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Comparative studies of innate host defense mechanisms against virulent and avirulent species of microsporidia

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COMPARATIVE STUDIES OF INNATE HOST DEFENSE MECHANISMS AGAINST VIRULENT AND AVIRULENT SPECIES OF MICROSPORIDIA

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Biological Sciences

by

Amber Lynn Mathews
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LIST OF ABBREVIATIONS

AIDS – Acquired Immunodeficiency Syndrome
CCL – C-C motif Ligand (chemokine)
CD – Cluster Differentiation
CTL – Cytotoxic T Lymphocyte
DC – Dendritic Cell
ERK – Extracellular signal-Regulated Kinase
GM-CSF – Granulocyte-Macrophage-Colony Stimulating Factor
HEK – Human Embryonic Kidney cells
HIV – Human Immunodeficiency Virus
IEL – Intraepithelial Lymphocyte
IFN-γ – Interferon Gamma
IL – Interleukin
iNOS – inducible Nitric Oxide Synthase
LPS – Lipopolysaccharide
MAPK – Mitogen-Activated Protein Kinase
MDM – Monocyte-Derived Macrophage
MKP – Mitogen-Associated Protein Kinase Phosphatase
NF-κB – Nuclear Factor kappa B
NO – Nitric Oxide
PAMP – Pathogen- Associated Molecular Pattern
PPR – Pattern Recognition Receptor
SAPK/JNK – Stress-Activated Protein Kinase/c-Jun N-terminal Kinase
SCID – Severe Combined Immunodeficiency
siRNA – small interfering RNA
Th – T-helper cell

TLR – Toll-like Receptor

TNF-α – Tumor Necrosis Factor alpha
ABSTRACT

Microsporidia are ubiquitous, obligate intracellular eukaryotes that cause chronic diarrhea and disseminated diseases in humans, especially in immunocompromised individuals. Macrophages, cellular components of the innate immune system, are believed to be the source of dissemination of this pathogen throughout the body. Little is known about the innate immune response to microsporidia. Macrophages are a source of interleukin (IL)-12 and IL-23 and play an essential role in the link between innate and adaptive immunity. The focus of this thesis is the investigation of the p38 mitogen-activated protein kinase (MAPK) signaling mechanisms and IL-12 and IL-23 production regulated by Toll-like receptor (TLR) 2 and TLR4 engagement with pathogenic and nonpathogenic species of microsporidia. IL-12 and IL-23 production by primary human macrophage were induced in response to challenge with avirulent but not virulent species from 12 to 24 hour time points. Using western blot, we found that activated p38α MAPK is continuous from three to 24 hours post infection of human macrophages with avirulent species. Activation of p38α MAPK is transient when infected with pathogenic species as we only detected phosphorylation at three hours and six hours post infection. These data suggest that activation of p38 MAPK may be necessary for the proper innate immune response to microsporidia to control infection. Using small interfering RNA, p38α, γ, and δ MAPK were knocked down in primary human macrophages and resulted in a decrease in IL-12/IL-23 p40 production when infected with nonpathogenic species. Thus, additional isoforms of p38 MAPK may regulate the production of IL-12 and/or IL-23 which is a novel finding to the field of microsporidian research and immunology as a whole. MAPK phosphatases (MKP) 1
and/or MKP5 may be negative regulators of this IL-12 and/or IL-23 response. Increased expression of MKP5, but not MKP1, was observed in MDMs challenged with pathogenic species for six hours. The deactivation of p38 MAPK by MKPs may result in the diminished levels of IL-12 and IL-23 observed in virulent infections and thus leading to host susceptibility to microsporidian infection.
Abstract

Microsporidia are obligate intracellular, eukaryotic fungi, which have gained recognition as opportunistic parasites in immunocompromised patients. Resistance to lethal microsporidia infections requires a Th1 immune response; how this protection is initiated against *Encephalitozoon* species is the focus of this review article.

Keywords: Innate immunity; *Encephalitozoon*; Microsporidia; Macrophage; Dendritic cells

Introduction

Microsporidia are eukaryotic, intracellular parasites that comprise over 1200 species and infect a wide range of hosts, such as invertebrates, fish, and many mammals, including both human and non-human primates. Once considered as protozoa, recent genomic characterization has supported their reclassification as fungi. Microsporidians were first proven to cause prebrine or pepper disease in silkworms in the mid-nineteenth century when Louis Pasteur identified a protozoan, later to be named *Nosema bombycis*, that was devastating the silk industry in Europe. In 1959, the first case of microsporidiosis in humans was diagnosed (Monaghan et al., 2009). Since then, 14 species have been reported to infect humans resulting in more severe symptoms in immunocompromised individuals as compared with their immunocompetent counterparts (Didier et al., 2004). Diarrhea is considered the most

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common symptom, but disseminated disease may occur, triggering other conditions which include, but are not limited to, encephalitis, keratoconjunctivitis, and hepatitis (Hale-Donze, 2007). Infection has been shown to occur through a variety of sources, but the ingestion of contaminated food and water continue to be the most prominent transmission route (reviewed in Didier et al., 2004).

Of the 14 microsporidia species infecting humans, Enterocytozoon bieneusi causes infection most frequently but rarely is the cause of disseminated infection, unlike the Encephalitozoons (Encephalitozoon cuniculi, Encephalitozoon hellem and Encephalitozoon intestinalis) which induce multi-organ pathogenesis. Encephalitozoons can infect most tissues and E. intestinalis is the second most common pathogen leading to microsporidiosis. Other species reported to cause infections in humans include Brachiola algerae, Brachiola connori, Brachiola vesicularum, Microsporidium africanum, Microsporidium ceylonensis, Nosema ocularum, Pleistophora ronneafiei, Trachipleistophora anthropopthera, Trachipleistophora hominis, and Vittaforma corneae (reviewed in Didier, 2005).

**The Encephalitozoon spore: the infectious unit**

The infective stage of the microsporidian life cycle is the spore, which is also the only life cycle stage capable of survival outside of host cells (Vavra and Larsson, 1999). Microsporidia are unique, unicellular organisms and these features are highlighted in the contents of the spores (figure 1). Spores enclose the sporoplasm which contains several universal organelles, including nuclei, ribosomes, and membranes forming the endoplasmic reticulum. The number of nuclei varies among species and life cycle stages, with some being diplokaryon (Vavra and Larsson, 1999); however,
Encephalitozoons are monokaryons. Organelles contributing to the distinctiveness of microsporidia include mitosomes (thought to be reduced mitochondria), a polar filament (tubule) originating at an anterior anchoring disk, and an atypical Golgi apparatus. All microsporidia are distinguished by the presence of a polar filament that is thought to play a key role in infection. Along with the unique polar filament, microsporidia spores contain a membranous polaroplast as well as a posterior vacuole (Hale-Donze, 2007) thought to be involved in polar filament extrusion (Findley et al., 2005).

Figure 1. Generalized Encephalitozoon spore.
The life cycle of the *Encephalitozoon*

The initial stage of infection includes contact between the spore and host cell. Following ingestion of *Encephalitozoon* species through contaminated food or water, interactions between spores and intestinal epithelia occur. Other means of transmission, such as inhalation, involve other cell types such as the respiratory tract epithelia (Didier, 2005). Upon spore–cell contact and certain environmental conditions (i.e. ion levels, pH, or osmotic conditions), the eversion of the polar filament is triggered. Whether the firing mechanism is dependent upon specific receptors or surface molecules is not yet known. For the *Encephalitozoon* species (figure 2), the sporoplasm travels through the everted polar filament into the host cytoplasm where it undergoes merogony within a parasitophorous vacuole in the host cell (Cali and Takvorian, 1999). During this replication stage, nuclear division takes place, which may be immediately followed by cytokinesis. The membrane enclosing the meront becomes more electron dense, as does the interior of the parasite, due to the accumulation of endoplasmic reticulum membranes and ribosomes. This transition marks the onset of sporogony. The newly formed sporonts divide further to give rise to sporoblasts. For *Encephalitozoon* species, each sporont gives rise to two sporoblasts. Sporoblasts undergo maturation to form the spore stage of the life cycle. During this maturation period, the polar filament, polaroplast, and other intracellular organelles are formed. When the mature spore is formed, it contains both an exospore coat (the electron dense layer added during the merogony/sporogony transition) and an endospore coat (added along with the posterior vacuole during the final stages of maturation). Mature spores are released from the host cell via lysis (Cali and Takvorian, 1999; Hale-Donze, 2007).
While the generalized life cycle is known, detailed mechanisms such as triggering of the polar filament eversion and induction of host lysis are not well understood.

Figure 2. The life cycle of *Encephalitozoon* in epithelial cells. 1. The spore everts its polar filament when in contact with the host cell and penetrates through the plasma membrane. 2. The spore injects sporoplasm into host. 3. The sporoplasm undergoes merogony by binary fission within a parasitophorous vacuole. 4. Meronts mature into sporonts that divide into sporoblasts. 5. Sporoblasts undergo sporogony to develop into mature spores that are released from the host cell.

**Diseases**

Infection with the three *Encephalitozoon* species has been shown to originate in either the intestinal or respiratory epithelia. Dissemination to other parts of the body may occur, due to the ability of these species to infect macrophages. Specifically, dissemination of *E. intestinalis* can cause nephritis, as well as secondary infections of
the sinuses, urinary bladder, and skin. *E. cuniculi* has been shown to cause keratoconjunctivitis and is capable of infecting the heart, brain, kidneys, and even the tongue. Similarly, *E. hellem* has been shown to cause keratoconjunctivitis, and has been found infecting the sinuses, urinary bladder, and prostate (Orenstein, 2003). Although infection may occur in most tissues of the body leading to symptoms based on infection site, by far the most common presentation of disease is diarrhea. In immunocompromised individuals, chronic diarrhea may persist and lead to malabsorption and wasting (Hale-Donze, 2007). While inclusion of individuals (HIV seronegative or seropositive) who reported to health centers with chronic diarrhea was the focus of previous population studies (Mathis et al., 2005), a more inclusive sampling of the general population was conducted in Cameroon. This project included individuals with and without clinical symptoms or HIV infections. The survey revealed that the majority of the healthy population had subclinical microsporidial infections and were shedding infectious spores. This study reported a prevalence rate of 87% in teenagers, and 68% in all healthy asymptomatic individuals which was higher than the 53% rate reported for the HIV-infected cohort (Nkinin et al., 2007). Furthermore, new associations of microsporidia parasites with common infections are being reported as researchers and clinicians become more aware and capable of diagnosing these pathogens. One such investigation from India linked multiple cases of self-limiting, seasonal epidemic keratoconjunctivitis in immunocompetent individuals to microsporidia (Das et al., 2008). Together, this new information would suggest that the incidence of microsporidia infections is much higher than the reported projections of 1.2–13 million affected individuals in the Sub-Saharan with AIDS and may represent an underlying
etiological agent for more common diseases for which the pathogen is not commonly investigated. While it is not known how extensive such silent infections are in humans, asymptomatic carriers in other mammals are believed to be the reservoir for these parasites (Didier et al., 2000).

**Immune responses**

During the last few years, several reviews on adaptive immune responses to microsporidial infection have been published (Khan et al., 2001); (Khan and Didier, 2004); (Franzen et al., 2005b). Using mice as the experimental model suggested a genetic basis for innate resistance (Khan et al., 2001). In experiments where the animal was immunosuppressed with cortocosteroids or the strain is genetically immunocompromised (SCID), infection of these animals with *E. cuniculi* led to overt disease with the production of ascites, infiltration of macrophages containing parasites, and eventually death (Snowden et al., 1999). Initial studies showed that these mice could be rescued with the adoptive transfer of activated T cells. Furthermore, it was concluded that resistance to infection was mediated by one or more cytokines, but not antibody responses. Similarly, human studies have demonstrated that recovery of T cell levels via protease inhibitor (antiretroviral) therapy lead to resolution of microsporidiosis in patients infected with HIV (Pozio and Morales, 2005) and (Goguel et al., 1997). Data obtained from murine models have shown that cytotoxic T lymphocytes (CTL) are critical for protection and that their activation does not appear to be dependent upon CD4+ T cells (Khan et al., 1999). However, a caveat to this interpretation is that the CTLs must become activated prior to killing; therefore, resistance or susceptibility to *Encephalitozoon* infection is interdependent upon
interactions of CTL with the innate arm of immunity. Treatment of CD8 deficient mice (which normally succumb to infection) with a p38 mitogen-activated protein kinase (MAPK) inhibitor was sufficient to increase the survival rate. While the authors proposed this was due to inhibition of the microsporidal p38 signaling pathways (Wei et al., 2007), an alternative interpretation is that the innate immune responses may be adequate enough to induce resistance to disease. In this study, the MAPK inhibitor was given to the CTL-deficient animal more likely affecting the host response, thus resulting in increased survival against *Encephalitozoon* infection rather than the parasite themselves.

**The role of Th1 and Th2 cytokines in *Encephalitozoon* infections.** The prominence of a Th1 response has been implicated in resistance to microsporidial infection. The first indication of the importance of Th1-type responses in clearance of these parasites came from reports that interferon (IFN)-γ null mice could not clear microsporidian infection. The second showed that interleukin(IL)-12 deficient mice succumb to *E. cuniculi* or *E. intestinalis*, suggesting that in addition to IFN-γ, IL-12 is required for clearance of these fungi (reviewed in Khan and Didier, 2004)). While several reports show that in a permissive environment, IL-10 is elevated, secreted by macrophages, and correlates with infection, no further mechanism or signaling pathway has been elucidated (Franzen et al., 2005b); (Braunfuchsova et al., 1999); (El Fakhry et al., 2001; Moretto et al., 2004); therefore it is unclear whether or not IL-10, which is known to reduce Th1 responses, is critical for induction of microsporidia-induced pathogenesis.
Innate immune responses against microsporidia. The picture of which components of the adaptive immune response are required for protection against microsporidia has become more focused in recent years. It is clear that cell-mediated immunity and those cytokines leading to activation of these responses are critical for protection. Because IL-12 and IFN-γ can also be secreted from cells of the innate immune system in response to invasion, it is important to understand how these first responders recognize and direct Th1 responses leading to CTL activation and parasitic resistance.

The role of innate immunity in regulating infectious disease is becoming more prominent as researchers discover its contributions to clearance of infections. Innate immunity is often the only form of immunity needed to deal with initial invasions and is the only system left after acquired immunodeficiencies like AIDS. Understanding its contribution in microsporidia infections is essential to elucidating how these pathogenic fungi evade the immune defenses. This arm of the immune system serves as the first line of defense against foreign pathogens by responding immediately and ensuring survival until an adaptive immune response is generated. The function of the innate arm is to recognize the diversity of pathogens, activate and recruit effector cells to rapidly dispose of the intruder(s), and communicate with the adaptive arm to generate memory against encountered pathogens (Janeway, 2001; Vasselon and Detmers, 2002). This expeditious response is generated primarily by monocytes, neutrophils, dendritic and endothelial cells (Vasselon and Detmers, 2002).

The role of the macrophage. Macrophages are a critical link between innate and adaptive immunity. They are part of the initial response against pathogens
because they are resident in portals of entry from the outside environment (Tailor et al., 2006). These local macrophages can quickly recognize foreign invaders through several classes of receptors, including pattern recognition receptors (PRR), on their surface. This recognition results in a milieu of host defense mediators including chemokines, cytokines, nitric oxide (NO), inducible nitric oxide synthase (iNOS) and radical oxygen species. They respond to IFN-γ secreted by activated T cells to kill phagocytized intracellular pathogens (Sebastian et al., 2005) by initiating a respiratory burst. When pathogens like microsporidia evade these protective responses, macrophages often become “Trojan horses”, carrying these pathogens throughout the body and infecting new cells.

**The role of macrophages in disseminated microsporidiosis.** In individuals with multifocal organ involvement, infiltrates of microsporidian-infected macrophages are evident in lesions, microabscesses, and granulomas (Orenstein, 2003). Dissemination is believed to occur in two steps. The first step is an initial infection which often occurs in resident macrophages of the intestine for *E. cuniculi* or *E. intestinalis*. These macrophages recognize the pathogen and respond by secreting chemoattractants that recruit new cells, including monocytes, to resolve the infection. If the macrophages fail to kill the intracellular intruder, then in the second phase, the infected macrophages migrate from the initial infection sites into the lymphatic system and ultimately into the blood and tissues.

**Host uptake of microsporidia.** While microsporidia classically gain entry into the host through eversion of the polar filament penetrating the cell membrane, phagocytosis is another mechanism used for uptake of spores (Franzen, 2004). It has
been reported that the events subsequent to phagocytosis may affect the ability of the parasites to survive within the macrophage. Early studies suggested that microsporidia were able to inhibit phagosome and lysosome fusion (Franzen, 2004) and more recent findings show that spores are found in the phagolysosomal compartment in both professional and non-professional phagocytes (Couzinet et al., 2000; Franzen et al., 2005c). However in one of the first studies to try and dissect what happens after phagocytosis, it was suggested that spores that remain in the phagolysosomes are killed and that viable parasites are derived from those which escape from the early endosome by polar filament eversion into the cytoplasm (Franzen et al., 2005c). We have observed everted parasites along with intact spores within early vesicles. This observation supports the claims that the parasites that remain within the initial phagolysosome, are capable of evading the immune responses of macrophages, and can complete their life cycles. These findings are more likely due to the differences in the sensitivity of the detection methods employed to visualize these intracellular pathogens (Fischer et al., 2008b).

**Intracellular macrophage responses to Encephalitozoon**s. Early work investigating the role of the macrophages in host responses to *Encephalitozoon* infection focused on the respiratory burst and led to different conclusions based upon the model used. In ex-vivo studies of murine peritoneal macrophages, levels of both iNOS and NO were elevated and inhibition of parasite replication was observed; whereas, infection of naïve human monocyte-derived macrophages (MDM) produced neither an augmentation of NO nor reduction of the parasitic burden despite elevated levels of tumor necrosis factor (TNF)-α or IFN-γ (Franzen et al., 2005a). These
conflicting findings most likely reflect the pre-activation state of cells, as prior treatment of either human MDM or mouse macrophages with IFN-γ and lipopolysaccharide (LPS), known inducers of the respiratory burst, were shown to lower parasitic burden in infected macrophages (Didier and Shadduck, 1994; Fischer et al., 2008b). If prior activation of the cells results in protection, then this would also indicate that microsporidia may also have a survival mechanism for evading the induction of a respiratory burst in resting macrophages. One such example was reported in fish. Cultures of ayu head kidney macrophages were shown to engulf *Glugea plecoglossi* spores leading to the inhibition of a respiratory burst and allowing replication of these pathogens (reviewed in (Monaghan et al., 2009)). Both phagocytosis and induction of the respiratory burst have been shown to be receptor-mediated events; therefore, it is likely that engagement of specific host receptors and subsequent signaling pathways may vary between cells where parasitic replication is unabated or is controlled.

**Macrophage recognition of *Encephalitozoon*.** Specific host protein interactions with microsporidia are scarcely reported. Two groups of glycosylated proteins have been linked with spore attachment to host cells. The first are glycosaminoglycans, which were shown to be involved in the recognition of *E. intestinalis* and dependent upon adherence to heparin-binding motifs of the spore wall protein EnP1 (Southern et al., 2007). The second are proteins that can recognize O-glycans on the spores and are associated with host attachment (Taupin et al., 2007). The types of host receptors that most likely would recognize these glycosylated proteins are included in PRR.
The concept of PRR and Pathogen-Associated Molecular Proteins (PAMP) founded in *Drosophila* research revolutionized studies in entomology and mammalian immunology. PRR rapidly recognize a broad spectrum of repetitive moieties (PAMPs) found on a variety of pathogens (Medzhitov et al., 1997). PRR can bind to proteins, glycoproteins, lipids, and complex carbohydrates such as peptidoglycan, bacterial lipoproteins, zymosans, and mannose (Netea et al., 2004). One group of PRR is the Toll-like receptors (TLR) which in mammalian systems initiate innate immune responses to foreign invaders (Akira, 2003). *Encephalitozoon* spp. utilize TLR2 for induction of immune responses and immune evasion in naïve human macrophages (Fischer et al., 2008a). Analysis of HEK293-transfected cell lines expressing either TLR2, or TLR4/MD2/CD14 signaling complex, revealed that TLR2 was activated by both *E. cuniculi* and *E. intestinalis* resulting in activation of nuclear factor kappa B (NF-κB) and TNF-α secretion. TLR2 appears to be important for activation of these innate immune responses as siRNA knock-down for TLR2, but not TLR4, inhibited nuclear translocation of NF-κB and reduced TNF-α levels in naïve human macrophages. Furthermore, TLR2 knock-down also affected the production of the chemokines IL-8, CCL3 and CCL4, which are critical chemokines involved in recruitment of phagocytes important for dissemination of pathogens (Fischer et al., 2008a). From this research, *ex vivo* naïve macrophages represent a permissive environment for microsporidial replication. Because LPS is a ligand for TLR4 and is known to reduce parasitic burden, TLR4 signaling mechanisms may be important in understanding pathways leading to resistance as TLR4 has been linked to induction of Th1 responses in fungal infections (van de Veerendonk et al., 2008).
Macrophage production of chemokines and cytokines influencing immune outcome in microsporidiosis. While it is difficult to directly link macrophages with disseminated microsporidiosis, clinical observations and in vitro models taken together support such hypotheses. Using a co-culture system, microsporidia-infected human macrophages were able to recruit naïve monocytes into the lower chamber. This recruitment was dependent upon the chemokines CCL2, CCL3, CCL4, and CCL5 (Fischer et al., 2007). While only the recruitment of monocytes was tested, the production of these chemokines along with IL-8 suggest that microsporidial infection of macrophages can influence the recruitment of monocytes, dendritic cells, neutrophils and IL-2-dependent T cells into areas of infection, thereby influencing adaptive immunity (Fischer et al., 2007).

The role of the dendritic cell. Dendritic cells (DC), the most effective antigen presenting cells, play a critical role in both innate and adaptive immune responses. Antigen presentation by dendritic cells to T cells stimulates the adaptive arm of immunity by activating naïve lymphocytes into effector T cells that function to control infections. In addition to their role as antigen presenting cells, dendritic cells secrete cytokines such as IL-12 and IFN-γ in response to microbial invasion, (Mellman and Steinman, 2001) promoting Th1 responses. Very little is known about the DC response to *Encephalitozoon*. All of the published work to date has been performed in murine models. In mice, the production of the Th1 cytokine IFN-γ by mucosal DCs is critical in priming of intraepithelial lymphocytes (IEL) in response to *E. cuniculi* infection. A diminished IEL response in IFN-γ knock-out mice resulted in failed protection against lethal challenge with *E. cuniculi*. Lack of clonal expansion of the IEL population was
also observed when dendritic cells in mice were treated with anti-IFN-γ antibodies. Collectively, these data demonstrate that the production of IFN-γ by the DC population plays a role in triggering protective immune response to intestinal microsporidian infection (Moretto et al., 2007). Additional evidence for the role of DC in protective immunity against microsporidia addition was revealed in studies of immunosenescence. These investigations showed that aged mice infected with *E. cuniculi* demonstrated aberrant T cell priming by dendritic cells; whereas, the restoration of adaptive immunity was observed when these mice were reconstituted with DCs from younger mice (Moretto et al., 2008). While current studies are limited to mice, it is known that the elderly are more susceptible to microsporidial infections; therefore, more investigations into the role of DC in priming adaptive immunity against *Encephalitozoon* are needed.

**Other components of innate immunity.** Innate immunity has many defense systems in place for host protection. While the majority of research concerns the classic cellular responses initiated against *Encephalitozoon*, there are two other areas important for host defense that are discussed below.

**The role of non-professional phagocytes.** Specialized epithelial cells like enterocytes of the intestinal tract are a primary target for *Encephalitozoon*, yet no clear mechanism of host defense generated by these cell types have been demonstrated. Non-professional phagocytes were reported to have the ability to internalize microsporidian spores in an actin-mediated process, albeit less efficiently than macrophages (Couzinet et al., 2000; Franzen et al., 2005c). It was suggested that these epithelial cells may contribute to dissemination events in host organisms (Carlson et al., 2004; Gunnarsson et al., 1995) as a result of the lack of production of nitrogen
and oxygen intermediates (Couzin et al., 2000). However, others did not observe phagocytosis by human colonic cell lines but instead noted that the majority of cells had been penetrated by the polar filament after the spores attached via interactions through sulfated glycoaminoglycans (Leitch et al., 2005). It is still unclear how epithelial cells defend against microsporidia or if contact with host molecules can directly trigger spore firing.

The role of antimicrobial molecules. Finally, we must conclude with a note about antimicrobial molecules that are found in the secretions of mucosal tissues and are used as a primary defense against pathogens. In an attempt to distill which naturally occurring antimicrobials might inhibit microsporidial infection of epithelial cells, the anti-microsporidial properties of the naturally occurring defensins – lactoferrin, lysozyme, human beta defensin 2, human alpha defensin 5, and human alpha defensin 1 – were investigated. It was reported that different defensins play a role in preventing infection by certain but not all species of microsporidia. It was proposed that this differential inhibition may contribute to the tissue distribution observed for various microsporidial species. For the microsporidia which induce intestinal infections, lactoferrin or human alpha defensin 1 seem to be the two tested defensins important for controlling these pathogens (Leitch and Ceballos, 2009). While these types of studies are difficult to assess due to the complexity of the temperature, pH, and buffer requirements, the contributions of such molecules are critical to host defense in the acute stages of microsporidial infections.
Synopsis

The true extent of microsporidian infections is unknown. Microsporidia were, and perhaps still are, often overlooked and underdiagnosed because a) they are not specifically looked for in most diagnostics labs, b) organisms are small, and c) organisms fail to stain with hematoxylin and eosin. With increased awareness and improved diagnostics, infections due to microsporidia have been more frequently reported, and often in immunocompetent individuals. While adaptive immunity is clearly essential for clearance of these parasites, evidence is mounting that the response initiated by the innate arm of immunity may ultimately define whether or not the parasite can survive. Current research has focused on elucidating the mechanisms of resistance and susceptibility. It is important to keep in mind that innate immune responses are very much dependent upon cell type, activation, species and genetic background of the model system used as disparate reports can often be attributed to one of these factors. Understanding how naïve macrophages can be stimulated to kill intracellular pathogens even in the absence of adaptive immune responses or how aging affects resistance to microsporidial infections will lead to better designs of drugs that can reverse aging of DC or switch these innate immune cells into promoting cell-mediated immunity for clearance of infection.
Microsporidia and microsporidiosis

Microsporidia are obligate, intracellular eukaryotes that cause microsporidiosis, an infectious disease that has emerged in immunocompromised individuals, including HIV/AIDS patients, organ transplant recipients, children, contact lens wearers and travelers (Didier et al., 2004; Hale-Donze, 2007). Although these fungi are pathogenic to a variety of hosts, 14 of the 1200 species of microsporidia are known to infect humans via ingestion of contaminated food or water (Didier, 2005). After ingestion, the spore uses its unique polar extrusion to penetrate the host membrane and inject its contents into host cells; however, infection can also occur via phagocytosis of the spore by the host particularly demonstrated by macrophages (Fischer et al., 2008b; Franzen et al., 2005c). Chronic diarrhea is the most common symptom of microsporidiosis which leads to malabsorption and wasting (Hale-Donze and Didier, 2007). In addition, monocytes/macrophages have been identified as vehicles for dissemination of certain species of these fungi in humans leading to infection in most tissue types, resulting in pneumonia, pancreatitis, sinusitis, keratoconjunctivitis, and nephritis to name a few (reviewed in (Hale-Donze and Didier, 2007); (Mathews et al., 2009)).

Immune responses

Adaptive immunity, especially the activation of the T cells, is critical for clearance of infection (Khan et al., 2001). T cells are divided into phenotypically and functionally different subgroups, including CD4+ (T helper or Th) and CD8+ T cells (cytotoxic T cell) (Kindt et al., 2007). CD8+ T cells are involved in direct killing of intracellularly infected cells. CD4+ T cells mediate immune responses by secreting various cytokines that are
classified as different Th subsets (e.g. Th1 or Th2) and thus affecting other cell mediators involved in the immune response (Kindt et al., 2007). Differentiation of and production of certain cytokines by Th cells are under the influence of cytokines produced by innate immune cells in response to different stimuli. As illustrated in figure 1, Th1 cytokines, including interferon gamma (IFN-γ) and interleukin-12 (IL-12), are

**Figure 1.** In response to different stimulants, macrophages secrete cytokines that elicits the appropriate adaptive immune response needed for protection against pathogens.
involved in host protection against intracellular infection, while Th2 cytokines, such as IL-4, IL-5 and IL-10 are effective in extracellular infection (Netea et al., 2005). IFN-\(\gamma\) activates phagocytic macrophages, components of innate immunity, to not only secrete cytokines (e.g. IL-12) that induce the differentiation and proliferation of Th cells into the Th1 subset, but also to destroy the engulfed microbe (Kindt et al., 2007). Studies have shown that CD8+ knock-out mice (Moretto et al., 2000) and mice unable to produce IFN-\(\gamma\) (Khan and Moretto, 1999) are susceptible to pathogenic *Encephalitozoon cuniculi* infection, which suggests the need of CD8+ T cells and a Th1 response for protective immunity (Khan and Moretto, 1999; Moretto et al., 2000). The focus of this thesis is the examination of the innate immune responses responsible for this protection.

**Macrophages and the immune response**

Macrophages, an essential link between innate and adaptive immunity, are a source of IL-12 (Kindt et al., 2007; Langrish et al., 2004). Composed of subunits p40 and p35, this cytokine is structurally similar to IL-23, a cytokine recently discovered to comprise of subunits IL-12/IL-23 p40 and p19. Both IL-12 and IL-23 can be secreted by antigen presenting cells (APCs) and are essential players in regulating an appropriate host immune response against infection by inducing the maturation of Th subsets (Langrish et al., 2004). Early studies indicated that these two cytokines act on CD4+ T cells and can induce the development of Th1 cells and production of IFN-\(\gamma\) - a finding one would expect based on their structural similarities (Hunter, 2005). More recent work has uncovered functional differences between IL-12 and IL-23 (Boniface et al., 2008). As reviewed by Hunter (2005), it has been established that effective immunity against intracellular pathogens such as *Leishmania major*, *Toxoplasma gondii*, and
Listeria monocytogenes is dependent upon IL-12 in murine models (Hunter, 2005). On the other hand, there are few reports that show the requirement of IL-23 for resistance against intracellular parasites. Reports have shown IL-23, and not IL-12, is required for the establishment of autoimmune diseases such as inflammatory bowel disease, experimental allergic encephalomyelitis, and collagen-induced arthritis in mice (Hunter, 2005). Supportive studies indicate that IL-23 promotes the production of IL-17 by Th17 cells, a recently discovered Th subset. IL-23 also contributes to the expansion of Th17 cells [reviewed in (Dong, 2009)] thus influencing the mobilization of other cell mediators to the site of infection (Dong, 2009; McKenzie et al., 2006).

How IL-23 contributes to resistance or susceptibility to infectious disease is less clear (Hunter, 2005). IL-23 is needed for protection against the bacterium Klebsiella pneumoniae as IL-23 p19 deficient mice have increased susceptibility to infection (Happel et al., 2005). With regards to microsporidia, p40 knock-out mice succumbed to infection with Encephalitozoon spp. (Khan and Moretto, 1999; Salat et al., 2004), suggesting that IL-12 and/or IL-23 production is necessary to overcome microsporidial infection. However, the role of IL-23 in microsporidiosis has not been examined. Because of the similarities between IL-12 and IL-23, it is necessary to investigate the importance of both cytokines in the immune response to microsporidia and whether these two cytokines have divergent roles in host defense against microsporidian infection.

**TLRs and the immune response**

Our laboratory is investigating the role of macrophage responses to both pathogenic and nonpathogenic species of microsporidia to determine differences in host
recognition, signaling, and subsequent cytokine profiles. Our studies identified toll-like receptors (TLRs) as the host receptors responsible for recognition of microsporidia by human macrophages (Fischer et al., 2008a). Toll-like receptors are pattern recognition receptors that bind conserved microbial components and trigger numerous inflammatory responses induced by signaling cascades within immune cells (Akira, 2003; Kopp and Medzhitov, 2003). TLR activation has been associated with recognition of several parasites that are structurally similar to microsporidia (Akira and Takeda, 2004; Debierre-Grockiego et al., 2007; Xu et al., 2006). Our previous studies indicated that *E. cuniculi* and *E. intestinalis* are preferentially recognized by TLR2 in primary human macrophages leading to NF-κB activation; this TLR2 stimulation is linked to the initial inflammatory response (TNF-α and IL-8 production)(Fischer et al., 2008a). There is strong evidence that suggest Th2-type responses and small amounts of IL-12 are produced upon TLR2 stimulation while stimulation of TLR4 induces Th1 (IL-12) cytokine production (Netea et al., 2005). Here, we begin to investigate whether TLR2 and TLR4 differentially recognize pathogenic and nonpathogenic species of microsporidia and examine how this may contribute to immune function during infection.

**MAPK/p38 signaling and cytokine production**

TLR activation induces signaling of several pathways, including mitogen-activated protein kinase (MAPK) (Akira, 2003), which leads to production of inflammatory cytokines essential for clearance of infections (Netea et al., 2005). The MAPK family, including extracellular signal-regulated kinase (ERK), stress-activated protein kinase/c-Jun N-terminal Kinase (SAPK/JNK), and p38 MAPK, is activated by dual phosphorylation within its activation loop. Regulation of these kinases are
important to both innate and adaptive immune responses (Zhang and Dong, 2005). It has been shown that TLR4-mediated IL-12 production is dependent upon p38α MAPK and JNK activation (Agrawal et al., 2003; Kim et al., 2005). Specifically, reduced IL-12 production in LPS-stimulated macrophages was observed upon inhibition of p38α (Zhang and Dong, 2005). The role of MAPK in microsporidiosis is limited to a small study showing decreased parasitic load in CD8+ knock-out mice treated with p38α MAPK inhibitors when compared to wild-type CD8+ knock-out mice (Wei et al., 2007), indicating p38 MAPK involvement in susceptibility to microsporidial infection. However, this study neither showed a mechanism nor identified whether the administered inhibitors targeted the host, the parasite or both nor the extent of these effects. Therefore, it is important to determine the effects of p38 MAPK activation in the immune response against microsporidia in which this thesis begins to investigate.

**Additional isoforms of p38 MAPK**

In addition to p38α MAPK, p38β, γ, and δ MAPK have been identified (reviewed in (Cuenda and Rousseau, 2007; Huang et al., 2009)) Based on amino acid sequences, sensitivity to different p38 MAPK inhibitors, and substrate preference, these isoforms can be divided into two groups. p38α and β have more similar amino acid sequence identity to each other and are inhibited by substances like SB203580 and SB202190 whereas p38γ and δ are more identical and are uninhibited by these chemicals (Cuenda and Rousseau, 2007; Huang et al., 2009)). Most immunological reports have been focused on how p38α regulates the synthesis of pro-inflammatory cytokines (Kang et al., 2005). Therefore, determining whether or not additional p38 MAPK isoforms play a role in regulation of pro-inflammatory cytokine production would not only be novel within
the area of microsporidiosis but also within the broad field of immunology. Here, we present preliminary data indicating that p38α, γ and δ may regulate IL-12 and/or IL-23 production.

**MAPK phosphatases as negative regulators p38 MAPK**

One mechanism that negatively regulates p38 MAPK is dephosphorylation by MAP kinase phosphatases (MKP). Both MAPK phosphatase 1 (MKP1) and MKP5 were shown to have preference for p38 MAPK in vitro (Zhang et al., 2004; Zhang and Dong, 2005). It has been shown that MKP1 knock-out mice showed sustained p38α activation (Salojin and Oravecz, 2007) and that MKP5 knock-out mice had increased p38α MAPK activation (Qian et al., 2009). It has also been reported that MKP5 negatively regulates innate pro-inflammatory responses (Zhang et al., 2004). These studies present the need to consider the role of MKP5 in addition to MKP1 as potential negative regulators of macrophage production of IL-12 and/or IL-23 in response to microsporidia. Here we begin to explore the role of MKP1 and MKP5 in pathogenesis of microsporidiosis.

By examining the mechanism induced by nonpathogenic infections, one can better understand what is necessary for protection against these fungal pathogens. There is a direct relationship between TLR2 recognition of pathogenic spores and the subsequent production of immune mediators in response to pathogenic *Encephalitozoon* infections (Fischer et al., 2008a). In this study, we are investigating the signaling cascades regulated by TLR2 and TLR4 engagement with both pathogenic and nonpathogenic species of microsporidia. We know that TLR4 engagement triggers MAPK activation leading to IL-12/IL-23 p40 production (Agrawal et al., 2003; Kim et al., 2005). As compared to pathogenic infections, we hypothesize that differences in TLR
activation by nonpathogenic species of microsporidia will lead to continual activation of p38α MAPK and IL-12 and/or IL-23 production – a response that may be critical for clearance of infection. We hypothesize that this sustained p38 activation may be due to distinct MKP regulation. In addition, we suspect that p38γ and δ MAPK differentially regulate in part the production of these cytokines. We also hypothesize that MKP induced by distinct TLR activation by pathogenic species negatively regulate IL-12 and/or IL-23.
CHAPTER 3: MATERIALS AND METHODS

Reagents

Pam₃CSK₄ (a TLR2 agonist) was obtained from Axxora (San Diego, CA), lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 and protease inhibitor cocktail were obtained from Sigma Chemical Co. (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) reagents and antibodies, cell extraction buffer and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), L-glutamine, streptomycin/penicillin, and gentamicin were purchased from Lonza Walkersville, Inc. (Walkersville, MD). Lymphocyte separation medium (LSM), phosphate-buffered saline (PBS), Dulbecco’s modified Eagle’s medium (DMEM), and Greiner Bio-One Cellstar tissue culture plates were purchased from VWR International LLC. p38 MAPK siRNA for p38α, γ, and δ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific p38α MAPK antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Cell Culture

Gradient centrifugation on LSM was used to isolate peripheral blood mononuclear cells (PBMCs) from buffy coats of healthy donors (Our Lady of the Lake Regional Blood Bank, Baton Rouge, LA) according to guidelines established by the Internal Review Board (Louisiana State University, Baton Rouge, LA). Monocyte-derived macrophages (MDMs) were obtained by using adherence assays. Monocytes were plated onto 6-well (2 x 10⁶ cells/well) and 96-well (1 x 10⁵ cells/well) culture plates and cultured for 4 h in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 μg/ml gentamicin at 37°C in 5% CO₂. Nonadherent
PBMCs were washed out with PBS while the adhered monocytes were allowed to differentiate for 10 days in supplemented DMEM with 10% FBS at 37°C in 5% CO₂.

**Parasites**

Pathogenic species *Encephalitozoon cuniculi* III and *E. intestinalis* (donated by Elizabeth Didier, Tulane National Primate Research Center, Covington, LA and purchased from ATCC) were grown in a rabbit kidney cell line (ATCC CCL-37) in DMEM supplemented with 10% FBS at 37°C in 5% CO₂ and harvested from tissue culture supernatants. Spores were washed once in PBS containing 0.2% Tween 20, rinsed and then were resuspended in supplemented DMEM, and counted with a hemacytometer (Didier et al., 1991). Virulent spores were used at a 5:1 parasite-to-MDM ratio as determined (Fischer et al., 2007).

*Vairimorpha necatrix*, a parasite of moths and butterflies (Becnel and Andreadis, 1999), was provided by Dr. Charles Vossbrink (Connecticut Agricultural Experiment Station, New Haven, CT). *Antonospora locustae*, parasite of grasshoppers (Becnel and Andreadis, 1999), was provided by Dr. Yuliya Sokolova (Louisiana State University, Baton Rouge, LA). *Spraguea lophii*, a parasite of fish (Freeman et al., 2004), and an undescribed *Thelohania-like* species were donated by Dr. Earl Weidner (Louisiana State University, Baton Rouge, LA). There have been no reports of pathogenicity in humans due to these species used throughout this thesis for the purpose of establishing a model for resistance to infection; these nonpathogenic species are used interchangeably throughout as our data shows consistent responses among them (Figure 3). Table 1 below summarizes the microsporidian species used in this thesis.
Table 1. Species of Microsporidia Used

<table>
<thead>
<tr>
<th>Pathogenic species</th>
<th>Nonpathogenic species</th>
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<tbody>
<tr>
<td><em>Encephalitozoon cuniculi</em></td>
<td><em>Vairimorpha necatrix</em></td>
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<tr>
<td><em>Encephalitozoon intestinalis</em></td>
<td><em>Antonospora locustae</em></td>
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<tr>
<td><em>Spraguea lophii</em></td>
<td></td>
</tr>
<tr>
<td><em>Thelohania</em>-like species</td>
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**TLR-transfected HEK cell lines**

Human embryonic kidney (HEK) cells transfected with TLR2, TLR4/MD2/CD14, or null plasmids (InvivoGen, San Diego, CA) were grown in 96-well culture plates in supplemented DMEM with 10% FBS at 37°C in 5% CO₂. Cultures were inoculated with spores of pathogenic *E. cuniculi* and nonpathogenic *V. necatrix*, *Thelohania*-like sp. and *S. lophii* for 18 hours. TLR4 agonist LPS (10 ng/ml) or TLR2 agonist Pam₃CSK₄ (50 ng/ml) were used as controls in some cultures. Supernatants were collected and TLR activation was determined by analyzing IL-8 production by using ELISA as suggested by the manufacturer (Invitrogen).

**siRNA**

In 96 well plates with supplemented, serum-free DMEM, p38α, γ, and δ MAPK knock-downs in MDMs were achieved by transfection of cells with 20 pmol of p38α, γ, or δ siRNA sequences or a negative control siRNA (Santa Cruz Biotechnology, Inc.) and 1 μl of Lipofectamine 2000 (Invitrogen) per well. Cells were incubated with siRNA-Lipofectamine mixture for 4h at 37°C in 5% CO₂, washed with PBS, and then placed in
supplemented DMEM with 10% FBS for 48 hours of recovery before performing experiments.

Protein extraction and western blotting

Cells were rinsed with PBS and whole-cell extracts were harvested using cell extraction buffer (Invitrogen) containing 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, and protease inhibitor cocktail (2 mM AEBSF, 14 µM E-64, 130 µM Bestatin, 0.9 µM Leupeptin, and 0.3 µM Aprotinin) (Sigma Aldrich). Samples were extracted for 30 minutes on ice and centrifuged at 13,000 RPM for 10 minutes at 4°C. Lysates were analyzed for protein quantity using bicinchoninic acid analysis. Proteins were separated using SDS-PAGE and proteins were transferred to Immobilon polyvinylidene fluoride membranes (Millipore).

Using the SnapID (Millipore), membranes were blocked using 0.05% non-fat dry milk at room temperature. Membranes were incubated with primary antibody and then secondary antibody in 0.05% non-fat dry milk. Detection was performed using enhanced chemiluminescence reagents (Millipore) and exposed to light film (VWR) for 5-30 minutes.

Real-time quantitative PCR (RT-qPCR)

In six well plates, MDMs were infected with *E. cuniculi* at a 5:1 ratio and *V. necatrix* at a 1:1 ratio for 6 or 12 hours as indicated in the figures below. Total RNA was isolated from spore-infected MDMs using Qiagen RNeasy minikit (Valencia, CA) according to the manufacturer’s instructions and was quantified using a NanoDrop ND-1000 spectrophotometer, and reverse transcribed using SuperScript III First-Strand Synthesis
Supermix (Invitrogen). Real-time quantitative PCR was performed using a Bio-Rad iCycler according to manufacturer’s instructions with primers (Integrated DNA Technologies, Coralville, IA) indicated below using RT SYBR Green Fluorescein Master Mix according to manufacturer’s instructions. Data was analyzed using the compative method $2^{\Delta\Delta C_T}$ method where $\Delta\Delta C_T = C_T$ gene of interest $- C_T$ control and $C_T = C_T$ of $\beta$-actin $- C_T$ of gene of interest. $C_T$ is cycle threshold at which detection signal has passed an arbitrary threshold. The following primers were used (Teng, Huang, & Meng, 2007):

MKP1 – Forward: 5’ – TTTGAGGGTCACTACCAG -3’; Reverse: 5’-GAGATGATGCTTCGCC -3’; MKP5 – Forward: 5’ - CTGAACATCGGCTACG -3’; Reverse: 5’ – GGTGTAAGGATTCTCGGT -3’

ELISA

Monocyte-derived macrophages (MDMs) cultures were challenged with *E. cuniculi*, *E. intestinalis*, *V. necatrix*, or *A. locustae* for specified time points. Supernatants were collected and IL-12/IL-23 p40, IL-12 p70, and IL-23 p19 ELISA was performed in triplicate according to manufacturer’s (Invitrogen) instruction.
CHAPTER 4: RESULTS

Nonpathogenic, but not pathogenic, species induces p40, p70 and p19 cytokine production. ELISAs were performed to determine if there are differences in MDMs IL-12/IL-23 p40, IL-12 p70, and IL-23 p19 responses to microsporidian spores. We determined the induction kinetics of these cytokines (Figure 2) post infection with virulent and avirulent species. We observed no significant increase in IL-12/23 p40, IL-12 p70 or IL-23 p19 at any time point in pathogenic infections, whereas MDMs IL-12/23 p40, IL-12 p70 and IL-23 p19 responses were augmented overtime post-nonpathogenic infection. MDMs infections with avirulent species induce a significant IL-12/IL-23 p40 response 12 - 18 hours post-challenge which strongly correlates with the IL-23 p19 increase observed during those time points. An increased IL-12 p70 is also observed, but at lower levels; the higher levels of the p19 subunit suggests a preferred generation of the IL-23 complex rather than IL-12.

![Graph](image-url)
MDMs were infected with either pathogenic (E. intestinalis or E. cuniculi) or nonpathogenic (A. locustae or V. necatrix) spores, supernatants were collected over time and an ELISA was performed for either the IL-12/IL-23 p40 (A), IL-12 p70 (B) or the IL-23 p19 (C) subunits. Levels of IL-12 or IL-23 were augmented in avirulent infections but failed to be elicited with pathogenic microsporidia. At least three independent experiments (each ran in triplicate) were performed with both nonpathogenic and pathogenic infections at time points indicated (n ≥ 3 (n=number of donors) for each time point). Data expressed as means ± S.E.M.
Avirulent species are recognized by both TLR2 and TLR4 while virulent species are recognized by TLR2 only. Fisher et al. (2008a) reported that TLR2-transfected but not TLR4/MD2/CD4-transfected HEK293 cells responded to Encephalitozoon spp. To investigate the involvement of TLR2 and TLR4 recognition of avirulent species of microsporidia, HEK293 cells transfected with either null plasmids, TLR2-expressing plasmids or TLR4/MD2/CD14–expressing plasmids were inoculated with both virulent (E. cuniculi) and avirulent (V. necatrix, S. lophii, or Thelohania-like sp) species and the IL-8 response was measured (Figure 3). Neither of the agonists nor the microsporidian spores had an effect on IL-8 production by HEK293 null cell line. However, both TLR4/MD2/CD14-transfected and TLR2- transfected cells generated a response when stimulated with all three non-pathogenic species, though higher levels of IL-8 was induced through TLR4/MD2/CD14 activation as compared to levels produced through TLR2. Consistent with the previous report (Fischer et al., 2008a), an IL-8 response was generated in TLR2-transfected HEK stimulated with E. cuniculi but not with TLR4/MD2/CD14-transfected cells. These data suggest that the utilization of both TLR4 and TLR2 may be an important factor to the MDMs recognition of avirulent species of microsporidia and the subsequent cytokine profile.
**Figure 3. Differential TLR utilization by microsporidian species.**

HEK293 cells transfected with plasmids encoding (A) null, (B) TLR2, or (C) TLR4/MD2/CD14 were challenged with spores of pathogenic microsporidia (*E. cuniculi* spores) non-pathogenic species (*S. lophii, Thelohania sp.*, or *V. necatrix*, LPS (10 ng/ml), or Pam3CSK4 (50 ng/ml)) for 18 hours. Culture supernatants were collected and assessed for IL-8 production by ELISA. For each experiment, n=3.
**p38α MAPK activation was down regulated in response to pathogenic species of microsporidia.** MDMs were challenged with *E. cuniculi* (virulent species) and *A. locustae* (avirulent species) for time points indicated (Figure 4). Extracts were prepared and western blot analysis was performed using a dual phospho-specific p38α MAPK antibody. Here we show that activated p38α is transient in MDMs when infected with *E. cuniculi* as we only detected activation at 3h and 6h post infection. p38α MAPK is continuously activated from 3h to 24h post infection of human macrophages with *A. locustae*. These data suggest that continuous activation of p38α MAPK may be necessary for the appropriate innate immune response to microsporidia and for control of infection. How this activation is regulated will be an area of future investigation.

![Phospho-specific p38 MAPK](image)

**Figure 4.** p38α MAPK activation is induced but not sustained in response to pathogenic infections. Human primary macrophages were infected for indicated time points with either pathogenic (*E. cuniculi*) or non-pathogenic (*A. locustae*) microsporidia and total cell lysates were collected. Protein was quantified and western blot was performed using phospho-specific antibodies for p38α MAPK. This experiment was repeated independently; western blot shown here is provided by Jeff Fischer.
p38α MAPK contributes to both IL-12/IL-23 p40 production in avirulent infections.

To directly link p38α MAPK activation to differed cytokine profiles, MDMs transfected with either negative control or p38α MAPK siRNA were challenged only with nonpathogenic species of microsporidia as there was little induction of IL-12 and IL-23 observed in response to pathogenic species (Figure 2). siRNA-treated cells were stimulated with nonpathogenic spores for 24 hours – a time point where IL-12 and IL-23 production was observed. Diminished levels of IL-12/23 p40 were detected with p38α MAPK siRNA-treated cells to negative control siRNA-treated cells 24 hour post-infection as compared (Figure 5). This suggests the need for p38α is necessary for the appropriate IL-12 and/or IL-23 response to microsporidia.

**Figure 5.** Inflammatory cytokine production in p38α knockdown-MDMs infected with nonpathogenic species. Using siRNA, p38α MAPK was knocked down in human macrophages and resulted in a decrease in IL-12/IL-23 p40 production when infected with *V. necatrix* for 24 hours. LPS (50 ng/mL) was used a positive control (n≥1). Fold change is the ratio of p38α MAPK siRNA-infected cells to negative control siRNA-infected cells.
Other isoforms of p38 MAPK may play a role in IL-12/23 p40 production in nonpathogenic infections. Evaluation of whether or not p38 MAPK regulates IL-12 and/or IL-23 production is critical to understanding protective immune responses in microsporidiosis. Thus far, the work described here focused on the examination of p38α MAPK. In addition to p38α, p38β, δ, and γ MAPK have been identified. The preliminary data shown here suggest that two additional p38 MAPK isoforms may have an effect on IL-12/IL-23 p40 production in response to challenge with different species of microsporidia (Figure 6). Primary human macrophage IL-12/IL-23 p40 production decreases when both p38 δ and γ are knocked down using siRNA transfection; this

![IL-12/IL-23 p40 Production Graph](image)

**Figure 6. Regulation of IL-12 and/or IL-23 by p38γ and δ MAPK.** Knock-downs of p38γ or δ MAPK in primary human macrophages were achieved by transfection of cells with Lipofectamine 2000 and p38γ or δ MAPK siRNA or a negative control siRNA followed by a recovery of 48 hours as indicated above. These cells were then challenged with LPS (50 ng/mL) or *V. necatrix* for 12 hours. Supernatants were collected and IL-12/IL-23 p40 ELISA was performed.
diminished response is more evident in nonpathogenic-infected macrophages treated with p38γ MAPK siRNA as compared to those treated with p38δ MAPK siRNA. This may be a result of differential TLR activation. Further experiments are needed to validate the presented data and to identify how these isoforms regulate IL-12 and/or IL-23 production in response to microsporidia.

**MAPK phosphatases as negative regulators of IL-12 and IL-23 production in microsporidian infection.** Because there is limited activation of p38α MAPK observed when human macrophages are infected with virulent species as compared to sustained p38α MAPK activation with avirulent infections (Figure 4), we investigated whether or not MKPs were induced as one potential mechanisms of immunosuppression. After challenging primary human macrophages with pathogenic species for six hours, we observed the loss of p38α MAPK phosphorylation (Figure 4). However, at this time point, there are very little differences in MKP1 expression between pathogenic and nonpathogenic infections as shown in Figure 7A. In contrast, pathogenic stimulation elicited much higher expression levels of MKP5 than nonpathogenic infection at six hours post challenge (Figure 7B). This difference in expression level may be responsible for diminished IL-12/IL-23 production observed by macrophages post challenge with virulent species. In addition to induction of MKP expression, these phosphatases can be regulated by post-translational modifications as well (Liu et al., 2007). One would suspect that these mechanisms would also need to be evaluated to understand how MKP1 and MKP5 regulate p38 MAPK in microsporidian infection.
Figure 7. Expression of MKP1 and MKP5 in response to virulent and avirulent species of microsporidia. MDMs were infected with E. cuniculi and V. necatrix and stimulated with LPS (100ng/mL) for six hours. Total RNA was isolated from spore-infected MDMs, quantified, and reverse transcribed. Real-time quantitative PCR was done using a Bio-Rad iCycler and RT SYBR Green Fluorescein Master Mix with primers indicated above. Data was analyzed using the comparative method $2^{-\Delta\Delta C_T}$ method where $\Delta\Delta C_T = C_T$ gene of interest $- C_T$ control and $C_T = C_T$ of β-actin $- C_T$ of gene of interest. $C_T$ is cycle threshold at which detection signal has passed an arbitrary threshold.
CHAPTER 5: DISCUSSION

The contribution of macrophages to microsporidian clearance is poorly understood. The current study investigates toll-like receptor-regulated signaling mechanisms used by nonpathogenic species of microsporidia to induce a pro-inflammatory cytokine profile by primary human macrophages. These cytokines, such as IL-12, are critical for resistance to infection. Functional IL-12 is a heterodimer composed of p40 and p35 while biologically active IL-23 also is composed of the p40 subunit but instead is covalently linked to p19 subunit (Langrish et al., 2004; Oppmann et al., 2000). Both IL-12 and IL-23 can be secreted by APCs and are necessary in regulating an appropriate host immune response against infection by inducing the maturation of Th cells into distinct subsets (i.e. Th1 or Th17 respectively) (Langrish et al., 2004). It is known that IL-12/IL-23 p40 is needed for host protection against microspridia. Here, we propose a protective role for IL-23. The data shown here indicates that the presence of Th1 cytokines (IL-12 p70) does not affect the Th17 (IL-23) response which, in addition to IL-12, may also contribute to the Th1 response (Hunter, 2005). Other studies have shown that these responses promote T cell IFN-γ production and the effector phase of memory T-cell-mediated immunity (Oppmann et al., 2000) which may be involved in control of microsporidian infection but must be further investigated.

In addition, macrophages/monocytes have been suggested as the principal cells involved in dissemination of microsporidia (Orenstein, 2003; Orenstein et al., 1997). In mice, studies have shown that IL-23, but not IL-12, induce T-cell GM-CSF (granulocyte-macrophage-colony stimulating factor) production (Aggarwal et al., 2003) which would
induce monocyte differentiation into mature macrophage phenotype. From the present study, one could speculate that the lack of macrophage IL-23 production detected in response to pathogenic species may result in a lack of growth factor GM-CSF (granulocyte-macrophage-colony stimulating factor) response by T-cells thus reducing the ability of recruited monocytes to fully differentiate into effector macrophages. Further examination is necessary to substantiate this hypothesis. However, these data do suggest that macrophage immune responses to virulent microsporidia may contribute to pathogenesis of the disease by suppressing Th1 and Th17 responses. It is also possible that IL-23 is required for an adequate mucosal production of IL-17 in early host defenses against microsporidia

Macrophage activation can occur through TLRs, evolutionarily conserved signaling receptors known to recognize molecular patterns found on pathogens (Netea et al., 2005). The ligation of TLRs to PAMPs similar to those found on microsporidia has been identified. For example, glycosylphosphatidylinositol (GPI) isolated from Toxoplasma gondii and Trypanosoma cruzi are recognized by and signal through TLR2 and TLR4 (Akira and Takeda, 2004; Debierre-Grockiego et al., 2007); GPI was also identified on the spore coat of Encephalitozoon spp. (Xu et al., 2006). The production of inflammatory cytokines needed for clearance of infection is induced by several signaling pathways activated by TLR engagement. We know that LPS stimulation of TLR4 induces Th1 (e.g. IL-12) cytokine production while small amounts of IL-12 are produced upon TLR2 stimulation (Agrawal et al., 2003; Netea et al., 2005). The adaptive immune response is highly influenced by the innate response induced by stimulation of different TLRs (Netea et al., 2005), thus it is necessary to examine these differences in response
to microsporidian infection. Our previous work indicated that NF-κB activation induced by TLR2 recognition of Encephalitizoons is linked to the initial inflammatory response observed (Fischer et al., 2008a). The TLR2 recognition of both pathogenic and nonpathogenic species and the TLR4 recognition of nonpathogenic species suggest that both TLRs are involved in the mechanism of host defense against these parasites. Here, the data shows that there is differential activation (IL-8) of TLR4- and TLR2-transfected HEK 293 cells infected with either virulent or avirulent strains of microsporidia. This current study supports the need to further evaluate the link between TLR2 versus TLR4 recognition of pathogenic species of microsporidia and the diminished MDM production of IL-12 and IL-23 observed in response to challenge with these spores.

Data presented in this thesis also shows p38α MAPK activation was down-regulated in response to pathogenic species whereas avirulent species sustained the p38α MAPK, which may be required for adequate IL-12 and/or IL-23 production. Additionally, at 24 hour post-infection with avirulent species, a decrease of IL-12/IL-23 p40 was observed for p38α and γ MAPK knock-down MDMs. This abatement of cytokine response observed in nonpathogenic infections when p38α and γ MAPK was knocked down suggest that the down regulation of p38α activation and p38γ expression and/or activation in MDMs infected with pathogenic species may result in suppression of Th1 and/or Th17 responses observed in challenge with virulent spores. As illustrated in the predicted model in figure 8, MKP1 and/or MKP5 gene expression of activation induced by TLR2 recognition of pathogenic species of micropsoridia may lead to
decreased p38α and γ and production of IL-12 and IL-23. In contrast, TLR2 and TLR4 recognition of nonpathogenic species leads to continuous activation of p38α and

![Diagram of regulatory pathways induced by pathogenic and nonpathogenic species of microsporidia.](image)

**Figure 8.** Predicted model of distinct regulatory pathways induced by pathogenic and nonpathogenic species of microsporidia.

increased p38γ expression and/or activation which results in augmented production of IL-12 and IL-23 and resistance to infection. Initial recognition of both species by TLR2 may activate p38α but recognition by TLR4 may be needed for sustained activation as observed in avirulent infections. Although these speculations are aligned with TLR More conclusive experiments are necessary to validate these speculations.
In conclusion, we observe differences in host recognition and responses to pathogenic and nonpathogenic infections. Resistance to infection appears to be dependent upon IL-12, and here, we suggest, maybe more importantly, protective IL-23 responses. This response may be dependent upon (1) the use of different PRR by these strains and (2) the activation and/or increased expression of different isoforms of p38 MAPK which is a novel mechanism for pro-inflammatory cytokine production. Our in vitro system with pathogenic microsporidia represents a model to investigate mechanisms for permissive environments as observed in immunocompromised individuals. Analyzing immune responses to nonpathogenic microsporidia, an approach not yet used in the microsporidian field of research, could be used to predict the mechanisms required for protection against these fungal pathogens. Such a model is less expensive as compared to animal models. In addition, this model directly reveals signaling mechanisms in humans while avoiding (1) the biases of Th immune responses associated with different strains of mice and (2) the developmental complications that sometimes accompany the use of gene knockouts in animals. Thus, this comparative system allows for the identification of regulatory proteins involved in susceptibility or resistance to microsporidian infection. From these studies, chemotherapeutics can be derived that can switch the signaling cascades towards producing a resistant environment.
REFERENCES


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