ACS Infectious Diseases

Article

Cite This: ACS Infect. Dis. 2019, 5, 1295−1305

pubs.acs.org/journal/aidcbc

Comparative Proteomic Analysis of Murine Cutaneous Lesions Induced by *Leishmania amazonensis* or *Leishmania major*

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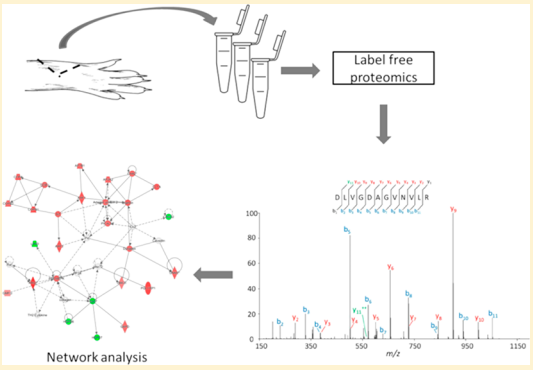
Supporting Information

ABSTRACT: Cutaneous leishmaniasisis is the most common clinical form of leishmaniasis and one of the most relevant neglected diseases. It is known that the progress of the disease is species specific and the host's immune response plays an important role in its outcome. However, the pathways that lead to parasite clearance or survival remain unknown. In this work, skin tissue from mice experimentally infected with *L. amazonensis*, one of the causative agents of cutaneous leishmaniasis in the Amazon region, *L. major*, another causative agent of cutaneous leishmaniasis in Africa, the Middle East, China, and India, or lipopolysaccharides from *Escherichia coli* as an inflammation model were investigated using label-free proteomics to unveil *Leishmania*-specific protein alterations. Proteomics is a powerful tool to investigate host−pathogen relationships to address biological questions. In this work, proteins from mice skin biopsies were identified and quantified using nano-LC coupled with tandem mass spectrometry analyses. Integrated Proteomics Pipeline was used for peptide/protein identification and quantification. Western blot was used for validation of protein quantification by mass spectrometry, and protein pathways were predicted using Ingenuity Pathway Analysis. In this proteomics study, several proteins were pointed out as hypothetical targets to guide future studies on *Leishmania*-specific modulation of proteins in the host. We identified hundreds of exclusively modulated proteins after *Leishmania* spp. infection and 17 proteins that were differentially modulated in the host after *L. amazonensis* or *L. major* infection.



KEYWORDS: *cutaneous leishmaniasis, host−pathogen interaction, label-free proteomics, mass spectrometry*

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The neglected tropical disease Leishmaniasis is a parasitic disease caused by infection with *Leishmania* parasites, which are spread by the bites of phlebotomine sand flies.[1] Leishmaniasis comprises multiple clinical forms, including cutaneous, mucosal, and visceral forms, which result from infection of macrophages in the dermis, in the naso-oropharyngeal mucosa, and throughout the reticuloendothelial system, respectively. For all three forms, the infection can range from asymptomatic to severe.[2] Cutaneous leishmaniasis (CL) is the most common form of the disease, both in general and in U.S. travelers, causing skin lesions that can persist for months or years. The lesions usually are painless; however, the healing process is quite challenging, and it typically results in atrophic scarring. The number of new CL cases may vary over time, but it is estimated to be 700,000 to 1.2 million annually.[2]

We have focused on two etiological agents of cutaneous leishmaniasis, *L. amazonensis* and *L. major*, to search for protein modulations in the host that are species specific. *L. amazonensis* is one of the causative agents of cutaneous leishmaniasis that is usually limited to South America and is spread throughout the Brazilian Amazon region.[3] *L. major* is another causative agent of cutaneous leishmaniasis that is exclusively found in the eastern hemisphere, northern Africa,[4] the Middle East, northwestern China, and Northwestern India.[5] Using a murine infection model combining traditional histopathology and matrix assisted laser desorption ionization (MALDI) imaging, our group previously demonstrated that *L. amazonensis* leads to more aggressive infection than *L. major*.[6] However, since *in situ* top-down proteomics technologies are/were still under development at the time, we were unable to identify the proteins involved in the pathogenesis of CL.

Proteomic approaches have become powerful tools to understand how host−pathogen interactions impact the outcome of infections.[7] Improvements in the resolution, mass accuracy, sensitivity, and scan rate of mass spectrometers used to analyze proteins have significantly improved proteomic

Received: December 20, 2018
Published: May 16, 2019

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DOI: 10.1021/acsinfecdis.8b00370
ACS Infect. Dis. 2019, 5, 1295−1305

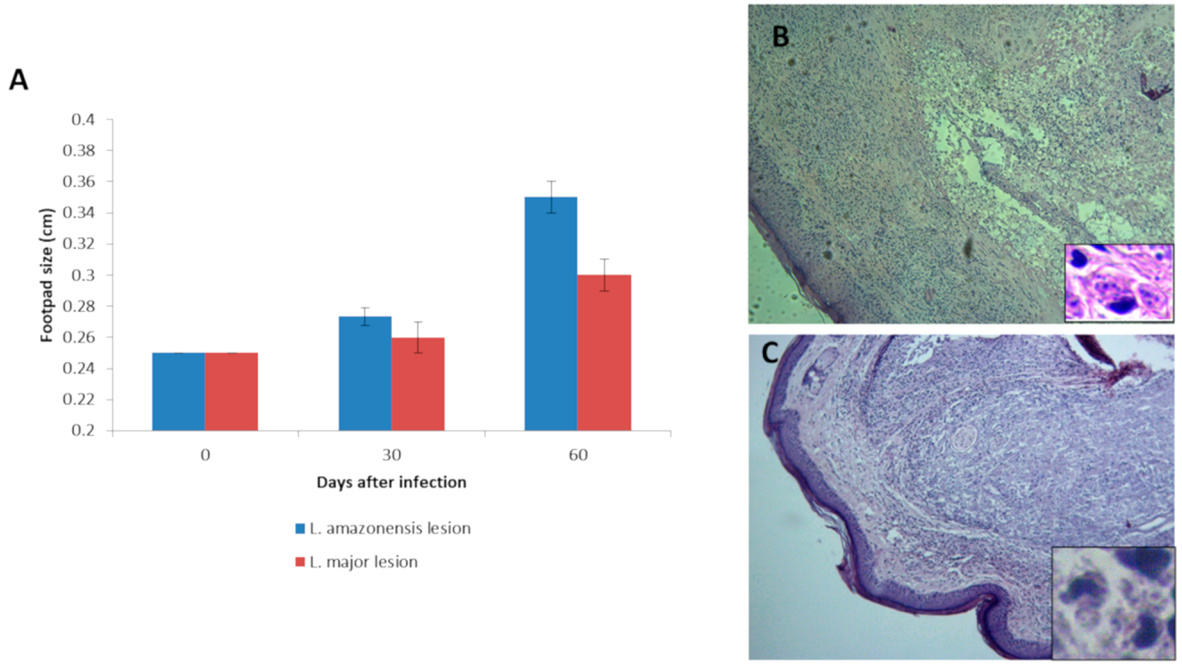

Figure 1. Footpad measurement after *Leishmania* spp. infection (A). Panoramic view of histological analysis of footpads at 60 days after infection with *L. amazonensis*(B) and *L. major* (C) (40×). In both cases, epidermis is preserved, and dermis is severely comprised by necrosis and inflammatory cells. Infected macrophages are shown at the right corner (1000×). The results are representative data from biological quintuplicates.

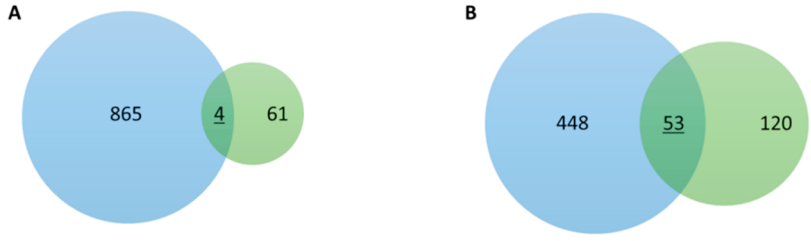

Figure 2. Venn diagrams representing the number of altered proteins in skin biopsies after *L. amazonensis* infection (in blue) and after LPS stimulation (in green) (A) and after *L. major* infection (in blue) and after LPS stimulation (in green) (B).

analysis.[8] In addition, increased awareness from the microbiology community has increased the application of such innovative approaches. The integration of proteomics with other molecular biology methods has expanded the number of tools available to study infections.[7]

The parasite *Leishmania* has already been studied using proteomics,[9−12] but only a few studies used proteomics to investigate the host−pathogen relationship or compare how different species may lead to different outcomes of the disease.[13,14] To the best of our knowledge, this is the first study to employ a proteomics approach to reveal *Leishmania*-specific protein alterations in skin biopsies using mass spectrometry. Proteins were identified and quantified in skin biopsies from mice footpads that were infected with *L. amazonensis* or *L. major* (after 60 days of infection) or were injected with LPS (LPS has been used in animal models to induce inflammation). Biological network analysis was employed to predict altered networks and to address which proteins were involved. In this proteomics study, we identified 17 shared proteins that were differentially modulated between *L. amazonensis* and *L. major* infected skin and hundreds of proteins that were exclusively modulated in infection versus inflammation. Those differentially expressed proteins are believed to have fundamental roles in the outcome of the infections. Additionally, we identified proteins as potential targets for further investigation that would contribute to discover new approaches to control the infection.

RESULTS AND DISCUSSION

Disease Progression and Skin Biopsies. The course of CL was monitored using a dial caliper through the measurements of footpad enlargement after *Leishmania* spp. infection (Figure 1A). According to our previous work, histological analysis had shown that the disease progression was more aggressive for *L. amazonensis* than it was for *L. major*.[6] After 30 days of infection, the dermis of footpads was still preserved in mice infected with *L. major*, whereas mice infected with *L. amazonensis* had revealed signs of necrosis into the dermis. At 60 days post-infection, necrosis had started to progress in *L. major* infection (Figure 1B,C). A positive control of inflammation was also analyzed by injecting LPS into the footpad dermis of another set of animals.[15]

Label-Free Proteomics of Skin Lesions. Label-free proteomics of skin lesions from Balb/c mice footpads were performed to search for *Leishmania*-specific protein modulation in the host after *L. amazonensis* or *L. major* infection (both compared to an inflammation model using LPS). Five biological replicates of each condition (LPS stimulated, noninfected,

DOI: 10.1021/acsinfecdis.8b00370
ACS Infect. Dis. 2019, 5, 1295−1305

L. amazonensis, or *L. major* infected footpads) were analyzed. The entire skin biopsy was used to obtain the tissue homogenate so that microenvironment changes might be present. Table S1 shows the total number of peptides and protein IDs that were identified and quantified for each sample, demonstrating the reproducibility of the analysis. The census output table is available as Supporting Information. *t* tests were performed as a univariate statistical analysis, and fold changes were independently calculated to access changes in protein modulation to provide descriptive information from data sets. Venn diagrams compare the number of proteins altered in uninfected versus infected skin versus LPS stimulated skin (Figure 2). Using label free quantitative proteomics, a total of 865 proteins were found to be modulated (250 up- and 615 downregulated) after *L. amazonensis* infection. On the other hand, after LPS stimulation, only 65 proteins were modulated, 4 of which were shared with infection and showed opposite regulation (Figure 1A). Table 1 reveals fold change (FC) of protein IDs that were shared between *L. amazonensis* infection and inflammation. Our results revealed that 448 proteins were regulated after *L. major* infection (424 up- and 24 downregulated), while after LPS stimulation, 173 proteins were modulated and 53 of them were shared with infection (Figure 1B). Table 2 reveals FC and opposite modulation of protein IDs that were shared between *L. major* infection and inflammation. Western blots of proteins PCNA and ARG1 were performed to validate its quantification (Figure 3). PCNA was differentially modulated after the infections caused by *L. amazonensis* and *L. major*, while Arg1 was upregulated in the host after *Leishmania* spp. infections (see Table 3).

Table 1. List of Proteins Oppositely Modulated in the Host after *L. amazonensis* Infection Compared to LPS Inflammation

			FC	
gene	Uniprot ID	protein name	LA[a]	LPS[b]
Pcna	P17918	proliferating cell nuclear antigen	0.78459	1.28744
Ryr1	E9PZQ0	ryanodine receptor 1	0.750908	1.38926
Cox6a1	P43024	cytochrome c oxidase subunit 6A1	0.78295	1.543484
Hnrnpul2	Q00PI9	heterogeneous nuclear ribonucleoprotein U-like protein 2	0.785404	1.352445

[a]LA: *L. amazonensis* infected tissue. [b]LPS: Lipopolysaccharide stimulated tissue.

IPA-Ingenuity Systems analysis was used as a tool to indicate the networks most affected after infection. Analysis is based on the *z*-score, a statistical parameter that is used to infer likely activation states of upstream regulators based on comparison with a model that assigns random regulation directions (Figure 4A–F).[16] Our results revealed activation of beta-2 microglobulin (B2m) and RhoGTPase Cdc42 (Cdc42) and decorin (Dnc) and inhibition reticulon-4 (Rtn4), myotrophin (Mtpn), and myocilin (Myoc) after *L. amazonensis* infection. B2m, a component of the class I major histocompatibility complex (MHC I), is involved in the presentation of peptide antigens to the immune system.[17] B2m levels also rise during infection with some viruses, including cytomegalovirus and human immunodeficiency virus (HIV).[18] Cdc42 is known to be important in several aspects of B-lymphocyte cell biology, including B-lymphocyte cell development and activation and plasma cell differentiation.[19]

After *L. amazonensis* infection, we observed activation of Dcn, a protein that is known to activate the proinflammatory immune response by binding to Toll-like receptors (TLRs) 2 and 4. Activation of TLRs stimulates programmed cell death and results in decreased release of the anti-inflammatory cytokine interleukin-10 (IL-10).[20] Stimulations of TLRs are known to play species-specific roles after leishmaniasis infection.[21] The outcomes of TLR2 and -4 stimulation could be either parasite persistence or clearance based on the *Leishmania* species. In *L. amazonensis*, binding at TLR2 has been shown to promote parasite persistence by inducing superoxide dismutase.[21] Even though Dcn does not change expression after *L. major* infection, TLRs can be important candidates for immunotherapy and vaccine development based on their role in influencing the outcome of the infection. The role of Rtn-4 in inflammation is still largely unknown. However, it is recognized that the loss of proteins from the Rtn-4 family suppresses the acute inflammatory response.[22] We found inhibition of Rtn-4 after 60 days of infection, thus confirming suppression of acute inflammatory response and leading to parasite persistence (chronicity). Proteins from the Rtn-4 family could be interesting targets to stimulate the acute inflammatory response, especially during the early phase of the infection to promote effective parasite clearance.

The role of Mtpn in infection and inflammation is still unknown. Nevertheless, our results reveal decreased expression of Mtpn after *L. amazonensis* infection. Mtpn is known to play the essential regulatory function in stabilizing the integrity of the cytoskeleton. It is most likely that its degradation leads to the loss of its function, leading to reorganization and destabilization of actin filaments, which is essential for the apoptosis-related morphological changes. However, beyond the specific degradation process, it is still unclear whether the disruption of cytoskeleton proteins is an amplifying step for the apoptotic process or if their degradation is a consequence of apoptosis.[23]

We demonstrated that *L. amazonensis* infection led to decreased expression of Myoc, a protein that was revealed to be associated with the regulation of cell growth and survival by promoting cell proliferation and resistance to apoptosis via the ERK1/2 MAPK signaling pathway.[24,25] After *L. major* infection, IPA-Ingenuity Systems upstream analysis revealed activation of B2m and Cdc42 as well as signal transducer and activator of transcription (Stat1) and plasminogen (Plg) (Figure 4A,B,G–

Table 2. List of Proteins Oppositely Modulated in the Host after *L. major* Infection Compared to LPS Inflammation

			FC	
gene	Uniprot ID	protein name	LM[a]	LPS[b]
Nampt	Q99KQ4	nicotinamidephosphoribosyltransferase	1.265782	0.731764
Ube2l3	P68037	ubiquitin-conjugating enzyme E2 L3	1.245238	0.696653

[a]LM: *L. major* infected tissue. [b]LPS: Lipopolysaccharide stimulated tissue.

DOI: 10.1021/acsinfecdis.8b00370
ACS Infect. Dis. 2019, 5, 1295–1305

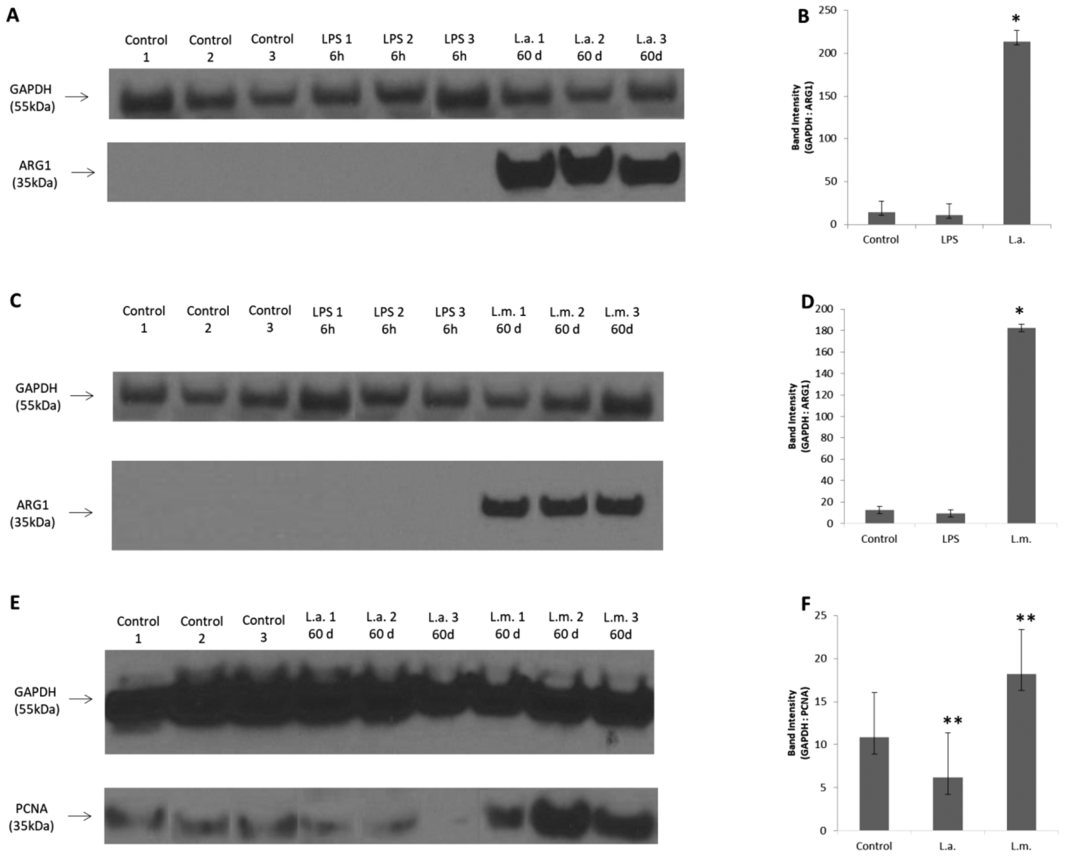

Figure 3. Western blot validation of protein quantification. The extracts of uninfected skin (control) or skin infected with *L. amazonensis* (L.a.) or LPS stimulated (A) or *L. major* (L.m.) (B). The proteins were then immunostained using anti-Arg1 chain or anti-GAPDH as a loading control, followed by incubation with antimouse and antirabbit secondary antibodies, sequentially. Bands at 35 kDa represent protein Arg1, and bands at 55 kDa correspond to GAPDH. Western blot images were acquired on a scanner station and analyzed for mean intensity above background. GAPDH: Arg1 ratio (B, D) represents normalized band intensities after *L. amazonensis* or *L. major*, respectively. (E) The extracts of uninfected skin (control) or skin infected with *L. amazonensis* (L.a.) or *L. major* (L.m.) The proteins were then immunostained using anti-PCNA or anti-GAPDH as a loading control, followed by incubation with antirabbit secondary antibodies. Bands at 35 kDa represent protein PCNA, and bands at 55 kDa correspond to GAPDH. the GAPDH/PCNA ratio (F) represents normalized band intensities after *L. amazonensis* or *L. major*. The results represent data from triplicates, and the statistics validation using the Mann−Whitney Test revealed a *p*-value of 0.003* and 0.04** after infection.

H). Since Stat1 expression is enhanced after both infection and inflammation, it does not point to *Leishmania* specific alterations. However, it may play an important role in the pathophysiology of general inflammation and inflammation triggered by the presence of the parasite. *L. major* infection promoted increased expression of Plg in the host, which is known to bind to plasmin from the promastigote form of *Leishmania* spp. Likewise, a previous study reported that Plg activation was enhanced by the presence of *L. mexicana* promastigotes, raising the question whether this interaction is involved in the virulence of the parasite.[26] The Plg binding by plasmin from the parasite could thus be part of the pathogenesis mechanism, similar to many other pathogenic organisms that degrade the extracellular matrix to facilitate access to macrophages.[27]

We revealed proteins that were exclusively downregulated after *L. amazonensis* infection and upregulated after LPS stimulation (Table 1). Proliferating cell nuclear antigen (Pcna), ryanodine receptor 1 (Ryr1), cytochrome c oxidase subunit 6A1 (Cox6a1), and heterogeneous nuclear ribonucleoprotein U-like protein 2 (Hnrnpul2) were downregulated after infection and upregulated after inflammation.

Pcna, a nuclear factor involved in DNA replication and repair of proliferating cells, is known to be a key regulator of neutrophil survival.[28] Neutrophils are among the first-responders of inflammatory cells and migrate toward the site of inflammation. It is important to understand the strategic pathways used by neutrophils that control their survival during parasite clearance.[29] After *L. amazonensis* infection (60 days), histology showed that the main population in infected tissue is composed of macrophages,[6,30] and we saw a decreased expression of Pcna. We also revealed a Pcna increase after inflammation, where the main population is composed by *polymorphonuclear cells* (i.e., neutrophils), suggesting that Pcna may stimulate sustained inflammation by neutrophils and that this increase in Pcna expression may be ideal for parasite clearance. A previous study reported that the activation of Ryr1, a protein expressed by dendritic cells, leads to an enhanced MHC class II response.[31] Our results reveal downregulation of Ryr1 after *L. amazonensis* infection that might impair the immune response of the host. It has also been reported that the overexpression of Cox6a1in yeast and U373MG cells has a protective effect against reactive oxygen species (ROS)-induced cell damage.[32] Our results revealed decreased expression of Cox6a1 after *L. amazonensis* infection that may impair cellular function caused by ROS-induced cell damage and therefore parasite killing. Likewise, Hnrnpul2, involved in RNA binding,[33] had also shown decreased expression after *L. amazonensis* infection and increased

DOI: 10.1021/acsinfecdis.8b00370
ACS Infect. Dis. 2019, 5, 1295−1305

Table 3. Most Statistically Relevant Proteins Shared after *Leishmania* spp. Infection[a]

gene	Uniprot ID	protein name	FC		
			LA[b]	LM[c]	LPS[d]
Arg1	Q61176	arginase-1	1.700315	1.807856	–
Mybpc2	Q5XKE0	myosin-binding protein C	0.366403	0.664556	–
Myot	Q9JIF9	myotilin	0.494938	0.782542	–
Ldb3	Q9JKS4-6	LIM domain-binding protein 3	0.45933	0.565528	0.536713
Tpm3	P21107	tropomyosin alpha-3 chain	0.450544	0.703612	0.697754
Pdlim5	Q8CI51-3	PDZ and LIM domain protein 5	0.465507	0.729645	–
Synpo2	Q91YE8	synaptopodin-2	0.492965	0.597402	–
Angptl7	Q8R1Q3	angiopoietin-related protein 7	0.529889	0.643825	0.750165
B2m	P01887	beta-2-microglobulin	1.657486	1.702183	–
Kera	O35367	keratocan	0.47419	0.707123	–
Myoz2	Q9JJW5	myozenin-2	0.466593	0.709803	0.795126
Hspb7	P35385	heat shock protein beta-7	0.474423	0.567254	–
Rap1b	Q99JI6	Ras-related protein Rap-1b	1.468064	1.344776	–
Pdlim3	O70209	PDZ and LIM domain protein 3	0.470542	0.769031	–
Tnnt1	O88346	troponin T	0.476769	0.707094	–
H2-D1	P01900	H-2 class I histocompatibility antigen	1.560654	1.736072	–
Hspb6	Q5EBG6	heat shock protein beta-6	0.441072	0.749809	–
Acsl1	P41216	long-chain-fatty-acid−CoA ligase 1	0.552194	0.775141	–
H2-L	P01897	H-2 class I histocompatibility antigen	1.564021	1.386619	–
Gbe1	Q9D6Y9	1,4-alpha-glucan-branching enzyme	0.427248	0.619501	0.706621
Ear1	P97426	eosinophil cationic protein 1	1.650182	1.650515	–
Ctsz	Q9WUU7	cathepsin Z	1.478789	1.683503	–
S100a9	P31725	protein S100-A9	1.74318	1.915599	–
Serpina3g	Q5I2A0	serine protease inhibitor A3G	1.652822	1.763581	–
Ndufb10	Q9DCS9	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	0.4708	0.712756	0.629384
Arpc1b	Q9WV32	actin-related protein 2/3 complex subunit 1B	1.522804	1.670217	–
Ctsh	P49935	pro-cathepsin H	1.563842	1.693833	–
Pdia4	P08003	protein disulfide-isomerase A4	1.46126	1.592716	–
Xdh	Q00519	xanthine dehydrogenase/oxidase	1.471983	1.57876	–
H2-Ab1	P01921	H-2 class II histocompatibility antigen, A-D beta chain	1.598611	1.774666	–
Rab5c	P35278	Ras-related protein Rab-5C	1.471153	1.42828	–
Psap	Q61207	prosaposin	1.468435	1.682252	–
Sh3bgr	Q9WUZ7	SH3 domain-binding glutamic acid-rich protein (SH3BGR protein)	0.461401	0.575635	0.538676
Rplp2	P99027	60S acidic ribosomal protein P2	1.592227	1.379012	–
Ngp	O08692	neutrophilic granule protein	1.624425	1.810628	–
Ctsc	P97821	cathepsin C	1.483931	1.662364	–
Esyt1	Q3U7R1	extended synaptotagmin-1	1.466252	1.459658	–
Mrc1	Q61830	macrophage mannose receptor 1	1.415654	1.471413	–
Tpd52	Q62393	tumor protein D52	1.637316	1.855927	–
Hk3	Q3TRM8	hexokinase-3	1.343152	1.370757	–
H2-K1	P01902	H-2 class I histocompatibility antigen	1.473553	1.521056	–
Mpo	P11247	myeloperoxidase	1.456884	1.575845	–
Itgb2	P11835	integrin beta-2	1.422160	1.493969	–

[a]Blanks (−) represent nonsignificant fold change (FC) compared to healthy macrophages. [b]LA: *L. amazonensis* infected tissue. [c]LM: *L. major* infected tissue. [d]LPS: Lipopolysaccharide stimulated tissue.

expression after inflammation. Moreover, we revealed proteins that were exclusively upregulated after *L. major* infection and downregulated after LPS stimulation (Table 2). Nicotinamide phosphoribosyltransferase (Nampt) and ubiquitin-conjugating enzyme E2 L3 (Ube2l3) were upregulated after infection and downregulated after inflammation.

Nampt plays a role in many different human diseases, and a new study reports that the incubation of neutrophils from healthy volunteers with recombinant Nampt resulted in dose-dependent inhibition of apoptosis.[34,35] Our results revealed increased expression of Nampt in Balb/c mice lesions after *L. major* infection, but more studies are necessary to confirm the molecular and cellular mechanisms affected by Nampt in leishmaniasis.

Ube2l3 was reported to reduce levels of chikungunya infection without altering RNA genome levels, suggesting that Ube2l3 might mediate the pathogenesis of chikungunya fever.[36] Our results, however, demonstrated increased expression of Ube2l3 after *L. major* infection and decreased expression after inflammation caused by LPS suggesting *L. major* specific alteration of this protein. In this case, Ube2l3 inhibition would be a strategy that could lead to parasite clearance.

Comparison of Leishmania-Specific Protein Regulation in Skin Lesions and Network Analysis. Since the outcome of leishmaniasis depends on the host response and on

DOI: 10.1021/acsinfecdis.8b00370
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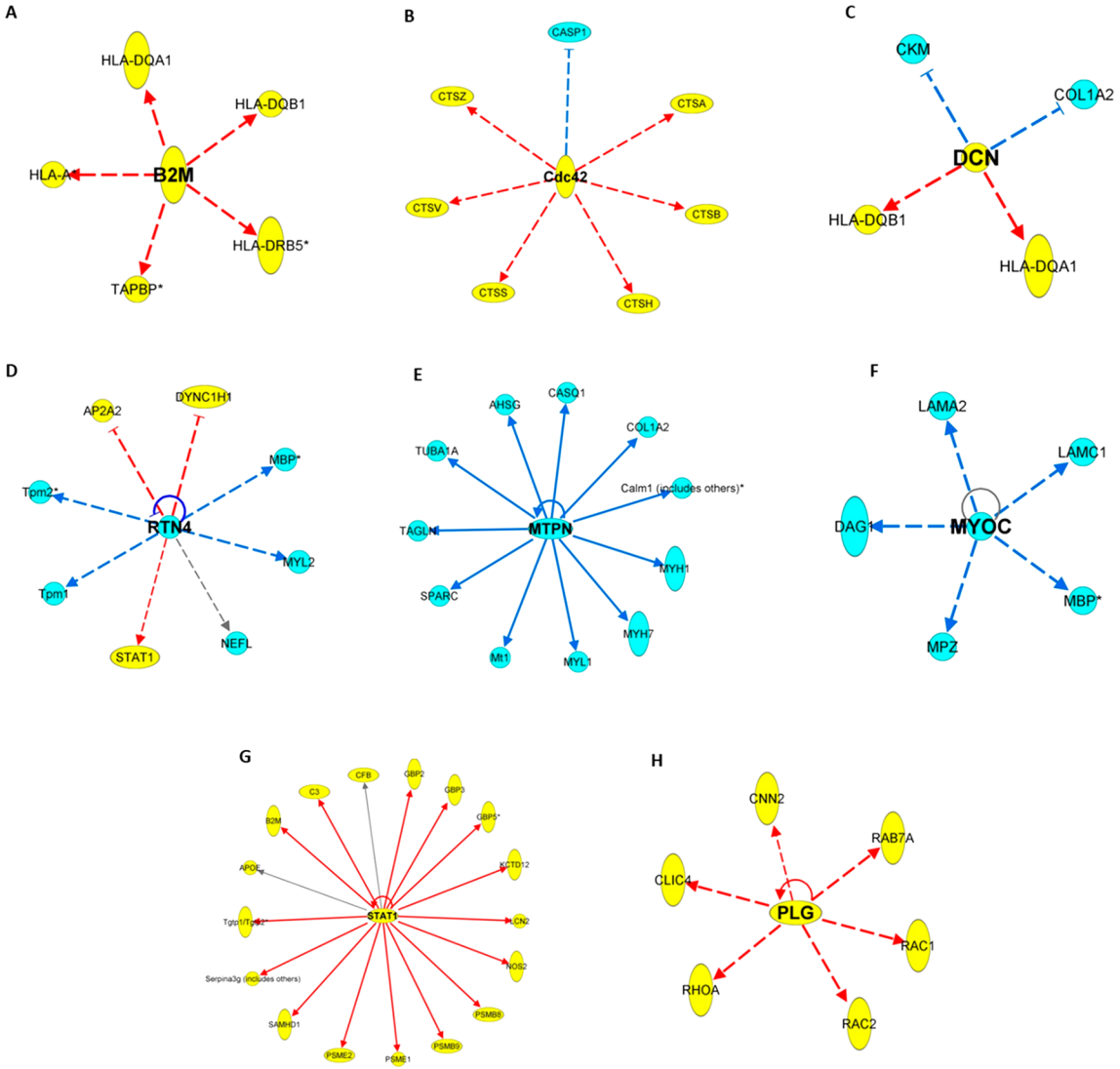

Figure 4. IPA-Ingenuity Systems upstream analysis for (A) beta-2 microglobulin (B2M), (B) rho GTPase Cdc42 (Cdc42), (C) decorin (Dnc), (D) inhibition reticulon-4 (Rtn4), (E) myotrophin (Mtpn), (F) myocilin (Myoc), (G) signal transducer and activator of transcription (Stat1), and (H) plasminogen (Plg). The regulators (nodes) are colored by their predicted activation state: activated (yellow) or inhibited (blue). The edges connecting the nodes are colored red when leading to activation of the downstream node and blue when leading to its inhibition.

the species,[21] it is not surprising that these differences are also observed at the molecular level. A total of 730 proteins were exclusively modulated after *L. amazonensis* infection (54 upregulated and 676 downregulated) and 219 after *L. major* infection (215 upregulated and 4 downregulated). Tables S2 and S3 show the complete list of exclusively modulated proteins after *L. amazonensis* and *L. major* infection and shared proteins between infections, respectively. Shared proteins were used to build network analysis using IPA-Ingenuity Systems. Figure 5 represents proteins that indicate activation of an infectious diseases network.

Arginase-1 (Arg1) is the most upregulated protein after infection. It influences its own upregulation and is induced by mitogen-activated protein kinase (p38 MAPK or MAPK14) signaling. p38 MAPKs are known to respond to environmental stress and inflammatory cytokines.[37] It was previously demonstrated that selected anti-inflammatory drugs inhibit LPS-stimulated cytokine production, and this was linked to the inhibition of p38 MAPK and, therefore, cytokine synthesis. This finding pointed out that p38 MAPK inhibitors are potential anti-inflammatories.[38] p38 MAPK is known to induce IL-12 in *L. major* infected macrophages, and this interleukin suppresses the infection.[39]

Further experiments on the proteins highlighted in this study are encouraged. We believe that Arg1 plays a fundamental role through the course of the infection by inducing MAPK14 signaling. Therefore, we hope to stimulate researchers to evaluate Arg1 expression and its distribution through the lesion at different time points (immunohistochemistry), to evaluate disease progression by inhibiting Arg1 expression (gene knockout experiments or local inhibition of Arg1), and to validate these findings in other mice lines.

L. major infection has also been shown to induce MAPK phosphatase 1 (MPK1), which dephosphorylates p38 MAPK and ultimately leads to ERK1- or ERK2-dependent secretion of IL-10,[40] an interleukin known to enhance *L. major* infection.[39] Our results reveal increased expression of p38 MAPK after infection with *L. amazonensis* or *L. major*, leading to Arg1 upregulation, which is involved in cell signaling and inflammation as will be discussed. Moreover, some heat shock proteins are

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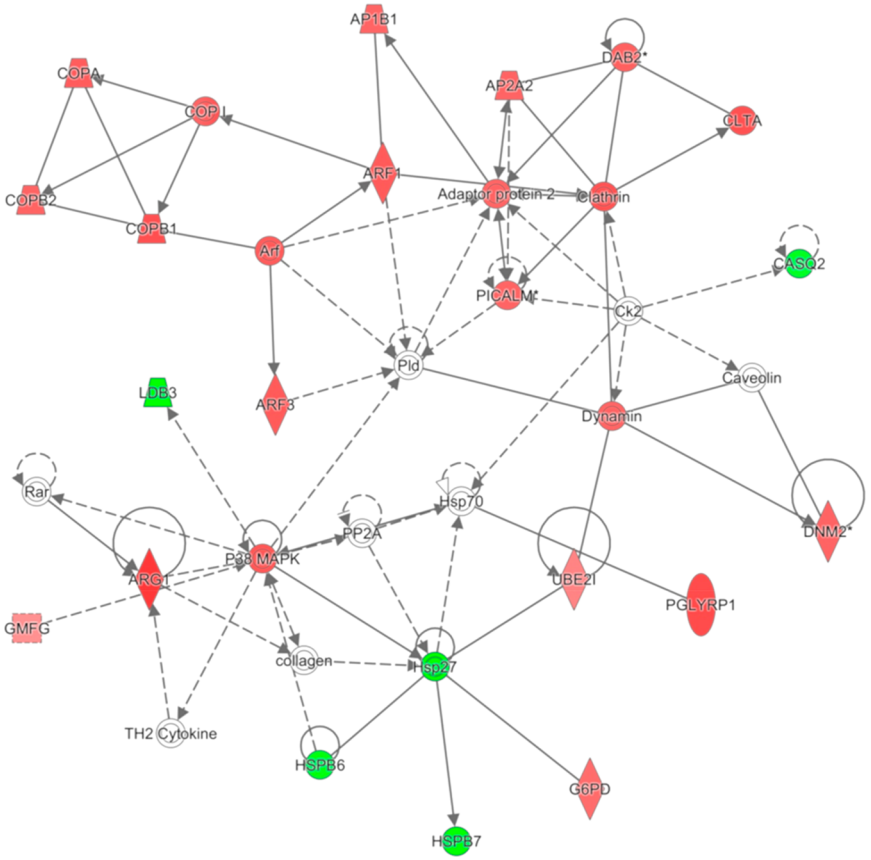

Figure 5. Network analysis for *Leishmania* spp. infected skin involving proteins related to infectious diseases. Network is displayed as a series of nodes (proteins) and strings, corresponding to biological relationships between nodes. Nodes in green had decreased expression after *Leishmania* infection, whereas those in red had increased expression after infection. Unmarked nodes were added through IPA, reflecting a high degree of probability of involvement in a given network. Node color intensity indicates the degree of the up- or downregulation of the proteins observed in the biological network in response to infection. The solid lines denote direct interactions, whereas the dotted lines represent indirect interactions between the proteins represented in this network. Except by the protein glia maturation factor gamma (Gmfg), which is downregulated after *L. amazonensis* infection and upregulated after *L. major* infection, the other ones represented in this network have the same modulation after both infections.

Table 4. List of Differentially Modulated Proteins in Infected Skin after *L. amazonensis* or *L. major* infection[a]

gene	Uniprot ID	protein name	FC		
			LA[b]	LM[c]	LPS[d]
Blvra	Q9CY64	biliverdin reductase A	0.782371	1.540299	–
Ly6c1	P0CW02	lymphocyte antigen 6C1	0.750927	1.461284	–
Mtpn	P62774	myotrophin	0.754762	1.534118	–
Asna1	O54984	ATPase Asna1	0.756631	1.440172	–
Twf1	Q91YR1	twinfilin-1 (protein A6)	0.771688	1.451588	–
Pcna	P17918	proliferating cell nuclear antigen	0.78459	1.661816	1.287439
Ube2i	P63280	SUMO-conjugating enzyme UBC9	0.767996	1.440801	–
Url40fp	A8C9X2	ubiquitin/ribosomal L40 fusion protein-like protein	0.777604	1.967482	–
Eif3i	Q9QZD9	eukaryotic translation initiation factor 3 subunit I	0.741926	1.407393	–
Ppt1	O88531	palmitoyl-protein thioesterase 1	0.78784	1.572093	–
Rtcb	Q99LF4	tRNA-splicing ligase RtcB homologue	0.798028	1.521801	–
Hnrnpul2	Q00PI9	heterogeneous nuclear ribonucleoprotein U-like protein 2	0.785404	1.517389	1.352444
Nans	Q99J77	sialic acid synthase	0.785087	1.477043	–
Itpa	Q9D892	inosine triphosphate pyrophosphatase	0.791197	1.533648	–
Gmfg	Q9ERL7	glia maturation factor gamma	0.432165	1.579511	–
Pnkp	Q9JLV6	bifunctional polynucleotide phosphatase/kinase	0.798143	1.560319	–
Trim28	Q62318	transcription intermediary factor 1-beta (tripartite motif-containing protein 28)	0.73376	1.442516	–

[a]Blanks (−) represent nonsignificant fold change (FC) compared to healthy macrophages. [b]LA: *L. amazonensis* infected tissue. [c]LM: *L. major* infected tissue. [d]LPS: Lipopolysaccharide stimulated tissue.

DOI: 10.1021/acsinfecdis.8b00370
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downregulated in the host after *Leishmania* spp. infection. Table 3 reveals the most statistically relevant proteins that were shared after *L. amazonensis* or *L. major* infection (see Table S4 for the complete list of shared proteins between infections).

IPA-Ingenuity Systems analysis reveals that increased expression of Arg1, protein S100-A9 (S100a9), and integrin beta-2 (Itgb2) is involved in inflammation. Along with serine protease inhibitor A3G (Serpina3g) and heat shock protein beta-6 (Hspb6), these proteins are involved in the apoptotic processes. Our results revealed upregulation of Serpina3g and downregulation of Hspb6 in infected skin. Upregulation of Arg1, H-2 class II histocompatibility antigen A-D beta chain (H2-Ab1), S100a9, H-2 class I histocompatibility antigen (H2-L), H-2 class I histocompatibility antigen (H2-D1), B2m, Serpina3g, H-2 class I histocompatibility antigen (H2-K1), and procathepsin H (Ctsh) is involved in the activation of the immune response. Downregulation of long-chain-fatty-acid−CoA ligase 1 (Acsl1) is involved in lipid metabolism and cell signaling. Increased expression of Arg1, Itgb2, H2-Ab1, Ras-related protein Rap-1b (Rap1b), Ctsh, xanthine dehydrogenase/oxidase (Xdh), cathepsin Z (Ctsz), and tumor protein D52 (Tpd52) is related to enhanced cell proliferation.

We have also looked at 17 proteins that were divergently modulated after *L. amazonensis* or *L. major* infection (Table 4) and compared these results to expression in inflammation to identify proteins exclusively modulated after *Leishmania* spp. infection. IPA-Ingenuity Systems analysis shows that eukaryotic translation initiation factor 3 subunit I (Eif3i) and transcription intermediary factor 1-beta (Trim28) enhances apoptotic events. It was previously described that early stages of *L. major* infection (24 h) have an inhibitory effect on induced apoptosis, while showing partial induction of apoptosis at later stages (48−72 h).[25] Likewise, Eif3i and Trim28 were upregulated after *L. major* infection. However, some of the molecular pathways are species specific.[21] In our results, Eif3i and Trim28 were downregulated after *L. amazonensis* infection, suggesting that these proteins might be involved in inhibition of apoptosis for *L. amazonensis* infection.

It was demonstrated that the enzyme biliverdin reductase A (Blvra) induces proinflammatory responses in macrophages and thus elevates levels of TNFα and enhances expression of toll-like receptors.[41] To the best of our knowledge, the role of this enzyme has not yet been described for leishmaniasis. Our results revealed downregulation of Blvra after *L. amazonensis* infection and upregulation after *L. major* infection, thus indicating species-specific regulation of this enzyme.

Another enzyme, inosine triphosphate pyrophosphatase (Itpa), was revealed to be downregulated after *L. amazonensis* infection and upregulated after *L. major* infection. Itpa is known to be an important factor in maintaining the stability of the genome since its absence results in accumulation of nucleotides that ultimately leads to cell dysfunctions.[42] The downregulation of Itpa after *L. amazonensis* infection is notable since the progress of infection caused by *L. amazonensis* is more aggressive than for *L. major*, in which Itpa is upregulated.

It has been reported that Glia maturation factor gamma (Gmfg) negatively regulates TLR4-induced proinflammatory signaling, but its function in macrophage response to infections remains unclear.[43] This study also revealed that knockdown of Gmfg resulted in decreased expression of ferroportin (iron exporter) and increased iron storage ferritin-L protein levels in Raw264.7 macrophages infected with *Salmonella* as a host defense strategy to limit microbes from accessing iron. Gmfg-knockdown macrophages also showed enhanced expression of proinflammatory TNF-alpha and anti-inflammatory IL-10 after infection. Even though our results do not show statistically significant alteration of this protein after LPS stimulation, they do show opposite modulation after *L. amazonensis* (downregulation) or *L. major* infection (upregulation). Knowing that Gmfg antagonizes TLR4 signaling and that Gmfg is upregulated after *L. major*, one can conclude that *L. major* infection leads to decreased proinflammatory response compared to *L. amazonensis* infection.

CONCLUSIONS

We have compared how *L. amazonensis* or *L. major* specifically affect protein expression in infected skin relative to inflammation induced by LPS. Upstream analysis revealed that *L. amazonensis* infection activates the expression of B2m, Cdc42, and Dcn that are involved in MHC class I activation, B-lymphocyte development, and binding to TLRs, respectively, and suppresses Rtn-4, Mtpn, and Myoc expression, the former leading to suppression of acute inflammation and the latter two leading to resistance to apoptosis. Likewise, *L. major* infection also activated expression of B2m, Cdc42, Stat1, and Plg. Moreover, 865 and 448 proteins were exclusively modulated in infected skin after *L. amazonensis* and *L. major* infection, respectively. Compared to the inflammation model, *L. amazonensis* infection specifically presented decreased Pcna that impairs neutrophil functions, decreased Ryr1 that impairs proper activation of MHC II, and decreased Cox6a1 that impairs protective ROS-cell damage. *L. major* infection specifically showed increased Nampt, which inhibits neutrophil apoptosis, and increased Ube2l3, which has no role yet reported for leishmaniasis. However, since an increase in Ube213 is a *Leishmania*-specific alteration, it could be an interesting target for further investigation.

Several proteins involved in inflammation, apoptotic processes, activation of immune response, lipid metabolism, and cell proliferation were found in common after leishmaniasis infections, and some were differentially modulated depending on the species. For instance, Arg1 leads to p38 MAPK activation, which subsequently leads to ERK1- or ERK2-dependent secretion of IL-10 for both leishmaniasis infections. However, our results reveal that an increase in Arg1 modulates Gmgf expression differentially for *L. amazonensis* or *L. major* infection. Thus, selected *Leishmania*-mediated alteration of proteins may influence the immunoregulatory function in the host. Our results have revealed some interesting proteins/pathways that may contribute to a better understanding of the pathogenesis of different species of *Leishmania*. Therefore, we encourage further studies on the proteins highlighted in this work to elucidate several mechanisms of *Leishmania* spp. infection.

METHODS

Parasite Cultures. *L. amazonensis*, strain MHOM/BR/67/M2269, and *L. major*, strain *Friedlin*, were maintained by regular passage in BALB/c mice in accordance with the Ethics Committee on the Use of Animals number 4041-1 (CEUA/UNICAMP Universidade Estadual de Campinas). *L. amazonensis* and *L. major* promastigotes were kept in RPMI medium containing 10% FBS and 50 μg/mL gentamicin at pH 7.4. Parasite cultures were maintained at 26 °C. The starter culture contained 10^5 promastigotes in 5 mL of medium, and the parasites were used for inoculation in the Balb/c mice footpad.

DOI: 10.1021/acsinfecdis.8b00370
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Skin Lesion Biopsies. Five-week-old female BALB/c mice, obtained from the Centro de Bioterismo/UNICAMP, were infected through one of their footpads with 5×10^6 promastigotes of *L. amazonensis* or *L. major* in accordance with the Ethics Committee on the Use of Animals number 4041-1 (CEUA/UNICAMP Universidade Estadual de Campinas). Positive controls of inflammation were obtained after inoculation of 50 μg of lipopolysaccharide (LPS) solution (1 μg/μL) in the footpad. Footpads from uninfected mouse were used as negative controls. Mice were sacrificed by cervical dislocation after 60 days of infection and after 3 h of LPS inoculation. Biological experiments were performed in quintuplicate. Haematoxylin and eosin staining was used for the histological analysis of the tissue sections, which was performed to confirm the infection.[44]

Protein Extraction and Digestion Protocol. Skin biopsies were snap frozen in liquid N_2 and ground; protein extraction and digestion followed. Protein extraction was performed in 300 μL of lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris, and 2% CHAPS) supplemented with PMSF as protease inhibitor. All reagents were from Sigma-Aldrich. The solutions were homogenized for 240 min and centrifuged at 13 800*g* for 20 min. As a cleanup step, proteins were precipitated using chloroform/methanol precipitation (3:4 v/v) before digestion.

Samples were resuspended in 100 mM TEAB buffer, and protein concentration was determined using the Pierce BCA Protein Assay Kit from Thermo Scientific. A total of 100 μg of protein per sample was used for digestion. TCEP was added to a 5 mM final concentration to reduce solubilized proteins, and proteins were alkylated by adding 100 mM chloroacetamide to 55 mM final concentration followed by 15 min of incubation in the dark. 100 mM of calcium chloride was added to a 1 mM final concentration, and proteins were digested by incubating with 1:50 (enzyme/protein, w/w) trypsin overnight at 37 °C with vigorous orbital shaking. Digestion was interrupted by adding 90% trifluoroacetic acid solution to a 5% final concentration.

Data Acquisition. Data was acquired using a nano LC-MS/MS coupled to an LTQ-OrbitrapVelos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were randomly injected using an autosampler and eluted onto a self-packed C18 emitter column (100 μm inner diameter × 3 μm × 20 mm). Chromatographic gradient ran from 1% to 80% solvent B (acetonitrile) over 120 min at 400 nL/min flow rate. To avoid carry over effects, blanks were injected between samples. An LTQ-OrbitrapVelos was operated in data-dependent acquisition mode with the XCalibur software. MS survey scans within a 400−2000 m/z range were acquired on the Orbitrap with resolution set to 60 000 (fwhm). The 20 most intense ions per survey scan were selected for collision-induced dissociation (CID) fragmentation, and the resulting fragments were analyzed in the linear trap (LTQ). Dynamic exclusion was employed within 120 s to prevent selection of the same peptide. Raw data is available at ftp://massive.ucsd.edu/MSV000083623; ftp://massive.ucsd.edu/MSV000083622; ftp://massive.ucsd.edu/MSV000083621; https://massive.ucsd.edu/ProteoSAFe/status.jsp?task=c9b7cbb933fc45f88eb1b757938eac2f.

Protein Identification, Quantification, and Normalization. Raw files were extracted into ms1 and ms2 files from raw files using Raw Converter 1.1.0.19. Protein identification was performed using the Integrated Proteomics Pipeline-IP2 (Integrated Proteomics Applications. Using a minimum length of six amino acids, mass spectra were searched for peptide identifications with ProLuCID.[45] ProLuCID results were filtered using DTASelect2[46] to a false discovery rate (FDR) at the protein level of 1%. All tandem mass spectrometry (MS/MS) spectra were compared using the search algorithm ProLuCID[45] against theoretical mass spectra calculated from the *in silico* digested reference database using a decoy strategy.[47] In order to differentiate between macrophage and *Leishmania* proteins, the MS/MS spectra were searched against a combined database (UniprotKB/*Mus musculus* (mouse); *Leishmania amazonensis*; *L. major* release 2018_04). Only proteins belonging exclusively to the *Mus musculus* database were considered for further analysis. Precursor mass tolerance was set to 50 ppm and fragment ion tolerance, to 600 ppm for CID spectra with carbamidomethylation of cysteine as static modification. The FDR was calculated on the basis of the number of Peptide Spectrum Matches (PSMs) that matched sequences in the reverse decoy database of *Mus musculus*.

Census[48] was employed for the quantification of proteins at the MS1 level using an intensity based approach that involves integration of chromatographic peak areas of all the peptides of a given protein. Peptides were evaluated after first searching with ProLuCID, and a peptide needs only to be identified in one of the replicates to be quantified (missing values were retrieved in the aligned chromatogram). MS1 scans were used by Census for chromatogram reconstruction and alignment using dynamic time warping.[48] Thus, the process for quantification of proteins is based on the *area under the curve* (AUC) by measurement of ion abundance at specific retention times for given ionized peptides. Protein abundances were normalized by the sum of all peptides measured for a given sample, and log base 2 transformation was applied.

Statistical Analysis. Five biological replicates were measured for each biological condition (control, *L. amazonensis* or *L. major* infected footpads, and LPS stimulated footpads). Normalized data were accessed by fold change and univariate analysis. Fold change was determined by the ratio *group x/group y*, where "*group x*" is the average of normalized intensity for condition *x* and "*group y*" is the average of normalized intensity for a healthy macrophage. *p*-values were calculated to access changes in protein expression in macrophages after different stimulations (*Leishmania* spp. or LPS). Using *t* tests as univariate analysis, *p*-values were individually calculated by comparing two conditions at a time (control versus infection; control versus inflammation). *p*-values were corrected using the Benjamini−Hochberg procedure.[49] First, each individual *p*-value was ranked from smallest to largest (from 1 to n); then, the correction was calculated by the equation: $(i/m) \times Q$, where i is the rank, m is the total number of tests, and Q is the false discovery rate that was set as 5% (0.05). The largest *p*-value that had $p < (i/m) \times Q$ was considered as significant, and all of the *p*-values smaller than it are also significant. Corrected *p*-values < 0.05 were considered as statistically significant. Fold changes ± 1.2 and corrected *p*-values < 0.05 were considered as statistically significant.

Validation of Protein Quantification Using Western Blot Analysis. Western blot analysis was performed to validate the proteins whose quantification pattern suggested differential expression in the host after *L. amazonensis* or *L. major* infection (PCNA) and another one which was exclusively modulated after *Leishmania* spp. infection (ARG1). A total of three new lysates, other than the ones used for mass spectrometry quantification, were used for the Western blot assay. For ARG1, a total of 10 μg of lysates from uninfected, LPS stimulated, or *L. amazonensis* or *L. major* infected footpads was loaded onto a Bolt 4−12% Bis-

DOI: 10.1021/acsinfecdis.8b00370
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Tris Plus Gel (Thermo Scientific) and transferred to a nitrocellulose membrane (Thermo Scientific). For PCNA, a total of 20 μg of lysates was loaded. PCNA was upregulated after *L. major* infection and downregulated after *L. amazonensis* infection; ARG1 was upregulated after infections and showed no modulation after the inflammation. First, the proteins were immunostained using anti-ARG1 antigen (from Proteintech, 16001-1-AP) diluted to 1:1000 with blocking buffer (5% nonfat dry milk in Tris buffered saline with Tween 20 (Sigma-Aldrich)) followed by incubation with the secondary antibody (HRP, Thermo Scientific). In a separate membrane, proteins were immunostained with anti-PCNA antigen (from Santa Cruz Biotechnology, Inc., PC10) diluted to 1:200 with blocking buffer (5% nonfat dry milk in Tris buffered saline with Tween 20 (Sigma-Aldrich)) followed by incubation with the secondary antibody antirabbit HRP (ab205718, Abcam) diluted to 1:5000 with blocking buffer. Sequentially, Anti-GAPDH (ab181602, Abcam) diluted 1:1000 with blocking buffer was used as a loading control followed by incubation with a secondary antibody antirabbit HRP (ab205718, Abcam) diluted to 1:5000 with blocking buffer. The ECL Western Blotting kit (#32106, Thermo Scientific) was used as the chemiluminescence reagent. ImageJ[50] was used to access the fold change by densitometry (triplicate), and statistical analysis was performed using the Mann−Whitney U Test.

IPA Network Analysis. Network prediction was achieved using Ingenuity Systems Pathway Analysis v8.8 software (IPA-Ingenuity Systems, Redwood City, CA, USA). Predicted networks were scored and modeled considering proteins identities (IDs) that revealed p-values < 0.05 and a fold change of ±1.2 after protein quantification.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00370.

Table S1 (total number of protein identities for each analyzed sample); Table S2 (list of proteins exclusively modulated in the host after *L. amazonensis* infection); Table S3 (list of proteins exclusively modulated in the host after *L. major* infection); Table S4 (list of shared proteins modulated in the host after *L. amazonensis* or *L. major* infection) (PDF)

Information on protein sequence coverage, raw intensities, spectral counting, normalized abundance of all identified proteins across samples, and original and corrected p-values (FDR) for each comparison (XLSX)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

F.N. and M.N.E. acknowledge FAPESP (2017/22651-4). S.G. acknowledges FAPESP 2015/23767-0, and J.R.Y. acknowledges NIH grant P41 GM103533.

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