for Asc<sup>−/−</sup> hosts, with concurrent increases in disseminated fungal levels observed. Given that ASC is used as an adaptor for other Pyrin-domain containing inflammasomes, it is not surprising these mice have greater disease severity. The esophagi and stomachs of these animals exhibited similar patterns of sustained increases in fungal loads, particularly at later timepoints. Examination of the small intestines revealed that mice began to
exhibit increased infection levels after 7 days. Interestingly, the levels of detectable *Candida* in the small intestinal tissue began to increase in *Asc*<sup>−/−</sup> mice after Day 7 of infection which contrast with the diminishing levels observed in the absence of NLRP3 indicating a possible NLRP3-independent role for ASC in the small intestines. It was also found that caspase-1 deficiency resulted in complete abrogation of *Candida* induced IL-1β from macrophages and increased susceptibility to infection. *Casp1*<sup>−/−</sup> mice exhibited significantly elevated oral and disseminated fungal loads as early as Day 3. Oral fungal loads returned to WT levels by Day 21 however an increase in kidney fungal loads was still evident, mimicking what was seen in *Nlrp3*<sup>−/−</sup> mice. These data identify the NLRP3/ASC/Caspase-1 axis as critical for control of both oral and disseminated candidiasis. While both NLRP3 and ASC deficient hosts exhibit modest mortality in this model, loss of caspase-1 resulted in substantial mortality following *Candida* infection. This, along with the presence of IL-1β in the serum of *Nlrp3*<sup>−/−</sup>, lead to the conclusion that NLRP3 is not the sole inflammasome mediating IL-1β release following *Candida* infection.

Collectively these findings point to the critical role that IL-1β plays during the initiation of mucosal host defenses to the fungal pathogen *Candida albicans*. Dectin-1, and to a lesser extent TLR2, are the key innate immune receptors which recognize *Candida* infection. Loss of these receptors caused a significant reduction of *in vitro* and *in vivo* IL-1β release and concurrent increases in susceptibility to disease. Elimination of IL-1β signaling, through genetic knockout of both the protein and the receptor, resulted in elevated infection levels and
mortality. NLRP3 was identified as a crucial cog in the inflammasome machinery necessary to produce IL-1β following *Candida* stimulation. Intact formalin fixed *Candida* was shown to drive IL-1β release in the absence of prior PRR priming. These results not only represent one of the few pathogens identified as sufficient to provide both signaling steps required for IL-1β release but are also key proof that viability is not a requirement for pathogen-induced inflammasome activity. This somewhat contradicts the idea that pathogen-mediated inflammasome activation is due to active infection and points, rather, to the central role for these proteins in sensing general cell dysfunction and damage. Further studies will need to be conducted to identify the additional NLR(s) responsible for IL-1β production *in vivo*. 
Chapter 3

NLRC4 functioning in resident mucosal cells is a critical component in combating oral *Candida* infection
The release of IL-1β during *Candida* infection is not solely dependent on the action of the NLRP3 inflammasome. Here it is clearly shown that the NLRC4 inflammasome plays a critical role during oral candidiasis. In spite of having no role in IL-1β release from macrophages, *Nlrc4*−/− animals have markedly reduced circulating IL-1β in their serum. These mice are predisposed to early and sustained increases in mucosal *Candida* colonization when compared to WT mice. Significantly elevated kidney CFU were also detected at early timepoints. Bone marrow chimeric mice revealed that this NLRC4-dependent immunity was being mediated by a component of the resident mucosal tissue in contrast to NLRP3 which was clearly shown to have a more prominent role in the hematopoietic compartment. One cause of increased *Candida* infection in the oral cavity was a severe defect in neutrophil infiltration observed in NLRC4-deficient hosts. mRNA expression analysis revealed that NLRC4, in addition to NLRP3 and ASC, expression was required for the proper transcriptional induction of a range of key innate immune components including antimicrobial peptides and cytokines like IL-17A and IL-1β with known importance in antifungal immunity. Taken together, these data clearly elucidate a role for non-hematopoietic NLRC4 on the induction of mucosal immunity to *Candida* and opens the doorway for future research into the role of this protein in response to other common mucosal pathogens.
INTRODUCTION

To date, over 10 NLR protein family members have been discovered in the human genome. NLRP3 has been the most well studied and been found to respond to an array of stimuli. This leaves open the question of what the role of these other NLRs are. It is possible that the other NLRs function to recognize a specific molecule, in a traditional receptor fashion. While this would explain the high number of these proteins, it would seem somewhat improbable that all members of the family but NLRP3 recognize specific targets. It is also plausible, and my contention, that these additional inflammasomes function is specific tissue and/or cellular manners to provide additional inflammatory activation capacity to the different organs and cells involved in the immune system. Since the majority of NLR research, and immune research in general, has focused on dissecting molecular mechanisms in infiltrating leukocytes, especially monocytes/macrophages and dendritic cells, it would be quite easy to fail to identify key roles for these proteins if their function is not as crucial in leukocytes.

One of the major advantages to the use of an in vivo screen is that it allows researchers to identify proteins which are critical to generation of immune responses but mediate their effects in cells/tissues not readily studied in vitro. Serum data (Figure 2.5D) shows that mice lacking NLRP3 still have detectable levels of IL-1β in their serum, demonstrating an additional mechanism is activated in response to Candida. The OPC model in the lab allows for the screening of NLRs which could be functioning to promote in vivo IL-1β but not macrophage
IL-1β release. Since IL-1β was still detectable in Asc−/− mice, this indicated utilization of a CARD containing inflammasome to mediate this NLRP3-independent IL-1β. A number of inflammasome proteins contain a CARD domain, including NLRP1, NLRC4, and NOD1/2. NOD1/2 are traditionally associated with driving NF-kB response elements following bacterial stimulation\textsuperscript{134,135}. NLRP1 has been implicated in responses to anthrax lethal toxin and in a subset of autoimmune conditions including vitiligo but also contains a PYD domain so this protein was not immediately investigated\textsuperscript{228-233}. NLRC4, previously known as IPAF, has been found to be a potent responder to the presence of bacterial products in the cytosol of macrophages\textsuperscript{193-195}. NLRC4 recognizes portions of flagellin and has been found to respond to certain members of the Type III secretion system, a molecular needle capable of penetrating host cell membranes and directly delivering toxic effector proteins into the host cell. It is believed that due to high structural homology between the basal body of the polar flagellum and the membrane spanning portion of the Type III secretion apparatus, flagellin can be aberrantly secreted into host cytosol during infection. Due to its association with IL-1β processing independent of ASC in response to known pathogens, we investigated the possible role for NLRC4 in response to Candida.
METHODS

Animals

C57BL/6 mice (originally derived from Jackson Laboratories Bar Harbor, ME) were bred in microisolator cages. *Nlrc4*−/−, *Nlrp3*−/− and *Asc*−/− mice were generated by Millenium Pharmaceuticals (Cambridge, MA) and bred for studies. Animals were housed in filter-covered microisolator cages in ventilated racks. All animal studies have been approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and University of Massachusetts.

Murine model of Oral candidiasis

Susceptibility to oral infection is assayed through our model of oral candidiasis. Mice are treated for 5 days with tetracycline containing water. On the day of infection, mice are anesthetized and three superficial scratches are made on the dorsal layer of the tongue. After 3 hrs of recovery, mice are inoculated with 100 μL of a 5x10^7 yeast/mL solution soaked into a cotton ball. Cotton balls are removed after 4 hrs and infection carried out for 2-28 days. Mice are sacrificed and the tongues, esophagi, stomachs, duodenums, jejuns, ilea, and kidneys are harvested. Organs are homogenized in sterile PBS using a bead-beater homogenizer (Retsch). Serial dilutions of organ homogenates are plated into SD agar and colony forming units determined after 72 hrs at 37°C. CFU counts are logged and graphed as logCFU/organ.
Fungal preparations

To assess the impact of *C. albicans* on immune cell functioning *in vitro*, formalin-fixed preparations of GDH2346 were generated. GDH2346 yeast, isolated following overnight growth, were placed into RPMI1640 (Thermo Scientific) containing 10% FBS (Atlanta Biologicals) for 0, 2, 4, 6, 12, and 24 hrs. Cells were then isolated by centrifugation and fixed with 10% formalin. Following fixation, cells were washed 2x with PBS and resuspended in PBS. Preps were stored at 4°C for immediate use and -20°C for long-term storage.

Primary macrophage cell culture

To ascertain the role of NLRC4 in macrophages, primary peritoneal macrophages were isolated after thioglycollate injection. Isolated WT and *Nlrc4−/−* macrophages were centrifuged, resuspended in RPMI1640+10%FBS, and 2x10⁶ macrophages were plated in a 6 well plate. After allowing for 4 hrs for adherence, media was replaced with media containing formalin fixed GDH2346 preparations. After overnight (O/N) stimulation, supernatants were harvested and IL-1β levels in diluted samples assayed by ELISA (R&D Systems).

Cytokine levels in circulating serum

Circulating blood was obtained by orbital bleeding of *Candida* infected WT and *Nlrc4−/−* mice. After centrifugation, serum was separated from the cellular components and pooled. Serum was stored at -20°C for future cytokine determination by ELISA.
Bone marrow chimeric mice

To test the role of non-hematopoietic NLR expression in mucosal candidiasis, bone marrow chimeric mice were generated. WT, \textit{Nlrc4}^{−/−}, \textit{Nlrp3}^{−/−}, and \textit{Asc}^{−/−} mice were exposed to a 900 rad lethal dose of gamma radiation from a Cesium-137 source. Bone marrow progenitor cells were isolated from non-irradiated mice. Femurs and tibia were removed from mice with sterilized scissors and remaining tissue removed by forceps and #10 scalpel. Ends of the bones were cut off and the end-exposed bones placed into a 500 μL centrifuge tube (Eppendorf). The top and bottom of the tube were punctured using a 18 gauge needle (BD Biosciences) prior to bones being placed in them. The centrifuge tube and bone were placed into a 1.5 mL centrifuge tube and spun at 2000xG for 5 minutes. Bone marrow cells will centrifuge through the hole in the bottom of the small tube and collect in the 1.5 mL tube. After centrifugation, bones were discarded and pelleted marrow resuspended in 150 μL of RBC lysis buffer (eBiosciences). Samples from syngeneic animals were pooled. After 10 minutes on ice, cell suspensions were placed into 15 mL conical tubes and 10 mLs of RPMI1640 (Thermo) media added. Cells were pelleted at 3000 rpm for 10 min at 4°C. Bone marrow cells were counted on a hemocytometer and a final concentration of 1.5x10^7 cells/mL was made. 3x10^6 cells were injected into each irradiated host. After allowing a minimum of 4 weeks for reconstitution, animals were infected in our OPC model and level of disease was assessed after 7 days.
Histological analysis of *Candida* infected tongues

WT, *Nlrc4*<sup>−/−</sup>, *Nlrp3*<sup>−/−</sup>, and *Asc*<sup>−/−</sup> tongues were harvested two days after *Candida* infection. Tongues were placed into freezing cartridge (TissueTek) and embedded in OCT solution (TissueTek). Samples were flash frozen using liquid nitrogen and blocks stored at -80°C until sectioning. 5 μM sections were obtained by cryo-sectioning. Serial sections were stained for histological analysis. Gross histology was visualized by Periodic Acid-Shiff’s (PAS) reagent with haematoxylin counterstain (H), or PAS/H (Sigma-Aldrich). PAS reacts to the presence of polysaccharide residues and stains purple/magenta. Haematoxylin is a blue colored basic stain used to identify nuclei. PAS/H staining allows for the visualization of tissue architecture, as well as the identification of fungal cells and debris is the outer epithelial layer. Coverslips were placed on slides using Permount. For direct identification of neutrophil infiltration, frozen sections were stained with NIMP R14 which binds to GR-1 on the cell surface. Sections were then treated with a secondary antibody conjugated to the Alexa-488 fluorophore. Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) was used to mount coverslips. DAPI is a blue fluorescent dye which binds to A-T rich regions of DNA and allows for visualization of nuclei. Coverslips were permanently sealed using clear commercial nail polish. Stained sections were visualized using a Leica DMI 6000B inverted scope with the assistance of Dr. Scott Howell, Ph.D. 40x images were taken spanning the entire tongue and individual pictures spliced into a complete 40x image of the tongue using MetaMorph software (MDS Analytical Technologies). For NIMP stained sections, quantification of neutrophils was
performed using MetaMorph software. A minimal threshold of fluorescent intensity corresponding to a neutrophil was set. The number of pixels at or above that threshold was measured and divided by the total number of pixels in the section. This analysis was performed for the epithelial layer alone and whole tongue.

**Quantitative real-time PCR**

To assess inflammatory responses in the oral mucosa, buccal tissue was excised from mice infected with GDH2346 and PBS treated controls. Following euthanasia, tissue is excised along the gum lines using sterile scissors. The resulting piece of cheek tissue is pinned to a dissection board using 25 gauge needles (BD Biosciences). A dissection microscope is used to identify the small section of raised tissue corresponding to the buccal mucosa. A sterilized #10 scalpel and forceps are used to excise the tissue sample. Excised plugs are placed into a DNA, RNA, and pyrogen free 500 μL tube containing 100 μL of sterile RNALater (Qiagen). Samples are stored at 4°C for immediate use or frozen on dry ice and stored at -80°C for future isolation. Buccal samples are transferred to a 2 mL DNAse, RNAse, nucleotide, and pyrogen free tube (Eppendorf) containing 500 μL of RNA lysis buffer (USB/Affymetrix). A sterilized metal bead is added to each sample and tubes are homogenized using TissueLyser homogenizer (Retsch) for 1 min at a frequency of 30 Hz. Samples are transferred to RNA solution and mRNA was extracted using the published protocol in the PrepEase
RNA Spin Kit (USB/Affymetrix). Concentration of isolated RNA samples was determined by Nanodrop 1000 Spectrophotometer (Thermo). Level of DNA contamination was determined by 260/230 ratio. cDNA was then generated for qualitative real-time PCR using HighThroughput cDNA synthesis kit (Roche). 1000 ng of RNA for each sample was mixed with kit reagents and PCR reaction performed as detailed in the protocol. Total volume of the reaction is 20 μL. Newly generated cDNA samples are then diluted 3 fold in sterile, RNase, DNase, nucleotide free PCR grade water. Samples are stored at 4°C for future use. Gene specific primer sequences were designed to span exons and selected on the basis of low self complementarity. Primers were purchased from Invitrogen and gene-specific primers are listed in Table 2.1. Primers were resuspended at a stock concentration of 100 nM in sterile, PCR grade water. Primers were diluted to 20 nM for use in qRT-PCR. Stock primers stored at -20°C and diluted primers were stored at 4oC. qRT-PCR was performed using SybrGreen master mix (Roche). 3 μL of diluted cDNA is mixed with 4 μL PCR grade water, 0.25 μL of each of forward and reverse gene-specific primers, and 7.5 μL of SybrGreen master mix. PCR reaction was performed on a 7300 Real Time PCR System (Applied Biosystems). Sample reactions were performed in duplicate and 3 μL of water in place of cDNA was used for controls. A total of 40 amplification cycles were performed. Dissociation curves were obtained for each sample to confirm gene specific amplification was observed. Ct values were exported. Expression levels were then determined using the $2^{-\Delta\Delta Ct}$ method. Data is expressed as fold induction of gene expression over mock (PBS) infected samples.
Table 2.1 Quantitative real-time polymerase chain reaction (qRT-PCR) primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-</td>
<td>Forward: CGCTCCGCTAGACAAAAATGTGGTT</td>
<td>Ccr5</td>
<td>Forward: AACGGAGAAGAAAGACGACAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAATTTGCCGCTGACOGTGGG</td>
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<td>Reverse: GACGGACGAGCAGAGAGAG</td>
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<tr>
<td>Nix-</td>
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<td>IL-1β</td>
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</tr>
<tr>
<td></td>
<td>Reverse: CACGGGCGACATGAAATGGAAC</td>
<td></td>
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</tr>
<tr>
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<td>IL-6</td>
<td>Forward: CGGAGGAGGAGTTCAGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACCGTCAACAGAOGGTGACAC</td>
<td></td>
<td>Reverse: TGCTGACGATCTGATGATG</td>
</tr>
<tr>
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<td>defB1</td>
<td>Forward: ACTGATTTTCTTTCCTG</td>
</tr>
<tr>
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<td></td>
<td>Reverse: ACTACGTGCAACTTCCAAG</td>
</tr>
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<td>Forward: AAGTATTGGATACGAGG</td>
</tr>
<tr>
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<td>Reverse: CCGTGAGCTTGGGAGCAGTCTAG</td>
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<td>Forward: GGAACATTGCTCCACCAAA</td>
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<td>Reverse: ACTGCAAGATCTGCTGTCGATTC</td>
<td></td>
<td>Reverse: TCTCTGTTGATCAAGGAAGT</td>
</tr>
</tbody>
</table>

Statistical Analysis

Statistical analysis were performed using GraphPad Prism software (). Mann-Whitney U-tests were performed for quantitative fungal burdens, Gross Clinical Score, and gene expression by qPCR. Student t-tests were used to analyze in vitro macrophage data.
RESULTS

The NLRC4 inflammasome is dispensable for macrophage induced IL-1β but contributes to systemic IL-1β release in *Candida* infected mice

In order to ascertain the role of the NLRC4 inflammasome in response to *Candida* infection, primary peritoneal macrophages from WT and *Nlrc4*−/− mice were elicited by thioglycollate injection. Stimulation of these cells with formalin-fixed preparations of *C. albicans* revealed no defect in IL-1β secretion in the absence of NLRC4 (Figure 3.1A). Knockout cells also responded normally to treatment with LPS followed by ATP, indicating intact NLRP3 signaling in these cells in response to stimuli. The innate immune system is also comprised of cells that do not derive from the hematopoietic compartment. To determine the possible role of non-hematopoietic cells in NLRC4-induced IL-1β, we tested the circulating serum from *Nlrc4*−/− mice. Despite no discernable role in macrophage studies, the serum from deficient mice exhibited a substantial reduction in circulating IL-1β (Figure 3.1B). Interestingly, this level of IL-1β reduction nearly matched the amount of detectable IL-1β in the serum of *Nlrp3*−/− (Figure 2.5D). These data indicated that not only is NLRC4 responsible for mediating IL-1β release in response to *in vivo* *Candida* infection but it also plausible NLRC4 is the sole inflammasome responsible for NLRP3-independent IL-1β processing in response to *Candida*. 
The NLRC4 inflammasome is a key mediator in the control of mucosal candidiasis

The *in vivo* role for NLRC4 in the accumulation of systemically active IL-1β led us to assess the impact of this protein on mucosal fungal loads in our model of OPC. The oral fungal loads of NLRC4 deficient mice became significantly elevated by 7 days post-infection, with sustained increases in infection detected out to 21 days (Figure 3.1A). Disease severity, as measured by gross inspection of the oral cavity, mimicked this trend of significant exacerbation by 7 days with sustained increases still observed after 21 days (Figure 3.2B). To more fully elucidate the mucosal role of NLRC4, additional organs from the gastrointestinal tract were studied. Increased fungal loads in the esophagi were observed as early as 3 days and consistent through 14 days with a return to WT levels by 21 days (Figure 3.2C). Stomachs of infected *Nlrc4*−/− demonstrated substantial elevation in fungal levels at Days 3-14 but returned to WT levels by
Day 21 (Figure 3.2D). The duodenum, jejunum, and ileum all exhibited increased fungal loads at Days 7 and 14 with no elevation detected at 21 days following infection (Figure 3.2E-G).

To ascertain the impact of NLRC4 activation on prevention of dissemination, kidneys were harvested. Increases in fungal dissemination were evident as early as Day 3, peaked by Day 7 and returned to WT levels by Day 21 (Figure 3.2H). A small increase in mortality was seen in \textit{Nlrc4}$^{-/-}$ mice when infected with GDH2346, however the mortality rate increased to 33% when infection was carried out with a more virulent strain, CA90234, known for its capacity to disseminated (Figure 3.2I-J).

**NLRC4 expression in resident mucosa is required for protective immunity to \textit{Candida} infection**

Given the prominent role for NLRC4 \textit{in vivo} but no identified role in leukocytes tested, we hypothesized that NLRC4 activation in resident epithelial cells was responsible for control of early infection. To assess the contribution of non-hematopoietic cells during anti-\textit{Candida} defenses, bone marrow chimeric mice were generated. Following bone marrow transplant and recovery time, mice were infected with GDH2346 orally for 7 days and fungal loads assessed. In order to confirm the bone marrow transplant did not predispose mice to greater levels of \textit{Candida} infection, WT bone marrow was injected into WT mice (WT$\rightarrow$WT) with no appreciable difference observed in fungal loads of the tongues or kidneys of
Figure 3.2. NLRC4 is a critical protein mediating protection from Candida infection and mortality. WT and Nlr4-/- mice were infected orally with GDH2346 for 3-21 days. CFUs for (A) Tongue, (C) Esophagi, (D) Stomach, (E) Duodenum, (F) Jejunum, (G) Ileum, and (H) Kidney were determined. (B) Visual disease severity was assessed by Gross Clinical Score. Mortality following oral infection with (I) C. albicans GDH2346 and (J) ATCC 90234. Statistical analysis was performed by Mann Whitney U-test for CFU and Logrank test for survival (*p<0.05, **p<0.01, ***p<0.001)
these mice when compared to historical WT fungal loads (Figure 3.3A). Gross disease severity and levels of disseminated *Candida* were also unaffected (Figure 3.3B,C). When *Nlrc4*⁻/⁻ bone marrow was transplanted into WT hosts (NLRC4→WT) oral fungal loads were nearly identical to those in WT→WT mice (Figure 3.3A). Those data are in stark contrast to WT→NLRC4 mice which had significantly elevated fungal loads as compared to WT→WT and NLRC4→WT mice (Figure 3.3A). The gross clinical scores of NLRC4→WT mice were elevated compared to WT→WT mice but not to the significant level seen in WT→NLRC4 (Figure 3.3C). Susceptibility to disseminated candidiasis was also assayed and it was found that both NLRC4→WT and WT→NLRC4 mice have heightened fungal loads in their kidneys, with a significant increase observed in WT→NLRC4 mice (Figure 3.3B). These data confirm our proposed role for NLRC4 in non-hematopoietic resident mucosal cells and provides direct evidence for tissue specific NLR functioning.

**The NLRP3/ASC axis in hematopoietic cells is indispensible for protection from oral and disseminated candidiasis**

Earlier studies identified a critical role for NLRP3 in *Candida* induced IL-1β from macrophages, so the contribution of hematopoietic NLRP3 was investigated by generation of bone marrow chimeras. In contrast with what was observed for NLRC4 chimeras, *Nlrp3*⁻/⁻ bone marrow transplant into a WT host (NLRP3→WT) resulted in significant susceptibility to oral fungal infection.
(Figure 3.3A). Not surprisingly, ASC→WT mice exhibited similar susceptibility to oral infections (Figure 3.4A). Both WT→NLRP3 and WT→ASC mice had minimally elevated oral fungal loads as compared to WT→WT, demonstrating the critical function of NLRP3 and ASC in hematopoietic cells. Interestingly, all NLRP3/ASC chimeric mice had elevated gross clinical scores despite obvious differences in oral fungal loads (Figure 3.3C). Similar to previous observations with NLRP3 and ASC deficient hosts, infectious fungal loads are not an adequate determinant of disease severity and vice-versa. Similar trends in susceptibility were observed for disseminated candidiasis. Both NLRP3→WT and ASC→WT groups had significantly elevated kidney fungal loads at Day 7 (Figure 3.3B). In contrast to oral loads, WT→NLRP3 mice had significantly increased disseminated fungal loads. WT→ASC mice had no discernable difference in disseminated fungal loads compare to WT→WT, identifying a potential mechanism for non-hematopoietic NLRP3 in preventing dissemination that is dispensable for mucosal control.

**Inflammasome activity is required for proper neutrophil infiltration into Candida infected tongues**

The NLRP3 and NLRC4 inflammasome proteins have both been identified as critical components of the mucosal immune response to *Candida* infection. In spite of this identification, a difference in the state of immune activation in these deficient mice has not yet been determined. Neutrophil
Figure 3.3 NLRC4 is required in resident mucosal cells to combat oral and systemic candidiasis. WT, Nlrc4-/-, Nlrp3-/-, and Asc-/- were given a lethal dose of gamma radiation. Mice were injected with bone marrow progenitor from donor WT, Nlrc4-/-, Nlrp3-/-, or Asc-/- mice. Mice were later infected with GDH2346 for 7 day and animals sacrificed for harvest. CFUs for (A) Tongues and (B) Kidneys were quantified. (C) Gross Clinical Score was used to assess visual disease severity. Highlighted chimeric mice in red. Statistical analysis was performed by Mann Whitney U-test (*p<0.05, **p<0.01, ***p<0.001)
infiltration into tissue is one of the earliest events in innate immune activation and a crucial step in combating infectious fungi. To assess neutrophil infiltration into the tongue of OPC-infected mice, tongues were harvested 2 days post infection and frozen in liquid nitrogen. Serial sections were then stained with Periodic Acid-Schiff’s reagent counterstained with haematoxylin (PAS/H) for gross histology or with an anti-GR1 antibody (NIMP), specific for the cell surface of neutrophils, and the nuclear stain DAPI. PASH staining of WT tongue revealed substantial thickening of the mucosal epithelium due to edema and subsequent cellular infiltration (Figure 3.4A). These areas of massive inflammation correspond to evidence of active tissue disruption as determined by erosion of the outer stratum corneum. Closer examination of the cellular infiltrate in the epithelium reveals the formation of abscess-like structures accompanied by large amounts of what appear to polymorphonuclear neutrophils (black arrows, Figure 3.4A). The presence of large numbers of neutrophils in the inflamed tissue of WT mice was confirmed by NIMP staining (Figure 3.4B). Neutrophil positive staining was observed for areas previously identified for cellular infiltration (yellow arrows, Figure 3.4B). Histological examination of Nlrc4−/− tongue showed a pronounced reduction in inflammation when compared to WT mice (Figure 3.4C). Despite the presence of obvious tissue disruption, minimal cellular infiltrate is observed into the area under the epithelium. Fluorescent staining confirms the absence of neutrophil accumulation at the epithelial surface, however some neutrophils are evident in the underlying tissue as was seen for WT
(white arrows, Figure 3.4D). Whether this neutrophil defect is temporal or due to an inability to properly traffic in tissue remains to be investigated.

Given the importance of NLRP3 for leukocyte mediated cytokine responses, we next tested whether susceptibility to oral infection in Nlrp3<sup>−/−</sup> was due to defective cellular infiltration. Gross histological examination of Nlrp3<sup>−/−</sup> mice revealed a moderate defect in cellular infiltration (Figure 3.4E). This represents an intermediate phenotype to what was seen with NLRC4 deficiency. Evident tissue disruption can be seen associated with inflammatory infiltrates. Asc<sup>−/−</sup> mice have a nearly identical histological phenotype to Nlrp3<sup>−/−</sup> (Figure 3.4G). The infiltrating cells were confirmed as neutrophils (Figure 3.4F,H). The formation of abscesses can clearly be seen in both knockout mice, which differs starkly from Nlrc4<sup>−/−</sup> which have no such abscess formations (yellow arrows, Figure 3.4F,H). As seen with other mice studied, neutrophils were also detectable in the sub-mucosal layer indicating continued extravasation and chemotactic trafficking to infected sites (white arrows, Figure 3.4F,H).

To quantify the level of neutrophil infiltration into the tongues of WT and NLR knockout mice, the percent of NIMP positive pixels were determined for both the epithelium alone and the whole tongue. 66.94% of pixels from the WT epithelium were positive for NIMP staining (Figure 3.4I). Nlrp3<sup>−/−</sup> and Asc<sup>−/−</sup> had substantially reduced levels of neutrophils infiltrating their epithelial layer, with values of ~20%. As expected from visual inspection, neutrophil infiltration into the epithelium of Nlrc4<sup>−/−</sup> hosts was almost completely abrogated. When the whole tongue was analyzed, it was found that over 35% of WT tongue pixels were
positive for NIMP (Figure 3.4J). The NLR knockout mice exhibited the same trend as for the epithelium, with a near complete loss observed in Nlrc4−/− and an intermediate phenotype for NLRP3/ASC deficient animals. The percentage of NIMP positive pixels was increased in the whole tongue versus epithelium alone in the absence of NLRC4, demonstrating that the majority of neutrophils remain below the epithelial barrier at 2 days post infection.

Inflammasome components and IL-1 family members show increased expression in buccal mucosa following infection

Having established that NLRC4, NLRP3, and ASC are all required for control of mucosal fungal infections and neutrophil infiltration into infected tongues, we next sought to determine the level of immune activation in the oral cavity of these mice 3 days post infection. To accomplish this, mRNA was isolated from buccal tissue of infected mice and cDNA generated from this RNA was assayed for gene expression using quantitative real-time polymerase chain reaction (qPCR). Expression levels of target genes following infection were then normalized to GAPDH and expression levels expressed as fold increase over mock infection. Given their central role in fungal defense, expression levels of NLRC4 and NLRP3 were the initial genes quantified. NLRC4 transcript levels were found to be elevated over 5-fold in the buccal tissue of WT mice (Figure 3.5A). This upregulation was reduced in both NLRP3 and ASC deficient hosts, with expression barely detectable in Asc−/− mice. NLRP3 expression levels were
Figure 3.4. Neutrophil infiltration into infected tongues is nearly abrogated in the absence of NLRC4. WT (A,B), Nlrc4-/- (C,D), Nlrp3-/- (E,F), and Asc-/- (G,H) tongues were harvested 2 days post inoculation. Frozen 5 μM sections were stained with (A,C,E,G) Periodic Acid-Schiff’s Reagent counterstained with haematoxylin or (B,D,F,H) NIMP R14 antibody (green) and DAPI (blue). Quantification of neutrophil infiltration in represented sections are displayed as NIMP+ pixels/total pixels for (I) Epithelial layer alone and (J) whole tongue.
highly upregulated, greater than 100-fold, in WT mice whereas this increase in expression was absent from both \textit{Nlrc4}\textsuperscript{−/−} and \textit{Asc}\textsuperscript{−/−} animals (Figure 3.5B). This loss of NLRP3 expression in NLRC4 knockout mice might be explained by the near complete abrogation of cellular infiltration observed previously. Due to the well established role for inflammasomes on the production of IL-1β, we next assessed IL-1β expression levels. As expected, substantial upregulation of the IL-1β gene was observed in WT mice following infection (Figure 3.5C). This upregulation was significantly decreased in \textit{Nlrc4}\textsuperscript{−/−} mice and completely lost in the absence of NLRP3 or ASC. This severe defect in IL-1β expression means that even if other inflammasomes are capable of maturing IL-1β following \textit{Candida} treatment such inflammasomes would likely be incapable of reconstituting normal immunity to \textit{Candida}.

Given that the biological activity of IL-1β requires binding to the IL-1R, expression levels of the IL-1R subunit IL-1R1 were determined. A mild upregulation in receptor expression was observed in WT mice (Figure 3.5D). No discernable defect was observed in the absence of NLRC4 and ASC, however loss of NLRP3 did result in a reduction in IL-1R1 expression following infection. One of the consequences of IL-1R activation, in addition to inflammatory gene expression, is the production of a natural receptor antagonist, IL-1Ra, capable of competitively inhibiting the binding of IL-1α/IL-1β to the receptor resulting in diminished intracellular signaling. This pathway is used as a negative regulator to ensure aberrant inflammation in response to IL-1 does not occur. As with IL-1R, a mild upregulation in IL-1Ra was seen in WT buccal tissue (Figure 3.5E). All
three knockout strains has reduced IL-1Ra expression, likely due to decreased IL-1β production, with Nlrp3−/− exhibiting the most severe reduction.

In addition to mediating the cleavage of IL-1β, inflammasomes also mediate the cleavage of two other cytokines IL-18 and IL-33. The role of IL-18 in pathogenic responses has been poorly characterized. Limited studies have identified a role for this protein in protection from disseminated candidiasis. We were able to find a minor increase in IL-18 gene expression in the infected buccal tissue of WT mice (Figure 3.5F). This increase was absent in the NLR knockout mice. The consequence of reduced IL-18 expression in this model of OPC remains to be assessed in the future.

**Induction of key cytokines depends on the presence of a functional inflammasome**

A major mechanism of action for IL-1β is the induction of cytokine gene expression in target cells. Since a number of these downstream cytokines play critical roles in response to pathogens including fungi, expression levels in the buccal mucosa were determined following infection. The role of IL-17 family members has been well documented in response to a range of extracellular pathogens including *Candida*, with one of the genetic conditions associated with candidiasis linked to a mutation in Stat3 which results in diminished Th17 responses. IL-17A has been shown to be critical for immune responses to
extracellular pathogens due to its capacity to activate infiltrating leukocytes. IL-17A levels were highly augmented following *Candida* infection (Figure 3.6A). This upregulation was nearly abrogated in *Nlrc4*<sup>−/−</sup>, *Nlrp3*<sup>−/−</sup>, and *Asc*<sup>−/−</sup> mice clearly showing the importance of IL-1β production on the subsequent generation of IL-17A. This profound defect in a cytokine known to be critical for control of mucosal candidiasis led us to investigate the susceptibility of *Il17a*<sup>−/−</sup> mice to our OPC
model. Oral fungal loads were slightly elevated in Il17a−/− mice at Day 3, with significant augmentation after 7 days of infection (Figure 3.6B). Fungus was undetectable in the kidneys of Il17a−/− animals at Day 3 however significantly elevated systemic infection was detected at Day 7. Slight, but not significant, increases in fungal loads were found gastrointestinal tissue (data not shown). The data directly demonstrate the crucial role for IL-17A in combating mucosal and disseminated candidiasis.

IL-17F was also found to be highly expressed in the buccal tissue of WT mice following infection (Figure 3.6D). Slight reductions in expression were observed in NLRC4 and ASC deficient hosts, with almost no increase in expression found in Nlrp3−/− mice. Thus IL-17F, like IL-1R1, represents a unique ASC-independent, NLRP3-dependent alteration in gene expression. Like other cytokines, the action of IL-17A and IL-17F require activation of a surface bound receptor. One subunit of the IL-17R, IL-17RA, was found to have increased expression in WT mice, with similar levels observed for both NLRP3 and ASC knockout mice (Figure 3.6E). In contrast, no increase in expression of IL-17RA was found in the buccal tissue of Nlrc4−/−. This is the first evidence of an NLRC4-specific defect in cytokine responses and demonstrates that even though NLRC4 deficient mice are capable of producing some IL-17A and IL-17F they likely fail to respond to the presence of these cytokines in infected tissues.

In addition to IL-17 family members, a range of additional inflammatory cytokines can be produced downstream of IL-1β signaling. One important cytokine mediating neutrophil chemotaxis is the chemokine CXCL1, or KC.
Expression of this gene was found to be increased greater than ~350 fold in infected WT tissue (Figure 3.6F). Less than 5-fold increases in expression were observed in all three knockout mice, potentially explaining the substantial neutropenia observed in these mice. IL-6, a potent and systemically active cytokine associated with induction of fever, was also found to be highly upregulated at the mRNA level in the buccal mucosa of WT mice (Figure 3.6G). Expression levels were reduced to less than 5-fold for Nlr4⁻/⁻ mice with no detectable levels found in either Nlrp3⁻/⁻ or Asc⁻/⁻ animals.

**Oral antimicrobial peptide production is dependent on the concerted actions of the NLRP3 and NLRC4 inflammasome**

Cytokine signaling is traditionally linked with increased immune cell activation including enhanced effector functioning such as cytotoxicity and ROS production. Another mechanism by which a host responds to infection is through production of antimicrobial peptides, or AMPs. AMPs can be secreted constitutively from mucosal surfaces in the absence of infection for proper maintenance of the mucosal microbiome but expression can also be induced and/or augmented following infection to promote pathogenic killing. One class of AMPs called β-defensins are defined by enrichment of cationic peptide residues, disulfide bonds, and beta-sheets and these molecules have been associated with protection from *Candida* infection in humans. mRNA expression analysis revealed that β-Defensin 1 (mBD1) levels were not significantly upregulated
following infection in WT tissue (Figure 3.7A). Similar expression levels were observed for Nlrc4<sup>−/−</sup> mice, however a decrease in mBD1 expression on a per cell basis was seen for NLRP3 and ASC knockout animals. β-Defensin 2 (mBD2)

![Graphs and images of experiments](image)

**Figure 3.6. Key antifungal cytokine responses depend on inflammasome activity.**

WT, Nlrp4<sup>−/−</sup>, Nlrp3<sup>−/−</sup>, and Asc<sup>−/−</sup> buccal tissue mRNA was analyzed by qPCR for specific gene expression. Values are normalized to GAPDH and expressed as fold induction over PBS mock treatment. (A) IL-17A, (D) IL-17F, (E) IL-17RA, (F) CXCL1, or KC, and (G) IL-6. Susceptibility of Il17a<sup>−/−</sup> mice to oral Candida infection was assessed. (B) Tongues, and (C) Kidneys were harvested for CFU. Statistical analysis was performed by Mann Whitney U-test (*p<0.05, **p<0.01, ***p<0.001)

levels were severely augmented in WT following infection (Figure 3.7B). All three inflammasome deficient mouse strains tested demonstrated a minimal
upregulation in expression of the mBD2 gene. β-Defensin 3 (mBD3) expression was also substantially increased in WT mice after Candida treatment (Figure 3.7C). Expression was significantly reduced in both Nlrp3\(^{-/-}\) and Asc\(^{-/-}\) mice, with NLRC4 deficient hosts exhibiting an intermediate increase in expression. A similar pattern of expression was observed for β-Defensin 4 (mBD4) as was seen with mBD2 (Figure 3.7D). Like mBD1, β-Defensin 14 (mBD14) had minimal upregulation in WT mice however all three inflammasome deficient strains had reduced expression of mBD14 following infection, with both Nlrp3\(^{-/-}\) and Asc\(^{-/-}\) mice having significantly reduced upregulation when compared to WT and to Nlrc4\(^{-/-}\) mice (Figure 3.7E).

CAMP, or LL-37 in humans, is an AMP belonging to a different class from β-Defensins. A recent report has implicated CAMP as capable of binding to the P2X7R and augmenting IL-1β production by mimicking the role of endogenous ATP as a DAMP\(^{234}\). CAMP levels were substantially increased in infected WT tissue (Figure 3.7F). This increased expression was absent for Nlrc4\(^{-/-}\), Nlrp3\(^{-/-}\), and Asc\(^{-/-}\) identifying another mechanism that may exacerbate the IL-1β deficiency observed in these mice.
Figure 3.7. IL-1β is necessary for proper antimicrobial peptide expression.

WT, Nlr4−/−, Nlrp3−/−, and Asc−/− buccal tissue mRNA was analyzed by qPCR for specific gene expression. Values are normalized to GAPDH and expressed as fold induction over PBS mock treatment. (A) mBD1, (B) mBD2, (C) mBD3, (D) mBD4, (E) mBD14, and (F) CAMP. Statistical analysis was performed by Mann-Whitney U-test (*p<0.05, **p<0.01, ***p<0.001).
DISCUSSION

The persistence of IL-1β in the serum of *Candida* infected mice in the absence of a functional NLRP3 inflammasome led to the investigation of alternative NLR family members which could be invoked following infection. The fact that the remaining IL-1β was also ASC independent required targeting of one of a select few NLRs which contain an intrinsic CARD domain. NLRC4, previously named IPAF, is an NLR possessing a CARD domain that has been shown to respond to pathogenic components of a number of bacterial species. In spite of *Candida* having no readily identifiable structural or sequential homologues to the bacterial proteins known to activate NLRC4, it was hypothesized that this protein was contributing to the release of IL-1β during infection with *Candida*.

Stimulation of *Nlrc4*−/− macrophages with *Candida* revealed no role for this protein, which confirmed our previous findings that NLRP3 was the inflammasome mediating IL-1β downstream of *C. albicans* in macrophages. Serum isolated from NLRC4 deficient hosts, however, was found to contain reduced levels of IL-1β providing the first evidence for a role of this protein in any response to a fungal pathogen. The level of reduced IL-1β in *Nlrc4*−/− was nearly equal to the amount of IL-1β that persisted in the circulation of either NLRP3 or ASC knockout animal pointing towards these two inflammasome proteins as the mediators of IL-1β release following *Candida* infection. Quantitative fungal burden analysis of *Nlrc4*−/− identified a severe susceptibility to mucosal candidiasis. CFUs were significantly elevated in knockout mice at Day 7
post infection and persisted at elevated levels throughout the 21 day timecourse of study. Similar trends were observed in the other mucosal tissues through 14 days of infection, with tongues being the only organ possessing elevated fungal loads at Day 21. Despite having no identifiable role in macrophages, loss of NLRC4 signaling resulted in significant systemic dissemination that peaked at Day 7 and returned to WT levels after 21 days. Minimal alterations in survival were observed during GDH2346 infection however oral inoculation of Nlrc4−/− mice with a virulent, disseminated strain of Candida (CA90234) resulted in a 33% reduction in survival. These clearly identify NLRC4 as a critical component of competent immune responses to mucosal pathogens and demonstrate that in the context of more pathogenic fungal species this protein is crucial for the prevention of Candida mediated mortality.

The facts that NLRC4 is critical for control of Candida infection and IL-1β release in vivo but dispensable in macrophage induced IL-1β led to the hypothesis that NLRC4 activation in resident tissue cells, specifically epithelial cells, was responsible for the drastic mucosal phenotype observed. Further evidence for this hypothesis came upon closer examination of the fungal loads of Nlrp3+/− and Nlrc4+/− mice. While both strains exhibited a pronounced increase in oral infection at Day 7, only the Nlrc4+/− had sustained increases at the later timepoints whereas Nlrp3+/− were able to clear infection to WT levels. The exact opposite was seen for disseminated candidiasis, with sustained increases found in NLRP3 deficient animals while their NLRC4 deficient counterparts were able to control disseminated infection at later times. These data together hint at a mucosal
tissue specific role for NLRC4, with NLRP3 mediating its effects through infiltrating cells.

To test this, bone marrow chimeric mice were generated. Full body gamma irradiation followed by reconstitution with exogenous bone marrow progenitor cells generates a mouse whose hematopoietic compartment is of a different genotype than the rest of their tissue. When WT bone marrow was transplanted into \( \text{Nlrc4}^{-/-} \) mice, oral fungal loads were nearly identical to those observed for our historical, full \( \text{Nlrc4}^{-/-} \) mice. NLRC4 deficient bone marrow, by contrast, was unable to recapitulate the susceptibility to oral disease seen in \( \text{Nlrc4}^{+/+} \) mice. Kidney fungal loads revealed similar phenotypes as seen with oral infection, though transplanted \( \text{Nlrc4}^{+/+} \) bone marrow did predispose WT mice to slightly elevated levels of dissemination. As predicted by our hypothesis, NLRP3 behaved in the opposite manner. Injection of bone marrow from NLRP3 deficient mice into WT recipients resulted in oral disease severity that is comparable to fully NLRP3 deficient hosts. \( \text{Asc}^{-/-} \) bone marrow injection into WT mice was also able to reconstitute the oral disease severity observed in \( \text{Asc}^{+/+} \). Transplantation of WT bone marrow into either NLRP3 or ASC knockout animals resulted in an amelioration of oral disease severity to that observed in WT mice. Investigation of disseminated \( \text{Candida} \) levels revealed the same trends as seen for oral fungal loads, with one exception. WT bone marrow injection into \( \text{Nlrp3}^{+/+} \) resulted in increased disseminated fungal loads, despite no observable mucosal phenotype. This elucidates the possibility of an ASC-independent, NLRP3-dependent mechanism involved in prevention of tissue dissemination in non-hematopoietic
cells. Future studies will seek to identify the nature of this ASC-independent signaling cascade, specifically aimed at the capacity for NLRP3 to associate with other NLRs.

The finding that mucosal expression of NLRC4 is required for control of an infectious pathogen is revolutionary to the field of innate immunology as it represents the first substantial experimental evidence for a tissue specific role of an NLR. Limited studies have been performed on expression patterns of NLRs other than NLRP3 and the majority of NLR research still focuses on the role of these proteins in hematopoietic cells. The finding that hematopoietic expression of NLRC4 is dispensable for proper functioning not only opens up many new avenues of research on these molecules but also supports the emerging realization that global immunity cannot be solely attributed to the efforts of hematopoietic cells but is rather the result of the concerted efforts of nearly every cell type in the body, with certain immune pathways seemingly critical only in non-hematopoietic cells.

Having identified a critical difference in tissue function of NLRC4 and NLRP3, further characterization of the nature of the immune responses elicited by both proteins was required. Early and robust neutrophil infiltration into infected tissues is known to be crucial to the generation of competent immune responses, especially to extracellular pathogens like *Candida* which can be difficult to phagocytose. Histological examination of tongues 2 days post infection reveal the presence of significant inflammation and cellular infiltration associated with areas of epithelial erosion in WT mice. Fluorescent staining of the tissue revealed the
infiltrating cells are composed primarily of neutrophils. Nlrc4-/- mice did not exhibit close to the same level of tissue inflammation and cellular infiltration as seen in WT mice, in spite of the observable presence of tissue erosion. As expected, minimal neutrophil staining was seen in the tissue underlying the epithelium however neutrophils were observable in the tongue but were lined up along the border of the epithelial layer having failed to infiltrate further as seen in WT mice. Tissue inflammation and cellular infiltration was present in the epithelium of both Nlrp3-/- and Asc-/-, though not to the levels seen in WT. Unlike their Nlrc4-/- counterparts, both strains exhibited the presence of infiltrating neutrophils into the epithelial layer and visible abscess formation. Quantification of neutrophil infiltration confirmed the observed phenotype with severely reduced neutrophils in the epithelium alone or whole tongue of Nlrc4-/- compared to WT, with Nlrp3-/- and Asc-/- having intermediate levels of neutrophil infiltration. The near complete abrogation of neutrophil infiltration is believed to be a dominant driver of exacerbated infection levels in Nlrc4-/- mice while reduction in neutrophils coupled with proposed defective functioning due to loss of NLRP3 or ASC is thought to drive the early susceptibility to infection observed in mice deficient in those proteins. Future studies will determine if the defect in neutrophils observed in NLR knockout mice are sustained defects or temporal in nature.

Transcriptional upregulation of key immune genes in resident and infiltrating cells is the principle mechanism by which IL-1β induces a state of immune activation. To ascertain the impact of IL-1β production on downstream
transcriptional activity, buccal tissue was harvested from mice and mRNA isolated. The first set of genes looked were NLRs and IL-1 family members. Expression of both NLRP3 and NLRC4 was increased in WT following *Candida* infection. Upregulation of NLRP3 expression was almost absent in both *Nlrc4*<sup>−/−</sup> and *Asc*<sup>−/−</sup> samples with NLRC4 expression showing minimal changes following infection for both *Nlrp3*<sup>−/−</sup> and *Asc*<sup>−/−</sup>. Reduced NLRP3 expression in NLRC4 deficient tissue can be explained by an absence of infiltrating cells known to express this protein at high levels. The loss of increased NLRC4 in *Nlrp3*<sup>−/−</sup>, on the other hand, might be due to some transcriptional crossregulation between the two NLRs and would constitute some of the earliest evidence for such a relationship.

As expected mRNA levels were substantially upregulated in WT mice following infection, a phenomenon absent in all three knockout animals. *Nlrc4*<sup>−/−</sup>, in contrast with *Nlrp3*<sup>−/−</sup> and *Asc*<sup>−/−</sup>, mice had a minor increase in IL-1β expression following infection. Analysis of IL-1R1 expression found minimal upregulation in receptor expression following infection for WT, *Nlrc4*<sup>−/−</sup>, *Nlrp3*<sup>−/−</sup>, and *Asc*<sup>−/−</sup> mice however *Nlrp3*<sup>−/−</sup> had discernibly lower increases than the other three mouse strains. The use of IL-1R1 by IL-1α to mediate its effects means that NLRP3 deficiency may not only result in a reduction in IL-1β signaling but also IL-1α helping to explain early disease severity. IL-1 signaling results in the upregulation of an IL-1R antagonist, IL-1Ra, designed to negatively regulate inflammatory signaling by preventing association of IL-1 with the receptor. As seen with IL-1R1, minimal upregulation of IL-1Ra was observed in WT mice. All 3 knockout strains had diminished, if not absent, upregulation in IL-1Ra when compared to WT. The
more pronounced defect observed in Nlrp3<sup>−/−</sup> may be due to decreased IL-1RI expression in these mice which is required for IL-1Ra upregulation. It was discovered that two other cytokines, IL-18 and IL-33, require proteolytic cleavage by inflammatory caspases. IL-18 has been experimentally linked with susceptibility to intravenous *Candida* infection. A minor increase in IL-18 expression levels was seen in WT that was diminished in Nlrc4<sup>−/−</sup> and completely absent in Nlrp3<sup>−/−</sup> and Asc<sup>−/−</sup>. Future studies will seek to elucidate the contributions of this IL-18 to prevention of dissemination following oral infection.

A multitude of cytokines become expressed following IL-1β signaling and a number of these have been implicated as playing critical roles in antifungal immunity. Members of the IL-17 family have been associated with combating extracellular pathogens since their discovery and genetic deficiency in IL-17 signaling is associated with severe susceptibility to recurrent mucosal candidiasis. IL-17A expression was highly upregulated in WT mice following infection, with almost a complete loss of upregulation in NLR knockout mice. Il17a<sup>−/−</sup> mice had significantly elevated oral and disseminated fungal levels at Day 7, corroborating the importance of this cytokine in anti-*Candida* responses. IL-17F levels were also increased in WT mice with the only discernable defect in expression observed in Nlrp3<sup>−/−</sup> samples. The IL-17 receptor is composed of multiple subunits and is responsible for signaling of both IL-17A and IL-17F. It was found that upregulation of the alpha subunit was present in all mouse strains except for Nlrc4<sup>−/−</sup>. The lack of increased IL-17RA expression indicates that even though they mice are capable of producing some IL-17A and WT levels of IL-17F, they
likely fail to respond properly to the presence of either cytokine. KC, a potent neutrophil chemotactic factor, was also found to have significantly reduced expression in all three knockout strains tested partially explaining the lack of neutrophil infiltration observed by histology. Like IL-17A and KC, IL-6 levels significantly increased in WT mice but not NLR knockout mice. These defects in key cytokines involved in combating both local and systemic infections underlie the pronounced disease phenotypes observed in these genetic knockout mice.

In addition to the release of inflammatory mediators, early mucosal immune responses involve the release of increased levels of antimicrobial peptides which can not only function to directly kill cells but also mediate chemotaxis of immune cells by binding to chemokine receptors. The first AMP studied was mBD1 and it was found to be unchanged following infection with Candida in both WT and Nlrc4−/− mice however reduced expression was observed in Nlrp3−/− and Asc−/− samples. mBD2 and mBD4 were both found highly upregulated in WT but not knockout mice. mBD3 was highly upregulated in WT mice with intermediate expression in Nlrc4−/− and significantly reduced expression in Nlrp3−/− and Asc−/− mice. mBD14 exhibited a similar profile as mBD3 with respect to alterations in expression in knockout mice, although expression of WT samples did not change following infection meaning the absence of IL-1β results in decreased mBD14 expression. Another antimicrobial peptide CAMP has been implicated as an activator of IL-1β release via action of the P2X7R. mRNA levels of this protein increased dramatically in WT mice following infection with
minimal upregulation observed in all the knockout strains. An illustration of the overall model of mucosal defense to Candida is presented in Figure 3.8.

All three knockout mouse strains tested exhibited substantial defects in cytokine and antimicrobial peptide responses following Candida infection. Despite observable difference in fungal loads and disease severity, these three strains had similar expression patterns for many of the proteins tested. Studies of expression patterns at later times of infection, 10-14 days, may reveal additional differences between mouse strains which can explain the sustained oral infection observed in Nlrc4−/−. One interpretation of why both NLRC4 and NLRP3 deficient hosts have similar protein expression profiles is the majority of these proteins derive from leukocytes. Since Nlrc4−/− have demonstrably lower levels of early neutrophil infiltration, initiation of innate immune pathways may either be absent or delayed early during infection. While Nlrp3−/− animals do have substantial neutrophil accumulation early on, it is still reduced from WT levels and the lack of downstream immune activation likely reflects a defect in the functioning of the cells that do infiltrate rather than their absence as with Nlrc4−/−. Interpretation of expression profiles in Asc−/− is more complicated due to the recent revelation that ASC can directly inhibit the activity of DUSP10, a phosphatase involved in shutting off MAPK signaling. Researchers found almost no MAPK signaling downstream of PRR stimulation in ASC deficient cells, meaning some of the lack of upregulation of gene expression seen in these mice may be due to IL-1β independent mechanisms. The findings that NLRC4 is mediating its protective effects during fungal infections in non-hematopoietic cells is one that will begin
**Figure 3.8. Integrated model of the mucosal immune response to Candida.** Epithelial cells encountering Candida release cytokines and chemokines which activate endothelial cells. Endothelial cells upregulate adhesion molecules, enabling circulating leukocytes to extravasate. Infiltrating leukocytes travel along the chemokine gradient in tissue to the site of infection and release key immune effector molecules (cytokines, ROS, proteases) upon contact with Candida.
to change the way immunologists approach the idea of inflammasome functioning. The bulk of NLR research has focused on hematopoietic cells with many NLRs having minimal to no established functioning at this stage. The revelation that an NLR can function solely in a non-hematopoietic cell should spur future research into the 10+ NLRs that have minimal or no identified function yet as many of these may work via unconventional mechanisms and/or in non-traditional immune cells. The fact that NLRP3 is able to identify such a wide range of molecules and yet evolution has created so many additional inflammasome proteins indicates many must function in more constrained contexts.
Chapter 4

Murine β-defensin 1 is required for control of oral candidiasis and the induction of inflammatory immune responses *in vivo*
SUMMARY

Antimicrobial peptide production is a critical component of mucosal immunity, in particular for the culling of commensal populations and to induce potent mortality during microbial infections. Murine β-defensin 1 was found to play a critical role during early mucosal immune responses. Oral and mucosal Candida infection levels were drastically increased through Day 7 but returned to normal levels by Day 14, confirming a key role in early innate immunity. Levels of disseminated candidiasis were also elevated at these early time points. These mice also had significant drops in body weights before Day 3, perhaps a result of decreased food consumption due to infection. Histological examination revealed substantial reduction in the levels of neutrophils infiltrating the epithelial layer of mBD1-deficient mice. Buccal gene expression of key AMPs were also reduced in these mice, pointing to a possible role as a master regulator of AMP production. As expected by decreased inflammation histologically, expression levels of key cytokine and chemokines including IL-17A and KC were reduced. NLR and IL-1 family members previously shown to be crucial for mucosal immune responses were also found to be at reduced levels in the absence of DEFB1, exacerbating their susceptibility to oral infection. These data clearly identify mBD1 as not only an important AMP due to its direct antimicrobial activity, but a key inducer of inflammatory immune responses in the mucosa following Candida infection.
INTRODUCTION

An essential component of the normal maintenance of mucosal homeostasis is the release of antimicrobial peptides (AMPs) which can act to cull normal commensal populations and induce killing of pathogens prior to recognition by the immune system. These proteins are most commonly found in the tears, saliva, and secreted into the lumen of mucosal cavities\textsuperscript{198}. Many AMPs are released by mucosal epithelial cells or epithelial cells lining the tear and salivary ducts. Antimicrobial peptides can either be expressed constitutively, induced after infection, or both. There are four major classes of AMPS: 1) Small, anionic peptides which require the cofactor zinc, 2) small α-helical cationic peptides (<40 amino acids) lacking cysteines, 3) cationic, linear peptides rich in proline or tryptophan but lacking in cysteines, and 4) defensins, which are β-sheeted peptides containing disulfide bonds which are critical for function\textsuperscript{198}. A number of murine and human β-defensins are associated with direct anti-\textit{Candida} activity, including recombinant mBD\textsubscript{1}\textsuperscript{214,208}. Certain defensins have also been shown to bind to chemokine receptors, presumably activating these receptors and directly altering the migratory pattern of innate immune cells\textsuperscript{203,218}. Despite the identification of mBD\textsubscript{1} as a direct anti-\textit{Candida} defensin, almost no research has been conducted on the role of this protein during live \textit{Candida} infection in mice.
METHODS

Animals

C57BL/6 mice (originally derived from Jackson Laboratories Bar Harbor, ME) were bred in microisolator cages. Defb1\(^{-/-}\) were originally obtained from Dr. James Wilson (University of Pennsylvania). Animals were housed in filter-covered microisolator cages in ventilated racks. All animal studies have been approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and University of Massachusetts.

Murine model of Oral candidiasis

Susceptibility to oral infection is assayed through our model of oral candidiasis. Mice are treated for 5 days with tetracycline containing water. On the day of infection, mice are anesthetized and three superficial scratches are made on the dorsal layer of the tongue. After 3 hrs of recovery, mice are inoculated with 100 μL of a 5x10\(^7\) yeast/mL solution soaked into a cotton ball. Cotton balls are removed after 4 hrs and infection carried out for 2-28 days. Mice are sacrificed and the tongues, esophagi, stomachs, duodenums, jejunums, ilea, and kidneys are harvested. Organs are homogenized in sterile PBS using a bead-beater homogenizer (Retsch). Serial dilutions of organ homogenates are plated into SD agar and colony forming units determined after 72 hrs at 37\(^\circ\)C. CFU counts are logged and graphed as logCFU/organ.
Fungal preparations

To assess the impact of C. albicans on immune cell functioning in vitro, formalin-fixed preparations of GDH2346 were generated. GDH2346 yeast, isolated following overnight growth, were placed into RPMI1640 (Thermo Scientific) containing 10% FBS (Atlanta Biologicals) for 0, 2, 4, 6, 12, and 24 hrs. Cells were then isolated by centrifugation and fixed with formalin. Following fixation, cells were washed 2x with PBS and resuspended in PBS. Preps were stored at 4°C for immediate use and -20°C for long-term storage.

Cytokine levels in circulating serum

Circulating blood was obtained by orbital bleeding of Candida infected WT and Defb1−/− mice. After centrifugation, serum was separated from the cellular components and pooled. Serum was stored at -20°C for future cytokine determination by ELISA.

Histological analysis of Candida infected tongues

WT and Defb1−/− tongues were harvested two days after Candida infection. Tongues were placed into freezing cartridge (TissueTek) and embedded in OCT solution (TissueTek). Samples were flash frozen using liquid nitrogen and blocks stored at -80°C until sectioning. 5 μM sections were obtained by cryo-sectioning. Serial sections were stained for histological analysis. Gross histology was visualized by Periodic Acid-Shiff’s (PAS) reagent with haematoxylin counterstain.
(H), or PAS/H. PAS reacts to the presence of polysaccharide residues and stains purple/magenta. Haematoxylin is a blue colored basic stain used to identify nuclei. PAS/H staining allows for the visualization of tissue architecture, as well as the identification of fungal cells and debris is the outer epithelial layer. Coverslips were placed on slides using Permount. For direct identification of neutrophil infiltration, frozen sections were stained with NIMP R14 which binds to GR-1 on the cell surface. Sections were then treated with a secondary antibody conjugated to the Alexa-488 fluorophore. Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) was used to mount coverslips. DAPI is a blue fluorescent dye which binds to A-T rich regions of DNA and allows for visualization of nuclei. Coverslips were permanently sealed using clear commercial nail polish. Stained sections were visualized with the assistance of Dr. Scott Howell, Ph.D. 40x images were taken spanning the entire tongue and individual pictures spliced into a complete 40x image of the tongue using MetaMorph software. For NIMP stained sections, quantification of neutrophils was performed using MetaMorph software. A minimal threshold of fluorescent intensity corresponding to a neutrophil was set. The number of pixels at or above that threshold was measured and divided by the total number of pixels in the section. This analysis was performed for the epithelial layer, underlying tissue, and whole tongue.
**Neutrophil Lavage**

To test extravasation capacity of \( \text{Defb1}^{-/-} \) neutrophils, mice were injected with 2 mL of thioglycollate into their peritoneal cavity. After 24 hours, the peritoneal cavity of mice was lavaged with 10 mL of DMEM. Neutrophils were isolated by Ficoll-Plaque Plus gradient centrifugation (Amersham Biosciences). After pelleting, cells were counted via hemocytometer and percentage of neutrophils determined by DiffQuik staining protocol.

**Quantitative real-time PCR**

To assess inflammatory responses in the oral mucosa, buccal tissue was excised from mice infected with GDH2346 and PBS treated controls. Buccal tissue was harvested from WT and \( \text{Defb1}^{-/-} \) mice. mRNA was extracted by column purification and cDNA generated using High Throughput cDNA synthesis kit (Roche). Quantitative real time PCR was performed using gene specific primers. Resulting Ct values were then analyzed using the \( 2^{-\Delta\Delta Ct} \) method. Data is expressed as fold induction of gene expression over mock (PBS) infected samples. Gene specific primers listed in Table 2.1.
**Statistical Analysis**

Statistical analysis were performed using GraphPad Prism software (). Mann-Whitney U-tests were performed for quantitative fungal burdens, Gross Clinical Score, and gene expression by qPCR.
RESULTS

Murine β-defensin 1 deficiency results in a dramatic susceptibility to early oral candidiasis

OPC infection of Defb1<sup>-/-</sup> revealed these mice were highly susceptible to candidiasis. Defb1<sup>-/-</sup> mice had significantly elevated fungal loads in their tongues by Day 3 which was sustained at Day 7 (Figure 4.1A). A similar trend of early susceptibility to fungal infection was observed in other mucosal organs tested including esophagi, stomach, and small intestines (Figure 4.1B-F). As expected, gross clinical scores of disease severity mimicked the trend of drastic and early susceptibility (Figure 4.1G). Defb1<sup>-/-</sup> mice were also highly susceptible to early disseminated candidiasis, with significantly increased dissemination observed at Day 3 with a near complete loss of disseminated fungus by Day 14 (Figure 4.1H). Defb1<sup>-/-</sup> mice had a small increase in mortality when compared to WT mice and lost significantly more weight during the first 3 days of infection, returning to WT levels by 14 days (Figure 4.1I,J). Given the crucial role for mBD1 in early innate responses, with no prior implication on the induction of adaptive immune responses, it is not surprising the phenotype of these mice is dramatic at early timepoints and nearly absent by Day 14.

mBD1 is critical for early recruitment of neutrophils to infected tissue

Neutrophil recruitment to sites of infection is a critical stage in the development of early antifungal immune responses. Though AMPs were
originally discovered and described based upon their capacity for antimicrobial activity, it has since been found that these proteins are also capable of engaging chemokine receptors to mediate chemotactic cellular trafficking.
To address the role of mBD1 in neutrophil chemotaxis, histological examination of tongues at 2 days post infection was performed. As seen before, PAS/H staining revealed significant tissue damage on the dorsal side of WT tongues (Figure 4.2A). Tissue inflammation and inflammatory cellular infiltrate was associated with this tissue damage. This contrasted starkly with \textit{Defb1}^\text{−/−} mice which, like their WT counterpart, had visual tissue damage but markedly reduced cellular infiltration (Figure 4.2E). Fluorescent staining for neutrophils revealed this defective cellular infiltration was due to an absence of neutrophils. (Figure 4.2B,F). 40x magnified images of the epithelium clearly demonstrate the presence of high levels of neutrophils in WT mice that are diminished in the absence of mBD1 (Figure 4.2C,G). Due to the possible role as a chemotactic factor, we also investigated the presence of neutrophils in the underlying submucosal tissue. Closer examination revealed the presence of substantial neutrophils in the underlying tissue, immediately below the epithelium, of \textit{Defb1}^\text{−/−} mice that were not as evident in WT mice (Figure 4.2D,H).

Quantitative analysis of neutrophil infiltration confirmed these observations. Analysis of the entire tongue revealed minimally different levels of neutrophil infiltration between WT and \textit{Defb1}^\text{−/−} mice (Figure 4.2I). While there was not a substantial difference in overall neutrophil infiltration levels, \textit{Defb1}^\text{−/−} had nearly 50% fewer neutrophils in the epithelium.

To ascertain whether these decreases in neutrophil numbers were the result of defective extravasation or defective trafficking, neutrophil counts were obtained from peritoneal lavage after thioglycollate injection. No discernable
difference was observed between WT and Defb1Δ/Δ mice for either neutrophils alone or bulk cell count (Figure 4.2J,K). IL-1R1Δ/Δ, used as a control for defective neutrophil extravasation, had ~50% reduced neutrophil extravasation following thioglycollate injection. These data indicate that mBD1 deficient neutrophils are fully capable of normal extravasation into the peritoneal cavity and that defective levels present in the epithelium are likely the result of improper chemotaxis.
mBD1 expression is required for normal antimicrobial peptide production following *Candida* infection

Though previous studies have demonstrated a minimal increase in mBD1 expression following *Candida* infection, *Defb1*−/− mice have severely increased susceptibility to infection. To elucidate possible mechanisms leading to this exacerbation, expression levels of other AMPs known to be important for pathogenic clearance were tested. *Defb1*−/− had significantly decreased levels of mBD2 RNA in the buccal mucosa following *Candida* infection (Figure 4.3A). mBD3 expression increased following *Candida* infection in WT and *Defb1*−/− mice, with slightly increased expression on average for *Defb1*−/− samples (Figure 4.3B). mBD4 levels were not increased in mBD1 deficient hosts as compared to the significant upregulation that was found in WT mice (Figure 4.3C). Increased expression of mBD14 was not observed in either mouse strain following infection with *Candida* (Figure 4.3D). CAMP, an antimicrobial peptide belonging to the α-defensins, was induced in WT mice but diminished in *Defb1*−/− mice (Figure 4.3E).

**Induction of inflammatory and chemotactic mediators following *Candida* infection requires mBD1**

The release of inflammatory and chemotactic factors is considered the most important aspect of innate immune functioning. Cytokines such as IL-17 and IL-6 are known to be of critical importance in immune responses to pathogenic
organisms. Given the known role for IL-17 family members on the secretion of AMPs from epithelial cells, and our data demonstrating perturbation of a range of AMPs in the absence of mBD1, we sought to determine the induction of protective innate cytokine responses following oral inoculation with *Candida*. Expression levels of both IL-17A and IL-17F were highly upregulated in WT mice following *Candida* treatment, with marked reductions observed in expression levels in *Defb1*⁻/⁻ mice (Figure 4.4A,B). This decrease in IL-17 expression in the buccal mucosa may explain the severe defect in AMP expression observed as IL-17 is known to be involved in AMP production. A potent neutrophilic chemotactic protein KC, or CXCL1, had highly increased
mRNA expression following *Candida* (Figure 4.4C). This increase was significantly lowered in *Defb1*−/−, providing potential causation for the defective neutrophil trafficking observed histologically in these mice. Upregulation of IL-6, a systemically active inflammatory cytokine, levels were observed in WT but not *Defb1*−/− mice (Figure 4.4D).

Previous work has shown similar cytokine defects in infected animals deficient in proteins responsible for IL-1β production. Both of these proteins,
NLRC4 and NLRP3, were found to have reduced upregulation following *Candida* infection in mBD1-deficient hosts, with NLRC4 having significantly reduced expression (Figure 4.5A,B). Expression levels of IL-1β were also significantly lessened in these animals (Figure 4.5C). In conjunction with decreased IL-1β transcript, *Defb1*−/− mice had lowered levels of circulating IL-1β at 3 days post infection (Figure 4.5D). The IL-1R antagonist, a negative regulatory molecule produced in response to IL-1R1 signaling, also had reduced expression compared to WT hosts after infection (Figure 4.5E). IL-18 levels did not increase on a per cell basis following infection in WT mice however *Defb1*−/− mice had significantly decreased expression levels compared to WT mice (Figure 4.5F).

**Figure 4.5. Regulation of NLR and IL-1 family expression by DEFB1**

WT and *Defb1*−/− mice were infected orally with GDH2346 and buccal tissue harvested after 3 days. Isolated mRNA was converted to cDNA and gene expression ascertained by qPCR. Gene expression is normalized to GAPDH and values graphed as fold induction over mock infection. (A) NLRC4, (B) NLRP3, (C) IL-1β, (E) IL-1Ra, and (F) IL-18 expression was quantified. (D) Circulating serum levels of IL-1β were measured in WT and *Defb1*−/− mice at Statistical analysis was performed by Mann Whitney U-test (*p*<0.05, **p**<0.01)
DISCUSSION

Antimicrobial peptides secreted into the saliva and lumen of mucosal organs represents perhaps the most basic and early innate immune response. Constitutively expressed AMPs provide a mechanism to control the native microbiome populations and ensure microbial overgrowth does not readily occur. Certain AMPs can also become expressed during the course of pathogenic infection which function to directly control microbial growth and promote wound healing through chemotaxis of infiltrating leukocytes to areas of active infection. A number of these AMPs are inducible following Candida infection and have been shown by others to have direct anticandidacidal activity. One antimicrobial peptide in particular, mouse β-defensin 1, has recently been found to possess anti-Candida activity\textsuperscript{208}. This AMP appears to be constitutively produced with minimal upregulation following infection, however NLR genetic knockout mice have diminished levels of mBD1 expression which may contribute to their severe oral disease phenotypes.

To test the role of mBD1 during oral candidiasis, genetically deficient mice were tested for their capacity to combat Candida infection. Significantly augmented oral infection was observed at 3 and 7 days post infection with a return to WT levels by Day 14, confirming the proposed role for AMPs during early infection. Other mucosal organs of the gastrointestinal tract had the same pattern of exacerbated disease severity at early timepoints, with no discernable phenotype after extended infection. These mice also had significant elevated levels of disseminated Candida at these early timepoints. This early predisposition to
disseminated candidiasis points to a more critical role in early innate immune activation for mBD1 than simply functioning to kill fungus on the luminal surface of mucosal organs. Mortality levels were slightly increased in Defb1−/− mice. Assessment of body weight during infection revealed significant reductions in the overall body weights of DEFB1 deficient hosts compared to WT animals at early timepoints. A known consequence of Candida infection in humans is dysphagia, or the inability to swallow, resulting in decreased oral nutrient intake. A reduction in food consumption could explain these drastic drops in body weight. In spite of identifying no upregulation in mBD1 expression during active infection, these data clearly identify this protein as a crucial mediator of early protective mucosal immune responses.

Early and robust neutrophil infiltration is known to be a critical component in protection from fungal infections. It was hypothesized that the severe susceptibility to oral infection in Defb1−/− mice was due to impaired neutrophil infiltration. Histological examination with PAS/H staining of WT and Defb1−/− tongues after 2 days of infection showed active tissue erosion and damage of the dorsal epithelium in both strains. Associated with this erosion was inflammation and thickening of the epithelial layer of these mice. As seen before, WT mice has substantial cellular infiltration associated with this inflammatory state whereas Defb1−/− had markedly reduced cellular infiltrate underlying areas of active infection. A fluorescent stain specific for a cell surface marker on neutrophils clearly showed the cells infiltrating the inflamed tissue of WT mice were neutrophils and fungal abscess formation was evident in the tissue. In
contrast, neutrophils infiltrating the epithelium were substantially reduced in the absence of mBD1. Quantification of neutrophil infiltration highlighted this defect, with almost 50% fewer neutrophils present in the epithelium of Defb1−/−. Gross examination of the tongue revealed an interesting observation. Unlike NLRC4 or NLRP3 knockout mice which had substantially reduced neutrophil numbers throughout their tongues, mBD1 deficiency resulted in a specific defect in neutrophil numbers in the epithelial layer. Quantification of the tongue minus epithelium revealed nearly identical numbers of neutrophils between WT and Defb1−/− mice. These finding are of particular interest as they demonstrate that the defect in neutrophil numbers is likely due to defective trafficking into the epithelium as opposed to delayed or defective extravasation. Neutrophil numbers in the peritoneal cavity of mice following thioglycollate injection was used as a measure of extravasation. No discernable differences existed in either neutrophil cell count or bulk cell count in the peritoneal lavage fluid providing evidence that mBD1 deficiency may not impact absolute levels of extravasation but rather functions at the level of inducing trafficking from the submucosal tissue into inflamed epithelial tissue.

Upregulation in innate immune gene expression is one of the earliest events in infection and is crucial for the proper activation of subsequent infiltrating cells to combat microbes. Given the marked reduction in infiltrating cells observed in Defb1−/− mice, we wanted to determine the transcriptional upregulation occurring in the oral cavity of these animals. Given the fact mBD1 is not the only antimicrobial peptide expressed during infection with known capacity
to combat *Candida*, buccal mRNA expression levels of other key AMPs was determined. mBD2 expression was almost ~1000 fold higher in WT mice following infection, however this upregulation was almost completely abrogated in *Defb1<sup>−/−</sup>* samples. mBD3 levels were also upregulated in WT mice with this increase somewhat exacerbated in *Defb1<sup>−/−</sup>* mice. Like mBD2, increased levels of mBD4 mRNA were nearly absent in mBD1 deficient samples. mBD14 expression levels did not change significantly in either mouse strain following infection. CAMP expression, an AMP implicated in the augmentation of IL-1β production, was substantially elevated in WT samples with a marked decrease in levels detected in *Defb1<sup>−/−</sup>*. A prior report found that early upregulation of β-Defensin expression in epithelial cells requires active tissue damage since hyphal mutant *Candida* was incapable of inducing defensin expression<sup>236</sup>. It is possible that early epithelial lysis by *Candida* releases mBD1 and that this release is critical for the induction of downstream immune responses.

These data show that key AMPs in combating mucosal candidiasis are also decreased in the absence of mBD1 however it remains to be determined if the lower expression level is due to a perturbation of downstream inflammatory signaling or an as of yet unidentified ability for mBD1 to directly contribute to the expression of other key AMPs. This will be examined in detail in future studies as the identification of mBD1 as a master regulator of AMP expression would be a significant alteration in our understanding of the function of AMPs.

With the reduced levels of neutrophils infiltrating the infected epithelium of *Defb1<sup>−/−</sup>*, it was necessary to ascertain the impact of mBD1 deficiency on
inflammatory gene expression. Analysis of IL-17A and IL-17F demonstrated that upregulation in gene expression following Candida infection was reduced in Defb1−/− compared to WT samples. The known role of IL-17 family members, and downstream signaling, on the induction of AMP expression could help explain the significant defects observed in AMP expression in these mice. The chemokine KC was increased in WT samples and significantly reduced in infected Defb1−/− tissue following infection. This is consistent with a prior report identifying an upregulation in KC expression following gastric candidiasis\textsuperscript{210}. IL-6 expression following infection was also reduced in mBD1 knockout animals which may be linked to the increased susceptibility of these mice to disseminated candidiasis given the systemic and pleiotropic nature of this cytokine.

Much of my previous work has focused on the role of IL-1β and its regulators on the control of candidiasis. Due to the central role identified for IL-1β in combating Candida in our model, expression levels of IL-1 family members and key NLRs were quantified. Expression of IL-1β was significantly reduced in Defb1−/− buccal samples, with a concurrent decrease in circulating IL-1β observed at 3 days post infection. IL-1β serum levels in Defb1−/− animals exceeded that observed in WT mice at 7 days, providing rationale for the decrease in oral and disseminated phenotype seen by 14 days of infection. IL-18, another cytokine requiring proteolytic activation, was found to have similar expression levels in WT mice before and during infection, however IL-18 expression levels did decrease in Defb1−/− mice following infection. IL-18 has been shown to be involved in control of disseminated Candida infection so loss of this protein may
contribute to the disseminated candidiasis found in these mice. Expression levels of the inflammasome protein NLRP3 were upregulated during infection, with reduced increases observed in Defb1−/− samples. On the other hand, the high levels of NLRC4 upregulation observed in WT mice were completely absent from knockout samples. The precise cause of this substantial decrease in NLRC4 remains to be determined however our previous results would indicate such a substantial decrease in expression is likely a driving factor in the mucosal phenotype seen in these mice.

Antimicrobial peptides were originally characterized solely for their capacity to induce microbial death, especially in salivary secretions and the lumen of the intestines. Recent evidence has begun to expand our understanding of the role of these small peptides in immune responses. Our data clearly identify mBD1 as a critical component to early immune responses, both locally in mucosal organs as well as preventing dissemination. A novel role for mBD1 in neutrophil trafficking was identified with a marked reduction of neutrophils in the epithelial layer of Defb1−/−. Interestingly, the effects of mBD1 deficiency extended into the expression pattern of key innate immune cytokines and other AMPs known to be induced following infection. The potential capacity for mBD1 to directly impact downstream gene expression is a notion that would be revolutionary to our understanding of the multiple functions of AMPs.
Chapter 5

Future Directions
Delineate the role of NLRC4 on the induction of protective mucosal immune responses to pathogenic infection

Data in Chapter 3 clearly identify NLRC4 as a key mediator of mucosal immune responses to *Candida albicans*. It remains to be determined whether NLRC4 is responding directly to *Candida* or if it is responding to the presence of bacterial components that become introduced into tissue following *Candida* disruption of epithelial integrity. Even though mice are treated with tetracycline drinking water, mucosal bacteria are presumably still present in these mice. In order to determine whether mucosal NLRC4 responses depend on the presence of bacteria, immortalized murine cell lines, including 3T3 fibroblasts and primary oral epithelium, will be transfected with siRNA directed against NLRC4. WT and NLRC4 silenced cells will then be stimulated with *Candida* alone and IL-1β production determined by ELISA and Western Blot and cell death determined by LDH release and Annexin V staining. Initial studies will be conducted using formalin-fixed preparations and live *Candida* in case NLRC4 activation requires live infection of cells. If no defect in IL-1β production is observed, it is possible that epithelial cells require stratified culture conditions, which mimic *in vivo* tissue stratification, in order to function properly. In order to eliminate any artifact associated with plating cells on a flat plastic surface, an organotypic model of reconstituted epithelium will be employed. After growing cells using this protocol, the apical surface of the organotypic model will be treated with fixed and live *Candida*. IL-1β release will be assessed as before. An additional advantage to this system is it will allow for the addition of key immune cells,
including neutrophils and macrophages, to the media underlying the organotypic culture. Infiltration of these cells can be directly visualized in the presence and absence of NLRC4. If no difference in IL-1β release is observed in any of these models, stimulation will be repeated in the presence of common mucosal bacteria.

If a decrease in IL-1β in response to Candida alone is seen in the absence of NLRC4, in vivo infection of WT and Nlrc4<sup>−/−</sup> raised in germ-free conditions will be conducted with GDH2346. Infection in these mice will be assessed by organ CFU, Gross Clinical Score, histology, and mucosal RNA expression. Initial studies will focus on Day 3 and 7. If a defect is still observed in Nlrc4<sup>−/−</sup>, infections will be carried out for longer timepoints. A major caveat to using germ-free mice is that it is known the microbiome of the mucosa has direct impacts on the immune functioning in mucosal organs. It is possible that a defect will not be observed in Nlrc4<sup>−/−</sup> due to intrinsic issues with germ-free raised mice despite the identification of Candida-specific activation in vitro. It is also possible that even though Candida is capable of inducing NLRC4 activation during in vitro experiments, in vivo activation still requires the presence of bacteria. Co-infection of germ-free mice with Candida and bacterial species, including Streptococcus, will help reveal if a lack of phenotype is due to an intrinsic defect that results from being raised in germ-free conditions or if mucosal immune responses in hosts is dependent on bacterial components.

Identification of NLRC4 activation by Candida alone in vitro and in vivo will lead to investigation of the mechanism by which activation occurs. It is known that bacterial components linked with NLRC4 activation induce the
activation by binding to NAIP5\textsuperscript{238-240}. It is then the binding of NAIP5 to NLRC4 which is required for NLRC4 activation. Epithelial cells, either grown in monolayer or in a stratified model depending on results of prior experiments, will have NAIP5 expression silenced by siRNA. Cell death and IL-1β release will be measured. If no defect is detectable, experiments will be repeated using silencing of other identified NAIPs. If a NAIP is identified as critical, knockout mice defective in this protein will be obtained or generated. It is possible that generation of that knockout mouse is not possible due to embryonic or post-natal fatality however it seems likely elimination of a single NAIP will not produce adverse impacts on mortality. Knockout mice will be orally infected with GDH2346 and disease severity determined as previously described.

If no NAIP is identified as critical, research into the phosphorylation status of NLRC4 after \textit{Candida} treatment will be conducted. It was recently discovered that NLRC4 activation in response to \textit{Salmonella typhimurium} required phosphorylation of Ser533\textsuperscript{241}. Mutagenesis of this residue to alanine resulted in loss of NLRC4 activation and phosphomimetic mutation to aspartic acid resulted in NLRC4 activity in the absence of any activating stimuli. \textit{Candida} treated cells would be lysed and proteins prepared for Western Blot analysis. Samples will be probed for the presence of phospho-NLRC4. If phospho-NLRC4 is detected following \textit{Candida} treatment, stimulation of cells transfected with S533A mutated NLRC4 will be conducted. Levels of IL-1β and cell death will be assessed. If this phosphodefective NLRC4 fails to induce IL-1β following \textit{Candida}, further research will be conducted on the kinase(s) involved. Phosphorylation following
Salmonella was found to involve PKCδ so this protein would be the initial target of research.

Having elucidated the mechanism underlying Candida-induced NLRC4 activation, further studies delineating the consequences of NLRC4 activation on downstream immunity remain to be performed. Based upon our previous studies, it is hypothesized that epithelial cells are the resident mucosal cells which require NLRC4 expression for proper functioning. To directly interrogate the role of mucosal epithelial cell NLRC4, NLRC4^{loxP/loxP} mice will be crossed with recently generated Cre-recombinase mice driven by a Keratin-19 specific promoter. Progeny will be specifically deficient in NLRC4 in Keratin-19, which is a keratin expressed in non-keratinized squamous epithelial cells. Mice will be orally infected with C. albicans GDH2346. Infection levels will be assessed by quantitative CFU and Gross Clinical Score assessment. Neutrophil and macrophage infiltration into infected tissue will be assessed by flow cytometry and IHC examination of tissue sections. Buccal and intestinal inflammatory gene expression will be determined by qPCR. Defects observed would be direct evidence for an epithelial-specific role of NLRC4 in mucosal candidiasis. If mucosal epithelial knockdown of NLRC4 is found to have minimal impact on immune responses or only partial defects in immune functioning compared to full Nlrc4^{+/}, it will be necessary to generate additional mice that lack NLRC4 is other resident mucosal cells such as fibroblasts and endothelial cells. It may also be necessary to induce a specific NLRC4 deficiency in intestinal epithelial cells in order to recapitulate increased dissemination seen previously. If NLRC4
deficiency in mucosal epithelial cells is capable of recapitulating the phenotype observed in \( Nlrc4^{−/−} \), these animals will be used for future studies aimed at the role of NLRC4 in immune activation. If the phenotype is partial or absent in these mice, fully NLRC4 deficient animals will be used.

One of the key determinants of control of fungal infections is known to be recruitment and activation of neutrophils. Examination of neutrophil infiltration 2 days post infection revealed a near abrogation in neutrophil infiltration into the tongues of \( Nlrc4^{−/−} \) animals. The fact these mice fail to adequately clear infection to WT levels as late as 28 days, despite clearing disseminated infection by 14 days, indicates a sustained defect in mucosal immune functioning. This defect could either be due to sustained decreases in infiltrating inflammatory cells or a result of infectious \( Candida \) loads reaching too great a level to adequately combat by the time sufficient cellular infiltration has occurred. In order to determine the temporal nature of cellular infiltration, infected tongues will initially be harvested from WT and \( Nlrc4^{−/−} \) mice at Days 3-7 of infection. Levels of neutrophil, macrophage, dendritic cell, and T lymphocyte infiltration will be assessed by flow cytometry of digested tongues. Functionality of cells will be assessed by probing flow cytometry samples for production of key antifungal proteins including IL-17A, IL-1β, and IFNγ. Cryosections will also be obtained from infected tongues at these timepoints and fluorescent antibody staining will be used to determine the tissue location of infiltrating cells. Promising results in oral tissue will lead to investigation of the temporal nature of cellular infiltration into other gastrointestinal tissues such as the stomach, small intestines, and colon. If a defect
Neutrophil infiltration into infected tissue is not only important for the control of oropharyngeal candidiasis but also critical in combating another form of mucosal Candida infection, vulvovaginal candidiasis. Vaginal candidiasis is the most common form of mucosal candidiasis, yet little to no research into the role of NLRs has been performed in this disease process. The critical role for mucosal NLRC4 in the induction of neutrophil infiltration allows the possibility that NLRC4 activation is critical for the control of vaginal candidiasis. I hypothesize that NLRC4 activation in vaginal tissue is required for proper neutrophil recruitment and control of Candida infection. WT and Nlrc4−/− mice
will be infected using the model of vaginal candidiasis developed by Dr. Paul Fidel. Levels of fungal infection will be assessed at by CFU at Days 3 and 7 post-infection. If no role for NLRC4 is identified, additional studies will be conducted with Nlrp3$^{-/-}$, Il1r1$^{-/-}$, and Il1b$^{-/-}$ mice to determine the impact of these proteins on the control of vaginal candidiasis. If a role is identified for NLRC4, mice will be harvested at additional timepoints. Vaginal tissue will be harvested at Days 2, 3, and 7 for determination of cellular infiltration by flow cytometry and histology. The presence of neutrophils, macrophages, and T-cells would be determined by flow cytometry, along with the presence of key markers of activation in these cells. The tissue distribution of infiltrating cells will be determined by PAS/H staining and fluorescent antibody staining. Alteration in gene expression of key immune proteins will be determined in vaginal tissue following Candida infection. Bone marrow chimeric mice will be infected and the contribution of hematopoietic and non-hematopoietic NLRC4 in the process determined. One of the advantages to the identification of a disease phenotype in a vaginal candidiasis model is that it will allow for the easy testing of topical treatments for Candida infection. Topical treatment of the vagina would not require extensive immobilization for dosing and would not run into issues associated with the solution being swallowed or spit up as could be the case with oral treatments.

A key component to the resolution of skin and mucosal infections is the closure of the wound. Failure to close the wound can result in recurrent infection and development of more severe infections. Many of the same cytokines that
induce inflammatory responses have been implicated as important for wound healing. *Nlrc4*−/− mice had significantly reduced early inflammation in their tongues as assessed by histology (Figure 3.4). These mice also exhibited reduction in cytokines involved in wound healing including IL-1 family members \( ^{244-247} \), KC \( ^{247,248} \), and IL-17 family members \( ^{249,250} \). Two modes of wound healing will be utilized in order to assess the impact of NLRC4 deficiency on this process. First, a biopsy punch will be used to excise tissue from the dorsal portion of the tongue of an anesthetized mouse. Mice will be sacrificed at 12, 24, 48, 72, and 96 hours post biopsy. Wound size will be directly measured after excision of the tongue. Excised tongues will then be frozen in OCT media and 5 μM sections made for histological examination. Haematoxylin and Eosin (H&E) staining will be performed to assess gross tissue architecture surrounding the wound. Cellular infiltration and inflammation will be determined by fluorescence microscopy. Antibodies against key cytokine and cellular components will be used to probe tissue sections. The second method will involve physical disruption of skin, forming a lesion. A section of the back of WT and *Nlrc4*−/− mice will be shaved to expose the skin. A biopsy punch of the skin will be made and wound size will be measured on a daily basis. If a defect in healing is found, future mice will be sacrificed at key points and histological examination of the surrounding tissue will be performed. If a phenotype is observed in a model, experiments will be repeated in germ-free conditions to assess the impact of NLRC4 in sterile vs. non-sterile wound closure.
These studies will help further delineate the role that NLRC4 plays during mucosal immune responses, specifically to *Candida*. Identification of the mechanism and consequences of NLRC4 activation will not only provide greater understanding for the role of this protein in *Candida* but will also provide the basis for research on this protein in other mucosal pathologies. A number of pathological conditions are associated with improper or failed wound closure and identification of a role for NLRC4 in this process would allow for the development of new therapies aimed at augmenting the function of this pathway to induce more rapid wound closure.
Contribution of NLRP1 proteolysis on the induction of inflammation and estrogen-induced NLRP1 activation in autoimmune disorders

NLRP1 is an inflammasome that possesses a distinct structure when compared to other inflammasomes. NLRP1 contains two types of effector domains whereas other inflammasomes only contain one type. In humans, NLRP1 contains a C-terminal CARD domain and an N-terminal Pyrin domain. In mice, there are three dominant paralogous NLRP1 genes on chromosome 11 which have a N-terminal domain of unrecognized function and a C-terminal CARD domain\(^{251}\). Additionally, NLRP1 in both mice and humans contains a Function-to-Find (FIIND) domain. Originally mistaken as a portion of the leucine rich repeat, it has recently been shown that NLRP1 undergoes a proteolytic cleavage at a conserved SF/S motif in the FIIND domain and that this is required for NLRP1 function\(^{252,253}\). That report also identified the FIIND domain as sharing homology with the ZU5-UPA domain which is known to have proteolytic function.

Little research has been performed to determine the role of NLRP1 in mediating immune responses. Research on this protein is complicated by the fact that murine and human NLRP1 have different effector domains and that mice can express up to three different paralogous forms of NLRP1 which makes it difficult to completely eliminate the protein from mice. There have been a number of studies linking genetic mutations in NLRP1 with human autoimmune disorders including vitiligo\(^{229,231,254,255}\), Addison’s Disease\(^{230,256}\), Type 1 diabetes\(^{230}\), autoimmune thyroid disease\(^{257}\), Celiac disease\(^{232}\), and rheumatoid arthritis\(^{233}\). Mutant alleles associated with vitiligo result in increased IL-1β production\(^{258}\).
Research done on murine NLRP1b has revealed an important role in response to *Bacillus anthracis*, with one report identifying Anthrax Lethal Factor as capable of cleaving murine and rat NLRP1 in a required step for inflammasome activity\(^{228,259}\). This finding is particularly intriguing in light of the identification of human NLRP1 as a protein that undergoes catalytic cleavage for proper activation. Anthrax is the only pathogen to date identified to activate NLRP1 and it is likely due to mimicking the normal activation event which is autocatalytic cleavage. NLRP1 expression has been observed in multiple tissues involved in the immune response against *Candida* including glandular and columnar epithelial cells of the gastrointestinal tract, myeloid cells, and in secondary lymphoid tissue such as lymphnodes and tonsils\(^{260}\). With little being known about the capacity for NLRs to interact in the formation of inflammasomes, NLRP1 may be playing a critical role in the intestinal mucosal immune response and the induction of downstream adaptive immunity to *Candida* infection. Studies into the mechanisms involved in NLRP1 activation may shed light into the pathways by which *Candida*, and other inflammasome activators, are capable of inducing inflammasome formation.

Initial proposed studies will be aimed at dissecting the consequences of proteolytic cleavage on downstream caspase activity. In addition to IL-1\(\beta\) and IL-18 cleavage, NLRP1 activation has been linked to the development of pyroptosis\(^{261,262}\) and the anti-apoptotic proteins Bcl-2 and Bcl-XL are known to inhibit NLRP1 by suppressing ATP binding to NLRP1 and subsequent oligomerization\(^{263,264}\). It is my hypothesis that cleavage of the FIIND domain will
release the CARD domain from the complex and allow it to functionally interact with and activate cellular caspases. To test this, I will generate stably transfected HEK cells that express NLRP1 with a truncated CARD domain incapable of interacting with other CARD domains or NLRP1 with no CARD domain. Cells will be primed to induce expression of other inflammasome components and NLRP1 activated. Cell supernatants will be assessed for cytokine release and LDH for necrotic cell death and Annexin V staining for the induction of apoptosis. If diminished cytokine release and/or cell death are observed downstream of mutated NLRP1 proteins, an HA tagged FIIND-CARD protein will be generated, expressed in HEK cells under a CMV promoter, and purified from cell lysates. After purification, this CARD containing fragment(s) will be transfected into primed cells, in the absence of any other inflammasome activating signal, and IL-1β release measured. If cytokine release is seen, purified CARD fragments will be tested in a cell free system to assay for the capacity of the CARD domain of NLRP1 to directly activate caspase-1, with activity measured by fluorogenic substrate conversion and/or cytokine cleavage as detected by Western Blot. If cell death is induced upon transfection, tranfections will be repeated in the absence of priming to determine if NLRP1 induced cell death requires PRR priming. Additionally, cells will be pretreated with a pharmacological inhibitor of apoptosis to ascertain whether cell death following NLRP1 activation occurs through mechanisms of apoptosis or necrosis. Further studies would be required to discern the precise mechanism of apoptotic death if apoptosis is found to be a substantial component of NLRP1-induced cell death.
Should a role for the released CARD domain prove to be absent or minor, studies will be conducted to determine the role of the Pyrin domain that remains attached to the NACHT-domain. It is possible that this released NACHT is capable of interacting with other inflammasome proteins to form the traditional multiprotein complex associated with inflammasome formation. One very intriguing aspect is that the role of Cathepsin B in mediating NLRP3 activation in response to certain stimuli has been well documented but the actual mechanism has yet to be identified. It is known that Cathepsin B requires a histidine in the catalytic pocket in order to function and the report that identified NLRP1 FIIND as an autoprotolytic domain identified a key histidine reside near the SF/S cleavage site that was required for autoproteolysis. It is possible that Cathepsin B is also able to cleave NLRP1 and that the way Cathepsin B is involved in NLRP3 activation is through the release of an unrepressed NLRP1 NACHT-PYD (NLRP1 N-P) protein that interacts with NLRP3 and overcomes its repressed state. This would partially mimic the recent finding that NAIP5 binding to NLRC4 appears to be the actual trigger for the formation of the NLRC4 inflammasome. One reason a role for NLRP1 in Cathepsin B mediated NLRP3 activation has not been identified is that nearly all studies have been performed in murine cells which possess three paralogous NLRP1 genes and to my knowledge no mouse or cell line has been generated that lacks all 3 forms. To test the role of NLRP1 N-P in NLRP3 activation, N-terminal myc-tagged NLRP1 will be generated and *in vitro* cleavage achieved as described before. Purified NLRP1 N-P will then be transfected into WT and NLRP3 siRNA silenced and primed THP-1 cells.
Caspase-1 activation will be determined. If a difference is observed, further studies will be conducted to determine the nature of this interaction. Colocalization studies will be performed to directly visualize the interaction of NLRP1 N-P and NLRP3. FRET analysis of NLRP1 N-P and NLRP3 will be conducted by transfecting HEK cells with NLRP1 N-P-CFP under an endogenous promoter and NLRP3-YFP under an inducible promoter. The endogenous promoter will need to be chosen based upon ability to mimic similar expression levels to WT NLRP1 expression. If a direct molecular interaction occurs, emission will be detected by confocal microscopy. Given that FRET analysis requires an interaction distance of under 10 nM and the fact that placement of fluorophores on the NLRP1 N-P and NLRP3 may not bring these two fluorophores into close proximity despite an actual interaction, direct visualization of the CFP and YFP will be also performed by confocal microscopy. Highly magnified images of the cells may allow for the observance of colocalization even if FRET analysis is negative. If no role for NLRP1 N-P is identified in NLRP3 dependent inflammasome functioning, this protein will be assayed for its capacity to induce IL-1β release alone. Purified myc-tagged NLRP1 N-P will be combined with ASC in a cell free system and caspase-1 activity determined as before. The ability for this protein to induce cell death in the absence of IL-1β will be determined by transfection of unprimed THP-1 cells with NLRP1 N-P. I have personally hypothesized that cell death induced by NLRs is a mechanism to ensure that activated cells do not survive in the absence of continued PRR stimulation. That is to say that once the normal inflammatory cytokine substrates
for inflammatory caspases are removed, these activated proteases will accumulate in the cytosol and since apoptotic effector caspases are cleaved by other caspases the active caspase-1 will begin to cleave inactive caspases inducing death. This makes sense with the notion that pyroptotic cell death is an intermediate form of cell death, partially mimicking both necrosis and apoptosis. In a scenario in which a cell is dying due to removal of once existent inflammatory stimuli, it is logical to induce a form of cell death that is somewhat inflammatory but does not involve the lysis of all cellular contents.

All proposed studied involve the use of human cell lines and human proteins to delineate the mechanisms governing NLRP1 activation. This is because the structures of murine and human NLRP1 are distinct enough that it is possible they share only some overlap in function. Technical restrictions may require the use of murine cell lines for initial studies and careful examination will have to be performed to ensure that results found in murine cells are applicable to human cells. The fact NLRP1 has yet to be linked with substantial autoimmunity in mice provides evidence that the functional role of these proteins may not fully overlap.

Identification of human mutations in proteins that result in immune pathologies is a key step in determining the role these proteins play in in vivo immunity. NLRP1 mutations have been associated with Addison’s disease, vitiligo, and Type 1 diabetes among others. Estrogen-responsive B-box protein, or EBBP, is a member of the TRIM family of proteins that is turned on by estrogen signaling\textsuperscript{265}. It was shown that EBBP was capable of directly interacting with
NLRP1, caspase-1, and IL-1β and introduction of exogenous EBBP resulted in augmented IL-1β cleavage. These findings are of particular importance for two reasons. Other members of the TRIM family of proteins have been associated with the development of autoimmune disorders\(^ {266-270}\) and women are more highly predisposed to a range of autoimmune conditions when compared to men. Closer examination of the forms of autoimmunity elucidates an interesting distinction. Most male autoimmune disorders are characterized by heightened inflammatory pathology whereas many female autoimmune disorders have strong antibody components\(^ {271}\). IL-33 is an inflammasome substrate that is transcribed in a constitutive manner, with expression highest in endothelial and epithelial cells\(^ {272}\), and IL-33 has been linked with increased antibody responses and Th2 T-cell induction\(^ {273-281}\). Thus is it is possible that NLRP1 activation in the absence of additional inflammatory signaling (traditional signal 1 through PRR) drives IL-33 mediated Th2 immunity that becomes directed against host tissues resulting in auto-antibody production. The ability for a protein downstream of estrogen signaling to activate the NLRP1 inflammasome may provide the basis for the susceptibility to autoimmunity observed in women. It has been proposed that women are at risk for autoimmunity due to increased estrogen but studies have been inconclusive in proving this. One of the problems is that NLRP1 has a restricted tissue expression and many of these studies may have failed to identify its functioning in critical cell types.

In order to ascertain the role of estrogen signaling and EBBP on the induction of IL-1β, human THP-1 cells will be treated with various PRR stimuli
and the expression of the estrogen receptor will be assessed. It has previously been shown that PMA stimulation of THP-1 cells will induce estrogen receptor expression, so PMA in combination with a known inducer of signal 1 will be used as an alternative should traditional immune activating ligands be incapable of driving estrogen receptor expression. Once primed for estrogen receptor expression and inflammasome function, cells will be treated with increasing quantities of recombinant human estrogen. Depending on availability and cost, it may be necessary to use a chemical analog that is capable of binding the estrogen receptor. Cells treated with estrogen will be assayed for the induction of IL-1β release and cell death. Differing concentrations will be used to generate a curve identifying the optimal dose of estrogen to use in future experiments.

If cell death is observed as a result of estrogen signaling, it will be determined if PMA treatment alone results in IL-1β expression. If PMA treatment does not upregulate IL-1β, THP-1 cells will be treated with PMA alone followed by estrogen and cell death will be assayed. The capacity for NLRP1 to induce cell death in the absence of PRR priming would provide foundation to how autoinflammatory events can begin in the absence of microbial infection. In such a scenario, aberrant estrogen signaling would result in NLRP1 activation and eventually lead to cell death and/or IL-33 release which could begin the cascade that leads to the autoinflammatory pathology observed. These released components, in conjunction with estrogen signaling, could result in inflammatory signaling by neighboring cells which would serve as a signal for the recruitment and activation of additional immune cells. To confirm a role for NLRP1, THP-1
cells will be transfected with siRNA directed against NLRP1. Cells will then be stimulated to increase estrogen receptor signaling and followed by exogenous estrogen. Cytokine release and cell death will be measured and compared to NLRP1-replete cells. Many of the identified mutations in NLRP1 that result in autoimmunity have yet to be characterized in vitro and cell lines expressing these mutated NLRP1 proteins will be tested for capacity to induce cell death and cytokine release.

Limited research has been done on post-translational modifications on inflammasome components prior to and after activation. Given that related family members, including NOD2, are known to undergo post-translational modification which affects their function it is plausible that similar modifications occur on inflammasome components and may be critical for their functioning. A number of other TRIM proteins have been identified as E3-ubiquitin ligases. TRIM16, or EBBP, has been shown to mediate ubiquitination and histone acetylation. These prior results, taken together with the discovery that NLRC4 requires phosphorylation for normal activity, lead me to hypothesize that NLRP1 activation also requires post-translational modification. In order to test this, THP-1 cells will be isolated prior to and after estrogen receptor activation. Total protein lysates will be analyzed by tandem mass spectrometry. Particular interest will be paid to modification occurring on NLRP1, ASC, Caspase-1, and IL-1β. Any targets identified as possible post-translational modification sites will be mutated to silence modifications at this site. THP-1 cells transfected with these mutated genes will then be assessed for the capacity to induce caspase-1 activation.
following NLRP1 activation. Results will be compared to transfection with WT NLRP1. Additionally, THP-1 cells treated with siRNA against EBBP will be assessed for post-translation modification following estrogen signaling to determine the impact of EBBP on the modification of NLRP1. Positive identification of modifications involved in NLRP1 activation will be further investigated to identify the protein(s) which mediate these modifications. These proteins would provide additional therapeutic targets for the limiting of NLRP1 activation in autoimmune disorders. These studies can be expanded to include post-translational modifications occurring on NLRP3 and would provide additional insight into the molecular events involved in the function of that protein.

Confirmation of these in vitro findings in in vivo models would be ideal. The significant differences between murine NLRP1 proteins and human NLRP1 will likely make the study of NLRP1 in mice problematic. Other commonly used research animals including ferrets, rabbits, and pigs will be tested for structural homology to human NLRP1. If an animal is identified that expresses NLRP1 with the same domains as human NLRP1, it can be used as a model for determining the impact of this protein in vivo. Additionally, circulating PBMCs can be obtained from healthy individuals and individuals suffering from autoimmune disorders. These cells can be tested for their capacity to induce caspase-1 activation following estrogen treatment. Ideally, cells will also be obtained from patients with identified mutations in NLRP1. Collaboration with researchers at other institutes may be necessary to find patients with NLRP1 mutations.
Experimental evidence directly linking estrogen signaling to inflammatory cytokine production and/or cell death would provide the foundation for future therapeutics that may significantly reduce or eliminate a number of common autoimmune conditions. One of the major advantages to identifying NLRP1 as a target is that it has yet to be identified as a critical component of responses to common human pathogens. This means that therapeutic targeting of this protein may not lead to the pronounced defects in immunity to foreign pathogens seen with current immunotherapies such as TNFα neutralizing antibodies. Studies have found that TRIM16, or EBBP, is increased in synoviocytes in an arthritis model and that a mutation in NLRP1 is a risk factor for rheumatoid arthritis. Some of the most effective drugs at ameliorating autoimmune arthritis are drugs that reduce TNFα signaling. These drugs, however, leave individuals predisposed to numerous forms of pathogenic infections and blood malignancies. It is possible that targeting NLRP1 could have similar effectiveness in treating arthritic patients without the potential side effects associated with a number of current arthritis treatment.

NLRP1 was found to have a partially overlapping but distinct expression profile from the well documented NLRP3 inflammasome. While expression tended to overlap in hematopoietic-derived cells, distinct differences could be seen in human tissues. In particular NLRP1, but not NLRP3, was detected in the glandular and columnar epithelium in the respiratory, gastrointestinal, and reproductive systems whereas NLRP3 was predominantly expressed in squamous mucosal epithelium. NLRP1 was also found to be expressed in brain tissue,
including pyramidal neurons and oligodendrocytes, whereas minimal NLRP3 expression was detected in normal brain tissue. Lymphnodes, tonsils, and skin also stained positive for NLRP1 but not NLRP3. This differential tissue distribution may explain how NLRP1 has been found associated with tissue-specific autoimmune disorders, including those of the brain and skin.

A recent report on traumatic brain injury in rats identified injection of anti-ASC antibodies as capable of eliminating the NLRP1-dependent neuronal damage. While this therapy did not directly target NLRP1 and targeting ASC would likely have profound impacts on global immunity, it does demonstrate the possibility for anti-NLRP1 antibody treatment to be effective in reducing symptoms. Extensive research would need to be conducted into the best therapeutic approach for manipulating NLRP1 levels. Nanoparticle delivery of siRNA against NLRP1 is an additional possibility to reduce expression. There are a number of caveats associated with pursuing anti-NLRP1 therapy. Given that NLRP1 has been previously found to associate with APAF, it is possible this protein is a critical component of normal cell homeostasis. Reduction in this protein may result in cells that fail to undergo apoptosis in response to certain conditions. Such a defect in apoptosis could result in the development of malignancies, impact the functioning of hematopoiesis, impairment in restoration of normal homeostatic levels of various immune cells following infection, or general dysfunction in cellular overturn in normal organs. It is also possible that since NLRP1 is highly expressed in T-cells, including those in the thymus, that targeting NLRP1 could result in defective T-cell selection in the thymus. Such a
defect could result in greater autoimmune occurrence, as opposed to a reduction in symptoms.

The potential benefits of therapies directed at silencing NLRP1 function are not solely limited to autoimmunity. NLRP1 activation has been found to be a causative agent of neuronal dysfunction following various models of neuronal injury\textsuperscript{290-293} and NLRP1 variants have been associated with Alzheimer’s disease\textsuperscript{294}. Given the stark rise in the occurrence of traumatic brain injuries, especially in veterans and athletes, it is possible that blocking NLRP1 could prove a useful therapy in reducing neuronal degeneration following injurious stimuli.

The identification of NLRP1 as an estrogen responsive gene involved in the etiology of autoimmune disorders would be of great importance to our understanding of autoimmunity. The majority of autoimmune cases present in females and being able to demonstrate an experimental link to estrogen signaling would not only provide these individuals with the knowledge for what has contributed to their susceptibility to autoimmunity, it would open up new avenues of research aimed at ameliorating these diseases. The fact that this protein has been linked to the development of neurodegeneration further highlights the importance of understanding how this protein is activated and how it can be targeted for therapy in humans. As the age of the population increases, it only stands to reason that the incidence of neurodegenerative disorders will continue to rise. Added to the recent revelations of how concussive injury to the brain, even asymptomatic concussive events, can lead to the development of neuronal
degeneration and dementia research into the functioning of this poorly characterized inflammasome is absolutely necessary.

Future studies identifying crucial components of the immune response to *Candida*

Elucidate the capacity for NLRs to regulate the expression of other NLRs

Buccal mRNA expression studies clearly demonstrated substantial reductions in NLRP3 expression in *Nlrc4*−/− and NLRC4 expression in *Nlrp3*−/− mice. To my knowledge, this is the first evidence for the possibility of cross regulation between NLR family members. In order to assess the impact of NLRP3 and NLRC4 on the transcriptional regulation of one another, WT and gene knockout macrophages will be isolated by peritoneal lavage. Cells will be stimulated with ligands for PRRs including LPS, Pam3CSK, flagellin, CpG DNA, and β-glucan. Expression levels of NLRP3 and NLRC4 will then be determined by absolute qPCR using cloned standards to determine copy number. Results will be normalized to GAPDH and expressed as fold change over untreated. Given that inflammasome activation may be required, expression levels will also be
determined in cells additionally stimulated with known activators of NLRP3 and NLRC4, such as ATP or uric acid and flagellin. If decreased expression is observed in these knockout macrophages following stimulation, immortalized THP-1 cells will be transiently transfected with siRNA pools against NLRP3 or NLRC4. Knockdown of expression will be confirmed by Western Blot, and if necessary PCR. Cells will be stimulated as before and NLRP3/NLRC4 expression levels determined. Studies will be repeated with human cell lines to ascertain whether the same effects occur in humans. The identification of a cross-regulatory role would be novel and further research required to elucidate the precise mechanism(s) governing this phenomenon. One possible explanation is that in addition to their function as caspase-1 activators, NLRP3 and/or NLRC4 may be able to directly impact the transcription of genes by acting as nuclear factors in transcription complexes. The NLR protein CIITA is known to have a nuclear distribution and has been identified as a regulator of MHC class II gene expression \textit{in vivo}^{295,296}. The fact that the known function for a member of the NLR family is as a transcription factor, it stands to reason that it is plausible other members are capable of acting in this manner as well. If no discernable role is found for either NLRP3 or NLRC4 to directly affect the transcription of genes, WT, \textit{Nlrc4}^{-/-}, and \textit{Nlrp3}^{-/-} samples will be assessed for the expression of other NLR members including CIITA. Defective expression will lend new targets for research on the role of NLRs in directing gene transcription.
The role of additional pyrin-containing NLRs in the development of mucosal candidiasis

Given our findings that ASC deficiency resulted in greater susceptibility to oral and intestinal *Candida* infection than NLRP3 deficiency, it was posited that an additional NLRP(s) were involved in the control of mucosal infection. NLPR6 deficient mice were examined at 7 days post *Candida* infection. Loss of NLRP6 resulted in no difference in oral colonization however increased intestinal infection was observed as well as a slight increase in dissemination (Figure 5.1A-G). This is in agreeance with a few prior reports which have identified NLRP6 as functioning in intestinal tissue^{297-299}.

Further characterization of this intestinal phenotype will be conducted by prolonging infection time to 10, 14, and 21 days. Fungal loads as well as histology will be performed on portions of the small intestines. Additionally, the association of NLRP6 with colon inflammation will lead to the assessment of fungal loads in the large intestinal tissue of mice. One previous report identified NLRP6 as required for epithelial cell self-renewal, so WT and *Nlrp6*−/− will be assessed for intestinal permeability. Mice will be fed a diet consisting of exogenous lactulose and mannitol. The presence of these sugars will be measured in the urine of mice using a metabolic chamber and differences in intestinal permeability will be assessed. These experiments will be repeated following *Candida* infection to assess the impact of *Candida* infection on permeability and ascertain if lack of NLRP6 results in sustained increases in intestinal permeability following infection.
If a pronounced phenotype exists in NLRP6 KO animals, further dissection of the inflammatory activation in the bowels will be performed. qPCR analysis of mRNA expression in the small intestines and colon will be performed. Special attention will be paid to cytokines previously identified to be driven by NLR activation and that are known to be important for anti-fungal immune mechanisms. If perturbations in permeability are observed, known mediators of epithelial proliferation and wound closure will be assayed. Generation of bone-

![Figure 5.1. NLRP6 is specifically required for control of intestinal Candida infection.](image)

WT and Nlrp6−/− mice were infected orally with GDH2346. Fungally infected organs were harvested and quantitative fungal loads were determined. Early susceptibility to fungal loads were determined at 3 and 7 days for (A) Tongues, (B) Esophagi, (C) Stomachs, (D) Duodenum, (E) Jejunum, (F) Ileum, and (G) Kidneys. Data generated by Kruppen Patel. Statistical analysis was performed by Mann Whitney U-test (*p<0.05, **p<0.01, ***p<0.001)
marrow chimeric mice will allow for the determination of the general cell type involved in this NLRP6-dependent process. Once it is determined whether NLRP6 is required in the hematopoietic cells or the resident cells, *in vitro* assessment of implicated cell types will be performed. Cell types of interest will have NLRP6 expression silenced, either through acquisition of primary cells from *Nlrp6*−/− mice or through siRNA silencing of NLRP6 in immortalized cells such as epithelial cells and fibroblasts. The capacity for these cells to respond to *Candida* will be determined via caspase activation and cell death assessment. Given the limited amount of work on NLRP6, it would also be beneficial to assess the capacity for these cells to respond to bacterial ligands and whole bacteria. It is possible that the role of NLRP6 in the intestines is recognition of bacteria and modulation of subsequent responses. In this case, little to no effect would be expected with *Candida* treatment alone but bacterial stimulation may illuminate the function of this protein. If no discernable defect is noticed in any cell type tested, *in vivo* infected will need to be performed again using mice raised in germ-free conditions. It is possible that the role of NLRP6 in *Candida* infection has nothing to do with early immune activation but the predisposition to infection in these animals is due to perturbations in local gut flora which allow for more robust *Candida* overgrowth. Infecting mice in germ-free conditions will allow for the elimination of the effect of the microbiome on the functioning of NLRP6. If NLRP6 is not directly involved in *Candida* sensing, one would expect a mild or non-existent phenotype under germ-free conditions. As always, there are numerous caveats to the use of germ-free mice however it is the easiest procedure
to utilize that can help eliminate the contribution of the gut microbiome to immune responses.

NLRP6 is not the only additional NLR implicated in anti-Candida responses. It has been found that NLRP12 deficiency predisposes mice to significantly elevated mucosal and disseminated candidiasis at 7 days (Figure 5.2A-G). NLRP12 mutations have previously been associated with the development of periodic fever syndromes, presumably via release of IL-1β, and has been found to recognize the bacteria responsible for the Black Plague,
Initial studies will involved expanded OPC studies to more accurately describe the susceptibility of these mice. Studies will include CFU analysis at additional timepoints, histology of tongue sections 2-7 days post infection, and bone marrow chimerics will be used to determine which cell types are involved in this NLRP12-dependent Candida control.
One report identified NLRP12 as a negative regulator of colon tumorigenesis through inhibition of non-canonical NF-kB signaling. Though it seems unlikely that loss of NF-kB inhibition will result in greater *Candida* infection, initial studies in macrophages will assess the capacity for *Nlrp12* knockout cells to produce important cytokines including IL-1β, IL-6, CXCL1, and TNFα following stimulation with fixed *Candida*. Since NLRP12 was shown to affect non-canonical NF-kB, it is possible that this negative regulatory function is absent in response to *Candida*. If decreased cytokine production, especially IL-1β, is observed the capacity for this protein to interact with NLRP3 will be assessed.

NLRP3 deficiency was previously shown by my data to result in a complete loss of macrophage derived IL-1β following *Candida* treatment. While these results highlighted the critical role for NLRP3 in this process, they did not eliminate the possibility that other NLR family members are involved in the inflammasome complex downstream of *Candida*. Given the known relationship of NLRC4 and NAIP5, it is possible that NLRP12 is crucial for sensing the presence of *Candida* and that removal of this protein from the downstream inflammasome complex results in a reduction in proper functioning. Molecular studies will be conducted to ascertain the role of NLRP12 in inflammasome functioning. FRET analysis, or as an alternative confocal microscopy, can be utilized to directly visualize the interaction of NLRP12 and other NLRs, with NLRP3 being the initial target for screening. If neither of these methods is effective, a yeast two-hybrid screen involving the NACHT domains of NLRP3 and NLRP12 will be performed. The major limitation to the use of a yeast two-hybrid screen is that overexpression of...
these two domains may result in interaction, due solely to homology, that does not occur during normal physiological functioning. If an interaction is identified, future studies would seek to delineate the precise role NLRP12 plays in the NLRP3 inflammasome. It is possible NLRP12 is required for the activation of NLRP3 and it is also possible it is a scaffolding component in the complex designed to promote/stabilize complex formation without a direct effect on the initial activation of NLRP3.

If increased cytokine production is observed, either in vitro or in the serum/tissue of Candida infected mice, the mechanism by which increased cytokine production predisposes mice to increased infection will be assessed. It is quite easy to imagine how increased cytokine production can result in a stark increase in immune-mediate tissue pathology but a link to increased fungal colonization is less evident. In depth analysis of mRNA expression in these mice will need to be performed to identify if key antifungal cytokines identified as increased during in vitro studies continue to be elevated during OPC infection. It is possible that defensins or members of the IL-17 family, both of which are known to be important for Candida clearance, are reduced during in vivo infection in spite of an increased inflammatory phenotype of macrophages. If no reduction in known immune mediators is identified during OPC infection, in depth thought will need to be placed on how increased cytokine production results in increased fungal colonization. One possibility is that increased inflammatory signaling results in extensive tissue damage, releasing components that can be utilized by Candida for growth. In this scenario, even though immune cells are fully capable
of fighting the infection, the *Candida* may grow out of control in spite of this increased inflammation. Another possibility is that significantly increased inflammatory signaling in infected tissues impairs the normal trafficking of cells to the site of active infection. If loss of NLRP12 results in a hyper-activated phenotype, it is possible that key chemokines will be released aberrantly in underlying tissue where infection is less prevalent. Such release could affect the chemotactic gradient(s) utilized by immune cells to home to site of infection. Thus, even though inflammatory cell infiltrate and activation status of cells may be increased, many of these cells fail to associate directly with *Candida* which would limit their capacity to control infection.

The role of IL-18 in combating *Candida* infection

IL-18 is another cytokine that requires proteolytic cleavage to become biologically active. Gene expression studies from genetic knockout mice susceptible to oral candidiasis indicated reduced IL-18 expression post-*Candida* infection when compared to WT mice. Despite previous studies which demonstrated IL-18 was important in controlling *Candida* infection following intravenous injection, almost no research has been conducted on the role of this protein in mucosal immune responses\(^\text{303-307}\). One report found IL-18 upregulated in human mucosal epithelial cells grown *in vitro* and stimulated with *Candida*\(^\text{308}\). To test the role of IL-18 in mucosal *Candida*, *Il18*\(^{-/-}\) mice will be purchased from Jackson Laboratories (Bar Harbor, ME). Mice will be infected using the
GDH2346 OPC model and quantitative fungal loads determined for organs. In addition to the standard mucosal organs tested, the brain, liver, and spleen will also be harvested as additional markers for dissemination given the established role for IL-18 in controlling bloodborne infections. Histological sections will be prepared to assess the location of IL-18 production in infected tissues and levels of infiltrating immune cells including neutrophils, macrophages, and T lymphocytes. Cells identified by histology as likely producers of IL-18 will be assessed for capacity to produce IL-18 \textit{in vitro}. Identification of a single or select cells which produce IL-18 will allow future research aimed at characterizing the impact of specific deficiency in those cell types on the induction of immunity. Serum will be isolated from mice throughout the course of infection and key cytokines including IL-1β, IL-18, IL-6, and TNFα. Given that IL-18 requires the activity of inflammasomes to become activated, the ability for intravenous injection of recombinant murine IL-18 to overcome susceptibility observed in $Nlrp3^{-/-}$ and $Nlrc4^{-/-}$ mice will be assessed. Intravenous injection of \textit{Candida} has been shown to cause mortality, even in WT strains. Simultaneous injection of rmIL-18 along with a lethal inoculum of \textit{Candida} will be performed to assess the capacity for this cytokine to ameliorate \textit{Candida}-induced mortality. If an increase in survival is observed, additional tests will be performed to determine how soon following inoculation IL-18 must be introduced to protect against survival. Positive results may have significant impacts on the way patients are treated in hospitals following a positive blood culture for the presence of fungus in the bloodstream.
Capacity for recombinant mBD1 to ameliorate oral *Candida* infection

A major goal of fungal immune research is to identify pathways and proteins that are targets for the development of therapies. Given the known anti-*Candida* activity of mBD1 and our findings of a crucial role in control of mucosal candidiasis, this protein represents a novel prophylactic treatment in mucosal *Candida* infections. To test the efficacy of mBD1 as a topical treatment for oral candidiasis, WT mice will either be infected with *Candida* alone or following inoculation, receive topical dorsal tongue treatment with a solution containing recombinant mBD1. Recombinant murine β-defensin 1 will be generated by inserting FLAG-tagged mBD1 into a CMV promoter construct. N-terminal and C-terminal tagging will both be tried, separately, given the small size of mBD1 and potential for effect on confirmation. The plasmid will be stably transfected into HEK293T cells and protein purified by antibody-affinity columns. FLAG tag will then be cleaved off using enterokinase. Treatments will be given 3x daily. The level of dose will be calculated based upon *in vitro* studies assaying the capacity for fungal killing at various concentration levels. Given that WT mice are not particularly susceptible to oral candidiasis and clear the infection on their own, it may be necessary to conduct studies in a susceptible strain such as *Il1r1*−/− to more clearly delineate the effectiveness of treatment. Measures to assess effectiveness of mBD1 treatment include quantitative CFU loads for the tongues, visual disease severity, and gross histology of tongues. CFU can also be measured for other mucosal organs in the gastrointestinal tract to determine the impact oral treatment
has on general mucosal colonization. The treatment can be repeated in a model of vulvovaginal candidiasis to test the effectiveness of topical mBD1 treatment on control of vaginal yeast infections.
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