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Cytokine Production during Microsporidia Infection of Macrophages

by

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Undergraduate honors thesis under the direction of

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the Upper Division Honors Program.

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Abstract

Microsporidiosis is a disease that has shown emerging importance as an infection in immunocompromised individuals. Fourteen species of microsporidia are capable of infecting humans, causing chronic diarrhea that could lead to malabsorption. Both pathogenic and non-pathogenic species of microsporidia can be studied to observe the immune responses elicited by macrophages when faced with a microsporidia infection. Cytokines serve as a basic quantitative tool to assess the signaling pathways involved in immune responses to microsporidia. Testing the cytokine production of both a cell line and primary cells allows for the understanding of which type of cell is better to use in the laboratory setting for certain studies. Liver X receptors (LXR) have been shown to partially regulate the immune response to the bacterial cell wall component lipopolysaccharide (LPS), which signals through toll-like receptor 4. Some species of microsporidia have been shown to signal similarly to LPS. The investigation of LXR involvement in immune responses to microsporidia infection could help elucidate more information about some functions of LXR.

Introduction

Microsporidia

Microsporidia are eukaryotic, intracellular parasites that comprise over 1200 species and infect a wide range of hosts, such as invertebrates, fish, and many mammals, including both human and non-human primates. Fourteen species are currently known to infect humans, and infection by these species brings about more severe symptoms in immunocompromised individuals than in their immunocompetent counterparts [1]. Cases in HIV/AIDS patients have identified opportunistic microsporidia infections as a source of increasing significance. Diarrhea is considered the most common symptom, but disseminated infection may occur, triggering other conditions which include, but are not limited to, encephalitis, keratoconjunctivitis, and hepatitis [2]. Infection has been shown to occur

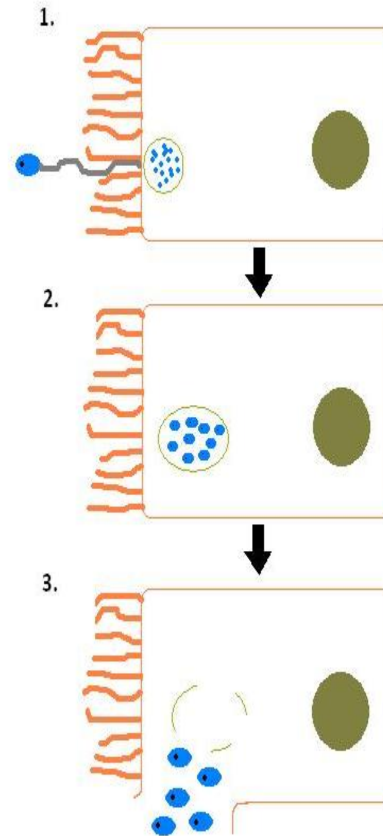
through a variety of sources, but ingestion of contaminated food and water continue to be the most prominent transmission routes [1].

The infective stage of the microsporidian life cycle is the spore, which is also the only life cycle stage capable of survival outside of host cells [3]. Microsporidia are unique, unicellular organisms, and these features are highlighted in the contents of the spores. Spores enclose several universal organelles, including nuclei, ribosomes, and membranes forming the endoplasmic reticulum. The number of nuclei varies among species and life cycle stages, with some exhibiting the monokaryon configuration and others exhibiting the diplokaryon configuration [3]. Organelles contributing to the distinctiveness of microsporidia include mitosomes (thought to be reduced mitochondria), a polar filament originating at an anterior anchoring disk, and an atypical Golgi apparatus. The polar filament plays a key role in infection, as it is the source of ejection of spore contents including both the nucleus and cytoplasm. Along with the unique polar filament, microsporidia spores contain a membranous polaroplast as well as a posterior vacuole [2].

Of the fourteen microsporidia species infecting humans, *Enterocytozoon bieneusi* causes infection most frequently. Other species known to infect humans include *Brachiola algerae*, *B. connori*, *B. vesicularum*, *Microsporidium africanum, M. ceylonensis, Nosema ocularum, Pleistophora ronneafiei, Trachipleistophora anthropopthera, T. hominis,* and *Vittaforma corneae*. Along with these species, three species of *Encephalitozoon* are also known to infect humans. *Encephalitozoon intestinalis* is the second most common species to infect humans, and *E. cuniculi* and *E. hellem* were shown to infect humans as well [4]. Because of their importance in both human and other mammalian infections, the *Encephalitozoon* species have been studied such that a generalized life cycle is known (Figure 1).

The initial stage of infection includes contact of the spore and host cell. Following ingestion of *Encephalitozoon* species through contaminated food or water, interactions between spores and intestinal epithelia occur. Other means of transmission, such as inhalation, involve other cell types

(respiratory tract epithelia) [4]. Upon spore-cell contact and certain environmental conditions (i.e. ion levels, pH, or osmotic conditions), the spore undergoes polar filament eversion. For the *Encephalitozoon* species, the sporoplasm travels through the everted polar filament and enters a parasitophorous vacuole in the host cell [5]. Inside the parasitophorous vacuole, merogony begins. During this replication stage, nuclear division takes place, which may or may not be immediately followed by cytokinesis. At this point the organisms are in the meront form. Upon thickening of the membrane surrounding the meront, the sporont stage is achieved. The membrane enclosing the parasite becomes more electron dense during this stage, as does the interior of the parasite, due to the accumulation of endoplasmic reticulum membranes and ribosomes. With the onset of sporogony, the newly formed sporonts divide further to give rise to sporoblasts. For *Encephalitozoon* species, each sporont gives rise to two sporoblasts. Sporoblasts undergo maturation to form the spore stage of the life cycle. During this maturation period, the polar filament, polaroplast, and other intracellular organelles are formed. When the mature spore is formed, it contains both an exospore coat (the electron dense layer added during the merogony/sporogony transition) and an endospore coat (added along with the posterior vacuole during the final stages of maturation). Mature spores are released from the host cell via lysis [2, 5].



1. Spore comes into contact with host epithelial cell and everts its polar filament. Spore contents are injected into host cell and remain in a parasitophorous vacuole.

2. Inside the vacuole, the parasite undergoes merogony and sporogony.

3. Mature spores are released from the parasitophorous vacuole and host cell via cell lysis.

Figure 1. General *Encephalitozoon* Life Cycle

The three *Encephalitozoon* species infecting humans have been shown to originate infection in either the intestinal epithelia or respiratory epithelia. Dissemination to other parts of the body may occur, due to the ability of these species to infect macrophages. Specifically, dissemination of *E. intestinalis* has caused nephritis, as well as infections of the sinuses, urinary bladder, and skin. *E. cuniculi* has been shown to cause keratoconjunctivitis, and is capable of infecting the heart, brain, kidneys, and even the tongue. Similarly, *E. hellem* has also been shown to cause keratoconjunctivitis, and has been found infecting the sinuses, urinary bladder, and prostate. These aforementioned sites of infection are just a few of the locations that these intracellular parasites have been found [6]. Although infection may occur in all of these locations and lead to symptoms based on infection site, by far the most common symptom of infection is diarrhea. In immunocompromised individuals, chronic diarrhea (lasting at least three months) may persist and lead to malabsorption and wasting [2].

Host Immunity

Macrophages are often thought of as one of the first line of defense against intruding pathogens. They serve as defense in host tissues, where they reside after differentiating from monocytes found in the blood. It is these cells that serve to sequester and kill the invading organism, and in doing so, allow the host to receive as little damage as possible. Generally speaking, when a macrophage encounters an invading intracellular parasite, it ingests the organism and degrades it through the use of an organelle referred to as the lysosome. Lysosomes provide environments for the denaturation of proteins and nucleic acids, and provide a compartment were the macrophage can kill the invader without damaging itself in the process. Macrophages that have become activated cause the phagosome, which contains the harmful material, to fuse with a lysosome. Along with killing already ingested particles, macrophages are also activated so that they will send out chemical signals and cause the immune response to proliferate. These signals include cytokines, such as interleukin 10 or 12 (IL-10, IL-12). The downstream effects of emitting cytokines and other molecules include further activation of

immune system cells, such as natural killer cells, as well as the aggregation of immune cells to the area of need [7]. Some organisms are able to circumvent the host mechanisms of macrophages, and in doing so, cause disease and stress to the host organism. Microsporidia are a perfect example of this type of organism. Because these intracellular invaders use macrophage signaling pathways to their advantage, they are an interesting organism to study. Understanding just how they alter macrophage function may be a key in unlocking prevention or treatment of microsporidiosis.

In order for macrophages to recognize and engulf harmful material, they need to have ways of recognizing those materials. Therefore, the surfaces of macrophages often contain multiple types of receptors for just that purpose. One type of receptor present is the Toll-like receptors. TLRs get their name from their relation to the *toll* gene found in *Drosophila melanogaster* [8]. Interaction of ligands with the multiple TLRs present on human cells leads to many downstream events, including upregulation of compounds that take part in the host immune response. Different TLRs can activate different signaling pathways, and in turn, elicit different host responses. A major example of this can be seen in the push towards a T helper type 1 or type 2 response. The two types of T helper cells cause the activation of different cell types. Activation of T_H1 cells result in the further activation of cells involved in phagocytic protection of the host. When T_H1 cells are activated, they release interferon gamma (IFN-γ), which causes phagocytic cells to degrade molecules that they have ingested and marked as dangerous. In contrast, the activation of T_H2 cells does not trigger the activation of phagocytic cells. Instead, several of the molecules secreted by T_H2 cells actually act to suppress some of the immune response initiated by the T_H1 cells. As stated above, the use of different TLRs may lead to a different downstream immune response. For example, the main cytokine involved in the T_H1 response is interleukin 12 (IL-12) [9]. IL-12 is a heterodimer made of two subunits, IL-12p40 and IL-12p35. The two subunits are regulated independently and together make up the whole particle, IL-12p70. IL-12 is induced following signal transduction mechanisms that occur after a ligand binds to TLR-4 on the cell

surface. After IL-12 is produced, it aides in the production of other signaling molecules, such as IFN-γ [10]. It is in this manner that the cell and its system are pushed towards a T_H1 type mechanism. Conversely, signaling that begins with TLR-2 has been shown to cause a lean towards the T_H2 type of response. In his review, O'Neill discusses the cytokine production that occurs following ligand binding to TLR-2. He mentions that cytokines such as IL-10 are produced, which in turn lead to the T_H2 type response. Interestingly, it is also noted that only one of the subunits that makes up IL-12 is induced by this pathway [8]. Because of the lack of the other subunit, the IL-12 particles are not fully functional [10].

Specific Immune Responses to Microsporidia

Previously, it was shown by Fischer et al. that TLR-2 is capable of recognizing both *Encephalitozoon cuniculi* and *E. intestinalis* during infection of human cells. This is significant because both of these species are pathogenic to humans. These species were shown to cause increased gene expression of TLR-2 [11]. Based on the information about human immune responses that is already known, it would make sense that these pathogenic species attempt to push the host immune response toward the T_H2 side. Other findings by the same lab group showed that non-pathogenic species of microsporidia, such as *Antonospora locustae* and *Vairimorpha necatrix* do not signal through the same mechanisms as the pathogenic species. Unpublished data have provided evidence that links infection of human cells with these non-pathogenic species to the activation of TLR-4. This evidence includes the induction of IL-12 following infection, a sign that the non-pathogenic microsporidia are causing the cells to shift towards a T_H1 type response. These findings are important because they could signify the difference between infections that produce disease (pathogenic species) and those that do not (non-pathogenic species). Terms that could be used to describe the types of responses to these infections are protective in the case of non-pathogenic species and permissive in the case of pathogenic species

infection. Therefore, it is important to continue to study the differences in downstream signaling that occurs following infection with both pathogenic and non-pathogenic species of microsporidia.

Liver X Receptors

The liver X receptor (LXR) is a nuclear receptor, involved in transcription regulation for molecules including cholesterol. LXRs act in conjunction with retinoid X receptors (RXR) in order that regulation occurs at its greatest efficiency [12]. It has been shown recently that LXR is involved in the immune response provoked by macrophage interaction with bacterial lipopolysaccharide (LPS). Because LPS is known to interact with TLR-4, and LXR induction was shown to downregulate some of the LPS response (in the form of reduced cytokine production), the inference could be made that LXR could downregulate the entire set of products of TLR-4 activation [13]. Downregulating the TLR-4 response may ultimately lead to the dampening of a T_H1 response. Because the two types of helper T cell responses are exquisitely balanced, a dampening of the T_H1 response would mean an increase in the T_H2 response. By back coordinating, this would also lead to an increase in the activity of TLR-2 and events that occur downstream of TLR-2 activation.

As stated above, the dichotomy of the TLR-2/TLR-4 is seen during infections of macrophages with microsporidia. Because of the dichotomy seen there, some connections can be made between the liver X receptor and effects of microsporidia infection. More specifically, adding LXR agonist or antagonist before infecting cells with microsporidia may affect the type of cytokine production (and thus signaling pathways) that occur within the cells. Adding LXR agonist to upregulate the receptor would likely cause an increase in the TLR-2 response. Conversely, adding LXR antagonist to downregulate the receptor would likely cause an increase in the TLR-4 response. The amount of cytokine produced in these infections can be directly measured and used to deduce the direction that the immune response would go. IL-12 is an ideal cytokine to test for because of its involvement in the TLR-4 signaling cascade and in the T_H1 immune response.

Specific Aims

This project had two specific goals. The first was to compare cytokine production in primary human cells derived from donated blood to that in a cell line after both were infected with microsporidia. Previous reports showed that cell lines and primary cells do not always give the same cytokine profiles under like conditions, due to the preactivation of cell line cells [14]. In this case the activation of the cells was even more augmented, due to the fact that the cell line in question (THP-1) is a leukemia human monocyte line, which is already activated. Also, a chemical (phorbol 12-myristate 13-acetate – PMA) that also causes activation of the cells was added in order that the cells from culture would adhere to a culture plate. These two features would likely create skewed cytokine profiles after microsporidia infection. The goal was to study production of two different cytokines (IL-10 and IL-12) known to be produced after microsporidia infection.

The second goal was to investigate the effects of LXR upregulation and down regulation on the immune responses to microsporidia. As stated previously, analysis of the production of IL-12 would be used to determine the activation of TLRs. Information from this would give insight into the role of LXR in the immune response.

Methods and Materials

Reagents

Liver X receptor (LXR) agonist (T0901317, or T09) and antagonist (geranyl geranyl pyrophosphate) were donated by Dr. Kirsten Prufer-Stone. LPS from *Escherichia coli* O111:B4 was obtained from Sigma Chemical Company (St. Louis, MO). All tissue culture media and reagents were obtained from VWR International (West Chester, PA). Enzyme linked immunosorbent assay (ELISA) reagents and kits were obtained from Invitrogen (Carlsbad, CA).

Cell Culture

Buffy coats of healthy donors (Our Lady of the Lake Regional Blood Bank, Baton Rouge, LA) were used to isolate peripheral blood mononuclear cells (PBMCs) via gradient centrifugation on lymphocyte

separation medium. Following isolation, monocyte derived macrophages were attained. PBMCs were initially plated onto 6 well plates at a concentration of 2×10^6 cells per well, and following a three hour incubation in Dulbecco's modified Eagle's medium (DMEM) with additives (100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 µg/mL gentamicin, and 2mM L-glutamine), they were washed with phosphate buffered saline (PBS). The purpose of the wash was to remove any monocytes that had not adhered to the wells. After washing, the monocytes were incubated for seven days in DMEM plus 10% fetal calf serum (FCS) and the additives mention above. This incubation at 37°C in 5% CO_2 allowed the monocytes to differentiate into macrophages (MDM – monocyte derived macrophages).

The THP-1 cells were collected from a continuous cell line maintained in the lab. In culture, the cells are maintained with DMEM containing 10% FCS as well as the additives mentioned above. For experiments, cells are collected from culture and treated with phorbol 12-myristate 13-acetate (PMA) for adherence to plates.

Parasites

Encephalitozoon cuniculi III, *E. intestinalis* (donated by Elizabeth Didier, Tulane National Primate Research Center), *Vairimorpha necatrix* (donated by Charles Vossbrinck, The Connecticut Agricultural Experiment Station), and *Antonospora locustae* (donated by Yuliya Sokolova, Russia) were grown in a rabbit kidney cell line (ATCC CCL-37) in DMEM plus 10% FCS, and incubated at 37°C in 5% CO_2. Upon spore harvesting from tissue culture supernatants, they were washed in PBS containing 0.2% Tween 20 and resuspended with supplemented DMEM before being counted with a hemacytometer. *E. cuniculi* and *E. intestinalis* were used at a 5:1 spore to cell ratio. *V. necatrix* and *A. locustae* were used at a 1:1 spore to cell ratio.

ELISA

Supernatants were collected from cells after they had been infected with *E. cuniculi, E. intestinalis, V. necatrix,* or *A. locustae* for certain timepoints. Some cells were pretreated with T09 or geranyl geranyl pyrophosphate. These supernatants were analyzed for the presence of IL-12p40, IL-

12p70, or IL-10 using Cytosets ELISA kits (Invitrogen). All sample analyses were performed in duplicate according to the protocols established in the kits.

Results and Discussion

For all of the experiments performed, few replicates (2 or 3) provided usable data. When using human cells, at least 6-10 replicates are needed in order to get results with statistical significance. Therefore, no error bars are shown on the graphical representations of the data. The numbers used are mean concentrations of the two or three replicates.

Comparison of cell line to primary cell cytokine production.

To exhibit the propensity of THP-1 cells as models for macrophage signaling in response to microsporidia, cells were plated into 96-well plates and infected with both pathogenic (*Encephalitozoon cuniculi*) and non-pathogenic (*Antonospora locustae*) species of microsporidia. Following infection, both IL-10 and IL-12p70 responses were analyzed using enzyme linked immunosorbent assay (ELISA). Data was compared to that collected from previous laboratory experiments testing for the production of both cytokines in supernatants collected from plates of monocyte derived macrophages (MDM) after they had been infected (unpublished data). In the case of the MDM, two species of pathogenic microsporidia were tested (*E. cuniculi* and *E. intestinalis*), while just the one non-pathogenic species was used.

When THP-1 were challenged with pathogenic species and analyzed for the concentration of IL-10 following infection, the results followed a similar pattern to that set by infection of MDM. There was a low concentration (below 40pg/ml) of IL-10 present in the supernatants from all time points. These low concentrations did not differ very much from the concentrations seen in the supernatants of uninfected control cells. In contrast, the concentration of IL-10 present in the supernatants from non-pathogenic species challenge of both MDM and THP-1 was higher than that of the control. The trend of both infections (MDM and THP-1) was about the same, with IL-10 concentration peaking at the 12 hour

time point. Graphs showing the IL-10 production by MDM and THP-1 from various time points and infections are shown in figures 2 and 3.

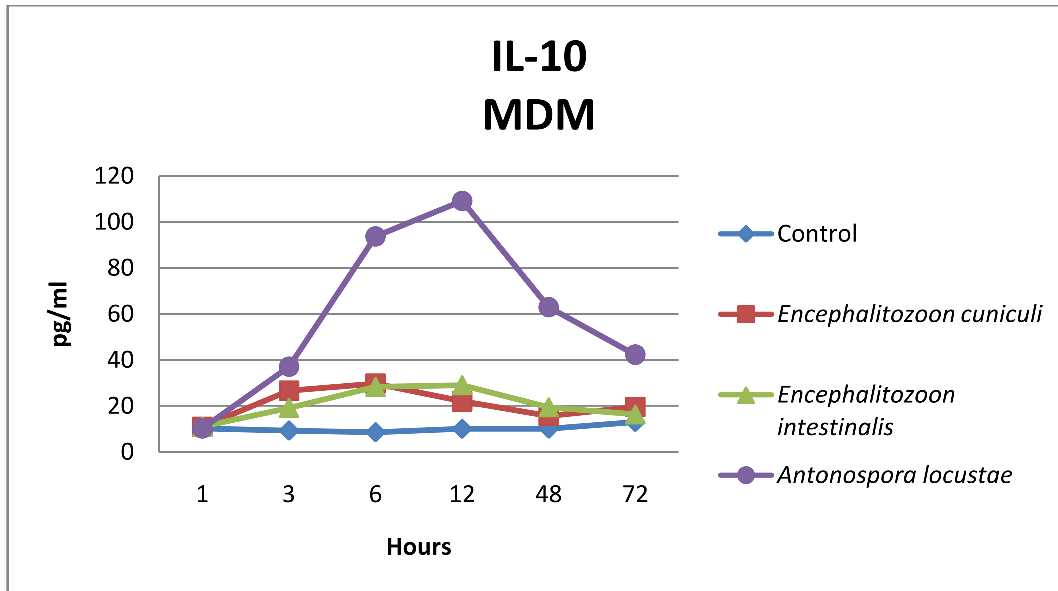


Figure 2. IL-10 production by MDM at certain time points postinfection. (n=3)

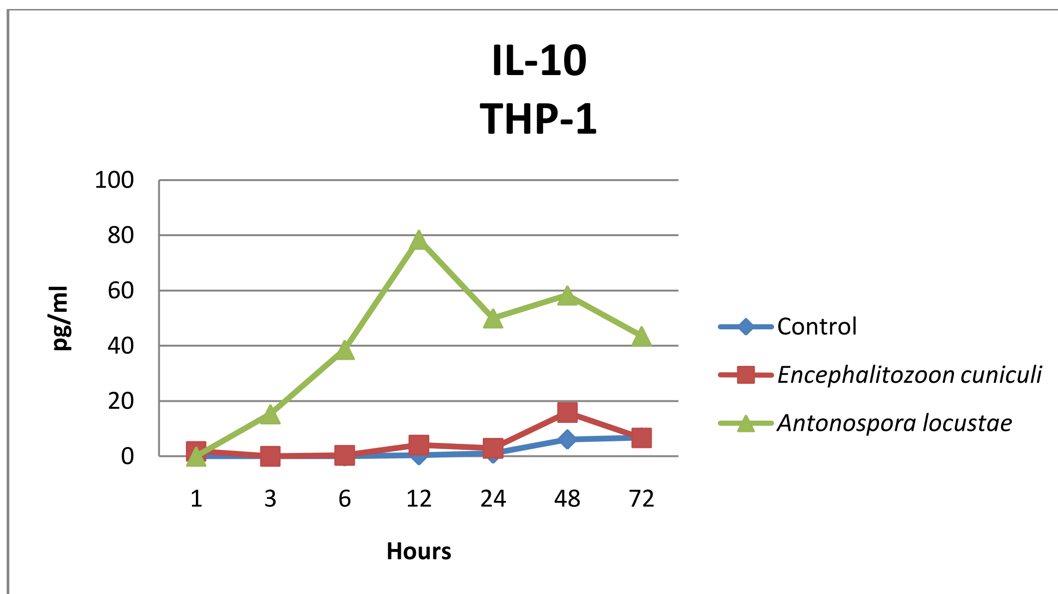


Figure 3. IL-10 production by THP-1 at certain time points postinfection. (n=2)

The IL-12p70 responses of MDM and THP-1 did not show the same similarities as those seen with IL-10 concentration. When THP-1 cells were infected with either pathogenic or non-pathogenic microsporidia, the amount of IL-12p70 produced did not differ from uninfected control cells. Similarly, when MDM were infected with pathogenic species, the levels of IL-12p70 did not differ from the controls. Interestingly, the amount of IL-12p70 produced by MDM infected with non-pathogenic species increased over time and differed greatly from the trend seen in the control cells. Graphs indicating the production of IL-12p70 in both THP-1 and MDM at each time point and infection are shown in figures 4 and 5.

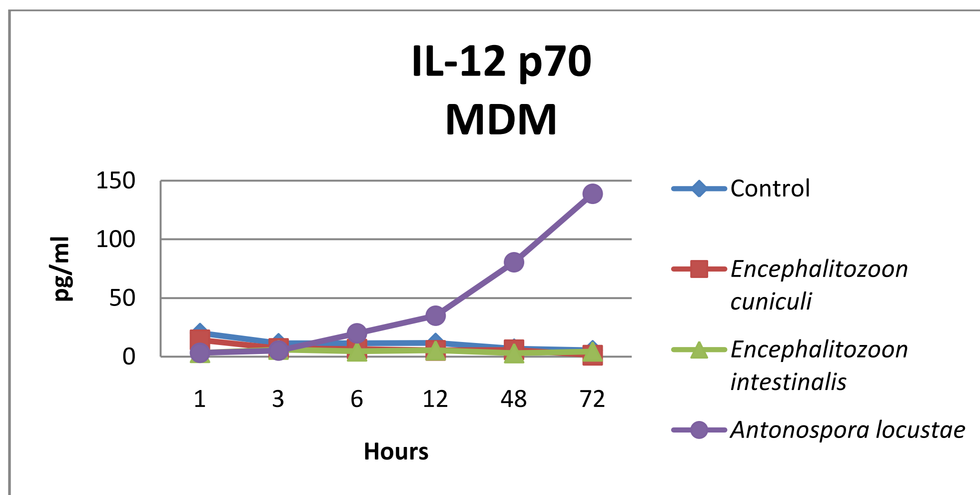


Figure 4. IL-12p70 production by MDM at certain time points postinfection. (n=3)

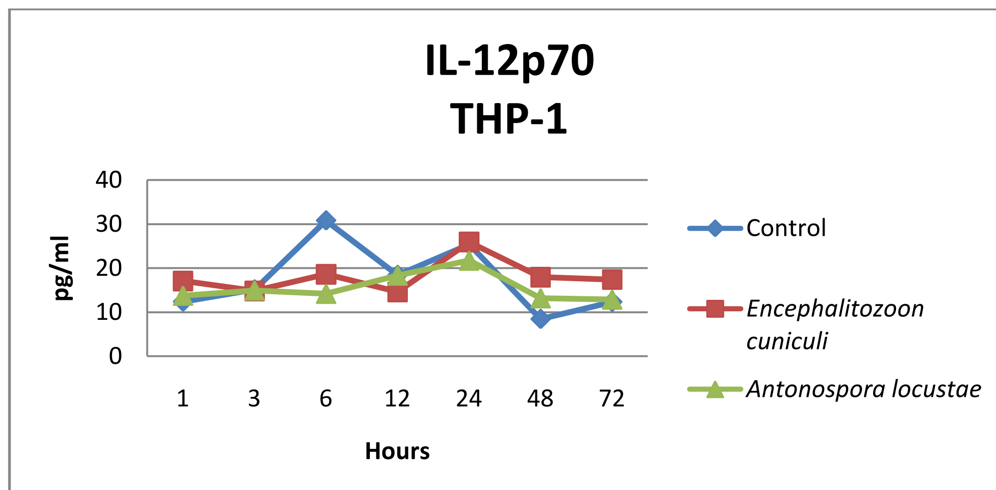


Figure 5. IL-12p70 production by THP-1 at certain time points postinfection. (n=2)

These results, which show contrasting results of infection with THP-1 and MDM cells, support the idea that THP-1 cells are difficult to use when studying cell activation and signaling pathways. Because the cells are part of a leukemia cell line, they were previously activated before infection even began. In addition, to use THP-1 cells in the same manner as plated MDM cells, the THP-1 have to be treated with PMA, which also serves to either activate or dampen certain signaling pathways [14]. The pre-activation of this cell line deems them unworthy for studies involving cell signaling pathways that are affected by any of the reagents used. IL-10 may not have been affected by the addition of PMA, possibly because PMA does not affect signaling pathways involved in the production of IL-10. Future studies may address to what extent the pre-treatment of the THP-1 cells is affecting the results of cytokine analysis.

LXR activation/deactivation in microsporidia infection of macrophages.

Compounds known to be either an LXR agonist or LXR antagonist were used to pretreat MDM before they were infected with both pathogenic and non-pathogenic microsporidia. In this study, the goal was to see the effects of the LXR agonist and antagonist on infection. Following infection, supernatants were collected at various time points and analyzed via ELISA for the production of IL-12p40. For these experiments, the pathogenic species of microsporidia was *E. intestinalis,* while the non-pathogenic species used was *Vairimorpha necatrix*. Data is shown separated based on virulence of the microsporidia species, since both were expected to produce drastically different amounts of IL-12.

<u>Pathogenic Species</u>

In the infections involving *E. intestinalis*, a few trends could be seen. Uninfected control cells produced a comparable amount of IL-12p40 over all time points, and this concentration was very low. Cells infected with *E. intestinalis* that were pretreated with the LXR agonist showed IL-12p40 production similar to the control for all time points. Interestingly, both the cells challenged with only *E. intestinalis* and the cells pretreated with LXR antagonist and then challenged with *E. intestinalis* had slightly

increased concentrations at 12 hours after infection (compared to control cells). At the 16 hour time point, the levels of IL-12p40 for those two conditions dropped to equal those of the control. At the 24 hour time point, the amount of IL-12p40 in the cells treated with either the LXR antagonist or agonist was similar to the controls, while that of the cells infected only with *E. intestinalis* increased, following the decrease at 16 hours. The results are shown graphically in figure 6.

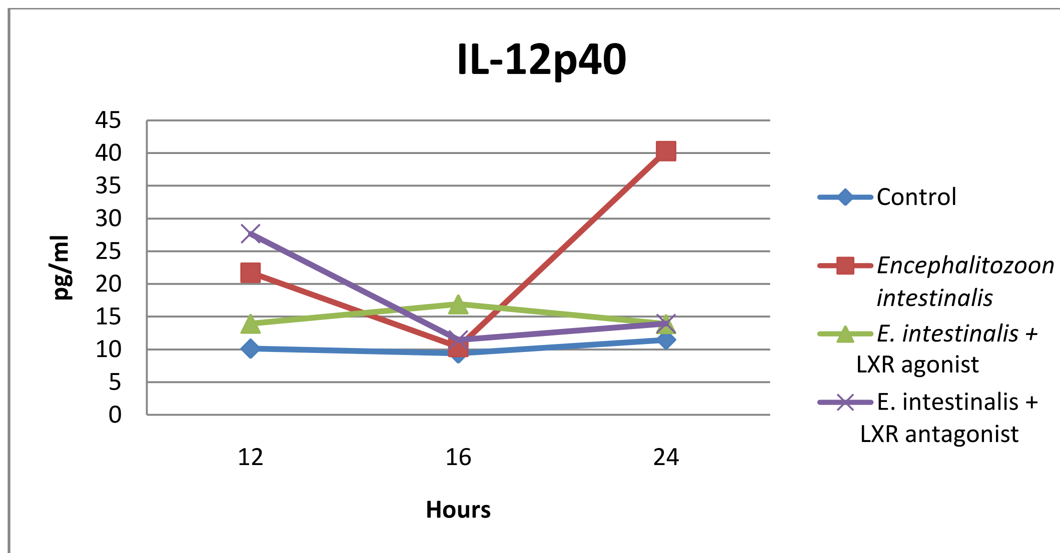


Figure 6. IL-12p40 production by MDM after infections and treatments noted. (n=2)

Because only three time points were investigated, it is difficult to make conclusions on the activities of the cells. The higher concentration of IL-12p40 at the 12 hour time point in the infections with *E. intestinalis* and the LXR agonist may be a peak in the activity of the cells, but without data from time points prior to 12 hours, this is unclear. At this time point, however, the increased concentration shown with the cells pretreated with the LXR antagonist does agree with the hypothesis. As a whole, the trends here do not completely agree with the hypothesis that the LXR agonist and *E. intestinalis* should both decrease the macrophage TLR-4 response (quantitatively, the amount of IL-12). At the same time, the trends do not discount the hypothesis. With so few replicates and time points it is difficult to see the complete picture, so more experiments need to be performed.

<u>Non-pathogenic Species</u>

As a whole, the infections with *V. necatrix* caused the MDM cells to produce higher amounts of IL-12p40 than those challenged with *E. intestinalis*. This supports previous work in the lab showing that non-pathogenic species cause increased IL-12 production (unpublished data). For these experiments, none of the challenged cells produced as little IL-12p40 as the control cells. The cells pretreated with LXR antagonist before being infected had levels of IL-12p40 similar to those that were not pretreated with anything prior to infection; this was consistent across all three time points. Those cells that were pretreated with LXR agonist prior to infection gave results different from all other treatments. Those cells that were treated with T09 before challenge with *V. necatrix* showed cytokine levels similar to the other treated cells at 12 hours, but at 16 and 24 hours, the levels in these cells increased well above those seen in the other treatments. A graph displaying these results is shown in figure 7.

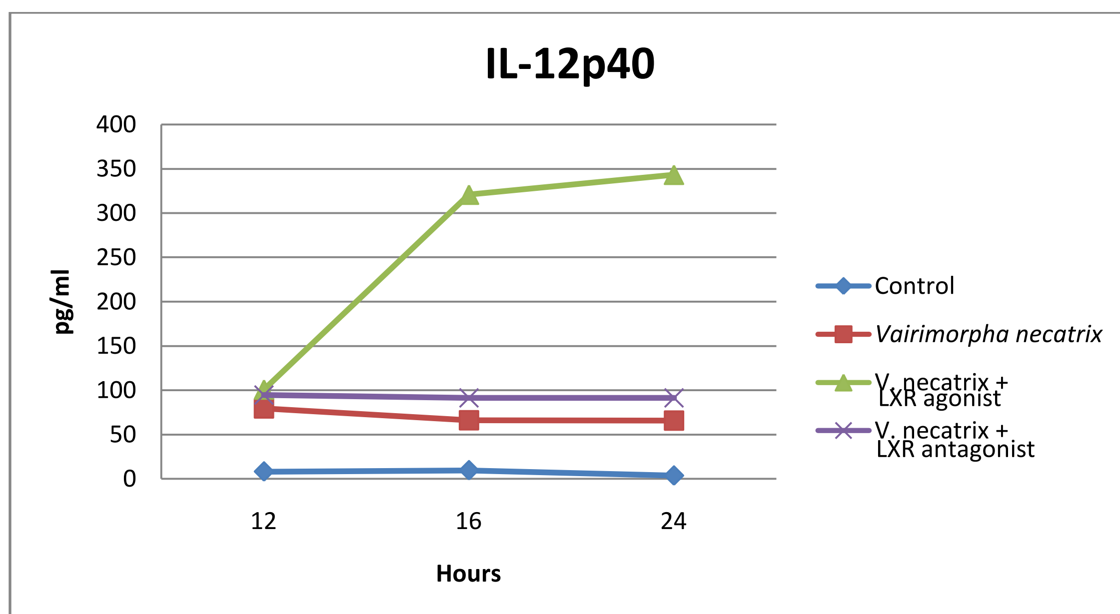


Figure 7. IL-12p70 production by MDM after infection and treatments noted. (n=2)

As with the results for the pathogenic species, these results do not fit with the hypothesis. The hypothesis states that addition of the LXR antagonist should increase signaling through TLR-4 and in

turn, the production of IL-12. The data from this part of the experiments show results opposite to this hypothesis. Again, because only three time points were tested, it is difficult to see complete trends in the levels of IL-12p40. Further investigation would need to be made to encompass other time points both before 12 hours and after 24 hours. Investigation into these time points would enable comparison with previous data relating to the concentration of IL-12p40 present during infections of various lengths. Along with the addition of other time points, a higher number of replicates would be necessary to see more concrete trends.

Conclusions

Overall, the results of the above experiments did not give conclusive evidence to either support or refute any of the hypotheses set forth early on. This was mostly due to the fact that there were not a lot of replicates for each experiment. This could be resolved by doing future experiments with more patients, and extending the time points. For the LXR experiments, the time points could be extended to cover the time points covered in the first experiments. Another problem that needs to be addressed is the confirmation of the up- and downregulation of LXR. Because the agonist and antagonist do not always function to their full potential, a portion of future experiments should be devoted to determining whether or not the receptor is activated. The agonist and antagonist may not have actually caused LXR to be induced or held back, and a method would need to be used to determine the effect of each compound. This is currently a tricky task, because a lack of antibodies against LXR proves any immunological tests difficult. Beyond those tests, it would also be necessary to investigate more closely the link between LXR, TLRs, and the immune response.

As for the differences in cytokine production in a cell line versus primary cells, more tests could be done to see if other cytokines also incur the same differences. Finding out exactly what is affected could help point out what experiments could be performed with the cell line over the primary cells or

primary cells over the cell line. Another way in which the experiment could be amended would be to test if other forms of causing cell adherence would alter the cytokine production of the cells. In the end, this would really just be a laboratory protocol asset, but it would be important nonetheless.

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