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Activity of trypanosome lytic factor: a novel component of innate immunity

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Abstract

Trypanosome lytic factors (TLFs) are high-density lipoproteins and components of primate innate immunity. TLFs are characterized by their ability to kill extracellular protozoon parasites of the genus *Trypanosoma*. Two subspecies of *Trypanosoma brucei* have evolved resistance to TLFs and can consequently infect humans, resulting in the disease African sleeping sickness. The unique protein components of TLFs are a hemoglobin-binding protein, haptoglobin-related protein and a pore-forming protein, apoL-I. The recent advances in our understanding of the roles that these proteins play in the mechanism of TLF-mediated lysis are highlighted in this article. In light of recent data, which demonstrate that TLFs can ameliorate infection by the intracellular pathogen *Leishmania*, we also discuss the broader function of TLFs as components of innate immunity.

Keywords

African sleeping sickness; apolipoprotein L-I; apoL-I; haptoglobin-related protein; Hpr; innate immunity; *Leishmania*; pore-forming protein; serum resistance-associated protein; SRA protein; *Trypanosoma brucei*; trypanosome lytic factor; TLF

Trypanosome lytic factors

Several *Trypanosoma* species, including *Trypanosoma brucei brucei*, are transmitted by the bite of an infected tsetse fly and cause disease in livestock and other animals. Humans cannot be infected by *T. brucei brucei* because of trypanosome lytic factors (TLFs) present only in the blood of humans and several nonhuman primate species. Only two subspecies of *T. brucei* can cause disease in humans, *T. brucei rhodesiense* in East Africa and *T. brucei*

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gambiense in West Africa, precisely because they are resistant to TLF-mediated lysis [1,2]. According to the WHO, 60 million people living predominantly in poor rural areas are at risk from human African trypanosomiasis, or sleeping sickness, and the total cost, both human and economic, incurred by this disease must not be underestimated [3]. Human TLFs are comprised of two high-density lipoprotein (HDL) subfractions termed TLF1 and TLF2 [4,5]. Both fractions contain the primate-specific proteins haptoglobin-related protein (Hpr) [6] and apoL-I [7], and both proteins are implicated in the killing of trypanosomes by different mechanisms. The mechanism of lysis by TLF is not fully agreed upon but recent findings, which will be reviewed here, have provided fresh insight. First we will introduce the a*poL-I* and *Hpr* gene families.

ApoL-I: a primate-specific paralog of a rapidly evolving multigene family

The *apoL-I* gene is one member of the apoL family of genes, of which there are six known members in humans and as many as 14 in mice [8,9]. In humans, the apoL gene family is split between a 127 kb cluster containing *apoL-I–IV* and a second cluster, containing *apoL-V* and *apoL-VI*, both of which are found on chromosome 22 [10]. The apoL-I–IV cluster arose recently via tandem gene duplications during primate evolution. Smith and Malik recently provided evidence of positive selection in all members of the apoL gene family, in multiple primate species [9], suggesting that these genes are rapidly evolving and, therefore, may have a role in the host defense against pathogens. ApoL-VI can induce apoptosis, whereas apoL-I causes autophagy when overexpressed in cancer cells, indicating a possible role for apoL proteins in host-cell death [11,12]. ApoL-I is the only member of the apoL gene family that contains a canonical N-terminal signal peptide; all other apoL proteins are localized inside the cell. The apoL-I protein has so far only been found in the trypanolytic serum of human and gorilla, despite the presence of TLF activity in baboon, mandrill and sooty mangabey sera [5,13]. The genome of the baboon *Papio anubis* contains a divergent *apoL-I* gene, which has not been completely sequenced [9]. Thomson *et al.* have recently cloned *apoL-I* from *Papio hamadryus* and shown that it encodes a full-length protein with 60% identity to human apoL-I. When used to generate transgenic mice by hydrodynamic gene delivery, baboon apoL-I effectively killed human-infective trypanosomes [14]. This observation is novel and suggests that the other primates that are able to kill human-infective trypanosomes [5,13], such as sooty mangabeys and mandrills, may have similar *apoL-I* genes to baboons, which are different from the *apoL-I* genes of humans and gorillas.

Hpr: a primate-specific duplication at the haptoglobin locus

Detailed molecular analyses of the haptoglobin (Hp) locus have revealed several expansion and contraction events that have occurred during primate evolution [15]. The human genome contains two Hp genes: *Hp* and *Hpr*. The most obvious difference between the *Hp* and *Hpr* genes is the presence of a retrovirus-like sequence in the first intron of *Hpr*, which contributes to the decreased (6%) expression of *Hpr* compared with *Hp* in humans [16]. Both Hp and Hpr proteins are formed from the covalent association of an α- and β-chain dimer, which in the case of Hp can be further disulfide linked to form covalent tetramers or oligomers, owing to the presence of one or more α-chain cysteines, which are not found in Hpr [5,17]. Human Hp and Hpr are 91% identical and both are high-affinity hemoglobin (Hb)-binding proteins, though Hp has a sixfold higher affinity than Hpr [18]. Hp ameliorates toxicity associated with free Hb at vascular and extravascular sites and enables the removal of Hp–Hb complexes from the circulation via the macrophage Hp–Hb scavenger receptor, CD-163 [17,19]. Clusters of surface-exposed amino acids, which are unique to Hpr, were found to abrogate high-affinity binding of Hpr–Hb to CD-163 [20]. Importantly, this prevents the clearance of Hpr–Hb-associated TLF particles from circulation during intravascular hemolysis in human patients [18]. Hpr protein has been found in the serum HDLs of all primates known to have TLF activity

(it is absent in primates that do not have TLF) and is specifically associated with TLF1 and TLF2 complexes [5,21].

Mechanism of trypanosome lysis by TLF

As a first step, trypanosome lysis by TLF requires the uptake of the lytic particle by receptor-mediated endocytosis [22]. As lipid auxotrophs, trypanosomes obtain lipids in the form of host plasma-derived HDL and low-density lipoprotein, which are endocytosed by a parasite lipoprotein scavenger receptor [23]. Binding experiments indicate that TLF1 and HDL compete for the same low-affinity lipoprotein receptor but that TLF also has an additional low-capacity, high-affinity TLF receptor [24]. These observations have been extended with the recent discovery of a high-affinity trypanosome Hp–Hb receptor (THpHbR), which is used by the parasite to scavenge heme, for which they are auxotrophs [25]. Receptor-knockout parasites are unable to endocytose recombinant forms of Hp–Hb and Hpr–Hb, and exhibit attenuated lysis by normal human serum. These results suggest that Hpr–Hb-bound TLF is a 'Trojan horse' that piggybacks on the parasite-specific Hp–Hb endocytic pathway to enhance its uptake. Importantly, trypanosomes in a TLF-transgenic mouse model lacking Hpr, or in Hp/Hpr-null human serum, are still killed at physiologically relevant TLF concentrations, confirming that uptake of TLF1 also occurs independently of the THpHbR, presumably via engagement of the lipoprotein scavenger receptor [25–27].

Following endocytosis, TLF is trafficked to the lysosome, which is the primary site of TLF activation. Acidification of the lysosome, pore formation by TLF and subsequent trypanosome lysis is blocked by the weak bases ammonium chloride or chloroquine [28,29]. Studies using reconstituted lipid bilayers and liposomes made from trypanosome lipids revealed that low-pH activation of TLF resulted in pore formation, which allowed the selective passage of cations [30]. Recently, data by Harrington *et al.* further detailed the membrane association of TLF proteins in a reconstituted liposome system; association was dependent on low pH, the presence of anionic phospholipids and physiological ionic strength [31]. The selectivity of delipidated apoL-I for membrane binding was consistent with that of whole TLF, suggesting that apoL-I drives the initial membrane interaction and insertion. Thereafter, at acidic pH, Harrington *et al.* propose that there is an Hpr–Hb (Hp–Hb)-catalyzed generation of oxygen free radicals, leading to lipid peroxidation and loss of lysosomal membrane integrity [32,33]. However, this oxidative pathway is not required for the lysis of trypanosomes, because human serum genetically devoid of Hpr and Hp still has physiologically relevant trypanolytic activity and transgenic mice producing human Hpr have no detectable TLF activity in their serum *in vivo* or *ex vivo* [26,27].

Most importantly, apoL-I is sufficient to kill *T. brucei brucei in vitro* [7] and to protect mice from infection with *T. brucei brucei* in a transgenic mouse model [26]. When added to nonlytic serum (fetal calf serum), recombinant apoL-I trafficked to the lysosome, which resulted in lysosome membrane depolarization, swelling of the lysosome (only readily apparent when the parasite was 'immobilized' in semi-solid agar) and lysis of the parasite [34]. Furthermore, an N-terminal fragment with limited homology to the pore-forming domain of bacterial colicins, comprising amino acids 90–235 of apoL-I, formed anion-selective pores in artificial planar lipid bilayers [34,35]. By contrast, TLF1 formed cation selective pores in planar trypanosome lipid bilayers and liposomes [30]. These results suggest that the ion selectivity of the pore-forming domain may be modulated by additional components present in TLF1, including but not limited to the C-terminus of apoL-I, which is essential for lysis of trypanosomes *in vivo* [26]. Subsequent to lysosomal activation of TLF, it has been established that trypanosome lysis involves the initial influx of Cl^- ions into the lysosome, followed by the influx of Na^+ and Cl^- ions from the extracellular milieu into the cytoplasm of the parasite, which is then followed

by a compensatory influx of water [30,34]. Overall, the data suggest that Na^+ permeability of the plasma membrane precedes Cl^- influx owing to the following observations:

- At the time of addition of TLF, lysis could be delayed by the replacement of NaCl with Na gluconate (gluconate$^-$ replaces and reduces influx of Cl^-) and choline Cl (choline$^+$ replaces and reduces influx of Na^+) [30];
- After 60 min preincubation with human serum (TLF), lysis could be delayed only by the replacement of NaCl with Na gluconate (reduces influx of Cl^-) but not choline Cl (reduces influx of Na^+);
- Cl^- influx into the whole parasite could be prevented by di-isothiocyanostilbene disulfonate, which blocks the Cl^- channels in the plasma membrane, but it does not block the monovalent ion pores generated by apoL-I in liposomes [34].

Although the pore is initially generated in the lysosome and potentially late endosomal compartments, we speculate that membrane containing the pores ultimately recycles back to the plasma membrane. Indeed, small peptide fragments derived from endocytosed host transferrin were found to be released into the medium by trypanosomes, suggesting that endocytosed proteins can be recycled back to the surface, even after trafficking to a compartment for degradation [36]. Furthermore, the glycosylphosphatidylinositol-anchored surface coat VSGs are constantly recycled by the parasite [37,38].

Although it is undisputed that Hpr–Hb enhances high-affinity uptake of TLF by trypanosomes, the relevance of Hpr–Hb to killing via the generation of oxygen free radicals is less clear. The Hajduk group demonstrated that TLF1-mediated lysis in the presence of Hb could be partially blocked by antioxidants and by an iron chelator, N,N´-diphenyl-p-phenylene diamin, which prevented the production of hydroxyl radicals by the Fenton reaction [32,33]. By contrast, in an essentially Hb-free study, oxygen radicals were not critical for lysis, because lysis by TLF1 could not be blocked by any relevant inhibitors, including an iron chelator and several antioxidants [39]. In addition, the stimulation of TLF1 killing observed in the presence of Hb can be completely blocked by Hp, a protein which is present at an approximate 50–100-fold greater concentration than Hpr in normal human serum [33]. Thus, the significance of Hpr–Hb-mediated lipid peroxidation during lysis by human serum may be most relevant in the context of reduced Hp and the amount of Hb attached to TLF, which is a ball of oxidizable lipids. Hb-bound TLF could be prevalent when red blood cells lyse, releasing Hb [40,41], and in ahaptoglobinemic individuals [6].

Serum resistance mechanism of *T. brucei rhodesiense* provides clues to the molecular mechanism of lysis by TLF

Trypanosoma brucei subspecies *T. brucei gambiense* and *T. brucei rhodesiense* are distinguished from the morphologically identical *T. b. brucei* by their resistance to human TLF [2]. Although *T. b. gambiense* accounts for up to 90% of all reported cases of human sleeping sickness in Africa, the mechanism by which this parasite resists human serum is completely unknown [42]. The human serum resistance phenotype of *T. brucei rhodesiense* depends on the expression of a single gene, termed serum resistance associated (*SRA*) [43]. Significantly, *SRA* expression is sufficient to confer human serum and TLF resistance to *SRA*-transgenic *T. brucei brucei* parasites [44]. The *SRA* gene is considered a marker of *T. brucei rhodesiense* since it is not found in *T. brucei gambiense*, *T. brucei brucei* or any other trypanozoan tested [1,45]. The SRA protein is a truncated VSG homolog, with a core VSG-like fold containing a canonical C-terminal glycosylphosphatidyl inositol anchor-signal sequence but lacking the exposed VSG variable loops [46]. SRA mediates resistance to TLF via a specific interaction with human serum apoL-I, although the *in vivo* site of this interaction has been difficult to establish [7]. In one study, SRA protein was detected in a *T. brucei rhodesiense* compartment

containing the lysosome marker p67 [7] along with endocytosed recombinant apoL-I, leading to the conclusion that SRA binds to and prevents apoL-I pore formation in the lysosome. In another study using *T. brucei brucei* expressing ty-tagged SRA, SRA alone or SRA and endocytosed TLF1 were observed in an uncharacterized nonlysosomal vesicular compartment [22], leading to the conclusion that SRA reroutes TLF1 away from the site of activity in the lysosome, potentially into recycling endosomes. It is likely that both mechanisms operate to maximally prevent TLF1 activation.

Regardless of where apoL-I interacts with SRA, disruption of this interaction could hypothetically render human-infective trypanosomes susceptible to lysis by TLF. To confirm this hypothesis, a truncated mutant of recombinant apoL-I (Tr-apoL-I), which lacked the 60 C-terminal amino acids, was generated; Tr-apoL-I did not bind to SRA and could kill human-infective trypanosomes *in vitro* [7]. In addition, Tr-apoL-I was protective against human-infective trypanosome infection *in vivo*, but only if it was conjugated to trypanosome VSG-specific nanobody, which serves to bind Tr-apoL-I to the entire surface of trypanosomes [47]. This likely results in supraphysiological amounts of Tr-apoL-I accumulating in the parasite, as the clearance of antibody from the surface of trypanosomes is very fast, continuous and efficient [37]. In contrast to these data, Tr-apoL-I produced in a transgenic mouse model and incorporated into mouse HDLs could not protect against *T. brucei* infection, human infective or otherwise, suggesting that the C-terminus does indeed have an essential role in trypanolysis by native apoL-I-containing HDLs. Given that the C-terminal domain is the most conserved domain throughout the apoL family of proteins and there is genomic evidence of positive selection in primate apoL-I, II, IV and VI C-terminal domains, it has been concluded that it is not dispensable [9]. With all the current data taken together, we conclude that Tr-apoL-I is a hypomorph; a protein with partial but not full activity. We propose that the C-terminal domain of apoL-I, which contains a hydrophobic α-helix comprising residues 340–362 with membrane fusogenic activity [7,48] and a coiled–coil amphipathic α-helix (residues 365–395), may, under acidic conditions, serve to concentrate apoL-I by oligomerization and/or directly mediate the interaction of TLF with a trypanosome protein that facilitates the proximity to, and interaction with, trypanosome membranes in addition to the previously proposed membrane-addressing domain (A248–P291) [35].

TLF is a novel component of innate immunity

Based on our understanding of the mechanism of trypanolysis by TLF, it seemed plausible that TLF could potentially kill other microbes if the correct conditions existed. TLF requires low pH to activate the pore-forming toxin; therefore, the pathogen would have to either take up TLF and transport it to an acidic intracellular organelle, or TLF would have to be taken up by the host cell and co-localized with an intracellular pathogen in an acidic organelle.

To test this hypothesis, we evaluated the action of TLF against intracellular *Leishmania* parasites, which are the causative agents of leishmaniasis, a disease that ranges from mild cutaneous and mucocutaneous lesions to fatal visceral infections. Infection of the vertebrate host is initiated by the deposition of nondividing metacyclic promastigotes in the skin by sand flies. The parasites are initially taken up by neutrophils [49], which apoptose and release parasites that are then engulfed by macrophages. In macrophages, promastigotes develop within an acidic compartment, the parasitophorous vacuole, and differentiate into amastigotes, which multiply, and eventually rupture the cell and spread to uninfected cells [50,51].

Trypanosome lytic factor was shown to accumulate within macrophage endosomes and lysosomes including the parasitophorous vacuole, where it killed approximately 50% of the intracellular parasites without activating the macrophages. The action of TLF was shown to be directly on the parasite. *Leishmania*-infective metacyclics bound TLF and, when incubated

under acidic conditions, were visibly swollen and as a result had significant reduction in their ability to infect macrophages. By contrast, the dividing intracellular amastigotes did not bind TLF and were resistant to TLF [52]. Finally, it was demonstrated that apoL-I and Hpr were both required for maximal reduction of *Leishmania* lesions in a transgenic-TLF animal model *in vivo*. Overall, the data suggest that Hpr is a ligand for an as yet unidentified receptor on macrophages that enhances the uptake of TLF, and apoL-I is probably the pore-forming, parasite-damaging component. The mechanism of action of TLF against *Leishmania* is not yet clearly defined; however, it is likely that TLF damages *Leishmania* by pore formation directly in the plasma membrane of the susceptible metacyclics. In view of these data, we hypothesize that TLF can damage other microbes that reside within acidic compartments of a host cell to which TLF can be delivered.

Many microbes parasitize professional phagocytic cells (i.e., neutrophils, dendritic cells and macrophages). A key to their survival is their ability to either adapt within the hostile environment of the phagolysosome or to evade phagolysosome fusion. We propose that TLF is one of the antimicrobial components of the phagolysosome that has pressured pathogens to evolve in order to avoid TLF's damaging activity. We surmise that the most susceptible microbes would be:

- Microbes residing in cells that: endocytose HDL particles (i.e., harboring appropriate lipoprotein scavenger receptors, such as scavenger receptor type B (SR-B)I [53], SR-BII [54] or CD36 [55]), endocytose Hp via receptors such as the human leukocyte adhesion glycoprotein MAC-1 [56] and, thus, endocytose TLF that contains Hpr;
- Intracellular microbes living in an acidic environment, because TLFs are activated at acidic pH. Lysis of African trypanosomes by TLF is dependent on acidic pH [57], as is TLF activity against *Leishmania* [52];
- Microbes living in dynamic compartments, where TLFs could be delivered upon fusion with endosomes or lysosomes;
- Microbes that bind TLF to their surface, such that TLF (500 kDa complex) is brought into close proximity to the membrane. Upon acidification, apoL-I (42 kDa) will be released and insert into the membrane, creating monovalent ionic pores.

Obligate intracellular pathogens, such as the parasite *Leishmania* or the Gram-negative bacterium *Coxiella burnetti*, satisfy all the above requirements to be targeted by TLFs *in vivo*, as they live in dynamic acidic compartments [50,58] and their host cells (macrophages) can take up TLF particles [52]. Some facultative intracellular pathogens arrest the organelle's maturation at an early stage in order to avoid encountering lysosomal content; nonetheless, they still have contact with the upstream endocytic machinery (e.g., *Mycobacteria tuberculosis* [59] and *Histoplasma capsulatum* [60,61]), or eventually allow phagolysosomal fusion (e.g., *Salmonella enterica* Serovar Typhimurium [62]). The pathogen *Cryptococcus neoformans* resides in fully acidified phagosomes, but eventually extrudes the phagosomes from living cells [63]. Therefore, TLF delivery to these pathogens remains possible, though the conditions may not be optimal for lytic effect owing to either the poor acidification of the compartment in the case of *M. tuberculosis* and *H. capsulatum*, or modified delivery of TLF in the case of the other pathogens [64].

By contrast, pathogens that rapidly escape from phagolysosomes, disrupting the membrane of the maturing organelle to develop in the cytoplasm, should be resistant to TLF *in vivo* (e.g., the Gram-negative bacterium *Shigella flexneri* [65] or the parasite *Trypanosoma cruzi* [66]). In fact, any effect of TLF against *T. cruzi in vivo* was not detected [52]. Other pathogens, such as the parasite *Toxoplasma gondii* [67,68] and the Gram-negative bacterium *Brucella*

abortus [69] are taken up by host cells but rapidly block fusion of their pathogen/host vacuole with endocytic pathways, precluding any potential TLF delivery.

In conclusion, we consider TLF to be one of the first barriers of defense against microbes. The effect and specificity of TLF varies on different microbes depending on their exposure: time of contact, quantity of particles delivered, pH conditions and stage/vulnerability of the microbe itself must all be taken into account. We speculate that humans harboring defective TLF activity may be more susceptible to select infections. Individuals susceptible to a nonhuman-infective African trypanosome, *Trypanosoma evansi*, have been identified in India with mutations in their *apoL-I* gene [70]. Interestingly, a genome-wide linkage study with visceral leishmaniasis performed in eastern Sudan [71] showed a significant linkage with markers on chromosome 22q12 and susceptibility to the disease. This region includes the *apoL-I* gene, which encodes the pore-forming protein of TLF.

Future perspective

It is anticipated that a greater understanding of the mechanism of TLF activity will be achieved with the molecular dissection of the process of membrane interaction and pore formation by apoL-I. We believe this will only be physiologically relevant if studies are carried out on the membrane-interactive properties of apoL-I and genetically modified variants thereof in their native, lipoprotein-bound state. These results will also have important implications for understanding the largely unexplored functions of the apoL family, all of which are potentially involved in human disease processes. Molecules and/or proteins that protect self from endogenous apoLs and also regulatory molecules and/or proteins that keep the apoL family of proteins inactive until they are required are expected to be discovered. Furthermore, we predict that the future discovery of TLF-susceptible intracellular pathogens will enlighten a previously unrecognized effector arm of primate-specific innate immunity. This unexplored host–pathogen interaction will reveal mechanisms elaborated by pathogens to evade TLF-mediated lysis, and the host's drive to overcome pathogen resistance mechanisms by evolution of the components of the TLFs. The gene expressed by *T. brucei rhodesiense* to evade killing TLF has been defined, but *T. brucei gambiense* does not have this gene; nonetheless, research will ultimately discover how this parasite evolved to evade TLF. One example of host evolution that has recently been delineated at the molecular level is the ability of some Old World monkeys, including baboons, to evade the trypanosome-encoded antagonist, SRA, and kill human-infective parasites. This discovery has led to endeavors to create transgenic cows, which will be resistant to both animal- and human-infective trypanosomes. This would reduce, if not eliminate, disease in livestock and, thereby, help alleviate poverty and hunger in sub-Saharan Africa.

Executive summary

ApoL-I: a primate-specific paralog of a rapidly evolving multigene family

- ApoL-I is a pore-forming antimicrobial protein.
- ApoL-I is activated at low pH and binds to negatively charged microbe/membrane surfaces.
- The C-terminus of apoL-I is required for activity *in vivo*.

Haptoglobin-related protein: a primate-specific duplication at the haptoglobin locus

- Haptoglobin-related protein (Hpr), when bound to hemoglobin (Hb), is a ligand for a high-affinity heme scavenger receptor on African trypanosomes.

- Trypanosome lytic factor (TLF)1-bound Hb utilizes the heme scavenger receptor, whereas TLF2, an immunocomplex of TLF1 and polyclonal IgM (autoantibody), does not utilize the Hpr–Hb receptor. The receptor for TLF2 remains unidentified.
- Hpr is a ligand for an unidentified haptoglobin receptor on macrophages.

Mechanism of trypanosome lysis by TLF

- TLF binds to high- and low-affinity receptors on trypanosomes and macrophages, which facillitate uptake of the antimicrobial complex.
- TLFs are activated in the lysosome of the trypanosome and macrophage by low pH.
- TLFs form very small pores in the membrane of trypanosomes that allow the flux of cations and anions down their concentration gradients. Dysregulation causes osmotic imbalance and the flux of water into the parasite.

Serum resistance mechanism of *Trypanosoma brucei rhodesiense* provides clues to the molecular mechanism of lysis by TLF

- Serum resistance-associated gene binds to apoL-I and recycles TLF out of the parasite and/or directly neutralizes the pore-forming activity within the lysosome; this is an example of pathogen evasion.
- Baboon, mandrill and mangabey TLFs evade neutralization by SRA.

TLF is a novel component of innate immunity

- TLF is endocytosed by macrophages and delivered to lysosomes.
- TLF is delivered to the *Leishmania* phagolysosome and kills the infective metacyclic promastigote at low pH.
- TLF binds to the surface of metacyclic promastigotes, and at low pH causes them to swell, probably owing to pore formation, thereby reducing their infectivity.
- Amastigotes, the intracellular proliferative forms of *Leishmania* in mammals, do not bind TLF and are resistant to TLF; this is an example of pathogen evasion.

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