Chemotactic and inflammatory responses to and recognition of Encephalitozoon spp. of microsporidia

Jeffrey Lynn Fischer
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CHEMOTACTIC AND INFLAMMATORY RESPONSES TO AND RECOGNITION OF
ENCEPHALITOZOON SPP. OF MICROSPORIDIA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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Doctor of Philosophy

in

The Department of Biological Sciences

by
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August 2008
ACKNOWLEDGEMENTS

How I have arrived at this point and completed such an enormous task is all a little blurry, but the people who have stood by me, guided me, and encouraged me are now clearer than ever and will forever be remembered for their support.

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ABSTRACT

Microsporidiosis is an emerging disease among immunocompromised individuals who often present with chronic diarrhea. The intracellular, eukaryotic parasites responsible for this pathology can often disseminate, causing multiorgan infections. Dissemination of these pathogens is believed to occur through vehicular spread by macrophages. The macrophage response to microsporidia is poorly understood. The information, described herein, is focused on defining the host-pathogen interaction and subsequent inflammatory response in human monocyte-derived-macrophages (MDM) against *Encephalitozoon spp.* of microsporidia. Initial studies were designed to better define the infection kinetics in MDM using various microscopic analysis and novel staining approaches. Spore adherence and uptake occurs within the first 6 hr and parasitophorous vacuole formation within 24 hr after infection. Replication was shown to peak at 72 hr as measured by bromodeoxyuridine incorporation and spore formation by 120 hr. Treating the MDM with interferon gamma and bacterial lipopolysaccharide reduced parasitic burden. The role of MDM in initiating monocyte recruitment after infection was evaluated using co-culture chemotaxis assays, limited gene and protein arrays, ELISA, and neutralizing antibody assays. These studies identified three major monocyte chemoattractants, CCL2, CCL3, and CCL4 that were upregulated, produced, and secreted in response to Encephalitozoon infections. Furthermore, these were necessary for monocytic infiltration. Finally, investigations into the receptors involved in initiating host recognition and regulating chemokine production were examined. Toll-like receptor (TLR) 2 was shown to be activated by Encephalitozoon spores. Using siRNA gene knock-downs in MDM, TLR2 was revealed to activate NF-κB within 1 hr after
parasite exposure resulting in the production of not only CCL3 and CCL4 but also two 
pro-inflammatory cytokines, TNF-α and IL-8. These results indicate that microsporidia 
are recognized by TLR2 and induce the production of chemotactic and inflammatory 
mediators needed for the recruitment of monocytes/macrophages, which allow for 
parasitic proliferation.
CHAPTER 1
LITERATURE REVIEW

Introduction

Microsporidia are a unique group of obligate intracellular eukaryotic parasites found virtual everywhere in nature and are known to infect a wide variety of unicellular organisms, multicellular invertebrates, and all classes of vertebrates. There are more than 1200 species and 140 genera of microsporidia and the group is characterized by their diverse structural, physiological, and genetic characteristics that transcend borders of traditional taxonomical classification, making it difficult to place them in any one category. However, two defining characteristics of microsporidia unite all species of these organisms together: 1) they all exist as spores in the extracellular environment, and 2) they all contain a structurally similar polar filament (tube) which is everted during the invasion of a host cell (Bigliardi and Sacchi, 2001; Didier, 2005; Didier et al., 2004; Franzen and Muller, 1999; Weiss, 2001; Wittner, 1999).

The detrimental effects of microsporidiosis have been reported in several industries of economic importance, including fisheries, honeybee, and silkworm, and are a known cause of infection in laboratory animals (Wittner, 1999). However, human infections have gained greatest recognition since the advent of HIV/AIDS in the early 1980’s and with the improvement of diagnostic techniques (Bryan and Schwartz, 1999; Didier, 2005). While the disease has primarily been associated with immunocompromised individuals, such as HIV/AIDS patients, organ recipients and cancer patients, the parasite is becoming reported more frequently as the cause of enteric and ocular infections in healthy people (Didier, 2005; Nkinin et al., 2007).
Currently, there are 14 species of microsporidia that have been described in human infections (Table 1.1), of which, the most prevalent cases described are enteric infections from *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Encephalitozoon cuniculi* (Didier et al., 2004). Infections primarily occur through the ingestion of spores and infect enterocytes of the small intestine, however, infections of lung epithelial tissues have also been reported and believed to occur through the inhalation of aerosolized spores (Didier, 2005). *E. bieneusi* is known to cause infections in the small intestine and hepatobiliary tree, but is mostly limited to these sites. Similarly, *E. cuniculi* and *E. intestinalis* are also known to infect the small intestine and have been identified in disseminated disease affecting the kidneys, liver, spleen, brain, and sinus mucosae (Kolter and Orenstein, 1999).

<table>
<thead>
<tr>
<th>Microsporidia species</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Identified</th>
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<tbody>
<tr>
<td><em>Brachiola algerae</em></td>
<td>Visvesvara et al. (1999)</td>
</tr>
<tr>
<td><em>Brachiola connori&lt;sup&gt;a&lt;/sup&gt;</em></td>
<td>Matsubayashi et al. (1959)</td>
</tr>
<tr>
<td><em>Brachiola vesicularum</em></td>
<td>Cali et al. (1998)</td>
</tr>
<tr>
<td><em>Encephalitozoon cuniculi</em></td>
<td>Bergquist et al. (1984)</td>
</tr>
<tr>
<td><em>Encephalitozoon hellem</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Friedberg et al. (1990)</td>
</tr>
<tr>
<td><em>Encephalitozoon intestinalis</em></td>
<td>Cali et al. (1993)</td>
</tr>
<tr>
<td><em>Enterocytozoon bieneusi</em></td>
<td>Desportes et al. (1985)</td>
</tr>
<tr>
<td><em>Microsporidium africanum</em></td>
<td>Pinnolis et al. (1981)</td>
</tr>
<tr>
<td><em>Microsporidium ceylonensis</em></td>
<td>Ashton &amp; Wirasinh (1973)</td>
</tr>
<tr>
<td><em>Nosema ocularum</em></td>
<td>Bryan et al. (1991)</td>
</tr>
<tr>
<td><em>Pleistophora ronneafiei</em></td>
<td>Ledford et al. (1985)</td>
</tr>
<tr>
<td><em>Trachipleistophora anthropopthera</em></td>
<td>Yachnis et al. (1996)</td>
</tr>
<tr>
<td><em>Trachipleistophora hominis</em></td>
<td>Field et al. (1996)</td>
</tr>
<tr>
<td><em>Vittaforma cornea</em></td>
<td>Davis et al. (1990)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Species was described as *E. cuniculi*, later determined by Weiser (1993) to be *N. connori*.

<sup>b</sup> First cases were believed to be *E. cuniculi*. 

---
Historical Perspective

During the mid-1800’s, Louis Pasteur was enlisted to help identify preventative techniques needed to stop the spread of pebrine disease, which was threatening to destroy much of the silk industry in France and Italy. Infected silkworms were described by their tegument being covered, or “peppered” with blackish marks and tissues containing oval inclusions. Pasteur recognized the importance of removing infected silkworms and mulberry leaves from silk cultivation farms to prevent further spread of the disease. The agent of pebrine disease was identified by Nägeli in 1857 as the first microsporidian and assigned the name *Nosema bombycis*. Since this initial discovery and classification, microsporidia have been identified in the infections of many insects, fishes, and mammals and a cause of negative economic impact in associated industries (Didier et al., 2004; Wittner, 1999). In 1922, Wright and Craighead reported the first mammalian microsporidian infection in rabbits by *Encephalitozoon cuniculi*; affecting neurological systems and causing motor paralysis (Wright and Craighead, 1922).

The first documented case of human microsporidiosis was described by Matsubayashi et al. (1959) in a 9-year old boy as *Encephalitozoon cuniculi* and was later determined to be *Brachiola connori* (Didier, 2005). In 1973, disseminated microsporidiosis was diagnosed in an infant with thymic aplasia (Margileth et al., 1973) and a case of ocular infection was detected in an 11-year-old boy (Ashton and Wirasinha, 1973). Human cases of microsporidiosis remained infrequent until the mid 1980’s. In 1985, *Enterocytozoon bieneusi* was established as the etiological agent of diarrheal disease in an AIDS patient (Desportes et al., 1985), and, thereafter, cases of microsporidiosis became more frequently detected in immunocompromised individuals.
Currently, cases of microsporidiosis have been described in immunocompromised individuals, including HIV/AIDS patients, organ recipients prescribed immunosuppressants, and cancer patients undergoing chemotherapeutic treatments. Additionally, microsporidians have been identified in immunocompetent individuals, including the very young, elderly, travelers, and contact lens wearers (Didier et al., 2004).

**Taxonomy of Microsporidia**

Microsporidia have had a long history of being classified and reorganized into various groupings based on classical morphological studies and more recent phylogenetic analyses. In 1857, Nägeli placed the newly named *Nosema bombycis* in with the group Schizomycetes, which at that time consisted of mostly yeast and bacteria. To make a clearer distinction between the *Nosema sp.* and other organisms in this group, in 1882 the order Microsporida was created (Didier et al., 2004; Franzen and Muller, 1999; Wittner, 1999). During the 1900’s, several classification systems were devised based on ultrastructural studies, number of nuclei in each organism, spore morphology, and differences in developmental stages; however until recently, the most widely accepted classification proposed by Sprague in 1976 was based on membrane delimited or direct cytoplasmic development of the parasites. This system was again modified to incorporate the nuclear state as the primary defining characteristic, such as diplokaryon or haplokaryon, in organizing specific species of microsporidia (Franzen and Muller, 1999).

While microsporidia have been classified as eukaryotic protozoa, new phylogenetic studies suggest these organisms are more closely related to fungi, and
therefore should be reclassified as such (Gill and Fast, 2006; Thomarat et al., 2004). Initial phylogenetic studies of ribosomal RNA and elongation factors would argue that these organisms were primitive eukaryotes that diverged early in history, prior to gaining the endosymbiont \( \alpha \)-proteobacteria and should be placed with Archezoa (Cavalier-Smith, 1991; Gill and Fast, 2006). However, later studies that focused on the genes of \( \alpha \)- and \( \beta \)-tubulin, mitochondrial heat-shock protein 70, TATA-box binding protein, the large subunit of RNA polymerase II, and subunits of pyruvate dehydrogenase proposed a stronger relationship between microsporidia and fungi (Gill and Fast, 2006). Additionally, structural and physiological similarities between fungi and microsporidia have been noted, such as the abilities to form spores and synthesize chitin, a component of the spore coat (Thomarat et al., 2004). The structural, biological, and recent phylogenetic similarities between microsporidia and fungi have strengthened the idea that these organisms are related to fungi; however, the exact placement within this kingdom has not been resolved. Current studies have placed them with the zygomycetes (Keeling, 2003) and chytrids (James et al., 2006), while another report suggests that they are a sister to a combined basidiomycetes/ascomycetes group (Gill and Fast, 2006). At this time, additional studies must be completed to identify the complete taxonomic placement of these eukaryotes.

**Biology of Microsporidia**

**Structure.** Microsporidia have a unique ultrastructural morphology and molecular biology that easily distinguishes them from other enteric organisms; however, they are small enough that technical and expensive diagnostic techniques, such as electron microscopy, are needed to determine their presence from other pathogens
The infectious stage for all microsporidia is an oval-shaped, environmentally resistant spore, typically measuring from 1 to 12 µm in length (Cali and Takvorian, 1999); however, those that infect humans range in size from 1 to 3 µm (Didier, 2005). The spore coat is comprised of two layers: an electron-dense, proteinaceous exospore and an electron lucent endospore composed of chitin (Bigliardi and Sacchi, 2001; Didier, 2005; Franzen and Muller, 1999; Vavra and Larsson, 1999). Closer observations of the spore layers of *Encephalitozoon* reveal that the exospore consists of three layers of varying densities and the endospore is a space with bridges of chitin that connect the exospore to the plasma membrane of the sporoplasm (Bigliardi and Sacchi, 2001; Vavra and Larsson, 1999). The spore coat functions to protect the integrity and viability of the sporoplasm from environmental pressures including acidic or alkaline conditions that range from pH 4-9, freezing temperatures, dehydration, and changes in tonicity (Didier, 2005). However, inactivation of spore viability can occur by various chemicals, including extreme acidic and alkaline solutions, 70% ethanol, sodium hypochlorite (Santillana-Hayat et al., 2002), and UV radiation (Marshall et al., 2003).

A classical plasma membrane delimits the sporoplasm from the spore coat. The intracellular contents of the sporoplasm include several organelles needed for eukaryotic survival, which include a membrane-defined nucleus, observed in a monokaryon or dikaryon configuration depending upon the species of microsporidia, a modified endoplasmic reticulum usually adorned with ribosomes, free cytoplasmic ribosomes, and modified golgi bodies (Vavra and Larsson, 1999). Some cytoplasmic characteristics separate this group of organisms from other eukaryotes, such as atypical ribosomes and golgi bodies. The ribosomes are prokaryote-like having a 70S
sedimentation rate and lack the 5.8S rRNA. The golgi bodies are not composed of stacked lamellar cisternae, but are a membrane bound aggregation of vesicles. In addition, these organisms have been shown to lack mitochondria (Franzen and Muller, 1999; Vavra and Larsson, 1999), however, a mitochondria-like organelle, called the mitosome, has recently been identified (Williams et al., 2002). Newer evidence shows that some elements of the classical mitochondria are preserved in the mitosome, yet its function is largely not understood (Burri et al., 2006; Goldberg et al., 2008).

Unlike most eukaryotic organisms, microsporidia also contain unique elements used during the infection process, which include the posterior vacuole, polaroplast membranes, a polar sac-anchoring disk, and a polar filament (tube) (Fig. 1.1A). The polar filament is found in the resting spore coiled around the cytoplasmic contents within the plasma membrane. The number of coils in the spore is used to differentiate between species of microsporidia. Upon induction of polar filament extrusion, the tube turns inside out as it fires out of the apical region of the spore (Fig 1.1B) (Bigliardi and Sacchi, 2001; Didier et al., 2004; Franzen, 2004; Vavra and Larsson, 1999). The base of the polar tube is hinged to the polar sac-anchoring disk, a bell-shaped complex located at the spore’s apex, which functions to maintain the attachment of the polar tube to the spore after eversion. The polaroplast is a membrane system of lamellae that unite continuously with the polar sac and outer membrane of the polar filament (Bigliardi and Sacchi, 2001). It is proposed that the polaroplast is pushed through the polar tube and forms the new plasma membrane of the meront at the tip of the filament. The posterior vacuole is believed to be the force behind the extrusion and ejection of spore contents, because this vacuole swells when the spore has been activated (Bigliardi and
Sacchi, 2001; Franzen, 2004). A universal trigger for activation of the spore has not been identified, but several conditions are known to induce activation for individual species of microsporidia, such as spore exposure to acidic and alkaline conditions, temperature changes, cations, ultraviolet light, and biological complexes, such as mucin (Keohane and Weiss, 1999).

**Figure 1.1 Major structures of the microsporidian spore.** (A) A diagram of a microsporidian spore showing the spore coat layers, nucleus, and major organelles utilized during infection*. (B) A DIC light microscopy image of an *E. cuniculi* spore (s) after the polar filament (arrows) has fired and has forced out the sporoplasm (sp).

**Life Cycle.** The life cycle of microsporidia begins after activation of the spore and eversion of the polar tube results in successful penetration of a host cell and the intracellular entry of the sporoplasm. The newly acquired sporoplasm proceeds to replicate by merogony, increasing numbers of meronts and sporonts, and differentiates

into sporoblasts and mature spores by sporogony (Fig 1.2). Upon spore maturation, the host cell is lysed and the newly formed spores are released into the extracellular environment, where they are able to infect additional nearby host cells or are excreted into the extracorpeal environment. (Bigliardi and Sacchi, 2001; Franzen, 2004; Vavra and Larsson, 1999).

**Figure 1.2 Microsporidia life cycle.** Infection begins by extrusion of the polar tube from the spore, penetrating the host membrane, and expulsion of the sporoplasm into the host cytoplasm (A). Merogony proceeds in a membrane bound parasitophorous vacuole (*Encephalitozoon* spp.) with meront reproduction by binary fission in direct contact with the membrane and the development of sporonts (B). During sporogony, newly formed sporonts develop into sporoblasts (C), and finally mature spores, which are released by lysis of the host cell (D).

The exact process of division and development depends upon the species of microsporidia. *Enterocytozoon bieneusi*, the most prevalent cause of intestinal infections in humans, begins development within the host cell in direct contact with the cytoplasm. The uninucleate meront divides to produce a multinucleate meront which
then undergoes sporogony to generate a multinucleate sporont. The final development of independent spores is completed during cytokinesis. In contrast, *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis*, causes of intestinal infection and disseminated diseases in humans, begin development in the host cell inside a membrane bound vacuole, called the parasitophorous vacuole (PV), and avoids contact with the host cytoplasm. Meronts develop in contact with the inside membrane of the parasitophorous vacuole and replicate by binary division. Sporonts detach from the membrane and undergo development in the center of the vacuole to produce mature spores. Both methods of spore development result in host cell lysis and the release of spores into the environment (Bigliardi and Sacchi, 2001; Didier, 1998).

Although polar filament extrusion is thought to be the predominant method of parasitic invasion, actin-dependent phagocytosis has also been suggested and shown to occur in macrophages and lung fibroblast cells (Couzinet et al., 2000; Franzen et al., 2005b). The internalization and the intracellular fate of the spores has been a constant topic of discussion. TEM studies of *E. cuniculi* infected murine macrophages showed that ferritin and acid phosphatase labeled lysosomes do not fuse with the parasitophorous vacuoles of developing parasites (Weidner, 1975). Supporting evidence from Fausshauer et al. (2005) showed that pre-labeled endocytic vesicles did not fuse with the PV, nor was the late endocytic lysosomal marker LAMP-1 found in the PV membrane. In contrast, other studies have suggested that phagosomes fuse immediately with lysosomes, as demonstrated by the vacuoles capability to acquire markers of phagolysosomes, including LAMP-1, LAMP-2, CD68, and cathepsin D (Couzinet et al., 2000; Franzen et al., 2005b) and from observations of rhodamine-
dextran labeled lysosomal compartments ability to fuse with spore vacuoles (Couzin et al., 2000). Taken together, these studies suggest that internalized spores must escape the lysosome to proliferate within the cell and support Franzen et al. (2005b) findings that some internalized spores fire the polar tube out of the phagolysosomes, allowing for the potential escape of the sporoplasm.

**Epidemiology**

Human cases of microsporidiosis have been reported world-wide and it is only recent that the prevalence of this disease has been better understood with increased diagnostic technologies. However, this data still varies greatly due to differences in diagnostic methods and a lack of random sampling (Bryan and Schwartz, 1999). Early prevalence rates among AIDS patients with diarrhea were reported to range between 5% and 50% with an overall suggested prevalence of 15%, whereas, rates amongst healthy individuals with diarrhea were seemingly lower and reported to range between 1.3% and 22% (Didier, 2005). Additional reports in Niger and Vietnam showed a prevalence of 10.5% and 9.5%, respectively, amongst HIV-infected individuals (Espern et al., 2007) and 76.9% in HIV-infected children (Tumwine et al., 2005), whereas, a study of three United States cities reported only a prevalence of 1.5% amongst HIV patients (Dworkin et al., 2007). These contrasts reflect possible differences in water sanitation and food handling practices and the availability of effective chemotherapeutics amongst countries. Most reports have focused on sampling from symptomatic individuals; however, a study of microsporidiosis in Cameroon of sub-Saharan Africa surprisingly revealed the prevalence in healthy people without diarrhea.
was higher (67.5%) than in immunocompromised individuals (24%-35.7%) (Nkinin et al., 2007).

Age related incidences have also been reported for healthy individuals exhibiting symptoms and shown to have higher prevalence rates amongst young children and the elderly. Studies of HIV negative children have reported ranges between 4.1% to 6.6% (Leelayoova et al., 2005; Tumwine et al., 2005) with the highest reports amongst children between 12-23 months-old (Leelayoova et al., 2005). In HIV negative elderly patients with an average age of 75 years, the prevalence was 17.05% (Lores et al., 2002). These occurrences support the hypothesis that susceptibility to infection may be also associated with special age-related characteristics of the immune system (Lores et al., 2002).

Transmission

Various modes of transmission to humans have been suggested based on environmental detection of microsporidia and the prevalence of the disease. The parasite is known as a natural cause of infection in domestic and feral animals and has raised concern about its zoonotic potential to cause cases of human microsporidiosis (Didier, 2005; Wasson and Peper, 2000). Direct evidence of zoonotic transmission from domestic animals to children has been reported (Cama et al., 2007; McInnes and Stewart, 1991) and further studies suggest that birds participate in the airborne and water transmission of spores (Graczyk et al., 2007). The potential for transmission by contaminated food and water sources has been supported by studies detecting human-virulent strains of microsporidia in slaughterhouse animals, dairy from cows, fresh produce, and water sources for consumption (Buckholt et al., 2002; Dowd et al., 2003;
Jedrzejewski et al., 2007; Lee, 2008). In addition, the microsporidia *Brachiola algerae*, once believed to have potential as a mosquito bioinsecticide (Becnel et al., 2005), was diagnosed as the cause of fatal myositis in a diabetic patient (Coyle et al., 2004), raising concern for the potential vector-borne spread of disease.

**Disease and Treatment**

The clinical manifestation of microsporidiosis is dependent upon the initial infection site, immune status of the host, and the species of microsporidia. Disease typically results from the ingestion of spores resulting in the infection of enterocytes of the small intestine, but respiratory infections from inhalation of spores and ocular disease have also been confirmed (Didier, 2005). While milder symptoms of gastrointestinal disease have been reported for immunocompetent individuals, more severe disease symptoms, such as chronic enteric infections and disseminated disease, have been reported for immunocompromised individuals. Immunocompetent individuals with intestinal disease typically develop self-limited cases of diarrhea lasting 2-3 weeks, which results in the clearance of pathogen (Didier and Bessinger, 1999). Healthy individuals have been diagnosed with ocular infections that required treatment for resolution (Sagoo et al., 2007). In addition, data suggests that some immunocompetent individuals are asymptomatic carriers of persistent infections (Nkinin et al., 2007).

In contrast to healthy hosts, immunodeficient individuals can develop serious cases of intestinal microsporidiosis that result in chronic diarrhea and is associated with blunting of intestinal microvilli and decreased nutrient absorption. The chronic diarrhea can lead to wasting and death. While chronic diarrhea is the most prevalent pathology, disseminated disease in immunocompromised individuals infected with *Encephalitozoon*
spp. has been shown to affect the liver, gall bladder, kidneys, heart, and bladder (Kolter and Orenstein, 1999).

Many treatments have been prescribed for microsporidiosis, but a limited number of therapies have been successful (Conteas et al., 2000). Albendazole, an inhibitor of \( \beta \)-tubulin polymerization, has been shown to be effective against intestinal Encephalitozoon spp. infections (Blanshard et al., 1992), but is only variably effective against \textit{E. bieneusi} (Dieterich et al., 1994). Fumagillin, an antibiotic produced by \textit{Aspergillus fumigatus}, has been effective in treating Encephalitozoon ocular infections (Rosberger et al., 1993), as well as intestinal \textit{E. bieneusi} infections (Molina et al., 1997). In addition, antiretroviral therapy was shown to increase immune competency among HIV patients and indirectly decrease cases of microsporidiosis (Conteas et al., 2000).

**Adaptive Immune Responses to Microsporidia**

**Role of T Cells in Adaptive Immunity.** T cells are critical for host adaptive responses to infections. CD4\(^+\) T cells provide cytokines that help to activate either cell-mediated immunity or humoral responses. Activation of these two adaptive responses is dependent upon CD4\(^+\) T cell differentiation into Th1 or Th2 cells. These two sub-populations of CD4\(^+\) T cells differ in the profiles of cytokines that will be released, which ultimately determines the function of the adaptive response (Janeway et al., 2005).

Cell-mediated immunity is promoted by the differentiation of CD4\(^+\) T cells into Th1 cells and the release of Th1 type cytokines, including interferon-gamma (IFN-\(\gamma\)). The effects of these Th1 mediators are most notably seen on CD8\(^+\) T cells and macrophages. When activated by Th1 responses, CD8\(^+\) T cells deliver cytotoxic proteins to target cells upon recognition of antigen. Perforin is one cytolytic protein that
is released and forms pores in the membranes of target cells, allowing for intracellular contents to leak out. In addition, granzymes, which induce apoptosis, are released and enter the target cell using the perforin pore (Janeway et al., 2005). Macrophages are also activated by Th1 responses and induce an array of intracellular killing mechanisms. Some of these mechanisms include phagosome acidification, lysosomal fusion, and generation of reactive oxygen and nitrogen species. Activation of macrophages also induces the secretion of IL-12 which directs the differentiation of CD4+ T cells into Th1 effector cells (Beutler, 2004).

The differentiation of CD4+ T cells into Th2 cells and subsequent release of Th2 cytokines induces humoral immunity, activating the production of antibodies and complement proteins. Th2 cytokines involved in humoral effects include IL-4, IL-5, IL-10, and IL-13. Th2 responses enhance B cell activation and differentiation, while inhibiting the activation of macrophages. The effects of humoral immunity involve coating pathogens with antibodies and proteins that neutralize their intracellular entry, opsonize them to enhance phagocytosis, and activate the complement system (Janeway et al., 2005).

**Role of T Cells in Microsporidiosis.** T lymphocytes and cell-mediated immune responses against microsporidia have been proven to promote host resistance to microsporidiosis. Early evidence for cell-mediated immunity was gathered from experiments on athymic and severe combined immunodeficiency (SCID) mice that acquired lethal microsporidia infections after challenge with *E. cuniculi*. Resistance to infections was achieved by adoptive transfer of sensitized T cells into these immunocompromised mice (Schmidt and Shadduck, 1983), whereas the transfer of B
lymphocytes did not promote survival (Enriquez, 1997). As well, supernatants from infected lymphocytes were shown to activate macrophages to destroy microsporidia (Schmidt and Shadduck, 1984).

The role of T lymphocytes and the subtypes of T cells have been further defined in murine microsporidiosis. Khan et al. observed that CD8+ T cell knock out mice succumb to *E. cuniculi* infection (Khan et al., 1999), whereas, CD4+ T cell knock out mice survive and retain a normal antigen-specific cytotoxic T cell response (Moretto et al., 2000). Although CD4+ T cells do not affect disease outcome in mice (Moretto et al., 2000), they are believed to serve a role in protection in humans. Susceptibility to microsporidia increases as CD4+ T cell counts decline even in the presence of normal CD8+ T cells (Kotler and Orenstein, 1998). Furthermore, treatment with anti-retroviral therapy, which increases CD4+ T cell counts, reduces parasitic load (Hale-Donze and Didier, 2007).

To further delineate the mechanism of killing used by CD8+ T cells, perforin gene knock out mice were challenged with *E. cuniculi*. Perforin molecules are secreted by CD8+ T cells and exhibit their cytolytic effects on target cells by forming holes in the plasma membrane. Lethal infections were observed in perforin knock out mice and a greater parasitic burden was found in tissues (Khan et al., 1999).

**Role of Th1 Cytokines in Microsporidiosis.** Th1 cytokine responses have been shown to play an important role in host protection to microsporidia infections. Increased levels of IFN-γ have been observed in immunocompetent mice challenged with *E. cuniculi* (Khan and Moretto, 1999) and *E. intestinalis* (El Fakhry et al., 2001). The *in vitro* effects of IFN-γ have been observed in cultures of murine macrophages that
decreased parasite burden (Didier and Shadduck, 1994) and in vivo effects in IFN-γ receptor knock out mice challenged with E. intestinalis, which resulted in chronic infections (Achbarou et al., 1996). The protective nature of IFN-γ and IL-12 in mice was observed by an in vivo antibody neutralization experiment, in which, IFN-γ and IL-12 neutralized mice succumbed to infection. IL-12 is known to stimulate IFN-γ production, therefore, the neutralization of this cytokine was believed to decrease IFN-γ responses leading to fatal infections (Khan and Moretto, 1999). Susceptibility to infection was observed in IFN-γ and IL-12 knock out mice and further supported a requirement for IFN-γ in resistance to infection (Khan and Moretto, 1999). In a recent study, the requirement for IFN-γ in microsporidia clearance was assessed by administering IFN-γ therapy to SCID mice, which resulted in their prolonged survival (Salat et al., 2008).

**Role of Humoral Immunity in Microsporidiosis.** The production of antibodies against microsporidia infections is well documented in animal models of infection and in the human population; however, the protective role of antibodies is not well understood (Omura et al., 2007; Sak and Ditrich, 2005). Neiderkorn et al. first suggested that antibodies produced in response to infection may enhance phagocytosis of the parasite by macrophages and induce intracellular killing by lysosomal fusion with the parasitophorous vacuole (Niederkorn and Shadduck, 1980). A supporting study observed a decrease in infected cells when spores were incubated with anti-microsporidial exospore monoclonal antibody prior to challenge (Enriquez et al., 1998). More recently, a novel anti-exospore antibody was used in vivo to prolong the survival of CD4+ reconstituted SCID mice, resulting in decreased parasite burden in post-mortem peritoneal smears (Sak et al., 2006).
In contrast, full resistance did not ensue when B lymphocytes were transferred to athymic or SCID mice, as all mice succumbed to infection (Enriquez, 1997). Passive transfer of immune sera also did not promote resistance to infection in athymic mice (Schmidt and Shadduck, 1984). While the antibody response has been documented, it is proposed that it does not play a pivotal role in resistance to infection, but may enhance the efficiency of the immune response.

**Innate Immune Responses to Microsporidia**

**Role of Macrophages in Innate Immune Responses.** Macrophages are found throughout the body of the host and are central in defending epithelial barriers from entry by microorganisms, as they are the first cells to encounter pathogens. Although these cells are primarily recognized for their immune functions, they also contribute to host homeostasis and overall maintenance by scavenging and removing dead cells during embryonic development, bone remodeling, and tissue repair. Macrophages are derived from pluripotent haematopoietic stem cells found in bone marrow, which mature into blood monocytes. Monocytes migrate into tissues becoming tissue macrophages and make up a diverse population of cells in the body, including alveolar macrophages in the lungs, Kupffer cells in the liver, osteoclasts in bone, and microglial cells in nervous tissue. Macrophages primary functions in host defense are to recognize, engulf, and destroy pathogens, but they are also considered a link to adaptive immunity and function as antigen-presenting cells that can activate T cell responses (Janeway et al., 2005).

**Macrophages as Phagocytes.** Macrophages role in host immunity was first recognized by Ellie Metchnikoff when he observed cells in a sea star surround and
engulf a thorn that was introduced into the invertebrate (Metchnikov, 1884). The interaction between the host and microbe activated host surface receptors and intracellular signaling mechanisms which induced cytoskeletal rearrangements, the extension of the plasma membrane, and promoted engulfment. Receptors found on phagocytes involved in uptake can either identify microbes opsonized with antibody and complement proteins, such as the Fc and complement receptors, or can directly recognize surface molecules of the organism (Underhill and Ozinsky, 2002).

**Recognition of Pathogens by Macrophages.** Macrophage sensing is achieved through an array of germline encoded receptors found on the surface of the cell and inside endosomal compartments known as pattern recognition receptors (PRR). These receptors are triggered by the recognition of pathogen-associated molecular patterns (PAMPs) found on bacteria, viruses, protozoans, and fungi. Activation of these receptors results in initiation of innate immune responses. More recently, intracellular, non-compartmentalized molecules have been identified and found to recognize PAMPs and stimulate immune responses (Medzhitov, 2007; Takeda and Akira, 2005). While PRR are a large, diverse family of receptors and includes the β-glucan receptor, dectin-1 and the macrophage mannose receptor, the best characterized PRR are the toll-like receptors (Medzhitov, 2007; Underhill and Ozinsky, 2002).

**Toll-like Receptors.** The discovery of Toll and its function in innate immune responses was first identified in Drosophila, but soon lead to the identification of Toll homologues in mammals (Takeda and Akira, 2005). There have been 11 members of TLR identified in mammals, of which, 10 TLR are found in humans. Characteristically, these type I transmembrane receptors contain an extracellular leucine rich repeat (LRR)
domain used to recognize conserved molecular products and an intracellular Toll/IL-1 receptor (TIR) domain to recruit adaptor proteins that initiate signaling cascades. Although TLR are believed to form homodimers, some TLR are reported to heterodimerize with each other, such as TLR2 with TLR1, and TLR2 with TLR6. In addition, TLR4 is known to collaborate with molecules CD14 and MD2 in the recognition of lipopolysaccharide. TLR have been shown to recognize a variety of organisms, however, individual TLR are specific for molecular components (Table 1.2). These include triacyl lipopeptides by TLR1; peptidoglycan, zymosan, and lipoarabinomannan by TLR2; double-stranded RNA by TLR3; lipopolysaccharide by TLR4; flagellin by TLR5; diacyl lipopeptides and zymosan by TLR6; single-stranded RNA by TLR7 and TLR8; and CpG DNA by TLR9 (Medzhitov, 2001; Takeda and Akira, 2005).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
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<tr>
<td>TLR1</td>
<td>Triacyl lipopeptides</td>
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<td>TLR2</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td></td>
<td>Lipoteichoic acid</td>
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<td>Glycoinositolphospholipids</td>
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<td>Zymosan</td>
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<td>TLR3</td>
<td>Double-stranded RNA</td>
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<td>TLR4</td>
<td>Lipopolysaccharide</td>
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<td>TLR5</td>
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<td>TLR6</td>
<td>Diacyl lipopeptides</td>
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<td>Zymosan</td>
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<tr>
<td>TLR7</td>
<td>Single-stranded RNA</td>
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<td>TLR8</td>
<td>Single-stranded RNA</td>
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<tr>
<td>TLR9</td>
<td>CpG-containing DNA</td>
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While triggering TLR results in divergent gene products, the initial adapter proteins in these different cascades are often shared (Fig. 1.3). The MyD88 pathway

**Figure 1.3 TLR signaling pathways.** TLR triggering stimulates the recruitment of the adapter molecule MyD88 to the TIR domain of the receptor. Association of IRAK1, IRAK4, and TRAF6 induces the phosphorylation of IκB and results in activation of NF-κB. Additionally, TLR signaling has been shown to activate MAPK signaling and TRIF-dependent IRF3 pathways.

was first shown to be important in establishing an inflammatory response that included the production of TNF-α and IL-12 cytokines (Takeda and Akira, 2005). The recruitment of the adapter protein MyD88 to the TIR domain of the activated TLR leads to recruitment of IRAK-4 and the phosphorylation of IRAK-1. IRAK-1’s association with TRAF6 leads to the diversion of two distinct signaling pathways. One path induces
mitogen-activated protein kinase (MAPK) signaling, while the other path ultimately leads to the phosphorylation of inhibitor of nuclear factor kappa B (IκB). IκB functions to bind cytosolic nuclear factor kappa B (NF-κB) and prevent its translocation into the nucleus. Phosphorylation of IκB leads to the ubiquitination and degradation of this molecule, and ultimately results in the release and nuclear translocation of NF-κB (Medzhitov, 2001; Takeda and Akira, 2005). NF-κB activation has been described in mediating the transcription of a number of inflammatory cytokines, chemokines, and co-stimulatory molecules (Medzhitov, 2001). Additionally, TLR4 and TLR3 have been shown to signal independently of MyD88 by recruiting the adapter molecule TRIF. TRIF signaling activates the transcription factor interferon regulatory factor 3 (IRF-3) leading to the production of type I interferons (IFN-α and IFN-β) and late phase activation of NF-κB (Medzhitov, 2001; Takeda and Akira, 2005).

**Intracellular Killing of Pathogens.** Once the pathogen has been recognized, many of the receptors activate phagocytosis (Underhill and Ozinsky, 2002). The newly developed phagosome that surrounds the internalized microbe quickly becomes extremely toxic and begins destroy and degrade the organism. This process serves two purposes in immunity: killing of the organism preventing further progression of potential disease, and processing antigens allowing for co-presentation with MHC II class molecules on the cell surface leading to the activation of CD4+ T cell responses. The microbe-containing phagosome quickly becomes acidic and binds to lysosomes containing degradative enzymes and antimicrobial peptides, which are released into the phagolysosome (Beutler, 2004; Janeway et al., 2005). Classical activation of macrophages induces additional killing mechanisms within the cell. Activation of
macrophages can occur through direct interaction between the microbe and host cell surface molecules, such as reported interactions between bacteria and host toll-like receptors, or by host secreted stimulatory molecules, such as the cytokine IFN-γ. These additional mechanisms include the generation of toxic superoxide and hydrogen peroxide molecules by phagosome bound NADPH oxidase and the production of reactive nitrogen molecules by nitric oxide synthase (Beutler, 2004; Janeway et al., 2005; Underhill and Ozinsky, 2002).

**Induction of Inflammatory Cytokines.** Macrophage-microbe interactions lead to the release of cytokines into surrounding tissues, which contribute to the overall activation of acute phase responses and effector cells which limit microbial spread (Janeway et al., 2005). TNF-α and IL-1 are two common cytokines produced in response to infection. These mediators have been documented to induce the production of vascular occluding proteins, the release of opsonizing acute phase proteins, and an increase body temperature (Beutler, 2004; Janeway et al., 2005). Additionally, these two cytokines initiate the expression of adhesion molecules, such as E-selectin and VCAM-1, in vascular tissues, which contribute to cellular recruitment (Janeway et al., 2005).

The chemotactic cytokines, or chemokines, are a family of signaling proteins secreted to induce the recruitment of immune effector cells to sites of injury or infection. Chemokines bind to seven-transmembrane, trimeric G-protein coupled receptors on target cells. Activation of the chemokine receptor induces cell migration by intracellular actin reformation of the lamellipodia and increases in surface integrins that function in adherence (Baggiolini, 1998). CCL2 (monocyte chemoattractant protein 1, MCP-1),
CCL3 (macrophage inflammatory protein 1α, MIP-1α), CCL4 (macrophage inflammatory protein 1β, MIP-1β), and CXCL8 (interleukin-8, IL-8) are known chemokines produced by macrophages in response to microbes in an effort to recruit immune effector cells (Laing and Secombes, 2004). The gene expression of these four chemokines can be regulated by the transcription factor NF-κB (Grove and Plumb, 1993; Kunsch and Rosen, 1993; Teferedegne et al., 2006; Widmer et al., 1993). Chemokines are promiscuous molecules; typically able to bind to more than one type of chemokine receptor and recruit a diverse profile of immune cells. CCL2, CCL3, and CCL4 predominately induce migration of monocytes, whereas IL-8 is classically known as a recruitment factor for neutrophils (Laing and Secombes, 2004).

**Intracellular Killing of Microsporidia.** Nitric oxide responses have proven to be important mediators of intracellular killing and disease resistance for a number of microbial pathogens (James, 1995). The role of nitric oxide in host resistance to microsporidia has been studied by a limited number of groups and their interpretation of results provide conflicting views on the importance of this response to infection. Franzen et al. (2005a), reported that the levels of nitrate and nitrite, which correlates to nitric oxide production, did not increase in naïve primary human macrophage cultures challenged with *Encephalitozoon spp*. Didier et al. (1994), showed that cultures of murine macrophages activated with LPS, IFN-γ, LPS + IFN-γ, or TNF-α prior to infection with spores of *E. cuniculi* resulted in decreased number of spores after 48 hr in comparison to untreated cultures. In further studies, cultures activated with IFN-γ or the combination of LPS and IFN-γ resulted in the detection of increased levels of nitrite and microbiostatic activity, whereas the addition of the nitric oxide synthase inhibitor N3
monomethyl-L-arginine (NMMA) decreased the production of nitric oxide and prevented microbial killing (Didier, 1995). Classical activation of resting macrophages by bacterial lipoproteins or cytokines is important in the initiation of nitric oxide responses (James, 1995). While these studies support a role for classical activation of macrophages in nitric oxide killing of microsporidia, Khan et al. (1999), showed that mice deficient in inducible nitric oxide synthase, the enzyme responsible for nitric oxide production, were able to survive a high dose of microsporidia challenge suggesting that this molecule does not participate in resistance to infection.

In addition to nitric oxide responses, Didier et al. (2001), investigated the role of reactive oxygen intermediates in resistance to infection. They observed that mice with defective phagocytic oxidative respiratory burst challenged with spores of E. cuniculi were susceptible to longer infections and a higher parasitic burden. Five weeks after challenge, infection was still observed in oxidative burst deficient mice, whereas, infection was not observed in wild type mice. However, deficient mice did not succumb to infection. This suggests that oxidative respiratory bursts may contribute to resistance and a more efficient resolution of infection, but is ultimately not responsible for microbial clearance.

**Cytokine Response to Microsporidia.** Studies investigating the cytokine responses to microsporidia infections have provided limited insight. Franzen et al. (2005a), reported elevated levels of TNF-α, IFN-γ, and IL-10 cytokines from human macrophages in response to human virulent species of microsporidia. In support of its protective function, TNF-α was shown to inhibit E. cuniculi replication when added to murine cultures of macrophages (Didier and Shadduck, 1994). In addition, elevated
levels of TNF-a have been detected in fecal samples of AIDS patients with microsporidiosis and is believed to contribute to the disease pathology (Sharpstone et al., 1997).

**Statement of Research Problem and Hypothesis**

The immunobiology of microsporidiosis is not well understood and there is limited information about the role of macrophages in this disease. From the analyses of pathology reports, macrophages have been suggested as the facilitator of dissemination of the parasite from initial infection sites (Orenstein, 2003; Soule et al., 1997). Internalization of microsporidia has been observed in macrophages, however, there is some debate whether the parasite is able to replicate within the cell and produce mature spores or is degraded by the cell (Didier and Shadduck, 1994; Franzen et al., 2005b; Weidner, 1975). The focus of chapter 2 is the delineation of parasite proliferation within primary human macrophages and the effects of IFN-γ priming of macrophages on parasite development. A second aspect of the work centered on the production of chemokines due to the host-parasite interaction and the recruitment of monocytes. Parasite dissemination is believed to occur via macrophages (Orenstein, 2003), therefore, the functional chemokine gradient produced in response to infection was determined and a co-culture chemotaxis assay system was utilized to establish key chemokines needed for monocyte recruitment. The functional role of chemokines and the recruitment of additional monocytes to sites of infection are the focus of chapter 3. Encephalitozoon interactions with primary human macrophages have been shown to mediate the production of several cytokines (Franzen et al., 2005a). However, a receptor required to initiate parasite recognition and subsequent production of these
mediators has not been described. The focus of chapter 4 is the elucidation of the role of toll-like receptor recognition, intracellular signaling activation, and cytokine and chemokine production via this mechanism.

**Statement of Research Objectives**

The goal of this research was to gain a greater understanding of the host-pathogen relationship and the innate immune responses produced during this interaction between species of the Encephalitozoon and primary human macrophages. The specific objectives of this research were 1) to establish if *Encephalitozoon* spp. can cause an active infection in primary human macrophages that leads to parasite proliferation and the production of mature spores, 2) to determine if infection induces the production of a functional chemokine gradient that leads to the recruitment of additional monocytes to sites of infection, and 3) to elucidate the role of toll-like receptors in the recognition of *Encephalitozoon* spp. which leads to the production of pro-inflammatory responses.

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CHAPTER 2
KINETICS OF ENCEPHALITOZOOON SPP. INFECTION OF HUMAN MACROPHAGES*

Introduction

For many years, microsporidians have been recognized as pathogens of invertebrate and vertebrate species, including silkworms, honeybees, mosquitoes, mice, rabbits, foxes, pigs, and cows (Didier et al., 2004; Weiss, 2001), but have only recently gained greater notoriety for their ability to cause chronic and fatal disseminated infections in humans diagnosed with HIV/AIDS or in patients on immunosuppressive therapies (Carlson et al., 2004; Didier et al., 2004; Orenstein, 2003; Schottelius and da Costa, 2000). Of the more than 1,200 microsporidian species, only 14 have been reported to infect humans (Didier et al., 2004). When left untreated, immunocompromised patients suffer from chronic diarrhea, leading to dehydration and malnutrition. Often, in these patients, disseminated disease has been reported to cause keratoconjunctivitis, sinusitis, tracheobronchitis, encephalitis, interstitial nephritis, hepatitis, cholecystitis, osteomyelitis, and myositis (Didier, 2005; Orenstein, 2003; Schottelius and da Costa, 2000).

Microsporidians are obligate intracellular, eukaryotic parasites that were once classified as protozoans, but are now suggested to be more closely related to fungi, based on phylogenetic analysis (Gill and Fast, 2006). Microsporidians are found worldwide and are shed in the feces and urine of infected animals, thereafter, residing as environmentally resistant spores. Microsporidian infections are believed to occur

when the spore is ingested from contaminated water or food sources (Didier et al., 2004). Infections of epithelial and endothelial cells and macrophages are common (Khan et al., 2001). Classically, cellular invasion occurs when the spore encounters a host cell and everts a polar tube, thereby penetrating the cell membrane and injecting the sporoplasm. Alternatively, spores can be internalized through phagocytosis or endocytosis. Further proliferation and spore production occur through merogony and sporogony within a parasitophorous vacuole (PV), followed by lysis of the host cell and release of mature spores (Franzen, 2004).

The microsporidians, *Encephalitozoon* spp., have been described in several case reports that have culminated in disseminated infections (Carlson et al., 2004; Gunnarsson et al., 1995; Soule et al., 1997) and have been reported as having a promiscuous tropism for a variety of mammalian tissues and cell lines (Visvesvara, 2002). Some reports have suggested that dissemination is the result of migrating macrophages that act as reservoirs and allow for the vehicular dispersal of the parasite, all the while providing host metabolites for their replication (Fischer et al., 2007; Orenstein, 2003; Soule et al., 1997). To elucidate the role of macrophages in microsporidioses including dissemination events, it is critical to establish the timing of parasitic entry, replication, and lysis in primary human macrophages. The infection kinetics and production of mature spores have been well established in several non-human cell lines and animal models (Cox et al., 1979; Didier et al., 2001; Gannon, 1980; Salat et al., 2001; Wasson and Barry, 2003), whereas, most studies on infection kinetics in human cell lines and primary cells are limited (Couzinet et al., 2000; Fasshauer et al., 2005; Franzen et al., 2005a; Franzen et al., 2005b; Franzen et al.,
While these studies suggest that a variety of cell types can act as permissive hosts for microsporidian replication, the role of primary human macrophages in these infections is not well defined.

Studies on infections of murine models with *Encephalitozoon* spp. have described methods of parasitic evasion in macrophages (Weidner, 1975) and the consequent increase of mature spores (Didier and Shadduck, 1994) which suggests that parasite replication is capable in these cells. Furthermore, it was established that host resistance to the parasite could be induced by supplementing the cells with interferon-gamma (IFN-\(\gamma\)) or tumor necrosis factor-alpha (TNF-\(\alpha\)) (Didier and Shadduck, 1994; Khan and Moretto, 1999), which lead to the subsequent release of reactive nitrogen intermediates that contributed to the success of parasitic killing (Didier, 1995).

Recently, studies conducted on *Encephalitozoon* spp. infections of resting human macrophages reported elevated levels of various cytokines needed for pathogenic clearance, i.e., IFN-\(\gamma\), TNF-\(\alpha\), however failed to detect increases in nitric oxide that may lead to microbial killing (Franzen et al., 2005a). Further investigations determining the kinetics of infection in primary human macrophages showed that these infections did not result in the production of mature spores when followed for 12 days (Franzen et al., 2005c). However, in the discussion and subsequent review, it was proposed that parasites could escape from the phagolysosome yielding a productive infection (Franzen, 2005; Franzen et al., 2005c). Taken together, these studies suggest that microsporidians activate resting macrophages to become microbicidal and do not allow for the pathogen to gain an intracellular stronghold. Both human and murine studies of infection agree that pathogenic clearance is mediated through the induction of
cytokines, such as IFN-γ and TNF-α; however, the same studies disagree about the likelihood of *Encephalitozoon* spp. directly activating resting macrophages to invoke the microbicidal response.

Using 3 different methods to identify the adherence and uptake of the spore, the active replicating stages of the vegetative meronts and the formation of PV, the present study uniquely defines the life cycle for *Encephalitozoon* spp. in primary human macrophages. Additionally, we confirm the role for IFN-γ and bacterial lipopolysaccharide (LPS) treatment of resting human macrophages in reducing parasite replication.

**Materials and Methods**

**Cell Culture.** Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors (Our Lady of the Lake Regional Medical Center, Baton Rouge, Louisiana) by gradient centrifugation on lymphocyte separation media (Cambrex, Walkersville, Maryland). Monocyte-derived-macrophages (MDM) were obtained by adherence assays, as previously reported (Fischer et al., 2007). Briefly, monocytes were plated onto 12-well culture plates (1 x 10^6 monocytes/well), containing coverglasses, (Greiner Bio-One, Cellstar, Monroe, North Carolina) and cultured for 3 hr in Dulbecco’s modified Eagle’s medium (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. Cells were stringently washed with PBS to remove non-adhered PBMC and then allowed to differentiate for 7 days in complete media with 10% fetal calf serum (FCS) (Cambrex) at 37 °C in 5% CO₂. Unless stated, all tissue culture media was purchased from VWR (West Chester, Pennsylvania).
Parasites. *Encephalitozoon cuniculi* III and *E. intestinalis* (a generous gift from Elizabeth Didier, Tulane National Primate Research Center, Covington, LA) were grown in a rabbit kidney cell line (ATCC CCL-37) and harvested from tissue culture supernatants. Spores were washed once in PBS containing 0.2% Tween-20, resuspended in supplemented DMEM, and counted with a hemacytometer (Didier et al., 1991). Some spores were inactivated by treatment with 10% bleach solution for 30 min and washed once with water, while others were heat-inactivated in a 95 °C water bath for 30 min. Spores were used at a 5:1 spore-to-MDM ratio (Fischer et al., 2007), and infected cultures were washed free of unadhered spores at 6 hr and maintained at 37 °C in 5% CO₂.

Scanning Electron Microscopy (SEM). SEM was used to monitor immediate adherence of spores to MDM. Cells grown on culture plates were challenged with spores of *E. intestinalis* for 5 min and fixed with 2% glutaraldehyde and 1% formaldehyde in 0.75% sucrose and 0.1 M sodium cacodylate buffer for 1 hr; washed 4 times with 1.5% sucrose and 0.02 M glycine in 0.1 M sodium cacodylate buffer; post-fixed with 2% osmium tetroxide for 1 hr and rinsed with distilled water. Cells were dehydrated with a graded series of ethanol and air dried. Well bottoms were cut from culture dishes and mounted on aluminum specimen holders with conductive tape, coated with gold/palladium (60/40) in an Edwards S-150 sputter coater, and imaged with a Cambridge S-260 SEM.

Adherence, Uptake, and Proliferation Assays. Kinetics of adherence and uptake were measured by enumerating macrophages that were positive for spores and contained a peri-nuclear parasitophorous vacuole (PV) of spores, respectively. In
adherence assays, macrophages were challenged with spores for 1, 3, or 6 hr, washed with PBS and fixed in methanol for 10 min. Cells were stained with a 0.2% solution of SCRI Renaissance Stain 2200 chitin label (Renaissance Chemicals Ltd., North Yorkshire, U.K.) for 20 min at room temperature to allow for visualization of the spores. Coverglasses were mounted in Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, California). MDM with fluorescent blue spores attached were counted.

In uptake assays, spores were pre-labeled with PKH26 Fluorescent Cell Linker, per manufacturer’s instructions, (Sigma, St. Louis, Missouri). MDM were challenged for 3, 6, 12, 24, 48, or 72 hr. MDM challenged for 3 hr were washed of free spores and fixed. All other cultures were washed with PBS at 6 hr post-challenge to remove free spores. Cells were fixed in 10% formalin for 30 min and coverglasses were mounted in Prolong Gold antifade reagent with DAPI to detect nuclear staining of MDM. MDM with peri-nuclear organized vacuoles of red spores were counted.

Microsporidia proliferation and infection were monitored using the bromodeoxyuridine (BrdU) assay system (Invitrogen). MDM were challenged with spores for 24, 48, 72, 120, 168, or 240 hr and fixed in 4% paraformaldehyde. Free spores were washed from cultures with PBS at 6 hr post-infection (PI) and media was replaced. Twenty-four hr prior to fixation, 100 µM of BrdU was added to each well. Cells were washed with 1% Triton X-100 and treated with 1 N HCl for 10 min on ice, 2 N HCl for 10 min at room temperature, and 2 N HCl for 20 min at 37 °C. Cells were neutralized in a 0.1 M borate buffer and washed with 1% Triton X-100. MDM were incubated on ice with a 1/50 dilution of Alexa Fluor 488 anti-BrdU (Invitrogen) in 0.5% bovine serum albumin and 1% Triton X-100 for 90 min. Cells were washed with 1%
Triton X-100 and mounted in Prolong Gold antifade reagent. In some cultures, MDM were stimulated with various concentrations of human recombinant IFN-γ (eBioscience, San Diego, California) or LPS (Sigma) 24 hr prior to spore challenge. MDM containing green fluorescent vacuoles of replicating parasite were counted.

The kinetics of spore production was monitored with Differential Interference Contrast (DIC) microscopy by counting MDM that contained mature parasitophorous vacuoles of spores.

Ten fields of view with an average number of 115 MDM per experiment were counted with a 63x objective in all assays, unless otherwise stated. Cells were viewed using a Leica DMI 6000 B inverted fluorescence microscope, and captured and analyzed with a Leica DFX300 FX CCD camera and Image Pro Plus v5.1 software (MediaCybernetics, Silver Spring, Maryland).

**Statistical Analysis.** Three experiments were performed at each time point and for each parasite or condition, unless otherwise stated. Each experiment consisted of a single donor and was measured in duplicate. Statistical significance was determined by the unpaired, 2-tailed t-test, and values of \( P \leq 0.05 \) were considered significant. All error bars shown in this paper are SEM. Analyses were performed using InStat software (GraphPad 3.0, San Diego, California).

**Results**

*Encephalitozoon spp. Rapidly Adhere to Macrophages and Are Internalized by a Majority of the Cells within the First 24-48 hr.* Spore adherence to macrophages is a rapid process as seen by SEM. MDM exposed to *E. intestinalis* spores for 5 min prior to fixation and mounting demonstrate that minimal time was
needed for some of the cells to acquire several spores on their surface (Fig. 2.1A). Typically, aggregates of spores were seen bound on MDM, which suggests that cells may cluster specific surface molecules needed for microsporidia adherence (Fig. 2.1B). Host membranes were observed intimately associated with the spores.

Figure 2.1  Scanning electron micrographs of spores attached to macrophages. SEM was used to monitor the immediate attachment of spores to MDM. Cells were exposed to *E. intestinalis* spores for 5 min prior to fixation and mounting for SEM. (A) The rapid association between spores and macrophages is observed as clusters of spores attach to the pseudopodia of this macrophage (bar = 5 µm). (B) Spores immediately contact the host cell and are rapidly surrounded by cell membrane (arrow) (bar = 2 µm).

Using fluorescence microscopy, we established that MDM exposure to spores for 6 hr is required to generate greater than 75% of MDM with adhered spores. Cells cultured with *E. cuniculi* or *E. intestinalis* for 1, 3, or 6 hr were washed free of spores, fixed, and stained with a fluorescent chitin label to visualize the spore wall (Fig. 2.2A). Nearly 50% of MDM had at least 1 spore attached after only 1 hr of challenge with either species (Fig. 2.2B). The percentage of MDM with attached spores and the number of spores per macrophage (Fig. 2.2C) continued to increase over time up to 6 hr. At 6 hr, approximately 79% of MDM showed adherence of *E. cuniculi*, while 85% of
Figure 2.2  Kinetics of spore adherence. MDM challenged with spores of *E. cuniculi* or *E. intestinalis* were fixed and treated with DAPI nuclear stain to visualize MDM nucleus (blue, n) and a chitin stain to label endospores (blue, arrows) at various times. DIC and fluorescent images were captured at 630x and merged (A). MDM positive for spores were counted to determine the kinetics of adherence. Both species of parasites showed similar rates of adherence with greater than 75% of MDM staining positive for spores at 6 hr post-challenge (B). The average number of spores per MDM resulted in a greater accumulation of spores by 6 hr for both species (C).
cells were positive for *E. intestinalis*. The average number of spores per macrophage observed at 6 hr was approximately 11 and 17 for *E. cuniculi* and *E. intestinalis*, respectively. However, some MDM did not have any spores attached. This suggests that a population of MDM may be present that does not express a surface molecule needed for immediate attachment. These initial results determined that greater than 75% of MDM would have attached spores after 6 hr of exposure. Therefore, in all performed experiments, non-adhered spores were washed out at this time point, unless otherwise stated.

To monitor the kinetics of microsporidian uptake, MDM were challenged with spores pre-labeled with an amphipathic lipid fluorescent dye that binds plasma membranes and allows one to follow the parasitic membranes during intracellular development. In previous reports that studied kinetics (Couzin et al., 2000; Didier and Shadduck, 1994; Franzen et al., 2005c), parasites have been detected with fluorescent chemicals or antibodies directed against the spore coat, which is subsequently removed or lost during parasite replication. MDM were monitored for peri-nuclear vacuoles containing red labeled spores at various times (Fig. 2.3A). By 6 hr PI (Fig. 2.3B), approximately 27% of MDM contained vacuoles and demonstrated minor increases in uptake at 12 hr and 24 hr. Comparison of uptake data at time points consecutive to each other revealed significant increases in spore uptake occurring at both 48 hr and 72 hr, with 56% and 69% of MDM observed to contain labeled vacuoles, respectively.

**Pronounced Replication of *E. cuniculi* in MDM Begins at 72 hr PI.** MDM infected with *E. cuniculi* spores at various times were observed for microsporidia
Figure 2.3 Kinetics of spore uptake. MDM challenged with pre-labeled *E. cuniculi* spores (red) were observed for peri-nuclear, organized vacuole formation and counted to determine the kinetics of uptake. All cultures were washed free of unattached spores by 6 hr post-challenge, except for 3 hr cultures, which were washed at this time. MDM were fixed at various time points and stained with DAPI, a nuclear label (blue). Images were analyzed at 400x magnification, which allows for determining between MDM with and without spore vacuoles (A). The percentage of MDM containing red vacuoles of spores continuously increased from 3 hr through to 72 hr post-challenge (B), resulting in greater than 50% of MDM with spores by 48 hr and almost 70% by 72 hr.

replication using the BrdU assay. The nucleotide analog (BrdU) was added to infected cultures 24 hr prior to fixation, and stained with an anti-BrdU antibody conjugated to a fluorescent dye, to visualize vacuoles of replicating parasites (Fig. 2.4A). MDM are terminally differentiated cells and therefore, do not incorporate BrdU into the DNA, whereas the actively replicating meront stage of the parasite will utilize this nucleotide. Little replication was observed at 24 hr and 48 hr, while approximately 32% and 34% of MDM were positive for vacuoles with BrdU labeling at 72 hr and 120 hr, respectively.
(Fig. 2.4B). By 168 hr and 240 hr, a majority of the cells contained replicating vacuoles of microsporidians as detected by BrdU labeling.

![Figure 2.4 Replication of microsporidia in human macrophages.](image)

**Figure 2.4 Replication of microsporidia in human macrophages.** *Encephalitozoon cuniculi* kinetics of replication in human macrophages was measured by the incorporation and detection of BrdU. DIC and fluorescent images were visualized at 630x magnification and merged to show peri-nuclear microsporidian replication (A). At 72 hr PI (B), approximately 32% of MDM were observed as containing vacuoles of fluorescent green replicating microsporidians (n=5). DIC microscopy revealed MDM to contain mature vacuoles of newly created spores (C). A significant percentage of MDM were observed to contain spores at 120 hr (D). Arrows indicate parasite vacuoles, n represents host cell nucleus.

The production of mature spores is analogous with the formation of mature PV containing easily identifiable spores by light microscopy. Using DIC microscopy, MDM that contained PV with mature spores were counted at various times (Figs. 2.4C-D). By 120 hr, 15% of MDM contained vacuoles of spores, increasing to 27% and 22% by 168
hr and 240 hr, respectively. It was first noted that a modest amount of MDM appeared to have ruptured and were releasing spores by 168 hr and was also observed at 240 hr. The release of spores and destruction of the MDM could account for an unexpected lower percentage of cells containing mature PV. This also signifies the end of one round of the life cycle.

In control studies, some MDM were infected with chlorine treated or heat-inactivated *E. cuniculi* spores in which approximately 1% of MDM stained positive for chlorine treated spores and 1.6% for heat-inactivated spores at 72 hr. These positive results from the chlorine treated and heat-inactivated studies could be attributed to minor emissions of cellular auto-fluorescence. The production of mature PV was not observed in either treatment.

**Replication Kinetics of *Encephalitozoon* spp. Are Similar in MDM.** To determine if both species of microsporidians are able to replicate in MDM and have similar kinetics of infection, MDM were infected with *E. intestinalis* and observed for proliferation using the BrdU assay. Like *E. cuniculi*, *E. intestinalis* was able to replicate and complete sporogony as evaluated by PV formation in human MDM. Approximately 26% of the MDM were positive for replicating parasites at 72 hr and this number increased to 47% of cells by 120 hr. The BrdU incorporation for *E. intestinalis* was statistically significant from that reported for *E. cuniculi* at 120 hrs, however, the percent of cells with *E. intestinalis* PV did not differ at the same time point (Table 2.1).

**MDM Treated with IFN-γ and LPS Decrease Microsporidia Replication.** To determine if treatment of MDM with compounds known to increase intracellular killing
Table 2.1 Comparison of replication kinetics between *Encephalitozoon* spp.

<table>
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<th>% MDM BrdU Positive</th>
<th>% MDM with P.V.</th>
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<tr>
<td></td>
<td>72 hr</td>
<td>120 hr</td>
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<tr>
<td><em>E. cuniculi</em></td>
<td>32.43 ± 3.55</td>
<td>34.03 ± 1.52</td>
</tr>
<tr>
<td><em>E. intestinalis</em></td>
<td>26.17 ± 1.74</td>
<td>47.45 ± 3.03*</td>
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*P < 0.05 in comparison to *E. cuniculi* at 120 hr

could decrease replication events of microsporidians, IFN-γ and LPS were added to MDM cultures at various doses, independently and in combination, 24 hr prior to infection with *E. cuniculi* spores (Fig. 2.5). Cells on coverglasses were stained for BrdU incorporation to determine the percentage of MDM containing replicating microsporidian vacuoles. Treatment of MDM with IFN-γ yielded modest, but not statistically significant decreases of replicating parasites in host cells with concentrations of 10, 100, and 1,000 U/ml. Comparable results were observed when MDM were treated with 10, 100, and 1,000 ng/ml of LPS. In experiments where MDM were treated with either IFN-γ or LPS alone, a noticeable decrease in replication was observed when concentrations of 100 U/ml or 100 ng/ml, respectively, were added to cultures. Therefore, some MDM cultures were pre-treated with a combination of the compounds at these specific concentrations. This resulted in nearly a 44% decrease in MDM that contained replicating microsporidians in comparison to *E. cuniculi* infected MDM cells not exposed to IFN-γ and LPS, which confirms that these pro-inflammatory mediators help induce a resistant environment with subsequent reduction in parasitic proliferation.
Figure 2.5 Reduction of *E. cuniculi* replication. IFN-γ and LPS activation of MDM decreased the percentage of MDM with replicating *E. cuniculi*. MDM were stimulated with IFN-γ and LPS for 24 hr prior to infection with spores of *E. cuniculi*. Modest decreases in replication were observed when MDM were treated with IFN-γ or LPS independently; however, a significant decrease was seen in MDM treated with both IFN-γ (100U/ml) and LPS (100ng/ml).

**Discussion**

Macrophages have been described as essential players for parasitic clearance in many model systems and for various pathogens (James, 1995). Resting macrophages require signals from Th1 cells, such as IFN-γ and CD40L, which result in their activation and increased intracellular microbicidal effects (Janeway et al., 2005). Cases of microsporidiosis in HIV/AIDS patients and organ recipients receiving immunosuppressive therapies, who have significantly lower numbers of T cells, report that full clearance of the pathogen is difficult, and in some cases impossible, leading to dissemination and death (Salat et al., 2001; Schottelius and da Costa, 2000; Soule et al., 1997). In our system, *Encephalitozoon* spp. could successfully exploit the host surface molecules and intracellular environment of human macrophages in the absence...
of Th1 activation signals, and achieve a productive infection resulting in the accumulation of mature spores.

To address critical questions about infection dynamics within a larger population and to establish a more defined time between spore attachment and parasitophorous vacuole formation, this study has used different approaches to better define the infectious process in primary human macrophages. Prior methods to detect the spore coat with UV brighteners or antibodies are problematic in that the sporoplasm will eventually dissociate from the spore coat to undergo merogony. Because the meront stage has only previously been shown using TEM, limited information about the total population dynamics could be addressed. This study, using a variety of methods, specifically addresses the need to determine adherence, uptake, replication, and spore formation in primary human macrophage, as no clear kinetics/methods have been established for following the infectious process, which is critical for looking at immune parameters related to entry versus replication.

Our results indicate that spore adherence to the host cell is a rapid process, as adherence could be detected by SEM within 5 min after exposure of MDM to spores (Fig. 2.1) and three-quarters or more of MDM were adorned with numerous microsporidia by 6 hr post-challenge (Fig. 2.2). This work is in agreement with several other research groups (Couzin et al., 2000; Franzen et al., 2005b; Leitch et al., 2005), that have reported that spore uptake is a rapid, actin-dependent process. Additionally, we observed a subset of macrophages that did not contain any spores on their surface, which may represent a population of cells that do not express a surface molecule required for spore adherence. Recently, Hayman et al. (2005), has shown that spore
adherence can be mediated by the expression of sulfated glycosaminoglycans, specifically, heparan sulfate, which has been established as an abundant component of the activated human MDM cell surface (Clasper et al., 1999). Based on these reports, the disparity in number of spores we observed in association with MDM may reflect the activation status of the cells.

In experiments with pre-labeled spores, we looked to define the amount of MDM present that contained organized vacuoles of microsporidia and were peri-nuclear in the host cell at various times (Fig. 2.3). Similar to Franzen et al. (2005c), greater than 30% of MDM were observed to contain these vacuoles at 24 hr PI. In contrast to Franzen et al. (2005c) results, which show that the number of MDM internalized spores decreases over the next 48 hr and that new developing parasites were not observed for the remainder of a 12-day infection, we report that 50% of MDM contain vacuoles of the parasite between 24-48 hr and nearly 70% at 72 hr. This discrepancy could be due to our differences in staining techniques used to follow the parasite’s uptake and intracellular fate. Our staining technique utilizes an amphipathic dye that incorporates into cell membranes, thereby allowing us to follow the sporoplasm of the parasite, not the spore coat, which must be removed prior to replication.

Furthermore, proliferation in MDM was confirmed by BrdU incorporation as early as 72 hr PI, while ruptured and broken cells were observed at 168 hr PI (Fig. 2.4). This indicates that these microsporidians are capable of completing their life cycle within MDM as early as 5 days post-infection and commit to successive rounds of re-invasion. Differences in kinetics between Encephalitozoon spp. were not detected and, therefore, demonstrate that both species have similar infection kinetics (Table 2.1). Knowledge of
the kinetics of replication can be used to identify differences in host cell gene expression between initial, pre-proliferative events and those that occur during replication, as well as for studies of meront genomics. This information could lead to a better understanding of what genes and proteins are being expressed or suppressed to produce a permissive environment and foster parasite development.

Treatment of macrophage with extracellular signals, such as IFN-\(\gamma\) and LPS, has been shown to prevent various intracellular pathogens from proliferating and to promote parasitic clearance. These signals sensitize and activate the cells to release reactive oxygen and nitrogen species into membrane bound vesicles containing the parasite and promote fusion of degradative lysosomes (Janeway et al., 2005). Our studies suggest that an extracellular signal must be present in order to reduce parasitic replication (Fig. 2.5), as IFN-\(\gamma\) production was not detected upon spore exposure to resting MDM (data not shown). Cultures pretreated with IFN-\(\gamma\) or LPS showed moderate decreases in parasitic replication, whereas, cultures treated with a combination of the 2 produced a significant decrease in MDM with replicating parasites; approximately a 44% decrease was observed. Although a decrease in parasitic burden was observed, none of the treatments completely inhibited replication. Similar results were reported by Didier and Shadduck (1994), when activating a murine macrophage cell line with either IFN-\(\gamma\) or LPS decreased the number of intracellular \textit{E. cuniculi} spores over time in a dose-dependent response. Additionally, a combination of the 2 signals had an even greater decrease in the number of spores observed, but did not result in complete clearance of the spores (Didier and Shadduck, 1994). In another study, SCID mice infected with \textit{E. cuniculi} were reported to have 90% of their macrophages infected with spores by day
14, whereas spores were not observed in macrophages of wildtype mice (Salat et al., 2001). Taken together, these reports indicate a necessity for a diverse immune response, possibly including CD8$^+$ and $\gamma\delta$ T cells (Khan et al., 1999; Moretto et al., 2001), for a full recovery.

The intracellular fate of microsporidia has been the topic of debate in the literature (Franzen, 2004; Weidner, 1975). Our studies clearly indicate that *Encephalitozoon* spp. can avoid destruction by naïve macrophages and replicate within these permissive cells resulting in the formation of mature spores. We have recently reported that infected macrophage can induce a functional chemokine gradient leading to the recruitment of additional macrophages (Fischer et al., 2007), which correlate to the onset of parasitic replication. Taken together, these studies suggest that microsporidia have a means of utilizing phagocytes responding to sites of infection as reservoirs for proliferation and as a media for the continuous growth and dissemination of the infection.

**References**


CHAPTER 3

INDUCTION OF HOST CHEMOTACTIC RESPONSE BY ENCEPHALITOZOOON SPP.*

Introduction

Microsporidiosis is a disease that is caused by an obligate intracellular, eukaryotic parasite and has gained recognition as an opportunistic infection in AIDS patients, commonly causing chronic diarrhea leading to malabsorption of nutrients and wasting (Kotler and Orenstein, 1994; Orenstein et al., 1990). The true extent of microsporidiosis is difficult to determine, because it is often under-reported due to a lack of proper facilities to diagnose this disease (Grant and De Cock, 2001). Epidemiological reports indicate that anywhere from 5 to 50% (Khan et al., 2001) of patients with chronic diarrhea are positive for microsporidia, most notably Enterocytozoon bieneusi and Encephalitozoon spp. (Didier et al., 2004). Based upon the projections that in the course of HIV/AIDS about 93% of individuals have at least one bout of chronic diarrhea (Gazzard, 1988), these pathogens could conservatively account for chronic diarrhea in approximately 1.3 to 13.5 million people suffering from a life-threatening loss of nutrients and liquids in the Sub-Saharan region alone (The number of cases of microsporidiosis was estimated using previously published reports of the incidence [5 to 50%] of infection in individuals who have chronic diarrhea and are HIV positive. An estimated 93% of AIDS patients develop chronic diarrhea in regions, such as sub-Saharan Africa, where water treatment and highly active antiretroviral therapy are

limited. According to the 2005 UNAIDS/WHO AIDS report, 29 million individuals are infected with HIV in this region.

More recently, cases of microsporidiosis in patients having received immunosuppressive therapies (Carlson et al., 2004; Goetz et al., 2001; Guerard et al., 1999) or in immunocompetent individuals (Muller et al., 2001) having presented with symptoms of chronic diarrhea have been published. Although the most common symptoms of the disease are enteric, there are accumulating reports of more severe disseminated disease, such as keratoconjunctivitis, sinusitis, tracheobronchitis, encephalitis, interstitial nephritis, hepatitis, cholecystitis, osteomyelitis, and myositis (Didier et al., 2004; Kotler and Heymsfield, 1998).

Microsporidia are ubiquitous in nature and known to infect a variety of vertebrate and invertebrate organisms (Didier, 2005; Didier et al., 2000; Snowden et al., 1999; Visvesvara, 2002). Once classified as protozoa, new evidence based on phylogenetic analysis suggests that these organisms are more closely related to fungi (Didier, 2005; Gill and Fast, 2006; Thomarat et al., 2004). Of the more than 1200 species of microsporidia, only 14 have been reported to infect humans (Didier, 2005). Microsporidia infections are believed to occur when a spore is ingested from contaminated water or food (Didier, 2005). Infections of epithelial and endothelial cells and macrophages are common (Khan et al., 2001). Classically, cellular invasion occurs when a spore encounters a host cell and everts a polar tube, thereby penetrating the cell membrane of the host cell and injecting the sporoplasm. Alternatively, spores can be internalized through phagocytosis or endocytosis (Franzen, 2004). Further proliferation and spore production occur through merogony and sporogony within a
parasitophorous vacuole, followed by lysis of the host cell and release of mature spores (Franzen et al., 2005).

While most studies of microsporidia have focused on their genome and life cycle (Didier et al., 2004), limited data exist concerning the host response to these opportunistic pathogens and especially their role in human macrophage infection and disseminated disease (Franzen, 2004; Orenstein, 2003). Some reports indicate that macrophages/monocytes are the source of the disseminated pathogen (Orenstein et al., 1997). In individuals with multifocal organ involvement, infiltrates of infected macrophages are evident in lesions, microabscesses, and granulomas (Shadduck and Orenstein, 1993). In animal models, injection of infectious spores results in a peritoneal infiltrate with predominately monocytes/macrophages, followed by disappearance of these cells, presumably spreading to the lymph nodes and into other tissues (Niederkorn et al., 1981; Salat et al., 2001). Determining which chemokines are present is critical in the development of therapeutics that prevent the dissemination of the pathogen and consequent disease. Based upon these reports, we have investigated the infections of two species of microsporidia that are known to cause disseminated diseases, *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis*, and defined the subsequent production of a chemotactic gradient that is induced by an infection with these intracellular parasites. This work provides the first description of the innate immune response in disseminating infections regarding chemokines and provides a foundation for describing the initial host reaction to these pathogens by human macrophages.
Materials and Methods

Reagents and Antibodies. Unless stated otherwise, all tissue culture media and plasticware were purchased from VWR (West Chester, PA), all neutralizing antibodies and protein array kits from R&D systems (Minneapolis, MN), all ELISA reagents from Biosource (Invitrogen, Carlsbad, CA), and all gene microarray materials from SuperArray Bioscience Corp (Frederick, MD).

Cell Culture. Peripheral blood mononuclear cells (MNC) were isolated from buffy coats from healthy donors (Our Lady of the Lake Regional Medical Center, Baton Rouge, LA) by gradient centrifugation on lymphocyte separation media (Cambrex). Monocyte-derived-macrophages (MDM) were obtained by adherence assays. Briefly, monocytes were plated into 75 cm² flasks (1 x 10⁷ monocytes), 6-well culture plates containing coverglass, or 24-well (1 x 10⁶ monocytes/ml) culture dishes (Greiner Bio-One, Cellstar) for 3 hours in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 µg/ml gentamicin, and 0.25 µg/ml fungizone. Cells were stringently washed with PBS to remove non-adhered MNC and then allowed to differentiate for seven days in complete media with 10% FCS. Monocytes used as target cells for recruitment in chemotaxis assays were isolated from mononuclear cells by magnetic bead separation using Dynal Monocyte Negative Isolation Kit (Invitrogen) and resuspended in complete media with 10% FCS.

Microsporidians. *E. cuniculi* III and *E. intestinalis* (a generous gift from Elizabeth Didier, Tulane National Primate Research Center, Covington, LA) were grown in a rabbit kidney cell line (ATCC CCL-37) and harvested from tissue culture supernatants. Spores were washed once in PBS containing 0.2% Tween-20,
resuspended in supplemented DMEM, and counted with a hemacytometer (Didier et al., 1991). Some spores were inactivated by treatment with 10% bleach solution for 30 minutes and washed 1x with water. All spores were used at a 5:1 spore-to-MDM infection ratio (5x10^6 spores/1x10^6 macrophages), unless otherwise stated.

**Microscopy.** MDM, adhered to coverglass, were monitored for spore uptake by challenging them with either *E. cuniculi* or *E. intestinalis* spores labeled with PKH26 Fluorescent Cell Linker, per manufacturer’s instructions, (Sigma, St. Louis, MO) for various times at 37 °C in 5% CO₂. Cells were fixed in 10% formalin (Sigma) and labeled with PKH67 Fluorescent Cell Linker (Sigma), general cell membrane label. Coverglasses were mounted in Prolong Gold antifade reagent with DAPI (Invitrogen) to detect nuclear staining of MDM.

Microsporidia proliferation and infection were monitored using the Bromodeoxyuridine (BrdU) assay system (Invitrogen). MDM were challenged with spores for 3 days and fixed with 4% paraformaldehyde. 24 hr prior to fixation, 100 μM of BrdU was added to each well. Cells were washed with 1% Triton X-100 and treated with 1N HCl for 10 min. on ice, 2N HCl for 10 min. at RT, and 2N HCl for 20 min. at 37 °C. Cells were neutralized in a 0.1M borate buffer and washed with 1% Triton X-100. Coverslips were incubated on ice with a 1/50 dilution of anti-BrdU (Invitrogen) in 0.5% BSA and 1% Triton X-100 for 90 min. Coverslips were washed with 1% Triton X-100 and mounted in Prolong Gold antifade reagent (Invitrogen).

Cells were viewed using a Leica DMI 6000 B inverted fluorescence microscope, and captured and analyzed with a Leica DFX300 FX CCD camera and Image Pro Plus v5.1 software (MediaCybernetics, Silver Spring, MD).
**Chemotaxis Assay.** Co-culture chemotaxis assays were performed as previously described (Hale-Donze et al., 2002). Briefly, 7-day MDM were infected with *E. cuniculi* or *E. intestinalis* for 24 hr at 37 °C in 5% CO₂. Sterile 3-µm polycarbonate transwell inserts (Corning Costar) were placed in 24-well plates that contained infected MDM. Target monocytes were labeled with red fluorescent cell linker (Sigma) following manufacturer’s instructions and added to the top of the transwell inserts (1 x 10⁶) and co-cultured with infected MDM for 24 hr at 37 °C in 5% CO₂. After co-culture, supernatants were removed and cells were fixed with 10% formalin. In some of the cultures, supernatants were removed and collected after 24 hr infection and replaced with complete DMEM with 10% FCS containing one of the following neutralization antibodies: anti-CCL2 (0.9 µg/ml), -CCL3 (0.5 µg/ml), -CCL4 (0.3 µg/ml), or mouse IgG₁/IgG₂b (1.0 µg/ml). Migration of fluorescently labeled target cells was viewed and quantified using an inverted fluorescence microscope (Leica DMI 6000 B) and Image Pro Plus v5.1 software (Mediacybernetics). Each experiment was completed in duplicate and ten fields of view were counted for each condition. Ten donors were analyzed for infection with *E. cuniculi* and three donors with *E. intestinalis*. Fold change in monocyte recruitment was calculated by dividing the number of cells migrating in the experimental condition by the number of cells in the untreated wells.

**Focused Microarray.** All focused microarray analyses were performed per manufacturer’s instructions (R&D systems). Total RNA was isolated from 6 hr *E. cuniculi* infected MDM grown in 75 cm² flasks using a Qiagen RNeasy Mini Kit (Valencia, CA), quantified with a NanoDrop ND-1000 Spectrophotometer, and assessed for quality with an Experion Automated Electrophoresis System (Bio-Rad). Total RNA
was amplified and labeled with Biotin-16-UTP (Roche Applied Science) using the TrueLabeling-AMP 2.0 Kit (SuperArray). Amplified cRNA was, again, quantified and equally pooled in groups of three donors. Pools of oligo cRNAs were hybridized to the Chemokines and Receptors Oligo GEArray (OHS-022) overnight in a hybridization oven at 60 °C. Expressed genes were detected using a CDP-Star chemiluminescence kit (SuperArray) and imaged using the Bio-Rad Gel Doc 2000 system and Quantity One software (Bio-Rad). Analysis of the data was performed using GEArray Expression Analysis Suite (SuperArray). All data were normalized to the housekeeping genes GAPDH and β-actin, and background corrected to blank values. Fold changes in gene expression are represented as the mean of two experiments.

**Focused Protein Array.** All focused protein arrays were performed per manufacturer’s instructions. Supernatants from overnight infections with *E. cuniculi* were equally grouped in pools of six donors and incubated overnight to the Human Cytokine Array Panel A (R&D systems). HRP substrate (Bio-Rad) was used to detect protein expression and captured by exposure to Kodak BioMax Light film. Arrays were scanned into a computer and optical density measurements taken with Image Pro Plus v 5.1 software (Media Cybernetics). Data is represented as the mean fold change of two experiments.

**ELISA.** Supernatants were collected from MDM cultures at specified time intervals, post-infection with *E. cuniculi* or *E. intestinalis*, and analyzed for CCL2, CCL3, and CCL4 chemokine production by ELISA. Samples were assayed in duplicate.
Statistical Analysis. Student’s paired, two-tailed t test was used. Values of p ≤ 0.05 were considered significant. All error bars shown in this paper are SEM. Analyses were performed using InStat software (GraphPad).

Results

Infection of Primary Human Macrophages with *Encephalitozoon* spp. Recruits Monocytes *in vitro*. To evaluate the host response generated to the initial spore entry, studies were conducted to determine the proper kinetics for obtaining cultures of primary macrophages in which the majority of cells contained visible vacuoles of microsporidia. MDM grown on coverglass were challenged with labeled spores (red) and fixed in 10% buffered formalin at various times. Host cells were labeled with a green membrane stain and a blue nuclear stain. Using fluorescence microscopy, cells positive for spores were counted in 10 consecutive fields. In agreement with the kinetics previously reported (Franzen et al., 2005), it was established that more than 50% of cells contained peri-nuclear vacuoles of spores between 24 and 48 hr post-infection (Fig. 3.1A). Distinct vacuoles containing labeled spores could be readily observed by 48 hr post-infection (Fig. 3.1B). To determine if spore uptake resulted in microsporidia proliferation and productive infection, a BrdU assay system was performed. Since MDM are terminally differentiated, only replicating meronts would incorporate the BrdU nucleotide. At 72 hr post-infection, approximately 27% of cells contained a peri-nuclear vacuole that stained positive for BrdU (Fig. 3.1C).

To determine whether an infection of macrophages by *Encephalitozoon* spp. induces a recruitment of monocytes, a co-culture chemotaxis system, which was
Figure 3.1 *Encephalitozoon* spp. uptake and replication in macrophage. MDM were challenged with labeled *E. cuniculi* spores (red) and the number of cells containing vacuoles of spores were counted in 10 fields over several time points (A). By 48 hr post-challenge a majority of MDM are positive for parasitophorous vacuoles. (B) Arrows indicate the location of labeled spores contained in vacuoles that are peri-nuclear (blue) (n=3). (C) By 72 hr post-infection, approximately 27% of MDM are positive for vacuoles containing replicating microsporidia as seen by the incorporation of BrdU (green) (n=3). Replication was not seen in MDM challenged with chlorine treated spores.
previously described (Hale-Donze et al., 2002), was used to detect a functional chemotactic gradient. Initial experiments were conducted to determine the ratio of spores to MDM (2:1, 5:1, 10:1) needed to provide reproducible infection rates and immune responses. An infection ratio of 5:1 gave consistent responses among all donors and was used as the infection ratio in all experiments presented (Fig. 3.2A). The fluorescent-labeled target cells in the transwell system allowed the times of optimal cell migration to be monitored. MDM were infected for 24 hr prior to performing the analysis. At the end of 24 hr, the supernatants containing any free spores were removed and new media were added. Monocytes were labeled and placed into the upper chamber of a co-culture plate. The cells were analyzed over the course of an additional 24 hr for prime chemotaxis response. In initial experiments, the co-culture incubations were monitored at 30 minute intervals for up to 2 hr, and subsequently by 1 hr increments up to 8 hr, and at 24 hr (data not shown). The 24 hr incubation yielded the optimal response. Infection of macrophages with either *E. cuniculi* or *E. intestinalis* induced a 2.9-fold increase in monocyte migration after a total of 48 hr post-infection (Fig. 3.2B). To ensure that the observed monocyte migration was induced by a MDM-generated gradient, only *E. cuniculi* spores were added to some wells. Spores by themselves, do not induce migration (Fig. 3.2B).

**Focused Gene Array Reveals Upregulation of Chemokines and Receptors by *E. cuniculi* Infection.** To establish which chemokines and receptors may be responsible for initiating the monocytic infiltration, a limited, pathway-focused gene array for chemokine signaling was performed to screen for possible candidates. Total RNA was isolated from MDM in medium alone or infected with *E. cuniculi* for 6 hr, converted
to biotinylated oligo cRNA and amplified. The amplified cRNA was hybridized overnight and detected using a chemiluminescent substrate.

Figure 3.2 Encephalitozoon spp. infection of human macrophages induce monocyte migration. MDM were infected with E. cuniculi spores for 24 hr at a spore-MDM ratio of 2:1, 5:1, and 10:1 to determine optimal spore infection ratios for monocyte recruitment (A). At 24 hr, labeled monocytes were added to transwell inserts and co-cultured with infected MDM for an additional 24 hr. A ratio of 5:1 spore-MDM resulted in consistent infection rates with human donors (A). In co-culture chemotaxis assays, infection of MDM with either E. cuniculi or E. intestinalis resulted in a 2.9-fold recruitment of naïve monocytes (B). There were no significant differences in chemotactic response among Encephalitozoon spp. Wells that contained only spores and no MDM did not induce a chemotactic response. * p ≤ 0.05.

Stimulation with E. cuniculi spores induced a 2-fold or greater increase in 40 out of 128 genes. Included among the 40 genes that were augmented were several key chemokines and chemokine receptors, presented in Table 3.1. E. cuniculi infection of MDM elicited expression of several important chemokines which are capable of recruiting the granulocytes [CXCL1, CXCL2, CXCL3, CXCL5, CXCL8], providing supporting evidence for other cell types present at sites of infection (Orenstein, 1999; Shadduck and Orenstein, 1993). However, the vast majority of genes which were augmented by microsporidia infection were shown to recruit monocytes, including
CCL1, CCL2, CCL3, CCL4, CCL4L1, CCL5, and CCL7. It is important to note that these chemokines can also act upon lymphocytes such as \( \text{T}\,1 \) cells, and dendritic cells to influence their migration. Additionally, increases in two vital monocyte receptors, CCR1 and CCR5, were also observed.

Table 3.1 Microsporidia infection increases chemokine and receptor gene expression.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Common name</th>
<th>Refseq no.</th>
<th>Avg. Fold ∆a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C-C Chemokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccl1</td>
<td>I-309</td>
<td>NM_002981</td>
<td>11.2</td>
</tr>
<tr>
<td>Ccl2</td>
<td>MCP-1</td>
<td>NM_002982</td>
<td>2.3</td>
</tr>
<tr>
<td>Ccl3</td>
<td>MIP-1α</td>
<td>NM_002983</td>
<td>2.3</td>
</tr>
<tr>
<td>Ccl4</td>
<td>MIP-1β</td>
<td>NM_002984</td>
<td>12.3</td>
</tr>
<tr>
<td>Ccl4l1</td>
<td>LAG-1</td>
<td>NM_207007</td>
<td>22.7</td>
</tr>
<tr>
<td>Ccl5</td>
<td>RANTES</td>
<td>NM_002985</td>
<td>25.6</td>
</tr>
<tr>
<td>Ccl7</td>
<td>MCP-3</td>
<td>NM_006273</td>
<td>2.9</td>
</tr>
<tr>
<td>Ccl15</td>
<td>MIP-1δ</td>
<td>NM_004167</td>
<td>8.4</td>
</tr>
<tr>
<td>Ccl20</td>
<td>MIP-3α</td>
<td>NM_004591</td>
<td>6.2</td>
</tr>
<tr>
<td><strong>C-X-C Chemokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cxcl1</td>
<td>GROα</td>
<td>NM_001511</td>
<td>20.7</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>GROβ</td>
<td>NM_002089</td>
<td>21.4</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>GROγ</td>
<td>NM_002090</td>
<td>5.2</td>
</tr>
<tr>
<td>Cxcl5</td>
<td>ENA-78</td>
<td>NM_002994</td>
<td>5.7</td>
</tr>
<tr>
<td>Cxcl8</td>
<td>IL-8</td>
<td>NM_000584</td>
<td>9.9</td>
</tr>
<tr>
<td>Cxcl16</td>
<td></td>
<td>NM_022059</td>
<td>6.4</td>
</tr>
<tr>
<td><strong>C-C Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccr1</td>
<td></td>
<td>NM_001295</td>
<td>2.9</td>
</tr>
<tr>
<td>Ccr5</td>
<td></td>
<td>NM_000579</td>
<td>4.5</td>
</tr>
<tr>
<td>Ccr7</td>
<td></td>
<td>NM_001838</td>
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<tr>
<td>Ccr12</td>
<td></td>
<td>NM_003965</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>C-X-C Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cxcr4</td>
<td></td>
<td>NM_003467</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*aAverage fold increases in MDM gene expression were determined at 6 hr post-infection with \( E.\, cuniculi \). Each array experiment represents cRNA pooled from three donors (n=2).

Proteomic Profiling Confirms Increased Levels of Chemokines After \( E.\, cuniculi \) Infection. To verify the results obtained with the DNA arrays, a focused protein array, which includes several, but not all, of the chemokines of interest was performed. Supernatants from MDM cultures infected for 6 hr with \( E.\, cuniculi \) were pooled in equal amounts from six donors and incubated overnight with the Human
Cytokine Array Panel A. Detection using chemiluminescence showed significant increases (≥ 2-fold) in only a few of the proteins being screened for by the limited array (Table 3.2). Both CCL3 and CCL4, monocyte chemoattractants, had greater than a 15.0 fold increase, and CCL5, a potent chemokine for lymphocytes, had approximately a 25.0 fold increase. Moderate increases (≥ 1.5-fold) were seen in CCL1, CCL2, and CXCL1. The modest difference seen in CXCL1 can be attributed to high levels in both the control and treated arrays, as was also true for the involvement of CXCL8.

**Table 3.2 Proteome Profiler Array**

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Common name</th>
<th>Avg fold expression in expression</th>
<th>Protein array</th>
<th>Gene array</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL1</td>
<td>I-309</td>
<td>1.6</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>Monocyte chemoattractant protein 1</td>
<td>1.9</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>Macrophage inflammatory protein 1α</td>
<td>16.4</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>Macrophage inflammatory protein 1β</td>
<td>23.3</td>
<td>35.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>25.2</td>
<td>25.6</td>
<td></td>
</tr>
<tr>
<td>CXCL1</td>
<td>Growth-regulated oncogene α</td>
<td>1.5</td>
<td>20.7</td>
<td></td>
</tr>
<tr>
<td>CXCL8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Interleukin-8</td>
<td>1.1</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>CXCL10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Interferon-inducible protein 10</td>
<td>0.9</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>CXCL11&lt;sup&gt;d&lt;/sup&gt;</td>
<td>I-TAC</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>CXCL12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Stromal cell-derived factor 1</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>G-CSF&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Colony-stimulating factor 3</td>
<td>1.0</td>
<td>6.9</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Average fold change calculated by dividing the mean optical densities of infected MDM by uninfected MDM (n=2).

<sup>b</sup> Average fold changes of gene expression from Table 1.

<sup>c</sup> Equivalent levels of protein was detected in both experimental conditions.

<sup>d</sup> Protein was not detected in either condition.

<sup>e</sup> Sum of the avg. fold increase of both CCL4 and CCL4L1 gene expression. These two protein products are represented as MIP-1β on the protein array.
Sustained Chemotactic Levels Differ Between *Encephalitozoon* spp.

Because microsporidia dissemination is thought to be associated with monocytic infiltrates, the data presented focuses on those chemokines that primarily recruit monocytes, namely CCL2, CCL3, and CCL4. To establish the time at which these selected chemokines are elevated in *Encephalitozoon* infections, kinetic studies at 1, 3, 6, 12, 24, 48 and 72 hr were conducted and protein levels measured by ELISA (Fig. 3.3). Significant differences in all three chemokine levels were detected at 6 hr post-infection for both *E. cuniculi* and *E. intestinalis*. Infections with *E. cuniculi* resulted in peak levels of CCL3 and CCL4 at 24 and 12 hr, respectively, followed by a decline. These levels appeared to undulate, but remained moderately high after their initial peak. CCL2 production continued to increase until 48 hr and then appeared to remain at this elevation. Similarly, *E. intestinalis* resulted in peak levels of CCL3 and CCL4 at 24 hr, followed by a decrease. The amount of CCL2 that was produced in response to *E. intestinalis* peaked by 12 hr and remained elevated up to 72 hr. Levels of all chemokines produced were similar among the *Encephalitozoon* spp. through the kinetics of infection.

Inhibiting CCL4 Significantly Reduces Cells Responding to *Encephalitozoon* Infection. To evaluate whether any or all of the chemokines evaluated by ELISA contributed significantly to the infiltration of monocytes in the co-culture system, MDM were infected for 24 hr with *E. cuniculi* or *E. intestinalis*. The spent media were removed and fresh complete media containing antibodies against CCL2, CCL3, CCL4, a combination of the three antibodies, or isotype match control
were added prior to introducing the responding monocytes to the co-culture chemotaxis system.

Figure 3.3 Levels of CCL2, CCL3, and CCL4 are elevated over time. ELISA confirmed the results of the gene array and protein array for CCL2, CCL3, and CCL4. Supernatants were collected from co-cultures of MDM and *E. cuniculi* or *E. intestinalis* at multiple time points during infection. Both *Encephalitozoon* spp. induce a strong chemotactic profile at approximately 24 hr of infection. Thereafter, both CCL3 (n=6) and CCL4 (n=6) begin to decline in expression, whereas CCL2 (n=5) levels remain present after 24 hours. * P<0.05.
In assays containing *E. cuniculi*, neutralizing antibodies against CCL2 or CCL3 yielded a modest but significant decline in recruited monocytes by 25% and 31%, respectively. In contrast, the inhibition of CCL4 markedly decreased the number of migrating cells by 49% (Fig. 3.4A). Similar results were obtained in cultures infected with *E. intestinalis* (Fig. 3.4B), in which, CCL2, CCL3, and CCL4 decreased migration by 28%, 36%, and 50%, respectively. Furthermore, *E. cuniculi* cultures inhibited with combinations of two antibodies together resulted in a modest decrease with CCL2-CCL3 (38%) or CCL2-CCL4 (45%) in infiltrating monocytes, while a CCL3-CCL4 grouping further reduced recruitment by half. Cultures containing a mixture of all three antibodies reduced the population of migrating cells by 58%.

**Figure 3.4 Neutralization of chemokines results in decreased monocyte recruitment.** Neutralizing CCL2 or CCL3 shows small but significant decreases in monocyte recruitment, whereas blocking CCL4 results in a more than 50% decline in monocytic infiltration. Significant differences in cellular recruitment due to infection with either *E. cuniculi* (n=10) or *E. intestinalis* (n=3) were not observed. * P<0.05.

**Discussion**

Limited data exist about the innate immune response against microsporidia. Increasingly, reports of microsporidian infections not only in immunocompromised but also in immunocompetent individuals are published, indicating the incidence of infection
may be more common than typically reported (Boldorini et al., 1998; Carlson et al., 2004; Goetz et al., 2001; Guerard et al., 1999; Muller et al., 2001). Histological studies of microsporidiosis show that infiltrates may often be composed of several cell types, including monocytes, granulocytes and lymphocytes (Boldorini et al., 1998; Didier, 1998; Orenstein, 1991; Weber et al., 1994), however, infected macrophages have been observed in disseminated disease (Orenstein, 2003) and are thought to be the vehicle for multi-organ infections.

The production of chemokines to recruit various immune cells during parasitic infections is important in innate immunity (Laing and Secombes, 2004). Our data indicate that MDM infected with *Encephalitozoon spp.* induce a functional chemokine gradient that supports the recruitment of monocytes to sites of infection. In these studies, *E. cuniculi* and *E. intestinalis* infected primary human macrophages induced an approximate three-fold increase in migration of naive monocytes after 24 hr incubation, in a chemotaxis system. This delay in migration may reflect the parasitic evasion of the immune response providing time to invade and replicate (Fig. 3.1C).

Focused microarray analyses of MDM infected with *E. cuniculi* revealed increases in expression of several critical chemokines and receptors needed to recruit a variety of effector cells in order to mount a successful immune response. A case study, in a patient with AIDS, described an *Encephalitozoon sp.* infection of kidney epithelium that was surrounded by lymphocytes, plasma cells, and macrophages (Boldorini et al., 1998), suggesting a role for these cells clearing infection. We show several genes involved in neutrophil recruitment, namely the C-X-C chemokines, that were strongly induced, as well as, genes involved in the chemotaxis of dendritic and T<sub>H</sub>1 cells, namely
CCL1 and CCL5 (Laing and Secombes, 2004). In a murine model, Khan et al. reported that a Th1 response was critical against microsporidia infections (Khan and Moretto, 1999), and later, further defined a role for CD8+ and γδ T cells in resolving these infections in mice (Khan et al., 2001; Khan et al., 1999). CCL5 is a potent chemoattractant for memory T cells, IL-2 activated T cells and eosinophils (Laing and Secombes, 2004). Among the genes upregulated that are important in monocyte recruitment (Laing and Secombes, 2004), included CCL2, CCL3, CCL4 and CCL4L1. These four chemokines have been identified as agonists for the key monocyte receptors CCR1 and/or CCR5 (Murdoch and Finn, 2000), both of which showed increased expression in our model of infection. This similar response has also been demonstrated in *Candida albicans* infection of human monocytes, where increased gene expression in CCL2, CCL3, and CCL4 chemokines, and CCR1 and CCR5 receptors has been observed (Kim et al., 2005). Interestingly, as also seen in our model, *C. albicans* induces CCL4 expression many folds greater than either CCL2 or CCL3 at 6 hr post-infection (Kim et al., 2005).

To determine which of these genes give rise to protein products, a focused protein analysis was performed using proteome profiler arrays. Data obtained from the limited array revealed that the chemokines CCL3, CCL4, and CCL5 had greater than a 15-fold increase (Table 3.2). Both, CCL3 and CCL4 are known to be strong inducers of monocytes to areas of infection and are also ligands for CCR5, a HIV co-receptor, whereas CCL5 predominately recruits T cells. In addition, CCL2, which is another potent monocyte chemoattractant, displayed moderate, but significant levels of protein expression.
The secretion kinetics of monocyte chemokines most likely to participate in the spread of infection were further analyzed by ELISA to determine whether their expression coincide with the delayed recruitment observed. Increased levels began around 6 hr and peaked between 12 and 24 hr. These time points corresponded to the observed chemotactic response. It has yet to be elucidated whether or not microsporidians can dampen immediate immune responses during host uptake until they have established the meront stage within the macrophage. Delayed responses to other fungal pathogens including *Aspergillus fumigatus*, have been attributed to altered host response based upon the recognition of conidia verses hyphal forms or with *Candida albicans* yeast and hyphal forms which are thought to contribute to the pathology observed in these opportunistic infections (Roeder et al., 2004).

To determine the individual role of these chemokines in the recruitment of potential new hosts for microsporidians, neutralizing antibodies were employed in the co-culture system. This analysis revealed that CCL2 and CCL3 contributed to the migration of monocytes, but inhibiting CCL4 in the cultures resulted in the most dramatic reduction (Fig. 3.4). The neutralization of all three major chemokines reduced the levels of migrating monocytes near that of uninfected MDM, suggesting that these three chemokines were the chemoattractants responsible for the monocytic infiltration and, therefore, could be potential targets of chemotherapeutic agents in controlling microsporidiosis (Pease and Williams, 2006). In comparison, Huffnagle *et al.* showed in a murine model, that *Cryptococcus neoformans*, a yeast forming fungi known to cause disseminated disease, could envoke a MCP-1 and MIP-1α response. In neutralization studies, the cryptococcal burden increased in the lungs of mice, while a decrease in
macrophage and CD4\(^+\) T cell recruitment was observed, resulting in inhibited clearance of the infection (Huffnagle et al., 1997; Huffnagle et al., 1995).

While the immune response generated against *Encephalitozoon spp.* can result in the recruitment of monocytes, it also has the potential to mediate the adaptive immunity. However, in individuals with impairment of the adaptive arm of the immune system, as seen in AIDS, organ transplant recipients, the young and the very old, these same recruited monocytes could serve to amplify the infection. Understanding how macrophages function in propagating disease and how the life cycle of microsporidia can influence their responses is critical in developing anti-microsporidial compounds or anti-inflammatory interventions. Further works to define the host recognition of the parasite, signaling pathways and subsequent cytokine profiles are under current investigation.

**References**


CHAPTER 4

TLR2 RECOGNITION OF THE MICROSPORIDIA ENCEPHALITOZOOON SPP. INDUCES NUCLEAR TRANSLOCATION OF NF-κB AND SUBSEQUENT INFLAMMATORY RESPONSES

Introduction

Microsporidiosis has been reported as the cause of chronic and life-threatening diseases in HIV/AIDS patients and organ recipients, and therefore, garnered a greater medical importance amongst individuals who have an impaired immune status (Didier, 2005; Orenstein, 2003; Schottelius and da Costa, 2000). Increasingly, infections involving immunocompetent individuals, particularly affecting the elderly or young, have also been reported (Carlson et al., 2004; Didier, 2005; Didier et al., 2004; Leelayoova et al., 2005; Nkinin et al., 2007). Infections are believed to primarily occur through the ingestion of microsporidia spores and proceed through the invasion of enterocytes in the small intestine (Didier, 2005). Clinical reports of microsporidiosis have described a range in severity of the disease, consisting of intestinal infections leading to chronic diarrhea and disseminated diseases thought to occur as a result of migrating macrophages, including keratoconjunctivitis, sinusitis, tracheobronchitis, encephalitis, interstitial nephritis, hepatitis, cholecystitis, osteomyelitis, and myositis (Didier, 2005; Hale-Donze and Didier, 2007; Orenstein, 2003; Schottelius and da Costa, 2000).

Microsporidia comprise a group of more than 1200 species of obligate intracellular pathogens that can be found virtually everywhere in nature and are able to infect a wide variety of vertebrate and invertebrate organisms (Bigliardi and Sacchi, 2001; Didier, 2005; Franzen, 2005; Gill and Fast, 2006). These parasites were once classified as protozoa, however, recent phylogenetic analysis has revealed their close
association with fungi (Didier, 2005; Gill and Fast, 2006). The infective stage of the parasite is an environmentally resistant spore that is believed to infect cells through the eversion of a unique polar filament, which structurally distinguishes microsporidia from other spore-forming organisms (Bigliardi and Sacchi, 2001; Didier, 2005; Franzen, 2005). The polar filament functions similar to a syringe and needle by penetrating the host membrane and forcing the sporoplasm through the tube and into the host cell (Bigliardi and Sacchi, 2001; Didier, 2005; Franzen, 2005; Xu and Weiss, 2005). For macrophages, infection has been reported to occur via phagocytosis of the infectious spore (Fischer et al., 2008). However, the specific mechanism of macrophage recognition of these spores has not been reported.

Host recognition of pathogens is accomplished by a diverse repertoire of germ-line encoded proteins called pattern recognition receptors (PRR). Toll-like receptors (TLR) represent a unique collection of evolutionarily conserved PRR known to recognize pathogen-associated molecular patterns (PAMPs) found on viruses, bacteria, parasites, and fungi. Ten TLR have been identified in humans and are known to recognize molecular moieties, such as peptidoglycan and zymosan by TLR2 or lipopolysaccharide by TLR4 (Lee and Kim, 2007; Takeda and Akira, 2005). Engagement of TLR has been shown to activate a variety of intracellular signaling pathways, including the MyD88-dependent pathway which ultimately activates the transcriptional factor NF-κB (Lee and Kim, 2007; Medzhitov, 2001; Takeda and Akira, 2005). Activation and nuclear translocation of NF-κB is implemented in the transcription of many inflammatory cytokines and chemokines needed for successful clearance of the
pathogen and recruitment of immune cells, including TNF-α and IL-8 (Collart et al., 1990; Kunsch and Rosen, 1993).

Since the discovery of TLR, their recognition of molecular patterns on bacteria and viruses has been well documented. More recently a number of protozoan and fungal organisms that share structural and/or biological similarities with microsporidia have also been linked with TLR activation. *Cryptosporidium parvum*, an oocyst-forming protozoan that typically infects the small intestine, recruits both TLR2 and TLR4 and activates NF-κB signaling in human cholangiocytes (Chen et al., 2005). Genomic analysis of *Encephalitozoon spp.* has predicted that the spore coats may contain glycosylphosphatidylinositol (GPI)-anchors (Peuvel-Fanget et al., 2006; Xu et al., 2006) which recently have also been considered key molecules isolated from *Toxoplasma gondii* and *Trypanosoma cruzi* and shown to signal through TLR2 and TLR4 (Campos et al., 2001; Debierre-Grockiego et al., 2007). Additionally, TLR have been shown to recognize highly conserved fungal cell wall components such as glucans, chitin, and mannoproteins from Candida and Aspergillus organisms, which share some similarities to recently identified microsporidian spore coat molecules (Netea et al., 2006a; Netea et al., 2006b).

To date, there is limited information about the host innate immune response to microsporidia and no information exists about the role of TLR in the recognition of these parasites. However, studies involving microsporidian infection have provided some evidence that suggests an involvement of NF-κB. Analyses of culture supernatants from primary human macrophages challenged with *Encephalitozoon spp.* spores revealed increases in the levels of the cytokine TNF-α (Franzen et al., 2005) and the
chemokines CCL2, CCL3, and CCL4 in response to infection (Fischer et al., 2007). Taken together, these reports of inflammatory mediator production and the previously described biological similarities between microsporidia and pathogens that are known to activate TLR, suggest that microsporidia may also be recognized by and elicit a TLR response.

The present study has explored the role of TLR in macrophage host defense against microsporidian parasites. We report that *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis* are both recognized by TLR2. In addition, parasite challenge leads to the nuclear translocation of NF-κB, which is required for TNF-α and IL-8 production. To our knowledge, this is the first study that demonstrates a role for TLR in the innate immune response to any species of microsporidia.

**Materials and Methods**

**Reagents.** Lymphocyte separation media (LSM), fetal calf serum (FCS), L-glutamine, gentamicin, penicillin, and streptomycin were purchased from Cambrex (Walkersville, Maryland), and Dulbecco’s modified Eagle’s medium (DMEM), phosphate-buffered saline (PBS), and Greiner Bio-One, Cellstar tissue culture plates were from VWR International (West Chester, Pennsylvania). Lipopolysaccharide (LPS) from *E. coli* 0111:B4 was from Sigma Chemical Co. (St. Louis, Missouri) and Pam3CSK4 was from Axxora (San Diego, California). ELISA reagents and antibodies, goat anti-rabbit Alexa Fluor 488, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, California) and anti-NF-κBp65 was from Santa Cruz Biotechnology, Inc., (Santa Cruz, California). NF-κB inhibitor (Bay11-7085), all validated RT-qPCR primers, and RT2 SYBR Green/Fluorescein master mix were from SuperArray (Frederick,
Maryland). Predesigned TLR2 and control oligonucleotides were purchased from Ambion (Foster City, California).

**Cell Culture.** Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors (Our Lady of the Lake Regional Blood Bank, Baton Rouge, Louisiana) by gradient centrifugation on lymphocyte separation media. Monocyte-derived-macrophages (MDM) were obtained by adherence assays, as previously reported (Fischer et al., 2007). Briefly, monocytes were plated onto 6-well (2 x 10^6 cells/well), 24-well (5 x 10^5 cells/well), and 96-well (1 x 10^5 cells/well) culture plates and cultured for 3 hr in supplemented DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1 µg/ml gentamicin at 37 °C in 5% CO₂. Cells were stringently washed with PBS to remove non-adhered PBMC and then allowed to differentiate for 7 days in supplemented DMEM with 10% FCS at 37 °C in 5% CO₂. Some MDM were plated in 24-well culture plates with coverglasses.

**Parasites.** *Encephalitozoon cuniculi III* and *E. intestinalis* (a generous gift from Elizabeth Didier, Tulane National Primate Research Center, Covington, LA) were grown in a rabbit kidney cell line (ATCC CCL-37) in DMEM supplemented with 10% FCS at 37 °C in 5% CO₂, and harvested from tissue culture supernatants. Spores were washed once in PBS containing 0.2% Tween-20, resuspended in supplemented DMEM, and counted with a hemacytometer (Didier et al., 1991). Spores were used at a 5:1 spore-to-MDM ratio (Fischer et al., 2007). Some spores were heat-inactivated in a 95 °C water bath for 30 min.

**TLR Transfected HEK Cell Lines.** HEK cell lines transfected with TLR2, TLR4, TLR4/MD2/CD14, or Null plasmids (Invivogen, San Diego, California) were grown in 96
well culture plates in supplemented DMEM with 10% FCS at 37 °C in 5% CO₂. Confluent cultures were challenged with spores of *E. cuniculi* or *E. intestinalis* and supernatants were collected and analyzed by ELISA for TLR activation via IL-8 production as suggested by the manufacturer. As controls, some cultures were stimulated with the TLR4 or TLR2 agonists LPS (10 ng/ml) or Pam₃CSK₄ (50 ng/ml), respectively.

**Real-time Quantitative PCR (RT-qPCR).** Total RNA was isolated from 1 x 10⁷ *E. cuniculi* or *E. intestinalis* infected MDM grown in 6 well culture plates using the Qiagen RNeasy Mini Kit (Valencia, California) according to the manufacturer’s instructions, quantified with a NanoDrop ND-1000 Spectrophotometer (Wilmington, Delaware), and assessed for quality with an Experion Automated Electrophoresis System (Bio-Rad, Hercules, California). Reverse transcription was performed with SuperScript III First Strand Synthesis Supermix (Invitrogen). RT-qPCR was performed using a BioRad iCycler according to the manufacturer’s instructions. The amplification of TNF-α, TLR2, and TLR4 cDNA was completed using validated primers and RT2 SYBR Green/Fluorescein master mix. All data were normalized to the β-actin housekeeping gene and gene expression was calculated as previously described (Khaleduzzaman et al., 2007). Briefly, the cycle threshold (*C*₇) value (log₂) for β-actin minus the *C*₇ value of the gene of interest for each sample (Δ*C*₇ = *C*₇ β-actin − *C*₇ gene of interest) were calculated and presented as the percentage of β-actin.

**Immunofluorescence Detection of Nuclear NF-κB.** Adherent cells on coverglasses were challenged with spores or LPS (10 ng/ml) for 1 or 6 hr, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were
then incubated with Image FX Signal Enhancer (Invitrogen) for 30 min at RT followed by polyclonal rabbit anti-mouse NFκBp65 (1:50 dilution) overnight at 4 °C. The cells were extensively washed in PBS and then incubated with goat anti-rabbit Alexa Fluor 488 (1:200 dilution) for 60 min at RT, washed again, and mounted in ProLong Gold media (Invitrogen). Cells were examined using a Leica DMI 6000 B inverted fluorescence microscope, and captured and analyzed with a Leica DFX300 FX CCD camera and Image Pro Plus v5.1 software (MediaCybernetics, Silver Spring, Maryland).

**ELISA.** Supernatants were collected from MDM cultures at specified time intervals, post-infection with *E. cuniculi* or *E. intestinalis*, and analyzed for TNF-α, IL-8, CCL3, and CCL4 cytokine/chemokine production as per manufacturer’s instruction. Nuclear extracts were collected and assayed for the translocation of NF-κB following the manufacturer’s protocol. Samples were assayed in duplicate. In some experiments, 20 µm of the NF-κB inhibitor, Bay11-7085, was added to cell cultures 1 hr prior to infection. Colorimetric determination of values was performed on VersaMax microplate reader (Molecular Devices, Sunnyvale, California) and analyzed using SoftMax Pro software (Molecular Devices).

**siRNA.** Three TLR2 siRNA sequences were equally mixed in each transfection and experiments were performed as previously described (Wang et al., 2005). Briefly, MDM were seeded in 96-well plates at 1 x 10⁵ cells per well in serum-free, supplemented DMEM. MDM were transfected with 20 pmol of siRNA and 1 µl of Lipofectamine 2000 per well. Cultures were allowed to incubate with siRNA-Lipofectamine complexes for 4 hr at 37 °C in 5% CO₂, washed, and placed in fresh
supplemented DMEM with 10% FCS for 48 hr prior to performing experiments. siRNA knockdown of the specific gene was confirmed by RT-qPCR.

**Statistical Analysis.** Three experiments were performed at each time point and for each parasite or condition, unless otherwise stated. Each experiment consisted of a single donor and was measured in duplicate. Statistical significance was determined by one-way ANOVA followed by Dunnett’s post test, and values of \( p \leq 0.05 \) were considered significant. All error bars shown in this paper are SEM. Analyses were performed using InStat software (GraphPad 3.0, San Diego, California).

**Results**

**TLR2 Mediates the Recognition of *Encephalitozoon* spp.** To delineate a role for TLRs in the recognition of microsporidian parasites, HEK293 cells stably transfected with plasmids containing null, TLR2, TLR4, or the complex TLR4/MD2/CD14 (confirmed in LPS recognition and signaling), were stimulated with spores from two *Encephalitozoon* spp. HEK293 cells do not express TLR, but do contain the intracellular machinery to provide a response if the receptor was available. When stimulated, these transfected cells provide an IL-8 response (Raffatellu et al., 2005). The HEK293-null cell line did not respond to either *Encephalitozoon* spp., or TLR agonist (Fig. 4.1A) indicating that no native IL-8 responses were generated against the spores. Detection of IL-8 was observed in TLR2 transfected cells challenged with spores from both species and heat-killed *E. cuniculi* spores (Fig. 4.1B). Neither TLR4 alone (Fig. 4.1C) nor the TLR4/MD2/CD14 (Fig. 4.1D) complex generated a signal in response to the challenge with Encephalitozoon spores. As well, heat-killed spores did not generate a strong signal. These data suggests that spores of the *Encephalitozoon*
spp. are recognized by TLR2 which could initiate subsequent inflammatory signaling and production of mediators.

Figure 4.1. *Encephalitozoon* spp. are recognized by TLR2. HEK293 cells transfected with plasmids encoding Null (A), TLR2 (B), TLR4 (C), or TLR4/MD2/CD14 (D) were challenged with spores of *Encephalitozoon* spp., heat-inactivated (∆) *E. cuniculi*, LPS (10ng/ml), or Pam₃CSK₄ (50ng/ml) overnight. Culture supernatants were collected and assessed for IL-8 production by ELISA (n=3). *, p ≤ 0.05.

**Encephalitozoon Stimulation Increased TLR2 Expression in Human Primary Macrophages.** While HEK293 cell lines are widely used to determine TLR responses to pathogens, it is important to establish the role of these receptors in cells which are involved in recognition of Encephalitozoons *in vivo*. Macrophages are not only involved in innate immune responses against microsporidia, but can also serve as vessels for the
dissemination of infection in the immunocompromised host, therefore primary human macrophages were analyzed for altered gene expression of TLR in response to *Encephalitozoon spp*. MDM were incubated with spores for 6 hr and total RNA was collected. Gene expression of TLR2, TLR4, and TNF-α (a known product of parasite challenge) was assessed using RT-qPCR. While the parasite challenge caused the predicted increase in TNF-α expression (Fig. 4.2A), an approximate 2-fold increase in TLR2 mRNA levels was also observed (Fig. 4.2B). Similar induction of TLR2 was observed for both *E. cuniculi* and *E. intestinalis*. Interestingly, TLR4 gene expression was not significantly augmented (Fig. 4.2C). These results along with the TLR2 transfection studies, strongly suggest that the infectious spores can induce responses to increase the host receptor required for their recognition.

**NF-κB Translocation Is Activated in Macrophages Challenged with Spores.**

Little is understood about signaling events in response to microsporidian infections. Since many pro-inflammatory cytokines are at least under partial regulation of the transcription activator NF-κB, our HEK studies and previously published reports of TNF-α would suggest that NF-κB is activated during these infections. We next investigated whether *Encephalitozoon* spores could induce the translocation of NF-κBp65 into the nuclear compartment of primary human macrophages. Nuclear extracts were collected from cells at 1, 3, and 6 hr post-challenge with spores, and measured for total NF-κBp65. Translocation of NF-κBp65 was detected as early as 1 hr post-challenge and continued to decrease at 3 and 6 hr (Fig. 4.3A). To confirm the nuclear localization of NF-κBp65 in macrophages, immunostaining was performed on cells after challenge with
spores from both species. Again, NF-κBp65 was observed in the nucleus optimally at 1 hr (Fig. 4.3B) and continued to decline out to 6 hr.

**Figure 4.2.** Microsporidia induce increased gene expression of TLR2, but not TLR4, in primary human macrophages. Total RNA was extracted from MDM cultures after challenge with spores for 6 hr and gene expression of TLR2, TLR4, and TNF-α was measured by RT-qPCR. Data represented as the means of 6 (TLR2, TLR4) and 3 (TNF-α) experiments. *, p ≤ 0.05.
Figure 4.3. Nuclear translocation of NF-κBp65 in primary human macrophages is initiated early after challenge with spores of *Encephalitozoon spp.* MDM cultures in 6-well plates were challenged with spores for 1, 3, or 6 hr. Nuclear proteins were collected and analyzed for levels of NF-κBp65 subunit by ELISA. (A) Significant levels were observed at both 1 and 3 hr post-challenge, while levels returned back to that of control by 6 hr (n=4). To confirm NF-κBp65 activity, some MDM cultures were plated on coverglass and immunostained for nuclear localization. Nuclear NF-κB (green) was clearly observed at 1 hr (B) in the nucleus, but not 6 hr post-challenge. *, *p* ≤ 0.05.

*Encephalitozoon spp.* Induce the Production of Pro-inflammatory Cytokines in Primary Human Macrophages. To substantiate the role of macrophage in recognition of Encephalitozoons in inducing inflammatory responses, the temporal expression of two inflammatory mediators, TNF-α and IL-8, was established. TNF-α and IL-8 are key inflammatory cytokines/chemokines that are produced in response to numerous types of intracellular pathogens. TNF-α and IL-8 were measured in
supernatants from MDM cultures challenged with spores at various time points. Both species of microsporidia induced a strong, significant TNF-α response by 3 hr post-challenge, which peaked at 12 hr and sharply fell after 24 hr (Fig. 4.4A). In contrast, the production of IL-8 increased significantly by 6 hr post-challenge and continued to increase overtime out to 72 hr (Fig. 4.4B).

**Figure 4.4.** Microsporidia induce the production of TNF-α and IL-8 by primary human macrophages. MDM cultures in 96-well plates were challenged with spores and stopped at various times by collecting the supernatants. TNF-α (A) and IL-8 (B) levels in supernatants were determined by ELISA. Data represented as the means of 6 (TNF-α) and 3 (IL-8) experiments. At time points 3, 6, 12, 24 hr for TNF-α and 6, 12, 24, 48, 72 hr for IL-8, \( p \leq 0.05 \).
Encephalitozoon Induced NF-κB Translocation in Macrophages Is Required for the Production of TNF-α and IL-8. To establish the role of NF-κB in the production of the inflammatory mediators TNF-α and IL-8 in response to Encephalitozoon spores, the NF-κB inhibitor, Bay11-7085, was added to MDM cultures for 1 hr prior to challenge with microsporidia spores. This inhibitor is known to prevent the phosphorylation and degradation of IκB, thus, preventing the nuclear translocation of NF-κB (Pierce et al., 1997). A significant reduction in both TNF-α and IL-8 levels at 12 hr was observed in cultures treated with Bay11-7085 (Fig. 4.5A-D). As anticipated, NF-κB activation is required for producing an effective inflammatory response against the parasites.

![Figure 4.5](image)

**Figure 4.5.** Blocking NF-κB activity prevents the production of microsporidia induced TNF-α and IL-8 from primary human macrophages challenged with spores. Cultures of MDM in 96-well plates were pre-incubated for 60 min. with 20 μm of the NF-κB inhibitor, Bay11-7085, followed by a 12 hr challenge with Encephalitozoon spores or LPS (10ng/ml). Levels of TNF-α and IL-8 were measured by ELISA. TNF-α (A-B) and IL-8 (C-D) were abrogated in cultures of both MDM in comparison to cultures without the addition of inhibitor (n=3). *, p ≤ 0.05.
TLR2 Knockdown in Macrophages Inhibits NF-κB Nuclear Translocation and Subsequent Cytokine and Chemokine Responses Against Microsporidia. To directly show that the engagement of TLR2 by Encephalitozoon spp. in primary human macrophages results in NF-κB translocation, and the production of inflammatory mediators, MDM were transfected with either control or TLR2 siRNA. Treatment with siRNA inhibits TLR2 mRNA expression, which could not be detected by RT-qPCR. TLR2 siRNA altered NF-κBp65 translocation, which was observed in control cells at 1 hr challenge with E. cuniculi, but not in TLR2 siRNA treated cells (Fig 4.6A). After a 12 hr challenge with the parasites, cell culture supernatants were collected and measured for levels of TNF-α and IL-8 mediators, as well as, two NF-κB driven chemokines, CCL3 and CCL4, previously described as being produced in response to Encephalitozoon challenge (Fischer et al., 2007). The knockdown of TLR2 resulted in an approximate 2-fold decline in the production of TNF-α (Fig. 4.6B) and IL-8 (Fig. 4.6C) in comparison to control siRNA transfected MDM when challenged with either species of parasite. A reduction was also observed in siRNA TLR2 transfected MDM stimulated with the TLR2 agonist, Pam₃CSK₄, whereas the siRNA knockdown had no effect on the cytokine response to the TLR4 agonist LPS. Similar results were also observed in supernatants analyzed for levels of CCL3 (Fig. 4.6D) and CCL4 (Fig. 4.6E). Levels of both chemokines were decreased in TLR2 transfected MDM as compared to control transfects. Taken together, these data show a direct reduction in these four inflammatory mediators as a result of Encephalitozoon spp. ligation of TLR2 and subsequent NF-κB activation in primary human macrophages.
Figure 4.6. siRNA gene silencing of TLR2 reduced Encephalitozoon-mediated NF-κB nuclear translocation and inflammatory responses. MDM transfected with TLR2 siRNA were challenged with Encephalitozoon spores, LPS (10ng/ml), or Pam3CSK4 (50ng/ml) for 12 hr. Cells on coverslips were analyzed by immunofluorescence microscopy for nuclear NF-κB (A) and culture supernatants analyzed by ELISA for the production of TNF-α (B), IL-8 (C), CCL3 (D), and CCL4 (E) inflammatory mediators. For Encephalitozoon challenge, data represented as the means of 9 (TNF-α; IL-8) and 5 (CCL3; CCL4) experiments. *, p ≤ 0.05.
Discussion

*Encephalitozoon* spp. have been recognized as emerging pathogens, however very little knowledge about the immunobiology of microsporidiosis in humans is available. Pathology and epidemiological reports have identified immune effector cells at sites of infection (Orenstein, 2003) and detected increased levels of serum antibodies for microsporidia in humans (Halanova et al., 2003; van Gool et al., 2004). Recent *in vitro* studies have shown that infections lead to the production of numerous cytokines and chemokines including TNF-α, CCL2, CCL3, and CCL4 (Fischer et al., 2007; Franzen et al., 2005). Although these responses have been well-documented, to date no known receptor involved in microsporidia recognition and signaling has been identified.

TLR have been shown to mediate the responses of many host-pathogen interactions. This family of receptors was first identified in Drosophila Toll-mutants that succumbed to fungal infections and soon after their receptor homologues were identified in mammals (Lemaitre et al., 1996; Medzhitov, 2001; Rock et al., 1998; Takeda and Akira, 2005). Many insects, including Drosophila, are natural host for entomogenous microsporidia (Becnel and Andreais, 1999). Given these receptors’ involvement in inducing innate immune mechanisms leading to inflammatory responses, such as those previously reported against microsporidia, and their presence in all major natural hosts for microsporidia, toll-like receptors were investigated for their role in host recognition of *Encephalitozoon* spp. We report here, that *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis* are recognized by TLR2. Furthermore, the TLR2-parasite
interaction mediates the nuclear translocation of NF-κB, which initiates the production of inflammatory mediators, such as TNF-α, IL-8, CCL3, and CCL4.

Although the cytokine and chemokine responses to *Encephalitozoon* infection have been described in previous studies, intracellular signaling molecules involved in the induction of this cascade, such as NF-κB, have not been reported. Similar to the reports of others (Franzen et al., 2005), we found that both species of *Encephalitozoon* elicit a very strong inflammatory response when introduced to primary human macrophages, and here we show that this interaction results in a rapid nuclear translocation of NF-κB (Fig. 4.3) and robust TNF-α and IL-8 production (Fig. 4.4) that can be inhibited by blocking IκB phosphorylation (Fig. 4.5). Interestingly, our temporal studies reveal that NF-κB translocation begins as early as 1 hr post-challenge, but continuously decreases thereafter and returns to levels of that of control by 6 hr (Fig. 4.3). The decrease in nuclear NF-κB is reflected in our TNF-α data which shows increases in secreted protein levels to 12 hr, followed by a sharp decrease at 24 hr (Fig. 4.4). Increases in TNF-α mRNA at 6 hr post-challenge (Fig. 4.2) followed by declining levels overtime (data not shown) were also observed. Unlike that of the TNF-α response, IL-8 protein levels (Fig. 4.4) continue to increase overtime out to 72 hr post-challenge, regardless of nuclear NF-κB amount, however, the initial production of IL-8 is altogether abrogated when NF-κB translocation is blocked (Fig. 4.5). Similarly, Mpiga et al. (2006) showed that IL-8 expression was sustained, while TNF-α levels peaked and decreased in cultures infected with *Chlamydia trachomatis*. Control of IL-8 expression has been shown to be regulated by several signaling pathways and transcriptional regulators, including, but not limited to NF-κB. Additional pathways may
be required for the stabilization of IL-8 mRNA allowing for sustained levels of IL-8 expression (Hoffmann et al., 2002). Taken together, our studies illustrate a mechanism involving the nuclear translocation of NF-κB which initiates an immediate inflammatory response against the parasite, but requires a second mechanism to sustain levels of IL-8. This mechanism may be used to prevent an over reaction of the inflammatory response, but continues to produce a potent chemokine that will attract additional cells for pathogenic clearance.

These studies show that TLR are involved in the recognition of Encephalitozoon spores, not only in classically transfected cell lines, but also in primary cells. Studies using transfected HEK293 cells showed that TLR2 alone was sufficient to induce IL-8 production, whereas TLR4 alone or TLR4 in collaboration with the accessory molecules MD2 and CD14 were not (Fig. 4.1). Additionally, heat-inactivated *E. cuniculi* stimulated a TLR2 mediated response observed at levels lower than that observed with viable spores. This may simply be attributed to the method of parasite inactivation which may cause molecular alterations to the spore coat that lessens the ability of TLR2 recognition. Similar differential cytokine responses were observed between viable and heat-inactivated *Candida albicans*, and so it was suggested that increased temperature may manipulate structural moieties found on the yeast surface that influence receptor recognition and responses (Gantner et al., 2005; Netea et al., 2006b). Alternatively, it may indicate that the PAMP is located on a parasite surface which is only exposed during or after leaving the exospore. This would predict that the spores would have to be viable to trigger TLR2 pathways.
Macrophages are professional antigen-presenting cells found at mucosal barriers and are equipped with TLR to recognize a variety of pathogens (Janeway et al., 2005). To provide evidence for the direct role of TLR in the recognition of *Encephalitozoon* spp., siRNA was utilized to knock down the expression of TLR2 in primary human macrophages. Our results indicate that NF-κBp65 nuclear translocation is inhibited and TNF-α and IL-8 production is reduced by almost 50% in knockdown cultures stimulated with either species of microsporidia (Fig. 4.6). In addition, a reduction in the chemokines CCL3 and CCL4 was also observed in these cultures. Although we observed decreases in TNF-α, IL-8, CCL3, and CCL4, the production of these inflammatory mediators were not completely abrogated via our siRNA model, and thus, it remains possible that the pathogens can be recognized by additional receptors that have not been described in this study.

Although no specific agonists from microsporidia spore coats or sporoplasms have been defined in TLR recognition, molecules from the exospore, endospore, polar filament and sporoplasma are rapidly being identified, some of which have a striking resemblance to molecules from other microorganisms that are TLR agonists. Cell wall components of fungi, such as zymosan and β-glucan, have proven to be potent stimulants of inflammatory responses by signaling through TLR. Recently, linear O-linked mannosylated glycans derived from the spore coat were described in *E. cuniculi* (Taupin et al., 2007) These microsporidia glycans closely resemble molecules found on the surface of *C. albicans* that trigger TLR activation (Netea et al., 2006b). Additionally, proteins identified in the endospore are believed to be attached to the membrane by GPI-anchors. GPI-anchors derived from *Trypanosoma cruzi* and *Toxoplasma gondii*
have been identified in stimulating TLR2 (Campos et al., 2001; Debierre-Grockiego et al., 2007). This innate recognition has been portrayed as one way that our defenses can identify self from non-self, and begin an immediate response that will result in pathogenic clearance. It has also been described as a mechanism of immune evasion for pathogens (Netea et al., 2005).

Our data presented show that the opportunistic pathogens of the *Encephalitozoon* spp. induce TLR2 signaling, which leads to the immediate nuclear translocation of NF-κB and subsequent production and secretion of TNF-α and IL-8 inflammatory mediators. These studies have also raised further questions about additional unidentified receptors potentially involved in microsporidia recognition and/or signaling molecules involved in immunosuppression, which will be addressed in future studies. Defining the host-parasite interaction at the molecular level is helpful in understanding the pathology of the disease and developing possible methods of treatment and prevention.

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Summary

Microsporidians are a significant cause of disease in immunocompromised individuals, however, existing information regarding the immunobiology of the disease in humans is limited and not well understood (Didier and Bessinger, 1999; Khan et al., 2001). *Encephalitozoon* spp. of microsporidia are known to infect enterocytes and macrophages in humans and have been reported in disseminated infections (Didier et al., 2004; Khan et al., 2001; Orenstein, 2003). Dissemination of the disease is believed to occur through the migration of infected macrophages, as suggested by pathology reports (Kolter and Orenstein, 1999; Orenstein, 2003). The contributions of macrophages to either resolution of or progression of disease have been underreported and often conflicting when published. The work included in this dissertation is focused on determining the role of primary human macrophages in response to parasite challenge with *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis* in a well defined *in vitro* system.

**Kinetics of Encephalitozoon spp. Infection of Human Macrophages.** While *in vivo* pathogenesis reports clearly indicated macrophages were found in tissues with parasitophorous vacuoles containing spores (Kolter and Orenstein, 1999), previous *in vitro* modeling has produced conflicting evidence of microsporidia entry and intracellular fate (Couzin et al., 2000; Franzen et al., 2005b; Weidner, 1975). Using several different methods which label spore coat, sporoplasm, and replicating meronts, we established a well defined *in vitro* model that clearly delineates the kinetics of infection,
including parasite adherence, uptake, replication, and spore production in primary human macrophages.

Classical entry mechanisms of microsporidia require the eversion of the polar filament and expulsion of the sporoplasm into the host cell; however, an alternative method of infection in macrophages has been described as an actin-dependent phagocytic process (Couzin et al., 2000; Franzen et al., 2005b). Supporting the alternative method of infection, our SEM observations indicated a lack of polar filament firing, but an intimate association of host membrane partially surrounding the spores. Using a novel spore coat dye, we identified that this strong microbial-host association was quickly developed as nearly half of MDM were tightly adorned with spores by 1 hr post-challenge. In agreement with Franzen et al. (2005a), spores were rapidly internalized by macrophages and an observed 50% of MDM by 48 hr post-challenge contained peri-nuclear vacuoles of sporoplasms.

Parasite manipulation of intracellular signaling pathways in macrophages leading to host cell susceptibility and microbial proliferation is a common phenomenon (Pieters, 2001) that has been suggested to occur with microsporidia (Weidner, 1975). More recent studies have focused on the intracellular fate of human virulent strains of microsporidia in macrophages and suggest that the parasite is susceptible to intracellular killing mechanisms that limit microbial proliferation (Couzin et al., 2000; Franzen et al., 2005b). Using the bromodeoxyuridine (BrdU) assay to monitor parasite replication in terminally differentiated monocyte-derived-macrophages (MDM), we observed definitive microsporidia replication that resulted in the production of mature spores and confirmed our hypothesis that macrophages are susceptible host cells for
microsporidia development. Greater than 30% of MDM contained vacuoles of parasite that stained positive for BrdU by 72 hr post-challenge and increased at later times. In agreement with Didier et al (1994), treatment of MDM with IFN-\(\gamma\) and LPS, two known inducers of classical macrophage activation, resulted in decreased parasite replication, which suggests a need for Th1 responses to induce parasite killing. However, total parasite replication was not ablated; therefore, activated macrophages may contribute by limiting the disease, but may require additional responding effector cells to establish disease resistance.

**Induction of Host Chemotactic Response by Encephalitozoon spp.** Many host-pathogen interactions result in the production of chemokines which lead to the localization of effector cells to these sites in order to establish microbial clearance (Chensue, 2001). Pathology reports have shown that enteric infections of microsporidia cause an increase in numbers of macrophages in the lamina propria, some of which were shown to contain spores (Kolter and Orenstein, 1999; Soule et al., 1997). In disseminated cases of microsporidiosis, Infiltrates of spore containing macrophages have been identified in lesions of affected organs (Shadduck and Orenstein, 1993; Yachnis et al., 1996), however extracellular parasites have not been reported in the blood or lymph. These reports suggest that macrophages migrate to localized areas of infection to contribute to pathogenic clearance, but are instead, manipulated to act as a vehicle for the spread of the organism.

While reports of disseminated disease suggest the potential involvement of chemokines, previous evidence for the production of and functional recruitment by these factors has not been investigated. We describe here that microsporidia infected MDM
express a strong and diverse chemokine profile and show that these infections lead to the recruitment of monocytes. Analysis of focused gene and protein arrays of infected MDM revealed increases in the expression of several chemokines involved in leukocyte trafficking. A closer look at the temporal expression of three monocyte attracting chemokines, which were upregulated on the microarrays, showed peak levels of CCL2, CCL3, and CCL4 between 12-24 hr post-challenge and remained elevated out to 72 hr, which, based upon our infection kinetics, show that these data correlate to the merogony stages. In chemotaxis assays, the neutralization of CCL2 and CCL3 chemokines resulted in a significant decrease of monocyte recruitment, whereas the greatest decrease was observed with the neutralization of CCL4. These data suggest that the recruitment of monocytes to infected sites is partially induced by the production of CCL2, CCL3, and CCL4 and results in the recruitment of microsporidia susceptible host cells that have the potential for dissemination.

**TLR2 Recognition of the Microsporidia *Encephalitozoon* spp. Induces Nuclear Translocation of NF-κB and Subsequent Inflammatory Responses.**

Pattern recognition receptors (PRR) have been identified in the recognition of a number of pathogenic organisms; however, no molecules have been found which recognize microsporidia and stimulate an immune response. In macrophages, the immediate initial inflammatory responses can be generated by microbial interactions with germline encoded PRR that are able to identify particular structural and chemical moieties found on the organism (Medzhitov, 2007). The toll-like receptors (TLR), one family of PRR, have been shown to recognize molecules found on a number of parasites and fungal organisms that are similar in chemical structure to those identified on microsporidia.
(Debierre-Grockiego et al., 2007; Netea et al., 2006; Peuvel-Fanget et al., 2006; Xu et al., 2006). Additionally, TLR are known to regulate chemokine expression via NF-κB pathways (Grove and Plumb, 1993; Kunsch and Rosen, 1993). These data suggest that TLR may contribute to microsporidia recognition and subsequent immune responses.

In this study, we show that both *E. cuniculi* and *E. intestinalis* are recognized by TLR2 and that this interaction triggers inflammatory responses in macrophages. We observed that microsporidia-challenged MDM resulted in the nuclear translocation of NF-κB and the production of TNF-α and IL-8. The direct role of TLR2 parasite recognition in the activation of NF-κB and the production of cytokines and chemokines was confirmed by siRNA knockdown of TLR2 expression levels in primary human macrophages. This knockdown resulted in decreased nuclear amounts of NF-κB and lower levels of TNF-α, IL-8, CCL3, and CCL4. Taken together, these experiments suggest that TLR2 is a key molecule in the activation of initial inflammatory responses in primary human macrophages after challenge with *Encephalitozoon spp.* and may be partially responsible for the recruitment of monocytes/macrophages to infected tissues. Potentially, the development of host-tolerant molecules that target and disrupt TLR2-*Encephalitozoon* interactions may lead to the discovery of more effective chemotherapeutics that contribute to the prevention of disseminated disease.

In conclusion, the work described in this dissertation has provided a greater understanding for the host-parasite interactions between macrophages and microsporidia of the *Encephalitozoon spp.* and the subsequent innate immune responses. These studies have better defined an *in vitro* model to look at
Encephalitozoon spp. infections of primary human macrophages. Our investigations have confirmed the supporting role of macrophages in parasite proliferation and established the potential for their part in initiating extraintestinal dissemination. These studies are the first to describe a host receptor family involved in the recognition of microsporidia. Specifically, these data show that TLR2 recognizes Encephalitozoon spp. and generates inflammatory responses, which consist of known chemokines needed to recruit additional monocytes for potential proliferation and spread of microsporidia.

Future Research Goals

Reports of disseminated cases of microsporidiosis and the lack of information regarding macrophages potential role in parasitic spread make this research a very interesting and important area of further investigation. The prevalence of microsporidia in immunocompromised individuals and healthy people, together with the fact that effective treatment has yet to be discovered, pose a very real threat of continued multi-organ infections and fatality. By elucidating receptors and subsequent signaling pathways involved in microsporidiosis, these understandings may help to develop potential treatments against the newly recognized targets.

While our studies address the production of a functional chemokine gradient and recruitment of monocytes in response to infection, direct evidence for the role of macrophages in microsporidia dissemination to extraintestinal sites has not been shown. One method to investigate macrophages potential would require the use of a murine system, in which the macrophage population has been depleted. After inducing intestinal infection, animals could be monitored for the development of disseminated
multi-organ infections. The role of individual chemokines in dissemination could be addressed by challenging chemokine knock out mice with parasite and monitoring for the development of infection in organs. To establish a direct link between chemokines and macrophage dissemination, the adoptive transfer of individual chemokine receptor knock out macrophages into a murine model depleted of macrophages and analysis of disease development could be completed. Additionally, our data suggests that TLR2 activates chemokine responses. TLR2 knock out macrophages may be transferred into a previously described macrophage depleted murine model to assess the receptors role in parasite dissemination.

TLR are know to recognize a number of molecular moieties found on pathogens, and have more recently been shown to activate responses in concert with additional PRR (Mukhopadhyay et al., 2004; Takeda and Akira, 2005). Our data clearly shows a decrease in inflammatory immune responses when TLR2 is knocked down in MDM; however, cytokine levels produced by knock down cells are still elevated above controls. This suggests that microsporidia may be recognized by additional PRR to stimulate a full immune response. The collaboration of TLR2 with other PRR in microsporidia infections could be examined by constructing additional siRNA that target PRR of interest. Both, TLR2 and target PRR siRNA, could be transfected into MDM and infected cultures would be analyzed for cytokine levels. Understanding host receptor involvement in microsporidia infections would contribute to our knowledge of host-pathogen interactions and significantly progress microsporidia research.

References


APPENDIX

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Jeffrey L. Fischer was born in August 1976, to Ronald and Sue Fischer in Fairfield, Iowa. His interests in music began at age 5 when he started lessons on the piano, and continued to grow as he learned to play the saxophone, trumpet, and guitar. Jeffrey attended Simpson College in Indianola, Iowa, where he pursued an undergraduate degree in music education and began a career in teaching in 1998. In 2001, Jeffrey began to pursue his interest in the sciences and earned his Master of Natural Sciences from Louisiana State University in 2005. Jeffrey joined the laboratory of Dr. Hollie Hale-Donze in 2005 to pursue a doctoral degree in the research area of innate immunology and host-pathogen interactions.