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IRONY OF FATE: Role of iron-mediated ROS in *Leishmania* differentiation

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Abstract

The protozoan parasite *Leishmania* experiences extreme environmental changes as it alternates between insect and mammalian hosts. In some species, differentiation of insect promastigotes into mammalian-infective amastigotes is induced by elevated temperature and low pH, conditions found within macrophage parasitophorous vacuoles (PVs). However, the signaling events controlling amastigote differentiation remain poorly understood. Recent studies revealed a novel role for iron uptake in orchestrating the differentiation of amastigotes, in a process that involves production of reactive oxygen species (ROS) and is independent from pH and temperature changes. ROS are generally thought to be deleterious for pathogens, but it is becoming increasingly apparent that they can also function as signaling molecules regulating *Leishmania* differentiation, in a process that is tightly controlled by iron availability.

Keywords

Leishmania; differentiation; iron; virulence; ROS; FeSOD

Signaling events drive differentiation of *Leishmania* virulent forms

The protozoan parasite *Leishmania spp* causes a broad spectrum of human diseases referred to as leishmaniasis. These intracellular parasites currently infect about 12 million people, and threaten more than 350 million globally. Depending on the *Leishmania* species, the disease pathology varies from self-healing cutaneous lesions to severe visceralizing disease. If left untreated, the visceral form of the disease has a high fatality rate, ranking next to malaria as the most deadly protozoan human disease [1].

Leishmania alternates between insect and vertebrate hosts, with inter-species transmission occurring through sand fly bites (Figure 1). During their life cycle the parasites encounter dramatic changes in environment, as they transition from the midgut and proboscis of *Phlebotomus* insects (sand flies) to phagolysosomal vacuoles in mammalian macrophages. Shifts in temperature, pH, O₂/CO₂, and availability of nutrients coincide with morphological and metabolic changes associated with virulence. However, how environmental conditions trigger *Leishmania* differentiation remains unknown.

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A differentiation event of major clinical importance is the transformation of the flagellated metacyclic promastigotes, which are injected into the skin of mammalian hosts by sand flies, into rounded amastigotes, which replicate in macrophage phagolysosomes and can be generated axenically in some *Leishmania* species [2, 3, 4]. Transformation into amastigotes involves a shift to a higher energy production rate, thought to be required for driving proton pumps to counter the acidic environment of PVs [5]. Genome-wide changes in gene expression were observed between promastigotes and amastigotes of *L. mexicana* [6] and *L. donovani* [7], and proteomic studies revealed modulation of post-translational modifications [8, 9]. Understanding of the signaling pathways driving generation of *Leishmania* amastigotes has long been considered a ‘holy grail’ for the development of efficacious anti-leishmanial drug therapy. In this review we discuss recent advances in this area, including surprising new findings that point to iron acquisition as a process that drives differentiation of virulent amastigotes.

Metabolic and structural changes during amastigote differentiation are orchestrated at the post-transcriptional level

Leishmania amastigotes are highly adapted to function within the acidic environment of macrophage phagolysosomes, maintaining internal pH homeostasis and acquiring nutrients against a steep extracellular proton gradient. Residence inside host macrophages comes at a high cost, requiring significantly enhanced rates of ATP generation [5]. A major metabolic retooling appears to occur during amastigote differentiation, with the parasites replacing glucose by amino acids as the primary energy source and activating pathways such as amino acid catabolism, the tricarboxylic acid cycle, β -oxidation, the mitochondrial respiratory chain, and oxidative phosphorylation [10]. Protein synthesis is downregulated, a process proposed to be responsible for the slower growth rate of amastigotes [5].

Comparative genome-wide analysis of the transcriptome and proteome of promastigotes and amastigotes have been critical in understanding the differentiation process. Studies of amastigotes derived from infected animals provided valuable information on cell structure and role in disease development [6, 11]. However, studying parasites isolated from an *in vivo* system poses two main disadvantages: (i) the limited amount of purified parasites that can be obtained and (ii) the difficulty in separating amastigotes from mammalian tissue contaminants. These problems were overcome by the use of axenically-differentiated amastigotes, which can be induced in several species of *Leishmania* by conditions that simulate the elevated temperature and low pH encountered by the parasites within macrophages [5, 12]. The use of axenic amastigotes also allowed precise monitoring of the progression of differentiation over time. Four major phases were identified in the promastigote-to-amastigote axenic transformation of *L. donovani*: (i) signal perception with no significant morphological change (0-5 h); (ii) cessation of movement and cell aggregation (5-10 h); (iii) loss of flagella and morphological transformation to rounded shape (10-24 h); and (iv) maturation into replicating amastigotes (24-120 h) [5]. During the initial 10 h extensive changes in RNA processing and turnover were observed, resulting in rapid downregulation of several gene products and upregulation of others [9, 10]. In addition to pH and temperature, factors reported to influence the differentiation of amastigotes include nutrient availability, CO₂ levels, and hydrobiopterin concentration [13]. As discussed below, iron uptake was recently found to initiate differentiation of *L. amazonensis* amastigotes independently of the ‘classical’ pH and temperature cues [14].

Iron uptake regulates differentiation in *Leishmania amazonensis*

The Leishmania iron uptake machinery

Iron is a key trace element for virtually all organisms, functioning as an essential cofactor in many cellular processes. Due to its unique electrochemical properties, iron is an integral component of important physiological pathways such as oxygen delivery, acetyl CoA oxidation, ATP generation, nitrogen fixation, and photosynthesis, where iron is used as a sensor of the cellular redox status. However, iron is a double-edged sword. The soluble, free Fe^{2+} form can react with oxygen or nitrogen compounds generating highly toxic radicals: reactive oxygen species (ROS) and reactive nitrogen species (RNS) via the Fenton reaction. For this reason the cellular uptake, distribution, storage, and export of iron must be tightly regulated [15, 16].

Identification of the divalent cation transporter Nramp1 as a host susceptibility gene for infections with *L. donovani* and *L. infantum* [17] was among the first indications that *Leishmania* is critically dependent on iron availability for intracellular replication. Nramp1 translocates Fe^{2+} from late endocytic compartments into the cytosol [18], indicating that *Leishmania* amastigotes in these compartments have to compete with the host for the small amount of iron that enters by endocytosis. *Leishmania* can also influence the host cell's ability to internalize iron. Infection with *L. donovani* depletes the host cell labile iron pool and activates iron regulatory proteins, which in turn upregulate expression of transferrin receptors and stimulate iron uptake [19]. Preferential fusion of transferrin-containing endosomes with PVs was reported in *L. amazonensis*-infected macrophages, and proposed as a mechanism to ensure a constant supply of iron for replicating amastigotes [20]. Despite these strategies to increase their access to iron, the intracellular stages of *Leishmania* also must express specific surface transporters to acquire this essential element.

Leishmania parasites do not seem to express siderophore molecules capable of competing with host iron carrier proteins such as transferrin or lactoferrin or transferrin receptor-mediated uptake [21–22], leaving the question of how these parasites acquire iron unanswered until recently. We now know that *Leishmania* expresses two membrane proteins that play a central role in iron acquisition: (i) the ferric reductase LFR1, which reduces the insoluble Fe^{3+} to soluble Fe^{2+} [23] and (ii) the Fe^{2+} transporter LIT1, a member of the ZIP family of metal transporters (Figure 2) [24]. *L. amazonensis null* mutants in LFR1 and LIT1 are severely defective in intracellular replication as amastigotes, demonstrating the importance of this iron uptake machinery for survival in the iron-poor environment of macrophage phagolysosomes.

Significant similarities were identified between the *Leishmania* and plant iron acquisition machineries. The iron uptake strategy of *Arabidopsis thaliana* involves upregulation of AHA family H^+ -ATPases that promote acidification of the environment, thereby increasing the solubility of available iron [25]. This situation is comparable to the acidic environment where intracellular amastigotes of *Leishmania* replicate inside macrophages. In plants, Fe^{3+} is reduced to Fe^{2+} by a membrane-bound ferric-chelate reductase (AtFRO2 in *Arabidopsis* and PsFRO1 in peas) that shows significant homology to *L. amazonensis* LFR1. Reduction seems to be the rate-limiting step for iron uptake, as transgenic over-expression of ferric reductases in *Arabidopsis*, rice, tobacco, and soybean increases tolerance to low iron [25]. Similarly, over-expression of *LFR1* rescues the intracellular replication of *LIT1 null L. amazonensis* [23]. In *Arabidopsis* Fe^{2+} is taken up by IRT1, a divalent cation transporter from the ZIP family that shares significant sequence identity with the *L. amazonensis* Fe^{2+} transporter LIT1 [15]. *Arabidopsis IRT1 null* mutants are severely chlorotic and seedling-lethal unless supplied with large amounts of exogenous iron, resembling the intracellular

growth rescue of *L. amazonensis* *LIT1* null mutants observed after loading macrophages with cationic ferritin [23].

Leishmania can also obtain iron from heme sources, including hemoglobin [26-28]. Recent studies showed that heme uptake in *Leishmania* is mediated by LHR1, a transmembrane transporter with limited similarity to *C. elegans* HRG-4 [28].

Iron uptake regulates amastigote differentiation and virulence

L. amazonensis promastigotes lacking the ferric iron reductase *LFR1* are defective in generating infective metacyclic promastigotes and amastigotes [23]. This finding was the first indication that the iron acquisition pathway played an important role in signaling events regulating differentiation. Subsequent studies showed that specifically depleting the culture medium in iron (while maintaining all other divalent cations) induced strong upregulation in expression of the reductase *LFR1* and the ferrous iron transporter *LIT1*. These experiments revealed that *L. amazonensis* responds to iron deficiency by rapidly upregulating the iron uptake machinery, which results in an increase in their iron content. Remarkably, the spike in iron uptake triggered by low iron was associated with growth arrest and a gradual change in morphology, with the generation of rounded forms expressing specific markers of the amastigote stage. Importantly, this transformation was also associated with expression of parasite genes regulating autophagy, a cell remodeling mechanism known to participate in promastigote-to-amastigote differentiation (Box 1) [29]. These were surprising findings, since all prior protocols for the generation of axenic amastigotes in *L. mexicana*/*amazonensis* involved a shift to acidic pH and 37°C, conditions mimicking the mammalian phagolysosome. Remarkably, the iron-regulated process of amastigote differentiation occurred at the neutral pH and low temperature (26°C) normally utilized to cultivate the insect promastigotes. Mouse infection experiments demonstrated that the amastigotes generated in these iron-deprived cultures were fully virulent, while parasites maintained in iron-containing medium as promastigotes were not, as expected, able to form cutaneous lesions [14].

Unlike wild type promastigotes that respond to iron deprivation by increasing their iron content, slowing replication and differentiating into amastigotes, promastigotes lacking the iron transporter *LIT1* maintain low intracellular iron and continue to grow exponentially, followed by a sudden catastrophic cell death. These findings indicated that the ability to acquire iron is necessary for signaling events that inhibit promastigote replication and trigger differentiation into virulent amastigotes [14]. These studies also suggested that entering a non-replicative stage is probably a necessary intermediate in the process of differentiation of promastigotes into amastigotes. Additional results revealed that the *L. amazonensis* iron uptake machinery is tightly regulated, consistent with the need to avoid toxicity from excessive intracellular iron accumulation: (i) expression of the *LFR1* ferric reductase and the *LIT1* ferrous iron transporter is very low in promastigotes and not required for their replication in iron-rich media; (ii) expression of *LFR1* and *LIT1* is upregulated in promastigotes exposed to low-iron conditions, causing an increase in intracellular iron concentration that slows growth and drives amastigote differentiation; and (iii) over-expression of the ferric reductase *LFR1* is toxic for promastigotes [24, 23, 14].

Role of iron-dependent redox signaling in *Leishmania* differentiation

ROS as signaling molecules controlling the balance between proliferation and differentiation

ROS produced *in vivo* are largely viewed as deleterious for eukaryotic cells [30, 31]. However, it is becoming increasingly apparent that superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) also function as critical intermediates in cellular signaling pathways

[32-35]. In particular, the intracellular balance between $O_2^{\bullet-}$ and H_2O_2 can influence cellular fate [36-38]. During normal cell growth, a tight balance between these two ROS species is maintained by the cells' antioxidant systems, and when these systems fail, a pro-oxidant environment is generated. Evidence from a variety of model systems underscores the relationship between a pro-oxidant state and cancer progression, and highlights a role for ROS in the control of cell proliferation and differentiation [39, 40, 41]. In *Drosophila melanogaster*, ROS prime hematopoietic progenitors for differentiation [34]. In *Arabidopsis thaliana*, superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) exhibit distinct patterns of distribution in root tissues; proliferation of root cells requires $O_2^{\bullet-}$ accumulation, whereas cellular differentiation depends on elevated levels of H_2O_2 [42, 43]. Additional studies have confirmed a role for reduced superoxide dismutase (SOD) activity and increased $O_2^{\bullet-}$ in promoting cell proliferation [32], and of H_2O_2 in driving cellular differentiation [44]. Thus, it is becoming increasingly clear that the relative concentrations of $O_2^{\bullet-}$ and H_2O_2 can determine cell fate by altering the balance between cell proliferation and differentiation. As discussed below, recent findings indicate that ROS play an important role in the iron-regulated promastigote-to-amastigote differentiation of *Leishmania*.

Role of ROS in the regulation of *Leishmania* differentiation

Evidence has been accumulating that ROS generated during promastigote-to-amastigote differentiation is a key signaling event driving differentiation. Exposure to elevated temperature, a stimulus that induces promastigote-to-amastigote differentiation in *L. infantum*, triggers hyperpolarization of the parasite's mitochondrion, an increase in respiratory rate, and a ROS surge. Promastigotes in the stationary phase of growth in culture can tolerate this ROS surge and transform into amastigotes, whereas log-phase promastigotes accumulate higher levels of ROS and enter apoptosis, a process that can be reversed by over-expression of mitochondrial SOD [45].

The progression of promastigotes from logarithmic to stationary phase in culture is accompanied by an increase in resistance to oxidative stress [45, 46], increased virulence [47], and enhanced levels of SOD activity [48]. This was mostly interpreted as a protective measure to counter production of ROS by the host macrophage [49]. However, oxidative stress caused by heat shock or by treatment with H_2O_2 or the $O_2^{\bullet-}$ -generating drug menadione was found to increase the virulence of *L. infantum (chagasi)* for mice [47]. The importance of redox regulation in the differentiation of virulent forms was also suggested by studies of *L. major* mutants deficient in ascorbate-dependent peroxidase (APX) [50] or in components of the trypanothione reductase system [51, 52]. Interestingly, it is becoming increasingly clear that ROS plays an important role in the activation of autophagy [53], a process involved in *Leishmania* differentiation (Box 1). Consistent with these findings, recent work found a correlation between autophagy and ROS levels in *L. mexicana* [54], reinforcing the view that the regulation of oxidative signaling is critical for controlling life cycle transitions in these parasites.

Iron-dependent H_2O_2 generation drives amastigote differentiation in *L. amazonensis*

Direct evidence for ROS-mediated differentiation was recently obtained while exploring the responses of *L. amazonensis* to changes in iron availability [14]. The fact that SODs in trypanosomatid parasites exclusively contain iron as an essential co-factor (Box 2) suggested that this class of ROS-regulating enzymes might be particularly susceptible to changes in the intracellular iron content of *L. amazonensis*. Investigation of this issue revealed a sharp increase in SOD activity immediately preceding the promastigote-to-amastigote differentiation process that is triggered by iron removal from the culture medium. In agreement with an essential role for iron uptake in the activation of FeSOD capable of converting $O_2^{\bullet-}$ into H_2O_2 , exposure of promastigotes to the $O_2^{\bullet-}$ -generating drug

menadione initiated amastigote differentiation in wild type, but not in *LIT1 null* parasites. Importantly, bypassing the requirement for FeSOD by exposing the parasites to H₂O₂ alone was sufficient to initiate amastigote differentiation in both wild type and LIT1-deficient promastigotes [14], highlighting the key role played by this stable and diffusible ROS molecule.

A recent examination of changes in the proteome after oxidative stress provided independent evidence for ROS-induced differentiation in another *Leishmania* species, *L. donovani*. Promastigotes exposed to sub-lethal doses of menadione as a source of ROS and S-nitroso-N-acetylpenicillamine as a source of RNS showed upregulation of proteins involved in redox homeostasis including FeSODA and APX, and proteins of the amastin family that include amastigote-specific markers. Also consistent with the onset of amastigote differentiation, the observed changes in protein abundance were consistent with a shift from glucose to fatty acid oxidation as the main source of metabolic energy [55].

Collectively, these recent findings revealed an unexpected role for iron uptake and ROS generation in initiating autophagy and differentiation in *Leishmania*. Although the exact mechanism is still incompletely understood, a stress response triggered by low extracellular iron may set the process in motion by altering the parasite's mitochondrial redox balance. Iron deprivation may disrupt Fe-S clusters causing a leaky electron transport chain (ETC), generating O₂^{•-}. These highly reactive molecules may then further disrupt ETC Fe-S clusters and exacerbate O₂^{•-} production, unless the situation is remedied by SOD. Since all *Leishmania* SOD enzymes require iron as co-factor for activity, the ability to dismutate O₂^{•-} to H₂O₂ and restore the ETC depends critically on iron availability. Upregulation of the LIT1 iron transporter triggered by low extracellular iron could then provide a mechanism by which the parasites obtain the necessary iron for FeSOD assembly, fully consistent with the surge in activity of this enzyme that is observed following iron deprivation [14]. FeSOD-mediated dismutation of O₂^{•-} generates H₂O₂, which would correspond to the diffusible signaling molecule triggering differentiation (Figure 3).

Concluding remarks and future directions

A stress-related response has long been assumed to initiate differentiation events in *Leishmania*, as the parasites transition from the insect to the mammalian environment. However, the underlying molecular mechanism triggering differentiation has remained very poorly understood. Recent work investigating iron uptake in *Leishmania amazonensis* led to the surprising discovery that changes in intracellular iron concentration activate a ROS-dependent signaling pathway that induces promastigotes to transform into infective amastigotes. This finding provided an unexpected connection between evidence for redox signaling in the development of infectivity, and the recent characterization of the *Leishmania* iron uptake machinery.

One aspect that still remains to be clarified is how *Leishmania* parasites sense low extracellular concentrations of iron, activating expression of genes required for iron uptake. Sensors that regulate expression of iron responsive genes have been extensively characterized in several systems [25, 56, 57]. However, how this process is achieved in trypanosomatid protozoa is still unclear [58]. Given that mechanisms for transcript stabilization play a major role in regulating gene expression in *Leishmania*, the identification of iron-responsive *cis*-elements and *trans*-factors involved in this process should now be pursued to clarify this issue.

Another outstanding question is how *Leishmania* parasites store iron. Ferritin-like proteins that bind iron intracellularly and release it in a controlled fashion are found in bacteria, algae, higher plants, and animals, but are apparently absent in the *Leishmania* genome.

Yeast cells also lack ferritin but contain frataxins, small mitochondrial proteins that bind and supply iron for assembly of Fe-S clusters and heme [59]. Frataxin orthologs are present in all trypanosomatids including *Leishmania* (*LmjF.25.1050*, *LmxM.25.1050*, *LinJ.25.1090*). In *Trypanosoma brucei*, RNAi-mediated knockdown of frataxin (*Tb927.3.1000*) rapidly decreased the activity of essential Fe-S dependent enzymes but did not cause iron accumulation in mitochondria, suggesting that this protein may not function in iron storage in trypanosomatids [60]. The role of frataxin in *Leishmania* still needs to be investigated, but the potential absence of a bona-fide iron sequestration mechanism in these parasites may have been overcome by the development of a tightly regulated iron transport machinery.

When appropriately controlled, ROS function as signaling molecules by modulating the activity of targets by oxidation. Thus, an important and still unanswered question is what are the targets of oxidation in the iron-dependent, H₂O₂-mediated promastigote-to-amastigote differentiation of *Leishmania*. Under tightly regulated concentrations, H₂O₂ can reversibly inhibit several enzymes, including cellular phosphatases that regulate major eukaryotic signaling cascades [61]. Analysis of the TriTryp database reveals the presence of more than 80 phosphatases in *L. major*. Kinetoplastids, when compared to humans, *Saccharomyces cerevisiae*, and plants, have a reduced number of tyrosine-specific phosphatases and an expanded number of serine/threonine phosphatases [62]. Several of these kinetoplastid phosphatases have novel domain architectures that suggest potentially novel functions. Despite their low number (two in *T. brucei*, two in *Trypanosoma cruzi* and three in *L. major*), phosphotyrosine protein phosphatases (PTPs) have been associated with infectivity in trypanosomatids. *L. major* PTP1 (LmPTP1), which has an ortholog in *T. cruzi* but not in *T. brucei*, is needed for virulence and intracellular survival of amastigotes [63]. A similar role for calcineurin, a Ca²⁺/calmodulin dependent serine/threonine-specific phosphatase was recently reported in *L. major* [64]. It remains to be determined if specific *Leishmania* phosphatases are targets for H₂O₂ oxidation under conditions that trigger amastigote differentiation. This possibility is reinforced by recent studies showing that a glycosomal tyrosine-phosphatase (TbPTP1) acts as a master-switch controlling differentiation in the African trypanosome *T. brucei*. TbPTP1 activity blocks the development of transmissible ‘stumpy’ bloodstream forms until the parasites enter the tsetse fly, where citrate and cis-aconitate signaling inactivates TbPTP1, triggering differentiation [62]. The recent developments in *Leishmania* linking iron uptake, FeSOD activity, H₂O₂ generation, and the potential oxidation of target enzymes provide an exciting new avenue of research that should rapidly expand our understanding of how environmental changes drive cellular remodeling in trypanosomatid parasites.

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Highlights

1. Iron uptake regulates differentiation of *Leishmania* promastigotes into virulent amastigote forms.
2. Iron uptake is essential for FeSOD activation and generation of H₂O₂.
3. H₂O₂ promotes differentiation of avirulent promastigotes into virulent amastigotes.

Box 1**Role of autophagy in *Leishmania* remodeling during differentiation**

Autophagy serves a dual purpose: (i) a pathway for nutrient acquisition activated by cellular starvation, and (ii) a pathway for protein and organelle degradation during cellular differentiation [65]. The genesis of autophagosomes requires the activity of two protein conjugation systems, one involving the ubiquitin-like protein Atg8 that is proteolytically processed by the cysteine peptidase Atg4, and a second involving the ubiquitin-like protein Atg12, covalently attached to Atg5. In yeast and mammalian systems, formation of the Atg5-Atg12 complex is critical for generating the curved pre-autophagosomal membrane known as the phagophore. Conjugation of Atg5 and Atg12 is a multi-step process requiring ATP and interaction with several additional Atg proteins, which ultimately promote closure of the autophagosome and fusion with lysosomal compartments [66].

The autophagic machinery was shown to be involved in two *Leishmania* differentiation events: the transformation of procyclic promastigotes into metacyclic promastigotes within the sand fly, and the remodeling of metacyclic promastigotes into amastigotes within the macrophage [29]. Unlike yeast and humans that have only one Atg8 family, *Leishmania* has four families of Atg8-like genes. When over-expressed as GFP-fusions in *L. major* promastigotes, GFP-ATG8 and GFP-ATG8A showed a punctate pattern that was enhanced under nutrient starvation, consistent with a role of these isoforms in autophagosome formation. The function of Atg8 and Atg8A could not be investigated directly by creating *null* mutants because of multiple gene copies. However, mutants deficient in the Atg4.2 cysteine peptidase that activates Atg8 proteins show a pronounced virulence defect, and incomplete transformation into amastigotes [54]. Further highlighting the role of autophagy in *Leishmania* differentiation, a functional ATG5-ATG12 system was identified in *L. major* and *atg5 null* promastigotes were defective for differentiation and infectivity for macrophages and mice [67]. Furthermore, inhibition of the *Leishmania* lysosomal cysteine peptidases CPA and CPB also impaired autophagy and markedly reduced metacyclogenesis and differentiation into amastigotes [29].

There is evidence that ROS can activate autophagy in eukaryotic cells, which in turn protects cells by removing oxidized proteins [53]. In *L. major*, an increase in intracellular ROS coincided with the onset of autophagy during metacyclogenesis [54]. *atg5* and *atg4.2 null* strains deficient in autophagy showed dysfunctional mitochondria and higher levels of ROS [54, 67], but the antioxidant N-acetyl cysteine reversed this phenotype restoring mitochondrial integrity and decreasing autophagosome formation. Thus, in *Leishmania* ROS also appear to regulate autophagy, a cell remodeling process that is essential for differentiation.

Box 2**Iron-dependent superoxide dismutases and *Leishmania* redox control**

Iron plays a crucial role in regulating redox balance in all trypanosomatids, due to its essential role in the activity of superoxide dismutases (SODs). SODs are metalloenzymes that catalyze dismutation of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). Unlike mammals, which contain Cu/Zn and Mn-dependent SOD in the mitochondrial matrix and cytoplasm respectively, SODs in trypanosomatids are exclusively dependent on iron for activity [16]. For this reason, modulation in iron availability is expected to have a direct impact in cellular functions dependent on SOD and H_2O_2 generation, as recently demonstrated for the differentiation of *Leishmania amazonensis* amastigotes [14].

Of the three functionally characterized FeSODs in *Leishmania*, two isoforms have been localized in the glycosomes, compartments that house enzymes involved in the glycolytic pathway [16, 68]. The glycosomal FeSOD-B1 and FeSOD-B2 are developmentally regulated in *Leishmania infantum (chagasi)*, with SODB1 transcript levels being more abundant in amastigotes and SODB2 in promastigotes. *L. infantum (chagasi)* SODB1 *null* mutant promastigotes were not viable, and single allele deletion strains showed reduced viability in macrophages or when exposed to the pro-oxidant paraquat [48]. Interestingly, in *Trypanosoma brucei*, which undergo pronounced glycosomal turnover during differentiation, both SODB1 and SODB2 were found to be essential for survival [16, 68].

The physiological role of *Leishmania* mitochondrial FeSOD A (LdFeSODA) has not yet been fully elucidated, but there is evidence that it can protect the parasites from oxidative stress and programmed cell death [69]. The FeSODA ortholog in *T. brucei* was found not to be essential for the bloodstream form of the parasites [16], but in *T. cruzi* upregulation of antioxidant enzymes including FeSODA observed in the infective metacyclic stage of were proposed to represent an important defense mechanism against the oxidative burst of host phagocytes, ROS production, and programmed cell death [70, 71].

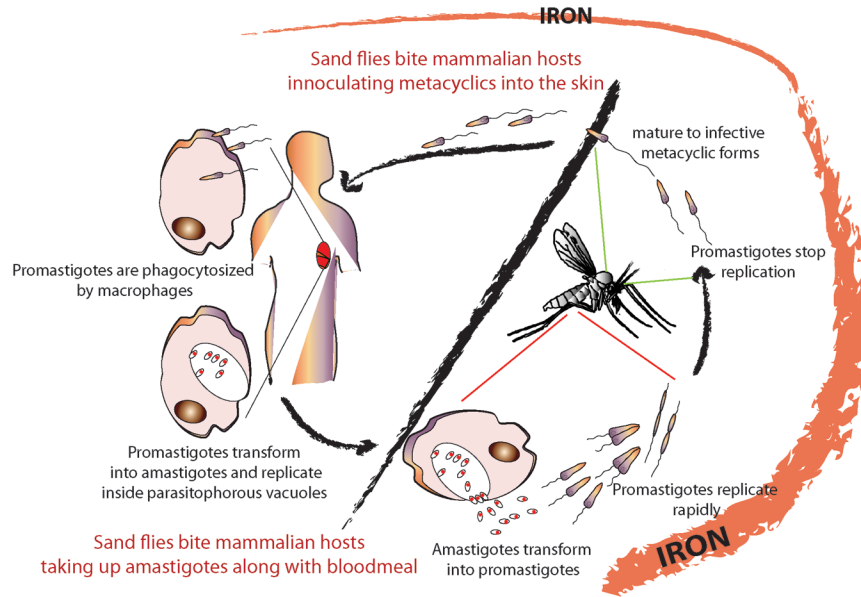


Figure 1. The availability of iron changes during the *Leishmania* life-cycle

Iron is abundant in the sand fly midgut following blood meals, as result of the breakdown of hemoglobin. Amastigotes taken up in a blood meal transform into promastigotes within the digestive tract and multiply by binary fission. A few days after the initial feeding the parasites cease to replicate and transform into infective metacyclic promastigotes, during the 'sugar-meal phase', when iron availability becomes limited.. The transmissible metacyclic forms are regurgitated into the skin of a mammalian host by the flies, entering macrophages where they transform into amastigotes within lysosome-like parasitophorous vacuoles. The low availability of iron inside PVs in macrophages triggers upregulation of proteins involved in iron acquisition (see Figure 2).

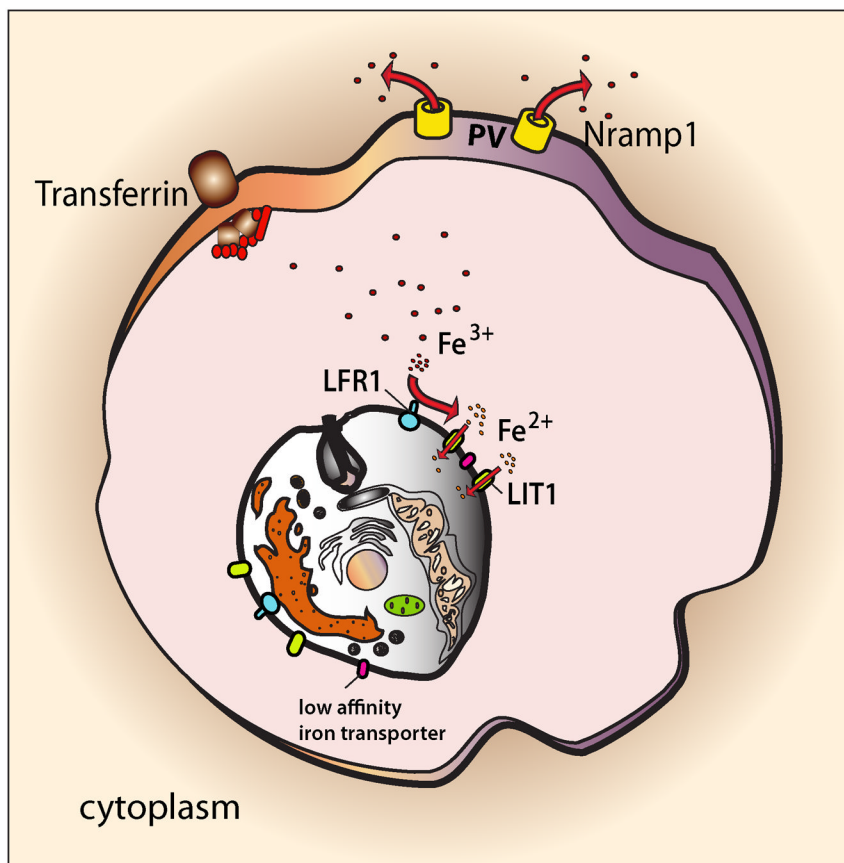


Figure 2. *Leishmania* must acquire iron from the host to survive inside PVs

Within PVs of macrophages *Leishmania* upregulates an iron transport machinery that allows it to compete with the host iron transporter Nrp1 for iron. Intracellular amastigotes of *Leishmania* express on their plasma membrane the ferric iron reductase LFR1, which converts Fe³⁺ that enters the macrophage as a complex with transferrin into Fe²⁺, the soluble iron form. Fe²⁺ is then translocated across the membrane by LIT1, a ferrous iron transporter that is also upregulated in the iron-poor environment of the PV. When higher concentrations of iron are available inside the parasitophorous vacuole, low affinity iron transporters can compensate for the absence of LFR1 and LIT1, promoting parasite replication. Abbreviation: PV, parasitophorous vacuole.

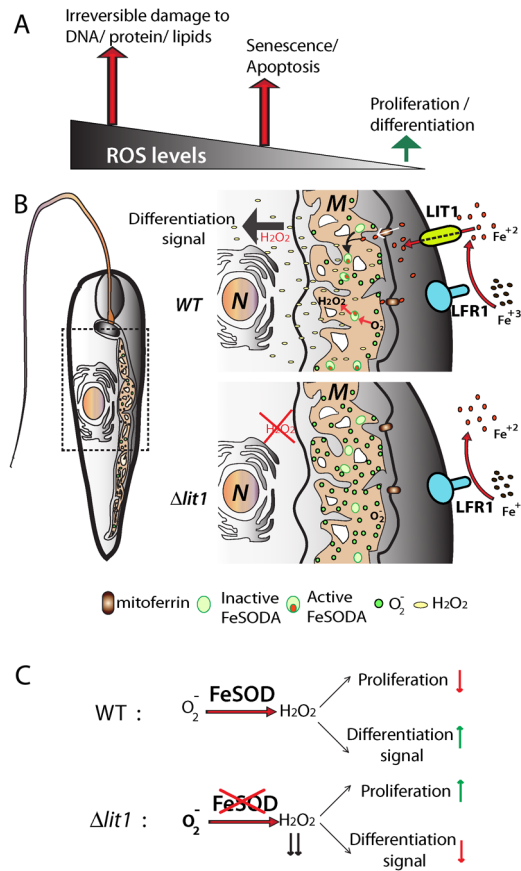


Figure 3. Model for the iron-regulated mechanism for ROS generation that triggers amastigote differentiation

(A) Mitochondrial ROS levels determine cell fate. At low concentrations ROS molecules act as potent cell signals regulating the balance between proliferation and differentiation. Increasingly higher concentrations of ROS molecules trigger senescence and apoptotic death, and then irreversible damage to DNA, protein and lipid cellular components of the cell. (B) Low iron conditions cause an initial drop of intracellular iron, affecting Fe-S clusters and impairing the function of the mitochondrial electron transport chain, resulting in leaking of electrons that in the presence of molecular oxygen generates superoxide ($O_2^{\bullet-}$). Iron transport is then activated by upregulation of the ferric reductase LFR1 and the ferrous iron transporter LIT1 on the parasite's plasma membrane. This results in an increase in intracellular iron concentration, activating FeSOD activity, that converts $O_2^{\bullet-}$ into H_2O_2 . Being a highly diffusible molecule, H_2O_2 can oxidize cellular targets to initiate differentiation into amastigotes. In the absence of LIT1 mediated iron import, *LIT1 null* promastigotes are unable to activate FeSOD and H_2O_2 production, not generating a differentiation signal. M: Mitochondria; N: Nucleus. (C) FeSOD-mediated generation of H_2O_2 controls the balance between proliferation and differentiation.