Chapter 1

Transferrin Binding Proteins as a Means to Obtain Iron in Parasitic Protozoa

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1. Introduction

Iron is the fourth most abundant element on Earth and is essential for almost all living organisms. However, it is not accessible to cells in every environment. Ferric iron solubility is low at physiological pH, and in aerobic environments, ferrous iron is highly toxic. Thus, iron is not free but bound to proteins [Clarke *et al.*, 2001; Taylor and Kelly, 2010]. In complex organisms, the majority of iron is intracellularly sequestered within heme-compounds or iron-containing proteins or is stored in ferritin.

Extracellular ferric iron is bound to lactoferrin (LF) and transferrin (TF). Lactoferrin is found mainly in secretions such as milk, saliva, mucosal secretions, and other secretory fluids. TF is the iron transporter that allows cellular iron uptake. Additionally, TF and LF maintain Fe^{3+} in a soluble and stable oxidation state, avoiding the generation of toxic free radicals through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} OH^- + OH$), which are deleterious to most macromolecules [Clarke *et al.*, 2001; Wandersman and Delepelaire, 2004; Halliwell and Gutteridge, 2007; Gkouvatsos *et al.*, 2012].

1.1. Transferrin and the transferrin receptor: An overview

TF is mainly found in serum and lymph. It binds two atoms of Fe^{3+} with high affinity (Ka of 10^{-23} M). TF is a single-chain glycoprotein with a molecular mass of approximately 80 kDa and two homologous lobes. Its saturation is indicative of body iron stores; under normal conditions, only 30% of the TF iron-binding sites are saturated. TF and LF maintain the free iron concentration at approximately 10^{-18} M in body fluids, a concentration too low to sustain bacteria and parasite growth [Bullen, 1981]. The relative low TF saturation and high affinity for iron allows TF to maintain a low iron concentration in the serum, thus acting as

the first line of defense against infections in that fluid by preventing invading microorganisms from acquiring the iron essential for their growth [Kaplan, 2002; Wandersman and Delepelaire, 2004; Halliwell and Gutteridge, 2007; Gkouvatsos *et al.*, 2012].

Virtually all cells express a transferrin receptor (TFR) on their surface; the quantity of receptor molecules reflects the cellular iron requirement. Human TFR (*Hs*TFR) is a glycoprotein of 180 kDa formed by two disulfide-bonded homodimers. The TFR/TF complex is endocytosed inside clathrin-coated vesicles in practically all cell types. In early endosomes, the content of the vesicle is acidified to approximately pH 5.5. This low pH weakens iron-TF binding; then, the iron is removed, reduced by a ferrireductase (Steap3), and transported out of the vacuole via the divalent metal ion transporter-1 (DMT1) to form the cellular labile iron pool (LIP); this pool consists of a low-molecular-weight pool of weakly chelated iron (ferrous and ferric associated to ligands) that rapidly passes through the cell. Both apoTF (TF without iron) and TFR return to the cell membrane to recycle the TF back to the bloodstream to bind iron in another cycle. At physiological pH, TFR has a much higher affinity for iron-loaded TF (holoTF) than for apoTF [Halliwell and Gutteridge, 2007; Sutak *et al.*, 2008; Gkouvatsos *et al.*, 2012]. There are two different TF receptors, TFR1 and TFR2. TFR1-mediated endocytosis is the usual pathway of iron uptake by body cells. TFR2 participates in low-affinity binding of TF, supporting growth in a few cell types, but the true role of TFR2 is unknown [Halliwell and Gutteridge, 2007; Gkouvatsos *et al.*, 2012].

2. Transferrin and pathogens

The effective acquisition of iron is indispensable for the survival of all organisms. To survive, bacteria, fungi and parasitic protozoa in particular require iron to colonize multicellular organisms. In counterpart, their hosts have to satisfy their own iron requirements and simultaneously avoid iron capture by pathogens. It is very important to the host iron-control strategy to keep this element away from invading pathogens: intracellular and extracellular iron stores are meticulously maintained so that they are unavailable for invaders. As a consequence, pathogens have evolutionarily developed several strategies to obtain iron from the host, e.g., specialized iron uptake mechanisms from host iron-binding proteins, such as TF, through the use of specific TF binding proteins or receptors [Wilson and Britigan, 1998; Wandersman and Delepelaire, 2004; Halliwell and Gutteridge, 2007; Sutak *et al.*, 2008; Weinberg 2009].

2.1. Prokaryotic pathogens

Although it is out of the scope of this chapter, it is important to briefly mention as a reference what has been found in other pathogens such as prokaryotes. Bacteria have evolved specific and efficient mechanisms to obtain iron from various sources that they may contact in their diverse habitats and to compete for this element with other organisms sharing the same space. Some pathogenic bacteria can produce and secrete siderophores, which are low molecular-weight compounds with more affinity than the host proteins for Fe^{3+}; iron-charged siderophores are recognized by bacterial-specific receptors that deliver

iron into the cell. Other bacteria directly bind iron from host iron compounds and proteins such as heme, hemoglobin, LF, TF and ferritin [Wooldridge and Williams, 1993; Wilson and Britigan, 1998; Wandersman and Delepelaire, 2004]. Studies in Gram negative bacteria describe their interactions with host iron-containing proteins through outer membrane (OM) receptors; the iron goes through the inner membrane (IM) and is subsequently stored. Iron regulates genes encoding receptor biosynthesis and the uptake of iron proteins [Wandersman and Delepelaire, 2004; Halliwell and Gutteridge, 2007].

Species of the *Neisseriaceae* and *Pasteurellaceae* families are the most studied. They acquire iron directly from host TF, through a receptor on the OM that contacts holo-TF and extracts its iron and transports it across this membrane. The receptor is formed by two proteins: TF-binding protein A (TbpA) and TF-binding protein B (TbpB). TbpA is similar to a classical receptor; it is an integral membrane protein that depends on TonB for energy transduction between the OM and IM. TbpA transports ferric ions across the OM [Cornelissen *et al.*, 1992]. TbpB is a surface-exposed lipoprotein that binds TF independently [Gray-Owen and Schryvers, 1995]. Participation of TbpB is essential for colonizing the host and acquiring iron from TF and displays specificity by binding only TF from the infected animal species [Calmettes *et al.*, 2011]. Once the Fe^{3+} is in the periplasm, it is transported to the cytosol through the FbpABC transporter, which is composed of FbpA, a periplasmic iron-binding protein, and an ABC transporter, formed by the permease FbpB and the ATP-binding protein FbpC [Khun *et al.*, 1998; Nikaido, 2003; Wandersman and Delepelaire, 2004].

TbpB-deficient mutants of *Actinobacillus pleuropneumoniae*, a pathogen of the pig respiratory tract, are neither virulent nor able to colonize its host; thus TbpB is required for iron acquisition *in vivo* [Baltes *et al.*, 2002; Wandersman and Delepelaire, 2004]. Surface lipoproteins such as TbpB have been targeted for vaccine development because they elicit a strong immune response, and antibodies (Abs) to this specific surface lipoprotein are bactericidal. Nevertheless, there is an insufficient cross-protective response induced by an individual receptor protein to be considered as a suitable vaccine antigen [Calmettes *et al.*, 2011]. The abundance of iron acquisition systems present in most pathogenic species undoubtedly reflects the diversity of the potential iron sources in the various niches. Some studies have shown that the iron acquisition systems are important determinants of virulence and that the inactivation of only one system decreases virulence. Bacterial OM receptors can show variability, enabling the pathogen to escape from the host immune system [Wandersman and Delepelaire, 2004].

2.2. Unicellular eukaryotic pathogens

Binding proteins to host iron-containing proteins are also important determinants of virulence in protozoa, as has been deduced from the diversity of iron acquisition systems that have been identified in these protists. In this review, we discuss the current knowledge of transferrin binding proteins (Tbps) in some important parasites. These pathogens possess elaborate control systems for iron uptake from the mammalian hosts that they invade, and these systems ensure their success as parasites. Intracellular parasites are able to live inside

of a number of body cells and obtain iron from these sites; for example, in erythrocytes, parasites have free access to hemoglobin as an iron source, debilitating the host by causing anemia and other major problems. Parasites that are phagocytosed by macrophages need to avoid the oxidative stress response of these cells; one of these responses is the production of toxic radicals derived from the oxygen metabolism, and ferrous iron is responsible for their production by Fenton's reaction. However, some parasites not only evade oxidative stress but are also able to survive and multiply inside macrophages; these parasites need to acquire iron for their own growth and to produce the enzyme superoxide-dismutase (SOD), which protects the parasites against toxic radicals. One macrophage's strategy to prevent iron availability to parasites is to sequester this metal through different cleavage mechanisms, such as by reducing the expression of TFR1, the main cellular iron-uptake protein [Mulero and Brock, 1999]. Other mechanisms include increasing the synthesis of ferritin, the main iron-storage protein of the cell, and increasing the expression of ferroportin, the main protein that releases iron from the cell [Das *et al.*, 2009]. Nevertheless, as we will see next, pathogenic parasites have evolved several counterstrategies to stay inside macrophages and acquire cellular iron.

2.2.1. Trypanosomatids

Trypanosomatid parasites face different challenges in their fight for iron in the diverse niches that they inhabit inside a host. In extra- and intracellular parasitic forms, iron plays roles in infection as well as in metabolism. Studies of parasite iron acquisition have led to extraordinary therapeutic possibilities of interfering with parasite survival inside the host.

2.2.1.1. *Trypanosoma brucei*

T. brucei is most likely the most-studied parasitic protozoan with respect to iron acquisition from host TF. This parasite is responsible for producing sleeping sickness or human African trypanosomiasis, a disease widespread throughout the African continent. It causes at least 50,000–70,000 cases every year, which can be fatal if not treated correctly [Kinoshita, 2008]. The transmission vector is the tsetse fly, which inoculates *T. brucei* parasites in the blood of its mammalian host during feeding. Trypanosomiasis presents two stages: first, trypanosomes are observed in the hemolymphatic system, producing fever, splenomegaly, adenopathies, endocrine disarrays, and cardiac, neurological and psychological disorders. In this stage, trypanosomes multiply rapidly, infecting the spleen, liver, lymph nodes, skin, heart, eyes and the endocrine system. In the second stage, trypanosomes are distributed in the central nervous system (CNS) leading to several sensory, motor and psychic disorders and ending in death [Kennedy, 2005; de Sousa *et al.*, 2010].

Use of host transferrin by *T. brucei*

In mammals, *T. brucei* lives as a trypomastigote in the bloodstream and tissue fluids [Bitter *et al.*, 1998; Subramanya, 2009; Taylor and Kelly, 2010; Johnson and Wessling-Resnick, 2012]. As an extracellular parasite, it depends on endocytosis to take up nutrients from the host blood [Subramanya, 2009]. This organism uses host TF as the main iron source for growth

and has the ability to bind TF from several origins, thus increasing its capacity to colonize a large range of mammals [Salmon *et al.*, 2005]. This ability is important because by taking up different TFs, the parasite favors its own growth without being affected by the host immune system due its variability, leading to chronic infection; in this way, the ability to switch between different TFR genes allows *T. brucei* to cope with the large sequence diversity in the TFs of its hosts [Bitter *et al.*, 1998; Van Luenen *et al.*, 2005]. In contrast, *T. equiperdum* presents a restricted host range, infecting only horses [Isobe *et al.*, 2003; Witola *et al.*, 2005].

T. brucei **transferrin receptor (TbTFR)**

T. brucei binds TF through a transferrin receptor, *Tb*TFR. Although *Tb*TFR and human transferrin receptor (*Hs*TFR) bind the same iron transport protein (TF), they have no detectable amino acid homology [Borst, 1991; Schell *et al.*, 1991; Taylor and Kelly, 2010]. *Tb*TFR is present in only bloodstream forms and not in insect forms of the *T. brucei* life cycle. In fact, *T. evansi*, a derivative of *T. brucei*, does not appear to have a life cycle stage in an insect vector; it presents similar TFR to *T. brucei* [Kabiri and Steverding, 2001]. *Tb*TFR is encoded by two of the expression-site associated genes (*ESAGs)*, *ESAG6* and *ESAG7*, of the variant surface glycoprotein (VSG), the major surface antigen of the bloodstream form of *T. brucei*. ESAG6 and ESAG7 proteins evolved to bind TF [Salmon *et al.*, 1994; Salmon *et al.*, 1997]. The *VSG* gene is at a telomeric expression site (ES) that contains at least seven expression-site associated genes. Each strain of *T. brucei* contains 20 different copies of *ESAG* with a corresponding 20 copies of *Tb*TFR, but only a single ES is active at a time. The receptor expression occurs independently of the ES employed for antigenic variation [Borst, 1991; Schell *et al.*, 1991; Salmon *et al.*, 1994; Salmon *et al.*, 1997; Salmon *et al.*, 2005; Van Luenen *et al.*, 2005]. Antigenic variation prevents receptors from being recognized by the immune system and allows parasites to use TF from different mammalian hosts [Borst, 1991; Bitter *et al.*, 1998]. The surface of the parasite bloodstream form is covered with VSG protein, which is required for nutrient uptake; its variability provides protection from the mammalian immune system [Schell *et al.*, 1991; Taylor and Kelly, 2010]. When some parasites in the population switch VSG gene expression, they produce resistant phenotypes. VSG are powerful antigens, and the initial set of Abs is no longer useful for controlling trypanosomiasis. A proliferation of survivors is produced with posterior infection of the CNS, when parasites move across the blood-brain barrier [Kinoshita, 2008].

*Tb*TFR is a heterodimer consisting of ESAG7, a 42 kDa soluble protein attached to the membrane by the 50-60 kDa ESAG6 protein through a glycosyl-phosphatidylinositol (GPI) residue in the C-terminal tail [Borst, 1991; Schell *et al.*, 1991; Ligtenberg *et al.*, 1994; Salmon *et al.*, 1994; Steverding *et al.*, 1995; Salmon *et al.*, 1997; Steverding, 2000; Maier and Steverding, 2008; Taylor and Kelly, 2010]. ESAG6 and ESAG7 can homodimerize, but only heterodimers bind TF; thus, each subunit provides a necessary component for the specific ligand-binding site [Salmon *et al.*, 1994; Salmon *et al.*, 1997]. The two subunits show differences in their C-terminal region in the four blocks of 5-16 amino acids that generate the ligand binding site. The sequence of the N-terminal half is highly conserved [Salmon *et al.*, 1997]. Near the middle part of the gene is a hypervariable region of approximately 32 nucleotides [Pays, 2006].

Affinity binding of *Tb*TFR for TF is important when the host begins to make a significant Ab response against invariant regions of the receptor that could interfere with TF uptake [Borst, 1991; Salmon *et al.*, 1994; Steverding *et al.*, 1995; Steverding, 2003; Steverding, 2006; Stijlemans *et al.*, 2008]. In some cases, these Abs compete with TF for the receptor binding site, and only a high-affinity receptor could maintain the required iron level for trypanosome replication [Bitter *et al.*, 1998]. Nevertheless, during the course of trypanosomiasis, Abs produced against the *Tb*TFR are too low to deprive the parasite of iron [Steverding, 2006]. This factor could be important for the characteristic anemia observed in chronic illness, in which TF levels are decreased. Because iron is sequestered by macrophages and bloodstream pathogens can obtain iron, the "anemia of chronic infection" results, and erythropoiesis diminishes because there is no available iron to produce hemoglobin. Then, parasites produce a high affinity receptor to TF, which is present in very low quantities [Taylor and Kelly, 2010].

There is a controversy surrounding the purpose of the TFR variability. Some authors report that each TFR encoded by trypanosomatids is slightly different and that these differences affect the binding affinity to TF from different hosts [Van Luenen *et al.*, 2005; Pays, 2006]. Other researchers propose that each receptor with low or high affinity allows trypanosome growth independent of the *in vitro* or *in vivo* TF levels [Salmon *et al.*, 2005]. After the synthesis and heterodimer formation of *Tb*TFR, this receptor is transported to the flagellar pocket by the conventional route of glycoproteins. The flagellar pocket is the site for exocytosis and endocytosis in bloodstream trypanosomes, and it is formed by an invagination of the plasma membrane at the arising flagellum. This pocket protects the parasite from Abs and cell-mediated cytotoxic mechanisms directed against important functionally conserved proteins such as the TFR (Fig. 1) [Balber, 1990; Borst, 1991; Schell *et al.*, 1991; Van Luenen *et al.*, 2005].

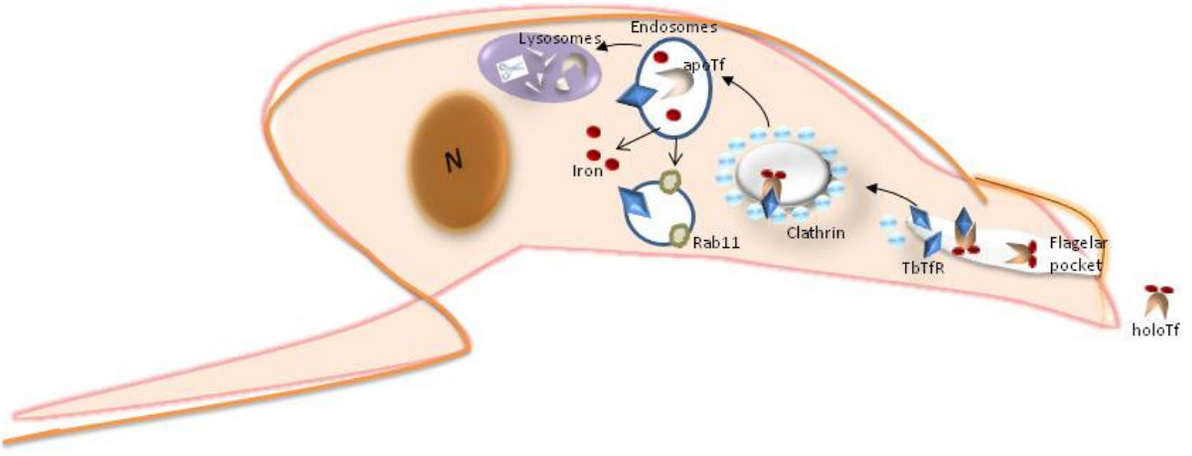

Figure 1. Transferrin endocytosis and iron acquisition in *Trypanosoma brucei*. Transferrin is bound by the *Tb*TFR localized at the flagellar pocket; the complex is then internalized in clathrin-coated pits. The pH is acidified in the endosomes, and the iron is released and transported to the cytoplasm. Apotransferrin is degraded in lysosomes, and the TFR is recycled to the membrane by Rab11-positive vesicles.

VSG proteins leave the flagellar pocket and spread from there to cover the surface, but receptors such as *Tb*TFR are prevented from spreading [Borst, 1991; Mussmann *et al.*, 2004].

Apparently, TFR is retained in the flagellar pocket by the single GPI anchor, while those that present two GPI anchors are targeted to the cell surface [Schwartz *et al.*, 2005; Taylor and Kelly, 2010]. Then, GPI is essential for the correct formation of the VSG coat, for the expression of *Tb*TFRs on the flagellar pocket, and to signal for clathrin-coated endocytosis [Allen *et al.*, 2003]. The lack of TFR leads to lethality; for this reason, some authors have proposed the GPI biosynthetic pathway as a target for the development of anti-trypanosome drugs [Kinoshita, 2008].

Retention of the receptor in the flagellar pocket is a very regulated and saturable process. *Tb*TFR expression depends on the host in which the trypanosome finds itself and on the quantity of iron present. Upregulation of TFR gene expression produces a mislocalization of the receptor onto the cytoplasmic membrane, most likely resulting in binding to more TF molecules. The upregulation of the receptor expression implies that the parasite can sense the reduction in TF availability by sensing cytosolic iron [Van Luenen *et al.*, 2005].

Signal transduction and endocytosis of transferrin by clathrin-coated vesicles

On the flagellar pocket membrane, *Tb*TFR captures TF, and the complex is endocytosed in clathrin-coated pits in a saturable way [Borst, 1991; Schell *et al.*, 1991; Salmon *et al.*, 1994; Taylor and Kelly, 2010] . TF endocytosis is a temperature- and energy-dependent process (Fig. 1) [Ligtenberg *et al.*, 1994; Steverding *et al.*, 1995]. Other proteins that participate in the endocytosis of TF are dynamin, epsin, the adaptor AP-2 [Allen *et al.*, 2003], and small GTPases such as *Tb*Rab5A, β-adaptin [Morgan *et al.*, 2001; Pal *et al.*, 2003], and phosphatidylinositol-3 kinase (PI-3K), *Tb*VPS34 [Hall *et al.*, 2005]. Interestingly, *Tb*TFR does not discriminate between apoTF and holo-TF [Steverding *et al.*, 1995; Steverding, 2003]. TF endocytosis is activated by diacylglycerol (DAG), a diffusible second messenger produced in GPI digestion by the GPI-phospholipase C (GPI-PLC) expressed in bloodstream *T. brucei*. GPI-PLC can cleave intracellular GPIs, producing DAG and inositolphosphoglycan. DAG receptors in trypanosomatids contain a divergent C1_5 domain and DAG signaling pathway that depends on protein tyrosine kinase (PTK) for the activation of proteins in the endocytic system by the phosphorylation of clathrin, actin, adaptins, and other components of this machinery. TF uptake depends on PTK because TF endocytosis diminishes when Tyrphostin A47, an inhibitor of PTK, is used in *T. brucei* and *Leishmania mayor,* another member of the trypanosomatid family [Subramanya and Mensa-Wilmot, 2010].

When the ligand-receptor complex is delivered into the endosomes, the acidic pH triggers the release of iron from TF and the formed apo-TF dissociates from the receptor [Steverding, 2000]. The TFR is recycled into the flagellar pocket via *Tb*Rab11 vesicles [Steverding *et al.*, 1995; Jeffries *et al.*, 2001]. TF is delivered into the lysosomes, where it is degraded by the cathepsin-like protein, *Tb*catB. A small reduction in *Tb*catB produces the accumulation of TFR within the flagellar pocket and the upregulation of TFR levels as a response to iron starvation [Maier and Steverding, 1996; O'Brien *et al.*, 2008]. Later, degraded fragments are exocytosed by the same Rab11 vesicles (Fig. 1) [Steverding *et al.*, 1995; Pal *et al.*, 2003; Hall *et al.*, 2005]. *Tb*TFR has a long half-life, so the receptor is not degraded with TF but is recycled

back to the flagellar pocket in approximately 11 min [Kabiri and Steverding, 2000; Kabiri and Steverding, 2001].

The mechanism by which iron crosses to the cytoplasm from the endolysosomal system has not yet been determined; it could be through a ferric reductase. In the *T. brucei* genome, two putative ferric reductases have been found, a cytochrome b561-type (*Tb*927.6.3320) and an NADPH-dependent flavoprotein (*Tb*11.02.1990). These enzymes could act in cooperation with some divalent putative cation transporters, but none of them have been related with iron transport [Taylor and Kelly, 2010].

Iron storage

Depending on the growth conditions, *Tb*TFR can be found at very low concentrations of approximately 1.0 – 2.3 x 10^3 molecules per cell [Borst, 1991; Steverding *et al.*, 1995] or 1.88 – 2.71 x 10^4 molecules per cell [Salmon *et al.*, 1994]; thus, the parasite is very efficient at taking iron from TF. TF is taken up at rates 100–1000 times higher than those for phase fluid endocytosis [Borst, 1991]. The iron necessity is approximately 85,000 Fe^{3+} ions/parasite/generation [Steverding *et al.*, 1995] to 1.4 x 10^6 atoms/trypanosome [Schell *et al.*, 1991], but its requirements are approximately 40,000 Fe^{3+} per generation [Steverding, 2003]. For this reason, it is possible that *T. brucei* accumulates iron in some way [Steverding *et al.*, 1995]. When iron provisions are depleted due to TF starvation, a rapid increase in *Tb*TFR takes place, and the capacity to capture TF increases [Mussmann *et al.*, 2004]. During chronic trypanosomiasis in cattle, anemia occurs, in which the host TF level is decreased and the bloodstream pathogens develop the ability to grow at very low iron concentrations [Steverding *et al.*, 1995]. It is in this stage of iron deprivation and chronic infection when a TFR other than *Tb*TFR, with higher affinity for its ligand, is produced; this occurs because *Tb*TFR is not able to discriminate between holo- and apo-TF [Taylor and Kelly, 2010].

Iron chelation and therapeutic improvement

In the absence of iron, the parasite DNA synthesis rate decreases, oxidative stress levels increase, electron transfer stops, and other functions are affected, all of them leading to death. Iron chelation affects *T. brucei* growth; thus, it could be a therapeutic method for combating the infection. The iron chelator deferoxamine (DFO) prevents iron incorporation in newly synthesized enzymes, decreasing the growth rate and oxygen consumption [Taylor and Kelly, 2010]. Acute iron starvation leads to a rapid increase in *Tb*TFR, allowing an increased capacity to uptake TF [Mussmann *et al.*, 2004].

*Tb*TFR is immunologically important, and it has been studied for its antigenic potential in the production of vaccines. Using the complete collection of TFRs as a vaccine, the proliferation of trypanosomes was blocked; however, some authors are not convinced and suggest that antigenic variation makes the production of a vaccine against sleeping sickness improbable [Kinoshita, 2008]. *ESAG*s could also be targets for immune attack. Flagellar pocket proteins were used for immunization of mice and were able to confer protection against superinfection with trypanosomes [Olenick *et al.*, 1988]. A functional *Tb*TFR was expressed in insect cells and could be helpful in crystallographic studies to determine the

structure and characterize the interface between TF and its receptor, which could lead to a new approach to combat infection [Maier and Steverding, 2008]. TF uptake is very important in trypanosomes for obtaining iron, so endocytic uptake systems were developed earlier in evolution compared with TF endocytosis in mammalian cells. Nevertheless, this process has numerous similarities between the two groups.

2.2.1.2. *Trypanosoma cruzi*

This parasite causes human Chagas disease, a chronic and debilitating condition affecting 40 million people in Africa, South America, Europe, and Asia, according to data of the World Health Organization (WHO). *T. cruzi* is transmitted either by an insect vector that has access to the host via breaches in the skin or through mucosal membranes, mainly the conjunctiva or the gastric mucosa. It is an obligate intracellular parasite that disseminates from the initial infection site to the heart and smooth muscle, with several rounds of invasion, growth and egression from infected cells during acute infection. Very little is known regarding the early interaction between the parasite and its host that facilitates the establishment of infection [Mott *et al.*, 2011].

T. cruzi transferrin receptor (TcTFR) and endocytosis

It has been suggested that the internalization of TF is mediated by a receptor in *T. cruzi*. However, until now, there is no biochemical evidence of the presence of a TFR. Epimastigote forms of *T. cruzi* could use a TFR to obtain iron and transport TF through uncoated vesicles formed in the most posterior portion of the cytostome/cytopharynx system, a plasma membrane invagination that penetrates deeply into the cytoplasm towards the nucleus. All the endocytic vesicles formed in the cytostome are uncoated and are associated with lipid raft markers in detergent membrane-resistant (DMR) domains [Correa *et al.*, 2007]. Endocytic vesicles originate either from the cytostome or from the flagellar pocket, and they fuse with early endosomes and then with reservosomes (prelysosomal compartments); endocytosed TF is taken into the reservosomes, which are structures that present numerous proteases [Correa *et al.*, 2008; Cunha-e-Silva *et al.*, 2010; Rocha *et al.*, 2010]. Other proteins that participate in endocytosis have been identified, such as *Tc*Rab7, an indicator of high traffic between the Golgi apparatus and reservosomes, and *Tc*Rab11, which is involved in the recycling process [Cunha-e-Silva *et al.*, 2010; Rocha *et al.*, 2010]. Amastigote forms replicate in the host cell cytoplasm, where TF is almost absent, so the relevance of these forms during infection is not clear. The importance of the receptor is observed in trypomastigotes in the bloodstream and epimastigotes in bloodmeal, where TF was observed in the reservosome [Soares and de Souza, 1991; Soares *et al.*, 1992].

Iron chelation

There is not enough information about how cytoplasmic iron is taken up by *T. cruzi* parasites. They replicate in macrophage cytoplasm; therefore, the macrophage iron-withholding response would benefit the parasite, allowing access to iron [Taylor and Kelly, 2010]. An increase of parasitemia and mortality associated with high levels of iron were observed, as was a reduction in parasitemia with the use of chelants such as DFO or

benznidazole [Lalonde and Holbein, 1984; Taylor and Kelly, 2010; Johnson and Wessling-Resnick, 2012]. The obtained iron is stored in specialized electron-dense organelles; these organelles are different from lysosomes and reservosomes [Scott *et al.*, 1997]. The infection in mouse models involves the production of anemia. This anemia is due to interference with the stimulation of the IFN-induced GTPase LRG-47, which produces severe effects in the hematopoietic system [Taylor and Kelly, 2010]. When the parasite is extracellular, it must obtain nutrients from host proteins. The possibility of infecting several organisms makes it possible that this parasite could use different iron sources, including TF. Because TF accumulation reported an organelle in which TF could be accumulated could exist. Very little is known about the *T. cruzi* iron uptake mechanisms either in its different extracellular or intracellular forms of its life cycle.

2.2.2. Entamoeba histolytica

E. histolytica is the causal protozoan agent of amoebiasis in humans, a disease characterized by dysentery and intestinal ulcers. The parasite is able to invade and destroy tissues, affecting not only the large intestine but also other extra-intestinal organs such as the liver; these infections can be fatal. Amoebiasis shows high level of morbidity and mortality worldwide, particularly in developing countries. Worldwide, 500 million people are infected with *E. histolytica,* causing disease in 50 million and 100,000 deaths each year [Ali *et al.*, 2008; Anaya-Velázquez and Padilla-Vaca, 2011].

Iron and *E. histolytica*

Iron is essential for *E. histolytica* trophozoites living inside the human host because these parasites require a high quantity of iron (approximately 100 μM) for growing *in vitro* and are able to use iron from several iron-binding proteins [López-Soto *et al.*, 2009b]. High amoebic damage was caused in the liver of hamsters that were fed with ferrous gluconate. In addition, there is a significant relationship between amoebic growth and the mechanisms of iron acquisition modulated by determinants of virulence [Diamond *et al.*, 1978; Smith and Meerovitch, 1982]. Within the host, amoebae face the hostility of nonspecific defense systems such as oxidative stress and the lack of nutrients. Several protective mechanisms have been developed by *E. histolytica,* such as the induction of the superoxide dismutase (SOD) gene under iron-limited conditions; this enzyme defends amoeba from the toxicity and damage caused by oxygen metabolites. Thus, SOD is useful during tissue invasion, when amoebae are exposed to great amounts of superoxide radicals [Bruchhaus and Tannich, 1994a].

If iron is reduced in the culture medium to < 20 μM, amoebae do not survive. Several studies have shown responses of the parasite to the absence or excess of iron and to the presence of iron-containing proteins [Serrano-Luna *et al.*, 1998; Reyes-López *et al.*, 2001; León-Sicairos *et al.*, 2005; López-Soto *et al.*, 2009a]. The concerted use of strategies to bind and use iron from different sources provides the parasite with the ability to use various host proteins for its benefit. In the absence of iron, *E. histolytica* expresses several genes that

encode for cysteine proteases CP1, CP2 and CP3; these proteases are virulence factors, as they degrade the mucus barrier in the intestinal epithelium. However, there is no information concerning the mechanisms for iron regulation in this parasite. Genes involved in translation were identified to be expressed in the absence of iron [Park *et al.*, 2001], as occurs with the ferric uptake regulator (Fur) in bacteria and in iron responsive element (IRE) and IRE-binding proteins of mammalian cells [Wang *et al.*, 2007].

Use of host iron-containing proteins

E. histolytica has developed specific mechanisms to obtain iron from host iron-containing proteins. This assertion is based on the parasite growth *in vitro* in media depleted of iron and to which different iron proteins have been added. Trophozoites have been tested in cultures with hemoglobin, LF, TF, ferritin, and as the sole iron sources, and all of them have been utilized by the parasite for growth [Serrano-Luna *et al.*, 1998; Reyes-López *et al.*, 2001; León-Sicairos *et al.*, 2005; López-Soto *et al.*, 2009b]. In this way, amoebae could ensure the presence of iron for the colonization of the different organs and tissues involved in amoebic infection.

***E. histolytica* transferrin binding proteins, EhTFbps**

Iron-loaded TF (holoTF) but not apoTF binds to the *E. histolytica* trophozoite surface. Interestingly, this parasite has two methods of obtaining iron from TF: one is mediated by receptor-independent internalization [Welter *et al.*, 2006] and the other is through three specific TF-binding proteins (*Eh*TFbps) of 70, 96 and 140 kDa of molecular mass, identified by overlay assays with holoTF. The 140 kDa protein is recognized by an anti-*Hs*TFR mAb B3/25 (Boehringerheim cat. No. 1118-048), and the 96 kDa protein is recognized by the anti-*Hs*TFR mAb H68.4 (Zymed cat. No. 13-6800). Apparently, the *Eh*TFR forms a complex with TF to be endocytosed (Fig. 2). Using pharmacological and immunofluorescence microscopy studies, the participation of clathrin protein in the endocytic process was demonstrated. Once inside the vacuoles, TF is transported into the endolysosomal system [Reyes-López *et al.*, 2001; Reyes-López *et al.*, 2011]. However, when the endocytic process was followed using high TF concentrations, the TF was internalized independently of the binding protein [Reyes-López *et al.*, 2011]. This result is in agreement with the observation that TF internalization is unsaturable [Welter *et al.*, 2006]. The presence of clathrin has been demonstrated in some protozoa [Morgan *et al.*, 2001] and in *E. histolytica* [León-Sicairos *et al.*, 2005; López-Soto *et al.*, 2009a; Reyes-López *et al.*, 2011]; clathrin may be important in parasites for the acquisition of nutrients. The gene encoding the clathrin protein has been identified in the *E. histolytica* genome [Loftus *et al.*, 2005]. Once inside the lysosomes, TF could be degraded by specific cysteine proteases (Fig. 2) (our unpublished data), as was observed in *T. brucei.*

In addition to the phagocytosis of erythrocytes to use hemoglobin, the direct binding of host TF to specific proteins on the amoeba surface may be another strategy used to capture iron in the blood and liver, which is important in the human host invasion process of this parasite. The 96 kDa protein was identified as the enzyme acetaldehyde/alcohol

dehydrogenase-2 (*Eh*ADH2) by mass spectrometry after its isolation by immunoprecipitation with the mAb H68.4 [Reyes-López *et al.*, 2011]; to our knowledge, this report is the first in which an enzyme was shown to bind TF in parasitic protozoa. Internalization of TF through a receptor is a fast, saturable, and temperature-, time-, and concentration-dependent process. It is possible that the *Eh*ADH2 protein, which requires iron for its activity, participates in the regulation of iron-Tf uptake and utilization. *Eh*ADH2 enzyme is essential for amoeba survival and is able to discriminate between iron-loaded TF and apoTF, possibly because iron is the enzyme cofactor of the protein [Espinosa *et al.*, 2009].

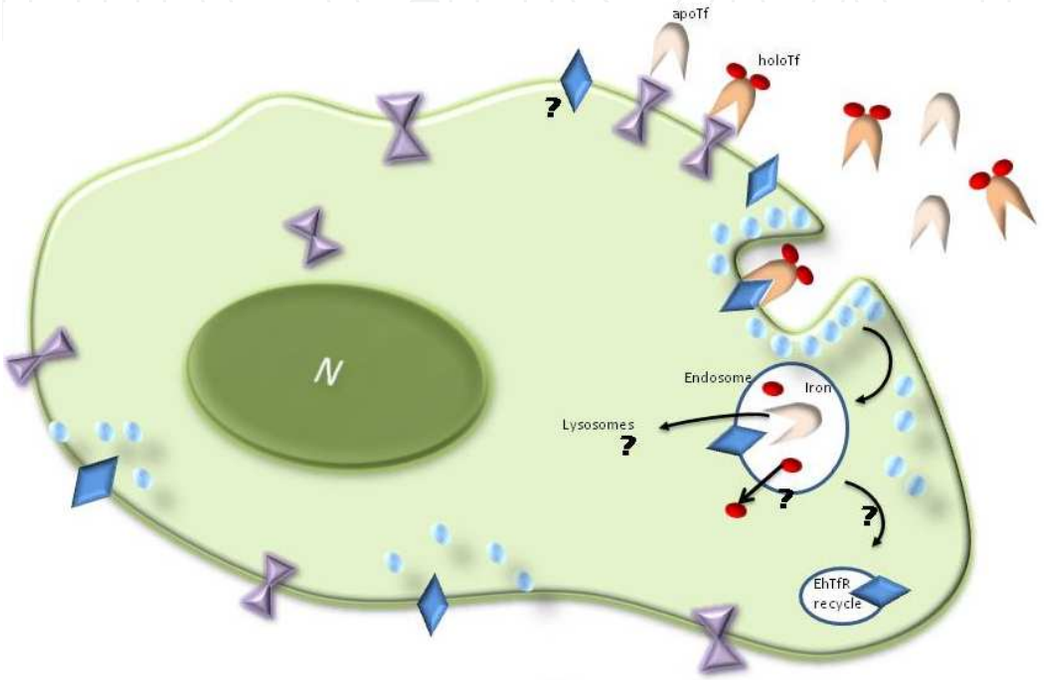

Figure 2. Transferrin endocytosis in *Entamoeba histolytica*. HoloTF is detected by the *Eh*Tbp and the TFR and internalized in clathrin-coated pits.

It has been reported that bacteria such as *Staphylococcus aureus* and S*taphylococcus epidermidis* use the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to bind TF [Modun and Williams, 1999], and the parasite *Trypanosoma brucei* uses an organelle in which TF could be accumulated could exist.GAPDH for binding LF rather than TF [Tanaka *et al.*, 2004]. Apparently, glycolytic enzymes have several functions; an example is enolase, which also regulates the activity of cytosine 5-methyltransferase 2 (Dnmt2), an enzyme that catalyses DNA and tRNA methylation in amoeba [Tovy *et al.*, 2010]. The *Eh*ADH2 amino acid sequence and that predicted for the *Hs*TFR are not similar, so the recognition of both proteins by the mAb could be explained by a structural connection. *Eh*ADH2 is an essential enzyme used for obtaining energy by glucose fermentation [Bruchhaus and Tannich, 1994b; Yang *et al.*, 1994; Flores *et al.*, 1996; Espinosa *et al.*, 2001; Avila *et al.*, 2002; Chen *et al.*, 2004; Espinosa *et al.*, 2004; Espinosa *et al.*, 2009]. Due to the properties of *Eh*ADH2, such as its ability to bind to host extracellular matrix proteins, its presence on the cell membrane, and its requirement for iron, the blocking of this enzyme with iron chelators as a therapeutic strategy against *E. histolytica* is an interesting future perspective [Espinosa *et al.*, 2009].

*Eh*TFbp is able to bind TF with either high or with low affinity (1.81 and 1.1-5.7 x 10^{-9} M). This observation could be due to the presence of two binding proteins or only one protein with two different affinities [Reyes-López *et al.*, 2011]. Comparing the affinity for TF in bacteria (0.7 a 4 x 10^{-7} M) [Pintor *et al.*, 1993] and *Trypanosoma cruzi* (2.8 X 10^{-6} M) [Testa, 2002], the amoebic receptor presents the higher affinity. The fact that *E. histolytica* trophozoites possess a variety of mechanisms to obtain iron from TF is advantageous to the parasite. However, in amoebiasis, the host usually has lower iron levels and TF saturation than that showed in uninfected people; this defense is a normal response to limit iron from pathogens during infection, a phenomenon known as hypoferremia of infection [Van Snick *et al.*, 1974; Otto *et al.*, 1992; Jurado, 1997; Griffiths *et al.*, 1999; Weinberg, 1999; Weinberg 2009]. Further studies are necessary to comprehend the role of all the proteins that participate in the iron acquisition system of TF and in the iron metabolism of this important parasite.

2.2.3. Tritrichomonas foetus

T. foetus is a venereal protozoan pathogen of cattle that infects the female genital tract, resulting in abortion, endometritis, and infertility [Manning, 2010; Pereira-Neves *et al.*, 2011]. This parasite has a worldwide distribution and causes significant economic losses to cattle producers. Strains of *T. foetus* have also been recognized that cause diarrhea in cats [Gookin *et al.*, 1999] and mild rhinitis in swine [Lun *et al.*, 2005]. As an obligate parasite, *T. foetus* depends on endogenous bacteria and host secretions for nutrients such as iron. This organism has high iron requirements for *in vitro* cultivation (50–100 μM) [Tachezy *et al.*, 1996], surpassing those of eukaryotic cells, although comparable to other anaerobic amitochondriate protists. *T. foetus* inhabits the vagina, cervix, and the lumen of the bovine uterus, with the last one being characterized as rich in TF [Roberts and Parker, 1974]. Therefore, TF could be an important source of iron for *T. foetus.*

The involvement of iron and holo-TF in *T. foetus* virulence has been examined in experimental infection of mice with the moderately virulent KV-1 strain (~5% mortality rate). Administration of ferric ammonium citrate to infected mice increased the mortality rate to the level associated with the highly virulent LUB-1MIP strain (~80% mortality rate) [Kulda *et al.*, 1999]. When examined *in vitro*, the KV-1 strain showed significantly lower iron acquisition from holo-TF and low molecular mass complexes than the highly virulent strain. These data indicate a correlation between strain virulence and iron acquisition from holo-TF [Kulda *et al.*, 1999]. Growth of parasites using holoTF as a sole iron source has been reported *in vitro* [Tachezy *et al.*, 1996]. Accordingly, iron from ^{59}Fe-TF was efficiently accumulated into *T. foetus*, specifically in the labile iron pool (LIP). Interestingly, the concentration of protein-bound iron that restored 50% cell growth (5 μM for Fe-TF) was approximately 5-fold lower than that of low molecular weight iron complexes [Tachezy *et al.*, 1996; Suchan *et al.*, 2003], indicating that *T. foetus* uses TF iron more efficiently. This finding agrees with results in studies of other pathogens that require higher iron concentrations from these complexes than those from host proteins (holoLF and HG) [Wilson *et al.*, 1994; Jarosik *et al.*, 1998].

Retrieval of iron from TF may depend on the extracellular release of iron from this ligand caused by the acidification of the microenvironment by *T. foetus* [Tachezy *et al.*, 1996]. This hypothesis is based on the observation that the pH of the conditional media decreased from pH 7.4 to 5.6 after incubation with *T. foetus*. As predicted at this pH, there was a marked release of iron from holoTF (up to 47%) measured in the cell-free medium (Fig. 3) [Tachezy *et al.*, 1996]. Iron uptake from TF was almost exponential, which possibly reflected the accelerated release of iron from the protein by the acidification of the cellular microenvironment [Tachezy *et al.*, 1996]. Nevertheless, further studies are needed to demonstrate the actual role of microenvironmental acidification in iron uptake, for example, by measuring the iron uptake by *T. foetus* using a stronger buffered medium to prevent acidification.

Iron uptake from transferrin in *T. foetus*

Iron uptake from TF is a process dependent on the energy produced by glycolysis, as sodium fluoride affected the uptake [Tachezy *et al.*, 1998]. The mechanism also involves extracellular iron reduction from holo-TF. This idea is supported by the inhibitory effect of BPSA (a membrane impermeable, ferrous-iron specific chelator) on iron uptake from holo-TF, as iron is originally in the ferric state in this molecule. Additionally, the presence of ascorbic acid, a strong reducing agent, stimulated iron accumulation by *T. foetus* from holo-TF [Tachezy *et al.*, 1998]. Which mechanism is actually used by *T. foetus* to reduce holo-TF iron is unknown. Iron released from holo-TF could be acquired by a mechanism related or identical to that used for acquisition from the low molecular weight iron chelator nitrilotriacetic acid (Fe-NTA) because these processes displayed similar kinetics and susceptibility to various agents [Tachezy *et al.*, 1998]. Iron uptake from Fe-NTA by this microorganism also depends on iron reduction and is better characterized. Extracellular iron reduction from Fe-NTA seems to be non-enzymatic, as the reduction activity is thermo-labile and unaffected by proteases, and the majority is filterable through a membrane with a cut-off of 3 kDa. Additionally, iron acquisition is not enhanced by the presence of NADH, a nucleotide reported to provide electrons to ferrireductases [Low *et al.*, 1986; Berczi and Faulk, 1992; Riedel *et al.*, 1995]. In fact, trichomonads are able to produce reducing volatile agents such as H_2S [Thong and Coombs, 1987] or methanethiol [Thong *et al.*, 1987], which have been suggested to participate in oxygen detoxification [Thong *et al.*, 1986]. It could be that trichomonads are able to take advantage of their reducing environment to take up iron from holo-TF (Fig. 3). This hypothesis needs to be tested and does not completely rule out the possibility that a ferrireductase may also participate.

The extracellular release of iron from holo-TF could be independent of proteolysis because less than 40% of the parent molecule was digested even after 24 h of contact with extracellular *T. foetus* proteases [Talbot *et al.*, 1991]. Iron acquisition from TF seems to be independent of endocytosis because lysosomotropic bases such as ammonium chloride and chloroquine acting as inhibitors of endosome acidification did not decrease iron accumulation from TF [Tachezy *et al.*, 1998]. However, work from Affonso shows that

endocytosis of TF by *T. foetus* actually takes place [Affonso *et al.*, 1994]. It was shown that TF binds to the parasite surface, and because unlabeled TF does not compete with labeled TF, this binding does not seem to be through specific surface receptors. In agreement with this result, holo-TF binding does not display saturable kinetics [Tachezy *et al.*, 1996]. The initial binding of gold-labeled human TF may be due to low-affinity interactions, as occurs with *T. vaginalis* [Peterson and Alderete, 1984]. Gold-labeled TF is internalized by the parasite through endocytic vesicles and concentrated into vacuoles of variable dimension, peripheral tubular and tubulovesicular structures all without a typical clathrin coat. The absence of a specific receptor suggests a principal role for fluid phase endocytosis [Tachezy *et al.*, 1996].

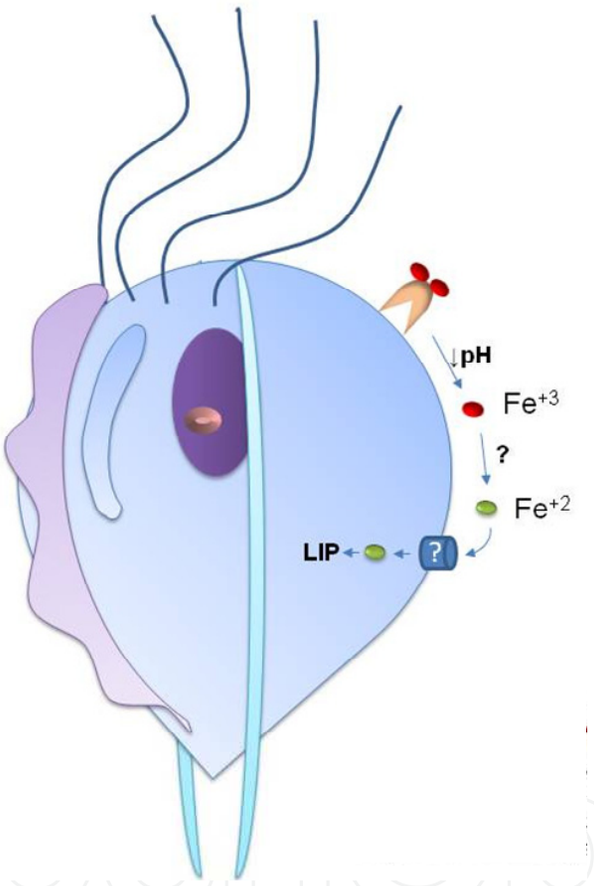

Figure 3. Transferrin acquisition in *Plasmodium falciparum*. The parasite produces its receptor by an unknown mechanism. The receptor is transported to the erythrocyte membrane, where it is able to bind TF. Then the iron from TF is transported back to the parasite by an unknown mechanism.

Further studies are necessary to fully understand the mechanism of iron acquisition from holoTF by *T. foetus*, specifically to characterize the mechanism of iron reduction and of iron transport through the membrane and to clarify the role of holo-TF endocytosis in iron acquisition. Moreover, due to its unusually high nutritional requirement for iron, the inhibition of iron uptake from holo-TF might be an attractive therapeutic strategy against *T. foetus*.

2.2.4. Plasmodium spp.

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasite protozoa of the genus *Plasmodium*. The disease results from the multiplication of parasites inside red blood cells (erythrocytes), causing fever, headache, splenomegaly, cerebral ischemia, hepatomegaly, hypoglycemia, and hemoglobinuria with renal failure, progressing in severe cases to coma and death [Trampuz *et al.*, 2003]. It is widespread mainly in the tropical and subtropical regions of Sub-Saharan Africa, Asia, and America. Five species of malaria can be transmitted to humans. Severe disease is largely caused by *P. falciparum*, while the diseases caused by *P. vivax*, *P. ovale* [Sutherland *et al.*, 2010] and *P. malariae* are generally milder and rarely fatal. *P. knowlesi* is a zoonosis that causes malaria in macaques but sometimes can infect humans [Fong *et al.*, 1971; Singh *et al.*, 2004]. Malaria has been a widely prevalent disease throughout human history. The World Health Organization has estimated that malaria annually causes 250 million cases [WHO, 2008]. In 2010, it was estimated that 655,000 people died from the disease [WHO, 2010]. However, a 2012 meta-study published in *The Lancet* reported 1,238,000 people dying from malaria in 2010 [Murray *et al.*, 2012]. The majority of cases occur in children under 5 years old [Greenwood *et al.*, 2005]; pregnant women are also especially vulnerable. *P. falciparum* is responsible for the vast majority of deaths associated with the disease [Snow *et al.*, 2005].

The life cycle of malaria parasites in the human body begins when a mosquito infects a person by taking a blood meal. Malaria develops via two phases: an extra-erythrocytic and an intra-erythrocytic phase. The extra-erythrocytic phase involves infection of the hepatic system, whereas the intra-erythrocytic phase involves infection of erythrocytes. When an infected mosquito pierces a person's skin, sporozoites in the mosquito's saliva enter the bloodstream and migrate to the liver, infecting hepatocytes, multiplying asexually and asymptomatically for a period of 8–30 days. After this dormant period in the liver, parasites differentiate to yield thousands of merozoites, which, following rupture of their host cells, escape into the blood and infect red blood cells [Bledsoe, 2005]. The parasite escapes from the liver undetected by wrapping itself with the host cellular membrane. Within the red blood cells, the parasites multiply further, again asexually, periodically breaking out of these cells to invade fresh red blood cells. Several such amplification cycles occur [Sturm *et al.*, 2006]. The parasites are relatively protected from attack by the body's immune system because they reside within the liver and blood cells and are relatively invisible to immune surveillance for most of their life cycle in humans. However, circulating infected blood cells are destroyed in the spleen [Chen *et al.*, 2000].

P. falciparum parasites need iron to support their growth

Treatment with iron supplementation in *Plasmodium*-infected patients increases malaria morbidity [Oppenheimer, 1989]. Interestingly, despite the fact that the intra-erythrocytic parasite is surrounded by hemoglobin, it is unable to utilize this ferrous molecule, and therefore, heme accumulates in hemozoins (crystalline particles) within the parasites [Roth *et al.*, 1986; Goldberg *et al.*, 1990]. The delivery of extracellular iron from serum TF to infected erythrocytes has been postulated [Pollack and Fleming, 1984; Haldar *et al.*, 1986; Rodriguez and Jungery, 1986]. The uptake of ^{125}I- or ^{55}Fe-labeled human TF has been detected in

parasitized cells during several days of culture [Pollack and Fleming, 1984]. Furthermore, two independent studies have reported the identification of proteins on the surface of *P. falciparum*-infected erythrocytes that have an affinity for ferric TF [Haldar *et al.*, 1986; Rodriguez and Jungery, 1986]. Rodriguez and Jungery [Rodriguez and Jungery, 1986] described the presence of a 93 kDa protein that bound to a TF affinity-column. These authors claim that this protein could be a parasite-derived TFR, synthesized by *P. falciparum* (*Pf*TFR), because the vast majority of mature erythrocytes lack the expression of TFR (CD71) [Marsee *et al.*, 2010]. Almost at the same time, Haldar et al. [1986] identified another probable *Pf*TFR of 102 kDa synthesized by the intracellular parasite and inserted in the erythrocyte membrane of mature infected cells. This protein recognizes only holoTF. Biochemical analysis indicated that this protein is acylated via 1,2-diacyl-sn-glycerol, which may be important for its association with the membrane. Fry [1989] described a diferric reductase activity in *P. falciparum*-infected erythrocytes. This activity was absent in uninfected mature erythrocytes, suggesting its synthesis and incorporation by *P. falciparum*. The author suggests that the presence of the diferric TF reductase together with the parasite–derived TFR in the erythrocyte membrane could form a TFR –mediated uptake mechanism.

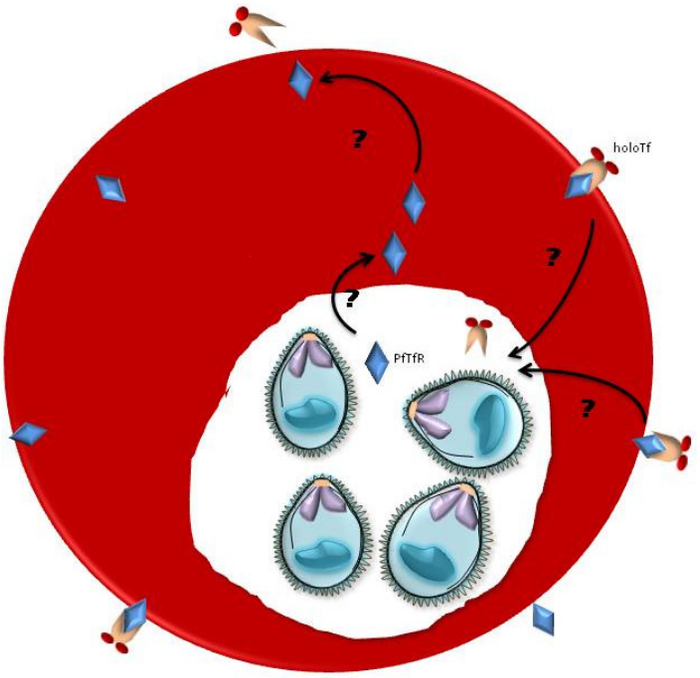

Figure 4. *Tritrichomonas foetus* uptake of iron from Transferrin by a reducing mechanism. HoloTF binds *Tritrichomonas* surface most likely through low-affinity interactions, and then iron is released due to the microenvironment acidification. Ferric iron is reduced to the ferrous form by an unknown mechanism, most likely non-enzymatic, and is then internalized by the parasite to become part of the labile iron pool (LIP).

In contrast, a controversial study performed by Pollack and Vera Schnelle in [1988] was unable to detect a TFR in *P. falciparum*-infected erythrocytes. This study concluded that the binding of TF to the erythrocyte surface was not specific because it was neither saturable nor limited to TF, as LF and albumin were also bound to the parasitized cells. These authors

suggested that TF was non-specifically bound and in this way endocytosed and degraded inside the parasite. Furthermore, in [1992], Sánchez-López and Haldar described a TFR-independent iron uptake activity in *P. falciparum*, and this activity was also apparent in uninfected erythrocytes. These authors demonstrated that normal levels of TF in human serum were not required for intra-erythrocytic *P. falciparum* growth. However, although the iron uptake activity was not parasite specific in parasitized erythrocytes, apparently radiolabeled iron (^{55}Fe) was found in association with parasites mechanically released from the infected erythrocyte, indicating that it was delivered to the intracellular organism.

In view of the controversial state of the research regarding to *Plasmodium* TF iron uptake, we think that more careful studies have to be performed to determine whether there is a complete *Pf*TFR in parasitized erythrocytes, in which state the iron travels through the erythrocyte cytoplasm until reaching the parasitic surface, and lastly, how iron is internalized by the parasite (Fig. 4). Another interesting question is that if *P. falciparum* can obtain iron from different sources, which of these sources are important in parasitic iron uptake

2.2.5. Leishmania spp.

Leishmania species are dimorphic-protozoa that cause leishmaniases, a range of diseases displaying a large spectrum of clinical symptoms in mammals. Approximately 2 million new cases occur every year, with an estimate of 150 million people infected around the world [Kaye and Scott, 2011]. Five main species of *Leishmania* can infect human beings: *L. tropica, L. major, L. donovani, L. braziliensis* and *L. mexicana*. There are mainly three clinical forms of infection: the self-healing cutaneous leishmaniasis (CL), the mucocutaneous leishmaniasis (MCL), and the often fatal visceral leishmaniasis (VL) that affects people of the South American continent. The severity of symptoms depends on the parasite species and strain; exposure dose; and genetic, health and immune status of the host [Anstead *et al.*, 2001; Marquis and Gros, 2007; Kaye and Scott, 2011].

Leishmanias can live in two stages: flagellated promastigotes and non-flagellated amastigotes. When promastigotes are inoculated in the host human dermis by the vector insect (sandfly), they are phagocytosed by macrophages, and then transform into amastigotes within a membrane-rounded organelle named the parasitophorous vacuole (PV) that belongs to the endocytic route, and progressively acquires characteristics of a late endosome/lysosome [Courret *et al.*, 2001; Courret *et al.*, 2002]. Inside the PV, the parasites replicate leading to cell lysis, and free parasites infect the surrounding cells. Leishmanias surviving intracellularly produce multiple effects in phagocytes: inhibition of the respiratory burst, prevention of apoptosis, chemotaxis inhibition in both macrophages and neutrophils, and suppression of the Th1 type protective response [Olivier *et al.*, 2005].

Some species of *Leishmania* possess specific transferrin binding proteins

More than 20 years ago, a putative TF binding protein in *L. infantum* promastigotes was reported [Voyiatzaki and Soteriadou, 1990]. The binding of human iron-free ^{125}I-TF to *Leishmania*-purified membrane preparation was found of high affinity (K_d 2.2 x 10^{-8} M); in addition, this binding was

saturable and specific for TF. The affinity of the *Li*Tbp for TF is comparable with that reported in mammalian cells. Interestingly, the anti-human TFR mAb B_3/25, which recognizes a Tbp of 140 kDa in *E. histolytica*, did not recognize the *Li*Tbp, suggesting that the *Hs*TFR and *Li*Tbp do not share epitopes. Binding of human apo-TF was also tested on living avirulent *L. infantum* promastigotes, and on *L. mexicana* amastigotes obtained from infected mice with promastigotes. In both cases the binding was specific and saturable, suggesting that the *Li*Tbp is functionally similar to the human TFR. Although the experiments were performed with iron-free TF, these data suggest that both stages of *Leishmania* are able to bind human TF, an important iron protein for the parasite iron requirement; indeed, TF-mediated uptake of iron was observed in these parasites. Later, the same authors isolated and identified the *Li*Tbp as an integral membrane monomeric glycoprotein of 70 kDa [Voyiatzaki and Soteriadou, 1992]. The purification of this receptor was carried out through the use of affinity chromatography with human TF from membrane preparations of *L. infantum* and *L. major* promastigotes.

Figure 5. Iron uptake from Transferrin by *Leishmania chagasi* promastigotes. A non-specific receptor, *Lc*Tbp, binds TF, allowing a parasite-associated or secreted reductase to reduce the ferric iron from holoTF, and in this way, the TF affinity for iron diminishes, allowing ferrous iron to be internalized by the parasite.

L. chagasi also showed a 70 kDa protein that binds TF (*Lc*Tbp); however, this protein is not specific for TF because LF and albumin were also bound. Apparently, a parasite-associated or secreted reductase is needed to reduce the ferric iron from holoTF, and in this way, the affinity of TF for iron diminishes allowing iron be internalized by the parasite (Fig. 5)

[Wilson *et al.*, 2002]. *L. chagasi* promastigotes require a lower iron concentration than other parasites (8 μM hemin). Lactoferrin, as an extracellular protein, interacts with promastigotes, and it can be used by them as an iron source *in vitro* [Wilson *et al.*, 1994]. Promastigotes were able to take up ^{59}Fe-LF more rapidly than that from hemin or holoTF, suggesting that iron uptake from holoTF and holoLF occurs via a non-specific receptor because apoLF, apoTF and holoTF competed with holoLF for the uptake. In other experiments [Britigan *et al.*, 1998], it was demonstrated that the binding to TF is markedly greater if this protein is iron-charged; also, if *L. chagasi* does not excrete proteases that cleave TF, then the proteolytic cleavage is not a mechanism to obtain iron in this parasite.

Leishmania can live inside macrophages

The access of iron inside the macrophage's phagosome plays a central role in *Leishmania* infection. Nramp1 protein is located in macrophage lysosomes and in tertiary granules of neutrophils, and it is rapidly recruited towards the membrane of leishmania-containing phagosomes. In that membrane, the iron transporter protein Nramp1 chelates Fe^{2+} in the intraphagosomal environment and in this way, Nramp1 avoids the parasite multiplication and activation of macrophages. However, *L. amazonensis* upregulates the expression of its own ferrous iron transporter LIT1 after being endocytosed by the macrophage. Mutations in *Nramp1* (*Slc11a1*) gene are responsible of mouse propensity to be infected with *Leishmania* because macrophages permit its replication [Forbes and Gros, 2001; Marquis and Gros, 2007; Huynh and Andrews, 2008; Jacques *et al.*, 2010].

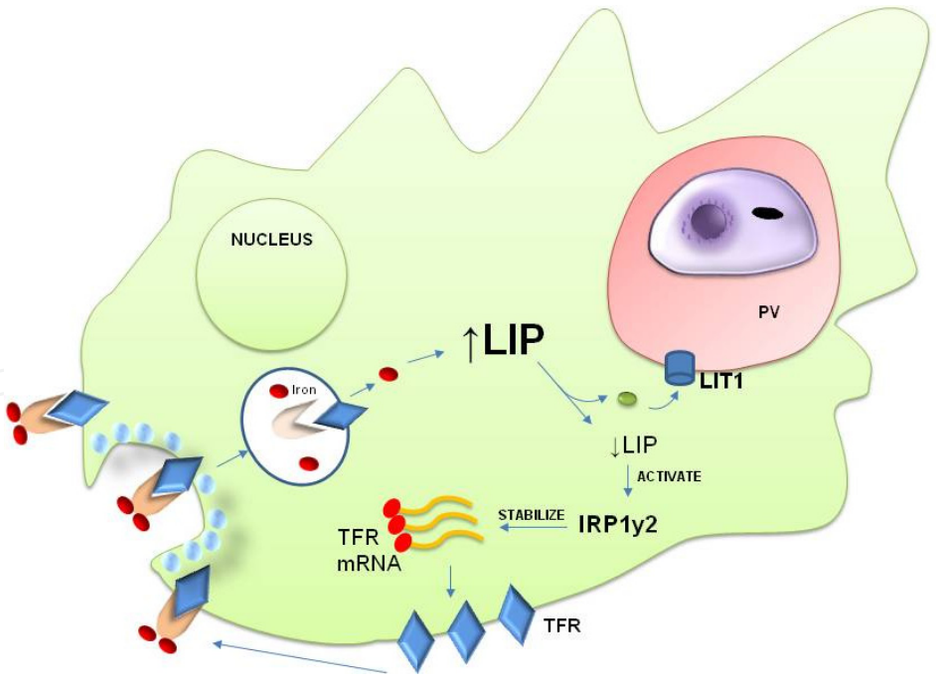

Figure 6. Iron uptake by intracellular *Leishmania donovani* amastigotes. From its parasitophorous vacuole (PV), *Leishmania* expresses its ferrous iron transporter (LIT1) to scavenge iron and deplete the macrophage labile-iron pool (LIP); this activates the host cytosolic iron-responsive element sensor proteins IRP1 and IRP2. These proteins increase the stability of the TFR mRNA , increasing macrophage iron uptake from TF and the intracellular iron parasite needs for survival.

Interestingly, studies with *L. donovani* suggest that there is an intraphagosomal competition for free ferrous-iron between the iron transporters from host and those from the parasite. This competition may, by depleting the macrophage labile-iron pool (LIP) (Fig. 6), activate the host cytosolic iron-responsive element sensor proteins IRP1 and IRP2. These proteins increase the stability of the mRNA of the TFR by binding to iron-responsive elements (IREs) present in the 3′UTR of the TFR1 gene, which in turn leads to increased production of the TFR1 and, thus, to TF-mediated iron uptake [Das *et al.*, 2009]. In this study, the authors clearly demonstrate that instead of macrophages sequester iron, virulent parasites directly scavenge iron from the host LIP (Fig. 6), which activates the interaction IRE-IRP leading to an up-regulation of the macrophage′ TFR1, increasing the intracellular iron needed for parasite survival.

2.2.6. Toxoplasma gondii

Toxoplasma gondii is an intracellular obligate protozoan that belongs to the phylum *Apicomplexa* and is unique in invading a large diversity of mammals and birds [Ossorio *et al.*, 1994; Ajioka *et al.*, 1998; Joiner and Roos, 2002]. It is thought that approximately 25% of the world human population is infected by *Toxoplasma* [Tenter *et al.*, 2000]. Toxoplasmosis can produce severe damage in humans with often fatal results, mainly in immunosuppressed patients suffering from AIDS or cancer and in people undergoing immunosuppressive treatments. The most frequent damages include chorioretinitis with consecutive loss of vision and damage to the CNS, lungs, and heart, and when infection occurs during pregnancy, parasites reach the placenta and infect the fetus, causing abortion [Luft and Remington, 1992; Barragan and Sibley, 2002]. However, in immune competent people, infection occurs in a transitory and asymptomatic fashion. The success of *T. gondii* as an intracellular pathogen is based on its high capacity for invasion and dissemination in practically all tissues due to its migration through biological barriers such as intestinal, hematic-encephalic, hematic-ocular, and placental; parasites can be detected in amniotic, cerebrospinal, bronchoalveolar, ocular, pleural and ascitic fluids, as well as in urine and peripheral blood [Derouin and Garin, 1991; Barragan and Hitziger, 2008; Unno *et al.*, 2008]. Due to the high incidence of toxoplasmosis in AIDS patients, in the last few years, much attention has been placed on the pathology caused by this parasite.

The *T. gondii* life cycle involves two types of hosts: definitive hosts, which include members of the *Felidae* family such as the domestic cat in which the sexual reproduction cycle takes place, and intermediate hosts, which include warm-blood animals such as cattle, sheep, pigs, and humans in which asexual reproduction occurs [Dubey, 1998; Tenter *et al.*, 2000]. Parasite replication takes place in the gut, resulting in the production of oocysts, which are shed in the feces. After sporulation, the resultant sporozoites are infective when ingested by humans and other mammals, and tachyzoites multiply and enter into all host nucleated cells, creating the parasitophorous vacuole (PV), a highly specialized non-fusogenic compartment [Martin *et al.*, 2007] delimited by a membrane that allows the passage of small molecules [Gail *et al.*, 2004]. After the parasite undergoes repeated replication rounds, the host cells lyse and tachyzoites are disseminated via the blood and lymph. When the host immune system becomes activated

due to the presence of the parasite, immune cells such as macrophages and lymphocytes respond with proliferation, activation, and the release of diverse cytokines including IFN-γ. The presence of IFN-γ induces tachyzoite differentiation in bradyzoites and modification of the infected host cell in tissue cysts, in which this form of the parasite remains in latency for several years, giving rise to chronic infections [Dimier and Bout, 1998].

Role of iron in cell invasion by *T. gondii*

Iron is an essential component in the intracellular survival and multiplication of *T. gondii*. Obtaining iron from the invaded host cell is a key process that *Toxoplasma* has to regulate to secure an adequate provision at the intravacuolar level. As a strategy, the parasite activates the increase of iron regulator proteins (IRPs), which apparently function to stabilize the TFR mRNA of host cells; therefore, a high expression of TFR is induced at the membrane in the invaded cells, leading to TF-iron capture, which is taken up by intracellular tachyzoites to aid in proliferation (Fig. 7). This positive regulation seems to be mediated by soluble factors secreted by *Toxoplasma* that are not yet identified [Gail *et al.*, 2004]. As occurs with other parasites, iron chelating agents such as deferoxamine can limit tachyzoite development. This effect is reverted through the addition of exogenous holo-TF or ferrous sulfate as sources of Fe, conditions that allow successful intracellular replication of the parasite and demonstrate the importance of iron in the intracellular development of this parasite [Dimier and Bout, 1998; Mahmoud, 1999].

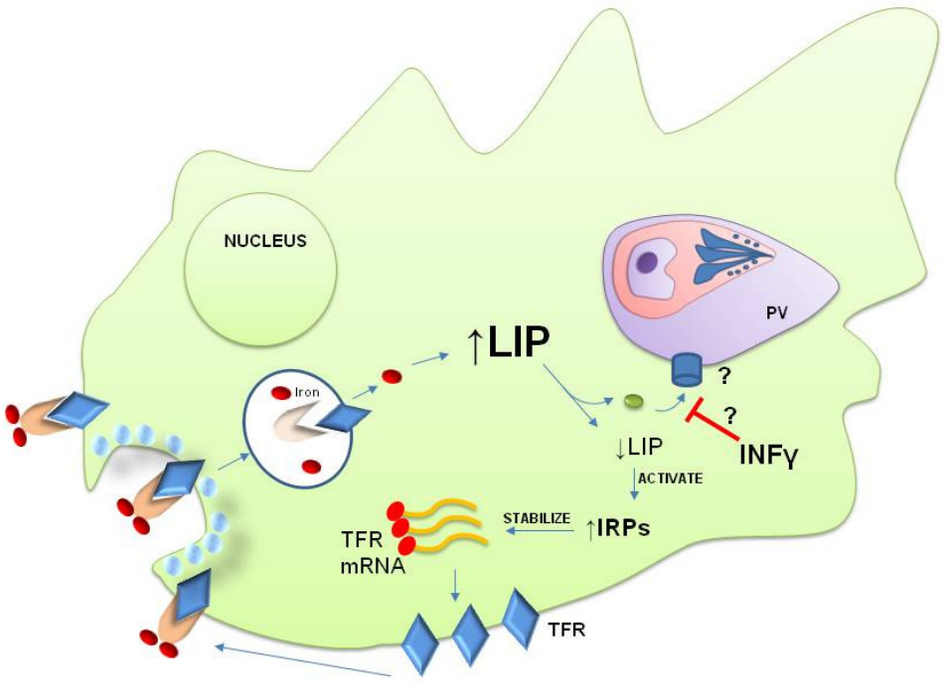

Figure 7. Iron uptake by intracellular *Toxoplasma gondii*. *T. gondii* lives inside a parasitophorous vacuole (PV) within the host cell; from there, it sequesters iron and depletes the macrophage labile-iron pool (LIP), activating the host cytosolic iron-responsive element sensor proteins (IRPs). These proteins increase the stability of the TFR mRNA, increasing host cell iron uptake from TF. This positive regulation system increases the intracellular iron that the parasite requires for survival. Iron uptake by the parasite is limited by TNFγ through an unknown mechanism.

In addition to the participation of macrophages and neutrophils in the immune response to *Toxoplasma*, fibroblasts, endothelia, and intestinal cells might also protect against this pathogen, most likely through a mechanism that involves the incorporation of iron from plasma TF, consequently limiting the availability of Fe resources to the parasite. An alternative strategy is the participation of exogenous IFN-γ in the inhibition of intracellular tachyzoite replication, a phenomenon observed in a dose-dependent manner in primary cultures of rat enterocytes. Interestingly, the exogenous addition of ferrous sulfate or holo-TF neutralizes entirely the effect of IFN-γ on the enterocytes. Although the precise molecular events initiated in enterocytes as a result of IFN-γ exposure are not clear, it has been suggested that IFN-γ inhibits tachyzoite replication by a mechanism that involves the limitation of available intracellular Fe (Fig. 7) [Dimier and Bout, 1998].

Dziadek et al. reported in [2005] that *T. gondii* tachyzoites of the BK strain bind to human holoLF but not holoTF, suggesting the presence of specific membrane receptors of *Toxoplasma* to host mucosal LF. Tanaka studied the expression of Lbps on tachyzoites of the RH-strain maintained through Vero cells incubated with labeled bovine LF and bovine TF. Both iron-carrier proteins were recognized by a single protein of 42 kDa, suggesting a non-specific binding to a common receptor [Tanaka *et al.*, 2003]. In additional studies, it was determined that both the absence and the excess of Fe produce an inhibition of the intracellular proliferation of RH-strain tachyzoites grown in cultured host cells, however, the mechanism of action by which this phenomenon occurs is unknown [Tanaka *et al.*, 1997].

3. Concluding remarks

Our knowledge of iron-uptake mechanisms from host TF by parasitic protozoa has improved in the past few years. These new insights have demonstrated the importance of effective iron uptake for virulence and increased the understanding of several mechanisms. Although substantial progress has been made, there is surprisingly little information available, including information about *T. brucei* and *T. cruzi*, which have been extensively studied, present interesting differences in their iron internalization mechanisms, and have enormous therapeutic potential. In other pathogens with serious medical implications as *Leishmania, P. falciparum* and *E. histolytica*, there is very limited information available, although the importance of iron for their survival is evident.

It is necessary to obtain more knowledge on the iron acquisition mechanisms in unicellular eukaryotic pathogens in order to develop new chemotherapeutic strategies that avoid the utilization of host iron by these parasitic organisms that have intense iron requirements.

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