The effect of YakA deficiency in *T. marneffei* infection of THP-1 and J774 macrophage cell lines.

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ABSTRACT

The goal of this study was to examine the host cytokine response to a mutant strain (*ΔyakA*) of *T. marneffei*. *T. marneffei* is a fungus that causes the disease talaromycosis in AIDS patients. The fungus is dimorphic and switches between growth forms depending upon temperature. *T. marneffei* exhibits mold growth at 25C but transforms into yeast cells inside of the host which become intracellular pathogens, living inside a variety of cells including macrophages. Previously, our laboratory discovered a mutant, designated *ΔyakA*. The protein encoded by the *yakA* gene is a type of molecular sensor. Studies showed that the *ΔyakA* mutant of *T. marneffei* has a weakened cell wall, which may affect its pathogenicity. Therefore, conidia of the *ΔyakA* mutant were co-cultured with the human monocyte cell lxine THP-1 or the macrophage-like murine cell line J774 to investigate whether the weakened cell wall has an effect on cytokine production, since cytokines play a vital role in host response to infection. The levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were measured from cell lines infected with the *ΔyakA* and wild-type conidia. The THP-1 human macrophages produced significantly more IL-6 and TNF-α when co-cultured with WT compared with *ΔyakA* but IL-1β showed no significant difference. There were no significant differences in cytokines measured from J774 mouse macrophages exposed to WT versus mutant conidia. Although some differences were noted between cytokine levels of macrophages incubated with WT versus *ΔyakA* conidia, the differences were not uniform enough to presently conclude that the *yakA* gene affects host cytokine response.

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CHAPTER 1: INTRODUCTION

1.1 Background

Talaromyces (previously designated *Penicillium* [Samson et al., 2011]) *marneffei* is a thermally dimorphic fungus and is an important human pathogen. The disease caused by *T. marneffei* is traditionally termed penicilliosis (though other names include penicilliosis marneffei and talaromycosis). Talaromycosis is endemic to Southeast Asia and southern China, and in some regions it has become the third most common AIDS-defining opportunistic infection after tuberculosis and *Cryptococcus* (Supparatpinyo et al., 1998). Despite its reputation, the discovery of *T. marneffei* was relatively recent with its first documented appearance in Dalet, South Vietnam less than sixty years ago (Capponi et al., 1956). The fungus was originally isolated from the hepatic lesions of a bamboo rat (*Rhisomys sinensis*) which died unexpectedly from the infection (Capponi et al., 1956). One of the first researchers to extensively work with *P. marneffei*, professor Gabriel Segretain, named the fungus after the director of the Pasteur Institute during his time, Dr. Hubert Marneffe (Segretain, 1959).

In addition to giving *T. marneffei* its name (originally as *P. marneffei*), Segretain would also become famous for becoming the first documented case of *T. marneffei* infection in a human host. While performing experiments on rodents, he accidently inoculated his own finger which formed a small nodule at the injection site (Segretain, 1959). The first naturally-occurring case of human *T. marneffei* infection was recorded many years later in 1973, involving an American minister suffering from Hodgkin's lymphoma who lived in Southeast Asia (Disalvo et al., 1973). Although a few more sporadic cases had occurred in Hong Kong, Thailand, and southern China, the incidence

rate of *T. marneffei* did not increase until after the HIV/AIDS epidemic, which happened in 1988 in Southeast Asia (Vanittanakom, et al., 2006). Reports of *T. marneffei* infection were documented globally, appearing in Europe (Borradori et al., 1994; Depraetere et al., 1998; Garbino et al., 2001; Hilmarsdottir et al., 1993, 1994; Hulshof et al., 1990; Julander & Petrini, 1997; Kok et al., 1994; Kronauer et al., 1993; Mcshane et al., 1998.; Peto et al., 1988; Rimek et al., 1999; Sobottka et al., 1996; Vilar et al., 2000), Asia (Chang et al., 1995; Chariyalertsak et al., 1997; Hien et al., 2001; Huynh et al., 2003; Liao et al., 2002; Mohri et al., 2000; Ranjana et al., 2002; Tsunemi et al., 2003), Australia (Heath et al., 1995), Cambodia (Bailloud et al., 2002), and the United States (Nord et al., 1998).

AIDS patients are the most susceptible to *T. marneffei* infection, but other immunosuppressive conditions can make non-AIDS patients susceptible (Chan et al., 2016). Actually, the occurrence of talaromycosis in people who have HIV/AIDS has been decreasing due to antiretroviral therapy whereas the occurrence in people without HIV/AIDS has increased in parts of Asia since the 1990's (Chan et al., 2016; Vanittanakom et al., 2006). According to Chan et al. (2016), this may be explained by improved diagnosis of talaromycosis and an increasing number of people with immunosuppressive conditions other than AIDS, particularly those with compromised cell-mediated immunity. Comorbidities that cause non-AIDS related adult-onset immunosuppression include anti-interferon-gamma autoantibodies and secondary immunosuppressive conditions including hematopoietic stem cell and solid organ transplantations, immunosuppressive drugs that deplete T-lymphocytes, novel anti-cancer targeted therapies, and other autoimmune diseases (Chan et al., 2016). According to Vanittanakom et al. (2006) and Wong et al. (1999), infection severity varies with the

degree of immunosuppression. *Talaromyces marneffei* infection in HIV-infected patients is frequently disseminated, involving multiple organs. According to Chan et al. (2016), the infection may be focal or disseminated in non-HIV-infected individuals, depending on the type and severity of immunosuppression and how early the patient is diagnosed. *Talaromyces marneffei* infection has a higher case-fatality rate in non-HIV-infected individuals as opposed to HIV-infected individuals even though it is typically more severe in HIV-infected patients because the infection is frequently disseminated and involves multiple organs. The higher fatality rate noted in non-HIV-infected individuals may be associated with delayed diagnosis caused by an absence of clinical suspicion (Chan et al., 2016). AIDS or no AIDS, scientists estimate that more than 75% of patients with talaromycosis will die without proper treatment with an antifungal medicine (Supparatpinyo et al., 1994; Supparatpinyo et al., 1993; Vanittanakom et al., 2006).

Despite there being plentiful isolates and well-documented cases, the natural reservoir of *T. marneffei* has yet to be discovered (Capponi et al., 1956). Later studies found that bamboo rats (*Rhizomys* sp. and *Cannomys* sp.) in addition to soil from the rats' burrows were significant reservoirs of *T. marneffei* but exposure to or consumption of these rats has not shown to be a risk factor for infection (Capponi et al., 1956; S Chariyalertsak et al., 1996; Chariyalertsak et al., 1997; Deng et al., 1986; Gugnani et al., 2004). Interestingly, young farmers in certain parts of Southeast Asia, southern China, or eastern India appear to be more likely to contract talaromycosis (Chariyalertsak et al., 1997). Regardless, no additional soil or plant-based reservoir has been discovered. Supported by the fact that most human cases that have been documented occurred in cities where these species of rodents rarely frequent, it is thought that non-isolated, soil-

based conidia may gather and enter human hosts through inhalation during Thailand's rainy season (Chariyalertsak et al., 1996; Liu et al., 2011).

1.2 Dimorphism

Like a number of other pathogenic fungi, *T. marneffei* has evolved the ability to alternate growth forms, a property termed dimorphism. The dimorphic stages are hyphal growth at 25°C and single-celled yeast at 37°C. The hyphae are composed of filamentous bodies that grow through lateral branching and apical extension and produce asexual spores, or conidia, which are the infectious agents that are inhaled into the host lung (Boyce & Andrianopoulos, 2013). Conidia inhaled into the lung become yeast cells that replicate by binary fission. The dimorphic capability of *T. marneffei* is thought to serve an important role in the virulence of this pathogenic fungus and an inability to make this transition to the yeast phase has been shown to result in a loss of virulence (Andrianopoulos, 2002; Boyce et al., 2009; Cánovas & Andrianopoulos, 2006; Todd et al., 2003).

1.3 Pathogenesis, virulence, and host defenses

Alveolar macrophages seem to be the first immune cells involved in *T. marneffei* infection. Alveolar macrophages are the phagocytes that guard the alveolar-blood interface, serving as the first line of defense against respiratory pathogens, including aspirated *T. marneffei* conidia (Sibille & Reynolds, 1990). The macrophage kills the ingested conidia and other microbes by entrapping them in phagosomes which destroy the invading pathogens through enzymatic attack, oxidative burst, nutrient-limitation, and acidic condition (pH 4–5) (Becker et al., 2015; Nyberg et al., 1992) Alveolar

macrophages also function as regulators of innate defenses by synthesizing and secreting a wide selection of cell signal molecules called cytokines (Roilides et al., 2003). According to Haslett (1999), some cytokines initiate inflammatory responses and recruit other immune cells, such as neutrophils, into the area of infection. Examples of pro-inflammatory cytokines include interleukin-1β, tumor necrosis factor-α, and interleukin-6 (Haslett, 1999). *T. marneffei* is also susceptible to killing by neutrophils. The neutrophils recruited by the macrophages are stimulated to kill *T. marneffei* yeast cells by granulocyte macrophage colony-stimulating factor (GM-CSF) secreted by the alveolar macrophages (Becker et al., 2015).

In healthy human and murine hosts, macrophages can control *T. marneffei* growth and eliminate intracellular yeast cells (Vanittanakom et al., 2006). Therefore, in healthy hosts, *T. marneffei* is usually cleared within 3 weeks, dependent on the size of inoculum, whereas in T-cell-depleted mice or CD4+ T-cell depleted AIDS patients, infection is fatal without proper treatment (Kudeken et al., 1996, Kudeken et al., 1997, Viviani et al., 1993, Supparatpinyo et al., 1992). These results demonstrated that CD4+ T cells are essential for eradicating this fungal infection in mice and humans. T-cells secrete cytokines, such as gamma interferon (IFN-γ), that activate macrophages, which becomes vital in the clearance of intracellular pathogens (Cogliati et al., 1997, Kudeken et al., 1998, Kudeken et al., 1999, Kudeken et al., 1999, Levitz, 1992, Taramelli et al., 2000). It appears that T-cells are also vital in eradicating talaromycosis.

Despite the existence of various protective immune mechanisms, *T. marneffei* is able to survive the harsh intracellular conditions of macrophages and evade other host

immune responses. To comprehend the pathogenesis of *T. marneffei*, more studies of the virulence factors associated with the fungi are needed. One presumed virulence attribute of *T. marneffei* is its dimorphic capability. An inability to make this transition to the yeast phase has been shown to result in a loss of *T. marneffei* virulence (Andrianopoulos, 2002; Boyce et al., 2009; Cánovas & Andrianopoulos, 2006; Todd et al., 2003). It is known that adhesion to host cells is the first step in colonization and biofilm formation. A laminin-binding protein appearing to be a sialic acid-specific lectin may function as a virulence factor identified in *Aspergillus fumigatus* (Bouchara et al., 1997; Gil et al., 1996; Tronchin et al., 1997). Laminin functions as an extracellular matrix glycoprotein that is located in basement membranes, and tissue damage might expose it (Knibbs et al., 1989). *T. marneffei* conidia also utilize a laminin-binding dependent adhesion mechanism (Hamilton et al., 1999; Hamilton et al., 1998). Lau et al. (2013) identified one of the surface molecules involved in the attachment of *T. marneffei* to laminin. By using inhibition assays involving rGAPDH proteins and anti-rGAPDH antibodies, researchers demonstrated that glyceraldehayde-3-phosphate dehydrogenase (GAPDH) mediates binding of conidia to laminin and human pneumocytes cultured *in vitro*. This suggests that GAPDH may play a key role in establishing *T. marneffei* infection by mediating adherence to host bronchoalveolar epithelium (Lau et al., 2013).

Another virulence factor is the production of DOPA-melanin or melanin-like substance by *T. marneffei* which enhances the fungi's resistance to phagocytosis by macrophages. Strong evidence has also been provided for the role of fungal laccases in host responses to *T. marneffei.* Laccases expressed by fungi have been previously linked to stress resistance, nutrient acquisition, pathogenicity, detoxification processes, and

morphogenesis (Baldrian, 2006). Laccase has been best characterized in the pathogenic yeast *Cryptococcus neoformans* in which it is an important virulence factor (Williamson, 1997; Zhu & Williamson, 2004). Laccase expression is stimulated by environmental stress such as exposure to low pH, nutrient deprivation, and oxidative stress, the exact conditions encountered by *T. marneffei* living inside of macrophages (Baldrian, 2006; Piscitelli et al., 2011). Sapmak A. et al. (2016) demonstrated that laccase production in *T. marneffei* significantly decreases phagocytosis and release of cytokines by macrophages as well as increases intracellular survival of conidia in THP-1 cells. A last virulence factor is the secretory mannoprotein Mp1p which has been shown by Sze et al. (2017) to sequester the pro-inflammatory lipid arachidonic acid and effectively dampen host immune responses.

1.4 Agrobacterium tumefaciens-Mediated Transformation

Researchers have employed *Agrobacterium tumefaciens*-Mediated Transformation (ATMT) to genetically characterize *T. marneffei* and to explore those genetic mechanisms that regulate dimorphism (Zhang et al., 2008; Kummasook et al., 2010; Chandler et al., 2008). This method was developed by Zhang et al. (2008) and improved upon by Kummasook et al. (2010). *Agrobacterium tumefaciens* is a Gram-negative bacterium that possesses a tumor-inducing plasmid. The plasmid is known to cause crown gall tumors in plants by guiding random DNA insertion into the plant's genome and inducing uncontrolled growth (Brencic et al., 2005). *Agrobacterium tumefaciens* can be used to stimulate random mutagenesis in *T. marneffei*.

The *A. tumefaciens* plasmid has been used in research to transform genetically

tagged, random inserts into the *T. marneffei* genome. Although these transformations are random, they tend to disrupt a single gene. The transformants are then cultured alongside wild-type *T. marneffei* to monitor phenotypic differences. The DNA of strains of interest is isolated, digested to target the tagged insertion sites, and subjected to inverse PCR for amplification. The amplified sites around the ATMT insertions are electrophoretically isolated, sequenced, and analyzed via the online program Basic Local Alignment Search Tool (BLAST). Since the genome of *T. marneffei* has already been sequenced, the exact gene that was disrupted is often identifiable for a particular ATMT-generated sample of interest (Kummasook et al., 2010).

1.5 Talaromyces marneffei Mutant I231 – Δ*yakA*

Using the ATMT system, Suwunnakorn et al. (2014) concluded that the *P. marneffei* mutant strain I231 contained a defective *yakA* gene. The *yakA* gene codes for a dual-specificity protein kinase, regulated by tyrosine phosphorylation. Homologues to the *yakA* gene function in carbon source response, growth regulation, and stress response, particularly the regulation of the stress response transcription factors *hsf1* and *msn2/4* (Aranda et al., 2011; Hartley et al., 1994; Lee et al., 2012). The gene *msn2/4* functions in various cell wall-based stress responses and *hsf1* functions in thermotolerance. (Elfving et al., 2014; Lee et al., 2008; Sadeh et al., 2012; Verna et al., 1997; Garrett et al., 1991; Goyard et al., 2008; Malcher et al., 2011; Moriya et al., 2001).

The *yakA* mutant strain, designated *ΔyakA*, produces fewer conidia and phialides (Suwunnakorn et al., 2014). Supplementation of the growth medium with glucose slightly restores this condition, as it allows for a bypass mechanism that promotes conidiogenesis

by effectively increasing mRNA levels of *abaA*, a conidial production gene (Bulik et al., 2003; Borneman et al., 2000). Although conidia levels are decreased, the mutant with a defective *yakA* gene developed more rapid conidial germination, which usually signifies a weakened and more flexible fungal cell wall. It was determined previously that this compromised cell wall integrity in the *ΔyakA* strain resulted in hypersensitivity when cultured with the cell-wall stressors ionic detergent sodium dodecyl sulfate (SDS) and the β-1,3-glucan synthase inhibiting antifungal drug caspofungin (CAS). On the other hand, the *ΔyakA* strain was resistant to the anionic glucan-binding dye Congo Red (CR) (Kopecká and Gabriel, 1992; Verwer et al., 2012; Roncero & Durán, 1985). Further studies of the *ΔyakA* cell-wall using the chitin-binding agent Calcoflour White (CW) revealed abnormal distribution of chitin, suggesting that *yakA* plays a role in determining cell-wall ultrastructure (Hill et al., 2006). In addition, the mutant was found to possess higher levels of cell-wall chitin when compared to wild-type. This most likely occurs because chitin synthases, a large group of enzymes responsible for synthesis and distribution of cell-wall chitin, will attempt to compensate for a lack of proper-cell wall functionality by over-producing chitin (Beauvais et al., 2013; Latgé, 2007; Lee et al., 2012; Ram et al., 2004; Fuchs and Mylonakis, 2009).

The higher chitin content in *ΔyakA* prompted a study of chitin synthase gene expression and the results indicated that this mutant strain may be activating an alternative chitin biosynthetic pathway to compensate for its weakened cell wall (Levin et al., 2011; Liu et al., 2011). Engle (2015) measured expression levels of seven known chitin synthases in *T. marneffei* when exposed to the cell-wall stressors CR, SDS, and Calcofluor White and determined that the *ΔyakA* strain produced no significant

differences in chitin-synthesis gene expression under stressed conditions. The mutant did however produce significantly higher expression levels of chitin synthase genes in the *ΔyakA* control conditions at 25ºC and 37ºC. Although these results reveal the importance of this gene and its corresponding kinase, additional testing revealed no apparent role of *yakA* in virulence.

1.6 Cytokine studies with *T. marneffei*

Prior cytokine studies with *T. marneffei* yielded noteworthy results about fungi virulence. A mutant strain of *T. marneffei* lacking four different laccase genes was used to infect the human monocyte cell line, THP-1. The levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α secreted by THP-1 cells infected with the quadruple laccase mutant were significantly higher compared with the wild type. Overexpression oof pro-inflammatory cytokines have been related to successful immune responses to other thermally dimorphic fungi, such as *A. fumigatus* (Stevens 2006). The *T. marneffei* wild-type conidia exhibited a less robust host pro-inflammatory response compared with the response to the quadruple disruptant. This demonstrates that *T. marneffei* production of laccases promotes the fungi's resistance to host defenses (Sapmak et al., 2016).

In another study, it was discovered that an abundant secretory mannoprotein Mp1p in *T. marneffei*, previously shown to be a virulence factor, causes down-regulation of the cytokines TNF-α and IL-6 in *T. marneffei*-infected J774 macrophages (Sze et al., 2017). TNF-α targets vasculature to cause inflammation and also targets the liver to induce the production of acute phase proteins, which are chemical mediators released in response to tissue damage. TNF-α can induce death in many cell types and activates

neutrophils. IL-6 is secreted by both macrophages and endothelial cells to induce an antiviral state in most nucleated cells, increases MHC class I expression, and activates natural killer cells (all important in the defense against intracellular pathogens). IL-1β causes inflammation and induces acute phase proteins. Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 function as regulators of innate response, meaning that when macrophages are overwhelmed with the number of infectious particles, these cytokines induce powerful antifungal cellular responses to eradicate the pathogen. A down-regulation of host pro-inflammatory responses may impede the competence of the antifungal defense mechanisms (Sapmak et al., 2016).

CHAPTER 2: SPECIFIC AIMS AND HYPOTHESIS

The specific aim of this study is to compare the pathogenicity of *ΔyakA* conidia with wild-type conidia when both are used to infect two macrophage cell lines: J774A.1 and THP-1. It is important to note that these two cell lines are not primary cells isolated from subjects; these are cells purchased from the American Type Culture Collection. J774A.1 originates from mouse BALB/c monocyte macrophages and was chosen because rodents are an important reservoir of *T. marneffei*—Segretain used rodents for his studies when he became the first documented case of *T. marneffei* infection—and *T. marneffei* was first isolated from the hepatic lesions of bamboo rats. THP-1 originates from a human monocytic leukemia line established in 1980 by Tsuchiya et al. (1980). THP-1 cell line has been extensively used to study monocyte and macrophage functions, mechanisms, nutrient and drug transport, and signaling pathways, and is a suitable in vitro cell model to study modulation of monocyte/macrophage activities (reviewed in Chanput et al., 2014). Both THP-1 and J774 cell lines have been used as infection hosts in vitro (Taramelli et al., 2000, Taramelli et al., 2001, Sapmak et al., 2016; Sze et al., 2017; Wasylnka and Moore, 2003; Zhang J. et al., 2013;). Both cell lines have also been used to measure cytokine profiles (Woodfork K.A. et al., 2001; Sapmak et al., 2016; Sze et al., 2017; Raza H. et al., 2014).

The pathogenicity of the wild-type and mutant conidia will be measured using a set of three ELISAs to determine the role of the *yakA* gene in cytokine production. The cytokines TNF-α, IL-6 and IL-1β were chosen because all are induced in macrophages post-infection. It is hypothesized that *ΔyakA* conidia will induce a greater production of

macrophage cytokines when compared with wild-type conidia.

CHAPTER 3: MATERIALS AND METHODS

3.1 *Talaromyces marneffei* Strains and Fungal Culturing

This study utilized two strains of *T. marneffei* including the F4 strain originally isolated from a Chiang Mai AIDS patient (wild-type; CBS 119456) (Pongpom et al., 2005) and the Δ*yakA* strain (Suwunnakorn et al., 2014). In order to ensure strain stability, both of these strains were continually cultured on a weekly basis in 75cm^2 Nunc EasYFlasks (ThermoScientific Roskilde, Denmark) containing potato dextrose agar (PDA) (BD Difco; Franklin Lakes, New Jersey) and allowed to grow at room temperature.

3.2 *T. marneffei* preparation for assays

Ten days before the planned infection, wild-type and mutant strains were streaked onto ten separate 75cm^2 flasks (5 for each strain) containing PDA supplemented with glucose (40g per 1L). On the day macrophages were infected, conidia were harvested by suspension in sterile water. The conidial suspension was filtered through sterile glass wool (Corning) where fungal hyphae were trapped on the surface while conidia passed through the glass wool. The eluate containing mycelium-free conidia was centrifuged for 10 minutes at 600xg for a total of three cycles and the pellets were washed twice in phosphate buffered saline (PBS, pH 7.2). The conidial suspension, at an initial concentration of 6 x 10^7 conidia/ml, was used for the experiments. No more than 2 hours before infection, colonies of the wild-type (strain F4) and mutant (Δ*yakA*) strains were suspended in DMEM (for J774) or RPMI 1640 plus 2 g per L sodium bicarbonate (tissue-culture tested) (for THP-1) with 10% fetal bovine serum (FBS).

3.3 Cell Cultures

The human macrophage cell line THP-1 (ATCC TIB-202) was continually cultured in RPMI 1640 medium (Gibco 31800-022) supplemented with 2.0g/L tissue-culture tested sodium bicarbonate and 10% heat-inactivated FBS at 37°C + 5% CO_2. A total number of 4 x 10^6 cells per well was used for the experiments. The cells were seeded into 24 well culture plates 72 hours before infection and differentiated with 100ng/ml phorbol 12-myristate 13-acetate (PMA). PMA is an activator of protein kinase C and causes the THP-1 cells to differentiate into mature, adherent macrophages (Sigma-Aldrich). Undifferentiated THP-1 cells (no PMA) in RPMI 1640 with conidia and THP-1 cells in PMA without conidia were used as controls. The undifferentiated THP-1 cells exposed to conidia was used as a negative control because undifferentiated THP-1 cells are not expected to contribute cytokine signatures. THP-1 cells differentiated with PMA but without conidia were used to establish a baseline for cytokine secretion.

The murine macrophage-like J774 cells were continually cultured in DMEM (Sigma Aldrich D5796) supplemented with 10% heat-inactivated FBS at 37°C + 5% CO_2. A total number of 4 x 10^6 cells per well was used for the experiments. On the day of infection, the cells were washed three times with PBS and resuspended in 24-well tissue culture plates with DMEM supplemented with 10% FBS. J774 cells without conidia served as a control. All of the experiments were performed in duplicate.

3.4 Induction of cytokines in cell lines THP-1 and J774

THP-1 (4×10^6) or J774 (4×10^6) cells were cultured with 3×10^6 WT or mutant conidia and incubated for 16 or 24 hours at 37°C. Data were collected at an additional timepoint of 2 hours post infection but were excluded from the study because supernatants were stored before proper centrifugation and pellet removal. After incubation, the supernatants were collected and kept at -20°C until the cytokine assays were performed. The pro-inflammatory cytokines TNF-α, IL-6, and IL-1β were measured by commercial ELISA kits according to the manufacturer's instructions (Invitrogen Ready-Set-Go by ThermoFisher). The concentrations of cytokines in the experimental samples were calculated according to the optical densities at 450 nm obtained from the wells containing cytokine standard.

3.5 Statistical Data Analysis

All data analyses were performed using SPSS software (Version 17.0; IBM; Amonk, New York). After infection of THP-1 and J774 macrophages with wild-type and *ΔyakA* fungal strains, the levels of secretion of cytokines TNF-α, IL-6, and IL-1β were examined by 2 X 2 two-factor ANOVA to determine any statistically significant relationships of the variable cytokine concentration (pg/ml) between and among fungal strain and time since infection. Cytokine expressions were analyzed separately rather than as multivariate data because they were generated by independently run ELISAs, and also because degrees of freedom were likely insufficient for a MANOVA. Data were not analyzed by repeated measures ANOVA because different levels of the time factor (16 and 24 hours) occupied separate culture wells. The type of macrophage cell line used was

not included as an ANOVA factor because of the difficulty in comparing cell lines especially across species (murine vs human). This is due to dramatic changes in the genes related to proliferation and control of the cell cycle that arise from immortalization steps required to make cell lines. Thus, huge differences in gene expression profiles exist across cancer cell lines, even those classified into the same organ types (Lee et al., 2018).

Any significant results in cytokine expression generated by the macrophage X time ANOVA were subsequently analyzed by one-factor ANOVA and Tukey's post-hoc test in order to determine significant differences in cytokine release between experimental groups and a no conidia control. It is important to analyze the no conidia control in order to obtain a base-line of cytokine secretion, because the J774 and THP-1 cell lines secrete a normal amount of cytokine without stimulation. Then this baseline can be compared with the experimental groups of cell line stimulated with wild-type or *ΔyakA* fungal strains.

CHAPTER 4: RESULTS

General Experimental Information. We analyzed the secretion of IL-1β, TNF-α, and IL-6 by both THP-1 and J774 cells. Figures 1-3 depict cytokine release by THP-1 cells infected with fungal strains and Figures 4-6 depict cytokine release by J774 cells. The bar graphs for each figure were generated using Microsoft Excel software. All figures have the same scale to facilitate comparison of results.

Each experimental condition was performed in duplicate and the results averaged. It should be noted that the ELISA measuring IL-1β secreted by THP-1 cells was performed a second time because the first time yielded absorbances that were too high to be read by the spectrophotometer. For the second trial the sample that was too concentrated was diluted by half and the ELISA was repeated.

Secretion of Cytokines by THP-1 Cells. Figure 1 depicts the release of IL-1β by THP-1 cells infected with conidia of the *ΔyakA* and wild-type strains. There were not any significant differences in IL-1β secretion by THP-1 cells infected with wild-type versus mutant strains ($p=0.113$). However, there was a significant decrease (1.04 fold-decrease) in the overall release of this cytokine at the 16- and 24-hour time-points ($p=0.032$). There was a significant interaction term between strain and time ($p=0.042$). There were significant increases in the levels of IL-1β released at 16- and 24-hour time-points (2.34 and 2.26 fold-increases, respectively) containing conidia when compared with the no conidia control group ($p=0.000$ and $p=0.000$, respectively).

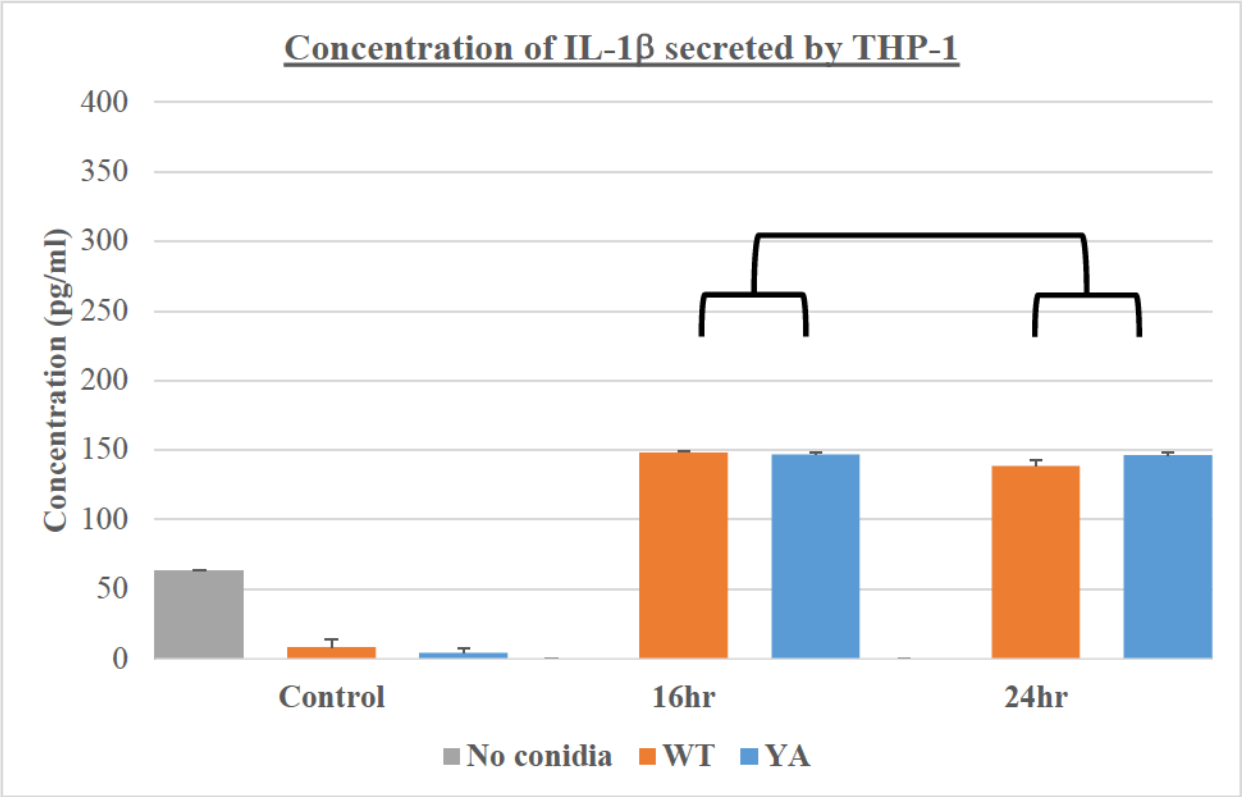


Figure 1. Concentration of IL-1β secreted by THP-1 macrophages when exposed to wild-type and *ΔyakA* conidia. Cytokine levels were measured at 16- and 24-hours after initial exposure with conidia, as well as under non-exposed (control) conditions measured at 24 hours. The grey control bars indicate the macrophages were differentiated with PMA, whereas the orange and blue control bars indicate undifferentiated THP-1 (no PMA) cells exposed to wild type or *ΔyakA* conidia, respectively. Statistical analyses were not done comparing these no PMA controls exposed to conidia with other data because cytokine signatures were found to be negligible. Absorbances from a blank well containing buffer only was subtracted from all samples before concentrations were calculated. Concentrations were calculated using a standard curve yielding the highest R-value (Appendix A). All results were performed in duplicate. Error bars represent SD. Statistical analyses can be found in Appendix D.

Figure 2 depicts the release of TNF-α by THP-1 cells infected with conidia of the *ΔyakA* or wild-type strains. There was a significant decrease (1.27-fold decrease) in TNF-α secretion by THP-1 cells infected with *ΔyakA* conidia versus wild-type ($p=0.001$). There was a significant decrease (1.17-fold decrease) in the overall release of this cytokine at the 16- and 24-hour time-points ($p=0.004$) but the interaction term between strain and time was not significant ($p=0.129$). There were significant increases in the levels of TNF-α released at 16-hour wild-type ($p=0.000$; 3.47-fold increase), 16-hour *ΔyakA* ($p\leq0.0001$; 2.91-fold increase), 24-hour wild-type ($p\leq0.0001$; 3.15-fold increase), and 24-hour *ΔyakA* ($p=0.001$; 2.29-fold increase) groups when compared with the no conidia control group.

Figure 3 depicts the release of IL-6 by THP-1 cells infected with conidia of the *ΔyakA* or wild-type strains. There was a significant decrease (1.67-fold decrease) in IL-6 secretion by THP-1 cells infected with *ΔyakA* conidia versus wild-type ($p=0.037$). There were no significant differences in cytokine release between 16- and 24-hour time points ($p=0.842$) nor was there a significant interaction term between strain and time ($p=0.840$). There was a significant increase (2.72-fold increase) in the level of IL-6 released in groups containing WT conidia when compared with the no conidia control group ($p=0.004$). There was no significance in the level of IL-6 released in groups containing *ΔyakA* conidia when compared with the no conidia control group ($p=0.229$).

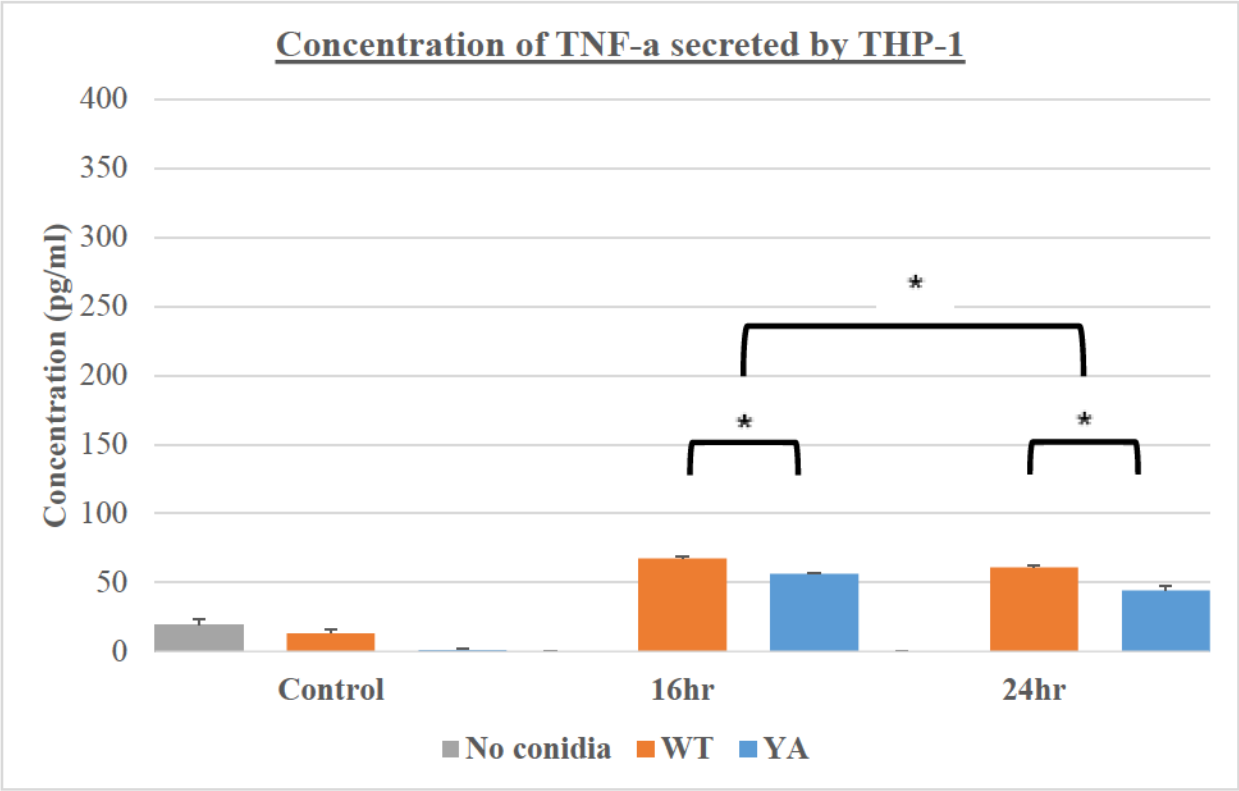


Figure 2. Concentration of TNF-α secreted by THP-1 macrophages when exposed to wild-type and *ΔyakA* conidia. Cytokine levels were measured at 16- and 24-hours after initial exposure with conidia, as well as under non-exposed (control) conditions measured at 24 hours. The grey control bars indicate the macrophages were differentiated with PMA, whereas the orange and blue control bars indicate undifferentiated THP-1 (no PMA) cells exposed to wild type or *ΔyakA* conidia, respectively. Statistical analyses were not done comparing these no PMA controls exposed to conidia with other data because cytokine signatures were found to be negligible. Absorbances from a blank well containing buffer only was subtracted from all samples before concentrations were calculated. Concentrations were calculated using a standard curve yielding the highest R-value (Appendix A). All results were performed in duplicate. Error bars represent SD. Statistical analyses can be found in Appendix D.

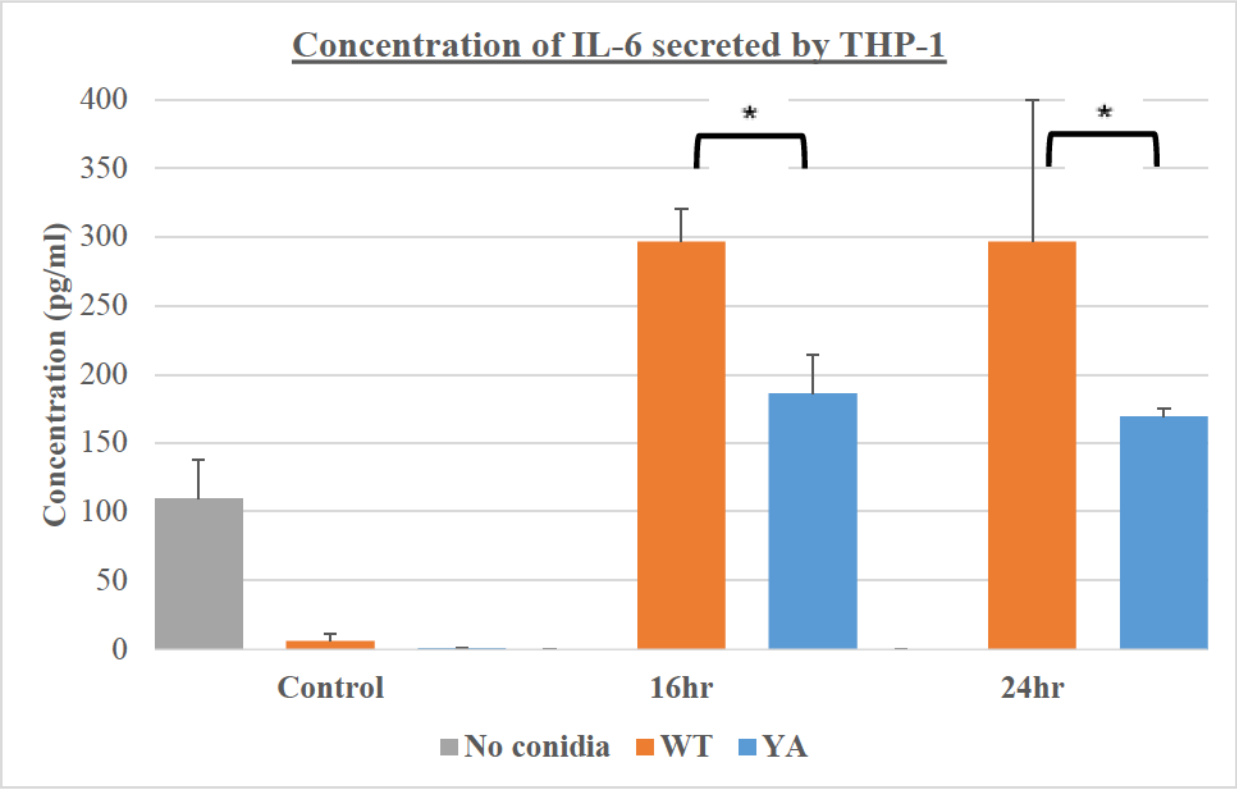


Figure 3. Concentration of IL-6 secreted by THP-1 macrophages when exposed to wild-type and *ΔyakA* conidia. Cytokine levels were measured at 16- and 24-hours after initial exposure with conidia, as well as under non-exposed (control) conditions measured at 24 hours. The grey control bars indicate the macrophages were differentiated with PMA, whereas the orange and blue control bars indicate undifferentiated THP-1 (no PMA) cells exposed to wild type or *yakA* conidia, respectively. Statistical analyses were not done comparing these no PMA controls exposed to conidia with other data because cytokine signatures were found to be negligible. Absorbances from a blank well containing buffer only was subtracted from all samples before concentrations were calculated. Concentrations were calculated using a standard curve yielding the highest R-value (Appendix A). All results were performed in duplicate. Error bars represent SD. Statistical analyses can be found in Appendix D.

Secretion of Cytokines by J774 Cells. Figure 4 depicts the release of IL-1β by J774 cells infected with conidia of the *ΔyakA* or wild-type strains. There were no significant differences in IL-1β secretion by J774 cells infected with *ΔyakA* conidia versus wild-type (p=0.537). There were no significant differences in cytokine release between 16- and 24-hour time points (p=0.496) nor was there a significant interaction term between strain and time (p=0.619).

Figure 5 depicts the release of TNF-α by J774 cells infected with conidia of the *ΔyakA* and wild-type strains. There were no significant differences in TNF-α secretion by J774 cells infected with *ΔyakA* conidia versus wild-type (p=0.945). There were no significant differences in cytokine release between 16- and 24-hour time points (p=0.058) nor was there a significant interaction term between strain and time (p=0.848).

Figure 6 depicts the release of IL-6 by J774 IL-6 cells infected with conidia of the *ΔyakA* or wild-type strains. There were no significant differences in IL-6 secretion by J774 cells infected with *ΔyakA* conidia versus wild-type (p=0.194). There were no significant differences in cytokine release between 16- and 24-hour time points (p=0.479) nor was there a significant interaction term between strain and time (p=0.815).

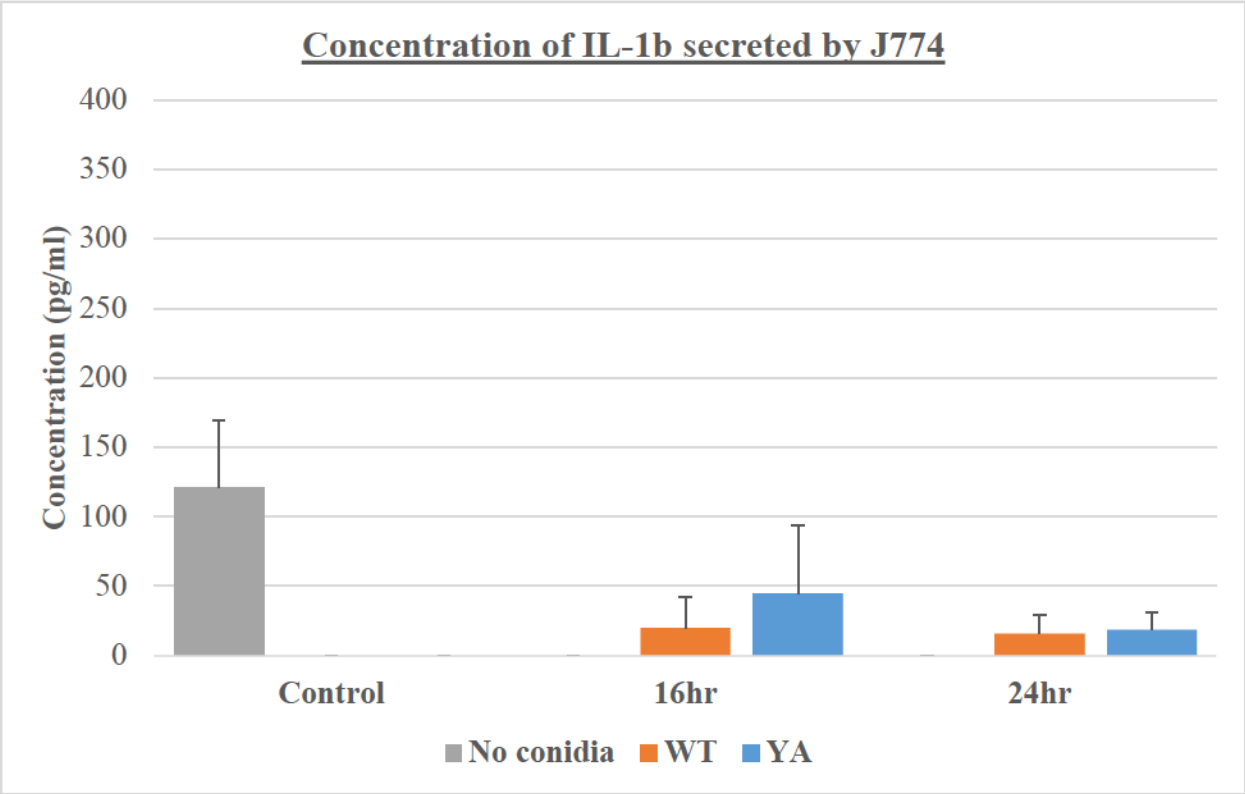


Figure 4. Concentration of IL-1β secreted by J774 macrophages when exposed to wild-type and *ΔyakA* conidia. Cytokine levels were measured at 16- and 24-hours after initial exposure with conidia, as well as under non-exposed (control) conditions measured at 24 hours. The grey bar indicates macrophages incubated without conidia. Absorbances from a blank well containing buffer only was subtracted from all samples before concentrations were calculated. Concentrations were calculated using a standard curve yielding the highest R-value (Appendix A). All results were performed in duplicate. Error bars represent SD. Statistical analyses can be found in Appendix D.

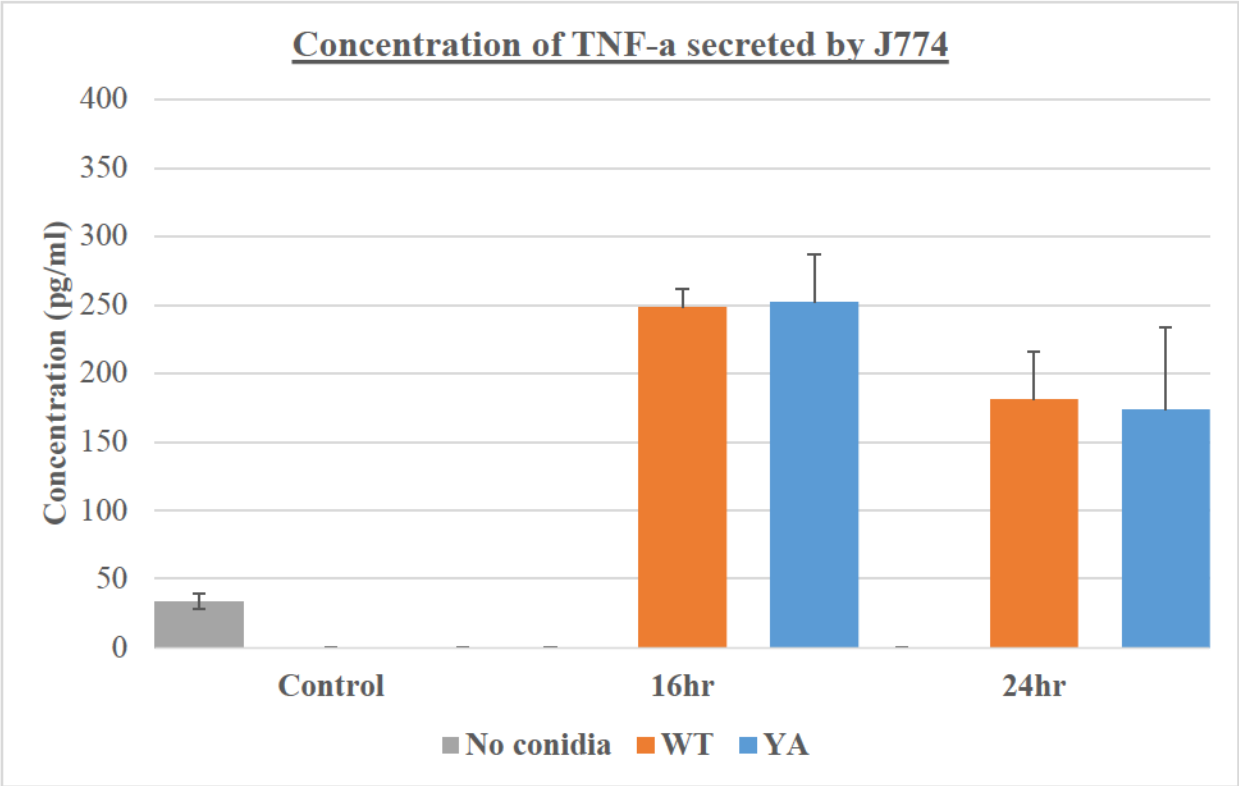


Figure 5. Concentration of TNF-α secreted by J774 macrophages when exposed to wild-type and *ΔyakA* conidia. Cytokine levels were measured at 16- and 24-hours after initial exposure with conidia, as well as under non-exposed (control) conditions measured at 24 hours. The grey bar indicates macrophages incubated without conidia. Absorbances from a blank well containing buffer only was subtracted from all samples before concentrations were calculated. Concentrations were calculated using a standard curve yielding the highest R-value (Appendix A). All results were performed in duplicate. Error bars represent SD. Statistical analyses can be found in Appendix D.

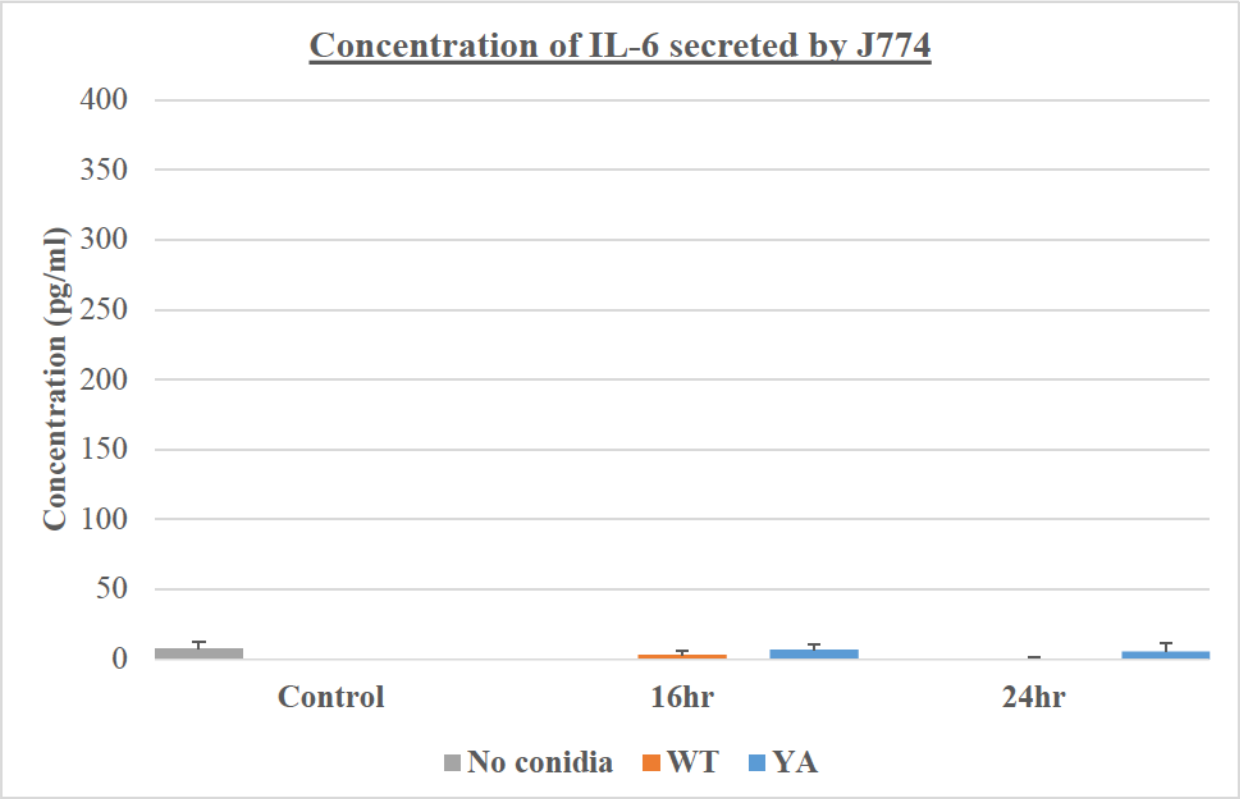


Figure 6. Concentration of IL-6 secreted by J774 macrophages when exposed to wild-type and *ΔyakA* conidia. Cytokine levels were measured at 16- and 24-hours after initial exposure with conidia, as well as under non-exposed (control) conditions measured at 24 hours. The grey bar indicates macrophages incubated without conidia. Absorbances from a blank well containing buffer only was subtracted from all samples before concentrations were calculated. Concentrations were calculated using a standard curve yielding the highest R-value (Appendix A). All results were performed in duplicate. Error bars represent SD. Statistical analyses can be found in Appendix D.

CHAPTER 5: DISCUSSION

Talaromycosis has traditionally been identified as an AIDS-defining illness in endemic areas. Through better control of HIV infection using highly active antiretroviral therapy, the incidence rate of *T. marneffei* infection associated with HIV infection has been on the decline (Chan et al., 2016). On the other hand, talaromycosis is increasingly reported in patients with other immunodeficiencies (Chan et al., 2016; Vanittanakom et al., 2006). Enhanced genetic testing for a variety of immunodeficiency syndromes has increased the identification of *T. marneffei* infection in children without HIV. A study by Chan et al., (2016) also showed the mortality rate of non-HIV-infected individuals with *T. marneffei* infection (27.7%) was higher than that of HIV-infected patients (20.7%). This difference in mortality rate might reflect overdue diagnosis of the fungal infection caused by an absence of clinical suspicion (Chan et al., 2016).

Most of these non-HIV-infected patients were misdiagnosed and treated as tuberculosis since both infections occur in Southeast Asia, have overlapping early symptoms, and similar predisposing factors (Chan et al., 2016). After misdiagnosis, appropriate diagnosis with *T. marneffei* infection often wasn't established until weeks had passed when the patient hadn't improved with anti-tuberculosis treatment (Chan et al., 2016). Moreover, some of the patients that had visited endemic areas from non-endemic areas only became symptomatic months to years after initial exposure (De Monte et al., 2014; Hart et al., 2012).

For whatever reason the mortality rate is higher for non-HIV-infected individuals with talaromycosis. Prophylaxis drug development would benefit immunocompromised travelers to *T. marneffei* endemic areas. Even if prophylaxis drugs aren't a suitable path

of treatment for talaromycosis, patients would benefit from a treatment that is more specific to *T. marneffei*. The current treatment regimen is comprised of the antifungal drugs amphotericin B and itraconazole for a total of 12 weeks. These antifungal drugs, along with many others, cause many side effects, as it is difficult to target the fungi without affecting human cells. Therefore, development of a drug to specifically target *T. marneffei* could decrease treatment time and the amount of side effects seen.

Identifying *T. marneffei* genes that are important to the initial infection in humans or phase-dependent proteins that are important to the transformation of hyphae to yeast *in vivo* could help in the development of drugs to eliminate *T. marneffei* as early as first exposure. In this study, identifying virulence genes is attempted by investigating the potential involvement of *yakA* in *T. marneffei* infection by comparing the cytokine response of J774 and THP-1 macrophage cell lines exposed to *ΔyakA* mutant and wild-type *T. marneffei* conidia. It was originally hypothesized that the *ΔyakA* conidia would induce a greater production of macrophage cytokines when compared with wild-type conidia but analysis led to a failure to reject the null hypothesis due to variable differences in cytokine levels when comparing cells infected with mutant versus wild-type conidia.

Through ELISAs testing for the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, we demonstrated that IL-6 and TNF-α concentrations significantly decreased when THP-1 cells were exposed to *ΔyakA* conidia as compared with wild-type. Both the *ΔyakA* and wild-type conidia stimulated more IL-6 and TNF-α cytokine release when compared with the no conidia control (Figures 2 & 3) Measurement of IL-1β did not reveal any significant differences in production when comparing the two types of conidia

(Figure 1). Since THP-1 cells released more cytokine with the mutant than with no conidia, the mutant conidia are successfully infecting the macrophages. However, the mutant doesn't cause as much cytokine release as the wild-type, which could indicate that the *yakA* gene is either more effective than the wild type at suppressing a cytokine response or that the *yakA* gene is less effective at causing infection. Since the goal of the conidia is to inhibit immune recognition, it's to be expected that with the more virulent conidia, the macrophages will not be alerted to fungal presence and therefore fewer cytokines will be released by the macrophages. It should be noted that while TNF-α comparison between the two types of conidia had a P value <0.05, this may not be a reflection of biological significance but rather small sample size with a single data point that influenced the P value.

In a similar study by Sapmak et al. (2016) THP-1 cells were infected with *T. marneffei* conidia from wild-type and a quadruple *lac* gene deletion strain and secreted IL-1β, IL-6, and TNF-α were measured using ELISAs after 2, 8, and 24 hours post-infection. Every experiment was repeated at least 3 times and each set was performed in duplicate. The average secretion levels measured by THP-1 cells infected by their wild-type at 24 hours were drastically different from cytokines measured in this study. The study by Sapmak et al. measured 57 times more IL-1β and 12 times less IL-6 than the levels measured in this study but levels of TNF-α were comparable between the two studies. These differences could possibly be explained by the different wild-type strain this previous study used compared with the halotype F4 strain used in the current study. The wild-type used in their study was the G681 strain, which is a derivative of the neotype strain FRR2161, that was generated from G526 (*ΔpkuA pyrG−*) strain in which

the *kuA* gene is deleted to prevent non-homologous DNA end joining repair. Results showed that the *T. marneffei* quadruple laccase mutant conidia stimulated significantly more pro-inflammatory cytokine production by THP-1 than wild-type conidia and it was concluded that *T. marneffei* laccases modulate the pro-inflammatory cytokine response. Whereas their study results were uniform in order to definitively conclude that laccases modulate host cytokine responses, the current study was unable to conclude whether or not YakA modulates host cytokine responses.

Time since infection was also considered a factor in the current study examining the *ΔyakA* strain, mostly to examine a possible interaction between time since initial infection and strain of fungi. Even though a significant interaction term was discovered when measuring IL-1β in THP-1 macrophages, no other cytokine profiles revealed interactions. Consequently, there is likely no interaction between time since initial infection and strain of fungi.

Cytokine profiles of J774 cells yielded no statistical significance. In addition, the detection of IL-6 secretion by J774 cells was negligible. It was indicated previously that J774 cells do secrete IL-6 (Sze et al., 2017). The study by Sze et al. (2017) measured TNF-α and IL-6 production by ELISA and demonstrated a statistically significant down-regulation of these cytokines when exposed to wild-type compared to the *MP1* knockout strain. *MP1* codes for Mp1p which researchers proposed would downregulate IL-6 and TNF-α by sequestering a critical pro-inflammatory signaling lipid. Because downregulation of these cytokines was observed in J774 cells exposed to the wild-type compared to the *MP1* knockout, it was concluded that Mp1p represents a novel virulence factor for *T. marneffei* in a mouse model.

In this study, the experiments were performed in duplicate with no replicates. Therefore, standard deviations were very high and fluctuations between trials may have influenced the analysis. The graphical representations of this data look promising, so it is possible that a study design with more trials could result in statistically significant data analysis. If statistically significant analyses are still not achieved, it may be advised to choose a different murine cell line for future studies or to collect primary alveolar macrophages from study mice. It's also possible that the fungal strains used in the study were human-specific, since both were originally isolated from human tissue. For future studies, it may be sensible to use a fungal strain isolated from rodents when using it to infect rodent cells.

For future experiments, further cytokine profiling should be done with ELISAs performed in at least triplicate in order to minimize the standard deviations. Also, a phagocytosis assay should be done to assess whether the cell wall defects in *ΔyakA* affect the interaction of *T. marneffei* with host cells and to provide direct evidence that the conidia are successfully entering the macrophages. Differences noted in cytokine production from this study only provide indirect evidence of infection. Lastly, a conidial survival assay should be done to compare intracellular killing of *T. marneffei* WT and mutant strains by both cell lines and to provide direct evidence that the conidia can survive inside the macrophages while avoiding detection.

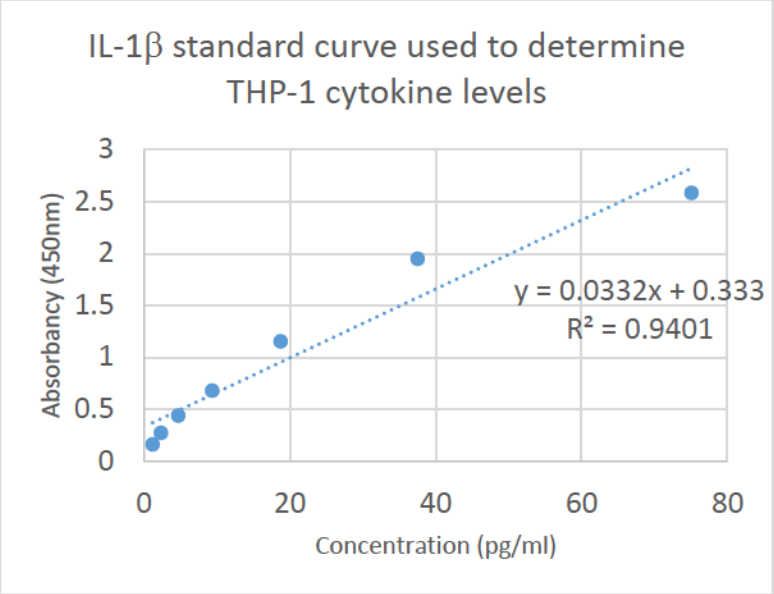
Phagocytosis assays and conidial survival assays were performed by Sapmak et al. (2016) using THP-1 macrophages that had been exposed to conidia from wild-type and a quadruple *lac* deletion strain. For the phagocytosis assay, they found that phagocytosis was significantly higher when THP-1 had been infected with the deletion strain when

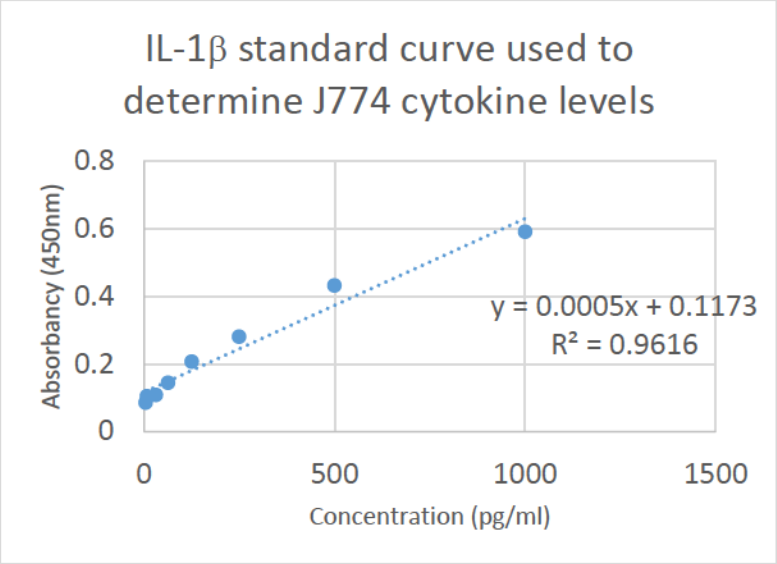
compared to the wild-type. The conidial survival assay showed that THP-1 macrophages more effectively killed conidia of the deletion strain than the wild-type after 2 and 4 hours of incubation. Together, these results demonstrated that laccase production significantly impacts phagocytosis and conidial survival inside macrophages. In future studies by this laboratory, the conidial survival assay should be modified by incubating released conidia at 37°C rather than 25°C, because forcing the yeast to revert back to hyphal growth may induce greater stress on the fungi. This is especially true for the *ΔyakA* strain, since homologs for this gene function in stress response (Aranda et al., 2011; Hartley et al., 1994; Lee et al., 2012).

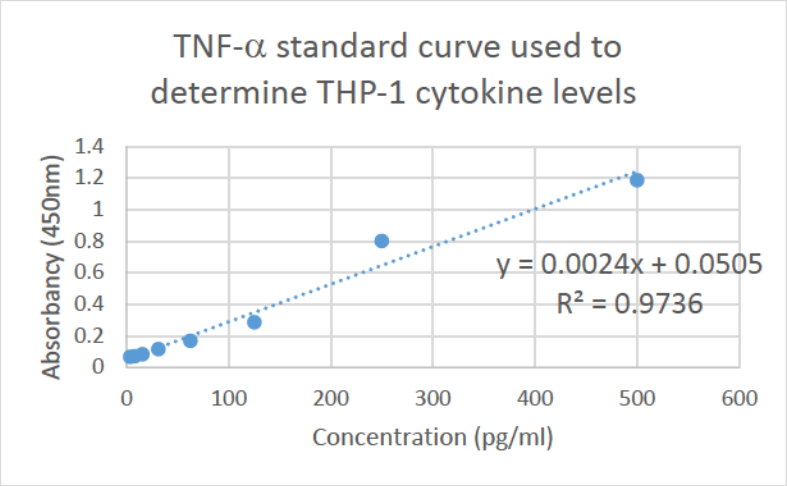
The data from this study reveals what may be a suggestive pattern to drive further research. The most promising data to suggest a role of *ΔyakA* in virulence is noted in the cytokines measured in THP-1 cells, more specifically IL-6 and TNF-α. Differences in cytokine release were noted when the cells were exposed to *ΔyakA* versus wild-type conidia. Although these differences were noted between cytokine levels of THP-1 cells incubated with WT versus *ΔyakA* conidia, the differences were not uniform across all cytokines and cells tested. Even though at present it cannot be decided whether the *yakA* gene affects host cytokine responses, investigation of newly discovered mutants such as *ΔyakA* are important to pursue in order to decide whether these genes' products should be tested as suitable drug targets or should be dismissed.

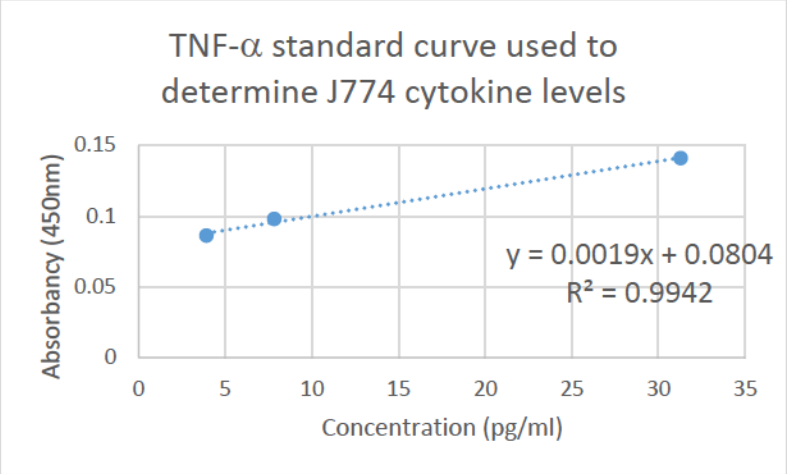
APPENDICES

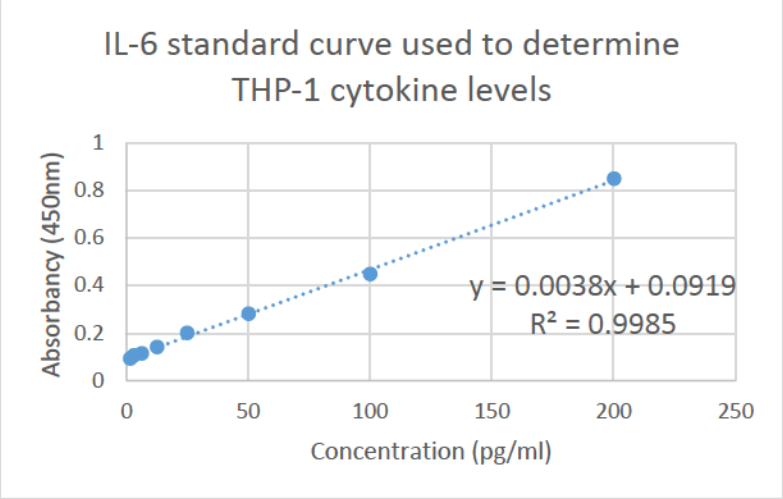
Appendix A: Standard curves produced for calculating concentration of cytokine using lyophilized antigen provided with ELISA kit. Equations and R^2 values are displayed on chart.

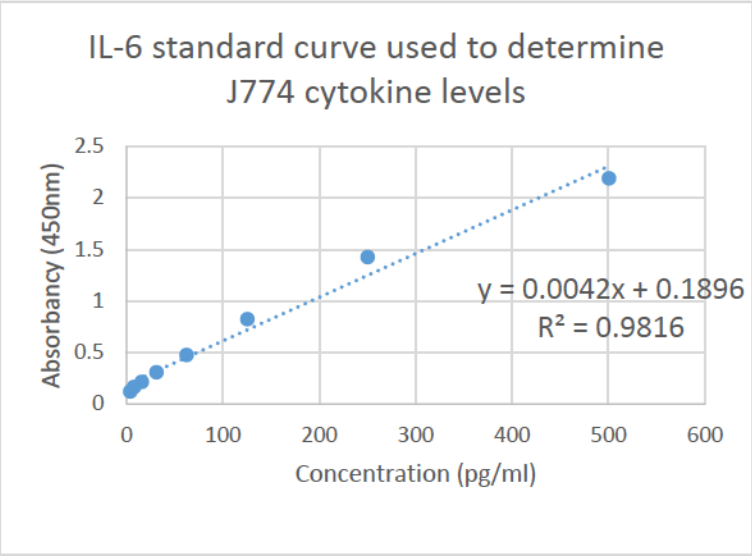





TNF-α standard curve used to determine THP-1 cytokine levels
Absorbancy (450nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
Concentration (pg/ml)
y = 0.0024x + 0.0505
R² = 0.9736


TNF-α standard curve used to determine J774 cytokine levels
Absorbancy (450nm)
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
Concentration (pg/ml)
y = 0.0019x + 0.0804
R² = 0.9942


IL-6 standard curve used to determine THP-1 cytokine levels
Absorbancy (450nm)
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
Concentration (pg/ml)
y = 0.0038x + 0.0919
R² = 0.9985


IL-6 standard curve used to determine J774 cytokine levels
Absorbancy (450nm)
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
600
Concentration (pg/ml)
y = 0.0042x + 0.1896
R² = 0.9816

Appendix B: Plate map used for ELISAs

Term	Meaning
HS	Human cytokine standard
MS	Mouse cytokine standard
NC	No conidia
NP	No PMA
WT	Wild-type
YA	yak-A

	1	2	3	4	5	6	7	8	9	10	11	12
A	HS	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	blank		
B	MS	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	blank		
C	NC	NP WT	2hr WT	16hr WT	24hr WT		NC	2hr WT	16hr WT	24hr WT		
D	NC	NP WT	2hr WT	16hr WT	24hr WT		NC	2hr WT	16hr WT	24hr WT		
E		NP YA	2hr YA	16hr YA	24hr YA			2hr YA	16hr YA	24hr YA		
F		NP YA	2hr YA	16hr YA	24hr YA			2hr YA	16hr YA	24hr YA		
G												
H												

Appendix C: Manufacturer's instructions for ELISAs (Invitrogen; Carlsbad, California).

1. Dilute coating buffer by making a 1:10 dilution of PBS (10X) in deionized water
2. Coat Nunc™ MaxiSorp™ 96-well plates (Cat. No. 44-2404) with 100 µL/well of capture antibody in Coating Buffer (1X). Seal plate and incubate overnight at 4°C.
3. Aspirate wells and wash 3 times with >250 µL/well Wash Buffer. Allowing time for soaking (~1 minute) during each wash step. Blot plate on absorbent paper to remove any residual buffer.
4. Dilute Diluent Concentrate (5X) 1:5 in deionized water.
5. Block wells with 200µL of ELISA/ELISASPOT Diluent (1X). Incubate at room temperature for 1 hour.
6. Prepare Standard by addition of distilled water according to the reconstitution volume on the label of the standard vial. Allow standard to reconstitute for 10-30 minutes. Swirl or mix gently to ensure complete and homogeneous solubilization. Mix well prior to making dilutions. The standard has to be used immediately after reconstitution and cannot be stored.
7. Perform 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points. For that add 100µL of ELISA/ELISASPOT Diluent (1X) to the wells leaving the first wells empty. Add 200µL/well of top standard concentration to the first empty wells A1/A2. Transfer 100µL of top standard from wells A1/A2 to wells B1/B2. Mix the contents of the wells B1 and B2 by repeated aspiration and ejection and transfer 100µL to wells C1/C2. Take care not to scratch surface of the microwells. Continue this procedure 5 times.
8. Add 100µL/well of samples to the appropriate wells.
9. Add 100µL of ELISA/ELISASPOT Diluent (1X) to the blank well.
10. Seal the plate and incubate at room temperature overnight at 4°C.
11. Prepare the Detection Antibody by diluting it 1:250 in ELISA/ELISASPOT Diluent (1X).
12. Aspirate and wash as in Step 2. Repeat for a total of 3-5 washes. Allow time for soaking ~1 minute during each wash step. Blot plate on absorbent paper to remove any residual buffer.
13. Add 100µL/well diluted Detection Antibody to all wells.
14. Seal the plate and incubate at room temperature for 1 hour.
15. Prepare the Avidin-HRP by diluting it 1:250 in ELISA/ELISASPOT Diluent (1X).
16. Aspirate and wash as in Step 2. Repeat for a total of 3-5 washes. Allow time for soaking (~1 minute) during each wash step. Blot plate on absorbent paper to remove any residual buffer.
17. Add 100µL/well of diluted Avidin-HRP.
18. Seal the plate and incubate at room temperature for 30 minutes.
19. Aspirate and wash as in Step 2, making sure to allow time for soaking for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes.
20. Add 100µL/well of 1X TMB Solution.
21. Incubate at room temperature for 15 minutes.

22. Add 50 µL/well of Stop Solution.
23. Calculate the concentrations of cytokines in the experimental samples according to the optical densities at 450 nm obtained from the wells containing cytokine standard.

Appendix D: Statistical analyses for ELISA data including significant Tukey's post-Hoc.

Tests of Between-Subjects Effects Human IL-6

Dependent Variable: Conc

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	28540.367[a]	3	9513.456	3.163	.148
Intercept	450725.278	1	450725.278	149.873	.000
Gene	28265.503	1	28265.503	9.399	.037
Time	135.244	1	135.244	.045	.842
Gene * Time	139.620	1	139.620	.046	.840
Error	12029.529	4	3007.382		
Total	491295.174	8			
Corrected Total	40569.896	7			

a. R Squared = .703 (Adjusted R Squared = .481)

Multiple Comparisons Human IL-6

Dependent Variable: concentration

Tukey HSD

(I) exp_group	(J) exp_group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
No conidia	WT	-187.4870*	37.52227	.004	-297.9923	-76.9817
	YA	-68.6058	37.52227	.229	-179.1110	41.8995
WT	No conidia	187.4870*	37.52227	.004	76.9817	297.9923
	YA	118.8813*	30.63681	.015	28.6541	209.1084
YA	No conidia	68.6058	37.52227	.229	-41.8995	179.1110
	WT	-118.8813*	30.63681	.015	-209.1084	-28.6541

Tests of Between-Subjects Effects Human TNF-alpha

Dependent Variable: Conc

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	563.214[a]	3	187.738	40.051	.002
Intercept	26258.757	1	26258.757	5601.868	.000
Gene	378.153	1	378.153	80.673	.001
Time	168.049	1	168.049	35.851	.004
Gene * Time	17.012	1	17.012	3.629	.129
Error	18.750	4	4.688		
Total	26840.721	8			
Corrected Total	581.964	7			

a. R Squared = .968 (Adjusted R Squared = .944)

Multiple Comparisons Human TNF-alpha

Dependent Variable: concentration

Tukey HSD

(I) exp_group	(J) exp_group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
No conidia	16hr WT	-47.9170*	2.67461	.000	-58.6462	-37.1878
	16hr YA	-37.0830*	2.67461	.000	-47.8122	-26.3538
	24hr WT	-41.6670*	2.67461	.000	-52.3962	-30.9378
	24hr YA	-25.0000*	2.67461	.001	-35.7292	-14.2708
16hr WT	No conidia	47.9170*	2.67461	.000	37.1878	58.6462
	16hr YA	10.8340*	2.67461	.048	.1048	21.5632
	24hr WT	6.2500	2.67461	.270	-4.4792	16.9792
	24hr YA	22.9170*	2.67461	.002	12.1878	33.6462
16hr YA	No conidia	37.0830*	2.67461	.000	26.3538	47.8122
	16hr WT	-10.8340*	2.67461	.048	-21.5632	-.1048
	24hr WT	-4.5840	2.67461	.500	-15.3132	6.1452
	24hr YA	12.0830*	2.67461	.032	1.3538	22.8122
24hr WT	No conidia	41.6670*	2.67461	.000	30.9378	52.3962
	16hr WT	-6.2500	2.67461	.270	-16.9792	4.4792
	16hr YA	4.5840	2.67461	.500	-6.1452	15.3132
	24hr YA	16.6670*	2.67461	.008	5.9378	27.3962
24hr YA	No conidia	25.0000*	2.67461	.001	14.2708	35.7292
	16hr WT	-22.9170*	2.67461	.002	-33.6462	-12.1878
	16hr YA	-12.0830*	2.67461	.032	-22.8122	-1.3538
	24hr WT	-16.6670*	2.67461	.008	-27.3962	-5.9378

Tests of Between-Subjects Effects Human IL-1beta

Dependent Variable: Conc

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	120.440[a]	3	40.147	7.702	.039
Intercept	168144.615	1	168144.615	32258.175	.000
Gene	21.383	1	21.383	4.102	.113
Time	54.023	1	54.023	10.364	.032
Gene * Time	45.035	1	45.035	8.640	.042
Error	20.850	4	5.212		
Total	168285.905	8			
Corrected Total	141.290	7			

a. R Squared = .852 (Adjusted R Squared = .742)

Multiple Comparisons Human IL-1beta

Dependent Variable: concentration

Tukey HSD

(I) exp_group	(J) exp_group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
No conidia	16 hr	-84.4897*	3.05842	.000	-93.4970	-75.4825
	24 hr	-79.2925*	3.05842	.000	-88.2997	-70.2853
16 hr	No conidia	84.4897*	3.05842	.000	75.4825	93.4970
	24 hr	5.1972	2.49719	.163	-2.1571	12.5516
24 hr	No conidia	79.2925*	3.05842	.000	70.2853	88.2997
	16 hr	-5.1972	2.49719	.163	-12.5516	2.1571

Tests of Between-Subjects Effects Mouse IL-6

Dependent Variable: Conc

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	45.805[a]	3	15.268	1.035	.467
Intercept	143.067	1	143.067	9.694	.036
Gene	35.909	1	35.909	2.433	.194
Time	8.978	1	8.978	.608	.479
Gene * Time	.919	1	.919	.062	.815
Error	59.036	4	14.759		
Total	247.908	8			
Corrected Total	104.841	7			

a. R Squared = .437 (Adjusted R Squared = .015)

Tests of Between-Subjects Effects Mouse TNF-alpha

Dependent Variable: Conc

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10717.576[a]	3	3572.525	2.324	.216
Intercept	366601.297	1	366601.297	238.473	.000
Gene	8.366	1	8.366	.005	.945
Time	10644.645	1	10644.645	6.924	.058
Gene * Time	64.565	1	64.565	.042	.848
Error	6149.147	4	1537.287		
Total	383468.020	8			
Corrected Total	16866.723	7			

a. R Squared = .635 (Adjusted R Squared = .362)

Tests of Between-Subjects Effects Mouse IL-1beta

Dependent Variable: Conc

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1075.503[a]	3	358.501	.435	.740
Intercept	4948.483	1	4948.483	6.002	.070
Gene	375.558	1	375.558	.455	.537
Time	461.244	1	461.244	.559	.496
Gene * Time	238.700	1	238.700	.290	.619
Error	3298.042	4	824.510		
Total	9322.028	8			
Corrected Total	4373.545	7			

a. R Squared = .246 (Adjusted R Squared = -.320)

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