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Current Opinion in
Microbiology

The impact of arginase activity on virulence factors of *Leishmania amazonensis*

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The outcome of *Leishmania* infection depends on the parasite species and the host immune response. Virulence factors have been extensively studied over the years in an effort to find efficient vaccines and/or treatments for *Leishmania* infection. Arginase activity in *Leishmania* has been described as an essential player for the polyamines pathway, impacting parasite replication and infectivity. Considering previous studies showing that the absence of arginase activity leads to low infectivity of *Leishmania amazonensis*, we reanalyzed transcriptomic data comparing both promastigotes and axenic amastigotes from *L. amazonensis* wild type (*La*-WT) and *L. amazonensis* arginase knockout (*La*-arg^{-}) backgrounds. The analysis produced a new compilation of modulated transcripts that indicated the role of arginase not only in the polyamines pathway but also in the modulation of virulence factors involved in parasite recognition, growth and differentiation.

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Current Opinion in Microbiology 2019, **52**:110–115

This review comes from a themed issue on **Host-microbe interactions: parasites**

Edited by **Norma Windsor Andrews** and **Dario SimoÄ es Zamboni**

For a complete overview see the Issue and the Editorial

Available online 21st July 2019

https://doi.org/10.1016/j.mib.2019.06.003

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Introduction

L-arginine has been described as an essential amino acid for *Leishmania* survival and infectivity [1•]. Arginine is converted by arginase into urea and ornithine, providing the substrate for polyamine production [2]. It was shown that the absence of arginase impairs parasite growth, revealing a central role for arginase in the fate of infection during host–parasite interaction [3••].

Transcriptome sequencing produces a large amount of data that can be analyzed in depth from various viewpoints. A previous RNA-seq analysis identified 1268 genes that were differentially expressed in a comparison of promastigotes and axenic amastigotes from *La*-WT and *La*-arg^{-} [4••]. Revisiting that RNA-seq data in the present study, we evaluate and describe the impact of arginase activity on the expression of important virulence factors, such as plasma-membrane anchored proteins and others related to growth, differentiation and survival of the parasite.

The transcriptomic data revealed modulation of the transcript levels of several virulence markers present both in promastigote and axenic amastigote comparisons between *La*-arg^{-} versus *La*-WT (Table 1).

Parasite recognition and host cell signaling

Several *Leishmania*-related molecules that are secreted and/or expressed on the parasite membrane are characterized as virulence factors, which contribute to *Leishmania* pathogenesis and alter host cell signaling molecules, such as lipophosphoglycan (LPG), glycoprotein 63 (GP63), proteophosphoglycan (PPG) and GPI-anchored proteins, among others [5,6] These virulence factors have been shown to play essential roles on the interaction with the host for some *Leishmania* species, but not for all of them [6].

LPG is one of the major surface glycoconjugates in promastigotes and is directly involved in the initial stages of infection. *Leishmania major* mutants lacking lpg1 and lpg2 are susceptible to the host complement system; thus, they are unable to survive either both sandflies or mammalian host cells [7,8].

GP63 cluster genes are part of a set of surface proteins abundant in promastigotes, but these genes have been shown to be downregulated in amastigotes [9]. Considering that GP63 is expressed in both parasite forms, its role depends on the parasite stage. In promastigotes, GP63 is related to protection against the insect's digestive enzymes, while in the mammalian host it is related to macrophage interaction, prevention of complement activation and modulation of other immune cell activity [10,11]. In amastigotes, GP63 is involved in protection against phagolysosomal degradation [12]. Additionally, its expression in the amastigote form can be directly corelated to low survival rate in attenuated variants [13].

Table 1

List of selected genes related to virulence and differentially modulated in the comparisons of La-WT and La-arg^{-} promastigotes and axenic amastigotes.

Predicted Function	Gene ID	Product	pro *La*-arg^{-} *vs.* pro *La*-WT		ama *La*-arg^{-} *vs.* ama *La*-WT	
			FC	*p*-value	FC	*p*-value
Membrane proteins	LmxM.10.0405	GP63	0.76	0.0291	1.03	ns
	LmxM.10.0460	GP63	0.58	0.0008	1.08	ns
	LmxM.10.0465	GP63	0.61	$<$0.0001	0.99	0.0097
	LmxM.28.0570	GP63	1.10	ns	1.55	0.0246
	LmxM.30.2000	GP63	1.95	0.0003	0.93	ns
	LmxM.26.0550	LPG1	0.81	0.0026	1.03	ns
	LmxM.33.3120	LPG2	1.39	0.0065	0.89	ns
LmxM.08_29.0760	LPG3	0.96	ns	0.82	0.0099	
	LmxM.34.0550	PPG1	1.74	ns	0.50	0.0046
	LmxM.24.0380	PPG3	0.64	0.0324	1.03	ns
Differentiation related	LmxM.19.1420	CPA	0.29	$<$0.0001	0.48	0.0027
	LmxM.09.0180	ATG8	0.57	0.0315	0.98	ns
	LmxM.19.0820	ATG8	0.29	0.0080	0.77	ns
	LmxM.19.0830	ATG8	0.29	0.0147	0.76	ns
	LmxM.19.0840	ATG8	0.29	0.0089	0.76	ns
	LmxM.19.0860	ATG8	0.32	0.0384	0.66	ns
	LmxM.19.0870	ATG8	0.62	0.0022	1.02	ns
	LmxM.19.0880	ATG8	0.29	0.0082	0.89	ns
	LmxM.19.1630	ATG8	0.61	0.0029	0.72	ns
	LmxM.29.0980	ATG5	0.56	0.0232	1.22	ns
	LmxM.08.0290	FeSODA	0.50	0.0011	0.75	ns
	LmxM.31.1820	FeSODB1	1.27	ns	0.67	0.0095
	LmxM.31.1830	FeSODB2	1.15	ns	0.70	0.0099
	LmxM.33.0070	APX	1.41	0.0422	0.65	0.0021
Amastigote markers	LmxM.28.1400	amastin α	0.75	0.0499	0.73	ns
	LmxM.08.0720	amastin δ	0.17	0.0001	1.31	ns
	LmxM.30.0450	amastin β	0.17	0.0342	0.72	0.0135
	LmxM.30.0451	amastin β	0.31	0.0005	1.25	0.0041
	LmxM.24.1250	amastin γ	1.25	0.0339	0.65	0.0020
	LmxM.24.1260	amastin γ	0.46	0.0005	0.97	ns
	LmxM.24.1270	amastin γ	1.28	ns	0.71	0.0018
	LmxM.24.1280	amastin γ	1.22	ns	0.70	0.0020
	LmxM.30.0452	amastin β	0.25	$<$0.0001	0.90	0.0088
	LmxM.08.0750	amastin δ	0.22	0.0021	0.79	ns
	LmxM.08.0760	amastin δ	0.16	0.0067	0.85	ns
	LmxM.08.0770	amastin δ	0.14	0.0471	1.21	ns
	LmxM.08.0800	amastin δ	0.35	0.0185	0.73	0.0461
	LmxM.08.0840	amastin δ	0.34	$<$0.0001	0.98	ns
	LmxM.08.0850	amastin δ	0.31	$<$0.0001	0.85	ns
	LmxM.29.0850	amastin δ	1.75	$<$0.0001	0.98	ns
	LmxM.29.0860	amastin δ	1.75	0.0069	0.98	ns
	LmxM.33.0500	amastin δ	0.59	0.0022	0.90	ns
	LmxM.33.0960	amastin δ	0.52	0.0031	0.68	$<$0.0001
	LmxM.33.0961	amastin δ	0.58	0.0038	0.67	0.0001
	LmxM.33.0980	amastin δ	0.85	0.0036	0.82	ns
	LmxM.33.1560	amastin δ	0.22	0.0002	0.65	0.0003
	LmxM.33.1720	amastin δ	0.192	$<$0.0001	1.05	ns
	LmxM.33.1721	amastin δ	0.16	0.0002	1.03	ns
	LmxM.33.1820	amastin δ	0.18	$<$0.0001	1.42	0.0213
	LmxM.33.1920	amastin δ	0.20	0.0001	0.63	0.0002
	LmxM.33.1920a	amastin δ	0.21	0.0001	0.62	0.0002
	LmxM.33.1920b	amastin δ	0.21	0.0001	0.62	0.0001
	LmxM.33.1920c	amastin δ	0.19	0.0002	0.61	0.0002
	LmxM.33.1920d	amastin δ	0.21	0.0001	0.62	0.0002
	LmxM.33.1920e	amastin δ	0.21	0.0016	0.62	0.0022
	LmxM.33.1980	amastin δ	0.06	0.0032	1.28	ns
	LmxM.36.1270	amastinδ	0.17	0.0009	0.82	0.0158

Fold change and *p*-value of the transcripts related to virulence based on FPKM values in the comparisons of *L. amazonensis* wild type (*La*-WT) and *L. amazonensis* arginase knockout (*La*-arg-) promastigotes (pro) and axenic amastigotes (ama). ns, not significant.

PPG is expressed in both promastigotes and amastigotes, and it is present on the parasite surface, flagellar pocket, Golgi and lysosome [14]. PPG facilitates binding of the parasite to macrophage receptors and appears to contribute to the maintenance of the parasitophorous vacuole, improving survival of the parasite during infection [15].

The downregulation of the analyzed membrane markers indicated that the previously observed reduced infectivity of *La*-arg$^-$ not only could be the result of diminished polyamine production [3••] but could also be due to impairments in parasite recognition and host cell signaling.

Autophagy machinery and parasite metacyclogenesis

Leishmania differentiation into the infective metacyclic form, which occurs in the gut of the insect, depends on autophagy [16]. During autophagy, cells form autophagosomes that sequester proteins or organelles to be degraded; this allows cells to eliminate specific components as a protective mechanism in response to multiple stressors or as a remodeling process for cell differentiation [17,18]. In yeast and mammals, the canonical pathway for autophagosome genesis requires two ubiquitin-like conjugation systems. A multistep ATP-dependent process, with successive events involving proteins of the ATG family, leads to the attachment of ATG8 to phosphatidylethanolamine (PE) and ultimately to the cleavage of ATG8 from the surface of mature autophagosomes before they fuse with the lysosomal network [19]. In *Leishmania*, these processes may be different since some genes that encode proteins required for the ATG5–ATG12 conjugation system appear to be absent [20]. Despite these missing components, ATG8 lipidation to PE has still been observed in *L. major*, which also presents genes encoding proteins similar to ATG5, ATG7, ATG10 and ATG12 [16,21]. Furthermore, *L. major* mutants lacking atg5 present an important differentiation defect and reduced infectivity, confirming that ATG5 is essential for ATG8-dependent autophagy [19].

In yeast, peptidases such as proteinase A and B (PEP4 and PRB1, respectively) are involved in the degradation step of autophagy; however, the *Leishmania* genome lacks orthologs to these proteins [19]. Studies suggest that *Leishmania mexicana* lysosomal cysteine peptidases (CPA and CPB) perform similar roles to the yeast PEP4 and PRB1. Inhibition of CPA and CPB was shown to interfere with autophagy and prevent parasite differentiation [19]; thus, these peptidases play a critical role in pathogenesis as virulence factors [22].

The downregulation of CPA, ATG5 and ATG8 transcript levels indicated that the absence of arginase could impact the autophagy machinery assembly, impairing differentiation. Therefore, the observed reduced metacyclogenesis of parasites lacking arginase could be not only a result of the increased nitric oxide synthase-like expression [23] but could also be the result of impairment of the parasite's autophagy-dependent cell remodeling process in the differentiation to the infective metacyclic form.

Iron-dependent redox balance and parasite infectivity

Another important feature in *Leishmania* differentiation is related to changes in temperature, pH and nutrient availability, such as L-arginine [24] and iron [25–27]. Iron is an essential cofactor of several parasite enzymes; therefore, *Leishmania* must acquire iron from the host to survive inside the parasitophorous vacuole. It was shown that *Leishmania* Ferric Reductase 1 (LFR1) is a parasite plasma membrane protein that reduces iron to be translocated across the parasite membrane by *Leishmania* Iron Transporter 1 (LIT1) [28]. Once acquired by the parasite, iron can be translocated into the mitochondria of the parasite by *Leishmania* Mitochondrial Iron Transporter 1 (LMIT1) [29]; in the mitochondria, iron can be used as a cofactor for several enzymes, including superoxide dismutase A (SODA) [30]. Iron is also the cofactor of the glycosomal isoforms of superoxide dismutase B (SODB1 and SODB2) [31], but how iron is translocated into the glycosome is still unknown.

Superoxide dismutases (SODs) are metalloenzymes that detoxify superoxide anions by converting them into hydrogen peroxide (H_2O_2) and oxygen. In *Leishmania*, SODs exclusively use iron as an essential cofactor [32]. Reactive oxygen species (ROS) are described as deleterious for eukaryotic cells; however, in the last few decades, a new role for ROS in cellular signaling pathways has emerged [33–35]. Mittra *et al.* showed that iron uptake affects parasite differentiation to the amastigote infective form through regulation of ROS levels by modulating FeSOD activity and H_2O_2 generation [36]. Other studies using single allele knockouts revealed that both SODA and SODB are important for *Leishmania* virulence [30,31]. Another oxidative stress-related enzyme implicated in *Leishmania* differentiation is ascorbate peroxidase (APX), a mitochondrial hemoprotein involved in H_2O_2 detoxification [37]. APX was also shown to control parasite differentiation by the modulation of intracellular levels of H_2O_2 [36,38].

The modulation of APX, FeSODA, FeSODB1 and FeSODB2 transcripts levels indicated that arginase absence could also impact intracellular redox balance. Therefore, the observed reduced infectivity of parasites lacking arginase could not only be a result of diminished polyamine production [3••] but could also be due to an impairment in parasite differentiation caused by a dysregulated intracellular redox balance.

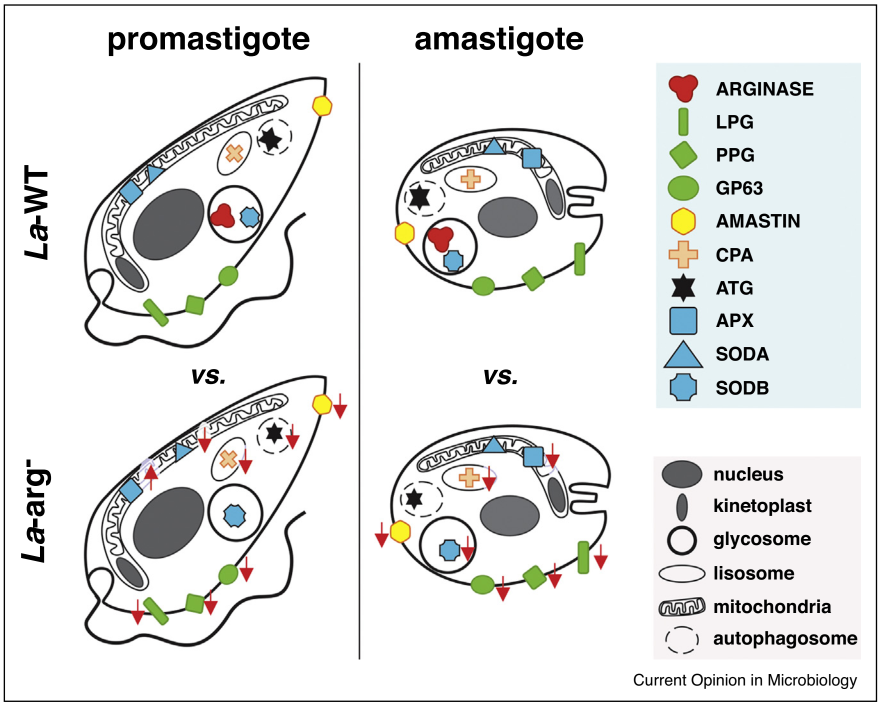
In addition, previously characterized iron transporters are also able to translocate manganese [39], like iron-regulated transporter 1 (IRT1) from *Arabidopsis thaliana* that shares similarity the *Leishmania* iron importer LIT1 [28]. Thus, *Leishmania* iron transporters could potentially translocate manganese, as indicated by a recent study showing that the *Leishmania* iron exporter LIR1 (*Leishmania* Iron Regulator 1) can protect the parasite from the toxic effects of high concentrations of both iron and manganese [40]. Like iron, manganese is a transition metal that is an essential cofactor for arginase activity [41]. Similar to SODBs, arginase is a glycosomal protein [3••,42]; therefore, we speculate that manganese and iron could both be translocated by the same transporters. Further studies addressing iron and manganese transport in *Leishmania* could confirm a cross-regulation of the arginase pathway and the iron-dependent redox signaling in the modulation of *Leishmania* virulence.

Amastins expression and amastigote survival and replication

Amastins belong to the largest conserved gene family in *Leishmania* that is regulated during the parasite life cycle [43]. These genes have been associated with parasite virulence and pathogenesis [44,45]. Amastins are associated with the amastigote intracellular life stage [46•] and replication within the host cell [47,48]. They are organized into subfamilies: α, β, γ and δ, according to secondary structure and chromosomal localization [43,44,47]. The δ-family comprises the vast majority of amastins, and studies indicate that the increased amastins gene repertoire in *Leishmania* is due to the expansion of the δ-family [43,47].

Amastins are described as abundant surface antigens with stage-specific expression in *Leishmania braziliensis*, *Leishmania donovani*, *Leishmania infantum* and *L. major* [44,47,49] that induce an immune response in mice and humans [45,50]. Amastins are not involved in the attachment of promastigotes to the macrophage, but they most likely play a role in the following: (1) stability during stimulated acidic conditions, thereby protecting the parasite from exposure to lysosomal contents, and (2) efficiency in response to increased temperature [46•,49]. A knockdown of the δ-amastin gene in *L. braziliensis* showed impaired infection in *in vitro* and *in vivo* mice models. Additionally, the essential role in host-interaction is demonstrated by significant alterations in the tight contact between amastigotes and the parasitophorous vacuole membrane. Besides, this lipid bilayer membrane appears to be disrupted in macrophages infected with δ-amastin knockdown parasites [47].

Figure 1



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Schematic representation of virulence factors modulated by arginase activity in *L. amazonensis*. Comparisons of the *L. amazonensis* wild type (*La*-WT) and arginase knockout (*La*-arg^{-}) promastigotes and the *La*-WT and *La*-arg^{-} axenic amastigotes; these comparisons identify proteins whose transcripts appeared differentially modulated according to their subcellular localization. The red arrows indicate downregulation or upregulation of the indicated transcripts in *La*-arg^{-}.

The downregulation of most amastins transcripts indicated that amastigote survival and replication inside macrophages could be modulated by arginase due to both diminished polyamine production and expression of amastins.

Conclusions

Transcriptomic data provide a large amount of information concerning the life stage of an organism at the time the RNA was obtained. In addition, the comparison of data corresponding to different stages associated with different mutant strains can provide a picture of the importance of a particular gene and its product during the life cycle of an organism. Arginase activity is important to *Leishmania* development and its ability to establish infection. The main role of this enzyme consists of providing a substrate for polyamine synthesis. However, analysis of the transcriptomic data comparing *La*-arg^{-} versus *La*-WT promastigotes, as well as *La*-arg^{-} versus *La*-WT axenic amastigotes, showed that arginase activity can impact several other cellular processes implicated in infectivity and virulence of the parasite (Figure 1). Therefore, our analysis pointed to a more complex role for arginase in the parasite virulence and life cycle scenarios, which need further exploration to indicate new targets to interfere in the infection process.

Conflict of interest statement

Nothing declared.

Funding

This work was supported by São Paulo Research Foundation Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [2014/50717-1, 2016/19815-2, 2016/03273-6, 2017/23933-3], Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [307587/2014-2 and 406351/2018-0] and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgement

We would like to thank Juliane Cristina Ribeiro Fernandes for suggestions and Stephanie Maia Acuña for artwork and suggestions.

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- • of special interest
- •• of outstanding interest

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