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Haptoglobin-hemoglobin receptor independent killing of African trypanosomes by human serum and trypanosome lytic factors

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The haptoglobin-hemoglobin receptor (HpHbR) of African trypanosomes plays a critical role in human innate immunity against these parasites. Localized to the flagellar pocket of the veterinary pathogen *Trypanosoma brucei brucei* this receptor binds Trypanosome Lytic Factor-1 (TLF-1), a subclass of human high-density lipoprotein (HDL) facilitating endocytosis, lysosomal trafficking and subsequent killing. Recently, we found that group 1 *Trypanosoma brucei gambiense* does not express a functional HpHbR. We now show that loss of the *Tbb*HpHbR reduces the susceptibility of *T. b. brucei* to human serum and TLF-1 by 100- and 10,000-fold, respectively. The relatively high concentrations of human serum and TLF-1 needed to kill trypanosomes lacking the HpHbR indicates that high affinity *Tbb*HpHbR binding enhances the cytotoxicity; however, in the absence of *Tbb*HpHbR, other receptors or fluid phase endocytosis are sufficient to provide some level of susceptibility. Human serum contains a second innate immune factor, TLF-2, that has been suggested to kill trypanosomes independently of the *Tbb*HpHbR. We found that *T. b. brucei* killing by TLF-2 was reduced in *Tbb*HpHbR-deficient cells but to a lesser extent than TLF-1. This suggests that both TLF-1 and TLF-2 can be taken up via the *Tbb*HpHbR but that alternative pathways exist for the uptake of these toxins. Together the findings reported here extend our previously published studies and suggest that group 1 *T. b. gambiense* has evolved multiple mechanisms to avoid killing by trypanolytic human serum factors.

Keywords: trypanosome lytic factor, innate immunity, African sleeping sickness, haptoglobin hemoglobin, high density lipoprotein, haptoglobin related protein, apolipoprotein L-1

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Introduction

African trypanosomes are eukaryotic pathogens that cause important human and animal diseases. These parasites have evolved a variety of mechanisms to escape innate and acquired immunity, including the use of the variant surface glycoprotein (VSG) coat to cover the plasma membrane of the parasite providing a barrier against attack by complement.[1] The VSG coat also serves as the molecular decoy during antigenic variation, presenting an ever-changing target to the adaptive immune system of the mammal, thus allowing the parasites to evade antibody-mediated killing.[2] The subspecies of trypanosomes that infect humans face the additional challenge of encountering a unique innate defense mechanism mediated by two related serum proteins complexes. In the circulation of humans, TLF-1 is a minor subclass of HDL containing apolipoprotein A-1 (apoA-1), the defining protein of all HDLs, and two primate specific proteins, apolipoprotein L-1 (apoL-1) and haptoglobin-related protein (Hpr).[3-7] In addition to these apolipoproteins, Hpr binds free hemoglobin (Hb) in the circulation, which is likely released from erythrocytes during early infection.[8] The heterodimeric Hpr/Hb complex is proposed to be bifunctional, serving both as the ligand for the *T. b. brucei* HpHbR[9,10] and directly contributing to high specific activity killing by catalyzing the peroxidation of lysosomal membrane lipids.[6,7,11] The other primate specific apolipoprotein in TLF-1, apoL-1, is also directly involved in *T. b. brucei* killing.[5,12,13] An ion channel forming protein, apoL-1 undergoes conformation

changes at lysosomal pH and can integrate into membranes.[5,12,14] The combined action of Hpr/Hb and apoL-1 results in the osmotic lysis of the parasite.[15,16] The other trypanolytic serum complex is called TLF-2 and, while largely devoid of lipids it contains Hpr and apoA-1[17] and apoL-1 (this paper) suggesting that these complexes share a common origin and perhaps have a similar mechanism of trypanosome killing.[18]

The two subspecies of human sleeping sickness trypanosomes have evolved distinct mechanisms to survive in the human host. *Trypanosoma brucei rhodesiense* produces the serum resistance associated (SRA) protein that binds and inhibits TLF-1.[19-21] SRA, an intracellular protein largely found in endosomes, co-localizes with TLF-1 in early endosomes and traffics to the lysosome.[22] Thus, *T. b. rhodesiense* survives in humans largely because it is able to produce an antidote to TLF-1. While untested, it is likely that TLF-2 is inhibited by SRA by the same mechanism since both serum complexes contain apoL-1. In contrast, we recently showed that group 1 *T. b. gambiense* does not bind or take up TLF-1, suggesting that these cells have evolved a different mechanism to avoid the cytotoxicity of TLF-1.[23] The underlying basis for reduced TLF-1 uptake is 2-fold. First, *Tbg*HpHpR is expressed at very low levels by group 1 *T. b. gambiense* and second, *Tbg*HpHbR contains a number of point mutations within the coding sequence that render the receptor non-functional.[23] The combination of mutations to the *Tbg*HpHbR and reduced expression abolished TLF-1 binding and uptake resulting in resistance to TLF-1. Thus, in contrast to *T. b. rhodesiense*, it appears the mechanism of group 1 *T. b. gambiense* resistance to TLF-1 involves reduced uptake and avoidance of the toxin. To date no evidence for an inhibitory protein with SRA-like characteristics has been described in *T. b. gambiense*. In this short addendum to the Kieft et al. paper[23] we now show that while the *Tbb*HpHbR enhances susceptibility to human serum, TLF-2 and TLF-1 other receptors or fluid phase endocytosis also contribute to trypanosome killing. Further, our results suggest that the resistance of group 1 *T. b. gambiense* to human serum and TLF involves other mechanisms beyond the simple loss of a single receptor.

Materials and Methods

TLF-1 and TLF-2 purification. Total serum was obtained from a healthy human donor. As previously described, two sequential flotations on sodium bromide gradients (ϱ = 1.063 and 1.26 g/ml) resulted in an HDL-rich fraction (TLF-1; top third of the gradient) and a lipoprotein-deficient fraction (TLF-2; bottom third of the gradient).[4] The TLF-1 fraction was passed over an anti-IgM column (Sigma, A9935). The unbound material was then passed over an anti-Hpr column, washed with PBSE (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 3 mM EDTA) and bound protein was eluted in 100 mM glycine (pH 2.5) and neutralized with 1 M Tris (pH 7.5). The TLF-2 fraction was passed over an anti-Hpr column and washed with PBSE. Bound protein was eluted in 100 mM glycine (pH 2.5), neutralized with 1 M Tris (pH 7.5) and immediately added to an anti-IgM column and washed with PBSE. Bound protein was eluted in 100 mM glycine (pH 2.5), neutralized with 1 M Tris (pH 7.5). All protein samples were aliquoted and stored at −80°C.

Size exclusion chromatography and protein gel blot analysis. Size exclusion chromatography was performed on a 1 X PBSE equilibrated Superose 6 10/300 GL column (GE Healthcare). Individual protein standards were used to estimate the molecular weights of TLF-1 and TLF-2. Samples of TLF-1 and TLF-2 from immuno-affinity purification (70 μg) were run on the Superose 6 column at a flow rate of 0.5 ml/min. Fractions were collected (0.5 ml), proteins concentrated 6-fold with microspin S-300HR columns (GE, 27513001) and the distribution of Hpr, apoL-1 and IgM determined by SDS-PAGE, silver staining and protein gel blot analysis. Characterization of antibodies against Hpr and apoL-1 has previously been described.[7] Anti-IgM was purchased from Sigma and used according to the manufacturer's recommendation (Sigma, I0759).

Results

TLF-1 resistant *T. b. brucei*. During the course of our studies on the mechanism of TLF-1 resistance in group 1 *T. b. gambiense* we developed a laboratory model for TLF-1 resistance using well-characterized clonal cell lines of *T. b. brucei* that had been selected for resistance to human HDLs.[23,24] We isolated TLF-1 resistant (R) or susceptible (S) *T. b. brucei* lines expressing either the VSG800 or VSG060.[23] The *T. b. brucei* 427-800^R and *T. b. brucei* 427-060^R lines showed reduced uptake of TLF-1 relative to the TLF-1 susceptible parental *T. b. brucei* 427-221^S cells and TLF-1 susceptible cells expressing either VSG800 or VSG060. In addition, we showed that the expression of *Tbb*HpHbR mRNA was reduced approximately 20-fold in resistant cells.[23] These findings led us to examine group 1 *T. b. gambiense* where we found that not only was expression of the *Tbg*HpHbR mRNA reduced but that mutations to the gene abolished function.[23]

Purification and characterization of TLF-1 and TLF-2. In order to determine whether loss of *Tbb*HpHbR was sufficient to provide complete protection from human serum, TLF-1 and TLF-2 activity we developed a purification protocol exploiting physical and compositional differences in these human serum innate immune complexes (**Fig. 1**). Freshly collected human plasma was initially separated by density gradient ultracentrifugation to produce HDL-enriched (ϱ 1.063–1.26 g/ml) and lipoprotein-deficient fractions ($\varrho < 1.063$ g/ml) that were used as the starting materials for TLF-1 and TLF-2 purification respectively. During the purification of TLF-1 small amounts of contaminating TLF-2 were removed from the HDL-containing fraction by absorption with anti-IgM. TLF-1 was subsequently bound to anti-Hpr resin, washed extensively at neutral pH to remove human HDLs lacking Hpr and eluted at low pH. TLF-2 was purified from the lipoprotein-deficient serum by sequential affinity chromatography with anti-Hpr followed by binding and elution from an anti-IgM column. The purity of TLF-1 and TLF-2 was evaluated by size exclusion chromatography on Superose 6 and protein gel blot analysis with anti-Hpr, apoL-1 and IgM (**Fig. 1A and B**). Based on size exclusion chromatography, TLF-1 and TLF-2 have estimated relative sizes of 576 kDa and 1.6 MDa

respectively.[17] Superose 6 chromatography of purified TLF-1 and TLF-2 revealed somewhat dispersed distributions consistent with particle heterogeneity but there was minimal overlap of the TLF-1 and TLF-2 absorbance peaks at 280 nM (**Fig. 1A**). Protein gel blot analysis revealed no contaminating TLF-2 in our purified TLF-1 preparations based on the lack of anti-IgM reactive material on protein gel blots (**Fig. 1B**; data not shown). TLF-2 preparations were highly enriched in particles containing Hpr, apoL-1 and IgM; however, these preparations also contained small amounts of IgM deficient complexes with an elution time (~28 min) from the Superose 6 column consistent with TLF-1. Based on the distribution of the Hpr dimer and IgM across the size exclusion fractions we estimate the amount of contaminating TLF-1 in these preparations to be ~10%.

Susceptibility of *T. b. brucei* to human serum, TLF-1 and TLF-2. Our previous studies compared the short-term killing of trypanosomes to TLF-1.[23] Here we have re-examined the susceptibility of these *T. b. brucei* lines using a long-term growth assay (**Fig. 2**). Consistent with previous studies, the parental *T. b. brucei* 427-221^{S},

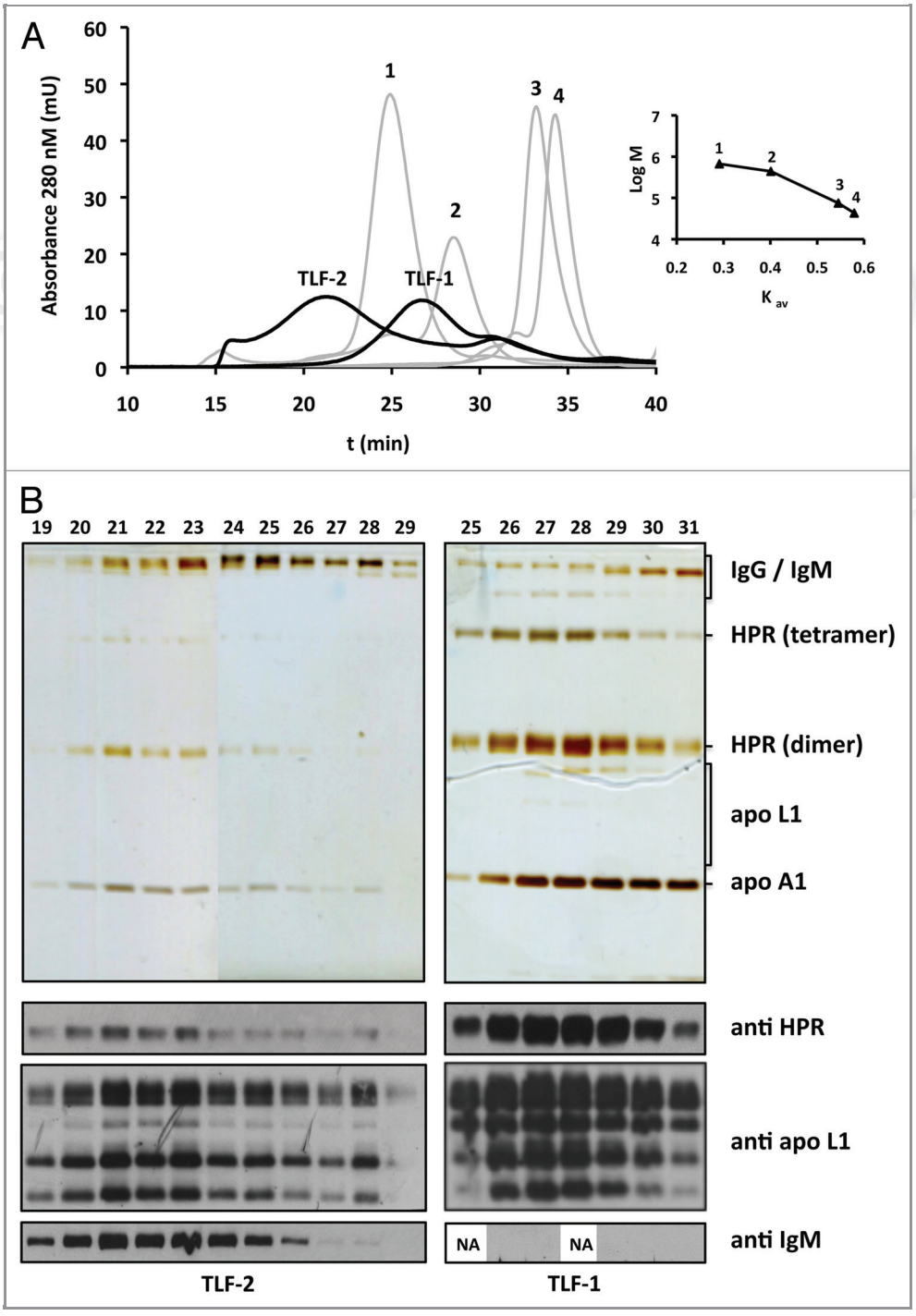

Figure 1. Characterization of purified TLF-1 and TLF-2. (A) Superose 6 size exclusion chromatography of TLF-1 and TLF-2. Absorbance profiles (280 nM) of TLF-1 and TLF-2, superimposed on individually ran marker proteins [1, thyroglobulin (660 kDa); 2, apoferritin (480 kDa); 3, conalbumin (67 kDa); 4, ovalbumin (45 kDa)]. (B) Analysis of individual Superose 6 column fractions of TLF-1 and TLF-2 separated on non-denaturing 12% SDS-PAGE and silver stained (top panel). Hpr, apoL1 and IgM were detected by protein gel blot. NA, not analyzed.

T. b. brucei 427-800^{S}, and *T. b. brucei* 427-060^{S} were highly susceptible to TLF-1 with a calculated LG_{50} of 0.8–6 ng/ml. *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} were > 10,000-fold more resistant to TLF-1 than wild-type *T. b. brucei*, suggesting the *Tbb*HbHbR is important in TLF-1 susceptibility. However, concentrations of > 10 μg/ml overcame the *Tbb*HpHbR deficiency leading to reduced survival (**Fig. 2A**). Since the concentration of TLF-1 needed to kill *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} is similar to that found in human serum it is likely that *Tbb*HpHbR-independent mechanisms of TLF-1 uptake play a significant role in trypanosome killing.

Based on our studies with both the group 1 *T. b. gambiense* and the TLF-1 resistant *T. b. brucei* lines we predicted that loss of a functional *Tbg*HpHbR played a critical role in human infection by African trypanosomes.[23] The dramatic reduction in susceptibility to TLF-1 in the *Tbb*HpHbR-deficient cell lines supports this prediction (**Fig. 2A**). However, the possibility remained that human serum contained additional innate immune factors, such as TLF-2, that might not require the *Tbb*HpHbR. To test this possibility, we treated TLF-1 resistant and susceptible *T. b. brucei* lines with human serum (**Fig. 2B**). We found that *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} were approximately 100-fold more resistant to human serum killing than either *T. b. brucei* 427-800^{S} or *T. b. brucei* 427-060^{S} (**Fig. 2B**). Based on these results, we conclude that loss of *Tbb*HpHbR expression contributes to the overall resistance of these cells to human serum; however the level of resistance is much less than the high level of resistance seen for TLF-1 (10,000-fold). A possible interpretation of these findings is that other human serum components, such as TLF-2, are less dependent on *Tbb*HpHbR binding than TLF-1.

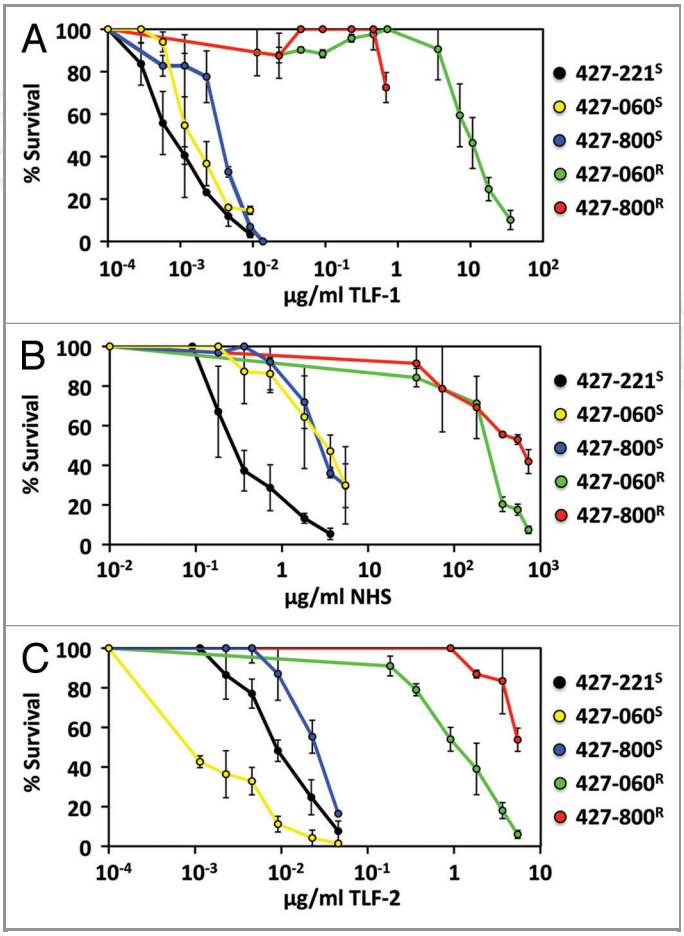

Figure 2. In vitro activity of human serum, TLF-1 and TLF-2. TLF-1 resistant (R) and susceptible (S) clonal cell lines of bloodstream form *T. b. brucei* Lister 427 expressing VSG221, 800 and 060 were prepared as previously described.[24,25] The percentage surviving cells was determined, using phase contrast microscopy, 14 h following the addition of TLF-1, TLF-2 or complete human serum to exponentially growing cultures at 37°C. (A) TLF-1 susceptibility of *T. b. brucei* 427-221^{S} (black), *T. b. brucei* 427-800^{S} (blue), *T. b. brucei* 427-800^{R} (red), *T. b. brucei* 427-060^{S} (yellow) and 427-060^{R} (green). (B) Normal human serum (NHS) susceptibility of *T. b. brucei* 427-221^{S} (black), *T. b. brucei* 427-800^{S} (blue), *T. b. brucei* 427-800^{R} (red), *T. b. brucei* 427-060^{S} (yellow) and 427-060^{R} (green). (C) TLF-2 susceptibility of *T. b. brucei* 427-221^{S} (black), *T. b. brucei* 427-800^{S} (blue), *T. b. brucei* 427-800^{R} (red), *T. b. brucei* 427-060^{S} (yellow) and 427-060^{R} (green).

It has been proposed that TLF-2 can bind to *T. b. brucei* in the absence of the *Tbb*HpHbR.[18] We tested whether highly purified TLF-2 was able to kill *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} (**Fig. 2C**). Similar to our findings with complete human serum, these *Tbb*HpHbR-deficient cells were more resistant to TLF-2 relative to the wild-type *Tbb*HpHbR-expressing cell lines. Thus, reduced expression of *Tbb*HpHbR expression caused a reduced susceptibility of TLF-2 killing suggesting that TLF-2 can bind to the *Tbb*HpHbR. However, the toxic concentration of TLF-2 is >10-fold less than TLF-1, indicating that *Tbb*HpHbR-independent mechanisms may play a greater role in TLF-2 binding, uptake and killing.

Discussion

In the studies presented here human serum, TLF-1 and TLF-2 susceptibility was examined in isogenic lines of *T. b. brucei* differing in *Tbb*HpHbR expression. Cells deficient in *Tbb*HpHbR expression were 10,000-fold more resistant to TLF-1 relative to wild-type susceptible cells. However, at concentrations of TLF-1 typically found in serum (>10 μg/ml), both resistant and susceptible cell lines were killed. Human serum killing was also reduced approximately 100-fold in cell lines expressing reduced levels of *Tbb*HpHbR. However, significant killing was still observed at human serum

concentrations above 100 μg/ml. Since the only difference in the susceptible and resistance cell lines is the levels of expression of *Tbb*HpHbR, it seems likely that human serum contains a second trypanolytic activity that interacts with *T. b. brucei* independently of the *Tbb*HpHbR. Based on this interpretation of the human serum killing results we decided to investigate whether TLF-2 killing of *T. b. brucei* was independent on the level of expression of *Tbb*HpHbR. We found that TLF-2 killing was reduced 500–1000-fold in cell lines with reduced levels of *Tbb*HpHbR, suggesting that TLF-2 also binds *Tbb*HpHbR. These results are in apparent contrast to previous studies on TLF-2 showing that TLF-2 killing was not inhibited by the addition of haptoglobin, an inhibitor of HpHb binding to the *Tbb*HpHbR.[17] These results have been used subsequently to argue that TLF-2 does not bind the *Tbb*HpHbR.[10] It is possible that our results are influenced by the small amount of contaminating TLF-1 in our TLF-2 preparations. Clearly, a detailed characterization of the TLF-2 binding, uptake and cellular location is needed.

Our results are consistent with previous findings indicating the importance of the *Tbb*HpHbR in TLF-1 killing but also suggest that other mechanisms of TLF-1 binding and uptake may contribute to trypanosome killing. The most likely pathways for *Tbb*HpHbR-independent uptake of TLF-1 is either by fluid phase endocytosis or the trypanosome lipoprotein scavenger receptor[18,25]. The findings presented here further support the findings of others that TLF-2 killing is less dependent on the *Tbb*HpHbR than is TLF-1[10,17]. Finally, we propose that group 1 *T. b. gambiense* have evolved multiple mechanisms, including but not limited to the loss of a functional HpHbR, to avoid the cytotoxicity of the trypanosome lytic factors. We are currently exploring these mechanisms.

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