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JB Review

Access and utilization of host-derived iron by *Leishmania* parasites

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Yasuyuki Goto[1,†], Tatsumi Ito[1], Souradeepa Ghosh[2] and Budhaditya Mukherjee[2]

[1]Laboratory of Molecular Immunology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan; and [2]School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

[†]Yasuyuki Goto, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Tel.: +81-3-5841-5196, email: aygoto@g.ecc.u-tokyo.ac.jp

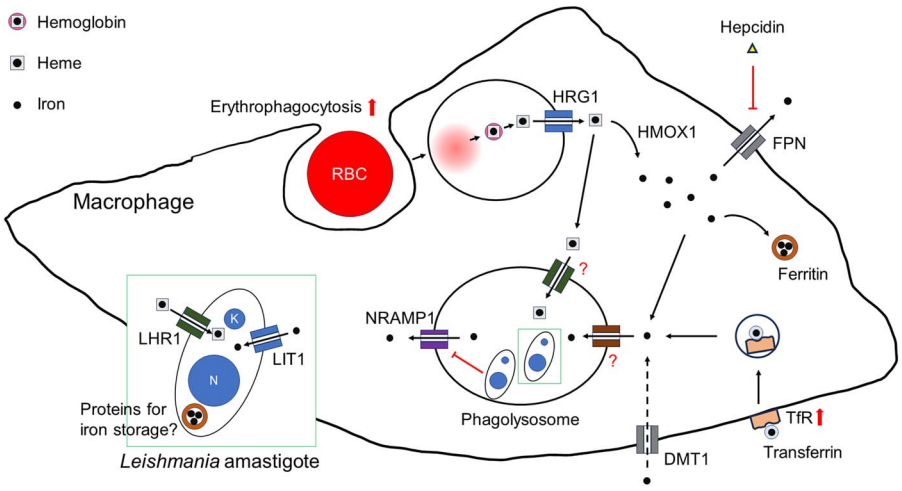
Iron is involved in many biochemical processes including oxygen transport, ATP production, DNA synthesis and antioxidant defense. The importance of iron also applies to *Leishmania* parasites, an intracellular protozoan pathogen causing leishmaniasis. *Leishmania* are heme-auxotrophs, devoid of iron storage proteins and the heme synthesis pathway. Acquisition of iron and heme from the surrounding niche is thus critical for the intracellular survival of *Leishmania* inside the host macrophages. Moreover, *Leishmania* parasites are also exposed to oxidative stress within phagolysosomes of macrophages in mammalian hosts, and they need iron superoxide dismutase for overcoming this stress. Therefore, untangling the strategy adopted by these parasites for iron acquisition and utilization can be good targets for the development of antileishmanial drugs. Here, in this review, we will address how *Leishmania* parasites acquire and utilize iron and heme during infection to macrophages.

Keywords: leishmania, macrophage, iron, heme.

Leishmaniasis is a parasitic disease caused by protozoan parasite of the genus *Leishmania*. Depending on the infecting species, human patients represent three clinical forms of the disease, cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). CL is the most common form of the disease with an estimated 600,000 to 1 million new cases annually (World Health Organization website, as of March 2023) and is characterized by skin lesions often with ulcers. MCL is characterized by lesions on mucosal tissues including nose, mouth and throat, which are often destructive to the tissues. VL is the most severe form caused by *Leishmania donovani* complex. Countries endemic to VL include India, Bangladesh, Nepal, Brazil, Ethiopia, and Sudan. There are estimated 50,000 to 90,000 new cases of VL annually and the fatality rate of this disease can be as high as 100% if left untreated (World Health Organization). Currently, the treatment of leishmaniasis relies mostly on chemotherapy. Pentavalent antimonials, liposomal amphotericin B and miltefosine are often used for chemotherapy of the disease, but each of them has some drawbacks including the emergence of drug resistance, high cost and teratogenicity leading to treatment failure *(1, 2)*. Therefore, the quest for new drug candidates is a crucial issue.

In mammalian hosts, *Leishmania* parasitizes macrophages and proliferates as amastigotes inside a compartment called phagolysosomes (Figure 1). Since macrophages are professional immune cells for eliminating foreign bodies through phagocytosis, *Leishmania* parasites need to overcome the killing activities by macrophages for their survival. In addition, phagolysosomes have limited access to nutrition as they are compartmentalized from macrophage cytosol or the extracellular fluid. One of such critical nutrients that these parasites need for proliferation is iron, which needs to be collected inside phagolysosomes where *Leishmania* resides for making it accessible.

Graphical Abstract



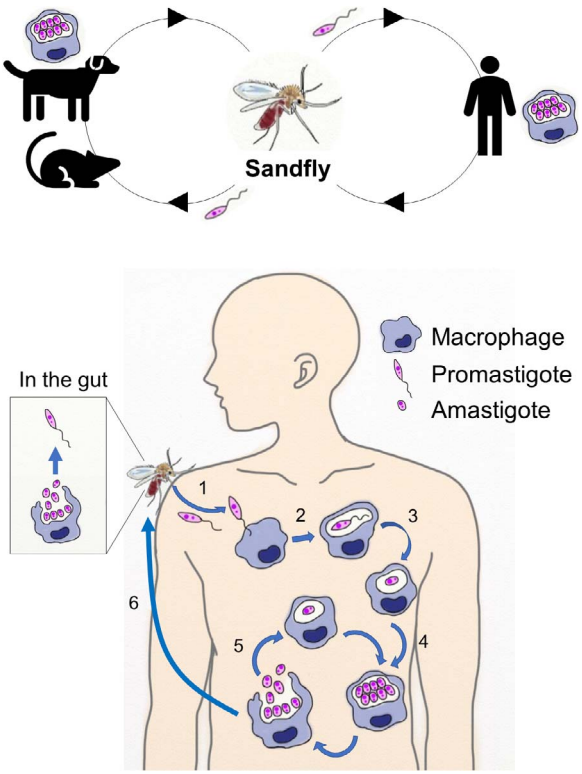


Fig. 1. The life cycle of *Leishmania.* *Leishmania* parasites are transmitted by the bites of blood-sucking sandflies. In sandflies, the parasites proliferate as promastigotes, a flagellated developmental form, and are delivered into mammalian hosts through sandfly bites (1). Once entered mammalian hosts, the parasites are phagocytosed by macrophages (2), and within phagolysosomes of macrophages promastigotes transform into amastigotes, an aflagellated developmental form (3). Amastigotes can proliferate within phagolysosomes (4), and eventually rupture the infected macrophages to exit and to enter new macrophages (5). Amastigotes can be taken up by sandflies during their blood meal (6), and then transform into promastigotes in the gut of sandflies. Both anthroponotic and zoonotic transmissions are known for *Leishmania* parasites, and dogs and rodents serve as the major reservoirs for zoonotic leishmaniasis.

Therefore, amastigotes require a set of unique molecules for their intracellular survival and drugs targeting those molecules are expected to be highly effective and specific.

Roles of Iron and Heme in *Leishmania* Survival

Iron and heme are involved in many biochemical processes, including oxygen transport, ATP production, synthesis and repair of DNA and antioxidant defense *(3)*. However, during evolution, Kinetoplastida has lost the complete pathway for heme biosynthesis. Few trypanosomatid parasites (except *Trypanosoma*), such as *Leishmania,* have partially reacquired the genes encoding the last three enzymes of this pathway by horizontal gene transfer from a γ-proteobacteria thus making this parasite auxotroph for heme *(4, 5)*. Iron is an essential nutrient also for *Leishmania* parasites, while obtaining sufficient iron or iron-containing molecules in macrophages may be challenging for the parasites due to their secluded niche within phagolysosomes. Consequently, there are studies focusing on the acquisition and functioning of iron and iron-containing molecules in *Leishmania*. For example, there is a study that has reported the essentiality of an iron transporter of *Leishmania amazonensis* for parasite replication within phagolysosomes of macrophages *(6)*. As of March 9, 2023, a PubMed search with keywords *Leishmania* and 'iron' has retrieved 240 articles. Prominent keywords in titles of the articles include 'superoxide dismutase' (25 articles), 'heme' (12 articles) and 'transport(er)' (10 articles).

Once the iron is transported inside phagolysosomes of infected macrophages, the iron becomes easily accessible to the parasites. The *Leishmania*-specific genes responsible for iron mobilization and utilization can be categorized into the following types:

1. Iron reductase

Dietary iron can be categorized into heme and ionic forms. Non-heme iron primarily exists in non-bioavailable oxidized-Fe^{3+} form which remains complexed with transferrin or lactoferrin and must be reduced to the ferrous Fe^{2+} form to make it accessible for these parasites *(7)*. Wilson *et al.* in their study have shown the presence of nicotinamide adenine dinucleotide phosphate reduced (NADPH)-dependent iron reductase activity in promastigotes of *Leishmania chagasi (8)*. According to this report, promastigotes reduce the oxidized chelated form of iron in order to get internalized for utilization by these parasites *(8)*. Another group identified and functionally characterized one of the members of the *Leishmania* iron acquisition pathway, ferric iron reductase 1 (LFR1) *(9)*. The knock-out of LFR1 has no defect in the procyclic promastigotes but rather shows defects in vertebrate-infective forms, i.e. metacyclic promastigotes and amastigotes *(9)*.

2. Iron and heme transporters

Reduced iron (Fe^{2+}) and heme can be directly uptaken by *Leishmania* parasites. Jacques *et al.* have identified and characterized a ZIP-family ferrous iron transporter (LIT1) in *L. amazonensis* based upon homology with IRT1, a ferrous iron transporter from *Arabidopsis thaliana (10)*. Interestingly, LIT1 contains two regions that are not found in IRT1 and deletion of those regions affects the iron transport capacity, as well as intracellular growth of the parasite *(10)*.

On the other hand, *Leishmania* can utilize heme bound to host hemoglobin as well as direct hemoglobin *(11)*. Once receptor-mediated and clathrin-dependent hemoglobin endocytosis occurs, hemoglobin is digested via early Rab5 and late Rab7-containing endosomes. The digested heme moiety gets released into the cytosol which can be translocated to different cellular organelles to perform its function *(12–15)*.

Huynh *et al*. have identified and characterized a heme transporter present in plasma membrane of *L. amazonensis* named as *Leishmania* heme response-1 (LHR1), the transcript level of which gets activated upon heme deficiency *(16)*. LHR1 is a homolog of *Candida elegans* heme transporter heme-responsive gene 4 and is required for the virulence of this parasite *(16)*. Cabello-Donayre *et al*, have identified and characterized a novel porphyrin receptor feline leukemia virus subgroup C receptor in *L. major*, LmFLVCRb that was exclusively found in metazoans *(17)*.

LmFLVCRb functions as a heme importer and is responsible for imparting virulence in parasites *(17)*.

3. Superoxide dismutase (SOD)

Macrophages produce reactive oxygen species (ROS) to control invasive microorganisms. The ROS include superoxide anion, hydrogen peroxide and hydroxyl radical. *Leishmania* amastigotes are exposed to the ROS attack inside macrophages and therefore need to detoxicate the ROS. Phagocytes such as neutrophils and macrophages produce superoxide anion during through activation of NADPH oxidase *(18)*. An *in vivo* study demonstrated exacerbated skin lesion development in mice defective in NADPH oxidase *(19)*. Another study showed that $gp91^{phox\ -/-}$ mice have a higher liver parasite burden than wild-type mice when infected with *Leishmania donovani (20)*. Some reports demonstrated its antileishmanial effect *in vitro* using superoxide inducers or NADPH oxidase inhibitor *(21–24)*, while some report demonstrates its minimal effects in reducing the number of *Leishmania* parasites *in vitro* and *in vivo (19, 25, 26)*. Of relevance, there is a report that infection with *Trypanosoma cruzi*, another Kinetoplastid protozoan pathogen like *Leishmania*, exhibits higher organ parasite load in presence of prooxidants *(27)*. These conflicting results imply that macrophages produce superoxide for sure but *Leishmania* parasites have systems to mitigate the stress.

SOD is an enzyme to catalyze the conversion of superoxide to hydrogen peroxide and oxygen *(28–30)*. In mammals, there are three types of SODs, i.e. SOD1 (cytosolic), SOD2 (mitochondrial) and SOD3 (extracellular). Mammalian SOD1 and SOD3 are Cu, ZnSOD, while SOD2 is MnSOD. FeSOD can be observed in primitive eukaryotes, the plastids of plants and in bacteria, and FeSOD and MnSOD appear to have evolved from a common ancestral gene *(29, 31)*. FeSOD is the sole SOD found in trypanosomatid suggesting that this category of ROS-regulating enzymes may be particularly vulnerable to changes in iron content.

Leishmania parasites have at least two FeSOD genes, i.e. FeSODA and FeSODB *(32)*. According to the genome information on TriTrypDB, both FeSODA and FeSODB are well conserved across *Leishmania* species, with FeSODA as a single-copy gene while FeSODB as a multi-gene family as originally identified *(32)*. The two FeSODs have low sequence identity (~40%), partially due to the extra amino acid extension at the N-terminus of FeSODA *(32)*. Later, FeSODB is further classified into FeSODB1 and FeSODB2; the former is identical to the previously characterized FeSODB, whereas the latter, despite the high similarity to FeSODB1 in sequence, has an opposite trend in expression, i.e. higher in early log-phase promastigotes and lower in amastigotes *(33)*. In *Leishmania*, both FeSODB1 and FeSODB2 are located in glycosomes *(33)*, whereas FeSODA is located in mitochondria *(34, 35)*. FeSODA and FeSODB1, the two isoforms upregulated in amastigotes, are indeed required for the developmental stage; FeSODA is required for transformation of promastigotes to amastigotes, consequently for virulence in mice *(35)*, and FeSODB1 demonstrates a beneficial role in intracellular survival of amastigotes *(33, 36)*.

4. Heme proteins

In *Leishmania*, many heme proteins have been identified and characterized to be involved in various biological processes including respiration, oxidative/nitrosative stress defense, signal transduction and sterol synthesis (reviewed in *(5)*). Heme also serves as a source of iron to the parasites *(11)*. As being involved in such the fundamental biological processes, defect in heme uptake results in impairment of parasite survival both in promastigotes and amastigotes *(16, 37)*. Because many of the biological processes involving heme are conserved widely in many organisms, we would like to introduce only one of them, ascorbate peroxidase, in this section.

As mentioned earlier, SODs catalyze conversion of superoxide anion to hydrogen peroxide. The product is still a part of ROS and is toxic to *Leishmania* parasites *(38–40)*. Heme peroxidases are known to function in scavenging hydrogen peroxide and *L. major* ascorbate peroxidase (LmAPX) is one of such enzymes. LmAPX, which locates in mitochondria of promastigotes, shows an upregulated expression in response to hydrogen peroxide and its overexpression in the parasites leads to increased tolerance to hydrogen peroxide *(41)*. On the contrary, although LmAPX-null promastigotes are more sensitive to hydrogen peroxide-mediated growth inhibition, the null parasites have superior ability to infect and survive within macrophages and higher virulence in experimental murine infection *(42)*. Although the mechanism is not clear, the other hydrogen peroxide-catalyzing peroxidases, e.g. tryparedoxin peroxidases *(43, 44)* may be more important compared to APX for intracellular survival of *Leishmania* parasites.

How Does the Parasite Acquire Iron-Containing Molecules from the Host?

In this review, we would like to discuss acquisition of iron, heme and hemoglobin with a focus on amastigotes within macrophages. As *Leishmania* amastigotes reside within phagolysosomes of macrophages, successful delivery of iron-containing molecules to the parasites may be sequentially categorized into three steps: acquisition of the iron-containing molecules by macrophages and their delivery to the cytoplasm, mobilization of the molecules from macrophage's cytoplasm to phagolysosome and acquisition of the molecules in phagolysosome by *Leishmania*.

Macrophages uptake iron in three forms: non-transferrin-bound iron (NTBI), transferrin-bound iron (TBI) and heme/hemoglobin. NTBI can be acquired by divalent metal transport 1 (DMT1), also known as natural resistance-associated macrophage protein 2 (NRAMP2), in enterocytes *(45)*. Although a recent study showed that bone marrow-derived macrophages lacking DMT1 showed less resistance to *Salmonella* infection, possibly due to reduced uptake of NTBI *(46)*, the significance of its contribution to iron acquisition in *Leishmania*-infected macrophages remains unclear.

TBI, carried by holo-transferrin in the blood, can be delivered into macrophages through the transferrin receptor (TfR) *(47, 48)*. As many intracellular pathogens require iron for their survival, macrophages rather regulate iron uptake by suppressing expression of TfR to avoid iron

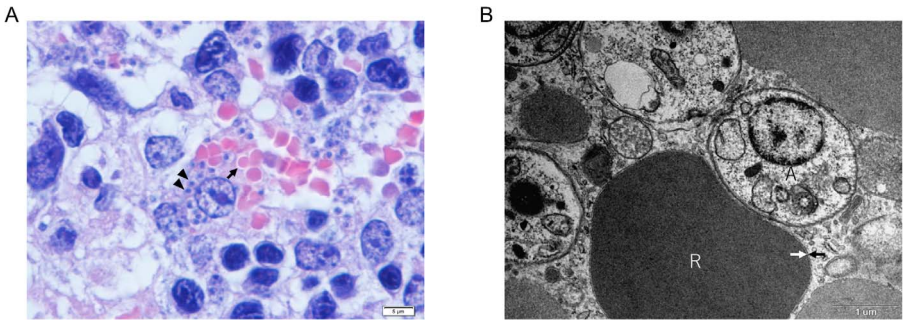


Fig. 2. Phagocytosis of erythrocytes by *L. donovani*-infected splenic macrophages. (A) HE-stained section of the red pulp of spleen from a *L. donovani*-infected mouse. Erythrocytes (arrow) are individually packed in phagosomes of macrophages and do not share the compartments with *L. donovani* amastigotes (arrowheads). Scale bar, 5 μm. (B) Transmission electron microgram of the red pulp of spleen from a *L. donovani*-infected mouse. As the membrane of an erythrocyte (marked as R) is indicated with a white arrow and the phagosomal membrane is indicated with a black arrow, the erythrocyte is separated by membrane structures from *L. donovani* amastigotes (marked as A). Scale bar, 1 μm.

sequestration by the invading organisms *(49, 50)*. On the other hand, *L. donovani* can induce increased expression of TfR by macrophages *(51)*. This upregulation is triggered by the depletion of labile iron pool in macrophages by *Leishmania* parasites as does an iron chelator deferoxamine *(51)*. Intracellular survival of *Leishmania* is hampered by deferoxamine-mediated depletion of labile iron pool while addition of holo-transferrin to the culture medium can compensate for the depleted labile iron pool *(51)*, which *Leishmania* seem to secure by promoting transferrin uptake by infected macrophages.

Another source of iron, especially to splenic macrophages, is heme in hemoglobin. Splenic macrophages engulf senescent or damaged erythrocytes and serve as the key players for iron recycling. Spleen is also the important organ for parasitization of VL-causing *Leishmania* species, including *L. donovani* and *L. infantum*. One of the intriguing features of *L. donovani* is to augment the hemophagocytosis of infected macrophages. In the spleen of *L. donovani*-infected mice, multinucleated giant cells engulfing erythrocytes are prominent *(52)*. Because those hemophagocytes are characterized by heavy infection with amastigotes *(52)*, it seems *L. donovani* actively transforms infected macrophages into hemophagocytes *in vivo*. This is supported by an *in vitro* experiment that macrophages infected with *L. donovani* engulf undamaged erythrocytes as opposed to uninfected macrophages *(53)*. Hemophagocytosis can be found in human VL cases *(54–58)*, suggesting that this phenomenon is not an artificial event only in the mouse model.

Erythrocytes engulfed by splenic macrophages are digested in their phagolysosomes resulting in release of heme from hemoglobin. The heme in the phagolysosome is transported to the cytosol by heme-responsive gene 1 (HRG1) *(59)* and catabolized into biliverdin, ferrous ion and carbon monoxide by heme oxygenase 1 (HMOX1). The resulting iron can be stored by ferritin in the cytoplasm of the macrophage. Because phagocytosed erythrocytes are often individually packed and found in compartments distinct from those for *Leishmania* amastigotes (Figure 2), the forementioned phagosome-to-cytoplasm transportation seems required before the parasites in the separate compartments have access to the erythrocyte-derived heme/iron. In other words, no matter how heme/iron molecules are acquired by macrophages, they might come to the cytoplasm before being transported to phagolysosomes where *Leishmania* parasites resides. It remains unclear what kind of transporters are responsible for this cytosol-to-vacuole delivery but it can be hypothesized that probably this iron is delivered as Fe^{2+} ion which can be utilized by *Leishmania* amastigotes using LIT1. In a recent report, it has been observed that infection with *L. major* induces proteasomal degradation of natural resistance-associated macrophage protein 1 (NRAMP1), which increases bio-available phagolysosomal iron *(60)*. Interestingly apart from *Leishmania*, other intracellular pathogens like *Mycobacterium* and *Salmonella (61)* have also been reported to cause defect in NRAMP1 benefitting their intracellular survival. Once ferrous iron and heme exist in the fluid surrounding *Leishmania* amastigotes within the phagolysosomes of macrophages, the parasites can acquire the molecules using either the ferrous iron transporter LIT1 *(6)* and the heme transporter LHR1 *(37)*, respectively. Although *Leishmania* are known to possess receptors for both transferrin and hemoglobin *(12, 62)*, holo-transferrin or hemoglobin may not to be the major forms of iron supply to intracellular amastigotes within the phagolysosomes.

For *Leishmania*, not only securing iron in phagolysosomes but also storing cytoplasmic iron by macrophages and inhibiting its export to the extracellular compartment should be beneficial. Ferroportin (FPN) is involved in release of iron by macrophages after erythrophagocytosis, and the expression of FPN is downregulated by hepcidin *(63)*. Therefore, the parasites regulate the expression of FPN by macrophages directly or indirectly through hepcidin to increase the bio-available iron *(64, 65)*. In human VL cases, patients with lower blood hemoglobin levels showed higher hepcidin expression levels in peripheral blood mononuclear cells *(66)*. Considering that upregulated hepcidin results in suppression of FPN leading to inhibition of iron export in macrophages, it is possible that hepcidin helps *Leishmania* grow more efficiently within the phagolysosomes of macrophages by providing better iron supply to the parasites. Also as forementioned, ferritin serves as iron storage in macrophage cytoplasm and excess iron in cytoplasm can be loaded into ferritin, and poly(rC)-binding proteins act as chaperones for the loading *(67)*. A recent study demonstrated that *L. donovani* interferes with iron sequestration into ferritin by cleaving the chaperones

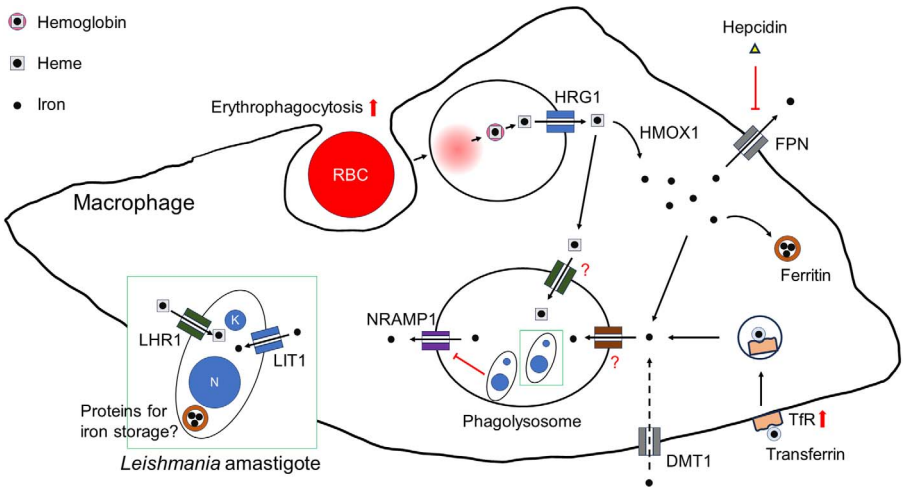


Fig. 3. Schematic illustration of *Leishmania*-macrophage interfaces for intracellular iron acquisition. Macrophages uptake iron in 3 forms: NTBI, TBI, and heme/hemoglobin. NTBI can be acquired by DMT1, whereas the significance of its contribution to iron acquisition in *Leishmania*-infected macrophages remains unclear. TBI can be delivered into macrophages through the TfR, and *Leishmania donovani* can induce increased expression of the TfR on macrophages. Another source of iron, especially to splenic macrophages, is heme in hemoglobin. *L. donovani* augments erythrophagocytosis by infected macrophages. Erythrocytes engulfed by splenic macrophages are digested in their phagolysosomes resulting in release of heme from hemoglobin. The heme in the phagolysosome is transported to the cytosol by HRG1 and catabolized into biliverdin, ferrous ion and carbon monoxide by HMOX1. The resulting iron can be stored as labile iron pool in the cytoplasm of macrophages, and if excess, the iron is either stored in ferritin or expelled from the cell by FPN. The parasites regulate the expression of FPN by macrophages directly or indirectly through hepcidin to increase the bio-available iron. Although it remains unclear what kind of transporters are responsible for cytosol-to-phagolysosome delivery of iron or heme, *Leishmania* parasites possess transporters for both, i.e., LIT1 and LHR1, respectively. NRAMP1 may act to export iron from the phagolysosome, and *L. major* induces proteasomal degradation of NRAMP1, which increases bio-available phagolysosomal iron. So far, no iron storage protein like ferritin in mammalian cells has been identified in *Leishmania*.

(68), suggesting another strategy of the parasites for securing available iron.

Is Iron Acquisition a Good Target for Control of the Parasite Infection?

Treatment of leishmaniasis currently relies on pentavalent antimonial, amphotericin B and its liposomal formulation, miltefosine and paramomycin *(2)*. Although modes of action for these drugs are fully elucidated, their major targets may not be iron acquisition by *Leishmania* parasites. As mentioned above, iron is an indispensable nutrition for intracellular survival of *Leishmania* parasites within macrophages *(51)*. A study has shown that trans-chalcone, a common precursor of flavonoids, acts as an antileishmanial agent possibly modulating iron acquisition by macrophages, although specific target is not defined *(69)*. Another study has shown that quercetin serves an iron chelator and has an antileishmanial effect *in vivo (70)*. However, treatment with iron chelators, such as deferoxamine and deferiprone, may bring detrimental effects, and more specific targets are needed for leishmaniasis treatment with minimal adverse events. A recent study has also reported that *L. major* infection in mice causes accumulation of iron at the infection site that is associated with decreased iron storage in the liver *(71)*. Intriguingly, oral iron supplementation to the infected mice resulted in restoration of systemic iron homeostasis and reduction of parasite load *(71)*. On the other hand, changes in liver iron levels were not found in *L. amazonensis* infection *(72)*. Also, outcomes of systemic iron overload differ between *L. major* and *L. infantum* infections *(73)*. Together, these indicate a complicated relationship between local iron at the infection site and systemic iron and difficulty of systemic iron regulation as a treatment option for antileishmanial treatment. Because the parasites have various unique strategies to secure the resource in the contained environment and because there are many iron-related molecules not highly conserved between *Leishmania* parasites and the mammalian hosts, targeting iron acquisition by *Leishmania* parasites may be a good strategy for development of antileishmanial drugs. Nonetheless, target-based drug development against such leishmanial molecules does not seem to be very progressive and few articles with success can be found in the literature so far.

Host-directed therapies correcting the parasite-modulated functions of macrophages may be another choice in treating leishmaniasis. Although hemophagocytosis is a phenomenon that splenic macrophages engulf senescent or injured erythrocytes under normal condition *(74)*, enhanced engulfment of erythrocytes by hyper-activated macrophages can be observed during VL *(52, 53, 75)*. Splenic macrophages limit phagocytosis of intact erythrocytes by an 'don't-eat-me' signal through signal regulatory protein α (SIRPα) *(76)*. Intriguingly, *L. donovani* can suppress the expression of SIRPα in macrophages by post-transcriptional control *(53, 77)*. Identification of *Leishmania* molecules responsible for this downregulation of SIRPα may lead to control of enhanced erythrophagocytosis by the infected macrophages resulting in limiting iron availability to the parasites.

In conclusion, intracellular amastigotes manipulate their host macrophages to acquire iron and heme from outside and keep those molecules within the cells. With good access to iron and heme in the phagolysosomes, the parasites have multiple transporters to uptake those molecules

into their bodies and utilizes them for various functions (Figure 3). One of the missing pieces in iron homeostasis of *Leishmania* is the storage; no iron storage protein like ferritin in mammalian cells has been identified in *Leishmania* yet. The finding that detrimental effects of iron depletion in culture medium appear not acutely but appear slowly *(78)* indicates the existence of the iron storage mechanism in *Leishmania*. Identification of leishmanial proteins involved in the iron storage may facilitate target-based drug development for leishmaniasis together with the other *Leishmania*-specific proteins for iron homeostasis.

Acknowledgments

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