Leishmaniasis: immunologic indicators of clinical progression and mechanisms of immune modulation

by

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Leishmaniasis is a group of vector-borne diseases caused by obligate intracellular protozoan parasites of the genus *Leishmania*. *Leishmania* transmission primarily occurs via the bite of infected female sandflies, which inoculate *Leishmania* promastigotes into mammalian hosts. Promastigotes are internalized by phagocytic cells of the immune system, primarily macrophages and dendritic cells (DC), and begin the differentiation process into amastigotes, the parasite form which persists in the host. Disease outcome is dependent on both the host’s immune status and the species of parasite. Clinical manifestation can range from self-healing cutaneous lesions to fatal disseminated visceral disease. Leishmaniasis is endemic in 88 countries affecting regions in the tropics and subtropics, with an estimated 350 million people are at risk for infection and disease and of the 1.5-2 million new cases each year, 70,000 of which are fatal.

To date no effective vaccine against *Leishmania* for humans is available and although a variety of drugs are available to treat leishmaniasis, treatment efficacy remains subpar. In most cases parasites are never completely eliminated and disease recrudescence is very common. Additionally, the emergence of parasite resistance, increasing co-infections—especially with the increasing incidence of HIV—and the high cost of treatment all decrease the number of treatment options available and the chances of effective cure. Novel therapeutic options for leishmaniasis are in dire need.

In cases of visceral leishmaniasis, early detection is key if successful therapeutic treatment is to be achieved. In endemic areas, canine infection with *Leishmania infantum* is considered the most important risk factor for human infection. Recently, canine visceral leishmaniasis, caused by *Leishmania infantum*, has been reported in the United States.
Disease manifestation and progression in *L. infantum*-infected Foxhounds in the U.S. parallels visceral leishmaniasis in naturally- and experimentally-infected animals as well as humans. This makes the Foxhounds an attractive model to study natural *L. infantum* infection and visceral leishmaniasis disease progression. The objective of the first study of the dissertation is to characterize the temporal humoral and cellular immune responses to natural *L. infantum* infection in Foxhounds. The goals are to determine the immune parameters that characterize the different clinical stages of disease in infected animals: (1) CD4+ lymphoproliferative responses, (2) cytokine production profile, and (3) antibody responses. The information obtained from these studies describes for the first time the temporal immune response to *L. infantum* in naturally-infected Foxhounds in the US in the absence of vector transmission. In addition, this work identifies immune parameters that can prognosticate visceral leishmaniasis disease progression.

Current treatment options, based solely on chemotherapy, are not always effective. Novel treatment options that target the parasite and work with the host immune response should be considered. The murine model of cutaneous leishmaniasis using *L. amazonensis* infection provides an excellent model to study host-parasite interactions that may lead to a non-productive immune response. Understanding these interactions may allow us to develop therapeutic strategies that directly enhance the host immune response against *Leishmania*. Dendritic cells play a critical role as inducers of adaptive immune responses and serve as host cells for *Leishmania* parasites. The goals of the second study of the dissertation are to understand the mechanism of DC modulation following *L. amazonensis* infection. More specifically, this work will determine (1) the maturation phenotype of infected DC *in vivo* and *in vitro*, and (2) will explore changes in MAP kinase ERK activation and its role in DC maturation.
The objective of the third study in this dissertation will characterize the endosomal mechanism of *L. amazonensis*-mediated ERK activation. In the previous chapter we show that following infection with *L. amazonensis* promastigotes, ERK1/2 activation is delayed for several hours, at which time most parasites are found intracellularly within infected cells. Modulation of signaling pathways by *Leishmania* parasites has been previously described. However, little is known regarding the mechanisms employed by *Leishmania* parasites to modulate host cell signaling from the parasitophorous vacuole—the intracellular, membrane-bound compartment in which they reside inside the host cell. The work in this section will determine (1) whether live parasites are required to trigger ERK1/2 activation from infected DC, (2) characterize the endosomal compartment in which *L. amazonensis* parasites are found during the time of ERK1/2 activation, and (3) determine if ERK1/2-specific scaffold proteins associate with *L. amazonensis*-containing organelles. The information obtained from the first set of studies furthers our understanding of host-parasite amastigote-specific interactions and provides evidence for the use of ERK inhibitors as immunomodulators to directly enhance the host immune response after *Leishmania* infection. The second set of studies characterizes a *L. amazonensis*-mediated, scaffold-specific mechanism of ERK1/2 activation. The information from this work bring forth the possibility of developing treatment options that specifically modulate pathogenic ERK1/2 phosphorylation, sparing more universal functions. These therapeutic treatments could serve to treat not only *L. amazonensis* infection, but other diseases where aberrant activation of ERK1/2 leads to disease.
LITERATURE REVIEW

Leishmaniasis

Epidemiology and Ecology

Leishmaniasis is a group of vector-borne diseases caused by obligate intracellular protozoan parasites of the genus *Leishmania*. Twenty one different species of *Leishmania* have been characterized as disease-causing agents (20). Disease outcome is dependent on both the host’s immune status and the infecting parasite species. Clinical manifestation ranges from self-healing cutaneous lesions to fatal visceral disease. Leishmaniasis is endemic in 88 countries affecting regions in the tropics and subtropics, and a variety of settings including rain forests, deserts, rural and peri-urban areas (54). Currently, an estimated 350 million people are at risk for infection and disease and of the 1.5-2 million new cases each year, 70,000 are fatal (111).

*Leishmania* transmission primarily occurs via the bite of infected female sandflies belonging to one of two genuses: *Phlebotomous* (Old World) and *Lutzomyia* (New World). There are approximately 600 known species of sandflies, but of these only 30 are competent vectors (130). Sandflies are found in tropical and sub-tropical climates and live in desert or semi-arid regions. In the Old World (Europe, North Africa, Middle East, and Asia) sandfly contact with humans results from insect breeding in peridomestic areas, while in the New World (the Americas), sandfly contact is associated with humans living or working near forests (130). Female sandflies require blood to obtain the necessary proteins for egg development. Feeding occurs mostly at dawn and dusk and on a variety of animals including mammals, birds and reptiles.

Classically, *Leishmania* parasites have a dimorphic life cycle alternating between the vector and the mammalian host (Figure 1). Infected sandflies can inoculate a host during a
blood meal. The predominant form of the parasite within the vector is the extracellular, highly motile, flagellated promastigote. Promastigotes are long and slender (15-30μm by 2-3μm), covered in a thick glycocalyx composed of a variety of extensively-branched, glycosylphosphatidylinositol (GPI)-anchored glyco-conjugates, including lipophosphoglycan (LPG) and the metalloprotease glycoprotein (gp) 63 (34). Once introduced into a host, promastigotes are internalized by phagocytic cells of the immune system, including macrophages, dendritic cells and neutrophils (119). Following phagocytosis, promastigotes are contained within membrane-bound compartments that mature from early phagosomes to late phagosomes. Ultimately these late phagosomes will fuse with lysosomes to become phagolysosomes. These parasite-containing organelles are referred to as parasitophorous vacuoles (PV) (71). Once inside host cells, promastigotes begin differentiating into the intracellular form of the parasite, the amastigote. This process takes place over a period of 24-72 hours. Amastigotes are smaller (2-6μm), have a vestigial flagellum and are covered by a densely packed glycocalyx primarily composed of glycoinositolphospholipids and glycosphingolipids (34). Amastigotes are able to survive and multiply within the harsh, highly acidic (pH 4.0), environment of the phagolysosome, but maintain a neutral internal pH (15). When a sandfly takes a blood meal from an infected host, amastigote-containing macrophages can be ingested. Amastigotes are released in the sandfly midgut during digestion and begin the process of differentiation into promastigotes.

Disease presentation

Infection with Leishmania parasites can lead to a variety of clinical presentations in the infected host including cutaneous, diffuse cutaneous, muco-cutaneous or visceral leishmaniasis. Disease manifestation depends on the infecting Leishmania species and the immune status of the host. Incubation periods can range from days to years and infection
can remain asymptomatic for the life of the host. Cutaneous leishmaniasis (CL) can present as a small erythematous nodule that develops at the site of the sandfly bite. This lesion progressively develops into an ulcer over a period of 2 weeks to 6 months. Ulcer development (size), progression (healing time), and clinical appearance (localized, diffuse, muco-cutaneous) differ from patient to patient (111). During localized CL, lesions can be large (up to 5 cm) and ulceration rarely penetrates into the subcutaneous tissue, except in the cartilage of the ear (4). These lesions are normally painless and often spontaneously self-heal resulting in lifelong protection from re-infection.

Diffuse CL is the rarest and most severe manifestation of cutaneous disease (50). During diffuse CL, non-ulcerating lesions disseminate from the initial inoculation site and may affect the entire body. Lesion spread can occur locally or hematogenously and may result in deep tissue damage (4). Diffuse CL is difficult to treat and patients do not self cure (111). In the case of muco-cutaneous leishmaniasis (MCL), hematogenous or lymphatic dissemination of the parasite affects the mucosal surfaces of the mouth, nose, and pharynx. MCL can manifest 1-5 years after localized CL has healed, although cases of concurrent localized CL and MCL have been reported (4, 111). MCL does not heal spontaneously, is severely disfiguring, treatment is very difficult and in certain cases, can potentially be fatal (111).

Visceral leishmaniasis (VL), or kala azar, can manifest in a broad range of clinical spectrum from asymptomatic to chronic disease. Parasites primarily infect the lymphoreticular system, which can lead to life-threatening disease if left untreated. Classic clinical signs include lymphadenopathy, hepato- and splenomegaly, pancytopenia, and hypergammaglobulinaemia, and renal disease (54).

*Treatment options*
Several treatment options for leishmaniasis are available but their usage and efficacy depend on a variety of factors. These factors include parasite species, clinical presentation of the disease and the immune status of the host. While most localized cutaneous lesions self heal, drug therapy for cutaneous leishmaniasis (CL) is often used to increase healing time, reduce scarring and prevent potential parasite dissemination to other areas (111). For visceral leishmaniasis (VL), treatment is always recommended due to the fatal nature of the disease.

The only treatment currently available for leishmaniasis relies on chemotherapy (33). Classical, first-line drug treatments include pentavalent antimonials compounds, stibogluconate (Pentostam, Glaxo-Smith-Kline) or meglumine antimonite (Glucantime). Pentavalent antimonials have been in use for more than 50 years and are the recommended treatment by the World Health Organization (WHO) (43, 54, 111). Other common drugs include amphotericin B, pentamidine, imidazoles, allopurinol (Zyloric), miltefosine (Impavido or Miltex) and paromomycin among others.

However, several obstacles remain in the treatment of leishmaniasis. Most treatments require extensive (weeks to months) and invasive (intramuscular or intravenous) modes of administration and there is a high rate of significant drug-related toxic side effects. The cost of treatment is often well beyond the means of many patients and severe toxicity may require secondary treatment, adding to the already high cost of therapy (23, 111). These factors often deter many patients from continuing and completing their therapies. Most importantly, these drugs may only have selective efficacy as a result of drug-resistant parasite strains or patient immunosuppression, or may not be efficacious at all (4, 23, 54).

While orally-available drugs (imidazoles, allopurinol, miltefosine) facilitated administration and have shown limited toxicity, their efficacy remains limited (4, 54). Non-traditional, physical therapies including cryo- and therapeutics are alternative treatment
options used for CL lesions (73, 112). However, they have not gained much recognition due to limited availability of equipment, increased secondary infections, parasite dissemination, and the appearance of larger scars following treatment (4).

Although a variety of drugs are available to treat leishmaniasis, treatment efficacy for MCL and VL remains subpar. In most cases parasites are never completely eliminated and disease recrudescence is very common. Additionally, the emergence of parasite resistance, increasing co-infections—especially with the increasing incidence of HIV—and the high cost of certain treatments all decrease the number of options available to patients. While current drugs all target the parasite itself, novel treatment options that target the parasite and work with the host immune response should be considered. Developing vaccines against Leishmania parasites has proven a difficult task and although a canine vaccine has been developed and it is in use in South America (reviewed in (32), at present no immunization options are available against leishmaniasis in humans.

**Visceral leishmaniasis**

With an estimated 500,000 new cases of visceral leishmaniasis (VL) and 59,000 annual deaths, VL is second only to malaria in annual worldwide fatalities due to protozoal infections (8, 132). Over 90% of VL cases occur in six countries: Bangladesh, India, Nepal, Sudan, Ethiopia, and Brazil (23). The main factors driving VL incidence include migration, urbanization, lack of vector and/or reservoir control measures, opportunistic co-infections, and civil war (8, 23).

VL is caused by infection with L. *donovani* parasite species in East Africa and India, and by L. *infantum* infection in Europe, North Africa, and Latin America (23). Various *Phlebotomous* and *Lutzomyia* species of sandflies have been shown to transmit parasites in the Old and New Worlds, respectively. There are two types of VL, anthroponotic, transmitted
between humans and the vector, and zoonotic, transmitted from animal to vector to human. *L. donovani* transmission is anthroponotic while *L. infantum* is found in areas of zoonotic.

Zoonotic visceral leishmaniasis (VL) is caused by *L. infantum* and both dogs and humans are natural hosts (115). In endemic regions, canine infection with *L. infantum* has a prevalence of 63-80% of the population, but shows a low rate of clinical disease (7). Infected dogs are considered the primary peridomestic reservoir for zoonotic VL and the most significant risk factor predisposing humans to *L. infantum* infection (23, 132). VL in dogs parallels both immunologic alterations and the pathophysiology of human disease (13).

**Host immune response to visceral leishmaniasis**

Host protection against VL requires a pro-inflammatory, T helper (T<sub>H</sub>) 1 immune response, as characterized by the production of interleukin (IL)-12 by antigen presenting cells and IL-2, tumor necrosis factor alpha (TNF-α) and interferon (IFN)-γ by T cells (94, 95). Infected macrophages are activated by IFN-γ and TNF-α to kill intracellular amastigotes via the L-arginine nitric oxide pathway (88, 93, 146). Cured or subclinical patients are able to mount antigen-specific IFN-γ responses following *Leishmania* antigen stimulation *in vitro*. Treatment-cured individuals can be resistant to re-infection and become leishmanin skin test positive, suggesting no inherent defect in the *Leishmania*-dependent T<sub>H</sub>1 response (19, 45, 149).

In experimental models of cutaneous leishmaniasis, susceptibility can be associated with a T<sub>H</sub>2 humoral-mediated immune response and production of IL-4 or the lack of a T<sub>H</sub>1 immune response. However, VL does not show a clear T<sub>H</sub>1/T<sub>H</sub>2 dichotomy pattern (137, 138), as both T<sub>H</sub>1 and T<sub>H</sub>2 cytokine responses have been reported in both asymptomatic and symptomatic dogs (22, 122). One feature of susceptibility to developing clinical VL is the appearance of *Leishmania*-specific lymphoproliferative unresponsiveness and decreased
production of IFN-γ following *Leishmania* antigen stimulation (48, 120). This loss of lymphoproliferation and T<sub>H</sub>1 polarization can be attributed to the induction of an immunosuppressive response, geared towards preventing damaging immune-mediated pathology (84).

Active disease in humans is associated with elevated IL-10 levels in serum and enhanced IL-10 mRNA in lesional tissues (47, 84). In canine VL (CVL), IL-10 production has been positively correlated with parasitic load and progression of clinical disease in naturally infected dogs (72). IL-10 is an immunoregulatory cytokine produced by a variety of cells including T cells, B cells, macrophages, and dendritic cells. The regulatory functions of IL-10 extend to both innate and adaptive immune responses. IL-10 modulates the expression of inflammatory mediators, cytokines, and surface molecules in cells of myeloid origin, thus interfering with their ability to promote and maintain inflammatory responses (81). The presence of IL-10 is one factor that leads to a shift in the balance from a pro-inflammatory and effective immune response to a regulatory and dysfunctional immune response not capable of controlling disease progression.

Another immunological parameter associated with disease progression and suppression of the immune response to VL is hypergammaglobulinemia (109). Dogs with clinical disease produce higher levels of *Leishmania*-specific immunoglobulin (Ig) G as compared to clinically healthy dogs (2, 57, 132). In addition there is a correlation between high *Leishmania*-specific IgG levels and low *Leishmania*-specific cell-mediated immunity (41, 76, 95, 114). Four IgG subclasses have been described in the dog (IgG1-4), with IgG1 and IgG2 being the most studied in regards to CVL. However, a relationship between IgG subclass profile and disease resistance versus susceptibility is unclear. Thus to date the role for IgG1 and IgG2 production during infection and disease development remains controversial (103, 109, 116).
Production of other immunoglobulins, such as IgM and IgA, is also increased in symptomatic dogs as compared to asymptomatic dogs (109, 116). However, no protective role of these antibodies has been shown. The overall increase in IgG, IgM, and IgA antibodies suggests the development of a strong but non-protective *Leishmania*-specific humoral immune response in dogs with clinical CVL.

The mechanisms contributing to CVL susceptibility are not fully understood. It has been shown that certain dog breeds such as Boxers, Cocker spaniels, Rottweiler and German shepards seem to be more susceptible to disease, while other breeds such as the Ibizian hound are more resistant to CVL (132). Several genetic polymorphisms in TNF-α, certain MHC class II alleles, and solute carrier family 11 member a1 (N-RAMP1)—an iron and divalent cation antiporter involved in innate resistance to intraphagosomal pathogens—have been associated with susceptibility (65, 104). Other factors such as age, gender, nutrition, co-infections, immunosuppressive conditions, parasite burden, virulence of *Leishmania* strain, previous infections and mode of transmission can affect the course of disease development (132).

**Canine visceral leishmaniasis in the United States**

Although VL is not endemic in the United States (US), canine visceral leishmaniasis (CVL) has recently been described in this country within the Foxhound population. The most recent outbreak of CVL in the US occurred in 1999 in a foxhound kennel in New York (46). By 2005, it was reported that 60 kennels in 22 states and two Canadian provinces had *L. infantum*-seropositive Foxhounds, and that autochthonous infection in canines was limited to the Foxhound population (38).

Disease transmission within the Foxhound population has not been shown to be vector-dependent. Fourteen species of *Lutzomyia* sandflies are found within the United
States, of which three (*Lutzomyia anthopora*, *Lutzomyia diabolica*, and *Lutzomyia shannoni*) can transmit at least one *Leishmania* species, *L. mexicana* (66). *Lutzomyia shannoni* could serve as a potential vector for *L. infantum* and is currently found in the Southern, Midwestern and Southeastern US (38). Anecdotal data indicates that *Lutzomyia shannoni* sandflies within the US can become infected with *L. infantum*, however whether these flies can support metacyclogenesis (parasite development within the insect vector) and transmit infection is still unknown.

Other means of transmission have been postulated for autochthonous CVL in the United States including horizontal (dog to dog) and vertical (transplacental or transmammary) mechanisms. Transmission via packed red blood cell transfusions from infected foxhounds have been documented (87). Housing practices in Foxhound kennels place large numbers of animals together where potential horizontal transmission (direct blood to blood contact) can occur. Transplacental transmission in Beagles after experimental infection with *L. infantum* has been reported (117) and recently, transplacental transmission in *L. infantum* and *L. chagasi* naturally-infected dogs in endemic countries has been shown (30, 89). Analysis of fetal tissues from symptomatic and asymptomatic female dogs revealed the presence of *L. chagasi* amastigotes via immunodetection and PCR, indicating that vertical transmission does occur. Our own studies have shown, via PCR, the presence of *L. infantum* DNA in multiple tissues of neonatal puppies (lymph nodes, bone marrow, liver, lung, thymus and spleen) born to a sero-positive, *L. infantum*-infected Foxhound female (unpublished data).

Despite potential alternative modes of transmission, the clinical presentation and disease pathology of infected Foxhounds is equivalent to that of infected dogs in endemic areas (49). Physical examination findings of infected Foxhounds include depression, loss of muscle mass, mildly distended abdomen, epistaxis, dull hair coat, hepatosplenomegaly and
generalized lymphoadenopathy, consistent with findings in endemic regions (reviewed in (132)). Clinical findings including decreased hematocrit, thrombocytopenia and signs of renal failure and hepatic compromise (reviewed in (92)). Gross and histopathological examination of tissues from infected Foxhounds in the US revealed liver and spleen enlargement, systemic lymphoadenopathy, glomerulonephritis, and the presence of *Leishmania* amastigotes in a variety of tissues (13, 49).

While CVL presentation and pathology in Foxhound mirrors disease in endemic regions, to date, the host immune response to natural *L. infantum* infection in the US has not been described. The Foxhound model of CVL not only presents a unique opportunity to study the host immune response to *L. infantum* infection in a traditionally non-endemic region, but also under conditions of non-vector transmission.

**Cutaneous leishmaniasis**

The number of human cutaneous leishmaniasis cases globally has increased in the last decade as documented both in the Old and New Worlds (111). Approximately 350 million people are at risk for infection with a world-wide prevalence of 12 million and an annual incidence of 1.5 million cases of CL (4). The increased number of cases can be explained, in part, by improved diagnostics. However, inadequate vector and/or reservoir control, CL associated with opportunistic co-infections, and the emergence of anti-leishmanial drug resistance have contributed to the observed increased dissemination of CL (111). Increased incidence of CL can also be attributed to migration of people into or from endemic areas, deforestation and urbanization, tourism, natural disasters, armed conflict and global warming—allowing spread of vector habitat (4, 54, 111, 125).

CL is primarily found in Africa, Asia and in Latin American countries. In the Old World several species of *Leishmania* can cause CL including *L. major*, *L. aethiopica* and
dermotropic *L. infantum* (50), with *L. major* having the largest geographical distribution. *L. major* is widely distributed in arid and savannah regions, surviving in several rodent reservoirs. *Plebotomous* species are well characterized vectors for *L. major*, with *P. papatasi* acting as the principal vector. Infection with *L. major* primarily causes localized, self-healing CL. Epidemics of CL caused by *L. major* were reported in endemic countries in the 1980s and coincided with rodent migrations or fluctuations in rodent populations (50).

In the New World, over 11 species of *Leishmania* have been characterized as causative agents of CL, including *L. amazonensis*, *L. braziliensis* and *L. panamensis*. *L. amazonensis* has one of the widest geographical distributions affecting Brazil, Bolivia, Colombia, Ecuador, Peru, French Guyana, Panama and Venezuela (50). Forest rodents are the main reservoir, with marsupial species acting as secondary hosts. *L. amazonensis* parasites are mainly transmitted by *Lutzomyia flaviscutellata*. Infection with *L. amazonensis* can cause localized or diffuse CL and unlike most cases of *L. major* infection, *L. amazonensis* can result in non-healing chronic disease.

*Murine cutaneous leishmaniasis: T11/T12 balance?*

Protective immunity against *Leishmania* infection in mice is dependent on the development of a T1 cell mediated immune response (reviewed in (118)). This is characterized by the production of interleukin (IL)-12 by antigen presenting cells (APC), interferon gamma (IFN-γ)-producing CD4⁺ T cells and activation of macrophages to produce nitric oxide (NO) and peroxinitrite to eliminate intracellular parasites. Classically, disease susceptibility in mice is thought to result from the induction of T12 responses, characterized by APC that fail to produce IL-12 but release IL-4 or IL-10, leading to the priming of IL-4-producing CD4⁺ T cells, resulting in alternatively-activated macrophages that do not effectively promote parasite killing.
Studies of murine models of *L. major* infection provided the first direct evidence of the importance of Th1/Th2 balance to the regulation of disease resistance versus susceptibility (108, 118). Early studies in this field demonstrated the role for CD4+ T cells and the production of IFN-γ as key regulators of resistance and parasite clearance (55, 74). Via an adoptive transfer model, it was shown that while Th1 cells protected susceptible mice from *L. major* infection, Th2 cells promoted disease (126). This protective response was directly linked to the production of IFN-γ, as depletion of this cytokine in vivo led to disease susceptibility, while neutralization of the Th2 cytokine IL-4 led to increased resistance in susceptible mice (10, 52).

Infection of either *L. major* resistant (C3H and C57BL6) mouse strains with *L. amazonensis* results in non-healing, chronic disease. The immune response associated with susceptibility to *L. amazonensis* infection is not dependent on the development of a Th2 type of immune response, but rather the lack of a Th1 immune response. Infection with *L. amazonensis* shows low to undetectable levels of both IFN-γ and IL-4 (1, 61). In these animals, susceptibility is thus associated with the absence of an efficient Th1 immune response rather than the presence of a Th2 response. The lack of a Th1 response persists in the absence of IL-4 and IL-10. Treatment with exogenous IL-12, a Th1-polarizing cytokine, to promote parasite clearance also fails to promote healing (1, 60, 61). Furthermore, analysis of antigen-responsive CD4+ T cells from C3HeB/FeJ mice chronically infected with *L. amazonensis*, indicate that these cells have an impaired ability to transition from a naïve to an effector phenotype (106). Altogether, these data suggest that while a population of antigen-specific CD4+ T cells are present during *L. amazonensis* infection of C3HeB/FeJ mice, improper Th1-polarized priming may responsible for the non-healing phenotype.

**Promoting a Th1 immune response**
The induction of protective T\(_h\)1 cell-mediated immune responses is driven by the cytokine IL-12, a heterodimer composed of p35 and p40 subunits. During the initiation of an immune response, IL-12 is produced by antigen-presenting cells (APC), such as macrophages and dendritic cells (DC). While p35 is expressed constitutively at low levels, p40 gene expression is inducible in response to microbial stimuli via engagement of Toll-Like Receptors (TLR) (143, 148) and activation of nuclear factor-κB (NF-κB) family members. IL-12 binds the IL-12 receptor (IL-12R) complex composed of IL12-Rβ1 and IL-12Rβ2, and signals through the transcription factor STAT4 to mediate biological responses. IL-12 plays a critical role in the priming of naïve T cells to proliferate and differentiate into T\(_h\)1 cells that secrete IFN-γ (148). In addition, production of IL-12 prevents T\(_h\)2 polarization and the production of T\(_h\)2-related cytokines.

However, microbial signals alone are not enough to trigger production of the bioactive IL-12 heterodimer. Additional activating signals are necessary to enhance its production, including IFN-γ secretion and direct cell to cell contact (82, 143, 148). One such cell to cell interaction occurs via the secondary receptor CD40 expressed on the APC and CD40 ligand (CD40L) expressed on the surface of lymphocytes. CD40 is a 43-50kDa protein belonging to the tumor necrosis factor (TNF) family of receptors originally identified on B cells. It is now known that CD40 is widely expressed by a variety of cell types including monocytes, DC, hematopoietic progenitors, endothelial and epithelial cells (124). CD40-CD40L interaction is a bidirectional event providing activating signals to both APC and lymphocytes (91). CD40-CD40L stimulation promotes T cell proliferation and IL-2 and IFN-γ production and enhances the production of cytokines from APCs, including IL-12 via activation of the nuclear factor-kappa B (NF-κB) pathway (91, 153).

The role of IL-12 during *Leishmania* infection has been extensively studied and deficiencies in IL-12 or IL-12R subunits are associated with disease susceptibility to *L. major*. 
infection in genetically-resistant mouse strains (21, 78). IL-12 is also required not only in the initiation but also in the maintenance of T_1 immune response to _L. major_ infection (90).

Genetic deficiencies in CD40 or CD40L have proven to be detrimental to the host during the initiation of protective immune responses against both intracellular and extracellular pathogens (77). These findings indicate the importance of CD40 in activating both T_1 and T_2 responses to provide host protection against infection. Several reports have shown that during _Leishmania_ infection the absence of CD40 or CD40L results in disease susceptibility (17, 63, 133) owing to decreased IL-12 production and an inability to mount a protective T_1-mediated immune response.

**Dendritic cell biology**

Dendritic cells (DC) are unique antigen presenting cells (APC) because of their efficiency in translating innate immune messages into adaptive immunity (6, 110, 136). These messages can be either immunogenic or tolerogenic in nature. The properties that allow these cells to perform different activities rely on the ability of DC to sense their environment via antigen-sampling, appropriately respond to activating cues, and deliver messages during antigen (Ag) presentation to T cells.

DC are classically described as being either immature or mature, referring to both phenotypic and functional characteristics. A key feature of immature DC is their ability to efficiently capture Ag (80) via a variety of pathways including macropinocytosis, receptor-mediated endosytosis and phagocytosis (5). Ag is taken up and targeted to major histocompatibility complex (MHC) class II-positive lysosomes, where it will be retained for future use as immunogenic peptides (56, 144). Upon Ag encounter, immature DC undergo phenotypic and functional changes, collectively known as DC maturation. These changes
will provide the “immunological context” under which adaptive responses—immunogenic or tolerogenic—will be initiated.

Mature DC have a reduced capacity for Ag uptake, but have an enhanced ability for Ag presentation, and therefore, greater capacity for priming immune responses (5, 6). This process is characterized by their ability to generate functional peptide-major histocompatibility complex (MHC) class II complexes (56, 144), redistribute these complexes to the plasma membrane, upregulate surface co-stimulatory molecules (i.e. CD40, CD80, and CD86) and produce APC-related factors (i.e. cytokines) (5). These changes transform DC from an Ag-sampling cell to an Ag-presenting cell, able to promote the expansion and differentiation of T cells into appropriate effector cells (110). The maturation process also results in upregulation of chemokine receptors that facilitate DC migration into secondary lymphoid organs where encounter with naïve T cells will occur.

During DC-T cell interactions, three signals are delivered from the APC to the naïve lymphocyte. The first activating signal, ‘signal one’ is delivered during peptide-MHC binding to cognate T cell receptors (TCR). This signal transmits the molecular identity of the Ag and determines the specificity of the response (59, 62, 64). Co-stimulatory molecules on the APC, such as CD40, deliver ‘signal two’ by engaging counter-receptors on the T cell. This second signal is critical for T cell survival and proliferation, as in the absence of co-stimulatory signals, antigen presentation via the TCR leads to anergy or deletion (64). The third signal is delivered by mediators produced by DC, such as cytokines, and provides polarization of the immune response (27, 59).

*Dendritic cell subsets*

In the bone marrow, two progenitors have been shown to give rise to DC, lymphoid and myeloid. The DC population that arises from these progenitors is a sparsely distributed,
heterogenous population composed of multiple subtypes of DC with distinct life spans and functions (150). In the steady-state, myeloid progenitors give rise to immature DC that migrate through the circulation into peripheral tissues and become resident DC, also known as conventional DC (cDC). cDC can be further characterized into different subsets depending on their location: Langerhans cells in the skin, mucosal-associated DC in mucosal tissues, interstitial DC in the interstitial spaces of an organ, lymphoid-tissue associated DC, etc. Myeloid progenitors may also give rise to monocyte-derived DC and tumor necrosis factor (TNF)-producing and nitric oxide synthase (iNOS)-expressing DCs in response to inflammation (5, 27, 150). Lymphoid progenitors give rise to all DC subtypes in the lymphoid tissues and to type I interferon-producing plasmacytoid DC (pDC).

T cell polarization

Given the different DC subsets that have been characterized and the distinct microenvironment where they reside, it was proposed that DC function was pre-determined by lineage. Several initial studies suggested that distinct DC subsets might regulate TH responses differentially, more specifically, that myeloid-derived DC preferentially primed TH1 responses while lymphoid-derived DC primed TH2 or tolerogenic responses (27, 101). However, this data was later challenged by reports showing that DC had a high degree of functional plasticity in response to pathogens, and that their ability to induce TH1 versus TH2 responses was dependent on the nature of the microbial stimulus, various environmental factors (i.e. local inflammatory or anti-inflammatory mediators), tissue of residence, and in vitro culture conditions under which DC were being studied. These data suggested that while genetic pre-programming may affect the TH polarizing function of DC to an extent, the dynamic environment under which DC become activated affects its effector phenotype and the ensuing adaptive immune response (62).
It is now clear that immature DC can become mature DC with different effector functions capable of priming distinct T cell fates including T helper as well as T regulatory responses (110). This is largely determined by environmental stimuli, both of exogenous (microbial) and of endogenous origins (cytokines, chemokines, hormones, apoptotic/necrotic cells) in which DC become activated. Alteration of DC function can enhance pathogen survival and persistence within the host. Suppression of DC maturation, antigen presentation, and altered DC migration have been shown to be modulated by pathogens including *Leishmania* parasites (reviewed in (119)).

**Signals that trigger DC maturation**

Dendritic cells express a variety of pattern recognition receptors (PRR) on their surface responsible for sensing the presence of pathogens. The stimuli received through ligand-receptor interactions trigger signaling pathways that mediate host-cell responses (i.e. maturation) and can alter the biological response of the host (67). The balance of particular molecular signaling cascades has been shown to affect the maturation process of DC (5, 26, 113). Two such pathways are the NF-κB and mitogen activated protein kinase (MAPK) signaling cascades. MAPK pathways terminate in three primary kinases, p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2). Activation of NF-κB, p38 and JNK positively regulate DC maturation and promote the production of a variety of inflammatory cytokines including TNF-α, IL-1, IL-6 and IL-12 (154). In contrast, activation of the ERK1/2 pathway negatively regulates DC maturation resulting in decreased expression of maturation markers and production of pro-inflammatory cytokines (100, 113). ERK1/2 activation can also enhance release of IL-10 from DC promoting a pro-tolerogenic DC phenotype (18).
The Mitogen Activated Protein Kinase Pathway

The mitogen activated protein kinases (MAPK) are a family of intracellular kinases involved in the regulation of a wide variety of cellular processes including embryogenesis, cell differentiation, proliferation, migration, apoptosis, and innate and adaptive immune responses. There are three main families of MAPK, the extracellular signal-regulated kinases (ERK) 1 and 2 (Figure 2), the stress-activated protein kinase (SAPK) 1, also known as the c-Jun N-terminal Kinase (JNK1-3), and the SAPK-2, p38α/β/γ.

All MAPKs consist of a Thr-X-Tyr motif in their activation loop, and phosphorylation of both threonine and tyrosine residues are required for their activation (154). Phosphorylation of MAPK is accomplished through a triple kinase cascade. Dual phosphorylation of MAPK is carried out by upstream MAPK kinases (MKKs), which are dual-specificity kinases that themselves are activated by MKK kinases (MKKKs) via phosphorylation of their Ser/Thr residues (154).

Despite the simple architecture of MAPK activation, these pathways can respond to a variety of stimuli leading to different and specific outcomes in gene expression and ultimately cellular function. While it is not completely understood how these responses are fine-tuned, it has become clear that signal kinetics (activation/deactivation), subcellular localization, substrate availability, and the formation of signaling complexes play a role in this regulation (105, 152, 154).

Raf-MEK1/2-ERK1/2 Pathway

ERK1/2 are ubiquitously expressed 44 and 42kDa proteins activated by a variety of stimuli including growth factors, serum, ligands for heterotrimeric G-coupled protein receptors (GPCR), cytokines, stress factors, microtubule depolymerization, and pattern recognition receptors (PRRs) (24, 105, 154). ERK1/2 is also ubiquitously distributed
intracellularly and found to be associated with plasma membrane receptors and transporters, cytoplasmic microtubules, and on intracellular compartments (i.e. Golgi, endosomes). In addition to the microtubule cytoskeleton, ERK1/2 interactions with its upstream kinase, MEK1/2, have been shown to play a critical role in maintaining cytoplasmic distribution of ERK1/2 (105).

Activation of ERK1/2 requires dual phosphorylation which is exclusively carried out by the upstream MKKs, MEK1 and MEK2. MEK1/2 contain a kinase domain which itself requires dual phosphorylation on both serine and threonine residues for their activation. In addition to its kinase domain, MEK1/2 contains three non-enzymatic domains necessary for their function: an ERK1/2 binding domain, a proline-rich domain, and a nuclear export sequence (NES). The ERK1/2 binding domain, also known as a D domain, interacts with common docking (CD) motifs on ERK. These CD motifs are necessary for ERK regulation as mutations that modify or delete this domain not only interfere with ERK phosphorylation they also affect subcellular distribution of ERK (24). The proline-rich domain is absent from other MEK family members and may serve as docking sites for other proteins, including scaffolding proteins MEK partner 1 (MP1). The NES of MEK1, as mentioned above, plays a critical role in maintaining cytoplasmic distribution of ERK2.

Dual phosphorylation of MEK1/2 is carried out by the MKKK, RAF. All isoforms of RAF, A-RAF, B-RAF, C-RAF and Raf-1 have been shown to activate the ERK1/2 pathway. RAF appears to be the major, if not the exclusive, activator of MEK1/2. However, RAF-1 is the most widely distributed protein and the most commonly studied (24, 105, 154). In its inactive state, RAF is found in the cytosol and bound to regulatory proteins (14-3-3 and possible connector enhancer of KSR (CNK)) maintaining RAF as a closed, catalytically inactive form (107). Upon stimulation, RAF translocates to the plasma membrane where it
can interact with GTP-loaded RAS. This interaction causes conformational changes leading to the release of the regulatory proteins, and subsequent activation (85, 107).

**MAPK pathway and therapeutic intervention**

A large number of cellular processes are regulated by the RAS-MAPK pathway and aberrant signaling through this cascade has been associated with a variety of conditions from cancer to inflammatory diseases (reviewed in (128)). The central role of MAPK in regulating cell growth and survival has made this pathway an attractive target for anti-cancer therapies. Due to the specificity of the RAS-RAF-MEK signaling components, it is possible to selectively inhibit this pathway without affecting other MAPK signaling cascades.

Pharmacological interventions are now focusing on exploiting the unique properties in these signaling cascades. Small-molecule inhibitors are low molecular weight organic compounds that bind proteins, nucleic acids, or polysaccharides with high affinity. Their small size, no bigger than 800 Daltons, allows these molecules to easily diffuse through cell membranes and reach targets within intracellular sites. Small-molecule inhibitor drugs are currently being utilized to target RAS, RAF and MEK for therapeutic intervention. Several small-molecule, non-ATP binding inhibitors have been developed for MEK inhibition, starting with PD98059 to the more recently, orally-available inhibitors CI-1040 and its related compounds (37, 127, 128).

Currently a variety of small-molecule inhibitors for the Ras-MAPK pathway are available for anti-cancer treatment and several more are undergoing clinical trials. These inhibitors can potentially be utilized in the treatment of other diseases, including infectious diseases, whose pathogenesis is mediated by the MAP kinase pathways. However, given the large repertoire of MAP kinase ERK substrates including transcription factors, kinases, phosphatases, cytoskeletal proteins, apoptotic proteins, and others (152), one important
aspect in the development of new therapeutic drugs is the idea of targeting specific signals within this pathway, rather than global suppression of ERK activation. Recently, scaffolding proteins have been described, which regulate the temporal and spatial regulation of ERK signaling (reviewed in (14, 35, 70)). These findings have raised interest in utilizing scaffold proteins as therapeutic targets.

**Leishmania host cell interactions**

**Modulation of DC maturation**

Studies of low dose infection in mice with *L. major* promastigotes revealed an immunologically silent phase of infection before the onset of an immunological response. During this time, parasite replication occurred with minimal production of IL-12 or IFN-γ suggesting the absence of an ongoing immune response (9). These data corroborated *in vitro* studies showing that *Leishmania* could actively inhibit immune activation of their host cells including Ag-presentation, and decreased expression of both MHC class II and co-stimulatory molecules (CD80, CD86, CD54, and CD40) (11, 39, 68, 97, 98, 121).

*Leishmania* spp. can also differentially regulate cytokine production by infected DC. While infection with *L. major* leads to the secretion of IL-12, infection with *L. donovani* and *L. tropica* leads to inhibition of IL-12 production in response to activating stimuli such as lipopolysaccharide (LPS) and IFN-γ (79). *L. amazonensis* internalization via Fc receptors results in the release of anti-inflammatory cytokines such as IL-10 (147). Altered migration has also been reported following infection with *L. major in vitro* (58, 96). Decreased DC migration has also been observed *in vivo* following *L. donovani* infection, as a result of decreased expression of the chemokine receptor CCR7 (3).

*Leishmania modulation of host cell signaling pathways*
*Leishmania* parasites have been shown to alter host-cell signaling pathways to promote their survival. Activation of host Src homology 2 domain (SH2) containing tyrosine phosphatases (SHP-1) by *Leishmania* infection results in global dephosphorylation of tyrosine residues, leading to deactivation of a variety of signaling pathways including JAK/STAT, NF-κB, IRF-1, and MAP kinases (reviewed in (67, 86)). Increased concentrations of secondary messengers including calcium (Ca^{2+}), inositol lipids, inositol phosphatases, and PKC have also been observed following *Leishmania* infection. Induction of the negative regulatory proteins, suppressors of cytokine signaling (SOCS), have been characterized following *L. donovani* infection and interfere with cytokine signaling and host cell activation (12). *L. mexicana* cysteine peptidases have been shown to degrade NF-κB leading to modulation of this pathway (16). Proteosome-mediated degradation of signaling components, specifically STAT1α, has also been observed to play a role in *Leishmania*-mediated modulation of host signaling pathways (42). More recently, cleavage of the NF-κB p65 subunit by the *Leishmania* proteolytic glycoprotein (gp) 63 has been observed following *L. donovani*, *L. major*, *L. mexicana*, and *L. braziliensis* infection of macrophages *in vitro* (51).

Most studies aimed at understanding the regulation of signaling pathways triggered by *Leishmania* parasites have focused on the initial events of host-parasite interactions. However, *Leishmania* parasites reside intracellularly within parasitophorous vacuoles (PV) and it is from within these intracellular compartments that parasites continue to engage cell signaling pathways and alter the host’s biological responses (67). This has been demonstrated by the presence of products of the phosphoinositide 3-kinase (PI3K) pathway on PV following *L. amazonensis* infection. This observation suggests that these organelles could serve as activation sites for downstream kinases of this pathway, such as protein kinase (PK)B/Akt (67).
Signaling from endosomes

The endocytic pathway

Entry into host cells through the endocytic pathway is a common feature of most intracellular pathogens, including *Leishmania*. Some pathogens enter the endocytic pathway via phagocytosis, an endocytic pathway through which large particles (>0.5μm in size) are internalized and contained within membrane-bound organelles known as phagosomes. Internalized material via phagocytosis follows a pre-determined pathway ultimately leading to the degradation of its content. This process known as phagosome maturation results in the sequential acidification of these compartments, ultimately leading to fusion of phagosomes with lysosomes. The stages of phagosome maturation can be classified and tracked via their associations with Rab proteins (GTPases), lipids (phosphoinositides), and glycoprotein (lysosomal-associated membrane protein LAMP) composition which not only phenotypically distinguish between different maturation stages, but are also important in the maturation process (69).

The parasitophorous vacuole (PV) in which *Leishmania* resides is composed of host plasma membrane, phagosomal organelles, and parasite-derived molecules (53). PV display phagolysosomal features characterized by a highly acidic luminal pH, the presence of proteases, phosphatases, and cytosolic surface expression of Rab7, LAMP1 and LAMP2 (29, 71). These organelles are highly dynamic in their structure and composition. Acquisition of endosomal components—and their content—is an ongoing process, as fusion of newly internalized materials and recycled cytoplasmic molecules are delivered into lysosomal compartments, including *Leishmania*-containing PV (15). Based on recent work suggesting the important role of endocytosis in signal transduction (reviewed in (134)), signaling components (i.e. extracellular receptors, lipids, kinases, adaptor and scaffold proteins) could
be delivered to parasite-containing PV. Once associated with PV it is feasible that these signaling molecules could be utilized by the parasite to trigger signaling cascades.

*Endosomal signal transduction*

Receptor internalization upon ligand binding is a common regulatory mechanism for receptor desensitization (134). A variety of receptors including G-protein coupled receptors (GPCR) and receptor tyrosine kinases (RTK) are rapidly internalized upon ligand binding and are either recycled back to the plasma membrane or sent to lysosomes for degradation. Although this process was initially thought to play a role in signal attenuation, the presence of activated epidermal growth factor receptors (EGFR) and downstream effectors on endosomes, first suggested the possibility of signaling from endosomes (36). The idea of endosomes serving as signaling platforms for RTK and GPCR has since been strengthened by subsequent studies showing the myriad of signaling proteins found associated with endosomal compartments, attenuated activation of signaling pathways when endocytosis is inhibited or when endosomal compartments are mis-localized inside the cell (reviewed in (31, 135)) (139, 145).

Most of these studies have focused on the activation of the mitogen-activated protein kinases (MAPK) pathway ERK1/2 following RTK or GPCR trigger. ERK1/2 activation has been shown to occur both at the plasma membrane and from within endosomal compartments following receptors internalization (reviewed in (134)). The presence of activated MAP kinases on endosomal membranes and the distinct pattern of ERK activation from this subcellular location (140) supported the concept that intracellular signaling could serve to control MAPK signal specificity. The characterization of scaffold proteins provided a mechanism for the recruitment and subcellular localization of MAPK signaling components.
Scaffold proteins in signal transduction regulation

Scaffolding proteins are molecules that can bind to two or more signaling proteins, regulate signal transduction, and localize signaling molecules at specific sites within the cell (i.e. plasma membrane, golgi, endosomes). The first scaffolding proteins to be identified were characterized using receptor tyrosine kinases, platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) (28). These initial studies showed recruitment of then unidentified proteins that bound to phosphorylated SRC homology 2 (SH2) domains on the intracellular domain of the receptors. These sites appeared to serve as docking or scaffolding domains for other proteins and/or substrates. The proteins recruited at these sites were hypothesized to determine the strength and type of signal triggered.

At least four different functions have been characterized including the idea that these proteins serve as platforms for assembly of signaling cascades, coordinate positive and negative feedback signals, compartmentalize signals to specific sites within the cell, and protect activated molecules from inactivation or degradation (131). Although further roles of many scaffold proteins are not yet understood, in general, scaffold proteins may provide a critical level of regulation during signal transduction shedding light on how cells can differentiate between messages within an ubiquitous signaling cascade.

Scaffolding proteins of the MAPK ERK Pathway

Scaffolding proteins in the MAPK signaling pathway were first identified in the budding yeast S. cervisiae (reviewed in (35)). Using the pheromone signaling mating response as a stimulus, activation of the S. cervisiae equivalent of the MAPK pathway was analyzed, revealing the requirement of a protein encoded by Ste5. In its absence, activation of this pathway was abolished (25, 75, 99). Yeast-two-hybrid analysis and co-purification
studies indicated that the different domains of Ste5 specifically interact with Ste11, Ste7 and Fus3 (MAP3K, MAP2K, and MAPK) forming a signaling module that provides specificity to the mating signal by co-localizing Ste11-Ste7-Fus3 and optimizing the orientation of Ste11 so as to be phosphorylated by its upstream kinase (40, 102).

To date seven different scaffold proteins have been identified in the regulation of ERK1/2 signaling: IQ motif-containing GTPase-activating protein 1 (IQGAP1), kinase suppressor of Ras (KSR), β-arrestins, paxillin, similar expression of FGF (Sef), MEK partner-1 (MP1), and MAPK organizer 1 (MORG1). Compartmentalization in MAPK/ERK signaling has emerged as a critical component in the spatial arrangement and regulation of this pathway (14, 70). The importance of compartmentalization is highlighted by the variety of these scaffold proteins involved not only in localization but also regulating nucleocytoplasmic distribution of activated MAPK/ERK (70).

KSR (Figure 3) is one of the best characterized scaffold proteins of the MAPK signaling cascade, interacting with c-Raf, MEK1/2 and ERK1/2 (14). Association with MEK is constitutive while interaction with ERK1/2 occurs only after stimulation. KSR localizes to the plasma membrane, thus regulating extracellular activation of the MAPK pathway. Studies in KSR-deficient mice have revealed a defect in cytokine production, peripheral T cell proliferation, and defective activation of ERK in response to TNF-α and IL-1 stimulation (44). KSR enhances the generation of activated/phosphorylated ERK, which can phosphorylate both nuclear and cytoplasmic substrates.

β-arrestins are known regulators of G protein coupled receptors (GPCRs). β-arrestins have been shown to interact with Raf, MEK, and ERK, and recruit these proteins to the activated receptor. β-arrestins promote the internalization of GPCRs and recruitment of MAPK signaling components to early endosomes (142). Interestingly, MAPK activation via
β-arrestin association leads to cytosolic activation of ERK1/2, but does not promote its nuclear translocation (14).

MP1 (Figure 3) is a widely expressed scaffold protein for ERK1/2 signaling originally isolated from a two-hybrid screen conducted to identify non-enzymatic components of the MAPK signaling cascade (123). MP1 is a 13.5kDa protein that specifically interacts with MEK1 and ERK1, but not MEK2 or ERK2. MP1 was shown to co-localize with late endosomes via the adaptor protein p14, which is associated with the cytosolic side of late endosomes (151). More recently, the lipid raft adaptor protein p18 has been identified as an anchor for the p14-MP1-MEK complex on late endosomes (83).

While in vitro studies have shown that MP1, p14, and p18 can augment MEK1 phosphorylation and therefore subsequent ERK1 phosphorylation, the importance of these adaptor and scaffold proteins in ERK signaling are evident as depletion of any of these proteins leads to deficiencies in ERK activation and kinase activity. Using epidermal growth factor (EGF) stimulation, it has been demonstrated that silencing of MP1 and p14 via short interfering (si) RNA leads to reduced activation of ERK1 (141). In the absence of MP1, p14 does not interact with MEK1 or ERK1 and in the absence of p14, MP1 is mis-localized to the cytoplasm, leading to decreased phosphorylation of ERK from late endosomes. In p18-/- cells, the p14-MP1 complex is excluded from late endosomes, again resulting in decreased ERK activation from these compartments (83). Plasma membrane-mediated activation of ERK following EGF stimulation is not affected by the absence of MP1, p14 or p18, further demonstrating the role of these proteins in the endosomal-mediated activation of ERK.

Similar to MP1, MORG1 (Figure 3) was characterized via a two-hybrid screen in order to identify binding partners for MP1 (129). MORG1 can also associate with Raf-1, B-Raf, MEK1/2, and ERK1/2. MORG1 appears to mediate MAPK signaling in response to GPCR-triggering, and not RTKs, making the role of MORG1 scaffold protein ligand/receptor-
specific. MORG1 enhances ERK activation in response to lisophosphatidic acid (LPA), phorbol ester, and serum but not in response to EGF stimulation (129). Recruitment of MORG1 to late endosomes and its association with MP1 may provide an additional level of regulation from upstream activators.

The additional level of regulation provided by specific scaffold proteins during signal transduction provides a very specific target for modulation of signaling pathways. The myriad of scaffold proteins characterized within the ERK pathway provide a unique activation environment that leads to a specific cellular function. Targeting of these scaffold proteins (upregulation or downregulation) may enable direct and specific modulation of ERK activation. Therapeutically, this can lead to very specialized, effective treatment options where regulation of ERK signaling only occurs under pathological cascade stimulation, without affecting steady-state ERK function.

Overview of this thesis

Infection with *Leishmania infantum* and *Leishmania amazonensis* lead to very different, yet chronic, disease manifestations. While infection with *L. infantum* may initially promote an effective CD4+ Th1 immune response, disease progression has been correlated to the appearance of an immuno-suppressive response. In contrast, infection with *L. amazonensis* does not promote a clear Th1/Th2 polarization, and more rapidly results in a tolerogenic response. The factors that promote chronic disease following *Leishmania* infection and the mechanisms utilized by these parasites to modulate the adaptive immune response remain poorly understood. The objectives of this dissertation are to characterize immune parameters that could be indicative of immune dys-regulation and therefore disease progression following *L. infantum* infection, to understand the *L. amazonensis*-mediated modulation of dendritic cell maturation phenotype that could lead to the induction of a non-
polarized immune response, and to characterize the molecular mechanism by which *L. amazonensis* triggers endosomal activation of ERK1/2.

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Figure 1 Life cycle of *Leishmania major* infection. *Leishmania* parasites are transmitted by the bites of infected female sandflies, which inject a small number of infectious-stage, metacyclic promastigotes into the skin. These forms are opsonized efficiently by serum components and taken up by macrophages, where they reside in phagolysosomes and transform into replicating amastigotes. Infected macrophages are taken up by sandflies during blood feeding; they are lysed in the fly midgut, releasing parasites that transform into rapidly dividing, non-infectious-stage promastigotes. These forms undergo a process of attachment to the midgut wall, release and anterior migration that is accompanied by their differentiation to non-dividing, metacyclic promastigotes that can be transmitted when the sandfly takes another blood meal. Figure and legend reprinted with permission from Nature Publishing Group: *Nature Reviews Immunology*, Sacks, D. and Noben-Trauth, N. 2(11):845-858, copyright 2002.
Figure 2 The ERK/MAPK pathway. Most cell-surface receptors activate Ras GTPases. Ras GTPases comprise a large family of mostly membrane-resident proteins that shuttle between an inactive GDP-bound and active GTP-bound conformation. The best-characterized family members are K-Ras, H-Ras and N-Ras, which are encoded by potent proto-oncogenes that are mutated in ~30% of human tumours. The oncogenic mutations prevent them from hydrolysing GTP, which maintains them in the activated state. In its activated conformation, Ras-GTP can bind to a number of effector molecules, including the serine/threonine kinase Raf, phosphatidylinositol 3-kinase, RalGDS and others. Ras-GTP recruits these proteins to the membrane compartment, which is crucial for their activation and signaling function. All three Raf family members, A-Raf, B-Raf and Raf-1, bind Ras-GTP as the first step in their activation process. Whereas Ras-GTP association might suffice to activate B-Raf, both Raf-1 and A-Raf undergo a complex series of activation steps that have not been entirely elucidated and involve changes in phosphorylation levels and protein interactions. All Raf isoforms can activate MAPK and ERK kinase (MEK) by phosphorylating two serines in the MEK activation loop, although B-Raf is much more effective at doing so than Raf-1, which is better than A-Raf. The gene that encodes B-Raf is also mutated in many cancers, mainly in melanoma and cancers of the thyroid, colon and ovaries. MEK is a dual-specificity kinase, the only known substrate of which is extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), the prototypical MAPK that is activated by phosphorylation of both the threonine and tyrosine residues in a TEY motif that is in the activation loop. Both MEK and ERK/MAPK have two isoforms in mammals, which, in most instances, are co-regulated but might have different functions. ERK/MAPK is considered the main effector of this pathway and has more than 70 known substrates that include nuclear transcription factors, cytoskeletal proteins, signaling proteins and receptors. Figure and legend reprinted with permission from Nature Publishing Group: Nature Reviews Molecular Cell Biology, Kolch, W., 6(11):827-837, copyright 2005.
Figure 3 Scaffold proteins KSR1, MP1, and MORG1. MEK partner-1 (MP1) is a scaffold that coordinates the interaction between extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and MAPK and ERK kinase (MEK). The MP1–MEK–ERK/MAPK complex is targeted to late endosomes by p14. MP1 also binds to MAPK organizer-1 (MORG1). The exact composition and localization of MORG1 complexes are unknown. MORG1 also binds Raf-1 in addition to MP1, MEK and ERK/MAPK, and co-localizes with vesicles in cells. The MORG1-bound ERK/MAPK complex is selectively stimulated by serum and lysophosphatidic acid (LPA), which are agents that function mainly through G-protein-coupled receptors (GPCRs), but not through epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which signal through receptor tyrosine kinases (RTKs). By contrast, ERK/MAPK complexes that are coordinated by the scaffold MP1–p14 on endosomes in the absence of MORG1 or by KSR1 at the cell membrane respond to both GPCR and RTK signals. Figure and legend reprinted with permission from Nature Publishing Group: Nature Reviews Molecular Cell Biology, Kolch, W., 6(11):827-837, copyright 2005.
CHAPTER 2: IMMUNOLOGIC INDICATORS OF CLINICAL PROGRESSION DURING CANINE LEISHMANIA INFANTUM INFECTION

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Abstract

In both dogs and humans Leishmania infantum infection is more prevalent than disease, as infection often does not equate with clinical disease. Previous studies additively indicate that advanced clinical visceral leishmaniasis (VL) is characterized by increased production of anti-Leishmania antibodies, Leishmania-specific lymphoproliferative unresponsiveness, and decreased production of IFN-γ with concomitant increase of IL-10. In order to differentiate infection vs. progressive disease for better disease prognostication, we temporally evaluated humoral and cellular immunologic parameters of naturally infected dogs. The work presented here describes for the first time the temporal immune response to natural autochthonous L. infantum infection in Foxhounds within the United States. Several key changes in immunological parameters should be considered to differentiate infection versus clinical disease, including a dramatic rise in IgG production, progressive increases in antigen-specific PBMC proliferation, and IFN-γ production. Polysymptomatic disease is precluded by increased IL-10 production and consistent detection of parasite kinetoplast DNA in whole blood. This clinical presentation and immuno-dysregulation mirrors that

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observed in human patients indicating that this animal model will be very useful for testing immunomodulatory anti-IL-10 or other therapies.

**Introduction**

Leishmaniasis is a group of vector-borne diseases caused by intracellular protozoan parasites of the genus *Leishmania*. Disease manifestation can range from localized, self-healing cutaneous ulcers to disseminated disease, referred to as visceral leishmaniasis (VL). VL is fatal if left untreated. It is primarily caused by *Leishmania* (*L.*) *donovani* in Africa and India, and by *L. infantum/chagasi* in the Mediterranean basin, Asia and Central and South America.

VL, as caused by *L. infantum* infection, is zoonotic (4). Both dogs and humans are natural hosts (27), and in endemic regions, infected dogs are the primary domestic reservoir for zoonotic VL and the most significant risk factor predisposing humans to infection (9). *L. infantum* infection often does not equate with clinical disease (18). Typical clinical signs of VL include fever, weight loss, anemia, lymphadenopathy, and hepato- and splenomegaly (4, 22, 27). Clinical stages of infection can be classified by the severity of clinical signs, humoral and cell-mediated responses, and parasite load (33). We propose that these parameters can also be used to determine the best window for treatment and in some cases predict the appearance of clinical signs and prognosis (24).

Host protection against *Leishmania* infection requires a proinflammatory, T<sub>H1</sub> immune response, as characterized by the production of interleukin (IL)-12 by antigen presenting cells and interferon (IFN)-γ by T cells (reviewed in (22)). Advanced clinical VL in human patients is characterized by *Leishmania*-specific lymphoproliferative unresponsiveness and decreased production of IFN-γ following *in vitro* *Leishmania* antigen
restimulation (11, 31). Active disease is associated with elevated IL-10 levels in serum and enhanced IL-10 mRNA in lesional tissues (reviewed in (22)). Cured or subclinical individuals are able to mount antigen-specific IFN-γ responses following *Leishmania* antigen restimulation *in vitro*. Cured patients are also resistant to reinfection and are leishmanin skin test positive, suggesting no inherent defect in the antigen-dependent Tₘ₁ response (3, 7, 34).

Canine visceral leishmaniasis (CVL) in endemic areas mimics both the immunologic alterations and pathophysiology of human disease. Autochthonous *L. infantum* infection in the United States Foxhound population has been recently described (5, 8). Despite a potentially different means of transmission, i.e. non-vector borne (12, 24, 29), symptomatic disease and pathologic findings in naturally infected Foxhounds parallels that observed in both canines and humans endemic regions (12). For these studies, we hypothesized that the immunopathology of primarily non-vector mediated *L. infantum* CVL would reflect the changes observed in humans, including increased anti-*Leishmania* antibodies in sera, and decreased lymphoproliferative IFN-γ-mediated responses with increased IL-10 production. Here we follow a cohort of U.S. born, naturally-infected canines to determine their immunopathology and clinical presentation(s) of autochthonous *L. infantum* infection. Analysis of clinical signs, serology, and kinetoplast-specific qPCR categorized these animals into four different groups: 1) non-infected, 2) infected-resistant, 3) infected-susceptible and 4) clinical, as previously described in (33). Animals in the 4th clinical state had increased production of IgG1 and IgG2, decreased lymphoproliferative responses and IFN-γ production, and increased IL-10 production. The appearance of any of these immunological parameters correlated with disease progression.
The work presented here describes for the first time the temporal immune response to natural autochthonous *L. infantum* canine infection in United States. We show that even in the likely absence of vector-mediated transmission (32) clinical presentation and immunodysregulation mirrors that observed in endemically-infected dogs and humans (1, 22). The ongoing antigen-specific immune response to *L. infantum* infection wanes as disease progresses and production of anti-*Leishmania* antibodies and IL-10 are key immunologic features of disease manifestation and progression.

**Materials and Methods**

*Description of Animals*

Although VL is not endemic in the United States, canine visceral Leishmaniasis (CVL) has recently been described as an epidemic within the Foxhound population in this country. The first report of Foxhound CVL epidemic in the U.S. was in 1999 in a foxhound kennel in New York (8). By 2005, it was reported that 60 kennels in 22 states and two Canadian provinces had *L. infantum*-seropositive Foxhounds, and that autochthonous infection in canines was for the most part limited to Foxhounds (5).

Dogs used in this study, all Foxhounds, ranged in age from six months to seven years of age. These animals or their tissues were donated to Iowa State University College of Veterinary Medicine by two different Midwestern Foxhound kennels. Nine of the dogs were donated based on positive serological indirect immunofluorescence assay test (IFAT) results (>1:64) and presentation of clinical signs. The remaining four dogs were born to an IFAT positive (> 1:256) female. All animals were housed at Iowa State University Veterinary College and the Institutional Animal Care and Use Committee at Iowa State approved all protocols involving animals. Prior to arrival, all dogs were vaccinated for core canine
Once under the care of laboratory animal resources (LAR) at Iowa State University, blood samples were obtained for complete blood count (CBC) and chemistry, and stool samples were collected for enteric parasite assessment. All animals were treated for ecto- and intestinal parasites (Giardia, roundworms, and Coccidia) via treatment with Strongid (5mg/kg), Baytril (¼ tablet), Albon (55mg/kg), Panacur (2ml/kg), Clavamox 13.75mg/kg, and Cephalexin (25mg/kg).

**Clinical Evaluation**

Upon arrival to Iowa State University College of Veterinary Medicine, all animals were clinically assessed via physical examination, complete blood count, chemistry panel analysis, L. infantum kDNA specific qPCR and IFAT serologic analysis. Based on these parameters, animals were classified into four distinct categories: non-infected, showing no clinical signs of disease and qPCR and IFAT negative; infected-resistant, showing none to mild clinical signs and IFAT and qPCR positive/negative; infected-susceptible, showing mild to moderate clinical signs and qPCR and IFAT positive; and clinical, showing severe, disseminated disease, and IFAT and qPCR positive.

**Parasites**

Leishmania infantum (LIVT-2) (30) was grown to stationary phase in complete Grace’s medium (Incomplete Grace’s supplemented with 20% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin and 2mM L-glutamine). Freeze-thawed whole antigen was prepared as described previously (13).
Peripheral blood mononuclear cell (PBMC) Isolation and Carboxyfluorescein succinyl ester (CFSE) Staining

All animals were allowed to acclimate for one week prior to immunological studies. PBMC were isolated from heparinized blood samples using Ficoll-Histopaque 1077 (Sigma, St. Louis, MO) gradient centrifugation. Red blood cells were removed using ACK lysis buffer (0.15M NH₄Cl, 1.0mM KHCO₃, 0.1mM Na₂EDTA, pH 7.4). PBMC were labeled with CFSE (Molecular Probes, Eugene, OR) as described previously (14). PBMC were washed twice in phosphate-buffered saline (PBS) and resuspended in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin, 2mM L-glutamine, and 25mM HEPES buffer). PMBC were counted and adjusted to 4x10⁶/ml for further analysis.

PBMC Proliferation Assay

CFSE-labeled PBMC (4x10⁵/well) were plated on 96-well plates and incubated with media alone, stimulated with concavanalin A (ConA) (5ug/ml) for 4 days or with freeze-thawed, whole L. infantum antigen (10ug/ml) for 7 days, or Distemper vaccine (Vanguard Plus 5, Pfizer) control for 10 days, at 37°C with 5% CO₂. Cells were harvested, washed in FACS buffer (0.1% albumin, 0.1% sodium azide in PBS) and labeled with PE-conjugated anti-canine CD4 antibody (Serotec, Raleigh, NC). Cells were fixed in 1% paraformaldehyde and analyzed using the FACSCanto flow cytometer (BD Pharmingen, San Diego, CA). Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

IFN-gamma and IL-10 ELISA
Unlabeled PMBC (2x10^5) were plated and incubated as described above. Supernatants were collected at the indicated time points and stored at -20ºC until analysis. IFN-γ and IL-10 production were measured using R&D ELISA kits (Minneapolis, MN), according to manufacturer’s recommendations.

**Serology and Real Time qPCR**

Serum samples were collected from all animals, stored at -20ºC and sent to the Centers for Disease Control and Prevention for IFAT testing for antibodies to *Leishmania* spp. as previously described (5). DNA from whole blood samples collected in heparinized tubes (BD Pharmingen, San Diego, CA) was isolated using the Qiagen blood DNA isolation kit according to manufacturer’s instructions. DNA quality and quantity was measured using a NanoDrop spectrophotometer ND1000 (Wilmington, DE). *L. infantum* kinetoplast DNA (kDNA)-specific primers and probe  F 5’-CCGCCCGCCTCAAGAC, R 5’-TGCTGAATATTGGTGGTTTTGG, (Integrated DNA Technologies, Coralville, IA), Probe 5’-6FAM-AGCCGCGAGGACC-MGBNFQ (Applied Biosystems, Foster City, CA) were used. (FAM: laser-activated reporter dye; MGBNFQ: 3’-minor-groove binder non-fluorescent quencher). Blood DNA samples were assayed via qPCR in duplicate of three dilutions (straight, 1:10, 1:20) using a Stratagene Mx3005P® qPCR System via a 96-well format and Platinum qPCR SuperMix-UDG Master Mix (Invitrogen, Carlsbad, CA). Primers were used at 775 nM and probe at 150 nM with thermocycling at 50°C for 2 minutes, 95°C for 2 minutes, and 50 cycles of [95°C for 30 seconds, 57°C for 1 minute and 60°C for 1 minute]. Results were analyzed via MxPro™ QPCR software version 4.01 in conjunction with Microsoft Excel. *L. infantum*-specific IgG ELISA
High-affinity plates were coated overnight at 4°C with 10µg/well of freeze-thawed *L. infantum* antigen in 50mM carbonate-bicarbonate buffer. Plates were blocked with 200µl of blocking buffer for 1 hour at room temperature and washed. Serum samples (100µl) were diluted 1:100 and incubated for 2 hours at room temperature. Plates were developed with HRP-conjugated anti-canine IgG1 or IgG2 (1:20,000) (Bethyl Laboratories, Montgomery, TX) for 1 hour and Absorbance was read at 405nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

**Statistical analysis**

Statistical significances were analyzed using Prism4 (GraphPad Software Inc., La Jolla, CA). Differences between groups were determined using Mann-Whitney U-test. *P*-values below 0.05 were considered significantly different.

**Results**

**Clinical evaluation**

Clinical assessment included a complete blood (cell) count (CBC) and chemistry panel (Table 1, fourth column). Following euthanasia, necropsy was performed by a veterinary pathologist and a complete set of tissues was collected for each animal and evaluated histologically (Table 1, last two columns). Lymphocytosis (elevated lymphocyte numbers in the blood) was consistently present in all dogs tested. Persistent lymphocytosis is indicative of chronic antigen stimulation, which we would attribute to the presence of *Leishmania* parasites in infected animals. However, since non-infected dogs also show lymphocytosis, we cannot rule out the possibility of increased circulating lymphocyte number due to other infections including gastrointestinal or ecto-parasitism, which has been observed in these Foxhounds previously (data not shown). In all infected animals we
observed a moderate to marked hyperglobulinemia. Serum chemistry and histopathologic findings in the infected-susceptible dogs indicated the onset of systemic disease consistent with visceral leishmaniasis, including elevated blood urea nitrogen (BUN), creatinine and phosphorous, anemia, lymphoplasmacytic portal hepatitis, histiocytic splenitis, and membranous glomerulonephritis. CBC and serum chemistry evaluation of clinical dogs indicated these animals had signs of nonregenerative anemia, renal compromise (elevated BUN, creatine and phosphorus), and hepatic injury (elevated alanine transferase). Despite their clinical state, lymphocytopenia was not observed in clinical dogs. Histopathologic examination confirmed these findings and also showed systemic histiocytic inflammation with a myriad of intracellular *Leishmania* amastigotes.

**Serology and qPCR**

To confirm infection and disease status, each dog was evaluated for *L. infantum* serostatus and qPCR for *L. infantum* kinetoplast (k) DNA. Serum samples from all Foxhounds in the study were sent to the Centers for Disease Control and Prevention (CDC) for IFAT analysis of antibodies against *Leishmania* spp (Table 1). All dogs in the control (non-infected, non-Foxhound), non-infected group and two dogs from the infected-resistant group were seronegative (≤1:16). The two remaining dogs within the infected-resistant group had seropositive titers (1:64). Dogs within the infected-susceptible group (3 dogs) had titers of 1:256, and dogs within the clinical group (4 dogs) had strong sero-reactivity to *Leishmania* antigen (1:512).

*L. infantum*-specific kDNA amplification was observed in all clinical and infected-susceptible dogs, and in two of the infected-resistant group. As expected, no amplification was observed in the non-infected Foxhounds (Table 1) and in the control, non-Foxhound
dogs. These data indicate that increased parasitemia is found during later stages of infection.

**L. infantum-specific IgG1 and IgG2 production**

Chemistry findings in serum samples from clinical dogs indicated these animals had pan-elevation of immunoglobulins (Ig): IgA $>$500mg/dl, IgG $>$5000mg/dl, IgM 400mg/dl; normal range 20-150 mg/dl; 1000-2000 mg/dl; and 70-270 mg/dl, respectively. Hypergammaglobulinemia has been associated with CVL disease progression pathophysiology (12) and suppression of the immune response to *L. infantum* (26). However, a relationship between immunoglobulin (Ig) G isotype profile and disease resistance versus susceptibility remains to be established. Conflicting reports fail to provide a clear role for IgG1 or IgG2 production in disease development (25, 28). Based on our findings of detectable circulating parasites as disease progressed, we wanted to determine if this observation correlated with detection of specific antibody levels. Using whole parasite antigen we found that sera from control and non-infected groups contained minimal IgG1 and IgG2 antibodies when measured by ELISA, as OD values observed were similar to background readings (OD ~0.01). Highest levels of anti-*L. infantum* IgG1 (Figure 1A) and IgG2 (Figure 1B) were produced by the infected susceptible and clinical groups. Overall, IgG levels increase as disease progresses, however, we did not observe a direct correlation between either IgG isotype and clinical status. Other, non-antibody, effector functions may therefore be more predictive of disease progression.

**L. infantum-specific PBMC proliferative response**
A key immunologic feature of late clinical VL is the inability of PBMC to generate a protective, *L. infantum*-specific immune response (31). This is characterized by the loss of the antigen-specific lymphoproliferative response and the loss of IFN-γ production. To identify if this lack of antigen-responsiveness as disease progresses occurs in our canine cohort, we analyzed the antigen-specific proliferative response of PBMC CD4⁺ T cells from all four groups. Blood samples were collected every four weeks during a period of at least three months for each dog. PBMC were isolated from whole blood samples, stained with CFSE, and stimulated with concavanalin A (ConA), *L. infantum* antigen, distemper vaccine or left untreated. PMBC were then analyzed for CD4⁺ T cell proliferation via flow cytometry. CD4⁺ T cells from all dogs proliferated in response to stimulation with ConA, indicating that the CD4⁺ T cell compartment was not mitogenically deficient (Figure 2B). In response to distemper vaccine stimulation all groups except for the clinical dogs had a proliferative response indicating that although mitogenically competent, clinical dogs were not capable of initiating antigen-specific proliferative responses (Figure 2A, B). In response to *L. infantum*-antigen stimulation, control (uninfected, non-Foxhound) and non-infected dogs showed a minimal level of proliferation in response to antigen restimulation (Figure 2). While a significantly greater percentage of CD4⁺ T cells from infected-resistant dogs proliferated in response to antigen restimulation as compared to non-infected dogs, infected-susceptible dogs demonstrated the greatest percentage of proliferative CD4⁺ T cells, significantly higher than infected-resistant animals (Figure 2A). In contrast, the antigen-specific CD4⁺ T cell proliferative response from clinical animals was significantly decreased as compared to that of infected-susceptible dogs. These data suggest that as disease progresses, there is an initial increase in antigen-specific lymphoproliferative responsiveness of CD4⁺ T cells that eventually dwindles. Appearance of clinical disease correlates with the loss of the antigen-
specific lymphoproliferative response. Based on this observed loss of proliferative response in late disease, we wished to determine if cytokine production, specifically IFN-γ and IL-10, could be correlated with this lympho-suppressive change.

*Disease progression and antigen-specific PBMC IFN-γ and IL-10 production*

Treated individuals develop a cell-mediated immune response capable of offering protection from reinfection, as characterized by antigen-specific IFN-γ responses (7, 34). In contrast, individuals with advanced VL show a decrease in antigen-specific IFN-γ production and elevated levels of the immunoregulatory cytokine IL-10 in serum and increased IL-10 mRNA expression in lesional tissue (6, 10). The correlation between VL disease progression and IL-10 production in humans is now well established (22). In CVL, IFN-γ-mediated responses seem to predominate in *L. infantum*-infected but asymptomatic dogs (23). Similar to human disease, IL-10 mRNA expression has been positively correlated with parasitic load and progression of clinical disease in naturally infected dogs (15). In order to determine the correlation between disease and cytokine production in our cohort, culture supernatants from PBMC restimulated with *L. infantum* antigen were assayed for IFN-γ (Figure 3A) and IL-10 (Figure 3B) production. Production of IFN-γ and IL-10 from PBMC in the control group (Figure 3A and B) was below the detection limit of the assay (16pg/ml and 10pg/ml respectively). PMBC from infected-resistant and infected-susceptible animals produced comparable levels of IFN-γ (Figure 3A). PBMC from infected-resistant dogs produced significantly higher levels of IFN-γ as compared to non-infected animal PMBC. PBMC from clinical dogs, however, produced significantly lower amounts of IFN-γ as compared to infected-susceptible and infected-resistant dogs.
Analysis of IL-10 production from culture supernatants indicated a significant increase in the production of this cytokine with disease progression. PBMC from dogs in the clinical group produced the greatest amount of IL-10 as compared to all other groups (Figure 3B), with decreasing amount detected from infected-susceptible and then infected-resistant dogs. All three groups were significantly different from one another. PBMC from non-infected dogs produced levels of IL-10 that were below the detection limit of the assay (10pg/ml). These data demonstrate that clinical progression and loss of antigen-specific T cell proliferation in our cohort were associated with decreased levels of antigen-specific IFN-γ production, and increased production of IL-10 in response to L. infantum antigen restimulation.

**Discussion**

During CVL, susceptibility to symptomatic infection has been associated with increased antibody production and loss of L. infantum-specific CD4+ T cell function with a concomitant increase in immunosuppressive mechanisms. However, little is known regarding the mechanisms that control the balance between resistance to infection and susceptibility. Characterization of measurable immunopathological endpoints may provide a means to better predict disease development in infected dogs. Our studies using a cohort of naturally infected dogs show how changes in IgG production, lymphoproliferative responses, and effector cytokine production correlate with the appearance of clinical signs and disease progression.

In our study increases in serologic titer were associated with disease progression (Table 1). The highest titers (1:256 and 1:512) were observed in dogs displaying mild to severe clinical disease within the infected-susceptible and clinical groups. Moreover, high
antibody titers also correlated to the detection of *L. infantum* parasites in peripheral blood samples via qPCR (Table 1), indicating an increase in circulating parasites later in disease. Analysis of antigen-specific IgG1 and IgG2 in sera of the four groups of dogs showed an increase in both isotypes with disease progression (Figure 1A and B). Infected-susceptible and clinical dogs exhibited the highest O.D. values indicating increased IgG1 and IgG2 levels as compared to non-infected and infected-resistant dogs but there was no clear difference between isotypes regarding clinical state or progression.

During human VL increased levels of anti-*Leishmania* IgG have been shown to have a negative correlation with delayed-type hypersensitivity (DTH) responses (17). Here we show that along with increased IgG in sera, *L. infantum*-antigen responsiveness of PBMC CD4+ T cells significantly decreased in the clinical group of animals (Figure 2). This loss of lymphoproliferation has been described as “immune exhaustion” due unchecked levels of pathogen antigen (2, 19). Infected-susceptible animals showed the most robust proliferative response compared to all other groups. Proliferation in the non-infected Foxhound group may be attributed to non-specific proliferation or perhaps a dwindling recall response. Animals in this group were donated as part of a litter of puppies born to a seropositive, qPCR-positive female. It is therefore possible they may have been exposed to *L. infantum* parasites *in utero* at a very low dose, leading to exposure and some T cell activation but perhaps not patent infection. Altogether, our data shows that PBMC CD4+ T cells from *L. infantum*-infected dogs respond to antigen stimulation during the earlier stages of infection, but lose that ability as they progress to clinical disseminated disease, negatively correlating with the increased levels of IgG in sera.

Antibody production is an important contributor to VL pathology due to antigen-antibody complex deposition. B cell activation and increased IgG production are observed in
conjunction with IL-10 overproduction during VL (22). To determine what effector cytokines were produced by the dampened T cells with limited antigen-responsiveness in our cohort, we assessed IFN-γ and IL-10 production in cultured PMBC. We found that decreased proliferative responses in the clinical group were accompanied by significantly decreased IFN-γ production (Figure 3A) and significantly increased IL-10 production (Figure 3B). This profile matches observed changes in cytokine production in endemic human cohort studies (10, 20, 21, 31) and dogs (23). Our infected-resistant and infected susceptible groups produced similar levels of IFN-γ, however, the infected-susceptible group showed significantly increased production of IL-10 compared to the infected-susceptible group. The observed increase in IL-10 production, along with increased blood parasite burden, may be specific factors which promote clinical disease.

The factors that determine disease progression in CVL remain poorly understood. It is clear that no one clinical parameter can be used to predict which infected dogs will likely become clinically ill. Our studies using our canine cohort of progressive CVL indicate that several key changes in clinical parameters should be considered, including a rise in IgG production, a progressive increase of antigen-specific PBMC proliferation followed by a decreased IFN-γ-mediated response, a dramatic increase in IL-10 production, and consistent detection of parasite kDNA in whole blood. Further studies are needed to fully understand the relationship between increased IgG, IL-10 production and parasite load. While it has been shown that all three of these events precede clinical disease (16, 17, 23), the causal relationship between them is yet to be determined. Understanding which event drives the others may provide insight into the mechanisms leading to VL and provide thought for future immuno-therapies.
Acknowledgments

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References


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<th>Gross pathological findings (no. with finding/total no. tested)</th>
<th>Histopathologic finding(s) (no. with finding/total no. tested)</th>
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<td>1:512</td>
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<td>Thin to emaciated (4/4)</td>
<td>Marked systemic lymphadenomegaly (2/2), splenomegaly (2/2), hepatomegaly (2/2)</td>
<td>Lymphohistiocytic portal hepatitis (2/2), membranous glomerulonephritis (2/2), histiocytic splenitis (2/2), systemic histiocytic inflammation with myriad intracellular <em>Leishmania</em> amastigotes (2/2)</td>
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* Only two of four dogs were assessed for CBC, chemistry, and necropsy changes.
Figure 1. Anti-*L. infantum* IgG1 and IgG2 responses increase with CVL disease progression. *L. infantum*-specific IgG responses were measured using sera from control (1 dog, ■), non-infected (2 dogs, □), infected-resistant (4 dogs, ♦), infected susceptible (2 dogs, ○) and clinical (3 dogs, ●) animals via ELISA. Blood samples were collected and centrifuged to clarify serum. Shown are O.D. values from antigen-specific (A) IgG1 and (B) IgG2 ELISA. Lines indicate mean value for each group.
Figure 2. Decreased lymphoproliferative response in PBMC from *L. infantum*-infected, clinical dogs. (A) PBMC proliferative responses from control (1 dog, ■), non-infected (2 dogs, □), infected-resistant (4 dogs, ♦), infected susceptible (2 dogs, ○) and clinical (2 dogs, ●) animals repeated monthly over three to six months. PBMC were isolated, stained with CFSE, restimulated with freeze-thawed *L. infantum* antigen, and incubated for seven days at 37°C. Cells were then harvested and stained with a PE-conjugated anti-CD4 antibody. PMBC CD4⁺ T cell proliferation was assessed via CFSE dilution using flow cytometry. Each point is indicative of a blood draw from each animal over a three to six month period and subsequent proliferation assay. At least four separate proliferation assays were carried out over time on each dog in every group. Lines indicate the mean response for each group. (*) Indicates significant difference, p< 0.05. (B) PBMC proliferative response to *L. infantum* (black), Distemper vaccine (DHPP) (gray), and ConA (white) stimulation for control (1 dog), infected-susceptible (3 dogs) and clinical dog (2). PBMC were isolated and processed as in (A) and stimulated with *L. infantum* Ag for seven days, DHPP for 10 days and ConA for 4 days. CD4⁺ T cell proliferation was assessed via CFSE dilution using flow cytometry. At least 3 separate experiments were carried out for each dog in every group. Bars indicate average proliferation for each group, ±SEM.
Figure 3. Disease progression correlates with decreased IFN-γ and increased IL-10 production. Shown are PBMC effector cytokine responses from control (1 dog, ■), non-infected (2 dogs, □), infected-resistant (4 dogs, ♦), infected susceptible (2 dogs, ○) and clinical (2 dogs, ●) animals. Culture supernatants were collected from PBMC cultures stimulated with *L. infantum* antigen for seven days and analyzed via ELISA for (A) IFN-γ and (B) IL-10. Each point is indicative of one experiment. At least three separate experiments were carried out for each dog in every group. Lines indicate the mean response for each group. (*) Indicates significant difference, *p* < 0.05.
CHAPTER 3: ALTERED DENDRITIC CELL PHENOTYPE IN RESPONSE TO 
LEISHMANIA AMAZONENSIS AMASTIGOTE INFECTION IS MEDIATED BY THE 
MAP KINASE, ERK

A paper published in The American Journal of Pathology

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Abstract

Dendritic cells (DC) are professional antigen presenting cells, critical for induction and regulation of T cell immune responses against pathogens, including Leishmania. Leishmania, intracellular protozoan parasites, are the causative agent for a group of diseases that range from benign to fatal. Initiation of productive immune responses against this pathogen depends on the successful transition of DC from an immature to a mature phenotype, characterized by high CD40 surface expression and IL-12 production. This productive immune response is frequently seen in response to L. major infection.

Characterization of draining lymph node CD11c+ DC from L. amazonensis or L. major promastigote-infected mice revealed that by 7 days post-infection, CD11c+ cells from the draining lymph node of L. amazonensis promastigote-infected mice have significantly reduced CD40 surface expression. At 7 days post-infection the number of IL-12p40-producing cells is drastically decreased as compared to DC from L. major-infected mice. Analysis of MAP kinase signaling revealed that infection of DC in vitro with L. amazonensis resulted in increased ERK phosphorylation. Inhibition of ERK in vitro or in vivo led to enhanced DC surface CD40 expression and in vitro increased IL-12p40 production. In

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conclusion, *Leishmania amazonensis* amastigote-mediated regulation of DC maturation occurred via increased phosphorylation of the MAP kinase ERK.

**Introduction**

Leishmaniasis is a vector-borne disease caused by intracellular protozoan parasites of the genus *Leishmania*. The intracellular amastigote stage of *Leishmania* predominates in the mammalian host while the vector stage exists as an extracellular, flagellated promastigote. Both promastigotes and amastigotes are capable of initiating mammalian infection. *Leishmania* amastigotes have been shown to interfere with host cell function, including modulation of signaling pathways, suppression of antimicrobial and pro-inflammatory mediators, and induction of cytokines that promote disease progression.\(^1\), \(^2\)

Infection of C3HeB/FeJ mice with *Leishmania major* leads to the development of a healing TH\(_1\) immune response characterized by high levels of interleukin-12 and interferon (IFN)-\(\gamma\)-producing CD4\(^+\) T cells.\(^3\) In contrast, infection with *L. amazonensis* results in a non-healing immune response, leading to chronic disease and high parasite loads. Multiple studies have investigated and identified differences in the T cell response to these two parasites.\(^4\)\(^-\)\(^6\) Studies from our laboratory have revealed that antigen-responsive CD4\(^+\) T cells from C3HeB/FeJ mice chronically infected with *L. amazonensis* are impaired in their ability to transition from a naïve to an effector phenotype.\(^6\)

*Leishmania* spp. infect phagocytic cells of the immune system, primarily macrophages and dendritic cells (DC). DC are antigen presenting cells (APC) which efficiently initiate antigen-specific immune responses by inducing the differentiation of naïve T cells.\(^7\) DC maturation can be characterized *in vitro* and *ex vivo* by the upregulation of co-stimulatory molecules including CD40 and the production of T cell-polarizing cytokines such
as interleukin (IL)-12. Clearance and resistance to *Leishmania* infection is dependent on the development of a T\(_h\)1 response\(^3,^8\) and susceptibility to *Leishmania* infection has been associated with deficiencies in CD40, CD40 ligand\(^9\text{-}^11\) and IL-12 or IL-12 receptor subunits.\(^12,^13\) Infection of C57BL/6 mice bone marrow-derived dendritic cells (BMDC) with *L. amazonensis* promastigotes indicated a significant reduction in CD40 surface expression and IL-12p40 production as compared to *L. major* promastigote-infected BMDC.\(^14\) Moreover, a recent publication indicated that *L. amazonensis* amastigote infection of BMDC significantly down-regulated CD40 expression and suppressed IL-12p40 and IL-12p70 production, leading to an impairment in APC function, characterized by an inability of *L. amazonensis*-amastigote infected BMDC to prime naïve CD4\(^+\) T cells or re-stimulate antigen-specific CD4\(^+\) T cells.\(^15\)

Improper or insufficient activation of dendritic cells (DC) can promote a non-polarized, often tolerogenic, T cell response. DC maturation depends on the balance of particular molecular signaling cascades.\(^7,^16,^17\) One such pathway is mitogen activated protein kinase (MAPK) signaling cascades composed of three primary kinases, p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). A recent report has linked ERK activation in macrophages with the induction of IL-10, a critical cytokine involved in host susceptibility to *Leishmania* infection.\(^18\) Work from our own laboratory has shown that inhibition of ERK *in vitro* increases the ability of *L. amazonensis*-infected macrophages to kill the intracellular parasites.\(^19\) Unlike p38 and JNK, ERK has been shown to play a role in preventing proper maturation of DC.\(^16,^20\)

As *L. amazonensis*-infected mice fail to develop an effective T\(_h\)1 response,\(^4\text{-}^6\) and *L. amazonensis*-infected BMDC *in vitro* have been shown to have an altered maturation phenotype,\(^14,^15\) we hypothesized that *L. amazonensis* infection *in vitro* promotes an
immature DC phenotype consistent with the observed lack in T cell polarization. In this study, we present novel *ex vivo* evidence that *L. amazonensis* infection promotes an immature CD11c⁺ DC phenotype characterized by significantly low CD40 surface expression and significantly decreased IL-12p40 production as compared to *L. major* infection. Furthermore, we explored the molecular mechanisms that may lead to impaired DC maturation and found that *in vitro*, BMDC infection with *L. amazonensis* amastigotes resulted in rapid and significant phosphorylation of the MAP kinase ERK1/2, observed within minutes of exposure to the parasite. Infection with *L. amazonensis* promastigotes led to increased ERK1/2 phosphorylation as compared to *L. major* infection; however, this phosphorylation was delayed several hours. This delay in phosphorylation correlated with promastigote transformation into amastigotes within infected DC, as confirmed by microscopic analysis of parasite stage prior to and after initiation of robust ERK phosphorylation. *In vitro* inhibition studies determined that treatment of DC with a MEK-specific inhibitor, PD98059, led to enhanced surface CD40 expression and IL-12p40 production following *L. amazonensis*-amastigote infection as compared to non-treated cells. Treatment of *L. amazonensis*-infected mice with the highly-specific MEK inhibitor, CI-1040, enhanced surface CD40 expression. Together, this data indicates that *L. amazonensis* amastigotes, through activation of the MAP kinase ERK1/2, inhibit the ability of DC to undergo proper maturation *in vivo*. This is the first report of use of a biochemical inhibitor, here targeted to the MAPK ERK, which restores the immune phenotype of dendritic cells after pathogen infection both *in vitro* and *in vivo*.

**Materials and Methods**

*Mice*
C3HeB/FeJ mice were bred in house or obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in a specific-pathogen-free facility. The IACUC at Iowa State University approved all protocols involving animals. Six- to eight-week old females were inoculated with $1 \times 10^6$ stationary-phase promastigotes in 50 μl of phosphate-buffered saline (PBS) in the left hind footpad.

C3H severe combined immunodeficiency (SCID) mice (C3SnSmn.CB17-Prkdc<sup>scid</sup>/J) were inoculated with 1-2 $\times 10^7$ stationary-phase promastigotes in 50 μl of PBS in the left hind footpad. These mice were later sacrificed for tissue-derived amastigotes.

For in vivo ERK inhibitor treatment, L. amazonensis- and L. major-infected C3HeB/FeJ mice were orally gavaged twice daily with 100mg/kg of CI-1040 (kind gift from Pfizer Global Health, Groton, CT) in dimethyl sulfoxide (DMSO) supplemented with 0.5% hydroxypropyl methyl cellulose (HPMC) and 0.2% Tween 80, for a total of 7 days and sacrificed.

*Isolation and preparation of bone marrow-derived dendritic cells (BMDC)*

BMDC were cultured in vitro in the presence of 10 ng/ml of murine granulocyte-macrophage colony-stimulating factor (PeproTech Inc., Rocky Hill, NJ) according to the method of Lutz et al. At day 10 of culture, approximately 90% of the BMDC were positive for the DC marker CD11c. For in vitro studies, 10-day-old BMDC were incubated for 24 h at 37°C, 5% CO₂ with fresh, tissue-derived amastigotes at a cell to parasite ratio of 1:3.

*Parasites and infection*

Culture of L. amazonensis (MHOM/BR/00/LTB0016) and L. major (MHOM/IL/80/Friedlin) parasites was performed as previously described. For in vitro
promastigote experiments, stationary phase *L. amazonensis* or *L. major* promastigotes were used. Where indicated, parasites were labeled using the dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) as previously described\textsuperscript{22} with some modifications. Amastigotes were directly recovered from footpad lesions of infected C3H SCID mice. Infected feet were disinfected with 70% ethanol, and extraneous tissue dissected away. The remaining lesion was then homogenized with a Tenbrock tissue homogenizer. Cellular debris was removed by centrifuging at 180 x g for 15 min at 4\(^\circ\)C. The resulting supernatant was centrifuged at 3000 x g for 15 min at 4\(^\circ\)C. The number of viable amastigotes in the pellet was assessed by fluorescent microscopy with fluorescein diacetate (Acros Organics, Morris Plains, NJ) and propidium iodide (Sigma, St. Louis, MO). For *in vitro* infection of BMDC, cells were infected with either *L. amazonensis* or *L. major* at a multiplicity of infection (MOI) of 3. Where indicated, as a positive control for maturation, some BMDC were stimulated with 500ng of LPS (Sigma, St. Louis, MO) and 200iU of IFN-\(\gamma\) (Pharmigen, San Diego, CA), or treated with the MEK inhibitor PD98059 (Sigma, St. Louis, MO).

*Flow cytometry*

For flow cytometry analysis of surface molecule expression, 1 x 10\(^6\) BMDC were washed in 2 ml of FACS buffer (0.1% sodium azide and 0.1% BSA in phosphate buffer saline). Fc\(\gamma\) receptors were blocked with 10% purified rat anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA) in 1mg/ml rat IgG for 20 min at 4\(^\circ\)C to prevent nonspecific binding. BMDC were then incubated with the appropriate antibody or isotype control for 30 min on ice. The antibodies used include FITC-labeled CD11c (HL3), PerCP-Cy5.5-labeled CD11b (M1/70), PE-labeled CD40 (3/23), APC-labeled CD80 (16-10A1), PE-Cy7-labeled CD86 (GL-1), APC-Cy7-labeled CD19 (1D3), and APC-Cy7-labeled CD3e (145-2C11).
CD11c, CD11b, CD40, CD19 and CD3 were purchased from BD Pharmigen (San Diego, CA). CD80 and CD86 were purchased from Biolegend (San Diego, CA). Following staining, cells were washed in 2ml of FACS buffer and fixed in 200ul of 1% paraformaldehyde and stored at 4°C until analysis. Analysis was performed on a BD FACScanto flow cytometer (Becton Dickinson, San Jose, CA), and data analyzed using FlowJo software V8.5.2 (Tree Star, Inc., Ashland, OR).

**Isolation of cell extracts**

To make whole cell lysates, 3 x 10⁶ BMDC were resuspended in 400 μl of 1x cell lysis buffer (Cell Signaling Technologies, Beverly, MA), supplemented with 1mM phenylmethylsulphonyl fluoride (PMSF) and a protease inhibitor cocktail (Roche, Indianapolis, IN) immediately prior to use. Samples were incubated on ice for 15 min and then centrifuged at 16,000 x g for 5 min at 4°C. Supernantants were collected as whole cell lysates and stored at -80°C.

**Immunoblot analysis**

Protein content of all cell extracts was determined via BCA protein assay (Pierce, Rockford, IL) according to manufacturer’s recommendations, and all samples were normalized to 1mg/ml using distilled water. Samples (20-30 μg of protein) were heated for 4 min at 95°C in 1x loading buffer and electrophoresis was performed on a 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. Gels were electroblotted onto polyvinylidene fluoride membranes (PVDF), blocked with 5% bovine serum albumin, and probed with antibodies specific for phospho-ERK, p38 and JNK, total-ERK1/2, p38, JNK (1:1000) (Cell Signaling, Beverly, MA) and β-actin (1:5,000) (Sigma, St.
Signals were detected with horseradish-peroxidase (HRP)-conjugated goat anti-rabbit antibodies (1:20,000) (Jackson ImmunoResearch, West Grove, PA) using the SuperSignal West chemiluminescent substrate (Pierce, Rockford, IL) and expressed to autoradiography film (Midsci, St. Louis, MO).

**Indirect Immunofluorescence and hematoxylin and eosin stain**

5x10^5 BMDC were plated onto 24-well plates containing tissue cover slips. BMDC were infected with *L. amazonensis* promastigotes at an MOI of 3:1 and incubated at 34ºC with 5% CO₂. Tissue cover slips were then harvested and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature and washed three times with PBS. BMDC were permeabilized with 0.1% saponin in PBS for 10 minutes at room temperature. Cells were then incubated for 1 hour at room temperature with mouse anti-LPG C7AE monoclonal antibody (A kind gift from Dr. Jeffery Beetham) at a 1:100 dilution in 0.1% saponin. After incubation, cover slips were washed three times with PBS and incubated for 1 hour at room temperature with donkey anti-mouse Cy2-conjugated antibody (A kind gift from Dr. Bryan Bellaire) (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:200 dilution in 0.1% saponin. BMDC were then counter stained with propidium iodide according to manufacturer’s instructions (Molecular Probes, Eugene, OR). Cover slips were then mounted onto slides using MOWIOL (Calbiochem, La Jolla, CA) and viewed using an Olympus IX71 inverted epifluorescence scope (Olympus America Inc., Center Valley, PA).

For morphological analysis, cover slips were harvested at the indicated time points, fixed in 100% methanol for 5 minutes and stained with hematoxylin and eosin stain (H&E) according to manufacturer’s instructions (Fisher Diagnostics, Middletown, VA). Cover slips
were analyzed using a Nikon Eclipse 50i light microscope (Nikon Instruments Inc., Melville, NY).

**IL-12p40 ELISA and ELIspot**

Supernatants from BMDC cultures were harvested at indicated time points following amastigote or promastigote infection, and IL12-p40 ELISA was performed using commercially available antibodies (BD Pharmigen, San Diego, CA), peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) and ABTS microwell peroxidase substrate (Roche, Indianapolis, IN). IL-12p40 ELIsspots were performed on total lymph node cells using commercially available purified IL-12p40 and biotinylated anti-IL12p40 antibodies (BD Pharmigen, San Diego, CA), and developed using 2-amino-2-methyl-1-propanol (2A2M1P) (ICN Biomedicals Inc., Aurora, OH) and 5-bromo-4-chloro-3-indoly-phosphate (BCIP) (Fisher, Fair Lawn, NJ).

**Statistics**

Statistical analysis between two values was determined via Student’s t-tests. \( P \) values of < 0.05 were considered as statistically significant.

**Results**

*Dendritic cells in the draining lymph node of L. amazonensis-infected mice have an immature phenotype*

Recent studies demonstrated that BMDC infected with *L. amazonensis* amastigotes have an immature phenotype, as determined by CD40 expression and IL-12p40 and IL-12p70 production, and are impaired in their ability to prime naïve CD4\(^+\) T cells as compared
to *L. amazonensis* promastigote-infected BMDC. Our laboratory has shown that CD4+ T cells from mice chronically infected with *L. amazonensis* are impaired in their ability to transition into an effector phenotype. We propose that these differences in BMDC phenotype previously observed to be elicited by *L. amazonensis* promastigotes and amastigotes *in vitro*, would be reflected during *in vivo* infection as the infection progresses from peracute (2 days post infection (dpi)) to acute (7 dpi). Mice were infected subcutaneously in the left footpad with *L. amazonensis* or *L. major* promastigotes and sacrificed at 2 and 7 dpi. DC, as designated by CD11c+ cells from the draining lymph node (DLN) of infected mice, collected based on a CD19 and CD3 double negative events (Fig. 1A), were analyzed for DC maturation markers. As shown previously, infection with *Leishmania* not only increases the number of CD11c+-events in the draining lymph node, but also increases CD40 expression in these cells. CD11c+ DC surface expression of CD40 (Fig. 1B and D), CD80 and CD86 (data not shown) was similar between *L. amazonensis*- and *L. major*-infected mice at 2 dpi. By 7 dpi, CD11c+ DC from *L. amazonensis*-infected mice had significantly lower CD40 surface expression as measured by FACS analysis as compared to DC from *L. major*-infected mice (Fig 1C and E). No differences were observed in the surface expression of CD80 and CD86 (data not shown) between *L. amazonensis*- and *L. major*-infected mice at 7 dpi, suggesting that CD40 expression is specifically targeted for down regulation.

The number of DLN cells producing IL-12p40 *ex vivo* was analyzed at 2 and 7 dpi via ELIspot. At two 2 dpi, no significant differences were observed in IL-12p40 production from the DLN of *L. amazonensis*- or *L. major*-infected mice (Fig. 1F). However, by 7 dpi the number of IL-12p40-producing cells was significantly decreased in response to *L. amazonensis* infection compared to *L. major*-infected mice (Fig. 1G). These data suggest
that early during infection, *L. amazonensis* and *L. major* promastigotes elicit similar DC phenotypes in the DLN, as measured by CD40 surface expression and IL-12p40 production. However, once the *in vivo* infection transitions to the acute stage (7 dpi) when the amastigote form of the parasite predominates, we observe a DC phenotype with significantly decreased CD40 surface expression and IL-12p40 production suggesting that *L. amazonensis* amastigotes can modulate the maturation phenotype of DC *in vivo* as it has been observed *in vitro*.

*Increased ERK phosphorylation following infection with *L. amazonensis* amastigotes*

In order to determine a potential mechanism by which *L. amazonensis* could inhibit CD40 surface expression and IL-12 production during DC maturation, we determined which of several signaling pathways are differentially upregulated in DC following infection with *L. amazonensis* and not with *L. major* infection. Several reports indicate that activation of mitogen-activated protein kinase ERK can prevent proper DC maturation. Recent published work from our laboratory and others has shown that activation of ERK1/2 following infection with *L. amazonensis* amastigotes leads to decreased ability of mouse macrophages to kill these intracellular parasites *in vitro*, and promotes a non-healing response in infected Balb/c mice. Based on these findings, we were interested in determining whether ERK phosphorylation occurs in C3HeB/FeJ-derived BMDC after infection with either *L. amazonensis* amastigotes or promastigotes. BMDC infected with *L. amazonensis* amastigotes (Fig. 2A) or promastigotes (Fig. 2B and C) at a 3:1 parasite-cell ratio were lysed at the indicated time points and analyzed via western blot for phosphorylated ERK1/2. Following *L. amazonensis* amastigote infection, a significant increase in ERK1/2 phosphorylation was observed within minutes of exposure to the
parasite (Fig. 2A), compared to *L. major* amastigote infection. In contrast, *L. amazonensis* promastigote infection did not result in significantly increased phosphorylated ERK1/2 until at least 3 hours post infection (Fig 2B and C) as compared to *L. major* promastigote-infected BMDC. Phosphorylation of ERK1/2 after *L. amazonensis* amastigote infection was unique to ERK1/2 as other MAP kinases, p38 and JNK, were not upregulated following infection (Fig. 2D and E).

We hypothesized that the observed delay in ERK1/2 phosphorylation following *L. amazonensis* promastigote infection may correlate with transformation of the parasite into the amastigote stage. We analyzed *L. amazonensis*-infected BMDC at 2 and 4 hours post-infection to determine which parasite form, promastigote or amastigote, predominated at these time points in infected cells. Using both immunofluorescence with an anti-LPG antibody (Fig. 3A) and morphology analysis via hematoxylin and eosin stain (H&E) (Fig.3B), we show that by 4 hours post-infection the percentage of promastigotes significantly decreases correlating with the rise of increased ERK1/2 phosphorylation after promastigote infection. These data suggest that *L. amazonensis* amastigotes specifically induce ERK1/2 phosphorylation.

*ERK inhibition restores CD40 surface expression both in vitro and in vivo following* *L. amazonensis* *infection*

We have shown that MAPK ERK1/2 is selectively and rapidly phosphorylated following *L. amazonensis* amastigote infection of BMDC. To examine if increased ERK1/2 phosphorylation affects BMDC maturation in response to activating stimuli following *Leishmania* infection, BMDC were pre-treated with the specific MEK inhibitor PD98059 and then infected with either *L. amazonensis* or *L. major* amastigotes and activated with LPS.
and IFN-γ two hours post-infection. Following overnight culture, BMDC were analyzed for CD40 surface expression via FACS and resultant culture supernatants were analyzed to determine IL-12p40 production via ELISA. ERK inhibition of *L. amazonensis* amastigote-infected BMDC significantly increased CD40 surface expression MFI (Fig. 4A) and IL-12p40 production (Fig. 4B) as compared to non-treated *L. amazonensis* amastigote-infected BMDC. Treatment with PD98059 had no effect on the ability of *L. major*-infected BMDC to express surface CD40 as measured by FACS analysis or to produce IL-12p40 (data not shown).

Based on these *in vitro* findings, where inhibition of ERK prior to *L. amazonensis* amastigote infection of BMDC enhanced CD40 expression and IL-12p40 production, we sought to determine the effect of ERK inhibition *in vivo* using a different ERK inhibitor, CI-1040 (100mg/kg). Mice were inoculated in the left footpad with stationary phase *L. amazonensis* or *L. major* promastigotes. Starting day 0 mice were treated with CI-1040 via oral gavage. Twice daily treatment continued for a total of 7 days. Mice were sacrificed one week after infection, DLN harvested and CD11c⁺ DC phenotypes analyzed via FACS as described previously (Fig. 1). ERK inhibitor treatment of *L. amazonensis*-infected mice resulted in increased CD11c⁺ DC surface expression of CD40 (Fig. 4C (left panel) and D) as determined by FACS analysis. ERK inhibitor treatment significantly decreased the number of IL-12p40-producing cells as measured by ELIspot (Fig. 4F). CI-1040 treatment of *L. major*-infected mice had no effect on CD40 surface expression of CD11c⁺ DC (Fig. 4C (right panel) and E) or on the number of IL-12p40 producing cells from the DLN of infected mice (Fig. 4G). Western blot analysis of splenic lysates showed that ERK1/2 phosphorylation was inhibited in CI-1040-treated animals (Fig. 4H). These data indicate that *in vitro* *L. amazonensis* infection modulates DC maturation via ERK-mediated down regulation of
CD40 surface expression and decreased IL-12p40 production, and that in vivo, ERK activation down regulates CD40 surface expression of CD11c⁺ DC.

Discussion

Here we describe a novel L. amazonensis amastigote-dependent mechanism modulating DC maturation via activation of the MAP kinase ERK and for the first time recover DC phenotype by inhibiting ERK phosphorylation. Alteration of surface marker expression, cytokine production, maturation and function of DC following L. amazonensis infection has been previously characterized in vitro.¹⁵,²⁴ Defects in DC maturation may, in part, contribute to the un-polarized T cell phenotype observed during L. amazonensis infection,⁶ and lead to a non-healing immune response. As early as 7 dpi CD11c⁺ DLN cells from L. amazonensis-infected C3HeB/FeJ mice have significantly reduced CD40 surface expression and a decreased number of IL-12p40-producing cells, as compared to L. major-infected mice. Consistent with another report indicating the activation of the MAP kinase ERK during L. amazonensis infection,¹⁸ we found that L. amazonensis amastigote infection of DC leads to phosphorylation of the MAP kinase ERK1/2. When ERK phosphorylation was inhibited, DC surface expression of CD40 increased both in vitro and in vivo, and production of IL-12p40 was enhanced in vitro.

Several pathways that alter DC maturation have been characterized, including MAP kinase pathways.¹⁶,²⁰ p38 and JNK phosphorylation were not different between L. amazonensis- and L. major-infected BMDC (Fig. 2D and E). ERK1/2 phosphorylation in BMDC increased 4-fold within minutes of contact with L. amazonensis amastigotes compared to L. major amastigotes (Fig 2A). ERK1/2 phosphorylation was also observed after L. amazonensis promastigote infection of BMDC but not until 3-4 hours post infection.
Early ERK1/2 phosphorylation observed with *in vitro* following *L. amazonensis* amastigote infection of BMDC (Fig. 2A) and microscopic analysis revealing that the amastigote form of *L. amazonensis* predominates within infected BMDC by 4 hours post infection (Fig. 3), suggest a correlation between ERK1/2 phosphorylation and amastigote predominance. We postulate that the amastigote form of *L. amazonensis* activates the ERK1/2 pathway.

ERK1/2 has multiple cytosolic and nuclear targets. The role of ERK1/2 in cellular function ranges from cell survival and cell cycle regulation, to modulation of additional signaling pathways and regulation of transcription. Both CD40 and IL-12p40 transcription can be negatively regulated by activation of ERK1/2. ERK1/2 has also been demonstrated to play a role in the DC survival rather than maturation. Moreover, in tumor cells, aberrant activation of ERK1/2 is implicated in inhibition of differentiation and apoptosis. Activation of ERK1/2 in infected DC by *L. amazonensis* amastigotes may provide two critical mechanisms to ensure parasite success within the mammalian host. First, by targeting specific genes for regulation, namely CD40 and IL-12p40, *L. amazonensis* could interfere with proper DC maturation thereby preventing immune detection and induction of a proper adaptive immune response. Second, increased activation of host ERK1/2 would promote survival of the host cell therefore maintaining a viable host cell for an extended period of time.

ERK1/2 activation as an immunomodulatory mechanism for leishmaniasis has been previously described in other cells systems. *Leishmania* phosphoglycan has been shown to inhibit IL-12 production by macrophages via ERK1/2 activation. Recent work has demonstrated that antibody-opsonized *L. amazonensis* amastigotes induce ERK1/2 activation in Balb/c macrophages. Our work using a C3HeB/FeJ mouse model complement
these findings, providing further support for ERK in the pathogenesis of *L. amazonensis* infection. We demonstrate that *L. amazonensis* amastigote infection of BMDC promotes increased ERK1/2 activation in the absence of additional activating stimuli. In contrast to these findings, Xin et al. observed that both *L. amazonensis* promastigotes and amastigotes both reduce ERK1/2 phosphorylation in the presence or absence of activating stimuli.\(^{15}\) These differences could be explained by the time points chosen for analysis. Based on our data, the 6.5 hour time point occur after the observed peak in ERK1/2 phosphorylation by both *L. amazonensis* amastigotes (7 minutes) and promastigotes (4.5 hours).

We report here that inhibition of ERK phosphorylation with the ERK inhibitor PD98059 prior to BMDC infection with *L. amazonensis* amastigotes leads to an enhanced CD40 surface expression and IL-12p40 production (Fig. 4A and B). *In vivo*, treatment of mice with the orally available ERK inhibitor CI-1040 enhanced CD40 surface expression of CD11c\(^+\) DC (Fig. 4C and D), but did not result in an increased number of IL-12p40-producing cells collected from the DLN of *L. amazonensis*-infected mice (Fig. 4F). We hypothesize that the observed decrease in the number of IL-12p40-producing cells from *L. amazonensis*-infected mice, in the presence of ERK inhibition, may be a result of the lower number of CD11c\(^+\) cells found in the draining lymph node (CD11c\(^+\) events with no treatment, 2818 vs. CD11\(^+\) events with CI-1040, 1225). CI-1040-treatment of Raf-transformed hematopoietic cells leads to increased sensitivity to apoptosis.\(^{37}\) We suggest that systemic ERK inhibition via treatment with this inhibitor may adversely affect the survival of monocytes migrating into the site of infection and later into the draining lymph node. A closer analysis of IL-12p40 production of individual CD11c\(^+\) events from the DLN of infected mice may be required to determine the effects of ERK inhibition on the production of this cytokine.
Our observation of a more mature DC phenotype following treatment of *L. amazonensis*-infected C3HeB/FeJ mice with the ERK inhibitor CI-1040 complements work from our laboratory showing that ERK inhibition can promote parasite killing in *L. amazonensis*-infected macrophages *in vitro*.\(^\text{19}\) The data is also consistent with work indicating that inhibition of ERK slows disease progression of *L. amazonensis*-infected Balb/c mice.\(^\text{18}\) Although we did not observe a restoration in IL-12p40 production during *in vivo* *L. amazonensis* infection, ERK inhibition did enhance CD40 surface expression. Previously, it has been shown that CD40-CD40L interactions are important during *L. amazonensis* infection, as deficiencies in this interaction lead to increased susceptibility to infection.\(^\text{10}\) The work presented here indicates that *L. amazonensis* specifically targets CD40 expression *in vivo*, and that we can restore CD40 surface expression by inhibiting phosphorylation of ERK. We suggest that the mature phenotype restored to CD11c\(^+\) DC via ERK inhibition would promote the development of a productive CD4\(^+\) T cell response during *L. amazonensis* infection; however, further studies are necessary. The work presented here furthers our understanding of host-parasite amastigote-specific interactions and provides evidence for the use of ERK inhibitors as immunomodulators to directly enhance the host immune response after *Leishmania* infection.

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Figure 1. Decreased CD40 expression and IL-12p40-producing cells from DC subsets of total draining lymph node cells from *L. amazonensis*-infected mice at 7 days post-infection. Mice were infected with *L. amazonensis* or *L. major* promastigotes and sacrificed at 2 and 7 days post infection. CD11c+ DC subsets from total draining lymph node cells, collected on CD19 and CD3 double negative events (A) were analyzed for CD40 surface expression via FACS scan. Bar graphs indicate percent CD40 positive (gray, isotype control; black, CD40) and mean fluorescence intensity for CD11c+CD11b+ cells at 2dpi (B and D) and 7 dpi (C and E). Shown are number of IL-12-producing cells from DLN of mice infected with *L. amazonensis* or *L. major* at 2dpi (F) or 7 dpi (G) measured via ELispot. The data is from 3 separate experiments; error bars indicate ± standard error of the mean (S.E.M.); (*) denotes significant differences, *p* ≤ 0.05.
Figure 2. Robust ERK1/2 phosphorylation in *L. amazonensis* amastigote-infected BMDC and delayed ERK1/2 activation following *L. amazonensis* promastigote infection. BMDC were infected with *L. amazonensis* or *L. major* amastigotes (A) or promastigotes (B and C). Whole cell lysates were collected at the indicated time points. Samples were analyzed via western blot for phosphorylated ERK1/2 (A-C), p38 (D), JNK (E) and total ERK1/2, p38 and JNK. Densitometry values were normalized to t-ERK, t-p38 or t-JNK and then to non-infected controls. Densitometry analysis for at least 3 different experiments and representative blots are shown; error bars denote ± standard deviation (S.D.); (*) denotes significant differences, *p* ≤ 0.05.
Figure 3. *L. amazonensis* amastigotes predominate within infected cells at 4 hours post infection. BMDC were infected with *L. amazonensis* promastigotes within 24 well plates. Cover slips were recovered at the indicated time points, fixed and stained. (A) Epifluorescent microscopy analysis of BMDC, (100x, oil). Infected cells were determined by propidium iodide (PI) nuclear and kinetoplast staining, and promastigotes were characterized via C7AE anti-LPG antibody and a secondary Cy2-conjugated antibody. Data representative of n = 2. (B) Cover slips were analyzed via light microscopy, (100x, oil). Parasite stage was identified by presence or absence of flagellum and by size. Graphs indicate the percentage of promastigote-infected cells from total infection rate. Data from at least 3 separate experiments; error bars denote ± S.E.M; (*) denotes significant difference, p≤ 0.05.
Figure 4. ERK inhibition enhances DC maturation phenotype following *L. amazonensis* infection  (A-B) BMDC were pretreated with the ERK inhibitor PD98059 (20mM) for 30 minutes and then infected with *L. amazonensis* amastigotes, and activated with LPS and IFN-γ. 24 hours-post infection cells were harvested and processed for (A) surface CD40 expression and analyzed via FACS scan and (B) supernatants were collected to determine IL-12p40 production via ELISA.  (C-H) Mice were infected with *L. amazonensis* or *L. major* promastigotes and then treated with 100mg/kg of CI-1040 via oral gavage started on day 0 post infection and for the next seven days, twice daily. On day 7 mice were sacrificed; draining lymph nodes were harvested to assess CD40 surface expression on DC populations.  (C) Representative histograms based on CD11c⁺CD11b⁺ population, (gray line, isotype control; black CD40).  Mean fluorescence intensity of CD40 surface expression on CD11c⁺ cells in *L. amazonensis* (D) or *L. major*-infected mice (E) treated with CI-1040 or DMSO-mock control. Number of IL-12p40-producing cells (F and G) as determined by Elispot analysis of total draining lymph node cells. Western blot of splenic lysates probed with anti-phosphorylated ERK1/2 and anti-total ERK1/2. Data from at least 3 different experiments; error bars denote ± S.E.M.; (*) denotes significant differences, p≤0.05.
CHAPTER 4: TARGETED EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) ACTIVATION LOCALIZED TO *LEISHMANIA AMAZONENSIS*-CONTAINING PARASITOPHOROUS VACUOLES

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Abstract

*Leishmania amazonensis* infection of dendritic cells (DC) activates the extracellular signal-regulated kinase (ERK)1/2, a member of the mitogen-activated protein kinase (MAPK) family. This activation has been shown to lead to an altered DC maturation phenotype, which may contribute to non-healing cutaneous leishmaniasis. ERK1/2 activation following *L. amazonensis* promastigote infection peaks 4 hours post-infection *in vitro*, when parasites are primarily present within intracellular phagolysosome-based parasitophorous vacuoles. Studies using non-pathogen-based means of ERK1/2 activation in cell lines have shown that robust intracellular activation of ERK1/2 from phagolysosomes requires the recruitment and association of ERK1/2-specific scaffolding proteins, including p14/MP1 and MORG1, on the surface of this intracellular compartment. Here we identify scaffolding module co-localization, specifically the endosomal ERK-related proteins MP1 and MORG1 with *L. amazonensis*-containing, LAMP2 positive-phagolysosomes in DC. This is the first time pathogen ERK1/2 activation is correlated to intracellular-induced ERK activation and identifies novel signaling cascade components which interact with the *L. amazonensis* parasitophorous vacuole.

Introduction

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*Leishmania* parasites have a dimorphic life cycle alternating between the vector and the mammalian host. Once inoculated into the mammalian host, promastigotes are internalized by phagocytic cells of the immune system including macrophages and dendritic cells (DC). After phagocytosis, promastigotes are found within membrane-bound organelles (i.e. phagosomes) of the endocytic pathway. These parasite-containing organelles, also referred to as parasitophorous vacuoles (PV), progressively acquire characteristics of late endosomal/lysosomal compartments. During this time promastigotes begin the differentiation process into amastigotes, the parasite form that persists in the infected mammalian host. As intracellular parasites residing in phagocytic cells of the immune system, amastigotes must continue to subvert the host cell immune response in order to survive.

*Leishmania* parasites have been shown to alter host cell processes including host cell signaling to promote their survival (reviewed in (17)). Most studies aimed at understanding the alteration of host signaling pathways triggered by *Leishmania* parasites have focused on the initial events of host-parasite interactions occurring on the host cell plasma membrane. However, *Leishmania* parasites reside intracellularly within mammalian host cells, and it is from these intracellular compartments that they continue to engage host cell signaling pathways and interfere with host cell function (12). The molecular mechanisms by which *Leishmania* parasites continue to target host cell processes from within the PV remain poorly understood.

PV are highly dynamic structures, which contain components of host plasma membrane, endosomal organelles, and parasite-derived molecules (10). These organelles are characterized by an acidified environment and are enriched in late endosomal/lysosomal proteins including the lysosomal associated protein 2 (LAMP-2) (2). Classically, late
endosomes/lysosomes are the endpoint step in the endocytic pathway and serve as sites for degradation of pathogens and other materials (16). Because PV share characteristics of late endosomes/lysosomes, newly internalized material and recycled cytoplasmic molecules can be delivered to the PV for degradation (6). This provides *Leishmania* parasites with a constant supply of host-derived molecules, including signaling components (receptors, kinases, adaptor proteins) and maintains the PV in continuous communication with the endocytic pathway of the host cell.

The concept of the endocytic pathway serving as sites for signal transduction has gained increased interest as endocytosis and signal transduction appear to be bidirectionally regulated (24). Endocytic internalization of receptors upon ligand binding serves as a common regulatory mechanism for receptor desensitization for a variety of receptors, including receptor tyrosine kinases (RTK) and G-protein coupled receptors (GPCR) (25). Although this process was initially thought to solely serve for signal attenuation, the presence of activated epidermal growth factor receptors (EGFR) and its downstream effectors on endosome membranes, suggested that signaling may also be initiated from the endocytic pathway (9). Endosomes serving as signaling platforms for a variety of receptors has been proven by the myriad of signaling proteins found to be associated with endosomal compartments and signal attenuation secondary to inhibition of endocytosis or subcellular mis-localization of endosomes (reviewed in (7, 25), 26, 28). Critical in mediating activation of signal transduction pathways from endocytic compartments are site-specific scaffold proteins. Scaffold proteins not only provide targeted location sites for the formation of signaling complexes but can also enhance signal transduction (13, 23).

We have previously demonstrated that *Leishmania amazonensis* infection of DC promotes increased phosphorylation of the extracellular-regulated kinase (ERK) 1/2, which
results in an impaired DC maturation phenotype (2). Infection with *L. amazonensis* promastigotes results in ERK1/2 activation at around 3-4 hours post-infection, when parasites are predominately found intracellularly within infected cells. This delay in ERK1/2 activation suggests *L. amazonensis* is triggering ERK1/2 phosphorylation from within the host cell PV. Activation of the ERK pathway from subcellular locations, including endosomal organelles, has been previously described (reviewed in (8)). Mediating this process are two scaffolding proteins, MEK partner (MP) 1 and MAPK organizer (MORG) 1 (21, 22). We sought to determine the mechanism by which *L. amazonensis* could trigger ERK1/2 phosphorylation from within the host cell. We tested the hypothesis that MP1 and MORG1 scaffolding molecules were involved in *L. amazonensis*-mediated ERK1/2 phosphorylation from an intracellular host cell compartment. We find that *L. amazonensis*-dependent ERK1/2 enhanced phosphorylation is mediated by live parasites, which are able to undergo stage transformation, as heat-killed promastigotes are unable to mediate ERK1/2 activation. We find that *L. amazonensis* parasites are found within late endosomal compartments, characterized by association with LAMP-2, and that at time points during peak ERK1/2 activation, amastigotes predominate within infected cells. We demonstrate intracellular phosphorylated ERK1/2, and the ERK1/2-specific scaffold proteins MP1 and MORG1 co-localizing with parasite-containing organelles. Altogether, our data describes a novel *Leishmania*-dependent mechanism of host cell signaling modulation from subcellular compartments, mediated through host cell MAP kinase ERK1/2 scaffolding proteins.

**Materials and Methods**

*Bone marrow-derived dendritic cells (BMDC)*
BMDC were cultured *in vitro* in the presence of 10 ng/ml of murine granulocyte-macrophage colony-stimulating factor (PeproTech Inc., Rocky Hill, NJ) according to the method of Lutz et al. (5, 15). At day 10 of culture BMDC were harvested for use and approximately 90% of the cells were CD11c+ as analyzed via flow cytometry. Where indicated, BMDC were treated with Pertussis toxin (100ng/ml) (Calbiochem, San Diego, CA).

Parasite culture and infection

Culture of *L. amazonensis* (MHOM/BR/00/LTB0016) parasites was performed as previously described.(11) For *in vitro* promastigote experiments, stationary phase *L. amazonensis* promastigotes were used. Where indicated, parasites were labeled with CellTracker Orange (Invitrogen, Carlsbad, CA). Parasites in PBS were incubated with 0.1uM solution of CellTracker dye for 15 minutes at room temperature, washed, and resuspended in PBS. For heat-killed experiments, *L. amazonensis* promastigotes were diluted to the desired concentration and then incubated at 65°C for 1 hour and placed on ice for at least 20 minutes prior to use. For *in vitro* studies, 10-day-old BMDC were incubated at 34°C, 5% CO₂ with *L. amazonensis* promastigotes at a cell to parasite ratio of 1:3.

Electrophoresis and immunoblotting

To make whole cell lysates, 3 x 10⁶ BMDC were resuspended in 400 μl of 1x cell lysis buffer (Cell Signaling Technologies, Beverly, MA), supplemented with 1mM phenylmethylsulphonyl fluoride (PMSF) and a protease inhibitor cocktail (Roche, Indianapolis, IN) immediately prior to use. Samples were incubated on ice for 15 min and then centrifuged at 16,000 x g for 10 min at 4°C. Supernatants were collected as whole cell lysates and stored at -80°C.
**Immunoblot analysis**

Protein content of all cell extracts was determined via BCA protein assay (Pierce, Rockford, IL) according to manufacturer’s recommendations, and all samples were normalized to 1mg/ml using distilled water. Samples (20-30 μg of protein) were heated for 4 min at 95°C in 1x loading buffer and electrophoresis was performed on a 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. Gels were electroblotted onto polyvinylidene fluoride membranes (PVDF), blocked with 5% bovine serum albumin, and probed with antibodies specific for phospho-ERK, total-ERK1/2 (1:1000) (Cell Signaling, Beverly, MA) and β-actin (1:5,000) (Sigma, St. Louis, MO). Signals were detected with horseradish-peroxidase (HRP)-conjugated goat anti rabbit antibodies (1:20,000) (Jackson ImmunoResearch, West Grove, PA) using the SuperSignal West chemiluminescent substrate (Pierce, Rockford, IL) and expressed to autoradiography film (Midsci, St. Louis, MO).

**Intracellular immunofluorescence staining and microscopy analysis**

1x10^6 BMDC were plated onto 24-well plates containing tissue cover slips. BMDC were infected with CellTracker Orange-labeled *L. amazonensis* promastigotes at an MOI of 3:1 and incubated at 34ºC with 5% CO₂. Cover slips were harvested and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature and washed three times with PBS. BMDC were permeabilized with 0.1% saponin in PBS for 20 minutes at room temperature. Cells were incubated for 1 hour at room temperature with rabbit anti-MP1 (Santa Cruz Biotechnology, Santa Cruz, CA), MORG1 (Abcam, Cambridge, MA), pERK1/2 (Cell Signaling, Beverly, MA), and LAMP-2 (eBiosciences) at a 1:100 dilution in 0.1% saponin. After incubation, cover slips were washed three times with PBS and
incubated for 1 hour at room temperature with rabbit anti-mouse Cy2-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:200 dilution in 0.1% saponin. BMDC were counter-stained with DAPI according to manufacturer’s instructions (Molecular Probes, Eugene, OR). Cover slips were mounted onto slides using MOWIOL (Calbiochem, La Jolla, CA) and viewed using an Olympus IX71 inverted epifluorescence scope (Olympus America Inc., Center Valley, PA). Co-localization analyses were carried out using NIH Image software (1) and confirmed by sequential scanning confocal microscopy using an Olympus IX81 inverted scope (Olympus America Inc., Center Valley, PA).

Statistical analysis

Statistical significances were analyzed using Prism4 (Graphpad Software Inc., La Jolla, CA). Differences between groups were determined using Student’s t-tests and two-way ANOVA. P-values below 0.05 were considered statistically significant.

Results

Live L. amazonensis promastigotes are required to trigger ERK phosphorylation

To demonstrate that the ERK1/2 phosphorylation observed after L. amazonensis infection requires live parasites and was not a time-dependent response to parasite internalization, we treated bone marrow-derived dendritic cells (BMDC) with either live or heat-killed L. amazonensis promastigotes and collected whole cell lysates at the indicated time points. Using western blot analysis we measured ERK1/2 phosphorylation induced by these two treatments. As previously demonstrated (5), infection of BMDC with live L. amazonensis promastigotes induced significantly increased ERK1/2 phosphorylation as compared to BMDC treated with heat-killed parasites (Figure 1). This indicates that L.
amazonensis-mediated activation of ERK1/2 is dependent on cellular interaction with live parasites.

L. amazonensis promastigotes reside in late endosomal compartments and undergo expected developmental progression into amastigotes

Upon phagocytosis, Leishmania promastigotes are contained within a double membrane-bound compartment, referred to as a parasitophorous vacuole (PV). During the process of phagosome maturation, promastigotes undergo developmental and morphological changes becoming amastigotes. It has been previously shown that newly formed phagosomes following Leishmania internalization in DC rapidly acquire late endosomal markers including LAMP-1 and -2 (14). We wanted to further characterize the phagosomal compartment and developmental stage of the parasite during the time of observed ERK1/2 phosphorylation. Using L. amazonensis promastigotes stained with a fluorescent dye, CellTracker orange, we infected BMDC and harvested cells at 2, 3 and 4 hours post-infection and characterized the phagosomal compartment via immuno-fluorescence analysis and epifluorescent microscopy. Green, punctuate cytosolic staining indicated presence of LAMP-2 (Figure 2A, second panel). At the time points analyzed, L. amazonensis parasites were found within membrane-bound compartments that tightly delineated the shape of the parasite (Figure 2A, third panel). LAMP-2 was also observed to closely follow parasite outline at all time points analyzed, suggesting association with the organelles containing L. amazonensis (Figure 2A, second and fourth panels, white arrows). These data support previous findings indicating that PV maturation occurs within 1 hour after infection (14). In addition, the data indicates that at the time of ERK1/2 phosphorylation, L. amazonensis is located within late endosomal/lysosomal compartments.
To further characterize parasite development within infected BMDC, we sought to determine the percentages of promastigotes versus amastigotes just before (2hr post-infection) and during ERK1/2 activation (4hr post-infection). BMDC cultured on coverslips were infected with *L. amazonensis* promastigotes and then harvested at 2 and 4 hours post infection and analyzed via light microscopy. Promastigotes and amastigotes were differentiated based on morphology, presence or absence of a flagellum, and size (amastigotes, <5μm). As expected, while promastigotes predominated within infected cells at 2 hours post-infection, there was a significant increase in the number of intracellular amastigotes at 4 hours post-infection (Figure 2B). Altogether these data suggest that BMDC ERK1/2 phosphorylation occurs when *L. amazonensis* parasites are found within late endosomal/lysosomal compartments and differentiating into amastigotes.

*Intracellularly-phosphorylated ERK co-localizes with L. amazonensis*

We have previously shown that when BMDC are infected with *L. amazonensis* promastigotes there is significant and robust ERK1/2 phosphorylation observed 3-4hours post-infection (5). As shown in Figure 2A, at this point, parasites are intracellular, within late endosomal compartments. We hypothesized that *L. amazonensis*-mediated ERK1/2 activation is initiated from this intracellular compartment. To test this hypothesis we infected BMDC with *L. amazonensis* promastigotes stained with a fluorescent dye and detected the location of phosphorylated ERK1/2 using intracellular immuno-fluorescent analysis and epifluorescence microscopy. Green cytosolic staining indicated the presence of phospho-ERK1/2 in BMDC (Figure 3A, second panel). Immunofluorescence, particle co-localization analysis showed that phopho-ERK1/2 co-localized with *L. amazonensis* promastigotes (red) at all time points analyzed, 2-4 hrs (Figure 3A, fourth panel, 3B). We confirmed these
findings via sequential scanning confocal microscopy. This data suggests that ERK1/2 phosphorylation is occurring from intracellular, parasite-containing, organelles.

**Scaffold proteins MP1 and MORG1 associate with L. amazonensis-containing organelles**

MP1 and MORG1 have been recently characterized as scaffold proteins for late endosomal-mediated ERK1/2 phosphorylation (21, 29). Moreover, while MP1 directly localizes with late endosomes via its interaction with p14, MORG1 can only form a complex on late endosomes in the presence of MP1, as the two scaffold proteins directly interact (27, 29). Given that *L. amazonensis* parasites are found within late endosomal compartments, and that phospho-ERK1/2 co-localizes with intracellular parasites, we hypothesized that scaffold protein(s) may function to mediate activation of ERK1/2 from intracellular parasite-containing organelles.

To analyze the role of MORG1 in our system, we utilized intracellular immunofluorescence analysis and epifluorescence microscopy to determine any potential association between MORG1 and parasite-containing organelles following infection. Immunofluorescence analysis indicated green cytosolic distribution for MORG1, along with co-localization of this scaffold protein with intracellular parasites (Figure 4A). Particle co-localization quantitative analysis shows co-localization between MORG1 and *L. amazonensis* during all time points studied (Figure 4B). Our findings were confirmed by sequential scanning confocal microscopy.

Since MORG1 requires MP1 to form a scaffolding complex on late endosomes, we hypothesized that MP1 must also be recruited to parasite-containing organelles. Again using immunofluorescence, and particle co-localization analysis, we show that MP1 co-localizes with *L. amazonensis* in infected cells (Figure 4C and D). Altogether these data suggest that
MORG1 and MP1 are recruited to parasite-containing organelles and are likely to mediate intracellular *L. amazonensis*-dependent activation of ERK1/2 within infected cells.

**Pertussis toxin treatment reduces *L. amazonensis*-dependent ERK1/2 phosphorylation**

Little is known regarding the regulation and recruitment of MP1 or MORG1 to late endosomal compartments. However, of the two, MORG1 appears to show more selectivity as it is only recruited after triggering of G protein-coupled receptor (GPCR) signaling (24). In order to further test the role of MORG1 in our system, we pre-treated BMDC with Pertussis toxin (PTX), which binds to the Gα (G-i/o subfamily) subunit of G proteins and prevents their activation. When BMDC were pre-treated with PTX and then infected with *L. amazonensis* promastigotes, we observed a significant decrease in ERK1/2 phosphorylation as compared to untreated, infected cells (Figure 5A). In addition, confocal microscopy analysis of the location of MORG1 in BMDC infected with *L. amazonensis* following PTX treatment shows no co-localization of this scaffold protein and the parasite (Figure 5B). Similar to the effect on MORG1 recruitment, no co-localization of MP1 and *L. amazonensis*-containing organelles was observed (Figure 5C). In addition, PTX treatment altered the cytosolic distribution of MP1 (Figure 5C) as compared to non-treated cells (Figure 4C). These data suggest that activation of the Gα-i/o subfamily of GPCR may be involved in mediating enhanced *L. amazonensis*-dependent ERK1/2 activation, possibly through recruitment of MORG1 to late endosomal compartments.

**Discussion**

Our understanding of the mechanisms employed by *Leishmania* parasite to modulate host cell signaling is limited (17). Previous works assessing the host cell response to
Leishmania infection have focused on the events taking place during initial host-pathogen interactions at the host’s plasma membrane. However, in the mammalian host, Leishmania parasites reside intracellularly within parasitophorous vacuoles (PVs), and it is from within these membrane-bound compartments that they continue to modulate the host cell response (12). Understanding how Leishmania harness the host cell immune response may provide insight into therapeutic approaches to combat infection.

As potent antigen presenting cells, dendritic cells (DC) play a critical role in the initiation of adaptive immune responses (4). Interfering with proper DC maturation and function can be detrimental to the ensuing immune response. The balance of particular molecular signaling cascades has been shown to affect the maturation process of DC (3, 18, 19). Pathogen-mediated modulation of these signaling pathways could lead to improperly activated DC that subsequently promote a non-polarized adaptive immune response. We have previously shown that L. amazonensis infection can impair proper DC maturation via activation of ERK1/2 (5). Our data indicated that following infection with L. amazonensis promastigotes, robust ERK1/2 activation was not observed until hours post infection, when parasites were predominantly found intracellularly. This suggested that L. amazonensis could be mediating activation of ERK1/2 from within the PV. Recent work has shown that infection with L. amazonensis promastigotes leads to activation of the phosphoinositide 3-kinase (PI3K) pathway (20). Interestingly, PVs were observed to contain lipid products of the PI3K pathway for at least 12 hours post infection, suggesting that these endosomal organelles could serve as activation sites for downstream kinases of this pathway, such as protein kinase (PK)B/Akt (12). These data support the possibility of other pathways being activated from PVs during Leishmania infection. In this paper, we sought to characterize a mechanism by which L. amazonensis parasite could trigger ERK1/2 activation from within
PVs. The data presented here point to a potential molecular mechanism of *L. amazonensis*-dependent ERK1/2 signaling via recruitment of host scaffold proteins, MP1 and MORG1.

While *L. amazonensis* amastigote infection triggers ERK1/2 phosphorylation within minutes of parasite exposure, *L. amazonensis* promastigote infection of bone marrow-derived dendritic cells (BMDC) results in delayed ERK1/2 phosphorylation, not observed until hours post-infection (5). Treatment of BMDC with heat-killed *L. amazonensis* promastigotes resulted in a significant decrease in ERK1/2 phosphorylation as compared to BMDC treated with live parasites (Figure 1). This data indicated that ERK1/2 phosphorylation is dependent on the presence of viable parasites, which can undergo stage transformation, and is not a time-based response of promastigote phagocytosis. Moreover, the data suggests that ERK1/2 signaling is actively modulated by intracellular *L. amazonensis* parasites. While both promastigotes and amastigotes can initiate infection, the amastigote form of the parasite predominates and persists within host cells, and must subvert the host immune response from within an intracellular compartment. Amastigotes are capable of altering multiple host cell processes, including host cell signaling, to establish infection and persist within phagocytic cells of the immune system (reviewed in (12)). It could be suggested that the delay in ERK1/2 phosphorylation following promastigote infection is due to a requirement for a specific amastigote-derived ligand and/or specific time or dose-dependent conditions within the PV that promote signaling through this MAP kinase pathway.

In order to assess parasite localization within BMDC, we characterized parasite-containing organelles via immunofluorescence using the lysosomal-associated membrane protein 2 (LAMP-2) (Figure 2A). LAMP-2-positive organelles are characterized as late endosomal/lysosomal compartments, which is where *Leishmania* parasites have been
previously shown to reside (reviewed in (6)). Lysosomal compartments contain a highly hydrolytic, highly acidic environment, specialized in the degradation of material within membrane-trafficking pathways, including pathogens (16). While promastigotes are not well adapted to survive within lysosomes, amastigotes can survive and multiply under these harsh conditions. Increased temperature and reduced pH are important triggers for amastigote differentiation (31). The presence of *L. amazonensis* within LAMP-2-positive compartments would suggest a stage of infection when promastigotes are undergoing the differentiation process into amastigotes.

Using light microscopy we assessed amastigote differentiation within infected cells. Promastigotes predominated within infected cells at 2 hours post-infection, while by 4 hours post-infection, the amastigote form predominated within infected BMDC (Figure 2B). This switch from promastigotes to amastigotes correlates with the onset of ERK1/2 activation observed following *L. amazonensis* promastigote infection. Taken together, these data suggest that an amastigote-derived ligand may be required to trigger the ERK1/2 pathway. This is further supported by previous findings of our laboratory showing the almost immediate ERK1/2 phosphorylation following *L. amazonensis* amastigote infection.

We and others have previously reported activation of the MAP kinase ERK pathway during *L. amazonensis* infection (5, 30). Here we demonstrate for the first time that phosphorylated ERK1/2 is found to co-localize with intracellular parasites (Figure 3A and B), suggesting activation of this kinase from an intracellular compartment. While the plasma membrane is the canonical site for initiation of signal transduction, MAP kinase signaling from intracellular organelles has been previously described (reviewed in (8)). Scaffold proteins have emerged as critical mediators in regulation of ERK1/2 signaling from subcellular compartments. These molecules act by binding one or more signaling
components (kinases) to regulate their association with other kinases or substrates, enhance their activity, and determine specific subcellular localization of the signaling complex (13, 23).

MP1 and MORG1 are two such scaffold proteins shown to mediate ERK1/2 activation from late endosomal compartments. We hypothesized that either MP1, MORG1 or both, could be recruited to parasite-containing organelles and mediate activation of the ERK1/2 pathway. Here we show that both MP1 and MORG1 co-localize with *L. amazonensis* within infected BMDC from 2-4 hours post-infection (Figure 4A and B), when robust ERK1/2 phosphorylation was previously demonstrated (5). Previous studies demonstrated that MORG1 is recruited under specific signaling conditions, specifically, when G-protein coupled receptors (GPCR) are stimulated (29). Treatment of *L. amazonensis*-infected BMDC with Pertussis toxin (PTX), a known Gαi/o subunit inhibitor, led to a decrease in ERK1/2 phosphorylation as compared to non-treated cells (Figure 5A). This observation suggested a potential role for Gαi/o signaling during *L. amazonensis*-dependent ERK1/2 activation. Our data would suggest that stimulation of Gαi/o during internalization of *L. amazonensis* may promote MORG1 recruitment to late endosomal compartments and subsequent enhanced ERK1/2 phosphorylation. This is supported by the finding that during PTX treatment we observe a lack of MORG1-*L. amazonensis* co-localization via confocal microscopy (Figure 5B, top panel).

Altogether, our data describes a novel *L. amazonensis*-mediated mechanism of ERK1/2 activation from an intracellular compartment. Intracellular *L. amazonensis* parasites actively utilize host scaffold proteins to trigger activation of the MAP kinase ERK1/2 pathway. We have previously shown that activation of this pathway following *L. amazonensis* infection leads to impaired DC maturation and that inhibiting ERK1/2 activation
leads to restoration of the DC maturation phenotype in vitro and partial recovery in vivo (5).

Given the ubiquitous nature of ERK1/2 signaling and the myriad cellular processes in which it is involved, systemic inhibition of ERK could lead to non-desirable host side effects. Through characterization of a specific, scaffold-mediated mechanism of ERK1/2 activation, it is feasible to envision the development of treatment options to specifically modulate pathogenic ERK1/2 phosphorylation, sparing more universal functions. These therapeutic treatments could serve to treat not only L. amazonensis infection, but other diseases where aberrant activation of ERK1/2 leads to disease.

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References


interacts with components of the ERK cascade and links ERK signaling to specific agonists. Proc Natl Acad Sci U S A 101:6981-6.


Figure 1: Live *L. amazonensis* promastigotes are required to promote phosphorylation of the MAP kinase ERK1/2. BMDC were treated with live or heat-killed *L. amazonensis* promastigotes and incubated at 34°C with 5% CO₂. BMDC were harvested at the indicated time points and total cell lysates were made. Samples were analyzed via western blot for phosphorylated and total ERK1/2. Densitometry values were normalized to total ERK and then to non-infected controls. Densitometry analysis of at least three different experiments and representative blot are shown; error bars denote ±SEM, (*) denotes statistically significant differences, p<0.05.
Figure 2 *L. amazonensis* parasites are found within LAMP-2-positive compartments and amastigotes predominate within infected cells at four hours post-infection. BMDC in 24 well plates containing glass coverslips were infected with CellTracker orange-labeled or unlabeled *L. amazonensis* promastigotes. Coverslips were recovered at the indicated time points, fixed and stained. (A) Sequential scanning confocal microscopy (x60, oil) analysis of BMDC infected with *L. amazonensis* (red) and intracellular LAMP-2 (green) (upper panel) at 3 hours post infection. LAMP-2 can be observed delineating the outline of *L. amazonensis* (top panel, second column, white arrows) and co-localizing with the parasite (top panel, fourth column, areas of yellow). Bottom panel shows control staining for LAMP-2 with secondary antibody only. Data representative of at least three different experiments. (B) Coverslips were analyzed via light microscopy (x100, oil). Parasite stage was determined by presence of absence of flagellum and by size. Graph indicates the percentage of cells infected by either or both forms. Representative images for both time points are shown. Data from at least three different experiments; error bars denote ±SEM; (*) denotes statistically significant differences, p<0.05.
Figure 3 Co-localization of *L. amazonensis* and intracellular phosphorylated-ERK1/2.

BMDC in 24 well plates containing glass coverslips were infected with CellTracker orange-labeled *L. amazonensis* promastigotes. Coverslips were recovered at the indicated time points, fixed and stained. (A) Sequential scanning confocal microscopy (x60, oil) analysis of *L. amazonensis* (red) and intracellular phospho-ERK1/2 (green) at 3 hours post-infection. Co-localization of *L. amazonensis* and phospho-ERK1/2 is observed (top panel, fourth column, areas of yellow). Secondary only control for phosphor-ERK1/2 staining (bottom panel, second column). (B) Immunofluorescence, particle co-localization analysis of *L. amazonensis* and phospho-ERK1/2 during time course of infection. Graph indicates mean gray value (sum of the gray values of all the pixels in a selected area divided by the number of pixels in that area) of phospho-ERK1/2 signal where parasites were localized. Values were normalized to secondary antibody only control. Data from at least three separate experiments; error bars denote ±SEM.
Figure 4 Co-localization of the scaffold proteins MORG1 and MP1 with *L. amazonensis* within infected BMDC. BMDC in 24 well plates containing glass coverslips were infected with CellTracker orange-labeled *L. amazonensis* promastigotes. Coverslips were recovered at the indicated time points, fixed and stained. Sequential scanning confocal microscopy (x60, oil) analysis of *L. amazonensis* (red) and intracellular (A) MORG1 or (C) MP1 (green) at 3 hours post-infection. Images indicate areas of co-localization between parasite and MORG1 and MP1, as indicated by areas of yellow (top panels, fourth column, white arrows). Immunofluorescence, particle co-localization analysis of *L. amazonensis* and (B) MORG1 or (D) MP1. Graphs indicate mean gray value of MORG1 or MP1, signal in areas where parasites were localized, as described in Figure 3. Values were normalized to secondary antibody only control. Data from at least three separate experiments; error bars denote ±SEM.
Figure 5 Pertussis toxin treatment reduces *L. amazonensis*-dependent ERK1/2 phosphorylation and MORG1 co-localization. (A) BMDC were infected with *L. amazonensis* promastigotes and left untreated or treated with Pertussis toxin (100ng/ml) and incubated at 34°C with 5% CO₂. BMDC were harvested at the indicted time points and total cell lysates were made. Samples were analyzed via western blot for phosphorylated and total ERK1/2. Densitometry values were normalized to total ERK and then to non-infected controls. Densitometry analysis of at least three different experiments and representative blot are shown; error bars denote ±SD. (B) BMDC in 24 well plates containing glass coverslips were infected with CellTracker orange-labeled *L. amazonensis* promastigotes and left untreated or treated with Pertussis toxin (100ng/ml). Coverslips were recovered at the indicated time points, fixed and stained. Images of confocal microscopy (x60, oil) analysis of *L. amazonensis* (red) and intracellular MORG1 (top panel) or MP1 (second panel) (green), at 3 hours post-infection. PTX treatment inhibits MORG1 co-localization with *L. amazonensis*; no areas of yellow are present (top panel, fourth column). Secondary only antibody control for MORG1 (bottom panel).
CHAPTER 5: GENERAL CONCLUSIONS

Summary

The first part of the work presented in this dissertation describes for the first time the temporal immune response to natural autochthonous *Leishmania infantum* infection in the United States. Clinical disease progression within the Foxhound population of the United States recapitulates immune dys-regulation described in endemic patients. Our data indicates that infected animals may initially develop an antigen-specific, IFN-γ-producing, lymphoproliferative CD4⁺ T cell response, which can control infection. This is supported by the low to undetectable levels of blood parasites and the overall subclinical status of these animals. In contrast, animals showing signs of disease progression demonstrate a waning CD4⁺ T cell proliferative response, decreased IFN-γ production and a concomitant increase of production IL-10 and immunoglobulins. In these animals, there is an increased parasite burden detectable in blood, which may be directly correlated to a non-productive immune response.

In endemic regions, dogs are the primary reservoir for *L. infantum* and are considered the most significant risk factor predisposing humans to infection. Early detection and treatment of infected dogs are important in limiting potential human transmission. Our work indicates that given the temporal variability in the immune response to *L. infantum* infection, a variety of immune parameters (i.e. antigen-specific proliferation, levels of IFN-γ and IL-10, antibody production, and parasite burden) should be considered to better prognosticate resistance versus susceptibility to infection and clinical disease. Ultimately understanding the causal relationship between these parameters may provide insights into
the mechanisms leading to disease progression and consequent development of immunotherapies.

The second part of the work presented in this dissertation utilizes a different model of *Leishmania* infection, murine cutaneous leishmaniasis, to dissect host-parasite interactions that lead to disease susceptibility. We demonstrate that infection of dendritic cells (DC) with *L. amazonensis* leads to an impaired maturation phenotype characterized by decreased surface expression of CD40 and production of IL-12p40, as compared to *L. major*-infected DC. Moreover, this immature phenotype results specifically from *in vitro* infection with the amastigote form of the parasite. These data are consistent with the observed lack of T cell polarization observed following *L. amazonensis* infection resulting in non-healing, chronic disease.

Initial assessment of DC maturation following *L. amazonensis* amastigote infection *in vitro*, indicated that CD40 expression and IL-12p40 production were specifically targeted for modulation, as other maturation markers, CD80 and CD86, were expressed in comparable levels to *L. major*-infected DC. This impaired maturation phenotype was mediated by the amastigote form of the parasite, as promastigote infection did not affect CD40 expression or IL-12p40 production. *Ex vivo* analysis of DC from the draining lymph node of either *L. amazonensis* or *L. major*-infected mice supported our *in vitro* findings. Decreased CD40 surface expression and IL-12p40 production from CD11c⁺ cells from *L. amazonensis*-infected mice was observed as early as 7 days post-infection as compared to *L. major*. In contrast, at 2 days post-infection, no differences were observed between the two infections. The temporal difference (2 days vs. 7 days) observed *in vivo* were taken to emphasize times when promastigote versus amastigote infection is likely to predominate. This data supports
our *in vitro* findings suggesting that the amastigote form of the parasite is responsible for modulation of CD40 expression and IL-12p40 production.

Characterization of the molecular mechanisms leading to impaired DC maturation indicated that *L. amazonensis* amastigotes induced rapid and significant phosphorylation of the MAP kinase ERK1/2, observed within minutes of exposure of DC *in vitro* to the parasite. Infection with *L. amazonensis* promastigotes also led to increased ERK1/2 phosphorylation as compared to *L. major* infection; however, this phosphorylation was delayed several hours, between 3-4.5 hours post-infection.

*In vitro* inhibition studies determined that treatment of DC with a MEK—upstream kinase of ERK inhibitor—PD98059, led to enhanced surface CD40 expression and IL-12p40 production following *L. amazonensis*-amastigote infection as compared to non-treated cells. Treatment of *L. amazonensis*-infected mice with the highly-specific MEK inhibitor, CI-1040, enhanced surface CD40 expression. Together, this data indicates that *L. amazonensis* amastigotes, through activation of the MAP kinase ERK1/2, inhibit the ability of DC to undergo proper maturation *in vivo*.

The delay in ERK1/2 phosphorylation following *L. amazonensis* promastigote infection indicated that the parasite-mediated signaling was being triggered from an intracellular compartment within the infected host. Exposure of DC to heat-killed promastigote parasites did not result in ERK1/2 phosphorylation as compared to live *L. amazonensis* promastigote infection. This data suggested that the delayed ERK1/2 phosphorylation was not a pre-determined effect of parasite internalization. Moreover, immunofluorescence analysis of intracellular staining for phosphorylated-ERK1/2 demonstrated a co-localization between the parasite and activated ERK1/2, suggesting ERK1/2 phosphorylation was occurring around the parasite and not from the plasma
membrane. ERK1/2 activation from intracellular compartments requires the recruitment of scaffold proteins to these specific sites. Immunofluorescence analysis indicates that two scaffold proteins specific for late endosomal compartments, MP1 and MORG1, co-localize with *L. amazonensis*-containing organelles.

Altogether, our data suggest that a possible mechanism of immune evasion by which *L. amazonensis* directly utilizes host-derived signaling components to trigger intracellular ERK1/2 activation. Activation of the ERK1/2 pathway negatively affects the maturation phenotype of DC, and thus negatively affects the ensuing immune response.

**Discussion**

**North American canine visceral leishmaniasis**

Although endemic in many parts of the world, canine visceral leishmaniasis (CVL) is an emerging disease in the United States Foxhound population. Infection with *L. infantum* does not always equate with clinical disease. When disease progression occurs, clinical presentation manifests in a broad spectrum ranging from asymptomatic to disseminated, polysymptomatic disease. Moreover, the observed disease progression and the immune response of *L. infantum*-infected Foxhounds are very similar to naturally- and experimentally-infected dogs (5, 13), making natural, autochthonous *L. infantum* infection in this country a unique opportunity to study VL.

Host protection against VL requires a pro-inflammatory, T helper (T\(_H\)) 1 immune response, as characterized by the production of interleukin (IL)-12 by antigen presenting cells and IL-2, tumor necrosis factor alpha (TNF-\(\alpha\)) and interferon (IFN)-\(\gamma\) by T cells (29, 30). Infected macrophages are activated by IFN-\(\gamma\) and TNF-\(\alpha\) to kill intracellular amastigotes via the L-arginine nitric oxide pathway (26, 28, 41). In contrast, susceptibility to disease is
characterized by the appearance of Leishmania-specific lymphoproliferative unresponsiveness, decreased production of IFN-γ following Leishmania antigen stimulation, and a concomitant immune-suppressive response (12, 36). Susceptibility to L. infantum infection is unique in that the susceptible response is often secondary to an established productive immune response. The factors that control the balance between susceptible and protective immune responses remain poorly understood.

In our studies of L. infantum-infected Foxhounds, we were able to characterize the temporal immune response to this parasite as described in Figure 1. In our cohort of animals we were able to observe three different stages in the immune response to L. infantum as infection progressed from asymptomatic to oligosymptomatic and to full clinical VL. Our data indicates that infected animals may initially develop a productive, IFN-γ-mediated immune response, able to control infection. During this stage, parasite-specific antibodies and blood parasites in these animals are low to undetectable. While some infected animals may remain asymptomatic for years, showing no signs of disease, other animals begin to show signs of disease progression. During this second phase, infected animals have an antigen-specific proliferative response and IFN-γ production, in addition to increased production of IL-10 and antigen-specific antibodies. Blood parasites are also more readily detectable, indicating increased levels of circulating parasites. In the third stage of infection, during clinical disease, the initial protective, Th1-mediated immune response wanes, while levels of IL-10 and antigen-specific antibodies are significantly increased. Loss of the antigen-specific CD4+ T cell proliferative response and IFN-γ production have been previously described as one hallmark of disease progression (24). In the absence of IFN-γ, macrophages are not properly activated to kill intracellular parasites. In addition, increased production IL-10 leads to alternately activated macrophages, which are also unable to mediate parasite killing. With
decreased parasite clearance, there is an increase in parasite burden in tissues and blood. During this non-productive, immune-suppressive response, systemic parasite dissemination goes uncontrolled leading to full-blown clinical disease.

Based on the spectrum of immune responses we observed in the *L. infantum*-infected animals studied here, it is clear that no one clinical parameter can be used to predict in advance which infected dogs will likely become clinically ill. Our data indicates that several key changes in clinical parameters should be considered including a rise in antibody production, a decrease in the proliferative and IFN-γ-mediated CD4+ T cell response, a progressive increase in IL-10 production, and increased parasite burden. While this information is critical during diagnosis and prognostication of disease progression, the cause-and-effect relationship between these parameters remains uncharacterized.

The initial TH1 immune response observed in our cohort of *L. infantum*-infected dogs suggests that these animals are capable of inducing a protective response and controlling infection. This has been previously observed both in dogs and humans in previous studies, showing that subclinical and cured individuals are capable of mounting antigen-specific IFN-γ responses following *Leishmania* stimulation *in vitro*, are resistant to reinfection and become leishmanin skin test positive, suggesting no inherent defect in the antigen-dependent TH1 immune response (7, 11, 42). The appearance of an immune-suppressive response characterized by increased production of IL-10 in serum and lesional tissues has been proposed to be responsible for the immunological changes that result in disease progression (24). Since animals can remain infected for years, it has been suggested that IL-10 production is triggered as a homeostatic response to prevent self-mediated tissue damage due to the ongoing inflammatory immune response. IL-10 has pleotropic
deactivating effects on the immune response and can be produced by a variety of cell types including macrophages, dendritic cells, B cells and T cells.

To date, the cellular source of IL-10 during canine VL has not been determined. In humans, a recent study implicated a population of CD25\(^{-}\)Foxp3\(^{-}\) T cells in the production of IL-10 (23). These T cells arise in the periphery, are antigen driven and produce large amounts of IL-10, different from naturally-occurring, CD25\(^{+}\)Foxp3\(^{+}\) T regulatory cells. The generation of such cells has been previously described in an experimental model involving repeated antigenic peptide stimulation (39). It is feasible that IL-10-producing, antigen-specific T cells are playing a role in our model of canine VL. While we have not tested this hypothesis directly, we do observe an antigen-specific IL-10 response from PBMC stimulated with _L. infantum_ antigen, and an increase in blood-parasite burden. Higher antigenic load would promote continual antigen presentation in the presence of inflammation, which may in part lead to the generation of adaptive tolerogenic T cells (2, 15, 40).

High levels of antigen have also been associated with the induction of T cell exhaustion (4, 21). Exhausted T cells are characterized by decreased antigen-specific proliferation and reduced effector function, including cytokine production. Most models of T cell exhaustion have focused on CD8\(^{+}\) T cells following viral infection and less is known regarding this effect on CD4\(^{+}\) T cells. Recently, using a mouse model of visceral leishmaniasis, it was shown that following _L. donovani_ infection; CD8\(^{+}\) T cells became less functional, characterized by decreased IFN-γ production, and weak proliferative responses (17). Clinical disease in our _L. infantum_-infected Foxhounds is characterized by a loss in CD4\(^{+}\) antigen-specific lymphoproliferation and IFN-γ production, which can be explained in part by exhaustion of the T cell compartment.
While a loss in the cellular immune response to *L. infantum* signals disease progression, the appearance of a measurable humoral response during visceral leishmaniasis is associated with failure to control infection. There is a strong correlation between the levels of immunoglobulins (Ig), clinical status, and parasite burden (33). The humoral immune response observed in *L. infantum*-infected Foxhounds recapitulates these findings (5). The marked increased in antigen-specific IgG production in infected animals suggests the generation of T cell help associated with antibody production (TH2). Yet, infected animals transitioning into disease progression do not show a clear TH1/TH2 dichotomy pattern as mixed TH1/TH2 responses have been observed in both subclinical and clinical animals, albeit susceptible animals showing slightly higher expression of IL-4 and IL-10 mRNA (27). To date, production of IL-10 is thought to promote a humoral response by enhancing plasma cell survival and subsequent antibody production (24). However, the exact mechanism leading to the increased IgG production in infected animals remains poorly understood. We would propose that again increased antigen burden could explain the observed immune-dysregulation. It has been postulated that the amount and type of antigen can regulate the type of immune response (44). During disease progression and concomitant higher parasite burden, it is feasible that antigens are more readily presented to B cells in secondary lymphoid organs leading to their activation (3).

We would hypothesize that immune dysregulation observed in *L. infantum*-infected Foxhounds results from an initial TH1 immune response which is not completely efficient at parasite clearance (i.e. no sterile cure). Under these conditions, parasites would slowly accumulate in infected tissues even under anti-*Leishmania*, inflammatory conditions. Over time, the immune response, either due to chronic antigen stimulation and/or inflammation-induced tissue damage, would transition into an alternative immune response. In the case of
L. infantum infection, the alternate immune response leads to immune-suppression, which leads to further increases parasite burden and promotion of disease progression.

So far, we have assumed that increased antigen burden and chronic antigen exposure may lead to changes in the CD4+ T cell and humoral responses. However, it is also feasible that increased antigen burden may be a secondary outcome to an already pre-disposed condition, where immune-suppression is inherent in the initial response of susceptible individuals. While we propose a one-way regulation of antigen and immune dysregulation, we cannot discard the possibility of the opposite playing a role, or both acting side-by-side. Other factors including genetic predisposition and/or environmental conditions may also be responsible for controlling disease outcome. Ultimately, understanding how these immunological parameters interact with each other would provide insight into the mechanisms leading to disease susceptibility during VL and other similar chronic diseases.

L. amazonensis-mediated modulation of DC signaling pathways and maturation

Infection of C3HeB/FeJ mice with L. amazonensis leads to chronic, non-healing disease, characterized by a non-polarized immune response with low to undetectable levels of both IFN-γ and IL-4 (1, 16). Analysis of antigen-responsive CD4+ T cells from C3HeB/FeJ mice chronically infected with L. amazonensis, indicate that these cells have an impaired ability to transition from a naïve to an effector phenotype (32). Defects in DC maturation may, in part, contribute to the non-polarized T cell phenotype observed during L. amazonensis infection (32), and lead to a non-healing immune response.

L. amazonensis infection both in vitro and in vivo results in DC with an impaired maturation phenotype characterized by decreased surface expression of CD40 and IL-12p40 production. CD40 and IL-12 are critical in the induction of TH1 immune responses and
defects in both have been shown to lead to disease susceptibility during *Leishmania* infection (8, 19, 20, 37). DC with deficiencies in both CD40 surface expression and IL-12p40 production may be unable to properly prime CD4+ T cells and promote Th1 polarization. Improper or insufficient activation of DC can promote non-polarized, often tolerogenic, immune responses like those observed following *L. amazonensis* infection.

Both morphological forms of *Leishmania* parasites, promastigotes and amastigotes, are capable of initiating mammalian infection. However, *Leishmania* amastigotes predominate as the life form within the mammalian host and therefore to survive need to avoid host detection and elimination. *Leishmania* amastigotes have been shown to interfere with host cell function, including modulation of signaling pathways, suppression of antimicrobial and pro-inflammatory mediators, and induction of cytokines that promote disease progression (14, 25). In support of these findings, we found *L. amazonensis* amastigotes to be responsible for modulating DC maturation, as promastigote infection did not lead to the observed impaired phenotype.

Consistent with other reports indicating an immunomodulatory role for MAP kinase ERK during *L. amazonensis* infection (10, 43), we found that *L. amazonensis* amastigote infection of DC leads to phosphorylation of ERK1/2. ERK activation has been shown to negatively regulate DC maturation, characterized by decreased expression of surface co-stimulatory molecules and cytokine production (31, 34). Both CD40 and IL-12p40 expression can be negatively regulated by activation of ERK1/2 (22). When ERK phosphorylation was inhibited, DC surface expression of CD40 increased both in vitro and in vivo, and production of IL-12p40 was enhanced in vitro. While ERK inhibition in vitro fully restored this CD40/IL-12p40 maturation phenotype of infected DC, in vivo inhibition only resulted in partial restoration of phenotype. While these findings support the pathogenic role
of ERK during *L. amazonensis* infection, they also suggest that during *in vivo* infection other
*L. amazonensis*-mediated immune regulatory mechanisms may be at work, which are not
responsive to ERK inhibition. In addition, during our *in vivo* analysis of mice treated with an
ERK1/2 inhibitor, we observed an overall decrease in the number of CD11c⁺ cells in the
draining lymph node. Reduction in IL-12p40-producing cells may be partially explained by
the overall decrease in CD11c⁺ cells in the draining lymph node. The observed decrease in
these cells may point to not-accounted-for side effects of systemic ERK1/2 inhibition,
including diminished cell survival, as ERK1/2 has been demonstrated to play a critical role in
DC survival (34). Together these data point to an important role for ERK1/2 activation during
the modulation of DC maturation mediated by *L. amazonensis* amastigotes. However, given
the ubiquitous nature of this pathway, the data also point to the need for targeted versus
systemic ERK inhibition as a potential anti-leishmania therapy.

Based on the finding that *L. amazonensis* promastigote infection resulted in delayed
phosphorylation of ERK1/2 as compared to amastigote infection, we explored the dynamics
and potential mechanism of intracellular ERK1/2 activation. The delayed response in
ERK1/2 activation could be explained by two mechanisms, requirement for a stage-specific
ligand and/or specific signaling mediators (e.i. scaffold proteins). Since *L. amazonensis*
amastigote infection leads to rapid ERK1/2 phosphorylation is it possible that an amastigote-
specific ligand (membrane-bound or secreted) is necessary to trigger this pathway. The
variety and complexity of glycoproteins and glycolipids coating the surface of promastigotes
and amastigotes are very different in these two stages, supporting the idea of a ligand
requirement. We would hypothesize that our observed delayed ERK1/2 phosphorylation
following promastigote infection is dependent on the generation of an amastigote-derived
molecule that is expressed and/or secreted once the promastigote beings its transformation
into an amastigote. Moreover, the appearance of the ligand may not be sufficient to trigger ERK1/2 activation from endosomal compartments. Along with a specific ligand, it is possible that recruitment of specific signaling mediators is necessary to trigger promastigote-mediated ERK1/2 activation. We would suggest that promastigote internalization recruits signaling mediators, specifically scaffold proteins, to the parasitophorous vacuole (PV) that can then mediate activation of the MAPK ERK signaling cascade.

Based on previous work which indicated that internalized growth factor receptors could continue to signal via the ERK1/2 pathway from late endosomal compartments (reviewed in (9, 38)), we hypothesized a similar mechanism of endosomal ERK1/2 activation could be occurring during *L. amazonensis* promastigote infection. Late endosomal-mediated activation of ERK1/2 has been shown to be facilitated by scaffold proteins, specifically MP1 and MORG1.

Immunofluorescence analysis of endosomal *L. amazonensis*-mediated ERK1/2 phosphorylation revealed intracellular phospho-ERK1/2 co-localizing with internalized parasites. This data suggested that activation of this kinase from the same location as parasite-containing organelles. In addition, we observed co-localization of both MP1 and MORG1 with internalized *L. amazonensis*. Altogether, the data suggest a model (Figure 2) of *L. amazonensis*-mediated ERK1/2 activation from intracellular compartments facilitated by scaffold proteins MP1 and MORG1. Following parasite phagocytosis at the plasma membrane specific receptors may be triggered leading to the subsequent recruitment of scaffold proteins. The parasite-containing early endosome—or parasitophorous vacuole (PV)—proceeds through its maturation process becoming a late phagosome and eventually fusing with lysosomes to become a phagolysosome. Once at the late phagosome/phagolysosome stage, specific scaffold proteins associate with these
organelles, serving as platforms for the ERK signaling pathway. We know from our own work that activation of ERK does occur during *L. amazonensis* infection, leading to the modulation of CD40 surface expression and IL-12p40 production.

*Leishmania* parasites have been shown to alter host-cell signaling pathways to promote their survival. Most studies aimed at understanding the regulation of signaling pathways triggered by *Leishmania* parasites have focused on the initial events of host-parasite interactions. However, *Leishmania* parasites reside intracellularly within parasitophorous vacuoles (PV) and it is from within these intracellular compartments that parasites continue to engage cell signaling pathways and alter the host’s biological responses (18). This has been demonstrated by the presence of products of the phosphoinositide 3-kinase (PI3K) pathway on PV following *L. amazonensis* infection (35).

PV are highly dynamic in their structure and composition. Acquisition of endosomal components—and their content—is an ongoing process, as fusion of newly internalized materials and recycled cytoplasmic molecules are delivered into lysosomal compartments, including *Leishmania*-containing PV (6). *Leishmania* parasites acquire many of their nutrients from the host cell via the host cell’s endocytic network. Based on recent work suggesting the important role of endocytosis in signal transduction (reviewed in (38)), signaling components (i.e. extracellular receptors, lipids, kinases, adaptor and scaffold proteins) could be delivered to parasite-containing PV giving the parasite access to the host’s signaling networks.

The model presented here provides for the first time a description of a pathogen-mediated mechanism utilizing host-derived molecules that trigger signaling pathways intracellularly. The co-localization of phosphorylated ERK1/2 and the scaffold proteins MP1 and MORG1 suggest that such a mechanism may be at work in our model of *L.*
amazonensis infection. It remains to be determined if a specific receptor/ligand interaction is necessary to trigger ERK1/2 activation. It is also possible that parasite-induced changes in the PV membrane alone may be sufficient trigger the ERK pathway once its components are bound to recruited scaffold proteins. In addition, the dynamic nature of PV and their steady acquisition of endosomal components may provide a constant source signaling components, which can be directly used by the parasite.

The requirement for MP1 and/or MORG1 to facilitate ERK1/2 activation following L. amazonensis infection remains to be determined. However, if such a mechanism is being utilized by L. amazonensis, targeting of these scaffold proteins (upregulation or downregulation) may enable direct and specific modulation of ERK1/2 activation. Therapeutically, this can lead to very specialized, effective treatment options where regulation of ERK1/2 signaling occurs only during parasite-induced pathological cascade activation, without affecting steady-state ERK1/2 function.

Recommendations for future studies

One of the future goals of the research presented in this dissertation is to understand how a non-productive immune response evolves during L. infantum infection. Additional experiments are necessary to demonstrate the mechanisms behind increased production of IL-10, antigen-specific antibodies and antigen load during disease progression. While it is evident that changes in these parameters are indicators of clinical disease, the causal relationship between them remains poorly understood. Characterization of the CD4+ T cell response to antigen from subclinical and clinical dogs should be more closely analyzed. We have proposed that T cell exhaustion and/or adaptive tolerance mechanisms may be at play during the immune response to L. infantum. It would be interesting to characterize whether
the disease-associated immuno-suppressed immune response results from initially-activated T cells that become exhausted or tolerant, or from newly-activated naïve T cells that are primed later in disease. CD4+ T cell subpopulations should be characterized based on activation/effector marker expression (CD25, CD69, CD44, CD62L), markers associated with adaptive tolerance (PD-1, OX40), and effector function (IFN-γ, IL-10, TGF-β).

Additionally, in vitro studies to determine if IL-10 production is responsible for the observed decrease in lymphoproliferation and IFN-γ production are also warranted. The marked increase in antigen-specific antibody production is another point of interest that requires further study. It is likely that protective antibodies are found at early stages of infection but produced at low levels, below the detection limit of current assays. Western blot analysis of serum hybridization onto SDS-PAGE-electrophoresed L. infantum antigen may provide a more sensitive method of characterizing antigen-specific antibodies in subclinical animals.

The murine cutaneous leishmaniasis model also used in this dissertation has allowed for a closer dissection of the mechanisms leading to immune dysregulation during infection with another Leishmania parasite, L. amazonensis. The work presented here describes a molecular mechanism by which L. amazonensis modulates DC maturation phenotype by targeting a host signaling pathway and potentially utilizing host cell signaling components to do this. Additional experiments are needed to further characterize the involvement of scaffold proteins during L. amazonensis infection. In order to achieve this, a new cell system, where genetic manipulation is more readily available, is warranted. Initial studies should focus on cloning of tagged (i.e. FLAG, GFP) MP1 and MORG1 to facilitate co-immunoprecipitation studies to validate the immunofluorescence studies. Continuing studies should also determine the requirement for MP1 and/or MORG1 during ERK1/2 phosphorylation. Silencing inhibitory (si) RNA technology is available for both MP1 and
MORG1, and would be recommended for these studies. Western blot analysis can then be used to determine ERK1/2 phosphorylation in the absence of these proteins following *L. amazonensis* infection.

By better understanding how the immune-dysregulation observed during *L. infantum* and *L. amazonensis* infection, we may be better able to develop therapeutic strategies to aid the immune system in fighting infection, not only against these parasites, but other chronic infectious disease with similar immune-regulatory patterns.

References


Figure 1. Different *Leishmania* diagnostics are more or less effective because of alterations in parasite load and immune response. Because of cost and materials required, serology is the most common means of diagnosing *Leishmania* infection, both in the United States and elsewhere. The antibody response measured by serology can be against the whole parasite, for example the CDC IFA assay (CDC, light blue bar) or to a specific antigen, for example the Cornell-developed kELISA (kELISA, dark blue bar). This difference, whole parasite response versus specific antigen, in this case may lead to different sensitivity and specificity to detecting infection. Whole parasite-based serology can also cross-react with antibodies to *Trypanosoma cruzi*, for instance. kELISA has been shown to identify infection earlier, as portrayed by the x-axis, and may detect a different subset of antibodies (nonproductive and productive antibodies are portrayed by the bottom 2 sets of dashed lines). T-cell–based responses, for instance T-cell proliferation to parasite antigen (TCP, tan bar), detects infection very early after infection and is able to consistently indicate infection throughout the course of infection until very late in disease when the T-cell response wanes (small dashed line). Unfortunately, this assay is currently only performed on an experimental basis because of the time and materials required for successful analysis of this cellular response. Measurement of actual parasites in the bloodstream (solid line) via detection of parasite kinetoplast DNA via qPCR (PCR, orange bar) is another highly sensitive means of diagnosing parasite infection. This assay requires very rigid quality control to assure accurate results and, because of the nature of the test, can lead to occasional false-positive results. Figure and legend reprinted with permission from Elsevier Limited: *Topics in Companion Animal Medicine*, Vol. 24(4), Petersen, C.A., Leishmaniasis, an Emerging Disease Found in Companion Animals in the United States, pp.182-188, Copyright Elsevier, 2009.
Figure 2. Proposed model for endosomal ERK activation following *Leishmania amazonensis* infection. *L. amazonensis* promastigotes are phagocytosed by dendritic cells and (A) may initially signal through different receptor types which influence the formation of unique signaling scaffolds for the mitogen-activated protein kinase, ERK. (B) Parasite-containing phagosomes proceed through the endosomal maturation process becoming late endosomes/lysosomes. (C) *L. amazonensis*-containing late endosomes recruit the p14-MP1-MORG1 scaffolding complex. *L. amazonensis* infection of dendritic cells, using a MORG1 signaling scaffold leads to ERK phosphorylation and nuclear translocation of activated ERK. MORG1 signaling scaffold leads to ERK phosphorylation and nuclear translocation of activated ERK.
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