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RECEPTOR-MEDIATED PHAGOCYTOSIS AND PARASITOPHOUS VACUOLE MATURATION OF *LEISHMANIA AMAZONENSIS* PROMASTIGOTES

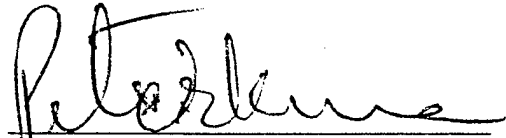
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2001, 60 pages

MS, University of Florida

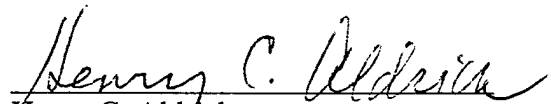
This study ascertains the importance of opsonin dependant receptors CR3 and FcγR to the phagocytosis of the intracellular parasite *Leishmania amazonensis*. Additionally, it assesses differences in phagosome composition and maturation as a result of internalization through these receptors. RAW 264.7 macrophages were infected with non-opsonized, complement opsonized, or anti-parasite antibody opsonized *L. amazonensis* promastigotes. The composition of parasite-containing phagosomes was monitored by immunofluorescence microscopy up to 480 min post-infection. Promastigotes firmly established infection by 30 min and were 40% more efficient at cellular entry when opsonized with complement, 13% more efficient when antibody opsonized. By 5 min post-infection, 88% of internalized complement opsonized parasites were in vacuoles transiently reactive with anti-CR3 antibody and most exhibited characteristic association with vinculin. In contrast, only 29% of antibody opsonized parasite vacuoles were anti-CR3 reactive and 68% were positive for vinculin. LAMP1 colocalization steadily increased throughout the infection under all opsonization conditions and nearly all phagosomes contained this molecule by 75 min post-infection. Rab5a association persisted with approximately 40% of phagosomes harboring complement and non-opsonized parasites for at least 90 min, compared to rab7, which was present in negligible amounts throughout the experiments. In contrast, antibody opsonized promastigotes showed approximately 9% rab5a association and 5% rab7 association with similar kinetics. Cathepsin S was detected in association with phagosomes without the involvement of CI-M6PR. After 60 min post-infection, phagosomes harboring antibody opsonized parasites began to rapidly accumulate cathepsin S (27% association by 180 min) compared to non-opsonized or complement opsonized parasites which presented with marginal levels of cathepsin S. The significance of receptor entry on endocytic marker association kinetics was reinforced through identical experiments using CR3 knockout cells, zymosan A particles, and fixed promastigotes. These findings expand our current knowledge of *Leishmania* pathogenesis and may have implications in developing leishmaniasis treatments and controls.

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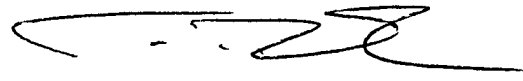
Peter E. Kima, Chair
Assistant Professor of Microbiology
and Cell Science

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Henry C. Aldrich
Professor of Microbiology and Cell
Science

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This thesis was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Master of Science.

August 2001

Dean, College of Agricultural and
Life Sciences

Dean, Graduate School

RECEPTOR-MEDIATED PHAGOCYTOSIS AND PARASITOPHOUS VACUOLE
MATURATION OF *LEISHMANIA AMAZONENSIS* PROMASTIGOTES

By

JAMES BENJACK

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2001

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Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

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MATURATION OF *LEISHMANIA AMAZONENSIS* PROMASTIGOTES

By

James Benjack

August 2001

Chair: Peter Kima
Major Department: Microbiology and Cell Science

Leishmania parasites gain access to their intracellular niche through phagocytosis. Phagocytosis is a receptor-mediated process that has been shown to involve the use of opsonin dependent as well as opsonin independent receptors. This study ascertains the importance of the opsonin dependant receptors CR3 and FcγR to the cellular entry of *Leishmania amazonensis*. Furthermore, it assesses differences in phagosome composition and maturation as a result of internalization through these receptors. RAW 264.7 macrophages were infected with non-opsonized, complement opsonized, or anti-parasite antibody opsonized *L. amazonensis* promastigotes. The composition of parasite-containing phagosomes was monitored by immunofluorescence microscopy up to 480 min post-infection. Promastigotes firmly established infection by 30 min and were 40% more efficient at cellular entry when opsonized with complement, 13% more efficient when antibody opsonized. By 5 min post-infection, 88% of internalized complement opsonized parasites were in vacuoles transiently reactive with anti-CR3 antibody and

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CHAPTER 1 INTRODUCTION AND REVIEW

Background and Significance

Members of the *Leishmania* genus are obligate intracellular hemoflagellate parasites that infect cells of the mononuclear phagocyte lineage. With over 20 known species, this genus is geographically widespread with the capability of infecting a variety of vertebrate hosts. Transmitted to the host by a sandfly vector, the *Leishmania* spp. establish a myriad of disease conditions (Herwaldt 1999). Collectively known as leishmaniasis, these conditions can have numerous clinical manifestations in humans. Cutaneous leishmaniasis is a common form of the disease caused by some *Leishmania* species that occurs as a result of the parasite remaining at the site of the vector's bite. This condition is often self-limiting and is characterized by a localized lesion and/or ulcer in the infected area. Visceral leishmaniasis, also referred to as kala-azar, is an often fatal form of the disease caused by parasite infected cells spreading throughout the body to sites such as the liver, spleen, lymphatic system, and bone marrow. Mucocutaneous leishmaniasis is similar to the visceral disease in that it involves spread from the initial infection site. This form of leishmaniasis progresses into the mucous membrane and may result in disfigurement or scarring.

Leishmaniasis awareness is escalating worldwide despite the fact that it was once considered a health issue solely of the Third World and developing nations. Unexpected instances of leishmaniasis have raised force protection concerns of US Armed Forces

assigned to the Middle East and Southwest Asia (Centers for Disease Control 1992; Magill et al. 1993). Additionally, significant public health implications may be on the horizon. Since 1993, the geographic regions considered to be *Leishmania* endemic areas have experienced significant expansion due to increased travel and various developmental factors (World Health Organization 2000; Olliaro and Bryceson 1993). Indeed, questions are currently being raised as to whether leishmaniasis has become endemic to North America (Enserink 2000). Long thought to be devoid of the parasite, the discovery of over 1000 hunting dogs infected with *Leishmania* in the US and Canada suggest the presence of a sylvatic cycle and does not preclude the possibility of an increase in human instances of the disease.

In some areas, the increasing incidence of HIV/*Leishmania* co-infection has had two-fold implications. First, immunosuppression makes it more likely that a *Leishmania* infection, which typically may have remained latent, will exaggerate itself into a fatal visceral manifestation of the disease (Olliaro and Bryceson 1993). Second, stimuli from parasite infections may induce HIV replication, thus promoting AIDS progression (Wolday et al. 1999). A related epidemiological concern is that new avenues of person-to-person transmission, such as through needle sharing among intravenous drug users, may soon challenge *Leishmania's* natural transmission cycle (Alvar et al. 1997). Greater understanding of this parasite, to include the immunology of how it establishes and maintains infection, will undoubtedly prove essential in dealing with these issues and in developing effective disease controls.

***Leishmania* Biology**

Many instances of leishmaniasis are zoonotic (Alvar et al. 1997), with humans becoming infected through accidental exposure to the natural transmission cycle. Alternately, the disease can be transmitted in the anthroponotic form where human-to-human transmission occurs through the sandfly vector (Alvar et al. 1997). Regardless of infection route, the general life cycle of the *Leishmania* parasite remains the same. *Leishmania* spp. are digenetic organisms that exist extracellularly as motile flagellated promastigotes in the lumen of the female sandfly (genus *Phlebotomus* for Old World and *Lutzomyia* for New World leishmaniasis) gut. When the sandfly obtains a blood meal from its vertebrate host, promastigotes are deposited into the host's skin where they are rapidly engulfed by host macrophages. Once inside the macrophage, the promastigote transforms into the aflagellated amastigote form with its characteristic ovoid shape. Asexual reproduction of amastigotes within the macrophage ultimately results in the cell's rupture and the release of large numbers of amastigotes that are subsequently internalized by other phagocytes, thereby continuing the infection cycle.

Phagocytosis and Intracellular Pathogenesis

Classical phagocytosis, as it occurs in "professional phagocytes" of mononuclear lineage, is the cellular uptake of particles generally larger than 0.5 μm into a vacuole (Tjelle et al. 2000). The process can be visualized as occurring in four steps: interaction of a particle with the cell surface, internalization of the particle into a vacuole designated a phagosome, maturation of the phagosome into a phagolysosome, and digestion of the particle within the phagolysosome.

Phagocytic Uptake into the Cell

The initiation of particle attachment to a cell is mediated by the interaction of a plasma membrane receptor with a specific ligand on the particle surface. These receptors are classified as either opsonin-independent or opsonin-dependent relative to the method employed to interact with the particle surface. Opsonin-independent receptors are considered a more primitive type of receptor and generally function by pattern recognition with a specific structural determinant on the particle surface. The mannose and scavenger receptors are examples of this type of receptor. The mannose receptor initiates cellular entry through recognition of branched α -linked oligomannoses, whereas scavenger receptors bind chemically modified LDL. The best-characterized opsonin-dependent receptors also happen to be the major receptors used in phagocytosis: the receptor that binds the Fc portion of IgG (Fc γ R) and complement receptor 3 (CR3), which binds complement protein C3bi.

Although upon gross examination the end result of phagocytosis may appear the same for the typical particle, procedural differences occur depending on the receptor used to mediate entry. The morphological differences between CR3 and Fc γ R are the best characterized. Phagocytosis mediated by Fc γ R occurs through a zipper mechanism whereby pseudopods appear to reach out and surround the particle, binding it in a tight phagosome (Tjelle et al. 2000). By comparison, CR3 bound particles sink directly into the cell with little, if any, pseudopod extension, yielding a particle that is loosely bound to the phagosome membrane (Tjelle et al. 2000).

Following cell-particle interaction and formation of a receptor-ligand complex, signaling events are triggered which result in the local rearrangement of the phagocyte

actin cytoskeleton to drive particle engulfment. Besides the macroscopic differences between CR3 and Fc γ R mediated phagocytosis, each has its own array of unique molecular and signaling interactions that function to accomplish particle ingestion. These differences are illustrated by the observation that inhibitors of tyrosine kinases prevent Fc γ R mediated uptake, but not that of CR3 (Brown 1995; Kwiatkowska and Sobota 1999). Accordingly, inhibitors of threonine and serine kinases block CR3 mediated phagocytosis (Brown 1995; Kwiatkowska and Sobota 1999). Phagocytosis is initialized by the crosslinking of multiple receptors, which form clusters (Kwiatkowska and Sobota 1999). In the case of Fc γ R, this clustering in turn triggers the phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM). For CR3, the clustering process results in the phosphorylation of serine residues by PKC on the β 2 subunit of the receptor.

Historically, the role of mediating initial phosphorylation of ITAM has been assigned to members of the Src family of tyrosine kinases. Recent studies have shown, however, that the exact role of Src kinases is yet to be determined. Crowley et al. (1997) demonstrated that ITAM phosphorylation and phagocytosis occurs at a significantly reduced level in macrophages lacking normally present Src kinases (*lyn*, *hck*, *fgr*). More recently, it has been suggested that Src kinases participate differentially, with only some members (*lyn* and *hck*) being able to fully trigger phagocytic signaling. Irrespective of the initial activation step, once ITAM is phosphorylated, it becomes a docking site for Syk tyrosine kinase (Suzuki et al. 2000). Syk has the ability to autophosphorylate in addition to phosphorylating additional ITAMs. Syk deficient cells have been shown to be

defective in Fc γ R mediated phagocytosis although the particle can still engage the receptor (Crowley et al. 1997).

The end result of Syk/PKC phosphorylation is rearrangement of the actin cytoskeleton around the ligated receptor, followed by particle internalization and phagosome formation. Although the exact phagocytic signal transduction pathway has not been elucidated, members of the Rho family of GTPases have been implicated in cytoskeletal signaling. Specifically, CR3 mediated internalization has been shown to be dependent on RhoA, while Fc γ R phagocytosis requires RacI and Cdc42 (Caron and Hall 1998; Greenburg 1999).

Phagosome Maturation

After formation, the phagosome goes through a maturation process in which it is exposed to organelles of the endocytic pathway and transformed into a digestive phagolysosome. Essentially, the phagosome membrane progresses from resembling the cell's plasma membrane to becoming similar to that of a late endosome or lysosome. The earliest models described this process as a direct fusion; a simple process in which the nascent phagosome directly merges with a lysosome with no intermediate steps (Beron et al. 1995). Later, the maturation process was conceived to involve the shuttling of internalized material between a series of pre-existing endocytic organelles (Tjelle et al. 2000). The currently held model views the maturation process as a gradual modification of the phagosome into a terminal phagolysosome. The most recent extension of the maturation model is the "kiss-and-run" hypothesis. This hypothesis explains the mechanism of maturation as a series of transient fusion/fission encounters of the phagosome with endocytic organelles; instead of complete fusion between the

phagosome and endocytic organelles, a limited exchange of fluid and membrane occurs between the two compartments during a brief fusion and fission process (Desjardins 1995; Storrie and Desjardins 1996).

Although the complexity of the process and subtle variations between cell lines can make it difficult to discern all of the molecular events and kinetics associated with phagosome maturation, phagosomes have been seen to interact with endocytic organelles in a specific directional pattern (Desjardins et al. 1997). Studies of membrane traffic using internalized inert particles have identified molecular markers to denote the various stages along the endocytic pathway (Desjardins et al. 1994; Pitt et al. 1992). Indeed, both phagocytosis and endocytosis illustrate themselves as dynamic processes characterized by the temporal appearance and disappearance of key proteins.

In choosing markers to facilitate studies of phagocytosis, it is useful to note that a phagosome's initial composition is similar to that of the plasma membrane from which it originates (Muller et al. 1980). Modification of this composition begins nearly as soon as the compartment is formed with the rapid recycling of membrane receptors, such as Fc, mannose, and transferrin receptors, back to the cell surface (Beron et al. 1995; Pitt et al. 1992). Progressive enrichment of the phagosome with vacuolar ATPase and lysosome associated membrane proteins (LAMP) follows as well as the transient appearance of late endosomal markers such as the mannose 6-phosphate receptor (Beron et al. 1995; Pitt et al. 1992). The process is completed with the loss of specific late endosomal markers and the entrance of lysosomal hydrolases, such as the cathepsins (Beron et al. 1995).

Several molecules are known to comprise the machinery that mediates vesicle docking and fusion. While not necessarily integral membrane proteins, these molecules

can be used to reveal a compartment's age within the endocytic pathway. Among the best studied are the Ras-related Rab small GTPases. These GTPases recruit tethering and docking factors that allow for a firm connection between two compartment membranes (Schimmoller et al. 1998). Rab5 is seen to be essential for early endosomal fusion, whereas rab7 is necessary for late endosomal fusion and the biogenesis of lysosomes (Bucci et al., 2000; Gorvel et al., 1991).

Intracellular Survival

Common biological uses for phagocytosis include cellular remodeling and inflammation, as well as defense. Interestingly, it is also a common mechanism for certain pathogens to invade host cells, as it provides for avoidance of direct destruction by serum antibodies, complement, and/or cytotoxic cells. Since a major phagocytic function is the elimination of infectious agents, intracellular pathogens have devised strategies to interfere with the phagocytic process in order to promote survival inside cells.

One possible way of surviving inside a phagocytic cell is to avoid exposure to the digestive conditions of the phagolysosome. The bacteria *Shigella flexneri* and *Listeria monocytogenes* and the protozoan *Trypanosoma cruzi* accomplish this by transient vacuole residence; pathogen-directed lysis of the vacuolar membrane allows them to enter the host cytosol where they survive and replicate (Bogdan and Rollinghoff 1999; High et al. 1992; Smith et al. 1995).

Some pathogens facilitate survival by interfering with the maturation process in order to prevent the phagosome from acidifying. *Toxoplasma gondii* is a protozoan parasite that employs this method of survival. While internalization is a host-mediated

process for most intracellular pathogens, *T. gondii* takes an active approach with an entry process requiring the extension of actin-based membranous tubules by the *T. gondii* tachyzoite (Bogdan and Rollinghoff 1999). In forming its own "phagosome," the parasite eliminates certain proteins from the plasma membrane. The resulting compartment does not acidify, likely due to a disruption in normal endocytic trafficking caused by the vacuole membrane proteins originating from the parasite and not the host cell. Certain other pathogens invade cells in a host-mediated manner, yet still manage to restrict vacuole fusion activity. For example, the protein SpiC secreted by intracellular *Salmonella typhimurium* has been implicated in inhibiting cellular trafficking, leaving a bacteria-containing vacuole that lacks some lysosomal markers (Uchiya et al. 1999). Similarly, *Mycobacterium bovis* recruits and retains a host protein, termed TACO, on its phagosome that prevents lysosomal fusion (Ferrari et al. 1999).

Leishmania spp. do not display interference with the phagocytic process in a way that is as readily evident as the above examples. Curiously, *Leishmania* parasites are internalized through conventional phagocytosis to reside in what appears to be a classic phagolysosome (Chang 1979). Here, the amastigote form is able to resist the inhospitable environment of low pH and lysosomal enzymes. Despite containment in what is essentially a digestive vacuole, *Leishmania* survives and replicates.

Appropriately, this raises the question as to why an organism would choose to parasitize a hostile cell that has evolved to provide clearance of cellular debris and foreign entities. Perhaps the answer lies in the phagocytic mechanism itself. By exploiting phagocytosis, *Leishmania* is not required to provide its own entry mechanism; given the proper ligand

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on the parasite surface it can bind to the respective cellular receptor and gain convenient cell entry (Russell 1995).

Considerations with *Leishmania* Phagocytosis

The process of parasite entry and any parasite activities that may cause deviation in routine cellular processes are possible keys to understanding a parasite's pathogenesis and developing effective preventive measures. Reports of *in vitro* binding studies have surmised the importance of phagocytic receptors in *Leishmania* cell entry, but definitive roles for these receptors have not been clearly identified. The literature proposes that CR3 is perhaps the main mechanism in the uptake of *Leishmania* promastigotes (Brittingham and Mosser 1996; Mosser and Brittingham 1997). This conclusion mainly stems from observations that *Leishmania* is adept at both fixing complement and binding to CR3 (Mosser et al. 1992; Mosser and Edelson 1985). Further evidence is seen in the substantial decrease in cellular infection associated with the inhibition of parasite CR3 binding by site specific blocking (Mosser and Edelson 1985; Wilson and Pearson 1988).

Pattern recognition based receptors, such as mannose-fucose receptor (MFR), may also play an important role in *Leishmania* phagocytosis. Similar to CR3, blocking of the MFR results in decreased attachment of promastigotes to the macrophage surface and decreased cellular ingestion (Wilson and Pearson 1986, 1988). Both CR3 and MFR initially appear to play independent and important parts in *Leishmania* pathogenesis (Wilson and Pearson 1988). Indeed, the observation has been made that both receptor types must be available on the segment of macrophage membrane to which the parasite binds for phagocytosis to occur (Blackwell et al. 1985).

Little is known about how *Leishmania* may affect normal cellular activities. An intriguing consideration is whether the composition of the parasitophorous vacuole (PV) varies with the receptor employed to enter the cell. Evidence has been presented citing initial physical differences in the phagosomes formed by internalization with different receptors. When comparing the phagocytosis of opsonized and unopsonized inert particles, it has been noted that the cytoskeletal proteins vinculin and paxillin associate differently with the nascent phagosome depending on the entry receptor used (Allen and Aderem 1996). With complement receptor entry, vinculin and paxillin are distributed in discrete foci around the phagosome. In contrast, IgG mediated entry results in a uniform distribution of vinculin and paxillin around the phagosome, while mannose receptor entry displays no vinculin or paxillin phagosome association at all.

After initial phagocytosis, the resulting phagosomal membrane may have signaling properties that are dependent on the entry receptor. In turn, this could result in alternate vesicular trafficking or antigen processing pathways. Over time, the molecular content of PVs created through different means of entry could differ. A recent report examining the selectivity of fusion of the *Leishmania* vacuole with other particle containing vacuoles demonstrates that the receptor employed during ingestion may indeed influence vacuole fusion (Collins et al. 1997). This study showed that when live *Listeria monocytogenes* was introduced into *Leishmania mexicana* infected macrophages, the *Listeria* vacuole readily fused with the *Leishmania* compartment. However, *Listeria* opsonized with IgG no longer displayed this activity, suggesting that internalization by Fc receptor results in a vacuole composition that no longer permits fusion with the *Leishmania* PV. An additional finding in this study was that acquisition of the molecule

annexin I during maturation of the *Leishmania* phagosome suspended its ability to fuse with some vacuoles. While *Leishmania* vacuole formation has received comparatively little research attention, it should be noted that studies of other protozoan parasites have indicated differences in vacuole content and disposition depending on the parasite's mode of entry (Hall et al. 1991; Mordue et al. 1999).

Research Objectives

This study addresses cellular entry and vacuole maturation of *Leishmania amazonensis* promastigotes. Progressing beyond historical binding and attachment studies by examining functional engagement and receptor internalization, it identifies CR3 as the dominant receptor employed by promastigotes to gain cellular entry. Existing reports on *Leishmania* PVs focused on their characteristics days, or even weeks, following infection. This work looks at the crucial first hours of infection when most travel along the endocytic pathway occurs. To this end, the endocytic markers monitored throughout the course of these experiments indicate that the receptor employed for parasite entry determines the course of PV maturation and the final characteristics of the mature vacuole.

CHAPTER 2 MATERIALS AND METHODS

Reagents

Unless otherwise stated, chemicals and reagents were obtained from Sigma (St. Louis, MO).

Antibodies

Primary antibodies and dilutions used are as follows: Vinculin (1:100 in binding buffer without saponin), rab5a (1:100), rab7 (1:100), Cathepsin S (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA); mAB 1D4B (mouse LAMP1) (1:50) (Developmental Studies Hybridoma Bank, Iowa City, IA); mAb HB198 (mouse F4/80) (1:100) and 5C6 (mAb to CR3) (1:100) (American Type Cell Culture, Rockville MD); polyclonal CI-M6PR (1:3000) (courtesy of Dr. P. Lobel, University of Medicine and Dentistry of New Jersey, Piscataway NJ).

The following fluorophore conjugated secondary antisera were used diluted 1:200 in binding buffer with saponin: Alexa Fluor 546 rabbit and goat anti-mouse IgG. Alexa Fluor 488 or 568 donkey anti-goat IgG, Alexa Fluor 488 or 546 goat anti-rabbit IgG, or Alexa Fluor 488 goat anti-rat IgG (Molecular Probes, Eugene, OR).

Parasites

Leishmania amazonensis (MHOM/BR/77/LTB0016) promastigotes were maintained in complete medium (Schneider's *Drosophila* medium (GIBCO BRL, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY) and 10 µg/ml gentamicin) at 23°C. Infectivity of parasites was maintained by periodic passage through BALB/c mice. All parasites were used in the stationary phase of growth.

Mice

CR3 knockout mice that are deficient in the integrin CD11b on a C57BL/6 background were kindly provided by Dr. C. M. Ballantyne (Baylor College of Medicine, Houston TX). Normal C57BL/6 mice and BALB/c mice were acquired from a breeding colony in the Department of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida.

Cell Culture

The RAW 264.7 murine macrophage-like cell line (American Type Culture Collection, Rockville MD) was maintained at 37°C in complete medium (RPMI 1640 (Mediatech, Inc., Herndon, VA) supplemented with 10% heat inactivated FBS and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml, and gentamicin 10 µg/ml)) under a 5% CO₂ atmosphere.

Peritoneal macrophages were obtained from both normal and CD11b^{-/-} C57BL/6 mice for certain experiments. These mice were challenged by injection of 1.5 ml thioglycolate into the peritoneal cavity. After four days, the mice were sacrificed and

macrophages were harvested by flooding the peritoneal cavity with phosphate-buffered saline (PBS) under aseptic conditions. Collected macrophages were treated with red blood cell lysis solution for 10 minutes at room temperature, washed twice with PBS, and resuspended to 1.5×10^6 cells per ml in RPMI 1640 complete medium.

For immunofluorescence experiments, macrophages were seeded on 12 mm round coverslips in six well cell culture plates (Costar, Corning, NY) at a density of 10^6 cells per well. Cells were allowed to adhere to the cover slips overnight before proceeding with an infection.

Opsonization

Parasites

L. amazonensis promastigote aliquots were washed twice with PBS prior to opsonization. Complement opsonization was achieved by incubating the washed promastigotes in fresh mouse serum diluted 1:2 with Dulbecco's modified minimal essential medium (DMEM) (Mediatech, Inc., Herndon, VA) for 30 min at room temperature. Likewise, parasites were opsonized with IgG by incubating for 30 min at room temperature with heat inactivated mouse polyclonal antisera (diluted 1:5 in DMEM) to *Leishmania* (tested for reactivity to the promastigote surface). A negative opsonization control was accomplished by incubating an aliquot of parasites with 5% BSA/PBS or heat inactivated normal mouse serum (1:2 dilution with DMEM). Following incubation, all parasites were washed twice and resuspended in DMEM in preparation for infection of macrophages.

Zymosan A

Aliquots of zymosan A BioParticles (Molecular Probes, Eugene, OR) were washed twice with PBS prior to opsonization. For complement opsonization, the BioParticles were incubated for 30 min at room temperature in fresh mouse serum diluted 1:2 with DMEM. To achieve antibody opsonization, the particles were incubated for 60 min at 37°C in a 1:1 dilution of BioParticles opsonizing reagent (Molecular Probes, Eugene, OR). A negative opsonization control consisted of BioParticles incubated for 30 min at room temperature in 5% BSA/PBS or heat inactivated normal mouse serum diluted 1:2 in DMEM. Following opsonization, BioParticles were washed twice and resuspended in DMEM in preparation for phagocytosis experiments.

Infection and Phagocytosis

Parasites

Live promastigotes. Macrophage monolayers were treated with 200 nM phorbol 12-myristate 13-acetate (PMA) for 30 min before infection, followed by washing twice with DMEM. Promastigotes resuspended in DMEM were added to cell culture plate wells at a 40:1 parasite-to-cell ratio. All studies were carried out in a volume of 750 μ l per well. Synchronized infection of cells was achieved by allowing parasites to attach to cells for 10 min at 37°C under a 5% CO₂ atmosphere. Plates were then washed to remove unattached parasites and returned to the incubator for defined infection intervals. Infection was terminated at each experimental time point by adding 2% paraformaldehyde/PBS to the well. Paraformaldehyde fixation was allowed to proceed for at least 30 minutes before continuing with immunofluorescent staining.

Fixed promastigotes. Promastigote pellets were incubated in 4% paraformaldehyde for 30 min at room temperature following opsonization. Following incubation, the fixed parasites were washed twice in PBS and incubated in 0.1M lysine/PBS for 20 min at room temperature to quench unreacted aldehyde groups. The parasites were then washed twice and resuspended in the required amount of DMEM for infection. Infection of macrophages was carried out in the same manner as with live promastigotes.

Zymosan A

Macrophage monolayers were pretreated with PMA as described above with promastigote infections. Zymosan A BioParticles resuspended in DMEM were added to the culture plate wells at a 5:1 zymosan-to-cell ratio, using an infection volume of 750 μ l per well. Macrophages were allowed to internalize the BioParticles for 10 min at 37°C under a 5% CO₂ atmosphere before the plates were washed to remove any unattached zymosan. Plates were then returned to the incubator for defined infection intervals. At the end of each experimental time point, fluorescence of extracellular zymosan was quenched by adding trypan blue (250 μ g/ml in PBS, pH 4.4) for 1 min. After 1 min, the dye was removed and the coverslips were washed twice before adding 2% paraformaldehyde in PBS to terminate the infection. Paraformaldehyde fixation was allowed to proceed for at least 30 minutes before continuing with immunofluorescent staining.

Immunofluorescence

Indirect Fluorescent Antibody Staining

Paraformaldehyde was removed from culture plate wells and coverslips were washed twice with PBS. Unreacted aldehyde groups were quenched by incubating the coverslips in 50 mM NH₄Cl at room temperature for 15 min. Coverslips were then washed and incubated for 20 min in binding buffer (2% BSA/PBS) to reduce non-specific binding of antibodies in preparation for anti-parasite staining.

Macrophages were first stained under non-permeabilizing conditions to allow phagocytized parasites to be distinguished from those externally bound to cells. To accomplish this, coverslips were incubated for 30 min at room temperature with mouse polyclonal anti-*Leishmania* surface protein serum obtained from mice previously infected with viable *Leishmania amazonensis* promastigotes. Coverslips were next washed three times with PBS, followed by a 30 min room temperature incubation with secondary Alexa Fluor antiserum. The nucleic acid dye 4',6-diamidino-2 phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR), was dissolved in methanol to a final concentration of 1mg/ml and added to this secondary antibody/binding buffer at 1:1000.

With the exception of cells stained for vinculin, macrophage monolayers were permeabilized with binding buffer containing 0.05% saponin/PBS for 20 min. In the case of vinculin staining, cell permeabilization was achieved by incubating cover slips with 0.1% Triton X-100/PBS for 5 min at room temperature followed by washing three times with PBS.

Following permeabilization, coverslips were incubated for 30 min at room temperature with primary antibody diluted in binding buffer with saponin. Coverslips were next washed with buffer three times followed by a 30 min room temperature incubation with the appropriate secondary antisera. After secondary antibody staining, coverslips were washed three times, inverted, and mounted on glass slides with Gel Mount (Biomedica Corp, Foster City, CA).

Microscopic Analysis

Determination of the ratio of infected to non-infected macrophages provided an indication of the effectiveness of opsonization in facilitating parasite entry. At every experimental time point, 200 cells were examined for internalized parasites on two different coverslips each. A parasite was considered to have infected a macrophage if its nuclear material could be visualized by DAPI staining without the parasite outline being visible through anti-parasite staining.

The colocalization or disappearance of the various molecules of interest with the *Leishmania* PV was determined through immunofluorescence microscopy by scoring 100 *Leishmania*-containing vacuoles per cover slip on two coverslips at each experimental time point. Vacuoles were scored as positive if a bright, distinct rim of fluorescence was apparent around the parasite.

Slides were examined using a Nikon Optiphot-2 or Olympus BX 50 microscope. Images were captured on a Zeiss Axioplan 2 microscope using Spot v. 3.1 imaging software. Each experiment was repeated two or more times with results reported as means +/- s.d.

CHAPTER 3 RESULTS

Phagocytosis of *Leishmania* Parasites

Internalization Kinetics

The internalization of *Leishmania* promastigotes was determined by examining macrophage monolayers stained under non-permeabilizing conditions. Uptake of promastigotes by RAW 264.7 macrophages was seen to be a fairly rapid process with the observed number of infected cells gradually increasing between 5 and 30 minutes of incubation (Fig. 3-1). After the 30 min post infection, the infection ratio reached a plateau and remained essentially constant throughout the remainder of the observed infection. These kinetics held for all experimental groups (non-opsonized, complement opsonized, and antibody opsonized parasites), with the noticeable difference being the highest level of infection achieved under each opsonization condition. Comparison of the plateau area of the infection ratio curves revealed complement opsonized promastigotes as having the highest infectivity. Accordingly, this group averaged 40% greater efficiency of infection than the non-opsonized control. Antibody opsonized promastigotes did not establish infections comparable to that of complement opsonized promastigotes, but did display an average infection ratio 13% greater than the non-opsonized control.

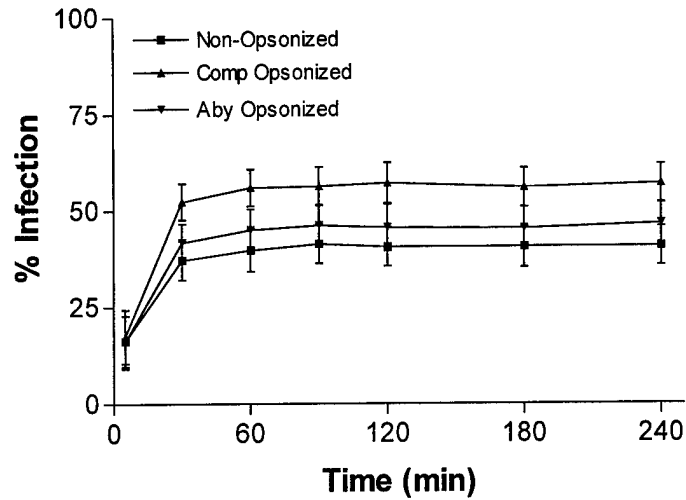


Figure 3-1. Infection of *L. amazonensis* in RAW 264.7 macrophages. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. % infection is the ratio of infected cells to total cells. Error bars represent standard deviations.

Infection kinetics in C57BL/6 normal macrophages were comparable to those illustrated by the promastigotes in RAW 264.7 cells (Fig. 3-2A). However, a considerable difference was seen with the infection profiles created by the promastigotes in C57BL/6 *cd11b^{-/-}* macrophages (Fig. 3-2B). Not only did absence of functional CR3 reduce the infection ratio of complement opsonized parasites to values consistent with the non-opsonized parasites, but the overall infectivity for all parasite treatment groups fell to levels lower than that seen in either normal C57BL/6 or RAW 264.7 macrophages.

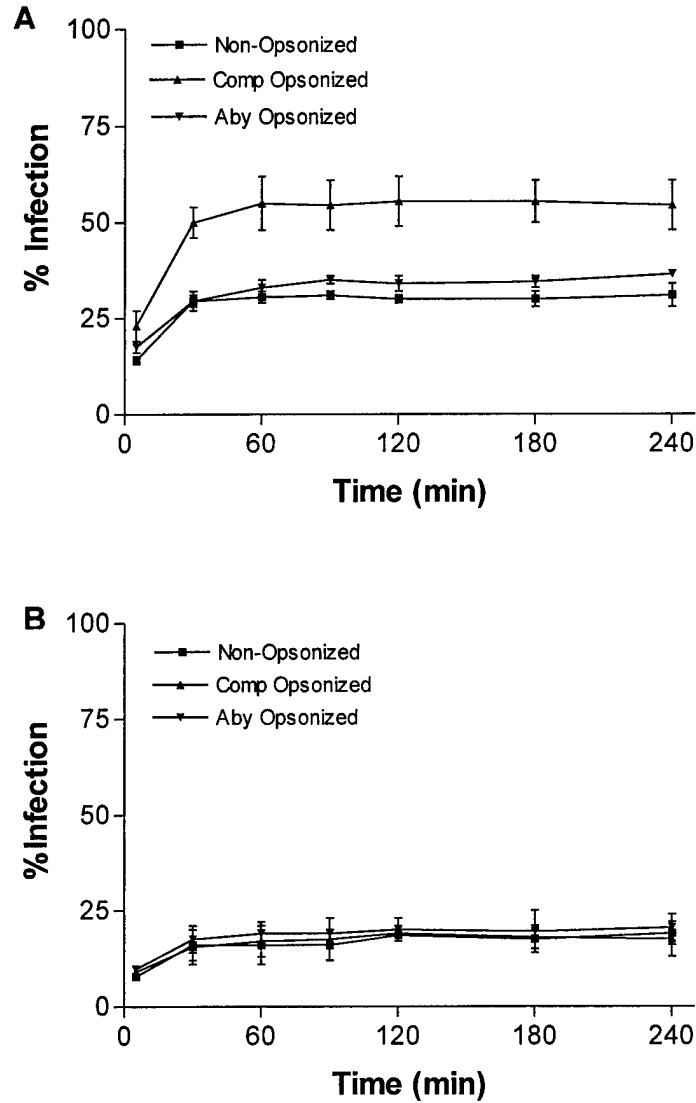


Figure 3-2. Infection of *L. amazonensis* in C57BL/6 macrophages. A) Infection in normal cells; B) Infection in *cd11b^{-/-}* cells. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. % infection is the ratio of infected cells to total cells. Error bars represent standard deviations.

It is essential to note when examining figures 3-1 and 3-2 that emphasis should be placed not on the overall infection ratios, but on the differences between the infections established under the different opsonization conditions. The differences noted between opsonization groups remained comparable between infections. However, upper limits of the infection ratios can vary significantly from infection to infection due to individual

characteristics of the parasite and cell passages or the media batch used. Likewise, the infection kinetics of RAW 264.7 cells vs. those of C57BL/6 macrophages are similar; however, slight differences are not uncommon between cell lines and primary cells.

Receptor-Mediated Entry

A primary goal of this study was to identify the preferred receptor for promastigote entry into the macrophage. Since complement opsonized parasites yielded the greatest internalization and infectivity, the next step was to verify functional engagement of the complement receptor. Considering that the composition of the nascent phagosomal membrane is similar to that of the cellular plasma membrane, parasites internalized via CR3 should initially contain higher amounts of CR3 in their vacuoles than those not internalized by CR3. To determine the presence of complement receptor in the nascent phagosome, immunofluorescence staining was used to detect CR3 in the phagosomal membrane using antisera to epitope 5C6 of CR3.

Approximately 90% of all phagosomes containing complement opsonized promastigotes were initially found to contain CR3 in RAW 264.7 macrophages (Fig. 3-3) (See appendix for representative images of selected molecules.). By comparison, vacuoles harboring non-opsonized and antibody opsonized promastigotes initially displayed positive 5C6 staining at 33% and 29% colocalization respectively. The amount of CR3 association was seen to rapidly diminish, leaving the receptor essentially undetectable by 30 min post-infection under all opsonization conditions.

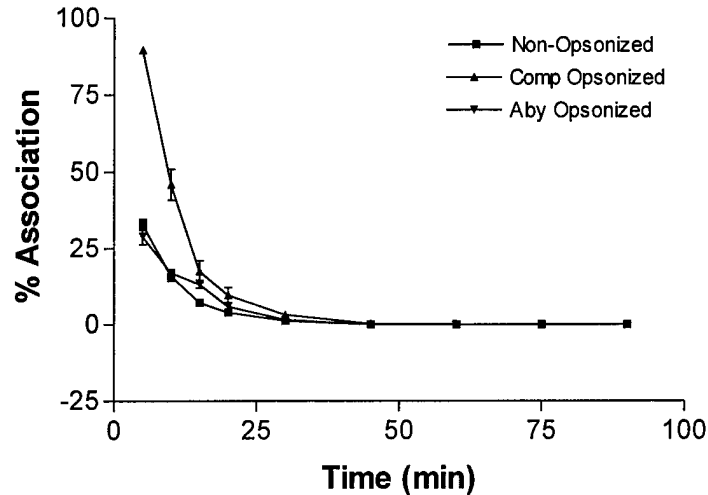


Figure 3-3. Kinetic association of 5C6 with *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

As CR3 is spread throughout the plasma membrane, it was important in this study to distinguish between the 5C6 colocalization seen as active receptor recruitment as opposed to passive incorporation following parasite engagement with the plasma membrane. Association kinetics of the macrophage surface molecule F4/80 were used as a tool to establish a pattern for passive incorporation into the *Leishmania* PV. While the general kinetics and disappearance of F4/80 was similar to that of 5C6 under all opsonization conditions, the maximal association with nascent vacuoles ranged from 22-25% (Fig. 3-4).

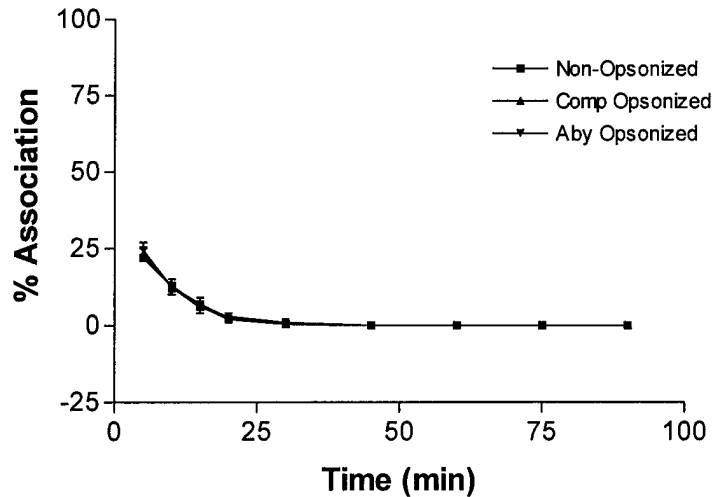


Figure 3-4. Kinetic association of F4/80 with *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

Although phagocytosis in general requires actin polymerization at the internalization site, recent work has shown differences in the internalization of complement vs. antibody opsonized particles at the level of actin binding cytoskeletal proteins (Allen and Aderem 1996). As an additional means to verify internalization via CR3, nascent phagosomal association with the cytoskeletal protein vinculin was examined in RAW 264.7 cells (Fig. 3-5). With 88% association at 5 min post infection, complement opsonized promastigotes showed the greatest vinculin phagosomal association. With 66% and 68% association respectively, phagosomes containing non-opsonized and antibody opsonized promastigotes also showed a significant amount of vinculin. Vinculin association decreased with time for all opsonization treatments until it was effectively absent at 30 min post-infection in a manner similar to that seen with 5C6.

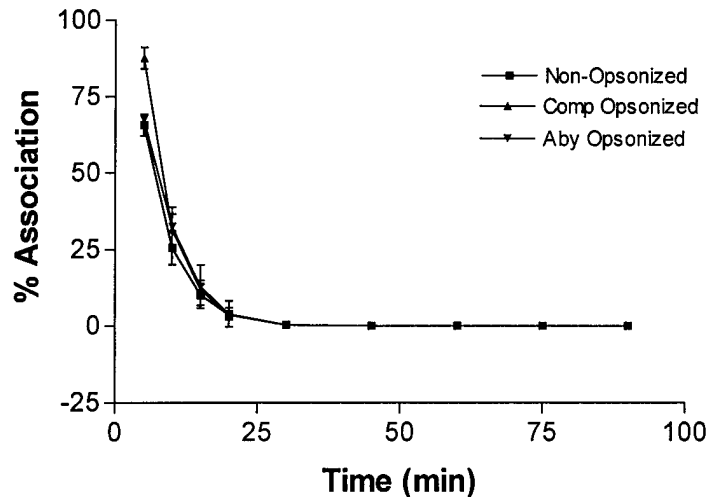


Figure 3-5. Kinetic association of vinculin with *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

While vinculin was recruited to PVs created under all opsonization conditions, the association pattern differences seen by Allen and Aderem (1996) was not present. All phagosomes that associated with vinculin did so in the uniform manner seen by Allen and Aderem with Fc mediated phagocytosis. The defined foci seen with complement-mediated phagocytosis was not observed under any condition.

Experiments using normal C57BL/6 cells yielded comparable vinculin association kinetics to those seen with RAW 264.7 cells (Fig. 3-6A). However, studies with CD11b^{-/-} cells showed differences in vinculin association (Fig. 3-6B). Vinculin colocalization with the promastigote PV was maximal at 5 min post infection under all opsonization conditions. However, non-opsonized and complement opsonized promastigotes displayed considerably lower rates of initial association (23% for non-opsonized and 26% for complement opsonized promastigotes).

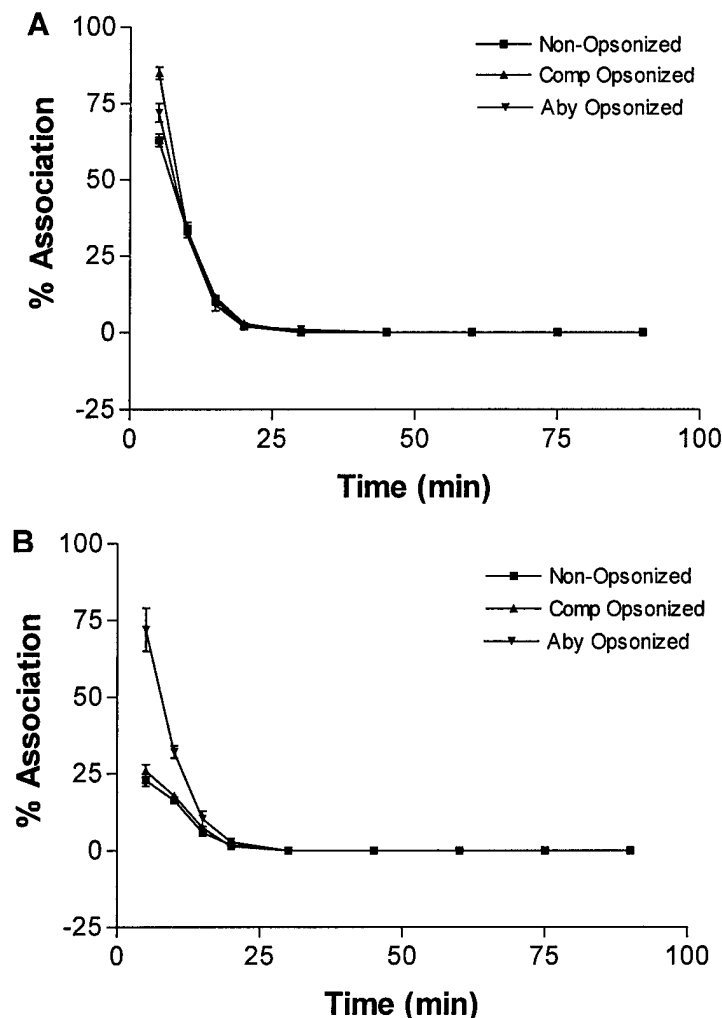


Figure 3-6. Kinetic association of vinculin with *L. amazonensis* phagosomes in C57BL/6 macrophages as determined by immunofluorescent microscopy. A) Infection in normal cells; B) Infection in *cd11b*^{-/-} cells. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

Distribution of Endocytic Markers on Maturing Phagosomes

Rab5a

Rab5a was used to identify early endosomal characteristics in *Leishmania* PVs.

The staining pattern seen with rab5a presented itself as a diffuse localized area of association around the phagosome.

Phagosomes containing both complement opsonized and non-opsonized promastigotes displayed similar rab5a association kinetics in RAW 264.7 macrophages (Fig. 3-7). For both groups, rab5a persisted at low levels well into the infection. For the first 90 min of infection, 36-42% of complement opsonized PVs associated with rab5a, whereas 40-45% of non-opsonized control PVs were colocalized with the GTPase. Rab5a association gradually decreased after 90 min, leaving no detectable staining in either group by 360 min. By comparison, only 8-10% of PVs containing antibody opsonized promastigotes associated with rab5a throughout the first 90 min of infection, with association decreasing to nearly undetectable by 150 min of infection.

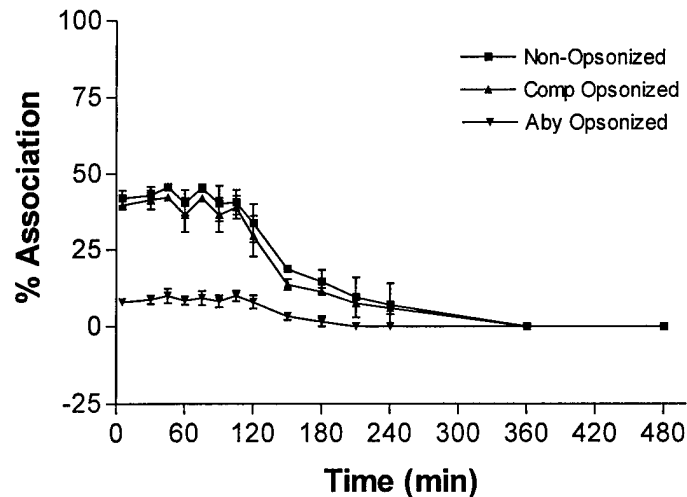


Figure 3-7. Kinetic association of rab5a with *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

Fig. 3-8A illustrates the colocalization of rab5a found when C57BL/6 normal macrophages were infected with promastigotes. Results were similar to the profile displayed by RAW 264.7 cells with the notable exception that localization of rab5a with PVs containing complement opsonized and non-opsonized parasites was reduced

throughout the first 90 min of infection relative to the RAW264.7 cells. Elimination of functional CR3 in C57BL/6 CD11b^{-/-} cells yielded significantly different patterns of association with rab5a (Fig. 3-8B). While 27% of complement opsonized and 24% of non-opsonized PVs were seen to be associated with rab5a at early times, the molecule disappeared at a sharper rate, being only barely detectable after 60 min. Rab5a in PVs of antibody opsonized promastigotes was comparable to that seen in other cell types.

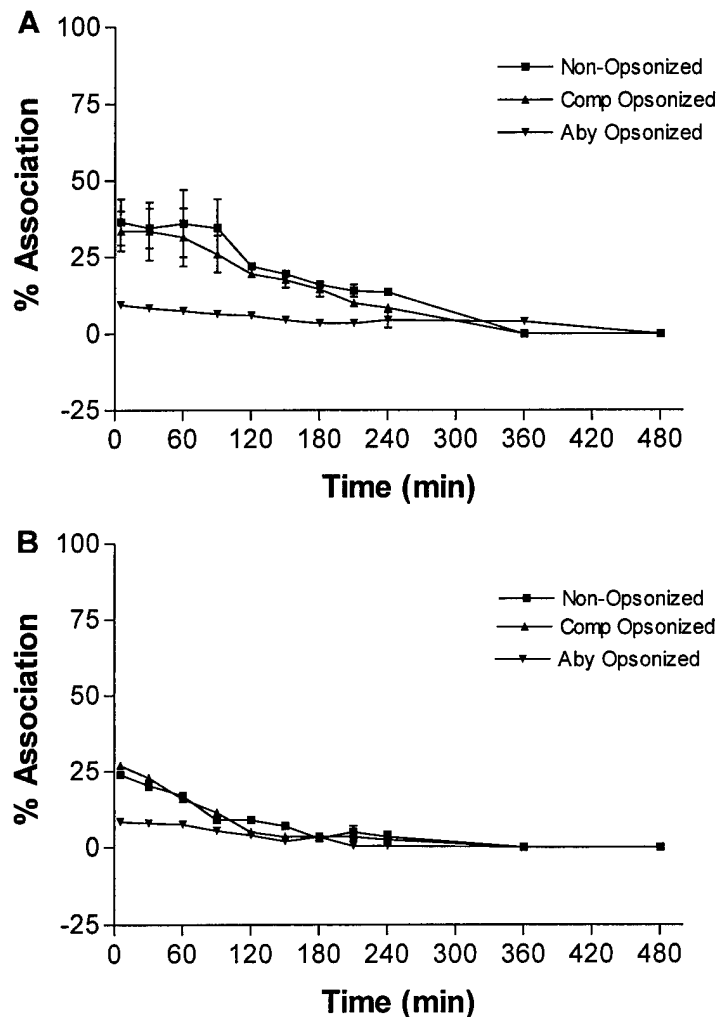


Figure 3-8. Kinetic association of rab5a with *L. amazonensis* phagosomes in C57BL/6 macrophages as determined by immunofluorescent microscopy. A) Infection in normal cells; B) Infection in cd11b^{-/-} cells. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

Rab7

To further establish the course of phagosome maturation, rab7 was employed as a marker to assess the presence of late endosomal compartment characteristics. Visual staining pattern was similar to that seen in rab5a. Although the colocalization of rab7 under all opsonization conditions was barely detectable, association was distinctly present in positive scoring phagosomes. Phagosomes harboring antibody opsonized parasites contained only slightly more rab7 (4-5% maximum association) than those containing non-opsonized or complement opsonized parasites (Fig. 3-9). Peak association was achieved after 60 min and was maintained throughout the remainder of the experiments.

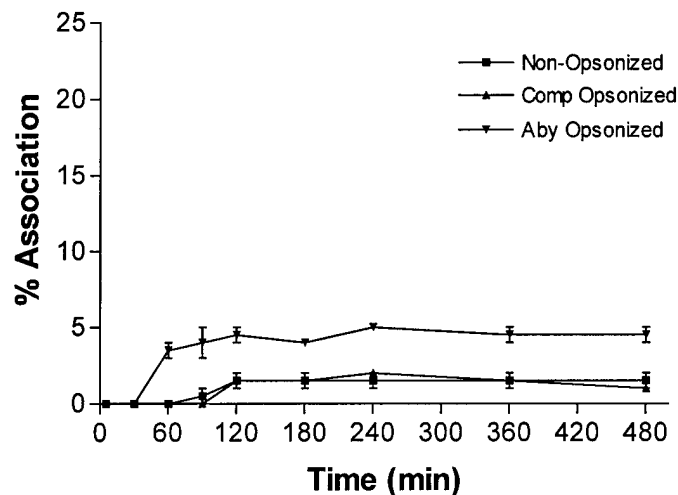


Figure 3-9. Kinetic association of rab7 with *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

LAMP1

Acquisition of the glycosylated lysosomal membrane protein LAMP1 was primarily used to verify the *L. amazonensis* PV's maturation into a phagolysosome. It

also served as an additional marker in monitoring the PV's transition through the late endocytic to lysosomal portions of the endocytic pathway. LAMP1 was detected in increasing amounts on phagosomes throughout the infection of RAW 264.7 macrophages (Fig. 3-10).

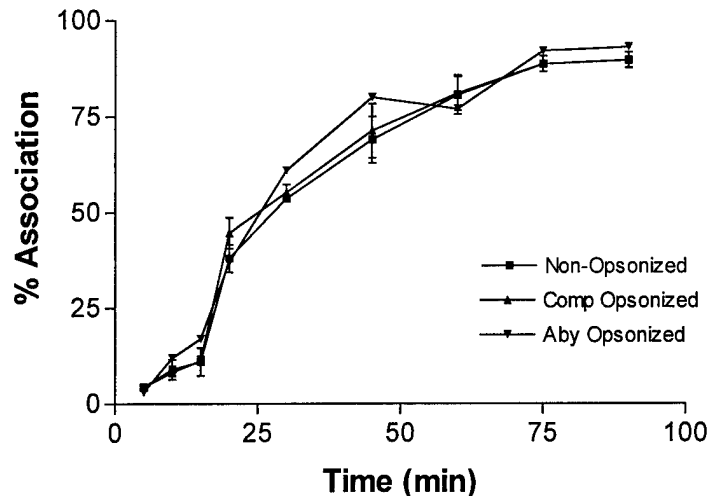


Figure 3-10. Kinetic association of LAMP1 with *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

The distribution of LAMP1 on phagosomes aged 5-15 min gradually increased from 3% to 17% association. By 20 min post infection, LAMP1 colocalization with the PV membrane more than doubled from its 15 min value. This was followed by a gradual increase in association until 75 min post-infection, at which time nearly all phagosomes were positive for LAMP1 staining. All opsonization treatments displayed similar LAMP1 association kinetics. Phagosomes enumerated from infections performed in both C57BL/6 normal and CD11b^{-/-} macrophages displayed LAMP1 association kinetics similar to those seen in RAW 264.7 cells (Fig. 3-11).

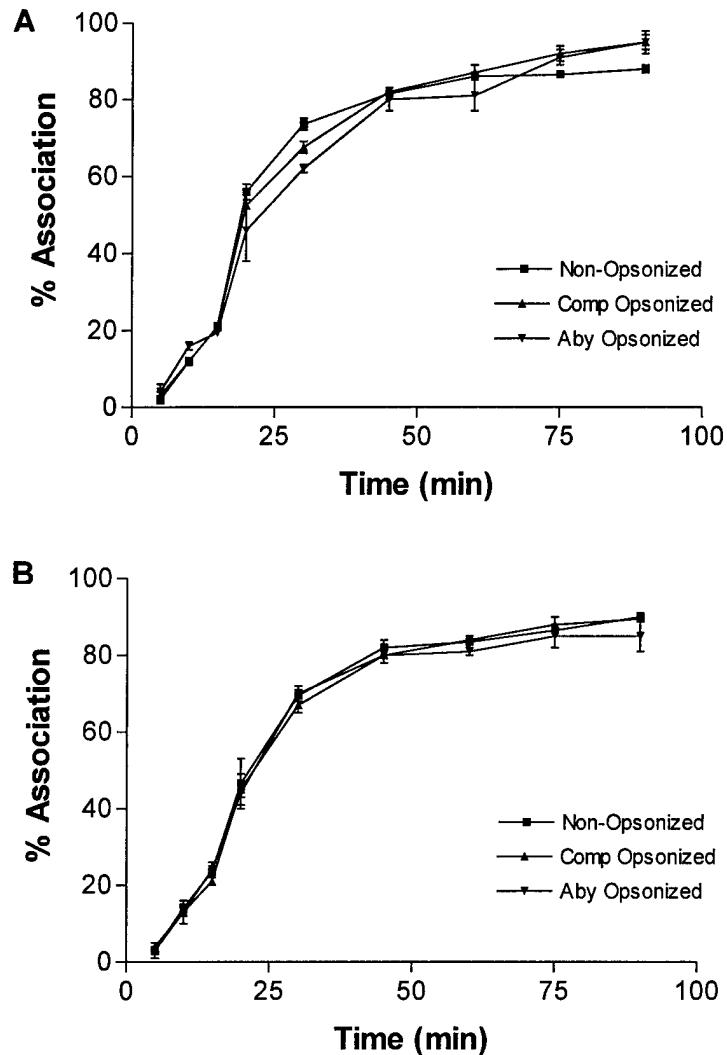


Figure 3-11. Kinetic association of LAMP1 with *L. amazonensis* phagosomes in C57BL/6 macrophages as determined by immunofluorescent microscopy. A) Infection in normal cells; B) Infection in *cd11b*^{-/-} cells. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

Cation-Independent Mannose 6-Phosphate Receptor

Phagosomal distribution of the cation-independent mannose 6-phosphate receptor (CI-M6PR) was studied with the possibility of identifying a temporal window where PVs can be seen to transition from late endosomal to lysosome-like compartments. While stained CI-M6PR vesicles occurred in a stippling pattern throughout the macrophage

cytoplasm, a negligible amount was witnessed in association with parasite-containing phagosomes.

Cathepsin S

The lysosomal proteinase cathepsin S was used as a marker to better define lysosomal activity within the PV. The general staining pattern of cathepsin S around the PV was seen to have similarities to LAMP1 – an ample bright ring around the phagosome membrane, although perhaps somewhat more diffuse than that of the glycoprotein.

Antibody opsonized promastigote PVs showed marked cathepsin S association in RAW 264.7 macrophages (Fig. 3-12). Significant association was first detected in PVs aged 105 min, followed by a steady increase until 180 min post-infection. With 47% of PVs staining positive for cathepsin S, the association level did not increase for the remainder of the infection after this point. Non-opsonized and complement opsonized promastigotes displayed similar cathepsin S association kinetics. Colocalization was seen to be a more gradual process than that displayed by antibody opsonized parasites, with the association level for both peaking at 24% at 480 min.

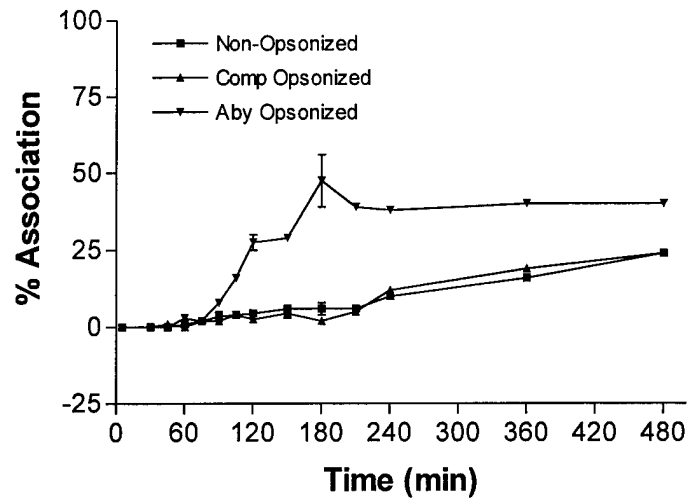


Figure 3-12. Kinetic association of cathepsin S with *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

As seen with rab5a, cathepsin S association with PVs in infected C57BL/6 normal macrophages was similar to that observed in RAW 264.7 macrophages (Fig. 3-13A). PVs containing antibody opsonized promastigotes rapidly acquired cathepsin S, reaching 39% colocalization by 240 min. Colocalization with complement opsonized and non-opsonized PVs was negligible through 240 min, gradually increasing to 14% and 23% respectively by 480 min. This prolonged and gradual association was removed when CD11b^{-/-} macrophages were challenged with complement opsonized and non-opsonized promastigotes (Fig. 3-13B). Both groups displayed cathepsin S association curves analogous to that created by antibody opsonized promastigote PVs. PVs containing antibody opsonized parasites accumulated cathepsin S in CD11b^{-/-} macrophages as rapidly as seen in normal cells; however, 65% of these PVs presented positive cathepsin S staining at 240 min.

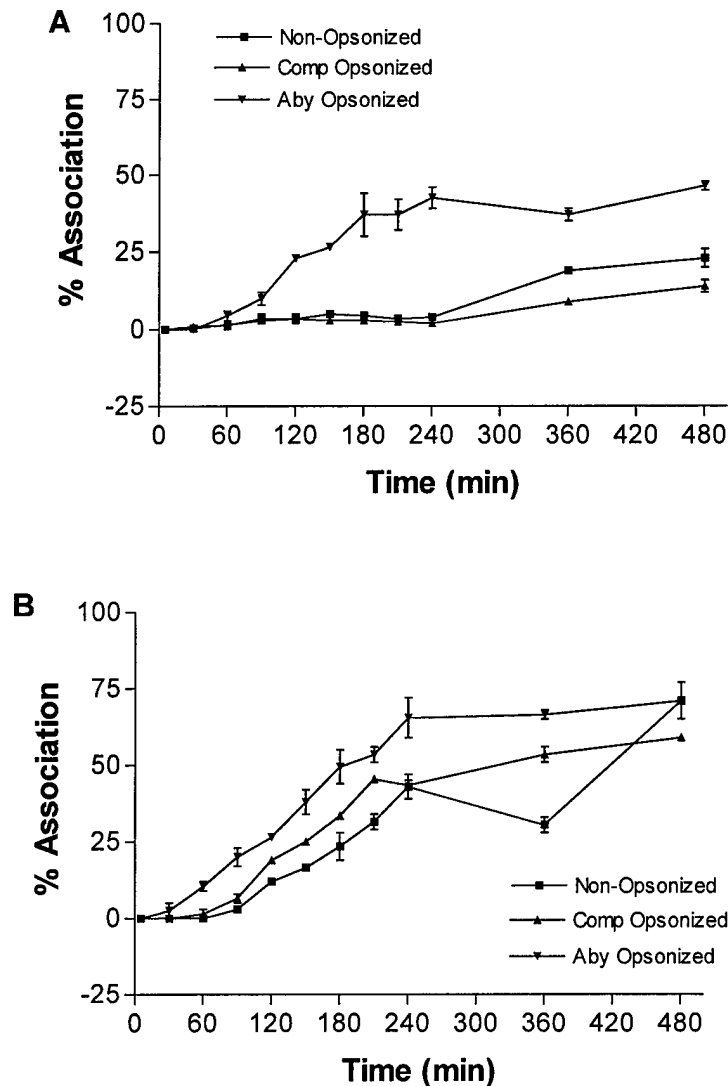


Figure 3-13. Kinetic association of cathepsin S with *L. amazonensis* phagosomes in C57BL/6 macrophages as determined by immunofluorescent microscopy. A) Infection in normal cells; B) Infection in *cd11b^{-/-}* cells. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

Distribution of Endocytic Markers on Control Particles

Most markers of the endocytic pathway examined in this study have been looked at by other researchers with inert particles, hence providing a basis of reference from which to compare the *Leishmania* data. However, comparatively little research has been

done with cathepsin S, let alone extensively determine the trafficking of this molecule. Because of this, and in part as a general control, the phagosome distribution of cathepsin S and rab5a were examined using both internalized zymosan and paraformaldehyde fixed promastigotes.

The pattern of cathepsin S distribution with zymosan-containing phagosomes was strikingly different from that seen with *Leishmania* in RAW 264.7 macrophages. Association with zymosan-containing phagosomes was nearly identical under all opsonization conditions. However, colocalization occurred much later in the infection process than seen with *L. amazonensis*, with barely detectable levels of cathepsin S only beginning to appear at 240 min post-infection (Fig. 3-14).

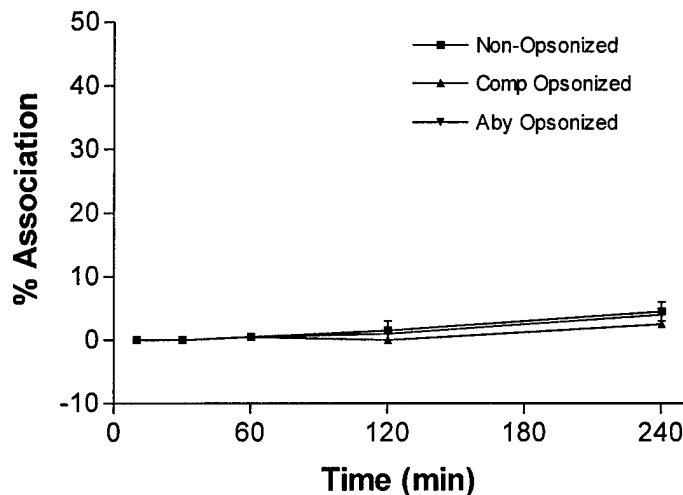


Figure 3-14. Kinetic association of cathepsin S with zymosan A vacuoles in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 5:1 with non-opsonized, complement opsonized, and anti-zymosan antibody opsonized zymosan particles. Error bars represent standard deviations.

Similarly, rab5a association was distinctly different from that observed with phagosomes containing *L. amazonensis* promastigotes. Rab5a was maximal at 5 min post-infection under all opsonization conditions, with approximately 9% of zymosan-containing

vacuoles scoring positive for association (Fig. 3-15). Considering the nature of rab5a as a docking mediator of the plasma membrane and early endosomes, it is quite possible that the level of rab5a was significantly higher at some point prior to this 5 min point (the same may be applicable with *L. amazonensis* promastigotes). However, practical limitations of the experimental methodology prevent examination of time points earlier than 5 min post-infection. Regardless, this modest amount of rab5a quickly dissipated, leaving essentially no vacuoles associated with the molecule by 60 min post-infection.

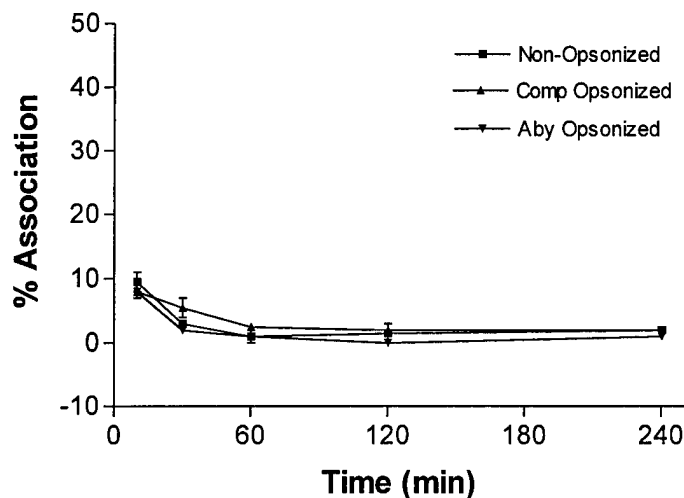


Figure 3-15. Kinetic association of rab5a with zymosan A vacuoles in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 5:1 with non-opsonized, complement opsonized, and anti-zymosan antibody opsonized zymosan particles. Error bars represent standard deviations.

Cathepsin S and rab5a association kinetics data illustrated that phagosome colocalization with these molecules does not vary with the receptor used for cellular entry when zymosan is internalized. As a means of further comparison, the association kinetics of rab5a and cathepsin S were examined in phagosomes contained dead *L. amazonensis* promastigotes that had been fixed with paraformaldehyde after opsonization.

Cathepsin S association with the fixed promastigotes was negligible and remained comparable to that of zymosan-containing phagosomes (Fig. 3-16). The kinetics displayed were essentially identical under all opsonization conditions. The first appearance of meaningful cathepsin S association by 120 min post-infection indicated a minor departure from the zymosan data.

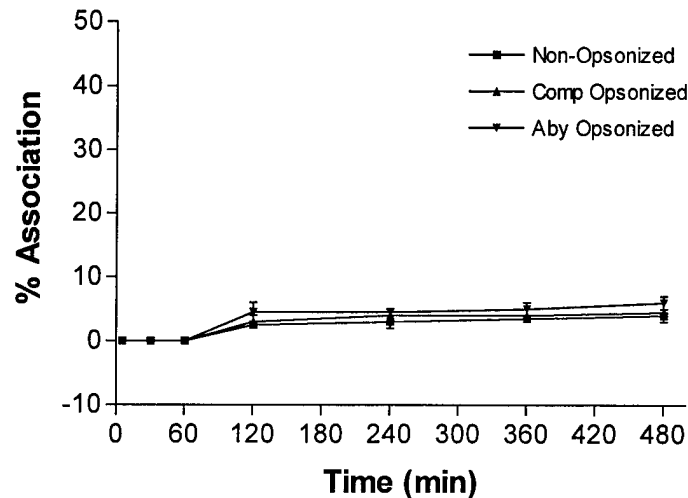


Figure 3-16. Kinetic association of cathepsin S with paraformaldehyde-fixed *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

Initial peak levels of rab5a association with phagosomes containing fixed promastigotes were similar to that seen with zymosan under non-opsonized and complement opsonized conditions (Fig. 3-17). Unlike zymosan, compartments containing antibody opsonized fixed promastigotes presented with negligible rab5a association. Another difference seen in the overall association kinetics of rab5a was that the molecule persisted slightly longer throughout the infection times monitored.

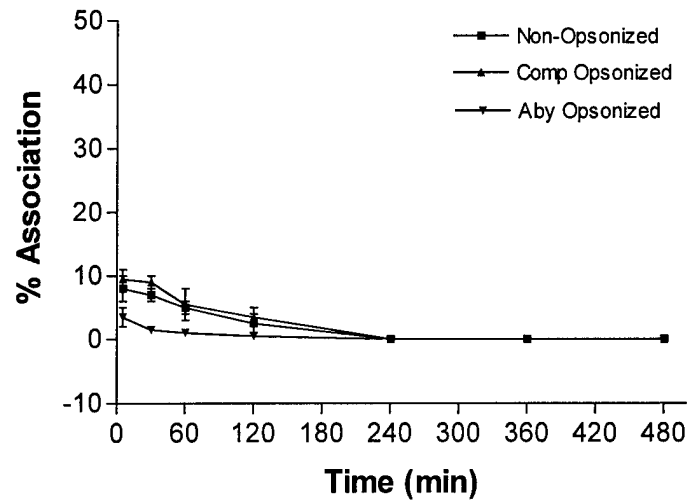


Figure 3-17. Kinetic association of rab5a with paraformaldehyde-fixed *L. amazonensis* phagosomes in RAW 264.7 macrophages as determined by immunofluorescent microscopy. Cells were infected 40:1 with non-opsonized, complement opsonized, and anti-parasite antibody opsonized promastigotes. Error bars represent standard deviations.

The departure of the association kinetics seen with fixed parasites from that of live promastigotes in normal macrophages hints at a difference in cellular recognition between the two particles. The disparity may reflect the absence of an active pathogenic function mediated by live promastigotes or some alteration of the parasite surface during the fixation process that prevents regular association with the macrophage endocytic machinery. Taken together, the zymosan and fixed parasite data demonstrate that the association kinetics witnessed with live promastigotes are unique events and are not artifacts of the experimental system.

CHAPTER 4 DISCUSSION

Receptor-Mediated Entry

Because *Leishmania* parasites do not mediate entry into the cells they infect, but rely on host directed phagocytosis for uptake, identifying the phagocytic receptors employed during entry is important to understanding *Leishmania* pathogenesis. The initial results reported in this study provide evidence based on immunofluorescence methods that *L. amazonensis* promastigotes are most efficient at establishing infection in mouse macrophages when entering the cells via CR3. This extends historical observations showing a preferential binding of *Leishmania* to complement receptors. Furthermore, low incidences of colocalization with the macrophage surface molecule F4/80 and nearly identical kinetics for all opsonization treatment groups demonstrated that 5C6 staining was indeed indicative of active receptor use. Although antibody opsonization enhanced promastigote macrophage entry, this observation may have only marginal significance *in vivo*, as the presence of *Leishmania*-specific antibody circulating at the site of infection is unlikely in uninfected hosts.

Staining for the presence of vinculin in the nascent promastigote PV was intended to be an additional indicator of entry using the complement receptor. While vinculin was seen to be present in the PV, the discrete foci seen with complement-mediated phagocytosis by Allen and Aderem (1996) was not observed under any condition. The relevance of this observation remains questionable. It is quite possible that the foci were

indeed present, only they could not be resolved because the condition/quality of the optics used may not have permitted clear viewing of such fine detail. Nonetheless, coupling vinculin colocalization kinetics with the 5C6 association data for PVs harboring complement opsonized parasites contributes to the case for CR3 use. This argument is reinforced by the data from the CD11b^{-/-} cells. The overall decrease in infection levels along with the dramatic drop in vinculin association leads to the hypothesis that promastigote uptake in these cells is occurring via a more primitive pattern-recognition system in the absence of functional complement receptor.

Use of CR3 is logical from the standpoint of parasite survival, as this method of entry is able to promote phagocytosis without triggering the oxidative burst (Wright and Silverstein 1983). Additionally, it has been indicated that *Leishmania* spp. may have physical adaptations that facilitate interaction with the host complement system. The most notable of these is the dominant surface molecule of the promastigote, the glycoconjugate lipophosphoglycan (LPG). Several functions related to the parasite's survival and pathogenesis in both its insect vector and vertebrate host have been attributed to LPG. Regarding cellular entry, LPG has been shown to be an acceptor molecule for C3 and its cleavage product C3bi in various promastigote species (Turco and Descoteaux 1992). To a lesser extent, the glycoprotein gp63, another major promastigote surface molecule, has been seen to bind C3 as well (Russell 1988). With such evolved structures, it is not a surprising finding that promastigotes are most adept at utilizing CR3 for both cellular attachment and entry.

Studies of the CR3 molecule have shown that it has a capacity to recognize a variety of diverse ligands in addition to C3bi. A structurally defined I-domain in the α -

subunit of the receptor has been shown to recognize ICAM-1 and fibrinogen in addition to C3bi (Diamond et al. 1993). Interestingly, a lectin-like domain has also been identified within the α -subunit with the capacity to bind certain β -glucans (Thornton et al. 1996). The finding that approximately 30% of phagosomes containing non-opsonized promastigotes display association with 5C6 indicates that a certain number of parasites are able to internalize through CR3 regardless of opsonization treatment. Aside from its role in parasite survival, *Leishmania* LPG has been implicated in direct binding to the α -subunit of CR3 (Turco and Descoteaux 1992). Although gp63 has been indicated to directly bind to CR3 in studies using purified forms of the proteins (Russell and Wright 1988), its polymannose content has led to speculation that it would be more likely to promote *in vivo* entry via MFR (Turco and Descoteaux 1992). Regardless, these surface proteins may offer an explanation for non-opsonized CR3 entry either by exploiting the pattern-recognition feature of the β -glucan site or the broad substrate recognition of the I-domain. More importantly, the presence of major surface proteins with both the ability to directly attach to CR3 or fix its primary ligand provides the wherewithal to make CR3 the main receptor for promastigote phagocytosis.

Examination of the complement receptor in this study has shed light on the nature of promastigote entry, but the importance of other receptor types in this process remains undisclosed. While entry of non-complement opsonized parasites through CR3 may be surmised through interaction with alternate lectins, the mode of parasite entry when CR3 knockout cells are used remains largely undetermined. The possibility is raised that the entry of a certain percentage of parasites (regardless of opsonization) may be attributable to either alternative receptors or the potentially additive effects between receptor types.

In addition to devising methods to improve opsonization efficiency, entry studies focusing on FcγR, MFR and scavenger receptors are necessary (pending antisera availability) to comprehensively describe cellular entry of the *L. amazonensis* promastigote and to define the roles of all receptors involved.

Intracellular Trafficking and Phagosome Maturation

Position of the *L. amazonensis* PV along the Endocytic Pathway

Survival of an intracellular parasite is dependent upon its ability to live within an acidic compartment, obtain nutrients through the host cell, and to circumvent certain aspects of the host immune response. Accomplishing this often involves some feature of the parasite itself or a unique characteristic of the compartment in which it resides.

Leishmania is viewed as somewhat of an anomaly in that its method of subverting the host machinery has not been clearly defined. Whereas intracellular pathogens such as *Mycobacterium*, *Legionella*, and *Toxoplasma* have been shown to affect the fusogenic properties of their vacuoles to assist survival (Portillo 1999), *Leishmania* unexplainably exists within a degradative compartment. As little information exists on the maturation of the *Leishmania* PV, much of the assumptions about the parasite's intracellular existence are inference. The data presented in this study on the trafficking of a variety of endocytic markers illustrate that *L. amazonensis* inhabits a compartment that matures along a unique pathway as compared to what has been described for model phagosomes.

Analysis of the *L. amazonensis* PV demonstrated that the vacuolar membrane was associated with LAMP1. Presence of this molecule is consistent and expected for a compartment with late endocytic and/or lysosomal characteristics and has been seen previously with other *Leishmania* species (Lang et al. 1994; Russell et al. 1992).

However, where *Leishmania* required approximately 75 min for maximal LAMP1 association, phagosomes created by a variety of inert particles have shown >90% association with this molecule within 7-15 min, depending on the methodology used for analysis (Oh and Swanson 1996; Pitt et al. 1992).

Although a specific marker does not exist for the detection of lysosomes, the absence of rab7 combined with the presence of LAMP1 can be used to identify lysosome-like organelles (Rabinowitz et al., 1992). Interestingly, rab7, a regulator of fusion between late endosomal and lysosomal compartments, was only barely detectable throughout this study. Therefore, by Rabinowitz's definition, *L. amazonensis* promastigotes exist in a true lysosomal compartment throughout the first 8 hr of infection. This reduction of rab7 association is in agreement with Scianimanico et al. (1999) who noted that phagosomes containing *L. donovani* promastigotes were able to acquire and lose early endocytic markers, yet failed to recruit rab7 at high levels. Additionally, slower kinetics were involved with LAMP1 recruitment in similarity to the findings reported here.

Considering the relative absence of rab7, it was interesting to observe the persistence of the GTPase rab5a surrounding PVs harboring non-opsonized and complement opsonized promastigotes in normal macrophages. The body of information compiled on rab5 implicates it as a regulator of fusion between early endocytic compartments. Recruitment and dissociation with inert particles has been seen to occur very rapidly (within 5 min in some cases) (Hashim et al. 2000; Mordue and Sibley 1997; Mosleh et al. 1998). Therefore, the prolonged association of rab5a with the non-opsonized and complement opsonized promastigote PVs further suggests that maturation

is impaired in these compartments. Despite the presence of LAMP1 and absence of rab7, the persistence of rab5a places *L. amazonensis* in a compartment that detours from the characterized endocytic pathway and does not fit the generally accepted definition of either a truly late endosomal or lysosomal compartment. As rab5 has been found to act as a timer for endocytic membrane fusion (Rybin et al. 1996), the continuing presence of the molecule may be directly involved in the delay in rab7 recruitment. Current work has further connected rab5 to both phagosome-endosome fusion regulation and *Leishmania* survival (Ducios et al. 2000). Mutant cells expressing active rab5 displayed uncontrolled fusion events that induced the formation of giant phagosomes containing *L. donovani*. Promastigotes in these giant vacuoles enjoyed a 5 to 10 fold increase in intracellular survival, possibly due to the size of the vacuole diluting microbicidal molecules (e.g., lysosomal hydrolases) to below their effective concentrations. Although the PVs of various *Leishmania* species studied have shown common features, not all display identical morphology (Antoine et al. 1998). Some species, to include *L. amazonensis*, transcend initially confining phagosomes to eventually become observed in giant PVs. The possibility cannot be ignored that the persistence of rab5a seen in this study may find a purpose in contributing to the formation of *L. amazonensis*'s larger PV.

A host of functions have been attributed to the *Leishmania* LPG as a virulence factor. Not surprisingly, it has been proposed that LPG is the entity responsible for the alteration in phagosome maturation seen with rab7 and LAMP1 trafficking (Scianimanico et al. 1999). This idea stems from several observations. Experiments using a LPG-defective mutant strain of *L. donovani* promastigotes have implicated LPG as an inhibitor of phagosome-endosome fusion at the onset of infection with the observation that the

PVs of parasites with defective LPG fused extensively with endocytic organelles, while those phagosomes containing wild type promastigotes remained non-fusogenic (Desjardins and Descoteaux 1997). The method by which LPG may be able to modulate phagosome maturation is not understood. However, studies indicate that the LPG may insert itself into the phagosomal membrane, stabilizing the lipid bilayer from forming an inverted hexagonal structure (Miao et al. 1995). The resulting reduction in fusogenicity would be a consequence of steric hindrance between the modified phagosomal membrane and endosomal vesicles. The seemingly early endosome-like characteristics of the promastigote compartment are in contrast to that seen with amastigotes. Curiously, it should be noted that a downregulation of LPG expression accompanies the promastigote to amastigote transformation. Work done by Lang et al. (1994) has shown the presence of rab7 with the amastigote PV. Russell et al. (1992) verified that the *L. mexicana* amastigote inhabits a PV with varying lysosomal and late endosomal characteristics. Apparently, the delay in vacuole maturation seen with the promastigote is part of its survival strategy, allowing the parasite time to affect transformation into an amastigote, which is more adapted to intracellular life.

Cathepsin S and Immune Response Implications

It has long been established that phagosome maturation ultimately leads to the acquisition of a full complement of lysosomal hydrolases (i.e., cathepsins) that are viewed as the mediators of terminal protein degradation. The method used by *Leishmania* spp. to withstand destruction by these enzymes remains an unresolved question. Indeed, it has been determined that infection with *L. amazonensis* does not interfere with the overall protein degradation capacity of the host cell (Prina et al. 1990).

Not only do the lysosomal enzymes remain active, but their production even seems to be stimulated in infected macrophages. Cathepsin S was studied here as a previously uncharacterized indicator of lysosomal activity in conjunction with *L. amazonensis* infection. Incorporation of cathepsins into the late endosome to create a lysosome is routinely accomplished through interaction with mannose 6-phosphate receptors on the compartment surface (Ludwig et al. 1995). Thus, the appearance of substantial cathepsin S association in the possible absence of CI-M6PR is intriguing. Perhaps the simplest rationalization is for cathepsin S distribution to occur by a mannose 6-phosphate-independent mechanism, since evidence exists for delivery of newly synthesized hydrolases to early endosomes in clathrin-coated vesicles originating from the trans-Golgi network (Ludwig et al. 1991). Studies in various macrophage lines report cathepsin S distribution ranging from mostly late endosomal, to active forms being found along the entire endocytic pathway (Claus et al. 1998; Driessen et al. 1999). Whatever the mechanism used for cathepsin S trafficking, the early distribution seen here is apparently not extraordinary and lends support to the idea of a mannose 6-phosphate-independent entry mechanism.

The distinctly rapid appearance of cathepsin S around PVs containing antibody opsonized promastigotes in normal cells raises interesting questions concerning the immune response to parasites internalized in this manner. These questions stem from evidence implicating cathepsin S as a key regulator of the MHC class II antigen presentation pathway to include degrading the invariant chain (Ii) chaperone of the class II molecule (Driessen et al. 1999; Riese et al 1998). Investigations into the role of antibody in antigen presentation to T cells have concluded that internalization via the Fc

receptor can result in modification of endocytic transport, leading to enhanced presentation of certain epitopes (Amigorena and Bonnerot 1998; Simitsek et al. 1995). Possible scenarios include the interaction of opsonized promastigotes with the Fc receptor leading to differential cytokine production, which in turn could affect disease progression at the onset of infection. Indeed, cathepsin S is known to be upregulated by IFN- γ (Riese et al. 1998), a Th1 response cytokine that contributes to the clearance of intracellular pathogens in response to immune stimulation.

As is the case with cellular entry, cathepsin S considerations with antibody opsonized promastigotes may be moot, as anti-*Leishmania* antibodies do not circulate in the typical uninfected host. However, the case may be different with amastigotes, which are antibody opsonized when tissue derived. Indeed, impaired maintenance of *Leishmania* infections has been shown in the absence of circulating antibody (Kima et al. 2000). Although this implies a role for the Fc receptor in amastigote phagocytosis, it does not necessarily extend to a role in enhancing the immune response to the parasite. Experimental data has shown that activated macrophages present little endogenous parasite antigen to CD4⁺ T cells when infected with *Leishmania* amastigotes (Kima et al. 1996; Prina et al. 1996). This contrasts with cells infected with promastigotes, which were fully competent at antigen presentation. The promastigote's differentiation into an amastigote may reduce the parasite's recognition by the MHC class II antigen presentation pathway in addition to allowing it to better tolerate the hostile intracellular environment. Nevertheless, modulation of the immune response by cathepsin S certainly warrants further investigation with amastigotes as a basis for comparison to that seen here with promastigote infections.

CHAPTER 5 CONCLUSION

The experimental data presented in this study show that *Leishmania amazonensis* promastigotes achieve the greatest infectivity when internalized via CR3 into mouse macrophages *in situ*. It can be inferred from this that CR3 is the main, or preferred, receptor employed by the parasite in establishing infection. However, future research should emphasize determining the collective contribution of all major phagocytic receptors to firmly establish the role of CR3 in *Leishmania* phagocytosis.

Historical reports on *Leishmania* LPG solidify the molecule's position as a virulence factor to some degree. However, the phagosome remodeling data presented here indicate that LPG alone cannot fully explain the maturation of the *L. amazonensis* PV; certain characteristics of the phagosomal membrane are imparted by the receptor used for phagocytosis of the promastigote. This was clearly evident from the divergent rab5a and cathepsin S association kinetics seen under different opsonization conditions and was further reinforced in experiments using CR3 knockout mice. Furthermore, deviations seen with docking mediators and late endosomal markers from that reported for inert particles indicate that the *L. amazonensis* PV occupies a unique niche along the endocytic pathway. The rapid colocalization of cathepsin S seen only with antibody opsonized PVs raises additional questions surrounding possible use of CR3 as a method to reduce exposure of the parasite to the host immune system. Whether the use of CR3 by

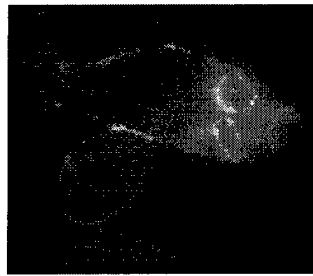
the promastigote is merely a coincidence of evolutionary adaptation or an active pathogenic mechanism has yet to be determined.

It is hoped that this work fosters better understanding of *Leishmania* pathogenesis. By elucidating *Leishmania* host-parasite interactions, more effective treatments and controls can be developed for leishmaniasis.

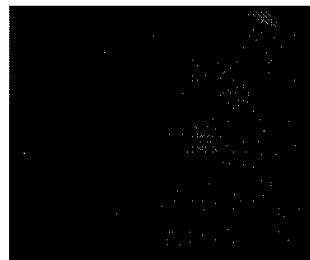
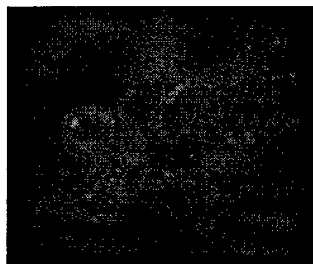
APPENDIX
IMMUNOFLUORESCENCE IMAGES

The following images represent typical immunofluorescent staining patterns of selected cellular markers seen in association with *Leishmania amazonensis*-containing phagosomes. Target molecules were visualized using fluorescein isothiocyanate-labeled secondary antibodies (green). Macrophage and parasite nuclear material was stained with 4',6-diamidino-2 phenylindole dihydrochloride (blue).

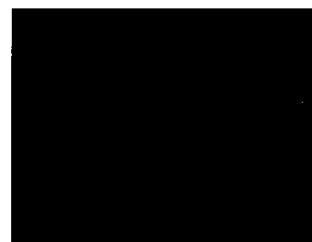
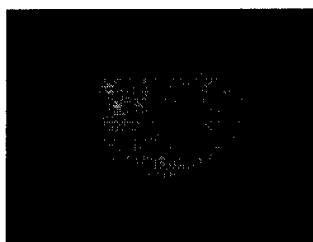
CR3



Vinculin



rab5



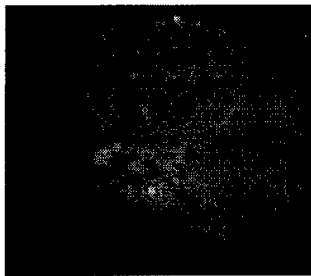
rab7



LAMP1



Cathepsin S



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BIOGRAPHICAL SKETCH

James Benjack is a U.S. Air Force Biomedical Laboratory Officer attending the University of Florida through an Air Force Institute of Technology civilian institutions program. His prior educational endeavors include earning a Bachelor of Science degree in microbiology and a Master of Arts degree in management. Upon completion of this work, he will continue in military service wherever the Air Force may send him.

*Thesis
Proposal*

Student's name: Capt Matthew D.
Civilian institution: Washington
Degree level: Master's
Academic field: Kinesiology

*for
Maj Russo*

Spokane

Statement of the subject area: Shoulder Rehabilitation. Testing the hypothesis that a newly designed self-assisted rotator cuff machine will significantly increase external and internal range of motion when compared to common static stretch shoulder exercises in a healthy populace of research participants.

Identification of the expected sources of research data: University supplied goniometer to measure range of motion, shoulder machine to use for stretching, and EMG for measure muscle response to stretch.

Description of the travel required, if any, to support the research, number of trips, location, number of days, and estimated expense (Travel should be held to a minimum):

1. TDY to a Seattle sports medicine clinic to learn how to measure shoulder range of motion with a goniometer. Must have this skill to access study participant range of motion before, during, and after treatment.

-One trip

-Seattle, WA

-Two days

-Plane ticket=\$90, Per diem=military off-base rate, Transportation=\$20
ESTIMATED COST=\$200

2. American College of Sports Medicine Regional Northwest Chapter Conference to present thesis work.

-One attendance

-Spokane, WA

-Two days

-ESTIMATED COST=\$0

3. American Sports Medicine Annual Meeting to present thesis work.

-One attendance

-St. Louis, MO

-May 29 - June 1, 2002 (4 days)

-\$110 for meeting enrollment, \$400 for plane ticket, Per diem=military off-base rate, Lodging=military rate off-base rate, Transportation=\$10/day ESTIMATED COST=\$1000

Endorsement by the student's advisor:

Sally E. Rank, Ph.D.