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Iron acquisition in *Leishmania* and its crucial role in infection

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SUMMARY

Iron is an essential cofactor for many basic metabolic pathways in pathogenic microbes and their hosts. It is also dangerous as it can catalyse the production of reactive free radicals. This dual character makes the host can either limit iron availability to invading microbes or exploit iron to induce toxicity to pathogens. Successful pathogens, including *Leishmania* species, must possess mechanisms to circumvent host's iron limitation and iron-induced toxicity in order to survive. In this review, we discuss the regulation of iron metabolism in the setting of infection and delineate the iron acquisition strategies used by *Leishmania* parasites and their subversions to host iron metabolism to overcome host's iron-related defences.

Key words: iron, *Leishmania*, iron acquisition, subversion.

INTRODUCTION

Leishmaniasis, which is caused by the intracellular protozoan parasite *Leishmania*, is reported to be the ninth largest infectious disease burden worldwide. Its global incidence is 2 million new cases per year (Alvar *et al*. 2012). The disease's manifestation, which varies depending on the species involved and host, ranges from self-healing cutaneous lesions to very severe visceral disease (McCall *et al*. 2013). Mortality due to visceral leishmaniasis can be as high as 100% within 2 years if without treatment (McCall *et al*. 2013). *Leishmania* parasites have a digenetic life cycle, alternating between the promastigote stage in the sandflies' gut and the amastigote stage in macrophages of mammalian hosts (Kaye and Scott, 2011). The intracellular stage of *Leishmania* have specialized mechanisms that allow them to survive and acquire nutrients from host cells. Iron is one of such essential nutrients. Iron is the fourth most common element in the earth's crust and present in the vast majority of living organisms. The ability of iron to readily lose and gain an electron thereby oscillating between the ferrous and ferric states is critical to the activity and is a structural determinant of a plethora of enzymes and proteins. In parasites, these proteins, including iron superoxide dismutase (SOD), ascorbate peroxidase, cytochrome b5 (CytB5) and cytochrome p450 (CYP), are involved in detoxification of reactive oxygen species, fatty acid desaturation and ergosterol synthesis (Taylor and Kelly, 2010; Tripodi *et al*. 2011) (Fig. 2B). Moreover, iron is a component of ribonucleotide reductase and iron clusters in the mitochondrial respiratory chain, which suggests that iron is also critical for energy metabolism and DNA synthesis in *Leishmania* (Sen *et al*. 2008; Tripodi *et al*. 2011; Phan *et al*. 2015). Of course, its participation in haeme and iron-sulphur cluster containing proteins is also essential for host in a variety of vital biological functions (Gozzelino and Arosio, 2016). Thus, iron is an object of extreme competition between pathogens and their hosts.

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THE METABOLISM OF IRON IN HOST

Dietary iron is found in haeme and ionic forms, and their absorption occurs in the proximal portion of the duodenum, carried out by enterocytes via different mechanisms (Andrews and Schmidt, 2007). Dietary non-haeme iron primarily exists in an oxidized (Fe^{3+}) form that is not bioavailable and must be reduced to the ferrous Fe^{2+} form before it is transported across the intestinal epithelium. The responsible ferrireductase is a membrane bound haemoprotein called duodenal cytochrome B (DCYTB), expressed in the brush border of enterocytes (McKie *et al*. 2001). Then, a protein called divalent metal transporter 1 (DMT1) transports Fe^{2+} into enterocytes (Fig. 1A) (Morgan and Oates, 2002). After being transported into the enterocytes, Fe^{2+} can be accumulated inside the cell, used for cellular processes, or exported to the circulatory system by the basolateral membrane transporter ferroportin 1 (Abboud and Haile, 2000; Donovan *et al*. 2000). Once exported by ferroportin 1, iron must be transformed in process coupled by reoxidation of Fe^{2+} to Fe^{3+} by ferroxidases, such as hephaestin, and followed by the loading of Fe^{3+} onto transferrin (Tf)

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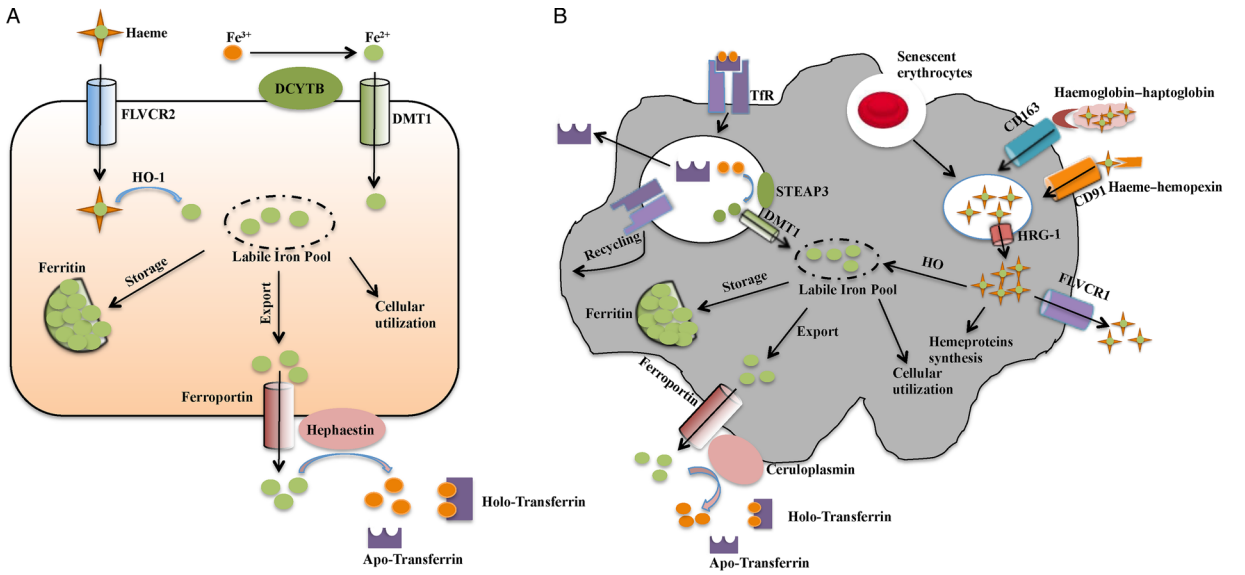

Fig. 1. Iron transport and homeostasis in human cells. (A) Prior to transport into intestinal enterocytes, dietary ferric iron is reduced by ferric reductase duodenal cytochrome B (DCYTB), followed transported into cells by DMT1. Haeme absorption is performed by the haeme receptor FLVCR2, and the iron is extracted from haeme by haeme oxygenase. After absorption, iron can be used for cellular processes, stored in ferritin, or exported out of cells by ferroportin in partnership with hephaestin. (B) Macrophages gain iron via phagocytosis of senescent erythrocytes, TFR-mediated endocytosis of iron-loaded transferrin, or uptake of haeme–haemopexin and haemoglobin–haptoglobin complexes. Haeme in the phagolysosome is transported into the cytosol by HRG-1, where it can be degraded by HO to iron. This haeme may also be effluxed from the cell by the haeme exporter FLVCR1. Cytoplasmic iron be used for cellular processes, stored in ferritin, or exported out of macrophages by ferroportin coupled with ceruloplasmin.

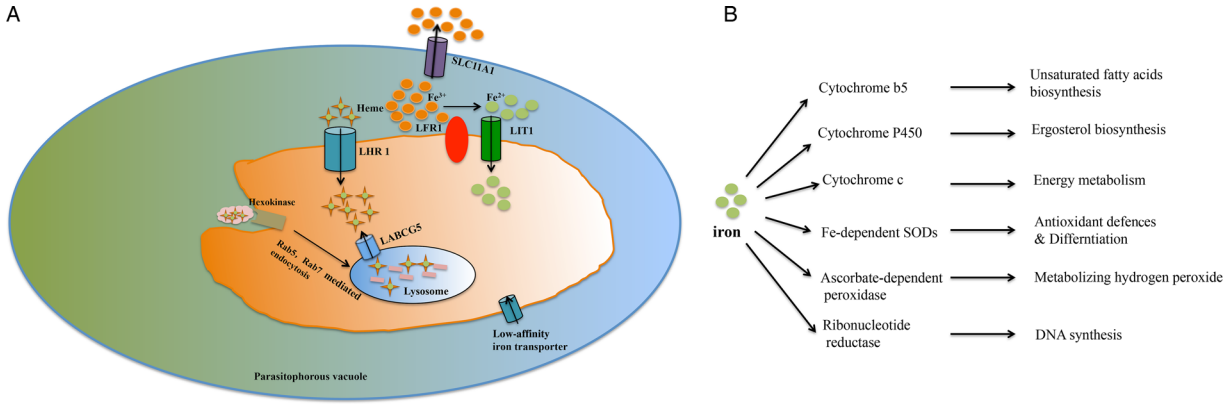

Fig. 2. Iron acquisition pathways and its role in *Leishmania*. (A) Iron acquisition in *Leishmania*. The host transporter SLC11A1 (Nramp1) transports iron into cytoplasm from PVs, thereby limiting iron availability to *Leishmania*. To compete with host, intracellular leishmanial parasites upregulate the expression of ferric iron reductase LFR1 on their plasma membrane, converting Fe^{3+} into Fe^{2+}. Fe^{2+} is then transported into leishmanial cytosol by LIT1. Iron-containing haeme can be transported into leishmanial cytosol by hexokinase-mediated endocytosis pathway or by the haeme transporter LHR1 directly. When high concentrations of iron are available, low-affinity iron transporters contribute to the iron acquisition of *Leishmania*. (B) The role of iron in *Leishmania*. Cytochrome b5 that is small haeme-binding protein acts as an electron-transfer component in the desaturation reaction, which catalysed by fatty acid desaturases. An important step in the synthesis of ergosterol, an essential component of parasite membranes, is carried out by a sterol-14-α-demethylase CYP51. Haeme-binding protein cytochrome p450 is as cofactor of CYP51 accepting electrons. Mitochondrial cytochrome c is a haemeprotein and plays a role in the electron transfer during oxidative phosphorylation. Iron-dependent superoxide dismutases (Fe-SODs) in *Leishmania* help to protect parasites from oxidative stress by catalysing the dismutation of superoxide radicals into H_2O_2 and O_2, the H_2O_2 then being metabolized by peroxidases. Iron is also a cofactor of ribonucleotide reductases in *Leishmania*, which catalyse *de novo* biosynthesis of deoxynucleotides that are used in the synthesis of DNA.

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(Ganz and Nemeth, 2012; Musci *et al*. 2014). A transferrin can bind two atoms of ferric iron, which can limit iron-catalysed free radical production and facilitate transport to target cells (Fig. 1B) (Reyes-Lopez *et al*. 2015). Iron-loaded transferrin can bind with high affinity to the transferrin receptor 1 (TFR1) expressed ubiquitously at cell surfaces, and is then internalized by clathrin-dependent endocytosis (El Hage Chahine *et al*. 2012; Harris, 2012). The endosomal acidification facilitates release of iron, and the TFR complex is recycled to the cell surface. Ferric iron released from transferrin is reduced by the ferrireductase six-transmembrane epithelial antigen of the prostate 3 (STEAP3) protein in erythrocytes and other STEAP proteins in non-erythroid cells (Ohgami *et al*. 2005, 2006), and subsequently transported into the cytoplasm by DMT1 (Graham *et al*. 2007). From this point, iron can be incorporated into metalloproteins in complexes with haeme (e.g. catalase, cytochromes, haemoglobin and myoglobin), as mono and dinuclear iron (e.g. ribonucleotide reductase), or as Fe–S clusters (e.g. aconitase, succinate dehydrogenase) (Rouault and Tong, 2008; Dlouhy and Outten, 2013). Iron that is not needed for immediate use or export can be stored in ferritin, a spherical heteropolymer capable of storing up to 4500 iron atoms (Harrison and Arosio, 1996).

The mechanism of dietary haeme uptake remains to be clarified. A candidate intestinal haeme transporter, haeme carrier protein 1 (HCP1), was proposed (Shayeghi *et al*. 2005), but later evidence suggests it is most likely a folate transporter (Qiu *et al*. 2006). The haeme responsive gene-1 (HRG-1), which was first identified in *Caenorhabditis elegans* as a haeme importer (Rajagopal *et al*. 2008), appears to transport haeme in human, but rather from the lysosome into the cytosol (Delaby *et al*. 2012). FLVCR2 (feline leukaemia virus, subgroup C receptor 2) was also recently reported to mediate the endocytosis of haeme by mammalian cells (Duffy *et al*. 2010). Once internalized in the enterocyte, iron is released from haeme by haeme oxygenases (HO) and then either stored or transported out of the enterocyte via mechanisms similar to that of ionic iron.

Although iron has critical functions in many cellular processes, iron overload is harmful because Fe^{2+} can generates highly reactive hydroxyl radicals in the presence of oxygen by the Fenton and Haber–Weiss reaction (Wessling-Resnick, 2000; Weiss, 2002). For this reason, the iron metabolism *in vivo* is tightly regulated. Intracellular iron levels are regulated by an elegant machinery involving the iron regulatory proteins (IRPs) and the iron-responsive elements (IREs). In low iron conditions, the binding of IRPs to IREs represses mRNA translation when located in 5′-untranslated regions (e.g., ferritins) and stabilizes the mRNA when in 3′-untranslated regions (e.g, TFR1) (Torti and Torti, 2002). In conditions of iron excess, the expression of ferritins is promoted and TFR1 is downregulated (Torti and Torti, 2002). In addition to IRP-mediated regulation in cellular level, iron metabolism is also regulated systemically. The hormone hepcidin is regarded as the central regulator of systemic iron homeostasis. Hepcidin is a 25 amino acid peptide that is mainly produced by hepatocytes. It can bind to the iron exporter ferroportin, leading to its internalization and degradation. Thus, it reduces the iron entry into plasma after enterocyte absorption and the iron export after recycling by the reticuloendothelial system (Nicolas *et al*. 2002).

Macrophages play a central role in the maintenance of iron homeostasis, delivering approximately 95% of the iron required for *de novo* haeme synthesis during erythropoiesis (Korolnek and Hamza, 2015). Apart from gaining iron from iron-loaded transferrin as described above, macrophages can engulf senescent erythrocytes via phagocytosis, and degrade their haemoglobin content in phagolysosome. Free haemoglobin and haeme in blood, which are from ruptured erythrocytes, can be trapped by haptoglobin and haemopexin, and taken up by macrophages via the CD163 receptor and CD91 receptor, respectively (Kristiansen *et al*. 2001; Hvidberg *et al*. 2005). Labile haeme in phagolysosome is transferred to the cytoplasm by HRG-1 (Rajagopal *et al*. 2008; White *et al*. 2013), and then can be secreted from macrophages via haeme exporters or degraded by HO, which extracts iron from the tetrapyrrole ring of haeme, resulting in the reutilization of iron (Korolnek and Hamza, 2015). The labile iron in macrophages can be excreted via ferroportin-1 or stored in macrophages by ferritin (Knutson *et al*. 2005; Gozzelino and Soares, 2014). Iron export from macrophages is controlled systemically by hepcidin as described above. Hepcidin expression is mainly regulated at the transcriptional level, being induced or repressed in response to multiple stimuli (Nemeth *et al*. 2003; Armitage *et al*. 2011; Loreal *et al*. 2014). The release of iron by HO induces ferritin synthesis via increasing the production of labile iron, which can block the capacity of iron regulatory proteins to repress translation of ferritin mRNA (Eisenstein *et al*. 1991).

The regulation of macrophages intracellular iron content affects innate and adaptive immune responses. Although the underlying mechanisms have not been entirely elucidated, iron perturbations regulate macrophages' response to interferon-γ (IFN-γ) (Weiss *et al*. 1992; Oexle *et al*. 2003), which is a central cytokine for the regulation of antimicrobial effector mechanisms of macrophages and can promote antigen presentation (Nathan *et al*. 1983; Mach *et al*. 1996). Decreased iron availability also inhibits translation of proinflammatory cytokines TNF-α and IL-6 and reduces LPS-induced

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cytokine expression in macrophages (Wang *et al*. 2008, 2009). Via its inhibitory potential on IFN-γ, iron affects T-helper (Th) cell differentiation favouring the expansion of Th-2 cells that produce a number of macrophage-deactivating cytokines such as IL-4 or IL-13 (Weiss, 2002). Accordingly, iron overload and iron chelation have been reported to modulate the ratio of Th-1/Th-2 cells in mice infected with different microbes (Mencacci *et al*. 1997; Ibrahim *et al*. 2007; Nairz *et al*. 2013). Lactoferrin was demonstrated to protect the host against pathogens by influencing systemic immune responses through regulation of the Th-1/Th-2 cytokine balance (Fischer *et al*. 2006). Iron deficiency also significantly decreases hepatic T cell and natural killer T cell activation (Bonaccorsi-Riani *et al*. 2015). Furthermore, both NADPH oxidase (gp91phox) and inducible nitric oxide synthase (iNOS), which are critical for microbicidal activity of activated macrophages (Mastroeni *et al*. 2000; Vazquez-Torres *et al*. 2000), are haemoproteins that require the insertion of haeme to catalyse the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Nathan and Shiloh, 2000). This argues that expression of these enzymes must be associated with mechanisms regulating haeme metabolism to support their activity. In support of this notion, haeme catabolism by HO-1 reduces haeme availability required to NADPH oxidase activity, acting therefore as a negative regulator of ROS production (Taille *et al*. 2004). Haeme catabolism by HO-1 also inhibits cyclooxygenase-2 (COX2) expression and prostaglandin synthesis (Haider *et al*. 2002), which play an important role in host inflammatory responses (Rouzer and Marnett, 2009).

IRON LIMITATION IS AN INNATE IMMUNE DEFENCE

Another reason for tightly regulating iron metabolism is related to the fact that regulation of iron distribution can serves as an innate immune mechanism against invading pathogens. Successful colonization by pathogens in host requires that these must gain access to the required amount of iron. However, even in the absence of infection, the concentration of free ionic iron is very low in host (Cassat and Skaar, 2013). Several facets contribute to this outcome. First, the majority of iron in humans is sequestered intracellularly, complexed within haemoglobin inside erythrocytes (Haley and Skaar, 2012). Second, extracellular iron is bound with iron-binding proteins transferrin, which have very high association constants for ferric iron and are normally only 30–40% saturated with iron (Bullen *et al*. 2005). Furthermore, even if transferrin-binding capacity is exceeded, iron also can be chelated by lower affinity molecules in plasma, including albumin, citrate and amino acids (Cassat and Skaar, 2013).

During infection, additional fortification of iron-withholding defence occurs. Hepcidin is not only a major orchestrator in the integration of iron metabolism but it also provides a critical connection with immune response to infection and inflammation. In fact, hepcidin was initially characterized by an antimicrobial peptide in human urine and blood ultrafiltrate (Krause *et al*. 2000; Park *et al*. 2001). Upon infection with pathogens, proinflammatory cytokines, TLR activation and ER stress can induce hepcidin expression and release from the liver, which results in hypoferraemia as described above (Drakesmith and Prentice, 2012). This systemic iron retention serves to deplete circulating iron that would otherwise be available to extracellular pathogens. Furthermore, hepcidin can also be produced by myeloid cells in response to pathogens in a TLR4-dependent fashion, allowing for regulating the iron availability at the infectious focus (Peyssonnaux *et al*. 2006). Some hepcindin-independent hypoferraemic mechanisms also exist, which further strengthen iron-withholding defences in response to infection (Weiss, 2005; Nairz *et al*. 2010).

In addition to systemic induction of hypoferraemia, some innate immune effectors further sequester iron locally at infectious foci. Lactoferrin is an antimicrobial peptide, which can bind two ferric ions with a high affinity (Baker and Baker, 2012). Constitutively high levels of lactoferrin, which produced by neutrophils and epithelial cells, are found in most surface secretions, including tears, saliva, bile and breast milk (Ward *et al*. 2002). Consequently, lactoferrin functions as a first line defence molecule against invading pathogens through its ability to sequester iron. Moreover, proinflammatory cytokines such as TNF-α also induce the release of lactoferrin from neutrophilic granules at the site of infection (Afeltra *et al*. 1997). Activated phagocytes at infection foci also limit iron availability to intracellular pathogens. Several proinflammatory cytokines, including IFN-gama, downregulate the expression of TFR1 in macrophages, thus reducing the intramacrophage access of iron (Byrd and Horwitz, 1989; Zhong *et al*. 2001; Olakanmi *et al*. 2002). Besides, the activation of macrophages enhances the expression of the SLC11A1 protein (solute carrier family 11 member a1, formerly termed Nramp1) (Vidal *et al*. 1995; Jabado *et al*. 2000). SLC11A1 is intracellular iron transporter, which will be recruited to the late endosomal and phagosomal membrane. It can pump iron out of phagosome into the cytosol, thereby limiting availability to intracellular pathogens in this compartment (Zwilling *et al*. 1999). It has been reported that polymorphisms in SLC11A1 is associated with susceptibility to tuberculosis, suggesting that iron limitation in phagosome is an important innate immune defence (van Crevel *et al*. 2009).

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IRON METABOLISM IN LEISHMANIA INFECTION

Several studies have reported an association between susceptibility to leishmaniasis and polymorphisms in the SLC11A1 gene that codes for an intracellular iron transporter as mentioned before, which suggests that the host iron status may influence the outcome of the *Leishmania* infection (Bucheton *et al*. 2003; Sanchez-Robert *et al*. 2008; Blackwell *et al*. 2009; Castellucci *et al*. 2010). However, the results concerning the impact of iron depletion on *Leishmania* infection are frequently contradictory. Some studies supported that the iron deprivation could be an effective strategy to control infections, similarly to what is found for other pathogens such as *Mycobacterium tuberculosis*. Incubation of *Leishmania major* promastigotes with iron-chelating compounds significantly suppresses parasite growth in a dose-dependent manner (Soteriadou *et al*. 1995). A study about *Leishmania chagasi* infection in BALB/c mice showed that the iron chelator desferrioxamine (DFO) caused significant reduction in haemoglobin concentration of treated mice and reduction in parasite load in spleen and liver (Malafaia *et al*. 2011). Similarly, DFO has shown an inhibitory effect on the intracellular growth of *Leishmania donovani* (Segovia *et al*. 1989) and *Leishmania amazonensis* (Borges *et al*. 1998). DFO treatment also leads to the reduction of parasite levels of *Trypanosoma cruzi*, a phylogenetic relative of *Leishmania*, and increases the efficacy of drug benznidazol (Arantes *et al*. 2007; Francisco *et al*. 2008). However, using the same iron chelator, Murray *et al*. found iron depletion did not affect the intracellular growth of *L. donovani* in unstimulated human macrophages (Murray *et al*. 1991). And dietary iron deficiency did not influence *Leishmania infantum* proliferation in the mouse model (Vale-Costa *et al*. 2013). In a *L. major* infection study, treatment with DFO just resulted in a very slight delay of the development of cutaneous lesions on the mouse footpad whereas systemic iron delivery significantly reduced *L. major*-caused pathology and parasite loads (Bisti *et al*. 2000). The impact of iron supplementation is also not consistent. Thus iron supplementation enhances the growth of *L. donovani* in macrophages and hamsters (Das *et al*. 2009). By contrast, the iron administration to susceptible mice clearly leads to inhibition of the growth of *L. major* in the skin and *L. infantum* in the liver and spleen (Bisti and Soteriadou, 2006; Vale-Costa *et al*. 2013). Undoubtedly, iron acquisition is essential for leishmanial pathogenicity based on its fundamental role and iron deprivation could be an effective host strategy to control leishmanial infections, which supported by the fact that these parasites are equipped with efficient iron acquisition systems and these systems are essential for their intracellular growth (Huynh *et al*. 2006; Renberg *et al*. 2015). However, on the other hand, the iron status of macrophages is also important to the host defence. Adequate concentrations of iron are required to support macrophage-killing mechanisms during infection that were correlated to oxidative burst (Collins *et al*. 2002; Vale-Costa *et al*. 2013). Intracellular iron accumulation is also involved in pro-inflammatory activation of macrophages and development of Th-2-type immune response as described above. Therefore, the host defence mechanism regarding macrophage iron sequestration against pathogen proliferation has to maintain a difficult balance. The apparent discrepancy regarding the role of iron in *Leishmania* infection may reflect the different sensitivity of *Leishmania* species to iron deprivation and iron-induced host killing like reactive oxygen and nitrogen species. Indeed, It has been reported that *Leishmania tropica* promastigotes were considerably more susceptible than *L. donovani* to hydrogen peroxide, showing a 50% lethal dose (LD50) of 0·5 and 1·5 nM min^{-1} respectively (Murray, 1981). *Leishmania braziliensis* and *L. infantum* also exhibited different susceptibility to hydrogen peroxide and antimony tartrate in a parallel study (Andrade and Murta, 2014). And the sensitivities of *Leishmania* species to nitric oxide *in vitro* are different as well (Lemesre *et al*. 1997). Furthermore, another point which we should note is that the chelating/loading efficiency of different iron depletion/supplementation protocols may be different.

HOW DO LEISHMANIA ACQUIRE IRON?

Like many other intracellular pathogens, *Leishmania* are also equipped with diverse iron acquisition mechanisms and are capable of utilizing various iron sources in order to thrive. Initial studies reported that *Leishmania* expressed a 70 kDa protein acting as TFR (Voyiatzaki and Soteriadou, 1992), but subsequent studies suggested that the binding between this protein and transferrin is not specific (Wilson *et al*. 2002). In the same report, it was reported that *L. chagasi* exhibits a NADPH-dependent iron reductase activity, capable of converting oxidized Fe^{3+} into the Fe^{2+} (Wilson *et al*. 2002). This discovery strongly suggested the potential existence of a ferrous iron transport mechanism in *Leishmania* because there is extensive evidence that ferric iron reduction is usually coupled to ferrous iron transport in prokaryotic and eukaryotic cells (Kosman, 2010; Caza and Kronstad, 2013). Indeed, a membrane Fe^{2+} transporter (LIT1) in *L. amazonensis* was identified and characterized subsequently (Huynh *et al*. 2006). As with other members of ZIP (Zrt/IRT-like proteins) transporter family, LIT1 has eight predicted transmembrane domains (Huynh *et al*. 2006). By the year 2011, a

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membrane protein with ferric reductase activity in *L. amazonensis* (LFR1) was identified (Flannery *et al*. 2011). LFR1 is a predicted 11 transmembrane protein with a molecular mass of 119 kDa, including an apparent signal peptide in N-terminal and the FAD- and NADPH-binding sites in tis C-terminal (Flannery *et al*. 2011). Together, LIT1 and LFR1 provide *Leishmania* with an inorganic iron acquisition pathway (Fig. 2A). These two proteins are localized to the plasma membrane reflecting that *L. amazonensis* can obtain iron directly from the lumen of the phagosome. Both LFR1 and LIT1 null mutants lost the ability to replicate in macrophages, indicating that this pathway of iron acquisition is essential for the ability of *Leishmania* to cause disease (Huynh *et al*. 2006; Flannery *et al*. 2011). Moreover, the overexpression of LFR1 was found to have toxic effects on the wild-type parasites, but was not toxic for LIT1 null mutants, which suggested that the toxicity of LFR1 overexpression is likely a direct consequence of increased intracellular Fe^{2+} concentration (Flannery *et al*. 2011). Interestingly, LFR1 overexpression also rescues the growth defect of LIT1 null mutants in macrophages by likely increasing available Fe^{2+} concentration (Flannery *et al*. 2011). This iron uptake mechanism is also a major trigger for the differentiation of *L. amazonensis* because both LIT1 and LFR1 null mutants are defective in differentiation of promastigotes into infective amastigotes after inducing by iron depletion, unlike the wild-type parasite (Flannery *et al*. 2011; Mittra *et al*. 2013). Iron depletion leads to ROS production in parasites by affecting Fe–S clusters and impairing the function of the mitochondrial electron transport chain. Followed up-expression of LFR1 and LIT1 induced by low iron conditions results in an increase of intracellular iron concentration and activation of Fe-SOD activity that converts the superoxide into H_2O_2, a molecule which is sufficient to trigger *Leishmania* differentiation signal (Mittra and Andrews, 2013). Recently, a mitochondrial iron transporter LMIT1 was identified and was demonstrated to be crucial for *Leishmania* mitochondrial function and virulence (Mittra *et al*. 2016). This study also supported that iron-dependent ROS signals generated in the mitochondria regulate differentiation of *Leishmania* amastigotes (Mittra *et al*. 2016). It was reported that the procyclic form of *Trypanosoma brucei*, whose bloodstream form acquires iron from transferrin by receptor-mediated endocytosis, takes up ionic iron via a reductive mechanism, perhaps similar with LFR1-LIT1 system in *Leishmania* (Mach *et al*. 2013). This study suggests that parasites may activate different iron acquisition systems in their different life cycle stages.

The growth of *Leishmania* also can be supported by iron derived from heme. Heme also functions an essential group for many enzymes, involved in a variety of critical cellular functions. However, *Leishmania* lack a complete haeme biosynthetic pathway and only can perform the last three steps catalysed by coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase, localized in the mitochondria (Alves *et al*. 2011). To survive, these parasites must acquire haeme from the environment. The data available to date indicates that *Leishmania* can acquire haeme by two mechanisms, haemoglobin receptor-mediated endocytosis and direct transmembrane transport. An initial report revealed that haemoglobin endocytosis in *Leishmania* is mediated through a 46-kDa protein located in the flagellar pocket, which was identified as a hexokinase subsequently (Krishnamurthy *et al*. 2005). But how this glycolytic enzyme is exported to cell surface remained elusive until more recently. After initial binding with hexokinase, the haemoglobin is internalized into discrete intracellular compartment and subsequently targeted to lysosome for degradation. Haemoglobin endocytosis in *Leishmania* is a clathrin dependent process (Agarwal *et al*. 2013). Its early step is regulated by a Rab5 homologue whereas transport of haemoglobin to the lysosomal compartment is controlled by Rab7 (Singh *et al*. 2003; Patel *et al*. 2008). The *L. donovani* ATP-Binding Cassette G5 (LABCG5) was proposed to be involved in haeme salvage after breakdown of haemoglobin in lysosome (Campos-Salinas *et al*. 2011). The direct transport of extracellular haeme into the parasites's cytosol is mediated by LHR1 protein in *L. amazonensis*, which shares homology with HRG-4, a plasma membrane haeme importer in *C. elegans* (Huynh *et al*. 2012). The LHR1 protein, with a molecular mass of about 20 kDa, localizes dually to the plasma membrane and lysosomes, regulating the intracellular pool of haeme in the parasites (Huynh *et al*. 2012). The LHR1 null strain could not be generated, suggesting that this transporter is essential for the survival of promastigote forms of *L. amazonensis* (Huynh *et al*. 2012). And the LHR1-single-knockout strain was not able to replicate in macrophages and was severely defective in the development of cutaneous lesion in mice (Miguel *et al*. 2013). Recently, Renberg *et al*. demonstrated that three key tyrosine residues within predicted transmembrane domains of LHR1 are required for efficient haeme uptake and *L. amazonensis* virulence (Renberg *et al*. 2015). As these tyrosine residues are unique to LHR1, not existing in its corresponding homologue in human, this study also implied that LHR1 could be an effective target for developing anti-leishmanial drugs. A recent study demonstrated that a mitochondrial ABC half-transporter LmABCB3 in *L. major*, which is essential for *Leishmania*, is involved in mitochondrial haeme biosynthesis from cytosolic protoporphyrin IX and required for the

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maturation of cytosolic iron/sulphur clusters (Martinez-Garcia *et al*. 2016).

As mentioned above, high iron concentrations can generate highly toxic hydroxyl radicals, which suggest that the iron uptake machinery should be tightly regulated. Indeed, the expression of LFR1 ferric reductase and LIT1 ferrous iron transporter is very low in promastigotes exposed to iron-rich condition and just upregulated in the response to iron deprivation although how *Leishmania* parasites sense extracellular iron concentrations remains to be clarified (Mittra *et al*. 2013). Moreover, overexpression of LFR1 is toxic for parasites when LIT1 is functional (Flannery *et al*. 2011). However, how *Leishmania* regulate their iron responsive genes is unclear. *Trypanosomatid* parasites do not regulate these genes expression by classic transcriptional promoters, utilizing instead post-transcriptional strategies such as regulation of mRNA stability or of translation initiation, which is thought to be mediated by control elements in the 5′ and 3′-untranslated regions (Flannery *et al*. 2013). A protein which binds mammalian iron response elements (IREs) was identified in *L. tarentolae* (Meehan *et al*. 2000). However, whether the mechanism of regulating iron-responsive genes in *Leishmania* is similar with the mammalian IRE/IRP paradigm remains elusive and future studies are needed. Another interesting question is how *Leishmania* parasites store iron. Although ferritins are found in bacteria, higher eukaryotes, but it has failed to yield a putative ferritin orthologue in published genomes of various *Leishmania* species. Yeast cells also lack ferritin but contain frataxins, which can bind and supply iron for assembly of Fe-S clusters (Adamec *et al*. 2000). Genes encoding frataxin-like proteins are present in all trypanosomatids including *Leishmania*. However, although frataxin is essential for Fe-S cluster biogenesis in *Trypansoma brucei*, it seems not play a role in iron storage as *T. brucei* frataxin does not form large complexes and knockdown of frataxin did not cause iron accumulation in mitochondria (Long *et al*. 2008). The role of frataxin in *Leishmania* still needs to be investigated. Another possibility is that *Leishmania* may store iron in a mineralized form like the *Ectocarpus siliculosus* that also lack ferritin (Bottger *et al*. 2012). Furthermore, it was reported that moderate amount of ionic iron can inhibit the growth or even kill leishimanial promastigotes and amastigotes (Mittra *et al*. 2013; Vale-Costa *et al*. 2013). Thus, the possibility that *Leishmania* have not an efficient iron storage mechanism may also exist. Anyway, additional studies are needed to clarify the alternative mechanism used by *Leishmania* for iron storage. These mechanisms may also contribute to differential sensitivity to environment iron concentrations reported for different *Leishmania* species.

SUBVERTING HOST'S IRON SYSTEMS

Apart from the mechanisms of iron uptakes, *Leishmania* parasites also are armed to subvert the host's iron metabolism system to their own advantage. An early study revealed that the *Leishmania pifanoi* infection promotes the delivery of human transferrin to the parasitophorous vacule, leading to acquisition transferrin-carried iron by parasites (Borges *et al*. 1998). Furthermore, *L. donovani* can directly deplete labile iron pool of macrophages to activate iron-sensing proteins (IRPs). IRPs then increase the TFR1 expression to increase host iron uptake, so that *L. donovani* could use the iron for its growth (Das *et al*. 2009). The decrease of labile iron pool caused by *L. donovani* was subsequently thought to be due to the decreased expression of SLC11A1 by a secretory peroxidase Prx (Singh *et al*. 2013). This peroxidase also can significantly decrease reactive oxygen/nitrogen species levels and proinflammatory cytokines levels in LPS activated macrophages (Singh *et al*. 2013). Hepcidin is a key regulator of host iron homeostasis. Recently, a study demonstrated that *L. amazonensis* infection can increase the hepcidin levels and lower the ferroportin content of macrophages in a TLR4-dependent manner, and thus increased total macrophage iron content for stimulating parasite intracellular replication (Ben-Othman *et al*. 2014). The mechanism by which *Leishmania* regulates hepcidin levels remains unknown. The zinc metalloprotease GP63, a leishmanial pathogenicity factor, has shown a capacity of augmenting TNF and IL-6 release probably by degrading Synaptotagmin XI (Arango Duque *et al*. 2014). IL-6 is the necessary and sufficient cytokine for the induction of hepcidin during inflammation (Nemeth *et al*. 2004). Thus, the GP63-mediated IL-6 secretion possibly is responsible for the higher hepcidin levels in *Leishmania*-infected macrophages. Moreover, in infected host cells, *Leishmania* parasites are sheltered within parasitophorous vacuoles (PVs), which raises a question that how parasites make iron enter the PVs. Iron acquisition from extracellular transferrin by *M. tuberculosis* occurs through delivery of Fe-transferrin to the phagosome via receptor-mediated endocytosis and fusion of the early endosome with the *M. tuberculosis*-containing phagosome (Olakanmi *et al*. 2002). The co-localization of human transferrin with the parasitophorous vacule was observed in *Leishmania*-infected macrophages, which suggests that the similar mechanism may exist. It has also been known that the iron stored within cytosolic ferritin can be mobilized and released through lysosomal proteolysis following autophagy from the cytosol into lysosomes (Linder, 2013). Whether PVs can fuse with these lysosomal vacuoles is an interesting question and need to be investigated. *Leishmania* has shown capabilities to manipulate intracellular vesicle

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trafficking. For example, *Leishimania* GP63 can inhibit recruiting gp91phox and thus hinder antigen cross-presentation by degrading VAMP8, which is a key protein that mediate vesicle trafficking (Matheoud *et al*. 2013). *Leishmania* surface glycolipid lipophosphoglycan (LPG), which can cause periphagosomal accumulation of F-actin and disruption of phagosomal lipid microdomains, functionally inhibited phagosome maturation by an impaired assembly of the NADH oxidase and the exclusion of the vesicular proton-ATPase from phagosome (Lodge and Descoteaux, 2005; Lodge *et al*. 2006; Vinet *et al*. 2009). However, whether and how *Leishmania* interfere with iron-relating vesicle trafficking in macrophages remain to be studied.

Concluding remarks

Despite considerable efforts to define the host and leishmanial determinants of iron homeostasis during infection, several important questions remain to be answered. First, although we have identified some critical proteins in leishmanial iron homeostasis, which are the additional components and how do they work? Second, it seems that *Leishmania* can acquire iron effectively in macrophages. Which mechanisms of *Leishmania* are involved in subverting the host's iron homeostasis systems? Third, unlike the case of mycobacterial infections, which it is well-established that iron deprivation inhibits pathogen proliferation. It is not clear completely that how the host controls parasites infection using iron. Providing or depriving? Given that iron is essential for *Leishmania*, the mechanism of acquisition and use are potential targets for new therapeutic approaches.

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