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|                               |  |

## **The trypanolytic factor of human serum: many ways to enter the parasite, a single way to kill**

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### **Summary**

Humans have developed a particular innate immunity system against African trypanosomes, and only two *Trypanosoma brucei* clones (*T. b. gambiense*, *T. b. rhodesiense*) can resist this defence and cause sleeping sickness. The main players of this immunity are the primate-specific apolipoprotein L-I (apoL1) and haptoglobin-related protein (Hpr). These proteins are both associated with two serum complexes, a minor subfraction of HDLs and an IgM/apolipoprotein A-I (apoA1) complex respectively termed Trypanosome Lytic Factor (TLF) 1 and TLF2. Although the two complexes appear to lyse trypanosomes by the same mechanism, they enter the parasite through various modes of uptake. In case of TLF1 one uptake process was characterized. When released in the circulation, hemoglobin (Hb) binds to Hpr, hence to TLF1. In turn the TLF1-Hpr-Hb complex binds to the trypanosome haptoglobin (Hp)-Hb receptor, whose original function is to ensure heme uptake for optimal growth of the parasite. This binding triggers efficient uptake of TLF1 and subsequent trypanosome lysis. While Hpr is involved as TLF ligand, the lytic activity is due to apoL1, a Bcl-2-like pore-forming protein. We discuss the *in vivo* relevance of this uptake pathway in the context of other potentially redundant delivery routes.

Running title: The trypanosome lytic factor

Key words: apolipoprotein L-I, haptoglobin-related protein, human innate immunity, sleeping sickness, pore-forming proteins, BH3-only proteins, haptoglobin-hemoglobin receptor.

## A brief history of the Trypanosome Lytic Factor

### *HDLs as TLF carriers*

In the early 20<sup>th</sup> century, Laveran reported on the natural immunity of humans against *T. brucei* infection (Laveran, 1902). This immunity was found to depend on a trypanocidal factor in normal human serum (NHS), termed trypanosome lytic factor (TLF) (Hajduk et al., 1989). Early reports disclosed the association of TLF with HDL particles (Rifkin, 1978), and subsequent work concluded that TLF is restricted to the densest fraction called HDL<sub>3</sub> (Hajduk et al., 1989). In addition to the main apolipoprotein apoA1, this fraction was shown to contain haptoglobin-related protein (Hpr), a primate-specific protein derived from haptoglobin through tandem gene duplication (Maeda, 1985). Eventually the existence of two independent trypanolytic particles was established: besides HDL-bound TLF redefined as TLF1, a large lipid-poor apoA1/IgM/Hpr-containing complex called TLF2 was also found to kill trypanosomes (Tomlinson et al., 1995; Raper et al., 1999).

### *Haptoglobin-related protein as possible toxin*

Hpr was presented as the trypanosome toxin, notably because anti-Hpr antibodies and competing amounts of Hp both inhibited TLF-mediated trypanosome lysis (Smith et al., 1995; Smith and Hajduk, 1995). This hypothesis was also supported by the phylogenetic match between presence of Hpr and trypanolytic potential in different old world monkeys such as gorillas (*Gorilla*) or baboons (*Papio*), and the absence of both from chimpanzee serum (Lugli et al., 2004; Poelvoorde et al., 2004). Trypanolysis was shown to depend on Hpr-mediated binding of TLF1 to ~350 high affinity sites of the parasite surface (Drain et al., 2001), followed by intracellular trafficking of TLF1 to the lysosome (Hager et al., 1994; Shimamura et al., 2001). However, the mechanism of lysis by Hpr was debated (Molina Portela et al., 2000; Bishop et al., 2001).

### *Apolipoprotein L-I as alternative toxin*

Two *T. brucei* subspecies (*T. b. gambiense* and *T. b. rhodesiense*) resist TLF and cause sleeping sickness. While resistance of *T. b. gambiense* is constitutive, *T. b. rhodesiense* resistance appeared to be inducible under selective conditions. This character was found to result from the particular mode of expression of a gene encoding resistance to TLF, termed SRA for Serum Resistance Associated (Xong et al., 1998). The SRA gene encodes a truncated version of the major surface antigen of the parasite, the Variant Surface Glycoprotein or VSG, and is contained in one of the multiple genomic sites allowing expression of the VSG, which exhibit strict mono-allelic activity. The presence of NHS was found necessary to trigger the selection of the unique *SRA*-containing expression site, leading to transcription of this gene

under these conditions only. Expression of SRA was found sufficient to confer the resistance phenotype (Xong et al., 1998). In contrast with the VSG, SRA is located in the endocytic pathway (Vanhamme et al., 2003; Oli et al., 2006). This differential sorting might result from the internal sequence truncation differentiating SRA from regular VSGs. The key determinant for resistance to TLF was found to be the N-terminal  $\alpha$ -helix of SRA (Vanhamme et al., 2003). As in VSGs this helix is interactive, it was postulated that SRA might physically neutralise TLF through coil-coiling interaction. Affinity chromatography of detergent-solubilised NHS on immobilised SRA revealed the specific and strong binding of apolipoprotein L-I (apoL1). Recombinant versions of apoL1 exhibited a trypanolytic potential closely mimicking that of NHS, including neutralisation by SRA (Vanhamme et al., 2003). Therefore, apoL1 was presented as the trypanosome toxin of NHS. Accordingly, apoL1 was found to be present in both TLF1 and TLF2 (Molina-Portela et al., 2005; Shiflett et al., 2005), but absent from the non-trypanolytic serum of chimpanzee (Poelvoorde et al., 2004; Lugli et al., 2004).

ApoL1 is expressed from a recent gene copy of a multigene family of unknown function (Vanhollebeke and Pays, 2006). It is the only member to be secreted in the serum, where it associates with HDL<sub>3</sub> (Duchateau et al., 1997). This association suggested a role in lipid transport and metabolism, but a distinct picture emerged from studies on the trypanolytic activity of this protein (Pérez-Morga et al., 2005). ApoL1 was shown to generate ionic pores both *in vitro* and *in vivo*, due to its N-terminal domain that shares structural and functional similarity with the pore-forming domain of bacterial colicins. Adjacent to this domain resides a pH-sensitive domain that allows the pore-forming domain to reach the target biological membranes. Based on these results, a model was proposed for trypanolysis (Pays et al., 2006) (Fig. 1). Upon uptake, HDL-bound apoL1 proceeds through the endocytic pathway where progressive acidification will induce conformational changes in the membrane-addressing domain. These changes allow the toxin to dissociate from the HDL carrier and to insert into endosomal membranes, where the pore-forming domain triggers an influx of chloride ions from the cytoplasm. This *primo evens* induces osmotic swelling of the lysosome. Depletion of chloride ions from the cytoplasm would activate compensatory entry of chloride ions from the extracellular medium through plasma membrane channels. Once initiated, the process irreversibly leads to parasite death.

As recombinant apoL1 alone entirely mimicked the phenotype of trypanosome lysis by NHS, the possible role of Hpr in this process was not obvious. Analysis of the lytic potential of human sera naturally lacking either apoL1 or Hpr suggested that Hpr is actually devoid of

trypanolytic activity, but is involved in the binding of TLF to the parasite (Vanhollebeke et al., 2006, 2007b), as previously suggested (Drain et al., 2001). In the absence of Hpr trypanolysis appeared to be slowed down only because of a default in TLF uptake (Vanhollebeke et al., 2007b). The lack of Hpr trypanolytic activity *in vivo* was further confirmed in transgenic murine models (Hatada et al., 2002; Molina-Portela et al., 2008).

#### *TbHpHbR as TLF1 receptor*

The ligand of the TLF1 receptor was identified as being not Hpr alone, but the complex formed by Hb with either Hp or Hpr (Widener et al., 2007; Vanhollebeke et al., 2008). The receptor for this complex, termed TbHpHbR, turned out to be a glycosyl-phosphatidyl-inositol (GPI)-anchored glycoprotein present in ~300 copies in the flagellar pocket of bloodstream forms (Vanhollebeke et al., 2008). TbHpHbR was not essential for the parasite. However, through the supply of heme derived from Hb it appeared to contribute to the growth of the parasite in mice. As TbHpHbR cannot discriminate between Hp-Hb complexes and TLF-Hpr-Hb particles, this receptor was found responsible for the binding and uptake of Hb-charged TLF1 complexes (Fig. 1). The existence of a mammalian receptor analogue to TbHpHbR termed CD163, which is responsible for the rapid clearance of Hp-Hb complexes by macrophages, explained the necessity for *T. brucei* to evolve a specific receptor to access nutritional Hb.

#### **A function for heme in bloodstream forms**

*T. brucei* develops in a heme-poor ecological niche. Indeed diverse host scavenging mechanisms collaborate to ensure low Hb/heme concentrations in the plasma and intercellular spaces where trypanosomes thrive, to avoid the membrane destabilising and oxidative properties of heme. The dominant Hb scavenging mechanism relies on high affinity binding of Hb to Hp and further clearance by the monocyte/macrophage receptor CD163. Like other trypanosomatids, *T. brucei* is a heme auxotroph. Therefore, the fact that this parasite evolved a receptor able to specifically capture the Hp-Hb complex with high affinity means that it must require Hb/heme. This finding was puzzling for two main reasons. Firstly, heme is a potentially toxic compound that was suspected to kill trypanosomes (Meshnick et al., 1978) and secondly, heme was considered absent from bloodstream forms, essentially due to the lack of obvious hemoproteins like catalase and cytochrome c. Regarding the first point, bloodstream form trypanosomes could alleviate heme toxicity through heme oxygenase, as this activity was reported in the closely related *Leishmania* (Srivastava et al., 1997) and *T. cruzi* parasites (Cicarelli et al., 2007). Moreover, heme uptake via TbHpHbR appears to be

highly regulated (BV, unpublished data). Concerning the second point, evidence was obtained for incorporation of heme into bloodstream hemoproteins (Vanhollebeke et al., 2008). Moreover, at least two hemoproteins are suspected to be present in bloodstream forms. An oleate desaturase appears to be involved in fatty acid desaturation, and the electrons required for this activity might originate from a cytochrome b5 (Petrini et al., 2004). In addition, both protein presence and activity were demonstrated for the cytochrome P450 isoform lanosterol demethylase (Lepesheva et al., 2009).

### **ApoL1 is a Bcl2-like protein**

ApoL1 belongs to a family of proteins whose original function is exerted intracellularly (Page et al., 2001; Vanhollebeke and Pays, 2006). These proteins resemble pro- and anti-apoptotic members of the Bcl2 family as they share the same type of multi-helical ionic pore-forming domain with a Bcl2-homology signature (BH3), the presence of a membrane-associating domain and the capacity to trigger cell death *in vitro* (Vanhollebeke and Pays, 2006; Wan et al., 2008). As the expression of several apoLs, including apoL1, is increased with inflammation (Vanhollebeke and Pays, 2006), it would seem as if in humans and some primates, proteins originally designed to organize cell death under inflammatory conditions were also used to kill extracellular pathogens. Secretion of apoL1 appears linked to the presence of an N-terminal signal peptide in this protein only (Page et al., 2001).

### **ApoL1 internalization pathways**

After an initial incompressible lag period required for apoL1 routing towards the acidic endocytic compartments, the speed of the lysis process is positively correlated with the amount of toxin entering the cell (Vanhollebeke et al., 2007b). As such, the rate of apoL1 internalization dictates the rate of cell destruction and hence, the trypanolytic potential of a particular serum or experimental preparation. However, the rate of apoL1 internalization is not simply proportional to its extracellular concentration. Instead, a complex picture integrating two toxic complexes and four distinct pathways emerges (Fig. 2). In this section we provide a detailed discussion on their relative contribution to the actual lysis process, depending on *in vivo* or *in vitro* conditions.

#### *The TbHpHbR pathway*

The physiologically relevant ligand of TbHpHbR was shown to be Hp-Hb in mice and both Hp-Hb and Hpr-Hb in humans. This receptor is involved in TLF1 uptake, since TLF1-associated Hpr-Hb constitutes the dominant ligand of the TLF1 particles under physiological

conditions. Although it is not known if TLF2 can also bind to this receptor, full susceptibility to TLF2 was conserved in TbHpHbR KO trypanosomes, suggesting that the TLF2 dominant uptake is independent from TbHpHbR (Vanhollebeke et al., 2008).

The rate of apoL1 uptake through TbHpHbR is a function of the relative ratio between TLF1-Hpr-Hb and competing Hp-Hb, which are both formed *in vivo* upon intravascular release of Hb from maturing, aged or damaged erythrocytes (Fig. 3). Hp and TLF1-bound Hpr exhibit the same affinity for Hb, but Hp concentrations usually exceed those of Hpr by a factor of 100. So, excess Hb added to NHS *in vitro* will set the Hp-Hb to TLF1-Hpr-Hb ratio close to 100, resulting in reduced engagement of TbHpHbR by TLF1-Hpr-Hb and hence, limited trypanolysis (Fig. 3, case 1). Such conditions are frequently encountered when trypanolysis by NHS is assayed *in vitro*, as foetal bovine serum from the growth medium contains large amounts of Hb but not Hp (Hp is an acute-phase protein whose levels are very low in foetuses). This situation contrasts with that prevailing *in vivo*. The continuous but modest amount of Hb that is released in human circulation through intravascular hemolysis indistinctly binds to both Hpr and Hp in the same 100:1 ratio (Nielsen et al., 2006). However, *in vivo* Hb-bound Hp is maintained extremely low, as it is quickly cleared ( $T_{1/2} \sim 5\text{min}$ ) from the circulation upon endocytosis mediated by the monocyte/macrophage receptor CD163 (Fagoonee et al., 2005). In contrast, TLF1-Hpr-Hb is not recognized by CD163 (Vanhollebeke et al., 2008). Therefore, in the vascular compartment Hp-Hb and TLF1-Hpr-Hb complexes will compete for binding to TbHpHbR, but while Hp-Hb is concurrently depleted through engagement by CD163, TLF1-Hpr-Hb is not. It results that in human blood, the ratio of Hp-Hb over TLF1-Hpr-Hb might be low enough to authorize TLF1 uptake by the parasite (Fig. 3, case 2).

TLF1 delivery to the parasite might be particularly important in extravascular tissues such as the lymphatic system and interstitial fluids, because in those compartments the concentration of blood-derived components drops dramatically (up to 3 logs), so that TbHpHbR is probably not saturated anymore. As a result, TLF1-Hpr-Hb uptake is not influenced by Hp-Hb, with a net increase in specific trypanolytic activity. Support for this idea is provided *in vitro*, when trypanosomes are incubated with diluted concentrations of NHS or when they overexpress TbHpHbR, both conditions where the TbHpHbR pathway is the only one active (Vanhollebeke et al., 2007, 2008).

Usually, the detected trypanolytic activity is only a small fraction of the overall TLF1 trypanolytic potential of NHS. Under some experimental conditions or pathological situations, this full lytic potential can be revealed (Fig. 3, case 3). When TLF1 particles are isolated from



competing Hp through either ultracentrifugation or gel filtration, the activity increases dramatically (Smith and Hajduk, 1995). Similarly, under pathological conditions where Hp levels are strongly reduced, such as drepanocytose that is frequently encountered in tsetse-infested regions, TLF1 access to trypanosomes is largely increased (Raper et al., 1996b).

In the bloodstream the actual fate of TLF1-Hpr is unknown, but its turnover is probably slower than that of Hp-Hb as it is independent from Hb binding. Under pathological situation where excess Hb is released from red blood cells (drepanocytosis), the Hp levels drop dramatically to undetectable levels as a result of their clearance by CD163, but TLF1 levels appear to be unaffected (Raper et al., 1996b). So, *in vivo* TLF1-Hpr-Hb does not seem to be specifically cleared from the circulation. It follows that the steady-state proportion of TLF1-Hpr-Hb, the active TLF1 particles, will be dictated by both the rate of Hb release and the unknown turnover rate of this particular HDL particle.

In naïve serum, the very large majority of TLF1 is free of Hb (Widener et al., 2007; Vanhollebeke et al., 2008). So, to what extent could the TbHpHbR pathway contribute to human immunity? TLF1 relevance to *in vivo* trypanosome killing must cope with two factors. The first one is the extraordinary high trypanolytic capacity of NHS, which contains enough TLF1 to affect trypanosomes after a  $10^5$ -fold dilution (Vanhollebeke et al., 2007b). Thus, provided the proportion of Hb-bound versus Hb-free TLF1 exceeds 0.001%, TLF1 is likely to contribute to *in vivo* trypanolysis. Focused approaches supported this notion: TLF1-Hpr-Hb could be pulled-down with TbHpHbR from unperturbed human plasma (Vanhollebeke et al., 2008), and purified TLF1 was able to affect parasitemia when injected into mice (Barker et al., 2001). However, global TLF1 purification approaches failed to detect this minor population of particles (Widener et al., 2007). The second factor is hemolysis induced by trypanosome infection (Widener et al., 2007). It has been proposed that this infection-induced hemolysis would actually be necessary to arm TLF1 by both providing Hb to the TLF1 particles and decreasing the Hp-Hb levels through their CD163-mediated clearance. However, in experimental models trypanosome-induced hemolysis is only detectable after seven days of infection, and mainly proceeds through extravascular hemolysis, *i.e.* erythrophagocytosis by macrophages, and not by intravascular hemolysis. So, this scenario might be limited to the cure of long-lasting infections that would not have been targeted by the other described pathways. As discussed above, during the initial steps of infection the serum levels of Hb might be very low, but nevertheless still sufficient for TLF1 activity.

*The TLF2 pathway*

Several lines of experimental evidence indicate that apoL1 in the form of TLF2 does not enter trypanosomes just by fluid-phase endocytosis and that efficient TLF2-specific uptake is active during trypanolysis: (i) When TbHpHbR KO cell lines were incubated with equivalent amounts of TLF1 and TLF2, TLF2 activity largely exceeded that of TLF1. (ii) Under conditions where TLF1 binding to its receptor was inhibited by excess Hp, TLF2 still efficiently bound to the trypanosome surface. (iii) When other uptake pathways were competed out, TLF2-mediated lysis occurred significantly faster than what could be accounted by fluid-phase uptake.

So far the TLF2 pathway is not characterized and no direct evidence for its activity *in vivo* has been provided. In particular, it is so far unknown whether purified TLF2 particles are able to alleviate parasitemia when injected in mice. However, some information has been gained from *in vitro* studies. At high, and thus physiologically relevant NHS concentrations (over 10%), the TLF2 pathway largely dominates the others. Upon dilution of NHS all trypanolytic activity was found to switch to the TbHpHbR-dependent, Hp-Hb completable pathway (Vanhollebeke et al., 2007b). Even sub-physiological concentrations of Hp were sufficient to fully inhibit TLF1 *in vitro*, suggesting that TLF2 could be the main lytic factor in normal blood (Raper et al., 1996b). Some authors observed a negative correlation between Hp levels and total lytic activity, discrediting the TLF2 dominant role and rather favouring TLF1 (Smith and Hajduk, 1995), but this study involved a limited sample size and was contradicted by others (Raper et al., 1996a). Strain variability and Hb influence might explain part of those discrepancies.

Studies involving anhaptoalbuminemic serum revealed that Hpr is required for the binding of TLF2 to the trypanosome surface (Vanhollebeke et al., 2007b). However, the fact that Hp(r)-Hb does not compete for TLF2 activity suggests that the role of Hpr in TLF2 is distinct from involvement as a ligand for the trypanosome surface. An alternative function could be direct or indirect involvement in physical linkage between the apoL1 toxin and the unknown trypanosome ligand of this complex. Regarding this ligand, our data suggest that TLF2 binding sites on the trypanosome surface are so numerous or so rapidly recycled that saturation is never achieved at 37°C within reasonable ligand concentration ranges (BV, unpublished data). We propose that TLF2 uptake relies on low affinity/high capacity interaction with the VSG coat. Support to this idea comes from the observation that while the level of TLF1-mediated lysis is similar between different antigenic variants, lysis by TLF2 is highly variable as could be expected from differences between VSGs. The rapid turnover of the VSG would explain both TLF2 efficiency and absence of competition. The nature of the

TLF2 ligand is unknown, but polyclonal IgM, the dominant component of TLF2, represents an interesting candidate. Firstly, it is the only known component differentiating TLF2 from TLF1. Secondly, IgMs appear to bind non-specifically and weakly to VSG. Finally, uptake of fluorescently labelled IgMs, although much less efficient than transferrin or Hp-Hb, exceeds that of fluid-phase markers like bovine serum albumin, and is interestingly not competed by an excess of unlabelled IgM. Possibly the high-mannose residues of IgMs (Arnold et al., 2005), similar to those of VSG, could be involved in the binding process.

#### *The lipoprotein receptor pathway*

A so far uncharacterized lipoprotein receptor appears to be involved in sterol supply to the parasite (Green et al., 2003). Being an HDL particle, TLF1 could target the cell via this pathway. Accordingly, some TLF1 binding to the trypanosome surface could be inhibited by an excess of competing lipoprotein particles (Green et al., 2003). However, TLF1 constitutes only a minor subset of the total lipoprotein pool in NHS (most conservative estimation range around 1 percent), so that the contribution of the lipoprotein receptor pathway to the lysis process is probably low, both in vascular and other compartments. As TLF2 contains some lipids and apoA1, it is possible that those particles also bind to the lipoprotein receptor. However, as in the TLF1 case this uptake pathway is probably largely masked by a more efficient one.

#### *Fluid-phase uptake*

Although initiated from a minor fraction of the cellular surface, the endocytic rate of African trypanosomes is high, with an average of  $0.2 \mu\text{m}^3$  entering each cell per minute and a net uptake value 10 times lower (Engstler et al., 2004). Given the mean concentration of apoL1 in human plasma ( $10 \mu\text{g/ml}$ ), 3 to 30 molecules should accumulate per minute in each cell, as either TLF1 or TLF2. Experiments with physiological levels of recombinant protein free in solution have shown that apoL1 uptake through this pathway is sufficient for complete parasite killing, while a ten-fold lower concentration was only trypanostatic, i.e. the population remained stable over time (Vanhollebeke et al., 2007b). These results illustrate the effectiveness of fluid-phase alone in NHS.

### **What is the physiologically relevant apoL1 delivery pathway?**

The respective contribution of each TLF uptake pathway to trypanosome lysis can only be hypothesized, because the reconstitution of murine models recapitulating the subtleties of the different complexes seems to be out of reach. Conditions prevailing in the bloodstream should

clearly be distinguished from those prevailing in the other extravascular tissues where trypanosomes develop (lymphatic system, cerebrospinal fluids, interstitial fluid).

In the bloodstream, this debate is probably irrelevant regarding the outcome of infection. Indeed, fluid-phase endocytosis and lipoprotein receptor-mediated uptake are efficient enough to condemn a trypanosome population within a few hours. In 100% anhaploglobinemic serum, where TLF1 and TLF2-specific pathways are missing, a population of trypanosomes is destroyed in as little as 3 h, thus just 1 h longer than in 100% NHS (Vanhollebeke et al., 2007b). However, as far as can be extrapolated from *in vitro* studies, if the TLF2 pathway was operational *in vivo* it could dominate the process. In this context the lipoprotein receptor pathway is most probably outcompeted and the contribution of TLF1 should be minor, but significant. It is possible that this last contribution depends on the relative affinity of the VSG type for IgMs. Moreover, as human populations facing trypanosome infection appear to have increased occurrence of Hp and TLF2 deficiencies (Raper et al., 1996b; Widener et al., 2007), TLF1-TbHpHbR could represent a potent physiological uptake pathway in the field.

In conditions where trypanosomes face reduced concentration of lytic particles, both the low affinity TLF2 pathway and the fluid-phase delivery might lack potency. Instead, Hb-charged TLF1 particles should still be able to deliver sufficient apoL1 molecules to stop infection. It is also possible that the lipoprotein uptake pathway might participate. Finally, as African trypanosomes are injected intradermally by the tsetse fly and thrive for some days in a superficial inflamed tissue patch called “chancre” before gaining the systemic circulation, one could speculate that extravasated TLF1 particles might offer the first efficient line of immune defence.

### **Control of apoL1 toxicity**

The factor neutralizing apoL1 in *T. b. rhodesiense*, the VSG-like protein SRA, was initially proposed to interact with the C-terminal helix of apoL1 in the lysosome of the parasite (Vanhamme et al., 2003). Functional data presented in two recent papers have fully confirmed this view: due to sequence differences with human apoL1 in the C-terminal helix, baboon apoL1 no longer interacts with SRA, and accordingly the baboon-specific apoL1 efficiently kills *T. b. rhodesiense* (Thomson et al., 2009; Lecordier et al., 2009). When assayed in *E. coli*, the intrinsic pore-forming activity of apoL1 did not require the presence of the C-terminal helix (Pérez-Morga et al., 2005), but this activity was completely inhibited following interaction of SRA with this helix (Lecordier et al., 2009). Thus, it is conceivable that in the trypanosome, inactivation of apoL1 would result from direct interaction with SRA and not

from eventual SRA-mediated alteration of the intracellular trafficking of apoL1 (Oli et al., 2007). Experimental apoL1 mutants where the C-terminal helix was deleted or disrupted were found to lose their trypanolytic potential (Molina-Portela et al., 2008; Lecordier et al., 2009). Therefore, the C-terminal helix of apoL1 is probably involved in the control of the pore-forming activity of this protein, and either interaction with parasite antidotes or disruption of the helix would equally inactivate the toxin.

### **Out of Africa**

Trypanolytic activity is only found in African primates. The common ancestor that gave rise to humans, gorillas and baboons (~35 Mo years) appeared long after African trypanosomes (~100 Mo years) in tsetse infested regions (Stevens et al., 1999). Is it conceivable that trypanosome-elicited Darwinian selective pressures would have favoured the spread of immune organisms? This type of pathogen-driven selection is not infrequent: *Plasmodium falciparum*, for instance, has increased the frequency of sickle Hb coding sequences in some human populations because this  $\beta$ -globin variant provides heterozygotes with dominant resistance to the parasite, as infected sickle erythrocytes are removed by the spleen. TLF presence in human blood would be a vestige attesting of its recent African origin. In agreement with this idea, some humans living in tsetse-free areas have lost the key components of the lytic complex without seemingly overt negative consequences (Koda et al., 1998; Vanhollebeke et al., 2007b). Moreover, the acquisition of trypanolytic capacity in old world monkeys strikingly parallels exposure to the parasite. All positive animals are diurnal non arboreal central African primates. This predisposes them preferentially to the bite of tsetse flies. Chimpanzees lack trypanocidal activity, but their dominantly arboreal way of life might reduce their exposure to the tsetse fly.

The phylogenetic distribution of Hpr was proposed to match trypanolytic capacity (Lugli et al., 2004). However, scrutinizing the genome drafts of various primates including *Macaca mulata* and *Pongo pygmaeus* (BV, unpublished data) revealed that Hpr coding sequences are present in all old world monkeys, trypanolytic or not, as initially suggested (McEvoy and Maeda, 1988). So it is probable that the appearance of Hpr-bearing HDL preceded the secretion of apoL1 in the serum. In turn, apoL1 clearly defines primate trypanolytic activity in general, since it was found to be responsible for this activity not only in humans, but also in baboons (Thomson et al., 2009).

### **A novel innate immunity system**

Although TLF could have evolved in Africa to fight trypanosomes, it might play additional functions related to innate immunity, as the building blocks of TLF all appear to be derived from or linked to immunity proteins. Hp is indeed an acute-phase protein, i.e. a plasma protein whose concentration is modulated by inflammation. Moreover, Hp is derived from a complement-associated protein, the mannose-binding lectin-associated serine proteinase (Wicher and Fries, 2006). In TLF2 the Hp-derived Hpr is associated with another immunity component, IgM. Finally, apoL1 itself is strongly induced by inflammation (Vanhollebeke and Pays, 2006). So, recycling and assembly of non-specific innate immune components led to the development of a highly toxic immune complex. Given the pH dependency of apoL1 toxicity (Lecordier et al., 2009) it can be anticipated that potentially affected pathogens must either, like trypanosomes, be affected after endocytosis of TLF in their lysosomal compartment, or reside inside acidic compartments of host cells such as microbes that thrive in the phagolysosome of macrophages. Accordingly, TLF was found to attenuate parasitaemia by the intracellular pathogen *Leishmania* (Samanovic et al., 2009).

## Conclusions

Humans and related primates have assembled different innate immunity components into efficient anti-trypanosome toxic complexes. That these complexes might have arisen under trypanosome-elicited selection prevailing in the African belt during primate speciation represents a seducing hypothesis.

Various pathways converge to deliver these complexes to the invading parasites. Numerous parameters are known to influence the relative contribution of each of these pathways and one can expect others to be disclosed when all pathways will be defined in molecular terms. It is anticipated that depending on infection conditions (infection site, additional affection, trypanosome strain), different uptake pathways could dominate the immune response and offer the first line of protection.

In recent years, a combination of gain-of-function experiments in mice and loss-of-function observation in humans has indicated that primate immunity to trypanosome infection strictly relies on the presence in plasma of the Bcl-2 like protein apoL1. Even in the absence of specific uptake mechanisms apoL1 was found to be necessary and sufficient to provide immunity against the parasite. The observation that a mammalian Bcl2-like protein can trigger cell death in lower eukaryotes that lack downstream cell death executioners like caspases, is particularly remarkable and reveals a so far unknown aspect of Bcl-2 physiology.

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### Figure legends

Fig. 1. Model of trypanolysis by TLF1. (1) ApoL1 is bound to HDL<sub>3</sub> particles (dotted circle) via its membrane-addressing domain (black) located close to the N-terminal pore-forming domain (white). The HDL<sub>3</sub> particles bind to the *T. brucei* receptor TbHpHbR through their Hpr-Hb complex. This triggers HDL<sub>3</sub> uptake and intracellular traffic to the lysosome. (2) Upon acidification the membrane-addressing domain of apoL1 undergoes conformational change that allows apoL1 dissociation from HDL and insertion in the lysosomal membrane, whereas Hpr and Hb are degraded to release heme for incorporation in bloodstream form hemoproteins. (3) The pore-forming domain of apoL1 triggers a flux of chloride ions from the cytoplasm to the lysosome lumen, due to the high concentration of chloride in the cytoplasm (0.1 M). This flux is accompanied by water, leading to uncontrolled osmotic swelling of the lysosome. (4) The reduction of cytoplasmic chloride concentration triggers the entry of chloride ions from the extracellular medium through plasma membrane channels (hatched oval). This process can be inhibited by the anion channel blocker DIDS.

Fig. 2. ApoL1 uptake pathways. ApoL1 circulates in the form of two high molecular weight (lipo)proteic complexes termed TLF1 and TLF2. These complexes engage in various cellular uptake pathways. TLF1 is mainly taken up through the Hp-Hb surface receptor TbHpHbR and relies on Hb priming. TLF2 is mainly taken up *via* another potent but uncharacterised mechanism. Other uptake routes, i.e. lipoprotein receptor pathway and fluid-phase uptake, may contribute under particular situations (see text).

Fig. 3. Involvement of TLF1 in different trypanolysis conditions. While the Hp-Hb complex exhibits the same affinity as TLF1-Hp-Hb for TbHpHbR, Hp-Hb also efficiently binds to the monocyte-macrophage receptor CD163, but the TLF1-Hp-Hb complex does not. Therefore, the trypanolytic potential of TLF1 strongly depends on the relative contribution of these different factors.



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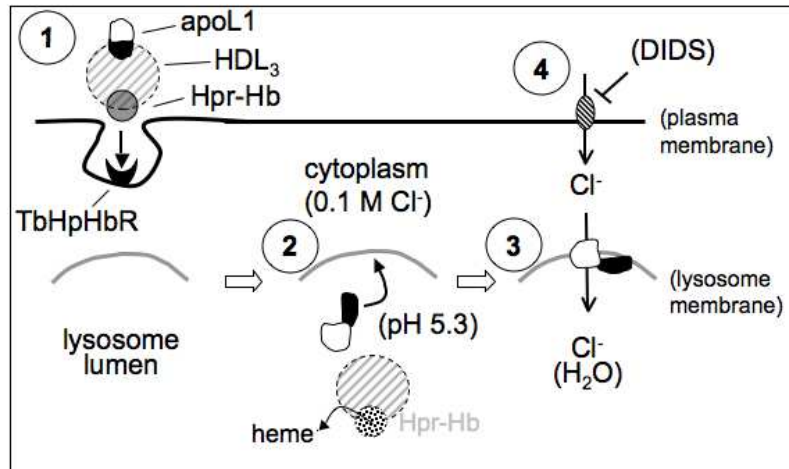
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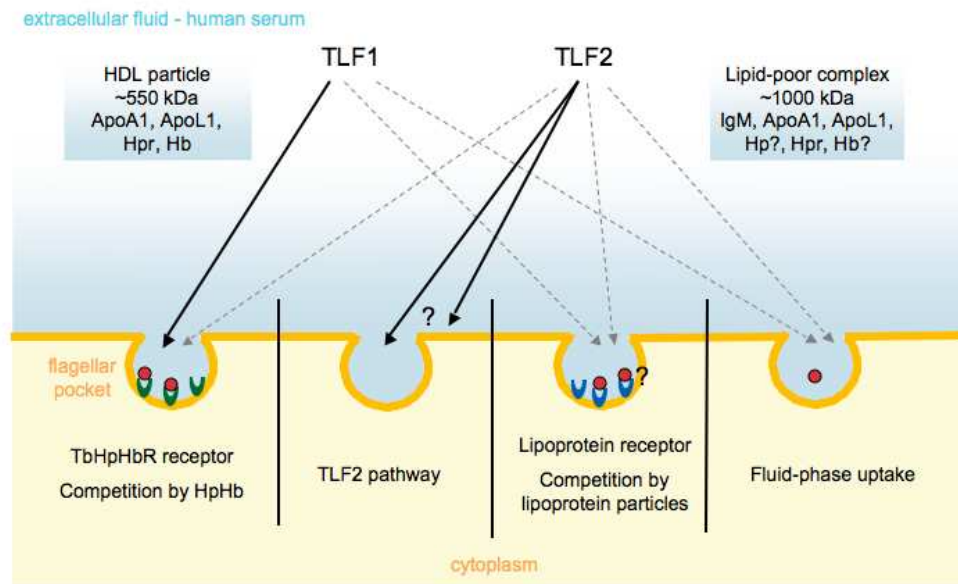


Vanhollebeke, Pays. Fig. 1

Fig. 1. Model of trypanolysis by TLF1. (1) ApoL1 is bound to HDL3 particles (dotted circle) via its membrane-addressing domain (black) located close to the N-terminal pore-forming domain (white).

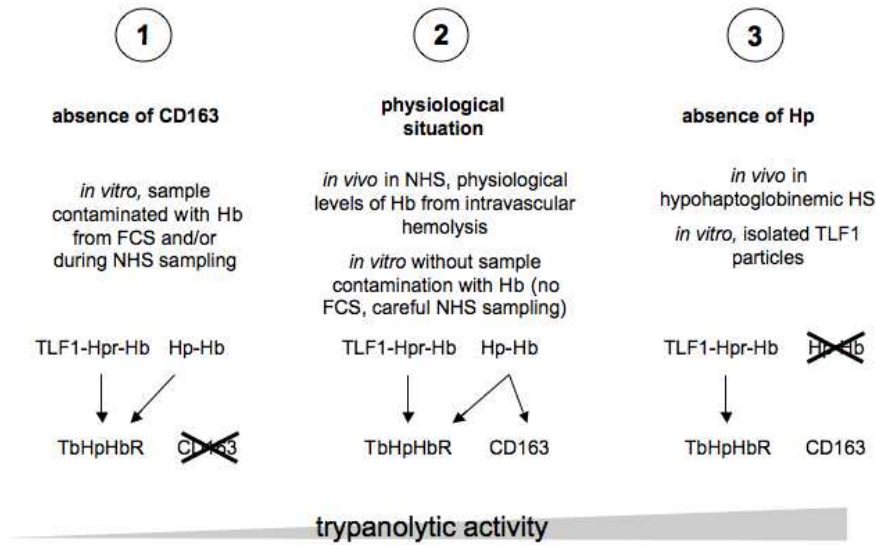
The HDL3 particles bind to the *T. brucei* receptor TbHpHbR through their Hpr-Hb complex. This triggers HDL3 uptake and intracellular traffic to the lysosome. (2) Upon acidification the membrane-addressing domain of apoL1 undergoes a conformational change that allows apoL1 dissociation from HDL and insertion in the lysosomal membrane, whereas Hpr and Hb are degraded to release heme for incorporation in bloodstream form hemoproteins. (3) The pore-forming domain of apoL1 triggers a flux of chloride ions from the cytoplasm to the lysosome lumen, due to the high concentration of chloride in the cytoplasm (0.1 M). This flux is accompanied by water, leading to uncontrolled osmotic swelling of the lysosome. (4) The reduction of cytoplasmic chloride concentration triggers the entry of chloride ions from the extracellular medium through plasma membrane channels (hatched oval). This process can be inhibited by the anion channel blocker DIDS.





Vanhollebeke, Pays. Fig. 2

Fig. 2. ApoL1 uptake pathways. ApoL1 circulates in the form of two high molecular weight (lipo)proteic complexes termed TLF1 and TLF2. These complexes engage in various cellular uptake pathways. TLF1 is mainly taken up through the Hp-Hb surface receptor TbHpHbR and relies on Hb priming. TLF2 is mainly taken up via another potent but uncharacterised mechanism. Other uptake routes, i.e. lipoprotein receptor pathway and fluid-phase uptake, may contribute under particular situations (see text).



Vanhollebeke, Pays. Fig. 3

Fig. 3. Involvement of TLF1 in different trypanolysis conditions. While the Hp-Hb complex exhibits the same affinity as TLF1-Hpr-Hb for TbHpHbR, Hp-Hb also efficiently binds to the monocyte-macrophage receptor CD163, but the TLF1-Hpr-Hb complex does not. Therefore, the trypanolytic potential of TLF1 strongly depends on the relative contribution of these different factors.

## **The trypanolytic factor of human serum: many ways to enter the parasite, a single way to kill**

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### **Summary**

Humans have developed a particular innate immunity system against African trypanosomes, and only two *Trypanosoma brucei* clones (*T. b. gambiense*, *T. b. rhodesiense*) can resist this defence and cause sleeping sickness. The main players of this immunity are the primate-specific apolipoprotein L-I (apoL1) and haptoglobin-related protein (Hpr). These proteins are both associated with two serum complexes, a minor subfraction of HDLs and an IgM/apolipoprotein A-I (apoA1) complex respectively termed Trypanosome Lytic Factor (TLF) 1 and TLF2. Although the two complexes appear to lyse trypanosomes by the same mechanism, they enter the parasite through various modes of uptake. In case of TLF1 one uptake process was characterized. When released in the circulation, hemoglobin (Hb) binds to Hpr, hence to TLF1. In turn the TLF1-Hpr-Hb complex binds to the trypanosome haptoglobin (Hp)-Hb receptor, whose original function is to ensure heme uptake for optimal growth of the parasite. This binding triggers efficient uptake of TLF1 and subsequent trypanosome lysis. While Hpr is involved as TLF ligand, the lytic activity is due to apoL1, a Bcl-2-like pore-forming protein. We discuss the *in vivo* relevance of this uptake pathway in the context of other potentially redundant delivery routes.

Running title: The trypanosome lytic factor

Key words: apolipoprotein L-I, haptoglobin-related protein, human innate immunity, sleeping sickness, pore-forming proteins, BH3-only proteins, haptoglobin-hemoglobin receptor.

## A brief history of the Trypanosome Lytic Factor

### *HDLs as TLF carriers*

In the early 20<sup>th</sup> century, Laveran reported on the natural immunity of humans against *T. brucei* infection (Laveran, 1902). This immunity was found to depend on a trypanocidal factor in normal human serum (NHS), termed trypanosome lytic factor (TLF) (Hajduk et al., 1989). Early reports disclosed the association of TLF with HDL particles (Rifkin, 1978), and subsequent work concluded that TLF is restricted to the densest fraction called HDL<sub>3</sub> (Hajduk et al., 1989). In addition to the main apolipoprotein apoA1, this fraction was shown to contain haptoglobin-related protein (Hpr), a primate-specific protein derived from haptoglobin through tandem gene duplication (Maeda, 1985). Eventually the existence of two independent trypanolytic particles was established: besides HDL-bound TLF redefined as TLF1, a large lipid-poor apoA1/IgM/Hpr-containing complex called TLF2 was also found to kill trypanosomes (Tomlinson et al., 1995; Raper et al., 1999).

### *Haptoglobin-related protein as possible toxin*

Hpr was presented as the trypanosome toxin, notably because anti-Hpr antibodies and competing amounts of Hp both inhibited TLF-mediated trypanosome lysis (Smith et al., 1995; Smith and Hajduk, 1995). This hypothesis was also supported by the phylogenetic match between presence of Hpr and trypanolytic potential in different old world monkeys such as gorillas (*Gorilla*) or baboons (*Papio*), and the absence of both from chimpanzee serum (Lugli et al., 2004; Poelvoorde et al., 2004). Trypanolysis was shown to depend on Hpr-mediated binding of TLF1 to ~350 high affinity sites of the parasite surface (Drain et al., 2001), followed by intracellular trafficking of TLF1 to the lysosome (Hager et al., 1994; Shimamura et al., 2001). However, the mechanism of lysis by Hpr was debated (Molina Portela et al., 2000; Bishop et al., 2001).

### *Apolipoprotein L-I as alternative toxin*

Two *T. brucei* subspecies (*T. b. gambiense* and *T. b. rhodesiense*) resist TLF and cause sleeping sickness. While resistance of *T. b. gambiense* is constitutive, *T. b. rhodesiense* resistance appeared to be inducible under selective conditions. This character was found to result from the particular mode of expression of a gene encoding resistance to TLF, termed SRA for Serum Resistance Associated (Xong et al., 1998). The SRA gene encodes a truncated version of the major surface antigen of the parasite, the Variant Surface Glycoprotein or VSG, and is contained in one of the multiple genomic sites allowing expression of the VSG, which exhibit strict mono-allelic activity. The presence of NHS was found necessary to trigger the selection of the unique SRA-containing expression site, leading to transcription of this gene

under these conditions only. Expression of SRA was found sufficient to confer the resistance phenotype (Xong et al., 1998). In contrast with the VSG, SRA is located in the endocytic pathway (Vanhamme et al., 2003; Oli et al., 2006). This differential sorting might result from the internal sequence truncation differentiating SRA from regular VSGs. The key determinant for resistance to TLF was found to be the N-terminal  $\alpha$ -helix of SRA (Vanhamme et al., 2003). As in VSGs this helix is interactive, it was postulated that SRA might physically neutralise TLF through coil-coiling interaction. Affinity chromatography of detergent-solubilised NHS on immobilised SRA revealed the specific and strong binding of apolipoprotein L-I (apoL1). Recombinant versions of apoL1 exhibited a trypanolytic potential closely mimicking that of NHS, including neutralisation by SRA (Vanhamme et al., 2003). Therefore, apoL1 was presented as the trypanosome toxin of NHS. Accordingly, apoL1 was found to be present in both TLF1 and TLF2 (Molina-Portela et al., 2005). Moreover, in two distinct cases of apoL1 absence from serum (chimpanzee and a mutant human individual), both TLF1 and TLF2 were absent, implying the role of apoL1 in both cases (Poelvoorde et al., 2004; Lugli et al., 2004; Vanhollebeke et al., 2006).

ApoL1 is expressed from a recent gene copy of a multigene family of unknown function (Vanhollebeke and Pays, 2006). It is the only member to be secreted in the serum, where it associates with HDL<sub>3</sub> (Duchateau et al., 1997). This association suggested a role in lipid transport and metabolism, but a distinct picture emerged from studies on the trypanolytic activity of this protein (Pérez-Morga et al., 2005). ApoL1 was shown to generate ionic pores both *in vitro* and *in vivo*, due to its N-terminal domain that shares structural and functional similarity with the pore-forming domain of bacterial colicins. Adjacent to this domain resides a pH-sensitive domain that allows the pore-forming domain to reach the target biological membranes. Based on these results, a model was proposed for trypanolysis (Pays et al., 2006) (Fig. 1). Upon uptake, HDL-bound apoL1 proceeds through the endocytic pathway where progressive acidification will induce conformational changes in the membrane-addressing domain. These changes allow the toxin to dissociate from the HDL carrier and to insert into endosomal membranes, where the pore-forming domain triggers an influx of chloride ions from the cytoplasm. This *primo evens* induces osmotic swelling of the lysosome. Depletion of chloride ions from the cytoplasm would activate compensatory entry of chloride ions from the extracellular medium through plasma membrane channels. Once initiated, the process irreversibly leads to parasite death.

Recombinant apoL1 alone entirely mimicked the phenotype of trypanosome lysis by NHS, in particular the typical swelling of the lysosome in NHS-sensitive trypanosomes only.

Lysosome swelling was not documented in the early publications of Rifkin (1984), but this could result from the extreme fragility of swollen cells, which after fixation for microscopy would only allow the presence of cells with a transitory multivesicular phenotype (Vanhollebeke et al., 2007a). Given the phenotype induced by apoL1 the possible role of Hpr was not obvious. Analysis of the lytic potential of human sera naturally lacking either apoL1 or Hpr suggested that Hpr is actually devoid of trypanolytic activity, but is involved in the binding of TLF to the parasite (Vanhollebeke et al., 2006, 2007b), as previously suggested (Drain et al., 2001). In the absence of Hpr trypanolysis appeared to be slowed down only because of a defect in TLF uptake (Vanhollebeke et al., 2007b). The lack of Hpr trypanolytic activity *in vivo* was not only observed in human serum devoid of apoL1 (Vanhollebeke et al., 2006), but also in two independent transgenic murine models (Hatada et al., 2002; Molina-Portela et al., 2008). Nevertheless, it has been argued that native Hpr is trypanolytic *in vitro* (Shiflett et al. 2005; Harrington et al., 2009). As such *in vitro* effect was not observed with NHS that was experimentally or naturally deprived of apoL1 only (Vanhamme et al., 2003; Vanhollebeke et al., 2006), it might result from artefactual toxicity of purified Hpr under *in vitro* conditions. The relevance of these observations regarding the natural process of trypanolysis remains to be determined.

#### *TbHpHbR as TLF1 receptor*

The ligand of the TLF1 receptor was identified as being not Hpr alone, but the complex formed by Hb with either Hp or Hpr (Widener et al., 2007; Vanhollebeke et al., 2008). The receptor for this complex, termed TbHpHbR, turned out to be a glycosyl-phosphatidyl-inositol (GPI)-anchored glycoprotein present in ~300 copies in the flagellar pocket of bloodstream forms (Vanhollebeke et al., 2008). TbHpHbR was not essential for the parasite. However, through the supply of heme derived from Hb it appeared to contribute to the growth of the parasite in mice. As TbHpHbR cannot discriminate between Hp-Hb complexes and TLF-Hpr-Hb particles, this receptor was found responsible for the binding and uptake of Hb-charged TLF1 complexes (Fig. 1). The existence of a mammalian receptor analogue to TbHpHbR termed CD163, which is responsible for the rapid clearance of Hp-Hb complexes by macrophages, explained the necessity for *T. brucei* to evolve a specific receptor to access nutritional Hb.

#### **A function for heme in bloodstream forms**

*T. brucei* develops in a heme-poor ecological niche. Indeed diverse host scavenging mechanisms collaborate to ensure low Hb/heme concentrations in the plasma and intercellular

spaces where trypanosomes thrive, to avoid the membrane destabilising and oxidative properties of heme. The dominant Hb scavenging mechanism relies on high affinity binding of Hb to Hp and further clearance by the monocyte/macrophage receptor CD163. Like other trypanosomatids, *T. brucei* is a heme auxotroph. Therefore, the fact that this parasite evolved a receptor able to specifically capture the Hp-Hb complex with high affinity, together with the facts that Hp-Hb appeared to be the only ligand of this receptor and that the absence of either ligand or receptor triggered growth reduction (Vanhollebeke et al., 2008), mean that *T. brucei* requires Hb/heme. This finding was puzzling for two main reasons. Firstly, heme is a potentially toxic compound that was suspected to kill trypanosomes (Meshnick et al., 1978) and secondly, heme was considered absent from bloodstream forms, essentially due to the lack of obvious hemoproteins like catalase and cytochrome c. Regarding the first point, bloodstream form trypanosomes could alleviate heme toxicity through heme oxygenase, as this activity was reported in the closely related *Leishmania* (Srivastava et al., 1997) and *T. cruzi* parasites (Cicarelli et al., 2007). Moreover, heme uptake via TbHpHbR appears to be highly regulated (BV, unpublished data). Concerning the second point, evidence was obtained for incorporation of heme into bloodstream hemoproteins (Vanhollebeke et al., 2008). Moreover, at least two hemoproteins are suspected to be present in bloodstream forms. An oleate desaturase appears to be involved in fatty acid desaturation, and the electrons required for this activity might originate from a cytochrome b5 (Petrini et al., 2004). In addition, both protein presence and activity were demonstrated for the cytochrome P450 isoform lanosterol demethylase (Lepesheva et al., 2009).

### **ApoL1 is a Bcl2-like protein**

ApoL1 belongs to a family of proteins whose original function is exerted intracellularly (Page et al., 2001; Vanhollebeke and Pays, 2006). These proteins resemble pro- and anti-apoptotic members of the Bcl2 family as they share the same type of multi-helical ionic pore-forming domain with a Bcl2-homology signature (BH3), the presence of a membrane-associating domain and the capacity to trigger cell death *in vitro* (Vanhollebeke and Pays, 2006; Wan et al., 2008). As the expression of several apoLs, including apoL1, is increased with inflammation (Vanhollebeke and Pays, 2006), it would seem as if in humans and some primates, proteins originally designed to organize cell death under inflammatory conditions were also used to kill extracellular pathogens. Secretion of apoL1 appears linked to the presence of an N-terminal signal peptide in this protein only (Page et al., 2001).



### ApoL1 internalization pathways

After an initial incompressible lag period required for apoL1 routing towards the acidic endocytic compartments, the speed of the lysis process is positively correlated with the amount of toxin entering the cell (Vanhollebeke et al., 2007b). As such, the rate of apoL1 internalization dictates the rate of cell destruction and hence, the trypanolytic potential of a particular serum or experimental preparation. However, the rate of apoL1 internalization is not simply proportional to its extracellular concentration. Instead, a complex picture integrating two toxic complexes and four distinct pathways emerges (Fig. 2). In this section we provide a detailed discussion on their relative contribution to the actual lysis process, depending on *in vivo* or *in vitro* conditions.

#### *The TbHpHbR pathway*

The physiologically relevant ligand of TbHpHbR was shown to be Hp-Hb in mice and both Hp-Hb and Hpr-Hb in humans. This receptor is involved in TLF1 uptake, since TLF1-associated Hpr-Hb constitutes the dominant ligand of the TLF1 particles under physiological conditions. Although it is not known if TLF2 can also bind to this receptor, full susceptibility to TLF2 was conserved in TbHpHbR KO trypanosomes, suggesting that the TLF2 dominant uptake is independent from TbHpHbR (Vanhollebeke et al., 2008).

The rate of apoL1 uptake through TbHpHbR is a function of the relative ratio between TLF1-Hpr-Hb and competing Hp-Hb, which are both formed *in vivo* upon intravascular release of Hb from maturing, aged or damaged erythrocytes (Fig. 3). Hp and TLF1-bound Hpr exhibit the same affinity for Hb, but Hp concentrations usually exceed those of Hpr by a factor of 100. So, excess Hb added to NHS *in vitro* will set the Hp-Hb to TLF1-Hpr-Hb ratio close to 100, resulting in reduced engagement of TbHpHbR by TLF1-Hpr-Hb and hence, limited trypanolysis (Fig. 3, case 1). Such conditions are frequently encountered when trypanolysis by NHS is assayed *in vitro*, as foetal bovine serum from the growth medium contains large amounts of Hb but not Hp (Hp is an acute-phase protein whose levels are very low in foetuses). This situation contrasts with that prevailing *in vivo*. The continuous but modest amount of Hb that is released in human circulation through intravascular hemolysis indistinctly binds to both Hpr and Hp in the same 100:1 ratio (Nielsen et al., 2006). However, *in vivo* Hb-bound Hp is maintained extremely low, as it is quickly cleared ( $T_{1/2} \sim 5\text{min}$ ) from the circulation upon endocytosis mediated by the monocyte/macrophage receptor CD163 (Fagoonee et al., 2005). In contrast, TLF1-Hpr-Hb is not recognized by CD163 (Vanhollebeke et al., 2008). Therefore, in the vascular compartment Hp-Hb and TLF1-Hpr-Hb complexes will compete for binding to TbHpHbR, but while Hp-Hb is concurrently



depleted through engagement by CD163, TLF1-Hpr-Hb is not. It results that in human blood, the ratio of Hp-Hb over TLF1-Hpr-Hb might be low enough to authorize TLF1 uptake by the parasite (Fig. 3, case 2).

TLF1 delivery to the parasite might be particularly important in extravascular tissues such as the lymphatic system and interstitial fluids, because in those compartments the concentration of blood-derived components drops dramatically (up to 3 logs), so that TbHpHbR is probably not saturated anymore. As a result, TLF1-Hpr-Hb uptake is not influenced by Hp-Hb, with a net increase in specific trypanolytic activity. Support for this idea is provided *in vitro*, when trypanosomes are incubated with diluted concentrations of NHS or when they overexpress TbHpHbR, both conditions where the TbHpHbR pathway is the only one active (Vanhollebeke et al., 2007, 2008).

Usually, the measured trypanolytic activity is only a small fraction of the total TLF1 trypanolytic potential of NHS. Under some experimental conditions or in pathological situations, this full lytic potential can be revealed (Fig. 3, case 3). When TLF1 particles are isolated from competing Hp through either ultracentrifugation or gel filtration, the activity increases dramatically (Smith and Hajduk, 1995). Similarly, under pathological conditions where Hp levels are strongly reduced, such as sickle cell anemia that is frequently encountered in tsetse-infested regions, the potential of TLF1 to kill trypanosomes is largely increased (Raper et al., 1996b).

In the bloodstream the actual fate of TLF1-Hpr is unknown, but its turnover is probably slower than that of Hp-Hb as it is independent from Hb binding. Under pathological situation where excess Hb is released from red blood cells (sickle cell anemia), the Hp levels drop dramatically to undetectable levels as a result of their clearance by CD163, but TLF1 levels appear to be unaffected (Raper et al, 1996b). So, *in vivo* TLF1-Hpr-Hb does not seem to be specifically cleared from the circulation. It follows that the steady-state proportion of TLF1-Hpr-Hb, the active TLF1 particles, will be dictated by both the rate of Hb release and the unknown turnover rate of this particular HDL particle.

In naïve serum, the very large majority of TLF1 is free of Hb (Widener et al., 2007; Vanhollebeke et al., 2008). So, to what extent could the TbHpHbR pathway contribute to human immunity? TLF1 relevance to *in vivo* trypanosome killing must cope with two factors. The first one is the extraordinary high trypanolytic capacity of NHS, which contains enough TLF1 to affect trypanosomes after a  $10^5$ -fold dilution (Vanhollebeke et al., 2007b). Thus, provided the proportion of Hb-bound versus Hb-free TLF1 exceeds 0.001%, TLF1 is likely to contribute to *in vivo* trypanolysis. Focused approaches supported this notion: TLF1-Hpr-Hb

could be pulled-down with TbHpHbR from unperturbed human plasma (Vanhollebeke et al., 2008), and purified TLF1 was able to affect parasitemia when injected into mice (Barker et al., 2001). However, global TLF1 purification approaches failed to detect this minor population of particles (Widener et al., 2007). The second factor is hemolysis induced by trypanosome infection (Widener et al., 2007). It has been proposed that this infection-induced hemolysis would actually be necessary to arm TLF1 by both providing Hb to the TLF1 particles and decreasing the Hp-Hb levels through their CD163-mediated clearance. However, in experimental models trypanosome-induced hemolysis is only detectable after seven days of infection, and mainly proceeds through extravascular hemolysis, *i.e.* erythrophagocytosis by macrophages, and not by intravascular hemolysis. So, this scenario might be limited to the cure of long-lasting infections that would not have been targeted by the other described pathways. As discussed above, during the initial steps of infection the serum levels of Hb might be very low, but nevertheless still sufficient for TLF1 activity.

#### *The TLF2 pathway*

So far the TLF2 pathway is not characterized and no direct evidence for its activity *in vivo* has been provided. In particular, it is so far unknown whether purified TLF2 particles are able to alleviate parasitemia when injected in mice. However, some information has been gained from *in vitro* studies. At high, and thus physiologically relevant NHS concentrations (over 10%), the TLF2 pathway largely dominates the others. Upon dilution of NHS all trypanolytic activity was found to switch to the TbHpHbR-dependent, Hp-Hb competable pathway (Vanhollebeke et al., 2007b). Even sub-physiological concentrations of Hp were sufficient to fully inhibit TLF1 *in vitro*, suggesting that TLF2 could be the main lytic factor in normal blood (Raper et al., 1996b). Some authors observed a negative correlation between Hp levels and total lytic activity, discrediting the TLF2 dominant role and rather favouring TLF1 (Smith and Hajduk, 1995), but this study involved a limited sample size and was contradicted by others (Raper et al., 1996a). Strain variability and Hb influence might explain part of those discrepancies.

Studies involving anhaptoalbuminemic serum revealed that Hpr is required for the binding of TLF2 to the trypanosome surface (Vanhollebeke et al., 2007b). However, the fact that Hp(r)-Hb does not compete for TLF2 activity suggests that the role of Hpr in TLF2 is distinct from involvement as a ligand for the trypanosome surface. An alternative function could be direct or indirect involvement in physical linkage between the apoL1 toxin and the unknown trypanosome receptor of this complex. Regarding this receptor, we propose that TLF2 uptake relies on low affinity/high capacity interaction with the VSG coat. The rapid turnover of the

VSG would explain TLF2 efficiency. The nature of the TLF2 ligand is unknown, but polyclonal IgM, the dominant component of TLF2, represents an interesting candidate, since it is the only known component differentiating TLF2 from TLF1. Possibly the high-mannose residues of IgMs (Arnold et al., 2005), similar to those of VSG, could be involved in the binding process.

#### *The lipoprotein receptor pathway*

A so far uncharacterized lipoprotein receptor appears to be involved in sterol supply to the parasite (Green et al., 2003). Being an HDL particle, TLF1 could target the cell via this pathway. Accordingly, some TLF1 binding to the trypanosome surface could be inhibited by an excess of competing lipoprotein particles (Green et al., 2003). However, TLF1 constitutes only a minor subset of the total lipoprotein pool in NHS (Duchateau et al., 1997; most conservative estimation range around 1 percent), so that the contribution of the lipoprotein receptor pathway to the lysis process is probably low, both in vascular and other compartments. As TLF2 contains some lipids and apoA1, it is possible that those particles also bind to the lipoprotein receptor. However, as in the TLF1 case this uptake pathway is probably largely masked by a more efficient one.

#### *Fluid-phase uptake*

Although initiated from a minor fraction of the cellular surface, the endocytic rate of African trypanosomes is high, with an average of  $0.2 \mu\text{m}^3$  entering each cell per minute and a net uptake value 10 times lower (Engstler et al., 2004). Given the mean concentration of apoL1 in human plasma ( $10 \mu\text{g/ml}$ ; Duchateau et al., 1997), 3 to 30 molecules should accumulate per minute in each cell, as either TLF1 or TLF2. Recombinant apoL1 does not appear to spontaneously reconstitute with lipoprotein carriers unless a specific procedure is used (Vanhamme et al., 2003). Experiments with physiological levels of such free recombinant protein have shown that fluid-phase apoL1 uptake is sufficient for complete parasite killing, while a ten-fold lower concentration was only trypanostatic, i.e. the population remained stable over time (Vanhollebeke et al., 2007b). These results illustrate the effectiveness of fluid-phase alone in NHS.

### **What is the physiologically relevant apoL1 delivery pathway?**

The respective contribution of each TLF uptake pathway to trypanosome lysis can only be hypothesized, because the reconstitution of murine models recapitulating the subtleties of the different complexes seems to be out of reach. Conditions prevailing in the bloodstream should

clearly be distinguished from those prevailing in the other extravascular tissues where trypanosomes develop (lymphatic system, cerebrospinal fluids, interstitial fluid).

In the bloodstream, this debate is probably irrelevant regarding the outcome of infection. Indeed, fluid-phase endocytosis and lipoprotein receptor-mediated uptake are efficient enough to condemn a trypanosome population within a few hours. In 100% anhaptoalbuminemic serum, where TLF1 and TLF2-specific pathways are missing, a population of trypanosomes is destroyed in as little as 3 h, thus just 1 h longer than in 100% NHS (Vanhollebeke et al., 2007b). However, as far as can be extrapolated from *in vitro* studies, if the TLF2 pathway was operational *in vivo* it could dominate the process. In this context the lipoprotein receptor pathway is most probably outcompeted and the contribution of TLF1 should be minor, but significant. It is possible that this last contribution depends on the relative affinity of the VSG type for IgMs. Moreover, as human populations facing trypanosome infection appear to have increased occurrence of Hp and TLF2 deficiencies (Raper et al., 1996b; Widener et al., 2007), TLF1-TbHpHbR could represent a potent physiological uptake pathway in the field.

In conditions where trypanosomes face reduced concentration of lytic particles, both the low affinity TLF2 pathway and the fluid-phase delivery might lack potency. Instead, Hb-charged TLF1 particles should still be able to deliver sufficient apoL1 molecules to stop infection. It is also possible that the lipoprotein uptake pathway might participate. Finally, as African trypanosomes are injected intradermally by the tsetse fly and thrive for some days in a superficial inflamed tissue patch called “chancre” before gaining the systemic circulation, one could speculate that extravasated TLF1 particles might offer the first efficient line of immune defence.

### **Control of apoL1 toxicity**

The factor neutralizing apoL1 in *T. b. rhodesiense*, the VSG-like protein SRA, was initially proposed to interact with the C-terminal helix of apoL1 in the lysosome of the parasite (Vanhamme et al., 2003). Functional data presented in two recent papers have fully confirmed this view: due to sequence differences with human apoL1 in the C-terminal helix, baboon apoL1 no longer interacts with SRA, and accordingly the baboon-specific apoL1 efficiently kills *T. b. rhodesiense* (Thomson et al., 2009; Lecordier et al., 2009). When assayed in *E. coli*, the intrinsic pore-forming activity of apoL1 did not require the presence of the C-terminal helix (Pérez-Morga et al., 2005), but this activity was completely inhibited following interaction of SRA with this helix (Lecordier et al., 2009). Thus, it is conceivable that in the trypanosome, inactivation of apoL1 would result from direct interaction with SRA and not

from eventual SRA-mediated alteration of the intracellular trafficking of apoL1 (Oli et al., 2006). Experimental apoL1 mutants where the C-terminal helix was deleted or disrupted were found to lose their trypanolytic potential (Molina-Portela et al., 2008; Lecordier et al., 2009). Therefore, the C-terminal helix of apoL1 is probably involved in the control of the pore-forming activity of this protein, and either interaction with parasite antidotes or disruption of the helix would equally inactivate the toxin.

### **Out of Africa**

Trypanolytic activity is only found in African primates. The common ancestor that gave rise to humans, gorillas and baboons (~35 Mo years) appeared long after African trypanosomes (~100 Mo years) in tsetse infested regions (Stevens et al., 1999). Is it conceivable that trypanosome-elicited Darwinian selective pressures would have favoured the spread of immune organisms? This type of pathogen-driven selection is not infrequent: *Plasmodium falciparum*, for instance, has increased the frequency of sickle Hb coding sequences in some human populations because this  $\beta$ -globin variant provides heterozygotes with dominant resistance to the parasite, as infected sickle erythrocytes are removed by the spleen. TLF presence in human blood would be a vestige attesting of its recent African origin. In agreement with this idea, some humans living in tsetse-free areas have lost the key components of the lytic complex without seemingly overt negative consequences (Koda et al., 1998; Vanhollebeke et al., 2007b). Moreover, the acquisition of trypanolytic capacity in old world monkeys strikingly parallels exposure to the parasite. All trypanolytic animals are diurnal non arboreal central African primates. This predisposes them preferentially to the bite of tsetse flies. Chimpanzees lack trypanocidal activity, but their dominantly arboreal way of life might reduce their exposure to the tsetse fly.

The phylogenetic distribution of Hpr was proposed to match trypanolytic capacity (Lugli et al., 2004). However, scrutinizing the genome drafts of various primates including *Macaca mulata* and *Pongo pygmaeus* (BV, unpublished data) revealed that Hpr coding sequences are present in all old world monkeys, trypanolytic or not, as initially suggested (McEvoy and Maeda, 1988). So it is probable that the appearance of Hpr-bearing HDL preceded the secretion of apoL1 in the serum. In turn, apoL1 clearly defines primate trypanolytic activity in general, since it was found to be responsible for this activity not only in humans, but also in baboons (Thomson et al., 2009).

### **A novel innate immunity system**

Although TLF could have evolved in Africa to fight trypanosomes, it might play additional functions related to innate immunity, as the building blocks of TLF all appear to be derived from or linked to immunity proteins. Hp is indeed an acute-phase protein, i.e. a plasma protein whose concentration is modulated by inflammation. Moreover, Hp is derived from a complement-associated protein, the mannose-binding lectin-associated serine proteinase (Wicher and Fries, 2006). In TLF2 the Hp-derived Hpr is associated with another immunity component, IgM. Finally, apoL1 itself is strongly induced by inflammation (Vanhollebeke and Pays, 2006). So, recycling and assembly of non-specific innate immune components led to the development of a highly toxic immune complex. Given the pH dependency of apoL1 toxicity (Lecordier et al., 2009) it can be anticipated that potentially affected pathogens must either, like trypanosomes, be affected after endocytosis of TLF in their lysosomal compartment, or reside inside acidic compartments of host cells such as microbes that thrive in the phagolysosome of macrophages. Accordingly, TLF was found to attenuate parasitaemia by the intracellular pathogen *Leishmania* (Samanovic et al., 2009).

## Conclusions

Humans and related primates have assembled different innate immunity components into efficient anti-trypanosome toxic complexes. That these complexes might have arisen under trypanosome-elicited selection prevailing in the African belt during primate speciation represents a seducing hypothesis.

Various pathways converge to deliver these complexes to the invading parasites. Numerous parameters are known to influence the relative contribution of each of these pathways and one can expect others to be disclosed when all pathways will be defined in molecular terms. It is anticipated that depending on infection conditions (infection site, additional affection, trypanosome strain), different uptake pathways could dominate the immune response and offer the first line of protection.

In recent years, a combination of gain-of-function experiments in mice and loss-of-function observation in humans has indicated that primate immunity to trypanosome infection strictly relies on the presence in plasma of the Bcl-2 like protein apoL1. Even in the absence of specific uptake mechanisms apoL1 was found to be necessary and sufficient to provide immunity against the parasite. The observation that a mammalian Bcl2-like protein can trigger cell death in lower eukaryotes that lack downstream cell death executioners like caspases, is particularly remarkable and reveals a so far unknown aspect of Bcl-2 physiology.

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For Peer Review



### Figure legends

Fig. 1. Model of trypanolysis by TLF1. (1) ApoL1 is bound to HDL<sub>3</sub> particles (dotted circle) via its membrane-addressing domain (black) located close to the N-terminal pore-forming domain (white). The HDL<sub>3</sub> particles bind to the *T. brucei* receptor TbHpHbR through their Hpr-Hb complex. This triggers HDL<sub>3</sub> uptake and intracellular traffic to the lysosome. (2) Upon acidification the membrane-addressing domain of apoL1 undergoes conformational change that allows apoL1 dissociation from HDL and insertion in the lysosomal membrane, whereas Hpr and Hb are degraded to release heme for incorporation in bloodstream form hemoproteins. (3) The pore-forming domain of apoL1 triggers a flux of chloride ions from the cytoplasm to the lysosome lumen, due to the high concentration of chloride in the cytoplasm (0.1 M). This flux is accompanied by water, leading to uncontrolled osmotic swelling of the lysosome. (4) The reduction of cytoplasmic chloride concentration triggers the entry of chloride ions from the extracellular medium through plasma membrane channels (hatched oval). This process can be inhibited by the anion channel blocker DIDS.

Fig. 2. ApoL1 uptake pathways. ApoL1 circulates in the form of two high molecular weight (lipo)proteic complexes termed TLF1 and TLF2. These complexes engage in various cellular uptake pathways. TLF1 is mainly taken up through the Hp-Hb surface receptor TbHpHbR and relies on Hb priming. TLF2 is mainly taken up *via* another potent but uncharacterised mechanism. Other uptake routes, i.e. lipoprotein receptor pathway and fluid-phase uptake, may contribute under particular situations (see text).

Fig. 3. Involvement of TLF1 in different trypanolysis conditions. While the Hp-Hb complex exhibits the same affinity as TLF1-Hp-Hb for TbHpHbR, Hp-Hb also efficiently binds to the monocyte-macrophage receptor CD163, but the TLF1-Hp-Hb complex does not. Therefore, the trypanolytic potential of TLF1 strongly depends on the relative contribution of these different factors.



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