Research Article

Endocytosis and Sphingolipid Scavenging in Leishmania mexicana Amastigotes

Hayder Z. Ali,¹ Clare R. Harding,¹ and Paul W. Denny¹,²

¹ Biophysical Sciences Institute, Department of Chemistry and School of Biological and Biomedical Sciences, Durham University, Durham DH1 3LE, UK
² School of Medicine and Health, Durham University, Queen’s Campus, Stockton-on-Tees TS17 6BH, UK

Correspondence should be addressed to Paul W. Denny, p.w.denny@durham.ac.uk

Received 28 June 2011; Revised 18 July 2011; Accepted 22 July 2011

Academic Editor: Terry K. Smith

Copyright © 2012 Hayder Z. Ali et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Leishmania species are the causative agents of the leishmaniases, a spectrum of neglected tropical diseases. Amastigote stage parasites exist within macrophages and scavenge host factors for survival, for example, Leishmania species utilise host sphingolipid for synthesis of complex sphingolipids. In this study L. mexicana endocytosis was shown to be significantly upregulated in amastigotes, indicating that sphingolipid scavenging may be enhanced. However, inhibition of host sphingolipid biosynthesis had no significant effect on amastigote proliferation within a macrophage cell line. In addition, infection itself did not directly influence host biosynthesis. Notably, in contrast to L. major, L. mexicana amastigotes are indicated to possess a complete biosynthetic pathway suggesting that scavenged sphingolipids may be nonessential for proliferation. This suggested that Old and New World species differ in their interactions with the macrophage host. This will need to be considered when targeting the Leishmania sphingolipid biosynthetic pathway with novel therapeutics.

1. Introduction

Leishmania species are insect vector-borne kinetoplastid protozoan pathogens causing a wide spectrum of neglected tropical diseases (the leishmaniases—cutaneous, mucocutaneous, and visceral) in humans across the globe. Moreover, the spread and severity of disease are exacerbated by its status as an important coinfection in AIDS patients [1]. Leishmania species exhibit a digenetic lifecycle: (a) existing as flagellated promastigotes within the sand fly vector; (b) following an insect bite and uptake by professional phagocytic cells, especially macrophages, differentiating into the aflagellate amastigote form. Within the host macrophage the parasite proliferates within acidic, fusogenic, endosome-derived phagolysosomes [2]. This compartment intersects with the phagosomal and autophagosomal pathways meaning that the parasites have access to the rich mix of nutrients resulting from degradation of phagocytosed macromolecules within the lytic environment of the phagolysosome. However, residency within such a compartment could also lead to contact with components of the host major histocompatibility complex (MHC), subsequent macrophage activation, and parasite killing. To overcome this certain species of Leishmania sequester and degrade MHC molecules [3, 4]; presumably this occurs via endocytosis although the mechanism has not been characterised. Endocytosis is also the process by which nutrients can be taken into the parasite, for example, L. amazonensis amastigotes were reported to endocytose transferrin from the phagolysosome [5]. However other species, including L. mexicana, do not show this behaviour [6]. Low-density lipoprotein [7] and haemoglobin [8] have also been shown to be endocytosed by Leishmania promastigote forms.

Sphingolipids are amphipathic lipids consisting of a sphingosine backbone with a long-chain fatty acid and a polar alcohol as attachments. Ceramide is an unmodified sphingolipid that functions as a secondary messenger in ubiquitous, eukaryotic signalling mechanisms. Modified (complex) sphingolipids are major components of the outer leaflet of eukaryotic plasma membranes believed to be
involved, with sterols, in the formation of microdomains or lipid rafts. Rafts are proposed to function in a diverse array of processes from the polarised trafficking of lipid-modified proteins, to the assembly of signal transduction complexes [9]. The first, rate-limiting, enzyme in sphingolipid biosynthesis is serine palmitoyltransferase (SPT). SPT catalyses the condensation of L-serine and palmitoyl CoA to form 3-ketodehydrophinganine. Subsequently, N-acetylation of sphingoid base in the endoplasmic reticulum (ER) leads to the formation of ceramide, which is converted to complex sphingolipids (e.g., sphingomyelin SM or inositol phosphorylceramide—IPC) via sphingolipid synthases. The animal sphingolipid synthase, SM synthase (SMS), transfers phosphorylcholine from phosphatidylcholine (PC) to ceramide to give SM; in contrast yeast, plants, and the kinetoplastid protozoan parasite (MNYC/BZ/62/M379) promastigotes were maintained at 26°C in Schneider’s media (Sigma Aldrich) pH7, supplemented with 15% foetal bovine sera (FBS, Biosera Ltd). Promastigotes were differentiated to amastigote forms in Schneider’s media with 20% FBS at pH 5.5 and 32°C according to the published protocol [20]. The continuous murine macrophage cell line RAW264.7 was maintained in DMEM (Gibco-BRL) with 10% FBS, at 37°C and 5% CO₂. The cytotoxicity of myriocin (Sigma Aldrich) was established using the AlamarBlue (Invitrogen) assay according to manufacturer’s protocol and as previously [21, 22]. The efficacy of myriocin was confirmed using a yeast diffusion assay [23].

2.2. Metabolic Labelling. L. mexicana axenic promastigotes and amastigotes (10⁷ mL⁻¹) were incubated in serum-free Schneider’s media for 30 minutes before labeling in the same with 5 μM of BSA conjugated BODIPY FL C₅-ceramide (Invitrogen) at 26°C and 32°C, respectively, for 2 hours. Promastigote parasites were similarly labeled for 16 hours in DMEM (ICN) supplemented with 10% FCS (Gibco BRL) and 20 mCi mL⁻¹ [myo-³H]-inositol (102 Ci mmol⁻¹ Amersham) [14]. For serine-containing lipid analysis axenic amastigotes (5 × 10⁷ mL⁻¹) were incubated for 45 minutes in MEM Eagle (Sigma Aldrich) then labeled for 8 hours in the same medium containing 20 mCi mL⁻¹ [³H]-L-serine (20 Ci mmol⁻¹, ICN) [14]. Lipids were extracted and analysed as previously described [23].

2.3. Endocytosis Assay. L. mexicana cells were washed three times using warm serum-free media and counted using a Neubauer haemocytometer. 10⁷ cells were then incubated with 50 μg mL⁻¹ 3 k Texas Red Dextran (Invitrogen) in 500 μL of serum-free media for 2 hours at 32°C. Controls were incubated on ice. Cells were subsequently washed 5 times with ice cold PBS and fixed with 3.7% formaldehyde. After washing, cells were resuspended in 500 μL of PBS and the fluorescence quantified using a plate reader (FLx800TM, BioTek; 590/20 Ex 645/40 Em). Observation of cells by fluorescence microscopy indicated that the dextran had been taken up by the parasites.

2.4. Macrophage Infection. 1 × 10⁵ RAW264.7 murine macrophages in DMEM were allowed to adhere to coverslips within each well of a 24-well tissue culture plate (Nunc) and then incubated for 24 hours in appropriate media (DMEM with 10% FBS or with 1% Nutridoma, Roche) with or without myriocin. L. mexicana amastigotes were then applied for 45 minutes to macrophages in DMEM were allowed to adhere to coverslips within each well of a 24-well tissue culture plate (Nunc) and then incubated for 24 hours in appropriate media (DMEM with 10% FBS or with 1% Nutridoma, Roche) with or without myriocin. L. mexicana amastigotes were then applied for 48 hours at 32°C and 5% CO₂, with daily changes of media with myriocin where appropriate.

2.5. Expression Analyses. Denatured parasite lysates were separated and immunoblotted as described [24] and the filters probed with mouse anti-LmjFLC2B or rabbit anti-LmjNMT polyclonal primary antibodies [14] at 1:1000, followed by horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich). Complexes were detected using the ECL system (Amersham Pharmacia). For the mRNA analyses, total RNA from equivalent numbers of 48 hours infected, or noninfected, RAW264.7 cells were extracted using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. Following DNase treatment (RQ1,
Promega) cDNA was synthesized using the ImProm-II Reverse Transcriptase System (Promega) according to manufacturer’s protocol. Quantitative PCR was performed in a Rotor-Gene RG3000 (Corbett Research) using SYBR Green Jump-Start Taq Ready Mix (Sigma Aldrich) according to the manufacturer’s instructions. The murine MmLcb2 (encoding subunit 2 of SPT) was amplified using the primer pair 5’-AGGTGGATATCATGGAGAGA 3’ and 5’-GATCCAGTTCTTCGCG 3’. The reference gene, MmCasc3, was amplified using primers as previously [25]. The qPCR was carried out in triplicate on 3 replicates with annealing temperature 52°C for MmLcb2 and 55°C for MmCasc3.

3. Results and Discussion

3.1. Endocytosis in Promastigote and Amastigote L. mexicana. Like Leishmania species, Trypanosoma brucei (which causes the neurological NTD African sleeping sickness) is a kinetoplastid parasite. However, whereas the pathogenic amastigote forms of Leishmania species shelter within macrophages, the mammalian form of T. brucei proliferates extracellularly within the bloodstream of the host. Here it must avoid the full force of the immune system, and the switching of the predominant surface molecule, Variable Surface Glycoprotein (VSG), is key to this. Endocytosis of VSG is an important part of this antigenic variation and immune evasion. Notably, the rate of endocytosis in bloodstream form (BSF) T. brucei is 10 times higher than in the insect stage procyclic form (PCF), and the entire VSG coat is replaced every 7 minutes [26]. This allows the internalisation and degradation of bound antibodies and complement factors which helps protect the free-living parasite from the humoral immune response. This upregulation of endocytosis is facilitated by an increase in expression of factors involved in the uptake of VSG and its subsequent trafficking and processing [27, 28]. Before this study it remained unknown whether Leishmania species increased their rate of endocytosis on differentiation to the mammalian amastigote form. This may be predicted to facilitate the observed uptake of MHC and immune avoidance [6] or allow the acquisition of host sphingolipid for use in IPC synthesis [15, 18].

To address this the New World species L. mexicana was employed. Unlike L. major, this species can be grown in both promastigote and amastigote stages in axenic cell culture [20] allowing direct comparative analyses to be undertaken [29]. To quantify endocytosis fluorescently labelled dextran was utilised. Previously, dextran has been demonstrated to be taken up, via the endocytic pathway, by L. donovani promastigotes [30]. Here, axenic cell equivalents of promastigotes (both noninfective, proliferating procyclids, and infective, nonproliferating metacyclics) and amastigotes were labelled under identical conditions (32°C in serum-free media). Dextran is measurably taken up by procyclic promastigotes; however the quantity endocytosed by amastigotes is more than 4 times greater (Figure 1). Interestingly the nondiving metacyclic forms demonstrate a similar up-regulation of this activity.

These data indicate that endocytosis is up regulated in amastigote forms, and this could be speculated to facilitate efficient immune evasion or substrate scavenging as discussed. The observation of similar levels of endocytic activity in metacyclic promastigotes was surprising given their apparent quiescence [20]. However, one may speculate that this indicated that the machinery for this process is acquired at this stage ready for the establishment of amastigote infection in a mammalian host.

3.2. The Role of Host Sphingolipid Biosynthesis in L. mexicana Invasion and Proliferation. To investigate IPC synthase activity in L. mexicana, axenic promastigotes and amastigotes were metabolically labeled with fluorescent BODIPY FL-ceramide (Figure 2). Both cell types produced a labelled product that comigrated with IPC, although the level in amastigotes was relatively low reflecting the slower growth of this form. These data indicated that L. mexicana has an active IPC synthase in both lifecycle stages, as does L. major [15].

As discussed above, host sphingolipid must be acquired as a source of ceramide substrate for the IPC synthase of amastigote L. major [15]. In support of this several studies have identified host cell glycosphinoglipids in intramacrophage amastigotes of Leishmania species [31–33]. In addition, it has been reported that fumonisin B1 (an inhibitor of dihydroceramide synthase and ceramide
Figure 2: HPTLC analysis of axenic procyclic promastigotes (aP) and amastigotes (aA) metabolically labelled with BODIPY FL C5-ceramide. IPC: inositol phosphorylceramide; Cer: ceramide.

synthesis) inhibits the replication of intramacrophage \( L. \) \( \text{donovani} \) [34]. Utilising myriocin (a potent, specific SPT inhibitor [35]) the role of host synthesized sphingolipids was investigated in \( L. \) \( \text{mexicana} \) infected RAW264.7 cells, a continuous murine macrophage cell line. The viability of axenic promastigotes and amastigotes (data not shown) was unaffected by the presence of 50 \( \mu \)M myriocin as previously reported [36]. Similarly, the RAW264.7 host cells were viable at 50 \( \mu \)M myriocin; levels above this lead to detachment of cells from the tissue culture well (data not shown).

In this study 50 \( \mu \)M of myriocin was applied to RAW264.7 cells for 24 hours prior to challenge with \( L. \) \( \text{mexicana} \) axenic amastigotes at a ratio of 10 : 1. With daily application of the drug the infection was allowed to continue for 48 hours prior to fixation, staining, and counting as described in Section 2. This experiment was conducted either in the presence or absence of the SPT inhibitor myriocin and with (FBS-DMEM) or without (FBS-reduced DMEM) exogenous serum in the media. Results of three independent experiments.

Figure 3: Invasion, % infected macrophages, determined 48 hours after infection in the presence or absence of the SPT inhibitor myriocin and with (FBS-DMEM) or without (FBS-reduced DMEM) exogenous serum in the media. Results of three independent experiments.

3.3. Host and Parasite Serine Palmitoyltransferase (SPT) Expression during Macrophage Infection. Notably host de novo sphingolipid synthesis is up regulated during \( L. \) \( \text{donovani} \) proliferation within macrophages [16], and it is possible that a similar effect during \( L. \) \( \text{mexicana} \) infection could mask the efficacy of the SPT inhibitor myriocin. To test the hypothesis that an increase in host sphingolipid biosynthesis, via the up regulation of biosynthetic enzymes, is elicited by the parasite, the levels of host \( \text{MmLcb2} \) (encoding subunit 2 of SPT) transcript with and without \( L. \) \( \text{mexicana} \) infection were established using real-time qPCR. To do this normalisation to a reference was required; of nine commonly used reference genes in RAW264.7 cells only \( \text{Casc3} \) was previously found to be suitable and sufficient for this purpose on infection with \( \text{Mycobacterium avium} \) [25]. Normalised to \( \text{MmCasc3} \) expression, \( \text{MmLcb2} \) mRNA levels were unchanged 48 hours after...
FBS-DMEM
FBS-reduced DMEM

0 µM myriocin 50 µM myriocin

Parasites/macrophage

Figure 4: Proliferation, parasites per infected macrophage, determined 48 hours post infection in the presence or absence of the SPT inhibitor myriocin, and with (FBS-DMEM) or without (FBS-reduced DMEM) exogenous serum in the media. Results of three independent experiments.

infection with *L. mexicana* (Table 1) under the conditions described. This indicated that host sphingolipid biosynthesis is not up regulated on infection.

These results were perhaps surprising given the previously observed down-regulation of the *Lm*LCB2 protein in *L. major* amastigote forms [14, 38], coupled with the maintenance of IPC biosynthesis [15] and the requirement for catabolism of scavenged SM [17, 18]. In light of these data it was chosen to examine the expression profile of the *L. mexicana* SPT. Utilising the cross reactive anti-*Lmj*LCB2 antibody [14, 39] the expression of *Lmx*LCB2 was probed using Western blotting (Figure 5). The levels of the constitutively expressed *N*-myristoyltransferase (NMT) were used as a loading control [24].

This indicated that *Lmx*LCB2, and so *Lmx*SPT, is constitutively expressed throughout the lifecycle of *L. mexicana*. These data have been recently confirmed by proteomic analyses of isolated, intra-macrophage *L. mexicana* amastigotes [40]. Furthermore, metabolic labelling of axenic amastigotes demonstrated the incorporation of tritiated serine into the primary complex sphingolipid, IPC (Figure 6).

Taken together, these data indicated that this New World species has a complete and active *de novo* sphingolipid biosynthetic pathway in the amastigote stage, and this contrasts with Old World *L. major* [14, 38]. In addition, the possibility that myriocin may inhibit *Lmx*SPT in intra-macrophage *L. mexicana* amastigotes needs to be considered in relation to the data shown in Figure 4. If this compound is able to access and inhibit the parasite enzyme in these assays, then the results obtained would imply that both host and parasite SPT activity are nonessential for *L. mexicana* proliferation, a similar scenario to that seen in *L. major* [15].

### Table 1: Expression of *MmLcb2* in infected and noninfected macrophages.

| Serum | Normalised *MmLcb2* infected: noninfected ± standard deviation |
|-------|---------------------------------------------------------------|
| +     | 1.04 ± 0.12                                                  |
| −     | 0.94 ± 0.12                                                  |

3.4. Summary. These data are the first to demonstrate that the endocytic rate of the pathogenic, amastigote stage of *Leishmania* species is raised when compared with insect stage procyclic promastigotes. This is reminiscent of the situation in *T. brucei*, where endocytosis is up regulated on differentiation to mammalian BSF parasites in order to facilitate immune evasion [36]. This dramatic adaptation is facilitated by an increase in expression of factors involved in the uptake of molecules and their subsequent trafficking and degradation [27, 28]. In *Leishmania* species proteolytic activity is highly up regulated during differentiation from promastigotes to amastigotes, and this coincides with the appearance of multivesicular lysosomes (megasomes) [41]. This correlates with the uptake of host MHC molecules [41] and, as shown here, an increase in endocytosis. However, unlike in *T. brucei*, none of the known molecular machinery of endocytosis so far studied in *Leishmania* species has demonstrated any increased expression on differentiation [24, 42]. However, a RAB-like GTPase in *L. major* has been shown to have increased expression at the level of mRNA in amastigote forms, although the role of this factor in the cell remains unknown [43].

Both promastigotes and amastigotes of *L. major* [15] and *L. mexicana* are able to synthesize the primary complex sphingolipid, IPC. Old World *L. major* is able to do this in the absence of *de novo* SPT activity, therefore is the observed increase in endocytic activity related to an ability to scavenge host sphingolipid for use as substrate [15]? The
data presented here suggest that *L. mexicana* amastigotes can invade and proliferate normally when host sphingolipid biosynthesis is inhibited and/or when the exogenous source of lipid (serum) is removed. This suggested that the parasite is not wholly dependent on ongoing host sphingolipid synthesis, although residual host complex sphingolipid may remain and be sufficient for the pathogen. Analyses of host *M. leibbrandti* and parasite *L. mexicana* expression indicated that infection does not influence host sphingolipid biosynthesis and that promastigote and amastigotes stages of *L. mexicana* equivalently express SPT, the first enzyme in sphingolipid biosynthesis [40]. This is in contrast to *L. major* where *LmjLCB2* is down regulated and nonessential for pathogenesis and complex sphingolipid biosynthesis [14, 38]. This implies that there are profound differences between the Old and New World species in terms of the relationship between the *Leishmania* parasite and its mammalian host cell. Further knowledge of these differences will be required when considering the targeting of lipid biosynthesis for the development of novel therapies.

**Acknowledgments**

This work was supported by an Iraqi Ministry of Higher Education and Scientific Research scholarship to H. Z. Ali, a Wellcome Trust vacation scholarship to C. R. Harding and a Wolfson Research Institute Small Grants Award to P. W. Denny. The authors also thank Diane Hart and Jen Topping for technical support and Professor Deborah Smith (University of York) for the anti-*LmjLCB2* and anti-*LmjNMT* antibodies and for helpful discussions.

**References**

[1] R. Molina, L. Gradoni, and J. Alvar, "HIV and the transmission of *Leishmania*," *Annals of Tropical Medicine and Parasitology*, vol. 97, no. 1, pp. S29–S45, 2003.

[2] J. Nyalwidhe, U. G. Maier, and K. Lingelbach, "Intracellular parasitism: cell biological adaptations of parasitic protozoa to a life inside cells," *Zoology*, vol. 106, no. 4, pp. 341–348, 2003.

[3] J. C. Antoine, T. Lang, E. Prina, N. Courret, and R. Hellio, "H-2M molecules, like MHC class II molecules, are targeted to parasitophorous vacuoles of *Leishmania*-infected macrophages and internalized by amastigotes of *L. amazonensis* and *L. mexicana*," *Journal of Cell Science*, vol. 112, no. 15, pp. 2559–2570, 1999.

[4] S. De Souza Leao, T. Lang, E. Prina, R. Hellio, and J. C. Antoine, "Intracellular Leishmania amazonensis amastigotes internalize and degrade MHC class II molecules of their host cells," *Journal of Cell Science*, vol. 108, no. 10, pp. 3219–3231, 1995.

[5] V. M. Borges, M. A. Vannier-Santos, and W. De Souza, "Subverted transferrin trafficking in *Leishmania*-infected macrophages," *Parasitology Research*, vol. 84, no. 10, pp. 811–822, 1998.

[6] D. G. Russell, S. Xu, and P. Chakraborty, "Intracellular trafficking and the parasitophorous vacuole of *Leishmania mexicana*-infected macrophages," *Journal of Cell Science*, vol. 103, no. 4, pp. 1193–1210, 1992.

[7] V. V. Andrade-Neto, N. N. T. Cicco, E. F. Cunha-Junior, M. M. Canto-Cavalheiro, G. C. Atella, and E. C. Torres-Santos, "The pharmacological inhibition of sterol biosynthesis in *Leishmania* is counteracted by enhancement of LDL endocytosis," *Acta Tropica*, vol. 119, no. 2–3, pp. 194–198, 2011.

[8] N. Patel, S. B. Singh, S. K. Basu, and A. Mukhopadhyay, "Leishmania requires Rab7-mediated degradation of endocytosed hemoglobin for their growth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 10, pp. 3980–3985, 2008.

[9] K. Simons and E. Ikonen, "Functional rafts in cell membranes," *Nature*, vol. 387, no. 6633, pp. 569–572, 1997.

[10] K. Hanada, "Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism," *Biochimica et Biophysica Acta*, vol. 1632, no. 1–3, pp. 16–30, 2003.

[11] M. M. Nagiec, E. E. Nagiec, J. A. Baltisberger, G. B. Wells, R. L. Lester, and R. C. Dickson, "Sphingolipid synthesis as a target for antifungal drugs. Complementation of the inositol phosphorylceramide synthase defect in a mutant strain of Saccharomyces cerevisiae by the AUR1 gene," *Journal of Biological Chemistry*, vol. 272, no. 15, pp. 9809–9817, 1997.

[12] J. G. Mina, S. Y. Pan, N. K. Wansadhipathi et al., "*The Trypanosoma brucei* sphingolipid synthase, an essential enzyme and drug target," *Molecular and Biochemical Parasitology*, vol. 168, no. 1, pp. 1–6, 2009.

[13] S. S. Sutterwala, F. E. Hsu, E. S. Sevova et al., "Developmentally regulated sphingolipid synthesis in *African* trypansosomes," *Molecular Microbiology*, vol. 70, no. 2, pp. 281–296, 2008.

[14] P. W. Denny, D. Goulding, M. A. J. Ferguson, and D. F. Smith, "Sphingolipid-free *Leishmania* are defective in membrane

---

**Figure 6: HPTLC analysis of axenic amastigotes (aA) metabolically labelled with tritiated serine (Ser; 1 × 10^6 cell equivalent). Axenic promastigotes (aP) similarly labelled with tritiated inositol (Inos; 5 × 10^6 cell equivalent) served as markers. Following fluorography the plate was exposed to film for 15 days. PIP: phosphatidylinositol phosphate; IPC: inositol phosphorylceramide; PI: phosphatidylinositol; Cer: ceramide, migrating at solvent front.**
trafficking, differentiation and infectivity," *Molecular Microbiology*, vol. 52, no. 2, pp. 313–327, 2004.

[15] K. Zhang, F. F. Hsu, D. A. Scott, R. Docampo, J. Turk, and S. M. Beverley, "Leishmania salvage and remodelling of host sphingolipids in amastigote survival and acidocalcisome biogenesis," *Molecular Microbiology*, vol. 55, no. 5, pp. 1566–1578, 2005.

[16] S. Ghosh, S. Bhattacharyya, S. Das et al., "Generation of ceramide in murine macrophages infected with Leishmania donovani alters macrophage signaling events and aids intracellular parasitic survival," *Molecular and Cellular Biochemistry*, vol. 223, no. 1-2, pp. 47–60, 2001.

[17] W. Xu et al., "Sphingolipid degradation by Leishmania is required for its resistance to acidic pH in the mammalian host," *Infection and Immunity*, vol. 79, no. 8, pp. 3377–3387, 2011.

[18] O. Zhang, M. C. Wilson, W. Xu et al., "Degradation of host sphingomyelin is essential for Leishmania virulence," *PLoS Pathogens*, vol. 5, no. 12, Article ID e1000692, 2009.

[19] R. A. Heinzen, M. A. Scidmore, D. D. Rockey, and T. Hackstadt, "Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*," *Infection and Immunity*, vol. 64, no. 3, pp. 796–809, 1996.

[20] P. A. Bates, "Complete developmental cycle of Leishmania mexicana in axenic culture," *Parasitology*, vol. 108, no. 1, pp. 1–9, 1994.

[21] J. G. Mina, J. A. Mosely, H. Z. Ali, P. W. Denny, and P. G. Steel, "Exploring Leishmania major Inositol Phosphorylceramide Synthase (*LmjIPCS*): insights into the ceramide binding domain," *Organic and Biomolecular Chemistry*, vol. 9, no. 6, pp. 1823–1830, 2011.

[22] J. G. Mina, J. A. Mosely, H. Z. Ali et al., "A plate-based assay system for analyses and screening of the Leishmania major inositol phosphorylceramide synthase," *International Journal of Biochemistry and Cell Biology*, vol. 42, no. 9, pp. 1553–1561, 2010.

[23] P. W. Denny, H. Shams-Eldin, H. P. Price, D. F. Smith, and R. T. Schwarz, "The protozoan inositol phosphorylceramide synthase: a novel drug target that defines a new class of sphingolipid synthase," *Journal of Biological Chemistry*, vol. 281, no. 38, pp. 28200–28209, 2006.

[24] P. W. Denny, S. Lewis, J. E. Tempero et al., "Leishmania RAB7: characterisation of terminal endocytic stages in an intracellular parasite," *Molecular and Biochemical Parasitology*, vol. 123, no. 2, pp. 105–113, 2002.

[25] D. L. Taylor, P. C. Thomson, K. de Silva, and R. J. Whittington, "Validation of endogenous reference genes for expression profiling of RAW264.7 cells infected with *Mycobacterium avium* subsp. paratuberculosis by quantitative PCR," *Veterinary Immunology and Immunopathology*, vol. 115, no. 1-2, pp. 43–55, 2007.

[26] S. K. A. Natesan, L. Peacock, K. Matthews, W. Gibson, and M. C. Field, "Activation of endocytosis as an adaptation to the mammalian host by trypomastigotes," *Eukaryotic Cell*, vol. 6, no. 11, pp. 2029–2037, 2007.

[27] G. W. Morgan, B. S. Hall, P. W. Denny, M. C. Field, and M. Carrington, "The endocytic apparatus of the kinetoplastida. Part II: machinery and components of the system," *Trends in Parasitology*, vol. 18, no. 12, pp. 540–546, 2002.

[28] G. W. Morgan, B. S. Hall, P. W. Denny, M. Carrington, and M. C. Field, "The kinetoplastida endocytic apparatus. Part I: a dynamic system for nutrition and evasion of host defences," *Trends in Parasitology*, vol. 18, no. 11, pp. 491–496, 2002.

[29] F. L. Chadbourne et al., "Studies on the antileishmanial properties of the antimicrobial peptides temporin A, B and 15a," *Journal of Peptide Science*. In press.

[30] E. Ghedin, A. Debrabant, J. C. Engel, and D. M. Dwyer, "Secretory and endocytic pathways converge in a dynamic endosomal system in a primitive protozoan," *Traffic*, vol. 2, no. 3, pp. 175–188, 2001.

[31] M. J. McConville and J. M. Blackwell, "Developmental changes in the glycosylated phosphatidylinositols of Leishmania donovani," *Journal of Biological Chemistry*, vol. 266, no. 23, pp. 15170–15179, 1991.

[32] P. Schneider, J. P. Rosat, A. Ransijn, M. A. J. Ferguson, and M. J. McConville, "Characterization of glycoinositol phospholipids in the amastigote stage of the protozoan parasite Leishmania major," *Biochemical Journal*, vol. 295, no. 2, pp. 555–564, 1993.

[33] G. Winter, M. Fuchs, M. J. McConville, Y. D. Sterhof, and P. Overath, "Surface antigens of Leishmania mexicana amastigotes: characterization of glycoinositol phospholipids and a macrophage-derived glycosphingolipid," *Journal of Cell Science*, vol. 107, no. 9, pp. 2471–2482, 1994.

[34] S. Ghosh, S. Bhattacharyya, M. Sirkar et al., "Leishmania donovani suppresses activated protein 1 and NF-kB activation in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase," *Infection and Immunity*, vol. 70, no. 12, pp. 6828–6838, 2002.

[35] K. Hanada, M. Nishijima, T. Fujita, and S. Kobayashi, "Specificity of inhibitors of serine palmitoyltransferase (SPT), a key enzyme in sphingolipid biosynthesis, in intact cells. A novel evaluation system using an SPT-defective mammalian cell mutant," *Biochemical Pharmacology*, vol. 59, no. 10, pp. 1211–1216, 2000.

[36] J. E. Dalton, K. A. Mullin, and M. J. McConville, "Intracellular trafficking of glycosphatidylinositol (GPI)-anchored proteins and free GPIs in Leishmania mexicana," *Biochemical Journal*, vol. 363, no. 2, pp. 365–375, 2002.

[37] K. Hanada, M. Nishijima, M. Kiso et al., "Sphingolipids are essential for the growth of Chinese hamster ovary cells. Restoration of the growth of a mutant defective in sphingoid base biosynthesis by exogenous sphingolipids," *Journal of Biological Chemistry*, vol. 267, no. 33, pp. 23527–23533, 1992.

[38] K. Zhang, M. Showalter, J. Revollo, E. F. Hsu, J. Turk, and S. M. Beverley, "Sphingolipids are essential for differentiation but not growth in Leishmania," *EMBO Journal*, vol. 22, no. 22, pp. 6016–6026, 2003.

[39] S. Besteiro, G. H. Coombs, and J. C. Mottram, "A potential role for ICP, a leishmanial inhibitor of cysteine peptidases, in the interaction between host and parasite," *Molecular Microbiology*, vol. 54, no. 5, pp. 1224–1236, 2004.

[40] D. Paape, M. E. Barrios-Llerena, T. Le Bihan, L. Mackay, and T. Aeberscher, "Gel free analysis of the proteome of intracellular Leishmania mexicana," *Molecular and Biochemical Parasitology*, vol. 169, no. 2, pp. 108–114, 2010.

[41] N. Courret, C. Frehel, E. Prina, T. Lang, and J. C. Antoine, "Kinetics of the intracellular differentiation of Leishmania amazonensis and internalization of host MHC molecules by the intermediate parasite stages," *Parasitology*, vol. 122, no. 3, pp. 263–279, 2001.

[42] P. W. Denny, G. W. Morgan, M. C. Field, and D. F. Smith, "Leishmania major: clathrin and adaptin complexes of an intra-cellular parasite," *Experimental Parasitology*, vol. 109, no. 1, pp. 33–37, 2005.
[43] M. Chenik, N. Chaabouni, Y. B. Achour-Chenik et al., “Identification of a new developmentally regulated Leishmania major large RAB GTPase,” Biochemical and Biophysical Research Communications, vol. 341, no. 2, pp. 541–548, 2006.