Microbes and Infection 15 (2013) 579–591

www.elsevier.com/locate/micinf

Original article

Proteomic analysis reveals differentially expressed proteins in macrophages infected with *Leishmania amazonensis* or *Leishmania major*

J.P.B. Menezes [a,1], T.F. Almeida [a,1], A.L.O.A. Petersen [a,b], C.E.S. Guedes [a], M.S.V. Mota [a,b], J.G.B. Lima [a,b], L.C. Palma [a,b], G.A. Buck [c], M.A. Krieger [d], C.M. Probst [d], P.S.T. Veras [a,*]

[a] *Laboratory of Pathology and Biointervention, Gonçalo Moniz Research Center (CPqGM)-FIOCRUZ, Bahia, Brazil*
[b] *Federal University of Bahia, Salvador, Bahia, Brazil*
[c] *Center for the Study of Biological Complexity, Virginia Commonwealth University, Richmond, VA, USA*
[d] *Functional Genomic Laboratory, Carlos Chagas Institute (ICC)-FIOCRUZ, Paraná, Brazil*

Received 28 December 2012; accepted 18 April 2013
Available online 28 April 2013

Abstract

CBA macrophages effectively control *Leishmania major* infection, yet are permissive to *Leishmania amazonensis*. Employing a transcriptomic approach, we previously showed the up-regulation of the genes involved in the classical pathway of macrophage activation in resistant mice. However, microarray analyses do not evaluate changes in gene expression that occur after translation. To circumvent this analytical limitation, we employed a proteomics approach to increase our understanding of the modulations that occur during infection and identify novel targets for the control of *Leishmania* infection. To identify proteins whose expression changes in CBA macrophages infected with *L. major* or *L. amazonensis*, protein extracts were obtained and digested and the peptides were characterized using multi-dimensional liquid chromatography coupled with tandem mass spectrometry analyses. A total of 162 proteins were selected as potentially modulated. Using biological network analyses, these proteins were classified as primarily involved in cellular metabolism and grouped into cellular development biological networks. This study is the first to use a proteomics approach to describe the protein modulations involved in cellular metabolism during the initial events of *Leishmania*–macrophage interaction. Based on these findings, we hypothesize that these differentially expressed proteins likely play a pivotal role in determining the course of infection.

Keywords: *Leishmania amazonensis*; *Leishmania major*; Proteomics; Macrophages

1. Introduction

Leishmaniasis is a complex of parasitic diseases that constitute a worldwide public health challenge, ranking among the most serious endemic diseases, with a broad spectrum of clinical manifestations. Murine infection models have been utilized to elucidate host immune response mechanisms leading to resistance or susceptibility. When infected with *Leishmania major*, CBA mice develop an inflammatory response characteristic of lesion control, whereas this same strain, when infected with *Leishmania amazonensis*, exhibits a histopathological profile similar to that observed in highly susceptible BALB/c mice [1,2]. The CBA murine model of infection facilitated the identification of the mechanisms involved in *Leishmania* infection, reflecting the static genetic background of these animals [2].

Leishmania are protozoan parasites that predominantly survive and replicate inside host macrophages [3]. Following internalization, parasite promastigotes transform into amastigotes within macrophage phagolysosomal compartments [3].

* Corresponding author. Laboratory of Pathology and Biointervention, CPqGM, Oswaldo Cruz Foundation, Rua Waldemar Falcão, 121, Candeal, Salvador/BA 40296-710, Brazil. Tel.: +55 71 3176 2263; fax: +55 71 3176 2290.
E-mail addresses: pveras@bahia.fiocruz.br, pstveras@gmail.com (P.S.T. Veras).
[1] These authors contributed equally to this work.

1286-4579/$ - see front matter © 2013 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.micinf.2013.04.005

Previous studies have shown that the control of *Leishmania* infection depends on the ability of the host macrophages to destroy the intracellular parasites [4]. Thus, *Leishmania* infection activates macrophages, thereby leading to parasite destruction [5]. Furthermore, there is ample evidence that these parasites suppress and modulate the host intracellular immune response [6,7] through the inhibition of cell functions, including microbicidal molecule production, antigen presentation, apoptosis, cytokine and chemokine secretion, and alterations in the host cell signaling pathways [8–10].

It has been demonstrated that CBA macrophages effectively control *L. major* infection yet are permissive to *L. amazonensis* infection, suggesting that these versatile cells might play a crucial role in the outcome of *Leishmania* infection [11]. To identify markers associated with resistance and susceptibility using a murine model of parasite infection, we assessed the discrepancies in CBA macrophage responses to *L. major* and *L. amazonensis*.

In a previous study, we employed cDNA microarray analysis to demonstrate that CBA macrophages infected with *L. amazonensis* exhibit a reduced expression of the genes involved in the activation of macrophage pathways compared to the expression in C57BL/6 cells, which control *L. amazonensis* infection [10]. Microarray technology is a useful tool for identifying molecules at the transcriptional level, but primary transcription levels do not always correlate with the corresponding levels of protein expression due to post-translational regulation events.

Comparative proteomic studies are fundamental to the identification of potential markers of pathogen virulence, such as those involving *Mycobacteria* sp. [12] and *Helicobacter pylori* [13]. Many proteomic studies focusing on *Leishmania* infection have considered a variety of aspects related to parasite biology and host interactions, including parasite differentiation, drug resistance mechanisms, and the identification of immunogenic proteins for vaccine development [14–21]. However, the events related to *Leishmania*–host cell interactions have received little consideration [17,18,22,23].

Henriques et al. (2003) [22] used a classic proteomic technique to detect the expression of parasitophorous vacuolar membrane proteins and lipids of *Leishmania*-infected macrophages. These authors observed that promastigote and amastigote forms of *L. amazonensis* induce different patterns of protein and lipid expression in parasitophorous vacuole membranes; however, they did not attempt to identify these molecules.

Therefore, the aim of the present study was to evaluate the macrophage response to *Leishmania* infection using a proteomic approach. To our knowledge, this study represents the first attempt to employ large-scale proteomic analysis to identify host cell protein expression in response to *Leishmania* infection. We hypothesized that *L. major*-infected CBA macrophages express proteins associated with infection control compared to *L. amazonensis*-infected CBA cells. Using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), the levels of protein expression were assessed in uninfected and infected macrophage cell cultures at intervals of 6 and 24 h after infection with *L. major* or *L. amazonensis*. We employed qualitative and bioinformatics analyses to determine whether these two *Leishmania* species induced the expression of specific sets of proteins in macrophages in response to infection. Based on the results obtained from our proteomic analysis, we utilized biological network analysis to assess the distinct responses observed in the CBA macrophage infection in the two *Leishmania* species. These network models revealed that the proteins with elevated levels of differential expression in CBA macrophages belong to a biological network pertaining to cellular development and cellular metabolism.

2. Materials and methods

2.1. Ethics statement

The Animal Care Facility at CPqGM/FIOCRUZ provided male and female CBA and C57BL/6 mice. The animals were housed under specific pathogen-free conditions, fed commercial rations, and provided water *ad libitum*. Both CBA and C57BL/6 strains of mice were used at 6–12 weeks of age. The animal husbandry, experimentation, and welfare were performed in compliance with the International Guiding Principles for Biomedical Research Involving Animals and approved through the Animal Care Ethics Committee from CPqGM/FIOCRUZ.

2.2. Cell cultures

Inflammatory peritoneal CBA murine macrophages were obtained and maintained according to the modified protocols of Gomes et al. (2003). All cells were recovered in heparinized saline (5 UI/mL) and centrifuged at 300 × *g* for 10 min at 4 °C. Subsequently, cultures plated with 5×10^6 cells were maintained in an incubator supplemented with 5% CO_2 at 37 °C overnight. All cell cultures were washed and reincubated until further analysis.

2.3. Parasites and infection

L. major (strain MHOM/RI/-/WR-173) and *L. amazonensis* (strain MHOM/Br88/Ba-125) parasites were isolated from the lymph nodes of infected C57BL/6 mice. Dr. Lucile Flöeter-Winter recharacterized the parasites in accordance with previously described protocols [24]. CBA mouse peritoneal macrophages were harvested and distributed at 5×10^6 cells per well in six-well plates. Uninfected macrophages were used as controls. In parallel, CBA macrophages were infected with *L. major* or *L. amazonensis* stationary-phase promastigotes, at a parasite per macrophage ratio of 10:1. After a 12-h infection period, the control and infected cells were washed and reincubated for an additional 6 or 24 h at 37 °C. After culturing, the proteins were extracted from uninfected and infected macrophages. Each experimental group was cultured in

duplicate, and each biological experiment was repeated five times.

2.4. Protein extraction and LC–MS/MS

Following either 6 or 24 h of incubation, the cells were harvested and protein extraction was performed in 150 μL of lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris and 4% CHAPS), supplemented with a Complete Mini, EDTA-free Protease Inhibitor cocktail (Roche, IN, USA). The solutions were homogenized for 30 min and centrifuged at 13,800 × *g* for 20 min.

After determining protein concentrations using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), the contaminants were removed using a 2D clean-up kit (GE Healthcare, Waukesha, WI, USA). Following purification, the samples were resuspended in a solution of 8 M urea and 5 mM dithiothreitol (DTT) and incubated for 1–2 h. Subsequently, a solution containing 200 mM iodoacetamide (IAA) in 1 M ammonium bicarbonate (NH_4HCO_3) was mixed into the sample solution and incubated for 45 min at room temperature in total darkness. Next, the samples were incubated with 200 mM of DTT for 20 min at room temperature. All samples were then diluted in 50 mM of NH_4HCO_3 and 2 mM of $CaCl_2$, and then a trypsin solution was added for protein digestion and the generation of tryptic peptides at a ratio of 1:30 (trypsin:-protein). The digestion occurred over 16–18 h at 37 °C, interrupted through the addition of a 10% trifluoroacetic acid solution and the subsequent pH adjustment to 5.0. The resulting tryptic peptides were desalted on C8 cartridges (Michrom BioResources, Auburn, CA, USA) and subjected to 2D Nano LC–MS/MS analysis using a Dionex nano LC system (Dionex Corporation, Sunnyvale, CA, USA). To perform first-dimension separation, a 300 μm ID SCX column (PolyLC Polysulfoethyl A, 150 × 0.3 mm, 5 μm, 200 A) was used with a 15-step gradient (0–100%, pH 3.6–6.5) of ammonium formate (generated in-house, 0.8 M solution), for 1 h each step, at a flow rate of 5 μL/min. The peptides eluted from the SCX column were trapped on a C4 precolumn (Dionex PepMap300, 5 μm, 300 A, 300 μm ID × 5 mm), desalted [0.1% formic acid, 2% acetonitrile (ACN)] and separated on a 75-μm ID C18 column (Dionex NAN75-15-15-03-C18 PepMap100 stationary phase, 3 μm) using an acetonitrile gradient at a flow rate of 200 nL/min and finally electrosprayed, with a potential of 1.8–2.2 kV, onto an LCQ Deca XP(Plus) ion trap mass spectrometer (Thermo-Finnigan Corporation, San Jose, CA, USA) in data-dependent mode. A full-scan MS spectra survey was acquired from *m*/*z* 350 to 2000, and the four most abundant ions were selected and fragmented to produce tandem mass spectra. The target ions previously selected twice for MS/MS were dynamically excluded for 3 min. A normalized collision energy of 35% was used for peptide dissociation, and the MS/MS spectra were recorded in profile mode.

2.5. Bioinformatics analysis

In order to differentiate between macrophage and *Leishmania* proteins, the MS/MS spectra were searched against both a *Mus musculus* and *L. amazonensis* local protein database using the Sequest algorithm incorporated into the Bioworks v3.2 software [25–27]. The X_{corr} and ΔC_n threshold values for a 1% false discovery rate (FDR) were used to obtain the peptide ID list. The FDR was calculated using a reversed database. The modification parameters were set to +57.02146 for cysteine alkylation and +15.99492 for methionine oxidation. The spectra were searched allowing a maximum mass deviation of 3 amu and two missed cleavage sites. Only peptides identified as possessing fully tryptic termini with cross-correlation scores greater than 1.9 for single charged, 2.3 for double charged, and 3.75 for triple charged, and a delta-correlation score larger than 0.1 and a probability score lower than 1×10^{-5} were selected for further analyses. The peptides that passed the criteria listed above were used for the differential expression analysis, where a qualitative (non-quantitative) metric was applied. For each protein, we counted the number of replicates, where the number of identified peptides passing the above criteria was larger than 0 (n_p) for each time point and population (uninfected, *L. amazonensis* or *L. major* infected). Subsequently, for further analysis of each comparison made, we selected only those proteins whose difference between both countings (dn_p) was equal or greater than two (±2).

2.6. Western blot analysis

Western blot analysis was performed to validate the proteins whose proteomics identification pattern suggested differential expression in *L. amazonensis*- and *L. major*-infected macrophages. The myosin light chain was selected for validation from a selected group of 15 proteins with the greatest dn_p. Western blot analysis was performed after loading 85 μg of lysate from uninfected control macrophages and macrophages infected with *L. amazonensis* or *L. major* onto a 12% polyacrylamide gel. The proteins were separated through SDS–PAGE for 1 h 50 min at 150 V/20 mA and transferred to nitrocellulose membrane (Hybond ECL, 0.2 μm – Amersham Biosciences, Piscataway, NJ, USA) for 1 h 10 min at 50 V/300 mA using the Blot module system for the miniVE electrophoresis unit (Hoefer®, Inc., Holliston, MA, USA). To perform immunostaining, the blot was incubated with 1.36 μg/mL of anti-Myosin Light Chain (GeneTex Inc., Irvine, CA, USA) antibody diluted in PBS containing 3% BSA (Sigma, Warrensburg, MO, USA) overnight, followed by incubation with 11.2 μg/mL of anti-rabbit antibody conjugated to peroxidase (Sigma). To visualize the protein bands, a Super-Signal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used, in addition to Amersham Hyperfilm ECL film (Amersham Biosciences). The images were acquired on a scanner, and densitometry analysis was performed for determining the band intensity using the Image Processing and Analysis Java (ImageJ) v.1.45 program according to the manufacturer's software guide.

2.7. Immunofluorescence assay

To validate the differential expression of the 15 proteins in *L. amazonensis*- and *L. major*-infected macrophages, an

immunofluorescence assay was also performed. Hypoxia inducible factor (HIF)-1α was selected for validation from a selected group of proteins with the greatest dn_p. The uninfected control and *L. amazonensis*- or *L. major*-infected macrophages were fixed in 4% paraformaldehyde (Sigma) for 20 min at room temperature. The fixed cells were washed three times with phosphate buffered solution (PBS) at room temperature for 5 min, and the free-aldehyde groups were quenched after incubating the cells in PBS containing 50 mM NH_4Cl for 15 min at room temperature. Subsequently, the cells were washed once with PBS following permeabilization with PBS-2%Tween (PBST) for 30 min at room temperature. Subsequently, the macrophages were washed with 0.1% PBST at room temperature for 5 min and incubated in 0.1% PBST containing 10% normal donkey serum supplemented with 0.1% gelatin (blocking solution) for 40 min at room temperature. After blocking, the cells were incubated overnight at 4 °C with specific rabbit anti-mouse HIF (Novus Biologicals, Littleton, CO, USA), diluted in 0.1% PBST containing 0.1% gelatin at a final concentration of 1 μg/mL or with control rabbit immunoglobulin diluted at the same concentration. The cells were then washed twice with 0.1% PBST and incubated for 30 min at room temperature with donkey anti-rabbit IgG (Alexa Fluor 488; Molecular Probes Inc., Eugene, OR, USA) diluted in 0.1% PBST containing 0.1% gelatin, diluted at 1:200. The macrophages were rinsed three times in 0.1% PBST and three times in PBS, and the coverslips were mounted with DAPI Vectashield® (Vector, Burlingame, CA, USA). The images were obtained using a confocal microscope (IX-81, Fluo View 1000; Olympus America Inc., Center Valley, PA, USA), and in each experimental group, uninfected macrophages, and *L. amazonensis*- and *L. major*-infected macrophages, at least five fields were randomly captured at 6 and 24 h after infection. The images were analyzed using Image Pro-Plus version 6.0 software (Media Cybernetics, MD, USA). To determine HIF-1α expression in uninfected and infected macrophages, the Integrated Optical Density (IOD) of each labeled cell was calculated, representing the average intensity/density of each object (object = labeled cell). The values on the graph represent the IOD means ± standard deviation (SD) from each experimental group described. One-way analysis of variance (ANOVA) and the Newman–Keuls test were used to compare the HIF-1α expression among groups using Graph-Pad Prism software (GraphPad Prism version 5.0, GraphPad Software, Inc., La Jolla, CA, USA), and the differences were considered statistically significant when $p < 0.05$.

2.8. IPA Analysis

Ingenuity Systems Pathway Analysis v8.8 software (IPA-Ingenuity Systems®, Redwood City, CA, USA) was employed to model the possible canonical pathways and networks involving the selected proteins under *L. amazonensis* or *L. major* infection [10]. The potential networks were scored and modeled considering the sets of proteins with dn_p derived from the comparisons described above. The canonical pathway analysis identified the pathways, which were most significant to the input dataset. For the analyses, Fisher's exact test was used to measure the probability of proteins with dn_p in a given network, which have also been identified together in functional networks as a result of chance with a 0.05 threshold value.

3. Results

3.1. Leishmania modulates macrophage response

Using a proteomic approach, we endeavored to identify markers of resistance and susceptibility in *Leishmania* infection. Through the analysis of protein profiles, a total of 1352 proteins were identified as expressed in uninfected and infected macrophages. The number of proteins identified in this study was similar or even higher than that identified in macrophages using proteomics [28,29]. First, to assess the differences in protein expression, the proteins exclusively identified in infected cells were determined. Sixty-two proteins were identified in infected but not uninfected macrophages (Table 1). Among this group of proteins, some proteins were implicated in cell metabolism, such as protein subunits of the protein degradation complex, proteasomes (proteasome 26S subunit, non-ATPase, 10, proteasome 26S ATPase subunit 6; proteasome 26S non-ATPase subunit 11; proteasome beta 3 subunit; proteasome 26S non-ATPase subunit 13), cellular detoxification (sulfiredoxin 1 homolog; antioxidant protein 1), and enzymes (glucuronidase-beta; adenylosuccinate synthetase 1). In addition, carrier proteins (fatty acid binding protein 3, solute carrier family 25, and solute carrier family 37), immune receptors (scavenger receptor class B), and proteins associated with immune receptors (TNF receptor-associated protein 1) and proteins involved in cell signaling (N-acetylglucosamine kinase, adenylate kinase 2 isoform a, and protein phosphatase 1 regulatory subunit 9B) were also identified.

3.2. L. amazonensis *and* L. major *modulate protein expression differently in CBA macrophages*

A previous study compared the parasite burden in CBA macrophages infected with *L. major* or *L. amazonensis* parasites by determining the percentage of infected macrophages, as well as the number of parasites per infected macrophage [11]. These authors demonstrated that, at early time periods following infection, the percentage of infected cells was similar between the *L. major*- and *L. amazonensis*-infected macrophages. However, at later time points, a greater proportion of CBA macrophages were infected with *L. amazonensis* in comparison with *L. major*. These findings allowed us to suggest that the CBA mouse macrophage in vitro model is suitable to identify markers involved in resistance and susceptibility to *Leishmania* infection.

A total of 62 proteins were exclusively expressed in infected macrophages (Table 1). Among them, only 10 proteins were identified in *L. major* infection: ribosomal protein S13; glutamate receptor ionotropic; guanine nucleotide

Table 1
Proteins identified exclusively in macrophages infected by *Leishmania*.

GI	Protein	Macrophage					
		La		Lm		NI	
		6 h	24 h	6 h	24 h	6 h	24 h
6671519	Adenylosuccinate synthetase 1	+	−	+	+	−	−
31560349	Solute carrier family 37 (glycerol-3-phosphate transporter), 2	+	−	+	+	−	−
30409998	Solute carrier family 25 (mitochondrial carrier; ADP/ATP), member 31	+	+	+	−	−	−
31980811	Proteosome (macropain) 26S subunit, nonATPase, 10	+	−	+	+	−	−
12181182	Cytochrome c oxidase, subunit Va	+	−	+	−	−	−
16716343	Cytochrome c oxidase, subunit VIc	+	−	−	+	−	−
6753810	Fatty acid binding protein 3, muscle and heart	+	+	−	+	−	−
6755110	Procollagen-lysine, 2-oxoglutarate 5dioxygenase 3	+	+	−	+	−	−
27754103	Proteasome 26S ATPase subunit 6	+	−	−	+	−	−
74315975	Proteasome 26S non-ATPase subunit 1	+	+	−	+	−	−
21313268	Sulfiredoxin 1 homolog	+	+	−	+	−	−
30520133	Transportin 1	+	−	+	−	−	−
6753136	Antioxidant protein 1	+	−	−	+	−	−
94368817	PRD: similar to Ribosome-binding protein 1 (mRRp)	−	+	+	−	−	−
50053703	Protein phosphatase 1, regulatory subunit 9B	−	+	+	+	−	−
7305095	Golgi autoantigen, golgin subfamily a, 5	−	+	+	−	−	−
31543239	Microtubule-associated protein, RP/EB family, 2	−	+	+	+	−	−
13386034	Ribosomal protein S13	−	−	+	+	−	−
6678053	Small nuclear ribonucleoprotein B	−	+	+	−	−	−
77020262	Adenylate kinase 2 isoform a	−	+	−	+	−	−
31982541	Biliverdin reductase A	−	+	+	−	−	−
17975508	Glucan (1,4-alpha-), branching enzyme 1	−	+	+	+	−	−
28076961	Hypothetical protein LOC70617	−	+	+	+	−	−
6678952	Methylenetetrahydrofolate dehydrogenase (NAD+ dependent)	−	+	+	+	−	−
33859763	Myosin IF	−	+	−	+	−	−
13385392	N-ethylmaleimide sensitive fusion protein attachment protein α	−	+	+	+	−	−
6679134	Neuropilin 1	−	+	+	+	−	−
28849889	Oxysterol binding protein-like 11	−	+	+	+	−	−
40254624	Platelet-activating factor acetylhydrolase, isoform 1b, α2 subunit	−	+	+	+	−	−
6680878	Scavenger receptor class B, member 2	−	+	+	+	−	−
29789012	Cytochrome P450, family 2, subfamily c, polypept 38	−	+	−	+	−	−
6680101	Glutamate receptor, ionotropic, NMDA2D (épsilon4)	−	−	+	+	−	−
21327669	Guanine nucleotide binding protein (G protein), γ 8 subunit	−	−	+	−	−	−
83649762	Hypothetical protein LOC68767	−	+	−	+	−	−
68299824	Myosin IE	−	−	−	+	−	−
6755202	Proteasome beta 3 subunit	−	−	+	+	−	−
6680726	Ras homolog gene family, member B	−	−	+	−	−	−
34328286	Succinate dehydrogenase Ip subunit	−	+	−	−	−	−
6671672	Capping protein (actin filament) muscle Z-line, alpha 2	−	+	−	+	−	−
13385006	Cytochrome c-1	−	−	−	+	−	−
9506739	N-acetylglucosamine kinase	−	−	−	+	−	−
42415473	Protein phosphatase 3, catalytic subunit, alpha isoforms	−	+	+	−	−	−
6679217	Protein-L-isoaspartate (D-aspartate) O-methyltransferase 1	−	+	−	+	−	−
13385998	TNF receptor-associated protein 1	−	−	+	+	−	−
6755899	Translin	−	−	+	−	−	−
6755210	Proteasome 26S non-ATPase subunit 13	+	+	−	+	−	−
40254163	Hypothetical protein LOC75734	+	+	+	+	−	−
6754214	Heme oxygenase (decycling) 2	+	+	+	−	−	−
6753552	Phosphate cytidylyltransferase1, choline, alpha isoforms	+	+	+	−	−	−
94364433	PRD: similar to Cell surface glycoprotein gp42 precursor	+	+	+	−	−	−
29789343	Eukaryotic translation initiation factor 3, subunit 9	+	+	+	−	−	−
22267442	Ubiquinol cytochrome c reductase core protein 2	+	+	+	+	−	−
51871123	Renin binding protein	+	+	+	+	−	−
109809757	Hydrogen voltage-gated channel 1	+	−	+	+	−	−

(*continued on next page*)

Table 1 (*continued*)

GI	Protein	Macrophage					
		La		Lm		NI	
		6 h	24 h	6 h	24 h	6 h	24 h
31559920	Hypothetical protein LOC109168	−	+	+	+	−	−
31542440	Cytochrome b-245, beta polypeptide	+	+	+	+	−	−
28313453	Adenine phosphoribosyl transferase	−	+	+	+	−	−
40254521	Preimplantation protein 3	−	+	+	+	−	−
84794631	Tubulin, alpha-like 3	+	+	+	+	−	−
149263303	PRD: similar to endothelial monocyte-activating polypeptide	+	+	+	+	−	−
6754098	Glucuronidase, beta	+	+	+	+	−	−

Proteins expressed by infected macrophages were identified using the MudPIT LC–MS/MS method as described in Materials and methods. Proteins were considered to be + in an experimental group when at least one peptide was identified in at least two replicates. GI is the electronic reference protein from the NCBI database. La and Lm represent the experimental groups of infected macrophages for each respective species of Leishmania. NI represents the uninfected macrophage control group.

binding protein (G protein, gamma 8 subunit); myosin IE; proteasome beta 3 subunit; ras homolog gene family, member B; cytochrome c-1; N-acetylglucosamine kinase; TNF receptor-associated protein 1 (TRAP1); and translin. Moreover, a single protein was identified only in infection through *L. amazonensis*: succinate dehydrogenase Ip subunit.

Subsequently, we selected only the differentially expressed proteins identified in both *L. major*- and *L. amazonensis*-infected cells that showed a difference between both countings (dn_p) equal to or greater than two (± 2). A total of 162 proteins were selected according to this criterion. Of these, 40 proteins were preferentially identified in *L. amazonensis* infection (Table S1), while 122 proteins were preferentially identified in *L. major* (Table S2). A total of 15 proteins showed greater differences in expression, with dn_p values varying from +5 to −6 (Table 2). Eleven of the 15 proteins exhibited reduced expression under *L. amazonensis* infection (negative dn_p values), and four proteins exhibited increased expression.

Among the 15 proteins with the greatest differences in expression, six proteins were modulated through *L. amazonensis* infection, two proteins exhibited increased expression of 6-phosphogluconate dehydrogenase (6PGDH) ($dn_p = +5$), and phospholipase D1 (PLD1) ($dn_p = +3$), and four proteins showed reduced expression of PYDCARD ($dn_p = -4$), the programmed cell death protein 5 (PDCD5) ($dn_p = -5$), a protein with the SH3 domain ($dn_p = -6$), and the peripheral benzodiazepine receptor (TSPO) ($dn_p = -4$). In contrast, all seven proteins modulated through *L. major* infection were up-regulated in response to infection (negative dn_p values), light-chain myosin kinase ($dn_p = -5$), coronin-1B ($dn_p = -3$), the regulatory subunit of phosphatase 2 (PP2) ($dn_p = -5$), protein RAB1 ($dn_p = -4$), the subunit VI of cytochrome C oxidase

Table 2
Proteins with greater differences in expression between CBA macrophages infected with *L. major* or *L. amazonensis*.

GI	Protein	Total number of peptides identified						La − Lm
		La		Lm		Macrophages		
		6 h	24 h	6 h	24 h	6 h	24 h	
47059071	Phospholipase D1	9	9	13	5	5	1	**+3**
6679601	RAS-related C3 botulinum substrate 2	3	2	0	0	1	1	**+3**
6754098	Glucuronidase, beta	2	5	0	7	0	0	**+3**
94374018	PRD: similar to 6-phosphogluconate dehydrogenase, decarboxylating isoform 2	3	4	0	1	2	3	**+5**
6753494	Coronin, actin binding protein 1B	2	0	0	6	0	1	**−3**
31560222	PYD and CARD domain containing	0	4	2	7	6	3	**−4**
31981875	Peripheral benzodiazepine receptor (TSPO)	1	1	7	4	4	1	**−4**
6679587	RAB1, member RAS oncogene family	0	0	1	10	3	5	**−4**
9790259	Programmed cell death 5	0	0	2	4	5	3	**−5**
29789016	Myosin, light polypeptide 1	0	1	1	5	0	1	**−5**
31542333	Hypoxia up-regulated 1	1	2	9	8	2	1	**−5**
13385090	Cytochrome c oxidase, subunit VIb polypeptide 1	0	0	1	6	1	4	**−5**
8394027	Alpha isoform of regulatory subunit A, protein phosphatase 2	3	0	11	4	6	3	**−5**
22267440	SH3 domain protein 3	0	0	4	10	2	6	**−6**
13385280	SERPINE1 mRNA binding protein 1	5	7	10	21	12	12	**−6**

Differentially Expressed Proteins by infected macrophages were identified using the MudPIT LC–MS/MS method as described in Materials and methods. The difference between countings (dn_p) equal or greater and equal or lower than two (± 2) for each expressed protein, respectively, highly expressed in *L. amazonensis*- or *L. major*-infected macrophages was determined (La − Lm). *L. amazonensis* (La) and *L. major* (Lm) represent the experimental groups of infected macrophages for each respective species of *Leishmania*. Macrophage represents the uninfected control group.

(Cox6b) ($dn_p = -5$), SERPINE ($dn_p = -6$), and hypoxia-inducible factor-1 alpha (HIF-1α) ($dn_p = -5$) (Table 2).

3.3. Network analysis of differentially expressed proteins following L. amazonensis or L. major infection

To elucidate how the proteins that were differentially expressed in macrophages infected with *L. amazonensis* or *L. major* might be associated with specialized functions or pathways, we employed IPA-Ingenuity Systems® to build models of potential networks and connections among 162 differently expressed proteins.

3.3.1. L. amazonensis and L. major distinctly modulate signaling pathways, cellular organization and lipid metabolism in macrophages

The networks that contain proteins modulated through *Leishmania* infection were involved in the cell signaling and cell death network (score 48), cellular movement and organization network (score 46), and the lipid metabolism and molecular transport network (score 36).

The signaling and cell death network comprised 35 proteins. Of these, 25 proteins were differentially expressed. Seventeen proteins had the highest expression levels under infection with *L. major* (shown in green), and eight proteins were more highly expressed under *L. amazonensis* infection (shown in red) (Fig. 1A).

The second network associated with cell organization and cell movement was comprised of 35 proteins. This network contained 18 proteins with increased expression in macrophages infected with *L. major*, while six proteins exhibited increased expression levels under *L. amazonensis* infection (Fig. 1B).

Finally, the lipid metabolism and molecular transport network was comprised of 35 proteins, with 10 proteins showing increased expression in macrophages infected with *L. major* and three proteins showing increased expression under *L. amazonensis* infection (Fig. 1C).

3.3.2. L. amazonensis and L. major modulate the cellular development pathway differently

To better understand the modulations induced through *L. amazonensis* and *L. major* infections in CBA macrophages, the 15 proteins with the greatest differences in expression between these two types of infection (Table 2) were submitted to IPA-Ingenuity Systems® for biological network modeling. Interestingly, 14 of these 15 proteins were organized into a single cell development network (Fig. 2).

To confirm the differences in protein expression identified through mass spectrometry, Western blot and immunofluorescence analyses were performed. Two randomly selected proteins out of the 15 proteins with marked differences in expression between *L. amazonensis*- and *L. major*-infected cells were selected for validation. As shown in Fig. 3, the myosin light chain expression was two times higher in *L. major*-infected cells compared with *L. amazonensis*-infected cells at 24 h of incubation time. In addition, immunofluorescence staining for HIF-1α also corroborates the results observed from mass spectrometry, with 1.5–1.8 times higher expression levels of this protein identified in *L. major*-infected cells compared to those infected with *L. amazonensis* for 6 and 24 h, respectively (Fig. 4).

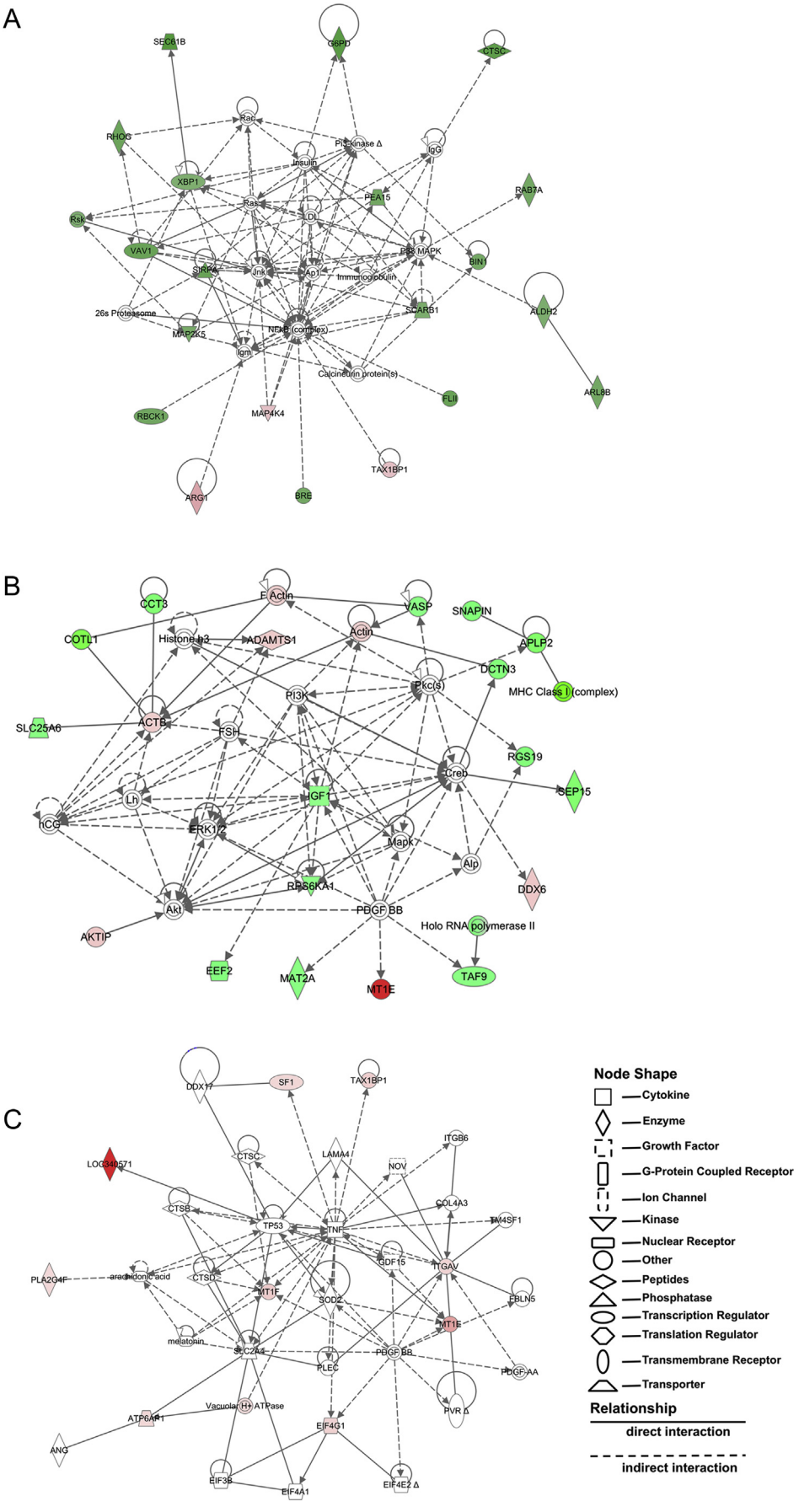
4. Discussion

Large-scale studies have been fundamental to both the identification of new gene or protein functions and networks and achieving a more comprehensive understanding of the responses of cells and/or organisms to a variety of stimuli. In addition, these studies open the possibility to identify novel targets for vaccination and chemotherapeutic strategies. Notably, that among the large-scale methodologies, the analysis of protein expression is essential, as the exclusive identification of messenger RNA using microarray analysis does not always correspond to the levels of protein expression [30].

To date, only one study has evaluated the profiles of proteins expressed in response to *Leishmania*–macrophage infection [22]. These authors demonstrated differences in the protein and lipid composition of parasitophorous vacuole membranes induced through promastigote and amastigote forms of *L. amazonensis*. However, these authors did not identify the host cell proteins and cellular pathways that were modulated through infection. Furthermore, no additional proteomic studies have been performed to identify differences in the protein expression in macrophages of a unique genetic background when challenged with distinct species of *Leishmania*.

The present study comparatively evaluated the protein expression profiles in uninfected CBA macrophages, and cells infected with *L. amazonensis* or *L. major*. Sixty-two proteins were identified only in infected macrophages (Table 1). The fact that these proteins were not identified in uninfected macrophages does not necessarily mean that these proteins were not expressed but does suggest that they might be expressed to a lesser extent. Surprisingly, among the 62 proteins identified in infected macrophages, only one protein was exclusively identified in *L. amazonensis* infection and 10 proteins were exclusively identified in *L. major* infection, while 51 proteins were identified in both macrophages infected with *L. major* and *L. amazonensis*. These results are consistent with recently published studies employing DNA microarray analysis, in which a small number of genes were modulated in macrophages infected with distinct *Leishmania* species [10,31].

Large-scale analytical techniques, whether based on functional genomics or proteomics, generate an enormous amount of data, creating challenges for traditional methods of analysis [32]. Using biological network analysis (IPA software) to organize differentially expressed proteins in potential canonical pathways and functional metabolic networks modulated through infection with *L. amazonensis* or *L. major*. This large-scale data analysis is a powerful technique to evaluate this type dataset and facilitates the formulation of new hypotheses [33].


A
SEC61B
GBP2
CTSC
Rac
RHOG
PI3-kinase Δ
IgG
Insulin
XBP1
RAB7A
Ras
PEA15
Rak
LDL
VAV1
ERK MAPK
BIN1
SIRK4
Jnk
Ap1
Immunoglobulin
ALDH2
26s Proteasome
SCARB1
MAP2K5
NFkB (complex)
Igm
Calcineurin protein(s)
FLII
ARL8B
RBCK1
MAP4K4
TAX1BP1
ARG1
BRE
B
F Actin
CCT3
VASP
SNAPIN
COTL1
Histone h3
ADAMTS1
Actin
APLP2
DCTN3
Pkc(s)
MHC Class I (complex)
PI3K
SLC25A6
ACTB
FSH
RGS19
Creb
SEP15
Lh
IGF1
hCG
ERK1/2
Mapk
Alp
RPS6KA1
DDX6
Akt
PDGF BB
Holo RNA polymerase II
AKTIP
EEF2
MAT2A
MT1E
TAF9
C
DDX17
SF1
TAX1BP1
ITGB6
CTSC
LAMA4
LOC340571
NOV
COL4A3
CTSB
TP53
TNF
TM4SF1
GDF15
ITGAV
PLA2G4F
arachidonic acid
CTSD
MT1F
SOD2
FBLN5
MT1E
melatonin
SLC2A4
PBGF BB
PLEC
PDGF-AA
PVR Δ
Vacuolar H+ ATPase
ATP6AP1
EIF4G1
ANG
EIF3B
EIF4A1
EIF4E2 Δ
Node Shape
Cytokine
Enzyme
Growth Factor
G-Protein Coupled Receptor
Ion Channel
Kinase
Nuclear Receptor
Other
Peptides
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Relationship
direct interaction
indirect interaction

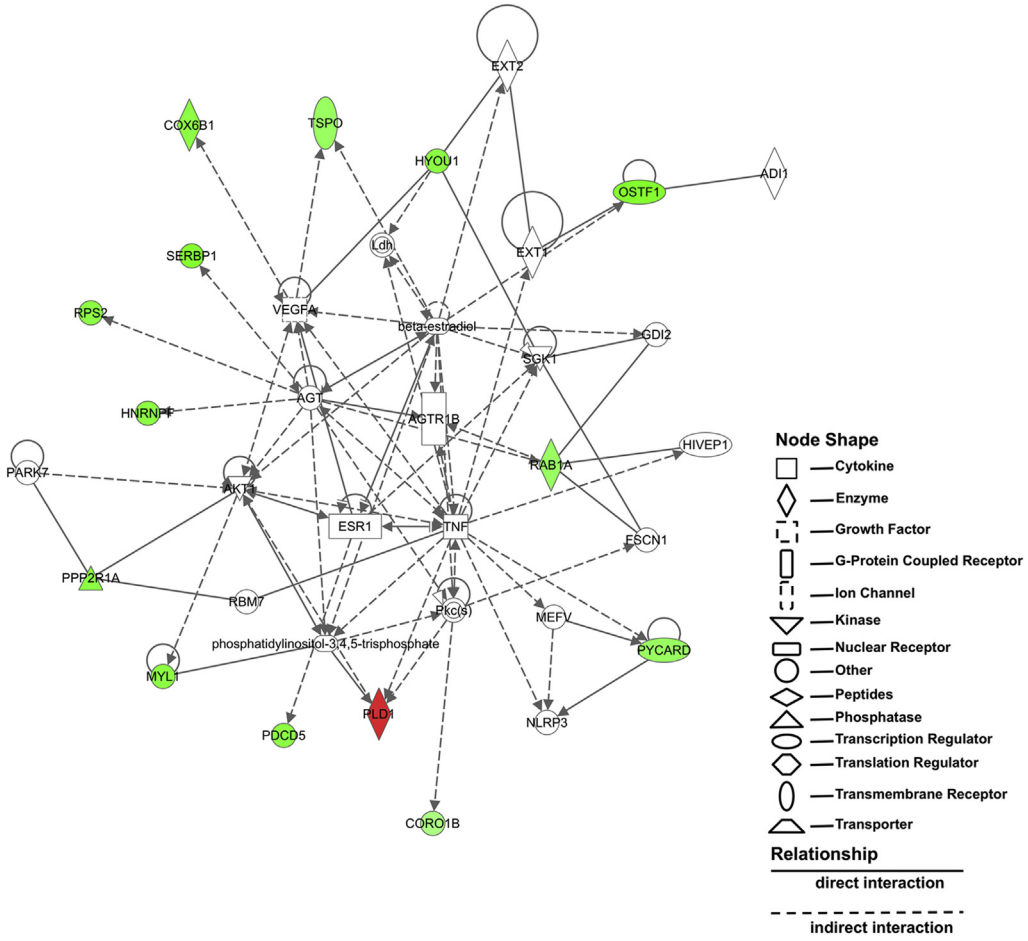


Fig. 2. Cellular development network modeled using proteins with the greatest differences in expression between *L. amazonensis*- and *L. major*-infected macrophages. CBA macrophages were cultured separately, infected with *L. amazonensis* or *L. major*, and processed for proteomics analysis as described in the Materials and methods section. The 15 proteins with the most significant differences in expression among *L. amazonensis*- and *L. major*-infected cells were analyzed and modeled in the cellular development network using software v8.8 (IPA-Ingenuity Systems®). As in Fig. 1, the above network is displayed as a series of nodes (proteins) and edges (or lines, corresponding to biological relationships between nodes). The nodes are displayed using shapes, as indicated in the key. The nodes indicated in green were highly expressed *in L. major*-infected macrophages, and those indicated in red were highly expressed in *L. amazonensis*-infected macrophages. Unmarked nodes were added through IPA®, reflecting a high degree of probability of involvement in a given network. The node color intensity is an indication of the degree of the up- or down-regulation of the proteins observed in the biological network analysis from both *L. amazonensis*- and *L. major*-infected macrophages in response to infection. The solid lines denote direct interactions, whereas the dotted lines represent indirect interactions between the proteins represented in this network.

Using a qualitative approach, we identified a total of 162 differentially expressed proteins in infected cells. The biological network analysis of these proteins revealed that cellular processes were modulated differently in macrophages depending on the strain of infecting parasite, i.e., *L. amazonensis* and *L. major* differently modulated the signaling pathways, cellular organization, and lipid metabolism in macrophages (Fig. 1). Different modulation profiles of host proteins might induce distinct responses in macrophages, leading to susceptibility or control of the disease. The most significant proteins and their potential biological importance are discussed.

Fig. 1. Networks built using 162 differentially expressed proteins by *L. amazonensis*- and *L. major*-infected CBA macrophages. CBA macrophages were cultured separately, infected with *L. amazonensis* or *L. major*, and processed for proteomics analysis as described in the Materials and methods section. The cell signaling and cell death network (A), cell organization and cell movement network (B), and the lipid metabolism network (C) were modeled using IPA software v8.8 (Ingenuity Systems®). The above networks are displayed as a series of nodes (proteins) and edges (or lines, corresponding to biological relationships between nodes). The nodes are displayed using shapes that represent the functional class of the proteins, as indicated in the key. The nodes indicated in green were highly expressed in *L. major*-infected macrophages compared to *L. amazonensis*-infected macrophages, and those indicated in red were highly expressed *in L. amazonensis*-infected macrophages compared to *L. major*-infected macrophages. The unmarked nodes were not identified in our samples; however, IPA® added these nodes to the networks due to their high probability of involvement in a given network. The node color intensity is an indication of the degree of the up- or down-regulation of the proteins observed in the biological network analysis from *L. amazonensis*-infected cells compared with *L. major*-infected cells. The solid lines denote direct interactions, whereas the dotted lines represent indirect interactions between the proteins represented in these networks.

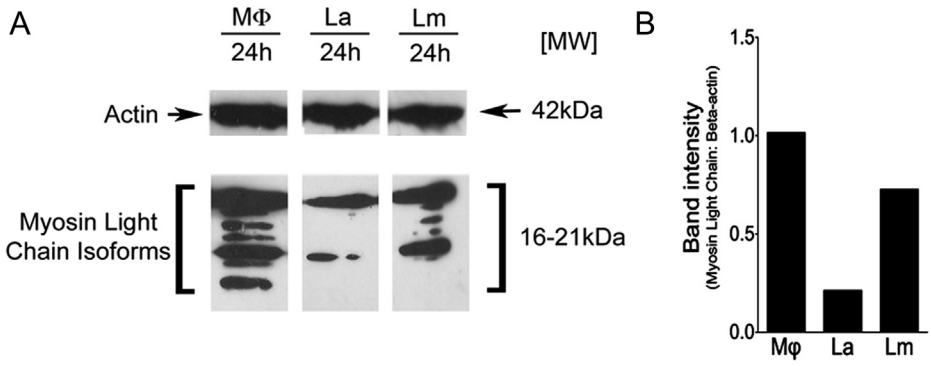


Fig. 3. Western blot confirmation of differences in myosin light chain expression. The extracts of uninfected control macrophages (MΦ) or cells infected with *L. amazonensis* (La) or *L. major* (Lm) were obtained after 24 h of incubation time, and 85 μg of macrophage lysates were loaded onto single lanes. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as described in the Materials and methods section and transferred to nitrocellulose membrane. The proteins were then immunostained using anti-myosin light chain or anti-actin, followed by incubation with anti-rabbit secondary antibody. Western blot images were acquired on a scanner station and analyzed for mean intensity above background. (A) The bands with molecular weights between 16 and 21 kDa represent myosin light chain isoforms, and bands of approximately 42 kDa correspond to actin. (B) A graph of the mean band intensity of myosin light chain isoforms associated with the intensity of actin bands from each extract analyzed using ImageJ 1.45. The results are representative data from one of two independent experiments.

By analyzing the functions of the 15 proteins with elevated degrees of differential expression under infection with the two *Leishmania* strains analyzed (Table 2), we observed that 14 out of the 15 proteins were surprisingly organized within a single network involved in cellular development (Fig. 2). This finding suggests that these proteins should be involved in directing the response to *Leishmania* infection, and some proteins were of particular interest. Among these 15 proteins, four proteins showed increased expression under *L. amazonensis* infection. One of the proteins with higher expression in *L. amazonensis* infection was PLD1. PLD1 acts on phosphatidylcholine, releasing phosphatidic acid [34]. PLD1 has been associated with the recruitment of additional membrane for the formation of nascent phagosomes and the maintenance of phagosomes through fusion with endocytic vesicles [35]. It is possible that PLD1 contributes to the formation and

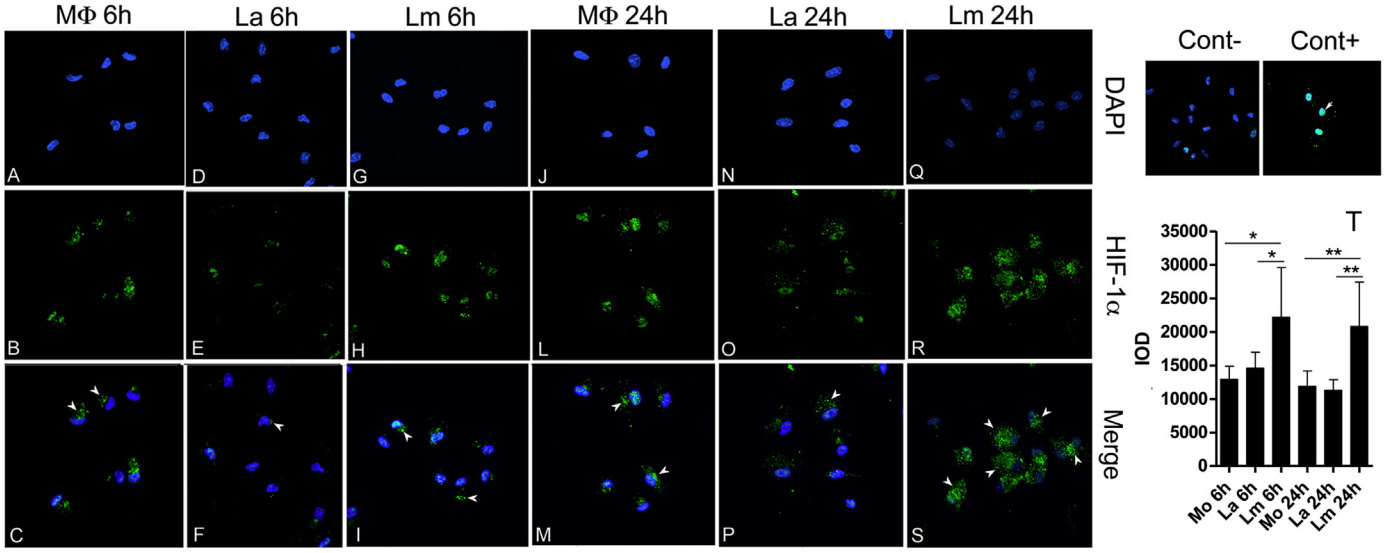


Fig. 4. Expression and distribution of HIF-1α in macrophages infected by *L. amazonensis* or *L. major*. Macrophages were infected for 12 h and washed to remove non-internalized parasites. The cells were fixed with 4% paraformaldehyde at 6 and 24 h post-infection. Fixed macrophage cultures were incubated with polyclonal antibody against HIF-1α, followed by incubation with anti-rabbit conjugated to IgG-Alexa 488 (green). The nuclei were indicated with 4′,6-diamidino-2-phenylindole (DAPI) (blue) staining. All images were acquired using a confocal microscope (1000×), and at least five fields were randomly captured for each experimental group: uninfected macrophages at 6 (A, B, C) and 24 h (J, L, M), *L. amazonensis*-infected macrophages at 6 (D, E, F) and 24 h (N, O, P), and *L. major*-infected macrophages at 6 (G, H, I) and 24 h (Q, R, S) after infection. The arrowheads represent HIF-1α distribution in uninfected and *Leishmania*-infected cells. Uninfected cells treated with cobalt chloride were used as positive control of HIF-1α expression (Cont+), which is predominantly concentrated in cell nuclei (arrow). Infected cell groups labeled with the immunoglobulin isotype, instead of the anti-HIF-1α antibody, were used as an immunolabeling negative control (Cont−). In the graph (T), the bars represent the means ± SD of the IOD of each group of cells labeled with anti-HIF-1α (one-way ANOVA, Newman–Keuls Multiple Comparison Test; $*p < 0.05$; $**p < 0.005$). The results are representative data from one of two independent experiments.

maintenance of large parasitophorous vacuoles, characteristic of intracellular infection with *L. amazonensis* but not *L. major* [36]. Moreover, it is possible that PLD1 contributes to lipid-mediated signaling, as phosphatidic acid can be converted into diacylglycerol through the action of phosphoesterase [37].

With significant levels of differential expression, 13 out of the 15 proteins were down-modulated in *L. amazonensis* or up-modulated in *L. major*-infected macrophages, including coronin 1B, cox6B, HNRPF, HIF-1α, OSTF1, PDCD5, PP2, PYCARD, RAB1, RPS2, Serpin, PBR, and myosin light chain. The myosin light chain is involved in the phagocytosis of particles and maintenance of tight vacuoles around phagocytosed particles [38]. Additionally, it has been demonstrated that the phagocytosis of particles via CR3 is reduced when myosin light chain kinase is blocked [39]. The internalization of several species of *Leishmania* (*Leishmania donovani*, *L. major*, *Leishmania mexicana*, and *Leishmania braziliensis*) is initiated through the interaction of these parasites with receptors, including CR3, FcγR and the mannose receptor [40–44]. It has been previously demonstrated that parasitophorous vacuoles induced through *L. major* remain small and individualized throughout the maturation process [36]. However, *L. amazonensis* induces large parasitophorous vacuoles that typically contain more than one *Leishmania* parasite, in addition to other particles [36,45]. Based on the identification of myosin light chain expression under *L. major* infection (Fig. 3), we hypothesized the involvement of myosin light chain in the maintenance of small, individual vacuoles during infection with *L. major*, and its lower expression could be associated with the large size of the vacuoles harboring *L. amazonensis*.

Our group has previously demonstrated that CBA macrophages infected with *L. major* express twice as much TNF-α as these same macrophages infected with *L. amazonensis* in response to stimulation through IFN-γ. Furthermore, the addition of TNF-α to cells infected with *L. major* promotes increased parasite destruction, reinforcing the protective role of TNF-α in this particular murine infection model [11]. Currently available proteomic data facilitate the identification of TRAP1 and HIF-1α in macrophages infected with *L. major* and the identification of Serpin and PYDCARD proteins, which exhibit reduced expression levels under *L. amazonensis* infection. The HIF-1α protein is a regulator of the response to hypoxia [46], but it has been shown that NO and TNF-α stabilize the expression of HIF-1α in macrophages activated under normoxic conditions [47]. In the present study, HIF-1α was highly expressed in *L. major*-infected macrophages (Table 2 and Fig. 4). TRAP1 is associated with the maintenance of cellular viability under H_2O_2 -induced oxidative stress [48] in addition to Serpin, another protein induced through TNF-α, which participates in the cascade of inflammation together with IL-1α [49]. The PYDCARD adapter protein is involved in regulating the activation of NFκ-B and the regulation of caspases [50], which is primarily achieved through interactions with members of the family of receptors for TNF-α. In summary, considering the data obtained in the present study, together with findings from previous studies, there is abundant evidence to hypothesize that the response to TNF-α provides favorable conditions for the control of infection through *L. major*, in contrast to *L. amazonensis*. The potential involvement of TNF-α during infection with *L. major* would likely be associated with the induction of TRAP1 and HIF-1α, thereby regulating the effects of increased peroxide production, including H_2O_2. Previously, the authors demonstrated that this leishmanicidal molecule has been expressed at higher levels through *L. major*-infected macrophages compared to those infected with *L. amazonensis* [11].

In conclusion, the present study represents an initial attempt at making direct comparisons between the global protein expression profiles of mouse macrophages infected with two distinct species of *Leishmania*. Previous studies have demonstrated that CBA macrophages can control infection with *L. major* while being permissive to *L. amazonensis*, which suggests that these cells might play a central role in determining the outcome of *Leishmania* infection. Using a proteomics approach, we showed for the first time that distinct parasite species modulate proteins involved in cell metabolism during the initial events of *Leishmania*–macrophage interactions. The proteins involved in host cell metabolism influence the course of infection [21]. The set of proteins with dn_p identified herein might be responsible for the distinct phenotypic macrophage responses observed, leading to infection control under *L. major* and parasite survival under *L. amazonensis*. We have identified for the first time proteins that are differentially modulated between *L. amazonensis* and *L. major* infection using a qualitative approach. These proteins are being investigated in further detail to increase our understanding of the macrophage response to *Leishmania* infection. Nevertheless, we plan to improve our analyses in the future using a quantitative approach based on the SILAC technique. Because cellular processes and proteins modulated through *Leishmania* infection were involved in host cell metabolism, we propose that future studies are required to obtain evidence of whether these differentially expressed proteins can be used as novel markers and targets for the control of *Leishmania* infection.

Acknowledgments

The authors would like to thank Andris K. Walter for providing English revision and consulting services. Additionally, the authors acknowledge Dr. Ricardo Ribeiro dos Santos, Carine Azevedo, and Bruno Solano from the Center of Biotechnology and Cell Therapy at São Rafael Hospital (Salvador-Bahia/Brazil) for support to acquire fluorescence images. Finally, the authors would also like to thank Dr. Lucile Flöeter-Winter for recharacterizing *L. amazonensis* parasites.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micinf.2013.04.005.

References

[1] Z.A. Andrade, S.G. Reed, S.B. Roters, M. Sadigursky, Immunopathology of experimental cutaneous leishmaniasis, Am. J. Pathol. 114 (1984) 137–148.

[2] V. Lemos de Souza, J. Ascencao Souza, T.M. Correia Silva, P. Sampaio Tavares Veras, L.A. Rodrigues de-Freitas, Different *Leishmania* species determine distinct profiles of immune and histopathological responses in CBA mice, Microbes Infect. 2 (2000) 1807–1815.

[3] J.C. Antoine, E. Prina, C. Jouanne, P. Bongrand, Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH, Infect. Immun. 58 (1990) 779–787.

[4] C.A. Nacy, A.H. Fortier, M.G. Pappas, R.R. Henry, Susceptibility of inbred mice to *Leishmania* tropica infection: correlation of susceptibility with *in vitro* defective macrophage microbicidal activities, Cell. Immunol. 77 (1983) 298–307.

[5] C. Bogdan, H. Moll, W. Solbach, M. Rollinghoff, Tumor necrosis factor-alpha in combination with interferon-gamma, but not with interleukin 4 activates murine macrophages for elimination of *Leishmania major* amastigotes, Eur. J. Immunol. 20 (1990) 1131–1135.

[6] D. Nandan, R. Lo, N.E. Reiner, Activation of phosphotyrosine phosphatase activity attenuates mitogen-activated protein kinase signaling and inhibits c-FOS and nitric oxide synthase expression in macrophages infected with *Leishmania donovani*, Infect. Immun. 67 (1999) 4055–4063.

[7] J.L. Stafford, N.F. Neumann, M. Belosevic, Macrophage-mediated innate host defense against protozoan parasites, Crit. Rev. Microbiol. 28 (2002) 187–248.

[8] J. Mauel, Y. Buchmuller-Rouiller, Effect of lipopolysaccharide on intracellular killing of *Leishmania enriettii* and correlation with macrophage oxidative metabolism, Eur. J. Immunol. 17 (1987) 203–208.

[9] M. Olivier, K.G. Baimbridge, N.E. Reiner, Stimulus–response coupling in monocytes infected with *Leishmania*. Attenuation of calcium transients is related to defective agonist-induced accumulation of inositol phosphates, J. Immunol. 148 (1992) 1188–1196.

[10] C.M. Probst, R.A. Silva, J.P. Menezes, T.F. Almeida, I.N. Gomes, A.C. Dallabona, L.S. Ozaki, G.A. Buck, D.P. Pavoni, M.A. Krieger, P.S. Veras, A comparison of two distinct murine macrophage gene expression profiles in response to *Leishmania amazonensis* infection, BMC Microbiol. 12 (2012) 22.

[11] I.N. Gomes, A.F. Calabrich, S. Tavares Rda, J. Wietzerbin, L.A. de Freitas, P.S. Veras, Differential properties of CBA/J mononuclear phagocytes recovered from an inflammatory site and probed with two different species of *Leishmania*, Microbes Infect. 5 (2003) 251–260.

[12] S.G. Kumar, A.K. Venugopal, A. Mahadevan, S. Renuse, G.H. Harsha, N.A. Sahasrabuddhe, H. Pawar, R. Sharma, P. Kumar, S. Rajagopalan, K. Waddell, Y.L. Ramachandra, P. Satishchandra, R. Chaerkady, K.T. Prasad, S.K. Shankar, A. Pandey, Quantitative proteomics for identifying biomarkers for tuberculous meningitis, Clin. Proteomics 9 (2012) 12.

[13] R.A. Lock, S.J. Cordwell, G.W. Coombs, B.J. Walsh, G.M. Forbes, Proteome analysis of *Helicobacter pylori*: major proteins of type strain NCTC 11637, Pathology 33 (2001) 365–374.

[14] M.A. Dea-Ayuela, L. Ordonez-Gutierrez, F. Bolas-Fernandez, Changes in the proteome and infectivity of *Leishmania infantum* induced by *in vitro* exposure to a nitric oxide donor, Int. J. Med. Microbiol. 299 (2009) 221–232.

[15] J. Drummelsmith, V. Brochu, I. Girard, N. Messier, M. Ouellette, Proteome mapping of the protozoan parasite *Leishmania* and application to the study of drug targets and resistance mechanisms, Mol. Cell. Proteomics 2 (2003) 146–155.

[16] M. Forgber, R. Basu, K. Roychoudhury, S. Theinert, S. Roy, S. Sundar, P. Walden, Mapping the antigenicity of the parasites in *Leishmania donovani* infection by proteome serology, PLoS ONE 1 (2006) e40.

[17] S.K. Gupta, B.S. Sisodia, S. Sinha, K. Hajela, S. Naik, A.K. Shasany, A. Dube, Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of *Leishmania donovani* promastigotes, Proteomics 7 (2007) 816–823.

[18] D. Paape, C. Lippuner, M. Schmid, R. Ackermann, M.E. Barrios-Llerena, U. Zimny-Arndt, V. Brinkmann, B. Arndt, K.P. Pleissner, P.R. Jungblut, T. Aebischer, Transgenic, fluorescent *Leishmania mexicana* allow direct analysis of the proteome of intracellular amastigotes, Mol. Cell. Proteomics 7 (2008) 1688–1701.

[19] D. Rosenzweig, D. Smith, F. Opperdoes, S. Stern, R.W. Olafson, D. Zilberstein, Retooling *Leishmania* metabolism: from sand fly gut to human macrophage, FASEB J. 22 (2008) 590–602.

[20] G. Kumar, P. Sharma, G. Rathore, D. Bisht, U. Sengupta, Proteomic analysis of outer membrane proteins of *Edwardsiella tarda*, J. Appl. Microbiol. 108 (2010) 2214–2221.

[21] N.F. Luz, B.B. Andrade, D.F. Feijo, T. Araujo-Santos, G.Q. Carvalho, D. Andrade, D.R. Abanades, E.V. Melo, A.M. Silva, C.I. Brodskyn, M. Barral-Netto, A. Barral, R.P. Soares, R.P. Almeida, M.T. Bozza, V.M. Borges, Heme oxygenase-1 promotes the persistence of *Leishmania chagasi* infection, J. Immunol. 188 (2012) 4460–4467.

[22] C. Henriques, G.C. Atella, V.L. Bonilha, W. de Souza, Biochemical analysis of proteins and lipids found in parasitophorous vacuoles containing *Leishmania amazonensis*, Parasitol. Res. 89 (2003) 123–133.

[23] K. Hassani, E. Antoniak, A. Jardim, M. Olivier, Temperature-induced protein secretion by *Leishmania mexicana* modulates macrophage signalling and function, PLoS ONE 6 (2011) e18724.

[24] T.M. Castilho, L.M. Camargo, D. McMahon-Pratt, J.J. Shaw, L.M. Floeter-Winter, A real-time polymerase chain reaction assay for the identification and quantification of American *Leishmania* species on the basis of glucose-6-phosphate dehydrogenase, Am. J. Trop. Med. Hyg. 78 (2008) 122–132.

[25] D. Fenyo, Identifying the proteome: software tools, Curr. Opin. Biotechnol. 11 (2000) 391–395.

[26] R.G. Sadygov, D. Cociorva, J.R. Yates 3rd, Large-scale database searching using tandem mass spectra: looking up the answer in the back of the book, Nat. Methods 1 (2004) 195–202.

[27] M.P. Washburn, D. Wolters, J.R. Yates 3rd, Large-scale analysis of the yeast proteome by multidimensional protein identification technology, Nat. Biotechnol. 19 (2001) 242–247.

[28] L. Liu, J. Zhou, Y. Wang, R.J. Mason, C.J. Funk, Y. Du, Proteome alterations in primary human alveolar macrophages in response to influenza A virus infection, J. Proteome Res. 11 (2012) 4091–4101.

[29] Q. Lu, J. Bai, L. Zhang, J. Liu, Z. Jiang, J.J. Michal, Q. He, P. Jiang, Two-dimensional liquid chromatography–tandem mass spectrometry coupled with isobaric tags for relative and absolute quantification (iTRAQ) labeling approach revealed first proteome profiles of pulmonary alveolar macrophages infected with porcine reproductive and respiratory syndrome virus, J. Proteome Res. 11 (2012) 2890–2903.

[30] S.P. Gygi, Y. Rochon, B.R. Franza, R. Aebersold, Correlation between protein and mRNA abundance in yeast, Mol. Cell. Biol. 19 (1999) 1720–1730.

[31] S. Zhang, C.C. Kim, S. Batra, J.H. McKerrow, P. Loke, Delineation of diverse macrophage activation programs in response to intracellular parasites and cytokines, PLoS Negl. Trop. Dis. 4 (2010) e648.

[32] N. Gehlenborg, S.I. O'Donoghue, N.S. Baliga, A. Goesmann, M.A. Hibbs, H. Kitano, O. Kohlbacher, H. Neuweger, R. Schneider, D. Tenenbaum, A.C. Gavin, Visualization of omics data for systems biology, Nat. Methods 7 (2010) S56–S68.

[33] R.F. Deighton, L.E. Kerr, D.M. Short, M. Allerhand, I.R. Whittle, J. McCulloch, Network generation enhances interpretation of proteomic data from induced apoptosis, Proteomics 10 (2010) 1307–1315.

[34] L. Wang, R. Cummings, P. Usatyuk, A. Morris, K. Irani, V. Natarajan, Involvement of phospholipases D1 and D2 in sphingosine 1-phosphate-induced ERK (extracellular-signal-regulated kinase) activation and interleukin-8 secretion in human bronchial epithelial cells, Biochem. J. 367 (2002) 751–760.

[35] M. Corrotte, S. Chasserot-Golaz, P. Huang, G. Du, N.T. Ktistakis, M.A. Frohman, N. Vitale, M.F. Bader, N.J. Grant, Dynamics and function of phospholipase D and phosphatidic acid during phagocytosis, Traffic 7 (2006) 365–377.

[36] J.C. Antoine, E. Prina, T. Lang, N. Courret, The biogenesis and properties of the parasitophorous vacuoles that harbour *Leishmania* in murine macrophages, Trends Microbiol. 6 (1998) 392–401.

[37] R. Cummings, N. Parinandi, L. Wang, P. Usatyuk, V. Natarajan, Phospholipase D/phosphatidic acid signal transduction: role and physiological significance in lung, Mol. Cell. Biochem. 234–235 (2002) 99–109.

[38] N. Araki, T. Hatae, A. Furukawa, J.A. Swanson, Phosphoinositide-3-kinase-independent contractile activities associated with Fcgamma-receptor-mediated phagocytosis and macropinocytosis in macrophages, J. Cell Sci. 116 (2003) 247–257.

[39] A. Wiedemann, J.C. Patel, J. Lim, A. Tsun, Y. van Kooyk, E. Caron, Two distinct cytoplasmic regions of the beta2 integrin chain regulate RhoA function during phagocytosis, J. Cell Biol. 172 (2006) 1069–1079.

[40] J.M. Blackwell, R.A. Ezekowitz, M.B. Roberts, J.Y. Channon, R.B. Sim, S. Gordon, Macrophage complement and lectin-like receptors bind *Leishmania* in the absence of serum, J. Exp. Med. 162 (1985) 324–331.

[41] R. Chakraborty, P. Chakraborty, M.K. Basu, Macrophage mannosyl fucosyl receptor: its role in invasion of virulent and avirulent *L. donovani* promastigotes, Biosci. Rep. 18 (1998) 129–142.

[42] R.A. Guy, M. Belosevic, Comparison of receptors required for entry of *Leishmania major* amastigotes into macrophages, Infect. Immun. 61 (1993) 1553–1558.

[43] D.M. Mosser, P.J. Edelson, The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes, J. Immunol. 135 (1985) 2785–2789.

[44] D.G. Russell, P. Talamas-Rohana, J. Zelechowski, Antibodies raised against synthetic peptides from the Arg–Gly–Asp-containing region of the *Leishmania* surface protein gp63 cross-react with human C3 and interfere with gp63-mediated binding to macrophages, Infect. Immun. 57 (1989) 630–632.

[45] P.S. Veras, C. de Chastellier, M. Rabinovitch, Transfer of zymosan (yeast cell walls) to the parasitophorous vacuoles of macrophages infected with *Leishmania amazonensis*, J. Exp. Med. 176 (1992) 639–646.

[46] G.L. Semenza, L.A. Shimoda, N.R. Prabhakar, Regulation of gene expression by HIF-1, Novartis Found. Symp. 272 (2006) 2–8. discussion 8–14, 33–36.

[47] J. Zhou, J. Fandrey, J. Schumann, G. Tiegs, B. Brune, NO and TNF-alpha released from activated macrophages stabilize HIF-1alpha in resting tubular LLC-PK1 cells, Am. J. Physiol. Cell Physiol. 284 (2003) C439–C446.

[48] J.W. Pridgeon, J.A. Olzmann, L.S. Chin, L. Li, PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1, PLoS Biol. 5 (2007) e172.

[49] S. Mishra, T. Fujita, V.N. Lama, D. Nam, H. Liao, M. Okada, K. Minamoto, Y. Yoshikawa, H. Harada, D.J. Pinsky, Carbon monoxide rescues ischemic lungs by interrupting MAPK-driven expression of early growth response 1 gene and its downstream target genes, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 5191–5196.

[50] J.C. Reed, K. Doctor, A. Rojas, J.M. Zapata, C. Stehlik, L. Fiorentino, J. Damiano, W. Roth, S. Matsuzawa, R. Newman, S. Takayama, H. Marusawa, F. Xu, G. Salvesen, A. Godzik, Comparative analysis of apoptosis and inflammation genes of mice and humans, Genome Res. 13 (2003) 1376–1388.