Evidence for a Trypanosoma brucei Lipoprotein Scavenger Receptor

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Running Title: Trypanosome Lipoprotein Scavenger Receptor*
African trypanosomes are lipid auxotrophs that live in the bloodstream of their human and animal hosts. Trypanosomes require lipoproteins in addition to other serum components in order to multiply under axenic culture conditions. Delipidation of the lipoproteins abrogates their capacity to support trypanosome growth. Both major classes of serum lipoproteins, LDL and HDL, are primary sources of lipids, delivering cholesterol esters, cholesterol and phospholipids to trypanosomes. We show evidence for the existence of a trypanosome lipoprotein scavenger receptor, which facilitates the endocytosis of both native and modified lipoproteins, including HDL and LDL. This lipoprotein scavenger receptor also exhibits selective lipid uptake, whereby the uptake of the lipid components of the lipoprotein exceeds that of the protein components. Trypanosome lytic factor (TLF1), an unusual HDL found in human serum that protects from infection by lysing *Trypanosoma brucei brucei*, is also bound and endocytosed by this lipoprotein scavenger receptor. HDL and LDL compete for the binding and uptake of TLF1 and thereby attenuate the trypanosome lysis mediated by TLF1. We also show that a mammalian scavenger receptor facilitates lipid uptake from TLF1 in a manner similar to the trypanosome scavenger receptor. Based on these results we propose that HDL, LDL, and TLF1 are all bound and taken up by a lipoprotein scavenger receptor, which may constitute the parasite’s major pathway mediating the uptake of essential lipids.
Introduction

Exogenous lipids play indispensable roles in trypanosome cell structure and metabolism. African bloodstream-form trypanosomes are single-celled parasites that appear not to synthesize fatty acids de novo (1-3), with the exception of myristate (C14 fatty acid). Trypanosomes have an atypical type II fatty acid synthase that utilizes exogenously supplied butyrate to generate myristate which is used exclusively for glycosylphosphatidylinositol anchor biosynthesis (4,5). Despite having a variety of enzymes that catalyze metabolic lipid-modifying pathways (6-9), trypanosomes are lipid auxotrophs. They require lipoproteins in addition to other serum components in order to multiply under axenic culture conditions (10,11). Delipidation of the lipoproteins abrogates their capacity to support trypanosome growth. Both major classes of serum lipoproteins, LDL and HDL, are primary sources of lipids, delivering cholesterol esters, cholesterol and phospholipids to trypanosomes (12,13).

Trypanosomes endocytose HDL and LDL through their flagellar pocket (10,13). All endocytosis and exocytosis in trypanosomes occurs at this site. It is a specialized invagination in the cell membrane, which is not lined with the microtubule network that encases the rest of the cell that precludes any vesicular fusion or fission. At physiological concentrations (~1 mg/ml), specific binding and uptake of the protein component of both LDL and HDL has been demonstrated (12-14). In contrast, at sub-physiological concentrations (1-50 µg/ml) there was no detectable uptake of the apolipoproteins themselves, whereas the lipid components of HDL and LDL were taken up at rates that exceeded fluid phase endocytosis by 1000-fold, suggesting that “specific binding sites were probably involved” (15). A putative LDL receptor protein has been
Trypanosome lytic factors are HDL related particles found in human plasma. TLF1 contains lipid, apolipoprotein A-I (apoA-I), paraoxonase and haptoglobin related protein (Hpr) (18), while TLF2 is a lipid-poor molecule that contains apoA-I, Hpr, and IgM (19). Both high and low affinity binding sites for TLF1 on trypanosomes have been reported in experiments using purified preparations of TLF1 (20). The low affinity binding site can be competed by HDL whereas the high affinity binding site is partially competed by reconstituted nonlytic HDL containing Hpr (21), which led to the proposal that Hpr can mediate TLF1 binding to trypanosomes through a haptoglobin-like receptor.

Many lipoprotein receptors have been characterized in eukaryotes, to date only cubilin (22) and members of the CD36 superfamily of scavenger receptors (23-25) bind native HDL (without requiring ApoE as a component). The CD36 superfamily of scavenger receptors bind and take up both native HDL and LDL as well as other polyanionic ligands, including oxidized and acetylated LDL (26). Some of these scavenger receptors mediate bi-directional lipid flux and exhibit a process called selective lipid uptake. In polarized cells selective lipid uptake is characterized by receptor-mediated uptake of the lipoprotein, distribution of the lipid within the cell, and recycling of the apolipoprotein to the cell surface (27). In non-polarized cells there does not appear to be any uptake of the holo-particle, rather binding to the surface of the cell via lipoprotein scavenger receptors facilitates the transfer of lipid from the lipoprotein into cell membranes and intracellular vesicles (28). After lipid transfer, the lipid-depleted particle is
released intact from the cell. One of the members of this family, SR-BI (scavenger receptor class BI), mediates the highest level of selective lipid uptake analyzed to date (29,30).

While studying trypanosome lytic factors, which are by definition lipoproteins, we decided to revisit lipoprotein receptors. We found evidence that *T. b. brucei* has a lipoprotein scavenger receptor that mediates the selective uptake of lipid over the protein component of both HDL and LDL. The same receptor can also mediate the uptake of oxidized lipoproteins. TLF1 is also bound and endocytosed by this lipoprotein scavenger receptor. We show that HDL and LDL compete for the binding and uptake of TLF1 and therefore attenuate the trypanosome lysis mediated by TLF1.

**Experimental Procedures**

**Materials**

The fluorescent probes: Alexa Fluor 488, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol), NBD-PtdCho, NBD-PtdEth, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and Calcein-AM were purchased from Molecular Probes (Eugene, OR). Tissue culture reagents: Ham's F12, DMEM, cell stripper solution and G418 were obtained from Cellgro. Rhodamine-concanavalin A and Vectashield mounting medium with DAPI were obtained from Vector Labs. Protease inhibitors were purchased from Roche. Polyclonal rabbit anti-mouse SR-BI was obtained from Novus Biologicals. [*125*I]Iodine was purchased from Amersham. Sephadex G-25 columns were bought from Isolab Inc. (Ohio). Mouse monoclonal anti-human haptoglobin, anti-rabbit IgG-FITC (F-0511) and all other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis,
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MO). *T. brucei brucei* Etat 1.9s were kindly provided by Dr. Miki Rifkin. CHO ldlA clone 7 and ldlA[mSR-BI] cells were generously provided by Dr. Monty Krieger.

**Methods**

*Purification of lipoproteins*-Normal human serum, plasma or bovine serum was adjusted to a density of 1.25 g/ml with KBr and ultracentrifuged in a near vertical NVTi 65 rotor (Beckman) for 16 hours at 49K, 10°C (31). The top 2 ml (ρ=1.0-1.26 g/ml) were collected and size fractionated on a Superose 6 16/50 column equilibrated with TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl). Proteins from fractions were separated on a 4-15% Tris-HCl gel and stained with Coomasie Blue. Fractions containing either ApoA-I (HDL) or Apo-B (LDL) were separately pooled and concentrated.

*Purification of trypanosome lytic factors*-Isolation of human HDL and purification of TLF1 from normal human serum (Hp 1-1) was performed as described by Raper *et al.* (19).

*Fluorescent labeling of lipoproteins*-Purified lipoproteins were incubated with Alexa Fluor 488 Protein Labeling Kit (Ex 494, Em 519) according to manufacturers instructions. In order to label lipoprotein lipids, twenty ml of normal human serum were incubated with 50 µl of 1 mg/ml NBD-cholesterol (Abs 469, Em 537) in DMF, NBD<sub>c12</sub> PtdCho (Abs 465, Em 534) in DMSO, or NBD PtdEth (Abs 463, Em 536) in MeOH for 16 hours at 37°C. Lipoproteins were purified according to above protocol. The specific activity (Absorbance/mg protein) of each fluorophore incorporated into HDL was determined in a 96 well fluorometer (Labsystems Fluoroskan II).
Labeling of human HDL with DiI was carried out according to Calvo et. al. (24) . Briefly, lipoproteins were incubated with the DiI probe in lipoprotein-deficient serum for 12 h at 37°C, using the following relative amounts: 300 µg DiI, 3 mg of lipoprotein, and 2 ml of lipoprotein deficient serum. The labeled lipoproteins were subsequently re-isolated by ultracentrifugation at 100K for 2.3 h at 10°C in a Beckman table top ultracentrifuge (TLA 1.3 rotor). DiI-labeled HDL was then sized on a Superdex HR 200 column (Pharmacia). DiI-labeled TLF1 was obtained by affinity purification of DiI-labeled HDL using a mouse anti-human haptoglobin monoclonal (H-6395,Sigma) coupled to a HiTrap column (Pharmacia). The fractions containing Hpr were pooled and concentrated.

**Radiolabeling lipoproteins**—Purified TLF1 was radiolabeled by the [125I]ICl technique with 125I to a specific activity of 300-600 dpm/ng. Bound 125I was separated from unbound by gel filtration on a PD10 column. TCA precipitable counts were > 95%. The radiolabeled proteins were used on the day they were labeled.

**Trypanosome Isolation**—Swiss Webster mice were inoculated intraperitoneally with serum sensitive *T. brucei brucei* ETat 1.9S, and the trypanosomes were harvested 2 days later from infected mouse blood as described previously (32). Parasites were resuspended at 2x10^7/ml in Dulbecco’s modified medium (DMEM) supplemented with 0.2-2 % BSA.

**Uptake Analyses**—Increasing concentrations of labeled lipoprotein were incubated at 37°C for various times with 2 x 10^7 trypanosomes in DMEM containing 0.2-2% BSA supplemented with the following protease inhibitors: 0.3 mg/ml antipain-HCl, 0.05 mg/ml bestatin, 0.1 mg/ml
chymostatin, 0.3 mg/ml E-64, 0.05 mg/ml leupeptin, 0.05 mg/ml pepstatin, 0.3 mg/ml phosphoramidon, 2.0 mg/ml Pefabloc SC, 1.0 mg/ml EDTA, and 0.05 mg/ml aprotinin (miniTab, Boehringer Manheim). Cells were washed twice with bicine buffered saline with glucose (BBSG), transferred to a black 96 well plate, and lysed in 0.5% SDS. Lysates were read in a Fluoroskan I at the wavelengths for the specific fluorophore.

**Competition Analyses**-The ligand under study was mixed with competitors at 30-100 fold excess in DMEM/0.2-2% BSA. Then prewarmed *T. b. brucei* (2 x 10⁷) were added and incubated at 37°C for 30 min. For radioactive ligands, cells were washed three times with DMEM/BSA and transferred to a clean tube prior to quantitating radioactivity in a gamma counter. Fluorescently labeled cells were washed twice with BBSG, transferred to black 96 well plates, and lysed and read as above.

**Fluorescence Microscopy**-Live trypanosomes were incubated in DMEM/0.2% BSA with either 300 µg/ml of NBD cholesterol/cholesterol ester-labeled plasma HDL or Alexa-labeled plasma HDL, 150 µg/ml of rhodamine-concanavalin A, or 300 µg/ml Alexa-HDL combined with 150 µg/ml rhodamine-concanavalin A at 37°C for 30 min. Cells were washed 3 times in PBSG, and resuspended in 3%paraformaldehyde/PBSG. Cells were washed with PBSG and dried onto 12 well slides (Erie Scientific Co.). DAPI-containing mounting medium was used to adhere coverslips to slides. Slides were viewed on a Nokia fluorescent microscope.

**Trypanolytic assays**-To measure lytic activity, 2 x 10⁶ trypanosomes were incubated in the presence of TLF1 (1-1.5 LU (5-40 µg/ml), or TLF1 combined with human LDL (0.75-1 mg/ml)
or bovine HDL (1-1.6 mg/ml). Following incubation for 150 min at 37°C, parasite lysis was
determined using a previously described calcein fluorescence-based assay (33).

**Cell culture and Maintenance**-LdlA[mSR-BI] and ldlA7 cells were grown in Ham's F-12
medium supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine with or without 500
µg/ml G-418, respectively. All cells were maintained in a 37°C humidified 95% air, 5% CO₂
incubator.

**Flow cytometry**-LdlA[mSR-BI] and parental ldlA7 cells were plated overnight in 6 or 12 well
plates washed and incubated for 2-4 hours in Ham's medium supplemented with 0.2% BSA. The
cells were then incubated at 37°C for 2 h with 2.5 µg/ml DiI-HDL or DiI-TLF1 in Ham's F12
supplemented with 0.2% BSA. Cells were washed twice with PBS, detached from the plate with
Cell stripper solution and resuspended in PBS, then fixed with an equal volume of 4%
paraformaldehye.

For immunofluorescence, LdlA[mSR-BI] and ldlA7 cells were resuspended in 100 µl of
Ham's F12 , 5% FCS to 10⁶/ml. Anti SR-BI antibody (100 µl) to a final dilution 1:1000 was
added and incubated for 30 minutes on ice. Cells were then washed 3 times, resuspended to
100 µl in PBS 0.1% BSA, and 100 µl of anti rabbit IgG-FITC labeled antibody was added to
final dilution of 1:50. After 30 min incubation on ice, the cells were washed 3 times with PBS,
then resuspended to 100 µl in PBS and fixed with 100 µl of 4% paraformaldehyde. Samples were
subjected to flow cytometric analysis using a Becton Dickinson FACSscan flow cytometer. The
following excitation/emission were used: 488/525 nm for FITC-conjugated antibody and
488/575 nm for DiI-labeled HDL and TLF1. Mean relative fluorescence of cells was recorded and results are expressed as a percentage of control.
RESULTS

_HDL and LDL compete for HDL uptake by trypanosomes._

We labeled HDL and LDL with Alexa, a fluorophore that conjugates to the free amino groups in the protein components of these lipoproteins. We found that trypanosomes accumulated HDL protein (2.25 pmol, calculated based on a molecular mass of 350,000 Da by size exclusion chromatography, 50% of which is protein) and LDL protein (2 pmol, calculated based on the molecular mass of apoB, 550,000 Da) (Fig. 1 A) with similar kinetics, approximating steady state within 30 minutes. Trypanosomes also accumulated NBD-cholesterol/cholesterol ester (NBD-C/CE) from HDL (lipid uptake equivalent to 350 pmol protein) and LDL (lipid uptake equivalent to 425 pmol protein; Fig. 1 B), approximating steady state within 30 minutes. TLC analysis indicated that the NBD cholesterol labeled both free cholesterol and cholesterol esters in the lipoproteins (data not shown). Therefore, results shown in Fig. 1 B represent total cholesterol uptake. As HDL and LDL have similar kinetics of protein and lipid uptake (Figs. 1 A, 1 B), they were used in competition assays. The competition was assessed at 30 minutes, such that the uptake was close to steady state (Figs. 1 A and 1 B). Both unlabeled HDL and LDL were effective competitors of protein (Fig. 1D) and lipid (Fig. 1C) uptake from labeled HDL. Fig. 1 _panel D_ shows that 4 times more HDL than LDL was needed to give a 50% reduction in the uptake of HDL protein. This suggests that the putative lipoprotein receptor has higher affinity for LDL than HDL. Although Fig. 1 _panel C_ displays a similar trend in LDL vs. HDL competition, we found that some of the NBD-cholesterol was transferred to the non-labeled lipoprotein, which may contribute to the enhanced competition of lipoprotein lipid uptake. This desorption diffusion process may also occur to some extent when NBD-cholesterol HDL is incubated with trypanosomes.
HDL lipids are selectively taken up over HDL protein

To date the only characterized eukaryotic lipoprotein receptors that bind both native HDL and LDL have the unique characteristic of selective lipid uptake over protein. In light of the above findings we examined if there was a differential uptake of other lipid components over the protein components of HDL. We extended this study to include HDL labeled with fluorescent phospholipids NBD-phosphatidylcholine (NBD-PtdCho), NBD-phosphatidylethanolamine (NBD-PtdEth) as well as NBD-cholesterol/cholesterol ester (NBD-C/CE). The specific activity (fluorescence/µg protein) of each fluorescently labeled HDL was taken into consideration, such that the uptake of each fluorescent lipid was calculated from a standard curve of µg protein vs. fluorescent units. The results are expressed as µg of protein equivalents taken up rather than fluorescent units. We found that all classes of lipid molecules in HDL were taken up 50-100 fold more than the protein component (Fig. 2). It is apparent that the uptake of free cholesterol and cholesterol ester exceeds that of phospholipids. In addition NBD-PtdEth was taken up almost two fold more than NBD-PtdCho. The uptake of individual lipid species does not correlate with their concentrations found in a typical human HDL, which are 55, 7 and 27 weight % for phosphatidylcholine, cholesterol and cholesterol esters respectively (34).

Localization of lipid vs. protein

Since all classes of lipid molecules were taken up more avidly than protein, we examined the distribution of the fluorescent lipid and protein molecules by fluorescent microscopy. NBD-cholesterol and cholesterol esters (NBD-C/CE) were rapidly distributed throughout the parasite and all of the parasites were labeled (Fig. 3 A). Confocal analysis revealed diffuse staining
throughout the cytosol of the parasites (data not shown). The labeled phospholipids, NBD-PtdCho and NBD-PtdEth also demonstrated a staining pattern similar to that of NBD-C/CE (data not shown). In contrast, the apolipoprotein (Alexa-HDL) uptake by trypanosomes (all of the parasites accumulated Alexa-HDL) was visualized in the flagellar pocket and intracellular vesicles (Fig. 3 B) with a distribution similar to endocytosed concanavalin A (conA) (Fig 3 C). Concanavalin A has been shown to distribute within endocytic vesicles of trypanosomes when endocytosed by live trypanosomes (35,36), in contrast conA labels the VSG coat when used on fixed trypanosomes presumably due to the exposure of carbohydrate epitopes upon fixation.

Coincubation with rhodamine-conA and Alexa-HDL revealed colocalization in some endocytic vesicles (yellow) near the flagellar pocket but not all endocytic vesicles (red) (Fig. 3 D).

**HDL competes for the binding of TLF1 to trypanosomes**

All of the data thus far points to the presence of a lipoprotein receptor in trypanosomes that can facilitate the uptake of both native HDL and LDL. TLF1 is a subclass of HDL, and is composed of lipids, apolipoprotein A-I, paraoxanase, and haptoglobin-related protein. Lipids and apolipoprotein A-I are the common components of all HDLs, whereas Hpr is unique to TLF particles. There are studies documenting the specific and saturable binding of TLF1 and HDL to the flagellar pocket of African trypanosomes (14,20). We found that TLF1 (20 µg/ml (60 pmoles, calculated based on a molecular mass of 550,000 Da by size exclusion chromatography, 60% of which is protein)) and HDL (200 µg/ml (1440 pmoles) could compete for binding of [¹²⁵I]TLF1 to *T. b. brucei* (Fig. 4). We did not investigate the effect of LDL on the binding of TLF1 to *T. b. brucei*, because LDL takes 6 hours to reach equilibrium binding to trypanosomes whereas HDL
and TLF take 30 min. Therefore we could not have a fair competition for binding, and evaluated uptake only.

**HDL and LDL can attenuate TLF1 mediated trypanosome lysis.**

Given that we observed competition for binding of TLF1 to trypanosomes by HDL, we evaluated the effect of non-lytic bovine HDL on TLF1-mediated trypanolysis. We observed that non-lytic bovine HDL was able to attenuate trypanosome lysis by purified TLF1 (Fig. 5). Non-lytic human LDL was also effective in attenuating trypanolysis by TLF1.

**TLF1 binds to mouse Scavenger Receptor Class B type I and donates lipids**

Our results support the presence of a lipoprotein scavenger receptor in trypanosomes that can bind multiple lipoprotein ligands such as TLF1, HDL, LDL, and oxidized LDL (not shown) and exhibit a process called selective lipid uptake similar to eukaryotic lipoprotein scavenger receptors. To directly address whether TLF1 could bind to a eukaryotic lipoprotein scavenger receptor with the same ligand binding characteristics as the trypanosome lipoprotein receptor, we examined the binding of TLF1 to a CHO cell line that overexpresses mouse Scavenger Receptor-Class BI (mSR-BI). The parental CHO ldlA[clone 7] cells do not express the LDL receptor, and were stably transfected with a vector expressing mouse scavenger receptor-class BI to create ldlA[mSR-BI] cells (37). These cell lines were first validated with antibodies raised against mSR-BI; the parental ldlA cells showed little staining, while the transfected ldlA[mSR-BI] cells stained readily with anti-mSR-BI (Fig. 6, insert). HDL labeled with the fluorescent lipid DiI exhibited lipid uptake into cells expressing mSR-BI that was 30-fold greater than the uptake by the parental ldlA cells (Fig. 6). TLF1 labeled in the lipid component with DiI to the same specific
activity as DiI-HDL, was taken up to a 5-fold greater extent by cells expressing mSR-BI than that shown by the parental ldlA cells (Fig.6).
DISCUSSION

Lipoprotein receptors that can bind more than one ligand are known as lipoprotein scavenger receptors. Our results suggest that bloodstream-form trypanosomes have a single lipoprotein scavenger receptor that can facilitate the uptake of all major lipoprotein classes including HDL, LDL, oxidized LDL (results not shown), and TLF1. Moreover, like eukaryotic scavenger receptors, the trypanosome receptor shows selective uptake of lipid over protein from the lipoprotein particle. Trypanosomes are lipid auxotrophs, and host lipoproteins are required for their survival. This putative lipoprotein scavenger receptor may well constitute the primary pathway by which the parasite acquires essential host lipids, and would therefore represent an important therapeutic target. Very few bloodstream-form trypanosome receptors have been previously characterized biochemically. These include an LDL receptor (10) and a HDL receptor (13) both of which may be identical to the scavenger receptor described here (see below), a haptoglobin-like receptor which may also be a TLF receptor (21), and a receptor for transferrin which has been molecularly cloned (38-43).

The characterization of this trypanosome scavenger receptor serves to unify a variety of disparate data regarding the utilization of lipoproteins and TLF by the parasite. Vandeweerd et al., showed that the uptake at 37°C of radiolabeled lipid components in either HDL or LDL was inhibited (50-85%) by unlabeled HDL or LDL (15). It was concluded that the uptake process did not discriminate between HDL or LDL. In this study we have confirmed and extended these observations. We also found that accumulation of HDL labeled protein or HDL labeled lipid, was inhibited by increasing concentrations of HDL and LDL (Fig 1C and 1D). Previous
investigations of lipoprotein binding to a different isolates of trypanosomes indicated that HDL binding could be partially competed by LDL (13) although LDL binding could not be competed by HDL (17). Although our data for lipoprotein uptake at 37°C suggests that competition should have been reciprocal, it is possible that binding at 4°C gives different results from uptake experiments performed under physiological conditions at 37°C. The observation that the number of estimated HDL and LDL receptors in trypanosomes are roughly equivalent (30,000-52,000 LDL receptors/cell (10,17,44), 22,000-64,000 HDL receptors/cell (10,13,20,21)) supports the notion that a single scavenger receptor can mediate uptake of both HDL and LDL. Interestingly, anionic phospholiposomes, which have been shown to be effective inhibitors of trypanolysis mediated by TLF1 (45), are also ligands for eukaryotic lipoprotein scavenger receptors (46,47). Taken together, these results suggest the presence of a lipoprotein scavenger receptor in trypanosomes that can bind multiple ligands.

The trypanosome lipoprotein scavenger receptor shares characteristics with certain subclasses of mammalian scavenger receptors. Members of the CD36 superfamily can bind native HDL and LDL and exhibit selective lipid uptake from both lipoproteins. Binding appears to be mediated by a combination of apolipoprotein and lipid. These characteristics most resemble what we have found for the putative trypanosome lipoprotein scavenger receptor. We find that when we correct for the specific activity of each labeled lipoprotein, the lipid components are selectively accumulated more than the protein component (Fig. 2). It is worth noting that although cholesterol/cholesterol ester is taken up 3-4 fold more than phospholipid, it only comprises 36% of native HDL lipids relative to 55% for phosphatidyl choline. The selective uptake of
lipoprotein cholesterol/cholesterol ester over phospholipid has also been characterized in SR-BI scavenger receptors, which are a subclass of the CD36 superfamily (28,48).

Ligands other than native HDL and LDL have been identified for eukaryotic lipoprotein scavenger receptors, such as oxidized lipoproteins (46). Oxidized LDL is a ligand for the trypanosome lipoprotein receptor, in that native HDL and LDL or oxidized lipoproteins (not shown) were effective competitors for uptake. Native HDL was a consistently better competitor than native LDL when measuring oxidized LDL uptake by trypanosomes. On the other hand, native LDL was a better competitor than native HDL when measuring HDL uptake in trypanosomes (Figs. 1C and 1D). It has been shown for CD36 that oxidized LDL competes more effectively than LDL for HDL binding to CD36 (24).

The similar biochemical properties of the trypanosome lipoprotein scavenger receptor and mammalian CD36 superfamily members compelled us to analyze the interaction of TLF1 with the prototypical class B eukaryotic lipoprotein scavenger receptor, SR-BI, which exhibits the highest degree of selective lipid uptake (29,30). We found that like HDL, TLF1 is able to donate lipids via this eukaryotic lipoprotein scavenger receptor (Fig. 6), indicating that TLF1 can bind to and productively interact with SR-BI. Although there was a specific association of TLF1 with CHO cells expressing SR-BI we did not observe any obvious toxicity at physiological concentrations of TLF1 (~20 µg/ml).

Both apolipoprotein and lipid are taken up by the parasite. The lipid is selectively removed from the lipoprotein and distributed throughout the cell (Fig. 3A). The apolipoprotein localizes to
endocytic vesicles (Fig. 3D). The distribution of protein is very different from that seen for lipid, suggesting that at some point after interaction with a receptor the lipid is selectively removed and dispersed throughout the cell. The current hypotheses for HDL uptake in eukaryotic cells involve either retro-endocytosis or the formation of a non-polar channel, created by the binding of the apolipoprotein at the cell surface (28), through which lipids are delivered. We have demonstrated that HDL apolipoprotein is found inside the trypanosome (Fig. 3D). Because we detect intracellular HDL and we do not detect degradation (not shown), the majority of the endocytosed apolipoprotein may be recycled back to the cell surface. Other researchers have found that trypanosome endocytosis of HDL (13) and more recently TLF1 (49) do not result in the degradation of the apolipoproteins. In contrast, apolipoprotein B of LDL is rapidly degraded during its transit through the endocytic machinery (50). The reason for this difference in proteolytic processing is not known, it may be due to differential routing of the ligands in the endocytic pathway or differential sensitivity to endosomal and lysosomal proteases.

Physiological concentrations of HDL can compete for at least ~80% of the binding of TLF1 to *T. b. brucei* (Fig. 4). It has been proposed that the remaining ~20% of TLF1 is taken up by another trypanosome receptor that recognizes haptoglobin (21). Irrespective of whether there are one or more receptors for TLF, if there is competition for binding of TLF, there should be competition for uptake. It has been reported that TLF1 exhibits both high affinity (0.75-3.6 µg/ml) and low affinity (80-175 µg/ml) binding to trypanosomes, and that only the low affinity sites are competed by HDL (20,21). We believe, as has been proposed by others for LDL binding to trypanosomes (10,51), that the low affinity sites for TLF1 may represent single receptors along the flagellum and within the pocket, whereas high affinity sites represent
dimerized or clustered receptors within the flagellar pocket. Complete inhibition of binding or uptake at the receptor level, requires the competing ligand to be at least 100 fold above its own Kd in order to saturate all of the available receptors (52). Therefore complete inhibition of TLF1 binding and uptake at the receptor level would require 2.7-8 mg/ml of HDL (13,21), and 13-33 mg/ml LDL (44). These concentrations are above the physiological levels found in plasma which are ~1 mg/ml. Therefore, in vivo as in our assay, the lipoproteins would be able to attenuate the killing by TLF1 but they would not inhibit the killing. This is illustrated by the attenuation of TLF1 mediated lysis (Fig. 5) in the presence of HDL (1-1.6 mg/ml) and LDL (0.75-1 mg/ml).

Oxidized lipoproteins were in themselves trypanolytic, and we therefore could not evaluate their capacity to attenuate TLF mediated lysis.

Overall our results suggest that trypanosomes have a lipoprotein scavenger receptor that can bind HDL, LDL, and TLF despite their distinct apolipoprotein content. The characteristics of the lipoprotein interactions with trypanosomes most closely resemble those of the class B type lipoprotein scavenger receptors, which are the only receptors to date that bind native HDL. Members of the CD36 superfamily of proteins are found in eukaryotes ranging from mammals to fungi (53), and generally have 30% amino acid sequence identity (26). However, extensive searching of the trypanosome databases has not yet revealed an identifiable homologue. This is not entirely surprising as trypanosome proteins are often very divergent; extensive searching of the trypanosome databases with the eukaryotic transferrin receptor reveals nothing, yet there is a non-homologous but well-characterized transferrin receptor in trypanosomes (38-43). Other scavenger receptor domains (SRCR, Pfam 00530) have been identified in Plasmodium sp. (54). It
is quite probable that scavenger receptors exist in other lipid auxotrophic parasites, and these may become apparent as the genomes of parasites are completed and fully annotated.
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Footnotes

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1 The abbreviations used are: apoA-I, apolipoprotein A-I; Hpr, haptoglobin related protein; Hp 1-1, haptoglobin type 1-1; TLF, trypanosome lytic factor; HDL, high density lipoprotein; LDL, low density lipoprotein; SR-BI, scavenger receptor class B type I; mSR-BI, murine scavenger receptor class B type I; ldlA7, ldlA (clone 7) LDL receptor negative CHO cell mutant clone; CHO, Chinese hamster ovary; PBSG, phosphate-buffered saline with glucose; TBS, Tris-HCl buffered saline; BBSG, bicine buffered saline with glucose; Kd, dissociation constant (ligand concentration at which 50% of the receptors are occupied); ConA, concanavalin A; C/CE, cholesterol/ cholesterol ester; PtdEth, Phosphatidylethanolamine ; PtdCho, Phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; TCA , trichloro acetic acid; bHDL, bovine HDL; DMEM, Dulbecco's modified medium; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; LU, lytic unit wherein 50% of 2 x 10^6 trypanosomes are lysed in 150 min. at 37°C in a final volume of 200 µl.
2 Manuscript in preparation
Figure Legends

**Fig. 1. HDL and LDL compete for HDL uptake in trypanosomes.**

Trypanosomes (300µl of 2 x 10⁸ parasites/ml) were incubated at 37°C in the presence of 50 µg/ml (A) Alexa-labeled HDL (285 pmoles; squares) or LDL (90 pmoles; diamonds) or (B) NBD-C/CE labeled HDL or LDL. Uptake was determined by reading fluorescence of cell lysates at 0, 5, 15, 30, 45 and 60 min. Fluorescent units were converted to moles of protein using a standard curve for each fluorophore. Data are representative of two independent experiments.

Trypanosomes (100 µl of 2 x 10⁸/ml) were incubated at 37°C in the presence of (C) 50 µg/ml (285 pmoles) NBD-C/CE labeled HDL or (D) 50 µg/ml (285 pmoles) Alexa-labeled HDL and increasing concentrations of unlabeled HDL (squares) or LDL (diamonds). Panel C: 100% ~ 1.9 µg (10 pmole); Panel D: 100% ~20 µg (114 pmole).

**Figure 2. Trypanosomes exhibit selective lipid uptake.**

Trypanosomes (2 x 10⁷) were incubated in a final volume of 150 µl in the presence of increasing concentrations of Alexa-HDL (squares), NBD-PtdEth-HDL (diamonds), NBD-PtdCho-HDL (triangles), or NBD-C/CE-HDL (circles) for 30 min. at 37°C. Cells were washed and fluorescence was determined.

**Figure 3. HDL lipid and protein are distributed differently.**

Trypanosomes (3 x 10⁶) were incubated for 30 min. at 37°C in a final volume of 100 µl in the presence of A) 300 µg/ml (1.7 nmoles) of NBD-C/CE labeled HDL; B) 300 µg/ml (1.7 nmoles)
of Alexa labeled plasma HDL; C) 150 μg/ml (0.7 nmoles) Concanavalin A; D) 300 μg/ml of Alexa labeled plasma HDL and 150 μg/ml ConA. Cells were washed, nuclei and kinetoplast (mitochondrial DNA) stained with DAPI and fixed and then viewed by fluorescent microscopy.

**Figure 4. HDL and TLF1 compete for TLF1 binding to T. b. brucei.**

200 ng/ml (0.06 pmoles) \[^{125}\text{I}]\text{TLF1} (\text{*TLF1}) was mixed with 20 μg/ml (60 pmoles) TLF1 or 200-250 μg/ml (1.1-1.4 nmoles) HDL. Trypanosomes (1 x 10^7) were added and binding was allowed to reach equilibrium at 4°C. Each value for bound TLF is the mean of 2 independent experiments done in triplicate.

**Figure 5. Lipoproteins attenuate trypanolysis by TLF1.**

Trypanosomes (2x10^6) were incubated in a final volume of 200 μl in the presence of TLF1 (1-1.5 LU, 5-40 μg/ml, 15-121 pmoles) alone or TLF1 plus human LDL (0.75-1 mg/ml, 1.45-1.8 nmoles) or bovine HDL (1-1.6 mg/ml, 5.7-9.1 nmoles) for 150 min at 37°C. Cell lysis was determined microscopically and as described in Methods. Results are expressed as % lysis and represent the mean of 5 independent experiments.

**Figure 6. SR-BI mediates lipid uptake from TLF1.**

Ldl 7[mSR-BI] and ldl A7 cells were analyzed with fluorescent anti-mSR-BI to confirm the over expression of mSR-BI. The cells lines were then incubated in the presence of 2.5μg/ml (14 pmoles) DiI-HDL or 2.5μg/ml (7.5 pmoles) DiI-TLF1 for 2 hours at 37°C as described under Methods. Red fluorescence (DiI) or green fluorescence (anti SR-BI FITC) was measured by flow
cytometry. Shown is mean relative fluorescence expressed as % of the control (control = 100%, see Methods)
Figure 1

(A) Transport (pmol / nmol added) over time (mins.) for Alexa HDL and Alexa LDL.

(B) Transport (pmol / nmol added) over time (mins.) for NBD C/CE LDL and NBD C/CE HDL.

(C) Percent NBD C/CE-HDL uptake vs. competitor (nmoles) for HDL and LDL.

(D) Percent Alexa-HDL uptake vs. competitor (nmoles) for HDL and LDL.
Figure 2

μg protein equivalents transported/
2x10^7 trypanosomes/30 minutes

Concentration (μg/ml)

NBD C/CE HDL
NBD PE HDL
NBD HPC HDL
Alexa HDL
Figure 6

Fluorescent intensity (% of control)

Di I-HDL  Di I-TLF1

Fluorescence of bound anti-m SR-BI (% of control)

- ldIA[mSR-BI]
- ldIA7
Evidence for a trypanosoma brucei lipoprotein scavenger receptor
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