Published in final edited form as:

Microbes Infect. 2011 November ; 13(12-13): 1033–1044. doi:10.1016/j.micinf.2011.05.014.

The effects of macrophage source on the mechanism of phagocytosis and intracellular survival of *Leishmania*

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Abstract

Leishmania spp. protozoa are obligate intracellular parasites that replicate in macrophages during mammalian infection. Efficient phagocytosis and survival in macrophages are important determinants of parasite virulence. Macrophage lines differ dramatically in their ability to sustain intracellular *Leishmania infantum chagasi* (*Lic*). We report that the U937 monocytic cell line supported the intracellular replication and cell-to-cell spread of *Lic* during 72 hours after parasite addition, whereas primary human monocyte-derived macrophages (MDMs) did not. Electron microscopy and live cell imaging illustrated that *Lic* promastigotes anchored to MDMs via their anterior ends and were engulfed through symmetrical pseudopods. In contrast, U937 cells bound *Lic* in diverse orientations, and extended membrane lamellae to reorient and internalize parasites through coiling phagocytosis. *Lic* associated tightly with the parasitophorous vacuole (PV) membrane in both cell types. PVs fused with LAMP-1-expressing compartments 24 hours after phagocytosis by MDMs, whereas U937 cell PVs remained LAMP-1 negative. The expression of one phagocytic receptor (CR3) was higher in MDMs than U937 cells, leading us to speculate that parasite uptake proceeds through dissimilar pathways between these cells. We hypothesize that the mechanism of phagocytosis differs between primary versus immortalized human macrophage cells, with corresponding differences in the subsequent intracellular fate of the parasite.

Keywords

MACROPHAGES; PHAGOCYTOSIS; LEISHMANIA; CR3 RECEPTOR; U937 CELLS

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1. Introduction

Leishmania infantum chagasi (*Lic*) is an obligate intracellular protozoan parasite that causes visceral leishmaniasis in Latin America, a disease that can be fatal if untreated [1, 2]. *Leishmania* spp. are transmitted to susceptible mammalian hosts during the bite of a phlebotomine sand fly, after which they are taken up by resident or infiltrating phagocytic cells in the skin. The majority of parasites reside intracellularly in macrophages of different tissues during the different forms of leishmaniasis. Parasites can be killed intracellularly by activated macrophages *in vitro*, even though some parasites survive long-term within the host cell. The current study is based on the hypothesis that the initial events during internalization of leishmania by the macrophage may be determinants of ultimate parasite fate within the cell [3].

Leishmania bind to the macrophage surface and stimulate their own phagocytosis [4, 5]. Although early studies described the polarization of leishmania parasites during attachment and phagocytosis by macrophages as random [5], subsequent reports show a preferential orientation of *L. mexicana*, *L. tropica*, *L. braziliensis*, *L. major* or *L. aethiopica* promastigotes during macrophage entry. These differences may to some extent be species-specific. For example, *L. donovani* promastigotes adhere through either their flagellar tip or aflagellar (posterior) pole [4, 6-9], whereas *L. enrietti* promastigotes enter mouse macrophages primarily from the posterior pole [10].

Attachment of leishmania to host macrophages is mediated by interactions between specialized receptors on the phagocyte surface and presumed complementary ligands on the parasite surface [4-9, 11]. In particular, the third complement receptor, CR3, has been implicated in the safe uptake of leishmania promastigotes, guiding them toward a pathway that delays phagolysosome maturation [12]. The parasite subsequently undergoes phagocytosis as a passive partner requiring host cell, but not parasite, cytochalasin-inhibitable actin [4, 5, 13]. Microscopic analyses have documented variably shaped pseudopods engulfing leishmania [14]. These include funnel-shaped extensions of either human or murine macrophage membranes when internalizing *L. donovani*, *L. tropica*, *L. major* or *L. aethiopica* promastigotes [6, 8, 9]. In contrast, one study described the formation of coiling rather than tubular pseudopods around *L. major* or *L. aethiopica* promastigotes in human peripheral blood monocytes [8]. The authors speculated that this process might lead to cytosolic localization of promastigotes, similar to the intracellular pathogenesis of *Borrelia burgdorferi* [15]. Indeed some studies have claimed that *Leishmania* spp. attain a cytosolic location after phagocytosis, despite the conventional belief that these protozoa remain exclusively in phagolysosomes [7, 8, 16].

Based upon the above observations, we hypothesized that the conformation of the initial interaction between leishmania promastigotes and host macrophages varies between cells with different microbicidal potential. We additionally questioned whether a “zipper” or “coil” phagocytic pattern would lead to cytosolic, endosomal and/or lysosomal localization of the parasite. Our observations showed differences between the mechanisms of *Lic* promastigote adhesion to the host cell surface, modes of phagocytosis, localization in the parasitophorous vacuole (PV), and maturation of the PV in immortalized human macrophage-like cell line (U937) versus human primary macrophages. Importantly, there were corresponding differences in the intracellular replication of the parasite. The data reveal key differences in the conformation of phagocytosis, and support the notion that the earliest stages of *Lic* internalization are integral determinants of whether or not a successful macrophage infection will be established.

2. Materials and Methods

2.1. Parasites

A Brazilian strain of *Lic* (MHOM/BR/00/1669) was originally isolated from a patient with visceral leishmaniasis. Parasites were passed through male golden hamsters to maintain their virulence and used within 3 weeks of isolation from the animal. Promastigotes were cultured at 26°C in hemoflagellate-modified minimal essential medium, pH 7.4, supplemented with 10% heat-inactivated fetal calf serum (HI-FCS) [17]. Promastigotes used for phagocytosis assays were either bulk stationary phase cultures or purified metacyclic promastigotes, isolated from stationary phase cultures using a modified Ficoll gradient [18, 19]. Promastigotes were opsonized by incubation with either 2.5% fresh autologous human serum (metacyclic) or 2.5% C7 deficient human serum (stationary) before they were used in experiments.

2.2. Macrophages

Human peripheral blood leukocytes from healthy human platelet donors were obtained from the DeGowan Blood Center at the University of Iowa (Iowa City, IA). Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation [20]. Monocytes (5×10^5) were isolated by adherence to glass coverslips in 24-well plates [21]. Monocytes were maintained at 37°C, 5% CO₂ in RPMI 1640 with 10% HI-FCS, 2 mM L-glutamine, and penicillin (100 U/ml) / streptomycin (100 µg/ml) for 5 days to promote differentiation to monocyte-derived macrophages (MDMs) before exposure to parasites.

The human monocytic U937 cell line was obtained from American Type Culture Collection (Manassas, VA), and maintained in suspension culture in RPMI 1640 supplemented with 10% (v/v) HI-FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C, 5% CO₂. U937 cells were treated for 48 hours with 10 ng/ml of phorbol myristate acetate (PMA; Sigma-Aldrich Co., St. Louis, MO) to induce differentiation toward macrophages.

2.3. Macrophage phagocytosis assays and electron microscopy

Five $\times 10^5$ MDMs or U937 cells in 300 µl media without (MDM) or with (U937) PMA were allowed to adhere to glass coverslips in 24 well plates (SEM) or to the bottom of 6 well plates (TEM). Because metacyclic promastigotes enter more slowly yet survive and multiply more avidly in MDMs than stationary phase promastigotes, a multiplicity of infection (MOI) of 5 or 20 opsonized parasites per macrophage were added, depending on whether samples were prepared for imaging adherence/pseudopod extension or for intracellular localization and growth over time. Phagocytosis was synchronized by centrifugation at 250 xg for 3 minutes. Macrophages incubated for more than 1 hour were carefully rinsed at 1 hour to remove extracellular promastigotes. Cells were harvested and fixed at 4°C after 1, 5, 10, 20 or 30 minutes, or 4, 24 or 48 hours some for SEM or TEM, respectively.

2.3.1. Scanning and transmission electron microscopy (SEM, TEM)—SEM

samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hour, post-fixed with 1% osmium tetroxide for 1 hour, dehydrated through sequential incubation in 25%, 50%, 75%, 95% and 100% ethanol, and dried using the CO₂ Sorvall Critical Point Drying System (DuPont, Wilmington, DE). Coverslips were mounted on aluminum stubs, sputter coated with gold-palladium and examined by an S-4800 scanning electron microscope (Hitachi High Technologies America Inc., Pleasanton, CA).

TEM samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and dehydrated in a series of ethanol, and embedded in Eponate 12 resin (Ted Pella Inc., Redding, CA). Ultrathin (70 nm) sections were cut with an ultramicrotome, counterstained with uranyl acetate and lead citrate, and viewed by JEM-1230 transmission electron microscope (Japan Electron Optics Laboratory Co., Peabody, MA). All electron microscopy was performed at the University of Iowa Central Microscopy Research Facility (CMRF).

2.5. Live-cell imaging

MDMs and U937 cells were allowed to adhere to glass bottom Petri dishes and exposed to opsonized metacyclic promastigotes at an MOI of 5, so as not to overcrowd the imaging field with rapidly moving parasites. Petri dishes were immediately placed on the microscope stage, housed inside a 37°C CO₂ incubator. Living cells were photographed every 3 seconds after inoculation of parasites using an IX-81 Inverted Microscope (Olympus America Inc., Center Valley, PA), University of Iowa CMRF. Slidebook 5.0 software was used to compile the images into a time lapse video. ImageJ 1.4 software was used to prepare the videos for playback at 8 frames per second.

2.6. Confocal microscopy

Metacyclic promastigotes were fluorescently labeled with 5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA) as described [22], then opsonized and added to MDMs or U937 cells on coverslips at an MOI of 10. The MOI was lower than that in EM studies as more parasites were visible in one imaging field. Phagocytosis was synchronized by centrifugation at 250 xg for 3 minutes and rinsed at 1 hour to remove extracellular promastigotes. After 5 minutes, 2, or 24 hours, coverslips for confocal microscopy were fixed in 2% paraformaldehyde, permeabilized with 0.2% Triton X-100 and blocked with 5% normal goat serum. Samples were incubated with either 1:100 affinity purified anti-human lysosomal-associated membrane protein-1 (LAMP-1) H4A3 mAb (Developmental Studies Hybridoma Bank, University of Iowa) or 1:100 anti-human early endosome antigen-1 (EEA-1) mAb 1391-1410 (Calbiochem, San Diego, CA). Secondary antibodies were 1:200 Alexafluor 568 goat anti-mouse, or Alexafluor 568 goat anti-rabbit (Molecular Probes, Carlsbad, CA) [12]. Coverslips were mounted to slides with Vectashield H-1000 (Vector Labs, Burlingame, CA) and examined within 48 hours under the 60 x objective lens of an MRC-1024 Confocal Microscope (Bio-Rad Laboratories, Hercules, CA), University of Iowa CMRF. 0.5 μ m confocal Z-sections were sequentially acquired and imported into ImageJ 1.4 software, which was used to prepare the figures. For quantification, PVs were considered to co-localize with EEA-1 or LAMP-1 if there was specific enrichment of EEA-1 or LAMP-1 fluorescence surrounding the fluorescent parasite, or if there was direct pixel overlay of parasites and marker. At least 200 parasites across over 180 micrographs in ImageJ were scored.

2.7. Intracellular replication assays

Adherent MDMs or U937 cells on coverslips were exposed to metacyclic *Lic* promastigotes at MOI of 5, and incubated for 5 minutes, 2, 24, 48, or 72 hours. Coverslips were fixed with methanol, air-dried, and stained with Protocol Hema 3 Solutions 1 and 2 (Thermo Fischer Scientific, Waltham, MA). For time points after 5 minutes, coverslips were rinsed at 1 hour to remove extracellular promastigotes. Coverslips were mounted onto slides with Permount (Thermo Fischer Scientific) and visualized on a BX41 light microscope (Olympus America Inc.). At least 700 macrophages on each triplicate coverslip were scored per time point.

2.8. Flow cytometry

MDMs or U937 cells were incubated in 10 ng of PMA/ml in Teflon jars at 37°C, 5% CO₂ for 48 hours. Non-specific binding was blocked in 5% A/J mouse serum (Jackson Labs, Bar Harbor, ME). Macrophages were incubated with APC-conjugated anti-human CR3 and/or FITC-conjugated anti-human CD14 monoclonal antibodies (BD Pharmingen, San Jose, CA). APC-conjugated anti-MOPC-21 mouse IgG1 κ and FITC-conjugated anti-TNP mouse IgG2a κ antibodies (BD Pharmingen, San Jose, CA) were used as isotype controls. Samples were fixed in 2% paraformaldehyde and resuspended in PBS supplemented with 0.05% sodium azide and 1% HI-FCS. Flow cytometry was performed on an LSRII (Becton Dickinson, Mountain View, CA), University of Iowa Flow Cytometry Facility. FlowJo 3.0 software was used for scatterplot analysis.

3. Results

3.1. *Lic* promastigotes bind to MDMs via their anterior/flagellar end, but attachment to the U937 cells is less directional

Both primary human cells and macrophage cell lines have been used to study leishmania phagocytosis. We compared the phagocytic process between primary and immortalized cells, in order to learn how closely these mimic each other. We used SEM to compare the points of attachment of serum-opsonized *Lic* promastigotes to MDMs versus U937 cells (Fig. 1). The initial contact between the parasites and the MDM surface occurred primarily through the parasite flagellar tip, following which parasites were engulfed through an open funnel-like macrophage projection that lacked microspikes (Fig. 1A, B, C, D). Rarely, parasites adhered to MDMs through the posterior pole or at the flagellar base (not shown). In contrast, parasites phagocytosed by U937 cells became enveloped in a lamellar sheath that extended from the host cell surface (Fig. 1E, F, G, H). Contacting U937 cells via the lateral surface of the parasite resulted in the formation of local ruffling of the macrophage membrane (Fig. 1G). Occasionally, the lamellar sheaths surrounded the anterior end of the parasite body (Fig. 1H).

Because SEM provides a momentary snapshot of parasite-macrophage contact, live cell time lapse differential interference contrast (DIC) imaging was also utilized to evaluate the orientation of promastigotes throughout the attachment and entry into MDMs or U937 cells. Macrophages adherent to glass-bottom Petri dishes were synchronously exposed to 5:1 metacyclic *Lic* promastigotes and maintained in a heated chamber at 5% CO₂. Time lapse photography was used to capture images of the initial promastigote-macrophage interactions through the first 40 minutes of exposure to parasites (Supplemental Videos V1, V2).

The orientation of *Lic* promastigotes during initial contact with macrophages prior to phagocytosis was quantified from both SEM and time lapse data (Table 1). Only promastigotes that remained attached for 10 seconds or longer were included in the analysis of time lapse images.

According to both methods, most promastigotes attached to MDMs through their anterior (flagellar) end. Observations by SEM showed that most of the 80.8% of parasites with anterior end attachment contacted MDMs via the flagellar tip (73.8%), but some attached at the base of the flagellum (6.9%). The small percentage that attached through the posterior (aflagellar) end was observed only by live cell imaging in (arrows in Video V1). Overall, promastigotes were phagocytosed by MDMs in one preferred orientation, i.e. the anterior end.

In contrast to MDM attachment, only one fourth or one third of parasites attached to U937 cells through the anterior (flagellar) poles according to SEM or live cell imaging,

respectively. SEM images showed 24.9% attachment at the anterior end, with 21.1% attached via the flagellar tip and 3.8% attached at the flagellar base. According to SEM, 61.4% of promastigotes ultimately anchored to U937 cells via the posterior end whereas only a minority (7.6%) anchored via the lateral parasite surface. Nonetheless, video microscopy showed that half of promastigotes initially encountered U937 cells via the lateral surface, and some reoriented and extended membrane sheets to achieve ultimate anchoring through the posterior end. We presume the posterior contact visualized in SEM images may reflect the net result after U937 contact with the lateral parasite body, triggering changes in the macrophage membrane that lead to reorientation prior to phagocytosis. Very few promastigotes contacted U937 cells via the flagellum (Video V2, arrows).

According to Chi square analysis, the differences in promastigote posterior versus anterior end orientation toward U937 cells versus MDMs was statistically significant ($p < 0.0001$).

3.2. *Lic* promastigotes are phagocytosed via symmetrical pseudopod extension by MDMs and via coiling phagocytosis by U937 cells

“Coiling” phagocytosis differs from symmetrical phagocytosis in that one asymmetric pseudopod curls around an incoming particle, forming the characteristic ultrastructural multilayered coil. In some instances, the process is thought to lead to cytosolic localization of microbes [7, 15, 23]. Although both MDMs and U937 cells extended long pseudopods toward incoming leishmania, the pattern of pseudopod extension differed, as observed by TEM between 1 minute and 4 hours after addition of promastigotes (Fig. 2).

MDMs extended symmetrical complex pseudopods during phagocytosis, sometimes appearing as elaborate networks. Most commonly, MDMs surrounded the incoming promastigote with symmetrical membrane extensions, starting with the tip of the parasite flagellum or posterior end (Fig. 2A, B). Rarely there was a single “whorl” or circular configuration reminiscent of coiling phagocytosis (Fig. 2C).

In contrast, U937 cells frequently wound layers of asymmetric membrane sheets around the incoming parasite (Fig. 2D, E, F), similar to prior reports of other *Leishmania* species [8]. Characteristic of coiling phagocytosis, points of adherence between the U937 cell membrane and the incoming promastigote were most frequently visualized on one side of the parasite (Fig. 2E). Fully internalized parasites were often wrapped in multiple layers of macrophage membrane (Fig. 2F), forming a stack of pseudopods.

3.3. Metacyclic *Lic* promastigotes remain tightly associated with the phagosome membrane, and do not escape to the cytosol

The intracellular localization of a parasite would undoubtedly affect subsequent communication between the intracellular microbe and the host cell. Most reports describe *Leishmania* spp. in PVs, although a few studies have described apparent cytosolic localization [7, 15, 23]. In addition, many published TEM images of *Leishmania* spp. show points of tight adhesion between internalized promastigotes and their PV membranes. We therefore examined the PV character from 30 minutes to 48 hours following phagocytosis by MDMs or U937 cells (Fig. 3) in order to (1) to assess the possibility of cytosolic localization; and (2) to determine the association between the intracellular parasite membrane and the PV membrane.

Three types of interactions between parasite plasma membrane and host PV membrane were observed: (1) tight, (2) separated, and (3) fused (Fig. 3A). In all sections we observed two membranes in very close proximity: one corresponding to the parasite surface (Fig. 3A, arrowheads) and another belonging to the PV itself (Fig. 3A, arrows). Consistently, for all time points observed, *Lic* metacyclic and stationary phase promastigotes were located in

individual compartments. PVs containing metacyclic promastigotes were tight-fitting around the parasite body (Fig. 3B), whereas some PVs were looser when initiated with stationary phase promastigotes (Supplemental Fig. S1). For almost every internalized parasite, the parasite membrane appeared tightly apposed to the PV membrane through at least one zone of contact. Similar interactions with the PV membrane were observed in MDMs and U937 cells (representative images in Fig. 3B and Fig. S1). Interestingly, a very small number of parasites from unpurified stationary-phase cultures were seen in spacious parasite-containing vacuoles with no apparent contact between the parasite and the vacuolar membranes (Fig. S1F). Also in experiments initiated with bulk stationary parasites, a small number of PVs were seen in which we could not distinguish a surrounding vacuolar membrane (not shown). We could not distinguish between cytosolic localization and merely tightly apposed parasite-PV membrane surrounding the entire parasite in these images. The infrequency of such observations led us to conclude that the overwhelming majority of parasites remained inside the PV following phagocytosis.

Quantitative analysis of tight versus and separated membranes at 4, 24, and 48 hours post-inoculation of parasites is shown in Fig. 3C, derived from counting more than 300 PVs from MDMs and U937 cells. The majority of PVs in MDMs (69.7% considering all time points) or U937 cells (70.2%) contained parasites that were closely associated with the PV membrane. Fewer PVs contained a zone of separation, and there was no substantial change in proportions over time. Taken together, the data suggested that *Lic* remain within the PV space, parasite membranes associate tightly with the PV membrane in both MDMs and U937 cells, and parasites do not escape into the cytosol in either cell type.

3.4. Maturation of the phagosome occurs after a delay in MDMs, but does not occur in U937 cells after phagocytosis of metacyclic *Lic* promastigotes

Immunofluorescence confocal microscopy was used to explore whether metacyclic promastigotes entered the endosomal pathway in MDMs and in U937 cells, and whether the subsequent kinetics of phagosome maturation was similar between the two cell types. To this end, CFSE-loaded metacyclic parasites were incubated with adherent macrophage cultures (Fig. 4), which were then fixed and stained with antibodies to detect EEA-1 or LAMP-1.

Vesicles positive for EEA-1 are expected to fuse with parasitophorous vacuoles early after phagocytosis [24]. Indeed, we observed accumulation of EEA-1 around some invading promastigotes 5 minutes after parasite addition to either cell type (Fig. 4, top row). The peripheral localization of PVs surrounded by EEA-1 in both MDMs and U937 cells could reflect the fact that sufficient time had not passed for the parasites to progress further into the host cell. The distribution of EEA-1 in unexposed macrophages differed dramatically from infected cells, showing diffuse staining with slight perinuclear enrichment (Fig. S2).

One proposed virulence mechanism of metacyclic *Leishmania* spp. promastigotes is the ability to delay phagosome maturation and fusion with lysosomes during macrophage infections, documented by a delay in the recruitment of late endosome/lysosome markers such as LAMP-1 [25]. As predicted by these studies, PVs containing metacyclic *Lic* promastigotes had not acquired the LAMP-1 marker by 2 hours after promastigote addition to MDMs (Fig. 4, center left panel), a time when phagosome fusion with lysosomes would be expected to occur [25]. However, 24 hours after parasite addition to MDMs, many intracellular parasites were surrounded by a distinct “rim” of LAMP-1 staining reflecting heavy accumulation of LAMP-1 at the PV membrane and fusion with late endosomes or lysosomes (Fig. 4, bottom left panel). U937 cells incubated in parallel with metacyclic promastigotes were remarkable for a conspicuous absence of LAMP-1 staining of the PV membrane surrounding parasites at both 2 and 24 hours (Fig. 4, center right and bottom right

panels). LAMP-1 in unexposed macrophages was distributed evenly through the cytosol, similar to EEA-1 (Fig. S2). Some of the diffuse LAMP-1 stain in U937 cells overlay intracellular parasites, but the absence of either LAMP-1 accumulation around PVs or concentration of the LAMP-1 stain at the site of parasites indicated a lack of PV fusion with LAMP-1 positive compartments (Fig. 4, center row and bottom row, LAMP-1 panels).

Quantitative analysis of more than 200 PVs for each condition at each time point revealed there were no significant differences between the rates of EEA-1 recruitment to the PV at 5 minutes, or the rates of LAMP-1 recruitment to the PV at 2 hours after parasite addition to MDMs versus U937 cells (Fig. 5A). However, at 24 hours, LAMP-1 had accumulated at the periphery of greater than 2 fold more PVs in MDMs compared to U937 cells ($P < 0.01$). These data suggest that there are inherent differences between the maturation of parasite-containing phagosomes in primary MDMs versus the immortalized U937 cell line. Maturation occurred after an initial delay in MDMs, whereas no fusion with LAMP-1 positive compartments was observed in U937 cells incubated for at least 24 hours with *Lic*.

3.5. Entry of *Lic* metacyclic promastigotes results in amastigote replication in U937 cells but not in MDMs

To investigate a possible functional outcome of the observed differences in phagocytosis and phagosome maturation between MDMs versus U937 cells, we compared the rates of *Lic* entry into, and intracellular replication in MDMs or U937 cells over the course of 72 hours after parasite addition (Fig. 5 panels B, D, C). Metacyclic *Lic* promastigotes were added to macrophages at an MOI of 5 and synchronized by centrifugation. After 1 hour, macrophages were rinsed extensively to remove extracellular promastigotes. Macrophages were fixed and stained at subsequent time points, and parasite loads were determined microscopically by counting more than 700 macrophages in each of the triplicate coverslips at each time point. Data were examined by t test between macrophage cell types, and by two-way ANOVA to assess changes in parasite loads at different time points for each cell type. Asterisks in Figure 5 indicate statistically significant differences between the numbers of parasites associated with MDMs versus U937 cells at individual time points. Significant differences in parasite numbers at different time points after entry into each macrophage type are described in the paragraph below.

Five minutes after parasite addition, there were no statistically significant differences between the numbers of promastigotes associated with MDMs versus U937 cells. At this time point, metacyclic promastigotes may associate with host cells but many may not have undergone phagocytosis yet. The data at 5 minutes suggest that there were not differences in the rates of attachment to the cell types. Similarly, there was no change in the percent of macrophages containing associated or intracellular parasites between 5 minutes and 2 hours after parasite addition to either cell type (Fig. 5D). Nonetheless both MDMs and U937 cells illustrated a slight yet statistically significant ($p < 0.001$) increase in the total number of promastigotes in the macrophage population (parasites / 100 MΦ) and per *Lic*-associated macrophage (parasites / MΦ with *Lic*) between the 5 minute and 2 hour times (Fig. 5B, C). As most attached promastigotes would be expected to have undergone internalization by the 2 hour time point, this difference could indicate that for MDMs and U937 cells, the number of loosely anchored promastigotes increased similarly from 5 minutes to 1 hour, when all extracellular parasites were washed, and the remaining promastigotes were subsequently phagocytosed from 1 hour to 2 hours. Overall the data seem to indicate that the efficiency of anchoring to the macrophage surface and the rate of internalization were not significantly different between MDMs and U937 cells.

Promastigotes undergo morphologic conversion from promastigote to amastigote by 24-48 hours after phagocytosis by U937 cells [26]. After their conversion, there was evidence of

Lic replication between 24 and 48, and between 48 and 72 hours in U937 cell monolayers (Fig. 5B; $p < 0.001$ for both intervals). This increase in parasites per 100 macrophages was associated with both increased numbers of parasites within each infected macrophage (Fig. 5C, $p < 0.01$ comparing 48 to 72 hours), and significant spread to additional macrophages in the monolayer (Fig. 5D, $p < 0.01$ comparing 24 to 48 hours, and comparing 48 to 72 hours). These data suggest both intracellular replication and cell-to-cell spread occurred in U937 cells.

Contrastingly, although MDMs behaved similarly to U937 cells at 5 minutes and 24 hours, there was no significant change in the number of intracellular amastigotes in MDMs (Fig. 5B, C, $p > 0.01$), or in the numbers of macrophages harboring parasites (Fig. 5D, $p > 0.1$), between 24 and 72 hours after parasite addition to MDMs. By 72 hours after parasite addition, the combined effect of a three fold difference in parasite burden within each infected macrophage (Fig. 5C, $p < 0.001$) and two fold difference in the percent of macrophages containing one or more intracellular parasites (Fig. 5D, $p < 0.01$) resulted in over a five-fold difference between the number of *Lic* in the total MDM versus U937 cell population (Fig. 5B, $p < 0.001$).

3.6. Differences between surface receptor expression by MDMs versus U937 cells

Coiling phagocytosis of bacteria correlates with a non-uniform distribution of surface markers, with differential density of receptors on different aspects of the invaginating macrophage membrane [15, 23, 27]. We hypothesized that the heterogeneous orientation of promastigote-U937 cell attachment, triggering extension of coiling membranes that re-orient and engulf the invading promastigote, would correlate with heterogeneous distribution of macrophage surface receptors. Furthermore we hypothesized that the symmetrical pattern of promastigote phagocytosis by MDMs might correspond to binding one predominant macrophage surface receptor type. Many investigators have documented the importance of complement receptor CR3 in phagocytosis of *Leishmania* spp. promastigotes by primary murine or human macrophages [12, 28-30], although other receptors clearly are also involved [28, 30-34]. We used flow cytometry to assess whether the density of this major macrophage receptor for *Leishmania* differs between the two macrophage types. MDMs or PMA-differentiated U937 cells were labeled with fluorochrome conjugated antibodies, fixed and analyzed by flow cytometry (Fig. 5). Single cells were gated based on forward and side scatter of the isotype Ab control, and CD14+ was used as a marker for fully differentiated MDMs or U937 cells (78.2% or 79.7% of the total sample, respectively). 18.9% of all U937 cells, or 21.6% of CD14+ U937 cells expressed CR3. This compares to 71.9% of all, or 82.6% of CD14+ MDMs expressing CR3, nearly a four-fold difference (Fig. 5E).

4. Discussion

Biological activities of macrophages derived from different body sites, including expression of surface receptors and microbicidal capacities, can exhibit diverse characteristics [35-37]. It follows that immortalized macrophage cell lines might also show different characteristics compared to primary host-derived cells. Rather than discard some *in vitro* models as poor representations of natural leishmania infection, we reasoned that comparisons between macrophages of different sources could provide clues to the host determinants that permit parasite infection. The purpose of this study, therefore, was to correlate the rates of intracellular replication with the contrasting mechanisms of *Leishmania* spp. phagocytosis by a primary human macrophage (MDM) versus an immortalized human macrophage cell line (U937).

Fundamental differences between MDMs and the U937 cell line were illustrated by their differential expression levels of CR3, a macrophage surface membrane receptor that

participates in leishmania phagocytosis [31, 34]. Consistent with their contrasting surface compositions, the conformational changes exhibited during attachment and internalization of *Lic* promastigotes differed between the cell types. SEM, TEM and video microscopic observations showed that most *Lic* promastigotes contacted MDMs at their flagellar (anterior) poles, stimulating the macrophage to extend symmetrical pseudopods that maintained the directional entry of the parasite. This would be in agreement with symmetrical binding of abundant CR3 lining the macrophage pseudopods to an abundant complementary ligand lining the promastigote surface. In contrast, promastigote adherence and entry into U937 cells were most often initiated through contact at the lateral parasite body, followed by macrophage membrane lamellae enveloping the parasite, reorientation of promastigotes, and ultimately entry via the parasite's posterior end. Such asymmetry of parasite entry could correspond with ligation of several receptor types, each with different affinities for parasite surface receptors.

Once intracellular, the PV membrane surrounding metacyclic promastigotes frequently remained in close apposition with much of the parasite surface membrane, creating the tight, parallel alignment of membranes observed by TEM in both MDMs and U937 cells. The direct association between parasite and vacuolar membranes could facilitate critical intracellular needs such as nutrient acquisition and proper fusion/separation of membranes.

Maturation of phagosomes with eventual fusion with lysosomes results in delivery of microbicidal factors to the phagosome [38, 39]. LAMP-1 is expressed in late endosomes and lysosomes, and LAMP-1 recruitment to phagosomes often heralds lysosomal fusion. Several groups have shown that acquisition of the LAMP-1 marker is delayed by 24 or more hours after phagocytosis of *Leishmania* spp. promastigotes, allowing time for the conversion from the promastigote into the more oxidant-resistant amastigote form [12, 24, 38].

Using confocal microscopy, we observed that metacyclic *Lic* promastigotes entered the endosomal pathway in both MDMs and U937 cells, documented by a surrounding ring of EEA-1 in the PV membrane five minutes after parasite addition to the macrophages. Consistent with the above predictions, lysosomal fusion with PVs was delayed for at least 24 hours after parasite addition to MDMs. In marked contrast, PVs that surrounded metacyclic *Lic* in U937 cells were not observed to acquire the LAMP-1 marker during the time frame studied. The lack of PV-lysosome fusion in U937 cells suggests abnormal phagosome biogenesis, and correlated with parasite replication in U937 cells but not in MDMs.

It should be noted that the rates of promastigote attachment or internalization (5 minutes and 2 hours PI) by MDMs versus U937 cells were not different. Nonetheless there was significant amastigote replication in U937 cells between 24 and 72 hours after parasite addition, whereas there was no evidence of replication or cell-to-cell spread in MDMs. Taken together with the absence of LAMP-1 recruitment to the PV, U937 cells appeared hospitable to amastigote multiplication and highly permissive for subsequent invasion of neighboring cells [40]. Although U937 cells demonstrated the amastigotes' avid replicative potential in this intracellular niche, a similar effect was strikingly absent in MDMs, correlating with the positive accumulation of LAMP-1 to PVs in MDMs.

It is clear from our studies that MDMs are able to limit the parasite's intracellular replication and spread to neighboring macrophages. In contrast, U937 cells are incapable of limiting its parasite burden to the initial low level, which likely leads to greater amplification and subsequent cell-to-cell spread across the cell monolayer. These observations raise the possibility that insufficient microbicidal potential in the immortalized cell line, whether due to a different path of entry or defects in microbicidal functions, make U937 cells favorable for *Lic* proliferation.

Receptors reported to participate in uptake of *Leishmania* spp. promastigotes by macrophages include CR3, CR1 and mannose receptors (MR) amongst others [28, 31-34]. In addition to CR3, it has been shown that expression of other surface receptors differs between macrophage cell types [41, 42]. Our data do not implicate a particular receptor to be responsible for any specific consequence to *Lic* following ingestion by U937 versus MDM cells, and it must be noted that other studies suggest that phagocytosis through CR3 facilitates intracellular survival [12]. Nonetheless, our findings lead to the hypothesis that the two macrophage cell lines differ in the accessibility of surface receptors and the intracellular trafficking that follows phagocytosis. These differences could reflect the parasite's ligation of distinct receptors or groups of receptors, triggering entry through different pathways. Other reports document that different species of *Leishmania* promastigotes attach to host macrophages in preferred orientations, leading investigators to hypothesize that ligands become focally concentrated on the parasite surface at the sites of initial contact [6-9]. Our data highlight an additional role for the macrophage in guiding promastigote orientation and subsequent entry into macrophages.

In contrast to the observations of *Lic* phagocytosis presented herein, Courret et al. [43] reported that phagocytosis of either *L. major* or *L. amazonensis* metacyclic promastigotes by primary BALB/c mouse bone marrow macrophages most often occurred via the lateral parasite cell body, sometimes followed by envelopment by macrophage plasma membranes. Attachment that initiated at the tip of the flagellum was followed by reorientation prior to entry into the macrophage. As a result, only 3% of internalized promastigotes were oriented with the flagellum toward the interior of the cell, whereas the majority had their posterior ends facing inward [43].

The observations of Courret et al. were similar to the predominant posterior end uptake of *Lic* promastigotes by immortalized human U937 cells reported herein. They differed from our observation that primary human MDMs most frequently phagocytosed promastigotes through their flagellar (anterior) pole. Conclusions from studies of murine cells cannot be directly extrapolated to our studies of a different species of *Leishmania* interacting with human macrophages, other than to point out that the pathways leading to phagocytosis by different macrophages, if correlated with parasite survival and intracellular killing, may illuminate potential routing within the macrophage that determines parasite fate.

It can be debated whether the flagellum itself is an important source of ligands for receptor-mediated *Lic* entry into human MDMs, or whether the flagellum serves merely as a motor directing parasites toward a highly phagocytic cell [44]. Supporting a lack of importance of the flagellum itself are studies of *L. amazonensis* mutants overexpressing a constitutively active small GTPase encoded by *LdARL-3A*, which contain very short or no flagellae. Such mutants are defective in their ability to parasitize sand flies, but they undergo normal phagocytosis and short-term survival in murine macrophages [44].

Variability of particle shape influences the efficiency of phagocytosis. In particular, elongated bodies such as promastigotes, when positioned tangent to the macrophage membrane, are less efficiently internalized than bodies that are perpendicular [45, 46]. The ability of U937 cells to re-orient promastigotes after initial lateral attachment may therefore be critical to parasite uptake. Reorientation could in turn be associated with the preference of U937 cells to phagocytose promastigotes via coiling phagocytosis rather than the zipper-like phagocytic mechanism observed in MDMs. Furthermore, asymmetrical (or coiling) phagocytosis of particles is thought to result from the stereologic effects of asymmetric receptor aggregation [23]. Additional studies could address whether heterogeneous complexes of phagocytic receptors lead to the asymmetric distribution of receptors around

the incoming parasite, and extension of unilateral instead of bilateral pseudopods during phagocytosis [14, 23].

These early interactions between leishmania promastigotes and primary or immortalized macrophages, and their downstream consequences, reflect a summation between strategies of the parasite to establish intracellular residence in a host niche devoid of microbicidal activity, and the strategies of the host cell to efficiently phagocytose and kill invading microbes. Subsequent investigations could determine whether additional factors known to be critical for the intracellular life of *Leishmania* spp., including the accumulation of actin around the PV and release of exosomes by the parasite into the host cell, are consequences or causes of the initial mode of phagocytosis [47, 48].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to Chantal Allamargot for technical support of EM studies. All microscopic studies were performed in the University of Iowa Central Microscopy Facility. We thank Andrew Wiemer, Ph.D. for helpful discussions.

This work was supported by grants AI32135 and AI059451 from the National Institutes of Health (JED and MEW), NIH grants AI045540, AI067874, AI080801 and AI076233 (MEW), and by a Merit Review grant and RFA from the Department of Veterans' Affairs (MEW).

Abbreviations

CFSE	carboxyfluorescein diacetate succinimidyl ester
EEA-1	early endosomal antigen 1
LAMP-1	lysosomal-associated membrane protein-1
Lic	<i>Leishmania infantum</i> chagasi
MDM	monocyte derived macrophage
MOI	multiplicity of infection
PMA	phorbol myristate acetate
PV	parasitophorous vacuole
SEM	scanning electron microscopy
TEM	transmission electron microscopy

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Figure 1. SEM of MDMs and U937 cells exposed to *Lic* promastigotes. Stationary phase *Lic* promastigotes were opsonized and synchronously added to adherent MDMs or PMA-treated U937 cells at MOI 5 for 1 to 30 minutes. Samples were fixed and processed for SEM. (**A, B, C, D**) Examples of parasite – MDM contact via their flagellar pole (arrows). (**E, F**) U937 cells making contact with the posterior ends of promastigotes (arrows). (**G**) Example of the lateral surface of a promastigote contacting a U937 cell (arrow). (**H**) A promastigote contacting a U937 cell at its anterior end (base of the flagellum; arrow). Bars: 10 μ m.

Figure 2.

TEM of MDMs (left panels) and U937 cells (right panels) exposed to *Lic* promastigotes. Metacyclic *Lic* promastigotes were opsonized and synchronously added to adherent MDMs or PMA-treated U937 cells at an MOI of 5. Four hours after parasite addition, samples were fixed, dehydrated, and embedded in resin prior to microtomy. (A, B) Examples of *Lic* promastigotes undergoing phagocytosis via symmetrical macrophage pseudopod extension (arrow). (C) Asymmetrical pseudopod extension by MDMs (arrow) was very rarely observed. (D, E, F) Stationary phase *Lic* promastigotes were engulfed by an asymmetrically extended macrophage membrane (arrows), which wrapped (“coiled”) around the parasite, forming multiple visible layers. Bars: 2 μm.

Figure 3.

TEM analysis of intracellular *Lic* promastigotes and their PV junction morphology in MDM and U937 cells. Purified metacyclic promastigotes were opsonized and synchronously added to adherent MDMs or PMA-treated U937 cells at MOI 20. Samples were fixed after 4, 24, and 48 hours, dehydrated, and embedded in resin prior to microtomy. **(A)** Junctions with the PV membrane were characterized into three types. Tight: the PV membrane closely outlines the parasite membrane, with separation less than approximately 10 nm. Separated: one or more zones of separation of over 100 nm are observed between the PV membrane and the parasite body. Fusion: Organelle is fusing with (or budding from) the PV, but the junction is scored as “tight”. Arrows: parasite membrane. Arrowheads: PV membrane. Bars: 100 nm. **(B)** Metacyclic *Lic* promastigotes remain in individual, tightly bound compartments with no observed evidence of cytosolic escape. Bars: 500 nm. **(C)** Quantitative analysis of PV junction types in MDM or U937 cells incubated with metacyclic *Lic* promastigotes. The percent of PV junctions that were either “tight”, “separated” as described above were calculated. Very few PVs characterized as “fusion” were observed. More than 300 PVs were scored by TEM.

Figure 4.

Confocal microscopy of endocytic organelle trafficking in MDMs and U937 cells exposed to metacyclic *Lic* promastigotes. Purified metacyclic promastigotes were labeled with CFSE (green), opsonized with fresh human serum, and synchronously added to adherent MDMs or PMA-treated U937 cells at an MOI of 10. Cells were fixed and stained after 5 minutes (anti-human EEA-1), 2 hours, or 24 hours (anti-human LAMP-1), followed by secondary anti-IgG labeled with alexa Fluor 568 (red). Cellular actin was labeled with Alexa Fluor 647 phalloidin (blue). (Top row) 5 minutes after adding metacyclic *Lic* promastigotes to macrophages, EEA-1 stain surrounded 21.97% or 28.3% of the PVs in MDMs or U937 cells, respectively. Arrows point to parasites co-localized with EEA-1. The small right-hand panels contain enlargements of regions with arrows, with the pseudo-colors separated. (Center row) LAMP-1 did not surround metacyclic *Lic* promastigotes 2 hours following inoculation in either MDMs or U937 cells. (Bottom row) Metacyclic *Lic* promastigotes co-localized with LAMP-1 in MDMs, but not U937 cells. Representative photomicrographs from 5 experiments are shown.

Figure 5.

Quantitative analysis of endosomal/lysosomal marker recruitment (A), parasite burden (B, C, D), and CR3 surface expression (E) in MDMs or U937 cells. (A) The percent of metacyclic *Lic*-induced PVs that co-localized with EEA-1 or LAMP-1 was calculated as the percentage of internalized parasites that either were surrounded by a rim of EEA-1 or LAMP-1 or co-localized with these markers, as observed by confocal microscopy. (B, C, D) The numbers of attached (5 min) or internalized parasites (2, 24, 48, 72 hr) after synchronous addition of metacyclic *Lic* promastigotes to either MDMs or U937 cells was assessed microscopically in Wright-Giemsa stained slides. Data were calculated as (B) the total numbers of parasites per 100 macrophages, (C) numbers of parasites in macrophages that had attached or internalized parasites, and (D) the percent of macrophages containing either attached or intracellular parasites. Panels A, B, C, and D show the means $\pm$ SE of 3 phagocytosis assays of MDMs or U937 cells, respectively. At least 200 PVs (A) or 700 macrophages (B, C, D) in triplicate coverslips were scored for every time point. Statistical analyses utilized the one-tailed t test with 95% confidence (A) and two-way ANOVA (B, C, D). (E) CR3 expression on the surface of MDMs or U937 cells was assessed by flow cytometry. 10^6 MDMs or PMA-treated U937 cells prior to *Lic* exposure were stained with anti-human CR3 Ab conjugated to APC and/or anti-human CD14 Ab conjugated to FITC prior to fixation. Quadrants were set such that fewer than 2% of the cells stained with isotype control Abs were positive for CR3 or CD14. Results are shown as scatter plots of CR3/CD14 double stained MDMs or U937 cells and histograms of gated CD14+ populations showing APC mean fluorescence intensity representing CR3+ expression. Light-gray shaded peak: CD14+ cells without APC-anti-CR3 Ab. Dark gray shaded peak: CD14+ cells incubated with APC-Mouse IgG1 κ isotype control antibody. Black unshaded peak: CD14+ cells stained with APC-anti-CR3 Ab.

Table 1

Orientation and points of contact between *Lic* promastigotes and either monocyte-derived macrophages (MDMs) or U937 cells as documented by SEM (stationary) or live cell imaging (metacyclic).

	MDMs		U937 cells	
Column number	1	2	3	4
Point of parasite contact with macrophage	Quantification by SEM: number (%)	Quantification by live cell imaging: number (%)	Quantification by SEM: number (%)	Quantification by live cell imaging: number (%)
Flagellar pole *	105 (80.8)	226 (76.5)	65 (24.9)	73 (31.5)
Posterior end [†]	25 (19.2)	40 (14.4)	129 (61.4)	28 (14.7)
Lateral contact	0	21 (9.1)	16 (7.6)	119 (51.1)
P (columns compared) [‡]	0.216 (1 to 2)	<0.0001 (2 to 4)	<0.0001 (1 to 3)	0.0012 (3 to 4)

* Parasites binding to the tip of the flagellum and the parasite's anterior end (base of flagellum) were counted together.

[†] The posterior end is the aflagellar pole.

[‡] Chi square test