

Biogenesis of Trypanosome Lytic Factor and Mechanism of Trypanosome Killing

By

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Widener J, Hajduk S (2007). TLF-Hb Complex As A Cell Specific Toxin With No Primate Side Effects. Patent Pending

Shiflett AM, Faulkner SD, Cotlin LF, **Widener J**, Stephens N, et al. (2007) African trypanosomes: intracellular trafficking of host defense molecules. J Eukaryot Microbiol 54: 18-21.

Faulkner SD, Oli MW, Kieft R, Cotlin L, **Widener J**, et al. (2006) In vitro generation of human high-density-lipoprotein-resistant *Trypanosoma brucei brucei*. Eukaryot Cell 5: 1276-1286.

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Presentations

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Preface

I have executed all of the experiments contained in this body of work in the laboratory of Stephen Hajduk, with the following exceptions:

- In Chapter 2, figure 1, April Shiflett conducted the binding studies using AlphaScreen binding technology.
- In Chapter 3, figure 3, Marianne Jensby Nielsen and Søren Kragh Møestrup conducted surface plasmon resonance studies and TLF purification studies using hemoglobin coupled to sepharose beads.
- In Chapter 3, figure 7, April Shiflett conducted inhibition studies with DPPD (figure 7b) and dextran studies (figure 7c).

Acknowledgements

After college, I took a job at a small biotechnology company outside of Boston. As an experience, I learned two things. First, I worked in a miserable environment. Second, I wanted to do real science. To explain my second bit of garnered knowledge, my employers considered the science it based its technology on “phenomenology.” In so saying, they admitted that they did not care what was happening, only that they might succeed in making billions of dollars. Not understanding the science behind the technology probably contributed to the eventual failure of the company, but more importantly for the work presented here, the lack of understanding the scientific basis drove me nuts. How can you fix a problem if you don’t know what’s going wrong? Duct tape won’t even work. So I decided that I wanted to enter the halls of academia, where people strive to understand biology, among other things. Chris Lambert, my supervisor of all things science convinced me to hit the literature and identify people doing science I found interesting.

Some background. Parasites are cool. After playing with copepods on the gills of Red Fish growing up, and doing a bit of work on parasite-induced gigantism in college with Patrick Reynolds, I was hooked. I should say of Pat that talking science, drinking guinness, and hanging out with his Irish folk band spurred my interest in parasites. People that studied parasites were cool too. So after Chris, in his English accented, smart-assed questions, prompted me to read, I did. And I found a paper describing a lytic HDL that killed trypanosomes. What the hell is a trypanosome? I consulted “Parasite Rex” and found that it’s a parasite that evades the host immune response by shedding its skin every few days. The new skin is not recognized by the immune response. Every few days the

parasite sheds its skin so that the immune system can never catch up. Really cool. So I sent a letter.

Steve Hajduk spoke with me on the phone a few days later. After spending an hour speaking with me, he invited me down to Alabama. I sent other letters, but no one responded. No one took my calls. Steve took the time to speak with me, and over the two days I spent visiting with the “Duk” lab, I realized that this was a great place to work. Not only did the lab have interesting personalities, but it was led by a man that cared about science in the greater scheme of things. Steve was passionate about extending science to the general public, most specifically younger people. His passion created an environment that made doing science fun. It harkened back to drinking guinness with Pat and his band. Instead of a band though, I drank with fellow Ducklings. I did some experiments along the way and they are compiled here. Along the way, fellow Ducklings provided tons of support. Dan Golden and I brewed guinness. April Shiflett, along with tons of advice and scientific discussions, showed me what it means to be organized. Organization is an art unto its own, and one I’ll never master. April also married Jason Hall, and Jason, Mike Cipriano, Adam Lazarus, and Seth Bordenstein, all wonderful colleagues, also gave me enumerable welts during the best paintball matches ever played on Cap Cod. Rudo has provided several new words to my vocabulary including “HUP” and “Dewy,” And Rudo provided a view of assless chaps that will stay with me forever. Melissa, thanks for the education in wine. Rach and I hope to have many more before you or I go. Nats educated me on the ways of Jamaica. I owe many thanks to Viking John. And perhaps the biggest influence on my development as a scientist, and to whom

much thanks is owed, Torsten. All Ducklings, and there are more Ducklings, many not mentioned, but they have all given advise and contributed to fun times.

There are non-ducklings that deserve mentioning. First and foremost, my committee, Andrew Campbell, George Yap, Eduardo Nillni, and Rob Greenberg have been wonderful. Special thanks to Richard Bennett for joining my committee with short notice. Catfish, John and Cyclone, what an experience. Dr. Davis, Fred, Giaco, Ricco, Flem and Jenny, how do I thank you for your invaluable but intangible contribution to this work? And Bill Bestermann, how do I thank you for showing me that words don't mean shit? Well, to each of you, I extend my thanks.

I owe special thanks to a few individuals. Mom and Dad, even when other folks thought I was mentally retarded, believed in me. They spent every night forcing me to do math problems and edit English essays during middle school. I hated it. So did they, but they did it anyway. Mom, at the worst possible time in her life, sent me to live with a strange family in a distant town. I owe her for that, too. Mom has always believed in me and supported me in everything. And she doesn't care that I'll never be able to pay her back for everything. The family in a strange town, the Coggiolas, first introduced me to Republican lies. Every year since then, both parties have lied incessantly. I suppose they lied before but I wasn't paying attention. My life long friend and brother has always been present, and I'm grateful to you Colin.

Finally, I owe much to my wife Rach, who moved to Cape Cod and lived in a basement for me. I love you for being you, but I owe you thanks for putting up with me and this pursuit of a degree. Thanks for commuting to Brown for a year. And thanks for moving to Athens. I've been dragging you everywhere that Duk wanted to go and

eventually we'll settle down somewhere. Thanks also for feeding me so well and lending me that ear whenever I needed it. Oh yeah, thanks for reading this work for me. It's better for it. I love you.

I know I have forgotten to mention people that need mentioning, but I'm working under a deadline so forgive me. Now to the work.

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List of Abbreviations

HDL	High density lipoprotein
LDL	low density lipoprotein
VLDL	very low density lipoprotein
TLF	Trypanosome Lytic Factor
ApoA-1	Apolipoprotein A-1
Hpr	Haptoglobin-related protein
ApoL-1	Apolipoprotein L-1
Hb	Hemoglobin
PON1	Paraoxonase arylesterase 1
PAI-2	plasminogen activation inhibitor type 2
SRA	Serum resistance associated protein
CD163	scavenger receptor cysteine-rich domain protein
BSA	Bovine serum albumin
FBS	fetal bovine serum
DMEM	Dulbecco's modified eagle medium
ABCG1	ATP-binding cassette G1
ABCG4	ATP-binding cassette G4
ABCG5	ATP-binding cassette G5
ABCG7	ATP-binding cassette G7
ABCA1	ATP-binding cassette A1
ATP	Adenosine triphosphate
FAO	Food and Agriculture Organization of the UN

UN	United Nations
g	grams
mg	milligram
ml	milliliter
min	minute
hr	hour
mCi	millicurie
μg	microgram
ng	nanogram
μl	microliter
M	molar
mM	millimolar
μM	micromolar
nM	nanomolar
kDa	kilodaltons
NaCl	sodium chloride
mg	milligrams
mg/ml	milligrams per milliliter
g/ml	grams per milliliter
<i>T.b. rhodeseinse</i>	<i>Trypanosoma brucei rhodeseinse</i>
<i>T.b. brucei</i>	<i>Trypanosoma brucei brucei</i>
<i>T.b. gambiense</i>	<i>Trypanosoma brucei gambeinse</i>
L1	deferriprone

DPPD	N,N'-diphenyl-1,4-Benzenediamine
DIDS	4,4-diisothiocyanatostilbene-2,2-disulfonic acid
PNGase F	N-Glycosidase F
SDS	Sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
ER	endoplasmic reticulum
LCAT	lecithin cholesterol acyltransferase
Sr-b1	Scavenger receptor beta 1
β	beta
α	alpha
CHO	Chinese hamster ovary
HEK	human embryonic kidney
HepG2	Hepatocyte G2
O-linked	monosaccharide N- Acetylgalactosamine
N-linked	N-Acetylglucosamine
PLTP	phospholipids transfer protein
%	percent
C	Celsius
°	degree
LC	liquid chromatography
MS	mass spectroscopy
SPR	surface plasmon resonance
K_d	dissociation constant

H ₂ O ₂	Hydrogen peroxide
rpm	rotations per minute
1000 x g	1000 time gravity
PBSE	phosphate buffered saline – EDTA
EDTA	ethylenediaminetetraacetic acid
KCl	potassium chloride
Na ₂ HPO ₄	dibasic sodium orthophosphate
KH ₂ PO ₄	potassium phosphate monobasic
TTBS	Tris-Bicine solution with Tween 20
HRP	horseradish peroxidase
NCBI	National Center for Biotechnology Information
DAPI	4, 6 diamidino-2-phenylidole
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CaCl ₂	Calcium chloride
MgCl ₂	Magnesium chloride
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DNA	deoxyribonucleic acid
Cys	cysteine
Met	methionine
³⁵ S	sulfur-35
NP-40	nonidet P-40

Abstract

Trypanosomes are the causative agent of human African sleeping sickness and nagana in cattle. The bovine parasite, *Trypanosoma brucei brucei* (*T. b. brucei*), is unable to infect humans because of a small subclass of High Density Lipoprotein (HDL) known as trypanosome lytic factor (TLF) [1,2]. TLF is composed of several proteins including apolipoprotein A-1 (ApoA-1), apolipoprotein L-1 (ApoL-1), and haptoglobin-related protein (Hpr). Both ApoL-1 and Hpr have been shown to be trypanolytic [3,4]. When these two proteins are reconstituted into an HDL particle, the lytic activity increases 100-fold, indicating that the proteins have a synergistic relationship [3]. Furthermore, Hpr and ApoL-1 co-purify from plasma when an antibody to either Hpr or ApoL-1 is used, suggesting that the proteins co-assemble. Our findings suggest, however, that Hpr and ApoL-1 do not have direct interactions. To understand how Hpr and ApoL-1 are co-assembled, we have examined the biogenesis of TLF in human liver cells. Hpr and ApoL-1 localize to the surface of liver cells. Addition of HDL to the media results in scavenging of Hpr. We propose that Hpr and ApoL-1 traffic to the cell surface of liver cells, where they are scavenged by HDL.

Once co-assembled, Hpr and ApoL-1 containing HDL are still not lytic *in vitro*. Only after TLF binds Hb is TLF “armed” for killing. We show that Hb is required for binding and endocytosis of TLF. In addition, an iron chelator and free radical scavenger inhibit lysis by TLF-Hb. Using 500 kDa dextrans, we also show that TLF-Hb causes lysosomal membrane breakdown. Taken together, TLF-Hb generates free radicals by an iron dependent mechanism. The free radicals lead to lipid peroxidation and membrane breakdown.

We now have a model of TLF that begins with its biogenesis and ends with its killing of trypanosomes. Hpr and ApoL-1 are assembled into HDL by a cell-mediated process. Upon infection by *T. b. brucei*, an acute phase response causes severe hemolysis. Hpr binds hemoglobin, activating the toxin. TLF is then endocytosed by the parasite, leading to cell death and clearance of the infection.

Introduction

TLF prevents infection of humans by *T. b. brucei*. To confer protection, two proteins must assemble into TLF; Hpr and ApoL-1. If either protein is absent, the toxicity of TLF decreases substantially, and may not prevent infection by the parasite. The experiments presented here attempt to understand both how TLF is assembled and the biochemical mechanism by which TLF kills trypanosomes.

Specific Aims

Aim 1: Characterize interactions between Hpr and ApoL-1 by:

- a. Alpha screen experiments and immunoprecipitations to determine stable protein interactions within the lipid environment of TLF.
- b. Determine protein content of native TLF by MS/MS.

Interactions between different proteins, particularly Hpr and ApoL-1, may influence the assembly of TLF.

Aim 2: Determine location of Hpr and ApoL-1 association by:

- a. Producing and localizing Hpr and ApoL-1 in human hepatocytes.
- b. HDL scavenging studies.

HDL biogenesis and remodeling occur both intracellularly and on the cell surface. TLF biogenesis may also occur within the cell or on the cell surface.

Aim 3: Determine the role that hemoglobin plays in TLF killing of trypanosomes by:

- a. Developing a lysis assay that does not contain hemoglobin.
- b. Use pharmacological agents to verify nature of TLF-Hb killing.
- c. Develop flow cytometry assay to determine role Hb plays in TLF binding to trypanosomes.

Hpr binds hemoglobin and these studies will determine if Hb plays a role in lysis. Hb - TLF complexes may stimulate binding and uptake of the particle, or Hb may be directly involved in toxicity.

Background

1. General Background

The species *Trypanosoma brucei* contains several subspecies with a wide mammalian host range. *T. b. rhodesiense* can infect humans and causes African sleeping sickness. It persists in the bloodstream of the host by employing antigenic variation [5,6,7]. *T. b. brucei* is a second subspecies that is indistinguishable from *T. b. rhodesiense* by microscopy. *T. b. brucei* cannot infect humans because the parasite is lysed by a minor subclass of human high density lipoprotein (HDL) termed trypanosome lytic factor (TLF). *T. b. brucei* causes a similar disease to African sleeping sickness, called nagana, in cattle (Program Against African Trypanosomiasis, FAO). Transmission of the parasites occurs through the infected tsetse fly during a blood meal.

2. Pathogenesis

African sleeping sickness begins as a seemingly innocuous general malaise. Symptoms include fever, chills, and headache and are often described as flu-like. The tsetse fly bite site frequently swells and becomes hard and painful during a trypanosome infection [8]. The parasites remain at the bite site for up to 10 days prior to migrating to the blood stream. Once in the blood stream, the cells exhibit rapid replication and then periodic, near eradication by the immune system that corresponds with periodic surface coat changes in the parasite population [5,6,7]. As the infection progresses, an acute phase response leads to fever and a rash, followed by anemia, hepatitis, a swollen spleen, and myocarditis and renal failure. Encephalitis resulting in death will occur without treatment [8].

3. Trypanosome Lytic Factor

TLF is a small subclass of HDL particles that contain phospholipid, cholesterol and several proteins including ApoA-1, Hpr, and ApoL-1 (Figure 1). TLF migrates at about 500 kDa by gel filtration, is about 200 Angstroms in diameter, and has a density between 1.21-1.26 g/ml [1]. Rifkin [2] first showed that trypanolytic activity fractionated with HDLs purified from serum, and that injection of trypanosome infected mice with human HDLs resulted in near eradication of infection within 48 hours, while control mice died from parasitemia in less than 20 hours. Later it was shown that the lytic activity was attributable to a small subclass of HDLs termed Trypanosome Lytic

Factor (TLF). Antibodies raised against TLF inhibited lysis of trypanosomes *in vitro*, probably by preventing uptake [1]. It was shown that TLF trafficked to the lysosomal compartment of *T.b.*

brucei. Inhibition studies with chloroquine indicated that TLF requires a low pH in the lysosome to exhibit killing activity.

Furthermore studies with a lipophilic iron chelator L1 and the antioxidant DPPD suggested that iron was needed for killing and that free radicals were generated during

Trypanosome Lytic Factor-1

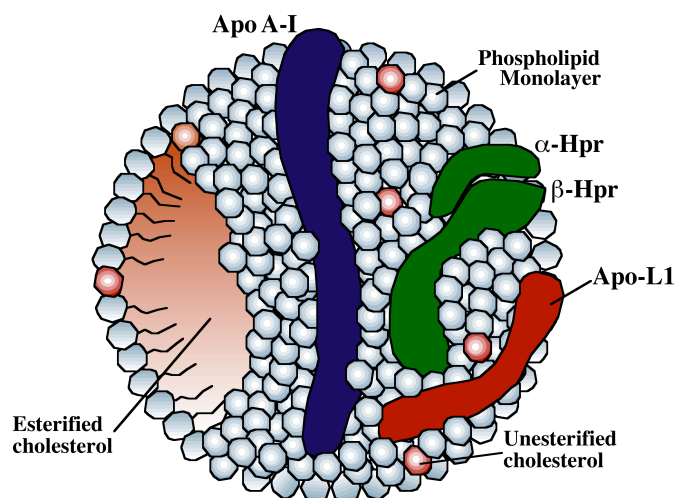


Figure 1. Cartoon of TLF. This depicts the essential molecules present in TLF including phospholipids, cholesterol, Apolipoprotein A-1, Apolipoprotein L-1, and Haptoglobin-related protein.

killing [9,10,11,12]. In addition, lipid peroxidation was evident in TLF treated but not control cells. From these studies, a mechanism of lysis was proposed by which TLF induced free radical generation followed by lipid peroxidation and subsequent lysosomal membrane breakdown [9]. The mechanism through which TLF induces such a reaction is not known.

4. The toxins

Hpr was shown to be present in TLF [1]. Antibodies to haptoglobin that cross-react with Hpr inhibited lysis by TLF, suggesting that the toxin in TLF was Hpr [12]. Several other proteins were identified in TLF and it was reported that one of these proteins, ApoL-1, was the actual trypanotoxin [4]. This was confirmed by detergent solubilization of TLF to purify Hpr and ApoL-1. Both proteins turned out to be toxic to trypanosomes [3].

4a. Apolipoprotein L-1

ApoL-1 is a 398 amino acid long protein that is expressed from chromosome 22 [13,14,15]. Vanhamme *et al.*, purified ApoL-1 from serum using SRA coupled to agarose beads for affinity purification [4]. SRA is the *T.b. rhodesiense* protein that confers resistance to TLF, and since SRA binds ApoL-1, ApoL-1 was postulated to be the toxin [4,16,17]. Upon cloning and expressing ApoL-1, lysis assays verified that recombinant ApoL-1 was trypanolytic [4]. Homology searches revealed a colicin A-like domain within ApoL-1 (Figure 2), and replacing the colicin pore-forming domain with

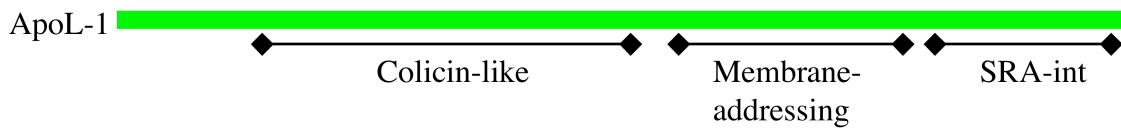


Figure 2. ApoL-1 functional domains. ApoL-1 contains a Colicin A-like domain with activity in Bacteria [12]. Additionally, a membrane-addressing domain and an SRA-interacting domain were experimentally shown.

the homologous region from ApoL-1 resulted in equivalent activity. Based on the similarities between ApoL-1 and Colicin A, it was postulated that ApoL-1 lytic activity occurs when ApoL-1 localizes to the trypanosome lysosome and forms a pore in the lysosome. Another domain, the membrane-addressing domain, is required for function, and probably allows ApoL-1 to associate with the membrane. When ApoL-1 colicin A-like domains were added to lipid bilayer membranes *in vitro*, anions but not cations, moved along the concentration gradient, suggesting that ApoL-1 forms an anion specific pore [18]. 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS), an anion pore inhibitor, had no affect on anion movement *in vitro*, although it did inhibit lysis by ApoL-1 *in vivo*. ApoL-1 associates with lipid by means of its lipid membrane-addressing domain (Figure 2), and the colicin A-like domain forms a pore in the lysosomal membrane of *T. b. brucei* that leads to ion imbalances and subsequent lysis of trypanosomes. The SRA interaction domain prevents *T. b. rhodesiense* lysis by ApoL-1 because SRA binds and removes ApoL-1 from the trypanosome.

4b. Haptoglobin-related protein

Hpr is a 348 amino acid protein that is transcribed from human chromosome 16 [19]. The Hpr gene arose from a gene duplication event of haptoglobin (Hp), and differs from Hp by 28 amino acids (Figure 3). Hp and Hpr share about 35% sequence similarity with the serine protease family and based on this we constructed homology models of Hp and Hpr using the trypsin crystal structure. Hpr is mainly expressed in liver tissue [20,21,22]. Hp is an acute phase serum protein that binds and clears hemoglobin in the circulation [23]. Hp inhibits lysis of *T. b. brucei* by TLF and is not associated with TLF [24].

```

Hpr (1)  -----MSDLGAVISLLLWGRQLFALYSGNDVTDISDDRFPKPPEIANGYVEHLF
Hp  (1)  -----MSALGAVIALLLWG-QLFVDSGNDVTDIADDGCPKPPEIAHGYVEHSV
                                     ↑
Hpr (50)  RYQCKNYYRLRTEGDGVYTLNDKKQWINKAVGDKLPECEAVCGKPKNPANPVQRILGGHL
Hp  (60)  RYQCKNYYRLRTEGDGVYTLNDKKQWINKAVGDKLPECEAVCGKPKNPANPVQRILGGHL
                                     ↓

Hpr (110) DAKGSFPWQAKMVSHHNLTTGATLINEQWLLTTAKNLFNLHSENATAKDIAPTLTLYVGK
Hp  (120) DAKGSFPWQAKMVSHHNLTTGATLINEQWLLTTAKNLFNLHSENATAKDIAPTLTLYVGK

Hpr (170) KQLVEIEKVVLHPNYHQVDIGLIKQKQVLVNERVMPICLPSKNYAEGVRGVYVSGWGQS
Hp  (180) KQLVEIEKVVLHPNYSQVDIGLIKQKQVSVNERVMPICLPSKDYAEGVRGVYVSGWGRN

Hpr (230) DNFKLTDHLKYVMLPVADQYDCITHYEGSTCPKWKAPKSPVGVQPILEHTFCVGMISKYQ
Hp  (240) ANFKFTDHLKYVMLPVADQDQCIRHYEGSTVPEKKTTPKSPVGVQPILEHTFCAGMISKYQ

Hpr (290) EDTCYGDAGSAFAVHDLEEDTWYAGILSFDKSCAFAEYGVYVKVTSIQDWVQKTIEN
Hp  (300) EDTCYGDAGSAFAVHDLEEDTWYATGILSFDKSCAFAEYGVYVKVTSIQDWVQKTIEN

```

Figure 3. Alignment of Hp and Hpr amino acid sequences. The two proteins differ by less than 9 percent. The signal sequence cleavage site of Hp is indicated by the empty arrow and the alpha / beta cleavage site is indicated by a solid arrow.

Hpr and Hp form dimers that are composed of one disulfide linked alpha and beta subunit. Hp exists as a tetramer while Hpr exists in both a dimer and tetramer form. The alpha and beta subunits of both proteins are transcribed as one message. In the case of Hp, the alpha and beta subunits are cleaved by an unknown protease after formation of the intermolecular disulfide bridge and it appears that the proteolytic cleavage occurs both in the secretory pathway and in the serum [25,26]. N-Glycosidase F (PNGase F)

treatment of both Hp and Hpr result in faster migrating proteins by SDS-PAGE, indicating that both Hp and Hpr are glycosylated [22]. Hp and Hpr contain an amino acid signal sequence. While the signal sequence of Hp is cleaved at the expected position and secreted without the signal peptide, the signal sequence of Hpr remains uncleaved. This is most likely due to two mutations at the signal peptide processing site of Hpr (Figure 3) [12]. Hpr toxicity is proposed to occur through generation of Fenton Chemistry within the trypanosome lysosome [9,12].

Fenton Chemistry

Fenton chemistry occurs when reduced iron or copper react with H_2O_2 to generate hydroxyl radicals [27]. The rate of the reaction depends on the source of iron, the pH, concentration of H_2O_2 , and the temperature; temperature and H_2O_2 concentration are directly proportional to rate while pH is inversely proportional, so reaction rates increase substantially at acidic pH [28]. Hydroxyl radicals produced from Fenton chemistry react rapidly with several substrates, including, proteins, polynucleotides, and lipids [28].

Trypanosomes lack catalase and have elevated levels of H_2O_2 [29]. This creates an environment within the trypanosome lysosome that is ideal for Fenton chemistry, i.e. low pH and high hydrogen peroxide levels. It has been proposed that TLF supplies iron through Hpr mediated binding to Hb [12]. Additionally, TLF has been shown to have peroxidase activity *in vitro*. To test whether the mechanism of TLF killing of trypanosomes was due to Fenton chemistry, lysis assays were conducted in the presence of free radical scavengers and iron chelators [9]. Both inhibit killing of trypanosomes by TLF. Additionally, lipid peroxides are produced when trypanosomes are killed by TLF,

but not under osmotic lysis of trypanosomes. This suggests that iron is required and generates free radicals that lead to lipid peroxidation. Furthermore, TEM images of trypanosomes incubated with gold-conjugated TLF show lysosomal breakdown and release of TLF-gold particles into the cytoplasm [10]. These results are consistent with TLF inducing Fenton chemistry that leads to lipid peroxidation and membrane breakdown of the trypanosome lysosome.

5. HDL assembly and remodeling

High density lipoproteins play a critical role in cholesterol metabolism [30]. They are the primary molecule responsible for reverse cholesterol transport, and they are becoming recognized as important signaling molecules [31].

Two proteins play a key role in HDL assembly, the ATP-binding cassette transporter A1 protein (ABCA1) and ApoA-1. Initially, ABCA1 was discovered as a genetic defect that resulted in a deficiency of serum HDL. The low HDL levels resulted from the inability of nascent (cholesterol poor) HDLs to interact with cells possibly leading to their clearance or instability [32]. Several groups discovered mutations in the ABCA1 gene of Tangiers patients (who lack HDL), and ABCA1 knockout mice were also deficient in HDL [33,34,35,36]. Likewise, patients with mutations to ApoA-1 can result in an absence of the protein, resulting in an absence of HDL in serum [37]. Knockout studies in mice resulted in dramatic decreases in HDL cholesterol and HDL particles [38].

Although ABCA1 and ApoA-1 play a critical role in HDL synthesis, the assembly of ApoA-1 into a lipid-containing particle is just now being clarified. ApoA-1 is phospholipidated upon synthesis into the ER [39]. Cholesterol addition is ABCA1 dependent, and to some extent occurs in the Golgi, while further lipidation happens upon secretion at the cell surface. Typically, the nascent ApoA-1/HDL particles are discoidal in shape, and cholesterol esterification is required for maturation of the HDL particle [40,41]. In addition to lecithin cholesterol acyltransferase (LCAT), other proteins and HDL receptors play a critical role in HDL maturation; scavenger receptor-B1 (Sr-b1), and several members of the ATP-binding cassette (ABC) family of proteins including ABCG1, ABCG4, ABCG5, and ABCG7 [42,43,44,45,46]. It is believed that these receptors remodel HDL, resulting in the great diversity of HDL particles. Little is known about when and where additional HDL proteins assemble into the particle.

6. HDL assembly of Paraoxonase/arylesterase (PON1)

PON1, like Hpr, is secreted without processing of its signal peptide. PON1 is an HDL lipoprotein that reduces oxidized lipid in lipoprotein particles in the serum. There is also evidence that PON1 associates with atherosclerotic plaques [47,48]. N-terminal sequencing of PON1 revealed that it is secreted without cleavage of its signal sequence, probably because PON1 lacks two bulky amino acids at the site of cleavage [49,50]. Mutating the bulky amino acids at the cleavage site to alanine residues resulted in signal sequence cleavage and secretion as a lipid free protein. Without the signal sequence, PON1 could not assemble into lipid particles, suggesting that the N-terminal signal sequence is necessary for lipoprotein association [51]. Furthermore, PON1 is trafficked

to the cell surface of Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells, and addition of HDL removes PON1 activity from the cell surface to the media [52]. Media analyzed by gel filtration and SDS polyacrylamide electrophoresis and western blot showed PON1 elutes with ApoA-1. LDLs and VLDLs do not scavenge PON1. Perhaps HDLs dock with cell membranes via the Sr-b1, providing a platform for PON1 assembly into the HDL [52].

How proteins are secreted without cleavage of their signal sequence is unknown. Several genetic disorders, however, result from mutations at the signal peptide cleavage site. For example, if the signal peptide of coagulation factor X is not cleaved, it is degraded and presents as hemophilia [53]. On the other hand, plasminogen activation inhibitor type 2 (PAI-2) is secreted without signal sequence cleavage [54]. Apparently it has an internal signal sequence and PAI-2 is O and N-link glycosylated, suggesting that it traffics through the constitutive pathway [55]. The secretory pathway of Hpr is not known, although evidence suggests that PON1 and Hpr may traffic through the constitutive pathway.

Cell-Mediated Assembly of Trypanosome Lytic Factor

Running Title: Trypanosome Lytic Factor Biogenesis

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remodeling, Trypanosome lytic factor (TLF), Scavenger receptor B1, Caveolar/lipid rafts.

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Abstract

Tyranosome lytic factor (TLF) is a low abundance subclass of high density lipoprotein (HDL) that confers innate protection to humans and old world primates against the parasite *Trypanosoma brucei brucei*. Two TLF associated proteins, apolipoprotein L-1 (ApoL-1) and haptoglobin-related protein (Hpr), have been shown to be lytic to *T. b. brucei*. TLF can be purified directly from human serum by immuno-affinity chromatography using monoclonal antibodies to either ApoL-1 or Hpr, demonstrating that these proteins reside in the same HDL in the circulation. ApoL-1 and Hpr are several hundred fold more toxic to *T. b. brucei* when assembled into the same particle, suggesting that formation of TLF is critical for maximal trypanosome killing activity. How Hpr and ApoL-1 assemble into HDL is not known. Binding studies show that Hpr and ApoL-1 do not have direct protein interactions. Immunofluorescence studies indicated that Hpr and ApoL-1 were expressed on the cell surface of human liver cells (HepG2 cells). Addition of human HDL to HepG2 cells reduced the cell-surface associated Hpr, indicating that HDL scavenges Hpr from the cell surface. These results suggest that Hpr and ApoL-1 assemble into human HDL after being presented on the cell surface, and may provide a pathway for Hpr and ApoL-1 co-assembly into TLF in humans.

Introduction

Humans and old world primates possess a minor subclass of anti-parasitic high density lipoprotein particle (HDL) termed trypanosome lytic factor (TLF) [1,2,3]. As its name implies, TLF is toxic to the parasite *Trypanosoma brucei brucei* (*T.b. brucei*), and TLF confers complete protection to humans against the parasite. Two proteins found in TLF distinguish it from other HDL particles, haptoglobin-related protein (Hpr) and apolipoprotein L-1 (ApoL-1) [4,5,6]. TLF constitutes less than 0.1% of total HDL. This minor subclass contains better than 99% of the lytic activity in serum [4]. Approximately 50% of Hpr and ApoL-1 do not coexist in HDL, and these two subclasses of HDL contain less than 1% of the lytic activity of serum. Particles that contain both Hpr and ApoL-1 (TLF) are at least 100 times more lytic than particles containing only Hpr or only ApoL-1. This suggests that their presence within the same particle is functionally important and that the proteins have evolved a mechanism to promote co-assembly [4].

Three general possibilities exist for how Hpr and ApoL-1 are co-assembled. First, direct protein-protein interactions between Hpr and ApoL-1 may cause co-assembly. Second, cofactors may actively insert the two proteins into the same HDL particle, and third, a combination of direct protein interactions and assistance from cofactors may occur. We examined the first possibility by testing for direct protein-protein interactions but did not detect binding. Addition of HDL did not stimulate binding. In mice transiently transfected by a DNA vaccine method, these proteins only form into the same HDL when expressed from the same plasmid [7], which suggests that the assembly of these proteins is dependent on being expressed from the same cell. Taken together, the

assembly of Hpr and ApoL-1 into the same particle is likely mediated by cellular processes.

Hpr is secreted without cleavage of its signal peptide. Paraoxonase arylesterase (PON1) is one of three known proteins to be secreted without cleavage of its signal peptide [8,9]. In the case of PON1, the protein traffics to the cell surface and is scavenged by HDL [10]. We examined the localization of native Hpr and ApoL-1 in HepG2 cells to determine if are presented on the cell surface. We also examined the affect of HDL on cell surface localization of Hpr.

Results

Protein Binding Studies

To test whether Hpr and ApoL-1 interact directly, we employed AlphaScreen binding technology. We covalently linked Hpr and ApoL-1 to donor beads and acceptor beads, respectively. When the donor bead is excited, it releases a singlet oxygen that can react with an acceptor bead and induces a fluorescent signal if the beads are in close proximity. After incubating Hpr and ApoL-1 linked to their beads for one hour, we measured the fluorescence emitted from the acceptor beads. We did not detect a significant increase in fluorescence as the concentration of ApoL-1 increased relative to Hpr (**Figure 1A and B**). Neither Hp nor BSA interacted with ApoL-1 in control experiments, whereas streptavidin and biotin exhibited a strong interaction. Since Hpr and ApoL-1 reside in a lipid environment under native conditions, we added HDL to determine whether the interaction between Hpr and ApoL-1 was lipid dependent. Addition of the lipid did not stimulate an interaction between Hpr and ApoL-1 (**Figure 1C**).

Cellular localization

Paraoxonase arylesterase 1 (PON1) is secreted without cleavage of its signal peptide, by way of the cell surface. It is scavenged from the cell surface by HDL. Hpr, like PON1, lacks the signal peptide cleavage site. We postulated that Hpr remains cell associated until it is scavenged by HDL. AlphaScreen data suggest that it is unlikely that Hpr and ApoL-1 interact in the plasma. To test if ApoL-1 and Hpr localize on the cell surface, we applied immunofluorescence microscopy and deconvolution analysis using a monoclonal antibody to Hpr and ApoL-1. Based on co-localization with the known cell surface HDL

receptor Sr-b1, Hpr and ApoL-1 localize to the cell surface of HepG2 cells (**Figure 2A, 3**).

Based on the interaction between PON1 and HDL at the cell surface, we asked whether Hpr was exposed at the cell surface. After trypsinizing the cells to remove cell surface proteins, we repeated the IFA experiments. The signal from Hpr and Sr-b1 was lost, suggesting that Hpr is presented on the cell surface.

HDL Scavenging

To test whether Hpr is removed from the cell surface of HepG2 cells by HDL, we labeled cells with ³⁵S cysteine and methionine. After harvesting, the media and cell lysates were purified over a polyclonal anti-haptoglobin affinity column that recognizes both Hp and Hpr. Analysis of the column eluates by SDS-PAGE showed Hp purified from the media as expected. Three bands, one of which migrates at 45kDa, is the size of the Hpr dimer band were purified from the cell lysate. Two additional bands, one which migrates at the size of ApoL-1 at around 42 kDa, was also purified from the cell lysate (**Figure 4A**). In pulse chase experiments, the 45kDa band, presumably Hpr, remained cell associated, suggesting that Hpr is stably associated with the cell (**Figure 4B**). Addition of HDL to the cell removed the 45 kDa Hpr band from the cell lysate (**Figure 4C**). The ApoL-1 band cannot be seen in this experiment but the larger band is present.

Discussion

In order for TLF to kill a trypanosome, Hpr associated with TLF binds to a Hp receptor in the trypanosome flagellar pocket [11]. TLF traffics to the lysosome where the low pH change induces toxicity through membrane disruption [12]. Purified Hpr and purified ApoL-1 alone are inefficient toxins, and at their concentrations in human serum may not effectively prevent infection [4]. When the two proteins are packaged together in the same HDL, the toxicity increases at least 100 fold. Understanding how these two toxins co-assemble into TLF is important for elucidating the relationship between Hpr and ApoL-1 and will provide insight into how HDL subclasses are formed in general.

AlphaScreen binding studies indicate that Hpr and ApoL-1 do not interact directly. It is unlikely that the proteins are secreted independently and then associate in the serum because the proteins do not interact under the conditions tested using the AlphaScreen binding assay, and because they must be expressed in the same plasmid in mice [7]. AlphaScreen technology is a powerful tool to analyze protein-protein interactions. In our case, however, the presence of alkenes in HDL might quench the reaction and abolish a possible signal if the proteins interact in the presence of lipid [13].

Recent studies provide evidence that cholesterol efflux occurs when HDL associates with cholesterol rich regions or lipid rafts [14,15]. Caveolae / lipid rafts begin to form in the ER when caveolin oligomerizes and associates with lipid [16]. As caveolin traffics from the ER and through the Golgi, additional lipids and proteins associate as the membrane matures. In several cell types raft formation is important in cell sorting, and the rafts transport specific proteins to different regions at the cell surface [17,18]. Hpr and ApoL-1 may associate through direct interactions while they are folding in the ER.

Then, Hpr and ApoL-1 may associate with rafts via the signal peptide of Hpr. HDL receptors ABCG1 and Sr-b1 also associate with rafts as the rafts mature and raft-mediated protein sorting may co-localize HDL remodeling proteins [14,19,20]. Once the rafts reach the cell surface, Hpr, ApoL-1, and HDL receptors are ready to interact with HDL.

Our data showing scavenging of Hpr from the cell surface by HDL suggests that HDL remodeling requires addition of protein factors as well as changes in lipid content [14,15,19,21]. In the case of PON1, HDL removes the protein but other lipoprotein particles do not, suggesting that the interaction is also specific [10]. Removal of the signal peptide of PON1 prevents both cell surface localization and HDL association, suggesting that the signal peptide is critical for both trafficking and HDL association of PON1 [10,21]. Recombinant Hpr (rHpr) expressed in HEK 293 cells, and unlike native Hpr, is secreted from cells into media after accurate processing of its signal peptide. Furthermore, rHpr does not kill a trypanosome, probably because it does not associate with HDL (Widener et. Al., in preparation). This suggests that the signal peptide of Hpr is critical for secretion and scavenging of Hpr and ApoL-1 into HDL and probably follows a similar pathway as that of PON1. The pathway that allows HDL associated proteins to escape the ER without processing of the signal peptide is critical for HDL remodeling and the biogenesis of functional TLF.

TLF is one example of an HDL subclass, and TLF must contain both Hpr and ApoL-1 to have maximum trypanosome killing activity. Since receptor mediated maturation and remodeling of HDLs frequently occurs at the cell surface, it is attractive to speculate that TLF is constructed when HDL associates with remodeling receptors. At

these receptors, cholesterol and lipid exchange occurs, as does addition of protein to HDL. Hpr and ApoL-1 transition from the cell surface to the HDL at these remodeling centers. Following remodeling of the HDL, it is able to confer protection against *T.b. brucei* infection.

Methods and Materials

Protein binding studies

AlphaScreen binding technologies along with the EnVision Multilabel Reader (Perkin Elmer Life Sciences, Waltham, MA, USA) were used to probe the interactions between Hpr and ApoL1. Alpha Screen works by energy transfer from an excited “donor” bead to an acceptor bead that emits light at a shorter wavelength than the excitation energy [22]. For our studies, we covalently linked 17µg Hpr and Hp to 660 µg of donor beads as described in the protocol. 17 µg of ApoL-1 was linked to 660 µg/ml of AlphaScreen acceptor beads. A dilution series of ApoL-1 from 1 µg to 0.1 µg final amount (4.125 µg to 0.4125 µg of beads were incubated with 1 µg of Hpr or Hp (4.125 µg of beads) for 75 minutes. We varied the concentration of HDL devoid of TLF to determine if lipid had an affect. A caveat of using lipid in these reactions is that the singlet oxygen attacks the alkene in cholesterol [13]. As a positive control, we examined the interaction of biotin labeled IgG with Protein A-conjugated acceptor beads. We then added streptavidin conjugated donor beads (AlphaScreen™ IgG (Protein A) Detection Kit, Perkin Elmer Life Sciences).

Cell Culture

HepG2 and HEK293 cells were purchased from ATCC and grown in DMEM high glucose etc (Gibco). Cells were passaged when they achieved about 90% confluency and media was typically changed after four days of culture. After no more than 15 passages, cells were discarded and new cultures initiated. For ^{35}S Cys Met labeling experiments, DMEM high glucose was removed, cells were washed with PBS, and 10 ml DMEM high glucose without Cysteine and Methionine (Gibco) was added to a T75 culture flask (Corning Inc., Corning, NY) and 0.25 mCi of ^{35}S Cys Met was added.

Immunofluorescence

Immunofluorescence studies were conducted on HepG2 cells and HEK 293 cells. In most experiments, cells were grown on a coverslip for between 4 and 6 days. Coverslips were washed three times with PBS, and then fixed with 1% paraformaldehyde in PBS for 10 minutes. Cells were washed 1X with PBS, then incubated in PBS Glycine for five minutes to block the fixation process. After two additional washes in PBS the cells were blocked in 5% BSA. Anti-Hpr monoclonal was used at 1/100 concentration in 5% BSA in PBS. A monoclonal anti-ApoL-1 antibody was used at 1/200 concentration in the same conditions and an Sr-b1 polyclonal antibody (Novus Biologicals, Littleton, CO) was used at 1/1000 concentration in the same conditions.

Immunoprecipitation

^{35}S labeled cells were harvested by addition of cell lysis buffer (20mM CHAPS, 500mM NaCl, 0.1% SDS, 0.5% NP-40, and 0.5% Deoxycholate) and incubated for five minutes in the presence of protease inhibitors. Media was harvested and Complete Mini-EDTA

protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) were immediately added. Both cell lysate and media were purified by anti-body spin columns that contained a polyclonal anti-Hp antibody (DAKO Cytometrix, Denmark), covalently linked to Affigel 15 (BioRad, Hercules, CA, USA). The eluate was analyzed by SDS-PAGE and a Storm 840 phospho-imager (GE Healthcare, Piscataway, NJ). In pulse-chase experiments, cells were labeled overnight and then the media was removed and the cells were washed two times with PBS prior to adding DMEM with high glucose. Cultures were then harvested at the times indicated in figure 4. HDL scavenging studies were conducted by replacing FBS in DMEM with high glucose with 10% HDL or fresh 10% BSA and incubated for four hours. At this time both media and cells were harvested, purified, and analyzed as described above.

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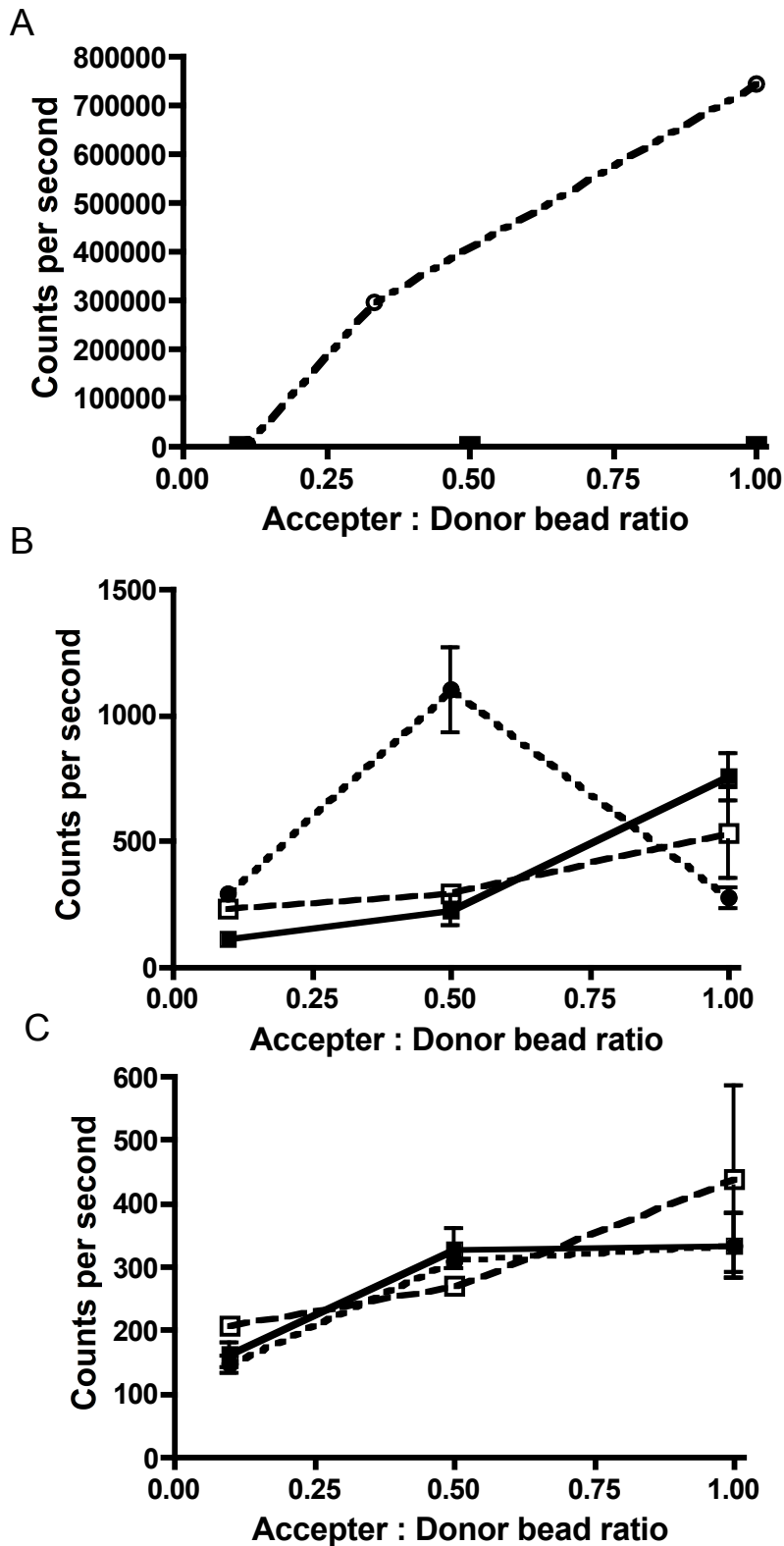


Figure 1. AlphaScreen binding studies to determine if Hpr and ApoL-1 interact. ApoL-1 was covalently linked to Alphascreen acceptor beads and Hpr (closed square), Hp (closed circle), BSA (open square) was unconjugated as a negative control. As a positive control, streptavidin and biotin were labeled with donor and acceptor beads, respectively (A). Streptavidin and biotin produced a strong signal (A), whereas Hpr, Hp, and BSA did not interact appreciably with ApoL-1 (B). Addition of unlabeled HDL did not alter binding of ApoL-1 to Hpr (C). As expected, Hp nor BSA showed affinity for ApoL-1.

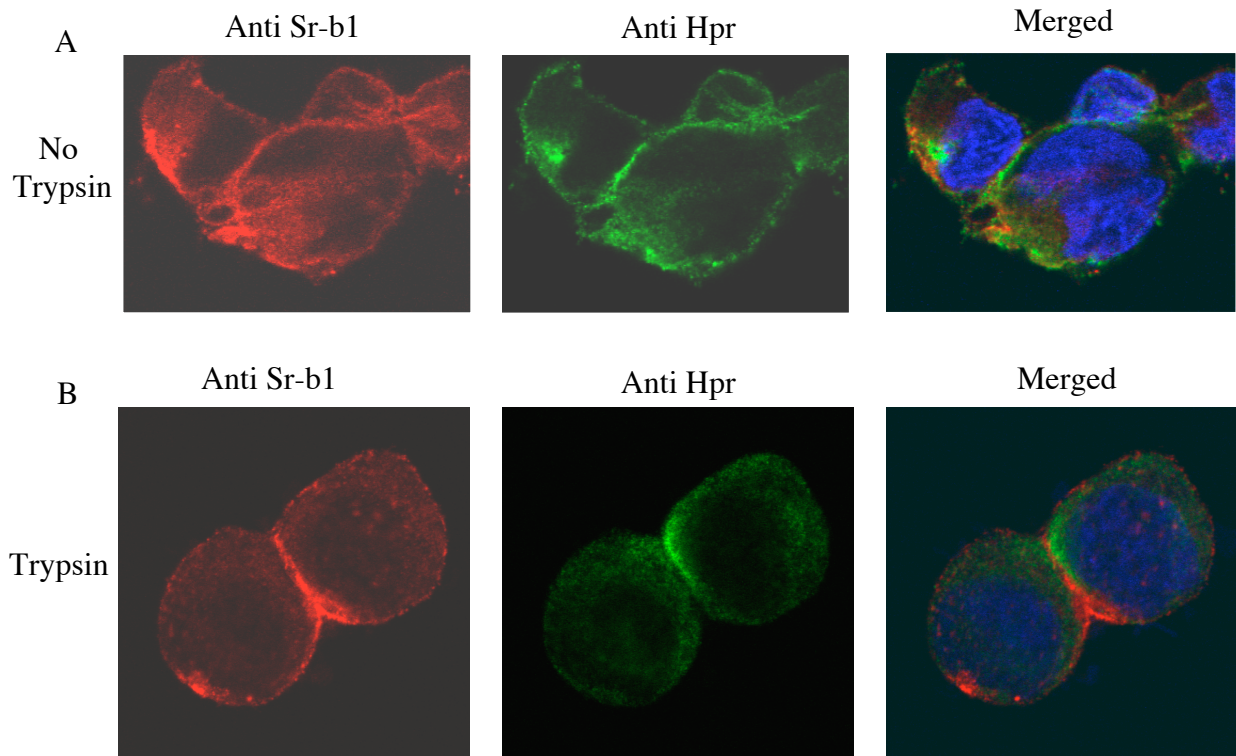


Figure 2. Hpr localizes to the cell surface in HepG2 cells. In panel A, HepG2 cells were incubated with monoclonal antibody to Hpr (green) and a polyclonal antibody to the surface HDL receptor Sr-b1 (red). In panel B, cells were treated with 0.25% trypsin, 2mM EDTA for 20 minutes to remove surface proteins. Cells were then treated as in A.

A

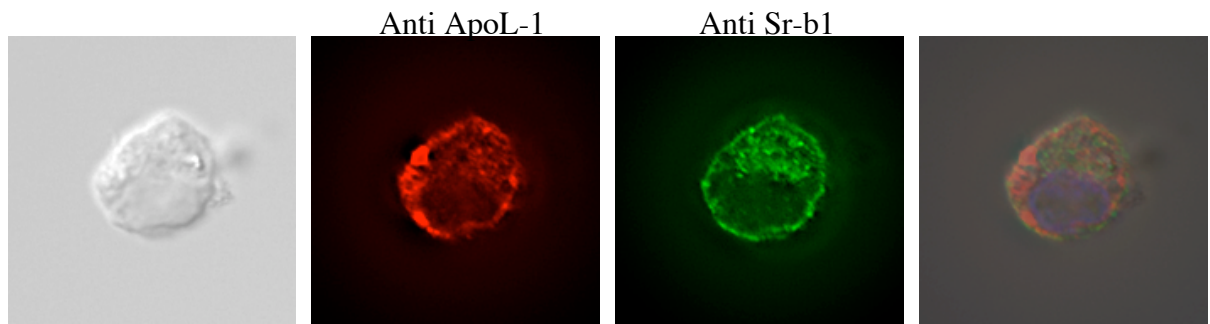


Figure 3. ApoL-1 localizes on the cell surface. In panel A, HepG2 cells were released from the culture flask with 3mM EDTA and 0.25 % trypsin and incubated with a monoclonal antibody to ApoL-1 (red) and a polyclonal antibody to the surface HDL receptor Sr-b1 (green).

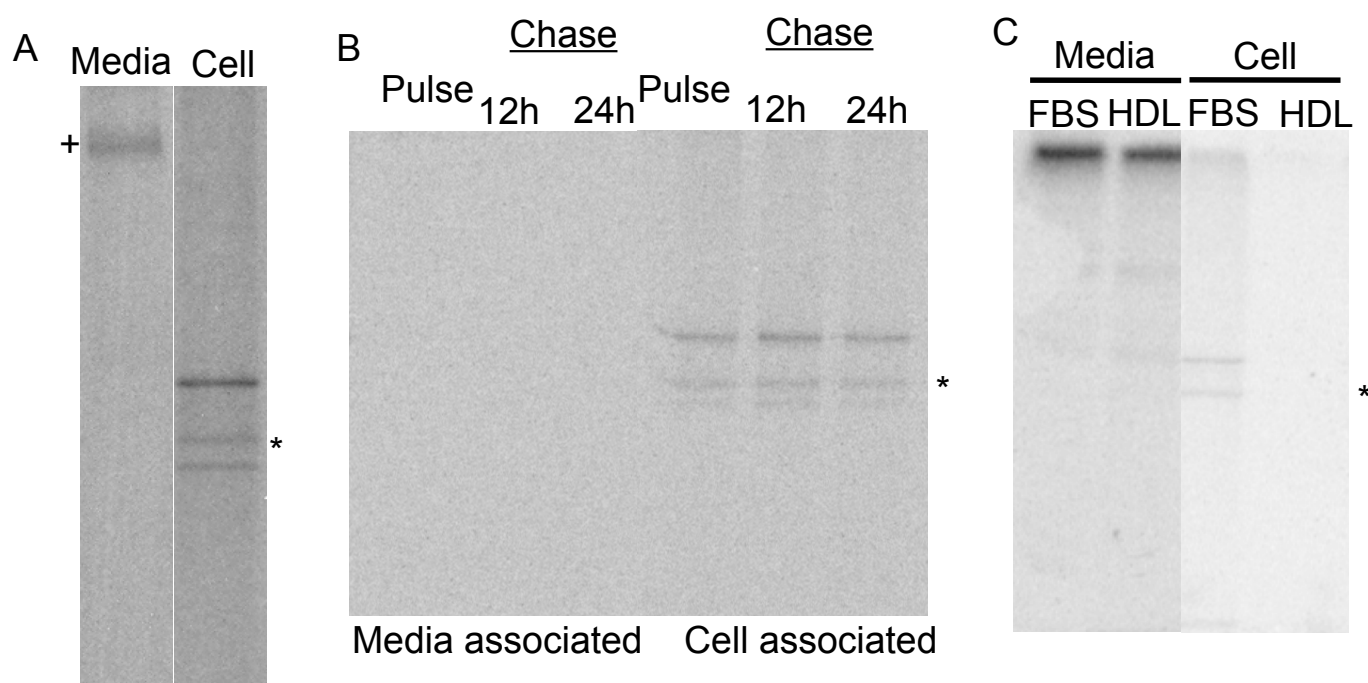


Figure 4. HDL scavenges Hpr from the cell surface. HepG2 cells were labeled with ^{35}S Met/Cys overnight and both the media and cells were collected. After affinity purification with antibodies to Hp, proteins were analyzed by SDS-PAGE and imaged using a phosphoimager. Haptoglobin (+) purifies with the media and three bands purify with the cell lysate. The middle band (*) migrates at 45 kDa, or the same size as Hpr (A). Pulse chase experiments indicated that the cell associated bands are stable and not removed to the media (B). Upon addition of human HDL to the media, the bands are removed from the cell surface (C).

Hemoglobin is a Co-factor of Human Trypanosome Lytic Factor

Running Title: Trypanosome Lytic Factor

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Abstract

Trypanosome Lytic Factor (TLF) is a high-density lipoprotein (HDL) subclass providing innate protection to humans against infection by the protozoan parasite *Trypanosoma brucei brucei*. Two primate specific plasma proteins, haptoglobin-related protein (Hpr) and apolipoprotein L-1 (ApoL-1) have been proposed to kill *T. b. brucei* both singularly or when co-assembled into the same HDL. To better understand the mechanism of *T. b. brucei* killing by TLF, the protein composition of TLF was investigated using a gentle immunoaffinity purification technique that avoids the loss of weakly associated proteins. HDL particles recovered by immunoaffinity absorption, with either anti-Hpr or anti-ApoL-1, were identical in protein composition and specific activity for *T. b. brucei* killing. Here we show that TLF-bound Hpr strongly binds Hb and that addition of Hb stimulates TLF killing of *T. b. brucei* by increasing the affinity of TLF for its receptor, and by inducing Fenton chemistry within the trypanosome lysosome. These findings suggest that TLF in uninfected humans maybe inactive against *T. b. brucei* prior to initiation of infection. We propose that infection of humans by *T. b. brucei* causes hemolysis that triggers the activation of TLF by the formation of Hpr-Hb complexes leading to enhanced binding, trypanolytic activity and clearance of parasites.

Introduction

African trypanosomes are blood parasites of mammals in sub-Saharan Africa and cause chronic wasting diseases in both humans and domestic animals [1]. The three subspecies of *Trypanosoma brucei* are defined by their host range, geographical distribution and course of disease [1-3]. *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* infect humans and cause African sleeping sickness while *T. b. brucei* infects non-primate mammals and causes nagana in cattle. All African trypanosomes are able to evade the host adaptive immune system through a process called antigenic variation, which is a consequence of periodic changes in the variant surface glycoprotein that covers the entire parasite [4]. *T. b. brucei* does not cause human disease because of its susceptibility to an innate immune activity in human serum. This protection is conferred by Trypanosome Lytic Factor (TLF), a minor subclass of human HDL [5-7].

TLF contains apolipoprotein A-I (ApoA-1), a protein found in all subclasses of HDL, and two proteins, Hpr and ApoL-1 that are unique to primates [8-19]. Both Hpr and ApoL-1 have been reported to be toxic to *T. b. brucei* [8,14]. The cellular pathway for TLF killing of *T. b. brucei* initiates with binding of TLF to high affinity receptors located in the flagellar pocket of the parasite [20,21]. Bound TLF is endocytosed via coated vesicles and trafficks to the parasite lysosome. Within the acidified lysosome TLF is activated and causes parasite lysis [22-24]. ApoL-1 and Hpr have been proposed to have different mechanisms of toxicity and may act synergistically. ApoL-1 is a colicin-like protein that kills trypanosomes through the formation of ion-pores [10,25-29]. Hpr is an Hb binding protein that has been proposed to induce an iron dependent, Fenton-like reaction, within the acidic lysosome of *T. b. brucei* that leads to the formation of free

radicals and peroxidation of the lysosomal membranes [15,24,30]. When ApoL-1 and Hpr are present in the same HDL particle the specific activity for *T. b. brucei* killing is enhanced eight-hundred fold [14].

Hpr is 91% identical to Haptoglobin (Hp), an abundant (~0.45-3 mg/ml in normal human serum) acute phase serum protein, possessing very high affinity for Hb [31]. Complexes of Hp and Hb that form when Hb is released from erythrocytes undergoing intravascular hemolysis are removed from the circulation by the CD163 scavenger receptor [32]. In contrast to Hp-Hb, the Hpr-Hb complex does not bind CD163 [31,33] and the Hpr serum concentration appears to be unaffected by hemolysis [32,33]. A role for Hb in trypanolysis has previously been speculated but this view was subsequently debated [12,15,30]. In the light of this, the biological significance of the recently reported high affinity binding of recombinant Hpr to Hb remains enigmatic.

In the present study we have reinvestigated a potential function of Hb in relation to TLF and trypanolysis. Hemoglobin was absent from TLF purified from freshly collected human plasma using a gentle immuno-absorption protocol. Nevertheless, Hb bound to purified native Hpr, as well as TLF, with high-affinity and stimulated *T. b. brucei* killing *in vitro*. The association of Hb with Hpr significantly increased TLF binding to *T. b. brucei*. Furthermore, both an iron chelator and a free radical scavenger inhibited cell lysis suggesting a direct role for Hb derived iron in *T. b. brucei* killing by TLF. A combination of the ion pore forming activity of ApoL-1 and free-radical production of Hpr-Hb may account for the synergy observed in HDLs containing these proteins.

Results

Analysis of TLF protein components

In order to define the protein composition of native TLF, freshly prepared human plasma was fractionated by “one-step” immunoaffinity chromatography with antibodies against Hpr or ApoL-1. Unlike the “two-step” method that initially uses high-salt, density ultracentrifugation followed by immunoaffinity chromatography to purify TLF, the one-step method eliminates the possible loss of apolipoproteins during the high salt centrifugation step [34]. These methods allowed us to make two comparisons: first, a comparison of proteins purified by the one-step purification protocol with proteins purified by the two-step purification protocol. Second, it allowed us to compare the composition of TLF purified by the one-step purification method using antibodies to ApoL-1 or Hpr.

Previous analysis of the protein composition of TLF, purified by the two-step procedure, demonstrated the presence of ApoA-1, ApoL-1, Hpr dimer and Hpr tetramer (**Figure 1A**) [14]. Comparison of proteins purified by the two procedures by SDS-PAGE and western blot analysis showed that the protein compositions were similar in both cases (**Figure 1**). This shows that high salt density ultracentrifugation used in the two-step protocol does not dramatically change the protein composition of the TLF particle. Two differences were noticed. First, the ratio of Hpr dimer to tetramer was higher in samples purified using the single-step immunoabsorption method. Second, a reduction in sub-stoichiometric proteins in preparations of TLF purified by the two-step purification method. Specifically, a significant amount of Hp, albumin, and transferrin were detected by SDS-PAGE, western blotting, and LC-MS/MS in the one-step purified material

(Figure 1A-C, Table 1). In addition to these serum proteins, other human serum proteins including angiotensinogen and intelectin were detected at low abundance by LC-MS/MS analysis (data not shown). Notably, no hemoglobin was detected in any of the TLF preparations.

In order to determine whether the protein composition of HDLs containing Hpr or ApoL-1 differed significantly, samples were purified from human plasma by the one-step immunoaffinity method using antibodies to Hpr, ApoL-1 or ApoA-1. All samples were analyzed by coomassie staining, western blot and LC-MS/MS **(Figure 1 and Table 1)**. The protein composition of samples purified by anti-Hpr or anti-ApoL-1 absorption were indistinguishable. The similar ratio of Hpr and ApoL-1, in the samples purified with anti-Hpr and anti-ApoL-1, indicates that most serum Hpr and ApoL-1 are assembled into the same HDL. The major proteins present in samples purified by anti-ApoA-1 immunoaffinity chromatography were ApoA-1, Hp and transferrin. Only trace amounts of ApoL-1 and Hpr were detected in these samples consistent with TLF being a minor subclass of human HDL[6]. These findings rule out the possibility that a significant amount of human plasma Hpr or ApoL-1 is free in the circulation and suggests a pathway for co-assembly of these two apolipoproteins into the same HDL particle.

Analysis of differentially purified TLF lytic activity

The specific activity of TLF purified by the one-step method using antibodies to either Hpr or ApoL-1 was similar. In our standard lysis assay, 0.5 µg resulted in 40% lysis in 2 hrs at 37°C **(Figure 2A)**. Human plasma samples purified with anti-ApoA-1 were also trypanolytic but the specific activity for killing was reduced over 10-fold **(Figure 2A)**.

The low specific activity of the ApoA-1 purified samples was due to the relatively low ratio of TLF to non-lytic HDL in this preparation. This is consistent with previous estimates of TLF abundance suggesting it represents less than 0.1% of total human serum HDL [6,14].

When plasma samples were purified by the two-step protocol that included high-salt density ultracentrifugation followed by either anti-ApoL-1 or anti-Hpr immunoaffinity purification the specific activity for trypanosome killing was approximately 10 fold higher than samples purified by the one-step methods (0.025 μ g and 0.04 μ g giving ~50% lysis respectively) (**Figure 2B**). The increased lytic activity of the samples purified by the two-step procedure may be due to the increased purity of these samples. This is because gradient centrifugation employed in the two-step purification process reduces the amount of contaminating, high-abundance, serum proteins, such as albumin and transferrin (**Figure 1A**). In addition, Hp, a potent inhibitor of TLF killing of *T. b. brucei* [9], is weakly associated with all human HDL and is effectively removed by high-salt density ultracentrifugation (**Figure 1A**). The mechanism of Hp inhibition of human serum lysis of *T. b. brucei* is not known; based on results presented later, inhibition maybe a direct consequence of Hp binding to free Hb in serum.

Two different morphological phenotypes have been reported for human HDL killing. One is defined by lysosomal membrane breakdown, cell swelling and trypanosome death [13,24,30,35,36]. The other, recently reported by Pays and co-workers, involves extensive lysosome swelling, creating a large cytosolic vacuole that is proposed to exert enough pressure on the plasma membrane that the cell ruptures

[8,10,25]. Plasma samples purified by either anti-Hpr or anti-ApoL-1 immunoaffinity produced identical morphological changes in *T. b. brucei* preceding lysis (**Figure 2C-F**). After a lag phase of approximately 20 min the overall morphology of the cells changed. Swelling was accompanied by an overall change in the shape of the cells first resulting in a kite-like appearance followed by continued swelling and rounding of the cells until the cells ruptured. The formation of a large cytoplasmic vacuole was not observed. In live cell imaging, the nucleus, nucleolus, flagella, and other organelles are visible (**Figure 2 D and F**). The morphology of *T. b. brucei* treated with intact human plasma produces the same morphological changes leading to *T. b. brucei* lysis as those seen with purified TLF (**Figure 8C**). The reason for the discrepancies between the morphological changes observed by the Pays' lab and those observed by us and others following treatment with either purified TLF or intact human serum/plasma remains unresolved.

TLF binding to Hb

Previous studies suggested Hpr, despite being highly homologous to Hp, did not bind Hb [12,30]. It is likely that Hpr-Hb complexes were not detected in these studies because of the presence of mild, non-ionic detergents in the immunoprecipitation assays. More recently, it has been shown that native and recombinant Hp and recombinant Hpr bind Hb with high affinity and that Hb-coupled Sepharose can precipitate Hpr containing HDLs from plasma [31]. Although our analysis of TLF revealed the presence of Hpr no Hb was detected in any of the purified HDL preparations. In order to determine whether native TLF and purified native Hpr could bind Hb, Surface Plasmon Resonance (SPR) analysis was performed with purified preparations of native TLF, Hpr, and Hb. TLF and Hpr both

bound immobilized Hb with high affinity (the K_d for the Hpr-Hb interaction was 2-5 nM as estimated by SPR) (**Figure 3A**). The horizontal progress of the curves representing the dissociation phase (after arrows in **Figure 3A**) indicates an almost irreversible binding of Hpr as well as TLF to Hb.

To ensure that the SPR response observed upon incubation with TLF (**Figure 3A, right panel**) could not be attributed to binding between Hb and Hpr released from the TLF particle, binding of purified TLF to Hb was also studied in a pull down assay. As revealed in **Figure 3B**, Hb-coupled Sepharose beads specifically bound the entire TLF particle containing Hpr as well as ApoL-1 and ApoA-1. Consistent with the recent finding that recombinant Hpr in complex with Hb does not bind the human Hp-Hb receptor, CD163, the complex between native Hpr and Hb did not bind to immobilized purified human CD163 in the SPR analysis (data not shown) [31,33].

Hb involvement in TLF killing of *T. b. brucei*

The observation that Hb binds to purified Hpr and TLF led us to re-investigate the role of Hb in TLF mediated killing of *T. b. brucei*. Since fetal bovine serum (FBS), a component of our standard trypanosome assay, typically contains small amounts of Hb released by hemolysis during serum preparation, we modified the standard trypanosome lysis assay to eliminate FBS (**Figure 4A**). The killing activity of TLF was drastically reduced when bovine serum albumin (BSA) was substituted for FBS in our *in vitro* lysis assay. Addition of FBS restored maximal trypanosome killing activity in the assay (**Figure 4B and C**).

To determine whether Hb was the co-factor supplied by the FBS in the *in vitro* lysis reactions we titrated Hb into the modified, serum-free, assay (**Figure 5A**).

Hemoglobin restores the killing activity of TLF while the addition of Hb alone is non-toxic to *T. b. brucei* even at very high concentrations (**Figure 5A; Supplemental Figure 1**). Furthermore, if the Hb binding protein, Hp, is added to the reactions, it inhibits lysis in a concentration dependent fashion (**Figure 5B**). Together these results indicate that Hb is a necessary co-factor for maximal TLF killing of *T. b. brucei* and that direct binding of Hb to Hpr may be required.

TLF-Hb binding to trypanosomes

Hemoglobin could stimulate trypanosome killing either a) by increasing the affinity of TLF for the trypanosome receptor, b) by playing a direct role in the lytic process, or c) by a combination of the above. To test whether Hb was necessary for trypanosome binding, Alexa Fluor 488-labeled TLF was incubated with trypanosomes at 4° C for 1 hr, cells were washed and then cell-associated fluorescence was measured by flow cytometry (**Figure 6A**). Addition of Hb increased binding of TLF to *T. b. brucei*. Maximum stimulation of binding was reached at a concentration of approximately 30 µg/ml, which represents a 1:10 molar ratio of TLF to Hb assuming an average molecular mass of 500 kDa for TLF [6]. Addition of an equimolar concentration of Hp to Hb severely inhibited binding of TLF to cells, resulting in binding levels similar to the TLF binding observed in the absence of Hb, either by sequestering all available Hb or by competition for the trypanosome TLF-Hb receptor. These experiments suggest that Hb binding to TLF

stimulates receptor mediated binding to *T. b. brucei*, and that TLF-Hb binding to the receptor is specific and saturable.

Killing of *T. b. brucei* by TLF requires trafficking of the toxin to the acidic trypanosome lysosome [22,24]. To determine whether both TLF and Hb co-localize to the lysosome, Alexa Fluor 488-conjugated TLF and Alexa Fluor 594-conjugated Hb were incubated with trypanosomes. When TLF or Hb alone was incubated with cells no intracellular fluorescence was observed (**Supplemental Figure 2A**). Co-incubation of TLF and Hb resulted in the rapid uptake and intracellular co-localization to the lysosome (**Figure 6B; Supplemental Figure 2B**). These results illustrate that TLF needs to be associated with Hb in order to be endocytosed by *T.b. brucei*, and that both TLF and Hb traffic to the lysosome of the cell.

TLF-Hb toxicity to trypanosomes

Heme is toxic to trypanosomes presumably through production of free radicals that lead to lipid peroxidation of trypanosome membranes [37]. Previous studies suggested that the toxicity of TLF might involve lysosomal membrane peroxidation [15,24,30]. Since Hb trafficks with TLF to the trypanosome lysosome, we asked whether hemoglobin could initiate a Fenton-like reaction. Fenton chemistry requires ferrous iron, hydrogen peroxide, and low pH to produce hydroxyl radicals that peroxidate lipid and can lead to membrane degradation and cell death [38]. All of these conditions are present if TLF transports Hb to the trypanosome lysosome. To test whether TLF-Hb induces a Fenton reaction, we performed lysis assays in the presence of the iron chelator deferiprone and in

the presence of the free radical scavenger DPPD (**Figure 7A and B**). Both iron chelation and the scavenging of free radicals inhibited lysis by about 50%.

To further investigate the mechanism of TLF killing we returned to morphological analysis of TLF-Hb treated trypanosomes. We have shown that TLF killing of *T. b. brucei* results in cell swelling, changes in overall cell morphology and eventually lysis (**Figure 2C-F**) [24]. Consistent with earlier electron microscopy of cells treated with gold-conjugated TLF [24,36], these results suggest that TLF-Hb causes the breakdown of lysosomal membranes. To determine whether TLF-Hb caused extensive lysosomal membrane breakdown, *T. b. brucei* was incubated with fluorescein-labeled 500kDa dextrans, which trafficked to the lysosome by bulk phase endocytosis (**Figure 7 C**). After the cells were pre-loaded with dextrans for 30 min, TLF-Hb or human plasma was added to the cells. After 30 min exposure, the fluorescent dextrans were found predominately in a single vesicle co-localizing with the lysosomal membrane marker p67. By 120 min post-treatment with either TLF-Hb or human serum, the dextrans were visible throughout the cell, while the p67 remained predominantly associated with the singular lysosome. Since the fluorescently labeled dextrans escape the lysosome in the presence of TLF, the lysosomal membrane must be open to allow release of the large dextrans. The increased intensity of the fluorescein-labeled dextrans at 120 min post TLF treatment is likely due to their release into the neutral pH of the cytoplasm [39]. Incubation with normal human plasma also resulted in release of pre-loaded dextrans into the cytoplasm prior to trypanosome lysis (**Figure 7C**). These results show that TLF-Hb causes lysosomal membrane breakdown prior to cell lysis, as does normal human serum.

Discussion

We have identified Hb as a critical co-factor in the killing of *T. b. brucei* by human TLF. Analysis of purified TLF indicates that TLF is a subclass of human HDL containing ApoA-1, and two primate specific apolipoproteins Hpr and ApoL-1. Hpr binds human Hb with high affinity and the formation of the TLF-Hb complex enhances binding to the trypanosome TLF receptor and triggers lysosome breakdown by an iron dependent, free-radical mediated pathway. Despite the role of Hb in TLF binding and toxicity no Hb is detected in TLF prepared from healthy human donors. This indicates that activation of TLF occurs upon the release of free Hb in the circulation during intravascular hemolysis.

TLF contains both Hpr and ApoL-1

A major question concerning the killing of *T. b. brucei* by human serum has centered around whether the toxin was Hpr, ApoL-1 or both of these proteins [10,14,25,40]. In this paper, the protein composition of human TLF was determined by western blot and LC-MS/MS. While several studies have previously identified protein components of this subclass of trypanolytic human HDL, all have used samples that have been subjected to lengthy purification protocols involving high-salt density ultracentrifugation [6,34]. We were concerned that these procedures would dissociate proteins from TLF that might provide information on the native composition of this innate killing factor and the mechanism of killing.

When TLF was isolated directly from freshly prepared human plasma by immunoaffinity chromatography with antibodies against either human ApoL-1 or Hpr, the major protein components were identical. The specific activity and morphology of *T.*

b. brucei lysis was also identical. This analysis indicates that Hpr and ApoL-1 are assembled into the same HDL particle and that the amount of Hpr or ApoL-1 found free in the human circulatory system is extremely low. Thus, the native toxin in humans is not ApoL-1 or Hpr alone but is an HDL particle containing both of these apolipoproteins. This is consistent with previous studies showing that Hpr and ApoL-1 were present in the same HDL and both were required for maximal trypanosome killing [14]. In addition, recent analysis of the trypanolytic activity of human serum from individuals deficient in either ApoL-1 or Hpr suggests that both proteins play distinct and important roles to achieve maximal lytic activity [40].

The role of Hb in *T. b. brucei* killing by TLF

Several early studies reported that TLF did not bind Hb [12,30] but recently it was shown that recombinant Hpr bound Hb with high affinity [31]. Consistent with these results we found that purified native TLF and purified native Hpr bound Hb with high affinity (**Figure 3**). Nevertheless, MS analysis failed to detect Hb as a component of purified TLF, most likely because the low levels of Hb that are released into the plasma of individuals with normal erythrocyte turnover are instantly bound by Hp which is present in large excess compared to Hpr (the concentration of Hp is roughly 10-100-fold higher than Hpr in normal human serum) [12,31]. The rapidly formed Hp-Hb complex is then removed by CD163. Hence, despite its high affinity for Hb, Hpr may only compete well for free Hb under physiological conditions where Hp levels were low. As we discuss below, infection by trypanosomes causes declines in Hp levels in animals [41-44].

In order to evaluate the role of Hb as a co-factor in TLF mediated killing of *T. b. brucei* we developed an *in vitro* lysis assay without FBS, a standard component of our *in vitro* trypanosome lysis assays, and a source of contaminating free Hb (**Figure 4A**). We found that substitution of BSA for FBS in lysis assays dramatically reduced the activity of TLF, but that maximal activity could be restored by addition of human Hb to the assays (**Figure 5A**).

Hemoglobin stimulation of *T. b. brucei* killing by TLF is due to both enhanced binding to the trypanosome cell surface receptor for TLF and direct cytotoxicity of lysosomally localized Hb (**Figure 6; Supplemental 2B**). The basis for the enhanced trypanosome receptor recognition of the TLF-Hb complex is unknown but the mammalian CD163 receptor shows a similar binding preference for the Hp-Hb complex [32,33]. Our studies show that not only is trypanosome binding of TLF stimulated by Hb association but that the trafficking of both TLF and Hb to the lysosome is enhanced (**Figure 6 and Supplemental Figure 2B**).

The mechanism of TLF induced cell lysis is controversial, but studies from our lab have shown that both freshly prepared normal human serum and TLF produce peroxidated lipids in *T. b. brucei*, suggesting a mechanism of killing [30]. Based on the findings reported here we propose that Hb, bound to Hpr within the TLF particle, contributes directly to the established ApoL-1-mediated toxicity of TLF [8,10,28,29,40]. Following binding and lysosomal localization Hb may contribute iron that reacts with H₂O₂ in a Fenton-based reaction that leads to free-radical formation and lysosomal membrane breakdown (**Figure 7**) [9,15,24,45]. Interestingly, earlier studies using an *in*

vitro TLF assay containing 0.2% BSA, and no Hb, did not kill *T. b. brucei* by a Fenton-based mechanism ([46]).

The morphological analysis of *T. b. brucei* treated with TLF-Hb showed cells swelling into a kite-shape prior to the bursting. We were able to directly test the permeability of the lysosomal membrane following TLF-Hb treatment by pre-loading cells with defined size (500 kDa) fluorescein-conjugated dextrans then adding TLF-Hb (**Figure 7C**). Prior to lysis, dextrans were seen throughout the cytoplasm indicating that TLF-Hb caused the lysosomal membrane to breakdown releasing the large fluorescein-conjugated dextrans. Based on the pharmacological inhibition of TLF-Hb killing by deferiprone and DPPD we postulate that membrane lipid peroxidation plays a major role in lysosomal membrane breakdown followed by cell lysis. This does not argue against a role of ApoL-1 in TLF killing of *T. b. brucei* [8,10,19,25-28]. Recombinant and native ApoL-1 are toxic to *T. b. brucei* and the evidence that it creates ion-pores in trypanosome membranes is convincing [10].

Activation of the innate TLF response by Hb

Does Hb stimulation of TLF binding and killing of *T. b. brucei* occur in humans and is this important in protection against these parasites? *In vitro* studies reported here leave little doubt that Hb association with Hpr enhances TLF binding to and killing of *T. b. brucei*. However, when TLF is isolated from normal human donors Hb is absent. This apparent paradox can be explained when one carefully considers the early physiological events associated with trypanosome infection. During infection of animals by African trypanosomes, there is substantial hemolysis, and in some cases, the hematocrit can drop

by as much as 50% [42-44]. The exact cause of hemolysis is not known, but blood cell production increases during infection, so the decrease in hematocrit is not due to decreased erythrocyte production but rather is due to hemolysis [47]. Hemolysis results in the release of large quantities of Hb, most of which will be bound and removed by circulating Hp [42,48]. During a trypanosome induced acute phase response in calves, levels of Hp are reduced to undetectable levels eight days post-infection, presumably due to removal of Hp from the circulation after Hp-Hb complexes have formed [42]. In mice, Hp levels initially increase after trypanosome infection, and then decline [48,49].

Consistent with the studies in animals, human trypanosome infections also result in substantial hemolysis and release of free Hb [50]. While Hp initially binds all free Hb the clearance of the Hp-Hb complex would result in a significant drop in Hp levels to the point where Hpr would become a major Hb binding protein. Thus, as Hp levels decrease and TLF-Hb levels increase, there maybe a substantial stimulation of TLF activity.

A second factor may influence the formation of TLF-Hb complexes in the circulation of humans. Previous studies have reported the incidence of both genetic and phenotypic ahaptoglobinemia in African populations, perhaps due to reduced severity of malaria infection in Hp negative individuals [51-53]. In some regions where malarial infections are prevalent, the frequency of ahaptoglobinemic individuals can be as high as 48% [51,54,55]. In these individuals, Hpr associated TLF could be the primary Hb binding protein.

We have previously postulated that ApoL-1 and Hpr act synergistically in *T. b. brucei* killing [14]. We now propose an avenue by which TLF activity is increased when Hpr and ApoL-1 are present in the same HDL particle. First, Hb binding to TLF

stimulates endocytosis of TLF-Hb by increased receptor affinity. Secondly, TLF-Hb provides iron that initiates Fenton chemistry leading to lysosomal membrane breakdown while ApoL-1 forms pores in membranes, disrupting the ability of the cell to regulate osmosis.

The results presented here support the hypothesis that formation of the Hpr-Hb complex within the TLF particle plays a role in human serum killing of *T. b. brucei*. Hb seems to induce increased binding of TLF to the parasite and contributes directly to toxicity in the lysosome. This suggests that parasite-induced hemolysis stimulates innate immunity against the parasite in humans.

Methods and Materials

Human serum preparation and immunoaffinity chromatography

Blood was collected from healthy human volunteers and was maintained on ice or at 4°C throughout the fractionation procedure. Plasma was separated from blood cells by centrifugation at 3500 rpm in a SLA-3000 rotor (Sorvall) for 10 min at 4°C. The plasma supernate was removed and re-centrifuged in an SS-34 rotor (Sorvall) at 9500 rpm for 10 min at 4°C to remove any residual blood cells. Plasma was kept on ice for no more than three hr prior to antibody affinity chromatography.

Monoclonal antibodies were raised against TLF particles and the specificity of the antibodies for Hpr and ApoL-1 has been previously described [14]. The monoclonal antibodies were purified from mouse ascites by Protein G affinity chromatography according to manufacturers recommendations (Pierce Biotechnology, Rockford, IL, USA). Affinity purified polyclonal antibodies against human ApoA-1 were purchased from Rockland Immunochemicals, Inc. (Philadelphia, PA, USA). The three antibodies (200 µgs) were coupled to Affigel 15 (50 µl) (BioRad, Hercules, CA, USA) according to the manufacturers recommendations and 100 µl of the AffiGel slurry (corresponding to approximately 200 µgs of antibody) was transferred to 1.5 ml microfuge tubes. One ml of human plasma was added to each antibody containing tube and incubated for 30 min at 4°C on a rotating platform. AffiGel/antibody resin was recovered by centrifugation for 90 sec at 1000 x g at 4°C and the immuno-depleted plasma discarded. Additional 1 ml samples of plasma were added to each sample and immuno-depletion steps were repeated until 5 ml of plasma had been treated. The AffiGel/antibody resin was then washed seven times with 1 ml of PBSE (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 10 mM

KH₂PO₄, 3 mM EDTA). Following the washes, the AffiGel/antibody resin was treated with 200 µl of 100mM glycine, pH 2.0 for 5 min at 4°C to elute bound human plasma proteins. The eluate was dialyzed against PBSE at 4°C prior to analysis for protein composition and trypanolytic activity.

Western blot analysis of TLF

Following elution from anti-ApoL-1, anti-Hpr and anti-ApoA-1 AffiGel resin, samples were precipitated with 100% ice-cold acetone and proteins were separated on a 10% SDS-polyacrylamide gel under non-denaturing conditions. Gels were either coomassie stained or western blotted onto nitrocellulose. For western blot analysis membranes were blocked with 5% milk in TTBS (20mM Tris, 500mM NaCl, 0.05 % Tween-20) and were probed with antibodies diluted with 5% milk in TTBS: Hpr (1:10,000), ApoL-1 (1:10,000), ApoA-1 (1:5000), Hb (1:2000) and transferrin-Hrp (1:2000). After incubation with secondary antibodies (goat anti-rabbit and goat anti-mouse at 1:10,000 in all cases except transferrin)(Pierce Biotechnology, Rockford, IL, USA) proteins were detected following reaction with ECL Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) and were visualized by autoradiography. Protein concentrations were determined by Bradford assay (BioRad, Hercules, CA, USA).

LC-MS/mass spectroscopy

For mass spectroscopy analysis, protein bands were excised from coomassie stained gels, trypsin digested, and fractionated by reverse phase chromatography (C-18 PepMap100, 75µm ID x 15mm, 3µm particle size, LC Packings/Dionex, Sunnyvale, CA, USA). The

column eluate was introduced onto a QSTAR XL mass spectrometer (Applied Biosystems, Foster City, CA and Sciex, Concord, Ontario, Canada) by electrospray ionization. Ions were selected and fragmented using a standard information dependent acquisition method. An ion had to be assigned a charge in the +2 to +4 range to be considered a candidate for fragmentation. Identification of proteins was performed using ProID software (Applied Biosystems, Foster City, CA) and experimental spectra were matched against *in silico* trypsinizations of the NCBI non-redundant database.

***T. b. brucei* cultivation and lysis assays**

Bloodstream *T. b. brucei* (Lister strain 427, MiTat 1.2) were cultured in HMI-9 media supplemented with 10% fetal bovine serum (FBS; heat inactivated, 58°C for 30 min). Trypanolytic activity of fractionated HDLs was determined by *in vitro* microscopic assays as previously described [6]. All samples were dialyzed exhaustively against PBSE, at 4°C, prior to incubation with the parasites (1×10^7 cells per 300 μ l assay) for 2 hr at 37°C in HMI-9 media containing 10% FBS. To determine whether FBS contributed to TLF mediated killing of *T. b. brucei* some lysis assays contained 1% bovine serum albumin (BSA) and 1% glucose without intact FBS. Hb A₀ and Hp 1-1 were obtained from Sigma-Aldrich. In this paper and in previous papers, we define a lytic unit of activity as the amount of material necessary to lyse 50% of the parasites, in a 300 μ l assay containing 1×10^7 cells after incubation for 2 hr at 37°C [8]. The iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (deferriprone) was used in the chelation inhibition studies and N,N'-diphenyl-1,4-Benzenediamine (DPPD) was the antioxidant used.

Cell imaging

Lysis of live parasites was visualized by the addition of TLF to *T. b. brucei* imbedded in 1% low melting point agarose (Sigma) made with PBSE containing 1% glucose following 2 hr of incubation at 37°C. Samples were prepared for microscopy by the addition of 10 µl of low melting point agarose to 10 µl of *T. b. brucei* at a final concentration of 1×10^7 /ml. Both the agarose and trypanosome samples were maintained at 37°C, gently mixed, and transferred to a microscope slide. To further reduce the motility of trypanosomes, the slide was chilled at 4°C for 10 min and then imaged using a motorized Zeiss Axioplan2 and an MRm camera interfaced with the Axiovisions 4.4 software (Zeiss, Germany). Methanol-fixed samples were also visualized. Cells incubated with TLF samples as above were washed with PBSE containing 10% FBS, then resuspended and smeared on a microscope slide. Slides were rapidly air dried, and cells were fixed for 5 min in methanol (-20°C). Following methanol fixation, slides were air dried and mounted with 4, 6 diamidino-2-phenylidole (DAPI) containing antifade reagent ProlongGold (Invitrogen, Carlsbad CA, USA) and imaged as above.

Lysosomal morphology studies

Trypanosomes were resuspended at a concentration of 1×10^7 /ml in F12-FBS were incubated with 300 µg/ml 500 kDa fluorescein labeled dextrans (Invitrogen, Carlsbad, CA) for 30 min at 37°C. Cells were then washed three times with 1X PBSE and were incubated at 3×10^6 /ml in F12-FBS with either 2 units of normal human serum (1.7% total human serum) or 2 units of purified TLF (0.017% TLF) and cells were examined after 30 min or 2 hr. Aliquots were treated with 0.001% formaldehyde on ice for 5 min,

rinsed with PBS, and resuspended in PBS-10% FBS. Cells were then smeared onto a slide and air-dried. For co-localization with anti-p67, cells were rehydrated in PBS-10% FBS and incubated in 1:1000 anti-p67 (a gift of Dr. Jay Bangs, University of Wisconsin, Madison, WI) for 1 hr at room temperature, followed by staining with a goat-anti-mouse secondary antibody labeled with Alexa Fluor-594 (Invitrogen, Carlsbad, CA). Slides were rinsed in PBS containing 1% glucose, air dried, and viewed using a motorized Zeiss Axioplan2 and an MRm camera interfaced with the Axiovisions 4.4 software (Zeiss, Germany).

Hb binding

Surface Plasmon Resonance (SPR) analysis was conducted essentially as described in [31] except that native TLF and Hpr were used in the binding assays instead of recombinant Hpr. Hpr and TLF were purified from fresh human plasma using the two step purification method [14]. In the case of Hpr purification, human HDLs were solubilized using 10mM CHAPS and purified using an anti-Hpr column as described [14]. The K_d for the Hpr-Hb interaction was estimated by the BIAevaluation 4.1 software using a Langmuir 1:1 binding model.

For precipitation experiments, purified TLF was incubated with Hb-coupled Sepharose, BSA-coupled Sepharose, or underivatized Sepharose. After extensive washing in a solution containing 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Hepes, and 140 mM NaCl, pH 7.8, bound proteins were eluted in SDS-containing sample buffer and visualized by SDS-PAGE.

TLF binding and localization

Binding studies of TLF to trypanosomes was conducted with Alexa Fluor 488 (Invitrogen, Carlsbad, CA) labeled TLF, and Hb and Hp noted above. Trypanosomes were incubated with labeled TLF, unlabeled Hb, and Hp at 4°C for 1 hr. The cells were then washed three times at 1400(xg) for 7 min at 4°C. The cells were resuspended in 2% formaldehyde at 4°C until measured by flow cytometry using the CyAn ADP (Dako, Ft. Collins, CO) and analyzed using FlowJo software (Tree Star, Inc. Ashland, Oregon). Immunofluorescence microscopy was employed to localize TLF and Hb in the cell. Alexa Fluor 488 (Invitrogen, Carlsbad, CA) labeled TLF and Alexa Fluor 594 (Invitrogen, Carlsbad, CA) labeled Hb were incubated with trypanosomes pre-incubated with 50 μ M chloroquine for 30 min. TLF and Hb were added at concentrations noted in Figure 7. LysoTracker Red DND99 (Invitrogen, Carlsbad, CA) was used to identify the lysosome. These studies were done on a Zeiss Axioplan and the images analyzed using IPLab Spectrum version 3.9.4r2 from Scanalytics-BD Biosciences (Rockville, MD, USA)

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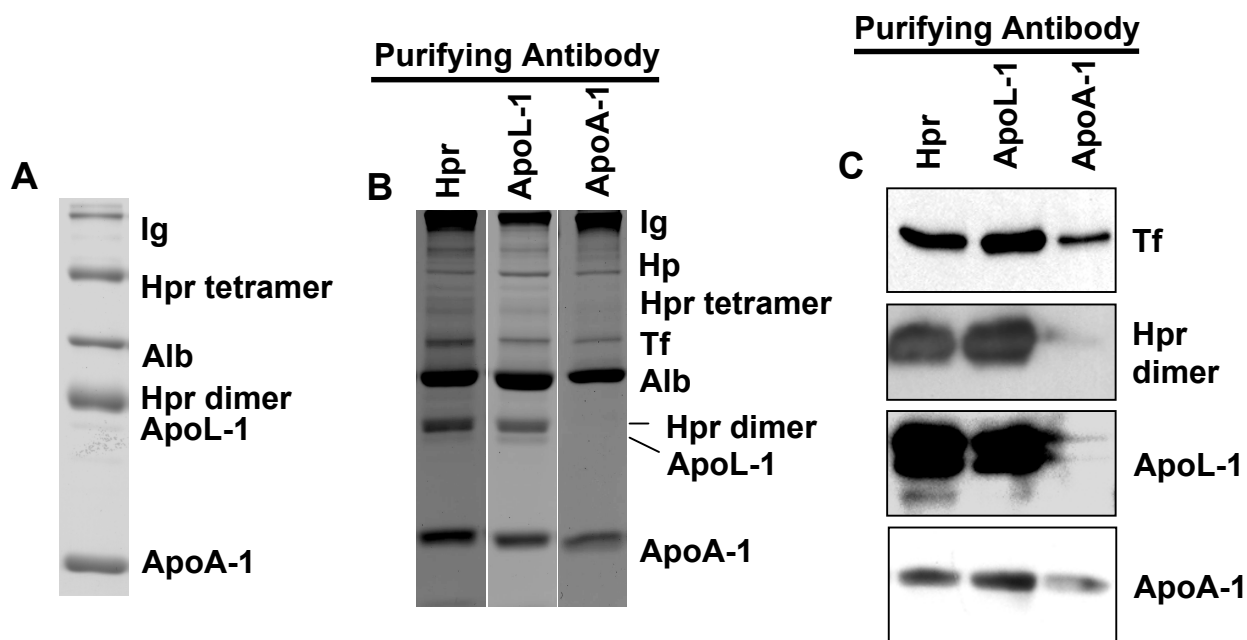


Figure 1. Analysis of differentially purified human serum HDLs. **(A)** TLF analyzed by non-reducing 10% SDS-PAGE and coomassie brilliant blue staining after purification by a two-step purification process that includes high-salt density centrifugation and antibody affinity chromatography with anti-Hpr. The presence of albumin (Alb) and immunoglobulin (Ig) are indicated. **(B)** HDL purified from serum using a single-step affinity purification method with antibodies to ApoL-1, Hpr, or ApoA-1 and analyzed by non-reducing 10% SDS-PAGE and stained with coomassie brilliant blue. **(C)** The same gel as in **(B)** transferred to nitrocellulose and probed with antibodies to transferrin (Tf), Hpr, ApoL-1 and ApoA-1.

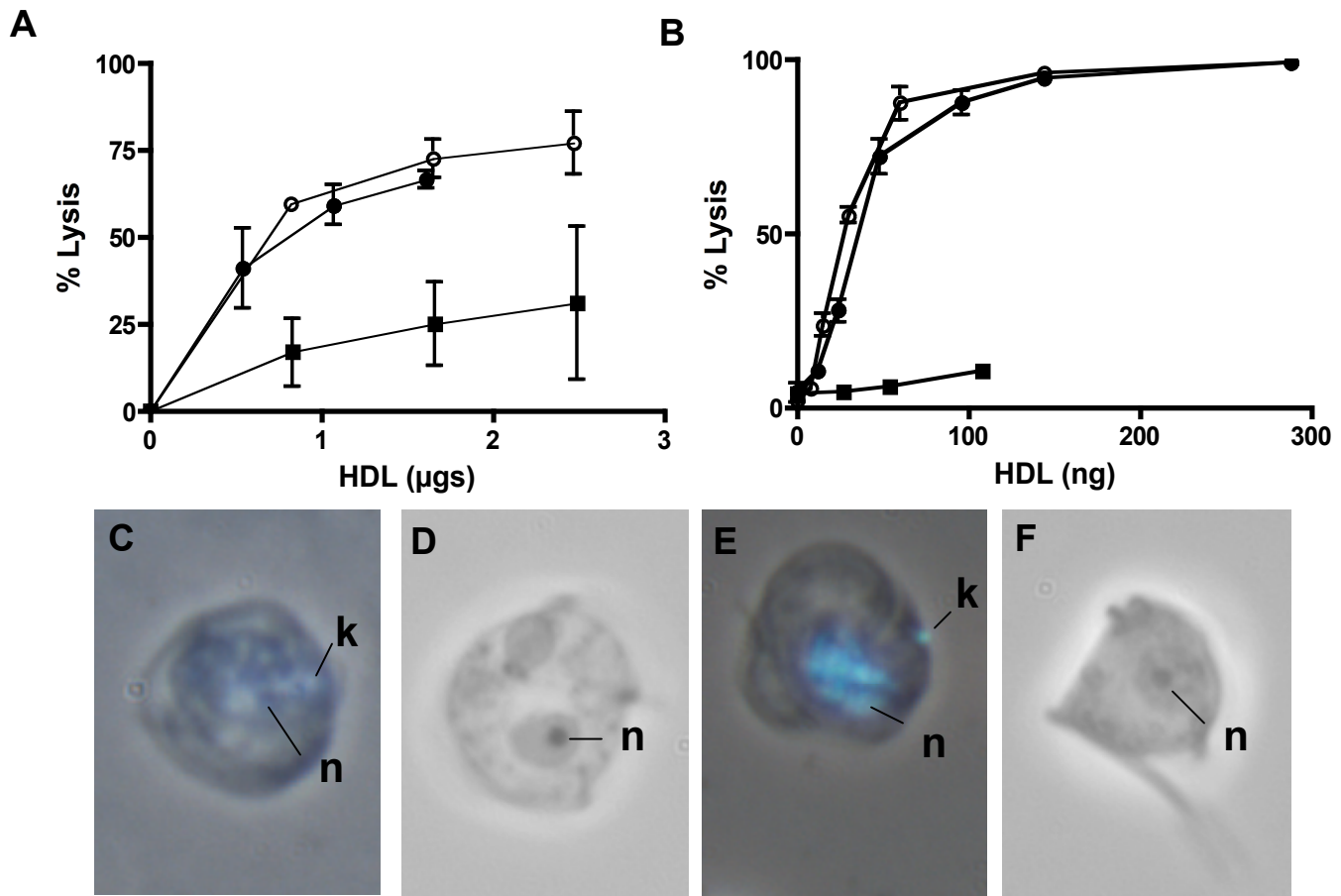


Figure 2. Lysis and morphology of *T. b. brucei* killed with purified HDL. **(A)** Human HDLs were purified by a single-step immunoaffinity method with either anti-Hpr, anti-ApoL-1 or anti-ApoA-1. The specific activity for *T. b. brucei* killing was similar for TLF purified with the single-step protocol with either anti-Hpr (closed circles) or anti-ApoL-1 (open circles) (~0.5 µg/ml killed 40% of the trypanosomes in our standard assay). The specific activity of samples purified with single-step immunoabsorption with anti-ApoA-1 (closed squares) was over 10 fold reduced in specific activity for *T. b. brucei* killing. **(B)** TLF purified by a two-step method using both high-salt density gradient ultracentrifugation and immunoaffinity absorption. TLF first subjected to high-salt gradient ultracentrifugation was 10 fold more toxic to *T. b. brucei* than TLF purified by the single-step immunoabsorption directly from serum. Similar specific activities were obtained when the second immunoaffinity step was conducted with either anti-Hpr (closed circles) or anti-ApoL-1 (open circles). The specific activity of HDLs purified with anti-ApoA-1 (closed squares) was over 100 fold lower. **(C-F)** Cell morphology of *T. b. brucei* treated with TLF purified by single-step immunoaffinity with anti-ApoL-1 or anti-Hpr. Following incubation at 37°C for 2 hrs, cells were either smeared on microscope slides and methanol fixed (**C and E**) or directly viewed following imbedding in 1% agarose (**D and F**). Incubation with TLF purified either by anti-Hpr (**C and D**) or anti-ApoL-1 (**E and F**) immunoabsorption showed similar morphologies. Prior to lysis all cells progressed through a series of morphological changes that included transition to a “kite-shaped” form and continued swelling until lysis. The position of the kinetoplast (k) and nucleus (n) was determined by DAPI staining in the fixed preparations (C and E). The nucleus (n) was clearly visible in the unfixed cells (D and F).

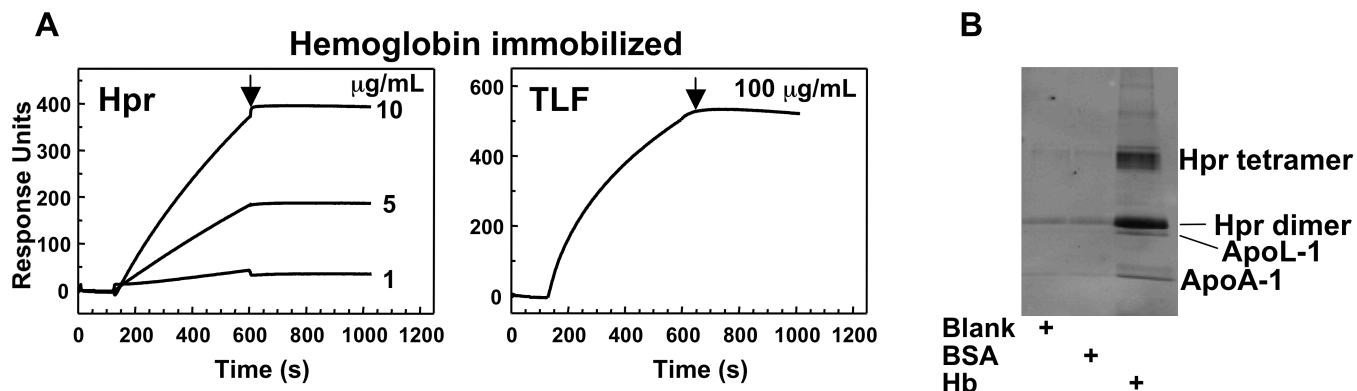


Figure 3. Binding of purified Hpr and TLF to Hb. **(A)** SPR analysis of the binding of purified Hpr (left panel) and TLF purified by the two-step procedure (right panel) to immobilized Hb A₀. The concentration of Hpr and TLF is shown to the right of the binding curves. The BIAevaluation 4.1 software estimated a K_d of 2-5 nM for the Hpr-Hb complex. Arrows indicate the beginning of the dissociation curves. **(B)** Silver-stained SDS-polyacrylamide (8-16% gradient) gel of proteins eluted from Hb-coupled Sepharose (Hb), BSA-coupled Sepharose (BSA), or underivatized Sepharose (blank) after incubation with purified TLF.

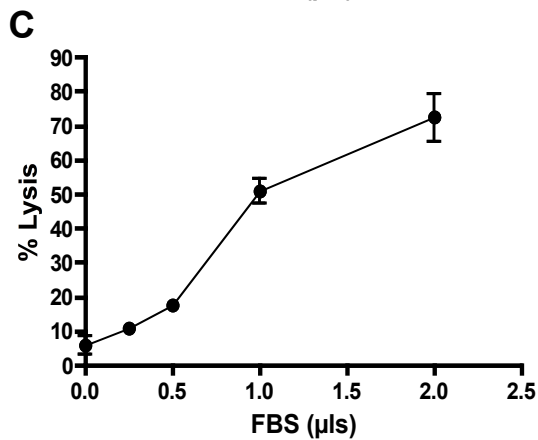
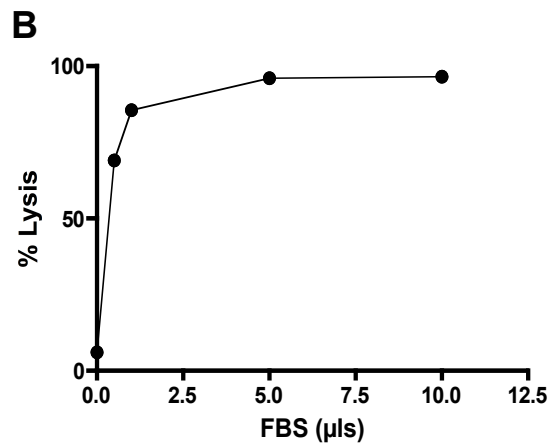
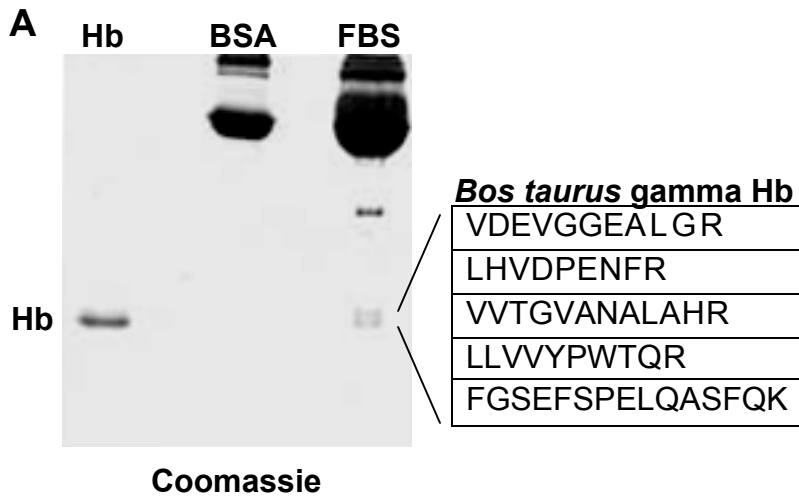


Figure 4. Effect of FBS on TLF killing of *T.b. brucei*. **(A)** FBS (1μl), BSA (1μg), and Hb (1.0μg) were fractionated on 15% SDS-PAGE gels and stained with coomassie. The presence of Hb in FBS was verified by mass spectroscopy of the appropriate sized bands. Both *Bos aaurus* alpha and gamma subunits of Hb were identified and five of the gamma globin tryptic fragments are shown. **(B)** FBS is required for maximum *in vitro* lysis of *T. b. brucei* with highly purified TLF. Titration of FBS into a 300 μl assay containing 1% BSA. Addition of 5 μl FBS restored lytic levels to those seen in standard lysis assays [8]. **(C)** The effect of FBS addition is concentration dependent. TLF used in these studies was purified by the two-step procedure.

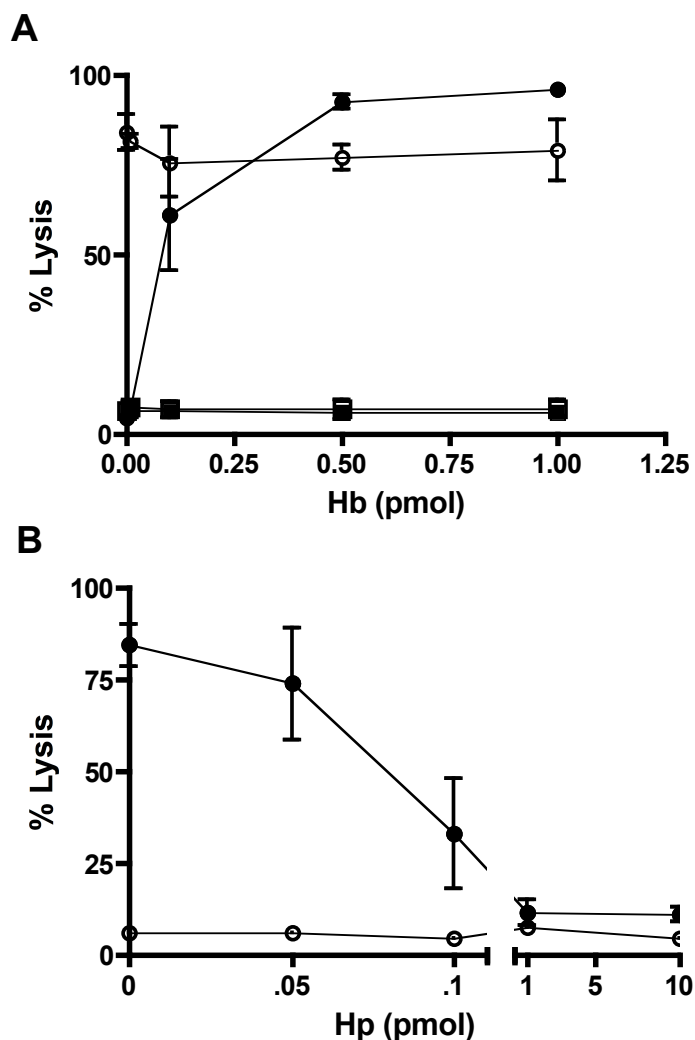


Figure 5. Hemoglobin is the co-factor in FBS necessary for maximal TLF killing of *T. b. brucei*. **(A)** Titration of Hb into *in vitro* lysis reactions containing highly purified TLF. Samples contained: TLF (1.5 units) in 10% FBS (open circles); TLF (1.5 units) in 1% BSA and 1% glucose (closed circles); Hp in 1% BSA and 1% glucose without TLF (closed squares); and no TLF (Hb only) (open squares). In the presence of TLF, Hb addition results in a concentration dependent increase in *T. b. brucei* killing. No increase is seen when Hb is added to FBS containing reactions because FBS contains approximately 0.5 $\mu\text{g}/\mu\text{l}$ of Hb **(B)** Addition of Hp inhibits Hb dependent killing by TLF. Hp was titrated into reactions containing TLF (1.5 units), 1% BSA and 1% glucose, and 0.1pmol of Hb (closed circles). Addition of Hp and Hb (0.1pmol) to *T. b. brucei* in the absence of TLF had no effect on cell viability (open circles). TLF used in these studies was purified by the two-step procedure.

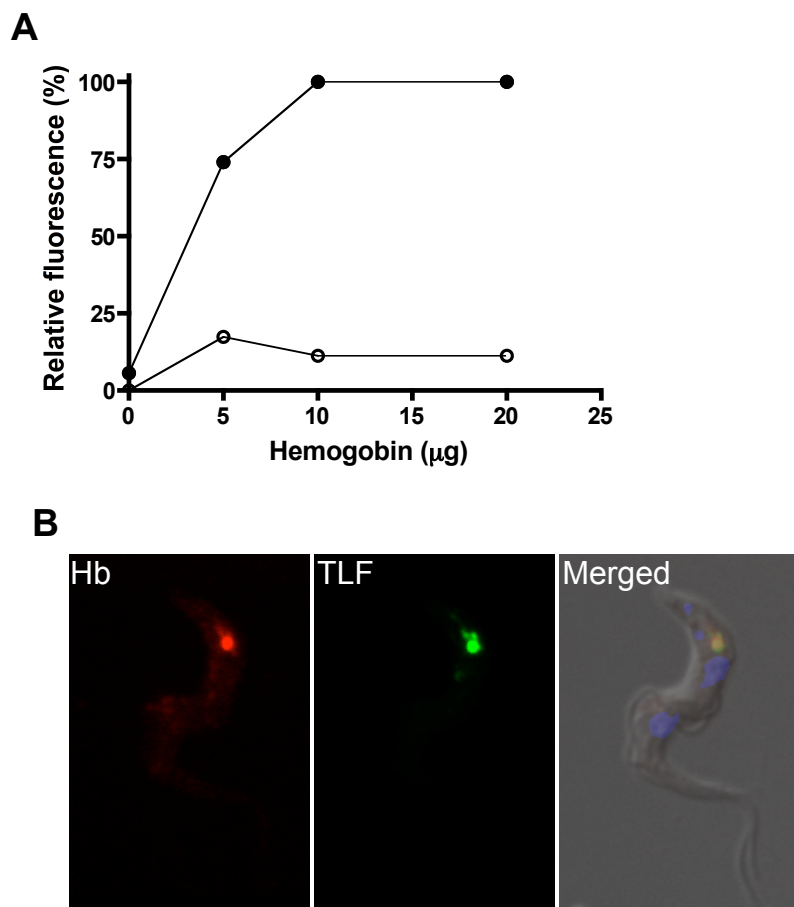


Figure 6. Hemoglobin is required for binding and uptake of TLF. **(A)** Alexa Fluor 488- labeled TLF was incubated with trypanosomes and increasing amounts of Hb. Binding was measured by flow cytometry and the relative mean fluorescence intensity determined following binding for 1 hr at 4°C. In the absence of Hp, there is a dose dependent increase of binding of Alexa Fluor 488-labeled TLF in the presence of Hb (closed circles). Addition of Hp inhibited TLF-Hb binding to *T. b. brucei* (open circles). **(B)** Uptake of TLF-Hb by *T. b. brucei*. Trypanosomes were pretreated with chloroquine for 30 min to block acidification of the lysosome and activation of TLF, followed by the addition of Alexa Fluor-594 labeled Hb and Alexa Fluor 488-labeled TLF. Cells were incubated for an additional hr at 37°C. The TLF-Hb complex was endocytosed and trafficks to the lysosome (**Supplemental Figure 2B**). TLF used in these studies was purified by the two-step procedure.

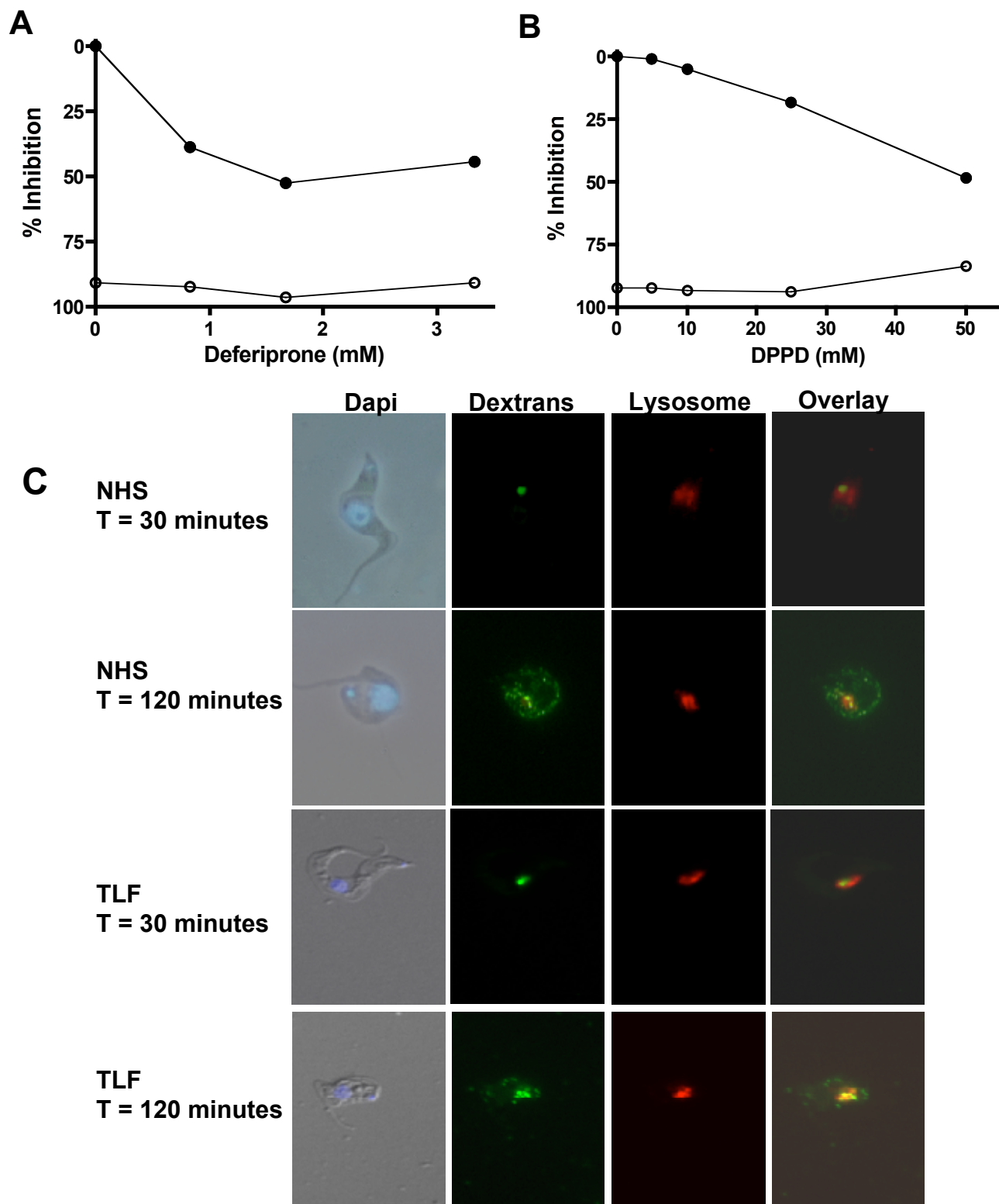
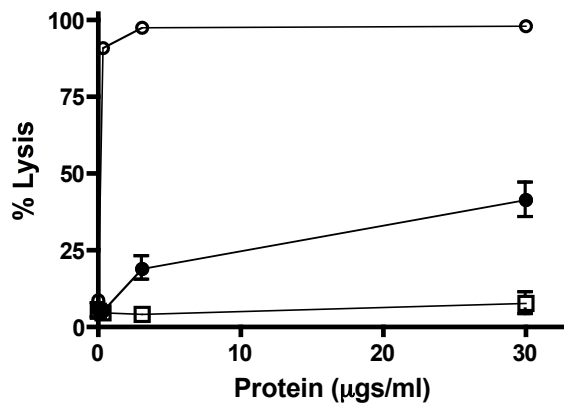
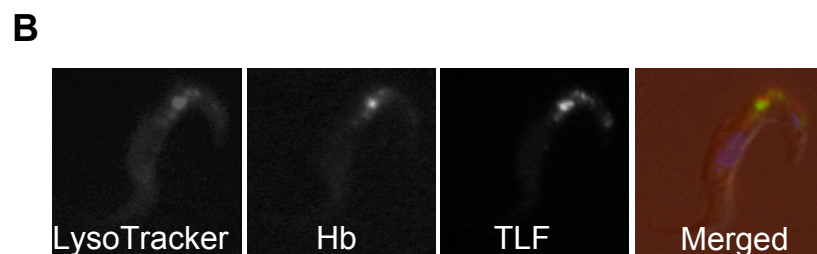
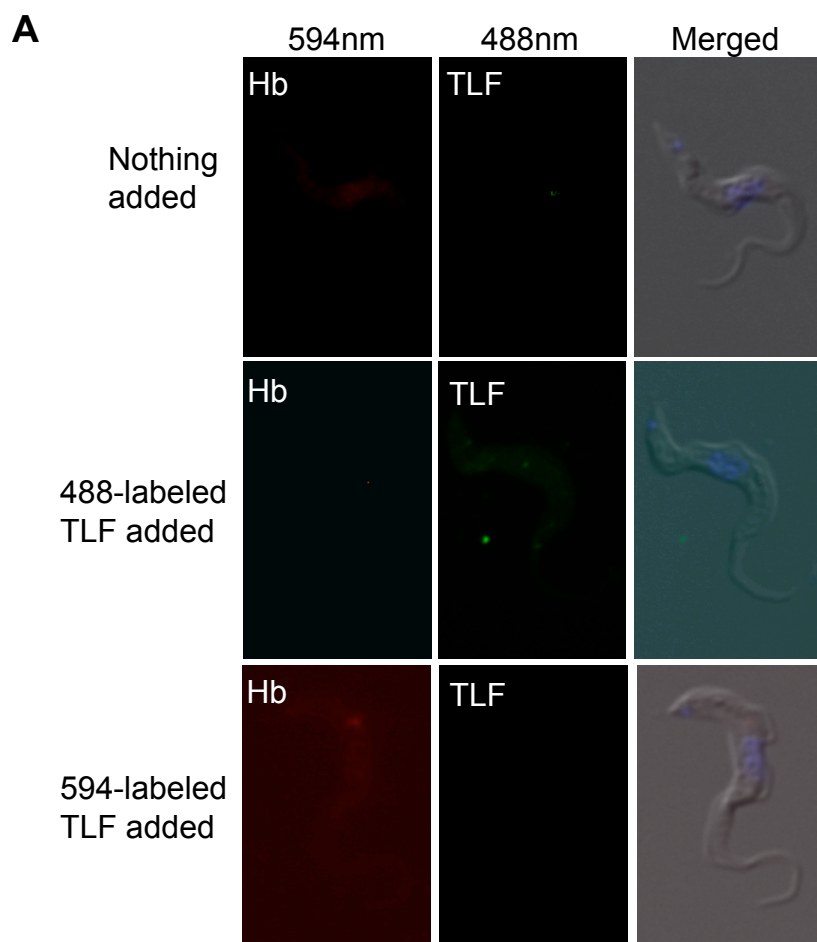


Figure 7. TLF-Hb indices iron dependent lysosomal membrane breakdown.
Continued on next page.

Figure 7. TLF-Hb induces iron dependent lysosomal membrane breakdown. **(A)** *T. b. brucei* was treated with increasing concentration of the iron chelator, deferiprone, in the presence (closed circles) and absence (open circles) of TLF-Hb. The inhibition of trypanosome lysis by deferiprone is indicated. We normalized the data to indicate that the addition of no deferiprone gave zero inhibition of TLF-Hb lysis after 2 hrs at 37°C. **(B)** Trypanosomes were treated with increasing concentrations of the antioxidant DPPD, in the presence (closed circles) or absence (open circles) of TLF-Hb. The inhibition of trypanosome lysis by DPPD is indicated. We normalized the data to indicate that the addition of no DPPD gave zero inhibition of TLF-Hb lysis after 2 hrs at 37°C. **(C)** Effect of human serum and TLF-Hb treatment on *T. b. brucei* lysosomal membrane structure. In order to label the lysosome with a size selective membrane permeable marker *T. b. brucei* was preincubated for 30 min at 37°C with fluorescein conjugated dextrans (500 kDa). Normal human serum or purified TLF-Hb was then added. After 30 or 120 min cells were formaldehyde fixed and stained with anti-p67 to localize the lysosomal membrane. After treatment with normal human serum or TLF-Hb for 30 min dextrans are localized to a single cytoplasmic structure that stained with an antibody against the lysosomal marker p67. Trypanosomes treated for 120 min with either normal human serum or TLF-Hb continued to show p67 staining largely restricted to a single structure in the cell but the 500 kDa dextrans are widely distributed in the cells indicating a breakdown in lysosomal membranes. TLF used in these studies was purified by the two-step procedure.



Supplemental Figure 1. Hemoglobin is necessary for maximal TLF killing of *T. b. brucei*. Trypanosome killing by TLF in the presence (open circles) and absence (closed circles) of Hb. Hb alone is not toxic to *T. b. brucei* at the concentrations tested (open squares). TLF used in these studies was purified by the two-step procedure.



Supplemental Figure 2. Neither Hb nor TLF alone are efficiently taken up by *T. b. brucei*. **(A)** Alexa Fluor-594 labeled Hb or Alexa Fluor 488-labeled TLF was incubated with *T. b. brucei* for one hr at 37°C. No uptake of was seen at either 488nm or 594nm excitation. **(B)** When cells were co-incubated with TLF and Hb, both TLF and Hb can be seen in the trypanosome lysosome by co-localization with LysoTracker. TLF used in these studies was purified by the two-step procedure.

Table 1. Mass Spectroscopy Analysis of TLF

Identified Protein ¹	Peptide Sequence ²	Purifying antibody ³
Hp	DIAPTLTLYVGK	Anti-Hpr, Anti-L1
	GSFPWQAK	Anti-Hpr, Anti-L1, Anti-A1
	ILGGHLDK	Anti-A1
	SCAVAEYGVYVK	Anti-Hpr
	SPVGVQPILNEHTFCAGMSK (unique to Hp)	Anti-Hpr, Anti-L1
	VGYVSGWGR (unique to Hp)	Anti-Hpr, Anti-L1, Anti-A1
	VOPICLPSK (unique to Hp)	Anti-Hpr
	VOPICLPSKDYAEVGR (unique to Hp)	Anti-Hpr
	VTSIQDWWQK	Anti-Hpr, Anti-L1, Anti-A1
	VTSIQHWWQKTIAEN	Anti-L1 (a variant)
Hpr	AVGDKLPECEAVCGKPK	Anti-L1
	DIAPTLTLYVGK	Anti-Hpr, Anti-L1
	GSFPWQAK	Anti-L1
	ILGGHLDK	Anti-L1
	LRTEGDGVYTLNDK	Anti-L1
	SCAVAEYGVYVK	Anti-Hpr, Anti-L1
	SPVGVQPILNEHTFCVGMMSK (unique to Hpr)	Anti-Hpr, Anti-L1
	TEGDGVYTLNDK	Anti-L1
	VGYVSGWGQSDNFK (unique to Hpr)	Anti-L1
	VMPICLPSK (unique to Hpr)	Anti-Hpr, Anti-L1
	VTSIQDWWQK	Anti-L1
	VVLHPNYHQVDIGLIK (unique to Hpr)	Anti-Hpr, Anti-L1
	YVMLPVADQYDCITHYEGSTCPK	Anti-L1
ApoL1	ANLQSVPHASASRPR	Anti-Hpr, Anti-L1
	ILQADQEL	Anti-L1
	LKSELEDNIR	Anti-L1
	LNILNNNYK	Anti-L1
	VNEPSILEMSR	Anti-Hpr, Anti-L1
	VTEPISAESGEQVER	Anti-Hpr, Anti-L1
	VTEPISAESGEQVERVNEPSILEMSR	Anti-Hpr
Tf	DGAGDVAFVK	Anti-L1
	ADRDQYELLCLDNTR	Anti-Hpr
	ASYLDCIR	Anti-Hpr
	DLLFRDDTVCLAK	Anti-Hpr
	EFQLFSSPHGK	Anti-Hpr, Anti-L1
	FDEFFSEGCAPGSK	Anti-Hpr
	EGYYGYTGAFR	Anti-L1
	HQTVPQNTGGKNPDPWAK	Anti-L1
	HSTIFENLANK	Anti-Hpr, Anti-L1
	SVIPSDGPSVACVK	Anti-Hpr
	YLGEEYVK	Anti-Hpr
	LHDRNTYEK	Anti-L1
	MYLGYEYVTAIR	Anti-L1
	SASDLTWDNLK	Anti-L1

¹The identified proteins.²The sequences for each tryptic peptide fragment.³The antibody purification method from which the fragments were identified by tandem LC-MS/MS.

Discussion

TLF assembly

Hpr and ApoL-1 are co-assembled into the same HDL. How HDL subclasses are manufactured and maintained is not well understood [40,41,43,45,46]. Several HDL receptors localize to lipid rafts, including ABCG1, and Sr-b1 receptors [56]. These receptors are important for lipid exchange between HDL and the cell surface [57]. This cell-mediated lipid exchange has been termed “remodeling.” While remodeling of HDL typically refers to the lipid content of HDL, the protein content is altered as well.

Phospholipid transfer protein (PLTP), for example, removes ApoA-1 from the HDL molecule and promotes fusion or destabilization of the molecules [58]. This illustrates the yin-yang relationship between lipid and protein. As nascent HDL collect cholesterol, LCAT is recruited and esterifies the cholesterol. As the HDL matures, the conformation of ApoA-1 changes [46]. Alterations in the lipid content alter protein conformations and content, while changes in the protein content will have consequences on lipid structure and content. PON1 is one such protein and is involved in maintaining the lipid content of HDL and may be upregulated during oxidative stress [47,59].

PON1 is secreted without cleavage of its signal peptide [49,50]. Addition of HDL to cells expressing PON1 results in HDL scavenging of PON1 from the cell surface, suggesting that both lipid and protein remodeling of HDL occurs at “remodeling centers” on the cell surface [52]. If the PON1 signal peptide is removed, the protein does not associate with HDL or lipid [51].

Similar to PON1, Hpr localizes to the cell surface with its signal peptide uncleaved and is scavenged by HDL [12]. Co-localization of Hpr and ApoL-1 on the cell

surface may facilitate the co-assembly in HDLs at remodeling centers. While Hpr and ApoL-1 do not interact directly, they may be sorted to lipid rafts and scavenged together from the cell surface. Caveolae / lipid rafts are involved in protein sorting [60,61]. Caveolin begins to oligomerize in the ER, and recent studies indicate that mature caveolae develop in the Golgi and traffic as rafts to the cell surface [62]. We propose that Hpr and ApoL-1 are translated into the ER, and as caveolin oligomerizes to form nascent rafts and begins to recruit additional proteins to the rafts, Hpr and ApoL-1 co-localize. Association with Hpr-containing rafts prevents ApoL-1 from being secreted. Once the raft is presented on the cell surface, Hpr and ApoL-1 are available for HDL scavenging (Figure 1).

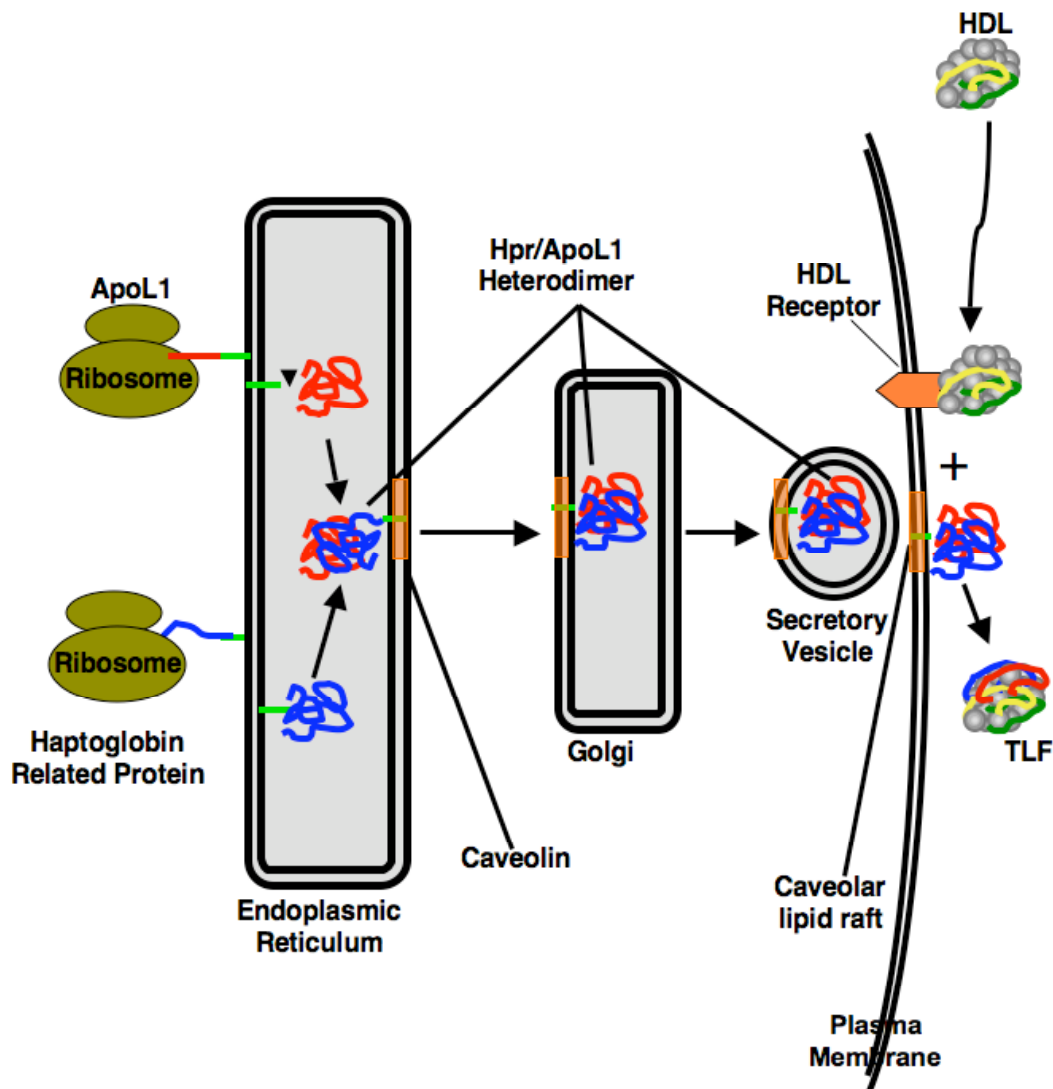


Figure 1. TLF biogenesis pathway. ApoL-1 and Hpr are translated into the ER. The proteins may associate while folding in the ER but the proteins do not have direct interactions under the conditions tested. Hpr contains two mutations at the signal sequence cleavage site that prevent cleavage of the signal peptide and are likely the reason Hpr remains membrane associated. Hpr and ApoL-1 localize with nascent Caveolar/lipid rafts and traffic through the secretory pathway. Once they are presented on the cell surface, Hpr and ApoL-1 are removed by HDL.

TLF killing of trypanosomes

TLF confers sterile immunity against *T.b.brucei* infection. TLF, however, does not kill trypanomes in the absence of Hb. In normal, healthy individuals, Hb levels are low because Hp rapidly binds Hb and the complex is removed from circulation [63]. During severe anemia, serum Hb levels increase and Hp-Hb complexes are cleared by scavenger receptors leading to reduction in serum Hp concentrations. [64,65]. In the absence of Hp, Hpr associated with TLF is the primary Hb binding protein (Figure 2).

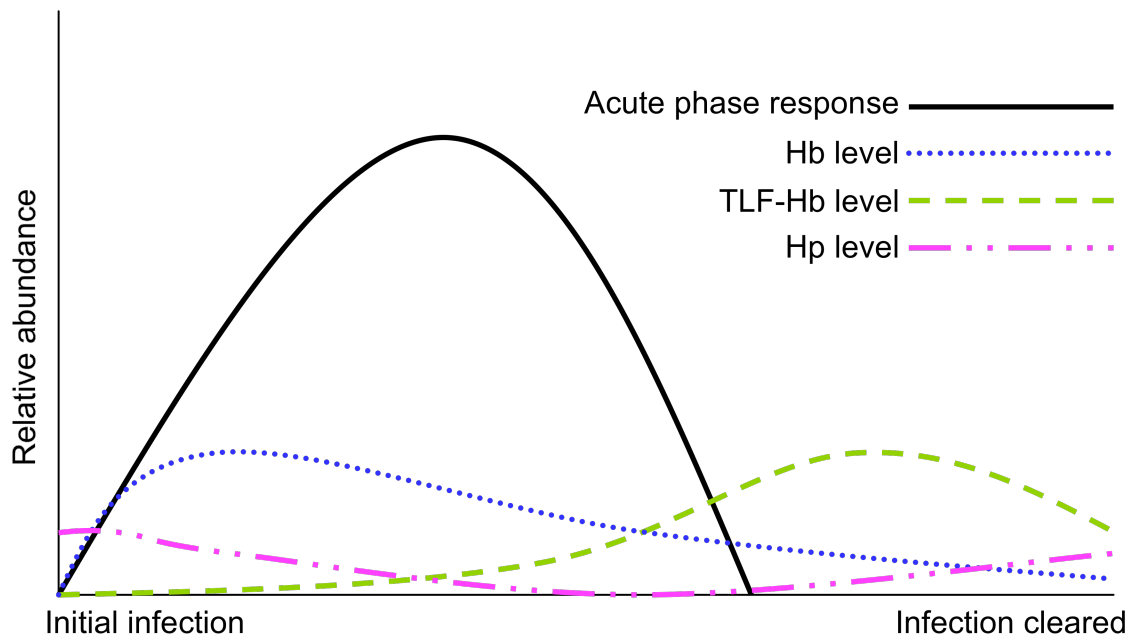


Figure 2. Hypothetical effect of *T.b. brucei* infection in humans on serum protein levels. During an acute phase response (solid black), severe hemolysis results in significant levels of free hemoglobin (blue dot). Hp (pink dash-dot) rapidly binds to and clears Hb from the circulation and levels of Hp soon drop. At this point TLF binds Hb (green dash) and the trypanosome infection is rapidly cleared. This figure loosely represents data compiled from references 60-69.

Acute anemia is a symptom of trypanosome infection in animals and humans. In animals, hematocrit levels can drop by as much as 50% [66,67]. Hp levels decrease after trypanosome-induced hemolysis in cattle [68]. Human trypanosome infections likewise have severe anemia and hemolysis [69,70,71]. In goats infected with *T.b. brucei*, mean packed cell volume begins to decline almost immediately after infection, and by day 12 is decreased by at least 33% [72]. This suggests that hemolysis induced by the acute phase response begins early in *T.b. brucei* infection. Release of hemoglobin into the circulation leads to increased binding to Hp (Figure 3A). This complex is then removed from the circulation, which leads to a decrease in Hp, an inhibitor of trypanosome killing by TLF (Figure 3B). The remaining free Hb then binds to TLF, allowing the trypanosome receptor to recognize the toxin (Figure 3C).

The only known function of TLF is as a trypanoside. TLF contains two trypanolytic proteins, Hpr and ApoL-1. Neither are efficient toxins by themselves, and it is critical that both proteins be packaged in the same HDL for proper function. One remaining question is what was the evolutionary pressure selecting for the association between ApoL-1, Hpr and HDL? Is trypanosomiasis pressure enough? Perhaps not, but TLF is armed by the acute phase response, and recent evidence suggests that TLF is toxic not only to trypanosomes, but also inhibits malarial growth, and is toxic to *Leishmania* [73,74]. If TLF is a general anti-parasitic agent, the selective pressure is amplified many fold and suggests a broader role in the function of TLF that needs to be understood.

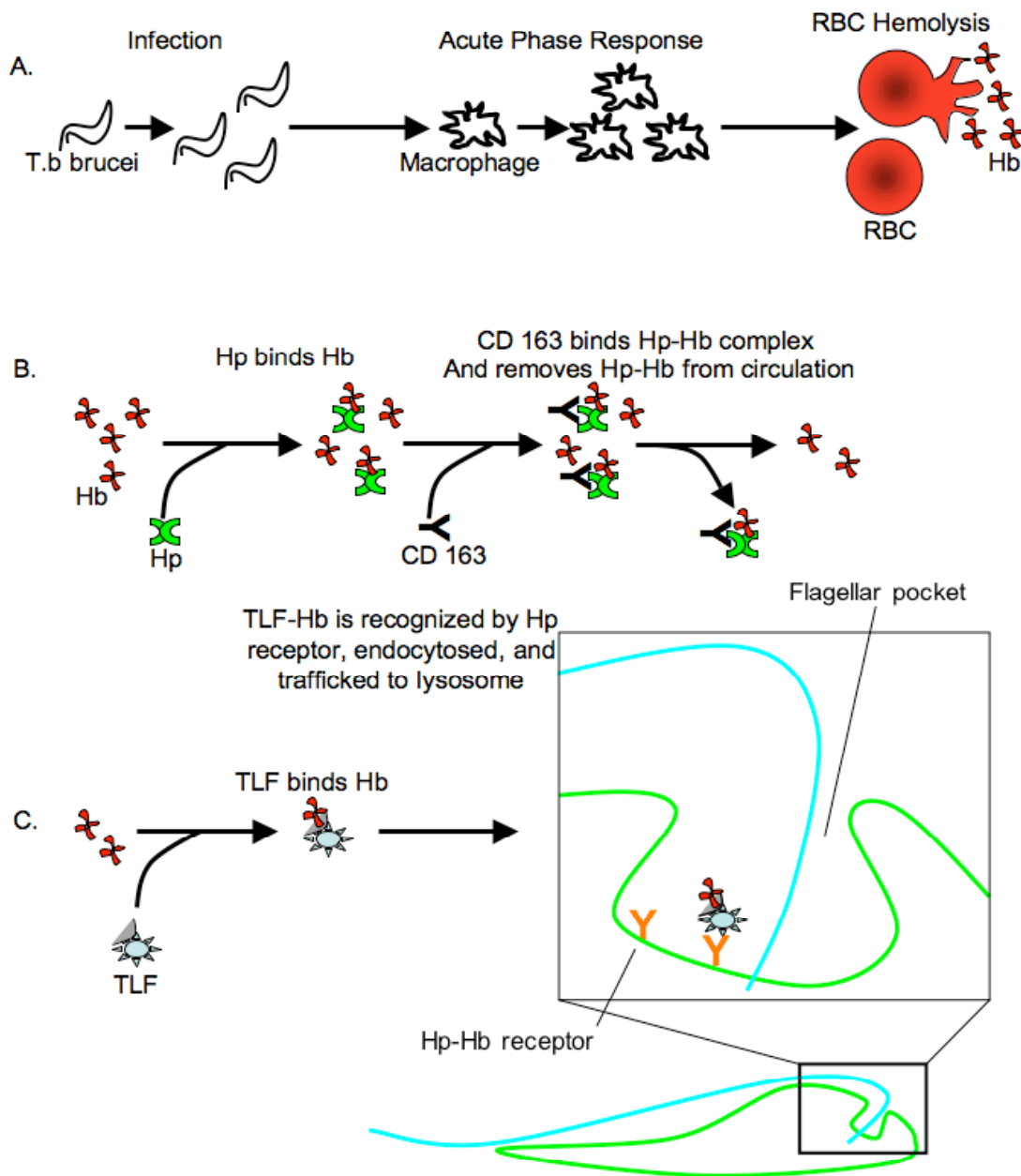


Figure 3. Human African Trypanosomiasis (HAT) results in hemolysis that activates TLF. Shortly after trypanosome infection, an acute phase response results in acute anemia and release of Hb (A). Severe hemolysis overwhelms the body's Hp capacity to clear Hb and results in extremely low concentrations of Hp. In the absence of Hp, TLF binds Hb and is activated (B). Endocytosis of TLF-Hb by the trypanosome leads to cell death and the infection is cleared (C).

TLF is a complex molecule, the biology of which we are only beginning to understand. At present we know that the assembly of TLF is critical for its function, and requires cellular processes. Once TLF has been assembled, it is not trypanolytic, and needs to be armed. Hemolysis and binding of Hb by TLF complete the process creating a unique class of HDL with anti-trypanosome and anti-parasitic function.

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