A Single Amino Acid Substitution in the Group 1 Trypanosoma brucei gambiense Haptoglobin-Hemoglobin Receptor Abolishes TLF-1 Binding

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Abstract

Critical to host innate immunity against African trypanosomes is a minor subclass of human high-density lipoproteins, termed Trypanosoma Lytic Factor-1 (TLF-1). This primate-specific molecule binds to a haptoglobin-hemoglobin receptor (HpHbR) on the surface of susceptible trypanosomes, initiating a lytic pathway. Group 1 Trypanosoma brucei gambiense causes human African Trypanosomiasis (HAT), escaping TLF-1 killing due to reduced uptake. Previously, we found that group 1 T. b. gambiense HpHbR (TbgHpHbR) mRNA levels were greatly reduced and the gene contained substitutions within the open reading frame. Here we show that a single, highly conserved amino acid in the TbgHpHbR ablates high affinity TLF-1 binding and subsequent endocytosis, thus evading TLF-1 killing. In addition, we show that over-expression of TbgHpHbR failed to rescue TLF-1 susceptibility. These findings suggest that the single substitution present in the TbgHpHbR directly contributes to the reduced uptake and resistance to TLF-1 seen in these important human pathogens.

Introduction

Primate specific innate immunity plays a decisive role in defining the host range of African trypanosomes. Trypanosoma brucei brucei, Trypanosoma congolense and Trypanosoma vivax infect both domesticated and wild mammals but are unable to infect most primates, including humans, because of their susceptibility to two primate specific innate immune complexes, Trypanosoma Lytic Factor-1 (TLF-1) and TLF-2 [1–3]. TLF-1 and TLF-2, isolated from humans, have similar protein compositions. Both complexes contain apolipoprotein A-1 (apoA-1), a characteristic protein of high-density lipoproteins (HDLs), and two primate-specific proteins, apolipoprotein L-1 (apoL-1) and haptoglobin related protein (Hpr) [3–8]. Despite similarities in protein composition, the two complexes differ significantly. TLF-2 containing IgM and having little associated lipid, while TLF-1 is a minor sub-class of HDL (ρ = 1.21–1.26 g/ml) which is ~40% lipid by mass [9].

TLF-1 killing of T. b. brucei requires high affinity binding within the flagellar pocket, a specialized region of the trypanosome cell surface, followed by endocytosis and lysosomal localization [10]. Within the acidic lysosome, TLF-1 is activated leading to disruption of the lysosome and cell lysis [5,10–13]. Critical to initiating the lytic pathway is the binding of TLF-1 to the T. b. brucei haptoglobin-hemoglobin receptor (TbHpHbR) [11,14,15]. Haptoglobin (Hp) is an acute phase protein produced at high levels in all mammals, which binds and detoxifies free hemoglobin (Hb) by facilitating its clearance from the circulation [15]. Since African trypanosomes are heme auxotrophs, TbHpHbR has been proposed to function as a nutrient receptor providing heme to these parasites [14]. Unlike the mammalian HpHb scavenger receptor (CD163) the TbgHpHbR also binds Hpr present in TLF-1 when complexed with Hb [16].

Two mechanisms of trypanosome resistance to TLF-1, and therefore human infectivity, have been described [17]. Trypanosoma brucei rhodesiense, the cause of acute human African trypanosomiasis (HAT), has evolved the human serum resistance associated protein (SRA), which binds and neutralizes TLF-1 killing [16–19]. A member of the variant specific glycoprotein (VSG) family, SRA, is a glycophatidylidyinositol-anchored protein that is synthesized in the endoplasmic reticulum and transiently presented on the surface of the trypanosome within the flagellar pocket. However, its steady state distribution suggests it is rapidly endocytosed and localized predominately to endosomes in T. b. rhodesiense [20–22]. SRA tightly binds the apoL-1 component of TLF-1, providing complete protection against TLF-1 killing [5]. It is assumed that SRA also binds apoL-1 in TLF-2 and inhibits its activity. Trypanosoma brucei gambiense, the causative agent of chronic HAT, lacks SRA. We recently reported that expression of the T. b. gambiense HpHbR (TbgHpHbR) in the group 1 subtype of T. b. gambiense, suggesting that decreased expression of the receptor contributed to TLF-1 resistance and human infectivity [23]. We also observed that the TbgHpHbR gene, from four distinct geographic isolates of group 1 T. b. gambiense, contained four non-synonymous amino acid substitutions within the coding sequence for the mature protein [23]. A more extensive analysis of a large number isolates further revealed a single leucine (L) to serine (S) substitution, at amino acid 210 of TbgHpHbR which was conserved in all group 1 T. b. gambiense
isolation catalyzes a wide variety of mammals; however, only two sub-species, Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense, are able to infect humans. A human innate immune molecule, trypanosome lytic factor-1 (TLF-1), is responsible for this selective protection. TLF-1 killing requires high affinity binding to the trypanosome haptoglobin-hemoglobin receptor (HpHbR), which initiates endocytosis and lysosomal localization of the toxin. T. b. gambiense infects humans because it does not bind TLF-1, and several amino acid changes in the HpHbR are conserved in group 1 T. b. gambiense. To better understand the mechanism of resistance in these parasites, we analyzed TLF-1 binding to trypanosomes expressing the T. b. gambiense HpHbR (TbgHpHbR) and variants in which single amino acids were changed. Our studies showed that a single, highly conserved, amino acid substitution in the TbgHpHbR was sufficient to ablate high affinity TLF-1 binding and contributed to TLF-1 resistance. This likely plays a key role in human infectivity by group 1 T. b. gambiense.

Methods

Trypanosomes, growth, and transfections

Bloodstream form T. b. brucei Lister 427(MiTat 1.2) were grown at 37°C under 5% CO2 in HMI-9 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 10% Serum-Plus (Sigma-Aldrich). HpHbR KO constructs were generated after cloning HpHbR flanking sequences onto blasticidin and hygromycin resistance genes [23]. All primers used in the studies reported here are listed in Table S1. 5 × 10^7/ml trypanosomes were transfected with 5 µg of NotI digested DNA using the Amaxa Nucleofector Kit (program X-001). Transfected cells were then allowed to recover for 24 hours before addition of blasticidin (2.5 µg/ml) or hygromycin (2.5 µg/ml). Cell lines were clonally selected prior to a second round of transfection. To obtain HpHbR double knockout cell line, we transfected the second HpHbR drug resistance construct into the single allele TbgHpHbR^−/− line. To examine the effects of amino acid substitution on HpHbR function, stable cell lines expressing ectopic copies of the TbgHpHbR or the individual TbgHpHbR substitutions were prepared by targeting to the tubulin locus and selection with phleomycin (2.5 µg/ml) [23]. To determine growth rates, cells were grown to mid-log phase and diluted to 1 × 10^7/ml. Cell counts, determined by hemocytometer, were carried out until stationary phase. Growth curve data is in triplicate.

Epitope tagging of HpHbR

An HA-epitope tag was cloned into the TbgHpHbR construct via a three-step PCR method. The HA-tag was added downstream of the signal peptide. Once completed, the construct was sequenced and digested with NotI and Apal (5 µg total) prior to transfection. Transfections and cloning were carried out as described above.

TbgHpHbR mutant cell lines

The construct used to generate the TbgHpHbR cell line [23] was subjected to site-directed mutagenesis to generate the four TbgHpHbR substitutions of S210L, V293A and GA369-370EG. Mutagenesis was carried out according to manufactures instructions (Agilent Technologies). TbgHpHbR^−/− cells were transfected independently with the mutagenized constructs. Transfections and cloning were carried out as described above. Mutant TbgHpHbR constructs were sequenced with HpHbR sequence primers (sense and antisense). To prepare an HpHbR over-expressing cell line, PCR products were generated with Platinum High Fidelity Taq Polymerase (Invitrogen), gel purified, digested with EcoRI and cloned into the pURAN over-expression constructs [27]. Prior to transfection into TbgHpHbR^−/− cells, pURAN HpHbR constructs were linearized with BstXI. Both strands were sequenced with HpHbR sequence primers (sense and antisense).

TLF-1 purification, lytic activity and binding

TLF-1 purification, labeling and survival assays were performed as previously described [28]. Briefly, for the survival assays, trypanosomes were harvested from mid-log phase cultures, washed and re-suspended at a final concentration of 1 × 10^7/ml in complete HMI-9 media. Susceptibility to hemoglobin (Hb) bound TLF-1 was determined over a range of TLF-1 concentrations following incubation at 37°C for 16 hours. The number of surviving cells was determined by hemocytometer count with phase contrast microscopy. All survival assays were done in triplicate.

Southern analysis

For Southern analysis, 5 µg genomic DNA was digested with EcoRI. DNA was fractionated on a 0.6% agarose gel and fractionated for 3 hours in a 1 x 10^-7/ml complete HMI-9 media. Susceptibility to hemoglobin (Hb) bound TLF-1 was determined over a range of TLF-1 concentrations following incubation at 37°C for 16 hours. The number of surviving cells was determined by hemocytometer count with phase contrast microscopy. All survival assays were done in triplicate.
Table S1. Blots were then washed in a solution containing 3 x SSC/0.1% SDS at 55°C for 30 minutes then a final stringency of 0.3 x SSC/0.1% SDS at 65°C for 20 minutes. Blots were exposed to a storage phosphor screen (Molecular Dynamics) and analyzed on a STORM-860 PhosphorImager (GE Healthcare).

TLF-1 binding and uptake studies

All TLF-1 binding and uptake studies were carried out with Alexa-Fluor 488 TLF-1 that was labeled according to manufacture instructions (Invitrogen). Alexa-488 TLF-1 was incubated with an excess of Hb for 10 minutes on ice prior to analysis of binding. The binding and uptake of Alexa-488 TLF-1 was examined using either fluorescence microscopy or FAC analysis. To measure the amount of binding by fluorescence microscopy, the fluorescence intensity values from AxioVision v4.6 software (www.zeiss.com) was plotted versus TLF-1 concentrations. Imaging was carried out using a Zeiss Axio Observer inverted microscope. Quantification of Alexa-488 TLF-1 was done on compressed images.

To measure TLF-1 uptake by FAC analysis, cells were grown to mid-log phase, collected, washed and resuspended (1 x 10^7/ml) in HMI-9 supplemented with 1% bovine serum albumin (BSA), 1% glucose. Alexa-488 TLF-1, and excess Hb, were added to the cells followed by incubation at 37°C for 30 minutes. Uptake was stopped by placing the tubes on ice followed by two washes with ice-cold phosphate buffered saline buffer (PBS) (10 mM NaPi, 137 mM NaCl, pH 7.4). The amount of TLF-1 uptake was determined using CytoSoft cytometer (DAKO) and FlowJo software. Uptake was also measured by fluorescence microscopy. Following incubation, cells were washed two times with ice cold PBS. Following the washes, cells were spread onto glass slides, methanol-fixed for 5 min, at -20°C, and analyzed by fluorescence microscopy. Images were captured with the same exposure and were contrasted to the same extent. To analyze only binding in the flagellar pocket, PFA fixed and methanol treated binding to the flagellar pocket, PFA fixed and methanol treated samples were transferred to 3°C for 2 hours. Cells were then transferred to ice, washed with ice-cold 1 x PBS and analyzed by CytoSoft cytometer and FlowJo software. For studies without Hb, competitors were added to Alexa-488 TLF-1/Hb (6 nM) in the same increasing molar concentrations and taken through the same protocol as previously described. All competition studies were done in triplicate.

RT-PCR of expressed HpHbR and qPCR

Total RNA was isolated with TriPure Isolation Reagent (Roche). cDNA was generated in a Reverse Transcription (RT) reaction (Promega). Control reactions were performed with enolase, as well as reactions without added RT. Real time PCR was performed with and iCycler (iQ5 multicolor real-time PCR detection system; Bio-Rad) using cDNA from an equivalent of 20 ng of total RNA. 6 pmol sense primer, 6 pmol antisense primer, 10 µl SYBR green PCR master mix (Fermentas) in a final volume of 20 µl. Real time PCR conditions were: one cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The relative amounts of specific cDNA between samples were calculated using CT values calculated with the iQ5 optical detection system software. All qRT-PCR reactions were carried out with a splice leader RNA sense primer and gene specific anti-sense primers. Triplicate analyses were performed for each parasite line. All primers were designed using Integrated DNA Technologies software.

Immunoblotting analysis

For western analysis of HA-tagged HpHbR, total cellular protein was prepared from TbbHpHbR^-/-, Rab5aHA and TbgHpHbRHA cell lines and analyzed, based on cell equivalents, as previously described [22]. Rat monoclonal anti-HA-horseradish peroxidase (Roche Diagnostics, Indianapolis, IN) was used at a dilution of 1:1,000, with streptavidin-HRP conjugate (Invitrogen, Camarillo, CA) used for secondary detection at 1:5000.

Results

Generation of an HpHbR^-/- cell line

Previously, we described the isolation of a TLF-1 resistant line of T. b. brucei following in vitro selection for growth in the presence of human HDLs [25,29]. The resistance phenotype correlated with reduced expression of the TbbHpHbR, susceptibility being restored by ectopic expression of the TbbHpHbR from a different chromosomal locus. We also observed that ectopic expression of the TbgHpHbR failed to restore TLF-1 uptake or susceptibility, suggesting that substitutions to the TbgHpHbR might contribute to TLF-1 resistance in this important human pathogen [23]. Initial sequence analysis of four T. b. gambiense isolates revealed five non-synonymous amino acid substitutions, four in the coding sequences of the mature protein, when compared to TbbHpHbR [23]. To test whether these substitutions lead to loss of TLF-1 binding, we generated a T. b. brucei HpHbR^-/- knockout cell line and then systematically tested the ability of each of the four substitutions to restore TLF-1 binding to the TbgHpHbR.

The HpHbR knockout cell lines were prepared in T. b. brucei 427-221 (Lasser 427) cells by sequentially replacing the complete coding sequence for each TbbHpHbR allele with the coding sequences for hygromycin and/or blasticidin (Figure 1A). Replacement of the coding sequence for TbbHpHbR, in both single TbbHpHbR^-/- (sKO) and double TbbHpHbR^-/- (KO) knockouts, was verified by Southern blot hybridization of genomic DNA digested with EcoRI. The expected size restriction fragments were

For each image set.

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detected when blots were hybridized with a probe complementary to the 5' flanking sequence of \(Tbb\)HpHbR. A 7.0 kb fragment was detected in untransfected \(Tbb\)HpHbR while EcoRI sites in both the blasticidin and hygromycin gene constructs gave rise to smaller fragments (3.9 kb and 4.2 kb respectively) (Figure 1A). PCR analysis of genomic DNA from WT and \(Tbb\)HpHbR\(^{-/-}\) cells, with oligonucleotide probes complementary to coding sequences in the \(Tbb\)HpHbR, showed that the \(Tbb\)HpHbR gene had been deleted in the \(Tbb\)HpHbR\(^{-/-}\) cells (Figure S1). Furthermore, (q)RT-PCR with total RNA from WT \(T. b. brucei\) and \(Tbb\)HpHbR\(^{-/-}\) showed that double knockout cells do not express \(Tbb\)HpHbR (Figure 1B, Table S2). The generation of a stable

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**Figure 1. Generation of \(Tbb\)HpHbR\(^{-/-}\) cells.** (A) Both allelic copies of the \(Tbb\)HpHbR were replaced by homologous recombination with the blasticidin (B) and hygromycin (H) resistance cassettes. Southern analysis was carried out with DNA from \(T. b. brucei\) (WT), blasticidin \(HpbR\) single knockout line (sKO) of the \(Tbb\)HpHbR and a line with both alleles replaced (KO). Because of internal EcoRI (E) sites in the drug resistance constructs, fragments of 3.9 kb and 4.2 kb are generated in contrast to the 7 kb \(Tbb\)HpHbR in WT cells. (B) Expression levels of HpHbR mRNA were determined by RT-PCR from WT and KO cells. Enolase was used as the loading control. (C) In vitro growth of WT and KO cell lines at 37°C. (D) DIC and fluorescence microscopy of the WT and KO cell lines after incubation with Alexa-488 TLF-1 (37°C for 30 minutes). Higher magnification images (right side of panel) were analyzed with the position of the kinetoplast (k), and nucleus (n) was visualized by DAPI staining. (E) \(T. b. brucei\) WT cells were incubated with Alexa-488 TLF-1 for 1 hour at 3°C over a range of 0 to 66 nM. Binding was localized to the flagellar pocket and was concentration dependent. (F) Susceptibility of \(T. b. brucei\) (WT) and \(Tbb\)HpHbR\(^{-/-}\) cells (KO) to TLF-1 killing was determined following a 16 hour incubation at 37°C. In these studies a TLF-1 killing unit = 0.019 nM.

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HpHbR<sup>H</sup>-/- cell line showed that this gene was non-essential in the bloodstream stage of \textit{T. b. brucei} used in these studies. In addition, only a very slight reduction in growth rate was observed (Figure 1C).

**TbbHpHbR<sup>H</sup> is necessary for TLF-1 binding, uptake and killing**

We examined whether the \textit{TbbHpHbR<sup>H</sup>-/-} cells were deficient in TLF-1 uptake. When WT \textit{T. b. brucei} was incubated at 37°C with Alexa-488 conjugated TLF-1 (10 nM), cells rapidly accumulate TLF-1 in endosomes and lysosomes, whereas no detectable Alexa-488 TLF-1 internalization was observed in \textit{TbbHpHbR<sup>H</sup>-/-} cells (KO) (Figure 1D). As previously reported, uptake of TLF-1 was dependent on the addition of Hb prior to incubation with trypanosomes [28]. Additionally, it was shown that at 3°C, Alexa-488 TLF-1 localized specifically to the flagellar pocket [22]. To determine whether TLF-1 uptake at this low concentration was dependent on flagellar pocket binding, WT and \textit{TbbHpHbR<sup>H</sup>-/-} cells were incubated at 3°C with Alexa-488 TLF-1 (Figure 1E). At concentrations as low as 0.6 nM, TLF-1 binding to the flagellar pocket was detectable and was concentration dependent up to 66 nM. No TLF-1 binding to \textit{TbbHpHbR<sup>H</sup>-/-} cells was observed at concentrations up to 66 nM (Figure 1E). In addition, \textit{TbbHpHbR<sup>H</sup>-/-} cells were highly refractory to TLF-1 killing at concentrations of 0.1 nM (Figure 1F). These studies showed that the \textit{TbbHpHbR<sup>H</sup>} was required for high affinity TLF-1 binding and further supports the role of this receptor in trypanosome infection.

**Functional analysis of the \textit{TbgHpHbR}**

In order to determine whether the \textit{TbgHpHbR} was functional in TLF-1 binding and subsequent killing, stable cell lines, ectopically expressing the \textit{TbbHpHbR} and \textit{TbgHpHbR} genes, were prepared in the \textit{TbbHpHbR<sup>H</sup>-/-} background. In addition to verify HpHbR expression, an HA-epitope tagged variant of the \textit{TbgHpHbR} (\textit{TbgHpHbR<sup>HA</sup>}), in the \textit{TbbHpHbR<sup>H</sup>-/-} background, was also prepared (Figure 2A). Expression of \textit{TbbHpHbR}, \textit{TbgHpHbR} and \textit{TbgHpHbR<sup>HA</sup>} was determined by nested RT-PCR allowing detection of both endogenous and HA-tagged HpHbR mRNAs (Figure 2A). The level of HpHbR mRNA was comparable in all cell lines (Table S2). The expression of \textit{TbgHpHbR<sup>HA</sup>} was also evaluated by western blot with antibodies specific to the HA-tagged HpHbR (Figure 2B). A single band, migrating around 80 kDa, was visible in \textit{TbgHpHbR<sup>HA</sup>} cells, but not in the \textit{TbbHpHbR<sup>H</sup>-/-} cell line (Figure 2B). Specificity of the anti-HA antibody was verified with a cell line expressing a HA-tagged Rab5A (Figure 2B) [22]. To determine whether the \textit{TbgHpHbR<sup>HA</sup>} was functional in TLF-1 binding and uptake, cells were examined for TLF-1 binding and subsequent killing (Figures 2C and 2D). \textit{TbbHpHbR<sup>H</sup>-/-}, but not \textit{TbgHpHbR} or \textit{TbgHpHbR<sup>HA</sup>}, restored both TLF-1 binding and killing in the \textit{TbbHpHbR<sup>H</sup>-/-} background (Figures 2C and 2D). We also prepared a HA-epitope tagged variant of the \textit{TbbHpHbR} (\textit{TbbHpHbR<sup>HA</sup>}), in the \textit{TbbHpHbR<sup>H</sup>-/-} background. However, perhaps due to the positioning of the HA-epitope within the highly structured cytosolic domain of the receptor, no detectable signal was seen on western blots (unpublished data). It is unlikely that the \textit{TbbHpHbR<sup>HA</sup>} was not expressed since mRNA levels were comparable to the other receptor knock-in lines and TLF-1 binding and susceptibility were restored to wild type levels in these cells. Together these results indicate that \textit{TbgHpHbR<sup>HA</sup>} retains the ability to bind TLF-1 while \textit{TbgHpHbR} and \textit{TbgHpHbR<sup>HA</sup>}, while expressed at comparable levels, do not function in TLF-1 binding, uptake or trypanosome killing.

**Functional analysis of sequence polymorphisms in the \textit{TbgHpHbR}**

Sequence analysis of a small number of isolates of group 1 and 2 \textit{T. b. gambiense}, \textit{T. b. rhodesiense} and \textit{T. b. brucei} led to the initial hypothesis that a limited number of amino acid substitutions may contribute to reduced uptake of TLF-1 by cells expressing the \textit{TbgHpHbR} [23] (Figure 3A). In a more comprehensive geographic and taxonomic analysis of HpHbR sequences, a single substitution replacing a leucine with a serine at position 210 was observed in all group 1 \textit{T. b. gambiense} and was not observed in TLF-1 susceptible \textit{T. b. brucei or in T. b. rhodesiense} [24]. To test whether any of the substitutions in the \textit{TbgHpHbR} could individually restore TLF-1 binding and killing, each of the \textit{T. b. gambiense} specific amino acid substitutions were changed back to the amino acid found in the \textit{TbgHpHbR} (Figure 3A). The steady state levels of \textit{TbgHpHbR}, \textit{TbgHpHbR<sup>S210L</sup>}, \textit{TbgHpHbR<sup>A293D</sup>}, \textit{TbgHpHbR<sup>G295A</sup>} and \textit{TbgHpHbR<sup>A293D-G295A</sup>} mRNAs were evaluated by qRT-PCR (Figure 3B, Table S2). The levels of expression of these ectopically expressed genes were comparable in all five analyzed cell lines.

In order to determine whether amino acid changes in the \textit{TbgHpHbR} affected TLF-1 uptake, each cell line was examined by fluorescence microscopy and flow cytometry following incubation with Alexa-488 TLF-1 for 30 minutes at 37°C (Figure 3C and 3D). Fluorescence microscopy showed that \textit{TbgHpHbR} expressing cells endocytosed TLF-1 and that most was localized to the posterior region of the cells between the kinetoplast and nucleus consistent with lysosomal trafficking (Figure 3C). Flow cytometry indicated that the amount of TLF-1 taken up by the \textit{TbgHpHbR} cells was similar to that seen in WT \textit{T. b. brucei} (Figure 3D). In contrast, \textit{TbgHpHbR} cells showed no detectable uptake of TLF-1 either by fluorescence microscopy or flow cytometry analysis (Figure 3C and 3D). Similarly, \textit{TbgHpHbR<sup>G295A</sup>} and \textit{TbgHpHbR<sup>A293D-G295A</sup>} did not take-up TLF-1 and appeared identical to \textit{TbgHpHbR} cells. However, the single amino acid change at position 210 of the \textit{TbgHpHbR}, from serine to leucine, restored TLF-1 uptake and localization to levels seen in the \textit{TbgHpHbR} cells (Figure 3C and 3D). The specificity of TLF-1 binding in \textit{TbbHpHbR<sup>H</sup> and TbgHpHbR<sup>G295A</sup>} was examined by competition binding studies with unlabeled TLF-1 or Hp1-1 in the presence or absence of Hb (Figure 3E and 3F, respectively). When complexed with Hb both unlabeled Hp-1 and TLF-1 effectively competed for TLF-1 binding. These results indicated that the HpHbR mediated all TLF-1 binding in these cells.

To determine whether susceptibility to TLF-1 killing was also influenced by the changes to the HpHbR, cell lines expressing \textit{TbbHpHbR}, \textit{TbgHpHbR, TbgHpHbR<sup>S210L</sup>}, \textit{TbgHpHbR<sup>G295A</sup>} and \textit{TbgHpHbR<sup>A293D-G295A</sup>} were incubated with increasing concentrations of TLF-1 and the percentage of cells surviving after 16 hours was determined (Figure 3G and 3H). As expected, based on uptake studies, cells expressing \textit{TbbHpHbR} and \textit{TbgHpHbR<sup>G295A</sup>} were fully susceptible to TLF-1. \textit{TbgHpHbR<sup>S210L</sup>} and \textit{TbgHpHbR<sup>A293D-G295A</sup>} were resistant to TLF-1 killing. Together these studies show that the single amino acid change of serine to leucine at position 210 of \textit{TbgHpHbR} is sufficient to restore both TLF-1 uptake and killing to levels seen in cells expressing the \textit{TbbHpHbR}. This finding is consistent with the substitution to the HpHbR in \textit{T. b. gambiense} playing a critical role in human infectivity.

**TLF-1 binding affinities of cells expressing variant HpHbR**

To evaluate the effect of the amino acid substitutions in the \textit{TbgHpHbR} on the binding affinity for TLF-1, a live cell-binding assay was developed with Alexa-488 TLF-1. Cells were incubated
at 3°C for 2 hours with varying concentrations of Alexa-488 TLF-1. Unbound TLF-1 was removed by washing in ice-cold 1× PBS and the amount and location of TLF-1 binding evaluated by FAC analysis and fluorescence microscopy (Figure 4, Figure S3). Alexa-488 TLF-1 localized exclusively to the flagellar pocket (Figure 4D) and cell associated fluorescence was concentration dependent in WT T. b. brucei, TbbHpHbR and TbgHpHbR/S210L cell lines (Figure 4A–C). No detectable TLF-1 binding was seen in TbbHpHbR/G293A, TbgHpHbR (Tbg) and TbgHpHbR/G293A (TbgHA) cell lines (Figure 4C and D). To determine whether the affinity for TLF-1 differed in the TbbHpHbR/G293A cell lines expressing TbbHpHbR, TbgHpHbR, TbgHpHbR/S210L, TbgHpHbR/G293A and TbgHpHbR/G293A-370EG we performed saturation binding studies with Alexa-488 TLF-1 (Figure 4A–C). The binding affinity was estimated based on half-maximal binding. Both WT T. b. brucei and TbbHpHbR had high affinity for TLF-1 (3.96±0.31 nM and 4.12±0.25 nM, respectively). The TbgHpHbR/G293A cells also bound TLF-1 with similar affinity (3.96±0.35 nM). Consistent with the results obtained by microscopic analysis, TLF-1 binding was not observed in the TbgHpHbR/KO, TbgHpHbR (Tbb), TbgHpHbR (Tbg) and TbgHpHbR/G293A (TbgHA) cell lines. Based on these results, the highly conserved amino acid substitution in the TbgHpHbR at position 210 is responsible for decreased binding affinity for TLF-1.

In flow cytometry studies, a small amount of TLF-1 binding to the TbgHpHbR was detected (Figure 4B). To determine whether this represented binding to the TbgHpHbR or a low level of background binding we over-expressed the TbgHpHbR and the TbbHpHbR through ectopically expressing the HpHbR, driven by a ribosomal promoter, in the TbbHpHbR/G293A cell lines. This resulted in a 15-fold increase in expression of TbgHpHbR and the TbbHpHbR mRNAs as measured by qRT-PCR (Table 1).
Figure 3. Effects of amino acids substitutions in TbgHpHbR on TLF-1 uptake and killing. (A) Sequence alignment of T. b. brucei and T. b. gambiense HpHbR. Four amino acids in the TbgHpHbR, positions 210, 293 and 369–370, within the mature coding sequence. (B) Expression levels of HpHbR mRNA were determined by RT-PCR from TbbHpHbR \( ^{-/-} \) cells ectopically expressing the TbbHpHbR (Tbb), TbgHpHbR (Tbg), TbgHpHbR\( ^{V293A} \) (V293A), TbgHpHbR\( ^{GA/EG} \) (GA/EG) and TbgHpHbR\( ^{S210L} \) (S210L) genes. (C) Fluorescence microscopy of the TbbHpHbR \( ^{-/-} \) cells ectopically expressing the Tbb, Tbg, V293A, GA/EG and S210L genes after incubation with 20 nM Alexa-488 TLF-1 at 37°C for 30 minutes. Kinetoplast (k), Nucleus (n), Flagellum (f). (D) FAC analysis of Alexa-488 TLF-1 uptake by wild type T. b. brucei (WT), TbbHpHbR \( ^{-/-} \) (KO), and the TbbHpHbR \( ^{-/-} \) cell lines
Overexpression of the *Tbb* HpHbR also resulted in a large increase in TLF-1 binding (16-fold) and sensitivity to TLF-1 killing (18-fold). However, over-expression of *Tbg* HpHbR, to similar levels, had no effect on TLF-1 binding or susceptibility (Table 1). Together these results indicate that the *Tbg* HpHbR is unable to bind TLF-1 and that a single amino acid change in the *Tbg* HpHbR is sufficient to spare *T. b. gambiense* from TLF-1 killing.

**Discussion**

Previous studies have shown that the level of HpHbR expression can influence the susceptibility of African trypanosomes to TLF-1 and human serum [4,23]. Analysis of mRNA levels in five field isolates of group 1 *T. b. gambiense* showed that TbgHpHbR expression was reduced 20-fold relative to *T. b. brucei* [23]. In addition to reduced mRNA levels, four non-synonymous substitutions present in the *Tbg* HpHbR and not in *Tbb* HpHbR were identified [23]. A more extensive analysis of HpHbR gene sequences from 67 isolates of *T. b. brucei*, *T. b. gambiense* group 1 and group 2 and *T. b. rhodesiense* supported these findings and further narrowed conserved substitutions in *Tbg* HpHbR. This led to the suggestion that substitution of leucine with serine at position 210 might abolish TLF-1 binding [23–25].

To directly test the consequence of amino acid substitutions within the *Tbg* HpHbR, on TLF-1 binding, uptake and trypanolytic activity we established a *Tbb* HpHpR/2 cell line by replacement of both alleles with drug resistance markers (Figure 1A). Using this stable cell line, we tested each amino acid substitution in the *Tbg* HpHbR individually by ectopic expression (Figures 3B). By systematically changing each of the amino acid substitutions in the *Tbg* HpHbR to the most common sequence in *Tbb* HpHbR, we showed that the S210L change restores high affinity TLF-1 binding, uptake and trypanosome killing (Figure 3, 4). Based on these new findings and our previous results we propose that group 1 *T. b. gambiense* has evolved two mechanisms to avoid uptake of TLF-1. First, the abundance of HpHbR mRNA was reduced 20-fold in all group 1 *T. b. gambiense* isolates tested [23]. Secondly, as shown in the studies presented here, the *Tbg* HpHbR had reduced affinity for TLF-1 due to an amino acid substitution that was highly conserved in all members of this
subgroup. It is likely that both reduced HpHbR expression and TLF-1 affinity contribute to the overall resistance of group 1 T. b. gambiense to TLF-1.

Recent crystallographic studies of the T. congolense HpHbR have allowed a detailed structural analysis of the trypanosome HpHbR [26]. These studies revealed a hydrophobic core head domain predicted to be important in receptor-ligand interaction and further predicted that the hydrophobic core of the ligand-binding domain would be disrupted by the S210L substitution described in Figure 3. We found that addition of a HA-epitope within the disrupted head domain of the TbgHpHbR was accessible for antibody detection (Figure 2B). In contrast, the HA-epitope, in the stabilized head domain of the TbbHpHbR, was inaccessible to antibody binding yet retained TLF-1 binding and facilitated killing (unpublished data). The in vivo binding results presented in Figures 3 and 4, were also consistent with SPR binding assays with recombinant HpHbR, which showed that the leucine to serine substitution significantly reduced TLF-1 and HpHb binding to the HpHbR [26].

A potentially important difference in TLF-1 binding was revealed in the in vitro binding studies with recombinant HpHbR [26] and the in vivo studies reported here (Figure 3). The SPR binding results showed a striking difference in the affinity for TLF-1 and HpHb for the TbbHpHbR (5–10 μM and 4.5 nM respectively) [26]. This is inconsistent with our findings showing that TLF-1, when saturated with bound Hb, binds with a similar affinity as HpHb to the TbgHpHbR (Figure 3). The relatively low affinity binding of TLF-1 may result from sub-saturating levels of Hb in the TLF-1 samples used in their studies. Alternatively, the higher affinity measured in vivo may reflect a role for secondary binding proteins on the trypanosome surface that increase the affinity as HpHb to the HpHbR [26].

Table 1. Over expression of TbbHpHbR and TbgHpHbR.

| Cell Line          | HpHb mRNA | TLF-1 binding | TLF-1 killing |
|--------------------|------------|---------------|---------------|
| T. b. brucei       | 1.0        | 1.0           | 1.0           |
| KO                 | N.D.       | N.D.          | N.D.          |
| TbbHpHbR           | 1.1±0.2    | 1.0           | 1.0           |
| TbbHpHbR (over-expressed) | 15.8±0.2^a | 17.0±0.2^a | 17.8±2.2^a |
| TbgHpHbR           | 0.9±0.1    | N.D.          | N.D.          |
| TbgHpHbR (over-expressed) | 11.2±0.4^b | N.D.         | N.D.         |

N.D. = Not Detectable.

1) determined by qRT PCR (Fig. 1B).
2) determined by FAC analysis (Fig. 2D).
3) determined by 16 hr. survival assays (Fig. 2G, 2H).
4) determined by SPR analysis (data not shown).
5) determined by qRT PCR (data not shown).
6) determined by FAC analysis (data not shown).

The HpHbR has been proposed to be an essential nutrient receptor in African trypanosomes functioning in hemoglobin scavenging in these heme auxotrophs [14]. The near wild type growth rate of T. b. brucei HpHbR^-/- cell line showed that the receptor was not essential for survival in vitro (Figure 1). Furthermore, this suggests that heme scavenging by the HpHbR may not be necessary in bloodstream African trypanosomes. An attractive alternative is that the T. b. brucei HpHbR^-/- cell lines have other mechanisms for heme uptake that can compensate for the loss of the HpHbR under in vitro growth conditions. Recently, a heme transporter has been described in Leishmania that is partially localized to the plasma membrane suggesting that heme may be transported into kinetoplastids in the absence of the HpHbR [31].

It is not surprising that group 1 T. b. gambiense has evolved diverse mechanisms for protection against TLF-1 and 2. These parasites have a long and intimate involvement with the human host. Largely lacking wild game or domesticated animal reservoirs, these parasites have had ample opportunities to develop both redundant and augmenting mechanisms of resistance. It is likely that the observed reduced expression and loss of function substitution to the HpHbR gene in group 1 T. b. gambiense, though seemingly redundant processes, heightens the collective resistance of these cells to the more complex assault by the human innate immune systems. Group 2 T. b. gambiense is genetically more diverse than group 1 and has evolved a novel HpHbR independent mechanism for inhibition of TLF-1 killing [24]. Since group 2 T. b. gambiense express a functional HpHbR, resistance requires inhibition of TLF-1 killing. It is appealing to speculate that group 1 T. b. gambiense may share this mechanism but its effect on TLF-1 killing is largely masked by reduced TLF-1 uptake.

Supporting Information

Figure S1 Genomic PCR analysis. HpHbR-specific primer PCR analyzed the presence of DNA for the HpHbR in both wild type T. b. brucei (WT) and TbbHpHbR^-/- (KO). Transcript presence is indicated by PCR band appearance with β-tubulin used as the loading control. (TIF)

Figure S2 Saturation of TLF-1 binding by Hb. To ensure that all TLF-1 was saturated with Hb in the competition binding assays, Hb was added to TLF-1 (3 nM constant) in increasing concentrations. The percentage of maximum binding measured from FAC analysis is plotted versus the concentration of Hb. (TIF)

Figure S3 Comparative binding curve for TLF-1 measured by fluorescence microscopy and FAC analysis. For both analyses a Kd of 4.06±0.08 nM (microscopy) and 3.42±0.52 nM (FAC analysis) was determined. (TIF)

Methods S1 Supporting Methods. (DOCX)

Table S1 Oligonucleotides used in (RT) PCR experiments. (TIF)

Table S2 Relative HpHbR mRNA levels. (TIF)

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Author Contributions
Conceived and designed the experiments: ED RK SLH. Performed the experiments: ED RK BA NAS. Analyzed the data: ED RK BA SLH. Contributed reagents/materials/analysis tools: ED RK. Wrote the paper: ED RK SLH.

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