Review

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The trypanolytic factor–mechanism, impacts and applications

Richard J. Wheeler

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, UK, OX1 3RE

The *Trypanosoma brucei* subspecies *T. brucei brucei* is non-human infective due to susceptibility to lysis by trypanolytic factor (TLF) in human serum. Reviewed here are the advances which have revealed apolipoprotein L1 (ApoL1), found in high density lipoprotein, as the lysis-inducing component of TLF, the means of uptake via haptoglobin-related protein receptor and the mechanism of resistance in *T. b. rhodesiense* via its serum resistance-associated (SRA) protein. The first practical steps to application of these discoveries are now in progress; transgenic animals expressing either baboon or minimally truncated human ApoL1 show resistance to both *T. b. brucei* and *T. b. rhodesiense*. This has major implications for treatment and prevention of human and animal African trypanosomiasis.

The issue of trypanosomiasis

African trypanosomiasis is a major mammalian disease in sub-Saharan Africa with significant human health and economic impacts. It is caused by protozoan parasites of the genus *Trypanosoma* which are transmitted by flies from the genus *Glossina* (tsetse) and follow a lifecycle through the fly and mammalian host (Figure 1) [1,2]. There are three main livestock pathogens, *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei* [3]. Two *T. brucei* subspecies, *T.b. rhodesiense* and *T.b. gambiense*, are also human pathogens [4]. The extent of infection is not well known owing to the remote nature and limited health resources of affected areas; however, the World Health Organisation estimates human infection, which is fatal if not treated, at a minimum of 50 000 new cases worldwide per year [5].

Prevention of trypanosomiasis has been unsuccessful because the established methods of vector control, trapping of flies and release of sterile males, work in principle but face significant logistical and financial difficulties [6]. Furthermore, antigen-targeting vaccine development is prevented by the variable surface glycoprotein (VSG) coat of the bloodstream form which allows the parasite to avoid the antibody-mediated immune response [7,8]. Drug treatment of trypanosomiasis is difficult because available drugs are often toxic and resistance to them is increasing [9].

Difficulties in traditional approaches to disease control demand an understanding of the differences between human infective and non-infective *T. brucei* subspecies, which might allow targeted development of drugs and vaccines for humans and livestock. Both the human

Corresponding author: Wheeler, R.J. (richard.wheeler@path.ox.ac.uk)

Glossary

Apolipoprotein: a lipid-binding protein found associated with lipoprotein particles in the bloodstream. An example is apolipoprotein A1 (ApoA1), a cofactor of lecithin cholesterolacyltransferase, which recruits free cholesterol for esterification to allow its addition to the hydrophobic lipid core of the lipoprotein particle.
Colicins: a family of bactericidal proteins produced by some strains of *Escherichia coli*. They are composed of three domains, one of which causes death by nuclease or pore-forming activity. Humans possess a cluster of genes that encode proteins with a colicin-like pore forming domain, including the widely expressed apolipoprotein L1 (ApoL1), which appears to be the only one of the cluster secreted to the extracellular environment.
Endosomes: a membrane bound compartment in eukaryotic cells involved in membrane and cargo trafficking between the cell surface and the lysosome. Like the lysosome, endosomes also have a low internal pH. In *T. brucei* all endo- and exocytosis occurs in the flagellar pocket.
Expression site associated genes: the set of genes co-expressed from the polycistronic transcript of a particular variable surface glycoprotein (VSG) expression site (ES). The expression site associated genes (ESAGs) are thought to help 'optimise' a *T. brucei* cell for growth in a particular mammalian host.
Flagellar pocket: the large invagination at the base of a *T. brucei* cell's single flagellum proximal to the golgi apparatus. It is the only region of the cell where the plasma membrane is not supported by a dense corset of microtubules, the subpellicular array, and therefore is the only site at which vesicle invagination can occur for endocytosis. It is thought the evolutionary pressure for the confinement of endocytosis and exocytosis to this region is for immune evasion; membrane proteins in the flagellar pocket appear not to be immunogenic.
High density lipoproteins: a heterogeneous population of approximately spherical blood-borne particles consisting of a lipid core (particularly triglycerides and cholesterol derivatives) with an outer hydrophilic layer (phospholipids, cholesterol and apolipoproteins), which solubilise the lipid cargo. The term 'high density' refers to high protein content relative to other lipoproteins. Their major role is the collection of cholesterol from peripheral tissues and transport to the liver.
Iron Fenton chemistry: the catalysis of hydrogen peroxide breakdown to the hydroxyl radical (OH·) by ferric iron(III). OH· is a powerful oxidising agent capable of damaging virtually all biological molecules, thus causes oxidative stress.
Phagolysosome: the specialised membrane bound compartment formed when a phagosome, carrying a cargo from phagocytosis, fuses with a lysosome. The mammalian lifecycle stage of *Leishmania* spp. is an intracellular parasite within the macrophage phagolysosome where ApoL1 might also have ion pore forming activity.
Trypanolytic factor: a component of mammalian serum that causes trypanosome lysis. In humans the trypanolytic factor is a subcomponent of the densest sub-fraction of HDL, HDL3, which contains apolipoprotein L1 (ApoL1) and haptoglobin related protein (Hpr). More specifically it can refer to the particular protein responsible for lysis.
Tsetse fly: the insect host of *T. brucei* which occupies much of sub-Saharan Africa. Trypanosomes can be found in several organs: the crop, where a blood meal is initially stored; the midgut, where digestion occurs; the proventriculus, a valve responsible for connecting the oesophagus to the crop and the crop to the midgut; and the salivary glands.
Variable surface glycoprotein coat: a highly variable physical barrier made up of approximately 10^7 identical glycosylphosphatidylinositol (GPI) anchored molecules, which shield the plasma membrane and other surface proteins from interaction with antibodies and toxins. The extracellular surface loops of the molecules are where most variation is found. At any time, only one of a library of approximately 1000 VSG genes is expressed from a VSG gene expression site (ES); a combination of genetic recombination and VSG ES switching is responsible for regular changes in VSG expression.
Variable surface glycoprotein expression sites: a group of approximately 20 telomeric expression sites (ESs) used for variable surface glycoprotein (VSG) expression for antigenic variation in *T. brucei*. The VSG variant expressed depends on a combination of ES switching and VSG recombination with the library of VSG genes and pseudogenes elsewhere in the genome.

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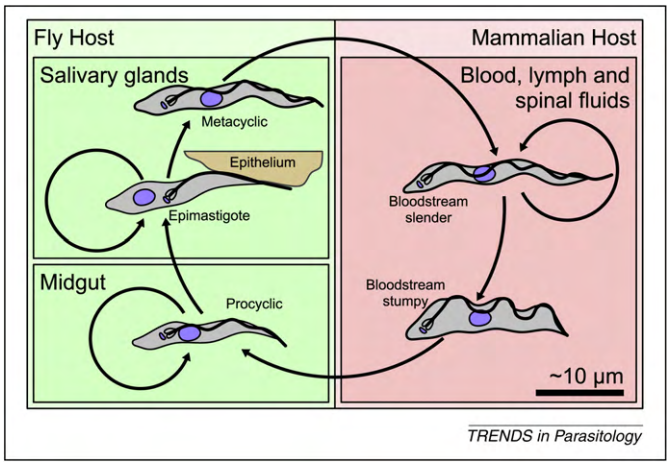

Figure 1. Lifecycle and cellular forms of *Trypanosoma brucei*. The lifecycle of *T. brucei* involves several cellular forms in various areas of two hosts, and the different morphologies are shown approximately to scale. The slender bloodstream form proliferates in the blood, lymph and spinal fluid. As the population increases some slender bloodstream cells differentiate into non-proliferative stumpy form cells. A tsetse fly takes a blood meal and ingests stumpy form cells, which transform into procyclic form cells in the midgut of the fly. Some cells migrate, via the proventriculus, to the salivary gland and differentiate into the epimastigote via an asymmetric division. The epimastigote attaches to the epithelium of the salivary gland (shown in brown) by the flagellum. Some cells detach and transform into non-proliferative metacyclic cells ready for injection into a mammal's bloodstream during a blood meal. Proliferative lifecycle stages are indicated by the arrowed loops. The VSG coat is only found on bloodstream and metacyclic form cells.

protein responsible for serum-induced lysis of *T.b. brucei* and the protein conferring *T.b. rhodesiense* resistance to lysis were identified, as has the receptor and ligand that is at least partly responsible for lytic factor uptake. Although some aspects of the mechanism involved still require clarification, these discoveries have clear implications for new treatments and preventative measures.

Early research

Humans have an innate resistance to many trypanosome species; unlike most other mammals, this includes *T.b. brucei*. For over 100 years it has been known normal human serum (NHS) and some primate sera have trypanolytic activity [10]. Resistance to lysis by NHS is the key distinguishing feature of the morphologically identical *T.b. rhodesiense* and *T.b. gambiense* from the non-human infective *T.b. brucei* [11]. The endemic and chronic nature of trypanosome infection coupled with the parasite's ability to avoid the adaptive immune system has driven the evolution of innate resistance in many species, including humans [12].

Laying the foundations: 1970s to 1990s

The trypanolytic factor (TLF) was originally identified as a class of high density lipoprotein (HDL) particles [13,14]. There are two subclasses of trypanolytic HDL, TLF1 in the HDL3 fraction (the densest HDL subfraction) and TLF2, which is arguably too large and dense to be considered a normal HDL particle [15–17]. Endocytosis and trafficking of TLF to the low pH of the lysosome is required for lytic activity; inhibition of uptake prevents lysis [18–20].

The mechanism of action and active component of trypanolytic HDL has been heavily debated and was initially investigated by direct characterisation. The two separate classes of HDL particles with lytic activity, TLF1 and TLF2, share several protein components, including apolipoprotein A1 (ApoA1) and haptoglobin related protein (Hpr), indicating that one of these would prove to be responsible for TLF activity [21]. ApoA1, which is found almost universally in HDL, was shown to lack trypanolytic activity [21–23].

Hpr was therefore thought to be responsible for trypanolysis; competition with anti-Hpr antibody or haptoglobin (Hp, with 91% sequence identity to Hpr) inhibited lysis [24,25], and Hpr appeared to be recognised by a trypanosome surface receptor [26]. The mode of action for Hpr-induced lysis was proposed to be via oxidative damage (by peroxidation) to the lysosomal membrane, possibly caused by hydroxyl radicals produced by iron Fenton chemistry [24,27]. A trypanolytic role of Hpr was supported by genetic and serum western blot studies of primates from *T. brucei* affected areas. Hpr is found in gorilla, baboon and sooty mangabey sera, primates which are resistant to *T.b. brucei* infection, but not chimpanzee serum, and chimpanzees are susceptible to infection [28].

Some evidence for the role of Hpr as the TLF has proved unreliable. Reports that addition of protease inhibitors and antioxidants prevent lysis by NHS [24,27,29], that peroxidated lipids can be detected during lysis [27] and that lysosomal membranes are disrupted [29] have proved hard to replicate [30,31]. Accordingly, the role of Hpr as the lytic component has been under heavy debate [14].

Identification of the lysis inducing protein: 2000 to 2006

An alternative route of investigation, considering the mode of resistance of *T.b. rhodesiense* to NHS, identified apolipoprotein L1 (ApoL1), not Hpr, as the lytic component in TLF. *T.b. rhodesiense* carries an unstable resistance to lysis by NHS which can be lost following serial passage of bloodstream form cells through mice [10,32] and is associated with the mechanisms responsible for VSG-based antigenic variation. The serum resistance associated (SRA) gene, first identified in 1989 [33], was shown to be a VSG expression site (ES) associated gene (ESAG), which is found at only one ES [33–35]. ESAGs are thought to contain genes that allow adaptation to optimise growth in different mammalian hosts [33,36]. As SRA is expressed only when VSG expression is from one particular ES, this provides a simple selection pressure explanation as to how resistance is quickly lost in non-human hosts. Transfection of SRA into *T.b. brucei* confers resistance to NHS lysis; therefore SRA is both necessary and sufficient for resistance to TLF [37].

SRA is an abnormal VSG lacking the surface loops [38], which is found in the endosomes [39], not on the cell surface [40]. SRA binds strongly and specifically to ApoL1, implying ApoL1 is responsible for NHS lytic activity [40]. Analysis of binding of truncated ApoL1 and SRA showed an interaction between SRA and the C-terminal α-helix of ApoL1; this is proposed to be via a coiled α-helix interaction and is supported by directed point mutations that disrupted the α-helix and abolished binding (Figure 2) [40,41]. Both trypanolytic sub-fractions, TLF1 and 2, include ApoL1; ApoL1 is vital for trypanolytic activity of NHS [42,40], and recombinant ApoL1 has trypanolytic

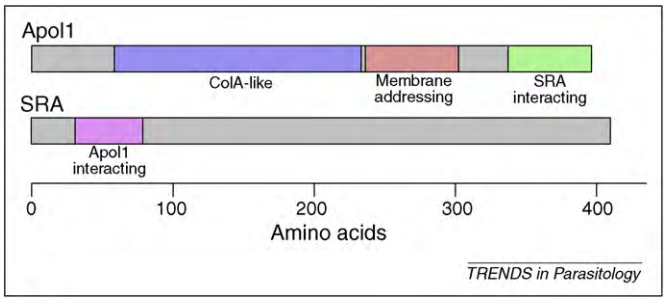

Figure 2. Functional domains of ApoL1 and SRA. ApoL1 possesses three domains of known function: **(i)** the ColA-like pore forming domain, which is predicted to consist of a bundle of α-helices surrounding two hydrophobic α-helices, which insert into a membrane to generate an anion pore; **(ii)** the membrane addressing domain, which is predicted to consist of two amphipathic α-helices, which untwist from a hairpin conformation to reveal a large hydrophobic surface under acidic conditions, allowing binding to a lipid membrane; **(iii)** the SRA interacting domain, which is predicted to consist of a single α-helix which can bind to the ApoL1 interacting domain of SRA in an α-helix-α-helix interaction. SRA possesses one domain of known function, the ApoL1 interacting domain; the remainder of the molecule has no known function but is similar in sequence to VSGs.

activity [40]. Prior to 2009, ApoL1 had only been detected in the sera of humans and gorillas, the only two primates which are resistant to *T.b. brucei* infection, yet also susceptible to *T.b. rhodesiense* [28,43]. SRA is accepted as the defining feature of the *T.b. rhodesiense* subspecies [11,44–46], though *T.b. brucei* and *T.b. gambiense* do not possess the gene [47,48]. Evidence for the key *in vivo* role of ApoL1 comes from humans lacking either ApoL1 or Hpr; ApoL1 is required for trypanolytic activity [42,49], whereas lack of Hpr only delays the trypanolytic response [42].

ApoL1 uptake can be tracked by immunofluorescence, and ApoL1 co-localises with the marker ligand tomato lectin, which is also taken up via the endocytic compartments [39]. SRA is not found in the *T. brucei* lysosome, as shown by limited co-localisation with the lysosomal marker p67 [39], in contrast with the lysosomal location previously described [40]. The interaction of SRA and ApoL1, and the point of action of ApoL1, is therefore likely to be in the endocytic compartments [39].

The N-terminal domain of ApoL1 shows similarity to colicin A anion pore-forming proteins, suggesting a similar activity. Expression of ApoL1 in *Escherichia coli* increases membrane permeability and reduces viability, confirming that it disrupts membrane integrity. ApoL1 increases the flux of anions across a purified lipid bilayer, and Cl^- (tracked with ^{36}Cl) is among the ions transported. In *T. brucei*, using the membrane potential-sensitive dye RH414, exposure to NHS was shown to reduce the membrane potential in the area of the lysosome, consistent with opening of ion channels in the lysosomal membrane. ApoL1 mediated anion transport in a purified lipid bilayer increases at low pH, suggesting that the low pH of the lysosome is required in order to insert the channel into the membrane or open the channel [41]. Unlike some Cl^- channels, ApoL1 is not inhibited by 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS). DIDS does, however, inhibit the transport of Cl^- across the *T. brucei* surface membrane and prevents lysis by NHS. This indicates that the increase of intracellular Cl^- by influx of Cl^- from the extracellular environment is involved in lysis. The resulting osmotic stress is consistent with observations of enlarged lysosomes in NHS affected cells [41]. ApoL1 is found associated with HDL3 particles in low quantities along with ApoA1, Hpr and haemoglobin (Hb) [50]. Consequently, uptake of HDL via an Hpr receptor is likely to be one of the routes for ApoL1 to enter the cell [41].

Debate over the identity and mode of action of the lytic factor(s) (and the timing of its effect) has been confused by the different assays and conditions used to assess killing. The pathologies of death described have different emphases on lysosome swelling [14,41,42] or the creation of multiple non-lysosomal vesicles [51,52]. Lysosome swelling seems incompatible with the membrane oxidative damage model as a damaged membrane might have difficulty supporting the osmotic stress, whereas multiple vesicle swelling is not fully compatible with the ApoL1 pore formation model, as ApoL1 requires the acidic environment of the lysosome for activity [41]. A clear indication of the mechanism of lysis comes from direct time-lapse observations of a population of immobilised cells, which indicate lysosomal swelling is the cause of lysis due to NHS. It was proposed that the past disagreement over the morphological changes in the lead up to lysis stem from the fact that cells with a swollen lysosome are very delicate and thus not observed by some analyses, leaving only unusual cells with multiple swollen vesicles [53]. Alternatively, normal cell movement can disrupt the formation of a single swollen vesicle. The study of immobilised cells shows slower cell death than in liquid media [53], supporting the idea that motility assists in uptake [54], or flagellar motion assists in damaging the cell. The membranes of endosomal organelles are disrupted during lysis allowing release of large molecules into the cytoplasm [50], but how this is consistent with lysosome swelling is unclear. Use of multiple assays, different time courses and variation in methods of quantification of cell death and lysis are likely to continue to complicate interpretations in this field.

The mechanism of SRA-mediated resistance is not, as might be expected, the loss of ability to internalise HDL particles [55]. The current model states SRA interacts with and neutralises ApoL1 in the endosomes prior to ApoL1 reaching the lysosome [39,40]. Degradation of ApoL1 is not required to prevent lysis. First, the presence of ApoL1 along with SRA in the lysosome does not cause lysis [40]. Second, the lysate of TLF-exposed, NHS-resistant *T. brucei* has trypanolytic activity [55], and the TLF has not been degraded as part of the resistance pathway.

Unravelling the roles of Hpr and Hb: 2007 to 2009

Uptake of TLF is partly mediated by an Hp-Hb receptor that also recognises Hpr-Hb. It has long been assumed that *T. brucei* possesses an Hpr receptor that has a role in TLF uptake [26,42]. Initial evidence for an Hp-Hb co-receptor came from a gentle, single-step anti-ApoL1 and anti-Hpr immunoprecipitation purification, identifying Hb as a TLF component that presumably binds via Hpr [50]. Later work confirmed Hpr is a high affinity Hb-binding protein [56], and Hb was shown to be a key cofactor for TLF activity, increasing sensitivity of *T.b. brucei* to TLF by increasing the rate of uptake [50].

Affinity chromatography of Hp-Hb identified the glycoprotein receptor, *T. brucei* haptoglobin-haemoglobin receptor (TbHpHbR), which binds and promotes uptake of both Hp-Hb and Hpr-Hb and performs a similar role to the macrophage Hp-Hb receptor CD163. Unlike TbHpHbR, CD163 does not bind Hpr-Hb, and therefore cannot clear Hpr-Hb from the blood following haemolysis [57]. TbHpHbR is the product of the gene Tb927.6.440, and has a putative GPI anchor site, indicating a membrane bound localisation. The receptor is found in the flagellar pocket of bloodstream form *T. brucei* [58], the site at which endocytosis occurs and is typical of surface receptors [59]. TbHpHbR is a co-receptor; Hb cannot be internalised without either Hp or Hpr. Excess recombinant Hp reduces internalisation of TLF, confirming that TLF uptake is partially mediated by this receptor [58].

The principal role of TbHpHbR appears to be haem uptake. *T. brucei* is deficient in haem biosynthesis [47], and in a non-trypanolytic host lacking Hp (an $Hp^{-/-}$ mouse) or following knockout of TbHpHbR, growth rate is reduced [58]. Inhibition of macrophage oxidative responses recovers the reduction in growth rate; therefore an important role of haem proteins in *T. brucei* is for resistance of damage by the macrophage oxidative burst. Interestingly TbHpHbR-like genes are only found in trypanosome species with a complete lifecycle stage in a host's bloodstream [58].

TbHpHbR has a major, but not exclusive, role in the uptake of trypanolytic ApoL1 by cotransport on the surface of ApoL1 and Hpr containing HDL particles. Accordingly, knockout of TbHpHbR reduces *T.b. brucei* sensitivity to lysis by NHS owing to prevention of lysis by TLF1, but TLF2 can still cause lysis [58]. The cotransport of ApoL1 with Hpr implies structural integrity of the TLF1 particles is required for effective uptake of ApoL1 and trypanolytic activity; degradation of the phospholipid component of TLF1 was previously shown to reduce lytic activity [60].

TbHpHbR is not the only receptor involved in TLF uptake; TbHpHbR improves, but is not required for, the lytic activity of TLF. There is evidence for lipoprotein scavenger receptors that would act in parallel to TbHpHbR for TLF uptake. Prior to discovery of TbHpHbR, the TLF binding properties of *T. brucei* were modelled as two putative receptors capable of binding TLF: one high affinity, low copy number (K_d ~12 nM, ~350 per cell) and one low affinity, high copy number (K_d ~1 μM, ~60 000 per cell) [26]. The properties of TbHpHbR match those of the first receptor [58]; the molecular identity of the second receptor, likely to be a lipoprotein scavenger receptor [61], is currently unknown.

The principal role of the putative low affinity lipoprotein scavenger receptor is likely to be in lipid uptake from low density lipoprotein (LDL) and HDL. *T. brucei* is a lipid auxotroph and requires the lipid cargo of lipoproteins to survive in culture conditions. The scavenger receptor is responsible for uptake of some TLF; competition with non-lytic HDL reduces *T. brucei* lysis by TLF1 [26,61], and human serum that entirely lacks Hpr does have some, albeit delayed, trypanolytic activity [42]. These pathways must be independent of TbHpHbR.

Combinatorial analysis of human ApoA1, ApoL1 and Hpr in a transgenic mouse model highlights the synergistic function of these proteins in producing HDL particles with efficient trypanolytic activity. ApoL1 alone is sufficient to confer resistance to infection by *T.b. brucei,* whereas Hpr alone does not. The level of trypanolytic activity of transgenic mouse serum is increased when Hpr is expressed with ApoL1, consistent with receptor-mediated uptake of ApoL1 and Hpr containing HDL particles. Expression of human ApoA1 is required to maximise lytic activity of the mouse serum and appears to alter HDL particle sizes to more closely match that of humans [62].

Seeking the most convincing model

Three human proteins are thought to be responsible for one mode of NHS lytic activity: Hpr, Hb and ApoL1. ApoL1 and Hpr are found associated with specific subfractions of HDL3 [13]. TLF1 and 2, and Hb is weakly associated with trypanolytic HDL via Hpr [50]. The mechanism by which lysis occurs has been heavily debated, but presently the most convincing model is that lysis is caused by the uncontrolled expansion of the lysosome [53], is dependent on uptake of ApoL1 to the lysosome [40] and is driven by insertion of ApoL1 into the lysosomal membrane at low pH, which forms anion pores [41,63]. Hpr and Hb are recognised by TbHpHbR, which is partly responsible for trypanolytic HDL uptake, thus introducing ApoL1 to the lysosome (Figure 3) [58]. The roles of these proteins have been confirmed *in vivo* in a transgenic mouse model [62]. There are still modes of NHS lytic activity that are not fully understood, although they are likely to involve ApoL1. Not all TLF uptake is TbHpHbR mediated; HDL lacking Hpr but including ApoL1 is trypanolytic [62,42], and TLF is known to bind *T. brucei* via a putative lipoprotein scavenging receptor as an alternative form of uptake [61,26]. These pathways have relevance *in vivo* [42].

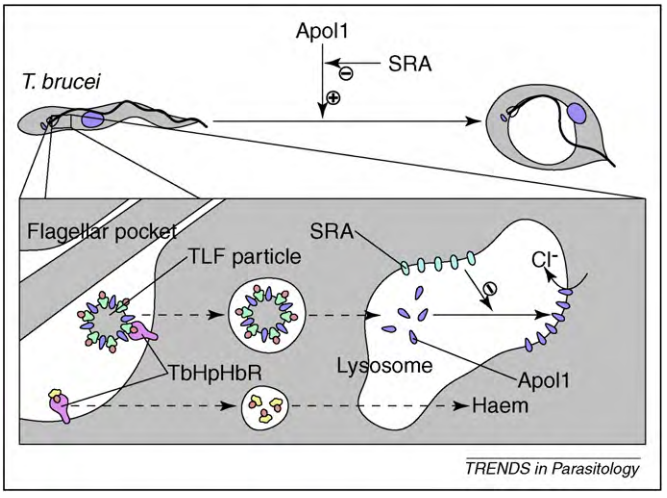

Figure 3. Role of TbHpHbR, Hp and Hb and the mechanism of action of ApoL1 and SRA. TbHpHbR, a receptor normally involved in the uptake of haem via Hp, is partly responsible for the uptake and endocytosis of TLF particles. Normal function of TbHpHbR (pink) is the uptake of haem from Hb (red) via uptake with Hp (yellow). TbHpHbR is also responsible for the uptake of TLF particles carrying the protein ApoL1 (blue) via Hpr (green) and Hb. Once in the endosome the low pH promotes formation of pores in the endosome membrane by ApoL1, which leads to an influx of Cl^-. The following osmotic imbalance results in the characteristic swelling of the lysosome and cell death. SRA, expressed by some sub-species of *T. brucei* prevents the action of ApoL1. The large and small blue structures within the *T. brucei* cells are the nucleus and kinetoplast respectively.

The sera of many species of African mammals have trypanolytic activity, of which primates, owing to their similarity to humans, have been most studied [28,43]. Hpr is found in all primate sera with *T.b. brucei* lytic activity, whereas only humans, gorillas [43] and baboons [64] have detectable ApoL1. Therefore ApoL1 is not the only primate TLF. The ApoL gene family does, however, have many members that could have related trypanolytic activity, and the gene family is under strong selective pressure, possibly indicative of a role in host–pathogen interaction [65]. It is possible the Hpr/TbHpHbR uptake pathway is used by many primate innate immune systems to target *T. brucei*. The ubiquity of TbHpHpR-like proteins in bloodstream-dwelling trypanosomes also suggests the Hpr/TbHpHbR uptake pathway would be a general target to provide resistance to multiple *Trypanosoma* species.

The requirement of Hb in the blood plasma to allow TLF uptake was proposed to imply an 'arming mechanism' for the innate immune system [66]. Trypanosome infection is linked with erythrocyte damage releasing Hb into the plasma, allowing association with Hpr on trypanolytic HDL and trypanosome uptake via TbHpHbR (Figure 4) [50]. Nonetheless, erythrocyte damage normally only occurs late during infection via extravascular erythrophagocytsis by macrophages, and there is no evidence for an evolutionary pressure that would select for such an arming mechanism. It might be the case that this is actually a limitation of this innate immune response and that the earliest stages of infection are not affected by TLF as the free Hb concentrations have not reached high enough levels, although the normal serum haemoglobin concentrations is probably sufficient to drive some trypanolysis. Alternatively, there simply might not be enough selective pressure to improve Hpr-mediated TLF uptake since TbHpHbR-independent routes of uptake (as seen in the absence of Hpr) are effective enough in its absence. Despite these caveats, there is the intriguing possibility that other chronic infections which cause haemolysis, for example malaria, influence anti-trypanosome innate immunity during co-infection.

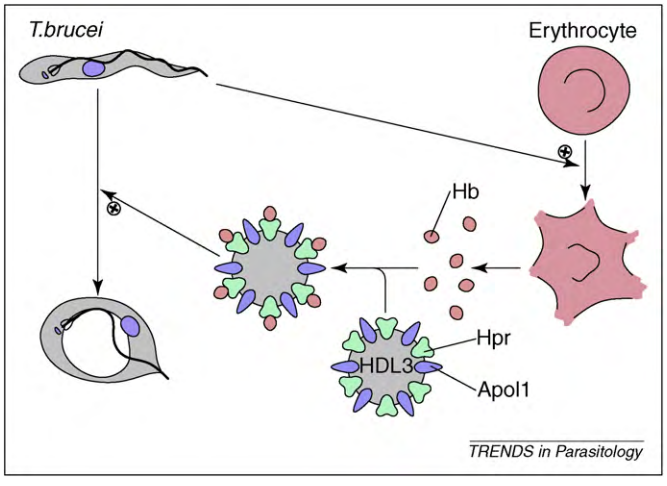

Figure 4. The proposed 'arming mechanism' of TLF. *T. brucei* infection causes erythrocyte damage releasing Hb into the plasma. Hb binds HDL3-bound Hpr, which in turn allows *T. brucei* receptor binding, uptake and lytic activity. *T. brucei* requires the haem from haemoglobin for the production of proteins to resist the oxidative burst response of macrophages as *T. brucei* is deficient in the haem biosynthetic pathway.

Further work and applications

TLF is a complex multicomponent system and understandably clarity of its function has taken time. In particular, the phenotype and mechanism of lysis by TLF has been heavily debated. Hpr and ApoL1 have both been implicated as the primary cause of lysis by methods of oxidative damage/peroxidation of the lysosomal membrane [24] and anion pore formation in the lysosome membrane [40,41], respectively. The origin of this debate was from evidence of oxidative damage to the lysosomal membrane and the important role of Hpr in TLF activity. With the discovery of both ApoL1 and TbHpHbR, it now seems clear that Hpr does not have a primary lytic effect, but instead assists TLF uptake and full TLF activity; it is also now clear how the plausible (but incorrect) model of Hpr-mediated oxidative damage arose prior to knowledge of ApoL1, which acts as a passenger during uptake. Convincing evidence of non-Hpr mediated trypanosome lysis also exists [40–42], presumably by other routes of HDL uptake [26,61]. Hpr independent uptake of soluble ApoL1 might have a lytic role, but there is little evidence that it is independent of HDL since the latter has presumably always been present in the growth medium. The Hpr independent route of uptake is directly relevant to the trypanolytic activity of NHS, as serum entirely lacking Hpr has trypanolytic activity [42]. Therefore the mechanism is worthy of further study.

There is still some evidence for the direct trypanolytic activity of Hpr [67], although this role is minor *in vivo* [49]. In principle, the role of Hpr can be resolved by determining whether excess Hpr is toxic (as would be expected if it is a toxin) or inhibits lysis (as would be expected if it is the means of uptake), yet, there are reports of both these activities [42,67]. The reports differ in that toxicity is seen only when the N-terminal 19 amino acid hydrophobic signal peptide is present. It is not clear whether Hpr toxicity is an artefact, but a recombinant protein at non-physiologically high concentrations might have the potential for toxic effects. High concentration Hpr-containing HDL in the growth media of an organism known to require the uptake of HDL as a source of lipids, particularly cholesterol [61], would be an effective method for generating abnormally high lysosomal concentrations of Hpr. If Hpr has a toxic role under certain conditions, this might explain the observation of oxidative damage in ApoL1 induced lysis [50]. Insight into evidence for oxidative damage is necessary, either as a relevant effect of lysosomal disruption or as an artefact. Iron Fenton chemistry in the haem rich acidic environment of a damaged lysosome seems likely.

Current advances at the molecular level have not resolved some basic issues. Although the proteins required for serum lytic activity are associated with HDL the roles of the different trypanolytic HDL3 sub-fractions, TLF1 and 2, and the role of soluble ApoL1 *in vivo* is still lacking. The functional difference between TLF1 and 2 is in uptake; TLF2 uptake is not inhibited by competition with excess Hp or Hpr unlike TLF1 uptake [17,68]. Forming a consistent view of the roles of TLF1 and 2 is difficult owing to some apparent contradictions: (i) Hpr is not required for TLF2 uptake (only for TLF1) [17]; (ii) human serum lacking Hpr

has normal, but delayed, lytic activity [42]; and (iii) in apparent contradiction, NHS with TLF1 inhibited by excess Hp does not have the same lysis kinetics as serum entirely lacking Hpr [42]. Many analyses simply overlook the differences between TLF1 and 2 and the different roles the two factors could play; therefore evidence for different roles of TLF1 and 2 is too sparse to draw any meaningful conclusions. One possibility is that ApoAl causes the dissimilarities between TLF1 and 2 since ApoA1 is required to maximise serum lytic ability in transgenic mice [62]. Another possibility is that TLF2 is an immature, lipid poor, precursor of TLF1 but the origin of TLF1 and TLF2 dissimilarities might prove to be for homeostatic rather than immune purposes.

Interest in the mode of action of TLF1 is owing to the role of *T. brucei* in human disease. Of the three *T. brucei* subspecies, only *T.b. brucei* is susceptible to lysis by NHS, unlike *T.b. rhodesiense* (which causes acute human African trypanosomiasis (HAT) in southern and eastern Africa) and *T.b. gambiense* (which causes chronic HAT in western and central Africa) [40]. The pore forming activity of ApoL1 could be a powerful therapeutic agent against *T.b. brucei*, and genetic modification of livestock to express ApoL1 would make them resistant to infection. Unfortunately, this is not practical, as it would promote the generation of a reservoir of ApoL1 resistant, human infective, *T. brucei* subspecies in the livestock population, possibly increasing the number of HAT cases. To prevent this, alternative TLFs from non-human primate species that confer resistance to both animal and human infective *T. brucei* could be used. Baboon ApoL1, not detected until recently, has lytic activity and includes two C-terminal lysine residues whose positioning indicates they might block binding to SRA. Expression of baboon ApoL1 with baboon Hpr and human ApoA1 confers resistance to both *T.b. brucei* and *T.b. rhodesiense* in mice [64]. Similarly ApoL1, with the SRA interacting domain removed, can be used to treat *T.b. rhodesiense*. This principle was demonstrated as feasible using an ApoL1 pore-forming domain anti-VSG camel antibody fusion where the antibody targeted the pore forming domain for eventual endocytosis [69], and it has now been shown that expression of human ApoL1 lacking the nine C-terminal amino acids in mice confers resistance to *T.b. brucei* and *rhodesiense* infection [70].

Both of these methods could be used for generating *T.b. brucei* and *rhodesiense* resistant transgenic livestock but truncated ApoL1 does not protect from *T.b. gambiense* infection, and baboon ApoL1, which would be expected to confer resistance, is untested. Non-transient expression of exogenous ApoL1 has yet to be demonstrated in any animal. Resistant cattle would be effective at reducing livestock trypanosomiasis and shrinking the animal reservoir of human infective *T. brucei*. This is on the assumption that *T.b. gambiense* would not become prevalent in the absence of *T.b. rhodesiense* as currently *T.b. gambiense* is not widely carried by livestock. Caution is necessary because resistance to lysis by truncated or baboon ApoL1 in a strain of *T. brucei* could cause human infectivity. Reserving these ApoL1 variants for treatment of cases of human *T.b. rhodesiense* infection might be best, although the balance of the economic advantages of resistant cattle and the human health benefits of an effective drug for *T.b. rhodesiense* infection is complex.

Relatively little is known about TbHpHbR, including its structure and interaction with Hp/Hpr-Hb, and where on the surface TLF binding occurs. A TbHpHbR knockout demonstrated its importance for effective parasite growth *in vivo* as it is linked with resisting the macrophage oxidative burst. Targeting of TbHpHbR for therapeutic purposes might be worthwhile as a means of slowing growth, assisting macrophage attack or improving treatment of trypanosomiasis in a combinatorial manner; the ubiquity of TbHpHbR might give this more general applicability across several trypanosome species [58].

T.b. gambiense is resistant to NHS despite carrying a copy of the TbHpHbR gene and being able to endocytose TLF [71]. Therefore, it might be assumed that it neutralises ApoL1 during the endocytosis process in a manner analogous to *T.b. rhodesiense*, but there is no evidence for the mechanisms involved. The role of ApoL1 as a TLF is not limited to *T.b. brucei*. A rare case of human *Trypanosoma evansi* infection, normally the cause of surra in livestock, was linked to a frame shift in both ApoL1 alleles [49], and, in mice, ApoL1 expression reduces severity of *Leishmania* infection [72]. ApoL1 was indicated to have lytic activity wherever the organism is exposed to HDL in an acidic environment, including the phagolysosome.

In conclusion, it appears that, after over 100 years, the identity and mechanism of at least one of the human TLFs has been determined as ApoL1 acting via anion pore formation in the lysosomal membrane. The mechanism of uptake has also been identified as transport of an ApoL1-containing HDL particle by both Hpr-Hb/TbHpHbR mediated uptake and nonspecific HDL particle uptake. Unfortunately, there is still unresolved evidence for the possible role of Hpr as a separate lytic agent and oxidative damage to the cell by an unknown mechanism. The mechanism of trypanolysis by human serum is complex, though ApoL1 has emerged as an interesting protective molecule, with effects wider than previously considered and is a prime target for further study and development.

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