Trypanosome Lytic Factor-1 Initiates Oxidation-stimulated Osmotic Lysis of Trypanosoma brucei brucei

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Human innate immunity against the veterinary pathogen Trypanosoma brucei brucei is conferred by trypanosome lytic factors (TLFs), against which human-infective T. brucei gambiense and T. brucei rhodesiense have evolved resistance. TLF-1 is a subclass of high density lipoprotein particles defined by two primate-specific apolipoproteins: the ion channel-forming toxin ApoL1 (apolipoprotein L1) and the hemoglobin (Hb) scavenger Hpr (haptoglobin-related protein). The role of oxidative stress in the TLF-1 lytic mechanism has been controversial. Here we show that oxidative processes are involved in TLF-1 killing of T. brucei brucei. The lipophilic antioxidant N,N,N'-di-phenyl-p-phenylenediamine protected TLF-1-treated T. brucei brucei from lysis. Conversely, lysis of TLF-1-treated T. brucei brucei was increased by the addition of peroxides or thiol-conjugating agents. Previously, the Hpr-Hb complex was postulated to be a source of free radicals during TLF-1 lysis. However, we found that the iron-containing heme of the Hpr-Hb complex was not involved in TLF-1 lysis. Furthermore, neither high concentrations of transferrin nor knock-out of cytosolic lipid peroxidases prevented TLF-1 lysis. Instead, purified ApoL1 was sufficient to induce lysis, and ApoL1 lysis was inhibited by the antioxidant DPPD. Swelling of TLF-1-treated T. brucei brucei was reminiscent of swelling under hypotonic stress. Moreover, TLF-1-treated T. brucei brucei became rapidly susceptible to hypotonic lysis. T. brucei brucei cells exposed to peroxides or thiol-binding agents were also sensitized to hypotonic lysis in the absence of TLF-1. We postulate that ApoL1 initiates osmotic stress at the plasma membrane, which sensitizes T. brucei brucei to oxidation-stimulated osmotic lysis.

Humans and higher primates are unable to be infected by the veterinary pathogen T. brucei brucei because of a novel form of innate immunity mediated by trypanosome lytic factors (TLFs). Related parasites, T. brucei gambiense and T. brucei rhodesiense, have evolved resistance to the TLFs and cause the deadly disease human African trypanosomiasis. TLF-1 is a subclass of high-density lipoprotein (HDL) particles defined by two primate-specific apolipoproteins: ApoL1 (apolipoprotein L1) and Hpr (haptoglobin-related protein). Apolipoprotein L1 is an ion pore-forming protein that inserts into anionic membranes at low pH (1, 2). Haptoglobin-related protein has over 95% sequence identity to human haptoglobin (Hp), a non-HDL associated acute phase serum protein (3). The primary function of Hp is to bind free Hb, preventing heme-induced oxidative damage. Like Hp, Hpr binds to Hb dimers with high affinity (3). However, although the HbHp complex is cleared from the bloodstream via the macrophage CD163 receptor, Hpr-Hb in TLF-1 is not removed from circulation by CD163 because of four amino acid substitutions in the Hp-CD163 binding site (3, 4).

A haptoglobin-hemoglobin receptor (HpHbR) located within the flagellar pocket of T. brucei brucei binds to both HpHb and HprHb, facilitating high affinity endocytosis of Hb-bound TLF-1 into the parasite (5, 6). Following binding to the HpHbR, TLF-1 and Hb traffic through the endolysosomal pathway. Within an acidic compartment, ApoL1 inserts into the vesicle membrane (1, 2).

Until recently, it was thought that ApoL1 inserted into the lysosomal membrane, forming a chloride channel (2). However, a recent in vitro study of ApoL1 found a strong preference for cations (7). Surprisingly, they discovered that ApoL1 is an acid-gated channel that is not active at the low pH values found in the trypanosome lysosome (7). They proposed that after endocytosis, ApoL1 traffics through recycling endosomes to the flagellar pocket and plasma membrane, where it causes osmotic swelling of T. brucei brucei (7). In contrast, another recent study showed that ApoL1 traffics to the mitochondrial membrane of T. brucei brucei, inducing apoptosis-like cell death (8). Thus, the primary target organelle for ApoL1-induced lysis remains to be elucidated.

The mechanism of T. brucei brucei cell death caused by TLF-1 is disrupted. An observed phenotype of TLF-1 lysis is swelling of the plasma membrane of T. brucei brucei into a rounded “kite-shape” as shown in this paper (9–11). In contrast, one group has reported predominant lysosomal swelling induced by ApoL1 under certain conditions (2, 12). In contrast with osmotic swelling of the lysosome, TLF-1 has been shown to induce lysosomal membrane permeability to dextran, suggesting that the lysosomal membrane breaks down following TLF-1 treatment (8, 13, 14). In fact, recently published data
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indicate that neither lysosomal swelling nor lysosomal membrane permeability is responsible for TLF-1-induced lysis (8).

There is plentiful evidence for TLF-1 inducing osmotic stress at the *T. brucei brucei* plasma membrane. For instance, high concentrations of sucrose prevent TLF-1 lysis by preventing the influx of water into cells (9, 15). Similar to cells under hypotonic stress, TLF-1 incubation induces an efflux of potassium ions and an uptake of calcium ions in *T. brucei brucei* (9). In support of ApoL1 forming a sodium channel at the plasma membrane, TLF-1 lysis of *T. brucei brucei* is decreased in a sodium-free buffer (15).

The role of oxidative processes in the mechanism of TLF-1 lysis is also controversial. In favor of an oxidative mechanism of TLF-1-induced lysis, increased lipid peroxides were observed in TLF-1-treated versus control cells (16). Also, the addition of exogenous catalase or the antioxidant DPPD decreased lysis caused by TLF-1 (16, 17). Within the lysosome, the Hpr-Hb complex has been proposed to act as the TLF-1 peroxidase, initiating oxidative damage to the lysosomal membrane (13, 16, 17). In contrast, another group found no evidence for peroxide formation during TLF-1 lysis (18). The research presented here addresses the origin and role of oxidative stress in TLF-1-induced trypanosome lysis. We show that Hpr-Hb mediated lysosomal lipid peroxidation is not required for killing. Rather, we propose that oxidation of free thiols in *T. brucei brucei* at the plasma membrane. For instance, high density of 1 × 10^6 cells/ml in triplicate wells of a 6-well plate. Cells grew to mid-log phase during a 20-h incubation in the cell culture incubator. At the end point, cells were counted and graphed as a percentage of untreated cell number.

**Experimental Procedures**

**Trypanosome Growth and Preparation for Assays—Blood-stream form *T. brucei brucei* Lister 427(MiTat 1.2) cells were grown in cell culture medium made from HMI-9 with 10% fetal bovine serum (Sigma-Aldrich) and 10% Serum-Plus (Sigma-Aldrich). Cytosolic tryparedoxin peroxidase I and II double knock-out *T. brucei brucei* 449 cells, strain Lister 427 (Px KO) were generously donated by Luise Krauth-Siegel (Heidelberg University, Heidelberg, Germany) (19, 20). The Px KO cells were grown in the presence of 100 μM Trolox antioxidant. The HphBR knock-out *T. brucei brucei* (Lister 427MiTat 1.2) cell line has also been described previously (21). Unless otherwise noted, all trypanosome assays were performed in culture medium with mid-log phase cells grown at 37 °C with 5% CO_2.

**TLF-1 and ApoL1 Purification—**TLF-1 and ApoL1 were purified as described previously (22). Briefly, TLF-1 was purified from human HDLs from single donors. HDLs were eluted from an anti-Hpr column, dialyzed into phosphate-buffered saline solution with EDTA (PBSE), and concentrated by Centricon filtration with a 100-kDa cutoff size (Millipore). For ApoL1 purification, purified TLF-1 was solubilized in CHAPS, eluted from an anti-ApoL1 column, dialyzed into PBSE, and concentrated. SDS-PAGE gels and Western blots against Hpr and ApoL1 confirmed the identity and purity of TLF-1 and ApoL1 preparations.

**TLF-1 and ApoL1 Lysis Assays and Survival Assays—**Most lysis assays were performed as previously reported with 1 × 10^7 trypanosomes/ml in serum-containing cell culture medium (13). Unless otherwise specified, all TLF-1 assays were conducted following preincubation of TLF-1 on ice with an excess of Hb. For assays with reconstituted globins, cells were washed and resuspended in serum-free media composed of HMI9 with 1% by weight glucose and 1% by weight BSA. Before addition to the trypanosomes, 100 mol eq of globin were preincubated with TLF-1 on ice for several minutes. For ApoL1 assays, time courses were longer than 2 h as indicated in the figure legends.

Reagents used in lysis assays were purchased from Sigma-Aldrich: Human hemoglobin A (Hb), N,N′-diphenyl-p-phenylenediamine (DPPD), diethyl maleate (DEM), monobromobimane (BrBi), and N-ethylmaleimide (NEM). Differential interference contrast microscopy of live trypanosomes incubated with ApoL1, TLF-1, or hypotonic medium was performed using a Zeiss Axio Observer inverted microscope.

For survival assays, TLF-1 was added to 1 ml of cells at a density of 1 × 10^6 cells/ml in triplicate wells of a 6-well plate. Cells grew to mid-log phase during a 20-h incubation in the cell culture incubator. At the end point, cells were counted and graphed as a percentage of untreated cell number.

**DCF-DA Kinetic Measurements of Reactive Oxygen Species (ROS)—**DCF-DA was used to measure ROS in trypanosomes treated with H_2O_2 (23). 50 μM 2′,7′-dichlorofluorescein diacetate was incubated with *T. brucei brucei* cells (2 × 10^7 cells/ml) for 30 min at 37 °C before washing three times with HyClone® HyQ DMEM/high modified medium (without phenol red) with 10% FBS and resuspended at 1 × 10^7 cells/ml with 200 μl/well in a 96-well plate. The fluorescence was monitored in kinetic mode using an excitation of 485 nm (bandwidth 20 nm) and an emission of 528 nm (bandwidth 20 nm). The initial values for each sample at 0 min were set to 0 fluorescence units. For Fig. 1B, antioxidants were preincubated with the cells for 30 min at 37 °C before adding H_2O_2 at 0 min. For Fig. 9B, 100 μl of 2 × 10^7 cells/ml were added to the wells, and 100 μl of DMEM with 10% serum (isotonic) or H_2O (hypotonic) was added at time 0 with or without concurrent addition of 50 μM H_2O_2.

**Porphyrin Substituted Globins—**Hemoglobin was denatured in acid-acetone and reconstituted with iron or zinc protoporphyrin IX as described previously (24). First, 4 mg of Hb was dissolved in 200 μl of deionized water. A 20-μl aliquot of Hb was reserved for control experiments, and 180 μl was added to 14 ml of acid acetone (40 mM HCl in acetone) and incubated for 10 min in the −80 °C freezer. Globin was precipitated by centrifuging for 5 min at 4 °C and 3200 × g. Acetone-heme supernatant was removed, and the globin pellet redisolved in 180 μl of deionized water, suspended in 15 ml of acid acetone, and incubated 10 min at −80 °C. Globin was repelleted and washed a second time before resuspending the pellet in 1 ml of deionized water and dialyzed three times. The first dialysis was in 0.1% NaHCO_3 and 0.2 mM DTT buffer for at least 3 h at room temperature. The second dialysis was 50 mM KPi pH 7.5 buffer overnight at 4 °C. The third dialysis was 100 mM boric acid, pH 9.5, for at least 3 h at 4 °C. Globins were reconstituted with at least 4 equivalents of protoporphyrin IX (Fe^3+ or Zn^2+), which were mixed gradually into the globin solution. Contaminating aggregated porphyrins were pelleted by centrifuging at 4 °C. Free porphyrins were removed from the supernatant by elution by gravity through a Sephadex G-50 column. To make reconstituted reduced Fe^2+ -globin, sodium dithionite was placed at the top of the G50 column for reduction during purification. Globin-containing fractions were concentrated on a Centricon®
filter (10-kDa cutoff), and the protein concentration was determined by a Bradford Assay. Reconstituted globins were used within 24 h of synthesis.

MDA Peroxidation Assays—MDA was measured by the thio-barbituric acid reactive substances assay as previously reported using reagents from the Zeptometrix MDA assay kit (16, 18). TLF-1 (125 nm) was incubated with equimolar globin dimer in 25 μl of total PBS for 5 min at room temperature. To this mixture, 175 μl of citric acid buffer at pH 4.8 containing 70 μM H₂O₂ was added and incubated at 37 °C in a benchtop thermostirker set to 300 rpm for 30 min. The reaction was quenched with 30 μM SDS solution, and then 770 μl of thio-barbituric acid solution was added, and the mixture was incubated for 1 h at 95 °C. Fluorescence readings were taken on a PerkinElmer Life Sciences LS55 spectrophotometer (excitation, 530 nm; emission, 550 nm; slit widths, 3 and 10 nm, respectively). MDA equivalents were determined from a linear MDA standard curve. Negligible fluorescence from samples with only globin and no TLF-1 were background subtracted from the TLF-1 plus globin samples.

Blue Native PAGE Assay—Blue native PAGE was utilized to observe reconstituted globin binding to human Hp1-1 (25). Nonreducing native gel contained three layers: 16% polyacrylamide in the bottom layer, 10% in the middle layer (where the HpHb complex migrated), and a 4% stacking gel. Measured equivalents of globin in PBS were incubated with 5 nmol of human Hp1-1 per well (Sigma) at room temperature for 5–10 min before mixing with loading buffer before loading the gel and running at 150 V. After the solvent front had moved into the second layer of the gel, the central cathode buffer was replaced with imidazole anode buffer for better contrast. Gel was fixed with methanol (40% v/v) and acetic acid (10% v/v), stained with colloidal Coomassie dye, and destained in 8% acetic acid.

Alexa TLF Uptake Assay—Alexa 488-labeled TLF-1 (Alexa-TLF) was prepared from TLF-1 using the Alexa 488 protein/antibody labeling kit (Invitrogen) and dialyzed against PBS (21). For uptake assays, Alexa-TLF and the indicated globin (100 equivalents) were incubated with 300 μl of 1 × 10⁷ cells/ml for 20 min in serum-free medium at 37 °C, washed twice in ice-cold serum-free media, and analyzed by flow cytometry with a 488-nm laser and 530/30-nm BP filter on a Beckman Coulter Cyan instrument with a 405 laser excitation and a 450/50 BP filter for bromobimane fluorescence and 485/42 BP filter for PI. FlowJo software was utilized for data analysis. Cells positive for propidium iodide were excluded from thiol analysis, and cell thios were determined as percentages of control cell fluorescence after background subtraction of unstained cell fluorescence. For the thiol analysis during TLF-1 treatment, BrBi at 50 μM was added to TLF-1-treated cells for 10 min at 37 °C before quenching into 5 μM propidium iodide on ice 1 min before flow analysis. Again, PI-positive dead cells were excluded from the thiol analysis, and BrBi fluorescence was normalized to untreated cell fluorescence.

Thiol Oxidation Is Involved in TLF-1 Lysis—Consistent with older reports, we observed a role of oxidative stress in TLF-1 lysis (12–14). As shown previously, preloading T. brucei brucei with the lipophilic antioxidant DPPD ameliorated TLF-induced lysis (Fig. 1A) (16). To confirm a role for DPPD in decreasing oxidative stress to T. brucei brucei, ROS in DPPD-treated cells was monitored using a fluorescent indicator DCF-DA. As expected, T. brucei brucei treated with H₂O₂ experienced an initial increase in ROS, and DPPD addition significantly ameliorated the peroxide-induced increase in ROS (Fig. 1B). We next investigated whether addition of peroxides to the TLF-1-treated T. brucei brucei stimulated lysis. Consistent with a role for oxidation in TLF-1 lysis, a sublethal concentration of tert-butyl-hydroperoxide (+OOH) significantly increased TLF-1 lysis of T. brucei brucei (Fig. 1C). We next inhibited the peroxide metabolism of T. brucei brucei using DEM (28). Like peroxides, sublethal concentrations of DEM dramatically sensitized the cells to TLF-1 killing (Fig. 1D). These results indicate that TLF-1 induces oxidative stress to T. brucei brucei, which may be ameliorated by antioxidants or exacerbated by pro-oxidants.

In addition to inhibiting trypanothione-based hydrogen peroxide metabolism, DEM binds to free thiols in cells (28). A fluorescent BrBi assay was used to quantify free thiols in trypanosomes (29). DEM competed with BrBi for thiol binding, depleting total cellular free thiols by ~30% at a 0.4 mM concentration (Fig. 1E). Thus, the effects of DEM may reflect thiol
binding activity rather than direct effects on hydrogen peroxide metabolism.

Free thiols are some of the first targets of peroxide-induced oxidation in cells (30). To investigate the role of thiol oxidation in TLF-1 lysis, the promiscuous free thiol-binding molecules BrBi and NEM were tested on *T. brucei brucei* treated with TLF-1. TLF-1 lysis of *T. brucei brucei* was increased by BrBi or NEM addition (Fig. 1, A and B). In the absence of TLF-1, neither BrBi nor NEM were toxic to cells (Fig. 1, F and G).

**Hpr-Hb Is Not the Peroxidase Involved in TLF Lysis**—The Hpr-Hb complex was previously suggested to be the source of peroxides during TLF-1 lysis (17). To evaluate the role of Hpr-Hb as a peroxidase involved in TLF-1 lysis, we first determined whether Hb catalyzed peroxidation of endogenous TLF-1 lipids in vitro (2A). We measured the lipid peroxidation by-product MDA in TLF-1 samples incubated at low pH in the presence of H₂O₂ at 37 °C. To investigate the role of the heme iron in this reaction, Hb was denatured, the heme was removed, and the resulting apoglobin was reconstituted with zinc protoporphyrin IX to form Zn²⁺-globin (Zn-globin) or reconstituted with heme to form Fe³⁺-globin (methemoglobin). Only iron-containing globins (Fe³⁺-globin, reduced Fe²⁺-globin, and Hb), but not reconstituted Zn-globin or apo-globin, induced TLF-1 lipid peroxidation (Fig. 2A).

The reconstituted Zn-globin was not as stable as the native Hb, necessitating fresh reconstitution for each set of experiments. However, binding of freshly reconstituted Zn-globin and Fe-globin to haptoglobin1-1 (Hp) was observed by blue native PAGE, indicating that most of the Zn-globin was in the native conformation (Fig. 2B). Furthermore, *T. brucei brucei* efficiently bound and endocytosed Alexa 488-labeled TLF-1 with Hb, Zn-globin, or Fe-globin, through HpHbR-dependent uptake (Fig. 2C). As expected, globin-free TLF-1 was not efficiently endocytosed because of a lack of HpHbR binding. Similarly, porphyrin-free globin was not bound and endocytosed by *T. brucei brucei*. Although porphyrin-free globin monomers will bind to haptoglobin, the Hp1-1 apo-globin complex is less structured than the HpHb complex, which may explain its lack of binding and uptake in the parasite (31).

TLF-1 lysis assays in vivo showed no difference in lysis based on the oxidative capacity of the globin. Zn-globin and Hb-bound TLF-1 killed the trypanosomes equally effectively (Fig. 3A). Furthermore, the antioxidant DPPD rescued TLF-1 lysis with either iron or Zn-globin, suggesting that hemoglobin is not the source for peroxide stress (Fig. 3B). The chlorine channel inhibitor DIDS, which inhibits lysis of TLF-1-treated *T. brucei brucei*, also saved cells from lysis regardless of the type of globin added (Fig. 3B) (2).

**Lysosomal Lipid Peroxides Are Not the Source of Oxidative Stress in TLF Killing**—The role of lysosomal lipid peroxidation in the TLF-1 lytic mechanism was next investigated. In addition to Hb uptake, transferrin also delivers iron to the lysosome and is a major source of oxidative stress in bloodstream form trypanosomes (20). To determine whether transferrin iron uptake played a role in TLF-1 lysis, we preincubated cells in serum-free medium with either 100 μM transferrin or apo-transferrin, followed by a 2-h TLF-1 lysis assay. There was no difference in lysis between the three samples, indicating that iron delivery to the lysosome is not important for TLF-1 lysis (Fig. 4A).

Bloodstream form *T. brucei brucei* express two cytosolic tryptaredoxin peroxidases that detoxify lipid hydroperoxides.
arising from the lysosome (19, 20). We asked whether \textit{T. brucei} with both cytosolic peroxidases knocked out (Px KO) were more sensitive to TLF-1 lysis. The lethal phenotype of the Px KO cells is rescued by addition of an antioxidant, Trolox, without which the cells die within 1 h at 37 °C (19, 20). The antioxidant Trolox, a water-soluble vitamin E derivative, did not inhibit TLF-1 lysis of wild-type \textit{T. brucei} at concentrations up to 100 μM (Fig. 4B). This result contrasts with the lipophilic antioxidant DPPD, which prevented TLF-1 lysis (Fig. 4B with Fig. 1A). The two antioxidants have different mechanisms and lipid solubility, perhaps partitioning into different regions of the cell, accounting for differences in their ability to modulate TLF-1 activity (32, 33). When Trolox was titrated down to levels allowing ~50% spontaneous cell death of the Px KO cells, TLF-1 addition did not cause a synergistic increase in lysis, indicating that the Px enzymes are not important for metabolizing TLF-1-induced lipid peroxides (Fig. 4C). Furthermore, in lysis assays where sufficient Trolox was added to rescue maintain viability of the Px KO cells, there was no difference in TLF-1 lysis between Px KO cells and wild-type \textit{T. brucei} (Fig. 4D). There was no difference in survival of the Px KO cells compared with wild-type \textit{T. brucei} treated with TLF-1 in an overnight lysis assay (Fig. 4E).

**Apol1 Initiates Lysis Involving Oxidative Stress**—We next asked whether the TLF-1 protein Apol1 induced the oxidative stress involved in TLF-1 lysis. Apol1 was purified from the...
native TLF-1 particles as described previously and was tested for lytic activity in the presence of antioxidants and pro-oxidants (22). To prevent high affinity uptake of trace amounts of TLF-1 possibly contaminating the purified ApoL1 preparation, assays were conducted in a HpHbR KO line of *T. brucei brucei* (21). The HpHbR receptor is not involved in purified ApoL1 uptake, and therefore HpHbR KO cells are equally susceptible to ApoL1 lysis as wild-type *T. brucei brucei*. Both ApoL1- and TLF-1-treated HpHbR KO cells responded to addition of the antioxidant DPPD and the pro-oxidants DEM and \( \text{H}_2\text{O}_2 \) (Fig. 5). Higher concentrations of TLF-1 were needed to kill the HpHbR KO cells than wild-type *T. brucei brucei*. However, HpHbR KO cell lines responded to antioxidants and pro-oxidants in a similar manner to wild-type *T. brucei brucei*. Thus, ApoL1-induced *T. brucei brucei* lysis involves oxidation even when the TLF-1 or ApoL1 uptake is much slower because of the lack of receptor-mediated uptake.

**Kinetics of Sensitivity to Peroxides**—Hydrogen peroxide was next used to assess the time point during TLF-1 lysis that *T. brucei brucei* become sensitive to oxidation. Unlike \( \text{H}_2\text{O}_2 \), \( \text{H}_2\text{O}_2 \) is metabolized by *T. brucei brucei* within several minutes (28). The lifetime of ROS produced by peroxides extends longer than the \( \text{H}_2\text{O}_2 \) molecular itself. In Fig. 1B, the level of ROS plateaus \( \sim 15-20 \) min after \( \text{H}_2\text{O}_2 \) addition. Because of its rapid metabolism, \( \text{H}_2\text{O}_2 \) was utilized as a time-sensitive pro-oxidant, which only causes oxidative stress to cells for a limited window of time after its addition. TLF-1 lysis was only increased by \( \text{H}_2\text{O}_2 \) if added after the cells had been incubated with TLF-1 for 20 min (Fig. 6A).

We next investigated whether TLF-1 internalization and activation preceded sensitivity to oxidative stress. Blocking endosomal fusion by low temperature (15 °C) has been shown to prevent TLF-1 lysis in a reversible manner (34). Parasite endosomes were loaded with TLF-1 at 15 °C, the free TLF-1 was
T. brucei brucei was treated with TLF-1, ApoL1, or a short term hypotonic shock (60% diluted media for a few minutes), and live cells were imaged by differential interference contrast microscopy. TLF-1, ApoL1, and hypotonic treatment all caused cells to form a kite-shaped swollen morphology easily distinguishable from the normal cell morphology (Fig. 8A). Osmotic swelling seems to be required for TLF-1 lysis, because when the assay medium was made hypertonic with high concentrations of cell-impermeable sucrose, cells no longer lysed (Fig. 8B). Importantly, sucrose rescued TLF-1-induced lysis even when added halfway through the 2-h lysis assay, after cell swelling had initiated. Hypertonic sucrose may prevent TLF-1 lysis of T. brucei brucei by preventing the ultimate influx of water, which directly leads to cell death.

Although hypertonic rescue of lysis has been demonstrated before, no one has ever published the effect of hypotonic conditions on TLF-1 lysis of T. brucei brucei. Therefore, a hypotonic shock was developed. Cells were incubated in hypotonic media (culture media diluted with water). Normally, when exposed to hypotonic shock, cells undergo regulatory volume decrease (RVD), recovering cell volume and motility within 10 min (35). In this assay, most untreated cells (shown in Fig. 7C as the 0-min time points) recovered from 60 or 70% hypotonic shock for 5 min (111 and 82 mosM; see “Experimental Procedures” for the unit conversion). But after only 12 min of incubation with TLF-1, T. brucei brucei becomes hypersensitive to hypotonic shock (Fig. 8C). Thus, even before visible cell swelling or death caused by TLF-1, ApoL1 induces osmotic stress at the plasma membrane.

**Hypotonic Shock Confers Susceptibility to Oxidation-induced Osmotic Lysis**—We tested whether peroxides mediated the response of T. brucei brucei to hypotonic stress, similar to how peroxides modulated the response of T. brucei brucei to ApoL1-induced osmotic stress. In the absence of peroxides, most T. brucei brucei cells recovered from 5 min of 0–70% hypotonic shock. In contrast, cells exposed to 4–20 μM H₂O₂ lost their ability to volume regulate, and osmotic lysis occurred (Fig. 9A). The observed lysis was not due to extensive oxidative stress within the cells, because ROS production in T. brucei...
brucei following H$_2$O$_2$ addition was not higher in hypotonic than in isotonic medium (Fig. 9B).

Following hypotonic shock, cells initially swell with water and then rapidly return to normal volume through activation of volume regulatory channels in the plasma membrane. To probe whether T. brucei brucei were most sensitive to peroxides during initial cell swelling, a longer-term 30 min 50% hypotonic stress assay was performed. Hydrogen peroxide (10 $\mu$M) was spiked into the cells in hypotonic media every 5 min for the duration of the 30-min assay (Fig. 9C). At all the time points
where H$_2$O$_2$ was incubated with cells for at least 5 min in hypotonic buffer, H$_2$O$_2$ dramatically induced osmotic lysis. At the 30-min time point when cells were immediately quenched into isotonic buffer for immediate counting, the peroxide had no effect on the cells. Moreover, if peroxides were added prior to hypotonic shock, there was no effect on the cells. Thus, H$_2$O$_2$ only affects cells undergoing osmotic stress concurrently with peroxide addition.

In contrast to the peroxide effect, the antioxidant DPPD did not make T. brucei brucei more resistant to osmotic lysis (Fig. 9D). The thiol-binding inhibitor DEM, like H$_2$O$_2$, stimulated osmotic lysis, although in this case, preincubation of the T. brucei brucei cells was required for DEM uptake (Fig. 9E). Similarly, the promiscuous free thiol-binding molecules BrBi and NEM stimulated osmotic lysis of T. brucei brucei (Fig. 9, F and G).

**Discussion**

The results in this paper provide an integrated model for oxidative stress and osmotic swelling during TLF lysis of T. brucei brucei. Earlier work suggested that the Hpr-Hb complex initiated the peroxides involved in TLF-1 lysis (16). However, using zinc-substituted globin, we showed that the iron in Hb is dispensable for TLF-1 lysis (Fig. 3). In addition, none of our data support lysosomal damage caused by ApoL1 in T. brucei brucei (2). Rather, our data are consistent with the model that the Hpr-Hb-Hb mediated TLF-1 internalization, ApoL1 alone initiates T. brucei brucei lysis (Fig. 5) (36). Moreover, our results support the recent model that ApoL1 induces cell swelling by channel formation in the plasma membrane of T. brucei brucei (7). We propose that ApoL1-induced ionic disruption of plasma membrane of T. brucei brucei leads to oxidation-stimulated osmotic lysis.

Our data suggest that oxidation of sensitive thiol groups in osmotically stressed T. brucei brucei leads to increased cell swelling and lysis. Over time, ApoL1 from TLF-1 induces osmotic stress to T. brucei brucei by disrupting ion gradients at the plasma membrane. Therefore, TLF-1-induced osmotic stress may be modeled by hypotonic stress. T. brucei brucei treated with either TLF-1 or hypotonic medium are extremely sensitive to lysis in the presence of peroxides or thiol-binding agents (Fig. 9). It appears when thiol oxidation occurs in the context of osmotic stress, the cells become unable to undergo RVD, inducing uncontrolled cell swelling and death. In the case of TLF-1 treatment, T. brucei brucei were not sensitive to hydrogen peroxide until ~20 min after TLF-1 addition (Fig. 6). The kinetics suggest that TLF-1-induced osmotic stress develops over time, perhaps as more ApoL1 molecules traffic out of the endosomes, and that osmotic stress precedes peroxide sensitivity.

We found that TLF-1 lysis is not due to overwhelming the cell with oxidative stress, in agreement with a previous study (Fig. 7) (18). In other cell types, hypoxic shock leads to ROS production (37–39). In T. brucei brucei, however, osmotic regulation may be disrupted by peroxides produced at endogenous levels or levels of peroxides below the limits of detection in our assays. Alternatively, thiol oxidation by a non-peroxide oxidizing agent may induce osmotic swelling during TLF-1 lysis. The exact origin and chemical nature of the oxidations in T. brucei brucei treated with TLF-1 remain
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unknown. The commonly cited candidates for the source of ROS production in mammalian cells are the mitochondria and NADPH oxidase. Bloodstream form trypanosomes, however, do not contain NADPH oxidase or an active electron transport chain in the mitochondria. We propose that local oxidation of a small number of cellular thiol(s) involved in osmotic volume regulation leads to uncontrolled cell swelling and lysis caused by TLF-1. Three diverse thiol-binding molecules (DEM, NEM, and BrBi) increased TLF-1 lysis and stimulated hypotonic lysis (Figs. 1 and 9). Because total free thiols do not decrease during TLF-1 lysis, global redox imbalance does not appear to be necessary for induction of osmotic lysis of *T. brucei brucei*.

The specific target(s) that are oxidized in osmotically stressed trypanosomes are unknown, but some candidates can be selected from the literature. A multifunctional intracellular ATPase in trypanosomes, TbVCP, is sensitive to NEM inhibition (40). In bloodstream form *T. brucei brucei*, NEM caused depolarization of the plasma membrane potential, presumably through inhibition of H^+^-ATPases (41). In addition to ATPases, phosphatases often contain redox-active cysteine residues that are sensitive to oxidation (42). In a related parasite, Trypanosoma rangeli, H_2O_2 was shown to inhibit the activity of phosphatases in the outer membrane, and thiol-reducing agents reversed phosphatase inhibition (43). In various organisms, ROS have been shown to activate ion channels and aquaporins (44–47). More directly, aquaporins can transport peroxides and therefore may contribute to trypanosome peroxide uptake, especially in cells under osmotic stress (48). In erythrocytes and vascular smooth muscle cells, KCl co-transport is activated by oxidizing agents, sulphydryl reagents, or hypotonic stress, but the oxidized proteins in the signaling cascades are unknown (49).

Like *T. brucei brucei*, in the insect parasite Crithidia NEM inhibited RVD and amino acid efflux after hypotonic stress (50). Similarly, in rat neonatal cardiomyocytes, endogenously produced reactive oxygen species inactivate RVD during hypotonic conditions (38). In rabbit hearts, the proposed signaling cascade linking osmotic swelling and ROS production involves peroxides activating swelling-activated chloride channels (51). In rat pheochromocytoma cells (PC12), either hypotonic shock or 100 μM H_2O_2 activated an outward rectifying chloride channel, and incubation in media with a high α-mannitol concentration prevented channel activation (47). Like α-mannitol in rat cells, high sucrose concentrations prevent TLF-1 lysis in *T. brucei* (Fig. 8B) (10). Moreover, ROS activation of chloride channels may occur during TLF-1 lysis of *T. brucei brucei*. Incubating TLF-1- or ApoL1-treated cells with the chloride channel inhibitor DIDS prevents lysis (Fig. 3B) (2).

The synergy between oxidative stress and osmotic lysis of trypanosomes might be exploited for drug development. Combination therapy with drugs that have synergistic mechanisms is a goal for anti-parasitic drug development, but the current clinically available drugs show no synergy in field studies (52). The nifurtimox/efluonithine combination therapy used to treat central nervous system *T. brucei gambiense* infection shows no mechanistic synergy but is used to reduce costs and slow the development of resistant parasites (53, 54). Exploiting the synergy between oxidative and osmotic stress may be possible utilizing existing drugs. Thiol-modulating drugs like the clinically used anti-trypanosomal drug melarsoprol may synergize with ionophores like salinomycin, which kills trypanosomes via cytoplasmic swelling, resembling TLF-1 lysis (55–57).

Various models for TLF-1-induced lysis of *T. brucei brucei* have been proposed. Historically, the localization of TLF-1 and ApoL1 in the lysosome led to a model where lysosomal damage caused cell lysis. In the current work, however, oxidative damage to the lysosome was not observed as a result of TLF-1 treatment (2). Moreover, a recent study observed that ApoL1-induced damage to the lysosome is not sufficient to induce lysis (8). Although it is indisputable that a high proportion of TLF-1 and ApoL1 traffics to the lysosome, the lysosome may represent the parasite way of detoxifying TLF-1 rather than the site of trypanolytic activity.

Our model for TLF-1 lysis involves ApoL1 trafficking to the flagellar pocket of *T. brucei brucei*, causing ionic imbalance at the plasma membrane (Fig. 10). Supporting this model, cellular swelling caused by osmotic imbalance is a hallmark of TLF-1-induced lysis (Fig. 8) (9, 15). There are notable similarities at both the morphological and biochemical level between *T. brucei brucei* swelling caused by TLF-1 and swelling caused by hypotonic stress. The observed cytoplasmic volume increase observed in TLF-1-treated *T. brucei brucei* resembles cell swelling caused by hypotonic shock, although RVD after hypotonic shock facilitates recovery of cell volume after a short time (9, 58). Both TLF-1 treatment and hypotonic shock are associated with potassium ion efflux and calcium ion influx in *T. brucei brucei* (9, 59, 60).

Another recently published study suggests that ApoL1 causes apoptosis-like cell death by trafficking to the mitochondria (8). It is important to note that in bloodstream form trypanosomes, apoptosis has many divergent features caused by the lack of a ROS-producing electron transport chain in the mitochondria, no cytochrome c, and no annotated caspases. Osmotic swelling of *T. brucei brucei* during TLF-1 incubation was not specifically addressed in the apoptotic model (8). Importantly, apoptosis is canonically associated with volume decrease, not volume increase caused by water influx. For instance, in *Trypanosoma cruzi*, volume decrease is associated with apoptotic cell death induced by serum complement (61). Nevertheless, many common features exist between osmotic stress and apoptosis in trypanosomatids, suggesting that the osmotic response and cell death pathways are interconnected. Hypotonic stress leads to an influx of calcium, and increased cytosolic calcium is an inducer of apoptosis (60, 62). Calcium influx occurs following TLF-1 addition to *T. brucei brucei*, and calcium is important for the lytic activity of human serum (9, 63). Future studies should elucidate whether ApoL1-induced effects to the plasma membrane induce mitochondrial effects or vice versa.

In conclusion, in this study we resolved several important questions surrounding the mechanism that the human innate immune factor TLF-1 utilizes to kill *T. brucei brucei*. First, oxidative stress is important to TLF-1 lysis but does not arise from Hpr-Hb in the lysosome. Second, oxidation of cellular thiols causes deregulation of ApoL1-induced osmotic swelling, exac-
erbating lysis. Our discoveries advance the understanding of cell death pathways in T. brucei brucei, which may facilitate the development of better drugs for the deadly disease human African trypanosomiasis.

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FIGURE 10. Model for oxidation-stimulated osmotic lysis of T. brucei brucei via ApoL1 trafficking to the plasma membrane. TLF-1 is endocytosed via the HpHbR in the flagellar pocket of T. brucei brucei. Inside an acidic endosomal vesicle, ApoL1 inserts into the membrane. The sodium channel activity of ApoL1 is inhibited in acidic conditions; thus, an X is drawn over the channel. The series of steps leading to trypanosome lysis are indicated in the figure. First, after recycling to the plasma membrane (PM) ApoL1 causes early ionic disruption and osmotic stress to T. brucei brucei at the plasma membrane (shown in purple). Next, peroxide-induced oxidation of cellular thiols stimulates osmotic deregulation (shown in blue). Finally, lytic cell death is caused by excessive influx of water through the plasma membrane of T. brucei brucei (shown in green).

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