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Graduate Studies

NEUTROPHIL EXTRACELLULAR TRAP – FORMATION FOLLOWING  
*BLASTOMYCES DERMATITIDIS* YEAST STIMULATION

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the  
Degree of Master of Science in Biology

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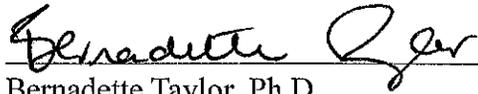
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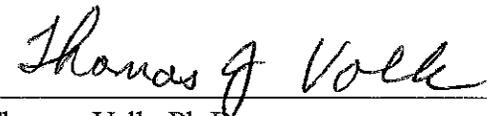
By Angela K. Ratekin

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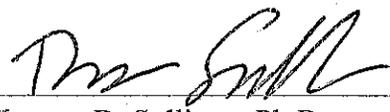
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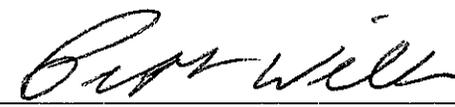
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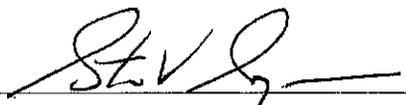
  
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## ABSTRACT

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Blastomycosis, a potentially fatal infection results from an infection developing from a thermo-dimorphic fungal pathogen *Blastomyces dermatitidis*. The pivotal points driving the pathogenesis associated with the varied clinical presentations are not understood. The invasive morphotype of *Blastomyces dermatitidis*, the yeast are large (up to 20µm diameter) making phagocytosis, an internal mechanism used by neutrophils difficult. Neutrophils, predominant immune response cells, also have an extracellular mechanism, Neutrophil Extracellular Trap (NET) formation. *Blastomyces dermatitidis* yeast express an essential virulence factor, *Blastomyces* Adhesin factor (BAD1). Neutrophils were stimulated with a wild type *B. dermatitidis* expressing the virulence factor  $BAD1^{+}$  or with knock out *B. dermatitidis*  $BAD^{-}$  not expressing the virulence factor to determine if BAD1 modulates NET-formation. Neutrophil elastase (NE) activity was measured to indirectly quantify NET-formation and a Colony Forming Unit (CFU) assay measured the percent surviving yeast allowing for assessment of the NET's fungicidal/fungistatic ability. The level of NET-formation and the percent surviving yeast for each yeast strain were compared when neutrophils from different donors were stimulated. The ability of yeast to evade a powerful extracellular mechanism used by the host immune response would allow continued pathogenesis.

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## TABLE OF CONTENTS

	PAGE
TABLE OF CONTENTS.....	v
LIST OF FIGURES .....	vii
INTRODUCTION .....	1
Blastomycosis .....	1
<i>Blastomyces dermatitidis</i> .....	7
Host Immune Response .....	11
Neutrophils.....	15
Neutrophil Extracellular Traps (NETs) .....	19
<i>Blastomyces</i> Adhesin factor (BAD1).....	23
RESEARCH OBJECTIVES .....	26
MATERIALS AND METHODS.....	27
Yeast Isolates .....	27
Human Neutrophils.....	29
Neutrophil Extracellular Trap-formation Assay .....	30
Table 1. Control and sample setup for the NET-formation assay.....	31
Neutrophil Elastase (NE) measurement.....	32
Table 2. Control and sample setup for the NET-formation assay using the NET assay kit.....	35
Colony Forming Unit (CFU) .....	36
Indirect Immunofluorescence Staining for BAD1 .....	37
RESULTS .....	39
Growing and Storage of <i>Blastomyces dermatitidis</i> yeast .....	39
Neutrophil Isolation from human blood .....	40
Neutrophil Extracellular Trap-formation.....	40
Quantification of NET-formation .....	41
Yeast Survival.....	42

Confirmation of BAD1 expression .....	45
DISCUSSION .....	47
REFERENCES .....	51
APPENDICES.....	56
Appendix A. Biological Materials and Recombinant DNA Registration.....	56
Appendix B. Institutional Review Board Review .....	63

## LIST OF FIGURES

FIGURE	PAGE
1. Pathogenesis of blastomycosis.....	3
2. Thermo-dimorphic <i>Blastomyces dermatitidis</i> .....	10
3. Peripheral blood smear. ....	15
4. Neutrophil Extracellular Trap (NET) - formation. ....	19
5. Separation of whole blood.. ....	29
6. An illustration of the NET-formation assay.....	33
7. <i>Blastomyces dermatitidis</i> broad-based budding yeast.. ....	39
8. Fluorescence micrograph of <i>Blastomyces dermatitidis</i> and NETs.. ....	40
9. Neutrophil elastase measurements.....	42
10. Percent Survival CFU of <i>Blastomyces dermatitidis</i> .....	43
11. Percent Survival CFU of NET related <i>Blastomyces dermatitidis</i> .....	44
12. Scatter plot comparing surviving yeast and neutrophil elastase levels.....	45
13. Fluorescence micrograph of BAD1 expression.. ....	46

## INTRODUCTION

The initial infection site for blastomycosis is in the lungs. Severe respiratory pathogenesis is associated with excessive neutrophil activity in the lungs. This clinical presentation has been observed in cases of blastomycosis with *Blastomyces dermatitidis* yeast expressing the essential virulence factor *Blastomyces* Adhesin factor (BAD1). Therefore, because BAD1-negative *B. dermatitidis* have less virulence our hypothesis was that BAD1 plays a role in triggering NET-formation potentially leading to severe respiratory pathogenesis in blastomycosis. To answer this question we developed the following goals:

- I. To study if *B. dermatitidis* yeast stimulate NET-formation by human neutrophils.
- II. To determine if BAD1 expression by *B. dermatitidis* yeast plays a role in NET-formation.
- III. To determine if NET-formation is capable of killing or inhibiting growth of *B. dermatitidis* yeast.

### **Blastomycosis**

Blastomycosis is a fungal disease resulting from a true fungal pathogen, *Blastomyces dermatitidis* that is potentially fatal and presents with a wide variety of clinical presentations. In North America *B. dermatitidis* is endemic in areas along the waterways found in regions including the Mississippi and Ohio River basins, areas around the Great Lakes, and also areas in the southeast region of the United States (Pfaller & Diekema, 2010). There are some hyperendemic areas found within these

regions, specifically in the north central part of the state of Wisconsin (Chapman et al., 2008). Blastomycosis occurs in outbreaks and in sporadic cases within these regions when the mold morphotype that is growing in the damp, acidic, enriched soil due to decaying organic material is disturbed and the airborne conidia are inhaled. The states that have mandatory reporting of blastomycosis are Arkansas, Louisiana, Michigan, Minnesota, Mississippi, and Wisconsin. Three of the five states that have the highest incidence of blastomycosis, Illinois, Kentucky, and Tennessee currently do not have mandatory reporting requirements when diagnosing blastomycosis (Seitz, Younes, Steiner, & Prevots, 2014). In the mortality report prepared by Khuu et al. 1,216 cases of blastomycosis-related deaths were identified during the years of 1990-2010. The highest incidence occurred in white males with a median age of 60.8 years. The observed bias in gender is attributed to occupational or recreational exposures. Examples are working in construction or forestry and recreational activities such as hunting. The authors note that death resulting from blastomycosis is preventable if diagnosis is early and appropriate treatment is administered. Itraconazole is used for mild or moderate infections and amphotericin B for severe cases (Khuu, Shafir, Bristow, & Sorvillo, 2014).

However, the first hurdle is to arrive at the correct diagnosis which can be challenging with cases of blastomycosis for several reasons. Blastomycosis needs to be included in the differential diagnosis after a lengthy incubation period that ranges from 3-15 weeks and 30%-50% of the cases are asymptomatic (Khuu et al., 2014). A positive culture confirming blastomycosis can take up to 30-days and other methods, including observation during histological examination are less sensitive. Antigen detection by serological testing or PCR are also less sensitive and may result in cross reactivity from

*Histoplasma capsulatum*, another true fungal pathogen that has overlapping endemic regions with *B. dermatitidis* (Khuu et al., 2014; Seitz et al., 2014).

Even when blastomycosis is included in the differential diagnosis the varied clinical presentations make exclusion difficult. The clinical manifestations of blastomycosis can be divided into pulmonary and extrapulmonary presentations. The

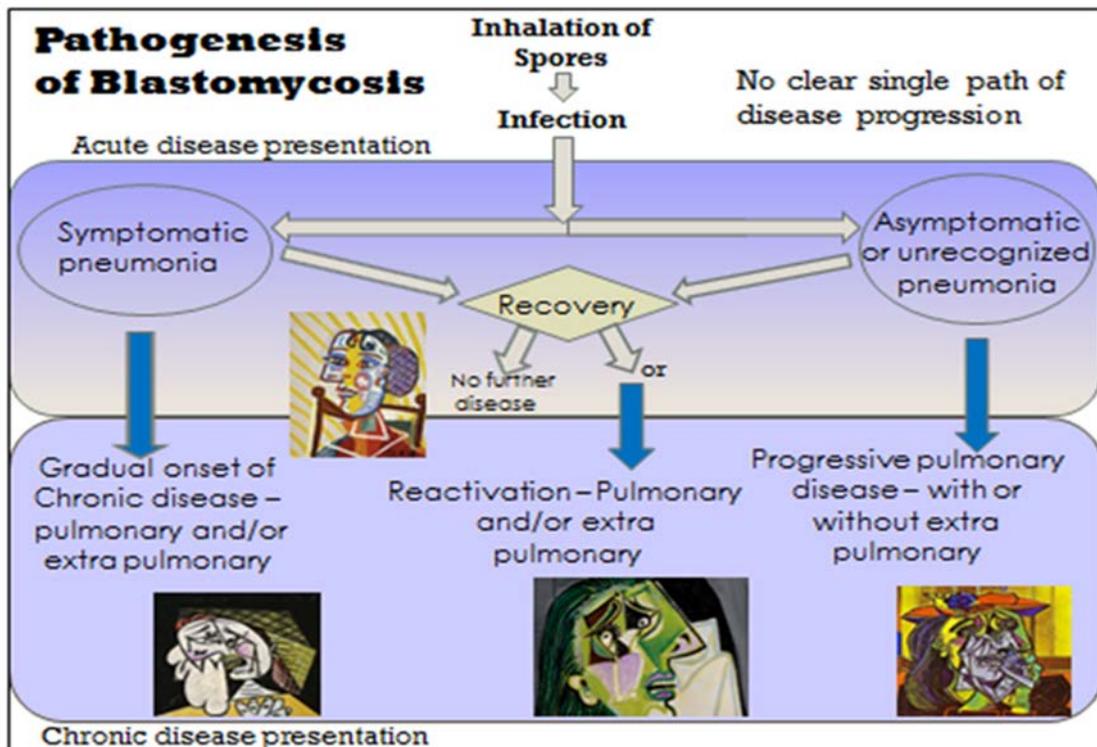


Figure 1. Pathogenesis of blastomycosis. The initial symptoms do not predict the path of pathogenesis and the path does not follow a progressive presentation that worsens with each stage of development. The symptoms may present in an ebb and way manner. The clinical presentation has a wide range of variability making prognosis difficult.

initial infection starts in the lungs and there are no known clear indications as to which cases will progress to severe pulmonary infection or which cases will progress to an extra-pulmonary infection (Figure 1). The pathogenesis of blastomycosis does not always follow a direct path that progressively leads to a more severe disease state. An initial respiratory presentation may progress to a more severe pulmonary presentation or it may

resolve only to reactivate months later, again as a pulmonary presentation or as an extrapulmonary presentation. The chronological presentation of blastomycosis often has more of an ebb and flow pattern that covers several months making an accurate patient history important when trying to reach the challenging diagnosis of blastomycosis.

In humans there are four general categories of blastomycosis (Saccante & Woods, 2010); asymptomatic, cutaneous, disseminated, and respiratory as described below.

Asymptomatic – the absence of symptoms or only a mild respiratory presentation with radiographic abnormalities. An asymptomatic infection is hypothesized in nearly 50% of patients, with the potential of progressing months later and presenting as a chronic cutaneous infection (Chapman et al., 2008; Khuu et al., 2014).

Cutaneous – skin ulcers present as verrucous or ulcerative lesions are found on the cooler parts of the body, the face, arms and legs, following a respiratory exposure. Verrucous ulcers are wart-like, raised and crusted lesions with irregular borders. The lesions grow out above subcutaneous abscesses and can mimic basal cell cancer, squamous cell cancer, and giant keratoacanthoma. Ulcerative lesions are cutaneous ulcers resulting from a subcutaneous abscess draining out through the skin forming a heaped-up border with or without an exudative base.

Disseminated – the common sites of disseminated blastomycosis are skin, bone, and genitourinary tissues (Saccante & Woods, 2010). The classic triad of blastomycosis is pulmonary, skin, and bone lesions (Davies & Sarosi, 1989). However, the yeasts can travel anywhere in the body using the lymphohematogenous system once they have escaped the lungs. Disseminated blastomycosis is never self-limiting (Davies & Sarosi,

1989) and cases with moderate to severe pneumonia or immunocompromised host require anti-fungal therapy to achieve the resolution of blastomycosis (Chapman et al., 2008; Saccente & Woods, 2010).

Respiratory – moderate to severe respiratory presentation are described in more detail below (Saccente & Woods, 2010).

Solely looking at respiratory/pulmonary blastomycosis one can artificially divide the pathogenesis into four presentations; respiratory infection with influenza-like symptoms, acute pneumonia with symptoms that are indistinguishable from bacterial pneumonia, chronic respiratory infection resembling tuberculosis that can be confused with lung cancer, or an acute rapidly progressive severe manifestation of acute respiratory distress syndrome (ARDS) associated with a high mortality rate (Davies & Sarosi, 1989; Wallace, 2002).

Influenza-like symptoms include fever, chills, myalgia, headache and a nonproductive cough may occur, often resolving within days. The symptoms are brief and self-limited and often go undiagnosed except with an investigation of an ongoing known outbreak when physicians are actively looking for cases of blastomycosis.

Acute pneumonia symptoms include high fever, chills, productive cough, pleurisy, and chest pain. Sputum is mucopurulent or purulent. The symptoms are indistinguishable from bacterial pneumonia but can be distinguished microscopically.

Chronic respiratory infection symptoms include low-grade fever, a productive cough, and night sweats, accompanied by weight loss. Sputum is mucopurulent or purulent and may present with blood. The symptoms resemble tuberculosis or lung cancer.

Acute Respiratory Distress Syndrome (ARDS) symptoms include fever, shortness of breath, tachypnea, and hypoxemia and diffuse pulmonary infiltrates.

The variability of the presentation creates difficulty with arriving at the correct diagnosis. The radiographic findings vary from lobar consolidation to miliary infiltration to large masses making the presentation difficult to distinguish from bacterial pneumonia, tuberculosis, histoplasmosis, acute respiratory distress syndrome, or bronchogenic carcinoma (Wallace, 2002). If misdiagnosed, antibacterial treatment may exacerbate the infection. The antibiotic alters the host's normal flora reducing host microbe competition against the fungus. Another scenario may occur whereby the antibacterial treatment is given inappropriate credit for resolution of the symptoms, thus allowing blastomycosis to progress to a systemic infection (Saccante & Woods, 2010). Even a mild or asymptomatic pulmonary presentation does not predict which cases will remain as a pulmonary presentation only, versus progressing to an extrapulmonary presentation.

Again another challenge is that the initial incubation period for blastomycosis is varied and can be as long as 3-15 weeks; it can then be months before the secondary symptoms occur. The long incubation contributes to the failure of environmental detection of *B. dermatitidis* at potential exposure sites and the patient's ability to accurately recall when a possible exposure may have occurred. An extra-pulmonary infection occurs 25%-40% of the time a lung infection is established (Chapman et al., 2008). Again, the first challenge is to have blastomycosis included in the differential diagnosis. A thorough patient history must be obtained, including outdoor activities within areas of known endemic blastomycosis. The second challenge is to correctly rule out the other etiologies that may be creating the observed symptoms. Health care

providers need to keep in mind that there is not a clear single path of progression with blastomycosis (Figure 1).

The vigilance of physicians in or near endemic regions is needed to improve the prognosis of blastomycosis. The degree of pathogenesis depends on virulence: toxin production, the ability to enter host tissue, the ability to colonize, the ability to hijack nutrients from the host and/or the ability to suppress the host's immune response as well as the exposure load. Once *B. dermatitidis* conidia are inhaled, the change in environment, especially temperature, triggers the mold morphotype to convert to the yeast morphotype. The invasive yeast morphotype expresses an essential virulence factor, *Blastomyces* ADhesin factor (BAD1) that manipulates the host's immune response by reducing the inflammatory response (T. T. Brandhorst, Rooney, Sullivan, & Klein, 2002; Romani, 2011). What drives each particular pathogenesis is a question that can only be answered by having a clear understanding of the host's immune response in general and the specific interactions with the etiologic agent, *Blastomyces dermatitidis*.

### ***Blastomyces dermatitidis***

Medically important fungi have developed diverse mechanisms to facilitate the establishment of an infection and not all medically important fungi are created equal. Fungi are widely found throughout the environment and are difficult if not impossible to completely eradicate. The clinical presentations of mycosis are highly variable, making a correct diagnosis difficult. Mycoses are difficult to prevent; there are few vaccines, there are a limited number of options available for treatment, and the treatment for an infectious eukaryotic agent within an eukaryotic host can result in harsh side effects for the host (Blanco & Garcia, 2008). Interestingly only a few of the well over 75,000

described species of fungi are considered true pathogens, capable of causing disease in immunocompetent hosts (Brown, 2011). These are the phylogenetically related members of the Ascomycota in the order Onygenales; *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides* spp., and *Paracoccidioides brasiliensis*. They are all dimorphic and ecologically restricted to specific niches.

For a fungal infection to develop beyond the subcutaneous layer of the host, fungi must overcome the primary immune defense barriers and be able to survive at 37°C.

There is a small group of thermo-dimorphic fungi whose primary exposure route is by inhalation of spores that germinate into hyphae. Upon exposure to body temperature, hyphae convert from mold morphology into a yeast morphotype. With this change *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* alter their cell wall glucan polymer linkage from  $\beta$ -(1,3) to  $\alpha$ -(1,3) glucan to evade detection and reduce their immunostimulatory impact. Other virulent species such as *Cryptococcus neoformans* are not dimorphic, but have a thick extracellular capsule consisting of glucuronoxylomannan (GXM) that reduces detection by the immune response cells. The capsule also reduces anti-inflammatory cytokines such as IL-10 from being released by monocytes, thus modulating the immune response (Levitz, 1992).

*Blastomyces dermatitidis* yeast cells are also capable of modulating the immune response when the virulence factor BAD1 is expressed. *Blastomyces* adhesin factor will be discussed in greater detail below. Along with the expression of a virulence factor the yeast morphotype of *B. dermatitidis* develops a thick wall that protects the cell from the direct attachment of complement thus lowering cell lysis, and reduces the ability of host immune response cells to successfully carry out phagocytosis. Along with the thick cell

wall, *B. dermatitidis* yeast morphotype cells are large, up to 20  $\mu\text{m}$  also making phagocytosis difficult (Zhang, Brandhorst, Kozel, & Klein, 2001). Fungi, such as *H. capsulatum* have developed the ability to survive within the phagocytic immune response cells by inhibiting the phagolysosome fusion and modulating the pH (Chai, Netea, Vonk, & Kullberg, 2009). The yeast morphotype of *Blastomyces*, *Paracoccidioides*, and *Histoplasma*, all true fungal pathogens, bind to macrophages via complement receptor 3 (CR3, CD18/CD11b). The CR3 receptor is found on several immune response cells, including neutrophils and macrophages. When the yeast morphotype attaches to the CR3 receptor found on the host immune cells there is a reduction in available sites for host antibody attachment to the yeast limiting opsonization. The ability of the immune cells to attach to additional yeast cells is also reduced. Both result in reduced phagocytosis of the organism by immune response cells (Rappleye & Goldman, 2008).

There are other challenges beyond cellular mechanisms that need to be considered when trying to understand any mycosis. The primary route of exposure is through the respiratory tract when aerosolized mycelial fragments or spores are inhaled (Gauthier & Klein, 2008). In the case of blastomycosis the mold morphotype of *B. dermatitidis* found in the environment is disturbed and the small conidia, 2-10  $\mu\text{m}$  are able to reach deep into the host's lungs where infection begins. Once in the lungs the temperature triggers conversion to the yeast morphotype.

Morphogenesis starts with conidia germinating into mycelium, and then the mycelium converts into the yeast morphotype. The conversion is triggered by an increase in temperature, changes in oxidative stress, carbon dioxide tension, and steroid hormone levels (Cooney & Klein, 2008; Gauthier & Klein, 2008; Saccente & Woods, 2010).

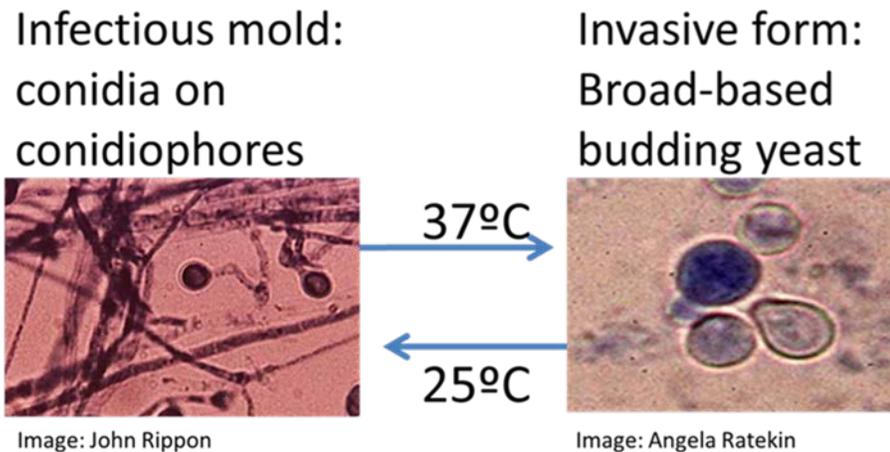


Figure 2. Thermo-dimorphic *Blastomyces dermatitidis*. Mold stained with safranin and the yeast stained with lactophenol-cotton blue viewed at 100X using a bright field microscope.

Dimorphic fungi do not require transition from one morphotype to the other in order to complete their life cycle. Yet they maintain the genetic information that allows the organism to directly interact with and regulate the mammalian host's response along with the information required for fungal growth and survival, independent of the host (Odds, Gow, & Brown, 2001). However, conversion to the yeast morphotype is required for pathogenesis (Klein, 1997). The large broad-based budding yeast is unique to *B. dermatitidis* (Figure 2). *Blastomyces dermatitidis* is the name used for the imperfect or asexual state of *Ajellomyces dermatitidis* (Figure 2). The fungus grows as a filamentous mold morphotype in the environment at ambient temperatures in acidic, moist, soil that has a high organic content due to decaying material. Microscopically the fruiting bodies of the mold are hyaline, ovoid to pyriform, one-celled, smooth-walled conidia (2-10  $\mu\text{m}$  in diameter) born on a short lateral or terminal hyphal branches. Exposure results from disturbing contaminated soil following weather that has provided the correct conditions for the mold morphotype to develop and release airborne conidia (Light et al., 2008). The

conidia must overcome drastic changes to survive in the challenging new environment of mammalian, specifically human lungs as compared to the soil.

As *B. dermatitidis* converts to a large (8-20  $\mu\text{m}$ ) broad-based budding yeast in the host, the cell wall content simultaneously develops a 95%  $\alpha$ -glucan layer that covers the predominantly antigenic  $\beta$ -glucan cell wall found on the mycelial form (T. Brandhorst, Wüthrich, Finkel-Jimenez, & Klein, 2003; Brown, 2011; Gauthier & Klein, 2008; Kanetsuna & Carbonell, 1971). The change in fungal cell size, the change in the composition of the outermost cell wall, and the expression of an essential virulence factor, BAD1, all aid in the success of *B. dermatitidis* establishing disease in the human host. We know that the interaction of specific factors presented by both the fungus and the host immune response during those first few days influence the resulting disease presentation, but the pivotal points that drive the pathogenesis are still not fully understood. One challenge with studying pathogens and immune response to those pathogens is to find ways to isolate one specific interaction within the context of the big picture all the while keeping in mind that *in vivo* these interactions do not occur independently. Therefore we need to consider the overall role that the host immune response plays in the resulting pathogenesis of blastomycosis before considering the specific factors involvement.

### **Host Immune Response**

Taking a step back and looking at the big picture one must realize that the mammalian immune response is the result of constant cycling of reciprocal mechanisms that trigger on/off responses. The immune response is somewhat artificially divided into two branches, the innate and the adaptive. There are redundancies within the response

and both branches work simultaneously. The ideal outcome is a balanced response that eliminates danger with minimal damage to the surrounding tissue – homeostasis. There are times when the host’s immune response contributes to pathogenesis. The enzymes and antimicrobial agents used to kill microbes also results in host tissue damage, considered to be collateral damage. Unfortunately, the response may overshoot what is needed adding to the severity of the pathogenesis. There are several factors that contribute to a less than ideal immune response: the level of microbe exposure, microbe adaptations that modulate the host immune response, and the timing of various triggers within the response. The various players of the immune response attempt to maintain homeostasis, minimizing host tissue damage while stopping the infection. Along with the complexity of pathogenic fungi we need to understand the complexity of the host immune response.

The innate branch of the immune system is a set of broad somewhat nonspecific defense mechanisms that develop shortly after birth without prior sensitization. The innate response carries out several tasks: recognizes a threat, initiates an attack, and gathers information to present to the adaptive branch. In short, the innate response is the initial attempt to contain and clear potential threats. The adaptive branch leads to the development of antibodies, memory, and potential immunity based on the information gathered and presented by the innate branch. There are many triggers that initiate an immune response, and they include cellular and non-cellular effectors. Once a threat is recognized, the effectors trigger a broad array of defense mechanisms. Specific effectors will be discussed in more detail below. The status of the response is continuously assessed and the resulting pathogenesis is, in part, the result of the type of and the degree

of the response mounted by the host. That being said, the initial evaluation of the situation by the innate branch establishes the primary attack against developing infections. Factors within the innate response are the first driving forces influencing the resulting pathogenesis.

When the innate branch detects a potential threat, interaction between the immune response cells and the microbe initiates a response. The interaction of the pathogen with the host's immune cells results from a limited list of germline encoded receptors, Pattern-Recognition Receptors (PRRs), found on the host cells, that recognize a group of conserved molecular patterns common to broad groups of microbial species, the Pathogen-Associated Molecular Patterns (PAMPs). Fungal PAMPs include glucans, chitin, and mannoproteins (Brown, 2011). Microbes need to attach to host tissue cells, thus enabling colonization. The host immune response cells, neutrophils and macrophages predominantly, need to attach to microbes to contain and destroy, as well as to gather information about the microbe. The recognition resulting from the attachment of the PAMPs to the PRRs triggers the release of non-cellular effectors, cytokines and chemokines, to signal the immune system as to what is needed to contain and destroy the invading microbes. The immune response is shaped by the secretion of cytokines and chemokines resulting from the interaction between the pathogen and the immune response cells.

The initial innate immune response resulting from a majority of pathogenic fungi occurs in the lungs after the mycelial fragments, including conidia, are inhaled. The innate response signals the possibility of a threat and initiates an attempt to contain the mycelial fragments and prevent colonization. Once the inhaled conidia are detected by

the host, and there are three opportunities to contain and eliminate dimorphic fungi, kill the inhaled conidia, prevent phase transition, or kill the invasive yeast morphotype. The pathogen attempts to establish a focus of infection within the epithelial layer of the alveoli. In the respiratory tract, disease results from the colonization of the mucosal surfaces and the ability of the pathogen to overcome or bypass the innate immune defenses associated with the cells lining the alveoli. The innate immune response includes activation of macrophages and neutrophils to release destructive compounds including reactive oxygen intermediates, reactive nitrogen intermediates, antimicrobial agents, the production of proinflammatory signals secreted by macrophages and neutrophils, and for phagocytosis to begin (Nicola, Casadevall, & Goldman, 2008; Rappleye & Goldman, 2008).

A successful pathogen must achieve a state that is less recognizable to avoid detection and then also develop a state that is able to resist being killed from either destructive compounds or phagocytosis, thus enabling the establishment of infection. As mentioned earlier, *B. dermatitidis* converts from the mold morphotype, very small conidia to the large yeast morphotype. During this conversion the outer cell wall consisting of highly antigenic component,  $\beta$ -glucan is covered with a new cell wall consisting predominantly of  $\alpha$ -glucan thus dampening the recognition of the organism (Brown, 2011). The initial reduction in antigenicity and the large size of *B. dermatitidis* yeast morphotype help the fungus resist phagocytosis. A primary player involved in detection, containment and the attempt to kill the invading microbe in the innate immune response is the neutrophil, the predominant population of white blood cells found circulating throughout the host's body.

## Neutrophils

Neutrophils are the predominant immune cell population found in human blood, making up sixty percent of circulating white blood cells, leukocytes, in the human host. They are often referred to as a polymorphonuclear leukocyte (PMN) because of their lobed nucleus (Figure 3). They passively circulate throughout the body on patrol ready to protect us from pathogens and the development of disease. Cases of neutropenia show us that they are indispensable in controlling infections. Neutrophils are professional



Figure 3. Peripheral blood smear. Neutrophil in a peripheral blood smear stained with Wright stain.

phagocytes, cells capable of engulfing microbes and destroying them within the neutrophil. Following the intracellular killing the neutrophil becomes a bag of information regarding the microbe. When the neutrophil is collected for removal the information about the invading microbe is presented by antigen presenting cells of the immune response to the adaptive branch of the immune response. The bone marrow of a healthy adult produces  $1 - 2 \times 10^{11}$  neutrophils per day (Borregaard, 2010). During development, granulopoiesis, in the bone marrow haematopoietic stem cells progress through stages of differentiation and during these different stages, groups of granules develop giving rise to the term granulocytes. The first group of granules to develop early in the differentiation of a neutrophil is the azurophilic or primary, followed by the specific or secondary, and then the gelatinase granules or tertiary (Borregaard, 2010). The granules contain  $>700$  proteins creating an armament to fight against pathogens and disease (Kruger et al., 2015). Ideally the cellular maturation of neutrophils is completed

in the bone marrow and mature cells are released to circulate throughout the body. The release of cells from the bone marrow is tightly regulated in healthy individuals and neutrophils have a short life span ranging from a few hours to a few days (Simon & Kim, 2010). The naïve neutrophils circulate throughout the body, making them readily available throughout the host's body. If danger is detected, they are pulled out of circulation and into the tissue to attack the imminent threat.

The immune response begins when conidia and hyphal elements of *B. dermatitidis* are inhaled into human lungs. The PRR on the immune response cells, namely dendritic cells and macrophages detect the PAMPs, chitin and  $\beta$ -(1,3) glucan found on the mold. Recognition of the threat and a call for help is sent with the release of cytokines from the macrophages. Inflammation develops at the site of detection and neutrophils are pulled from the circulation. Neutrophils are capable of generating signals to trigger four key actions: to retard their own accumulation, to suppress their own activation, to promote their own death, and to attract and program macrophages to stop the damage and orchestrate repair (Nathan, 2006).

Every breath taken by the host carries the potential for introducing a high enough exposure load of a microbe to result in an infection. Asymptomatic presentation and mild symptoms have been documented with cases of blastomycosis. This presentation may be the result of a low exposure load. However, sometimes blastomycosis progresses to a systemic presentation following mild respiratory symptoms. This would imply that there is more to the story than a simple dose response. In the lungs, depending on the exposure load, the alveolar macrophages and neutrophils may be able to contain the conidia of *B. dermatitidis* through phagocytosis, a mechanism that engulfs microbes and carries out

an intracellular destruction of the microbe (Saccente & Woods, 2010). Phagocytosis is one of the primary defense mechanisms used to contain and eliminate threats. The conidia not contained convert into yeast morphotype and the neutrophil's phagocytosis effort are unsuccessful when trying to deal with these large thick-walled yeast cells (Klein, Sondel, & Jones, 1992). As the yeast cells multiply one would hypothesize that the signal for the initial response utilizing phagocytosis has failed and that the site continues to request neutrophils to leave the circulation, enter the infected tissue, and activate to continue the fight against the pathogen. The mechanisms used by neutrophils for defense include oxidative burst, phagocytosis, degranulation, and Neutrophil Extracellular Trap (NET) formation (Nathan, 2006). Neutrophil extracellular traps are an extracellular mechanism utilized by neutrophils to contain and destroy microbes. The yeast morphotype of *B. dermatitidis* ranges in size from 8-20  $\mu\text{m}$  and neutrophils range in size from 12-15  $\mu\text{m}$  making phagocytosis difficult (Drutz & Frey, 1985). When considering the response from neutrophils against the relatively large yeast morphotype of *B. dermatitidis*, it is reasonable to accept that the failed attempts of phagocytosis leads to signaling for NET-formation. The signal of failing phagocytosis would trigger the request for another mechanism, an extracellular mechanism to be called into play. Extracellular microbes may trigger NET-formation more often than smaller microbes.

When cytokines, like Tumor Necrosis Factor (TNF) recruit neutrophils to sites of danger we don't know how the precise numbers of neutrophils needed is determined or how the decision regarding which mechanism to activate is determined. We do know that neutrophils are present and initially in large numbers. Although neutrophils express lower levels of cytokines than macrophages their presence in numbers certainly impacts the

resulting pathogenesis during a response. A majority of the immune cell research has looked at macrophage involvement. Macrophages and neutrophils are both professional phagocytes and they share some similar signaling abilities during inflammation/infectious settings. They also have similar antimicrobial and immunomodulatory abilities. However, during the development of these specialized and complementary mechanisms, the resulting antimicrobial ability and level of cytotoxicity differs in tissue location and the lifespan of each cell type (Silva, 2010).

Neutrophils and macrophages both have CR3 receptors and both release TNF-  $\alpha$  when activated. It is important to keep in mind how the partnership between neutrophils and macrophages manages the development and resolution of inflammation. Even though macrophages and neutrophils both develop from a common lineage the impact of similar mechanisms used by both cells may have different outcomes due to specific characteristics of each cell. The role of the neutrophil goes beyond recognition of a threat, signaling the alarm, and brute destruction; the response is modulated based on the neutrophil assessment, followed by a highly regulated customized response (Mantovani, Cassatella, Costantini, & Jaillon, 2011). Initial activation can be triggered and shut down continuously without any detectable symptoms. Neutrophils make an impact due to their sheer numbers and by the sophisticated roles they carry out during activation. One of those sophisticated roles is the ability to form Neutrophil Extracellular Traps (NETs) (Brinkmann et al., 2004), described in more detail in the next section. The formation of NETs may in turn trigger a new profile of cytokines creating new messaging to redirect the pathogenesis progression during the course of blastomycosis. The specifics of how, when, and which neutrophils are activated to form NETs is not fully understood. By

taking a closer look at the interaction occurring between neutrophils, specifically the extracellular mechanism of NET-formation and the yeast morphotype of *B. dermatitidis*, we will gain a better understanding of possible triggers involved in the immune response mounted against *B. dermatitidis*.

### **Neutrophil Extracellular Traps (NETs)**

Historically the mention of neutrophils would start and stop with phagocytosis.

Neutrophils have been regarded as a nonspecific brute force triggered to destroy invaders using phagocytosis and unsophisticated external dumping of toxic compounds leaving host cell damage in their wake. However, it is now recognized that neutrophils

deserve a closer look.

The observation of NETs has resulted in renewed interest with regards

to the neutrophil's role in the host's immune response. The ability of neutrophils to form NETs, which is not fully understood, is an intricate part of the immune response driving pathogenesis. Neutrophil extracellular traps were first observed using electron microscopy and their basic structure and function were defined by (Brinkmann et al., 2004). Smooth fibers with diameters of 15 to 17 nm and globular domains with diameters around 25 nm that aggregated into larger strands with diameters of up to 50 nm were thrown from neutrophils that had a de-lobed nucleus. The NETs are able to trap and kill

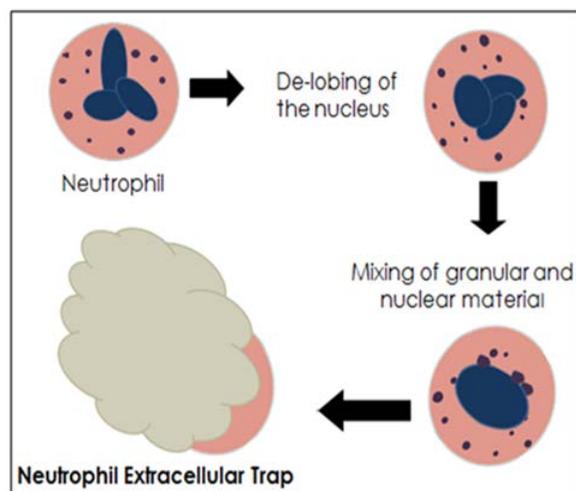


Figure 4. Neutrophil Extracellular Trap (NET) - formation. The nucleus de-lobes and combines with the granules forming a net of DNA decorated with the granular contents that is then thrown out of the cell.

bacteria extracellularly. When an activated neutrophil receives the signal, not yet defined, to start NET-formation, granular and nuclear components within the neutrophil combine and the nucleus loses its lobed shape, resulting in a NET consisting of a DNA backbone embedded with antimicrobial peptides and enzymes (Figure 4). As the acronym suggests the NETs are thrown out of the cell like a fisherman's net trapping the invading microbe (Brinkmann et al., 2004).

The antimicrobial peptides and enzymes are found in the granules of the neutrophils. Granules are categorized into groups based on the cell's stage of division when the granules develop. The primary or azurophilic granules develop first during the second stage of neutrophil differentiation, the promyelocyte stage. The azurophilic granules include neutrophil elastase (NE), a product found in NETs along with other antimicrobial compounds (Cowburn, Condliffe, Farahi, Summers, & Chilvers, 2008; Grommes & Soehnlein, 2011; Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012). When a neutrophil is activated the last granules formed, the gelatinase or tertiary are the first to be released. Once in the tissue activation leads to additional granules being released: the release of the tertiary granules is followed by the secondary and finally the primary or azurophilic granules (Kumar & Sharma, 2010). When we consider the first formed granules, the azurophilic granules are the last released it implies an extreme level of commitment needed from neutrophils once NET-formation is initiated. Interestingly all neutrophils do not activate to form NETs (Remijsen et al., 2011). Other antimicrobial compounds associated with NETs, such as histones, defensins, proteinase 3, heparin binding protein, cathepsin G, lactoferrin, myeloperoxidase along with neutrophil elastase decorate the strands of DNA that carry them out of the neutrophil forming a trap targeting

extracellular microbes (Papayannopoulos & Zychlinsky, 2009; Borregaard, 2010). The release of neutrophil elastase is associated with NET-formation (Papayannopoulos, Staab, & Zychlinsky, 2011).

The formations of NETs are the result of a complex mechanism that is organized and requires more development time than phagocytosis. Phagocytosis occurs within minutes and NET-formation requires 2-4 hours (Kaplan & Radic, 2012). The other subsets of granules have already been released resulting in an intense level of tissue damage to the host. It stands to reason that a small localized section of tissue damage is worth the cost if the microbes are killed. However, if the response requires a larger area of tissue or several smaller areas and the tissue damage is severe the secondary symptoms could result in additional harm to the host. The challenge for the immune system is to maintain homeostasis without adding to the pathogenesis when cytotoxic proteins are thrown from neutrophils. Understanding the details involved in NET-formation would help us understand pathogenesis. There are still many unanswered questions regarding how these tasks are carried out. What is the exact signaling profile and how a determination of which mechanism is called into action are still questions that need to be answered. Neutrophil extracellular traps may help explain the old classification of pus into viscous “pus bonum”, good pus containing NETs and liquid “pus malle”, bad pus not containing NETs as a prediction of favorable and poor outcomes of infection (Mantovani et al., 2011).

As with other immune response mechanisms there are microbes that have developed mechanisms to evade NETs. Gram positive bacteria including Group A *Streptococcus*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* release nucleases

that dissolve the NETs. *Mycobacterium tuberculosis* bacilli modulate increased neutrophil death resulting from NET formation referred to as NETosis, while *Pseudomonas aeruginosa* and *Aspergillus* spp. use molecular mimicry appearing as self, to avoid detection from NETs (Hahn, Giaglis, Chowdury, Hösli, & Hasler, 2013). However, the neutrophil cytosolic protein calprotectin associated with NETs has fungicidal activity against *Candida albicans* (Urban, Reichard, Brinkmann, & Zychlinsky, 2006). When two different strains of *Paracoccidioides brasiliensis* with different degrees of virulence were used to challenge neutrophils two unique patterns of NETs were observed microscopically (Della Coletta et al., 2015).

*Blastomyces dermatitidis* is considered to be a large extracellular microbe that expresses a virulence factor, BAD1, shown to modulate the immune response by reducing the expression of TNF- $\alpha$  from macrophages. This may affect the number of neutrophils being called to the site of infection. The impact of individual factors and the timing of these events define the resulting pathogenesis. Factors such as the initial concentration of *B. dermatitidis*, the level of each form of BAD1 expression, soluble and cell bound, and the number of neutrophils present at the site of infection when the signal is given for NET-formation, all play a role in determining the clinical presentation of blastomycosis. Interestingly not all neutrophils activate to form NETs (Remijsen et al., 2011). Understanding the interaction between NETs and the yeast morphotype of *B. dermatitidis* will help us understand the role NETs have in the innate response and the impact they have in driving the varied pathogenesis of blastomycosis.

There are situations when the ability to contain microbes and keep the host tissue damage localized is beneficial for the host. However, if the exposure level of *B.*

*dermatitidis* is high, the initial number of neutrophils is high, and the release of NETs is increased, the level of host tissue damage is great an acute respiratory distress may be the resulting presentation. Conversely if the exposure load is low, fewer neutrophils are called to the site of infection and the formation of NETs is low. If so, the pathogenesis may present as a mild respiratory infection allowing the yeast to survive and deepen the infection to a chronic respiratory presentation or even a systemic presentation. Exposure levels of the fungus and BAD1 expression are specific factors that drive the varying presentations of blastomycosis. Therefore understanding the specifics involved with the interaction of *B. dermatitidis* with NETs will provide a better understanding of the immune response and the resulting varied presentations of blastomycosis. Understanding the interplay between the *B. dermatitidis* virulence factor BAD1 and the host's immune response, specifically the formation of NETs and how this interplay affects the development of blastomycosis may lead to answers that will improve diagnosis and be used in treatment and possibly vaccine development.

### **Blastomyces ADhesin factor (BAD1)**

We know the presence of BAD1 is not required for the phase-conversion and is not found on hyphal elements or conidia (T. T. Brandhorst, Wüthrich, Warner, & Klein, 1999; T. Brandhorst & Klein, 2000; T. T. Brandhorst et al., 2002). However, once the conversion to yeast morphotype begins BAD1 is rapidly secreted. *Blastomyces* adhesin factor is detected as soluble BAD1 in the extra-cellular spaces and also attaches to chitin found on the yeast cell surface, essentially forming layers of BAD1 (T. Brandhorst et al., 2003). The trigger for secretion of BAD1 and what determines the level of expression as either cell bound versus soluble has not been fully ascertained. Unfortunately to-date

there has not been a good method developed to measure BAD1 expression. Another thing to keep in mind would be the value of understanding how the binding works and what it looks like. When the layers of BAD1 are disrupted and a total concentration is measured it doesn't tell the whole story. We need to understand the binding capacity and the interaction with all the immune response cells *in situ* to better understand the complete functionality of BAD1.

*Blastomyces* adhesin factor is a 120 kDa antigen/adhesin with three structural domains: an N-terminal hydrophobic domain that spans the cell membrane, a C-terminal epidermal growth factor-like domain, and a central domain of 24-amino acid tandem repeat array in tandem (T. T. Brandhorst et al., 1999). Interestingly the 24-amino acid tandem repeat adhesin region of BAD1 is 90% homologous to an adhesin virulence factor, invasion associated with gram-negative bacteria in the genus *Yersinia* (Klein, Chaturvedi, Hogan, Jones, & Newman, 1994). One of the roles of cell surface BAD1 is to promote binding of the yeast morphotype to the epithelial tissue cells of the alveoli.

Attachment promotes the establishment of an infection. The BAD1 expressed on the yeast morphotype as well as the soluble BAD1 in the extra-cellular space binds to the CR3 receptors found on phagocytic immune response cells, including neutrophils. The level of TNF- $\alpha$  released by macrophages is reduced when BAD1 binds to the CR3 receptor (T. T. Brandhorst et al., 1999). The result of BAD1 binding to CR3 receptors on macrophages decreases the expression of TNF- $\alpha$  but we don't know how the binding to the CR3 receptors found on neutrophils modulates the cytokine profile.

Tumor Necrosis Factor is one of the major cytokines involved in the innate immune response, stimulating the immune response by calling neutrophils to the site of

an impending infection. Remembering the redundancies found in the host's immune response it is interesting to note the abundant presence of neutrophils in the pus of the cutaneous lesions found with systemic blastomycosis. It is also interesting to consider the role of tumor necrosis factor, an endogenous pyrogen inducing fever, a generator of the signal for apoptotic cell death, and also the role TNF- $\alpha$  plays as a major factor in the development of inflammation. The clinical presentation of blastomycosis is a combination of pyogenic and granulomatous inflammation of varying degrees. We will need to understand the specific triggers, including how and by what means they are generated, in order to explain the wide variation of clinical presentations. A better understanding of the change in the signaling profile and how that affects the activation of NET formation would lead to answers regarding what drives the pathogenesis of blastomycosis. The expression of BAD1 and also the host's immune response, specifically the formation of NETs, both play important roles in the resulting pathogenesis.

## RESEARCH OBJECTIVES

To meet the goals listed in the introduction the following objectives were developed:

1. Obtain wild type  $BAD1^+$  and the isogenic knockout  $BAD1^-$  *B. dermatitidis* isolates, and learn to culture and store these isolates for future use.
2. Establish a protocol for isolation of neutrophils from human blood.
3. Develop methods for demonstrating and measuring NET-formation.
  - a. Neutrophil elastase assay
  - b. Fluorescence microscopy
4. Perform the NET-formation assay using *B. dermatitidis* yeast isolates, WT and KO as stimulants by co-culturing each independently with neutrophils from different human donors and then comparing the levels of NET-formation resulting from  $BAD1^+$  and  $BAD1^-$  strains of *B. dermatitidis*.
5. Find out if NETs were able to kill or inhibit growth of *B. dermatitidis* yeast using a colony forming unit assay and comparing the results from  $BAD1^+$  and  $BAD1^-$  strains of *B. dermatitidis*.

## MATERIALS AND METHODS

### Yeast Isolates

A Biological Materials and Recombinant DNA Registration Protocol was approved by the University of Wisconsin-La Crosse Institutional Biosafety Committee (Appendix A). *Blastomyces dermatitidis* is a true fungal pathogen capable of causing infection in immunocompetent host. The mold morphotype is highly infectious due to sporulation of conidia and a Bio-level III laboratory is required for cultivation of the mold morphotype. The yeast morphotype is considered pathogenic, not infectious and is classified as a Bio-safety level II microbe. Many of the microbiology teaching and research laboratories at the University of Wisconsin-La Crosse are classified as Bio-safety level II. However, *B. dermatitidis* was not an organism maintained on campus so a Biological Materials and Recombinant DNA Registration was submitted. Submitting the request provided an opportunity to consider a plan to handle the storage, the culturing, and the discarding of the yeast as well as how to handle the yeast during the experiments. The yeast needed to be held at 37°C in order to maintain the yeast morphotype.

Strains of *B. dermatitidis* received from the Marshfield Clinic were used for the preliminary work: successfully maintaining cultures, accurately counting yeast suspensions using a hemocytometer, and development of protocols used to safely manage any waste potentially containing yeast generated in the laboratory. Information regarding the source of the isolates was not available. A *Candida albicans* isolate was used for the

initial NET-formation assay to help determine if there were any additional safety issues that needed to be addressed.

Yeast isolates used in the final stages of the study included a BAD1-expressing wild type strain and a BAD1-negative knockout strain. The wild type (WT) BAD1<sup>+</sup> strain studied was ATCC strain 26199 (American Type Culture Collection, Manassas, VA), a virulent isolate of *B. dermatitidis* originally obtained from a human patient. The knockout (KO) BAD1<sup>-</sup> strain studied was #55 strain (T. T. Brandhorst et al., 1999) an isogenic, nonpathogenic isolate derived from ATCC strain 26199 of *B. dermatitidis*. The knockout strain has impaired binding ability, limiting entry into macrophages. The yeast have also lost the ability to adhere to lung tissue (T. T. Brandhorst et al., 1999). Isolates of both strains of yeast were provided by Thomas Sullivan and the Bruce Klein laboratory at the University of Wisconsin – Madison.

The yeast cultures were maintained following a strict protocol, holding cultures and waste containing yeast at 37° C to prevent conversion of the yeast morphotype back to the infectious mold morphotype. Once frozen aliquots of the isolates were received, the two strains were spread on separate brain heart infusion (BHI) agar plates. At the first sign of growth the yeast were transferred to separate 7H10 slants. The cultures were transferred every 2-days of growth, 2-3 times resulting in healthy yeast isolates with enough growth to prepare aliquots for storage. The yeast were suspended in of 1ml sterile water with 10% glycerol and stored in liquid nitrogen. An aliquot of each strain was thawed and transferred to a BHI agar plate and again at the first sign of growth the yeast were transferred to 7H10 slants. Both isolates were passaged after 2-days growth, 2-3 times before being used in an experiment. The yeast isolates were maintained on 7H10

slants at 37° C; with a maximum of 10 passages to ensure BAD1 was still being expressed with the WT strain. The yeast isolates were grown in liquid *Histoplasma* Macrophage Medium (HMM) in a shaking incubator at 37° C for 24-hours prior to being used in the NET-formation assay. This allowed the yeast to be in early log phase of growth. The yeast suspension was loaded onto a hemocytometer to count. There was variability in the budding and clumping among the *B. dermatitidis* isolates making it difficult to confidently count. An attempt was made to disrupt the clumps by aspirating and dispensing the suspension through a 25g needle on a 1 ml syringe. An attempt was also made to read the turbidity of the suspension after disrupting the clumps. However, the clumps were still too large even after aspirating them through the 25g needle to provide a consistent turbidity reading. The clumps were therefore disrupted and each clump was tallied as one using a hemocytometer.

### Human Neutrophils

Human neutrophils were isolated from whole blood collected from healthy adult volunteers. A written informed consent was obtained from each volunteer before blood was collected. The request form was approved by the University of Wisconsin-La Crosse Institutional Review Board (Appendix B). Two 10 ml tubes per human donor of fresh heparinized whole blood were collected using a vacutainer phlebotomy system. Neutrophils were isolated from the whole blood using two gradients of Histopaque,

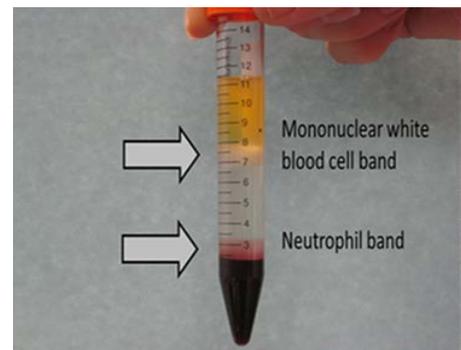


Figure 5. Separation of whole blood. Using two density gradients of Histopaque, H-119 and H-1077 heparinized whole blood is separated and two bands of leukocytes, the mononuclear leukocytes and the polymorphonuclear leukocytes (neutrophils).

H-1119 and H-1077 (Sigma Chemical Co., St. Louis, Mo). The two density gradients were brought to room temperature and then carefully layered in a 15ml conical screwcap centrifuge tube. The less dense layer of Histopaque, H-1077 was layer on top of a layer of H-1119 followed by a layer of heparinized whole blood. After centrifugation, the white blood cells formed two bands of leukocytes, the mononuclear leukocyte band and the desired polymorphonuclear leukocyte band (neutrophils) just above the red blood cells (Figure 5). Everything above the desired neutrophil band was aspirated and discarded. The neutrophil band was aspirated into a clean tube and the isolated cells were washed twice and suspended with sterile phosphate-buffered saline (PBS).

### **Neutrophil Extracellular Trap-formation Assay**

The initial NET-formation assays were run following a modified protocol received from Scott Cooper's laboratory UW-La Crosse Biology, utilizing microscopic observation of fluorescence-stained cells. Controls were setup following Table 1. The negative control well was seeded with neutrophils only, without a stimulant added and the positive control well was seeded with neutrophils and stimulated with Phorbol 12-Myristate 13-Acetate (PMA 20 nM). For the samples to be tested neutrophils were stimulated with yeast in the presence of PMA and in the absence of PMA.

A glass 12 mm coverslip pretreated with poly-L-Lysine was placed in each of the wells of a 24-well culture plate and seeded with human neutrophils at a concentration of  $3.5 \times 10^5$  cells/ml. Before the neutrophils were stimulated the plate was placed in a 37°C incubator at 5% CO<sub>2</sub> for 1-hour incubation. After stimulants were added the plate was returned to the incubator for an additional 2-hours.

Table 1. Control and sample setup for the NET-formation assay

	Neutrophils	PMA	yeast
Negative control	X		
Positive control	X	X	
N + yeast	X		X
N + PMA + yeast	X	X	X

N = neutrophils

PMA = phorbol 12-myristate 13-acetate

Yeast = ATCC 26199 *Blastomyces dermatitidis*

A stain solution was prepared resulting in the following final concentration for each of the three stains: Sytox green 0.5  $\mu$ M, Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) 4  $\mu$ M, and calcofluor white. Sytox green penetrates the compromised membrane of the neutrophil binding to nucleic acids. Orange CMTMR is able to pass through the cell membrane of living neutrophils and calcofluor white binds to chitin found in the cell wall of the yeast resulting in blue fluorescence.

After the final incubation the liquid media was carefully aspirated and discarded. Leaving the coverslips in the wells, they were stained using 300  $\mu$ l of prepared stain solution per well. The plate was held in the dark for 30-minutes for staining. The stain was carefully aspirated off and replaced with a media that utilizes a bicarbonate buffering system, Roswell Park Memorial Institute (RPMI). The RPMI buffer was replaced a second time as a wash. After the final aspiration the coverslips were carefully removed from the 24-well plate and mounted, cell side down on a slide for microscopic examination. Fluorescence microscopy showed that NETs were formed after the

neutrophils were stimulated with *B. dermatitidis* yeast. However, a method to quantify NET-formation was required.

### **Neutrophil Elastase (NE) measurement**

The measurement of Neutrophil Elastase (NE) would indirectly measure NET-formation. An attempt to develop an in-house NE measurement methodology was unsuccessful due to the lack of reproducibility. Quantitative fluorescence microscopy based upon methods reported by Brinkman et al. was explored but not all the necessary monoclonal antibodies were commercially available (Brinkmann, Goosmann, Kühn, & Zychlinsky, 2013). A newly released neutrophil extracellular trap (NET) assay kit (cat. No. 601010, Cayman Chemical Company, Ann Arbor, MI 48108) was then used following the diagram illustrated in Figure 6. This kit measures neutrophil elastase as a method of quantifying NET-formation.

The first part of the NET assay kit protocol was similar to the protocol used earlier for NET-formation using a known stimulus to challenge neutrophils. The known chemical stimulus, phorbol 12-myristate 13-acetate (PMA) was provided with the NET assay kit at a concentration of 50 nM. Neutrophils were co-cultured with PMA alone acting as a positive control for each run ensuring the neutrophils used were capable of NET-formation. The two strains of yeast were independently co-cultured with neutrophils in the presence of and the absence of PMA. The mechanisms involved with NET-formation have not been fully ascertained and by stimulating the neutrophils with a known stimulant along with the yeast allowed for the assessment of the fungicidal/fungistatic capabilities of the NETs using the CFU assay described below.

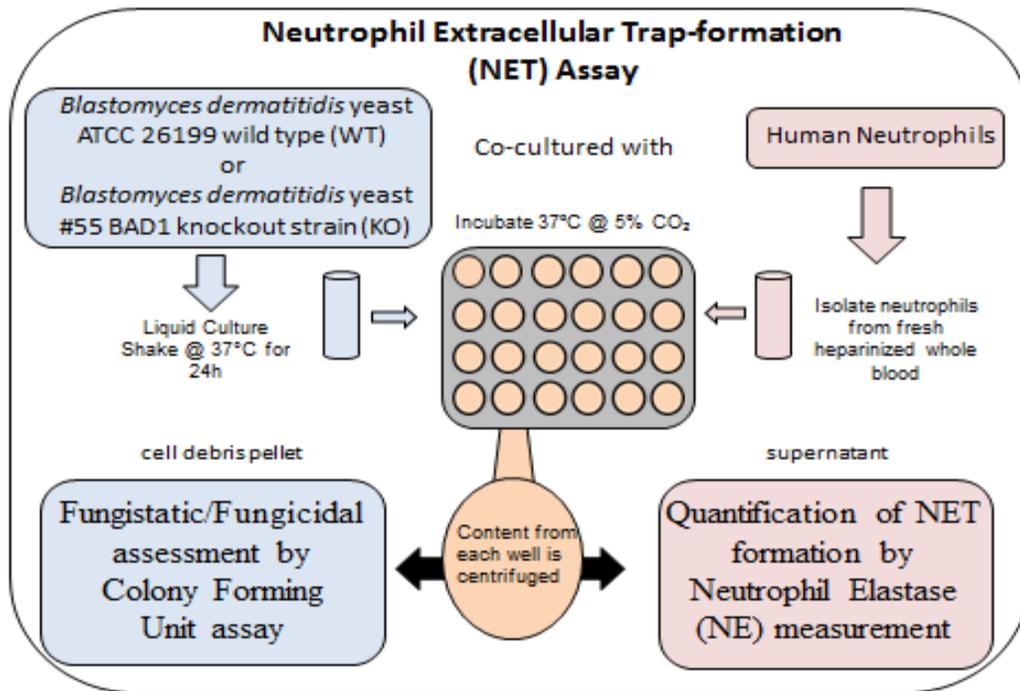


Figure 6. An illustration of the NET-formation assay. A flowchart of the NET-formation assay demonstrating the resulting products: supernatant to be used for the quantification of NET-formation and cell debris pellets to be used for the assessment of the fungistatic/fungicidal capabilities of NETs when human neutrophils are stimulated independently with two isogenic strains, WT BAD<sup>+</sup> and KO BAD<sup>-</sup> of *Blastomyces dermatitidis*.

Neutrophils were seeded in a 24-well tissue culture plate with a final concentration of  $1 \times 10^6$ /ml. This concentration is low enough to minimize spontaneous NET-formation (Brinkmann et al., 2004). Several assays were run using the same neutrophil donor to confirm reproducibility and then two additional donors were used to control for potential variability in human donor neutrophil function. Each control and each sample were tested using duplicate wells. An incubation at 37°C in 5% CO<sub>2</sub> for 2-hours allowed NETs to form. After the incubation the supernatant was carefully aspirated, saved and pooled with the subsequent washes, keeping each well separate. The wells were washed to remove soluble neutrophil elastase that is not NET-associated. The

pooled washes were centrifuged and the debris pellet used as part of the CFU assay to account for surviving yeast and to quantify yeast not trapped in the NETs.

After the washes the NETs were disrupted using S7 nuclease (15,000U/ml) releasing NET associated neutrophil elastase. The content of each well were transferred to sterile microfuge tubes; an EDTA solution was added to inactivate the nuclease. The microfuge tubes were centrifuged resulting in two samples, the supernatant to be used in the quantification of NET-formation/NE measurement assay and the cell debris pellet to be used in the yeast survival/CFU assay. The supernatant was carefully collected and stored at -20° C for testing at a later date to measure the NET-associated NE levels. The neutrophil elastase measurement was used to indirectly measure NET-formation and is described as follows. The yeast survival/CFU assay is described in the next section.

Controls and samples for the quantification of NET-formation/NE measurement assay was setup following Table 2. The negative control provided a measurement of possible spontaneous NET-formation. Previous studies have shown there can be a low level of spontaneous NET-formation without any form of stimulation, chemical or microbial (Brinkmann et al., 2013). A positive growth control for the yeast survival/CFU assay was setup using a well with yeast only.

Quantification of NET-formation was achieved by measuring the enzymatic activity of neutrophil elastase. As mentioned earlier, NE is found in the primary or azurophilic granules. The azurophilic granules are the first to form during the development of the cell and the last granules to be released from neutrophils. Elastase is essential for NET-formation; partially degrading histones and promoting chromatin to become decondensed (Papayannopoulos & Zychlinsky, 2009; Cheng & Palaniyar, 2013).

Table 2. Control and sample setup for the NET-formation assay using the NET assay kit

	Neutrophils	PMA	KO yeast	WT yeast
Negative control	X			
Positive control	X	X		
N + KO	X		X	
N + PMA + KO	X	X	X	
N + WT	X			X
N + PMA + WT	X	X		X

N = neutrophils

PMA = phorbol 12-myristate 13-acetate

KO = #55 BAD1-negative knockout *Blastomyces dermatitidis* yeast

WT = ATCC 26199 BAD1-positive wild type *Blastomyces dermatitidis* yeast

A specific elastase substrate, N-methoxysuccinyl-Ala-Ala-Pro-Val *p*-Nitroanilide, when selectively cleaved by elastase releases a 4-nitroaniline product that absorbs light at 405 nm. The frozen supernatant samples were thawed and transferred to a 96 well plate in duplicate; each control/sample was setup in duplicate wells in the NET-formation assay. Therefore, each control/sample would have four absorbance readings. All four absorbance readings for each control/sample were averaged and converted to NE mU/ml using the standard curve generated with each run.

The standard curve was generated by carrying out a serial dilution of neutrophil elastase to achieve eight concentration ranging from zero mU/ml – 36 mU/ml. The absorbance of the eight standards was plotted versus the known neutrophil elastase concentrations to establish the standard curve for the run. A milliunit is one thousandth of a unit of a specific enzyme that produces a certain amount of enzymatic activity that converts one micro mole of substrate per minute according to the General Conference on Weights and Measures. Each standard was setup in duplicate. Each well received the

addition of elastase substrate, and the plates were held at 37° C for 2-hours. After the incubation the plate was read at 405 nm. The absorbance data for each well was collected. The elastase concentration of each control and unknown sample was determined by converting the absorbance reading to mU/ml of NE using the standard curve established for that run.

### **Colony Forming Unit (CFU)**

A CFU assay was used to determine the fungistatic/fungicidal capabilities of NETs against the two stains of *B. dermatitidis* yeast, WT and KO. Throughout the NET-formation procedure following the protocol established with the NET Assay kit described above and diagramed in figure 6, the wells were seeded with neutrophils and stimulated with yeast in the presence of PMA or the absence of PMA. The washes for each well were collected and pooled keeping each well separate. The pooled washes were centrifuged and the debris pellet was diluted and plated. The CFU counts from the pooled washes were used to determine the percent of the total number of yeast plated that survived the procedure but were not trapped in the NETs produced by the neutrophils.

After the NETs were lysed the contents of each well was centrifuged and the supernatant was aspirated and stored for further testing to measure the NE levels as described above. The cell debris pellet was diluted and plated. The CFU counts from the debris pellet were used to determine the percent of the total number of yeast plated that survived the procedure and also survived being trapped in the NETs produced by the neutrophils.

Preliminary assays were run to ensure the lysate reagent used to disrupt the NETs did not alter the colony forming ability of the yeast. The assay was run as described

above with the addition of duplicate yeast only wells. Each strain had a well that received lysate reagent and a second well that received buffer only. A cell scraper was used to release the cells from the wells without lysate. It was determined that the lysate did not alter the results of the CFU assay. The well with yeast only served as a positive growth control ensuring viable yeast were present following all the steps of the NET assay. Appropriate dilutions to be used for the debris pellets in the CFU assay resulting in countable plates were determined. A 100  $\mu$ l of each suspended debris pellet sample was spread onto Brain Heart Infusion (BHI) plates. The same incubation environment for the NET-formation assay was continued for the CFU assay. The plates were held at 37° C, with 5% CO<sub>2</sub> for 5-days, screened for growth, and colony count data was collected.

### **Indirect Immunofluorescence Staining for BAD1**

Indirect immunofluorescence staining was used to confirm that the WT strain of *B. dermatitidis* was still expressing BAD1 at the time the experiments were setup. Following the 24-hour shaking incubation step used to prepare the yeast for the NET-formation assay with Donor 1 neutrophils each strain of yeast was transferred to fresh 7H10 slants to provide yeast to set the NET-formation assay with Donor 2 neutrophils using the same aliquots of yeast. After the second NET-formation assay with Donor 2 neutrophils was set the yeast suspensions were tested to confirm the expression of BAD1 using the Indirect Immunofluorescence staining protocol.

The procedure utilized glass coverslips pre-treated with poly-L-Lysine and placed in a 24-well culture plate prior to being seeded with the suspension of yeast cells. Incubation at room temperature for 10-15 minutes allowed the yeast to attach to the coverslips. Excess media was aspirated off and the cells were washed twice with Tris-

Buffered Saline (TBS) and then blocked with TBS 1% bovine serum albumin (BSA) for 10-minutes followed by two more washes with TBS. The excess TBS was aspirated off and the monoclonal mouse anti-adhesin antibody, DD5-CB4 Fab fragment that binds to BAD1 at a concentration of 50  $\mu\text{g}$  per  $1 \times 10^6$  yeasts was added. The plate was held for 60-minutes followed by two more washes with TBS. After the excess liquid was removed a secondary fluorescence tag of FITC conjugated rabbit anti-mouse IgG at a 1/2500 dilution was added. Because the secondary antibody is conjugated to a fluorochrome, it was necessary to minimize light exposure from this point forward; the plate was placed in the dark for 60-minutes. The excess liquid was aspirated off and the coverslips were mounted on a microscope slide and viewed using a fluorescence microscope. Both yeast isolates were tested. The KO BAD1<sup>-</sup> strain served as a negative control.

## RESULTS

### Growing and Storage of *Blastomyces dermatitidis* yeast

Following the Biological Materials and Recombinant DNA Registration Protocol approved by the University of Wisconsin-La Crosse Institutional Biosafety Committee (Appendix A) protocols were developed to safely maintain cultures and to safely handle the waste that was generated that might contain yeast. Appropriate dilutions to use in the CFU assay were determined. The CFU

assay was set using dilutions of each isolate to determine the appropriate dilution to use following the NET-formation assay. The BHI plate incubation continued to be at 37° C, in 5% CO<sub>2</sub>, the same as the incubation environment conditions used in the NET-formation assay. An incubation period of 5-days

allowed the yeast colonies to reach a large enough size to be counted accurately.

To confirm that the isolates were still in yeast form when being used in the experiments a drop of each yeast suspension from the MMH liquid inoculum used to plate the yeast for the NET-formation assay was mixed with a drop of Polyvinyl lactophenol cotton blue stain. The two were mixed and placed on a microscope slide and sealed with a coverslip. The polyvinyl in the stain creates a sealed permanent slide once

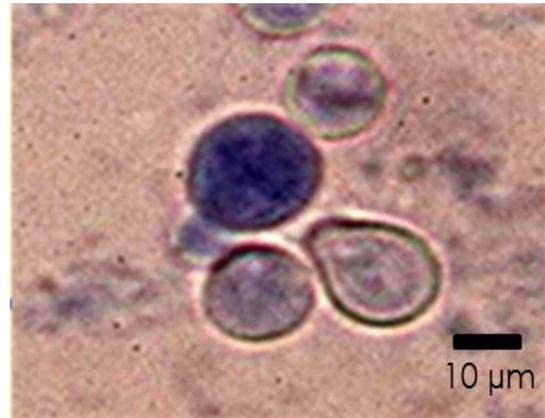


Figure 7. *Blastomyces dermatitidis* broad-based budding yeast. Stained with lactophenol cotton blue and viewed with a bright field microscope.

the prepared slide is oven dried. Broad based budding yeast was observed in the absence of any signs of the mold morphotype (Figure 7).

### **Neutrophil Isolation from human blood**

Neutrophils were isolated from human peripheral blood using density centrifugation. Using two concentrations of Histopaque was less cumbersome than other methods used in some of the literature and produced desirable results. Following isolation a smear of the isolated cells was prepared and stained with a modified Wright Giemsa stain and evaluated by microscopy to confirm the purity of the neutrophils resulting from the density gradient isolation. Greater than 95% of isolated cells showed morphologies consistent with neutrophils.

### **Neutrophil Extracellular Trap-formation**

Using the ATCC 26199 WT yeast isolate as an unknown stimulant the NET-formation assay was setup incorporating the use of coverslips being placed in the 24-well plate prior to being seeded with neutrophils. After staining the coverslips following the procedure described in the materials and methods section the coverslips were carefully mounted on a slide and observed microscopically using a confocal microscope. The presence of NET-formation, staining green when human neutrophils were co-cultured with

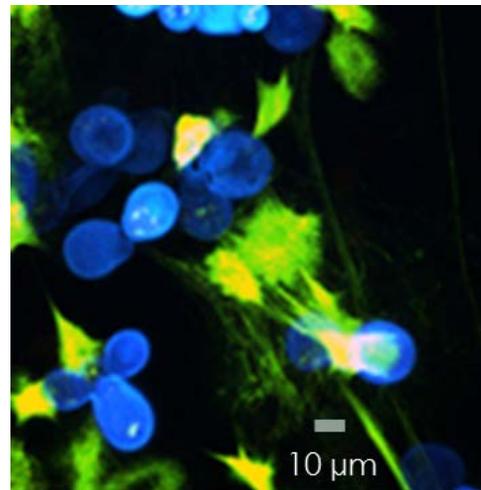


Figure 8. Fluorescence micrograph of *Blastomyces dermatitidis* and NETs. The broad based budding yeast (blue) stained with calcofluor white and neutrophils throwing NETs (green/yellow) stained with SYTOX green viewed on a confocal microscope. Scale bar is 10 $\mu$ m.

*B. dermatitidis* yeast, staining blue was confirmed (Figure 8).

The final set of experiments tested both the WT and KO strains of *B. dermatitidis* independently with two different neutrophil donors using the NET assay kit to generate the samples needed to quantify NET-formation, assess yeast survival, and to confirm the expression of BAD1 (Figure 6).

### **Quantification of NET-formation**

The NET-formation assay was set stimulating neutrophils independently with WT and KO yeast in the presence of or absence of PMA. The supernatant from the washes carried out throughout the NET-formation assay were collected and pooled. The supernatant from after the NETs were lysed was collected and frozen. Neutrophil extracellular trap-formation was quantified indirectly by measuring the NE level in the supernatant.

The NE measurement run was validated by the following controls: the known NE control (18 mU/ml) was 17.5 mU/ml, the negative controls (neutrophils only) for Donor 1 and Donor 2 confirming minimal spontaneous NET-formation were 3.5 mU/ml and 1.1 mU/ml respectively, and the positive controls (neutrophil plus PMA) for Donor 1 and Donor 2 showing that each of the donor's neutrophils were capable of NET-formation were 26.0 mU/ml and 36.0 mU/ml respectively.

Both the WT and KO yeast isolates were able to stimulate NET-formation in the absence of PMA, KO yeast and WT yeast with both donors resulted in NE levels higher than their respective negative controls. The levels of NE resulted in similar patterns for both donors. The yeast in the presence of PMA resulted in a higher level of NE for both donors than when using the yeast alone as the stimulant. The NE level of the yeast plus

PMA reflects both stimulants generated NET-formation. Interestingly, both yeast strains in the presence of PMA resulted in lower NE levels than neutrophils stimulated with just PMA, the positive control (Figure 9).

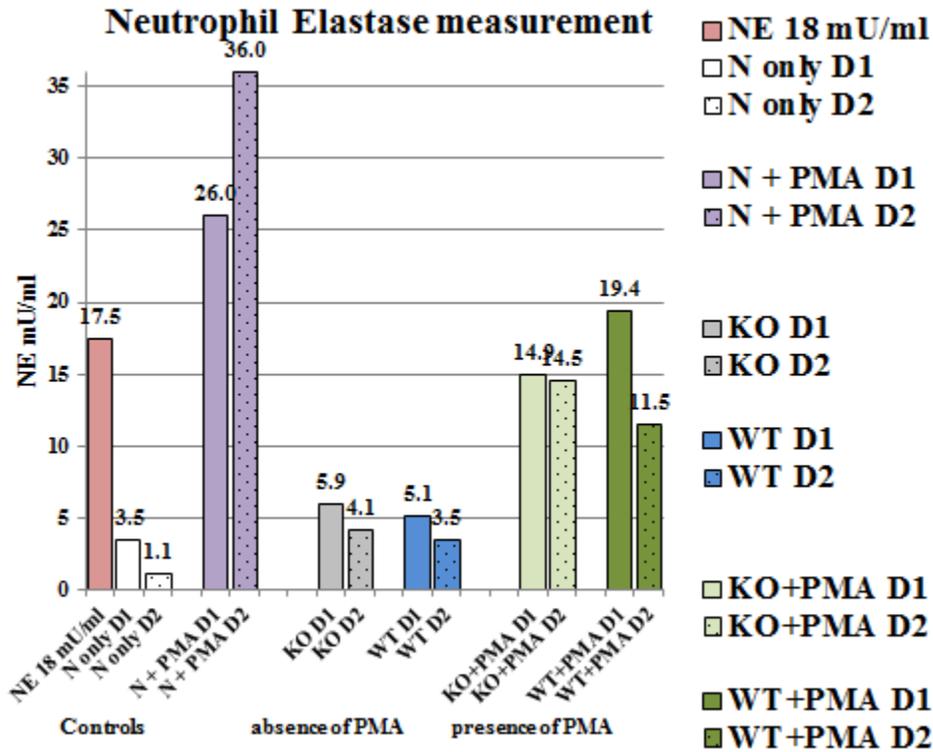


Figure 9. Neutrophil Elastase measurements. Neutrophils from two donors, D1 and D2 were stimulated for NET-formation using *Blastomyces dermatitidis* yeast in the absence of and the presence of Phorbol 12-myristate 13-acetate (PMA). Controls included a known neutrophil elastase (NE) concentration: 18 mU/ml, a negative control: neutrophils (N) only, a positive control: N + PMA. Yeast isolates were knockout strain #55  $BAD1^-$  (KO) and wild type strain ATCC 26199  $BAD1^+$  (WT).

### Yeast Survival

The NET-formation assay was set stimulating neutrophils independently with WT and KO yeast in the presence of or absence of PMA. A CFU assay was used to assess the fungistatic/fungicidal abilities of NETs by counting the colonies that grew after the debris pellets from the NET-formation assay were plated. Two sets of plates were set. One set

used the debris pellet from the pooled washes collected during the NET-formation assay and the other set used the cell debris pellet that resulted after NET lysis. The debris pellet from the pooled washes represents the surviving yeast not trapped in the NETs and the cell debris pellet from the lysed NETs represents the surviving yeast that were NET related. The CFU counts from both sets of plates together represent the total surviving yeast as a percent of the number of yeast plated at the beginning of the NET-formation assay. The surviving yeast levels are presented in Figure 10. Both the WT and KO strains were able to grow in the absence of and presence of PMA after being co-cultured with

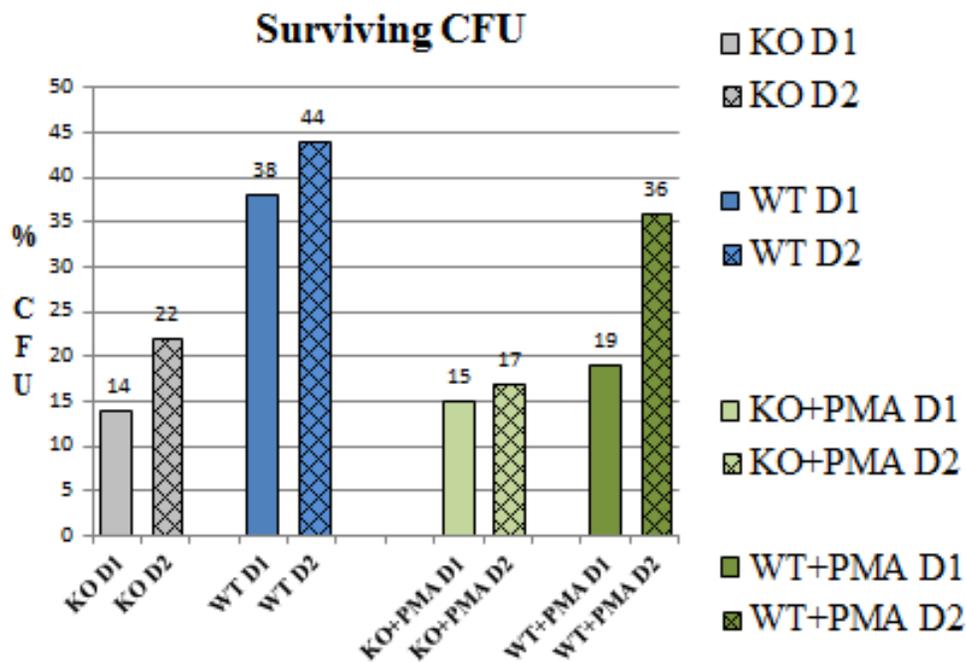


Figure 10. Percent Survival CFU of *Blastomyces dermatitidis* yeast following co-culture with neutrophils capable of Neutrophil Extracellular Trap-formation. Neutrophils from two donors, D1 and D2 were stimulated for NET-formation using *Blastomyces dermatitidis* yeast in the absence of and the presence of Phorbol 12-myristate 13-acetate (PMA). Yeast isolates were knockout strain #55  $BAD1^-$  (KO) and wild type strain ATCC 26199  $BAD1^+$  (WT). Two sets of CFU counts were collected. The CFU counts from a pooled wash debris pellet and the CFU counts from the lysed NET cell debris pellet. Together the CFU counts represent the % CFU of surviving yeast for each yeast isolate.

neutrophils capable of NET-formation. There was an indication that the WT yeast survived at a higher percent than the KO strain both in the absence of and the presence of PMA. The percent survival of each of the yeast strains in the presence of PMA appears to be lower than in the absence of PMA (Figure 10).

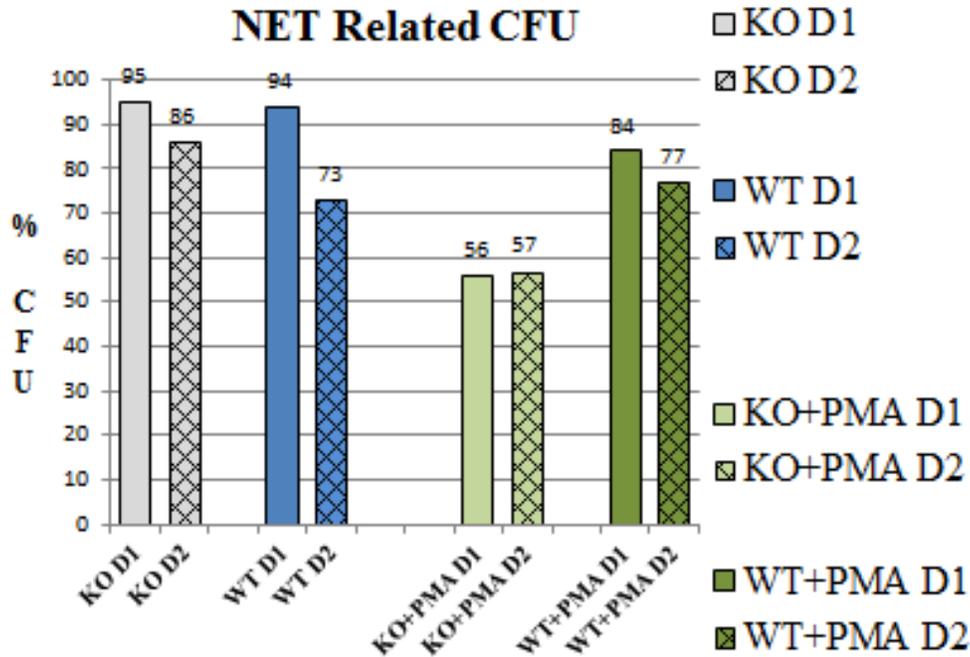


Figure 11. Percent Survival CFU of NET related *Blastomyces dermatitidis*. Neutrophils from two donors, D1 and D2 were stimulated for NET-formation using *Blastomyces dermatitidis* yeast in the absence of and the presence of Phorbol 12-myristate 13-acetate (PMA). Yeast isolates were knockout strain #55  $BAD1^-$  (KO) and wild type strain ATCC 26199  $BAD1^+$  (WT). Two sets of CFU counts were collected. The CFU counts from a pooled wash debris pellet and the CFU counts from the lysed NET cell debris pellet. The CFU counts from the NET cell debris pellet divided by the total CFU count of surviving yeast represents the % CFU of NET related surviving yeast for each isolate.

The NET related surviving yeast levels are presented in Figure 11. A majority of the surviving yeast, KO and WT were found to be NET related. The KO and the WT in the absence of PMA appear to survive at similar levels. The KO strain in the presence of PMA resulted in a lower percent NET related CFU possibly indicating that  $BAD1$  does provide protection from NETs (Figure 11).

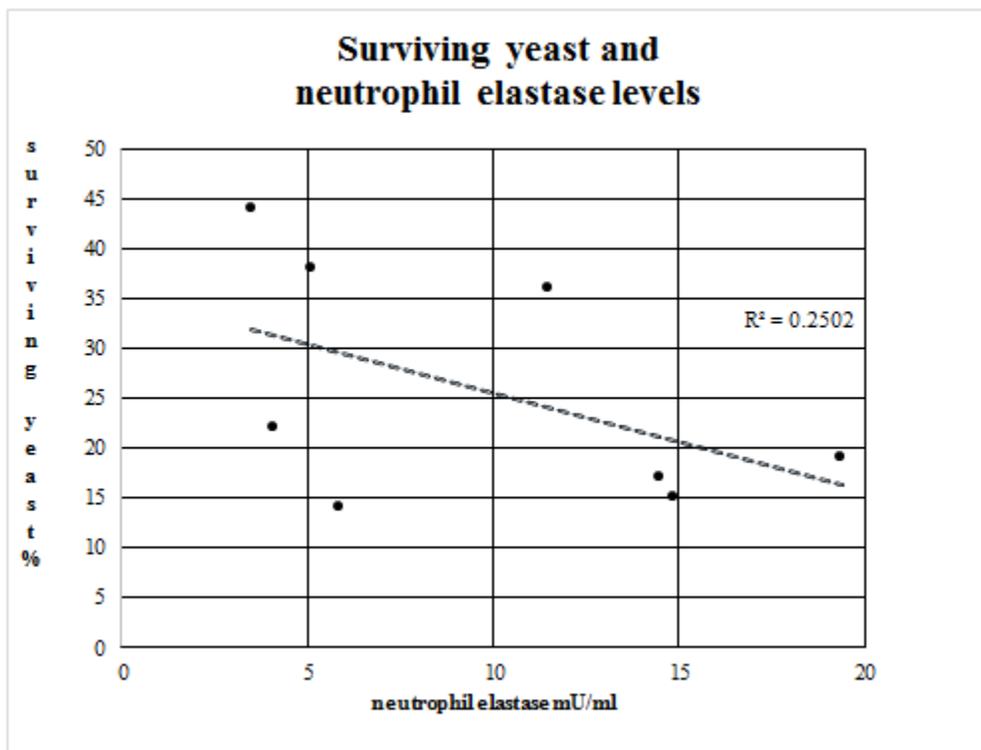


Figure 12. Scatter plot comparing surviving yeast and neutrophil elastase levels. When the percent of surviving yeast of the total number of yeast plated is plotted against the neutrophil elastase levels a trend line suggests an inverse correlation between surviving yeast and NET-formation indirectly measured by quantifying neutrophil elastase.

When the total CFU counts for the surviving yeast were plotted against the neutrophil elastase levels the trend line suggests an inverse correlation between NET-formation and surviving yeast. The higher the neutrophil elastase level indirectly measuring NET-formation resulted in a lower CFU count, fewer yeast survived. Conversely, a low level of NE resulted in a higher CFU count, more yeast survived.

### Confirmation of BAD1 expression

In order to ensure that the results were reflecting BAD1 expressing yeast compared to yeast not expressing BAD1 the ability of the WT yeast to still express BAD1 needed to be confirmed. Indirect immunofluorescence staining using a

monoclonal antibody specific for BAD1, DD5-CB4 and a FITC conjugated rabbit anti-mouse IgG fluorescence tag was performed with each strain of *B. dermatitidis* yeast, WT and KO following the setup of the final NET-formation assay. The WT strain showed fluorescence confirming the expression of BAD1 while the KO strain was negative for fluorescence due the absence of BAD1 expression (Figure 12).

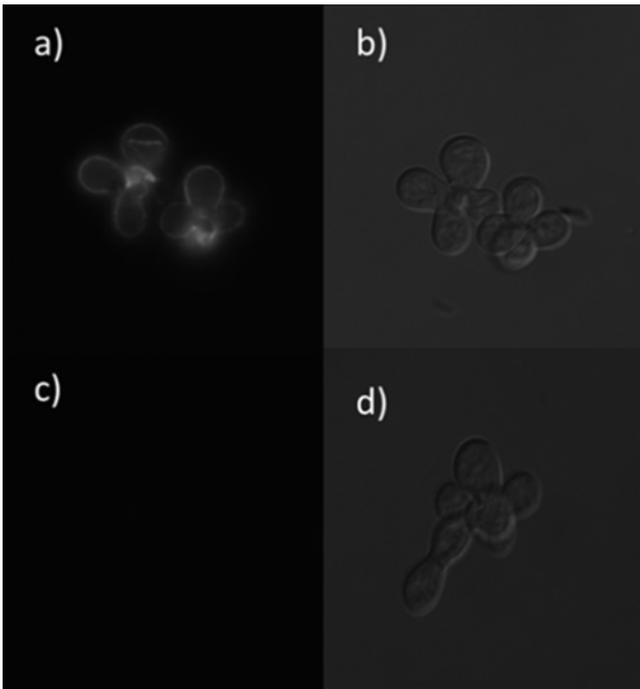


Figure 13. Fluorescence micrograph of BAD1 expression. An indirect Immunofluorescence staining procedure using a monoclonal antibody specific for BAD1, DD5-CB4 and a FITC conjugated rabbit anti-mouse IgG fluorescence tag was used to stain each isolate, WT and KO. ATCC 26199 WT a) showing fluorescence and b) fluorescence blocked. #55 KO c) no fluorescence and d) fluorescence blocked. 100X magnification.

## DISCUSSION

Learning to read and understand the characteristics of *B. dermatitidis* was the first task. The initial isolates of *B. dermatitidis* from the Marshfield Clinic grew at different rates and had subtle growth characteristics on solid media such as, color, colony morphology, and level of dryness. They also looked slightly different when viewed microscopically with regards to budding. A level of confidence needed to be developed to safely maintain the isolates in their yeast morphology. Developing a method of counting or measuring the concentration of the yeast suspension was another challenge. Aspirating the yeast suspension through a 25 gauge needle helped to break up the larger clumps.

Looking at the NE levels (Figure 9) there does not appear to be any difference between the levels of NE when comparing WT BAD1<sup>+</sup> to KO BAD1<sup>-</sup> *B. dermatitidis* yeast. The NE levels resulting from stimulating the neutrophils with yeast in the absence of PMA supports that *B. dermatitidis* yeast are capable of triggering NET-formation. One would expect the NE levels to be higher when the neutrophils were stimulated with yeast in the presence of PMA compared to when neutrophils were stimulated with yeast only. However, it was interesting to see a higher NE level when the neutrophils were stimulated with PMA only, positive control than when the neutrophils were stimulated with yeast in the presence of PMA. The results imply that the addition of the yeast created some inhibition. It was considered that the inhibition could have been from the media that the yeast were suspended in. However, the neutrophil only, used for the

negative control and the neutrophil + PMA, used for the positive control both received the same volume of yeast suspension media, only without the yeast.

One thing to consider would be to lower the concentration of the PMA used to stimulate NET-formation. A 50 nM/ml concentration of PMA was used following the protocol provided with the NET assay kit. However, the “activation of NET-formation” using PMA varies throughout the literature. Coletta et al. 2015 used 100 ng/ml concentration of PMA, a 50:1 neutrophil to fungus ratio when studying *Paracoccidioides* sp. and they did not include a test sample consisting of neutrophils + the fungus in the presence of PMA. When Urban et al. 2006 tested NET-formation following stimulation of neutrophils with *Candida albicans* they always pre-activated the neutrophils with a 25 nM/ml concentration of PMA and they tested with a 4:1 ratio of neutrophils to yeast. They did not look at neutrophils + yeast in the absence of PMA. We felt it was important to have both test samples: a sample with neutrophils + fungus and a sample with neutrophils + fungus + PMA to providing a more complete picture. The immune response has built in redundancies making it difficult to design experiments isolating one piece of the larger puzzle. Lowering the concentration of PMA to reach a NE level that would be higher than the negative control yet below the NE level achieved by neutrophils stimulated with the yeast only may present a different picture.

Although the NE measurement protocol did provide a quantifiable answer, it did not present the bigger picture. There is value in being able to visualize the physical attachment between the neutrophils and the yeast *in situ*. A possible option would be to use quantifying software and fluorescence microscopy to observe if there is steric hindrance or physical limitations of attachment between the neutrophils and the yeast in

the presence of PMA. Brinkmann et al. 2013 did use fluorescence microscopy and quantifying software. However, the monoclonal antibody used to measure the relaxed chromatin resulting from NET-formation was not commercially available. Being able to visualize the results of co-culturing neutrophils and *B. dermatitidis* may provide a better understanding of the interaction between the different cells.

The yeast survival/ CFU assay (figure 10) does provide a value directly associated with the neutrophil to yeast, cell to cell interaction. When the NET-formation assay was setup a well with yeast only for each strain of yeast was included. The CFU counts for the yeast only wells were not always equal between the two strains and the level of concentration was not reproducing from run to run. Due to the fact that the wells only contained yeast they may not have adhered to the wells and yeast was lost during the washing steps. The yeast did grow confirming that the reagents and media used in the experiments did not result in inhibition of the yeast ability to grow. A better control for quantification purposes would have been to plate the positive yeast control for the CFU assay straight from the yeast suspension used to inoculate the 24-well plate. The plating count of the yeast was used to calculate the surviving yeast percent.

However, using the data that was available there appears to be an inverse correlation between NE levels and yeast survival suggesting that NETs have a negative effect on *B. dermatitidis* yeast survival (Figure 12). When the percent survival is plotted against the NE level on a scatter plot the trend line suggests an inverse correlation between NE levels and yeast survival. The low r-square was not surprising due to the low number of neutrophil donors tested. Additional neutrophil donors would provide a stronger r-squared value.

Future work should also include using different concentrations of yeast. A 4:1 neutrophil: yeast ratio was the goal of these experiments. Using a higher ratio of neutrophils to yeast, 20:1 may allow a stronger fungistatic result from NET-formation. The NETs were not fungicidal using a 4:1 inoculum of yeast. The data was inconclusive to determine if NETs are fungistatic. Trying different concentrations of PMA and trying different ratios of neutrophil to yeast would be additional testing that should be considered. A visual testing platform would also provide additional information. Another benefit with being able to visualize the interaction is supported by Branzk et al. 2014 supporting the concept that neutrophils sense microbial size and therefore decides when to activate NET-formation. Visualization would also add to our understanding of the actions resulting from the expression of BAD1 in *situ*. As part of our initial quest we considered trying to find a way to measure the levels of cell bound BAD1 using a whole cell ELISA testing platform. Again, this would provide a closer in *situ* scenario possibly leading to a better understanding of how the levels of cell bound BAD1 layering back on the yeast cell after being expressed as a soluble protein might drive the pathogenesis of blastomycosis. This research gives us a basis for future research.

## REFERENCES

- Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., & Zychlinsky, A. (2012). Neutrophil function: from mechanisms to disease. *Annual Review of Immunology*, *30*, 459–489. <http://doi.org/10.1146/annurev-immunol-020711-074942>
- Blanco, J. L., & Garcia, M. E. (2008). Immune response to fungal infections. *Veterinary Immunology and Immunopathology*, *125*(1–2), 47–70. <http://doi.org/10.1016/j.vetimm.2008.04.020>
- Borregaard, N. (2010). Neutrophils, from Marrow to Microbes. *Immunity*, *33*(5), 657–670. <http://doi.org/10.1016/j.immuni.2010.11.011>
- Brandhorst, T., & Klein, B. (2000). Cell Wall Biogenesis of *Blastomyces dermatitidis* Evidence for a novel mechanism of cell surface localization of a virulence-associated adhesin via extracellular release and re-association with cell wall chitin. *Journal of Biological Chemistry*, *275*(11), 7925–7934. <http://doi.org/10.1074/jbc.275.11.7925>
- Brandhorst, T. T., Rooney, P. J., Sullivan, T. D., & Klein, B. S. (2002). Using new genetic tools to study the pathogenesis of *Blastomyces dermatitidis*. *Trends in Microbiology*, *10*(1), 25–30.
- Brandhorst, T. T., Wüthrich, M., Warner, T., & Klein, B. (1999). Targeted Gene Disruption Reveals an Adhesin Indispensable for Pathogenicity of *Blastomyces dermatitidis*. *The Journal of Experimental Medicine*, *189*(8), 1207–1216. <http://doi.org/10.1084/jem.189.8.1207>
- Brandhorst, T., Wüthrich, M., Finkel-Jimenez, B., & Klein, B. (2003). A C-terminal EGF-like domain governs BAD1 localization to the yeast surface and fungal adherence to phagocytes, but is dispensable in immune modulation and pathogenicity of *Blastomyces dermatitidis*. *Molecular Microbiology*, *48*(1), 53–65. <http://doi.org/10.1046/j.1365-2958.2003.03415.x>
- Brinkmann, V., Goosmann, C., Kühn, L. I., & Zychlinsky, A. (2013). Automatic quantification of in vitro NET formation. *Frontiers in Immunology*, *3*. <http://doi.org/10.3389/fimmu.2012.00413>
- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., ... Zychlinsky, A. (2004a). Neutrophil Extracellular Traps Kill Bacteria. *Science*, *303*(5663), 1532–1535. <http://doi.org/10.1126/science.1092385>

- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., ... Zychlinsky, A. (2004b). Neutrophil extracellular traps kill bacteria. *Science (New York, N.Y.)*, *303*(5663), 1532–1535. <http://doi.org/10.1126/science.1092385>
- Brown, G. D. (2011). Innate Antifungal Immunity: the Key role of phagocytes. *Annual Review of Immunology*, *29*, 1–21. <http://doi.org/10.1146/annurev-immunol-030409-101229>
- Chai, L. Y. A., Netea, M. G., Vonk, A. G., & Kullberg, B.-J. (2009). Fungal strategies for overcoming host innate immune response. *Medical Mycology*, *47*(3), 227–236. <http://doi.org/10.1080/13693780802209082>
- Chapman, S. W., Dismukes, W. E., Proia, L. A., Bradsher, R. W., Pappas, P. G., Threlkeld, M. G., & Kauffman, C. A. (2008). Clinical Practice Guidelines for the Management of Blastomycosis: 2008 Update by the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *46*(12), 1801–1812. <http://doi.org/10.1086/588300>
- Cheng, O. Z., & Palaniyar, N. (2013). NET balancing: a problem in inflammatory lung diseases. *Frontiers in Immunology*, *4*. <http://doi.org/10.3389/fimmu.2013.00001>
- Cooney, N. M., & Klein, B. S. (2008). Fungal Adaptation to the Mammalian Host: It's a New World, After All. *Current Opinion in Microbiology*, *11*(6), 511–516. <http://doi.org/10.1016/j.mib.2008.09.018>
- Cowburn, A. S., Condliffe, A. M., Farahi, N., Summers, C., & Chilvers, E. R. (2008). Advances in Neutrophil Biology. *Chest*, *134*(3), 606–612. <http://doi.org/10.1378/chest.08-0422>
- Davies, S. F., & Sarosi, G. A. (1989). Blastomycosis. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*, *8*(5), 474–479.
- Della Coletta, A. M., Bachiega, T. F., de Quaglia e Silva, J. C., Soares, Â. M. V. de C., De Faveri, J., Marques, S. A., ... Dias-Melicio, L. A. (2015). Neutrophil Extracellular Traps Identification in Tegumentary Lesions of Patients with Paracoccidioidomycosis and Different Patterns of NETs Generation In Vitro. *PLoS Negl Trop Dis*, *9*(9), e0004037. <http://doi.org/10.1371/journal.pntd.0004037>
- Drutz, D. J., & Frey, C. L. (1985). Intracellular and extracellular defenses of human phagocytes against *Blastomyces dermatitidis* conidia and yeasts. *The Journal of Laboratory and Clinical Medicine*, *105*(6), 737–750.
- Gauthier, G., & Klein, B. S. (2008). Insights into Fungal Morphogenesis and Immune Evasion. *Microbe (Washington, D.C.)*, *3*(9), 416–423.

- Grommes, J., & Soehnlein, O. (2011). Contribution of Neutrophils to Acute Lung Injury. *Molecular Medicine*, 17(3-4), 293–307.  
<http://doi.org/10.2119/molmed.2010.00138>
- Hahn, S., Giaglis, S., Chowdury, C. S., Höfli, I., & Hasler, P. (2013). Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. *Seminars in Immunopathology*, 35(4), 439–453.  
<http://doi.org/10.1007/s00281-013-0380-x>
- Kanetsuna, F., & Carbonell, L. M. (1971). Cell Wall Composition of the Yeastlike and Mycelial Forms of *Blastomyces dermatitidis*. *Journal of Bacteriology*, 106(3), 946–948.
- Kaplan, M. J., & Radic, M. (2012). Neutrophil extracellular traps: double-edged swords of innate immunity. *Journal of Immunology (Baltimore, Md.: 1950)*, 189(6), 2689–2695. <http://doi.org/10.4049/jimmunol.1201719>
- Khuu, D., Shafir, S., Bristow, B., & Sorvillo, F. (2014). Blastomycosis Mortality Rates, United States, 1990–2010. *Emerging Infectious Diseases*, 20(11), 1789–1794.  
<http://doi.org/10.3201/eid2011.131175>
- Klein, B. S. (1997). Role of cell surface molecules of *Blastomyces dermatitidis* in the pathogenesis and immunobiology of blastomycosis. *Seminars in Respiratory Infections*, 12(3), 198–205.
- Klein, B. S., Chaturvedi, S., Hogan, L. H., Jones, J. M., & Newman, S. L. (1994). Altered expression of surface protein WI-1 in genetically related strains of *Blastomyces dermatitidis* that differ in virulence regulates recognition of yeasts by human macrophages. *Infection and Immunity*, 62(8), 3536–3542.
- Klein, B. S., Sondel, P. M., & Jones, J. M. (1992). WI-1, a novel 120-kilodalton surface protein on *Blastomyces dermatitidis* yeast cells, is a target antigen of cell-mediated immunity in human blastomycosis. *Infection and Immunity*, 60(10), 4291–4300.
- Kruger, P., Saffarzadeh, M., Weber, A. N. R., Rieber, N., Radsak, M., von Bernuth, H., ... Hartl, D. (2015). Neutrophils: Between Host Defence, Immune Modulation, and Tissue Injury. *PLoS Pathog*, 11(3), e1004651.  
<http://doi.org/10.1371/journal.ppat.1004651>
- Kumar, V., & Sharma, A. (2010). Neutrophils: Cinderella of innate immune system. *International Immunopharmacology*, 10(11), 1325–1334.  
<http://doi.org/10.1016/j.intimp.2010.08.012>

- Levitz, S. M. (1992). Overview of Host Defenses in Fungal Infections. *Clinical Infectious Diseases*, 14(Supplement 1),S37S42.  
[http://doi.org/10.1093/clinids/14.Supplement\\_1.S37](http://doi.org/10.1093/clinids/14.Supplement_1.S37)
- Light, R. B., Kralt, D., Embil, J. M., Trepman, E., Wiebe, L., Limerick, B., ... MacDonald, K. (2008). Seasonal variations in the clinical presentation of pulmonary and extrapulmonary blastomycosis. *Medical Mycology*, 46(8), 835–841. <http://doi.org/10.1080/13693780802132763>
- Mantovani, A., Cassatella, M. A., Costantini, C., & Jaillon, S. (2011). Neutrophils in the activation and regulation of innate and adaptive immunity. *Nature Reviews Immunology*, 11(8), 519–531. <http://doi.org/10.1038/nri3024>
- Nathan, C. (2006). Neutrophils and immunity: challenges and opportunities. *Nature Reviews Immunology*, 6(3), 173–182. <http://doi.org/10.1038/nri1785>
- Nicola, A. M., Casadevall, A., & Goldman, D. L. (2008). Fungal killing by mammalian phagocytic cells. *Current Opinion in Microbiology*, 11(4), 313–317.  
<http://doi.org/10.1016/j.mib.2008.05.011>
- Odds, F. C., Gow, N. A., & Brown, A. J. (2001). Fungal virulence studies come of age. *Genome Biology*, 2(3), reviews1009.1–reviews1009.4.
- Papayannopoulos, V., Staab, D., & Zychlinsky, A. (2011). Neutrophil Elastase Enhances Sputum Solubilization in Cystic Fibrosis Patients Receiving DNase Therapy. *PLoS ONE*, 6(12), e28526. <http://doi.org/10.1371/journal.pone.0028526>
- Papayannopoulos, V., & Zychlinsky, A. (2009). NETs: a new strategy for using old weapons. *Trends in Immunology*, 30(11), 513–521.  
<http://doi.org/10.1016/j.it.2009.07.011>
- Pfaller, M. A., & Diekema, D. J. (2010). Epidemiology of Invasive Mycoses in North America. *Critical Reviews in Microbiology*, 36(1), 1–53.  
<http://doi.org/10.3109/10408410903241444>
- Rappleye, C. A., & Goldman, W. E. (2008). Fungal stealth technology. *Trends in Immunology*, 29(1), 18–24. <http://doi.org/10.1016/j.it.2007.10.001>
- Remijsen, Q., Kuijpers, T. W., Wirawan, E., Lippens, S., Vandenabeele, P., & Vanden Berghe, T. (2011). Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death and Differentiation*, 18(4), 581–588.  
<http://doi.org/10.1038/cdd.2011.1>
- Romani, L. (2011). Immunity to fungal infections. *Nature Reviews Immunology*, 11(4), 275–288. <http://doi.org/10.1038/nri2939>

- Saccante, M., & Woods, G. L. (2010). Clinical and Laboratory Update on Blastomycosis. *Clinical Microbiology Reviews*, 23(2), 367–381. <http://doi.org/10.1128/CMR.00056-09>
- Seitz, A. E., Younes, N., Steiner, C. A., & Prevots, D. R. (2014). Incidence and Trends of Blastomycosis-Associated Hospitalizations in the United States. *PLoS ONE*, 9(8), e105466. <http://doi.org/10.1371/journal.pone.0105466>
- Silva, M. T. (2010). When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *Journal of Leukocyte Biology*, 87(1), 93–106. <http://doi.org/10.1189/jlb.0809549>
- Simon, S. I., & Kim, M.-H. (2010). A day (or 5) in a neutrophil's life. *Blood*, 116(4), 511–512. <http://doi.org/10.1182/blood-2010-05-283184>
- Urban, C. F., Reichard, U., Brinkmann, V., & Zychlinsky, A. (2006). Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cellular Microbiology*, 8(4), 668–676. <http://doi.org/10.1111/j.1462-5822.2005.00659.x>
- Wallace, J. (2002). Pulmonary blastomycosis : A great masquerader. *Chest*, 121(3), 677–679. <http://doi.org/10.1378/chest.121.3.677>
- Zhang, M. X., Brandhorst, T. T., Kozel, T. R., & Klein, B. S. (2001). Role of Glucan and Surface Protein BAD1 in Complement Activation by *Blastomyces dermatitidis* Yeast. *Infection and Immunity*, 69(12), 7559–7564. <http://doi.org/10.1128/IAI.69.12.7559-7564.2001>

APPENDIX A

BIOLOGICAL MATERIALS AND RECOMBINANT DNA REGISTRATION

Protocol # \_\_\_\_\_  
(Assigned by IBC)

IBC-1

## Biological Materials and Recombinant DNA Registration

UNIVERSITY OF WISCONSIN-LA CROSSE  
Institutional Biosafety Committee

Return completed form to  
College of Science and Health  
105 Graff Main Hall, 785-8218

Name: Angela Ratekin Dept: Microbiology  
Title: Graduate Student Campus Addr: 1034 Cowley Hall  
Phone: 785-6451 Email Addr: aratekin@uwlax.edu  
Co-investigator(s): Thomas Volk and Bernadette Taylor  
Laboratory Personnel: \_\_\_\_\_

Project title: How bad is BAD1? Looking at the relationship between *Blastomyces dermatitidis* Virulence Factor BAD1 and Human Neutrophils, the "Infantry" of our Initial Immune Response

Granting agency: <u>UW-L RSEL</u>	Grant #: <u>1284361824</u>
_____	_____

### Location:

Where are experiments performed? Is there anything unique about the experiments or the location that requires the use of special precautionary measures such as containment facilities or biological safety cabinets? If so, have they been certified within the past year? Please give date.

Building	Room Number	Containment facilities or biosafety cabinets used	Certification date(s)
Cowley Hall	308	BSC	9/2011
Cowley Hall	310	BSC	9/2011
Cowley Hall	312	BSC	9/2011

### Occupational Health:

#### OSHA *Bloodborne Pathogens Standard*

If your research involves the use of human-derived substances (e.g., blood or blood components, tissues, secretions) or human-derived cell lines, it may be subject to the OSHA *Bloodborne Pathogens Standard*.

Could your research be subject to the OSHA Bloodborne Pathogens Program? • **yes**  
• **no**

If **yes**, please contact Environmental Health & Safety at 785-6800.

**Description of Research Elements**

If project does not include recombinant DNA (rDNA), please skip section **A** and begin with section **B**.

**A. Abstract of rDNA elements** (If subject to NIH *Guidelines*, please complete this section.)

DNA source(s)	
Nature of insert/protein expressed	
Vector(s)	
Host	
Cell/animal/plant recipients(s)	

**Description of rDNA construct (additional instructions on page 4).**

Please attach a description, in molecular terms (e.g., promoter[s], ORFs, selectable markers) of the rDNA construct.

**B. Microbiological Agents** (list agents and check appropriate categories.)

Agent	Human Pathogen	Animal Pathogen	Plant Pathogen (State whether indigenous to Wisconsin)
Yeast form of <i>Blastomyces dermatitidis</i>	X	X	

**C. Cell Cultures** (identify species and source, and describe how they are used).

Name	Species	Organ source	Usage

**D. Human-derived Substances**

Are blood components, secretions, or tissues, used in some phase of your work?

If so, briefly identify source and usage.

Substance	Source	Usage
Blood	Human volunteers	Isolation of neutrophils

**E. Hazardous Experimental Chemical Agents** (See Appendices).

Known/potential carcinogens/mutagens	Amount	Concentration

Other drugs/toxins	Amount	Concentration

Does your laboratory have an OSHA -required Chemical Hygiene Plan?  
If **no**, please contact Environmental Health and Safety at 785-6800

**yes**       **no**

**F. Experimental Animals**

Common name of species	Purpose of use	Building and room number where animals are housed

Material administered to animal(s)	Quantity	Method of administration

**G. Disposal**

Describe the method of disposal of hazardous substances, animal wastes and carcasses, and residual human substances (e.g., incineration, autoclaving, chemical disinfection). If chemical disinfectant is used, state kind and concentration. Is autoclave monitored with a biological indicator (e.g., spore strips)?

Substance	Disposal method	Description of procedure
Blood/body fluid—contaminated lab materials (blood vacutainer tubes, plastic pipettes, 12 well plates, gloves, and other items)  Biological yeast culture – culture medium will be held at 37°C to maintain yeast form until immediate autoclaving	Autoclaving. After checking that the autoclaving worked properly, the containers will be placed in black trash bags and placed in the regular trash.	Blood waste is kept in a biohazard bag inside a plastic container with a lid. When the bag is ¾ full, it is closed loosely with heat-sensitive tape and autoclaved at 121°C for 90 minutes (excluding exhaust time). 3M Comply Thermalog chemical integrator strips (attached to a string, and placed in the center of the load) are used to test decontamination effectiveness. The autoclave is serviced every three months.
Blood/body fluid—contaminated sharps (needles, & syringes, glass pipettes, slides, broken glass)	Biocertified sharps containers.	When the sharps container is almost full, it is autoclaved and picked up by Dan Sweetman for disposal.
Liquid waste (plasma, wash buffers), including flow cytometry waste.	Treated with bleach and poured down the sink.	Liquid bleach is added to reach 10% bleach solution, allowed to sit for 30 minutes and then discarded in the sink.

**G. Research Protocol**

Attach a brief description of the research objectives and methods for handling and disposing of hazardous substances and/or recombinant organisms. If you will be employing Biosafety Level 2, 3 or 4 pathogenic microorganisms, provide specific information about the following: investigator experience, adequacy of facility design and containment equipment, personnel practices, decontamination and disposal, staff training, and Chemical Hygiene considerations.

**I. Assessment of levels of physical and biological containment**

Cite the relevant sections of the current NIH Guidelines, January, 1996.

OSHA Regulations (Standards 29 CFR) Bloodborne Pathogens. 1910.10301 ([www.oshs.gov](http://www.oshs.gov))  
 Biosafety in Microbiological and Biomedical Laboratories, 5<sup>th</sup> edition, 2007  
 (<http://www.cdc.gov/od/ohs/biosfty/bml5/bml5toc.htm>)

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Principal Investigator	Date	Chair, Institutional Biosafety Committee	Date
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NOTE: If, after submitting this form, you (1) plan to make any significant changes in the research protocol that might affect biological safety, or (2) contemplate changing the location of research that involves either the movement of animals in containment, potential hazards, or release to the environment of rDNA molecules, you must obtain approval from the Institutional Biosafety Committee before making such changes.

3

### G. Research Protocol

#### Objectives

The project will investigate the human neutrophil response to the yeast form of *Blastomyces dermatitidis* and measure the survival rate of the yeast. Neutrophils will be isolated from whole blood collected from healthy adult volunteers, then co-cultured with the yeast form of *Blastomyces dermatitidis*.

#### Hazardous Substances

Hazardous substances used in this research are human blood, which could potentially harbor infectious agents such as hepatitis B virus, HIV or other blood borne pathogens, and the pathogenic fungus *Blastomyces dermatitidis*.

#### Handling

Safety glasses, disposable nitrile gloves, laboratory coats and closed-toe shoes are worn at all times. Work areas are wiped down with an approved biosafety laboratory cleaner. Biohazard signs are posted on the laboratory doors, refrigerators and incubators containing biohazardous material.

Universal precautions are followed (i.e. all blood and blood contaminated objects are treated as if they harbor agents capable of infecting the handler). Open tubes of human blood and blood-derived substances are handled only in a biosafety cabinet with laminar flow. Any transport of blood tubes between laboratories is performed with a plastic cooler to contain breakages or contamination. Centrifugation of blood and blood-derived substances is performed in a Beckman table-top swinging bucket centrifuge with safety screw-cap buckets to contain potential aerosolization.

Any media or container used for *Blastomyces dermatitidis* will be held at 37°C to maintain the non-infectious yeast form until autoclaving is carried out.

#### Disposal

Described in part G. Disposal

#### Personnel Training

I am a certified Medical Technologist (ASCP); I am educated and experienced in regard to Universal Precautions, microbial biosafety techniques, and general laboratory safety. It is also part of my job as a Microbiology Prep Room Manager at UW-L to train our student employees. I have received the Hepatitis B vaccination. Accidents are reported to the environmental health and safety officer.

#### Facilities

The Microbiology Department including the laboratory spaces I will be using is well equipped for safe handling and disposal of biohazardous waste. Microbiology has two autoclaves and Biology has a third one. All three laboratories listed have biosafety cabinets, supplies of biohazard bags, sharps containers, and biohazard signs.

#### Experience

I have been certified as a MT (ASCP) since 1984. I have worked in research and clinical settings for the past 28 years. I have worked as a Microbiology Prep Room Manager at UW-L since 2003. As a graduate student in the Clinical Microbiology program at UW-L I have completed my three clinical rotations, and I am under the supervision of Thomas Volk and Bernadette Taylor.

## DEFINITIONS

### Biological Materials

- Any plant, animal or microbial organism and any substance derived from these organisms (including DNA molecules) that is the subject of the research.
- Any substance derived from these organisms that are an ancillary reagent or indicator employed for the investigation.
- For biosafety purposes, chemicals that are biologically active, e.g., carcinogens, mutagens, or toxins.

### Significant Changes

- Change in research location, introduction of new techniques or methods, or increase in quantity or potency of hazardous substances.
- Use of pathogenic agents not previously described.
- Release of DNA materials to the environment.

## rDNA REGISTRATION

### Instructions for completing description of rDNA constructs.

Many aspects of rDNA research and its technology have become very complex and specialized. Thus, it is important that you clearly explain any of the following aspects that apply to your proposal **if your research is subject to the NIH Guidelines or other rDNA regulations**. This will aid the IBC to determine if your citation of the applicable section of the NIH Guidelines and assessment of containment levels are appropriate, thereby avoiding unnecessary delay and requests for more information.

- A) Research objectives.
- B) Source and species of genomic elements.
  - Do any sequences code for potent toxins (e.g., having an LD50 less than 100\  $\mu$ g/kg)?
  - Is the DNA source from a USDA-regulated article? If so, do you have a permit?
- C) Describe the vector system and provide a map, if available. Describe method of transfection.
  - If viral vectors will be used, are they replication defective?
  - Will helper viruses be employed?
  - Describe any packaging systems used.
  - Will the entire viral genome be involved? If not, what portion will be involved?
- D) Identify the intended cloning vehicle or recipient host.
- E) If genetic material is transferred to an animal or plant, will the organism become Transgenic? Will transferred material be stably transmitted to progeny?
- F) If oncogenes or other regulatory sequences are transferred, will pathogenic virulence, toxicity, antibiotic resistance, or immune competence be affected?
- G) Describe any measures taken to prevent or minimize expression of pathogenic/infectious sequences (e.g., deletion of viral LTRs or packaging instructions, vector disarming, etc.)
- H) If the DNA source is from a Class 2 or 3 infectious microorganism, exotic animal, plant pathogen, or any USDA-regulated article, discuss safety aspects of facility, containment, personnel practices, and staff training that will assure safe conduct of the investigation.

University of Wisconsin  
**LA CROSSE**

July 2, 2012

Angela Ratekin  
Department of Microbiology

Dear Ms. Ratekin,

I am writing to inform you that the Institutional Biosafety Committee has reviewed and approved IBC protocol 2012-02, as submitted for your project "How bad is BAD1? Looking at the relationship between *Blastomyces dermatitidis* Virulence Factor BAD1 and Human Neutrophils, the "infantry" of our Initial Immune Response"

Please contact me if you have any questions. Best wishes on the success of your project.

Sincerely,



Ray Abhold  
Associate Dean  
Chair, Institutional Biosafety Committee

Cc: Peg Maher  
Grants Office

**College of Science and Health  
Office of the Dean**

105 Graff Main Hall, University of Wisconsin-La Crosse  
1725 State Street, La Crosse, WI 54601  
Phone: (608)785-8218, Fax: (608)785-8221

APPENDIX B  
INSTITUTIONAL REVIEW BOARD REVIEW

**ATTACHMENT A - APPLICATION FOR UNIVERSITY IRB REVIEW**

(All submissions must be typewritten)

Date 4/10/2012

1. a. Principal Investigator/Project Director (If thesis or undergraduate research project, student's name):

Angela Ratekin

b. Applicant Status: (Check all that apply)

- Faculty  
 Academic Staff  
 Graduate Student  
 Undergraduate Student

c. Investigator/Project Director Local Address:

1034 Cowley Hall

d. Investigator/Project Director Local Telephone # 785-6451

E-mail: aratekin@uwflux.edu

2. a. Title of Proposed Project: Looking at the relationship between Blastomyces dermatitidis and human neutrophils

b. Project Period: Begin Date: April 2012 End Date: May 2013

c. If a student project of any type, Faculty Advisor's Name, Department, and Phone:

Name: Thomas Volk

Signature: Thomas J Volk

Department: Biology

Phone #: 785-6972

E-Mail: tvolk@uwflux.edu

\* Names and Signatures of Thesis Committee Members:

Bernadette Taylor

Name

Bernadette Taylor

Signature

Michael Hoffman

Name

Michael Hoffman

Signature

3. If the researcher believes his/her project may be reviewed under expedited procedures (p. 6-9) and/or falls within the exemptible category, (p.4-5) please check the appropriate box(es) below

- Expedited  
 Exemptible

a. If expedited, please indicate the number(s) of the categories listed on pages (6-9) Res 2a

b. If exemptible, please indicate the number(s) of the categories listed on pages (4-5) \_\_\_\_\_

4. By signing this application, I agree to comply with any decisions made by the University of Wisconsin-La Crosse IRB in regard to the above named research project, and or the standards of professional ethics in my field of study.

Angela Ratekin

Signature

4-13-12

Date

The IRB has reviewed the above research project and has determined that:

1. \_\_\_\_\_ APPROVAL IS GRANTED -as submitted or as modified per attached (check one)

- a. the protocol does not contain procedures which place human subjects at risk, or  
 b. the protocol contains procedures which place human subjects at minimal but acceptable risk, or  
 c. the protocol contains or is likely to contain procedures that may place human subjects at greater than minimal risk; however, the risk(s) are outweighed by the sum of the anticipated benefits of the research.

2. \_\_\_\_\_ APPROVAL NOT GRANTED

The following IRB members participated in this review:

\_\_\_\_\_

On behalf of the board:

\_\_\_\_\_  
IRB Chairperson or Coordinator Signature

\_\_\_\_\_  
Date

Narrative Statement for the University of Wisconsin-La Crosse IRB review

1. The research project will look at the survival of the yeast form of *Blastomyces dermatitidis* (fungus) after being co-cultured with human neutrophils (white blood cells). The research will begin May 2012 and finish May 2013. Over the course of the year phlebotomy will be performed on each of the healthy adult volunteer 6-10 times. A qualified phlebotomist will draw 3ml of blood using a vacutainer with heparin anticoagulant. Human neutrophils have a short life span and are not available commercially and cannot survive in cell culture, therefore they need to be fresh each day of testing.
2. The only characteristics of the subject population in the project will be that they are a healthy adult ensuring a normal neutrophil count. The project will be looking for differences in the yeast isolates and the neutrophils will need to be as consistent as possible. Therefore I will use only 4-6 volunteers.
3. N/A
4. Volunteers will be recruited from persons available during the testing days and agreeing to a standard consent form. Form is included.
5. Each donor will be assigned a number for identification.
6. Anticipated risks and/or inconveniences that are associated with a simple venipuncture are bruising at the site of puncture and/or becoming light headed. The time required will be the same as having blood drawn for a doctor's appointment requiring about 10 minutes of time.
7. A standard phlebotomy procedure will be followed. I will perform the venipuncture; I am a certified Medical Technologist (ASCP).
8. By using healthy normal neutrophils the beneficial knowledge I hope to obtain will be if there is a difference in *Blastomyces dermatitidis* strains resulting in a difference in clinical presentation of Blastomycosis when humans are exposed. The more we learn about pathogenic fungi and the immune response resulting from these organisms the better equipped we are to diagnose, treat, and prevent infection from *Blastomyces dermatitidis*.

## INFORMED CONSENT

Principal Investigator: Angela Ratekin  
Department of Microbiology  
University of Wisconsin - La Crosse  
1725 State Street, La Crosse, WI 54601

**Title: How bad is BAD1? Looking at the relationship between *Blastomyces dermatitidis* Virulence Factor BAD1 and Human Neutrophils, the “Infantry” of our Initial Immune Response**

*You need to be informed of the procedures, risks, possible benefits, and alternatives involved before you decide to take part in this study. The following will help you make an informed decision. You should also be informed that you are not giving up any legal rights to which you would otherwise be entitled by signing this consent document and agreeing to take part in this study.*

### ***Purpose of the Study***

I have been informed that the purpose of this study is to determine if the yeast form of *Blastomyces dermatitidis* survives co-culture with human neutrophils. The more we understand about the pathogen's relationship with neutrophils during an immune response the more knowledgeable we will be with regards to diagnosis, treatment, and prevention of infection in the future.

### ***What will happen to me if I agree to take part in the study?***

I have been informed that my participation will involve:

6-10 venepuncture events when 3 ml of blood will be drawn from my arm at each event by a qualified phlebotomist.

### ***What are the possible risks and discomforts of the study?***

I have been informed of the risks and inconveniences that may be associated with a simple venepuncture:

Possible bruising at the site of puncture

Possible light headedness

### ***What are the costs for taking part in this study?***

I have been informed that there will be no monetary cost to me to take part in this study.

### ***What are the benefits of taking part in this study?***

I have been informed that knowledge from this study may benefit others in the future by presenting new information with regards to the interaction between human neutrophils and the yeast form of *Blastomyces dermatitidis*.

### ***What if I decide not to participate in, or to withdraw from this study?***

I have been informed that my participation is entirely voluntary and that I can withdraw from the study at any time for any reason. I will not be able to withdraw scientific data generated from my ~~saliva and~~ blood samples because it will be incorporated into grouped data for statistical and presentation purposes.

### ***If I take part in this study, who will have access to my blood samples, records and research data?***

I have been informed that blood samples will be numerically coded by the principal investigator to ensure confidentiality. These samples will be used only for research into the relationship between human neutrophils and the yeast form of *Blastomyces dermatitidis*. This consent form and the human subject's code sheet will be the only records of your participation in the study. Only the principal investigator will know the human subject codes. The consent form may be inspected by the UW-La Crosse Institutional Review Board and governmental health regulatory agencies. All efforts will be made to ensure

confidentiality and your name will not be given to anyone not associated with the study unless required by law. The results of this study may be published in scientific literature or presented at professional meetings using grouped data only. Samples will be destroyed by addition of chlorine bleach and heat-sterilization.

***What happens, and who do I contact if I get sick or hurt as a result of this study?***

In the unlikely event that any injury or illness occurs as a result of this research, the Board of Regents of the University of Wisconsin System, and the University of Wisconsin-La Crosse, their officers, agents and employees, do not automatically provide reimbursement of medical care or other compensation. Payment for treatment of any injury or illness must be provided by you or your third-party payor, such as your health insurer or Medicare. If any injury or illness occurs in the course of research, or for more information, please notify the principal investigator. I have been informed that I am not waiving any rights that I may have for injury resulting from negligence of any person or the institution.

***Who do I contact if I want more information about this study?***

Questions regarding this study may be directed to the principal investigator, Angela Ratekin, Department of Microbiology, UW-La Crosse, Tel (608) 785 6451, E-mail: [aratekin@uwlax.edu](mailto:aratekin@uwlax.edu). Questions regarding the protection of human subjects may be addressed to Robert Hoar, the UW-La Crosse Institutional Review Board for the Protection of Human Subjects (608) 785 8124, [rhoar@uwlax.edu](mailto:rhoar@uwlax.edu).

***What does signing this consent form mean?***

My signature indicates that:

- < I have read the above
- < I have decided that I will take part in the study as described above.
- < The purpose of the study, the details of my involvement and possible risks and discomforts has been explained to my satisfaction.
- < I understand that I will receive a copy of this consent form once signed.
- < I understand that I do not give up any legal rights by signing this consent form.

Participant \_\_\_\_\_ Date

Researcher \_\_\_\_\_ Date

