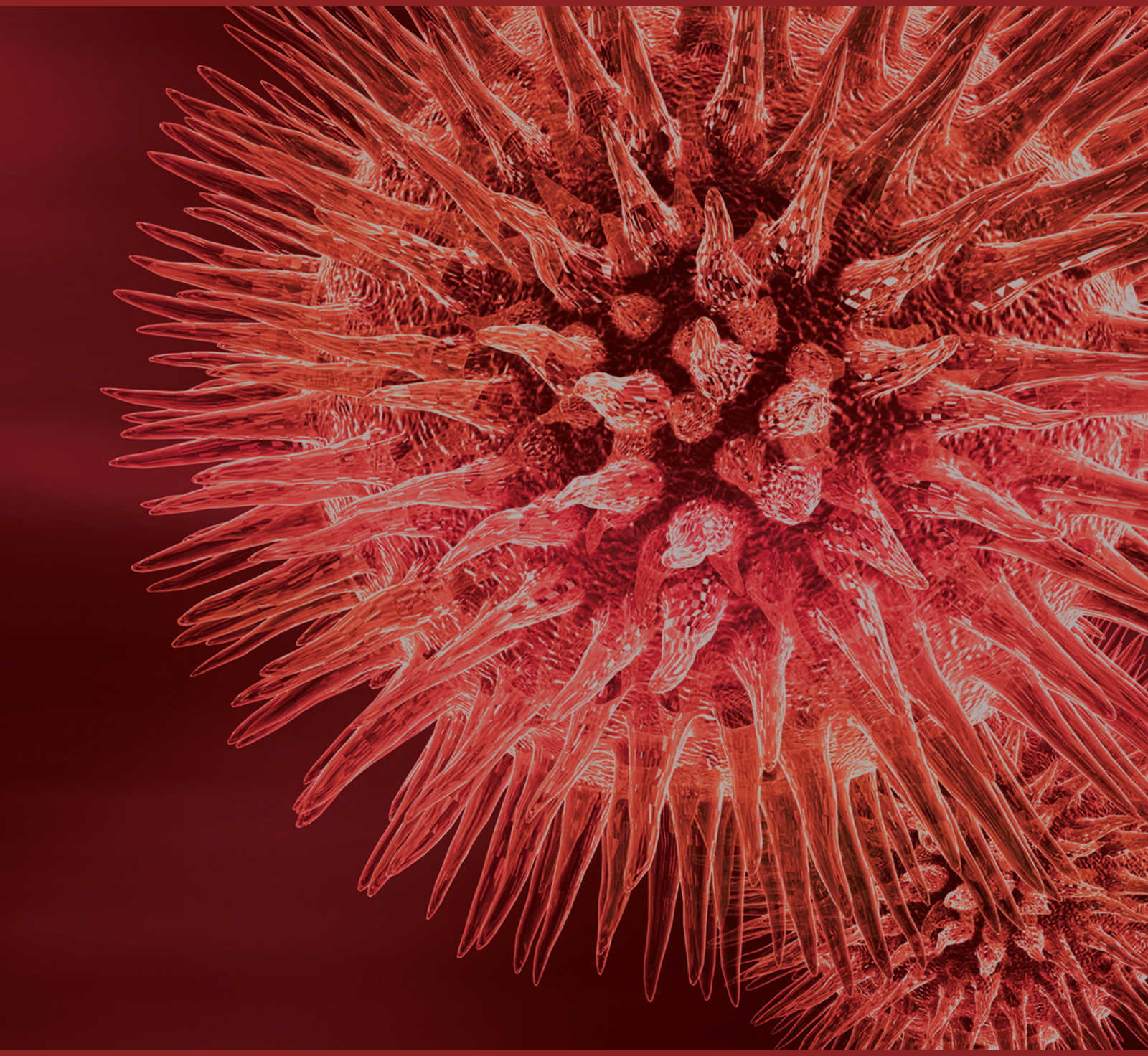
BioMed Research International

Iron and Parasites

Guest Editors: Rossana Arroyo, Theresa Ochoa, Jung-Hsiang Tai, and Mireya de la Garza



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Contents

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Editorial
Iron and Parasites

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In this special issue, we analyze the importance of iron in the host-parasite interplay. Iron is a transition element and the fourth most abundant element in the Earth's crust. Iron is vital for growth of nearly all living organisms, from prokaryotes to humans. Iron plays an important role in several cellular processes, such as respiration, photosynthesis, oxygen transport, and DNA synthesis. Iron is essential but it is not easily bioavailable; ferric iron solubility is low at physiological pH whereas ferrous iron, in aerobic environments, is highly toxic. Therefore, iron is normally bound to proteins and the whole body and cellular iron concentrations have to be regulated in all organisms.

Some iron-containing and iron-binding proteins are intracellular such as the oxygen-carrier hemoglobin, the iron-storing protein ferritin, and numerous enzymes. Others are extracellular, mainly transferrin (Tf) and lactoferrin (Lf). Tf and Lf are able to capture up to two Fe^{3+} atoms per molecule, maintaining iron in a soluble and stable oxidation state in fluids and avoiding the generation of toxic free radicals derived from Fe^{2+} through the Fenton reaction. Free radicals are deleterious to most macromolecules. Tf and Lf maintain the free iron concentration too low to sustain the parasites growth. Tf is the iron transporter that allows cellular iron uptake; it is mainly found in serum and lymph. Lf is secreted into mucosae and by the secondary granules of neutrophils, to chelate the Fe^{3+} and avoid its availability for parasites.

Therefore, during infection, there is a constant battle between the host and the invader for iron, in which the invader attempts to have access to host iron and the host arranges complex iron-withholding mechanisms to frustrate the iron stealing. Virtually, all iron-containing proteins in eukaryotes can be used as iron sources by iron-seeking parasites; for that, several elaborate strategies have been developed by parasites to obtain host iron. Thus, capture and uptake of host iron by parasites are considered as virulence determinants.

Little information regarding iron acquisition in free-living amoebae has been reported. In the research article "Iron-Binding Protein Degradation by Cysteine Proteases of *Naegleria fowleri*," M. Martínez-Castillo et al. report the cleaving of human hololactoferrin, hemoglobin, and holotransferrin by this parasite. *N. fowleri* causes primary amoebic meningoencephalitis. During the invasion, the microorganism interacts with different tissues such as olfactory neuroepithelium and olfactory bulbs that contain iron-binding proteins. The results show that this protozoan has several cysteine-secreted proteases that cleave iron-binding proteins. Using this strategy, *N. fowleri* could obtain iron from the host in the invaded tissues.

G. Ortíz-Estrada et al. address the issue about the possible way in which the human enteric parasite *Entamoeba histolytica* could have access to bovine lactoferrin,

a protein present in the milk mainly consumed by babies and infants fed with formula. In their research article "Binding and Endocytosis of Bovine Hololactoferrin by the Parasite *Entamoeba histolytica*," the authors compare virulent trophozoites recently isolated from hamster liver abscesses with nonvirulent trophozoites maintained for more than 30 years in *in vitro* cultures, regarding their interaction with bovine iron-charged Lf (B-holo-Lf). Interestingly, although both amoeba variants are able to use B-holo-Lf as an iron source and endocytosed this glycoprotein through clathrin-coated vesicles, the acquisition of iron, binding parameters, and number of protein-binding sites per amoeba are different. In addition, the virulent amoebae also endocytosed B-holo-Lf through a cholesterol-dependent mechanism; thus the B-holo-Lf endocytosis is more efficient in virulent amoebae.

In the minireview article "Strategies of Intracellular Pathogens for Obtaining Iron from the Environment," N. Leon-Sicairos et al. focus on how intracellular pathogens use multiple approaches to obtain nutritional iron from the intracellular environment, in order to use this element for replication. They explore the current knowledge about the process that occurs during infection by intracellular pathogens, where the iron is required by both the host cell and the pathogen that inhabits it. Intracellular microorganisms are destroyed by the host tissues through processes that usually involve phagocytosis and lysosomal disruption. However, some intracellular pathogens are capable of avoiding destruction by growing inside macrophages and other cells. Additionally, the implications of these mechanisms for iron acquisition in the host-pathogen relationship are discussed.

African trypanosomosis is caused by the parasitic protozoan *Trypanosoma brucei*. This is a chronic and debilitating disease suffered mainly by people of developing countries. In the review "Iron-Homeostasis and *Trypanosoma brucei* Associated Immunopathogenicity Development: A Battle/Quest for Iron," B. Stijlemans et al. analyze the different strategies that lead to a host immune response that results in iron deprivation, consisting in an iron modulation of the host myeloid phagocytic system that affects trypanosomosis-associated anemia development.

The review article "*Trichomonas vaginalis* Cysteine Proteinases: Iron Response in Gene Expression and Proteolytic Activity" by R. Arroyo et al. focuses on the iron response of *Trichomonas vaginalis* on gene family products as the cysteine proteinases (CPs) involved in virulence properties. In particular, it examines the effect of iron in gene expression regulation and function of cathepsin L-like and asparaginyl endopeptidase-like CPs as virulence factors. Aspects regarding CPs genomic organization are addressed to offer possible explanations to the fact that only few members of this large gene family are expressed at the RNA and protein levels. Also offers possible ways used to control these particular proteolytic activities. Moreover, all known iron regulatory mechanisms of CPs at transcriptional, posttranscriptional, and posttranslational levels along with new insights into the possible epigenetic and miRNA processes in *T. vaginalis* are also summarized.

Finally, in the review article "Transferrin: Endocytosis and Cell Signaling in Parasitic Protozoa," by M. Reyes-López et al., the authors describe the presence of specific receptors for Tf in protozoan parasites. The signal transduction initiated upon ligand binding at the parasite plasma membrane with the process in mammalian cells is compared, based on the large amount of information on the Tf endocytosis. Several signaling pathways participate in Tf trafficking, such as the insertion of membrane vesicles, and the signaling pathways mediated by the inositol-1,4,5-triphosphate and diacylglycerol, MAPK, or growth factors. Some components of these pathways also found in parasites are included, as well as the identification of signaling proteins, useful in the study of essential factors for the parasitic life and as potential targets for the development of chemotherapeutic approaches.

We hope that researchers enjoy the reading of this special issue related to parasites and one of the most important chemical elements, iron. Undoubtedly, the acquisition of host iron by a pathogen is a crucial step during the development of infection and is determinant in its outcome.

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Research Article

Iron-Binding Protein Degradation by Cysteine Proteases of *Naegleria fowleri*

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Naegleria fowleri causes acute and fulminant primary amoebic meningoencephalitis. This microorganism invades its host by penetrating the olfactory mucosa and then traveling up the mesaxonal spaces and crossing the cribriform plate; finally, the trophozoites invade the olfactory bulbs. During its invasion, the protozoan obtains nutrients such as proteins, lipids, carbohydrates, and cationic ions (e.g., iron, calcium, and sodium) from the host. However, the mechanism by which these ions are obtained, particularly iron, is poorly understood. In the present study, we evaluated the ability of *N. fowleri* to degrade iron-binding proteins, including hololactoferrin, transferrin, ferritin, and hemoglobin. Zymography assays were performed for each substrate under physiological conditions (pH 7 at 37°C) employing conditioned medium (CM) and total crude extracts (TCEs) of *N. fowleri*. Different degradation patterns with CM were observed for hololactoferrin, transferrin, and hemoglobin; however, CM did not cause ferritin degradation. In contrast, the TCEs degraded only hololactoferrin and transferrin. Inhibition assays revealed that cysteine proteases were involved in this process. Based on these results, we suggest that CM and TCEs of *N. fowleri* degrade iron-binding proteins by employing cysteine proteases, which enables the parasite to obtain iron to survive while invading the central nervous system.

1. Introduction

Naegleria fowleri is a free-living amoeba that causes primary amoebic meningoencephalitis (PAM) in humans. This protozoan gains access to the central nervous system (CNS) by penetrating the olfactory neuroepithelium and migrating through olfactory nerves and reaching the olfactory bulbs [1–5]. Immunohistochemical studies of the early events of infection in a murine model have shown that the amoebae induce intense mucus secretion and an inflammatory reaction in the nasal cavity [6]. At later stages of infection, tissue damage characterized by extensive lytic and necrotic areas, hemorrhage, and cellular debris has been reported. In hemorrhagic areas, the amoebae have been observed to carry several ingested erythrocytes [7]. The molecular mechanisms employed by *N. fowleri* to process and degrade cells or molecules are poorly studied. However, it is known that, during invasion, *N. fowleri* is able to release proteolytic proteins including naegleriapores A and B, phospholipases, glycosidases, neuraminidase, elastase, and other proteases, such as cathepsin B and mucinases [8–14]. Some of these proteases have been evaluated using *in vitro* systems. These studies evaluated specific human substrates such as IgA, IgG, IgM, collagen, fibronectin, hemoglobin, albumin, mucus, and elastin [2, 12, 13]. Other investigations have analyzed proteins associated with iron. In invasive pathogens such as bacteria,

the acquisition of iron is crucial to division and survival; additionally, previous studies have shown that bacteria can acquire iron from a substantial number of iron-binding proteins, including transferrin, lactoferrin, hemopexin, ferritin, hemoglobin, the hemoglobin/haptoglobin complex, and human serum albumin [15–17]. In some protozoans, such as *Tritrichomonas foetus*, *Trichomonas vaginalis*, *Toxoplasma gondii,* and *Entamoeba histolytica*, the expression of lactoferrin-binding proteins has been described, and these parasites use hololactoferrin as an iron source for *in vitro* growth [18]. Another mechanism involved in the acquisition of iron from lactoferrin has been reported in promastigotes of *Leishmania chagasi*, which use a surface reductase that recognizes and reduces ferric iron to the accessible ferrous form (Fe^{2+}) [19]. Cysteine proteases that cleave lactoferrin have also been reported in *E. histolytica* [20]. In contrast, there is less information regarding iron acquisition in the *Naegleria* genus; consequently, we analyzed the ability of *N. fowleri* to degrade molecules associated with iron. We determined that the proteases released from free-living amoebae were able to degrade iron-binding human proteins, including hololactoferrin, hemoglobin, and holotransferrin, but not ferritin. It is possible that the degradation of iron-binding proteins could play a role in PAM progression in both human and animal models of the disease.

2. Materials and Methods

2.1. Amoebic Cultures. The pathogenic strain *N. fowleri* (ATCC 30808) was used in all experiments. However, to maintain the amoebic virulence, trophozoites were instilled in mice; seven days later, the brains were recovered in Bacto Casitone medium with antibiotics. Finally, the culture was maintained in axenic conditions in 2% (w/v) Bacto Casitone medium supplemented with 10% (v/v) fetal bovine serum (FBS; Equitech-Bio, USA) at 37°C. The trophozoites were harvested during the exponential growth phase (48 h).

2.2. Sample Preparation. Total crude extracts (TCEs) were obtained as previously described with some modifications [21]. Briefly, *N. fowleri* trophozoites were removed from the culture flask surface by chilling in an ice bath for 20 min, centrifuged at 800 ×g for 10 min, and washed with phosphate buffered saline (PBS) (pH 7.2). Subsequently, the trophozoite pellets were incubated at 37°C for 30 min and then disrupted by five freeze-thaw cycles in PBS. The conditioned medium (CM) was prepared according to the following protocol. Six million trophozoites were placed in culture flasks containing 3 mL of fresh Bacto Casitone medium without FBS and incubated at 37°C for 24 h. The supernatant or CM was removed and centrifuged again at 1,500 ×g for 10 min and finally passed through a 0.22 μm Durapore membrane (Millipore, Bedford, MA). Next, the samples were precipitated with absolute ethanol (J.T. Baker, USA) at a 3 : 1 ratio and stored at −20°C for 2 h, and the CM was centrifuged at 6,000 ×g for 30 min. The protein concentrations of the TCEs and CM were quantified according to the Bradford method [22]; the TCEs and CM were stored at −80°C until use.

2.3. Protease Inhibitors. For the inhibition assays, TCE or CM was preincubated for 1 h at 37°C with protease inhibitors with constant agitation. The inhibitor concentrations were as follows: for cysteine proteases, 10 mM *p*-hydroxymercuribenzoate (*p*HMB); for serine and cysteine proteases, 5 mM phenyl-methyl sulfonyl fluoride (PMSF); and as a specific serine protease inhibitor, 1 mM aprotinin (Sigma-Aldrich, St. Louis, MO).

2.4. Substrate Gel Electrophoresis. Protease activities were determined by performing electrophoresis of the TCEs and CM in 10% SDS-PAGE copolymerized with bovine hololactoferrin (h-bLf), human hololactoferrin (h-hLf), human hemoglobin (hHb), human holotransferrin (h-hTf), equine ferritin (eqF), and porcine skin gelatin (Pg) as a protease substrate. All of the substrates had a final concentration of 1 mg/mL (Sigma-Aldrich).

To determine the protein patterns of the TCEs and CM, 40 μg of total protein was loaded per well. For the zymography assay, only 20 μg of protein was loaded per well. As an experimental control, Bacto Casitone medium was loaded in all assays (25 μL).

Electrophoresis was performed at 4°C in an ice bath and at a constant voltage (80 V) for 1 h; the gels were washed twice for 30 min with agitation in 2.5% (v/v) Triton X-100 solution (Sigma-Aldrich). The gels were then incubated overnight with 100 mM sodium acetate (pH 5.0), 100 mM Tris-HCl (pH 7.0), or 100 mM glycine (pH 9.0). All buffers contained 2 mM $CaCl_2$ with or without 2 mM DTT. Finally, the gels were stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 for 30 min. Protease activities were identified as clear bands on a blue background. All the assays were performed in triplicate. Inhibition analyses were performed using the ImageJ program (http://rsb.info.nih.gov/nih-image/).

3. Results

3.1. Proteases Present in Conditioned Medium and Total Crude Extracts in N. fowleri. CM (Figure 1) and TCEs (see Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/416712) were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. The CM protein pattern exhibited proteins between 100 and 30 kDa (Figure 1, second lane). We also evaluated the proteolytic activity of CM in 10% PAGE copolymerized with 0.1% porcine gelatin at pH 7 and 37°C. We observed four degradation bands, with calculated MWs of 172, 135, 80, and 60 kDa (Figure 1, lane 3). The Bacto Casitone medium was also analyzed as a control by 10% PAGE copolymerized with 0.1% gelatin; the medium did not produce any degradation bands (Figure 1, lane 4).

3.2. N. fowleri Proteases Can Degrade Hololactoferrin. To analyze the ability of *N. fowleri* to degrade iron-binding proteins, we performed zymography assays employing different substrates. We found that both CM and TCE (Supplementary Figures S1 and S2) were able to degrade bovine hololactoferrin (h-bLf) and human hololactoferrin (h-hLf).

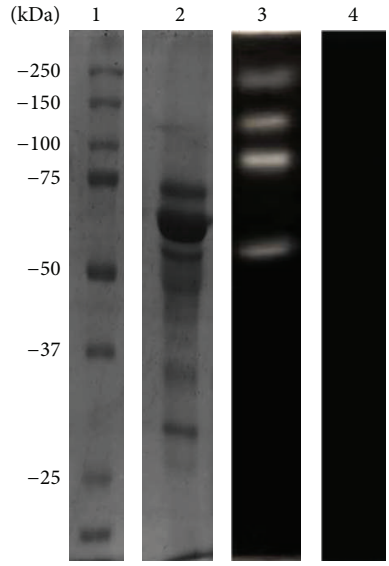

Figure 1: Conditioned medium proteins by SDS-PAGE and gelatin-PAGE 10%. Molecular weights (lane 1). Conditioned medium in the SDS-PAGE pattern protein (lane 2), zymogram in 0.1% porcine gelatin (lane 3). Bacto Casitone medium was loaded as a control (lane 4).

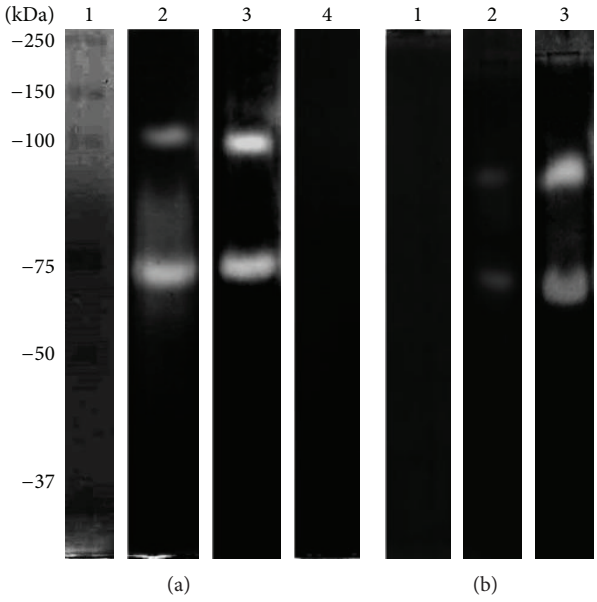

Figure 2: Zymography assay in 10% PAGE copolymerized with 0.1% (w/v) holo-bLf. (a) CM was evaluated at different pHs at 37°C: molecular weight marker (lane 1), pH 5 (lane 2), pH 7 (lane 3), and pH 9 (lane 4). (b) Effects of protease inhibitors evaluated at pH 7 37°C: pHMB (lane 1), PMSF (lane 2), and aprotinin (lane 3).

When CM was analyzed by h-bLf-PAGE at pH 5, two bands, with MWs of 100 and 75 kDa, were observed (Figure 2(a), lane 2); at pH 7, the same proteolytic pattern was found (Figure 2(a), lane 3). At pH 9, no degradation activity was detected (Figure 2(a), lane 4). In the case of h-hLf-PAGE, only two proteolytic bands (MW: 100 and 75 kDa) were found

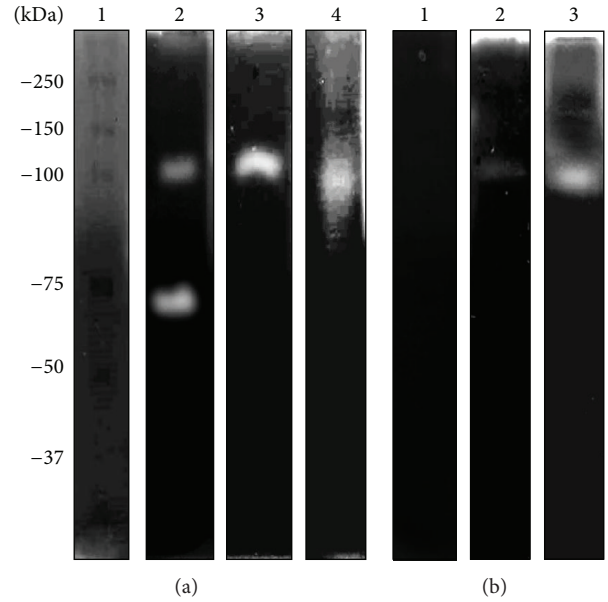

Figure 3: Zymography assay in 10% PAGE copolymerized with 0.1% (w/v) holo-hLf. (a) CM was evaluated at different pHs at 37°C: molecular weight marker (lane 1), pH 5 (lane 2), pH 7 (lane 3), and pH 9 (lane 4). (b) Effects of protease inhibitors evaluated at pH 7 and 37°C: pHMB (lane 1), PMSF (lane 2), and aprotinin (lane 3).

at pH 5 (Figure 3(a), lane 2) and only one band (100 kDa) was found at pH 7 (Figure 3(a), lane 3). In contrast with h-bLf-PAGE, we observed proteolytic activity at pH 9 and an MW of 100 kDa (Figure 3(a), lane 4). The activity bands of CM with all substrates are summarized in Table 1; those of TCE are given in Table 2.

Additionally, we evaluated whether the proteolytic pattern was produced by cysteine proteases. To elucidate this activity, we performed assays using specific (pHMB), partial (PMSF), and nonspecific (aprotinin) inhibitors of cysteine proteases. As anticipated, in both h-bLf and h-hLf, the degradative activity of CM at pH 7 and 37°C was abrogated only by the specific inhibitor of cysteine proteases, pHMB (Figures 2(b) and 3(b), lane 1). Moreover, we observed partial inhibition with PMSF (Figures 2(b) and 3(b), lane 2), and no effect was found with high concentrations of aprotinin (Figures 2(b) and 3(b), lane 3). Similar assays were performed for TCE, and similar results were obtained with pHMB, which inhibited all protease activities (Supplementary Figures S1 and S2).

3.3. Proteases Present in N. fowleri Degrade Human Holotransferrin (h-hTf). The results of the h-hTf zymography revealed activities at all the pH levels evaluated. At pH 5, we found one intense band of degradation with an MW of 100 kDa (Figure 4(a), lane 2); activation at pHs 7 and 9 revealed two activities at 180 and 100 kDa, respectively (Figure 4(a), lanes 3 and 4). In this case, we also evaluated the effect of inhibitors in CM and found a result similar to that described for hololactoferrin. Specific inhibition was achieved with cysteine protease inhibitor (pHMB) (Figure 4(b), lane 1).

Table 1: Conditioned medium (CM). Locations of bands present in the zymography assays using different substrates: (X) proteases at pH 7; (∗) proteases that appear at pH 5 and 9.

MW	Substrate																
	Pg		h-bLf			h-hLf			h-hTf			eqF			hHb		
pH	7	5	7	9	5	7	9	5	7	9	5	7	9	5	7	9
180									X	∗						
172	X															
135	X															
100		∗	X		∗	X	∗	∗	X	∗						
80	X													∗	X	
75		∗	X		∗											
65														∗		
60	X															

Table 2: Total crude extract (TCE). Locations of bands present in the zymography assays using different substrates: (X) proteases at pH 7; (∗) proteases that appear at pH 5 and 9.

MW	Substrate																
	Pg		h-bLf			h-hLf			h-hTf			eqF			hHb		
pH	7	5	7	9	5	7	9	5	7	9	5	7	9	5	7	9
150	X	∗	X					∗								
130	X			∗												
100	X	∗	X	∗	∗	X			X							
70	X	∗	X													
75								∗								
60	X							∗	X							
50					∗											

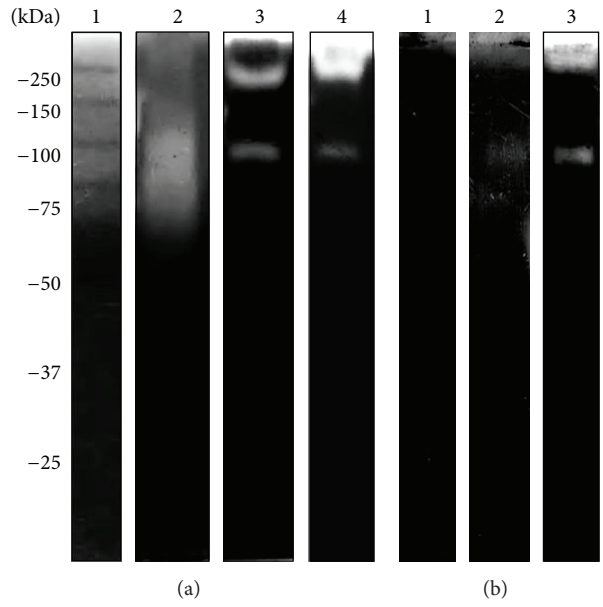

Figure 4: Zymography assay in 10% PAGE copolymerized with 0.1% (w/v) holo-hTf. (a) CM was evaluated at different pHs at 37°C: molecular weight marker (lane 1), pH 5 (lane 2), pH 7 (lane 3), and pH 9 (lane 4). (b) Effects of protease inhibitors evaluated at pH 7 and 37°C: pHMB (lane 1), PMSF (lane 2), and aprotinin (lane 3).

Similarly, we only evaluated the inhibition at a physiological pH and temperature (pH 7; 37°C). In the case of TCE activity, we detected degradation at pHs 5 and 7, and the inhibition was correlated with the results described for CM (Supplementary Figure S3).

3.4. N. fowleri Proteases Have No Effect on Ferritin. Ferritin, another iron-storing molecule, was also analyzed. This protein has been used as an indicator of iron availability in humans (liver and serum). To analyze the effect of *Naegleria* proteases on ferritin, we used equine ferritin as a substrate because this protein presents high homology with human ferritin. Interestingly, in the zymograms with ferritin as the substrate, we did not observe any degradation in CM (Figure 5) or TCE (Supplementary Figure S4).

3.5. N. fowleri Proteases Can Degrade Hemoglobin. Hemoglobin, a well-known iron-carrier protein that is present in erythrocytes, is another substrate possibly encountered by *N. fowleri* during its invasion process. Consequently, we also copolymerized hemoglobin with acrylamide (hHb-PAGE). The results revealed degradation activity in CM (Figure 6(a)) but not in TCE (Supplementary Figure S5). The degradation bands identified had MWs of 80 and 65 kDa at pH 5 and only one band of 80 kDa at pH 7; no activity was found at pH 9

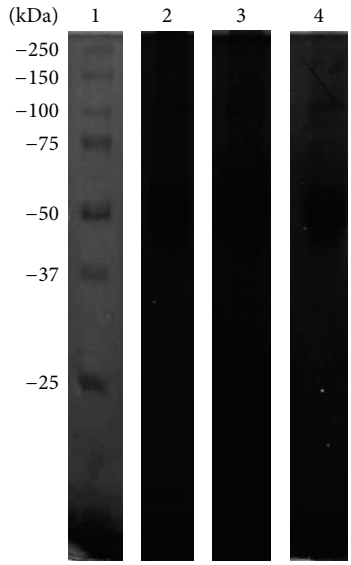

Figure 5: Zymography assay in 10% PAGE copolymerized with 0.1% (w/v) eqF. CM was evaluated at different pHs at 37°C: molecular weight marker (lane 1), pH 5 (lane 2), pH 7 (lane 3), and pH 9 (lane 4).

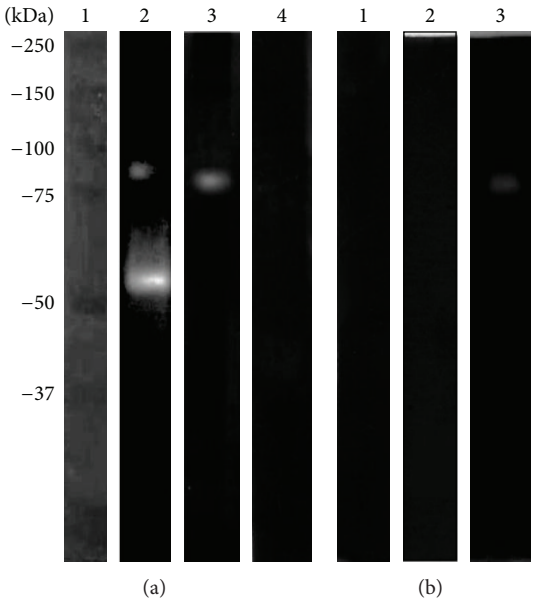

Figure 6: Zymography assay in 10% PAGE copolymerized with 0.1% (w/v) hHb. (a) CM was evaluated at different pHs at 37°C. Molecular weight marker (lane 1), pH 5 (lane 2), pH 7 (lane 3), and pH 9 (lane 4). (b) Effects of protease inhibitors: pHMB (lane 1), PMSF (lane 2), and aprotinin (lane 3).

(Figure 6(a), lanes 2–4). The main inhibition was observed with both pHMB and PMSF (Figure 6(b), lanes 1, 2) but not with aprotinin (Figure 6(b), lane 3).

4. Discussion

In nature, the distribution and proliferation of *N. fowleri* have been associated with the proportion of Fe^{2+} in the vertical distribution of the water column [23]. However, during the process of invading the nasal cavity, *N. fowleri* encounters an iron-deficient environment and an extracellular glycoprotein of the innate immune response (where the ferric iron is chelated by apolactoferrin) [24, 25]. Additionally, the deficiency of iron in *N. fowleri* cultures has been shown to be responsible for reduced proliferation of the parasite [26]. Furthermore, the addition of molecules that contain iron (e.g., Hb, hemin, and protoporphyrin IX) provides resistance to lysis by complement, increases motility, and induces proliferation in culture [27–29]. However, the mechanism by which *N. fowleri* obtains iron during PAM has not been described.

In the case of the parasitic protozoan *E. histolytica,* several mechanisms (such as receptors and proteases) are used to obtain iron [30]. In this context, we evaluated the ability of *N. fowleri* to degrade iron-binding proteins including hololactoferrin, hemoglobin, holotransferrin, and ferritin. The zymography assays revealed a degradation of the specific substrates; moreover, this strategy allowed us to estimate the molecular weight of the proteases involved in this process.

We observed proteolytic activity in gels copolymerized with both holo-hLf and holo-bLf; this is the first report of *N. fowleri* to demonstrate that this free-living amoeba degrades these proteins. Interestingly, we observed one activity band of 100 kDa conserved in human and bovine holo-Lf. In contrast, the presence of a 75 kDa activity band was predominant in bovine holo-Lf. This slight difference might be due to the homology of human and bovine Lf (69%) [31, 32]. In basal conditions, apolactoferrin is produced by serous cells in the nasal cavity (ratio 200–300 μg/mL) [33]; many reports confirm that the apo-Lf concentration increases during microbial infection or tissue damage [34, 35]. The main role of apo-Lf is its iron-chelating activity, and when this occurs, both globular domains of apo-Lf are occupied by iron ions (holo-Lf or h-Lf) [35]. It has recently been reported that *E. histolytica* can ingest human h-Lf; moreover, this protein is transported in acid vesicles. Additionally, the researchers performed PAGE copolymerized with h-hLf to elucidate the type of proteases present in these vesicles and found that total crude extracts of *E. histolytica* contained cysteine proteases; in contrast with our results, they did not observe activity in supernatants [20]. In the case of *N. fowleri,* we identified activity in both CM and TCEs; additionally, when we used ethanol to precipitate CM of *E. histolytica*, we observed bands of degradation (data not shown). These results support the hypothesis that *N. fowleri* can obtain iron by degrading h-hLf. The different patterns of CM degradation of h-hLf and h-bLf may be supported by the difference of homology in the sequence of amino acids in these proteins. The differential degradation by nonsecreted proteases suggests internalization and processing of hLf; unfortunately, little is known about endocytosis and vesicular trafficking in *N. fowleri*. Therefore, it is important to study the effect of h-hLf in *N. fowleri* and also to elucidate the mechanisms of uptake of hHb, h-Tf, and ferritin, which are other important sources of iron in the human body. Human transferrin is mainly synthesized by the liver, although other tissues and organs, such as the brain, are also able to produce transferrin [36, 37]. Both ferritin and transferrin are found

in oligodendrocytes; these cells are present in the optic nerve and in both the gray and white matter of the cerebral cortex, cerebellum, and olfactory bulbs. Astrocytes and glia cells are also positive for ferritin, transferrin, and iron [36, 38, 39]. These brain cells have been described to interact with *N. fowleri* during the later stages of PAM [7, 40]. Therefore, we evaluated whether *N. fowleri* could also degrade these iron-binding proteins, and we observed degradation of holo-hTf by CM and TCE, but these extracts had no effect on ferritin.

The effect of cysteine proteases in ferritin has been demonstrated in bacteria [41], fungi [42], and some protozoa [30, 43]. The differences between CM and TCE may depend on a specific process (endocytosis) or on degradation caused by a wide range of proteases [44]. In the present work, the nondegradation of ferritin was surprising because it is the main iron storage protein in the human body; furthermore, we expected that *N. fowleri* proteases would degrade this protein because *N. gruberi* has been reported to bind ferritin on the cell surface [45]. However, it is possible that coupling ferritin in *N. fowleri* only occurs in a transitory event that culminates in capping formation [2, 46]. Another possible mechanism involved in nondegradation is the high molecular weight of the protein [47].

The hemorrhagic and lytic zones in the brain during PAM progression include the presence of erythrocytes. The phagocytosis of red blood cells has been reported in *N. fowleri* [48, 49]. In erythrocytes, the iron is stored in a ferrous oxide form in the hemoglobin. Certain bacteria, such as *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Serratia marcescens,* and *Staphylococcus aureus*, have proteases that degrade Hb [15, 50, 51]. In *S. aureus*, it was reported that, during nasal colonization, invasion depends on the Hb concentration [50]. However, in *E. histolytica*, many studies have investigated erythrophagocytosis and the vesicular process involved in erythrocyte degradation. The proteolysis of red blood cells also includes the participation of cysteine proteases [18, 30, 52, 53]. Recently, in *N. fowleri*, the activity of secretion/excretion proteases against Hb was determined [12]. However, in our study, we evaluated the activity of CM and TCEs under physiological conditions (pH 7 at 37°C) and determined the MW of these hemoglobin proteases.

It is important to mention that in the zymography assays we evaluated the type of proteases involved in the degradation of each substrate evaluated. For this, we employed a cysteine proteases inhibitor, pHMB, serine and partially cysteine protease inhibitor, PMSF, and serine inhibitor, aprotinin [54, 55]. The results showed in all the substrates that the inhibition of proteases activities was mainly with pHMB; these data suggest that *N. fowleri* cysteine proteases are involved in the degradation of iron-binding proteins. Additionally, we evaluated the effect of the conventional E-64 over *N. fowleri* cysteine proteases to discriminate the possible family or clan of proteases [55, 56]. The results with E-64 inhibitor in CM revealed a partial inhibition in the case of holo-bLf and holo-hTf but total inhibition of h-hLf and hHb. In TCE only partial inhibition was observed in holo-bLf and total inhibition for holo-hLf and holo-hTf (data not shown). The results of inhibition suggest that family C1 (cathepsin-like) or clan CA and clan CD could be involved in the iron-binding proteins degradation [56]. However, it is important to mention that this hypothesis needs to be elucidated and it is subject of another study. Finally, it is important to mention that our research was focused mainly on secretion proteases involved in the invasive process. Thus, it will be important to perform new experiments using TCEs to evaluate the intracellular pathways involved in the degradation process of iron-binding proteins. This could be correlated with the different patterns observed in the CM and TCEs.

5. Conclusion

The present results support the hypothesis that *N. fowleri* can obtain iron during the initial and final stages of PAM. Our findings indicate that the proteases involved in this process vary over a wide range of MWs, from 50 to 150 kDa. Additionally, we hypothesize that *N. fowleri* depends on iron to survive and proliferate during PAM.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Trichomonas vaginalis Cysteine Proteinases: Iron Response in Gene Expression and Proteolytic Activity

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We focus on the iron response of *Trichomonas vaginalis* to gene family products such as the cysteine proteinases (CPs) involved in virulence properties. In particular, we examined the effect of iron on the gene expression regulation and function of cathepsin L-like and asparaginyl endopeptidase-like CPs as virulence factors. We addressed some important aspects about CPs genomic organization and we offer possible explanations to the fact that only few members of this large gene family are expressed at the RNA and protein levels and the way to control their proteolytic activity. We also summarized all known iron regulations of CPs at transcriptional, posttranscriptional, and posttranslational levels along with new insights into the possible epigenetic and miRNA processes.

1. Introduction

Almost all organisms require iron as a cofactor for many biochemical activities. Iron participates in all oxidation-reduction processes: that is, DNA synthesis, cellular detoxification, and oxygen transport [1]. To maintain an optimal balance, the cell tightly controls the intracellular levels of iron through "sensor" proteins that respond to changes in iron availability by transcriptional and posttranscriptional regulatory gene expression mechanisms [2, 3]. For many protist parasites iron is an essential nutrient for their survival in the host. Some of them have high-iron requirements (50–200 μM) such as the amitochondriate protists, *Tritrichomonas*, *Trichomonas*, *Giardia*, and *Entamoeba* sp., surpassing those of the majority of eukaryotic and prokaryotic cells (0.4–4 μM) [4].

The flagellated protist parasite *Trichomonas vaginalis* infects the urogenital tract and is responsible for human trichomoniasis, the most common nonviral sexually transmitted disease that has a strong impact on human health [5]. Trichomoniasis common symptoms include vaginitis, urethritis, and prostatitis and is associated with preterm delivery, low birth weight, pneumonia, increased infant mental retardation and mortality, and predisposition to HIV/AIDS infection and cervical and prostatic cancers. It is also responsible for pneumonia, bronchitis, and oral lesions in immunocompromised patients [6, 7]. *T. vaginalis* develops a chronical infection under different urogenital microenvironments, mainly affecting women, showing that it is able to respond accordingly to the hostile environment during infection by modulating the trichomonal pathobiology as an adaptative response.

To study the genetic diversity in *T. vaginalis*, Conrad et al. [8] found 27 polymorphic markers (21 microsatellites and 6 single-copy genes) using different *T. vaginalis* isolates from diverse geographical origins. These authors demonstrated that these isolates have a high degree of diversity distributed only in four of the six chromosomes. Thus, the presence of two population types in trichomonad isolates, Type 1 and Type 2, was demonstrated worldwide. Type 1 isolates are

located predominantly in Africa and Type 2 primarily in Mexico. The rest of the world has both types. The two types of trichomonad isolates show differences in the frequency of *T. vaginalis* virus infection by a double-stranded RNA virus (TVV) and metronidazole resistance. These differences could contribute to the ability of certain isolates to preferentially colonize the male urogenital tract in comparison with those found in the vagina. In addition to these, trichomonad isolates infected with TVV show different growth rates and virulence [9]. Furthermore, the presence of TVV has important implications in the disease pathogenesis and in the expression of trichomonad cysteine proteinases (CPs) [10].

In the urogenital tract, *T. vaginalis* is exposed to unfavorable conditions such as acidic pH, temperature, presence of lactobacilli, cyclic hormonal changes, epithelium desquamation, scarce nutrients, presence of zinc, fluctuation in polyamines and iron concentrations, menstrual blood flow, and other unknown factors. Thus, the parasite requires a great adaptive capacity to survive in this adverse environment. *T. vaginalis* modulates the expression of multiple virulence factors involved in cytoadherence, cytotoxicity, phagocytosis, hemolysis, immune evasion mechanisms, and induction of host cell apoptosis among others in order to survive, obtain nutrients, and maintain a chronic infection. Most of these properties and virulence factors are differentially regulated by iron [6, 7]. The regulatory effect of some environmental factors has been previously discussed in other reviews [7, 11, 12].

In this review, we describe the recent advances regarding the influence of iron on gene expression regulation and functions of cysteine proteinases as virulence factors and their endogenous inhibitors.

2. *Trichomonas vaginalis* and Iron

T. vaginalis has high requirements of exogenous iron (250–300 μM). Iron is an essential element for its survival, metabolism, and multiplication in culture [13, 14]. Iron also regulates some of the trichomonal virulence properties by known and unknown mechanisms. *T. vaginalis* uses multiple sources of iron in the ferrous free form: lactoferrin (Lf), hemoglobin (Hb), and heme. It has multiple iron uptake systems. One of them is through a 136 kDa receptor for binding the host holo-Lf. Other receptors bind the cytochrome C or Hb and heme [7, 14–16], using the adhesins AP65 and AP51 [7, 16] as heme- and hemoglobin-binding proteins [17]. This parasite also internalizes ferritin, but not transferrin. Other important sources of iron are erythrocytes and epithelial cells. Two erythrocyte-binding proteins of 12.5 and 27.5 kDa help *T. vaginalis* to acquire iron from Hb [15].

The absence of iron in the culture medium reduces cell growth and induces morphological changes in *T. vaginalis* from ellipsoid or amoeboid to rounded parasites followed by flagella internalization and axostyle invagination by a mechanism not yet understood. These rounded and irregular parasite forms resemble the *Tritrichomonas foetus* pseudocysts, which were observed among parasites that underwent stress conditions, that is, cold and starvation [18, 19]. However, these forms are rarely observed among trichomonads grown in axenic cultures [19–21]. Thus, iron has an important role in the general physiology and morphology of *T. vaginalis*. In addition, morphological alterations are also accompanied by an extensive change in their protein profiles. In particular, *T. vaginalis* actin proteins are upregulated under iron-depleted conditions and may participate in the morphological changes just described [22]. These observations show that under different iron conditions both growth and protein synthesis are differentially regulated in trichomonads [20–22].

3. Cysteine Proteinases (CPs) in *T. vaginalis*: Classification, Structure, and Processing

3.1. Proteinases. Proteinases, also known as peptidases or proteases, hydrolyze the peptide bond in proteins and peptides. Proteinases are widely distributed and can be found in biological systems from viruses to mammals [23]. Proteinases account for monomers of 10 kDa to multimeric complexes of hundreds of kDa. These enzymes disrupt the peptide bond either within the polypeptide chain (endopeptidases) or at the amino or carboxy ends (exopeptidases). Based on the catalytic mechanism and the nature of the residue involved in hydrolysis, proteinases are classified as serine, threonine, aspartic, glutamic, metallo-, or cysteine proteases; however, other proteases with unknown mechanism also exist [24].

Cysteine proteinases (CPs) are subdivided into families according to the statistically significant sequence similarity among them and biochemical specificity to small peptide substrates. Families are grouped into larger clans (CA, CD, CE, CF, CL, CM, CN, CO, CP, CQ, and an "unassigned" clan) with a common ancestral progenitor. Members of different clans are not evolutionarily related. However, members of different families within a clan share a common ancestor [24].

This review will focus on CPs and their iron regulation at transcriptional, posttranscriptional, and posttranslational levels and role in the virulence of *T. vaginalis*. According to the draft of its genome, *T. vaginalis* has more than 400 proteinase-coding genes; whereas 220 correspond to the cysteine type, only 23 CPs have been detected by two-dimensional (2D) substrate gel electrophoresis (zymograms) [25, 26] that only correspond to nine different gene products after being identified by mass spectrometry [27]. These CPs are distributed into clans: CA, CD, CE, CF, CO, and CP [25].

Two main clans, CA and CD, are the most well known due to their high expression levels in the parasite and their identification by two-dimensional substrate gel electrophoresis and proteomics studies [26–31]. Clan CA is dubbed "papain-like" due to the high sequence homology with the *Carica papaya* proteinase. All *T. vaginalis* papain-like proteinases characterized to date belong to family C1 (cathepsin L-like). Clan CD is another important clan of CPs in *T. vaginalis*, particularly family C13 (legumain-like). All *T. vaginalis* cysteine proteinases characterized to date belong to families C1 and C13 (cathepsin L-like and legumain-like, resp.) [27–31].

3.1.1. Papain-Like CPs. The catalytic site of papain-like CPs is highly conserved and formed by three residues denominated the catalytic triad: Cys25, His159, and Asn175 (papain numbering system). In the catalytic triad the Cys and the His residues form an ion pair stabilized by a hydrogen bond with Asn. The nucleophilic thiolate cysteine attacks the carbonyl carbon of the substrate and forms a tetrahedral intermediate, which transforms into an acyl enzyme with the simultaneous release of the C-terminal portion of the substrate. A water molecule hydrolyzes the acyl enzyme and a second tetrahedral intermediate is formed and cleaves into the free enzyme and the N-terminal portion of the substrate [31, 32]. The proteinase-binding region where the catalytic triad is located has binding pockets also known as subsites for the residues either side of the scissile bond in the substrate. Proteinase subsites in the N-terminal direction are named $S_1, S_2, S_3, \ldots, S_n$, and subsites in the C-terminal direction are called $S'_1, S'_2, S'_3, \ldots, S'_n$. In the substrate or inhibitor, the corresponding amino acids that bind to the subsites are named $P_1, P_2, P_3, \ldots, P_n$ and $P'_1, P'_2, P'_3, \ldots, P'_n$, respectively (Figure 1(a)). Members of the clan CA proteinases are either targeted to intracellular vesicle compartments such as lysosomes (cathepsin B, cathepsin L, and others) or are secreted if these possess a leader peptide. Clan CA proteinases are also sensitive to the irreversible inhibitor E-64 (L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane) and have substrate specificity defined by the S_2 pocket, in particular the amino acid residue 205 (papain numbering) [23]. For example, cathepsin L-like proteinases have alanine at this position, which cannot contribute to arginine binding; whereas cathepsin B-like proteinases have peptidyl-dipeptidase activity and an acidic group at this position that can preferentially bind to arginine (among any other residue), criteria used to distinguish them [23], using small peptides as substrates (Z-Phe-Arg-AMC and Z-Arg-Arg-AMC), where Z is an N-terminal blocking group, AMC is a fluorescent leaving group after hydrolysis. Mammalian cathepsin B can hydrolyse both substrates, whereas cathepsin L is limited to Z–FR–AMC only [23].

Nine *T. vaginalis* cathepsin L-like proteinases: TvCP1, TvCP2, TvCP3, TvCP4, TvCP4-like, TvCP12, TvCP25, TvCP39 (TvCPT), and TvCP65 have been partially characterized [7, 27, 33, 34] as virulence factors. All of them have a similar structure and motifs of cathepsin L-like CPs. In the parasite, family C1 members are expressed as zymogens, consisting of at least two regions: a prodomain and a catalytic domain (Figure 1(b)). In addition, TvCP2, TvCP3, and TvCP4 also have a signal peptide sequence [25, 35, 36]. Although structurally CA proteinases of *T. vaginalis* are cathepsin L-like, they possess substrate specificity that resembles cathepsin B and hydrolyze the fluorogenic substrates Z-FR-AMC and Z-RR-AMC (K_m values of 364 μM and 160 μM, resp.) [23].

Apart from TvCP3, all cathepsin L-like CPs of *T. vaginalis* characterized up to date have six conserved cysteine residues (Cys_{22}/Cys_{63}, Cys_{56}/Cys_{95}, and Cys_{153}/Cys_{200}, papain numbering) forming three disulfide bonds and all of them possess the ERFNIN motif (EX_3RX_2[Ile/Val]$FX_2NX_3IX_3N$), a characteristic of cathepsin L-like proteinases [25, 35]. Even though none of the crystal structures of the CPs of *T. vaginalis* have been elucidated, it is plausible they resemble that of the procathepsin L because the overall tridimensional fold of cathepsin L is highly conserved. The structure consists of two domains: L and R with the cysteine residue of the active site located in a structurally conserved α-helix of the L-domain where the histidine residue is in the R-domain. The propeptide occludes and runs in the opposite direction through the substrate binding cleft, which inhibits enzyme activity by sterically preventing the substrate from accessing the active site [37]. Recently, it was reported that the recombinant propeptide of the iron upregulated TvCP4 has a native-like conformation after *in vitro* refolding that works as an exogenous CP inhibitor of the proteolytic activity of certain *T. vaginalis* CPs from clan CA [38] (Figure 2(a)).

3.1.2. Legumain-Like CPs. To date, two CPs of clan CD family C13 have been identified in *T. vaginalis*: TvLEGU-1 and TvLEGU-2 [42, 43]. Due to their similarity with legumain, a protease of legume *Canavalia ensiformis*, these CPs are called legumain-like. These CPs have a signal peptide, a propeptide, and a catalytic domain, but unlike clan CA CPs, the propeptide is located at the C-terminus of the catalytic domain [44]. For example, human legumain is synthesized as a zymogen and has various processing steps: a precursor (56 kDa), intermediate product (47 kDa), and active form (36 kDa). It is processed at an aspartic acid (D) at the N-terminal and asparagine (N) at the carboxyl terminus [45, 46] (Figure 1(c)). In legumains, the propeptide acts as a chaperone and stabilizes the catalytic domain at neutral pH. At acidic pH, the propeptide is cleaved as legumain goes through conformational rearrangements during activation [47]. The catalytic dyad in a clan CD member such as legumain-like CPs is His and Cys (Figure 1(c)) [48]. This type of proteinase possesses tightly defined substrate specificities at P1 position. CPs of family C13 of clan CD exclusively hydrolyse peptides and proteins on the carboxyl side of asparagine residues and are known as asparaginyl endopeptidases (AEPs). CPs of family C13 also show sequence similarity with glycosylphosphatidylinositol (GPI): protein transamidases. Therefore, these legumain-like proteinases can play a role in the attachment of GPI anchors to precursor proteins in the endoplasmic reticulum [23, 48]. Through an acyl transferase reaction, a peptide bond is formed between the terminal amine of the ethanolamide phosphate group of the GPI anchor and the C-terminal carbonyl group at the ω site of the protein [48]. Due to their strict substrate specificity, legumain-like proteinases are not inhibited by E-64. However, general thiol-blocking reagents such as iodoacetamide and iodoacetic acid can inhibit their proteolytic activity [23, 48]. Legumain selective inhibitors such as aza-peptide epoxides have been also discovered [49]. This characteristic together with the high immunogenicity of legumain-like CPs of *T. vaginalis* leads to proposing these CPs as prospects for drug design and as diagnostic tools [27, 43]. Recently, human and mouse legumain crystal structures have been elucidated, with an overall architecture of a central six-stranded β-sheet

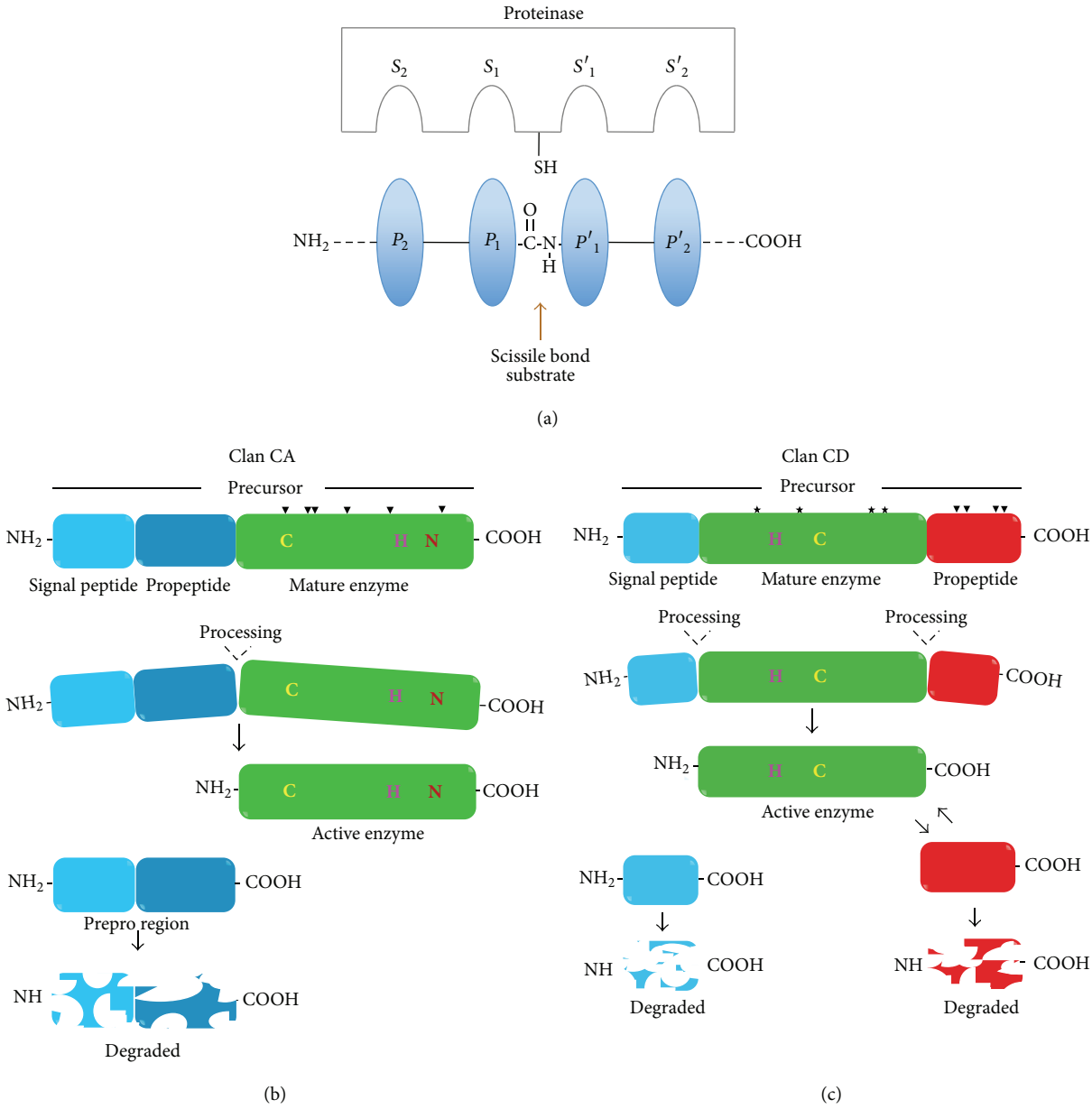

FIGURE 1: Processing involved in cysteine proteinase activation. (a) Representation of the interaction between substrate and the active sites of a cysteine proteinase. Subsites in the protease are denoted by "S" and subsites in the substrate by "P." The active cysteine sulfhydryl nucleophile is represented as –SH and the scissile bond is shown. Processing of clan CA (b) and clan CD (c) cysteine proteinases; the signal peptide for cellular trafficking (light blue), the propeptide located in the N-terminus (dark blue) of the catalytic domain (green) in clan CA and in the C-terminus (red) of the catalytic domain (green) for clan CD. The catalytic residues C (Cys), H (His), and N (Asn) are indicated. Arrowheads show conserved Cys residues that can form disulfide bonds. Asterisks show N-glycosylation sites.

[β1–β6], flanked by five major α-helices (α1–α5) [46, 50]. The theoretical 3D model of TvLEGU-1 is shown in Figure 2(b).

4. *T. vaginalis* CPs Involved in Virulence Properties Are Differentially Modulated by Iron

The pathogenesis of *T. vaginalis* is a multifactorial process and its virulence is differentially modulated by iron [51]. In this parasite iron modulates both the expression of crucial metabolic enzymes and several virulence factors such as adhesins, a cell-detaching factor, and CPs, among other molecules, directly affecting virulence properties, accordingly [6, 7, 14, 52, 53].

Some CPs are differentially modulated by iron [33, 36, 54] and play crucial roles in certain virulence properties of *T. vaginalis*, including cytoadherence [7, 43, 52, 55], cytotoxicity [7, 34, 52, 56, 57], hemolysis [58–61], complement resistance [62], immune evasion [7, 52, 63], and induction of

(a) (b) (c)

Figure 2: 3D molecular model of TvCP4 and TvLEGU-1 precursors, and endogenous inhibitor TC-2 from *T. vaginalis*. (a) 3D model of the TvCP4 precursor showing the signal peptide (dark blue), the propeptide (light blue), and the catalytic domain (green). Catalytic residues Cys112 (yellow), His251 (magenta), and N271 (orange) are shown as sticks. (b) 3D model of the TvLEGU-1 precursor showing the signal peptide (light blue), the catalytic domain (green), and the propeptide (red). Catalytic residues His119 (magenta) and Cys164 (yellow) are shown as sticks. The arrows show the processing cleavage sites: TvCP4 (N84-A85) and TvLEGU-1 (C10-D11 and N260-E261). (c) TC-2 showing the amino acids: Gln, Val, and Gly of cystatin motif as magenta sticks. 3D models were obtained by using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [39–41] and the models were visualized with the PyMOL Molecular Graphics System, Version 1.5.0.4 (Schrödinger, LLC, USA).

apoptosis in human cells [7, 52, 64–66] (Table 1). Virulence properties of *T. vaginalis* have been described in detail in recent reviews [7, 52, 67]. Moreover, trichomonad CPs are found in vaginal secretions of patients with trichomoniasis and some of them are immunogenic [7, 27, 52, 57, 68, 69]. Although the secretion pathway followed by CPs is still unknown in trichomonads, we could not discard that the presence of a signal peptide ensures the proteins to enter a secretory pathway via the endoplasmic reticulum as in any other eukaryote cell. Alderete and Provenzano [70] hypothesized that the *in vivo* synthesis of proteinases must somehow be under the control of environmental cues to modulate the number and amount of proteinases needed at any particular moment and microenvironmental condition during infection.

5. Only Few *T. vaginalis* CP-Encoding Genes Are Expressed: From the Genome to the Degradome of *T. vaginalis*

The publication of the draft of the *T. vaginalis* genome sequence was a breakthrough for research in this parasite, by providing an important platform for molecular and cellular studies. In a genome size comparison between *T. vaginalis* and other protist parasites it comes to light that this organism has one of the largest genomes with ~160 Mb spread in six haploid chromosomes [25]. The *T. vaginalis* genome sequence reveals that this parasite contains ~60,000 predicted protein-coding genes. At least ~65% of the genome sequence is repetitive and ~39 Mb corresponds to 59 repetitive families that can be classified as virus-like, transposon-like, retrotransposon-like, and other unclassified repetitive elements. Many gene families in *T. vaginalis* are represented by a high copy number. This conservative gene family expansion could facilitate the parasite adaptation to different environmental conditions. One of the largest families with ~880 genes corresponds to eukaryotic protein kinases (ePKs) and ~40 atypical protein kinases (aPKs), making it one of the largest eukaryotic kinomes known [24]. Several multigene families were found including some of the enzymes of the glycolytic pathway, cytoskeleton proteins, and Myb-like transcription factors with >400 genes [12].

T. vaginalis has ~440 peptidase-coding genes showing one of the most complex degradome described. This degradome includes proteolytic enzymes from different clans: aspartic AA (2), AD (4); cysteine, CA (185), CD (20), CE (9), CF (1), PC (C) (1), PB (C) (1), and U(-) (1); serine, SB (32), SC (36), SF (1), S- (9), and PC (S) (1); threonine, PB (T) (16), PB (T) (1); metallo-, MA (63), MC (11), ME (8), MG (13), MH (17), MK (1), and MP (7) (the number in parenthesis indicates the number of members in each clan) [25]. Half of the peptidase-coding genes (~220) are of the cysteine type (CPs), including ~48 members in family C1, which have sequences homologous to papain, and 10 members in family C13 of legumain-like CPs [25]. CPs are the major proteolytic enzymes expressed by this parasite (Figure 3).

T. vaginalis comparative transcriptomic analysis at large-scale gene expression level was performed as part of the collaborative work by several groups. It has generated an enormous collection of different expressed sequence tags (ESTs) from parasites cultured under defined conditions related to cell cycle, growth, iron depletion, restricted glucose starvation, cold, and pathogenesis. These data are available in the TrichDB genome sequence database (http://www.trichdb.org/) [25].

Table 1: Cysteine proteinases in virulence properties of *Trichomonas vaginalis*.

Proteinase	Virulence properties	Regulation by iron	Type of regulation	Ref.
CP (30 kDa)	Cytoskeleton disruption	ND	ND	[61]
TvCP4	Hemolysis	+	Posttranscriptional level	[36, 58]
TvCP12	Cytotoxicity	−	Posttranscriptional level	[7, 54, 57]
TvCP30	Cytoadherence Protein degradation	−	ND	[7, 52]
TvCP39	Cytotoxicity Igs Degradation	−	Posttranscriptional level Posttranslational level	[7, 52, 57]
TvCP62	Cytoadherence	+	ND	[52, 55]
TvCP65	Cytotoxicity	−	Transcriptional level Posttranscriptional level Posttranslational level	[7, 52, 56]
CP2, CP3, CP4, and CPT	Induction of host cell apoptosis	−	ND	[65, 66]
CP1	ND	ND	Transcriptional level	[27, 35, 71]
TvLEGU-1	Cytoadherence	+	Transcriptional level Posttranslational level	[7, 43, 52, 54]

ND: not determined; upregulated (+); downregulated (−).

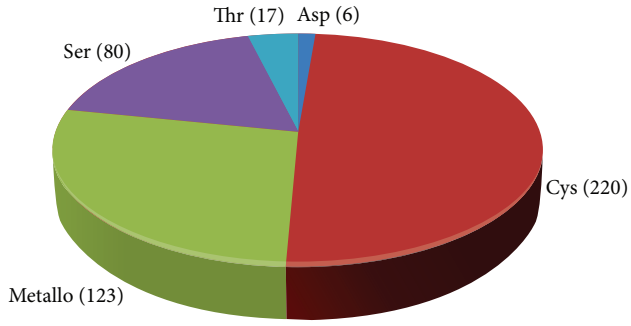

Figure 3: Classification of ~440 peptidase coding genes found in the *T. vaginalis* genome sequence database (http://www.trichdb.org/) as serine, threonine, aspartic, metallo-, or cysteine proteinase. The number of members per type of proteinases is shown in parenthesis: cysteine (220), metallo- (123), serine (80), threonine (17), and aspartic (6) [25].

Taking into account this genomic approach and in view of the different transcriptome and proteome data generated from parasites grown under different iron concentrations, we conducted a compilation of the information about cysteine proteinases belonging to cathepsin L-like (Table 2) and legumain-like CP families differentially modulated by iron (Table 3). In this review we also analyzed the EST collection together with the results of several transcriptomes and proteomes recently published [27–31, 71]. We also included in these tables other important aspects related to information in the genomic context and the known function for each CP.

The transcript levels measured based on the number of existing ESTs in the genome database showed that only few CPs are being expressed at the mRNA level (Figures 4 and 5). These findings are in agreement with the results shown in the transcriptomic data [71] and also with the proteomics and functional studies [7, 27–31, 34, 43, 52, 57, 58]. In addition, phylogenetic analysis based on protein sequences of cathepsin L-like and legumain-like CPs revealed that in each group the CPs that are highly expressed in the EST analysis are clustered into closely related clades that appear to diverge from a common ancestor (Figures 4(b) and 5(b)).

At the protein level, the majority of the expressed proteins including the proteolytic enzymes of *T. vaginalis* are acidic, as predicted by a bioinformatics approach [28, 30, 31]. This may suggest that the presence of abundant acidic proteinases in the *T. vaginalis* proteome reflects an adaptation to the acidic microenvironment of the vagina that has a reducing environment, where the iron concentration is constantly changing throughout the menstrual cycle. These reducing conditions are sufficient for activation of trichomonad proteinases [70] given that the substrate degradation by many cysteine proteinases requires breakage of disulphide bonds under reducing conditions [26, 72–74].

Some CPs are more abundant in the amoeboid than in the ovoid form, suggesting that CP profiles of *T. vaginalis* isolates exhibiting high- and low-virulence phenotypes and differences in CP expression indicate that papain-like CPs are one of the key factors in cellular damage by *T. vaginalis* [28–30, 75]. The heterogeneity in peptidase expression could suggest that *T. vaginalis* strains are constituted by two phenotypically distinct subpopulations of parasites that would express qualitatively and/or quantitatively different proteins or enzymes involved in pathogenicity [29]. This was recently confirmed by a genomic analysis by Conrad et al. [8, 9]. Comparative analysis of the proteinase patterns in different trichomonad isolates with distinct levels of cytoadherence and cytotoxicity show heterogeneity in the proteolytic activity patterns (Figure 6).

Table 2: Expression at the mRNA and protein levels of cathepsin L-like and papain-like cysteine proteinases of *Trichomonas vaginalis*.

Type of CP	ID Accession number	Name	bp	aa	Contig	Orientation	RE	EST		T[b]	Exp. reports	Proteome	Ref.
								Total	TvLI				
CB-like	**TVAG_488380** gb|XP_001323959.1 gi|123483120	NA	765	255	DS113314	3′-5′	4	0	0	NR	NR	NR	[25]
CL-like	**TVAG_485880** gb|XP_001321164.1 gi|123475979	NA	1356	452	DS113367	3′-5′	NP	10	1	NR	NR	NR	[25]
CL-like	**TVAG_484350** gb|XP_001304351.1 gi123413805	NA[a]	873	291	DS114036	5′-3′	NP	9	2	NR	NR	NR	[25]
CB-like	**TVAG_482410** gb|XP_001322190.1 gi|123478051	NA	864	288	DS113347	3′-5′	NP	0	0	NR	NR	NR	[25]
CL-like	**TVAG_46797** gb|XP_001326005.1[1] gi|123492185[1] gb|AAV98582[2] gi|56567186[2] gb|CAA54438[3] gi|454890[3]	TvCP4	915	305	DS113280	5′-3′	NP	215	19	NR	L/H	L	[7, 25, 27, 28, 35, 58, 65, 66, 71]
CL-like	**TVAG_465470** gb|XP_001328382.1 gi|123502829	NA[a]	915	305	DS113246	5′-3′	NP	0	0	NR	NR	NR	[25]
CL-like	**TVAG_461630** gb|XP_001297865.1 gi|123375585	NA	702	234	DS115136	3′-5′	NP	0	0	NR	NR	NR	[25]
CB-like	**TVAG_454200** gb|XP_001329727.1 gi|123508810	NA	759	253	DS113229	5′-3′	NP	0	0	NR	NR	NR	[25]
CL-like	**TVAG_437820** gb|XP_001310334.1 gi|123439119	NA[a]	915	305	DS113716	5′-3′	2	0	0	NR	NR	NR	[25]
CL-like	**TVAG_427120** gb|XP_001306414.1 gi|123423610	NA	405	135	DS113910	5′-3′	NP	0	0	NR	NR	NR	[25]
CL-like	**TVAG_427130** gb|XP_001306415.1 gi|123423613	NA	399	133	DS113910	5′-3′	NP	4	0	NR	NR	NR	[25]
CL-like	**TVAG_410260** gb|XP_001323249.1 gi|123480189	TvCP12	945	315	DS113327	5′-3′	NP	29	3	NR	L	NR	[25]
CL-like	**TVAG_405280** gb|XP_001325205.1 gi|123488591	NA[a]	918	306	DS113293	3′-5′	NP	0	0	NR	NR	NR	[25]
CL-like	**TVAG_398510** gb|XP_001304940.1 gi|123416650	NA	411	137	DS113996	3′-5′	3	0	0	NR	NR	NR	[25]
CL-like	**TVAG_355480** gb|XP_001310117.1 gi|123438675	TvCP4-like	915	305	DS113726	5′-3′	1	0	0	NR	NR	NR	[25, 27]
CL-like	**TVAG_328620** gb|XP_001313029.1 gi|123448602	NA	303	101	DS113614	3′-5′	NP	0	0	NR	NR	NR	[25]

Table 2: Continued.

Type of CP	ID Accession number	Name	bp	aa	Contig	Orientation	RE	EST		T[b]	Exp. reports	Proteome	Ref.
								Total	TvLI				
CL-like	**TVAG_325040** gb|XP_001330238.1 gi|123975201	NA[a]	873	291	DS113569	5′-3′	NP	5	1	NR	NR	NR	[25]
CL-like	**TVAG_302570** gb|XP_001320393.1 gi|123474420	NA	1479	493	DS113384	5′-3′	NP	3	0	NR	NR	NR	[25]
CL-like	**TVAG_298080** gb|XP_001316414.1[1] gi|123457373[1] gb|ABX56032.1[4] gi|161016200[4]	TvCPT/TvCP39	915	305	DS113482	3′-5′	1	168	8	L	L	N	[25, 27–29, 57, 65, 66]
CL-like	**TVAG_293170** gb|XP_001298580.1 gi|123381478	NA	399	133	DS114872	5′-3′	3	0	0	NR	NR	NR	[25]
CL- or K-like	**TVAG_267850** gb|XP_001579738.1 gi|154413416	NA[a]	960	320	DS113215	5′-3′	NP	0	0	NR	NR	NR	[25]
CL-like	**TVAG_257790** gb|XP_001313154.1 gi|123448858	NA	609	203	DS113609	3′-5′	NP	1	0	NR	NR	NR	[25]
CL-, S-, or H-like	**TVAG_242850** gb|XP_001311850.1 gi|123446194	NA	1419	473	DS113657	5′-3′	NP	5	1	NR	NR	NR	[25]
CL-like	**TVAG_228710** gb|XP_001580594.1 gi|154415137	NA	879	293	DS113206	5′-3′	NP	1	0	NR	NR	NR	[25]
CL-like	**TVAG_228450** gb|XP_001580568.1 gi|154415085	NA[a]	915	305	DS113206	5′-3′	1	0	0	NR	NR	NR	[25]
CL-like	**TVAG_218830** gb|XP_001300036.1 gi|123391254	NA	399	133	DS114510	3′-5′	NP	6	0	NR	NR	NR	[25]
CB-like	**TVAG_216350** gb|XP_001317882.1 gi|123469339	NA	723	241	DS113443	5′-3′	NP	0	0	NR	NR	NR	[25]
CL-like	**TVAG_202090** gb|XP_001327438.1[1] gi|123498602[1] gb|CAA54435.1[3] gi|452292[3]	CP1	927	309	DS113259	3′-5′	NP	69	12	L	L	N	[25, 27–29, 35, 65, 66, 71]
CH-like	**TVAG_181310** XP_001306268.1 GI:123422894	NA	1464	488	DS113918	5′-3′	NP	0	1	NR	NR	NR	[25]
CL-like	**TVAG_164120** gb|XP_001325526.1 giI123490067	NA[a]	915	305	DS113287	5′-3′	1	4	1	NR	NR	NR	[25]
CB-like	**TVAG_159150** gb|XP_001298125.1 gi|123377855	NA	405	135	DS115034	3′-5′	NP	0	0	NR	NR	NR	[25]
CL- or K-like	**TVAG_151460** gb|XP_001300085.1 gi|123391522	TvCP25	855	285	DS114500	5′-3′	NP	7	0	NR	NR	NR	[25, 33]

Table 2: Continued.

Type of CP	ID Accession number	Name	bp	aa	Contig	Orientation	RE	EST Total	EST TvLI	T[b]	Exp. reports	Proteome	Ref.
CL-like	**TVAG_146680** gb|XP_001579911.1 gi|154413764	NA	951	317	DS113213	5′-3′	1	2	0	NR	NR	NR	[25]
CL-like	**TVAG_135480** gb|XP_001290980.1 gi|123299807	NA[a]	792	264	DS122112	3′-5′	NP	1	1	NR	NR	NR	[25]
CL- or H-like	**TVAG_126090** gb|XP_001321671.1 gi|123477003	NA	1305	435	DS113357	5′-3′	NP	5	3	NR	NR	NR	[25]
P-like	**TVAG_096740** gb|XP_001313692.1 gi|123449986	CP65[a]	915	305	DS113590	5′-3′	NP	0	0	NR	NR	NR	[25]
P-like	**TVAG_090100** gb|XP_001314419.1[1] gi|123976011[1] gb|CAA54437.1[3] gi|452296[3]	CP3[a]	954	318	DS113554	3′-5′	NP	178	9	L	L	L	[25, 27, 28, 35, 65, 66]
P-like	**TVAG_057000** gb|XP_001319129.1[1] gi|123471864[1] gb|CAA54436.1[3] gi|452294[3]	TvCP2[a]	942	314	DS113412	3′-5′	3	198	56	L	L	N	[25, 27, 31, 35, 65, 66]
P-like	**TVAG_053440** gb|XP_001318243.1 gi|123470070	NA[a]	882	294	DS113434	5′-3′	NP	1	0	NR	NR	NR	[25]
P-like	**TVAG_052570** gb|XP_001308929.1 gi|123435098	NA	300	100	DS113779	3′-5′	NP	0	0	NR	NR	NR	[25]
P-like	**TVAG_047960** gb|XP_001330457.1 gi|123976147	NA[a]	900	300	DS113552	3′-5′	NP	2	0	NR	NR	NR	[25]
P-like	**TVAG_043620** gb|XP_001315639.1 giI123455797	NA[a]	915	305	DS113505	5′-3′	NP	0	0	NR	NR	NR	[25]
P-like	**TVAG_040870** gb|XP_001315160.1 gi|123454821	NA	1386	462	DS113520	3′-5′	NP	8	2	NR	NR	NR	[25]
P-like	**TVAG_034140** gb|XP_001318458.1 gi|123470506	NA[a]	951	317	DS113428	3′-5′	NP	20	0	NR	NR	NR	[25]
P-like	**TVAG_028720** gb|XP_001326142.1 gi|123492781	NA	723	241	DS113278	3′-5′	9	0	0	NR	NR	NR	[25]
P-like	**TVAG_003650** gb|XP_001324382.1 gi|123484966	NA[a]	930	310	DS113306	3′-5′	NP	1	0	NR	NR	NR	[25]
P-like	**TVAG_518810** gb|XP_001283092.1 gi|123194565	NA[a]	942	314	DS136773	5′-3′	NP	7	1	NR	NR	NR	[25]

CB-like: cathepsin B-like CP; CL-like: cathepsin L-like CP; P-like: papain-like CP. ID: gene identification in the TrichDB (http://www.trichdb.org/) genome database or in PubMed database; NA: nonassigned name; bp: gene size in base pairs (bp); aa: protein size in amino acids (aa); contig: contig number identification; orientation: gene orientation in the contig; RE: number of repetitive elements close to CP localized in the same contig; NP: not present; EST and TvLI: Total of EST sequence and ESTs reported in low-iron conditions in TrichDB database, respectively; NR: nonreported data; H: high-iron conditions; N: normal iron conditions: L: low-iron conditions: T: upregulated genes in H or L iron concentrations.

[a]Proteins presenting certain homology degree with TvCP4; [b]transcriptome information by Horváthová et al., 2012 [71]. [1]Accession number reported by Carlton et al., 2007 [25]; [2]accession number reported by Solano-González et al., 2007 [36]; [3]accession number reported by Mallinson et al., 1994 [35]; [4]accession number reported by Sommer et al., 2005 [65].

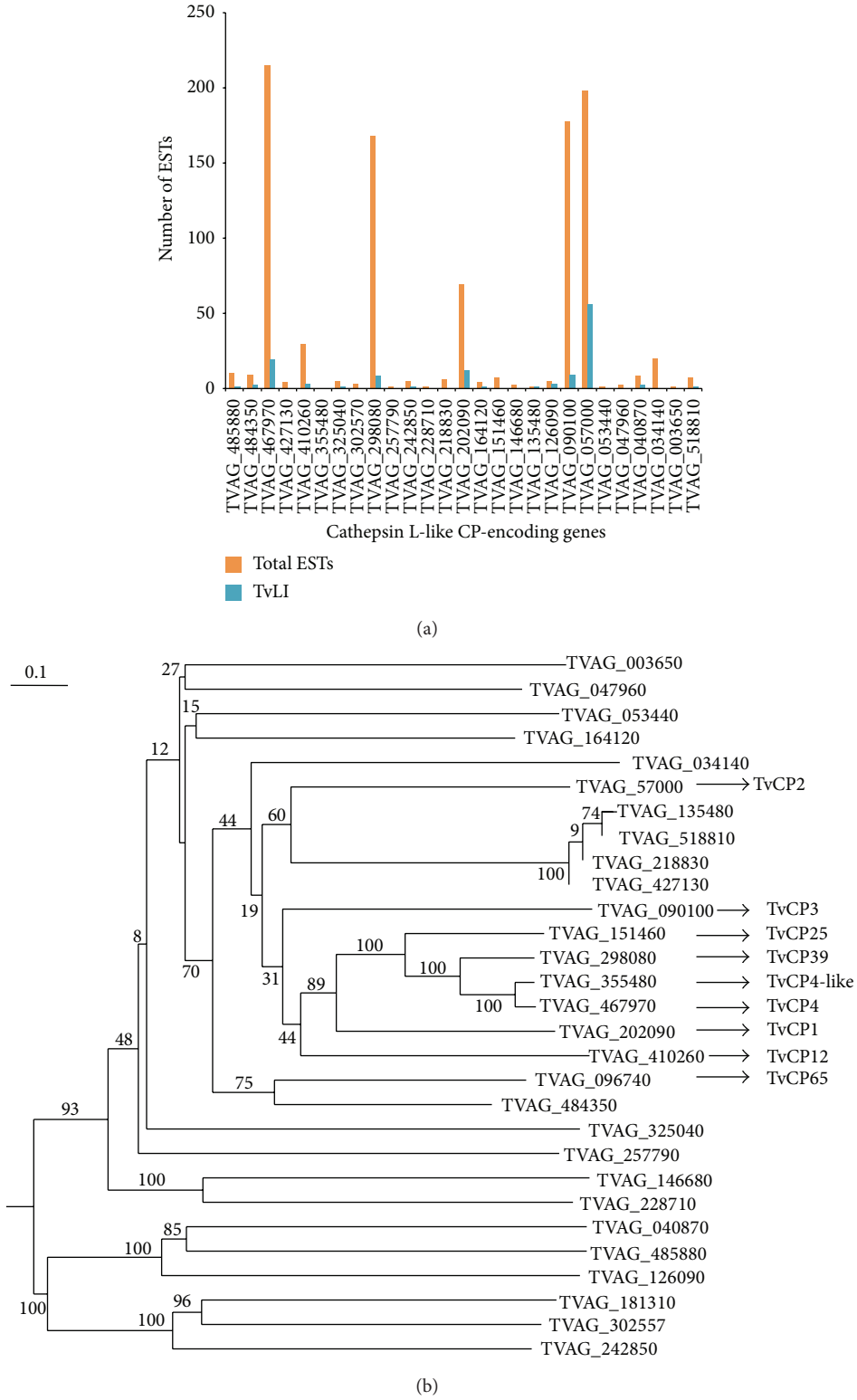

Figure 4: EST analysis and phylogenetic tree of cathepsin L-like CP-encoding genes expressed under different conditions. (a) Bar graph of the total ESTs (total EST, orange bars) found on the *T. vaginalis* genome database (http://www.trichdb.org/) compared with the number of ESTs expressed in parasites grown under iron-restricted conditions (TvLI, blue bars) (see Table 2). (b) Phylogenetic tree of expressed cathepsin L-like CPs using the DNAMAN program version 3.0 and a bootstrapping of 1000. The names of known cathepsin L-like CP-coding genes are shown.

Table 3: Expression at the mRNA and protein levels of legumain-like cysteine proteinases of *Trichomonas vaginalis*.

Type of CP	ID Accession number	Name	bp	aa	Contig	Orientation	RE	Total EST	Total TvLI	T[a]	Exp. reports	Proteome	Ref.
AEP-like	**TVAG_426660** gb|XP_001326695.1 gi|123495228[1] gb|AAQ93039.1[2] gi|39573850[2]	TvLEGU-1	1164	388	DS113270	3′-5′	NP	177	10	H	H	ID	[25, 27, 30, 42, 43, 71]
AEP-like	**TVAG_385340** gb|XP_001303267.1 gi|123408789	TvLEGU-2	1176	392	DS114117	3′-5′	1	33	1	NR	NR	N	[25, 29, 42]
AEP-like	**TVAG_328450** gb|XP_001313012.1 gi|123448568	TvLEGU-4	1176	392	DS113614	3′-5′	8	1	0	NR	NR	NR	[25]
AEP-like	**TVAG_305110** gb|XP_001299781.1 gi|123389835	TvLEGU-9	1245	415	DS114558	3′-5′	3	6	2	NR	NR	NR	[25]
AEP-like	**TVAG_277470** gb|XP_001304607.1 gi|123415014	TvLEGU-10	1140	380	DS114018	3′-5′	1	0	0	NR	NR	NR	[25]
AEP-like	**TVAG_185540** gb|XP_001584233.1 gi|154422442	TvLEGU-6	1134	378	DS113179	3′-5′	NP	4	0	NR	NR	NR	[25]
AEP-like	**TVAG_068410** gb|XP_001307303.1 gi|123427668	TvLEGU-8	1134	378	DS113861	3′-5′	NP	1	0	NR	NR	NR	[25]
AEP-like	**TVAG_060430** gb|XP_001321890.1 gi|123477445	TvLEGU-5	1179	393	DS113353	3′-5′	NP	0	0	NR	NR	NR	[25]
AEP-like	**TVAG_050390** gb|XP_001319446.1 gi|123472505	TvLEGU-3	1215	405	DS113405	5′-3′	1	13	1	NR	NR	NR	[25]
AEP-like	**TVAG_035520** gb|XP_001583515.1 gi|154421002	TvLEGU-7	1134	378	DS113183	5′-3′	1	0	0	NR	NR	NR	[25]

AEP-like: asparaginyl endopeptidase-like; ID: gene identification in TrichDB genome database [25] or in PubMed database; bp: gene size in base pairs (bp); aa: protein size in amino acids (aa); contig: contig number identification; orientation: gene orientation in the contig; RE: number of repetitive elements close to CP localized in the same contig; NP: not present RE; NR: no reported data; EST and TvLI: total of EST sequence and ESTs reported in low-iron conditions in the TrichDB database, respectively; H: high-iron conditions; T[a]: upregulated genes in H or L iron concentrations by transcriptomic analysis [71].

Moreover, in the *T. vaginalis* genome sequence, 58 genes encoding papain-like and legumain-like CPs have been found [25], but only up to 23 spots with proteolytic activity between 23 and 110 kDa and p*I* between 4.5 and 7.0 have been detected in different isolates by 2D substrate gel electrophoresis (zymogram) [26, 27].

Remarkably, all the CPs identified to date, in spite of using distinct trichomonad isolates, growth conditions, and distinct forms of sample preparation, are almost the same in all cases. The information obtained has been based on the G3 *T. vaginalis* genome sequence, showing that only few CP genes are being expressed in the different trichomonad isolates and strains analyzed [25, 26, 31, 33, 35, 42, 65, 68, 69]. Whether other CP genes have been expressed *in vivo* under other unknown environmental conditions found in the human genitourinary tract remains to be investigated.

Ramón-Luing et al. [27] showed that although *T. vaginalis* possesses an extremely complex degradome according to the genome sequence [25] only few CPs—seven cathepsin L-like CPs (TvCP1, TvCP2, TvCP3, TvCP4, TvCP4-like, TvCP12, and TvCP39) and two asparaginyl endopeptidase-like or legumain-like CPs (TvLEGU-1 and an uncharacterized AEP-like CP)—were identified in the active degradome of *T. vaginalis;* and some of these CPs have been characterized as virulence factors [7, 27, 52, 55–66].

For example, TvCP4, an iron upregulated CP, is a lysosomal and surface proteinase released *in vitro* by metabolically active parasites. It is a *T. vaginalis* virulence trait that plays a key role in hemolysis and expressed during infection. It can be considered as a potential biomarker for trichomoniasis [27, 58]. Like other genes in the *T. vaginalis* genome, *tvcp4* is a multicopy gene, and three TvCP4-like encoding genes have been reported [27, 35, 36, 65]. Although these CPs share high sequence identity (>96%), one is negatively regulated by iron and has been implicated in the induction of host cell apoptosis [65, 66], the iron upregulated TvCP4 is involved

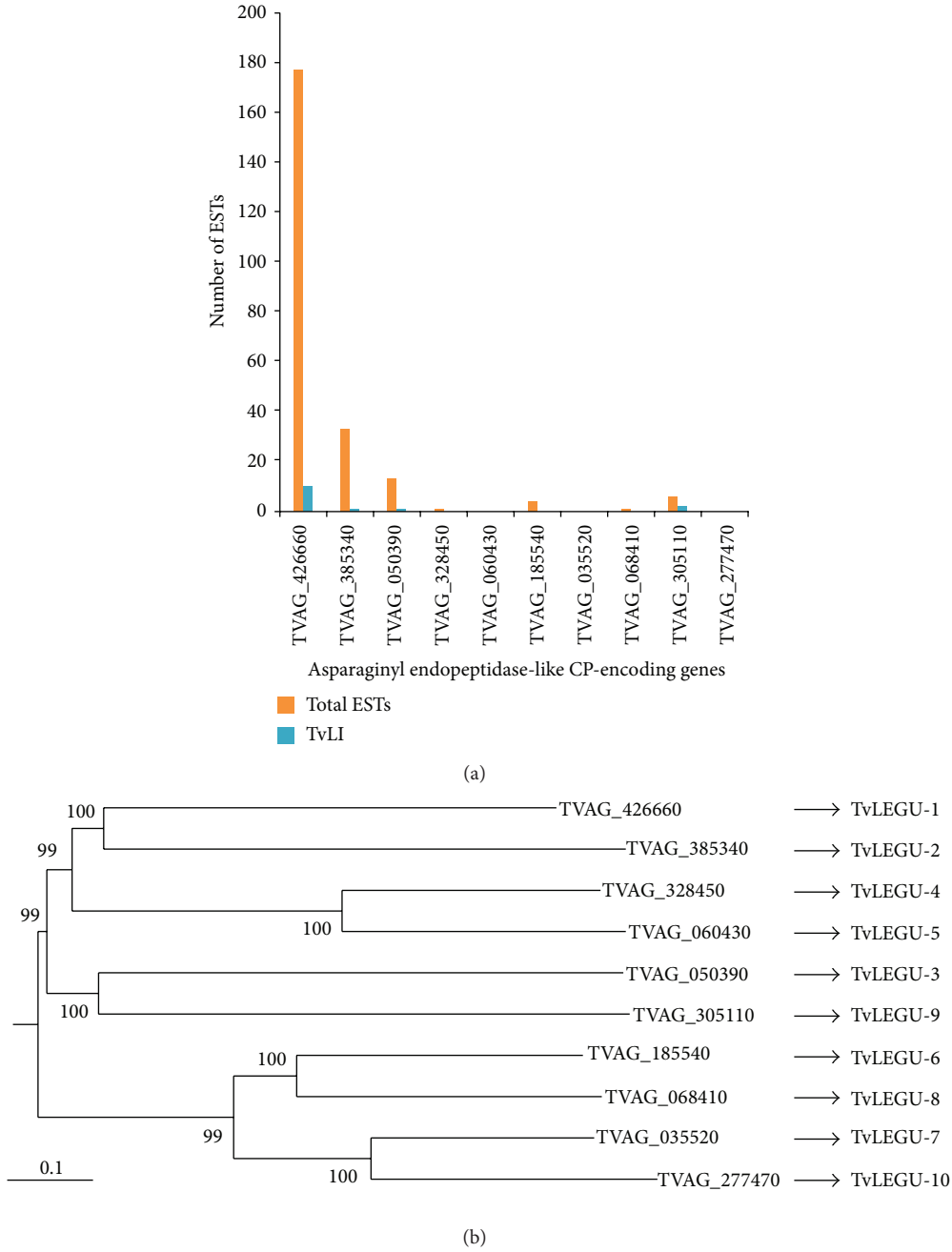

FIGURE 5: Total EST analysis and phylogenetic tree of asparaginyl endopeptidase-like (AEP-like) CP-encoding genes expressed under different conditions. (a) Bar graph of the total ESTs found on the *T. vaginalis* genome database (http://www.trichdb.org/) (Total EST, orange bars) compared with the number of ESTs expressed in parasites grown under iron-restricted conditions (TvLI, blue bars) (see Table 2). (b) Phylogenetic tree of legumain-like proteins using the DNAMAN program version 3.0 and a bootstrapping of 1000. The names of known AEP-like CP-coding genes are shown.

in hemolysis [58], and the iron regulation and function of the third TvCP4-like protein are still unknown. It appears to be a gene that is transcribed with an early stop codon, at least in the two *T. vaginalis* isolates from Mexican patients studied in our lab that may produce a smaller nonfunctional CP product (Lorenzo-Benito et al., our unpublished results) [27]. The presence of the three related *tvcp4* genes supports the hypothesis that the 48 genes coding for TvCPs belong to the cathepsin L-like group of the C1 family with genetic diversity, but with the same enzymatic active sites, conserved cysteine residues, and similar structural characteristics. In addition, these data suggest that all cathepsin L-like encoding genes in *T. vaginalis* may be the result of gene duplication and mutations derived from a single CP ancestor [76] as has also been shown in the phylogenetic analysis of expressed cathepsin L-like CPs (Figure 4(b)). Moreover, in the *T. vaginalis* degradome [27], TvCP4 was identified in five spots by 2D WB in the of 22 to 24 kDa region with different isoelectric

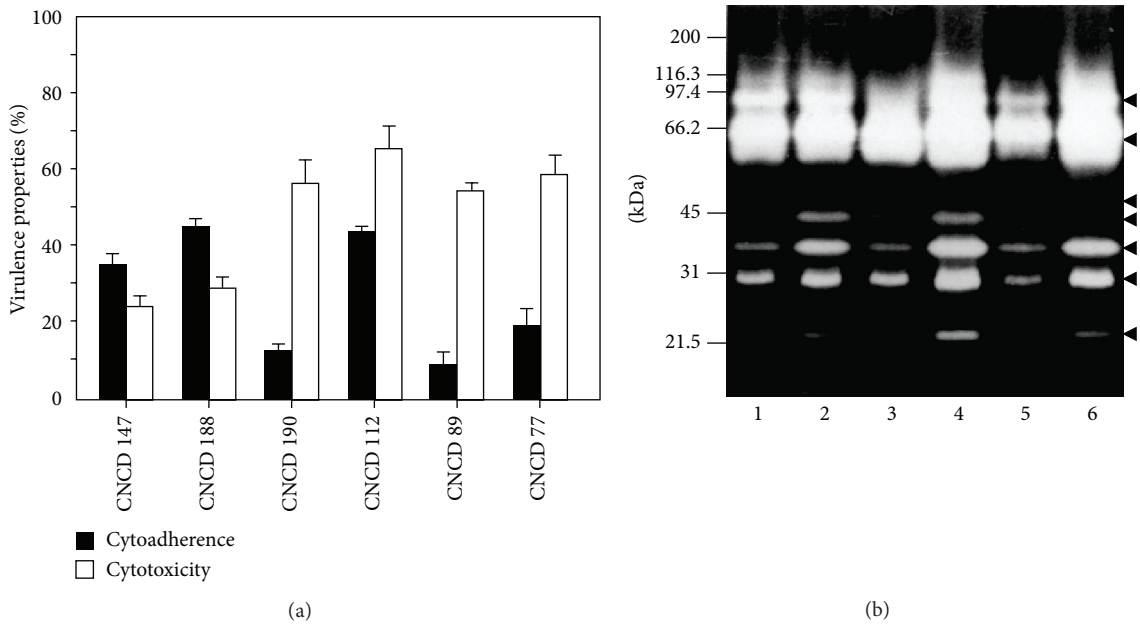

Figure 6: Cytoadherence and cytotoxicity levels in different *T. vaginalis* isolates and its differential proteolytical activity as zymogram profiles. (a) Percentage of the levels of cytoadherence and cytotoxicity of different fresh *T. vaginalis* isolates. Black bars: cytoadherence levels; white bars: cytotoxicity levels. (b) One-dimensional zymograms of protein extracts obtained from 4×10^4 parasites from different fresh *T. vaginalis* isolates: lane 1: CNCD147; lane 2: CNCD188; lane 3: CNCD190; lane 4: CNCD 112; lane 5: CNCD89; and lane 6: CNCD77. Arrowheads show the positions of proteolytic activity as clear bands against a dark background. kDa: broad range molecular weight standards, (Bio-Rad) in kilodaltons (kDa).

points [58]. In this low molecular weight region is where most of the identified *T. vaginalis* CPs of clan CA have been found [27, 29, 65].

It is noteworthy to mention the detection of CP proteolytic activity in the ~60–65 kDa region that participates in cytotoxicity as TvCP65 [7, 52]. TvCP65 is downregulated by iron [56] and zinc and requires polyamines for its expression [7, 52]. TvCP65 is active at pH and temperature found in the vagina during infection and degrades proteins of the vaginal milieu such as collagen (Coll) IV and fibronectin (Fn). It is also located at the parasite surface and is immunogenic [7, 52]. However, in the genome of *T. vaginalis,* no genes were found encoding for active ~60–65 kDa cathepsin L-like CPs [25]. Interestingly, Ramón-Luing et al. [27] by a proteomic approach identified the protein spots from the 60–65 kDa region formed as a combination of at least two low molecular weight CPs (TvCP4 and TvCP2, or TvCP4-like with TvCP2, or even TvCP4 and TvCP4-like).

Although the characterization of TvCP2 is still in progress, by Western blot the anti-TvCP2 antibody detected a 65 kDa protein spot; by indirect immunofluorescence assays, TvCP2 and TvCP4 colocalized on the parasite surface (Lorenzo-Benito et al., our unpublished results). These results are consistent with the proteomic data reported by Ramón-Luing et al. [27] and support the association between these two CPs detected by MS, forming an active high molecular weight CP that participates in cytotoxicity as TvCP65 [7, 52, 56]. We can also speculate that a protein splicing mechanism [77] unheard of in these type of microorganisms could explain the association of two low molecular weight CPs to form a new higher size active CP species with new function such as TvCP65 [7, 52, 56]. Therefore, work needs to be done to identify TvCP2 function, iron regulation, and the mechanism involved in CP-complex formation between CPs and the environmental conditions that trigger it.

TvCP39 is another proteinase of the cytotoxic surface proteinases that interacts with the surface of HeLa cells and is also downregulated by iron [34, 57] and zinc [7, 52] and requires polyamines for its expression and nuclear localization [78]. TvCP39 was identified as part of the *T. vaginalis* active degradome [27]. TvCP39 is detected as a single proteolytic spot of ~39 kDa and p*I* 4.5 in 2D substrate gel electrophoresis. It was identified by proteomic and mass spectrometry (MS) in several protein spots with different sizes (45, 37.5, 28, 27, and 24 kDa). However, this CP is encoded by a <1000 bp gene for a 34 kDa precursor cathepsin L-like CP. TvCP39 is glycosylated, degrades several extracellular matrix proteins (fibronectin, distinct types of collagen), immunoglobulin G (IgG), and IgA, and hemoglobin, is immunogenic, and can be found in vaginal secretions of patients with trichomoniasis. It has proteolytic activity at 37°C in a broad pH range, similar to the conditions found during infection in women and men [7, 34, 52, 57]. Interestingly, Sommer et al. [65] also found this peptidase as part of the secreted CPs of the 30 kDa region. It was named CPT (TvCPT) and was implicated in the induction of host cell apoptosis together with other CPs of the ~30 kDa region that are secreted by *T. vaginalis* grown under iron-restricted conditions [27, 65, 66]. The genomic sequence

helped to clarify that TvCP39 and TvCPT correspond to the same molecule that is encoded by a unique gene, *tvcp39*, with high identity to TvCP4; both were part of the secreted CPs that could cause cellular damage by inducing programmed cell death [57, 65].

Some of the CPs of the ~30 kDa region bind to the surface of HeLa cells and are necessary for cytoadherence (TvCP30) [7, 52]. This region is formed by at least six spots with proteolytic activity that corresponds to two distinct CP families: the papain-like family of clan CA, represented by four spots with p*I* between 4.5 and 5.5, and the legumain-like family of clan CD, represented by two spots with p*I* 6.3 and 6.5 [42] differentially regulated by iron at the transcript and proteolytic activity levels (Figures 7(b) and 7(c)) [54]. The family C13 of peptidases includes two distinct groups with different functions, the glycosylphosphatidylinositol (GPI): protein transamidase and the asparaginyl endopeptidase (AEP). Interestingly, TvLEGU-1 and TvLEGU-2 share ~30% amino acid identity with AEPs and ~26% with the GPI: protein transamidases [42]. We also showed that the amount of TvLEGU-1 transcript is positively regulated by iron, whereas the TvLEGU-2 mRNA is not affected by it [54] (Figure 7(a)). Of the ten legumain-like proteinases described in the *T. vaginalis* genome sequence [25], TvLEGU-1 [42] has been characterized at the functional level, playing a key role in trichomonal cytoadherence, and is located in lysosomes and Golgi complex and at the parasite surface in the presence of iron [43]. It also showed different levels of phosphorylation [43] and glycosylation (Rendón-Gandarilla et al., our unpublished results). Moreover, it is one of the most immunogenic CPs in patients with trichomoniasis and is detected in vaginal secretions during trichomonal infection [27, 43]. These data suggest that, during infection, *T. vaginalis* responds to different iron concentrations by differentially modulating the expression of several CPs [54], such as TvCP4, TvCP39, TvCP65, and TvLEGU-1 [7, 52]. Thus, it is reasonable to consider that both survival and the establishment of an infection in the host will depend on the ability of *T. vaginalis* to adapt to such environmental changes, including variations in host iron levels.

Furthermore, another important point to emphasize is some discrepancies between the theoretical and experimental p*I* and MW found in different proteins reported in proteomes, including CPs. These disparities could be explained as part of the processing steps during the maturation of the precursor CPs to remove the signal sequence and the N-terminal or C-terminal domain of cathepsin L-like or legumain-like, respectively, necessary for CPs activation [23–32, 48] as observed with the lower-size protein spots identified by MS as part of TvCP4 and TvCP39 [57, 58]. The differential posttranslational modification such as phosphorylation and glycosylation could also contribute to changes in p*I* and size as in TvLEGU-1 and TvCP39 [43, 58]. We can also speculate that the differences between experimental and predicted CP molecular masses could be due to unknown mechanisms for this parasite such as protein splicing [77]. Thus, TvCP65 is possibly formed by the combination of two lower-size CPs [27].

6. Molecular Mechanisms Involved in Gene Expression Regulation of CPs by Iron

To understand why few CPs are expressed at the mRNA and protein levels, in spite of the large number of genes encoding CPs as part of the extensive *T. vaginalis* degradome [25], it is necessary to review the possible molecular mechanisms involved in gene expression regulation at different levels. These mechanisms may include regulation at the transcriptional, posttranscriptional, and posttranslational levels or even some unexplored mechanisms that may include regulation by microRNAs (miRNA) and epigenetic mechanisms [7].

6.1. CP Regulation at the Transcriptional Level. The information about the mechanisms involved in gene expression regulation at the transcriptional level for CPs or other genes in *T. vaginalis* is limited. A stringent differential transcription regulation is suggested by the EST analysis carried on different types of genes, including housekeeping genes [25, 79].

The identification of the transcription start sites (TSS) of several trichomonad genes, including those encoding virulence factors, shows a highly conserved sequence surrounding the TSS with a consensus sequence T C A + 1 Py (T/A) that is similar to the metazoan initiator-like element (Inr, Motif 1) [80, 81] that function as an alternative core promoter element for gene transcription in some organisms. This sequence is present in ~75% of the genes in the *T. vaginalis* genome sequence. It is recognized by transcription factors associated with the RNA polymerase II (RNApol II) and is responsible for TSS selection [25, 81, 82]. The IBP39, a 39 kDa Inr-binding protein, recognizes the Inr element and binds the transcriptional factor IID (TFIID) and the RNApol II to initiate the transcription. The interaction between the IBP39 and the Inr sequence is depending on the presence of certain conserved nucleotides [83]. IBP39 has been crystallized and characterized as an Inr-binding protein [83, 84]. Moreover, at least 100 proteins with the identified Inr-binding domain and characteristics similar to IBP39 have also been found [81]. An *in silico* analysis shows that several CP genes have this specific motif in the upstream region. In many cases, CP genes have one or two Inr elements. The distal element is usually the functional one [80, 81]. For example, primer extension and 5′-RACE analyses of the *tvcp12* mRNA from parasites grown under different iron concentrations show that the distal TSS is the functional one also for this CP (Figure 8(b)) (León-Sicairos et al., 2015, under revision).

Moreover, the transcriptional regulation mediated by iron has been described only for the *ap65-1* gene (Figure 8(a)). This gene encodes the AP65 adhesin, a 65 kDa surface protein involved in cytoadherence with sequence homology to a malic enzyme [6, 7, 16]. The *ap65-1* gene contains an iron responsive promoter that includes a core promoter sequence with a single Inr and eight closely spaced regulatory elements including three Myb (a DNA-binding protein that functions as a transcription factor first identified in myeloblastosis) recognition elements (MRE): MRE-1/MRE-2r and MRE2f [85–87] (Figure 8(a)). These sequences are recognized by

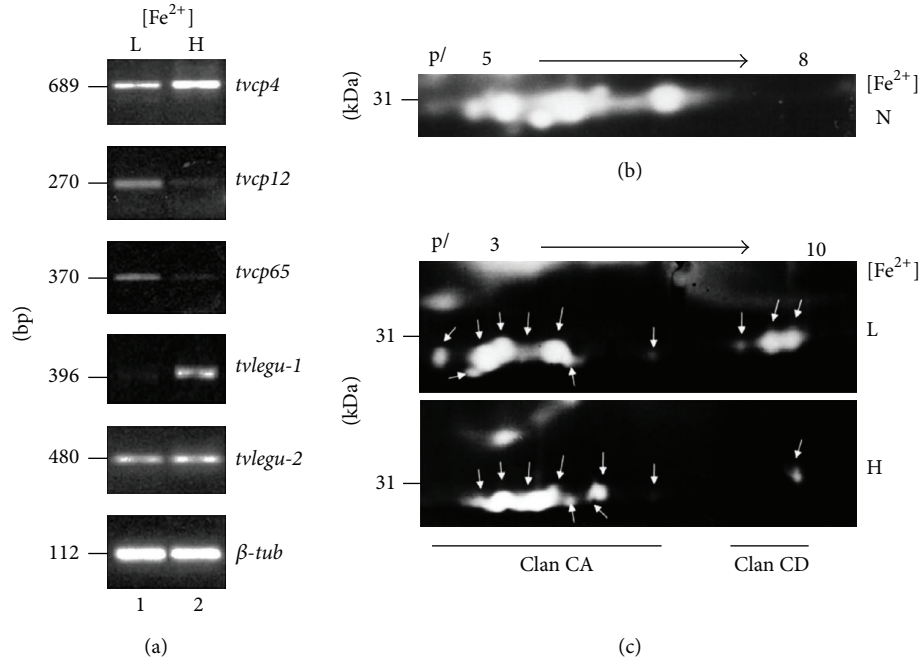

Figure 7: Effect of iron on the expression and proteolytic activity of *T. vaginalis* proteinases. (a) Semiquantitative RT-PCR using cDNA from parasites grown under iron-depleted (L) and iron-rich conditions (H) using specific primers to amplify several cathepsin L-like CP genes (*tvcp4*, *tvcp65*, and *tvcp12*) and AEP-like (*tvlegu-1* and *tvlegu-2)* CP genes. The *β-tubulin* gene was used as a loading control. (b) 2D zymograms of the 30 kDa region parasite proteinases obtained from trichomonads grown in normal iron conditions (N), (c) iron-depleted (L), and iron-rich conditions (H) separated over p*I* range 3–10. The arrows show the proteolytic spots of CPs from clan CA and clan CD that show differences depending on the iron conditions [7, 36, 41–43, 52].

three Myb-like transcription factors in *T. vaginalis*, TvMyb1, TvMyb2, and TvMyb3, and are responsible for the iron regulation of the *ap65-1* gene expression. These three proteins are responsible for the basal and iron-inducible transcription regulation through their interaction with MRE sequences. One of the most important features of this type of regulation is the Myb3 phosphorylation and nucleus translocation in response to iron concentration; please see below [88, 89].

The *T. vaginalis* genome contains ~400 Myb protein-encoding genes sharing 40–52% similarity. The three amino acids essential for DNA binding to the MRE sequences are present in all Myb-like proteins. Interestingly, by an *in silico* analysis, several MRE-like sequences have been identified in the 5′-region of genes encoding virulence factors. These MRE-like sequences can be recognized by various Myb proteins in response to iron or other physiological conditions, providing a higher plasticity in this type of regulation [81, 85–89].

Moreover, searching for the iron responsive promoter including the MRE-like elements in transcriptionally iron up- or downregulated CP genes revealed that none of them have all the regulatory elements identified in the *ap65-1* gene, but two CP genes have at least the eukaryotic MRE consensus sequence (C/T]AACG[G/T). One of these genes encodes for an unknown cathepsin L-, S-, or H-like CP TVAG_242850. Only 5 EST sequences were found for this gene; one of them is from low-iron condition library (Table 2). The other gene corresponds to the CP1 protein previously described by Mallinson et al. [35]. This protein is overexpressed under low-iron condition (Figure 8(a), Table 2). Thus, further work is required to solve whether these MRE-like motifs participate in a new transcriptional iron regulation mechanism using different Myb-like proteins, in addition to the one already described for *ap65-1* [85–89].

By an *in silico* analysis in search for alternative basal promoter sequences to the Inr motif 1 (M1), overrepresented motifs located at the 5′-region of some *T. vaginalis* genes were found and grouped into four additional motifs (M2, M3, M4, and M5). Motif 3 resembles the metazoan MRE element and is recognized by the nuclear protein M3BP, a Myb-like protein, and Motif 5 is reminiscent of the Inr element [81, 82]. Interestingly, the TvLEGU-1-coding gene that is upregulated by iron lacks the iron responsive promoter elements described for *ap65-1*. Instead, it has two putative Inr sequence and a Motif 3 (M3) (Figure 8(a)). 5′-RACE experiments using RNA from parasites grown in different iron concentrations show that none of the Inr sequences were used as transcriptional start site; instead, it was found in the M3 sequence. The EST analysis confirmed these results (Figure 9(a)) (Rendón-Gandarilla et al., our unpublished data). Therefore, we found genes that in spite of having Inr sequences used alternative motifs as promoters. More work is needed to determine whether these motifs participate in iron regulation.

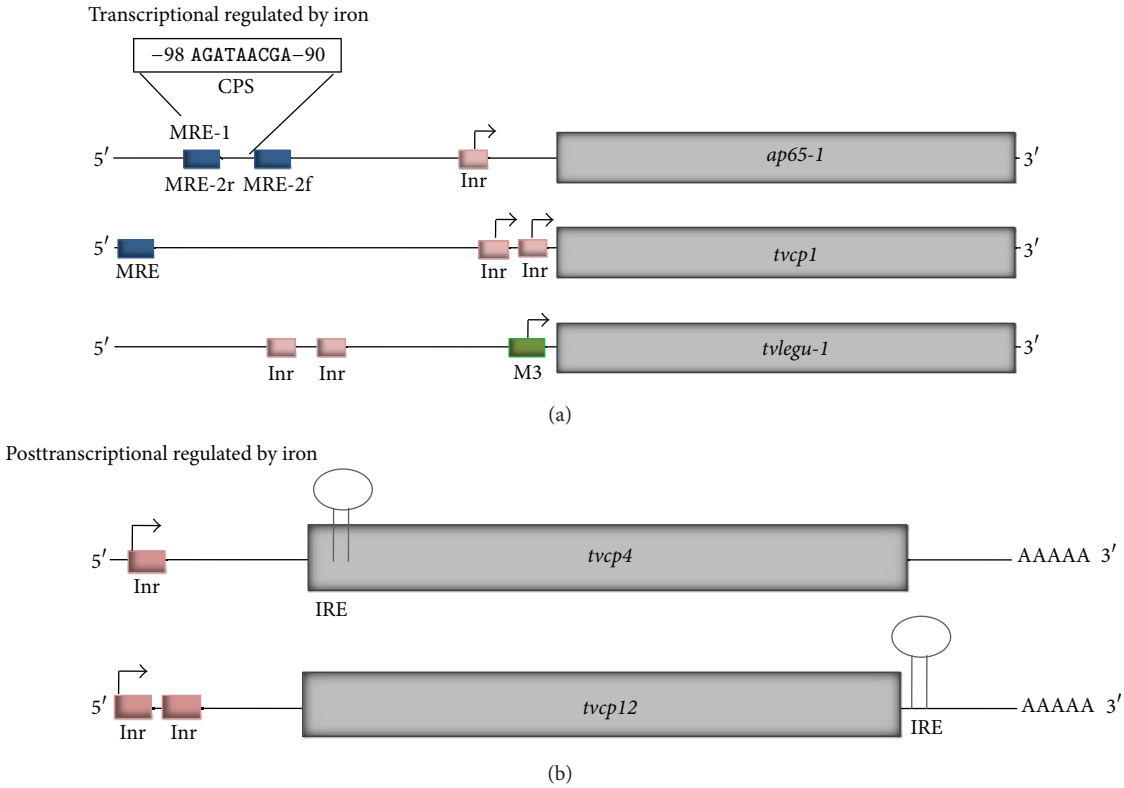

Figure 8: Genomic structure of different CP genes regulated by iron at the transcriptional or posttranscriptional level. (a) Transcriptional level. Comparison between the previously described iron inducible *ap65-1* gene promoter [85–87] and other two genes regulated by iron: the *tvcp1* gene promoter that responds to low-iron concentration. The *tvlegu-1* gene that responds to high-iron condition has a Motif 3 (M3, green box) element in the 5′-region, where the transcription start site (TSS) is also found (Figure 9). CPS: iron responsive core promoter sequence, MRE (blue boxes), Myb recognition element; Inr (pink boxes), initiator element; arrow: TSS. (b) Posttranscriptional level: comparison between two different genes with IRE hairpin loop elements located in the 5′-UTR, *tvcp4* mRNA, or in the 3′ UTR, *tvcp12* mRNA where the RNA-binding protein will bind under low-iron concentrations.

6.2. Posttranscriptional Regulation for CPs. Almost all organisms use iron as a cofactor for multiple biochemical activities. However, an excess of iron produces oxidative stress. To control intracellular iron levels and prevent its toxic effects, in vertebrates the iron homeostasis is regulated at the posttranscriptional level mediated by an IRE/IRP system. This mechanism is based on RNA-protein interactions between iron regulatory cytoplasmic proteins (IRPs) and stem-loop structures or iron responsive elements (IRE) located at the untranslated regions (UTRs) of certain iron-regulated mRNA [1, 91]. These RNA-protein interactions only occur under low-iron conditions. There are two possible scenarios depending on the location of the IRE element. (1) For genes that are upregulated by iron, that is, ferritin (FER), which is an iron-storage protein, in its mRNA, the IRE element is located at the 5′-UTR (IRE-*fer*). Under low-iron concentrations, IRP-1 and IRP-2 bind to the IRE-*fer* RNA, inhibiting its translation. In high-iron concentrations the IRP-1 is a multifunctional protein that acquires an aconitase activity instead, whereas IRP-2 is degraded; thus, the translation complex recognizes the mRNA and it is translated into the FER protein. (2) For genes that are downregulated by iron, that is, the transferrin receptor (TFR), in its mRNA, the IRE element is located at the 3′-UTR (IRE-*tfr*). Under low-iron conditions IRPs bind to the IRE-*tfr* RNA, preventing its degradation and increasing the half-life of the mRNA and the amount of translated TFR protein. In contrast, under high-iron concentrations, IRPs cannot bind to the IRE-*tfr* hairpin loops, the mRNA is degraded, and no TFR protein is synthesized [91].

Although the IRE/IRP system is a conserved iron regulatory mechanism throughout the evolution, *T. vaginalis* lacks aconitase activity and genes that encode for aconitase or IRP-like proteins. However, it has genes that are differentially regulated by iron at the posttranscriptional level such as those that encode for TvCP4 and TvCP12 CPs. The mRNA of these CPs contain a hairpin-loop structure at the 5′-UTR (*tvcp4)* or at the 3′-UTR (*tvcp12)*, respectively [36, 54] (Figure 8(b)). The RNA hairpin structures specifically bind to human IRPs [36] and to proteins present in cytoplasmic extracts from *T. vaginalis* grown under iron-restricted conditions [92]. Analysis of the *T. vaginalis* genome sequence revels that this parasite lacks genes coding for proteins with homology to the typical mammalian IRPs. Therefore, this parasite has an iron regulatory mechanism mediated by RNA-protein interactions that is parallel to the typical IRE-IRP system. The RNA-protein complexes are formed between

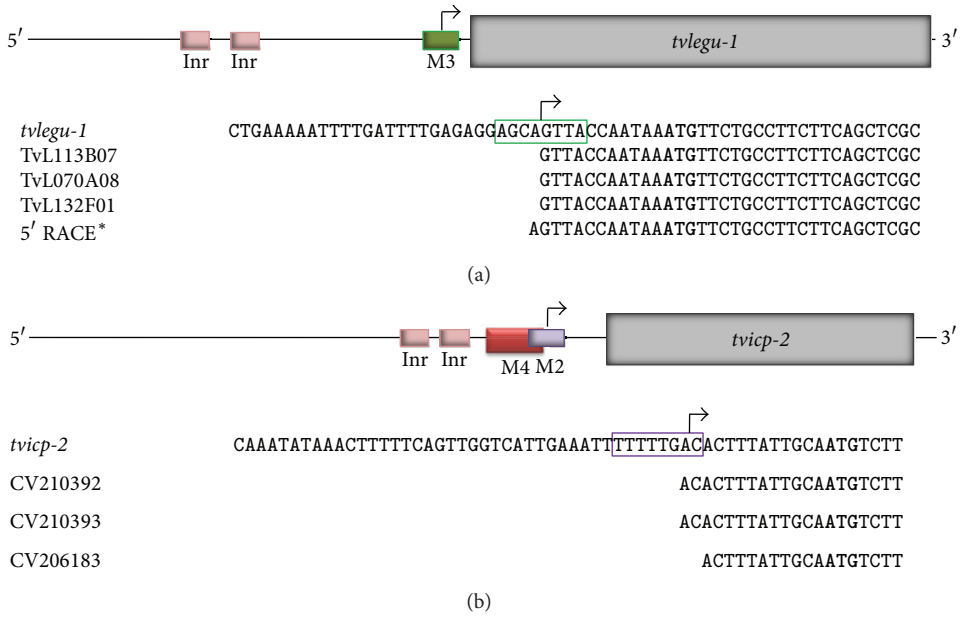

Figure 9: *tvlegu-1* and *tvicp-2* transcription start site identification. (a) Genomic organization compared with the ESTs (http://www.trichdb.org/) and 5′-RACE analysis of *tvlegu-1* mRNA to identify the possible TSS [42], Rendón-Gandarilla unpublished data. (b) Genomic organization of *tvicp-2* gene showing the putative Motif 4 (M4, red box) and Motif 2 (M2, purple box) compared with the ESTs (http://www.trichdb.org/) of *tvicp-2* mRNAs in different growth conditions to identify the possible TSS [90]. Motif 3 (M3, green box). Arrow: TSS.

atypical RNA IRE hairpin structures and multifunctional cytoplasmic proteins [54]. Recently, Calla-Choque et al. [92] reported the presence of four trichomonad cytoplasmic proteins that specifically bind to the IRE-*tvcp4* RNA. One of these proteins was identified by MS as the *T. vaginalis* α-actinin3 (TvACTN3) and characterized as an RNA-binding protein that could be involved in the iron posttranscriptional regulation in *T. vaginalis*. Functional assays demonstrate that this protein specifically interacts with the human IRE-*fer* and the trichomonad IRE-*tvcp4* RNAs [92].

6.3. CP Regulation at the Posttranslational Level by Iron. In addition to gene expression of CPs by a transcriptional or posttranscriptional regulation, other mechanisms are being studied to understand how the function of these proteins is regulated after being translated. The posttranslational regulation is frequently mediated by protein-protein interactions or protein modifications.

6.3.1. Cystatins in T. vaginalis (Trichocystatins). Trichocystatins are endogenous inhibitors of CPs in *T. vaginalis* that may participate in the posttranslational iron regulation mediated by protein-protein interactions. The best characterized inhibitors of cathepsin L are cystatins that belong to the MEROPS family I25 (clan IH) [93, 94]. Their members have structural and functional similarities and are classified into three main subfamilies: stefins, cystatins, and kininogens. The stefins (Type 1, subfamily I25A) are intracellular nonglycosylated single chain proteins (~11 kDa) and highly stable in a wide pH range [95]. The cystatins (Type 2, subfamily I25B) are extracellular proteins (~13 kDa) synthesized with a signal peptide [96], are nonglycosylated, and have two C-terminal disulfide bonds [93]. The kininogens (Type 3, subfamily I25C) are the largest CP inhibitors. They consist of an N-terminal heavy chain and a C-terminal light chain linked by a disulfide bridge with three tandemly repeated cystatin-like domains (D1, D2, and D3) [97]. All of them are potent, reversible, and competitive inhibitors acting in intracellular compartments and in the extracellular environment [98].

The principal function of cystatins is the protection of the cell from undesirable proteolysis [99]. The cystatins have been found in nematodes, platyhelminths, bacterial pathogens [100], and arthropods unlike parasitic protozoa where the cysteine protease inhibitors (ICPs) are commonly found instead [101], except for *Acanthamoeba* that has a cystatin-like inhibitor [102] involved in encystations and *T. vaginalis* that has three endogenous cystatin-like inhibitors, trichocystatins [25]. Interestingly, cystatins in parasites not only have the characteristic domains (a G domain in the N-terminal, the reactive Q × V × G domain in the central region, and the hairpin loop PW domain in the C-terminal domain) [98] necessary for inhibitory activity, but these inhibitors also perform a wide variety of specific functions as part of their biology.

In *T. vaginalis,* three genes encoding cystatin-like endogenous CP inhibitors, trichocystatins (TC-1, TC-2, and TC-3), have been identified in its genome sequence [25]. In the *T. vaginalis* active degradome the trichocystatin-2 (TC-2) inhibitor was identified by MS together with TvCP39 in a 45 kDa protein spot [90]. TC-2 belongs to the stefin subfamily of the cystatin family I25, is located in the cytoplasm and

lysosomes of the parasite, and inhibits the proteolytic activity of papain, cathepsin L, and some of the cathepsin L-like CPs of trichomonads mainly TvCP39 and TvCP65 as observed in the zymograms [90].

Trichocystatin-2 (TC-2) plays a key role in regulating the TvCP39 proteolytic activity affecting trichomonal cytotoxicity [90]. TvCP39 has been characterized as a virulence factor cytotoxic to the target cell [7, 34, 52, 57]. The gene expression regulation of this CP by iron and polyamines has been investigated [7, 34, 78]. Its regulation by the endogenous CP inhibitor TC-2 is under investigation (Puente-Rivera et al., 2015, under revision). TvCP39 and TC-2 are associated and colocalized in some cytoplasmic vesicles, possibly lysosomes, suggesting *in vivo* regulation through specific protein-protein interactions. Pretreatment of live parasites with recombinant TC-2 reduced the levels of the trichomonal cytotoxicity towards HeLa cells in a concentration-dependent manner [90]. Iron upregulates the expression of this inhibitor and its target CP at the transcript and protein levels and the complex formation with several CPs (Puente-Rivera et al., 2015, under revision). Thus, these protein-protein interactions between TC-2 and its target CPs could be one of the posttranslational regulatory mechanisms in trichomonads that may contribute to protecting the parasite from the unwanted CP proteolytic activity. However, we could not ignore the hypothesis that this CP inhibitor could also have a particular function in the host cells during the host-parasite interplay.

Trichocystatin-3, TC-3, is also being studied in *T. vaginalis* isolates from different phylogenetic groups (Type 1 and Type 2) [9]. Its expression appears to be downregulated by iron, an opposite behavior to TC-2. TC-3 expression under iron-restricted conditions is more prominent in Type 2 than in Type 1 isolates (Sánchez et al., our unpublished data).

The presence of three endogenous CP inhibitors in *T. vaginalis* and the expression of at least two of them (TC-2 and TC-3) may be another level of regulation, in addition to those described so far in *T. vaginalis*. However, we could not exclude that this parasite could use some of the mechanisms already described for other pathogens to carry out the successful parasitism to the host because some virulence properties are shared among pathogens and some of these functions are regulated by iron. The interaction of CP/cystatin can stimulate some of these functions as in *Streptococcus pyogenes* where the CP IdeS/cystatin C complex formation enhances the host IgG degradation [103]. Thus, we propose that in *T. vaginalis* iron could help in the selection of specific trichocystatin CP targets from the full range of expressed peptidases and that this protein-protein complex formation could modulate the appropriate biological effect, depending on the different locations where this interaction occurs.

6.3.2. Posttranslational Modifications (PTMs) Modulated by Iron May Help to Regulate the Specific Function of Each Trichomonad CP. PTMs play crucial roles in regulating the diverse protein-protein interactions involved in essentially every cellular process and therefore are required in every microorganism for its development. To date, PTM characterization in *T. vaginalis* has been reported for only a few proteins, for example, TveIF5a, cytoskeletal proteins, tubulin, and several virulence factors, P270, AP120, and TvCP39 [7, 52, 57, 104].

In silico analysis of several CP-encoding genes predicted distinct types of PTMs, glycosylation (O- or N-glycosylation), and phosphorylation among others that were also suggested after analysis of the proteome reference map of *T. vaginalis* [31]. Furthermore, based on these analyses, it is proposed that *T. vaginalis* has the machinery to perform both O- and N-glycosylation of proteins [105]. Protein phosphorylation is undoubtedly the most common and best studied of PTMs and *T. vaginalis* has one of the largest eukaryotic kinomes known [25, 79], suggesting that this parasite may perform protein phosphorylation reactions under different environmental conditions and through different signaling pathways. Surprisingly, *T. vaginalis* lacks PK receptors-coding genes that facilitate the transduction of extracellular signals [25].

The cytotoxic TvCP39 is N-glycosylated and is highly immunogenic [7, 27, 52, 57]. TvCP39 is the first glycosylated CP detected in *T. vaginalis*. However, we still do not know whether glycosylation is necessary for TvCP39 activation or modulates its proteolytic activity or even its interaction with the endogenous inhibitor TC-2, nor whether this is also modulated by iron and could help to explain changes in its molecular size as detected by MS.

6.4. Other Possible Mechanisms for CP Gene Regulation through Gene Silencing

6.4.1. By MicroRNAs. miRNAs are small, noncoding, double-stranded RNA found in many eukaryotic organisms that regulate different cellular process (proliferation, differentiation, apoptosis, and response to stress), modulating the mRNA translation efficiency, the mRNA degradation by binding to complementary sequences on the target mRNAs, and inducing posttranscriptional silencing. These types of RNAs because they are small interfering RNAs (siRNAs) activate the RNA interference machinery. These miRNA are transcribed by the RNApol II and processed into a 60-nucleotide precursor and exported from the nucleus to the cytoplasm by the exportin-5 and Ran-GTPase proteins. Cytosolic Dicer and Argonaute proteins process this precursor to a mature miRNA or siRNA.

In the *T. vaginalis* genome sequence there are Dicer- and Argonaute-encoding genes [25] as well as Exportin-5 and Ran-GTPase orthologues, and several miRNAs have been recently identified [106], suggesting that this parasite could employ these small RNAs transcribed from intergenic regions to regulate the expression of massively expanded gene families [25, 79]. These RNAs may play an important role in the regulation of several highly repeated gene families in the genome such as the cysteine proteinase families [76]. It is proposed that some genes that belong to multigene families could be transcribed and function as siRNAs. In these cases, the organisms could use the interference RNA machinery to modulate the expression of this type of multigene families.

So far, this type of regulation has been little explored in trichomonads and it is unknown whether iron can influence this type of regulation. However, it could be an explanation for understanding how genes encoding some CPs are expressed at low mRNA level (seven in low-iron conditions, Tables 2 and 3) and no proteins have been found yet. Recently, Woehle et al. [107] demonstrated the expression of intergenic loci including numerous transcribed pseudogenes and long noncoding RNAs that can act as regulatory RNAs too.

6.4.2. Repetitive Elements. As previously mentioned, *T. vaginalis* contains several repetitive elements in its genome. One of them is the *Tc1/mariner* transposable element (TE) superfamily (a type of DNA sequence that can change its position within the genome. It belongs to one of the most diverse and widespread class II TEs). Bradic et al. [108] investigated the abundance and distribution of a subset of 19 *Tvmar1* loci in different *T. vaginalis* isolates. This research group determined the effect of *Tvmar1* insertion on the *T. vaginalis* gene expression and found that mRNA expression positively correlates with an increase in the distance of the *Tvmar1* locus for genes that have a *Tvmar1* insertion located in the 5′-upstream region.

The *in silico* analysis of the CP genomic organization reveals the presence of mariner elements close to the 5′-region in some CP genes like the untranscribed CP (TVAG_218830) [25]. This analysis also shows that some genes that do not have reported mRNAs possess mariner elements. In addition, the other genes belonging to the same contig or located nearby are not transcribed either. Interestingly, most of these genes contain several repeated elements located at both ends of the contig. Thus, it will be very interesting to explore the other possible regulatory mechanism of gene silencing in *T. vaginalis* as selective for CP gene expression that could be related to the iron concentrations to release or maintain this blockage as Bradic et al. [108] reported.

6.4.3. Epigenetic Mechanisms. Another unexplored mechanism could be related to epigenetic factors that may control CP gene expression at the chromatin level. Chen et al. [109] demonstrate a novel DNA sequence periodicity signature of nucleosome organization in *T. vaginalis*, suggesting that nucleosomes present the right position and with regularity near to the 5′-end of transcripts. We conducted a search for potential chromatin-remodeling and histone-modifying proteins in the *T. vaginalis* genome database. We found the presence of several genes encoding histone acetylases and deacetylases. One of these genes (TVAG_319320) [25] is a member of the Sir2 family or sirtuins.

Sirtuins are NAD^+-dependent protein Nε-acetyl-lysine (AcK) deacetylases that could also have mono-ADP-ribosyltransferase activity. Although Sir2 main function is as a histone deacetylase able to downregulate the transcription of their target genes by controlling chromatin structure and function, it is also capable of deacetylating other nuclear and cytoplasmic proteins due to their multiple localizations. Sirtuins show function diversification mainly in four areas: chromatin organization, metabolic regulation, cell survival in stress conditions, and cell differentiation and development. Interestingly, a growing body of evidence suggests that, in a significant number of these new functions, the main effect of sirtuins is exerted via a direct effect on chromatin [110, 111].

The trichomonad Sir2-encoding gene (TVAG_319320) contains MRE2-r element in its 5′-region and its expression is negatively regulated by iron at the transcript level [71]. Thus, this type of trichomonad regulatory enzyme could play a key role in gene silencing of several CP genes in response to iron levels by a still unknown epigenetic regulatory mechanism. Work is in progress to explore this possibility.

7. Conclusion and Perspectives

This report shows that iron plays a key role in the general physiology, morphology, and pathogenesis of *T. vaginalis*. This cation differentially modulates growth and virulence properties such as cytoadherence, cytotoxicity, hemolysis, induction of apoptosis in the host cell, complement resistance, and immune evasion, through induction or repression of the expression of cysteine proteinases as virulence factors (Figure 10).

Interestingly, the 220 CP-coding genes are grouped into different clans and most of them belong to multigene families. Multiple CP proteolytic activities are detected in 2D zymograms but corresponded to few different cathepsin L-like and legumain-like CPs whose mRNAs were also detected in the transcriptomic and EST analyses. These CP genes appear to be highly transcribed. Some of these CPs are differentially regulated by iron at transcriptional, posttranscriptional, or posttranslational levels. Herein, we offer some explanations supporting the selectivity in gene expression of some members of this multigene family that could be related to different virulence degrees, the type of isolate, the presence of TVV, or other unknown characteristics.

Some of the possible molecular mechanisms involved in gene expression regulation mediated by iron could be through (1) *DNA-protein interactions* by an iron responsive promoter including an MRE or MRE-like motif and Myb-like proteins, (2) *RNA-protein interactions* by atypical IRE hairpin mRNA structures and atypical cytoplasmic proteins causing translational blockage and mRNA stabilization in the absence of iron as occur with *tvcp4* or *tvcp12* expression, (3) *protein-protein interactions* between trichocystatin endogenous CP inhibitors and the target CPs as in TC-2 and TvCP39 interaction to control the unwanted proteolytic activity in the parasite, (4) *posttranslational modifications* such as phosphorylation and glycosylation as in TvCP39 and TvLEGU-1 that could have an important role in CP activation and immunogenicity in the host during infection. Transcriptional blockage (5) by *Tvmar-1 repetitive elements* (presence, number, and position) and (6) by *miRNAs* that are carried on the specific mRNA degradation through the interference machinery, and (7) *an epigenetic mechanism* that could also be involved in iron regulation possibly through the differential expression of a NAD^+-dependent protein deacetylase *sir2*-encoding gene that could be expressed

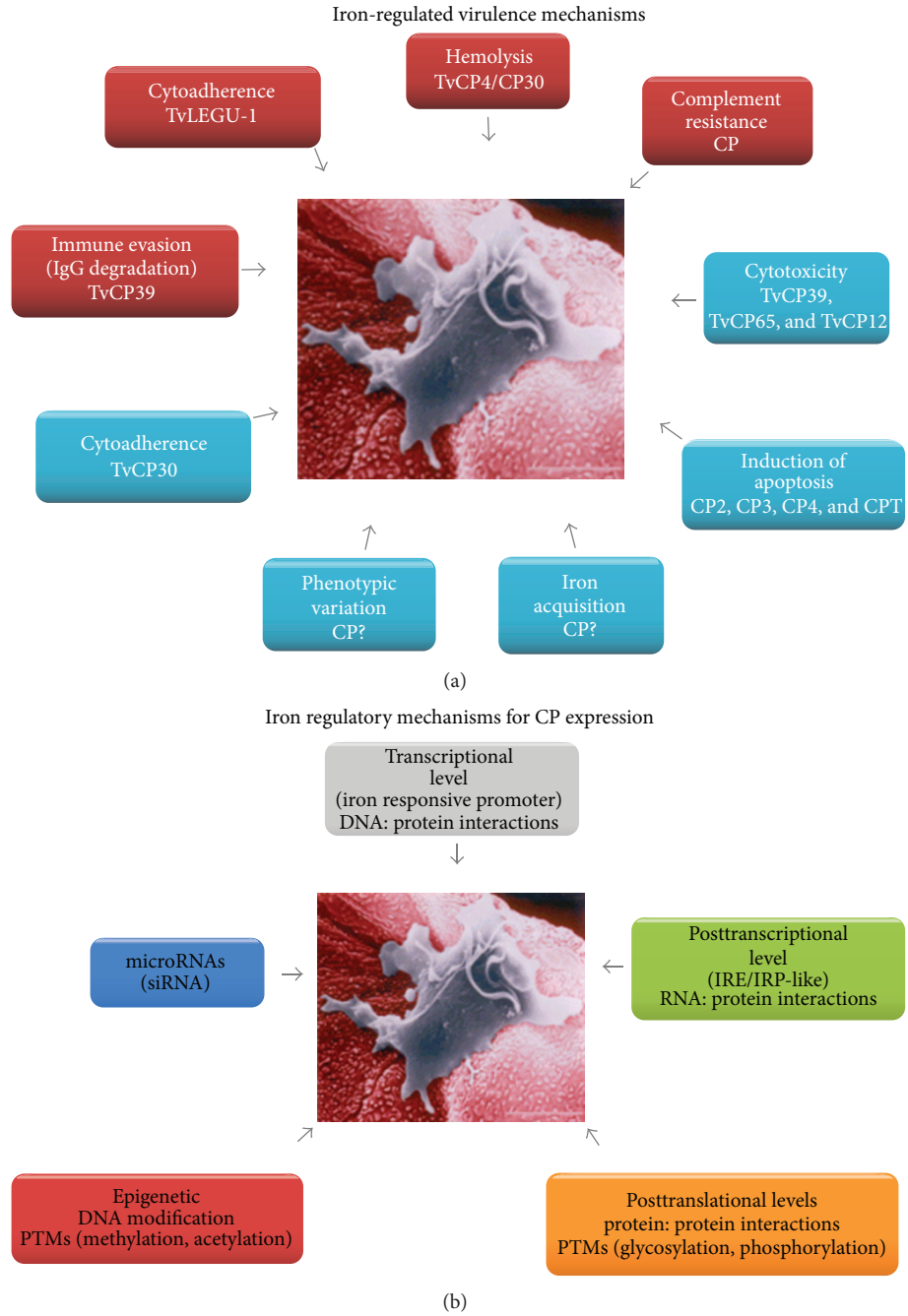

Figure 10: *T. vaginalis* virulence properties involving CPs and iron-depending mechanisms that regulate its expression. (a) Virulence mechanisms and CPs downregulated (blue) or upregulated (red) by iron. (b) Iron regulatory mechanisms implicated in CP gene expression at transcriptional, posttranscriptional, posttranslational, and epigenetic levels. Image modified from Arroyo et al. [112].

depending on the iron concentration and modulate gene expression by deacetylation of histones and other cytoplasmic regulatory proteins (Figure 10).

Thus, as shown herein, the transcriptomic and proteomic analysis in *T. vaginalis* is not enough to explain all the possible mechanisms involved in gene expression regulation of CPs mediated by iron due to the complexity observed for this early divergent protist, leaving many aspects of the parasite biology unexplained for future work to come in the following years.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Transferrin: Endocytosis and Cell Signaling in Parasitic Protozoa

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Iron is the fourth most abundant element on Earth and the most abundant metal in the human body. This element is crucial for life because almost all organisms need iron for several biological activities. This is the case with pathogenic organisms, which are at the vanguard in the battle with the human host for iron. The latest regulates Fe concentration through several iron-containing proteins, such as transferrin. The transferrin receptor transports iron to each cell that needs it and maintains it away from pathogens. Parasites have developed several strategies to obtain iron as the expression of specific transferrin receptors localized on plasma membrane, internalized through endocytosis. Signal transduction pathways related to the activation of the receptor have functional importance in proliferation. The study of transferrin receptors and other proteins with action in the signaling networks is important because these proteins could be used as therapeutic targets due to their specificity or to differences with the human counterpart. In this work, we describe proteins that participate in signal transduction processes, especially those that involve transferrin endocytosis, and we compare these processes with those found in *T. brucei*, *T. cruzi*, *Leishmania* spp., and *E. histolytica* parasites.

1. Iron

Iron (Fe) is a cofactor of a variety of proteins with important functions for almost all living organisms, prokaryotes, and eukaryotes. Fe is important for several biological processes, such as breathing, oxygen transport, the tricarboxylic acid cycle, gene regulation, and DNA synthesis [1]; however, this element presents high toxicity potential for biological macromolecules [2–6]. Therefore, maintaining cellular Fe concentration requires precise mechanisms to regulate its uptake and storage.

In a normal diet, Fe absorption is approximately 1.5 mg every day. Fe absorption is accomplished through complex mechanisms that are carried out by enterocytes in the upper part of the gut, the duodenum, and the proximal jejunum. The Fe absorbed can be nonhaem Fe or haem Fe. The Fe absorption mechanism involves several import proteins for the two ionic forms of iron, Fe^{2+} and Fe^{3+}. Haem Fe from haemoproteins is an important Fe source in omnivores; this is more easily absorbed than nonhaem Fe from vegetables and grains [3, 6].

In the enterocyte, Fe can have several destinations. The Fe destination depends on the iron pool inside the cell. Therefore, Fe can be exported from cells to the circulatory system or can be accumulated inside the cell. For Fe that is exported to the circulatory system, a protein specific for this purpose, ferroportin1, has been identified. Ferroportin1 is a multipass protein found in the basolateral membrane of enterocytes. Once exported by ferroportin1, Fe must be transformed in a process coupled by reoxidation of Fe^{2+} to Fe^{3+} by ferroxidases, such as ceruloplasmin, and followed by the loading of Fe^{3+} onto transferrin (Tf). These proteins are able to regulate iron efflux and consequently iron absorption, because overexpression of ferroportin1 is induced by cellular Fe and is suppressed by hepcidin, which inhibits Fe efflux through binding to and induction of the degradation of ferroportin1 [3, 6].

Cellular Fe metabolism is regulated by Fe itself. When Fe is at low concentrations, iron regulatory proteins 1 and 2 (IRP1 and IRP2), which are components of posttranscriptional regulation, bind to iron responsive elements (IREs) present in the untranslated regions of the mRNAs encoding TfR1, stabilizing it and increasing the number of receptors in

the membrane and Fe levels; when Fe levels are high, ferritin synthesis increases, and the receptor mRNA is destabilized, leading to low Fe entry [7–9].

In the blood, Fe bound to Tf, which is the main protein for transporting Fe in plasma, regulates Fe levels in biological fluids. Although Fe is the most essential nutrient for almost all organisms, it has a poor bioavailability and very low solubility and is not found free in nature; therefore, all organisms have invested significant efforts in obtaining Fe.

2. Transferrin

Fe exported to the serum is scavenged by Tf, a glycosylated Fe^{3+}-binding protein, which is found in blood plasma, lymph, and other body fluids and has as its primary function the transportation of Fe to all cells. Another function of Tf is to keep free Fe at a very low concentration, approximately 10^{-18} M, avoiding the high potential risk of damage and depriving pathogens of Fe, which they require for growth. Tf has an important impact in the defense against infections [10].

Tf is a single polypeptide of about 80 kDa with two homologous lobes (N- and C-terminal) connected by a short center region. Normally, only 30% of the binding sites of the protein are occupied by ferric Fe (Fe^{3+}). Tf binds one Fe^{3+} ion in each of the two lobes; the C-terminal lobe binds Fe more tightly and releases it more slowly. Iron binding requires binding of a carbonate/bicarbonate anion in a synergistic way. A complete series of reviews about Tf have been published recently in a special issue of Biochimica et Biophysica Acta (BBA), general subjects entitled Transferrins:Molecular mechanisms of iron transport and disorders 1820(3), 2012.

Serum Tf is synthesized in the liver, central nervous system, testes, ovaries, spleen, mammary glands, and kidneys. Tf is a very highly conserved protein found from bacteria to mammals, including algae [11–14]. Interestingly, Tf is absent in nematodes [15], and unfortunately there is no evidence of the presence of this protein in parasitic protozoa.

3. Transferrin Receptors from Mammalian Cells

Cells take up Fe bound to Tf using Tf receptors (TfR); thus, the biological function of the specific receptors is to bind Tf on the cell surface and ingest it. TfRs are a member of the family of tyrosine kinase-linked receptors that possess an intrinsic tyrosine kinase involved in signaling pathways.

Two TfRs have been described in mammals, TfR1 with high-affinity uptake for holoTf and currently the most studied and TfR2 that binds Tf with a 25-fold lower affinity; both TfRs are homodimeric transmembrane glycoproteins that are specific for Fe-loaded Tf (holoTf) [16]. TfR2 shares 45% amino acid sequence identity with TfR1 and plays a critical role in iron homeostasis, with a minor participation in Fe uptake [17].

At low Tf concentrations of <0.3 μmol/L, TfR1 mediates Tf internalization, but at high Tf concentrations, low affinity uptake of holoTf that is not mediated by TfR1 has been observed. TfR2 has been proposed as a receptor that participates in this low affinity uptake, but this receptor is expressed in only a few organs, and the low affinity uptake is found in more cells. Other proteins are responsible for binding and internalize Tf with low affinity, such as the proteoglycans via fluid phase endocytosis in hepatocytes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the macrophage cell surface [10, 18, 19]. It is important to note that several proteins, especially glycolytic enzymes, have been identified with multifunctional properties in both prokaryotic and eukaryotic cells. One of these new functions is to bind Tf in order to obtain Fe [20, 21].

TfR1 is a homodimer linked by two disulfide bridges with a molecular mass of 190 kDa. The receptor is formed by 3 domains: transmembrane, cytoplasmic, and extracellular, which is the larger one and contains the Tf binding site [9, 10, 22].

Tf internalized by both high and low affinity uptake receptors is transported to early endosomes as described below. Once Tf binds to the extracellular domain of the TfR on the plasma membrane, it changes conformation and dimerizes, and this change allows the activation of kinase activity, and it becomes phosphorylated. The Tf-TfR complex enters the endocytic pathway via endocytosis mediated by clathrin-coated pits. The action of dynamin is crucial for the fission of pits from the plasma membrane and the formation of coated vesicles. The Tf-TfR complex is transported to a unique endosomal compartment where acidification (pH lower than 5.6) leads to the release of ferric iron in 2-3 min [3, 23].

Successively, Tf without Fe (apoTf) bound to the receptor is transported to the plasma membrane via endocytic recycling compartments (ERC). The receptor becomes dephosphorylated, and apoTf is released outside the cell in order to bind new Fe [16, 17, 24]. All proteins involved in signal transduction depend on receptor activation produced by the binding of the ligand [25, 26].

Tf, through the TfR, acts as a growth factor. Therefore, its function is important for the regulation of embryogenesis, cell growth, motility, proliferation, differentiation, glucose metabolism, and apoptosis and is determined by its trafficking through the endosomal pathway [27].

In the same way as in mammalian cells, TfR from parasites increases the uptake of Tf, and in consequence of Fe, through the expression of a specific receptor or binding protein that is associated with the course of the infection. TfRs are very important determinants of virulence in pathogens, and depending from the environment where the infection takes place the receptor is or is not expressed. In this sense, parasites in blood vessels express TfR to bind Tf present in this environment, even more in other environments where the presence of this receptor will not be expected: like inside cells, some parasites express TfR, such as *Leishmania,* and it has been demonstrated that this parasite developed strategies to increase the presence of Tf inside the parasitophorous vacuole where *Leishmania* lives. These elaborated systems of Fe obtained by TfR expression ensure their success as parasites, host colonization, and the establishment of the infection.

4. TfR in Protozoan Parasitic Organisms

A successful infection by pathogens relies on the host colonization. Colonization depends on the availability of nutrients and growth factors, such as Fe. The relationship between parasitic organisms and their hosts is especially complex, because the hosts must obtain Fe from the diet and fulfil their own Fe needs and at the same time sequester this nutrient away from invading pathogens. Tf is the iron-containing protein that fulfils this activity, but protozoan parasites do not express Tf or Tf-like proteins that help them to acquire Fe. As these organisms are highly dependent on a plentiful supply of host Fe, they have developed mechanisms to acquire this metal by multiple and divergent pathways or steal it from host Fe deposition sites [1, 28].

These mechanisms include the secretion of specific Tf proteases, the presence of reductases that capture host Fe-containing proteins, or through specific TfR or Tf binding proteins [29]. The presence of TfR to piracy Fe must be effective enough to ensure parasites pathogenic potential and proliferation.

TfRs have been described in *Plasmodium* spp. [30, 31], *Tritrichomonas* [32], trypanosomatids such as *Trypanosoma brucei* [33], *Trypanosoma cruzi* [34] and *Leishmania* spp. [35], and the amoeba *Entamoeba histolytica* [36]. It is remarkable that these parasites express receptors that function similarly and recognize the same carrier proteins as the mammalian cell, even though some of them are structurally different and others utilise a completely different mechanism despite their similar function.

The use of specific receptors to obtain growth factors or nutrients ensures temporal prolongation of signal transduction initiated upon ligand binding at the plasma membrane and continued after internalization. In this review, the TfRs from parasites, mainly those with signal transduction studies, have been included and compared with what is known in mammalian cells.

Trypanosoma brucei. African trypanosomiasis is caused by the protozoan *Trypanosoma brucei*. This is a very important disease, because millions of people are at risk of infection and because current chemotherapies are toxic [37, 38].

The cell cycle of this protozoan consists of two general stages, one in humans and the other in the insect vector, the tsetse fly (genus *Glossina*). The initial infection is in the gut of the fly (procyclic stage). The infection travels to the salivary glands, and the parasite differentiates into the epimastigote stage and then to the metacyclic stage. This is the stage in which the parasite is injected into the mammalian host. The parasite lives within the mammalian bloodstream as its slender form, and when it is necessary, the parasite transforms into the stumpy form as a prelude to another insect infection [39]. In the bloodstream stage, this parasite is confronted by severe conditions of Fe scarcity. The sole source of iron provided by the host is available as Tf.

Therefore, bloodstream forms of *T. brucei* express a Tf receptor (*Tb*TfR) that mediates Tf endocytosis at the plasma membrane; this receptor has already been identified and is structurally completely different from the host TfR [33, 35, 40, 41]. *Tb*TfR is formed by a complex of the proteins encoded by two expression site-associated genes, ESAG6 and ESAG7. ESAG6 has a glycosylphosphatidylinositol (GPI) anchor that attaches the receptor to the plasma membrane. The binding of Tf requires the association of both ESAG proteins [42, 43]. ESAGs are cotranscribed with the gene encoding the variant surface glycoprotein (VSG) of the surface coat of the parasite. VSGs display the adaptation mechanism of antigenic variation [44]. This process allows the development of sustained infections.

Most trypanosomatid protozoa have a specific structure that allows uptake of nutrients at a specific membrane site, named the flagellar pocket. This is a cell membrane invagination from which the flagellum emerges. In this structure, endocytosis of Tf takes place [45]. The molecular mechanism for Tf internalization is through a dissimilar mechanism to that observed in mammals.

When Tf binds to the *Tb*TfR anchored to membrane by the GPI tail, it is internalized by clathrin-dependent endocytosis. The low pH of the endosome allows the release of Fe from Tf. Tf at this pH has a low affinity for the *Tb*TfR [40] and is released and transported to lysosomes for degradation by the action of the *T. brucei* cysteine-protease rhodesain or cathepsin L activity (*Tb*CATL) and *Tb*CATB (*T. brucei* cathepsin B) [33, 46]. *Tb*TfR is recycled to the cell surface to bind new Tf and the Fe associated with it [41, 47]. The main difference with mammalian cells is that the Tf is maintained attached to the receptor and transported to the extracellular medium in order to bind new Fe. However, the degradation of Tf in parasites could be for nutritional purposes.

The TfR is of great importance for parasite adaptability and for the ability to colonize several hosts. Because *Tb*TfR has a low-specificity for Tf, the parasite can use Tf from different sources providing the parasite the opportunity to increase its number of hosts, including humans and cattle [44, 48]. The use of Tf from different sources is important for the diversification of species that can be infected by parasite organisms.

Trypanosoma cruzi. *T. cruzi* is an intracellular protozoan, the causal agent of South American trypanosomiasis or Chagas disease, which infects 8 million people in Latin America [49].

Similar to the *T. brucei* parasite, *T. cruzi* infects humans and invertebrates hosts during defined stages of the life cycle. The invertebrate host is the triatomine bug that ingests trypomastigotes present in the bloodstream of an infected mammalian host when it feeds. In the gut of the vector, the parasites transform into epimastigotes and migrate to the posterior gut. Then, they transform into infective trypomastigotes, and the vector inoculates them subcutaneously into the mammalian host with infective feces. Once inside, parasites invade several kinds of cells through a lysosome-mediated mechanism, differentiating into amastigotes that replicate and transform into trypomastigotes causing host cell lysis that releases parasites into the bloodstream; the parasites are then capable of invading other cells or infecting vectors that make a meal of the host [50].

T. cruzi amastigotes growing in cell-free medium and epimastigotes require high concentrations of Fe to survive,

and curiously in these stages, they are able to obtain Fe from human Tf. Amastigotes present specific TfR in the flagellar pocket [51] that are not present in the trypomastigote form. *Tc*TfR presents structural homology with human TfR, with a 200 kDa molecular mass, and Tf is internalized by receptor-mediated endocytosis. However, in the epimastigote stage of the life cycle, the parasite ingests Tf at the cytostome/cytopharynx through a TfR [52]. This structure is a membrane invagination that is similar to the flagellar pocket, but the cytostome reaches deeply into the cytoplasm in the direction of the nucleus. The Tf receptor-mediated uptake is through small, uncoated vesicles to the reservosomes [52]. The participation of uncoated vesicles suggests that the TfR is not recycled to the membrane [34, 53].

Despite the fact that this parasite has clathrin that could participate in endocytosis [54, 55], morphological studies have demonstrated that Tf internalization is carried out through a clathrin-independent and cholesterol-dependent endocytosis pathway. This pathway was identified by the utilization of specific inhibitors of endocytic pathways. Clathrin-dependent internalization similar to that of *T. brucei* should not be excluded; the cholesterol-dependent pathway could be a secondary endocytic process, because inhibition of this pathway did not reduce cell proliferation [34, 56]. Correct Tf internalization requires the association of the cytostome with the flagellar complex in a way that is not well understood [57]. It would be interesting to know whether this route of Tf entry is constitutive or if it depends on the stage of the life cycle of the parasites, because the trypomastigote is the natural form that would confront human Tf.

Leishmania spp. The leishmaniases and fatal visceral leishmaniases are diseases with a large spectrum of clinical symptoms in mammals and are caused by at least 20 pathogenic obligate intracellular species that include *Leishmania major*, *L. infantum*, *L. braziliensis, L. mexicana*, *L. amazoniensis*, *L. tropica*, and *L. donovani*. Approximately 2 million new cases occur every year with an estimated 150 million people infected worldwide [58, 59].

The infection starts with the bite of an infected sand fly (dipteran insects) that inoculates metacyclic promastigotes (infective form) into a mammalian host. After being phagocytosed by macrophages, the parasites are found inside parasitophorous vacuoles (PVs); these acidic structures are similar to phagolysosomes and contain certain lysosomal enzymes. Inside the PVs, promastigotes transform into amastigotes. The parasites replicate and induce cell lysis; released parasites can be phagocytosed by adjacent macrophages or infect the surrounding cells. Sandflies become infected by ingesting infected cells during blood meals; amastigotes transform into promastigotes in the gut and then migrate to the proboscis for a new round of infection [60, 61].

Once the parasites are released and before the promastigotes are phagocytosed, they could be encountering Tf from the bloodstream; thus, the expression of a specific receptor would be useful. The presence of a specific and saturable TfR similar to the mammalian TfR was described in promastigotes. The TfRs of *L. infantum* (*Li*TfR) and *L. major* (*Lm*TfR) were described as an integral membrane monomeric glycoprotein of 70 kDa that is structurally different from the mammalian receptor [35]. In both developmental forms of *L. chagasi*, promastigotes and amastigotes, the binding of Tf is through nonspecific and saturable Tf binding proteins [62]. Unfortunately, the Tf endocytic process used by this parasite has not been described.

Leishmania amastigotes are usually the form internalized by the mammalian host cell, but in the case of *L. amazoniensis* promastigotes [60], they can also be internalized and then be able to survive and establish within PVs. Promastigotes and amastigotes inside the PV face conditions that include extremely restricted access to essential Fe, and *Leishmania* parasites have developed several strategies for surviving inside the mammalian host. One strategy consists of fusion of the PV with several individual vacuoles and fusion of the resultant vacuoles with compartments of the endolysosomal system. This was discovered because proteins specific for each of the vacuoles are found associated with PVs [60, 63]. In this form on the tenth day of infection, the mammalian Tf-TfR complex normally found in early and recycled endosomes is associated with the PV [64]; furthermore, Tf was found to be delivered to PV and then endocytosed by intracellular amastigotes, so it could be possible that infection time enhances the endosomal delivery to the PV [63]. The iron obtaining mechanisms could be different depending on the *Leishmania* species, because in *L. mexicana*-infected macrophages Tf was not present in the PV [64]; however, amastigotes survive in this environment suggesting the presence of an alternative iron source.

Leishmanial infection would also affect the TfR recycling regulation on macrophages [64], resulting in a Tf disorder where Tf could reach other late or lysosomal compartments and probably be transported to PVs.

Once the Tf is endocytosed by *Leishmania* intracellular parasites, it is delivered to the cysteine proteinase-rich compartments, where this protein is degraded [63].

Another strategy to obtain iron inside the acidic PV from the Tf-TfR complex could present a similar behaviour as in the endosomes; that is, the Tf loses affinity for Fe and remains attached to the receptor and iron release could be facilitated via Tf degradation by cysteine proteases secreted by living amastigotes or released by the lysis of the dead parasites [63]. Then, this element is transported by means of a parasite-associated or -secreted reductase like the Leishmanial iron transporter 1, LIT1, which plays an important role in Fe acquisition by converting Fe^{3+} into Fe^{2+} for transmembrane transport and allowing Fe to be internalized by the parasite [32, 61, 65]. This iron transporter provides enough Fe for the intracellular growth and virulence of *Leishmania*.

Entamoeba histolytica. *E. histolytica* is a parasitic protozoan of humans. It causes amoebiasis, a global disease characterized by dysentery and intestinal ulcer production. Under certain conditions, the parasite is able to invade the liver, lungs, and brain. *E. histolytica* infects 500 million people, causes disease in 50 million people, and causes death in 100,000 people each year [66].

E. histolytica has an absolute necessity of Fe. This need in the bowel can be sustained by bacteria or phagocytosed red blood cells or through endocytosis of Fe-containing proteins from the host. During invasive amoebiasis, the Fe source is Tf in the bloodstream and liver; in this organ, the use of ferritin, an Fe-storage protein, would be useful for the parasite.

The amoeba has developed two specific mechanisms for obtaining Fe from Tf [36, 67] to ensure it obtains the Fe needed for colonization and infection. One mechanism is by receptor-independent internalization which is active at high Tf concentrations (micromolar range) [68], and at low Tf concentrations between 1.1 and 5.6 nM, the internalization is through specific *Eh*Tfbps of 70, 96, and 140 kDa molecular mass as previously described [67]. Similar to mammalian cells, Tf is internalized by two mechanisms with differing affinity depending from the Tf concentration. More studies must be performed to determine the relationship between the presence of a specific receptor with low or high binding affinity for Tf and Fe necessity.

The *Eh*Tfbps identified present structural homology with the human TfR, because this is recognized with an anti-human TfR antibody. Similarly, *T. cruzi* Tf receptor is recognized with the same antibody, while *Tb*TfRs are different proteins not recognized by human TfR antibodies. *Eh*Tfbps form a complex with holoTf and are endocytosed with the participation of clathrin [67, 69, 70]. Tf is transported into the endolysosomal system (unpublished results).

The 96 kDa *Eh*Tfbp was identified as enzyme acetaldehyde/alcohol dehydrogenase-2 (*Eh*ADH2) [67]. The other *Eh*Tfbps have not been identified (70 and 140 kDa). This enzyme is essential for the growth and survival of *E. histolytica* and allows the parasite to obtain energy through glucose fermentation and to convert acetyl-CoA into ethanol. This enzyme binds extracellular matrix proteins and is found on the cell surface and in the cytoplasm [71]. This protein may be participating in binding Fe from Tf, because in the absence of Fe (apoTf), it does not bind Tf.

In this parasite, other glycolytic enzymes have been described with several functions, such as enolase, which interacts with the activity of the *Eh*meth enzyme that catalyzes DNA methylation [72].

As previously described, surface-localized GAPDH has a novel function with TfR in human and murine macrophage cell lines [19]. GAPDH is capable of interacting with Fe bound to Tf. This enzyme forms a complex with Tf and is taken to early endosomes. The same enzyme with a similar function was reported in *Staphylococcus aureus* and *Staphylococcus epidermidis*, bacteria capable of removing Fe from Tf via a receptor-mediated process [73]. Interestingly, these enzymes bind proteins from the extracellular matrix, like fibronectin and laminin, in addition to plasminogen, plasmin, lysozyme, myosin, and actin [19, 73, 74]. These proteins have been termed moonlighting or multifunctional proteins [21] due to their ability to have more than one function. Other glycolytic enzymes with multiple functions unrelated to their role in glycolysis are α-enolase, lactate dehydrogenase, and hexokinase [20].

The life cycle of protozoan parasites suggests why these organisms require an extensive network of cell surface signaling molecules. For example, *E. histolytica* has to compete with bacteria for Fe, other nutrients, and space in the intestinal microenvironment, and intra- or extracellular trypanosomatids in the mammalian host must obtain Fe and other nutrients that are present in very low concentrations. In addition, these parasites must sense several stressors to regulate the different stages of their life cycle to evade host defenses or control their invasive behaviour. Upon invasion, parasites continue to face a battery of challenges that require the ability to adhere and obtain sufficient nutrients. The survival of these parasites within their host requires a profound ability to sense and respond to environmental challenges, and utilization of an extensive signaling network may therefore be very useful.

5. Internalization Pathway and Signal Transduction Pathway

Despite the fact that there is much information about TfR signaling pathways in mammalian cells, very little information is available in protozoan parasites, despite the fact that this pathway regulates proliferation and cell growth. In describing these pathways, the emphasis will be placed on the Tf-TfR complex in mammalian cells and the way in which information travels from the cell surface to the cytosol in comparison with that observed in protozoa.

Tf trafficking of information inside the cell and initiation of several signaling pathways are very well defined in mammalian cells: (1) trafficking and insertion of membrane vesicles, (2) inositol-1,4,5-triphosphate and diacylglycerol signaling pathway, (3) MAPK signaling pathway, and (4) growth factors signaling pathway (Figure 1).

5.1. Trafficking and Insertion of Membrane Vesicles. The initial signal propagation is in the plasma membrane for endocytosis and then through the endocytic compartments [27, 75–79]. Endocytosis of the Tf-TfR complex is regulated by the concentration of phosphatidylinositol 4,5-bisphosphate (PI4,5-P_2) in the plasma membrane, which induces the recruitment of clathrin and its adaptor protein AP-2 [80, 81]. The processes of invagination and scission of the clathrin-coated pits are regulated by actin and actin-binding proteins [82–85] that increase the affinity for the dynamin 2 GTPase, Dyn2, which induces scission of the pit [84] to be posteriorly transformed into early endosomes [25, 27, 77, 78, 86].

Vesicle formation results in spatial and temporal compartmentalization that is controlled by Rab proteins, members of the small GTPase family, which are involved in transmitting signals and providing the identity of the endosome. Tf-TfR complexes accumulate in early endosomes that are specifically marked with Rab5, early endosome antigen 1 (EEA1) [25, 87], and phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3) [26, 75, 88, 89]. Later, complexes are transported to endocytic recycling compartments (ERCs), which present Rab4 [90] and Rab11 [91], where apoTf-TfR and other recycling proteins are concentrated. ERCs are concentrated in close proximity to the nucleus and around the microtubule-organizing center [92, 93].

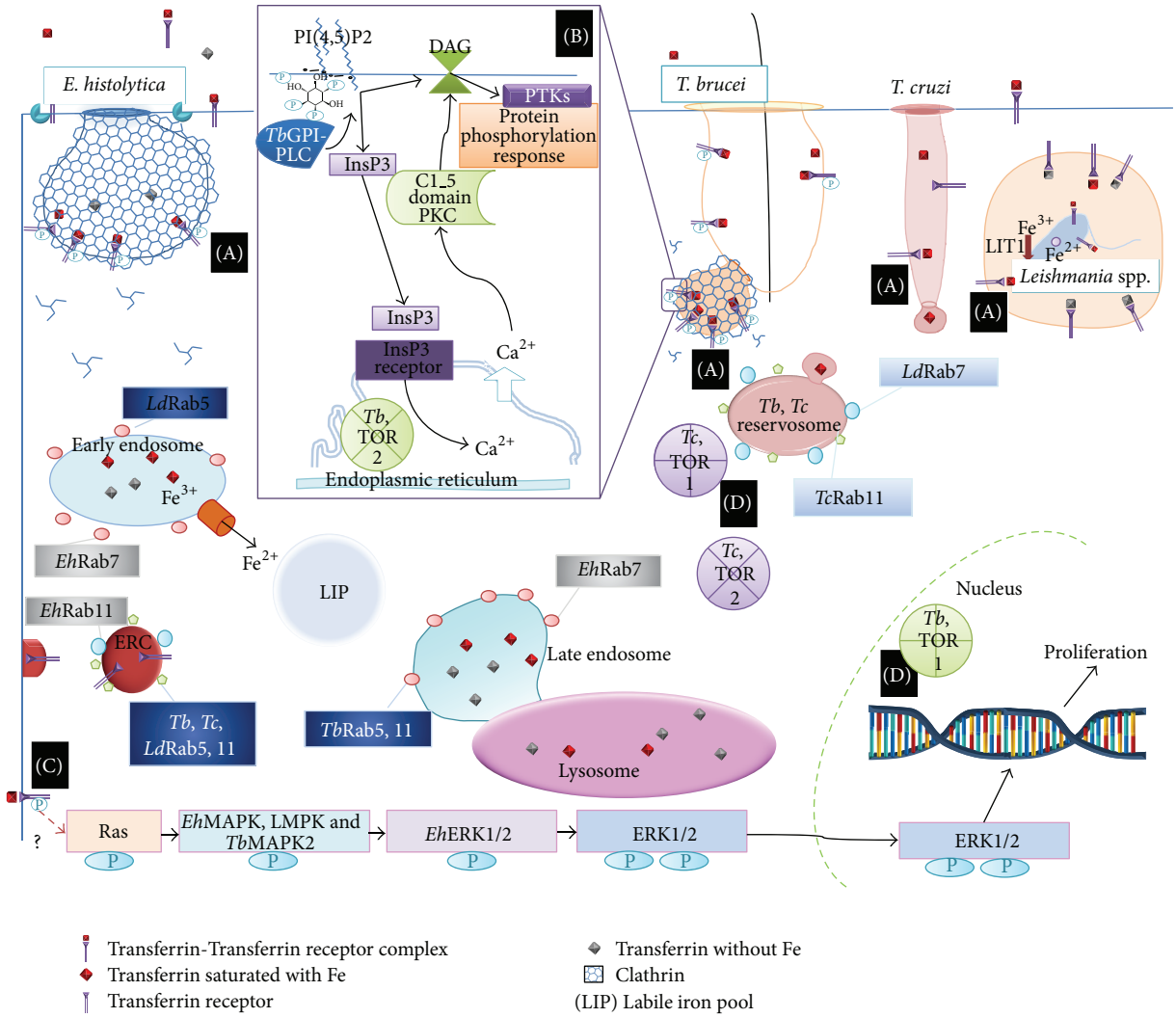

FIGURE 1: Transferrin endocytosis and signaling pathways in protozoan parasites. (A) Trafficking and insertion of membrane vesicles. The Tf-TfR complex is endocytosed in clathrin coated vesicles in *T. brucei* and *E. histolytica* but in noncoated vesicles in *T. cruzi*. The monomeric G proteins, Rabs, play a role in controlling the trafficking and insertion of new vesicles into endosomes or with endocytic recycling endosomes (ERCs) that recycle the receptor in *T. brucei* and *Leishmania*; in the case of *T. cruzi*, the receptor is not recycled back to the membrane. (B) Inositol-1,4,5-triphosphate and diacylglycerol signaling pathway. In *T. brucei* and *Leishmania*, TfR activation stimulates the formation of InsP3 and DAG through the action of GPI-PLC. Insp3 produces Ca^{2+} release from the endoplasmic reticulum to stimulate cell proliferation. Ca^{2+} in the cytoplasm binds to calmodulin (CaM) and translocates into the nucleus. DAG activates PKC, which then phosphorylates proteins that generate a specific response. (C) MAPK signaling pathway. TfR activated by Tf binding results in phosphorylation of MAPK, which has a central role in cell proliferation, and phosphorylation of the ERK1/2 kinases, which then translocate into the nucleus to activate transcription factors. These types of kinases are described in *E. histolytica*, *T. brucei,* and *Leishmania*. (D) Growth factor signaling pathway through TOR. Active PI3K takes information to TOR complexes that regulate protein synthesis by phosphorylation. TOR kinase functions are well conserved in eukaryotes with some differences in cellular localization in *T. brucei* and *T. cruzi*.

Actin regulators [92] and other proteins that function in membrane tubulation and fission [94], together with microtubules, are involved in endosome and ERC transportation [95, 96]. Also, specific vesicle-associated membranes (SNARE) proteins that mediate vesicle fusion [97] are important for binding membranes from different vesicles.

In *T. brucei*, the Tf-*Tb*TfR complex is endocytosed in clathrin-coated vesicles [45], and its adaptor protein, *Tb*EpsinR, instead of AP-2 from mammals, promotes clathrin assembly. Additionally, in trypanosomes the endocytosis and scission of the clathrin-coated pits are independent of dynamin [98] (Figure 1, (A)).

Endocytosis in unicellular parasitic protozoa is regulated by the Rab family of proteins. In *T. cruzi,* Tf is transported to reservosomes, structures that are similar to late endosomes, which present *Tc*RAb11 [54]. In *T. brucei,* the *Tb*TfR is recycled back to the flagellar pocket [99] in a recycling system that involves two isoforms of Rab5 (*Tb*Rab5A and *Tb*Rab5B), and *Tb*Rab11 [100]. A similar process occurs in *T. cruzi* and *Leishmania*, where Rab5, identified based on its homology

with *Tb*Rab5 [101] and Rab11, may be participating in the recycling of receptors [102], but it is not known in which kind of endosomes they are present exactly. Tf is transported to lysosomes for degradation. Interestingly, in the procyclic stage of *T. brucei* in the invertebrate host, the two Rab5 isoforms occupy the same compartment and have similar effects but in the fluid-phase endocytosis. The differences in endocytic regulation between the two stages of the parasite life cycle show the different mechanisms for surviving in two different hosts: insects and mammals [103, 104].

In the parasite *E. histolytica*, *Eh*Rab11A and *Eh*Rab11B may be participating in the recycling of receptors, because these Rab11s are observed in cells during Fe starvation conditions and in the beginning of the encystation process [105–108]; in addition, these *Eh*Rab11s participate in secretion of cysteine proteases [109].

In *Leishmania*, Rab7 protein promotes fusion with the late endosome during trafficking [110], but *Ld*Rab7 is present in Golgi cisternae, and *E. histolytica Eh*Rab7A is found in endosomes [111]. In the latter protozoan, a genetic screen established the presence of more than 100 Rabs [112], 75% of which are unique to the genus and called RabX, such as *Eh*RabX3 [113], for which the crystallization and preliminary X-ray diffraction analysis were performed. Despite the differences observed, Rab-mediated vesicular trafficking is a well-conserved process in parasitic protozoa.

5.2. Inositol-1,4,5-Triphosphate and Diacylglycerol Signaling Pathway. The inositol-1,4,5-triphosphate and diacylglycerol signaling pathway is another important signaling pathway activated by Tf internalization. In this pathway, Ca^{2+} signaling plays a key role in controlling the process of cell proliferation. Tf bound to TfR, a type of tyrosine kinase-linked receptor, stimulates the formation of inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) through the hydrolysis of phosphatidylinositol-4,5-diphosphate (PI-4,5-P2) by phospholipase C (PLC). The released DAG has an important role activating protein kinase C (PKC) and the $InsP_3$ diffuses into the cytosol to activate $InsP_3$ receptors to release Ca^{2+} stored in the endoplasmic reticulum. The $InsP_3/Ca^{2+}$ signaling system controls many different cellular processes, such as proliferation [114–116].

This kind of signaling pathway has been described only in *T. brucei* and *Leishmania*. In these parasites, Tf internalization is specifically regulated by glycosylphosphatidylinositol-phospholipase C (GPI-PLC). This enzyme is expressed in the bloodstream form of *T. brucei*. During transformation to the insect stage, GPI-PLC contributes to the release of VSG from the plasma membrane. A new function of the enzyme has been described as a signaling protein that stimulates endocytosis. Similar to that observed in mammalian cells, the products of the enzyme activity are DAG and inositolphosphoglycans (IPG). DAG regulation of Tf internalization depends on proteins with specific domains to act as DAG receptors with protein tyrosine kinase (PTK) and ubiquitin ligase domains [117]. Through the PTK domain, Tf endocytosis is regulated by phosphorylation of the components of the endocytic machinery, such as clathrin, actin, or SNARE proteins. In these organisms, phosphorylation depends on PTKs rather than the Ser/Thr kinases (PKCs) present in vertebrates [117, 118] (Figure 1, (B)).

5.3. MAPK Signaling Pathway. Early endosomes and ERCs function as structures for protein assembly in this signaling pathway. The classical example of a protein phosphorylation cascade, highly conserved in eukaryotic organisms, is the mitogen-activated protein kinase (MAPK) pathway that consists of activation of tyrosine kinase-linked receptors, resulting in the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) that then translocate into the nucleus. This pathway often begins with Ras, another member of the small GTPase family, and its function is the control of many cellular processes, particularly those related to cell proliferation.

Variability in the levels of expression or activity of MAPKs has been correlated with the proliferation, development, or cell cycle progression of many protozoan parasites. Along these lines, *T. brucei* and *Leishmania* MAPKs have been described: mitogen-activated protein kinase (LMPK) of *L. mexicana* [119] and *Tb*MAPK2 of *T. brucei* [39]. In the case of *E. histolytica*, two components of the MAPK signaling pathway have been identified in the *E. histolytica* genome [120]. MAPK belongs to the extracellular signal-regulated kinase (ERK) family, so it may conserve its biological role in regulating the response to the environment for cell proliferation [121] (Figure 1, (C)).

5.4. Growth Factors Signaling Pathway. This signaling pathway operates through phosphatidylinositol 3-kinase (PI3K) or PI4K and PI-related kinases with some functions in regulating cell proliferation, such as apoptosis, mitosis, cytokinesis, membrane trafficking, and cytoskeletal organization. The most important component, PI3K, generates the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI3P) that in turn activates both phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB) that translocate into the nucleus. PI3K confers the mobility needed for clathrin-coated membranes through the microtubule motor machinery [116, 122].

In this sense, PI3K contributes to sending information to the target of rapamycin (TOR) signaling pathway, named because it is inhibited by the drug rapamycin, which is a potent inhibitor of cell proliferation. TOR is a serine/threonine protein kinase, which operates as a nutrient-sensitive cell cycle checkpoint controlling protein synthesis. The activity of TOR is switched off and cell proliferation ceases under conditions of low concentrations of amino acids or when energy is limiting. This kinase is organized into two complexes, TOR1 and TOR2.

A genetic screen of the *T. brucei*, *T. cruzi*, *Leishmania major*, *L. braziliensis*, *L. infantum*, and *E. histolytica* genomes established the presence of several PIKs and PI3Ks, so they have been proposed as a novel signaling pathway [58, 112].

In the kinase TOR, the functions are well conserved in eukaryotes with some differences in cellular localization. The presence of the TOR1 and TOR2 complexes in *T. brucei* and *T. cruzi* was described, and cellular localization was determined

in order to define the function, because the localization of signaling molecules is related to their function and specificity. In *T. brucei*, *Tb*TOR1 was observed inside the nucleus and *Tb*TOR2 was associated with the endoplasmic reticulum and mitochondria. In *T. cruzi*, *Tc*TOR1 was absent from the nucleus and was observed close to reservosomes, and *Tc*TOR2 was found dispersed in the cytosol around *Tc*TOR1. These differences in localization suggest a new function of the TOR complex as a result of the high genome plasticity observed in *T. cruzi* originating from different events of intragenic recombination [123]. Different localization could suggest new functions of TOR complexes (Figure 1, (D)).

Several PI3Ks [124, 125], 307 putative PK [120], or hybrid kinases [126], more than 43 putative tyrosine kinases-linked receptors [120], and transmembrane kinases receptors (TMKs) that mediate responses to environment and immune evasion [127, 128] were identified in *E. histolytica*. Unfortunately, the role of these proteins in the endocytosis of Tf and signaling pathways has not been studied.

Further studies are necessary to comprehend the role of these proteins in order to understand the Fe acquisition system of Tf and Fe metabolism in these important parasites.

6. Conclusion

The importance of effective Fe uptake has been demonstrated for virulence in several pathogens, and although substantial progress has been made, there is surprisingly little information available about the signal transduction pathways induced by Tf endocytosis in order to obtain Fe. Although the broad picture suggests similarities with the mammalian host, there are many gaps in our understanding of these processes. The identification of signaling proteins will be useful to identify new factors that are essential for parasite adaptation to the host environment.

To date, it has been difficult to compare signal transduction processes in the studied organisms, but it is possible that they are very similar, and as was observed, this similitude may be conserved in intra- and extracellular parasites, despite the fact that they confront Fe absence in different ways.

The differences observed between several proteins and their equivalents in mammals could be used as therapeutic targets that may help treat diseases produced by these parasites, which has implications for biomedical research to develop new chemotherapeutic strategies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Strategies of Intracellular Pathogens for Obtaining Iron from the Environment

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Most microorganisms are destroyed by the host tissues through processes that usually involve phagocytosis and lysosomal disruption. However, some organisms, called intracellular pathogens, are capable of avoiding destruction by growing inside macrophages or other cells. During infection with intracellular pathogenic microorganisms, the element iron is required by both the host cell and the pathogen that inhabits the host cell. This minireview focuses on how intracellular pathogens use multiple strategies to obtain nutritional iron from the intracellular environment in order to use this element for replication. Additionally, the implications of these mechanisms for iron acquisition in the pathogen-host relationship are discussed.

1. Introduction

Intracellular pathogens are organisms that are capable of growing and reproducing inside host cells. These pathogens can be divided into facultative intracellular parasites and obligate intracellular parasites [1]. Intracellular microorganisms are very important because they cause many human diseases, resulting in significant morbidity and mortality. Some examples of infectious diseases of global importance that are caused by intracellular microorganisms include tuberculosis, leprosy, typhoid, listeriosis, Legionnaire's disease, malaria, leishmaniasis, Chagas' disease, and toxoplasmosis. The course of infection is frequently long lasting and eventually results in chronic disease [2–4]. Facultative intracellular parasites, for example, bacteria such as *Francisella tularensis*, *Listeria monocytogenes*, *Salmonella typhi*, *Mycobacterium* spp., and *Neisseria meningitidis*, are capable of living and reproducing either inside or outside host cells. Obligate intracellular parasites cannot reproduce outside their host cell, which means that the parasite's reproduction is entirely reliant on intracellular resources. Obligate intracellular parasites that infect humans include all viruses; certain bacteria such as *Chlamydia* and *Rickettsia*; certain protozoa such as *Trypanosoma* spp., *Plasmodium*, and *Toxoplasma*; and fungi such as *Pneumocystis jirovecii* [3]. Facultative intracellular bacteria invade host cells when they can gain a selective advantage in the host. Bacteria that can enter and survive within eukaryotic cells are shielded from humoral antibodies and can be eliminated only by a cellular immune response [5]. Moreover, once inside host cells, bacteria must utilize specialized mechanisms to protect themselves from the harsh environment of the lysosomal enzymes encountered within the cells. Some examples include the bacterium *Legionella pneumophila*, which prefers the intracellular environment of macrophages for growth so it induces its own uptake and blocks lysosomal fusion by an undefined mechanism [6];

Rickettsia, which destroys the phagosomal membranes (with which the lysosomes fuse); and *Salmonella* and *Mycobacterium* spp., which are resistant to intracellular killing by phagocytic and other cells [2]. Other facultative intracellular bacteria include enteroinvasive *Escherichia coli*, *Listeria monocytogenes, Neisseria* spp., and *Shigella* spp. [2, 7].

Obligate intracellular bacteria cannot live outside the host cell. Chlamydial cells are unable to carry out energy metabolism and lack many biosynthetic pathways and therefore are entirely dependent on the host cell to supply them with ATP (adenosine triphosphate) and other intermediate molecules [8]. Obligate intracellular bacteria cannot be grown in artificial media (agar plates/broths) in laboratories but require viable eukaryotic host cells (e.g., cell culture, embryonated eggs, and susceptible animals). Additional obligate intracellular bacteria include *Coxiella burnetii*, *Rickettsia* spp., and others [8, 9].

Microbial access to host nutrients is a fundamental aspect of infectious diseases. Pathogens face complex dynamic nutritional host microenvironments that change with increasing inflammation and local hypoxia. Because the host can actively limit microbial access to its nutrient supply, pathogens have evolved various metabolic adaptations to successfully exploit available host nutrients to facilitate their own proliferation [10]. Iron (Fe) is a key global regulator of cellular metabolism, which makes Fe acquisition a focal point of the biology of pathogen systems. In the host environment, the success or failure of Fe uptake processes impacts the outcome of pathogenesis [11]. After phagocytosis by macrophages, intracellular bacteria are located in a membrane-bound vacuole (phagosome), but the ensuing trafficking of this vacuole and subsequent bacterial survival strategies vary considerably. If the ingested bacteria have no intracellular survival mechanisms, the bacteria-containing phagosomes fuse with the lysosomal compartment, and bacteria are digested within 15–30 min. For this reason, the majority of intracellular bacteria and other parasites must keep host cells alive as long as possible while they are reproducing and growing [7, 9]. To grow, intracellular pathogens need nutrients such as the iron, that might be scarce in the cell, because this is usually retained or stored by proteins.

Pathogens that infect macrophages require Fe for growth, but, during infection, Fe is required by both the host cell and the pathogen that inhabits the host cell [12]. Macrophages require Fe as a cofactor for the execution of important antimicrobial effector mechanisms, including the NADPH- (nicotinamide adenine dinucleotide phosphate-oxidase-) dependent oxidative burst and the production of nitrogen radicals catalyzed by the inducible nitric oxide synthase [13]. On the other hand, intracellular bacteria such as *Legionella pneumophila*, *Coxiella burnetii*, *Salmonella typhimurium*, and *Mycobacterium tuberculosis* have an obligate requirement for Fe to support their growth and survival inside host cells [14]. In fact, it has been documented that deprivation of Fe *in vivo* and *in vitro* severely reduces the pathogenicity of *M. tuberculosis*, *C. burnetii*, *L. pneumophila*, and *S. typhimurium* [13–15].

2. Iron in the Human Host

Iron (Fe) is essential for the growth of all organisms. The human body contains 3–5 g of Fe distributed throughout the body in the protein hemoglobin, tissues, muscles, bone marrow, blood proteins, enzymes, ferritin, hemosiderin, and transport in plasma. Iron (approximately 75%) is contained in the protein hemoglobin (Hb) and in other iron-bound proteins that are important for cellular processes, and whatever remains in plasma (approximately 25%) is bound to plasma proteins such as transferrin (Tf) [16].

Dietary Fe has two main forms: heme and nonheme. Plants and iron-fortified foods contain nonheme Fe only, whereas meat, seafood, and poultry contain both heme and nonheme iron. Heme iron, which is formed when Fe combines with protoporphyrin IX, contributes about 10% to 15% of total Fe intakes in western populations [17]. Intestinal absorption is the primary mechanism regulating Fe concentrations in the body. Once ingested, Fe absorption occurs predominantly in the duodenum and upper jejunum. The mechanism of iron transport from the gut into the blood stream remains unknown. The first step of the pathway of iron absorption in the human host involves reduction of ferric Fe^{3+} to Fe^{2+} in the intestinal lumen by reductases or cytochrome b and transport of Fe^{2+} across the duodenal epithelium by the apical transporter DMT1 (divalent metal transporter). In nonintestinal cells most Fe uptake occurs via either the classical clathrin-coated pathway utilizing transferrin receptors or the poorly defined transferrin receptor independent pathway. Tf is the principal Fe storage protein that stores and releases Fe inside cells that express the transferrin receptor (TfR). The delivery of Fe from Tf is mediated by an acidic pH 5.5 of the endocytic vesicles carrying holo-Tf and TfR complexes. Fe is then transported across the endosomal membrane and utilized. Excess intracellular Fe is sequestered into the protein Ft [18, 19].

In a healthy individual Fe is largely intracellular, sequestered within Ft or as a cofactor of heme complexed to Hb within erythrocytes. Any extracellular free Fe is rapidly bound by circulating Tf. Hb or heme that is released as a result of natural erythrocyte lysis is captured by haptoglobin and hemopexin, respectively. Taken together, these factors ensure that vertebrate tissue is virtually devoid of free iron [21]. Maintaining cellular Fe content requires precise mechanisms for regulating its uptake, storage, and export. The iron response elements or iron-responsive elements (IRP1 and IRP2) are the principal regulators of cellular Fe homeostasis in vertebrates. IRPs are cytosolic proteins that bind to Fe-responsive elements (IREs) in the $5'$ or $3'$ untranslated regions of mRNAs encoding proteins involved in Fe uptake (TfR1, DMT1), sequestration (H-ferritin subunit (FTH1) and L-ferritin subunit (FTL)), and export (ferroportin). When cells are Fe deficient, IRPs bind to $5'$ IREs in ferritin and ferroportin mRNAs with high affinity to repress translation and to $3'$ IREs in TfR1 mRNA to block its degradation (Tf is involved in the transport or Fe). When Fe is in excess, IRPs do not bind to IREs, increasing synthesis of Ft and ferroportin (proteins involved in the storage of Fe), while promoting the degradation of TfR1 mRNA. The coordinated regulation of

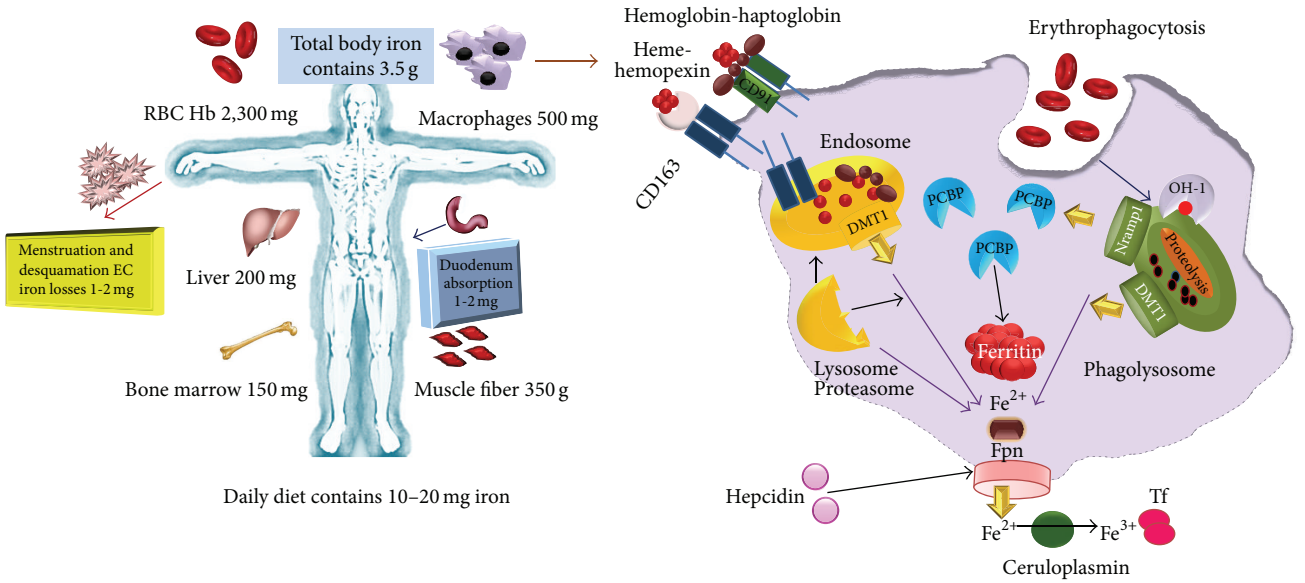

Figure 1: Iron content in the human body and iron-containing proteins in a macrophage. The average male adult contains approximately 3.5 g of iron. Approximately 2 g of iron is in hemoglobin: 1 g in body is stored predominantly in the liver and the rest in myoglobin and other iron-containing proteins. Approximately 1 to 2 mg of iron is lost each day by epithelial shedding in the gastrointestinal tract and the skin and through blood loss in menstruating women. Western diets contain a much greater amount of iron (10 to 20 mg) than what is absorbed daily under normal circumstances (1 to 2 mg). The macrophage is a key agent in iron homeostasis as well as in inflammatory hypoferremia. Macrophages in the spleen and in the liver (Kupffer cells) and perhaps elsewhere recognize damaged or senescent erythrocytes, phagocytize them, and digest them to extract heme and eventually iron. Macrophages can also scavenge heme and hemoglobin, usually complexed with hemopexin and haptoglobin, respectively, and endocytosed by CD163 and CD91, respectively. Whether phagocytosed in erythrocytes or endocytosed by scavenging, hemoglobin undergoes proteolysis to release heme. Heme is degraded by HO-1 to release iron, which is exported to the cytoplasm by DMT1 and probably also by Nramp1. Cytoplasmic chaperones family deliver iron for storage in the protein ferritin. Alternatively, iron from endosomes or phagolysosomes may also be delivered by an unknown carrier to ferroportin (Fpn) for export [20].

Fe uptake, storage, and export by the IRPs ensures that cells acquire adequate Fe for their needs without reaching toxic levels [22].

The ability of pathogens to obtain Fe from Tf, Lf, Ft, Hb, and other iron-containing proteins of their host is central to whether they live or die [14]. This is because these proteins are the main Fe sources for intracellular pathogens in the macrophage. Iron homeostasis in the macrophage is determined by uptake processes through Lf, Tf, DMT-1, and phagocytosis of senescent erythrocytes as well as by export through ferroportin (Fpn), as we have discussed before. Inside infected macrophages, a pathogen's access to Fe may be limited by natural resistance-associated macrophage protein 1 (SLC11A1, formerly Nramp1). SLC11A1 is a divalent metal transporter, recruited to the late endosomal and phagosomal membrane of macrophages and other professional phagocytes. Although SLC11A1 contributes to macrophages' efficiency in the recycling of erythrocyte-derived Fe, the main function of SLC11A1 seems to be the protection against microbes [20]. Its gene is present in inbred strains of mice in two allelic forms that determine the resistance or susceptibility to several intracellular pathogens such as *Mycobacterium* spp., *Salmonella* spp., and *Leishmania* spp. [23]. Some groups of researchers have suggested that Fe is transported via this protein into the pathogen-containing phagosome, causing the death of the pathogen by catalyzing the formation of reactive oxygen species (ROS), while others argue for Fe efflux from the phagosome, restricting pathogenic growth by Fe deprivation [23, 24]. Another Fe transporter that is expressed in macrophages is Fpn. This transporter is present in the macrophage cytoplasmic membrane and is responsible for Fe export. Overexpression of Fpn has been reported to inhibit the intramacrophagic growth of *M. tuberculosis* and *Salmonella enterica*, presumably through Fe deprivation. The details of this mechanism are unclear [25, 26]. A scheme of Fe sources in the human body and iron homeostasis inside the macrophage is shown in Figure 1.

3. Mechanisms Used by Intracellular Pathogens for Obtaining Iron: A General Point of View

During infection, pathogens are capable of altering the battlefield to increase the abundance of potential Fe sources. For example, bacterial cytotoxins damage host cells, leading to the release of Ft, while hemolytic toxins from bacteria can lyse erythrocytes, liberating Hb. The resulting inflammatory response includes the release of Lf from secondary granules contained with polymorphonuclear leukocytes (PMNs) [10, 21, 27]. Pathogens are capable of exploiting these diverse Fe sources through the elaboration of a variety of Fe acquisition systems. In the case of extracellular pathogens, they

can acquire Fe through receptor-mediated recognition of Tf, Lf, hemopexin, hemoglobin, or hemoglobin-haptoglobin complexes [19, 27]. Alternatively, secreted siderophores can remove Fe from Tf, Lf, or Ft, whereupon siderophore-iron complexes are recognized by cognate receptors at the bacterial surface. Siderophores are small ferric iron chelators that bind with extremely high affinity (iron formation constants K_d range from 10−20 to 10−50 M), some of which can extract iron from Tf and Lf [21]. Analogously, secreted hemophores can remove heme from Hb or hemopexin and deliver heme to bacterial cells through binding with hemophore receptors. Siderophore mediated Fe acquisition is inhibited by the innate immune protein siderocalin, which binds siderophores and prevents receptor recognition. This host defense is circumvented through the production of stealth siderophores that are modified in such a way as to prevent siderocalin binding [21, 27].

For proper use of Fe, extracellular or intracellular parasites must possess at least the following systems: (a) Fe sensors for monitoring Fe concentration in the intracellular environment, (b) synthesis and release of high-affinity compounds that can compete with host Fe binding proteins for Fe acquisition and storage, or proteases to degrade these host Fe binding proteins, (c) transportation of these Fe-loaded molecules and their assimilation, and (d) regulation of the expression of proteins involved in iron metabolism, in order to maintain iron homoeostasis [27, 28]. Once ingested by macrophages, many intracellular parasites are taken up by phagosomes through endocytosis. Thus, the success of intracellular parasites seems to be related mainly to their ability to take up Fe from the proteins Tf, Hb, hemoglobin-haptoglobin, free heme, and Ft. Figure 2 shows intracellular parasites and Fe sources inside a macrophage.

In order to take the Fe from Tf, these systems can be divided into three main categories: siderophore-based systems, heme acquisition systems, and transferrin/lactoferrin receptors.

Upon removing Fe from host proteins, iron-loaded siderophores are bound by cognate receptors expressed at the bacterial surface. The siderophore-iron complex is then internalized into the bacterium and the Fe is released for use as a nutrient source [21]. Heme acquisition systems typically involve surface receptors that recognize either heme or heme bound to hemoproteins such as hemoglobin or hemopexin. Heme is then removed from hemoproteins and transported through the envelope of bacteria into the cytoplasm. Once inside the cytoplasm, the iron is released from heme through the action of heme oxygenases or reverse ferrochelatase activity. Bacterial pathogens can also elaborate secreted heme-scavenging molecules that remove heme from host hemoproteins. These molecules, known as hemophores, are functionally analogous to siderophores but are proteins that target heme, whereas siderophores are small molecules that target iron atoms [29]. In addition to acquiring Fe from Tf and Lf through siderophore-based mechanisms, some pathogens are capable of direct recognition of these host proteins through receptors [21]. These receptors are modeled to recognize Tf or Lf, leading to Fe removal and subsequent transport into the bacterial cytoplasm. Additionally, acidification of the phagosome permits Fe release from Tf and probably Lf and, in this way, some pathogens can gain access to this element directly [19, 21, 30].

The following sections summarize the Fe acquisition systems used by some intracellular pathogens. Table 1 shows Fe sources, mechanism of uptake, transport and regulation, used by intracellular parasites.

4. Mechanism of Intracellular Pathogens for Obtaining Iron from Host Sources

4.1. Francisella tularensis. *F. tularensis*, the bacterial cause of tularemia, is a virulent intracellular pathogen that can replicate in multiple cell types. Acidification of the phagosome and acquisition of Fe is essential for growth of *F. tularensis* [31]. An acidic pH promotes the release of Fe from host cell Tf. To acquire the Fe from Tf, *F. tularensis* involves a receptor for this protein (Transferrin receptor 1, TfR1), induction of ferrireductases, an iron membrane transporter (DMT-1), and iron regulatory proteins (IRP1 and IRP2); this is an active Fe acquisition system associated with a sustained increase of the labile Fe pool inside the macrophage [31]. In addition, *F. tularensis* uses high-affinity transportation of ferrous Fe across the outer membrane via the proteins FupA and FslE. FsIe appears to be involved in siderophore-mediated ferric Fe uptake, whereas FupA facilitates high-affinity ferrous Fe uptake [32]. It has been hypothesized that *F. tularensis* uses the Fe from Lf to sustain its growth; however, the mechanism of Fe acquisition from LF remains undetermined [33]. It is most likely that *F. tularensis* can infect many types of cells because it contains several strategies for Fe acquisition. It has been reported that the expression of certain *F. tularensis* virulence genes is clearly regulated by Fe availability [34].

The expression of TfR1 is critical for the intracellular proliferation of *Francisella*. This contrasts with infection of macrophages by *Salmonella typhimurium*, which does not require expression of TfR1 for successful intracellular survival. Macrophages infected with *Salmonella* lack significant induction of DMT-1, Steap3, and IRP1 and maintain their labile Fe pool at normal levels [12]. Authors argue that this might be explained by *Salmonella's* intracellular localization within an endosomal structure or perhaps by more efficient Fe acquisition strategies compared to *Francisella* [12].

4.2. Salmonella spp. *Salmonella typhimurium* is an invasive pathogen that causes diseases ranging from mild gastroenteritis to enteric fever. To establish a systemic infection, *Salmonella* spp. must invade the epithelial wall of the intestine before the bacteria are ingested by immune effector cells and transported to lymph nodes, the spleen, and other organs. *Salmonella* spp. reside within modified phagosomes in macrophages, where replication is promoted and killing is evaded. Fe is an essential micronutrient for replication, and *Salmonella* spp. harbor various Fe acquisition systems, such as the siderophores enterobactin and salmochelin [35]. As iron sources, *Salmonella* spp. use Fe^{2+}, Fe^{3+}, heme, ovotransferrin, and Tf [35, 36]. *S. Typhimurium* acquires Fe^{2+} from

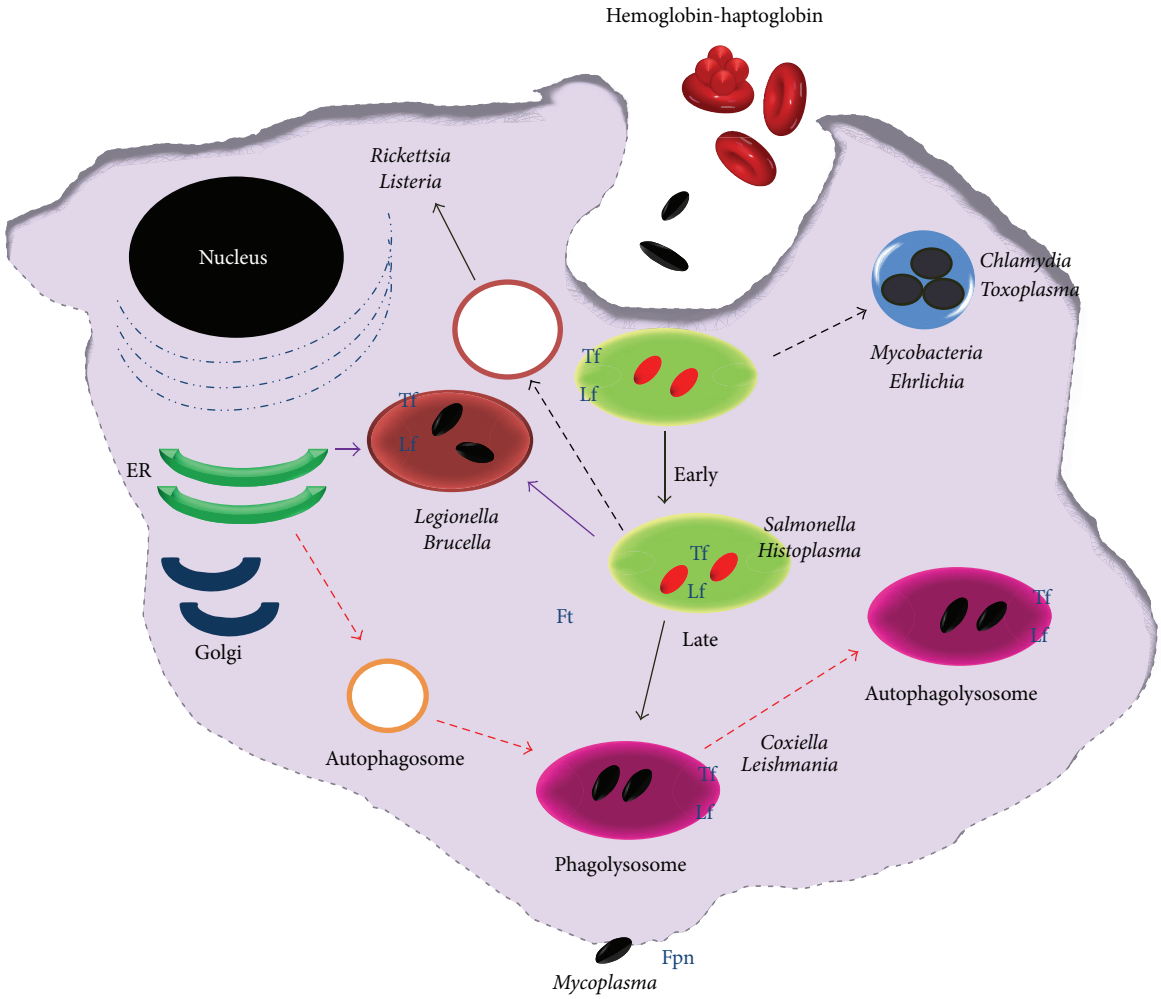

Figure 2: Intracellular parasites and iron sources inside of the macrophage. During infection with intracellular bacteria, iron is required by both the host cell and the pathogen that inhabits the host cell. Macrophages require iron as a cofactor for the execution of important antimicrobial effector mechanisms, and so forth. On the other hand, intracellular bacteria also have an obligate requirement for iron to support their growth and survival inside cells. Some pathogens are internalized into membranous compartments (endosomes/phagosome) and then subsequently trafficked to the lysosome for degradation. Intracellular pathogens have evolved specific mechanisms to survive within this intracellular environment, for example, *Salmonella* persists in the endocytic pathway, and others escape the endo-/lysosomal system and exist in the cytosol. Other bacteria remain within a membranous envelope that may be a modified version of the endoplasmic reticulum (*L. pneumophila*) or a membranous compartment generated by the bacteria (*Chlamydia*), and so forth. Intracellular pathogens can acquire iron because macrophages contain iron-proteins such as transferrin, lactoferrin, ferritin, hemopexin, hemoglobin, or hemoglobin-haptoglobin complexes, in its different compartments.

hemophagocytic macrophages and also secretes siderophores via IroC and EntS to bind Fe^{3+}, which is subsequently taken up by outer membrane receptors including IronN and FepA. ABC transporters such as FepBCDG are responsible for the transport of siderophores through the cytoplasmic membrane, whereas molecular iron is taken up via Feo-mediated transmembrane transport [35, 36].

During the infection process *in vivo*, *S. typhimurium* induces a number of virulence genes that are required to circumvent host defenses and/or acquire nutrients from the host. A putative Fe transporter in *Salmonella* called Pathogenicity Island 1, or sitABCD, has been characterized. The sitABCD operon is induced under Fe-deficient conditions *in vitro* and is repressed by Fur (ferric uptake regulator). This locus is specifically induced in animal models after invasion of the intestinal epithelium, suggesting that SitABCD plays an important role in Fe acquisition in the animal. To regulate its Fe content, *Salmonella enterica* serovar Typhimurium possesses four ferritins: bacterioferritin (Bfr), ferritin A (FtnA), ferritin B (FtnB), and Dps. The heme-containing Bfr accounts for the majority of stored Fe, followed by FtnA. Inactivation of Bfr elevates the free intracellular Fe concentration and enhances susceptibility to H_2O_2 stress. The DNA-binding Dps protein provides protection from oxidative damage without affecting the free intracellular Fe concentration at steady state. FtnB appears to be particularly important for the repair of Fe-sulfur clusters of aconitase that undergo oxidative damage, and, in contrast to Bfr and

Table 1: Iron sources and mechanisms of uptake, transport, and regulation used by intracellular parasites.

Parasite	Iron sources	Mechanisms of iron acquisition	Transport	Regulation
Francisella tularensis	Tf	Receptors (TfR1)	Ferrireductase/DMT-1	IRP1-IRP2
	Fe^{3+}	iron reductase, siderophores	FslE	
	Fe^{2+}	Receptors, iron reductase?	FupA	
	Lf	Receptors, iron reductase?	Ferrireductase/DMT-1?	
Salmonella spp.	Heme	Heme-oxygenase-1?	Porins?	FtnA, FtnB, FtnC, FtnD
	Tf	siderophores (enterobactin, enterochelin)/IronN and FepA	FepBCDG	sitABCD/Fur
	Fe^{3+}		Feo	
	Fe^{2+}	Phagocytosis		
Chlamydia	Tf	Receptors?	tonB analogue?	IRP-1/IRE?
	Ft	Receptors?, iron reductase?	Host Fe-transport pathways?	
		Receptors? Siderophores?	ABC transport systems?	
Neisseria spp.	Tf	Receptors (TbpA, TbpB)	FetA	Fur
	Lf	Receptors (LbpA, LbpB)	MpeR	
	haptoglobin-Hb	Siderophores (enterobactin Salmochelin)	HmbR	
Legionella pneumophila	Fe^{3+}	Siderophores	Feo AB/system	Fur?
	Fe^{2+}	iron reductase	PerR/Fur	
	Tf and Ft?	iron reductase?	Lpp_2867	
Shigella spp.	Tf	Siderophores	Feo	ArcA and FNR
	Fe^{3+}, Fe^{3+}	iron reductase	Feo, Sit, luc	Fur
	Heme	iron reductase	Shu	
	LF			
Listeria monocytogenes	Hb	HupDGC	Fhu	Fri/Fur
	Hemin	HupDGC	Fhu	PerR
	Ferric citrate	iron reductase	Ferric citrate systems	
	Fe-proteins?	Siderophores, iron reductase	Fur, ABC transporters	
Coxiella burnetii	?	?	?	Bacterioferritins Fur
Mycobacterium spp.	Ferric dicitrate	Salicylic acid	IrtAB	Bacterioferritins
	Tf, Lf, Ft	Citric acid	Mramp	FurA, FurB
		Siderophores		IdeR
Candida spp.	Heme	Heme-binding protein (Dap1)	Sit1 (iron transporter)	Sfu1
	Tf	Fe^{3+} reductases, Fe^{2+} transporter		
	Ft			
	Hemin			
Cryptococcus neoformans	Tf	Iron reductase, iron permeases	Cft1 and Cfo1	Cir1
	Heme	Hemophores, Heme receptors	Cig1 and the ESCRT	
	Ft			
Leishmania spp.	Heme, Hemin	LHR1	LIT1 transporter	?
	Tf?	Fe^{3+} reductase 1 (LFR1),		
	Lf			
Trypanosoma spp.	Tf	Receptors (TfR)/endocytosis	ESAG6, ESAG7	IRP/IRE

FtnA, is required for *Salmonella* virulence in mice. Moreover, FtnB and Dps are repressed by the Fe-responsive regulator Fur and induced under conditions of Fe limitation, whereas Bfr and FtnA are maximally expressed when Fe is abundant. The absence of a conserved ferroxidase domain and the potentiation of oxidative stress by FtnB in some strains that lack Dps suggest that FtnB serves as a facile cellular reservoir of Fe^{2+} [37].

4.3. Chlamydia spp. Chlamydia is an infection that is caused by the bacteria *Chlamydia trachomatis*. It is the most common sexually transmitted disease in the U.S., with nearly 3 million cases reported each year (the actual number of cases is likely much higher). The developmental cycle of *C. trachomatis* includes two forms: an infectious elementary body (EB) and a reticulate body that multiplies within the inclusion by binary fission. A third developmental form is the persistent form, which exists as a mechanism of survival under stressful conditions. Persistence is induced in response to changes in the culture medium, including amino acid or Fe deprivation, and in the presence of antibiotics or cytokines such as gamma interferon (IFN) [38]. It has been shown that Fe is an essential factor in the growth and survival of *C. trachomatis* and *C. pneumoniae* (this bacterium causes pneumonia) [39]. Although homologues for bacterial siderophores are missing in the genome of this bacterium, TfR expression does occur. *C. trachomatis* also appears to be missing a tonB analogue, which would span the periplasm and is crucial in energy transfer to substrate-specific outer membrane transporters that are used to bring Fe-siderophore complexes to the cell. Considering these apparent gaps in the genome, one could speculate that the *C. trachomatis* genome would need a reductase on the inclusion membrane to transport Fe^{2+} from the eukaryotic cytosol into the inclusion. *C. trachomatis* and *C. pneumoniae* appear to use the host's Fe transport pathways by attracting TfR and Ft to the phagosome [39]. A report from Vardhan et al. (2009) showed that *C. trachomatis* alters the Fe-regulatory protein-1 (IRP-1) binding capacity and modulates cellular iron homeostasis in HeLa-229 cells, suggesting that Fe homeostasis is modulated in CT-infected HeLa cells at the interface of acquisition and commensal use of Fe [40].

ATP-binding cassette (ABC) transport systems play a role in the acquisition of Fe and Fe-complexes, amino acids, sugars, and other compounds. They consist of a soluble periplasmic protein that binds the targeted molecule and changes conformation to close around the substrate. The periplasmic binding protein moves to and binds the transmembrane protein permease in receptor-ligand mechanisms. An ATP-binding lipoprotein binds to the ATP, creating a conformational change in the permease complex that transports the substrate into the cytoplasm. In other pathogenic bacteria, ABC transport systems that transport Fe, zinc, and manganese into the cytoplasm include Tro from *Treponema pallidum*, Yfe from *Yersinia pestis*, and Fbp from *Neisseria meningitidis* [40]. There is evidence that YtgA secretion occurs in *C. trachomatis*, and YtgA does have high homology with periplasmic binding proteins of the ABC transport systems. *yta*A is a gene of 978 bp that resides in an operon with *ytg*BVD. YtgB and Ytg have predictable membrane-spanning domains and most likely form the pore of the ABC transporter. YtgA contains similar metal-binding motifs (e.g., histidine, tyrosine) to other metal-binding periplasmic proteins, suggesting a role for YtgA as an Fe-binding periplasmic protein, in addition to its location on the chlamydial membrane [41].

4.4. Neisseria spp. Acquisition of Fe and Fe-complexes has long been recognized as a major determinant in the pathogenesis of *Neisseria* spp., and some of their high-affinity iron uptake systems are important virulence factors in bacteria. These have been shown to play a major role in promoting the survival of the meningococcus within the host. Most species are Gram-negative bacteria that are primarily commensal inhabitants or reside in the mucus membranes of mammals. There are 12 *Neisseria* species of human origin, with *N. meningitidis* and *N. gonorrhoeae* being important opportunistic pathogens. These intracellular pathogens contain high-affinity iron uptake systems, which allow meningococci to utilize the human host proteins Tf, Lf, Hb, and haptoglobin-hemoglobin as sources of essential Fe [29, 42]. Although the meningococci do not produce siderophores, studies indicate that meningococci may be able to use heterologous siderophores secreted by other bacteria. For some time, it has been reported that the gonococci could utilize ferric enterobactin, enterobactin derivatives, aerobactin, and salmochelin S2 in a FetA- and TonB-dependent manner [29]. In *N. gonorrhoeae*, an outer membrane protein named FetA (formerly FrpB) was recently described. FetA is an outer membrane transporter and is part of an iron-regulated operon that encodes a periplasmic binding protein and the components of a putative ABC transport system. FetA has demonstrated low binding affinity and the transport of ferric enterobactin. The binding contact of FetA for enterobactin was much lower than that for other enterobactin receptors, and it was therefore proposed that this receptor could interact with high affinity to an as-yet unidentified phenolate siderophore. A homologous protein, with 91% similarity to gonococcal FetA, has been identified in *N. meningitidis* and presumably functions in a similar manner [30, 43]. Only *fet*A and not the downstream genes require an iron-regulator MpeR for regulation. MpeR regulation is important because it may aid in gonococcal immune evasion. MpeR was suggested to modulate any change in *mtr*F expression that is needed for full hydrophobic agent resistance. AraC-like regulators of *N. meningitidis* are homologues of the *N. gonorrhoeae* type MpeR that is specific to the pathogenic *Neisseria* species. Both are induced during Fe limitation, and this regulation is also mediated by the Fur regulator. The presence of MpeR in a regulatory cascade downstream of the Fur master Fe regulator suggests that it is being expressed in the Fe limiting environment of the host, where it may in turn regulate a group of genes, including the divergent Fe transport locus, in response to signals that are important for infection [44].

Two proteins, transferrin-binding protein A (TbpA) and transferrin-binding protein B (TbpB), function as the transferrin receptor in *N. meningitidis*. TbpA and TbpB

are induced along with several other proteins in the outer membranes of *N. meningitidis* under Fe-restricted conditions [30]. Initially, an affinity isolation procedure using biotinylated transferrin was employed to demonstrate the presence of two transferrin-binding proteins in *N. meningitidis*. The proteins that bound transferrin were TbpA (formerly Tbp1), which is 98 kDa, and TbpB (formerly Tbp2), which is 68 kDa [45]. Among different meningococcal isolates, the molecular masses of TbpA and TbpB vary, with TbpA ranging from 93 to 98 kDa and the more heterogenetic TbpB varying from 68 to 85 kDa. TbpA can be found in all strains. Although it has not been characterized as well as the Tf receptor, the Lf receptor is believed to be an important meningococcal virulence factor [29]. The Lf receptor of *N. meningitidis*, like the Tf receptor, consists of two protein components, LbpA and LbpB. Initial experiments using affinity isolation by Lf identified a 98-kDa lactoferrin-binding protein named LbpA, formerly known as IroA [46].

4.5. Legionella pneumophila. *Legionella pneumophila*, the causative agent of Legionnaire's disease, is a facultative intracellular parasite of human macrophages and freshwater amoebae. This pathogenic bacterium is commonly found in water, thereby presenting a risk that it could be transmitted to humans via inhalation of contaminated aerosols. *L. pneumophila* resides in the phagosome, although this phagosome does not fuse with endosomes and lysosomes and is at nearly neutral pH during the early stages of the intracellular life cycle. It appears to fuse with low-pH cellular compartments during the later stages of the infection [47].

The ability of *L. pneumophila* to acquire host cell Fe is pivotal for the parasite to establish a successful intracellular infection. To occupy its intracellular niche, this pathogen has developed multiple Fe acquisition mechanisms: the ira AB locus, which encodes a transporter for Fe-loaded peptides; the cytochrome c maturation ccm genes; the Fe-regulated frgA, whose product is homologous to aerobactin synthetases; legiobactin siderophores; and two internal ferric reductases. Robey and Cianciotto (2002) identified and characterized *L. pneumophila* Feo AB, which bears homology to *E. coli* and *Salmonella enterica* serovar Typhimurium FeoAB. In those bacteria, FeoB has been shown to be a ferrous Fe transporter and FeoA is possibly involved in Fe^{2+} uptake [48].

In 2014, Portier and Cols discovered gene ipp_2867, which was highly induced in Fe-restricted conditions. A sequence analysis predicts that Lpp_2867 is a membrane protein involved directly or indirectly in Fe^{2+} transport and is also a virulence factor [49].

4.6. Shigella spp. *Shigella* is a Gram-negative bacterium of the Enterobacteriaceae family and is the etiological agent of bacillary dysentery or shigellosis. *Shigella* encompasses four subgroups (*S. flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii*), and all species are able to grow in a variety of environments, including intracellularly in host epithelial cells. *Shigella* has a number of different Fe transport systems that contribute to the bacterium's ability to grow in these diverse environments [50]. Siderophore Fe uptake systems, heme transporters, and Fe^{3+} and Fe^{2+} transport systems are present in these bacteria, and the genes encoding some of these systems appear to have spread among the *Shigella* species by horizontal transmission [50, 51]. Fe is not only essential for the growth of *Shigella* but also plays an important role in the regulation of metabolic processes and virulence determinants in *Shigella*. This regulation is mediated by the repressor protein Fur and the small RNA RyhB [52]. The only Fe transport system that appears to be common to all members of the *E. coli*/*Shigella* group is Feo. *Shigella* spp. have transport systems for both ferric and ferrous iron. The Fe can be taken up as free Fe or complexed with a variety of carriers. All *Shigella* species have both the Feo and Sit systems for acquisition of Fe^{2+}, and all have at least one siderophore-mediated system for transport of Fe^{3+} [53]. Several of the transport systems, including Sit, Iuc/IutA (aerobactin synthesis and transport), Fec (ferric di-citrate uptake), and Shu (heme transport), are encoded within pathogenicity islands. The presence and the genomic locations of these islands vary considerably among the *Shigella* species and even between isolates of the same species [53, 54]. The expression of the Fe transport systems is influenced by the concentration of Fe and by environmental conditions, including the level of oxygen. ArcA and FNR regulate Fe transport gene expression as a function of oxygen tension, with the sit and iuc promoters being highly expressed in aerobic conditions, while the feo Fe^{2+} transporter promoter is most active under anaerobic conditions [52]. The effects of oxygen are also observed in infection of cultured cells by *S. flexneri*; the Sit and Iuc systems support plaque formation under aerobic conditions, whereas Feo allows plaque formation to occur anaerobically [52, 53].

4.7. Listeria monocytogenes. *L. monocytogenes* is a Gram-positive, intracellular pathogen responsible for the fatal disease listeriosis. *L. monocytogenes* is recognized as a significant public health problem. The ability of this bacterium to acquire and utilize Fe is not only essential during infection but can also support its growth and survival in many diverse environmental niches.

L. monocytogenes possesses at least 4 mechanisms that enable Fe uptake: (1) acquisition of protein-bound Fe that involves the HupDGC protein (for the uptake of hemin, hemoglobin), or Fhu protein (involved in the uptake of ferrichrome siderophores); inside the cell, then Fe can be bound to the Fri protein (ferritin-like) Fur regulated; (2) extracellular and/or surface-bound Fe reductases; (3) a citrate inducible ferric citrate uptake system; and (4) siderophore and siderophore-like systems [55].

The *Listeria* life cycle involves escape from the phagosome, which is considered to be Fe-limiting and permits proliferation in the host-cell cytosol, where Fe-saturated Ft is stored. It has been hypothesized that *L. monocytogenes* has access to Fe through increased expression of the PrfA-regulated virulence factors listeriolysin (LLO) and ActA, which are used for phagosomal escape. Increased Fe concentrations result in the upregulation of internalin proteins InlA and InlB, which are required for invasion [56].

Fe homeostasis in *Listeria* is controlled by the regulatory protein Fur. It has been shown that expression of Fur is negatively regulated by PerR, a Fur homologue that is involved in the oxidative stress response. Fourteen Fur-regulated genes have been identified in *L. monocytogenes*, including genes that encode Fe^{2+} transporters and ferrichrome ABC transporters and proteins involved in Fe storage [56, 57].

4.8. Coxiella burnetii. *Coxiella burnetii* is the causative bacterial agent of Q fever in humans and is one of the most infectious pathogens known. Human infection with *C. burnetii* is generally a zoonosis that is acquired by inhalation of contaminated aerosols. Q fever typically presents as an acute, self-limiting flu-like illness accompanied by pneumonia or hepatitis. In 1% of cases, a severe chronic infection can occur, in which endocarditis is the predominant manifestation [58]. It is essential for most pathogenic bacteria to overcome the limitation of Fe in the intracellular host. To overcome this limitation, bacteria maintain cell storage systems under the tight control of Fur. It has been suggested that it is an absolute requirement for *C. burnetii*, similar to *L. pneumophila*, to regulate Fe assimilation via the Fur regulon. One study revealed that the Fur-regulon in *C. burnetii* consists of a Fur-like protein (CBU1766) and the putative iron-binding protein Frg1 (CBU0970) [59].

Iron plays a rather limited role in the pathogenesis of *C. burnetii*. Reports have described the expression of a thiol-specific peroxidase (CBU0963) in *C. burnetii* that belongs to the atypical 2-cysteine subfamily of peroxiredoxins, also designated as bacterioferritin comigratory proteins (BCPs). The implication is that this protein might protect DNA from the Fenton reaction [60]. Comparison to *L. pneumophila*, a phylogenetic relative, revealed that *C. burnetii* rarely encodes any known Fe acquisition or storage proteins, aside from some Fe dependent pathways, as well as the heme biosynthesis pathway and proteins such as SodB.

4.9. Mycobacterium spp. Mycobacterium is a genus of Actinobacteria, given its own family, the Mycobacteriaceae. The genus includes pathogens known to cause serious diseases in mammals, including tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*). Similar to most microorganisms, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, requires Fe for essential metabolic pathways. Like several other pathogenic bacteria, it has evolved an intricate mechanism of acquiring, assimilating, and storing Fe, which is a component that determines the fate of the pathogen inside the host [28]. Because Fe is not freely available in the host, Mycobacteria must actively compete for this metal to establish an infection, but they must also carefully control Fe acquisition, as excess free Fe can be extremely toxic. The molecules responsible for Fe acquisition in mycobacteria include simple molecules such as salicylic acid and citric acid, apart from the two classes of siderophores.

To acquire Fe, mycobacteria produce siderophores (high-affinity Fe chelators). The lipophilic siderophores that remain associated with the cell wall are called mycobactins, and the second class of siderophores includes polar forms that are released into the extracellular medium [28]. These are called carboxymycobactins (released by pathogenic mycobacteria) and exochelins (released by nonpathogenic mycobacteria). *M. tuberculosis* and *M. smegmatis* produce salicylate-containing siderophores known as mycobactins. There are two forms of mycobactins: carboxymycobactin, which is a water-soluble secreted molecule, and the cell-associated mycobactin, which is a hydrophobic molecule that is retained on the cell surface. In addition to mycobactins, *M. smegmatis* also produces a peptidic siderophore known as exochelin, which is the predominant siderophore secreted by this mycobacterium under Fe limitation [28].

The identification of two genes that are annotated as fecB and fecB2 and that code proteins similar to FecB of *Escherichia coli* suggests that *M. tuberculosis* may also utilize ferric dicitrate as an Fe source [61]. Siderophores avidly bind Fe^{+3} and can effectively compete with host Fe binding proteins for this metal. Fe^{+3}-carboxymycobactin can transfer Fe^{+3} to mycobactin or bring it into the cell via the iron-regulated transporter IrtAB. The putative transporter encoded by fxuABC may transport Fe^{+3}-exochelin complexes.

Previous work has linked the ESX-3 system with the ability of mycobacteria to adapt to Fe limitation. ESX-3 is one of the five type VII secretion systems encoded by the *M. tuberculosis* genome. Studies that examined an *M. smegmatis* exochelin synthesis mutant indicated an ESX-3 requirement for Fe^{+3}-mycobactin utilization. The precise role of ESX-3 in Fe acquisition in *M. tuberculosis* is unknown, but it is clear that ESX-3 is necessary for adaptation to low Fe conditions [62]. On the other hand, it has been documented that *M. tuberculosis* increases microvesicles production in response to Fe restriction and that these microvesicles contain mycobactin, which can serve as an iron donor and supports replication of Fe-starved mycobacteria. Consequently, the results revealed that microvesicles play a role in Fe acquisition in *M. tuberculosis*, and this can be critical for survival in the host. Recent studies have demonstrated that failure to assemble the Fe acquisition machinery or to repress Fe uptake has deleterious effects for *M. tuberculosis* [28].

A protein that was speculated to be a mycobacterial iron transporter is the Mramp, and this protein was able to increase the uptake of Fe^{2+} and Zn^{2+} in a pH dependent manner. Mramp was expected to be a cation transporter with no selective transport of Fe, although additional reports indicate that Mramp may act as a cation efflux pump [63].

Bacterioferritin-like molecules bfrA (a putative bacterioferritin) and bfrB (an Ft-like protein) have been identified in the *M. tuberculosis* genome and are the principal Fe storage molecules. Their expression is induced under Fe-rich conditions and repressed under Fe-deprived conditions. Therefore, it is speculated that this format allows the maintenance of basal levels of bacterioferritin inside the pathogen so that any amount of excess Fe can be immediately stored in a bound form [64]. Regulation of gene expression in *M. tuberculosis* includes that of regulatory proteins, stress response proteins, enzymes, and PE-PGR/PPE proteins. The genes that are upregulated under Fe-deprived conditions

included those that are responsible for acquisition of Fe, such as siderophores, biosynthesis gene clusters mbt1 and mbt2, and Fe regulated transporters of siderophores irtA, irtB, Rv2895c, and esx [28]. Genes that are upregulated under Fe-rich conditions include bacterioferritin and ferritin (bfrA and bfrB), as they serve to store excess Fe as catalase-peroxidase, or katG and its regulator, ferric uptake regulator A (FurA) [63].

There are two Fur proteins, FurA and FurB. After binding ferric iron, FurA recognizes and binds to a 19-base-pair pseudopalindrome sequence of a specific DNA motif called Fur Box that is present upstream to a gene and acts as a repressor. FurB, on the other hand, was later found to be regulated by zinc and not Fe and has been correctly referred to as Zur.

IdeR, an Fe-dependent repressor and activator, is the major regulatory protein involved in homeostasis in mycobacteria. Belonging to the Diphtheria toxin repressor family (DtxR), it acts as a homodimer, with each monomer possessing two binding sites for Fe. Two homodimers with four bound Fe ions recognize a 19-base-pair palindromic sequence and in Fe-replete conditions and negatively regulate the expression of proteins required in Fe-depleted conditions [65]. The genes or gene clusters essentially required during Fe starvation are effectively repressed by IeR. These include the siderophore synthesis gene cluster, mbt1, mbt2, irtA, irtB, and Rv2895c. Therefore, there are certain proteins that are differentially regulated by Fe in an IdeR-independent fashion. These include lipoprotein IprE, KatG, 50S ribosomal protein, L22, and ATP synthase c chain, two component response regulators, MTrA, PE-PGRS proteins, and NifU-like proteins [28]. Fur and Fe-dependent repressors and activators or IdeR are the two key proteins that regulate expression of other Fe-dependent genes [28, 63].

4.10. Candida spp. *Candida* is a genus of yeast and is the most common cause of fungal infection worldwide [66, 67]. Many *Candida* species are harmless commensals or endosymbionts of hosts including humans; however, when mucosal barriers are disrupted or the immune system is compromised they can invade tissues and cause disease [66]. Among *Candida* species, *C. albicans* is responsible for the majority of Candida bloodstream and mucosal infections. However, in recent years, there is an increasing incidence of infections caused by *C. glabrata* and *C. rugosa*, *C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis* [66]. Varied virulence factors and growing resistance to antifungal agents have contributed to their pathogenicity [66, 68].

Candida albicans can cause infections (candidiasis or thrush) in humans and other animals. Between the commensal and pathogenic lifestyles, this microorganism inhabits host niches that differ markedly in the levels of bioavailable iron. Once introduced into the bloodstream, *C. albicans* can acquire Fe from the molecules that are used by the host to sequester this metal [69]. For example, several groups have identified *C. albicans* hemolytic activity capable of releasing Hb from host erythrocytes. Free Hb or its heme/hemin metal-porphyrin ring is bound by a hemoglobin receptor, Rbt5, on the fungal cell surface, followed by endocytosis of Rbt5-hemoglobin complexes and release of Fe^{2+} by the heme oxidase, Hmx1 [69]. It has been reported that *C. albicans* encodes four additional homologs of Rbt5, of which Rbt51 has also been demonstrated to bind to hemin [69].

C. albicans can also utilize host Tf *in vitro* as a sole source of Fe, probably through the involvement of a transferrin receptor, similar to certain bacterial pathogens. It has been reported that the Fe^{3+} derived from Tf is taken up by a reductive iron uptake system that is conserved with the well-described high affinity iron uptake system of *Saccharomyces cerevisiae*. Fe^{3+} is first reduced to soluble Fe^{2+} by a cell surface-associated ferric reductase [69]. In coupled reactions, Fe^{2+} is then oxidized and imported into the fungal cytoplasm by a multicopper ferroxidase/iron permease complex. *C. albicans* encodes 17 putative ferric reductases, five putative multicopper ferroxidases, and four putative ferric permeases with potential functions in reductive Fe uptake, and different subsets of these enzymes are expressed under different *in vitro* conditions. Of the two ferric permeases, only Ftr1 is expressed when iron is limited, and FTR1 is essential in a murine bloodstream infection model of virulence [69].

In tissues, the Fe is mainly bound to Ft. The Ft is found inside of macrophages and epithelial cells. This protein binds 4500 Fe atoms, and cytoplasmic iron-ferritin complexes are generally extremely stable. It has been documented that *C. albicans* utilizes Ft as Fe source *in vitro*, or directly from epithelial cells in culture. When this yeast was cocultured with a human oral epithelial cell line, the protein Ft was found bound onto their surface. This Ft binding protein denominated Als3, is located in the hyphae from *C. albicans* [69]. Als3 also plays important roles in *C. albicans* biofilm formation [70] and adhesion to host epithelial and endothelial cells and induced endocytosis of hyphae [71]. Thus, Als3 integrates Fe uptake and virulence functions but only in oral epithelial infection models. This conclusion was obtained when deletion of ALS3 abrogated *C. albicans* virulence in the oral epithelial infection model, but not in a bloodstream infection model [69, 72]. Additionally, it has been reported that, *in vitro*, fungal-mediated acidification of the laboratory culture media is required to dissociate Fe^{3+} from ferritin. Fe^{3+} is transported into the fungal cytoplasm via the same reductive Fe uptake system described above for Ft [69]. Figure 3 shows the iron acquisitions systems in *C. albicans*.

C. albicans also possesses a third system of iron uptake based in the use of siderophores; however, it is unclear whether *C. albicans* synthesizes its own siderophores. Siderophore activity has been reported for this species but its genome does not encode the known fungal biosynthetic enzymes [69, 73]. Nevertheless, *C. albicans* has been demonstrated to utilize exogenous ferrichrome-type siderophores via the Sit1 siderophore importer. Similar to ALS3, deletion of SIT1 abolishes *C. albicans* virulence in a reconstituted human epithelial infection model but not in a bloodstream infection model [69, 74]. Finally, it has been recently reported that Hap43, Sfu1, and Tup1 act coordinately and regulate iron acquisition, iron utilization, and other iron-responsive metabolic activities in *C. albicans* [75].

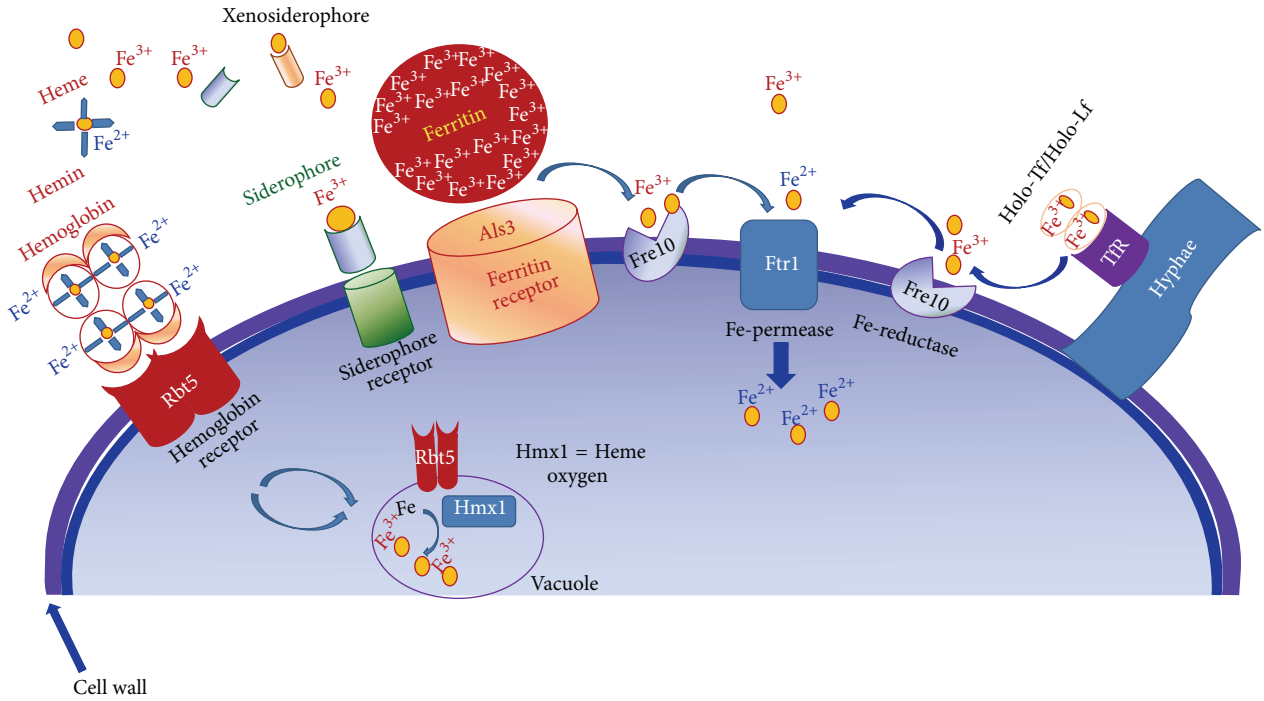

FIGURE 3: Iron acquisitions systems in *Candida albicans*. To acquire iron, *C. albicans* possesses three high-affinity iron acquisition systems: (1) a reductive system responsible for iron exploitation from transferrin or ferritin or from the environment; (2) a siderophore uptake system responsible for iron acquisition from a range of siderophores produced by other organisms; and (3) a heme-iron uptake and degradation system capable of acquiring iron from hemoglobin and probably from heme-proteins.

Candida glabrata is both a human fungal commensal and an opportunistic pathogen. It is the second most common cause of infection, surpassed only by *C. albicans*. This yeastis an intracellular pathogen that can survive phagocytosis and replicates within the host cell. *C. glabrata* infection is extremely difficult to treat due to its intrinsic antifungal resistance to azoles. The infections caused by this fungus are associated with a high mortality rate. Siderophore production is common among most microorganisms and is a major mechanism of Fe solubilization and acquisition. The very high Fe-binding contact observed for siderophores of fungal origin is approximately 10^{30} M at pH 7. Several bacteria and fungi do not produce siderophores but have evolved transporters that allow them to utilize siderophores they themselves do not produce. These are called xenosiderophores [76].

Computational analysis of Sit1 identified sequence signatures that are characteristic of members of the Major Facilitator Superfamily of Transporters. In a study by Nevitt and Thiele (2011), Sit1 is described as the sole siderophore Fe transporter in *C. glabrata*, and the study demonstrates that this siderophore is critical for enhancing their survival in the face of the microbicidal activities of macrophages [77]. Within the Sit1 transporter, a conserved extracellular siderophore transporter domain (SITD) was identified that is important for the siderophore-mediated ability of *C. glabrata* to resist macrophage killing and is dependent on macrophage Fe status [77]. They suggested that the host's iron status is a modifier of infectious disease that modulates the dependence on a distinct mechanism of microbial Fe acquisition. Iron-regulated CaSit 1 shares high homology with *S. cerevisiae* siderophore transporters and its deletion compromises utilization of fungal ferrichrome-type hydroxamate siderophores. The absence of an identifiable heme receptor in *C. glabrata* suggests that this pathogen may rely predominantly on the solubilization of the circulating exchangeable Fe pool to meet its requirements for Fe [76].

A study realized by Srivastava et al. (2014) described the molecular analysis of a set of 13 *C. glabrata* strains that were deleted for proteins and potentially implicated in Fe metabolism. The results revealed that the high-affinity reductive Fe uptake system is required for the utilization of alternate carbon sources and for growth under both *in vitro* Fe-limiting and *in vivo* conditions. Further, they showed for the first time that the cysteine-rich CFEM domain-containing cell wall structural protein CgCcw14 and the putative hemolysin CgMam3 are essential for maintenance of intracellular Fe content, adherence to epithelial cells, and virulence [78]. Additionally, they present evidence that the mitochondrial frataxin CgYfh1 is pivotal to Fe metabolism and conclude that high-affinity iron uptake mechanisms are critical virulence determinants in *C. glabrata* [78].

4.11. Cryptococcus neoformans. *Cryptococcus neoformans* is a fungal pathogen and a leading cause of pulmonary and central nervous systemic mycosis in immunocompromised individuals such as HIV-infected patients. For this reason,

C. neoformans is sometimes referred to as an opportunistic fungus. It is a facultative intracellular pathogen. In human infection, *C. neoformans* is spread by inhalation of aerosolized spores (basidiospores) and can disseminate to the central nervous system where it can cause meningoencephalitis [79]. In the lungs, *C. neoformans* are phagocytosed by alveolar macrophages. Macrophages produce oxidative and nitrosative agents, creating a hostile environment, to kill invading pathogens. However, some *C. neoformans* can survive intracellularly in macrophages. Intracellular survival appears to be the basis for latency, disseminated disease, and resistance to eradication by antifungal agents [80]. One mechanism by which *C. neoformans* survives the hostile intracellular environment of the macrophage involves upregulation of expression of genes involved in responses to oxidative stress. *C. neoformans* has been considered an excellent model fungal pathogen to study iron transport and homeostasis because of its intriguing connection with virulence. Growing evidence suggests that the fungus is able to utilize several different iron sources available in the host, and that the intracellular or extracellular localization of the pathogen influences its iron acquisition strategy [80]. *C. neoformans* infects alveolar macrophages; at this site, specifically in the acidic phagolysosome, free Fe^{2+} is released from the host Ft and Tf. The reductive high-affinity Fe uptake system mediated by Cft1 and Cfo1 was characterized, its function was closely associated with the reduction of Fe^{3+} at the cell surface by the reductase activity, and it was limited in the environment at neutral pH [79].

Therefore, *C. neoformans* could predominantly use an iron uptake system that is specifically responsive to the acidic intracellular niche, although Fe deprivation at an acidic pH no longer reduced the growth of the cft1 and cfo1 mutants. Moreover, a mutant lacking either CFT1 or CFO1 displayed attenuation of virulence and eventually caused disease in infected mice. These observations suggest that an as-yet unknown Fe uptake system, which is independent of the reductive high-affinity iron uptake system, may play a role in the acidic host microenvironment in a phagolysosome [79]. On the other hand, *C. neoformans* is able to utilize Tf through the reductive high-affinity iron uptake system and extracellular heme by Cig1 and the ESCRT complex; however, more studies should be carried out to understand how *C. neoformans* directly liberates Fe from Tf as well as Hb and other heme-containing proteins [80]. It has been suggested that the gene CIR1 (*Cryptococcus* iron regulator) shares structural and functional features with other fungal GATA-type transcription factors for iron regulation [81]. Figure 4 shows the iron acquisitions systems in *C. neoformans*.

4.12. Leishmania spp. Leishmaniasis is endemic in the tropics and neotropics. Clinical manifestations include skin lesions ranging from small cutaneous nodules to gross mucosal tissue destruction. The infection is transmitted to human beings and animals by sandflies. *Leishmania* parasites have a digenetic life cycle, alternating between the promastigote stage in the insect gut and the amastigote stage in macrophages of mammalian hosts. It has been postulated that *Leishmania* cells are equipped with diverse Fe acquisition mechanisms and are capable of utilizing various Fe sources, suggesting that Fe acquisition is essential for pathogenicity and that Fe deprivation could be an effective strategy for controlling leishmanial infections [82].

Like many other intracellular pathogens, *Leishmania* must be capable of acquiring Fe from the host milieu in order to thrive. In addition to Tf, the growth and survival of *L. infantum* and *L. amazonensis* amastigotes can be supported by Fe derived from hemoglobin and hemin [83]. The uptake of heme by intramacrophagic *L. amazonensis* amastigotes is mediated by the *Leishmania* heme response 1 (LHR1) protein. Furthermore, intracellular *L. amazonensis* also possesses a ferric reductase, the *Leismania* ferric iron reductase 1 (LFR1), which provides soluble Fe^{2+} for transport across the parasite plasma membrane by the ferrous iron transporter, *Leishmania* iron transporter 1 (LIT1) [83, 84]. Moreover, LIT1-mediated Fe acquisition seems to be essential for the differentiation of *L. amazonensis* parasites from the sandfly promastigote form to the macrophage-adapted amastigote form [85].

Apart from the mechanisms of direct iron internalization, *Leishmania* parasites can also subvert the host's Fe uptake systems to their own advantage. In fact, *L. amazonensis* amastigotes can obtain Tf by forcing the fusion of Tf-containing endosomes with the parasitophorous vacuole [86]. Alternatively, *L. donovani* is capable of decreasing the macrophage's labile Fe pool, a process that triggers an increased surface expression of transferrin receptor 1 and internalization of Tf, thus permitting continuous provision of Fe to the parasite. This decrease in the labile Fe pool of activated macrophages has recently been proposed to be the result of the downregulation of the expression of SLC11A1 by a *L. donovani*-secreted peroxidase. Also, in line with these data, it has been reported that the expression of ferroportin is downregulated in the spleen of *L. donovani*-infected mice, which may contribute to an increased accumulation of iron inside macrophages. In *Leishmania*, a transferrin receptor-based mechanism for Fe uptake was also initially postulated, but this mechanism was not confirmed by subsequent studies [87]. Tf can reach the lysosome-like parasitophorous vacuoles where Leishmania resides in macrophages, but it appears to function mainly as a source of Fe^{3+} for the sequential action of two surface-associated parasite molecules: the Fe^{3+} reductase LFR1 and the LIT1 transporter, which directly promote Fe^{2+} uptake. Intriguingly, the *T. cruzi* genome does not contain an obvious LIT1 orthologue, raising the possibility that this Fe^{2+}-transporter represents a specific *Leishmania* adaptation to the low Fe environment of phagolysosomes [88]. Mutations in the lysosomal Fe efflux pump NRAMP1 confer susceptibility to *Leishmania* and other intravacuolar pathogens, reinforcing the conclusion that *Leishmania* needs a high-affinity transporter such as LIT1 to compete effectively for Fe within its parasitophorous vacuole [89]. On the other hand, *L. amazonensis* directly interferes with the Fe export function of macrophages, by inhibiting cell surface expression of Fpn1, but the mechanism by which this is achieved is still unknown [90].

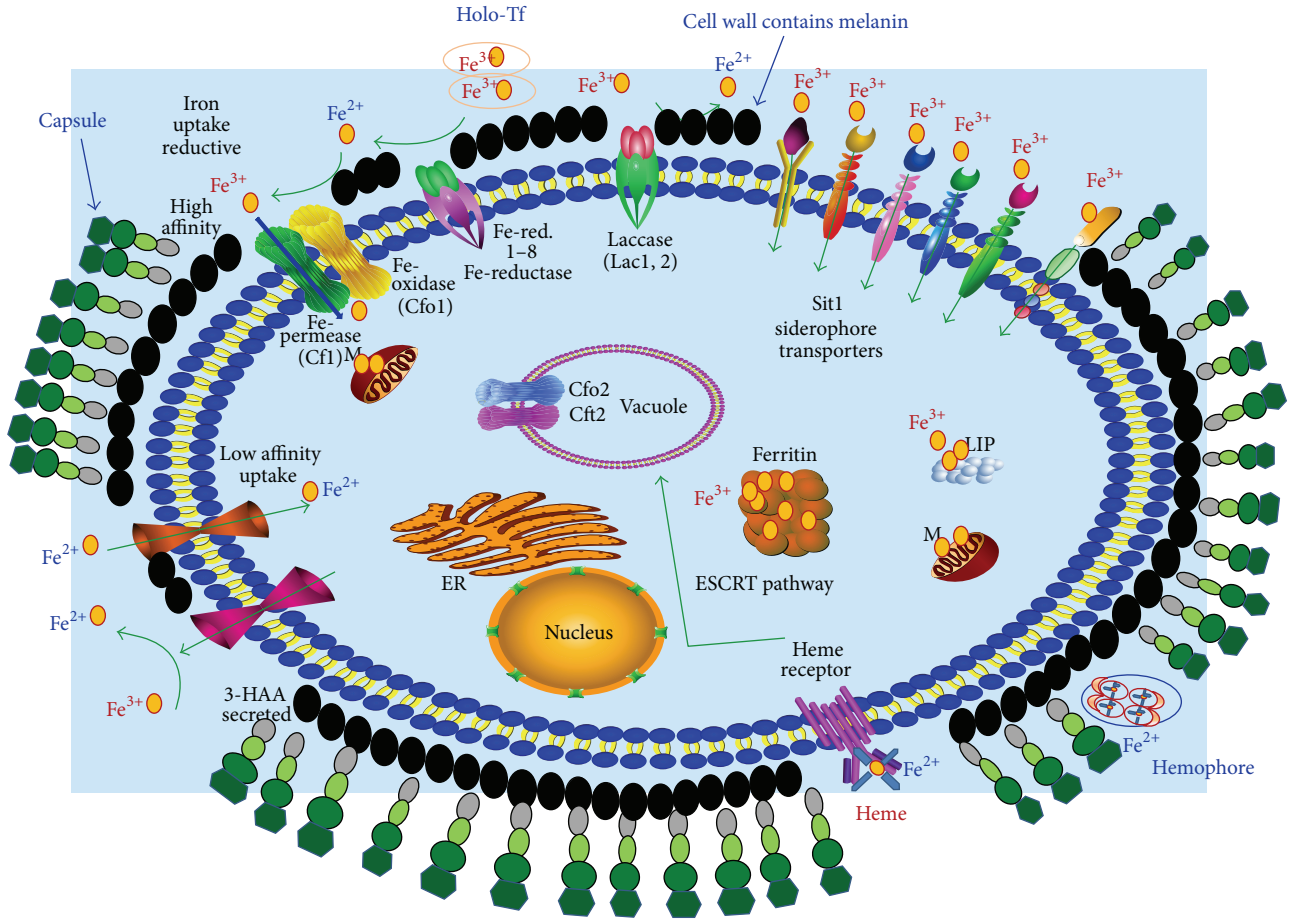

FIGURE 4: Iron acquisitions systems in *Cryptococcus neoformans*. *C. neoformans* infects alveolar macrophages; at this site, specifically in the acidic phagolysosome, free Fe^{2+} is released from the host Ft and Tf. The reductive high-affinity Fe uptake system mediated by Cft1 and Cfo1 plays a role in the reduction of Fe^{3+} at the cell surface by the reductase activity. In addition, *C. neoformans* is able to utilize Tf through the reductive high-affinity iron uptake system.Finally, for extracellular heme acquisition, *C. neoformans* relies on the complex Cft1/Cfo1, a xenosiderophore transporter (Sit1), and secreted and extracellular reductants (3-hydroxyanthranilic acid, melanin).

4.13. Trypanosoma spp. The amastigotes of the intracellular parasite *Trypanosoma* cruzi take up Fe-loaded Tf when grown *in vitro*, but the physiological significance of this process is unclear [91]. Tf is restricted to the lumen of the endocytic pathway and is therefore absent from the host cell cytosol, where intracellular amastigotes replicate. The bloodstream form of *Trypanosoma brucei* acquires Fe from Tf by receptor-mediated endocytosis by a process that is regulated by Fe availability. TrR is a heterodimeric complex encoded by two expression site-associated genes, ESAG6 and ESAG7, and shares no homology with the homodimeric mammalian Tf receptor. The binding of one molecule of Tf requires the association of both ESAG6 and ESAG7. In mammalian cells, the TfR mRNA is stabilized in iron-depleted cells due to the binding of IRPs to specific IREs. In *T. brucei*, this IRP-1 relation is not essential for Fe regulation of ESAG6 mRNA. In mammalian cells, the closely related IPR-2 can independently mediate the iron status via IREs. However, in trypanosomes, the presence of additional IRP-related proteins seems very unlikely. The *T. brucei* genome contains only one IRP-related gene, which suggests that a different mechanism, a different type of transacting factor, is responsible for Fe sensing and regulation of transferrin receptor mRNA in this protozoan [91, 92]. However, it is unknown how procyclic forms that cannot bind Tf acquire Fe. Additionally, the bloodstream-form of *T. brucei* acquires Fe by receptor-mediated endocytosis of host transferrin [93]. The mechanism(s) by which Fe is then transferred from the lysosome to the cytosol remains unresolved [94].

5. Conclusions

The use of Fe as a cofactor in basic metabolic pathways is essential to both pathogenic microorganisms and their hosts. It is also a pivotal component of the innate immune response through its role in the generation of toxic oxygen and nitrogen intermediates. During evolution, the shared requirement of micro- and macroorganisms for this important nutrient has shaped the pathogen-host relationship [14]. Two general mechanisms of Fe acquisition in intracellular parasites have been described: siderophore-mediated Fe acquisition by cognate receptors and receptor-mediated Fe

acquisition from host Fe-binding proteins [14]. Intracellular microorganisms have evolved a variety of siderochromes, which are special ligands that can dissolve insoluble Fe^{3+} and facilitate its transport into the cell in order to acquire Fe from Tf and other Fe-proteins in the host. The success of intracellular parasites seems to be related mainly to their ability to take up Fe from the protein Tf [12]. Once ingested by macrophages, intracellular parasites are taken up by phagosomes via endocytosis. Acidification of the phagosome permits the iron to be released from Tf, and, in this way, some pathogens can gain access to this element [12].

Bacteria use the protein ferritin or bacterioferritin to store Fe. These are ubiquitous Fe storage proteins that play a fundamental role in cellular Fe homeostasis and have similarities with Ft that is found in mammals. Bacterial Fts have the capacity to store very large amounts of Fe as a Fe^{3+} mineral inside its central cavity. In times of Fe deprivation, some bacteria require that iron be released from Ft mineral stores in order to maintain their metabolic rate and growth. In times of Fe repletion, intracellular microorganisms must regulate the genes required for Fe acquisition, but this mechanism has not been fully characterized [45, 61]. Transferrin and its receptor (TfR1) play an important role during infection of macrophages with bacterial pathogens that prefer an intracellular lifestyle. Expression of TfR1 can in turn be modulated by bacterial infections. Some pathogens actively recruit TfR1 to the bacterium-containing vacuole [29, 45].

The notion is conceivable that intracellular pathogens reside in phagosomal compartments to modulate Fe regulatory proteins, thereby increasing their Fe availability, but this notion is still speculative. The Fe acquisition process often begins when cell surface receptors recognize Fe^{3+} complexes and ultimately ends when cytoplasmic membrane (CM) transporters internalize and, in some cases, reduce the metal to Fe^{2+}, which then enters cytoplasmic metabolic pools [14]. Despite many advances, the exact role of Fe acquisition systems *in vivo* and their effects in pathogenic virulence remain to be determined.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Iron Homeostasis and *Trypanosoma brucei* Associated Immunopathogenicity Development: A Battle/Quest for Iron

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African trypanosomosis is a chronic debilitating disease affecting the health and economic well-being of developing countries. The immune response during African trypanosome infection consisting of a strong proinflammatory M1-type activation of the myeloid phagocyte system (MYPS) results in iron deprivation for these extracellular parasites. Yet, the persistence of M1-type MYPS activation causes the development of anemia (anemia of chronic disease, ACD) as a most prominent pathological parameter in the mammalian host, due to enhanced erythrophagocytosis and retention of iron within the MYPS thereby depriving iron for erythropoiesis. In this review we give an overview of how parasites acquire iron from the host and how iron modulation of the host MYPS affects trypanosomosis-associated anemia development. Finally, we also discuss different strategies at the level of both the host and the parasite that can/might be used to modulate iron availability during African trypanosome infections.

1. Introduction

Iron is a vital nutrient required by nearly every living organism ranging from archaea to eukaryotes. It is an essential cofactor present in heme groups and iron-sulphur clusters and impacts on a broad range of important biological/metabolic processes including host and pathogen cellular functions, erythropoiesis, and immunity. The capacity of iron to fluctuate between two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), makes it indispensable for many critical biological processes, including nucleic acid synthesis/DNA replication, lipid synthesis, protein translation, energy metabolism (cytochrome respiration), oxygen sensing/transport, and oxidant defense [1, 2]. Yet, the distinct oxidative state properties of iron which make iron indispensable also can contribute to toxicity to cells, because of its ability to promote the formation of damaging oxidative radicals. Indeed, the redox cycling of ferrous and ferric iron in the physiological presence of H_2O_2 in the cells results in the formation of reactive oxygen intermediates/free radicals (such as hydroxyl radicals) via the Fenton reaction which in turn can damage lipids, DNA, proteins, and other cellular components. Therefore, regulatory interactions between host iron homeostasis (quantity and subcellular location) and immune function are crucial, since both iron deficiency and iron excess can compromise cellular functions [3]. Access to iron is particularly important in the context of host-pathogen interactions. Indeed, when confronted with infection and inflammation the mammalian host reallocates its iron reservoirs in an effort to deprive invading intracellular or extracellular pathogens of iron [4, 5]. Thus, the control over iron homeostasis is a central battlefield in the course of an infectious disease [6, 7]. The host immune system can regulate iron availability for pathogens via activation of cytokines, cellular proteins/peptides, and hormones, hereby gaining control over pathogen proliferation and strengthening specific immune effector pathways, a strategy also termed "nutritional immunity" [6, 8, 9].

In this review we will give an overview of the role of iron homeostasis/modulation during African trypanosomosis, which is a chronic debilitating disease affecting the health

and economic well-being of many people in developing countries [10–12]. It is caused by strictly extracellular/free-living flagellated unicellular parasites, which are etiologic agents of highly disabling and often fatal diseases of humans (i.e., Human African trypanosomosis, HAT) and livestock (i.e., Animal African trypanosomosis, AAT, or nagana). As recently reviewed [13], African trypanosomes produce a number of components that modulate the mammalian host immune response. In particular, they manipulate cells of the myeloid phagocytes system (MYPS) which includes myeloid cells of the mononuclear phagocytes system (MPS, i.e., macrophages, monocytes, and immature DCs) as well as granulocytes (neutrophils) [14] and thereby affect the capacity of the host to (i) control parasite growth (referred to as resistance to infection) and (ii) to limit tissue pathogenicity caused by the immune response mounted for resistance to infection (referred to as (trypano)tolerance to infection). Trypanotolerance is associated with the sequential induction of IFN-γ and MyD88-dependent M1-type myeloid cells (i.e., classically activated myeloid cells) producing TNF and/or NO which reduce the fitness of the parasite and ensure parasite control, followed by a switch to IL-10 dependent M2-type myeloid cells (i.e., alternatively activated myeloid cells) ensuring pathogenicity control [15]. In contrast, trypanosusceptibility is associated with a persistence of M1-type myeloid cells and an inability to switch to M2-type myeloid cells, which culminates in pathogenicity. In natural and experimental hosts, the control of the African trypanosome load, and thus resistance to infection, is less of a problem than the control of the immune response to mount tolerance to the disease. In this review, we will focus on how African trypanosomes acquire iron within the mammalian host and how iron modulation in host myeloid cells affects trypanosomosis-associated pathogenicity development, whereby anemia development is one of the most prominent parameters.

2. Iron Homeostasis/Acquisition in the African Trypanosome

Trypanosomatids comprise a large group of flagellated unicellular protozoa with a free-living and parasitic lifecycle. The 3 major human diseases caused by trypanosomatids are African trypanosomosis (sleeping sickness caused by *Trypanosoma brucei* sp.), South American trypanosomosis (Chagas' disease caused by *Trypanosoma cruzi*), and Leishmaniasis (caused by different species of *Leishmania*). With respect to the parasites that are the main focus of this review, members of the *T. brucei* complex are transmitted by tsetse flies of the genus *Glossina* spp., which are only present in equatorial Africa. These members can be further divided into the (i) human pathogens *T. b. gambiense* and *T. b. rhodesiense* and (ii) animal pathogens causing either nagana, which is mainly caused by *T. b. brucei*, *T. congolense,* and *T. vivax,* or surra (*T. evansi*) or dourin (*T. equiperdum*). Of note, the trypanosomes of equines (*T. equinum* and *T. equiperdum*) and of camels (*T. evansi*) are not transmitted by tsetse flies but by direct contact during copulation of horses by biting insects such as horse flies (tabanids). The two HAT causing parasites, *T. b. rhodesiense* and *T. b. gambiense*, do not only differ at the level of their geographical distribution; they also differ biologically, clinically, therapeutically, and epidemiologically and cause separate diseases. Indeed, *T. b. gambiense* infection (accounting for over 95% of cases) is found in West and Central Africa and progresses at a more indolent pace (up to 3 years) than that of *T. b. rhodesiense* (accounting for the remainder of cases) causing an acute, rapidly progressive infection (within 6 months) in eastern and southern Africa [11, 21]. Both infections are characterized by two stages, whereby in the first stage parasites are observed in the hemolymphatic system, producing fever, splenomegaly, adenopathies, and cardiac, neurological, and psychological disorders. 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 if untreated [11, 22]. For *gambiense* HAT, human beings are the main reservoir and the predominant mode of transmission is by tsetse flies although sexual and congenital transmission was also reported [23]. *Rhodesiense* HAT, however, is a zoonosis, a "disease or infection naturally transmitted between vertebrate animals and humans," whereby the transmission cycle thus involves mainly transmission between nonhuman reservoirs by tsetse flies, with occasional animal-tsetse-human transfer. Since *T. b. rhodesiense* and *T. b. gambiense* are morphologically identical and also resemble *T. b. brucei* (a subspecies causing nagana), the majority of research used *T. b. brucei* as a model. However, this might not always reflect what is happening in the case of HAT. Identification of both HAT species is based on specific molecular diagnosis markers, that is, the serum resistance-associated (SRA) gene which is restricted to *T. b. rhodesiense* parasites and the *T. b. gambiense* specific gene (TGSGP) which is restricted to *T. b. gambiense* [24, 25]. Alternatively, *T. b. gambiense* can also be diagnosed based on antibodies. According to WHO (World Health Organization, specialized agency of the United Nations serving as the directing and coordinating authority for international health matters and public health), HAT brings about 6 million people at risk of infection within the 36 affected African countries, most of them in rural areas of extreme poverty [22, 26]. Around 300,000 people are currently infected with trypanosomes and 10,000–40,000 of them die every year [11, 27]. Regarding AAT, the economic losses are estimated to be about US$ 1.2 billion per year due to major problems in agricultural and nutritional development of endemic areas [28]. Furthermore, the lack of prospect for vaccine development against African trypanosomosis is strengthened by (i) the fact that pharmaceutical companies are less prone to engage/invest in drug discovery/development of diseases that affect the poorest people, (ii) the political instability of the affected regions, (iii) the fact that wild animals function as reservoir of the parasite and therefore hamper the control of the disease, and (iv) the inappropriate use of the available drugs resulting in the emergence of drug resistance [29–31]. Nevertheless, so far chemotherapy remains the only therapeutic choice for these diseases, whereby they target unique organelles of trypanosomes such as glycosomes and the kinetoplast that are absent in the mammalian host or trypanosome metabolic pathways that differ from the host

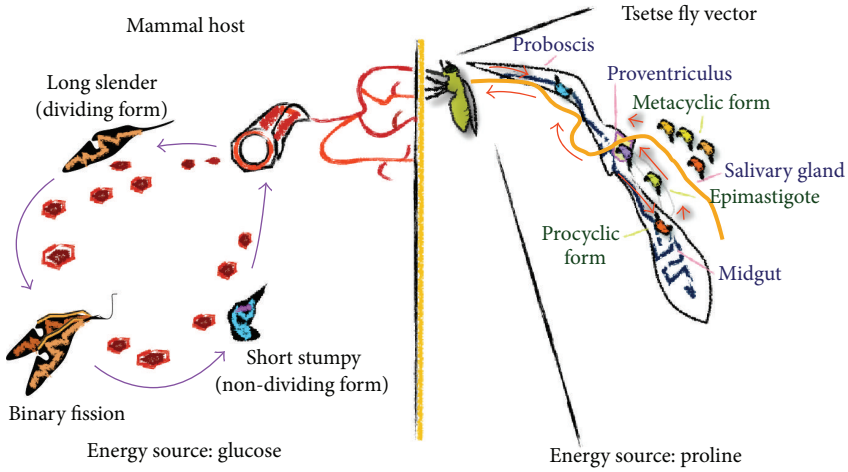

Figure 1: Lifecycle of African trypanosomes. Tsetse flies become infected following a blood meal taken from a trypanosome infected mammalian host. The parasites that are taken up reach the midgut together with the blood meal. Subsequently, only the short stumpy (non-dividing) forms of the parasite that are preadapted to the changed environment within the tsetse fly will be able to differentiate into procyclic forms. The multiplying procyclic forms colonize the ectoperitrophic space, after which they migrate to the salivary glands via the proventriculus lumen to move into the foregut and proboscis. During this migration, the procyclic forms in the fly differentiate into epimastigote forms that within the salivary glands attach to the epithelium and proliferate, giving rise to metacyclic forms which are preadapted for survival into the mammalian host [16, 17]. Upon a blood meal on a new host, the parasites will be inoculated and differentiate into a long slender (dividing) form. Within the mammalian host long slender forms multiply via binary fission, giving rise to a first peak of parasitemia. When the trypanosome population reaches a sufficiently high density, a quorum sensing-like mechanism elicits the differentiation of long slender forms into short stumpy forms that allow transmission following uptake by a new tsetse fly [18, 19]. During the entire lifecycle of trypanosomes, there is a continuous fight for iron acquisition at the level of both the parasites and the host. Image generated by Joar Pinto.

Table 1: Overview of the metabolic changes and differences in energy/iron source used by African trypanosomes during their lifecycle.

	Long slender form	Short stumpy form	Procyclic form
Stage	Proliferative	Quiescent	Proliferative
Surface coat	VSG	VSG	Procyclin (PE/GPEET)
Mitochondrium	Repressed	Repressed/enlarged	Active
Citric acid/respiratory chain enzymes	Absent	Present but not fully active	Present and activated
Energy source	D-Glucose	D-Glucose	L-Proline
Iron source	Tf, Lf, heme, and heme-containing proteins	Tf, Lf, heme, and heme-containing proteins	heme, heme-containing proteins

counterparts (carbohydrate metabolism, protein and lipid modifications, and programmed cell death) [32].

African trypanosomes have a strictly extracellular heteroxenous life cycle alternating between the intestine of the tsetse fly and the blood/tissues of the mammalian host (see Figure 1), whereby they exist as procyclic or trypomastigote forms, respectively [33]. Briefly, upon a bite of a trypanosome infected tsetse fly, metacyclic parasites are inoculated into the blood circulation of the mammalian host. The parasites immediately differentiate into long slender forms (dividing forms), which are adapted to survival in the glucose-rich and highly oxygenated blood of the host and multiply, thereby giving rise to a first parasitemia peak. Once a peak is reached, most likely due to quorum sensing [34], the long slender parasites differentiate into short stumpy forms (non-dividing forms), which are preadapted for survival in the tsetse fly vector (see Figure 1). Within the tsetse fly vector the parasites differentiate into procyclic forms which are adapted to survive in the proline-rich (carbon source) and low oxygenated environment. To this end, trypanosomes undergo important metabolic and morphological changes to adapt to the growth conditions imposed by the different hosts and environments they inhabit (Table 1) [33]. They have acquired elaborate mechanisms to adapt/survive in the different hosts such as fine tuning of energy metabolism, organelle reorganization, dedicated nutrient uptake, and biochemical and ultrastructural remodeling [35–38]. In particular, pathogenic trypanosomes have developed different mechanisms to guarantee iron supply from their host [39–41].

2.1. Iron Homeostasis/Acquisition by Bloodstream T. brucei Parasites. Trypanosomes in contrast to mammalian cells only require small amounts of iron [40], due to the fact that the bloodstream form lacks cytochromes and contains

only 4 iron-dependent enzymes (aconitase, alternative oxidase, ribonucleotide reductase, and superoxide dismutase) [42–46]. Within the bloodstream of the mammalian host, trypanosomes scavenge iron via a high affinity receptor-mediated endocytosis of iron-bound transferrin (Tf), which is referred to as holo-Tf [47, 48]. This heterodimeric transferrin receptor (Tf-R), which is unable to discriminate between holo- and apo-Tf (iron-free Tf) [39, 49, 50], is encoded by the expression-site-associated genes (ESAG) 6 and 7, whereby the Tf-R protein is composed of one molecule of pESAG6 containing a COOH-terminal glycosylphosphatidylinositol (GPI) membrane anchor and one molecule of pESAG7 devoid of this modification [51, 52]. The heterodimer binds one molecule of Tf giving rise to a ternary complex. This low abundant glycoprotein (about 3000 molecules/cell) is located in the flagellar pocket and its expression is regulated by iron availability and posttranscriptional control mechanisms that does not involve the IRE/IRP1 (iron regulatory proteins/iron responsive elements) system typical for mammals [52, 53]. It was shown that iron starvation (using iron chelators or species specific Tf) leads to a 3–10-fold upregulation of Tf-R with a concomitant redistribution of the receptor from the flagellar pocket to the entire parasites' surface [52, 54]. Furthermore, it was shown that during chronic trypanosomosis in cattle the host Tf level is decreased, yet the bloodstream pathogens develop the ability to grow at very low iron concentrations by increasing their Tf-R expression levels thereby allowing higher Tf uptake [54, 55]. In addition, *T. b. brucei* has about 15 VSG expression sites (VSG-ES); only one is transcribed at a given time, while the others remain repressed, providing the expression of a particular combination of ESAGs in a mutually exclusive manner. The transcriptional activation/inactivation of genes in the VSG-ESs is a highly regulated mechanism, potentially allowing the parasite to quickly respond to any environment change. Furthermore, the different copies of ESAG6/7 sequences are highly polymorphic in regions corresponding to Tf binding sites, whereby small changes in the amino acids present in the surface exposed-loops drastically affect the affinity of the receptor for a given Tf, thereby contributing to an additional mechanism of trypanosomes to acquire iron and to permit their rapid adaptation in distinct hosts [56, 57]. Importantly, similar observations as for the *T. b. brucei* parasites with respect to the ESAG6/7 were observed for the *T. b. rhodesiense* and *T. b. gambiense* parasites [58, 59]. This Tf-R polymorphism which allows selecting for high affinity Tf-Rs together with the rapid recycling of Tf-R and gene-specific activation events enables trypanosomes to efficiently compete for limiting substrate and withstand iron deprivation until a new set of Tf-R is expressed [55, 60, 61]. The Tf-R which has an affinity 50–1000 nM for mouse/human Tf is exclusively present in the mammalian bloodstream stage form of the parasite and its structural organization differs completely from the mammal counterpart [58, 62]. Following binding of iron-bound Tf, the Tf-R is endocytosed in a temperature- and energy-dependent manner, which involves proteins like dynamin, epsin, the adaptor AP-2, the small GTPase TbRab5A, β-adaptin, and clathrin [63–65]. In addition, it was suggested that the phosphatidylinositol-3 kinase (PI-3K) TbVPS34 also plays a role in Tf trafficking, possibly downstream of TbRAb5A GTPase [66]. Upon cleavage of the intracellular GPIs by the GPI-phospholipase C (GPI-PLC) expressed in bloodstream *T. brucei*, producing diacylglycerol (DAG) and inositolphosphoglycan, DAG receptors are activated. Subsequently, the DAG signaling pathway is activated 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 [67]. It was suggested that DAG stimulation of Tf uptake may contribute to parasite virulence by aiding *T. brucei* to acquire sufficient amounts of Tf (i.e., iron) to sustain its extracellular existence and compete with host cells for Tf in the blood [67]. Due to acidification within the endosomes, the iron is released from Tf, while the ESAG6/7 Tf-R complex loses affinity for apotransferrin [68]. Subsequently, while (apo)transferrin is delivered to the lysosome and proteolytically cleaved via a *T. brucei* cathepsin B-like protease TbcatB [53, 69, 70] and degraded fragments are exported/exocytosed from the cell via TbRAb11 positive recycling vesicles [71], the ESAG6/7 Tf-R is recycled back to the flagellar pocket via TbRAb11 positive recycling vesicles [72]. This is in contrast to the uptake of mammalian iron-bound Tf where the entire Tf-TfR complex is recycled to the cell surface (see later). How iron gets from the endolysosomal system to the cytoplasm is under investigation, but this involves possibly divalent metal ion transporters as in the mammalian host (see later). Given that Tf-bound iron (Fe^{3+}) is practically insoluble at physiological pH and temperature, it must first be reduced to Fe^{2+}, most likely through a ferric reductase, in order to be exported from the endolysosomal system into the cytoplasm. Interestingly, in the *T. brucei* genome, two putative ferric reductases have been found, a cytochrome b561-type (Tb927.6.3320) and an NADPH-dependent flavoprotein (Tb11.02.1990), which could act in cooperation with some divalent putative cation transporters [39]. In this context, recently, Taylor et al. [73] showed that a *T. brucei* Mucolipin-like protein TbMLP, orthologous to the mammalian endolysosomal cation channel Mucolipin 1, is involved in import of iron into the cytosol of African trypanosomes. It is expressed in both bloodstream and insect stages of the parasite and is confined to the endocytic system, with the highest expression being found in the p67-positive compartment (i.e., the lysosome). Yet, they also indicate that even when TbMLP expression is greatly reduced, there is sufficient iron import. Thus, an alternative mechanism to provide the parasite with an adequate supply of cytosolic iron needed for synthesis of iron-containing proteins is not excluded. For instance, it is suggested that intracellular iron is not homogeneously distributed. Excess iron is presumably transported to a storage compartment from which it can be released if cytosolic iron falls under a certain level. The signal for Tf-R upregulation could, therefore, come from a decrease in cytosolic iron. How the trypanosome monitors cytosolic iron is not known; however, cytosolic aconitase, the iron sensor in mammalian cells, is not involved in *T. brucei* iron sensing.

Besides the Tf-R, *T. brucei* parasites can also acquire iron via the uptake of iron-binding Lactoferrin (LF), a member

of the Tf family protein found in most biological fluids of mammals and secondary granules of polymorphonuclear cells (PMN) [74]. One of the receptors involved in LF binding was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [74], an enzyme typically involved in catalyzing several steps in glycolysis (i.e., breakdown of glucose for energy and carbon molecules) and also found to be involved in several nonmetabolic processes including transcription activation and initiation of apoptosis. It is considered to be a member of the moonlighting or multifunctional proteins [75]. Iron can also be acquired via the uptake of heme, which consists of a cyclic tetrapyrrole ring (protoporphyrin IX) that coordinates an iron atom which can adopt Fe^{3+} or Fe^{2+} oxidation states. This essential cofactor for proteins is involved in oxygen transport and storage (hemoglobin and myoglobin), mitochondrial electron transport (complexes II–IV), steroid metabolism (cytochromes), signal transduction (nitric oxide synthase), and transcription and regulation of antioxidant-defense enzymes (e.g., superoxide dismutase, catalase, and peroxiredoxins). Heme is also a regulatory molecule involved in gene transcription/translation [76, 77]. Given that trypanosomes are auxotrophic for heme [78], they have adapted their heme-dependent metabolic pathways (biosynthesis of sterols and polyunsaturated fatty acids, respiration, oxidative stress response, and detoxification) to fluctuations in nutritional availability across their life cycle [77]. Host heme cannot diffuse through the parasites' membrane due to the fact that it contains cationic carboxylate side chains. Thus, trypanosomes have evolved high affinity heme-binding proteins on their cell surface. To this end, the parasites express an haptoglobin-hemoglobin receptor (HpHbR), which is linked to the plasma membrane through a C-terminal GPI anchor and localized in the flagellar pocket of bloodstream parasites only [79, 80]. HpHbR also plays a central role in determining whether humans can be infected by trypanosomes [79]. Most species of trypanosomes, such as *T. b. brucei*, are unable to infect humans due to the trypanolytic serum protein apolipoprotein-L1 (APOL1) delivered via two trypanosome lytic factors (TLF-1 and TLF-2). Binding of TLF1 to the HpHbR results in endocytosis and lysosomal localization of the toxin, apoL-I, and subsequent death of the parasite. However, *T. brucei rhodesiense* and *T. brucei gambiense* have managed to resist this lysis mechanism. Indeed, *T. brucei rhodesiense* expresses the ApoL1 neutralizing serum resistance-associated (SRA) protein, which is a truncated version of the variant surface glycoprotein (VSG), which binds to APOL1 in the lysosome and hence prevents lysis [81]. *T. brucei gambiense* has besides a single highly conserved amino acid mutation in the TbgHpHbR thereby ablating high affinity TLF-1 binding and subsequent endocytosis, also a *T. b. gambiense* specific gene, *TgsGP*, which is also a truncated version of VSG [82]. With respect to heme transport and distribution in trypanosomes, these mechanisms remain so far elusive. Moreover, since African trypanosomes lack heme oxygenase and ferrochelatase required for heme catabolism and extraction of iron, the simplest explanation could be that scavenged heme is incorporated directly into heme-proteins which are distributed throughout different subcellular compartments without intermediate steps [78].

2.2. Iron Homeostasis/Acquisition by Insect Stage T. brucei Parasites. Switching from the mammalian host nutrient rich environment to the tsetse fly vector nutrient-poor environment requires a change in the energy metabolism of the parasites in order to survive. In contrast to the bloodstream form stage of trypanosomes where iron uptake occurs mainly via a Tf-R mediated mechanism, the iron uptake mechanisms in the procyclic insect stage form of *T. brucei* are less characterized. It is accepted that procyclic forms efficiently take up iron from ferric complexes via a reductive mechanism [83]. Surprisingly, although the insect stage requires more iron, the rate of endocytosis is greatly reduced compared to the bloodstream stage [35, 84]. This can be explained by the activation of a mitochondrial respiratory chain for energy metabolism in the insect life stage of the parasite [43, 85]; the bloodstream form of *T. brucei* shows a rudimentary mitochondrion but through the activity of an alternative oxidase and well-developed glycosomes depends on glycolysis for its energetic metabolism while the procyclic form of *T. brucei* presents a well-developed mitochondrion and low glycosomal activity. Given that the procyclic form (i) requires active mitochondria which are one of the most important heme-protein containing organelles and (ii) resides in the midgut of the tsetse fly where hemoglobin digestion following a blood meal results in massive release of free heme, uptake of heme or heme-containing proteins might be more important in this stage [86, 87]. The *T. brucei* vacuole protein sorting 41 (TbVPS41) plays an important role in the intracellular iron utilization system as well as the maintenance of normal cellular morphology in the procyclic form of the parasite [88]. In addition, it was shown that iron-sulphur (FeS) cluster proteins involved in a variety of cellular processes including electron transport and gene expression, such as the Rieske protein and cytochrome c reductase (complex III), in which iron atoms in different activation states are coordinated with inorganic sulphur in addition to cystein thiol groups, contribute to incorporation of heme into apo/heme-proteins [78, 89], which are involved in essential metabolic pathways like biosynthesis of sterols and polyunsaturated fatty acids (PUFAs) carried out in the endoplasmic reticulum (ER) and respiratory complexes in the mitochondrion [77].

3. Iron Homeostasis/Acquisition in the Mammalian Host during Steady-State Situation

Given that African trypanosomes multiply in the mammalian bloodstream as extracellular parasites, they continuously depend on host nutrient supply but are also confronted with the host's immune system. Hereby, the mammalian host immune system regulates iron availability for pathogens to gain control over pathogen proliferation and to strengthen the specific immune effector mechanisms via cytokines, cellular proteins/peptides, and hormones. Over the years, the molecular mechanisms involved in the regulation of iron homeostasis in the mammalian host have become increasingly clear [90, 91]. These mechanisms are summarized in the next paragraph since they provide the conceptual framework

for our investigations into the role of iron in host-African trypanosome interactions.

Free iron or heme-bound iron uptake/absorption occurs primarily in the duodenum across the apical mucosa of duodenal epithelial cells, whereby intestinal heme iron uptake occurs through the interaction with the heme carrier protein (HCP1). The iron in the heme is then released within the enterocytes via the action of the heme catabolizing enzyme heme oxygenase (HO-1) and the dietary ferric iron (Fe^{3+}) is reduced to the ferrous (Fe^{2+}) state by ferric reductases at the level of the enterocytes [92, 93]. Subsequently, ferrous iron is transported into the cell via the divalent metal ion transporter 1 (DMT1, Nramp2 (natural resistance-associated macrophages protein)/solute carrier family 11, member 2 (SLC11A2)), after which it can be used for cellular processes or stored intracellularly by ferritin or exported from the cell at the basolateral membrane to the plasma via the sole iron exporter ferroportin-1 (FPN-1, SLC40A1), depending on the hosts' requirements for iron. Associated with ferroportin is the enzyme hephaestin (a copper-containing ferroxidase with homology to ceruloplasmin (see later)) which oxidizes the ferrous form back to the ferric form. Once in the circulation, liver secreted Tf will bind one or two ferric iron molecules and transport iron in the serum and extravascular spaces where it serves as a source of iron for cells and tissues that are perfused by the systemic circulation, including liver, heart, muscle, kidney, and bone marrow. Hereby, two main factors determine iron absorption; (i) the amount of iron present in body stores and (ii) the need of iron for hematopoiesis/erythropoiesis [94]. Important to mention is that the amount of iron absorbed via alimentation is insufficient to meet the daily physiologic needs of the body. Therefore, the bulk of iron needed for homeostasis (mainly for red blood cell (RBC) production) is provided by the MYPS, more specifically the myeloid cells, that recycles senescent RBCs at the level of the liver, spleen, and bone marrow via erythrophagocytosis and allows their iron reutilization [95] (see Figure 2). The majority of the body iron in mammals is sequestered intracellularly and complexed within the heme moiety of hemoglobin inside RBCs. Catabolism of RBCs results in heme release which in turn is further processed via heme-oxygenase 1 (HO-1) to give rise to equal amounts of iron, biliverdin, and carbon monoxide [96]. Once iron is released from heme it follows the same pathway as iron released from the holo-Tf/Tf-R complex, whereby it is either stored in the cell by ferritin or exported via ferroportin-1 (see further). Under physiological conditions, iron recycling by macrophages accounts for approximately 95% of the daily needs of iron for erythropoiesis and other physiological processes [97, 98]. Given that there is no regulated pathway to excrete iron from the body, the iron balance is primarily preserved by the regulation of iron absorption from the duodenum and iron recycling from myeloid cells and other tissue stores (primarily within hepatocytes) [99].

Iron exported from enterocytes or cells from the MYPS is bound with high affinity to transferrin (Tf), a glycoprotein produced in the liver and able to bind one or two iron molecules. Tf has a dual function (i) limiting iron-catalyzed free radical production and (ii) facilitating iron transport to all cells within the host that requires iron [100]. Alternatively, circulating copper-carrying ceruloplasmin can also participate in iron transport [101]. Under steady state situations iron-bound Tf (holotransferrin) is taken up via endocytosis by cells that express the Tf receptor (Tf-R) in order to fulfill normal metabolism, DNA synthesis, and RBC production. Important to mention is that the mammalian Tf-R is a homodimeric transmembrane glycoprotein consisting of two identical monomers joined together by two disulphide bonds and composed of a short cytoplasmic NH2-terminal cytoplasmic region (residues 1–67), a single transmembrane pass (residues 68–88), and a large extracellular portion (residues 89–760) containing the Tf binding region. It can bind two molecules of Tf with an affinity of 10 nM [102–104]. There are two isoforms, Tf-R1 (expressed by all nucleated cells) and TfR-2 (restricted to hepatocytes and immature erythroid cells), present within the mammalian host that are different from the trypanosomal Tf-R. In addition, Tf-R1 levels are regulated by cellular iron levels while Tf-R2 levels are regulated by Tf saturation and reported to bind diferric Tf although with 25 times lower affinity than Tf-R1. Also at the level of the kidney, some Tf which is an essential growth factor in the development of kidney and differentiation of tubules normally enters glomerular filtrate, but it is mainly retrieved by specific receptor-mediated uptake in the kidney tubular system. The Tf-R is expressed on the apical membrane of proximal tubule and collecting duct cells. Also, in the proximal tubule, the cubilin receptor, which is highly expressed on the apical membrane of kidney proximal tubules, is thought to mediate uptake of Tf [105]. Of note, similar to trypanosomes, mammals are able to bind Tf via GADPH, albeit with lower affinity [106, 107].

Internalization of the iron-Tf-R complexes is initiated following receptor phosphorylation by protein kinase-C (PKC). Following uptake of holo-Tf, the lower pH of the endosome/phagolysosome triggers the release of iron and recycling of the apo-Tf/Tf-R complex to the cell surface, whereby the apo-Tf is released due to the neutral pH. Subsequently, ferric iron is reduced and transported into the cytoplasm by Nramp1 (a divalent metal transporter homologous to DMT1 expressed at the phagolysosomal membrane) where its faith depends on the cellular needs: (i) stored in ferritin (a large globular protein-complex able to bind up to 4500 iron molecules) or (ii) exported/released back into circulation via the basolateral transmembrane iron transporter ferroportin (FPN1/SLC40A1) followed by oxidization of the ferrous form back to the ferric form via ceruloplasmin. As mentioned before, to meet the cellular needs of the body and to prevent cellular damage by iron, the amount of iron in the body must be tightly regulated. Hereby, the liver is a central regulator of systemic iron homeostasis through secretion of the 25-amino-acid peptide hormone hepcidin mainly by hepatocytes [99, 108, 109]. Furthermore, the hepcidin production is also regulated via different triggers, whereby iron levels, proinflammatory cytokines (such as IL-1, IL-6, and IL-22), TLR activation, or endoplasmatic reticulum stress stimulate its production, whereas erythropoiesis, anemia, hypoxia, hormones (estrogen), and growth factors (epidermal growth factor, hepatocyte growth factor) decrease its production

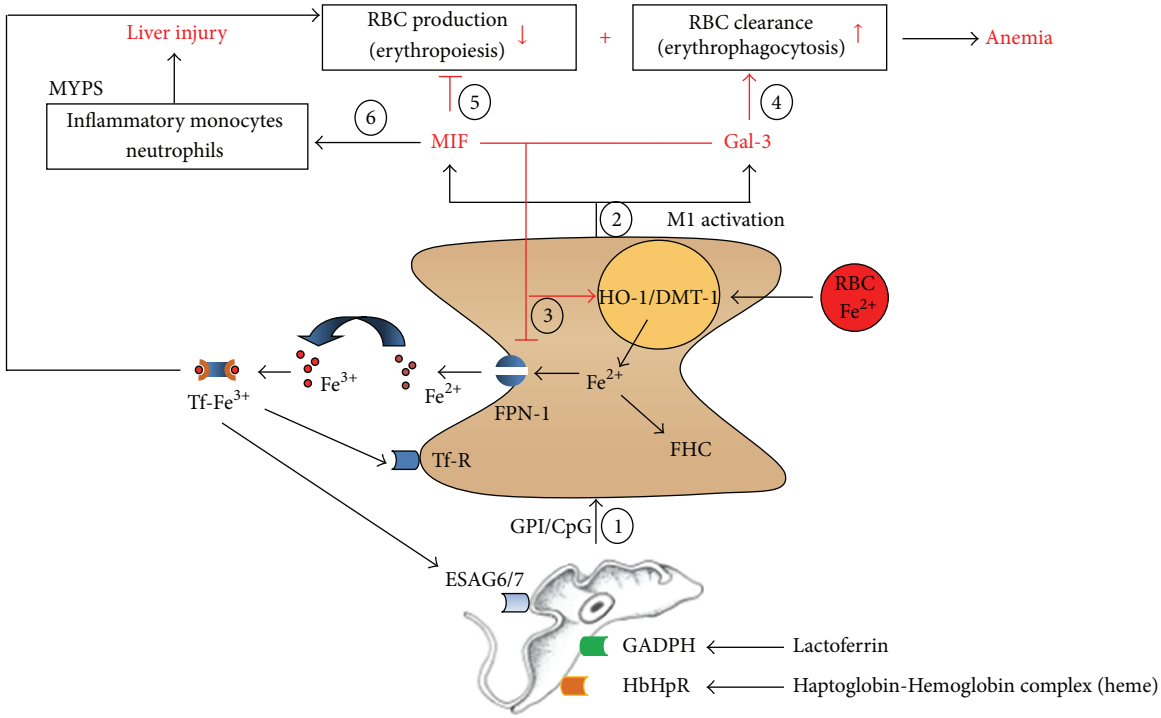

Figure 2: Iron modulation and pathogenicity development during *T. brucei* infection. Trypanosomes are equipped with different molecules to acquire iron from the mammalian host, namely, via their ESAG6/7 Tf-R (involved in Tf uptake), GADPH Lf-R (involved in Lf uptake), and the HbHpR (involved in heme/hemoglobin uptake). Parasites release molecules like the GPI-anchor or CpG-DNA to modulate/activate the host MYPS for their own benefit (1). Most of these molecules released by death or phagocytosed parasites trigger the release of proinflammatory molecules by M1-type myeloid cells, including Gal-3 and MIF (2). Both molecules stimulate iron-retention by inducing expression of HO-1, DMT-1, and FHC and by decreasing expression of FPN-1 within M1-type MYPS cells (3). Gal-3 by stimulating erythrophagocytosis (4) and MIF by suppressing erythropoiesis (5) contribute to anemia development. Due to their antiapoptotic effect, Gal-3 and MIF favor the persistence of the pathogenic M1-type MYPS. Moreover, MIF contributes to the recruitment of other pathogenic myeloid cells such as monocytes and neutrophils and further fuels the development of liver injury (6). GPI: glycosylphosphatidylinositol; Gal-3: galectin-3; MIF: macrophage migration inhibitory factor; Tf: transferrin; Tf-R: transferrin-receptor; Lf: lactoferrin; Lf-R: lactoferrin-receptor; HO-1: heme oxygenase 1; DMT-1: Divalent metal ion transporter 1; FPN-1: ferroportin-1; ESAG6/7 Tf-R: expression-site-associated genes (ESAG) 6 and 7; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HpHbR: haptoglobin-hemoglobin receptor; MYPS: myeloid phagocyte system. Figure adapted from [20].

[94, 110–114]. Of note, neutrophils and myeloid cells can also synthesize minute amounts of hepcidin in response to infectious agents, thereby allowing the modulation of iron availability in an autocrine fashion at the infectious focus [115, 116]. Following binding of hepcidin to the principal iron exporter FPN-1 at the cell surface, this latter is internalized and subsequently lysosomally degraded [117]. As a result, the export of iron is blocked, and iron is sequestrated at the level of the enterocytes, myeloid cells, and hepatocytes [109]. As mentioned before, limiting the iron availability for extracellular pathogens is considered to be a defense mechanism of the body, yet, reducing the levels of circulating iron can also culminate in anemia development [1, 118].

4. Iron Homeostasis/Acquisition in the Mammalian Host during African Trypanosome Infection

In livestock populations, anemia is considered the major cause of death during African trypanosomosis, and the capacity to limit anemia is critical in determining trypanotolerance [119]. The occurrence in infected cattle of hyper activated M1-type myeloid cells and massive erythrophagocytosis by tissue-associated MYPS cells as well as a modulated iron homeostasis suggests that these factors can be major causes of anemia [120, 121].

To unravel the mechanisms underlying African trypanosomosis-elicited anemia development, murine models (focusing on *T. brucei* trypanosomosis) exhibiting different degrees of anemia development were scrutinized. Although these mouse models show limitations, they have contributed significantly to our current understanding of trypanosomosis-associated anemia development [13, 20, 122]. In *T. brucei*-infected mice, anemia level does not correlate with parasitemia levels, antibody and T-cell responses, or survival time [119, 123], similar to bovine trypanosomosis [119, 124], suggesting that anemia is a consequence of the host immune response rather than of a direct influence of parasite products on RBC viability.

Anemia development during the course of experimental *T. brucei* infection can be divided into two phases

(reviewed in [20, 125]): (i) an acute phase whereby M1-activated myeloid cells (i.e., classically activated myeloid cells) eliminate/phagocytose RBCs mainly in the liver and the spleen, resulting in activation of pathways that govern iron homeostasis and (ii) a short-lived and partial recovery of RBC levels, which is most likely due to extramedullar erythropoiesis (at the level of the spleen and liver) in response to the acute anemia in an attempt to restore RBC numbers, followed by a chronic and progressive phase. During this phase, persistence of M1-type cells of the MYPS reduces iron bioavailability by retaining iron in storage sites within the MYPS, thereby diverting iron from erythropoiesis. In this chronic phase, the enhanced uptake of both RBCs and iron-containing compounds is maintained, which aggravates anemia development [125]. The upregulation in whole tissue and cells of the MYPS of molecules involved in import (HO-1/DMT-1) and storage (FHC) of iron and downregulation of the cellular iron export regulator (FPN-1) provides additional evidence for an augmented liver and spleen iron-metabolism and accelerated senescence of RBCs during African trypanosome infection in trypanosusceptible animals (Figure 2) [125, 126]. Moreover, erythropoiesis was shown to be suppressed during the course of trypanosome infections [126–128]. It should also be remarked that iron accumulation in M1-type cells of the MYPS can also contribute to oxidative stress and NF-κB activation [129–131], thereby contributing to liver pathogenicity occurring during African trypanosomosis [132, 133]. Thus, although limiting iron availability for pathogens during the acute phase of infection as an "iron withholding strategy" can prevent parasite development and benefit the host [1, 2], persistence of this response in the chronic phase of infection can disadvantage the host. Indeed, iron sequestration by MYPS cells can fuel their M1-type activation status and limit iron availability for erythropoiesis [134], thereby contributing to persistence of anemia.

5. Immune Modulation of Iron Homeostasis in the Mammalian Host during African Trypanosome Infection

The anemia induced during African trypanosomosis in mice, which is characterized by an imbalance between erythrophagocytosis and erythropoiesis and by an altered iron recycling and sequestration by MYPS cells, relates to the so-called anemia of chronic disease (ACD) [20, 90]. Studies over the past years aiming at unraveling the underlying mechanisms involved in anemia development during *T. brucei* infection have shown the following:

(i) IFN-γ, TNF, TNF-R2, and MyD88-deficient mice exhibit lower anemia levels as compared to control wild-type C57Black/6 mice [135–138]. Accumulated data suggest that the MyD88-dependent activation of the innate immune response results in the induction of IFN-γ by T cells. Subsequently, TNF production is triggered, whereby TNF-R2 signaling plays a key role in the induction of pathogenicity. In fact, the increased ratio of TNF over its soluble TNF-R2, not TNF levels per se, relates to the occurrence of infection-associated anemia [136].

(ii) The activation state of cells of the MYPS plays a detrimental role in pathogenicity development, whereby M1-type MYPS cells (classically activated myeloid cells) in trypanosusceptible animals (exhibiting severe anemia/ACD) triggered via IFN-γ and/or molecules acting via TLR signaling promote the production of proinflammatory cytokines (such as TNF and IL-6) and the sequestration of iron. In contrast, M2-type MYPS cells (alternatively activated myeloid cells) in trypanotolerant animals (exhibiting reduced anemia/ACD) induced via IL-10 or IL4/IL13 promote induction of anti-inflammatory cytokines like IL-10 (crucial for dampening the pathogenic effects of proinflammatory cytokines) and export of iron [139–141]. Moreover, trypanotolerant animals in contrast to trypanosusceptible animals exhibit a restored iron homeostasis and increased iron availability for erythropoiesis [127].

(iii) A comparison between trypanosusceptible and trypanotolerant animals allowed identification of two host-derived pleotropic molecules, Galectin-3 and macrophage migration inhibitory factor (MIF), which are strongly upregulated in *T. brucei* infected mice. Galectin-3 (Gal-3), a lectin contributing to the onset and persistence of type-1 inflammatory responses and phagocytosis, was shown to be involved in trypanosomosis-associated anemia development [142]. Hereby, *T. brucei* infected galectin-3 deficient mice exhibit greatly reduced anemia levels coinciding with a restored iron homeostasis and an increased IL-10 level that in turn leads to reduced liver destruction. With respect to MIF, we recently showed that MIF deficient animals feature limited anemia, which coincides with a reduced proinflammatory immune response, an increased iron bioavailability, improved erythropoiesis, reduced RBC clearance, and increased IL-10 levels associated with decreased liver injury during the chronic phase of infection [143]. In addition, neutrophil-derived MIF contributed more than monocyte-derived MIF to pathogenicity during *T. brucei* infection. Collectively, these results suggest that the M1-type MYPS cell associated molecules galectin-3 and MIF both promote the most prominent pathological features of experimental trypanosome infections (anemia and liver injury) (Figure 2).

Besides identifying host-derived molecules involved in triggering/maintaining M1 cells and consequently being detrimental in anemia development/iron sequestration, the identification of parasites-derived molecules triggering M1-type MYPS cell development could have potential therapeutic applications. In this context, the trypanosomal GPI-anchor was shown to be the most potent parasite-derived molecule able to trigger M1-type myeloid cells [144, 145]. Moreover, a GPI-based intervention strategy was found to alleviate trypanosomosis-associated anemia development by lowering

the proinflammatory cytokine production (including TNF, MIF, and Gal-3) and increasing IL-10 production [146]. In addition, this treatment strategy restores iron homeostasis at the level of the liver (increased iron export and reduced storage) and increases erythropoiesis in the bone marrow and the spleen [127]. This suggests that reprogramming MYPS cells towards an anti-inflammatory state can be a promising tool to alleviate ACD, normalize iron homeostasis, and restore erythropoiesis. Although most trypanosomes cannot be considered natural pathogens for rodents, murine models represent excellent tools to study trypanosome biology and their interaction with the mammalian immune response. Furthermore, research so far on HAT patients suggests that TNF is also involved in immunopathogenesis of late stage African trypanosomosis and that IL10 plays an important regulatory role in the disease [122]. In addition, chemokines documented to contribute to pathogenesis such as CCL2 and CXCL10 within the murine model were also found in cerebral spin fluid (CSF) of HAT patients [147–149]. Yet, more additional work should be devoted to determine whether results from the murine model reflect observations in HAT patients.

6. Blocking Iron Uptake/Homeostasis at the Level of the African Trypanosome

Iron deprivation may represent a new strategy for treatment of African trypanosomosis. However, so far there is only a limited amount of drugs documented to block trypanosomal iron uptake or metabolism. The iron chelator/siderophore deferoxamine/desferoxamine (DFO) produced by *Streptomyces pilosus* has been developed into the drug Desferal which is used for the treatment of acute iron poisoning and chronic iron-overload. DFO also stops *T. brucei* parasite growth *in vitro* [150, 151]. This compound does not inhibit iron-containing enzymes directly but acts by chelating cellular iron, thus compromising the activity of Fe^{3+}-containing enzymes such as ribonucleotide reductase, which is involved in DNA synthesis, and thereby preventing its incorporation into newly synthesized apoproteins. In their quest to identify novel iron-chelating molecules, the group of Merschjohann and Steverding [150] has shown that although most iron chelators tested so far also display some cytotoxicity to mammalian cells, only lipophilic iron-chelating agents represent potential as novel antitrypanosomal drugs. However, so far there are no published studies of the effect of direct iron chelation on *T. brucei* infection in animal models.

7. General Conclusions/Perspectives

Given that all organisms on earth depend on iron to fulfill vital cellular functions, there is a continuous quest of both pathogen and host to acquire this primordial metal. As far as parasites like African trypanosomes are concerned, their complex lifecycle alternating between the tsetse fly vector and the mammalian host adds an additional problem in the struggle for the supply of this metal.

At the level of the mammalian host, the concept that polarization of cells of the MYPS into distinct M1-type or M2-type activation states contributes to trypanosusceptibility or tolerance, respectively, suggests that reprogramming of MYPS cells may provide new therapeutical modalities in the treatment of infection-associated pathogenicity development [15]. However, additional research is required to dissect the exact contribution of the different liver and spleen associated MYPS cell subsets (Ly6c+ and Ly6c− monocytes, resident and Ly6c+ monocyte-derived macrophages, granulocytes, and dendritic cells) in erythrophagocytosis or modulation of iron homeostasis and to Gal-3 or MIF production. Recently, a mammalian MIF homologue D-dopachrome tautomerase (D-DT or MIF2) has been identified [152], but its role during African trypanosomosis-associated pathogenicity development (anemia) remains to be investigated.

At the level of the parasites, strategies able to block parasites' endocytosis of iron-containing molecules can form alternative approaches to control parasite development and survival [153–156]. Hereby, blockage of uptake of iron or of iron-containing compounds via antagonists or antibodies is promising. For instance, the specificity of the trypanosomal (ESAG6/7) Tf-R makes it a potential target to deliver toxic molecules inside the parasite [47]. In this context, a therapy based on nanobodies (Nbs) which are camelid derived single-domain antibody fragments [157] was found efficient in targeting drugs to African trypanosomes [158]. Also, Nbs able to block endocytic capacity of the parasite were found to block transferrin uptake thereby killing the trypanosome [156]. Moreover, a functional *T. brucei* Tf-R was expressed in insect cells which 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 [159]. Alternatively, the GPI-biosynthesis pathway which is crucial in parasites' viability may represent another therapeutic approach for trypanosomosis [160]. In addition, future efforts should also aim at improving the selectivity of iron chelators, for instance, by utilizing the wealth of information currently being generated in the development of cell-permeable iron chelators as cancer chemotherapeutic agents [161]. Moreover, iron chelators could be of interest for combination therapy with existing antitrypanosome drugs like, for instance, DFMO (eflornithine) [31]. Indeed, eflornithine (Ornidyl), by blocking polyamine (spermidine) biosynthesis and consequently by preventing the production of the antioxidant trypanothione synthesis by the African trypanosome [162], may cause oxidative stress by increasing the level of iron available for the Fenton reaction [163, 164]. However, it should be taken into consideration that the administration of iron chelators is not exempt from risks for the host due to their iron withholding activity which may lead to anemia. African trypanosomes are heme auxotrophs and are dependent on specialized transporters to import heme [165, 166]. Drug targeting of this transport pathway may be more valuable to target the insect stage of African trypanosomes where the heme import is more important than for the mammalian bloodstream stage of the parasite.

Despite the progresses achieved in the last years, more studies on the role of iron in the parasite development and on

modulation of iron metabolism in infected hosts are required before translation of this knowledge into effective treatments.

Conflict of Interests

The authors declare no conflicting financial interests.

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Research Article

Binding and Endocytosis of Bovine Hololactoferrin by the Parasite *Entamoeba histolytica*

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Entamoeba histolytica is a human parasite that requires iron (Fe) for its metabolic function and virulence. Bovine lactoferrin (B-Lf) and its peptides can be found in the digestive tract after dairy products are ingested. The aim of this study was to compare virulent trophozoites recently isolated from hamster liver abscesses with nonvirulent trophozoites maintained for more than 30 years in cultures *in vitro* regarding their interaction with iron-charged B-Lf (B-holo-Lf). We performed growth kinetics analyses of trophozoites in B-holo-Lf and throughout several consecutive transfers. The virulent parasites showed higher growth and tolerance to iron than nonvirulent parasites. Both amoeba variants specifically bound B-holo-Lf with a similar K_d. However, averages of 9.45×10^5 and 6.65×10^6 binding sites/cell were found for B-holo-Lf in nonvirulent and virulent amoebae, respectively. Virulent amoebae bound more efficiently to human and bovine holo-Lf, human holo-transferrin, and human and bovine hemoglobin than nonvirulent amoebae. Virulent amoebae showed two types of B-holo-Lf binding proteins. Although both amoebae endocytosed this glycoprotein through clathrin-coated vesicles, the virulent amoebae also endocytosed B-holo-Lf through a cholesterol-dependent mechanism. Both amoeba variants secreted cysteine proteases cleaving B-holo-Lf. These data demonstrate that the B-Lf endocytosis is more efficient in virulent amoebae.

1. Introduction

Entamoeba histolytica is an extracellular parasitic protozoan that causes amoebiasis, an infection that affects humans worldwide. Cysts are the infective stage transmitted via the fecal-oral route through the intake of contaminated food and water. When cysts are ingested, they tolerate the acidic pH of the stomach, and excystation occurs in the terminal ileum, producing the invasive stage or trophozoites (amoebae). *E. histolytica* trophozoites adhere to and invade the mucosa of the large intestine, ultimately causing dysentery, ulcers, fever, and abdominal pain. In addition, by an unknown mechanism, trophozoites occasionally travel to the liver via the portal vein, producing amoebic liver abscesses (ALA), which can be fatal if not treated. Amoebae also invade other organs, especially the brain and lungs [1]. Amoebiasis is the third most common cause of death by parasites, particularly in developing countries [2, 3].

During evolution, pathogens developed diverse strategies to obtain iron from host iron-containing proteins and with very high host specificity in several cases [4]. In Gram-negative bacteria, members of the *Neisseriaceae* family and the *Moraxellaceae* family express surface receptors that are capable of specifically binding host iron-charged Lf (holo-Lf)

and extracting the iron from this glycoprotein for growth. In mammalian cells, both iron-charged Lf (holo-Lf) and iron-lacking Lf (apo-Lf) can be taken up by the cell through the same receptor (LfR) on the cell surface. Research on both human Lf (H-Lf) and bovine Lf (B-Lf) has focused on the identification and characterization of the LfR in a variety of cell types. Specifically, the human lipoprotein receptor-related protein (HLRP) has been indicated to be a mitogenic receptor for B-Lf in osteoblastic cells [5]. Tanaka et al. [6] found that B-Lf and H-Lf bound to the same receptor in human Jurkat lymphoblastic T-cells. In fibroblasts, HLRP is required for B-Lf-enhanced collagen-gel contractile activity [7].

Parasites express diverse host iron uptake systems and pathogenicity mechanisms. In parasitic protozoa, iron acquisition systems have been poorly studied. *E. histolytica* trophozoites depend on host iron for their survival and expression of virulence. In axenic cultures, amoebae depend on iron from the medium for growth and use both Fe^{3+} and Fe^{2+} ions [8–10]. Interestingly, hamsters fed ferrous gluconate had high incidence and severity of liver lesions; therefore, iron is an important nutrient for this amoeba. In addition, patients suffering from ALA presented a hypoferremic state in the serum, confirming that nutritional immunity by iron is produced in amoebiasis [11]. Amoebae require approximately 100 μM iron for growth; therefore, the parasite has developed mechanisms to scavenge iron from host iron-containing proteins. We established that human holo-Lf (H-holo-Lf) supported the growth of a nonvirulent variant of *E. histolytica* through several consecutive culture passages. H-holo-Lf was recognized by two proteins (45 and 90 kDa) located at the amoebic membrane. Subsequently, H-holo-Lf was endocytosed by a mechanism inhibited by filipin and trafficked via the endosomal/lysosomal pathway. In acidic lysosomes, iron from H-holo-Lf was most likely released and the protein degraded [12].

B-Lf is present practically without degradation in the large intestine and feces of babies fed with milk formula and in infants. The B-Lf molecule is found in its complete form in a low percentage in adults who drink dairy products [13, 14]. In this study, we show that *E. histolytica* trophozoites use B-holo-Lf for their growth *in vitro* and analyze the binding and endocytosis of this glycoprotein by the parasite. Two amoeba variants, a strain that has been maintained in axenic culture for more than 30 years and is unable to produce ALA in hamsters (nonvirulent amoebae) and trophozoites derived from this strain that have been continuously passed through ALA in hamsters (virulent amoebae), were compared.

2. Materials and Methods

2.1. Iron-Containing Proteins Used in This Work. B-apo-Lf was obtained from NutriScience (USA) containing 4.1% iron and was subsequently iron-saturated to obtain B-holo-Lf as previously reported [16]. The homogeneity of B-holo-Lf was confirmed with 10% SDS-polyacrylamide gels. Iron-charged human lactoferrin (H-holo-Lf) (95–100% of iron), human holo-transferrin (H-holo-Tf) (100% iron), and human and bovine hemoglobin (H-Hb and B-Hb) were obtained from Sigma, St. Louis, MO, USA.

2.2. Culture of Nonvirulent Amoebae. Trophozoites of the *E. histolytica* HM-1:IMSS strain were axenically grown in BI-S-33 medium (Dibico, Mexico) [15] supplemented with 16% (v/v) heat-inactivated bovine serum (BS) (Microlab, Mexico) and Tween 80-vitamin mix (*In vitro*, Mexico). The cultures were grown in glass screw-cap tubes at 37°C for 48 h. The tubes were placed on an ice bath for 15 min, and the amoebae were harvested by centrifugation at 500 g and washed twice in PBS, pH 7.4. Glass materials were treated with 2.0 M HCl for 24 h and rinsed six times with double-distilled water before their use. All chemicals were obtained from Sigma.

2.3. Culture and Maintenance of Virulent Amoebae. The induction of ALA in hamsters was performed in accordance with the International Norms of Care and Use of Laboratory Animals (NOM 062-ZOO-1999). The experiments were conducted at the Animal Care Unit of CINVESTAV-IPN, Mexico. In all cases, 2-month-old male Syrian Golden hamsters (*Mesocricetus auratus*) with an average weight of 100 g were used. To activate the virulence of *E. histolytica*, three hamsters were intraperitoneally anesthetized with sodium pentobarbital (Anestesal, Smith Kline, Mexico), and a hepatic lobe was inoculated with 1.5×10^6 amoebae in 0.2 mL of BI-S-33 medium (from a culture of 48 h) [17]. The animals were euthanized at day 7. Subsequently, a liver fragment was excised under sterile conditions and transferred to a tube with culture medium supplemented with serum, streptomycin (500 μg/mL), and penicillin (500 U/mL) and cultured for 24–48 h at 37°C. These virulent trophozoites were reinoculated into hamsters six times and then resuspended in culture medium without serum or antibiotics prior to use in the assays with B-holo-Lf.

2.4. Growth of E. histolytica Trophozoites in B-holo-Lf. The culture media and iron concentrations used are shown in Table 1. The low-iron medium was BI-S-33 without ammonium ferric citrate (AFC) and BS, with vitamins, treated with 5 g/mL of Chelex-100 resin to remove iron from the trace reagents. The resin was subsequently removed by filtration, and the medium was sterilized. This medium contained 6.5 μM iron and is henceforth referred to as "low iron." With the purpose of determining whether B-holo-Lf sustains the trophozoite growth, the amoebae were maintained in low iron for 4 h to diminish their iron reserves and to synchronize the culture [18]. Trophozoites (10^4) were then inoculated into BI-S-33, into low iron plus serum (19 μM Fe), or into this medium with different concentrations of B-holo-Lf added. All of the cultures were incubated at 37°C for 96 h. Cell viability was determined every 24 h by the exclusion of trypan-blue dye and observed under a light microscope in a Neubauer chamber. For successive cultures, amoebae (10^4) were inoculated into BI-S-33, low iron, and low-iron containing BS and B-holo-Lf (100, 115, 125, and 135 μM total iron) for 48 h. Consecutive transfers were conducted at least three times in the same medium.

Table 1: Media used in this study.

Medium	Iron concentration (μM)*	Source of iron	Reference
BI-S-33	100 ± 4.68	AFC, serum, and iron traces from reagents	Diamond et al., 1978 [15]
Low-iron	6.5 ± 2.61	Iron traces from reagents	Serrano-Luna et al., 1998 [10]
Low-iron plus serum	19 ± 4.35	Serum and iron traces from reagents	Serrano-Luna et al., 1998 [10]
Low-iron plus serum and B-holo-Lf■	100 ± 2.5, 115 ± 1.5, 125 ± 1.95 and 135 ± 1.85	Bovine Lf-iron, serum, and iron traces from reagents	[This work]

*Iron concentration was measured by spectrophotometric method. Micro-Tec Laboratory, Mexico.
■B-holo-Lf was added to obtain the iron concentrations indicated.

2.5. Quantitative Determination of Iron. The BI-S-33 medium containing Fe^{3+} (from AFC) or low iron medium supplemented with B-holo-Lf was tested in order to determine the iron quantity. The iron was dissociated from the B-holo-Lf in acidic medium (50 mM citrate buffer, pH 2.2). Fe^{3+} released was reduced into Fe^{+2} by means of ascorbic acid (113.5 mM). Ferrous ions were complexed with tripyridyltriazine (9.6 mM) in a blue colour compound and absorbance was measured at 595 nm. The intensity of the coloured complex formed was proportional to the iron concentration in the sample [19].

2.6. Interaction between B-holo-Lf and E. histolytica Trophozoites. To study the binding and endocytosis of B-holo-Lf in *E. histolytica*, the amoebae were maintained in low-iron medium for 4 h, incubated for different periods of time in the same medium containing FITC-B-holo-Lf (100 μM Fe), and analyzed by flow cytometry and confocal laser-scanning microscopy. FITC-B-holo-Lf was prepared using fluorescein isothiocyanate (1.5 mg/mL; Sigma) in 50 mM sodium carbonate buffer (pH 9.5), which was added dropwise to the B-holo-Lf (10 mg/mL in 50 mM sodium bicarbonate, pH 8.3) with constant agitation for 2 h at room temperature (RT). To eliminate the unbound label, the conjugate was passed through a Sephadex G-25 column in PBS, pH 7.4.

Flow Cytometry. To first explore for the presence of a B-holo-Lf-binding protein in *E. histolytica* (*Eh*BholoLfbp) and test whether the binding/internalization depends on time and energy, virulent and nonvirulent amoebae (2×10^5) in low-iron medium were incubated with FITC-B-holo-Lf for 1–5, 10, and 15 min at 6°C (only binding) or 37°C (internalization). Next, parasites were washed with PBS and fixed with 2% (w/v) (final concentration) paraformaldehyde (PFA). The amoebae were processed for fluorescence quantification using a flow cytometer (FACScan; Becton Dickinson, USA).

Confocal Laser-Scanning Microscopy. To localize the *Eh*BholoLfbp, we followed the preceding protocol; however, the amoebae were incubated with FITC-B-holo-Lf for 30 s and 30 min. The samples were similarly washed and fixed. Nonspecific binding was blocked with 1 M glycine for 15 min. The amoebae were mounted in Vectashield on glass slides and examined under a Leica confocal TCS-SP2 microscope (at least 20 optical sections were observed in three independent experiments, each performed in triplicate).

2.7. Internalization of Iron-Containing Proteins in E. histolytica. To determine whether virulent amoebae have a different capacity to internalize other iron-containing proteins in addition to B-holo-Lf more than nonvirulent amoebae, trophozoites (2×10^5) were maintained in low-iron medium for 4 h, incubated for different periods of time at 37°C in the same medium containing 100 μM Fe derived from four FITC-labeled proteins, H-holo-Lf, H-holo-Tf, B-Hb, or H-Hb, and analyzed by flow cytometry. To determine the specificity of the *E. histolytica* B-holo-Lf binding sites and further internalization, virulent and nonvirulent trophozoites were preincubated at 37°C for 30 min with 40-fold excess of the following five unlabeled proteins: B-holo-Lf, H-holo-Lf, H-holo-Tf, B-Hb, or H-Hb. Next, the amoebae were washed, fresh medium containing FITC-B-holo-Lf (100 μM Fe) was added, and the mixture was incubated for 30 min. The samples were washed with PBS, fixed with PFA, and processed for fluorescence quantification by flow cytometry.

2.8. Effect of Ionic Strength and pH on B-holo-Lf Binding by E. histolytica. First, several cations concentrations and buffers with different pH values that would not affect cell viability were tested. The amoebae (10^6) in low iron were preincubated at 6°C for 30 min with the following cations: 0–25 mM of $CaCl_2$, $FeCl_3$, $MgCl_2$, and $FeSO_4$ and with pH 2–8 buffers. The amoebae were washed with PBS, fresh medium containing FITC-B-holo-Lf (100 μM total iron) was added, and the mixture was incubated for 30 min. The samples were washed, fixed with PFA, and analyzed by flow cytometry.

2.9. Determination of K_d, B_{max}, V_{max} and the Number of EhBholoLfbp. To determine whether *E. histolytica* binds and internalizes B-holo-Lf, we used whole cells that were maintained in low-iron medium for 4 h. Next, the cells were incubated with several concentrations of FITC-B-holo-Lf (1–12,000 nM) at 6°C or 37°C for 30 min. Subsequently, the amoebae were washed with PBS and fixed with PFA. The samples were processed for fluorescence quantification by flow cytometry. Finally, to determine K_d, $B_{\max}$, and $V_{\max}$ and estimate the average number of *Eh*BholoLfbp per cell,

saturation kinetics were analyzed and Scatchard plots were generated.

2.10. Determination of the B-holo-Lf Internalization Pathway Using Inhibitors. First, the maximal concentration of inhibitor that would not affect cell viability was determined. The amoebae (2×10^5) were preincubated at 37°C for 30 min in medium with the following inhibitors: 1–100 μM NH_4Cl, 0.5–10% (w/v) sucrose, 5–25 μM chlorpromazine, 5–10 μg/mL filipin, 25–100 μg/mL nystatin, 7.5–20 mM methyl-β-cyclodextrin, 100–300 nM wortmannin, 5 μM cytochalasin D, and 1-2 μM colchicine. Next, the amoebae were incubated at 37°C for 30 min with fresh medium containing FITC-B-holo-Lf (100 μM Fe). The samples were washed with PBS, fixed with PFA, and processed for fluorescence quantification by flow cytometry and confocal laser microscopy. At least 20 optical sections were observed in three independent experiments, each in triplicate. In all of the cases, the basal fluorescence was subtracted from the assayed values.

2.11. Determination of the Proteolytic Activity of E. histolytica Trophozoites against B-holo-Lf. To characterize the amoebic proteases that cleave B-holo-Lf, the previously reported method of López-Soto et al. [20] was used with several modifications. Both variants of amoebae (10^6) were maintained in BI-S-33 or low-iron medium lacking BS and AFC for 4 h at 37°C. Next, the amoebae were harvested, centrifuged, and washed twice with PBS (pH 7.4). The cells from the pellet were collected separately from the supernatant (SN). The cells were disrupted by five cycles of freeze-thawing in PBS, and the content corresponded to the crude cell extract (CCE). The protein concentration was quantified by the method of Bradford [21]. The SN was precipitated with absolute ethanol (1 : 1 v/v), centrifuged, and finally passed through a 0.22-μm Durapore membrane (Millipore, Bedford, MA). The CCE and SN proteins were maintained at 4°C and used immediately.

Protease activity was determined by electrophoresis of CCE and SN in 10% SDS-PAGE copolymerized with 0.1% (w/v) of B-holo-Lf as the substrate. The proteins from two variants of amoebae were loaded (40 μg per well). Electrophoresis was performed at 100 V, for 2.5 h at 4°C. The gels were rinsed and incubated for 1 h with orbital agitation in 2.5% (v/v) Triton X-100. Next, the gels were incubated overnight at 37°C with one of the following buffer solutions containing 2 mM $CaCl_2$: 100 mM sodium acetate-Tris/HCl (pH 5.0), 100 mM Tris/NaOH (pH 7.0), or 100 mM glycine-Tris/NaOH (pH 9.0). Finally, the gels were stained with 0.5% Coomassie brilliant blue R-250 for 30 min. The protease activities were identified as clear bands on a blue background. To discern the type of proteolytic activity, CCE and SN were incubated for 1 h at 37°C with inhibitors at a final concentration of 10 mM *p*HMB, 5 mM NEM, or 10 μM E-64 (cysteine-proteases); 2 mM EDTA (metallo-proteases); 5 mM PMSF (serine-proteases). The proteins were then separated by SDS-PAGE copolymerized with B-holo-Lf as mentioned above.

2.12. Statistical Analysis. All of the data are presented as the mean ± SD. The differences between the means in both groups of amoebae were compared using the *t*-test. The one-way analysis of variance (ANOVA) test was used to compare the difference between the means in more than two groups. A probability of $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Virulent Amoebae Are More Resistant to Stress Caused by Low and High Iron and Show Higher Growth in B-holo-Lf Than Nonvirulent Amoebae. To test the tolerance to stress caused by a low concentration of iron in *E. histolytica*, virulent and nonvirulent amoebae were grown in low-iron medium plus serum (19 μM Fe). The nonvirulent amoeba culture developed slowly until 48 h, and no viable cells remained by 72 h (Figure 1(a)), indicating that iron is an essential element for the survival of this parasite and that this iron concentration is insufficient for growth, which are supported by previous reports from our group [12, 20]. In contrast, the virulent amoeba cultures showed a gradual reduction of the viable cell number until 96 h (Figure 1(b)); therefore, these amoebae were more resistant to the absence of iron. With respect to the tolerance to high iron concentration, we used up to 900 μM Fe derived from AFC. Both amoeba variants tolerated this high iron concentration, but the virulent trophozoites used this metal in a more efficient manner (80–90% viability, data not shown) compared to the nonvirulent trophozoites (30% viability); this result supports previous results for nonvirulent amoebae [10].

To investigate whether B-holo-Lf supports the growth of *E. histolytica*, several concentrations of this glycoprotein were used as an iron source in growth kinetics analyses. B-holo-Lf (100–135 μM Fe) supported the growth of both nonvirulent and virulent variants over a period of at least 96 h; however, the virulent amoebae were more efficient in using the iron from B-holo-Lf because an increase of approximately 70% in the number of amoeba was observed in this culture. However, the cultures of both amoeba variants showed higher growth in BI-S-33, in which the iron source is primarily ferric citrate, not in B-holo-Lf (Figures 1(a) and 1(b)). In addition, B-holo-Lf sustained subcultures of both variants of *E. histolytica*; however, the percentages of viable nonvirulent amoebae (Figure 1(c)) were lower throughout three consecutive culture passages than virulent amoebae (Figure 1(d)). With respect to the B-Lf iron used, virulent amoebae developed almost normally (90% viability in the third passage) at the four concentrations tested (Figure 1(d)). Furthermore, virulent amoebae were more tolerant of the stress caused by the absence of iron, as 10% of the cells were still viable at the third passage (Figure 1(d), white bar). Together, these results indicate that both variants are capable of growing in B-holo-Lf as an iron source, but virulent amoebae resist the variations in the iron concentration of the environment and apparently use B-holo-Lf more efficiently than nonvirulent amoebae.

3.2. E. histolytica Trophozoites Bind and Internalize B-holo-Lf through a Receptor. The binding and internalization of FITC-B-holo-Lf in *E. histolytica* were evaluated by incubation

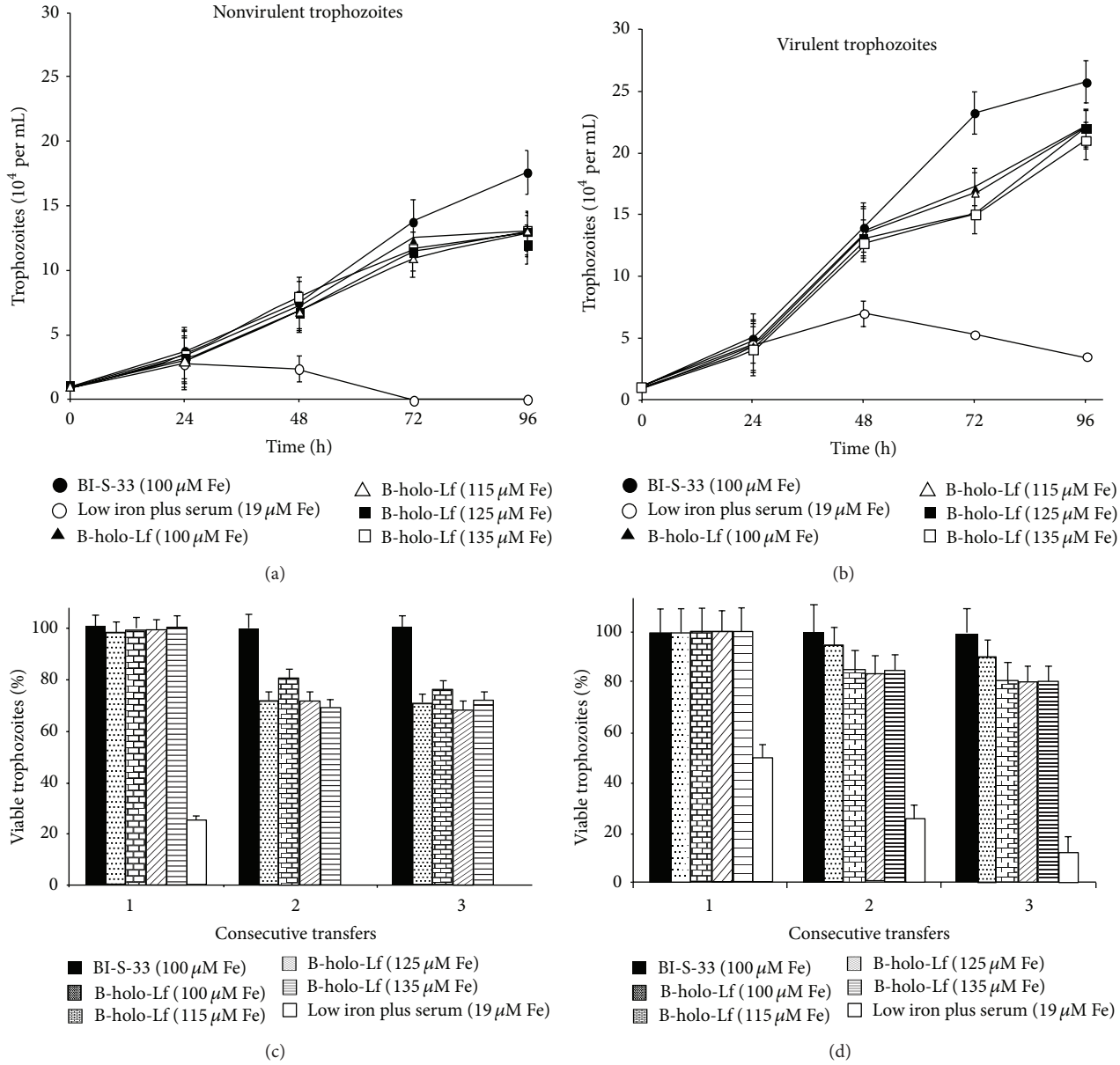

Figure 1: *E. histolytica* growth in B-holo-Lf as an iron source. ((a), (b)) Growth kinetics through 96 h in bovine holo-Lf (B-holo-Lf). Amoebic cultures were synchronized (see Methods section), 10^4 cells were inoculated into each medium, and viability was estimated every 24 h by trypan blue exclusion. ((c), (d)) Amoebas were grown for 48 h, through three passages, in the media indicated. Viability was measured by trypan blue exclusion. Data are means of three independent experiments performed in triplicate ± SD.

at 6°C and 37°C, respectively, using either flow cytometry (Figure 2(a)) or confocal microscopy (Figure 2(b)). In both amoeba variants, the binding and internalization were detected in the first minutes of incubation and reached saturation, suggesting the presence of B-holo-Lf-binding proteins (*Eh*BholoLfbp). The binding and saturation values were higher in the virulent amoebae (Figure 2(a), black squares). At 6°C and 30 minutes of incubation, we observed that FITC-B-holo-Lf bound to vesicle-like structures on the periphery (Figure 2(b), (panels 9 and 12)). FITC-B-holo-Lf is internalized at 30 minutes of incubation at 37°C (Figure 2(b), (panels 3 and 6)). Again, the virulent trophozoites showed higher binding and internalization of B-holo-Lf than the nonvirulent trophozoites. These results suggest that the B-holo-Lf internalization is time- and energy-dependent in *E. histolytica* and that an endocytosis mechanism could be involved in the uptake and utilization of B-holo-Lf by trophozoites. The B-holo-Lf internalization was continuous and saturable, and our results support the hypothesis that B-holo-Lf endocytosis is mediated by a protein receptor in *E. histolytica* and that this process may be regulated.

3.3. E. histolytica Trophozoites Specifically Endocytose B-holo-Lf. To determine whether the interaction of B-holo-Lf with

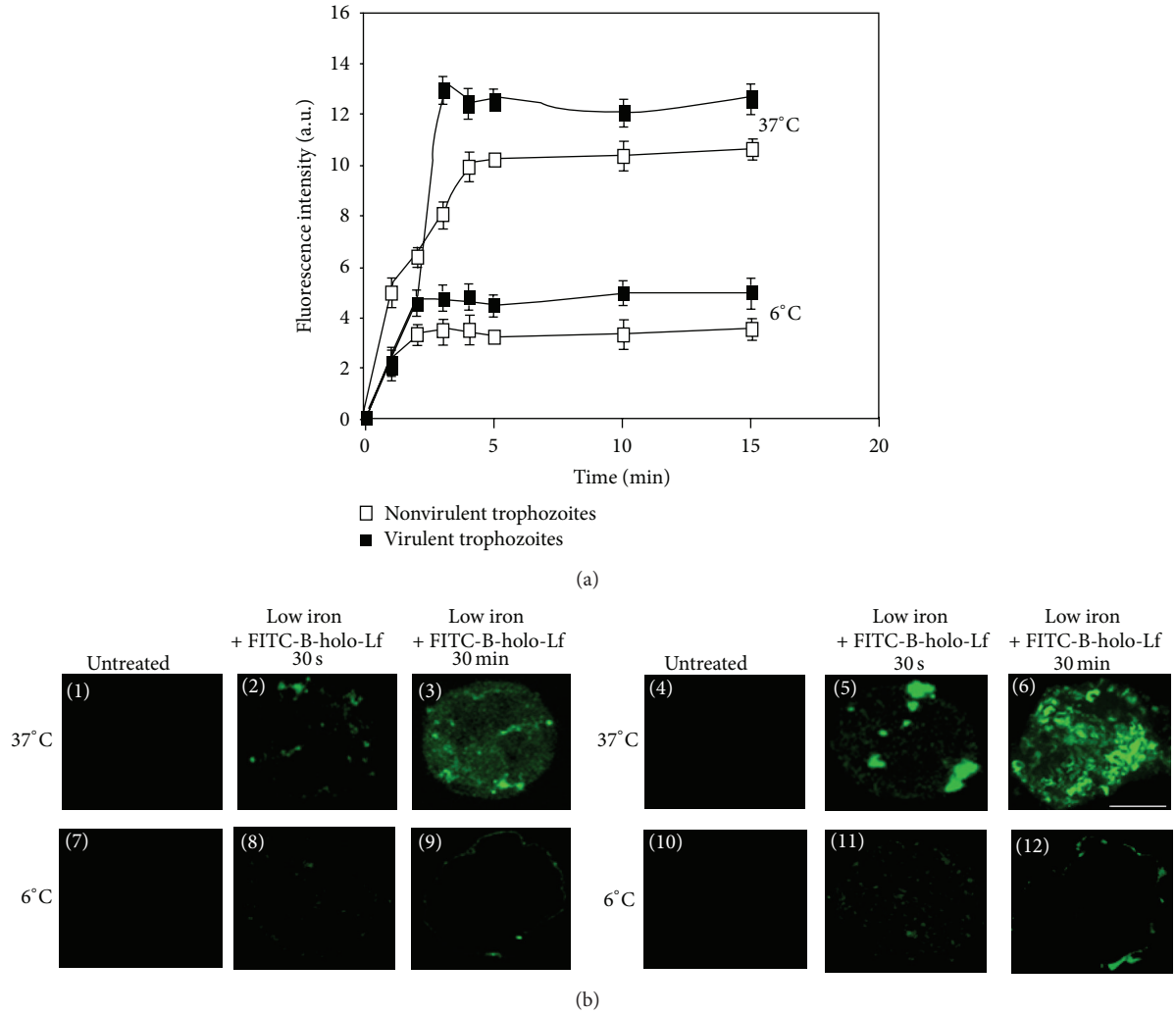

Figure 2: B-holo-Lf internalization in both variants of *E. histolytica* depends on time and energy. (a) Flow cytometry: amoebae were synchronized and incubated (2×10^5 cells) at 37°C or 6°C with FITC-B-holo-Lf for the periods of time indicated. The samples were then fixed with PFA and processed for fluorescence quantification. Data are means of three independent experiments performed in triplicate ± SD. (b) Confocal microscopy: amoebae were synchronized and 2×10^5 cells were incubated at 37°C or 6°C with FITC-B-holo-Lf for the periods of time indicated. 1–3 and 7–9 indicate the nonvirulent amoebae and 4–6 and 10–12 indicate the virulent amoebae. Bar, 10 μm.

both *E. histolytica* variants is specific for this glycoprotein, competition assays with other iron-containing proteins were developed. Virulent and nonvirulent amoebae were incubated with 40-fold excess of unlabeled B-holo-Lf, H-holo-Lf, H-holo-Tf, B-Hb, and H-Hb and then were incubated with FITC-B-holo-Lf at 37°C. Only unlabeled B-holo-Lf prevented the endocytosis of FITC-B-holo-Lf (Figure 3(a)), suggesting that nonvirulent and virulent *E. histolytica* trophozoites exhibit specific mechanisms to internalize B-holo-Lf, distinct from the other iron-proteins tested. Interestingly, through five experiments (and increasing to 50-fold excess of the B-holo-Lf concentration), nonvirulent amoebae showed almost 100% inhibition by unlabeled B-holo-Lf; however, only up to 80% inhibition of internalization was found for virulent amoebae, suggesting that B-holo-Lf may also be endocytosed by other nonspecific mechanisms in virulent amoebae.

3.4. Virulent E. histolytica Trophozoites Show Higher Endocytosis Capacity of Iron-Containing Proteins Than Nonvirulent Amoebae. To determine whether virulent trophozoites have higher capacity to internalize iron-containing proteins than nonvirulent trophozoites, internalization kinetics analyses at 37°C for four iron-proteins were performed by flow cytometry. The immediate internalization of FITC-B-Hb, FITC-H-Hb, FITC-H-holo-Tf, and FITC-H-holo-Lf was observed, achieving a maximum in the range of 5–10 min after the interaction with both variants of amoebae. Importantly, the internalization of these four iron-containing proteins was more rapid and efficient in the virulent amoebae than in nonvirulent amoebae in the following order: B-Hb and H-Hb (100% more at min 10), H-holo-Tf (50% more at min 10), and H-holo-Lf (30% more at min 15) (Figure 3(b)). In addition, in both variants, the internalization of all of these

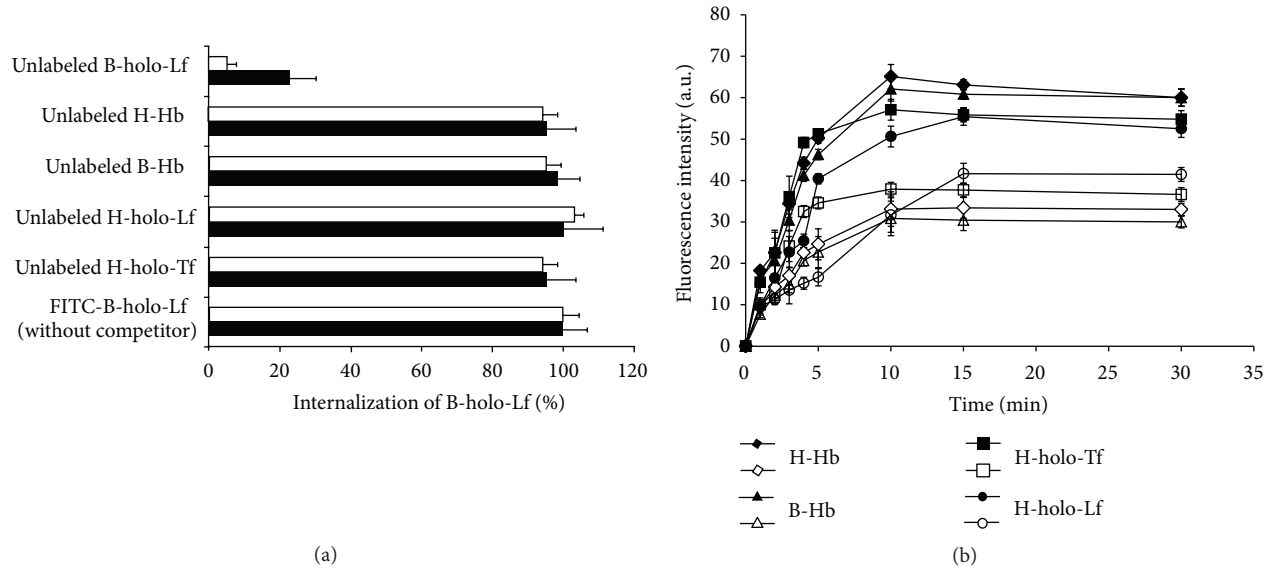

Figure 3: Both variants of *E. histolytica* endocytose B-holo-Lf specifically; however, virulent trophozoites show higher endocytosis level of iron-containing proteins than nonvirulent amoebae. (a) Amoebae (2×10^5 cells) were synchronized and incubated at 37°C for 30 min with 40-fold excess of nonlabelled H-Hb, B-Hb, H-holo-Tf, H-holo-Lf, or B-holo-Lf. Later, the amoebae were washed and incubated with FITC-B-holo-Lf for 30 min and then processed for fluorescence quantification by flow cytometry. White bars indicate the nonvirulent amoebae and black bars indicate the virulent amoebae. Data are means of three independent experiments performed in triplicate ± SD. (b) Amoebae (2×10^5 cells) were synchronized and incubated at 37°C with FITC-B-Hb, FITC-H-Hb, FITC-H-holo-Lf, and FITC-H-holo-Tf for the periods of time indicated. The samples were then fixed with PFA and processed for fluorescence quantification by flow cytometry. White symbols indicate the nonvirulent amoebae and black symbols indicate the virulent amoebae. Values are mean of three independent experiments performed in triplicate ± SD.

proteins was continuous and involved a prolonged saturation until 30 minutes of incubation. Together, these results suggest that virulent amoebae have a higher ability to bind and endocytose ferric- and ferrous-iron proteins to obtain iron, which may be an important virulence factor during infection.

3.5. E. histolytica Trophozoites Bind B-holo-Lf at Human Intestinal pH. To determine the optimum pH to reach the maximum ability of B-holo-Lf to bind in both variants of amoebae, the experiments were conducted at pH values between 2 and 8 at 6°C using whole cells and the binding was analyzed by flow cytometry. The optimum pH for B-holo-Lf binding ranged from 6.0 to 7.4 in both amoeba variants. In addition, B-holo-Lf binding drastically decreased in alkaline pH (pH = 8.0) (Figure 4(a)). This finding suggests that B-holo-Lf binding (and most likely endocytosis) by amoebae occurs at the pH of the human intestine, which should play an important role in the binding and use of B-holo-Lf by amoebae. These results also suggest a defined and optimum pH at which the maximum amount of B-holo-Lf binds to the amoebae, which supports the notion of specific binding proteins for this glycoprotein.

3.6. Calcium and Ferric Iron Increase the Binding of B-holo-Lf in E. histolytica. To determine the effect of the ionic strength and ion specificity on B-holo-Lf binding in both variants of amoebae, binding assays (6°C) in the presence of cations were developed, and the fluorescence was quantified by flow cytometry. In total, a 100% increase in B-holo-Lf binding in the presence of Ca^{2+} and Fe^{3+} was observed in both amoeba variants compared to the untreated amoebae. Fe^{2+} and Mg^{2+} did not have an effect on B-holo-Lf binding (Figure 4(b)). The binding of this glycoprotein was not affected when the amoebae were incubated with the monovalent cations Na^+ and K^+ (data not shown).

3.7. Virulent E. histolytica Trophozoites Show Higher B_{max}, V_{max}, and Number of Binding Sites for B-holo-Lf Than Nonvirulent Amoebae. The maximum binding capability of a ligand depends on the affinity of the cell binding proteins for the ligand, which is understood by saturation kinetics. To determine whether *E. histolytica* binds B-holo-Lf with constant kinetics and in a saturable manner, we used whole cells that were incubated with several concentrations of FITC-B-holo-Lf at 6°C and analyzed the binding by flow cytometry. As shown in Figure 5(a), the *E. histolytica* binding kinetics to B-holo-Lf were saturable with one kinetic component involved in each amoeba variant. The binding was similar and increased up to 3.25 μM for nonvirulent amoebae and up to 3.96 μM for virulent amoebae. Furthermore, during the internalization at 37°C using the same concentrations of B-holo-Lf, one kinetic component that participates in the accumulation of B-holo-Lf in nonvirulent amoebae was found; however, two kinetic components were involved in the internalization of this glycoprotein in virulent amoebae (Figure 5(c)). These results suggest the presence of a specific saturable mechanism that allows the binding/internalization of B-holo-Lf and supports

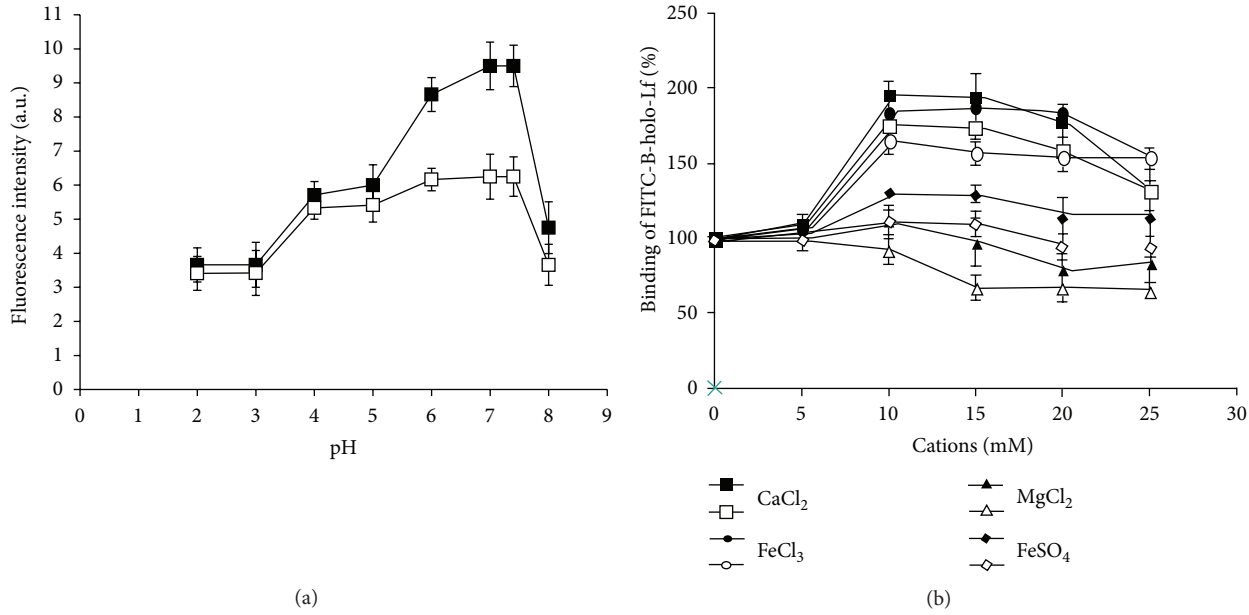

Figure 4: *Entamoeba histolytica* binding to B-holo-Lf is dependent of pH and it was increased in presence of Ca^{2+} and Fe^{3+}. (a) Amoebae were synchronized and preincubated (10^6 cells) with buffers (pH 2–8) at 6°C for 30 min. Next, fresh medium containing FITC-B-holo-Lf was added and incubated for 30 min. The samples were washed and fixed with PFA, and fluorescence quantification was analyzed by flow cytometry. (b) Amoebae (10^6 cells) in low iron were preincubated at 6°C for 30 min with one of the following cations: 0–25 mM of $CaCl_2$, $FeCl_3$, $MgCl_2$, or $FeSO_4$. Next, amoebae were washed with PBS, and fresh medium containing FITC-B-holo-Lf was added and incubated for 30 min. The samples were washed and fixed with PFA, and fluorescence quantification was analyzed by flow cytometry. White symbols indicate the nonvirulent amoebae and black symbols indicate the virulent amoebae. Values are means of three independent experiments performed in triplicate ± SD.

the results previously mentioned. Additionally, the virulent amoebae showed a more efficient system for the binding and internalization of B-holo-Lf than nonvirulent amoebae.

Subsequently, Scatchard plot transformation for FITC-B-holo-Lf was obtained from analyses of nonlinear regression, which is shown in Figures 5(b) and 5(d) and Table 2. Both amoebae specifically bound B-holo-Lf with a similar apparent K_d (1.85×10^{-6} M and 2.3×10^{-6} M for nonvirulent and virulent amoebae, resp.). However, the maximal binding at 6°C (B_{max}) for B-holo-Lf was higher in virulent amoebae (1.659 μmol/min) compared to nonvirulent amoebae (0.066 μmol/min) (Figure 5(b)). In addition, the maximal velocity of internalization at 37°C (V_{max}) was higher and showed two kinetic components in virulent amoebae (3.3 and 0.9 μmol/min) than in nonvirulent amoebae (0.4562 μmol/min), which only showed one component (Figure 5(d)). These data indicate that the binding affinity of B-holo-Lf was similar to other pathogenic protozoa; however, virulent amoebae showed higher B_{max} and V_{max} than nonvirulent amoebae, suggesting that virulent amoebae are capable of binding higher concentrations of B-holo-Lf, and this mechanism was more efficient for the binding of this glycoprotein than nonvirulent amoebae. Importantly, an average of 9.45×10^5 (nonvirulent amoebae) and 6.65×10^6 (virulent amoebae) binding sites for B-holo-Lf per amoeba were found (Table 2).

3.8. B-holo-Lf Is Primarily Endocytosed via Clathrin-Coated Vesicles by E. histolytica Trophozoites. To determine the cellular mechanism through which B-holo-Lf is taken up and internalized by both variants of amoebae, we used several types of inhibitors of endocytosis pathways, and B-holo-Lf internalization was evaluated by flow cytometry (Figure 6(a)). If the FITC-B-holo-Lf endocytosis in untreated amoebae is taken as 100%, the three clathrin-mediated endocytosis inhibitors, hypertonic sucrose solution, NH_4Cl, and the cationic amphiphilic drug chlorpromazine, alone or in combination, inhibited the internalization of B-holo-Lf by 60% in virulent as well as nonvirulent amoebae (Figure 6(a)). Furthermore, inhibitors of cholesterol-mediated endocytosis, such as methyl-β-cyclodextrin (MβCD), filipin, and nystatin, did not inhibit B-holo-Lf endocytosis in nonvirulent amoebae (<5%) (Figure 6(b), white bars). However, B-holo-Lf endocytosis was affected by these three inhibitors in all concentrations tested (60–70%) in the *E. histolytica* virulent variant (Figure 6(b), black bars). These results suggest that B-holo-Lf endocytosis occurs through both clathrin-coated-vesicles and a cholesterol-dependent mechanism in virulent amoebae. Wortmannin and cytochalasin D considerably affected B-holo-Lf endocytosis in both variants (43 and 40% inhibition, resp.) (Figure 6(c)). However, colchicine had a minor effect (15% inhibition). These results suggest that B-holo-Lf endocytosis may depend on PI3K activity and the actin cytoskeleton and not on microtubules.

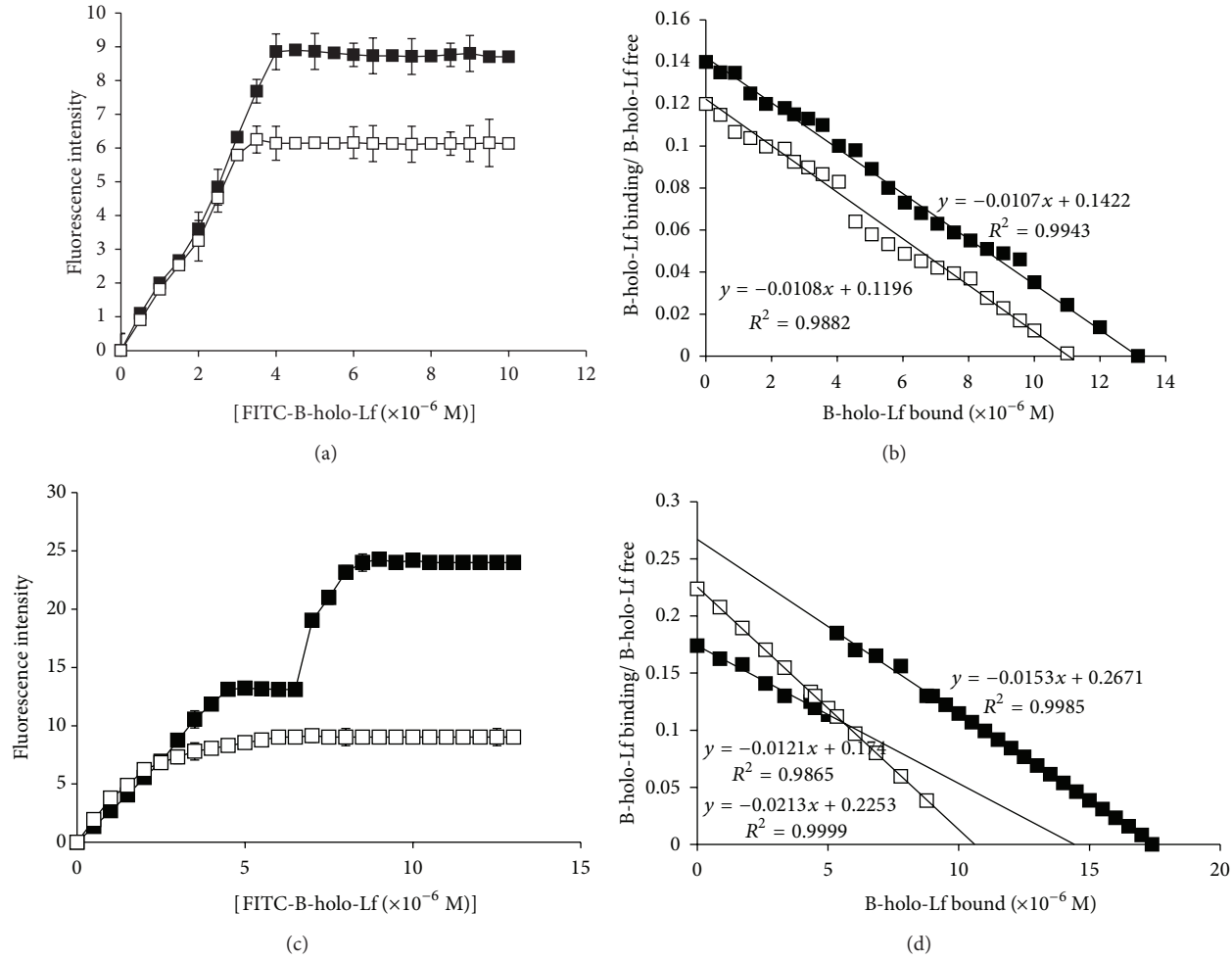

Figure 5: *E. histolytica* trophozoites bind and internalize B-holo-Lf with high affinity; however, virulent amoebae show higher V_{max} and number of *Eh*BholoLfbp than nonvirulent amoebae. ((a), (c)) Amoebae (5×10^5 cells) were synchronized and incubated with several concentrations of FITC-B-holo-Lf (1–12,000 nM) at 6 or 37°C for 30 min. The samples were washed and fixed with PFA, and fluorescence quantification was analyzed by flow cytometry. Data are means of three independent experiments performed in triplicate ± SD. ((b), (d)) Determination of K_d, B_{max}, V_{max}, and the number of *Eh*BholoLfbp/cell was estimated by the method of Scatchard. In all cases, white and black squares indicate the nonvirulent amoebae and virulent amoebae, respectively. Data are means of three independent experiments performed in triplicate ± SD.

3.9. E. histolytica Trophozoites Degrade B-holo-Lf through Cysteine Proteases. Proteins that were secreted into the culture supernatant (SN) and were present within the cells (CCE) were analyzed by electrophoresis in substrate gels in both variants of amoebae. B-holo-Lf was cleaved at a wide range of pH values by three proteases with MWs of 100, 75, and 60 kDa from the CCE and SN in both variants of amoebae maintained with Fe^{3+}-citrate (BI-S-33) and low-iron medium. The proteolytic pattern appears to be similar in CCE and SN in both variants of amoebae; therefore, they are most likely the same enzymes (activity at pH 5 is shown in Figure 7). Interestingly, our results show proteolytic activity against B-holo-Lf in the SN of *E. histolytica* cultures, unlike H-holo-Lf, where no proteolytic activity was detected in the SN of nonvirulent trophozoites [12]. Using B-holo-Lf as an in-gel substrate, the absence of iron increased the proteolytic activity of the three proteases in both variants of amoebae. Importantly, we found higher proteolytic activity (2-fold) in the CCE and SN of virulent amoebae compared with nonvirulent amoebae. Proteases against B-holo-Lf were cysteine-related, as NEM, E-64, and *p*HMB inhibited the cleavage activity of CCE and SN in both variants of amoebae (Figure 7). These data suggest that one of the mechanisms of *E. histolytica* to acquire iron from B-holo-Lf may be through internal and extracellular cysteine proteases.

4. Discussion

Iron (Fe) is an essential nutrient for both the host and the pathogens surviving inside the host. However, iron is toxic and leads to the production of free radicals via Fenton's reaction; thus, iron is generally bound to or forms part of proteins, and a less significant labile, low-molecular-mass pool of intracellular iron exists [22]. Due to the toxicity

Table 2: Biochemical properties of binding/internalization of B-holo-Lf in *E. histolytica*.

	6°C			37°C		
	K_d (M ± SD)[a]	B_{max} (μmol·min^{-1} ± SD)[a]	*Eh*BholoLfbp number (sites/cell ± SD)[a]	K_d (M ± SD)[a]	V_{max} (μmol·min^{-1} ± SD)[a]	*Eh*BholoLfbp number (sites/cell ± SD)[a]
Nonvirulent amoebae	$1.85 \pm 0.002 \times 10^{-6}$	0.066 ± 0.0001	$9.45 \pm 0.093 \times 10^{5}$	$2.5 \pm 0.002 \times 10^{-6}$	0.4562 ± 0.00012	$1.89 \pm 0.00023 \times 10^{7}$
Virulent amoebae	$2.3 \pm 0.0015 \times 10^{-6}$	1.659 ± 0.0013	$6.65 \pm 0.011 \times 10^{6}$	$2.48 \pm 0.0012 \times 10^{-6}$ $5.2 \pm 0.0027 \times 10^{-6}$	3.3 ± 0.0016 0.9 ± 0.00045	$1.97 \pm 0.00043 \times 10^{7}$

[a]Three independent experiments were done by triplicate.
SD: standard deviation.

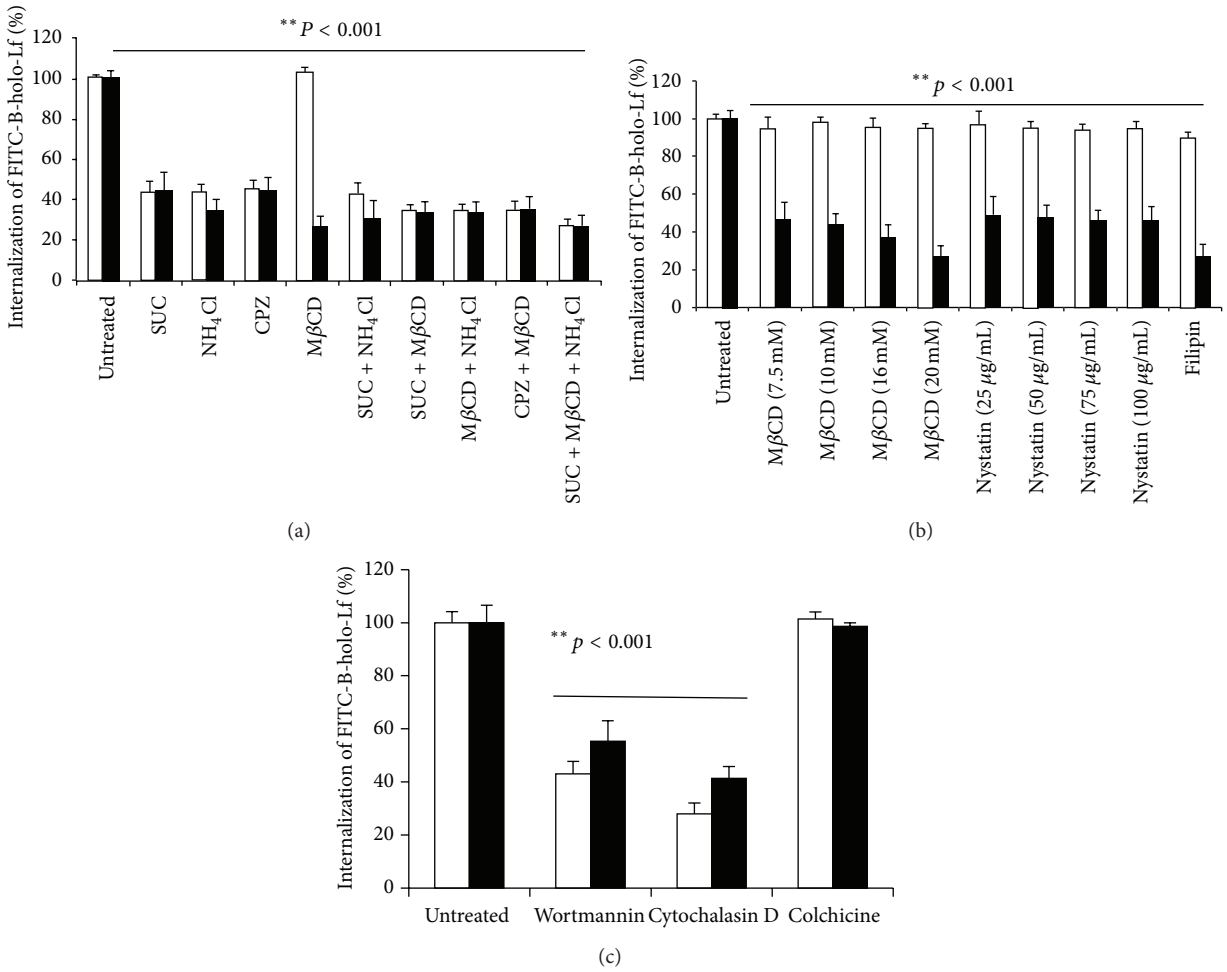

Figure 6: B-holo-Lf is primarily endocytosed via clathrin-coated vesicles in both variants of *E. histolytica*; however, virulent trophozoites also endocytose B-holo-Lf through a cholesterol-dependent route. ((a) and (c)) Amoebae (2×10^5 cells) in low iron were preincubated at 37°C for 30 min in medium with one of the indicated inhibitors. Next, they were incubated for 30 min with fresh medium containing FITC-B-holo-Lf. The samples were fixed and processed for fluorescence quantification by flow cytometry. (b) Amoebae were preincubated for 30 min at the concentration indicated of lipid-rafts inhibitors and processed as in (a). In all cases, white and black bars indicate the nonvirulent amoebae and virulent amoebae, respectively. Values are means of three independent experiments performed in triplicate ± SD.

and as a general strategy against pathogens, mammals have evolved complex iron-withholding systems to prevent microbial growth [23]. Therefore, pathogens that seek to colonize the host encounter an iron-limiting environment [24–26], and the free-iron concentration in fluids is approximately 10^{-18} M, an amount far too low to support their growth, which requires levels in the micromolar range [24]. Furthermore, several protozoa, such as amitochondriate protists (i.e., *Trichomonas, Tritrichomonas, Giardia,* and *Entamoeba*), require particularly high amounts of iron for *in vitro* growth (50–200 μM), surpassing the concentration in the majority of eukaryotic and prokaryotic cells (0.4–4 μM). As a result, competition for iron between the host and pathogen occurs during infections [27, 28].

According to our results, amoebae recently obtained from ALA (virulent) tolerated higher iron concentrations and were able to grow through several consecutive transfers in the absence of iron compared to nonvirulent amoebae, which strongly suggests that a highly efficient iron storage mechanism may be involved in their survival. In pathogenic protozoa, a ferritin-like molecule has not been described. In *E. histolytica,* the presence of a labile low molecular-mass iron pool or specific compartments for iron storage, such as those described in mammalian cells, has not been reported [22, 29]. Virulent amoebae were recently extracted from liver abscesses and apparently the iron utilization may be more efficient than in nonvirulent counterparts.

Bovine lactoferrin is present practically without degradation in the feces of babies fed with milk formula and in infants; therefore, the B-Lf molecule can resist the acidic pH of the stomach [13]. Based on our results *in vitro*, B-holo-Lf provides the required iron for the growth of *E. histolytica*, and the amoebae were able to bind and internalize this glycoprotein. Furthermore, trophozoites recently isolated from liver abscesses showed high efficiency in the binding and endocytosis of B-holo-Lf, which suggests that these

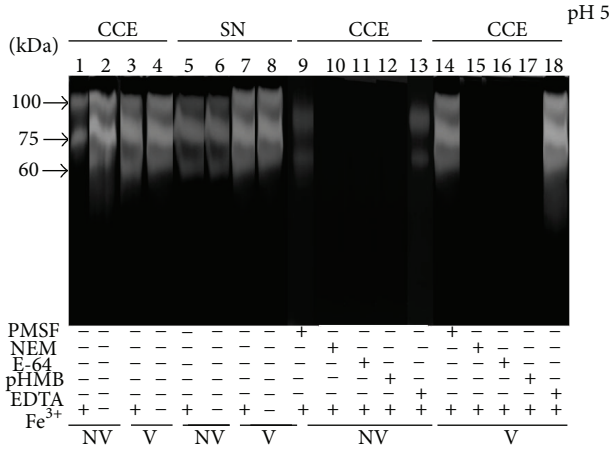

Figure 7: *E. histolytica* trophozoites degrade B-holo-Lf by means of cysteine proteases. Amoebae (10^6 cells) were maintained in BI-S-33 or low-iron medium for 4 h at 37°C. Next, the amoebae were harvested and washed and cells from the pellet were disrupted by freeze-thawing. Proteins from crude cell extract (CCE) and culture supernatant (SN) were separated by electrophoresis on a 10% SDS-PAGE copolymerized with 0.1% of B-holo-Lf. After that, the gels were incubated with a buffer (pH 5.0) and stained with Coomassie blue. CCEs of nonvirulent amoebae (NV) and virulent amoebae (V) were treated with the protease inhibitors indicated (lanes 9–18). Result is representative of three independent experiments.

amoebae possess more binding sites or a higher affinity for this iron source than the nonvirulent parasites. Virulent variant obtained from hamster ALA should be similar to the amoebae causing human infection. These results allow us to hypothesize that virulent trophozoites may efficiently use B-holo-Lf *in vivo*. We have measured the concentration of Lf by ELISA in samples of commercial pasteurized bovine products: milk, yogurts, and powder milk for infants [30]. The Lf concentration fluctuates, depending on the mark: 10–90, 20–70, and 2–14 μg/mL, respectively. In addition, in many countries, products such as cereals, milk, and cocoa are fortified with iron. Lönnerdal et al. [31] found 0.2-0.3 μg/mL of iron in bovine milk, and this value increased to 4–12 μg/mL in iron-fortified formula [32]. The normal iron saturation in Lf of milk is 30% and Lf could be more saturated in iron-fortified milk. In addition to lactoferrin, lactoperoxidase, casein, catalase and fat, bind iron. Iron from the diet and B-Lf could be increasing the possibility of infection.

The use of several iron-containing proteins such as H-holo-Tf, H-holo-Lf, H-Hb, HS-Ft, and Heme as sole sources of iron for the strain HM1-1:IMSS, which has been cultured *in vitro* for more than 30 years [12, 20, 33–36] has been described. In this work, we found that the binding of B-holo-Lf, H-holo-Lf, B-Hb, H-Hb, and H-holo-Tf increased in amoebae that recently passed through hamster livers. Several pathogenic protozoa are capable of recognizing iron-containing proteins through multifunctional proteins, including *Toxoplasma gondii* tachyzoites, which bind B-holo-Lf, B-holo-Tf, and chicken holo-ovo-Tf through one 42-kDa protein, suggesting low-specificity binding [37]. Regarding our results, we found that both variants of amoebae recognize B-holo-Lf with high specificity, and H-holo-Lf and H-holo-Tf did not compete with B-holo-Lf for the *Eh*BholoLf-binding sites despite the high homology in the amino acid sequence, which are approximately 77% between B-Lf and H-Lf and 60% between B-Lf and H-Tf [38, 39].

The maximum binding of B-holo-Lf by Lfbps or receptors in mammalian cells is dependent on environmental conditions such as pH, ionic strength, and temperature. In this work, the optimum pH for B-holo-Lf binding ranged from 6.0 to 7.4 in both amoeba variants. These results suggest that trophozoites may use B-holo-Lf in the human ileum and large intestine (pH = 7.2–7.6). In addition, the binding of Lf requires the presence of a calcium ion in some biological systems, especially in human enterocytes and rat hepatocytes, which bind H-Lf and B-Lf through Ca^{2+}-dependent intelectin. Importantly, we found that the binding of B-holo-Lf increased in the presence of Ca^{2+} and Fe^{3+} in both variants of amoebae. Perhaps the presence of Ca^{2+} is necessary to achieve maximal binding of this glycoprotein [40].

Holo-Lf-binding proteins (holoLfbps) have been reported in several protozoan species, and each parasite possesses one or several characteristic proteins for capturing iron from this glycoprotein. In this work, relevant information regarding the affinity of the amoebae to bind and endocytose B-holo-Lf is reported. We found that both variants of *E. histolytica* bind and internalize B-holo-Lf with high affinity although on the micromolar order. The K_d was similar to that of H-holo-Lf (0.47–5.27 μM) in *T. vaginalis* and to that of B-holo-Lf (3.6 μM) in *T. foetus* [41–43]. In addition, B-holo-Lf efficiently accumulated in both amoeba variants at 37°C, but the virulent variant showed higher endocytosis than the nonvirulent amoebae, which suggests that virulent amoebae are more capable of using the iron from B-holo-Lf than their nonvirulent counterparts and that expression of at least two classes of specific binding sites for B-holo-Lf may occur. Importantly, virulent amoebae showed a higher B_{max} (25-fold) and *Eh*BholoLfbps/per cell (7-fold) than nonvirulent amoebae. The number of sites/cell was comparable to the number determined in mammalian cells but higher as compared to other parasitic protozoa such as *T. vaginalis* and *T. foetus* [42, 43].

Higher eukaryotic cells take up nutrients by endocytic mechanisms, and clathrin is a protein that participates in the endocytic pathway of many molecules. Clathrin has recently been observed in protozoa such as *T. brucei* [44, 45], *T. cruzi* [46], *Leishmania major* [47], *Giardia lamblia* [48, 49], and *E. histolytica* [20, 34]. In this work, we found that clathrin-coated pit inhibitors blocked the B-holo-Lf internalization in both variants of amoebae. In addition, inhibitors of cholesterol-mediated endocytosis inhibited the B-holo-Lf endocytosis in the virulent variant of *E. histolytica*, suggesting that B-holo-Lf endocytosis is dependent on both endocytosis pathways only in virulent amoebae. In such amoebae, B-holo-Lf could also be internalized by using dynamin-independent or fluid-phase pathways, although to a lesser extent. Also, wortmannin, an inhibitor of the activity

of PI3K, inhibited the B-holo-Lf endocytosis. In *E. histolytica*, PI3K is involved in vital events such as phagocytosis and pinocytosis [50–53]. Furthermore, in this work, we found that B-holo-Lf endocytosis decreased in both variants of amoebae treated with cytochalasin D, an inhibitor of actin polymerization. During endocytosis, actin microfilaments are crucial for the formation and movement of vesicles. The connection between receptor-mediated endocytosis and the actin cytoskeleton during the formation and detachment of newly formed vesicles is well documented in other cells [54–56]. In *E. histolytica*, actin has been linked to phagocytosis [52], fluid-phase endocytosis [57], exocytosis [58], and erythrophagocytosis [53]. In contrast, microtubules appear not to be involved in B-holo-Lf endocytosis by amoebae. However, microtubules are involved in the endocytosis of holoLf in mammalian cells where clathrin-dependent vesicles are organized by microtubules [59]. In *E. histolytica*, microtubules have been studied to determine their involvement in cell division, but the data have not been able to correlate microtubules with the movement of vesicles [60]. Several investigators have reported the effect of cholesterol in the virulence of amoebae. Serrano-Luna et al. cultured nonvirulent and virulent variants of the strain HM1:IMSS in the presence of cholesterol and observed that the nonvirulent amoebae slightly increased the endocytosis of latex microspheres [61]. Virulent amoebae, which continuously are in contact with cholesterol, showed similar endocytosis. In addition, HSF cells stimulated with cholesterol increased the caveolae-dependent endocytosis [62]. Although the gene of caveolin has not been found in *E. histolytica*, H-holo-Lf is cholesterol-dependent endocytosed in nonvirulent amoeba [12] and according to our results, B-holo-Lf is endocytosed through clathrin- and cholesterol-dependent vias in virulent amoeba. More experiments are needed to understand the effect of cholesterol in the endocytosis of iron-containing proteins by *E. histolytica*.

Proteases secreted by pathogenic protozoa are essential for their biology, including development, immune evasion, and host tissue degradation for nutrient acquisition. Iron acquisition from B-holo-Lf and H-holo-Lf, respectively, has been reported in two protozoan species: *T. foetus* [63] and *E. histolytica* [12]. In this work, both *E. histolytica* variants degraded B-holo-Lf through three internal cysteine proteases, and proteolytic cleavage of holoLf caused the release of iron for cellular metabolism. These results allow us to hypothesize that after being endocytosed, B-holo-Lf proteolysis may occur in amoebic lysosomes (pH < 4) where B-holo-Lf is degraded by proteases and iron is released in these organelles as previously suggested for iron acquisition from H-holo-Lf in nonvirulent amoebae [12]. The acidic environment of amoebic vesicles and the presence of cysteine proteases may be factors that contribute to B-Lf-iron release to support amoeba growth *in vitro*; however, this mechanism may also likely occur with B-holo-Lf during intestinal amoebiasis and ALA. Interestingly, *E. histolytica* trophozoites also degraded B-holo-Lf through secreted proteases to the culture supernatant, suggesting that this substratum may be cleaved before endocytosis, providing iron for parasite growth.

5. Conclusion

In this study, we compare the utilization of B-holo-Lf as an iron source by *E. histolytica* trophozoites of different virulence, for their growth *in vitro*. The results suggest that B-holo-Lf is specifically and rapidly captured by amoebic Lfbps with a very high affinity, and virulent amoebae showed higher V_{max} and *Eh*BholoLfbps than nonvirulent amoebae. B-holo-Lf was internalized through constitutive and organized endocytic processes involving clathrin-coated pits and cholesterol. These data allow us to hypothesize that the endocytosis of iron-containing proteins from the human host as well as from bovine milk products may be important for the parasite.

Conflict of Interests

The authors declare that there is no conflict of interests regarding this paper and that they have no competing financial interests.

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