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SILAC-based quantitative proteomics reveals pleiotropic, phenotypic modulation in primary murine macrophages infected with the protozoan pathogen *Leishmania donovani*

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37 ABSTRACT

38 Leishmaniasis are major vector-borne tropical diseases responsible for great human morbidity
 39 and mortality, caused by protozoan, trypanosomatid parasites of the genus *Leishmania*. In the
 40 mammalian host parasites survive and multiply within mononuclear phagocytes, especially
 41 macrophages. However, the underlying mechanisms by which *Leishmania* spp affect their
 42 host, are not fully understood. Herein, proteomic alterations of primary bone marrow-
 43 derived, BALB/c macrophages are documented after 72 h of infection with *Leishmania*
 44 *donovani* insect-stage promastigotes, with the use of a SILAC-based, quantitative proteomics
 45 approach. The protocol was optimised by combining strong anion exchange and gel
 46 electrophoresis fractionation that displayed similar depth of analysis (>5500 proteins). Our
 47 analyses revealed 86 differentially modulated proteins (35 showing increased and 51
 48 decreased abundance) in response to *Leishmania donovani* infection. The proteomics results
 49 were validated by analysing the abundance of selected proteins. Intracellular *Leishmania*
 50 *donovani* infection led to changes in various host cell biological processes, including primary
 51 metabolism and catabolic process, with a significant enrichment in lysosomal organisation.
 52 Overall, our analysis allows new technical insight into the challenges of quantitative
 53 proteomics applied on primary cells, and establishes the first proteome of *bona fide* primary
 54 macrophages infected *ex vivo* with *Leishmania donovani*, revealing new mechanisms acting at
 55 the host/pathogen interface.

56

57 **Keywords:** quantitative proteomics, bone marrow-derived macrophages, *Leishmania*
 58 *donovani*, SILAC, LC-MS/MS

59

60 INTRODUCTION

61 The leishmaniasis include a group of diseases caused by more than 21 protozoan species of
 62 the trypanosomatid genus *Leishmania*, which are transmitted by the bite of phlebotomine
 63 sandflies [1]. These diseases, prevalent in 98 countries, have different clinical outcomes that
 64 range from localised skin ulcers (cutaneous leishmaniasis) to lethal systemic disease (visceral

65 leishmaniasis, VL)[2]. VL is the most serious form of the disease, with 200 000 to 400 000
66 new cases documented every year [2] and a lethal outcome if left untreated. VL is caused
67 mainly by strains of the *Leishmania (L.) donovani* complex and is characterised by systemic
68 symptoms, such as fever and weight loss [3].

69 All *Leishmania* parasites share a digenetic life cycle and live either as extracellular,
70 motile promastigotes within the midgut of a sand fly vector, or as intracellular, non-motile
71 amastigotes in professional phagocytes, with macrophage phagolysosomes representing the
72 primary niche for proliferation [4]. These phagocytic mononuclear cells carry important,
73 immuno-regulatory functions and accordingly can differentiate into phenotypically distinct
74 subtypes [5], including pro-inflammatory M1 cells with microbicidal properties and
75 alternatively activated, anti-inflammatory M2 cells involved in tissue repair (reviewed in [6]).
76 These M1 and M2 phenotypes represent the extremes of a continuous spectrum of
77 polarisation states [7]. Even though both the initial *Leishmania* infection through the bite of
78 infected sand flies and the later acute stages of the disease are characterised by inflammation
79 of the infected tissue [8, 9], *Leishmania* thrives in these environments by efficiently
80 dampening the pro-inflammatory response of its infected host cell, possibly by exploiting
81 developmental programs underlying macrophage phenotypic plasticity [10].

82 The prevention of *Leishmania* clearance and persistent infection of macrophages is
83 governed by the expression of specific parasite virulence factors, such as highly abundant
84 parasite surface glycolipids [lipophosphoglycan (LPG) and glycoinositol phospholipids
85 (GIPLs)], or the GPI anchored metalloprotease, GP63 [11-15]. These and other factors have
86 been shown to remodel the macrophage phenotype to establish permissive conditions for
87 *Leishmania* intracellular survival. Initially, following entry into the macrophage, *L. donovani*
88 promastigotes delay phagosomal/lysosomal fusion and phagosome maturation [16-18], and
89 subsequently modify host cell signaling and metabolic processes, inhibit apoptosis and
90 suppress antigen presentation (reviewed in [10]).

91 However, this ideal concept of ‘macrophage deactivation’ upon *Leishmania* infection
92 is clearly an oversimplification, as the macrophage response to infection under clinically more

relevant conditions can depend on many parameters, including the host cell differentiation state, the tissue context, and parasite species or even strain [19, 20]. Systems-level, ‘omics’ approaches revealed the complex interface between the parasite and the macrophage and shed important new light on the host cell response to *Leishmania* infection, particularly at the transcript level [8, 9, 19, 21-27]. However, transcript abundance does not always correlate with protein levels and thus may not give an accurate picture of the cellular phenotype. Proteome analyses certainly allows for a more relevant functional insight – but the few proteomics screens performed on *Leishmania* infected cells used either immortalised, macrophage-like cell lines [28] known to be phenotypically and functionally different from *bona fide* macrophages [29-32], or a semi-quantitative approach based on spectral counting [20] that lacks quantitative performance [33]. Here we overcame these limitations and for the first time quantified proteomic changes in primary, bone marrow-derived macrophages (BMDMs) after 72 h of infection with virulent *L. donovani* promastigotes using stable isotope labeling by amino acids in cell culture (SILAC). SILAC, a technique that relies on the metabolic replacement of a given natural amino acid (referred to as 'light') with a non-radioactive, stable isotope (referred to as 'heavy') [34], is considered one of the most accurate quantitative approaches when using cell culture systems to study dynamics of biological processes [33, 34]. Our analysis provides new insight into *L. donovani*-induced phenotypic changes of primary macrophages and mechanisms of intracellular parasite survival acting at the host/pathogen interface.

113

114 MATERIALS AND METHODS

115 *Ethics statement*

All animal experiments complied with the ARRIVE guidelines and were carried out according to the EU Directive 2010/63/EU for animal experiments. All animals were housed in an A3 animal facility of Institut Pasteur. Housing and conditions were in compliance with the protocol approved by the Pasteur Institute ethical committee for animal experimentation (CETEA: C2TEA 89) and procedures were conducted in agreement with the project licenses

HA0005 and #10587, issued by the Pasteur Institute CETEA and the Ministère de l'Enseignement Supérieur de la Recherche et de l'Innovation (MESRI) respectively.

123

Leishmania culture, BMDM differentiation and infection

L. donovani strain 1S2D (MHOM/SD/62/1S-CL2D obtained from Henry Murray (Weill Cornell Medical College, New York, USA) was maintained in female RjHan:AURA golden Syrian hamsters between four and six weeks of age (Janvier Labs) by serial *in vivo* passaging. Amastigotes were purified from hamster spleens as described [35] and differentiated into promastigotes in culture that were maintained for no more than 5 *in vitro* passages. Parasites were cultivated at 26°C in fully supplemented M199 medium (10% FCS, 25 mM HEPES, 4 mM NaHCO₃, 1 mM glutamine, 1 x RPMI 1640 vitamin mix, 0.2 μM folic acid, 100 μM adenine, 7.6 mM hemin, 8 μM bipterin, 50 U ml⁻¹ of penicillin and 50 μg ml⁻¹ of streptomycin, pH7.4).

Bone marrow cell suspensions were recovered from tibias and femurs of BALB/c mice (Janvier) and suspended in bacteriologic Petri dishes (Greiner, Germany) at a concentration of 1.35x10⁶ ml⁻¹ in complete medium i.e. RPMI 1640 supplemented with 50 μM β-mercaptoethanol, 15% heat-inactivated fetal calf serum (GIBCO), 50 U ml⁻¹ of penicillin and 50 μg ml⁻¹ of streptomycin and 75 ng mL⁻¹ of macrophage Colony Stimulating Factor-1 (mCSF-1) (Immunotools). Cells were incubated at 37°C and 5% CO₂. Three days after plating, 0.4 volume of fresh complete medium was added to the cells. Six days later, adherent bone marrow-derived macrophages (BMDMs) were washed with Dulbecco's phosphate buffered solution (PBS). Cells were detached by incubation with Dulbecco's PBS without Ca²⁺ and Mg²⁺ (Biochrom AG, Berlin, Germany) containing 25 mM EDTA for 30min at 37°C.

For *Leishmania* infection, recovered BMDMs were plated in flat-bottom 6-well plates (Tanner, Switzerland) at a density of 4x10⁶ cells per well and were maintained in complete medium. Six hours after plating, macrophages were infected with 4X10⁷ *L. donovani* stationary phase promastigotes (multiplicity of infection of 10:1, *in vitro* passage

149 2 after differentiation from splenic amastigotes) for 4 h and incubated at 37°C in a 5%
150 CO₂. Following infection, macrophages were washed four times with PBS to remove free
151 promastigotes. Infected and non-infected macrophages were then maintained for 72 h in
152 complete medium containing only 25ng mL⁻¹ mCSF-1.

153

154 *SILAC labelling and sample preparation*

155 For labelling cells by SILAC, equal numbers of BMDMs were differentiated and cultured as
156 described above, but using RPMI 1640 without Lysine and Arginine (Thermo Fisher
157 Scientific) that was supplemented either with natural amino acids (L-Lysine, 0.274 mM; L-
158 Lysine, 1.15 mM; Arginine, 1.15 mM) to obtain “light” cells (referred to as control) or
159 with amino acid isotopes ²H₄-Lysine (Lys4) and ¹³C₆-Arginine (Arg6) at the same
160 concentrations to obtain “heavy” cells (referred to as labelled) (Thermo Scientific). Fetal
161 calf serum (GIBCO) was dialysed over night against 100 volumes of sterile PBS, followed
162 by two new rounds of 3 h dialysis, each against a low cutoff membrane (3.5 kD, Gebaflex).
163 The light or heavy RPMI 1640 was supplemented with 15% (v/v) of dialysed and filter-
164 sterilised serum, 50 µM β-mercaptoethanol, 50 U mL⁻¹ penicillin, 50 µg mL⁻¹ streptomycin,
165 and 75 ng mL⁻¹ of mCSF-1.

166 BMDMs cultivated in SILAC medium were washed three times in PBS and were
167 lysed with a buffer containing 8M urea, 50 mM ammonium bicarbonate pH7.5, one tablet per
168 10 ml of cOmplete™ Protease Inhibitor Cocktail tablets (Sigma), and one tablet of the
169 phosphatase inhibitor cocktail PhosSTOP (Roche). Cell extracts were incubated on ice 30
170 min, sonicated 5 min, and centrifuged 15 min at 14,000 g to eliminate cell debris. Proteins
171 were quantified in the supernatants using the RC DC™ protein assay kit (Bio-Rad), according
172 to the manufacturer’s instructions. For protein identification and quantification, control
173 infected were mixed with labeled non-infected and labeled infected were mixed with control
174 non-infected at an equal ratio.

175

176 *SILAC Sample processing*

177 Samples supernatants as processed above were fractionated by two different methods: (i)
178 polyacrylamide gel electrophoresis fractionation (GEL) and (ii) Strong Anion eXchange
179 (SAX). For GEL fractionation, mixed samples were separated by polyacrylamide gel
180 electrophoresis (PAGE) with the use of Invitrogen NuPAGE 10% Bis-Tris 1.0mm*10 Well
181 gels. Proteins were recovered from 9 gel slices following in-gel digestion as described in
182 standard protocols. Briefly, following the SDS-PAGE and washing of the excised gel slices,
183 proteins were reduced by adding 10 mM DTT (Sigma Aldrich) prior to alkylation with 55
184 mM iodoacetamide (Sigma Aldrich). After washing and dehydrating the gel pieces with 100%
185 acetonitrile, trypsin (Sequencing Grade Modified, Roche Diagnostics) was added and proteins
186 were digested overnight in 25 mM ammonium bicarbonate at 30°C.

187 SAX-based samples were digested in solution prior to fractionation of peptides.
188 Briefly, the protein extracts obtained by urea extraction were mixed at a 1:1 ratio and
189 subjected to the following process: First, samples were reduced using 10mM DTT, alkylated
190 with 55mM iodoacetamide and digested using Trypsin overnight. Second, peptides were
191 desalted through Sep-Pak C18 cartridges (Waters) and dried down prior to their fractionation.
192 Peptide fractionation was carried out through SAX using in-house prepared microcolumns
193 (anion exchange disks, Empore) and a Britton - Robinson buffer (20mM acetic acid, 20mM
194 phosphoric acid, 20mM boric acid) at six decreasing pH values (11, 8, 6, 5, 4, 3) for the
195 elution of peptides [36].

196 Samples were loaded onto homemade C18 SepPak-packed StageTips for desalting
197 (principle by stacking one 3M Empore SPE Extraction Disk Octadecyl (C18) and beads from
198 SepPak C18 Cartridge (Waters) into a 200 µL micropipette tip). Peptides were eluted with
199 40/60 MeCN/H₂O + 0.1% formic acid and lyophilysed under vacuum. Desalted samples were
200 reconstituted in injection buffer (2% MeCN, 0.3% TFA) for LC-MS/MS analysis.

201

202 *LC-MS/MS analysis*

203 Online liquid chromatography (LC) was performed with an RSLCnano system (Ultimate
204 3000, Thermo Scientific) coupled online to an Orbitrap Fusion Tribrid mass spectrometer

(MS, Thermo Scientific). Peptides were trapped on a C18 column (75 μ m inner diameter $\times$ 2 cm; nanoViper Acclaim PepMapTM 100, Thermo Scientific) with buffer A (2/98 MeCN/H₂O in 0.1% formic acid) at a flow rate of 4.0 μ L/min over 4 min. Separation was performed on a 50 cm $\times$ 75 μ m C18 column (nanoViper Acclaim PepMapTM RSLC, 2 μ m, 100Å, Thermo Scientific) regulated to a temperature of 55°C with a linear gradient of 5% to 25% buffer B (100% MeCN in 0.1% formic acid) at a flow rate of 300 nL/min over 100 min for the SAX and the GEL samples. Full-scan MS was acquired in the Orbitrap analyzer with a resolution set to 120,000, a mass range of m/z 400–1500 and a 4×10^5 ion count target. Tandem MS was performed by isolation at 1.6 Th with the quadrupole, HCD fragmentation with normalised collision energy of 30, and rapid scan MS analysis in the ion trap. The MS² ion count target was set to 2×10^4 and only those precursors with charge state from 2 to 7 were sampled for MS² acquisition. The instrument was run in top speed mode with 3 s cycles.

217

218 *MS Data Processing and Protein Identification*

219 Data were searched against the Swiss-Prot *Mus musculus* database (012016) using Sequest^{HT}
220 through Thermo Scientific Proteome Discoverer (v 2.1). The mass tolerances in MS and
221 MS/MS were set to 10 ppm and 0.6 Da, respectively. We set carbamidomethyl cysteine,
222 oxidation of methionine, N-terminal acetylation, heavy ¹³C₆-Arginine (Arg6) and ²H₄-Lysine
223 (Lys4) as variable modifications. We set specificity of Trypsin digestion and allowed two
224 missed cleavage sites.

225 The resulting files were further processed by using myProMS (v 3.5) [37]. The
226 Sequest HT target and decoy search result were validated at 1% false discovery rate (FDR)
227 with Percolator. For SILAC-based protein quantification, peptides XICs (Extracted Ion
228 Chromatograms) were retrieved from Thermo Scientific Proteome Discoverer. Global Median
229 Absolute Deviation (MAD) normalisation was applied on the total signal to correct the XICs
230 for each biological replicate. Protein ratios were computed as the geometrical mean of related
231 peptides. To estimate ratio significance, a t-test was performed with the R package limma [38]

232 and the false discovery rate has been controlled using the Benjamini-Hochberg procedure
233 [39].

234

235 *RNA isolation and RT qPCR*

236 Total RNA was isolated from macrophages using the Nucleospin RNA isolation kit
237 (MACHEREY-NAGEL GmbH, Germany) according to the manufacturers' instructions. The
238 integrity of the RNA was assessed using the Agilent 2100 Bioanalyzer system. For RNA
239 reverse transcription to first strand cDNA, 1 µg of total RNA was mixed with random
240 hexamers (Roche Diagnostics) and MMLV-RT reverse transcriptase (Moloney Murine
241 Leukemia Virus, Invitrogen Life Technologies), and the reaction was carried according to the
242 manufacturer's instructions. qPCR was performed with the QuantiTect SYBR Green kit
243 (Qiagen) according to the manufacturers' instructions in a 10 µL reaction mixture containing
244 1 µL cDNA template and 0.5 µM forward/ reverse primers and 5 µl 2x QuantiTect master
245 mix. The reactions were performed in triplicates in white FrameStar® 384 PCR plates
246 (4titude) on a LightCycler® 480 system (Roche Diagnostics, Meylan, France) and gene
247 expression analysis was performed with the use of qBase [40]. The primers used are listed in
248 **Table S1**. Analysis of PCR data and normalisation over the reference genes Yhwaz and
249 RpL19, were performed as previously described [41].

250

251 *Western blot analysis*

252 Total protein extracts were separated on 4–12% Bis-Tris NuPAGE® gels (Invitrogen) and
253 blotted onto polyvinylidene difluoride (PVDF) membranes (Pierce). Western blot analyses
254 were performed as previously described [42]. The following primary antibodies were used at
255 1:1000 dilution: rabbit anti-GSTm (Abcam, ab178684), anti-Asah-1 (ab74469), anti-Rab3IL1
256 (Abcam, ab98839), anti-Plxna1 (Abcam, ab23391), anti-Hmox-1 (Abcam, ab13243) and anti-
257 Ctsd (Abcam, ab75852) and the monoclonal anti-β actin 13E5 (Cell Signaling Technologies,
258 #4970). The anti-rabbit and anti-mouse secondary antibodies (Pierce) were diluted 1:20000.

259 Blots were developed using SuperSignal™ West Pico (Pierce) according to the
260 manufacturer's instructions. Western blots were quantified with Image J software.

261

262 *Counting of intracellular parasites*

263 Infected and non-infected macrophages plated on coverslips were fixed for 20 minutes at RT
264 with 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS) and incubated for
265 15 min with PBS containing the nuclear dye Hoechst 33342 (0.5 µg/ml). Excess dye was
266 removed by rinsing coverslips in PBS before mounting on a microscope slide using Mowiol
267 (Sigma-Aldrich). Two different cover slips per condition were analysed with a Leica
268 DMI6000B microscope or an inverted microscope equipped with ApoTome (ZEISS).

269

270 *Functional enrichment analysis*

271 GO default annotations were parsed from the available NCBI GO information
272 (<http://www.ncbi.nlm.nih.gov/Ftp/>), using as background the whole annotation from Uniprot
273 *Mus musculus* reference proteome (UP000000589). The GO enrichment analysis for
274 biological process, molecular function and cell component was performed using the proteins
275 listed in **Table 1** and **2** as input. The multiple testing correction was selected to the Benjamini
276 and Hochberg false discovery rate at a significance level of 0.01. Results were visualised
277 using the Cytoscape 3.5.1 software package [43] and the BinGO plug-in [44].

278

279 **RESULTS AND DISCUSSION**

280 *Metabolic labeling of BMDMs in SILAC medium and experimental workflow for SILAC* 281 *experiments*

282 First, we tested the capacity of *L. donovani* promastigotes to infect and proliferate
283 within control and isotope-labelled BMDMs. Macrophages at day 6 post-differentiation in
284 SILAC culture medium were infected at a parasite-to-macrophage ratio of 10:1 with *L.*
285 *donovani* promastigotes at *in vitro* passage 2 after differentiation from lesion-derived

286 amastigotes. Intracellular parasite burden was microscopically assessed at 4 h, 48 h and 72 h
 287 post-infection (PI) (**Fig. 1A**). We observed a mean number of 8 parasites per macrophage at 4
 288 h PI, which transiently decreased at 48 h but then recovered at 72 h. More than 80% of
 289 macrophages were infected at all time points tested (4 h, 48 h and 72 h, data not shown),
 290 indicating robust infection and intracellular parasite growth. Infection was similar in both
 291 labelled and control macrophages, suggesting that the metabolic labelling did not influence
 292 the ability of parasites to survive and proliferate.

293 In a series of control experiments, we assessed the culture system for variations in
 294 host cell phenotype, SILAC label incorporation, infection efficiency and protein fractionation
 295 technique. Under our experimental conditions (see Materials and Methods), over 90% of the
 296 adherent cells differentiated into a homogeneous macrophage population as judged (i) by
 297 expression of the macrophage-specific F4/80 surface antigen as determined by fluorescent
 298 microscopy [45, 46], and (ii) the ability of the cells to phagocytose fluorescently labeled,
 299 yeast zymosan bioparticles (data not shown). We tested the isotope incorporation efficiency
 300 for the amino acids lysine and arginine using LC-MS/MS and established day 6 after the
 301 differentiation process, as the minimal culture period to achieve the required incorporation
 302 levels of above 95% (data not shown). As shown by the experimental layout in **Fig. 1B**, we
 303 performed label-swap experiments, where the experimental state and stable isotope labels are
 304 interchanged. Label swap enriches the list of proteins whose abundance is related to the
 305 experimental state as it allows for the identification of systematic errors due to labeling, and
 306 to experimental outliers. Bone marrow progenitor cells were differentiated into macrophages
 307 in medium supplemented with the ‘heavy’ amino acid isotopes, referred to as ‘labelled’ (L),
 308 and medium supplemented with the ‘light’, natural amino acids, referred to as ‘control’ (C).
 309 Cells were infected six days post-differentiation with *L. donovani* promastigotes for 72 h. *L.*
 310 *donovani*- infected (I) and non-infected (NI), labeled (L) and control (C) macrophages were
 311 lysed, mixed in equal amounts and their proteomes were analysed by LC/MS-MS. Our
 312 protocol further included two fractionation methods, SAX and GEL fractionation that are
 313 based on charge- or size-dependent separation, respectively. The reduction of extract

complexity by these methods increasing the number of quantifications and thus the quality of our proteomics analyses by increasing protein sequence coverage, which facilitates detection of low-abundance proteins.

317

318 *Analysis and validation of proteomics data*

319 The comparative analysis of macrophage proteomes from reciprocal L-I/ C-NI and C-I/L-NI
320 samples allowed the identification of 5322 and 5101 proteins using SAX and GEL
321 fractionation, respectively and a combined total 6189 proteins (**Fig. 2A** and **Fig. 2B**). Seventy
322 five percent of proteins identified by GEL and SAX fractionation (3871 and 4026 proteins,
323 respectively) were shared between L-I/C-NI and C-I /L-NI (**Fig. 2A**), indicating a very good,
324 qualitative reproducibility in the depth of our analysis. Moreover, the overlap between SAX
325 and GEL was also significant, with sixty five percent 3291 proteins of the proteins identified
326 were shared between the two groups (GEL, SAX: L-I/C-NI and GEL, SAX: L-NI/C-I) (**Fig.**
327 **2B**). Volcano plots using GEL and SAX fractionation revealed that a subset of proteins were
328 differentially expressed between in L-I/C-NI and C-I/L-NI (**Fig.1B**).

329 Amongst the proteins with modulated abundance, only macrophage proteins
330 identified in reciprocal (label swapped) experiments that conformed to filtering criteria raised,
331 were selected. To this end, we considered in both forward and reverse experiments a fold
332 change (FC) of 1.25 or higher, with at least 2 peptides identified per quantification. With the
333 exception of infinite quantifications, only quantifications with confidence level of 0.05 or
334 below ($p \leq 0.05$), in at least one of the experiments were accepted (**Table 1** and **Table 2**).

335 This analysis revealed increased abundance in infected macrophages for 35 proteins
336 (27 proteins with GEL, 8 proteins with SAX), with Glutathione S Transferase mu1
337 (Gstm1/P10649) common in the datasets (**Table 1**). The same analysis revealed reduced
338 abundance for 51 proteins in infected macrophages (42 proteins with GEL, 13 proteins with
339 SAX), with 4 proteins common in the two datasets [the macrophage-expressed gene 1 protein
340 (Mpeg-1/A1L314), Palmitoyl-protein thioesterase 1 (Ppt1/O88531), Cathespin D (Ctsd/
341 P18242) and the Mannose receptor C-type 1 (Mrc-1/Q61830)] (**Table 2**). Thus, for the

primary macrophage proteome, these results indicate that SAX and GEL fractionation procedures together increase the level of quantifications that pass the filtering criteria, increasing the depth of protein quantification.

We then validated our results by immunoblot analysis of infected and non-infected macrophages using β -actin for normalisation. We confirmed reduced abundance in response to infection for Acid ceramidase 1 (Asah1/Q9WV54), Guanine nucleotide exchange factor for Rab-3A (Rab3IL1, Q8TBN0), Heme oxygenase 1 (Hmox-1/P14901) and Cathepsin D (Ctsd/P18242). Additionally, we confirmed the higher abundance of Glutathione S-transferase Mu 1 (Gstm1/P10649) and Plexin A1 (PlxnA1/P70206) (**Fig. 3A, Table 1 and Table 2**). Total RNA of replica samples used for immunoblot analysis was extracted, and reverse transcription (RT) qPCR was performed for selected transcripts. The transcripts of 60S ribosomal protein L19 (RpL19/P84099) and 14-3-3 protein zeta/delta (Ywhaz/P63101) were used as normalisation controls, as these reference genes are considered to have stable expression [47, 48] in these culture conditions. Certain changes in transcript levels correlated with changes in protein abundance, including reduced abundance for Pppt1 and increased abundance for PlxnA1 and the copper-transporting ATPase 1 (Atp7/Q64430). Other transcripts did not show any correlation, e.g., Ctsd, Rab3IL1 and Hmox-1 mRNAs (**Fig. 3B**). These data suggest that *L. donovani* both affects transcriptional and post-transcriptional regulation of infected BMDMs.

Gene Ontology analysis illuminates the landscape of subverted pathways in L. donovani infected macrophages

The datasets of differentially modulated proteins were analysed for enriched biological processes, molecular functions and cellular components using the Gene Ontology (GO) and the *Mus musculus* Uniprot databases (<https://www.uniprot.org/proteomes/UP000000589>), and results were visualised with the BinGO plug-in of the Cytoscape 3.5.1 software package [43]. Up-regulated biological processes were related to metabolism, including lipopolysaccharide biosynthetic process (GO-ID: 9103), cellular ketone metabolic process (GO-ID: 42180),

370 oxoacid (GO-ID: 43436) and carboxylic acid (GO-ID: 19752) metabolism (**Fig. 4A, Table**
371 **S2**), the latter enrichment having previously been observed in THP-1 cells infected with *L.*
372 *donovani* promastigotes [28]. Enrichment was also observed for various molecular functions,
373 including intramolecular oxidoreductase activity transposing S-S bonds (GO-ID: 16864),
374 intramolecular oxidoreductase activity, interconverting keto and enol groups (GO-ID: 16862)
375 and protein disulfide isomerase activity (GO-ID: 3756)(**Fig. 4B, Table S2**). This last activity
376 is important for protein folding, stress response (balancing the effects of oxidative stress) and
377 phagocytosis [49]. Moreover, enrichment in cellular components included the cytoplasm
378 (GO-ID: 5737), intracellular membrane bounded organelle (GO-ID: 43231) and endoplasmic
379 reticulum (GO-ID: 5783), with many of the up-regulated endoplasmic reticulum (ER) proteins
380 involved in protein folding and post-translation modification (**Fig. 4C, Table S2**), revealing a
381 potential impact of *L. donovani* infection on endoplasmic reticulum stress and the unfolded
382 protein response as previously documented [50].

383 Using the data set of proteins showing reduced abundance, enrichment was observed
384 for biological processes involved in vacuole (GO-ID: 7033) and lysosome (GO-ID: 7040)
385 organisation and catabolic process (GO-ID: 9056) (**Fig. 5A, Table S3**). Enriched molecular
386 functions in the down-modulated dataset, included binding (GO-ID-5488), transcription
387 elongation regulator activity (GO-ID: 3711), hydrolase activity hydrolysing o-glycosyl
388 compounds (GO-ID: 4553) and cysteine type endopeptidase activity enriched in cysteine type
389 endopeptidases (GO-ID: 4197) (**Fig. 5B, Table S3**). Moreover, amongst the enriched cellular
390 components were lytic vacuole (GO-ID: 323), lysosome (GO-ID: 5764) and vacuole (GO-ID:
391 5773), and thus the very organelles and structures exploited by the parasite as intracellular
392 niche. Other enriched cellular components included cytoplasm (GO-ID: 5737), intracellular
393 membrane bounded organelle, (GO-ID: 43231) and integrin complex (GO-ID: 43231) (**Fig.**
394 **5C, Table S3**).

395

396 *L. donovani* modulate the abundance of proteins involved in the lysosome and lytic vacuole
397 organisation of BMDMs

Our study reveals that *L. donovani* promastigote infection has a profound effect on BMDM lysosomal biology, with remodeling of this organelle likely promoting parasite survival in its known intracellular niche, the phago-lysosome [4, 51]. Our data show that the ratios of a total of 15 lysosomal proteins in both forward [L-I/C-NI, ratio 1, (r_1)] and reverse experiments [C-I/L-NI, ratio 2, (r_2)] were below 0.8 (**Table 2** and **Table S3**). Down-regulated proteins included various hydrolases such as lysosomal cathepsins (Ctsb/P10605, $r_1=0.59$ $r_2=0.54$; Ctsc/P97821, $r_1=0.49$, $r_2=0.41$; Ctsd/P18242, $r_1=0.35$, $r_2=0.59$) or dipeptyl peptidase 2 (Dpp-2/Q9ET22, $r_1=0.4$, $r_2=0.75$) and the Lysosome-associated membrane glycoproteins, Lamp2 (Cd107b/P17047, $r_1=0.72$, $r_2=0.7$) and Lamp4 (Cd68/P31996, $r_1=0.68$, $r_2=0.49$) (**Table 2**).

Lysosomal proteases, including cathepsins may affect parasite survival as they carry microbicidal activity and are key regulators of the host immune response by modulating Major histocompatibility Class (MHC) II antigen presentation and the trafficking of Toll like receptors (TLRs) [52]. Down-modulation of different cathepsins may cause distinct effects on macrophage activation. For example, deletion or pharmacological inhibition of Ctsb results in inflammatory phenotypes [53, 54] and Ctsd inhibition blocks both Th1 and Th2 responses by strongly suppressing MHCII-associated invariant chain molecule [54]. Different *Leishmania* species and strains, parasite stages (promastigotes/amastigotes) and host species may modulate cathepsins in different ways. For example, Prina *et al* reported a time-dependent increase of Ctsb, Ctsh and Ctsd activity in rat bone marrow-derived macrophages infected with *L. amazonensis* [55]. On the other hand, the RNA transcripts of Ctsc and Ctss were reported to be down-modulated in monocyte derived human macrophages 24 h after *L. major* metacyclic promastigote infection [56].

Apart from lysosomal peptidases, a number of hydrolases involved in fatty acid metabolism were also affected in macrophages by *L. donovani* infection. Interestingly, the down-modulation of the lyase Palmitoyl-protein thioesterase 1 (Ppt1/O88531, $r_1=0.23$, $r_2=0.3$) involved in the removal of thioester-linked fatty acyl groups (like palmitate) is anticipated to disrupt lysosomal catabolism via the rapid accumulation of palmitoylated proteins [57]. Moreover, Ppt1 down-modulation impairs mTOR signaling [57] the repression of which has

426 been also linked to *Leishmania* intracellular survival [58]. Additionally, down-modulation of
 427 Acid ceramidase -1 (Asah-1/Q9WV54, $r_1 = 0.45$, $r_2 = 0.5$) (**Table 2**) - an enzyme that
 428 hydrolyses the sphingolipid ceramide into sphingosine and free fatty acid - may also impact
 429 the macrophage environment to favor parasite survival. *Leishmania* is favored by increase in
 430 ceramide concentration and in turn *Leishmania* infection, modulates the up-regulation of
 431 ceramide synthesis in a biphasic way [59]. At early time points after *Leishmania* infection,
 432 increase is mediated by Acid Sphingomyelinase activation, which catalyses the formation of
 433 ceramide from sphingomyelin, promoting the formation of cholesterol rich lipid
 434 microdomains [59], required for *L. donovani* uptake by macrophages [60]. In the second
 435 phase, the *de novo* biosynthesis of ceramide is induced via ceramide synthase, resulting in the
 436 displacement of cholesterol from the membrane and impacting MHC-II class antigen
 437 presentation [59]. Moreover, ceramide increase leads to severe host immune suppression via
 438 the reduction of nuclear translocation of NFkB and AP-1, the up-regulation of
 439 immunosuppressive cytokines TGF- β and IL-10, and reduced NO generation [61]. Thus
 440 increase in ceramide concentration is required for parasite survival, and the down-modulation
 441 of Asah-1 revealed in this screen, could be an additional mechanism contributing to this rise,
 442 and hence merits further investigation.

443 Overall these results suggest a major modification of lysosomal components that
 444 might aid the parasite to survive inside the hostile macrophage environment. The mechanism
 445 that contributes to lysosomal protein down-modulation after *L. donovani* infection, is elusive.
 446 One possible scenario would be a dysregulation of transcription factor EB, which is a master
 447 regulator of lysosomal biogenesis that mediates the transcription of many lysosomal
 448 hydrolases [62]. Another scenario would be that the lysosomal content is modified via the
 449 action of lysosomal exocytosis, which takes place to repair host cell plasma wounding caused
 450 by the parasite [63] or by the direct interaction of the parasites' exo-proteases with lysosomal
 451 proteins, leading to protein degradation.

452

453 *L. donovani* induce alterations in abundance of macrophage proteins involved in
454 immunomodulation

455 Our data demonstrate that *L. donovani* promastigotes inhibit host cell immune functions by
456 down-regulation of several macrophage proteins that participate in immunomodulation and
457 innate immunity (Cd180/Q62192, $r_1=0.66$, $r_2=0.76$; Tlr13/Q6R5N8, $r_1=0.66$, $r_2=0.5$;
458 Grn/P28798, $r_1=0.7$, $r_2=0.62$; Arrb2/Q91YI4, $r_1=0.61$, $r_2=0.4$; Gpr84/Q8CIM5, $r_1=0.64$ and
459 $r_2=1/\infty$; Hmox-1/P14901, $r_1=0.67$, $r_2=0.39$; Mpeg1/A1L314, $r_1=0.31$, $r_2=0.4$) (**Table 2**). These
460 results indicate that infection likely dampens the macrophage pro-inflammatory response
461 given the observed down-modulation of (i) Tlr13, which acts via Myd88 and Traf6 leading to
462 NF- κ B activation [64], (ii) Granulin (Grn) a soluble cofactor for Tlr9 signaling [65], and (iii)
463 the G-protein coupled receptor Grp84 known to be activated by medium-chain free fatty acids
464 to enhance inflammation and phagocytosis in macrophages [66].

465 Moreover, *L. donovani* promastigote infection results in increased abundance of
466 macrophage proteins that act as markers or as regulators of alternatively activated
467 macrophages (M2-like phenotype) (**Table 1**) in agreement with previous reports [67-72].
468 Amongst these proteins were: (i) the Cd9 antigen (P40240, $r_1=1.41$, $r_2=1.76$), a tetraspanin
469 family member known to form a complex with integrins and to negatively regulate LPS-
470 induced macrophage activation [73], (ii) the highly up-regulated urokinase-type plasminogen
471 activator (Plau/P06869, $r_1=6.02$, $r_2=5.41$) known to polarise macrophages towards a M2-like
472 phenotype [74], (iii) the monoamine oxidase A (Mao-A/Q64133, $r_1=1.31$, $r_2=2.24$) involved
473 in the breakdown of monoamines [75], (iv) the TNF alpha induced protein 8 like 2
474 (Tnfaip8l2/Q9D8Y7, $r_1=\infty$ and $r_2=1.4$) involved in phospholipid metabolism [76, 77], (v) the
475 Cd93 antigen (Cd93/O89103, $r_1=1.35$, $r_2=1.76$), a C-type lectin transmembrane receptor [78]
476 and (vi) the fatty acid-binding protein (Fabp4/P04117, $r_1=1.3$, $r_2=1.57$) that delivers long-
477 chain fatty acids and retinoic acid to their cognate receptors in the nucleus [79] (**Table 1**).
478 Interestingly, Fabp4 up-modulation in infected macrophages is in line with other studies after
479 macrophage infection with *L. major* promastigotes [26] and *L. amazonensis* amastigotes [24],

480 and suggest that the increased abundance of this protein maybe independent of the
481 *Leishmania* stage or species.

482 In contrast to proteins that control, or are enriched in alternatively activated
483 macrophages, two proteins less abundant in infected macrophages, Cd180 and Beta-arrestin-2
484 (Arrb2), are known to either promote or reduce inflammation depending on the cell type and
485 infection system [80-83]. Moreover a protein known to be enhanced in alternatively activated
486 macrophages, Hmox-1 that converts pro-oxidant heme to the antioxidant biliverdin and
487 bilirubin, restoring the redox environment [84] was found down-modulated in this screen in
488 response to *Leishmania* infection (**Table 2**). Other studies have shown that Hmox-1 is up-
489 regulated in mouse peritoneal macrophages infected with *L. infantum* (*L. chagasi*)
490 promastigotes and promotes the persistence of the parasite [85]. In our infection system,
491 despite the repressed protein levels, we find Hmox-1 mRNA to be up-regulated in
492 macrophages infected with *L. donovani*. The negative correlation between Hmox-1 RNA and
493 protein levels, amongst other factors, could be due to increased secretion of Hmox-1 in the
494 culture medium, as Hmox-1 can be a secreted protein [86].

495

496 *L. donovani* up-modulate the abundance of proteins involved in the defense against oxidative
497 and ER stress in BMDMs

498 Proteins related to oxidative stress and ER stress response were more abundant in BMDMs 72
499 h after *L. donovani* infection, including proteins involved in glutathione metabolism, such as
500 Glutamate-cysteine ligase regulatory subunit (Gclm/O09172, $r_1=1.28$, $r_2=1.29$), the
501 Glutathione transferase mu1 (Gstm1/P10649, $r_1=1.36$; $r_2=2.53$), ferritin light (Ftl1/ P29391,
502 $r_1=2.9$, $r_2=2.64$) and heavy (Fth1/P09528, $r_1=1.37$, $r_2=2.23$) chains, and Bola like 1
503 (Bola1/Q9D8S9, $r_1=1.73$ and $r_2=\infty$), a protein that prevents mitochondrial changes upon
504 glutathione depletion [87] acting as mitochondrial iron-sulfur cluster assembly factor [88]
505 (**Table 1**). All of these proteins are known to normalise the redox state of the cell or protect
506 the cell from oxidants [87, 89-91]. Interestingly, up-regulated transcripts involved in the
507 response against oxidative stress and glutathione metabolism were previously shown to be up-

regulated in human monocyte derived macrophages infected with *L. (Vianna) panamensis* promastigotes [9] and in mouse BMDMs infected with *L. major* promastigotes [26]. Moreover, up rise in cellular ROS, could be associated with ER stress, the unfolded protein (UPR) response and a concomitant up rise of ER proteins aiding protein folding, as a mechanism of the cell to repair oxidant damage [92]. In our system several of these proteins including disulfide-isomerase forms [Protein disulfide-isomerase A3 (Pdia3/ P27773, $r_1=1.39, r_2=1.27$), A4 (Pdia4/P08003, $r_1=1.35, r_2=1.27$) and A6 (Pdia6/Q922R8, $r_1=1.43, r_2=1.31$)] [93] and Hypoxia up-regulated 1 (Hyou1/Q9JKR6, $r_1=1.86, r_2=1.38$ [94], displayed higher levels in infected macrophages (**Table 1**). Likewise, ER stress and induction of the unfolded protein response was previously documented for RAW 264.7 cells infected with *L. amazonensis* [50]. Overall, these results suggest that *L. donovani* infection induces ROS production and ER stress [10, 95], with specific proteins involved in this process displaying higher abundance, and likely acting as a defense mechanism against the oxidants' deleterious effects.

L. donovani modulates the abundance macrophage proteins involved in intracellular trafficking and ion movement

The abundance of many macrophage proteins involved in intracellular trafficking was modified in *L. donovani* infected BMDMs. Previous studies have shown that *Leishmania* infection impairs intracellular trafficking by modulating small GTPase signal transduction [28]. This form of signaling is important in host/pathogen interactions as it regulates pathways involved in phagocytosis and oxidative burst, vesicle fusion and actin organisation [96, 97]. The deregulation of small GTPase signal transduction, likely alters these macrophage functions, and produces a favorable environment for intracellular parasite infection [28, 98, 99]. Our screening extends previous studies and provides evidence of altered protein expression data in proteins involved in small GTPase signalling, with four proteins showing reduced abundance during infection (Gmip Q6PGG2, $r_1=0.79, r_2=0.49$; Rgl2/Q61193, $r_1=1/\infty, r_2=0.35$; Arfp2/Q8K221, $r_1=1/\infty, r_2=0.64$; Rab3il1/Q8VDV3, $r_1=0.66, r_2=0.39$;

536 Nisch/Q80TM9, $r_1=0.66$, $r_2=0.46$) (**Table 2**), and one member showing increased abundance
 537 (Tbc1d22a/Q8R5A6, $r_1=\infty$ and $r_2=1.89$) (**Table 1**). Moreover, various other proteins affecting
 538 intracellular trafficking were modulated in response to *L. donovani* promastigote infection,
 539 including a signal recognition particle receptor subunit beta (Srprb/P47758; $r_1=1.36$, $r_2=1.25$)
 540 (**Table 1**) that likely restores trafficking imbalances to the ER [100], and two cytoskeletal
 541 organisation proteins (Peak1/Q69Z38, $r_1=0.45$, $r_2=0.46$; Ppp2r5e/Q61151, $r_1=1/\infty$, $r_2=0.73$)
 542 [101, 102]. Additionally, the abundance of macrophage mannose receptor 1 (Mrc-1) - a
 543 protein involved in endocytosis and phagocytosis - was down-modulated (Q61830, $r_1=0.63$,
 544 $r_2=0.33$), in agreement with previous studies performed with mouse peritoneal macrophages
 545 infected with *L. donovani* promastigotes [103]. With the exception of Mrc-1, the majority of
 546 these macrophage proteins have not been previously shown to be modulated by *Leishmania*
 547 infection. The modulation of these proteins merits further investigation, as they could be part
 548 of the parasite evasion strategy interfering with vesicular fusion events in the host cell,
 549 important for parasite survival.

550 Interestingly, another form of trafficking, ion movement, has been associated with
 551 inflammatory response [104-106]. For example, zinc/copper imbalance reflects immune
 552 dysregulation in human leishmaniasis [106]. In this study, some proteins involved in ion
 553 transport were modulated in infected BMDMs, including (i) Atp1a1 (Q8VDN2, $r_1=1.39$;
 554 $r_2=1.26$) a subunit of the sodium potassium ATPase [107](**Table 1**), (ii) stomatin
 555 (Stom/P54116, $r_1=0.78$, $r_2=0.48$) (**Table 2**) known to regulate ion channel activity and
 556 transmembrane ion transport [108] and (iii) copper-transporting ATPase 1 (ATP7a/P56542,
 557 $r_1=2.61$, $r_2=2.66$) that regulates copper efflux under conditions of elevated extracellular
 558 copper concentration [109] (**Table 1**). Overall, these changes may reflect immune function
 559 and their influence on host/pathogen interaction merits further investigation.

560

561 *L. donovani* modulate the abundance of extracellular matrix and adhesion proteins in
 562 BMDMs

Macrophage adhesion and cell-cell interactions with other immune cells play important roles in health and disease, with co-stimulatory molecules regulating T cell activation or cell migration enabling leukocyte recruitment to sites of inflammation. Our proteomics screen revealed changes in expression of extracellular matrix and adhesion proteins that could explain the reported modified adhesion of infected macrophages to connective tissue [110]. We confirmed down-modulation of the co-stimulatory molecule Itgax (Q9QXH4, $r_1=1/\infty$, $r_2=0.33$) (**Table 2**). Changes in abundance of the integrin subunit Itg β 5 (O70309, $r_1=0.73$ and $r_2=0.55$) which acts with Itga5 as a fibronectin receptor, were detected, as well as differences in other adhesion or adhesion signaling molecules (Nisch/Q80TM9, $r_1=0.66$, $r_2=0.56$; Plxna1/ P70206, $r_1=1.65$, $r_2=2.46$; Lpxn/Q99N69, $r_1=0.74$, $r_2=0.65$) (**Table 1** and **Table 2**). Thus, our data expand and further emphasise the impact of intracellular *Leishmania* infection on connective tissue remodeling that may modify host adaptive immune responses mediated by infected macrophages [110, 111].

L. donovani modulate the abundance of macrophage proteins involved in gene expression

It is well established that *Leishmania* infection modulates macrophage gene expression [21]. In our study, several proteins implicated at different levels of gene expression regulation, showed differential abundance in response to *L. donovani* BMDM infection, including transcriptional, epigenetic or post-transcriptional regulators (Hist4h4/P62806, $r_1=1.44$, $r_2=1.33$; Lpxn/Q99N69, $r_1=0.75$, $r_2=0.65$; Ddx42/ Q810A7, $r_1=0.74$, $r_2=0.65$; Psmc3/088685, $r_1=1.34$, $r_2=1.25$; Supt5h/O5520, $r_1=0.67$; $r_2=0.68$; Supt6h/Q62383, $r_1=0.59$, $r_2=0.64$), proteins that modify RNA (Cmtr1/Q9DBC3, $r_1=1.39$ and $r_2=\infty$; Dus3L/Q91XI1, $r_1=0.34$, $r_2=1/\infty$), as well as translation regulators (eIF-3/Q9QZD9, $r_1=0.77$, $r_2=0.73$; Rps6ka1-Rsk1/P18653, $r_1=0.43$, $r_2=1/\infty$) (**Table 1** and **Table 2**). The higher abundance of histone H4 (Hist4h4) in infected macrophages is in accordance with previous reports for *L. donovani* infected THP-1 cells [28], whereas up-regulation of histones is anticipated to have a negative effect on gene expression by the modulation of chromatin structure [112]. Moreover, the lower levels of focal adhesion regulator Lpxn [113, 114] and the increased abundance of the

591 26S proteasome ATPase component, Psmc3/TBP-1 [115], two proteins that display novel
 592 functions and are known to modulate transcription via specific nuclear receptors [116-118].
 593 Thus the modulation of abundance of these proteins in infected macrophages, may affect
 594 transcriptional regulation of *Leishmania* infected macrophages. In addition, Psmc3/TBP-1
 595 regulates the transcription of Class II trans-activator (CIITA), the master regulator of the
 596 MHC-II transcription complex and a critical factor for the initiation of the adaptive immune
 597 response [119]. In addition, the down-modulation of specific transcriptional elongation factors
 598 (Supt5h and Supt6h) [120-122] is anticipated to contribute to transcriptomic changes in
 599 infected macrophages. The down-modulation of the eukaryotic translation initiation factor
 600 eIF-3 and of protein S6 kinase alpha 1(Rps6ka1/Rsk1) - a kinase known to stimulate protein
 601 synthesis and a key mediator of mTOR function [123] - suggests that protein translation
 602 proceeds at lower rates in infected macrophages [123]. Rps6ka1/Rsk1 is known to “connect
 603 the stress-induced activation of transcription factors and mitogens to the ribosome” [124].
 604 This connection is mediated by the activation/phosphorylation of c-Fos, IκBα, cAMP-
 605 response element-binding protein (CREB) and CREB-binding protein [125], proteins
 606 involved in the inflammatory response. Thus, the modulation of this kinase likely participates
 607 in the reprogramming of *Leishmania*-infected macrophages to favor intracellular parasite
 608 survival.

609
 610 *L. donovani* modulate the abundance of macrophage proteins involved in metabolic processes
 611 Previous studies have shown that *Leishmania* parasites exploit host metabolism to survive
 612 inside the hostile macrophage environment. It has been demonstrated that, early during
 613 *Leishmania* infection, the aerobic glycolysis predominates with inhibition of the TCA cycle
 614 [26], whereas at later time points energy metabolism is shifted towards oxidative
 615 phosphorylation and TCA cycle [28, 126]. *Leishmania* infection also perturbs lipid
 616 metabolism, including sterol biosynthesis and triacylglyceride synthesis in BMDMs [24, 26].
 617 In our screen, the cellular ketone metabolic process (GO-ID: 42180) and oxoacid metabolic
 618 process (GO-ID: 43436) - a broad metabolic processes that includes TCA cycle and

aminoacid biosynthesis, were modified, with 5 proteins showing increased abundance (Fabp4/P04117, $r_1=1.3$, $r_2=1.57$; Suc1a2/Q9Z219, $r_1=1.27$, $r_2=1.37$; Phyh/O35386, $r_1=\infty$ and $r_2=1.25$; Cmas/Q99KK2; $r_1=1.27$, $r_2=1.72$; Gclm/O09172, $r_1=1.27$, $r_2=1.28$). Indeed, Succinate--CoA ligase [ADP-forming] subunit beta (Suc1a2) – an enzyme that couples the hydrolysis of succinyl-CoA to the synthesis of ATP [127] - was one of the TCA cycle proteins shown to be up-regulated in *L. donovani*-infected THP-1 cells [28]. Likewise, Sterol O-acyltransferase 1 (Soat1/Q61263, $r_1=1.29$, $r_2=1.31$) - a cholesterol metabolism enzyme known to form cholesteryl esters from cholesterol [128] – displayed higher levels in infected macrophages (**Table 1**). This change could be related to the increase in cholesterol biosynthesis of macrophages infected with *Leishmania* [24, 26].

Finally, fatty acid metabolism, including fatty acid elongation (Ppt1/ O88531, $r_1=0.23$, $r_2=0.3$), phospholipases of the ether lipid metabolic process [(Pla2g7/Q60963, $r_1=0.68$, $r_2=0.35$; Plbd2/ Q3TCN2, $r_1=0.71$, $r_2=0.49$) and hydrolases of the sphingolipid metabolism (Asah-1/Q9WV54, $r_1=0.45$, $r_2=0.5$; Gla/P51569, $r_1=0.63$, $r_2=0.75$; Glb1/P23780, $r_1=0.55$, $r_2=0.76$), were found to be less abundant in infected macrophages (**Table 2**). Fatty acid metabolism, including sphingolipid metabolism [129] has been shown to be modulated in macrophages infected with *Leishmania* [24, 26, 59] and is likely to play an important function in *Leishmania*/macrophage interaction.

L. donovani modulate the abundance of macrophage proteins that regulate glycan and glycoside protein post-translational modifications

In addition, proteins regulating protein sugar (glycan and glycoside) post-translational modifications were modulated in *L. donovani* infected macrophages, with decreased levels observed for (i) ribophorin I (RpnI/Q91YQ5, $r_1=1.65$, $r_2=1.28$), an essential subunit of the N-oligosaccharyl transferase (OST) complex which catalyses the N-glycosylation of proteins [130] (**Table 1**), and (ii) alpha and beta galactosidases (Gla/P51569, $r_1=0.63$, $r_2=0.75$; Glb1/Q91YQ5, $r_1=0.55$, $r_2=0.76$). In contrast, increased abundance was observed for cytidine monophospho-N-acetylneuraminic acid synthetase (Cmas/Q99KK2, $r_1=1.27$, $r_2=1.72$) that

catalyses the activation of N-acetylneuraminic acid (NeuNAc) to cytidine 5'-monophosphate N-acetylneuraminic acid (CMP-NeuNAc) [131], a substrate required by sialyltransferases for the addition of sialic acid to growing oligosaccharide chains. Conceivably, changes in protein glycosylation may affect protein function and alter immune responses and cell adhesion signaling [132, 133] in infected macrophages, that may favor parasite survival.

Conclusions

In conclusion, our analyses establish a novel experimental framework for the quantitative proteomics analysis of *Leishmania* infected primary macrophages. Our results draw a highly complex picture of *Leishmania*/macrophage interaction and highlight the pleiotropic modulation of biological processes and molecular functions in infected BMDMs that likely establish permissive conditions for intracellular parasite survival and chronic infection. The parasite seems to have developed mechanisms to subvert key macrophage functions and trigger changes in host cell metabolism, innate immunity and lysosomal function. Future experimental validation combining systems-level, phenotypic, and functional genetic analyses is required to correlate enrichment/activation or depletion/inhibition of identified host pathways with changes in intracellular *Leishmania* survival.

Data linking

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [134] with the dataset identifier PXD013448 (username:reviewer87022@ebi.ac.uk, password: APPQoJX0).

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1092 **FIGURE LEGENDS**

1093 **Fig. 1 *In vitro* macrophage infection model with *L. donovani* promastigotes and SILAC-**
1094 **based macrophage proteomics analysis** (A) Histogram plots showing the number of
1095 intracellular parasites per labeled and control macrophages after 4 h, 48 h and 72 h of
1096 infection. (B) Workflow diagram showing the experimental strategy used to reveal the
1097 variable proteome of *L. donovani* infected BMDMs. Initially, an equal number of bone
1098 marrow (BM) progenitors were cultured and differentiated in the presence of natural amino
1099 acids (light “control” medium) or amino acid with heavy isotopes (heavy “labeled” medium)
1100 supplemented with 75 ng/mL mCSF. After 6 days of differentiation, adherent cells were
1101 detached and plated with fresh control or labeled medium supplemented with 25 ng/mL
1102 mCSF. Labeled (red) and control (blue) macrophages were infected or not with stationary
1103 phase *L. donovani* promastigotes at a ratio of 10:1 for 4 h. Macrophages were lysed 72 h post-
1104 infection, protein extracts were quantified and mixed at a 1:1 ratio in pairs (control vs labeled,
1105 infected or not), fractionated by either polyacrylamide gel electrophoresis fractionation
1106 (GEL) or strong anion exchange fractionation (SAX), and processed by LC-MS/MS
1107 analysis.

1108

1109 **Fig. 2 Summary of proteomics results** (A) Venn diagram showing the overlap of identified
1110 proteins between L-I/C-NI and C-I/L-NI after GEL (left panel) or SAX fractionation (right
1111 panel). (B) Venn diagram showing the combined overlap identified proteins of L-I/C-NI,

1112 C-I/L-NI samples in both GEL and SAX fractionation conditions. (C) Volcano plots of the
1113 data presented in (A) showing the fold change (FC, x-axis, log2) of abundance for proteins
1114 with at least two identified peptides plotted against the p-value (y-axis, -log10). Red
1115 vertical and green horizontal lines reflect the filtering criteria, i.e. $FC \geq 1.25$ or $FC \leq 0.8$, and
1116 significance level of 0.05, respectively.

1117

1118 **Fig. 3 Differential abundance for BMDM proteins and transcripts at 72 h post *L.***
1119 ***donovani* infection** (A) Immunoblot analysis. 20 µg of total protein extracts from infected (I)
1120 and non-infected (NI) BMDMs were analysed for Asah-1, Rab3IL1, Plxna1, Hmox-1, Gstm,
1121 and Cttd proteins. β-actin expression was used as a loading control. The intensities of the
1122 bands were analysed using the Image J software. The fold overexpression, represented as a
1123 bar diagram, was calculated by dividing the band intensity representing the protein of interest
1124 with the band intensity of β-actin. Results are representative for two independent experiments.
1125 (B) RT-qPCR. Column diagrams showing the mean ratio from three different experiments
1126 of RNA abundance in infected versus non-infected macrophages for Plxna1, Ppt1,
1127 Rab3IL1, Asah-1, Atp-7A and Hmox-1 relative to Ywhaz and RpL19. Error bars represent
1128 the standard deviations of three experiments.

1129

1130 **Fig. 4 Gene Ontology analyses for up-regulated proteins in *L. donovani* infected BMDMs**
1131 (A) GO analysis for Biological processes. (B) GO analysis for Molecular functions. (C) GO
1132 analysis for Cellular component. The analyses were performed using the hypergeometric
1133 statistical test, a Benjamini & Hochberg false discovery rate and significance level of 0.01.
1134 The p-value is indicated by the color according to the legend.

1135 **Fig. 5 Gene Ontology analyses for down-regulated proteins in *L. donovani* infected**
1136 **BMDMs.** (A) GO analysis for Biological processes. (B) GO analysis for Molecular functions.
1137 (C) GO analysis for Cellular component. The analyses were performed using the

1138 hypergeometric statistical test, a Benjamini & Hochberg false discovery rate and significance

1139 level of 0.01. The p-value is indicated by the color according to the legend.

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1143 **Table 1 Macrophage proteins displaying increased abundance after *L. donovani* infection**

Description	Uniprot ID	Gene	GEL:L-I/C-NI			GEL:C-I-NI		
			Ratio 1	p-value	No. Peptides	Ratio 2	p-value	No. Peptides
Glutamate--cysteine ligase regulatory subunit	O09172	Gclm	1.28	0.16	9	1.29	0.02	10
Complement component C1q receptor	O89103	Cd93	1.35	0.19	8	1.76	0.02	10
Fatty acid-binding protein, adipocyte	P04117	Fabp4	1.3	0.02	10	1.57	0.001	8
Urokinase-type plasminogen activator	P06869	Plau	6.02	0.04	5	5.41	0.003	6
Protein disulfide-isomerase A4	P08003	Pdia4	1.35	0.01	23	1.27	0.12	26
Ferritin heavy chain	P09528	Fth1	1.37	0.03	23	2.23	1.47E-07	20
Glutathione S-transferase Mu 1	P10649	Gstm1	1.36	0.003	26	2.53	8.21E-07	22
Integrin alpha-5	P11688	Itga5	1.34	0.001	18	1.27	0.3	18
Protein disulfide-isomerase A3	P27773	Pdia3	1.39	1.60E-08	51	1.27	7.97E-07	59
Ferritin light chain 1	P29391	Ftl1	2.9	3.90E-07	18	2.64	0.009	5
CD9 antigen	P40240	Cd9	1.41	0.16	5	1.76	0.004	6
Signal recognition particle receptor subunit beta	P47758	Srprb	1.36	0.04	7	1.25	0.14	8
V-type proton ATPase subunit D	P57746	Atp6v1d	1.29	0.31	9	1.37	0.003	6
40S ribosomal protein S15a	P62245	Rps15a	1.31	0.006	11	1.34	0.499	7
Histone H4	P62806	Hist1h4	1.55	3.40E-06	12	1.33	0.15	14

Plexin-A1	P70206	Plxna1	1.65	3.00E-05	35	2.46	1.81E-10	35
Keratin, type I cytoskeletal 9	Q6RHW0	Krt9	∞		8	∞		3
Sterol O-acyltransferase 1	Q61263	Soat1	1.29	0.13	10	1.31	0.03	13
Transmembrane protein 214	Q8BM55	Tmem214	1.34	0.04	5	1.87	0.21	7
Procollagen galactosyl-transferase 1	Q8K297	Glt25d1	2.53	0.12	8	1.5	0.001	11
Na ⁺ /K ⁺ -transporting ATPase subunit alpha-1	Q8VDN2	Atp1a1	1.39	0.001	17	1.26	0.007	22
Ribophorin I	Q91YQ5	Rpn1	1.65	0.0003	26	1.28	0.009	32
Protein disulfide-isomerase A6	Q922R8	Pdia6	1.43	0.0004	19	1.31	0.05	19
N-acetylneuraminatase cytidyltransferase	Q99KK2	Cmas	1.27	0.37	11	1.72	0.002	10
BolA-like protein 1	Q9D8S9	Bola1	1.73	0.45	2	∞		2
Cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	Q9DBC3	Cmtr1	1.39	0.72	2	∞		4
Hypoxia up-regulated protein 1	Q9JKR6	Hyou1	1.86	2.90E-05	36	1.38	0.008	45
Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	Q9Z2I9	Sucla2	1.27	0.48	10	1.37	0.007	17
Description	Uniprot ID	Gene	GEL:L-I/C-NI			GEL:C-I-NI		
			Ratio 1	p-value	No. peptides	Ratio 2	p-value	No. peptides
Phytanoyl-CoA dioxygenase, peroxisomal	O35386	Phyh	∞		2	1.25	0.53	2
26S protease regulatory subunit 6A	O88685	Psmc3	1.34	0.56	17	1.25	0.03	21
Glutathione S-transferase Mu 1	P10649	Gstm1	1.41	0.14	16	2.18	0.002	22

Amine oxidase [flavin-containing] A	Q64133	Maoa	1.31	0.54	15	2.24	0.005	15
Copper-transporting ATPase 1	Q64430	Atp7a	2.61	0.63	3	2.56	0.03	4
Protein PRRC2A	Q7TSC1	Prrc2a	∞		3	3.86	0.08	3
TBC1 domain family member 22A	Q8R5A6	Tbc1d2 2a	∞		2	1.89	0.48	3
Tumor necrosis factor alpha-induced protein 8-like protein 2	Q9D8Y7	Tnfaip8l 2	∞		4	1.44	0.71	2

1144 **Table 2 Macrophage proteins displaying decreased abundance after *L. donovani* infection**

Description	Uniprot ID	Gene	GEL :L-I/C-NI			GEL: C-I/L-NI		
			Ratio 1	p-value	No. Peptides	Ratio 2	p-value	No. Peptides
Macrophage-expressed gene 1 protein	A1L314	Mpeg1	0.31	0.0006	12	0.4	1.00E-06	16
1.Transcription elongation factor SPT5	O55201	Supt5h	0.67	0.01	12	0.68	0.003	17
Integrin beta-5	O70309	Itgb5	0.73	0.31	9	0.55	0.01	5
Palmitoyl-protein thioesterase 1	O88531	Ppt1	0.23	0.001	8	0.3	2.00E-05	11
Tripeptidyl-peptidase 1	O89023	Tpp1	0.65	0.44	4	0.58	0.0181	8
Cathepsin B	P10605	Ctsb	0.59	4.00E-05	22	0.54	9.00E-08	22
Beta-glucuronidase	P12265	Gusb	0.69	9.00E-06	35	0.65	1.00E-05	32
Heme oxygenase 1	P14901	Hmox1	0.67	0.07	7	0.39	2.00E-06	15
Lysosome-associated membrane glycoprotein 2	P17047	Lamp2	0.72	0.04	9	0.7	0.4494	9
Cathepsin D	P18242	Ctsd	0.35	1.00E-13	35	0.59	4.00E-06	35
Ribosomal protein S6 kinase alpha-1	P18653	Rps6ka1	0.43	0.343	3	1/∞		2
Beta-galactosidase	P23780	Glb1	0.55	0.001	18	0.76	0.19	18
Lysosomal acid phosphatase	P24638	Acp2	0.41	0.003	5	0.75	0.36	4
Granulins	P28798	Grn	0.7	0.281	5	0.62	0.02	8
Beta-hexosaminidase subunit alpha	P29416	Hexa	0.74	0.009	20	0.62	0.02	21
Lamp4, Macrosialin	P31996	Cd68	0.68	0.006	8	0.49	0.07	8
Alpha-galactosidase	P51569	Gla	0.63	0.183	14	0.75	0.001	14

Erythrocyte band 7 integral membrane protein, Stomatin	P54116	Stom	0.78	0.057	11	0.48	0.01	6
Deoxyribonuclease-2-alpha	P56542	Dnase2	0.61	0.519	2	0.47	0.05	2
Acid sphingomyelinase-like phosphodiesterase 3a	P70158	Smpdl3a	0.44	0.0001	13	0.44	0.01	12
Dipeptidyl peptidase 1/ Cathepsin c	P97821	Ctsc	0.49	5.00E-05	14	0.41	0.004	10
Putative phospholipase B-like 2	Q3TCN2	Plbd2	0.71	0.009	11	0.49	0.05	9
Platelet-activating factor acetylhydrolase	Q60963	Pla2g7	0.68	0.2	6	0.35	1.00E-05	11
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform	Q61151	Ppp2r5e	1/∞		3	0.73	0.14	3
Macrophage mannose receptor 1	Q61830	Mrc1	0.63	1.00E-05	37	0.33	9.00E-16	41
CD180 antigen	Q62192	Cd180	0.66	0.012	15	0.76	0.0246	16
Transcription elongation factor SPT6	Q62383	Supt6h	0.59	0.018	7	0.64	0.02	7
Pseudopodium-enriched atypical kinase 1	Q69Z38	Peak1	0.45	0.127	6	0.47	0.01	9
Structural maintenance of chromosomes flexible hinge domain-containing prot. 1	Q6P5D8	Smchd1	0.66	0.172	12	0.51	8.00E-05	15
GEM-interacting protein	Q6PGG2	Gmip	0.79	0.48	7	0.49	0.004	9
Toll-like receptor 13	Q6R5N8	Tlr13	0.66	0.012	15	0.5	8.00E-06	20
E3 ubiquitin-protein ligase UBR2	Q6WKZ8	Ubr2	0.61	0.018	2	0.67	0.014	5
Nischarin	Q80TM9	Nisch	0.66	0.026	17	0.46	6.00E-07	25
ATP-dependent RNA helicase DDX42	Q810A7	Ddx42	0.74	0.122	10	0.65	0.02	7

Guanine nucleotide exchange factor for Rab-3A	Q8VDV3	Rab3il1	0.66	0.144	4	0.29	0.001	6
Beta-arrestin-2	Q91YI4	Arrb2	0.61	0.169	9	0.4	0.05	7
Leupaxin	Q99N69	Lpxn	0.74	0.049	12	0.65	0.003	12
Dipeptidyl peptidase 2	Q9ET22	Dpp7	0.4	0.011	8	0.75	0.23	6
Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	Q9QUR7	Pin1	0.68	0.198	5	0.5	0.04	3
Integrin alpha-X	Q9QXH4	Itgax	1/∞		2	0.33	0.001	4
Eukaryotic translation initiation factor 3 subunit I	Q9QZD9	Eif3i	0.77	0.399	6	0.73	0.03	9
Acid ceramidase	Q9WV54	Asah1	0.45	5.00E-06	15	0.5	0.1	14

Description	Uniprot ID	Gene	SAX:L-I/C-NI			SAX:C-I/L-NI		
			Ratio 1	p-value	No. Peptides	Ratio 2	p-value	No. Peptides
Macrophage-expressed gene 1 protein	A1L314	Mpeg1	0.43	0.32	7	0.45	0.01	6
Palmitoyl-protein thioesterase 1	O88531	Ppt1	0.29	0.03	7	0.22	0.02	8
Beta-2-microglobulin	P01887	B2m	0.61	0.39	4	1/∞		2
Cathepsin D	P18242	Ctsd	0.30	9.90E-07	26	0.77	0.21	20
Acid sphingomyelinase-like phosphodiesterase 3a	P70158	Smpdl3a	0.50	0.03	12	0.80	0.38	10

1145	Caspase-7	P97864	Casp7,Lice2	0.69	0.56	4	0.69	0.03	3
1146	Ral guanine nucleotide dissociation stimulator-like 2	Q61193	Rgl2,Rab21, Rlf	1/∞		2	0.71	0.74	2
1147	Macrophage mannose receptor 1	Q61830	Mrc1	0.67	0.09	24	0.31	0.01	17
	60S ribosomal protein L10	Q6ZWW3	Rpl10,Qm	0.35	0.7	4	0.6	0.01	3
	G-protein coupled receptor 84	Q8CIM5	Gpr84	1/∞		3	1/∞		2
	Arfaptin-2	Q8K221	Arfp2	1/∞		2	0.64		1
	tRNA-dihydrouridine(47) synthase [NAD(P)(+)]-like	Q91XI1	Dus3l	1/∞		2	0.68	0.60	2
	Transmembrane 9 superfamily member 1	Q9DBU0	Tm9sf1	1/∞		2	0.64	0.76	2

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